Chapter 14

Expression Profiling of Organellar Genes

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Summary

Due to their endosymbiotic origin, expression of plastid and mitochondrial genes retains several features of prokaryotes. Nevertheless, plant organelles acquired novel specific traits during evolution. Furthermore, due to the migration of many genes to the nucleus of the host

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cell, complex anterograde and retrograde signalling pathways evolved to coordinate gene expression in different subcellular compartments. Control of gene expression in plant organelles occurs at the transcriptional and posttranscriptional levels. In this chapter, we analyze the available data concerning the variability shown by both organelle genomes for different steps of gene expression in various genotypes or after environmental and developmental cues. Genotypic variability for the extent of RNA editing or transcript processing and stability in cytoplasmic organelles has been observed in natural populations at the interspecific and intraspecific level or in artificial CMS lines. The role of various plastid genes in global genome expression and chloroplast development has been highlighted in knock-out lines produced by plastid transformation. Significant differences in the transcriptome, editome and translatome have also been found comparing different plastid types in diverse organs or tissues. Similar differences have been found for mitochondrial genomes during the diurnal cycle or between cell suspensions and differentiated leaves. However, the precise level and mechanisms at which these changes are achieved and the signals necessary for their installation are barely understood.

I. Introduction

According to the endosymbiotic theory, plastids and mitochondria derive from early prokaryotic organisms engulfed by a proto-eukaryotic cell (Buchanan et al. 2000). The origin and the following evolution of both plant organelles have had important implications not only for the structure of their genomes, but also for the expression of their genes.

Expression of plastid and mitochondrial genes retain several features of prokaryotes, e.g. the common, albeit not exclusive, organization in operons, implying co-transcription of individual genes, similar RNA polymerases and promoters (in plastids), similar structure of mature mRNAs, the presence of 70S-type ribosomes, and others. Nevertheless, different from bacteria and other prokaryotes, plant organelles show novel traits, such as uncoupled transcription and translation, phage-type RNA polymerases, and frequent RNA editing and splicing of transcript precursors. Furthermore, during evolution, many genes originally present in the endosymbionts' genomes migrated to the nucleus of the host cell. Since in many cases nuclear and organelle genes encode subunits of the same protein complexes, their expression needs to be somehow co-regulated, implying complex anterograde and retrograde signalling pathways between different subcellular compartments (Bräutigam et al. 2007).

Control of gene expression in plant organelles occurs at the transcriptional and post-transcriptional levels, the latter including regulation of transcript maturation and stability, translation, protein stability and activity (Bollenbach et al. 2007; Liere and Börner 2007; Peled-Zehavi and Danon 2007; Schmitz-Linneweber and Barkan 2007). The recent development of novel technologies, in particular DNA arrays, allowed the genome-wide analyses of gene expression in different genotypes, tissues, and environmental conditions. In several cases, the concomitant analysis of nuclear genes involved in organelle gene expression allowed to dissect interorganellar regulatory pathways (Biehl et al. 2005).

In this chapter, after reviewing basic aspects of gene expression in plastids and plant mitochondria, we analyze the available data concerning the variability shown by both organelle genomes for different steps of gene expression in various genotypes or in response to environmental and developmental cues.

Abbreviations: ACCase – Acetyl-CoA carboxylase; – AOX – Alternative oxidase; CMS – Cytoplasmic male sterility; NEP – Nuclear encoded polymerase; PEP – Plastid encoded polymerase; PPR – Pentatricopeptide repeat; PSI – Photosystem I; PSII – Photosystem II; RNAP – RNA polymerase

II. Regulation of Gene Expression in Plant Organelles

A. Transcription

In plastids, the RNA-synthesizing activity is carried out by two enzymes of different evolutionary origins. A plastid-encoded RNA polymerase (PEP) is homologous to the eubacterial RNA polymerase also found in cyanobacteria, the closest extant bacterial relatives of plastids (Mereschkowsky 1905; Kaneko et al. 1996). The plastid genome encodes the core subunits of the bacterial-type RNA polymerase, consisting of the four proteins RpoA, RpoB, RpoC1 and RpoC2. The corresponding genes were identified in the first completely sequenced plastomes of *Marchantia polymorpha* and *Nicotiana tabacum* (Ohyama et al. 1986; Shinozaki et al. 1986). A second RNA-synthesizing activity is carried out through a nuclear-encoded RNA polymerase (NEP) with homology to phage-type RNA polymerases (Lerbs-Mache 1993). The genome of *Arabidopsis thaliana* contains three copies of *RpoT* genes designated as *RpoTm*, *RpoTmp*, and *RpoTp*, indicating sub-cellular localization in mitochondria (m) and/or plastids (p) (Hedtke et al. 1997, 2000; Chang and Stern 1999; Kobayashi et al. 2001). Although plastid genes encoding proteins involved in gene expression, including PEP, are preferentially transcribed by the NEP enzyme, several chloroplast genes are driven from promoters for both polymerases. The PEP enzyme transcribes predominantly photosynthesis-related genes (Allison et al. 1996; Hajdukiewicz et al. 1997; Silhavy and Maliga 1998; Liere and Maliga 1999). It was hypothesized that the NEP polymerase is activated early in chloroplast development resulting in transcription of PEP which, in turn, activates photosynthesis-related genes (Mullet 1993). Although a recent study could show that both enzymes are already present in seeds (Demarsy et al. 2006), transcripts encoding the gene expression machinery, which are predominantly transcribed by NEP, peak in their maximal abundance earlier during chloroplast development than the predominantly PEP-transcribed photosynthesis genes (Baumgartner et al. 1993).

Although it is known that auxiliary factors are required for efficient transcription initiation in vivo in plastids (Kühn et al. 2007), experiments to identify and characterize factors involved in NEP promoter recognition and transcription initiation have failed so far. By contrast, plastids require, like bacteria, additional σ-like factors for correct PEP promoter recognition. Whereas just one σ-like factor is known in *Chlamydomonas reinhardtii* (Carter et al. 2004; Bohne et al. 2006), six σ-factors, designated as Sig1-6, are encoded in the nuclear genome of *A. thaliana* (Isono et al. 1997b; Tanaka et al. 1997; Kanamaru et al. 1999; Fujiwara et al. 2000). They have a general role in transcription, recognize certain promoters or respond to environmental stimuli (for review see Shiina et al. 2005; Liere and Börner 2007).

Unlike plastids, plant mitochondria rely entirely on nuclear encoded RNA polymerases for transcription. Recent studies have shown that *RpoTmp* could be involved in the transcription of specific genes in mitochondria (Kühn et al. 2009) and requires additional protein partners to recognise specifically promoter sequences, as observed in human and yeast mitochondria (Tracy and Stern 1995). Contrary to vertebrates, where transcription is initiated at a single site on each DNA strand, plant mitochondrial transcription is initiated at multiple sites. Moreover, transcription of single genes can be initiated by multiple promoters (Lupold et al. 1999; Kühn et al. 2005). Promoter sequences of the *A. thaliana* mitochondrial genome often contain the consensus motif YRTA, although transcription can also be initiated at non-canonical sites that lack any kind of recognizable consensus motif (Binder and Brennicke 1993; Kühn et al. 2005; Remacle and Maréchal-Drouard 1996; Fey and Maréchal-Drouard 1999).

Inverted repeat sequences forming stem-loop structures in 3′-UTR of transcripts are present both in plastids and mitochondria. However, they were rather found to be

involved in RNA maturation than in transcription termination (Dombrowski et al. 1997; Hoffmann et al. 1999). Indeed, despite the complex transcription mechanisms, it appears that posttranscriptional processes have a major role in the regulation of gene expression in both organelles and often override changes at the transcriptional level (Giegé et al. 2000; Holec et al. 2006; Bollenbach et al. 2007; Liere and Börner 2007; Peled-Zehavi and Danon 2007; Schmitz-Linneweber and Barkan 2007).

B. RNA Editing

The term RNA editing comprises a variety of single nucleotide alterations which change the genetic information at the RNA level. Editing was initially described in trypanosome mitochondria (Benne et al. 1986) and includes alterations like nucleotide insertions or deletions as well as nucleotide modifications and replacements. In higher plant chloroplasts and mitochondria, RNA editing (see also Chap. 13) is restricted to C-to-U conversions (Covello and Gray 1989; Gualberto et al. 1989; Hiesel et al. 1989; Shikanai 2006; Liere and Börner 2007; Stern et al. 2010), while less frequent U-to-C conversions have been reported in fern, hornwort and lycophyte organelles (Malek et al. 1996; Knoop 2004; Wolf et al. 2004; Duff and Moore 2005; Shikanai 2006; Takenaka et al. 2008). Several common features of the editing process in chloroplasts and mitochondria suggest a common evolutionary origin of the two organelle editing systems (Freyer et al. 1997; Tillich et al. 2006).

To date, 34 editing sites are known in *A. thaliana* plastids (Chateigner-Boutin and Small 2007), representing a typical number of editing sites found in vascular plant chloroplasts. In comparison, RNA editing affects over 500 cytidines in mitochondria (Giegé and Brennicke 1999; Chateigner-Boutin and Small 2007; Zehrmann et al. 2008), although the number of editing sites per gene is highly variable. In Arabidopsis mitochondria, complex I and CCM (cytochrome *c* maturation) mRNAs have the highest RNA editing frequencies (Giegé and Brennicke 1999). There exist a few examples for editing occurring in non-coding regions such as introns (Chateigner-Boutin and Small 2007), but most editing events restore conserved amino acids and create start or stop codons (Chapdelaine and Bonen 1991; Hoch et al. 1991; Neckermann et al. 1994; Maier et al. 1995; Giegé et al. 2004; Miyata and Sugita 2004; Okuda et al. 2006). In addition, it often affects positions that appear to be essential for the respective protein functions (Bock et al. 1994b), although in 10% of cases RNA editing is "silent" since the third position of a codon is affected and the amino acid identity is unchanged (Giegé and Brennicke 1999). In maize mitochondria, the editing of a *nad7* intron is required for its proper folding and thus for efficient splicing (Carrillo and Bonen 1997). Similarly, RNA editing is required for the proper folding of mitochondrial tRNAs, which is a prerequisite for their maturation (Maréchal-Drouard et al. 1996a, b; Kunzmann et al. 1998).

The partial RNA editing at some sites and the consequent heterogeneous populations of transcripts (Chateigner-Boutin and Hanson 2003; Bentolila et al. 2008) could lead to the synthesis of different forms of individual proteins. Although both the edited and unedited versions of the plastid *ndhD* transcript are associated with ribosomes, edited transcripts are highly enriched in the most actively translated polysome fractions (Zandueta-Criado and Bock 2004). Other studies have shown that only the proteins resulting from fully edited transcripts accumulate in mitochondria or plastids and no examples exist showing that an unedited protein has a function within organelles (Grohmann et al. 1994; Lu and Hanson 1994; Phreaner et al. 1996). This suggests that translation of partially edited transcripts could be inhibited and/or that proteins resulting from partially edited RNA are instable and rapidly degraded. Hence, it has been hypothesized that instead of a regulatory role, the primary function of RNA editing could have been

to correct genomic mutations that appeared during the invasion of land by plants and thus to enable the translation of functional proteins (Shikanai 2006; Takenaka et al. 2008).

The exact editing mechanism still remains elusive. The analysis of the hundreds of editing sites present in the plant mitochondrial transcriptome (Giegé and Brennicke 1999; Bentolila et al. 2008) has not enabled to define specific consensus signals around editing sites. However, the distribution of nucleotides around the sites is not random because a strong preference for pyrimidines is observed for the two nucleotides immediately upstream of the sites (Giegé and Brennicke 1999). Both in plastids and mitochondria *trans*-acting factors are involved in recognition of the endogenous editing sites (Chaudhuri et al. 1995; Bock and Koop 1997). So far, several pentatricopeptide repeat (PPR) proteins, encoded by a gene family with more than 450 members in *A. thaliana* and characterized by repeated motifs of a degenerate 35 amino-acid consensus, were found to be involved in editing site recognition (Small and Peeters 2000; Kotera et al. 2005; Okuda et al. 2006, 2007; Kim et al. 2009; Zehrmann et al. 2009; Tasaki et al. 2010; Verbitskiy et al. 2010) although, so far, it could not be shown that these proteins actually carry out the deamination reaction, which converts the cytidine to a uridine (Hirose and Sugiura 2001).

C. RNA Processing

Plastid and mitochondrial genes are often, like in their bacterial ancestors, transcribed from operons resulting in polycistronic transcripts. Numerous rearrangements that occurred during the evolution of plant mitochondrial genomes have led to the loss of ancient syntenies of gene organisation into functional units (Schuster 1993; Giegé et al. 2000). As a consequence, co-transcription often does not involve genes of related function. This phenomenon is somewhat less widespread in plastids, where genes encoding subunits of the same protein complex are more frequently present in the same operon. Generally, prior to protein synthesis, transcripts are cleaved intercistronically and their 5′ and 3′ ends undergo maturation steps.

5′ ends of chloroplast transcripts are either unprocessed and then characterized by a 5′ di- or triphosphate or carry a 5′ hydroxyl group in the case of processed mRNAs. The enzymes catalyzing these processing steps are so far unknown (for review see Bollenbach et al. 2007). In contrast, the mechanisms of 3′ end maturation are known in much greater detail. Transcription termination is rather inefficient in plastids, resulting in the requirement of 3′ end processing. This involves the binding of a high molecular weight complex downstream of the stem-loop structures formed by inverted repeats (reviewed in Stern and Kindle 1993; Hayes et al. 1999). A second mechanism for mRNA stabilization is the binding of PPR proteins (Pfalz et al. 2009).

Similarly, the maturation of plant mitochondrial precursor transcripts involves 5′- and 3′-maturation steps. These maturations could be achieved through direct endoribonuclease activities and/or with 5′-to-3′ exoribonucleases and 3′-to-5′ exoribonucleases. Such enzymes are encoded in the nucleus and must be imported from the cytosol. In higher plant mitochondria, no 5′-to-3′ exoribonuclease has been identified yet. In contrast, two 3′-to-5′ exoribonucleases were characterised: RNase II is dually localised in mitochondria and plastids and a polynucleotide phosphorylase (PNPase) is found in mitochondria. Studies suggest that the 3′-processing of mitochondrial transcripts is at least a two-step phenomenon (Gagliardi et al. 2001; Perrin et al. 2004a, b). tRNAs are also transcribed as precursor molecules and have to be matured at their 5′- and 3′-ends. These maturation steps are performed by two ubiquitous endoribonuclease activities called RNase P and RNase Z, respectively (Vogel et al. 2005; Canino et al. 2009; Gobert et al. 2010; Jonietz et al. 2010).

Splicing is an essential process in RNA maturation in plant organelles as introns disrupt reading frames of important genes

involved in photosynthesis or gene expression. Twenty out of the 21 plastid introns found in land plants belong to group II introns and the remaining intron in the *trnL-UAA* is a group I intron (reviewed in Saldanha et al. 1993). Factors involved in plastid intron splicing are mostly encoded in the nucleus. A single maturase-like protein, MatK, is encoded in the *trn*K gene intron and is potentially involved in splicing of several group II introns (Liere and Link 1995; Jenkins et al. 1997). A rather unusual intron is one of the two introns in the *rps12* gene. This bipartite gene is encoded at two distant locations in the plastid genome, splitting this intron into two separate parts. Thus, two precursor mRNAs are generated which are joined together in a *trans*-splicing event (Hildebrand et al. 1988).

In seed plant mitochondria, group II introns are found in several genes (Unseld et al. 1997; Bonen and Vogel 2001), while only one example of a recently acquired group I intron has been found in the *cox1* genes of *Peperomia* and of some other plants (Vaughn et al. 1995; Cho et al. 1998; Grewe et al. 2009). The genes encoded in the Arabidopsis mitochondrial genome are interrupted by altogether 23 group II introns with sizes varying from 485 to about 4,000 nucleotides (Unseld et al. 1997). Some genes are interrupted by more than one intron, e.g. *nad7* has four introns. *Trans*-splicing is found in plant mitochondria in several instances (e.g. in the *nad1*, *nad2* and *nad5* genes), (Chapdelaine and Bonen 1991; Knoop et al. 1991; Wissinger et al. 1991; Binder et al. 1992; Glanz and Kück 2009). The highly conserved structure of the group II introns and specific protein factors, called maturases, are essential for splicing activity (Wank et al. 1999; Lambowitz and Zimmerly 2004; Meng et al. 2005; Fedorova and Zingler 2007). In plant mitochondria, one conserved gene encoding such a maturase, MatR, is located in the terminal *nad1* intron. Moreover, several nuclear genes and nucleus-encoded proteins, involved in splicing in chloroplasts and, putatively, in mitochondria were identified (Mohr and Lambowitz 2003; Nakagawa and Sakurai 2006; Keren et al. 2008).

Total RNA abundance also depends on the rate of transcript degradation. It has been shown in spinach and barley that plastid mRNA stability can highly vary during leaf development and therefore also accounts for transcript abundance (Klaff and Gruissem 1991; Kim et al. 1993). Lack of ribosome association can result in mRNA degradation which has been shown for the *rbcL* transcript, but this mechanism cannot be generalized as many other transcripts remain unaffected despite decreased ribosome association (Barkan 1993). The actual RNA degradation pathway in plastids involves polyadenylation (Kudla et al. 1996), a process which is known to be a stabilizing signal for nuclear mRNAs (for review see Dreyfus and Régnier 2002), but also acts as RNA instability signal in prokaryotes.

D. Translation

Plastid translation is related to translation in eubacteria. Both systems share homologous compounds, like initiation factors, rRNAs, tRNAs and 70S-type ribosomes (for review see Peled-Zehavi and Danon 2007). The tRNAs, rRNAs and some ribosomal proteins are encoded by the plastid genome, the remaining components are encoded in the nucleus and imported from the cytosol. Plant mitochondria also require a fully functional translation machinery to express the about 30 mRNAs encoded in the mitochondrial genome. Since only a few ribosomal proteins, rRNAs and an incomplete set of tRNAs are encoded by the mtDNA (Unseld et al. 1997), plant mitochondria must import most of the components of their translational apparatus, e.g. several tRNAs (Salinas et al. 2008) and all the required aminoacyl-tRNA synthetases (Duchêne et al. 2005).

Plastid ribosomes were characterized in proteomics studies in *C. reinhardtii* and spinach, which has led to the identification of 59 proteins. While 53 ribosomal proteins share homologues with *Escherichia coli*, six are specific to plastids and termed PRSP1-6 (Yamaguchi and Subramanian 2000; Yamaguchi et al. 2000, 2002, 2003;

Yamaguchi and Subramanian 2003). In eubacteria, the Shine-Dalgarno (SD) sequence plays a crucial role in the correct positioning of the ribosome during translation initiation (reviewed in Kozak 2005). In most plastid mRNAs, the SD-like sequence has a similar role, but the distance to the initiation codon is not as conserved as it is in *E. coli*. In addition, 30 of the 79 protein-coding genes in tobacco do not contain a SD-like sequence at all, indicating that alternative *cis*-elements and *trans*-acting factors may be responsible for correct translation initiation (Sugiura et al. 1998). In plant mitochondria, sequences resembling SD sequences are very rare and in the absence of an in vitro translation system, the function of these sequences in translation initiation could not be determined (Pring et al. 1992). Thus, the mechanism controlling translation initiation remains completely elusive in plant mitochondria. Potential translation regulation systems are also unknown. However, the function of PPR proteins might well be connected to plant mitochondrial translation as suggested by the involvement of CRP1 as a chloroplast translation regulator (Schmitz-Linneweber et al. 2005), by the requirement of Pet309 for translation in yeast mitochondria (Tavares-Carreón et al. 2008) and by the association of PPR336 to polysomes in plant mitochondria (Uyttewaal et al. 2008).

Sequence analysis has shown that translation is usually, but not always, initiated with an AUG codon in plant organelles. Alternative codons were found to be possible additional translation initiator triplets in mitochondria and plastids (Bock et al. 1994a; Unseld et al. 1997; Dong et al. 1998; Zandueta-Criado and Bock 2004). Moreover, plant mitochondrial genes can be expressed from mRNAs lacking canonical termination codons with no evidence that alternative termination codons had been created posttranscriptionally by either RNA editing or polyadenylation (Raczynska et al. 2006).

While in bacteria nascent transcripts are directly translated into proteins, the uncoupling of these two processes introduces a new level of regulation in organelles (Mayfield et al. 1995; Danon 1997; Zerges 2000; Peled-Zehavi and Danon 2007).

III. Technological Developments for the Expression Profiling of Organellar Genes

Northern blot analysis, in which a labeled probe is hybridized to a RNA target, was the first and most widely used technology to confirm and quantify gene expression. However, it can only be used to analyze the expression pattern of a limited number of genes under few experimental conditions. The recent development of "-omics" technologies enables researchers to carry out a genome-wide expression profiling, analyzing simultaneously up to thousands of genes. Most of these methods rely on the use of DNA arrays (macro- or micro-), although alternative methods such as differential display, expressed sequence tags (ESTs), serial analysis of gene expression (SAGE), quantitative RT-PCR (qRT-PCR), and others, can alternatively be used for such purposes. The above-mentioned transcriptional profiling technologies allow the analysis of complex RNA populations from different cells or tissues. Although DNA arrays produced a real advance in large-scale expression analysis and are currently widely used for transcript profiling, only limited datasets are as yet available for plant organelles and most of them are related to chloroplast genes or nuclear genes with chloroplast functions (Kurth et al. 2002; Legen et al. 2002; Richly et al. 2003; Kahlau and Bock 2008; Valkov et al. 2009).

In DNA arrays, DNA fragments or oligonucleotides corresponding to different genes or cDNAs are immobilized on a solid support (nylon membranes for macroarrays and glass slides for microarrays), and hybridized as probes to total RNA pools extracted from cells, tissues, whole organisms, etc. The hybridization signal detected for each spot can then be measured giving the relative abundance of the corresponding mRNA (Bouchez

and Höfte 1998; Meyers et al. 2004). The simplest and cheapest array systems use nylon membranes in combination with labeled (radioactive) cDNA probes, detected by Phosphorimager instruments (Kurth et al. 2002; Legen et al. 2002; Richly et al. 2003; Geimer et al. 2009). This method allowed to study, on a genome scale, the expression of the entire plastid chromosome of tobacco wild-type and mutant (PEP-deficient) plants (Legen et al. 2002) and *Euglena gracilis* under different culture conditions (Geimer et al. 2009), or nuclear genes related to chloroplast functions in *A. thaliana* under different environmental and genetic conditions (Kurth et al. 2002; Richly et al. 2003).

Initially, DNA microarrays were produced with cDNA fragments immobilized on microscope slides, but a competing approach, based on DNA oligonucleotides, has recently become the most widely used system (Bouchez and Höfte 1998; Stears et al. 2003; Meyers et al. 2004). Different fluorescent labeling and detection techniques are used to produce graphical images and numerical data corresponding to the measurement of spot intensities (Schulze and Downward 2001; Stears et al. 2003). The source of variation needs to be carefully controlled by replicating experiments at technical and biological levels (Schulze and Downward 2001; Meyers et al. 2004). In addition, it is often necessary to verify a subset of array results by alternative techniques, generally northern blot analysis and/or qRT-PCR (Schulze and Downward 2001). Several tools for array data analyses have been developed by both commercial and public suppliers (Schulze and Downward 2001; Stears et al. 2003).

Compared to cDNA arrays, arrays based on oligonucleotides offer several advantages: they can be synthesized either in plates or directly on solid surfaces, produce strong hybridization signals of superior specificity, also in the case of individual transcripts of multigene families that share sequence homology (by synthesizing oligonucleotides corresponding to regions of non-identity; Lemieux et al. 1998; Stears et al. 2003). Obviously, this method requires the availability of genome sequence for the organism under study, but this is usually no limitation in the case of organelles, considering the number of genomes continuosly released and the high degree of sequence conservation (http://megasun.bch.umontreal.ca/ogmp/projects/other/all_list.html). A plastome microarray, useable for different Solanaceae species, was recently developed (Kahlau and Bock 2008). This array is based on long (68–71 nucleotides) oligonucleotides and contains all genes and conserved open reading frames present in Solanaceae plastomes. It was designed using the complete tobacco, tomato and potato plastid genomes (Gargano et al. 2005; Yukawa et al. 2005; Daniell et al. 2006; Kahlau et al. 2006), and used to analyze the expression of different tomato and potato plastid genomes and identify regulatory expression patterns in different tissues and plastid types (Kahlau and Bock 2008; Valkov et al. 2009).

As an alternative to the array-based approach, a sequenced-based transcriptomic approach has been recently developed, with significant advantages, such as the potential to quantify the abundance of any transcript and the independence of the availability of a sequenced genome (Wang et al. 2010). Although these technologies have great potential, expression profiling studies based on Ultra High-Throughput Sequencing (UHTS) methods are still limited in plants (Cheung et al. 2006; Emrich et al. 2007; Weber et al. 2007; Schnable et al. 2009) and, so far, none of them has been applied to organelle transcriptomes.

IV. Expression Profiling in Plastids

A. Genotype-Specific Variation

Nuclear and plastid mutants have been used to study the effect of genotypic variability on differential plastid gene expression. Early studies about the profiling of gene expression in mutant genotypes involved the striped and albino mutants of maize and barley

(Han et al. 1993; Hess et al. 1993), which show altered chloroplasts in mutated tissues. In maize, the striped iojap 1 (*ij1*) and albino white 1 (*w1*) and white 2 (*w2*) mutants displayed alterations in the levels and sizes of several photosynthesis-related plastid transcripts. Furthermore, reduced protein accumulation was observed in *ij1*-affected plastids. Unchanged ptDNA content compared to wild-type plastids, and several other observations, suggested that *ij1* and *w1* mutants might have not only altered transcription rate, but also alterations in transcript processing and stability. On the other hand, the severe reduction of plastome copy number per plastid was likely responsible for the general reduction of transcripts in the *w2* mutant (Han et al. 1993). In the ribosome and plastid protein biosynthesis-deficient *albostrians* mutant of barley (Hess et al. 1993), the accumulation of transcripts for the photosynthesis genes *psbA*, *atpH*, *atpI*, and *rbcL* was strongly reduced, due to differential transcription rates and transcript stabilities in mutant and wild type plastids. In contrast, transcript accumulation for *rpo* and *rps* genes, encoding some subunits of the plastid-encoded RNA polymerase and small ribosome proteins, respectively, was enhanced, suggesting the involvement of NEP in their synthesis.

More recently, macro- and microarray analyses were carried out with mutants of the alga *C. reinhardtii* and the model Brassicaceae *A. thaliana* (Erickson et al. 2005; Cho et al. 2009). In the former case, using two RNA stability mutants (*mcd1-1* and *mcd1-2*), such analyses not only confirmed the gene-specific substrate (*petD*) of the nuclear *Mcd*1 gene, encoding an mRNA stability factor, but also allowed the discovery of an additional unlinked mutation (*mda1-2*) affecting accumulation of *atpA* mRNAs (Erickson et al. 2005). In Arabidopsis, the expression of 94 plastid genes was analyzed in a large set (75) of genotypes including albino mutants arrested at an early stage of chloroplast development, "high chlorophyll fluorescence" (*hcf*) mutants with impaired photosynthetic electron transport capacity and yellow/pale-green lines with altered, and often unknown, chloroplast functions (Cho et al. 2009). Transcriptomes were clustered in two main groups. Group I, including the majority of albino mutants, displayed up-regulation of non-photosynthetic genes transcribed by NEP and down-regulation of genes transcribed by PEP and encoding photosynthetic proteins. On the other hand, group II showed less evident expression changes and included all *hcf*, pale-green and the remaining albino mutants. Deviations from the expected profiles in the two groups allowed to identify 14 mutants specifically involved in plastid RNA metabolism.

Knock-out lines of Arabidopsis for the nuclear genes encoding PEP σ-like factors were used to analyze changes in global plastid gene expression and switch in promoter usage (Kanamaru et al. 2001; Nagashima et al. 2004; Schweer et al. 2006). In early experiments (Kanamaru et al. 2001), *sig2-1* mutants, showed reduced accumulation of chlorophyll and photosynthesis-related proteins, without significant reduction of the respective mRNAs. On the other hand, the observed phenotypic and biochemical defects were likely related to reduction in synthesis of some tRNAs encoded by genes (*trnE*-UUC, *trnV*-UAC, *trnM*-CAU, *trnQ*-UUG) with conserved eubacteria-type promoter sequences. Later, microarray analyses carried out on the same mutant line (Nagashima et al. 2004) showed that out of 79 protein coding genes, only the *psaJ* transcript was reduced in the mutant, whereas transcripts of 47 genes, many under the control of NEP, were increased, suggesting increase of NEP activity in the *sig2-1* genotype. In another σ-like factor knock-out line (*sig6-2*), the appearance of an unusually long transcript was observed in the *atpB-E* operon (Schweer et al. 2006), suggesting either a role of SIG6 in chloroplast RNA maturation or a differential usage of promoter sequences. Indeed, the authors detected motifs for NEP recognition far upstream of the *atpB* gene, between the *accD* and *rbcL* genes and thus devised a model for the developmentally regulated use

of alternative σ-like factors and promoter sequences.

Transgenic tobacco plants overexpressing a plastid-targeted bacteriophage T7 RNA polymerase (T7RNAP) were analyzed for their effects on plastid gene expression (Magee and Kavanagh 2002; Magee et al. 2007). In the former study, northern analyses showed an increase in transcript accumulation for several genes generally transcribed by NEP, but no variation in other genes (*psbD*, *ndhA*, *rrn16*) transcribed exclusively or predominantly by PEP in chloroplasts. These results could be explained by: (a) T7RNAP-mediated transcription from NEP promoters, (b) increased activity of NEP triggered by the presence of T7RNAP activity in chloroplasts of transgenic plants, and/or (c) differential increase in stability of some plastid mRNAs in transgenic plants (Magee and Kavanagh 2002). When the presence of the nuclear-encoded T7RNAP was associated with that of plastid transgenes driven by T7G10 5′ regulatory sequences, hybrid transplastomic plants showed reduced growth and altered expression of both plastid and nuclear genes (Magee et al. 2004). The profile of gene expression in mutant plants was analyzed using a customized array containing target sequences from all 124 tobacco plastid genes and 61 nuclear genes coding for photosynthetic proteins and components of the plastid translational apparatus. Significantly down-regulated plastid genes included those encoding subunits of the PSII, NADH dehydrogenase, ATP synthase, and cytochrome b_6f complexes as well as ribosomal RNAs and proteins. Genes co-transcribed in the same polycistronic operons were usually down-regulated similarly. At the same time, up-regulation was observed for the *accD* gene and the *ndhCKJ* operon, but while in the former case it was due to read-through transcription from the upstream promoters present in the construct used for transformation, in the latter case it was, as previously shown, likely due to non-specific recognition of a NEP promoter-like sequence by the plastid-directed T7RNAP. Furthermore, a reduction in the amount of the mature *clpP* mRNA was observed in mutant plants due to altered processing of the primary transcript (Magee et al. 2007).

The availability of *rpo* deletion mutants, derived by plastid transformation, and the comparative analysis of expression profiles in wild-type and mutant tobacco plants allowed to establish the existence of the nuclear-encoded RNA polymerase in higher plant plastids and to investigate the interplay of the two polymerase types (PEP and NEP) at several levels of gene expression (Allison et al. 1996; Hajdukiewicz et al. 1997; Serino and Maliga 1998; De Santis-Maciossek et al. 1999; Krause et al. 2000; Legen et al. 2002). Based on results of northern analyses and mapping of transcription initiation sites, early seminal studies (Allison et al. 1996; Hajdukiewicz et al. 1997) showed that, while some mRNA accumulation was always detected for all genes analyzed, plastid genes could be grouped in three classes on the basis of relative transcript accumulation levels in wild-type and mutant plants: (1) genes with high accumulation levels in wild-type leaves, but negligible levels in leaves of Δ*rpoB* plants; (2) genes with similar mRNA leaves in wild-type and mutant plants; (3) genes with significantly more transcript accumulation in mutant than in wild-type plants. Genes or operons with different physiological functions (e.g. photosynthesis or transcription/translation) belonged to different classes and displayed upstream sequences for recognition of either one polymerase type or both. It was thus proposed that transcription by NEP or PEP through differential promoter recognition could determine the pattern of group-specific gene regulation in plastids (Hajdukiewicz et al. 1997). Later studies, however, indicated a more complex picture, likely not based only on differential promoter usage but also on differential posttranscriptional mechanisms (Krause et al. 2000; Legen et al. 2002). In fact, the entire plastome was found to be transcribed in both wild-type and PEP-deficient plastids and, in genes specifying different functions, no correlation was found between transcription rates, transcript levels,

transcript patterns, and accumulation levels of derived polypeptides. A predominance of posttranscriptional regulation mechanisms over transcriptional ones was also found in a tobacco *psbA* gene deletion mutant showing changes in PSII protein accumulation levels and upregulation of the alternative electron transport pathways (Baena-González et al. 2003).

A microarray analysis of the expression profile of 108 plastid DNA fragments carried out in a chloroplast *trnR*-CCG gene knockout mutant of the moss *Physcomitrella patens* indicated that most plastid genes were expressed at comparable levels in mutant and wild-type genotypes, suggesting that the arginine *trnR*-CCG gene is not essential for plastid gene expression in *P. patens* (Nakamura et al. 2005).

As previously discussed, the RNA editing process occurs in all major lineages of land plants and only marchantiid liverworts do not seem to edit plastid transcripts (Table 14.1, Freyer et al. 1997; Duff and Moore 2005). By investigating editing patterns of the *ndhB* and *rbcL* transcripts, it could be observed that neither plastid editing frequencies nor the editing patterns of a specific transcript correlated with the phylogenetic tree of the plant kingdom (Freyer et al. 1997). Later genome-wide comparisons of the editotypes of one hornwort (*Anthoceros formosae*), one fern (*Adiantum capillus-veneris*) and five seed plants (*Arabidopsis thaliana*, *Nicotiana tabacum*, *Atropa belladonna*, *Zea mays* and *Pinus thunbergii*) showed that only 18 of the total 85 chloroplast editing sites in seed plants were present also in either one or both other taxa, while the latter shared 53 sites (Tillich et al. 2006). Tsudzuki et al. (2001) compared the conservation of editing sites among several higher plant species. The dicotyledonous plant tobacco and the monocots rice and maize have 12 editing sites in common, which may already have been present before divergence of both taxa (Hirose et al. 1999; Tsudzuki et al. 2001). The conservation of editing sites between gymnosperms and angiosperms is lower. Just five sites out of 26 are shared by the gymnosperm black pine (Wakasugi et al. 1996) and the so far analyzed angiosperms (Tsudzuki et al. 2001). The editing sites were also compared within more closely related taxa. The three Solanaceae tobacco (*N. tabacum*), tomato (*Solanum lycopersicum*) and deadly nightshade (*A. belladonna*) have 30 of the so far known sites in common. While 2–3 sites are always shared by two species, 1–2 appear to be species-specific (Schmitz-Linneweber et al. 2002; Kahlau et al. 2006). Differences in editing can even be observed at the subspecies level. The editotypes of three different ecotypes of *A. thaliana*, Columbia (Col-0), Cape Verde Islands (Cvi-0) and Wassilewskija (Ws-2) were characterized. One non-synonymous point mutation was detected in Cvi-0 at the first position of codon 17 of the *ndhG* gene. This codon is usually edited in all three ecotypes with a C-to-U transition at the second position. This mutation changes a serine codon in Col-0 and Ws-2 into an alanine codon in Cvi-0 at the DNA level, resulting at the RNA level in a phenylalanine or valine codon, respectively (Tillich et al. 2005).

By analyzing differences among various species, it is interesting to note how fast the capability to edit certain sites was lost during evolution. In *A. thaliana*, the *cis*-elements of *mat*K(2) and *ndhB*(11) editing sites show striking similarities, suggesting that they share the same *trans*-acting factor, a common mechanism which has been experimentally proven for other sites (Chateigner-Boutin et al. 2008; Hammani et al. 2009). However, while the *matK*(2) editing event restores a conserved tyrosine, the *ndhB*(11) does eliminate an evolutionary conserved serine. These results indicate that the *matK*(2) editing is the primary target whereas *ndhB*(11) editing might be secondary (Tillich et al. 2005). The potential of one *trans*-acting factor to recognize several similar *cis*-elemets might explain the capability of some species to edit foreign sites. The site *rps12*(74) is edited in tomato, but absent from tobacco, as the 'T' is already encoded at the DNA level (Kahlau et al. 2006). After introduction of the tomato editing site into the tobacco plastid genome, the transplastomic tobacco plant

Table 14.1. Examples of genome-wide analyses of RNA editing in plastid and mitochondrial transcripts of different plant species

Species	No. of edited sites (tissue/cell type)		References
	Plastids	Mitochondria	
Atropa belladonna	31 (leaf)[a]	–[b]	Schmitz-Linneweber et al. (2002)
Nicotiana tabacum	34 (leaf)[a]	–	Chateigner-Boutin and Hanson (2003)
Solanum lycopersicum	36 (leaf)[a]	–	Kahlau et al. (2006)
Arabidopsis thaliana	34 (leaf)	456 (cell suspension)[c]; 362 (leaf)[d]	Giegé and Brennicke (1999), Chateigner-Boutin and Small (2007), Bentolila et al. (2008)
Brassica napus	–	427 (leaf)[e]	Handa (2003)
Beta vulgaris	–	357 (seedling)	Mower and Palmer (2006)
Pisum sativum	27 (leaf)	–	Inada et al. (2004)
Oryza sativa	21 (leaf)[f]	491 (−)	Corneille et al. (2000), Notsu et al. (2002)
Zea mays	27 (leaf)	–	Peeters and Hanson (2002)
Pinus thunbergii	26 (leaf and stem)	–	Wakasugi et al. (1996)
Adiantum capillus-veneris	350 (frond)[g]	–	Wolf et al. (2004)
Isoetes engelmannii	–	1,782 (−)[g]	Grewe et al. (2010)
Physcomitrella patens	2 (−)	11 (−)	Rüdinger et al. (2009)
Anthoceros formosae	942 (thalli)[g]	–	Kugita et al. (2003)
Marchantia polymorpha	0	0	Rüdinger et al. (2009) and references therein

[a]Bioinformatics analyses detected additional sites in the three Solanaceous species, for a total of 35 sites in *A. belladonna*, 37 in *N. tabacum* and 36 in *S. lycopersicum*, most of them (30) shared by the three species (Kahlau et al. 2006)

[b]Not available

[c]Out of a total of 456 sites, 441 were identified in *orf*s, 8 in introns, and 7 in leader or trailer sequences (Giegé and Brennicke 1999)

[d]362 sites were detected in 33 genes, of which 67 sites had not been observed in suspension cultures, whereas 37 sites previously detected in suspension cultures were not observed in leaves (Bentolila et al. 2008)

[e]427 sites were identified in 33 genes, 358 of which are shared with *A. thaliana* (Handa 2003)

[f]21 sites were identified in 11 genes, 19 of which are shared with *Z. mays* (Corneille et al. 2000)

[g]About 10%, 12% and 46% of the total edited sites were U-to-C conversions in *A. capillus-veneris*, *I. engelmannii* and *A. formosae*, respectively

is able to edit this site with high efficiency. There are two possible explanations for this scenario: (1) either the responsible *trans*-acting factor is still present in the tobacco nuclear genome and was not lost during evolution or (2) the pre-existence of the *rpoB*(667) site facilitated the evolution of the *rps12* site, as both *cis*-elements share high similarity (Karcher et al. 2008). Other attempts to edit heterologous editing sites in vivo have not been successful so far. In contrast to the tomato *rps12* site, tobacco is not able to edit a foreign *psbF* site introduced from spinach (*Spinacia oleracea*, Bock et al. 1994b). But also more closely related species show nuclear-plastidial incompatibilities. By creating cybrids and introducing tobacco chloroplasts into deadly nightshade nuclear backgrounds, it became evident that the nuclear genome of nightshade is not able to support editing at all tobacco editing sites. The editing at site *atpA*(264) is absent, resulting in an albino phenotype. The most likely explanation is the absence of a nuclear-encoded editing factor in the nightshade which is responsible for correct processing of the site in tobacco (Schmitz-Linneweber et al. 2005).

B. Variation Due to Developmental and Environmental Cues

Most plastids are able to interconvert into other types following developmental and environmental cues (for review see Pyke 2007). Plastid gene expression and its regulation have been extensively studied in chloroplasts, which are present in photosynthetically active green tissues and generally develop from proplastids in meristems or etioplasts after illumination of dark-grown tissues. Non-green plastids, such as amyloplasts, chromoplasts and others, fulfill important functions in storage and pigmentation and are also the place of important metabolic pathways. The knowledge on gene expression in such plastid types, however, is still very limited.

Monocots are good model organisms to study changes in gene expression during chloroplast development. Cells at the leaf base contain proplastids which develop eventually into mature chloroplasts, present in the tip of the leaves. Using a custom maize chloroplast biogenesis cDNA microarray, it was shown that the abundance of most plastid transcripts in maize leaves (52 out of 63 analyzed) increases more than twofold during development (Cahoon et al. 2008). Ten transcripts which are present at similar levels in both plastid types are mostly involved in plastid gene expression and are transcribed by NEP (Cahoon et al. 2008). Since, in maize, transcription activity increases in developing chloroplasts, the latter transcripts are likely less stable in mature chloroplasts compared to proplastids at the leaf base (Cahoon et al. 2004, 2008). In the same study, a coordinated gene expression pattern in the nucleus and the plastids, likely based on a combination of anterograde and retrograde signalling between the two organelles, was found (Cahoon et al. 2008). Barley, another monocot, shows differences in transcript patterns during proplastid-to-chloroplast conversion compared to maize. At the leaf base, plastid transcriptional activity and transcript abundance are low. An increase in both can be seen in etioplasts, present in cells which already stopped dividing and entered the cell elongation phase. In contrast to maize, after illumination and further chloroplast maturation, transcript abundance and transcriptional activity decline again (Baumgartner et al. 1989, 1993). Another plastome-wide study in barley could not detect global quantitative changes in gene expression. During the de-etiolation process, no changes were found between etioplasts and chloroplasts, neither in relative transcription rates nor in transcript stability (Krupinska and Apel 1989). Differences between maize and barley could be caused by the differences in CO_2 fixation mechanisms. Barley uses the C3 fixation mechanism while maize is a C4 plant showing the typical Kranz anatomy. Maize bundle sheath and mesophyll cells have very different tasks during CO_2 fixation and also differ in their plastid transcript profiles. Transcripts for subunits of photosystem II are more abundant in mesophyll cells while *rbcL* is more abundant in bundle-sheath cells (Kubicki et al. 1994), the cell type in which concentrated CO_2 is fixed by Rubisco. It is possible that these two cell types also differ in their transcript patterns during proplastid-to-chloroplast development. In another monocot study based on macroarrays for studying chloroplast gene expression profiles, changes in gene expression levels were monitored using RNA isolated from germinating wheat seeds and seedlings at different stages of development (Siniauskaya et al. 2008). While transcript levels for PSI and PSII genes increased after imbibition until 1 week of development, the levels of other transcripts (e.g. those of *ndh* and *atp* genes) either did not change or decreased.

In the dicot *N. tabacum*, dark-grown etioplast-containing and illuminated chloroplast-containing seedlings were compared by using a microarray with 220 ptDNA fragments, each corresponding to a single known gene or an intergenic region and altogether covering the whole plastome (Nakamura et al. 2003). A clear trend of gene expression within the two functional groups of plastid-encoded genes was evident. The majority of

photosynthesis-related genes increased in their abundance in illuminated plants. On the other hand, the majority of genes involved in gene expression were expressed at similar levels in both plastid types. Furthermore, unexpected signals were found in several intergenic regions, suggesting the existence of novel transcripts (Nakamura et al. 2003). Similar studies conducted in the unicellular red alga *Cyanidioschyzon merolae* with a microarray containing almost all plastid protein coding genes, northern blot analyses and run-on transcription assays, showed differential activation of gene transcription by illumination (Minoda et al. 2005).

Results from microarray analyses in tobacco and Arabidopsis (MacLean et al. 2008) showed coordinated expression of nuclear and plastid genes encoding ribosomal proteins during seedling development. Transcript accumulation responded similarly to light and inhibitors of plastid signaling. In another study comprising the same two species, the effect of green light on seedling development and plastid gene expression was analyzed by using genome microarrays and RNA gel blot experiments (Dhingra et al. 2006). In both species, etiolated seedlings subjected to a short, dim, single pulse of green light showed stem elongation and concomitant decrease in a sub-set of plastid-encoded transcripts, including several ones known to be light inducible. The majority of plastid transcripts did not vary, while only three increased in abundance, indicating that the effect of green light on plastid gene expression is gene-specific.

As a representative for eukaryotes carrying secondary endosymbionts, plastid gene expression was analyzed in the protist *Euglena gracilis*. Similar to primary endosymbionts, *E. gracilis* plastids encode mainly genes involved in transcription, translation and photosynthesis (Hallick et al. 1993). Early work on *E. gracilis* using RNA-DNA hybridizations had already shown that plastid genes are transcribed in proplastids of dark-grown cells (Chelm and Hallick 1976; Rawson and Boerma 1976; Chelm et al. 1979). Although Dix and Rawson (1983) could not identify individual genes, they could distinguish between two major groups: (1) genes which are constitutively transcribed and (2) genes encoding transcripts which increase in their abundance during the greening process, as e.g. *psbA*, encoding a core subunit of photosystem II (Hollingsworth et al. 1984). A recent study analyzed the complete *E. gracili*s transcriptome under 12 different growth and stress conditions using a macroarray-based approach (Geimer et al. 2009). Overall, the organelle transcriptome showed pronounced global quantitative changes, but qualitative changes were negligible. After growth in darkness, the overall transcript abundance was much lower than in light-grown cells, but *psbA* transcription increased drastically. The *trnI*-CAU gene, involved in gene expression and an example for genes identified by Dix and Rawson (1983) as being constitutively transcribed, did not change in abundance (Geimer et al. 2009). As plastid gene expression patterns in *E. gracilis* remain more or less constant with quantitative changes on a global scale, these results suggest that fine-tuning of protein production might be regulated posttranscriptionally. In contrast to the limited global transcriptional changes happening in *E. gracilis* (Geimer et al. 2009), translational regulation is much more pronounced (Miller et al. 1983). Although this study did not identify single plastid-encoded proteins, it could clearly show the gap between transcriptional changes, which are just up to threefold, and the total rates of protein synthesis, which were increased to up to 100-fold. Different sets of proteins were expressed at different time points during proplastid-to-chloroplast development (Miller et al. 1983). Analyzing and comparing *psbA* transcription with protein accumulation during de-etiolation identified similar patterns in barley. Without noticeable changes in *psbA* mRNA, the encoded protein increased drastically once the plants were illuminated (Klein and Mullet 1987).

Eberhard and coworkers (2002) analyzed the ability of plastids to override transcriptional changes at the translational level more

systematically. *C. reinhardtii* cells were treated with rifampicin causing depletion of plastid transcripts by binding to and inhibiting the eubacterial-type RNA polymerase. Most of the analyzed transcripts dropped in their abundance to 10% compared to prior to the treatment. Despite these significant changes in RNA levels, the rate of protein synthesis measured with pulse-chase labeling experiments did not drop during the treatment (Eberhard et al. 2002).

Amyloplasts are present in storage organs like tubers or seed endosperm as well as in columella cells of root tips (for review see Pyke 2007). Several genes (16S rRNA, *atpB/E*, *psbA*, *rbcL*) were analyzed with respect to their abundance in *A. thaliana* and spinach root amyloplasts (Deng and Gruissem 1988; Isono et al. 1997a). All analyzed transcripts could be detected, but their abundance was highly decreased compared to leaf chloroplasts. Although plastid DNA levels were lower in Arabidopsis roots than in leaves, these differences could not explain the large differences in transcript abundances (Isono et al. 1997a). Similar observations were made by analyzing amyloplasts of potato tubers (Brosch et al. 2007; Valkov et al. 2009). The rather small reduction in plastid DNA content of approximately two- to threefold could not account for the reduction in transcript accumulation (Valkov et al. 2009). Run-on assays showed that the decrease in steady-state RNA levels was largely due to a decrease in transcriptional activity in amyloplasts (Sakai et al. 1992; Brosch et al. 2007; Valkov et al. 2009), although differences in stability could explain variable transcript accumulation levels. To get a more detailed insight into tissue versus plastid specificity, bell pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*) fruits, which convert chloroplasts (highly transcriptionally active in leaves) into red chromoplasts during fruit ripening, were analyzed. Surprisingly, chloroplasts in green fruits already show reduced transcript abundance and differ from their counterparts in leaves, pointing towards a developmental regulation of plastid transcription. Bell pepper fruit plastids show reduction in steady-state RNA levels which is due to a reduced transcriptional activity. However, no significant changes during ripening and chloroplast-to-chromoplast conversion in transcriptional activity could be detected (Kuntz et al. 1989). Similarly, in tomato and pumpkin (*Cucurbita pepo*), RNAs are present at lower levels in fruits compared to leaves, while transcriptional activity is already downregulated in green fruits and does not change significantly during ripening (Piechulla et al. 1985; Marano and Carrillo 1992; Obukosia et al. 2003; Kahlau and Bock 2008).

Although the functions and structure of the two plastid types are very different, amyloplasts and chromoplasts share striking similarities in their gene expression profiles (Fig. 14.1). In both plastid types, transcript abundance was highly reduced compared to leaf chloroplasts, but in both cases the differences could not be attributed to the absence of one of the two RNA polymerase activities present in plastids (Kahlau and Bock 2008; Valkov et al. 2009). In fact, although differences in promoter utilization were observed by comparing amyloplasts or chromoplasts to chloroplasts, both the nuclear-encoded and plastid-encoded RNA polymerases are active in non-green plastids. Plastome-wide expression profiling showed that two genes, *clpP* and *accD*, are expressed at similar high levels in leaf chloroplasts and amyloplasts or chromoplasts (Kahlau and Bock 2008; Valkov et al. 2009). *clpP*, a subunit of a protease, is essential in tobacco and important for plant development (Shikanai et al. 2001; Kuroda and Maliga 2003; Clarke et al. 2005; Adam 2007). Many nuclear-encoded proteins are imported into all plastid types (Baginsky et al. 2004; Siddique et al. 2006; Bancel et al. 2010; Barsan et al. 2010; Daher et al. 2010), indicating that the Clp protease is probably needed for the removal of damaged proteins (Zybailov et al. 2009). The *accD* gene is also essential and cannot be deleted from the plastid genome (Kode et al. 2005). The encoded protein is part of the plastid-localized Acetyl-CoA carboxylase

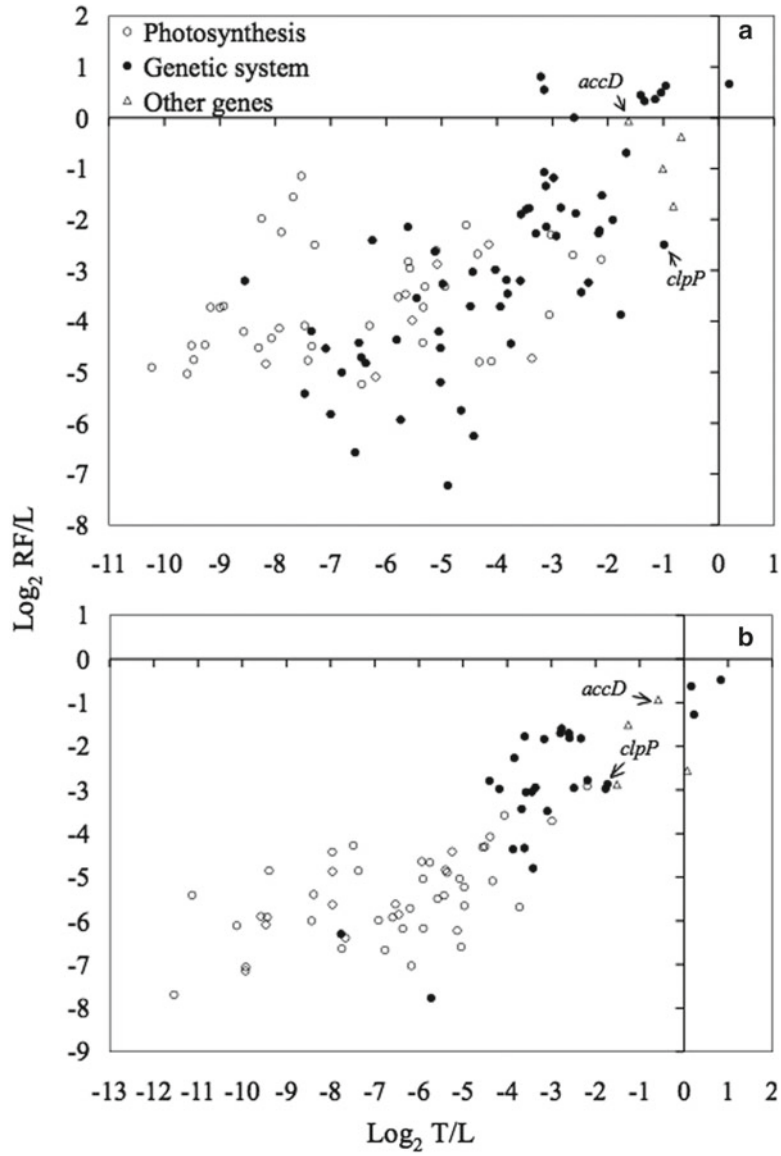

Fig. 14.1. Genome-wide analysis of total (**a**) and polysomal (**b**) RNA accumulation in potato tuber amyloplasts (Log_2 T/L) and tomato red fruit chromoplasts (Log_2 RF/L) compared to leaf chloroplasts (Kahlau and Bock 2008; Valkov et al. 2009). Based on their function, genes analyzed were grouped in three classes. The *accD* and *clpP* genes are highlighted.

(ACCase), catalyzing the first committed step in fatty acid biosynthesis. Residual expression of the plastid gene expression machinery may be necessary to produce the ACCase and secure therefore further production of lipids needed in all cell membranes (Kahlau and Bock 2008; Valkov et al. 2009).

Regulation of plastid translation at the level of polysome formation was investigated in various species and plastid types. In spinach root amyloplasts, representative transcripts involved in photosynthesis were detectable, but specifically depleted from polysomal fractions (Deng and Gruissem

1988). The situation is similar in potato tuber amyloplasts as well as in tomato fruit chromoplasts (Brosch et al. 2007; Kahlau and Bock 2008; Valkov et al. 2009). Both plastid types show a large reduction in polysome-associated mRNAs. In addition to the constantly low mRNAs levels in tomato fruit plastid-types, translation is increasingly down-regulated during chloroplast-to-chromoplast conversion for almost all mRNAs. The only genes which showed potentially similar translation levels in leaves and non-green plastids were genetic system genes like those encoding the subunits of the plastid-encoded RNA polymerase, *ycf1* and *ycf*2 (open reading frames of unknown function) and, interestingly, *clpP* and *accD* (Kahlau and Bock 2008; Valkov et al. 2009). Hence, also at the translational level, transcripts of the latter two genes differ in their regulation pattern from almost all other plastid mRNAs and the low level of plastid gene expression is probably maintained to secure the production of the Clp protease and the ACCase for fatty acid biosynthesis (Kahlau and Bock 2008; Valkov et al. 2009).

Both in higher and lower plant plastids, the RNA editing process was likewise shown to be affected by changes in the environmental conditions as well as the organ and plastid type analyzed (Bock et al. 1993; Hirose et al. 1996; Hirose and Sugiura 1997; Ruf and Kössel 1997; Karcher and Bock 1998; Hirose et al. 1999; Karcher and Bock 2002a, b; Peeters and Hanson 2002; Chateigner-Boutin and Hanson 2003; Miyata and Sugita 2004; Kahlau and Bock 2008; Valkov et al. 2009). However, results of studies analyzing individual sites as well as those of a comprehensive study in maize, involving 27 editing sites in 15 genes and 10 different tissues (Peeters and Hanson 2002), demonstrate that environmental and developmental effects on RNA editing efficiency are not consistent in different genes and/or editing sites. Furthermore, developmental co-variation of RNA editing extent in some editing sites was shown by surveying 34 editing sites in 15 tobacco genes (Chateigner-Boutin and Hanson 2003). In bell pepper chromoplasts, the *psbL* initiation codon is still edited although the product is obviously not needed in the non-photosynthesizing ripe fruits. These results suggest that editing is in this case not responsible for the regulation of PsbL protein expression (Kuntz et al. 1992). *psbL* and *psbF* editing were also analyzed in illuminated and etiolated leaf tissue as well as in roots and seeds of spinach (Bock et al. 1993). Editing was complete in leaf etioplasts as well as chloroplasts, indicating that light had no influence on editing extent in these two plastid-types. However, editing of these two sites in proplastids (seeds) and amyloplasts (roots) was incomplete. As unedited start codons render transcripts probably untranslatable, editing might be one mechanism controlling plastid gene expression (Bock et al. 1993). Several editing sites in the *ndhA*, *ndhB* and *ndhF* transcripts, encoding subunits of the NAD(P)H dehydrogenase complex, show incomplete editing in *A. thaliana* roots (Chateigner-Boutin and Hanson 2003). However, a functional significance of incomplete editing for regulation of gene expression is questionable in this case as the NdhD protein is completely absent in roots. The *ndhD* start codon is only partially edited in tobacco, tomato, potato and Arabidopsis leaf tissue. The incomplete editing in leaves is conserved across several species, but the editing extent in non-green plastid types varies considerably. Editing of *ndhD*(1) is completely absent in Arabidopsis roots and potato tubers, but is partial in tobacco and spinach roots as well as tomato fruits (Chateigner-Boutin and Hanson 2003; Kahlau and Bock 2008; Valkov et al. 2009). All available results suggest the importance of selective activation/inhibition of site-specific nuclear-encoded *trans*-factors (sometimes able to recognize more than one editing site) in explaining developmental and/or environmental differences in RNA editing efficiency. By comparing different species and plastid-types, however, no common pattern can be identified which could hint towards a role of editing in the regulation of plastid gene expression. In addition, no editing sites specific for non-green plastid types have been identified so far.

Although genome-wide studies on the effect of environment and/or development on transcript processing are missing, available data for a number of genes showed at least in some cases a reduction of transcript splicing in non-green plastids compared to leaf chloroplasts, suggesting a possible link with limited expression of the plastid genome in some tissues and plastid types (Barkan 1989; Kahlau and Bock 2008; Valkov et al. 2009).

V. Expression Profiling in Mitochondria

A. Genotype-Specific Variation

Only few studies investigated the mitochondrial expression profiling of natural and mutant plant populations. The *albostrians* mutant of barley, characterized by a very low expression level of photosynthesis-related plastid and nuclear genes, was used to study the influence of impaired chloroplast development on mitochondrial gene and transcript levels (Hedtke et al. 1999). The analysis of mitochondrial steady-state RNA levels in different tissues showed an enhanced transcript accumulation of all mitochondrial genes tested in white leaves, due to a threefold higher mitochondrial gene copy number. Further, because the increased transcript levels in mitochondria of white leaves could be caused by either the differentiation state of plastids or the direct action of the mutated nuclear *albostrians* allele, plants derived by reciprocal crosses between a green wild-type and a white (striped) *albostrians* parents were analyzed, showing that the enhanced transcript levels were a consequence of the impaired plastids and not of the nuclear mutant allele. These results highlight the crucial importance of inter-organellar crosstalk in plant cells.

In order to gain more knowledge about species-specific regulation of plant mitochondrial gene expression, Leino et al. (2005) compared transcriptional activity and RNA turnover in a cytoplasmic male-sterile (CMS) *Brassica napus* line, the corresponding male-fertile progenitors (*A. thaliana* and *B. napus*), and a fertility-restored line. The alloplasmic CMS line was obtained by protoplast fusion between *A. thaliana* and *B. napus* and contained mitochondrial DNA (mtDNA) mostly inherited from *A. thaliana* with some mtDNA fragments from *B. napus*, whereas the nucleus contained pure *B. napus* DNA (Leino et al. 2003). The fertility-restored line was isogenic for its mtDNA but had an additional pair of *A. thaliana* chromosome III in the nuclear genome (Leino et al. 2004). The analysis of transcriptional activities by run-on assays revealed a high variability between the parental species, with a higher transcript activity in *B. napus* than in *A. thaliana* for the *atp8*, *ccmB*, *rps7* and *rrn5* genes, and an opposite relationship for the *nad4L*, *nad9* and *cox1* genes. By contrast, the values obtained for the CMS and restored lines were very similar for all tested genes. The authors suggested that the differences observed in transcription activity could be due to differences in promoter strength, as already found in other species (Muise and Hauswirth 1992; Giegé et al. 2000). In comparison with transcription activities, the transcript steady-state levels were more homogeneous demonstrating that RNA turnover might act as a compensating mechanism.

In another study, the major transcript ends of all mitochondrial protein-genes were compared in three *A. thaliana* accessions (Forner et al. 2008). Authors identified mRNA polymorphisms for several genes (*nad4*, *nad9*, *ccmB*, *ccmC*, *rpl5-cob*), and linked them to variations at the 5′ ends that were conserved in all tissues analyzed. Since the polymorphisms observed could be caused by mitochondrial sequences or by differences in nuclear genes, they analyzed the inheritance of polymorphic mRNAs in reciprocal F_1 hybrids. These analyses showed a maternal (*ccmC*) or biparental (*nad4*, *nad9*, *ccmB* and *rpl5*) inheritance for polymorphic transcripts, suggesting that they could arise from differences in mtDNA or nuclear-encoded *trans*-factors, respectively. Despite intensive research in the past years, most of the *cis*-acting sequence elements and *trans*-factors

required to generate mature 5′ and 3′ ends of mtRNA of higher plants are still unknown, Forner et al. (2008) suggested that the analysis of reciprocal F_1 hybrids is a promising approach to identify mitochondrial *cis*-elements and nuclear-encoded *trans*-factors involved in 5′ end formation or mRNA stability.

Comprehensive studies were carried out on RNA editing in different ecotypes and tissues of Arabidopsis mitochondria (Table 14.1, Giegé and Brennicke 1999; Bentolila et al. 2008; Zehrmann et al. 2008). Giegé and Brennicke (1999) identified a total of 456 C-to-U conversions in suspension cultures of *A. thaliana*, of which 441 reside in open reading frames (orfs). Differences among Arabidopsis ecotypes both for the extent of RNA editing and accession-specific editing sites were found (Bentolila et al. 2008; Zehrmann et al. 2008). Dominance relationships and maternal effects were assessed for the most polymorphic sites by evaluating the degree of editing in reciprocal hybrids. Dominance was more common in non-silent than in silent sites, while additivity was observed only in silent sites. For more than half of the inspected sites, a significant difference depending on the direction of the cross was found (Bentolila et al. 2008). Quantitative variations among ecotypes suggested that the extent of editing can evolve more rapidly than the species (Zehrmann et al. 2008).

A comparative analysis of the mitochondrial genes and RNA editing sites of *B. napus* L. and *A. thaliana* was carried out by Handa (2003), identifying 427 editing sites in genes and orfs of *B. napus* compared with 441 sites in *A. thaliana* (Table 14.1, Giegé and Brennicke 1999). The number of editing sites shared by both plant mitochondria was 358, which correspond to 83.8% and 81.2% of the total editing sites in *B. napus* L. and *A. thaliana* transcripts, respectively. These percentages seem to be low considering that mitochondrial DNA nucleotide identity (for protein coding regions) between the two species was 99.2%. This means that, as already found in plastids, RNA editing variations in plant mitochondria evolve more rapidly than coding sequences. By contrast, in the moss model system *Physcomitrella patens*, only 11 editing sites in 9 mitochondrial genes (*atp9*, *cox1*, *cox2*, *cox3*, *nad3*, *nad4*, *nad5*, *rps14* and *ccm*FC) were found, and only the codon positions reconstituting highly conserved amino acids in the encoded proteins were subjected to C-to-U conversions (Rüdinger et al. 2009).

B. Variation Due to Developmental and Environmental Cues

The plant mitochondrial genome is far from being able to express all the required proteins for mitochondrial respiration and translation (Unseld et al. 1997). Various and precise communication mechanisms must be necessary for the biogenesis of mitochondrial protein complexes and especially for the modulation of this biogenesis. A number of studies have established that mitochondrial respiration can be modulated in the plant cell in response to environmental stimuli, at some particular developmental stages or in response to stress (Wood et al. 1996; Svensson and Rasmusson 2001; Giegé et al. 2005; Ribas-Carbo et al. 2005). If this modulation of respiration is due to changes in the number of respiratory complexes per cell, it means that the biogenesis of respiratory complexes can be adjustable as well. A coordinated expression must exist between mitochondrial and nuclear genes, between nuclear genes and between mitochondrial genes encoding subunits of the same respiratory complexes (Giegé et al. 2005; Welchen and Gonzalez 2006; Gonzalez et al. 2007). While many nuclear genes are clearly (co-)regulated at the transcriptional level, the mechanisms regulating coordination of mitochondrial gene expression are less clear.

A global study of the Arabidopsis mitochondrial transcriptome had shown that individual genes or transcription units are transcribed with distinct rates even if they encode components of the same multi-subunit complexes. These differences are at least partially counterbalanced at the steady-state

RNA level by posttranscriptional processes and different RNA stabilities (Giegé et al. 2000). Are the steady-state RNA levels obtained invariable or can they be regulated, e.g. during changing developmental stages? To address this question, Li-Pook-Than and colleagues (2004) examined RNA levels of wheat mitochondrial genes during the developmental period when seeds leave dormancy, germinate and develop into seedlings. Mitochondrial transcript levels from 0 h to 6 days post-imbibition were analysed. Stable and edited messengers were observed in dormant seeds and precursor RNAs were subsequently detected early in embryo germination. Respiratory chain genes showed mRNA profiles comparable to those of ribosomal RNAs, whereas ribosomal protein genes had proportionately lower steady-state mRNA levels in later stages of seedling development. The relative levels of precursors compared with the respective mRNAs decreased during development, consistent with transcription outpacing RNA processing in early stages of development. However, coordination was more effective several days after imbibition. In the case of multiply split genes containing group II introns, complex patterns of splicing intermediates were observed. This suggested an absence of strict polarity for splicing. Spliced introns were typically more abundant in embryos than in seedlings. These observations suggest a transient delay of the RNA processing mechanisms at the beginning of seed germination, a period where mitochondrial biogenesis is rapid and apparently demanding for the post-transcriptional machinery (Li-Pook-Than et al. 2004). In another global study, Howell and colleagues (2006) described mitochondrial biogenesis during imbibition of rice embryos both at the morphological and the molecular levels. For a subset of mitochondrial encoded subunits of the respiratory chain genes, they observed two different transcript expression profiles. While complex V *atp1* and complex IV *cox2* transcripts reached maximum levels at 48 h after imbibition, complex I *nad9* and complex III *cob* message levels peaked much earlier at 8 h (Howell et al. 2006). Similar to the previous case, this showed that gene expression does not seem to be synchronized in early developmental stages and could suggest that mitochondrial transcripts rather follow a defined expression pattern in early development for the biogenesis of mitochondrial complexes. A more comprehensive investigation of mitochondrial transcript profiles during germination and early seedling development in wheat gave similar results (Khanam et al. 2007). In this study, the mitochondrial transcripts were present in the initial dry embryo at variable levels. During early development, gene expression levels of individual genes were very variable. However, genes could be classified into four categories according to their expression patterns. Most mitochondrial respiratory genes were found in two categories. For one category, the timing of RNA accumulation corresponded to the activation of respiration, but not for the other one. Altogether, this work suggested that the initial respiratory burst during early development is supported by stored preexisting respiratory components, whereas *de novo* mitochondrial gene expression rather supports the subsequent seedling growth (Khanam et al. 2007). It also suggested that the availability of substrates might be a regulatory factor or a signal for the initiation of gene expression in plant mitochondria. Gene expression profiles were also monitored for later developmental stages. Mitochondrial encoded transcript levels, together with chloroplast and nuclear RNA levels, were followed along a maize leaf developmental gradient (Cahoon et al. 2008). Twenty-five out of the 27 mitochondrial transcripts investigated had at least twofold higher steady-state levels in the leaf base than in the rest of the leaf. This mitochondrial gene expression pattern is not surprising because the actively dividing and expanding base of maize leaves has high energy demands and is expected to contain highly active mitochondria (Cahoon et al. 2008). However, from this particular study, it is difficult to conclude whether mitochondrial gene expression had been up-regulated in response to a developmental signal

or whether the transcript level differences observed were due to an enriched content in mitochondria per cell at the maize leaf base.

The examination of plant mitochondrial transcript profiling studies has shown that specific transcript profiles emerge during development. Is this also the case in response to external stimuli? Variations for plant mitochondrial transcript profiles have also been observed during the day and night cycle (Okada and Brennicke 2006). In Arabidopsis mitochondria, these authors found that the transcription activity (measured by run-on RNA assays) varied during the diurnal cycle. In contrast, the steady-state transcript levels did not vary between light and dark phases and were stable throughout the diurnal as well as the circadian time course. From this, the authors concluded that the steady-state transcript levels available in plant mitochondria are sufficient to provide sufficient translation capacity at any time during the diurnal cycle (Okada and Brennicke 2006). This, together with previous work (Giegé et al. 2000), also illustrates that, in mitochondria, transcriptional variations are buffered at the level of posttranscriptional processes.

In a global study, where coordination of gene expression between the nucleus and mitochondria was investigated, authors have applied sugar starvation to Arabidopsis cells (Giegé et al. 2005). In this study, the overall mitochondrial transcript levels appeared to increase when sucrose was removed from the growth medium. On the other hand, the levels of mitochondrial transcripts drastically decreased when sugar was added back to the medium. These variations of RNA levels did not necessarily reflect adjustments in mitochondrial gene expression. The authors rather concluded from their results that the relative increase of mitochondrial transcript levels was due to an overall decrease of nuclear transcript levels in response to stress. Thus, after starvation, the proportion of mitochondrial RNA had increased among total RNA and vice versa, when sugar was added back, nuclear RNA expression had increased again and the proportion of mitochondrial RNA decreased among total RNA. Therefore, at least in this particular case, it appears that mitochondrial transcript levels had not been regulated in response to environmental demands. The required adjustment had rather been achieved by changes in nuclear gene expression and was reflected at the level of mitochondrial protein complexes assembly (Giegé et al. 2005). In another study, the effect of antimycin A treatment on mitochondrial function in wheat embryos was described. The transcript levels of five mitochondrial genes and two nuclear genes encoding mitochondrial proteins decreased in response to stress whereas the alternative oxidase (AOX) level increased (Naydenov et al. 2008). Although this study had not been conducted on a global scale, it suggested that in this case, antimycin A treatment had indeed been reflected at the level of mitochondrial gene expression. Finally, in a recent and very comprehensive study, the effects of low temperature, high salinity and high osmotic potential on the mitochondrial transcriptome have been monitored in wheat embryos (Naydenov et al. 2010). Most of the transcript level variations were stress specific. However, groups of genes could be defined with common responses to different stresses (Fig. 14.2). The authors predict from these results that common regulatory mechanisms must be active in response to some conditions whereas other regulatory processes appear to be active to specifically regulate the mitochondrial transcriptome in response to a particular situation (Naydenov et al. 2010).

The extent of editing in plant mitochondria was also found to be affected by developmentally-related effects. In fact, 67 new editing sites not previously observed in *A. thaliana* Col-0 cell-suspension cultures (Giegé and Brennicke 1999), were detected in rosette leaves (Bentolila et al. 2008). In contrast, 37 of the 441 editing events reported in suspension cultures were not observed in rosette leaves (Table 14.1). The proportion of silent sites in the two classes showing differential editing in the two tissues was similar: 48% (32/67) and 43% (16/37). These percentages were significantly higher than the proportion of silent sites found in the whole

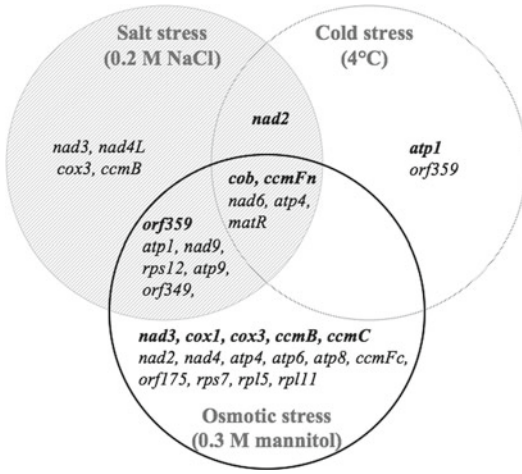

Fig. 14.2. Mitochondrial transcriptome variations in wheat embryos in response to stresses, as modified from Naydenov et al. (2010). Stresses were applied for 3 days. Up- and down-regulated genes (>1.5-fold) are shown in *bold* or *plain font*, respectively. Stress-specific responses are observed for some genes; however, other genes show common response patterns to two or three different stresses.

population of sites edited in either tissue (20%, Bentolila et al. 2008).

VI. Conclusions

Gene expression in plant organelles can be controlled either at the transcriptional or posttranscriptional level. The former is based on the differential use of multiple promoters and RNA polymerases (PEP in plastids, different NEP isoforms in plastids and mitochondria), and the action of various auxiliary factors. At the RNA level, the posttranscriptional regulation relates to differential editing, processing, stability and translatability of transcripts. Although the investigation of transcript profiling in plant organelles does not enable to draw general conclusions, available studies suggests that transcription itself is not highly regulated both in plastids and mitochondria, and that the steady-state levels of transcripts rather appear to be predominantly obtained through posttranscriptional processes.

Various steps of gene expression in plant organelles have been analyzed at a genome-wide scale by using DNA array-based technologies or others. Genotypic variability for the extent of RNA editing or transcript processing and stability in cytoplasmic organelles has been observed in natural populations at the interspecific and intraspecific level or in artificial CMS lines. The possibility to produce knock-out lines by plastid transformation has been particularly useful to highlight the role of various plastid genes on global genome expression and chloroplast development.

Specific transcript profiles can clearly be achieved also in response to developmental signals and environmental stimuli. Significant differences in the transcriptome, editome and translatome have been found comparing different plastid types in diverse organs or tissues. Similar differences have been found for mitochondrial genomes during the diurnal cycle or between cell suspensions and differentiated leaves. However, the precise levels and mechanisms at which these changes are achieved and the signals necessary to trigger them are barely understood.

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