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Novel biogenic plastid signals

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Light and plastid signals regulate different sets of genes in the albino mutant pap7-1

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AUTHOR CONTRIBUTIONS

B.G., L.M., R.B., S.R., T.P. designed the research and/or experiments; B.G., L.M., M.M., G.A., F.C., M.L., R.B. performed research; K.M. contributed new computational tools; B.G., L.M., S.T., S.L., S.R., T.P. analyzed data; T.P. wrote the article with the help of all co-authors.

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ONE-SENTENCE SUMMARY

The albino pap7-1 mutant of Arabidopsis reveals the relative impact of light and plastid developmental stage on the expression of nuclear genes involved in metabolism and photosynthesis.

ABSTRACT

Plants possessing dysfunctional plastids due to defects in pigment biosynthesis or translation are known to repress photosynthesis-associated nuclear genes (PhANGs) *via* retrograde signals from the disturbed organelles towards the nucleus. These signals are thought to be essential for proper biogenesis and function of the plastid. Mutants lacking plastid-encoded RNA polymerase-associated proteins (PAPs) display a genetic arrest in eoplast-chloroplast transition leading to an albino phenotype in the light. Retrograde signaling in these mutants, thus, could be expected to be similar as under conditions inducing plastid dysfunction. In order to answer this question we performed plastome- and genome-wide array analyses in the *pap7-1* mutant of *Arabidopsis.* In parallel, we determined the potential overlap with light-regulated expression networks. To this end we performed a comparative expression profiling approach using light- and dark-grown wild-type plants as relative control for the expression profiles obtained from light-grown *pap7-1* mutants. Our data indicate a specific impact of retrograde signals on metabolism related genes in *pap7-1* mutants reflecting the starvation situation of the albino seedlings. In contrast light regulation of PhANGs and other nuclear gene groups appears to be fully functional in this mutant indicating that a block in chloroplast biogenesis *per se* does not repress expression of them as suggested by earlier studies. Only genes for light harvesting complex proteins displayed a significant repression indicating an exclusive retrograde impact on this gene family. Our results indicate that chloroplasts and arrested plastids each emit specific signals that control different target gene modules both in positive and negative manner.

INTRODUCTION

The build-up of the photosynthetic machinery during photomorphogenesis of angiosperms requires a tight coordination of nuclear and plastid gene expression as the photosynthesis genes are distributed over both genetic compartments (Waters and Langdale, 2009; Arsovski et al., 2012; Pogson et al., 2015). This coordination is achieved by a mutual information exchange between nucleus and plastids that is called anterograde (from nucleus towards plastids) and retrograde (from plastids towards nucleus) signaling. This mutual signaling has been studied extensively, but is still far from being understood (Chi et al., 2013; Chan et al., 2016; de Souza et al., 2016; Kleine and Leister, 2016). The retrograde plastidial signals that are identified so far are numerous and of very diverse nature (see below). A recent proposal categorizes them according to their respective developmental context into i) biogenic signals that act during early chloroplast biogenesis (e.g. during germination and seedling development) controlling proper organelle establishment and ii) operational signals that are send from well-developed chloroplast in later plant stages in order to mediate acclimation responses to environmental changes (Pogson et al., 2008). This concept has been expanded by the proposal of a third category, degradational signals sent from chloroplasts during senescence and that mediate nutrient allocation when plastids are finally degraded (Pfannschmidt and Munne-Bosch, 2013).

Current models suggest the action of various plastid metabolites, protein factors, reactive oxygen species (ROS) as well as redox signals from photosynthesis as mediators of retrograde signaling. The list of identified metabolites and oxidation products includes haem, singlet oxygen or hydrogen peroxide, carotenoid oxidation products, 3´-phosphoadenosine-5´-phosphate, methylerythritol cyclodiphosphate and oxo-phytodienoic acid (OPDA) (Lee et al., 2007; Galvez-Valdivieso et al., 2009; Estavillo et al., 2011; Woodson et al., 2011; Ramel et al., 2012; Xiao et al., 2012; Park et al., 2013). Proteins proposed to act as plastid signals include envelope-tethered eukaryotic transcription factors TFIIB-like and PTM, both being released from the outer plastid membrane by targeted proteolysis (Lagrange et al., 2003; Sun et al., 2011) and a plastid localized Whirly1 protein that is released from plastids upon stress (Isemer et al., 2012). A very recent study, however, puts this particular function of PTM in retrograde signaling into question (Page et al., 2017). Most of these retrograde signaling molecules are discussed as stress signals operating from fully developed chloroplasts. However, their mode of action and their potential interactions are largely not understood and many questions concerning their function and interaction remain unanswered.

Biogenic signals that are proposed to be active only during proplastid-to-chloroplast conversion are even less understood and the retrograde signals that contribute to chloroplast

biogenesis still remain to be identified. One major difficulty in studying biogenic plastid signal(s) is the large functional and temporal overlap with the photoreceptor-controlled light signaling network. Already early studies in this research field revealed that it is difficult to separate the influences of plastid signals on nuclear gene expression from those initiated by light as both occur at the same time range and on the same target genes. Transgenic reporter gene approaches could demonstrate that plastid and light signals do even use the same promoter elements in front of their target genes (Kusnetsov et al., 1996; Sullivan and Gray, 2002; Brown et al., 2005). Recent studies suggest a close functional relationship between both control modes and it has been proposed that plastid signals can even remodel light signaling pathways from positive into negative signals and *vice versa* (Ruckle et al., 2007; Ruckle et al., 2012). Another recent study, however, suggests that light and plastid signaling routes act antagonistically in nuclear gene expression (Martin et al., 2016). Thus, the determined relative impact of light and retrograde signals on nuclear gene expression remains unclear.

Coordination of nuclear and plastid gene expression is also important for the establishment of the gene expression machinery in plastids and, in particular, for plastid localized RNA polymerases. A nuclear-encoded single-subunit phage-type RNA polymerase (NEP) and a plastid-encoded prokaryotic-type RNA polymerase (PEP) exist in plastids of green vascular plants. PEP is composed of four plastid-encoded subunits and 12 nuclear-encoded polymerase-associated proteins (PAPs). Furthermore, in *Arabidopsis* PEP requires interaction with six nucleus-coded sigma factors for promoter recognition (Lerbs-Mache, 2011; Borner et al., 2015; Pfannschmidt et al., 2015).

These RNA polymerases are key players in the coordination of the gene expression between plastids and the nucleus as they transcribe the genetic information within the plastids in a developmentally well-coordinated manner that is especially important during the early stages of seedling development (Liebers et al., 2017). Although not all details are known yet it is largely accepted that NEP represents the dominant plastid RNA polymerase activity in plastids of non-green embryonic and meristematic cells. Its activity is essential for the expression of the plastid *rpo* genes and for the establishment of the core enzyme of PEP (Liere et al., 2011). During the course of chloroplast biogenesis this basal PEP core enzyme becomes then decorated with PAPs. As far as known PAPs are induced in their expression by light and *in silico* analyses strongly suggest that they represent a tight regulon (Steiner et al., 2011; Pfannschmidt et al., 2015). The precise structural and functional roles of PAPs within the PEP complex and their regulatory relationship to plastid (and potentially nuclear) transcription are largely unknown and subject to current research.

Interestingly, all PAPs cause the same phenotypic consequences when their corresponding genes are inactivated. In *Arabidopsis* but also in maize or rice inactivation of *pap* genes result in albino, ivory or pale-green phenotypes with arrested plastid development. Plastids of such mutants do not develop a thylakoid membrane system and display enhanced transcript accumulation of NEP-dependent genes while transcript accumulation of PEP-dependent genes (including those for photosynthesis) is typically diminished (Pfalz and Pfannschmidt, 2013). This expression profile of plastid-encoded genes is reminiscent of those found in plastid *rpo* deletion mutants of tobacco (Hajdukiewicz et al., 1997; De Santis-Maclossek et al., 1999; Legen et al., 2002). The best possible explanation for this effect to date is that the lack of any of the PAPs either prevents the formation or compromises the stability of the PEP complex in developing chloroplasts. This, subsequently, leads to a lack of PEP-dependent processes and a concomitant arrest in chloroplast biogenesis since PEP is responsible for the expression of photosynthesis and tRNA genes (Williams-Carrier et al., 2014). It is important to note that the PAP assembly around the PEP core does not occur in the dark (Pfannschmidt and Link, 1994) and, consequently, *pap* mutants perform a normal skotomorphogenesis remaining undistinguishable from wildtype (Gilkerson et al., 2012). Expression and assembly of PAPs around the PEP core complex, thus, appear to represent a key initiation step in the formation of chloroplasts. The corresponding mutants represent, therefore, a useful tool to study the impact of a blocked transition from proplastids (or eoplasts) towards chloroplasts on the photomorphogenic program during seedling development.

Here, we present a study using the *Arabidopsis pap7-1* mutant in order to elucidate the relative impact of arrested plastid development and light on nuclear gene expression. Like other PAPs the PAP7/pTAC14 protein has been identified as a subunit of the plastid encoded RNA polymerase (PEP) (Pfalz et al., 2006; Steiner et al., 2011). In *Arabidopsis* the corresponding gene (At4g20130) codes for a protein of 55 kDa that contains a chloroplast transit peptide, a predicted SET domain characteristic of protein lysine methyltransferases and a putative Rubisco LSMT substrate-binding domain. The precise function of the protein within the PEP complex as well as any evidence for methylation activity is still elusive, but an inactivation of the *pap7-1/ptac14* gene in *Arabidopsis* results in an albino phenotype that is viable only on sucrose-supplemented medium (Gao et al., 2011; Steiner et al., 2011). The mutant displays all the molecular and structural features described for other *pap* mutants (Gao et al., 2011) that we proposed to name as the PAP syndrome. Our study provides a detailed catalogue of target gene modules at plastome and genome-wide levels and gives unexpected novel clues into the involvement of biogenic retrograde signaling in the regulation of nuclear genes for photosynthesis and metabolism.

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RESULTS

Arabidopsis pap7-1 mutants exhibit normal photomorphogenic development but never develop chloroplasts

Homozygous *pap7-1* mutant seedlings are known to develop an albino phenotype when grown in the light (Gao et al., 2011; Steiner et al., 2011). However, when grown in the dark homozygous mutants develop a fully normal etiolated phenotype that remains macroscopically undistinguishable from heterozygous mutant or WT seedlings. They can be identified only by the missing greening process upon subsequent illumination (Fig. 1A), while they otherwise demonstrate a WT-like morphology. Grown directly in light without preceeding dark phase homozygous mutants show light-induced repression of hypocotyl elongation, opening of the apical hook and separation and expansion of the cotyledons (Fig. 1B). When kept on standard growth medium without additional carbon source *pap7-1* mutant seedlings could not develop further than the cotyledon stage, started to bleach, became transparent and finally died (Fig. 1B). On sucrose supplemented medium, however, *pap7-1* mutants were able to generate a rosette of almost WT-like appearance. The leaf blades were slightly smaller and the petioles shorter than in WT (Fig. 1B). It has been observed that a transitory green stage with photosynthetic activities occurs in *Arabidopsis* embryos starting from the early torpedo stage (7-9 days after fertilization (daf)) until the end of the maturation (mature green stage at 23-27 daf) (Allorent et al., 2013). Checking this phase we found in siliques of heterozygous *pap7-1* mutants both green and white seeds with a segregation ratio of 3:1 indicating that already in homozygous embryos the chloroplast formation is prevented (Fig. 1C). Since these seeds, however, were capable to germinate with no bias in allelic transmission (3:1 ratio of homozygote mutants), this lack of chloroplast biogenesis appears to be not essential for proper seed development and maturation. When growing the progeny of such heterozygous *pap7-1* mutants on sugar-supplemented medium in short-day conditions under very dim light (approx. 8-12 µE WL) homozygous mutant plants developed a reasonable rosette that was even able to initiate the flowering programme after shifting the plant container to long-day-conditions (Fig. 1C, D). Architecture and size of the resulting inflorescence did not exhibit major differences in comparison to green plants as indicated by control plants grown in the same containers (Fig. 1D). In sum, these observations indicate that the major photomorphogenic programmes (and hence the action of the corresponding photoreceptors) are functional in the mutant.

Transcript accumulation in albino *pap7-1* plastids

All *pap* mutants analyzed so far display largely reduced accumulation of PEP-dependent transcripts while NEP-dependent gene transcripts do over-accumulate (Borner et al., 2015; Pfannschmidt et al., 2015). In all published reports, however, only a few representative

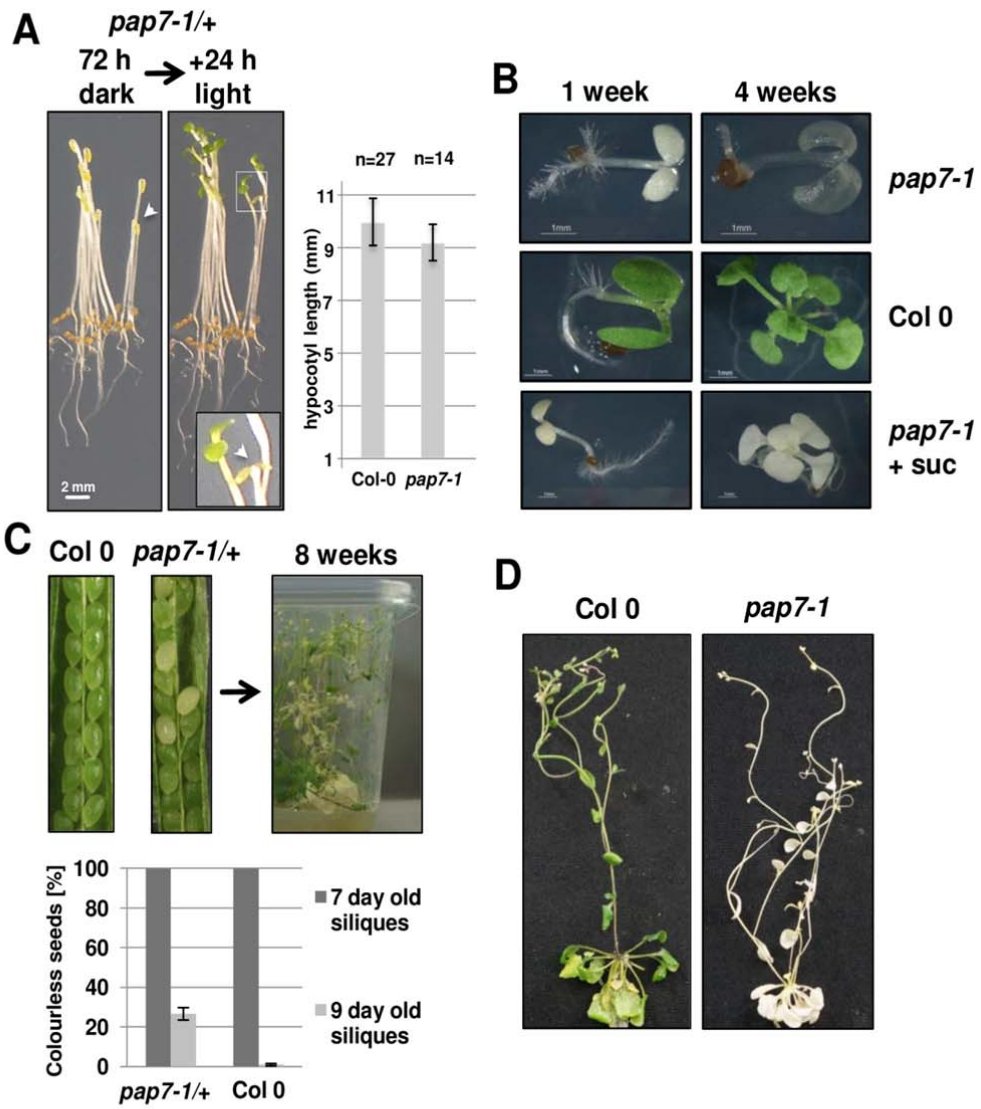

Figure 1. Developmental characteristics of the *pap7-1* mutant. A, 72h-dark-grown progeny of a *pap7-1*/+ heterozygote subjected to 24h of 20 µE white light to trigger photomorphogenesis. White arrowheads indicate a *pap7-1* homozygote mutant before and after subjection to light. Hypocotyl length +/- standard deviation measured after 72 h of dark growth. Genotypes were assigned after 24 h of light exposure (n= number of measurements). B, Growth of homozygous *pap7-1* seedlings and WT on ½ strength MS-medium in Petri-dishes without or with (+ suc) sucrose supplementation. C, Impact of *pap7-1* inactivation on chloroplast development during transient embryo greening and seed segregation in siliques of heterozygous *pap7-1* mutants (*pap7-1*/+) in comparison to WT (two left panels). Long-term growth (8 weeks) of the progeny of heterozygous mutants was performed on ½ strength MS-medium supplemented with 3% sucrose in transparent plastic containers in a short-day light period (right panel). Seed segregation into green and colourless seeds was counted in 7-10 siliques per measurement day (7 or 9 days after fertilization) in WT and *pap7-1*/+ mutants (bottom panel). D, Long-term grown plants in rosette stage were put into long-day conditions to induce flowering and photographed at the flowering stage.

genes per gene class were investigated. Here, we performed a comparative plastome-wide analysis in which we determined the accumulation of all plastidial mRNAs in light-grown

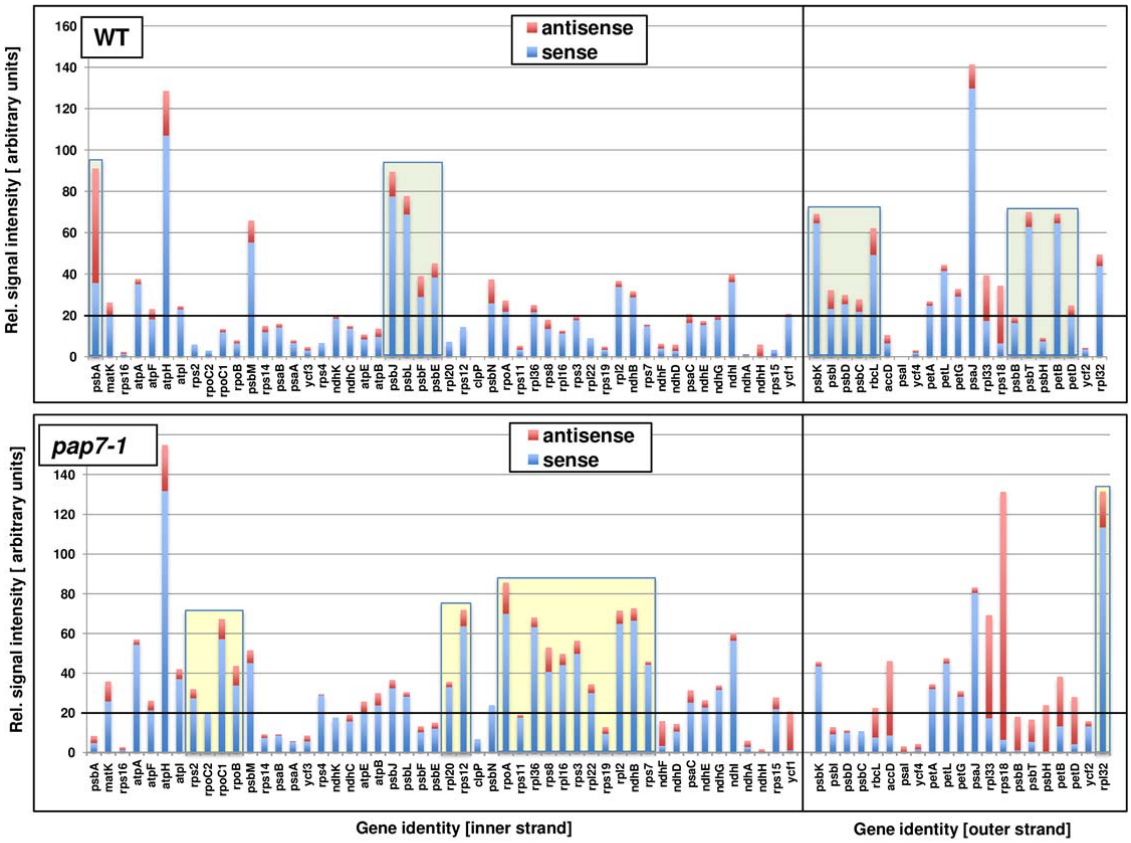

Figure 2. Macro-array analysis comparing plastid transcript accumulation in light-grown wild-type and *pap7-1 Arabidopsis* seedlings. Given is the transcript accumulation in wild-type (top panel) and mutant (bottom panel) seedlings both for sense (blue bars) and anti-sense (red bars) transcripts. Hybridization signals were normalized to the total signal intensity of the membrane and are given as arbitrary units in the left margin. Genes are labeled at the bottom of each panel according to accepted nomenclatures. Sequence of genes corresponds to their organization on the plastome separated between inner strand (left parts) and outer strand of the plastome (right parts). High transcript accumulation of PEP-dependent transcripts in WT is high-lighted by green boxes. High transcript accumulation of NEP-dependent transcripts in the *pap7-1* mutant is high-lighted by yellow boxes.

pap7-1 mutant plants and in WT plants grown in parallel (Fig. 2). To this end we used a custom-made macro-array that, in addition to sense RNAs, allowed also the detection of all corresponding antisense RNAs (Demarsy et al., 2012). Plastidial antisense RNAs were found in *Arabidopsis* to accumulate specifically in early phases of germination and radicle out-growth, two developmental steps preceding chloroplast biogenesis (Demarsy et al., 2012).

We observed reduced transcript accumulation for many PEP-dependent class 1 genes. However, mainly *psb* genes (encoding PSII components) were affected in the mutant (Fig. 2, light-green boxes) while for *psa* genes (encoding PSI components) the reduction was found to be less pronounced or even not existing. Genes for the cytochrome b_6f complex (*pet* genes) displayed mixed responses ranging from almost none to strong reduction of transcript accumulation. In contrast, all class 2 genes (genes with PEP and NEP promoters) displayed a stable or even enhanced transcript accumulation in the mutant. This class includes the *ndh* genes (encoding components of the NADH dehydrogenase complex), the genes encoding

the proteins of the small (*rps*) and large (*rpl*) ribosomal subunits and genes for components of the ATP synthase (*atp*). Enhanced accumulation could be also observed for class 3 genes (genes with NEP promoters only) *rpoA* and *rpoB/C1/C2* (encoding the PEP core subunits) (Fig. 2, yellow boxes), but not for the class 3 genes *ycf1* and *accD*. A general down-regulation of PEP-dependent genes and a corresponding up-regulation of NEP-dependent genes as suggested thus cannot be confirmed by our macroarray analysis.

These observations were independently supported by the microarray analysis that includes the full set of plastome located genes. In addition, the microarray also includes all tRNA genes that are not covered by the macroarray (Supplemental Table 1). These displayed all strongly reduced accumulation in the *pap7-1* mutant line suggesting that they are transcribed by PEP. This is in agreement with results obtained in a recent study on *pap* mutants of maize (Williams-Carrier et al., 2014).

Antisense RNA accumulation for most genes displayed no major differences between WT and *pap7-1* mutant plants. However, we observed specific over-accumulation of antisense transcripts for the *psbB/psbT/psbH/petB/petD* operon, *rpl33* and *rps18* genes and the two genes *ycf1* and *accD* (encoding import machinery subunit TIC214 and the β-subunit of the acetyl-CoA carboxylase complex, respectively) (Fig. 2). This differential accumulation suggests that antisense production is not just a concomitant by-product of read-through transcription, but that a distinct unknown mechanism is at its origin.

In summary, our macro-array experiment uncovered that the disturbance in PEP activity does not cause gene-class specific transcription changes but rather many gene-specific effects suggesting a much more complex transcription regulation in arrested albino plastids than current models anticipate.

Separation of light- and plastid-dependent gene regulation during photomorphogenesis by trilateral differential gene expression profiling

Chloroplast biogenesis is embedded into the general photomorphogenic programme of seedling development which strongly impedes a clear distinction of light-, development- and plastid-dependent signaling (Lopez-Juez et al., 1998; Lopez-Juez, 2007). However, since illuminated *pap7-1* mutants develop normally on sucrose-supplemented media (Fig. 1) (Gao et al., 2011; Steiner et al., 2011) indicating that chloroplast biogenesis can be separated from photomorphogenesis, we used it as a tool to separate the gene groups regulated by either light or plastid developmental stage. To this end we performed genome-wide differential gene expression profiling in *Arabidopsis* by microarray hybridization. In order to unravel truly

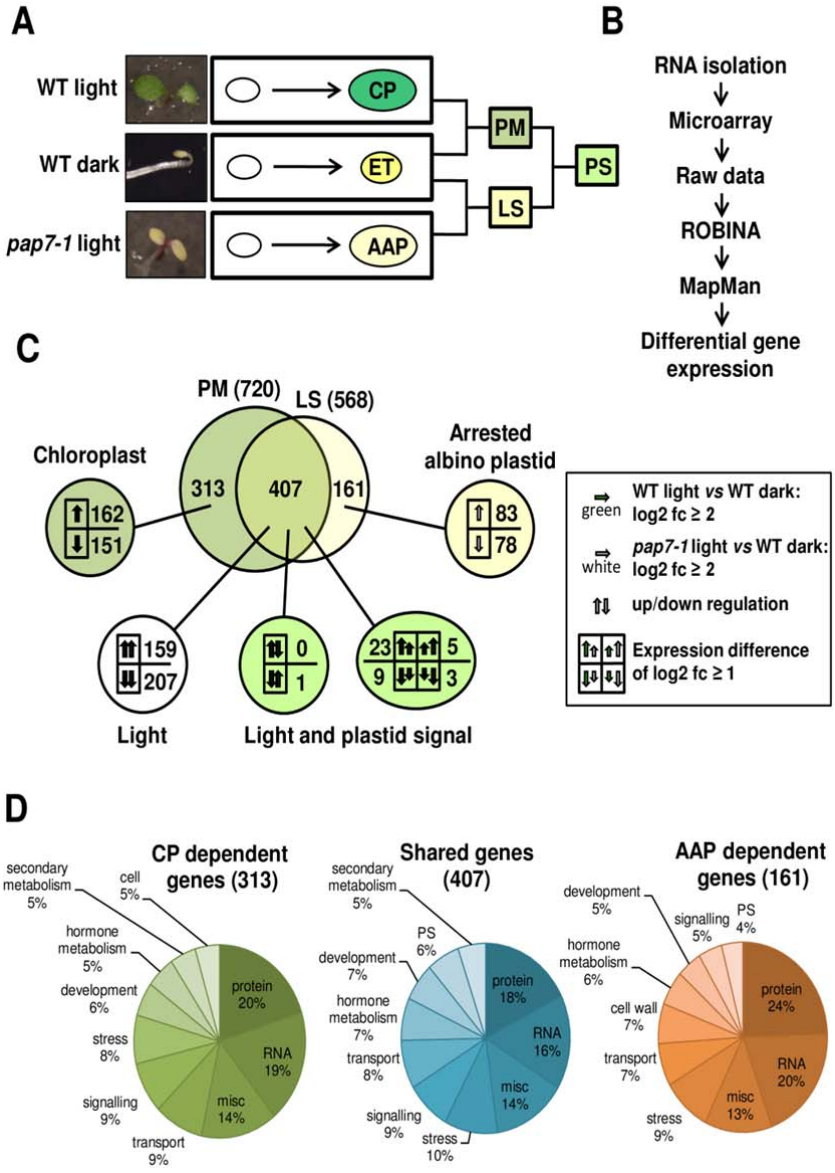

Figure 3. Identification of gene modules responsive to light and/or biogenic plastid signals. A, Strategy for differential expression profiling of 4-5-day-old *Arabidopsis* seedlings in wildtype (WT) and *pap7-1* mutants (*pap7-1*). Left panel displays photographs of seedlings with representative phenotypic appearance. The right panel indicates the corresponding developmental transition of the plastids in each of these seedlings. White small ovals represent undifferentiated pro-/eoplasts from seeds. Upon illumination they develop into green chloroplasts (CP) in WT (WT light) or arrested albino plastids (AAP) in *pap7* mutants (*pap7-1* light). Growth in the dark leads to development of yellow etioplasts (ET) in WT (WT dark). Analysis of differences between expression profiles in these plant samples (indicated by brackets) identify genes for photomorphogenesis (PM), for plastid-independent light signaling (LS) and for plastid signalling (PS) (for details see text). B, Flow diagramme of bioinformatic analysis done with primary expression data from the microarray analysis. C, Detailed comparison of differentially expressed genes (indicated by numbers) within the PM and LS expression modules according to their direction of expression change (indicated by arrows). Only genes exceeding a threshold of log2 fc ≥ 2 were taken into analysis. Genes found in both modules were further separated according to their direction and degree of gene expression change. Arrows of different size but same direction indicate genes that display the same direction of expression change but with a difference of at least log2 fc ≥ 1. Green arrows: Expression change in WT. White arrows: Expression change in *pap7-1* mutants. For more details see legend box. D, Proportional distribution of genes within the different gene groups defined in Fig. 3C according to their functional association within MapMan bins.

251 light- and plastid-dependent gene expression changes we did a trilateral comparative

252 profiling including light grown *pap7-1* mutant seedlings (*pap7-1*-light, containing white
253 plastids), light-grown WT seedlings (WT-light, containing chloroplasts) and dark-grown WT
254 seedlings (WT-dark, containing etioplasts), all at the two-cotyledon-stage (Fig. 3A). We
255 performed a supervised analysis of the expression data using the MapMan tool (Usadel et
256 al., 2005) and an unsupervised analysis by performing a weighted gene expression network
257 analysis (WGCNA) using gene ontology (GO) groups in order to combine the advantages of
258 both gene categorization tools (Klie and Nikoloski, 2012).

259 In our supervised strategy we assumed that a WT-light to WT-dark comparison reveals
260 expression changes controlled by the photomorphogenic program (PM, Fig. 3A). This should
261 identify all genes activated or inactivated by light and plastid signals. A parallel comparison of
262 *pap7-1*-light and WT-dark should identify genes being regulated only by light while the
263 developmental status of the plastid is negligible (light signaling, LS, Fig. 3A) since both
264 samples do not develop chloroplasts. Subsequent comparison of the significantly regulated
265 gene groups identified in PM and LS then should identify genes specifically regulated by
266 light, by plastid stage or by both (PS, Fig. 3A).

267 Significantly regulated genes in the data sets were identified (Supplemental Table 1) and
268 were imported into the MapMan visualization tool (Fig. 3B, Supplemental Figure 1). As
269 expected, the PM data set revealed large genome-wide changes known to be characteristic
270 for the photomorphogenic program (Ma et al., 2001). This included a massive up-regulation
271 of genes for photosynthesis, energy metabolism and tetrapyrrole biosynthesis as well as
272 sulfate reduction and a great number of other biosynthetic pathways (Supplemental Figure
273 1A). In the LS data set we observed an impact on mostly the same gene groups and with
274 similar strength indicating that the light-regulation in the *pap7-1* mutant works in a
275 comparable manner as in WT (Supplemental Figure 1B). Direct comparison of *pap7-1*-light
276 *versus* WT-light gene expression profiles demonstrated only limited differences
277 (Supplemental Figure 1C) indicating that the gene regulation in the light-grown *pap7-1*
278 mutant resembles much more to that of light-grown than to dark-grown WT. This indicates
279 that the lack of functional chloroplasts in the *pap7-1* mutant exerts only a minor impact on
280 light-regulated gene expression networks.

281 The quality of the expression profiles obtained in the microarray approaches was tested by
282 quantitative RT-PCR of selected genes being representative for different expression classes
283 found. To this end RNA was isolated from identically, but independently grown plant samples
284 from WT and *pap7-1* mutants. As a further independent control for the arrested plastid
285 development another *pap* mutant, *pap6-1*, was used (Gilkerson et al., 2012). All genes tested
286 in the qRT-PCR displayed highly reproducible expression data (correlation factors above 0.9)
287 when compared to the corresponding data obtained from the microarrays (Fig. 4,

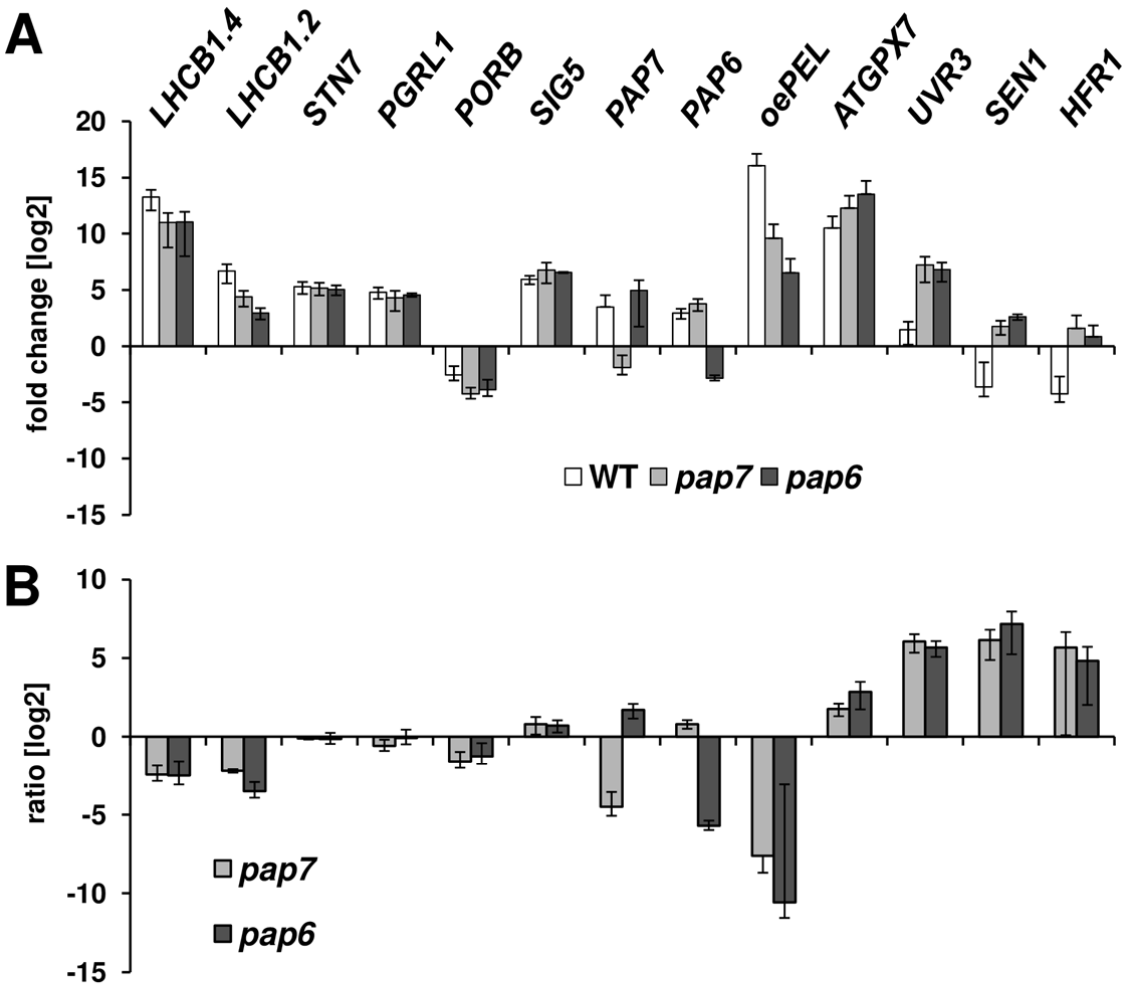

Figure 4. Expression profiles of selected genes from the microarray analysis tested by qRT-PCR. (a) Expression changes of genes representative for distinct expression classes in WT-light, *pap7-1* and *pap6-1* mutants compared to WT-dark. Genes for proteins Lhcb1.4, Lhcb1.2 and oePEL (light harvesting complex II proteins 1.4 and 1.2, "overexpression leads to pseudo-etiolation in light") represent genes displaying a reduced light induction in *pap7-1* when compared to WT. Genes encoding proteins STN7, PGRL1a and SIG5 (state transition kinase 7, PGR5-like protein 1a, sigma factor 5) represent genes displaying no effect of *pap7-1* on light induction, while the gene for PORB (protochlorophyllide oxidoreductase B) represents an example for strong light repression both in WT and *pap7-1* mutant. The genes for ATGPX7 and UVR3 (glutathione peroxidase 7 and (6-4) DNA photolyase) were used as examples displaying light induction in WT and further promotion in *pap7-1*. Genes for SEN1 and HFR1 (senescence-associated protein DIN1 and the transcription factor Long Hypocotyl after far-red 1) represent genes displaying repression in WT but promotion in the mutant. As additional genetic control *pap6-1/fln1 (*defective in the gene for a phospho-fructokinase like 1 protein) was used in order to detect potential mutation-specific responses. Expression of both *pap* genes was tested in all RNA samples as further control. (b) Difference in expression change between light grown WT and the *pap7-1* and *pap6-1* mutants. Negative values indicate lower expression, positive values higher expression than in WT. Data given represent means of three independent experiments. Primers used are given in Supplemental File 12.

Supplemental Figure 2). Furthermore, the expression data in the two different *pap* mutants displayed a remarkable low variation (Fig. 4) indicating that the observed expression profiles in the mutant background are robust and likely representative not only for *pap7-1* but also for other *pap* mutants.

Identification of gene groups responding either to light or to biogenic plastid signals

Next, we performed a more detailed analysis of the various regulated gene groups. In order to restrict the analysis to strongly regulated genes we introduced a threshold of log2 fold change (fc) ≥ 2 for definition of significantly regulated genes (in total 881 genes). Identities and expression changes of these genes are given in Supplemental Table 2. 720 genes in the PM data set and 568 genes in the LS data set met this criterion (Fig. 3C). 407 genes were identified in both data sets with a majority being regulated in the same direction in both conditions (Fig. 3C, 159 up-regulated and 207 down-regulated genes). These 366 equally regulated genes were not influenced by the developmental state of the plastid but by illumination only. They, thus, represent plastid-independent, light-regulated genes. Only 41 of the 407 genes displayed either opposite (1 gene) or different strength in their regulation where we regarded only differences of at least log2 fc ≥ 1 as significant. These genes appear to be partly affected by both, plastid stage and light. The largest group (23 genes) displayed up-regulation in WT-light *versus* WT-dark, but significantly less up-regulation in *pap7-1*- light *versus* WT-dark. This expression pattern corresponds to the regulation mode attributed to the classical definition of a plastid signal that causes a lower accumulation of light-induced nuclear gene transcripts when chloroplast development is inhibited by chemical or genetic means (Pfannschmidt 2010).

In addition, we identified 313 genes that were exclusively up- (162) or down- (151) regulated only when chloroplast biogenesis occurred (CP-dependent genes, Fig. 3). These genes, thus, may either cause chloroplast biogenesis or are related to specific functions exerted by this fully developed plastid type such as retrograde redox regulation from photosynthesis. Interestingly, we identified 161 genes that displayed exclusive up- (83) or down- (78) regulation only in the presence of the arrested albino plastid (AAP-dependent genes, Fig. 3). These genes are likely regulated because of the missing chloroplast biogenesis or function (i.e. photosynthesis or connected biosynthesis pathways). Both developmental plastid stages apparently send distinct, light-independent signals to the nucleus that either repress or enhance separate sets of genes. Because of their light-independency these signals are not identical with the “classical” plastid signal and imply the existence of plastid-type specific positive and negative signals not yet defined by current models of retrograde signaling.

In total, 41.5% of the 881 strongly regulated genes appear to be light-regulated, 4.6% are regulated by combined light and plastid signals and 53.8% (corresponding to 474 genes) are regulated by plastid signals only (CP and AAP). Decreasing the threshold for significant regulation to log2 fc ≥ 1 identified 3658 regulated genes, which is roughly four times more

than with log2 fc ≥ 2 as threshold (Supplemental Table 3). In this larger group 46.4% of the genes appear to be light-regulated, 3.9% are regulated by light and plastid signals and 49.5% are regulated by plastid signals only (Supplemental Figure 3). Thus, enlargement of the number of investigated genes did not result in major relative changes between the three regulation modes indicating that the arbitrarily chosen thresholds did not produce a bias for specific regulation pattern. The inclusion of dark-grown plants as additional reference point instead of doing a direct comparison of WT-light and *pap7-1*-light, thus, provides a much more precise separation of plastid- and light-regulated genes. Both factors control very distinct sets of genes and the over-lap between the two signaling pathways/expression networks appears to be very limited.

Among photosynthesis-associated nuclear genes only *LHCB* genes are affected by plastid signals in *pap7-1*

The CP-, light- and AAP-dependent genes were finally sorted according to their functional categories (MapMan bin) (Fig. 3D). The majority of functional categories were identical for all three groups comprising the MapMan bins “protein”, “RNA”, “stress”, “transport”, “signaling”, “development” and “hormone metabolism”. Interestingly, the group of CP-dependent genes did not include “photosynthesis genes”, while the group of AAP-dependent genes did not include the bin “secondary metabolism”, but instead the bin “cell wall”. The large overlap in functional categories, however, is not reflected at the level of individual gene identities (Table S1) and our analysis clearly demonstrates that each gene group represents a distinct set of regulated genes (see also results from WGCNA).

The lack of photosynthesis genes in the CP-dependent group was surprising with respect to the notion that photosynthesis associated nuclear genes (PhANGs) are considered to be a prime target for plastid signals during biogenic control. We, therefore, analysed these genes separately and compared their relative expression changes in the PM and LS data sets (Fig. 5A). In WT-light *versus* WT-dark 76 genes exhibited a light-induced expression increase of log2 fc ≥ 1. 93 photosynthesis genes (including all plastome localized genes) displayed expression variations that remained below this threshold suggesting that they are not or just mildly affected by light. Only 15 genes exhibited a strong light induction of log2 fc ≥ 2. The top five of these genes all encode proteins of the light-harvesting complex of PSII (*LHCB*) (framed in Fig. 5A). In the *pap7-1*-light *versus* WT-dark comparison we observed a similar expression profile for photosynthesis genes. Here, 62 genes exhibited light-induced expression of log2 fc ≥ 1 and 11 genes displayed expression changes of log2 fc ≥ 2. Ten of these genes were also found as top-regulated genes in WT. However, we observed that

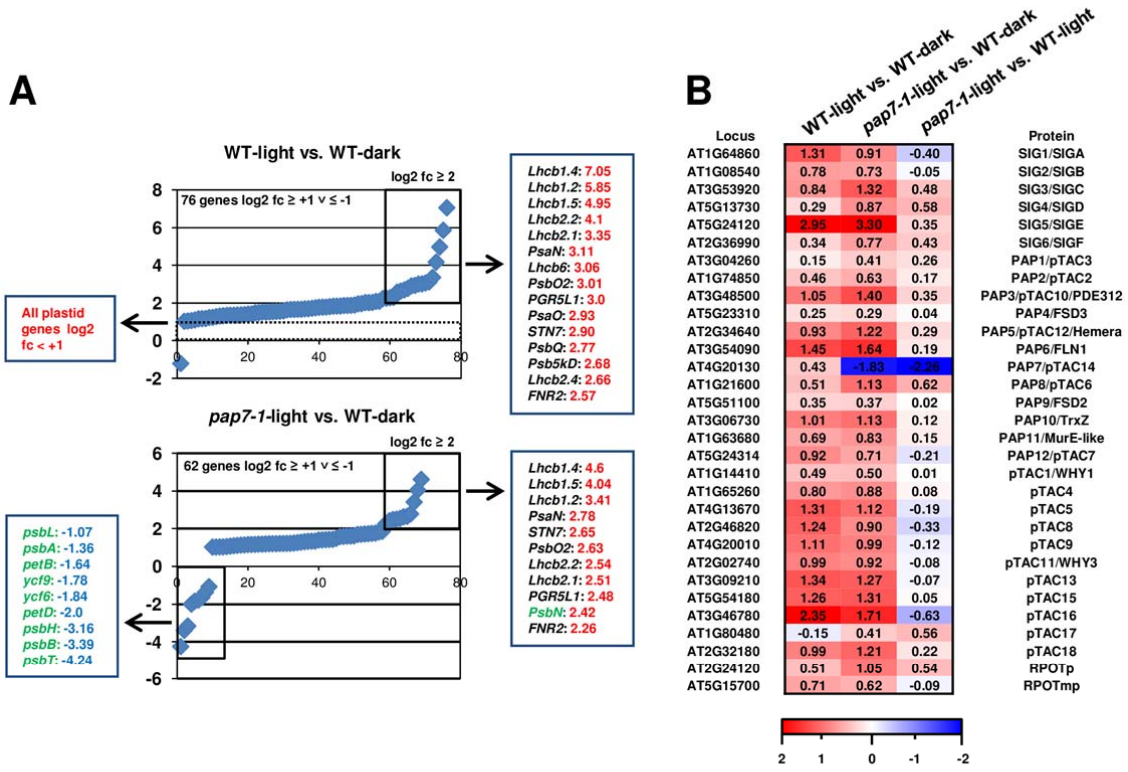

Figure 5. Light-induced expression changes of genes for photosynthesis and plastid transcription. A, The graphs display the expression values of significantly light-affected photosynthesis genes (log2 ≥ 1) detected by comparison of expression profiles WT-light *versus* WT-dark (top graph) and *pap7-1*-light *versus* WT-dark (bottom graph). From the 169 photosynthesis genes present in the corresponding MapMan bin 72 and 62 genes, respectively, exhibited an expression change of at least log2 ≥ 1. In the WT-light *versus* WT-dark comparison all plastid genes remained below this threshold. Genes exceeding an expression change of log2 ≥ 2 were boxed in the graph and listed in a panel aside. Nuclear encoded genes are given in black, plastid encoded genes are given in green. The difference in the expression change of a particular gene that occurs between the top and the bottom panel reflects the plastid influence on its expression. Strongly down-regulated genes in *pap7-1*-light *versus* WT-dark (log2 ≥ -1) are given in a left box. B, Expression changes for all 31 nuclear genes encoding components of the plastid gene transcription machinery in WT and *pap7-1*. The respective comparisons are indicated in the top panel, gene identities in the left and protein identities in the right panels. Given values represent log 2 fold changes, a corresponding color code is depicted at the bottom level.

specifically *LHCB* genes displayed significant less accumulation in *pap7-1* compared to WT while all other genes remained fairly constant. Comparable results were obtained in the independent qRT-PCR controls (Fig. 4). This reduced expression can be attributed to the impact of biogenic signals from the arrested albino plastid. In addition, we observed also nine significantly down-regulated genes in this comparison, all of them being plastome-localized (Fig. 5A). This confirms the observation using the MapMan visualization (Supplemental Figure 1) in which inhibition of chloroplast development had only limited impact on the overall nuclear gene expression profile. We conclude that inhibition of chloroplast development in

the *pap7-1* mutant represses specifically plastome-localized photosynthesis genes and of a small set of nuclear *LHCB* genes while all other PhANGs are either not affected or just mildly attenuated.

Another interesting question was whether or not other nuclear genes for components of the plastid transcription machinery were affected in their expression by the repressed PEP activity in *pap7-1* plastids. We, therefore, analyzed the expression data of genes for all sigma factors, PAPs, PTAC components and for NEP. The majority of these components displayed light induction in WT when compared to the dark control (Fig. 5B). This is in accordance with earlier bioinformatic analyses. The observed regulation patterns were largely maintained in the mutant with only minor deviations from the WT indicating that the developmental state of the plastid has also no significant impact on the expression of these genes while light appears to be a dominant regulator even in the mutant. The functional PEP deficiency, thus, does not exert a retrograde repressive control of other nuclear encoded PEP or NEP components.

Weighted gene expression correlation network analysis (WGCNA)

The supervised analysis (Fig. 3) used pre-settings based on assumptions drawn from literature and our own experiences. In order to avoid any unwanted bias we performed an additional un-supervised analysis of the gene expression data sets employing a weighted gene expression correlation network analysis (WGCNA) (Supplemental Figure 4). As in our supervised analysis the cluster analysis of the expression data revealed a much closer correlation between *pap7-1*-light and WT-light samples than between *pap7-1*-light and WT-dark samples (Fig. 6). Nevertheless, the gene expression profiles of *pap7-1*-light and WT-light samples displayed a number of specific differences. In total, six significant gene expression modules could be identified by WGCNA within the data set (Supplemental Figure 4). These modules describe characteristic similarities and differences between the three plant samples and largely correspond to the groups defined in the supervised differential analysis. Since the unsupervised analysis did not include expression thresholds, it, however, covered much larger gene numbers. The identified expression modules divide into two groups (Supplemental Figure 4B). Group1 comprises three modules (modules blue, yellow and green) in which two samples are highly similar while the third one is opposing. Group 2 comprises three modules (modules turquoise, brown and red) in which two samples are opposing while the third one is in an intermediate state between the two other.

Genes in module “blue” (in total 1838 genes) display highly similar expression in *pap7-1* light and WT light samples and an opposing expression in WT dark. The dominant regulating

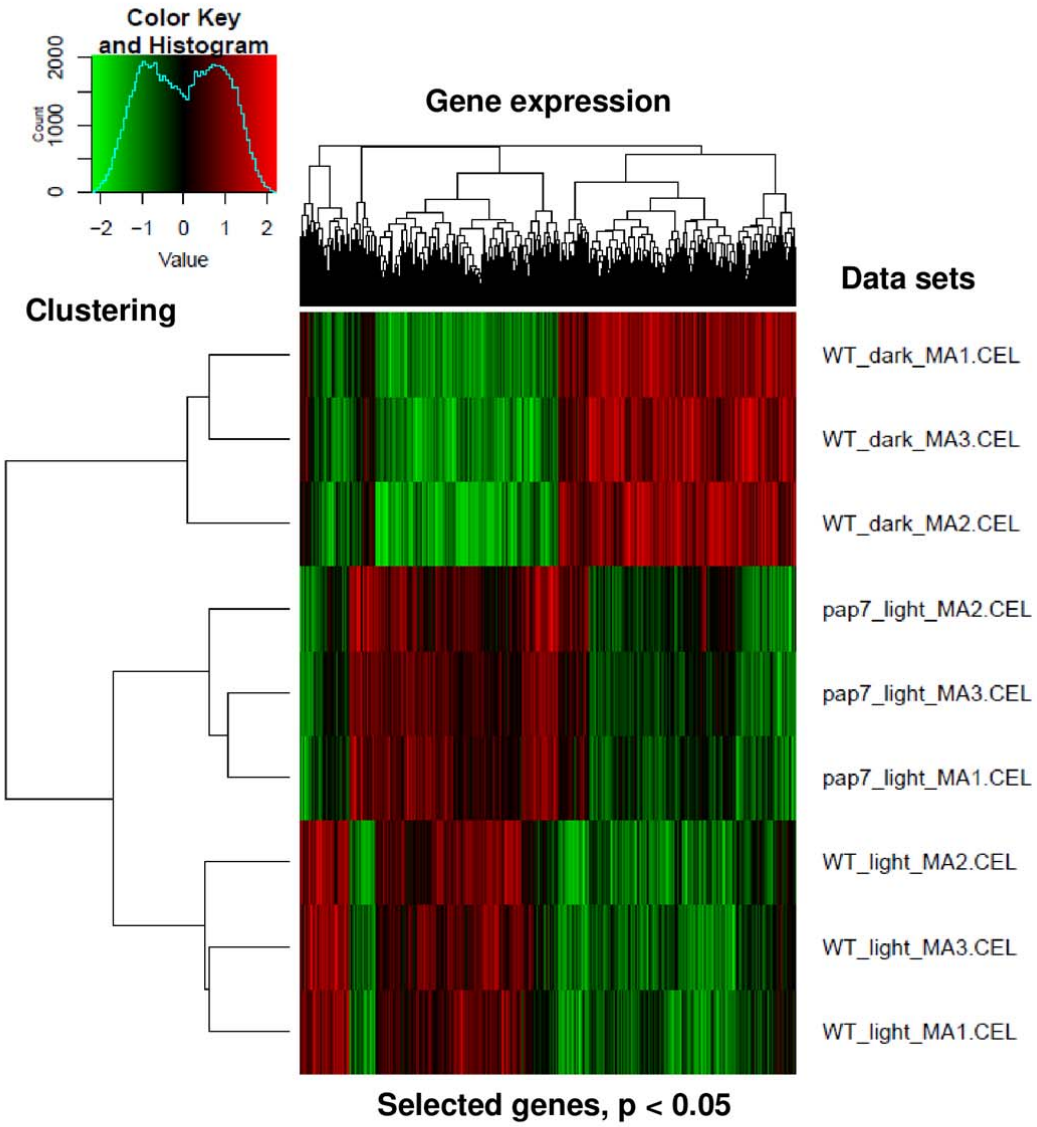

Figure 6. Cluster analysis of genes differentially regulated in the three growth set-ups as defined by ANOVA. Genes displaying a FDR of < 0.05 were included. The right margin identified the data sets from the microarray analysis, the left margin indicates a cladogramme defining the correlation in gene expression profiles between them. On top clustering of gene groups according to their expression is indicated. Diagramme in top left corner gives the color key and numbers of genes with corresponding gene expression values. Red: Up-regulation. Green: Down-regulation.

405 factor in this module, thus, is the light while the plastid state appears to play no or a very
406 minor role. Module “blue”, therefore, covers the light-regulated genes. Genes in module
407 “yellow” (in total 769 genes) display highly similar expression in *pap7-1* light and WT dark
408 samples and an opposing expression in WT light. The dominant regulating factor here is the

plastid state while light appears to have no major impact. Module “yellow”, thus, covers genes under plastidial regulation that is exerted from a normally developed chloroplast. Genes in module “green” (in total 671 genes) display a highly similar expression in WT light and WT dark samples and an opposing expression in *pap7-1* light. Light appears to be of no importance for the regulation of these genes, but the arrested plastid development and the genetic background of the mutant. Module “green”, therefore, covers genes that are mis-regulated because of the disturbance in plastid development and the *pap7-1* protein deficiency. The expression profiles of the genes covered by these three modules correspond to those in the three gene groups defined in our supervised analysis (Fig. 3C) and provide independent analytical confirmation for the correctness of our selection criteria. Understanding and interpretation of the modules in group 2 appeared to be much more difficult as the intermediate expression profiles in each module prevent an identification of the dominant regulating factor. Gene groups defined by these modules, thus, are likely under multi-factorial control and were, therefore, excluded from further analysis.

We analyzed the three gene modules for enriched GO groups (Supplemental Figures 5-7) and indicated most important GO groups with respect to the mutant phenotype below. Light-regulated genes in module “blue” included the GO groups for “Photosynthesis”, “Isoprenoid biosynthesis”, “Tetrapyrrole biosynthesis” and for “Anatomical structure and morphogenesis” (Fig. 7A). All these gene groups are apparently activated by light without displaying a major impact of the developmental state of the plastid. Like in the supervised analysis PhANGs occur under the light-regulated gene groups supporting the notion that the impact of the plastid stage on the expression of this gene group is very limited. Chloroplast-regulated genes in module “yellow” (Fig. 7B) contained GO groups for “Cellular metabolic processes”, “Photoperiodism and flowering”, “Fatty acid beta oxidation”, “Protein localization to peroxisome”, “Glucosinolate biosynthetic process” and “Indoleacetic acid metabolic processes”. While the first four groups appear to be repressed, the latter two are mainly activated by the presence of functional chloroplasts. Finally, in the *pap7-1*-regulated module “green” (Fig. 7C) we found GO groups for “Nitrogen compound metabolism processes”, “Circadian rhythm” and “Reproductive system development”. These gene groups were either activated or stayed active upon the genetic arrest of chloroplast biogenesis. Our triplicate analyses, thus, describe distinct gene sets that are specifically targeted by the three factors light, chloroplasts and arrested albino plastids both in positive and negative manner.

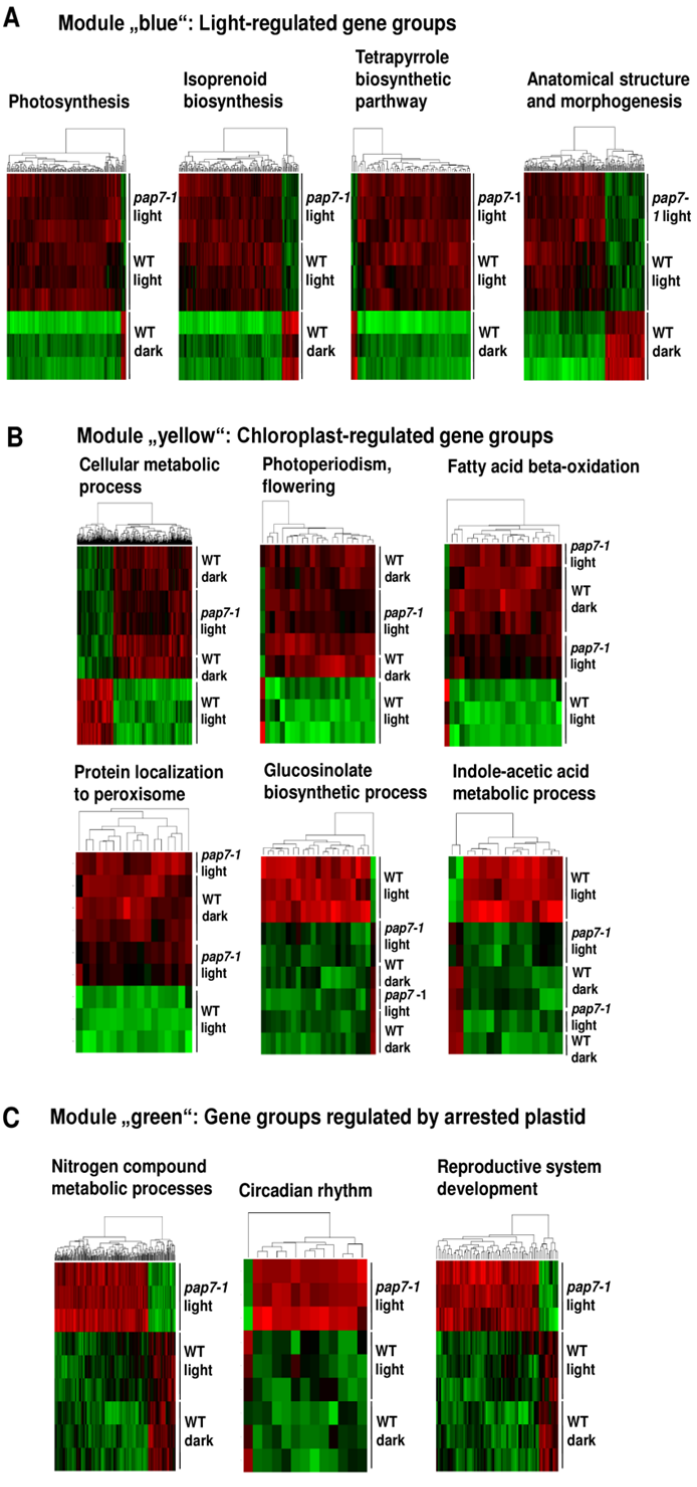

Figure 7. GO groups of differentially expressed gene sets within the gene modules identified by WGCNA. A, Gene module „blue“ with four major gene groups regulated by light. B, Gene module „yellow“ with six major gene groups regulated by the chloroplast. C, Gene module „green“ with three major gene groups specifically regulated by the arrested plastid. Genes displaying a FDR of < 0.05 were included. On top of each heat map the selected GO group is given, underneath a clustering of the genes in this group according to their expression is indicated. The right margin identifies the data sets from the microarray analysis. Red: Up-regulation. Green: Down-regulation. For further gene groups see Supplemental Files 8-10.

Our plastome- and genome-wide gene expression analysis in the *pap7-1* mutant provides the first detailed view of the disturbances occurring at the transcript level of this mutant. This revealed interesting and unexpected facts that help to better understand the causes for the albinism in this mutant. In addition, the obtained data provide important novel clues for our understanding of other *pap* mutants as well as retrograde biogenic signals and their target genes.

Impact of *pap7-1* deficiency on plastid gene expression

Elucidation of the plastid transcriptome in the *pap7-1* mutant uncovered that many PEP-dependent class I transcripts displayed only low accumulation while NEP-dependent *rpo* transcripts exhibited enhanced accumulation (Fig. 2). This is in coincidence with earlier reports. However, we observed significant differences between *psa* and *psb* gene groups suggesting differential effects on class I transcription. In addition, NEP-dependent *ycf1* and *accD* gene transcripts did not over-accumulate as *rpo* transcripts, but exhibited clearly reduced transcript accumulation (Fig. 2). Furthermore, class II transcripts revealed largely WT-like accumulation in the mutant (Fig. 2). These effects cannot be reconciled with the current model of plastid transcription in albino plastids of *pap* mutants which assumes a general inactivation of PEP activity while the NEP activities are up-regulated. Instead these data imply differential and gene-specific effects on transcription activities in the albino plastids. Since all class I transcripts did accumulate to a certain degree in *pap7-1* plastids it is likely that these arise from a basal activity of the PEP core-complex. Such a basal activity was identified in etioplasts of mustard and was shown to be able of faithful promoter recognition using sigma factors, however, in a different manner than the corresponding activity from chloroplasts (Eisermann et al., 1990; Tiller and Link, 1993; Pfannschmidt and Link, 1994). This scenario could provide a realistic explanation for the observed transcriptome in the *pap7-1* mutant plastids and suggest that the gene expression mechanisms in the albino plastids are likely arrested in a stage similar to etioplasts. Alternatively, the residual class I transcripts may arise from read-through transcription performed by NEP enzyme activities; however, such a model would lack an explanation for differential gene group transcription. Regardless of their origin, the residual class I transcripts apparently are not sufficient to elicit the formation of a photosynthetic apparatus suggesting the involvement of additional constraints for the chloroplast formation.

One such constraint could be the enhanced antisense transcript accumulation in the mutant which was observed notably for the *psbB/T/H/petB/D* operon, the *rpl33/rps18* operon and the *ycf1* and *accD* genes (Fig. 2). As this effect appears to be rather gene-specific it likely does

not represent just an arbitrary accumulation of read-through transcription of the respective opposite strand of the plastome. Mechanistically, one could expect that enhanced antisense transcript accumulation interferes with translation efficiency of the corresponding sense transcript due to duplex formation as it was suggested for the plastid *psbT* gene (Zghidi-Abouzid et al., 2011). This would provide a gene-group-specific mechanism that could prevent the formation of functional photosystem II and $Cytb_6f$ complexes. Whether this antisense production is based on a specific transcription event will be an interesting field of future research.

Our microarray analysis of *pap7-1* RNA samples indicated a strongly reduced accumulation of most plastidial tRNAs (Supplemental file 1). This is in agreement with a recent study performed in several maize *pap* mutants proposing that PEP activity has a major role in the expression of plastidial tRNAs (Williams-Carrier et al., 2014). Reduction in plastid tRNA accumulation restricts plastid translation as the tRNA molecules transfer the amino-acids. Furthermore, since the $tRNA^E$ is the precursor of amino-levulinic acid (ALA), tetrapyrrole biosynthesis and the generation of chlorophylls might be severely affected.

In sum the differential accumulation of gene-specific transcripts of all classes implies the existence of specific, yet unknown, transcription events in the arrested albino plastids suggesting a more defined and diversified division of labor between the PEP and NEP enzymes during early steps of chloroplast biogenesis than current models propose.

Retrograde control of nuclear gene expression by biogenic plastidial signals

A major improvement of our study when compared to earlier work in this field arose from our experimental set-up that included dark-grown WT plants as additional reference point. Inclusion of homozygous *pap7-1* mutants grown in the dark as further control was not feasible as homozygous mutant seedlings in this stage remain macroscopically indistinguishable from WT or heterozygous mutants (Fig. 1A).Discrimination between WT and mutant genotypes at this stage would be technically possible only at the molecular level of individual seedlings exacerbating largely the harvest of sufficient material for RNA preparation. The indistinguishability of dark-grown homozygous mutants is in accordance with our working hypothesis that the mutation becomes effective only under illumination while it does not affect the skotomorphogenic programme. Consequently, segregation of the progeny of heterozygous *pap7-1* mutant plants becomes macroscopically visible only in the light when the transition towards chloroplasts is arrested. Nevertheless the trilateral expression profiling allowed us for defining unambiguously distinct gene sets responding specifically to i) light, to ii) chloroplast signals and to iii) signals from arrested albino plastids. This distinction is impossible by a simple bilateral mutant-WT comparison in the light as this

provides only the relative differences between the two conditions. The impact of light in each of them requires the additional comparison with the dark control which then reveals whether light has a promoting, inhibiting or no effect and allows finally for the distinction between retrograde and light control.

The three gene modules identified by our approach contain both activated and repressed genes, thus, implying the existence of retrograde biogenic signals that are of either positive or negative nature. Since the gene sets regulated by chloroplasts and arrested plastids are different it is likely that the controlling signals are transmitted via separate pathways. Whether these are fully independent from each other or whether they do mutually exclude each other remains to be investigated. The question whether a weaker nuclear gene expression indicates the action of a negative signal or the missing action of a positive signal has been debated in the past (Pfannschmidt, 2010; Terry and Smith, 2013; Hills et al., 2015). A recent study (Page et al., 2017) and our data presented here indicate that the action of both, positive and negative signals, needs to be considered in current models.

The phenotypic analysis (Fig. 1) revealed that despite chloroplast deficiency the general photomorphogenic programme in the *pap7-1* mutant appears to be operational as long as an external carbon source is available. Older mutant plants generate a complete albino rosette that is comparable to those of dark-grown mutants with constitutively active phytochromes (Su and Lagarias, 2007) confirming that general plant development and chloroplast biogenesis represent largely separate processes. Even the initiation of the flowering transition was found to be functional, which corresponds to observations reported for *rpo* deletion mutants of tobacco (De Santis-Maclossek et al., 1999). We thus assume that illumination activates the photoreceptor systems in both WT and *pap7-1* mutant seedlings in the same manner suggesting that synthesis and action of the photoreceptors (including their chromophores) are fully functional in the albino mutant.

This explains the similarity of the expression profiles of light-grown WT and *pap7-1* mutants (Supplemental Figure 1) including the expression of the corresponding light-regulated gene module (module “blue”) (Fig. 8). The module contains the GO groups for isoprenoid and tetrapyrrole biosynthesis (Fig. 7) that produce important precursor for primary products such as carotenoids, chlorophylls, haem, phytochromobilin, plastoquinones or different plant hormones (Pulido et al., 2012). Both biosynthesis pathways provide essential metabolites for plant metabolism and development and, therefore, are likely active in the albino plastids with the apparent exception of Chl biosynthesis. For the same reasons the GO group for morphogenesis and anatomical structure (Fig. 7) may appear in this module because it is part of the light-initiation of photomorphogenesis. Unexpectedly, however, was the identification of PhANGs that, in contrast to general assumptions, did not exhibit any major

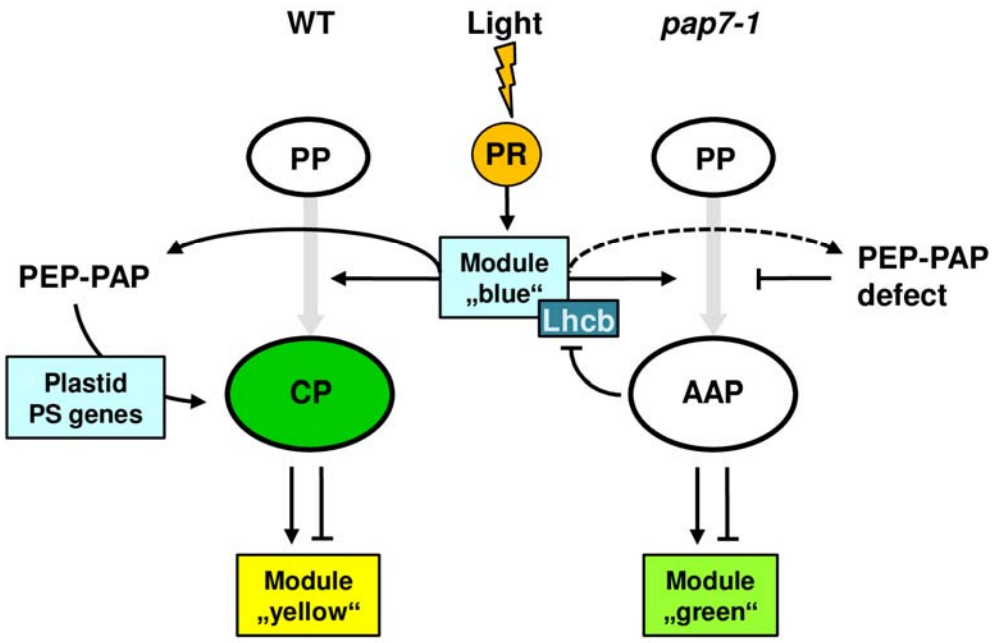

Figure 8. Model of anterograde and retrograde signalling during early steps of chloroplast biogenesis in Wt and *pap7-1* mutants. Light signals (flash) are perceived by photoreceptors (PR) (orange circle). Small white ovals represent undifferentiated pro-/eoplasts present in seeds. Large ovals in green and white represent the different plastid types in WT (CP) and *pap7-1* (AAP) seedlings. Grey bold arrows indicate the developmental process leading to these plastid types. Boxes indicate and name the genes or gene modules regulated during these processes. Thin black arrows represent positive regulation, lines with a blocking bar represent negative regulation.

repression by retrograde signals from the arrested plastid development. Only a few *Lhcb* genes appeared to be selectively targeted by plastid signals (Fig. 5) and these, in addition, seem to interact with light (Figs. 3, 5). This may explain the manifold connections identified between light and plastid signaling using promoters of these genes in genetic screens and corresponding mutants (Larkin, 2014). Interestingly, the structural and functional defect in the PEP-PAP complex in *pap7-1* mutants does not affect the transcript accumulation of other nuclear encoded components of the plastid transcription machinery (Fig. 5B) implying that these genes are not under retrograde control.

It remains to understand why chloroplast biogenesis does not work in the mutant. A major determinant of this developmental block certainly is the reduced expression of plastid photosystem II genes, but the finding of residual transcripts suggests that likely also other molecular reasons play a role such as the obvious inhibition of Chl biosynthesis (Gao et al., 2012) as well as further defects that are unknown to date. We conclude that in the *pap7-1* mutant especially plastidial photosystem II genes are strongly repressed while retrograde

565 signals from the arrested albino plastid neither modulate nor antagonistically counteract light
566 regulation of PhANGs. This is different from conclusions obtained in recent studies on
567 retrograde signaling using lincomycin, an inhibitor of plastid translation (Ruckle et al., 2007;
568 Martin et al., 2016). Since *pap7-1* mutants developed rather normal in the first few days even
569 without sugar we regard it as likely that either the genetic block in *pap7-1* mutants results in
570 milder effects than a lincomycin treatment or the observed differences are due to technical
571 differences in the respective set-ups, e.g. light intensity. Both senarios suggest that there
572 exists a threshold for the effectiveness of retrograde signals. Elucidating the molecular
573 nature for this will be an interesting topic for future research. The retrograde-controlled gene
574 groups identified in this study provide a useful first tool for such investigations.

575 An interesting result of our expression profiling was the identification of separate biogenic
576 signals from chloroplasts and albino plastids. Chloroplast signal-dependent GO groups were
577 mostly related to metabolism, likely because chloroplast biogenesis initiates the conversion
578 from a heterotrophic to autotrophic life style. This includes the down-regulation of beta-
579 oxidation of fatty acids that takes place in glyoxisomes. Oppositely GO groups for
580 glucosinolate biosynthesis and indoleacetic acid biosynthesis were activated, both being
581 highly important for pathogen defense and growth of green plants (Fig.7 B). GO groups
582 regulated by signals from the arrested albino plastid relate mostly to starvation processes
583 and the mobilization of storage energies (nitrogen compound metabolic processes,
584 reproductive system development) being indicative of the non-autotrophic metabolism in the
585 albino plantlets that requires the mobilization of all internal resources. Surprisingly a
586 significant impact on the GO group of circadian rhythm was observed that suggests an
587 influence of the plastid developmental stage on circadian clock genes. A circadian control
588 from the nucleus influencing chloroplast transcription has been recently reported (Noordally
589 et al., 2013). Furthermore, iron metabolism and plastid developmental stage were
590 demonstrated to have a significant influence on the period of the circadian clock (Salome et
591 al., 2013). These observations suggest that our results reflect a mutual influence between
592 plastids and the circadian clock of larger significance providing an interesting target for future
593 research (Fig. 7C).

594 In sum, the arrest of chloroplast development in the *pap7-1* mutant can be best explained by
595 a specific disturbance in the light-induced build-up of the PEP complex during the pro-
596 /eoplast-to-chloroplast transition. This leads to concomitant defects in the expression of PSII
597 components and tRNAs, which in turn limits Chl biosynthesis and (likely) translation. The
598 albinism of the mutant, thus, likely is the result of a multi-factorial syndrome that prevents
599 chloroplast biogenesis without destroying the plastid. Therefore, the functional and metabolic
600 state of the arrested albino plastid resembles that of an etioplast despite it is perceiving light.

This likely allows the mutant to develop normally when the lack of photosynthesis is complemented by an external carbon source. Correspondingly the overall gene expression profiles of *pap7-1* mutants do exhibit a chimerical character with metabolic genes regulated like in dark-grown seedlings and photo-regulated genes like in light-grown plants. In sum, the *pap7-1* mutant, but likely also other *pap* mutants provide an interesting tool for dissecting further the molecular processes during the early steps in chloroplast biogenesis.

MATERIALS AND METHODS

Plant material

We used *Arabidopsis thaliana* Columbia (Col) 0 as WT throughout the study. As null alleles for the genes *pap7-1/ptac14* and *pap6/fln1* we used the *Arabidopsis* T-DNA inactivation lines SAIL_566_F06 and GK-443A08, respectively. These lines (named *pap7-1* and *pap6-1* in this study) were characterized earlier in detail for singularity of T-DNA insertion and for causal connection between the albino phenotype and the corresponding gene defect (Arsova et al., 2010; Gao et al., 2011; Steiner et al., 2011; Gilkerson et al., 2012). Seeds of WT and heterozygous mutants were surface-sterilized and spread on half-strength standard MS medium supplemented with indicated amounts of sucrose in Petri dishes, stratified for 3 days and grown to the two-cotyledon stage at 21°C for further analyses. Light-grown plants used for array analyses were grown under permanent white light of 120-150 µE photon flux density. In long-term growth experiments the illumination intensity of the white light source was reduced to 8-12 µE.

Plastid macro-array analysis

Plant material was grown 6 days on MS medium supplemented with 0.5% sucrose. Around 500 mg each of green WT and albino mutant cotyledons were harvested separately and shock-frozen in liquid nitrogen. Total RNA was isolated following published procedures (Demarsy et al., 2006; Demarsy et al., 2012). Potential DNA contaminations were removed by DNase treatment and its absence was proven by PCR. For preparation of the hybridization probe 4 µg of each RNA preparation were reverse-transcribed using a gene-specific primer mix annealing to 80 protein-coding genes and their corresponding antisense sequences. Subsequently, a reverse transcription reaction was performed using Superscript II Reverse Transcriptase in presence of all four nucleotides and 100 µCi [α-^{32}P] dATP (Demarsy et al., 2012). Unincorporated nucleotides were finally removed from the labelled cDNAs by gel-filtration through a Sephadex G50 column. For evaluation of relative differences in plastid transcription between the biological backgrounds of the two samples we

measured the total radioactivity of each cDNA sample after synthesis and verified the cDNA profile on a sequencing gel. In the experiment described here radioactively labelled cDNAs of WT and *pap7-1* differed by just 3% (WT>*pap7-1*) in incorporation indicating that total plastid gene expression is not significantly different in WT and *pap7-1* plantlets. Subsequent hybridization of the radiolabelled probes with the macroarray, washing conditions, subsequent signal detection in a phosphorimager (Fujifilm FLA-8000) and final data analysis were done essentially as described (Demarsy et al., 2012). Construction of the plastome macroarray including description of the gene-specific sense and antisense probes as well as their spotting pattern were published elsewhere (Lerbs-Mache, 2011).

Genome-wide micro-array analysis

Plant material was grown on medium supplemented with 1% sugar in order harmonize the germination. Illuminated WT and homozygous *pap7-1* mutants were grown for 5 days for full expansion of the cotyledons while dark-grown WT seedlings were grown for 4 days in order to avoid mechanical stress imposed by physical contact with the lid of the Petri dishes. Plant materials were harvested 10:00 in the morning and shock frozen in liquid nitrogen. Dark grown material was harvested and shock-frozen at the same time point of the day, but under a green safe-light in order to exclude any light effects in the profiles. Total RNA from these materials then was basically prepared as described (Logemann et al., 1987). In brief, 250 mg of frozen plant material was ground in a mortar and purified with the Qiagen RNeasy purification kit. Concentration and purity of RNA samples were determined spectroscopically and intactness was proven by ethidium bromide staining after separation on 1.2% agarose gels. Purified samples from three biological replicates each were sent on dry ice to a commercial service (Kompetenzzentrum für Fluoreszente Bioanalytik (KFB) Regensburg, Germany) where a second quality check, cDNA synthesis and labelling was performed according to the GeneChip 3' IVT Express Kit protocol (Affymetrix). Hybridisation and reading of signals were performed using the Arabidopsis Genome Array ATH1 (Affymetrix, USA) and according to standard protocols of the service.

Quantitative reverse transcription PCR analysis (qRT-PCR)

Reverse transcription was performed with 1 µg total RNA isolated from three independent biological replicates of WT, *pap7-1* and *pap6* mutants grown identically as for the microarray analyses. cDNA was synthesized using oligo(dT) primers and the Invitrogen™ SuperScript™ II Reverse Transcriptase following the manufacturers´ recommendations. qRT-PCR was performed using the GoTaq® qPCR Master Mix (Promega) and the Rotor-Gene 3000™ equipment. Primer sequences for genes of interest were designed using ApE-A plasmid Editor (v2.0.47) and NCBI/ Primer-BLAST (Basic Local Alignment Search Tool) with

671 preference to intron spanning amplicons. Each primer pair was tested for amplification
672 efficiency using the synthesized cDNA. Only primer pairs with amplification efficiencies
673 between 90% and 110% were used for further analysis. For used primer sequences and
674 gene identities see Supplemental Table 4. The relative quantification was calculated
675 according to described methods (Pfaffl, 2001). The *Arabidopsis* genes for actin 7 and
676 ubiquitin 5 were used as internal control and reference genes for quantification.
677

678 **Bioinformatics**

679 In the supervised analysis the hybridization signal data from the microarray analysis
680 performed by a commercial service (KFB Regensburg, Germany) were analyzed with the
681 ROBINA (http://mapman.gabipd.org/web/guest/ robin-download) and MapMan
682 (http://mapman.gabipd.org/web/guest/robin-download) programmes (Usadel et al., 2005;
683 Usadel et al., 2009). Statistical analysis by t-test and subsequent calculation of the false
684 discovery rate were performed according to the ROBINA programme. Gene expression
685 changes with a FDR of $p \leq 0.05$ were regarded as statistically significant. The microarray
686 data given in the Supplemental file 1 are based on three biological replicates each. The data
687 discussed in this publication have been deposited in NCBI's Gene Expression Omnibus
688 (Edgar et al., 2002; Barrett et al., 2013) and are accessible through GEO Series accession
689 number GSE88988 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE88988).
690 Visualization of the cellular pathways and functional categories of the expression data was
691 carried out using the MapMan and Pegman package according to
692 Ath_AFFY_ATH1_TAIR8_Jan 2010 (http://mapman.gabipd.org) (Usadel et al., 2005). The
693 visualization tool of MapMan was used to identify similarities and differences in the different
694 metabolic pathways. A Wilcoxon rank sum test was used to visualize significantly expressed
695 genes in Pegman (Usadel et al., 2009). Venn diagrams were calculated using the expression
696 log values of Map-man package. In the un-supervised WGCNA the original CEL files
697 supplied by the commercial service were imported into R with Bioconductor (package oligo)
698 (Carvalho and Irizarry, 2010) followed by normalization and background correction of the raw
699 data using RMA. After selecting 27296 annotated genes a scaling and centering followed by
700 a cluster analysis was performed. Differentially expressed genes were then selected with
701 ANOVA and a FDR threshold of 0.05 followed by a WGCNA (Langfelder and Horvath, 2008)
702 and a GO enrichment analysis using the R package topGO (Alexa and Rahnenführer,
703 http://www.mpi-sb.mpg.de/~alexa).

704

705 **SUPPLEMENTAL MATERIALS**

706 **Supplemental Figure 1.** Relative gene expression profiles of WT and *pap7-1* mutants
707 visualized using the MapMan tool.

708 **Supplemental Figure 2:** Correlation of expression data from selected genes obtained from
709 microarrays and qRT-PCR.

710 **Supplemental Figure 3:** Identification of gene modules responsive to light and/or biogenic
711 plastid signals with a significance threshold of log2 fc ≥ 1**.** .

712 **Supplemental Figure 4:** Weighted gene co-expression network cluster analysis.

713 **Supplemental Figure 5:** Gene ontology groups within module “Blue”.

714 **Supplemental Figure 6:** Gene ontology groups within module “Yellow”.

715 **Supplemental Figure 7:** Gene ontology groups within module “Green”.

716 **Supplemental Table 1:** Gene expression changes of genes sorted according to the
717 encoding genomic compartment.

718 **Supplemental Table 2:** Gene sets with an expression change larger than the threshold 2
719 [log2].

720 **Supplemental Table 3:** Gene sets with an expression change larger than the threshold 1
721 [log2].

722 **Supplemental Table 4:** Nucleotide sequences of primers used for qRT-PCR.

723 **Supplemental Datasets 1-3**

724

725

726 **FIGURE LEGENDS**

727 **Figure 1.** Developmental characteristics of the *pap7-1* mutant. A, 72h-dark-grown progeny of
728 a *pap7-1*/+ heterozygote subjected to 24h of 20 µE white light to trigger
729 photomorphogenesis. White arrowheads indicate a *pap7-1* homozygote mutant before and
730 after subjection to light. Hypocotyl length +/- standard deviation measured after 72 h of dark
731 growth. Genotypes were assigned after 24 h of light exposure (n= number of
732 measurements). B, Growth of homozygous *pap7-1* seedlings and WT on ½ strength MS-
733 medium in Petri-dishes without or with (+ suc) sucrose supplementation. C, Impact of *pap7-1*
734 inactivation on chloroplast development during transient embryo greening and seed

segregation in siliques of heterozygous *pap7-1* mutants (*pap7-1*/+) in comparison to WT (two left panels). Long-term growth (8 weeks) of the progeny of heterozygous mutants was performed on ½ strength MS-medium supplemented with 3% sucrose in transparent plastic containers in a short-day light period (right panel). Seed segregation into green and colourless seeds was counted in 7-10 siliques per measurement day (7 or 9 days after fertilization) in WT and *pap7-1*/+ mutants (bottom panel). D, Long-term grown plants in rosette stage were put into long-day conditions to induce flowering and photographed at the flowering stage.

Figure 2. Macro-array analysis comparing plastid transcript accumulation in light-grown wild-type and *pap7-1 Arabidopsis* seedlings. Given is the transcript accumulation in wild-type (top panel) and mutant (bottom panel) seedlings both for sense (blue bars) and anti-sense (red bars) transcripts. Hybridization signals were normalized to the total signal intensity of the membrane and are given as arbitrary units in the left margin. Genes are labeled at the bottom of each panel according to accepted nomenclatures. Sequence of genes corresponds to their organization on the plastome separated between inner strand (left parts) and outer strand of the plastome (right parts). High transcript accumulation of PEP-dependent transcripts in WT is high-lighted by green boxes. High transcript accumulation of NEP-dependent transcripts in the *pap7-1* mutant is high-lighted by yellow boxes.

Figure 3. Identification of gene modules responsive to light and/or biogenic plastid signals. A, Strategy for differential expression profiling of 4-5-day-old *Arabidopsis* seedlings in wildtype (WT) and *pap7-1* mutants (*pap7-1*). Left panel displays photographs of seedlings with representative phenotypic appearance. The right panel indicates the corresponding developmental transition of the plastids in each of these seedlings. White small ovals represent undifferentiated pro-/eoplasts from seeds. Upon illumination they develop into green chloroplasts (CP) in WT (WT light) or arrested albino plastids (AAP) in *pap7* mutants (*pap7-1* light). Growth in the dark leads to development of yellow etioplasts (ET) in WT (WT dark). Analysis of differences between expression profiles in these plant samples (indicated by brackets) identify genes for photomorphogenesis (PM), for plastid-independent light signaling (LS) and for plastid signalling (PS) (for details see text). B, Flow diagramme of bioinformatic analysis done with primary expression data from the microarray analysis. C, Detailed comparison of differentially expressed genes (indicated by numbers) within the PM and LS expression modules according to their direction of expression change (indicated by arrows). Only genes exceeding a threshold of log2 fc ≥ 2 were taken into analysis. Genes found in both modules were further separated according to their direction and degree of gene expression change. Arrows of different size but same direction indicate genes that display

771 the same direction of expression change but with a difference of at least log2 fc ≥ 1. Green
772 arrows: Expression change in WT. White arrows: Expression change in *pap7-1* mutants. For
773 more details see legend box. D, Proportional distribution of genes within the different gene
774 groups defined in Fig. 3C according to their functional association within MapMan bins.

775 **Figure 4.** Expression profiles of selected genes from the microarray analysis tested by qRT-
776 PCR. A, Expression changes of genes representative for distinct expression classes in WT-
777 light, *pap7-1* and *pap6-1* mutants compared to WT-dark. Genes for proteins Lhcb1.4,
778 Lhcb1.2 and oePEL (light harvesting complex II proteins 1.4 and 1.2, "overexpression leads
779 to pseudo-etiolation in light") represent genes displaying a reduced light induction in *pap7-1*
780 when compared to WT. Genes encoding proteins STN7, PGRL1a and SIG5 (state transition
781 kinase 7, PGR5-like protein 1a, sigma factor 5) represent genes displaying no effect of *pap7-*
782 *1* on light induction, while the gene for PORB (protochlorophyllide oxidoreductase B)
783 represents an example for strong light repression both in WT and *pap7-1* mutant. The genes
784 for ATGPX7 and UVR3 (glutathione peroxidase 7 and (6-4) DNA photolyase) were used as
785 examples displaying light induction in WT and further promotion in *pap7-1.* Genes for SEN1
786 and HFR1 (senescence-associated protein DIN1 and the transcription factor Long Hypocotyl
787 after far-red 1) represent genes displaying repression in WT but promotion in the mutant. As
788 additional genetic control *pap6-1/fln1 (*defective in the gene for a phospho-fructokinase like 1
789 protein) was used in order to detect potential mutation-specific responses. Expression of
790 both *pap* genes was tested in all RNA samples as further control. B, Difference in expression
791 change between light grown WT and the *pap7-1* and *pap6-1* mutants. Negative values
792 indicate lower expression, positive values higher expression than in WT. Data given
793 represent means of three independent experiments. Primers used are given in Supplemental
794 Table 4.

795 **Figure 5.** Light-induced expression changes of genes for photosynthesis and plastid
796 transcription. A, The graphs display the expression values of significantly light-affected
797 photosynthesis genes (log2 ≥ 1) detected by comparison of expression profiles WT-light
798 *versus* WT-dark (top graph) and *pap7-1*-light *versus* WT-dark (bottom graph). From the 169
799 photosynthesis genes present in the corresponding MapMan bin 72 and 62 genes,
800 respectively, exhibited an expression change of at least log2 ≥ 1. In the WT-light *versus* WT-
801 dark comparison all plastid genes remained below this threshold. Genes exceeding an
802 expression change of log2 ≥ 2 were boxed in the graph and listed in a panel aside. Nuclear
803 encoded genes are given in black, plastid encoded genes are given in green. The difference
804 in the expression change of a particular gene that occurs between the top and the bottom
805 panel reflects the plastid influence on its expression. Strongly down-regulated genes in *pap7-*
806 *1*-light *versus* WT-dark (log2 ≥ -1) are given in a left box. B, Expression changes for all 31

807 nuclear genes encoding components of the plastid gene transcription machinery in WT and
808 *pap7-1*. The respective comparisons are indicated in the top panel, gene identities in the left
809 and protein identities in the right panels. Given values represent log 2 fold changes, a
810 corresponding color code is depicted at the bottom level.

811 **Figure 6.** Cluster analysis of genes differentially regulated in the three growth set-ups as
812 defined by ANOVA. Genes displaying a FDR of < 0.05 were included. The right margin
813 identified the data sets from the microarray analysis, the left margin indicates a cladogramme
814 defining the correlation in gene expression profiles between them. On top clustering of gene
815 groups according to their expression is indicated. Diagramme in top left corner gives the
816 color key and numbers of genes with corresponding gene expression values. Red: Up-
817 regulation. Green: Down-regulation.

818 **Figure 7.** GO groups of differentially expressed gene sets within the gene modules identified
819 by WGCNA. A, Gene module „blue“ with four major gene groups regulated by light. B, Gene
820 module „yellow“ with six major gene groups regulated by the chloroplast. C, Gene module
821 „green“ with three major gene groups specifically regulated by the arrested plastid. Genes
822 displaying a FDR of < 0.05 were included. On top of each heat map the selected GO group is
823 given, underneath a clustering of the genes in this group according to their expression is
824 indicated. The right margin identifies the data sets from the microarray analysis. Red: Up-
825 regulation. Green: Down-regulation. For further gene groups see Supplemental Files 8-10.

826

827 **Figure 8.** Model of anterograde and retrograde signalling during early steps of chloroplast
828 biogenesis in Wt and *pap7-1* mutants. Light signals (flash) are perceived by photoreceptors
829 (PR) (orange circle). Small white ovals represent undifferentiated pro-/eoplasts present in
830 seeds. Large ovals in green and white represent the different plastid types in WT (CP) and
831 *pap7-1* (AAP) seedlings. Grey bold arrows indicate the developmental process leading to
832 these plastid types. Boxes indicate and name the genes or gene modules regulated during
833 these processes. Thin black arrows represent positive regulation, lines with a blocking bar
834 represent negative regulation.

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836

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