RNA splicing and RNA editing in chloroplasts

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Abstract

During the evolution of chloroplasts from their bacterial ancestor traits emerged that are absent or rare in bacteria. Prominent among these acquired traits are RNA splicing and RNA editing. The numbers and distribution of introns and editing sites in different taxa suggest that editing and splicing have taken different evolutionary pathways in different chloroplast lineages. Both processes are dependent on nuclear-encoded factors and, intriguingly, PPR (pentatricopeptide repeat) proteins have recently been recognized as a common player. This review summarizes recent progress in understanding the mechanisms, regulation, and *trans*-acting factors for these two types of RNA processing.

1 Introduction

As endosymbiotic descendants of cyanobacteria, chloroplasts share many features of their metabolism and biogenesis with prokaryotes. However, their coevolution with the eukaryotic host genome has led to the pronounced modification of prokaryotic features and the acquisition of novel features not present in their prokaryotic ancestors. Understanding these plastid-specific attributes is critical for understanding how the organelle was integrated into the regulatory circuits of the plant cell. Two features of chloroplast gene expression exemplify these acquired features. First, the chloroplast genome is rich in intervening sequences, whereas introns are rare in bacteria. Even more striking, chloroplasts display an RNA processing event called RNA editing that does not exist at all in prokaryotes. The acquisition of these two RNA processing mechanisms in chloroplasts necessitated the recruitment of pre-existing proteins and/or the evolution of novel proteins to participate in these processes. This review summarizes recent advances in understanding the molecular mechanisms, evolution and regulation of these two RNA maturation steps in chloroplasts.

2 Plastid RNA splicing

Accurate splicing of plastid introns is essential for the biogenesis of the chloroplast, as introns disrupt plastid genes encoding components of the gene expression

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machinery and the photosynthetic apparatus. Moreover, RNA splicing can be exploited as an on/off switch for the expression of intron containing genes and provides opportunities for regulation of plastid gene expression by environmental and developmental cues. The machinery responsible for plastid splicing is encoded primarily by nuclear genes, with almost all characterized plastid splicing factors being – as expected – essential for chloroplast development. The growing number of such factors identified in recent years suggests a complexity that was not anticipated given the resemblance of plastid introns to "self-splicing" introns found in other organisms.

2.1 Intron classes and splicing mechanisms

Chloroplast introns are classified as either group I, group II, or group III introns by virtue of conserved features of primary sequence and predicted secondary structure (Michel and Dujon 1983; Michel et al. 1989; Copertino and Hallick 1993; Lambowitz et al. 1999; Bonen and Vogel 2001). Group I and group II introns are distributed broadly among prokaryotes and prokaryote-derived organelles, but they are particularly prevalent in the mitochondria and chloroplasts of plants and algae. Both group I and group II introns are considered to be ribozymes because some introns in each class exhibit self-splicing activity *in vitro.* Despite this similarity, group I and group II introns differ fundamentally in structure and catalytic mechanism. Group I introns are relatively small and uniform in structure, with two central helical domains that are stabilized by peripheral domains (Michel and Westhof 1990; Lehnert et al. 1996; Golden et al. 1998; Westhof 2002). Group II introns are larger and more structurally variable, consisting of six helical domains emanating from a central core, and inter-domain tertiary contacts that create a compact catalytic center (Michel et al. 1989; Michel and Ferat 1995; Qin and Pyle 1998). This canonical group II intron structure is often highly modified; for example, the chloroplast *psa*A mRNA in *C. reinhardtii* (Kuck et al. 1987; Choquet et al. 1988; Herrin and Schmidt 1988) and the land plant chloroplast *rps*12 mRNA (Fromm et al. 1986; Zaita et al. 1987) are transcribed in pieces that must then be “*trans*-spliced” (reviewed in Bonen and Vogel 2001). During *trans*-splicing, intron fragments are believed to assemble via RNA-RNA interactions to recreate an intact group II intron structure. Group III introns are believed to be still more highly degenerate group II introns, and are a specialized case in that they have been found only in Euglenoids (Christopher and Hallick 1989).

The chemistry of group I and group II intron splicing has been elucidated primarily from the study of self-splicing introns from fungal mitochondria (reviewed in Qin and Pyle 1998; Lambowitz et al. 1999; Bonen and Vogel 2001). Both intron classes splice via two consecutive *trans*-esterifications involving first the 5’ and then the 3’ splice junction, but the reactions otherwise differ. The splicing of group I introns is initiated by an exogenous guanosine that attacks the 5’ splice junction; the liberated 3’ hydroxyl group then attacks the 3’ splice junction, resulting in exon ligation and intron release. By contrast, group II splicing generally initiates when a 2’ hydroxyl group of a “bulged” adenosine residue in the domain 6

helix attacks the 5' splice junction. This yields a branched "lariat" structure in which the 2'hydroxyl group is covalently attached to the 5' phosphate at the 5' end of the intron. Splicing is completed during a second step during which the liberated 3'-hydroxyl group at the 5' splice junction attacks the 3' splice junction, resulting in exon ligation and release of an excised intron lariat. These canonical splicing mechanisms likely apply to most plastid introns, as excised group II intron lariats have been identified for many chloroplast introns (Kim et al. 1993; Vogel and Borner 2002) and mutagenesis of predicted functional elements of a chloroplast group I intron disrupted splicing *in vivo* and *in vitro* (Lee and Herrin 2003).

An alternative pathway for group II splicing *in vitro* is initiated by water or hydroxide rather than by a bulged intron adenosine (Daniels et al. 1996). In fact, a bulged adenosine is missing from domain six in land plant chloroplast *trnV-UAC* introns. The excised *trnV-UAC* intron was detected as a linear molecule but not as a lariat in barley chloroplasts (Vogel and Borner 2002), supporting the notion that this intron is, indeed, spliced via a hydrolytic pathway *in vivo*. Whether the use of this alternative pathway has any physiological significance is unclear.

Similarities between the chemistry of group II splicing and spliceosome-mediated splicing in the nucleus, together with structural similarities between specific snRNAs and specific group II intron domains have led to the intriguing speculation that spliceosomal snRNAs might be derived domains of ancestral self-splicing group II introns (Cech 1986; Hetzer et al. 1997; Shukla and Padgett 2002; Villa et al. 2002; Sashital et al. 2004). The evolutionary lability of group II introns in plant organelles, as exemplified by the *trans*-spliced introns in chloroplasts, lends credence to this idea. If true, endosymbiotic organelles could have been the initial donors of a proto-intron, thereby shaping eukaryotic genomes in a most fundamental way.

2.2 Intron distribution

Bryophytes, gymnosperms, angiosperms and their closest algal relatives (members of the charophyta) share a basic set of chloroplast introns, consisting of one group I intron and ~20 group II introns. 20 out of the 21 plastid introns represented in land plants have been detected in at least one charophyte lineage, indicating that these introns were acquired prior to the emergence of land plants; only the *clpP-2* group II intron was incorporated later, during the transition to land plants (Wakasugi et al. 2001; Turmel et al. 2002; Kugita et al. 2003a; Sugiura et al. 2003; Turmel et al. 2006). Thus, plastid introns were acquired early during plant evolution and are among the most stable features of the chloroplast genome. Land plants and charophyte algae (together called the streptophyta) contain a single group I intron, in the *trnL-UAA* gene. This intron is considered to be the most ancient of all plastid introns as it is represented in land plants, in both charophyte and chlorophyte green algae, in red algae and even in cyanobacteria (Xu et al. 1990; Simon et al. 2003).

Differences in plastid intron content among land plant species reflect lineage specific intron loss via either the complete loss of intron-containing genes (e.g. loss of the *ndhA* and *ndhB* genes in black pine chloroplasts, Wakasugi et al. 1994), or intron loss with retention of the host gene (e.g. introns disrupt the *clpP* and *rpoC* genes in dicots and ancestral embryophytes but not in monocot grasses). Thus, maize and rice chloroplasts have seventeen group II introns, whereas *Arabidopsis* and tobacco chloroplasts have twenty. The parasitic plant *Epifagus virginiana* provides an extreme case of intron loss, in that it retains only six chloroplast introns (Wolfe et al. 1992; Ems et al. 1995).

Group II introns are absent from the chloroplasts of the most basal species within the streptophyta, like the charophyte algae *Mesostigma viride* and *Chlorokybus atmophyticus* (Lemieux et al. 2000; Turmel et al. 2006), whereas the overall plastid gene organization in these species is highly conserved with land plants. This supports the idea that the acquisition of chloroplast group II introns within and outside the streptophyta were independent events. Accordingly, the chloroplasts of the chlorophyte *C. reinhardtii* harbors five group I introns and only two group II introns, none of which are found in land plants (Maul et al. 2002). *Euglena gracilis*, a photosynthetic protist, houses the most intron rich chloroplast genome described to date, with at least 155 introns; these introns fall into the group II and group III classes (Hallick et al. 1993).

2.3 Proteins involved in the splicing of chloroplast introns

2.3.1 Proteins are required for chloroplast intron splicing

Group I and group II introns are classified as ribozymes because representatives of both intron classes have been shown to self-splice *in vitro* (reviewed in Lambowitz et al. 1999). However, many introns with the characteristic features of group I or group II introns cannot be coerced to self-splice and, in fact, not one of the ~40 introns in the organelles of vascular plants has been reported to self-splice *in vitro*. Only two examples of self-splicing group II introns in chloroplasts have been described, one of them in a psychrophilic *Chlamydomonas* species (Odom et al. 2004), the other in *Euglena myxocylindracea* (Sheveleva and Hallick 2004). This latter intron is, however, an evolutionary oddity because it likely represents a recent horizontal transfer from a cyanobacterial donor. In *Chlamydomonas* and other algae, several plastid group I introns have been demonstrated to self-splice (Herrin et al. 1990, 1991; Deshpande et al. 1997; Kapoor et al. 1997; Simon et al. 2003), while the group I intron in higher plant *trnL* genes does not (Simon et al. 2003). Even where self-splicing has been detected, the reactions generally require non-physiological salt and temperature conditions. Moreover, a self-splicing group I intron from *C. reinhardtii* chloroplasts was more tolerant of mutations in core elements when expressed *in vivo* than during self-splicing *in vitro* (Lee and Herrin 2003). Together, these data strongly suggest that accessory factors facilitate the splicing of most or all group I and group II introns *in vivo.* Indeed, genetic data summarized below have provided evidence for the involvement of proteins in the

splicing of almost all of the group II introns represented in the chloroplasts of vascular plants and *C. reinhardtii*.

Proteins involved in group I and group II intron splicing fall into two general classes: conserved intron-encoded "maturase" proteins and diverse "host-encoded" factors (reviewed in Lambowitz et al. 1999). The majority of splicing factors in chloroplasts fall into this second category.

2.3.2 Intron-encoded maturases in chloroplasts

Group I and group II intron maturases have been studied primarily in fungal mitochondria and bacteria (reviewed in Lambowitz et al. 1999). Group I maturases are related to the LAGLIDADG class of homing endonucleases; some group I maturases promote both intron homing and splicing, whereas others have lost their DNA endonuclease function and are now specialized splicing factors. The single group I intron in land plant chloroplasts lacks a maturase open reading frame. However, three group I introns in *C. reinhardtii* chloroplasts encode maturase-like proteins; these have been shown to promote insertion of their host intron into intronless alleles (Durrenberger and Rochaix 1991; Holloway et al. 1999; Odom et al. 2001) but deletion of these open reading frames did not result in splicing defects (Thompson and Herrin 1991; Johanningmeier and Heiss 1993). Therefore, the available data suggest that these maturase-like proteins do not function in splicing.

Group II intron maturases are characterized by reverse-transcriptase and endonuclease domains involved in intron mobility, and a "domain X", which is implicated in RNA binding and splicing (reviewed in Lambowitz et al. 1999). The *C. reinhardtii* chloroplast genome lacks group II maturase open reading frames, whereas a single open reading frame related to group II maturases is encoded in land plant chloroplasts genomes; this gene is called *matK* and resides in the *trnK* intron (Neuhaus and Link 1987). MatK is a degenerate maturase-like protein, consisting of domain X fused to a remnant of the reverse transcriptase domain. Several lines of evidence suggest that MatK is involved not only in the splicing of its host *trnK* intron, but also in the splicing of other group II introns. First, MatK binds RNA *in vitro* (Liere and Link 1995) and the sequence encoding MatK is subject to an RNA editing event that increases its conservation with functional maturases (Vogel et al. 1997). Furthermore, the absence of MatK in maize and barley mutants lacking plastid ribosomes correlates with the failure to splice the *trn*K intron (Vogel et al. 1997) as well as several other group II introns (Jenkins et al. 1997; Vogel et al. 1999). Although the pleiotropic nature of the mutants used in these studies precludes firm conclusions about the role of MatK in splicing, these findings raised the possibility that MatK may facilitate the splicing of multiple introns, unlike canonical group II maturases which act specifically on the intron in which they are encoded (Lambowitz et al. 1999). Additional evidence that MatK promotes the splicing of multiple plastid introns arose from the sequence of the plastid genome of the non-photosynthetic angiosperm *Epifagus virginiana*. The Epifagus plastid genome lacks *trnK* but retains a stand-alone *matK* gene; it was proposed that retention of *matK* reflects a role for MatK in the splicing of one or

more of the six group II introns retained in the *Epifagus* chloroplast genome, all of which are accurately spliced *in vivo* (Wolfe et al. 1992; Ems et al. 1995). Still, proof that MatK promotes splicing is lacking. Initial attempts to delete *matK* from the chloroplast genome in tobacco resulted only in heteroplastomic plants (R. Maier, personal communication); this is consistent with a role for *matK* in splicing *trnK* and/or other essential plastid RNAs that contain group II introns. Biochemical approaches and the analysis of hypomorphic, non-lethal alleles of *matK* may help to clarify this issue.

2.3.3 Nucleus-encoded splicing factors in chloroplasts

Numerous nucleus-encoded proteins involved in the splicing of chloroplast introns in both vascular plants and algae have been identified in recent years, primarily through genetic screens for nuclear mutations that cause defects in chloroplast gene expression. In *C. reinhardtii*, several splicing factors involved in the maturation of the *psaA* mRNA have been described. Maturation of this mRNA is particularly complex, as it involves the *trans*-splicing of two group II introns: intron 2 is transcribed in two segments together with the flanking exons, whereas intron 1 consists of three pieces: 5' and 3' intron fragments that are cotranscribed with flanking exons and an internal intron fragment that is independently transcribed from a chloroplast locus called *tscA* (Kuck et al. 1987; Choquet et al. 1988; Herrin and Schmidt 1988; Roitgrund and Mets 1990; Goldschmidt-Clermont et al. 1991). The *tscA* RNA is proposed to bridge the 5' and 3' fragments of intron 1, but domain 1 of this mosaic intron appears to lack critical elements, suggesting that an additional intron fragment remains to be discovered (Turmel et al. 1995). As might be expected, a large number of accessory factors are required to assemble and splice the two *psaA* introns. In fact, mutations that disrupt this process define at least fourteen nuclear genes (Goldschmidt-Clermont et al. 1990); this gene set includes genes that function directly in splicing, as well as genes that affect splicing indirectly by promoting the maturation of the *tscA* RNA. One gene in the latter class, *Rat1,* has been cloned. *Rat1* codes for a protein with a domain that is related to NAD+-binding domains from eukaryotic organisms, and that can interact with the *tscA* RNA in a yeast 3-hybrid assay (Balczun et al. 2005).

The molecular cloning of three genes that seem likely to function directly in the *trans*-splicing of the *C. reinhardtii psaA* mRNA has been reported: *Raa2,* which is required for the *trans*-splicing of intron 2, *Raa3,* which is required for the *trans*-splicing of intron 1, and *Raa1,* which is required for the *trans*-splicing of both introns (Perron et al. 1999; Rivier et al. 2001; Merendino et al. 2006). Raa3 exhibits limited similarity to pyridoxamine 5'-phosphate oxidases and is found in a large complex in the chloroplast stroma, together with the *tscA* and *psaA* exon 1 precursor RNAs (Rivier et al. 2001). In contrast, Raa1 and Raa2 are associated with a chloroplast membrane fraction and are found, at least in part, in a complex with one another (Perron et al. 1999, 2004; Merendino et al. 2006). Raa2 resembles pseudouridine synthase enzymes; however, mutagenesis of amino acids that are essential for the catalytic activity of related bacterial enzymes did not disrupt *psaA*

Table 1. Nuclear-encoded proteins involved in plastid RNA splicing

Protein	Target Intron	Protein Class	Species	References
Raa1	psaA introns 1 and 2		*C. reinhardtii*	(Merendino et al. 2006)
Raa2	psaA intron 1	Pseudouridine synthase	*C. reinhardtii*	(Perron et al. 1999)
Raa3	psaA intron 2		*C. reinhardtii*	(Rivier et al. 2001)
Rat1	psaA intron 1 (tscA)	NAD+ binding	*C. reinhardtii*	(Balczun et al. 2005)
CRS1	atpF	CRM domain	*Z. mays* *A. thaliana*	(Jenkins et al. 1997; Till et al. 2001; Ostersetzer et al. 2005; Asakura and Barkan 2006)
CAF1	petD, trnG, rps16, rpl16, ycf3-intron 1,rpoC1*, clpP-intron 1*	CRM domain	*Z. mays* *A. thaliana*	(Ostheimer et al. 2003; Asakura and Barkan 2006)
CAF2	rps12- intron 1, petB, ndhB, ndhA, ycf3- intron 1	CRM domain	*Z. mays* *A. thaliana*	(Ostheimer et al. 2003; Asakura and Barkan 2006)
CRS2	All CAF1- and CAF2-dependent introns	Peptidyl-tRNA hydrolase	*Z. mays*	(Jenkins et al. 1997; Vogel et al. 1999; Jenkins and Barkan 2001)
PPR4	rps12-intron 1	PPR and RRM	*Z. mays*	(Schmitz-Linneweber et al. 2006)
HCF152	petB	PPR	*A. thaliana*	(Meierhoff et al. 2003; Nakamura et al. 2003)

* Introns present in *Arabidopsis* but not in maize

splicing *in vivo*, suggesting that pseudouridine synthase activity is not relevant to Raa2's role in splicing (Perron et al. 1999). Raa1 encodes a novel protein that includes repeated motifs that are reminiscent of tetratricopeptide (TPR) and pentatricopeptide (PPR) motifs (Merendino et al. 2006); it was speculated that these repeats might form an RNA binding surface analogous to the surface proposed for PPR proteins (Small and Peeters 2000); in fact, Raa1 resides in two high molecular weight complexes in chloroplasts, one of which contains RNA (Merendino et al. 2006). Mutational studies revealed that Raa1's C-terminal domain functions in the processing of the *tscA* RNA and the splicing of *psaA* intron 1, whereas the

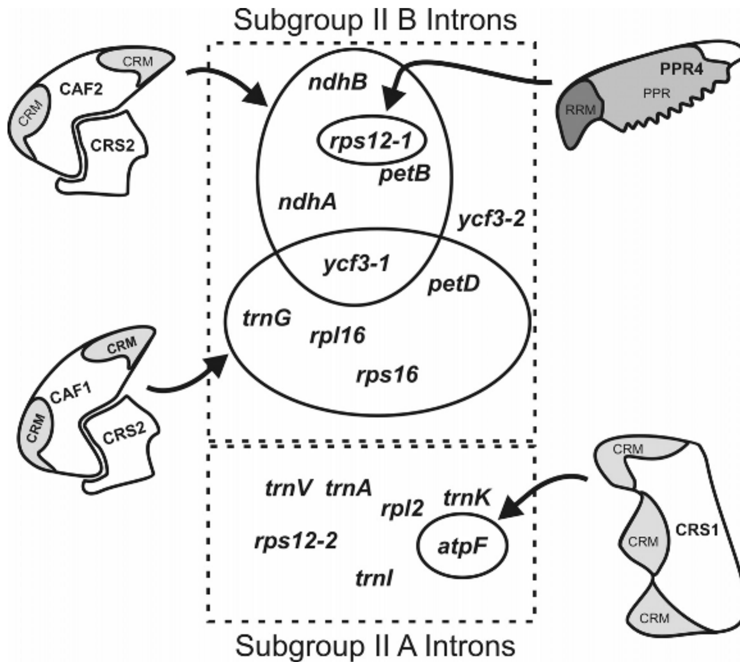

Fig. 1. Nucleus-encoded chloroplast splicing factors and their intron targets in maize. The intron targets indicated for each protein fail to splice in the corresponding mutant background and coimmunoprecipitate with the corresponding protein. CAF1, CAF2, and CRS1 are members of the CRM domain protein family (Barkan et al. 2007), CRS2 is a peptidyl-tRNA hydrolase homolog (Jenkins et al. 2001), and PPR4 contains a PPR tract and an RRM domain (Schmitz-Linneweber et al. 2006). All seven subgroup IIA introns fail to splice in mutant plastids lacking ribosomes, implicating a plastid translation product in their splicing. Results are summarized from Jenkins et al. 1997, Ostheimer et al. 2003, Vogel et al. 1999, Schmitz-Linneweber et al. 2005 and 2006. The functions of the *Arabidopsis* CAF1, CAF2, and CRS1 orthologs are conserved with those in maize, except that AtCAF1 promotes the splicing of two additional chloroplast introns that are not found in maize (*rpoC1* and *clpP*-intron 1; Asakura et al. 2006).

central domain mediates splicing of the second intron. Thus, Raa1 may serve to coordinate the two *trans*-splicing events during *psaA* maturation. This coordination may involve transient association between the predominantly stromal Raa3-containing complex and the predominantly membrane-bound Raa1/Raa2 complexes.

Analogous approaches have been used to identify nucleus-encoded proteins involved in the splicing of chloroplast introns in land plants. In maize, five proteins involved in the splicing of various subsets of its 17 chloroplast group II introns have been reported: CRS1, CRS2, CAF1, CAF2, and PPR4. For each of these proteins, splicing defects accompanying loss-of-function mutations have identified its intron targets, and RNA coimmunoprecipitation experiments have shown it to be associated *in vivo* with the corresponding intron RNAs (Jenkins et al. 1997; Jen-

kins and Barkan 2001; Till et al. 2001; Ostheimer et al. 2003; Schmitz-Linneweber et al. 2005b, 2006). Together, these results provide strong evidence for a direct role in splicing. These proteins are found in at least three distinct ribonucleoprotein complexes, all in the chloroplast stroma. CRS2 functions in complexes that contain either CAF1 or CAF2 to promote the splicing of nine introns, with CAF1 and CAF2 each required for the splicing of an overlapping subset of the CRS2-dependent introns (Table 1, Fig. 1). The CRS2-CAF complexes are bound to intron RNAs in the stroma, in complexes of ~500-600 kDa (Jenkins et al. 1997; Jenkins and Barkan 2001; Ostheimer et al. 2003; Schmitz-Linneweber et al. 2005b). CRS1 is required solely for the splicing of the *atpF* intron and is found in a distinct high molecular weight (~600-700 kDa) ribonucleoprotein complex that includes *atpF* intron RNA (Jenkins et al. 1997; Till et al. 2001; Ostheimer et al. 2003). PPR4 is required solely for the *trans*-splicing of the first intron of *rps12* and resides in stromal complexes that are heterogeneous in size, and that include both fragments of the split *rps12* intron (Schmitz-Linneweber et al. 2006). As noted above, a plastid translation product, possibly MatK, is required for the splicing of several chloroplast introns as well (Jenkins et al. 1997; Vogel et al. 1999). Taken together, the genetic data show that sixteen of the seventeen group II introns in maize chloroplasts rely on proteins for their splicing *in vivo* (Fig. 1). The splicing of the second intron in *ycf3* is not disrupted in any of the mutant backgrounds analyzed to date and is the only candidate for a truly self-splicing group II intron in the maize chloroplast genome.

The chloroplast splicing factors discovered in maize are unrelated to those identified in *C. reinhardtii*, which perhaps is not surprising, given the independent origin of chloroplast introns in land plants (e.g. maize) and chlorophyte algae (e.g. *C. reinhardtii*). CRS2 has strong sequence and structural similarity to bacterial peptidyl-tRNA hydrolases, but seems to lack peptidyl-tRNA hydrolase activity (Jenkins and Barkan 2001; Ostheimer et al. 2005). CRS1, CAF1, and CAF2 are related to one another in that they harbor several copies of the same conserved domain, which is represented as a stand-alone ORF in prokaryotes (Till et al. 2001; Ostheimer et al. 2003). The *E. coli* representative of this domain family, YhbY, is bound *in vivo* to 50S ribosomal subunit precursors and likely plays a role in ribosome maturation (Barkan et al. 2007); thus, the YhbY-like domain in the chloroplast splicing factors was named the chloroplast RNA splicing and ribosome maturation (CRM) domain (Ostheimer et al. 2003; Barkan et al. 2007). Structural and biochemical data show that CRM domains function as RNA binding domains: the crystal structure of YhbY shows structural similarity with a class of RNA binding proteins that includes IF3 (Ostheimer et al. 2002), and an isolated CRM domain from CRS1 binds RNA with high affinity *in vitro* (Barkan et al. 2007).

CRM domains are found in a protein family in vascular plants comprising 16 members in *Arabidopsis* and 14 members in rice (Barkan et al. 2007). A reverse-genetic approach in *Arabidopsis* showed that the splicing functions and intron specificities of the CRS1, CAF1, and CAF2 members of the CRM family are conserved between maize and *Arabidopsis*, indicating that these proteins were recruited to promote the splicing of plastid group II introns prior to the divergence of monocot and dicot plants (Asakura and Barkan 2006). The *Arabidopsis* CAF1

ortholog has additional functions in that it promotes the splicing of introns in *rpoC1* and *clpP*, which are found in *Arabidopsis* but not in maize (Asakura and Barkan 2006). Given that all three characterized members of the plant CRM family function in chloroplast group II splicing, it seems likely that additional group II intron splicing factors remain to be discovered among the uncharacterized CRM proteins. In fact, a CRS1 paralog has been shown to be bound to several group II introns in maize chloroplasts, and to be required for the splicing of the corresponding introns in *Arabidopsis* (Y. Asakura and A. Barkan, manuscript in preparation).

The PPR protein family, like the CRM family, is largely specific to plants and includes members that function in the splicing of chloroplast introns. PPR proteins are defined by tandem repeats of a degenerate 35 amino acid motif that is related to the TPR motif. The repeat tracts have been proposed to form an RNA-binding surface that is structurally similar to the protein-binding surface described for TPR tracts (Small and Peeters 2000). The maize protein PPR4 contains both a PPR tract and an RRM motif, and is required for the *trans*-splicing of the first intron in the chloroplast *rps12* mRNA, to which it is bound *in vivo* (Schmitz-Linneweber et al. 2006). *Arabidopsis* HCF152, another PPR protein, is required for the accumulation of spliced chloroplast *petB* RNA (Meierhoff et al. 2003) and binds *in vitro* to the *petB* precursor transcript (Nakamura et al. 2003); excised *petB* intron accumulates normally in *hcf152* mutants, however, suggesting that HCF152 may function to stabilize spliced *petB* mRNA rather than to promote splicing.

The nucleus-encoded chloroplast splicing factors described thus far are diverse in sequence and evolutionary origin, but a common theme is their derivation from RNA binding proteins that evolved in other contexts. For example, Raa2 was derived from a pseudouridine synthase (Perron et al. 1999), CRS2 was derived from a peptidyl-tRNA hydrolase (Jenkins and Barkan 2001), and CRS1, CAF1, and CAF2 were derived by duplication and diversification of a pre-ribosome binding protein (Barkan et al. 2007). This situation is mirrored in fungi, where derived tRNA synthetases promote the splicing of both group I and group II introns (reviewed in Lambowitz et al. 1999). The differences between these splicing factors and their ancestors can elucidate features responsible for their gain of intron splicing functions. For example, CRS2 maintains a three-dimensional structure that is highly similar to that of its peptidyl-tRNA hydrolase ancestor, but several amino acid substitutions result in a CRS2-specific hydrophobic surface that allows CRS2 to bind to its CAF1 and CAF2 partners (Ostheimer et al. 2005). Conversely, CAF1 and CAF2 acquired the corresponding CRS2-interaction motif: an amphipathic helix appended to their CRM domains that is lacking in their most closely-related paralogs (Ostheimer et al. 2006). These examples highlight how proteins with novel functions can emerge through minor evolutionary tinkering.

2.3.4 Biochemical functions of chloroplast splicing factors

Despite recent progress in identifying chloroplast splicing factors, little is known about the mechanisms by which they promote splicing. It is generally assumed that the catalytic activity of group I and group II introns is intrinsic to the intron RNAs, and that proteins facilitate their splicing by enhancing the productive fold-

ing of the introns into their catalytically-active structure. The folding of group I and group II introns, like that of other large and highly structured RNAs, is problematic because numerous non-native conformations are similar in stability to the active structures, so the RNAs can easily be trapped in inactive conformations (reviewed in Herschlag 1995; Weeks 1997). In addition, tertiary interactions that establish the three-dimensional architecture of the intron can be weak (Swisher et al. 2002). Proteins could potentially guide intron folding via high-affinity, sequence-specific interactions that stabilize an otherwise transient tertiary interaction, or that preclude competing non-productive folding pathways. Alternatively, proteins could act as “RNA chaperones” to resolve misfolded RNA structures through low-affinity non-specific interactions with unstructured RNA, or via an ATP-dependent helicase activity (Herschlag 1995; Lorsch 2002; Halls et al. 2007). The handful of group I and group II splicing factors that have been studied in detail (all from non-plant systems) act by promoting intron folding (reviewed in Lambowitz et al. 1999; Lambowitz and Zimmerly 2004), but it is likely that study of the diverse introns found in plant organelles will reveal additional mechanisms. For example, in the special case of *trans*-spliced introns, proteins such as PPR4, Raa1, Raa2, and Raa3 might assist in the assembly of intron fragments.

Among chloroplast splicing factors, details of protein-intron interactions have been reported only for the CRM-domain protein CRS1. CRS1 appears to function via the first of the general mechanisms outlined above, as it binds *in vitro* with high affinity and specificity to specific sequences in domains 1 and 4 of its *atpF* intron substrate (Ostersetzer et al. 2005). The results of hydroxyl-radical footprinting suggested that CRS1 binding promotes the internalization of intron elements that are expected to be at the core of the functional ribozyme. Thus, by making high-affinity contacts with two peripheral intron segments, CRS1 seems to act like a molecular scaffold to enhance the productive folding of internal intron segments (Ostersetzer et al. 2005).

In contrast to CRS1, CRS2 does not bind with high affinity to its target introns *in vitro* (Barkan lab, unpublished observations); therefore, it seems likely that CRS2 is recruited to specific introns via its interactions with its CRM-domain partners CAF1 and CAF2. A hypothesis for CRS2’s role in splicing was suggested by the structure and sequence of its derived peptidyl-tRNA hydrolase active site, which is highly conserved in CRS2 despite the fact that CRS2 did not exhibit peptidyl-tRNA hydrolase activity when expressed in *E. coli* (Jenkins and Barkan 2001; Ostheimer et al. 2005). These observations suggest the intriguing possibility that the ancestral active site may have been subtly modified in CRS2 so that it now contributes to a chemical step in splicing.

2.4 The regulation of chloroplast RNA splicing

RNA splicing is essential for the expression of intron-containing genes, and is therefore a potential regulatory step in chloroplast gene expression. In fact, unspliced chloroplast transcripts typically accumulate to high levels, so changes in splicing efficiency are likely to be reflected by changes in the abundance of ma-

ture transcripts. Tissue-dependent changes in the ratio of spliced to unspliced chloroplast RNAs have been described for the maize *atp*F, *pet*D, *pet*B, *rpl*16, and *ycf3* introns (Barkan 1989; McCullough et al. 1992), and for the mustard *trn*G intron (Liere and Link 1994). In each of these cases, a higher proportion of transcripts is spliced in mature chloroplasts than in immature chloroplasts or in non-photosynthetic plastid forms, consistent with the possibility that an increase in splicing rates early in chloroplast development contributes to the burst in synthesis of chloroplast-encoded subunits of the photosynthetic apparatus. Although light has no apparent effect on the splicing of several chloroplast introns in vascular plants (Barkan 1989; Liere and Link 1994), light does activate the splicing of the group I introns in the *C. reinhardtii* chloroplast *psb*A gene (Deshpande et al. 1997).

These observations suggest that splicing can be developmentally regulated in plants and light-regulated in *C. reinhardtii*. Still, varying ratios of spliced versus unspliced RNAs do not prove that the rate of splicing is regulated, as this could also result from changes in the stability of the unspliced precursor with respect to that of its spliced product. Even if plastid splicing rates do change, these changes will be regulatory only if the level of spliced mRNA limits the ultimate accumulation of the protein product. Some chloroplast mRNAs are in excess of the amount needed for maximal translation in *C. reinhardtii* (Eberhard et al. 2002), so small decreases in the synthesis of these mRNAs are not anticipated to impact the level of their gene product. Nonetheless, a mutation in a group I intron in the *C. reinhardtii psbA* pre-mRNA caused a twofold reduction in both the level of spliced mRNA and the rate of PsbA protein synthesis (Lee and Herrin 2003), indicating that, at least for this mRNA, small changes in splicing efficiency effectively change the rate of synthesis of the corresponding protein.

A variety of mechanisms could potentially regulate splicing in chloroplasts. One obvious possibility is that the synthesis or activity of nucleus-encoded chloroplast splicing factors is regulated, which in turn, regulates the splicing of chloroplast introns. Unfortunately, few studies have attempted to correlate changes in the abundance of chloroplast splicing factors with changes in the splicing of their substrate RNAs; in fact, only for CRS1 has such a correlation been demonstrated (Till et al. 2001). A protein-independent mechanism for splicing regulation could involve developmentally-regulated changes in stromal [Mg^{2+}], as the folding and catalysis of group I and group II introns is dependent on Mg^{2+} (Pyle 2002) and the concentration of free Mg^{2+} rises during chloroplast maturation in spinach (Horlitz and Klaff 2000). It is also plausible that chloroplast splicing in vascular plants could change during development as a consequence of the developmental switch in the plastid transcription machinery. A nucleus-encoded phage-like polymerase (NEP) predominates early in chloroplast development, whereas a chloroplast-encoded bacterial-like RNA polymerase (PEP) predominates in mature chloroplasts (reviewed in Weihe 2004). Based on the properties of the phage and bacterial polymerases to which these enzymes are related (Iost et al. 1992), it is likely that NEP elongates more quickly than PEP. A more rapid transcription elongation rate might hinder the productive folding of chloroplast introns by reducing the length of the kinetic window during which non-native RNA partners

are excluded from interaction with nascent intron segments. This general model could be tested by comparing splicing efficiencies in engineered tobacco chloroplasts that express the same intron-containing gene driven by either a NEP or PEP promoter.

2.5 Perspective

The findings summarized here raise numerous interesting questions: By what mechanisms do splicing factors promote the activity of chloroplast introns? How is chloroplast splicing coordinated with other steps in chloroplast gene expression and assembly of the photosynthetic apparatus? Is the rate of splicing in chloroplasts subject to regulation, and if so, how is this regulation accomplished? Did the "need" to promote the splicing of intrinsically poor chloroplast introns spur the evolution of plant-specific protein families such as the CRM and PPR families? What is the nature of the coevolutionary processes through which the degeneration of "self-splicing" group I and group II introns has been compensated by the recruitment and modification of pre-existing proteins to participate in splicing?

A thorough understanding of these issues cannot emerge without a more complete catalog of the proteins involved in the splicing of chloroplast introns. Results to date suggest a complexity that was not anticipated based on studies of protein-facilitated splicing in non-plant systems, where a single protein has, in several instances, been shown to be sufficient to reconstitute protein-facilitated splicing *in vitro* (reviewed in Lambowitz et al. 1999; Lambowitz and Zimmerly 2004). Reconstitution of protein-facilitated splicing of chloroplast introns has not been reported, suggesting that essential factors remain to be identified. Indeed, the large size of the particles harboring chloroplast intron RNAs and splicing factors *in vivo* cannot be accounted for by the identified components. Moreover, genetic screens for chloroplast splicing factors in land plants and *C. reinhardtii* are not yet saturating, and the genes underlying several known splicing mutants in maize and *Chlamydomonas* have not been identified (Goldschmidt-Clermont et al. 1990, Barkan lab, unpublished). Candidate gene approaches can be anticipated to play an increasingly important role in the efforts to identify more splicing factors. Candidates for reverse genetic analyses include nucleus-encoded group II maturase homologs in land plants (Mohr and Lambowitz 2003), paralogs of the plant CRM-domain splicing factors, and members of the PPR family: with more than 450 PPR proteins encoded in the genomes of vascular plants (Lurin et al. 2004), the PPR family constitutes a large pool of potential chloroplast splicing factors. Biochemical methods such as affinity purification of proteins that associate with known splicing factors and activity-based protein purifications can complement these efforts; indeed, two proteins were purified from *C. reinhardtii* chloroplasts by virtue of their ability to bind *in vitro* to chloroplast group II intron RNAs (Balczun et al. 2006; Glanz et al. 2006).

Studies addressing evolutionary questions are also limited by the restricted knowledge of organellar splicing machineries. It can be anticipated that the functions of splicing factors identified in one land plant species will generally be con-

served in other land plants, as has been demonstrated for maize and *Arabidopsis* CRS1, CAF1, and CAF2 (Asakura and Barkan 2006). However, the more interesting questions concern the evolution of these proteins: does the emergence of specific splicing factors coincide with the appearance of the chloroplast genome organization that is characteristic of land plants, and can factors present in *Chlamydomonas* still be found in basal taxa of land plants? The availability of nuclear genome sequences of various “lower” plants will be necessary to address these questions.

Finally, to understand the role of regulated splicing in chloroplast function, it will be necessary to more thoroughly catalog changes in chloroplast splicing under various conditions, to correlate the levels of the known splicing factors with these changes, and to generate engineered organisms in which the abundance of specific splicing factors can be manipulated such that their effects on protein output can be assessed. Recent advances in the tools available for genetic and genomic analyses in chloroplast-bearing organisms should enhance progress in understanding these issues during the coming years.

3 Plastid RNA editing

RNA splicing is embedded in a series of additional RNA processing events, among them RNA editing - the modification of single ribonucleotides such that the RNA sequence does not match that of its DNA template. Indeed, a link between splicing and editing has been demonstrated for an exonic nucleotide in *ndhA* close by the 3' intron/exon border, such that only spliced mRNAs are edited (Schmitz-Linneweber et al. 2001). However, few chloroplast introns have been analyzed for editing, and it is unclear whether intron-internal editing events are instrumental in the splicing of any chloroplast introns (e.g. Bonen and Vogel 2001; Vogel and Borner 2002; Kugita et al. 2003b). Much more information is available regarding the impact of RNA editing on exonic sequences.

After the initial discovery of RNA editing in trypanosome mitochondria (Benne et al. 1986), various examples of RNA editing were described in organisms from diverse taxa (Gott and Emeson 2000). These encompass a variety of alterations of RNA primary sequence that arise from base modifications, nucleotide insertions or deletions, and nucleotide replacements. Many of the editing processes discovered to date employ widely different mechanisms and are therefore believed to be polyphyletic (Smith et al. 1997; Gott and Emeson 2000).

In chloroplasts, RNA editing is restricted to nucleotide conversions (for recent reviews, see Bock 2000; Wakasugi et al. 2001; Shikanai 2006). Only changes from C to U or – less frequently – from U to C have been observed so far. This type of RNA editing usually affects the coding potential of the mRNA. Like any other RNA editing system, plastid conversional editing depends on *cis*-acting sequences that determine the base to be edited and *trans*-acting factors that carry out site recognition and catalysis. Since its discovery, substantial progress has been

made on understanding the *cis*-elements. In contrast, despite 15 years of research, very little is known about the executing machinery.

3.1 Editing sites impact protein function

Overwhelmingly, RNA editing restores evolutionarily conserved codons and thus conserved amino acids (Hirose et al. 1996; Inada et al. 2004; Tillich et al. 2005). Moreover, the most commonly observed codon conversions lead to amino acid substitutions that differ pronouncedly in their physico-chemical properties. In several instances it was found that if editing does not occur, the affected protein is severely impaired or altogether non-functional. For example, substitution of the unedited spinach-specific *psbF* editing site for the corresponding sequence in the *psbF* gene of tobacco led to tobacco plants in which the spinach editing site remained unprocessed and that had compromised photosynthesis (Bock et al. 1994). Presumably the aberrant PsbF protein encoded by this engineered gene led to reduced activity of photosystem II, of which PsbF is a subunit (Bock et al. 1994). Analogously, the introduction of the non-edited form of maize *petB* into the chloroplast genome of *Chlamydomonas*, which shows no editing at all, led to strains that were non-phototrophic, consistent with a lack of cytochrome b_6f activity (Zito et al. 1997). The mutant phenotype was due to defective assembly of cytochrome b_6f complexes, of which PetB is a subunit, confirming that the edited codon is essential for the functional interactions of PetB with that complex (Zito et al. 1997). Also, the carboxyltransferase subunit of the acetyl-CoA carboxylase showed no activity *in vitro* when translated from a message containing an unprocessed editing site from pea (Sasaki et al. 2001). In tobacco, mutation of an edited serine codon to a tryptophan codon in the plastid *atpA* gene led to albino plants, suggesting that this codon is essential for the function of the encoded alpha subunit of the plastid ATPase (Schmitz-Linneweber et al. 2005a). RNA editing sometimes is also necessary to create an initiation codon for translation; in such cases, it seems self-evident that the editing event plays an essential role in translation. This expectation was confirmed for the *ndhD* transcript in a tobacco *in vitro* translation system: only the edited version of the *ndhD* transcript gave rise to NdhD protein (Hirose and Sugiura 1997). Loss of editing at this site obliterated the function of the NDH complex, of which NdhD is a subunit (Okuda et al. 2006).

In summary, RNA editing is crucial for the proper expression or function of the encoded protein in every case in which this has been analyzed. This implies that edited codons generally code for amino acids that are critical for protein function.

3.2 Mechanism of RNA editing

3.2.1 Biochemistry

Initially, RNA editing was investigated by two methods: 1) direct sequencing of RNA and 2) sequencing of cloned or PCR-amplified cDNA. Thus, whether the

base resulting from editing is a U or a modified C that reverse transcriptase recognizes as a U, was unclear. More recently, however, *in vitro* editing techniques and single strand conformation polymorphism assays allowed the unequivocal demonstration that uridine bases are the product of editing (Fuchs et al. 2001; Hirose and Sugiura 2001). The next question, then, was how the U is produced from the genomically encoded C.

Three reactions could in principle underlie C-to-U and U-to-C conversions: *trans*-amination, base-exchange (transglycosylation), or nucleotide replacement. In plastids, biochemical data on enzymatic aspects of RNA editing are scarce, but in plant mitochondria, which are believed to have a phylogenetically related RNA-editing system (Maier et al. 1996), it seems that the N-glycosidic bond between the ribose and the pyrimidine base remains intact (Yu and Schuster 1995). Also, in both organelles, the sugar-phosphate backbone remains untouched by editing (Rajasekhar and Mulligan 1993; Hirose and Sugiura 2001). This clearly speaks against a nucleotide excision mechanism and has led to a search for cytidine deaminases or transaminases - that is, enzymes that modify the bases while leaving the RNA backbone intact- as editing enzymes.

Although *cis*-acting sequence requirements are defined for several editing sites (see Section 3.4), it is unclear whether these sequences are presented as single-stranded RNA, part of a stem-loop or as double-stranded RNA. This question is of particular interest in light of the editing system of trypanosome mitochondria, which uses complementary guide RNAs to direct editing events. Experiments involving antisense RNAs to the tobacco chloroplast editing site *rpoB*-2 (Hegeman et al. 2005a) suggested that the edited site itself must be single-stranded whereas the adjacent *cis*-element can be either single-stranded or double-stranded. Potential guide RNAs and complementary sections inside the same transcript were computationally predicted for tobacco and hornwort chloroplasts (Bock and Maliga 1995; Yoshinaga et al. 1997), but mutation of a putative guide RNA did not inhibit RNA editing (Bock and Maliga 1995).

In *in vitro* editing systems, processing of artificial templates is highly dependent on the magnesium concentration and on the presence of hydrolysable NTP (Hirose and Sugiura 2001; Hegeman et al. 2005b; Nakajima and Mulligan 2005). This reliance on an external energy source sets plastid RNA editing apart from other C-to-U editing systems like the mammalian APOBEC system, which functions *in vitro* without added nucleotides (Driscoll et al. 1989), and may point to the involvement of an ATP-dependent RNA helicase in plastid RNA editing (Nakajima and Mulligan 2005). Both APOBEC and chloroplast editing are dependent on free zinc (Navaratnam et al. 1993; Bhattacharya et al. 1994; Hegeman et al. 2005b). Whether this means that the chloroplast editase, like the APOBEC enzyme, is a zinc-dependent cytidine-deaminase, remains to be established.

3.2.2 Kinetics

It is unclear at what point during transcript maturation RNA editing occurs or whether this is uniform among different edited transcripts. Potentially, RNA editing could be co-transcriptional, either via the incorporation of U instead of C by

RNA polymerase, or by the action of a cytidine deaminase in close contact with the nascent transcript. Alternatively, editing might occur on transcripts that have been already released from the polymerase. No definite answer regarding this question can be given at the moment, but it is clear that editing is highly efficient as most sites are fully or almost completely edited (e.g. Maier et al. 1995; Hirose et al. 1999; Peeters and Hanson 2002). There are several exceptional editing sites, however, for which a large pool of unedited RNAs accumulates (Hirose et al. 1999; Peeters and Hanson 2002; Schmitz-Linneweber et al. 2002; Inada et al. 2004; Tillich et al. 2005). Taken together, this shows that for most sites, the capacity of the editing machinery is sufficient to cope with template abundance. Whether this means that nascent transcripts or fully transcribed, released transcripts are the substrates for editing remains an open question.

Another interesting question concerns how editing relates to other processing events like RNA splicing or endonucleolytic cleavage. Some editing sites seem entirely independent of other processing steps. For instance, the *petB* and *ycf3* transcripts were fully edited regardless of whether they were spliced or still part of a polycistronic precursor (Freyer et al. 1993; Ruf et al. 1994). Other sites show a strong or even obligate link to another processing step occurring on the same precursor. This is true for editing of the *rpl2* initiation codon, which is complete in mature, spliced RNA molecules but which is rare in uncleaved and unspliced *rpl2-rpl23* precursor molecules (Freyer et al. 1993). Similarly, unspliced *ndhA* mRNAs in spinach were not edited at all (Schmitz-Linneweber et al. 2001). For *ndhD* in *Allium porrum*, RNA editing is linked to intercistronic cleavage between *psaC* and downstream *ndhD* (Del Campo et al. 2002). The translational status of an mRNA can influence editing as well: heat induced reduction of plastid translation or mutational loss of plastid ribosomes leads to a reduction in editing efficiency at specific sites (Zeltz et al. 1993; Hess et al. 1994; Karcher and Bock 1998; Nakajima and Mulligan 2001; Karcher and Bock 2002b; Halter et al. 2004). It is unclear, however, whether plastid translation acts indirectly via (i) the synthesis of a translation product that functions in editing, (ii) the co-translational recruitment of editing factors, or (iii) a change in transcript abundance via alteration of the PEP/NEP ratio, or acts directly by influencing the accessibility of the editing site.

In summary, the timing of RNA editing events relative to other RNA maturation steps is specific to each site. While at least for some sites, a link between editing and translation, splicing and/or endonucleolytic cleavage has been established, editing of other sites appears to be indifferent to the processing state of the RNA. Data on how links between editing and other steps in gene expression might be reflected by commonalities among the different processing machineries is lacking.

Table 2. Requirements of *cis*-sequences for editing sites *in vitro* and *in vivo.*

site [a]	*in vivo* (v); *in vitro* (r)	species	cis-element for RNA editing [b]	Reference
ndhB-156	r	tobacco	-10 to -5	(Sasaki et al. 2006)
ndhB-246	r	tobacco	-22 to +9	(Hirose and Sugiura 2001)
ndhB-246	v	tobacco	-12 to -2	(Bock et al. 1997)
ndhB-249	v	tobacco	-21 to -11	(Bock et al. 1997)
ndhB-277	v	tobacco	-42 to +48	(Bock et al. 1996)
ndhB-279	v	tobacco	-48 to +42	(Bock et al. 1996)
ndhF-97	r	tobacco	-15 to +5 plus -40 to -35	(Sasaki et al. 2006)
petB-204	r	pea	-20 to -1	(Miyamoto et al. 2002; Nakajima and Mulligan 2005)
petB-204	r	tobacco	-20 to -1	(Miyamoto et al. 2002)
psbE-72	r	pea	-15 to -1	(Miyamoto et al. 2002)
psbE-72	r	tobacco	-15 to -1	(Miyamoto et al. 2002)
psbE-72	r	*Arabidopsis*	-13 to +15	(Hegeman et al. 2005b;
psbL-1	v	tobacco	-16 to +5	(Chaudhuri and Maliga 1996)
psbL-1	r	tobacco	-22 to +9	(Hirose and Sugiura 2001)
rpoB-158	v	tobacco	-20 to +6	(Reed et al. 2001b)
rpoB-158	r	tobacco	-27 to +11	(Hayes et al. 2006)
rpoB-158	r	tobacco [c]	-31 to +61	(Hayes et al. 2006)
rpoB-158	r	maize	-27 to +11	(Hayes et al. 2006)

[a] Numbers refer to the codon affected by RNA editing in tobacco (not necessarily the same in the other species listed here)
[b] Not all listed elements have been mapped down to the minimal *cis*-sequence required for editing; in most cases, the indicated sequence range defines a core element sufficient for editing, which is not to say that all nucleotides of an element are also essential for editing, and which also does not exclude that longer templates lead to higher editing efficiencies
[c] Template from maize

3.3 *cis*-elements involved in plastid RNA editing

Efforts to identify *cis*-elements for RNA editing started from the hypothesis that sequences surrounding the nucleotide to be edited participate in its recognition by *trans*-factors. For example, position -1 is likely to be critical for the editing of most mRNAs, since 29 of 31 tobacco editing sites include pyrimidines at this position (Maier et al. 1992a, 1992b; Hirose et al. 1999). Moreover, editing of *ndhB* mRNAs (site V) was impaired if the U at position -1 was converted to a G (Bock et al. 1996), confirming that bases adjacent to editing sites do play a role in the reaction. Several studies demonstrated that, in addition to the upstream nucleotide a minimum sequence context is necessary and sufficient to direct editing (summa-

rized in Table 2). The early studies of this nature involved laborious *in vivo* experiments, and revealed that the recognition of most editing sites relies on short sequences immediately upstream of the edited site, most of them less than 20 nt long (Chaudhuri et al. 1995; Bock et al. 1996; Chaudhuri and Maliga 1996; Reed et al. 2001b). No consensus sequence could be identified for these sites or any other sequences 5' to editing sites, nor could a consensus secondary structure be identified. Recently however, inter-site homologies were found in the 15 nt upstream of editing sites when all editing sites of *A. capsillus-veneris* and *A. formosae* were compared (Tillich et al. 2006a). These homologies do not allow generation of a consensus for all sites but rather point to small clusters of similar sites, at least in angiosperms (Chateigner-Boutin and Hanson 2002; Chateigner-Boutin and Hanson 2003; Tillich et al. 2005). Indirect evidence suggests that the members of each cluster of related *cis*-sequences are recognized by the same *trans*-factor (see Section 3.4).

Recently, *in vitro* editing systems have become available for four species: tobacco (Hirose and Sugiura 2001), pea (Miyamoto et al. 2002; Nakajima and Mulligan 2005), maize (Hayes et al. 2006), and *Arabidopsis* (Hegeman et al. 2005b). They have been used to dissect *cis*-elements at higher resolution. These studies confirmed the predominant role of 5' sequences over 3' sequences for determining editing efficiency, and showed further that nucleotides inside the *cis*-element do not contribute equally to editing. In particular, the nucleotides immediately preceding the editing site (one to four depending on the specific site) and the editing site itself are not essential for binding of the *trans*-factor(s), although they are required for the reaction itself (Miyamoto et al. 2002). Closer inspection of the proximal bases for the two editing sites in *psbL* and *petB* revealed that the sequence of these elements is recognized in a highly specific manner (Miyamoto et al. 2004). Thus, both binding of the site as well as catalysis after binding require sequence-specific interactions.

In addition to sites that require a short sequence element immediately upstream of the edited C, there are also reports of more complex *cis*-elements. For instance, the *cis*-element of the editing site in the tobacco *ndhF* mRNA is bipartite, with essential elements spaced by 19 nt (Sasaki et al. 2006). For other sites, editing efficiency increases with longer templates, although additional elements outside the usual -20 to +6 core are not essential (Hayes et al. 2006). Rarely, though, more distant putative elements can be essential as suggested by editing site *ndhB*-2 and -3, which were not edited *in vivo* despite 42 nt of both 5' and 3' adjacent sequences (Bock et al. 1996). In this context it is interesting that the more distant context of an editing site can determine how critical point mutations in the core element are: a point mutation 20 nt upstream of editing site *rpoB*-158 abolished editing in a construct stretching from -27 to + 6, but had little effect in a construct only little longer (-31 to +22, Hayes et al. 2006). This suggests that editing sites with short essential *cis*-elements have additional, non-essential elements farther away from the editing site that can compensate for mutations in the core elements. In fact, most editing sites are poorly edited *in vitro*, not reaching efficiencies greater than 10% (Sasaki et al. 2006) despite the high editing efficiency *in vivo*. This is also true for most studies in which short sequences around editing sites were intro-

duced into chloroplasts by biolistic transformation. In these experiments, editing of the short transgenes was low (Bock et al. 1996; Reed et al. 2001a). Whether this low editing efficiency is solely due to the overexpression of introduced editing sites, which overburdened the editing apparatus, or whether important distal sequence elements were lacking in these constructs remains to be determined.

In summary, essential elements for RNA editing are mostly situated immediately 5' to the edited site, but this does not exclude the possibility that additional elements contribute to editing efficiency. Given that the translational and processing status of the edited message contributes to editing efficiency (see Section 3.2), it seems likely that further sequence elements will play into determining editing efficiency.

3.4 *trans*-factors involved in plastid RNA editing

The finding that there is no clear consensus for editing site recognition led to the proposal that each site is served by its own specific factor, presumably an RNA binding protein. This was supported by titration studies, in which overexpression of an introduced site leads to a reduction in editing of the endogenous site, but not of any other site examined (Chaudhuri et al. 1995). Later, this conclusion was modified due to the finding that smaller clusters of related editing sites may exist and that titration of one factor could affect several related sites (Chateigner-Boutin and Hanson 2002, 2003). However, given the small size of these clusters (usually two to three sites), a substantial set of factors would still be needed to serve all sites. The nature of these factors has long been elusive. Experiments transferring plastids between different species demonstrated that at least some of these specificity factors are nuclear-encoded (Bock and Koop 1997). This finding was not entirely unexpected as the well-annotated and small plastid chromosome was unlikely to code for dozens of hitherto unidentified editing factors. Still, small RNAs functioning as editing specificity factors might be hidden in the chloroplast genome. In trypanosome mitochondria, small so-called guide RNAs (gRNAs) form Watson-Crick base pairs with pre-mRNAs thereby specifying RNA editing sites. To assess the involvement of gRNAs in chloroplast RNA editing, tobacco *in vitro* editing extracts were treated with RNAse. This did not abolish editing activity of the treated extracts, which suggests that editing factors are not ribonucleic acids but rather of a proteinacious nature (Hirose and Sugiura 2001). Attempts to identify putative guide RNAs by crosslink strategies were also unsuccessful (Hirose and Sugiura 2001), which further strengthens the supposition that it is proteins rather than RNAs that do the main job in plastid RNA editing (see Section 3.1).

A first major advance in identifying *trans*-factors for RNA editing came from studies on proteins bound in the sequence environment of editing sites. Using a stromal extract competent for *in vitro* editing, Sugiura and colleagues were able to UV-crosslink several proteins to short bait-RNAs containing editing sites (Hirose and Sugiura 2001). First, they found the highly abundant cpRNP proteins, which contain two RNA recognition motifs (RRMs) and an additional acidic domain.

Table 3. Potential and confirmed editing factors

factor	target site [a]	species	references
cp31	psbL-1, ndhB-246	tobacco	(Hirose and Sugiura 2001)
CRR4	ndhD-1	*Arabidopsis*	(Kotera et al. 2005)
p25	psbL-1	tobacco	(Hirose and Sugiura 2001)
p70	psbE-72	tobacco/pea	(Hirose and Sugiura 2001; Miyamoto et al. 2002)
p56	petB-204	tobacco/not in pea	(Hirose and Sugiura 2001; Miyamoto et al. 2002)

[a] Numbers refer to the codon affected by RNA editing

These proteins were bound to all editing sites they provided as targets suggesting that binding was nonspecific and had nothing to do with editing. Surprisingly however, after depleting their *in vitro* editing extract of one of these RRM proteins, cp31, they did observe inhibition of editing at the two sites tested (Hirose and Sugiura 2001). Depletion of other cpRNP proteins, some of them closely related to cp31, did not lead to this effect. In complementation studies, they could show that the acidic domain of cp31 is necessary for editing. In conclusion, cp31 appears to be a general editing factor, probably acting via its acidic domain.

In addition to cpRNPs, factors specific to selected editing sites were identified by UV crosslinking (Table 3). In tobacco, editing sites *psbL*, *psbE*, and *petB* were associated with proteins of 25, 56, and 70 kD, respectively. All three proteins could be titrated off the bait with a sequence-specific competitor, but not with unrelated sequences (Hirose and Sugiura 2001). Similarly, in pea, the *petB* editing site was also specifically associated with a 70 kD protein (Miyamoto et al. 2002). No sequence information for any of these proteins has been reported.

A breakthrough in the search for specificity factors involved in chloroplast RNA editing came from researchers originally interested in other features of chloroplast biogenesis. Shikanai and colleagues were studying the chloroplast NADH dehydrogenase (NDH) complex and isolated mutants affected in the activity of this complex. In an elegant forward genetic screen in *Arabidopsis*, they isolated numerous nuclear mutations that caused the loss of NDH complex activity (Hashimoto et al. 2003). Most of the subunits of the NDH complex are encoded on the chloroplast chromosome by *ndh* genes A through K, which contain several editing sites. One of the mutants isolated, *chlororespiratory reduction 4* (*crr4*), exhibited an editing defect of the *ndhD* start codon, while no other *ndh* editing site was affected (Kotera et al. 2005). Transcript patterns for the *ndh* genes in *crr4* mutants did not deviate from wild type, indicating that the encountered editing defect is likely not a secondary effect of the *crr4* mutation. Later, the authors provided *in vitro* evidence for a specific, direct interaction of CRR4 with the *ndhD* editing site (Okuda et al. 2006). Positional cloning revealed that the *crr4* gene encodes a member of the pentatricopeptide repeat (PPR) protein family (Kotera et al. 2005).

Intriguingly, PPR proteins had long been candidates for editing factors (Small and Peeters 2000; Lurin et al. 2004). These proteins are defined by the PPR motif (Small and Peeters 2000), which is discussed above in the context of chloroplast

RNA splicing. PPR family members have been found in diverse eukaryotic species with usually only a handful of genes per genome, but in embryophytes, the PPR lineage has greatly expanded, with over 450 members in *Arabidopsis* and rice. Most PPR proteins are predicted to be targeted to either mitochondria or chloroplasts, and a string of recent genetic studies suggests that they are generally involved in various aspects of organellar RNA metabolism (e.g. PPR4 and HCF152 in Section 2.3.3 above; reviewed by Small and Peeters 2000; Lurin et al. 2004). The common functions in RNA metabolism for many PPR proteins suggested that PPR proteins bind RNA, but for only a few plant PPR proteins has RNA association *in vivo* (Schmitz-Linneweber et al. 2005b, 2006) or RNA binding *in vitro* been demonstrated (Lahmy et al. 2000; Nakamura et al. 2003; Lurin et al. 2004). The editing factor CRR4 is one of these few: recombinant CRR4 binds to a short segment (-25 to +10) surrounding the *ndhD* start codon editing site in a sequence specific manner and with high affinity (Okuda et al. 2006). This suggests that CRR4 indeed is the factor conferring sequence specificity to this particular editing reaction. In tobacco, *ndhD*-1 has been clustered with two other editing sites, *rpoB*-3 and *ndhF*-2 (Chateigner-Boutin and Hanson 2002). Overexpression of *ndhF*-2 leads to a reduction in editing of the two related sites, suggesting that they share the same specificity factor (Chateigner-Boutin and Hanson 2002). Sites *ndhF*-2 and *ndhD*-1 are also present in *Arabidopsis*, while *rpoB*-3 has been lost. Although CRR4 is not essential for editing *ndhF*-2, it would be still interesting to test whether it binds to this site. In general, it remains an exciting prospect to test other PPR proteins that are evolutionarily or structurally related to CRR4 for a potential role in editing of other sites.

3.5 Models for the editosome

There are two competing models for the machinery responsible for editing site recognition and catalysis. Both models propose a host of specificity factors akin to CRR4 that dock to target *cis*-elements in a highly specific manner. The PPR family of RNA binding proteins is large enough to fill this job easily, but it is too early to exclude roles for other types of RNA binding proteins. The second pressing question is how catalysis occurs; this aspect is addressed differently by the two models.

The original model for the RNA editing apparatus proposed that site recognition factors like PPRs are only a platform for a common factor with enzymatic activity that serves all sites (Fig. 2). Such an activity has not been isolated so far, maybe because a knockout of a general editing enzyme would be gametophyte or embryo-lethal. Of course, cytidine deaminases have been on top of the candidate list for such a general editase, but the few studies on these enzymes did not find any evidence for their involvement in editing (Faivre-Nitschke et al. 1999). Another finding supporting the two-factor-model is that plastid-localized PPRs have been shown to reside in large ribonucleoprotein complexes, presumably together

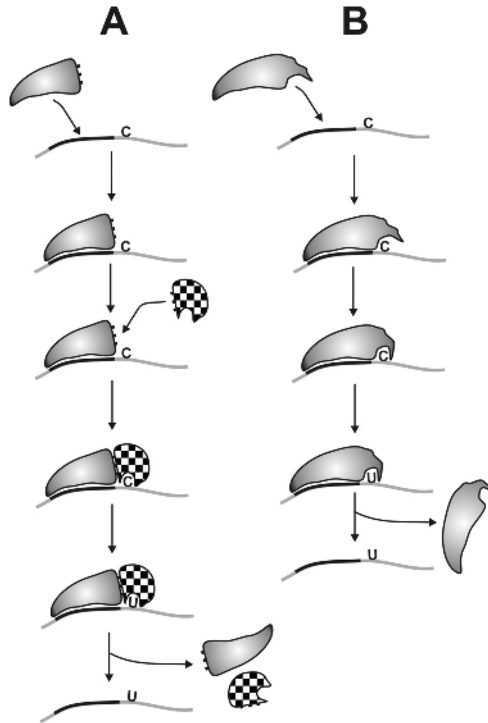

Fig. 2. Two models for the plastid editing machinery. A) Two-component model; a site specific factor (grey) recognizes a *cis*-element (black) upstream of an editing site (C). It forms a platform for an additional factor, the editase (checker pattern) that possesses an activity for converting Cs to Us, but is not necessarily an RNA-binding protein. After catalysis, factors might dissociate from the RNA. B) One-component model; the site-specific factor makes contacts with the *cis*-element, but also directly interacts with the editing site and is sufficient or at least required for catalysis.

with their RNA targets and additional proteins (Meierhoff et al. 2003; Williams and Barkan 2003; Schmitz-Linneweber et al. 2005b). Whether these additional proteins are important for editing is one of the more exciting questions in the field.

The second model for catalysis by the editosome was put forward by Sugiura's group after their finding that the specificity factor p56 makes contacts not only with the upstream *cis*-element, but also with the editing site and the adjacent nucleotides *in vitro*, although these latter interactions are comparatively weak (Hirose et al. 2004; Miyamoto et al. 2004). Consistent with this result, CRR4 also binds with a slight preference to non-edited rather than to pre-edited *ndhD* RNA (Okuda et al. 2006). It seems therefore possible that specificity factors work in a two-step mode, making first solid contact with upstream *cis*-elements and then in a second step also interact with the editing site itself to permit catalysis (Fig. 2). If this model is correct, specificity factors may have different functional protein domains, those for RNA binding and others for catalysis. In fact, many PPR proteins

have additional protein domains other than the PPR tract itself. A large subgroup of more than 87 proteins in *Arabidopsis* possesses for example a so-called DYW domain, to which no function has yet been assigned (Lurin et al. 2004). Other domains without known function are present in many PPRs as well. Potentially, these domains could carry out editing catalysis. In the end, both models may be correct: considering the number of sites to be served and the differences in *cis*-elements, there might well be different solutions for the recognition and catalysis of different sites.

3.6 Function and evolution of plastid RNA editing

3.6.1 Evolution of editing sites

Chloroplast RNA editing is widespread in land plants. Of the taxa studied so far, only the marchantiid liverworts do not seem to have RNA editing (Freyer et al. 1997; Duff and Moore 2005). Members of other ancient embryophyte taxa like *Adiantum capillus-veneris* of the ferns (Wolf et al. 2004), *Physcomitrella patens*, and *Takakia lepidozioides* of the mosses (Miyata et al. 2002; Sugita et al. 2006), or *Anthoceros formosae* and other hornworts (Yoshinaga et al. 1996; Yoshinaga et al. 1997; Kugita et al. 2003b; Duff and Moore 2005) each display chloroplast RNA editing. For example, 509 C-to-U and 433 U-to-C editing sites were found in the chloroplast of *A. formosae* (Kugita et al. 2003b). By contrast, spermatophytes exhibit only about 30 C-to-U editing events and no U-to-C editing (Maier et al. 1996; Tsudzuki et al. 2001). Some editing sites are conserved even between vastly divergent embryophyte taxa like ferns and dicots, but most editing sites are restricted to a more narrow taxonomic range (Tillich et al. 2006a). Even between species of the same genus, differences in editing sites were observed (Sasaki et al. 2003). This led to the conclusion that editing sites evolve rapidly (Freyer et al. 1997; Schmitz-Linneweber et al. 2002; Fiebig et al. 2004), at rates similar to those of synonymous codon positions (Shields and Wolfe 1997). This also means that no stabilizing selection acts on editing sites; that is, whether C-to-T editing occurs or whether a T is already encoded on the genomic level does not seem to influence chloroplast function. This apparent futility of RNA editing is reflected in the absence of any data that would support a regulatory role of RNA editing. Most sites are edited at high efficiencies in various tissues. Fluctuations in the ratio between edited and unedited messages over time and space or in response to environmental clues – a prerequisite for regulation – have only been rarely observed (Bock et al. 1993; Ruf and Kössel 1997; Hirose et al. 1999; Karcher and Bock 2002b, 2002a; Miyata and Sugita 2004). Even if quantitative changes in editing efficiency do occur, such effects are expected to be superceded by the much larger variations in abundance of the respective transcripts (Peeters and Hanson 2002). Thus, it is not surprising that the functional significance of quantitative differences in editing efficiency has in no case been established. Nor has the restoration of cryptic translational start codons by editing been shown to impact regulation of protein synthesis (Hirose and Sugiura 1997).

In summary, it is not regulation but simply the generation of conserved codons that makes RNA editing important for chloroplast gene expression. In fact, in the one case where the C of an editing site has been replaced by a T on the genomic level, no deviant phenotype was observed (Schmitz-Linneweber et al. 2005a). This raises the obvious question of why edited Cs are not ultimately substituted by Ts in the DNA. A potential answer is that these edited Cs are simply very stable in evolutionary terms. As a matter of fact, the plastid chromosome in its entirety is evolving rather sluggishly, with a mutation rate lower than that encountered in the nucleus (Palmer 1990; Lynch et al. 2006). In addition, certain sites are less likely to be mutated than others, depending on the identity of the immediate neighboring bases. For Cs in spermatophyte organelle DNA, the context with the lowest C-to-T transition rate is a preceding T and a trailing A: tCa (Morton et al. 1997, 2003). Intriguingly, there is a striking bias towards such a tCa context around editing sites (Tillich et al. 2006a). Apparently, editing sites occur mainly in places where regular point mutations are rare and it might be faster (in evolutionary terms) to evolve a *trans*-acting factor in the nucleus that disposes of an unwanted C at the RNA level. Hence, RNA editing would be compensating for a lack of variation at certain genomic sites, providing an alternative to regular point mutations (Tillich et al. 2006b). It has been calculated that this can only occur in genomes that are slowly evolving, because otherwise, the disadvantage of maintaining *cis*-sequences that are prone to mutation defects would be too great (Lynch et al. 2006). This is consistent with the fact that more rapidly evolving genomes like those of animal mitochondria do not support RNA editing.

3.6.2 Evolution of trans-factors

The paucity of data on *trans*-factors for RNA editing precludes any detailed delineation of trends in *trans*-factor evolution. Still, indirect data on the presence of editing activities in heterologous experiments allow some general conclusions on the evolution of nuclear-encoded *trans*-factors.

Editing sites have been artificially transferred between species by basically two methods: introduction via particle gun transformation or transfer of whole plastid genomes via cybridization. Here, only sites not present in the recipient's plastid genome, so-called foreign or heterologous sites, are considered. The first foreign sites introduced into tobacco were maize site *rpoB*-4 and spinach site *psbF*-1, neither of which was edited (Bock et al. 1994; Reed and Hanson 1997). Similarly, four sites introduced by cybridization in tobacco and *Atropa belladonna* remained unedited in the genomic background of the host species (Schmitz-Linneweber et al. 2005a). In addition, no editing of a tobacco-specific editing site was found in a pea *in vitro* editing system (Miyamoto et al. 2002). This was taken as evidence that the cognate editing factors are evolving rapidly and seemed in accordance with the rapid evolution of editing sites themselves. The picture became more complicated when four examples for the processing of heterologous editing sites were reported (Schmitz-Linneweber et al. 2001, 2005a; Tillich et al. 2006b). These unexpected findings demonstrated that there is a subgroup of editing factors that are conserved between plant taxa independently of their target sites. At the

moment, only speculative answers exist as to the reason for their survival despite the absence of their cognate target site. Possibly, such factors are retained because they edit additional sites in plastid transcriptomes as part of a related cluster of sites. Alternatively, these evolutionarily stable factors have additional functions unrelated to editing that provide a selective force for keeping them.

3.7 Perspectives

In comparison to what is known on RNA editing phenomena in humans and trypanosomes, research on plastid RNA editing is lagging far behind. A particularly serious gap is our lack of data on the editing apparatus, the plastid editosome. What are the factors, what is their chemistry, where did this machinery come from and how did it evolve? All these questions remain unanswered despite 15 years of research since the discovery of RNA editing in plastids (Hoch et al. 1991). The recent cloning of the first specificity factor for plastid RNA editing, a PPR protein, may mark the beginning of a rapid elucidation of the machinery behind this enigmatic processing step in the life of chloroplast RNAs.

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