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- 2 Biogenic signals from plastids and their role in chloroplast development
- 3 Authors: Monique Liebers¹, Carolina Cozzi¹, Finia Uecker¹, Louise Chambon², Robert
- 4 Blanvillain², Thomas Pfannschmidt¹
- 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, c.cozzi@botanik.uni-
- 10 <u>hannover.de</u>, <u>f.uecker@botanik.uni-hannover.de</u>, <u>louise.chambon.pro@gmail.com</u>,
- 11 robert.blanvillain@univ-grenoble-alpes.fr, t.pfannschmidt@botanik.uni-hannover.de
- 12
- 13 **Author for correspondence**: Thomas Pfannschmidt
- 14 Email: <u>T.Pfannschmidt@botanik.uni-hannover.de</u>, ORCID: 0000-0002-7532-3467
- 15
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27 Abstract

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

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 inner 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 Todays' 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 dictotyledonous Monocotyledonous 119 (mostly Arabidopsis thaliana). Angiosperms 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

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

meristematic stem cells. However, the tissue context of the cell decides which type of plastid is formed and, therefore, a given cell usually contains only one type of plastid. Independent of the respective form all plastid types contain the same plastome allowing them interconversions of these types in case developmental or environmental conditions change (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 divisionthe 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, $RpoB/C_1/C_2$). 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

polysomes on thylakoids, and protein translation of the photosynthetic apparatus while penetrating within the membrane. Subunits of <u>photosystem II (PSII)</u> appear and gradually accumulate throughout the development of the light-grown seedling. The flattened membranes then produce small pre-grana and serve as nucleation points for subsequent 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.

The genetic control of the dark-or-light developmental programme involves two major interacting pathways corresponding to two classes of isolated mutants; the long <u>hypocotyl</u> (*hy*) and the <u>constitutively photomorphogenic</u> (*cop*). Most important, in the dark, photomorphogenesis is inhibited by a degradational module (the COP9 signalosome) (Sullivan et al. 2003; Seluzicki et al. 2017; Lau and Deng 2012). The E3 ubiquitin ligase COP1 destabilizes the <u>basic domain/leucine zipper</u> (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) (Hug 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 phytochromes with the different PIFs allow a fine-tuning of the developmental responses to 248 249 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

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 development, leading to photo-oxidation. Lack of carotenoids in this very sensitive 284 285 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 phytochromobilin 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 phytochrome chromophore phytochromobilin (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 signals, such as ROS and <u>ab</u>scisic <u>a</u>cid (ABA) (Voigt et al. 2010). For haem the situation is 339 340 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.

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

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

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

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

The answers to each of these topics are tightly connected and the current progress in our 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.

Key activators of PhANG expression are GLK1 and GLK2 (Waters et al. 2009; Waters and Langdale 2009; Fitter et al. 2002). Especially GLK1 appears to be a target for retrograde control since it was also found within the core module displaying an expression behaviour like the PhANGs. Interestingly GLK1 and 2 overexpressing *Arabidopsis* lines exhibit a *gun* phenotype (Leister and Kleine 2016; Martin et al. 2016) and recent data indicate that GLK1 acts downstream of GUN1-mediated retrograde signalling suggesting the action of a 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 appear to result more in all-or-nothing phenotypes. During the build-up of the photosynthetic
 apparatus, thus, expression of TBP associated genes as well as PhANGs requires a high
 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.

More importantly, besides metabolites a number of proteins may act as retrograde signalling 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 <u>D</u> subunit of Mg-<u>chelatase</u> (CHLD) and <u>ferrochelatase I</u> (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).

ii) GUN1 has been shown to associate with the nucleoid and, thereby, is potentially able tointeract 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
GUN1 interacts with several ribosomal proteins and influences accumulation of the
<u>p</u>rokaryotic <u>r</u>ibosomal <u>S1</u> protein (PRPS1), which is involved in the recognition of mRNA
leader sequences and the binding of mRNAs to the ribosome during the translation initiation
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).

Protein homeostasis of plastids is also maintained by the ClpP complex, which derived from
 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 degradation does not take place during early chloroplast development or upon Lin or NF 703 704 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 the upregulation of the nuclear transcription factor HsfA2 and subsequent expression of 711 712 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 be required to verify the multiple protein interactions and to finally clarify the role of GUN1(and its potential interaction partners) in chloroplast biogenesis.

Mobile signalling proteins - This class of proteins resides temporarily inside or on the surface of the plastid compartment performing plastid-specific functions. Under defined conditions, however, they may leave the compartment, pass the cytosol and enter the 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 brakes 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 molecular size in immunoblots, suggesting that the nuclear version lacks the cTP and, thus, 769 770 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

strongly support the involvement of WHY1 in retrograde signalling. While *Arabidopsis why1*mutants do not exhibit a plastid-defective phenotype due to two paralogous gene copies,
maize and barley mutants display greening phenotypes indicating that WHY1 is required for
proper chloroplast biogenesis and therefore represent a genuine biogenic signal (Prikryl et al.
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 plastids (Pfalz et al. 2006). Thus, HEMERA/PTAC12 represents a dually localized protein 784 785 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 etiolating 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 photomorpogenesis, 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 an NLS). Indeed, PAP8 could be identified in high molecular subcomplexes in the nucleus (Chambon et al. 2022) and it is possible that these may interact with phytochromes. This hypothesis would place PAPs as new regulatory factors into the complex light signalling network providing a functional retrograde link between chloroplast biogenesis control and the photomorphogenic programme in seedling development. This tempting idea needs to be 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, pharmaceutics 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 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.

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 uncapable 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 maschinery). This disturbes plastid proteostasis, blocks formation of the photosynthesis 967 aparatus 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 brocken 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-

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 acivity. Expression of PAPs is repressed by COP and PIFs. After illumination phytochrome B is activated (PhyB^{fr}), represses the activity of COP and PIFs and activates photomorphogenic transcription factors such as HY5/HYH which drive transcription of PhANGS (through the action of GLKs) and PAPs. Formation of late photobodies (green filled circles), where these processes occur, requires the involvement of PAPs, NCP, RCB and likely other proteins sent from the plastid (way of export is unknown). Imported PhANGs assemble with PhAPs to build the photosynthetic apparatus. Coordination for production of stoichiometric amounts is achieved through RS. Stretched and piled up dark-green ovals represent thylakoids. For detailed explanations of functional connections see main text.

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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 identifies 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 uncapable 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 maschinery). This disturbes plastid proteostasis, blocks formation of the photosynthesis aparatus 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 brocken 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.



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 acivity. Expression of PAPs is repressed by COP and PIFs. After illumination phytochrome B is activated (PhyB^{fr}), represses the activity of COP and PIFs and activates photomorphogenic transcription factors such as HY5/HYH which drive transcription of PhANGS (through the action of GLKs) and PAPs. Formation of late photobodies (green filled circles), where these processes occur, requires the involvement of PAPs, NCP, RCB and likely other proteins sent from the plastid (way of export is unknown). Imported PhANGs assemble with PhAPs to build the photosynthetic apparatus. Coordination for production of stoichiometric amounts is achieved through RS. Stretched and piled up dark-green ovals represent thylakoids. For detailed explanations of functional connections see main text.