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RNA editing of cytochrome *c* maturation transcripts is responsive to the energy status of leaf cells in *Arabidopsis thaliana*

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ABSTRACT

Overexpression of AtPAP2, a phosphatase located on the outer membranes of chloroplasts and mitochondria, leads to higher energy outputs from these organelles. AtPAP2 interacts with seven MORF proteins of the editosome complex. RNA-sequencing analysis showed that the editing degrees of most sites did not differ significantly between OE and WT, except some sites on the transcripts of several cytochrome *c* maturation (Ccm) genes. Western blotting of 2D BN-PAGE showed that the patterns of $CcmF_{N1}$ polypeptides were different between the lines. We proposed that AtPAP2 may influence cytochrome *c* biogenesis by modulating RNA editing through its interaction with MORF proteins.

1. Introduction

Chloroplasts and mitochondria are the major ATP-producing organelles in plant leaves. Both organelles are derived from endosymbionts, each having their own separate genome. In addition to transcriptional control, posttranscriptional editing of RNA transcripts provides a control mechanism in plant organelles. After the organellar RNA is transcribed, some specific cytidines are deaminated to uridines and the editing may result in amino acid substitution, or the generation of start or stop codons (Kotera et al., 2005; Wintz and Hanson, 1991). In *Arabidopsis*, approximately 40 and 400 RNA editing sites have been identified in the plastid and mitochondrial transcripts, respectively (Bentolila et al., 2013; Germain et al., 2015). The edited sites from the organellar transcripts are recognized and determined by pentatricopeptide repeat (PPR) proteins, which are composed of tandem arrays of degenerate 35-amino-acid repeating units, the PPR motifs (Barkan and Small, 2014). Amino acids at three particular positions of each PPR motif were postulated to determine nucleotide specificity in a 1-motif to 1-nucleotide fashion (Barkan et al., 2012). Multiple PPR motifs in a PPR protein are thus able to recognize a stretch of RNA sequence and determine the RNA editing sites (Yagi et al., 2013).

PPR is a component of the 200–400 kDa protein complexes called editosomes, which carry out RNA editing in organelles (Bentolila et al., 2012; Hayes and Hanson, 2007). Editosomes were shown to be composed of PPR, Multiple Organellar RNA Editing Factor (MORF), organelle RNA Recognition Motif-containing proteins (ORRM), and organelle zinc (OZ) finger editing factor family proteins (Sun et al., 2015b). In the *Arabidopsis* genome, there are 469 *PPR*, 9 *MORF*, 15 *ORRM*, and 4 *OZ* genes (Sun et al., 2013b). To date, the enzyme responsible for deamination has not yet been identified. Although some PPR proteins contain an additional DYW domain with high homology to cytidine deaminase and the DYW domain of two PPR proteins (OTP84 and CREF7) are required for site-specific RNA editing (Hayes et al., 2015; Wagoner et al., 2015), no enzymatic activity has been shown for the DYW domain in the *in vitro* assays (Nakamura and Sugita, 2008; Okuda et al., 2009).

Interaction between MORF proteins and PPR proteins is required for RNA editing. For example, the interaction of PPR protein MEF10 with MORF8 is required for RNA editing at *nad2-842* in mitochondria (Hartel et al., 2013). Some PPR proteins interact with more than one MORF protein. For example, in plastids, PPR protein PDM1/SEL1 interacts with MORF2/8/9 and is involved in RNA editing and RNA splicing (Zhang et al., 2015). In the mitochondria, PPR protein MEF13 interacts with MORF3/8 and is involved in RNA editing at eight different sites (Glass et al., 2015). The situation becomes more complex when MORF proteins form homo- and heterodimers (Zehrmann et al., 2015). MEF35 interacts with the mitochondrial MORF1/8 proteins and is responsible for RNA editing at three sites on three mRNA transcripts, including ribosomal protein (*rpl16*-209), cytochrome *b* (*cob-286*), and subunit 4 of complex I (*nad4*-1373) (Brehme et al., 2015). It is not known whether different MEF35/MORF homodimers or MEF35/MORF heterodimers are responsible for the editing of different transcripts.

MORF and PPR proteins are encoded by the nuclear genome, and after their translation at the cytosolic ribosomes, they must be imported

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into chloroplasts or mitochondria to carry out their RNA editing functions. MORF2 and MORF9 are targeted to plastids and MORF1/3/4/5/7 are targeted to mitochondria, while MORF6/8 are dually targeted to both organelles (Law et al., 2015; Zehrmann et al., 2015). In addition, some PPR proteins are also dually targeted to both organelles. For example, the PPR protein AEF1 (ATPF Editing Factor 1) functions in the editing of *atpF* in plastids and *nad5* in mitochondria (Yap et al., 2015).

The mitochondrial and chloroplast genomes each encode ~100 protein coding genes and many nuclear-encoded precursor proteins translated in the cytosol are imported into these two energy-transforming organelles to maintain their functions. To be imported into organelles, cytosolic proteins are required to pass through the translocons of the outer membranes of the chloroplasts (TOC) and mitochondria (TOM), respectively (Schleiff and Becker, 2011). *Arabidopsis thaliana purple acid phosphatase 2* (AtPAP2) is a phosphatase, which is anchored on the outer membranes of both organelles by its hydrophobic C-terminal motif, and it plays a role in the import of selected nuclear-encoded proteins into these two organelles (Sun et al., 2012a; Zhang et al., 2016). Overexpression (OE) of AtPAP2 in *A. thaliana* accelerates plant growth and promotes flowering, seed yield, and biomass (Sun et al., 2012b). The leaves of the AtPAP2 OE lines contain higher sucrose and ATP contents (Liang et al., 2014; Sun et al., 2013a). It has been postulated that overexpression of AtPAP2 selectively enhances the import rate of certain proteins into these two organelles and modulates their energy harvest and conversion activities. AtPAP2 interacts with the presequences of a few MORF proteins (MORF2/3/5/6) and plays a role in the import of MORF3 into the mitochondria (Law et al., 2015). Therefore, it is possible that overexpression of AtPAP2 might affect the physiology of chloroplasts and mitochondria by modulating the RNA editing of these two organelles. In order to explore this possibility, we examined the interactions between AtPAP2 and the other MORF proteins and compared the degree of RNA-editing between the leaves of the OE line and wild-type (WT) plants at three time points.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotypes Columbia (Col-0) and AtPAP2 OE line (OE7) in Col-0 background (Sun et al., 2012b) were used in this study. Arabidopsis seeds were grown on MS (Murashige and Skoog) medium supplemented with 2% (w/v) sucrose for 10 d and then transferred to soil. The plants were grown under 16 h light (22 °C)/8 h dark (18 °C) in a growth chamber at a light intensity of 120–150 μmol $m^{-2} s^{-1}$. The rosette leaves of 20-day-old Arabidopsis were harvested at three different time points: t = 0 h (end of night), t = 1 h (1 h under illumination), and t = 8 h (8 h under illumination).

2.2. Yeast two-hybrid interaction

The coding sequence of mature bait protein AtPAP2 (25-613 a.a.), lacking its signal peptide and C-terminal transmembrane motif, was fused to the C-terminus of the GAL4 DNA-binding domain (BD) in the pGBKT7 vector. Full-length coding sequences of MORF1/4/7/8/9 fused with DNA-activating domain (AD) of the pGADT7 vector were co-transformed into yeast Y2HGold strain (Clontech) using the Yeastmaker™ Yeast Transformation System 2 (Clontech) according to the manufacturer's instructions. The transformed yeast culture was spread on double dropout medium without Trp or Leu (-WL); triple dropout medium without Trp, Leu, or His (-WLH); and quadruple dropout medium without Trp, Leu, His, or Ade (-WLHA; Clontech). Colonies were photographed after incubation for 5 days under 28 °C. Sequence data from this work can be found in the Arabidopsis Genome Initiative under the following accession numbers: AT1G13900 (AtPAP2), AT4G20020 (MORF1), AT5G44780 (MORF4), AT1G72530 (MORF7), AT3G15000 (MORF8) and AT1G11430 (MORF9). All nine pGADT7 vectors carrying AtMORF1–9 were deposited at The Arabidopsis Information Resource (TAIR).

2.3. Bimolecular fluorescence complementation (BiFC) assay

This method was performed according to Schweiger and Schwenkert (2014).

The full-length coding sequence of AtPAP2 was fused to the C-terminus of YFP^N on the pSPYNE vector. The full-length coding sequences of MORF1/4/7/8/9 were fused to the N-terminus of YFP^C on the pSPYCE vector. Both pSPYNE and pSYPCE vectors containing genes of interest were transformed into *Agrobacterium tumefaciens* using the freeze and thaw method (Weigel and Glazebrook, 2006). *Agrobacterium tumefaciens* containing pSPYNE and pSYPCE vectors were inoculated until OD_{600} = ~2.0 and adjusted to 1.0 by infiltration medium (10 mM $MgCl_2$, 10 mM MES/KOH (pH 5.7), 150 μM Acetosyringone). Equal volume of the *Agrobacterium tumefaciens* containing pSPYNE and pSYPCE vectors were gently mixed and were co-transformed into the three-week-old leaf cells of tobacco (*Nicotiana benthamiana*) by *Agrobacterium* infiltration. The transformed plants were kept in dark conditions for 1–2 days and the transfected leaves were visualized for fluorescence detection under an LSM710 confocal laser-scanning microscope (Carl Zeiss). MitoTracker® Red FM (ThermoFisher, USA) was used for live cell staining to localize mitochondria. All nine pSPYCE vectors carrying AtMORF1–9 were deposited at TAIR.

2.4. RNA sequencing and editing site identification

Six RNA libraries (WT0, WT1, WT8 and OE0, OE1, OE8) were constructed (insert size: 90 bp) and were then sequenced using Illumina HiSeq™2000, each producing 5 Gb bases of pair-end reads. All the raw data originated from NCBI GEO, with accession numbers GSE57790 and GSE57791 (Liang et al., 2016). After removal of reads with ≥ 10 nt aligned to adapter sequence, reads with Ns bases ≥ 5%, and reads with lower quality (> 30% of the sequencing read bases have a quality score ≤ 7) using SOAPfilter, clean reads were obtained for the six different RNA-seq samples and were then aligned against the full gene set of *Arabidopsis thaliana* genomes (TAIR10, including all of the predicted CDS sequences encoded from nucleus, mitochondria, and chloroplast, respectively). SOAPaligner/SOAP2 (Short Oligonucleotide Analysis Package) was used as the alignment tool with the following parameters: -m 0 -x 10,000 -s 40 -l 32 -v 5 -r 2 -p 6) (Li et al., 2009b). Only uniquely mapped reads were retained as inputs for Samtools (Li et al., 2009a) to call variations between RNA-seq reads and the corresponding DNA sequences. When running Samtools, -q (base quality) was set to 20, and –Q (mapping quality) was set to 10, the other parameters were left on the default setting to generate the first version of variation catalogs between DNA and RNA.

To generate a high-quality list of RNA editing sites, stringent criteria were implemented to define a final set of editing sites by taking a) editing sites supported by ≥ 10 reads in at least two of the six samples and b) editing sites with editing degrees ≥ 15% in at least two of the six samples. We found 34 editing sites in chloroplast transcripts and 510 editing sites in mitochondrial transcripts. Editing sites with a sample comparison (OE vs. WT, WT1 vs. WT0, etc.) with any differences ≥ 20% were regarded as differentially edited (yellow and blue in Supplementary Tables 1 and 2).

2.5. Prediction of transmembrane helices in proteins

Unedited amino acid sequences of the five Ccm proteins were downloaded from TAIR (https://www.arabidopsis.org/download/index.jsp) and sequences with 100% editing were generated manually based on the RNA-seq results (Supplementary Table 2). The prediction of the transmembrane secondary structure was performed by the

TMHMM Server v. 2.0 (Krogh et al., 2001). The helix information was recorded and then submitted to Protter (version 1.0) to compare the topology of Ccm proteins before and after RNA editing (Omasits et al., 2014).

2.6. RT-PCR and genomic DNA PCR validation

RNA editing sites were analysed by sequencing genomic DNA and RT-PCR products. DNA-free leaf RNA was reverse transcribed using M-MLV RT (200 U/μl) (Invitrogen) and random hexamers (Invitrogen) according to the manufacturer's instructions. RT-PCR products covering each editing site were generated with specific primers. Genomic DNA was extracted from 20-day-old leaves of WT and OE by CTAB method (Springer, 2010). PCR products were sequenced by BGI (Beijing Genomics Institute).

2.7. Mitochondria isolation and western blot analysis

Mitochondria were isolated from 14-day-old Arabidopsis seedlings grown on MS agar (Lister et al., 2007). 20 μg proteins were resolved by SDS-PAGE and transferred to Hybond-C nitrocellulose membranes, then immunodetected as previously described (Carrie et al., 2008). Antisera against Tom40 (Carrie et al., 2009), AtCcmF$_{N1}$, (Rayapuram et al., 2008) and AtPAP2 (Sun et al., 2012b) were employed for Western blotting.

2.8. Two dimensional blue native PAGE

Mitochondrial proteins were solubilized in 5% (w/v) digitonin extraction buffer (30 mM HEPES, 150 mM potassium acetate, 10% (v/v) glycerol, pH 7.4) for 20 min on ice before centrifugation at 13,000*g* for 20 min. The supernatants were transferred into new tubes and mixed with 5% (v/v) Serva blue G250 solution (750 mM aminocaproic acid, 5% (w/v) Coomassie brilliant blue G250). The solubilized mitochondrial proteins were then separated on 6% (w/v) blue native acrylamide gels, with anode buffer (50 mM Bis-Tris, pH 7.0) and cathode buffer (50 mM Tricine, 15 mM bis-Tris, 0.02% (v/v) Serva Blue G250, pH 7.0) for 6 h at 4 °C. For the second dimension, subunits of the various complexes were separated by SDS-PAGE. The separated mitochondrial proteins were then transferred to Hybond-P nitrocellulose membranes (Amersham, USA) for Western blot analysis using anti-AtCcmF$_{N1}$ antiserum (Rayapuram et al., 2008).

3. Results

3.1. AtPAP2 interacts with all MORF proteins except MORF4 and MORF7

Nine MORF proteins are encoded in the Arabidopsis genome. AtPAP2 was shown to interact with MORF2/3/5/6 in our previous study (Law et al., 2015). In this study, the interaction between AtPAP2 and MORF1/4/7/8/9 was tested using a yeast two-hybrid (Y2H) assay. Results obtained from this study showed that AtPAP2 can interact with MORF1/8/9, but not with MORF4/7 (Fig. 1).

Previous intracellular localization studies of the MORF proteins showed that MORF1/3/4/5 were located in the mitochondria, MORF2/9 were located in plastids, and MORF6/8 were located in both plastids and mitochondria (Law et al., 2015; Zehrmann et al., 2015). A BiFC assay was used to verify *in vivo* interactions between AtPAP2 and MORF1/4/7/8/9 (Fig. 2). The interaction between the two proteins will bring their fusion partners NYFP and CYFP to close proximity and generate a YFP signal. Our data showed that the co-expression of NYFP-AtPAP2 and MORF1-CYFP reconstituted a YFP signal in the mitochondria, the co-expression of NYFP-AtPAP2 and MORF8-CYFP reconstituted a YFP signal in both the chloroplasts and mitochondria, and the co-expression of NYFP-AtPAP2 and MORF9-CYFP reconstituted a YFP signal in the chloroplasts. No co-expression signal of NYFP-AtPAP2 and

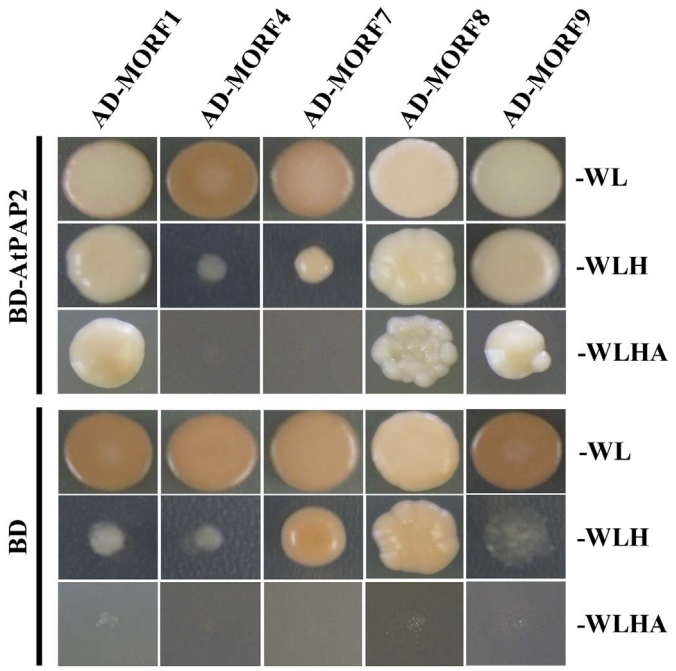

Fig. 1. Interactions between AtPAP2 and MORF proteins as demonstrated by yeast two-hybrid assay. AD-MORF1/8/9 proteins can interact with BD-AtPAP2 but not with BD only in the quadruple dropout medium without Trp, Leu, His, and Ade (-WLHA). AD, activation domain; BD, binding domain.

MORF4/7-CYFP was captured which indicated that AtPAP2 didn't interact with MORF4 and MORF7 (Fig. 2). The BiFC assay also indicated that AtPAP2 interacted with MORF1 in the mitochondria, with MORF8 in both chloroplasts and mitochondria, with MORF9 in the chloroplasts.

Within these organelles, MORFs interact with PPR proteins and form editosomes with ORRM and OZ proteins to recognize RNA strands and mediate RNA editing at specific sites (Barkan et al., 2012; Chaudhuri and Maliga, 1996; Cheng et al., 2016; Yin et al., 2013). AtPAP2 proteins are located on the outer membranes of chloroplasts and mitochondria and play a role in the import of pSSU into chloroplasts (Zhang et al., 2016) and MORF3 into the mitochondria (Law et al., 2015). According to Law et al. (2015) and our findings, AtPAP2 could interact with seven out of nine MORF family proteins. Over-expression of AtPAP2 may therefore alter the import kinetics of its interacting MORF proteins into chloroplasts and/or mitochondria. Transcripts of all 9 MORF and 460 PPR family genes were found in our RNA-seq data (Liang et al., 2015). Five out of nine MORF genes, (MORF 2/5/6/8/9) were highly expressed in all six samples (RPKM > 10) (Supplementary Table 3). At the middle-of-day (t = 8 h), the abundances of RNA transcripts of AtPAP2-interacting MORF1/2/5/6/8/9 were significantly lower in the OE line than the WT, whereas the abundances of RNA transcripts of MORF4/7, which do not interact with AtPAP2, were not found to be different between the OE and WT samples (Supplementary Table 3). The expression of 460 PPR genes are presented in Supplementary Table 4 and the OE/WT differential expression was calculated. Most of the transcription was stable among the six samples. Some divergent expression of *PPR* genes can be observed among samples (Supplementary Table 4) (Liang et al., 2015).

3.2. Assessment of the degree of RNA editing in leaf samples by RNA-seq

In Arabidopsis, around 40 and 400 C-to-U RNA editing sites have been reported in chloroplasts and mitochondria, respectively (Bentolila et al., 2013; Germain et al., 2015). We sequenced and mapped WT and OE RNA transcripts on the Arabidopsis mitochondrial and chloroplast genomes. After setting two strict filtering criteria (no < 10 reads were

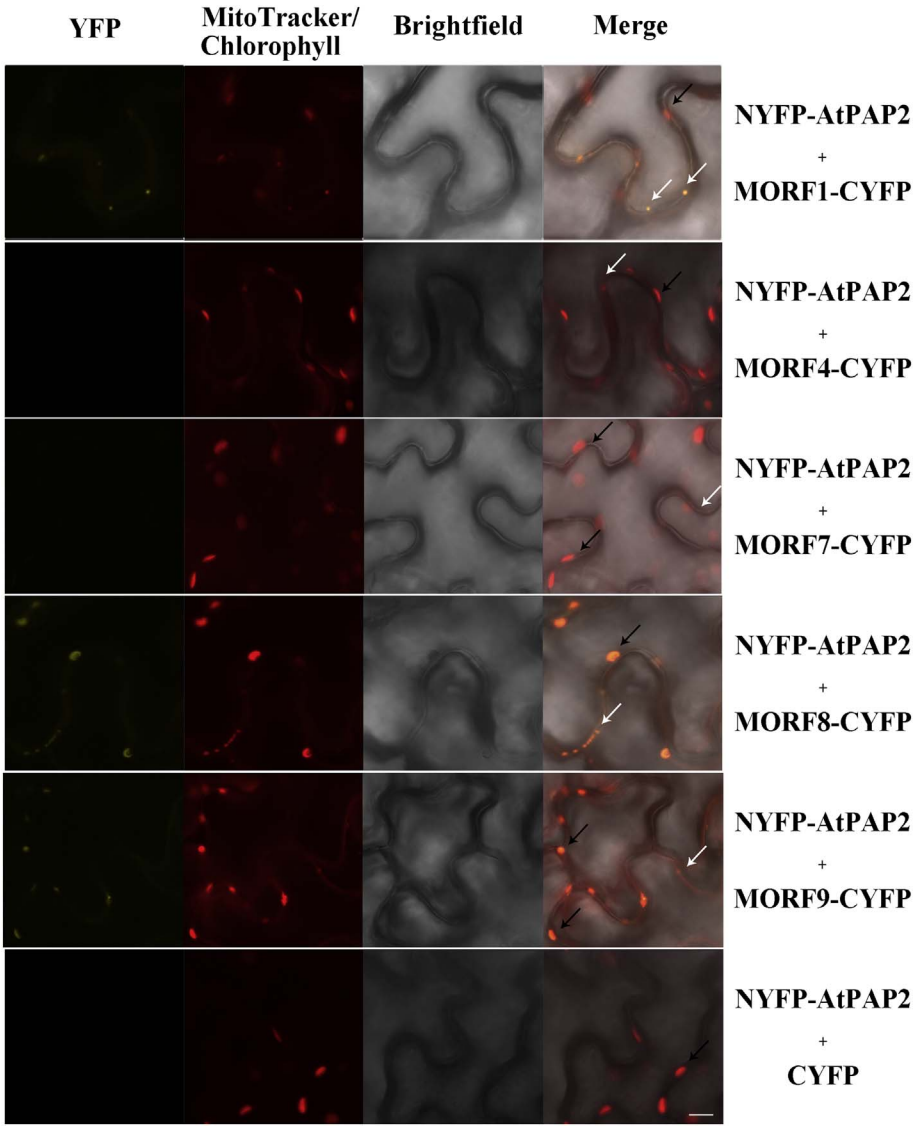

Fig. 2. Interactions of AtPAP2 and MORF proteins as demonstrated by bimolecular fluorescence complementation (BiFC) assay. The co-expression of NYFP-AtPAP2 and MORF1-CYFP reconstituted a YFP signal in the mitochondria. The co-expression of NYFP-AtPAP2 and MORF8-CYFP reconstituted a YFP signal in both chloroplasts and mitochondria. The co-expression of NYFP-AtPAP2 and MORF9-CYFP reconstituted a YFP signal in the chloroplasts. The white arrows indicate mitochondria and the black arrows indicate chloroplasts.

edited and no < 15% of reads were edited in at least two of the six samples), we identified 37 editing sites in chloroplast transcripts and 510 editing sites in mitochondria transcripts, of which 13 plastidal and 117 mitochondrial RNA editing sites were not reported previously (Table 1 and Fig. 3A). DNA sequencing showed that three sites on chloroplast transcripts (*psbA*-324, *rpl22*-231, and *ycf1.2*-5163) were caused by synonymous genome mutation in both OE lines (Supplementary Fig. 1). Hence, only 34 editing sites in chloroplast transcripts were actual editing sites, of which 10 were novel (Table 1 and Fig. 3A). Among the 146 mitochondrial genes and 133 chloroplast genes in *Arabidopsis thaliana* (TAIR 10), 47 mitochondrial genes and 21 chloroplast genes were identified as having editing events in this study (Table 1 and Fig. 3A). Chloroplast editing sites in *matK* (maturase K), *atpF*, *rpoB* (RNA polymerase subunit beta), *ycf9*, *rps14* (chloroplast ribosomal protein S14), *accD* (acetyl-CoA carboxylase carboxyl transferase subunit beta), *psbE* (photosystem II reaction center protein E), *orf31*, *clpP1* (plastid-encoded CLP P), and *ndh* (NADH-Ubiquinone/plastoquinone complex I protein) transcripts exhibited high editing rate (Fig. 4). Three chloroplast sites in *rpl16* and *rpoB* are differentially edited between WT/OE. Moreover, many mitochondrial transcripts, including *atp*, *ccm*, *cob*, *cox* (Cytochrome Oxidase), *nad*, *rps*, and *rpl16*, exhibited extensive editing (Fig. 4).

To validate the C-to-T editing events, cDNA and genomic DNA sequencing of both WT and OE lines was performed. We compared DNA sequences and RNA transcripts in some editing sites. For mitochondrial editing sites that we tested, Cs were detected in the genome sequencing but these sites were edited to Ts in the RNA transcripts (Fig. 4, Supplementary Figs. 2 and 3). Take $ccmF_{N2}$ as an example: in the positions of 176, 208, 226, 259, 277, 320, 344, 356, and 391, the sequencing data showed that these sites were Cs in both WT and OE DNA but were either partially or fully edited to Ts in the RNA transcripts (Supplementary Fig. 2). The editing rates of these sites were approximate to the RNA-seq data (Supplementary Figs. 2 and 3). Hence, the edited sites identified from RNA-seq were validated by RT-PCR.

3.3. Changes in the degree of RNA editing in chloroplast transcripts

We identified 34 editing sites (10 novel and 24 known) in chloroplast transcripts derived from 20 chloroplast genes (Supplementary Table 1). The 10 novel chloroplast RNA editing sites were found in *rpl16*, *rpoC1*, *rbcL*, *ndhH*, and *ndhF* transcripts. There were six novel non-synonymous editing sites and the other novel

Table 1
RNA editing sites on chloroplast transcripts and differential editing between OE and WT samples.

Symbol	mRNA_position	Amino acid change	OE0/WT0 (%)[a]	OE1/WT1 (%)	OE8/WT8 (%)	Function
accD	794	S → L	− 8	− 7	1	Acetyl-CoA carboxylase carboxyl transferase subunit beta
atpF	92	P → L	5	2	3	ATPase, F0 complex, subunit B/B′, bacterial/chloroplast
clpP1	559	H → Y	7	9	17	Plastid-encoded CLP P
matK	706	H → Y	5	9	4	Maturase K
ndhB.1	461,467	S → L; S → L	− 13, 0	0, 0	0, 0	NADH-ubiquinone/plastoquinone (complex I) protein
ndhD	20, 401, 692, 896, 905	T → M; S → L; S → L; S → L; P → L	0, 3, 7, 9, 3	6, − 1, − 3, 7, 4	− 5, 3, 8, − 1, − 2	NADH-Ubiquinone/plastoquinone (complex I) protein
ndhF	290, **826, 841, 844**	S → L; L → F; L → F; P → S	4, 2, 0, 0	8, − 2, − 4, − 3	1, 3, 6, 0	NADH-ubiquinone oxidoreductase (complex I), chain 5 protein
ndhG	50	S → F	12	− 8	6	NADH:ubiquinone/plastoquinone oxidoreductase, chain 6
ndhH	**396**	–	0	− 5	− 2	NAD(P)H dehydrogenase subunit H
orf31	5	P → L	− 4	− 2	− 2	Electron carriers
psbE	214	P → S	− 2	0	− 1	Photosystem II reaction center protein E
psbF	77	S → F	− 8	− 2	− 16	Photosystem II reaction center protein F
rbcL	**666, 731, 983**	(−); A → V; A → V	0, − 2, − 2	2, 2, 3	− 6, 15, − 4	Ribulose-bisphosphate carboxylases
rpl16	**intron, intron**		68, 1	70, − 4	69, 0	Ribosomal protein L16
rpoA	200	S → F	6	2	5	RNA polymerase subunit alpha
rpoB	338, 551, 2432	S → F; S → L; S → L	− 22, − 11, − 21	− 20, − 4, − 14	− 13, 1, − 10	RNA polymerase subunit beta
rpoC1	**488**	S → L	6	2	4	DNA-directed RNA polymerase family protein
rps14	80, 149	S → L; P → L	− 10, − 4	− 13, − 1	− 18, 3	Chloroplast ribosomal protein S14
ycf1.2	4342	E → K	5	− 6	5	Ycf1 protein
ycf9	50	S → L	0	1	6	Ycf9 protein

Numbers in bold represent novel RNA editing sites.
Editing sites with differences in editing degree ≥ 20% were underlined.
[a] Numbers represent the difference between OE and WT (editing degree OE - editing degree WT).

editing events only changed the 3rd position of codons without causing amino acid changes. At most of these novel editing sites, the degree of editing did not differ much between the OE and WT, reflecting that AtPAP2 overexpression did not affect the degree of editing at these novel sites in chloroplasts (Supplementary Table 1). Only three sites, one *rpl16*-82391 (genome location is 82391) and two *rpoB* (RNA polymerase subunit beta) sites, *rpob*-338 and *rpob*-2432, had over a 20% difference in editing degree between OE and WT. 70% of the *rpl16* transcripts were edited at 82391 in the OE samples while only 1% were edited in the WT samples. On the other hand, both non-synonymous editing sites in *rpoB*-2432 (S → L) and *rpoB*-338 (S → F) displayed lower editing degrees in OE than in WT. By contrast, the degrees of RNA editing at all 34 sites were not different between different time points in WT (0/1/8) or in OE (0/1/8) samples, indicating that the editing degrees at these sites are not affected by the light/dark cycle.

3.4. Changes in the degrees of RNA editing in mitochondrial transcripts

For mitochondrial transcripts, a total of 510 C-to-U editing sites (117 novel and 393 known sites) were identified. The 117 novel sites were found in ATP synthase subunits (*atp1*, *atp6*, *atp8* and *atp9*), NADH dehydrogenase (*nad1A/B/C*, *nad2A/B*, *nad3*, *nad4/4L*, *nad5A/C*, *nad6*, *nad7*, *nad9*), *ccm* transcripts (*ccmB*, *ccmC*, $ccmF_{N1}$, $ccmF_{N2}$ and $ccmF_C$; also named *ccb206*, *ccb256*, *ccb382*, *ccb203* and *ccb452*, respectively), *orf*, *rpl2/5/16*, *rps3/4/7/12*, *cob* and *cox2*, *cox3* and *matR* transcripts (Fig. 3A). Considering the distribution of all edited genes, most of the editing sites were found in *nad* and cytochrome related transcripts (*ccm*, *cob*, and *cox*) (Fig. 3A).

While the degrees of editing did not show many differences in chloroplast transcripts, the editing degrees of the 124 RNA editing sites in mitochondrial transcripts varied > 20% between OE and WT samples (Fig. 3B and Supplementary Table 2). In this study, we found that the RNA editing of five *ccm* transcripts was very active. There were 107 editing sites in the *ccm* transcripts, which contribute more than one-fifth of the total mitochondrial editing events (Fig. 3A). High editing degrees and variations between OE/WT were also observed among these *ccm* editing sites (Figs. 3B and 4). Moreover, the editing degrees of some sites in *nad7* and ribosomal protein transcripts (*rpl2*, *rpl5*, *rps3*, and *rps4*) varied > 20% between OE and WT samples (Fig. 3B). When the degrees of RNA editing were compared between WT0/1/8 samples, significant changes could be observed in some editing sites on *ccm*, *orfs*, and *rps4* transcripts (Fig. 3B). Most of the RNA editing sites in mitochondrial transcripts were not different between WT1 and WT0. The exception was in five sites of $ccmF_{N2}$, three sites of *ccmB*, three sites of *ccmC*, and two sites of *orf313* transcripts, in which the editing degrees were > 20% lower in the WT1 sample than in the WT0 sample (Supplementary Table 2). When the degrees of editing were compared between the OE and WT samples, most differences were observed on the sites of five *ccm*, *nad7*, *rpl2/5*, and *rps3/4* transcripts. The degrees of editing of most sites on *ccmC*, $ccmF_{N1}$, $ccmF_{N2}$, $ccmF_C$, *rpl2/5*, and *rps4* transcripts were higher in the OE samples than in the WT samples; but in *ccmB* and *nad7* transcripts, both editing sites with either elevated or reduced editing degrees could be seen in the OE samples (Supplementary Table 2). ORF regions contained a certain amount of editing sites, but they need more functional study. We validated this differential editing in $ccmF_{N1}$ and $ccmF_{N2}$ transcripts by RT-PCR (Supplementary Fig. 2).

3.5. Substitution of amino acids due to RNA editing

mRNA editing may lead to amino acid substitution and creation of translational start or stop codons. Comparing amino acid codons after editing, we found that 30 out of 34 editing events in chloroplasts and 400 out of 510 editing events in mitochondria can cause amino acid substitution. Serine (S) is the most substituted amino acid: there were 14 events in chloroplasts and 143 events in mitochondria in which serine (S) was edited to phenylalanine (F) or leucine (L). S → L events took place at 12 chloroplast editing sites of which six were located in the *ndh* transcripts (Supplementary Table 1). Meanwhile, 45 S → F events and 97 S → L events were observed in mitochondria (Supplementary Table 5). S → F/L non-synonymous editing events were largely spread in both *ccm* and *nad* transcripts and caused the

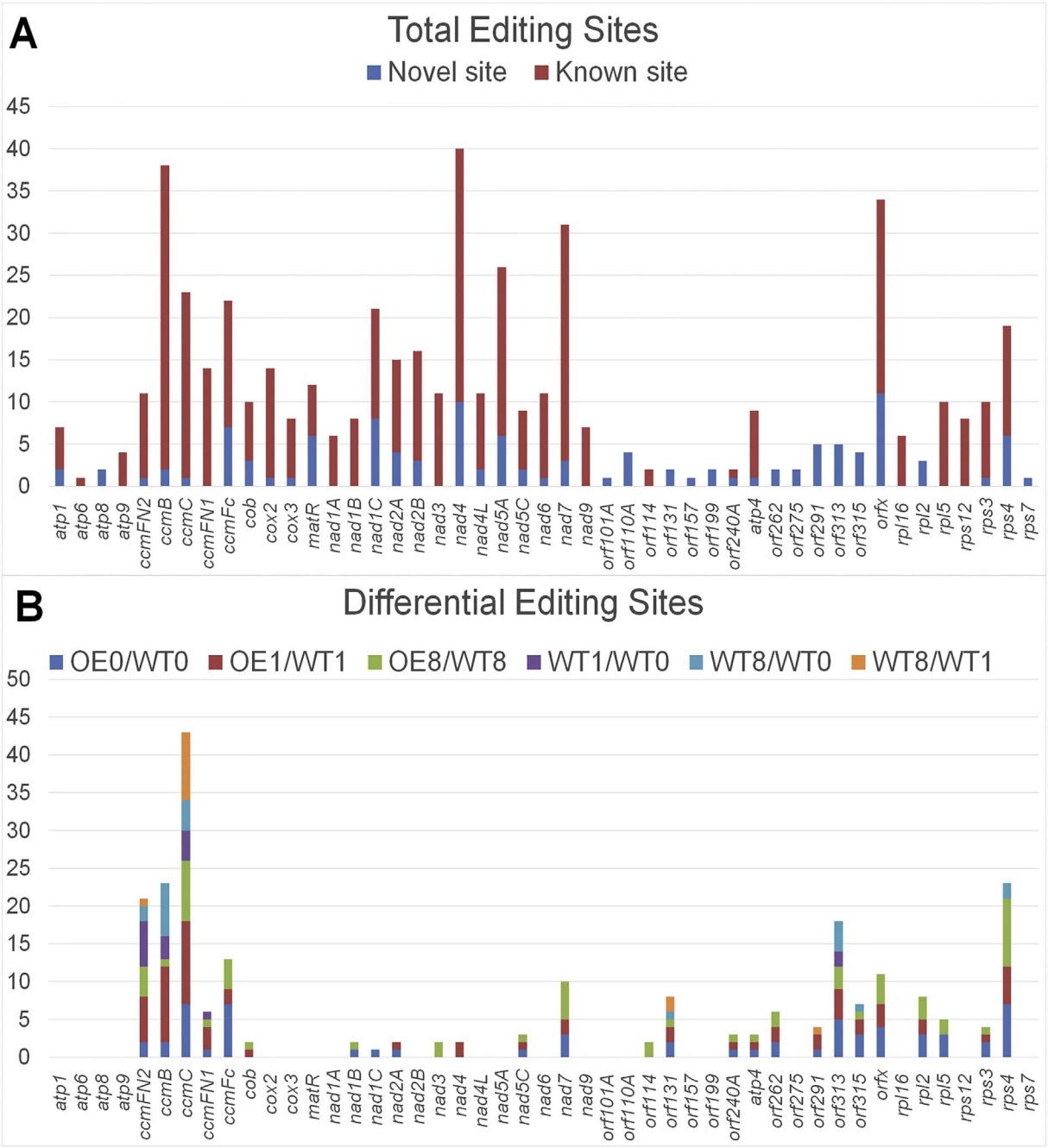

Fig. 3. RNA editing sites on mitochondrial transcripts. A. Distribution of RNA editing sites on mitochondrial transcripts. Cytochrome related (*ccm*, *cob*, and *cox*) and *nad* transcripts are the two major clusters. B. Differentially edited sites on mitochondrial transcripts. *ccm* transcripts were the major cluster.

substitution of a hydrophilic amino acid (S) for hydrophobic residues (F/L). In addition to serine, proline (P) was also a frequently substituted amino acid in RNA editing events. Proline (P) at six sites in chloroplasts and 133 sites in mitochondria was edited to leucine (L) or Serine (S). Of these 133 sites, 86 were P → L and 47 were P → S substitutions (Supplementary Tables 1 and 5). Interestingly, four sites were edited to TAG and TAA stop codons in which three sites in *rps3*, *rps4*, and *rpl16* transcripts were edited from glutamine (Q) to a TAG/TAA stop codon and one site in *ccmF*$_C$ was edited from arginine (R) to a TAA stop codon (Supplementary Table 5). Four stop codons were created in *rpl16*, *rps3*, *rps4* and *ccmF*$_C$. The stop codons occurred at the 21st amino acid residue of Rpl16 (179 a.a.), the 533th residues of Rps3 (556 a.a.), the 9th residue of Rps4 (362 a.a.), and the 443th residue of CcmF$_C$ (452 a.a.). The stop codons prematurely terminate the *rpl16* and *rps4* transcripts so that these transcripts cannot be translated into functional proteins (Supplementary Table 2). High editing degrees near the ends of *rps3* and *ccmF*$_C$ transcripts suggested that certain C termini-related functions of their proteins might be modulated by post-transcriptional editing in the mitochondria. The above three terminating editing sites in *rps3*, *rpl16*, and *ccmF*$_C$ were over 80% edited in all six samples and no differences were found between OE and WT. However, the editing degrees of the terminating codon in *rps4* were 30% greater in OE0/8 than in WT0/8 (Supplementary Table 2).

3.6. RNA editing sites assembled in functional regions of Ccm proteins

In the mitochondria, cytochrome *c* (cyt *c*) is a small, soluble hemeprotein that is loosely associated with the inner membrane. It delivers electrons between complex III and complex IV in the respiratory chain (Giege et al., 2008; Ow et al., 2008; Tafani et al., 2002). The

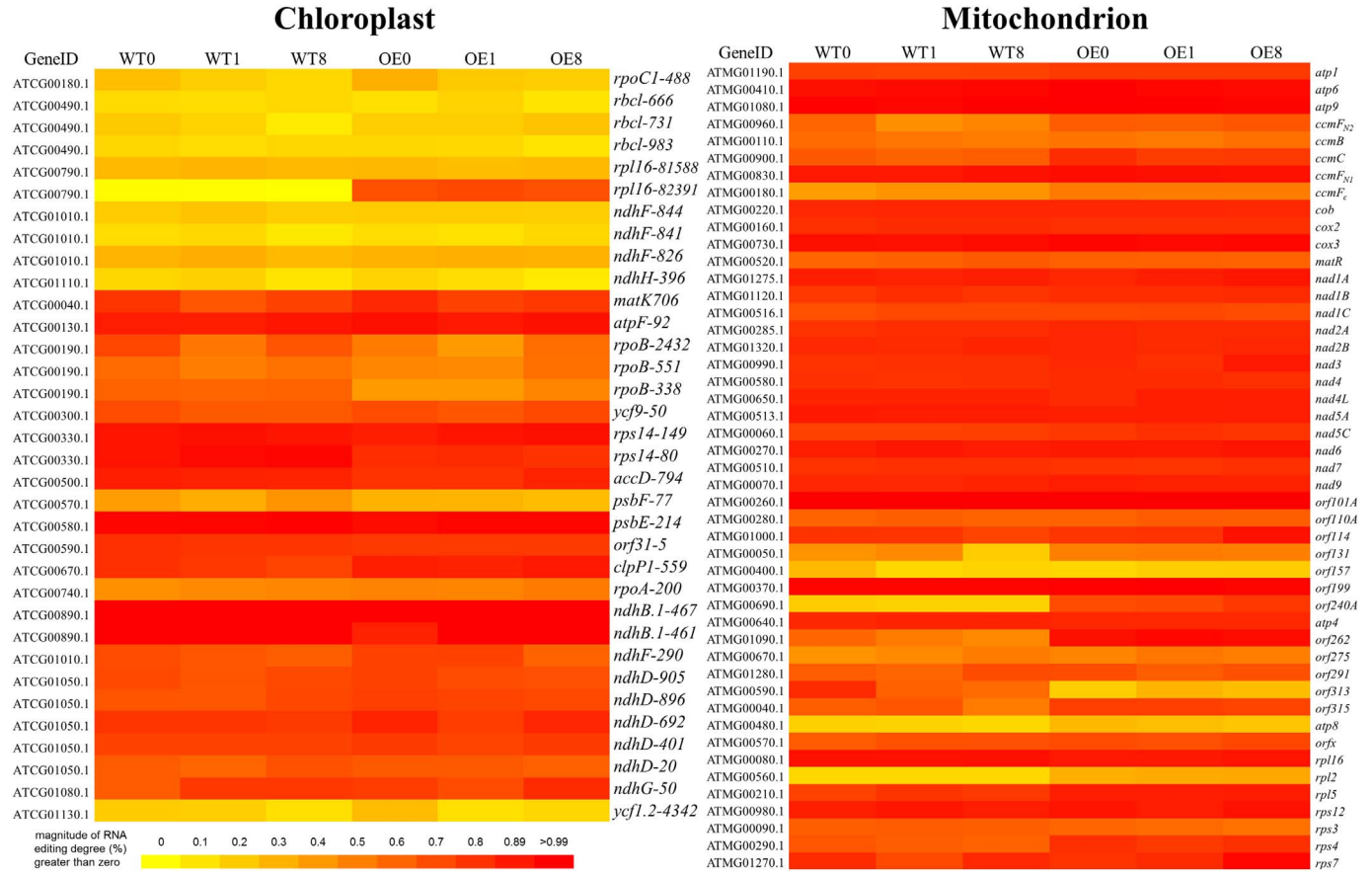

Fig. 4. Degrees of RNA editing of chloroplast and mitochondrial transcripts at different time points in OE and WT samples. In the chloroplasts, degrees of all identified sites are showed. In the mitochondria, sites in the same gene were averaged for presentation. Colors were scaled with 100% editing in red and 0% editing in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

electron gain/loss of heme in cyt *c* is the main reaction of the cyt *c* oxidation-reduction process (Ow et al., 2008). Ccm proteins (CcmA-H) constitute a heme delivery pathway in cyt *c* biogenesis (Rayapuram et al., 2008; Rayapuram et al., 2007; Richard-Fogal and Kranz, 2010; Sanders et al., 2010). $CcmF_{N2}$ and CcmC contain a conserved tryptophan-rich WWD domain that is a central part in the binding and delivery of heme to apocytochrome *c* (Goldman et al., 1998; Rayapuram et al., 2008; Ren et al., 2002; Richard-Fogal and Kranz, 2010). RNA editing of their transcripts at the WWD domains restores mutated codons to functional amino acids. Three key tryptophan residues of the WWD domain (W70, W76, and W87) of $CcmF_{N2}$ were encoded as arginine (R) in the mitochondrial genome (Fig. 5). If the protein was translated without RNA editing, the tryptophan-rich WWD domain would have lost three (W70, W76, W87) out of six Ws and most probably weaken or even lose the ability to bind apocytochrome *c* (Fig. 5, Supplementary Figs. 4 and 5). RT-PCR analysis showed that extensive RNA editing can be seen at all three sites in both WT and OE samples and that the editing degrees are higher in the OE than in the WT samples (Supplementary Fig. 2). On the other hand, a higher degree of editing was not seen at CcmC, another WWD-containing protein in the Ccm family (Supplementary Fig. 5).

We also carried out prediction of transmembrane helices for Ccm proteins using both edited and unedited amino acid sequences. In addition to WWD domains, we found that a lot of RNA editing sites assembled in helix regions in all five mitochondria encoded Ccm proteins (Supplementary Fig. 5). If these amino acids were not edited, the Ccm proteins probably would have had fewer transmembrane helices (Supplementary Fig. 4). Nearly all differentially edited amino acids from $CcmF_C$ were located in the transmembrane regions (Supplementary Fig. 5). Generally, OE samples registered higher editing degrees than WT samples (Supplementary Tables 1 and 2). Interestingly, although most editing sites in Ccm proteins were more highly edited in the OE samples, the editing degrees of seven serine and one arginine residue at the C terminus of CcmB were lower in the OE samples (Supplementary Fig. 5).

3.7. Ccm F_{N1} of OE and WT mitochondria have different mobility in BN-PAGE

Since a great number of the differentially edited sites were located on the transcripts of five mitochondrial *ccm* genes, SDS-PAGE and blue native PAGE of mitochondria were performed to examine the protein abundance of $CcmF_{N1}$ protein in supercomplexes as its antiserum is available (Fig. 6). Western blotting of SDS-PAGE showed that the content of $CcmF_{N1}$ was higher in the OE line at both t = 0 and t = 8 than in WT (Fig. 6A), which is consistent with the higher level of $CcmF_{N1}$ transcripts (2.63X and 1.99X at t = 0 and t = 8) in our previous transcriptome study (Supplementary Table 6) (Liang et al., 2015). Anti-AtPAP2 and anti-Tom40 were used as positive controls in Western blotting (Fig. 6A). $CcmF_{N1}$, $CcmF_{N2}$, and $CcmF_c$ are subunits of CcmF proteins. They form a 500 kDa complex that interacts with *c*-type apocytochromes in Arabidopsis (Rayapuram et al., 2008). A two-dimensional experiment was then performed to examine the supercomplexes containing $CcmF_{N1}$. Two $CcmF_{N1}$-containing supercomplexes (500 kDa and 1000 kDa) could be detected (Fig. 6B). In the second dimension electrophoresis (SDS-PAGE), both the 500 kDa and 1000 kDa supercomplexes could generate two (WT) or three (OE) antibody-reactive polypeptides. Interestingly, an additional band can be seen in the OE line. This differential processing of the $CcmF_{N1}$ proteins could arise from differential editing of the $CcmF_{N1}$ transcripts.

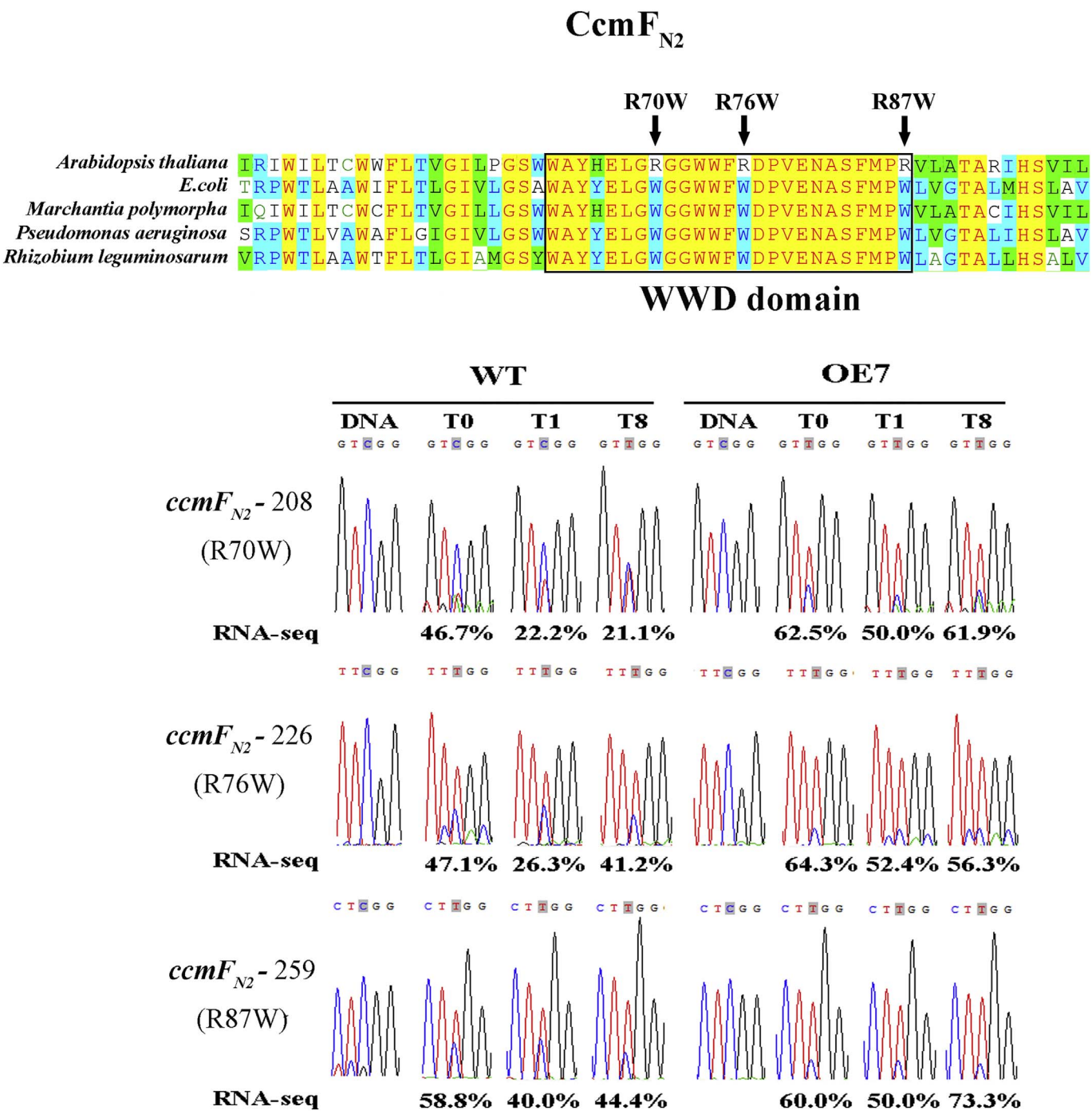

Fig. 5. WWD domain in different species. Upper part: protein alignment of $CcmF_{N2}$ (CCB203) protein of land plant *Arabidopsis thaliana* and *Marchantia polymorpha* with that of *E. coli*, *Pseudomonas aeruginosa* and *Rhizobium leguminosarum*. Black boxes show the conserved domain containing the tryptophan-rich WWD domain. Arrows indicate three edited amino acids. Lower part: RT-PCR and RNA-seq results of three sites in R70W, R76W and R87W. RNA editing ratio (edited reads/total reads) from RNA-seq data was presented under the RT-PCR sequencing signal. DNA sequencing was also performed for comparison.

RNA editing causes amino acid substitution, and the substitutions can result in changes in protein function. In the prediction of $CcmF_{N1}$ transmembrane helices, we found that three more helices can be predicted when using the edited amino acid sequences (Fig. 6C and D), which indicated that the RNA editing in $CcmF_{N1}$ transcripts could affect protein structure. Additionally, editing at the 237th codon, which converts proline (P) to phenylalanine (F), seemed important. The fifth helix may not exist (Fig. 6E) if this site is not edited, even when all other sites are 100% edited.

4. Discussion

RNA editing has been regarded as a post-transcriptional housekeeping function in plant organelles. In flowering plants, impaired RNA editing affects the physiology of plastids and mitochondria. For example, PPR protein CRR4 (chlororespiratory reduction 4) recognizes RNA editing site 1 (*ndhD*-1) of the plastid *ndhD* transcript (Kotera et al., 2005). The *ccr4* mutant has impaired RNA editing at this site and exhibited a decrease in cyclic electron flow (Kotera et al., 2005). Disruption mutants of *mef11*, a PPR protein required for the RNA editing of $ccmF_{N2}$ transcripts, exhibited an increase in drought tolerance

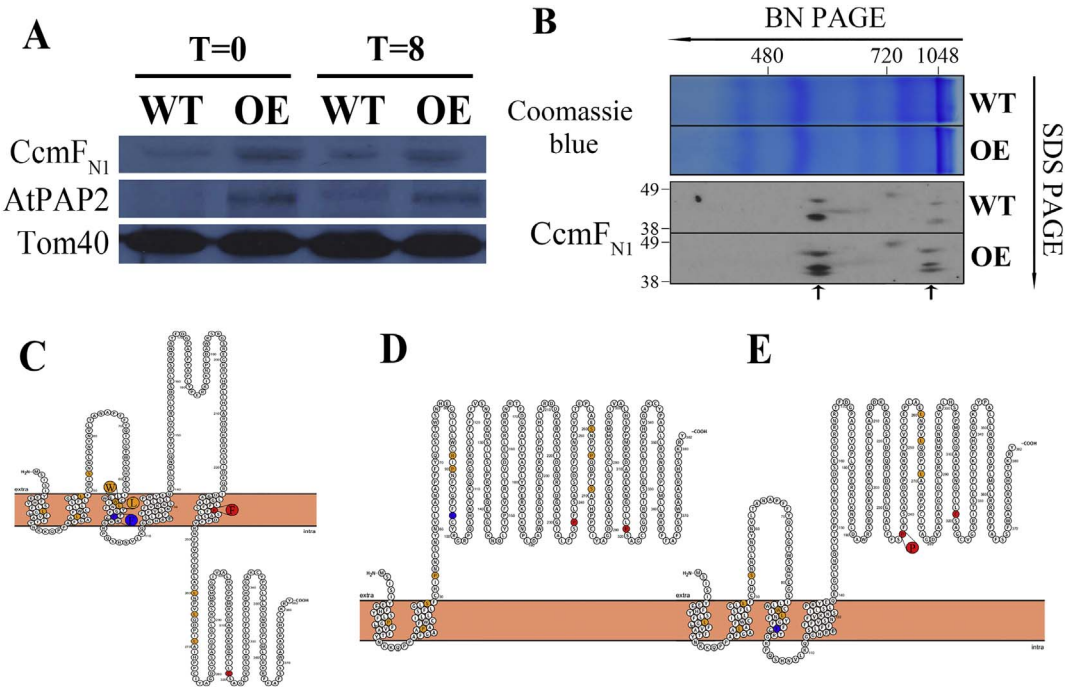

Fig. 6. Immunodetection of the $CcmF_{N1}$ protein and its supercomplexes. (A) Mitochondrial proteins were separated by SDS-PAGE. Anti-AtPAP2 and anti-Tom40 were used as positive controls. (B) Mitochondrial supercomplexes were separated by 6% (w/v) blue native PAGE in the first dimension, followed by SDS-PAGE in the second dimension. The arrows indicate the two major immunoreactive complexes. (C) Prediction of $CcmF_{N1}$ topology assuming 100% editing in all sites. Important amino acids of transmembrane helix were magnified. (D) Prediction of $CcmF_{N1}$ topology assuming 0% editing at all sites. (E) Prediction of $CcmF_{N1}$ topology assuming 100% editing at all sites except $CcmF_{N1}$-237 (0% editing). The result shows that single amino acid substitution of $CcmF_{N1}$-237 is sufficient to change the topology of $CcmF_{N1}$. The prediction was done using the TMHMM server and plotted using Protter (See Materials and methods). The sites with differential editing between OE and WT samples are marked in color. Red: editing degree in OE was at least 20% > WT. Blue: editing degree in OE was at least 20% < WT. Orange: no difference in editing degree. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and abscisic acid (ABA) sensitivity (Sechet et al., 2015). Disruption mutants of *slo2*, a PPR protein responsible for seven RNA editing sites in *mttB*, *nad1*, *nad4L* and *nad7*, were hypersensitive to ABA but insensitive to ethylene (Zhu et al., 2014). Mitochondrial and chloroplast genomes are highly conserved in land plants, but the amino acids generated from edited mRNAs are commonly better-conserved amino acids than those encoded by the genomic DNA (Gualberto et al., 1989). With this observation, it was speculated that RNA editing is a post-transcriptional engineering strategy in plants for repairing DNA mutations at the RNA level. However, RNA editing is a complex and energy-consuming process, if it does not have a biological function, it should not have evolved and been maintained in higher plant species. One possible biological function of RNA editing is to offer an additional regulatory mechanism to organelle physiology. This study on AtPAP2 OE line provides an example to support this hypothesis.

AtPAP2 proteins on the outer membranes of chloroplasts and mitochondria were shown to interact with MORF2/3/5/6 proteins and modulate the import of pMORF3 into the mitochondria (Law et al., 2015). MORFs and PPRs are integral components of the editosome (Glass et al., 2015; Hartel et al., 2013; Zehrmann et al., 2015; Zhang et al., 2015). Hence, it is possible that the interaction of AtPAP2 and MORF proteins modulate the degrees of RNA editing at some specific sites in organellar transcripts (Supplementary Tables 1 and 2). In this study, we reported changes in the degrees of RNA editing in chloroplasts and mitochondria upon illumination and AtPAP2 overexpression (Table 1 and Fig. 3). Generally, editing degrees at most sites did not exhibit significant changes in the OE lines, yet many of the differential editing events between OE and WT were clustered in *ccm* transcripts (Table 1 and Fig. 3).

RNA editing can directly affect production of functional complexes. The loss of editing at some sites can deleteriously influence protein function (Hammani et al., 2011; Hernould et al., 1993; Sung et al., 2010; Zhu et al., 2012). Taking $ccmF_N$ in maize as an example, a RNA editing defect solely at $ccmF_N$-*1553* due to the mutation of a PPR gene (*emp7*) leads to the loss of the $CcmF_N$ protein and a striking reduction in *c*-type cytochrome (Sun et al., 2015a). $ccmF_N$-*1553* in maize is homologous to $ccmF_{N2}$-391 in Arabidopsis, which, in our study, was edited 64%–84% and the editing degree of OE8 at this site was 20% higher than WT8 (Supplementary Table 2). If an editing defect at this site leads to protein dysfunction, higher editing degrees might generate more functional proteins and enhanced *c*-type cytochrome maturation. In this study, OE samples generally had higher editing degrees than WT samples (Supplementary Tables 1, 2, Supplementary Figs. 4 and 5). Western blotting also showed that $CcmF_{N1}$ protein content was higher in OE (both t = 0 and t = 8) than in WT (Fig. 6A). Two-dimensional PAGE also showed that $CcmF_{N1}$ supercomplexes were differentially processed between WT and OE lines (Fig. 6B). The changes in the degree of RNA editing of *ccm* transcripts might be a factor that affects Ccm supercomplex formation. Overexpression of AtPAP2 might influence cyt *c* biogenesis by modulating RNA editing of *ccm* transcripts through its interaction with MORF proteins. The leaf of AtPAP2 OE lines contains a higher level of ATP than the WT (Liang et al., 2015). Modulation of cyt *c* biogenesis may be required for higher ATP output from the mitochondria of the AtPAP2 OE lines.

Ccm proteins can be found in all main branches of eukaryotes. In bacteria, *ccmF* is encoded by a single gene instead of being split into two or three genes in land plants (Rayapuram et al., 2008). In Arabidopsis, *ccmF* is split into three genes named $ccmF_{N1}$, $ccmF_{N2}$, and $ccmF_C$. Large insertions between the regions of high sequence conservation make CcmF proteins in plants bigger (1027 codons) than CcmF proteins in *E. coli* (647 codons) (Rayapuram et al., 2008). The segregation of *ccmF* into three genes offers higher flexibility in transcriptional regulation (Supplementary Table 6), and possibly higher flexibility in supercomplex assembly/disassembly. In Arabidopsis, CcmB interacts with CCMA and forms a mitochondrial ABC transporter (Rayapuram et al., 2007). CcmC binds CCME and is responsible for heme delivery

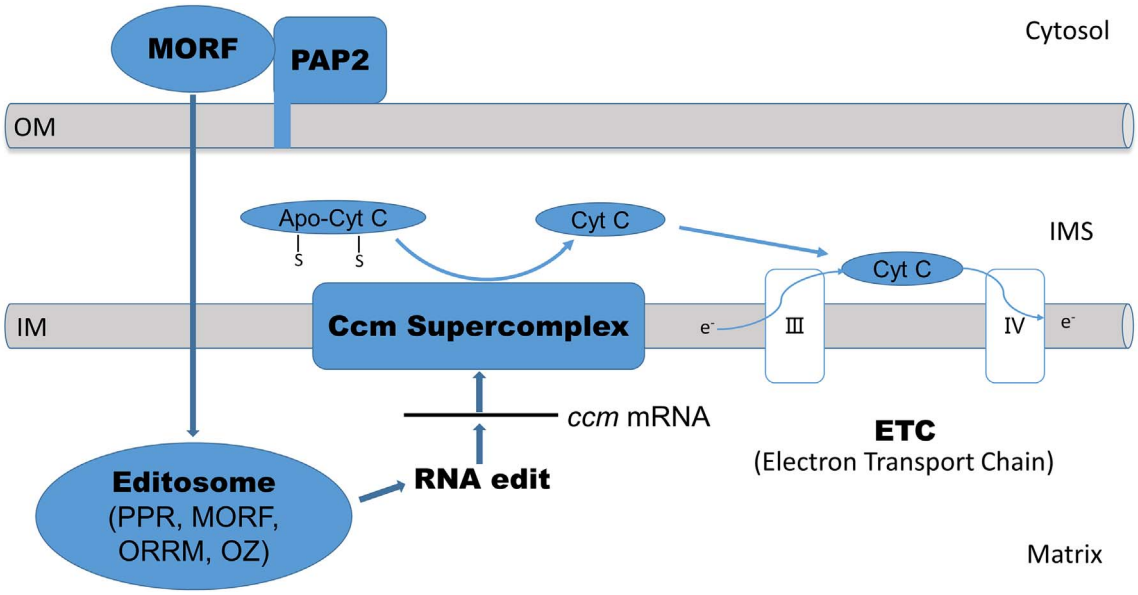

Fig. 7. A model on the action of AtPAP2 in mitochondrial RNA editing. Overexpression of AtPAP2 modulates the mitochondrial import of MORFs, which are the key components of the editosome supercomplex. This might cause differential RNA editing of *ccm* transcripts and affect cyt *c* biosynthesis. III and IV in white boxes represent complex III and complex IV in the electron transport chain (ETC). OM, outer membrane; IMS, intermembrane space; IM, inner membrane.

(Richard-Fogal and Kranz, 2010). $CcmF_{N1}/F_{N2}/F_C$ forms a complex that interacts with CCMH and executes the assembly of heme with *c*-type apocytochrome *c* into cyt *c* (Rayapuram et al., 2008). Other interactions probably also take place between Ccm proteins or between the Ccm machinery and the respiratory chain. Previous works showed that the Ccm machinery does not occur as a single stable complex, but suggests a series of dynamic protein interactions (Sanders et al., 2008; Travaglini-Allocatelli, 2013; Verissimo et al., 2013). According to our 2D BN-PAGE results, more than one complex containing $CcmF_{N1}$ protein could be detected (Fig. 6B). RNA editing of *ccmF* transcripts (Supplementary Figs. 4 and 5) may provide an additional regulatory mechanism of CcmF protein complex assembly.

Besides *ccm* transcripts, multiple sites at *nad7* transcripts were differentially edited between the fast-growing OE line and WT (Fig. 3), which might have an effect in Complex I activity or stability. NAD7 is a subunit of the mitochondrial respiratory complex I (NADH dehydrogenase). PPR proteins MTL1 and OTP87 were reported to be essential for the RNA editing of *nad7* transcripts: both *mtl1* and *otp87* displayed slow growth phenotype, which was due to certain unedited sites of *nad7* (Haili et al., 2016; Hammani et al., 2011). Another mutant with a similar phenotype, *slo3* (a PPR gene), was reported defective in the splicing of *nad7* intron 2 (Hsieh et al., 2015). A reduction in respiratory complex I activity might be the reason for the slow growth (Hsieh et al., 2015). Whether there is relationship between the OE line's fast growth and the differential editing in *nad7* transcripts will need further study.

Our previous small RNA study found that overexpression of AtPAP2 resulted in the elevation of miR173, which can induce tasiRNA (Trans-acting small interfering RNA) biogenesis from *TAS1* (Trans-acting siRNA 1 primary transcript) and *TAS2* transcripts. The 21 nt tasiRNAs can negatively regulate multiple *PPR* and *TPR* transcripts (Chen et al., 2007; Liang et al., 2014). A network of miR173-tasiRNAs-*PPR/TPR* that plays a role in regulating a certain group of *PPR* transcripts was proposed and differential expression of this network was observed in the leaves of AtPAP2 OE lines but not in their roots (Liang et al., 2014). Thus, in the OE lines, the higher energy outputs from the chloroplasts/mitochondria may induce miR173 expression from the nucleus and trigger the miR173-tasiRNAs-*PPR/TPR* network as a feedback mechanism to control RNA editing. Furthermore, the lower abundance of RNA transcripts of AtPAP2-interacting MORF1/2/5/6/8/9 proteins in the OE line during the middle-of-day (t = 8 h) may also be the result of a feedback mechanism (Supplementary Table 3).

Here we provide a hypothesis to link the observations in this study and the higher energy status of the AtPAP2 OE lines (Liang et al., 2015). Overexpression of AtPAP2 enhances energy outputs from chloroplasts and mitochondria by enhancing linear electron flow in chloroplasts and a higher capacity of mitochondria to convert reducing equivalents to ATP (Voon et al., submitted). A faster biogenesis of cytochrome *c* might therefore be required. Overexpression of AtPAP2 also enhances the import of certain MORF proteins into mitochondria (Law et al., 2015), and subsequently affect the RNA editing of some *ccm* transcripts (Fig. 7). Hence, RNA editing of cytochrome *c* maturation transcripts is responsive to the energy status of leaf cells in *Arabidopsis thaliana*.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mito.2017.04.006.

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Conflict of interest statement

Boon L. Lim is the inventor of a US patent (9,476,058). The subject of the patent application is AtPAP2. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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