Title: Chloroplast development in green plant tissues: the interplay between light, hormone, and transcriptional regulation

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Summary

Chloroplasts are best known for their role in photosynthesis, but they also allow nitrogen and sulphur assimilation, amino acid, fatty acid, nucleotide and hormone synthesis. How chloroplasts develop is therefore relevant to these diverse and fundamental biological processes, but also to attempts at their rational redesign. Light is strictly required for chloroplast formation in all angiosperms and directly regulates the expression of hundreds of chloroplast-related genes. Light also modulates the levels of several hormones including brassinosteriods, cytokinins, auxins and giberellins, which themselves control chloroplast development particularly during early stages of plant development. Transcription factors such as *GOLDENLIKE1&2 (GLK1&2)*, *GATA NITRATE-INDUCIBLE CARBON METABOLISM-INVOLVED* (*GNC*) and *CYTOKININ-RESPONSIVE GATA FACTOR 1* (*CGA1*) act downstream of both light and phytohormone signalling to regulate chloroplast development. Thus, in green tissues transcription factors, light signalling and hormone signalling form a complex network regulating the transcription of chloroplast- and photosynthesis-related genes to control the development and number of chloroplasts per cell. We use this conceptual framework to identify points of regulation that could be harnessed to modulate chloroplast abundance and increase photosynthetic efficiency of crops, and to highlight future avenues to overcome gaps in current knowledge.

Keywords: Chloroplasts, green tissues, biogenesis, plastid division, light signalling, hormone signalling

I. Introduction

Chloroplasts, the ancestral type of plastid, are thought to have evolved from cyanobacterial symbionts about 1.5 billion years ago (Yoon *et al*., 2004). Chloroplasts allow nitrate and sulphur assimilation, the biosynthesis of some amino acids, fatty acids, pigments, nucleotides and several plant hormones (Witte and Herde, 2020) and are important in sensing environmental stimuli and stressors (Spetea *et al*., 2014). However, the chloroplast compartment is best known for its role in photosynthesis. Of particular relevance to the photosynthetic process is the ability to assemble the photosynthetic apparatus in chloroplasts and control chloroplast number or the total chloroplast compartment per cell. This includes building the photosynthetic electron transport chain in the thylakoid membranes, targeting the components of the Calvin Benson Bassham cycle to the chloroplast stroma, but also manipulating chloroplast number or occupancy of the cell to optimise the volume available for photosynthesis. Given the fundamental nature of these processes, the control of chloroplast development and regulation of their number or total content per cell are considered critical to plant development and function.

The chloroplast content of the cell is primarily modulated through chloroplast biogenesis, the process by which chloroplasts develop from small, undifferentiated proplastids inherited from progenitor cells, and then through subsequent rounds of chloroplast division. The sequence of these two conceptually distinct processes varies depending on the organ and the conditions under which chloroplasts develop. Depending on the tissue, proplastids can differentiate into other types of plastids including chromoplasts, etioplasts, amyloplasts or elaioplasts (Lopez-Juez and Pyke, 2005). When dark-grown cotyledons containing abundant non-green chloroplast precursors called etioplasts initiate greening in the light, the conversion of etioplasts into chloroplasts is followed by the division of those chloroplasts as the cells harbouring them expand (Pipitone *et al*., 2021). In contrast, during development of linear monocotyledonous leaves, in which undifferentiated cells with a very small number of proplastids differentiate into photosynthetic cells with many chloroplasts, plastid division precedes the process of photosynthetic build-up (Loudya *et al*., 2021). The abundance of etioplasts in cells of dark-grown cotyledons probably reflects the fact that etioplasts may have also developed following division of proplastids in embryonic cells. Chlorophagy, the selective degradation of chloroplast material in the vacuole, can also impact on chloroplast numbers, particularly in response to internal and external stresses (Zhuang and Jiang, 2019). As such, the balance between biogenesis, division, and chlorophagy controls the size of the chloroplast compartment in any cell, and this outcome varies substantially between different cell types (Fig. 1A). In most plant species, mesophyll cells are the primary site of

photosynthesis and are tightly packed with chloroplasts such that they occupy the bulk of the cytoplasm between plasma membrane and the central vacuole. In contrast, other tissues such as the bundle sheath, mestome sheath and guard cells contain fewer and smaller chloroplasts, and in epidermal cells they are even less developed (Pyke and Leech, 1994).

Although light-independent greening takes place in gymnosperms, in angiosperms light is fundamental to chloroplast development because it is absolutely required for the expression of hundreds of chloroplast- and photosynthesis-related genes (Hills *et al*., 2015). However, light is not sufficient for this process. This is illustrated by the fact that not all cell types in a leaf exposed to light contain the same number of chloroplasts (Fig. 1A). In some cell types, these differences may be the result of differences in light quantity, quality, and/or perception. For example, for cells deep in a tissue, not only can less light be available but the response of gene expression to blue or red light can differ (Hendron and Kelly, 2020). In addition to light, other factors, including phytohormones such as brassinosteroids (BR), cytokinins (CK), auxins, and giberellins (GA) are involved in regulating chloroplast development (Müller and Munné-Bosch, 2021). These endogenous regulators carry out essential roles in co-ordinating hypocotyl elongation, unfolding of the apical hook, and expansion of the cotyledons as well as chloroplast biogenesis to allow photomorphogenesis and also modulate plant growth during later stages of development. It is thus noteworthy that phytohormones can affect the size of the chloroplast compartment in any cell indirectly by controlling the balance between cell division and expansion, as well as directly by impacting on chloroplast- and photosynthesis-related gene expression and chloroplast biogenesis and division. Also of note is that most hormones are modulated by light signalling, either through control of hormone biosynthesis pathways or activating hormones to induce downstream signalling pathways. Thus, a complex network of light and hormone signalling exists during plant and chloroplast development. Acting downstream of light and phytohormones, several families of transcription factors have been identified as playing key roles in controlling the size and abundance of chloroplasts.

In the next sections we first summarise our understanding of how plastids develop into the chloroplast compartment of a photosynthetic cell, with a specific focus on chloroplast- and photosynthesis-related gene expression and chloroplast biogenesis rather than any effect on cell development. We then address how transcription factor families, light signalling networks and phytohormones impact on this process, with a particular focus on BR, CK, auxins and GA. We consider areas that are poorly understood, but also how our understanding of this complex network may allow its rational redesign for crop improvement in the future.

II. Chloroplast biogenesis and division in green tissues

Biogenesis: The conversion of proplastids or etioplasts to chloroplasts

When seeds germinate in the dark, a developmental programme known as skotomorphogenesis is initiated. In species such as the dicotyledon *Arabidopsis thaliana* with hypogeal germination, increased hypocotyl growth promoted by cell elongation pushes the cotyledons through the soil to reach light. At the same time, a characteristic tightly closed apical hook is formed that protects the meristem and cotyledons from mechanical damage. During this process, proplastids, the progenitors of all plastids, proliferate and differentiate into etioplasts in cells of the cotyledon (Fig. 1B). Etioplasts contain a semi-crystalline agglomeration of membrane known as the prolamellar body composed of the structural building blocks of the photosynthetic apparatus consisting of prothylakoid membranes, protochlorophyllide and protochlorophyllide oxidoreductase, the light-requiring enzyme which will convert it into chlorophyllide (Liebers *et al*., 2017, Pipitone *et al*., 2021). Deficiencies in protochlorophyllide or any of these other components lead to absent or aberrant prolamellar bodies (Mascia and Robertson, 1978; Solymosi and Aronsson, 2013). Once the seedling emerges from the soil and is exposed to light, photomorphogenesis is initiated. Light triggers the conversion of etioplasts in cotyledons into mature chloroplasts (Fig. 1B). A recent elegant imaging and biochemical analysis provided a quantitative view of the processes involved (Pipitone *et al*., 2021). Structural lipids of chloroplast membranes overwhelmingly consist of essential galactolipids (Jarvis *et al.,* 2000). Within 12 hours of light, and continuing for abour four days, their accumulation increases as proteins of mature photosynthetic complexes appear and the surface area of thylakoids rapidly expands (Pipitone *et al.,* 2021). At the same time, cotyledons expand, light-regulated genes are activated, and hypocotyl growth is inhibited. Chloroplast biogenesis in cotyledons thus occurs efficiently upon light exposure because the formation of the prolamellar body in the dark has primed etioplasts for fast conversion into chloroplasts (Liebers *et al*., 2017).

In contrast to cotyledons, chloroplasts in true leaves develop in the light from proplastids in cells of primordia (Charuvi *et al*., 2012). These proplastids do not contain the prolamellar body and so are not primed for rapid conversion to chloroplasts (Hernández-Verdeja *et al*., 2020). During proplastid differentiation into chloroplasts (i.e. chloroplast biogenesis) thylakoids are formed and the photosynthetic machinery arranged within the thylakoid (Jarvis and López-Juez, 2013). Imaging, transcriptomic and proteomic analyses at increasing resolutions (Li *et al*., 2010, Majeran *et al*., 2010, Loudya *et al.,* 2021) have begun to define the processes and helped place genetically-identified components in context.

During the plastid build-up stage, machineries required for import of nuclear encoded proteins and for the synthesis of proteins encoded in the chloroplast DNA become established. This is followed by the expression and accumulation of membrane-synthesising and photosynthetic complex proteins. Expresssion of genes for pigment synthesis proteins are followed by those related to the light-dependent reactions of photosynthesis and eventually carbon metabolism (Loudya et *al.,* 2021). The three-dimensional assembly of thylakoids itself involves the activity of membrane remodelling proteins including the curvature-promoting CURT1 (Armbruster *et al.*, 2013) and the membrane remodelling motor VIPP1 (Ohnishi *et al.,* 2018). This structure is important for each thylakoid and for its relationship to others. Typically thylakoid membranes are divided into appressed regions (grana lamella) and non-appressed regions (stroma lamella) depending on whether they contact other thylakoid membranes or the stroma (Wietrzynski *et al.,* 2020). Photosystems are segregated between regions such that Photosystem II is primarily located in appressed (grana core) and Photosystem I is found in non-appressed (grana margin and stromal thylakoid) domains (Wietrzynski *et al.,* 2020). Despite recent improvements in our understanding of the processes underpinning thylakoid assembly, major gaps in knowledge include how it is regulated but also how the structures are arranged. In contrast, the chlorophyll biosynthesis pathway, another essential component of chloroplast biogenesis is well defined (Tanaka and Tanaka, 2007). One of the most important steps is light activation of protochlorophyllide oxidoreductase, which catalyses the conversion of protochlorophyllide into chlorophyllide *a* and *b*. These products are subsequently converted into chlorophyll *a* and *b* (Sperling *et al*., 1998, Liebers *et al*., 2017). At the same time integration of proteins, many of them imported from outside the chloroplast, involves chaperones of the chloroplast signal recognition pathway or direct insertion via a membrane anchor into the lipid bilayer (reviewed by Celedon and Cline, 2013). The fully formed thylakoid membrane therefore contains proteins and pigment-protein complexes including Photosystem II, the cytochrome b_6f complex, Photosystem I and the ATP synthase (Staehelin and DeWit, 1984). Whether from etioplasts in dark-grown cotyledon cells (Pipitone *et al.,* 2021) or from proplastids in meristematic cells (Loudya *et al.,* 2021), two stages of chloroplast biogenesis can be identified: the first has been referred to as the "plastid phase", or photosynthesis-enabling "structural establishment phase", while the second is the "chloroplast" or "greening phase", and during deetiolation this is also the "chloroplast proliferation phase".

Division: proliferation of green or non-green chloroplasts through fission

Depending on the tissue and conditions under which chloroplasts are developing chloroplast division can take place before and/or after greening (Fig. 1B). Moreover, genetic evidence demonstrates that division does not itself drive organelle biogenesis or cause the "filling" of cells with chloroplasts, implying that chloroplast division and biogenesis can be considered separately. For example, mutants with accelerated plastid division contain more chloroplasts but they are smaller, while loss of essential plastid division proteins results in one or two giant chloroplasts per cell. In either case the total proportion of the cell occupied by chloroplasts remains unaltered (reviewed by Pyke, 1999). In contrast, other mutants have been identified in which the total chloroplast occupancy of mesophyll cells is dramatically decreased but individual chloroplast size is perturbed only slightly (Larkin *et al.,* 2016). Thus, while plastid or chloroplast division are often associated with the process of establishing the final organelle compartment of the cell, organelle biogenesis and division are distinct and involve genetically separable components.

Although the process of chloroplast division is tightly correlated with cell size, it is considered largely independent of cell division as it continues even when cell division has stopped (Jarvis and López-Juez, 2013, Loudya *et al*., 2021). As several genes involved in chloroplast division are activated by light and repressed in the dark (Mohammed *et al*., 2018) it appears that along with chloroplast biogenesis, chloroplast division in developing leaves is regulated by light. The molecular machinery driving chloroplast division has been studied extensively (Chen *et al*., 2018). Briefly, ring-shaped contractile complexes positioned at the inner and outer chloroplast membranes lead to mid-plastid constriction. FtsZ1 and FtsZ2 proteins (tubulin-like cytoskeletal GTPases) form the inner contractile Z-ring (Olson *et al*., 2010). Plastid division protein 1 and 2 (PDV1 and PDV2) recruit the cytosolic dynamin-like component Dynamin related protein 5B (DRP5B; also called ARC5) to form the outer contractile ring which also contains polyglucan filaments (Holtsmark *et al*., 2013). Constriction of the Z- and DRP5B-containing rings result in division of the chloroplast, and membrane "pinching" activity of dynamins completes this process. In contrast to the abundance of FtsZ2 and DRP5B proteins, which do not change significantly during development in Arabidopsis, PDV protein abundance declines once division has taken place (Okazaki *et al*., 2009). Mutant alleles and overexpressors of PDV1/2 have fewer and more chloroplasts than wild type respectively (Okazaki *et al*., 2009). Taken together, it appears likely that PDV1 and PDV2 act as rate-limiting regulators of chloroplast division.

Nuclear- and chloroplast-encoded genes controlling chloroplast development

Although chloroplast division appears to be controlled entirely by nuclear-encoded genes, chloroplast biogenesis is determined by both nuclear- and chloroplast-encoded gene products. Approximately 3000 nuclear-encoded proteins localise to the chloroplast (Richly *et al*., 2003). These include proteins with roles in import, thylakoid biogenesis, RNA processing, protein maturation and degradation, plastid gene expression, chlorophyll biosynthesis, metabolite transport, and photosystem assembly (Waters and Langdale, 2009a). The genes encoding these proteins are broadly referred to as Chloroplast- and Photosynthesis Associated Nuclear Genes (Cp- and PhANGs). The majority of nuclear-encoded chloroplast-bound proteins are initially synthesised on cytosolic ribosomes as pre-proteins and imported into the chloroplast. For most, cleavable transit peptides at the N-terminus of pre-proteins direct movement into the chloroplast by interaction with chloroplast membrane complexes known as Translocon of the Outer Chloroplast membrane (TOC) and Translocon of the Inner Chloroplast membrane (TIC) (reviewed by Sjuts *et al*., 2017). The driving force for import is provided by chloroplast heat shock proteins, while the eventual processing is carried out by a stromal processing peptidase (Sjuts *et al*., 2017).

In contrast to the large nuclear genome, the chloroplast genome contains approximately 120 to 130 genes encoding only around 80 proteins, the majority of which relate to photosynthesis, transcription and translation (Daniell *et al*., 2016). Plastid transcription is carried out by both nuclear-encoded polymerases (NEPs) and plastid-encoded polymerases (PEPs) (Börner *et al*., 2015). A well-characterised group of plastidic *SIGMA* (*SIGs*) factors control initiation of PEP-mediated transcription of chloroplast genes (Chi *et al*., 2015). Transcription and translation of chloroplast genes is essential for chloroplast development. For example, mutant alleles for the PEP complexes are albino (Yang *et al*., 2019), *sig6* mutants are unable to produce sufficient chloroplast-encoded proteins and so are deficient in chloroplast biogenesis (Chi *et al*., 2010) and mutants fully defective in NEP are embryo lethal (Hricová *et al*., 2006).

Retrograde signalling from the chloroplast controls Cp- and PhANG transcription

Because most of the proteins needed for chloroplast function are nuclear-encoded, tight regulation between the functional status of the chloroplast and nuclear gene transcription is needed. Signalling from the chloroplast to control nuclear transcription is referred to as retrograde signalling and is controlled by factors such as light, chloroplast gene expression, chloroplast protein import, tetrapyrrole biosynthesis, redox-state and reactive oxygen species (Yurina and Odintsova, 2019). Biogenic retrograde signalling refers to signals from plastids during early steps of chloroplast biogenesis, to ensure the process is completed

287 safely, whereas operational retrograde signals derive from fully active chloroplasts and
288 adjust operation of the organelle in response to environmental conditions (Pogson *et al*.,
289 2008, Grübler *et al*., 2021). Several genes which belong to the pentatricopeptide repeat
290 (PRR) family have been shown to impact on retrograde signalling by altering mRNA
291 sequence, turnover, processing or translation (Barkan and Small, 2014). One such gene is
292 *GENOMES UNCOUPLED 1* (*GUN1*), a key player in biogenic signalling that interacts with,
293 and impacts on the function of other proteins involved in RNA editing and protein import via
294 the TIC-TOC system (Wu *et al*., 2019, Zhao *et al*., 2019, Tadini *et al*., 2020). GUN1 also
295 associates with enzymes involved in the synthesis of tetrapyrroles including haem and
296 chlorophyll (Shimizu *et al*., 2019) and modulates chloroplast homeostasis both in response
297 to environmental stresses and changes in development. For example, when chloroplast
298 integrity is disrupted by high light or lincomycin treatment, loss of a positive retrograde signal
299 (likely the tetrapyrrole haem) or induction of a negative one causes activation of GUN1. This
300 results in repression of the key chloroplast transcription factor *GOLDEN-LIKE 1* (*GLK1*)
301 through GUN1-mediated repression of an unknown *GLK1* transcriptional activator (Martin *et*
302 *al*., 2016). Furthermore, any existing GLK1 protein is targeted for ubiquitination and
303 proteasome-mediated degradation (Tokumaru *et al*., 2017). Although not directly linked with
304 chloroplast development GUN1 evidently contributes to the modulation of chloroplast
305 integrity during development and changing environments. We next address our
306 understanding of major transcription factors that activate Cp- and PhANGs and therefore
307 chloroplast biogenesis.
308

309 **III. Transcriptional regulators of chloroplast development**

310 Transcription factors such as *GOLDEN2-LIKE 1* and *2* (*GLK1&2*), *GATA NITRATE-*
311 *INDUCIBLE CARBON-METABOLISM-INVOLVED* (*GNC*) and *CYTOKININ-RESPONSIVE*
312 *GATA FACTOR 1* (*CGA1*) are involved in chloroplast biogenesis during all stages of plant
313 growth. Their expression levels differ between cell types and during leaf development, likely
314 contributing to observed differences in chloroplast development (Waters *et al*., 2008, Wang
315 *et al*., 2017a, Hua *et al.,* 2021). As these transcription factors have homologs in all plants
316 studied to date and act as key integrators of light and hormone signalling (Fig. 2 and 8) they
317 are considered the closest that we know of to master regulators of chloroplast development.
318

319 ***GLK1&2* regulate key chloroplast biogenesis genes and control cell-specific**
320 **chloroplast development in C_4 species**

GOLDEN2 (*G2*) and subsequently *GLK1* were identified in maize (Langdale and Kidner, 1994, Hall *et al*., 1998). *g2* mutants are pale because they have smaller chloroplasts with incorrectly formed thylakoids in bundle sheath cells (Langdale and Kidner 1994, Rossini *et al*., 2001). *GLK* genes belong to the Golden2, ARR-B and PSR1 (GARP) transcription factor family (Fitter *et al*., 2002) and have now been studied in many species including Arabidopsis, rice, tomato, sorghum, *Gynandropsis gynandra*, barley and the moss *Physcomitrium patens* (Fitter *et al*., 2002, Yasumura *et al*., 2005, Powell *et al*., 2012, Wang *et al*., 2013, Taketa *et al.,* 2021). In each of these species *GLK* genes exist as a homologous pair named *GLK1* and *GLK2* and *glk1 glk2* double mutants have a pale-green phenotype associated with smaller chloroplasts, reduced accumulation of thylakoid membranes and defects in grana stacking (Rossini *et al*., 2001, Fitter *et al*., 2002, Yasumura *et al*., 2005, Waters *et al*., 2008, 2009b). For example, mesophyll and bundle sheath chloroplasts of Arabidopsis *glk1 glk2* double mutants are 50% smaller in cross-sectional area than wild type (Fitters *et al*., 2002). As *glk* mutants exhibit no clear defects in cell or leaf development, the GLK proteins appear to impact specifically on the chloroplast. Overexpression of *GLKs* increases chlorophyll and chloroplast production and can lead to chloroplast development in tissues that would normally have a smaller chloroplast content (Nakamura *et al*., 2009, Kobayashi *et al*., 2012, Kobayashi *et al*., 2013, Wang *et al*., 2017b). GLKs appear to regulate and synchronize the expression of a suite of Cp- and PhANGs essential for the development of chloroplasts including chlorophyll biosynthesis genes *HEMA1, CHLH, GUN4, CAO*, *PORA, PORB* and *PORC* and others such as *LHCB1-6* and *RbcS* involved in light harvesting and carbon fixation (Fitter *et al*., 2002, Waters *et al*., 2008, 2009b). In most species the two *GLK* genes have redundant functions in controlling chloroplast development. However, in C_4 maize they have tissue-specific patterns of expression - while *ZmG2* is preferentially expressed in bundle sheath cells *ZmGLK1* is more strongly expressed in the mesophyll (Langdale and Kidner, 1994, Hall *et al*., 1998, Rossini *et al*., 2001). Thus, in maize the two genes appear to act independently, and due to restricted spatial domains of action associated with the compartmentation of photosynthesis in a C_4 leaf they have distinct roles during chloroplast biogenesis (Rossini *et al*., 2001). In fact, it was this specialisation that allowed their identification – functional redundancy in other species would have required a double mutant.

GNC and _CGA1/GNL_ play partially redundant roles in the regulation of chlorophyll biosynthesis and chloroplast development

The GNC and CGA1/GNL transcription factors belong to the GATA transcription factor family and were identified because of their strong induction in response to various

environmental perturbations, including light and nitrate (Bi *et al*., 2005, Manfield *et al*., 2007, Naito *et al*., 2007, Hudson *et al*., 2013). Their expression is also regulated by endogenous signals such as CK (Naito *et al*., 2007, Hudson *et al*., 2013) and GA (Richter *et al*., 2010). The *gnc* and *gnl* mutants in Arabidopsis have smaller chloroplasts and 10-30% less chlorophyll than wild type (Bi *et al*., 2005, Mara and Irish, 2008, Richter *et al*., 2010, Chiang *et al*., 2012). Moreover, chlorophyll content of the double *gnc gnl* mutant is reduced by 20-40% compared with wild type (Mara and Irish, 2008, Chiang *et al*., 2012). Due to the increased severity of the phenotype displayed by the double mutant, it has been proposed that both transcription factors play partially redundant roles in the regulation of chlorophyll biosynthesis and chloroplast development (Mara and Irish, 2008, Richter *et al*., 2010). Overexpression of *GNC* or *CGA1/GNL* in Arabidopsis and *CGA1/GNL* in rice leads to accelerated greening during photomorphogenesis, increased chlorophyll content in mature leaves, and activation of chloroplast development in non-green tissues such as the leaf epidermis and root cells (Richter *et al*., 2010, 2013, Köllmer *et al*., 2011, Chiang *et al*., 2012, Hudson *et al*., 2013, Kobayashi *et al*., 2017, Zubo *et al*., 2018). It is important to note that as with overexpression of GLKs, the activation of greening in such non-green tissues does not reach the degree normally seen in mesophyll cells. As modification of *CGA1/GNL* also affects germination, stomatal development, flowering, and senescence it appears that their function impacts on multiple processes at various developmental stages (Richter *et al*., 2010, 2013, Zubo *et al*., 2018). Whilst GNC and CGA1/GNL both act to positively regulate chloroplast development, their modes of action appear to differ such that they act as repressors or activators respectively (Fig. 2). Although overexpression of either GNC or CGA1/GNL leads to the induction of genes involved in chlorophyll biosynthesis including Mg-chelatase subunit genes, only CGA1/GNL has been shown to directly bind promoters of these genes suggesting that it activates their expression (Xu *et al*., 2017, Bastakis *et al*., 2018, Fig. 2). CGA1/GNL has also been proposed to regulate the expression of *SIG2* and *SIG6* that orchestrate gene expression in the chloroplast (Bastakis *et al*., 2018). In contrast, GNC represses expression of negative regulators of photosynthesis such as PHYTOCHROME INTERACTING FACTORs (PIFs) and genes involved in BR biosynthesis and signalling (Zubo *et al*., 2018 and Fig. 2). It is of course possible that CGA1/GNL and GNC can act as activators and repressors depending on target genes and interaction with other partners. This is illustrated by the finding that GNC binds to the promoter of *LHCB1.4* that is down-regulated in the *gnc* mutant, and so GNC likely acts as a transcriptional activator of *LHCB1.4* (Xu *et al*., 2017).

Analyses of *gnc cga1* and *glk1 glk2* double mutant alleles and a *gnc cga1 glk1 glk2* quadruple mutant revealed that these transcription factors act redundantly during chloroplast development (Bastakis *et al*., 2018, Zubo *et al*., 2018). However, the quadruple mutant still assembles chloroplasts. A small degree of *GLK1* expression remains present in the commonly studied *glk1* allele in Arabidopsis (Fitter *et al*., 2002) but it remains the case that knockout phenotypes do not cause albinism or embryo lethality in other species suggesting that additional regulators of chloroplast development exist. It has been proposed that members of the LLM-domain B-GATA transcription factor family fulfil this role (Behringer *et al*., 2014, Behringer and Schwechheimer, 2015, Ranftl *et al*., 2016). In summary, although additional players must be involved in chloroplast development there is compelling evidence that *GLK1&2*, *GNC* and *CGA1/GNL* are of fundamental importance. As these transcription factors are regulated by signals including light and phytohormones, we address this next.

IV. Light is necessary but not sufficient for chloroplast biogenesis

Phytochrome (Phy) and cryptochrome (Cry) light receptors play important roles in chloroplast development through their ability to sense red/far-red light and blue light respectively. Upon exposure to red light, the cytosolic, inactive Pr form of phytochrome is converted into active Pfr and translocated to the nucleus (Quail, 2002 and Fig. 3). There, Pfr induces changes in gene expression by regulating the activity of several classes of transcription factors including the PIFs, which are key regulators of chloroplast development and thus photosynthesis. PIFs belong to the basic helix-loop-helix family of transcription factors and in the dark inhibit transcription of multiple PhANGs. This includes genes encoding enzymes of chlorophyll biosynthesis (Moon *et al*., 2008, Shin *et al*., 2009, Gommers and Monte, 2018) and the GLK1 transcription factor (Martin *et al*., 2016). On perception of red light, Pfr inhibits PIF function by triggering phosphorylation and degradation at the proteasome (Bauer *et al*., 2004, Leivar and Monte, 2014). In addition to relieving repression of photosynthesis genes from PIFs, Pfr also inhibits another repressor of light signalling, the E3 ubiquitin ligase complex CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1)-SUPPRESSOR OF PHYA (SPA1) and in so doing allows accumulation of transcription factors which positively drive transcription of PhANGs (Lu *et al*., 2015, Sheerin *et al*., 2015).

Activation of cryptochromes by blue light also represses COP1 activity through interaction with SPA1 (Lian *et al*., 2011, Liu *et al*., 2011, Zuo *et al*., 2011). COP1-SPA1 and other proteins that are part of the ubiquitin proteasome system including DE-ETIOLATED (DET) and FUSCA (FUS) were identified because mutant alleles showed a light-grown phenotype

in the dark (Lau and Deng, 2012, Seluzicki *et al*., 2017). In the dark the COP1-SPA1/DET/FUS ubiquitin proteasome system targets a large number of transcription factors as well as activated phytochromes for degradation (Lau and Deng, 2012, Seluzicki *et al*., 2017). Of particular importance is ELONGATED HYPOCOTYL 5 (HY5), a bZIP transcription factor that promotes de-etiolation and chloroplast development (Oyama *et al*., 1997, Ang *et al*., 1998, Osterlund *et al*., 2000, Hardtke *et al*., 2000, Lee *et al*., 2007, Burko *et al*., 2020). As both PIFs and HY5 bind to G-boxes, it has been proposed that they compete for these motifs and so tune the extent to which light-regulated genes such as *LIGHT-HARVESTING COMPLEX 4* and *PHYTOENE SYNTHASE* are switched on (Lee *et al*., 2007, Zhang *et al*., 2011, Chen *et al*., 2013, Toledo-Ortiz *et al*., 2014). HY5 also regulates the expression of *DIGALACTOSYLDIACYLGLYCEROL SYNTHASE 1* that is important for the biogenesis of a major photosynthetic membrane lipid (Kobayashi *et al*., 2014). HY5 therefore acts as a central regulator of chloroplast development by integrating signals transduced from photoreceptors. However, HY5 activity is also modified by hormone signaling (Gangappa and Botto, 2016) with for example CK and GA regulating HY5 stability and activity (Vandenbussche *et al*., 2007, Alabadi *et al*., 2008). In addition, HY5 itself controls several hormone pathways by regulating genes involved in hormone signalling and biosynthesis (Gangappa and Botto, 2016).

V. Hormones: coordinators of cell and chloroplast development throughout plant growth

The hormonal control of chloroplast development in green tissues has been studied extensively during the transition from skotomorphogenesis to photomorphogenesis but also during chloroplast development in the shoot. BRs, CKs, auxins and GAs often have overlapping roles during both processes, highlighting the potential to use these phytohormones to alter the development of chloroplasts in plant tissue through engineering (as summarised in Table S1). We next summarise our understanding of how these phytohormones regulate chloroplast biogenesis before attempting to integrate this information with the information summarised above on light signalling and the transcription factors acting downstream of both responses.

Brassinosteroids (BRs) repress photomorphogenesis and chloroplast development in the dark

The *de-etiolated 2* (*det2*) mutant was the first BR-related mutant to be linked to chloroplast development. In the dark, the *det2* mutant has increased expression of PhANGs such as

460 *RbcS*, *RbcL, CAB* (*LHCB1*), *psaA-B* and *psb*A and several chlorophyll biosynthesis genes
461 (Chory *et al*., 1991). Nevertheless, chloroplasts of the *det2* mutant contain fewer granal
462 stacks. *DET2* encodes a steroid 5α-reductase, an enzyme that operates early in the BR
463 biosynthesis pathway, and so *det2* is unable to synthesise BRs (Fujioka *et al*., 1997).
464 Several other BR-related mutants have been isolated with similar perturbations to
465 phenotype, including the BR biosynthesis mutants *dwarf4* (*dwf4*) (Azpiroz *et al*., 1998) and
466 *constitutive photomorphogenesis and dwarfism* (*cpd*) (Szekeres *et al*., 1996), as well as BR
467 signalling- mutants *bri1* (Clouse *et al*., 1996) and *bin2* (Li *et al*., 2001). In the dark, although
468 they are unable to accumulate chlorophyll these mutants de-etiolate, accumulate chlorophyll
469 precursors, and initiate chloroplast biogenesis.

470 The negative control of photomorphogenesis and thus initial chloroplast development by
471 BRs is mediated by the BRASSINAZOLE RESISTANT1 (BZR1) transcription factor and its
472 homolog BZR2/BES1, both of which form homodimers to control transcription. In the dark
473 BZR1&2 repress genes involved in light-signalling and chloroplast biogenesis and so inhibit
474 photomorphogenesis (Fig. 4). For example, BZR1 represses the expression of
475 photoreceptors phytochrome B and phototropin1 (Sun *et al*., 2010), *GATA2* and *GATA4*
476 (Luo *et al*., 2010) and *GLK1&2* (Yu *et al*., 2011). BZR1 and PIF4 form a heterodimer to
477 regulate a large number of targets including repression of *GLK1&2* and several chlorophyll
478 biosynthesis genes during skotomorphogenesis (Oh *et al*., 2012). It is proposed that
479 repression of Cp- and PhANGs by BZR1-PIF4 avoids overaccumulation of
480 protochlorophyllide in the dark such that upon exposure to light photo-oxidative damage is
481 minimised and greening promoted (Wang *et al*., 2020). The repression of Cp- and PhANGs
482 by BR-signalling in the dark is reinforced by COP1-mediated degradation of inactive
483 (phosphorylated) BZR1. This increases the ratio of dephosphorylated to phosphorylated
484 BZR1 protein and thus makes it more likely for active and stable dephosphorylated BZR1
485 homodimers to form, and so for photomorphogenesis and chloroplast development to be
486 inhibited (Kim *et al*., 2014). In the light, BZR1 interacts with HY5 (Li and He, 2016) with HY5
487 specifically binding and inhibiting the active dephosphorylated form of BZR1. Therefore,
488 HY5 attenuates the activity of BZR1 such that chloroplast development is no longer inhibited
489 upon exposure to light (Li and He, 2016).

490 Further evidence for a central role of BR in the repression of chloroplast biogenesis
491 comes from the inhibitor of BR synthesis, brassinazole (Brz) that was used to identify two
492 novel chloroplast proteins known as BRZ-INSENSITIVE-PALE GREEN 2 (BPG2) and BPG3
493 (Komatsu *et al*., 2010, Yoshizawa *et al*., 2014). The *bpg2-1* mutant has pale green
494 cotyledons and is insensitive to the Brz-induced promotion of greening (Komatsu *et al*.,

2010). Additionally, the *bpg2-1* mutant has abnormal chloroplasts with fewer grana stacks, more starch granules, and larger plastoglobules. BPG2 is a chloroplast-localized protein which influences the accumulation of 16S and 23S rRNA derived from the chloroplast genome and is important for post-transcriptional and translational regulation in the chloroplast. Like *bpg2-1,* the *bpg3-1D* mutant has pale green cotyledons and is insensitive to Brz (Yoshizawa *et al*., 2014). As the *bpg3-1D* mutant allele shows lower rates of electron transport through Photosystem II, its pale green phenotype may be due to photoinhibition associated with reduced function of PSII (Yoshizawa *et al*., 2014). Although initial studies on BPG2 and 3 revealed an important role for BR signalling during chloroplast development (Komatsu *et al*., 2010, Yoshizawa *et al*., 2014) and their insensitivity to Brz treatment indicate that they are a target of BR action, to our knowledge it is not yet known how these two genes impact on either BR signalling or the control of chloroplast development.

Cytokinin (CK) positively regulates chloroplast biogenesis and division

A positive effect of CK on chlorophyll biosynthesis and chloroplast differentiation was reported more than 60 years ago (reviewed by Cortleven and Schmülling, 2015). While initial studies investigated the effect of CK on systems such as detached leaves and cultured tobacco tissue, later reports focused on its impact on chloroplast development and greening during de-etiolation. For example, while dark-grown Arabidopsis seedlings treated with exogenous cytokinin do not accumulate chlorophyll, they do display a developmental light-grown phenotype and exhibit larger etioplasts which contain thylakoid membranes (Chory *et al*., 1994). Later studies supported these findings and showed an acceleration of chloroplast differentiation in cytokinin-treated plants (Kusnetsov *et al*., 1998, Cortleven and Schmülling, 2015). In addition to affecting the ultrastructure of chloroplasts, CK also promotes chloroplast division (Boasson and Laetsch, 1969, Reutter *et al*., 1998, Okazaki *et al*., 2009). Furthermore, CK accelerates chlorophyll production by promoting several steps in chlorophyll biosynthesis, including the formation of 5-aminolevulinic acid (ALA, the first step in the biosynthesis pathway of tetrapyrroles) and the light-dependent conversion of protochlorophyllide into chlorophyllide (Fletcher *et al*., 1973, Masuda *et al*., 1994, Kuroda *et al*., 1996, Kusnetsov *et al*., 1998, Yaronskaya *et al*., 2006, Cortleven and Schmülling, 2015, Cortleven *et al*., 2016). These responses to CK are associated with the regulation of a large number of nuclear and plastid-encoded genes important for chloroplast function and development, including the small and large subunits of Rubisco and several components of the light-dependent reactions of photosynthesis (Abdelghani *et al*., 1991, Brenner *et al*.,

2005, Hirose *et al*., 2007, Zubo *et al*., 2008, Brenner and Schmülling, 2012, Bhargava *et al*., 2013, Cortleven and Schmülling, 2015).

CK is perceived by histidine kinases such as Arabidopsis Histidine Kinase 2 and 3 (AHK2&3) and Cytokinin Response1/Arabidopsis Histidine Kinase 4 (CRE1/AHK4) (Inoue *et al*., 2001, Suzuki *et al*., 2001). These histidine kinases transduce the signal to histidine phosphotransfer proteins named after the Arabidopsis Histidine Phosphotransfer proteins (AHPs) (Hutchison *et al*., 2006) and B-type Arabidopsis Response Regulators (B-type ARRs) (Fig. 5). The B-type ARRs then act as transcription factors and regulate early CK-responsive genes (Argyros *et al*., 2008, Ishida *et al*., 2008). ARR1, ARR10 and ARR12 were proposed to regulate chlorophyll biosynthesis because the *arr1 arr10 arr12* triple mutant is pale green (Argyros *et al*., 2008). It is now clear that ARR10 and ARR12 directly bind to promoters of chlorophyll synthesis and light harvesting complex genes *HEMA1* and *LHCB6*, and that the regulation of these and other genes involved in chlorophyll biosynthesis during de-etiolation is dependent on the CK receptors AHK2 and AHK3 (Cortleven *et al*., 2016). In addition to the B-type ARRs that act immediately downstream of CK signalling, several other transcription factors have been proposed to regulate genes associated with chloroplast development in response to CK. For example, one of the genes most strongly induced by CK is the APETALA2/ethylene-responsive element binding factors (AP2/ERF) transcription factor *CYTOKININ RESPONSE FACTOR 2* (*CRF2*) (Rashotte *et al*., 2006, Bhargava *et al*., 2013). Overexpression of *CRF2* accelerates chloroplast division, and it has been suggested that this is mediated by increased levels of the cytosolic component of the plastid division ring PDV2 (Okazaki *et al*., 2009). Moreover, the expression of several GATA transcription factors including *CGA1/GNL* and *GNC,* is also induced by CK (Manfield *et al*., 2007, Naito *et al*., 2007, Hudson *et al*., 2011, Bhargava *et al*., 2013, Ranftl *et al*., 2016). While *CGA1/GNL* is one of the most responsive CK-induced genes, up-regulation of *GNC* in response to CK is less pronounced (Naito *et al*., 2007, Chiang *et al*., 2012, Ranftl *et al*., 2016). Nevertheless, the expression of both *CGA1/GNL* and *GNC* is reduced in an *arr* mutant allele (Chiang *et al*., 2012). In roots, CK acts as a potent promoter of greening by inducing expression of *GNC, CGA1* and *GLK2* in a AHK2- and HK3-dependent manner (Kobayashi *et al*., 2012, Kobayashi *et al*., 2017, Ohnishi *et al*., 2018). However, it is currently not known whether the GLK family also responds to CK in shoots during photomorphogenesis. Together, these results suggest that the positive effect of CK on chloroplast development and function is mediated by a number of transcriptional regulators including the B-type ARRs as well as CRF2, CGA1/GNC, and possibly also the GLKs.

Auxin promotes cell elongation and controls root and shoot greening in older plants

Auxin can impact the proportion of a cell occupied by chloroplasts in two distinct ways. First, it controls cell expansion, and second it inhibits the proplastid to chloroplast transition in non-green root tissues. Auxin's central role in controlling leaf expansion could indirectly impact the chloroplast compartment of a cell. For example, cells such as the bundle sheath with a small chloroplast compartment tend to be larger than the mesophyll that is typically full of chloroplasts. It is thus plausible that an extended period of cell elongation is relevant to controlling size of the chloroplast compartment per bundle sheath cell, despite the fact that this is not considered the case for mesophyll where cells of different sizes both within, but also across species, maintain a constant chloroplast compartment (Pyke, 1999). Additionally, initial activity of local auxin and its consequent export from the shoot plays a central, switch-like role in the initiation of leaf primordia and subsequent cytokinin-requiring leaf development, which involves mesophyll cell differentiation and subsequent chloroplast development (Mohammed *et al*., 2018). During later stages of shoot and seedling growth, links between auxin and chloroplast development have been reported (Fig 6.). For example, chlorophyll content is increased in *S. lycopersicon* (tomato) and *C. camphora* (camphor) leaves treated with auxin (Khan *et al*., 2019, Zhou *et al*., 2020), and higher levels of auxin detected after *C. sinensis* (tea) was exposed to shade were associated with increased expression of chlorophyll biosynthesis and chloroplast biogenesis genes (Liu *et al*., 2020). In Arabidopsis, the abundance of 29 chloroplast-related proteins including CAB (LHCB1), LSU and LHCB2 responded to auxin treatment (Xing and Xue, 2012). In tomato plants, overexpression of *AUXIN RESPONSE FACTOR 10* (*SlARF10*) and *SlARF6A* results in leaves with increased chlorophyll content and rates of photosynthesis compared with wild type, whereas *SlARF10*-RNAi and *SlARF6A* knockdown lines had less chlorophyll (Yuan *et al*., 2018, 2019). *Sl*ARF6A, surprisingly, binds to the promoters of *CAB, RbcS* and *GLK1* genes to positively regulate their expression, thus providing insight into how auxin-modulated control of chloroplast development and photosynthesis in leaves could take place (Yuan *et al*., 2019). The action of auxin on chloroplast development in aerial organs is, therefore, negative in principle but can also be positive at later stages or in different contexts.

Meanwhile, auxin also regulates root greening by inhibiting the development of chloroplasts (Fig. 6). As roots develop, proplastids differentiate into non-photosynthetic amyloplasts. But, if the shoot is removed, chlorophyll accumulation and chloroplast development are initiated. Application of exogenous auxin to isolated roots inhibits chlorophyll accumulation and chloroplast development indicating that auxin can modulate root chloroplast development and that the greening effect of shoot removal is due in part to

599 the removal of the auxin source from young shoot tissues (Kobayashi *et al*., 2012). This is
600 controlled by the auxin signalling protein IAA14 and the auxin responsive transcription
601 factors ARF7 and ARF19 which repress genes involved in chloroplast development
602 including *GLK2, HY5, GNC/CGA1* (Richter *et al.,* 2013, Kobayashi *et al*., 2012, Kobayashi
603 *et al*., 2017). Taken together, current evidence indicates that auxin might modulate the
604 chloroplast compartment of cells by controlling cell division and expansion, but also that it
605 has the capacity to act more directly on Cp- and PhANGs. However, the majority of studies
606 have concentrated on auxin and fruit chloroplast development (Salazar-Iribe and De-la-
607 Peña, 2020) highlighting the need for further research on leaf tissue and specific cell types
608 in this organ.
609
610 **Giberellic acid (GA) balances cell and chloroplast development during skoto- and**
611 **photomorphogenesis**
612 GA promotes skotomorphogenesis in the dark by increasing hypocotyl elongation and
613 inhibiting PhANG expression. Seedlings with reduced levels of GA, such as GA biosynthesis
614 mutants, display a partially de-etiolated phenotype in the dark with induction of light-
615 regulated photosynthesis genes including *CAB2* and *RbcS* (Alabadi *et al*., 2004, De Lucas
616 *et al*., 2008, Feng *et al*., 2008, Gallego-Bartolome *et al*., 2011). The majority of GA-
617 modulated responses during skoto- and photomorphogenesis are closely linked with light
618 signalling. This is mediated by the DELLA proteins, negative regulators of GA signalling that
619 are degraded by the 26S proteasome pathway in response to GA (Alabadi *et al*., 2004,
620 Archard *et al*., 2007, Alabadi *et al*., 2008, De Lucas *et al*., 2008, Feng *et al*., 2008, Archard
621 and Genschik, 2009). Mutants that express a stabilized, GA-insensitive DELLA protein
622 display a photomorphogenic phenotype similar to GA biosynthesis mutants when grown in
623 the dark (Alabadi *et al*., 2004, De Lucas *et al*., 2008, Feng *et al*., 2008). When not degraded
624 in response to GA, DELLA proteins bind to and repress PIF activity (Fig. 7). This prevents
625 PIFs from binding and repressing target genes including multiple PhANGs (De Lucas *et al*.,
626 2008, Feng *et al*., 2008). DELLA proteins also affect PIF stability by inducing the degradation
627 of PIFs through the ubiquitin-proteasome pathway (Li *et al*., 2016). In short, GA responses
628 are the result of PIFs' action, GA causing the removal of the negative regulators of PIFs.
629 While high levels of GA prevent accumulation of DELLAs in cells of the hypocotyl in the
630 dark, in dark-grown cotyledons both DELLA transcripts and proteins accumulate to induce
631 synthesis of carotenoids and the chlorophyll precursor protochlorophyllide (Cheminant *et*
632 *al*., 2011). DELLA proteins can also induce expression of *POR*, which has been proposed
633 to be mediated independently of PIFs (Fig. 7) (Cheminant *et al*., 2011). Induction of pigment

634 and POR production in mutants with reduced GA levels or stabilized DELLA proteins results
635 in increased formation of prolamellar bodies in etiolated chloroplasts of cotyledons
636 (Cheminant *et al*., 2011). Unlike *pif* mutants, which show severe photobleaching upon
637 transfer to the light due to the the toxic, photodynamic effects of the accumulated
638 protochlorophyllide, the high levels of POR enzyme protect GA-deficient mutants from
639 photooxidative damage. Based on these results it has been proposed that DELLAs play a
640 central role in balancing the production of pigments and levels of POR in etiolated seedlings
641 to allow the rapid and safe conversion of etioplasts into fully photosynthetic chloroplasts in
642 the cotyledon upon light exposure (Cheminant *et al*., 2011). GA has also been shown to
643 modulate the expression of *GNC* and *CGA1*/*GNL*. *GNC* and *CGA1/GNL* expression is
644 negatively regulated by GA and consistent with this finding, GNC and GNL have been shown
645 to act downstream of DELLA proteins and PIFs, with the promoter sequences of both GNC
646 and GNL being directly targeted by PIF3 (Richter *et al*., 2010).

647 GA can also impact on development of the cellular chloroplast compartment because it
648 controls both cell expansion and cell division (Martínez *et al*., 2016). Arabidopsis and rice
649 plants deficient in GA biosynthesis show a reduction in cell number and size, which at a
650 whole leaf level coincides with a decrease in chloroplast division and thus total chloroplast
651 number (Jiang *et al*., 2012). Based on these findings, it has been proposed that GA positively
652 but indirectly regulates chloroplast division (Jiang *et al*., 2012). These authors showed that
653 the action of GA on cell elongation precedes the activation of chloroplast division genes,
654 consistent with the notion that chloroplast division is activated by the cellular mechanism
655 responsible for generating a constant chloroplast population of cells. However, such a
656 homeostatic mechanism is not completely effective - GA-deficient plants in Arabidopsis and
657 rice were found to have an increased chloroplast density per mesophyll cell, with higher
658 chlorophyll levels and an increased rate of photosynthesis per unit leaf area reflecting the
659 dark-green appearance of these mutants. In addition, chloroplasts in mesophyll cells show
660 increased granal stacking (Jiang *et al*., 2012). Overall, these results suggest that GAs are
661 important in the coordination of cell and chloroplast development in mesophyll cells of both
662 monocotyledonous and dicotyledonous leaves.

663

664 **VI. Conclusions and future prospects**

665 Chloroplast and plant development are tightly linked with light and hormones controlling
666 both processes through signalling cascades culminating in altered transcription of
667 development-related genes and Cp- and PhANGs (Fig. 8 and table S1). As expression of
668 the GLK and GNC/CGA1 transcription factors is regulated by multiple hormone- and light-

signalling pathways, they serve as an integration point for hormone- and light-driven chloroplast development. Thus, this site of integration is a logical starting point by which to engineer plants with modified chloroplast development (Wang *et al*., 2017b). For example, several studies have been performed to determine whether the cell-specific role of the two maize *GLK* genes could be harnessed to establish C_4-like phenotypes in rice to improve photosynthesis and yield. Overexpression of rice *GLK1* increased chloroplast biogenesis in calli, bundle sheath cells of coleoptiles and the leaf sheath (Nakamura *et al*., 2009), but enhanced chloroplast development was not maintained after the seedling stage. In contrast, overexpression of maize *G2* and *GLK1* in rice caused increased greening in shoots, and an enhanced chloroplast development was sustained in vascular sheath cells of mature rice leaves and in tissues where chloroplasts do not typically develop (Wang *et al*., 2017b). Moreover, constitutive expression of *ZmGLK* genes in rice enhanced leaf chlorophyll levels and pigment-antenna complexes leading to improved light harvesting efficiency, possibly due to improved repair and increased photosynthesis and vegetative biomass and grain yield (Li *et al*., 2020). Similarly, overexpression of *CGA1* in rice resulted in an increase in chloroplast numbers and chlorophyll levels thus providing another potential target for engineering crops with improved photosynthesis (Hudson *et al*., 2013, Ermakova *et al*., 2020, Lee et al., 2021). It may well be that tuning these responses such that they take place at specific developmental stages or under particular environmental conditions will lead to further improvements in photosynthesis and yield.

Although extensive research has shown that the GLK and GNC/CGA transcription factors are primary modulators of chloroplast development, other factors must contribute to chloroplast regulation. For example, although pale, the *gnc cga1 glk1 glk2* quadruple mutant develops viable green chloroplasts (Bastakis *et al*., 2018, Zubo *et al*., 2018) and the proteins responsible for this greening have not yet been identified. As several members of the GATA family of transcription factors to which GNC and CGA1 belong have been linked to chloroplast development, greening and photomorphogenesis they are considered candidates for contributing to this remaining regulation of chloroplast development. For example, in Arabidopsis mutant studies of four LLM-domain B-GATAs, *GATA15*, *GATA16*, *GATA17* and *GATA17-LIKE* revealed a role in the promotion of greening and hypocotyl elongation (Ranftl *et al*., 2016). Overexpression of *AtGATA2* caused constitutive photomorphogenesis in the dark whereas repression reduced photomorphogenesis in the light and during BR deficiency (Luo *et al*., 2010). In rice overexpressing *OsGATA12* increased leaf greenness and delayed senescence due to greater chloroplast numbers and more chlorophyll. Here, *OsGATA12* was shown to reduce the expression of genes such as

704 *STAY GREEN* that is involved in chlorophyll degradation (Lu *et al*., 2017). As the role of
705 most GATAs remains unclear (Behringer and Schwechheimer, 2015) they represent
706 interesting candidates for the control of chloroplast maturation. An additional source of
707 candidates may lie in chloroplast development in other tissues such as fruits. For example,
708 overexpression of the GLK-related tomato gene *ARABIDOPSIS PSEUDO RESPONSE*
709 *REGULATOR2-LIKE* (*APPR2-Like*) increases chloroplast number, area and chlorophyll
710 content in unripe fruits (Pan *et al*., 2013). The role of this gene has not been explored beyond
711 fruits of tomato and pepper.

712 Loudya *et al.* (2021) recently demonstrated that chloroplast proliferation, build-up of
713 chloroplast protein import and increases in chloroplast genetic machinery (genome
714 replication, transcription and translation) can all be completed before greening of the
715 chloroplast. Therefore, apart from the individual candidate genes mentioned above it should
716 be noted that identifying regulators that act prior to greening (during the first “plastid” or
717 “structural establishment” phase) is fundamental to complete our understanding of
718 chloroplast development. A limitation is the lack of transcriptomics datasets associated with
719 transitions from one type of plastid to another (e.g. from proplastid or etioplast to chloroplast
720 and the stages inbetween). Another limitation is the fact that such transitions, for example
721 during early leaf cell differentiation, occur simultaneously with other process that affect cells
722 regardless of their photosynthetic or alternative fate. This makes it difficult to discriminate
723 events specifically related to chloroplast development. Advances in techniques such as
724 single-cell isolation, sequencing and microscopy make the acquisition of such datasets
725 possible. With datasets such as these, as well as those currently available relating to
726 hormone, light and chloroplast responses, a systems approach could be used to help dissect
727 the networks and cross-talk occuring during chloroplast development in green plant tissue.

728 The work reviewed here emphasizes the central role that light, and plant hormones play
729 during chloroplast development, and how these pathways are linked through their control of
730 transcriptional regulators. Improved knowledge of how each component (transcription
731 factors, light and hormones) controls chloroplast development is undoubtedly valuable, but
732 it seems likely that more than one component of the network will need to be modified
733 simultaneously. The nodes and connections that are part of this complex system must vary
734 between cell types and result in cell-type-specific differences in chloroplast number and
735 function. Future investigations will therefore likely benefit from focusing on understanding
736 the regulation of chloroplast development in a tissue- and cell-specific context to define how
737 these networks are rewired in particular cell types. Once this is achieved, it should then be

738 possible to rationally redesign the size of the chloroplast compartment in each cell type of
739 the leaf and use this to improve current and future crops.
740

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742

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747

748

749 **Author contributions**

750

751 All authors contributed to the review of literature, conceptualisation of thinking, and writing.

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Figure legends

Figure 1: Chloroplast development in cotyledons and true leaves. A. Despite all cells of the leaf receiving light, the chloroplast compartment varies between cell types. Mesophyll cells (M) contain many large chloroplasts whilst chloroplast occupancy of the bundle sheath (BS) and mestome sheath (MS) is lower. Image of transverse section of a rice leaf taken with Transmission Electron Microscopy. Green, purple and blue colours indicate the M, BS and MS respectively. **B.** Schematic of skoto- and photomorphogenesis in dicotyledons (with epigeal germination) and monocotyledons with representative images illustrating differentiation of plastids to chloroplasts during these processes in Arabidopsis.

Figure 2: Summary of transcription factors known to primarily regulate chloroplast development. GATA NITRATE-INDUCIBLE CARBON METABOLISM-INVOLVED (GNC) promotes chloroplast development by removing the repression of phytochrome interacting factors (PIFs) and Brassinosteroid (BR) related genes on chloroplast biogenesis and division. GATA-LIKE (GNL) and GOLKENLIKE1/2 (GLK1/2) positively regulate Chloroplast- and Photosynthesis Associated Nuclear Genes (Cp- & PhANGs) to promote chloroplast biogenesis and division. Blue arrows and bars indicate transcriptional activation and repression respectively.

Figure 3: Schematic illustrating the impact of light on chloroplast development. A. In the dark PHYTOCHROME INTERACTING FACTORs (PIFs) repress Chloroplast- and Photosynthesis Associated Nuclear Genes (Cp- & PhANGs), and the CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1)/SUPPRESSOR OF PHYA (SPA1) E3 ubiquitin ligase complex degrades the positive regulator HYPOCOTYL 5 (HY5). Components that are inactivated in the dark are pale. **B.** On exposure to light phytochromes (PHYs) and cryptochromes (CRYs) are activated and accumulate in the nucleus. This inhibits PIF and COP1/SPA1 activity. Removal of PIF activity and degradation of COP1/SPA1 to allow accumulation of HY5 contribute to activation of Cp- and PhANGs. Asterisks indicate active protein. Inactivated components are pale. Blue arrows and bars indicate transcriptional activation and repression. Red arrows and bars indicate posttranslational activating and inhibitory effects on proteins.

Figure 4: Schematic illustrating how Brassinosteroids (BRs) inhibit photomorphogenesis and chloroplast development. A. In the dark, non-phosphorylated BRASSINAZOLE RESISTANT 1 (BZR1) represses *PHYTOCHROME B* (*PHYB*) and the *GATA* chloroplast development genes, and in so doing inhibits chloroplast development. Additionally, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)/SUPPRESSOR OF PHYA (SPA1) interacts with and degrades phosphorylated inactive BZR1 to increase the proportion of the non-phosphorylated active form of BZR1, further inhibiting photomorphogenesis and chloroplast development. BZR1 also interacts with

PHYTOCHROME INTERACTING FACTOR 4 (PIF4) to co-regulate several target genes including *GOLDENLIKE1&2 (GLK1&2)*, *GATAs, GATA NITRATE-INDUCIBLE CARBON METABOLISM-INVOLVED* (*GNC*), *CYTOKININ-RESPONSIVE GATA FACTOR 1* (*CGA1*) responsible for chloroplast development. **B.** In the light, HYPOCOTYL 5 (HY5) interacts with BZR1 to attenuate its activity and allow chloroplast development. Phy* and Cry* indicate active protein. Components that are inactivated are pale. Blue arrows and bars indicate transcriptional activation and repression. Red arrows and bars indicate posttranslational activating and inhibitory effects on proteins.

Figure 5: Schematic illustrating the impact of Cytokinin (CK) on chloroplast biogenesis and division. CK is perceived by Arabidopsis Histidine Kinases (AHK2, AHK3 and AHK4) which transduce the signal to activate the APETALA2/ethylene-responsive element binding factors (AP2/ERF) transcription factor, CYTOKININ RESPONSE FACTOR (CRF2), and the B-type Arabidopsis Response Regulators (B-type ARRs). The B-type ARRs and CRF2 transcription factors then positively regulate the expression of nuclear and plastid-encoded genes associated with chloroplast biogenesis and division. Blue arrows and bars indicate transcriptional activation and repression. Red arrows and bars indicate posttranslational activating and inhibitory effects on proteins.

Figure 6: Schematic illustrating the impact of auxin on greening in the shoot but inhibition of greening in the root. Auxin signalling acts to increase the degradation of Aux/IAA transcriptional repressors and therefore increase the activity of Auxin Responsive Transcription Factors (ARFs). **A.** In the shoot, after leaf initiation has taken place. ARFs increase the expression of *GOLDENLIKE1&2 (GLK1&2)* and Chloroplast- and Photosynthesis Associated Nuclear Genes (Cp- & PhANGs) to promote chloroplast development. Additionally, ARFs promote the expression of *EXPANSINS* (*EXPs*) which increase cell elongation and thus indirectly increase chloroplast development. **B.** In the root, ARFs repress the expression of *GATA NITRATE-INDUCIBLE CARBON METABOLISM-INVOLVED* (*GNC*)/ *CYTOKININ-RESPONSIVE GATA FACTOR 1* (*CGA1*) and GLK1/2 to inhibit chloroplast development in these tissues. Blue arrows and bars indicate transcriptional activation and repression. Red arrows and bars indicate posttranslational activating and inhibitory effects on proteins.

Figure 7: Schematic illustrating how Giberellic acid (GA) modulates hypocotyl elongation and pigment biosynthesis in the dark. In dark-grown hypocotyls, GA accumulation results in the degradation of DELLA proteins, which relieves their repression of PHYTOCHOME INTERACTING FACTOR 3/4 (PIF3/4). The PIF transcription factors then promote hypocotyl elongation and inhibit *GATA NITRATE-INDUCIBLE CARBON METABOLISM-INVOLVED* (*GNC*)/ *CYTOKININ-RESPONSIVE GATA FACTOR 1* (*CGA1*) and several other Chloroplast- and Photosynthesis Associated Nuclear Genes (Cp- & PhANGs). In parallel, DELLA proteins induce the expression of

the chlorophyll biosynthesis gene *PROTOPHOPHYRIN OXIDOREDUCTASE (POR)* either directly or indirectly, and thus control and balance pigment levels during de-etiolation. Blue arrows and bars indicate transcriptional activation and repression. Red arrows and bars indicate posttranslational activating and inhibitory effects on proteins.

Figure 8: Summary indicating the integration of hormone and light signalling pathways integrate to result in the regulation of chloroplast development. The four primary plant growth hormones; auxin, cytokinin (CK), brassinosteroid (BR) and giberellic acid (GA) form a regulatory network with light to regulate chloroplast biogenesis and division through regulation of gene expression and/or posttranslational modification of proteins. Blue arrows and bars indicate transcriptional activation and repression. Red arrows and bars indicate posttranslational activating and inhibitory effects on proteins.

Supplementary table 1: The nuclear and chloroplast encoded genes necessary for chloroplast biogenesis and division which are controlled by hormones. Genes are grouped by functional class.

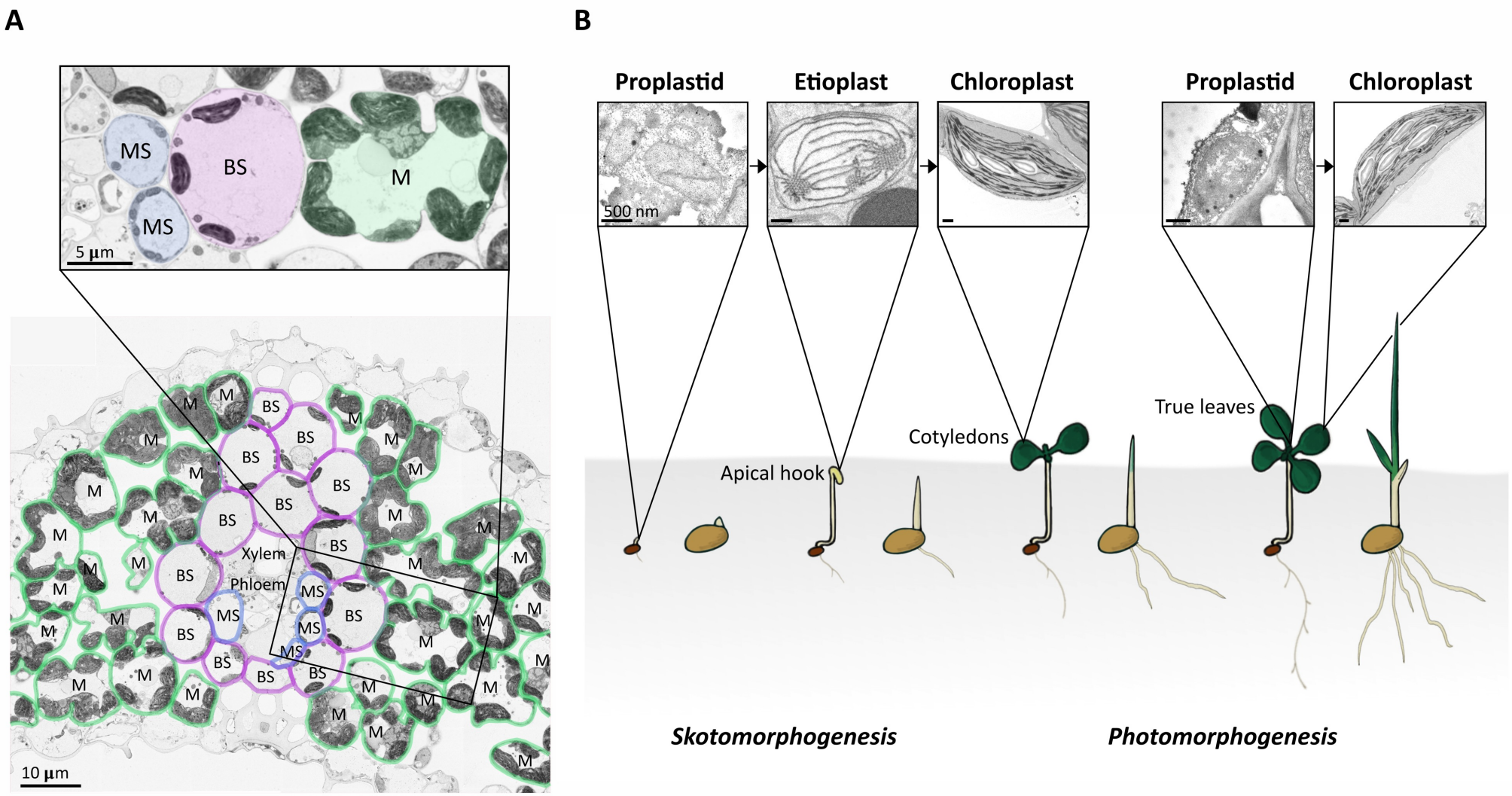
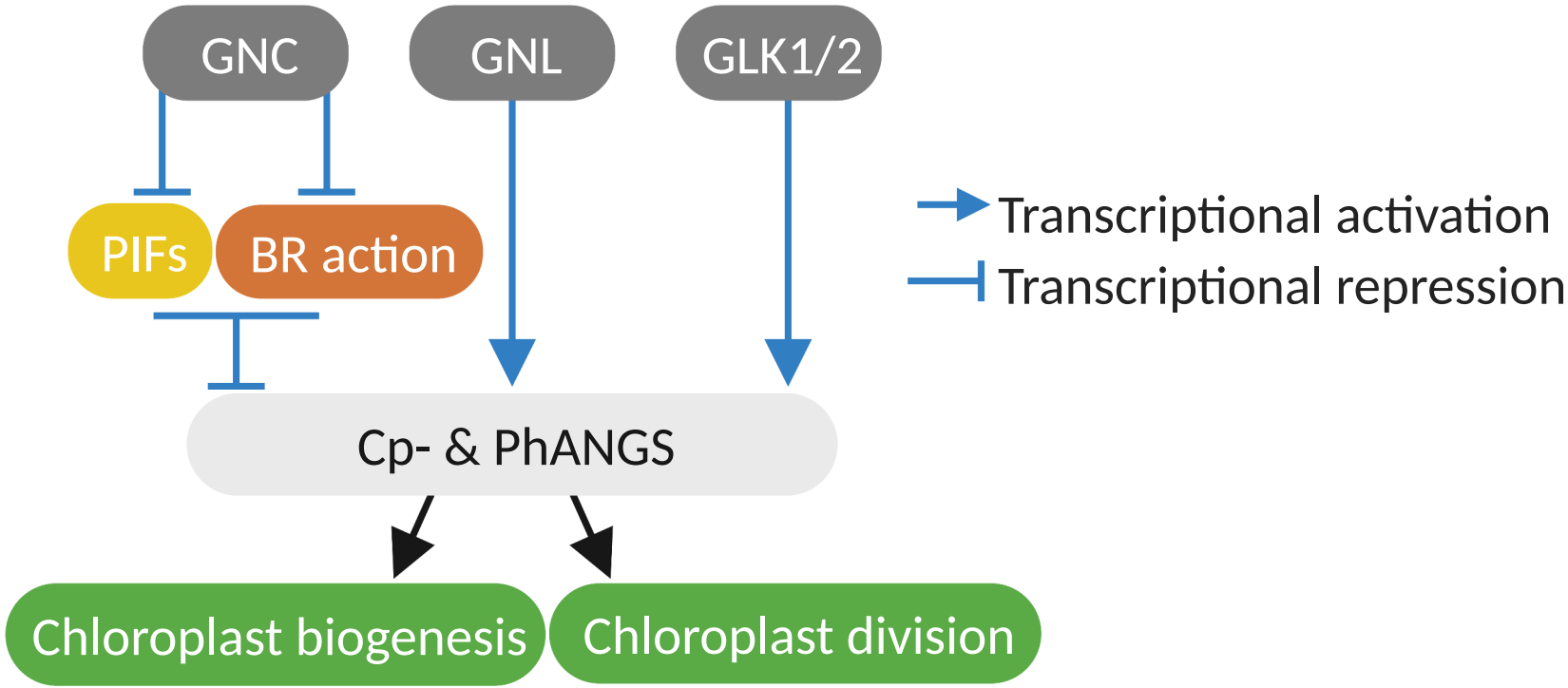
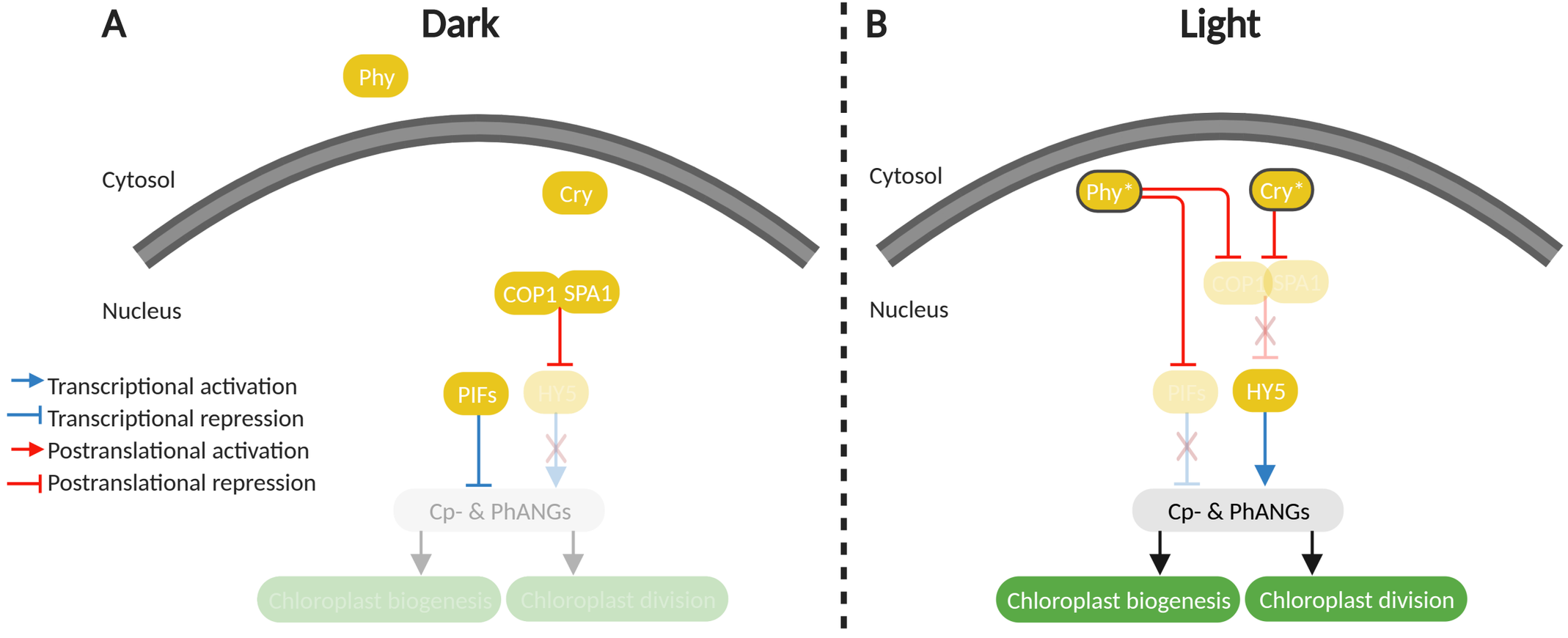
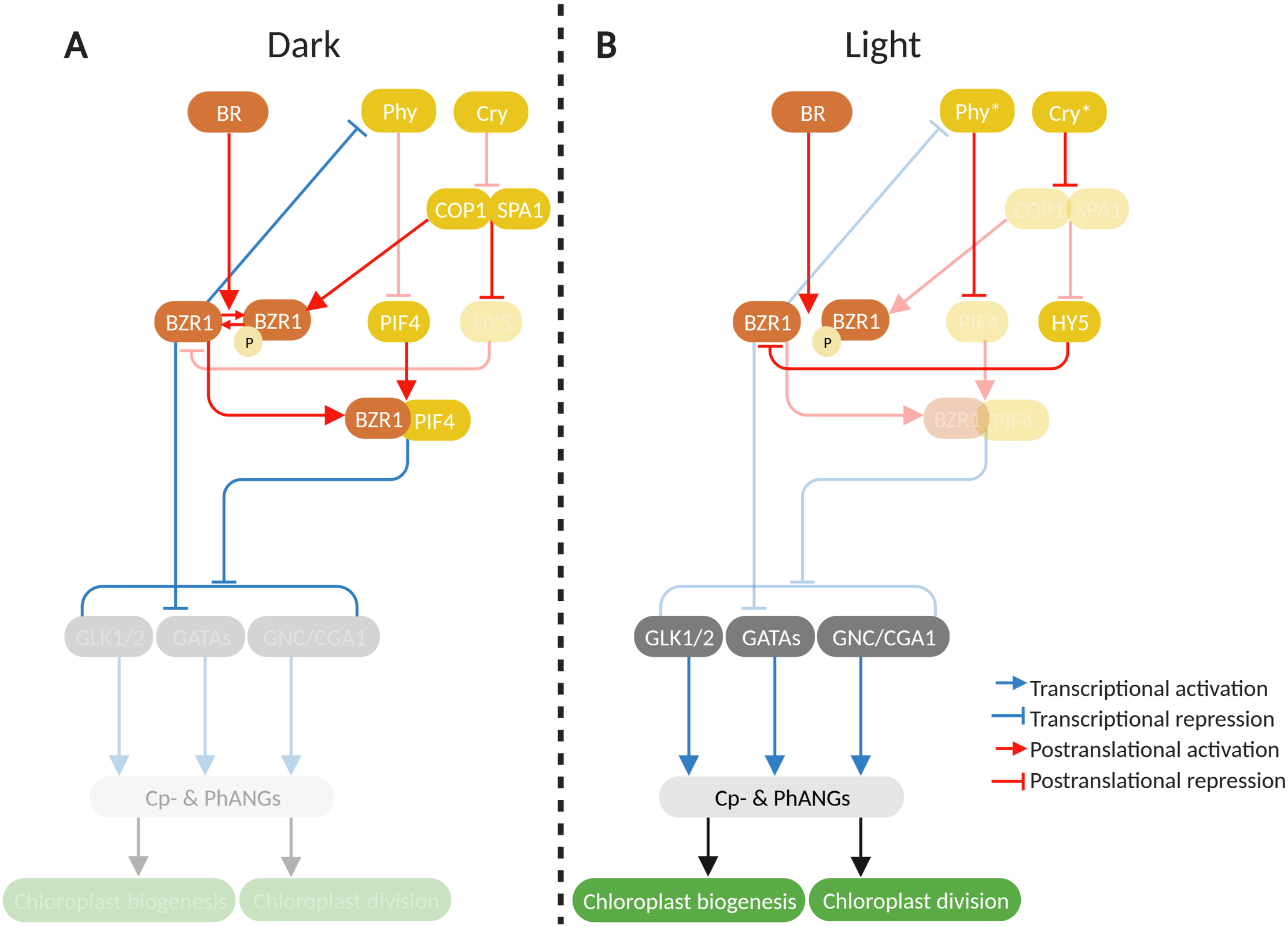
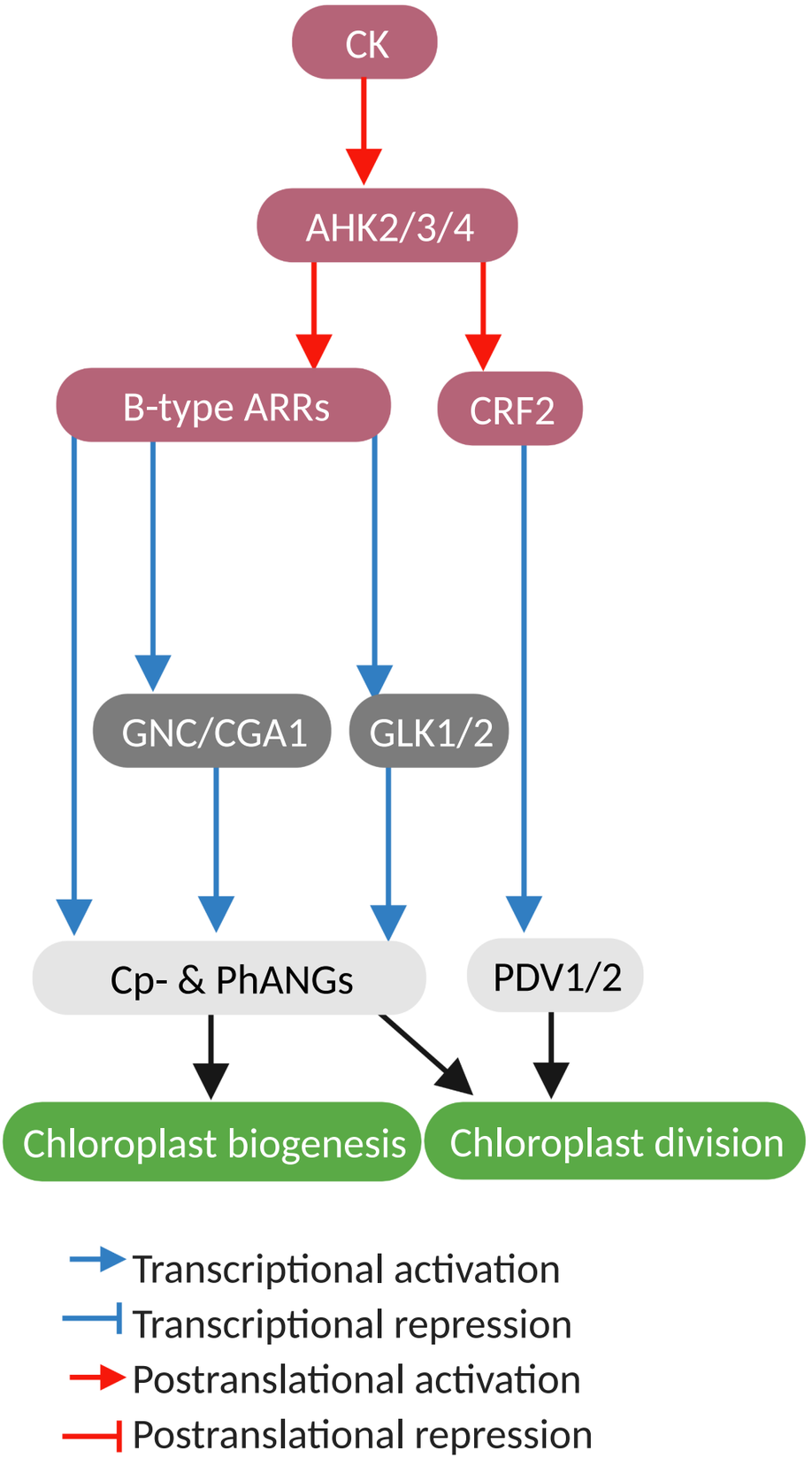


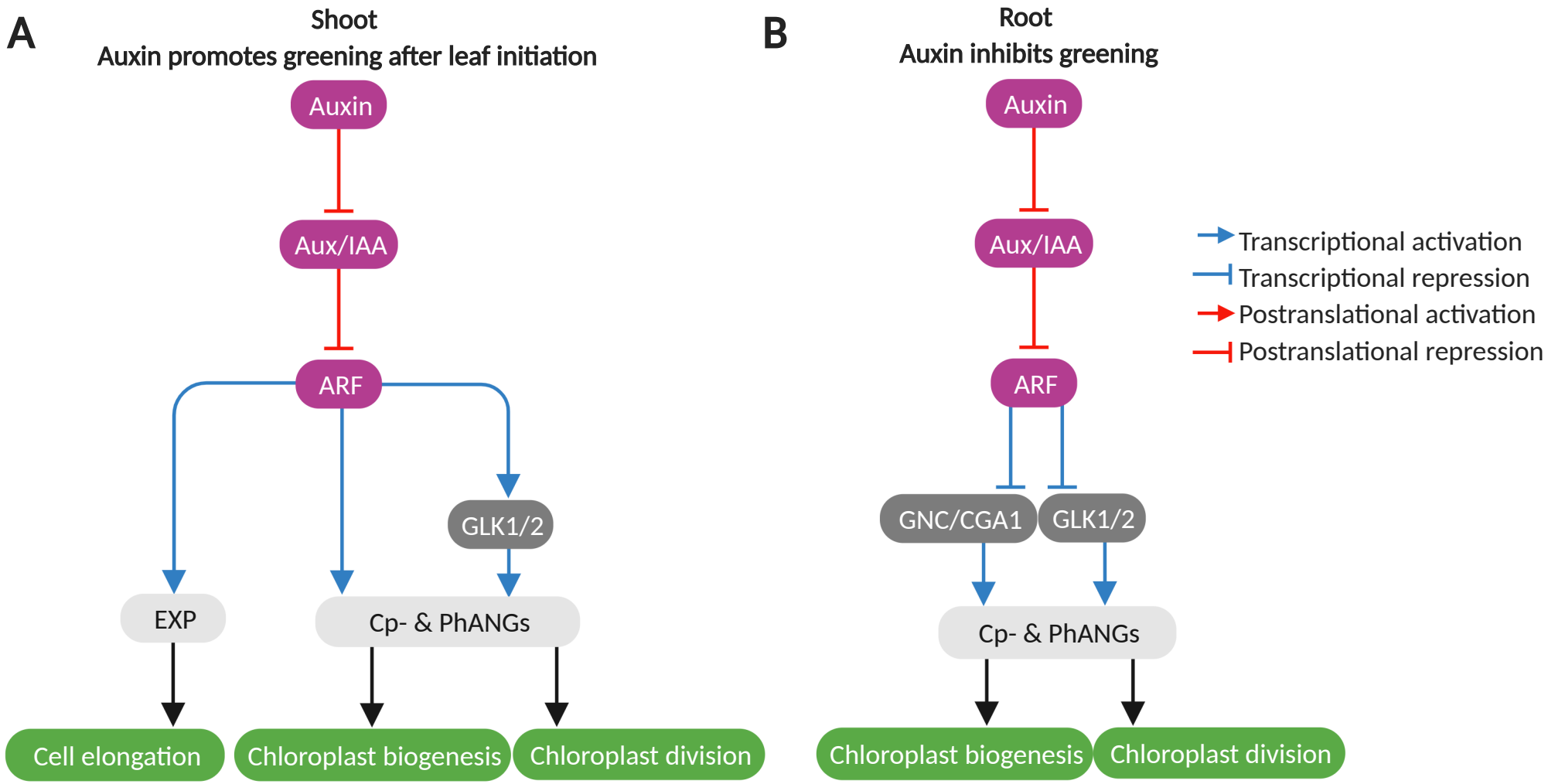
Figure 1: Chloroplast development in cotyledons and true leaves. A. Despite all cells of the leaf receiving light, the chloroplast compartment varies between cell types. Mesophyll cells (M) contain many large chloroplasts whilst chloroplast occupancy of the bundle sheath (BS) and mestome sheath (MS) is lower. Image of transverse section of a rice leaf taken with Transmission Electron Microscopy. Green, purple and blue colours indicate the M, BS and MS respectively. **B.** Schematic of skoto- and photomorphogenesis in dicotyledons (with epigeal germination) and monocotyledons with representative images illustrating differentiation of plastids to chloroplasts during these processes in Arabidopsis.

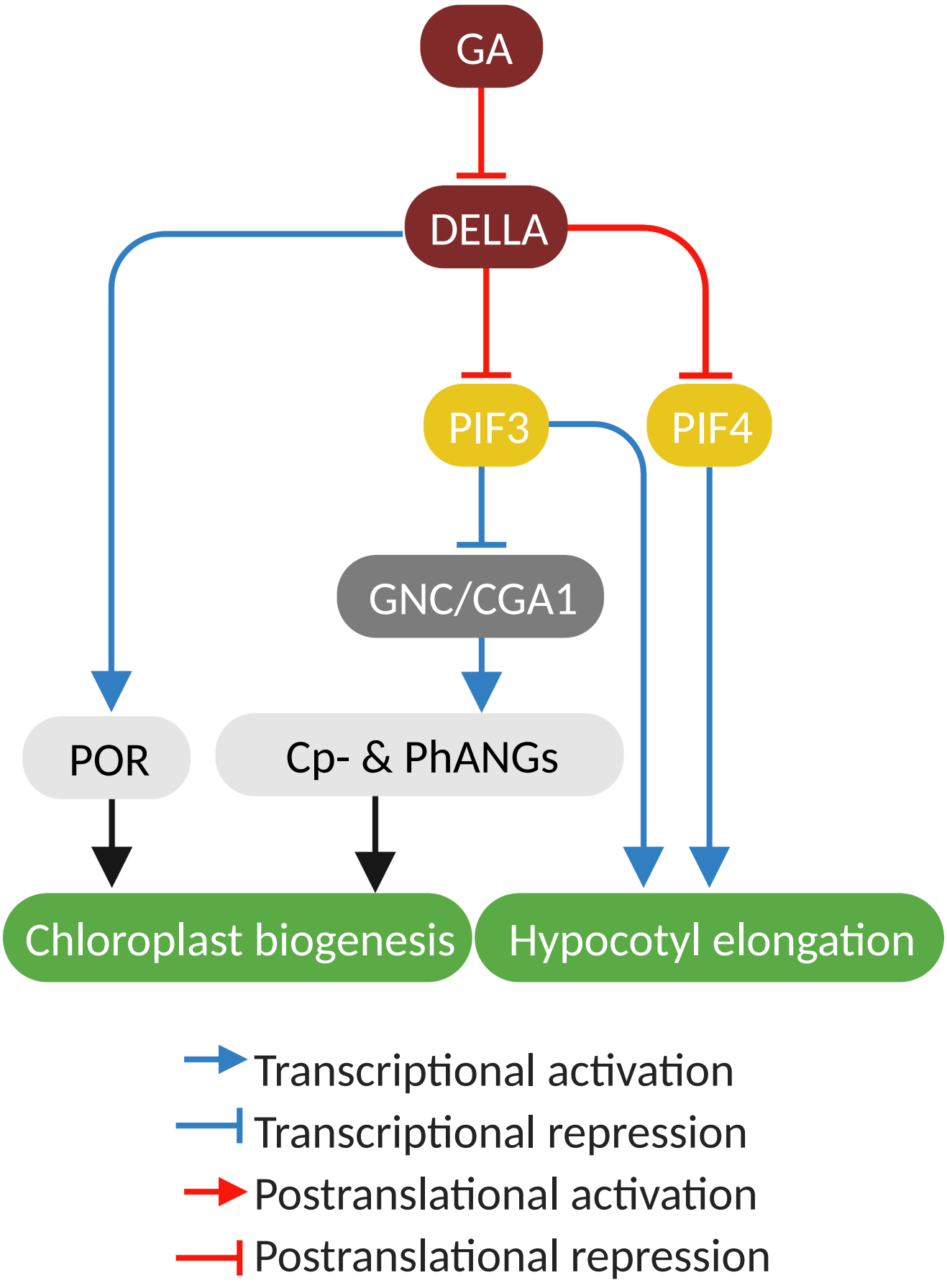

GNC
GNL
GLK1/2
PIFs
BR action
Cp- & PhANGS
Chloroplast biogenesis
Chloroplast division
Transcriptional activation
Transcriptional repression

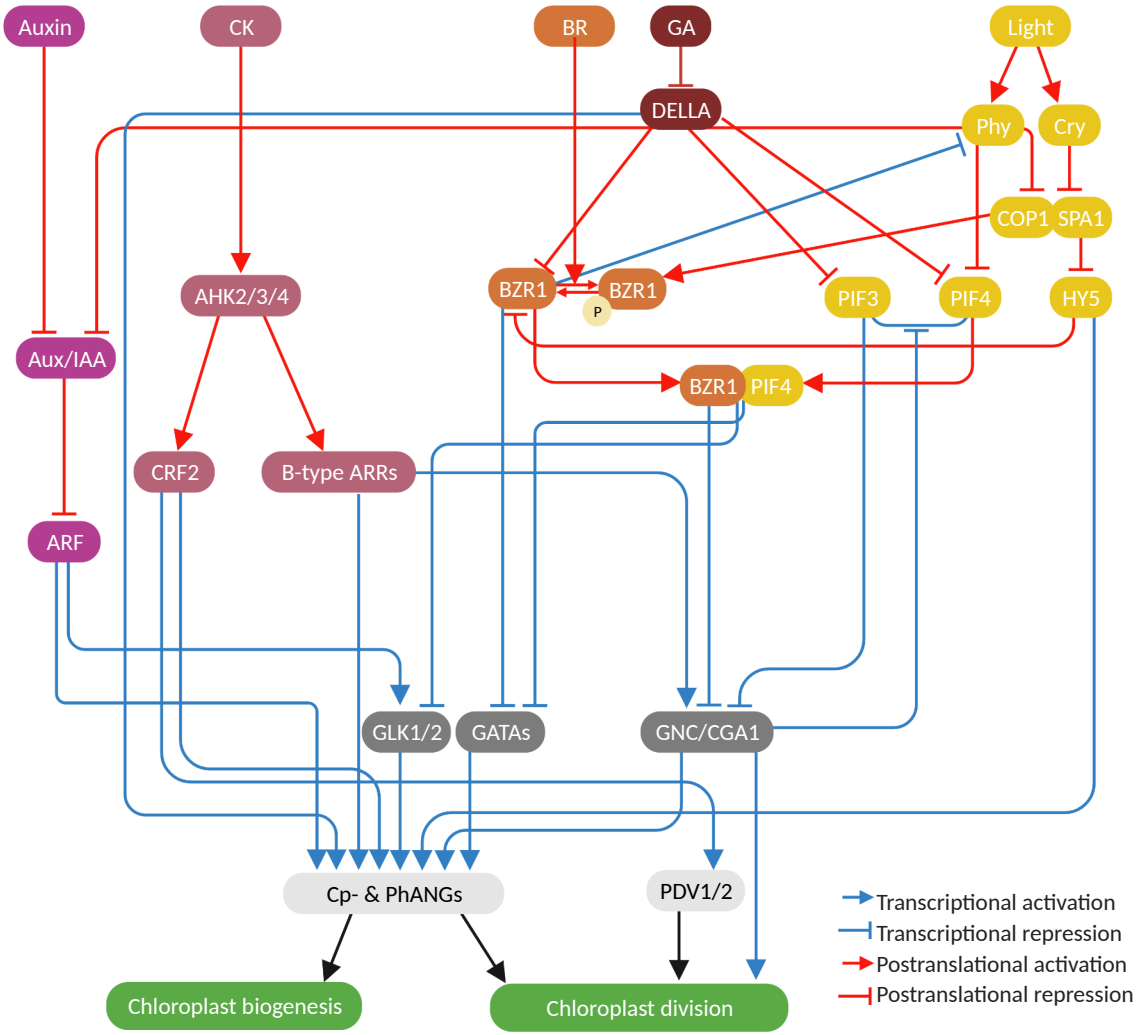

A
Dark
Phy
Cytosol
Cry
Nucleus
COP1
SPA1
PIFs
HY5
Cp- & PhANGs
Chloroplast biogenesis
Chloroplast division
Transcriptional activation
Transcriptional repression
Postranslational activation
Postranslational repression
B
Light
Cytosol
Phy*
Cry*
Nucleus
COP1
SPA1
PIFs
HY5
Cp- & PhANGs
Chloroplast biogenesis
Chloroplast division


A
Dark
BR
Phy
Cry
COP1
SPA1
BZR1
BZR1
P
PIF4
HY5
BZR1
PIF4
GLK1/2
GATAs
GNC/CGA1
Cp- & PhANGs
Chloroplast biogenesis
Chloroplast division
B
Light
BR
Phy*
Cry*
COP1
SPA1
BZR1
BZR1
P
PIF4
HY5
BZR1
PIF4
GLK1/2
GATAs
GNC/CGA1
Cp- & PhANGs
Chloroplast biogenesis
Chloroplast division
Transcriptional activation
Transcriptional repression
Postranslational activation
Postranslational repression


CK
AHK2/3/4
B-type ARRs
CRF2
GNC/CGA1
GLK1/2
Cp- & PhANGs
PDV1/2
Chloroplast biogenesis
Chloroplast division
Transcriptional activation
Transcriptional repression
Postranslational activation
Postranslational repression


A
Shoot
Auxin promotes greening after leaf initiation
Auxin
Aux/IAA
ARF
GLK1/2
EXP
Cp- & PhANGs
Cell elongation
Chloroplast biogenesis
Chloroplast division
B
Root
Auxin inhibits greening
Auxin
Aux/IAA
ARF
GNC/CGA1
GLK1/2
Cp- & PhANGs
Chloroplast biogenesis
Chloroplast division
Transcriptional activation
Transcriptional repression
Postranslational activation
Postranslational repression


GA
DELLA
PIF3
PIF4
GNC/CGA1
POR
Cp- & PhANGs
Chloroplast biogenesis
Hypocotyl elongation
Transcriptional activation
Transcriptional repression
Postranslational activation
Postranslational repression


Auxin
CK
BR
GA
Light
DELLA
Phy
Cry
COP1
SPA1
AHK2/3/4
BZR1
BZR1
P
PIF3
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