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2 **Biogenic signals from plastids and their role in chloroplast development**

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15

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27 **Abstract**

28 Plant seeds do not contain differentiated chloroplasts. Upon germination the seedling, thus,
29 need to gain photoautotrophy before storage energies are depleted. This requires the
30 coordinated expression of photosynthesis genes encoded in nuclear and plastid genomes.
31 Chloroplast biogenesis needs to be additionally coordinated with the light regulation network
32 that controls seedling development. This coordination is achieved by nucleus-to-plastid
33 signals called anterograde and plastid-to-nucleus signals coined retrograde. Retrograde
34 signals sent from plastids during initial chloroplast biogenesis are also called biogenic signals.
35 They have been recognized as highly important for proper chloroplast biogenesis and for
36 seedling development. The molecular nature, transport, targets and signalling function of
37 biogenic signals are, however, under debate. Several studies disproved the involvement of a
38 number of key components that were at the base of initial models of retrograde signalling.
39 New models now propose major roles for a functional feedback between plastid and cytosolic
40 protein homeostasis in signaling plastid dysfunction as well as the action of dually localized
41 nucleo-plastidic proteins that coordinate chloroplast biogenesis with light-dependent control
42 of seedling development. This review provides a survey of the developments in this research
43 field, summarizes the unsolved questions, high-lights several recent advances and discusses
44 potential new working modes.

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57 **General introduction**

58 Plastids are cell organelles that are characteristic for plant and algae cells. They originated
59 from an event in which a mitochondriated eukaryotic cell engulfed a cyanobacteria-like
60 photosynthetic prokaryote. This engulfment was of mutual benefit resulting in a stable
61 endosymbiosis (Archibald 2015). The circumstances leading to the establishment of this
62 endosymbiosis are far from fully understood, and phylogenomic analyses even suggest the
63 involvement of *Chlamydiae* as a third partner (Ball et al. 2016; Zeng and Dehesh 2021).
64 However, it is agreed that all plastid lineages trace back to one endosymbiotic event forming
65 a monophyletic group (Stoebe and Maier 2002; Delwiche 1999).

66 Plants and green algae derived from the green lineage and possess primary chloroplasts that
67 enable them to perform photosynthesis. Chloroplasts of even distant species share highly
68 conserved features including a double envelope membrane, a protein import machinery (the
69 TOC-TIC complex, translocon of the outer and innner membrane of the chloroplast,
70 respectively), an internal thylakoid membrane system embedding the photosynthetic
71 apparatus, a plastid-specific genome (the plastome) and fully functional transcription and
72 translation machineries for the expression of this plastome. Many of these features display
73 prokaryotic elements or structures that are signatures of the prokaryotic ancestry of
74 chloroplasts such as prokaryotic -35 and -10 promoter elements, 70S ribosomes or an *E.*
75 *coli*-like RNA polymerase (Pyke 2007; Jarvis and López-Juez 2013). However, during the
76 course of evolution the endosymbiont was re-shaped in many aspects, a process that
77 allowed the final functional and structural integration into the host cell. One important step in
78 this process was the transfer of the largest part of the genome of the endosymbiont into the
79 host nuclear genome. This gene transfer expanded the coding capacity of the host cell and
80 provided novel factors and elements for a functional enhancement of the biochemical and
81 regulatory properties in it (Martin et al. 2002; Bock and Timmis 2008; Criscuolo and Gribaldo
82 2011).

83 Today's plant chloroplasts contain a highly conserved genome of ~ 120 to 160 kbp that
84 carries around 120 genes encoding components of the photosynthetic apparatus and protein
85 subunits of the ribosomes and the RNA polymerase, ribosomal rRNAs and tRNAs (Bock
86 2007; Sugiura 1992). Proteomic analyses, however, identified 2,500-3,500 different proteins
87 in chloroplasts exceeding by far the coding capacity of the plastome (Ferro et al. 2010; von
88 Zychlinski et al. 2005; Zybailov et al. 2008). Thus, the vast majority of plastid-localized
89 proteins is encoded in the nucleus, translated in the cytosol as preproteins and finally
90 imported into the organelle (Bauer et al. 2000; Nakai 2018; Soll and Schleiff 2004;
91 Strittmatter et al. 2010). Intriguingly, all major protein complexes in chloroplasts are
92 composed of a mix of nuclear- and plastid-encoded subunits indicating that their expression

93 needs to be coordinated allowing a successful assembly of these protein complexes. For
94 many years it was believed that the nucleus exclusively controls chloroplast biogenesis by
95 providing all critical protein factors and structural components, however, in recent years it
96 became increasingly clear that the chloroplast itself also provides essential regulatory signals
97 that contribute to this coordination. In order to distinguish between these two types of
98 regulation the terms „anterograde control“ (describing the nucleus-to-chloroplast signalling)
99 and „retrograde control“ (describing the chloroplast-to-nucleus signalling) were coined
100 (Woodson and Chory 2008). Retrograde signalling from plastids has attracted much interest
101 in the last three decades and considerable progress has been made in its understanding. So
102 far, five different classes of signals can be distinguished: 1) plastid pigments or pigment
103 precursors (tetrapyrroles, apocarotenoids, carotenoids), 2) signals originating from plastid
104 gene expression (PGE), 3) reactive oxygen species (ROS), 4) photosynthesis-related redox
105 signals and 5) changes in metabolite pools or fluxes (Chan et al. 2016; de Souza et al. 2016;
106 Hernandez-Verdeja and Strand 2018). These signals do not all occur at the same time or in
107 the same developmental context and some signals may even appear under several
108 conditions but causing different effects. In order to respect the developmental and
109 environmental context in which these retrograde signals become active one can distinguish:
110 1) biogenic signals that are active during early steps of chloroplast biogenesis (e.g. during
111 proplastid-to-chloroplast or etioplast-to-chloroplast transition), 2) operational signals that are
112 active when the chloroplast is fully functional and responds to the environment and 3)
113 degradational retrograde signals that are active when plastids are degraded in response to
114 age or pathogen attack (Pogson et al. 2008; Pfannschmidt et al. 2020). In this review we
115 focus mainly on the field of biogenic signals where much progress has been obtained in the
116 last years. For detailed information about the other signals the interested reader is referred to
117 recent reviews (Chan et al. 2016; de Souza et al. 2016; Hernandez-Verdeja and Strand
118 2018). Most of the progress discussed below concerns studies using dicotyledonous
119 Angiosperms (mostly *Arabidopsis thaliana*). Monocotyledonous Angiosperms or
120 Gymnosperms were investigated much less frequently, but because of their distinct
121 differences in chloroplast developmental provide important additions to the field (Hills et al.
122 2015; Loudya et al. 2021).

123

124 **Plastid development during early steps of the plant life cycle**

125 Plastids are morphologically and functionally very diverse and chloroplasts represent only
126 one specific form of them. In multicellular plants one can observe chromoplasts,
127 amyloplasts, etioplasts or elaioplasts. They all develop from the same undifferentiated
128 precursor, the proplastid, that is inherited between generations and that is found in

129 meristematic stem cells. However, the tissue context of the cell decides which type of plastid
130 is formed and, therefore, a given cell usually contains only one type of plastid. Independent
131 of the respective form all plastid types contain the same plastome allowing them
132 interconversions of these types in case developmental or environmental conditions change
133 (Lopez-Juez and Pyke 2005; Liebers et al. 2017).

134 **Plastid development during embryogenesis and early seedling development, and the**
135 **impact of plastid transcription** - Plastids cannot be formed *de novo*, but are inherited from
136 parental progenitor cells (Pyke 2007). During their division the inherited proplastids are
137 randomly distributed to the daughter cells in which they subsequently divide and multiply by
138 fission, using a prokaryotic-type division apparatus (Osteryoung and Pyke 2014). In vascular
139 plants all types and interstages of plastid transmission, such as maternal, biparental or
140 paternal inheritance, as well as maternal inheritance with parental leakage were found
141 (Timmis et al. 2004; Pyke 2007; Greiner et al. 2015). Most Angiosperms inherit plastids
142 uniparentally but the underlying mechanisms vary largely, even between close taxa (Greiner
143 et al. 2015).

144 After fertilization, a characteristic developmental programme takes place that comprises
145 embryogenesis, maturation and desiccation ultimately leading to the formation of mature
146 embryos (Le et al. 2010). In oilseed plants, such as *Arabidopsis thaliana*, this includes an
147 transient formation of photosynthetically active chloroplasts, starting at the globular stage
148 (Tejos et al. 2010). With the progressing ripening of the seed, these plastids de-differentiate
149 to non-photosynthetic, colorless eoplasts that, similar to proplastids, are able to re-
150 differentiate into all plastid types (Liebers et al. 2017; Allorent et al. 2013). The conversion
151 between plastid types involves changes in nuclear gene expression and significant changes
152 in plastid transcriptional activities. There exist two types of plastid RNA-polymerases
153 (RNAPs): i) a nuclear encoded RNA-polymerase (NEP), which is a single-subunit T3/T7
154 phage-type polymerase and ii) a plastid encoded RNA-polymerase (PEP) that forms a multi-
155 subunit complex of prokaryotic origin (Lerbs-Mache 1993; Hedtke et al. 1997; Borner et al.
156 2015). The two types of RNA polymerases have distinct as well as over-lapping functions
157 and their relative contribution to the expression of the plastome varies largely over time and
158 developmental condition. The often-found notion that NEP is responsible for the expression
159 of house-keeping genes, while PEP is responsible for the expression of photosynthesis
160 genes, thus, is a simplification that does not reflect the complexity in the distribution of work
161 between the two plastid RNA polymerase activities. Plastidial genes are classified according
162 to the presence of NEP and/or PEP recognition sites in their promoters (Hajdukiewicz et al.
163 1997; Liere et al. 2011; Pfannschmidt et al. 2015). Class I genes are solely transcribed by
164 PEP (e.g. *PsbA*, *PsbB*, *PsbK*, *RbcL*, *PetB*, *NdhA*), class II genes are recognized by both

165 RNAPs (e.g.: *AtpB*, *AtpI*, *NdhB*, *NdhF*, *ClpP*, *Ycf1*) and class III genes are only NEP-
166 dependent (e.g.: *Ycf2*, *AccD*, *RpoB/C₁/C₂*). During the proplastid-to-chloroplast transition in
167 developing embryos both, NEP and PEP genes are upregulated. However, while NEP-
168 dependent transcripts only slightly increase, photosynthesis-associated genes are
169 tremendously up-regulated (Allorent et al. 2013; Kremnev and Strand 2014). A massive re-
170 programming, including a genome-wide upregulation of photosynthesis genes was also
171 observed during germination in long-day conditions as well as during the etioplast-to-
172 chloroplast transition, where the maximal transcript accumulation is reached after radicle
173 outgrowth or 10-44 h after a dark-to-light shift, respectively (Rodermel and Bogorad 1985;
174 Demarsy et al. 2012). Following the increase in transcript abundance, also protein
175 abundance is rising (Kanervo et al. 2008). However, RNAP activity as well as RNA stability
176 need to be considered as both change during chloroplast formation. This becomes especially
177 apparent in the developmental gradient along the leaf blade of monocotyledonous plants that
178 grow from a basal meristem. Cells at the base are the youngest with poorly developed
179 chloroplast. Towards the leaf tip cell age increases and chloroplasts become mature allowing
180 the determination of transcription rates and transcript stabilities at different developmental
181 stages (Cahoon et al. 2004; Mullet and Klein 1987; Baumgartner et al. 1993).

182 **Plastid development during photomorphogenesis** - In many Angiosperms, germination
183 occurs when a species-dependent combination of temperature, moisture and light is
184 perceived by the quiescent embryo. Whenever light is required to break dormancy, a
185 minimum of a few hours of exposure to specific wavelengths, such as red light, is necessary
186 to activate the phytochromes until germination is irreversibly launched. Then photoreceptors
187 can be converted back to their inactive forms; naturally and slowly in the dark (mimicking
188 night conditions) or artificially by far-red light exposure. The latter was defined as “true-dark
189 conditions” that corresponds to the photoconversion of phytochromes into the inactive stage;
190 although this is only achievable under laboratory conditions and not fully complete since Pr
191 absorbs also some FR light (Leivar et al. 2008). Once germination has started, the seedling
192 enters a direct photomorphogenetic programme, or engage into a dark developmental
193 programme called skotomorphogenesis. In direct photomorphogenesis, chloroplast
194 biogenesis has been followed through fluorescent studies (Yadav et al. 2019) showing that
195 cell-specificity of chloroplast biogenesis above ground tissues (mostly cotyledons) starts after
196 the emergence of the root tip from the seed coat. However, plastids of the cotyledons
197 transitorily lose their chlorophyll autofluorescence during early development while ribosomes
198 are present in the stroma (Liang et al. 2018) and the plastome expression machinery is
199 active (Dubreuil et al. 2018). The inner membrane of the plastid envelope invaginates to form
200 tubulovesicular thylakoids, then the plastids increase in size while the inner membranes
201 become flat and thin. This membrane remodeling is accompanied by the association of

202 polysomes on thylakoids, and protein translation of the photosynthetic apparatus while
203 penetrating within the membrane. Subunits of photosystem II (PSII) appear and gradually
204 accumulate throughout the development of the light-grown seedling. The flattened
205 membranes then produce small pre-grana and serve as nucleation points for subsequent
206 stacking (Liang et al. 2018).

207 From embryogenesis up to the first two days post-imbibition, the shoot apical meristem
208 (SAM) and epidermis are completely devoid of chlorophyll, regardless of their light
209 environment (Tejos et al. 2010; Yadav et al. 2019). In these areas, plastids are proplastids
210 with only a few vesicles, no thylakoid structure except for a few non-photosynthetic tubules,
211 and display residual photosynthesis proteins (Charuvi et al. 2012). In the course of
212 development depending on the specific cell lineages the SAM can differentiate rudimentary
213 chloroplasts, except in L2 central zone as well as some cells below the L3 layer (Charuvi et
214 al. 2012; Dalal et al. 2018; Yadav et al. 2019). In the peripheral zone, stacked internal
215 membrane systems develop within photosynthetic plastids that will either regress or further
216 develop, depending on their position and subsequent cell fate.

217 During skotomorphogenesis, etiolated tissues produce a typical round and small plastid that
218 contains starch, plastoglobuli, and an imposing structure named the prolamellar body (PLB)
219 as well as proto-thylakoids (PT). These etioplasts do not stack internal membranes; they are
220 devoid of chlorophyll, while the presence of carotenoids is largely responsible for the yellow
221 color of the cotyledons. Etioplasts are developmentally halted and can be regarded as a
222 precursor stage capable of acquiring a chloroplast structure in just a few hours after
223 illumination. Many studies of chloroplast biogenesis, therefore, use a dark-to-light shift
224 (Armarego-Marriott et al. 2020). The PLB has a unique lipid-protein-pigment composition in a
225 hexagonal para-crystalline structure sharing a continuous lumen (Floris and Kuhlbrandt
226 2021). Most of the prolamellar proteome corresponds to the light-dependent
227 protochlorophyllide oxidoreductase (LPOR) that is linked in a tertiary complex to NADPH and
228 protochlorophyllide (reviewed in (Solymosi and Schoefs 2010) as well as a some
229 photosynthesis precursor proteins (Blomqvist et al. 2008). The presence of these subunits in
230 etioplasts allow rapid synthesis of chlorophylls and construction of the photosynthetic
231 apparatus after illumination.

232 The genetic control of the dark-or-light developmental programme involves two major
233 interacting pathways corresponding to two classes of isolated mutants; the long hypocotyl
234 (*hy*) and the constitutively photomorphogenic (*cop*). Most important, in the dark,
235 photomorphogenesis is inhibited by a degradational module (the COP9 signalosome)
236 (Sullivan et al. 2003; Seluzicki et al. 2017; Lau and Deng 2012). The E3 ubiquitin ligase
237 COP1 destabilizes the basic domain/leucine zipper (bZIP) transcription factor elongated

238 hypocotyl 5 (HY5) and its closest homolog HYH (Holm et al. 2002; Osterlund et al. 2000).
239 Then light converts the cytosolic pool of inactive phytochrome B (Pr) into its active state (Pfr),
240 triggering its nuclear translocation (Yamaguchi et al. 1999; Chen et al. 2003). Specific
241 domains of the phytochromes interact with phytochrome-interacting factors (PIFs) (Huq et al.
242 2004) leading to mutual degradation or inactivation of the partners (Leivar and Monte 2014).
243 PIFs comprise four bHLH transcription factors (PIF1, 3, 4, and 5) acting as transcriptional
244 repressors of photomorphogenesis and activators of skotomorphogenesis (Leivar et al.
245 2008). The interaction of PIFs with photoactivated phytochromes lead to their degradation
246 (Al-Sady et al. 2006) subsequently releasing the repression of the photomorphogenic
247 programme (Jiao et al. 2007). The antagonistic and compounded roles of the different
248 phytochromes with the different PIFs allow a fine-tuning of the developmental responses to
249 their light environment including de-etiolation and shade avoidance. Furthermore, PIFs are
250 also negative regulators of chloroplast biogenesis, having direct effects on rate limiting steps
251 of the tetrapyrrole biosynthetic pathway (Stephenson et al. 2009). The phytochrome-
252 mediated light signaling represses the COP1-mediated degradation of HY5, thereby leading
253 to the accumulation of a different post-translationally modified HY5. This stabilized
254 transcription factor (together with further factors) is then responsible for a profound
255 reprogramming of the transcriptional activities with respect to its numerous target genes
256 (Hajdu et al. 2018). Notably HY5 initiates the expression of photomorphogenic factors (Lee et
257 al. 2007). Meanwhile, light exposure triggers the transcriptional activation of golden2-like
258 myb transcription factors 1 and 2 (GLK1 and 2) that are responsible for the proper
259 expression of nuclear photosynthesis genes (Waters and Langdale 2009; Waters et al. 2009)
260 required to build-up the photosynthetic machinery in the plastids. The rapidity of the
261 responses at gene expression and subsequent morphological levels prevented a precise
262 dissection of the sequence of events that lead eventually to a green photomorphogenic
263 seedling. However, recent detailed studies provided significant progress in this question (see
264 below for more details) (Dubreuil et al. 2018; Pipitone et al. 2021; Loudya et al. 2021). The
265 functional involvement of cytosolic and nuclear regulators in this particular step of seedling
266 development appears to be well understood, but several studies revealed that also
267 retrograde signals from plastids are required. The relative contribution of these signals as
268 well as the coordination with the nucleo-cytosolic system, however, remains to be elucidated
269 and is a major topic of this review.

270

271 **Retrograde biogenic signals in chloroplast biogenesis**

272 **Initial experimental identification of signals and mutants** - Retrograde biogenic signals
273 have been originally identified in plants with dysfunctional plastids. The first notion came from

274 studies with the barley mutant *albostrians* that exhibits defects in plastid ribosome formation
275 in cells of its basal leaf meristem. A just recently identified genetic defect in the nuclear gene
276 for a CCT domain protein (Li et al. 2019) results in white striped leaves with variable degrees
277 of variegation in this mutant. While the green tissues harbour fully functional chloroplasts
278 (making the mutant viable) white tissues cannot perform photosynthesis. Interestingly not
279 only the expression of plastid proteins was diminished by the ribosome defect but also that of
280 nuclear-encoded photosynthesis proteins such as RBCS leading to the proposal of a plastid
281 factor that reports the functional state of the plastids to the nucleus (Bradbeer et al. 1979).
282 This concept was supported by studies in plants in which carotenoid biogenesis was inhibited
283 either through pharmacological or genetic approaches during the very early steps of seedling
284 development, leading to photo-oxidation. Lack of carotenoids in this very sensitive
285 developmental stage in which thylakoid membranes still have to be established results in a
286 light-induced destruction of internal plastid structures and, thus, prevents the biogenesis of
287 chloroplasts resulting in dysfunctional plastids like those in the *albostrians* mutant. Maize and
288 mustard seedlings with such photo-oxidized, dysfunctional plastids displayed a repression of
289 the nuclear encoded genes *LHCB* (formerly called *CAB*) and *RBCS* (Mayfield and Taylor
290 1984; Oelmuller and Mohr 1986). Based on these results it was assumed that plastids could
291 communicate their developmental and functional status in order to adapt nuclear
292 photosynthesis gene expression (Oelmuller 1989; Taylor 1989).

293 Pharmaceutical inhibition of chloroplast biogenesis in many studies is achieved by the
294 application of *norflurazon* (NF), a potent inhibitor of the phytoene desaturase, the key entry
295 enzyme of carotenoid biosynthesis (Chamovitz et al. 1991). The inhibitor, in addition, has
296 been recently reported to inhibit also FAD2, an enzyme involved in fatty acid desaturation
297 (Abrous-Belbachir et al. 2009) and it, therefore, may have also a direct effect on the
298 formation of thylakoid membrane lipids. Dark or light grown seedlings treated with this
299 inhibitor exhibit white cotyledons demonstrating the effective inhibition of carotenoid
300 biosynthesis, however, photo-oxidative destruction occurs only in presence of illumination.
301 Alternatively, chloroplast biogenesis in germinating seedlings can be blocked by application
302 of inhibitors of prokaryotic gene expression. Chloramphenicol, erythromycin or *lincomycin*
303 (Lin) have been reported to block plastid translation resulting in seedlings of pale appearance
304 that display reduced levels of *LHCB* and *RBCS* transcripts (Oelmuller et al. 1986; Gray et al.
305 1995). Treatments with the transcription inhibitors tagetitoxin and rifampicin were found to
306 be equally effective (Rapp and Mullet 1991; Pfannschmidt and Link 1997). Such inhibitor
307 experiments revealed several important facts: 1) The plastid signal can originate both from
308 plastid translation or transcription, 2) the plastid signal is also required in the dark, i.e. before
309 first illumination (not observable with NF) (Pfannschmidt and Link 1997; Sullivan and Gray
310 1999) and 3) the plastid signal appears to be required in the first 48 to 72 h after germination

311 since later application of the inhibitors were found to be not effective anymore. It was,
312 therefore, concluded that the plastid signal involves a product of early PGE that affects a
313 specific developmental step essential for the initiation of chloroplast biogenesis (Gray et al.
314 2003).

315 Further progress in the understanding of the plastid signal came from a mutant screen in
316 *Arabidopsis* that revealed that the nuclear gene expression could be decoupled from the
317 developmental state of the plastid. These *genomes uncoupled* (*gun*) mutants displayed a
318 partial de-repression of *LHCB* expression despite photo-oxidation of plastids by NF
319 application (Susek et al. 1993). In subsequent years the *gun* mutants were studied in great
320 detail and *gun2* – *gun6* were all identified to contain defects in enzymes of the plastid
321 tetrapyrrole biosynthesis pathway (TBP). *GUN2* encodes the haem oxygenase and *GUN3*
322 the phytychromobilin synthase (Mochizuki et al. 2001). Both enzymes are located on the
323 haem branch of the TBP pathway and catalyse essential steps in the biosynthesis of the
324 phytyochrome chromophore phytychromobilin (Kohchi et al. 2001). *GUN4* encodes an
325 activating regulator of the Mg-chelatase and *GUN5* the H-subunit of Mg-chelatase complex
326 (Mochizuki et al. 2001; Larkin et al. 2003). Thus, both enzymes are involved in Chlorophyll
327 biosynthesis. The *gun6* mutant overexpresses the ferrochelatase 1 that forms haem from
328 protoporphyrin IX (Woodson et al. 2011) and, thus, belongs to the haem branch of TBP.
329 Because of the high proportion of TBP enzymes among the *GUN* components it was
330 assumed that metabolic intermediates of the TBP pathway i.e., Mg-protoporphyrin-IX (Mg-
331 proto-IX) or the endpoint product haem could serve as retrograde signals (Mochizuki et al.
332 2001; Strand et al. 2003; Woodson et al. 2011).. It was reported that amino-levulinic acid
333 (ALA)-feeding prior to NF treatment made Mg-proto-IX detectable in the cytosol (Ankele et al.
334 2007). However, further analyses could not identify significant correlations between changes
335 in nuclear gene expression and the accumulation of this intermediate ruling out that the
336 levels of Mg-proto-IX *per se* influence nuclear gene expression (Mochizuki et al. 2008;
337 Moulin et al. 2008). However, it cannot be excluded that Mg-proto-IX could function as a
338 transmitter of plastid signals by activation and/or interaction with other potential retrograde
339 signals, such as ROS and abscisic acid (ABA) (Voigt et al. 2010). For haem the situation is
340 somewhat different since it is known for long that haem is exported from plastids and
341 transported to the mitochondria where it serves as redox co-factor in mitochondrial electron
342 transport (Thomas and Weinstein 1990). Indeed, analysis of the *gun6-1D* mutant of
343 *Arabidopsis* indicated that increased flux through the ferrochelatase 1-mediated haem
344 synthesis pathway promoted PhANG expression supporting the assumption that the haem
345 pool (or a fraction of it) represents a retrograde signal (Woodson et al. 2011).

346 The *gun1* mutant is an exception within the *gun* mutant collection since it was found to be the
347 only mutant that exhibits decoupling of nuclear gene expression not only upon NF but also
348 upon LIN treatment. *GUN1* was identified to encode a plastid pentatricopeptide repeat (PPR)
349 protein that contains a small MutS-related domain (Koussevitzky et al. 2007; Cottage et al.
350 2010). PPR proteins are very numerous in plants and plastid PPR proteins are known to be
351 involved in various steps of RNA metabolism (Schmitz-Linneweber and Small 2008).
352 Furthermore, GUN1 was reported to interact with many proteins being probably involved in
353 protein homeostasis (Tadini et al. 2016) and also to bind tetrapyrroles in order to reduce
354 haem and protochlorophyllide synthesis (Shimizu et al. 2019) as well as enzymes of the TBP
355 pathway (Tadini et al. 2019). In fact, the precise molecular function of GUN1 is still under
356 investigation, but it appears to act as a central integrator of several signalling routes (see
357 below for more details) (Koussevitzky et al. 2007; Colombo et al. 2016).

358 In sum the *gun* mutant screen had, even though the precise molecular involvement in
359 retrograde signalling of these mutants is still debated, two important outcomes: i) it
360 demonstrated that the plastid signal can be genetically interrupted (thus the low expression
361 of nuclear target genes is not simply a general negative feedback effect of the dysfunctional
362 plastids in the affected cells) and ii) that genetic defects causing perturbations at different
363 sites in the plastid result in the same nuclear response (suggesting either merging of several
364 plastid signals into one pathway or a mutual molecular influence of the different affected
365 processes).

366 **Central open questions in the understanding of biogenic signals** - The initial studies
367 paved the avenue to more comprehensive approaches in recent years and the use of plant
368 models with dysfunctional plastids (either induced genetically or pharmaceutically) in
369 combination with physiological assays became a ~~sort of~~ standard approach for studying
370 biogenic signals in depth. The research field has seen tremendous progress in the last 15
371 years mostly focussing on five central aspects.

372 1) Initial studies used only a very limited number of nuclear genes as reporter for the activity
373 of the plastid signal, mostly *RBCS*, *LHCB* and few other photosynthesis associated nuclear
374 genes (PhANGs). This term became a common synonym for the target of biogenic signals. In
375 between it became clear that many other genes (also encoding non-plastid localized
376 proteins) are also under biogenic control and that in addition other levels of gene expression
377 such as translation or protein stability are affected. This indicates that retrograde signals
378 represent a major regulatory determinant in early seedling development, but its precise
379 position within the responsible regulation networks is far from understood.

380 2) Another discussion focussed on the qualitative action of the plastid signal(s), i. e. whether
381 lower nuclear gene expression in plants with dysfunctional plastids represents the result of i)
382 active negative signalling (that might be de-repressed in *gun* mutants, a molecular reaction in
383 recent studies often called the *gun* phenotype) or ii) the lack of (a) positive signal(s) (not
384 produced by the plastid due to its dysfunction).

385 3) The origin of biogenic retrograde signals has been attributed to two major processes, TBP
386 and PGE where the latter is comprised of two sub-processes: transcription and translation.
387 The question arose whether all processes initiate independent signals or whether they all
388 converge into one common signalling pathway as proposed for the function of GUN1, a
389 debate not yet finished.

390 4) Chloroplast biogenesis of Angiosperms is under strong light control and anterograde
391 signalling is a major determinant in the build-up of the photosynthetic apparatus during early
392 seedling development as well as in meristems of grown-up plants. Since retrograde biogenic
393 signals are equally important in chloroplast biogenesis, an interaction between anterograde
394 and retrograde signalling appears likely, but the potential interactions remain to be
395 understood representing an interesting field for future research.

396 5) Likely the most important question is the one about the physical nature of the biogenic
397 signal(s) that is/are transmitted by the plastid. Unambiguous identification of one or several
398 signal molecules would not only provide a better mechanistic understanding of the signalling
399 route/network, but also a means for further experimental approaches.

400 The answers to each of these topics are tightly connected and the current progress in our
401 understanding in each area, therefore, is subsequently discussed in an integrated manner.

402 **Gene targets and qualitative character of plastid signals** – Initial studies of retrograde
403 biogenic signals focussed on the expression behaviour of a limited number of PhANGs and
404 the propositions of positive or negative character of these signals in early models based
405 largely on the observed expression changes in these reporter systems. A major problem in
406 this context is the fact that only relative expression values could be analysed and the true
407 action of the signal remained ambiguous, e.g. low expression of PhANGs upon block in
408 chloroplast biogenesis in comparison to normal development could either indicate active
409 repression (a negative signal) or the lack of promotion (a positive signal) by the dysfunctional
410 plastid. At the same time only few transcriptional regulators could be assigned a potential
411 role in retrograde signalling and mechanistic regulatory models remained highly speculative
412 leading to a controversial discussion about the positive or negative character of plastid
413 signals (Pfannschmidt 2010, Terry and Smith 2013). Thus, without a detailed understanding
414 of the molecular mechanism a hypothesized positive or negative action of (a) plastid signal(s)

415 must be regarded as a pure conceptual categorization that, however, might be helpful in
416 generating working hypotheses. Furthermore, the “*gun* phenotype” as molecular read-out in
417 presence of NF or Lin was used for many mutants to test the potential (positive or negative)
418 involvement of the respective inactivated component in retrograde signalling. This way of
419 classifying mutants as “new” *gun* mutants, however, produced a number of cases with
420 controversial results leading to much debate about whether or not a specific component is
421 part of retrograde control (for more details see further below). The study of expression
422 behaviour of single genes without additional experimental lines, therefore, should be highly
423 disfavoured.

424 Much progress in this aspect was obtained by performing full genome transcriptomic
425 approaches in order to reveal the true regulatory impact of biogenic signals during early
426 seedling development of *Arabidopsis*. Virtually thousands of genes were found to be
427 changed in expression when chloroplast biogenesis was blocked genetically or by NF or LIN
428 treatments (Ruckle et al. 2012; Grubler et al. 2017; Page et al. 2017b) indicating that plastid
429 signals i) represent a major regulatory element in this early developmental stage, ii) affect
430 many more gene groups other than PhANGs and iii) control also genes that encode products
431 not located to plastids. The last aspect is largely unexplored, but appears highly interesting
432 with respect to potential influences of plastid signals on overall plant morphology (for
433 instance leaf shape) (Tiller and Bock 2014) and metabolism. A meta-analysis comparing data
434 sets from these studies identified a core module of nuclear genes responding under all three
435 conditions (Grubler et al. 2021). It is comprised of 152 genes that could be subdivided into
436 seven major functional categories including photosynthesis, carbohydrate metabolism and
437 transport, redox regulation, development, transcription, proteins and stress as well as lipids
438 and hormones representing the broad functional impact of biogenic signals at this stage of
439 seedling development. The group of PhANGs appeared to be regulated highly uniformly in all
440 three approaches while other groups revealed more differential responses suggesting that
441 one and the same plastid signal can cause gene-specific effects of both, positive or negative
442 response. A qualitative classification of retrograde signals, thus, can be done only in a gene-
443 specific manner.

444 Key activators of PhANG expression are GLK1 and GLK2 (Waters et al. 2009; Waters and
445 Langdale 2009; Fitter et al. 2002). Especially GLK1 appears to be a target for retrograde
446 control since it was also found within the core module displaying an expression behaviour
447 like the PhANGs. Interestingly GLK1 and 2 overexpressing *Arabidopsis* lines exhibit a *gun*
448 phenotype (Leister and Kleine 2016; Martin et al. 2016) and recent data indicate that GLK1
449 acts downstream of GUN1-mediated retrograde signalling suggesting the action of a
450 tentative GUN1/GLK1 module. This module is proposed to controls not only PhANG

451 expression in the nucleus but also developmental processes in skotomorphogenesis and
452 seedling de-etiolation providing a potential way by which chloroplast biogenesis and early
453 seedling development could be coordinated (Hernandez-Verdeja et al. 2022; Veciana et al.
454 2022). A recent study in addition could demonstrate a strong post-transcriptional effect on
455 GLK1 protein accumulation. While *GLK1* mRNA accumulates to normal levels in NF or LIN
456 treated *gun1-101* mutants, no GLK1 protein accumulated suggesting that GLK1 is controlled
457 by plastid signals at the protein level. Additional experiments with proteasome inhibitors
458 could confirm this assumption (Tokumaru et al. 2017) pointing to cytosolic protein
459 homeostasis as important target for retrograde signals. Several recent studies indeed
460 confirmed that a connection between plastid and cytosolic protein homeostasis exists and
461 that retrograde control is strongly exerted at this level (for more details see below) (Wu and
462 Bock 2021). A sole focus on transcript changes, therefore, might miss important aspects and
463 targets of retrograde control for a given gene and parallel analysis at different levels of
464 expression appears recommendable in future analyses.

465

466 **Functional connections between TBP and PGE derived signals** – The TBP pathway is
467 completely localized within plastids, however all enzymes of this pathway are encoded in the
468 nucleus and must be imported from the cytosol (Tanaka and Tanaka 2007). Thus,
469 establishment of the TBP enzyme activities do not require direct PGE activity. In turn, all
470 PGE activities as known so far can be performed without requiring any product from TBP. On
471 first sight, therefore, one could assume that both processes are independent from each other
472 and that retrograde biogenic signals emerging from them follow independent and distinct
473 signalling routes. This, however, is not the case since TBP and PGE affect each other in
474 multiple ways. This mutual influence starts already with the import of the TBP enzymes. The
475 TOC/TIC machinery involves one subunit that is encoded by *Ycf1*, a plastid class II gene
476 transcribed by NEP and PEP (Fig. 1) (Zhelyazkova et al. 2012; Lyska et al. 2013).
477 Furthermore, the plastid class III gene *Ycf2* that is transcribed only by NEP encodes a
478 component of the AAA-ATPase import motor complex that interacts with the TIC complex
479 and drives the ATP-dependent translocation of preproteins over the inner membrane. *Ycf2*
480 contains a NAD-malate dehydrogenase activity enabling ATP production also in the dark or
481 in non-photosynthetic plastids (such as in meristems or in dark grown seedlings) (Kikuchi et
482 al. 2018). Thus, establishment and function of the TOC/TIC complex is dependent on the
483 activity of PGE and as a consequence also the import of all cytosolic preproteins. There is
484 still some uncertainty about the possible existence of different sub-types of import complexes
485 that could possess different substrate specificities and it is yet not fully understood whether

486 or not Ycf1 is required for the import of all nuclear encoded components (de Vries et al.
487 2015; Nakai 2015).

488 Another important link between PGE and TBP relates to the production of the entrance
489 substrate of the TBP pathway, the glutamyl-tRNA (aminoacid glutamate linked to tRNA-
490 Glu/*trnE*) that serves as precursor for the production of ALA (von Wettstein et al. 1995). The
491 proper timely and quantitative expression of the *trnE* gene is absolutely essential for a
492 sufficient functioning of the TBP pathway (Fig. 1). The *trnE* gene is transcribed by the PEP
493 enzyme that is the dominant transcription machinery for the expression of plastid tRNA
494 genes (Williams-Carrier et al. 2014). PEP requires the interaction with the nuclear encoded
495 sigma factor 2 (Sig2) for recognition of the *trnE* promoter as demonstrated by the pale-green
496 phenotype of *sig2* inactivation mutants (Woodson et al. 2013; Hanaoka et al. 2003). PEP
497 transcription, thus, performs strong regulatory control over the substrate availability and the
498 metabolite flux through the TBP pathway. Therefore, *sig* mutants have been also regarded
499 as substitute mutants for TBP retrograde signalling (Woodson et al. 2013). Interestingly, a
500 feedback control has been proposed in which increasing levels of *trnE* production lead to
501 down-regulation of NEP activity by physical interaction, thereby providing a means for the
502 shift from NEP to PEP activity during early chloroplast biogenesis (Hanaoka et al. 2005).

503 The *trn* genes transcribed by PEP are, in addition, essential for effective functioning of plastid
504 translation (Fig. 1). Furthermore, PEP transcribes *rrn* genes as well as genes for the large
505 and small ribosome subunit proteins (*rpl* and *rps*, respectively) providing structural
506 components of the 70S ribosomes, and last but not least a high number of photosynthesis
507 genes. Since the RNA polymerase (Rpo) core subunits of PEP are plastid-encoded, in turn,
508 plastid translation is essential for the establishment of a high transcriptional PEP activity
509 especially during the critical early phases in the onset of chloroplast biogenesis. Plastid
510 transcription and plastid translation, therefore, are highly intertwined generating a chicken
511 and egg problem concerning the mutual dependency.

512 The faithful generation of a photosynthetic apparatus during chloroplast biogenesis requires
513 a high coordination in the production of photosynthesis proteins, chlorophylls and thylakoid
514 membranes. Any disturbance of this coordination can generate severe consequences
515 leading eventually to an interruption of the complete process as observed in various mutants
516 with defects in distinct regulatory steps of plastid gene expression, tetrapyrrole biosynthesis
517 or lipid formation (see Fig.1 and Supplemental Table 1). The plastid encoded genes *AccD*
518 and *ClpP* encode each one a single subunit of the multi-subunit acetyl-CoA carboxylase
519 (ACC) and Clp protease (ClpP) complexes, respectively. The ACC complex synthesizes
520 malonyl-CoA, an essential precursor for plastid lipid production (required for thylakoid lipid
521 production) while the ClpP complex is required for the maintenance of a proper protein

522 homeostasis (proteostasis) in plastids (required to remove damaged or misfolded proteins).
523 Correct expression and assembly of the plastid encoded subunits with the nuclear encoded
524 ones for both complexes have been shown to be achieved by retrograde signalling
525 (Babiychuk et al. 2011; Llamas et al. 2017). These processes, thus, are most likely coupled
526 to TBP (see below for more details). This is supported by the observation that the
527 transcriptional regulation of the nuclear encoded key enzymes of thylakoid membrane lipid
528 synthesis MGDG1 and DGD1 is coordinated with chlorophyll biosynthesis involving control
529 by light and plastid signals (Kobayashi et al. 2014) .

530 In sum, PGE and TBP pathways are tightly interlinked and a clear-cut separation between
531 retrograde signals from PGE and TBP remains often difficult. In fact, this close linkage may
532 explain why many mutants with defects in diverse steps within or related to PGE or TBP
533 reveal similar phenotypes with defects in proper chloroplast biogenesis. To obtain an
534 overview of the distribution of functional defects related to these phenotypes we performed a
535 database and literature survey and identified 122 mutants in *Arabidopsis* that exhibit an
536 albino or pale green phenotype sometimes with spatial (variegation) or timely (virescence)
537 variation (Supplemental Table 1). This survey might miss non-viable mutants leading to
538 embryo-lethality or mutants in which the gene defect can be compensated by paralogous
539 genes. Therefore, the total number of critical components is likely higher. Many of these
540 mutant alleles have orthologous alleles in maize which may survive because of the nuclear
541 location of the *ACCD* gene in monocotyledonous plants (Belcher et al. 2015). In all mutant
542 types, defects in PGE were found to be dominant in number, but especially in albino mutants
543 (Fig. 1). Detailed functional analyses indicate that intrinsic components of both the
544 transcription and translation complexes as well as plastid localized regulatory factors
545 controlling their respective expression are involved. The PGE system, thus, exhibits a
546 surprising low capability to compensate for functional deficiencies despite its high complexity
547 pointing to a low level of redundancy in the system. This might be explained by the
548 endosymbiotic origin of plastids that exerts a high selective pressure on autotrophy which, in
549 turn, may lead to a high specialisation in regulation and function of factors involved in PGE.
550 Consequently, such a high specialization in PGE results in a low flexibility towards defects,
551 dysfunctions or functional imbalances and a proper stoichiometric production of the
552 components required for chloroplast biogenesis requires a tight coupling to the nuclear gene
553 expression system. Precisely that is achieved by the biogenic retrograde control pathways.
554 Defects in pigment biosynthesis were found to be the second largest group causing albinism,
555 but apparently are less important in pale-green mutants where defects in photosynthesis
556 become more important (Fig. 1). This implies that in this mutant type probably the availability
557 of pigment binding proteins or complexes (assuming full functionality of the TBP) determines
558 the degree of coloration allowing also gradual variations while defects in pigment synthesis

559 appear to result more in all-or-nothing phenotypes. During the build-up of the photosynthetic
560 apparatus, thus, expression of TBP associated genes as well as PhANGs requires a high
561 coordination in order to avoid mismatches in the relative abundance of their products.

562 **Functional connections between biogenic signals and light regulation** – Due to the
563 strong impact of light on the biogenesis of chloroplasts it appears very likely that retrograde
564 signals and the light signalling network do interact (Hernandez-Verdeja et al. 2020). Indeed,
565 already early investigations of nuclear gene expression reported a convergence of signals
566 from plastids and the light regulation network at the level of promoter usage (Bolle et al
567 1996). Later transcriptomic approaches indicated that there exists a strong mutual influence
568 between light and retrograde signals even at the system level (Ruckle et al. 2007; Ruckle
569 and Larkin 2009; Ruckle et al. 2012; Grubler et al. 2017) and more recent studies confirmed
570 a convergence of both regulatory networks (Martin et al. 2016). In particular, it was proposed
571 that the GUN1/GLK1 module acts antagonistically to the phytochrome/PIF signalling hub and
572 inhibits the proper development of cotyledons when chloroplast formation is prevented or
573 compromised by the application of Lin or high light stress. Biogenic signals from plastids,
574 thus, can act as developmental triggers that impact the light-controlled seedling
575 morphogenesis besides their role as PhANG expression regulators (Martin et al. 2016).

576 The impact of light on the total transcriptome during seedling development is massive.
577 Almost one third of all genes exhibit significant expression changes upon the light-induced
578 shift from skoto- to photomorphogenesis (Ma et al. 2001). The resulting molecular and
579 developmental changes appear rapidly within hours rendering it difficult to dissect the
580 respective regulatory impact of each signalling system. However, recent studies on slowly
581 greening *Arabidopsis* single-cell cultures (Dubreuil et al. 2018) as well as careful analyses
582 along the developmental cell gradient in wheat leaves (Loudya et al. 2021) uncovered a bi-
583 phasic programme for chloroplast biogenesis. Although the two systems and the resulting
584 observations are not fully coherent a simplified two-step model for chloroplast biogenesis can
585 be proposed. The first phase begins with organelle proliferation followed by establishment
586 and build-up of the plastid genetic system in meristematic and non-differentiated cells. After
587 that a second build-up phase follows in which the photosynthetic apparatus is established
588 within more differentiated cells. Any severe disturbance of the first phase, thus, will prevent a
589 successful transition into the second phase resulting in developmentally arrested plastids
590 unable to perform efficient photosynthesis. This bi-phasic programme provides a reasonable
591 explanation for the observation that application of inhibitors of prokaryotic gene expression is
592 effective only in a small time window after germination (see above).

593 Interestingly, gymnosperms are able to perform greening already in the dark since they
594 possess a different type of protochlorophyllide reductase enzyme that works light-

595 independently (Forreiter and Apel 1993). Using pine seedlings as a natural tool to separate
596 plastid signals from the light signalling network, a recent study could provide independent
597 evidence for the assumption that retrograde control of nuclear genes evolved before the light
598 signalling network took the control over photomorphogenesis (Hills et al. 2015). Retrograde
599 biogenic signals, thus, need to be considered as an intrinsic component of the basal
600 developmental programmes triggering early seedling development rather than being a part of
601 the light signalling network. They, however, provide a potential connection point that couples
602 both signalling networks.

603

604 **The physical nature of retrograde biogenic signals**

605 Plastids are involved in most metabolic and biosynthesis pathways of plant cells and,
606 therefore, possess a huge variety of envelope-localized transporters that are involved in
607 shuttling of metabolic intermediates between the cytosol and the plastid stroma (Rolland et
608 al. 2012). Virtually in all developmental phases plastids are linked to cytosolic processes by
609 exchange of molecules providing many potential candidates that may act as retrograde
610 signals. This tight connection with the cytosol reflects the deep integration of plastids into the
611 cellular metabolism that was established during endosymbiosis and it has been discussed
612 that even metabolite signatures may have a signalling function (Pfannschmidt 2010). So far a
613 role as retrograde signal has been attributed to metabolites mostly in the context of
614 operational signalling. The functions of methyl-erythritol-cyclo-diphosphate (MEcPP),
615 phospho-adenosine-phosphate (PAP) and apocarotenoids as signalling molecules in stress
616 responses have been studied in great detail (Chan et al. 2016; Chi et al. 2013; de Souza et
617 al. 2016) and only a small number of observations suggest that metabolites may be also
618 involved in biogenic retrograde signalling. Of special interest in this context are sugars,
619 anthocyanins and the various precursor molecules for plant hormones that are produced in
620 plastids such as those for abscisic acid or jasmonates (Cottage et al. 2010; Richter et al.
621 2020). Chloroplast biogenesis is well known to be strongly promoted by cytokinins (Cortleven
622 et al. 2016) and an interplay with auxin and light (Kobayashi et al. 2012). Well defined
623 networks that describe the connections between light, hormones and nuclear transcriptional
624 regulators in early chloroplast biogenesis have been developed in recent years (Cackett et
625 al. 2022). Elucidating the input of biogenic retrograde signals into these networks will be of
626 highest interest for the understanding of chloroplast biogenesis representing a promising
627 prospect.

628 More importantly, besides metabolites a number of proteins may act as retrograde signalling
629 compounds although currently no specific protein export from plastids could be

630 demonstrated. Nevertheless a steadily increasing number of dually localized nucleo-plastidic
631 proteins has been identified (Krause and Krupinska 2009) and for several of them a direct
632 trafficking from the plastid towards the nucleus has been proposed (see below for detailed
633 discussion). These proteins are reported to affect the expression of PhANGs and other
634 genes either directly or indirectly. Two general scenarios were proposed so far: i) plastid
635 localized proteins or protein complexes that mediate or support the transition of a retrograde
636 signal (subsequently referred to as „stationary mediator proteins“) and ii) proteins that act as
637 genuine signal(s) by leaving the plastid or its surface and moving to the nucleus (referred to
638 as „mobile signalling proteins“).

639 **Stationary mediator proteins** - This class of proteins resides inside the plastid
640 compartment. These proteins may move within the compartment, but never leave it. The
641 most prominent representative of this protein group is GUN1, a highly investigated receiver
642 and transmitter protein for retrograde signals which interacts with several other mediator
643 proteins. The GUN1 transcript accumulates to significant amounts in all tissues, but the
644 protein is found to accumulate only in cotyledons during proplastid-to-chloroplast transition
645 and in the shoot apical meristem. Furthermore it accumulates in response to NF treatments,
646 thus the protein is present precisely at the developmental stage in which biogenic signals are
647 most important (Wu et al. 2018). Co-immunoprecipitation, yeast-two-hybrid and proteomic
648 analyses indicated that GUN1 is functionally connected to or involved in i) the TBP, ii) the
649 PGE, iii) plastid proteostasis and iv) protein import (Tadini et al. 2016; Wu et al. 2019; Wang
650 et al. 2021) (see Fig. 2).

651 i) GUN1 is able to bind haem and other porphyrins, as well as enzymes of the TBP, such as
652 the D subunit of Mg-chel₂atase (CHLD) and ferrochel₂atase I (FC1) and, thus, affects the flux
653 through the TBP, resulting in changes of the TBP-dependent retrograde signaling (Colombo
654 et al. 2016; Shimizu et al. 2019). Additionally, GUN1-mediated signals influence the
655 expression of GLK1 and GLK2, which modifies the expression of key TBP genes (Kakizaki et
656 al. 2009). Recent studies have extensively analysed especially the impact of GUN1 mediated
657 signals on GLK1 leading to the proposal of a GUN1/GLK1 module (compare above), that is
658 of importance for both seedling development and chloroplast biogenesis (Martin et al. 2016).
659 Moreover, GUN1 aids plastid protein import (see below) and proteins being imported in a
660 GUN1-dependent manner include TBP enzymes thus, underpinning an influence of GUN1 on
661 the level of haem and Mg-proto-IX (Wu et al. 2019). This may explain the proposed biogenic
662 signalling function of haem (Woodson et al. 2011).

663 ii) GUN1 has been shown to associate with the nucleoid and, thereby, is potentially able to
664 interact with RNAPs (Koussevitzky et al. 2007; Colombo et al. 2016; Tadini et al. 2020). In

665 perturbed plastids, GUN1 binds to NEP and promotes expression of NEP-dependent genes,
666 including the *Rpo*-genes coding for the PEP core enzyme (Tadini et al. 2019). GUN1 may
667 also interact with the PEP complex itself, since PTAC6/PAP8 was found among the protein
668 interaction partners of GUN1 (Tadini et al. 2016). Furthermore, GUN1 appears also to be
669 involved in RNA processing events. BSM/mTERF4, which is required for group II intron
670 splicing of some plastid transcripts shows the *gun*-phenotype and alters GUN1 accumulation
671 and distribution (Sun et al. 2016). Similarly, GUN1 interacts with RNA helicase 3 (RH3), a
672 protein that is involved in the splicing of group II introns from the transcripts of *rpl2*, *trnA*, *trnI*,
673 and *rps12* transcripts (Tadini et al. 2016; Colombo et al. 2016). Moreover, GUN1 interacts
674 with the RNA editing factor MORF2, whose overexpression also leads to a *gun* phenotype
675 (Zhao et al. 2019).

676 iii) GUN1 can manipulate the protein homeostasis of plastids. For instance, it was shown that
677 GUN1 interacts with several ribosomal proteins and influences accumulation of the
678 prokaryotic ribosomal S1 protein (PRPS1), which is involved in the recognition of mRNA
679 leader sequences and the binding of mRNAs to the ribosome during the translation initiation
680 step (Tadini et al. 2016), thus pointing to a regulation of the plastid translation machinery.

681 iv) GUN1 couples also proteostasis in the plastids and the cytosol through the action on the
682 protein import machinery (Tadini et al. 2019). A lack of GUN1 is associated with a decrease
683 in components of the plastid protein import machinery, such as TOC159 and TIC100 (Tadini
684 et al. 2020). This might be mediated by ubiquitination as shown for TOC34 in the *gun1*
685 mutant (Svozil et al. 2014). Moreover, GUN1 was found to aid the import of proteins during
686 early chloroplast biogenesis or in case of perturbations of the plastid, by binding to the
687 chaperon cpHSC70-1. This chaperon is a protein of the TIC complex that drives precursor
688 proteins through the TOC/TIC channel, thus, preventing accumulation of protein precursors
689 in the cytosol (Wu et al. 2019; Tadini et al. 2020). However, lack of GUN1 evokes an
690 accumulation of protein precursors in the cytosol and subsequently cytosolic folding stress in
691 LIN or NF-treated seedlings. This stress triggers the accumulation of the cytosolic chaperon
692 complex HSP90/HSP70. These chaperones aid plastid protein import on the cytosolic side
693 and HSP70-1, together with the E3 ligase CHIP mediate degradation of accumulating plastid
694 precursor proteins through the ubiquitin–proteasome system (Lee et al. 2009). The HSP90 is
695 thought to then interact with nuclear transcription factors and to either repress negative
696 transcription factors e.g. by mediating their degradation through the 26S proteasome or by
697 activating positive transcription factors e.g. through their proper folding or refolding and, thus,
698 support expression of *PhANGs* (Wu et al. 2019; Tadini et al. 2020).

699 Protein homeostasis of plastids is also maintained by the ClpP complex, which derived from
700 the ancient cyanobacterial endosymbiont, but gained more complexity during evolution

701 (Sjogren et al. 2006; Bouchnak and van Wijk 2021). The Clp proteases, in particular CLPC1
702 is responsible for the degradation of GUN1 in chloroplasts of mature plants. However, this
703 degradation does not take place during early chloroplast development or upon Lin or NF
704 treatment – conditions where the interaction of GUN1 with cpHSC70-1 is required for the
705 import of proteins and consequently the protein quality control (PQC). This demonstrates a
706 tight coupling of GUN1 and protein homeostasis *via* the Clp protease complex. Moreover, Lin
707 treatment represses PGE and reduces the activity of the Clp protease (see Fig. 1), thus
708 leading to protein misfolding and accumulation in the chloroplasts. This in turn triggers a
709 chloroplast unfolded protein response (cpUPR) by upregulation of nuclear encoded plastidial
710 chaperons through a yet unknown retrograde signal in order to maintain the PQC. It includes
711 the upregulation of the nuclear transcription factor HsfA2 and subsequent expression of
712 chaperones, such as Hsp21 and ClpB3 (Llamas et al. 2017). The existence of a cpUPR was
713 first demonstrated by the depletion of the plastid encoded Clp protease subunit ClpP1 in
714 *Chlamydomonas reinhardtii*, which led to the expression of chaperones, proteasomes,
715 proteins with ubiquitin-related functions and proteins involved in thylakoid membrane
716 assembly and lipid trafficking (Ramundo et al. 2014; Ramundo and Rochaix 2014). Among
717 the latter was the protein VIPP2 that was later used to screen for retrograde signalling
718 components. To this end VIPP2 was tagged with a fluorescence protein in the ClpP1-
719 depletion background, leading to VIPP2 expression *via* activation of the cpUPR. After
720 random mutagenesis, mutants with an inactivated VIPP2 were selected and a signal
721 transmitter mutant affected in chloroplast-to-nucleus retrograde signalling (MARS1), a
722 cytosolic kinase, was identified (Perlaza et al. 2019; Kessler and Longoni 2019). To date no
723 cytosolic signalling component of the cpUPR has been identified in higher plants. Yet, it
724 could be shown that despite a GUN1-independent expression of cpUPR target genes,
725 several interaction partners of GUN1 accumulated due to the repression of the Clp protease
726 (ClpP1 or CLPR2) in *Chlamydomonas reinhardtii* (e.g. HSP93, HSP70, RH3) and
727 *Arabidopsis thaliana* (e.g. HSP93, HSP70, CPN60, RH3), respectively (Ramundo et al. 2013;
728 Zybailov et al. 2009). Furthermore, *gun1/clp1* double mutants are seedling lethal, thus,
729 suggesting at least a partial involvement of GUN1 in the cpUPR.

730 The multiplicity of processes that were proposed to involve GUN1 renders it difficult to assign
731 a specific function to this protein. Many of these proposals are based on indirect mutant
732 studies combined with expression analyses of single genes that, as outlined above, were
733 observed to be difficult in reliability or stringency of causal relationship. Further, GUN1 was
734 found to interact with many proteins of different processes. However, GUN1 exhibits a PPR
735 domain, a motif typically found only in RNA interacting proteins and a very recent study
736 focussing on this structural aspect raises severe doubts about a direct involvement of GUN1
737 in retrograde biogenic signalling (Honaken and Small 2022). Thus, further investigations will

738 be required to verify the multiple protein interactions and to finally clarify the role of GUN1
739 (and its potential interaction partners) in chloroplast biogenesis.

740 **Mobile signalling proteins** - This class of proteins resides temporarily inside or on the
741 surface of the plastid compartment performing plastid-specific functions. Under defined
742 conditions, however, they may leave the compartment, pass the cytosol and enter the
743 nucleus where they perform a different function.

744 The first notion of such a potential signalling pathway came from a study reporting the
745 release of the plant-specific transcription factor *IIB* – related protein (PBRP) from the outer
746 envelope. It was hypothesised to transmit plastid-derived signals to the nucleus, where it
747 regulates nuclear gene expression through the recruitment of the RNAP machinery
748 (Lagrange et al. 2003). However, this signalling pathway was not further detailed in additional
749 follow-up studies and thus awaits further confirmation. Another protein reported to be
750 released from the envelope in response to plastid-derived signals is the plant homeodomain
751 (PHD) transcription factor (PTM) transcription factor that was proposed to be proteolytically
752 cleaved and set free by a yet unidentified peptidase. The processed shorter form of PTM
753 was reported to accumulate in the nucleus and modify nuclear gene expression through
754 activation of *ABI4*, thereby conveying GUN1-mediated signals to the nucleus (Sun et al.
755 2011). Reevaluation of PTM expression signatures and of the reported *gun* phenotypes of
756 *ptm* and *abi4* mutants by several laboratories, however, did not confirm the proposed roles
757 of PTM and *ABI4* in the transmission of *gun1* derived signals and thus, do not support a role
758 for PTM and *ABI4* in biogenic retrograde signalling (Kacprzak et al. 2019; Page et al. 2017a).
759 Whether proteins with signalling function are released from the outer chloroplast envelope,
760 therefore, remains an open question.

761 Another mode of protein-associated retrograde signalling is achieved by the release of
762 proteins from the inside of plastids and their subsequent migration to the nucleus (Fig. 3)
763 (Krupinska et al. 2020). The first identified candidate for this signalling mode was the single-
764 stranded DNA binding protein *whirly 1* (WHY1). It is involved in plastome genome stability
765 and might contribute to the repair of double strand breaks similar to WHY2 (Cappadocia et
766 al. 2010; Cappadocia et al. 2012; Marechal et al. 2009). Immunogold labelling demonstrated
767 a dual localization of WHY1 in plastids and the nucleus of the same cell (Grabowski et al.
768 2008). Interestingly, nuclear and plastid forms of WHY1 displayed the same apparent
769 molecular size in immunoblots, suggesting that the nuclear version lacks the cTP and, thus,
770 had to travel *via* chloroplasts to the nucleus in order to get the cTP removed (Grabowski et
771 al. 2008). This trafficking was further confirmed by stable transformation of tobacco plastids
772 with a recombinant HA-tagged *whirly1* protein, which was detected in the nucleus, although
773 the protein was only expressed in plastids (Isemer et al. 2012). Together these findings

774 strongly support the involvement of WHY1 in retrograde signalling. While *Arabidopsis why1*
775 mutants do not exhibit a plastid-defective phenotype due to two paralogous gene copies,
776 maize and barley mutants display greening phenotypes indicating that WHY1 is required for
777 proper chloroplast biogenesis and therefore represent a genuine biogenic signal (Prikryl et al.
778 2008; Krupinska et al. 2022).

779 A further protein behaving spatially like WHY1 is the *Arabidopsis* protein HEMERA (Fig. 3).
780 This protein was identified in a genetic screen for phytochrome signalling mutants and
781 exhibited nuclear localisation based on observations using GFP-tagging and immunoblots
782 (Chen et al., 2010). HEMERA is identical with PTAC12, a protein identified earlier in
783 biochemical approaches as subunit of the transcriptionally active chromosome (TAC) of
784 plastids (Pfalz et al. 2006). Thus, HEMERA/PTAC12 represents a dually localized protein
785 identified by independent approaches in plastids and nucleus. This dual localisation was later
786 also identified for the orthologous protein in maize (Pfalz et al. 2015) indicating that the dual
787 localisation is a conserved feature of the protein. The functions in nucleus and plastid,
788 however, appeared to be different. While the nuclear-localized version was proposed to act
789 in protein degradation the plastid version was proposed to interact with single stranded
790 nucleic acids (Pfalz et al. 2015; Chen et al. 2010). The final functions of this protein in the
791 two compartments are not yet understood, but the dual localisation raised the interest in
792 understanding how this distribution occurs. Intriguingly, HEMERA/PTAC12 displayed the
793 same molecular weight in nucleus and plastids both in *Arabidopsis* and maize suggesting
794 that the nuclear version needed to be processed in the same way as the plastid-localized
795 protein. More detailed studies uncovered that the nuclear localization of a truncated
796 HEMERA/PTAC12 that lacks the cTP was strongly reduced, while a fusion with the cTP from
797 RBCS rescued the plastid and nuclear localization and the function (Nevarez et al., 2017),
798 thus pointing to a passage through the plastid before a nuclear localization becomes
799 possible. By this HEMERA/PTAC12 meets all requirements for a plastid-to-nucleus traffic
800 and, thus, could represent also a retrograde signalling protein.

801 Another group of plastid proteins potentially trafficking towards the nucleus is comprised of
802 PEP associated proteins (PAPs) (Fig. 3). PAPs are nuclear encoded proteins that associate
803 with the PEP core enzyme upon first illumination of germinating seedlings (Pfannschmidt and
804 Link 1994). By this means the prokaryotic PEP is highly enlarged in size and surrounded by
805 a mantle of eukaryotic proteins during the initial phase of chloroplast biogenesis (Fig. 3,
806 bottom box) (Pfannschmidt et al. 2000; Suzuki et al. 2004; Steiner et al. 2011). PAPs are
807 structurally and functionally diverse and comprise proteins involved in DNA/RNA metabolism,
808 redox-regulations and ROS-protection (Steiner et al. 2011; Kindgren and Strand 2015; Yu et
809 al. 2014). Interestingly, functional inactivation by T-DNA insertion in any *PAP* gene leads to

810 an arrest in chloroplast biogenesis resulting in albino or ivory plants that are, however, viable
811 on media containing a carbon source. Thus, plant growth *per se* is not impaired in these
812 mutants, but the light-induced transition to autotrophy. Plastids of *pap*-mutants are smaller
813 and irregularly shaped, with no or only poorly developed internal membrane systems and
814 accumulate large vesicles and plastoglobuli (Pfalz et al. 2006; Myouga et al. 2008; Arsova et
815 al. 2010; Gao et al. 2011). Furthermore, they show characteristic changes in the expression
816 of plastid genes commonly referred to as the “NEP”-phenotype in which class I genes appear
817 to be strongly repressed, while class II genes and class III genes are either not affected or
818 up-regulated (Pfalz et al. 2006; Myouga et al. 2008; Arsova et al. 2010; Gao et al. 2011; Yagi
819 et al. 2012). Additionally, also *PhANG* expression is impaired as expected from the disturbed
820 chloroplast biogenesis, thus proving that a retrograde signalling occurs in those mutants.
821 However, microarray data show that contrary to the scientific consensus, retrograde
822 signalling does, at least in the *pap7-1* mutant background, not necessarily lead to a general
823 inhibition of *PhANG* expression, but rather leads to a specific repression of nuclear *LHCB*
824 genes, while other *PhANGs* are not or just mildly affected (Grubler et al. 2017).

825 Some PAPs were considered to convey a retrograde signal to the nucleus, due to the fact
826 that six out of the identified 12 PAPs contain a predictable nuclear localization signal (NLS),
827 which allows them to be potentially imported into the nucleus (Pfannschmidt et al. 2015).
828 HEMERA/PTAC12 is one of them and corresponds to PAP5. Note that PEP and TAC do
829 overlap in protein composition but are not identical structures (Pfalz and Pfannschmidt 2013).
830 Detailed studies of PAP8 confirmed that this protein is dually localized to plastids and
831 nucleus (Liebers et al., 2020). Similar to WHY1 and PAP5 it exhibited the same molecular
832 weight in both compartments, implying that the nuclear version needed to be processed in
833 the same way as the plastid-localized protein. Since the required proteases exist as known
834 so far only in plastids (Nishimura et al. 2016), these findings further support a model in which
835 a trafficking of NLS-containing PAPs into the nucleus occurs *via* the plastids.

836 Expression analyses of PAP2, PAP5 and PAP8 using promoter PAP::GUS transcriptional
837 fusions identified a strong and specific expression in cotyledons. In etiolated seedlings this
838 expression is restricted to the epidermis rendering it likely that the translocation of PAPs
839 occurs initially only in the epidermal layer, while later events spread to mesophyll cells of de-
840 etioloating seedlings (Fig. 3) (Liebers et al. 2018). The initial epidermal expression of PAPs
841 might be due to a sensory function of epidermal plastids that trigger a tissue-specific
842 response to light and/or stress signals (Dopp et al. 2021; Beltran et al. 2018). This tissue
843 specificity adds a new facette to our view of retrograde signalling in the control of chloroplast
844 biogenesis and seedling development.

845 The reason why PAPs have to translocate through the plastid before entering the nucleus in
846 the epidermal layer and how this retrograde signalling is achieved remains to be elucidated.
847 It was, however, shown that in the nucleus of etiolated seedlings, PAP5 and PAP8 interact
848 with phytochromes, resulting in the formation of phytochrome nuclear bodies that aid in the
849 degradation of PIF1 and PIF3. Concomitantly, the photomorphogenesis transcription factor
850 HY5 becomes stabilized, which will ultimately lead to the activation of photomorphogenesis
851 associated key transcription factors, such as *GLK1* and 2 (Galvao et al. 2012; Liebers et al.
852 2020). In case of PAP5, a nine amino acid transcriptional activation domain (TAD) at the C-
853 terminus mediates the degradation of PIFs and the expression of PIF target genes (Qiu et al.
854 2015). Upon induction of photomorphogenesis, HY5 was shown to bind to the promoter region
855 of *PAP8*, resulting in the up-regulation of *PAP* expression as confirmed by transient assays,
856 *in vitro* experiments, and *in vivo* genome-wide ChIP sequencing data (Liebers et al. 2020).
857 Cytosolically translated prePAP8 will be then imported into the plastid, processed and
858 assembled with the PEP-core in order to induce expression of photosynthetic-associated
859 plastid genes that together with the *PhANGs* will assure the proper build-up of the
860 photosynthetic apparatus. In *pap* mutants, however, the light induced formation of late
861 nuclear photobodies is prevented, thus, PIFs are less degraded, HY5 is not stabilized and
862 the signalling of GLKs is impaired. Concomitantly, PAPs are not increasingly expressed and
863 cannot assemble with the PEP core complex, thus, leading to the reduced expression of
864 PEP-dependent genes resulting in the albino phenotype characteristic for *pap* mutants. Thus,
865 PAPs represent a new group of dual-localized proteins that assure proper chloroplast
866 formation through changes of plastid and nuclear gene expression on the basis of combined
867 retrograde and anterograde signalling.

868 Recently, two more proteins belonging to the group of dually localized proteins were
869 identified using a forward genetic screen. Regulator of Chloroplast Biogenesis (RCB) and its
870 paralogue Nuclear Control of PEP activity (NCP) represent non-catalytic thioredoxin-like
871 proteins involved in phytochrome signalling and chloroplast biogenesis (Fig. 3) (Yoo et al.
872 2019; Yang et al. 2019). They were originally identified in two independent studies as
873 Mesophyll-cell RNAi Library line 7 (MRL7) and MRL7-like as well as Suppressor of
874 Variegation4 (SVR4) (Yu et al. 2011; Qiao et al. 2011). Both proteins are dually targeted to
875 nucleus and plastids. The nuclear forms are, similar to PAP5 and PAP8, involved in the
876 degradation of PIF1 and PIF3, while the plastid versions promote the assembly of the PEP
877 complex. Why plants evolved so many proteins involved in both, phytochrome signalling and
878 PEP function, remains elusive. One could speculate that this adds another level of light
879 regulation on chloroplast biogenesis. In this context it should be noted that PAP5 was shown
880 to be able to physically interact with PAP7 and PAP8 (Gao et al. 2011; Liebers et al. 2020)
881 suggesting the existence of a nuclear PAP subcomplex (including potentially all PAPs with

882 an NLS). Indeed, PAP8 could be identified in high molecular subcomplexes in the nucleus
883 (Chambon et al. 2022) and it is possible that these may interact with phytochromes. This
884 hypothesis would place PAPs as new regulatory factors into the complex light signalling
885 network providing a functional retrograde link between chloroplast biogenesis control and the
886 photomorphogenic programme in seedling development. This tempting idea needs to be
887 experimentally tested in the future.

888 How the nucleo-plastidic proteins presented above travel towards the nucleus remains a
889 question of debate and several trafficking modes were discussed, comprising diffusion or an
890 active transport *via* membrane contact, vesicles, stromules or through export complexes
891 (Krupinska et al. 2020; Krause et al. 2012). For instance the permeability of the envelope
892 could change providing a possibility of protein release. However, since the proteins need to
893 pass two membranes with different compositions, this appears to be unlikely. Another
894 possibility for a plastid-to-nucleus protein traffic would be a transport *via* stromules. These
895 are tubular stroma-filled protrusions of chloroplasts which were found to form upon diverse
896 biotic and abiotic stresses (Brunkard et al. 2015; Caplan et al. 2015; Erickson et al. 2014). It
897 was reported that stromule formation changes with plastid size, density and during the day-
898 night cycle (Waters et al. 2004; Brunkard et al. 2015). Stromules were found to interact with
899 the plasma membrane, the nucleus and the ER (Kwok and Hanson 2004; Schattat et al.
900 2011b). However, it is highly debated whether stromules transport proteins and if the
901 stromules are interconnected between themselves and with other organelles (Schattat et al.
902 2011a; Mathur et al. 2013; Delfosse et al. 2015). In sum, stromule dependent retrograde
903 signalling appears to be a very tempting, yet challenging hypothesis that awaits further
904 experimental proof.

905

906 **Concluding remarks**

907 The biogenesis of chloroplasts is an absolute essential process for the establishment of
908 autotrophy in developing and growing plants and algae. It is equally important for all
909 heterotrophic organisms in the biosphere of our planet that feed on plants, directly or
910 indirectly. Retrograde biogenic signals from plastids as a central intrinsic part of the
911 regulatory cellular network that coordinates and controls the proper build-up of chloroplasts
912 (and likely other plastid types), thus, are of eminent importance. While early studies used lots
913 of different plant species our recent understanding of molecular nature and functioning of
914 retrograde biogenic signals improved mostly by focussing on *Arabidopsis* as model organism
915 typically in test systems that investigate proplastid-to-chloroplast or etioplast-to-chloroplast
916 transitions in presence of chemical or genetic interferences. It will be of great interest to

917 expand those studies also to other plants and test systems in order to reveal whether the so-
918 far unravelled mechanisms follow uniform principles or rather represent species-specific
919 regulation pathways. This will largely improve our universal comprehension of plant
920 development and stress management and might be of great interest for the establishment of
921 climate change tolerant crops and biotechnologies. Future research may aim for instance to
922 understand whether the formation of chloroplasts in resurrection plants after re-watering
923 follows the same principles as the chloroplast-eoplast-chloroplast transition in embryos of
924 *Arabidopsis* seeds and whether this is relevant for drought tolerance (Ingle et al. 2008;
925 Allorent et al. 2013; Solymosi et al. 2013). Understanding the impact of retrograde signals
926 may also help to solve problems in current agricultural double-haploid breeding technologies
927 that often suffer from high proportions of albino plantlets that emerge during androgenesis
928 because of disturbed chloroplast biogenesis (Gajecka et al. 2020, 2021; Canonge et al.
929 2021). Finally, chloroplasts have been identified as a reliable and safe future production
930 platform for recombinant proteins, pharmaceuticals and many other bioproducts, but their
931 biotechnological potential is currently far from being efficiently used (Scharff and Bock 2014;
932 Newkirk et al. 2021). In the long-term, thus, control of chloroplast biogenesis in autotrophic
933 organisms grown in bioreactors, indoor farms and other biotechnology applications will be of
934 high economical value and understanding of retrograde biogenic signalling, therefore, is by
935 far more than just academic interest.

936

937 **Figure legends**

938 **Figure 1: Structural and functional integration of essential plastid processes during**

939 **chloroplast biogenesis.** The scheme depicts an overview of the molecular connections

940 between the major biosynthetic processes within the plastid required for the build-up of the

941 photosynthetic apparatus during chloroplast biogenesis. Light orange boxes: Transcriptional

942 activities. Dark orange boxes: Processes related to protein synthesis and homeostasis.

943 Yellow boxes: Envelope located processes. Green boxes: Processes directly involved in

944 formation of photosynthetic structures. Grey boxes: Genes transcribed by nuclear- and

945 plastid-encoded RNA polymerases (NEP and PEP). Green arrows: Delivery of proteins by

946 import through TOC-TIC from cytosol. Light orange arrows: Transcript maturation. Black

947 arrows: Delivery of plastid-synthesized products to subsequent processes. Broken black

948 arrows: Additional transcriptional involvement of PEP activity. Orange arrows: Translation of

949 plastid transcripts at 70S ribosomes. Red arrow: Negative feedback from *trnE* pool. Squares

950 indicate mutant alleles identified in *Arabidopsis* resulting in defects of the respective process

951 to which the squares are located. The phenotype caused by the genetic defect is indicated

952 by the color code of each square. The corresponding legend is given in the right margin. For
953 gene identities of the alleles refer to Supplemental Table 1. For details see main text.

954

955 **Figure 2: Retrograde signalling from dysfunctional plastids.** The scheme depicts a
956 cotyledon mesophyll cell of a seedling treated in the dark with NF or Lin and subsequently
957 transferred to light. The plastids are incapable to develop into functional chloroplasts
958 because of the inhibitors generating a severe disturbance of the general developmental
959 programmes of the seedling. NF blocks carotenoid biosynthesis (CarB) that usually prevents
960 oxidative damage through reactive oxygen species (ROS) that are generated by illumination
961 (yellow flash) of phototoxic protochlorophyllide (ProtoChlide), an intermediate of chlorophyll
962 (Chl) biosynthesis. Under NF treatment, thus, ROS production leads to irreversible
963 photobleaching of the developing plastid. Lin treatment blocks plastid translation and
964 interferes with the production of plastid encoded proteins causing an imbalance in
965 stoichiometry with nuclear encoded plastid proteins delivered by import (TOC-TIC
966 machinery). This disturbs plastid proteostasis, blocks formation of the photosynthesis
967 apparatus and activates a chloroplast unfolded protein response (cpUPR) that is connected by
968 unknown means to the cytosolic UPR (cytosolUPR). Ovals: Proteins; for abbreviations of
969 names see text. The GUN1 protein directly or indirectly interacts with multiple targets within
970 the plastid and affects corresponding processes. Ovals with broken surrounding: Unfolded
971 proteins. Blue arrows: Retrograde signals from dysfunctional plastid targeting nuclear gene
972 expression directly or indirectly by influencing cytosolic processes. Green arrows: Delivery of
973 nuclear encoded components (TFs, transcription factors; CPNs, chaperone and proteins
974 involved in proteostasis. For details of functional connections see main text.

975

976 **Figure 3: Retrograde signalling by dually-localized proteins during de-etiolation.** The
977 scheme depicts an overview of the proposed spatio-developmental actions of nucleo-
978 plastidic proteins in retrograde signalling. Bottom boxes: Left box, skoto- and
979 photomorphogenic development of *Arabidopsis* seedlings. Middle box, schematic crosscut
980 through the respective cotyledons that develop either etiolated or green mesophyll cells,
981 respectively. Right box: Basic working model depicting the light-induced subunit
982 reorganisation of the PEP complex. PEP-B representing the *E.coli*-like core enzyme is given
983 as simplified crystallographic structure of the *E. coli* RNA polymerase. Addition of PAPs
984 converts PEP-B into a structurally larger and more complex PEP-A given as 3D envelope
985 (Ruedas et al. 2022). Enlargements of representative cells from tissues indicated in the
986 middle box are given above. Epidermal cells in the dark may contain a fully assembled PEP-

987 A, the activity of it is yet untested. A potential retrograde signalling (RS) of nuclear localized
988 PAPs (N-PAPs) in the epidermis is unknown. Mesophyll cells in the dark contain etioplasts
989 with a prolamellar body (PLB) and a PEP-B with basal transcriptional activity. Expression of
990 PAPs is repressed by COP and PIFs. After illumination phytochrome B is activated (PhyB^{fr}),
991 represses the activity of COP and PIFs and activates photomorphogenic transcription factors
992 such as HY5/HYH which drive transcription of PhANGS (through the action of GLKs) and
993 PAPs. Formation of late photobodies (green filled circles), where these processes occur,
994 requires the involvement of PAPs, NCP, RCB and likely other proteins sent from the plastid
995 (way of export is unknown). Imported PhANGs assemble with PhAPs to build the
996 photosynthetic apparatus. Coordination for production of stoichiometric amounts is achieved
997 through RS. Stretched and piled up dark-green ovals represent thylakoids. For detailed
998 explanations of functional connections see main text.

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1006 **Literature**

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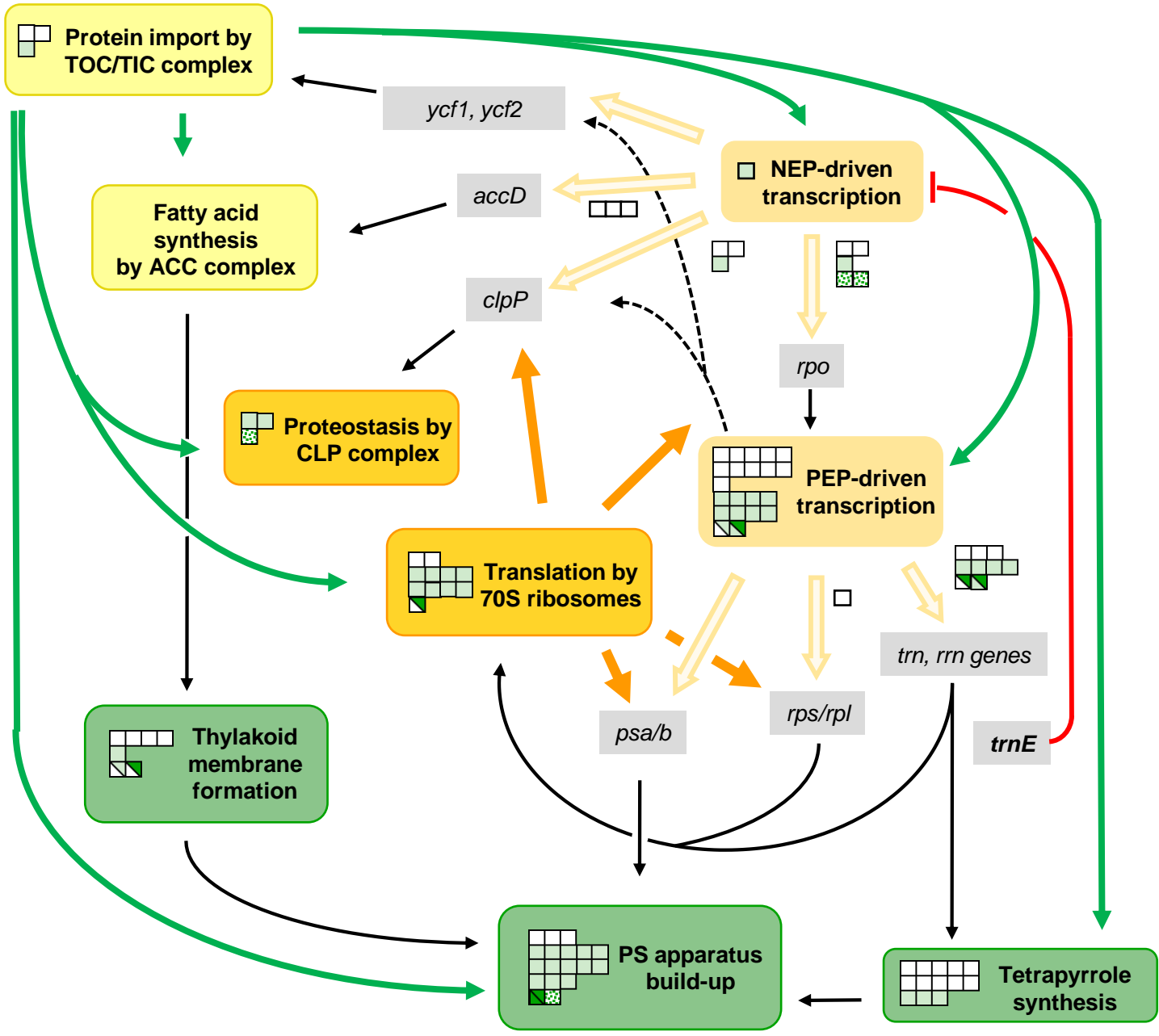
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Mutant phenotype legend

- ☐ albino
- ◻ pale-green
- ▤ variegated
- ▨ transient albino to pale-green
- ▧ transient albino to green
- ▩ transient pale-green to green
- stay-green

Figure 1: Structural and functional integration of essential plastid processes during chloroplast biogenesis. The scheme depicts an overview of the molecular connections between the major biosynthetic processes within the plastid required for the build-up of the photosynthetic apparatus during chloroplast biogenesis. Light orange boxes: Transcriptional activities. Dark orange boxes: Processes related to protein synthesis and homeostasis. Yellow boxes: Envelope located processes. Green boxes: Processes directly involved in formation of photosynthetic structures. Grey boxes: Genes transcribed by nuclear- and plastid-encoded RNA polymerases (NEP and PEP). Green arrows: Delivery of proteins by import through TOC-TIC from cytosol. Light orange arrows: Transcript maturation. Black arrows: Delivery of plastid-synthesized products to subsequent processes. Broken black arrows: Additional transcriptional involvement of PEP activity. Orange arrows: Translation of plastid transcripts at 70S ribosomes. Red arrow: Negative feedback from *trnE* pool. Squares indicate mutant alleles identified in *Arabidopsis* resulting in defects of the respective process to which the squares are located. The phenotype caused by the genetic defect is indicated by the color code of each square. The corresponding legend is given in the right margin. For gene identities of the alleles refer to Supplemental Table 1. For details see main text.

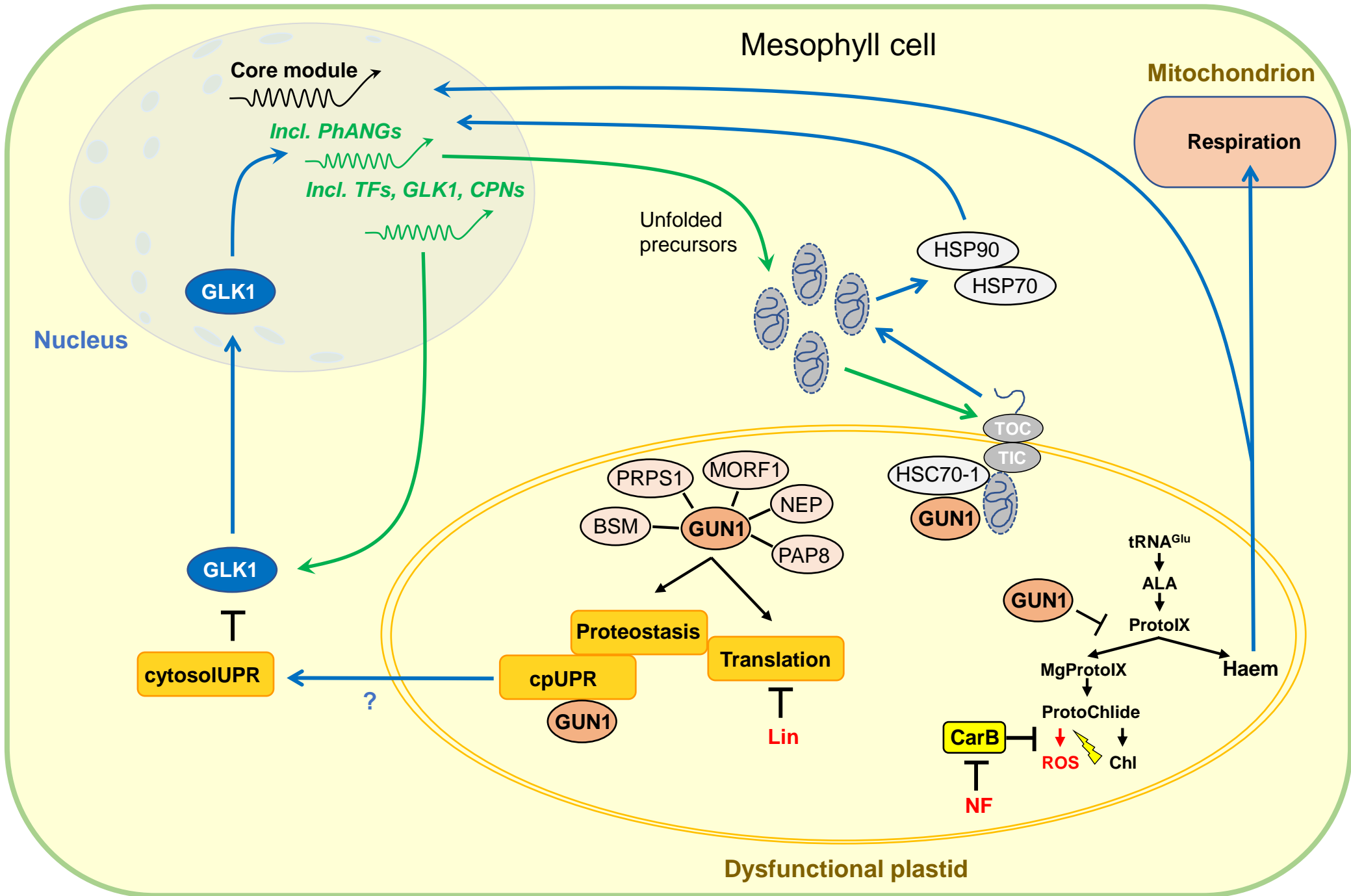
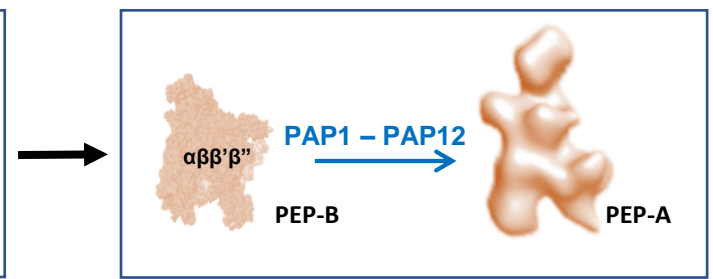
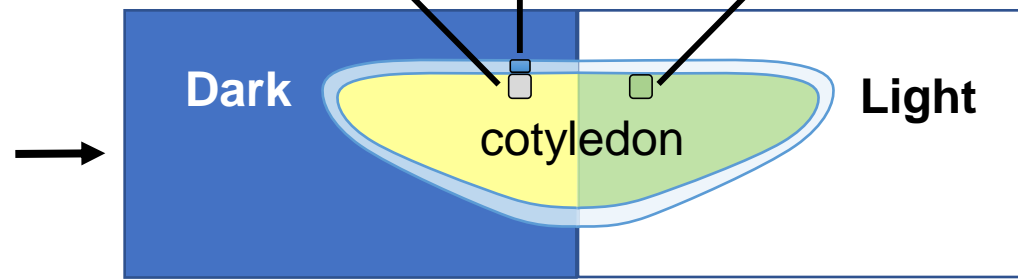
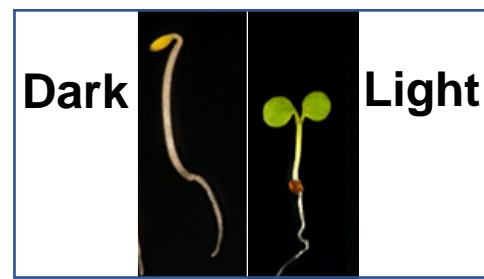
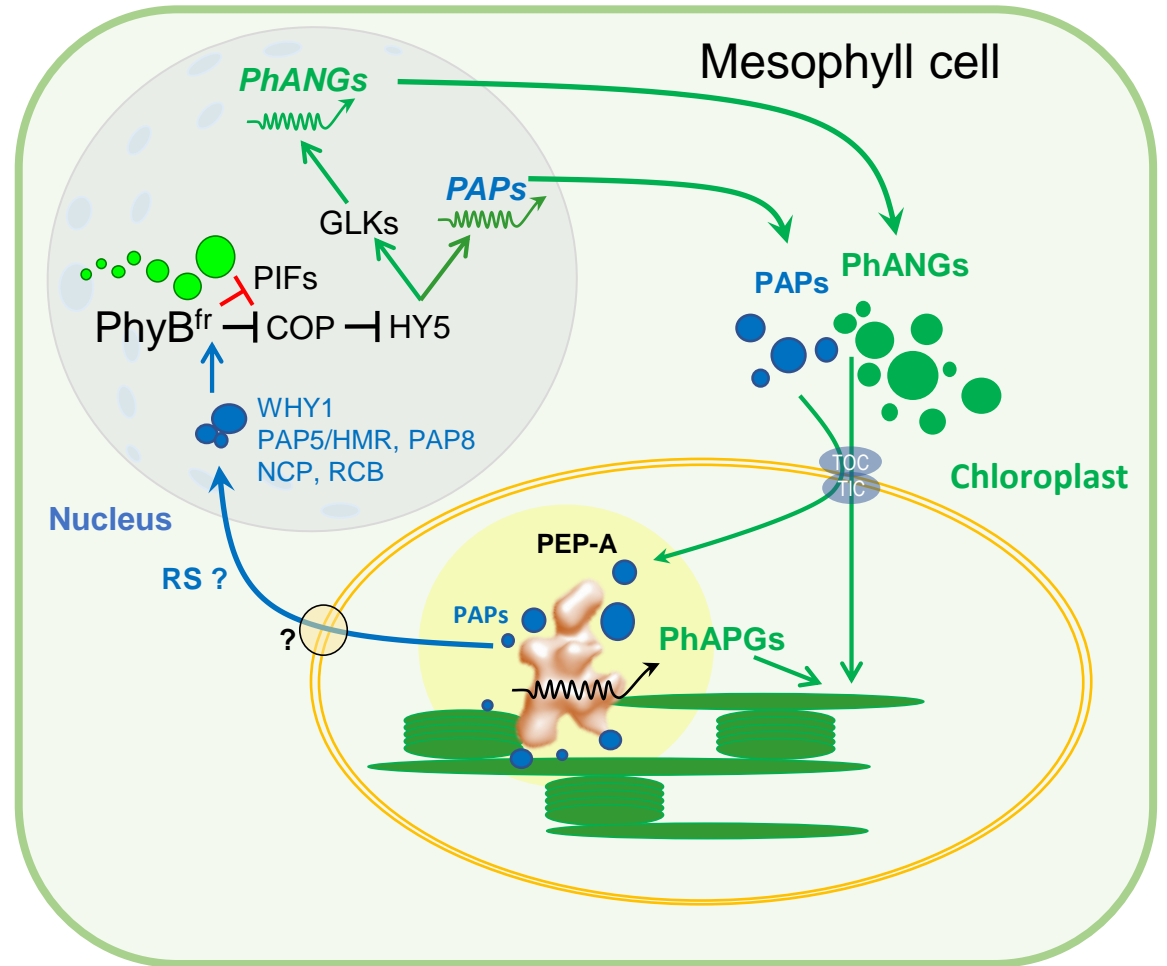
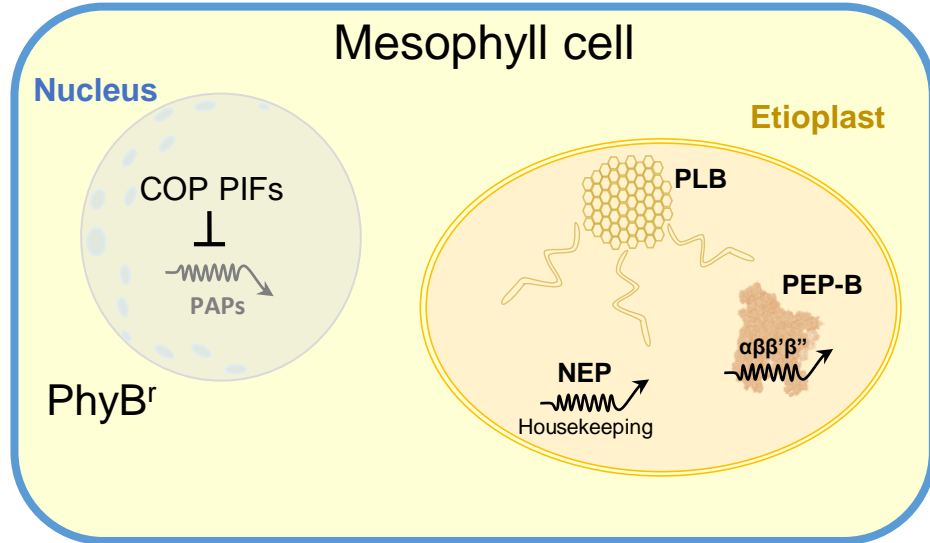
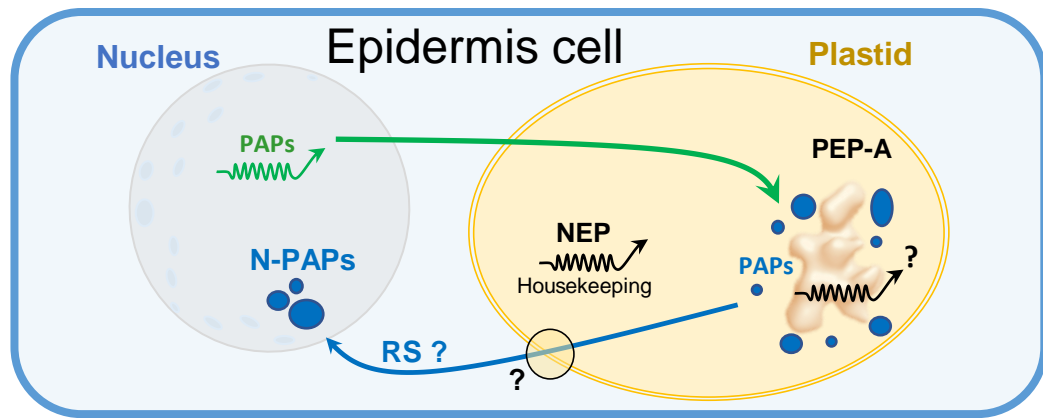


Figure 2: Retrograde signalling from dysfunctional plastids. The scheme depicts a cotyledon mesophyll cell of a seedling treated in the dark with NF or Lin and subsequently transferred to light. The plastids are incapable to develop into functional chloroplasts because of the inhibitors generating a severe disturbance of the general developmental programmes of the seedling. NF blocks carotenoid biosynthesis (CarB) that usually prevents oxidative damage through reactive oxygen species (ROS) that are generated by illumination (yellow flash) of phototoxic protochlorophyllide (ProtoChlide), an intermediate of chlorophyll (Chl) biosynthesis. Under NF treatment, thus, ROS production leads to irreversible photobleaching of the developing plastid. Lin treatment blocks plastid translation and interferes with the production of plastid encoded proteins causing an imbalance in stoichiometry with nuclear encoded plastid proteins delivered by import (TOC-TIC machinery). This disturbs plastid proteostasis, blocks formation of the photosynthesis apparatus and activates a chloroplast unfolded protein response (cpUPR) that is connected by unknown means to the cytosolic UPR (cytosolUPR). Ovals: Proteins; for abbreviations of names see text. The GUN1 protein directly or indirectly interacts with multiple targets within the plastid and affects corresponding processes. Ovals with broken surrounding: Unfolded proteins. Blue arrows: Retrograde signals from dysfunctional plastid targeting nuclear gene expression directly or indirectly by influencing cytosolic processes. Green arrows: Delivery of nuclear encoded components (TFs, transcription factors; CPNs, chaperone and proteins involved in proteostasis. For details of functional connections see main text.



Seedling

Cotyledon cross section

PEP transition

Figure 3: Retrograde signalling by dually-localized proteins during de-etiolation. The scheme depicts an overview of the proposed spatio-developmental actions of nucleo-plastidic proteins in retrograde signalling. Bottom boxes: Left box, skoto- and photomorphogenic development of *Arabidopsis* seedlings. Middle box, schematic crosscut through the respective cotyledons that develop either etiolated or green mesophyll cells, respectively. Right box: Basic working model depicting the light-induced subunit reorganisation of the PEP complex. PEP-B representing the *E.coli*-like core enzyme is given as simplified crystallographic structure of the *E. coli* RNA polymerase. Addition of PAPs converts PEP-B into a structurally larger and more complex PEP-A given as 3D envelope (Ruedas et al. 2022). Enlargements of representative cells from tissues indicated in the middle box are given above. Epidermal cells in the dark may contain a fully assembled PEP-A, the activity of it is yet untested. A potential retrograde signalling (RS) of nuclear localized PAPs (N-PAPs) in the epidermis is unknown. Mesophyll cells in the dark contain etioplasts with a prolamellar body (PLB) and a PEP-B with basal transcriptional activity. Expression of PAPs is repressed by COP and PIFs. After illumination phytochrome B is activated (PhyB^{fr}), represses the activity of COP and PIFs and activates photomorphogenic transcription factors such as HY5/HYH which drive transcription of PhANGS (through the action of GLKs) and PAPs. Formation of late photobodies (green filled circles), where these processes occur, requires the involvement of PAPs, NCP, RCB and likely other proteins sent from the plastid (way of export is unknown). Imported PhANGs assemble with PhAPs to build the photosynthetic apparatus. Coordination for production of stoichiometric amounts is achieved through RS. Stretched and piled up dark-green ovals represent thylakoids. For detailed explanations of functional connections see main text.