<https://helda.helsinki.fi>

CIA2 and CIA2-LIKE are required for optimal photosynthesis and stress responses in *Arabidopsis thaliana*

Gawronski, Piotr

2021-02

Gawronski , P , Burdiak , P , Scharff , L B , Mielecki , J , Gorecka , M , Zaborowska , M , Leister , D , Waszczak , C & Karpinski , S 2021 , ' CIA2 and CIA2-LIKE are required for optimal photosynthesis and stress responses in *Arabidopsis thaliana* ' , Plant Journal , vol. 105 , no. 3 , pp. 619-638 . <https://doi.org/10.1111/tpj.15058>

<http://hdl.handle.net/10138/335813>

<https://doi.org/10.1111/tpj.15058>

acceptedVersion

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.

1 **CIA2 and CIA2-LIKE are required for optimal photosynthesis and stress** 2 **responses in *Arabidopsis thaliana***

3 Piotr Gawroński¹, Paweł Burdiak¹, Lars B. Scharff², Jakub Mielecki¹, Magdalena Górecka³,
4 Magdalena Zaborowska¹, Dario Leister⁴, Cezary Waszczak⁵, and Stanisław Karpiński^{1*}

5 ¹Department of Plant Genetics, Breeding, and Biotechnology, Warsaw University of Life
6 Sciences, 02–776 Warsaw, Poland

7 ²Copenhagen Plant Science Center, Department of Plant and Environmental Sciences,
8 University of Copenhagen, 1871 Frederiksberg C, Denmark

9 ³Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-
10 106 Warsaw, Poland

11 ⁴Plant Molecular Biology, Faculty of Biology, Ludwig-Maximilians-University Munich,
12 Großhadernerstraße 2-4, 82152 Planegg-Martinsried, Germany

13 ⁵Organismal and Evolutionary Biology Research Programme, Faculty of Biological and
14 Environmental Sciences, and Viikki Plant Science Centre, University of Helsinki, 00014 Helsinki,
15 Finland

16 **ORCID IDs:** 0000–0002–9773–3109 (P.G.); 0000–0003–0252–7116 (P.B.); 0000–0003–
17 0210–3428 (L.B.S.); 0000–0003–1195–7118 (J.M.); 0000–0001–6585–4929 (M.G.); 0000–0002–
18 6550–0765 (M.Z.); 0000–0003–1897–8421 (D.L.); 0000–0002–5978–7560 (C.W.); 0000–0002–
19 4328–1207 (S.K.)

20 Correspondence to: Stanisław Karpiński: stanislaw_karpinski@sggw.pl

21 **Summary**

22 Chloroplast-to-nucleus retrograde signaling is essential for cell function, acclimation to
23 fluctuating environmental conditions, plant growth and development. The vast majority of chloroplast
24 proteins are nuclear-encoded and must be imported into the organelle after synthesis in the cytoplasm.
25 This import is essential for the development of fully functional chloroplasts. On the other hand,
26 functional chloroplasts act as sensors of environmental changes and can trigger acclimatory responses
27 that influence nuclear gene expression. Signaling *via* mobile transcription factors (TFs) has been
28 recently recognized as a way of communication between organelles and the nucleus. In this study, we
29 performed a targeted reverse genetic screen to identify dual-localized TFs involved in chloroplast
30 retrograde signaling during stress responses. We found that CHLOROPLAST IMPORT
31 APPARATUS 2 (CIA2) has a functional plastid transit peptide and can be located both in chloroplasts

32 and the nucleus. Further, we found that CIA2, along with its homolog CIA2-like (CIL) are involved
33 in the regulation of Arabidopsis responses to UV-AB, high light, and heat shock. Finally, our results
34 suggest that both CIA2 and CIL are crucial for chloroplast translation. Our results contribute to a
35 deeper understanding of signaling events in the chloroplast-nucleus cross-talk.

36 **Significance**

37 We found that a transcription factor CIA2 can be located in chloroplasts and nucleus and
38 together with its close homolog CIL is involved in protein translation in the chloroplasts and abiotic
39 stress responses.

40 **Keywords**

41 *Arabidopsis thaliana*, chloroplast retrograde signaling, CIA2, CIL, nonphotochemical
42 quenching, photosynthesis, thermo- and photooxidative stress tolerance, chloroplast translation

43 Introduction

44 In plants, intracellular communication between the nucleus, chloroplasts, and mitochondria is
45 essential for the regulation and coordination of physiological processes such as growth, development,
46 stress responses, photosynthesis, and respiration (de Souza *et al.*, 2017). Mechanisms that coordinate
47 organellar and nuclear gene expression enable responses to fluctuating or rapidly changing
48 environmental conditions. Besides photosynthesis, chloroplasts play an important role as redox
49 sensors of environmental conditions and trigger acclimatory responses (Li *et al.*, 2009b). Changes in
50 the developmental and metabolic states of chloroplasts or in the redox status of photosynthetic
51 electron carriers can trigger alterations in the nuclear gene expression in a process called retrograde
52 signaling (Chi *et al.*, 2013; Estavillo *et al.*, 2013; Guo *et al.*, 2016). Among the seminal studies that
53 contributed to the discovery of the retrograde signaling was the identification and characterization of
54 barley (*Hordeum vulgare*) *albostrians* mutant deficient in *HvCMF7* gene (Bradbeer *et al.*, 1979; Hess
55 *et al.*, 1994; Börner 2017; Li *et al.*, 2019). Mutation in the *HvCMF7* gene leads to the formation of
56 pigment-deficient plastids and the consequent development of leaf variegation manifested as white
57 stripes along the leaf blade. The green segments of the leaves contain functional chloroplasts, while
58 the white sectors contain ribosome-deficient chloroplasts unable of translation. Analysis of the
59 *albostrians* mutant demonstrated that the lack of chloroplast ribosomes can influence the expression
60 of nuclear genes encoding for chloroplast proteins, which laid the foundation for the existence of
61 chloroplast-to-nucleus communication (Bradbeer *et al.*, 1979; Hess *et al.*, 1994). Recently, the barley
62 *ALBOSTRIANS* (*HvCMF7*) gene was identified by positional cloning and found to encode for a
63 chloroplast-localized transcription factor with a conserved *CONSTANS*, *CO-like*, and *TOC1* (CCT)
64 domain (Li *et al.*, 2019).

65 It is now well established that the perturbation of multiple plastid processes, including
66 tetrapyrrole biosynthesis, protein synthesis, reactive oxygen species (ROS) metabolism, and dark and
67 light reactions of photosynthesis, influences the expression of nuclear genes encoding photosynthetic
68 proteins (Pesaresi *et al.*, 2006). Moreover, chloroplast retrograde signaling not only coordinates the
69 expression of nuclear and chloroplast genes, which is essential for chloroplast biogenesis, but also
70 ensures chloroplast vitality in changing environmental conditions (Barajas-López *et al.*, 2013) and
71 triggers the expression of nuclear-encoded genes for other cellular compartments such as the
72 cytoplasm and peroxisomes (Karpiński *et al.*, 1997; Karpiński *et al.*, 1999; Mateo *et al.*, 2004;
73 Mühlenbock *et al.*, 2008; Pogson *et al.*, 2008).

74 At the mechanistic level, multiple forms of retrograde signaling can be differentiated. Changes
75 in the absorbed light quality and intensity result in rapid changes in the redox state of photosynthetic
76 electron carriers and lead to unbalanced production of ROS such as hydrogen peroxide (H₂O₂), singlet
77 oxygen, and superoxide anions (Karpiński *et al.*, 1999; Mullineaux *et al.*, 2006; Mühlenbock *et al.*,

2008; Pogson *et al.*, 2008). Recently, it was proposed that H₂O₂ produced in chloroplasts can be directly transported to the nucleus to act as a signaling molecule as its accumulation in both compartments was observed immediately after exposure to light (Caplan *et al.*, 2015; Exposito-Rodriguez *et al.*, 2017). Moreover, H₂O₂ produced in chloroplasts under drought and excessive light conditions influences the metabolism of 3'-phosphoadenosine 5'-phosphate (PAP), which, upon accumulation, modulates the expression of nuclear stress-responsive genes (Estavillo *et al.*, 2011; Chan *et al.*, 2016). Increased singlet oxygen generation in chloroplasts can also trigger specific retrograde signals. However, due to the high reactivity of singlet oxygen, its half-life is too short to enable direct transport to the nucleus, and it was proposed that carotenoid oxidation product, β -cyclocitral, acts as a stress signal induced by singlet oxygen produced in grana stacks (Ramel *et al.*, 2012). Moreover, singlet oxygen can be also produced in grana margins where it induces retrograde signaling through two plastid localized proteins, EXECUTER 1 and 2 (Lee *et al.*, 2007; Wang *et al.*, 2016; Dogra *et al.*, 2019).

In addition to signaling via ROS and metabolites, many transcription factors (TFs) were shown to be controlled by signals generated in the organelles. There are two known TFs, ANAC013 and ANAC017, which respond to the mitochondrial redox status (De Clercq *et al.*, 2013; Ng *et al.*, 2013). These TFs are anchored in the endoplasmic reticulum membrane, and in response to signals from mitochondrial complex III, they are released to the nucleus by the proteolytic cleavage of their transmembrane domains. After translocation to the nucleus, ANAC013 and ANAC017 regulate the expression of mitochondrial dysfunction stimulon genes (De Clercq *et al.*, 2013; Ng *et al.*, 2013). Further, it was recently shown that RADICAL-INDUCED CELL DEATH1 (RCD1) interacts with ANAC013 and ANAC017 to integrate ROS signals from chloroplasts and mitochondria (Shapiguzov *et al.*, 2019). Thus, the dual localization of TFs presents a possibility of their function in retrograde signaling.

Early, *in silico* analyses of Arabidopsis genes encoding putative TFs predicted targeting of at least 48 TFs to the plastids (Wagner and Pfannschmidt, 2006). Later, another *in silico*-based screen approach predicted that 78 Arabidopsis TFs reside in the plastids (Schwacke *et al.*, 2007). Indeed, several proteins exhibiting dual nuclear-plastid localization might potentially be involved in signal transduction pathways involving regulatory protein storage in the plastids. It was shown that most of the dual-targeted (nucleus and organelle) proteins have functions in the maintenance of DNA, telomere structuring, gene expression, or innate immunity (Krause *et al.*, 2012; Caplan *et al.*, 2015). These *in silico* studies were supported by *in vivo* evidence with WHIRLY1 being the first protein to be identified in the nucleus and plastids of the same plant cell (Grabowski *et al.*, 2008); however, its molecular function appears to be compartment-specific. In the nucleus, WHIRLY1 is associated with WRKY53 promoter and acts as a suppressor of leaf senescence (Miao *et al.*, 2013; Huang *et al.*, 2018).

113 In chloroplasts, WHIRLY1 is present in nucleoid fractions where it might be involved in the
114 maintenance of plastid DNA stability (Maréchal *et al.*, 2009; Krupinska *et al.*, 2014). On the other
115 hand the association of WHIRLY1 with thylakoid membranes, led to formation of a model in which
116 WHIRLY1 was proposed to act as a redox sensor in chloroplast-to-nucleus retrograde signaling
117 (Foyer *et al.*, 2014). Another TF implemented in chloroplast retrograde signaling, the plant
118 homeodomain (PHD) transcription factor PTM (PHD-type TF with transmembrane domains) was
119 shown to accumulate in the nucleus after release from the plastid surface. In the nucleus, PTM was
120 suggested to activate the transcription factor ABA INSENSITIVE 4 (ABI4), thereby providing a way
121 to communicate the plastid status to the nucleus (Sun *et al.*, 2011). However, the roles of PTM and
122 ABI4 in chloroplast-to-nucleus communication were recently questioned (Page *et al.*, 2017; Kacprzak
123 *et al.*, 2019).

124 Chloroplasts contain a few thousand proteins that are involved in photosynthesis, intracellular
125 signaling, and biosynthesis of fatty acids, amino acids, hormones, vitamins, nucleotides, and
126 secondary metabolites (Yu *et al.*, 2008). Most of the chloroplast proteins are encoded by the nuclear
127 genome, synthesized by cytosolic ribosomes, imported into the chloroplast, and targeted to a specific
128 compartment within the chloroplast (Jarvis, 2008). Chloroplast genomes encode about 80 of these
129 proteins, most of which function in photosystems, photosynthetic electron transport, and the
130 organellar gene expression machinery (Scharff and Bock, 2014; Daniell *et al.*, 2016). Although
131 nuclear gene expression involves transcriptional control, it is generally believed that in the course of
132 evolution, the regulation of plastid gene expression has shifted from predominantly transcriptional to
133 predominantly posttranscriptional (Eberhard *et al.*, 2002), although significant transcriptional
134 regulation occurs in chloroplasts (Liere and Börner, 2007). Posttranscriptional control is exerted at
135 the level of mRNA stability and, most importantly, at the level of mRNA translation (Zoschke and
136 Bock, 2018). During chloroplast biogenesis, translational regulation is required for the differentiation
137 of chloroplasts from proplastids during the early development of stem and leaf tissues (Sugiura,
138 2014). At the later stages, in mature chloroplasts, translation is regulated mostly by light and controls
139 chloroplast growth for division in the expanding cells of green tissues. Translational regulation also
140 occurs in response to changing environmental conditions and quite often is essential to repair the
141 photosynthesis machinery (Chotewutmontri and Barkan, 2018). For example, the regulation of *psbA*
142 translation encoding for D1 core protein of photosystem II (PSII) is crucial for the repair of
143 photodamaged PSII complexes, whereas the repression of *rbcL* translation encoding for a large
144 subunit of RuBisCo occurs during oxidative stress (Nickelsen *et al.*, 2014).

145 In this paper, we aimed to characterize dual-localized TFs that can be involved in the
146 communication between the chloroplast and the nucleus. As a result of a targeted reverse genetic
147 screen, we focused on CHLOROPLAST IMPORT APPARATUS 2 (CIA2) and its homolog CIA2-

like (CIL), which encode TFs with conserved *CONSTANS*, *CO-like*, and *TOC1* (CCT) domain at the C-terminus and a predicted plastid transit peptide at the N-terminus. Our results suggest that CIA2 has a dual chloroplast-nuclear localization, and together with CIL plays an important role in the regulation of chloroplast translation, thus influencing photosynthetic electron transport, accumulation of photosynthetic pigments, and stress responses in *Arabidopsis thaliana*.

Results

Reverse genetic screen to identify chloroplast-targeted TFs involved in retrograde signaling

Because chloroplast-to-nucleus signaling pathways are not sufficiently understood, we decided to use the targeted reverse genetic screen to identify retrograde signaling components. To this end, we focused on the identification of dual-localized TFs due to their potential role in chloroplast retrograde signaling. To identify such TFs, we extracted and compared the gene identifiers obtained from three *Arabidopsis thaliana* plastid proteome databases (Schwacke *et al.*, 2003; Sun *et al.*, 2009b; Myouga *et al.*, 2013) and two transcription factor databases (Pérez-Rodríguez *et al.*, 2009; Jin *et al.*, 2014). This allowed us to identify a set of TFs with potential chloroplast localization. Further, we obtained T-DNA insertion lines for a selected set of genes (Data S1). These mutants were then subjected to conditions promoting chloroplast photooxidative stress, as we hypothesized that mutants lacking efficient communication between the chloroplast and the nucleus (possibly dependent on TFs) would show altered susceptibility to such treatments. During the primary screen, two types of stress treatments were applied: illumination with ultraviolet AB (UV-AB) and exposure to high light intensity in combination with cold (cHL) (Figure S1). UV-AB significantly impairs photosynthetic electron transport and the general function of photosynthetic machinery (Hollósy, 2002; Caldwell, 1993). At the molecular level, UV-AB damages the ribosomes by crosslinking the cytosolic and chloroplast ribosomal proteins to RNA, thereby transiently inhibiting translation *in vivo* (Casati and Walbot, 2004; Ferreyra *et al.*, 2010). On the other hand, cHL causes imbalances in photosynthetic reactions resulting in the photoinhibition of photosystem II (PSII) and oxidative stress (Yabuta *et al.*, 2002; Distelbarth *et al.*, 2013). Most of the analyzed mutants were not affected by treatment with UV-AB and cHL as compared to Col-0 (Figure S1); however, based on the phenotypes of mutant lines, we could select eight candidate genes for further investigation (Table S1). Next, the coding sequences of six of the eight candidate genes were cloned, and fusion proteins with C-terminal YFP were transiently expressed under the control of 35S promoter in *Arabidopsis* cotyledons to confirm their putative chloroplast localization (Table S1). Except for p35S::AT5G57180:YFP, which showed chloroplast and nuclear localization (Figure 1a), all of the analyzed fusion proteins were found exclusively in the nucleus (Table S1). AT5G57180 encodes CHLOROPLAST IMPORT

182 APPARATUS2 (CIA2) protein, which was previously found to play a role in protein import into
183 chloroplasts (Sun *et al.*, 2001) and protein synthesis in chloroplasts (Sun *et al.*, 2009a). Importantly,
184 Arabidopsis CIA2 is the closest homologue of barley *ALBOSTRIANS* (*HvCMF7*) (Li *et al.*, 2019).
185 The CIA2 protein contains a C-terminal conserved CCT motif, a characteristic feature of one family
186 of TFs involved in light signal transduction (Strayer *et al.*, 2000). The CCT domain contains a putative
187 nuclear localization signal (NLS), which is consistent with the nuclear localization of CIA2 observed
188 earlier (Sun *et al.*, 2001). However, our *in silico* analysis of CIA2 protein sequence predicted the
189 existence of an N-terminal 59 amino acid-long chloroplast transit peptide, that targets a fraction of
190 the CIA2 into chloroplasts (Figure 1a, Figure 2c and Table S1). The transient expression of full-length
191 CIA2 fused with YFP (p35S::CIA2:YFP) in Arabidopsis seedlings resulted in a relatively weak
192 fluorescence signal observed within chloroplasts (as compared to the one observed in the nuclei,
193 Figure 1a), therefore, we also transiently expressed a 100 amino acid-long N-terminal part of CIA2
194 fused N-terminally to YFP (p35S::CIA2¹⁻¹⁰⁰:YFP). As a result, we observed a notably higher
195 fluorescence intensity and confirmed the function of CIA2 transit peptide as the presence of the
196 CIA2¹⁻¹⁰⁰:YFP fusion protein was detected in chloroplasts (Figure 1b). To confirm our findings in an
197 independent system, we transiently expressed both constructs in tobacco (*Nicotiana benthamiana*)
198 leaves. In tobacco leaves, the CIA2:YFP localized to chloroplasts and the nucleus (Figure 1c, d), and
199 a similar localization was observed for CIA2¹⁻¹⁰⁰:YFP (Figure 1e). We did not manage to detect any
200 fluorescence signal in Arabidopsis plants stably expressing p35S::CIA2:YFP (discussed further),
201 therefore, as an additional confirmation of the functionality of CIA2 cTP we isolated total, stromal
202 and thylakoid protein fractions from Col-0 and plants stably expressing p35S::CIA2¹⁻¹⁰⁰:YFP and
203 separated them using SDS-PAGE followed by Western blot analysis (Figure 1f). Using antibodies
204 against GFP we detected processed CIA2¹⁻¹⁰⁰:YFP in total and stromal, but not in thylakoid fractions
205 (Figure 1f).

206 In our reverse genetic screening (Figure 2a,b), line SALK_045340 (hereafter referred to as
207 *cia2-4*) that harbors T-DNA insertion in the first intron of the CIA2 gene (Figure 2c) showed
208 increased susceptibility to UV-AB as indicated by the lower maximum efficiency of PSII (F_v/F_m) and
209 higher ion leakage resulting from the induction of cell death (Figure 2a). Similarly, the exposure to
210 cHL reduced the F_v/F_m of *cia2-4* to a higher extent than that in the exposure to Col-0 (Figure 2b).
211 Thus, based on the computational prediction of the chloroplast transit peptide (Sun *et al.*, 2009b;
212 Myouga *et al.*, 2013; Schwacke *et al.*, 2003), mutant phenotype observed during the initial screen,
213 and results of the protein localization experiments with the use of YFP-reporter, we decided to focus
214 on CIA2 that can localize in the nucleus and chloroplasts and is required for acclimatory responses
215 to UV-AB and cHL.

216 Functional analysis of CIA2 and CIL

217 To confirm the role of *CIA2* under the analyzed stress conditions, we isolated two additional
218 independent *cia2* mutant alleles, *cia2-2* (SALK_004037, Col-0 background) and *cia2-3* (SGT49,
219 Ler-0 background). In both mutants, the T-DNA was inserted into the first exon of *CIA2* (Figure 2c).
220 Because *cia2-4* is an intronic allele, we decided to focus our analysis on *cia2-2* and *cia2-3* alleles.
221 To test whether the incorporation of T-DNA into the *CIA2* gene inhibits the accumulation of *CIA2*
222 transcript, *cia2-2* and *cia2-3* mutants were subjected to quantitative RT-PCR analysis using two pairs
223 of primers (Figure 2c,f and Figure S2b,d). Although we did not detect *CIA2* transcript in *cia2-2* using
224 primers flanking the T-DNA insertion site, we detected a residual amount of PCR product using
225 primers specific to 3'UTR of the *CIA2* gene in *cia2-2* (Figure 2f). In *cia2-3*, we did not detect PCR
226 products in both tested primer pairs (Figure S2d). Taken together, these results suggest that full-length
227 *CIA2* transcript is not present in either of the analyzed mutants (i.e. *cia2-2* and *cia2-3*).

228 *CIA2* shares 54% identical amino acids with CIA2-LIKE (CIL, AT4G25990); moreover, CIL
229 also contains C-terminal CCT motif and NLS (Figure 2c) and has a predicted cTP (Figure 2c), albeit
230 the TargetP reliability class for CIL cTP is higher than that for *CIA2* (the lower value of reliability
231 class, the safer prediction). To investigate the subcellular localization of CIL we have transiently
232 expressed CIL:YFP under the control of 35S promoter, in Arabidopsis cotyledons and *Nicotiana*
233 *benthamiana* leaves and found that in Arabidopsis the majority of the CIL:YFP fusion protein could
234 be found in the nucleolus and nucleoplasm (Figure 3a), while in tobacco the CIL-YFP protein was
235 detected exclusively in nucleolus (Figure 3b). Similarly, no evidence for chloroplast localization was
236 found when CIL¹⁻¹⁰⁰:YFP was transiently expressed in Arabidopsis cotyledons (Figure 3c).
237 Therefore, we conclude that unlike *CIA2*, which has a dual localization, CIL can be found in the
238 nucleoplasm and nucleolus.

239 Because of the sequence similarity, CIL was speculated to act redundantly to *CIA2* (Sun *et al.*,
240 2001). To test this hypothesis, we isolated *cil-1* (SAIL_228_C01, Col-0 background) and *cil-2*
241 (SK14786, Col-4 background) mutants and introduced them into *cia2-2* background by crossing.
242 Despite complete lack of full-length *CIL* transcript in *cil-1* and *cil-2* mutants (Figure 2f and Figure
243 S2b), both mutants were phenotypically indistinguishable from their corresponding wild-type (WT)
244 plants. However, the double mutants *cia2-2 cil-1* and *cia2-2 cil-2* were paler than WT and the
245 corresponding single mutants (Figure 2d and Figure S2a). To confirm visual differences, we measured
246 the concentrations of photosynthetic pigments (Sumanta *et al.*, 2014). From this analysis, we deduced
247 that the content of chlorophyll *a*, chlorophyll *b*, and total carotenoids is significantly lower in *cia2-2*
248 and *cia2-3* (Figure 2e and Figure S2c) mutants, thus supporting previous results obtained for *cia2-1*
249 mutant (Sun *et al.*, 2009a). The introduction of *cil* mutations into *cia2-2* background further reduced

the concentration of photosynthetic pigments (Figure 2d,e and Figure S2a,b), suggesting that CIA2 and CIL synergistically contribute to the accumulation of photosynthetic pigments.

To further confirm that the lack of CIA2 is responsible for the observed phenotype, we introduced constructs that encode CIA2:YFP and YFP:CIA2 fusion proteins under CIA2 native promoter and CIA2:YFP under 35S promoter into *cia2-2* background. The complementation lines pCIA2::*CIA2:YFP_{cia2-2}* and pCIA2::*YFP:CIA2_{cia2-2}* exhibited CIA2 transcript level comparable to that of WT plants, whereas a ~7-fold increase was observed in p35S::*CIA2:YFP* plants (Figure 2g). The complementation and overexpression lines were visually indistinguishable from those of WT plants, and spectrophotometric measurement confirmed that the obtained transgenic lines had WT-like levels of photosynthetic pigments (Figure 2h). However, in contrast to results obtained with the use of transient expression systems, we were unable to detect the YFP signal in these complementation lines, which might be related to the high proteolytic turnover rate of CIA2. Taken together, our results strongly suggest that CIA2 and CIL synergistically contribute to accumulation of photosynthetic pigments and thus are required for the proper functioning of photosystems and photosynthesis.

Characterization of *cia2 cil* responses in chloroplast-targeted stress

Because *cia2-4* mutant was initially selected from the screening using stress conditions that target the photosynthetic apparatus (Figure 2a,b), we set out to test the importance of CIL and CIA2 under stress conditions using chlorophyll fluorescence as a readout. *cia2-4* was shown to be more susceptible to UV-AB that induced cell death (determined on the basis of ion leakage and chlorophyll fluorescence measurements) to a significantly higher extent than in Col-0 plants (Figure 2a). In agreement with these data, the exposure of *cia2-2* mutant to UV-AB resulted in similar changes in the photosynthetic parameters, which were significantly affected 1 and 2 days after the treatment (Figure 4a-c). Moreover, the introduction of *cil* mutant into *cia2-2* intensified the effect (Figure 4 and Figure S3). Measurements of ion leakage after UV-AB treatment confirmed chlorophyll fluorescence observations suggesting increased cell death in *cia2-2* and even stronger effect in *cia2-2 cil-1* and *cia2-2 cil-2* (Figure 4 and Figure S3).

To confirm the role of CIA2, and test whether CIL is required for the high light response, we exposed the plants to blue HL (bHL) and monitored F_v/F_m (Figure 5 and Figure S3). At all tested time points, we observed stronger photoinhibition of PSII as evidenced by decreased F_v/F_m in *cia2-2 cil-1* and *cia2-2 cil-2* (compared to that in WT plants) measured at a level of whole rosette with the strongest effect visible in young (not fully developed) leaves (Figure 5a,b and Figure S3a,b). As increased photoinhibition can result from either increased damage of PSII or decreased PSII repair (Miyata *et al.*, 2015), we monitored recovery after exposure to bHL (Figure 5c). We did not observe

284 differences between Col-0 and *cia2-2 cil-1* in the rate of F_v/F_m recovery suggesting that increased
 285 susceptibility to bHL in *cia2-2 cil-1* is not related to the repair of PSII but to increased damage. HL-
 286 induced damage of the photosynthetic electron transport chain is often related to the production of
 287 ROS such as singlet oxygen, which is mainly produced in PSII antenna, and superoxide anion and
 288 H_2O_2 , which are mainly produced in the vicinity of PSI (Smirnoff and Arnaud, 2019). To simulate
 289 conditions of photoinhibition at the PSII side and induce overproduction of ROS, we treated leaf disks
 290 of Col-0 and *cia2-2 cil-1* with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and methyl
 291 viologen (MV) and monitored F_v/F_m (Figure 5d). DCMU noncovalently binds to the quinone B
 292 binding side of PSII and inhibits the photosynthetic electron transfer to the plastoquinone (PQ) pool,
 293 thus keeping reduced quinone A and oxidized PQ pool. MV can induce the accumulation of
 294 superoxide anion in chloroplast stroma. Upon DCMU and MV treatments, F_v/F_m significantly more
 295 decreased in *cia2-2 cil-1* than in Col-0, suggesting that the double mutant is more susceptible to
 296 photoinhibition and ROS such as singlet oxygen and H_2O_2 (Figure 5d). Collectively, our results
 297 suggest that CIA2 and CIL are required for an adequate response to conditions that promote
 298 photooxidative stress in chloroplasts and trigger cell death signaling.

299 Role of CIA2 and CIL in the regulation of photosynthesis and NPQ

300 Increased susceptibility to HL and UV-AB can be a consequence of impaired nonphotochemical
 301 quenching (NPQ), which is responsible for the dissipation of excess light energy as heat (Kulasek *et*
 302 *al.*, 2016; Białasek *et al.*, 2017). Thus, we monitored NPQ using chlorophyll *a* fluorescence in Col-
 303 0, *cia2-2*, *cil-1*, *cil-2*, *cia2-2 cil-1*, and *cia2-2 cil-2* (Figure 6 and Figure S4). We observed that NPQ
 304 was slightly (but statistically significant) decreased in *cia2-2 cil-1* and *cia2-2 cil-2* at a level of the
 305 whole rosette. Differences were more pronounced when only young, not fully developed leaves were
 306 analyzed. In young leaves of *cia2-2 cil-1*, NPQ was decreased by 40% when compared to Col-0
 307 (Figure 6a,b). It is well established that the process of NPQ is controlled by a small PSII protein PsbS,
 308 which is activated by the acidification of the thylakoid lumen (Niyogi *et al.*, 2005). To check whether
 309 this process is impaired in *cia2 cil* mutants, we measured the proton gradient (ΔpH) and electric
 310 potential ($\Delta\psi$), which constitute proton motive force (PMF) (Figure 6c-e and Figure S4c-e) at two
 311 actinic light intensities (i.e., 160 and 660 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Although we did not observe differences in
 312 PMF between the analyzed genotypes, we noted that ΔpH in *cia2-2* as well as in *cia2-2 cil-1* and
 313 *cia2-2 cil-2* was significantly decreased as compared to that in Col-0 (Figure 6e and Figure S4e),
 314 which correlates well with decreased NPQ in these genotypes. Because the rosette size of *cia2-2 cil-*
 315 *1* was slightly smaller than that of Col-0 (Figure 2d, Figure 6b), we also measured CO_2 assimilation
 316 as a function of light intensity (20–2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and CO_2 concentration (20–1500 ppm) (Figure
 317 6f,g) and observed moderate but statistically significant decrease in CO_2 assimilation in *cia2-2 cil-1*

318 within the tested range of light intensity and CO₂ concentration. Taken together, our results strongly
319 suggest that CIA2 and CIL are required for optimal NPQ and proper formation of trans-thylakoid
320 proton gradient as well as for optimal CO₂ assimilation.

321 Chloroplast translation is attenuated in *cia2 cil*

322 It was shown that CIA2 binds to the promoters of genes encoding the chloroplast ribosomal
323 proteins, and decreased expression of these genes was observed in *cia2-1* mutant (Sun *et al.*, 2009a).
324 To check whether a similar phenomenon was observed in *cia2-2* and if the expression of these genes
325 was dependent on the activity of CIL, we checked the expression of *Rps6*, *Rpl11*, *Rpl18*, and *Rpl28*
326 nuclear genes encoding bS6c, uL11c, uL18c, and bL28c chloroplast ribosomal proteins using qRT-
327 PCR (Figure 7a). The expression of all analyzed genes was decreased in *cia2-2* as compared to that
328 in Col-0 and *cil-1*. The introduction of *cil-1* mutation into *cia2-2* led to stronger reduction in the
329 expression of all genes except *Rpl18*, whose transcript was at the same level as that in *cia2-2* (Figure
330 7a). To gain more comprehensive view of gene expression changes, we performed whole
331 transcriptome sequencing (RNA-seq) for Col-0, *cia2-2*, *cil-1*, and *cia2-2 cil-1* plants. First, we
332 focused on genes encoding for proteins involved in chloroplast translation. Our analysis revealed that
333 the expression of 21 out of 66 chloroplast ribosome genes is inhibited in *cia2-2 cil-1* (Figure 7b)
334 including two genes encoded by the chloroplast genome (i.e., *rpl2* and *rpl32*, encoding the ribosomal
335 proteins uL2c and bL32c). Pentatricopeptide repeat (PPR) proteins are involved in every step of
336 chloroplast gene expression, namely transcription, RNA metabolism, and translation (Barkan and
337 Small 2014). Thus, we checked the expression of chloroplast-targeted PPRs in *cia2-2 cil-1* and found
338 13 down- and 7 upregulated PPR genes (Figure 7b). Further, the expression analysis of other genes
339 possibly involved in plastid translation revealed the presence of both up- and downregulated genes
340 (Figure 7b). Taken together, the transcriptome profiling indicates that the lack of CIA2 and CIL leads
341 to altered expression of multiple genes encoding the components of the chloroplast translation
342 machinery.

343 To further confirm that CIA2 and CIL are required for optimal chloroplast translation, we
344 subjected Col-0 and *cia2-2 cil-1* to polysome loading assays. We examined the ribosomal loading of
345 *psbD* mRNA (encoding the D2 protein of PSII) using sucrose gradient fractionation followed by
346 northern blot analysis (Figure 7c). This analysis showed that *psbD* mRNA was underrepresented in
347 the polysomal fractions in *cia2-2 cil-1* compared to that in Col-0, and the mRNA was shifted toward
348 lighter (monosome) fractions, suggesting that *psbD* mRNA was associated with fewer ribosomes and
349 that its translation was reduced in *cia2-2 cil-1*. Furthermore, as multiple lines of evidence indicate
350 that CIA2 and CIL are involved in chloroplast translation, we also tested the growth of Col-0, *cia2-2*,
351 *cil-1*, and *cia2-2 cil-1* in media supplemented with spectinomycin, which inhibits this process.

352 Spectinomycin binds the 30S subunit of the 70S ribosome and prevents translocation of peptidyl-
353 tRNA from A to P site; consequently, many Arabidopsis translation mutants showed increased
354 susceptibility to this antibiotic (Parker *et al.*, 2014). In agreement with the results of ribosome loading
355 experiments, we observed severe inhibition of pigment accumulation in *cia2-2 cil-1* grown in media
356 supplemented with spectinomycin (Figure 7d), which suggests that CIA2 and CIL play a role in
357 plastid translation.

358 The decreased chlorophyll content in young leaves and inhibited translation in *cia2-2 cil-1* led
359 us to analyze chloroplast ribosomal RNA (rRNA) maturation. In Arabidopsis chloroplasts, all rRNAs
360 are encoded by the polycistronic *rrn* operon (Bollenbach *et al.*, 2007). Upon processing of the initial
361 transcript by distinct endo- and exonucleases, tRNAs, precursors of 16S and 5S rRNAs and bicistronic
362 23S-4.5S intermediate are created (Figure 8a). The 23S-4.5S precursor is cut to produce 4.5S and 23S
363 rRNA fragments. The maturation of 23S rRNA is followed by the introduction of two gaps (“hidden
364 breaks”) producing three distinct parts of 0.4, 1.1, and 1.3 kb; however, the functional relevance of
365 this postmaturation processing is not clear (Bollenbach *et al.*, 2007; Fristedt *et al.*, 2014). The
366 capillary electrophoresis of RNA isolated from young leaves showed that the 23S rRNA 1.1 and 1.3
367 kb species of and the 16S rRNA accumulate to lower levels in *cia2-2* and *cia2-2 cil-1* mutants as
368 compared to that in WT plants (Figure 8b). These results suggest that the expression and/or processing
369 of 16S and 23S rRNAs are dependent on CIA2 and CIL. To further characterize rRNA maturation,
370 we performed northern blot analysis with probes specific to 16S and 23S rRNAs (Figure 8c). We
371 observed only slightly lower level of 16S rRNA in *cia2-2 cil-1* without accumulation of its 1.7 kb
372 precursor suggesting that the processing of the 16S rRNA is not impaired (Figure 8c). In agreement
373 with the results of capillary electrophoresis, we observed decreased accumulation of 0.4, 1.1, 1.3, and
374 1.7 kb species and increased accumulation of 2.4 kb fragment of 23S rRNA in *cia2-2 cil-1* (Figure
375 8c). These results suggest that gap incorporation between 1.1 kb and 1.3 kb fragments is less efficient
376 in *cia2-2 cil-1* mutant. To confirm these results, we performed qRT-PCR analysis with primers
377 spanning hidden breaks (Figure 8d) and obtained confirmatory results.

378 Next, we tested whether the perturbations of chloroplast translation are linked to the decreased
379 NPQ observed in *cia2 cil* (Figures 6a and S5). For this, we measured NPQ in well-characterized
380 chloroplast translation mutants *prpl11-1*, *prps1-1*, *psrp5-R1*, and *rps17-1* (Pesaresi *et al.*, 2001;
381 Romani *et al.*, 2012; Tiller *et al.*, 2012; Tadini *et al.*, 2016) (Figure S5). However, in contrast to *cia2*
382 *cil*, NPQ in these mutants was increased as compared to that in Col-0 plants (Figure S5). These results
383 suggest that the observed decrease in NPQ in *cia2 cil* mutant is not related to inhibited chloroplast
384 translation. Taken together, our results suggest that the translation of chloroplast mRNA and the
385 maturation and accumulation of 23S rRNA are influenced by CIA2 and CIL.

386 Lack of CIA2 and CIL confers heat stress tolerance

387 To our surprise, RNA-seq analysis showed that many genes induced in *cia2-2 cil-1* were
388 annotated as heat shock proteins (HSPs) (Data S2). Further, the gene ontology (GO) enrichment
389 analysis demonstrated that GO terms related to heat response and acclimation were overrepresented
390 among genes induced in *cia2-2 cil-1* (Figure 9a). To confirm the RNA-seq results, we performed
391 qRT-PCR analysis to assess the transcript level of *HEAT SHOCK TRANSCRIPTION FACTOR A2*
392 (*HSFA2*) and *HEAT SHOCK PROTEIN 70-4* (*HSP70-4*) in *cia2-2*, *cia2-3*, *cil-1*, *cia2-2 cil-1*, and
393 corresponding WT plants (Figure 9b). Our results confirmed strong induction of both genes in *cia2*
394 mutants, and even stronger induction was observed in *cia2-2 cil-1* double mutant (Figure 9b). To
395 check whether increased expression of many HSPs can confer thermotolerance, we grew *cia2-2*, *cil-*
396 *1*, *cia2-2 cil-1*, and Col-0 seedlings on Petri dishes and exposed them to heat stress (45 °C) for 20–
397 40 min (Figure 9c,d). Exposure to 45 °C for 30 min resulted in almost complete bleaching and growth
398 inhibition of Col-0 plants (Figure 9c). A similar phenotype was observed in the case of *cia2-2* and
399 *cil-1* mutants; however, *cia2-2 cil-1* plants were affected to a significantly lower extent, and nearly
400 all individuals retained cotyledon and leaf pigmentation (Figure 9c,d). Furthermore, at other tested
401 time points, *cia2-2 cil-1* was more resistant to heat stress than the remaining genotypes (Figure 9c,d).
402 These results suggest that CIA2 and CIL synergistically contribute to the modulation of
403 thermotolerance presumably through the regulation of HSP expression.

404 Discussion

405 In the present study we have utilized a reverse genetic approach to identify regulators of
406 chloroplast retrograde signaling. With a similar aim, Ruckle *et al.*, (2012) utilized the transcriptome
407 profiling data to select the candidate genes, that were later subjected to phenotypical analysis to
408 identify genes potentially involved in plastid-to-nucleus communication. Our selection strategy was
409 specifically targeted at identification of dual-targeted transcription factors, therefore, we focused on
410 computational prediction of chloroplast transit peptides in known transcription factors. The initial
411 selection was followed by screening of a collection of T-DNA mutant lines for a selected set of
412 transcription factors (Data S1) under conditions promoting chloroplast stress (Figure S1).

413 As a result of our screening strategy we focused on CIA2 and found that it has a functional cTP
414 which, in transient expression experiments, targets a fraction of CIA2:YFP fusion protein to
415 chloroplasts of Arabidopsis seedlings and tobacco leaves (Figure 1c, Figure 2c). In agreement with
416 our observation, recently characterized barley CIA2 homolog, *HvCMF7*, was also shown to be
417 localized in chloroplasts (Li *et al.*, 2019). In the first attempt to determine the subcellular localization
418 of CIA2, the GUS:CIA2 fusion protein was found to reside exclusively in the nucleus (Sun *et al.*,
419 2001). We speculate that the discrepancy between our data and the previous work might be related to

the masking of CIA2 cTP by the GUS fused to CIA2 N-terminus, thus inhibiting the import of GUS:CIA2 fusion protein into chloroplasts (Sun *et al.*, 2001). From the results of our complementation test, we were unable to determine the significance of CIA2 chloroplast localization because the expression of both constructs, pCIA2::*CIA2:YFP* and pCIA2::*YFP:CIA2*, restored the accumulation of photosynthetic pigments in *cia2-2* mutant (Figure 2h). Thus, we speculate that at least with regard to the determination of the levels of photosynthetic pigments, the chloroplast localization of CIA2 is not crucial. Interestingly, despite clear complementation effect and restoration of *CIA2* transcript level, we did not observe YFP signal in stable transformants expressing CIA2:YFP fusion either under native or 35S promoter, suggesting that the CIA2 protein level might be subject to strict posttranslational control. Such stringent control of protein level was documented earlier for Golden 2-like (GLK) transcription factors that are involved in expression of nuclear-encoded chloroplast-localized proteins and photosynthesis-related genes in *Zea mays*, *Physcomitrella patens*, and *Arabidopsis thaliana* (Yasumura *et al.*, 2005; Waters *et al.*, 2009). According to the proposed model, damaged chloroplasts, send an unknown signal which activates the ubiquitin-proteasome system leading to GLK1 degradation, thereby optimizing the import of photosynthesis-related proteins into chloroplasts (Tokumaru *et al.*, 2017). A similar scenario i.e. the inability to detect the fluorophore-tagged proteins despite complementation of the mutant phenotype, was observed also for ABI4 (Finkelstein *et al.*, 2011) and RCD1 (Jaspers *et al.*, 2009), suggesting that certain regulatory proteins might be unstable and/or degraded by the proteasome, thus preventing their detection under standard conditions. A comparable phenomenon was observed in the case of another key player of retrograde signaling, GUN1, which accumulates in chloroplasts only at a very early stage of leaf development (Wu *et al.*, 2018). Interestingly, unlike CIA2:YFP, CIL:YFP was observed in nucleolus and nucleoplasm (Figure 3) suggesting that CIA2 and CIL have, at least to some extent, distinct molecular roles in the cell. However, both proteins can be localized in the nucleoplasm suggesting possible involvement in the same cellular process, which could explain observed synergistic effects in the *cia2 cil* double mutant.

Our data show that CIA2 and its close homolog CIL play a relevant function in the chloroplast biogenesis process (Figure 2). The *cia2 cil* double mutant exhibited a pale green phenotype, which was much more pronounced than that observed in single *cia2* mutants, suggesting that both of these proteins contribute to chloroplast biogenesis. Double mutant plants displayed a significantly lower concentration of chlorophyll *a*, chlorophyll *b*, and carotenoids (Figure 2 and Figure S2), suggesting impairment in the conversion of light into chemical energy. A similar phenotype was observed in plants lacking the activity of GLK transcription factors. Like *cia2 cil*, the *glk1 glk2* double mutants were pale green and deficient in the formation of the photosynthetic apparatus (Waters *et al.*, 2009). Our RNA-seq and qRT-PCR-based expression data revealed a significant transcriptional

455 downregulation of chloroplast ribosome genes in *cia2 cil* plants. Almost one-third of 66 chloroplast
456 ribosome protein genes were significantly repressed in *cia2-2 cil-1*. These data are consistent with
457 previous reports showing that CIA2 binds to the promoters of genes encoding chloroplast ribosomal
458 proteins (Sun *et al.*, 2009a) and support a positive regulatory role of CIA2 in chloroplast translation.
459 Chloroplast ribosomal proteins are encoded by both nuclear and chloroplast genomes (Zoschke and
460 Bock, 2018). Interestingly, the majority of ribosomal genes downregulated in *cia2 cil* are nuclear-
461 encoded. This, together with partial chloroplast localization of CIA2 (Figure 1), suggests that CIA2
462 might act as chloroplast sensor of the environmental stimuli and mediates chloroplast-dependent
463 adaptive responses (Estavillo *et al.*, 2013). On the other hand, the phenotypic similarities between
464 *cia2 cil*, *glk1 glk2* double mutant and *albostrians* appear to be related to the inhibition of chloroplast
465 translation which suggests that in addition to operational signaling in response to environmental
466 perturbations (i.e. HL and UV-AB) CIA2 might be involved in biogenic signaling. Similarly, *whirly1*
467 mutants show several chloroplast phenotypes including the lack of chloroplast ribosomes and reduced
468 compactness of nucleoids in chloroplasts (Prikryl *et al.*, 2008; Krupinska *et al.*, 2014). The
469 WHIRLY1 was also suggested to be involved in chloroplast to nucleus retrograde signalling (Foyer
470 *et al.*, 2014). Future research to determine the functional significance of the chloroplast localization
471 of CIA2, and its potential link with established retrograde signalling pathways is required to precisely
472 determine the function of CIA2.

473 More than 95% of the chloroplast proteins are nuclear-encoded (Jarvis and Robinson, 2004)
474 and transported across the double chloroplast membrane due to the activity of a specialized translocon
475 complex (Nakai, 2015; Paila *et al.*, 2015). This mechanism is essential for chloroplast biogenesis and
476 requires coordinated action of multiple proteins. Previous reports revealed a positive regulatory effect
477 of CIA2 on the transcription of the translocon genes *Toc33* and *Toc75* in leaves (Sun *et al.*, 2001;
478 Sun *et al.*, 2009a), suggesting the role of CIA2 in protein import into the chloroplast. Inefficient
479 chloroplast protein import causes cytosolic overaccumulation of preproteins, which results in the
480 activation of chaperones such as HSP70 and HSP90, which were recently postulated to be key
481 components of the chloroplast retrograde signaling pathway (Wu *et al.*, 2019). Accordingly, we
482 observed strong transcriptional induction of cytosolic chaperones and increased thermotolerance in
483 *cia2 cil* mutant (Figure 9). On the other hand, it was demonstrated that thermotolerance is regulated
484 by chloroplast signals that depend on the redox state of the PQ pool or hydrogen peroxide produced
485 in chloroplasts (Dickinson *et al.*, 2018). The redox state of the PQ pool and H₂O₂ are also involved
486 in high-light acclimatory responses (Karpiński *et al.*, 1999; Mühlenbock *et al.*, 2008; Gilroy *et al.*,
487 2016). Experimentally, it is almost impossible to separate foliar heat shock from high-light responses,
488 because exposure to high light for a few seconds significantly warms up *Arabidopsis* leaves due to
489 the dissipation of energy as heat (Kulasek *et al.*, 2016). Therefore, it is interesting that CIA2 and CIL

490 antagonistically influence high-light and UV-B acclimation *versus* thermotolerance in ambient light.
491 Abolished expression of *CIA2* and *CIL* genes in double mutant causes growth reduction, impaired
492 acclimation to high light and UV-AB stresses, and photosynthesis dysfunction, while it can cause
493 thermotolerance expressed as seedling survival rate at high temperatures. However, we were not able
494 to identify the precise role of *CIA2* and *CIL* in the antagonistic regulation of these processes, and we
495 plan to perform field experiments similar to those we did for cell death conditional regulators (i.e.
496 LSD1, EDS1, PAD4) (Wituszyńska *et al.*, 2013). These proteins, depending on growing conditions,
497 differently regulate chloroplast retrograde cell death signaling for growth, photosynthesis, high-light
498 and UV-B acclimation, water use efficiency, and seed yield (Wituszyńska *et al.*, 2013; Wituszyńska
499 *et al.*, 2015; Bernacki *et al.*, 2019).

500 Finally, our data indicate that *CIA2* and *CIL* influence chloroplast translation by the regulation
501 of ribosome assembly and maturation as *cia2 cil* double mutants displayed a disturbed accumulation
502 of 23S rRNA (Figure 8). In plant chloroplasts, 23S rRNA constitutes a component of a large (50S)
503 subunit of chloroplast ribosomes. The rRNA undergoes postmaturation processing, which includes a
504 site-specific cleavage that generates gapped, discontinuous rRNA molecules (Nishimura *et al.*, 2010).
505 The maturation process is followed by the removal of a specific region and introduction of a gap (the
506 so-called hidden break) into the 23S rRNA (Bollenbach *et al.*, 2005). This molecule is split into four
507 major fragments of 1.7, 1.3, 1.1, and 0.4 kb. Chloroplasts of all dicotyledonous plants have hidden
508 breaks at similar locations. Several proteins have been reported to bind to a 23S rRNA segment close
509 to the hidden break sites (Bieri *et al.*, 2017). We observed an increased accumulation of the 2.4 kb
510 23S rRNA species and a reduced occurrence of the hidden break in this species (Figure 8c,d).
511 Interestingly, the expression of the two genes *Rpl19.1* and *Rpl19.2* (Figure 6b), which encode the
512 ribosomal protein bL19c binding close to the hidden break (Bieri *et al.*, 2017), was reduced. This
513 indicates that the lack of bL19c could influence the rRNA structure in a way that makes it less
514 susceptible to the hidden break incorporation. The lack of bL19c could be one of the causes of the
515 impaired biogenesis of the 50S subunit as shown by the reduced accumulation of the 23S rRNA
516 (Figure 8c,d). The deficiencies in rRNA maturation observed in *cia2-2 cil-1* double mutant suggest
517 that *CIA* and *CIL* might play a role in this process, either directly by binding and processing 23S
518 rRNA precursor or more likely indirectly by regulating the expression of ribosomal proteins.

519 In conclusion, the presented results demonstrate high complexity and elegance of chloroplast
520 retrograde signaling and anterograde nuclear responses that depend on TFs such as *CIA2* and *CIL*.
521 This complexity is not only observed on the molecular and biochemical levels but also on the
522 physiological level of acclimatory responses as well as on the regulation of chloroplast biogenesis
523 and plant growth and development. To better understand the interdependence of these regulatory
524 processes, further interdisciplinary studies on the function of these proteins are needed.

525 Experimental procedures

526 Plant material and growth conditions

527 *Arabidopsis thaliana* ecotypes Columbia-0 (Col-0), Columbia-4 (Col-4), and Landsberg erecta
528 (Ler-0) were used as controls for analyzed mutants. *cia2-2* (SALK_004037), *cia2-3* (SGT49), *cia2-4*
529 4 (SALK_045340), *cil-1* (SAIL_228_C01), and *cil-2* (SK14786) were ordered from the Nottingham
530 Arabidopsis Stock Centre (NASC) and genotyped using primers shown in Table S2. The *cia2-2 cil-1*
531 and *cia2-2 cil-2* double mutants were generated by crossing. Plants were grown on Jiffy pots (Jiffy
532 Products) for 3–4 weeks in a growing chamber in long-day photoperiod (16 h/8 h) under constant
533 white light of 140–160 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at 22 °C.

534 Databases

535 In this work, we used information extracted from proteome databases to identify chloroplast
536 proteins [Plant Proteomics Database at Cornell (<http://ppdb.tc.cornell.edu/>) (Sun *et al.*, 2009b); The
537 Chloroplast Function Database II (<http://range-v2.psc.riken.jp/chloroplast/>) (Myouga *et al.*, 2013),
538 and ARAMEMNON (<http://aramemnon.uni-koeln.de>) (Schwacke *et al.*, 2003)] and TF databases to
539 look for annotated TFs in Arabidopsis [The Plant Transcription Factor Database
540 (<http://plntfdb.bio.uni-potsdam.de>) (Pérez-Rodríguez *et al.*, 2009) and PlantTFDB
541 (<http://planttfdb.cbi.pku.edu.cn/>) (Jin *et al.*, 2014)].

542 Vector constructions and Arabidopsis transformation

543 *CIA2* and *CIL* full CDSs were amplified with primers listed in Table S2 by PCR using cDNA
544 from Col-0 plants. PCR products of expected length were purified from agarose gel and inserted into
545 the entry clone using the pENTR/D-TOPO cloning kit (Invitrogen). DNA fragments encoding for N-
546 terminal 100 aa as well as predicted cTP were amplified from plasmids carrying *CIA2* and *CIL* CDSs.
547 Next, they were subcloned into pGWB641 vector (Nakagawa *et al.*, 2007) using Gateway® LR
548 Clonase™ II enzyme mix (Invitrogen). These constructs were introduced into *Agrobacterium fabrum*
549 (GV3101). The vector was transformed into Col-0 and/or *cia2-2* mutant by the floral dip method.
550 The T3 generation of homozygous transgenic plants was obtained and used for further experiments.
551 The complementation lines were obtained in a similar manner, but in the final step, the promoter,
552 CDS and YFP sequences were cloned into the pK7m34GW binary vector and transformed into
553 *Agrobacterium*.

554 In parallel, whole *CIA2* and the *CIA2* fragment encoding for the 100 aa N-terminal part of *CIA2*
555 were amplified from cDNA using USER compatible (Nour-Eldin *et al.*, 2006) primers and the

improved Pfu X7 polymerase (Nørholm, 2010). PCR fragments were cloned into the pLIFE001 vector (Silvestro *et al.*, 2013) and introduced into *Agrobacterium* (GV3101) for generation of stably transformed line expressing p35S::CIA¹⁻¹⁰⁰:YFP construct.

Chlorophyll *a* fluorescence, CO₂ assimilation, and ΔpH measurements

Chlorophyll *a* fluorescence parameters were measured in dark-acclimated (30 min) plants using PAM FluorCam 800 MF PSI device (Brno, Czech Republic) as described earlier (Gawronski *et al.*, 2014). CO₂ assimilation was measured as described earlier (Burdiak *et al.*, 2015). The electrochromic pigment shift (ECS) was measured using DUAL-PAM 100 equipped with P515/535 module (Walz), which allows the simultaneous measurement of the dual beam 550–515 nm signal difference (Schreiber and Klughammer, 2008). Before measurement, the plants were dark acclimated for 30 min and subsequently illuminated with red actinic light of 160 μmol photons m⁻² s⁻¹ for 20 min. At the beginning of each measurement, the actinic light was turned off for 23 s, and three single turnover (ST) flashes were applied. Next, the red actinic light (160 μmol photons m⁻² s⁻¹) was turned on, and after 4 min, it was again turned off to determine total ECS, ΔpH, and ΔΨ as described previously (Herdean *et al.*, 2016). Immediately after the first measurement, the ECS signal was recorded using the same protocol but at a higher actinic light intensity (660 μmol photons m⁻² s⁻¹). The ECS signal was normalized to the highest ST peak recorded at the beginning of each measurement.

Photosynthetic pigment analysis

For pigment analysis, approximately 15–20 mg of tissue was collected from the sixth leaf of 3–4-week-old plants. Tissue was immediately frozen in liquid nitrogen and ground in the presence of methanol. Absorbance was measured using Multiskan GO (Thermo Fisher Scientific) spectrophotometer after the clarification of the supernatant by centrifugation. Photosynthetic pigment concentrations were calculated as reported previously (Sumanta *et al.*, 2014).

Stress treatments and ion leakage

High-light stress was applied to whole plants using blue light (455 nm) of 1100 μmol photons m⁻² s⁻¹ for the indicated time. As a light source, SL3500-B-D LED array was used (PSI, Brno, Czech Republic). After illumination, the plants were dark acclimated for 20 min, and chlorophyll *a* fluorescence was measured as described above. For recovery experiments, we used blue light of 2500 photons m⁻² s⁻¹ for 2.5 h. After this treatment, the plants were placed in the growing chamber for an indicated period of time followed by 20 min dark acclimation and chlorophyll *a* fluorescence measurement. For UV-AB stress, UVC 500 Crosslinker (Hoefer Pharmacia Biotech, USA) equipped with UV-A (TL8WBLB, Philips) and UV-B lamps (G8T5E, Sankyo Denki) was employed. The

588 plants were exposed to single UV-AB episode at a dose of 800 mJ cm⁻², and chlorophyll a
589 fluorescence was analyzed 24 and 48 h after stress. Ion leakage was also used to analyze cell death
590 48 h after UV-AB stress as described before (Burdiak *et al.*, 2015) with the following modification:
591 total ion leakage was evaluated after freezing at -80 °C overnight.

592 Herbicide and antibiotic treatments

593 Leaf disks cut from 4-to 5-week-old plants were treated with 62.5 µM of 3-(3,4
594 dichlorophenyl)-1,1-dimethylurea (DCMU) and 5 µM of N,N'-dimethyl-4,4'-bipyridinium dichloride
595 (methyl viologen, MV) with 0.05% Tween-20 and were kept in the growing chamber under ambient
596 light between measurements. The maximum efficiency of PSII (F_v'/F_m') of leaf disks was measured
597 using FluorCam 800MF (Photon Systems Instruments, Czech Republic). As a control, we used leaf
598 disks incubated in identical conditions without herbicides.

599 For spectinomycin-resistance experiments, seeds were surface sterilized in 50 % (v/v)
600 commercial bleach, washed five times with sterile water and stratified for 2-4 days at 4 °C in 0.1 %
601 (w/v) agarose solution. Next, seeds were sowed onto ½ MS media supplemented with 1 % sucrose
602 and 1.25 mg/L spectinomycin (Duchefa, S0188.0025) and grown for two weeks in standard
603 conditions.

604 RNA isolation and transcriptome profiling

605 For RNA isolation, only young (not fully developed) leaves were used. In the middle of the
606 photoperiod, the leaves were detached and frozen in liquid nitrogen. For HL treatment, whole rosettes
607 were illuminated with blue light (length 1100 µmol photons m⁻² s⁻¹) for 1 h. Immediately following
608 the treatment, young leaves were detached and frozen in liquid nitrogen. Leaf samples represent three
609 biological replicates, and each contained leaves from at least three individual plants. Frozen leaves
610 were ground using mortar and pestle in liquid nitrogen, and RNA was isolated using Spectrum™
611 Plant Total RNA Kit (Sigma). The quality and quantity of total RNA were evaluated using
612 Experion™ StdSens RNA Kit (Bio-Rad), and the samples with RQI value higher than 9.0 were used
613 for RNA-seq library construction using TruSeq RNA Sample Prep Kit v2 (Illumina). RNA-seq
614 libraries were sequenced in 100 bp paired-end reads using Illumina HiSeq 4000. RNA-seq library
615 preparation and sequencing were conducted by Macrogen (Seoul, Korea). Reads were pseudomapped
616 to *Arabidopsis thaliana* cDNAs (Ensembl, TAIR 10, release 35) using Salmon software (Patro *et al.*,
617 2017). Transcript-level abundances were imported into R using tximport (Soneson *et al.*, 2016) and
618 analyzed using DESeq2 package (Love *et al.*, 2014). Significantly enriched GO terms were identified
619 in up-/downregulated gene sets using PANTHER Classification System at <http://geneontology.org/>.

620 Availability of supporting data

621 RNA-seq data are provided at (uploaded upon manuscript acceptance).

622 Quantitative real-time PCR

623 RNA for qRT-PCR was isolated as described above. RNA was treated with DNase (TURBO
624 DNA-freeTM Kit, Thermo Fisher Scientific) to remove residual DNA. Next, cDNA was synthesized
625 from 1 µg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).
626 PCRs were run on a 7500 Fast Real-Time PCR System (Applied Biosystems) using PerfeCTaTM
627 SYBR® Green FastMixTM, Low ROXTM (Quanta BioSciences) according to the manufacturer's
628 instructions. All primers used in qRT-PCR are listed in Table S2. The amplification efficiency of
629 primers was calculated on the basis of the amplification curve using LinRegPCR software (Ramakers
630 *et al.*, 2003). Relative expression was calculated using EasyqpcR package
631 (<http://www.bioconductor.org/packages/release/bioc/html/EasyqpcR.html>) with *PP2AA3* and
632 *TIP41L* as reference genes.

633 Northern blot and polysome analysis

634 Northern blot analysis and polysome analysis were done as described previously (Fristedt *et*
635 *al.*, 2014) but using Hybond-N membranes (GE Healthcare Life Sciences). Probes were amplified
636 from total plant DNA using gene-specific primers (Table S2), radioactively labeled using the
637 Megaprime DNA Labeling System (GE Healthcare Life Sciences), and hybridized at 65 °C.

638 Protein fractionation and Western blot

639 Chloroplasts were isolated from Col-0 and stable p35S::CIA¹⁻¹⁰⁰:YFP *Arabidopsis thaliana* plants by
640 differential centrifugation on 40% Percoll layer following published protocol (Klinkenberg 2014,
641 Klinkenberg *et al.*, 2014). Briefly, total protein extracts were obtained by grinding 100 mg of plant
642 tissue in 400 µl of PBS. Protein concentration in samples were quantified using Bradford Assay
643 (Sigma-Aldrich). Samples were mixed with 4x Laemmli Buffer (Bio-Rad) and denatured in 95°C
644 for 10 mins prior loading onto gel. Samples were separated by SDS-PAGE on 10% polyacrylamide
645 gel, each well was loaded with 20 µg of proteins. After electrophoresis samples were transferred from
646 gel to Immobilon P PVDF membrane (Merck) by semi-dry transfer. Next, 1:10 000 Dilution of
647 primary anti-GFP (G1544, Sigma-Aldrich), anti-RbcL (AS03 037, Agrisera), anti-D1 (AS05 084,
648 Agrisera) and secondary anti-rabbit antibodies (Thermo Fisher Scientific) were used. Signal from
649 chemiluminescence was detected using Pierce ECL Plus Western Blotting Substrate (Thermo Fisher
650 Scientific) and ChemiDoc Imaging Systems (Bio-Rad). To confirm that wells were loaded with same

651 amount of protein analogical gel were stained with QC Colloidal Coomassie Stain (Bio-Rad) and
652 PVDF membrane after transfer were stained with Ponceu S (Sigma-AldrichS) before blocking.

653 Transient expression in Arabidopsis seedlings and tobacco leaves

654 For transient expression CIA:YFP, CIA¹⁻¹⁰⁰:YFP, CIL:YFP or CIL¹⁻¹⁰⁰:YFP fusion proteins
655 in Arabidopsis, 4-days-old Arabidopsis seedlings were infiltrated using Agrobacterium (GV3101)
656 strain carrying appropriate constructs using the FAST method (Li *et al.*, 2009a).

657 For expression in tobacco leaves 4- to 5-week old plants were infiltrated with Agrobacterium
658 strain GV3101 carrying appropriate constructs. Suspensions for infiltration were prepared in
659 infiltration buffer (10 mM MgCl₂, 10 mM MES (pH 6.5), 100 μ M acetosyringone) from overnight
660 cultures supplemented with appropriate antibiotics and consisted of mixture of 19K strain for RNA
661 silencing suppression (Te *et al.*, 2005) at the final OD₆₀₀ = 0.2 and analysed strain with CIA:YFP,
662 CIA¹⁻¹⁰⁰:YFP, CIL:YFP or CIL¹⁻¹⁰⁰:YFP overexpression at the final OD₆₀₀ = 0.8. After infiltration
663 plants were kept in low light.

664 Confocal microscopy

665 Sections from central regions next to the mid-vein of fully expanded tobacco leaves were taken
666 for analyses 3 days after agro-infiltration. Observations were carried out using confocal laser scanning
667 inverted microscope Nikon TE2000E EZ-C1 equipped with 60x and 100x Plan-Apochromat oil-
668 immersion objectives. Fluorescence was excited with the 488-nm argon ion laser. Signals were
669 detected using the 515/30 and 610LP emission filters for YFP and chlorophyll autofluorescence,
670 respectively.

671 For Arabidopsis transformations, cotyledons were taken for analysis 3 days after infiltration.
672 Confocal microscopy observations were performed using Zeiss LSM700 microscope equipped with
673 20x and 40x EC Plan-Neofluar objectives. YFP and chlorophyll fluorescence was excited using 488
674 nm laser. Signals were detected using 507-561BP (YFP) and 652-682BP (chlorophyll) filters with
675 beam splitter set at 601 nm.

676 Thermotolerance tests

677 Arabidopsis seeds of WT, *cia2-2*, *cil-1*, and *cia2-2 cil-1* plants were surface sterilized by
678 soaking in a 50% (v/v) commercial bleach solution for 7 min. The seeds were then rinsed five times
679 with sterile water and resuspended in 1 ml of 0.1% (w/v) agarose solution. The seeds were stratified
680 for 2 days at 4 °C. They were then placed evenly onto plates with 35 ml of ½ MS medium with agar
681 at pH 5.8. Each plate was divided into four equal parts, one part per genotype, and approximately 25

seeds of every genotype were sown on a single plate. The plates were parafilm sealed to prevent dehydration of growing plants. The seeds were germinated and cultivated for 7 days under standard growth conditions.

After 7 days of *in vitro* cultivation, the seedlings were subjected to thermotolerance assay according to a previously described procedure (Silva-Correia *et al.*, 2014). Heat treatment was performed under ambient laboratory light after 3 h of light photoperiod. Parafilm-sealed plates with grown seedlings were set in a water bath preheated to 45 °C for 20, 30, or 40 min. After the treatment, the plates were transferred again to the growth chamber where the plants were cultivated for subsequent 7 days.

The survival rate of seedlings of individual genotypes was evaluated from the heat-treated plants' ability to produce new green leaves 7 days after the treatment. The plants were imaged, and the proportion of seedlings survived after heat treatment and all germinated plants was calculated for each genotype at every timepoint.

Accession numbers

The following gene names are used in Figure 7: *Rps6* (AT1G64510), *Rpl11* (AT1G32990), *Rpl18* (AT1G48350), *Rpl28* (AT2G33450), *Rpl3* (AT2G43030), *Rpl21* (AT1G35680), *PSRP2* (AT3G52150), *Rpl9* (AT3G44890), *Rps17* (AT1G79850), *Rps5* (AT2G33800), *Rps10* (AT3G13120), *Rpl17* (AT3G54210), *Rpl13* (AT1G78630), *PSRP4* (AT2G38140), *Rps9* (AT1G74970), *Rps21* (AT3G27160), *rpl2* (ATCG01310 and ATCG00830), *Rps20* (AT3G15190), *Rpl19.1* (AT4G17560), *Rpl19.2* (AT5G47190), *Rpl29* (AT5G65220), *rpl32* (ATCG01020), *DG1* (AT5G67570), *PPR2* (AT3G06430), *SVR7* (AT4G16390), *PPR5* (AT4G39620), *EMB2279* (AT1G30610), *PDM1/SEL1* (AT4G18520), *PPR596* (AT1G80270), *HCF152* (AT3G09650), *PSRP1* (AT5G24490), *PBR1* (AT1G71720), *IF3-4* (AT4G30690), *IF3-2* (AT2G24060), *cpHSC70-2* (AT5G49910), and *SVR3* (AT5G13650).

Acknowledgments

We thank Prof. Ralph Bock for *psrp5-R1* and *rps17-1* seeds. We thank Dr. Peter Robert Kindgren for help with *PPR* gene analysis and Dr. Anna Kozłowska-Makulska for technical help during the cHL screening. P.G., P.B., J.M., M.G., M.Z., and S.K. acknowledge financial support from the Polish National Science Center (Narodowe Centrum Nauki; OPUS6, UMO-2013/11/B/NZ3/00973, MAESTRO6 UMO-2014/14/A/NZ1/00218 given to S.K. and SONATA14 UMO-2018/31/D/NZ3/03296 given to M.G.). L.B.S. acknowledges financial support from the Independent Research Fund Denmark (Danmarks Frie Forskningsfond; 7014-00322B). C.W. acknowledges financial support from the Academy of Finland (Decision 294580), D.L. is grateful for support from the German Science Foundation (DFG, grant TR175 C05).

716 **Short legends for Supporting Information**

717 **Table S1.** List of genes selected from targeted reverse genetic screen for subcellular localization
718 experiments.

719 **Table S2.** List of primers used in this study.

720 **Figure S1.** Examples of results obtained from the initial reverse genetic screening in UV-AB
721 and high-light experiments.

722 **Figure S2.** Isolation and characterization of *cia2-2 cil-2* double mutant.

723 **Figure S3.** High-light (HL) and UV-AB susceptibility of *cia2-2 cil-2*.

724 **Figure S4.** CIA2 and CIL are required for optimal photosynthesis in Arabidopsis.

725 **Figure S5.** Analysis of rRNA and NPQ in plastid translation mutants.

726 **Data S1.** List of T-DNA mutants used in targeted reverse genetic screen.

727 **Data S2.** List of genes differentially expressed in *cia2-2 cil-1* compared to Col-0.

728 **Conflict of interest**

729 No conflicts of interest.

730 **Author contributions**

731 P.G., C.W., and S.K. formulated the hypothesis and conceived the research plan. P.G., P.B.,
732 L.B.S., M.Z., M.G. and J.M. performed the experiments; P.G., P.B., and L.B.S. analyzed the data;
733 P.G., C.W., D.L. and S.K. supervised the analysis; and P.G., P.B., C.W., L.B.S., and S.K. wrote the
734 article, with contributions from all authors.

735 **References**

- 736 **Barajas-López, J. de D., Blanco, N.E. and Strand, Å.** (2013) Plastid-to-nucleus communication,
737 signals controlling the running of the plant cell. *Biochim. Biophys. Acta*, **1833**, 425–437.
- 738 **Barkan, A. and Small, I.** (2014) Pentatricopeptide repeat proteins in plants. *Annu. Rev. Plant Biol.*,
739 **65**, 415–442.
- 740 **Bernacki, M.J., Czarnocka, W., Szechyńska-Hebda, M., Mittler, R. and Karpiński, S.** (2019)
741 Biotechnological potential of LSD1, EDS1, and PAD4 in the improvement of crops and
742 industrial plants. *Plants*, **8**, 290.
- 743 **Białasek, M., Górecka, M., Mittler, R. and Karpiński, S.** (2017) Evidence for the involvement of
744 electrical, calcium and ROS signaling in the systemic regulation of non-photochemical
745 quenching and photosynthesis. *Plant Cell Physiol.*, **58**, 207–215.

746 **Bieri, P., Leibundgut, M., Saurer, M., Boehringer, D. and Ban, N.** (2017) The complete
747 structure of the chloroplast 70S ribosome in complex with translation factor pY. *EMBO J.*, **36**,
748 475–486.

749 **Bollenbach, T.J., Lange, H., Gutierrez, R., Erhardt, M., Stern, D.B. and Gagliardi, D.** (2005)
750 RNR1, a 3'-5' exoribonuclease belonging to the RNR superfamily, catalyzes 3' maturation of
751 chloroplast ribosomal RNAs in *Arabidopsis thaliana*. *Nucleic Acids Res.*, **33**, 2751–2763.

752 **Bollenbach, T.J., Schuster, G., Portnoy, V. and Stern, D.B.** (2007) Processing, degradation, and
753 polyadenylation of chloroplast transcripts. *Topics in Current Genetics*. pp. 175–211.

754 **Börner T.** (2017) The discovery of plastid-to-nucleus retrograde signaling—a personal perspective.
755 *Protoplasma*, **254**, 1845–1855.

756 **Bradbeer, J.W., Atkinson, Y.E., Borner, T., and Hagemann, R.** (1979). Cytoplasmic synthesis
757 of plastid polypeptides may be controlled by plastid-synthesised RNA. *Nature* 279, 816–817.

758 **Burdiak, P., Rusaczonek, A., Witoń, D., Glów, D. and Karpiński, S.** (2015) Cysteine-rich
759 receptor-like kinase CRK5 as a regulator of growth, development, and ultraviolet radiation
760 responses in *Arabidopsis thaliana*. *J. Exp. Bot.*, **66**, 3325–3337.

761 **Caldwell, C.R.** (1993) Ultraviolet-induced photodegradation of cucumber (*Cucumis sativus* L.)
762 microsomal and soluble protein tryptophanyl residues *in vitro*. *Plant Physiol.*, **101**, 947–953.

763 **Caplan, J.L., Kumar, A.S., Park, E., Padmanabhan, M.S., Hoban, K., Modla, S., Czymmek,**
764 **K. and Dinesh-Kumar, S.P.** (2015) Chloroplast stromules function during innate immunity.
765 *Dev. Cell*, **34**, 45–57.

766 **Casati, P. and Walbot, V.** (2004) Crosslinking of ribosomal proteins to RNA in maize ribosomes
767 by UV-B and its effects on translation. *Plant Physiol.*, **136**, 3319–3332.

768 **Chan, K.X., Mabbitt, P.D., Phua, S.Y., et al.** (2016) Sensing and signaling of oxidative stress in
769 chloroplasts by inactivation of the SAL1 phosphoadenosine phosphatase. *Proc. Natl Acad. Sci.*
770 *USA*, **113**, E4567–E4576.

771 **Chi, W., Sun, X. and Zhang, L.** (2013) Intracellular signaling from plastid to nucleus. *Annu. Rev.*
772 *Plant Biol.*, **64**, 559–582.

773 **Chotewutmontri, P. and Barkan, A.** (2018) Multilevel effects of light on ribosome dynamics in
774 chloroplasts program genome-wide and *psbA*-specific changes in translation. *PLOS Genet.*, **14**,
775 e1007555.

776 **Clercq, I. De, Vermeirssen, V., Aken, O. Van, et al.** (2013) The membrane-bound NAC
777 transcription factor ANAC013 functions in mitochondrial retrograde regulation of the
778 oxidative stress response in *Arabidopsis*. *Plant Cell*, **25**, 3472–3490.

779 **Daniell, H., Lin, C.-S., Yu, M. and Chang, W.-J.** (2016) Chloroplast genomes: diversity,
780 evolution, and applications in genetic engineering. *Genome Biol.*, **17**, 134.

781 **Dickinson, P.J., Kumar, M., Martinho, C., et al.** (2018) Chloroplast signaling gates
782 thermotolerance in *Arabidopsis*. *Cell Rep.*, **22**, 1657–1665.

783 **Distelbarth, H., Nägele, T. and Heyer, A.G.** (2013) Responses of antioxidant enzymes to cold and
784 high light are not correlated to freezing tolerance in natural accessions of *Arabidopsis thaliana*.
785 *Plant Biol.*, **15**, 982–990.

786 **Dogra, V., Li, M., Singh, S., Li, M. and Kim, C.** (2019) Oxidative post-translational modification
787 of EXECUTER1 is required for singlet oxygen sensing in plastids. *Nat. Commun.*, **10**, 2834.

788 **Eberhard, S., Drapier, D. and Wollman, F.A.** (2002) Searching limiting steps in the expression
789 of chloroplast-encoded proteins: Relations between gene copy number, transcription, transcript
790 abundance and translation rate in the chloroplast of *Chlamydomonas reinhardtii*. *Plant J.*, **31**,
791 149–160.

792 **Estavillo, G.M., Chan, K.X., Phua, S.Y. and Pogson, B.J.** (2013) Reconsidering the nature and
793 mode of action of metabolite retrograde signals from the chloroplast. *Front. Plant Sci.*, **3**, 1–9.

794 **Estavillo, G.M., Crisp, P. a., Pornsiriwong, W., et al.** (2011) Evidence for a SAL1-PAP
795 chloroplast retrograde pathway that functions in drought and high light signaling in
796 *Arabidopsis*. *Plant Cell*, **23**, 3992–4012.

797 **Exposito-Rodriguez, M., Laissue, P.P., Yvon-Durocher, G., Smirnoff, N. and Mullineaux,**
798 **P.M.** (2017) Photosynthesis-dependent H₂O₂ transfer from chloroplasts to nuclei provides a
799 high-light signalling mechanism. *Nat. Commun.*, **8**, 49.

800 **Ferreira, M.L.F., Pezza, A., Biarc, J., Burlingame, A.L. and Casati, P.** (2010) Plant L10
801 ribosomal proteins have different roles during development and translation under ultraviolet-B
802 stress. *Plant Physiol.*, **153**, 1878–1894.

803 **Finkelstein, R., Lynch, T., Reeves, W., Petitfils, M. and Mostachetti, M.** (2011) Accumulation
804 of the transcription factor ABA-insensitive (ABI)4 is tightly regulated post-transcriptionally. *J.*
805 *Exp. Bot.*, **62**, 3971–3979.

806 **Foyer, C.H., Karpinska, B. and Krupinska, K.** (2014) The functions of WHIRLY1 and REDOX-
807 RESPONSIVE TRANSCRIPTION FACTOR 1 in cross tolerance responses in plants: a
808 hypothesis. *Philos Trans R Soc Lond B Biol Sci* **369**, 20130226.

809 **Fristedt, R., Scharff, L.B., Clarke, C. a, Wang, Q., Lin, C., Merchant, S.S. and Bock, R.** (2014)
810 RBF1, a plant homolog of the bacterial ribosome-binding factor RbfA, acts in processing of
811 the chloroplast 16S ribosomal RNA. *Plant Physiol.*, **164**, 201–15.

812 **Gawronski, P., Witon, D., Vashutina, K., Bederska, M., Betlinski, B., Rusaczek, A. and**
813 **Karpinski, S.** (2014) Mitogen-activated protein kinase 4 is a salicylic acid-independent
814 regulator of growth but not of photosynthesis in *Arabidopsis*. *Mol. Plant*, **7**, 1151–1166.

815 **Gilroy, S., Bialasek, M., Suzuki, N., Górecka, M., Devireddy, A.R., Karpiński, S. and Mittler,**
816 **R.** (2016) ROS, calcium, and electric signals: Key mediators of rapid systemic signaling in
817 plants. *Plant Physiol.*, **171**, 1606–1615.

818 **Grabowski, E., Miao, Y., Mulisch, M. and Krupinska, K.** (2008) Single-stranded DNA-binding
819 protein Whirly1 in barley leaves is located in plastids and the nucleus of the same cell. *Plant*
820 *Physiol.*, **147**, 1800–1804.

821 **Guo, H., Feng, P., Chi, W., et al.** (2016) Plastid-nucleus communication involves calcium-
822 modulated MAPK signalling. *Nat. Commun.*, **7**, 1–15.

823 **Herdean, A., Teardo, E., Nilsson, A.K., et al.** (2016) A voltage-dependent chloride channel fine-
824 tunes photosynthesis in plants. *Nat. Commun.*, **7**, 11654.

825 **Hess, W.R., Müller, A., Nagy, F., and Börner, T.** (1994). Ribosome- deficient plastids affect
826 transcription of light induced nuclear genes: Genetic evidence for a plastid derived signal. *Mol.*
827 *Gen. Genet.* **242**, 505–512

828 **Hollósy, F.** (2002) Effects of ultraviolet radiation on plant cells. *Micron*, **33**, 179–197.

829 **Huang, D., Lan, W., Li, D., Deng, B., Lin, W., Ren, Y. and Miao, Y.** (2018) WHIRLY1
830 occupancy affects histone lysine modification and *WRKY53* transcription in *Arabidopsis*
831 developmental manner. *Front Plant Sci.* **19**, 1503.

832 **Jarvis, P.** (2008) Targeting of nucleus-encoded proteins to chloroplasts in plants. *New Phytol.*, **179**,
833 257–285.

834 **Jarvis, P. and Robinson, C.** (2004) Mechanisms of protein import and routing in chloroplasts.
835 *Curr. Biol.*, **14**, 1064–1077.

836 **Jaspers, P., Blomster, T., Brosché, M., et al.** (2009) Unequally redundant RCD1 and SRO1
837 mediate stress and developmental responses and interact with transcription factors. *Plant J.*,
838 **60**, 268–279.

839 **Jin, J., Zhang, H., Kong, L., Gao, G. and Luo, J.** (2014) PlantTFDB 3.0: A portal for the
840 functional and evolutionary study of plant transcription factors. *Nucleic Acids Res.*, **42**, 1182–
841 1187.

842 **Kacprzak, S.M., Mochizuki, N., Naranjo, B., Xu, D., Leister, D., Kleine, T., Okamoto, H. and**
843 **Terry, M.J.** (2019) Plastid-to-nucleus retrograde signalling during chloroplast biogenesis does
844 not require ABI4. *Plant Physiol.*, **179**, 18–23.

845 **Karpiński, S., Escobar, C., Karpinska, B., Creissen, G. and Mullineaux, P.M.** (1997)
846 Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase
847 genes in *Arabidopsis* during excess light stress. *Plant Cell*, **9**, 627–640.

848 **Karpiński, S., Reynolds, H., Karpinska, B., Wingsle, G., Creissen, G. and Mullineaux, P.M.**
849 (1999) Systemic signaling and acclimation in response to excess excitation energy in
850 *Arabidopsis*. *Science*, **284**, 654–657.

851 **Klinkenberg, J.** (2014). Extraction of chloroplast proteins from transiently transformed *Nicotiana*
852 *benthamiana* leaves. *Bio-protocol*, **4**, e1238.

853 **Klinkenberg, J., Faist, H., Saupe, S., Lambertz, S., Krischke, M., Stingl, N., Fekete, A.,**
854 **Mueller, M. J., Feussner, I., Hedrich, R. and Deeken, R.** (2014). Two fatty acid desaturases,
855 STEAROYL-ACYL CARRIER PROTEIN Delta9-DESATURASE6 and FATTY ACID
856 DESATURASE3, are involved in drought and hypoxia stress signaling in *Arabidopsis* crown
857 galls. *Plant Physiol.*, **164**, 570-583.

858 **Krause, K., Oetke, S. and Krupinska, K.** (2012) Dual targeting and retrograde translocation:
859 Regulators of plant nuclear gene expression can be sequestered by plastids. *Int. J. Mol. Sci.*,
860 **13**, 11085–11101.

861 **Krupinska, K., Oetke, S., Desel, C., Mulisch, M., Schäfer, A., Hollmann, J., Kumlehn, J. and**
862 **Hensel G.** (2014) WHIRLY1 is a major organizer of chloroplast nucleoids. *Front Plant Sci.*, **5**,
863 432.

864 **Kulasek, M., Bernacki, M.J., Ciszak, K., Witoń, D. and Karpiński, S.** (2016) Contribution of
865 PsbS function and stomatal conductance to foliar temperature in higher plants. *Plant Cell*
866 *Physiol.*, **57**, 1495–1509.

867 **Lee, K.P., Kim, C., Landgraf, F. and Apel, K.** (2007) EXECUTER1- and EXECUTER2-
868 dependent transfer of stress-related signals from the plastid to the nucleus of *Arabidopsis*
869 *thaliana*. *Proc. Natl Acad. Sci. USA*, **104**, 10270–10275.

870 **Li, J.F., Park, E., Arnim, A.G. Von and Nebenführ, A.** (2009a) The FAST technique: A
871 simplified *Agrobacterium*-based transformation method for transient gene expression analysis
872 in seedlings of *Arabidopsis* and other plant species. *Plant Methods*, **5**, 1–15.

873 **Li, M., Hensel, G., Mascher, M., et al.** (2019) Leaf variegation and impaired chloroplast
874 development caused by a truncated CCT domain gene in *albostrians* Barley. *Plant Cell*, **31**,
875 1430–1445.

876 **Li, Z., Wakao, S., Fischer, B.B. and Niyogi, K.K.** (2009b) Sensing and responding to excess light.
877 *Annu. Rev. Plant Biol.*, **60**, 239–260.

878 **Liere, K. and Börner, T.** (2007) Transcription and transcriptional regulation in plastids. In *Systems*
879 *Biology*. pp. 121–174.

880 **Love, M.I., Huber, W. and Anders, S.** (2014) Moderated estimation of fold change and dispersion
881 for RNA-seq data with DESeq2. *Genome Biol.*, **15**, 550.

882 **Maréchal, A Parent, J-S., Véronneau-Lafortune, F., Joyeux, A., Lang, F. and Brisson, N.**
883 (2009) Whirly proteins maintain plastid genome stability in *Arabidopsis*. *Proc. Natl Acad. Sci.*
884 *USA*, **106**, 14693-14698.

885 **Mateo, A., Mühlenbock, P., Rustérucci, C., Chang, C.C., Miszalski, Z., Karpinska, B., Parker,**
886 **J.E., Mullineaux, P.M. and Karpinski, S.** (2004) LESION SIMULATING DISEASE 1 is
887 required for acclimation to conditions that promote excess excitation energy. *Plant Physiol.*,
888 **136**, 2818–2830.

889 **Miao, Y., Jiang, J., Ren, Y. and Zhao, Z.** (2013) The single-stranded DNA-binding protein
890 WHIRLY1 represses *WRKY53* expression and delays leaf senescence in a developmental
891 stage-dependent manner in *Arabidopsis*. *Plant Physiol.*, **163**, 746-756.

892 **Miyata, K., Ikeda, H., Nakaji, M., Kanel, D.R. and Terashima, I.** (2015) Rate constants of PSII
893 photoinhibition and its repair, and PSII fluorescence parameters in field plants in relation to
894 their growth light environments. *Plant Cell Physiol.*, **56**, 1841–1854.

895 **Mühlenbock, P., Szechynska-Hebda, M., Plaszczyca, M., Baudou, M., Mullineaux, P.M.,**
896 **Parker, J.E., Karpinska, B. and Karpiński, S.** (2008) Chloroplast signaling and LESION
897 SIMULATING DISEASE1 regulate crosstalk between light acclimation and immunity in
898 *Arabidopsis*. *Plant Cell*, **20**, 2339–2356.

899 **Mullineaux, P.M., Karpiński, S. and Baker, N.R.** (2006) Spatial dependence for hydrogen
900 peroxide-directed signaling in light-stressed plants. *Plant Physiol.*, **141**, 346–350.

901 **Myouga, F., Akiyama, K., Tomonaga, Y., Kato, A., Sato, Y., Kobayashi, M., Nagata, N.,**
902 **Sakurai, T. and Shinozaki, K.** (2013) The chloroplast function database II: A comprehensive
903 collection of homozygous mutants and their phenotypic/genotypic traits for nuclear-encoded
904 chloroplast proteins. *Plant Cell Physiol.*, **54**, 1–10.

905 **Nakagawa, T., Kurose, T., Hino, T., et al.** (2007) Development of series of gateway binary
906 vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J.*
907 *Biosci. Bioeng.*, **104**, 34–41.

908 **Nakai, M.** (2015) The TIC complex uncovered: The alternative view on the molecular mechanism
909 of protein translocation across the inner envelope membrane of chloroplasts. *Biochim. Biophys.*
910 *Acta*, **1847**, 957–967.

911 **Ng, S., Ivanova, A., Duncan, O., et al.** (2013) A membrane-bound NAC transcription factor,
912 ANAC017, mediates mitochondrial retrograde signaling in *Arabidopsis*. *Plant Cell*, **25**, 3450–
913 3471.

914 **Nickelsen, J., Bohne, A.-V. and Westhoff, P.** (2014) Chloroplast gene expression—translation. In
915 *Plastid Biology*. New York, NY: Springer New York, pp. 49–78.

916 **Nishimura, K., Ashida, H., Ogawa, T. and Yokota, A.** (2010) A DEAD box protein is required
917 for formation of a hidden break in *Arabidopsis* chloroplast 23S rRNA. *Plant J.*, **63**, 766–777.

918 **Niyogi, K.K., Li, X.P., Rosenberg, V. and Jung, H.S.** (2005) Is PsbS the site of non-
919 photochemical quenching in photosynthesis? *J. Exp. Bot.*, **56**, 375–382.

920 **Nørholm, M.H.** (2010) A mutant Pfu DNA polymerase designed for advanced uracil-excision
921 DNA engineering. *BMC Biotechnol.*, **10**, 21.

922 **Nour-Eldin, H.H., Hansen, B.G., Nørholm, M.H.H., Jensen, J.K. and Halkier, B.A.** (2006)
923 Advancing uracil-excision based cloning towards an ideal technique for cloning PCR
924 fragments. *Nucleic Acids Res.*, **34**, e122–e122.

925 **Page, M.T., Kacprzak, S.M., Mochizuki, N., Okamoto, H., Smith, A.G. and Terry, M.J.** (2017)
926 Seedlings lacking the PTM protein do not show a *genomes uncoupled* (*gun*) mutant phenotype.
927 *Plant Physiol.*, **174**, 21–26.

928 **Paila, Y.D., Richardson, L.G.L. and Schnell, D.J.** (2015) New insights into the mechanism of
929 chloroplast protein import and its integration with protein quality control, organelle biogenesis
930 and development. *J. Mol. Biol.*, **427**, 1038–1060.

931 **Parker, N., Wang, Y. and Meinke, D.** (2014) Natural variation in sensitivity to a loss of
932 chloroplast translation in *Arabidopsis*. *Plant Physiol.*, **166**, 2013–2027.

933 **Patro, R., Duggal, G., Love, M.I., Irizarry, R.A. and Kingsford, C.** (2017) Salmon provides fast
934 and bias-aware quantification of transcript expression. *Nat. Methods*, **14**, 417–419.

935 **Pérez-Rodríguez, P., Riaño-Pachón, D.M., Corrêa, L.G.G., Rensing, S.A., Kersten, B. and**
936 **Mueller-Roeber, B.** (2009) PlnTFDB: Updated content and new features of the plant
937 transcription factor database. *Nucleic Acids Res.*, **38**, 822–827.

938 **Pesaresi, P., Masiero, S., Eubel, H., Braun, H.-P., Bhushan, S., Glaser, E., Salamini, F. and**
939 **Leister, D.** (2006) Nuclear photosynthetic gene expression is synergistically modulated by
940 rates of protein synthesis in chloroplasts and mitochondria. *Plant Cell*, **18**, 970–991.

941 **Pesaresi, P., Varotto, C., Meurer, J., Jahns, P., Salamini, F. and Leister, D.** (2001) Knock-out
942 of the plastid ribosomal protein L11 in *Arabidopsis*: Effects on mRNA translation and
943 photosynthesis. *Plant J.*, **27**, 179–189.

944 **Prikryl, J., Watkins, K. P., Friso, G., Wijk, K. J. and Barkan A.** (2008) A member of the
945 Whirly family is a multifunctional RNA- and DNA-binding protein that is essential for
946 chloroplast biogenesis. *Nucleic Acids Res.*, **36**, 5152–5165.

947 **Pogson, B.J., Woo, N.S., Förster, B. and Small, I.D.** (2008) Plastid signalling to the nucleus and
948 beyond. *Trends Plant Sci.*, **13**, 602–9.

949 **Ramakers, C., Ruijter, J.M., Deprez, R.H.L. and Moorman, A.F.** (2003) Assumption-free
950 analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.*, **339**,
951 62–66.

952 **Ramel, F., Birtic, S., Ginies, C., Soubigou-Taconnat, L., Triantaphylides, C. and Havaux, M.**
953 (2012) Carotenoid oxidation products are stress signals that mediate gene responses to singlet
954 oxygen in plants. *Proc. Natl Acad. Sci. USA*, **109**, 5535–5540.

955 **Romani, I., Tadini, L., Rossi, F., Masiero, S., Pribil, M., Jahns, P., Kater, M., Leister, D. and**
956 **Pesaresi, P.** (2012) Versatile roles of *Arabidopsis* plastid ribosomal proteins in plant growth
957 and development. *Plant J.*, **72**, 922–934.

958 **Ruckle, M.E., Burgoon, L.D., Lawrence, L.A., Sinkler, C.A. and Larkin, R.M.** (2012) Plastids
959 are major regulators of light signaling in *Arabidopsis*. *Plant Physiol.*, **159**, 366–390.

960 **Scharff, L.B. and Bock, R.** (2014) Synthetic biology in plastids. *Plant J.*, **78**, 783–798.

961 **Schreiber, U. and Klughammer, C.** (2008) New accessory for the DUAL-PAM-100: The
962 P515/535 module and examples of its application. *PAM Appl. Notes*, **1**, 1–10.

963 **Schwacke, R., Schneider, A., Graaff, E. Van Der, Fischer, K., Catoni, E., Desimone, M.,**
964 **Frommer, W.B., Flügge, U.I. and Kunze, R.** (2003) ARAMEMNON, a novel database for
965 *Arabidopsis* integral membrane proteins. *Plant Physiol.*, **131**, 16–26.

966 **Schwacke, R., Fischer, K., Ketelsen, B., Krupinska, K., Krause, K.** (2007) Comparative survey
967 of plastid and mitochondrial targeting properties of transcription factors in *Arabidopsis* and
968 rice. *Mol. Genet. Genomics.*, **277**:631–646.

969 **Shapiguzov, A., Vainonen, J.P., Hunter, K., et al.** (2019) Arabidopsis RCD1 coordinates
970 chloroplast and mitochondrial functions through interaction with ANAC transcription factors.
971 *Elife*, **8**, 1–35.

972 **Silva-Correia, J., Freitas, S., Tavares, R.M., Lino-Neto, T. and Azevedo, H.** (2014) Phenotypic
973 analysis of the Arabidopsis heat stress response during germination and early seedling
974 development. *Plant Methods*, **10**, 7.

975 **Silvestro, D., Andersen, T.G., Schaller, H. and Jensen, P.E.** (2013) Plant sterol metabolism. $\Delta 7$ -
976 sterol-C5-desaturase (STE1/DWARF7), $\Delta 5,7$ -sterol- $\Delta 7$ -reductase (DWARF5) and $\Delta 24$ -sterol-
977 $\Delta 24$ -reductase (DIMINUTO/DWARF1) show multiple subcellular localizations in *Arabidopsis*
978 *thaliana* (Heynh). *PLoS One*, **8**, e56429.

979 **Smirnoff, N. and Arnaud, D.** (2019) Hydrogen peroxide metabolism and functions in plants. *New*
980 *Phytol.*, **221**, 1197–1214.

981 **Soneson, C., Love, M.I. and Robinson, M.D.** (2016) Differential analyses for RNA-seq:
982 transcript-level estimates improve gene-level inferences. *F1000Research*, **4**, 1521.

983 **Souza, A. de, Wang, J.-Z. and Dehesh, K.** (2017) Retrograde signals: integrators of
984 interorganellar communication and orchestrators of plant development. *Annu. Rev. Plant Biol.*,
985 **68**, 85–108.

986 **Strayer, C., Oyama, T., Schultz, T., Raman, R., Somers, D., Mas, P., Panda, S., Kreps, J. and**
987 **Kay, S.** (2000) Cloning of the *Arabidopsis* clock gene *TOC1*, an autoregulatory response
988 regulator homolog. *Science*, **289**, 768–771.

989 **Sugiura, M.** (2014) Plastid mRNA translation. In *Chloroplast Biotechnology*. pp. 73–91.

990 **Sumanta, N., Haque, C., Nishika, J. and Suprakash, R.** (2014) Spectrophotometric analysis of
991 chlorophylls and carotenoids from commonly grown fern species by using various extracting
992 solvents. *Res. J. Chem. Sci.*, **4**, 2231–606.

993 **Sun, C.-W., Chen, L.-J., Lin, L.-C. and Li, H.** (2001) Leaf-specific upregulation of chloroplast
994 translocon genes by a CCT motif-containing protein, CIA2. *Plant Cell*, **13**, 2053–2061.

995 **Sun, C.-W., Huang, Y.-C. and Chang, H.-Y.** (2009a) CIA2 coordinately up-regulates protein
996 import and synthesis in leaf chloroplasts. *Plant Physiol.*, **150**, 879–888.

997 **Sun, Q., Zybaïlov, B., Majeran, W., Friso, G., Olinares, P.D.B. and Wijk, K.J. van** (2009b)
998 PPDB, the Plant Proteomics Database at Cornell. *Nucleic Acids Res.*, **37**, D969–D974.

999 **Sun, X., Feng, P., Xu, X., Guo, H., Ma, J., Chi, W., Lin, R., Lu, C. and Zhang, L.** (2011) A
1000 chloroplast envelope-bound PHD transcription factor mediates chloroplast signals to the
1001 nucleus. *Nat. Commun.*, **2**, 477.

1002 **Tadini, L., Pesaresi, P., Kleine, T., et al.** (2016) GUN1 controls accumulation of the plastid
1003 ribosomal protein S1 at the protein level and interacts with proteins involved in plastid protein
1004 homeostasis. *Plant Physiol.*, **170**, 1817–30.

1005 **Te, J., Melcher, U., Howard, A. and Verchot-Lubicz J.** (2005) Soilborne wheat mosaic virus
1006 (SBWMV) 19K protein belongs to a class of cysteine rich proteins that suppress RNA
1007 silencing. *Viro. J.*, **2**, 18.

1008 **Tiller, N., Weingartner, M., Thiele, W., Maximova, E., Schottler, M.A. and Bock, R.** (2012)
1009 The plastid-specific ribosomal proteins of *Arabidopsis thaliana* can be divided into non-
1010 essential proteins and genuine ribosomal proteins. *Plant J.*, **69**, 302–316.

1011 **Tokumaru, M., Adachi, F., Toda, M., et al.** (2017) Ubiquitin-proteasome dependent regulation of
1012 the GOLDEN2-LIKE 1 transcription factor in response to plastid signals. *Plant Physiol.*, **173**,
1013 524–535.

1014 **Wagner, R. and Pfannschmidt, T.** (2006) Eukaryotic transcription factors in plastids -
1015 Bioinformatic assessment and implications for the evolution of gene expression machineries in
1016 plants. *Gene*, **381**, 62–70.

1017 **Wang, L., Kim, C., Xu, X., Piskurewicz, U., Dogra, V. and Singh, S.** (2016) Singlet oxygen- and
1018 EXECUTER1-mediated signaling is initiated in grana margins and depends on the. *Proc. Natl*
1019 *Acad. Sci. USA*, **113**, E3792–E3800.

1020 **Waters, M.T., Wang, P., Korkaric, M., Capper, R.G., Saunders, N.J. and Langdale, J. a**
1021 (2009) GLK transcription factors coordinate expression of the photosynthetic apparatus in
1022 *Arabidopsis*. *Plant Cell*, **21**, 1109–1128.

1023 **Wituszyńska, W., Ślesak, I., Vanderauwera, S., et al.** (2013) LESION SIMULATING
1024 DISEASE1, ENHANCED DISEASE SUSCEPTIBILITY1, and PHYTOALEXIN
1025 DEFICIENT4 conditionally regulate cellular signaling homeostasis, photosynthesis, water use
1026 efficiency, and seed yield in *Arabidopsis*. *Plant Physiol.*, **161**, 1795–1805.

1027 **Wituszyńska, W., Szechyńska-Hebda, M., Sobczak, M., Rusaczek, A., Kozłowska-
1028 Makulska, A., Witoń, D. and Karpiński, S.** (2015) LESION SIMULATING DISEASE 1
1029 And ENHANCED DISEASE SUSCEPTIBILITY 1 differentially regulate UV-C-induced
1030 photooxidative stress signalling and programmed cell death in *Arabidopsis thaliana*. *Plant,*
1031 *Cell Environ.*, **38**, 315–330.

1032 **Wu, G.-Z., Chalvin, C., Hoelscher, M.P., Meyer, E.H., Wu, X.N. and Bock, R.** (2018) Control
1033 of retrograde signaling by rapid turnover of GENOMES UNCOUPLED 1. *Plant Physiol.*, **176**,
1034 2472–2495.

1035 **Wu, G., Meyer, E.H., Richter, A.S., et al.** (2019) Control of retrograde signalling by protein
1036 import and cytosolic folding stress. *Nat. Plants*, **5**, 525–538.

1037 **Yabuta, Y., Motoki, T., Yoshimura, K., Takeda, T., Ishikawa, T. and Shigeoka, S.** (2002)
1038 Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems
1039 under photo-oxidative stress. *Plant J.*, **32**, 915–925.

1040 **Yasumura, Y., Moylan, E.C. and Langdale, J.A.** (2005) A conserved transcription factor
1041 mediates nuclear control of organelle biogenesis in anciently diverged land plants. *Plant Cell*,
1042 **17**, 1894–1907.

1043 **Yu Q-B., Li G., Wang G., et al.** (2008) Construction of a chloroplast protein interaction network
1044 and functional mining of photosynthetic proteins in *Arabidopsis thaliana*. *Cell Res.*, **18**, 1007-
1045 1019

1046 **Zoschke, R. and Bock, R.** (2018) Chloroplast translation: structural and functional organization,
1047 operational control and regulation. *Plant Cell*, **30**, 745-770.

1048

1049 **Figure legends**

1050 **Figure 1.** Sub-cellular localization of CIA2 in Arabidopsis and tobacco. CIA2:YFP (**a, c, d**) and
1051 CIA2¹⁻¹⁰⁰:YFP (**b, e**) fusion proteins were transiently expressed in Arabidopsis seedlings (**a, b**) and
1052 in *Nicotiana benthamiana* leaves (**c-e**) under control of the 35S constitutive promoter. **d** presents
1053 chloroplast localized CIA2:YFP at larger magnification. Pictures were taken 3 days post infiltration
1054 using confocal microscopy. Magenta and green colors represent chlorophyll and YFP fluorescence,
1055 respectively. White scale bars indicate 10 μ m. (**f**) Localization of CIA2¹⁻¹⁰⁰:YFP in stroma
1056 (p35S::CIA2¹⁻¹⁰⁰:YFP was expressed in Col-0). Total protein extracts, chloroplast stroma and
1057 thylakoid membranes (TM) were isolated from Col-0 and plants expressing CIA2¹⁻¹⁰⁰:YFP under the
1058 control of 35S promoter. Protein extracts were separated by SDS-PAGE and analyzed on Western
1059 blots using antibodies against GFP, large subunit of RuBisCo (RbcL) and D1 protein of PSII.

1060 **Figure 2.** Isolation and characterization of *cia2-2 cil-1* double mutant. (**a, b**) Isolation of *cia2-4*
1061 mutant in reverse genetic screening using UV-AB (**a**) and high light in combination with low
1062 temperature (cHL) (**b**). (**c**) In the upper part, a schematic representation of CIA2 and CIL genes is
1063 presented. Black and white rectangles represent exons and untranslated regions, respectively. The
1064 lower part shows the analysis of functional domains in CIA2 and CIL proteins: cTP–chloroplast
1065 transit peptide (functional – dark green, predicted – light green), NLS–nuclear localization signal,
1066 CCT–putative active domain in CIA2 and CIL. The CCT domain contains NLS; however, it is not
1067 shown in the diagram for simplicity. (**d**) Phenotypes of 4-week-old plants grown in long-day
1068 conditions. *cia2-2* and *cia2-2 cil-1* plants were paler than the rest of analyzed genotypes; thus, the
1069 content of photosynthetic pigments was measured and shown in (**e**). (**f**) Expression of CIA2 and CIL
1070 in the analyzed genotypes was measured using qRT-PCR with gene-specific primers. Locations of
1071 primers are depicted in panel (**c**). (**g, h**) Complementation of *cia2-2* phenotype with ectopic
1072 expression of CIA2 under 35S and native promoter. (**g**) Relative expression of CIA2 in analyzed lines.
1073 (**h**) Relative content of photosynthetic pigments in complementation lines. In (**a**), (**e**) and (**f**) statistical
1074 significance (ANOVA and Tukey HSD test) is shown relative to Col-0 (** $p < 0.01$; *** $p < 0.001$).
1075 In (**g**) and (**h**) statistical significance (ANOVA and Tukey HSD test) is shown relative to *cia2-2* (** p
1076 < 0.01 ; *** $p < 0.001$) and to Col-0 ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$; $^{\wedge\wedge\wedge}p < 0.001$).

1077 **Figure 3.** Sub-cellular localization of CIL in Arabidopsis and tobacco. CIL:YFP (**a, b**) and CIL¹⁻
1078 ¹⁰⁰:YFP (**c**) fusion proteins were transiently expressed in Arabidopsis seedlings (**a, c**) and in *Nicotiana*
1079 *benthamiana* leaves (**b**) under control of the 35S constitutive promoter. Pictures were taken 3 days
1080 post infiltration using confocal microscopy. Magenta and green colors represent chlorophyll and YFP
1081 fluorescence, respectively. White scale bars indicate 10 μ m.

1082 **Figure 4.** CIA2 and CIL are required for resistance to UV-AB. Mature plants were exposed to UV-
 1083 AB, and the plants' performance was assessed using chlorophyll *a* fluorescence (a and b) and ion
 1084 leakage (c). (a) Maximum efficiency of PSII (F_v/F_m), nonregulated energy dissipation (Y(NO)), and
 1085 nonphotochemical quenching (NPQ) were measured before and after UV-AB treatment (1 and 2
 1086 days). Each point represents mean $\pm$ SEM of at least eight plants. (b) F_v/F_m of control (UV-) and UV-
 1087 AB-treated (UV +) plants of Col-0 and *cia2-2 cil-1* plants. Values of F_v/F_m are shown in pseudocolor
 1088 scale. (c) Ion leakage of control (UV-, n = 5) and UV-AB-treated (UV +, n = 18) plants is shown as
 1089 a percentage of total ion leakage. Statistical significance (ANOVA and Tukey HSD test) is shown
 1090 relative to Col-0 (* p < 0.05; *** p < 0.001) and to control conditions (^^^ p < 0.001).

1091 **Figure 5.** High-light (HL) susceptibility of *cia2-2 cil-1*. (a) Representation of Arabidopsis rosette
 1092 with the young leaves marked with blue, dashed line. (b, c, d) Maximum efficiency of PSII (F_v/F_m)
 1093 measured in plants (b) exposed to blue HL (1100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for a specified time (n = 4–8 plants),
 1094 (c) during recovery after HL stress, n = 6–8, (d) and treated with inhibitors influencing the production
 1095 of ROS in chloroplasts (n = 12–22). In each plot, points represent mean $\pm$ SEM measured in
 1096 independent plants (b and c) or leaf disks (d). Statistical significance (ANOVA and Tukey HSD test)
 1097 is shown relative to Col-0 (* p < 0.05; ** p < 0.01; *** p < 0.001).

1098 **Figure 6.** CIA2 and CIL are required for optimal photosynthesis in Arabidopsis. (a)
 1099 Nonphotochemical quenching (NPQ) in analyzed genotypes. Points represent mean $\pm$ SEM of the
 1100 whole rosette and only young leaves. (b) NPQ of Col-0 and *cia2-2 cil-1*. (c) Analysis of
 1101 electrochromic pigment shift (ECS, P515) at 160 and 660 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of actinic light. For simplicity,
 1102 only Col-0 and *cia2-2 cil-1* are shown. ST—single turnover flash. Total ECS (ECS_t) (d) and ΔpH (e)
 1103 in analyzed genotypes at 160 and 660 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Box plots represent values of 10 independent
 1104 plants. (f) CO₂ assimilation as a function of light intensity. (g) CO₂ assimilation as a function of CO₂
 1105 concentration. In (f) and (g), values represent mean $\pm$ SEM of 7–9 plants. Statistical significance
 1106 (ANOVA and Tukey HSD test) is shown relative to Col-0 (* p < 0.05; ** p < 0.01; *** p < 0.001).

1107 **Figure 7.** Characterization of chloroplast translation in analyzed genotypes. (a) Expression analysis
 1108 of genes encoding chloroplast ribosomal proteins. Levels of analyzed transcripts were determined
 1109 using qRT-PCR and normalized to two house-keeping genes (*PP2AA3* and *TIP41L*). Bars indicate
 1110 mean values $\pm$ SD (three independent biological replicates). (b) Expression profile (RNA-seq) of genes
 1111 involved in the regulation of plastid translation. (c) Polysome analysis reveals slower translation of
 1112 *psbD* mRNA in *cia2-2 cil-1* double mutant as compared to Col-0. Gray triangles indicate the density
 1113 of sucrose gradient. Methylene blue-stained 18S rRNA is shown as a loading control. (d)
 1114 Susceptibility to chloroplast translation inhibitor (spectinomycin, 1.25 mg/L) of analyzed genotypes.

1115 statistical significance (ANOVA and Tukey HSD test) is shown relative to Col-0 (* $p < 0.05$; ** $p <$
1116 0.01; *** $p < 0.001$).

1117 **Figure 8.** Maturation of plastid rRNAs. (a) Plastid rRNA operon. Mature forms of 16 and 23S rRNA
1118 are shown. Precursor of 23S rRNA is processed and cleaved into three parts: 0.4, 1.1, and 1.3 kb. (b)
1119 Relative abundance of precursor 23S and 16S rRNAs was measured using capillary electrophoresis
1120 and normalized to the cytoplasmic 18S rRNA. Values represent mean values $\pm$ SEM ($n = 3$). (c)
1121 Maturation and abundance of plastid rRNA were measured using Northern blot indicating that the
1122 2.4 kb form of 23S rRNA is accumulated in *cia2-2 cil-1*. (d) Determination of relative amounts of
1123 23S rRNA forms with qRT-PCR and primers (depicted in panel (a)) specific to each of cleaved
1124 fragments (primers A, B, and C) and flanking “hidden breaks” (primers AB and BC). Values were
1125 normalized to the level of 16S rRNA and represent mean values $\pm$ SD ($n = 3$). Statistical significance
1126 (ANOVA and Tukey HSD test) is shown relative to Col-0 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

1127 **Figure 9.** CIA2 and CIL negatively regulate tolerance to heat shock. (a) Gene ontology (GO) analysis
1128 of genes significantly induced in *cia2-2 cil-1* compared to Col-0 in the RNA-seq experiment. Ten
1129 most significantly overrepresented GO terms are shown. The number of genes in each group is shown
1130 in color of the bar. (b) Validation of expression of heat shock marker genes using qRT-PCR in
1131 analyzed genotypes and additional allele (*cia2-3*) in Ler-0 background ($n = 3$). Error bars represent
1132 $\pm$ SD. (c and d) Thermo tolerance was tested in seedlings grown *in vitro*. Plants were exposed to 45
1133 °C for a specific period of time. (c) Pictures of plants grown in control conditions 7 days after heat
1134 shock. (d) Survival rate of control and heat shock-treated plants. Error bars represent $\pm$ SEM ($n \geq 3$
1135 plates) from two independent experiments (in total at least 75 seedlings were analyzed per genotype
1136 and treatment). Statistical significance (ANOVA and Tukey HSD test) is shown relative to
1137 corresponding WT (*** $p < 0.001$).

1139

1140 **Figure 1.** Sub-cellular localization of CIA2 in Arabidopsis and tobacco. CIA2:YFP (a, c, d) and

1141 CIA2¹⁻¹⁰⁰:YFP (b, e) fusion proteins were transiently expressed in Arabidopsis seedlings (a, b) and

1142 in *Nicotiana benthamiana* leaves (c-e) under control of the 35S constitutive promoter. d presents

1143 chloroplast localized CIA2:YFP at larger magnification. Pictures were taken 3 days post infiltration

1144 using confocal microscopy. Magenta and green colors represent chlorophyll and YFP fluorescence,

1145 respectively. White scale bars indicate 10 μm. (f) Localization of CIA2¹⁻¹⁰⁰:YFP in stroma

1146 (p35S::CIA2¹⁻¹⁰⁰:YFP was expressed in Col-0). Total protein extracts, chloroplast stroma and

1147 thylakoid membranes (TM) were isolated from Col-0 and plants expressing CIA2¹⁻¹⁰⁰:YFP under the

1148 control of 35S promoter. Protein extracts were separated by SDS-PAGE and analyzed on Western
1149 blots using antibodies against GFP, large subunit of RuBisCo (RbcL) and D1 protein of PSII.
1150

1151

1152 **Figure 2.** Isolation and characterization of *cia2-2 cil-1* double mutant. (**a**, **b**) Isolation of *cia2-4*
 1153 mutant in reverse genetic screening using UV-AB (**a**) and high light in combination with low
 1154 temperature (cHL) (**b**). (**c**) In the upper part, a schematic representation of *CIA2* and *CIL* genes is
 1155 presented. Black and white rectangles represent exons and untranslated regions, respectively. The
 1156 lower part shows the analysis of functional domains in *CIA2* and *CIL* proteins: cTP–chloroplast
 1157 transit peptide (functional – dark green, predicted – light green), NLS–nuclear localization signal,
 1158 CCT–putative active domain in *CIA2* and *CIL*. The CCT domain contains NLS; however, it is not
 1159 shown in the diagram for simplicity. (**d**) Phenotypes of 4-week-old plants grown in long-day
 1160 conditions. *cia2-2* and *cia2-2 cil-1* plants were paler than the rest of analyzed genotypes; thus, the
 1161 content of photosynthetic pigments was measured and shown in (**e**). (**f**) Expression of *CIA2* and *CIL*
 1162 in the analyzed genotypes was measured using qRT-PCR with gene-specific primers. Locations of
 1163 primers are depicted in panel (**c**). (**g**, **h**) Complementation of *cia2-2* phenotype with ectopic
 1164 expression of *CIA2* under 35S and native promoter. (**g**) Relative expression of *CIA2* in analyzed lines.
 1165 (**h**) Relative content of photosynthetic pigments in complementation lines. In (**a**), (**e**) and (**f**) statistical
 1166 significance (ANOVA and Tukey HSD test) is shown relative to Col-0 (** $p < 0.01$; *** $p < 0.001$).

1167 In (**g**) and (**h**) statistical significance (ANOVA and Tukey HSD test) is shown relative to *cia2-2* (** p
1168 < 0.01 ; *** $p < 0.001$) and to Col-0 ($p < 0.05$; $p < 0.01$; $p < 0.001$).
1169

Figure 3. Sub-cellular localization of CIL in Arabidopsis and tobacco. CIL:YFP (a, b) and CIL¹⁻¹⁰⁰:YFP (c) fusion proteins were transiently expressed in Arabidopsis seedlings (a, c) and in *Nicotiana benthamiana* leaves (b) under control of the 35S constitutive promoter. Pictures were taken 3 days post infiltration using confocal microscopy. Magenta and green colors represent chlorophyll and YFP fluorescence, respectively. White scale bars indicate 10 μm.

1177 **Figure 4.** CIA2 and CIL are required for resistance to UV-AB. Mature plants were exposed to UV-
 1178 AB, and the plants' performance was assessed using chlorophyll *a* fluorescence (**a** and **b**) and ion
 1179 leakage (**c**). (**a**) Maximum efficiency of PSII (F_v/F_m), nonregulated energy dissipation (Y(NO)), and
 1180 nonphotochemical quenching (NPQ) were measured before and after UV-AB treatment (1 and 2
 1181 days). Each point represents mean $\pm$ SEM of at least eight plants. (**b**) F_v/F_m of control (UV-) and UV-
 1182 AB-treated (UV +) plants of Col-0 and *cia2-2 cil-1* plants. Values of F_v/F_m are shown in pseudocolor
 1183 scale. (**c**) Ion leakage of control (UV-, n = 5) and UV-AB-treated (UV +, n = 18) plants is shown as
 1184 a percentage of total ion leakage. Statistical significance (ANOVA and Tukey HSD test) is shown
 1185 relative to Col-0 (* $p < 0.05$; *** $p < 0.001$) and to control conditions (^^^ $p < 0.001$).

1186

1187 **Figure 5.** High-light (HL) susceptibility of *cia2-2 cil-1*. **(a)** Representation of Arabidopsis rosette
 1188 with the young leaves marked with blue, dashed line. **(b, c, d)** Maximum efficiency of PSII (F_v/F_m)
 1189 measured in plants **(b)** exposed to blue HL (1100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for a specified time ($n = 4-8$ plants),
 1190 **(c)** during recovery after HL stress, $n = 6-8$, **(d)** and treated with inhibitors influencing the production
 1191 of ROS in chloroplasts ($n = 12-22$). In each plot, points represent mean $\pm$ SEM measured in
 1192 independent plants **(b and c)** or leaf disks **(d)**. Statistical significance (ANOVA and Tukey HSD test)
 1193 is shown relative to Col-0 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

1194

Figure 6. CIA2 and CIL are required for optimal photosynthesis in Arabidopsis. (a) Nonphotochemical quenching (NPQ) in analyzed genotypes. Points represent mean $\pm$ SEM of the whole rosette and only young leaves. (b) NPQ of Col-0 and *cia2-2 cil-1*. (c) Analysis of electrochromic pigment shift (ECS, P515) at 160 and 660 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of actinic light. For simplicity, only Col-0 and *cia2-2 cil-1* are shown. ST—single turnover flash. Total ECS (ECS_i) (d) and ΔpH (e) in analyzed genotypes at 160 and 660 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Box plots represent values of 10 independent plants. (f) CO₂ assimilation as a function of light intensity. (g) CO₂ assimilation as a function of CO₂ concentration. In (f) and (g), values represent mean $\pm$ SEM of 7–9 plants. Statistical significance (ANOVA and Tukey HSD test) is shown relative to Col-0 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 7. Characterization of chloroplast translation in analyzed genotypes. (a) Expression analysis of genes encoding chloroplast ribosomal proteins. Levels of analyzed transcripts were determined using qRT-PCR and normalized to two house-keeping genes (*PP2AA3* and *TIP41L*). Bars indicate mean values $\pm$ SD (three independent biological replicates). (b) Expression profile (RNA-seq) of genes involved in the regulation of plastid translation. (c) Polysome analysis reveals slower translation of *psbD* mRNA in *cia2-2 cil-1* double mutant as compared to Col-0. Gray triangles indicate the density of sucrose gradient. Methylene blue-stained 18S rRNA is shown as a loading control. (d) Susceptibility to chloroplast translation inhibitor (spectinomycin, 1.25 mg/L) of analyzed genotypes. statistical significance (ANOVA and Tukey HSD test) is shown relative to Col-0 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 8. Maturation of plastid rRNAs. **(a)** Plastid rRNA operon. Mature forms of 16 and 23S rRNA are shown. Precursor of 23S rRNA is processed and cleaved into three parts: 0.4, 1.1, and 1.3 kb. **(b)** Relative abundance of precursor 23S and 16S rRNAs was measured using capillary electrophoresis and normalized to the cytoplasmic 18S rRNA. Values represent mean values $\pm$ SEM ($n = 3$). **(c)** Maturation and abundance of plastid rRNA were measured using Northern blot indicating that the 2.4 kb form of 23S rRNA is accumulated in *cia2-2 cil-1*. **(d)** Determination of relative amounts of 23S rRNA forms with qRT-PCR and primers (depicted in panel **(a)**) specific to each of cleaved fragments (primers A, B, and C) and flanking “hidden breaks” (primers AB and BC). Values were normalized to the level of 16S rRNA and represent mean values $\pm$ SD ($n = 3$). Statistical significance (ANOVA and Tukey HSD test) is shown relative to Col-0 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 9. CIA2 and CIL negatively regulate tolerance to heat shock. **(a)** Gene ontology (GO) analysis of genes significantly induced in *cia2-2 cil-1* compared to Col-0 in the RNA-seq experiment. Ten most significantly overrepresented GO terms are shown. The number of genes in each group is shown in color of the bar. **(b)** Validation of expression of heat shock marker genes using qRT-PCR in analyzed genotypes and additional allele (*cia2-3*) in Ler-0 background ($n = 3$). Error bars represent $\pm$ SD. **(c and d)** Thermo tolerance was tested in seedlings grown *in vitro*. Plants were exposed to 45 °C for a specific period of time. **(c)** Pictures of plants grown in control conditions 7 days after heat shock. **(d)** Survival rate of control and heat shock-treated plants. Error bars represent $\pm$ SEM ($n \geq 3$ plates) from two independent experiments (in total at least 75 seedlings were analyzed per genotype and treatment). Statistical significance (ANOVA and Tukey HSD test) is shown relative to corresponding WT ($***p < 0.001$).