New insights into plastid nucleoid structure and functionality

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Abstract Investigations over many decades have revealed that nucleoids of higher plant plastids are highly dynamic with regard to their number, their structural organization and protein composition. Membrane attachment and environmental cues seem to determine the activity and functionality of the nucleoids and point to a highly regulated structure–function relationship. The heterogeneous composition and the many functions that are seemingly associated with the plastid nucleoids could be related to the high number of chromosomes per plastid. Recent proteomic studies have brought novel nucleoid-associated proteins into the spotlight and indicated that plastid nucleoids are an evolutionary hybrid possessing prokaryotic nucleoid features and eukaryotic (nuclear) chromatin components, several of which are dually targeted to the nucleus and chloroplasts. Future studies need to unravel if and how plastid–nucleus communication depends on nucleoid structure and plastid gene expression.

Keywords Plastid nucleoid-associated proteins · Plastid nucleoid structure · Plastid gene expression · Transcriptionally active chromosome

Abbreviations

ptNAP Plastid nucleoid-associated protein
NEP Nuclear-encoded plastid RNA polymerase

PEP Plastid-encoded plastid RNA polymerase
ptDNA Plastid DNA
TAC Transcriptionally active chromosome

Introduction

Plastid DNA is assembled in complex structures of high molecular weight that are attached to intraplastidial membranes and contain proteins as well as RNA. These structures, termed plastid nucleoids or plastid nuclei (Sakai et al. 2004), are associated with numerous enzymatic activities such as DNA repair, DNA replication, recombination, transcription and post-transcriptional control of gene expression. Microscopic studies showed that the localization and composition of the complexes are dynamic and undergo age-dependent changes that could be coupled to changes in the overall activity and specificity of RNA polymerases and components of other regulatory levels of plastid gene expression.

For a long time, knowledge about the protein components of the plastid nucleoid was limited to information from biochemical studies, which was summarized in excellent reviews by Sato and co-workers (Sato et al. 2003) as well as by Sakai and co-workers (2004). The improvement of bioinformatic targeting predictions, GFP-based analysis of subcellular protein localization and last but not least the increasing sensitivity of proteome analyses have led to a recent sharp increase in our knowledge of the composition of the plastid nucleoids. Among the newly identified proteins are DNA binding proteins with eukaryotic motifs for which a dual targeting activity to the plastids and the nucleus has either been demonstrated or is postulated. The current review will concentrate mainly on

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these recent advances and will refer to older knowledge only when necessary for the integrated picture of the whole nucleoid. We therefore apologize to those whose work could not be cited due to space limitations.

Dynamics in the organization of the multicopy chloroplast genome in higher plants

As indicated by the formation of nucleoids, plastids have kept many of the structural and organizational features of their prokaryotic ancestors. On the other hand, they have also evolved several unique features. For example, the conformation of the plastid DNA exhibits a much greater structural complexity and variability than that of their bacterial counterparts. Investigations have revealed that in addition to the circular forms also linear as well as branched monomers and multimers with defined ends can occur (Bendich 2004; Bock 2007). The fundamental difference between genome organization in plastids versus that in bacteria is that the plastids are multi-nucleated as opposed to the single-nucleated organization of their prokaryotic counterparts (Sakai et al. 2004). In higher plants, the plastid genome occurs in high copy numbers which differ in various organs and cell types (Boffey et al. 1979; Mullet 1993). In mesophyll cells of green leaves, numbers in the range from 2,000 to 50,000 genomes per cell were estimated (Kuroiwa et al. 1982; Bendich 1987; Coleman and Nerozzi 1999). On average, 10–20 copies of the plastid genome are organized into a variable number of structures that originally were named plastid nucleoids due to their resemblance to their bacterial counterparts (Kuroiwa 1991). Plastid nucleoids exhibit tremendous differences with respect to number, shape and distribution that parallel the developmental changes in structure and function of plastids (Sato et al. 2003). Differences in nucleoid number and morphology seem to occur also in different sectors of variegated leaves (Sakamoto et al. 2009) and during plastid division.

In yeast mitochondria, there is evidence for a functional variance among the multiple nucleoids within one mitochondrion. An actively replicating type can be distinguished from a non-replicating type by its association with a proteinaceous structure spanning the inner and the outer membrane (Meeusen and Nunnari 2003). Given the significant morphological differences among plastid nucleoids, structural and functional variance among the multiple nucleoids of a single chloroplast is also likely. In fact, sizes and positions of different nucleoid sub-populations could very well reflect the different requirements for enzymatic activities within the plastid nucleoids during plastid biogenesis. First support for this comes from co-localization studies of different plastid nucleoid-associated proteins (ptNAPs) (Melonek et al. 2010).

Sub-nucleoid domains

A functional diversification in different zones was reported for nucleoids of mitochondria (Shutt et al. 2010) and bacteria (Dillon and Dorman 2010) as well as for the chromatin in the nucleus (Shaw and Brown 2004; Matera et al. 2009). The benefit of these sub-compartmental structures is concentration of DNA-binding proteins and DNA-regions to certain areas, which might enhance the rates of biochemical reactions associated with gene expression and might confer an evolutionary advantage over a random chromatin architecture (Matera et al. 2009).

Evidence for discernible sub-domains with different specific functions has also been found within plastid nucleoids. Initial reports from electron microscopic studies, which showed that the de-proteinized spinach plastid chromosome is organized in a folded form around a central body composed of proteins which are not extractable by usual procedures (Herrmann et al. 1974; Yoshida et al. 1975), were soon followed by biochemical data that confirmed the observation that proteins are either firmly bound to DNA in a central region or are more loosely bound to the peripheral DNA fibrils. These observations led to the proposal of a layered structure of plastid nucleoids (Briat et al. 1982; Hansmann et al. 1985; reviewed by Sakai et al. 2004) (see Fig. 1). An estimated 30–50 % of the ptDNA is incorporated in the central body. Surprisingly, ultrastructural studies revealed that the DNA loops protruding from the core of chloroplast nucleoids are lacking in nucleoids from chromoplasts of *Narcissus pseudonarcissus* (Hansmann et al. 1985). Even though this indicates that the layered structure is not static but rather under developmental control, it is unclear what determines the relative proportions and degrees of compaction in the nucleoid layers and whether the observed changes have functional consequences.

Some authors have compared the dense nucleoid core with eukaryotic heterochromatin and the more dispersed peripheral regions with eukaryotic euchromatin where transcription is active (Sato and Ohta 2001; Sato et al. 2003). However, this contrasts with the observation that even highly condensed plastid DNA can be actively transcribed (Kuroiwa et al. 1990). The latter, in fact, corresponds well with the current model of mitochondrial nucleoid architecture in which replication and transcription were suggested to occur in the central core whereas translation and complex assembly occur in peripheral regions (Bogenhagen et al. 2008). Sakai and co-workers (Sakai et al. 2004) in fact suggested that the central body is involved in membrane binding and that this is a prerequisite for active transcription. On the other hand, at least one type of plastid RNA polymerase, namely the NEP encoded by the *RPOTmp* gene seems to be sensitive to

Fig. 1 Model for the layered structure of plastid nucleoids. Plastid nucleoids consist of a dense layer, or nucleoid core, where protein–DNA interactions are insensitive to salt or detergent treatments (see text) and where transcriptional activity is particularly high (Sakai et al. 2004). Several membrane anchor proteins in this layer can mediate the attachment of the DNA to plastidial membranes. The nucleoid core is surrounded by a second layer where DNA–protein interactions and possibly DNA compaction are less tight. Switching

between the “core DNA” conformation and the “surrounding DNA” conformation could be mediated by chromatin remodeling proteins like SWIB-4 that are depicted here exemplarily at the interface between the two layers. Other membrane-associated proteins like the NEP interacting protein NIP can selectively control the activity of the NEP polymerase by preventing its association with plastid DNA (Azevedo et al. 2008)

membrane binding and was proposed to be switched off when it is brought into close proximity of the thylakoid membranes. This sensitivity might be conferred by a thylakoid membrane-embedded RING protein, NIP1 (NEP interacting protein 1), that showed specific interaction with RPOTmp (Azevedo et al. 2008), reflecting the complex interplay between structure, distribution and activity.

Considering that the two plastid nucleoid layers or sub-domains could have different functionality, they would also be expected to have different sets of proteins. Whether they, in addition, attract different chromosomal regions, has not been answered conclusively but there is some evidence for this. For example, the analysis of transcripts synthesized by highly purified transcriptionally active chromosomes (TAC) that resemble the central body, revealed that ribosomal RNA transcripts were enriched (Krause and Krupinska 2000). This might indicate a spatial organization of different transcription foci similar to what has been found in bacteria (Cabrera and Jin 2003, 2006).

Analysis of the protein composition of plastid nucleoids

Plastid nucleoids can be prepared by different methods. Most preparations were done by either sucrose density gradient centrifugation (Nemoto et al. 1988; Cannon et al. 1997) or by gel filtration of extracts prepared from plastid

membranes treated with detergents (Hallick et al. 1976; Krause and Krupinska 2000). The DNA containing fraction prepared by gel filtration was called TAC, because it has been used initially to determine the activity and composition of the transcriptional apparatus (see reviews of Grussem and Tonkyn 1993; Igloi and Kössel 1992; Sakai et al. 2004, and references therein). The TAC fraction is prepared from plastid membranes by release of the nucleoids with detergents and by subsequent purification involving sepharose gel filtration and other steps (Krause and Krupinska 2000; Pfalz et al. 2006). Recent proteomic analyses of these (Pfalz et al. 2006; Melonek et al. 2012) and other nucleoid-enriched fractions (Phinney and Thelen 2005; Majeran et al. 2012) have revealed that several of the newly identified DNA binding proteins were not inherited from the prokaryotic ancestors. Plastid proteins homologous to the abundant prokaryotic HU protein, on the other hand, were not detected in higher plants which stands in contrast to their identification in some algal plastids (Karcher et al. 2009), in the apicoplasts of apicomplexans (Sato et al. 2003) as well as in some dinoflagellates (Chan et al. 2006).

In analogy to the nomenclature proposed for bacterial nucleoid-associated proteins (NAPs) (Dillon and Dorman 2010), proteins of the plastid nucleoids are here designated plastid nucleoid-associated proteins (ptNAPs). NAPs contribute to both nucleoid structure and gene regulation and

an according classification has been discussed (Dillon and Dorman 2010). In this review we follow a simplified classification into enzymatic ptNAPs (including transcription, post-transcriptional levels of gene expression, DNA replication and repair) and architectural ptNAPs (including membrane anchoring and DNA compaction). It should be kept in mind, however, that the architecture can have an influence on transcriptional activity and on the formation of transcriptional foci in bacteria (Berger et al. 2010) and that the same is likely the case in plastids.

Enzymatic ptNAPs

Transcription

A number of functional activities have been associated with the nucleoids of plastids. These include the transcription of plastid genes. Accordingly, core subunits of the plastid-encoded RNA polymerase, PEP, have been identified in most of the proteomic studies of plastid nucleoid fractions of dicots (i.e. Arabidopsis, mustard, spinach, pea) (Phinney and Thelen 2005; Pfalz et al. 2006; Melonek et al. 2012) as well as monocots (i.e., maize) (Majeran et al. 2012). A preparation, where only the core PEP and very tightly associated subunits were preserved, contained an additional ten proteins that were termed PEP-associated proteins (for PAPs) (Steiner et al. 2011). These PAPs seem to be closely associated accessory subunits of the PEP core. In a recent detailed study, one of them, the PAP1/pTAC3 protein, was confirmed to be an essential component of the chloroplast PEP complex during transcription initiation, elongation and termination (Yagi et al. 2012).

Besides PEP, a nuclear-encoded plastid RNA polymerase, NEP, has been postulated to be involved in plastid gene transcription (for a recent review see Liere et al. 2011). So far, this NEP has neither been identified in the TAC fraction nor in the soluble protein fraction (Pfannschmidt and Link 1994; Krause and Krupinska 2000) that has recently been subjected to proteomic analysis (Schröter et al. 2010) nor in any of the nucleoid or TAC proteomes. It is, therefore, likely that its amount is below the detection limit even of the currently available analytical methods.

Interestingly, only one sigma factor (Sigma 2) could be identified in the nucleoid preparations performed with maize seedlings (Majeran et al. 2012) while no sigma factors were detected in preparations of the soluble RNA-polymerase fraction (Suzuki et al. 2004; Schröter et al. 2010) including fractions enriched in PEP (Steiner et al. 2011). The reason for this failure might be that the proteins escaped detection due to very low abundance. However, it is equally possible that the transient interactions of these

factors with the DNA are too weak to grant a co-purification. The same could be true for the putatively dually targeted transcription factors with eukaryotic binding motifs that were identified in bioinformatic screens (Wagner and Pfannschmidt 2006; Schwacke et al. 2007) as well as for the Sigma factor binding proteins (SIB) 1 and 2 (Morikawa et al. 2002; Lai et al. 2011). For the SIBs, experimental data were recently provided that they are, in fact, dually targeted to the nucleus and the plastids (Lai et al. 2011).

Post-transcriptional levels of gene expression

In the TAC fraction of spinach (Melonek et al. 2012) two members of the cpRNP family (CP29B, CP31A) have been found. In the TAC proteome analysis described by Pfalz et al. (2006) a PPR protein with a molecular weight of 97 kDa (pTAC2) was identified. Interestingly, Phinney and Thelen (2005) identified a pea protein of similar size in their nucleoid analysis which matched the monoisotopic masses of the same PPR protein, corroborating its presence in chloroplast nucleoids. In a recent analysis of spinach TAC, two further PPR proteins of 98 and 67 kDa were identified (Melonek et al. 2012). Since cpRNPs and PPRs are both involved in organellar RNA processing and translation (Schmitz-Linneweber and Small 2008; Ruwe et al. 2011), this indicates that post-transcriptional levels of gene expression such as RNA stability and turnover are at least in part closely connected to the nucleoids. This is in line with the notion that ribosomes seem to be quite closely associated with the nucleoids of plastids (Phinney and Thelen 2005; Pfalz et al. 2006). This close association of ribosomal subunits with the DNA–protein complexes is, in fact, a common trait between plastid and mitochondrial nucleoids (see Kucej and Butow 2007; Nosek et al. 2006, and references cited within), and also explains the occurrence of proteins involved in translation in all plastid nucleoid fractions containing subunits of PEP (Phinney and Thelen 2005; Pfalz et al. 2006; Majeran et al. 2012; Melonek et al. 2012) (Table 1). Their systematic occurrence in diverse preparations and organisms suggests that post-transcriptional processing steps and translational activity are probably closely associated with the nucleoids (Majeran et al. 2012). This is also in accordance with reports from bacterial nucleoids (Gowrishankar and Harinarayanan 2004).

DNA methylation

Methylation of plastid DNA and its potential effects on gene expression has in the past been discussed controversially. While early reports could convincingly demonstrate that DNA methylation is crucial for the regulation of transcription during differentiation of amyloplasts in

Table 1 Examples for ptNAPs with a metabolic function in plastids

NAP	Arabidopsis homolog	Metabolic function	Evidence for nucleoid association
NmrA-like/pTAC16	At3g46780	NADP-dependent reductase/dehydrogenase	Proteomic (Phinney and Thelen 2005; Pfalz et al. 2006; Majeran et al. 2012)
FSD2, FSD3	At5g51100 At5g23310	Iron superoxide dismutase (Myouga et al. 2008) ^a	Proteomic (Pfalz et al. 2006; Myouga et al. 2008)
TRX Z	At3g06730	Thioredoxin (Arsova et al. 2010) ^a	Proteomic (Pfalz et al. 2006; Majeran et al. 2012)
SiR		Ferredoxin:sulfite reductase precursor (Kang et al. 2010; Sekine et al. 2007) ^a	Proteomic, Western blot, immunofluorescence, gene silencing (Phinney and Thelen 2005; Kang et al. 2010; Sekine et al. 2007)
MurE	At1g63680	Mur ligase	Proteomic (Pfalz et al. 2006; Majeran et al. 2012)
Rca	At2g39730	Rubisco activase	Proteomic (Phinney and Thelen 2005; Melonek et al. 2012)
ClpC1/C2, ClpD	At5g50920 At3g48870 At5g51070	Protease; chaperonin	Proteomic (Phinney and Thelen 2005; Majeran et al. 2012)
ATPase subunits	Chloroplast encoded	ATP generation	Proteomic (Phinney and Thelen 2005; Pfalz et al. 2006; Melonek et al. 2012)
Aminotransferase-like protein 1	At3g05190	Branched-chain amino acid biosynthesis	Proteomic (Melonek et al. 2012)
CND41	At5g10760	DNA-binding aspartate protease (Murakami et al. 2000) ^a	Immunoblot (Nakano et al. 1997)

^a Functions which have been experimentally demonstrated

sycamore cells (Ngerprasisirsiri et al. 1988a, 1990) and during chromoplast formation in ripening tomato fruits (Ngerprasisirsiri et al. 1988b; Kobayashi et al. 1990), more recent analyses came to a different conclusion. Two investigations by Jaffé et al. (2008) and Ahlert et al. (2009), respectively, reported a methylation of plastid DNA upon integration of different genes encoding DNA methyltransferases into the tobacco chloroplast genome. This methylation was shown to have no effect on plastid gene expression in chloroplasts. This is, however, not surprising because changes in methylation were also not observed during chloroplast development in barley (Krupinska 1992), but might rather be important in other plastid types. Although methylation is likely to occur in plastids, a DNA methyltransferase was so far not identified in any of the TAC or nucleoid proteomes.

Replication and repair

Nucleoids have been shown to be active in replication of DNA (Kuroiwa 1991; Heinhorst and Cannon 1993) and some candidates for these activities have been identified in the newly published nucleoid proteomes but so far very few reports have analyzed these activities more carefully. The polymerase that is responsible for the replication of the plastid chromosome has originated from the mitochondrial

RNA polymerase, Pol I or POP (reviewed by Moriyama et al. 2010). Along with DNA gyases, it belongs to the enzymes whose presence in TAC proteomes was shown repeatedly (Phinney and Thelen 2005; Pfalz et al. 2006; Ono et al. 2007).

In the proteomic study of thylakoid membranes isolated from pea chloroplasts, a homolog of the bacterial RecA protein (RecA1) could be identified (Phinney and Thelen 2005). Members of the Rec family are associated in general with recombinational activities. One protein with a RecF/RecN/SMC domain has also been identified in the spinach TAC proteome (Melonek et al. 2012). In rice, two RecQ-like helicases, OsRecQ1 and OsRecQsim, were found to be targeted to the plastids and were discussed as components of a plastid-specific DNA-repair system (Saotome et al. 2006). It has also been reported, that the two plastid-located Whirly proteins AtWhy1 and AtWhy3 that have been identified in TAC proteomes (Pfalz et al. 2006) function as anti-recombination proteins contributing to safeguard plastid genome integrity (Marechal et al. 2009). Whirly proteins can bind to single stranded DNA and RNA (Desveaux et al. 2000; Prikryl et al. 2008) and seem to assume a number of important functions in nucleoid metabolism. Like the Whirly group of proteins, the organellar single stranded DNA binding proteins (OSBs) belong to a small family of proteins involved in ptDNA

stability and recombination surveillance (Marechal and Brisson 2010). OSB2 alias pTAC9 was identified in the Arabidopsis TAC proteome (Pfalz et al. 2006) while OSB1 was found to be associated with nucleoids in maize (Majeran et al. 2012). Other proteins proposed to be involved in plastid DNA repair are Arp1, Nth2 and MutS. MutS is dually localized to plastids and mitochondria and has been postulated to be involved in DNA recombination and to influence genome stability and plastid development (Xu et al. 2011).

Architectural ptNAPs

Membrane anchoring

While bacterial nucleoids are associated with the cytoskeleton (Travers and Muskhelishvili 2005), organellar nucleoids are attached to intraplastidial membranes that were proposed to serve as a platform for DNA synthesis and transcription (Sakai et al. 2004). Consequently, many of the architectural nucleoid proteins that have so far been identified in plastids are involved in anchoring the nucleoids to either envelope membranes (PEND, PD1, PD3) or thylakoid membranes (MFP1, TCP34, pTAC16).

PEND

The plastid envelope DNA binding protein (PEND) is a 70 kDa membrane-spanning protein with a basic region plus leucine zipper (bZIP) domain that forms dimers in vivo which seem to tether the nucleoids to the plastid inner envelope (Sato et al. 1993, 1998). The PEND protein was initially discovered in developing pea chloroplasts (Sato et al. 1993). Homologs were later detected in other angiosperms, e.g., in *Brassica napus* (Waldmüller et al. 1996) while functional homologs in algae and in non-flowering plants are still not known (Terasawa and Sato 2005a). The cbZIP domain of PEND was shown to bind selectively to AT-rich regions of plastidic DNA containing the canonical sequence TAAGAAGT (Sato and Ohta 2001). Interestingly, both the PEND protein from *Brassica napus* and its rapeseed homolog, GSBF1, were found to repress the expression of the nuclear-encoded *rbcS* gene (Waldmüller et al. 1996; Wycliffe et al. 2005). That PEND indeed fulfills a direct role in the nucleus was supported by the recent observation that a PEND:GFP fusion protein is targeted to the nucleus when the N-terminal presequence is deleted (Terasawa and Sato 2009). It has been proposed that the PEND protein might be first targeted to plastids where the N-terminal presequence is cleaved and that it might be relocated to the nucleus when the chloroplast envelope is degraded by accident or by some kind of

physiological process (Terasawa and Sato 2009). Interestingly, to date PEND has been only identified by proteome analysis of a Triton-insoluble fraction of nucleoids (Phinney and Thelen 2005). This could indicate that the integration of PEND inside the envelope membrane is so stable that it was not extracted by any detergent used by the other methods enriching nucleoids.

PD1 and PD3

PD1 and PD3 (plastid DNA binding 1 and 3, respectively) were detected during research on PEND (Sato et al. 1995) and are both characterized by the possession of AT-hook motifs. These motifs were originally associated with the high mobility group I (HMG-I) of nuclear proteins and seem to interact with the minor groove of AT-rich regions of nuclear DNA (Grasser 1995). In addition to the two or five AT-hooks of PD1 and PD3, respectively, PD3 possesses eight CxxC motifs, which are presumed to be metal binding sites of the protein (Sato et al. 1995). PD3 also has a Jumonji C (jmc) domain at the C-terminus which was proposed to be involved in chromatin remodeling (Kodama 2007). Structural data suggest that these plastid DNA binding proteins have evolved from nuclear transcription factors containing AT-hook motifs (Kodama 2007). The localization of the PD1 and PD3 proteins to the plastid nucleoids was shown by immunological analysis with specific antibodies. In contrast to PEND which was shown to be distributed between membrane and stroma fractions, PD1 and PD3 were exclusively found in membrane fractions including the attached nucleoids (Sato et al. 1995). Similarly as PEND, neither of the two proteins was detected in the proteomes of recent nucleoid or TAC preparations from chloroplasts.

MFP1, TCP34 and pTAC16

The MAR binding filament-like protein 1 (MFP1) mediates the attachment of nucleoids to thylakoid membranes. MFP1 was first described in tomato as a nuclear DNA binding protein that connects chromatin with the nuclear envelope via matrix attachment regions (MAR) (Meier et al. 1996). MFP1, as an anchor protein, has an additional N-terminal hydrophobic membrane-spanning domain (Meier et al. 1996). Its targeting to the thylakoid membranes of chloroplasts was detected some years later and MFP1 is now known to be involved in anchoring the nucleoids to the thylakoid membranes with the C-terminal DNA-binding domain orientated towards the stroma (Jeong et al. 2003) (see model in Fig. 1). Another protein possibly involved in anchoring of plastid nucleoids to thylakoid membranes is the tetratricopeptide-containing chloroplast protein of 34 kDa (TCP34), which was shown to stably

associate with thylakoid membranes (Weber et al. 2006). Immunological analysis revealed that the protein is a TAC component (Weber et al. 2006; Melonek et al. 2010). The recombinant TCP34 protein showed specific binding to chloroplast DNA isolated from spinach (Weber et al. 2006). Very recently, it was postulated that next to MFP1 and TCP34 also the pTAC16 protein could anchor DNA to the thylakoid membranes (Ingelsson and Vener 2012; Majeran et al. 2012).

Nucleoid/DNA compaction

SiR

Sulfite reductase (SiR) is one of the most abundant proteins of nucleoids with a molecular weight of 70 kDa. It was shown to repress the transcriptional activity in isolated nucleoids by compacting their DNA (Sekine et al. 2002). In contrast to the PEND protein its binding to DNA is apparently sequence-independent. Nevertheless, it was shown in a recent study that the depletion of SiR leads to a significant downregulation of the expression of some chloroplast genes whereas others were unaffected or even upregulated (Kang et al. 2010). This provides important evidence that DNA compaction and gene expression are linked and that the level of compaction is not homogenous in the nucleoids. The 68 kDa protein DCP68 which an earlier report linked to a suppression of ptDNA replication (Cannon et al. 1998) was found to correspond to SiR. SiR, which initially has been identified as a sulfite reductase is therefore a bifunctional (Sato et al. 2001) or even multifunctional protein. It has been shown that binding to DNA does not influence the sulfite reductase activity (Sekine et al. 2007) but, whether, vice versa, the enzymatic activity modulates the DNA compacting activity is not known. Such interplay would be of particular interest, as it would potentially link gene expression to chloroplast metabolism. So far, SiR has been detected in the proteome of a Triton-insoluble plastid fraction that is enriched in NAPs but is devoid of membranes (Phinney and Thelen 2005).

CND41

The chloroplast nucleoid DNA binding protein of 41 kDa (CND41) is another protein that binds non-specifically to plastid DNA via a helix-turn-helix motif and a putative zinc finger motif. It was identified initially in nucleoids isolated from cultured tobacco cells (Nakano et al. 1993). CND41 has been regarded as a negative regulator of transcription because its abundance in various tissues negatively correlates with the levels of certain plastid mRNAs (Nakano et al. 1997). Its sequence is similar to those of aspartic proteases and it exhibits strong proteolytic

activity at an acidic pH (Murakami et al. 2000). It could be shown that CND41 is involved in Rubisco degradation and in the translocation of nitrogen during senescence (Kato et al. 2004). In senescent leaves of barley its expression was shown to be down-regulated (Parrott et al. 2007). This suggests that the activity of CND41 is associated with the maintenance of chloroplast function and not to dismantling of chloroplasts.

YLMG1

A protein involved in nucleoid distribution, AtYLMG1-1 was discovered recently by Kabeya and co-workers (Kabeya et al. 2010). While plastids of wild-type *Arabidopsis thaliana* contain a filamentous network of multiple nucleoids during chloroplast division (Terasawa and Sato 2005b), overexpression or knockdown of *AtYLMG1-1* caused the formation of an irregular network of chloroplast nucleoids or aggregation of the nucleoids as larger structures, respectively (Kabeya et al. 2010). Immunofluorescence studies revealed that AtYLMG1-1 forms a punctuate pattern in close proximity to thylakoid membranes and possibly co-localizes with nucleoids (Kabeya et al. 2010).

SWIB-4

Among the 46 proteins identified in the highly enriched TAC-II fraction from spinach chloroplasts a small molecular weight protein containing a SWIB (for SWI/SNF complex b) domain was identified (Melonek et al. 2012). The SWIB domain represents a conserved region found in human BAF60b proteins (Bennett-Lovsey et al. 2002) that are known components of SWI/SNF (for switching defective/sucrose non fermenting) chromatin remodeling complexes originally identified in yeast (Burns and Peterson 1997). Sequences for 20 SWIB domain-containing proteins are present in the genome of *Arabidopsis thaliana*, six of which are predicted to localize to mitochondria and/or chloroplasts and the nucleus. By fusion with GFP, the chloroplast localization for four of them (SWIB-2, SWIB-3, SWIB-4 and SWIB-6) could be confirmed (Melonek et al. 2012). Interestingly, out of the four plastidic SWIBs, only SWIB-4 has a histone H1 like motif (KKPAAKPKAK AKPKPKAKSDSPAK). A SWIB-4:GFP fusion protein was located in plastids and the nucleus (Melonek et al. 2012), placing SWIB-4 alongside several other dually targeted nucleoid proteins like PEND, MFP1, Whirly1 and others (Krause and Krupinska 2009). Overexpression of the SWIB-4 protein in *Escherichia coli* led to complete arrest of bacterial cell growth caused by compaction of the nucleoid (Melonek et al. 2012). In a complementation study of an *E. coli* mutant lacking HNS, an abundant NAP

in bacteria, the compacting function of SWIB-4 could be confirmed (Melonek et al. 2012).

Proteins replacing HU and histones

The SWIB domain proteins identified in chloroplasts were shown to have low molecular masses, high isoelectric point and high lysine content (Melonek et al. 2012). They hence belong to those DNA-binding proteins biochemically detected in thylakoids and nucleoid fractions (Briat et al. 1984; Bülow et al. 1987; Nemoto et al. 1988; Yurina et al. 1988; Baumgartner and Mullet 1991) that could have functionally replaced the bacterial histone-like HU (for histone-like protein from *E. coli* strain U93) proteins which are involved in formation of nucleosome-like structures in bacterial chromosomes (Rouviere-Yaniv and Gross 1975). Among these proteins are several low molecular weight proteins (10–20 kDa) detected by cross-reaction with antibodies directed toward the bacterial HU (Briat et al. 1984). Such basic low molecular weight proteins of eukaryotic origin could have replaced the bacterial histone-like proteins as has been hypothesized earlier (Kodama 2007).

Bifunctionality and dual targeting of ptNAPs

An intriguing aspect is the association of metabolic/bio-synthetic chloroplast functions with nucleoid fractions that have been found, as detailed in the corresponding chapters above. In this context, the repeated discovery of NAPs such as SiR and CND41 with homology to proteins possessing known metabolic functions (see Table 1) seems to corroborate that in nucleoids a tight linkage between gene expression and metabolism exists. While on one hand the level of gene expression determines the metabolic activity, metabolic enzymes on the other hand may directly give feed back to the genetic machinery by influencing, for example, accessibility of transcription factors and core enzymes to promoters. How exactly the bifunctional ptNAPs contribute to the regulation of nucleoid structure and gene expression is in most cases unknown and awaits future investigations. The association of yeast and human mitochondrial nucleoids with metabolic enzymes whose occurrence seems to be highly conserved between different fungal and mammalian species (Nosek et al. 2006) has triggered the hypothesis that such activities could mediate signal transduction from metabolic stimuli to the nucleoids and invoke structural changes (Kucej and Butow 2007). Well documented examples of bifunctional proteins associated with chloroplast nucleoids are SiR, FSD2/FSD3 or the CND41 protein (Table 1).

In several nucleoid preparations, subunits of the chloroplast Clp protease system could be identified (Table 1). This fits with the recent notion that ATP-dependent proteases (AAA+ proteases) appear to be associated with eubacterial and mitochondrial nucleoids (Ambro et al. 2012). It is postulated that AAA+ proteases could control the level of several abundant DNA-binding proteins and in this way regulate many nucleoid-associated processes. Moreover, they could be involved directly in the regulation of gene transcription by controlling the cellular level of transcriptional regulators and thereby could influence the formation of active transcription complexes (Ambro et al. 2012). For two *E. coli* proteases (Lon and ClpA) a direct binding to DNA could be shown (Zehnbauer et al. 1981; Kubik et al. 2012). In a proteomic study of the Clp protease complexes from *Arabidopsis thaliana* three Clp AAA+ chaperones (C1, C2, D) that possess chloroplast targeting sequences and are similar to the *E. coli* ClpA could be identified (Peltier et al. 2004). The homolog of the *Arabidopsis* ClpC1 was identified as a component of the TAC fraction isolated from spinach (Melonek et al. 2012) as well as nucleoid-enriched fractions from pea (Phinney and Thelen 2005) and maize (Majeran et al. 2012). In the latter case also the ClpD subunit could be found (Majeran et al. 2012) (Table 1). Further investigations will be needed to unravel a possible role of AAA+ proteases in the regulation of plastid nucleoid dynamics and metabolism.

Several of the plastid DNA binding proteins that were experimentally identified possess motifs that are typically found in nuclear transcription factors (Kodama 2007). Examples are PEND, CND41, PD1, PD3 (Kodama 2007) as well as Whirly1 (Krause et al. 2005; Grabowski et al. 2008) and the newly identified ptNAP SWIB-4 (Melonek et al. 2012). It has been suggested that these proteins have changed their subcellular localization during evolution and became plastid or dually targeted proteins. For some of these proteins experimental evidence for a dual localization in plastids and in the nucleus has already been obtained (Grabowski et al. 2008; Terasawa and Sato 2009; Chen et al. 2010; Isemer et al. 2012) and functions of the plastidic and nuclear isoforms have been analyzed (Table 2). Two in silico studies performed on eukaryotic transcription factors have revealed that a considerable number of these proteins with a nuclear localization sequence also have an N-terminal plastid targeting sequence, increasing the number of putative dually targeted DNA-binding proteins significantly (Wagner and Pfannschmidt 2006; Schwacke et al. 2007). Among the DNA binding proteins predicted to be dually located, some were initially described to have functions in nuclear chromatin remodeling. This occurrence of chromatin remodeling proteins with functions in the nucleus and plastids could imply that communication between plastids and the nucleus occurs by dynamic

Table 2 Examples for identified or putative ptNAPs from *Arabidopsis* that are dually targeted to plastids and the nucleus

NAP	AGI code	Plastid function	Nuclear function
PEND	At3g52170	Anchorage to envelope membranes (Sato et al. 1993; Sato and Ohta 2001)	Unknown (Terasawa and Sato 2009)
MFP1	At3g16000	Anchorage to thylakoid membranes (Jeong et al. 2003)	Chromatin attachment to the nuclear envelope (Meier et al. 1996)
Whirly1	At1g14410	SsDNA and RNA binding; recombination (Marechal and Brisson 2010; Melonek et al. 2010; Pfalz et al. 2006; Prikryl et al. 2008)	Transcription factor and telomere homeostasis (Desveaux et al. 2000; Yoo et al. 2007)
SWIB-4	At3g03590	Unknown (Melonek et al. 2012)	Component of SWI/SNF chromatin remodeling complexes (Melonek et al. 2012)
pTAC12/HEMERA/PAP5	At2g34640	Component of the plastid transcriptional apparatus, subunit of plastid-encoded RNA-polymerase complex (Gao et al. 2011; Pfalz et al. 2006; Steiner et al. 2011)	Phytochrome signaling (Chen et al. 2010)
AGI <i>Arabidopsis</i> gene identifier ^a SIB1 and 2 were not identified by current nucleoid proteomes	SIB1 + 2 ^a At3g56710 At2g41180	Interacting with Sigma factor Sig1 (Morikawa et al. 2002)	Activation of WRKY33, pathogen response (Lai et al. 2011)

changes in both plastid nucleoid structure and nuclear chromatin architecture. Whether a differential distribution of the proteins between the two compartments could induce changes in structure and gene expression awaits further investigations.

Conclusions and prospects

The proteomic studies with chloroplast nucleoids performed so far show that nucleoids are not isolated entities within plastids, but are connected to structures performing different pivotal metabolic functions such as photosynthesis and fatty acid biosynthesis. Sophisticated biochemical purification methods will be required to get further insight into the substructure of nucleoids in order to discriminate between core components and different layers at the periphery of nucleoids and to understand why some ptNAPs like PEND, NEP or Sigma-like factors are so conspicuously underrepresented in the current analyses. The investigation of protein–protein interactions among the ptNAPs might help to get insight into the organization of nucleoids within the plastid. Of particular importance for future investigations are comparative analyses with biochemically purified nucleoids from different stages of chloroplast development. This will extend the insight into the roles of nucleoids within the overall context of development dependent plastid functions that has been initiated recently (Majeran et al. 2012).

Environmental impact on nucleoid morphology represents another intriguing open question. Chloroplasts can be

regarded as sensors of environmental changes (Bouvier et al. 2009) that are perceived as imbalances in electron flow resulting in changes in the redox state of the plastoquinone pool and other redox systems of chloroplasts (Pfannschmidt et al. 2009). These imbalances are known to alter the transcription of a number of plastid genes (Pfannschmidt et al. 1999). Considering the tight association of nucleoids and thylakoids, a direct perception of redox changes in the photosynthetic apparatus by redox sensitive proteins in the nucleoids as also reported for a soluble RNA polymerase preparation from chloroplasts (Schröter et al. 2010) must be expected.

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