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► **To cite this version:**

Monique Liebers, Carolina Cozzi, Finia Uecker, Louise Chambon, Robert Blanvillain, et al.. Biogenic signals from plastids and their role in chloroplast development. *Journal of Experimental Botany*, 2022, 73, pp.7105-7125. 10.1093/jxb/erac344 . hal-03819575

**HAL Id: hal-03819575**

**<https://hal.science/hal-03819575v1>**

Submitted on 18 Oct 2022

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1 **Darwin Review for Journal of Experimental Botany**

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

3 **Authors: Monique Liebers<sup>1</sup>, Carolina Cozzi<sup>1</sup>, Finia Uecker<sup>1</sup>, Louise Chambon<sup>2</sup>, Robert**  
4 **Blanvillain<sup>2</sup>, Thomas Pfannschmidt<sup>1</sup>**

5 **Addresses:** 1) Gottfried-Wilhelm-Leibniz-Universität Hannover, Naturwissenschaftliche  
6 Fakultät, Institut für Botanik, Pflanzenphysiologie, Herrenhäuser Str. 2, 30419  
7 Hannover, Germany; 2) Univ. Grenoble-Alpes, CNRS, CEA, INRA, IRIG-LPCV, F-  
8 38000 Grenoble

9 Email addresses: [m.liebers@botanik.uni-hannover.de](mailto:m.liebers@botanik.uni-hannover.de), [c.cozzi@botanik.uni-](mailto:c.cozzi@botanik.uni-hannover.de)  
10 [hannover.de](mailto:f.uecker@botanik.uni-hannover.de), [louise.chambon.pro@gmail.com](mailto:louise.chambon.pro@gmail.com),  
11 [robert.blanvillain@univ-grenoble-alpes.fr](mailto:robert.blanvillain@univ-grenoble-alpes.fr), [t.pfannschmidt@botanik.uni-hannover.de](mailto:t.pfannschmidt@botanik.uni-hannover.de)

12

13 **Author for correspondence:** Thomas Pfannschmidt

14 **Email:** [T.Pfannschmidt@botanik.uni-hannover.de](mailto:T.Pfannschmidt@botanik.uni-hannover.de), **ORCID:** 0000-0002-7532-3467

15

16 **Running title:** Retrograde biogenic signals

17 **Keywords:** Plastids, biogenic signals, cellular communication, light regulation, gene  
18 expression, PEP associated proteins, photomorphogenesis

19 **Word count:** 11455 (main text)

20 **Number of figures and tables:** 3 figures, 1 Supplemental table

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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/C<sub>1</sub>/C<sub>2</sub>*). 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-chel<sub>2</sub>atase (CHLD) and ferrochel<sub>2</sub>atase 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/clpr1* 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<sup>fr</sup>),  
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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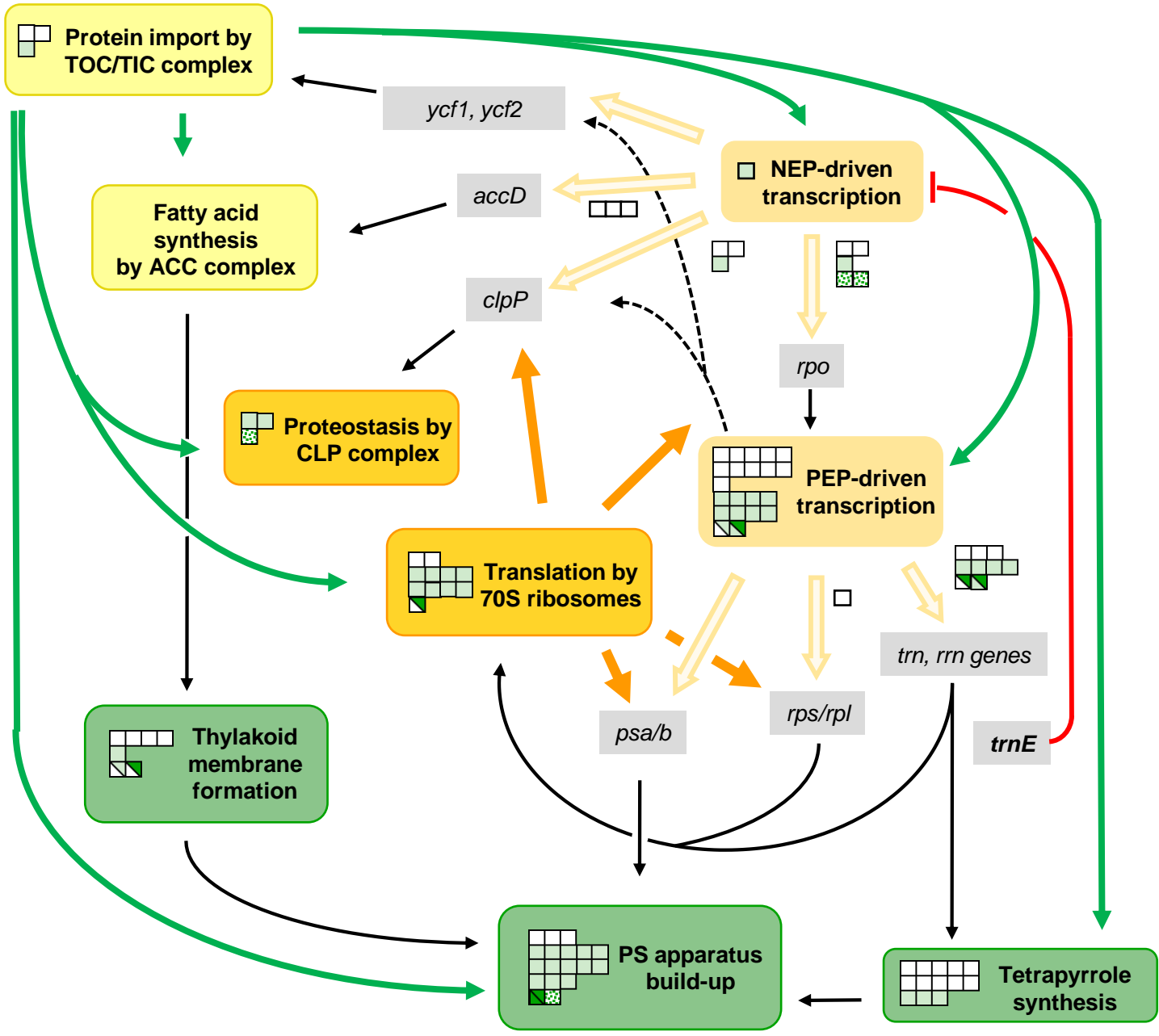
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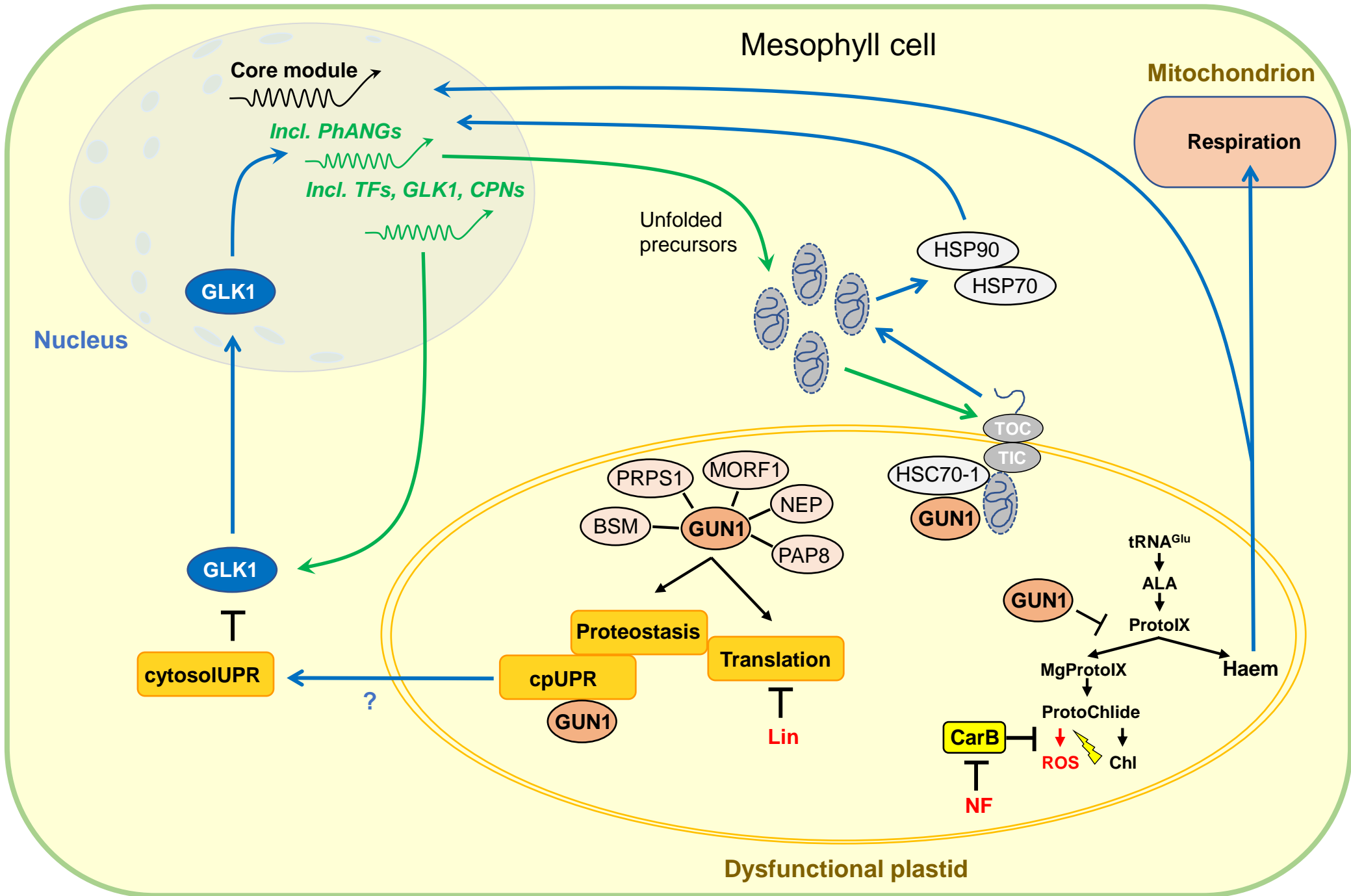




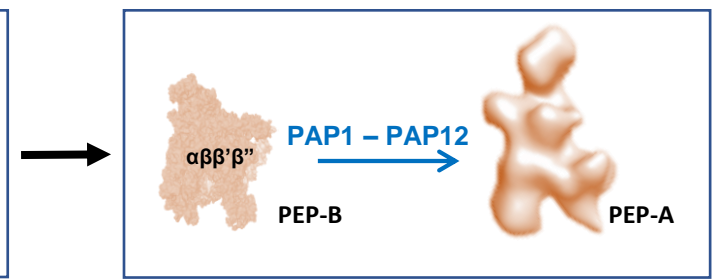
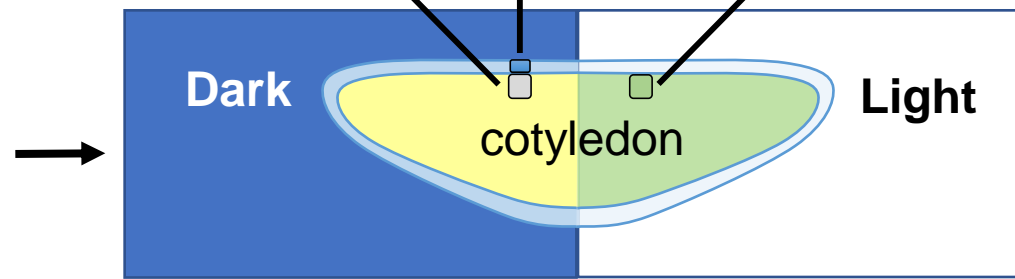
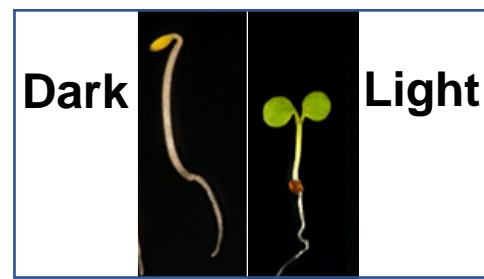
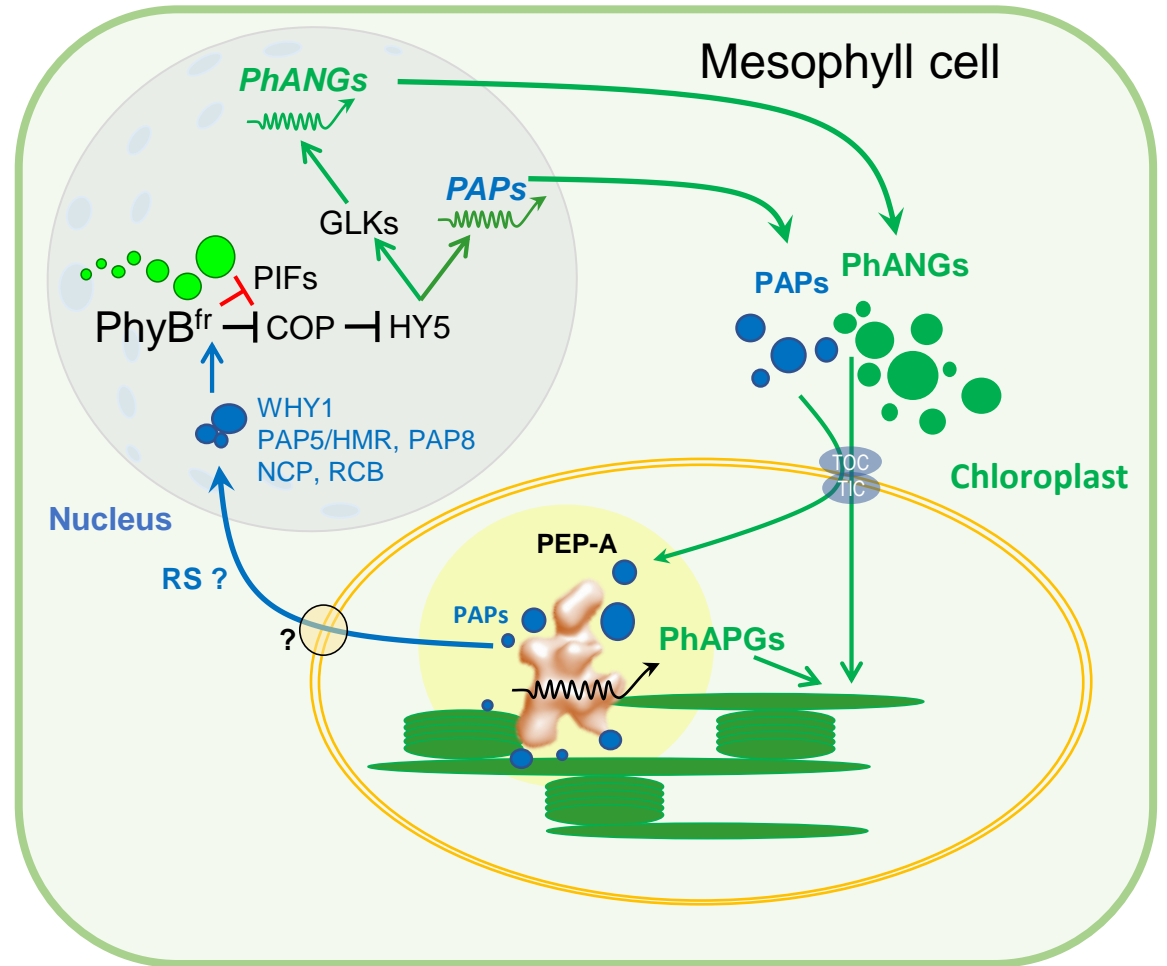
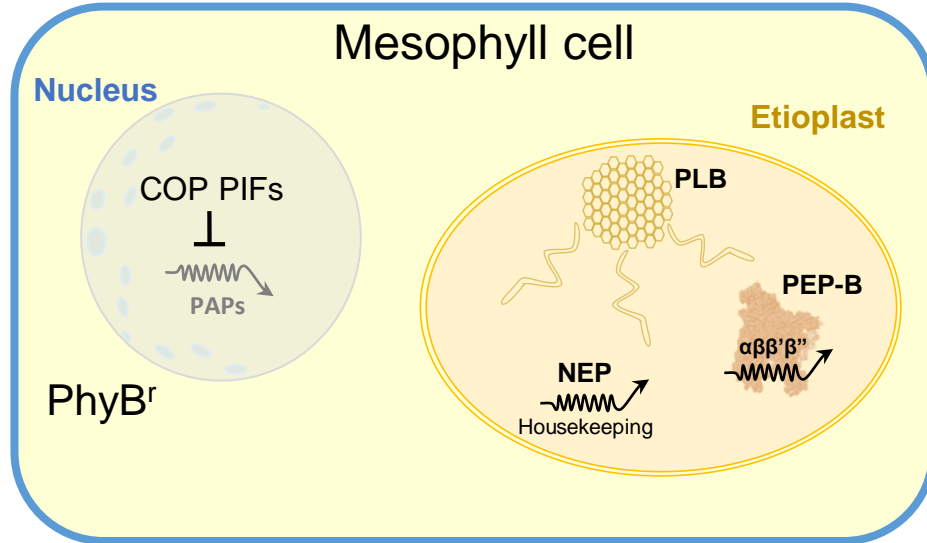
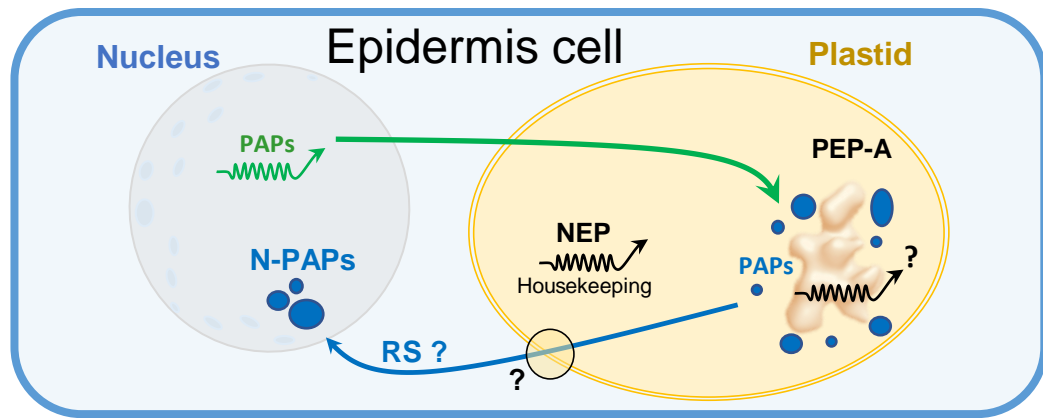
### Mutant phenotype legend

- 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<sup>fr</sup>), 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.