MEFs und MORFs: Charakterisierung von Vertretern zweier Arten von RNA-Editing-*trans*-Faktoren

Dissertation

zur Erlangung des Doktorgrades Dr. rer. nat.

der Fakultät für Naturwissenschaften der Universität Ulm

vorgelegt von

Barbara Härtel

aus Kempten

Ulm 2013

Die Arbeiten im Rahmen der vorgelegte Dissertation wurden in der Zeit von März 2010 bis Januar 2013 am Institut für Molekulare Botanik der Universität Ulm durchgeführt und von Herrn Prof. Dr. Axel Brennicke betreut.

Amtierender Dekan der Fakultät für Naturwissenschaften:

Prof. Dr. Joachim Ankerhold

Erstgutachter:

Prof. Dr. Axel Brennicke, Institut für Molekulare Botanik, Universität Ulm

Zweitgutachter:

PD Dr. habil. Mizuki Takenaka, Institut für Molekulare Botanik, Universität Ulm

Tag der Promotion: 18.03.2013

“Science is wonderfully equipped to answer the question ‘How?’ but it gets terribly confused when you ask the question ‘Why?’ “

(Erwin Chargaff, 1977)

Inhaltsverzeichnis

I Abkürzungsverzeichnis 4

II Zusammenfassung 7

III Summary 8

IV Einleitung 9

IV.1 RNA-Editing in Landpflanzen 9

IV.2 Potentielle Vorteile des RNA-Editing in Pflanzen 10

IV.3 Evolution des RNA-Editing in Pflanzen 12

IV.4 Erkennung der RNA-Editingstellen 13

IV.5 Ansätze zur Identifizierung von RNA-Editing-*trans*-Faktoren 14

IV.6 PPR-Proteine 15

IV.7 Der „PPR-Code“ 16

IV.8 DAL- bzw. MORF-Proteine 17

IV.9 Mögliche Mechanismen des RNA-Editings in Pflanzen 19

IV.10 Aufklärung der potentiellen Funktion des Maturase-ähnlichen Gens *matR* durch RNA-Editing-Mutanten 21

IV.11 Zielsetzung dieser Arbeit 23

V Material und Methoden 25

V.1 Material 25

V.1.1 Organismen 25

V.1.2 Vektoren 25

V.1.3 Chemikalien, Verbrauchsmaterialien und kommerzielle Kits 26

V.1.4 Enzyme 26

V.1.5 Mono- und Oligonukleotide 26

V.1.6 Geräte 27

V.1.7 Datenbanken und Analyseprogramme 27

V.2 Methoden 28

V.2.1 Standardmethoden der Molekularbiologie 28
V.2.2 EMS-Mutanten 29
V.2.3 T-DNA-Insertionslinien 29
V.2.4 Protoplastentransformation 29
V.2.5 Stabile Transformation von *Arabidopsis thaliana* 30
V.2.6 Bestimmung der Editingeffizienz 30
V.2.7 Transiente Transformation von Epidermiszellen von *Nicotiana benthamiana* 30
V.2.8 Konstrukte für die bimolekulare Fluoreszenz-Komplementation (BiFC) 31
V.2.9 Untersuchung des Splicing-Status mitochondrialer Transkripte 31
VI Ergebnisse 33
VI.1 Charakterisierung mehrerer PPR-Proteine als RNA-Editing-*trans*-Faktoren 33
VI.1.1 Identifizierung durch EMS-Mutanten: MEF10, MEF12 und MEF13 33
VI.1.2 Identifizierung durch T-DNA-Insertionslinien: MEF28 und MEF30 41
VI.2 Untersuchung der RNA-Editing-Effizienz aller chloroplastidären Stellen in den Mutanten von *morf2*, *morf9* und *morf5* 44
VI.3 Analyse potentieller Interaktionen verschiedener RNA-Editing-*trans*-Faktoren durch BiFC 47
VI.3.1 Intrazelluläre Lokalisation der MORF-Proteine 47
VI.3.2 Bildung von Homo-und Heterodimeren zwischen MORF-Proteinen 49
VI.3.3 Interaktionen einiger PPR-Proteine mit MORF-Proteinen 54
VI.4 Analyse des Splicing-Status mitochondrialer Transkripte in *matR*-Editing-Mutanten 58
VII Diskussion 61
VII.1 Charakterisierung mehrerer PPR-Proteine als RNA-Editing-*trans*-Faktoren 61
VII.1.1 Identifizierung durch EMS-Mutanten: MEF10, MEF12 und MEF13 61
VII.1.2 Identifizierung durch T-DNA-Insertionslinien: MEF28 und MEF30 63

VII.2 Untersuchung der RNA-Editing-Effizienz aller chloroplastidären Stellen in den Mutanten von *morf*2, *morf*9 und *morf5* 65

VII.3 Analyse potentieller Interaktionen verschiedener RNA-Editing-*trans*-Faktoren durch BiFC 67

VII.3.1 Intrazelluläre Lokalisation der MORF-Proteine 67

VII.3.2 Bildung von Homo-und Heterodimeren zwischen MORF-Proteinen 67

VII.3.3 Interaktionen einiger PPR-Proteine mit MORF-Proteinen 69

VII.4 Analyse des Splicing-Status mitochondrialer Transkripte in *matR*-Editing-Mutanten 70

VIII Literaturverzeichnis 72

IX Publikationsliste 82

IX.1 Publikationen 82

IX.2 Reviews 82

IX.3 Kongressvorträge 82

IX.4 Poster 83

X Publikationen 85

XI Curriculum Vitae 86

XII Danksagung 87

XIII Eidesstattliche Erklärung 88

I Abkürzungsverzeichnis

A	Adenin
aox	kernkodiertes Gen, welches für die alternative Oxidase kodiert; AOX wird in die Mitochondrien importiert
ATP	Adenosintriphosphat
atp9	Gen, kodiert für F0-ATPase Proteolipid
BiFC	*bimolecular fluorescence complementation;* bimolekulare Fluoreszenzkomplementation
bp	Basenpaare
C	Cytosin
cDNA	*complementary* Desoxyribonukleinsäure
CDS	*Coding Sequence*
Col	*Arabidopsis thaliana*- Ökotyp Columbia
C24	*Arabidopsis thaliana*- Ökotyp C24
DEPC	Diethylpyrocarbonat
ddNTP	Didesoxynukleosidtriphosphat
DNA	Desoxyribonukleinsäure
dNTP	Desoxynukleosidtriphosphat
EMS	Ethylmethansulfonat ($C_3H_8O_3S$)
F1	erste Tochtergeneration
F2	zweite Tochtergeneration
G	Guanin
GFP	*green fluorescent protein;* grün fluoreszierendes Protein aus der Qualle *Aequorea victoria*

gRNA	*guide* RNA
kb	Kilobase
kDa	Kilodalton
L*er*	*Arabidopsis thaliana*- Ökotyp Landsberg *erecta*
Mb	Megabase
MCS	*Multiple Cloning Site*
MEF	mitochondrialer RNA-Editing-Faktor
MORF	*multiple sites organellar RNA editing factor*
mRNA	*messenger* Ribonukleinsäure
nad5	mitochondrial kodiertes Gen, welches für die NADH-Dehydrogenase, Untereinheit 5 kodiert
nt	Nukleotid
NTP	Nukleosidtriphosphat
orf	*Open Reading Frame*
PCR	*Polymerase Chain Reaction*
PPR-Protein	*Pentatricopeptide Repeat* Protein
RNA	Ribonukleinsäure
rRNA	ribosomale Ribonukleinsäure
RT-PCR	reverse Transkription Polymerase-Kettenreaktion
SNP	*Single Nucleotide Polymorphism*
tRNA	Transfer-Ribonukleinsäure
T	Thymin
U	Uracil

WT	Wildtyp
YFP	*yellow fluorescent protein;* gelb fluoreszierendes Protein
YFP-N	N-terminale Hälfte des YFP
YFP-C	C-terminale Hälfte des YFP

II Zusammenfassung

MEF- und MORF-Proteine sind zwei Gruppen von RNA-Editing-*trans*-Faktoren. In dieser Arbeit wurden Vertreter beider Gruppen näher charakterisiert. Zum einen konnten durch zwei verschiedene Ansätze -die Analyse von EMS-Linien und die Untersuchung von T-DNA-Insertionslinien von PPR-Genen- fünf weitere PPR-Proteine als RNA-Editing-Faktoren in Mitochondrien von *Arabidopsis thaliana* identifiziert werden. MEF10 ist am Editing einer Stelle in *nad2* beteiligt, MEF12 an einer Stelle in *nad5*, MEF13 an sechs Stellen in den Transkripten von *ccmFc*, *cox3*, *nad2*, *nad4*, *nad5* und *nad7*. Transgen in der Linie *mef13-1* eprimiertes MEF13 erhöht zudem das Editing einer weiteren Stelle in *nad5*, die im Wildtyp nur zu einem sehr geringen Teil editiert wird. MEF28 ist für das Editing einer von zwei oder beider benachbarter Stellen in *nad2* nötig und MEF30 für zwei Stellen in *nad4* und *cyt*. In Mutanten der beiden in den Chloroplasten lokalisierten MORF-Proteine, MORF2 und MORF9, wurden die chloroplastidären RNA-Editingstellen untersucht. Die beiden Proteine sind für das Editing fast aller plastidärer Stellen erforderlich. Zur weiteren Chrakterisierung beider Gruppen, wurde zuerst durch Fluoreszenzmikroskopie die intrazelluläre Lokalisation der neun MORF-Proteine untersucht. Durch BiFC-Analysen in Tabakepidermiszellen wurden Interaktionen zwischen MORF-Proteinen, sowie MORF- und PPR-Proteinen nachgewiesen. Außerdem wurden zwei RNA-Editing-Mutanten mit defektem Editing an zwei Stellen des *matR*-Transkripts verwendet, um die potentielle Funktion von matR als Maturase zu entschlüsseln.

III Summary

MEF and MORF proteins are two groups of RNA editing *trans* factors. In this thesis representatives of both groups were further charaterized. In two different approaches - via EMS mutant lines and via T-DNA insertion lines of so far uncharacterized PPR proteins- five new PPR proteins could be identified as required for RNA editing at one to six sites in mitochondria of *Arabidopsis thaliana*. MEF10 is needed for editing of a site in *nad2*, MEF12 of a site in *nad5*, MEF13 of six sites in transcripts of *ccmFc, cox3, nad2, nad4, nad5* und *nad7*. MEF13 transgenically expressed in *mef13-1* plants additionally increases editing at another site which is only weakly edited in wildtype plants. MEF28 is responsible for editing of one of two or both neighbouring sites in the *nad2* transcript. MEF30 is required for editing of two sites in *nad4* und *cyt.* In mutants of the two chloroplast targeted MORF proteins, MORF2 and MORF9, all chloroplast editing sites were examined. Both proteins are needed for editing of almost all plastidic sites. First, the intracellular localization of all nine MORF proteins was examined by fluorescence microscopy to further characterize both groups of RNA editing actors. In BiFC assays in tobacco epidermal cells interactions between MORF proteins as well as between MORF and PPR proteins were shown. Furthermore, editing mutants with defects in editing at two sites in the *matR* transcript were used to elucidate the potenial function of matR as a maturase.

IV Einleitung

Die Veränderung einer RNA-Sequenz gegenüber der Sequenz, die im Genom kodiert ist, wird als „RNA-Editing“ bezeichnet. Seit den fünfziger Jahren des 20. Jahrhunderts galt das Dogma der Molekularbiologie, dass aus der in der DNA kodierten Information über die Transkription in RNA unmittelbar das entsprechende Protein synthetisiert wird. Dieses musste bis 1986 durch die Entdeckung von Mechanismen wie alternativem Splicing, reverser Transkription und Introns nur geringfügig erweitert werden. Benne *et al*. fanden dann jedoch in den mRNAs in Mitochondrien von Trypanosomen Veränderungen der genetischen Information auf RNA-Ebene. Nicht in der DNA kodierte Uridine werden hierbei in die mRNA eingefügt oder -seltener- aus ihr entfernt (Benne *et al*., 1986).

Zu diesen Modifikationen werden inzwischen sowohl co- als auch posttranskriptionelle Prozesse gezählt, welche die Sequenz von messenger-RNAs (mRNAs), transfer-RNAs (tRNAs) und ribosomalen RNAs (rRNAs) verändern (Gott und Emeson, 2000).

Beim RNA-Editing können grundsätzlich zwei Mechanismen unterschieden werden. Durch das Modifikations-Editing werden einzelne Basen verändert, was zu Änderungen der Aminosäuren in den resultierenden Proteinen oder zur Einführung von Start- bzw. Stopcodons führen kann. Dies geschieht vor allem bei der Konversion von Cytidinen zu Uridinen in Säugetieren und Landpflanzen (Powell *et al.,* 1987; Chen *et al.,* 1987). Das Insertions-/Deletions-Editing hingegen ruft durch Entfernen oder Einfügen einzelner Nukleotide Leserasterverschiebungen hervor. Dieser Editing-Mechanismus ist nicht nur in den Mitochondrien der Trypanosomen (Benne *et al.,* 1986; Feagin *et al.,* 1988) sondern auch in verschiedenen Viren (Vidal *et al.,* 1990; Cattaneo *et al.,* 1989) und in den Mitochondrien des Schleimpilzes *Physarum polycephalum* (Mahendran *et al.,* 1991) zu finden.

IV.1 RNA-Editing in Landpflanzen

In den Mitochondrien und Plastiden von allen bisher untersuchten Blütenpflanzen treten zahlreiche posttranskriptionelle Konversionen von Cytidinen zu Uridinen auf. Dies wurde erstmals 1989 in Mitochondrien (Covello und Gray, 1989; Gualberto *et al.,* 1989; Hiesel *et al.,* 1989) und 1991 in Chloroplasten (Hoch *et al*., 1991) beschrieben.

In Pflanzen werden drei Arten von Editing gefunden. Am seltensten tritt dabei die Umwandlung der ersten Anticodon-Base in cytosolischen und in die Mitochondrien importierten tRNAs von Adenin zu Inosin auf (Pfitzinger *et al.,* 1990; Delannoy *et al.,* 2009). Des Weiteren kommt es in den mRNAs von Hornmoosen, Farnen und Lycopoden zu Aminierungen von Uridin zu Cytidin (Malek *et al*., 1996) Dieses U-zu-C-Editing tritt nur sehr selten in den Mitochondrien von Blütenpflanzen auf und kann in deren Plastiden überhaupt nicht beobachtet werden. Das häufigste Editing-Ereignis ist jedoch das in den mRNAs und einigen tRNAs aller Landpflanzen -außer den Marchantiiden- auftretende C-zu-U-Editing (Schuster *et al*., 1990; Gualberto *et al.,* 1990; Hiesel *et al.,* 1990, Freyer *et al.*, 1997). Vor allem in den Mitochondrien des Bärlappgewächses *Isoetes engelmanii* kommt es sehr häufig zu C-zu-U Konversionen in tRNAs (Grewe *et al.*, 2009).

Während in den Plastiden von Blütenpflanzen nur etwa 35 Editing-Ereignisse stattfinden (Tillich *et al.,* 2005), kommt es in den Mitochondrien zu ca. 450 Konversionen. So werden z. B. in den proteinkodierenden Regionen der Modellpflanze *Arabidopsis thaliana* 441 Cytidine zu Uridinen umgewandelt (Giegé und Brennicke, 1999).

Jedoch wurden bisher weder in ribosomalen RNAs von Mitochondrien noch von Plastiden Editing-Ereignisse entdeckt. Es bleibt unklar, warum diese RNAs vom Editing ausgeschlossen sind (Takenaka *et al*., 2008b).

IV.2 Potentielle Vorteile des RNA-Editing in Pflanzen

Zum einen stellt RNA-Editing in Pflanzenmitochondrien einen notwendigen Schritt für die Synthese funktionsfähiger Proteine dar. Diese zeigen nach dem Editing eine höhere Sequenzähnlichkeit mit ihren Homologen in anderen Spezies. Es ist jedoch unklar, warum für die Wiederherstellung konservierter Aminosäuren neue kernkodierte Genfamilien, die für die Komponenten des Editingkomplexes kodieren, geschaffen wurden, statt Mutationen in der DNA anderweitig zu reparieren (Kugita *et al.*, 2003) .

Die Einführung von Editing-Ereignissen könnte jedoch auch eine Kontrollmöglichkeit der Organellen durch das Kerngenom darstellen, anstatt nur eines Reparatur-mechanismus, um DNA-Mutationen wieder auszugleichen. Diese Kontrolle kann z. B. durch Umwandlung eines ACG-Codons in ein AUG-Startcodon oder durch Einführung von Stopcodons geschehen, wodurch verschiedene mRNA-Varianten und somit mehrere Polypeptide von einem Gen gebildet werden können. Allerdings weisen pflanzliche

Chondrome genügend Kapazität auf, um zusätzliche Gene oder Genvarianten zu kodieren. Zudem benötigt der RNA-Editing-Prozess an sich eine noch nicht genau bekannte Anzahl zusätzlicher Polypeptide (Bock, 2001; Tillich *et al*., 2006; Maier *et al*., 2008; Jobson und Qiu, 2008).

Zudem wurde ein Zusammenhang zwischen der Frequenz, mit der Editing auftritt und dem GC-Gehalt eines Genes festgestellt. Editingereignisse könnten also auf Ebene der RNA die genetische Drift hin zu einem GC-reichen Genom wieder ausgleichen (Malek *et al.*, 1996). Diese Erklärung stimmt auch mit der Hypothese von Covello und Gray (1993) überein, wonach zuerst ein Desaminierungs-/Aminierungs-Enzym mutierte, welches nun nicht mehr nur Mononukleotide, sondern auch Polynukleotide als Substrate akzeptierte. Danach wurde es möglich, dass in der mitochondrialen DNA in den Bereichen, die durch das neu entstandene Editing-Enzym korrigiert werden konnten, eine genetische Drift von T zu C stattfand.

Langfristig gesehen kann durch RNA-Editing der Transfer mitochondrialer und plastidärer Gene in das Kerngenom behindert oder sogar vermieden werden. Da keine Hinweise auf RNA-Editing im Nukleus oder Cytoplasma bestehen, könnte im Nukleus eine Gensequenz nicht sofort aktiv werden, die in den Mitochondrien erst durch Editing zu einem funktionalen Protein führt (Brennicke *et al*., 1993; Blanchard und Schmidt, 1995; Stupar *et al.,* 2001; Bergthorsson *et al.,* 2003, Castandet und Araya, 2012).

Bei der Reifung von tRNAs ist RNA-Editing für die korrekte Faltung nötig. Die tRNAs werden erst dadurch funktionsfähig. (Binder *et al.,* 1994).

Außerdem wurde beobachtet, dass durch RNA-Editing der Anteil hydrophober Aminosäure-Codons erhöht und die Funktionalität der Proteine sichergestellt wird. Editing-Ereignisse zugunsten erhöhter Hydrophilie kommen hingegen äußerst selten vor (Giegé und Brennicke, 1999; Kugita *et al.,* 2003).

Das von Mulligan (2004) postulierte Bürokratie-Modell erklärt die Entstehung des RNA-Editings damit, dass Editing etabliert wurde als die erste Mutation auftrat, die eine kompensierende Korrektur notwendig machte. Fortan musste der RNA-Editing-Prozess beibehalten werden. In diesem Modell wird RNA-Editing als Zeit- und Energie-verschwendung betrachtet, da alle für das Editing benötigten Prozesse unnötig wären, wenn die Sequenz der reifen mRNA bereits im Genom kodiert wäre.

IV.3 Evolution des RNA-Editing in Pflanzen

Die Desaminierung von Cytidinen zu Uridinen scheint gleichzeitig mit dem Übergang der Embryophyten vom Wasser ans Land aufgetreten zu sein (Covello und Gray, 1993; Steinhauser *et al.,* 1999; Jobson und Qui, 2008; Maier *et al.,* 2008), da RNA-Editing in Algen nicht vorkommt, jedoch in allen Landpflanzen außer in den Marchantiiden gefunden wird (Hiesel *et al.,* 1994; Freyer *et al.,* 1997; Groth-Malonek *et al.,* 2007). Wie bereits eingangs beschrieben, weisen die Chondrome von Blütenpflanzen etwa 300-500 Editingstellen auf (Giegé und Brennicke, 1999; Notsu, 2002; Handa 2003; Mower und Palmer, 2006), während in den plastidären Genomen nur 30-40 Editing-Stellen zu finden sind (Sasaki *et al.,* 2003; Tillich *et al.*, 2005). Im Moos *Physcomitrella patens* gibt es zwar nur elf mitochondriale RNA-Editingstellen (Rüdinger *et al.,* 2009), im Lebermoos *Haplomitrium mnioides* sind jedoch allein in einem mitochondrialen Gen (*nad7*) mehr als 20 Editing-Stellen zu finden (Groth-Malonek *et al.,* 2007). In der ältesten Linie der Tracheophyta sind hingegen sehr hohe Editingfrequenzen zu finden. So dokumentierten z. B. Grewe *et al.* (2011) mehr als 1700 Editingstellen im Brachsenkraut *Isoetes engelmanii*.

Diese Beobachtungen führen zu der Hypothese, dass RNA-Editing ein sehr dynamischer evolutionärer Prozess ist. RNA-Editing könnte als Adaptation an die Vielzahl mutagener Umwelteinflüsse beim Übergang vom Wasser ans Land betrachtet werden (Fujii und Small, 2011; Tillich *et al.,* 2006), da alle Landpflanzen RNA-Editing zeigen und angenommen wird, dass in einigen der phylogenetisch sehr alten Lebermoosen alle RNA-Editingereignisse sekundär verloren gingen (Rüdinger *et al.,* 2012). Allerdings lässt sich aus den sehr geringen Editingfrequenzen in Lebermoosen und Moosen, wie z. B. *Physcomitrella patens* (Rüdinger *et al.,* 2009) und den extrem hohen Frequenzen wie in *Isoetes engelmanii,* annehmen, dass Editing im Laufe der Evolution in den jeweiligen Linien unabhängig expandiert oder reduziert wurde (Gray, 2003).

Eine weitere Erklärungsmöglichkeit für die Evolution des RNA-Editing in Pflanzen liefert das Konzept der „*constructive neutral evolution*“, der konstruktiven neutralen Evolution (Stoltzfus, 1999; Gray *et al.,* 2010; Lukeš *et al.,* 2011; Doolittle *et al.,* 2011). Dieses Modell basiert darauf, dass die biochemischen Voraussetzungen für RNA-Editing gegeben sein müssen, bevor die Notwendigkeit des Editing entsteht. Davon ausgehend erweitert dieses Modell das von Covello und Gray (1993) vorgeschlagene Konzept ebenso wie das Bürokratie-Modell. Hierbei könnte Komplexität in Abwesenheit eines positiven

Selektionsdrucks dadurch entstehen, dass ein autonomes Organell solche Mutationen anreichert, die es von der Funktion eines anderen bestehenden zellulären Systems abhängig machen (Gray, 2012). Dies würde bedeuten, dass RNA-Editing-Systeme durch neutrale Mutationen aus Proteinen entstanden sind, die bereits eine Rolle im Zellstoffwechsel spielten, bevor sie für Editing-Ereignisse rekrutiert und notwendig wurden. Durch die Möglichkeit des RNA-Editing, nicht-funktionale Mutationen auf RNA-Ebene zu kompensieren, werden solche Einschränkungen auf Genebene gelockert, sodass sich Mutationen anhäufen können, die ansonsten beseitigt werden müssten. RNA-Editing könnte wieder verloren gehen, wenn die Zahl solcher Mutationen so klein ist, dass diese auf Genebene gleichzeitig rückgängig gemacht werden können. Wenn die Zahl dieser Mutationen jedoch so groß wird, dass die Wahrscheinlichkeit ihrer gleichzeitigen Eliminierung verschwindend gering wird, bleibt RNA-Editing bestehen und wird zu einem unverzichtbaren Teil der Genexpression (Gray, 2012).

IV.4 Erkennung der RNA-Editingstellen

Für das RNA-Editing in Pflanzen sind im Gegensatz zu den Editingprozessen in Trypanosomen und Säugern bisher weder die beteiligten Enzyme noch die Reaktionsmechanismen bekannt.

Aus dem *in vivo* Vorkommen nur teilweise editierter mRNAs lässt sich auf eine posttranskriptionelle Reaktion mit individueller Erkennung jeder Editingstelle schließen (Takenaka *et al*., 2008b).

Beleg für die posttranskriptionelle Editingreaktion lieferte auch die Etablierung von *in vitro* Systemen, in denen zugegebene RNA-Moleküle korrekt editiert werden (Hirose und Sugiura, 2001; Takenaka *et al*., 2004)

Durch solche *in vitro, in vivo*- und *in organello*-Ansätze wurden die beteiligten *cis*-Elemente in der RNA für einige Editingstellen identifiziert (Miyamoto *et al.,* 2002; Takenaka *et al.,* 2004; Neuwirt *et al.,* 2005; van der Merwe *et al.,* 2006).

In silico-Vergleiche der Sequenzumgebungen um alle bekannten Editingstellen lieferten zwar keine erkennbar übereinstimmenden Motive, zeigten aber, dass Guanosine nur sehr selten in direkter Nachbarschaft zum zu editierenden C zu finden sind (Giegé und Brennicke, 1999).

Für die erste Editingstelle im *atp9* Gen wurde z. B. mit *in vitro* RNA-Editingsystemen in Erbsen- und Blumenkohl-Mitochondrienlysaten das benötigte 5‘ *cis*-Element auf die Nukleotide -5 bis -20 5‘ der Editingstelle eingeschränkt. Im Bereich 3‘ der Editingstelle sind keine für die Erkennung essentiellen Nukleotide vorhanden (Takenaka *et al.,* 2004).

Zusätzlich können im Blumenkohl-Mitochondrienlysat bis zu 40 Nukleotide 5‘ der Editingstelle die *in vitro*- Editingeffizienz nicht nur dieser, sondern auch einer anderen, noch weiter 3‘ gelegenen Editingstelle positiv beeinflussen (Neuwirt *et al*., 2005; van der Merwe *et al*., 2006). Die entsprechende Region aus Erbsen hemmt das Editing der weiter 3‘ gelegenen Stelle im Blumenkohl-Mitochondrienlysat. Dies deutet darauf hin, dass die grundsätzlichen Eigenschaften des RNA-Editing-Apparates in Blütenpflanzen konserviert sind, aber dennoch speziesspezifische Unterschiede auftreten (Neuwirt *et al*., 2005; van der Merwe *et al*., 2006).

Da bisher keine gRNA-Moleküle gefunden werden konnten, welche wie bei Trypanosomen bei der Erkennung der einzelnen Editingstellen als *trans*-Faktoren wirken könnten, liegt die Vermutung nahe, dass in Pflanzenorganellen nur Proteine als *trans*-Faktoren aktiv sind (Takenaka *et al*., 2008b). Zur Identifizierung dieser Proteine werden die in IV.5 beschriebenen Verfahren angewandt.

IV.5 Ansätze zur Identifizierung von RNA-Editing-*trans*-Faktoren

Zur Identifizierung von RNA-Editing-*trans*-Faktoren werden drei Ansätze verfolgt.

MEF1, der erste mitochondriale *trans*-Faktor, wurde von Zehrmann *et al.* (2009) durch ökotypspezifische Unterschiede identifiziert. Sieben der 379 untersuchten RNA-Editing-Stellen in Mitochondrien von *Arabidopsis thaliana* zeigen unterschiedliche Editing-effizienzen in den Ökotypen Columbia, C24 und Landsberg *erecta* (Zehrmann *et al*., 2008). Der u.a. für das Editing der Stelle *rps4*-956 zuständige kernkodierte Faktor MEF1 wurde durch Kartierung von F2-Pflanzen einer Kreuzung von C24 x Col identifiziert (Zehrmann *et al*., 2009).

Eine weitere Möglichkeit zur Identifizierung von MEFs ist die Analyse von chemisch mit Ethylmethansulfonat (EMS) mutierten Pflanzen. Durch SNaPshot-Analysen (Takenaka und Brennicke, 2009; Takenaka, 2011; Takenaka und Brennicke, 2012) werden RNA-Editing-Mutanten identifiziert und in diesen der durch die EMS-Mutation betroffene Locus in der F2-Generation in einer Kreuzung von L*er* und den EMS-mutierten Col-

Pflanzen kartiert (Verbitskiy *et al*., 2010; Takenaka, 2010; Verbitskiy *et al*., 2011; Zehrmann *et al*., 2012; Härtel *et al*., 2013).

Ebenso wie EMS-mutierte Pflanzen können durch eine SNaPshot-Analyse auch Pflanzen mit T-DNA-Insertionen in für Kandidaten-Proteine kodierenden Genen auf Defekte im RNA-Editing hin untersucht werden. Hierbei entfällt die Kartierung, da bekannt ist, in welchem Gen sich die jeweilige T-DNA-Insertion befindet (Takenaka *et al*., 2010). Alle so bisher identifizierten Proteine, spezifisch für eine oder wenige Editingstellen, gehören zu den PPR-Proteinen.

IV.6 PPR-Proteine

Die 2000 zum ersten Mal von Small und Peeters und Aubourg *et al.* beschriebenen PPR-Proteine tragen ihren Namen wegen eines Elementes von 35 Aminosäuren, welches in einem solchen Protein bis zu 30 Mal wiederholt wird (**p**entatrico**p**eptide **r**epeat). PPR-Proteine binden direkt an RNA und sind an vielen posttranskriptionellen Prozessen wie RNA-Editing, RNA-Splicing, RNA-Spaltung und der Translation in Mitochondrien und Chloroplasten von Landpflanzen beteiligt (Andrés *et al*., 2007; Schmitz-Linneweber und Small, 2005; Beick *et al.,* 2008). Während in tierischen Genomen normalerweise weniger als zehn solcher Proteine kodiert sind, kommen in den Genomen von Landpflanzen oft mehr als 450 für PPR-Proteine kodierende Gene vor (Lurin *et al.,* 2004). Basierend auf der Art ihrer PPR-Motive können diese Proteine in zwei Hauptgruppen unterteilt werden, die P-Klasse und die PLS-Klasse. Die P-Klasse besteht nur aus einem Typ dieser Wiederholungssequenz von 35 Aminosäuren. Die PLS-Klasse besitzt zusätzlich zu den in der P-Klasse vorkommenden 35mer Wiederholungssequenzen noch lange (long, L; 36-38 Aminosäuren) und kurze (short, S; 32-34 Aminosäuren) Motive. Innerhalb der PLS-Klasse können zudem noch die E-Subklasse und die DYW-Subklasse unterschieden werden. Diese Unterteilung in Subklassen stützt sich auf die charakteristischen C-terminalen Motive der PPR-Proteine, welche in Abbildung 1 schematisch dargestellt sind (Schmitz-Linneweber und Small, 2008). Das DYW-Motiv ist nach den letzten drei Aminosäuren Aspartat, Tyrosin, Tryptophan benannt und enthält konservierte Aminosäuren, welche mit der katalytischen Domäne der Cytidin-Desaminasen übereinstimmen. Phylogenetisch ist das Auftreten von DYW-Domänen mit dem Auftreten von RNA-Editing verbunden (Salone *et al.,* 2007).

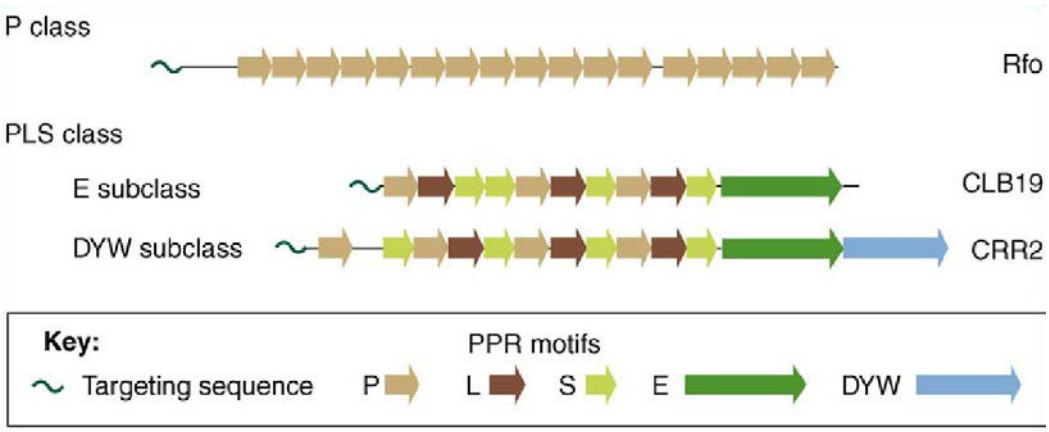


Abbildung 1: Symbolische Darstellung der Unterklassen der PPR-Proteine. Die P-Klasse besteht nur aus einem Typ einer Wiederholungssequenz von 35 Aminosäuren. Die PLS-Klasse besitzt zusätzlich zu den in der P-Klasse vorkommenden 35mer Wiederholungssequenzen noch lange (long, L mit 36-38 Aminosäuren) und kurze (short, S mit 32-34 Aminosäuren) Motive. Innerhalb der PLS-Klasse können aufgrund der charakteristischen C-terminalen Motive zudem noch die E-Subklasse und die DYW-Subklasse unterschieden werden (Schmitz-Linneweber und Small, 2008).

Mitglieder der PLS-Klasse wurden als RNA-Editing *trans*-Faktoren in Chloroplasten (CRR4: Kotera *et al.,* 2005) und Mitochondrien (MEF1: Zehrmann *et al.,* 2009) identifiziert. In Mitochondrien wurden diese Faktoren **M**itochondriale RNA-**E**diting-**F**aktoren (MEF) genannt.

IV.7 Der „PPR-Code“

Kobayashi *et al.* (2012) haben einen Mechanismus für die Interaktion des PPR-Proteins HCF152 von *Arabidopsis thaliana* mit RNA postuliert, in welchem die Aminosäuren 1, 4, 8, 12 des einen PPR-Motivs und die Aminosäure, die an der Stelle -2 vor dem folgenden PPR-Motiv steht, an der Oberfläche des PPR-Proteins exponiert sind und die RNA-Bindungsfläche beeinflussen. In diesem durch SELEX-Analysen erstellten Modell bindet HCF152 antiparallel, d.h. mit dem C-Terminus an das 5‘-Ende der RNA, an Adenosin-reiche RNA-Sequenzen (Kobayashi *et al.,* 2012).

Barkan *et al*. (2012) untersuchten ebenfalls, wie PPR-Proteine spezifisch an RNA-Sequenzen binden. Sie postulierten hierfür einen kombinatorischen Code aus zwei Aminosäuren. Hierdurch lässt sich die Wahrscheinlichkeit der Bindung eines PPR-Motivs an eine Base als entweder höher für Adenosin, Guanosin, oder Uracil gegenüber Cytosin, für Cytosin bevorzugt gegenüber Uracil, oder für Uracil und Cytosin gleich wahrscheinlich einstufen (Barkan *et al*., 2012). Da jedoch die PPR-Proteine für das RNA-

Editing eine C-terminale Domäne besitzen, die für die Editingaktivität nötig ist, liegt in diesem Modell die RNA parallel zum Protein. Zudem werden hier die sechste Aminosäure des einen PPR-Motivs und die erste Aminosäure des folgenden Motivs als signifikant mit der Sequenz der RNA korreliert erachtet. Barkan *et al.* erklären diese Unterschiede damit, dass die SELEX-Analysen mit HCF152 (Kobayashi *et al*., 2012) mit einer nicht spezifischen Bindestelle durchgeführt wurden.

IV.8 DAL- bzw. MORF-Proteine

1996 wurde von Chatterjee *et al*. ein nukleäres Gen (*DAG, **d**ifferentiation **a**nd **g**reening*) identifiziert, welches in *Antirrhinum majus* für die Differenzierung von Chloroplasten und die Entwicklung von Palisadengewebe zuständig ist. Eine Mutation im *DAG*-Locus führt zu weißen, und z.T. panaschierten Blättern. Es wurden zudem Hinweise gefunden, dass DAG zu einer Proteinfamilie in Pflanzen mit bis dahin unbekannter Funktion gehört (Chatterjee *et al.,* 1996). Sieben Jahre später wurde in *Arabidopsis thaliana* ebenfalls ein nukleäres Gen identifiziert (*DAL, **DA**G-**l**ike*), welches essentiell für die Plastidenreifung und Chloroplastendifferenzierung ist (Bisanz *et al.,* 2003).

Durch die Analyse einer EMS-mutierten *Arabidopsis thaliana*-Population mittels SNaPshot (Takenaka und Brennicke, 2009; Takenaka, 2011; Takenaka und Brennicke, 2012) wurde eine Mutante identifiziert, welche im Gegensatz zu Mutanten mit defekten PPR-Proteinen mit ein bis sechs betroffenen Stellen, an mehr als 40 mitochondrialen Stellen reduzierte Editingeffizienzen aufweist (Takenaka *et al*., 2012). Durch Kartierung der F2-Generation einer Kreuzung von WT-L*er* mit mutierten Col-Pflanzen konnte der in Frage kommende Locus auf einen Bereich eingeschränkt werden, in dem sich keine für PPR-Proteine kodierenden Gene befinden. Dieses nun *MORF1* (***m**ultiple **o**rganellar **R**NA editing **f**actor*) genannte Gen gehört zu einer Familie von neun Genen, von denen, wie in Abbildung 2 dargestellt, zwei nur in die Chloroplasten importiert werden (MORF2 und MORF9), eines sowohl in die Chloroplasten als auch in die Mitochondrien transportiert wird (MORF8: Takenaka *et al*., 2012, bzw. RIP1: Bentolila *et al*., 2012) und sechs nur in die Mitochondrien gelangen (MORF1, MORF3, MORF4, MORF5, MORF6, MORF7).

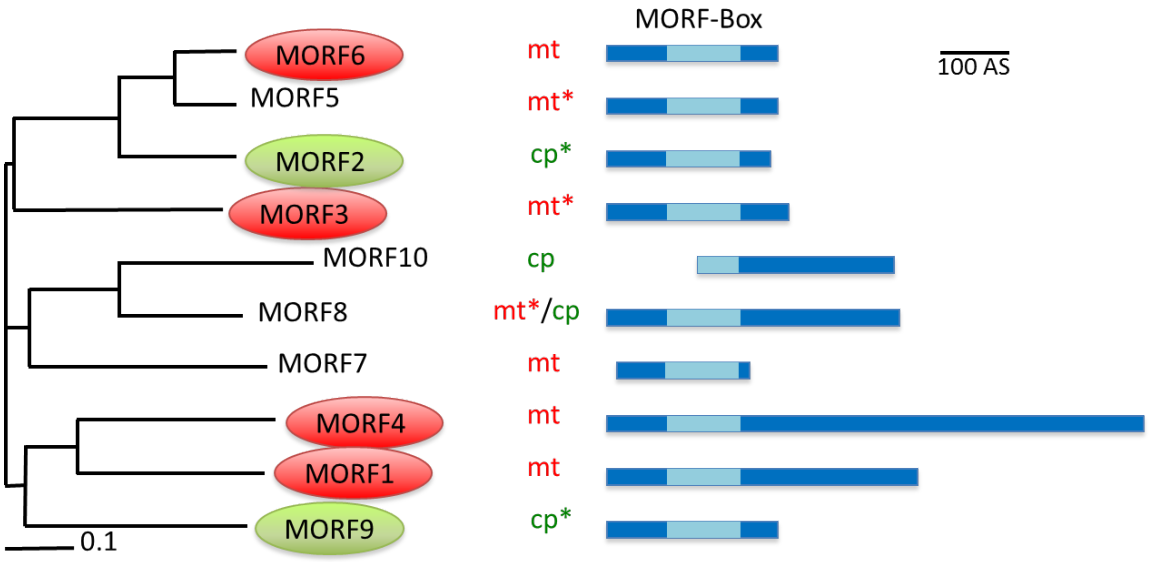


Abbildung 2: Die MORF-Proteinfamilie besteht aus neun Genen (MORF1-9) und einem Pseudogen (MORF10), dem die N-terminale Hälfte der konservierten MORF-Box fehlt. Die Anordnung nach Ähnlichkeit im linken Teil zeigt die Verwandtschaftsverhältnisse der MORF-Proteine untereinander. Vorhersagen über die Lokalisation sind mit „mt" für Mitochondrien oder „cp" für Chloroplasten gekennzeichnet. Experimentell durch GFP-Fusionsproteinlokalisation oder MS-Analysen ermittelte Lokalisationen tragen ein Sternchen. Bisher funktional untersuchte Proteine sind eingerahmt. Die MORF-Box ist ein ungefähr 100 Aminosäuren (100 AS) großer Bereich, der in allen MORF-Proteinfamilienmitgliedern konserviert und hier hell dargestellt ist (modifiziert nach Takenaka *et al.*, 2012). Näheres ist dem Text zu entnehmen.

Während in den Mutanten von MORF1 und MORF 3 jeweils mehr als 40 unterschiedliche mitochondriale Editingstellen betroffen sind, stören Mutationen in MORF4 und MORF6 je nur eine mitochondriale Stelle. Jedoch sind sich, wie dem Stammbaum in Abbildung 2 zu entnehmen, MORF4 und MORF1 sowie MORF6 und MORF5 jeweils sehr ähnlich, sodass diese Proteine möglicherweise einander ersetzen können (Takenaka *et al.*, 2012).

Die Mutanten der beiden chloroplastidären Proteine MORF2 und MORF9 zeigen beide an fast allen Stellen in Chloroplasten verringertes Editing. MORF2 ist identisch mit dem von Bisanz *et al.* (2003) beschriebenen DAL-Protein.

MORF-Proteine kommen in Blütenpflanzen vor, aber nicht im Moos *Physcomitrella patens*. Jedoch besitzt dieses Moos nur PPR-Proteine mit DYW-Domänen (Rüdinger *et al.*, 2011; Ohtani *et al.,* 2010). Daraus kann gefolgert werden, dass MORF-Proteine also in Blütenpflanzen den Verlust der DYW-Domäne mancher PPR-Proteine ausgleichen können (Takenaka *et al.*, 2012).

IV.9 Mögliche Mechanismen des RNA-Editings in Pflanzen

Grundsätzlich ließe sich die Umwandlung von Cytosin in Uracil durch fünf biochemische Reaktionen erklären.

Diese sind ein Basenaustauch durch Exzision und Insertion, der Einbau von Uracil ohne Substrat während der Transkription, ein posttranskriptioneller Basenaustausch durch Transglykosilierung, eine kovalente Modifizierung des Cytosins oder eine Trans- bzw. Desaminierung.

Aktive *in vitro*-Systeme mit zugegebenen RNA-Molekülen schließen die ersten vier Reaktionen als Mechanismen der RNA-Editingreaktion aus. Ein Basenaustausch durch Exzision und Insertion wurde widerlegt, da Rajasekhar und Mulligan (1993) nachweisen konnten, dass das Zucker-Phosphat-Rückgrat intakt bleibt. Da in *in vitro*-RNA-Editing-Systemen die Umwandlung von C zu U posttranskriptionell stattfindet, ist auch der Einbau von Uracil ohne Substrat als unwahrscheinlich anzusehen (Sasaki *et al.*, 2006; Takenaka und Brennicke, 2003). Bei einer Transglykosylierung bliebe zwar das Zucker-Phosphat-Rückgrat intakt, jedoch konnte von Yu und Schuster (1995) gezeigt werden, dass kein Cytosin freigesetzt wird und somit kein posttranskriptioneller Basenaustausch stattfinden kann.

Die nach der Editingreaktion vorliegende Base ist kein modifiziertes Cytosin mit den Paarungseigenschaften von Uracil, sondern tatsächlich Uracil (Yu und Schuster, 1995). Somit kann auch einer der beiden von Bock (2001) vorgeschlagenen Modifikations-mechanismen ausgeschlossen werden, bei welchen die Cytidin-Base kovalent so modifiziert wird, dass eine Base mit den Paarungseigenschaften von Uracil gebildet wird.

Als mögliche Reaktionsmechanismen bleiben also die Desaminierung (Blanc *et al.*, 1995) oder die Transaminierung von Cytosin zu Uracil, welche in Abbildung 3 dargestellt sind.

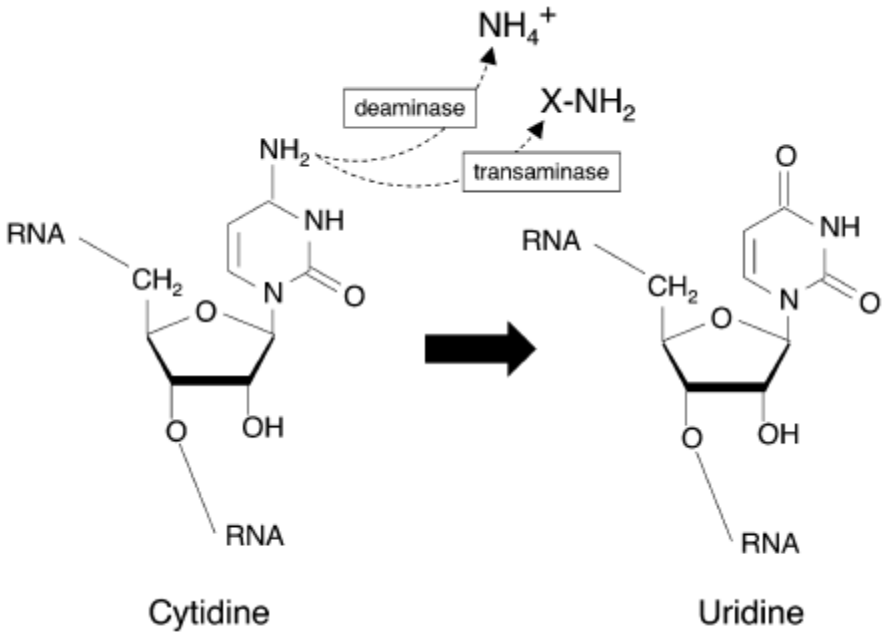


Abbildung 3: Möglicher Reaktionsmechanismus des RNA-Editings in Mitochondrien und Plastiden von Pflanzen durch eine Cytidin-Desaminase oder Transaminase (nach Takenaka *et al.,* 2008a).

Bei der Aminosäuresynthese benötigen Transaminierungsreaktionen Oxaloacetat oder α-Ketoglutarat als Aminogruppenakzeptoren. Jedoch werden die Lysate der Organellen dialysiert, bevor sie bei *in vitro*-Systemen zur Untersuchung des Editings verwendet werden (Chloroplasten: Hirose und Suigura, 2001; Mitochondrien: Takenaka und Brennicke, 2003; Neuwirt *et al*., 2005). Hierdurch sollten alle Moleküle kleiner als 10 kDa entfernt werden und solche bekannte Aminogruppenakzeptoren wären nicht mehr vorhanden (Takenaka *et al*., 2008a), was eine Transaminierungsreaktion unwahrscheinlich macht.

Zwei Argumente sprechen aber gegen eine modifizierte Cytidin-Desaminase als für das Editing verantwortliches Enzym. Einerseits hat die Zugabe von Zink-Chelatbildnern, welche klassische Cytidin-Desaminasen mit einem Zinkion im Reaktionszentrum inaktivieren, auf die *in vitro*-Editingreaktion keinen Einfluss (Takenaka und Brennicke, 2003). Jedoch könnte das RNA-Editing-Enzym auch einen Schutz für das Zinkion in seinem Reaktionszentrum entwickelt haben. Andererseits kann das bei Farnen und Moosen häufig beobachtete reverse U-zu-C-Editing energetisch nicht durch eine Cytidin-Desaminase katalysiert werden (Takenaka *et al.*, 2008a). Das Vorliegen zweier verschiedener Enzyme für die U-zu-C-Reaktion und die C-zu-U-Reaktion ist eher unwahrscheinlich. Eine Transaminase könnte beide Reaktionen katalysieren.

Eine Beteiligung der sieben klassischen Cytidin-Desaminasen, die im Genom von *Arabidopsis thaliana* kodiert sind, kann ausgeschlossen werden, da diese nicht in die Organellen importiert werden, keine Affinität für RNA und keine Desaminase-Aktivität bei RNA-Substraten aufweisen (Faivre-Nitschke *et al*., 1999; Delannoy *et al.,* 2009).

Wie bereits beschrieben, ist jedoch in den DYW-Domänen vieler am RNA-Editing beteiligter PPR-Proteine ein Motiv konserviert, das dem klassischen Cytidin/Deoxycytidylat Desaminase Motiv ähnelt: C/HXE(X)nPCXXC. Da das Auftreten der DYW-Domäne zudem mit dem Auftreten von RNA-Editing in Landpflanzen korreliert ist, wurde postuliert, dass die DYW-Domäne das RNA-Editing katalysieren könnte (Salone *et al*., 2007; Rüdinger *et al.*, 2008).

Zwar gehören nicht alle PPR-Proteine, die als RNA-Editing-Faktoren identifiziert wurden zur DYW-Klasse (z. B. Zehrmann *et al.*, 2009; Okuda *et al.,* 2009), sondern sehr viele zur reinen E-Klasse (z. B. Takenaka, 2010; Takenaka *et al*., 2010; Chateigner-Boutin *et al.*, 2008), jedoch ist nach Entdeckung der MORF-Proteine (Takenaka *et al*., 2012) als zweite Gruppe von RNA-Editing *trans*-Faktoren neben den PPR-Proteinen folgendes Modell vorstellbar:

Ein PPR-Protein erkennt das *cis*-Element der betreffenden Editing-Stelle, MORF-Proteine binden ebenfalls an die RNA und vor allem an das PPR-Protein und ermöglichen dadurch dem Enzym den Zugang zum zu editierenden C. Die enzymatische Aktivität wird hierbei entweder durch die DYW-Domäne des PPR-Proteins, durch die DYW-Domäne eines zweiten beteiligten PPR-Proteins oder ein noch zu identifizierendes Enzym geliefert (Takenaka *et al*., 2012).

IV.10 Aufklärung der potentiellen Funktion des Maturase-ähnlichen Gens *matR* durch RNA-Editing-Mutanten

Die beiden Faktoren MEF4 und MEF14 sind für das Editing der Stelle 1771 bzw. 1895, ausgehend vom A im ATG-Startcodon, des Gens *matR* verantwortlich (Verbitskiy *et al.,* 2011).

Die Funktion dieses Gens, welches im vierten Intron des mitochondrialen Gens *nad1* kodiert ist, ist jedoch noch nicht aufgeklärt. *nad1* besteht, wie in Abbildung 4 dargestellt, aus drei *trans*- und zwei *cis*-Splicing Exons (Wissinger *et al.,* 1992).

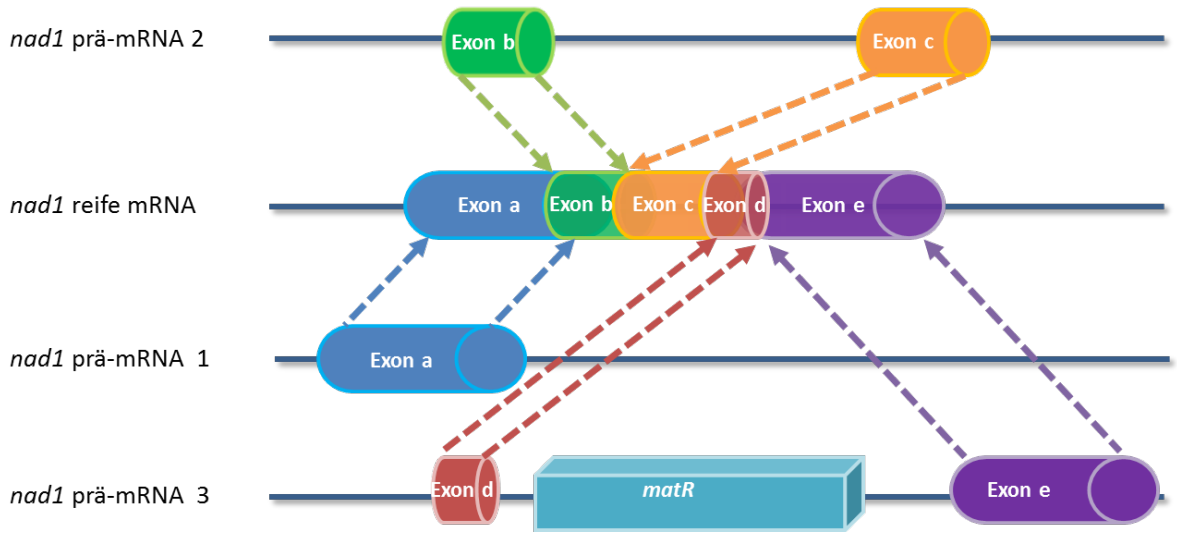


Abbildung 4: Modell der Exon-Intron-Struktur von *nad1.* Die Exons a/b und c/d werden sowohl in *Arabidopsis thaliana* als auch in Weizen durch *trans*-Splicing, die Exons d/e werden in *Arabidopsis thaliana* durch *cis*- und in Weizen mit *trans*-Splicing verbunden. In *Arabidopsis thaliana* sind zur Reifung der mRNA zwei *trans*- und zwei *cis*-Splicing-Ereignisse nötig (modifiziert nach Wissinger *et al.*, 1992).

MatR weist zwei Domänen auf, die charakteristisch für Maturasen sind. In Bakterien und in den Mitochondrien von Hefe sind Maturasen Proteine, die in Gruppe-II-Introns kodiert sind und das Splicing dieser Introns vermitteln (Bonen, 2011). In den Mitochondrien von Pflanzen kommen hingegen 20 Gruppe-II-Introns vor, jedoch nur ein Maturase-ähnliches Protein, matR. In Chloroplasten wurde matK als Maturase identifiziert und im Nucleus sind vier Maturase-ähnliche Gene kodiert: AtnMat1, AtnMat2, AtnMat3.1 und AtnMat4 (Bonen, 2011; Keren *et al.,* 2009).

Maturasen besitzen zwei bzw. drei Domänen (Abbildung 5): die Reverse Transkriptase-Domäne (RT), welche nicht in jeder Maturase vorhanden sein muss. Diese bindet die prä-mRNA zusammen mit der zweiten Domäne X, welche 3‘ der RT-Domäne liegt und nur gering konserviert, aber in allen bekannten Gruppe-II-Intron-Maturasen vorhanden und vermutlich für das Splicing zuständig ist. Die dritte Domäne ist die C-terminale DNA-Endonuklease (Bonen, 2011).

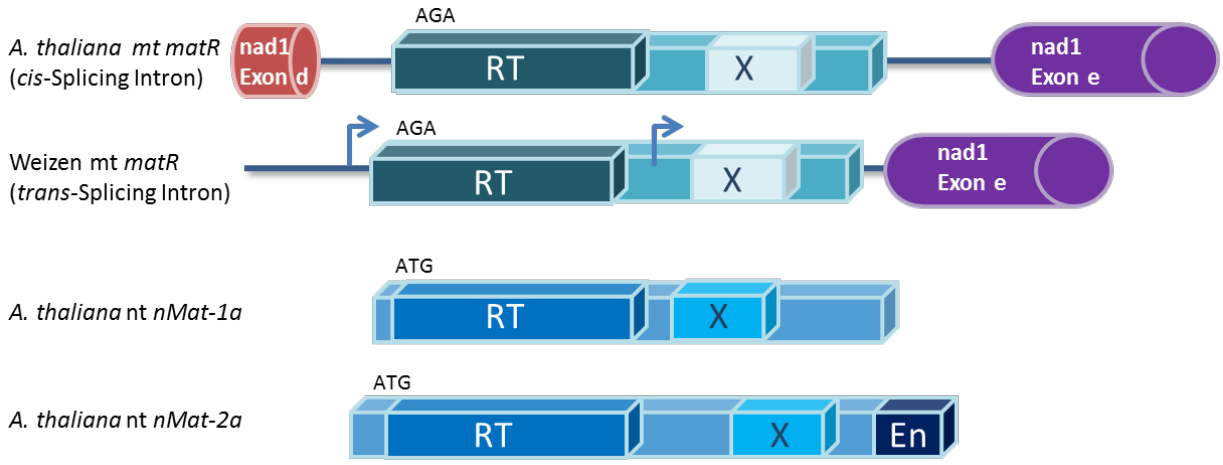


Abbildung 5: Schematische Darstellung verschiedener nukleärer (nt) und mitochondrialer (mt) Maturase-ähnlicher Gene in *Arabidopsis thaliana* und Weizen. „RT" symbolisiert die Reverse Transkriptase-Domäne (dunkel), „X" die Domäne X (hell) und „En" die Endonuklease-Domäne (dunkler). Die Pfeile in matR aus Weizen symbolisieren die identifizierten Promotoren (modifiziert nach Bonen, 2011).

MatR besitzt zwar Domänen homolog zu Reverser Transkriptase (RT) und RNA-Maturase (Domäne X), welche beim klassischen Splicing-Mechanismus von Gruppe-II-Introns die prä-mRNA binden, es fehlen jedoch andere Domänen, die für die Mobilität des Introns bei identifizierten Gruppe-II-Retroelementen zuständig sind wie die Endonuklease-Domäne. Zudem ist der Expressionsmechanismus dieses Intron-kodierten Leserahmens rätselhaft, da ein AUG-Startcodon fehlt (Abbildung 5) (Bonen, 2011). In Weizen wurden aber 5' und innerhalb dieses matR-Leserasters Promotoren identifiziert (Farré und Araya, 1999). Möglicherweise wird die Domäne X als unabhängiges Protein *in trans* exprimiert (Bonen, 2011).

IV.11 Zielsetzung dieser Arbeit

Auch mehr als 20 Jahre nach Entdeckung des RNA-Editings in Pflanzen ist der genaue zugrundeliegende Mechanismus und das verantwortliche Enzym noch nicht aufgedeckt. Im weitesten Sinne war daher das Ziel dieser Arbeit, RNA-Editing in Pflanzen weiter zu charakterisieren.

Hierzu wurden zum einen weitere *trans*-Faktoren aus der Gruppe der PPR-Proteine identifiziert, was z.T. auch den angehängten Publikationen zu entnehmen ist und ihre Interaktion mit den in dieser Arbeitsgruppe neu entdeckten *trans*-Faktoren, den MORF-Proteinen mit der Einführung der Methode der bimolekularen Fluoreszenz-

komplementation untersucht. Zur weiteren Charakterisierung der MORF-Proteine wurden die chloroplastidären Editingstellen in den Mutanten von MORF2, MORF9 und MORF5 untersucht sowie für weitere Untersuchungen verschiedene *morf*-Mutanten miteinander gekreuzt. Durch Fluoreszenzmikroskopie wurde die subzelluläre Lokalisation überexprimierter MORFs in Epidermiszellen von Tabak gezeigt und die Interaktion von MORF-Proteinen nicht nur mit *trans*-Faktoren der PPR-Familie sondern auch mit MORFs in Form von Homo- und Heterodimeren nachgewiesen.

Zudem wurde versucht, die Funktion des Maturase-ähnlichen Gens *matR* mittels einer RNA-Editing-Doppelmutante aufzuklären.

V Material und Methoden

V.1 Material

V.1.1 Organismen

V.1.1.1 Pflanzenmaterial

Es wurden Pflanzen verschiedener *Arabidopsis thaliana* Ökotypen wie Columbia (Col-0), Landsberg *erecta* (L*er*), C24, Bay-0, Bur-0, Fei-0, Got-7, Lov-5, Shakdara (Sha) und Tamm-2 verwendet. Die Samen der T-DNA-Insertionslinien (SAIL, SALK, GABI-Kat, FLAG) wurden über NASC (European Arabidopsis Stock Centre) bezogen. Die Pflanzen wurden auf Erde mit Phosphat-Nitrat-Kalium-Dünger bei 21 °C und 65 % Luftfeuchtigkeit unter Langtagbedingungen (16 h Licht, 8 h Dunkelheit) in Klimaschränken angezogen. Für DNA bzw. RNA-Präparationen wurden nach drei bis vier Wochen von den Pflanzen grüne Rosettenblätter von ca. 1,5 cm Länge geerntet und direkt verarbeitet oder bei -80 °C eingefroren.

Für bimolekulare Fluoreszenzkomplementations- (BiFC-) Studien wurde *Nicotiana benthamiana* unter den gleichen Bedingungen wie *Arabidopsis thaliana* angezogen.

V.1.1.2 Bakterienstämme

Die Transformation von *Escherichia coli* wurde mit hitzekompetenten Zellen des Stammes K12, welcher *rec*A und *end*A Mutationen trägt, durchgeführt. Diese stammen ursprünglich von der Firma Clontech Laboratories Inc. (Mountain View, USA).

Für die Transformation der vier unter V.1.2 beschriebenen Vektoren in *Agrobakterium tumefaciens* wurde der Stamm GV2260 (Deblaere *et al.*, 1985) verwendet, welcher das nicht-onkogene Ti-Plasmid pGV2260 mit Rifampicin- und Carbenicillin-Resistenz trägt.

V.1.2 Vektoren

Zur Transformation von Arabidopsis-Protoplasten und -Pflanzen wurde der Vektor pAD41 verwendet. Dieser basiert auf dem Vektor pMDC123 (Curtis und Grossniklaus, 2003) mit der GFP-Kassette des Vektors psMGFP4 (Forner und Binder, 2007) und der *Multiple Cloning Site* (MCS) aus dem Vektor pET41 (Merck Millipore Novagen®).

Die BiFC-Analysen wurden mit den Vektoren pYFP, pYFP-N und pYFP-C durchgeführt. Diese basieren auf dem oben beschriebenen Vektor pAD41, tragen jedoch statt der GFP-

Kasette die vollständige YFP-Kasette (pYFP) bzw. nur die N-terminale Hälfte der YFP-Kasette (Aminosäuren 1 bis 166) (pYFP-N) oder nur die C-terminale Hälfte (pYFP-C).

V.1.3 Chemikalien, Verbrauchsmaterialien und kommerzielle Kits

Die eingesetzten Chemikalien und Verbrauchsmaterialien stammten von den Firmen Bayer CropScience Deutschland GmbH (Monheim, Deutschland), Becton Dickinson GmbH (Heidelberg, Deutschland), Bio-Budget Technologies GmbH (Krefeld, Deutschland), Carl Roth GmbH & Co. KG (Karlsruhe, Deutschland), Duchefa Biochemie B.V. (Haarlem, Niederlande), Eppendorf AG (Hamburg, Deutschland), Merck KGaA (Darmstadt, Deutschland), MP Biomedicals GmbH (Eschwege, Deutschland), PEQLAB Biotechnologie GmbH (Erlangen, Deutschland), Sarstedt AG & Co. (Nümbrecht, Deutschland), SERVA Feinbiochemica GmbH & Co. (Heidelberg, Deutschland), Sigma-Aldrich Chemie GmbH (Taufkirchen, Deutschland) und VWR International GmbH (Darmstadt, Deutschland). Die Pufferlösungen wurden in bidestilliertem, bei Arbeiten mit RNA mit Diethylpyrocarbonat (DEPC) behandeltem Wasser angesetzt.

Kommerzielle Kits zur Plasmid-Aufreinigung, Gelextraktion von DNA, RNA-Präparation aus Blättern oder Klonierung mit dem InFusion-System wurden von GE Healthcare (München, Deutschland), Roche Diagnostics GmbH (Mannheim, Deutschland), Macherey-Nagel GmbH & Co. KG (Düren, Deutschland), und Clontech Laboratories Inc. (Mountain View, USA) bezogen.

V.1.4 Enzyme

Die verwendeten Enzyme stammen von den Firmen Fermentas GmbH (St. Leon-Rot, Deutschland), Genaxxon Bioscience GmbH (Ulm, Deutschland), Clonetech Laboratories Inc. (Mountain View, USA) und Promega GmbH (Mannheim, Deutschland).

V.1.5 Mono- und Oligonukleotide

Desoxy-Mononukleotide (dNTPs) und Größenstandards wurden von der Firma Genaxxon (Ulm, Deutschland) bezogen.

Die Oligonukleotide stammen von Sigma-Genosys (Steinheim, Deutschland) und biomers.net GmbH (Ulm, Deutschland). Sequenzdetails stehen auf Anfrage zur Verfügung.

V.1.6 Geräte

Es wurden die zur Standardausstattung eines molekularbiologischen Labors gehörenden Geräte, wie Thermocycler, Waagen, Bakterienschüttler, Inkubatoren, Thermoblöcke, Zentrifugen, Klimaschränke, Elektrophoresekammern mit zugehörigen Spannungsgeräten, Geldokumentationsanlage, Speed Vac etc. diverser Hersteller benutzt.

Die fluoreszenzmikroskopischen Aufnahmen wurden an einem Leica TCS SP5, einem spektralen konfokalen Laser-Mikroskop, mit einem 63x Glycerin-Immersionsobjektiv durchgeführt (Leica Micorsystems, Heidelberg). YFP wurde mit einem Argon-Laser der Wellenlänge 488 nm bei 514 nm angeregt und die Emissionsspektren wurden zwischen 525 und 600 nm aufgenommen. RFP wurde mit 561 nm angeregt und die Emission zwischen 576 und 629 nm detektiert. Die Chlorophyll-Autofluoreszenz wurde ebenfalls mit 514 nm angeregt und das Emissionsspektrum im dunkelroten Bereich zwischen 650 und 704 nm aufgenommen. Die drei Kanäle wurden aufgrund der Überlagerung der Wellenlängen von YFP und RFP sequenziell gescannt.

V.1.7 Datenbanken und Analyseprogramme

Die Lage von Polymorphismen, die Gen- und Proteinsequenzen, die Editingstellen in Chloroplasten und Mitochondrien verschiedener Spezies sowie orthologe und homologe Proteine wurden mit Hilfe folgender Datenbanken und online-Ressourcen bestimmt:

www.arabidopsis.org (Swarbreck, *et al.*, 2007)

http://urgv.evry.inra.fr/projects/FLAGdb++/ (Dèrozier *et. al.*, 2011)

polymorph-clark20.weigelworld.org (Clark *et al.*, 2007; Zeller *et al.*, 2008)

http://biologia.unical.it/py_script/REDIdb/ (Picardi *et al.*, 2007)

Stammbäume wurden mit dem Programm *MEGA,* Version 5 von Tamura *et al.* (2011) erstellt.

Die Auswertung von Daten aus Sequenzierungen erfolgte mit dem Programm DNADynamo von Blue Tractor Software Ltd. (North Wales, Großbritannien).

V.2 Methoden

V.2.1 Standardmethoden der Molekularbiologie

Molekularbiologische Standardmethoden, wie Gelelektrophoresen, Nukleinsäure-extraktionen, Amplifizierungen, cDNA-Synthesen, Klonierungen etc. wurden entsprechend der Standardprotokolle (Sambrook und Russel, 2001), bzw. bei Verwendung kommerzieller Kits, nach Hersteller-Angaben durchgeführt.

V.2.1.1 Nukleinsäurepräparationen

DNA wurde aus entsprechendem Pflanzenmaterial mit der CTAB-Methode (Sambrook und Russell, 2001) isoliert.

Für Anwendungen, bei denen kein hoher Reinheitsgrad der DNA benötigt wurde wie z. B. Kartierungen oder Genotypisierung von T-DNA-Insertionslinien, wurden Blätter zuerst zermörsert, mit einem Extraktionspuffer aus 200 mM Tris-HCl (pH 7,5), 250 mM NaCl, 25 mM EDTA und 0,5 % SDS versetzt, gut durchmischt, nach Zugabe von 2 Volumen 100%igem Ethanol zentrifugiert, das Pellet getrocknet und die DNA in TE-Puffer (pH 7,8) aufgenommen.

Die Isolierung der Gesamt-RNA für die Analyse des Editing-Phänotyps bzw. des Splicing-Status erfolgte mit dem illustra RNAspin Mini Kit der Firma GE Healthcare (München, Deutschland). cDNA wurde mit spezifischen Primern mit der M-MLV Reversen Transkriptase der Firma Promega (Mannheim, Deutschland) synthetisiert und mit der GoTaq® DNA-Polymerase der Firma Promega (Mannheim, Deutschland) amplifiziert. Zur Kontrolle auf Kontaminationen der cDNA mit genomischer DNA wurde ein Ansatz mit bidestilliertem Wasser statt Reverser Transkriptase durchgeführt. Konnte nach anschließender Amplifikation kein Produkt nachgewiesen werden, war die RNA-Präparation frei von genomischer DNA.

Für die Durchführung von Northern-Blots wurde zur RNA-Präparation das Spectrum™ Plant Total RNA Kit von Sigma-Aldrich Chemie GmbH (Steinheim, Deutschland) verwendet, um eine höhere Ausbeute zu erzielen.

Zur Analyse von Single Nucleotide Polymorphisms (SNPs) und zur Genotypisierung von T-DNA-Insertionslinien wurden die entsprechenden Regionen im Genom mit der Taq S DNA-Polymerase der Firma Genaxxon (Ulm, Deutschland) amplifiziert.

DNA-Fragmente zur Klonierung in Expressionsvektoren wurden mit der Phusion™ High-Fidelity DNA-Polymerase der Firma Thermo Fisher Scientific (Waltham, USA) erzeugt.

Die Sequenzierungen wurden von den Firmen 4base lab GmbH (Reutlingen, Deutschland), LGC Genomics GmbH (Berlin, Deutschland), Macrogen Korea (Seoul, Korea) und Macrogen Europe (Amsterdam, Niederlande) durchgeführt.

V.2.2 EMS-Mutanten

Zur Identifizierung von *MEF10*, *MEF12* und *MEF13* wurden EMS-Mutanten des Ökotyps Col herangezogen.

Columbia-Pflanzen (Col), bei denen ein Allel mittels EMS mutiert wurde, wurden mit Wildtyp Landsberg *erecta*-Pflanzen (WT L*er*) gekreuzt. Heterozygote Pflanzen der F1-Generation, welche ein WT-L*er*-Allel sowie ein mutiertes Col-Allel trugen, wurden geselbstet und das Editing der betroffenen Stelle in Pflanzen der F2-Generation untersucht.

V.2.3 T-DNA-Insertionslinien

T-DNA-Insertionslinien wurden zum einen dafür genutzt, mit EMS-Mutanten identifizierte Editing-Faktoren zu bestätigen, zum anderen, um direkt durch Editingdefekte in homozygoten Pflanzen neue Editing-Faktoren, wie *MEF27, MEF28* und *MEF30* zu identifizieren, sowie zur Untersuchung des Editingstatus der chloroplastidären RNA-Editing–Stellen in Mutanten von MORF2, MORF5 und MORF9. Die Insertionslinien wurden über das *European Arabidopsis Stock Centre* bezogen und mit den entsprechenden Oligonukleotiden auf die Präsenz der Insertion hin untersucht. Hierzu bindet ein Oligonukleotid im Bereich der T-DNA-Insertion, ein zweites in entgegengesetzter Richtung im Gen.

V.2.4 Protoplastentransformation

Protoplasten von *Arabidopsis thaliana* und *Nicotiana benthamiana* wurden entsprechend des Protokolls von Yoo *et al.* (2007) gewonnen und transformiert. Die zu transformierenden Gene wurden hierzu unter die Kontrolle des CaMV-35S-Promotors in den Vektor pAD41 für *A. thaliana* bzw. pYFP, pYFP-N und pYFP-C für *N. benthamiana* mittels des In-Fusion™ HD Cloning Kits der Firma Clontech Laboratories Inc. (Mountain View, USA) eingebracht. Im Falle von *A. thaliana* wurde nach 24 h bzw. 48 h Inkubation

bei Raumtemperatur die Gesamt-RNA isoliert. Die durch RT-PCR mit spezifischen Primern erzeugten cDNA-Fragmente wurden anschließend sequenziert, um die Editing-effizienz zu bestimmen. Parallel wurden als Negativkontrolle Protoplasten mit dem leeren, ungeschnittenen Vektor und mit bidestilliertem Wasser transformiert und ebenfalls für 24 h bzw. 48 h bei Raumtemperatur inkubiert.

V.2.5 Stabile Transformation von *Arabidopsis thaliana*

Die *Agrobacterium*-vermittelte Transformation von T-DNA-Insertionslinien bzw. EMS-Mutanten von *A. thaliana* erfolgte nach der Methode von Clough und Bent (1998) durch *Floral Dip*. Die zu transformierenden Gene wurden dafür ebenso wie für die Protoplasten-Transformation unter die Kontrolle des CaMV-35S-Promotors in den Vektor pAD41 kloniert. Die Samen der behandelten Pflanzen wurden geerntet und nach mindestens 14-tägiger Vernalisation bei 4 °C ausgesät. Nach Bildung der ersten beiden Rosettenblätter wurde mit Basta® (Hoechst Schering AgrEvo GmbH) auf transformierte Pflanzen selektioniert. Nach dreimaliger Basta® -Behandlung wurden von resistenten Pflanzen Blätter geerntet, RNA isoliert und analog zur Analyse transformierter Protoplasten die durch RT-PCR mit spezifischen Primern erzeugten cDNA-Fragmente sequenziert, um die Editingeffizienz zu bestimmen. Im Fall von MEF28 war die T-DNA-Insertionslinie bereits gegen Basta® und Kanamycin resistent. Deshalb wurden mittels PCR transformierte Pflanzen identifiziert. Beim ersten Primerpaar bindet das revers-orientierte Oligonukleotid im T-DNA-Insertionsbereich und das vorwärts-orientierte in *MEF28.* Bei einem zweiten Primerpaar binden beide Oligonukleotide in *MEF28.* Eine PCR mit dem zweiten Primerpaar sollte aufgrund der Länge der Elongationszeit nur bei transformierten Pflanzen zu einem Produkt führen.

V.2.6 Bestimmung der Editingeffizienz

Nach Zehrmann *et al.* (2009) kann aus dem Verhältnis der Fläche unter dem C- Peak zur Fläche unter dem T-Peak in der Sequenzanalyse einer Editingstelle auf die Editingeffizienz geschlossen werden.

V.2.7 Transiente Transformation von Epidermiszellen von *Nicotiana benthamiana*

Für BiFC-Untersuchungen wurden Epidermiszellen von *N. benthamiana* nach dem Protokoll von Sparkes *et al.* (2006) mit den unter V.2.8 angeführten Konstrukten infiltriert. Agrobakterienkulturen mit den gewünschten Konstrukten wurden bei 28 °C

angezogen und nach zwei Tagen geerntet. Nach Waschschritten im Infiltrationsmedium zur Entfernung der Antibiotika wurde die optische Dichte der Kultur bei 600 nm ($OD_{600\ nm}$) gemessen.

Für jedes neue Konstrukt wurde zunächst die OD_{600nm} ermittelt, bei der die stärkste Fluoreszenz zu beobachten war. Hierfür wurden jeweils zwei Blätter einer Tabakpflanze mit einer $OD_{600\ nm}$ von je 0,01, 0,05 und 0,1 infiltriert. Die Kulturen von P19 und AOX-RFP wurden mit einer $OD_{600\ nm}$ von je 0,05 eingesetzt.

Nach zweitägiger Inkubation bei 21 °C und 65 % Luftfeuchtigkeit wurden ca. 1-2 cm^2 große Stücke aus den infiltrierten Blättern ausgeschnitten, in Wasser eingebettet und unter dem konfokalen Mikroskop Leica TCS SP5 mit den unter V.1.6 beschriebenen Einstellungen betrachtet.

V.2.8 Konstrukte für die bimolekulare Fluoreszenz-Komplementation (BiFC)

Das mitochondriale Transitpeptid der alternativen Oxidase (AOX) diente mit RFP fusioniert als Marker für die Mitochondrien. P19 ist ein Protein des *Tomato Bushy Stunt Virus*, das die Genexpressionshemmung unterdrückt.

Alle MORF-Proteine sowie verschiedene mitochondriale und chloroplastidäre RNA-Editing-Faktoren wurden zum einen als Fusionsproteine mit dem Vollängen-YFP (im Vektor pYFP) untersucht, um die Lokalisation zu bestimmen und die Transformationseffizienz zu überprüfen. Zudem wurden verschiedenste Kombinationen von MORF-Proteinen untereinander, MEF-Proteinen untereinander und MORF- und MEF-Proteinen auf ihre Interaktion hin getestet. Hierfür wurden die jeweiligen Proteine mit der N-terminalen bzw. der C-terminalen Hälfte des YFPs fusioniert (in den Vektoren pYFP-N bzw. pYFP-C). Nur wenn die beiden getesteten Proteine interagieren, treten die beiden nicht fluoreszierenden YFP-Hälften in räumliche Nähe zueinander, sodass das YFP-Protein rekonstituiert und dessen Fluoreszenz detektiert werden kann.

V.2.9 Untersuchung des Splicing-Status mitochondrialer Transkripte

Um zu analysieren, ob das matR-Protein Maturase-Aktivität besitzt und am Splicing mitochondrialer Transkripte beteiligt ist, wurden Mutanten von *MEF4* und *MEF14* gekreuzt. MEF4 ist für das Editing der Stelle *matR*-1771 und MEF14 für die Stelle *matR*-1894 (Verbitskiy *et al.*, 2011) notwendig.

Da in dieser ersten Kreuzung aus *mef4-1* und *mef14-2* keine Doppelmutanten gefunden werden konnten, wurden die Pollen von Pflanzen untersucht, welche homozygot für die T-DNA-Insertion in *MEF14* und heterozygot für die Insertion in *MEF4* waren.

Um RNA aus Pollen zu gewinnen, wurden ca. 5 geöffnete Blüten in Reaktionsgefäßen geschüttelt, sodass sich die Pollen lösten und statisch an der Wand des Reaktionsgefäßes hafteten. Anschließend wurde eine RNA-Präparation mit dem illustra RNAspin Mini Kit der Firma GE Healthcare (München, Deutschland) durchgeführt. Die Behandlung mit DNase erfolgte während der Präparation auf der Säule und anschließend nochmals mit der RQ1 DNase von Promega (Mannheim, Deutschland).

Für die cDNA-Synthese wurden die von Koprivova *et al.* (2010) entworfenen Oligonukleotide verwendet.

Die Amplifizierung der cDNA erfolgte zunächst in einer PCR mit limitierter Zyklenzahl von 10, 15, 20, 25 und 30 Zyklen.

VI Ergebnisse

VI.1 Charakterisierung mehrerer PPR-Proteine als RNA-Editing-*trans*-Faktoren

VI.1.1 Identifizierung durch EMS-Mutanten: MEF10, MEF12 und MEF13

Für EMS-Mutationslinien der Gene für die drei PPR-Proteine MEF10, MEF12 und MEF13 waren SNaPshot-Analysen und anschließend eine Kartierung der entsprechenden Loci durchgeführt worden. In dieser Arbeit wurden dann stabile bzw. transiente Transformationen zur Bestätigung des jeweiligen PPR-Proteins als RNA-Editing-Faktor durchgeführt. Als weiterer Beweis wurde der Editing-Status in T-DNA-Insertionslinien der betreffenden Gene untersucht.

VI.1.1.1 MEF10

Der RNA-Editing-Faktor MEF10 ist für das Editing der Stelle *nad2*-842 notwendig. Die Identifizierung von MEF10 als At3g11460 wurde unter dem Titel „MEF10 is required for RNA editing at *nad2*-842 in mitochondria of *Arabidopsis thaliana* and interacts with MORF8.“ veröffentlicht. Diese Publikation ist im Anhang zu finden. Die Ergebnisse lassen sich folgendermaßen zusammenfassen:

MEF10 wird im Locus At3g11460 codiert. Eine EMS-Mutation des Nukleotides 1333 (ausgehend vom A im ATG-Startcodon) dieses Gens führt zu einem kompletten Verlust des Editings an der Stelle *nad2*-842. Eine T-DNA-Insertion in der 5‘ untranslatierten Region von At3g11460 führt zu einer Verringerung der Editingeffizienz auf 60 %. Das PPR-Protein MEF10 besteht aus 12 PPR-Wiederholungen, einer E-, einer E^+- und einer DYW-Domäne. Durch die EMS-induzierte Mutation von Guanin an Position 1333 zu Adenin wird die Aminosäure 445 von Alanin zu Threonin verändert. Diese Aminosäure befindet sich in der E-Domäne des PPR-Proteins.

VI.1.1.2 MEF12

Durch SNaPshot-Analyse (Takenaka und Brennicke, 2009; Takenaka, 2011; Takenaka und Brennicke, 2012) einer Population EMS-mutierter *A. thaliana* Pflanzen wurde eine Mutante mit Verlust des RNA-Editings an der Stelle 374 der mRNA für die Untereinheit 5 im Komplex I der Atmungskette (*nad5*) identifiziert. Diese Mutante wurde *mef12-1* genannt. Abbildung 6 zeigt die Editingeffizienz der Stelle *nad5*-374 im Columbia-Wildtyp und in der Mutante *mef12-1*.

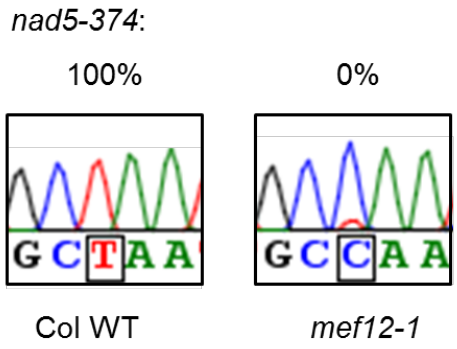


Abbildung 6: In Columbia-Wildtyppflanzen wird das genomisch codierte C an der Stelle 374 der mRNA der Untereinheit 5 der NADH-Dehydrogenase zu U verändert. In der EMS-Mutante *mef12-1* wird diese Stelle nicht editiert.

Durch Kartierung in der F2-Generation einer Kreuzung von L*er* mit EMS-mutierten Col-Pflanzen konnte der betroffene Locus auf einen Bereich von 1,3 Mb auf Chromosom 3 eingeschränkt werden (Abbildung 7). In diesem Bereich befinden sich sechs für PPR-Proteine kodierende Gene. Zwei von diesen kodieren für PPR-Proteine der P-Klasse, drei für Proteine der DYW-Unterklasse und eines für ein Protein der E-Unterklasse. Zwei dieser PPR-Proteine der DYW-Unterklasse wurden bereits als RNA-Editingfaktoren identifiziert.

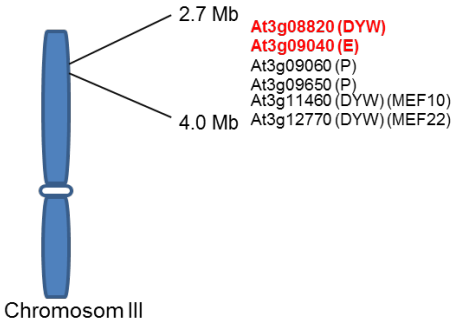


Abbildung 7: Schematische Darstellung des durch Kartierung eingeschränkten Bereichs auf Chromosom 3, in dem die EMS-Mutation (*mef12-1*) liegt. In diesem Bereich sind sechs PPR-Proteine kodiert, von denen zwei bereits als RNA-Editing-Faktoren identifiziert wurden, MEF10 und MEF22. Zuerst wurden die Gene sequenziert, die für PPR-Proteine der PLS-Klasse kodieren und mit der Sequenz aus dem Columbia-Wildtyp verglichen. Die beiden in Rot dargestellten Gene At3g08820 und At3g09040 wiesen jeweils eine für EMS typische Punktmutation von G zu A auf.

Die Sequenzen der Gene At3g08820 und At3g09040 aus *mef12-1* wiesen jeweils eine EMS typische Punktmutation von G zu A im Vergleich zu der Sequenz aus dem Wildtyp

auf. In der Sequenz von At3g08820 aus *mef12-1* ist das Nukleotid 1470 betroffen, wodurch statt der Aminosäure Serin ein Asparagin eingebaut wird. Bei At3g09040 aus *mef12-1* ist das Guanin an Position 2925 zu Adenin mutiert, wodurch statt der Aminosäure Tryptophan an Position 975 ein Stopcodon entsteht.

Beide Wildtyp-Gene sollten in den Vektor pAD41 kloniert werden und transient in Protoplasten von *mef12-1* eingebracht werden. Die Klonierung gelang nur für At3g08820, jedoch erhöhte sich durch Einbringen dieses Gens die Editingeffizienz der Stelle *nad5*-374 nicht (Abbildung 8).

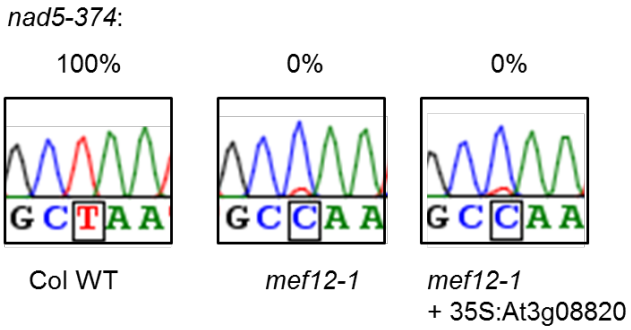


Abbildung 8: Das Columbia-Wildtyp-Gen At3g08820 wurde unter Kontrolle des CaMV-35S-Promotors in Protoplasten der Editingmutante *mef12-1* eingebracht. Das Editing der Stelle *nad5*-374 wurde durch Anwesenheit des intakten Proteins nicht wiederhergestellt.

Für das Gen At3g09040 konnte eine homozygote T-DNA-Insertionslinie (FLAG_394H02) gefunden werden. Die Analyse der *nad5* cDNA aus dieser *mef12-2* genannten Mutante ergab, dass die Stelle *nad5*-374 nur zu 20 % editiert wird (Abbildung 9).

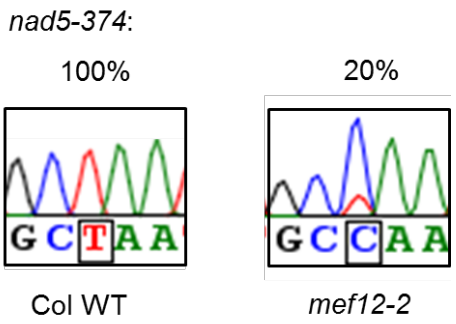


Abbildung 9: In einer T-DNA-Insertionslinie des Gens At3g09040 (*mef12-2*) wird die Stelle *nad5*-374 nur zu 20 % editiert.

Die Positionen der T-DNA-Insertion und der EMS-Mutation in At3g09040 sind in Abbildung 10 schematisch dargestellt.

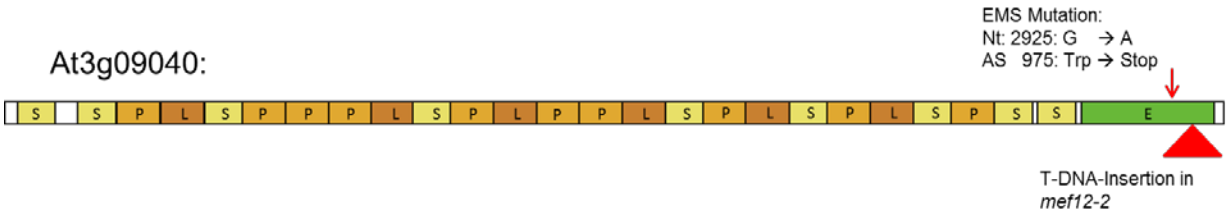


Abbildung 10: Schematische Darstellung der PPR-Wiederholungen und der C-terminalen Domäne des Proteins des Locus At3g09040. Sowohl die EMS-Mutation (roter Pfeil) in der Mutante *mef12-1* als auch die T-DNA-Insertion (rotes Dreieck) in der Mutante *mef12-2* liegen in dem für die E-Domäne kodierenden Genabschnitt.

VI.1.1.3 MEF13

Die Mutante für MEF13, *mef13-1* wurde ebenfalls durch SNaPshot-Analyse (Takenaka und Brennicke, 2009; Takenaka, 2011; Takenaka und Brennicke, 2012) einer Population EMS-mutierter *A. thaliana* Pflanzen identifiziert. In dieser Mutante ist das Editing an sechs Stellen betroffen: *ccmFc*-50, *cox3*-314, *nad2*-59, *nad4*-158, *nad5*-1914 und *nad7*-213. Die Stellen sind jeweils relativ zum A des ATG-Startcodons angegeben. In Abbildung 11 sind die Editingeffizienzen der betroffenen Stellen in der Mutante *mef13-1* im Vergleich zum Columbia-Wildtyp dargestellt.

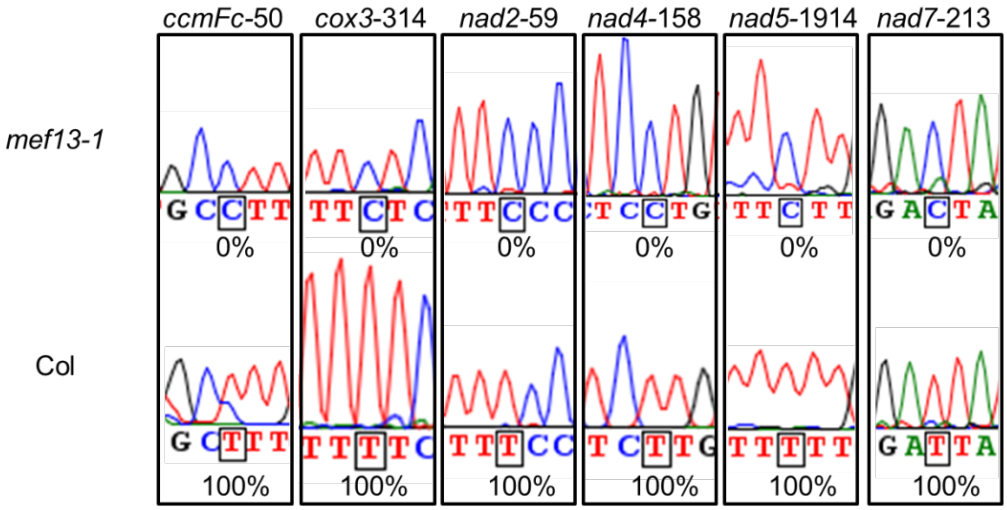


Abbildung 11: In Columbia-Wildtyppflanzen wird das genomisch codierte C an den Stellen *ccmFc*-50, *cox3*-314, *nad2*-59, *nad4*-158, *nad5*-1914 und *nad7*-213 in der mRNA zu U verändert. In der EMS-Mutante *mef13-1* werden diese Stellen nicht editiert.

Bei dieser Mutante konnte durch Kartierung in der F2-Generation einer Kreuzung von L*er* und EMS-mutierten Col-Pflanzen der betroffene Locus auf einen Bereich von 700 kb auf Chromosom 3 eingeschränkt werden (Abbildung 12). In diesem Bereich befinden sich drei für PPR-Proteine der PLS-Klasse kodierende Gene. Zwei von diesen kodieren für Proteine der E⁺-Unterklasse, eines für ein Protein der DYW-Unterklasse.

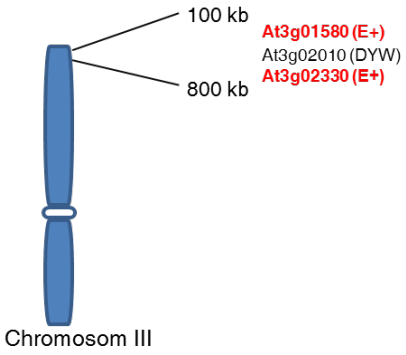


Abbildung 12: Schematische Darstellung des durch Kartierung eingeschränkten Bereichs auf Chromosom 3, in dem die EMS-Mutation liegt. In diesem Bereich sind drei PPR-Proteine kodiert. Die für diese Proteine kodierenden Gene aus der Mutante *mef13-1* und dem Columbia-Wildtyp wurden sequenziert und verglichen. Die beiden in Rot dargestellten Gene At3g01580 und At3g02330 wiesen jeweils eine für EMS typische Punktmutation von G zu A auf.

In den Sequenzen der Gene At3g01580 und At3g02330 aus *mef13-1* befindet sich jeweils eine EMS typische Punktmutation von G zu A im Vergleich zu der Sequenz aus dem Columbia-Wildtyp. Die Wildtyp-Sequenzen beider Gene wurden jeweils in den Vektor pAD41 kloniert und durch *Floral Dip* stabil in *A. thaliana*-Pflanzen (*mef13-1*) eingebaut. Das intakte Gen At3g01580 hatte keinen Effekt auf das Editing der Zielstellen von MEF13, aber die Wildtyp-Sequenz des Gens At3g02330 stellte das Editing an allen sechs betroffenen Stellen wieder her (Abbildung 13).

In der Sequenz von At3g02330 in *mef13-1* ist das Nukleotid 200 verändert, wodurch statt der Aminosäure Glycin an Position 67 in der ersten PPR-Einheit (L) Aspartat eingebaut wird (Abbildung 15).

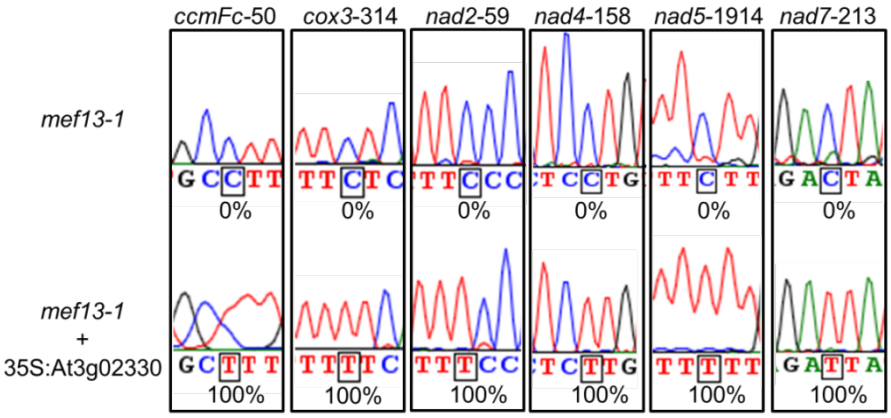


Abbildung 13: Das Columbia-Wildtyp-Gen At3g02330 wurde unter Kontrolle des CaMV-35S-Promotors stabil in die Editingmutante *mef13-1* eingebracht. Das Editing aller sechs in der Mutante betroffenen Stellen wird durch das intakte Gen wiederhergestellt.

Zehrmann *et al.* (2008) fanden ökotypspezifische Unterschiede der RNA-Editingfrequenz an sieben Stellen zwischen Columbia, C24 und Landsberg *erecta*. Die Stelle *nad7*-213 wird in Col und L*er* zu 100 % editiert, in C24 jedoch nur zu 60 % (Zehrmann *et al.*, 2008). Um zu untersuchen, ob MEF13 auch für diesen ökotypspezifischen Unterschied verantwortlich ist, wurde die Columbia-Sequenz von At3g02330 im Vektor pAD41 unter Kontrolle des CaMV-35S-Promotors transient in Protoplasten aus C24 eingebracht. Dadurch wurde die in diesen C24-Pflanzen auf 70 % verringerte Editingeffizienz auf 100 % erhöht (Abbildung 14).

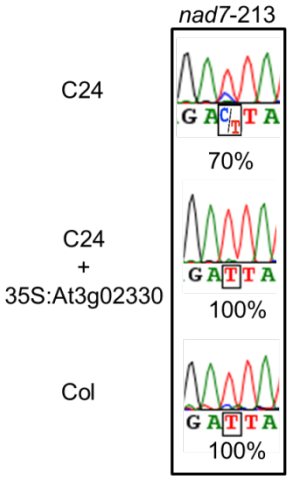


Abbildung 14: Das Columbia-Wildtyp-Gen At3g02330 wurde unter Kontrolle des CaMV-35S-Promotors in Protoplasten des C24-Wildtyps eingebracht. Die Anwesenheit des Proteins aus Col erhöht das Editing der Stelle *nad5*-374 in den transgenen C24-Protoplasten.

Abbildung 15 zeigt die Domänenstruktur im Protein MEF13. Die Positionen der EMS-Mutation und des durch Sequenzierung der Gensequenzen aus den beiden Ökotypen ermittelten Polymorphismus (SNP) zwischen Col und C24 sind eingezeichnet. In der für *MEF13* zu Verfügung stehenden T-DNA-Insertionslinie SALK_097270C konnten nur Wildtyp-Pflanzen gefunden werden.

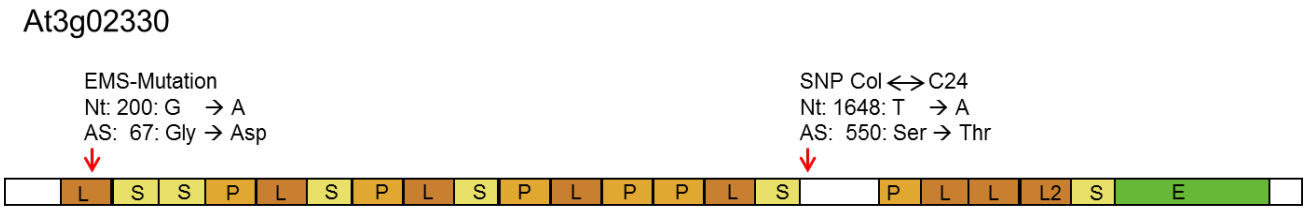


Abbildung 15: Schematische Darstellung der PPR-Struktur und der C-terminalen Domäne im Protein MEF13, Locus At3g02330. Die Lage der EMS-Mutation (roter Pfeil; EMS-Mutation) in der Mutante *mef13-1* und die Lage des Einzelnukleotidaustauschs zwischen den Ökotypen Col und C24 (roter Pfeil; SNP Col↔C24) sind im ersten L-Motiv bzw. im Sequenzbereich zwischen dem fünften S- und dem sechsten P-Motiv eingezeichnet.

Interessanterweise wird durch die transgene Expression des Wildtyp-Gens At3g02330 in *mef13-1* im Transkript von *nad5* die Stelle 1663 zu 60 % editiert (Abbildung 16). In der Mutante *mef13-1* und den Wildtyppflanzen der beiden Ökotypen wird diese Stelle nur zu 20 % editiert.

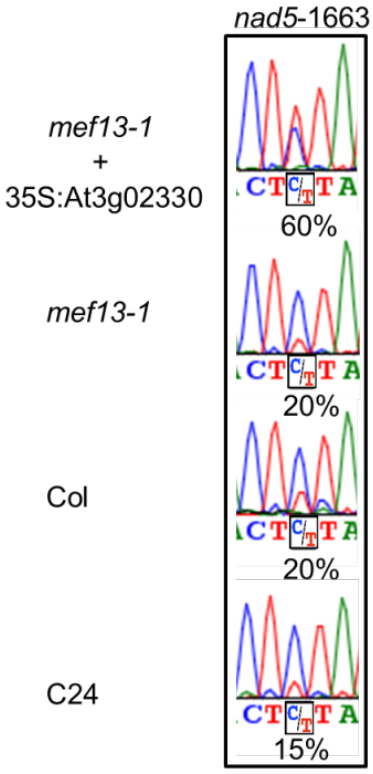


Abbildung 16: Durch Expression des Gens At3g02330 mit der Columbia-WT-Gensequenz in der Mutante *mef13-1* erhöht sich die Editingeffizienz der Stelle *nad5*-1663 auf 60 %. In der nicht komplementierten Mutante und den beiden Ökotypen C24 und Col ist diese Stelle ungefähr zu 20 % editiert.

Ein Vergleich der in Abbildung 17 dargestellten *cis*-Elemente an den sechs betroffenen Editingstellen zeigt als einzige Gemeinsamkeiten drei Uridine an den Stellen -9, -12 und -17 relativ zur Editingstelle. Betrachtet man nur die *cis*-Elemente der vier Editingstellen, die auch Ziele von MORF3 sind, *ccmFc*-50, *nad2*-59, *nad5*-1916 und *nad7*-213 (Takenaka *et al.,* 2012), so kommen zwei weitere gemeinsame Uridine an den Stellen -15 und -22 hinzu.

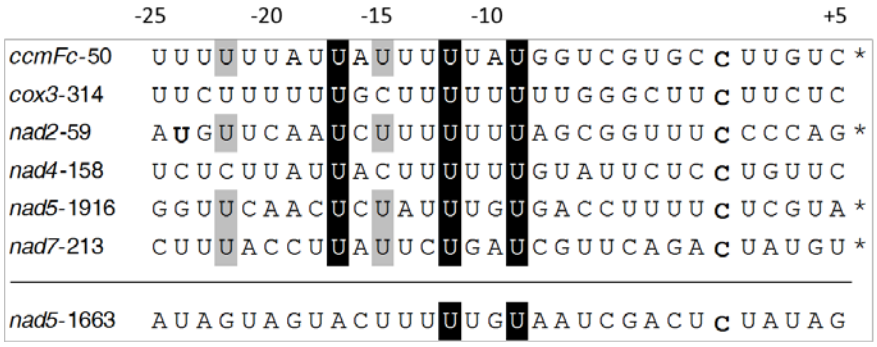


Abbildung 17: Sequenzalignment der *cis*-Elemente an den sechs Editingstellen von MEF13. In allen sechs *cis*-Elementen identische Nukleotide sind schwarz hinterlegt. Die Sternchen am Ende der Sequenzen

markieren Editingstellen, die auch Ziel von MORF1 sind. Nukleotide die in den *cis*-Elementen dieser vier Stellen zusätzlich identisch sind, sind grau hinterlegt. Die Positionen sind relativ zur Editingstelle (fett gedrucktes C) angegeben. Eine weitere Editingstelle in *nad2*-59 ist als fettes U markiert. Durch eine Linie abgetrennt ist das *cis*-Element an der Stelle *nad5*-1663 dargestellt, welches in *mef13-1* Pflanzen mit transgenem MEF13 höheres Editing als in den Wildtypen Columbia und C24 zeigt.

Auch das *cis*-Element an der durch das transgene MEF13 editierten Stelle *nad5*-1663 zeigt als einzige Gemeinsamkeit jeweils ein U an Stelle -9 und an Stelle -12.

VI.1.2 Identifizierung durch T-DNA-Insertionslinien: MEF28 und MEF30

SNaPshot-Analysen (Takenaka und Brennicke, 2009; Takenaka, 2011; Takenaka und Brennicke, 2012) einiger Linien mit T-DNA-Insertionen in für PPR-Proteine kodierenden Genen haben in zwei Linien RNA-Editing-Defekte festgestellt. Zur Bestätigung der betroffenen PPR-Proteine als Editingfaktoren wurden stabile bzw. transiente Transformationen der T-DNA-Insertionslinien mit den Wildtyp-Genen durchgeführt.

VI.1.2.1 MEF28

Die T-DNA-Insertionslinie SAIL_77_E03 enthält eine Insertion im Gen At5g06540 (Abbildung 18). Dieses kodiert für ein PPR-Protein der DYW-Subklasse. In dieser Insertionslinie, die *mef28-1* genannt wurde, ist das Editing zweier direkt benachbarter Stellen (89 und 90 relativ zum A im ATG-Startcodon) in der mRNA der Untereinheit 2 des Komplex I der Atmungskette (*nad2*) betroffen.

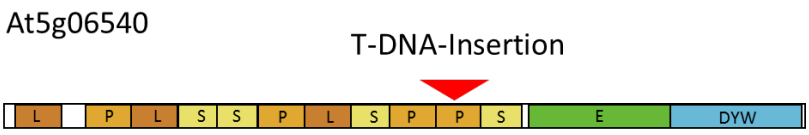


Abbildung 18: Schematische Darstellung der PPR-Elemente und der C-terminalen Domänen des im Locus At5g06540 kodierten PPR-Proteins. Die T-DNA-Insertion befindet sich im Bereich der für das vierte P-Motiv kodierenden Region (rotes Dreieck).

Durch stabile Transformation von *mef28-1*-Pflanzen mit dem intakten Gen At4g38010 aus Col konnten die Editingdefekte an beiden Stellen ausgeglichen werden (Abbildung 19).

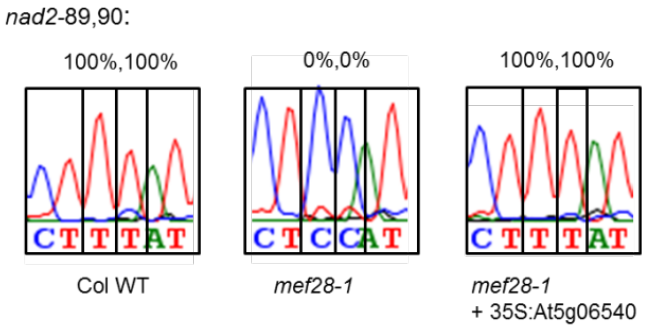


Abbildung 19: In Columbia-Wildtyppflanzen wird das genomisch codierte C an den Stellen *nad2*-89 und *nad2*-90 in der mRNA zu U verändert. Eine T-DNA-Insertion im Gen At5g06540 führt zum Verlust des Editings dieser Stellen. Das Columbia-Wildtyp-Gen At5g06540 wurde unter Kontrolle des CaMV-35S-Promotors stabil in die Editingmutante *mef28-1* eingebracht. Das Editing der beiden in der Mutante betroffenen Stellen wurde durch das intakte Gen wiederhergestellt.

Um zu untersuchen, ob die Anwesenheit der DYW-Domäne für das Editing beider benachbarter Stellen essentiell ist, wurde eine Deletionsmutante des Gens *MEF28* (At5g06540ΔDYW) konstruiert, die nach der E-Domäne endet. Dieses deletierte Gen wurde ebenfalls in den Vektor pAD41 kloniert und damit transient *mef28-1*-Protoplasten transformiert. Als Transformationskontrolle wurden in einem weiteren Ansatz *mef28-1*-Protoplasten mit dem Volllängenkonstrukt transformiert. Durch das Deletionskonstrukt konnte das Editing an keiner der beiden Stellen erbracht werden. Das Volllängen-MEF28 konnte das Editing beider Stellen auf 70 % erhöhen (Abbildung 20).

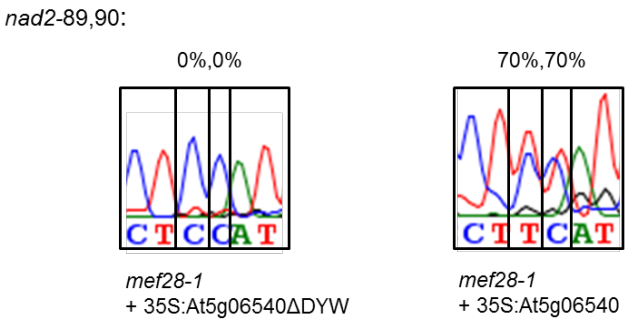


Abbildung 20: Das Columbia-Gen At5g06540 wurde einmal ohne die für die DYW-Domäne kodierende Sequenz (35S:At5g06540ΔDYW) und einmal als Volllängenkonstukt (35S:At5g06540) unter Kontrolle des CaMV-35S-Promotors in Protoplasten der Editingmutante *mef28-1* eingebracht. Das Editing der beiden Stellen *nad2*-89 und *nad2*-90 wurde nur durch Anwesenheit des Proteins mit DYW-Domäne zu 70 % wiederhergestellt. Das Protein ohne DYW-Domäne hatte keinen Einfluss auf das Editing der beiden Stellen.

VI.1.2.2 **MEF30**

In der Linie SALK_062576C befindet sich die T-DNA im Gen At4g38010 (Abbildung 21). Der Locus At4g38010 kodiert ein PPR-Protein der E-Subklasse. In dieser Insertionslinie *mef30-1* ist das Editing der Stelle 1033 (relativ zum A im ATG-Startcodon) in der mRNA der Untereinheit 4 des Komplex I der Atmungskette (*nad4*) und der Stelle 982 im Transkript des Apocytochroms b (*cyt*) betroffen.



Abbildung 21: Schematische Darstellung der PPR-Einheiten und der C-terminalen Domäne des im Locus At4g38010 kodierten PPR-Proteins. Die T-DNA-Insertion (rotes Dreieck) befindet sich im Bereich der für das dritte L-Motiv kodierenden Region.

Die Col-Sequenz des Gens At4g38010 wurde unter Kontrolle des CaMV-35S-Promotors in den Vektor pAD41 kloniert. Da keine stabil transformierten *mef30-1*-Pflanzen gewonnen werden konnten, wurden *mef30-1*-Protoplasten transient transformiert. Durch Anwesenheit des intakten Proteins konnten die Editingeffizienzen der Stelle *nad4*-1033 von 0 % auf 50 % und der Stelle *cyt*-982 von 0 % auf 70 % erhöht werden (Abbildung 22).

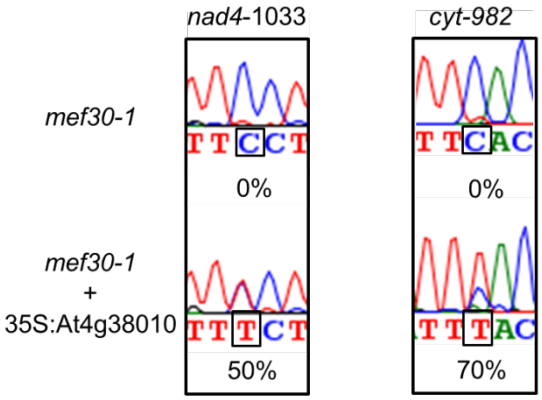


Abbildung 22: Das Columbia-Wildtyp-Gen At4g38010 wurde unter Kontrolle des CaMV-35S-Promotors in Protoplasten der Editingmutante *mef30-1* eingebracht. Das Editing der Stellen *nad4*-1033 und *cyt*-982 wurde durch Anwesenheit des intakten Proteins wieder möglich.

VI.2 Untersuchung der RNA-Editing-Effizienz aller chloroplastidären Stellen in den Mutanten von *morf2*, *morf9* und *morf5*

MORF1 war der erste von Takenaka *et al.* (2012) identifizierte RNA-Editing-*trans*-Faktor dieser Proteinfamilie. Durch Sequenzvergleiche waren weitere Mitglieder dieser Familie gefunden worden. Die Lokalisation von MORF2 in den Chloroplasten wurde von Chatterjee *et al.* (1996) und Bisanz *et al.* (2003) experimentell durch *in vitro* Importstudien nachgewiesen. MORF2 und MORF9 wurden in MS-Proteom-Analysen zusammen mit plastidären Proteinen gefunden (Zybailov *et al.*, 2008). Deshalb wurden in den T-DNA-Insertions-Mutanten von MORF2 (SALK_094930C) und MORF9 (SALK_013483) alle chloroplastidären RNA-Editingstellen untersucht und mit den Editingeffizienzen dieser Stellen im Col-Wildtyp verglichen. Die Mutante *morf2-1* musste aufgrund von Defekten der Chlorophyllsynthese auf zuckerhaltigem MS-Medium angezogen werden. Die Kotyledonen der Mutante *morf 9-1* sind grün und erst die ersten echten Rosettenblätter weisen ein panaschiertes Muster auf. RNA wurde nur aus weißen Blattteilen gewonnen. Um sekundäre Effekte als Grund für Editingdefekte auszuschließen, wurden auch die Editingstellen in der *psy*-Mutante untersucht (Kugelmann *et al.,* 2013). Wie Tabelle 1 zu entnehmen ist, waren fast alle Stellen in beiden *morf*-Mutanten betroffen. In der zur Kontrolle untersuchten *psy*-Mutante weisen einige Stellen geringere Editingeffizienzen als der Col-Wildtyp auf, jedoch immer höhere Effizienzen als die MORF-Mutanten.

Tabelle 1*:* RNA-Editingeffizienzen der chloroplastidären Stellen in den Mutanten *morf2-1*, *morf9-1* und *psy* im Vergleich mit dem Wildtyp Columbia. Die Editingstelle ist als Anzahl der Nukleotide Entfernung relativ zum A des ATG-Startcodons des betroffenen Gens angegeben, bzw. im Falle der 3'UTR von *accD* und des Introns von *rps12* als Anzahl der Nukleotide auf dem Plastidengenom. Für „n. d." wude kein Sequenzierungsergebnis erhalten.

Locus	Editingstelle	Col	*morf2-1*	*morf9-1*	*psy*
ndhB	149	90 %	0 %	10 %	n. d.
	467	90 %	5 %	15 %	90 %
	586	100 %	20 %	10 %	90 %
	746	100 %	50 %	50 %	100 %
	830	100 %	0 %	0 %	80 %
	836	100 %	0 %	0 %	70 %
	872	85 %	0 %	0 %	100 %
	1255	100 %	10 %	15 %	75 %
	1481	100 %	10 %	10 %	n. d.
ndhD	2	45 %	0 %	0 %	0 %
	383	90 %	50 %	40 %	95 %
	674	80 %	50 %	50 %	100 %
	878	80 %	0 %	10 %	75 %
	887	100 %	5 %	10 %	80 %
psbE	214	100 %	30 %	100 %	100 %
psbF	65	100 %	0 %	70 %	100 %
atpF	92	100 %	10 %	10 %	75 %
clpP	559	80 %	60 %	40 %	35 %
accD	794	89 %	5 %	5 %	95 %
	3' UTR (58642)	20 %	0 %	0 %	65 %
matK	706	70 %	45 %	50 %	85 %
ndhF	290	100 %	0 %	40 %	90 %
ndhG	50	100 %	40 %	0 %	90 %
petL	5	100 %	65 %	0 %	40 %
rpoB	338	90 %	70 %	70 %	95 %
	551	90 %	80 %	60 %	95 %
	2432	100 %	80 %	80 %	n. d.
rps14	80	100 %	0 %	50 %	80 %
	149	95 %	50 %	50 %	65 %
rpoA	200	50 %	80 %	0 %	25 %
psbZ	50	100 %	0 %	30 %	90 %
rps12	Intron (69553)	20 %	15 %	10 %	40 %
rpl23	89	70 %	0 %	0 %	n. d.

In Abbildung 23 sind die Chromatogramme ausgewählter Beispiele für die vier unterschiedlichen Situationen dargestellt. Für das Editing von insgesamt sieben Stellen, wie z. B. auch *ndhD*-2, sind beide MORF-Proteine essentiell. *psbZ*-50 ist ein Beispiel für die sechs Stellen, für deren Editing MORF2 benötigt wird. Drei Stellen, wie z. B. *petL*-50 können nicht ohne MORF9 editiert werden. Die restlichen 18 Editing-Stellen, z. B. *ndhB*-1255, zeigen verringerte Editingeffizienzen in Abwesenheit jeweils eines der beiden Faktoren, benötigen also beide Proteine für vollständiges Editing. Jedes Editingereignis in Plastiden benötigt mindestens eines der beiden MORF-Proteine.

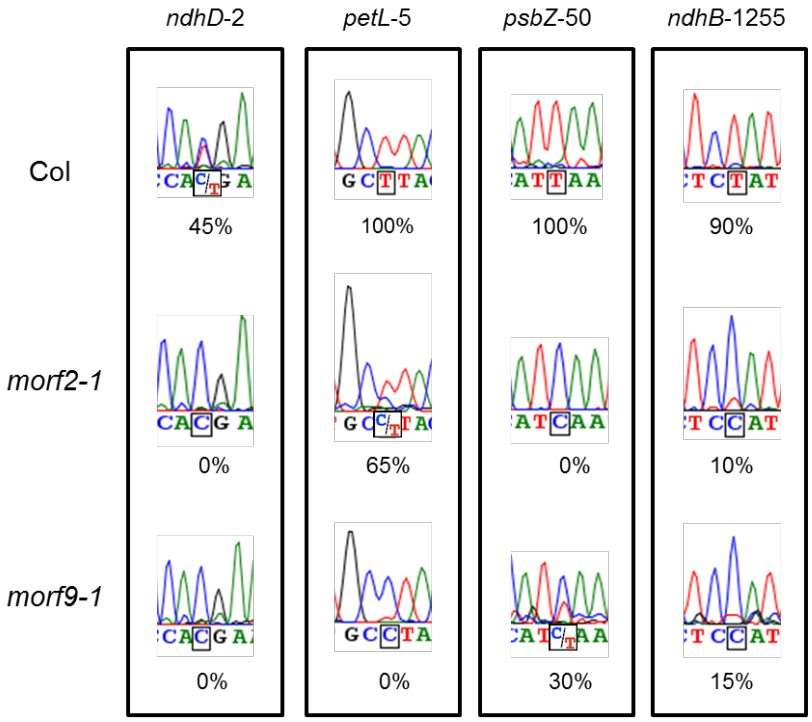


Abbildung 23: Beispiele für die in den *morf2-1* und *morf9-1* Mutanten auftretenden unterschiedlichen Editingdefekte im Vergleich mit der jeweiligen Editingeffizienz im Col-Wildtyp. Einige Stellen (u.a. *ndhD*-2) benötigen die Anwesenheit beider Proteine, manche Stellen werden nur in Abwesenheit eines der beiden Proteine überhaupt nicht editiert: *petL*-50 benötigt MORF9, *psbZ*-50 MORF2. Die meisten Stellen benötigen beide Proteine für effizientes Editing und werden in der jeweiligen Mutante nur teilweise editiert (z. B. *ndhB*-1255).

Das Wirken von MORF2 und MORF9 in Form von Heterodimeren würde erklären, warum die meisten Stellen in Abwesenheit eines der beiden Faktoren nur noch teilweise editiert werden. Um dies zu überprüfen, wurden die unter VI.3 beschriebenen BiFC-Experimente durchgeführt. Bei den Lokalisationsstudien stellte sich heraus, dass das YFP-Signal von MORF5 sowohl in den Mitochondrien als auch den Chloroplasten zu

finden ist. Deshalb wurde auch der Editingstatus der chloroplastidären Stellen in der *morf5-1*-Mutante (SALK_016801C) untersucht. In dieser Mutante sind die Stellen *petL*-5 und *rpl23*-89 betroffen. *petL*-5 ist zu 40 % editiert, *rpl23*-89 zu 0 %.

VI.3 Analyse potentieller Interaktionen verschiedener RNA-Editing-*trans*-Faktoren durch BiFC

Mit der Methode der bimolekularen Fluoreszenzkomplementation (BiFC) kann die Interaktion verschiedener Proteine in ihrem natürlichen Umfeld untersucht werden. BiFC wurde hier verwendet, um die Interaktion verschiedener RNA-Editingfaktoren zu analysieren. Zunächst wurde die Lokalisation der MORF-Proteine in Mitochondrien oder Chloroplasten betrachtet und die verschiedenen MORF-Proteine wurden untereinander auf die Bildung von Homo- oder Heterodimeren getestet.

VI.3.1 Intrazelluläre Lokalisation der MORF-Proteine

Zur Bestimmung der intrazellulären Lokalisation wurde das vollständige YFP an die jeweiligen MORFs fusioniert. Abbildung 24 gibt eine Übersicht der detektierten YFP-Signale in den jeweiligen Organellen. Das YFP-Signal von MORF2 und MORF9 wurde nur in den Chloroplasten der transient transformierten Tabakepidermiszellen detektiert, das von MORF5 und MORF8 in Chloroplasten und Mitochondrien und das von MORF1, MORF3, MORF4 und MORF6 nur in den Mitochondrien. Für MORF7 konnte kein YFP-Signal detektiert werden.

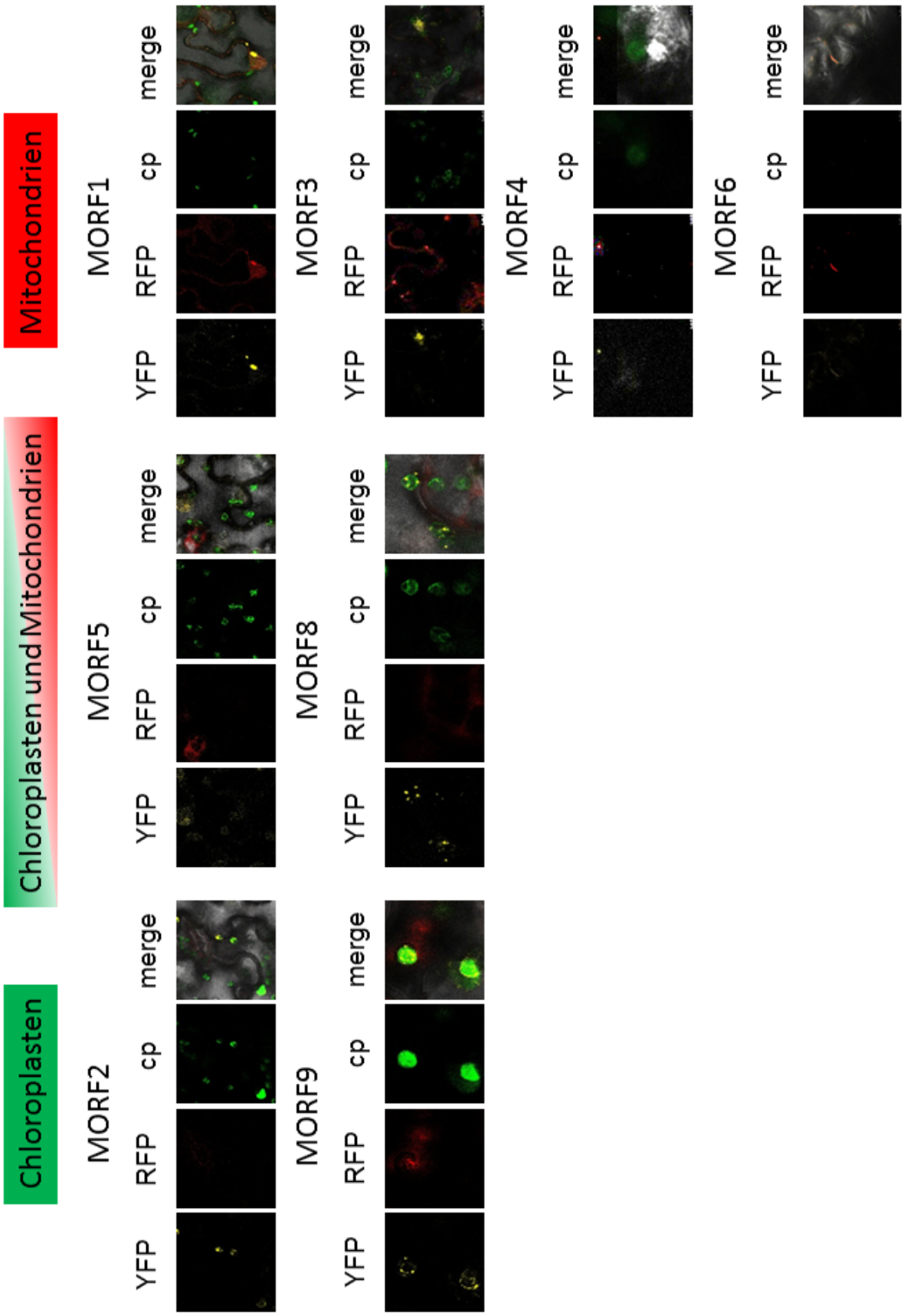


Abbildung 24: Intrazelluläre Lokalisation der neun mit YFP fusionierten MORF-Proteine. Das YFP-Signal von MORF2 und MORF 9 ist nur in den Chloroplasten zu sehen, das von MORF5, MORF6 und MORF8 sowohl in den Chloroplasten als auch in den Mitochondrien und das YFP-Signal von MORF1, MORF3,

MORF4 und MORF7 in den Mitochondrien. „YFP“ bezeichnet den Kanal, mit dem nur das YFP-Signal detektiert wurde (525-600 nm) (gelb), „RFP“ den Kanal, mit dem das RFP-Signal detektiert wurde (576-629 nm) (rot). Die mitochondriale Zielsequenz der alternativen Oxidase (AOX) diente an RFP fusioniert als Marker für die Mitochondrien. „cp“ bezeichnet den Kanal, mit dem die Chlorophyll-Autofluoreszenz detektiert wurde (650-704 nm) (grün). „merge“ überlagert die Signale dieser drei Kanäle und das im Hellfeld gewonnene Bild der Tabakepidermiszellen.

VI.3.2 Bildung von Homo-und Heterodimeren zwischen MORF-Proteinen

Durch BiFC wurde zunächst die Interaktion der MORF-Proteine untereinander untersucht.

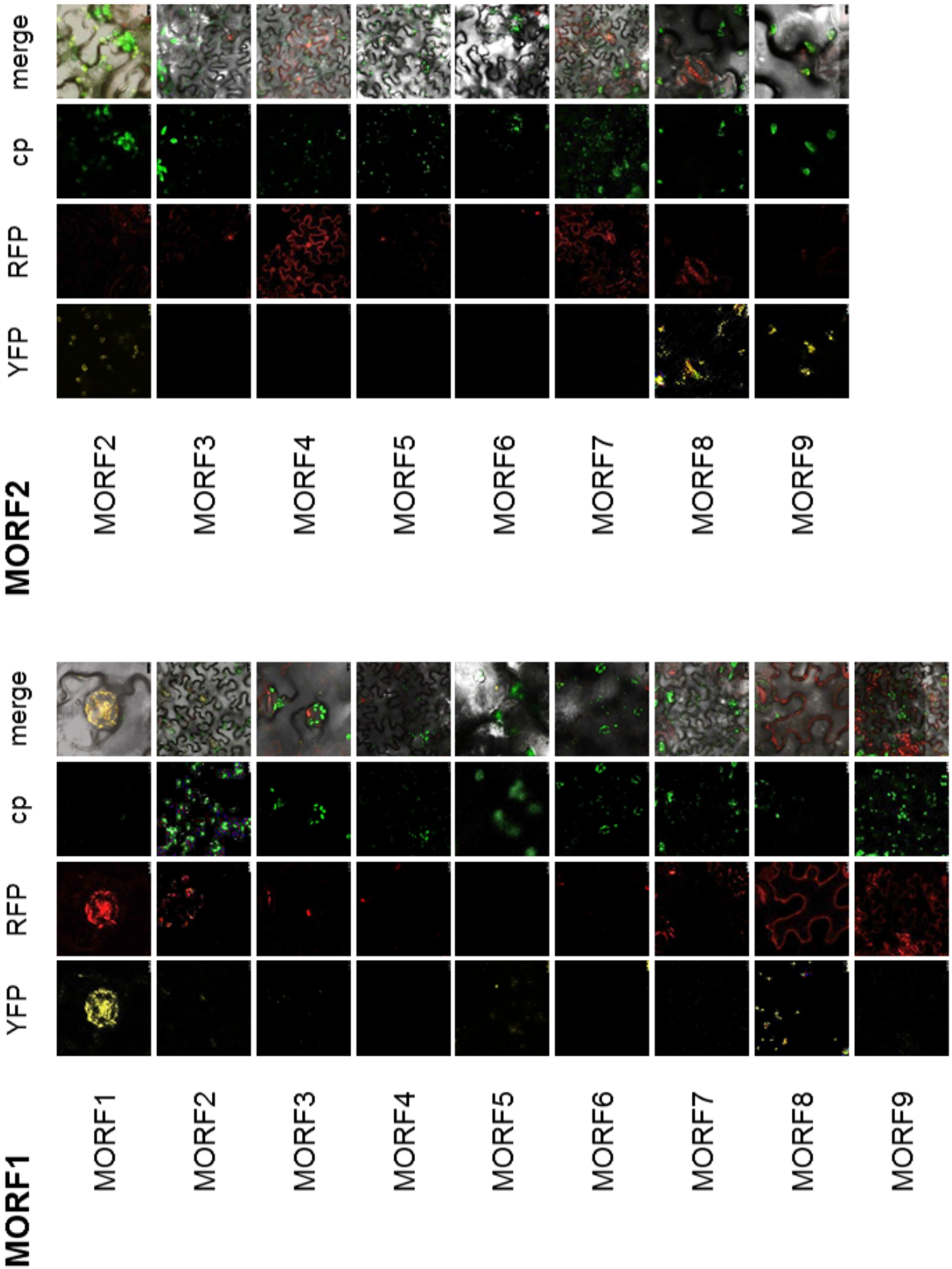
Für MORF1:YFP-N mit MORF1:YFP-C, MORF2:YFP-N mit MORF2-YFP-C und MORF9:YFP-N mit MORF9-YFP-C konnte das YFP-Signal bei MORF1 in den Mitochondrien und MORF2 und MORF9 in den Chloroplasten detektiert werden.

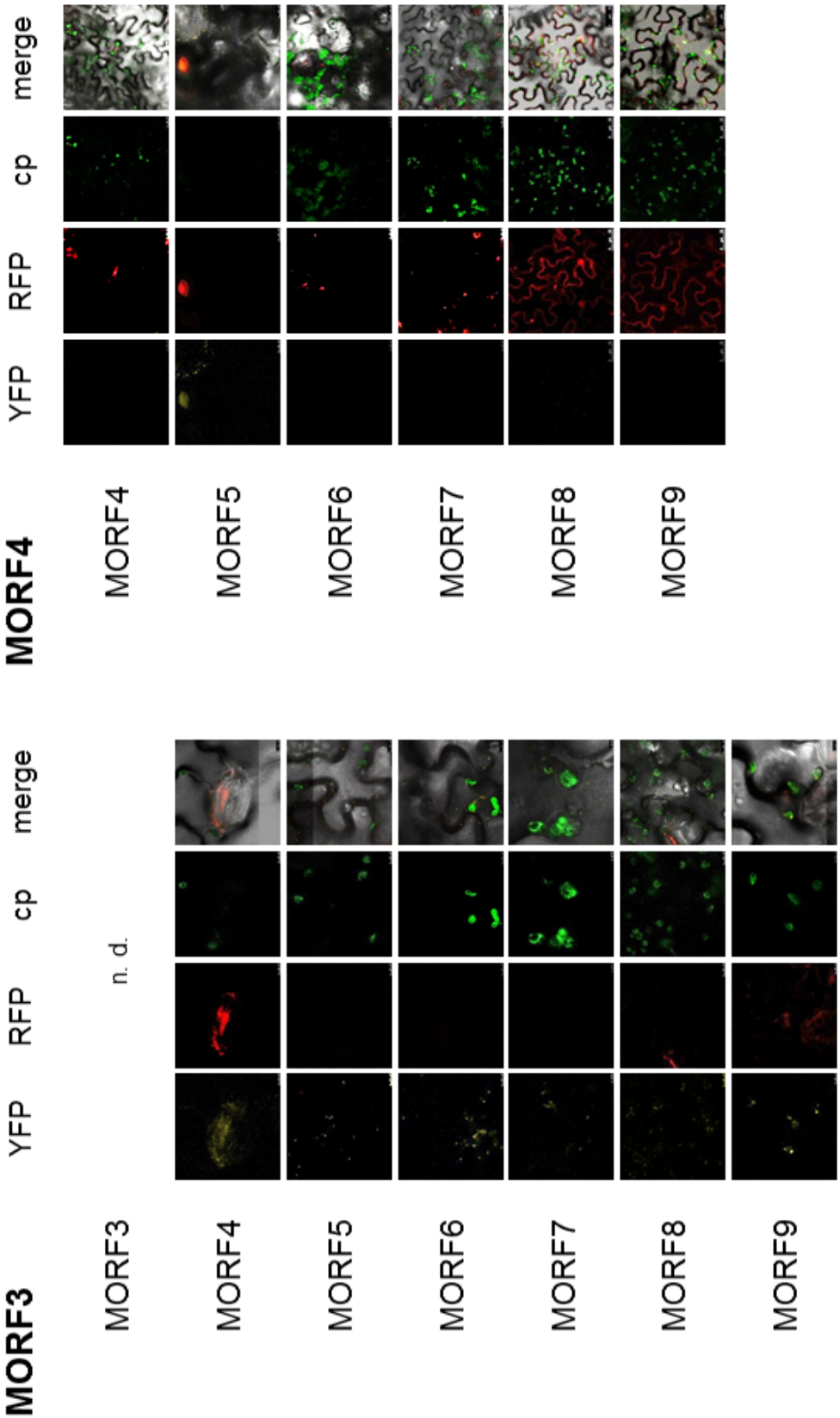
Zudem zeigten die Kombinationen MORF1 mit MORF8, MORF2 mit MORF8, MORF2 mit MORF9, MORF5 mit MORF6, MORF5 mit MORF7, MORF5 mit MORF8, MORF5 mit MORF9, MORF6 mit MORF7, MORF6 mit MORF8, MORF6 mit MORF9 und MORF7 mit MORF8 ein YFP-Signal.

Diese Ergebnisse sind in Tabelle 2 und Abbildung 25 zusammengefasst.

Tabelle 2: Zusammenfassung der Interaktion der MORF-Proteine untereinander. „mt“ gibt die Lokalisation des YFP-Signals in den Mitochondrien, „cp“ in den Chloroplasten an.

	MORF1	MORF2	MORF3	MORF4	MORF5	MORF6	MORF7	MORF8	MORF9
MORF1	YFP in mt								
MORF2	kein YFP	YFP in cp							
MORF3	YFP im Cytosol	kein YFP	n. d.						
MORF4	kein YFP	kein YFP	YFP in mt	kein YFP					
MORF5	kein YFP	kein YFP	YFP im Cytosol	YFP im Cytosol	YFP in mt + Cytosol				
MORF6	kein YFP	kein YFP	YFP an Membran	kein YFP	YFP in mt	n. d.			
MORF7	kein YFP	kein YFP	YFP an Membran	kein YFP	YFP in mt	YFP in cp + mt	kein YFP		
MORF8	YFP in mt	YFP in cp + mt	YFP im Cytosol	kein YFP	YFP in cp + mt	YFP in mt	YFP in cp	YFP in mt	
MORF9	kein YFP	YFP in cp	kein YFP	kein YFP	YFP in cp	YFP in cp	kein YFP	kein YFP	YFP in cp


MORF2
YFP
RFP
cp
merge
MORF2
MORF3
MORF4
MORF5
MORF6
MORF7
MORF8
MORF9
MORF1
YFP
RFP
cp
merge
MORF1
MORF2
MORF3
MORF4
MORF5
MORF6
MORF7
MORF8
MORF9


MORF4
YFP
RFP
cp
merge
MORF4
MORF5
MORF6
MORF7
MORF8
MORF9
MORF3
YFP
RFP
cp
merge
n. d.
MORF3
MORF4
MORF5
MORF6
MORF7
MORF8
MORF9

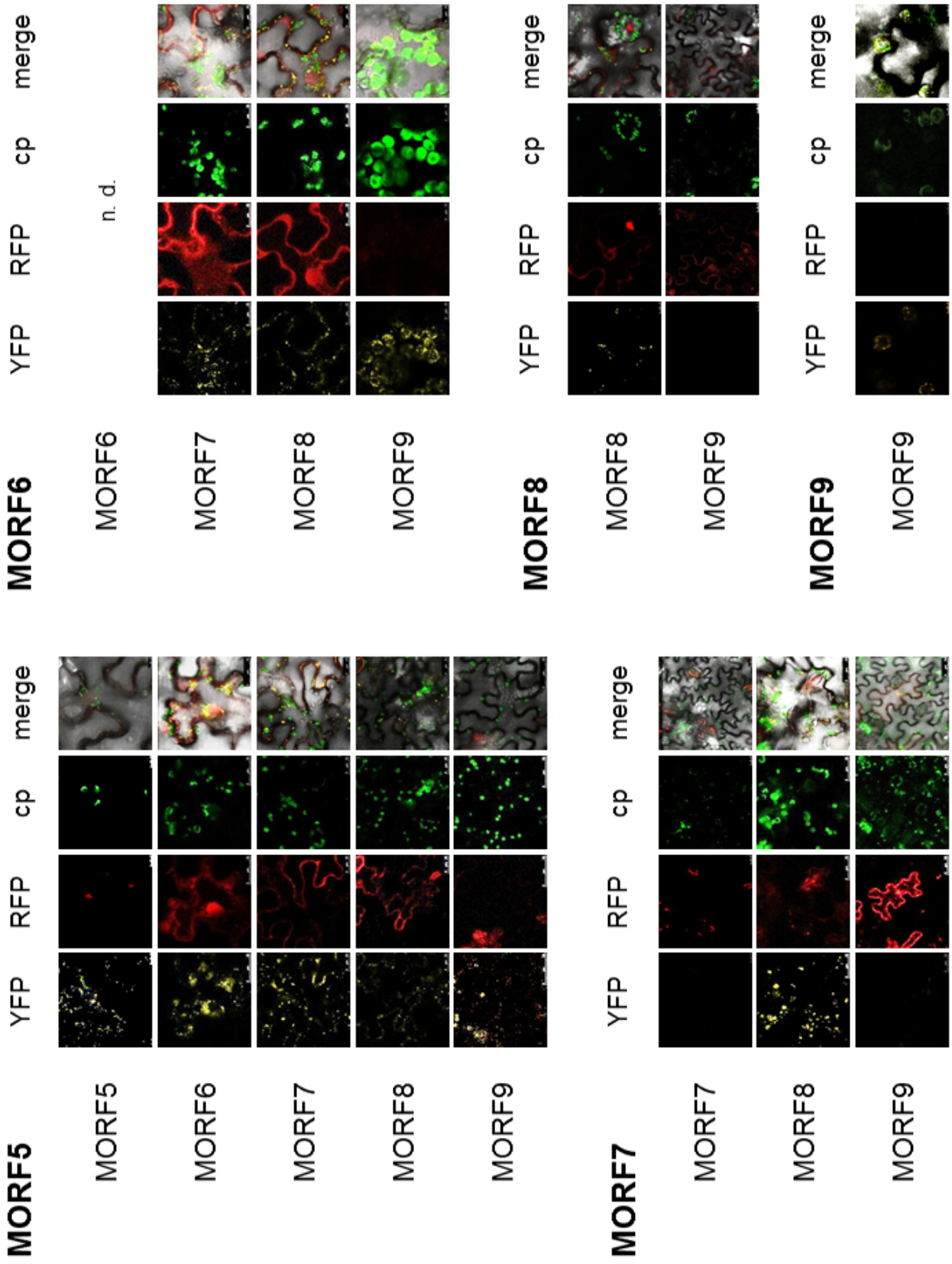


Abbildung 25: Interaktion der neun mit der N-terminalen bzw. C-terminalen Hälfte des YFP fusionierten MORF-Proteine. Für MORF1:YFP-N mit MORF1:YFP-C, MORF2:YFP-N mit MORF2:YFP-C, MORF8:YFP-N mit MORF8:YFP-C und MORF9:YFP-N mit MORF9:YFP-C konnte das YFP-Signal bei MORF1 und MORF8 in

den Mitochondrien und MORF2 und MORF9 in den Chloroplasten detektiert werden. MORF5:YFP-N mit MORF5:YFP-C zeigte in den Mitochondrien und im Cytosol ein Signal. Die Kombinationen MORF1 mit MORF8, MORF1 mit MORF3, MORF2 mit MORF8, MORF2 mit MORF9, MORF3 mit MORF4, MORF3 mit MORF5, MORF3 mit MORF6, MORF3 mit MORF7, MORF3 mit MORF8, MORF4 mit MORF5, MORF5 mit MORF6, MORF5 mit MORF7, MORF5 mit MORF8, MORF5 mit MORF9, MORF6 mit MORF7, MORF6 mit MORF8, MORF6 mit MORF9 undMORF7 mit MORF8 zeigten ebenfalls ein YFP-Signal.
„YFP“ bezeichnet den Kanal, mit dem nur das YFP-Signal detektiert wurde (525-600 nm) (gelb), „RFP“ den Kanal, mit dem das RFP-Signal detektiert wurde (576-629 nm) (rot). Die mitochondriale Zielsequenz der alternativen Oxidase (AOX) diente an RFP fusioniert als Marker für die Mitochondrien. „cp“ bezeichnet den Kanal, mit dem die Chlorophyll-Autofluoreszenz detektiert wurde (650-704 nm) (grün). „merge“ überlagert die Signale dieser drei Kanäle und das im Hellfeld gewonnene Bild der Tabakepidermiszellen.

In Abbildung 26 ist zur besseren Sichtbarkeit die fluoreszenzmikroskopische Aufnahme der Interaktionen von MORF2 mit MORF2 als Beispiel vergrößert dargestellt.

MORF2:YFP-N + MORF2:YFP-C:

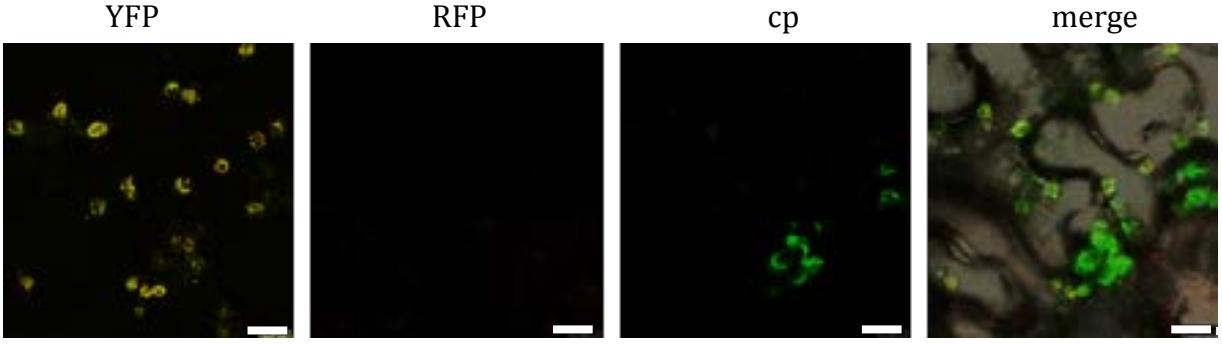


Abbildung 26: Vergrößerte Darstellung des YFP-Signals von MORF2 mit MORF2 in Chloroplasten**.** Dargestellt ist der fokussierte Bereich, in dem das YFP-Signal zu sehen ist, deshalb ist in diesem Bildausschnitt kein RFP-Signal zu sehen**.** Die Länge der Maßstabsleiste beträgt 10 µm. „YFP“ bezeichnet den Kanal, mit dem nur das YFP-Signal detektiert wurde (525-600 nm) und gelb eingefärbt ist, „RFP“ den Kanal, mit dem das RFP-Signal detektiert wurde (576-629 nm) (rot). Die mitochondriale Zielsequenz der alternativen Oxidase (AOX) diente an RFP fusioniert als Marker für die Mitochondrien. „cp“ bezeichnet den Kanal, mit dem die Chlorophyll-Autofluoreszenz detektiert wurde (650-704 nm) (grün). „merge“ überlagert die Signale dieser drei Kanäle und das im Hellfeld gewonnene Bild der Tabakepidermiszellen.

VI.3.3 Interaktionen einiger PPR-Proteine mit MORF-Proteinen

Da einige der als RNA-Editing-Faktoren identifizierten PPR-Proteine in Mitochondrien und Chloroplasten dieselben Stellen betreffen wie MORF-Proteine, wurden auch mögliche Interaktionen dieser beiden Kategorien von Editingfaktoren untersucht.

Zuerst wurden die in Chloroplasten lokalisierten PPR-Proteine LPA66 (Cai *et al.,* 2009) und CRR4 (Kotera *et al.,* 2005) auf Interaktionen mit MORF2 und MORF9 getestet. LPA66 besitzt eine DYW-Domäne und ist für das Editing der Stelle *psbF*-65 nötig. Diese Stelle wird in *morf2-1* überhaupt nicht, in *morf9-1* zu 70 % editiert. CRR4 ist ein PPR-Protein mit E-Domäne und am Editing der Stelle *ndhD*-2 beteiligt. Diese Editingstelle wird weder in *morf2-1* noch *morf9-1* editiert.

In Abbildung 27 ist das YFP-Signal der Interaktion von MORF2 mit LPA66 und MORF2 mit CRR4 in den Chloroplasten zu sehen.

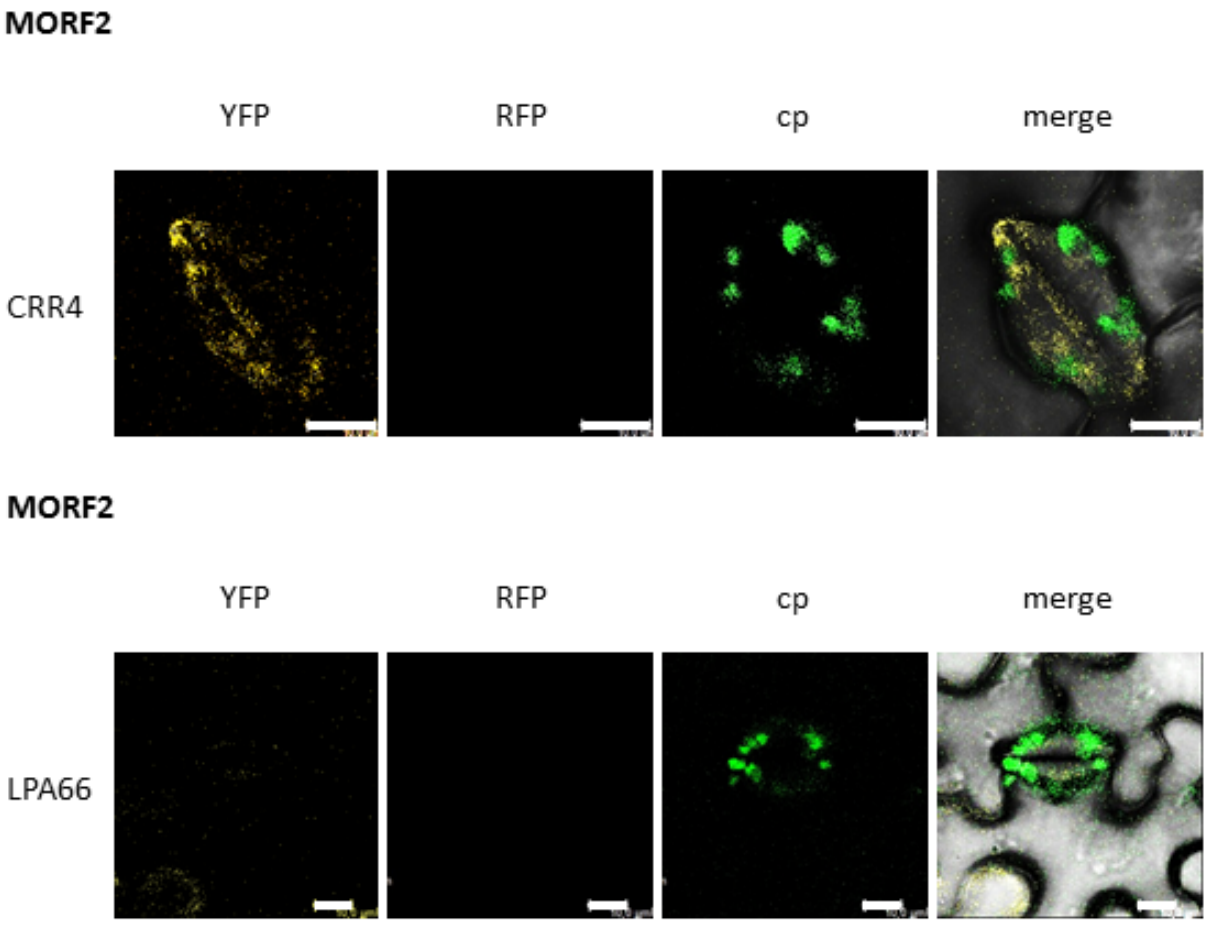


Abbildung 27: Vergrößerte Darstellung des YFP-Signals von MORF2 mit CRR4 (oben) und LPA66 (unten) in Chloroplasten**.** Dargestellt ist der fokussierte Bereich, in dem das YFP-Signal zu sehen ist, deshalb ist in diesem Bildausschnitt kein RFP-Signal zu sehen**.** Die Länge der Maßstabsleiste beträgt 10 µm. „YFP" bezeichnet den Kanal, mit dem nur das YFP-Signal detektiert wurde (525-600 nm) und gelb eingefärbt ist,

„RFP“ den Kanal, mit dem das RFP-Signal detektiert wurde (576-629 nm) (rot). Die mitochondriale Zielsequenz der alternativen Oxidase (AOX) diente an RFP fusioniert als Marker für die Mitochondrien. „cp“ bezeichnet den Kanal, mit dem die Chlorophyll-Autofluoreszenz detektiert wurde (650-704 nm) (grün). „merge“ überlagert die Signale dieser drei Kanäle und das im Hellfeld gewonnene Bild der Tabakepidermiszellen.

MORF9 interagiert sowohl mit LPA66 als auch mit CRR4 in den Chloroplasten (Abbildung 28).

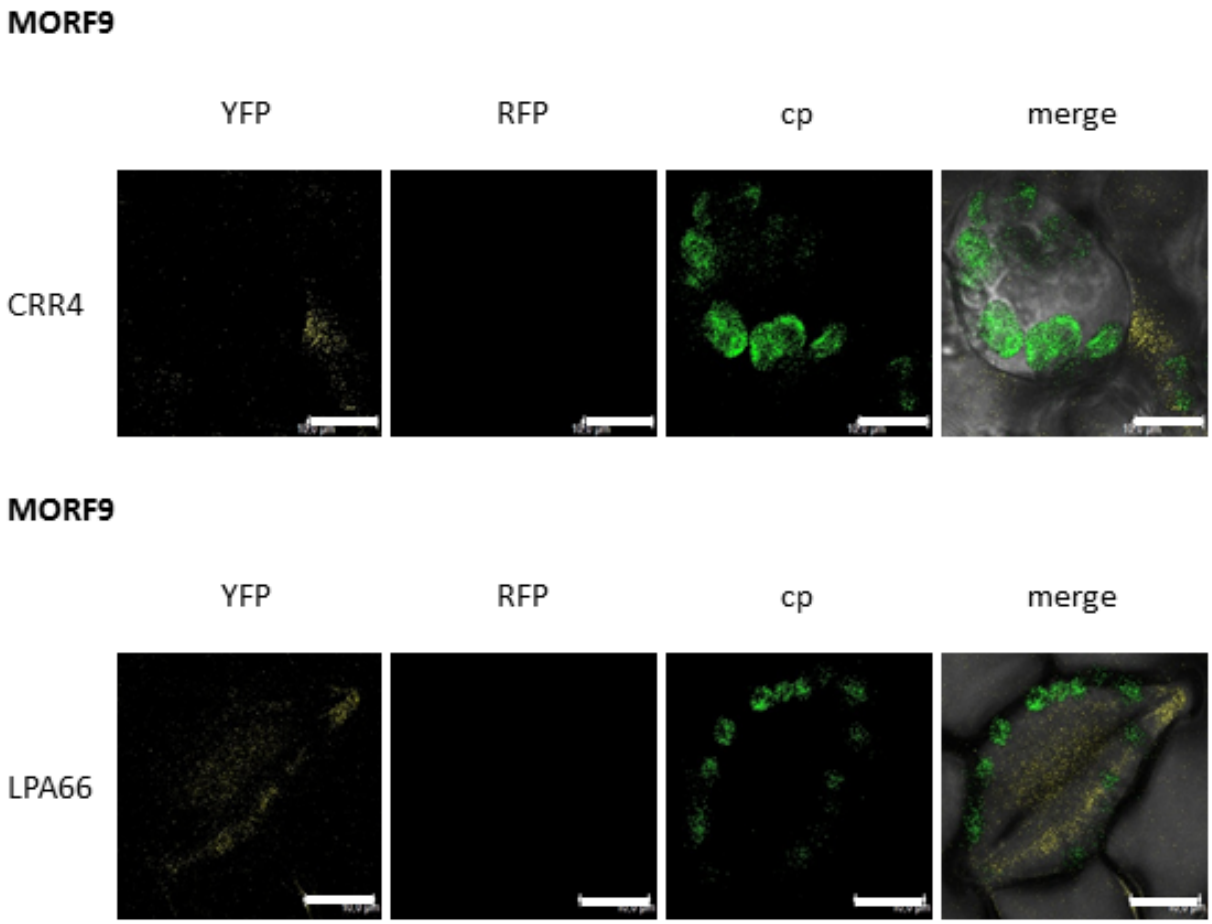


Abbildung 28: Vergrößerte Darstellung des YFP-Signals von MORF9 mit CRR4 (oben) und LPA66 (unten) in Chloroplasten**.** Dargestellt ist der Bereich, in dem das YFP-Signal zu sehen ist, deshalb ist in diesem Bildausschnitt kein RFP-Signal zu sehen**.** Die Länge der Maßstabsleiste beträgt 10 µm. „YFP“ bezeichnet den Kanal, mit dem nur das YFP-Signal detektiert wurde (525-600 nm) und gelb eingefärbt ist, „RFP“ den Kanal, mit dem das RFP-Signal detektiert wurde (576-629 nm) (rot). Die mitochondriale Zielsequenz der alternativen Oxidase (AOX) diente an RFP fusioniert als Marker für die Mitochondrien. „cp“ bezeichnet den Kanal, mit dem die Chlorophyll-Autofluoreszenz detektiert wurde (650-704 nm) (grün). „merge“ überlagert die Signale dieser drei Kanäle und das im Hellfeld gewonnene Bild der Tabakepidermiszellen.

Da fast alle MORF-Proteine in Yeast-Two-Hybrid-Analysen mit MEF1 (Zehrmann *et al*., 2009) interagieren (A. Zehrmann, persönliche Mitteilung) wurden MORF1, MORF2, MORF4, MORF5, MORF6, MORF8 und MORF9 auf ihre Interaktion mit MEF1 in Tabakepidermiszellen untersucht (Abbildung 29).

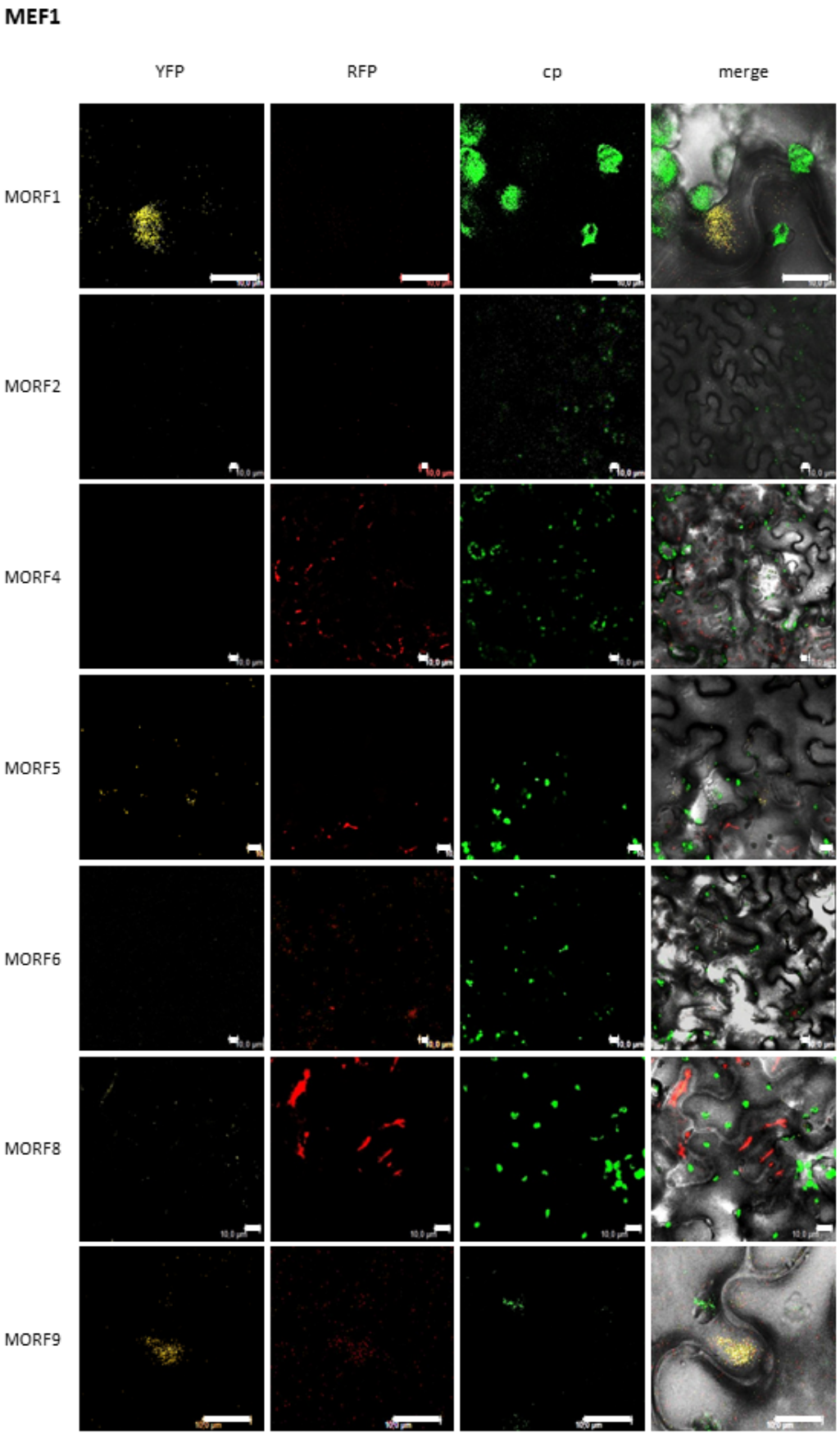


Abbildung 29: Darstellung des Auftretens eines YFP-Signals für die Kombination von MEF1 mit MORF1, MORF2, MORF3, MORF5, MORF6, MORF8 und MORF9. Für MORF1+MEF1, MORF5+MEF1, MORF8+MEF1

und MORF9+MEF1 ist ein YFP-Signal zu sehen. In diesen Aufnahmen ist der Bereich mit dem YFP-Signal vergrößert dargestellt. Die Länge der Maßstabsleiste beträgt 10 µm. „YFP“ bezeichnet den Kanal, mit dem nur das YFP-Signal detektiert wurde (525-600 nm) und gelb eingefärbt ist, „RFP“ den Kanal, mit dem das RFP-Signal detektiert wurde (576-629 nm) (rot). Die mitochondriale Zielsequenz der alternativen Oxidase (AOX) diente an RFP fusioniert als Marker für die Mitochondrien. „cp“ bezeichnet den Kanal, mit dem die Chlorophyll-Autofluoreszenz detektiert wurde (650-704 nm) (grün). „merge“ überlagert die Signale dieser drei Kanäle und das im Hellfeld gewonnene Bild der Tabakepidermiszellen.

Für MORF2, MORF4 und MORF6 wurde kein YFP-Signal mit MEF1 detektiert. Für die Kombinationen mit MORF1, MORF5, MORF7, MORF8 und MORF9 wurde ein YFP-Signal aufgenommen. Das YFP-Signal der Kombination MORF9 und MEF1 ist in den Chloroplasten zu sehen, die YFP-Signale der Interaktion von MEF1 mit den anderen untersuchten MORFs in den Mitochondrien bzw. im Cytosol.

VI.4 Analyse des Splicing-Status mitochondrialer Transkripte in *matR*-Editing-Mutanten

Die beiden Stellen 1771 und 1895 im *matR*-Transkript, die in T-DNA-Insertionslinien von MEF4 bzw. MEF14 nicht mehr editiert werden, liegen in der Domäne X der putativen Maturase. Da in Northern-Blots mit RNA aus den *mef14-1*- und *mef14-2*-Mutanten keine Veränderung des Splicing-Status des *nad1*-Transkripts festgestellt werden konnte (Verbitskiy *et al*., 2011), wurden *mef4-1* und *mef14-2*-Pflanzen gekreuzt und die F1-Generation geselbstet, um eine Doppelmutante zu erhalten und in dieser die mögliche Beteiligung von matR am Splicing von *nad1* zu untersuchen.

Es wurden jedoch in der F2-Generation keine Pflanzen gefunden, die für die T-DNA-Insertionen in beiden Genen homozygot waren. 52 % der untersuchten Pflanzen waren homozygot für die Insertion in *MEF14* und hatten keine Insertion in *MEF4*, 24 % waren homozygot für die Insertion in *MEF14*, aber heterozygot für die Insertion in *MEF4*, 18 % trugen die Insertion in *MEF14* heterozygot und keine Insertion in *MEF4* und 6 % waren für beide Insertionen heterozygot.

Die weiteren Untersuchungen wurden deshalb mit der RNA aus Pollen von drei Pflanzen, die homozygot für die Insertion in *MEF14*, aber heterozygot für *MEF4* waren, durchgeführt.

Zunächst wurde die RNA in cDNA umgeschrieben und diese mit den Paaren aus Intron-überspannenden und jeweils im Intron und benachbarten Exon liegenden Oligonukleotiden von Koprivova *et al.* (2010) amplifiziert. Für das dritte Intron in *nad1* wurden unterschiedliche Ergebnisse für die RNA aus Blättern von C24, Col und L*er*, aus Blättern von *mef14-2*, *mef4-1* und *mef14-1* und aus Pollen der für *mef14-2* homozygoten und für *mef4-1* heterozygoten Kreuzungen (*mef4-1* x *mef14-2* F2-1 bis 3) (Abbildung 30) erhalten. In der EMS-Mutante *mef14-1* ist die Stelle *matR*-1895 zu 20 % editiert (Verbitskiy *et al.,* 2011). Nach 20 Zyklen sind für die cDNA aus allen untersuchten Pflanzen nur Signale mit dem Primerpaar, welches in Exon 4 und Intron 3 liegt, zu sehen. Nach 25 Zyklen zeigen C24, Col, L*er* und *mef14-2* zusätzlich eine leichte Bande für das Primerpaar in Exon 3 und Exon 4. Dieses Signal wird bei Erhöhung auf 30 Amplifikationszyklen noch stärker. Dieses Signal für die in den beiden Exons gelegenen Primer tritt bei *mef4-1* x *mef14-2* F2-1, *mef4-1* x *mef14-2* F2-2, *mef4-1* x *mef14-2* F2-3 auch nach 30 Amplifikationszyklen nicht auf. Für *mef14-1* und *mef4-1* tritt nur das Signal für die beiden in den Exons gelegenen Primer auf.

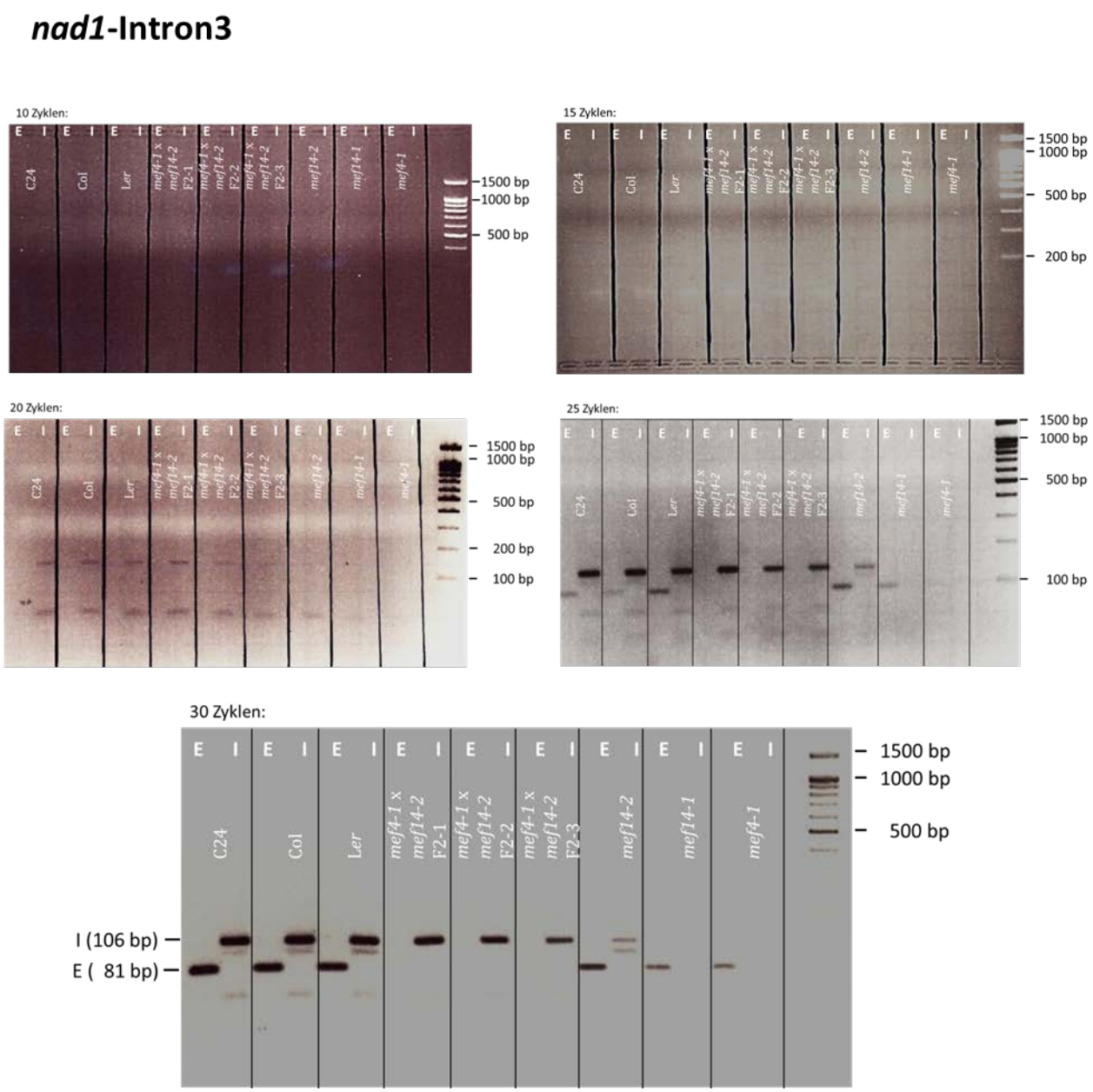


Abbildung 30: Ergebnisse der RT-PCR mit limitierter Zyklenzahl mit RNA aus Blattmaterial von C24, Col, L*er*, Pollen aus der F2-Generation der Kreuzung von *mef4-1* x *mef14-2* (heterozygot für die T-DNA-Insertion in *MEF4*, homozygot für die T-DNA-Insertion in *MEF14*), und Blattmaterial aus *mef14-2*, *mef14-1* und *mef4-1*. Die PCR zur Amplifikation der cDNA wurde jeweils mit einem Primerpaar in Exon 3 und Exon 4 („E“) und einem Primerpaar in Intron 3 und Exon 4 („I“) durchgeführt. Für die PCR mit Primerpaar E wurde ein Produkt von 81 bp Größe erwartet, mit Primerpaar I von 106 bp.

VII Diskussion

VII.1 Charakterisierung mehrerer PPR-Proteine als RNA-Editing-*trans*-Faktoren

Die Untersuchung von fünf Mutationslinien mit defektem RNA-Editing an jeweils ein bis sechs Stellen führte zur Identifizierung von fünf PPR-Proteinen, die am RNA-Editing in Mitochondrien beteiligt sind.

VII.1.1 Identifizierung durch EMS-Mutanten: MEF10, MEF12 und MEF13

Drei der Editingfaktoren wurden durch EMS-Mutanten identifiziert. Die EMS-induzierten Mutationen lagen bei zwei Faktoren in den für die E-Domänen, bei einem Faktor in dem für die L-Einheit kodierenden Sequenzabschnitten.

VII.1.1.1 **MEF10**

Die Zielstelle von MEF10, *nad2*-842, ist in pflanzlichen NAD2-Proteinen konserviert. Editing dieser Stelle führt zum Codon für die Aminosäure Phenylalanin statt der genomisch codierten Aminosäure Serin. Die Sequenzumgebung der Editingstelle ist jedoch sowohl in nicht pflanzlichen Proteinen als auch in Pflanzen kaum erhalten. Es ist also möglich, dass der Einbau von Serin, welches ohne Editing aus der mRNA entsteht, toleriert wird und sich deshalb auch kein Phänotyp der *mef10-1*-Mutante zeigt (Härtel *et al.,* 2013).

Durch den Aminosäureaustausch von Alanin zu Threonin in der E-Domäne von MEF10 wird die Stelle *nad2*-842 nicht mehr editiert. Möglicherweise kann das PPR-Protein dadurch nicht mehr an die RNA binden oder seine Interaktion mit MORF-Proteinen (Takenaka *et al*., 2012) wird erschwert.

VII.1.1.2 **MEF12**

Das von dem Locus At3g09040 kodierte Protein wurde als MEF12 identifiziert. Dieser Editingfaktor MEF12 ist für das Editing der Stelle *nad5*-374 verantwortlich. Ein Beweis durch Komplementation des fehlenden Editings in *mef12-1*-Protoplasten konnte nicht durchgeführt werden, da dieses Gen nicht in *E. coli* kloniert werden konnte. At3g09040 hat eine sehr lange Sequenz und ist im mittleren Teil sehr GC-reich, was die Amplifikation und Klonierung erschwert. MEF12 ist mit 25 PLS-Einheiten und einer E- und E^+-Domäne eines der längsten PPR-Proteine. Diese Identifizierung als MEF12 liefert

eine T-DNA-Insertionslinie dieses Gens, die eine Reduktion des Editings der Stelle *nad5*-374 auf 20 % zeigt. Zudem liegt in *mef12-1* eine EMS-Mutation nur in diesem und einem weiteren Gen für ein PPR-Protein in dem durch Kartierung ermittelten Bereich vor. Das zweite Gen At3g08820 wurde bereits ausgeschlossen, da sich durch Transformation von *mef12-1*-Protoplasten mit der Col-Wildtyp-Variante dieses Gens das Editing der Stelle *nad5*-374 nicht erhöht. Somit kann davon ausgegangen werden, dass es sich bei At3g09040 um MEF12 handelt. Durch das Editing der Stelle *nad5*-374 (CCA →CTA) entsteht statt des Codons für Prolin ein Codon für Leucin. Das verbleibende Editing von 20 % an der MEF12-Zielstelle in *mef12-2* könnte durch den Ort der Insertion der T-DNA erklärt werden. Wie aus Abbildung 10 ersichtlich, ist die T-DNA in die für die E-Domäne kodierenden Sequenz eingebaut. Es wäre also möglich, dass noch ein Teil-Protein gebildet wird, welches ein partielles Editing leisten kann.

Wie schon bei MEF10 bestätigt sich auch bei MEF12 die Bedeutung der C-terminalen Domänen E bzw. E und DYW für die Funktionalität der PLS-PPR-Proteine (Chateigner-Boutin und Small, 2010). Eine Mutation in diesem Bereich ist wohl ausreichend, dass das PPR-Protein nicht mehr an die RNA oder seine Interaktionspartner, wie die MORF-Proteine, bindet.

VII.1.1.3 **MEF13**

MEF13 weist mit sechs betroffenen Stellen die bisher meisten detektierbaren Ziele eines PPR-Proteins auf (Abbildung 11). Ebenso wie bei der Kartierung von MEF12 wurden auch hier zwei für PPR-Proteine kodierende Gene mit je einer EMS-Mutation im kartierten Bereich gefunden. Die stabile Transformation von *mef13-1*-Pflanzen mit jeweils einem der beiden Kandidatengene für *MEF13* zeigte für At3g02330 eine Komplementation der Editingeffizienzen aller sechs betroffenen Stellen (Abbildung 11). Bisher konnte für At3g02330 noch keine homozygote T-DNA-Insertionslinie gefunden werden, welche zusätzlich unabhängig bestätigen würde, dass At3g02330 der Editingfaktor MEF13 ist. Ein zusätzlicher Beweis für die Identifizierung von At3g02330 als MEF13 liefert aber die Beobachtung, dass das Editing an der Stelle *nad7*-213 im Ökotyp C24 von 50 % im Wildtyp auf 100 % erhöht wird. MEF13 ist wahrscheinlich noch an weiteren, nicht erkennbaren RNA-Editing-Prozessen beteiligt, da die geringe Editingeffizienz an Stelle *nad5*-1663 von 20 % auf 60 % durch das transgene At3g02330 in stabil transformierten *mef13-1*-Pflanzen erhöht wird (Abbildung 16). Zwei der sechs RNA-Editingereignisse, *nad5*-1914 (TTC → TTT) und *nad7*-213 (GAC → GAT), führen

nicht zu einer Veränderung der genomisch codierten Aminosäuren. Editing in den *ccmFc*- und *nad4*-Transkripten führt jeweils zum Codon für Leucin statt Prolin, durch Editing der Stellen *cox3*-314 und *nad2*-59 wird jeweils Phenylalanin statt Serin codiert.

Ein Vergleich der jeweiligen *cis*-Elemente an den sechs Editingstellen zeigt nur drei in diesen Sequenzen gemeinsame Uridine an den Stellen -9, -12 und -17 (Abbildung 17). Bezieht man das *cis*-Element der nur durch das transgene MEF13 editierten Stelle mit ein, sind an Position -9 und -12 ebenfalls Uridine vorhanden. Ein einzigartiges Muster von ca. 20 Nukleotiden bestimmt im Bereich 5‘ des zu editierenden Cs die Bindestelle eines PPR-Proteins (Chaudhuri und Maliga, 1996; Bock und Koop, 1997; Farré *et al.,* 2001; van der Merwe *et al.,* 2006). Im Gegensatz zum *cis*-Element an der Zielstelle von MEF10, das viele gemeinsame Nukleotide mit den *cis*-Elementen an den RNA-Editing-Stellen anderer Faktoren hat (Härtel *et al.,* 2013), bilden diese drei Us relativ wenig Gemeinsamkeiten. Jedoch sind auch bei MEF10 nur vier Nukelotide eindeutig der Erkennungssequenz von MEF10 zuzuordnen. Dies stimmt auch mit dem von Barkan *et al.* (2012) veröffentlichten PPR-Code überein, wonach sich durch den kombinatorischen Code zweier Aminosäuren die Wahrscheinlichkeit der Bindung eines PPR-Motivs an eine bevorzugte Base einstufen lässt. Diese geringen Übereinstimmungen zwischen den Zielsequenzen von MEF13 könnten einerseits darauf hindeuten, dass MEF13 relativ wenig Spezifität zur RNA-Sequenz zeigt. Das bedeutet, dass möglicherweise noch weitere Stellen von MEF13 erkannt werden, die in den Mutanten aber nicht auftauchen, da andere MEF-Proteine die MEF13-Funktion ersetzen können. Eine solche Redundanz ist z. B. an der Stelle *nad5*-1663 eine mögliche Erklärung für die restliche Reaktion in *mef13-1*. Andererseits lässt sich die niedrige Ähnlichkeit zwischen den Zielstellen vielleicht auch durch unterschiedliche Beteiligung von einzelnen PPR-Elementen erklären, da MEF13 mit 20 PPR-Motiven relativ groß ist (Abbildung 15).

VII.1.2 Identifizierung durch T-DNA-Insertionslinien: MEF28 und MEF30

Alle RNA-Editing-*trans*-Faktoren in Mitochondrien und Chloroplasten außer den neun MORF-Proteinen (Takenaka *et al*., 2012) gehören zur PLS-Klasse der PPR-Proteine (z. B. Kotera *et al*., 2005; Chateigner-Boutin *et al.,* 2008; Zehrmann *et al.,* 2008; Takenaka *et al.,* 2010). Deshalb wurden in T-DNA-Insertionslinien von Genen, die für bisher nicht charakterisierte PPR-Proteine kodieren, die Editingeffizienzen mitochondrialer Stellen durch SNaPshot-Analysen untersucht (Takenaka und Brennicke, 2009; Takenaka, 2011; Takenaka und Brennicke, 2012).

VII.1.2.1 **MEF28**

Die Funktion von MEF28 als RNA-Editingfaktor wurde durch fehlendes Editing an den Stellen *nad2*-89 und *nad2*-90 in der T-DNA-Insertionsmutante *mef28-1* gezeigt (Abbildung 19). Ein Beweis für die Wirkung von MEF28 als Editingfaktor ist die Wiederherstellung des Editing dieser beiden Stellen durch das intakte *MEF28* in stabil transformierten *mef28-1*-Pflanzen. Die T-DNA-Insertion liegt in der Sequenz, die für die zehnte PPR-Einheit, das vierte P-Motiv, kodiert (Abbildung 21).

MEF28 ist der bisher einzige identifizierte RNA-Editing-Faktor, in dessen Mutante das Editing zweier benachbarter Stellen reduziert ist. Durch das Editing beider Nukleotide wird statt Serin Phenylalanin gebildet. Die Zielstelle von MEF19, *ccb206*-566, wird zu 100 % editiert, während die direkt benachbarte Stelle, *ccb206*-565, ein C bleibt (Takenaka *et al*., 2010). Das gleiche gilt für die Stelle *nad1*-308, die von MEF25 zu 100 % editiert wird, deren Nachbar *nad1*-309 jedoch nicht (M. Takenaka, persönliche Mitteilung). Im Fall von SLO2 wird die Stelle *mttB*-144 zu 50 % editiert, die benachbarte Stelle *mttB*-145 zu 100 % (Zhu *et al.*, 2012). MEF13 editiert nur eines von vier aufeinanderfolgenden genomischen Cs in der *nad2*-mRNA, *nad2*-59. Die Stellen *nad2*-60, *nad2*-61 und *nad2*-62 bleiben C. Bei all diesen mitochondrialen Editing-Faktoren handelt es sich um PPR-Proteine mit E-Domäne. MEF28 ist dagegen ein PPR-Protein mit DYW-Domäne. Durch das Deletionskonstrukt At5g06540ΔDYW sollte überprüft werden, ob MEF28 ohne DYW-, nur mit E-Domäne nur noch eine der beiden Stellen in der *mef28-1*-Mutante editieren kann. Interessanterweise konnte jedoch keine der beiden Stellen editiert werden. Dies ist der zweite Fall nach MEF1 (Zehrmann *et al.,* 2010), in dem die DYW-Domäne essentiell für die Funktion als Editing-Faktor zu sein scheint. Möglicherweise lässt sich das Editing einer der beiden Stellen in *mef28-1* erhöhen, wenn statt eines Deletionskonstruktes ein Konstrukt mit fehlender DYW-Domäne, aber E-Domäne eines anderen Faktors, z. B. SLO2, eingebracht wird.

MEF28 könnte jedoch auch nur für das Editing der Stelle *nad2*-89 verantwortlich sein und die Stelle *nad2*-90 von einem zweiten Faktor editiert werden, der erst nach Editing der ersten Stelle binden kann. Um dies zu überprüfen, müssten eine Vielzahl von *nad2*-cDNAs sequenziert und der Editingstatus der beiden Stellen analysiert werden. Wenn dabei vereinzelt cDNAs gefunden werden, in denen nur die erste Stelle editiert ist und andere, in denen nur die zweite Stelle editiert ist, müsste MEF28 für das Editing beider Stellen verantwortlich sein. Wenn jedoch neben cDNAs, in denen beide Stellen editiert

sind, bei manchen nur die erste Stelle editiert ist, ist MEF28 wahrscheinlich nur für das Editing der ersten Stelle verantwortlich und ein weiterer Faktor für die zweite Stelle. Dieser weitere Faktor würde dann ein U vor dem zu editierenden C benötigen, könnte also in *mef28-1* nicht an das *cis*-Element der Stelle *nad2*-90 binden.

VII.1.2.2 **MEF30**

Für die Bestätigung von MEF30 als RNA-Editingfaktor sollten stabil transformierte *mef30-1*-Pflanzen konstruiert werden. Diese wurden aber auch in mehrmaligen Versuchen nicht erhalten. Dies könnte ein Problem der Stelle des Einbaus von *MEF30* ins Genom oder eine Inkompatibilität einer solchen transgenen Überexpression sein. Durch Editing der Stellen in der *nad4*-mRNA und *cyt*-mRNA werden Serin bzw. Tyrosin gebildet. In Protoplasten konnte durch das intakte MEF30 das Editing der Stelle *cyt*-982 von 0 % auf 70 % und der Stelle *nad4*-1033 von 0 % auf 50 % erhöht werden (Abbildung 22). Diese partielle Komplementation lässt sich wahrscheinlich durch eine niedrige Transformationseffizienz der Protoplasten erklären. Um eine Erhöhung auf 100 % zu sehen, hätte wahrscheinlich nur aus transformierten Protoplasten RNA gewonne, und deren jeweilige cDNA für *nad4* und *cyt* sequenziert werden dürfen.

VII.2 Untersuchung der RNA-Editing-Effizienz aller chloroplastidären Stellen in den Mutanten von *morf2*, *morf9* und *morf5*

Erstaunlicherweise sind in den beiden Mutanten *morf2-1* und *morf9-1* fast alle chloroplastidären Editingstellen betroffen (Tabelle 1). Allerdings sind in Chloroplasten auch nur 34 Editingstellen vorhanden (Chateigner-Boutin und Small, 2010), im Gegensatz zu den 300 – 400 Stellen in Mitochondrien (Giegé und Brennicke, 1999). Einige Stellen werden in beiden Mutanten *morf2-1* und *morf9-1* überhaupt nicht editiert, an den meisten Stellen ist die Editingeffizienz reduziert und manche Stellen zeigen nur in einer der beiden Mutanten einen Verlust der Editingaktivität. Beide Proteine, MORF2 und MORF9 werden also für das Editing fast aller Stellen in den Chloroplasten benötigt. Wenn man die Anzahl der betroffenen Stellen jedoch mit denen in *morf1-1* vergleicht, ist dies nicht mehr so überraschend hoch. In *morf1-1* sind mehr als 40 mitochondriale Editingstellen betroffen (Takenaka *et al.,* 2012). Aufgrund der betroffenen Stellen in den Chloroplasten lässt sich vermuten, dass MORF2 und MORF9 an den meisten Stellen als Heterodimere zusammenarbeiten und sich in den Mutanten teilweise gegenseitig

ersetzen können. An einigen Stellen, wie *petL*-5 und *psbZ*-50 (Tabelle 1, Abbildung 23), die in Abwesenheit eines der beiden Faktoren auch nicht mehr partiell editiert werden, wirkt das jeweils abwesende MORF-Protein wohl als Homodimer. Zur Kontrolle wurden auch die Editingeffizienzen der chloroplastidären Editingstellen in der *psy*-Mutante (Kugelmann *et al.,* 2013) untersucht. Die meisten Stellen sind in dieser Mutante zu 75 % bis 100 % editiert, was im Rahmen der experimentellen Abweichung liegt. Manche Stellen, wie *ndhD*-2, *clpP*-559, *petL*-5 und *rpoA*-200 scheinen eher durch sekundäre Effekte, die durch die defekte Chloroplastenentwicklung erklärbar sind, ihr Editing in *morf2-1* und *morf9-1* verloren zu haben, da sie auch in der *psy*-Mutante kein oder deutlich reduziertes Editing zeigen (Tabelle 1).

Durch die Identifizierung der MORF-Proteine (Takenaka *et al.,* 2012) und der Annahme, dass diese untereinander in direkter Interaktion stehen und einander ersetzen können, muss das bisherige Modell eines Pflanzeneditosoms erweitert werden. Bisher wurde angenommen, dass PPR-Proteine die *cis*-Elemente an der RNA-Editingstelle erkennen, die RNA binden und entweder durch die Deaminase-Aktivität ihrer DYW-Domäne die Stelle editieren oder die Bindestelle für ein noch zu identifizierendes Enzym bereitstellen, welches die Editingaktivität ausführt (Zehrmann *et al.,* 2011; Salone *et al.,* 2007). Dieses Modell muss dahingehend erweitert werden, dass ein PPR-Protein das cis-element an der Editingstelle erkennt und bindet und dadurch die Bindestelle für ein MORF-Protein schafft. Die Anwesenheit des MORFs-Proteins bzw. der als Heterodimere wirkenden Proteine, könnte dann die Voraussetzung für das Binden des Editingenzyms sein (Takenaka *et al*., 2012).

Um dieses Modell und die Möglichkeit der Interaktion von MORF-Proteinen untereinander und mit PPR-Proteinen zu überprüfen, wurden von Takenaka *et al.* (2012) Yeast-Two-Hybrid-Analysen durchgeführt. Diese bestätigten eine Bindung von MORF-Proteinen an andere MORF-Proteine und an PPR-Proteine in Hefezellen. Darüber hinausgehend sollte in dieser Arbeit überprüft werden, ob diese Interaktionen auch in der natürlichen Umgebung der MORF- und PPR-Proteine in Pflanzenzellen zu beobachten sind.

VII.3 Analyse potentieller Interaktionen verschiedener RNA-Editing-*trans*-Faktoren durch BiFC

Zum einen wurden hier die z. T. nur vorhergesagten Lokalisationen der MORF-Proteine in Mitochondrien und/oder Plastiden in der Pflanzenzelle durch Fluoreszenzmikroskopie untersucht. Zum anderen wurden die Interaktionen der MORF-Proteine untereinander und mit PPR-Proteinen in ihrem physiologischen Umfeld analysiert.

VII.3.1Intrazelluläre Lokalisation der MORF-Proteine

Für MORF2 wurde der Import in Chloroplasten von Chatterjee *et al.* (1996) und Bisanz *et al*. (2003) experimentell nachgewiesen. Bentolila *et al*. (2012) haben MORF8 bzw. RIP1 durch Immunopräzipitation mit dem chloroplastidären RNA-Editing-Faktor RARE1 (Robins *et al*., 2009) identifiziert. MORF2 und MORF9 wurden in MS-Proteom-Analysen zusammen mit chloroplastidären Proteinen gefunden, MORF3, MORF5 und MORF8 zusammen mit mitochondrialen Proteinen. Durch Fusion aller neun MORF-Proteine an YFP wurden in transient transformierten Tabakepidermiszellen Lokalisationsstudien durchgeführt. Für MORF1, MORF3, MORF4 und MORF6 wurde das YFP-Signal ausschließlich in den Mitochondrien detektiert. Die YFP-Signale für MORF5 und MORF8 zeigten sich sowohl in den Chloroplasten als auch in den Mitochondrien, für MORF2 und MORF9 nur in den Chloroplasten. Diese Lokalisationen stimmen sowohl mit den bereits durchgeführten Lokalisationsstudien (Chatterjee *et al.,* 1996; Bisanz *et al*., 2003; Bentolila *et al.,* 2012) als auch mit den Vorhersagen überein. Zudem konnten Bentolila *et al.* (2012) in der Mutante von MORF8 (RIP1) sowohl in Mitochondrien als auch in Chloroplasten betroffene Stellen identifizieren. Aufgrund der Detektion des YFP-Signals von MORF5 in Chloroplasten wurden in der Mutante *morf5-1* die chloroplastidären Stellen untersucht. Das Editing der Stelle *petL*-5 ist jedoch nur um 60 % reduziert, nur die Stelle *rpl23*-89 ist gar nicht mehr editiert. Diese beiden Stellen sind auch in den Mutanten von MORF2 und MORF9 betroffen. Es könnte sich also auch um sekundäre Effekte handeln, da zum einen die Zahl der betroffenen Stellen wesentlich geringer als in den ausschließlich in Chloroplasten lokalisierten MORF-Proteinen ist und zum anderen auch die als Kontrolle untersuchte *psy*-Mutante verringertes Editing der Stelle *petL*-5 zeigt (Tabelle 1).

VII.3.2 Bildung von Homo-und Heterodimeren zwischen MORF-Proteinen

Da für einige Editing-Stellen in den Plastiden beide MORF2 und MORF9-Proteine notwendig sind, diese also möglicherweise interagieren und ferner in der *Arabidopsis*

Interactome Datenbank (Cui *et al*., 2008) eine Interaktion von MORF9 mit MORF6 vorhergesagt wird, wurden von Takenaka *et al.* (2012) mögliche Interaktionen von MORF-Proteinen in Yeast-Two-Hybrid-Analysen untersucht. In Hefezellen sind die meisten MORF-Proteine in der Lage, miteinander als Homo- und Heterodimere zu interagieren. Um zu untersuchen, ob diese Interaktionen auch *in vivo* in Pflanzenzellen auftreten, wurden hier entsprechende BiFC-Analysen durchgeführt (Tabelle 2, Abbildung 25). MORF2 und MORF9 zeigen für eine Interaktion als Heterodimere das stärkste YFP-Signal in Chloroplasten. Dies unterstützt die These, dass beide Proteine im Editosom zur Editierung einiger chloroplastidärer Stellen vorhanden sind. MORF2 zeigt auch als Homodimer ein YFP-Signal in Chloroplasten, jedoch ebenso wie bei MORF9 als Homodimer etwas schwächer als als Heterodimer. Die Interaktion von MORF2 mit dem ebenfalls z. T. in Chloroplasten lokalisierten MORF8 ist wiederum schwächer als bei MORF2-Homodimeren. Ebenso ist die Interaktion von MORF9 mit MORF5 erkennbar, aber relativ schwach. Zudem kann für MORF8 mit MORF5 und für MORF9 mit MORF6 ein YFP-Signal in Chloroplasten und Mitochondrien detektiert werden. MORF6 ist in der Lokalisationsstudie jedoch nicht in Chloroplasten beobachtet worden. Diese Interaktion könnte aufgrund der Überexpression der beiden *MORF*s durch den CaMV-35S-Promotor in den Tabakepidermiszellen zustande kommen. Es könnten so viele Protein-Moleküle gebildet werden, dass nicht mehr alle korrekt in die jeweiligen Organellen importiert werden. Dadurch könnten MORF-Proteine, die sich unter den Expressionsbedingungen des nativen Promotors nie begegnen würden, interagieren und eventuell auch ihren nicht nativen Bindungspartner mit in das andere Organell ziehen. Dies könnte auch eine Begründung für das YFP-Signal von MORF2 mit MORF8 in Mitochondrien sein. Die Unterschiede der Signalstärken von MORF2 und MORF9 als Homodimere bzw. Heterodimere können auch ein Hinweis auf die jeweils spezifischen Interaktionen an den unterschiedlichen RNA-Editingstellen in Chloroplasten sein. Die in den Mitochondrien lokalisierten MORF-Proteine zeigen für einige Interaktionen klare YFP-Signale in Mitochondrien, für die meisten Interaktionen lässt sich das Signal jedoch im Cytosol oder an Membranen detektieren. In diesen Fällen sind wahrscheinlich nur aufgrund der hohen Zahl an vorhandenen MORF-Proteinmolekülen Interaktionen möglich, die unter physiologischen Bedingungen mit den nativen Promotoren nicht zu sehen wären. Eindeutige Interaktionen sind für MORF1 mit MORF1 und für MORF1 mit MORF8 in Mitochondrien, für MORF2 mit MORF2, MORF2 mit MORF8 und für MORF2 mit MORF9 in Chloroplasten, für MORF3 mit MORF4 in Mitochondrien, MORF5 mit

MORF6 und MORF5 mit MORF7 in Mitochondrien, MORF6 mit MORF8 in Mitochondrien und MORF9 mit MORF9 in Chloroplasten zu sehen. Eventuell können die Partner der schwächeren Interaktionen zwischen MORF-Proteinen auch die eigentlich bevorzugten Interaktionen ersetzen, wenn einer der Partner der eigentlich stärkeren Interaktion fehlt. Dies würde auch erklären, warum in den Mutanten von MORF1 und MORF3 jeweils über 40 Stellen betroffen sind, in den Mutanten von MORF4 und MORF6 jedoch nur jeweils eine Stelle betroffen ist (Takenaka *et al.,* 2012). Wahrscheinlich können ein oder mehrere der anderen MORF-Proteine in diesen Fällen die Rolle von MORF4 und MORF6 in den jeweiligen Heterodimeren übernehmen. Bei MORF6 könnte das aufgrund der engen Verwandtschaft (Abbildung 2) MORF5 sein, MORF4 könnte durch MORF1 ersetzt werden und umgekehrt.

VII.3.3 Interaktionen einiger PPR-Proteine mit MORF-Proteinen

Für einige Stellen in Chloroplasten und Mitochondrien, deren Editingeffizienz in MORF-Mutanten reduziert ist, waren bereits PPR-Proteine als *trans*-Faktoren identifiziert worden. In Chloroplasten werden für das Editing der Stelle *ndhD*-2 sowohl das PPR-Protein CRR4 (Kotera *et al.,* 2005) also auch MORF2 und MORF9 benötigt. Das Editing der Stelle *psbF*-65 ist sowohl in der Mutante von LPA66 (Cai *et al.,* 2009) als auch in *morf2-1* abwesend, in *morf9-1* jedoch nur um 30 % von 100 % auf 70 % reduziert. In BiFC-Analysen können sowohl für die Interaktion von CRR4 mit jeweils MORF2 und MORF9 als auch von LPA66 mit jedem dieser beiden in Chloroplasten lokalisierten MORF-Proteine YFP-Signale detektiert werden. Die verbleibenden 70 % Editing an der Stelle *psbF*-65 in der *morf9-1* Mutante spiegeln sich jedoch nicht in einem schwächeren YFP-Signal für die Interaktion von LPA66 mit MORF9 wider.

Der mitochondriale Editingfaktor MEF1 (Zehrmann *et al.,* 2009) scheint mit einigen MORF-Proteinen zu interagieren, mit denen keine gemeinsamen Editingstellen gefunden wurden. Diese Interaktionen sind jedoch trotzdem spezifisch, da sie zum einen mit MORF9 nur in Chloroplasten, mit MORF1, MORF5, MORF7 und MORF8 in Mitochondrien auftreten. Andererseits werden für die Kombinationen von MEF1 mit MORF2, MORF4 und MORF6 keine YFP-Signale detektiert. Zusammenfassend lässt sich sagen, dass MORF- und PPR-Proteine mehr oder weniger spezifisch miteinander interagieren können und so gemeinsam in den Editosomen für die jeweiligen Editingstellen vorliegen können.

VII.4 Analyse des Splicing-Status mitochondrialer Transkripte in *matR*-Editing-Mutanten

Ziel dieser Analyse war, die Funktion der Maturase (matR) aufzuklären, die in Intron 4 der *nad1*-mRNA kodiert ist. Dazu wurden RNA-Editing-Mutanten mit Defekten im Editing der *matR*-mRNA auf die Prozessierung der nad1 mRNA hin untersucht. In Abbildung 30 sind bereits nach 25 Zyklen und noch deutlicher nach 30 Amplifikationszyklen Signale für das *trans*-Splicing Intron 3 von *nad1* in den drei Pflanzen, die homozygot für die Insertion in MEF14 und heterozygot für die Insertion in MEF4 sind (*mef4-1* x *mef14-2* F2), zu sehen. Für die drei Wildtypen-Ökotypen Col, C24 und L*er* treten diese Signale nach 20 Zyklen auf. Nach 25 Zyklen treten hier jedoch auch Signale für die Produkte der in den Exons 3 und 4 gelegenen Primerpaare auf. Diese Signale werden nach 30 Zyklen stärker. In den drei Ökotypen liegen also sowohl reife als auch unreife Transkripte von *nad1* vor. In den Pollen von *mef4-1* x *mef14-2* F2 ist wahrscheinlich nur die unreife Form der *nad1*-mRNA vorhanden. Intron 3 ist gar nicht oder nur sehr wenig prozessiert, sodass keine reife funktionsfähige mRNA entsteht. Es ist jedoch verwunderlich, dass in der F2 Generation der Kreuzung keine Doppelmutanten gefunden wurden, da Defekte in der NADH-Ubichinon-Oxidoreduktase nicht letal sein sollten (Sledwo und Umbach, 1995). Dieser Befund könnte andeuten, dass matR auch am Splicing von anderen Introns beteiligt ist, die in mRNAs für essentielle Proteine liegen. Die Mutanten *mef14-1* und *mef4-1* zeigen nach 25 und 30 Zyklen jeweils nur ein Signal für die cDNA, die von der reifen *nad1*-mRNA stammt. Diese Signale sind jedoch schwächer als die für die drei Wildtypen detektierten. Eigentlich würde in diesen beiden Mutanten entweder dasselbe Signalmuster wie für den WT-Ökotypen erwartet werden, wenn matR nicht an der Reifung der *nad1*-mRNA beteiligt ist oder jeweils eine der nicht editierten Stellen in der Domäne X keine Auswirkungen auf die potentielle Maturaseaktivität hat. Bei einer Auswirkung des durch fehlendes Editing an Stelle 1771 eingebauten Arginins statt Cysteins oder an Stelle 1895 Serins statt Leucins auf die matR-Funktion, müsste für die Amplifikation der cDNA mit den Primern in den Exons 3 und 4 („E“) kein oder nur ein schwaches Signal und für die Primer in Intron 3 und Exon 4 („I“) ein starkes Signal zu sehen sein. Eventuell hat in diesen Fällen auch die Amplifikation mit dem Primerpaar „I“ trotz Wiederholungen nicht funktioniert. Zur Klärung dieser Ergebnisse und zur Bestätigung der Beteiligung von matR als Maturase an der Reifung der *nad1*-mRNA müssen Northern Blot-Analysen mit Sonden für das Intron 3, die verbundenen Exons 3 und 4 und die verbundenen Exons 4

und 5 durchgeführt werden. Ferner sollten die RT-PCR-Ergebnisse mit anderen Primerpaaren überprüft werden.

VIII Literaturverzeichnis

Andrés, C., Lurin, C., and Small, I.D. (2007). The multifarious roles of PPR proteins in plant mitochondrial gene expression. Physiol. Plant. **129:** 14-22.

Aubourg, S., Boudet, N., Kreis, M., and Lecharny, A. (2000). In *Arabidopsis thaliana*, 1 % of the genome codes for a novel protein family unique to plants. Plant Mol. Biol. **42**: 603-613.

Barkan, A., Rojas, M., Fujii, S., Yap, A., Chong, Y. S., Bond, C. S., and Small, I. (2012). A Combinatorial Amino Acid Code for RNA Recognition by Pentatricopeptide Repeat Proteins. PLoS Genet. **8**: e1002910. DOI 10.1371/journal.pgen.1002910.

Beick, S., Schmitz-Linneweber, C., Williams-Carrier, R., Jensen, B., and Barkan, A. (2008). The pentatricopeptide repeat protein PPR5 stabilizes a specific tRNA precursor in maize chloroplasts. Mol. Cell. Biol. **28:** 5337-5347.

Benne, R., Van den Burg, J., Brakenhoff, J. P., Sloof, P., Van Boom, J. H. and Tromp, M. C. (1986). Major transcript of the frameshifted *coxII* gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. Cell **46**: 819-826.

Bentolila, S., Heller, W. R., Sun, T., Babina, A. M., Friso, G., Van Wijk, K. J., and Hanson, M. (2012). RIP1, a member of an *Arabidopsis* protein family, interacts with the protein RARE1 and broadly affects RNA editing. Proc. Natl. Acad. Sci. USA **109**: E1453-E1461.

Bergthorsson, U., Adams, K., Thomason, B., and Palmer, J. D. (2003). Widespread horizontal transfer of mitochondrial genes in flowering plants. Nature **424**: 197-201.

Bisanz, C., Bégot, L., Carol, P., Perez, P., Bligny, M., Pesey, H., Gallois, J.-L., Lerbs-Mache, S., and Mache, R. (2003). The Arabidopsis nuclear DAL gene encodes a chloroplast protein which is required for the maturation of the plastid ribosomal RNAs and is essential for chloroplast differentiation. Plant Mol. Biol. **51**: 651-663.

Blanc, V., Litvak, S., and Araya, A. (1995). RNA editing in wheat mitochondria proceeds by a deamination mechanism. FEBS Lett. **373**: 56-60.

Blanchard, J. L., and Schmidt, G. W. (1995). Pervasive migration of organellar DNA to the nucleus in plants. J. Mol. Evol. **41**: 397-406.

Binder, S., Marchfelder, A. and Brennicke, A. (1994) RNA editing of $tRNA^{Phe}$ and $tRNA^{Cys}$ in mitochondria of *Oenothera berteriana* is initiated in precursor molecules. Mol. Gen. Genet. **244**: 67-74.

Bock, R., and Koop, H. U. (1997). Extraplastidic site-specific factors mediate RNA editing in chloroplasts. EMBO J **16**: 3282-3288.

Bock, R. (2001). RNA editing in plant mitochondria and chloroplasts. In: Bass, B.L. (Ed.), RNA editing, Oxford University Press, Oxford. Pp. 38-60.

Bonen, L. (2011). RNA Splicing in Plant Mitochondria. In: Kempken, F. (Ed.), Plant Mitochondria, Advances in Plant Biology I, Springer Science+Business Media. Pp. 131-150.

Brennicke, A., Grohmann, L., Hiesel, R., Knoop, V., and Schuster, W. (1993). The mitochondrial genome on its way to the nucleus: Different stages of gene transfer in plants. FEBS Lett. **325**: 140-145.

Cai, W., Ji, D., Peng, L., Guo, J., Ma, J., Zou, M., Lu, C., and Zhang, L. (2009). LPA66 is required for editing *psbF* chloroplast cranscripts in *Arabidopsis*. Plant Physiol. **150**: 1260-1271.

Castandet, B., and Araya, A. (2012). The nucleocytoplasmic conflict, a driving force for the emergence of plant organellar RNA editing. IUBMB Life **64**: 120-125.

Cattaneo, R., Kaelin, K., Baczko, K., and Billeter, M. A. (1989). Measles virus editing provides an additional cysteine-rich protein. Cell **56**: 759-764.

Chateigner-Boutin, A.-L., Ramos-Vega, M., Guevara-Garcia, A., Andrés, C., Gutierrez-Nava, M. d. l. L., Cantero, A., Delannoy, E., Jimenez, L. F., Lurin, C., Small, I. D., and León, P. (2008). CLB19, a pentatricopeptide repeat protein required for editing of *rpoA* and *clpP* chloroplast transcripts. Plant J. **56:** 590-602.

Chateigner-Boutin, A. L., and Small, I. D. (2010) Plant RNA editing. RNA Biol. **7**:213–219.

Chatterjee, M., Sparvoli, S., Edmuns, C., Garosi, P. Findlay, K., and Martin, C. (1996). DAG, a gene required for chloroplast differentiation and palisade development in Antirrhinum majus. EMBO J. **15**: 4194-4207.

Chaudhuri, S., and Maliga, P. (1996). Sequences directing C to U editing of the plastid *psbL* mRNA are located within a 22 nucleotide segment spanning the editing site. EMBO J. **15**: 5958-5964.

Chen, S. H., Habib, G., Yang, C. Y., Gu, Z. W., Lee, B. R., Wen, S. A., Silberman, S. R., Cai, S. J., Deslypere, J. P., and Rosseneu, M. (1987). Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon. Science **238**: 363-366.

Clark, R. M., Schweikert, G., Toomajian, C., Ossowski, S., Zeller, G., Shinn, P., Warthmann, N., Hu, T. T., Fu, G., Hinds, D. A., Chen, H., Frazer, K. A., Huson, D. H., Schölkopf, B., Nordborg, M., Rätsch, G., Ecker, J. R., and Weigel, D. (2007). Common sequence polymorphisms shaping genetic diversity in Arabidopsis thaliana. Science **317**: 338-42.

Clough, S. J., and Bent, A. F. (1998). Floral Dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735-743.

Covello, P.S., and Gray, M.W. (1989). RNA editing in plant mitochondria. Nature **341**: 662-666.

Covello, P.S. and Gray, M.W. (1993). On the evolution of RNA editing. Trends Genet. **9**: 265-268.

Cui, J., Li, P., Li, G., Xu, F., Zhao, C., Li, Y., Yang, Z., Wang, G., Yu, Q., Li, Y., and Shi, T. (2008) AtPID: Arabidopsis thaliana Protein Interactome Database-An integrative platform for plant systems biology. Nucl. Acids Res. **36**: D999–D1008.

Curtis, M. and Grossniklaus, U. (2003). A Gateway™ cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiology **133**: 462-469.

Deblaere, R., Bytebier, B., De Greve, H., Deboek, F., Schell, J., Van Montagu, M. and Leemans, J. (1985). Efficient octopine Ti plasmid-derived vectores for *Agrobacterium*-mediated gene transfer to plants. Nucl. Acids Res. **13**: 4777-4788.

Delannoy, E., Le Ret, M., Faivre-Nitschke, E., Estavillo, G. M., Bergdoll, M., Taylor, N. L., Pogson, B. J., Small, I., Imbault, P., and Gualberto, J. M. (2009). Arabidopsis tRNA adenosine deaminase arginine edits the wobble nucleotide of chloroplast $tRNA^{Arg}$(ACG) and is essential for efficient chloroplast translation. Plant Cell **21**: 2058-2071.

Dèrozier,S., Samson,F., Tamby,J.-P., Guichard, C., Brunaud, V., Grevet, P., Gagnot, S., Label, P., Leplé, J.-C., Lecharny, A., and Aubourg, S. (2011). Exploration of plant genomes in the FLAGdb++ environment. Plant Methods **7**: 8.

Doolittle, W. F., Lukeš, J., Archibald, J. M., Keeling, P. J., and Gray, M. W. (2011). Comment on "Does constructive neutral evolution play an important role in the origin of cellular complexity?". BioEssays **33**: 427-429.

Faivre-Nitschke, S. E., Grienenberger, J. M., and Gualberto, J. M. (1999). A prokaryotic-type cytidine deaminase from *Arabidopsis thaliana* gene expression and functional characterization. Eur. J. Biochem. **263**: 896-903.

Farré, J.-C., and Araya, A. (1999). The *matR*-open reading frame is transcribed from a non-canonical promoter and contains an internal promoter to co-transcribe exons *nad1e* and *nad5III* in wheat mitochondria. Plant Mol. Biol. **40**: 959-967.

Farré, J.-C., Leon, G., Jordana, X., and Araya, A. (2001). *Cis* recognition elements in plant mitochondrion RNA editing. Mol. Cell Biol. **21**: 6731-6737.

Feagin, J. E., Abraham, J. M., and Stuart, K. (1988). Extensive editing of the cytochrome c oxidase III transcript in *Trypanosoma brucei*. Cell **53:** 413-422.

Forner, J. and Binder, S. (2007). The red fluorescent protein eqFP611: application in subcellular localization studies in higher plants. BMC Plant Biol. **7**: 28.

Freyer, R., Kiefer-Meyer, M. C., and Kossel, H. (1997). Occurrence of plastid RNA editing in all major lineages of land plants. Proc. Natl. Acad. Sci. USA **94**: 6285-6290.

Fujii, S., and Small, I. D. (2011). The evolution of RNA editing and pentatricopeptide repeat genes. New Phytologist **191**:37-47.

Giegé, P. and Brennicke, A. (1999). RNA editing in *Arabidopsis* mitochondria affects 441 C to U changes in ORFs. Proc. Natl. Acad. Sci. USA **96**: 15324-15329.

Gott, J. M., and Emeson, R. B. (2000). Functions and mechanisms of RNA editing. Annu. Rev. Genet. **34**: 499–531.

Gray, M. W. (2003). Diversity and evolution of mitochondrial RNA editing systems. IUBMB Life **55**: 227-233.

Gray, M. W., Lukeš, J., Archibald, J. M., Keeling, P. J., and Doolittle, W., F. (2010). Irremediable complexity? Science **330**: 920-921.

Gray, M. W. (2012). Evolutionary origin of RNA editing. Biochemistry. **51**: 5235-5242.

Grewe, F. Viehöver, P., Weisshaar, B., and Knoop, V. (2009). A trans-splicing group I intron and tRNA-hyperediting in the mitochondrial genome of the lycophyte *Isoetes engelmanii*. Nucl. Acids Res. **37**: 5093-5104.

Grewe, F., Herres, S., Viehöver, P., Polsakiewicz, M., Weisshaar, B., and Knoop, V. (2011). A unique transcriptome: 1782 positions of RNA editing alter 1406 codon identities in mitochondrial mRNAs of the lycophyte *Isoetes engelmanii*. Nucl. Acids Res. **39**: 2890-2902.

Groth-Malonek, W. Wahrmud, U., Polsakiewicz, M., and Knoop, V. (2007). Evolution of a pseudogene: Exclusive survival of a functional mitochondrial *nad7* gene supports Haplomitrium as the earliest liverwort lineage and proposes a secondary los of RNA editing in Marchantiidae. Mol. Biol. Evol. **24**: 1068-1074.

Gualberto, J. M., Bonnard, G., Lamattina, L. and Grienenberger, J. M. (1989). RNA editing in wheat mitochondria results in the conservation of protein sequence. Nature **341**: 660-662.

Gualberto, J. M., Weil, J. H. and Grienenberger, J. M. (1990). Editing of the wheat *cox*III transcript: evidence for twelve C to U and one U to C conversions and for sequence similarities around editing sites. Nucl. Acids Res. **18**: 3771-3776.

Härtel, B., Zehrmann, Verbitskiy, D., A., van der Merwe, J. A., Brennicke, A., and Takenaka, M. (2013). MEF10 is required for RNA editing at *nad2*-842 in mitochondria of *Arabidopsis thaliana* and interacts with MORF8. Plant Mol. Biol. DOI 10.1007/s11103-012-0003-2.

Handa, H. (2003). The complete nucleotide sequence and RNA editing content of the mitochondrial genome of rapeseed (*Brassica napus* L.): comparative analysis of the mitochondrial genomes of rapeseed and *Arabidopsis thaliana*. Nucl. Acids Res. **31**: 5907-5916.

Hiesel, R., Wissinger, B., Schuster, W. and Brennicke, A. (1989) RNA editing in plant mitochondria. Science **246**: 1632-1634

Hiesel, R., Wissinger, B. and Brennicke, A. (1990). Cytochrome oxidasae subunit II mRNAs in *Oenothera* mitochondria are edited at 24 sites. Curr. Genet. **18**: 371-375.

Hiesel, R., Combettes, B., and Brennicke, A. (1994). Evidence of RNA editing in mitochondria of all major groups of land plantsexcept the Bryophyta. Proc. Natl. Acad. Sci. USA **91**:629.

Hirose, T., and Sugiura, M. (2001). Involvement of a site-specific *trans*-acting factor and a common RNA-binding protein in the editing of chloroplast RNA: development of an *in vitro* RNA editing system. EMBO J. **20:** 1144-1152.

Hoch, B., Maier, R. M., Appel, K., Igloi, G. L. and Kössel H. (1991). Editing of a chloroplast mRNA by creation of an initiation codon. Nature **353**: 178-180.

Jobson, R. W., and Qiu, Y. L., (2008). Did RNA editing in plant organellar genomes originate under natural selection or through genetic drift? Biol. Direct **3**: DOI 10.1186/1745-6150-3-43.

Keren, I., Bezawork-Geleta, A., Kolton, M., Maayan, I., Belausov, E., Lavy, M., Mett, A., Gidoni, D., Shaya, F., and Ostersetzer-Biran, O. (2009). AtnMat2, a nuclear-encoded maturase required for splicing of group-II introns in *Arabidopsis* mitochondria. RNA J. **15**: 2299-2311.

Kobayashi, K., Kawabata, M., Hisano, K., Kazama, T., Matsuoka, K., Sugita, M., and Nakamura, T. (2012). Identification and characterization of the RNA binding surface of the pentatricopeptiderepeat protein. Nuc. Acids Res. **40**: 2712-2723.

Koprivova, A., Colas des Francs-Small, C., Calder, G., Mugford, S.t., Tanz, S., Lee, B.-R., Zechmann, B., Small, I., and Kopriva, S. (2010). Identification of a Pentatricopeptide Repeat Protein Implicated in Splicing of Intron1 of Mitochondrial *nad7* Transcripts. J. Biol. Chem. **285**: 32192-32199.

Kotera, E., Tasaka, M., and Shikanai, T. (2005). A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature **433**: 326–330.

Kugelmann, M., Fausser, A., Ossenbühl, F., and Brennicke, A. (2013). Phenotypes of Alb3p and carotenoid synthesis mutants show similarities regarding light sensitivity, thylakoid structure and protein stability. Photosynth. **51**: DOI 10.1007/s11099-012-0078-7

Kugita, M., Yamamoto, Y., Fujikawa, T., Matsumoto, T., and Yoshinaga, K. (2003). RNA editing in hornwort chloroplasts makes more than half the genes functional. Nucl. Acids Res. **31**: 2417-2423.

Lukeš, J., Archibal, J. M., Keeling, P. J., Doolittle, W. F., and Gray, M. W. (2011). How a neutral evolutionary ratchet can build cellular complexity. IUBMB Life **63**: 528-537.

Lurin, C., Andrés, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyère, C., Caboche, M., Debast, C., Gualberto, J.M., Hoffmann, B., Lecharny, A., Le Ret, M., Martin-Magniette, M.-L., Mireau, H., Peeters, N., Renou, J.-P., Szurek, B., Taconnat, L., and Small, I.D. (2004). Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell **16**: 2089–2103.

Mahendran, R., Spottswood, M. S., Ghate, A., Ling, M. L., Jeng, K., and Miller, D. L. (1991). Editing of the mitochondrial small subunit rRNA in *Physarum polycephalum*. EMBO J. **13**: 232-240.

Maier, U. G., Bozarth, A., Funk, H. T., Zauner, S., Rensing, S. A., Schmitz-Linneweber, C., Börner, T., and Tillich, M. (2008). Complex chloroplast RNA metabolism: just debugging the genetic programme? BMC Biol. **6**: 36-40.

Malek, O., Lättig, K., Hiesel, R., Brennicke, A. and Knoop, V. (1996). RNA editing in bryophytes and a molecular phylogeny of land plants. EMBO J. **15**: 1403-1411.

Mower, J. P., and Palmer, J. D. (2006). Patterns of partial RNA editing in mitochondrial genes of *Beta vulgaris*. Mol. Genet. Genomics **276**: 285-293.

Mulligan, M. (2004). RNA editing in plant organelles. In: Daniell, H., Chase, C.D. (Eds.), Molecular Biology and Biotechnology of Plant Organelles. Springer-Verlag, Dordrecht. Pp. 239-260.

Miyamoto, T., Obokata, J., and Sugiura, M. (2002). Recognition of RNA editing sites is directed by unique proteins in chloroplasts: biochemical identification of cis-acting elements and trans-acting factors involved in RNA editing in tobacco and pea chloroplasts. Mol. Cell. Biol. **22**: 6726-6734.

Neuwirt, J., Takenaka, M., van der Merwe, J. A., and Brennicke, A. (2005). An *in vitro* RNA editing system from cauliflower mitochondria: editing site recognition parameters can vary in different plant species. RNA **11**: 1563-1570.

Ohtani, S., Ichinose, M., Tasaki, E., Aoki, Y., Komura, Y., and Sugita, M. (2010). Targeted gene disruption identifies three PPR-DYW proteins involved in RNA editing for five editing sites of the moss mitochondrial transcripts. Plant Cell Physiol. **51**: 1942-1949.

Okuda, K., Chateigner-Boutin, A.-L., Nakamura, T., Delannoy, E., Sugita, M., Myouga, F., Motohashi, R., Shinozaki, K., Small, S., and Shikanaia, T. (2009). Pentatricopeptide Repeat Proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in *Arabidopsis* chloroplasts. Plant Cell **21**: 146-156.

Pfitzinger, H., Weil, J. H., Pillay, D. T., and Guillemaut, P. (1990). Codon recognition mechanisms in plant chloroplasts. Plant Mol. Biol. **14**: 805-814

Picardi, E., Regina, T. M., Brennicke, A., Quagliariello, C. (2007) REDIdb: the RNA editing database. Nucl. Acids Res. **35**: D173-D177

Powell, L. M., Wallis, S. C., Pease, R. J., Edwards, Y. H., Knott, T. J., and Scott, J. (1987). A novel form of tissue-specific RNA processing produces apolipoprotein-B48 in intestine. Cell **50**: 831-840.s

Rajasekhar, V. K. and Mulligan, R. M. (1993) RNA editing in plant mitochondria- a phosphate is retained during C-to-U conversion in mRNAs. Plant Cell **5**: 1843-1852.

Robbins, J. C., Heller, W. P., Hanson, M. R. (2009). A comparative genomics approach identifies a PPR-DYW protein that is essential for C-to-U editing of the Arabidopsis chloroplast *accD* transcript. RNA **15**: 1142–1153.

Rüdinger, M., Polsakiewicz, M., and Knoop, V. (2008). Organellar RNA editing and plant-specific extensions of pentatricopeptide repeat proteins in jungermaniid but not in marchantiid liverworts. Mol. Biol. Evol. **25**: 1405-1414.

Rüdinger, M., Funk, H. T., Rensing, S. A., Maier, U. G., and Knoop, V. (2009). RNA editing: only eleven sites are present in the *Physcomitrella patens* mitochondrial transcriptome and a universal nomenclature proposal. Mol. Genet. Genomics **281**: 473-481.

Rüdinger, M., Szövényi, P., Rensing, S. A., and Knoop, V. (2011). Assigning DYW-type PPR proteins to RNA editing sites in the funariid mosses *Physcomitrella patens* and *Funaria hygrometrica*. Plant J. **67**: 370-380.

Rüdinger, M., Volkmar, U., Lenz, H., Groth-Malonek, M., and Knoop, V. (2012) Nuclear DYW type PPR gene families diversify with increasing RNA editing frequencies in liverwort and moss mitochondria. J. Mol. Evol. **74**: 37-51.

Salone, V., Rüdinger, M., Polsakiewicz, M, Hoffmann, B., Groth-Malonek, M., Szurek, B., Small, I.D., Knoop, V., and Lurin, C. (2007). A hypothesis on the identification of the editing enzyme in plant organelles. FEBS Lett. **581**: 4132-4138.

Sambrook, J., and Russell, D. W. (2001). Molecular cloning. A laboratory manual. 3 rd edition, Cold Spring Harbor Laboratory Press, New York.

Sasaki, T., Yukawa, Y., Miyamoto, T., Obokata, J., and Sugiura, M. (2003). Identification of RNA editing sites in chloroplast transcripts from the maternal and paternal progenitors of tobacco (*Nicotiana tabacum*): comparative analysis shows the involvement of distinct *trans*-factors for *ndhB* editing. Mol. Bio. Evol. **20**: 1028-1035.

Sasaki, T., Yukawa, Y., Wakasugi, T., Yamada, K., and Sugiura, M. (2006). A simple *in vitro* RNA editing assay for chloroplast transcripts using fluorescent dideoxynucleotides: distinct types of sequence elements required for editing of ndh transcripts. Plant J. **47**: 802-810.

Schmitz-Linneweber, C., and Small, I. (2008). Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends Plant Sci. **13**: 663-670.

Schmitz-Linneweber, C., Williams-Carrier, R. E., and Barkan, A. (2005). RNA immunoprecipitation and microarray analysis show a chloroplast pentatricopeptide repeat protein to be associated with the 5' regions of mRNAs whose translation it activates. Plant Cell **17:** 2791-2804.

Schuster, W., Hiesel, R., Wissinger, B. and Brennicke, A. (1990). RNA editing in the cytochrome b locus of the higher plant *Oenothera berteriana* includes a U-to-C transition. Mol. Cell Biol. **10**: 2428-2431.

Sledow, J. N., and Umbach, A. L. (1995). Plant Mitochondrial Electron Transfer and Molecular Biology. Plant Cell **7**: 821-831.

Small, I., and Peeters, N. (2000). The PPR motif- a TPR-related motif prevalent in plant organellar proteins. Trends Biochem. Sci. **25**: 46-47.

Sparkes, I. A., Runions, J., Kearns, A. and Hawes, C. (2006). Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. Nat. Protoc. **4**: 2019-2025.

Steinhauser, S., Beckert, S., Capesius, I., Malek, O., and Knoop, V. (1999). Plant mitochondrial RNA editing. J. Mol. Evol. **48**: 303-312.

Stoltzfus, A. (1999). On the possibility of constructive neutral evolution. J. Mol. Evol. **49**: 169-181.

Stupar, R. M., Lilly, J. W., Town, C. D., Cheng, Z., Kaul, S., Buell, C. R., and Jiang, J. (2001). Complex mtDNA constitutes an approximate 620-kb insertion on *Arabidopsis thaliana* chromosome 2: implication of potential sequencing errors caused by large-unit repeats. Proc. Natl. Acad. Sci. USA **98**: 5013-5099.

Swarbreck, D., Wilks, C., Lamesch, P., Berardini, T. Z., Garcia-Hernandez, Foerster, H., Li, D., Meyer, T., Muller, R., Ploetz, L., Radenbaugh, A., Singh, S., Swing, V., Tissier, C., Zhang, P., and Huala, E. (2007). The Arabidopsis Information Resource (TAIR): gene structure and function annotation. Nucl. Acids Res. **36:** DOI 10.1093/nar/gkm965

Takenaka, M. and Brennicke, A. (2003). *In vitro* RNA editing in pea mitochondria requires NTP or dNTP, suggesting involvement of an RNA helicase. J. Biol. Chem. **278**: 130-136.

Takenaka, M., Neuwirt, J., Brennicke, A. (2004) Complex *cis*-elements determine RNA editing site in pea mitochondria. Nucl. Acids Res. **32**: 413-4144.

Takenaka, M., van der Merwe, J. A., Verbitskiy, D., Neuwirt, J., Zehrmann, A. and Brennicke, A. (2008a). RNA editing in plant mitochondria. In:Göringer, H.U. (Ed.) RNA editing. Nucleic acids and Molecular Biology **20**, Springer-Verlag, Berlin, Heidelberg. Pp. 105-122.

Takenaka, M., Verbitskiy, D., van der Merwe, J. A., Zehrmann, A., and Brennicke, A. (2008b). The process of RNA editing in plant mitochondria. Mitochondrion **8:** 35-46.

Takenaka, M. and Brennicke, A. (2009) Multiplex single base extension typing to identify nuclear genes required for RNA editing in plant organelles. Nucl. Acids Res. **37**: e13.

Takenaka, M. (2010). MEF9, an E-Subclass Pentatricopeptide Repeat Protein, Is Required for an RNA Editing Event in the nad7 Transcript in Mitochondria of *Arabidopsis*. Plant Physiol. **152:** 939-47.

Takenaka, M., Verbitskiy, D., Zehrmann, A., and Brennicke, A. (2010). Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. J. Biol. Chem. **285**: 27122-27129.

Takenaka, M. (2011). Identifying specific *trans*-factors of RNA editing in plant mitochondria by multiplex single base extension typing. In: RNA and DNA editing. (R. Aphasizhev, ed.) Humana Press, Springer, Heidelberg. Pp. 151-161.

Takenaka, M., Zehrmann, A., Verbitskiy, D., Kugelmann, M., Härtel, B., and Brennicke, A. (2012). Multiple organellar RNA editing factor (MORF) family proteins are required for RNA editing in mitochondria and plastids of plants. Proc. Natl. Acad. Sci. USA **109**: 5104-5109.

Takenaka, M., and Brennicke, A. (2012). Using multiplex single base extension typing to screen for mutants defective in RNA editing. Nat. Protoc. **7**: 1931-1945.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., and Kumar S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. **28**: 2731-2739.

Tillich, M., Funk, H. T., Schmitz-Linneweber, C., Poltnigg, P., Sabater, B., Martin, M., and Maier, R. M. (2005). Editing of plastid RNA in *Arabidopsis thaliana* ecotypes. Plant J. **43**: 708-715.

Tillich, M., Lehwark, P., Morton, B. R., and Maier, U. G. (2006). The evolution of chloroplast RNA editing. Mol. Biol. Evol. **23**: 1912-1921.

van der Merwe, J. A., Takenaka, M., Neuwirt, J., Verbitskiy, D., and Brennicke, A. (2006). RNA editing sites in plant mitochondria can share *cis*-elements. FEBS Lett. **580**: 268-272.

Verbitskiy, D., Zehrmann, A., van der Merwe J. A., Brennicke A. and Takenaka M. (2010). The PPR protein encoded by the LOVASTATIN INSENSITIVE 1 gene is involved in RNA editing at three sites in mitochondria of *Arabidopsis thaliana*. Plant J. **61**: 446-455.

Verbitskiy, D., Zehrmann, A., Härtel, B., Brennicke, A., and Takenaka, M. (2011). The DYW-E-PPR protein MEF14 is required for RNA editing at site *matR*-1895 in mitochondria of *Arabidopsis thaliana*. FEBS Lett. **585**: 700-704.

Vidal, S., Curran, J., and Kolakofsky, D. (1990). A stuttering model for paramyxovirus P mRNA editing. EMBO J. **9**: 2017-2022.

Wissinger, B., Brennicke, A., and Schuster, B. (1992). Regenerating good sense: RNA editing and *trans* splicing in plant mitochondria. Trends Genet. **8**: 322-328.

Wolf, P. G., Rowe, C. A., and Hasebe, M. (2004). High levels of RNA editing in vascular plant chloroplast genomes: analysis of transcripts from the fern *Adiantnum capillus-veneris*. Gene **339**: 89-97.

Yoo, S.-D., Cho, Y.-H., and Sheen, J. (2007). *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. **2**: 1565-1572.

Yu, W. and Schuster, W. (1995). Evidence for a site-specific cytidine deamination reaction involved in C to U RNA editing of plant mitochondria. J. Biol. Chem. **270**: 18227-18233.

Zehrmann, A., van der Merwe, J. A., Verbitskiy, D., Brennicke, A., and Takenaka, M. (2008). Ecotype-specific variations in the extent of RNA editing in plant mitochondria. Mitochondrion **8:** 319-327.

Zehrmann, A., van der Merwe, J. A., Verbitskiy, D., Brennicke, A. and Takenaka, M. (2009). A DYW-domain containing PPR-protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. Plant Cell **21**: 558-567.

Zehrmann, A., Verbitskiy, D., Härtel, B., Brennicke, A., and Takenaka, M. (2010). RNA editing competence of trans-factor MEF1 is modulated by ecotype-specific differences but requires the DYW domain. FEBS lett. **584**: 4181-4186.

Zehrmann, A., Verbitskiy, D., Härtel, B., Brennicke, A., and Takenaka, M. (2011) PPR proteins network as site-specific RNA editing factors in plant organelles. RNA Biol **8**: 67–70.

Zehrmann, A., van der Merwe, J. A., Verbitskiy, D., Härtel, B., Brennicke, A., and Takenaka, M. (2012). The DYW-class PPR protein MEF7 is required for RNA editing at four sites in mitochondria of *Arabidopsis thaliana*. RNA Biol. **9**: 1-7.

Zeller, G., Clark, R. M., Schneeberger, K., Bohlen, A., Weigel, D., and Rätsch, G. (2008). Detecting Polymorphic Regions in the *Arabidopsis thaliana* Genome with Resequencing Microarrays, Genome Res. DOI 10.1101/gr.070169.107.

Zhu, Q., Dugardeyn, J., Zhang, C., Takenaka, M., Kühn, K., Craddock, C., Smalle, J., Karampelias, M., Denecke, J., Peters, J., Gerats, T., Brennicke, A., Eastmond, P., Meyer, E. H., and Van Der Straeten, D. (2012). SLO2, a mitochondrial pentatricopeptide repeat protein affecting several RNA editing sites is required for energy metabolism. Plant J. **71**: 836 - 849.

Zybailov B., Rutschow, H., Friso, G., Rudella, A., Emanuelsson, O., Sun, Q., and van Wijk, K. J. (2008) Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. PLoS One **3**:e1994

IX Publikationsliste

IX.1 Publikationen

8) **Härtel, B**., Zehrmann, Verbitskiy, D., A., van der Merwe, J. A., Brennicke, A., and Takenaka, M. (2013). MEF10 is required for RNA editing at *nad2*-842 in mitochondria of Arabidopsis thaliana and interacts with MORF8. Plant Mol. Biol. DOI 10.1007/s11103-012-0003-2.

7) Verbitskiy, D., Zehrmann, A., **Härtel, B.**, Brennicke, A., and Takenaka, M. (2012). Two related RNA editing proteins target the same sites in mitochondria of Arabidopsis thaliana. J. Biol. Chem. DOI 10.1074/jbc.M112.397992.

6) Takenaka, M., Zehrmann, A., Verbitskiy, D., Kugelmann, M., **Härtel, B**., and Brennicke, A. (2012). Multiple organellar RNA editing factor (MORF) family proteins are required for RNA editing in mitochondria and plastids of plants. Proc. Natl. Acad. Sci. USA **109**, 5104-5109.

5) Zehrmann, A., van der Merwe, J. A., Verbitskiy, D., **Härtel, B.**, Brennicke, A., and Takenaka, M. (2012). The DYW-class PPR protein MEF7 is required for RNA editing at four sites in mitochondria of Arabidopsis thaliana. RNA Biol., **9**, 1-7.

4) Verbitskiy, D., van der Merwe, J. A., Zehrmann, A., **Härtel, B.**, and Takenaka, M. (2012). The E-class PPR Protein MEF3 of Arabidopsis thaliana can Function in Mitochondrial RNA editing also with an Additional DYW Domain. Plant Cell Physiol. **53**, 358-367.

3) Zehrmann, A., Verbitskiy, D., **Härtel, B.**, Brennicke, A., and Takenaka, M. (2011). PPR proteins network as site-specific RNA editing factors in plant organelles. RNA Biol. **8**, 67-70.

2) Verbitskiy, D., **Härtel, B.**, Zehrmann, A., Brennicke, A., and Takenaka, M. (2011). The DYW-E-PPR protein MEF14 is required for RNA editing at site matR-1895 in mitochondria of Arabidopsis thaliana. FEBS Lett. **585**, 700-704.

1) Zehrmann, A., Verbitskiy, D., **Härtel, B.**, Brennicke, A., and Takenaka, M. (2010). RNA editing competence of trans-factor MEF1 is modulated by ecotype-specific differences but requires the DYW domain. FEBS Lett. **584**, 4181-4186.

IX.2 Reviews

1) Zehrmann, A., Verbitskiy, D., **Härtel, B**., Brehme, N., Takenaka, M., and Brennicke, A. (2012). RNA-Editing: Das Gen ist nicht alles. Naturw. Rundsch. **65**, 281-287.

IX.3 Kongressvorträge

1) „MEFs and MORFs: site-specific and multiple sites organellar RNA editing factors"
1st Summer Academy in Plant Molecular Biology
Freudenstadt, 09.07.2012 - 11.07.2012

IX.4 Poster

16) „MEFs & MORFs: two types of RNA editing factors"
Barbara Härtel, Anja Zehrmann, Daniil Verbitskiy, Mizuki Takenaka, Axel Brennicke
Endosymbiosis from Prokaryotes to Eukaryotic Organelles- International Meeting
München, 10.10.2012 – 13.10.2012

15) „Mapping the binding site between MEF and MORF RNA editing factors"
Eszter Czászár, Anja Zehrmann, **Barbara Härtel**, Daniil Verbitskiy, Axel Brennicke, Mizuki Takenaka
Endosymbiosis from Prokaryotes to Eukaryotic Organelles- International Meeting
München, 10.10.2012 – 13.10.2012

14) „Interactions between various editing factors"
Anja Zehrmann, Daniil Verbitskiy, **Barbara Härtel**, Eszter Czászár, Axel Brennicke, Mizuki Takenaka
Endosymbiosis from Prokaryotes to Eukaryotic Organelles- International Meeting
München, 10.10.2012 – 13.10.2012

13) „MORF proteins are novel RNA editing factors in plant organelles"
Mizuki Takenaka, Matthias Kugelmann, Daniil Verbitskiy, Anja Zehrmann, **Barbara Härtel,** Axel Brennicke
Endosymbiosis from Prokaryotes to Eukaryotic Organelles- International Meeting
München, 10.10.2012 – 13.10.2012

12) „Mapping the binding site between MEF and MORF RNA editing factors"
Eszter Czászár, Anja Zehrmann, **Barbara Härtel**, Daniil Verbitskiy, Axel Brennicke, Mizuki Takenaka
XV. Annual Meeting of the German Section of the International Society for Endocytobiology
Martinsried, 08.10.2012 – 10.10.2012

11) „Interactions between various editing factors"
Anja Zehrmann, Daniil Verbitskiy, **Barbara Härtel**, Eszter Czászár, Axel Brennicke, Mizuki Takenaka
XV. Annual Meeting of the German Section of the International Society for Endocytobiology
Martinsried, 08.10.2012 – 10.10.2012

10) „MEFs & MORFs: two types of RNA editing factors"
Barbara Härtel, Anja Zehrmann, Daniil Verbitskiy, Mizuki Takenaka, Axel Brennicke XV. Annual Meeting of the German Section of the International Society for Endocytobiology
Martinsried, 08.10.2012 – 10.10.2012

9) „RNA-binding propertis of factors involved in RNA-editing"
Matthias Kugelmann, Daniil Verbitskiy, Anja Zehrmann, **Barbara Härtel**, Axel Brennicke, Mizuki Takenaka
5th Regio Plant Science Meeting Stuttgart-Tübingen-Ulm
Ulm, 05.11.2011

8) „The ecotype specific editing factor MEF4 and the potential role of matR"
Barbara Härtel, Anja Zehrmann, Daniil Verbitskiy, Mizuki Takenaka, Axel Brennicke
5th Regio Plant Science Meeting Stuttgart-Tübingen-Ulm
Ulm, 05.11.2011

7) „RNA editing factor MEF5 causes ecotype specific editing effects at site *ccb382*-709"
Barbara Härtel, Anja Zehrmann, Daniil Verbitskiy, Rhonda Meyer, Thomas Altmann, Axel Brennicke, Mizuki Takenaka
International Conference for Plant Mitochondrial Biology
Hohenroda, 14.05.2011 – 19.05.2011

6) „Screening for interactors of plant mitochondrial RNA editing factors"
Anja Zehrmann, **Barbara Härtel**, Daniil Verbitskiy, Axel Brennicke, Mizuki Takenaka
International Conference for Plant Mitochondrial Biology
Hohenroda, 14.05.2011 – 19.05.2011

5) „Two similar MEFs (PPRs) share the function"
Daniil Verbitskiy, Anja Zehrmann, **Barbara Härtel**, Axel Brennicke, Mizuki Takenaka
International Conference for Plant Mitochondrial Biology
Hohenroda, 14.05.2011 – 19.05.2011

4) „Screening for interaction partners of mitochondrial editing factors"
Anja Zehrmann, **Barbara Härtel**, Daniil Verbitskiy, Axel Brennicke, Mizuki Takenaka
24. Tagung Molekularbiologie der Pflanzen
Dabringhausen, 22.02.2011 – 25.02.2011

3) „Role of PPR proteins in mitochondrial RNA editing"
Barbara Härtel, Anja Zehrmann, Daniil Verbitskiy, Axel Brennicke, Mizuki Takenaka
24. Tagung Molekularbiologie der Pflanzen
Dabringhausen, 22.02.2011 – 25.02.2011

2) „The DYW domain of MEF11 is required for efficient RNA editing"
Daniil Verbitskiy, Anja Zehrmann, **Barbara Härtel**, Axel Brennicke, Mizuki Takenaka
4th Regio Plant Science Meeting Stuttgart-Ulm-Tübingen
Tübingen, 7.10.2010

1) „Role of PPR proteins in RNA editing in plant mitochondria"
Barbara Härtel, Anja Zehrmann, Daniil Verbitskiy, Mizuki Takenaka, Axel Brennicke
4th Regio Plant Science Meeting Stuttgart-Ulm-Tübingen
Tübingen, 07.10.2010

X Publikationen

Copyright Notice:
Reprinted from FEBS Letters 548 (2010),
Zehrmann, A., Verbitskiy, D., Härtel, B., Brennicke, A., Takenaka, M.
„RNA editing competence of *trans*-factor MEF1 is modulated by ecotype-specific differences but requires the DYW domain.“
pp. 4181-4186,
Copyright 2010 Elsevier, with kind permission from Elsevier.

Author's personal copy

FEBS Letters 584 (2010) 4181–4186

FEBS Letters

journal homepage: www.FEBSLetters.org

RNA editing competence of *trans*-factor MEF1 is modulated by ecotype-specific differences but requires the DYW domain

Anja Zehrmann, Daniil Verbitskiy, Barbara Härtel, Axel Brennicke, Mizuki Takenaka *

Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany

ARTICLE INFO

Article history:
Received 19 April 2010
Revised 19 August 2010
Accepted 31 August 2010
Available online 7 September 2010

Edited by Tamas Dalmay

Keywords:
RNA editing
Plant mitochondria
PPR protein
MEF1
Ecotypes
DYW domain

ABSTRACT

RNA editing in plant mitochondria posttranscriptionally changes multiple cytidines to uridines. The RNA editing *trans*-factor MEF1 was identified via ecotype-specific editing polymorphisms in *Arabidopsis thaliana*. Complementation assays reveal that none of the three amino acid changes between Columbia (Col) and C24 individually alters RNA editing. Only one combination of these polymorphisms lowers editing at two of the three target sites, suggesting additive effects of the involved SNPs. Functional importance of the C-terminal DYW domain was analysed with DYW-truncated and extended constructs. These do not recover RNA editing in protoplasts and regain only low levels in stable transformants. In MEF1, the DYW domain is thus required for full competence in RNA editing and its C-terminus has to be accessible.
© 2010 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Since the detection of RNA editing in plant mitochondria more than 20 years ago, a lot of research has been done with the aim to identify the mechanism of the C to U-alteration and to determine the requirements of targeting the sites to be edited. Although the exact mode of action has not yet been revealed, it seems that the identity of the nucleotide is changed by a deamination reaction [1,2]. Concerning the *cis*-elements around the editing sites, a region between 20 nts upstream and only 3 nts downstream appears to be sufficient to identify a C-nucleotide target [3–5]. For the RNA editing in plastids several *trans*-factors have already been identified during the last few years [6–10], while the first *trans*-factors acting in mitochondria have been identified only recently [11–14].

All RNA editing *trans*-factors in plastids and mitochondria known so far belong to the class of pentatricopeptide repeat proteins (PPR proteins). Characteristic of these proteins is a repeated motif of about 35 amino acids [15]. The approximately 450 members of the nuclear-encoded protein family in flowering plants can be classified into different categories on the basis of the nature of the repeats and of various C-terminal extensions [16]. So far all factors involved in RNA editing exhibit at their C-terminus at least one extension, the so-called E-domain. Some possess in addition a region which is known as DYW domain. For two of the DYW-class PPR proteins involved in RNA editing in plastids, CRR22 and CRR28, it has been shown that their DYW domains are interchangeable and can be even completely removed without influence on the RNA editing efficiency of the respective target sites [8]. This observation suggests that the DYW domains are in vivo dispensable for correct function of these *trans*-factors. Removal of the E domain of the plastid editing factor CRR4 however resulted in significantly reduced editing efficiency of its target site in transgenic plants, indicating that the E domain is required for RNA editing. Exchanging the E domains of *trans*-factors CRR4 and CRR21 yielded functional chimeric proteins, suggesting that these E domains have a common function in RNA editing [7].

The first mitochondrial factor MEF1 was discovered via ecotype-specific editing polymorphisms in *Arabidopsis thaliana*. Two sites in mitochondrial transcripts, *rps4*-956 and *nad7*-963, show a lower editing efficiency of 40–50% in ecotype C24 when compared to 100% C to U alteration in ecotype Col [17]. In two independent EMS mutant lines no detectable editing is observed at these two sites and in addition RNA editing at a site in the *nad2* transcript (*nad2*-1160) is strongly reduced. The nuclear-encoded editing factor was identified by linkage-based cloning and verified by complementation of C24 and mutant protoplasts. While in the mutant plants single amino acid changes in MEF1 inactivate RNA editing, the reduced editing of *rps4*-956 and *nad7*-963 in C24 is connected with three SNPs between the ecotypes Columbia (Col)

* Corresponding author. Fax: +49 731 502 2626.
E-mail addresses: anja.zehrmann@uni-ulm.de (A. Zehrmann), daniil.verbitskiy@uni-ulm.de (D. Verbitskiy), barbara.haertel@uni-ulm.de (B. Härtel), mo.bo@uni-ulm.de (A. Brennicke), mizuki.takenaka@uni-ulm.de (M. Takenaka).

0014-5793/$36.00 © 2010 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2010.08.049

Author's personal copy

and C24 which alter the encoded amino acid sequence of MEF1 [11]. We here investigate the influence of each of these SNPs on the editing efficiency of the affected sites. Furthermore we examine the functional requirement for the DYW domain in this RNA editing *trans*-factor.

2. Materials and methods

2.1. Plant material

Seeds for the *Arabidopsis thaliana* ecotypes C24 and Col-0 were kind gifts of J. Forner and S. Binder (Universität Ulm). The two mutant lines *mef1-1* and *mef1-2* are derived from an EMS mutant population of *Arabidopsis thaliana* ecotype Col obtained from Lehle Seeds (http://www.arabidopsis.com). These had been identified by a multiplexed SNaPshot approach [18]. All plants were grown as described previously [17].

2.2. Protoplast complementation assays

Preparation of protoplasts from 3- to 4-week-old individual plantlets and transfection was performed by the method of Yoo et al. [19]. Transfected genes were expressed from the 35S promoter in vector pSMGFP4 [20]. The C24 ecotype-specific mutations were introduced into the Col MEF1 reading frame by site-directed mutagenesis [21]. Deletion of the region coding for the DYW domain was achieved by inverse PCR [22]. Efficiency of the transfections was monitored as the RNA editing levels obtained in control transfections with the intact Col MEF1 reading frame. Total RNA was prepared after 20–24 h incubation at room temperature with the illustra RNAspin Mini Kit (GE Healthcare). Specific cDNA fragments were generated by RT-PCR amplification by established protocols [23]. The cDNA sequences (4base lab; Macrogen) were compared for differences in C to T ratios resulting from RNA editing. RNA editing levels were estimated by the relative height of the respective nucleotide peaks in the sequence analyses [11]. All assays were performed at least four times and interpreted according to the replicate results. These all agreed within the typical experimental variance of such biological assays. Seven assays were performed with different preparations of protoplasts from different plants. In three of these assays, one or the other data point (of a total of 16 parallel transfections and RNA preparations, and 48 RT-PCRs with sequence analyses in each assay series) had failed and was not interpretable. Thus four complete series of protoplast transfection assays could be used for the statistics in Fig. 2. The efficiency of parallel control transfections with the wt Col gene was taken as 100% in each separate set of assays to which each mutant was compared.

2.3. Plant transformation

To obtain transgenic plants, respective DNA sequences were cloned under control of the 35S promotor into the binary vector pMDC123 [24] and introduced into *mef1-1* mutant plants via *Agrobacterium tumefaciens* GV2260 by the method of Clough and Bent [25].

3. Results

3.1. In uence of the SNPs between Col and C24 in MEF1 on RNA editing of the target sites

In the DYW-class PPR protein MEF1 three amino acids differ between Col and C24, caused by three SNPs between the two ecotypes (Fig. 1A). The polymorphism in the first S-domain (nucleotide position 214) changes an Ala in the Col sequence to Thr in C24, the SNP at nucleotide position 314 alters a Lys to an Arg residue. In the E domain a conserved Gly is altered to a Ser in C24 by the polymorphism at nucleotide position 1297. The ecotype C24-specific MEF1 variant reduces the editing efficiency at two of the mitochondrial target sites, *rps4*-956 and *nad7*-963, to 40–50% which are edited to 100% in Col. This effect can be caused by amino acid alterations from either of these SNPs individually or by a combination of them.

To address this question and to investigate the influence of each of these non-synonymous SNPs on the editing efficiency of the affected sites, we monitored the recovery of RNA editing in cells of the mutants *mef1-1* and *mef1-2*, in which editing at the respective two target sites *rps4*-956 and *nad7*-963 is absent. In the first series of experiments, we transfected mutant protoplasts with three different constructs of the Col-*MEF1* gene mutated individually at each of the variant C24 nucleotide positions 214, 314 and 1297, respectively. Each of these variants recovered RNA editing at sites *rps4*-956 and *nad7*-963 in the transfection assays and increased the RNA editing efficiency at the third target site *nad2*-1160 (Fig. 1B). At this latter site, MEF1 does not seem to be required *per se* for editing, but enhances the reaction and is needed for complete C to U conversion in all steady state *nad2* mRNA molecules. The editing levels achieved by each of the three SNP-constructs were similar to the recovery of editing by complementation with the Col wild-type *MEF1*. These results show that single mutations of the nucleotides in positions 214, 314 and 1297, respectively, of the Col *MEF1* gene do not detract from the ability of the resulting MEF1 protein to complement editing deficient mutant protoplasts.

To analyse potential cumulative effects of the SNP-mutations, we next constructed derivates of *MEF1* with all possible combinations of the three non-silent SNPs between Col and C24 and transfected *mef1-1* and *mef1-2* EMS mutant protoplasts with each of these *MEF1* gene variants. The constructs with combinations of two altered nucleotides at SNP positions 1+2 and 1+3 still restore the ability for RNA editing at *rps4*-956 and *nad7*-963 and enhance RNA editing at *nad2*-1160 to levels comparable to the control transfections with the Col version of the gene (Fig. 2A, +*MEF1* C24-1+2, +*MEF1* C24-1+3; Fig. 2B). While the achieved levels of editing at *nad2*-1160 for all constructs tested are comparable to those after transfection with the Col version of the gene, the editing extents at *rps4*-956 and *nad7*-963 are lower in the protoplasts transfected with *MEF1* C24-2+3 than in protoplasts after introduction of the wild-type Col *MEF1* gene (Fig. 2A, *MEF1* C24-2+3; Fig. 2B).

Surprisingly, these lowered editing levels appear to slightly increase at *rps4*-956 and *nad7*-963, when mutant protoplasts are transfected with the C24 version of *MEF1*, almost up to the editing efficiency of protoplasts transfected with the Col version (+*MEF1* C24-1+2+3). However, these differences in the relative quantifications are not statistically significant and thus only suggest a trend (Fig. 2B)

3.2. Requirement of the DYW domain for MEF1 function

To investigate the role of the DYW domain for the function of the RNA editing *trans*-factor MEF1 we persued two lines of inquiries. The first was to test the requirement of the DYW domain for the editing activity, the second was to analyse the function of the highly conserved C-terminus of these proteins. For the first assays we deleted the DYW motif and tested the competence for the MEF1-ΔDYW to recover editing in mutant *mef1-1* protoplasts (*MEF1*-ΔDYW; Fig. 3A). RNA editing is not recovered at any of the three target sites: editing at the *rps4*-956 site is detectable, but very low at around the background limit, C to U conversion at *nad7*-963 is not detectable at all and the residual editing of

Author's personal copy

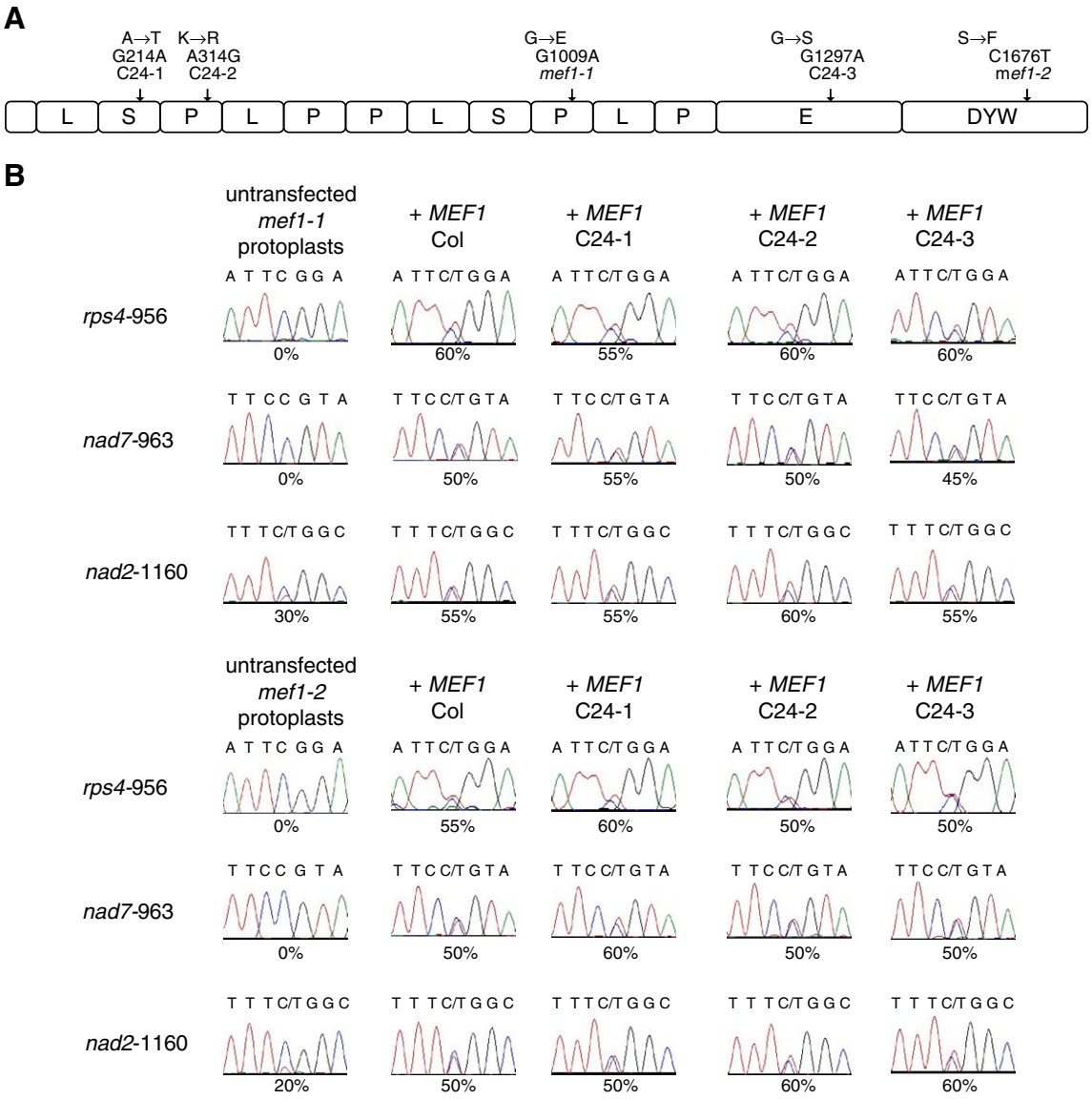


Fig. 1. Single mutations of the three non-silent nucleotide differences in the MEF1 gene between ecotypes Col and C24 do not change the ability of the resulting protein variants to complement mutant protoplasts. (A) MEF1 is a PPR protein of the DYW subclass. The positions of the SNPs (C24-1 to C24-3) between Col and C24 and of the point mutations in the EMS lines *mef1-1* and *mef1-2* are shown. (B) The editing efficiency at the three sites affected in mutant protoplasts is increased by transfection with the wild-type Col version of the gene *MEF1* (+*MEF1* Col traces). Introduction of *MEF1* gene versions mutated in nucleotide positions 214, 314 or 1297, respectively, found in the C24 version of this gene still leads to recovered RNA editing levels comparable to the wild-type Col version of *MEF1* (+*MEF1* C24-1 traces, *MEF1* C24-2 traces and *MEF1* C24-3 traces).

15–20% at site *nad2*-1160 is unaltered in the MEF1-ΔDYW transfected *mef1-1* protoplasts (Fig. 3B).

This apparent lack of function of the overexpressed MEF1-ΔDYW protein could be caused by the limited incubation time of the protoplasts. To exclude this parameter we generated transgenic plants stably overexpressing this truncated protein *MEF1*-ΔDYW. In these plants the RNA editing efficiencies at *rps4*-956, *nad7*-963 and *nad2*-1160 are increased at all target sites, but only to levels lower than the 100% editing achieved with the intact gene (Fig. 3C).

In the second series of DYW functional investigations we tested how important the accessibility of the DYW domain of MEF1 is for its function in RNA editing. Six histidines were attached to the C-terminus of the protein and the resulting protein was stably introduced in *mef1-1* plants. In contrast to editing efficiencies up to 100% attained in transgenic *mef1-1* plants with the wild-type Col MEF1, the MEF1+His protein with its C-terminal extension of six histidines has only a weak positive effect on the extent of RNA editing at either site (Fig. 3C).

4. Discussion

4.1. Interactions of several SNPs between Col and C24 in MEF1 lead to ecotype-speci c differences in RNA editing

The investigation of *trans*-factors involved in RNA editing in plant mitochondria has been considered to be challenging, as mutants defective in mitochondrial editing were suspected to cause severe phenotypes or to be even lethal. However, most of the mutants identified so far by screening of a collection of chemically mutagenized *Arabidopsis* Col plants show completely abolished editing of the respective target sites without causing severe phenotypic

Author's personal copy

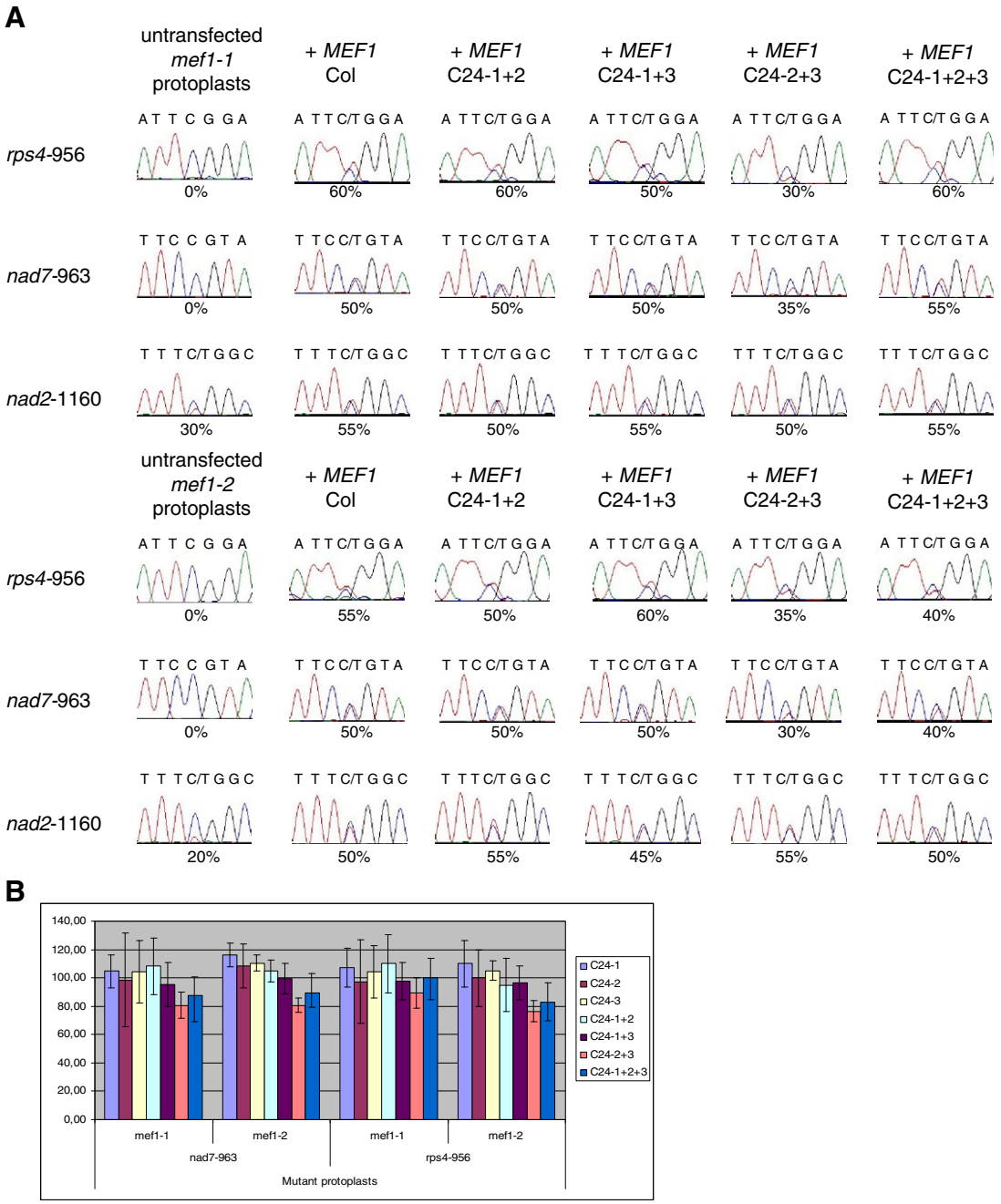


Fig. 2. Combinations of the ecotype C24-specific SNPs in *MEF1* affect RNA editing levels differentially at the three target sites. (A) Transfection of *mef1-1* (top part) and *mef1-2* (bottom part) mutant protoplasts with versions of *MEF1* after mutation of any of two or all three variant nucleotides still recovers editing and increases the editing efficiency at all three affected editing sites (+*MEF1* C24-1+2, *MEF1* C24-1+3, *MEF1* C24-2+3 and *MEF1* C24-1+2+3 traces). For site *nad2*-1160, editing recovery is with all constructs comparable to those achieved with the Col version of MEF1 (+MEF1 Col). In the experiment shown, the editing efficiencies at sites *nad7*-963 and *rps4*-956 reach the Col transfected levels in the protoplasts transfected with three of four versions of *MEF1* mutated in the variant nucleotides (+*MEF1* C24-1+2, *MEF1* C24-1+3, and *MEF1* C24-1+2+3 traces), while transfection with *MEF1* C24-2+3 leads to slightly lower levels. (B) Editing of sites *nad7*-963 and *rps4*-956 in protoplasts transfected with *MEF1* C24-2+3 is reduced in comparison to the transfection with the Col version of *MEF1*. Introduction of the C24 version (C24-1+2+3), however, appears to recover slightly higher editing levels. The error bars show that these interpretations are not statistically significant and thus only suggest possible trends. This graph summarizes the results of four independent experiments for each construct. RNA editing levels are shown relative to the levels recovered by transfection with the Col wt version of MEF1 (100%) in each series of assays to compensate for variations in protoplast quality and transfection. Individual transfections with other constructs may thus show better recovery of editing.

effects in the greenhouse [13,14]. Loss of editing sites may be detrimental under selective pressure in the wild, since in the analysis of ecotype-specific variations, RNA editing is never found to have been completely lost at any of the investigated sites between the three different ecotypes Col, L*er* and C24. Instead, only differing editing efficiencies between the ecotypes were observed at several editing sites [17]. These observations suggest that ecotype-specific polymorphisms are only tolerated when they have a moderate influence on protein structure and function and still allow sufficiently high levels of modification at the affected editing sites. In line with this reasoning we observe for MEF1 that each of the three ecotype-specific polymorphisms in the gene individually does not impair the function of the resulting MEF1 variant protein (Fig. 1B). Only when the last two of these amino acid identities

Author's personal copy

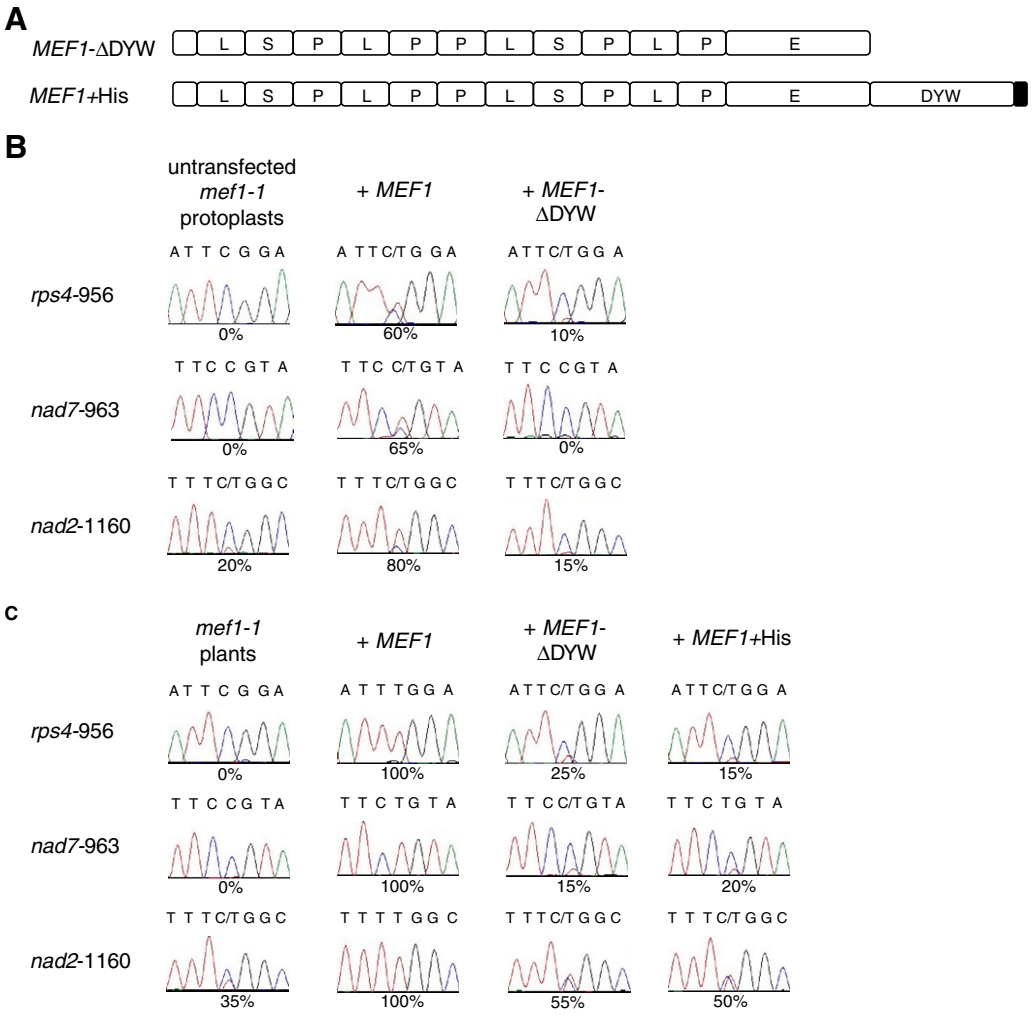


Fig. 3. The DYW domain of MEF1 is required for full competence in RNA editing at all three target sites. (A) Schematic of the MEF1 structure after deletion of the DYW domain and addition of 6 histidines, respectively. (B) Transfection with the truncated gene *MEF1*-ΔDYW does not remarkably increase RNA editing in mutant protoplasts at *rps4*-956, *nad7*-963 and *nad2*-1160 (+*MEF1*-ΔDYW) compared to wild-type transfected *mef1-1* protoplasts (+*MEF1*). (C) Introduction of the truncated gene into *mef1-1* plants does increase RNA editing at all target sites, but only to low levels (+*MEF1*-ΔDYW). Similar low editing recovery can be observed in transgenic *mef1-1* plants with an integrated *MEF1*+His gene (+*MEF1*+His), while the wild-type Col *MEF1* gene reconstitutes editing at all sites to 100% (+*MEF1*).

are altered to the C24 version, the mutated MEF1 protein is somewhat less active at the sites *nad7*-963 and *rps4*-956 than the Col wild-type version (Fig. 2A).

The reduced effectiveness in editing may originate from a lowered binding capacity to either of the target mRNAs or alternatively to other potential co-factors of the RNA editing reaction. Either scenario could be the result of a changed steric conformation caused by the altered amino acids. All three SNPs in C24 result in a decrease of the hydrophobicity of the encoded amino acid. This potentially leads to an aberrant solubility of parts of MEF1 and consequently may change folding of the C24 MEF1 protein and its editing activity.

Interesting is the differential effect of the ecotype-variations on the three editing targets:

The modification of the last two of the three C24 amino acids different from the Col MEF1 protein affects the editing efficiency at *nad7*-963 and *rps4*-956, but not at *nad2*-1160. This effect may be related to the observation that the three amino acids altered in C24 plants interfere less severely with the MEF1 activity in editing of this site than for sites *nad7*-963 and *rps4*-956. The fully converted C24-version of the MEF1 protein recovers nearly as much activity as the Col MEF1 version at all three target sites (Fig. 2A). The negative effects of two C24 amino acids in MEF1 on the editing efficiency thus appear to be partially neutralized by the third C24 amino acid. Here it has to be taken into account that a presently unsolvable potential source of variation may reside in the Col nuclear background of the *mef1-1* and *mef1-2* mutants. The C24 plants may have accumulated potentially compensatory mutations in factors interacting with MEF1 arisen in C24 to adjust to the three C24-SNPs in MEF1. These mutations may not be present in the respective factors in Col.

These site-specific effects of combined amino acid changes in MEF1 may be caused by a disturbed binding to the RNA or by an altered interaction with other protein(s). The first alternative can now be specifically investigated and differential RNA binding studies with the various MEF1 variants will clarify this possibility and, if yes, may show which domains of the MEF-PPR proteins are crucial for interaction with target RNAs and which of the ecotype-variations will disturb the MEF1-RNA interaction through altered binding to the *cis*-motifs in the three RNA editing targets of MEF1.

4.2. The DYW domain is necessary for ef cient function of MEF1

In the subclass of the E PPR proteins which contain an additional extension beyond the E-domain, most proteins terminate in the amino acids DYW, suggesting that this highly conserved

Author's personal copy

tripeptide and its accessibility is very important for the function of these proteins. Contrary to this theoretical consideration, the two plastid RNA editing *trans*-factors CRR22 and CRR28 are still fully functional without their C-terminal DYW domain [8]. The DYW domain in MEF1 may be more important, since already the mutation of a single nucleotide in the EMS-line *mef1-2* completely abolishes editing at two of the three target sites, *rps4*-956 and *nad7*-963, and results in a strong reduction at *nad2*-1160 [11]. Complete removal of the DYW motif from the MEF1 protein as assayed here could not recover or complement RNA editing at any of the three target sites in mutant protoplasts. Transgenic plants, however, in which the *MEF1*-ΔDYW gene is stably introduced, show during overexpression of the truncated protein recovery of or increased editing at these sites, although never reaching wild-type levels (Fig. 3C). This low level recovery in vivo may potentially be mediated by a recruitment of the mutant MEF1-1 protein, which is disabled in the region of the PPRs, but contains an intact DYW domain.

Similarly, masking of the DYW C-terminus by additional His-residues inhibits any increasing effect of the overexpressed *MEF1*+His protein on RNA editing efficiency in transgenic plants (Fig. 3C). These observations suggest that the DYW domain in MEF1 can be substituted to some extent by other (co-)factors, but that it is required for full function. While the deleted DYW domain of CRR22 and CRR28 is fully substituted by such additional, as yet unknown molecules, the activity of the *MEF1*-ΔDYW protein is not completely recovered in any of the assays, possibly due to a lower affinity or abundance of the co-factor of MEF1. The deleted DYW domain may be partially compensated for by either the high level of overexpression in the transgenic plants versus the protoplast assays, or alternatively the longer incubation time in the transgenic plants (versus the limited time frame in protoplast transfection assays) might be required to assemble the MEF1 editing complex with sufficient functionality. Other mutations in the DYW domain may destroy its binding activity to other proteins, but not the template RNA. Such mutations will in effect block the editing site (and concomitantly the editing reaction) and then cannot be compensated for or replaced by other co-factors.

Acknowledgements

We thank Dagmar Pruchner for excellent experimental help. We are grateful to Stefan Binder, Joachim Forner, Angela Hölzle and Christian Jonietz (all at Molekulare Botanik, Universität Ulm, Germany) for their gifts of plant lines and other materials. This work was supported by grants from the Deutsche Forschungsgemeinschaft to Mizuki Takenaka and Axel Brennicke.

References

[1] Rajasekhar, V.K. and Mulligan, R.M. (1993) RNA editing in plant mitochondria: α-phosphate is retained during C-to-U conversion in mRNAs. Plant Cell 5, 1843–1852.
[2] Yu, W. and Schuster, W. (1995) Evidence for a site-specific cytidine deamination reaction involved in C to U RNA editing of plant mitochondria. J. Biol. Chem. 270, 18227–18233.
[3] Farré, J.-C., Leon, G., Jordana, X. and Araya, A. (2001) *Cis* recognition elements in plant mitochondrion RNA editing. Mol. Cell. Biol. 21, 6731–6737.
[4] Neuwirt, J., Takenaka, M., van der Merwe, J.A. and Brennicke, A. (2005) An in vitro RNA editing system from cauliflower mitochondria: editing site recognition parameters can vary in different plant species. RNA 11, 1563–1570.
[5] van der Merwe, J.A., Takenaka, M., Neuwirt, J., Verbitskiy, D. and Brennicke, A. (2006) RNA editing sites in plant mitochondria can share *cis*-elements. FEBS Lett. 580, 268–272.
[6] Kotera, E., Tasaka, M. and Shikanai, T. (2005) A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature 433, 326–330.
[7] Okuda, K., Myouga, R., Motohashi, K., Shinozaki, K. and Shikanai, T. (2007) Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. Proc. Natl. Acad. Sci. USA 104, 8178–8183.
[8] Okuda, K., Chateigner-Boutin, A.-L., Nakamura, T., Delannoy, E., Sugita, M., Myouga, F., Motohashi, R., Shinozaki, K., Small, I. and Shikanai, T. (2009) Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in *Arabidopsis* chloroplasts. Plant Cell 21, 146–156.
[9] Chateigner-Boutin, A.-L., Ramos-Vega, M., Guevara-Garcia, A., Andrés, C., Gutierrez-Nava, M.d.l.L., Cantero, A., Delannoy, E., Jimenez, L.F., Lurin, C., Small, I.D. and León, P. (2008) CLB19, a pentatricopeptide repeat protein required for editing of *rpoA* and *clpP* chloroplast transcripts. Plant J. 56, 590–602.
[10] Zhou, W., Cheng, Y., Yap, A., Chateigner-Boutin, A.-L., Delannoy, E., Hammani, K., Small, I. and Huang, J. (2008) The Arabidopsis gene *YS1* encoding a DYW protein is required for editing of *rpoB* transcripts and the rapid development of chloroplasts during early growth. Plant J. 58, 82–96.
[11] Zehrmann, A., van der Merwe, J.A., Verbitskiy, D., Brennicke, A. and Takenaka, M. (2009) A DYW-domain containing PPR-protein is required for RNA editing at multiple sites in mitochondria of Arabidopsis thaliana. Plant Cell 21, 558–567.
[12] Kim, S.-R., Yang, J.-I., Moon, S., Ryu, C.-H., An, K., Yim, J. and An, G. (2009) Rice OGR1 encodes a pentatricopeptide repeat-DYW protein and is essential for RNA editing in plant mitochondria. Plant J. 59, 738–749.
[13] Takenaka, M. (2009) MEF9, an E subclass PPR protein, is required for an RNA editing event in the *nad7* transcript in mitochondria of *Arabidopsis thaliana*. Plant Physiol. 152, 939–947.
[14] Verbitskiy, D., Zehrmann, A., van der Merwe, J.A., Brennicke, A. and Takenaka, M. (2010) The PPR-protein encoded by the lovastatin insensitive 1 gene is involved in RNA editing at three sites in mitochondria of *Arabidopsis thaliana*. Plant J. 61, 446–455.
[15] Small, I.D. and Peeters, N. (2000) The PPR motif – A TPR-related motif prevalent in plant organellar proteins. Trends Biochem. Sci. 25, 46–47.
[16] Schmitz-Linneweber, C. and Small, I. (2008) Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends Plant Sci. 13, 663–670.
[17] Zehrmann, A., van der Merwe, J.A., Verbitskiy, D., Brennicke, A. and Takenaka, M. (2008) Seven large variations in the extent of RNA editing in plant mitochondria between three ecotypes of Arabidopsis thaliana. Mitochondrion 8, 319–327.
[18] Takenaka, M. and Brennicke, A. (2009) Multiplex single base extension typing to identify nuclear genes required for RNA editing in plant organelles. Nucleic Acids Res. 37, e13.
[19] Yoo, S.-D., Cho, Y.-H. and Sheen, J. (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. 2, 1565–1572.
[20] Forner, J. and Binder, S. (2007) The red fluorescent protein eqFP611: application in subcellular localization studies in higher plants. BMC Plant Biol. 7, 28.
[21] Weiner, M.P., Gackstetter, T., Costa, G.L., Bauer, J.C. and Kretz, K.A. (1995) Recent advances in PCR methodology (Griffin, A.M. and Griffin, H.G., Eds.), Molecular biology: Current innovations and Future trends, vol. 1, pp. 11–24, Horizon scientific press, Norfolk, UK.
[22] Imai, Y., Matsushima, Y., Sugimura, T. and Terada, M. (1991) A simple and rapid method for generating a deletion by PCR. Nucleic Acids Res. 19, 2785.
[23] Takenaka, M. and Brennicke, A. (2007) RNA editing in plant mitochondria: assays and biochemical approaches. Methods Enzymol. 424, 439–458.
[24] Curtis, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiol. 133, 462–469.
[25] Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16, 735–743.

Copyright Notice:
Reprinted from FEBS Letters 585 (2011),
Verbitskiy, D., Härtel, B., Zehrmann, A., Brennicke, A., Takenaka, M.
„The DYW-E-PPR protein MEF14 is required for RNA editing at site *matR*-1895 in mitochondria of *Arabidospis thaliana.*“
pp. 700-704,
Copyright 2011 Elsevier, with kind permission from Elsevier.

Author's personal copy

FEBS Letters 585 (2011) 700–704

journal homepage: www.FEBSLetters.org

The DYW-E-PPR protein MEF14 is required for RNA editing at site *matR*-1895 in mitochondria of *Arabidopsis thaliana*

Daniil Verbitskiy, Barbara Härtel, Anja Zehrmann, Axel Brennicke, Mizuki Takenaka *

Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany

ARTICLE INFO

Article history:
Received 23 December 2010
Revised 25 January 2011
Accepted 25 January 2011
Available online 1 February 2011

Edited by Tamas Dalmay

Keywords:
RNA editing factor
Plant mitochondria
PPR protein
Splicing maturase
MEF14

ABSTRACT

We here identify the PPR protein MEF14 of the DYW subclass as a specific *trans*-factor required for C to U editing of site *matR*-1895 by genetic mapping of an EMS induced editing mutant in *Arabidopsis thaliana*. The wild type Col *MEF14* gene complements mutant protoplasts. A T-DNA insertion in the *MEF14* gene abolishes detectable editing at the *matR*-1895 site. Lack of RNA editing at the *matR*-1895 site does not alter the level of mature and precursor *nad1* mRNA molecules. Such RNA editing mutants can be used to analyse the function of genes like this maturase related reading frame in plant mitochondria.

© 2011 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

RNA editing in plastids and mitochondria of plants changes selected cytidines to uridines in mRNAs. This process requires a precise targeting of the C to be altered against the background of the many more C nucleotides remaining unaltered. This is probably achieved by a cooperative binding of sequence elements located in *cis* in the unedited mRNAs by *trans*-acting proteins [1–7].

In the past few years several such *trans*-acting factors have been identified to target single or few specific sites. All of these factors belong to the class of the pentatricopeptide repeat proteins (PPR proteins). The PPR proteins form a large family with about 450 members encoded in the nuclear genomes of flowering plants [8–10]. According to the subtype of repeat of ±1 amino acid and additional C-terminal extensions, PPR subgroups have been recognized. The factors involved in RNA editing in mitochondria and plastids belong to the PLS family and have a C-terminal extension (termed E for extension).

Among these E class editing proteins are the mitochondrial MEF9 (mitochondrial editing factor) [11] and MEF18, MEF19, MEF20 and MEF21 proteins [12] and several plastid RNA editing factors [13–20]. Other *trans*-factors such as the *Arabidopsis* mitochondrial editing proteins MEF1, MEF11 and MEF22 as well as the OGR1 factor in rice [12,21–23] contain an additional about 100 amino acids long DYW region at the C-terminus beyond the E domain.

Several of these PPR proteins have been characterized through analysis of the physiological and growth phenotypes of their mutants and have only then been recognized as *trans*-factors for specific RNA editing events in plastids or mitochondria (e.g. [15]). For a direct forwards genetics approach to identify such nuclear encoded proteins required for individual RNA editing sites we have initiated a screening procedure of EMS mutagenized plants for mutants with defects in specific RNA editing events [24]. This approach is greatly facilitated by the identification of all of the *trans*-factors so far characterized being such E or DYW type PPR proteins. Now a rough mapping of the genomic locus often suffices to locate the responsible gene if only few such E or DYW type PPR proteins are encoded in the delineated region of a given chromosome.

Here we identify the DYW type PPR protein MEF14 to be required for RNA editing of the *matR*-1895 site in plant mitochondria by mapping of a single EMS mutated nucleotide. This mutation alters an amino acid in the MEF14 RNA editing factor in *Arabidopsis thaliana* and inhibits RNA editing specifically at this site.

2. Materials and methods

2.1. Plant material and preparation of nucleic acids

A. thaliana seeds for the Columbia (Col) ecotype were kind gifts of J. Forner and S. Binder (Universität Ulm). The EMS mutant

* Corresponding author. Fax: +49 731 502 2626.
E-mail addresses: daniil.verbitskiy@uni-ulm.de (D. Verbitskiy), barbara.haertel@uni-ulm.de (B. Härtel), anja.zehrmann@uni-ulm.de (A. Zehrmann), mo.bo@uni-ulm.de (A. Brennicke), mizuki.takenaka@uni-ulm.de (M. Takenaka).

0014-5793/$36.00 © 2011 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2011.01.037

Author's personal copy

population of *A. thaliana* ecotype Col was obtained commercially (Lehle Seeds, USA). The T-DNA insertion line of *A. thaliana* was obtained from the TAIR resources. Growth of the *A. thaliana* plants and preparation of DNA or RNA from the leaves were as described [25]. Seeds were sown as obtained, selfed and the T-DNA insertion sites were verified by PCR. Development of the homozygous plants was monitored and compared to wt Col plants.

2.2. SNaPshot assays and mutant analysis

The EMS mutant lines were screened by multiplexed single base extension [24] for plants with altered RNA editing at specific sites. Plants were first analysed in pools of 10 from which the deviant plants were recovered. In the identified individual plants, the compromised RNA editing phenotype was verified by cDNA sequence analysis. Sequences were obtained commercially from 4base lab, Reutlingen, Germany or from Macrogen, Seoul, Korea.

2.3. Analysis of RNA editing sites

Specific cDNA fragments were generated by RT-PCR amplification by established protocols [26]. The cDNA sequences were compared for C to T differences resulting from RNA editing.

2.4. Protoplast complementation assays

Protoplasts were prepared from 3 to 4 weeks old plantlets of the EMS mutant line *mef14-1* by the method of Yoo et al. [26]. Transfected genes, including the unrelated *MEF11* as control and the *MEF14* wt Col reading frame, were expressed from the 35S promoter in vector pSMGFP4. Efficiency of the transfection was monitored by signals from separately introduced or co-transfected GFP genes in the cytoplasm. Typically the GFP fluorescence was detected in more than 80% of the transfected protoplasts. Total RNA was isolated after 20–24 h incubation at room temperature. RNA editing levels were estimated by the relative areas under the respective nucleotide peaks in cDNA sequence analyses after RT-PCR.

2.5. Mitochondrial RNA analysis

For the investigation of splicing in the mitochondrially encoded *nad1* transcripts, total cellular RNA was purified by a standard protocol [25]. The RNA was size-fractionated on a glutaraldehyde gel, transferred to a nitrocellulose membrane and hybridized with a denatured ^{32}P-labelled RT-PCR product of the mature *nad1* mRNA and the *matR* coding sequence, respectively.

3. Results

3.1. Screening for a mutant defective in editing at site matR-1895

The gene coding for MEF14 (mitochondrial editing factor 14) was identified by a forwards genetic screen of RNA editing at specific sites in plant mitochondria in a population of chemically mutagenized *A. thaliana* ecotype Columbia plants. This search was done with a multiplexed single nucleotide extension protocol [24]. Among other sites we screened for mutant plant individuals deficient in editing at any of the identified sites in the mRNA from the *matR* gene, which codes for an open reading frame in plant mitochondria with similarity to intron maturases (Fig. 1). We identified one mutant plant, *mef14-1*, in which RNA editing at the *matR*-1895 site is lowered from about 100% in wt plants to about 30% (Fig. 2, right part *mef14-1*). The other sites in the same transcript, as an example *matR*-1807 is shown (Fig. 2), and about 360 sites in other mitochondrial mRNAs are edited normally in the mutant.

3.2. Identi cation of the MEF14 gene

To locate the gene locus affected by the mutation in *MEF14*, the mutant line *mef14-1* in the *A. thaliana* Columbia ecotype (Col) was crossed with wild type plants of ecotype Landsberg *erecta* (L*er*). All F1 plants show full editing at site *matR*-1895, suggesting that *mef14-1* is a recessive allele. From the F2 generation, about 100 plants were screened for individuals with the mutant phenotype at site *matR*-1895. The mutant and thus presumably homozygous plants were mapped with L*er* and Col specific markers for cosegregation during cross-over events. This analysis identified a region of about 2.5 Mb on chromosome 3 for the mutant allele (Fig. 1).

In this region 10 genes are annotated to code for PPR proteins, 4 of these are P type PPRs, 5 are characterized by an additional E extension and only one contains a DYW domain (Fig. 1). Since this latter, At3g26780, is one of the most likely candidate genes, it was sequenced first and the mutant and the respective wild type sequences were compared. A single nucleotide was found to be different from the wt sequence of Col. This C to T transition characteristic of EMS mutations alters an aspartic acid codon to an asparagine triplet (Asp455Asn) in the C-terminal PPR repeat in the mutant line *mef14-1* (Fig. 2; *mef14-1*).

For an independent confirmation that this protein encoded by At3g26780 indeed is the MEF14 factor, we analysed the status of RNA editing at this *matR* site in a T-DNA insertion line, SALK_131077. In this line, the reading frame encoded by At3g26780 is interrupted by a T-DNA insertion in one of the central PPR repeats (Fig. 2, lower part; *mef14-2*). RNA editing at site *matR*-1895 is completely absent in the homozygous plants (Fig. 2, sequence trace of *mef14-2*), confirming the identification of At3g26780 as the locus encoding MEF14. As in the EMS mutant *mef14-1*, another site 88 nucleotides upstream in the same transcript, *matR*-1807, is edited normally also in this mutant (Fig. 2, bottom part) which was accordingly named *mef14-2*.

3.3. The wild type MEF14 gene increases RNA editing in mutant mef14-1

To further confirm the *MEF14* gene as an RNA editing factor necessary for the *matR*-1895 site, we investigated the ability of this gene to complement the editing defect in protoplasts from the *mef14-1* mutant. The Col wild-type *MEF14* gene was cloned under the control of a 35S-CaMV promoter. When this construct was transfected into the mutant protoplasts, the ability for RNA editing at site *matR*-1895 was enhanced (Fig. 3, +At3g26780). The editing factor MEF11, which is involved in editing the three sites *nad4*-124, *cox3*-422 and *ccb203*-344 [23], does not alter the editing level of site *matR*-1895 in control transfections (Fig. 3, +*MEF11*). These results show that the *MEF14* gene is indeed involved in and required for RNA editing at site *matR*-1895.

3.4. Phenotypes of the mef14-1 and mef14-2 mutant plants

Both mutant lines *mef14-1* and *mef14-2* display a normal growth phenotype under standard growth chamber conditions. Comparison of general phenotypic parameters like sizes and shapes of leaves, their numbers at the time of bolting, flower shapes and numbers, times of bolting and flower set as well as seed set revealed comparable characteristics between both mutants and the Col wt plants within the range of normal variation. These observations suggest that the lack of editing at site *matR*-1895 does not strongly effect mitochondrial functions in the plant.

3.5. Orthologs of MEF14 in other plant species

A database screen for similar sequences revealed orthologs of *MEF14* in *Vitis vinifera* (vine; GSVIVG01033004001), *Oryza sativa*

Author's personal copy

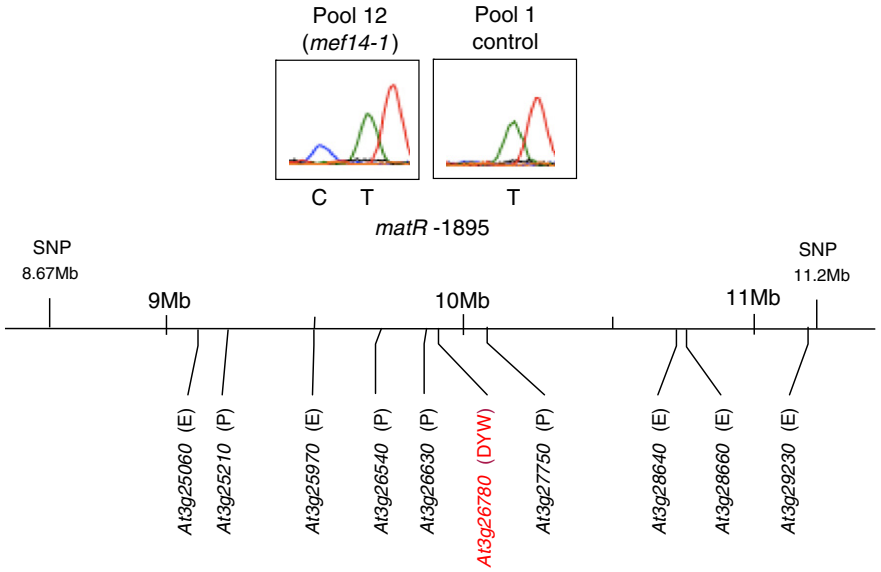


Fig. 1. Mapping of an EMS mutation in a plant line affected in RNA editing at one specific site in the *matR* mitochondrial RNA. Top part: A SNaPshot screen of an EMS mutated population of *Arabidopsis thaliana* plants identifies a mutant with diminished editing at site *matR*-1895. Shown is a second round SNaPshot of two pools of eight plants each, pool 12 containing the mutant plant as detected by the blue C signal. Pool 1 contains only wild type plants which are fully edited at site *matR*-1895 and thus show only the green T peak. The red signal comes from a different editing site also analysed in this assay. Lower part: Mapping of about 100 F1 plants narrowed the location of the EMS mutation responsible for the lower editing at *matR*-1895 to be located between two Ler/Col SNPs at 8.67 and 11.2 Mb on chromosome 3. Locus At3g26780 shown in red encodes the only DYW PPR protein in this region, the other nine PPR proteins encoded in this genomic window are either E or P type proteins as indicated in brackets.

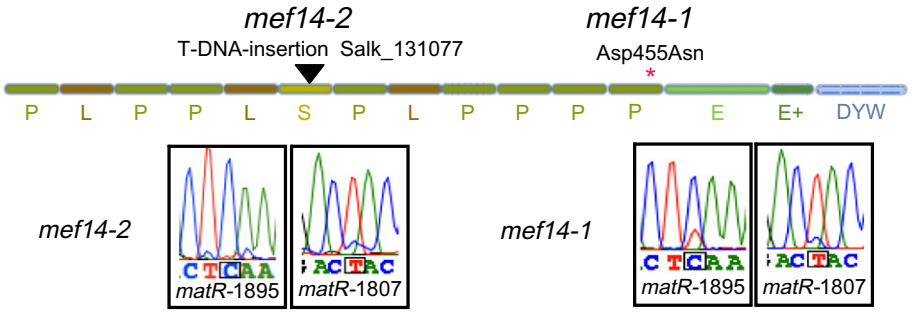


Fig. 2. Identification of the *MEF14* gene and the mutation in the EMS line *mef14-1*. Sequence analysis of the At3g26780 locus encoding a DYW PPR protein reveals in the mutant plant line an Asp to Asn amino acid change at residue 455 caused by a C to T transition typical for EMS induced lesions. This mutation reduces RNA editing in the *mef14-1* mutant to about 30% at site *matR*-1895 but does not affect site *matR*-1807. The T-DNA insertion in a SALK line renamed *mef14-2* completely disables MEF14 and no editing is detectable at site *matR*-1895. These two independent mutant lines confirm the identification of the gene for MEF14. Color traces are: G-black, A-green, T-red, C-blue.

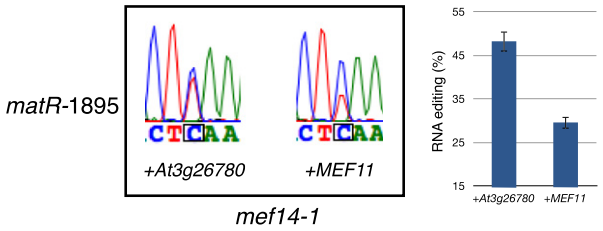


Fig. 3. Introduction of the Col wild type version of the *MEF14* gene into *mef14-1* mutant protoplasts enhances RNA editing in the mutant. Mutant protoplasts *mef14-1* transfected with the MEF11 gene which is involved in editing of other sites shows no detectable increase of the about 30% editing at the MEF14 target site *matR*-1895. Editing at this site is enhanced upon introduction of the *MEF14* wild type gene to more than 45% confirming the identification of the *MEF14* gene. A repeat of the assay yields very similar results as shown by the bar graph with standard deviation bars. Color traces are: G-black, A-green, T-red, C-blue.

(rice; OS07G20420) and in *Populus trichocarpa* (poplar; GW1.I.33 06.1). The most similar PPR protein sequence to the *Arabidopsis* MEF14 is the vine sequence which shows an overall percentage of about 60% identical and about 70% similar amino acids. While the genomic prediction for *Arabidopsis* identifies one intron, the vine annotations suggest in an additional intron just after the third repeat from the start codon. This would create a gap in the alignment of the MEF14 proteins from the two plants, which is not present if the coding sequence continues uninterrupted (not shown). We therefore conclude that vine MEF14 ortholog only contains the same single intron as do *Arabidopsis*, poplar and rice.

As noted in comparisons of orthologs of other MEF proteins and with plastid editing PPR proteins, the C-terminal PPR repeats just before the E domain are most highly conserved also in the MEF14 proteins. If – as suggested by the better evolutionary conservation – these repeats indeed have a special function, this may explain the strong effect of the mutation in the EMS mutant *mef14-1* on editing, since the amino acid altered is located in this terminal PPR repeat. Furthermore, the aspartic acid at position 455 is conserved in all four plant species and thus may be functionally important. The effect of the change to an asparagine in *mef14-1* on editing at *matR*-1895 confirms this conclusion.

Author's personal copy

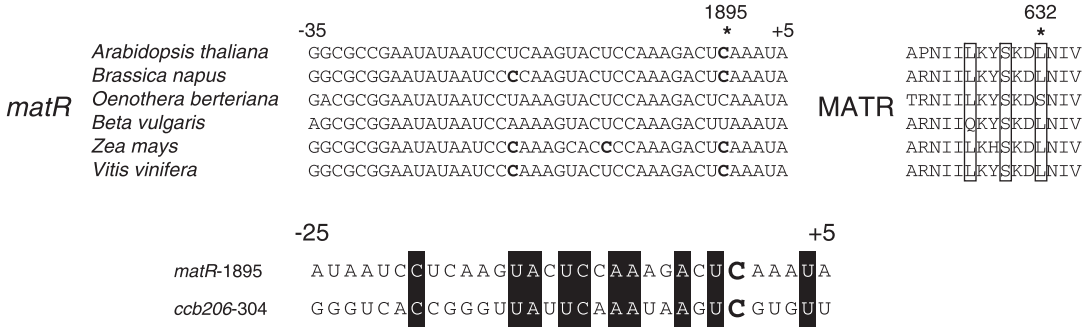


Fig. 4. Comparison of nucleotide and amino acid conservation around the *matR*-1895 editing site affected in the *mef14-1* and *mef14-2* mutants. The left alignment compares the nucleotide conservation around the editing site targeted by *MEF14* between *Arabidopsis* and other plants including four dicots and one monocot. Nucleotide 1895 is indicated by its position from the AUG. This and other documented RNA editing events are shown as bold unedited C-nucleotides. The amino acid alignment on the right shows the high conservation of shared amino acids between the different plant species at and around amino acid 632 which is affected in the *mef14* editing mutants. Amino acid residues altered by RNA editing events are boxed. Sequences are shown 5 to 3 or N- to C-terminus from left to right. In the lower part the target site *matR*-1895 is aligned with an editing site with ten additional identical nucleotides in the presumed *cis*-recognition region, nucleotide 304 in the *ccb206* mRNA (bold C). This site is however not affected in the *mef14* mutants and thus most likely not a target of MEF14. Identical nucleotide identities are shaded.

3.6. MEF14 may target only one editing site

To address all 400–500 RNA editing sites in mitochondria of flowering plants, the about 200 E and DYW PPR proteins will on average have to target more than one site. For several editing sites in mitochondria and plastids, in vitro and in organello investigations have identified the *cis*-elements in the RNA context to occupy the region between 20 or 25 nucleotides upstream (5′) to 1 or 3 nucleotides downstream (3′) of the edited C [2–7,27,28]. In extrapolation, for the site *matR*-1895 affected in the *mef14* mutants, a similar extent of the recognition sequences may be expected. We first investigated potential additional targets by looking for similar sequences in the −25 to +5 window around other editing sites as the most likely potential targets. Similar sites such as the editing event at nucleotide *ccb206*-304 (Fig. 4, bottom alignment) were tested in the *mef14* mutants, but none show any defect in editing.

Searching unbiased for editing sites in silico the sequence of the mitochondrial genome of *Arabidopsis* for motifs similar to the sequences surrounding *matR*-1895, scattered shared nucleotide identities are found but no common pattern. If further editing sites are addressed as targets by the MEF14 PPR protein, their roles or requirements are readily substituted for by other factors. In addition, other target sites may show less overall but crucial shared *cis*-motifs which escaped our analysis. It is therefore possible that MEF14 only targets a single editing site, while additional sites cannot be rigorously excluded at present.

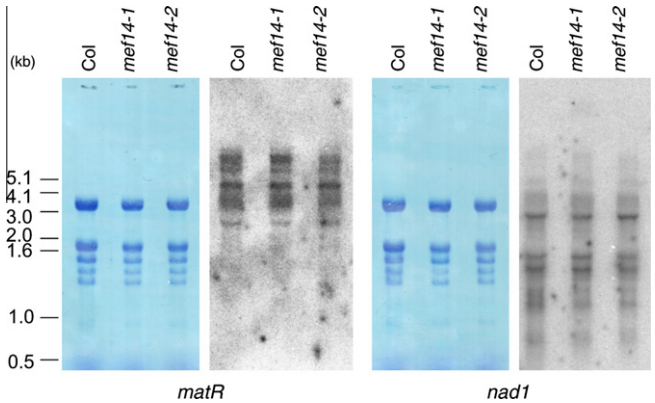


Fig. 5. Processing of the mitochondrial *nad1-matR* mRNA is not affected by the mutations in *MEF14*. The transcript patterns of the mitochondrial *nad1* gene are compared between Col wild type and the *mef14* mutants by RNA blot analyses. The *nad1* gene encodes the intron maturase related open reading frame *matR* in the group II intron between exons d and e. The hybridization signals of the *matR* (on the left hand) and the *nad1* exon specific probes (on the right hand) show comparable patterns and similar amounts of the respective precursors and mature transcripts in Col and the respective *mef14* mutants *mef14-1* and *mef14-2*. The source of the respective total cellular RNA preparation is given above each lane. The various rRNAs are visible upon staining with methylene blue (respective left panels) and the positions of DNA size standards are indicated alongside in kilobasepairs.

4. Discussion

4.1. The RNA editing site matR-1895 is conserved between plants

To evaluate the functional importance of this editing site *matR*-1895, we compared the degree of evolutionary conservation between different plant species (Fig. 4). This analysis shows that at the *matR*-1895 site the editing event is highly conserved in all flowering land plants aligned with the exception of *Oenothera berteriana*, in which no U generated by RNA editing has been reported. This may have to be re-evaluated, since the encoded amino acid sequence is well conserved and in sugarbeet, *Beta vulgaris*, a T resp. U is genomically encoded. Alignment of the amino acids surrounding the residue affected by editing event *matR*-1895 shows that the protein sequences are well conserved (Fig. 4, right hand alignment). Such a degree of conservation of the amino acid information suggests that this region is important for the functional protein products, the MATR proteins. Indeed, this leucin codon altered by the lost editing is located in the C-terminal region of a consensus motif within domain X in group II intron maturases which is conserved also outside of higher plants [29]. This domain X is probably involved in the excision of the intron by binding to the unspliced RNA.

4.2. RNA editing mutants may be tools to analyse the function of MATR

The function of the MATR proteins is still unclear. Their similarity with bona fide maturases in other organisms suggests analogous activities as intron maturases or splicing (co-)factors. Mutants in nuclear encoded specificity factors of RNA editing such as the here reported *mef14* mutants in effect represent point mutations in the targeted mitochondrially encoded protein(s). At highly conserved sites these nucleotide and concomitant amino acid changes can be expected to have an effect on the function of the protein. The *mef14* mutants do not show any gross physiological phenotype and northern analysis reveals no obvious defect in the transcript pattern of *nad1* and *matR* (Fig. 5). The *matR* gene is

Author's personal copy

encoded between *nad1* exons d and e and the likely candidate intron for MATR splicing support would be its own home intron.

Even if these mutants of MEF14 have no effect on any mitochondrial splicing event, mutants of other MEF proteins may yield insights into MATR functions. The other mutant affected in editing of a *matR* site unfortunately displays increased editing at one site [30]. Nevertheless this approach may circumvent the absence of primary mitochondrial mutants in the *matR* and other plant mitochondrial genes.

4.3. EMS populations yield soft RNA editing mutants

The residual RNA editing of about 30% in the EMS mutant *mef14-1* shows that the single amino acid exchange partially affects the function of the MEF14 PPR protein. This is the more unexpected since asparagine, the amino acid specified by the EMS mutated codon, is present at this position in many other PPR proteins. Both amino acids D and N are very similar in their properties and the lower level of editing will have to be explained. This may be due to disturbed RNA binding or as yet unidentified interaction(s) of MEF14 with other protein(s). Resolution of the step in the RNA editing process altered by soft mutations such as this *mef14-1* and of how editing is altered, through slowed initial connection, inhibited detachment or lower affinity has the potential to yield further detailed insights into the RNA editing process and its mechanistics.

Although the knock-out by the T-DNA insertion of *MEF14* is viable, it does confirm our rationale for investigating an EMS mutated population for RNA editing mutants. Mutant *mef14-1* shows that such EMS mutants may yield intermediate editing phenotypes and thereby can provide affected yet viable plants when knockouts are lethal and cannot be investigated directly. In these instances such EMS mutants will allow to determine the function of the affected *MEF* gene.

Acknowledgements

We thank Dagmar Pruchner for excellent experimental help. We are very grateful to the anonymous reviewers for their constructive comments. This work was supported by grants from the Deutsche Forschungsgemeinschaft to Mizuki Takenaka and Axel Brennicke.

References

[1] Verbitskiy, D., van der Merwe, J.A., Zehrmann, A., Brennicke, A. and Takenaka, M. (2008) Multiple specificity recognition motifs enhance plant mitochondrial RNA editing in vitro. J. Biol. Chem. 283, 24374–24381.
[2] Bock, R., Hermann, M. and Kössel, H. (1996) In vivo dissection of *cis*-acting determinants for plastid RNA editing. EMBO J. 15, 5052–5059.
[3] Bock, R. and Koop, H.U. (1997) Extraplastidic site-specific factors mediate RNA editing in chloroplasts. EMBO J. 16, 3282–3288.
[4] Farré, J.-C., Leon, G., Jordana, X. and Araya, A. (2001) *Cis* recognition elements in plant mitochondrion RNA editing. Mol. Cell. Biol. 21, 6731–6737.
[5] Kempken, F., Bolle, N. and Bruhs, A. (2009) Higher plant in organello systems as a model for RNA editing. Endocyt. Cell Res. 19, 1–10.
[6] Neuwirt, J., Takenaka, M., van der Merwe, J.A. and Brennicke, A. (2005) An in vitro RNA editing system from cauliflower mitochondria: editing site recognition parameters can vary in different plant species. RNA 11, 1563–1570.
[7] van der Merwe, J.A., Takenaka, M., Neuwirt, J., Verbitskiy, D. and Brennicke, A. (2006) RNA editing sites in plant mitochondria can share *cis*-elements. FEBS Lett. 580, 268–272.
[8] Small, I.D. and Peeters, N. (2000) The PPR motif – a TPR-related motif prevalent in plant organellar proteins. Trends Biochem. Sci. 25, 46–47.
[9] Schmitz-Linneweber, C. and Small, I. (2008) Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends Plant Sci. 13, 663–670.
[10] Lurin, C., Andrés, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyère, C., Caboche, M., Debast, C., Gualberto, J.M., Hoffmann, B., Lecharny, A., Le Ret, M., Martin-Magniette, M.-L., Mireau, H., Peeters, N., Renou, J.-P., Szurek, B., Taconnat, L. and Small, I.D. (2004) Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell 16, 2089–2103.
[11] Takenaka, M. (2009) MEF9, an E-subclass pentatricopeptide repeat protein, is required for an RNA editing event in the *nad7* transcript in mitochondria of *Arabidopsis*. Plant Physiol. 152, 939–947.
[12] Takenaka, M., Verbitskiy, D., Zehrmann, A. and Brennicke, A. (2010) Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. J. Biol. Chem. 285, 27122–27129.
[13] Sasaki, T., Yukawa, Y., Wakasugi, T., Yamada, K. and Sugiura, M. (2006) A simple in vitro RNA editing assay for chloroplast transcripts using fluorescent dideoxynucleotides: distinct types of sequence elements required for editing of ndh transcripts. Plant J. 47, 802–810.
[14] Shikanai, T. (2006) RNA editing in plant organelles: machinery, physiological function and evolution. Cell. Mol. Life Sci. 63, 689–708.
[15] Kotera, E., Tasaka, M. and Shikanai, T. (2005) A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature 433, 326–330.
[16] Okuda, K., Myouga, R., Motohashi, K., Shinozaki, K. and Shikanai, T. (2007) Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. Proc. Natl. Acad. Sci. USA 104, 8178–8183.
[17] Okuda, K., Chateigner-Boutin, A.-L., Nakamura, T., Delannoy, E., Sugita, M., Myouga, F., Motohashi, R., Shinozaki, K., Small, I. and Shikanai, T. (2009) Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in *Arabidopsis* chloroplasts. Plant Cell 21, 146–156.
[18] Chateigner-Boutin, A.-L., Ramos-Vega, M., Guevara-Garcia, A., Andrés, C., Gutierrez-Nava, M.d.l.L., Cantero, A., Delannoy, E., Jimenez, L.F., Lurin, C., Small, I.D. and León, P. (2008) CLB19, a pentatricopeptide repeat protein required for editing of *rpoA* and *clpP* chloroplast transcripts. Plant J. 56, 590–602.
[19] Zhou, W., Cheng, Y., Yap, A., Chateigner-Boutin, A.-L., Delannoy, E., Hammani, K., Small, I. and Huang, J. (2009) The Arabidopsis gene *YS1* encoding a DYW protein is required for editing of *rpoB* transcripts and the rapid development of chloroplasts during early growth. Plant J. 58, 82–96.
[20] Yu, Q.-B., Jiang, Y., Chong, K. and Yang, Z.-N. (2009) AtECB2, a pentatricopeptide repeat protein, is required for chloroplast transcript accD RNA editing and early chloroplast biogenesis in *Arabidopsis thaliana*. Plant J. 59, 1011–1023.
[21] Zehrmann, A., van der Merwe, J.A., Verbitskiy, D., Brennicke, A. and Takenaka, M. (2009) A DYW domain containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. Plant Cell 21, 558–567.
[22] Kim, S.-R., Yang, J.-I., Moon, S., Ryu, C.-H., An, K., Yim, J. and An, G. (2009) Rice OGR1 encodes a pentatricopeptide repeat-DYW protein and is essential for RNA editing in mitochondria. Plant J. 59, 738–749.
[23] Verbitskiy, D., Zehrmann, A., van der Merwe, J.A., Brennicke, A. and Takenaka, M. (2010) The PPR protein encoded by the lovastatin insensitive 1 gene is involved in RNA editing at three sites in mitochondria of *Arabidopsis thaliana*. Plant J. 61, 446–455.
[24] Takenaka, M. and Brennicke, A. (2009) Multiplex single base extension typing to identify nuclear genes required for RNA editing in plant organelles. Nucleic Acids Res. 37, e13.
[25] Takenaka, M. and Brennicke, A. (2007) RNA editing in plant mitochondria: assays and biochemical approaches. Methods Enzymol. 424, 439–458.
[26] Yoo, S.-D., Cho, Y.-H. and Sheen, J. (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. 2, 1565–1572.
[27] Chaudhuri, S. and Maliga, P. (1996) Sequences directing C to U editing of the plastid psbL mRNA are located within a 22 nucleotide segment spanning the editing site. EMBO J. 15, 5958–5964.
[28] Miyamoto, T., Obokata, J. and Sugiura, M. (2002) Recognition of RNA editing sites is directed by unique proteins in chloroplasts: biochemical identification of *cis*-acting elements and *trans*-acting factors involved in RNA editing in tobacco and pea chloroplasts. Mol. Cell. Biol. 22, 6726–6734.
[29] Mohr, G., Perlman, P.S. and Lambowitz, A.M. (1993) Evolutionary relationship among group II intron-encoded proteins and identification of a conserved domain that may be related to maturase function. Nucl. Acids Res. 22, 4991–4997.
[30] Bentolila, S., Knight, W. and Hanson, M. (2010) Natural variation in Arabidopsis leads to the identification of REME1, a pentatricopeptide repeat-DYW protein controlling the editing of mitochondrial transcripts. Plant Physiol. 154, 1966–1982.

Copyright Notice:
Reprinted from
Zehrmann, A., Verbitskiy, D., Härtel, B., Brennicke, A., and Takenaka, M.
„PPR proteins network as site-specific RNA editing factors in plant organelles.“
RNA Biology 8:1, 1-4
© 2011 Landes Bioscience, open access

RNA Biology 8:1, 1-4; January/February 2011; © 2011 Landes Bioscience

This manuscript has been published online, prior to printing. Once the issue is complete and page numbers have been assigned, the citation will change accordingly.

PPR proteins network as site-specific RNA editing factors in plant organelles

Anja Zehrmann,† Daniil Verbitskiy,† Barbara Härtel, Axel Brennicke and Mizuki Takenaka*
Molekulare Botanik; Universität Ulm; Ulm, Germany
†These authors contributed equally to this work.

©2010 Landes Bioscience. Do not distribute

RNA editing in flowering plant mitochondria targets several hundred C nucleotides mostly in mRNAs to be altered to U. Several nuclear encoded genes have been recently identified predominantly in *Arabidopsis thaliana* that code for proteins involved in specific RNA editing events in plastids or mitochondria. These nuclear genes code for proteins characterized by a stretch of 4–20 repeats of 34–36 amino acids each, accordingly classified as pentatricopeptide repeat (PPR) proteins. These repeats most likely participate in recognizing and binding the specific nucleotide motifs around editing sites which have been defined as essential cis-elements. All of the RNA editing PPR proteins contain at their C-termini an extension of as yet unclear function, the E domain, and some of these are further extended by another domain that terminates with the triplet DYW. While the E domain seems to be always required for their function in RNA editing, the DYW domain can sometimes be removed. At some editing sites a given PPR protein seems to be required, while at others their function can at least partially be compensated by presumably other PPR proteins. These observations suggest that the PPR proteins may act in a complex network to define and to target RNA editing sites.

Key words: RNA editing, plant mitochondria, PPR proteins, RNA editing factors

Submitted: 11/11/10

Accepted: 11/25/10

Previously published online: www.landesbioscience.com/journals/rnabiology/article/14298

DOI: 10.4161.rna.8.1.14298

*Correspondence to: Mizuki Takenaka; Email: mizuki.takenaka@uni-ulm.de

Processes of editing the genomic information content posttranscriptionally by RNA editing are found in animals and plants in very different evolutionary branches. These have no connections and it is clear that the diverse manifestations of RNA editing are derived traits, unrelated to an ancient primary RNA world. The independent lines of evolution are only convergent in the abstract final outcome, the change of the ultimately functional, often the translated, nucleotide sequence in the given RNA molecule. The biochemical molecular processes vary as much as their origins and thus have to be investigated independently.

RNA editing in flowering plants is unique in that it occurs in two compartments, in mitochondria and in plastids. It is very likely that the processes in both organelles co-evolved since their present occurences always coincide in the plant kingdom and the processes appear to be very similar.[1] Some of the molecular participants might even be shared between the two organelles, which is not uncommon as evidenced by the example of the RNA polymerases.[2] Nevertheless there may be differences and divergencies beyond the sheer asymmetry of the event numbers, 400–500 in mitochondria versus 35–40 in plastids of flowering plants. It is therefore necessary to clarify the RNA editing processes in both organelles to better understand their function, mechanisms and origins.

To date, several nuclear encoded specificity factors have been identified for editing in each organelle, so far none of them functional in both. These plastid[3-8] and mitochondrial editing factors[9-14] are required for specific RNA editing events and thus presumably recognize a sequence motif, the cis-element, generally located upstream of the editing-site in the affected RNA molecule.[15]

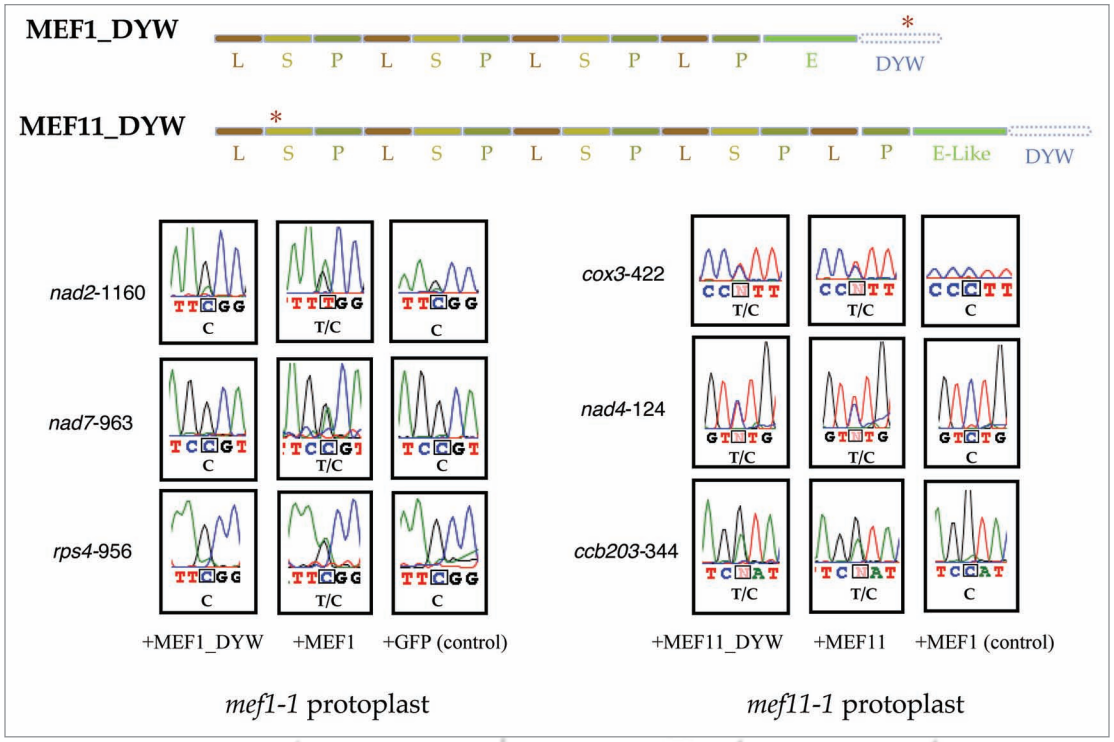


Figure 1. The DYW-domain is required in MEF1, but not in MEF11. Full length and truncated reading frames for MEF1 and MEF11 are compared for their ability to restore specific RNA editing events in the mutants *mef1-2* (left part) and *mef11-1* (right part), respectively. The top lines show the modular structures of the constructs MEF1ΔDYW and MEF11ΔDYW which are derived from the MEF1 and MEF11 proteins by deletion of the respective DYW-domain. The stars show the sites of the EMS mutations in the mutants *mef1-2* (left part) and *mef11-1*, respectively. The ability of the DYW deletion constructs to complement protoplasts of the respective mutants is compared to transfection assays with the respective full length proteins and as negative control the GFP protein, resp. MEF1 for the *mef11-1* mutant. For the RNA editing analysis, the specific cDNA sequences at the three target sites are compared for C to T differences. The *mef1-2* mutant plants (left part) have lost the ability of C to U editing at sites *rps4*-956 and *nad7*-963. In the third target site *nad2*-1160 editing is still occurring albeit strongly reduced. The *mef11-1* mutant plants (right part) show no detectable C to U editing at sites *nad4*-124 and *cox3*-422. While the MEF1ΔDYW cannot recover RNA editing at the target sites, the MEF11ΔDYW protein is competent to restore editing in protoplasts of the respective mutant.

All of the presently characterized plastid and mitochondrial editing factors in *Arabidopsis thaliana*, in rice and in the moss *Physcomitrella patens* are pentatricopeptide repeat proteins (PPR proteins) of the E subgroup, which are extended at their C-termini by the name-giving E domain.[3-14] Several of these specific nuclear-encoded factors in addition contain another C-terminal extension beyond this E domain, which nearly always ends with the amino acid triplet DYW. About 140 of the 450 PPR proteins encoded in the *Arabidopsis thaliana* nuclear genome are extended at their C-termini by such DYW domains.[16,17] The DYW domains contain signature amino acids characteristic of Zn-containing cytidine deaminases and have thus been proposed to be enzymatically involved in the C to U RNA editing.[18]

Differential Effects of Mutations in DYW Domains Suggest Different Functions of Individual DYW Domains in RNA Editing

The enigmatic function of the DYW motif in these PPR proteins has been further mystified by the intriguing observations that this domain in a PPR protein of as yet unknown function may have RNase activity,[19] that DYW domains can be deleted in PPR proteins involved in plastid editing with no effect on the processing of their editing targets,[20] and that these same DYW-regions can be exchanged between these proteins.[20] In the mitochondrial DYW-PPR protein MEF1 (mitochondrial editing factor 1), the DYW domain cannot be deleted without severe effects on its function in editing[21] (**Fig. 1**, left hand part). To determine whether this requirement for the DYW domain is a general feature of the mitochondrial editing proteins, we have now investigated another of these mitochondrial editing factors, the MEF11-PPR protein, for the involvement of its DYW domain. The nuclear encoded MEF11 is required for RNA editing at three distinct sites in three different mitochondrial mRNAs, sites *cox3*-422, *nad4*-124 and *ccb203*-344.[12,13] Surprisingly, in a knock-out mutant of *MEF11* all three target sites are functionally recovered by a MEF11 protein from which the DYW-domain has been deleted. This DYW-truncated protein shows the same level of increase in RNA editing in a complementation assay as when the complete wild type MEF11 protein is introduced (**Fig. 1**, right hand part).

This observation suggests that the no-editing effect of the T-DNA insertion in the DYW-domain in mutant line *mef11-2*, is due to a destabilisation of the *mef11-2*

mRNA or of the deviant protein and not caused by the disabled DYW domain. In this *mef11-2* mutant RNA editing is not detectable at the two sites *cox3*-422 and *nad4*-124, whereas editing at site *ccb203*-344 is diminished. The T-DNA insertion in line *mef11-2* has removed 26 amino acids from the C-terminus of the DYW region and has added 11 different amino acids.[22] Among the missing 26 amino acids are the name-giving DYW and other blocks of well conserved amino acids, while the amino acids proposed to resemble the cytidine deaminase signature are still present in the mutated protein.

Redundant E-type PPR Proteins for Recognition of Individual RNA Editing Events

These experimental data on the requirement of the DYW domains, dispensable in MEF11 but essential in MEF1, appear to be difficult to reconcile with a common function in the RNA editing process. Considering that the PPR-repeats are most likely involved in recognizing and binding the RNA target-motif,[23] the function of the DYW and the preceding E domain is probably the recruitment of and binding to accessory proteins such as the actual C-to-U editing enzyme. This function may in some of these DYW proteins, e.g., MEF11, be entirely mediated by the E domain, which would leave the DYW region redundant. In other such proteins like MEF1 the DYW moiety may participate in the contact to other cofactors and then be essential for a functional RNA editing event.

These differential results on the requirement of the DYW domains in RNA editing furthermore suggest that—if they have a function beyond the interaction with other proteins such as the suggested deaminase activity—this can at least partially be substituted by other factors. At present such substitutions of specific DYW-PPR proteins by others are suggested by observations that in several instances of apparent knock-out mutations some target sites cannot be edited any more, but others are only edited less efficiently. For example, in the *mef1* and *mef11* mutants editing is undetectable at two sites, but still seen at the third target.

Since DYW-editing factors such as MEF1,[9] and MEF11,[12] in Arabidopsis and OGR1,[14] in rice have been found to address several sites, there will be other such factors guided by similarly loosely defined target sequence motifs in the mitochondrial RNA population. In effect, their binding motifs may partially overlap which will result in overlapping and therefore complementing functions. These may have varying efficiencies so that a substituting factor may not be able to edit all cognate RNA molecules to 100%, but may result in lower yet sufficient levels of editing in the steady state RNA population. Such partially complementing functions could explain the differential observed residual editing activities of mutants of e.g., MEF11 at individual target sites. Sites without any detectable editing in the mutants, sites *cox3*-422 and *nad4*-124 for MEF11, can not be recognized by another MEF, while site *ccb203*-344 can be addressed by a different MEF albeit less efficiently and is thus seen partially edited in the knock-out of MEF11.

These observations suggest that the contact of the individual DYW- or E-PPR protein to its target RNA-motif is rather little defined, which in turn leads to the considerable overlap of targets between these proteins. Such a minimal specificity will in effect result in a network of PPR trans-factors which bind to RNA sequence elements at the minimal level of specificity to ensure that editing occurs at all sites required and possibly, as a side effect, at sites not harmful when accidentally edited. It is very possible that some or many of the so far identified RNA editing PPR proteins address more sites than those observed to be lost or greatly diminished in apparent knock-out mutants. Such additional targets cannot be detected and will thus escape definition in these mutants if other factors readily target these sites and complement the mutant. Such redundancy of the PPR specificity factors may ensure that the essential RNA editing sites are still modified even when one such PPR protein is mutated and lost. This scenario may be supported by the circumstantial argument that a surprisingly large number of PPR editing proteins can be mutated without any apparent phenotypic effect.[9-11] The target sites detected by their unedited status in the mutants may be those that are non-redundantly covered by different PPR proteins partly because they are not essential editing events. Additional targets may be the essential ones which will be lethal if not edited and which are therefore redundantly covered by several PPR proteins. No rule without exceptions in Biology: some sites non-redundantly covered are required for optimal growth parameters and their loss by the disabled cognate PPR protein will have a detectable phenotype.

Not Enough E-type PPR Proteins for One-on-one Recognition of All RNA Editing Events

Such a scenario of overlapping recognition parameters for individual PPR proteins appears at first site to exacerbate the obvious discrepancy between the number of RNA editing sites, particularly the more than 400 in plant mitochondria, and the number of PPR proteins available from the 140 genes for the E-type PPRs. On the other hand the assumed less-stringent selection of target-RNA motifs will result in a broader range of targets and can then explain how only 140 proteins can address more than 400 targets. This presumed network of PPR proteins resolves the problems that arose from the observation that in mitochondria as in plastids many PPR proteins appear to target only one or occasionally very few sites. Extrapolation to all the other editing-PPR proteins being likewise very selective and stringent in their choice of target site would leave many editing sites uncovered.

This consideration makes the assumption of many more hidden targets of individual PPR proteins much more attractive since the increased number of targets by a lowered specificity would resolve the need for yet further specific trans-factors. On the other hand, identification of the target site selection and the demarcation against non-target sites will become more complex if this interaction is presumed to be rather flexible and little defined. This low specificity threshold and the resulting variability in target selection may have originated as a prerequisite of an initial (and continuing) repair function and rescue mission of the PPR protein family and the RNA editing machinery as proposed in reference 24.

©2010 Landes Bioscience.
Do not distribute

The observed variability in DYW domain requirements in individual PPR proteins may contribute to this flexibility of the RNA-PPR protein interaction and be involved in the decision on which protein actually attaches to the RNA. Of course, the DYW domain may nevertheless participate in recruiting some or other of the presumed cooperating proteins and in positioning them on the RNA for proper and effective action. These may be other RNA binding proteins,[23] an actual deaminase, co-acting PPR proteins[25] and/or other networking DYW PPR proteins which may confer the deaminase activity.[18]

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft to Mizuki Takenaka and Axel Brennicke.

References

1. Covello PS, Gray MW. On the evolution of RNA editing. Trends Genet 1993; 9:265-8.
2. Hedtke B, Börner T, Weihe A. One RNA polymerase serving two genomes. EMBO Rep 2000; 1:435-40.
3. Kotera E, Tasaka M, Shikanai T. A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature 2005; 433:326-30.
4. Okuda K, Myouga R, Motohashi K, Shinozaki K, Shikanai T. Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. Proc Natl Acad Sci USA 2007; 104:8178-83.
5. Chateigner-Boutin AL, Ramos-Vega M, Guevara-García A, Andrés C, Gutiérrez-Nava MdlL, Cantero A, et al. CLB19, a pentatricopeptide repeat protein required for editing of rpoA and clpP chloroplast transcripts. Plant J 2008; 56:590-602.
6. Yu QB, Jiang Y, Chong K, Yang ZN. AtECB2, a pentatricopeptide repeat protein, is required for chloroplast transcript accD editing and early chloroplast biogenesis in *Arabidopsis thaliana*. Plant J 2009; 59:1011-23.
7. Zhou W, Cheng Y, Yap A, Chateigner-Boutin AL, Delannoy E, Hammani K, et al. The Arabidopsis gene YS1 encoding a DYW protein is required for editing of rpoB transcripts and the rapid development of chloroplasts during early growth. Plant J 2009; 58:82-96.
8. Hammani K, Okuda K, Tanz SK, Chateigner-Boutin AL, Shikanai T, Small I. A study of new Arabidopsis chloroplast RNA editing mutants reveals general features of editing factors and their target sites. Plant Cell 2009; 21:3686-99.
9. Zehrmann A, van der Merwe JA, Verbitskiy D, Brennicke A, Takenaka, M. A DYW domain containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. Plant Cell 2009; 21:558-67.
10. Takenaka M, Verbitskiy D, Zehrmann A, Brennicke A. Reverse genetic screening identifies five E-class PPR-proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. J Biol Chem 2010; 285:27122-9.
11. Takenaka M. MEF9, an E subclass pentatricopeptide repeat protein, is required for an RNA editing event in the nad7 transcript in mitochondria of Arabidopsis. Plant Physiol 2010; 152:939-47.
12. Verbitskiy D, Zehrmann A, van der Merwe JA, Brennicke A, Takenaka M. The PPR protein encoded by the lovastatin insensitive 1 gene is involved in RNA editing at three sites in mitochondria of *Arabidopsis thaliana*. Plant J 2010; 61:446-55.
13. Tang J, Kobayashi K, Suzuki M, Matsumoto S, Muranaka T. The mitochondrial PPR protein LOVASTATIN INSENSITIVE 1 plays regulatory roles in cytosolic and plastidial isoprenoid biosynthesis through RNA editing. Plant J 2010; 61:456-66.
14. Kim SR, Yang JI, Moon S, Ryu CH, An K, Kim KM, et al. Rice OGR1 encodes a pentatricopeptide repeat-DYW protein and is essential for RNA editing in plant mitochondria. Plant J 2009; 59:738-49.
15. Takenaka M, Verbitskiy D, van der Merwe JA, Zehrmann A, Brennicke A. The process of RNA editing in plant mitochondria. Mitochondrion 2008; 8:35-46.
16. Lurin C, Andrés C, Aubourg S, Bellaoui M, Bitton F, Bruyère C, et al. Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell 2004; 16:2089-103.
17. Schmitz-Linneweber C, Small ID. Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends Plant Sci 2008; 13:663-70.
18. Salone V, Rüdinger M, Polsakiewicz M, Hoffmann B, Groth-Malonek M, Szurek B, et al. A hypothesis on the identification of the editing enzyme in plant organelles. FEBS Lett 2007; 581:4132-8.
19. Nakamura T, Sugita M. A conserved DYW domain of the pentatricopeptide repeat protein possesses a novel endoribonuclease activity. FEBS Lett 2008; 582:4163-8.
20. Okuda K, Chateigner-Boutin AL, Nakamura T, Delannoy E, Sugita M, Myouga F, et al. Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in Arabidopsis chloroplasts. Plant Cell 2009; 21:146-56.
21. Zehrmann A, Verbitskiy D, Härtel B, Brennicke A, Takenaka M. RNA editing competence of transfactor MEF1 is modulated by ecotype-specific differences but requires the DYW domain. FEBS Lett 2010; 584:4181-6.
22. Verbitskiy D, Zehrmann A, Brennicke A, Takenaka M. The DYW domain in the MEF11 RNA editing protein seems to be involved in specific RNA binding rather than in the enzymatic reaction. Plant Signal Behav 2010; 5:1-3.
23. Okuda K, Nakamura T, Sugita M, Shimizu T, Shikanai T. A pentatricopeptide repeat protein is a site recognition factor in chloroplast RNA editing. J Biol Chem 2006; 281:37661-7.
24. Tillich M, Beick S, Schmitz-Linneweber C. Chloroplast RNA-binding proteins: Repair and regulation of chloroplast transcripts. RNA Biol 2010; 7:171-8.
25. Doniwa Y, Ueda M, Ueta M, Wada A, Kadowaki KI, Tsutsumi N. The involvement of a PPR protein of the P subfamily in partial RNA editing of an Arabidopsis mitochondrial transcript. Gene 2010; 454:39-46.

Landes Bioscience
Do not distribute

Copyright Notice:
Reprinted from:
Verbitskiy, D., van der Merwe, J. A., Zerhmann, A., Härtel, B., and Takenaka, M.
„The E-class PPR Protein MEF3 of Arabidopsis thaliana can also function in mitochondrial RNA editing with an additional DYW domain.“
Plant & Cell Physiology, 2012, 53(2), 358-367,
by kind permission of the Japanese Society of Plant Physiologists

PCP
PLANT & CELL PHYSIOLOGY

Regular Paper

The E-Class PPR Protein MEF3 of *Arabidopsis thaliana* Can Also Function in Mitochondrial RNA Editing With an Additional DYW Domain

Daniil Verbitskiy[1], Johannes A. van der Merwe[1,2], Anja Zehrmann[1], Barbara Härtel[1] and Mizuki Takenaka[1,*]

[1]Molekulare Botanik, Universität Ulm, D-89069 Ulm, Germany
[2]Present address: Institut für Molekulare Virologie, Uni Ulm, D.89069 Ulm, Germany
*Corresponding author: E-mail, mizuki.takenaka@uni-ulm.de; Fax, +49-731-502-2626
(Received November 4, 2011; Accepted December 12, 2011)

In plants, RNA editing is observed in mitochondria and plastids, changing selected C nucleotides into Us in both organelles. We here identify the PPR (pentatricopeptide repeat) protein MEF3 (mitochondrial editing factor 3) of the E domain PPR subclass by genetic mapping of a variation between ecotypes Columbia (Col) and Landsberg *erecta* (L*er*) in *Arabidopsis thaliana* to be required for a specific RNA editing event in mitochondria. The L*er* variant of MEF3 differs from Col in two amino acids in repeats 9 and 10, which reduce RNA editing levels at site *atp4*-89 to about 50% in L*er*. In a T-DNA insertion line, editing at this site is completely lost. In *Vitis vinifera* the gene most similar to *MEF3* continues into a DYW extension beyond the common E domain. Complementation assays with various combinations of PPR and E domains from the vine and *A. thaliana* proteins show that the vine E region can substitute for the *A. thaliana* E region with or without the DYW domain. These findings suggest that the additional DYW domain does not disturb the MEF3 protein function in mitochondrial RNA editing in *A. thaliana*.

Keywords: DYW domain • MEF3 • Plant mitochondria • PPR protein • RNA editing.

Abbreviations: GFP, green fluorescent protein; MEF, mitochondrial editing factor; PPR, pentatricopeptide repeat; RT–PCR, reverse transcription–PCR; SNP, single nucleotide polymorphism.

Introduction

The biochemical mechanisms of C to U RNA editing in plastids and mitochondria of plants are still largely unclear (Takenaka et al. 2008). Only recently, several proteins have been identified which are each required for a single or very few changes of selected cytidines to uridines in mRNAs in both organelles. These are pentatricopeptide repeat proteins (PPR proteins), a large family with at least 450 members encoded in the nuclear genomes of flowering plants (Lurin et al. 2004, O'Toole et al. 2008). Some of these proteins, such as the *Arabidopsis thaliana* mitochondrial editing factors MEF1, MEF11, MEF14 and MEF22 as well as the OGR1 factor in rice (Kim et al. 2009, Zehrmann et al. 2009, Takenaka et al. 2010, Verbitskiy et al. 2010, Verbitskiy et al. 2011), belong to the DYW subgroup which is characterized by an approximately 100 amino acids long DYW region at the C-terminus beyond a so-called extension region, the E domain. Others such as the mitochondrial proteins MEF9, MEF18–MEF21 and OTP87 do not contain the DYW C-terminus and terminate with the E domain (Takenaka 2010, Takenaka et al. 2010, Hammani et al. 2011). Similarly, several plastid RNA editing factors are PPR proteins with solely an E region, while others continue into a DYW domain (Kotera et al. 2005, Shikanai 2006, Okuda et al. 2007, Chateigner-Boutin et al. 2008, Hammani et al. 2009, Robbins et al. 2009, Yu et al. 2009, Zhou et al. 2009). The DYW regions are dispensable in some of the DYW PPR proteins such as OTP82 in plastids (Okuda et al. 2010) or MEF11 in mitochondria (Verbitskiy et al. 2010a), but not in MEF1 (Zehrmann et al. 2010).

This variable presence of DYW domains in the PLS subgroup of PPR proteins and their differing functional requirements remains an unresolved question. In flowering plants such as *A. thaliana*, genes for about 87 E + DYW type PPR proteins and another 107 genes for E only proteins are found, while six genes code for PPR proteins with only PLS-type repeats (Lurin 2004, O'Toole et al. 2008, Takenaka et al. 2010). In the moss *Physcomitrella patens*, only five genes for PLS and 10 for DYW PPR proteins have been identified, all of them coding for E + DYW-type proteins (O'Toole et al. 2008, Rüdinger et al. 2009). Assuming that the extant mosses represent modern versions of evolutionarily older forms of land plants which evolved and changed to a lesser extent than their flowering contemporaries, the DYW proteins have been suggested to be the ancient ancestral form of specific RNA editing proteins

Plant Cell Physiol. 53(2): 358–367 (2012) doi:10.1093/pcp/pcr182, available online at www.pcp.oxfordjournals.org
© The Author 2011. Published by Oxford University Press on behalf of Japanese Society of Plant Physiologists.
All rights reserved. For permissions, please email: journals.permissions@oup.com

Downloaded from http://pcp.oxfordjournals.org/ at University Ulm on January 29, 2013

for plastids and mitochondria (Rivals et al. 2006, Knoop and Rüdinger 2010). These authors suggested that with the increase of target editing sites from only 13 in both organelles in the moss (Knoop and Rüdinger 2010) to >400 in flowering plants (Giegé and Brennicke 1999), the number of the respective nuclear-encoded PLS PPR proteins also increased (or vice versa). Furthermore, they predicted that the E-type PPR proteins possibly arose secondarily by loss of the DYW domain.

In this study we have identified MEF3 as a sequence-specific RNA editing factor involved in editing of the *atp4*-89 site in plant mitochondria by ecotype-linked differences in the RNA editing efficiency at this site (Zehrmann et al. 2008). The MEF3 protein in *A. thaliana* terminates after the E domain, while the protein most similar in vine continues into a DYW domain.

Results

The ecotype-specific difference at site *atp4*-89

In our screen for deficiencies at one or more of 379 specific mitochondrial RNA editing sites between the three ecotypes Columbia (Col), Landsberg *erecta* (L*er*) and C24 in *A. thaliana* we had identified a lower editing efficiency at site *atp4*-89 in ecotype L*er* in comparison with Col and C24 (Zehrmann et al. 2008). In mature rosette leaves this site is edited to about 100% in C24 and Col, but only about 50% of the steady-state mRNA population contain the edited nucleotide in leaves of L*er* (**Fig. 1A**).

Reciprocal crosses between ecotypes L*er* and Col showed that the lower editing is caused by a nuclear locus inherited as a recessive Mendelian trait (Zehrmann et al. 2008). This nuclear locus *MEF3*, coding for MEF3, has now been identified by following the association of reduced editing at site *atp4*-89 with the L*er* genotype in a cross between the two ecotypes L*er* and Col.

Unique target sequence at the *atp4*-89 RNA editing site

For several editing sites in mitochondria and plastids, in vitro and in organello investigations have identified the *cis*-elements in the RNA context to occupy the region between 20 or 25 nucleotides upstream (5′) to one or three nucleotides downstream (3′) of the edited C (Bock et al. 1996, Chaudhuri and Maliga 1996, Bock and Koop 1997, Farré et al. 2001, Miyamoto et al. 2002, Hegeman et al. 2005, Neuwirt et al. 2005, Sasaki et al. 2006, van der Merwe et al. 2006, Verbitskiy et al. 2008). Therefore, for the site *atp4*-89 affected by MEF3, a comparable extent of the recognition sequence may be expected.

Searching the sequence of the mitochondrial genome of *A. thaliana* in silico for motifs similar to the sequence surrounding the edited C nucleotide, no common pattern even of scattered shared nucleotide identities was found in the entire mitochondrial genome sequence. Therefore, if further target editing sites are addressed by the L*er* mutant protein, their recognition must involve a rather distinct nucleotide pattern. In addition, no other editing sites appear to be affected specifically in ecotype L*er*. Nevertheless, additional targets besides *atp4*-89 cannot be rigorously excluded editing factor MEF3.

The *atp4*-89 editing site is also present in many other flowering plant species, and the altered amino acid codon as well as

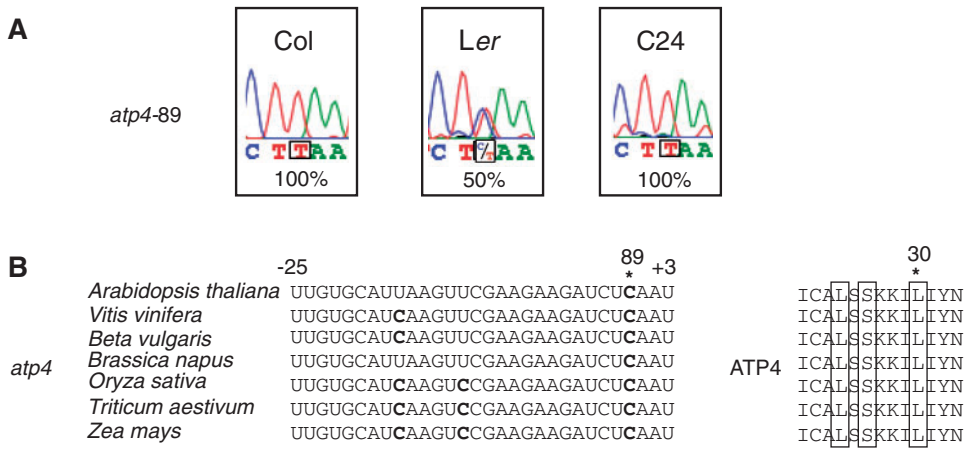


Fig. 1 An ecotype-specific difference in RNA editing is observed at a conserved nucleotide in mitochondrial RNAs. (A) The editing site at nucleotide 89 in the mRNA of the *atp4* gene (*atp4*-89) in mitochondria of *Arabidopsis thaliana* is processed to nearly 100% in ecotypes Columbia (Col) and C24, but only to about 50% in ecotype Landsberg *erecta* (L*er*). Color traces in the direct sequence analysis of the affected RNA editing site in the cDNAs of the three ecotypes are: C, blue; T, red; and A, green. (B) The nucleotide alignment of the −25 to +3 region surrounding this editing site between several species of flowering plants on the left and the respective amino acid alignment on the right show the high conservation of this region in the mitochondrially encoded subunit 4 of the ATPase. Editing sites are indicated by a bold C in the nucleotide sequences and amino acids at these sites are framed. The editing site *atp4*-89 targeted by MEF3 and the respective amino acid are marked with an asterisk and by the number above, counted from the first nucleotide or amino acid, respectively. All sequences are shown 5′ to 3′ or from the N- to the C-terminus from left to right.

Downloaded from http://pcp.oxfordjournals.org/ at University Ulm on January 29, 2013

the surrounding amino acid identities are well conserved, as would be expected for a subunit of the mitochondrial ATPase complex (Fig. 1B).

Identification of the nuclear gene responsible for the lowered mitochondrial RNA editing

The nuclear locus uniquely altered in L*er* was mapped by following the phenotype of reduced editing through a genetic screen of about 200 individual F_2 plants of a L*er*×Col cross and its co-segregating linkage to ecotype-specific sequence variations to an interval of 0.5 Mb on chromosome 1 (Fig. 2A). Since the previously identified RNA editing factors in plastids and in mitochondria (Kotera et al. 2005, Shikanai 2006, Okuda et al. 2007, Chateigner-Boutin et al. 2008, Kim et al. 2009, Okuda et al. 2009, Robbins et al. 2009, Takenaka 2010, Yu et al. 2009, Zehrmann et al. 2009, Zhou et al. 2009, Takenaka et al. 2010, Verbitskiy et al. 2010) are exclusively PPR proteins of the E or E + DYW group (besides the enhanced editing observed after knock-out of one P type protein; Doniwa et al. 2010), we first focused our search on genes encoding such proteins in this region. Besides three P-type PPR proteins, only two E group PPR protein-encoding genes (AGI code At1g06140 and At1g06145) are present in the mapped part of chromosome 1. Sequence comparison revealed several single nucleotide polymorphisms (SNPs) in one of these, At1g06140, to be L*er* specific among the three ecotypes (Fig. 2B). Thus only this gene variant shows a 100% correlation with the lowered editing phenotype in the plants analyzed, suggesting this gene At1g06140 as a candidate for being responsible for the lower RNA editing in L*er* which is thus caused by one or both of the L*er*-specific SNPs in this nuclear gene consequently termed *MEF3*.

For an independent verification of this identification, a T-DNA line of the gene At1g06140 was analyzed for its effect on RNA editing at the MEF3 target site. This Ds transposon insertion line from the RIKEN collection (Riken line name, PSH12413, 11-5869-1) is in the Noessen ecotype, and the editing status at this site was investigated in this ecotype. In wild-type Noessen plants, RNA editing at the *atp4*-89 site is, as in Col and C24, virtually complete in the steady-state mitochondrial RNA population, while in the mutant line no editing is observed (Fig. 3B). The lack of editing at this site does not seem to affect the overall growth parameters; in the greenhouse, mutant plants are indistinguishable from wild-type plants of the Noessen ecotype.

The nuclear gene At1g06140 complements mitochondrial RNA editing defects at the *atp4*-89 site

To corroborate further the connection between the reduced editing at the *atp4*-89 site and the At1g06140 locus *MEF3*, L*er* protoplasts were transfected with the wild-type Col version of the At1g06140 open reading frame under control of the 35S *Cauliflower mosaic virus* (CaMV) promoter. The L*er* protoplasts

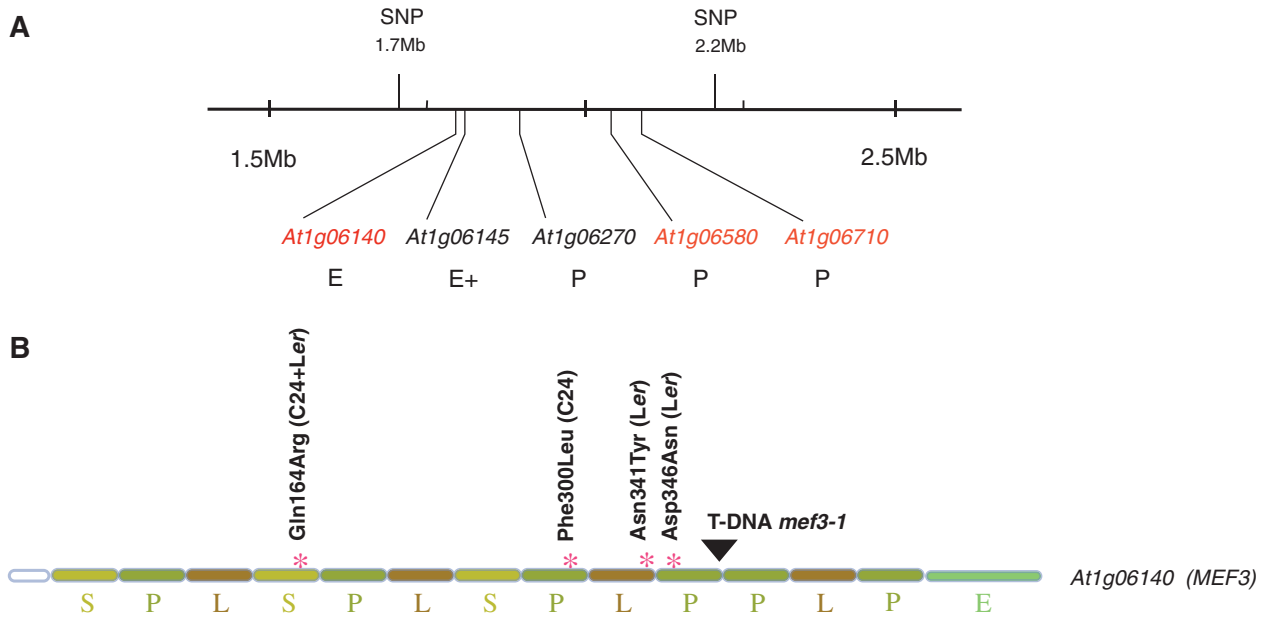


Fig. 2 Genomic mapping of the *Arabidopsis thaliana MEF3* gene. (A) Analysis of the offspring from a cross between L*er* and Col narrowed the locus connected with the lower mitochondrial editing on chromosome 1 of *A. thaliana* to a window of about 0.5 Mb. In this region, five PPR proteins are encoded, the most likely candidates for specific RNA editing factors. Three of these are P-type, one is an E+- and one an E-type PPR protein. Sequence analysis of these genes showed that the three PPR proteins shown in red have L*er* ecotype-specific SNPs while the two PPR proteins shown with black identifier numbers do not. (B) The SNPs in L*er* and C24 and the concomitant amino acid alterations against the reference sequence of Col are depicted for the E-type PPR protein deduced from the gene sequence of At1g06140 in the schematic structure. This locus encodes the MEF3 protein. The site of the T-DNA insertion in mutant *mef3-1* in repeat 10 is indicated by the arrowhead.

Downloaded from http://pcp.oxfordjournals.org/ at University Ulm on January 29, 2013

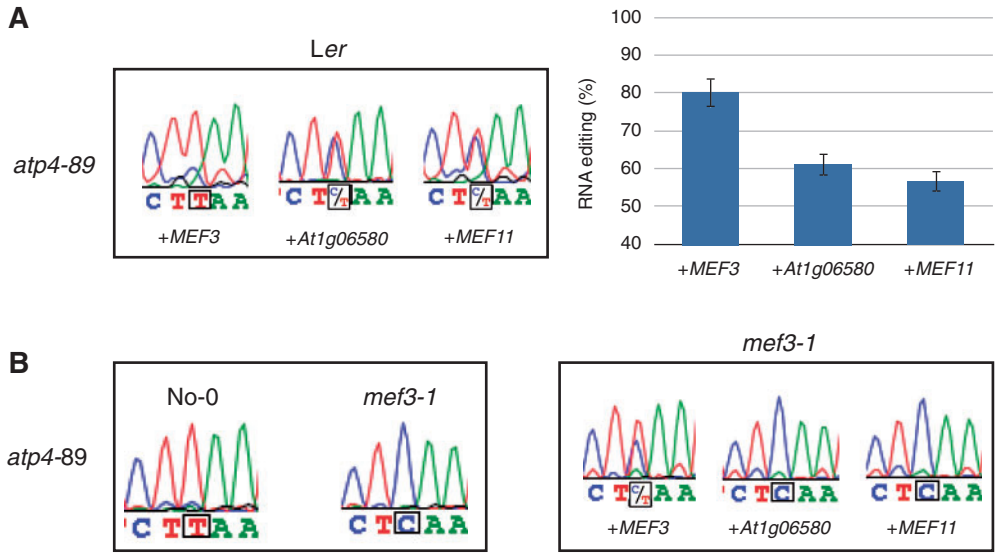


Fig. 3 Identification of the *Arabidopsis thaliana MEF3* gene. (A) Introduction of the *A. thaliana* Col versions of the two candidate genes for PPR proteins in the mapped genomic window into L*er* protoplasts shows differential effects. Sequence analysis of the *atp4* cDNA shows that gene At1g06140 strongly increases the level of RNA editing at the target site *atp4*-89 in the transfected protoplasts. Gene At1g06580 also alters the editing level in comparison with protoplasts transfected with the *MEF11* gene which is involved in RNA editing at different sites (Verbitskiy et al. 2010). The *MEF11* gene has no effect in comparison with editing in untransfected L*er* leaves (**Fig. 1A**). The right hand part shows the quantification of peak areas with the standard deviation obtained from three independent assays. (B) The T-DNA insertion mutant *mef3-1* is in ecotype Noessen plants. The left hand panel shows that wild-type Noessen mitochondrial RNA is completely edited at site *atp4*-89 while no editing is detected in the mutant. Complementation of mutant *mef3-1* protoplasts shows that only At1g06140, but not At1g06580, restores the ability to edit the target site and thus is confirmed to code for MEF3. Color traces are as in **Fig. 1**.

show RNA editing to be enhanced from ~50% editing in controls transfected with the unrelated *MEF11* gene to ~80% when transfected with At1g06140 (**Fig. 3A**). A control transfection with At1g06580, a P family PPR protein which maps in the same genomic interval and has some L*er*-specific nucleotide variations, also increased editing at the *atp4*-89 site, but much less so (**Fig. 3A**).

An analogous transfection assay with protoplasts from the T-DNA mutant plant line *mef3-1* and the *MEF3* gene at At1g06140 recovered the ability for RNA editing at *atp4*-89 (**Fig. 3B**). The control transfection with the P family PPR protein gene encoded at At1g06580 shows no recovery of RNA editing, suggesting that the alteration of the RNA editing level in the L*er* protoplasts was fortuitous and probably an unspecific secondary effect. These results confirm that the At1g06140 locus indeed codes for the MEF3 RNA editing factor specifically required for editing at the *atp4*-89 nucleotide.

Expression of the *MEF3* gene

The AtGenExpress analysis of *MEF3* gene expression shows the very low level of transcription that is typical for *MEF* genes (http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp; http://www.weigelworld.org/resources/microarray/AtGenExpress/). The steady-state transcript levels of *MEF3* in distinct tissues are very similar throughout the tissue types available and do not show any increase in, for example, anthers or pollen where often genes coding for mitochondrial household functions are more highly transcribed than in other tissues.

Comparison of the *MEF3* transcription level between the different ecotypes reveals an interesting correlation: while in Col and C24 the steady-state levels of the *MEF3* mRNA are very similar, transcripts of the variant *MEF3* gene in L*er* are more than twice as abundant (Winter et al. 2007). This may suggest some feedback signal which increases transcript abundance for the only partially functional L*er* version of MEF3 to compensate for the lowered level of editing. It may be interesting to see whether this correlation in mRNA abundance is a mere coincidence or if such a quality feedback control (potentially from the capacity of the ATPase) indeed exists and reaches from the mitochondrion through retrograde signaling into the nucleus.

The *MEF3* gene encodes a PPR protein of the E class

The genomic alterations responsible for the distinct editing phenotype at site *atp4*-89 in ecotype L*er* vs. Col and C24 are two nucleotide variations resulting in two amino acid changes. These two SNPs in ecotype L*er* alter an asparagine to tyrosine in one of the L-repeats (repeat number 9) and an aspartic acid is changed to asparagine in the next downstream P repeat (**Fig. 2B**). In ecotype C24, amino acid 164 is altered specifically compared with the Col reference by one SNP from a glutamine

Downloaded from http://pcp.oxfordjournals.org/ at University Ulm on January 29, 2013

to an arginine, which does not seem to affect the function of the MEF3 protein since editing in ecotype C24 at the *atp4*-89 target site of MEF3 is not different from that in Col. Otherwise the different ecotype-specific alleles of the *MEF3* gene (At1g06140) encode identical continuous open reading frames for a member of the E subgroup of the PPR protein family (Small and Peeters 2000, Lurin et al. 2004; **Fig. 2B**).

The protein most similar to the MEF3 E class PPR protein is a DYW PPR protein

The search for similar genes in other plant species reveals a very similar gene only in the *Vitis vinifera* (vine) genome (*V. vinifera* gene ID: GSVIVG01021021023001; **Fig. 4A**, VvMEF3). Homologs are present in the various *A. thaliana* accessions as well as in *Arabidopsis lyrata* and *Brassica napus*, but are not detectable in other species for which full sequence data are available. Identical and conserved amino acids are spread throughout the protein, with some of the repeats particularly in the C-terminal region showing a higher degree of conservation (**Fig. 4B**). This pattern of the three C-terminal repeats being better conserved has been found in most PPR proteins and has been interpreted to suggest a specific function of this region (Rivals et al. 2006). Intriguingly, PPR # 9, in which one of the amino acid changes between ecotypes L*er* and Col occurs, is also prominently conserved: almost 70% of the amino acids are identical and nearly 90% are similar between *A. thaliana* and *V. vinifera* (**Fig. 4C**). Comparison of the amino acid identities around the L*er* polymorphisms allows clear alignment of the amino acid sequences in this region. At the sites of the L*er* polymorphisms, the Col encoded amino acids are identical to those in vine, suggesting that the L*er* variants are indeed derived and tolerated, although they are less efficient in editing at site *atp4*-89. The altered amino acid 346, asparagine in L*er*, is still similar in its general properties to the aspartic acid encoded in Col and vine. The change of amino acid 341 from asparagine to the rather different tyrosine is more dramatic,

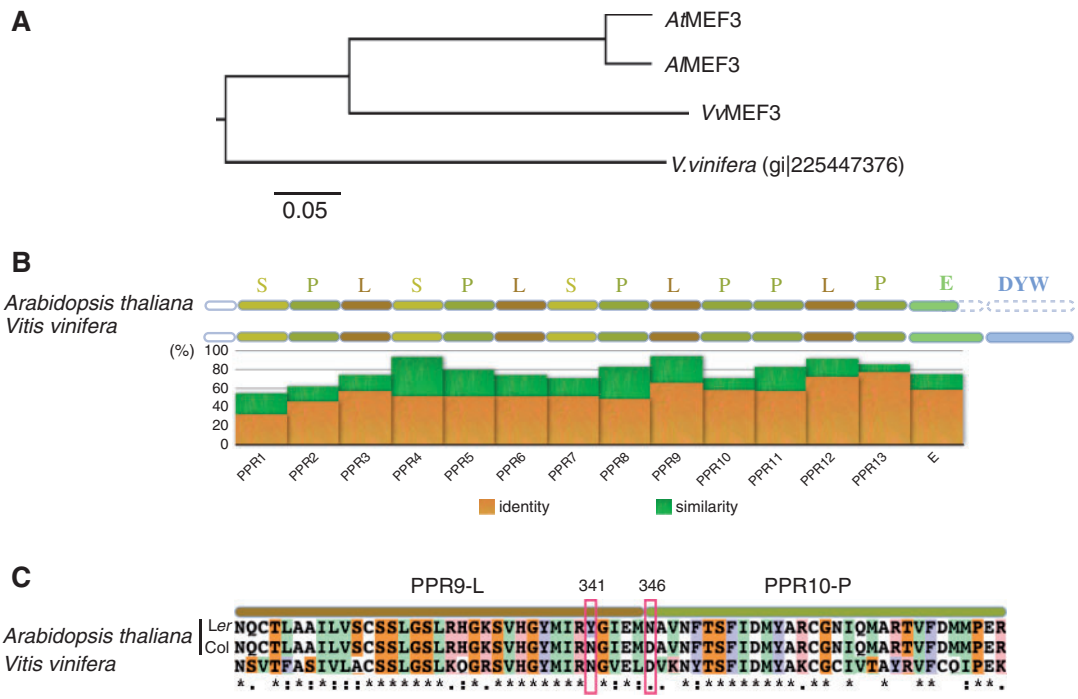


Fig. 4 Comparison of the modular structural composition of the protein MEF3 and the similar protein in vine. (A) A protein similar to the *Arabidopsis thaliana* (*At*) MEF3 is only identified in the vine genome (*Vv*MEF3) but not in other plants beyond *Arabidopsis lyrata* (Al). This vine protein is more similar to *At*MEF3 than the next most similar protein MEF22 in *A. thaliana* or the next most similar protein in vine (*Vv*; given is the NCBI Gene identification number) as the CLUSTALW similarity tree shows. (B) In *A. thaliana* the MEF3 protein terminates just after the (partial) E domain (filled light green oblong). The open reading frame of the MEF3-like protein in *Vitis vinifera* (vine) continues into an additional C-terminal adjacent DYW domain (blue oblong). The different types of PPRs are color coded and the putative mitochondrial target sequences are depicted as open ovals. Below the cartoon of the two protein structures, the similarity and identity, respectively, are shown for each PPR by the height of the columns as a percentage. The smaller, brown columns represent the percentages of identical amino acids, while the larger green columns show those of similar amino acids, including the identical moieties. (C) Alignment of the rather well conserved L repeat number 9 and the somewhat less conserved P repeat number 10. The L*er*-specific amino acid changes are framed in red and their positions are given. Comparison of the vine MEF3-like protein with the MEF3 proteins in the two *A. thaliana* ecotypes shows that L*er* deviates from the Col and vine consensus, suggesting that the lower editing phenotype in L*er* is the derived trait caused by these mutations. Dots indicate similar amino acids, double dots very similar residues and stars denote identical amino acids among the three protein sequences. Amino acids are background colored according to their biochemical properties.

Downloaded from http://pcp.oxfordjournals.org/ at University Ulm on January 29, 2013

suggesting that this latter alteration may be the cause of the lowered editing efficiency of MEF3 at its target site. On the other hand, only the combination of both alterations may cripple the MEF3 protein to a lower editing capacity. Such a situation has been observed with MEF1 where only all three SNPs of ecotype C24 together result in the reduction of the RNA editing efficiency (Zehrmann et al. 2010).

The E and DYW domains from the MEF3-like protein in vine fully support editing

In vine, the editing event *atp4*-89 in the mitochondrial RNA is conserved, suggesting that this may also be the specific target of the respective MEF3-like protein in this plant (**Fig. 1B**; Picardi et al. 2010). In vine, a total of 445 RNA editing sites have been reported (Picardi et al. 2010) and PPR domains have been detected in 605 proteins (Jaillon et al. 2007). Surprisingly, the vine PPR protein extends beyond the E extension region common to both plants into a perfect DYW domain. This observation raises the question of whether the vine protein really is a true ortholog and has the same function as MEF3 in *A. thaliana*. To investigate this, the vine gene was cloned and transfected into *mef3-1* protoplasts. In these assays, no recovery of editing by the vine protein was observed (**Fig. 5**, construct VvMEF3). The full-length vine protein thus appears to deviate too much from the MEF3 protein in *A. thaliana* to be able to substitute for it as a true functional ortholog.

To obtain further information about the parts of the protein which may be responsible for the inability to complement, we constructed several deletion variants and chimeras of the proteins from the two plants and tested these in protoplast complementation assays (**Fig. 5**). To investigate whether the DYW region from vine is inhibitory, it was deleted from the vine protein. This construct (VvMEF3ΔDYW) was not able to substitute for the MEF3 protein in the *mef3-1* mutant protoplasts. To investigate the function of the PPRs, this region from the vine protein was inserted into the *A. thaliana* MEF3 protein in the place of the original repeats between the putative mitochondrial target sequence and the E domain from *A. thaliana* [**Fig. 5**, construct AtMEF3/Vv(PPR)]. This chimeric protein was not able to target the *atp4*-89 site in *A. thaliana*, suggesting that the repeats from vine recognize a different RNA pattern or that they cannot funtion in conjunction with the *A. thaliana* E domain. To test the potential functional equivalence of the EE+ and DYW regions of the vine protein

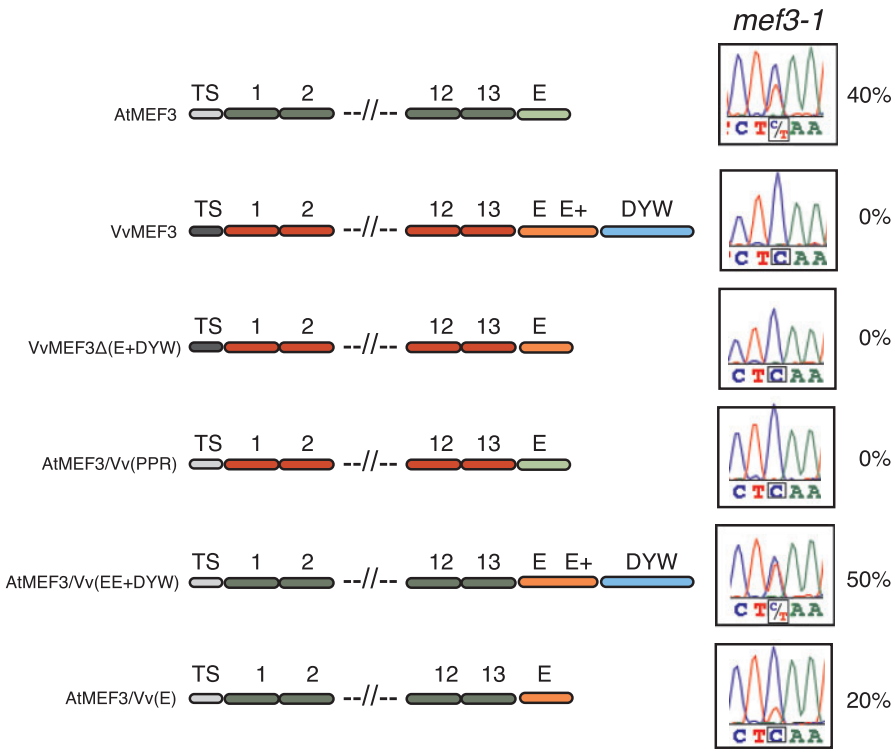


Fig. 5 The EE+ and DYW domains from vine can substitute for the *Arabidopsis thaliana* E domain. The structures of the various deletions and chimeras of *A. thaliana* MEF3 and the similar protein from *Vitis vinifera* are shown on the left. The complementation assays of mutant *mef3-1* protoplasts and the percentages of editing seen in these assays of the respective constructs are shown on the right. The pure vine protein with (VvMEF3) or without the E+ and the DYW domains [VvMEF3Δ(E+DYW)] cannot substitute for MEF3. Substitution of the *A. thaliana* PPR domain by the repeats from the vine protein [AtMEF3/Vv(PPR)] abolishes the ability to edit at the target site of MEF3. However, the E domain from vine [AtMEF3/Vv(E)] partially fulfills the function of the original *A. thaliana* E domain, and the vine EE+ and DYW regions together are as competent as the original *A. thaliana* E domain [AtMEF3/Vv(EE+DYW)]. The *A. thaliana* target sequence is shown in light gray and the vine sequence in dark gray. E and E+ domains are shown as one long oval, and the solitary E domain as a short oval as indicated.

Downloaded from http://pcp.oxfordjournals.org/ at University Ulm on January 29, 2013

with the E domain from *A. thaliana*, these were grafted onto the *A. thaliana* MEF3 PPRs in place of the original E domain. Both EE+ and DYW domains together regenerate the complete function of the native *A. thaliana* MEF3 protein [**Fig. 5**, construct AtMEF3/Vv(EE+DYW)]. However, the vine E domain alone is only partially able to substitute for the original *A. thaliana* E region [**Fig. 5**, construct AtMEF3/Vv(E)].

Discussion

Did the MEF3 E PPR editing factor evolve from a DYW protein?

While the MEF3 protein in *A. thaliana* terminates after the core E domain, the most similar protein found in vine continues into a full-length E and E+ domain and a DYW region (**Fig. 4**). In *A. lyrata*, the orthologous protein shows a structure similar to MEF3 in *A. thaliana*, suggesting that loss of the DYW domain occurred before the split of these two *Arabidopsis* species. A gain of the DYW region in vine seems mechanically unlikely since this would require some sort of recombination event with another DYW PPR protein-encoding locus which we cannot detect in vine or any other plant genome.

This suggested direction of the evolution of the MEF3 PPR protein has also been inferred from the observation that so far only DYW proteins have been connected with RNA editing events in the moss *P. patens*. The DYW configuration of the PPR proteins in plants can be considered as the ancient form, based on the recent confirmation that the DYW PPR proteins in moss are indeed involved in RNA editing (Ohtani et al. 2010, Tasaki et al. 2010, Rüdinger et al. 2011). Accordingly, it was proposed that the genes for DYW PPR proteins were amplified during the evolution of flowering plants to serve an increasing number of editing sites, and only subsequently lost their DYW region and in some instances also parts or all of the E+ extensions during the evolution of the flowering plants (**Fig. 4**; Rivals et al. 2006, Knoop and Rüdinger 2010). The potential deaminating activity of the DYW region in mitochondrial and plastid RNA editing (Salone et al. 2007) may then be provided by recruited co-acting DYW PPR proteins and thus free the site-specific PPR protein from the requirement for its own DYW extension. This scenario makes the DYW domains, at least in some of the site-specific PPR proteins, superfluous and allows their elimination as suggested here for MEF3.

Experimental evidence for such a flexible requirement for the DYW domains has been obtained in plastid and mitochondrial editing factors (Okuda et al. 2010). In the latter, the DYW domain can be deleted in MEF11, but not in MEF1 (Zehrmann et al. 2010).

In MEF3, the absence of the DYW domain in at least the two *Arabidopsis* species shows that this DYW domain is not required in MEF3 although it is (still) present in the otherwise similar vine protein. The substitution of the *A. thaliana* MEF3 E domain by the vine EE+ and DYW domains, and to some extent also by just the E domain from the vine protein (**Fig. 5**), suggests that the function of these regions is conserved. Furthermore, the additional E+ region and DYW domain from the vine protein do not inhibit the RNA editing process in *A. thaliana* and thus presumably do not interfere with the assembly of the MEF3 protein into the as yet hypothetical editosome.

The PPR repeats between MEF3 and the vine protein are not equivalent

Different from the E and optional E+ and DYW domains, the PPRs from the vine protein cannot substitute for the repeats in the *A. thaliana* MEF3, showing that these regions have functionally diverged. If, as has been proposed, these repeats do bind to specific nucleotides in the RNA *cis*-element, the specificity of the vine PPR protein may differ from that of the *A. thaliana* MEF3 protein. However, the *cis*-sequences upstream of the conserved *atp4*-89 editing sites are identical up to another editing site in vine. At this nucleotide, a U is already specified by the mitochondrial DNA in *A. thaliana* and, after editing in vine, the RNA sequences in both plants are identical. Therefore, even if the vine PPR protein would contact different individual nucleotides in this region, the *A. thaliana* site should still be addressed correctly. The vine protein thus seems to target a different editing site and thus would not be a true ortholog of MEF3 and/or it attracts different cofactors in the editosome. The first possibility would require another, very different PPR protein or other specificity factor, which is not present in *A. thaliana*. If an ortholog of such a hypothetical factor was present in *A. thaliana*, this factor should compensate at least partially for the absence of MEF3 in the *mef3-1* mutant plant. The second possibility of the vine protein attracting different cofactors would suggest that the PPR domain of the repeats is involved not only in binding to the RNA but also in the connection to other components of the editosome.

The absense of proteins similar to MEF3 in, for example, poplar and rice suggests that a different PPR protein indeed has taken over the role of MEF3 in these other plant species. Here the *atp4*-89 editing event as well as the *cis*-sequences upstream are identical and therefore have to be targeted by an only distantly related PPR protein. If this protein is also present in vine, the MEF3-like protein would be free to target a different site. Of course, the failure of the *A. thaliana* MEF3–vine PPR chimera [**Fig. 5**, construct AtMEF3/Vv(PPR)] and the vine gene (**Fig. 5**, construct VvMEF3) to complement each other is a negative result which may be due to any kind of interference.

The MEF3 E class PPR protein does not contain the E+ region

While many of the PPR proteins terminate after a 'full-length' E (and E+) domain, some—such as the MEF3 protein analyzed here—are truncated within the usually conserved E sequence near the border between the E and E+ subdomains. Alignment

Downloaded from http://pcp.oxfordjournals.org/ at University Ulm on January 29, 2013

of several of these shorter PPR proteins with partial E domains, MEF3, MEF9, MEF18, MEF19, MEF20 and OTP87, shows that all of them contain the N-terminal part and end at similar distances from the beginning of the E domain. This N-terminal region possibly contains an amino acid motif which is required for an important function such as the connection to other putative components of the 'editosome'. This inference is supported by the high degree of conservation of several amino acid identities and positions in this region. Furthermore, the five MEF proteins end at very closely spaced amino acid positions in the E domain which coincide with the border between the proposed E and E+ subregions (Lurin et al. 2004, Rivals et al. 2006, O'Toole et al. 2008). This coincidence supports a functional significance of the distinction into E and E+ subdomains, the E+ subregion being as dispensable as the originally adjacent DYW domain. Complete removal of the E domain may not be tolerated in PPR proteins essential for specific editing sites, while the truncated E domains seem to be sufficient in at least some MEF proteins.

Our finding reported here that the MEF3 RNA editing factor and a very similar protein exist as in vivo variants in two different plant species strengthens the theory of the evolutionary direction leading from DYW PPR proteins—via the loss of the DYW domain and in several instances also the deletion of the E+ subregion—to E-domain-only PPR proteins.

Materials and Methods

Plant material and preparation of nucleic acids

Arabidopsis thaliana seeds for the Col, L*er* and C24 ecotypes were kind gifts of J. Forner (Universität Heidelberg) and S. Binder (Universität Ulm). Growth of the *A. thaliana* plants and preparation of DNA or RNA from the leaves were as described (Takenaka and Brennicke 2007).

Analysis of RNA editing sites

Specific cDNA fragments were generated by reverse transcription–PCR (RT–PCR) amplification by established protocols (Takenaka and Brennicke 2007). The cDNA sequences were compared for C to T differences resulting from RNA editing. Most sequences were obtained commercially from 4base lab, Reutlingen, Germany or from Macrogen, Seoul, Korea.

Protoplast complementation assays

Protoplasts were prepared from 3- to 4-week-old plantlets by the method of Yoo et al. (2007). Transfected genes were expressed from the 35S promoter in the cloning site of vector pSMGFP4. The efficiency of the transfection was monitored by the signals from separately introduced or co-transfected green fluorescent protein (GFP) genes in the cytoplasm. Typically the GFP fluorescence was detected in >80% of the transfected protoplasts. Total RNA was isolated after 20–24 h incubation at room temperature. Sequences of cDNAs were determined after RT–PCR with the respective specific primers. RNA editing levels were estimated by the relative areas of the respective nucleotide peaks in the sequence analyses.

Funding

This work was supported the Deutsche Forschungsgemeinschaft; a Heisenberg award [to M.T.].

Acknowledgments

We thank Dagmar Pruchner and Angelika Müller for excellent experimental help. We are grateful to Stefan Binder and Christian Jonietz at Molekulare Botanik, Universität Ulm and Joachim Forner, Universität Heidelberg for their gifts of seeds, markers and other materials.

References

Bock, R., Hermann, M. and Kössel, H. (1996) In vivo dissection of *cis*-acting determinants for plastid RNA editing. *EMBO J.* 15: 5052–5059.

Bock, R. and Koop, H.U. (1997) Extraplastidic site-specific factors mediate RNA editing in chloroplasts. *EMBO J.* 16: 3282–3288.

Chateigner-Boutin, A.-L., Ramos-Vega, M., Guevara-García, A., Andrés, C., Gutiérrez-Nava, M.d.l.L. et al. (2008) CLB19, a pentatricopeptide repeat protein required for editing of rpoA and clpP chloroplast transcripts. *Plant J.* 56: 590–602.

Chaudhuri, S. and Maliga, P. (1996) Sequences directing C to U editing of the plastid *psbL* mRNA are located within a 22 nucleotide segment spanning the editing site. *EMBO J.* 15: 5958–5964.

Doniwa, Y., Ueda, M., Ueta, M., Wada, A., Kadowaki, K.-I. and Tsutsumi, N. (2010) The involvement of a PPR protein of the P subfamily in partial RNA editing of an Arabidopsis mitochondrial transcript. *Gene* 454: 39–46.

Farré, J.-C., Leon, G., Jordana, X. and Araya, A. (2001) Cis recognition elements in plant mitochondrion RNA editing. *Mol. Cell. Biol.* 21: 6731–6737.

Giegé, P. and Brennicke, A. (1999) RNA editing in Arabidopsis mitochondria effects 441 C to U changes in ORFs. *Proc. Natl Acad. Sci. USA* 96: 15324–15329.

Hammani, K., Okuda, K., Tanz, S.K., Chateigner-Boutin, A.-L., Shikanai, T. and Small, I. (2009) A study of new Arabidopsis chloroplast RNA editing mutants reveals general features of editing factors and their target sites. *Plant Cell* 21: 3686–3699.

Hammani, K., Takenaka, M., Tanz, S.K., Okuda, K., Shikanai, T., Brennicke, A. et al. (2011) The pentatricopeptide repeat protein OTP87 is essential for RNA editing of *nad7* and *atp1* transcripts in Arabidopsis mitochondria. *J. Biol. Chem.* 286: 21361–21371.

Hegeman, C.E., Hayes, M.L. and Hanson, M.R. (2005) Substrate and cofactor requirements for RNA editing of chloroplast transcripts in Arabidopsis in vitro. *Plant J.* 42: 124–132.

Jaillon, O., Aury, J.M., Noel, B., Policriti, A., Clepet, C., Casagrande, A. et al. (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature* 449: 463–467.

Downloaded from http://pcp.oxfordjournals.org/ at University Ulm on January 29, 2013

Kim, S.-R., Yang, J.-I., Moon, S., Ryu, C.-H., An, K., Kim, K.M. et al. (2009) Rice OGR1 encodes a pentatricopeptide repeat-DYW protein and is essential for RNA editing in mitochondria. *Plant J.* 59: 738–749.

Knoop, V. and Rüdinger, M. (2010) DYW-type PPR proteins in a heterolobosean protist: plant RNA editing factors involved in an ancient horizontal gene transfer? *FEBS Lett* 584: 4287–4291.

Kotera, E., Tasaka, M. and Shikanai, T. (2005) A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. *Nature* 433: 326–330.

Lurin, C., Andrés, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyère, C. et al. (2004) Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. *Plant Cell* 16: 2089–2103.

Miyamoto, T., Obokata, J. and Sugiura, M. (2002) Recognition of RNA editing sites is directed by unique proteins in chloroplasts: biochemical identification of cis-acting elements and trans-acting factors involved in RNA editing in tobacco and pea chloroplasts. *Mol. Cell. Biol.* 22: 6726–6734.

Neuwirt, J., Takenaka, M., van der Merwe, J.A. and Brennicke, A. (2005) An in vitro RNA editing system from cauliflower mitochondria: editing site recognition parameters can vary in different plant species. *RNA* 11: 1563–1570.

Ohtani, S., Ichinose, M., Tasaki, E., Aoki, Y., Komura, Y. and Sugita, M. (2010) Targeted gene disruption identifies three PPR-DYW proteins involved in RNA editing for five editing sites of the moss mitochondrial transcripts. *Plant Cell. Physiol.* 51: 1942–1949.

Okuda, K., Chateigner-Boutin, A.-L., Nakamura, T., Delannoy, E., Sugita, M., Myouga, F. et al. (2009) Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in *Arabidopsis* chloroplasts. *Plant Cell* 21: 146–156.

Okuda, K., Hammani, K., Tanz, S.K., Peng, L., Fukao, Y., Myouga, F. et al. (2010) The pentatricopeptide repeat protein OTP82 is required for RNA editing of plastid ndhB and ndhG transcripts. *Plant J.* 61: 339–349.

Okuda, K., Myouga, F., Motohashi, R., Shinozaki, K. and Shikanai, T. (2007) Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. *Proc. Natl Acad. Sci. USA* 104: 8178–8183.

O'Toole, N., Hattori, M., Andres, C., Iida, K., Lurin, C., Schmitz-Linneweber, C. et al. (2008) On the expansion of the pentatricopeptide repeat gene family in plants. *Mol. Biol. Evol.* 25: 1120–1128.

Picardi, E., Horner, D.S., Chiara, M., Schiavon, R., Valle, G. and Pesole, G. (2010) Large-scale detection and analysis of RNA editing in grape mtDNA by RNA deep-sequencing. *Nucleic Acids Res.* 38: 4755–4767.

Rivals, E., Bruyère, C., Toffano-Nioche, C. and Lecharny, A. (2006) Formation of the Arabidopsis pentatricopeptide repeat family. *Plant Physiol.* 141: 825–839.

Robbins, J.C., Heller, W.P. and Hanson, M.R. (2009) A comparative genomics approach identifies a PPR-DYW protein that is essential for C-to-U editing of the Arabidopsis chloroplast accD transcript. *RNA* 15: 1142–1153.

Rüdinger, M., Funk, H.T., Rensing, S.A., Maier, U.G. and Knoop, V. (2009) RNA editing: only eleven sites are present in the Physcomitrella patens mitochondrial transcriptome and a universal nomenclature proposal. *Mol. Genet. Genomics* 281: 473–481.

Rüdinger, M., Szövényi, P., Rensing, S.A. and Knoop, V. (2011) Assigning DYW-type PPR proteins to RNA editing sites in the funariid mosses *Physcomitrella patens* and *Funaria hygrometrica*. *Plant J.* 67: 370–380.

Salone, V., Rüdinger, M., Polsakiewicz, M., Hoffmann, B., Groth-Malonek, M., Szurek, B. et al. (2007) A hypothesis on the identification of the editing enzyme in plant organelles. *FEBS Lett.* 581: 4132–4138.

Sasaki, T., Yukawa, Y., Wakasugi, T., Yamada, K. and Sugiura, M. (2006) A simple in vitro RNA editing assay for chloroplast transcripts using fluorescent dideoxynucleotides: distinct types of sequence elements required for editing of ndh transcripts. *Plant J.* 47: 802–810.

Shikanai, T. (2006) RNA editing in plant organelles: machinery, physiological function and evolution. *Cell. Mol. Life Sci.* 63: 689–708.

Small, I.D. and Peeters, N. (2000) The PPR motif—a TPR-related motif prevalent in plant organellar proteins. *Trends Biochem. Sci.* 25: 46–47.

Takenaka, M. (2010) MEF9, an E subclass pentatricopeptide repeat protein, is required for an RNA editing event in the *nad7* transcript in mitochondria of *Arabidopsis*. *Plant Physiol.* 152: 939–947.

Takenaka, M. and Brennicke, A. (2007) RNA editing in plant mitochondria: assays and biochemical approaches. *Methods Enzymol.* 424: 439–458.

Takenaka, M., Verbitskiy, D., van der Merwe, J.A., Zehrmann, A. and Brennicke, A. (2008) The process of RNA editing in plant mitochondria. *Mitochondrion* 8: 35–46.

Takenaka, M., Verbitskiy, D., Zehrmann, A. and Brennicke, A. (2010) Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. *J. Biol. Chem.* 285: 27122–27129.

Tasaki, E., Hattori, M. and Sugita, M. (2010) The moss pentatricopeptide repeat protein with a DYW domain is responsible for RNA editing of mitochondrial ccmFc transcript. *Plant J.* 62: 560–570.

van der Merwe, J.A., Takenaka, M., Neuwirt, J., Verbitskiy, D. and Brennicke, A. (2006) RNA editing sites in plant mitochondria can share cis-elements. *FEBS Lett.* 580: 268–272.

Verbitskiy, D., van der Merwe, J.A., Zehrmann, A., Brennicke, A. and Takenaka, M. (2008) Multiple specificity-recognition motifs enhance RNA editing in plant mitochondria. *J. Biol. Chem.* 283: 24374–24381.

Verbitskiy, D., Zehrmann, A., Härtel, B., Brennicke, A. and Takenaka, M. (2011) The DYW-E-PPR protein MEF14 is required for RNA editing at site *matR*-1895 in mitochondria of *Arabidopsis thaliana*. *FEBS Lett.* 585: 700–704.

Verbitskiy, D., Zehrmann, A., van der Merwe, J.A., Brennicke, A. and Takenaka, M. (2010) The PPR-protein encoded by the lovastatin insensitive 1 gene is involved in RNA editing at three sites in mitochondria of *Arabidopsis thaliana*. *Plant J.* 61: 446–455.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V. and Provart, N.J. (2007) An 'Electronic Fluorescent Pictograph' browser for exploring and analyzing large-scale biological data sets. *PLoS One* 2: e718.

Yoo, S.-D., Cho, Y.-H. and Sheen, J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* 2: 1565–1572.

Yu, Q.-B., Jiang, Y., Chong, K. and Yang, Z.-N. (2009) AtECB2, a pentatricopeptide repeat protein, is required for chloroplast transcript

Downloaded from http://pcp.oxfordjournals.org/ at University Ulm on January 29, 2013

accD RNA editing and early chloroplast biogenesis in Arabidopsis thaliana. *Plant J.* 59: 1011–1023.

Zehrmann, A., van der Merwe, J.A., Verbitskiy, D., Brennicke, A. and Takenaka, M. (2008) Seven large variations in the extent of RNA editing in plant mitochondria between three ecotypes of Arabidopsis thaliana. *Mitochondrion* 8: 319–327.

Zehrmann, A., van der Merwe, J.A., Verbitskiy, D., Brennicke, A. and Takenaka, M. (2009) A DYW domain containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of Arabidopsis thaliana. *Plant Cell* 21: 558–567.

Zehrmann, A., Verbitskiy, D., Härtel, B., Brennicke, A. and Takenaka, M. (2010) RNA editing competence of trans-factor MEF1 is modulated by ecotype-specific differences but requires the DYW domain. *FEBS Lett.* 584: 4181–4186.

Zhou, W., Cheng, Y., Yap, A., Chateigner-Boutin, A.L., Delannoy, E., Hammani, K. et al. (2009) The Arabidopsis gene YS1 encoding a DYW protein is required for editing of rpoB transcripts and the rapid development of chloroplasts during early growth. *Plant J.* 58: 82–96.

Downloaded from http://pcp.oxfordjournals.org/ at University Ulm on January 29, 2013

Copyright Notice:
Reprinted from
Zehrmann, A., van der Merwe, J. A., Verbitskiy, D., Härtel, B., Brennicke, A., and Takenaka, M.
„The DYW-class PPR protein MEF7 is required for RNA editing at four sites in mitochondria of *Arabidopsis thaliana*."
RNA Biology 9:2, 155-161
© 2012 Landes Bioscience, open access

RESEARCH PAPER

RNA Biology 9:2, 155–161; February 2012; © 2012 Landes Bioscience

The DYW-class PPR protein MEF7 is required for RNA editing at four sites in mitochondria of *Arabidopsis thaliana*

Anja Zehrmann, Johannes A. van der Merwe,† Daniil Verbitskiy, Barbara Härtel, Axel Brennicke and Mizuki Takenaka*

Molekulare Botanik; Universität Ulm; Ulm, Germany

†Current affiliation: Institut für Molekulare Virologie; Uni Ulm; Ulm, Germany

Keywords: RNA editing, plant mitochondria, PPR protein, MEF7, specificity factor

In plant mitochondria and plastids, RNA editing alters about 400 and about 35 C nucleotides into Us, respectively. Four of these RNA editing events in plant mitochondria specifically require the PPR protein MEF7, characterized by E and DYW extension domains. The gene for MEF7 was identified by genomic mapping of the locus mutated in plants from EMS treated seeds. The SNaPshot screen of the mutant plant population identified two independent EMS mutants with the same editing defects as a corresponding T-DNA insertion line of the MEF7 gene. Although the amino acid codons introduced by the editing events are conserved throughout flowering plants, even the combined failure of four editing events does not impair the growth efficiency of the mutant plants. Five nucleotides are conserved between the four affected editing sites, but are not sufficient for specific recognition by MEF7 since they are also present at three other sites which are unaffected in the mutants.

Introduction

The selection of the individual C to U RNA editing events in plastids and mitochondria of plants against unedited C nucleotides has been puzzling for a long time. Recently, a number of proteins have been described which are each required for single or very few such editing events. Although the family of these proteins is rather large with at least 450 members in flowering plants,[1-4] they are hardly enough to specify all editing sites in a one-on-one modus. Especially since many of these proteins have other roles in RNA maturation, most of the editing specificity factors would have to be able to target several sites. Within the group of these pentatricopeptide repeat proteins (PPR proteins), the identified editing factors such as the *Arabidopsis thaliana* mitochondrial editing factors MEF1, MEF11 and MEF22 (mitochondrial editing factor) as well as the OGR1 factor in rice and others in a moss[5-8] contain a ~100 amino acids long DYW region at the C-terminus beyond the E domain. Others like the mitochondrial proteins SLO1, MEF9 and MEF18-21 terminate with the E domain.[9-11] Analogously, some plastid RNA editing factors are E type PPR proteins, others contain also a DYW domain.[12-20] The DYW region is optional in some of the DYW PPR proteins such as OTP82 in plastids[21] or MEF11 in mitochondria,[7,22] but is required in MEF1.[23,24]

All of the editing factors described so far belong to the PLS subgroup of PPR proteins and contain at least the E domain. In *Arabidopsis thaliana* genes for 194 E type PPR proteins are found, in addition to six genes for PPR proteins with only PLS repeats.[1,2,11] In order for these roughly 200 E class PPR proteins to address the about 500 editing sites in mitochondria and plastids in flowering plants,[25] each of them will have to target at least two sites. In contrast to this expectation, a number of the identified PLS PPR proteins seem specific to individual single sites.[10,11,13]

Here we identify the DYW PPR protein MEF7 as novel specific RNA editing factor which is required for editing of four sites in four different mRNAs in mitochondria of *Arabidopsis thaliana*.

Results and Discussion

Identification of the *mef7*-1 mutant deficient in editing at site *cob*-325. Our screen for mutants altered at one or more specific mitochondrial RNA editing sites in *Arabidopsis thaliana* identified a loss of RNA editing at site *cob*-325 in the SNaPshot analysis of an EMS mutated plant population.[26] This site is edited very efficiently in wild-type plants, and no residual unedited C signal is detected in the steady-state RNA from young leaves of accession Columbia (**Fig. 1**). When all other known editing sites were analyzed in the isolated homozygous mutant plants, now named mutant *mef7*-1, further three editing sites were found to be not detectably edited or at least strongly reduced, sites *ccb206*-28, *nad2*-1433 and *nad4L*-41 (**Fig. 1**). A second mutant plant was

*Correspondence to: Mizuki Takenaka; Email: mizuki.takenaka@uni-ulm.de
Submitted: 11/02/11; Accepted: 11/03/11
http://dx.doi.org/10.4161/rna.18644

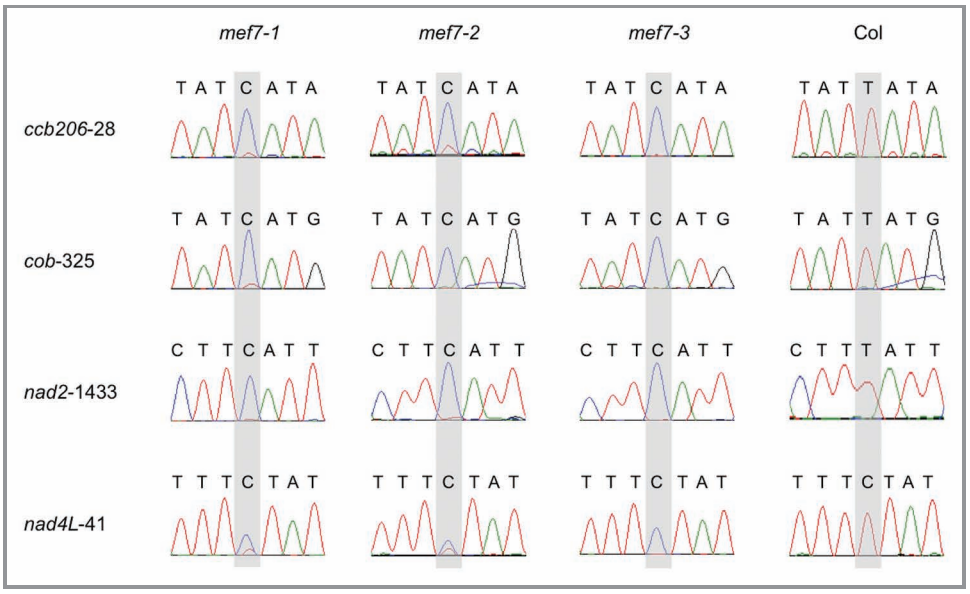


Figure 1. RNA editing is abolished or largely reduced at four editing sites in four different mitochondrial mRNAs. The two independent EMS mutant lines *mef7–1* and *mef7–2* show residual editing at nucleotide 41 in the mRNA of the *nad4L* gene (*nad4L*-41), whereas the T-DNA insertion line *mef7–3* completely lost the ability to edit this site similar to the other three target sites. All four target sites of MEF7 are altered to nearly 100% in mitochondria of wild type plants of *Arabidopsis thaliana* ecotype Columbia (Col). The affected RNA editing sites are shaded in the cDNA sequence analyses. In the sequence trace of *nad2*-1433, editing at the next downstream site at nucleotide *nad2*-1436 (the last T in the cDNAs) is unaffected by the loss of MEF7 and therefore targeted by a different specificity factor. Color traces are: C-blue, T-red, G-black, A-green.

found in the EMS population with similar defects in RNA editing at the same sites, which was accordingly named *mef7*-2. These coinciding RNA editing phenotypes in two plant lines, if they are indeed independent mutants, suggest a single locus to be responsible.

Identification of the nuclear gene coding for MEF7. The nuclear locus causing the absence of editing at the four sites was identified by following the association of reduced editing at these sites with the mutant Col genotype in a cross between the wild-type ecotype L*er* and the mutant plant *mef7*-1.

Analysis of editing of about 100 individual F2 plants of a L*er* x *mef7*-1 Col cross linked the mutation to an interval of about 1.75 Mb (Megabases) on chromosome 5 (**Fig. 2A**). In this region we focused our search for candidate genes on those encoding PPR proteins of the E or E+DYW group as being the most likely possibilities. So far all identified specific RNA editing factors in

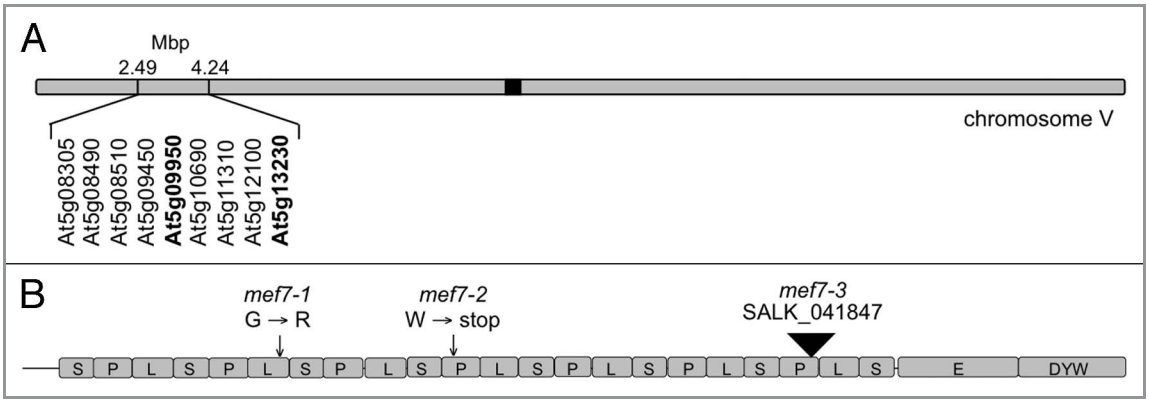


Figure 2. Identification of the *Arabidopsis thaliana MEF7* gene. (A) The locus of the *MEF7* gene was genomically mapped by analyzing the editing phenotype of the offspring from a cross between wild type L*er* and the *mef7–1* mutant plant. Cosegregation analysis with Col/L*er*-specific markers narrowed the locus connected with the reduced mitochondrial editing on chromosome 5 of *Arabidopsis thaliana* to a region of about 1.75 Mbp. In this region nine PPR proteins are encoded, two of which are DYW class proteins (shown in bold), the most likely candidates for specific RNA editing factors. Sequence analysis of these two genes in the two EMS mutant plants revealed specific SNPs in At5g09950 which is thus identified to encode the MEF7 protein. (B) These nucleotide changes and the concomitant amino acid alterations against the reference sequence of Col are shown in the schematic structure of the MEF7 DYW PPR protein deduced from the gene sequence. The T-DNA insertion site of a SALK line was determined, this mutant plant line is now named *mef7–3*.

plastids as well as in mitochondria belong to this family.[7-18] Four P group, three E type and two DYW class PPR proteins encoding genes are located in the identified interval on chromosome 5. We first sequenced the two DYW PPR protein coding genes in the two mutants *mef7*-1 and *mef7*-2 and found in both plant lines single, unique nucleotide differences to the wild type ecotype Col sequence only in At5g09950 (**Fig. 2B**). To confirm this observation and the phenotype-genotype linkage, we then analyzed the T-DNA insertion line SALK_041847 for RNA editing after we had confirmed the insertion site in this gene. This plant line, now named *mef7*-3, shows an RNA editing phenotype of a complete loss of editing at all four target sites altered also in *mef7*-1 and *mef7*-2 (**Fig. 1**). This correlation of genetic mutations and defects in RNA editing identifies the gene At5g09950 to encode the MEF7 protein.

Conserved nucleotides at the MEF7 target sequences. Alignment of the four target editing sites of MEF7 reveals five nucleotides to be identical in the putative *cis*-recognition regions (**Fig. 3A**). Such *cis*-elements have been delineated in several other RNA editing target sites to be located between 20 or 25 nucleotides upstream (5') to 1 or 3 nucleotides downstream (3') of the edited C by in vitro, in organello and in vivo investigations in mitochondria and in plastids respectively.[27-34] In extrapolation, we assume an analogous location of the specific target sequences for the sites addressed by MEF7.

The five nucleotide identities common between the four target editing sites are also found at three other bona fide editing sites in the mitochondrial transcriptome of *Arabidopsis thaliana* (**Fig. 3A**). These other sites are apparently not targeted by MEF7 but are fully edited in the *mef7*-1 and *mef7*-2 mutant plants. This finding suggests that these five nucleotide identities are not sufficient to identify the MEF7 targets, but that other parameters within the sequences surrounding the edited C nucleotides are involved. Discrimination of pyrimidines/purines by PPR proteins has been deduced from the analysis of other editing sites as a potential additional parameter besides the straightforward identity of a relevant base.[13] Such potential direct pyrimidine/purine differences discriminating between target and non-target sites arenot seen in the 30 nucleotides *cis*-elements (**Fig. 3A**). At positions where only pyrimidines are present in the presumed *cis*-elements of all four target sites, as for example at nucleotide positions –3, –6, –14 and –15 relative to the edited C, at least one of the non-target sites also displays a pyrimidine at this position.

Therefore, recognition by MEF7 will have to involve a rather more complex nucleotide pattern with at least two nucleotide identities contributing to such a selective pyrimidine/purine condition. Furthermore, inhibitory effects of e.g., G at positions –2 and –3 at two of the non-target sites and/or of a purine base at –14 or –15 and combinations thereof as found in the non-target sites may contribute to the characteristics of a target site. In addition to such conditional nucleotide identities, secondary structures of the mRNAs may influence the access of MEF7 to its respective targets to discriminate these from nucleotide configurations not to be altered.

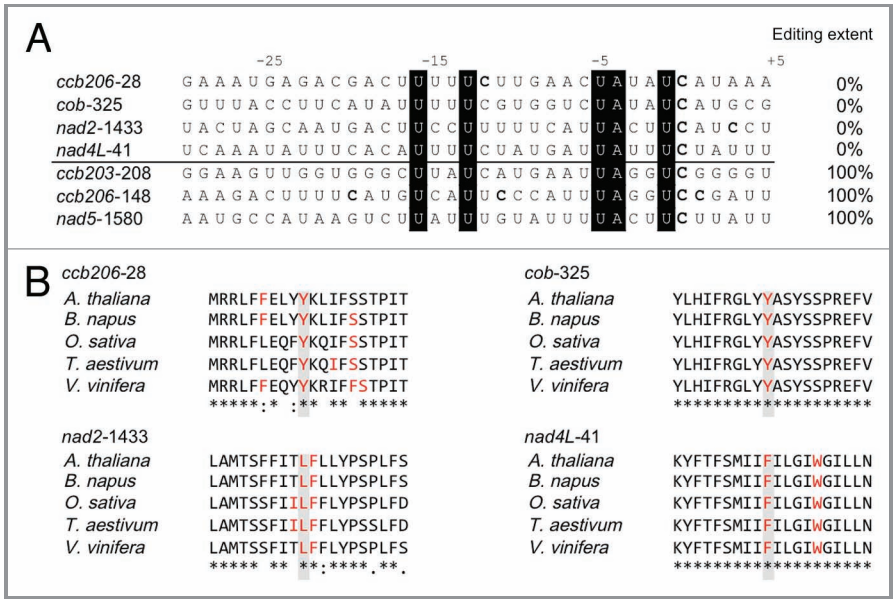


Figure 3. The RNA editing targets of MEF7 are conserved in different plant species. (A) Nucleotide alignment of the putative *cis*-elements, the –25 to +3 region surrounding the four target sites of MEF7 reveals only five nucleotides within these regions to be identical (inverse shading). These five nucleotides are also present in the nucleotide sequences of three other mitochondrially encoded mRNAs at the same distances from genuine editing sites which are however not targeted by MEF7. These sites are fully edited in the various mutants of *MEF7* which have lost editing at the MEF7 target sites. The editing sites targeted by MEF7 are aligned, all editing sites are given as C and indicated in bold. Sequences are shown 5′ to 3′ from left to right. (B) Comparison of the amino acids altered by editing in *Arabidopsis thaliana* with the respective protein sequences in other plant species. The amino acids encoded after editing with MEF7 (shaded) are all conserved in flowering plants, including the monocots rice (*Oryza sativa*) and wheat (*Triticum aestivum*). The divergent amino acids encoded by unedited mRNAs would therefore be expected to disturb the protein function, but the mutants of *MEF7* grow normally. Amino acids identical between all plants are indicated by stars, similar ones by double or single dots.

An RNA editing event three nucleotides downstream of a MEF7 target requires a different specificity factor. In the *nad2* transcript the next downstream site from the MEF7 target site at *nad2*-1433 is only three nucleotides away at *nad2*-1436 (**Fig. 3A**). This site is fully edited in all three *mef7* mutant plant lines (**Fig. 1**) and therefore must be addressed by a different specificity factor. The *cis*-recognition elements of MEF7 and this other factor have to overlap to a large extent, but should involve distinct nucleotide identities. Binding, editing and release of MEF7 and this second guided editing activity required for *nad2*-1436, respectively, must be fast and efficient to allow the respective other site also to be edited to the observed complete level of conversion.

The four editing events targeted by MEF7 are conserved in other plants. The amino acid codons generated by the four editing events specified by MEF7 as well as the surrounding amino acid identities are well conserved in many other flowering plant species as often observed for the proteins encoded by mitochondrial genomes in plants (**Fig. 3B**). The only amino acid difference in the databases is seen for *ccb206* in vine (*Vitis* spec.). However, editing sites especially in the *ccb* genes of this plant are sometimes incomplete.[35] We therefore sequenced this region in a cDNA from vine and observed that this nucleotide is in fact altered by editing to encode the conserved mitochondrial protein sequence like the other flowering plants compared here (Not shown).

Phenotypes of the *MEF7* mutants. The high degree of conservation of the amino acid sequences at and around the codons affected by the four editing events specified by MEF7 over millions of years suggests considerable functional constraints on these amino acid identities in the four different mitochondrially encoded proteins (**Fig. 3B**). Loss of these editing events would therefore be expected to lead to a detectable disadvantage for the affected plants, to cause considerable difficulties in growth and possible even disabled mitochondrial functions and to result in embryo lethality. Contrary to this expectation, both EMS mutant lines *mef7–1* and *mef7–2* with no detectable or very low residual editing at the four sites addressed grow normally under greenhouse conditions, are fully fertile and show no gross defects. Mutant plants of *mef7–3* grow slower than wild type plants but this may be attributed to further T-DNA insertions in other genes which affect plant vitality. Since both independent EMS mutant lines *mef7–1* and *mef7–2* do grow with normal vigor, this interpretation of the slowed growth of mutant *mef7–3* seems reasonable.

Alternatively the *nad4L*-41 site may be responsible for the slowed growth of *mef7–3* since in this mutant no editing can be observed at this site while *mef7–1* and *mef7–2* show residual C to U conversion only at this nucleotide but not at the other targets (**Fig. 1**). Whether this partial editing is effected by another MEF protein, access of which to this target is blocked by a truncated protein made from *mef7-3*, will have to be investigated. In the NAD4L protein the amino acid coded by the edited mRNA and surrounding residues are conserved in the plant species compared (**Fig. 3B**). This amino acid change may affect the functionality of the NAD4L protein somewhat and disturb the capacity of its destination, complex I, the NADH dehydrogenase. However, with a partially disabled complex I, plants are viable although detectably affected in their growth habitus and usually male sterile.[36] The effects of single amino acid changes like here caused by editing can range from increased activity via partial inhibition to a complete block of the protein complex. Therefore a reasonable prediction of the functional consequences of this editing event (and its loss) require detailed structural analyses of NAD4L and complex I. Similarly, the structural and functional effects of the other three target editing sites need to be investigated to determine potential consequences. However, this is not likely to yield great functional variations since the mutant plants do grow normally and the observed delayed growth of the *mef7-3* mutant plants may be caused by other mutations.

Expression of the *MEF7* gene and similar genes. The expression pattern and thus activity of the *MEF7* gene was analyzed in the expression data deposited in the AtGenExpress and Genevestigator web information (**Fig. 4A**). MEF7 shows the typical low level of transcription seen for many of the other *MEF* and PLS subfamily genes.[1] In the AtGenExpress expression levels display little variation in different tissues, the Genevestigator shows increased mRNA levels in the germinated seedling, but still very low. The low steady state transcript levels of *MEF7* in the various plant tissues are compatible with its function in mitochondrial RNA editing. For editing, small quantities of the specific PPR factors are probably needed and the low expression levels seem to be sufficient. Presumably, editing by MEF7 helps to sustain optimal activity of the respiratory chain and thus mitochondrial function in all cells and tissues.

Searching the *Arabidopsis thaliana* genome for similar proteins, we found the PPR protein encoded at locus At1g16480 to be the most similar to MEF7 in terms of amino acid sequence identity and similarity (**Fig. 4B**). This similar protein is most likely to have a function distinct from MEF7, since identical amino acids are only 34.4% and similar amino acids amount to merely 67.7%. The low similarity is reflected in the long branches in the similarity tree shown in **Figure 4B**. This PPR protein certainly cannot substitute for MEF7 since in all three mutants the disabled *MEF7* gene is not compensated for. The similarity in the pattern of transcription (**Fig. 4A**) is restricted to the above mentioned increase in vigorously growing seedlings and the low level generally found for PPR proteins and does not implicate any potential function for this protein in RNA editing. Coexpression analysis with the atted web-based program facility yielded scattered data points in developmental samples and more clustered expression pattern in other samples, the correlations expected for proteins of similar functions, but not allowing a decision between coordinated expression for RNA editing or for other organellar RNA metabolic functions.

Predictions of several targeting programs yielded a mixed batch for MEF7, TargetP could not decide between mitochondria and plastids while Predotar favors plastids (not shown). Since we have here shown the mitochondrial function of MEF7, this prediction has to be treated with caution. The PPR protein most similar to MEF7 (**Fig. 4B**), the one encoded by At1g16480, is predicted to the mitochondria by both analysis programs, which together

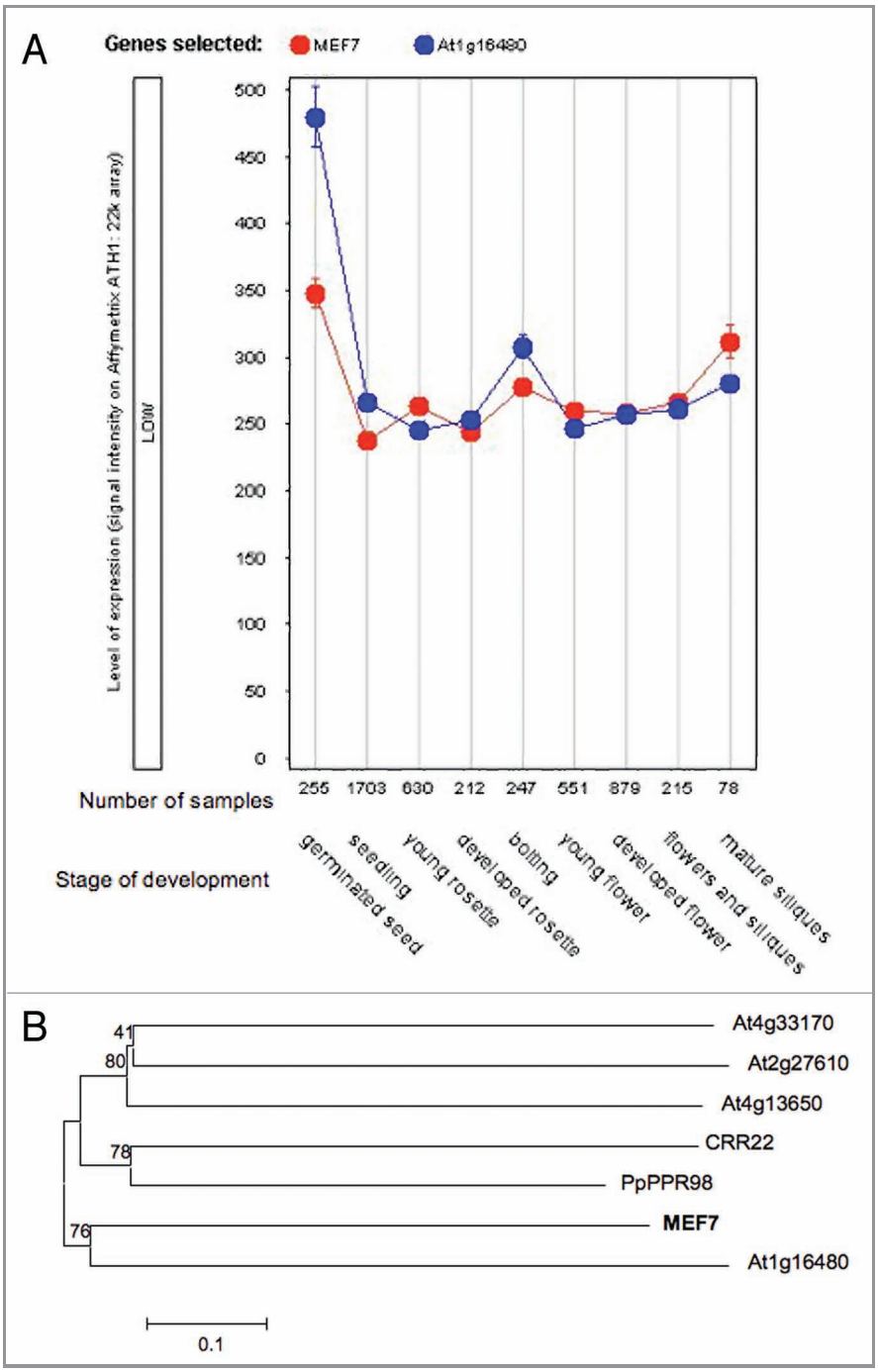


Figure 4. Expression of MEF7 and similar PPR proteins in *Arabidopsis thaliana*. (A) Expression pattern of the *MEF7* gene was analyzed by the Genevestigator web-based software.[37] Transcript levels are somewhat elevated in germinated seeds but still in the range of low expression. A similar pattern is seen for the gene most similar in the *Arabidopsis thaliana* genome (At1g16480). (B) Searching the *Arabidopsis thaliana* genomic sequence for proteins similar to MEF7 (At5g09950) identifies the PPR protein encoded by locus At1g16480 as the most similar. A neighbor-joining[38] tree displays the distance and the relationship to other PPR proteins such as CRR22, which is involved in RNA editing at specific sites in plastids and to the PpPPR_98 protein from *Physcomitrella patens*. The optimal tree with the sum of branch length = 3.45108573 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown above the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method[39] and are in the units of the number of amino acid substitutions per site. The analysis involved seven amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 749 positions in the final data set. Evolutionary analyses were conducted in MEGA5.[40]

with the similar expression levels may argue for a mitochondrial function, possibly also RNA editing, of this protein.

Screening genomic sequences from other plant species for an ortholog did not yield a clearly identifiable candidate even in the dicot plants (not shown). A functional ortholog must however be present since the same sites are edited in many species (**Fig. 3**). This suggests that in this MEF7 editing PPR, rapid evolution has altered the related open reading frames beyond recognizable similarity or that another PPR protein has mutated and has taken over the specificity of the *Arabidopsis thaliana* MEF7. Any of these scenarios supports the previously forwarded hypothesis that the PPR proteins form an interlaced network of specificities.[22] Which other PPR protein(s) provide the MEF7 activity to recognize specifically the target addresses in the respective mRNAs in other plant species will be interesting to investigate.

Materials and Methods

Plant material and preparation of nucleic acids. The EMS mutant population of *Arabidopsis thaliana* ecotype Col was obtained commercially (Lehle Seeds). The T-DNA insertion line of *Arabidopsis thaliana* was obtained from the TAIR resources. Growth of the *Arabidopsis thaliana* plants and preparation of DNA or RNA from the leaves were as described.[41] SALK line seeds were sown as obtained, selfed and the T-DNA insertion site was verified by PCR. Development of homozygous mutant plants of the three lines *mef7–3* was monitored and compared with wt Col plants.

SNaPshot assays and mutant analysis. The EMS mutant lines were screened by multiplexed single base extension[26] for plants with altered RNA editing at specific sites. Plants were first analyzed in pools of 10 from which the deviant plants were recovered. In the identified individual plants, the compromised RNA editing phenotype was verified by cDNA sequence analysis for the status of the respective investigated editing site. Sequences

were obtained commercially from 4base lab, Reutlingen, Germany or from Macrogen, Seoul, Korea.

Analysis of RNA editing sites. Specific cDNA fragments were generated by RT-PCR amplification by established protocols with respective specific primers.[41] The cDNA sequences were compared for C to T differences resulting from RNA editing. RNA editing levels were estimated by the relative heights of the respective nucleotide peaks in the sequence analyses.[8]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dagmar Pruchner and Angelika Müller for excellent experimental help. We are very grateful to the department of Human Genetics at the Universität Ulm for generously letting us use their sequencing machine for the SnaPshot analyses. We thank the anonymous reviewers for their constructive suggestion.

Financial support

This work was supported by grants from the Deutsche Forschungsgemeinschaft. M.T. is a Heisenberg fellow.

References

1. Lurin C, Andrés C, Aubourg S, Bellaoui M, Bitton F, Bruyère C, et al. Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell 2004; 16:2089-103; PMID:15269332; http://dx.doi.org/10.1105/tpc.104.022236
2. O'Toole N, Hattori M, Andres C, Iida K, Lurin C, Schmitz-Linneweber C, et al. On the expansion of the pentatricopeptide repeat gene family in plants. Mol Biol Evol 2008; 25:1120-8; PMID:18343892; http://dx.doi.org/10.1093/molbev/msn057
3. Rivals E, Bruyère C, Toffano-Nioche C, Lecharny A. Formation of the Arabidopsis pentatricopeptide repeat family. Plant Physiol 2006; 141:825-39; PMID:16825340; http://dx.doi.org/10.1104/pp.106.077826
4. Small ID, Peeters N. The PPR motif – A TPR-related motif prevalent in plant organellar proteins. Trends Biochem Sci 2000; 25:46-7; PMID:10664580; http://dx.doi.org/10.1016/S0968-0004(99)01520-0
5. Kim S-R, Yang J-I, Moon S, Ryu C-H, An K, Yim J, et al. Rice OGR1 encodes a pentatricopeptide repeat-DYW protein and is essential for RNA editing in plant mitochondria. Plant J 2009; 59:738-49; PMID:19453459; http://dx.doi.org/10.1111/j.1365-313X.2009.03909.x
6. Ohtani S, Ichinose M, Tasaki E, Aoki Y, Komura Y, Sugita M. Targeted gene disruption identifies three PPR-DYW proteins involved in RNA editing for five editing sites of the moss mitochondrial transcripts. Plant Cell Physiol 2010; 51:1942-9; PMID:20837503; http://dx.doi.org/10.1093/pcp/pcq142
7. Verbitskiy D, Zehrmann A, van der Merwe JA, Brennicke A, Takenaka M. The PPR-protein encoded by the lovastatin insensitive 1 gene is involved in RNA editing at three sites in mitochondria of Arabidopsis thaliana. Plant J 2010; 61:446-55; PMID:19919573; http://dx.doi.org/10.1111/j.1365-313X.2009.04076.x
8. Zehrmann A, van der Merwe JA, Verbitskiy D, Brennicke A, Takenaka M. A DYW-domain containing PPR-protein is required for RNA editing at multiple sites in mitochondria of Arabidopsis thaliana. Plant Cell 2009; 21:558-67; PMID:19252080; http://dx.doi.org/10.1105/tpc.108.064535
9. Sung T-Y, Tseng C-C, Hsieh M-H. The SLO1 PPR protein is required for RNA editing at multiple sites with similar upstream sequences in Arabidopsis mitochondria. Plant J 2010; 63:499-511; PMID:20497377; http://dx.doi.org/10.1111/j.1365-313X.2010.04258.x
10. Takenaka M. MEF9, an E subclass PPR protein, is required for an RNA editing event in the nad7 transcript in mitochondria of *Arabidopsis thaliana*. Plant Physiol 2010; 152:939-47; PMID:20018598; http://dx.doi.org/10.1104/pp.109.151175
11. Takenaka M, Verbitskiy D, Zehrmann A, Brennicke A. Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. J Biol Chem 2010; 285:27122-9; PMID:20566637; http://dx.doi.org/10.1074/jbc.M110.128611
12. Chateigner-Boutin A-L, Ramos-Vega M, Guevara-Garcia A, Andrés C, Gutierrez-Nava Mdl L, Cantero A, et al. CLB19, a pentatricopeptide repeat protein required for editing of rpoA and clpP chloroplast transcripts. Plant J 2008; 56:590-602; PMID:18657233; http://dx.doi.org/10.1111/j.1365-313X.2008.03634.x
13. Hammani K, Okuda K, Tanz SK, Chateigner-Boutin A-L, Shikanai T, Small I. A study of new Arabidopsis chloroplast RNA editing mutants reveals general features of editing factors and their target sites. Plant Cell 2009; 21:3686-99; PMID:19934379; http://dx.doi.org/10.1105/tpc.109.071472
14. Kotera E, Tasaka M, Shikanai T. A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature 2005; 433:326-30; PMID:15662426; http://dx.doi.org/10.1038/nature03229
15. Okuda K, Myouga R, Motohashi K, Shinozaki K, Shikanai T. Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. Proc Natl Acad Sci USA 2007; 104:8178-183; PMID:17483454; http://dx.doi.org/10.1073/pnas.0700865104
16. Robbins JC, Heller WP, Hanson MR. A comparative genomics approach identifies a PPR-DYW protein that is essential for C-to-U editing of the Arabidopsis chloroplast accD transcript. RNA 2009; 15:1142-53; PMID:19395655; http://dx.doi.org/10.1261/rna.1533909
17. Yu Q-B, Jiang Y, Chong K, Yang Z-N. AtECB2, a pentatricopeptide repeat protein, is required for chloroplast transcript accD editing and early chloroplast biogenesis in Arabidopsis thaliana. Plant J 2009; 59:1011-23; PMID:19500301; http://dx.doi.org/10.1111/j.1365-313X.2009.03930.x
18. Zhou W, Cheng Y, Yap A, Chateigner-Boutin A-L, Delannoy E, Hammami K, et al. The Arabidopsis gene YS1 encoding a DYW protein is required for editing of rpoB transcripts and the rapid development of chloroplasts during early growth. Plant J 2008; 58:82-96; PMID:19054358; http://dx.doi.org/10.1111/j.1365-313X.2008.03766.x
19. Okuda K, Chateigner-Boutin A-L, Nakamura T, Delannoy E, Sugita M, Myouga F, et al. Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in *Arabidopsis* chloroplasts. Plant Cell 2009; 21:146-56; PMID:19182104; http://dx.doi.org/10.1105/tpc.108.064667
20. Shikanai T. RNA editing in plant organelles: machinery, physiological function and evolution. Cell Mol Life Sci 2006; 63:698-708; PMID:16465445; http://dx.doi.org/10.1007/s00018-005-5449-9
21. Okuda K, Hammani K, Tanz SK, Peng L, Fukao Y, Myouga F, et al. The pentatricopeptide repeat protein OTP82 is required for RNA editing of plastid ndhB and ndhG transcripts. Plant J 2010; 61:339-49; PMID:19845878; http://dx.doi.org/10.1111/j.1365-313X.2009.04059.x
22. Zehrmann A, Verbitskiy D, Härtel B, Brennicke A, Takenaka M. PPR proteins network as site-specific RNA editing factors in plant organelles. RNA Biol 2011; 8:67-70; PMID:21289490; http://dx.doi.org/10.4161/rna.8.1.14298
23. Zehrmann A, Verbitskiy D, Härtel B, Brennicke A, Takenaka M. RNA editing competence of *trans*-factor MEF1 is modulated by ecotype-specific differences but requires the DYW domain. FEBS Lett 2010; 584:4181-6; PMID:20828567; http://dx.doi.org/10.1016/j.febslet.2010.08.049
24. Dahlke RI, Luethen H, Steffens B. ABP1: an auxin receptor for fast responses at the plasma membrane. Plant Signal Behav 2010; 5:1-3; PMID:20592799
25. Giegé P, Brennicke A. RNA editing in Arabidopsis mitochondria effects 441 C to U changes in ORFs. Proc Natl Acad Sci USA 1999; 96:15324-9; PMID:10611383; http://dx.doi.org/10.1073/pnas.96.26.15324
26. Takenaka M, Brennicke A. Multiplex single base extension typing to identify nuclear genes required for RNA editing in plant organelles. Nucleic Acids Res 2009; 37:e13; PMID:19059998; http://dx.doi.org/10.1093/nar/gkn975
27. Bock R, Hermann M, Kössel H. *In vivo* dissection of *cis*-acting determinants for plastid RNA editing. EMBO J 1996; 15:5052-9; PMID:8890178
28. Chaudhuri S, Maliga P. Sequences directing C to U editing of the plastid *psbL* mRNA are located within a 22 nucleotide segment spanning the editing site. EMBO J 1996; 15:5958-64; PMID:8918473
29. Farré J-C, Leon G, Jordana X, Araya A. Cis recognition elements in plant mitochondrion RNA editing. Mol Cell Biol 2001; 21:6731-7; PMID:11564858; http://dx.doi.org/10.1128/MCB.21.20.6731-6737.2001
30. Hegeman CE, Hayes ML, Hanson MR. Substrate and cofactor requirements for RNA editing of chloroplast transcripts in Arabidopsis in vitro. Plant J 2005; 42:124-32; PMID:15773858; http://dx.doi.org/10.1111/j.1365-313X.2005.02360.x
31. Miyamoto T, Obokata J, Sugiura M. Recognition of RNA editing sites is directed by unique proteins in chloroplasts: biochemical identification of cis-acting elements and trans-acting factors involved in RNA editing in tobacco and pea chloroplasts. Mol Cell Biol 2002; 22:6726-34; PMID:12215530; http://dx.doi.org/10.1128/MCB.22.19.6726-6734.2002
32. Neuwirt J, Takenaka M, van der Merwe JA, Brennicke A. An in vitro RNA editing system from cauliflower mitochondria: editing site recognition parameters can vary in different plant species. RNA 2005; 11:1563-70; PMID:16131591; http://dx.doi.org/10.1261/rna.2740905
33. van der Merwe JA, Takenaka M, Neuwirt J, Verbitskiy D, Brennicke A. RNA editing sites in plant mitochondria can share cis-elements. FEBS Lett 2006; 580:268-72; PMID:16364306; http://dx.doi.org/10.1016/j.febslet.2005.12.011

34. Verbitskiy D, van der Merwe JA, Zehrmann A, Brennicke A, Takenaka M. Multiple specificity-recognition motifs enhance RNA editing in plant mitochondria. J Biol Chem 2008; 283:24374-81; PMID: 18596040; http://dx.doi.org/10.1074/jbc.M803292200
35. Picardi E, Horner DS, Chiara M, Schiavon R, Valle G, Pesole G. Large-scale detection and analysis of RNA editing in grape mtDNA by RNA deep-sequencing. Nucleic Acids Res 2010; 38:4755-67; PMID:20385587; http://dx.doi.org/10.1093/nar/gkq202
36. Rasmusson AG, Heiser V, Zabaleta E, Grohmann L, Brennicke A. Physiological, biochemical and molecular aspects of mitochondrial complex I in plants. Biochim Biophys Acta 1998; 1364:101-11; PMID:9593845; http://dx.doi.org/10.1016/S0005-2728(98)00021-8
37. Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, et al. Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes. Adv Bioinformatics 2008; 2008:420747; PMID:19956698; http://dx.doi.org/10.1155/2008/420747
38. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4:406-25; PMID:3447015
39. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 1985; 39:783-91; http://dx.doi.org/10.2307/2408678
40. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 2011; 28:2731-9; PMID:21546353; http://dx.doi.org/10.1093/molbev/msr121
41. Takenaka M, Brennicke A. RNA editing in plant mitochondria: Assays and biochemical approaches. Methods Enzymol 2007; 424:439-58; PMID:17662853; http://dx.doi.org/10.1016/S0076-6879(07)24020-0

Copyright Notice:
Reprinted from:
Takenaka, M., Zehrmann, A., Verbitskiy, D., Kugelmann, M., Härtel, B., and Brennicke, A.
„Multiple organellar RNA editing factor (MORF) family proteins are required for RNA editing in mitochondria and plastids of plants“
© PNAS, 2012, with kind permission from PNAS.

Multiple organellar RNA editing factor (MORF) family proteins are required for RNA editing in mitochondria and plastids of plants

Mizuki Takenaka[1], Anja Zehrmann, Daniil Verbitskiy, Matthias Kugelmann, Barbara Härtel, and Axel Brennicke

Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany

Edited by Larry Simpson, University of California, Los Angeles, CA, and accepted by the Editorial Board February 21, 2012 (received for review August 26, 2011)

RNA editing in plastids and mitochondria of flowering plants changes hundreds of selected cytidines to uridines, mostly in coding regions of mRNAs. Specific sequences around the editing sites are presumably recognized by up to 200 pentatricopeptide repeat (PPR) proteins. The here identified family of multiple organellar RNA editing factor (MORF) proteins provides additional components of the RNA editing machinery in both plant organelles. Two MORF proteins are required for editing in plastids; at least two are essential for editing in mitochondria. The loss of a MORF protein abolishes or lowers editing at multiple sites, many of which are addressed individually by PPR proteins. In plastids, both MORF proteins are required for complete editing at almost all sites, suggesting a heterodimeric complex. In yeast two-hybrid and pull-down assays, MORF proteins can connect to form hetero- and homodimers. Furthermore, MORF proteins interact selectively with PPR proteins, establishing a more complex editosome in plant organelles than previously thought.

In all flowering plants, RNA editing alters more than 400 cytidines to uridines in the mRNAs of mitochondria and converts 30–40 cytidines in plastids (1, 2). In Lycopodiaceae, more than a thousand nucleotide identities in mitochondria and several hundred in plastids are changed (3). This process was recognized about 20 y ago (4–6), but only in recent years have the first determinants involved in the recognition of specific editing sites been identified (7). In target RNAs, the crucial sequence parameters that determine a nucleotide to be edited were identified by transgenic, in vivo, in vitro, and *in organello* assays to be similarly structured in the two organelles (8–10). These *cis* targets, located mostly 5–20 nucleotides 5′ of the target cytidine, are postulated to be recognized by specific *trans*-acting proteins of the 450 members strong pentatricopeptide repeat (PPR) protein family (11–13).

Roughly 30 individual PPR proteins have been assigned to one or several targets by connecting a dysfunctional gene with the loss of RNA editing at specific sites (14, 15). These proteins, which are essential for processing of single or very few RNA editing sites, belong to a subgroup within the PPR family characterized by their patterns of repeats and C-terminal extensions. Some are extended by only an extension (E) domain; others contain an additional conserved region terminating with the name-giving amino acids DYW. This subgroup can supply up to 200 proteins for editing at specific sites, providing an explanation of how the numerous RNA editing sites in flowering plant mitochondria and plastids can be specifically addressed (12, 13). For the enzymatic reaction of converting a cytidine to a uridine, a deaminating activity is required. Because a separate enzyme has not been identified so far, it was proposed that possibly one of the additional C-terminal domains directly contributes the enzymatic activity, in *cis* when present and in *trans* through heterodimer formation (16, 17). We now find that an entirely unexpected class of proteins constitutes an additional, essential component of the plant organellar editosome and is required for processing of almost all editing sites in plastids and of at least many sites in mitochondria.

Results

Mutation of *MORF1* Affects Numerous Editing Sites in Plant Mitochondria. Our forward genetic screen of an ethylmethanesulfonate (EMS)-mutated population of *Arabidopsis thaliana* ecotype Columbia (Col) plants with a multiplexed single-nucleotide extension protocol yielded a number of mutant plants that have lost detectable editing at specific sites (18). The mutations were mapped and the nuclear encoded genes identified several of the site-specific *trans* factors of the PPR family (19). One of the mutants, however, shows reduced RNA editing at more than 40 mitochondrial sites, very different from PPR proteins, which affect only one or several such sites (Fig. 1*A* and *SI Appendix*, Table S1). The effect of the mutation is specific to RNA editing defects; other RNA-processing steps and RNA stability are not affected (*SI Appendix*, Fig. S1). Genomic mapping in a cross of wild-type ecotype Landsberg *erecta* (L*er*) plants and the mutant ecotype Col plant narrowed the locus to a region where no PPR protein is encoded. Sequence analysis revealed an EMS-typical mutation in an unassigned reading frame, At4g20020 (Fig. 1*B*). To confirm this identification, protoplasts from the mutant plant were transfected with the wild-type gene. In these assays, editing at the target sites was increased but not fully restored. In mutant plants stably transformed with the intact Col gene under control of the 35S promoter, RNA editing was fully recovered at all affected sites (Fig. 1*C*). The complementation of the editing defects at the target sites confirms that indeed the right locus has been identified. This gene was named *MORF1* because it encodes a *m*ultiple *o*rganellar *R*NA editing *f*actor. Homozygous mutant plants with a T-DNA insertion in the *MORF1* gene are not viable (*morf1-2*; Fig. 1 *D* and *E*). This finding suggests that the EMS mutant (*morf1-1*) is a "soft" mutation, which only partially disables the function of the encoded MORF1 protein. Therefore, presumably further, essential editing sites are also targeted by the MORF1 protein, and/or those RNA editing sites that are still partially processed in the EMS mutant *morf1-1* are vitally required. The residual level of editing in this EMS mutant is sufficient for the viability of the plant.

MORF3, Another Member of the MORF Family, Is Required for Different RNA Editing Sites in Mitochondria. The *MORF1* gene belongs to a small family of nine genes and one potential pseudogene (Fig. 2*A* and *SI Appendix*, Fig. S2). Of the encoded proteins, four are predicted by Predotar to be targeted to plastids (MORF2, At1g53260, MORF8, and MORF9). In three different proteome analyses, however, fragments of MORF8 were identified in mitochondrial extracts, thus correcting the theoretical prediction. In one of these investigations, the MORF8 protein as well as MORF3 were found

Author contributions: M.T., A.Z., D.V., and A.B. designed research; M.T., A.Z., D.V., M.K., and B.H. performed research; M.T. contributed new reagents/analytic tools; M.T., A.Z., and D.V. analyzed data; and M.T. and A.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. L.S. is a guest editor invited by the Editorial Board.

[1]To whom correspondence should be addressed. E-mail: mizuki.takenaka@uni-ulm.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1202452109/-/DCSupplemental.

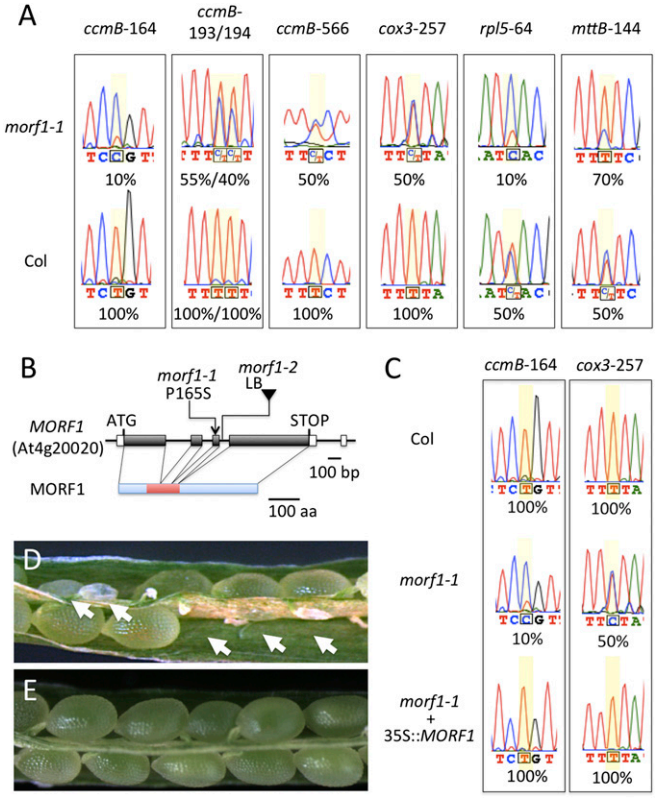
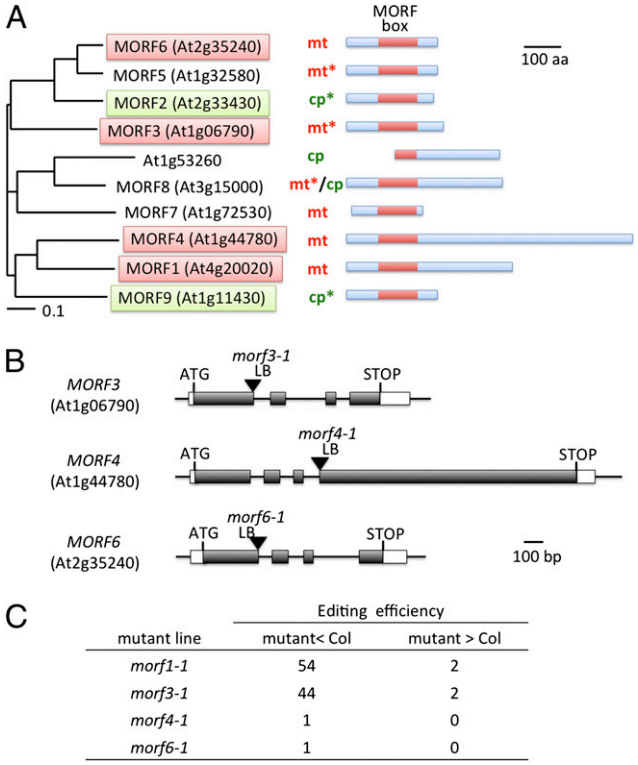


Fig. 1. The MORF1 protein is required for RNA editing at multiple sites. (*A*) Sample sequences of the more than 40 editing sites affected in *morf1-1* EMS mutant plants. The first five sites show editing reduced to different degrees. At the last site, editing increases in the mutant in comparison with wild-type plants of *A. thaliana* ecotype Columbia. (*B*) Structure of the *MORF1* gene and the MORF1 protein. The location of the *morf1-1* single-nucleotide alteration changing a proline to a serine codon and the T-DNA insertion site in *morf1-2* are indicated. LB denotes the location of the left border of the T-DNA. The darker shading in the MORF protein marks the conserved MORF domain. (*C*) Stable transformation of *morf1-1* mutant plants with the wild-type Col gene under control of a 35S promoter complements the editing defects. (*D*) The T-DNA insertion line *morf1-2* is homozygous lethal; homozygous seed growth is aborted (arrows) in pods on a selfed heterozygous plant. (*E*) Wild-type Col plants show the full seed set.



mutant line	Editing efficiency: mutant< Col	mutant > Col
morf1-1	54	2
morf3-1	44	2
morf4-1	1	0
morf6-1	1	0

Fig. 2. The MORF family of proteins contains nine genes and a potential pseudogene in *A. thaliana*. (*A*) The cladogram of similarities between the MORF proteins shows that the plastid editing factors MORF2 and MORF9 are rather distant from each other and more similar to the mitochondrial proteins MORF3 and MORF1, respectively. Predictions (marked mt or cp) and experimental data obtained by GFP-fusion protein localization (only MORF2) or proteomics MS data (marked with an asterisk) for the respective organellar locations are indicated. The MORF8 protein encoded by At3g15000 has been found in mitochondria in three independent assays. Proteins investigated here for their function are boxed. The conserved ~100-amino acids domain is shaded; the other sequences show much less conservation (*SI Appendix*, Fig. S2). The potential pseudogene (At1g53260) contains only the C-terminal part of this conserved region. (*B*) Exon structures of the *MORF3*, *MORF4*, and *MORF6* genes are similar to the *MORF1* locus and contribute similar fragments but differ in their C-terminal extensions. MORF3 is a mitochondrial editing factor involved at more than 40 sites. Locations of the T-DNA insertions in the mutants *morf3-1*, *morf4-1*, and *morf6-1* are shown. LB denotes the location of the left border of the T-DNA. (*C*) Numbers of editing sites affected by T-DNA insertions in the respective *MORF* genes. In the mutants *morf4-1* and *morf6-1*, only one noncoding site each shows somewhat reduced editing.

among mitochondrial proteins with affinity to cobalt ions (20). Genomic locus At1g53260 encodes a protein in which the first half of the otherwise conserved central domain of 100 amino acids (the MORF box) is missing and which is therefore not likely to be functionally competent (*SI Appendix*, Fig. S2). These findings leave MORF2 and MORF9 as functional plastid proteins and assign the seven other MORFs to mitochondria (MORF1 and MORF3–8).

We next investigated whether a second protein predicted for and found in a mitochondrial location, MORF3, is, like MORF1, involved in RNA editing. A T-DNA insertion in the first exon presumably disables the *MORF3* gene in a mutant plant line (Fig. 2*B*). Unlike mutant *morf1-2*, this T-DNA line, *morf3-1*, is viable as a homozygous plant. *morf3-1* plants grow a bit more slowly than wild-type plants but otherwise display no detectably altered morphological phenotype in the greenhouse. The analysis of about 400 mitochondrial editing sites showed that multiple sites are affected by the loss of MORF3. These are almost all different from the sites affected in *morf1-1* (*SI Appendix*, Table S1).

Analogous investigation of RNA editing in homozygous mutant lines of MORF4 and MORF6 showed diminished editing levels at only one site each (Fig. 2 *B* and *C*). These sites are silent; that is, they do not alter the encoded amino acids and are also in wild-type plants variably edited in different tissues of the plant. This finding does not exclude the participation of MORF4 and MORF6 in further editing events. Because MORF4 is similar to MORF1, and MORF6 is very similar to the MORF5 protein (Fig. 2*A*), the related proteins can potentially substitute for each other at some of their targets. Such functional substitutions seem to occur also between a number of mitochondrial PPR proteins at sites where editing in a knockout mutant of a given PPR gene is partially maintained, presumably by another specificity factor (21).

Mutants of Either MORF2 or MORF9 Are Affected at Almost All RNA Editing Sites in Chloroplasts. The protein MORF2 has been experimentally verified to be targeted to the plastid by in vitro import assays (22, 23), and dedicated proteomics analyses detected peptides of MORF2 and MORF9 in plastid proteins (24). Mutation of the *MORF2* gene has been reported to influence mRNA and rRNA accumulation in chloroplasts of *Arabidopsis* with a phenotype similar to an apparent ortholog in *Antirrhinum*

majus (22, 23). These genes have been tentatively assigned *DAG* (*d*ifferentiation *a*nd *g*reening) in *Antirrhinum* and *DAG-like* (*DAL*) in *Arabidopsis* (22, 23, 25).

To investigate whether the severe plastid developmental phenotypes of the *dag* and *dal* and of the *morf2* mutants are caused by deficiencies in plastid RNA editing and to see whether a mutation of *MORF9* also affects growth through RNA editing, we selfed the respective T-DNA insertion lines (Fig. 3*A*) and analyzed the homozygous plants *morf2-1* and *morf9-1* (Fig. 3 *B* and *C*–*E*). The *morf2-1* mutant has severe problems in chloroplast development similar to the allelic *dag* and *dal* mutants. The *morf2-1* mutant plants stay white, showing no sign of chlorophyll synthesis (Fig. 3*B*). The *morf9-1* mutant also exhibits defects in greening in light, but with features distinct from the *morf2-1* phenotype. When grown on sugar-supplying agar medium, the cotyledons of *morf9-1* are uniformly green, whereas the first true leaves are white. In these, scattered green flecks arise occasionally with advancing age. Subsequent leaves develop a variegated pattern with about 30% green islands, eventually sufficient to sustain autotrophic growth in soil (Fig. 3 *D*–*F*).

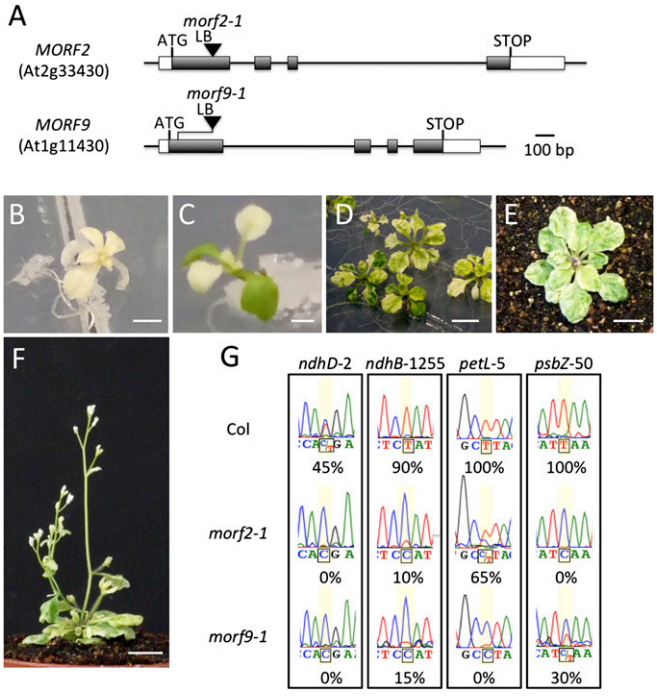


Fig. 3. MORF2 and MORF9 are required for RNA editing in plastid mRNAs. (*A*) Exons of the *MORF2* and *MORF9* genes yield similar-sized proteins, although the intron structures vary. Sites of the T-DNA insertions in the homozygous mutants *morf2-1* and *morf9-1* are shown. (*B*) Phenotype of the *morf2-1* mutant shows a complete lack of chlorophyll biosynthesis in light, and plantlets have to be grown on sugar-containing medium. This mutant is allelic to the *dag* and *dal* mutants described in *Antirrhinum* and *Arabidopsis*, respectively. (Scale bar, 1 mm.) (*C*) In the *morf9-1* mutant, the cotyledons are fully green but the leaves show a variegated appearance with spots of green on otherwise whitish leaves. (Scale bar, 1 mm.) (*D*–*F*) Sample plants of the *morf9-1* mutants show the variation of the green islands in intensity and distribution between individuals. These plants are able to grow autotrophically on soil. (Scale bars, 1 cm.) (*G*) Several of the affected editing sites are shown that document the differing influence of the *MORF2* and *MORF9* genes. Site *ndhD*-2 canonically requires both intact MORF proteins. Several sites cannot be edited without intact MORF2 (e.g., site *psbZ*-50); others require functional MORF9 proteins (e.g., site *petL*-5). Most of the sites show reduced editing in the absence of either factor, suggesting that the two MORF proteins act in concert at the same sites and that heterodimeric combinations of the two proteins are required for optimal editing.

For the analysis of RNA editing, white leaves were harvested from *morf2-1* and young white first true leaves were collected from *morf9-1*. From these samples, total cellular RNA was purified and the cDNA was investigated for all 34 plastid editing sites documented in *Arabidopsis*. Surprisingly, in both mutants, nearly all plastid editing sites are affected (Fig. 3*G* and *SI Appendix*, Table S2). At several sites editing is completely lost, most editing events are reduced by 10–70%, and some sites are less affected in each mutant. Some of the sites for which MORF2 is essential are different from those that canonically require MORF9, but several sites cannot be edited at all without either MORF. Both MORF2 and MORF9 are thus required for full editing at almost all plastid editing sites. At some sites the reduction in editing is rather small, at the level of experimental variation, that is, less than 10% reduction (*SI Appendix*, Table S2). These findings suggest that MORF2 and MORF9 act together at most editing sites in plastids and that at many sites one can compensate for (or substitute) the respective other factor, except at those sites that remain unedited when one MORF factor is missing. The most parsimonious and straightforward explanation is a direct interaction between the two proteins in a heterodimeric or—at some sites—a homodimeric configuration.

MORF Proteins Can Interact with Each Other. The *Arabidopsis* interactome database predicts MORF9 to interact with MORF6 (26), although MORF9 is plastid-located and MORF6 is a mitochondrial protein. To investigate the possibility of direct interactions between the MORF proteins, we cloned several of these into yeast two-hybrid bait and prey vectors and tested various combinations (Fig. 4*A*). Indeed, most MORF proteins are able to interact with others and also with themselves to form hetero- or homodimers. The physical formation of homodimers was also investigated by pull-down assays with MORF1 as bait (Fig. 5*A*). The bait MORF1 protein was able to retain prey MORF1 molecules, confirming the ability to form homodimers. Among the heterodimers observed in yeast cells, the interaction between the two plastid proteins MORF2 and MORF9 is particularly noteworthy. This observation supports their in vivo potential to act in a heterodimeric connection. Pull-down assays with MORF1 as bait confirmed the general ability of MORF proteins to form heterodimers; the MORF1 protein was able to retain prey MORF2 molecules, although much less effectively than prey MORF1 molecules in the homodimer assays (Fig. 5*A*). This promiscuous interaction between the mitochondrial MORF1 and the plastid MORF2 is also observed in the yeast two-hybrid assay (Fig. 4*A*). Furthermore, in a screen of an *Arabidopsis* cDNA expression library in yeast with mitochondrial MORF1 as bait, several clones of the plastid-located MORF2 were identified. The various combinations of interactions that are observed between plastid MORFs and mitochondrial MORFs suggest a flexible interactive binding that allows different combinations of MORFs in a given organelle. The slightly discriminating interactions of the plastid MORF2 and MORF9 proteins may still result in specific homo- and heterodimers of these two proteins because they seem to be the only MORFs present in this organelle.

In the mitochondrial compartment, the more than 40 sites affected by mutation of MORF1 and the likewise at least 40 sites addressed by MORF3 show almost no overlap; 92 of 95 sites are uniquely targeted (*SI Appendix*, Table S1). This observation suggests that one or more of the other as yet unassigned potential MORF proteins will be required for the remaining fully edited sites and may supply the residual activity for the partially affected sites. Alternatively, MORF1 and MORF3 substitute for each other at the partially affected sites as well as at the unaffected sites; for example, MORF1 potentially supplies the residual editing activities still available in the MORF3 mutant. These substitutions could be governed by the allowed MORF–

Multiple organellar RNA editing factor (MORF) family proteins are required for RNA editing in mitochondria and plastids of plants

Mizuki Takenaka[1], Anja Zehrmann, Daniil Verbitskiy, Matthias Kugelmann, Barbara Härtel, and Axel Brennicke

Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany

Edited by Larry Simpson, University of California, Los Angeles, CA, and accepted by the Editorial Board February 21, 2012 (received for review August 26, 2011)

RNA editing in plastids and mitochondria of flowering plants changes hundreds of selected cytidines to uridines, mostly in coding regions of mRNAs. Specific sequences around the editing sites are presumably recognized by up to 200 pentatricopeptide repeat (PPR) proteins. The here identified family of multiple organellar RNA editing factor (MORF) proteins provides additional components of the RNA editing machinery in both plant organelles. Two MORF proteins are required for editing in plastids; at least two are essential for editing in mitochondria. The loss of a MORF protein abolishes or lowers editing at multiple sites, many of which are addressed individually by PPR proteins. In plastids, both MORF proteins are required for complete editing at almost all sites, suggesting a heterodimeric complex. In yeast two-hybrid and pull-down assays, MORF proteins can connect to form hetero- and homodimers. Furthermore, MORF proteins interact selectively with PPR proteins, establishing a more complex editosome in plant organelles than previously thought.

In all flowering plants, RNA editing alters more than 400 cytidines to uridines in the mRNAs of mitochondria and converts 30–40 cytidines in plastids (1, 2). In Lycopodiaceae, more than a thousand nucleotide identities in mitochondria and several hundred in plastids are changed (3). This process was recognized about 20 y ago (4–6), but only in recent years have the first determinants involved in the recognition of specific editing sites been identified (7). In target RNAs, the crucial sequence parameters that determine a nucleotide to be edited were identified by transgenic, in vivo, in vitro, and *in organello* assays to be similarly structured in the two organelles (8–10). These *cis* targets, located mostly 5–20 nucleotides 5′ of the target cytidine, are postulated to be recognized by specific *trans*-acting proteins of the 450 members strong pentatricopeptide repeat (PPR) protein family (11–13).

Roughly 30 individual PPR proteins have been assigned to one or several targets by connecting a dysfunctional gene with the loss of RNA editing at specific sites (14, 15). These proteins, which are essential for processing of single or very few RNA editing sites, belong to a subgroup within the PPR family characterized by their patterns of repeats and C-terminal extensions. Some are extended by only an extension (E) domain; others contain an additional conserved region terminating with the name-giving amino acids DYW. This subgroup can supply up to 200 proteins for editing at specific sites, providing an explanation of how the numerous RNA editing sites in flowering plant mitochondria and plastids can be specifically addressed (12, 13). For the enzymatic reaction of converting a cytidine to a uridine, a deaminating activity is required. Because a separate enzyme has not been identified so far, it was proposed that possibly one of the additional C-terminal domains directly contributes the enzymatic activity, in *cis* when present and in *trans* through heterodimer formation (16, 17). We now find that an entirely unexpected class of proteins constitutes an additional, essential component of the plant organellar editosome and is required for processing of almost all editing sites in plastids and of at least many sites in mitochondria.

Results

Mutation of *MORF1* Affects Numerous Editing Sites in Plant Mitochondria. Our forward genetic screen of an ethylmethanesulfonate (EMS)-mutated population of *Arabidopsis thaliana* ecotype Columbia (Col) plants with a multiplexed single-nucleotide extension protocol yielded a number of mutant plants that have lost detectable editing at specific sites (18). The mutations were mapped and the nuclear encoded genes identified several of the site-specific *trans* factors of the PPR family (19). One of the mutants, however, shows reduced RNA editing at more than 40 mitochondrial sites, very different from PPR proteins, which affect only one or several such sites (Fig. 1*A* and *SI Appendix*, Table S1). The effect of the mutation is specific to RNA editing defects; other RNA-processing steps and RNA stability are not affected (*SI Appendix*, Fig. S1). Genomic mapping in a cross of wild-type ecotype Landsberg *erecta* (L*er*) plants and the mutant ecotype Col plant narrowed the locus to a region where no PPR protein is encoded. Sequence analysis revealed an EMS-typical mutation in an unassigned reading frame, At4g20020 (Fig. 1*B*). To confirm this identification, protoplasts from the mutant plant were transfected with the wild-type gene. In these assays, editing at the target sites was increased but not fully restored. In mutant plants stably transformed with the intact Col gene under control of the 35S promoter, RNA editing was fully recovered at all affected sites (Fig. 1*C*). The complementation of the editing defects at the target sites confirms that indeed the right locus has been identified. This gene was named *MORF1* because it encodes a *m*ultiple *o*rganellar *R*NA editing *f*actor. Homozygous mutant plants with a T-DNA insertion in the *MORF1* gene are not viable (*morf1-2*; Fig. 1 *D* and *E*). This finding suggests that the EMS mutant (*morf1-1*) is a "soft" mutation, which only partially disables the function of the encoded MORF1 protein. Therefore, presumably further, essential editing sites are also targeted by the MORF1 protein, and/or those RNA editing sites that are still partially processed in the EMS mutant *morf1-1* are vitally required. The residual level of editing in this EMS mutant is sufficient for the viability of the plant.

MORF3, Another Member of the MORF Family, Is Required for Different RNA Editing Sites in Mitochondria. The *MORF1* gene belongs to a small family of nine genes and one potential pseudogene (Fig. 2*A* and *SI Appendix*, Fig. S2). Of the encoded proteins, four are predicted by Predotar to be targeted to plastids (MORF2, At1g53260, MORF8, and MORF9). In three different proteome analyses, however, fragments of MORF8 were identified in mitochondrial extracts, thus correcting the theoretical prediction. In one of these investigations, the MORF8 protein as well as MORF3 were found

Author contributions: M.T., A.Z., D.V., and A.B. designed research; M.T., A.Z., D.V., M.K., and B.H. performed research; M.T. contributed new reagents/analytic tools; M.T., A.Z., and D.V. analyzed data; and M.T. and A.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. L.S. is a guest editor invited by the Editorial Board.

[1]To whom correspondence should be addressed. E-mail: mizuki.takenaka@uni-ulm.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1202452109/-/DCSupplemental.

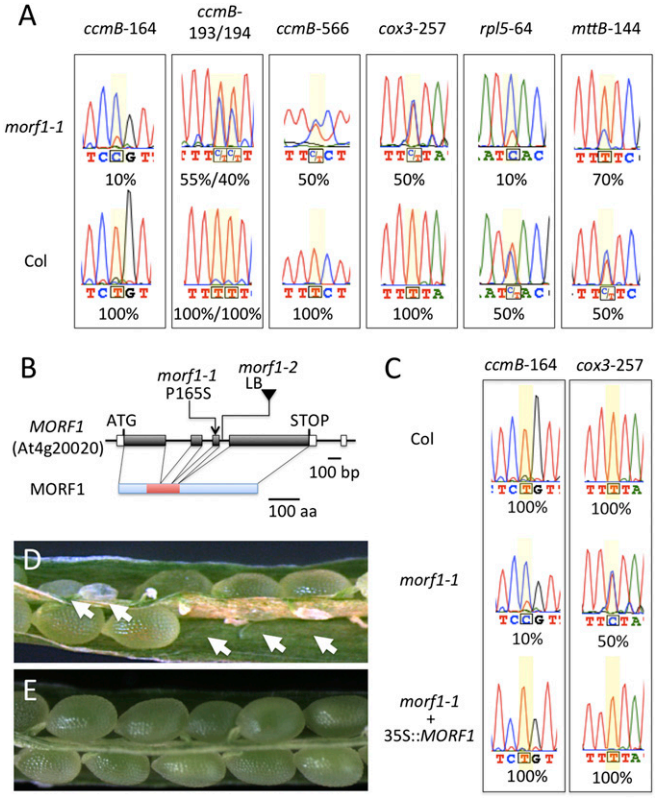
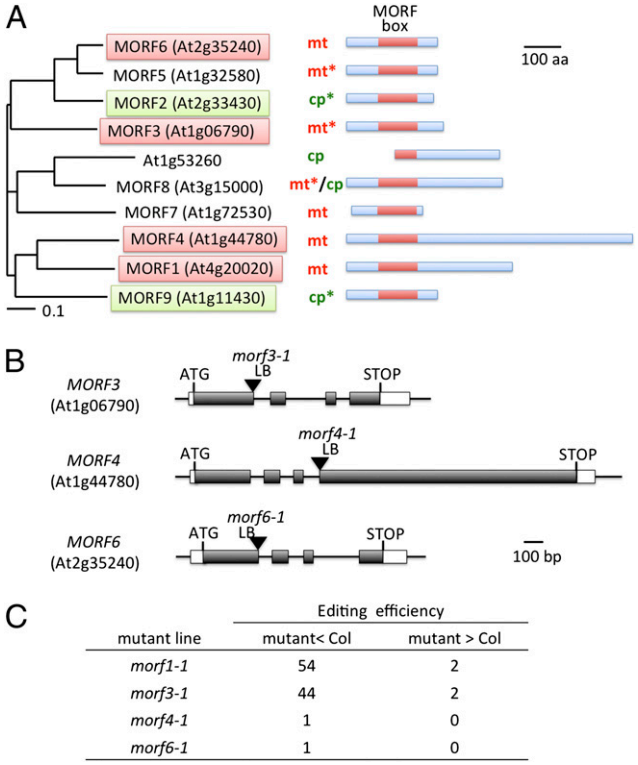


Fig. 1. The MORF1 protein is required for RNA editing at multiple sites. (*A*) Sample sequences of the more than 40 editing sites affected in *morf1-1* EMS mutant plants. The first five sites show editing reduced to different degrees. At the last site, editing increases in the mutant in comparison with wild-type plants of *A. thaliana* ecotype Columbia. (*B*) Structure of the *MORF1* gene and the MORF1 protein. The location of the *morf1-1* single-nucleotide alteration changing a proline to a serine codon and the T-DNA insertion site in *morf1-2* are indicated. LB denotes the location of the left border of the T-DNA. The darker shading in the MORF protein marks the conserved MORF domain. (*C*) Stable transformation of *morf1-1* mutant plants with the wild-type Col gene under control of a 35S promoter complements the editing defects. (*D*) The T-DNA insertion line *morf1-2* is homozygous lethal; homozygous seed growth is aborted (arrows) in pods on a selfed heterozygous plant. (*E*) Wild-type Col plants show the full seed set.



mutant line	Editing efficiency: mutant< Col	mutant > Col
morf1-1	54	2
morf3-1	44	2
morf4-1	1	0
morf6-1	1	0

Fig. 2. The MORF family of proteins contains nine genes and a potential pseudogene in *A. thaliana*. (*A*) The cladogram of similarities between the MORF proteins shows that the plastid editing factors MORF2 and MORF9 are rather distant from each other and more similar to the mitochondrial proteins MORF3 and MORF1, respectively. Predictions (marked mt or cp) and experimental data obtained by GFP-fusion protein localization (only MORF2) or proteomics MS data (marked with an asterisk) for the respective organellar locations are indicated. The MORF8 protein encoded by At3g15000 has been found in mitochondria in three independent assays. Proteins investigated here for their function are boxed. The conserved ~100-amino acids domain is shaded; the other sequences show much less conservation (*SI Appendix*, Fig. S2). The potential pseudogene (At1g53260) contains only the C-terminal part of this conserved region. (*B*) Exon structures of the *MORF3*, *MORF4*, and *MORF6* genes are similar to the *MORF1* locus and contribute similar fragments but differ in their C-terminal extensions. MORF3 is a mitochondrial editing factor involved at more than 40 sites. Locations of the T-DNA insertions in the mutants *morf3-1*, *morf4-1*, and *morf6-1* are shown. LB denotes the location of the left border of the T-DNA. (*C*) Numbers of editing sites affected by T-DNA insertions in the respective *MORF* genes. In the mutants *morf4-1* and *morf6-1*, only one noncoding site each shows somewhat reduced editing.

among mitochondrial proteins with affinity to cobalt ions (20). Genomic locus At1g53260 encodes a protein in which the first half of the otherwise conserved central domain of 100 amino acids (the MORF box) is missing and which is therefore not likely to be functionally competent (*SI Appendix*, Fig. S2). These findings leave MORF2 and MORF9 as functional plastid proteins and assign the seven other MORFs to mitochondria (MORF1 and MORF3–8).

We next investigated whether a second protein predicted for and found in a mitochondrial location, MORF3, is, like MORF1, involved in RNA editing. A T-DNA insertion in the first exon presumably disables the *MORF3* gene in a mutant plant line (Fig. 2*B*). Unlike mutant *morf1-2*, this T-DNA line, *morf3-1*, is viable as a homozygous plant. *morf3-1* plants grow a bit more slowly than wild-type plants but otherwise display no detectably altered morphological phenotype in the greenhouse. The analysis of about 400 mitochondrial editing sites showed that multiple sites are affected by the loss of MORF3. These are almost all different from the sites affected in *morf1-1* (*SI Appendix*, Table S1).

Analogous investigation of RNA editing in homozygous mutant lines of MORF4 and MORF6 showed diminished editing levels at only one site each (Fig. 2 *B* and *C*). These sites are silent; that is, they do not alter the encoded amino acids and are also in wild-type plants variably edited in different tissues of the plant. This finding does not exclude the participation of MORF4 and MORF6 in further editing events. Because MORF4 is similar to MORF1, and MORF6 is very similar to the MORF5 protein (Fig. 2*A*), the related proteins can potentially substitute for each other at some of their targets. Such functional substitutions seem to occur also between a number of mitochondrial PPR proteins at sites where editing in a knockout mutant of a given PPR gene is partially maintained, presumably by another specificity factor (21).

Mutants of Either MORF2 or MORF9 Are Affected at Almost All RNA Editing Sites in Chloroplasts. The protein MORF2 has been experimentally verified to be targeted to the plastid by in vitro import assays (22, 23), and dedicated proteomics analyses detected peptides of MORF2 and MORF9 in plastid proteins (24). Mutation of the *MORF2* gene has been reported to influence mRNA and rRNA accumulation in chloroplasts of *Arabidopsis* with a phenotype similar to an apparent ortholog in *Antirrhinum*

majus (22, 23). These genes have been tentatively assigned *DAG* (*d*ifferentiation *a*nd *g*reening) in *Antirrhinum* and *DAG-like* (*DAL*) in *Arabidopsis* (22, 23, 25).

To investigate whether the severe plastid developmental phenotypes of the *dag* and *dal* and of the *morf2* mutants are caused by deficiencies in plastid RNA editing and to see whether a mutation of *MORF9* also affects growth through RNA editing, we selfed the respective T-DNA insertion lines (Fig. 3*A*) and analyzed the homozygous plants *morf2-1* and *morf9-1* (Fig. 3 *B* and *C*–*E*). The *morf2-1* mutant has severe problems in chloroplast development similar to the allelic *dag* and *dal* mutants. The *morf2-1* mutant plants stay white, showing no sign of chlorophyll synthesis (Fig. 3*B*). The *morf9-1* mutant also exhibits defects in greening in light, but with features distinct from the *morf2-1* phenotype. When grown on sugar-supplying agar medium, the cotyledons of *morf9-1* are uniformly green, whereas the first true leaves are white. In these, scattered green flecks arise occasionally with advancing age. Subsequent leaves develop a variegated pattern with about 30% green islands, eventually sufficient to sustain autotrophic growth in soil (Fig. 3 *D*–*F*).

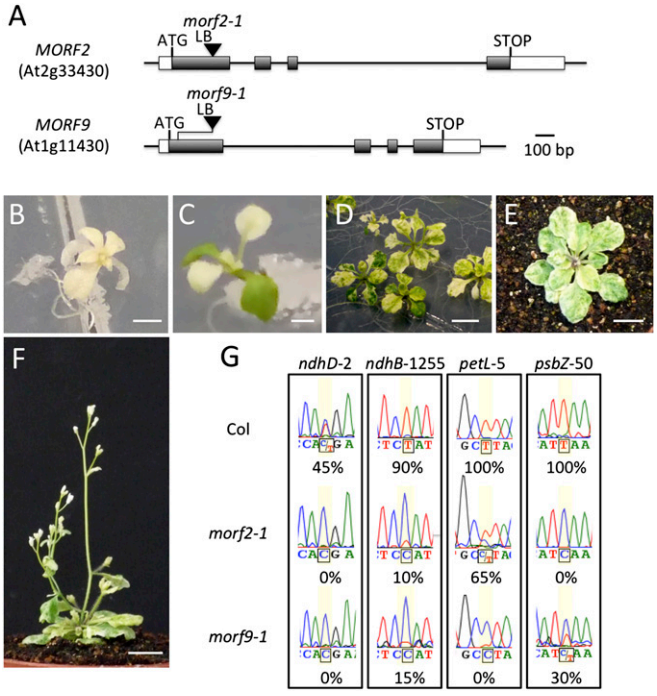


Fig. 3. MORF2 and MORF9 are required for RNA editing in plastid mRNAs. (*A*) Exons of the *MORF2* and *MORF9* genes yield similar-sized proteins, although the intron structures vary. Sites of the T-DNA insertions in the homozygous mutants *morf2-1* and *morf9-1* are shown. (*B*) Phenotype of the *morf2-1* mutant shows a complete lack of chlorophyll biosynthesis in light, and plantlets have to be grown on sugar-containing medium. This mutant is allelic to the *dag* and *dal* mutants described in *Antirrhinum* and *Arabidopsis*, respectively. (Scale bar, 1 mm.) (*C*) In the *morf9-1* mutant, the cotyledons are fully green but the leaves show a variegated appearance with spots of green on otherwise whitish leaves. (Scale bar, 1 mm.) (*D*–*F*) Sample plants of the *morf9-1* mutants show the variation of the green islands in intensity and distribution between individuals. These plants are able to grow autotrophically on soil. (Scale bars, 1 cm.) (*G*) Several of the affected editing sites are shown that document the differing influence of the *MORF2* and *MORF9* genes. Site *ndhD*-2 canonically requires both intact MORF proteins. Several sites cannot be edited without intact MORF2 (e.g., site *psbZ*-50); others require functional MORF9 proteins (e.g., site *petL*-5). Most of the sites show reduced editing in the absence of either factor, suggesting that the two MORF proteins act in concert at the same sites and that heterodimeric combinations of the two proteins are required for optimal editing.

For the analysis of RNA editing, white leaves were harvested from *morf2-1* and young white first true leaves were collected from *morf9-1*. From these samples, total cellular RNA was purified and the cDNA was investigated for all 34 plastid editing sites documented in *Arabidopsis*. Surprisingly, in both mutants, nearly all plastid editing sites are affected (Fig. 3*G* and *SI Appendix*, Table S2). At several sites editing is completely lost, most editing events are reduced by 10–70%, and some sites are less affected in each mutant. Some of the sites for which MORF2 is essential are different from those that canonically require MORF9, but several sites cannot be edited at all without either MORF. Both MORF2 and MORF9 are thus required for full editing at almost all plastid editing sites. At some sites the reduction in editing is rather small, at the level of experimental variation, that is, less than 10% reduction (*SI Appendix*, Table S2). These findings suggest that MORF2 and MORF9 act together at most editing sites in plastids and that at many sites one can compensate for (or substitute) the respective other factor, except at those sites that remain unedited when one MORF factor is missing. The most parsimonious and straightforward explanation is a direct interaction between the two proteins in a heterodimeric or—at some sites—a homodimeric configuration.

MORF Proteins Can Interact with Each Other. The *Arabidopsis* interactome database predicts MORF9 to interact with MORF6 (26), although MORF9 is plastid-located and MORF6 is a mitochondrial protein. To investigate the possibility of direct interactions between the MORF proteins, we cloned several of these into yeast two-hybrid bait and prey vectors and tested various combinations (Fig. 4*A*). Indeed, most MORF proteins are able to interact with others and also with themselves to form hetero- or homodimers. The physical formation of homodimers was also investigated by pull-down assays with MORF1 as bait (Fig. 5*A*). The bait MORF1 protein was able to retain prey MORF1 molecules, confirming the ability to form homodimers. Among the heterodimers observed in yeast cells, the interaction between the two plastid proteins MORF2 and MORF9 is particularly noteworthy. This observation supports their in vivo potential to act in a heterodimeric connection. Pull-down assays with MORF1 as bait confirmed the general ability of MORF proteins to form heterodimers; the MORF1 protein was able to retain prey MORF2 molecules, although much less effectively than prey MORF1 molecules in the homodimer assays (Fig. 5*A*). This promiscuous interaction between the mitochondrial MORF1 and the plastid MORF2 is also observed in the yeast two-hybrid assay (Fig. 4*A*). Furthermore, in a screen of an *Arabidopsis* cDNA expression library in yeast with mitochondrial MORF1 as bait, several clones of the plastid-located MORF2 were identified. The various combinations of interactions that are observed between plastid MORFs and mitochondrial MORFs suggest a flexible interactive binding that allows different combinations of MORFs in a given organelle. The slightly discriminating interactions of the plastid MORF2 and MORF9 proteins may still result in specific homo- and heterodimers of these two proteins because they seem to be the only MORFs present in this organelle.

In the mitochondrial compartment, the more than 40 sites affected by mutation of MORF1 and the likewise at least 40 sites addressed by MORF3 show almost no overlap; 92 of 95 sites are uniquely targeted (*SI Appendix*, Table S1). This observation suggests that one or more of the other as yet unassigned potential MORF proteins will be required for the remaining fully edited sites and may supply the residual activity for the partially affected sites. Alternatively, MORF1 and MORF3 substitute for each other at the partially affected sites as well as at the unaffected sites; for example, MORF1 potentially supplies the residual editing activities still available in the MORF3 mutant. These substitutions could be governed by the allowed MORF–

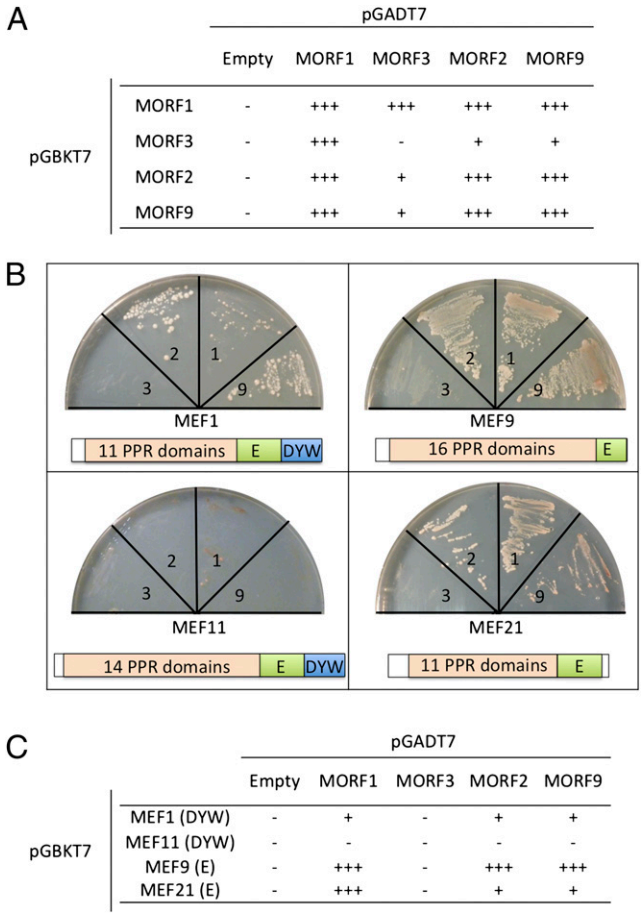


Fig. 4. MORF and MEF proteins can physically interact in yeast two-hybrid assays. (*A*) MORF proteins interact with each other. Reciprocal assays with the MORFs in bait (pGBKT7) or prey (pGADT7) vectors in a yeast two-hybrid analysis reveal that these proteins can interact with themselves in homodimers and with each other in heterodimers. The least specific appears to be the mitochondrial MORF1 protein, which can contact all other MORFs in either direction. Another mitochondrial protein, MORF3, forms strong heterodimers only with the likewise mitochondrial MORF1. The plastid proteins MORF2 and MORF9 interact with each other and with the mitochondrial MORF1, but only weakly with the mitochondrial MORF3. "Empty" is the control for autoactivation. +++ indicates a strong interaction; + represents fewer, slower-growing colonies formed; - indicates no colonies. (*B*) MORF and MEF proteins interact in yeast two-hybrid assays. Respective MEFs are indicated for each plate, and their protein structures are shown. The MORFs tested for binding are numbered in their respective quadrants. The mitochondrial editing protein MORF1, for example, interacts with the mitochondrial editing factors MEF1 (weakly), MEF9, and MEF21, but not with MEF11. These results show that principally MORF and MEF proteins interact rather unspecifically, as, for example, the binding of the plastid proteins MORF2 and MORF9 with the mitochondrial MEF1, MEF9, and MEF21 PPR proteins shows. However, some combinations are preferred, and others do not occur. No-growth quadrants show that there is no autoactivation. (*C*) The interactions between MEF and MORF proteins documented in *B* are interpreted as strong interactions (+++), weak (+), or no (-) binding. The results show that the two DYW-containing proteins, MEF1 and MEF11, interact weakly or not at all with the MORF proteins, whereas the MEF9 and MEF21 proteins, which terminate after the E domain and do not contain a DYW extension, connect more readily and promiscuously with MORF proteins in the yeast two-hybrid assays. The specific target site of MEF21 (*cox3*-257) also requires MORF1, and MEF21 indeed does interact strongly with the MORF1 protein. pGBKT7 is the bait and pGADT7 is the prey vector.

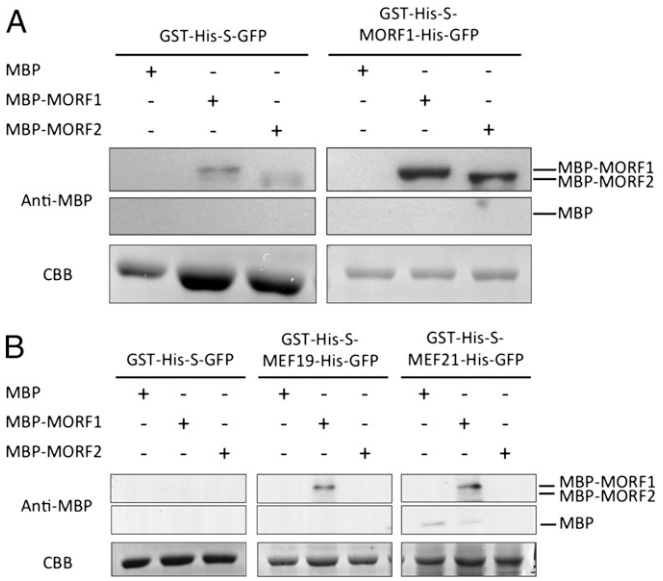


Fig. 5. MORF and MEF proteins interact in pull-down assays. (*A*) In the MORF-MORF pull-down experiment, the GST-His-S-tag-MORF1-His-GFP protein was bound to Ni-NTA agarose beads (*Right*). A parallel bound GST-His-S-tag-GFP protein served as control (*Left*). MORF1 and MORF2 proteins tagged N-terminally with a maltose binding protein (MBP) extension and, as a control, MBP only, were added in separate assays, washed, released, spread on an SDS/PAGE gel, and visualized with an MBP antibody system. (*Right*) The MORF1 protein binds efficiently to the immobilized MORF1 protein. The MORF2 protein binds less effectively: A 20-fold–higher amount of input protein is required to obtain a signal of comparable intensity. (*Left*) Weak interactions of the MBP-MORF1 and MBP-MORF2 proteins to the GST-His-S-tag-GFP control are revealed; to make this background detectable, an approximately fivefold excess of the GST-His-S-tag-GFP control was loaded, as documented by Coomassie stain (CBB; *Bottom*). MBP protein alone is not detectably retained by either the GST-His-S-tag-MORF1-His-GFP protein or the GST-His-S-tag-GFP protein. Agarose beads (400 μL) were loaded with 3.5 nmol of GST-His-S-tag-MORF1-His-GFP protein or 35 nmol of GST-His-S-tag-GFP protein. Input protein was 0.5 nmol of MBP-MORF1, 10 nmol of MBP-MORF2, and 10 nmol of MBP. (*B*) For this MORF-MEF pull-down analysis, the GST-His-S-tag-MEF19-His-GFP protein (*Center*) or the GST-His-S-tag-MEF21-His-GFP protein (*Right*) were immobilized on glutathione agarose beads and probed for interaction with the MBP-fused MORF1 and MORF2 proteins. Retained MORF proteins were detected in the gel blot with an MBP antibody system. Comparison with the control glutathione agarose-bound GST-His-S-tag-GFP (*Left*) shows that the MBP-tagged MORF1 and MORF2 proteins do not bind detectably to the GST-His-S-tag-GFP protein when present in amounts comparable to the MEF19 and MEF21 proteins; the weak signals obtained with excess amounts of the control are shown in *A*. Both MEF19 and MEF21 are able to bind and retain the mitochondrially located MORF1 but not the plastid-targeted MORF2. This result confirms the interaction pattern seen in the yeast two-hybrid assays (Fig. 4), where MEF21 strongly interacts with MORF1 but only weakly with MORF2. The interactions observed between MORF1 and MEF19, and MORF1 and MEF21, agree with the RNA editing site analysis, with MORF1 and MEF19 and MORF1 and MEF21 targeting the same respective RNA editing sites in mitochondria. The agarose beads (400 μL) were loaded with 3.5 nmol of the GST-His-S-tag-MEF19-His-GFP, the GST-His-S-tag-MEF21-His-GFP, or the GST-His-S-tag-GFP protein. Input protein was 0.5 nmol of MBP-MORF1 or MBP-MORF2 and in the control 1 nmol of MBP. In the Coomassie stain (*Bottom*), not all partial MEF proteins that contain the N-terminal GST-His-S tag but not the C-terminal His-GFP tag are documented. The weak signal seen of free MBP retained by immobilized MEF21 in the input lane of MBP-MORF1 is either a much shorter bacterial translation product or a result of protein cleavage before or during the protein preparation from the bacteria.

MORF heterodimer/homodimer combinations of which some may be less efficiently substituted by others and lead to loss of editing when one MORF is mutated. In yeast cells, MORF1 shows little discrimination in connecting to other MORFs, whereas MORF3 only interacts strongly with MORF1 but with none of the other MORF proteins (Fig. 4*A*). MORF3 does not seem to be able to form homodimers.

MORF Proteins Can Interact with Site-Specific RNA Editing PPR Proteins. In plastids as well as in mitochondria, RNA editing is affected by the loss of individual MORF proteins at sites that also require individual PPR proteins for processing. In plastids, for example, editing at site *ndhD*-2 is lost when either MORF2 or MORF9 is disturbed, but also when the PPR protein chlororespiratory reduction 4 (CRR4) is mutated (7). In mitochondria, editing site *ccmB*-566 requires mitochondrial editing factor 19 (MEF19) and MORF1; site *cox3*-257 needs MEF21 as well as MORF1 for processing (14). These coinciding requirements suggest that site-specific PPR proteins (e.g., CRRs and MEFs) are required in conjunction with one or more MORF proteins.

To directly investigate this potential connection between the specific editing factors and the MORF proteins, we tested whether MEF–PPR proteins can interact with MORF proteins in yeast two-hybrid assays (Fig. 4*B*) and in pull-down assays (Fig. 5*B*). Growth of yeast cells on respective selective media shows that most of the MEF proteins indeed interact with MORF proteins. Consistent with their "promiscuous" roles at most editing sites in their organelle, the plastid factors MORF2 and MORF9 interact with several mitochondrial MEFs. Similarly, the MORF1 protein, which is required for more than 40 RNA editing sites in mitochondria, interacts with MEF1, MEF9, and MEF21. This binding is, however, specific and selective, as the DYW–PPR protein MEF11 is not contacted by any of the MORFs investigated. Furthermore, MORF interaction with the second DYW domain-containing MEF protein, MEF1, seems to be rather weak, as suggested by slower establishment and growth of the yeast cells.

The potential of MEF and MORF proteins to interact is supported by the results of a screen of a cDNA library derived from RNA isolated from young *Arabidopsis* seedlings with MEF9 as bait. Several clones of the MORF8 protein were identified in the total plant cDNA library, which strengthens the findings from the yeast two-hybrid assays that MORF and MEF proteins can interact. Both MEF9 and MORF8 are mitochondrially located proteins and may interact in this organelle.

To investigate potential MORF–MEF interactions by another experimental approach, we tested whether MEF21 and MEF19 are able to bind the mitochondrially located MORF1 and the plastid-targeted MORF2 proteins in pull-down assays (Fig. 5*B*). MEF21 can retain MORF1 but not MORF2, as similarly observed in the yeast two-hybrid assays, where MEF21 interacts strongly with MORF1 but only weakly with MORF2 (Fig. 4*B*). In mitochondria, MEF21 and MORF1 are both required for editing at site *cox3*-257 (14). We also probed the potential for physical interaction between the MEF19 PPR protein and the MORF1 protein, which both target the RNA editing site at *ccmB*-566 (Fig. 5*B*). In the pull-down assay, the MEF19 bait protein bound to matrix beads retains the prey MORF1 molecules but not the plastid-targeted MORF2 protein (Fig. 5*B*). The physical interaction between MORF1 and MEF19 can thus connect the site-specific MEF19 PPR protein and the MORF1 protein for RNA editing at their common target site at *ccmB*-566. In summary, these lines of evidence support the potential of MEF and MORF proteins to interact more or less specifically with each other.

Discussion

MORF Proteins Are Unique Components of the Plant Organellar "Editosomes." The involvement of the family of MORF proteins suggests that the previous simple model of RNA editing in higher plant organelles has to be expanded to a more complex editosome model that contains more protein factors than envisaged: A PPR protein recognizes a specific sequence context in the RNA, binds there, and provides the attachment site for one or another of the MORF proteins. Or, vice versa, a MORF protein contacts an RNA molecule (provided MORF proteins bind RNA) and then attracts an MEF protein that then can play out its sequence specificity for binding to its cognate RNA motif. At the initiation step of MEF/MORF contacts to the RNA, additional, less specific ribonucleoproteins (RNPs) may be involved, such as CP31A and CP31B, which have been shown to be required for efficient RNA editing at several sites in plastids (27). These RNPs are partially redundant (27), a feature that seems to be common to MEF and MORF proteins. The interactions between PPR proteins and an RNA sequence appear to be specific yet fluid, because in some instances one PPR protein can be substituted by another: Those editing sites in mitochondria at which residual editing is still seen when a given PPR factor is disabled are not completely dependent on this single PPR protein; the remaining editing must be supported by another (PPR) protein (21).

MORF proteins must also partially overlap in their interactions with E or DYW–PPR proteins, as the numerous partial reductions in RNA editing at specific sites in plastids in the *morf2* and *morf9* mutants show. On the other hand, the complete loss of editing at several sites in the plastid when either MORF2 or MORF9 is disturbed indicates that at these sites both proteins are required and cannot substitute for each other. That both plastid MORFs are canonically required suggests that both should be contained within the editosome, potentially connecting each other directly in a heterodimeric arrangement. The observation that the MORFs interact selectively with each other corroborates this conclusion (Figs. 4*A* and 5*A*).

MORF Proteins Are Present in Plants with Numerous Editing Sites. In evolutionary terms, this type of RNA editing and editosome seems to be a requirement specific to the land plant lineage. PPR proteins and MORF proteins expanded in plants in parallel with an increase of RNA editing sites. MORF-like proteins appear to be absent from other organisms; their presence is correlated with the evolutionary burst of editing site numbers. Genes for MORF proteins are only detected in flowering plants (*SI Appendix*, Fig. S3), and not in the moss *Physcomitrella patens* (17, 28). In this plant, only PPR proteins with C-terminal extended DYW domains are involved in the few editing events. This correlation raises the possibility that MORF proteins may be involved in compensating the loss of the DYW domain in some editing PPRs.

In the editosome, a MORF protein would connect to the RNA-binding PPR protein, either an E or a DYW moiety, and to the cytidine-deaminating or -transaminating activity. The latter may be another PPR protein with a DYW domain that acts as a deaminase, or may be another protein that performs the reaction. Inclusion of a second, different yet specific, PPR protein could have consequences for the decoding of the RNA sequence. Each PPR protein would have to contact just very few nucleotide identities in the *cis* element of the RNA, and only the combination of both PPR proteins would need to have full affinity. In the moss *P. patens*, single PPR proteins may recognize a given RNA nucleotide pattern, because a knockout of one PPR factor always results in full or no editing but never leads to partial loss of editing. In addition, no MORFs are present in the moss.

The involvement of the MORF proteins with many editing events in both organelles of flowering plants shows that the RNA editing processes in plant mitochondria and in plastids are similar and probably coevolved (*SI Appendix*, Fig. S3). In plastids, the process seems to be more stable, with all PPR proteins so far identified being required for fixed sites with no overlapping specificities of the PPR proteins manifested by residual editing at some sites upon the loss of a given PPR factor. Although this is superficially similar to the situation in the organelles of *P. patens*, in flowering plants MORF proteins are required and, for example, in the instance of site *ndhD*-2, two MORFs are needed for any editing to occur (Fig. 3). In mitochondria, a more fluid and flexible editing complex may adapt more rapidly to novel sites and new specificities by small modifications of the activities involved. The connection of the MORF proteins may provide an additional

safety level to avoid deleterious unwanted editing events caused by the often rather loose PPR specificity that is required to address sites with little sequence similarity (14, 15, 29).

Although the actual editing process in plant organelles with "just" C-to-U (but also U-to-C) nucleotide transitions seems biochemically much less complex than the U insertion/deletion editing in trypanosome mitochondria (30–32), likewise more and specialized protein factors such as the MORF proteins are required to make up a functional RNA editing complex.

Materials and Methods

Plant culture, mutant screening, handling of nucleic acids, RNA editing analysis, transfection, and transformation were as described (18, 29). Protein target predictions were analyzed with the Predotar program (urgi.versailles.inra.fr/predotar). Details of yeast two-hybrid and pull-down analyses, full methods, and associated references are described in *SI Appendix, SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dagmar Pruchner, Angelika Müller, and Bianca Wolf for excellent experimental help; Christian Throm, Dorothea Kreuder, and Claudia Oecking at the Universität Tübingen for introducing us to the yeast two-hybrid system and for providing material and support; Alice Barkan for kindly providing cloning vectors and advice; Nadja Brehme for providing the MBP–MORF fusion proteins; and Chris Leaver for carefully editing the manuscript. We are very grateful to the Department of Human Genetics and to Stefan Britsch at Universität Ulm for the generous use of their facilities. This work was supported by grants to M.T. and A.B. from the Deutsche Forschungsgemeinschaft. M.T. is a Heisenberg Fellow.

1. Giegé P, Brennicke A (1999) RNA editing in *Arabidopsis* mitochondria effects 441 C to U changes in ORFs. *Proc Natl Acad Sci USA* 96:15324–15329.
2. Chateigner-Boutin AL, Small I (2010) Plant RNA editing. *RNA Biol* 7:213–219.
3. Grewe F, et al. (2011) A unique transcriptome: 1782 positions of RNA editing alter 1406 codon identities in mitochondrial mRNAs of the lycophyte *Isoetes engelmannii*. *Nucleic Acids Res* 39:2890–2902.
4. Covello PS, Gray MW (1989) RNA editing in plant mitochondria. *Nature* 341:662–666.
5. Gualberto JM, Lamattina L, Bonnard G, Weil JH, Grienenberger JM (1989) RNA editing in wheat mitochondria results in the conservation of protein sequences. *Nature* 341: 660–662.
6. Hiesel R, Wissinger B, Schuster W, Brennicke A (1989) RNA editing in plant mitochondria. *Science* 246:1632–1634.
7. Kotera E, Tasaka M, Shikanai T (2005) A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. *Nature* 433:326–330.
8. Bock R, Hermann M, Kössel H (1996) In vivo dissection of *cis*-acting determinants for plastid RNA editing. *EMBO J* 15:5052–5059.
9. Neuwirt J, Takenaka M, van der Merwe JA, Brennicke A (2005) An in vitro RNA editing system from cauliflower mitochondria: Editing site recognition parameters can vary in different plant species. *RNA* 11:1563–1570.
10. Farré J-C, Leon G, Jordana X, Araya A (2001) *cis* recognition elements in plant mitochondrion RNA editing. *Mol Cell Biol* 21:6731–6737.
11. Lurin C, et al. (2004) Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. *Plant Cell* 16:2089–2103.
12. Schmitz-Linneweber C, Small I (2008) Pentatricopeptide repeat proteins: A socket set for organelle gene expression. *Trends Plant Sci* 13:663–670.
13. Fujii S, Small I (2011) The evolution of RNA editing and pentatricopeptide repeat genes. *New Phytol* 191(1):37–47.
14. Takenaka M, Verbitskiy D, Zehrmann A, Brennicke A (2010) Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. *J Biol Chem* 285:27122–27129.
15. Hammani K, et al. (2009) A study of new *Arabidopsis* chloroplast RNA editing mutants reveals general features of editing factors and their target sites. *Plant Cell* 21: 3686–3699.
16. Salone V, et al. (2007) A hypothesis on the identification of the editing enzyme in plant organelles. *FEBS Lett* 581:4132–4138.
17. Rüdinger M, Szövényi P, Rensing SA, Knoop V (2011) Assigning DYW-type PPR proteins to RNA editing sites in the funariid mosses *Physcomitrella patens* and *Funaria hygrometrica*. *Plant J* 67:370–380.
18. Takenaka M, Brennicke A (2009) Multiplex single-base extension typing to identify nuclear genes required for RNA editing in plant organelles. *Nucleic Acids Res* 37:e13.
19. Takenaka M (2010) MEF9, an E-subclass pentatricopeptide repeat protein, is required for an RNA editing event in the *nad7* transcript in mitochondria of *Arabidopsis*. *Plant Physiol* 152:939–947.
20. Heazlewood JL, et al. (2004) Experimental analysis of the *Arabidopsis* mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. *Plant Cell* 16:241–256.
21. Zehrmann A, Verbitskiy D, Härtel B, Brennicke A, Takenaka M (2011) PPR proteins network as site-specific RNA editing factors in plant organelles. *RNA Biol* 8(1):67–70.
22. Chatterjee M, et al. (1996) DAG, a gene required for chloroplast differentiation and palisade development in *Antirrhinum majus*. *EMBO J* 15:4194–4207.
23. Bisanz C, et al. (2003) The *Arabidopsis* nuclear DAL gene encodes a chloroplast protein which is required for the maturation of the plastid ribosomal RNAs and is essential for chloroplast differentiation. *Plant Mol Biol* 51:651–663.
24. Zybailov B, et al. (2008) Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. *PLoS One* 3:e1994.
25. Babiychuk E, Fuangthong M, Van Montagu M, Inzé D, Kushnir S (1997) Efficient gene tagging in *Arabidopsis thaliana* using a gene trap approach. *Proc Natl Acad Sci USA* 94:12722–12727.
26. Cui J, et al. (2008) AtPID: *Arabidopsis thaliana* Protein Interactome Database—An integrative platform for plant systems biology. *Nucleic Acids Res* 36(Database issue): D999–D1008.
27. Tillich M, et al. (2009) Chloroplast ribonucleoprotein CP31A is required for editing and stability of specific chloroplast mRNAs. *Proc Natl Acad Sci USA* 106:6002–6007.
28. Ohtani S, et al. (2010) Targeted gene disruption identifies three PPR-DYW proteins involved in RNA editing for five editing sites of the moss mitochondrial transcripts. *Plant Cell Physiol* 51:1942–1949.
29. Zehrmann A, Verbitskiy D, van der Merwe JA, Brennicke A, Takenaka M (2009) A DYW domain-containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. *Plant Cell* 21: 558–567.
30. Grosjean H, Benne R (1998) *Modification and Editing of RNA* (ASM, Washington, DC).
31. Simpson L, Thiemann OH (1995) Sense from nonsense: RNA editing in mitochondria of kinetoplastid protozoa and slime molds. *Cell* 81:837–840.
32. Stuart K (1991) RNA editing in trypanosomatid mitochondria. *Annu Rev Microbiol* 45: 327–344.

Copyright Notice:
Reprinted from:
This research was originally published in the Journal of Biological Chemistry.
Verbitskiy, D., Zehrmann, A. Härtel, B., Brennicke, A., and Takenaka, M.
„Two related RNA-editing proteins target the same sites in mitochondria of *Arabidopsis thaliana*."
Journal of Biological Chemistry, 2012; 287 (45): 38064-38072.
© the American Society for Biochemistry and Molecular Biology, with kind permission from ASBMB

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 45, pp. 38064–38072, November 2, 2012
© 2012 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.

Two Related RNA-editing Proteins Target the Same Sites in Mitochondria of *Arabidopsis thaliana*[*]

Received for publication, July 4, 2012, and in revised form, September 13, 2012 Published, JBC Papers in Press, September 13, 2012, DOI 10.1074/jbc.M112.397992

Daniil Verbitskiy, Anja Zehrmann, Barbara Härtel, Axel Brennicke, and Mizuki Takenaka[1]

From the Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany

Background: Pentatricopeptide repeat (PPR) proteins act at specific RNA-editing sites in plant mitochondria.
Results: Two related PPR proteins with only five repeat units differentially influence RNA editing in pollen and leaves.
Conclusion: Two PPR proteins target the same two sites in RNA editing.
Significance: This is the first report on overlapping specificities of PPR proteins in RNA editing.

The facilitators for specific cytosine-to-uridine RNA-editing events in plant mitochondria and plastids are pentatricopeptide repeat (PPR)-containing proteins with specific additional C-terminal domains. Here we report the related PPR proteins mitochondrial editing factor 8 (MEF8) and MEF8S with only five such repeats each to be both involved in RNA editing at the same two sites in mitochondria of *Arabidopsis thaliana*. Mutants of *MEF8* show diminished editing in leaves but not in pollen, whereas mutants of the related protein MEF8S show reduced RNA editing in pollen but not in leaves. Overexpressed *MEF8* or *MEF8S* both increase editing at the two target sites in a *mef8* mutant. Double mutants of MEF8 and MEF8S are not viable although both identified target sites are in mRNAs for nonessential proteins. This suggests that MEF8 and MEF8S may have other essential functions beyond these two editing sites in complex I mRNAs.

RNA editing in mitochondria of flowering plants changes 400–500 selected cytosines to uridines mostly in coding regions of mRNAs and some tRNAs (1–3). Specific sequence contexts in the pre-mRNA act as *cis*-elements that distinguish an editing site from a cytosine remaining unedited (4–7). In *Arabidopsis thaliana*, these RNA sequences are presumably recognized by nuclear-encoded specificity factors such as mitochondrial editing factor 1 (MEF1),[2] MEF9, MEF11, MEF14, and MEF18–MEF22, which are required for correct editing of specific sites (8–13).

The generally one-to-one relationship between specific *cis*-elements in the mitochondrial RNA molecules and individual *trans*-factors, the MEF proteins, is most parsimoniously explained by the MEF proteins acting directly as RNA-binding proteins. This speculation is supported by the finding that some MEF proteins are required for editing at several sites that are preceded by similar *cis*-elements. Direct investigations of an analogous plastid RNA-editing factor, the CRR4 protein, have shown that this protein indeed binds to the specific RNA sequences at its cognate RNA-editing site (14). Related proteins involved in RNA processing such as PPR5 and PPR10 (15, 16) and others of unknown function (17) also bind to specific RNA sequences or at least contact polyribonucleotides.

The proteins required for specific RNA-editing events in plastids and in mitochondria, including for example CRR4, the MEFs, REME1 (18), and OGR1 in rice (19), are all pentatricopeptide repeat proteins (PPR proteins). Those identified to date are characterized by containing three types of repeats that vary between 31 amino acids in short, 35 in medium, and 37 in long repeat elements and contain an additional extension (E) domain. The single exception so far is a PPR protein with only medium-type repeats and no C-terminal extension; the absence of this protein enhances editing at several sites (20). A number of the mitochondria- and plastid-targeted RNA-editing PPR proteins are extended beyond the E domain by an additional approximately 100 amino acids long DYW region with the name-giving amino acid triplet DYW at the C terminus whereas others end with the E domain (18, 21–29). Direct investigations have shown that in some editing proteins, the DYW domain is essential but can be deleted in others (23, 30, 31). The presence of conserved features of cytidine deaminases in most DYW domains prompted speculations that these may contribute the as-yet-unidentified enzymatic activity (32), but an experimental investigation so far found only an RNA-degrading activity (33).

If the RNA-editing factors in the E subclass of the approximately 450 PPR proteins encoded in the nuclear genome of *Arabidopsis* (17, 34–37) indeed interact directly with specific RNA motifs at their cognate RNA-editing sites, one would expect that the loss of a given RNA-editing PPR protein would lead to the loss of editing at these target sites. This is in fact observed for many of these proteins, suggesting that there are usually no back-up factors that can substitute these specific functions (*e.g.* 8–13, 24, 25). In instances of partial residual editing in an apparent knockout of a given MEF protein (9, 12), another at least partially substituting editing factor must be postulated, but none has been identified.

We here report the identification of two novel PPR proteins of the DYW class with fewer PPRs than previously identified factors. These related MEF8 and MEF8S proteins are involved

* This work was supported by grants from the Deutsche Forschungsgemeinschaft (to M. T. and A. B.).
[1] Heisenberg Fellow. To whom correspondence should be addressed. Tel.: 49-731-502-2642; Fax: 49-731-502-2626; E-mail: mizuki.takenaka@uni-ulm.de.
[2] The abbreviations used are: MEF; mitochondrial editing factor; PPR, pentatricopeptide repeat; Col, Columbia; EMS, ethyl methanesulfonate.

Downloaded from www.jbc.org at UNIVERSITAETSBIBLIOTHEK Ulm, on January 29, 2013

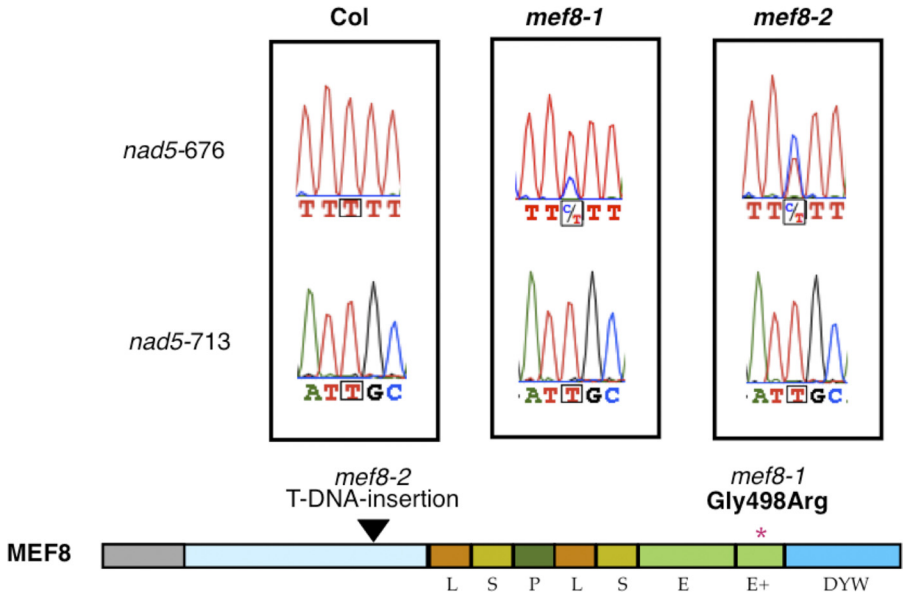


FIGURE 1. **Two independent mutants of the gene for MEF8 show reduced RNA-editing levels at the same site in the mitochondrial *nad5* mRNA.** The *mef8-1* EMS-induced *A. thaliana* mutant plant was identified in a cDNA SNaPshot analysis by its lower C-to-U-editing level at the mitochondrial *nad5*-676 RNA-editing site. Direct sequence analysis shows that the editing level is reduced from 100% in Col wild type plants to approximately 70%. In a second mutant plant with a T-DNA insertion, *mef8-2* (Salk_106391), a stronger reduction to approximately 40% is seen. In contrast, another site in the same mRNA, site *nad5*-713, is correctly edited to completion in wild type and in mutant plants. Color traces are C, *blue*; T, *red*; G, *black*; A, *green*. The *lower part* shows the predicted structure of the MEF8 protein. The different types of repeats, the E, E^+, and DYW elements, the predicted mitochondrial import sequence, and the unstructured region (*light blue*) are color-coded. The location of the EMS point mutation and the resulting change of a glycine-to-an arginine codon in *mef8-1* and the location of the T-DNA insertion in *mef8-2* are indicated.

in RNA editing at the same two specific sites in plant mitochondria.

EXPERIMENTAL PROCEDURES

Plant Material and Preparation of Nucleic Acids—*A. thaliana* seeds of wild type Columbia (Col) and the various mutants were grown as described (38). DNA or RNA from the leaves of the *A. thaliana* plants were prepared by published procedures (39). For pollen analysis, pollen was collected from flower buds just prior to their opening by manually opening them inside an Eppendorf tube. Pollen shaken out attached electrostatically to the wall of the tube. Usually pollen from seven flowers was collected. For RNA preparations, lysis buffer from commercial RNA kits (GE Healthcare) was added, and pollen was frozen and thawed twice.

Identification of the mef8 Mutants—Mutant *mef8-1* was identified in a screen of a population of ethyl methanesulfonate (EMS) mutagenized plants by its reduced editing at site *nad5*-676 (40–42). In parallel to the mapping of the gene mutated in this EMS plant, a screen of T-DNA insertion mutants for altered RNA editing identified site *nad5*-676 as a target of the locus At2g25580 (9). This mutant was accordingly named *mef8-2*.

Analysis of RNA-editing Sites—Specific cDNA fragments were generated by RT-PCR amplification following established protocols (39). The cDNA sequences were compared for C-to-T differences resulting from RNA editing. Most sequences were obtained commercially from 4base lab (Reutlingen, Germany), LGC Genomics (Berlin, Germany), or from Macrogen (Seoul, Korea). Evaluation of sequence data was done by measuring peak heights. Percentages of editing were obtained as the relationship of the peak height of the T signal to the sum of the T and C signals at the respective editing site (12).

Stable Transformation of Plants—Plants of the T-DNA mutant line *mef8-2* were transformed by floral dip (43) with the *MEF8* or *MEF8S* wild type Col reading frame under control of the 35S promoter in vector pMDC123 (44). Transgenic plants were selected by spraying with Basta®. For the analysis of RNA-editing levels, the respective cDNA fragments were sequenced, and relative peak heights were compared (12, 38).

RESULTS

Identification of MEF8 as a Factor of RNA Editing at Site nad5-676—With the recently developed multiplexed SNaPshot approach, 369 annotated editing sites were probed in 2,000 individuals of a population of EMS mutant plants to directly find mutants impaired in RNA editing at one or more of the investigated sites in plant mitochondria (40–42). This screen for deficiencies in RNA editing at specific sites identified a plant with reduced editing at site *nad5*-676. The homozygous mutant plant shows editing at this site to be diminished to approximately 70% in comparison with the wild type Columbia plants in which this site is altered to 100% from the genomically encoded C to an U (Fig. 1). The genomic locus responsible for this reduction was mapped by crosses between the mutant plants and wild type plants of ecotype L*er* to the site annotated At2g25580. This gene, now named *MEF8*, encodes the editing specificity factor MEF8.

To corroborate the connection between the EMS mutant line (now named *mef8-1*) and the editing defect, we next analyzed an independent mutation in *MEF8*, T-DNA insertion line SALK_106391 (now named *mef8-2*), for editing at the target

Downloaded from www.jbc.org at UNIVERSITAETSBIBLIOTHEK Ulm, on January 29, 2013

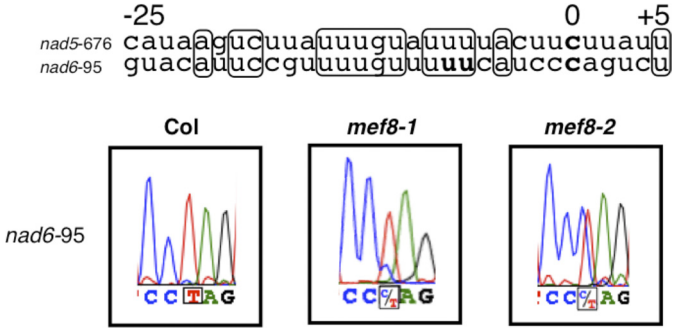


FIGURE 2. **A second site with a similar *cis*-sequence is also affected in the *mef8* mutants.** The *upper part* shows an alignment of the *nad5*-676 target site with the *nad6*-95 site with a similar *cis*-element in the region −25 to +5 relative to the edited C nucleotide (*bold C* at nucleotide 0). Nucleotides derived from other editing events are given as *bold U*, nucleotides identical between the two sequences are *framed*. The target editing site is labeled 0. The *lower part* shows a cDNA sequence analysis of the second site *nad6*-95, at which editing is reduced to approximately 80% in the *mef8-1* and to 50% in the *mef8-2* mutant from the nearly 100% editing observed in wild type plants.

site (Fig. 1). In this plant, editing at site *nad5*-676 is reduced to approximately 40%, confirming the involvement of MEF8 in RNA editing at this site. As a control the closest RNA-editing site in the same mRNA, site *nad5*-713 was analyzed (Fig. 1). This site is edited to apparent completion in both mutants as well as in WT plants, excluding any secondary influence on RNA editing by altered turnover or transcription rates and suggests that MEF8 is specifically involved in editing at site *nad5*-676.

The difference in residual editing in the two mutants (70 and 40%, respectively) suggests that the function of the mutant *mef*8-1 protein is only partially inhibited. In mutant *mef8-2* presumably no functional MEF8 protein is made, which reduces the remaining editing activity more strongly. If this scenario is correct, another factor must be responsible for the 40% remaining editing in mutant *mef8-2*.

A Second RNA-editing Site Is Targeted by MEF8—Sequence comparison of the target site *nad5*-676 in several plant species shows that this editing event of *A. thaliana* is conserved in only few species such as *Brassica napus*, whereas most other flowering plants code for a U at this position already in the genome. The amino acid phenylalanine at this position and the surrounding amino acids in the NAD5 protein are highly conserved, the only exception being *Oenothera berteriana*, where editing at this C has not been observed and a leucine may be encoded instead. Alternatively, the editing event may have been missed in this plant.

An *in silico* search of the mitochondrial transcriptome sequence of *A. thaliana* with the presumed *cis*-element of the *nad5*-676 target site yields the upstream region of editing site *nad6*-95 as the most similar sequence pattern (Fig. 2, *upper part*). Investigation of RNA editing at this site indeed shows an effect in leaves of the *mef8* mutants, editing being reduced from 100% in wild type RNA to approximately 80% in mutant *mef8-1* and to about 50% in mutant *mef8-2* (Fig. 2, *bottom part*).

A Gene Similar to the MEF8 Gene in the Arabidopsis Genome—The conclusion outlined above that another factor has to be able to compensate at least partially for the loss of MEF8 extends to the two editing sites, because both target sites show residual editing even in the T-DNA insertion line *mef8-2*. In this line the MEF8 protein is presumably disrupted and another factor must provide the residual activity. One possible candidate for such an alternative factor might be a protein with characteristics similar to MEF8. An *in silico* search in *A. thaliana* for genes encoding proteins similar to MEF8 identified a genomic locus (At4g32450) that codes for a protein in which overall approximately 25% of the amino acids are identical, this conservation increasing up to 80% similarity in some repeats (Fig. 3). Three related proteins with structures similar to the MEF8/MEF8S pair are encoded in the *Arabidopsis* genome, the two proteins encoded at loci At2g34370 and At1g29710 form another pair, and the one encoded at locus At2g15690 is more distantly related (Fig. 3*A*).

The MEF8 protein (and these related proteins) is unique in comparison with the other so far identified PPR proteins involved in RNA editing in that it contains an extremely reduced repeat domain with only five repeats (Figs. 1 and 3). Some of these repeats lack recognizable conservation of the amino acid moieties characteristic for PPRs and could therefore only be delineated by alignment with consensus patterns including evolutionary far distant species such as *Naegleria* (45). Degeneration is also seen in the E^-/E^+ region, including a >10 residues deletion. The MEF8-like protein ends with the EYW triplet instead of the highly conserved DYW motif.

For its high similarity, we renamed this MEF8-like protein MEF8S. To investigate the potentially similar function of the MEF8S protein, we analyzed RNA editing at the MEF8 target sites in a T-DNA insertion mutant of the *MEF8S* gene locus, now named *mef8s-1* (SALK_047005C; Fig. 4). Disruption of *MEF8S* has no detectable effect on processing of these sites in leaves, because, like in wild type plants, both sites are edited apparently completely in the steady-state mRNA population. Furthermore, 369 documented RNA-editing sites analyzed in leaves of the *mef8s-1* mutant by the SNaPshot procedure are all edited as in wild type plants.

Complementation of the MEF8 T-DNA Insertion Mutant—The connection between the *MEF8* gene and the reduced RNA editing at the *nad5*-676 and *nad6*-95 target sites was further assayed by exploring the ability of the WT Col *MEF8* and *MEF8S* genes to complement the *mef8-2* T-DNA insertion mutant. Transfection of the *MEF8* gene into *mef8-2* mutant protoplasts increased RNA editing significantly (data not shown). Protoplast complementation with the *MEF8S* gene also shows a slight increase of RNA editing at both sites that is however statistically not significant (data not shown).

To investigate this observation further, we stably transformed the *mef8-2* T-DNA insertion mutant with either the *MEF8* or the *MEF8S* gene under control of the 35S promoter to achieve (relatively) high levels of expression (Fig. 5). In the transgenic plants, the low levels of RNA editing at the target sites, *nad5*-676 and *nad6*-95, are significantly increased in leaves by either the *MEF8* or the *MEF8S* gene. In both instances, recovery is better by *MEF8* than by *MEF8S*.

Double Knockout of MEF8 and MEF8S Is Embryo-lethal—To further investigate the complementing functions of MEF8 and MEF8S in editing the two MEF8 target sites, we tried to establish a double knockout of *MEF8* and *MEF8S*. We crossed the two homozygous T-DNA insertion lines of *MEF8* and *MEF8S*

Downloaded from www.jbc.org at UNIVERSITAETSBIBLIOTHEK Ulm, on January 29, 2013

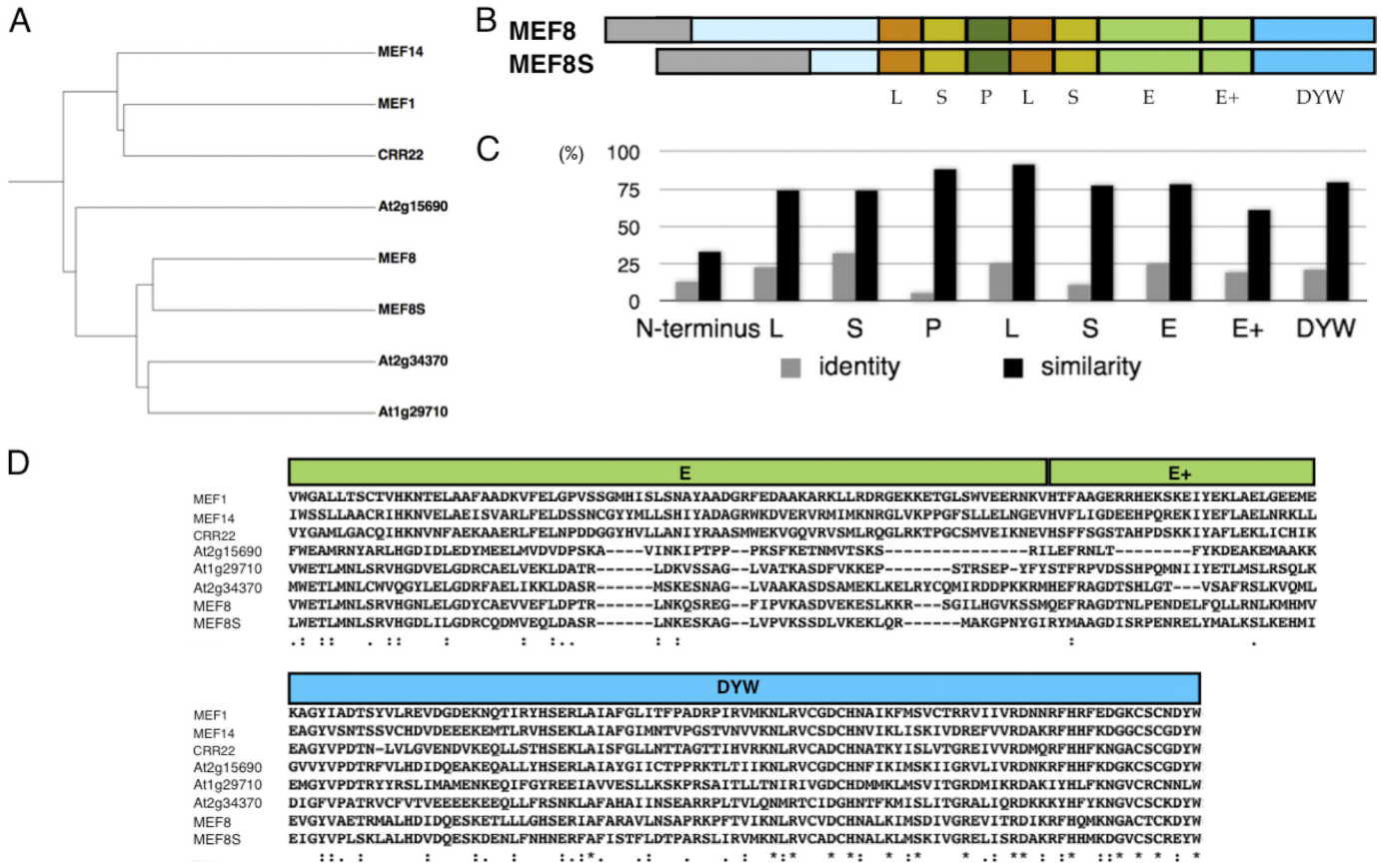


FIGURE 3. **Two similar proteins, MEF8 and MEF8S, are encoded in the *A. thaliana* nuclear genome.** *A,* a similarity tree with the MEF8 and MEF8S protein pair, three other proteins with related features, and the mitochondrial MEF14, MEF1, and the plastid CRR22 PPR proteins. This comparison shows that the two proteins encoded by At2g34370 and At1g29710 are closely related to each other and also similar to the MEF8/MEF8S pair. The PPR protein encoded by At2g15690 likewise has few PPRs and higher primary sequence similarity to these than to other PPR proteins encoded in the *Arabidopsis* genome. Sequences were aligned with the Clustal W program in the UniProt database. *B,* alignment of the schematic structure of the MEF8 and MEF8S PPR proteins encoded by locus At2g25580 and At4g32450, respectively. The N-terminal amino acid sequences are predicted to contain mitochondrial target peptides (*gray*) followed by amino acid stretches with no clear structure (*light blue*) before the first detectable PPRs. According to their sizes and conserved features, five PPR elements can be discerned as indicated. Color coding is as described in the legend to Fig. 1. *C,* percentages of amino acid identity (*gray bars*) and similarity (*black bars*) between the MEF8 and MEF8S proteins are given for each of the repeats, the N-terminal region up to the first repeat and the E, E^+, and DYW elements. The high degree of overall similarity suggests a common evolutionary origin of both genes and also a functional equivalence. *D,* amino acid alignment of the E, E^+, and DYW elements of the MEF8 and MEF8S proteins with the respective sequences from the three similar proteins and from the editing factors MEF1 (12), MEF14 (11), and CRR22 (55) compared in *A* shows the gaps in the E domain. The C-terminal DYW triplet is altered to EYW in MEF8S.

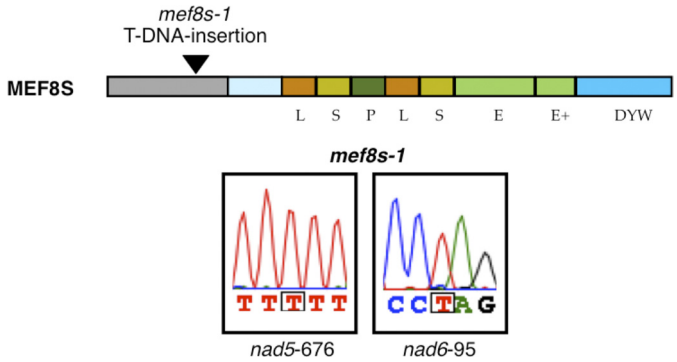


FIGURE 4. **Editing at the MEF8 target sites is not affected in leaves of a MEF8S mutant.** The *top part* shows the *mef8s-1* mutant with a T-DNA insertion in the open reading frame coding for the N-terminal region predicted as mitochondrial target sequence of the MEF8S protein. The sequence traces below show that both MEF8 target sequences are fully edited in leaves of the homozygous *mef8s-1* mutant plants.

and analyzed the offspring for the presence of the mutant genes. Surprisingly, we did not obtain any viable offspring in repeated crossings. No seeds developed, and no embryo grew detectably.

This is unexpected because the single mutants *mef8-2* and *mef8s-1* do not show any gross abnormalities in their growth habits. To exclude any problems potentially particular to the combination of the two homozygous mutant plants, we investigated the offspring of two different crosses (Fig. 6): For the first, plants homozygous for *mef8-2* and heterozygous for *mef8s-1* were generated by crossing the homozygous mutant of *mef8-2* with a plant heterozygous at *mef8s-1* and selection of respective individuals by PCR for the presence of the two T-DNA insertions. The second plant line was analogously obtained and is homozygous for *mef8s-1* and heterozygous for *mef8-2*. Both plant lines were selfed, and 160 randomly chosen individuals, respectively, were screened for offspring homozygous for both mutations. None was identified.

To see if (and if so at what stage) embryo development was compromised, seed pods of both crosses were analyzed (Fig. 6). Whereas control wild type selfings showed nearly full pods, each of the two crosses revealed approximately 25% aborted seed sites. The phenotypic appearance is very similar to embryo-lethal mutants as classified and described in detail (46, 47).

Downloaded from www.jbc.org at UNIVERSITAETSBIBLIOTHEK Ulm, on January 29, 2013

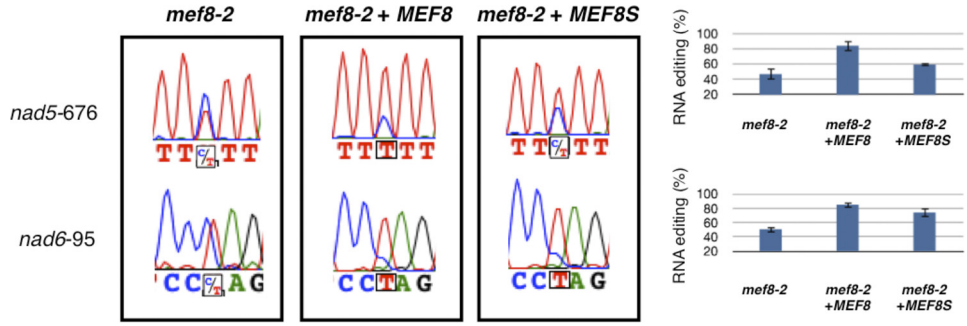


FIGURE 5. **Complementation of the T-DNA mutant line *mef8-2* by stable transformation with the Col *MEF8* or the Col *MEF8S* gene.** Sequence tracings of the cDNA analysis from stable transformants of the T-DNA mutant line *mef8-2* with the Col *MEF8* gene or the Col *MEF8S* gene under control of the 35S promoter show that both genes increase RNA-editing levels in leaves. The effect of MEF8S is greater at the *nad6*-95 editing site than at site *nad5*-676. The *bars* show data from two independently derived transgenic plants, the S.D. is indicated.

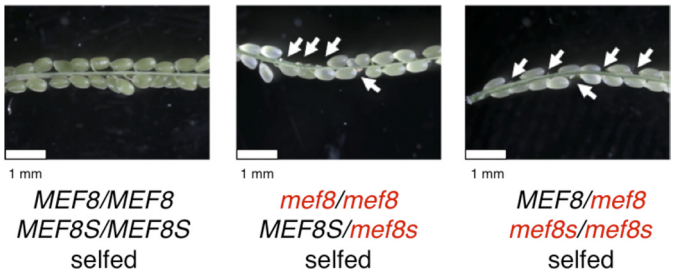


FIGURE 6. **The combination of homozygous disabled *mef8* and *mef8s* alleles is embryo-lethal.** Self-pollinations of plants with either homozygous *mef8-2* and heterozygous *mef8s-1* alleles (*center panel*) or of plants with homozygous *mef8s-1* and heterozygous *mef8-2* loci (*right panel*) show approximately 25% aborted seeds in the opened siliques (*arrows*). None of the approximately 160 plants recovered and analyzed of each cross is homozygous for both mutant genes. In wild type Col selfings, all seeds develop normally (*left panel*). Disabled alleles of the genes indicated are marked in *red*, intact alleles in *black* in the schematic below the photographs.

Counterregulated Expression of the MEF8 and MEF8S Genes in Different Tissues Results in Distinct RNA-editing Phenotypes in Their Mutants—The Atgenexpress analysis of *MEF8* and *MEF8S* gene expression patterns shows the overall very low level of transcription typical for *MEF* genes throughout the various tissues and growth conditions 48). The most striking differences between the steady-state transcript levels in distinct tissues between these two similar genes are seen in the floral organs (Fig. 7*A*). Whereas the transcript levels of *MEF8S* are elevated approximately 3-fold in stamen and pollen, the amount of steady-state transcripts of *MEF8* is reduced to about half of the level in other tissues. This observation suggests a potentially stronger influence of *MEF8S* during pollen development than that of *MEF8*.

To investigate this possibility, we compared the RNA-editing levels at the two target sites in pollen obtained from nearly mature but still closed flower buds from the three different genotypes: wild type plants, plants homozygous for *mef8-2*, and plants homozygous for *mef8s-1* (Fig. 7*B*). The average level of editing in pollen from plants without a functional *MEF8* gene, *i.e.* those homozygous for *mef8-2*, was similar to that in wild type plants and hardly affected. On the other hand, pollen from plants homozygous for *mef8s-1* without a functional *MEF8S* gene showed reduced levels of editing at both target sites (Fig. 7*B*).

The comparison of editing in leaves and in pollen shows that plants homozygous for *mef8-2* show the phenotype of reduced editing only in leaves but not in pollen, whereas plants homozygous for *mef8s-1* have reduced RNA editing in pollen but not in leaves (Fig. 7*B*).

Pollen kernels were analyzed for viability by the Alexander stain. All kernels from both mutants showed a percentage of positive staining identical to the wild type, suggesting that the altered mitochondrial RNA editing does not manifest in the phenotypic appearance and in the viability of the pollen as such.

DISCUSSION

RNA-editing Proteins with a Degenerated PPR Protein Skeleton—The two PPR proteins MEF8 and MEF8S are unusual in comparison with the previously assigned editing factors of mitochondria and plastids: both contain very short PPR domains with only five PPRs (Fig. 3). The assigned five medium, long, and short elements in MEF8 and MEF8S furthermore deviate from the characteristic amino acid signature with several of the usually conserved residues substituted by unconventional moieties (17, 45, 49, 50). The E domain shows a deletion of 11 amino acids in MEF8 and of 13 residues in MEF8S in comparison with the E domain consensus arrangement (Fig. 3*D*). The adjacent E^+ region and the following DYW domain are somewhat better maintained and have retained most conserved key elements.

Three further proteins with related features of few PPRs, a shorter E domain and high primary sequence similarity are present in the *Arabidopsis* genome (Fig. 3). Similar to the MEF8 and MEF8S pair, the two proteins encoded by At2g34370 and At1g29710 form another pair more closely related to each other than to other PPR proteins. The presence of shorter E and DYW domains in the three MEF8/MEF8S related proteins suggests that they may also be involved in RNA editing, but this will have to be investigated in detail.

MEF8 and MEF8S Proteins with Only Five PPRs Are Site-specific—The restricted number of PPRs in the MEF8 and MEF8S proteins raises the question of how this fits with the current model idea that the PPRs recognize and bind to a specific RNA sequence on a one-on-one basis, *i.e.* one repeat element attaching to one nucleotide. Precedence for such a connection between α-helical 35-amino acid units and individual nucleotides is found in several DNA-binding proteins, such as the TAL regulators (51).

Downloaded from www.jbc.org at UNIVERSITAETSBIBLIOTHEK Ulm, on January 29, 2013

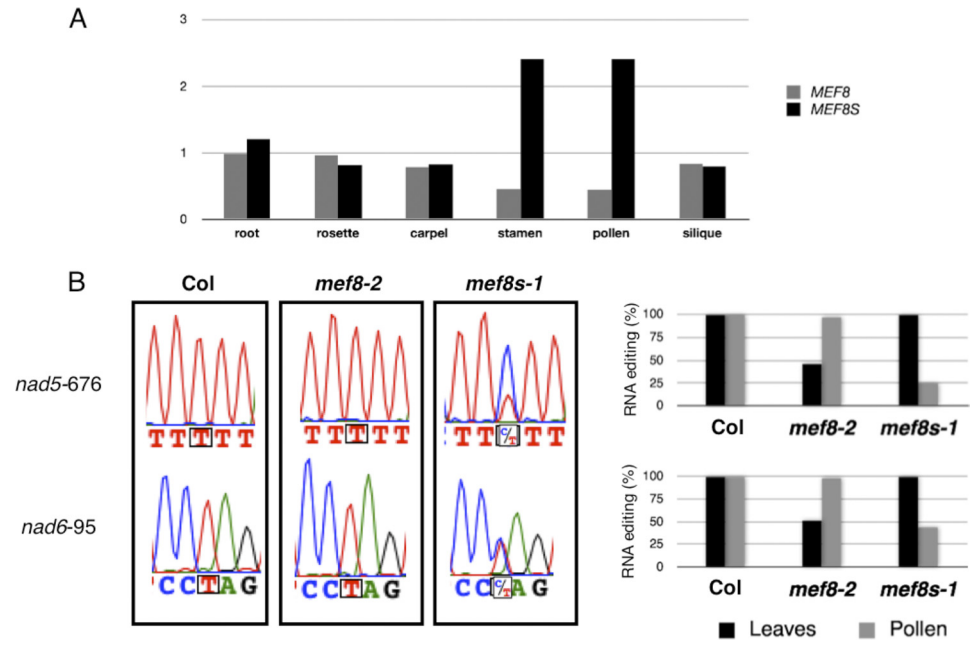


FIGURE 7. **RNA-editing levels at the MEF8 and MEF8S target sites in pollen and in leaves of the respective knock-out mutants correspond with the expression levels of the remaining *MEF8S* and *MEF8* genes.** *A*, Atgenexpress data (48) show that *MEF8S* expression levels (measured as quantities of steady-state RNA signals) increase in stamen tissues and in pollen, whereas transcript levels of *MEF8* are lower in these cells than in any other plant tissue. *B*, pollen kernels were collected from still closed flower buds, and the cDNA obtained from the young pollen was analyzed for editing at the two target sites. Pollen from the homozygous mutant plant *mef8-2* with intact *MEF8S* alleles (*center sequencing panels*; *gray bars* in histograms on the *right*) is hardly affected, and editing levels are similar to those in pollen from wild type Col plants (*left sequencing panel*). In leaves, editing at both target sites is reduced (*black bars* in histograms on the *right*; Figs. 1 and 2). Editing in pollen from the homozygous mutant plant *mef8s-1* (*right sequencing panels*; *gray bars* in histograms on the right) is severely reduced whereas editing in leaves is unaffected. In pollen, the intact *MEF8* alleles cannot compensate the loss of *MEF8S*, possibly because of the low expression of *MEF8* in these tissues.

With only a maximum of five such repeat units in the MEF8 and MEF8S proteins, even binding of all five repeats to specific nucleotides would statistically not be sufficient to yield unique interactions within the transcriptome of the 367-kb large mitochondrial genome of *Arabidopsis* (52). To achieve maximal specificity, all of the repeats in the MEF8 and MEF8S proteins should contact individual nucleotides in the target RNA. In larger RNA-editing specificity factors, the repeats may not all bind to nucleotides, but some may fulfill a function as spacer in the PPR proteins to allow gaps in the contacted nucleotide sequences. When several RNA target sites are addressed by individual PPRs, these RNAs often reveal some sort of consensus pattern only if gaps are allowed (12, 23, 30, 53–56).

Other proteins with similarly rather short tracts have been found to interact with RNA. The plant mitochondrial RNase P (57) recognizes tRNA structures and processes these at specific sites. The THA8 protein with only four copies of PPRs nevertheless binds to specific introns in the plastid and is essential for splicing of these sequences (58).

Both target sites of MEF8 and MEF8S are rather U-rich (Fig. 2), and at least some of the repeats contacting the RNA should be specific for U-nucleotides. A detailed analysis of the interaction of these unique five PPRs in MEF8 and MEF8S proteins with their RNA targets may provide experimental access to the mode and parameters of protein-RNA recognition by the RNA-editing PPR proteins.

MEF8 and MEF8S Proteins Target the Same Editing Sites—The initial identification of the EMS mutant *mef8-1* with lowered but not abolished editing can be interpreted as partial inactivation of the MEF8 protein by the single amino acid exchange in the E^+ domain (Fig. 1). The stronger reduced mitochondrial editing in the T-DNA insertion mutant *mef8-2*, which interrupts the MEF8 protein within the N-terminal region (Fig. 1), suggests that the single amino acid exchange in *mef8-1* does indeed disturb MEF8 function but does not abolish it completely. The T-DNA insertion in the *MEF8* reading frame in *mef8-2*, on the other hand, should prohibit expression of a functional MEF8 protein. Therefore, the observed residual editing in this mutant requires another factor to compensate for the destroyed MEF8 protein and to fulfill its role at least partially. The similar PPR protein MEF8S is a candidate for this second factor.

One crucial condition is that MEF8S targets the same editing sites as MEF8. This is confirmed by several lines of evidence. Stable transformation of *mef8-2* mutant plants with either *MEF8* or *MEF8S* increases the rate of editing at both target sites significantly (Fig. 5). The positive effect of the transformation with *MEF8S* indicates that this protein is indeed involved in editing of these sites. This result furthermore suggests that the low level of expression of *MEF8S* in leaves limits compensation of the loss of MEF8 in untransformed *mef8-2* mutant plants (Fig. 7).

The tissue-specific RNA-editing phenotype in the *MEF8S* knock-out mutant plant further confirms that MEF8 and MEF8S target the same editing sites. RNA editing at the MEF8 target sites is reduced in pollen from the *mef8s-1* mutant (Fig. 7*B*). The *MEF8* level of expression in leaves in this mutant is sufficient to accommodate the loss of *MEF8S*, but the reduced level of *MEF8* expression in pollen is not enough to compensate for the absence of *MEF8S*. These coinciding patterns of expres-

Downloaded from www.jbc.org at UNIVERSITAETSBIBLIOTHEK Ulm, on January 29, 2013

sion and RNA-editing levels (Fig. 7) confirm that both proteins are involved in editing the same two sites and that the expression levels of the *mef8* and *mef8s* genes limit the efficiency of RNA editing at these two sites.

These results confirm that the two proteins MEF8 and MEF8S can substitute for each other at least partially in RNA editing. Either MEF8 or MEF8S can presumably bind to the two target sequences and connect to other proteins in the hypothetical editosome, possibly a multiple organellar RNA-editing factor protein (59). Unfortunately, the substitution effect cannot be tested directly in a double mutant of *mef8* and *mef8s* because we find that this is not viable.

MEF8 and MEF8S Must Have Additional, Essential Functions—The observation that homozygous plants mutated in both *MEF8* and *MEF8S* genes are not viable remains as yet unexplained (Fig. 6). The embryo-lethal double knockout suggests that the two proteins can substitute for each other at one or more functions that are essential for survival of the plant. It is unlikely that the two MEF8 and MEF8S RNA-editing target sites in mRNAs for complex I subunits, *nad5*-676 and *nad6*-95, are these crucial functions because plants without functional complex I are viable.

Therefore, additional RNA-editing sites that are essential for survival of the embryo are likely to be targeted. These have not yet been identified, and further investigations will be required to answer this question. Mutations in some PPR proteins and the concomitant loss of their respective RNA-editing events in plastids or mitochondria result in severe phenotypes. The loss of a large number of editing sites has been found to be likewise embryo-lethal as homozygous T-DNA mutants of the mitochondrial editing cofactor multiple organellar RNA-editing factor 1 are not viable (59). Both crosses from self-pollinated plants, those homozygous for *mef8-2* and heterozygous for *mef8s-1* and those homozygous for *mef8s-1* and heterozygous for *mef8-2*, exhibit an embryo-defective phenotype in approximately 25% of the seed pods in immature siliques (Fig. 6). Similar embryo-lethal phenotypes have been observed with knock-out mutants of mitochondrial or chloroplast aminoacyl-tRNA-synthetases (46, 47). Accordingly, MEF8 and MEF8S seem to be required for proper development of the embryo.

Alternatively, it is possible that MEF8 and MEF8S have additional other essential functions beyond their involvement in RNA editing. Yet another explanation may be that either the NAD5 or the NAD6 proteins derived from mRNAs not edited at the *nad5*-676 and *nad6*-95 target sites of MEF8 and MEF8S result in mutant proteins that interfere with essential mitochondrial functions. It will be interesting to clarify the potential additional function of MEF8 and MEF8S and to determine whether indeed the specific RNA-binding preferences of the two proteins have begun to drift apart since their presumed separation.

Are the Genes for MEF8 and MEF8S Derived by Duplication?—Current models for the evolution of the PPR proteins involve an ancestral gene of the medium-long-short class to which E and DYW domains had been added (32, 34, 36). These original medium-long-short-E-DYW arrangements are the only editing factors in the moss *Physcomitrella patens* (49, 50, 53, 60). Subsequently, this ancestral gene was amplified, and the resulting large family of genes allowed establishment of the many RNA-editing sites observed in flowering plants and even more so in *Lycopodium* (61).

The MEF8 and MEF8S protein-coding genes most likely arose by such a gene duplication. This duplication has presumably taken place rather recently in evolution because both proteins are still recognizably similar and still target the same RNA sequence although possibly with slightly differing preferences. Furthermore, in other plant genomes, two genes coding for proteins similar to MEF8 and MEF8S are detected only in *Arabidopsis lyrata*, whereas in more distant plant species only single similar genes are retrieved.

These potential orthologs in vine, poplar, and rice match the consensus structure of MEF8 and MEF8S, with only five PPRs in the central part. These proteins show a similar degeneration of the C-terminal part of the E domain with an analogous deletion after the conserved N-terminal region.

Prediction programs suggest 64 amino acids for MEF8 and 110 residues for MEF8S as organellar target sequences with predicted mitochondrial locations. These rather long presequences leave unique N-terminal regions in both proteins up to the first PPRs for which little structural features are discerned. Their potential function in binding to RNA target sequences and in recruiting other editing factors needs to be determined experimentally.

In summary, our findings show that the two similar RNA-editing factors MEF8 and MEF8S can (partially) substitute for each other. This proves previous indirect surmises about additional editing factors acting in instances of partial editing mutants. In presumed knock-out mutants of MEF1, for example, two sites have completely lost editing whereas the third target is still edited to 20% (12). Substitution of the activity by another factor has been proposed, but none had been identified previously. The two PPR proteins MEF8 and MEF8S with only few PPRs and their presumed continuous contact to their RNA target sequences may allow direct advances toward deciphering the PPR-RNA code in plant organelles.

Acknowledgments—We thank Mareike Rüdinger and Volker Knoop at Molekulare Evolution, Universität Bonn, for analysis of the repeat structure and number in MEF8 and MEF8S; Rita Gross-Hardt at the ZMBP, Universität Tübingen, for help with the interpretation of the embryo-lethal phenotypes; Stefan Britsch for access to microscope facilities and Dagmar Pruchner and Angelika Müller for excellent experimental help.

REFERENCES

1. Giegé, P., and Brennicke, A. (1999) RNA editing in *Arabidopsis* mitochondria effects 441 C to U changes in ORFs. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 15324–15329
2. Handa, H. (2003) The complete nucleotide sequence and RNA-editing content of the mitochondrial genome of rapeseed (*Brassica napus L.*): comparative analysis of the mitochondrial genomes of rapeseed and *Arabidopsis thaliana*. *Nucleic Acids Res.* **31,** 5907–5916
3. Takenaka, M., Verbitskiy, D., van der Merwe, J. A., Zehrmann, A., and Brennicke, A. (2008) The process of RNA editing in plant mitochondria. *Mitochondrion* **8,** 35–46
4. Bock, R., Hermann, M., and Kössel, H. (1996) *In vivo* dissection of *cis*-acting determinants for plastid RNA editing. *EMBO J.* **15,** 5052–5059

Downloaded from www.jbc.org at UNIVERSITAETSBIBLIOTHEK Ulm, on January 29, 2013

5. Farré, J. C., Leon, G., Jordana, X., and Araya, A. (2001) cis-Recognition elements in plant mitochondrion RNA editing. *Mol. Cell. Biol.* **21,** 6731–6737
6. Neuwirt, J., Takenaka, M., van der Merwe, J. A., and Brennicke, A. (2005) An *in vitro* RNA-editing system from cauliflower mitochondria: editing site recognition parameters can vary in different plant species. *RNA* **11,** 1563–1570
7. van der Merwe, J. A., Takenaka, M., Neuwirt, J., Verbitskiy, D., and Brennicke, A. (2006) RNA-editing sites in plant mitochondria can share *cis*-elements. *FEBS Lett.* **580,** 268–272
8. Takenaka, M. (2010) MEF9, an E-subclass pentatricopeptide repeat protein, is required for an RNA-editing event in the *nad7* transcript in mitochondria of *Arabidopsis*. *Plant Physiol.* **152,** 939–947
9. Takenaka, M., Verbitskiy, D., Zehrmann, A., and Brennicke, A. (2010) Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. *J. Biol. Chem.* **285,** 27122–27129
10. Verbitskiy, D., Zehrmann, A., van der Merwe, J. A., Brennicke, A., and Takenaka, M. (2010) The PPR protein encoded by the lovastatin insensitive 1 gene is involved in RNA editing at three sites in mitochondria of *Arabidopsis thaliana*. *Plant J.* **61,** 446–455
11. Verbitskiy, D., Härtel, B., Zehrmann, A., Brennicke, A., and Takenaka, M. (2011) The DYW-E-PPR protein MEF14 is required for RNA editing at site *matR*-1895 in mitochondria of *Arabidopsis thaliana*. *FEBS Lett.* **585,** 700–704
12. Zehrmann, A., Verbitskiy, D., van der Merwe, J. A., Brennicke, A., and Takenaka, M. (2009) A DYW domain-containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. *Plant Cell* **21,** 558–567
13. Zehrmann, A., Verbitskiy, D., Härtel, B., Brennicke, A., and Takenaka, M. (2011) PPR proteins network as site-specific RNA-editing factors in plant organelles. *RNA Biol.* **8,** 67–70
14. Okuda, K., Nakamura, T., Sugita, M., Shimizu, T., and Shikanai, T. (2006) A pentatricopeptide repeat protein is a site recognition factor in chloroplast RNA editing. *J. Biol. Chem.* **281,** 37661–37667
15. Beick, S., Schmitz-Linneweber, C., Williams-Carrier, R., Jensen, B., and Barkan, A. (2008) The pentatricopeptide repeat protein PPR5 stabilizes a specific tRNA precursor in maize chloroplasts. *Mol. Cell. Biol.* **28,** 5337–5347
16. Pfalz, J., Bayraktar, O. A., Prikryl, J., and Barkan, A. (2009) Site-specific binding of a PPR protein defines and stabilizes 5′ and 3′ mRNA termini in chloroplasts. *EMBO J.* **28,** 2042–2052
17. Lurin, C., Andrés, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyère, C., Caboche, M., Debast, C., Gualberto, J., Hoffmann, B., Lecharny, A., Le Ret, M., Martin-Magniette, M. L., Mireau, H., Peeters, N., Renou, J. P., Szurek, B., Taconnat, L., and Small, I. (2004) Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. *Plant Cell* **16,** 2089–2103
18. Bentolila, S., Knight, W., and Hanson, M. (2010) Natural variation in *Arabidopsis* leads to the identification of REME1, a pentatricopeptide repeat-DYW protein controlling the editing of mitochondrial transcripts. *Plant Physiol.* **154,** 1966–1982
19. Kim, S. R., Yang, J. I., Moon, S., Ryu, C. H., An, K., Kim, K. M., Yim, J., and An, G. (2009) Rice OGR1 encodes a pentatricopeptide repeat-DYW protein and is essential for RNA editing in mitochondria. *Plant J.* **59,** 738–749
20. Doniwa, Y., Ueda, M., Ueta, M., Wada, A., Kadowaki, K., and Tsutsumi, N. (2010) The involvement of a PPR protein of the P subfamily in partial RNA editing of an *Arabidopsis* mitochondrial transcript. *Gene* **454,** 39–46
21. Chateigner-Boutin, A. L., Ramos-Vega, M., Guevara-García, A., Andrés, C., de la Luz, Gutiérrez-Nava, M., Cantero, A., Delannoy E., Jiménez, L. F., Lurin, C., Small, I., and León, P. (2008) CLB19, a pentatricopeptide repeat protein required for editing of *rpoA* and *clpP* chloroplast transcripts. *Plant J.* **56,** 590–602
22. Fujii, S., and Small, I. (2011) The evolution of RNA editing and pentatricopeptide repeat genes. *New Phytol.* **191,** 37–47
23. Hammani, K., Okuda, K., Tanz, S. K., Chateigner-Boutin, A. L., Shikanai, T., and Small, I. (2009) A study of new *Arabidopsis* chloroplast RNA-editing mutants reveals general features of editing factors and their target sites. *Plant Cell* **21,** 3686–3699
24. Kotera, E., Tasaka, M., and Shikanai, T. (2005) A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. *Nature* **433,** 326–330
25. Okuda, K., Myouga, F., Motohashi, R., Shinozaki, K., and Shikanai, T. (2007) Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 8178–8183
26. Robbins, J. C., Heller, W. P., and Hanson, M. R. (2009) A comparative genomics approach identifies a PPR-DYW protein that is essential for C-to-U editing of the *Arabidopsis* chloroplast *accD* transcript. *RNA* **15,** 1142–1153
27. Shikanai, T. (2006) RNA editing in plant organelles: machinery, physiological function and evolution. *Cell Mol. Life Sci.* **63,** 689–708
28. Yu, Q. B., Jiang, Y., Chong, K., and Yang, Z. N. (2009) AtECB2, a pentatricopeptide repeat protein, is required for chloroplast transcript *accD* RNA editing and early chloroplast biogenesis in *Arabidopsis thaliana*. *Plant J.* **59,** 1011–1023
29. Zhou, W., Cheng, Y., Yap, A., Chateigner-Boutin, A. L., Delannoy, E., Hammani, K., Small, I., and Huang, J. (2009) The *Arabidopsis* gene *YS1* encoding a DYW protein is required for editing of *rpoB* transcripts and the rapid development of chloroplasts during early growth. *Plant J.* **58,** 82–96
30. Verbitskiy, D., Zehrmann, A., Brennicke, A., and Takenaka, M. (2010) The DYW domain in the MEF11 RNA-editing protein seems to be involved in specific RNA binding rather than in the enzymatic reaction. *Plant Signal. Behav.* **5,** 558–560
31. Zehrmann, A., Verbitskiy, D., Härtel, B., Brennicke, A., and Takenaka, M. (2010) RNA editing competence of trans-factor MEF1 is modulated by ecotype-specific differences but requires the DYW domain. *FEBS Lett.* **584,** 4181–4186
32. Salone, V., Rüdinger, M., Polsakiewicz, M., Hoffmann, B., Groth-Malonek, M., Szurek, B., Small, I., Knoop, V., and Lurin, C. (2007) A hypothesis on the identification of the editing enzyme in plant organelles. *FEBS Lett.* **581,** 4132–4138
33. Nakamura, T., and Sugita, M. (2008) A conserved DYW domain of the pentatricopeptide repeat protein possesses a novel endoribonuclease activity. *FEBS Lett.* **582,** 4163–4168
34. O'Toole, N., Hattori, M., Andres, C., Iida, K., Lurin, C., Schmitz-Linneweber, C., Sugita, M., and Small, I. (2008) On the expansion of the pentatricopeptide repeat gene family in plants. *Mol. Biol. Evol.* **25,** 1120–1128
35. Small, I. D., and Peeters, N. (2000) The PPR motif: a TPR-related motif prevalent in plant organellar proteins. *Trends Biochem. Sci.* **25,** 46–47
36. Rivals, E., Bruyère, C., Toffano-Nioche, C., and Lecharny, A. (2006) Formation of the *Arabidopsis* pentatricopeptide repeat family. *Plant Physiol.* **141,** 825–839
37. Schmitz-Linneweber, C., and Small, I. (2008) Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends Plant Sci.* **13,** 663–670
38. Takenaka, M., and Brennicke, A. (2007) RNA editing in plant mitochondria: assays and biochemical approaches. *Methods Enzymol.* **424,** 439–458
39. Takenaka, M., and Zehrmann, A. (2011) in *RNA and DNA Editing* (Aphasizhev, R., ed) pp. 163–169, Springer, Heidelberg
40. Takenaka, M., and Brennicke, A. (2009) Multiplex single-base extension typing to identify nuclear genes required for RNA editing in plant organelles. *Nucleic Acids Res.* **37,** e13
41. Takenaka, M., and Brennicke, A. (2012) Using multiplex single-base extension typing to screen for mutants defective in RNA editing. *Nat. Protoc.* **7,** 1937–1945
42. Takenaka, M. (2011) in *RNA and DNA Editing*. (Aphasizhev, R., ed) pp. 151–161, Springer, Heidelberg
43. Clough, S. J., and Bent, A. F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16,** 735–743
44. Curtis, M. D., and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiol.* **133,** 462–469
45. Knoop, V., and Rüdinger, M. (2010) DYW-type PPR proteins in a heterolobosean protist: plant RNA-editing factors involved in an ancient hori-

Downloaded from www.jbc.org at UNIVERSITAETSBIBLIOTHEK Ulm, on January 29, 2013

zontal gene transfer? *FEBS Lett.* **584,** 4287–4291
46. Berg, M., Rogers, R., Muralla, R., and Meinke, D. (2005) Requirement of aminoacyl-tRNA synthetases for gametogenesis and embryo development in *Arabidopsis*. *Plant J.* **44,** 866–878
47. Kägi, C., Baumann, N., Nielsen, N., Stierhof, Y. D., and Gross-Hardt, R. (2010) The gametic central cell of *Arabidopsis* determines the lifespan of adjacent accessory cells. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 22350–22355
48. Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., Vingron, M., Schölkopf, B., Weigel, D., and Lohmann, J. U. (2005) A gene expression map of *Arabidopsis thaliana* development. *Nat. Genet.* **37,** 501–506
49. Rüdinger, M., Polsakiewicz, M., and Knoop, V. (2008) Organellar RNA editing and plant-specific extensions of pentatricopeptide repeat proteins in jungermanniid but not in marchantiid liverworts. *Mol. Biol. Evol.* **25,** 1405–1414
50. Rüdinger, M., Szövényi, P., Rensing, S., and Knoop, V. (2011) Assigning DYW-type PPR proteins to RNA-editing sites in the funariid mosses *Physcomitrella patens* and *Funaria hygrometrica*. *Plant J.* 67, 360–370
51. Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., and Bonas, U. (2009) Breaking the code of DNA-binding specificity of TAL-type III effectors. *Science* **326,** 1509–1512
52. Unseld, M., Marienfeld, J. R., Brandt, P., and Brennicke, A. (1997) The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. *Nat. Genet.* **15,** 57–61
53. Ohtani, S., Ichinose, M., Tasaki, E., Aoki, Y., Komura, Y., and Sugita, M. (2010) Targeted gene disruption identifies three PPR-DYW proteins involved in RNA editing for five editing sites of the moss mitochondrial transcripts. *Plant Cell Physiol.* **51,** 1942–1949
54. Okuda, K., Hammani, K., Tanz, S. K., Peng, L., Fukao, Y., Myouga, F., Motohashi, R., Shinozaki, K., Small, I., and Shikanai, T. (2010) The pentatricopeptide repeat protein OTP82 is required for RNA editing of plastid *ndhB* and *ndhG* transcripts. *Plant J.* **61,** 339–349
55. Okuda, K., Chateigner-Boutin, A. L., Nakamura, T., Delannoy, E., Sugita, M., Myouga, F., Motohashi, R., Shinozaki, K., Small, I., and Shikanai, T. (2009) Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in *Arabidopsis* chloroplasts. *Plant Cell* **21,** 146–156
56. Okuda, K., and Shikanai, T. (2012) A pentatricopeptide repeat protein acts as a site-specificity factor at multiple RNA-editing sites with unrelated *cis*-acting elements in plastids. *Nucleic Acids Res.* **40,** 5062–5064
57. Gobert, A., Gutmann, B., Taschner, A., Gössringer, M., Holzmann, J., Hartmann, R. K., Rossmanith, W., and Giegé, P. (2010) A single *Arabidopsis* organellar protein has RNase P activity. *Nat. Struct. Mol. Biol.* **17,** 740–744
58. Khrouchtchova, A., Monde, R. A., and Barkan, A. (2012) A short PPR protein required for the splicing of specific group II introns in angiosperm chloroplasts. *RNA* **18,** 1197–1209
59. Takenaka, M., Zehrmann, A., Verbitskiy, D., Kugelmann, M., Härtel, B., and Brennicke, A. (2012) Multiple organellar RNA-editing factor (MORF) family proteins are required for RNA editing in mitochondria and plastids of plants. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 5104–5109
60. Tasaki, E., Hattori, M., and Sugita, M. (2010) The moss pentatricopeptide repeat protein with a DYW domain is responsible for RNA editing of mitochondrial *ccmFc* transcript. *Plant J.* **62,** 560–570
61. Grewe, F., Herres, S., Viehöver, P., Polsakiewicz, M., Weisshaar, B., and Knoop, V. (2011) A unique transcriptome: 1782 positions of RNA editing alter 1406 codon identities in mitochondrial mRNAs of the lycophyte *Isoetes engelmannii*. *Nucleic Acids Res.* **39,** 2890–2902

Downloaded from www.jbc.org at UNIVERSITAETSBIBLIOTHEK Ulm, on January 29, 2013

Copyright Notice:
Reprinted from:
Springer Plant Molecular Biology, 81, 2013, 337-346,
„MEF10 is required for RNA editing at *nad2*-842 in mitochondria of *Arabidopsis thaliana* and interacts with MORF8“,
Härtel, B., Zehrmann, A., Verbitskiy, D., van der Merwe, J. A., Brennicke, A., and Takenaka, M.,
copyright and all rights are held exclusively by Springer Science+Business Media Dordrecht.
With kind permission from Springer Science and Business Media.

Plant Mol Biol
DOI 10.1007/s11103-012-0003-2

MEF10 is required for RNA editing at *nad2*-842 in mitochondria of *Arabidopsis thaliana* and interacts with MORF8

Barbara Härtel · Anja Zehrmann · Daniil Verbitskiy · Johannes A. van der Merwe · Axel Brennicke · Mizuki Takenaka

Received: 5 July 2012 / Accepted: 15 December 2012
© Springer Science+Business Media Dordrecht 2013

Abstract A forwards genetic screen of a chemically mutated plant population identified mitochondrial RNA editing factor 10 (MEF10) in *Arabidopsis thaliana*. MEF10 is a *trans*-factor required specifically for the C to U editing of site *nad2*-842. The MEF10 protein is characterized by a stretch of pentatricopeptide repeats (PPR) and a C-terminal extension domain ending with the amino acids DYW. Editing is lost in mutant plants but is recovered by transgenic introduction of an intact *MEF10* gene. The MEF10 protein interacts with multiple organellar RNA editing factor 8 (MORF8) but not with other mitochondrial MORF proteins in yeast two hybrid assays. These results support the model that specific combinations of MORF and MEF proteins are involved in RNA editing in plant mitochondria.

B. Härtel · A. Zehrmann · D. Verbitskiy · J. A. van der Merwe · A. Brennicke · M. Takenaka (✉)
Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany
e-mail: mizuki.takenaka@uni-ulm.de

B. Härtel
e-mail: barbara.haertel@uni-ulm.de

A. Zehrmann
e-mail: anja.zehrmann@uni-ulm.de

D. Verbitskiy
e-mail: daniil.verbitskiy@uni-ulm.de

J. A. van der Merwe
e-mail: dre.van-der-merwe@uni-ulm.de

A. Brennicke
e-mail: mo.bo@uni-ulm.de

Present Address:
J. A. van der Merwe
Institut für Molekulare Virologie, Uni Ulm, 89069 Ulm, Germany

Keywords RNA editing factor · Plant mitochondria · PPR protein · MEF10 · MORF8 · MEF-MORF interaction

Introduction

Maturation of many mRNAs in plastids and mitochondria of plants includes an editing step which alters selected cytidines to uridines. Altogether more than 500 editing sites have to be addressed in the two organelles in flowering plants. Processing of these many RNA editing sites is mediated by numerous specific *trans*-acting proteins which presumably recognize cognate sequences near to an editing site in the precursor mRNAs (Bock and Koop 1997; Fujii et al. 2010). The presently identified *trans*-acting factors specific for single or few sites are composed of sets of repeats of about 35 amino acids and are thus classified as pentatricopeptide repeat proteins (PPR proteins). The PPR proteins have been proposed to contact the RNA sequence with selected repeats at specific nucleotides (Fujii et al. 2010; Fujii and Small 2011; Kobayashi et al. 2012). The more than 500 editing sites in both organelles of flowering plants could thus be addressed in small groups of similar sequences by individual proteins from this protein family encoded by about 450 genes in the nuclear genomes (Small and Peeters 2000; Lurin et al. 2004; Schmitz-Linneweber and Small 2008). The RNA editing specificity factors presently assigned in flowering plants belong to a subgroup of the PPR family with at least one C-terminal extension, the E domain.

Terminating with this E domain are several editing proteins in plastids (Kotera et al. 2005; Okuda et al. 2007; Chateigner-Boutin et al. 2008) and in mitochondria (Takenaka 2009; Takenaka et al. 2010), most of these identified in *Arabidopsis thaliana*. Several other site-specific RNA editing

Published online: 04 January 2013

PPR proteins continue beyond the E domain into a region with the three amino acids DYW at the C-terminus (Kim et al. 2009; Okuda et al. 2009; Yu et al. 2009; Zehrmann et al. 2009; Zhou et al. 2009; Takenaka et al. 2010; Verbitskiy et al. 2010, 2011).

Recently, we developed a forward genetic procedure to analyse mitochondrial RNA editing sites in a population of *A. thaliana* plants with the aim to identify mutants with deviations in their RNA editing efficiency at one or more of these sites (Takenaka and Brennicke 2009, 2012). This approach identified several E and DYW type PPR proteins, but also novel RNA editing proteins like the members of the MORF protein family (Takenaka et al. 2012). Individual MORF proteins are required for editing at multiple sites, very different from the PPR proteins (Bentolila et al. 2012; Takenaka et al. 2012). Some MORF proteins bind selected PPR proteins in vitro, suggesting that MORF and PPR proteins may interact for the editing process.

In this communication we report the assignment of mitochondrial editing factor 10 (MEF10), a DYW type PPR protein, to be a mitochondrial RNA editing specificity factor which targets the *nad2*-842 nucleotide through mapping of an EMS induced mutation in the *MEF10* gene. Furthermore, we find that MEF10 interacts specifically with MORF8 (also named RIP1) which is also required for full editing at this target site (Bentolila et al. 2012).

Materials and methods

Plant material and preparation of nucleic acids

Growth of *A. thaliana* plants and preparation of DNA or RNA from leaves were as described (Takenaka and Brennicke 2007). The EMS mutant population of ecotype Col was purchased from Lehle Seeds, USA, and the T-DNA insertion line of *A. thaliana* came from the TAIR resources. Homozygous plants and T-DNA insertion sites were identified and confirmed by PCR with appropriate primers.

SNaPshot screening and analysis of mutants

The EMS mutant plants were analysed in batches of 10 individuals by multiplexed single base extension (Takenaka and Brennicke 2009, 2012) for deviations in RNA editing at any of about 400 sites in mitochondrial mRNAs. Individual plant lines with the relevant mutation were identified as described (Takenaka and Brennicke 2009, 2012), and the altered RNA editing was confirmed by cDNA sequence analysis. Sequences were determined commercially by LGC genomics, Berlin, Germany, 4base lab, Reutlingen, Germany or Macrogen, Seoul, Korea.

Investigation of RNA editing sites

Individual cDNA fragments covering one or more editing sites were obtained through RT-PCR following published protocols (Takenaka and Brennicke 2007) and sequences of wild type and mutant plants were compared for the presence of C or T at each RNA editing site.

Complementation by stable transformation

Plants of the EMS mutant line *mef10-1* were transformed by floral dip (Clough and Bent 1998) with the *MEF10* wt Col reading frame under control of the 35S promoter in vector pMDC123 (Curtis and Grossniklaus 2003) with a GFP cassette from psMGFP4 (Forner and Binder 2007) and a multiple cloning site from pET41 (Merck Millipore Novagen®, Darmstadt, Germany). Transgenic plants were selected by spraying with Basta®. For the analysis of RNA editing levels, the respective cDNA fragment was sequenced (Takenaka and Brennicke 2007).

Yeast two hybrid assays

The sequences coding for MEF10 and the various MORFs were cloned with the In-Fusion HD cloning system (Clontech Laboratories, Mountain View, USA). MEF10 inserts were integrated into the binding domain containing vector pGBKT7, MORFs into the activator domain containing vector pGADT7 of the GAL4 Two Hybrid System 3 (Clontech Laboratories, Mountain View, USA). The vectors were cotransfected for expression into yeast cells (PJ69-4A) according to the protocol. Yeast cells with both bait and prey vectors were cultured in synthetic dropout medium without Leu and Trp. 5 µl of suspended cells with an OD_{600} of 0.3 were dropped onto the various selection media plates.

Results

Identification of a mutant plant disturbed in editing at site *nad2*-842

A mutant deficient in RNA editing at a specific site in the mRNA for subunit 2 of complex I of the respiratory chain in plant mitochondria, *nad2*-842, was identified in a forward genetic screen (Takenaka and Brennicke 2009, 2012) of a population of chemically mutagenized *A. thaliana* plants (Figs. 1, 2). In the mutant plant individual, termed *mef10-1*, no RNA editing at the *nad2*-842 site is observed while wt plants alter the genomic encoded C to U (Fig. 2b, left panels). The absence of this editing event causes a serine to phenylalanine codon change in the mature RNA

which is presumably released for translation. Other sites in the *nad2* transcript, such as *nad2*-821 for example, are undisturbed by this mutation and are fully edited also in the mutant (Fig. 2b, right panels). These results confirm that a factor specifically required for the editing event at site *nad2*-842 has been mutated. All other 380 mitochondrial editing sites detected by the SNaPshot screen are unaltered in the *mef10-1* mutant.

The *MEF10* gene encodes a DYW PPR protein

The gene affected by the mutation in plant line *mef10-1* in the *A. thaliana* Columbia ecotype (Col) was mapped by crossing this mutant with wild type plants of ecotype Landsberg *erecta* (L*er*). In ecotype L*er* plants this site is fully edited (Zehrmann et al. 2008). In the F2 generation, the expected about 25 % of 100 individual plants showed the mutant phenotype of no editing at site *nad2*-842. L*er* and Col specific markers were used to map e.g. SNPs co-segregating with the editing phenotype. The closest identified cross-overs narrowed the coding region of the gene mutated in *mef10-1* to about 1.7 Mb on chromosome three (Fig. 2a).

The 450 genes for PPR proteins are spread throughout the *A. thaliana* nuclear genome and this region of 1.7 Mb encodes seven genes annotated for PPR proteins. Of these, two code for P type PPRs, two for E class PPRs and three for PPR proteins with DYW domains (Fig. 2a). The two candidate genes for E class PPRs and the three for DYW PPR proteins were sequenced in the mutant and compared

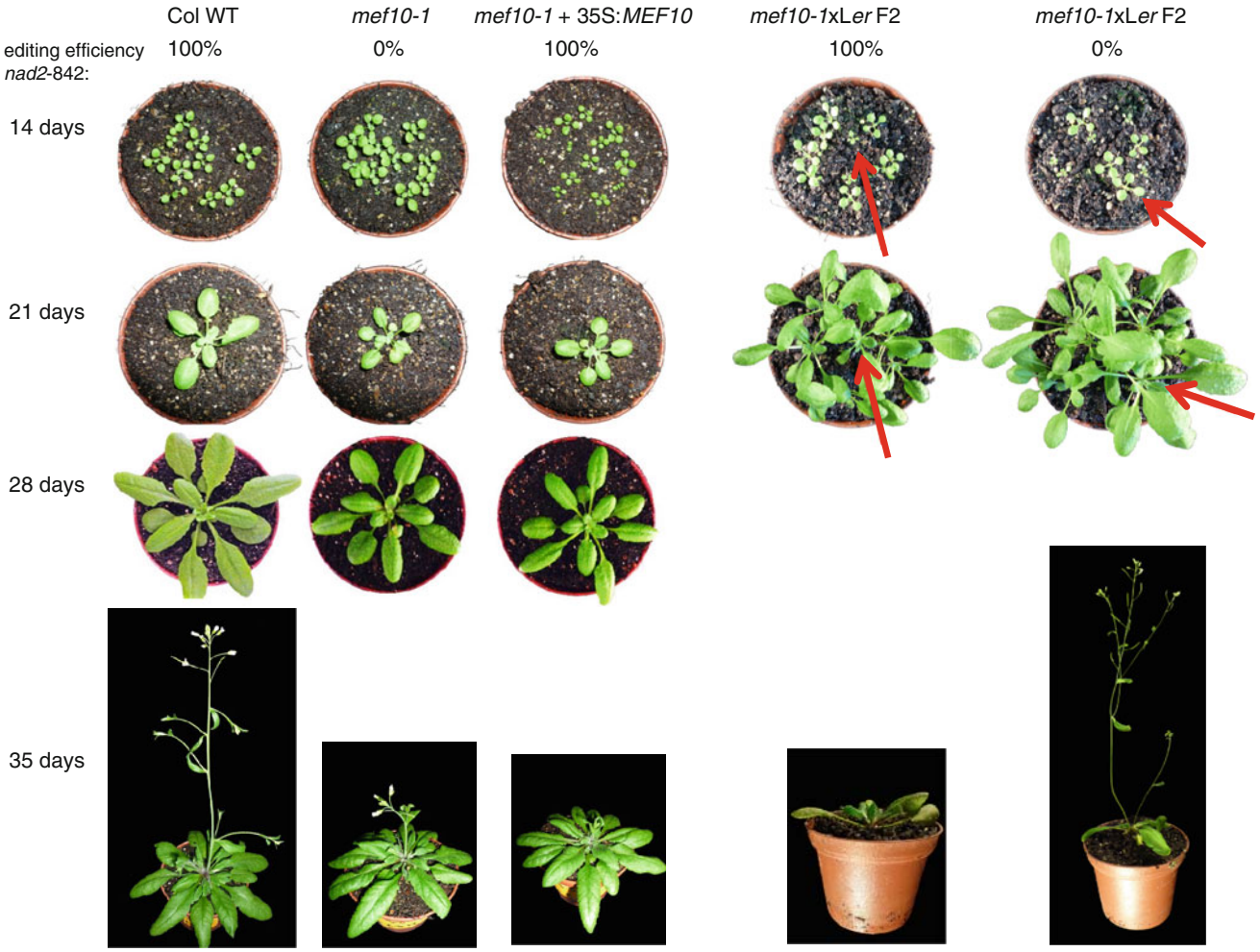


Fig. 1 Phenotype of the mutant plant *mef10-1*. Plants of the *Arabidopsis thaliana* Columbia (Col) ecotype, the mutant *mef10-1* in the Col genetic background and the complemented mutant (*mef10-1* + 35S:*MEF10*) were grown under identical conditions for the times indicated. Both mutant and complemented plants grow slower than the wild type, suggesting that the lack of RNA editing at site *nad2*-842 in the mRNA for subunit 2 of respiratory chain complex I is not responsible for this phenotype. This is confirmed in an outcrossing assay with the mutant *mef10-1* and wild type Landsberg *erecta* (L*er*) plants. In the F2 generation a plant is identified which shows the phenotype of delayed growth but is heterozygous for the mutant *mef10-1* and wild type *MEF10* L*er* alleles and is fully edited at the MEF10 target site (*red arrows*). Another plant is homozygous for the mutant *mef10-1* allele, is not edited at the MEF10 target site and shows a normal phenotype (*red arrows*)

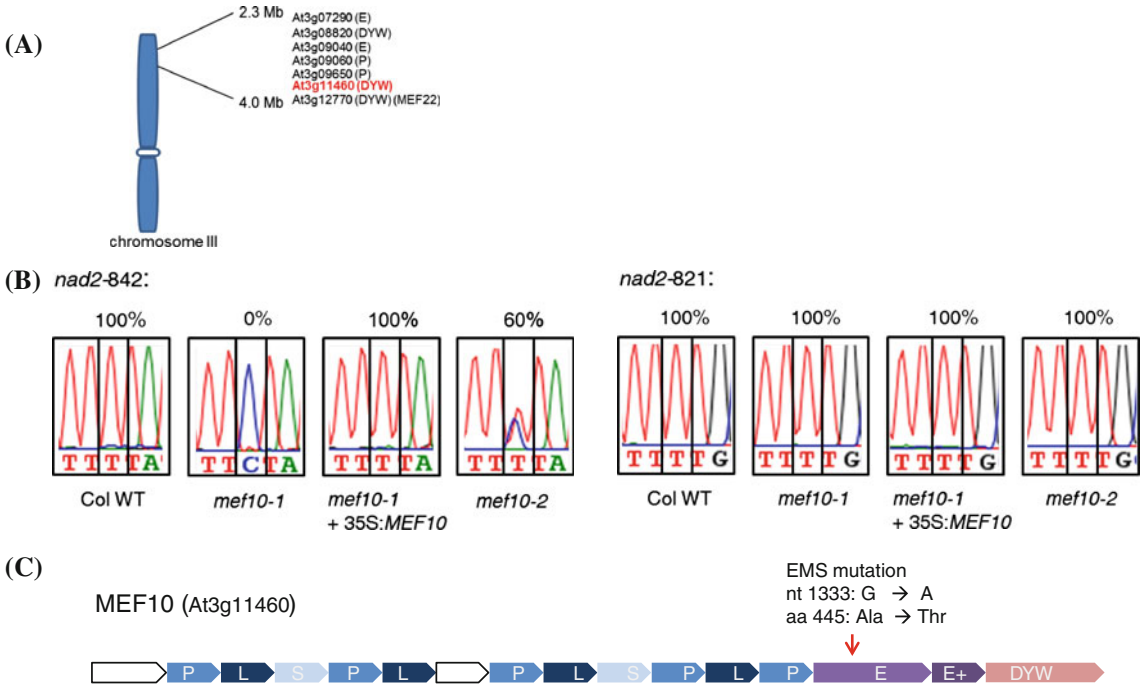


Fig. 2 The EMS mutation causing the lack of RNA editing at site *nad2*-842 identifies a nuclear gene encoding a DYW PPR protein. **a** Mapping of cross-over events in a cross between the mutant plant *mef10-1* in the Col genetic background and a wt Landsberg *erecta* (L*er*) plant narrows the chromosomal region of the EMS mutation causing the loss of editing at site *nad2*-842 to 1.7 Mb on chromosome 3. Locus At3g11460 (*red*) encoding one of three DYW PPR proteins in this window, contains a typical EMS induced single nucleotide alteration. **b** The Col wild type *MEF10* gene introduced into transgenic *mef10-1* mutant plants restores the ability for RNA editing at site *nad2*-842, confirming the identification of the *MEF10* gene at locus At3g11460. Furthermore, the T-DNA insertion in the 5′-transcribed but untranslated region of *MEF10* in mutant *mef10-2* reduces editing at the target site to 60 %. Another editing site 21 nucleotides upstream in the *nad2* mRNA, site *nad2*-821, is not affected in *mef10-1* plants and thus presumably not targeted by the MEF10 PPR protein. Colour traces are: G-*black*, A-*green*, T-*red*, C-*blue*. **c** The structural analysis of the MEF10 DYW PPR protein reveals 12 PPR repeats, an E and an E+ domain and a C-terminal DYW sequence. The predicted N-terminal mitochondrial target sequence is shown as an open arrow. P, L and S type repeats are differentially *shaded*, a central repeat of unclear prediction is in *white*. In the *mef10-1* mutant plant line a C to T (G to A on the other strand) transition at nucleotide 1333 causes an Ala to Thr amino acid change at residue 445

with their wild type counterparts. One of the here encoded DYW PPR proteins has been identified previously as MEF22 (Takenaka et al. 2010). In the open reading frame coded by locus At3g11460, a unique nucleotide alteration characteristic of EMS mutations was found in the mutant. The nucleotide difference changes an alanine codon to a threonine codon (Ala445Thr) in the E domain of MEF10 (Fig. 2c). Previous analysis of the intracellular location of the encoded protein with a GFP fusion assay has shown that its presequence indeed targets the attached protein to the mitochondrion, not to the predicted location in the plastid (Lurin et al. 2004).

From the TAIR resource three T-DNA insertion lines were obtained which are annotated to contain a T-DNA insertion in the *MEF10* gene. In line SALK_061966C we found that the T-DNA is inserted in the promoter region and that editing at site *nad2*-842 is not affected by this T-DNA insert. In line SAIL_579_DO1 we did not detect any insert in the *MEF10* gene. Our analysis of line SAIL_599_BO1 identified a T-DNA insert in the 5′-UTR of the *MEF10* gene. This insertion reduces RNA editing at the *nad2*-842 target site to 60 % but does not completely block MEF10 activity (Fig. 2b). Control site *nad2*-821 is fully edited also in this mutant. This mutant line has been consequently renamed *mef10-2*.

RNA editing is recovered in the mutant *mef10-1* by the wild type MEF10 gene

To further verify that *MEF10* codes for an RNA editing factor essential for maturation of the *nad2* mRNA, this candidate gene was tested in a complementation assay by stable transformation. Transgenic plants of the *mef10-1* mutant were generated by introducing the Col wild-type *MEF10* gene under control of a 35S-CaMV promoter. In the transgenic mutant plants, the ability to edit site *nad2*-842 is fully recovered (Fig. 2b, *mef10-1* + 35S:*MEF10*). This result confirms that the *MEF10* gene encodes an essential RNA editing factor specifically required for processing of the *nad2*-842 site.

RNA editing at the *nad2*-842 site is little altered in several ecotypes

Nucleotide alterations and the concommitant amino acid exchanges in MEF proteins can influence RNA editing levels between ecotypes (Zehrmann et al. 2008). To analyze potential differences in MEF10 editing between ecotypes, we investigated several candidate accessions of *A. thaliana* for which single nucleotide polymorphisms (SNPs) have been reported in the MEF10 open reading frame. The ecotypes analysed show nearly full editing at the MEF10 target site (Bay-0 [100 % editing], Bur-0 [90 % editing], C24 [100 % editing], Col [100 % editing], Fei-0 [100 % editing], Got-7 [100 % editing], L*er* [100 % editing], Lov-5 [95 % editing], Sha [100 % editing], Tamm-2 [87 % editing]). When we sequenced the *MEF10* gene in accessions Bur-0, Fei-0, Got-7, Lov-5 and Tamm-2 we did not see in our plants any of the SNPs annotated in comparison to Col in the respective databases (www.weigelworld.org). The only SNP we did observe between all accessions is in L*er*, where a G to A change at nucleotide 718 from the A in the ATG start codon alters an aspartic acid codon to an asparagine codon.

Lack of editing at site *nad2*-842 shows little if any phenotypic effect

The mutant plants of *mef10-1* grow slower than the wild type Col plants under normal growth chamber conditions (Fig. 1). To determine whether this altered phenotype is caused by the RNA editing defect, we compared the growth habitus of wild type, mutant and complemented mutant plants. In the latter, RNA editing is fully restored by the introduced *MEF10* gene, but plants still show the same slower growth and delayed bolting as the *mef10-1* mutant (Fig. 1). To further analyse a potential link between the slower growth and the mutation in *MEF10*, we crossed the *mef10-1* mutant in the Col background with wild type L*er* plants. The F1 offspring was selfed and the F2 generation was separated into slower and normally growing plants. In the slower growing plants, an individual was identified which is heterozygous for the L*er* wild type sequence of *MEF10* and the *mef10-1* mutant allele and is fully edited (Fig. 1). Among 60 normally growing plants, two were identified which are homozygous for the *mef10-1* mutant allele and which are unedited at the MEF10 target site *nad2*-842 (Fig. 1). The persistent slower growth of plants in which the *mef10-1* mutant has been either segregated out or compensated in the transgenic complemented plants suggests that the delayed growth is not caused by the mutation in the *MEF10* gene. Plants of the *mef10-2* mutant line also grow normally, further confirming that the delayed growth of *mef10-1* plants is not connected to the editing event *nad2*-842.

The phenylalanine amino acid introduced by this RNA editing event is conserved in higher plants (Fig. 3, centre panel). The only exception in the database is vine, where a C is annotated in the genomic sequence but no editing event reported (Picardi et al. 2011). When we resequenced vine genomic and cDNA, this site turned out to be genomically coded already as a T as it is also in sugar beet (Fig. 3, upper panel; the U given in normal type reflects the genomic T we found). This and other differences seen may be due to divergencies in the vine accessions used. In the sequence window between nucleotides 741–1500 from the A in the ATG codon in the grape cDNA, we observed only one editing event at nucleotide *nad2*-1200. The editing sites annotated in the REDIdb database at sites 788, 920, 1014, 1127, 1212, 1246, 1247, 1296, 1298, 1400, 1403 and 1416 were all encoded as T in the genomic mitochondrial DNA from the accession we analysed.

The monocot rice and the dicot *Oenothera berteriana* have additional editing events just 9 nucleotides upstream of site *nad2*-842 within the presumed specificity recognition region of the *nad2*-842 site. It would be interesting to determine whether this upstream event affects editing at site *nad2*-842.

The RNA editing target of MEF10 shares mostly uridines with other editing sites

In both mitochondria and plastids, the *cis*-elements identifying a specific C in the RNA sequence are located upstream (5′) of the edited C. There a unique pattern in the 20 or 25 nucleotides 5′ of the edited nucleotide defines a (PPR protein) binding site (Chaudhuri and Maliga 1996; Bock and Koop 1997; Farré et al. 2001; Miyamoto et al. 2002; van der Merwe et al. 2006; Kempken et al. 2009). The respective sequence at editing site *nad2*-842 reveals a surprising extent of similarity in this −25 to +5 window with other editing sites which are however all edited normally in the *mef10-1* mutant (Fig. 3, bottom panel). The similar nucleotides, which are mostly U nucleotides, are therefore not sufficient to identify the *nad2*-842 site. Consequently, contact of MEF10 to other (additional) nucleotides present at the *nad2*-842 site but not at any of the other sites is required.

There are only four unique nucleotide identities in the genuine MEF10 target site *nad2*-842 not found in one or more of the sites unaffected in *mef10-1*, the A at −13, the A at −16, the C at −18 and the G at −19. These nucleotides are therefore good candidates for a direct MEF10 interaction in which binding is discriminatory against other nucleotide identities. Alternatively or in addition unique combinatorial contacts may be involved, in which several

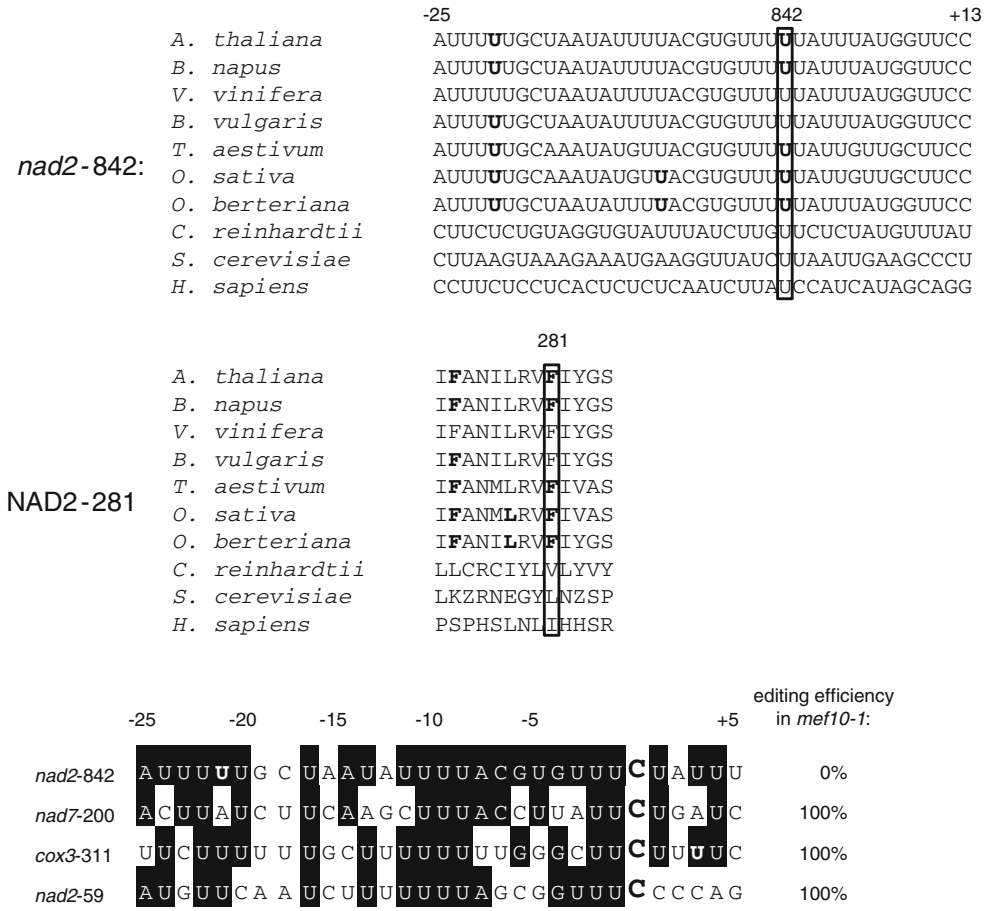


Fig. 3 Comparison of nucleotide and amino acid sequences around the *nad2*-842 editing site in different organisms. The *upper* alignment of the nucleotides around the *nad2*-842 editing site (*bold* U and *framed*) shows this nucleotide to be well conserved in plants. Other editing sites (bold U) are found in *Oryza* and *Oenothera* just 9 nucleotides upstream of the *nad2*-842 editing site. The amino acid alignment in the *center* shows that the amino acids in this region of the NAD2 protein are less conserved between the different plant species and other organisms than mitochondrially encoded proteins usually are. In the *bottom* alignment, the MEF10 target site *nad2*-842 is compared to other editing sites (*large bold* C) in *Arabidopsis thaliana* with similar nucleotide sequence patterns in the presumed *cis*-recognition region. The three editing sites in the *nad7*, *cox3* and *nad2* mRNAs are fully edited in the *mef10-1* mutant and are thus most likely not a target of MEF10. Site *nad7*-200 is not recognized by MEF10, but by another MEF protein, MEF9, which in turn does not recognize the MEF10 target site at nucleotide *nad2*-842 (Takenaka 2009). *Identical* nucleotides are inversely *shaded*. Sequences are shown 5′ to 3′ or N- to C-terminus from *left* to *right*

nucleotide identities have to interact for a successful contact.

In a different scenario, the U nucleotides common to the four aligned editing sites are sufficient to guide MEF10, but other PPR proteins can recognize the other three editing sites and compensate the loss of MEF10 there, but not at site *nad2*-842. However, this scenario is unlikely since the site *nad7*-200 is targeted by another editing factor, MEF9 (Takenaka 2009). In the *mef9* mutant, site *nad2*-842 and the two other sites aligned (Fig. 3) are not affected and are fully edited. That MEF9 cannot compensate for MEF10 and vice versa, implies that both PPR proteins recognize their own target site but not that of the respective other MEF. This further implies that several of the nucleotides differing between the two target sites have to be contacted by the MEF9 and the MEF10 proteins, respectively. These crucial contact nucleotides could also reside much further upstream or downstream outside of the assumed window of the *cis*-element. The example of the PPR5 protein, for which the RNA target site has been investigated in detail, shows that the nucleotides presumably bound by the PPR tract can be spread over a stretch of over 40 nucleotides with a loop-out of the internal nucleotides (Williams-Carrier et al. 2008). Although PPR5 is involved in intron splicing rather than RNA editing and the sizes of the individual PPR repeats are somewhat different, the basic mode of RNA binding should be similar between these PPR proteins.

Proteins similar to MEF10 in other plant species

BLAST searches of the databases reveal several proteins which are similar to MEF10 in other plant species (Fig. 4). Most similar to the MEF10 protein in *A. thaliana* (At3g11460) is the D7LAN3 protein in *Arabidopsis lyrata*, which is to be expected considering the close relationship between these two species. The respective most similar protein from *Vitis vinifera* (vine) is GSVIVG01033863001 given in the FLAG database and XP_002276416 indicated in the NCBI database as MEF10 like protein. This protein is annotated with an intron in the E domain in the FLAG database but the reading frame continues through and the deduced protein aligns throughout its length (Fig. 4). A reverse search with this vine protein identifed the MEF10 protein in *A. thaliana* as being most similar, suggesting that this vine protein may be a true ortholog. However, since the MEF10 target site does not need to be edited in vine, this vine protein may have evolved a different specificity and may actually target a different site.

MEF10 and MORF8 proteins interact

Recently, the small family of MORF proteins was identified to be involved in RNA editing in mitochondria and in plastids (Takenaka et al. 2012). In this analysis it was found that some MEF proteins which target the same sites as MORF1 interact with the MORF1 protein. To investigate whether one of the MORF proteins can bind to the MEF10 protein, we cloned *MEF10* and various *MORFs* in yeast bait and prey vectors and analysed their interaction in yeast cells (Fig. 5). MEF10 interacts most strongly with MORF8 and after prolonged incubation weakly also with MORF2 and MORF9. Of these, only MORF8 is mitochondrially located while MORF2 and MORF9 are plastid proteins (Takenaka et al. 2012).

Discussion

The E-domain is essential for MEF10 function in RNA editing

The single amino acid alteration induced by the *mef10-1* EMS mutation severely affects MEF10 function at least at the identified target editing site at *nad2*-842 with the caveats of the other more complex explanations discussed above. To evaluate the functional importance of this amino acid position, we aligned the respective E-domain sequences of the most similar proteins in different other plant species (Fig. 4a) and of other RNA editing PPR proteins in *A. thaliana* (Fig. 4b). These alignments show that the alanine amino acid is generally conserved in the E domains of these PPR proteins. The few exceptions encode as alternative the amino acid glycine, but never the threonine of the *mef10-1* mutant. The amino acid threonine is larger than the other two amino acids, which may influence interaction with the target RNA. Alternatively, interaction with other editing proteins such as MORF8 (Takenaka et al. 2012) may be disturbed. In any case, this *mef10-1* mutation confirms the importance of the E domain for the competence of the E- and E-DYW PPR proteins (Chateigner-Boutin and Small 2010). Mutants like these will be helpful in the analysis of the actual function of the E domain.

The RNA editing event at the *nad2*-842 target site may not be essential

As to be expected from the generally slow rate of evolution of mitochondrial genes and their encoded proteins in land plants, the amino acid identity affected by the MEF10 target editing site *nad2*-842 is evolutionarily well conserved in the NAD2 proteins in plants (Fig. 3, center alignment). The after editing encoded amino acid phenylalanine is conserved in all other plants. However, the alignment of amino acids surrounding the residue affected by this editing site reveals several amino acid alterations nearby, especially in the monocot-dicot comparison, and in the comparison with non-plant proteins, this region is not well conserved at all. Therefore, the NAD2 protein may in fact tolerate the incorporation of the serine encoded by the unedited mRNA in the *mef10-1* mutant, which would explain the absence of any gross phenotypic effect attributable to the *mef10-1* mutation. This conclusion is also supported by the severe effects observed in mutants of genes coding for proteins such as OTP43 which is required for splicing of *nad1* transcripts (de Longevialle et al. 2007). These mutant plants, in which no complex I activity is observed, require supplemented media to germinate and show a dramatically altered growth habitus. Analogous severe growth defects are seen in a mutant of the protein ABO5 which is involved in splicing of *nad2* transcripts (Liu et al. 2010). The absence of such dramatic effects in the *mef10-1* mutant thus suggests that the overall activity of complex I is little affected by the amino acid alteration of this RNA editing event.

MEF10 interacts with MORF8

In the yeast two hybrid analysis MEF10 interacts with MORF8 but not with other mitochondrially targeted MORFs (Fig. 5). Weak interaction of MEF10 with the plastid located MORF2 and MORF9 extends previous observations that these plastid proteins have a rather general affinity to PPR proteins which however has no functional consequence with mitochondrially located MEF

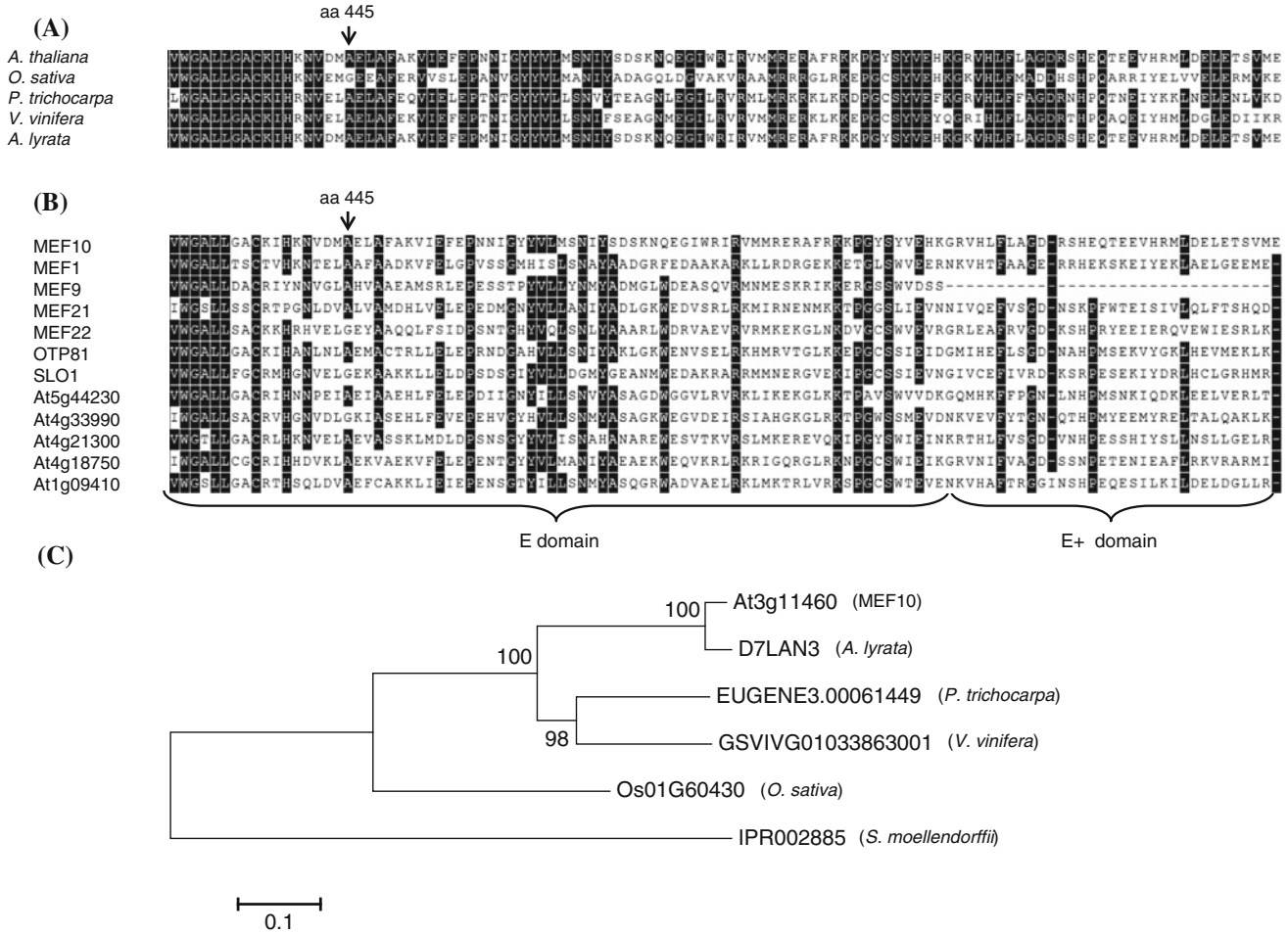


Fig. 4 Conservation of the E-domain and of proteins similar to MEF10 in other plant species. **a** Comparison of the MEF10 protein sequence around the *mef10-1* mutation with the most similar PPR proteins from other plant species, potential orthologs, shows this region to be highly conserved and thus to be potentially functionally important. Identical amino acids present in more than 70 % of the sequences aligned are shown in inverse *shading* in parts **a** and **b**. The mutation of alanine to threonine changes the biochemical properties of amino acid 445. **b** This amino acid alignment compares the E- and E+-domain sequences of several mitochondrial editing factors (MEFs), chloroplast RNA editing factors and other DYW- and E-PPR proteins from *Arabidopsis thaliana*. The *alignment* shows that at the site of the *mef10-1* mutation small, hydrophobic amino acids, alanine (A) and glycine (G) are conserved, which may explain why substitution by the polar and slightly larger amino acid threonine abolishes the RNA editing function. **c** Neighbour joining tree of proteins most similar to MEF10 in other plant species. The MEF10-like protein in *Arabidopsis lyrata* (D7LAN3) is as to be expected most similar to the MEF10 protein in *Arabidopsis thaliana* (At3g11460). The respective proteins from other plant species are less conserved and need to be evaluated to clarify whether they are true orthologs. Compared are proteins from *Populus trichocarpa* (poplar; EUGENE3.00061449), *Vitis vinifera* (vine; GSVIVG0 1033863001 annotated as most similar in the FLAG database which is XP_002276416 annotated as MEF10 like protein in the NCBI database) and *Oryza sativa* (rice; OS01G60430). The vine protein is aligned through the intron annotated in the databases. The PPR protein IPR002885 from *Selaginella moellendorffii* served as an outgroup in this alignment. Bootstrap values as given by the mega5.05 programme are shown (www.megasoftware.net; Tamura et al. 2011)

proteins as they are physically separated in the plant cell (Takenaka et al. 2012).

A recent analysis of the editing sites affected by disturbed expression of MORF8 also supports a functional connection between MEF10 and MORF8 (MORF8 is also termed RIP1; Bentolila et al. 2012). This investigation shows that editing at the *nad2*-842 target site of MEF10 is disturbed in a mutant of MORF8, indicating that both MORF8 and MEF10 are involved in editing of this target site. Analogous common target sites for MORFs and MEFs have been identified for MORF1 and MEF21 (site *cox3*-257), MORF1 and MEF19 (*ccmB*-566), MORF3 and SLO2 (*mttB*-144, *mttB*-145, *nad7*-739 and *nad4L*-110) respectively (Takenaka et al. 2010, 2012; Zhu et al. 2012). The control yeast two hybrid analysis of MORF proteins interacting with MEF21 supports the specific interaction observed, since MORF1 and MEF21 interact, but not MORF8 and MEF21 (Fig. 5). MEF21 also interacts with the chloroplast-located MORF2 and MORF9,

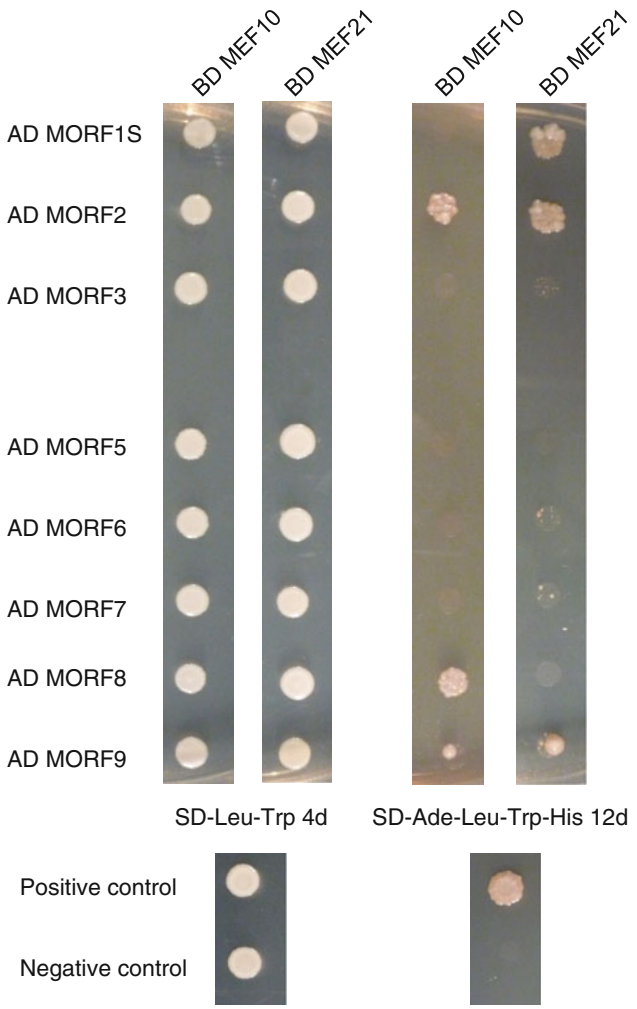


Fig. 5 Yeast two hybrid analysis of MEF10 interactions with MORF proteins. Yeast cells grown on selective media reveal that MEF10 can interact with MORF8 and weakly with the two plastid located MORF2 and MORF9 proteins but not with any of the other mitochondrial MORF proteins probed. The open reading frame for MEF10 was cloned in frame with the binding domain (BD in vector pGBKT7) and was co-transformed into yeast cells with the various MORF proteins in frame with the activation domain (AD in vector pGADT7). For MORF4, no clones were obtained. MORF1S contains a shortened open reading frame of MORF1. Growth on control medium for 4 days (SD-Leu-Trp 4d; *left part*) shows that equal numbers of cells were dropped onto the plates. On selective medium after 12 days of incubation (SD-Ade-Leu-Trp-His 12d; *right part*) an interaction with MORF8 is seen. Interaction is also detected as growing colonies with the plastid located MORF2 and MORF9 proteins after 12 days. As a control for specificity, interaction of the different MORF proteins was tested with MEF21 (Takenaka et al. 2010). This PPR protein interacts with MORF1 (and weakly with the two plastid located MORF2 anmd MORF9 proteins), but not with MORF8. Both MEF-MORF interacting pairs reflect the respective in vivo targets, MEF10 and MORF8 target the same editing events, MEF21 and MORF1 are required at their respective identical target editing sites. Positive control is a test of vectors pGBKT7 p35 with pGADT7 T and the negative control is pGBKT7 Lam with pGADT7 T

also confirming the rather sticky surface of the plastid MORF proteins attracting many mitochondrial PPR proteins. These observations showing that MEF10 and MORF8 (RIP1) can interact and that both are required for successful editing at the *nad2*-842 target site, support the model that MEF and MORF proteins need to interact for full editing at some sites in plant organelles.

Acknowledgments We thank Dagmar Pruchner, Bianca Wolf and Angelika Müller for excellent experimental help. This work was supported by grants from the Deutsche Forschungsgemeinschaft to Mizuki Takenaka and Axel Brennicke. Mizuki Takenaka is a Heisenberg fellow.

Conflict of interest The authors declare that they have no conflict of interest.

References

Bentolila S, Heller WP, Sun T, Babina AM, Friso G, van Wijk KJ, Hanson MR (2012) RIP1, a member of an *Arabidopsis* protein family, interacts with the protein RARE1 and broadly affects RNA editing. Proc Natl Acad Sci USA 109:E1453–E1461

Bock R, Koop HU (1997) Extraplastidic site-specific factors mediate RNA editing in chloroplasts. EMBO J 16:3282–3288

Chateigner-Boutin AL, Small I (2010) Plant RNA editing. RNA Biol 7:213–219

Chateigner-Boutin A-L et al (2008) CLB19, a pentatricopeptide repeat protein required for editing of *rpoA* and *clpP* chloroplast transcripts. Plant J 56:590–602

Chaudhuri S, Maliga P (1996) Sequences directing C to U editing of the plastid psbL mRNA are located within a 22 nucleotide segment spanning the editing site. EMBO J 15:5958–5964

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol 133:462–469

de Longevialle AF, Meyer EH, Andres C, Taylor NL, Lurin C, Millar AH, Small ID (2007) The pentatricopeptide repeat gene OTP43 is required for trans-splicing of the mitochondrial nad1 intron 1 in *Arabidopsis thaliana*. Plant Cell 19: 3256–3265

Farré J-C, Leon G, Jordana X, Araya A (2001) *Cis* recognition elements in plant mitochondrion RNA editing. Mol Cell Biol 21:6731–6737

Forner J, Binder S (2007) The red fluorescent protein eqFP611: application in subcellular localization studies in higher plants. BMC Plant Biol 7:28

Fujii S, Small I (2011) The evolution of RNA editing and pentatricopeptide repeat genes. New Phytol 191:37–47

Fujii S, Bond CS, Small I (2010) Selection patterns on restorer-like genes reveal a conflict between nuclear and mitochondrial genomes throughout angiosperm evolution. Proc Natl Acad Sci USA 108:1723–1728

Kempken F, Bolle N, Bruhs A (2009) Higher plant in organello systems as a model for RNA editing. Endocytobiosis Cell Res 19:1–10

Kim S-R, Yang J-I, Moon S, Ryu C-H, An K, Yim J, An G (2009) Rice OGR1 encodes a pentatricopeptide repeat-DYW protein and is essential for RNA editing in mitochondria. Plant J 59: 738–749

Kobayashi K, Kawabata M, Hisano K, Kazama T, Matsuoka K, Sugita M, Nakamura T (2012) Identification and characterization

of the RNA binding surface of the pentatricopeptide repeat protein. Nucl Acids Res 40:2712–2723

Kotera E, Tasaka M, Shikanai T (2005) A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature 433:326–330

Liu Y, He J, Chen Z, Ren X, Hong X, Gong Z (2010) *ABA overly sensitive 5* (*ABO5*), encoding a pentatricopeptide repeat protein required for *cis*-splicing of mitochondrial *nad2* intron 3, is involved in the abscisic acid response in Arabidopsis. Plant J 63:749–765

Lurin C et al (2004) Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell 16:2089–2103

Miyamoto T, Obokata J, Sugiura M (2002) Recognition of RNA editing sites is directed by unique proteins in chloroplasts: biochemical identification of cis-acting elements and trans-acting factors involved in RNA editing in tobacco and pea chloroplasts. Mol Cell Biol 22:6726–6734

Okuda K, Myouga R, Motohashi K, Shinozaki K, Shikanai T (2007) Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. Proc Natl Acad Sci USA 104:8178–8183

Okuda K, Chateigner-Boutin A-L, Nakamura T, Delannoy E, Sugita M, Myouga F, Motohashi R, Shinozaki K, Small I, Shikanai T (2009) Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in *Arabidopsis* chloroplasts. Plant Cell 21:146–156

Picardi E, Regina T, Verbitskiy D, Brennicke A, Quagliariello C (2011) REDIdb: an upgraded bioinformatics resource for organellar RNA editing sites. Mitochondrion 11:360–365

Schmitz-Linneweber C, Small I (2008) Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends Plant Sci 13:663–670

Small ID, Peeters N (2000) The PPR motif—a TPR-related motif prevalent in plant organellar proteins. Trends Biochem Sci 25:46–47

Takenaka M (2009) MEF9, an E-subclass pentatricopeptide repeat protein, is required for an RNA editing event in the *nad7* transcript in mitochondria of *Arabidopsis*. Plant Physiol 152:939–947

Takenaka M, Brennicke A (2007) RNA editing in plant mitochondria: assays and biochemical approaches. Meth Enzymol 424:439–458

Takenaka M, Brennicke A (2009) Multiplex single base extension typing to identify nuclear genes required for RNA editing in plant organelles. Nucleic Acids Res 37:e13

Takenaka M, Brennicke A (2012) Using multiplex single base extension typing to screen for mutants defective in RNA editing. Nat Protoc 7:1931–1945

Takenaka M, Verbitskiy D, Zehrmann A, Brennicke A (2010) Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. J Biol Chem 285:27122–27129

Takenaka M, Zehrmann A, Härtel B, Kugelmann M, Verbitskiy D, Brennicke A (2012) MORF family proteins are required for RNA editing in mitochondria and plastids of plants. Proc Natl Acad Sci USA 109:5104–5109

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

van der Merwe JA, Takenaka M, Neuwirt J, Verbitskiy D, Brennicke A (2006) RNA editing sites in plant mitochondria can share *cis*-elements. FEBS Lett 580:268–272

Verbitskiy D, Zehrmann A, van der Merwe JA, Brennicke A, Takenaka M (2010) The PPR protein encoded by the lovastatin insensitive 1 gene is involved in RNA editing at three sites in mitochondria of *Arabidopsis thaliana*. Plant J 61:446–455

Verbitskiy D, Zehrmann A, Härtel B, Brennicke A, Takenaka M (2011) The DYW-E-PPR protein MEF14 is required for RNA editing at site matR-1895 in mitochondria of *Arabidopsis thaliana*. FEBS Lett 585:700–704

Williams-Carrier R, Kroeger T, Barkan A (2008) Sequence-specific binding of a chloroplast pentatricopeptide repeat protein to its native group II intron ligand. RNA 14:1930–1941

Yu Q-B, Jiang Y, Chong K, Yang Z-N (2009) AtECB2, a pentatricopeptide repeat protein, is required for chloroplast transcript accD RNA editing and early chloroplast biogenesis in Arabidopsis thaliana. Plant J 59:1011–1023

Zehrmann A, van der Merwe JA, Verbitskiy D, Brennicke A, Takenaka M (2008) Seven large variations in the extent of RNA editing in plant mitochondria between three ecotypes of *Arabidopsis thaliana*. Mitochondrion 8:319–327

Zehrmann A, van der Merwe JA, Verbitskiy D, Brennicke A, Takenaka M (2009) A DYW domain containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. Plant Cell 21:558–567

Zhou W, Cheng Y, Yap A, Chateigner-Boutin A-L, Delannoy E, Hammani K, Small I, Huang J (2009) The Arabidopsis gene *YS1* encoding a DYW protein is required for editing of *rpoB* transcripts and the rapid development of chloroplasts during early growth. Plant J 58:82–96

Zhu Q, Dugardeyn J, Zhang C, Takenaka M, Kühn K, Craddock C, Smalle J, Karampelias M, Denecke J, Peters J, Gerats T, Brennicke A, Eastmond P, Meyer EH, Van Der Straeten D (2012) SLO2, a mitochondrial PPR protein affecting several RNA editing sites, is required for energy metabolism. Plant J 71:836–849

Copyright Notice:
Reprinted from:
Zehrmann, A., Verbitskiy, D., Härtel, B., Brehme, N., Takenaka, M., Brennicke, A.
„RNA-Editing: Das Gen ist nicht alles.“
Naturwissenschaftliche Rundschau | 65. Jahrgang, Heft 6, 2012
© 2013 Wissenschaftliche Verlagsgesellschaft Stuttgart, with kind permission

Anja Zehrmann, Daniil Verbitskiy, Barbara Härtel, Nadja Brehme, Mizuki Takenaka, Axel Brennicke, Ulm

RNA-Editing: Das Gen ist nicht alles

RNA-Editing bezeichnet gezielte Veränderungen im Informationsgehalt von RNA-Molekülen. Dadurch sagt die Basensequenz eines Gens in der DNA die codierte Information nicht mehr direkt vorher. Im Laufe der Evolution entstand eine Reihe verschiedener Strategien in vielen Organismen, um einzelne Bausteine in der RNA gezielt und kontrolliert zu verändern.

Ein Gen – eine mRNA – ein Protein" lautete eine der Maximen der Molekularbiologie. Die erste Erschütterung brachte die Entdeckung des alternativen Spleißens der von den Genen abgelesenen, primären Transkripte (prä-mRNA): Durch einen Reifungsprozess der prä-mRNA, das Spleißen, werden die nicht-codierenden Abschnitte (Introns) der Gensequenz herausgeschnitten, die zwischen den für Teile der Proteine codierenden Abschnitten (Exons) liegen. Bei manchen Genen könne die Exons beim Spleißen unterschiedlich kombiniert werden, z. B. durch Belassen von Introns oder Entfernen von Exons. Dieses alternative Spleißen bringt dann von einem Gen, also einem DNA-Abschnitt, mehrere verschiedene reife mRNAs und davon übersetzt verschiedene Proteine zur Produktion.

Vor etwa 30 Jahren kam das RNA-Editing hinzu. Dieses verändert einzelne Nucleotide (Konversion), fügt manchmal welche hinzu (Insertion) oder nimmt andere heraus (Deletion). Manchmal sieht die reife mRNA ganz anders aus als nach der DNA-Sequenz zu erwarten, von der die prä-mRNA abgelesen wurde. Von ein und derselben prä-mRNA können durch RNA-Editing auch mehrere verschiedene reife mRNAs und folglich unterschiedliche Proteine gebildet werden. Damit wächst die Zahl der codierten Proteine weit über die Zahl der Gene in einem DNA-Molekül hinaus (Abb. 1). Viele chemische Veränderungen modifizieren ein Nucleotid in der RNA, führen aber nicht zu einem Wechsel der Nucleotid-Identität. Diese Modifizierungen, z. B. Methylierungen in den tRNAs (Abb. 2) oder den ribosomalen RNAs, werden daher meist nicht dem RNA-Editing zugerechnet, obwohl viele für die richtige Funktion der jeweiligen RNA notwendig sind (s. auch S. ■■■).

RNA-Editing wurde bereits in einer ganzen Reihe von Organismen gefunden, wahrscheinlich existieren noch weit mehr solcher Prozesse, die bisher bloß noch nicht erkannt wurden. Die bekannten Beispiele werden durch sehr unterschiedliche biochemische Prozesse ausgeführt, was belegt, dass die einzelnen Wege des RNA-Editing unabhängig voneinander parallel in der Evolution entstanden sind.

Schauen wir uns die verschiedenen Prozesse in den weit auseinander liegenden Evolutionslinien entsprechend ihrer Biochemie geordnet an:

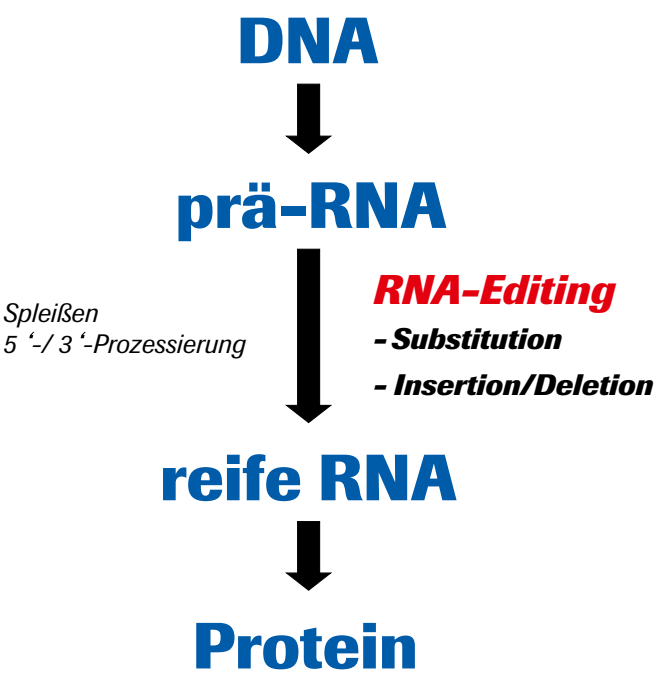


Abb. 1. Reifung der RNA. Die von der DNA abgelesene primäre oder prä-RNA durchläuft verschiedene Reifungsprozesse, bei denen zum Beispiel nicht-codierende Introns herausgeschnitten und die beiden Enden (5' und 3') auf ihre endgültigen Längen getrimmt werden. RNA-Editing verändert direkt die codierende Sequenz, entweder durch chemische Veränderung einzelner Nucleotide oder durch Einfügen und Herausschneiden ganzer Nucleotide nach exakten Vorgaben.

Veränderung einzelner Nucleotide in der RNA

Transfer-RNAs (Alle Lebewesen)

In allen transfer-RNAs (tRNAs), die bei der Proteinsynthese die Aminosäuren entsprechend der mRNA-Sequenz am Ribosom anordnen, werden nach der Transkription einzelne Nucleotide chemisch verändert (Abb. 2). Diese Modifizierungen können das Entfernen von Aminogruppen sein, dann entsteht aus Cytosin (C) ein Uracil (U) oder aus Adenin (A) ein Inosin (I). Methylgruppen können angefügt werden und vieles mehr [1]. Oft wird die tRNA erst mit der Abwandlung des Nucleotids funktionsfähig. Meist wird das zu verändernde Nucleotid durch seine Position in der dreidimensionalen-Struktur der prä-tRNA und der reifen tRNA vorgegeben. Alle tRNAs sind relativ klein und falten sich zu ähnlichen Strukturen, die manchmal erst durch die Veränderung eines Nucleotids möglich werden.

Apolipoprotein B (Säugetiere): C→U

Die Debatte über die Risiken erhöhter Blutfettwerte hat die Lipoproteine bekannt gemacht. Ihre Fähigkeit, Lipide für den Transport im Blut in Lösung zu halten, verdanken sie ihrem Gehalt an spezifischen Apolipoproteinen. Darunter ist Apolipoprotein B48 (kurz: Apo B48) typisch für die im Darm synthetisierten Chylomikronen, die Lipide aus der Nahrung aufnehmen, während sich Apo B100 vor allem in den von der Leber synthetisierten Lipoproteinen geringer Dichte (LDL) findet, die bei zivilisationsbedingt erhöhten Blutfettwerten mit Gefäßerkrankungen in Zusammenhang gebracht werden. Bemerkenswerterweise ist die Aminosäuresequenz von Apo B48 identisch mit dem aminoterminalen Teil des Apo B100, und für beide Proteine gibt es nur ein Gen. Die Nucleotid-Sequenzen der beiden mRNAs zeigen, wie diese beiden Proteine von einem Gen codiert werden: Für Apo B48 wird das Nucleotid C an Position 666 der prä-mRNA zu U editiert (Abb. 3). Dadurch entsteht aus einem CAA-Codon für die Aminosäure Glutamin ein UAA-Codon, also ein Stoppzeichen für die Translation – das Protein ist hier zu Ende [2].

Dies ist nur auf den ersten Blick sparsam: Für das RNA-Editing sind mindestens drei zusätzliche Gene notwendig, die für drei spezielle Proteine codieren: eines, das die Sequenz in der prä-mRNA an der editierten Stelle erkennt, weiterhin ein Enzym, das die Desaminierung von C zu U katalysiert, und ein drittes Protein, das beide miteinander verbindet. Ein nahe verwandtes Enzym desaminiert Cytosine in RNA-Viren, darunter auch die HIV-RNA, und dient so der Verteidigung gegen solche Retroviren [3].

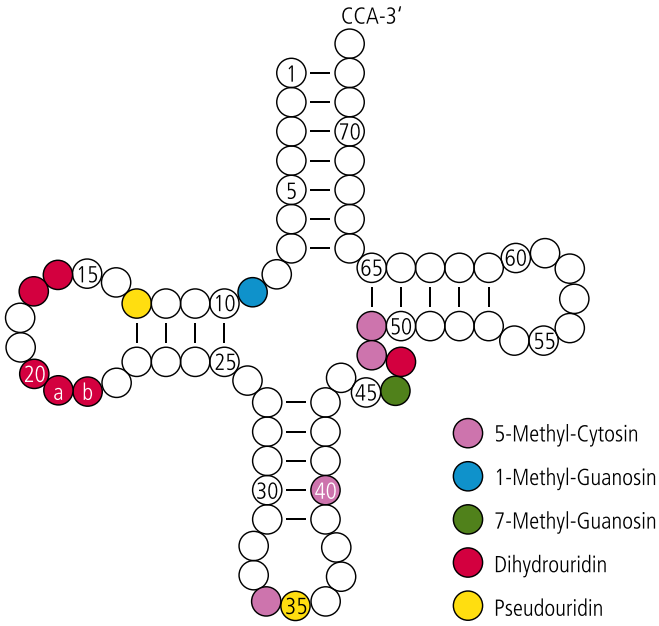


Abb. 2. Modifikation von Nucleotiden in transfer-RNAs (tRNAs). In tRNAs, die den Nucleotid-Triplett-Code der mRNA in Aminosäuren übersetzen, findet bei allen Lebewesen eine Vielzahl chemischer Abwandlungen statt. Einige davon verändern Seitengruppen, fügen Methylgruppen an oder etablieren Basenpaarungen an anderen Positionen. Viele dieser Änderungen sind für die räumliche Struktur (Tertiärstruktur) der tRNA wichtig. Führen diese Modifikationen zu Änderungen der Identität eines Nucleotids und damit auch seiner Paarungseigenschaften, sind sie dem RNA-Editing zuzuordnen. Besonders gravierend sind solche Abwandlungen an den Positionen 34–36, dem Anticodon, wo sie dazu führen können, dass von der mRNA ein anderes Triplett abgelesen wird. In einige Organismen werden die Positionen 1, 2 und manchmal sogar 3 nicht in der DNA codiert, sondern erst in der tRNA passend zu den Nucleotiden 70–73 eingefügt. Hier sind nur die gängigsten Modifikationen gezeigt. Verändert nach [1]

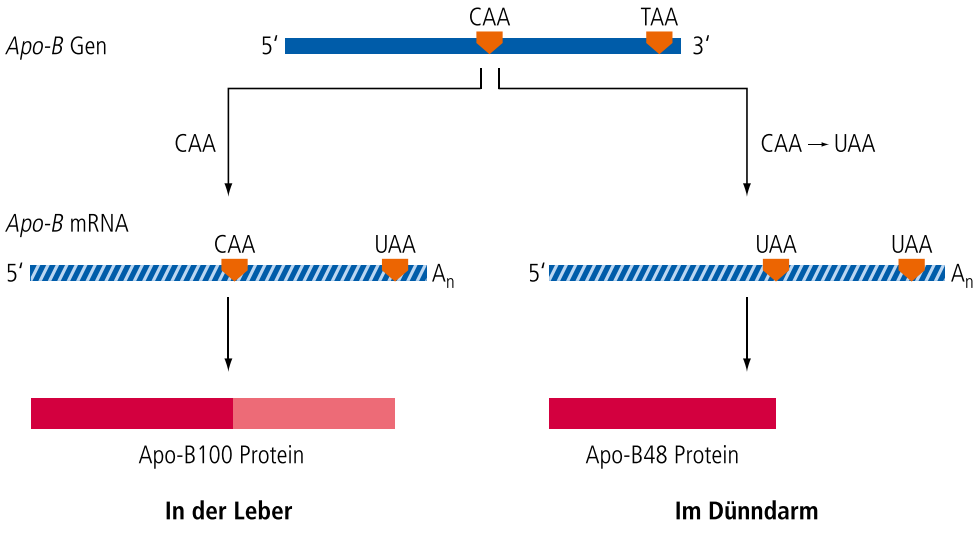


Abb. 3. RNA-Editing liefert durch Einführung eines Stoppcodons zwei Proteinvarianten unterschiedlicher Länge von einem Gen. Die beiden Apolipoproteine Apo B48 und Apo B100 werden von einem Gen codiert. Das 100 kDa große ApoB100 wird bei Menschen von der nicht editierten mRNA in der Leber synthetisiert. Im Darm entsteht durch die Desaminierung eines Cytosins zu Uridin aus einem CAA- ein UAA-Codon, ein Stoppsignal für die Translation. Dadurch wird ein verkürztes Protein synthetisiert, das 48 kDa große Apo B48. Verändert nach [2]

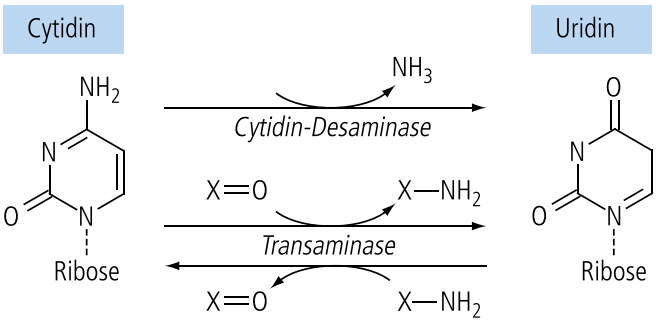


Abb. 4. Die Desaminierung von Cytosin zu Uridin ist eine häufige chemische Veränderung bei verschiedenen RNA-Editing-Prozessen. Die Aminogruppe kann entweder direkt durch Desaminierung abgespalten oder durch Transaminierung auf ein anderes Molekül übertragen werden.

Plastiden und Mitochondrien (Pflanzen): C→U

Das Editing eines C-Nucleotids zu U findet auch in allen Landpflanzen statt. Die Transkripte des großen Genoms im pflanzlichen Zellkern scheinen allerdings nicht durch RNA-Editing abgeändert zu werden. Dagegen werden in den mRNAs der beiden kleinen Genome in den Plastiden und Mitochondrien von Pflanzenzellen 30–40 bzw. 400–2.000 solcher Nucleotide umgewandelt [4]. Ob die biochemische Reaktion wie bei dem ApoB Editing eine Desaminierung ist oder eine Transaminierung, ist noch unklar (Abb. 4). Entsprechend der großen Zahl der editierten Nucleotide werden viele Proteine für die spezifische Erkennung benötigt, mindestens 200 Gene für Varianten einer großen Proteinfamilie mit ähnlichen Eigenschaften sind nur dafür abgestellt. Diese Proteine werden als PPR-Proteine bezeichnet, sie bestehen aus 5–30mal wiederholten Motiven von etwa 35 Aminosäuren (Pentatrico-Peptide-Repeats = PPR), die an unterschiedliche Nucleotide in der RNA binden und so die Spezifität eines RNA-Editing Ereignisses vermitteln. Sie sind allerdings grundverschieden von den am Apolipoprotein-mRNA-Editing beteiligten Faktoren und finden sich in dieser Zahl nur bei Pflanzen. Eine weitere, kleinere Familie von Proteinen ist ebenfalls notwendig für dieses RNA-Editing in beiden Organellen. Deren genaue Funktion ist noch nicht klar, wahrscheinlich fungieren sie als Bindeglieder zwischen den mRNA-sequenzspezifischen Proteinen und dem eigentlichen Enzym, das als Des- oder Transaminase aktiv sein muss [5].

Anders als bei dem Editing der Apolipoprotein-mRNA entstehen in den beiden Organellen in Pflanzen nicht mehrere Proteinvarianten von einem Gen, sondern erst nach dem vollständigen Editing der jeweiligen mRNA codiert diese für das funktionsfähige Protein (vgl. Abb. 5). Nicht oder nicht vollständig editierte mRNAs werden aussortiert, sofern mRNA-Moleküle noch vor dem Abbau in Protein übersetzt wurden, wird dieses verworfen. In den funktionalen Atmungsketten der Mitochondrien finden sich nur Proteine der vollständig editierten mRNAs. Diese synthetisierten Proteine zeigen deutlich größere Ähnlichkeit mit den entsprechenden Molekülen in Tieren und Pilzen als die zugehörigen Gene – ein Hinweis darauf, dass einige für die Funktion dieser Proteine wichtige Aminosäuren erst von der editierten mRNA codiert werden. Diese Schlussfolgerung bestätigen mutante Pflanzen, bei denen einzelne RNA-Editing-Ereignisse durch Mutation im entsprechenden PPR-Gen ausgefallen sind und die manchmal krank oder sogar nicht lebensfähig sind.

Ionenkanäle in Nervenzellen (Tiere): A→I

Auch Adenosin kann desaminiert werden, dabei entsteht Inosin [6]. Letzteres wird normalerweise nicht in DNA oder RNA benutzt, kann aber vom Ribosom bei der Translation der mRNA erkannt und gelesen werden. Allerdings fungiert Inosin im genetischen Code nicht als Adenin, sondern als Guanin, damit ändert sich der Informationsgehalt der RNA. Das A zu I-Editing wird von einer kleinen Familie von Enzymen katalysiert, den Adenosin-Desaminasen. Einige dieser Enzyme verändern die Sequenz in der DNA, zum Beispiel die aktivierungsinduzierte Desaminase AID bei der somatischen Hypermutation der Gene für die variablen Ketten der Immunglobuline. Andere Adenosin-Desaminasen editieren ganz gezielt ausgewählte mRNAs in spezifischen Zelltypen. Lebenswichtig ist das RNA-Editing von 10–50 Adenosinen zu Inosinen in den mRNAs für Kalium-Kanäle in einigen Neuronen unseres Gehirns.

Die spezifische Erkennung der zu verändernden Nucleotide wird durch die Sekundärstruktur der prä-mRNA vorgegeben: in dieser legen sich Intron- und Exon-Sequenzen durch Basenpaarung aneinander, das Muster der einzel- und doppelsträngigen Bereiche bestimmt die Positionen, die von der Adenosin-Desaminase erkannt und verändert werden sollen. Ein A zu I-RNA-Editing-Ereignis überführt ein Codon für Glutamin in ein Codon für Arginin und verändert so die Durchlässigkeit des Ionenkanals für K^+-Ionen. Fehlt diese Änderung bei Mäusen, neigen sie ab Geburt zu epileptischen Anfällen und sterben nach wenigen Tagen. Bei dem A zu I-RNA-Editing werden aber nicht nur Ja-Nein-Entscheidungen getroffen, sondern der Grad des Editing einer bestimmten Nucleotid-Position variiert von Zelle zu Zelle. Die Vielzahl der möglichen Kombinationen editierter und nicht editierter Nucleotide in der mRNA ergibt eine ganze Palette von K^+-Kanälen mit leicht

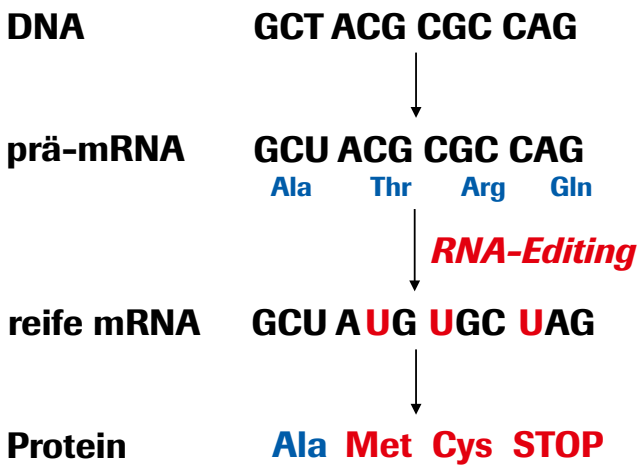


Abb. 5. In den Mitochondrien und Plastiden von Pflanzen werden durch Desaminierung von bestimmten Cytosinen zu Uridinen eine Vielzahl von mRNAs verändert. In einzelnen solcher RNAs kann durch Editing ein neues Start-Codon für die Translation entstehen, die codierte Aminosäure geändert oder ein neues Stopp-Codon etabliert werden.

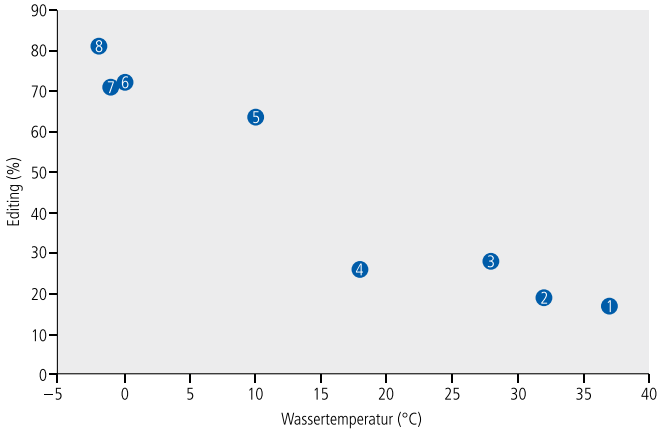


Abb. 6. Die Leitfähigkeit eines Kalium-Kanals in den Nervenzellen von Octopus ist abhängig von der Temperatur. Die chemische Desaminierung von Adenosin zu Inosin durch RNA-Editing an bestimmten Nucleotiden in der mRNA verändert das jeweilige Codon für eine Aminosäure und mit dieser die Leitfähigkeit des Kanals. Die Reaktionszeit der Zelle ist abhängig von dem Mischungsverhältnis der Proteinvarianten. Unterschiedliche Prozentsätze der editierten zur nicht editierten RNA in verschiedenen Spezies von Kraken, hier gezeigt am Beispiel des Editing des Codons für Isoleucin 321 zu Valin, ermöglichen ähnliche Reaktionszeiten der Nervenzellen bei tropischen, subtropischen und arktischen Temperaturen. Verändert nach [7]

unterschiedlichen Aminosäuresequenzen, die jeder Zelle ein anderes Muster an Leitfähigkeit und Signalweitergabe vermitteln. Um dieses bis ins Detail zu verstehen, müssten von jeder Nervenzelle die Gesamtheit der mRNAs für K^+-Kanäle untersucht und bezüglich der Leitfähigkeit der Kanäle und ihrer Verteilung in den Dendriten verglichen werden. Aber auch eine gröbere Analyse einer Gruppe von Zellen verrät eine Menge über die feine Regulation des A zu I-RNA-Editing und ihre physiologische Wirkung.

Eine kürzlich veröffentlichte Studie der K^+-Kanäle in den Ganglien von Kraken *(Octopus)* zeigte, dass deren mRNAs bei tropischen Spezies an bestimmten Nucleotiden viel weniger editiert sind als bei Spezies aus arktischen oder antarktischen Gewässern (Abb. 6) [7, 8]. Der Sinn dieses Unterschieds erschließt sich mit der Analyse der Temperaturabhängigkeit der Leitfähigkeit der K^+-Kanäle. Diejenigen Kanäle, die von editierten RNAs synthetisiert werden, zeigen sich bei Kälte aktiver als die von nicht editierten RNAs spezifizierten Kanäle. Bei den Kraken bestehen artspezifische Unterschiede im RNA-Editing, die sie für die Anpassung an verschiedene Umgebungstemperaturen nutzen. Dabei haben sich nicht nur zwei abgegrenzte Kalt-/Warm-Formen mit RNA-Editing Ja oder Nein etabliert, sondern graduelle Abstufungen im Verhältnis von editierter mRNA zu nicht editierter mRNA entsprechend dem globalen Temperaturgradienten, in dem die Kraken jeweils leben (Tab.). Das Mischungsverhältnis der unterschiedlichen Kanäle bestimmt dabei die Reaktionszeit der gesamten Zelle.

Klassisch führen wir Evolution (und auf lange Zeiträume scheinbare Anpassung) auf Mutation und Selektion genomischer DNA-Sequenzen zurück. Der Weg über die Steuerung durch RNA-Editing wurde bei diesen K^+-Kanälen in der Evolution offensichtlich anstelle einer genomischen Mutation etabliert: das graduell abgestufte RNA-Editing ist bei allen untersuchten, geographisch deutlich getrennten, arktischen und antarktischen Kraken Grundlage der Temperaturadaptation.

Austausch verschiedener Nucleotide (Schleimpilze, Myxomyceten-Mitochondrien)

Schleimpilze sind wenig bekannt und beliebt. Intensiv untersucht wurde besonders die weltweit verbreitete Gattung *Physarum*, deren Vertreter auf vermoderndem Holz leben. In den mRNAs ihrer Mitochondrien werden mit einer Mischung von Nucleotid-Desaminierungen, -Einbau und -Austauschen die Basensequenzen und damit die für Proteine codierenden Informationen stark verändert [9]. Neben dem C zu U-Austausch durch Desaminierung findet sich bei *Physarum* auch das Editing von Purinbasen zu Pyrimidinbasen (A→C oder G→U). Beide Veränderungen können nicht durch chemische Veränderung einer Base innerhalb der mRNA erklärt werden, hier wird jeweils ein Nucleotid ausgebaut und ein anderes eingesetzt. Wahrscheinlich über den gleichen Mechanismus werden manchmal auch zwei oder drei Nucleotide eingefügt oder herausgenommen. Diese kleinen Schleimpilze nutzen dabei mindestens drei Wege und enzymatische Prozesse: Einbau oder Austausch von Nucleotiden werden während der Transkription von der DNA zur mRNA vorgenommen, die chemische Abänderung von

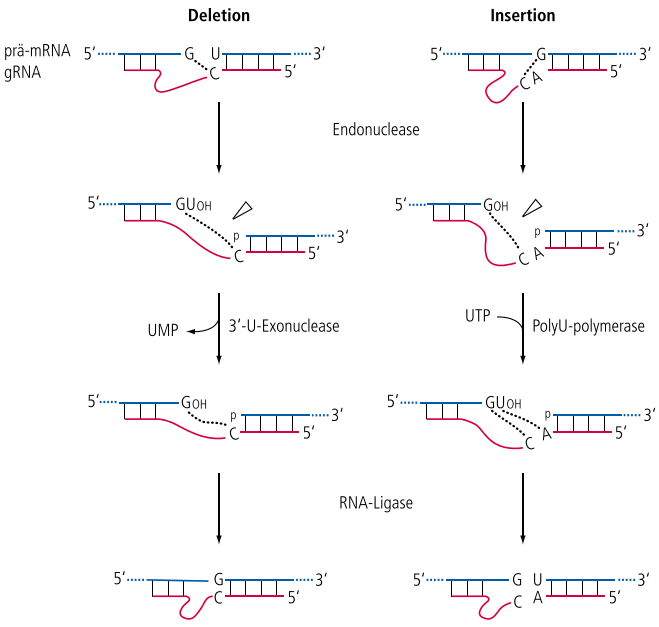


Abb. 7. Deletion und Insertion von Nucleotiden bei Trypanosomen. In den Mitochondrien der Erreger der Schlafkrankheit werden an einigen Stellen der prä-RNAs überzählige Uridine (U) herausgeschnitten, an anderen Stellen fehlende Uridine eingebaut. Die Information über die Zahl der endgültigen Uridine in der reifen mRNA wird durch kleine guide-RNA-Moleküle (gRNA) geliefert, die sich mit einer passenden Ankersequenz an die prä-RNA anlegen. Die Zahl der Adenosine in der gRNA gibt die endgültige Zahl der Uridine in der mRNA vor. Für das Editing wird die prä-RNA durch eine Endonuclease aufgeschnitten, anschließend schneidet eine Exonuclease überzählige Uridine ab, eine PolyU-Polymerase heftet fehlende an. Im letzten Schritt werden die beiden Enden der prä-RNA durch eine Ligase wieder zu einem RNA-Molekül verbunden. Verändert nach [11]

Nucleotiden wird in der RNA durchgeführt, darunter die C zu U-Änderung (wahrscheinlich durch eine Cytosin-Desaminase) und zum anderen die enzymatische Korrektur von Fehlpaarungen in tRNAs.

Ausbau und Einbau nicht-codierter Nucleotide

Einfügen zusätzlicher Nucleotide bei der Transkription (Viren)

Viele Viren codieren eine eigene RNA-Polymerase, die bei der Transkription der Virus-DNA an einigen Stellen hängen bleibt und sogar zurück rutscht. Dabei werden ein oder mehrere gleiche Nucleotide zusätzlich eingebaut [1]. Diese verändern die Stabilität der RNA oder bewirken, dass mehrere unterschiedliche Proteine von einem DNA-Stück hergestellt werden können. Die Spezifität wird durch bestimmte Nucleotidfolgen festgelegt, zum Beispiel wird die RNA-Polymerase an mehreren aufeinander folgenden Guanosinen in der DNA-Matrize gebremst. Dieses RNA-Editing geschieht während der Transkription, die meisten anderen Arten von RNA-Editing finden erst danach statt.

Entfernung überflüssiger und Einbau neuer Nucleotide (Trypanosomen, Kinetoplasten)

Erstmalig entdeckt und benannt wurde RNA-Editing in mRNAs bei der Untersuchung der Genome und ihrer Transkripte in den Kinetoplasten, den spezialisierten Mitochondrien, der einzelligen Parasiten *Trypanosoma brucei* und *Leishmania tarentolae,* den Erregern der Schlafkrankheit bei Kühen und auch Menschen [10, 11]. Einige Gene für lebenswichtige Proteine der Atmungskette in den Kinetoplasten enthalten innerhalb des codierenden Bereichs ein Stoppcodon der Translation, deshalb ist ihre prä-mRNA nicht funktionsfähig. Andere Gene sind also solche gar nicht erkennbar, da die direkte Übersetzung der DNA-Sequenz Polypeptide ergibt, die keine Ähnlichkeit mit anderen Proteinen in den Mitochondrien zeigen. Als man die mRNA zu einem solchen Gen mit einem Translationsstopp sequenzierte, stellte sich heraus, dass sie vier zusätzliche Uridin-Nucleotide enthält, die in der DNA nicht codiert werden. Damit verschiebt sich das Leseraster in der reifen mRNA und enthält kein Stoppcodon mehr, so dass von dieser RNA ein funktionsfähiges Protein in ganzer Länge synthetisiert wird. Die Untersuchung weiterer mRNAs zeigte, dass dies kein Einzelfall ist, sondern dass an vielen Stellen in die ursprünglich von der DNA abgeschriebenen mRNAs Uridine eingefügt wurden, die genomisch nicht vorhanden sind. Im extremsten Fall werden insgesamt fast 300 Uridine in ein mRNA-Molekül eingefügt, mehr als die etwa 250 Nucleotide, die für dieses Protein im Genom codiert sind.

Die Information, wie viele Uridine an welcher Stelle eingefügt (oder herausgeschnitten) werden sollen, ist an anderen Stellen in der Kinetoplasten-DNA codiert. Die prä-mRNAs werden von großen zirkulären DNA-Molekülen abgelesen, die Anweisungen für das RNA-Editing liegen auf kleineren DNA-Molekülen codiert, beide zusammen ergeben das gesamte Genom der Kinetoplasten. Von den Mini-DNAs werden kleine so genannte guide-RNAs (gRNAs) synthetisiert, die jeweils 20–30 Nucleotide komplementär passend zur reifen RNA enthalten. Dabei entsprechen die Adenosine in diesen gRNAs den endgültig einzubauenden oder zu entfernenden Uridinen. Am vorderen (5´-)Ende passen einige Nucleotide direkt zu der prä-mRNA, die als Anker für das Zusammenkommen der spezifischen gRNA mit der zu ihr passenden Region in der Gesamtheit der Kinetoplasten-RNAs dienen.

An dem nach der Verankerung beginnenden Editing sind eine ganze Reihe von Proteinen beteiligt (Abb. 7): Endonucleasen, die die ursprüngliche mRNA aufschneiden, RNA-Polymerasen, die einige Uridine einfügen, dann Exonucleasen, die von den angehefteten Uridinen so viele wieder herausschneiden bis die Zahl der restlichen Uridine in der reifenden mRNA zur Vorgabe der gRNA passt.

Insgesamt erfordert dieses RNA-Editing einen enormen Aufwand an Genen für RNAs und Proteine, der in keinem Verhältnis zu der Platzersparnis steht, die im Gen durch das spätere Einfügen selbst von vielen Uridinen erreicht wird.

Mehrfach unabhängige Entstehung des RNA-Editing

Die oben zusammengefassten Beispiele von RNA-Editing in so sehr verschiedenen Organismen und mit so unterschiedlichen biochemischen Abläufen machen offensichtlich, dass jede Art von RNA-Editing unabhängig entstanden ist. Einige sind relativ alt, andere erst sehr spät in der Evolution aufgetaucht. Das A zu I-RNA-Editing in den Kalium-Kanälen von Nervenzellen ist wahrscheinlich früh in der Entwicklung der Tiere entstanden, es findet sich in Wirbellosen wie *Octopus* ebenso wie in Säugetieren bis hin zum Menschen. Dagegen scheint sich

Tab. Die Verwandtschaftsgruppe der Kraken ist weltweit verbreitet, verschiedene Arten haben sich an unterschiedlichste Umgebungstemperaturen angepasst. Verändert nach [7]

Spezies	Herkunft	Wassertemperatur
Octopus digueti	Baja California, Flachwasser	37 °C
Octopus defilippi	Puerto Rico, Rio Grande, Flach-Riff	32 °C
Octopus vulgaris	Puerto Rico, Luquillo, Ufer-Riff	28 °C
Octopus bimaculata	Kalifornien, Isla Catalina, Uferfelsen	18 °C
Octopus rubescens	Kalifornien, Monterrey, Ufer	10 °C
Benthoctopus piscatorum	Norwegen, Svalberg, Netzfang	0 °C
Bathypolypus arcticus	Norwegen, Svalberg, Netzfang	-1 °C
Pareledone spec.	Antarktis, McMurdo Station	-2 °C

das C zu U-RNA-Editing in den mRNAs für Apolipoproteine erst spät in der Entwicklung der Säugetiere etabliert zu haben, es tauchte erst während der Aufspaltung der höheren Säuger auf, kurz vor der Abzweigung der Nagetiere.

Das C zu U-RNA-Editing in den Pflanzen findet sich nur in Landpflanzen, nicht in Algen. Es muss mit dem Übergang der Pflanzen auf das Land entstanden sein, da bis auf einige Lebermoose an der evolutionären Wurzel der Landnahme alle Landpflanzen mehr oder weniger intensives RNA-Editing zeigen. Dabei zeigen einige Zweige wie die Vertreter *Isoetes* und *Selaginella* aus der Verwandtschaft der Farnpflanzen extrem viele Editing-Ereignisse sowohl in Mitochondrien als auch in Plastiden, andere wie das Moos *Physcomitrella patens* [12] sehr wenige. Ebenso etablierte sich in einigen Pflanzengruppen wie den Hornmoosen viel U zu C-RNA-Editing, während Blütenpflanzen diese Reaktion kaum oder gar nicht im Programm haben.

Das hochkomplexe RNA-Editing in den Trypanosomatiden findet sich nur in nahe verwandten Einzellern, hat sich also erst bei der Spezialisierung auf die parasitäre Lebensweise etabliert. Dafür spricht auch die Beobachtung, dass in den im Blut lebenden Zwischenformen, in denen mitochondriale (kinetoplastidäre) Funktionen nicht benötigt werden, weniger RNA-Editing abläuft.

Ebenso spezialisiert ist das RNA-Editing in den Schleimpilzen, das nur in eng verwandten Spezies von *Physarum* gefunden wird. Diese komplexe Mischform von Insertions- und Konversions-RNA-Editing ist also auch erst relativ spät entstanden.

RNA-Editing als Mechanismus fein abgestufter Regulation

Die Entstehung des RNA-Editing kann wie andere Evolutionsprozesse nicht teleologisch beurteilt werden. Wie jede neue Entwicklung von Organismen haben sich die Mechanismen des RNA-Editing nach ihrer Entstehung durch einen Selektionsvorteil bewährt und konnten sich deshalb im Organismenreich etablieren. Dabei liegt das Potential des RNA-Editing offenbar in der fein abgestuften Regulation unterschiedlichster physiologischer Prozesse. So steuern die Trypanosomen die Stabilität ihrer RNAs in den Mitochondrien passend zu den veränderten Anforderungen an den Stoffwechsel ihrer im Blut bzw. in Insekten lebenden Entwicklungsstadien über das RNA-Editing. Beim Menschen wird der Fettstoffwechsel durch Regulation der verschiedenen Lipoproteinformen im Blut über das ApoB-mRNA-Editing sehr fein eingepegelt. Die Kraken nutzen das Editing der K^+-Kanäle in den Nervenzellen für die Anpassung an die Temperatur des Meerwassers und damit ihres eigenen Körpers. Auch beim Menschen werden die Leitfähigkeiten und Feuerungsgeschwindigkeiten der Axone über unterschiedliche Mischungsverhältnisse der durch das A zu I-mRNA-Editing abgewandelten Ionenkanäle fein reguliert.

Der Prozess des RNA-Editing wird aber noch viel mehr genutzt: In den letzten Jahren hat man gezielt nach weiteren A zu I-Editing-Ereignissen in Tieren gesucht. Besonders beim Menschen wurden Tausende solcher neuen Ziele entdeckt – viele davon in RNAs von transponierbaren Elementen [13]. Diese RNAs falten sich durchweg zu engen Sekundärstrukturen zusammen, bevorzugte Zielmoleküle der Adenosin-Desaminasen. Hier dient das RNA-Editing zur Kontrolle der Menge dieser Transkripte und zur Stilllegung unerwünschter RNAs von diesen Überbleibseln von RNA-Viren. Da das Genom des Menschen besonders viele transponierbare Elemente enthält, findet sich bei uns auch viel mehr solches A zu I-RNA-Editing als zum Beispiel bei Fliegen oder Fröschen.

Sicherlich kennen wir noch nicht alle Anwendungen und Einsätze des RNA-Editing. Wir können indirekt darauf schließen, dass das RNA-Editing an weiteren regulatorischen Prozessen direkt oder indirekt beteiligt ist. Fehlt das A zu I-RNA-Editing zum Beispiel durch Mutation des Enzyms, kann es je nach Gewebe zur Ausbildung von Tumoren kommen, besonders zu unkontrolliertem Wachstum im Gehirn, aber auch zu Leukämien [14]. Ein Teil dieser Krankheiten wird durch das dann fehlende RNA-Editing in kleinen, die Entwicklung und Zellidentität regulierenden RNAs ausgelöst. Diese Mikro-RNAs (miRNAs) von etwa 22 Nucleotiden Länge werden aus größeren RNA-Molekülen herausgeschnitten. Dazu falten sie sich zu doppelsträngigen Bereichen auf. Ebenfalls doppelsträngige RNA entsteht bei der Wirkung der miRNAs, wenn sie sich mit den passenden Ziel-RNA-Sequenzen paaren und diese so stabilisieren, abbauen oder für die Translation unzugänglich machen. Die doppelsträngigen RNA-Regionen sind nun wiederum die bevorzugten Ziele der A zu I-Desaminase. Nach dem RNA-Editing ist aber eine andere miRNA-Sequenz entstanden, die dann auch andere mRNAs als Ziele erkennt. Fällt dieses RNA-Editing aus, so kommt es zu Paarungen der miRNAs mit falschen mRNAs und die gesamte Steuerung der Zelle und ihre Entwicklung sind gestört – eine so veränderte Zelle kann die Funktion eines Organs behindern oder auch unkontrolliert wuchern.

RNA-Editing als sanfter Antrieb der Evolution

Das RNA-Editing bietet der Evolution eine neue, weichere Spielwiese neben der Ja-Nein Veränderung der DNA durch Mutation im Genom. Durch partielles RNA-Editing kann schon in der gleichen Generation das resultierende veränderte Protein neben dem ursprünglichen, die Funktion sicherstellenden ausprobiert werden. Dass beide Proteine nebeneinander in der Zelle vorliegen ist von Vorteil: Funktioniert die neue Form nicht so gut wie die alte, muss das nicht gleich letal für den Organismus sein, das RNA-Editing kann ebenso graduell wieder zurückgehen wie es eingeführt wurde. Ein gutes Beispiel dafür ist die stufenlose Anpassung des RNA-Editing an unterschiedliche Wassertemperaturen bei *Octopus*.

Auch auf DNA-Ebene ermöglichen Genduplikationen oder die verschiedenen Allele in diploiden Organismen, dass Neues nicht gleich dem scharfen Gegenwind der Selektion unterliegen muss. Doch das RNA-Editing ermöglicht auf einer zweiten molekularen Ebene, Varianten zu erzeugen und zu nutzen. Die einfache Sicht „Ein Gen – ein Protein“ erweist sich in einem noch viel größeren Umfang als bisher gedacht als unzureichend. Die häufig vorschnell als „Entschlüsselung“ des Genoms angesehene Sequenzierung der genomischen DNA ist nur ein Schritt auf dem Weg zum Verständnis der Proteinvielfalt, deren Erschließung weit schwieriger ist als geahnt.

Literatur

[1] H. Grosjean, R. Benne: Modification and editing of RNA. ASM Press. Washington DC 1998. – [2] N. O. Davidson, G. S. Shelness, Annu. Rev. Nutr. **20,** 169 (2000). – [3] C. E. Hamilton et al., RNA Biol. **7,** 1-10 (2010). – [4] M. Takenaka et al., Mitochondrion **8,** 35 (2008). – [5] M. Takenaka et al., Proc. Natl. Acad. Sci. U.S.A. **109,** 5104 (2012). – [6] B. L. Bass, Annu. Rev. Biochem. **71,** 817 (2002). – [7] S. Garrett, J. J. C. Rosenthal, Science **335,** 848 (2012). – [8] M. Öhman, Science **335,** 805 (2012). – [9] A. C. Rhee et al., RNA **15,** 1753 (2009). – [10] L. Simpson, O. H. Thiemann, Cell **81,** 837 (1995). – [11] K. D. Stuart et al., Trends Biochem. Sci. **30,** 97 (2005). – [12] M. Rüdinger et al., Plant J. **67,** 370 (2011). – [13] S. Maas et al., RNA Biol. **3,** 1 (2006). – [14] A. Gallo, S. Galardi, RNA Biol. **5,** 135 (2008).

Dr. **Anja Zehrmann** (Jahrgang 1981) studierte Biologie in Ulm, wurde dort promoviert und arbeitet jetzt als Wissenschaftlerin und Gruppenleiterin.

Dr. **Daniil Verbitskiy** (Jahrgang 1973) studierte Biologie in Irkutsk. Er wurde in Ulm promoviert und ist dort wissenschaftlicher Mitarbeiter.

Dipl.-Biol. **Barbara Härtel** (Jahrgang 1983) studierte Biologie in Ulm und ist Doktorandin.

Nadja Brehme (Jahrgang 1987) studierte Biologie in Ulm und ist Diplomandin.

PD Dr. **Mizuki Takenaka** (Jahrgang 1974) studierte Biologie in Kyoto und wurde dort promoviert. Er habilitierte in Ulm und ist Heisenberg Stipendiat.

Prof. Dr. **Axel Brennicke** (Jahrgang 1953), Studium der Biologie, Promotion und Habilitation in Tübingen, Professur und Institutsleitung.

Alle Autoren arbeiten am RNA-Editing in den Mitochondrien und Plastiden von Pflanzen. Institut für Molekulare Botanik der Universität Ulm, Albert-Einstein-Allee 11, 89069 Ulm.

http://www.biologie.uni-ulm.de/bio2/index.html

XI Curriculum Vitae

Name:	Barbara Härtel
Geburtsdatum:	20.12.1983
Geburtsort:	Kempten
Nationalität:	deutsch

Ausbildung:

1994-2003	Hildegardis-Gymnasium in Kempten Abschluss: Abitur
2003-2005	Studium der Biochemie an der TU München
2005-2010	Studium der Biologie an der Universität Ulm Hauptfach Molekularbiologie, Abschluss: Dipolm
04/2009-02/2010	Diplomarbeit am Institut für Molekulare Botanik, Universität Ulm: „Identifizierung der RNA-Editing-Faktoren für die Stellen *ccb382*-709 und *nad5*-374 in *Arabidopsis thaliana*“
03/2010 bis heute	Doktorarbeit in der Arbeitsgruppe von Prof. Dr. Axel Brennicke, Institut für Molekulare Botanik, Universität Ulm

XII Danksagung

Besonders möchte ich mich bei Herrn Prof. Dr. Axel Brennicke für die Möglichkeit bedanken, meine Doktorarbeit in seiner Abteilung durchzuführen. Außerdem vielen Dank für die Unterstützung und nicht zuletzt für die freundliche Aufnahme in seine Arbeitsgruppe.

Bei Herrn PD Dr. Mizuki Takenaka bedanke ich mich für seine Bereitschaft, das Zweitgutachten zu erstellen.

Ganz herzlichen Dank PD Dr. Mizuki Takenaka, Dr. Anja Zehrmann und Dr. Daniil Verbitskiy für die wertvollen Anregungen, Ratschläge und Diskussionen und ganz besonders die Freundlichkeit und Hilfsbereitschaft.

Vor allem auch herzlichen Dank an Dagmar Pruchner, Angelika Müller und Evelyn Laible-Schmid für die praktische Unterstützung im Labor, die Pflanzenanzucht, die Hilfe in bürokratischen Fragen und vor allem ihre Hilfsbereitschaft.

Außerdem natürlich vielen Dank an alle Mitglieder des Labors und der Abteilung Molekulare Botanik für die angenehme Atmosphäre.

Nicht zuletzt gilt meinen Eltern mein herzlicher Dank dafür, dass sie mir das Studium ermöglichten, immer großes Interesse für meine Arbeit zeigten und mich fortwährend unterstützten.
Danke Christian, Lisa und allen Freunden für ihr Verständnis und ihre Unterstützung.

XIII Eidesstattliche Erklärung

Ich versichere hiermit, dass ich die Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, sowie die wörtlich oder inhaltlich übernommenen Stellen als solche kenntlich gemacht habe.

Ulm, den

Barbara Härtel