Evidence for a contribution of ALA synthesis to plastid-to-nucleus signaling

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INTRODUCTION

Organellar genes translocated to the nucleus of host organisms during the process of evolution remain necessary for the function of organelles that now support important processes within their hosts. For example, several thousand nucleus-encoded proteins are required for chloroplast biogenesis, including the formation of complexes consisting of both nuclear and plastid-encoded subunits (Timmis et al., 2004). This bigenome-dependent assembly of functional protein complexes requires a finely tuned balance of anterograde and retrograde control. While anterograde control determines organellar metabolism and gene expression, nuclear gene expression (NGE) are under retrograde control responding to organelles. However, detailed knowledge about signaling molecules and pathways is still lacking (Papenbrock and Grimm, 2001; Brusslan and Peterson, 2002; Surpin et al., 2002; Gray et al., 2003; Pfannschmidt et al., 2003; Baier and Dietz, 2005; Beck and Grimm, 2006; Pogson et al., 2008; Kleine et al., 2009; Jung and Chory, 2010).

The formation of 5-aminolevulinic acid (ALA) in tetrapyrrole biosynthesis is widely controlled by environmental and metabolic feedback cues that determine the influx into the entire metabolic path. Because of its central role as the rate-limiting step, we hypothesized a potential role of ALA biosynthesis in tetrapyrrole-mediated retrograde signaling and exploited the direct impact of ALA biosynthesis on nuclear gene expression (NGE) by using two different approaches. Firstly, the Arabidopsis gun1, hy1 (gun2), hy2 (gun3), gun4 mutants showing uncoupled NGE from the physiological state of chloroplasts were thoroughly examined for regulatory modifications of ALA synthesis and transcriptional control in the nucleus. We found that reduced ALA-synthesizing capacity is common to analyzed gun mutants. Inhibition of ALA synthesis by gabaculine (GAB) that inactivates glutamate-1-semialdehyde aminotransferase and ALA feeding of wild-type and mutant seedlings corroborate the expression data of gun mutants. Transcript level of photosynthetic marker genes were enhanced in norflurazon (NF)-treated seedlings upon additional GAB treatment, while enhanced ALA amounts diminish these RNA levels in NF-treated wild-type in comparison to the solely NF-treated seedlings. Secondly, the impact of posttranslationally down-regulated ALA synthesis on NGE was investigated by global transcriptome analysis of GAB-treated Arabidopsis seedlings and the gun4-1 mutant, which is also characterized by reduced ALA formation. A common set of significantly modulated genes was identified indicating ALA synthesis as a potential signal emitter. The over-represented gene ontology categories of genes with decreased or increased transcript abundance highlighted a few biological processes and cellular functions, which are remarkably affected in response to plastid-localized ALA biosynthesis. These results support the hypothesis that ALA biosynthesis correlates with retrograde signaling-mediated control of NGE.

Keywords: ALA synthesis, retrograde signaling, gabaculine, gun mutants, microarray analysis

Abbreviations: ALA, 5-aminolevulinic acid; Chl, chlorophyll; fw, fresh weight; GAB, gabaculine; GluTR, glutamyl tRNA reductase; GO, gene ontology; GSAT, glutamate-1-semialdehyde aminotransferase; MgProto, Mg protoporphyrin IX; MgProtoMe, Mg protoporphyrin IX monomethyl ester; NF, norflurazon; NGE, nuclear gene expression; Pchlide, protochlorophyllide; PhANGs, photosynthesis-associated nuclear genes; POR, protochlorophyllide oxidoreductase; Proto, protoporphyrin IX; ROS, reactive oxygen species.

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**FIGURE 1 | Metabolic pathway of tetrapyrrole biosynthesis in plants.**

The color code comprise the five sections of the tetrapyrrole biosynthetic pathway: ALA synthesis purple, porphyrin synthesis blue, the Fe branch red, Mg branch green, siroheme synthesis yellow. The universal precursor of all tetrapyrroles 5-aminolevulinic acid (ALA) is synthesized from glutamate via a three-step reaction. ALA is further metabolized to protoporphyrin IX before the pathway branches into heme (and phytochromobilin) and chlorophyll biosynthesis. The ratio of chlorophyll a and b is balanced in the chlorophyll cycle. The end products of the pathway are underlined. Mutants of tetrapyrrole biosynthetic enzymes that play a role in plastid-to-nucleus signaling and inhibitors of certain enzymatic steps are shown. ALAD, ALA dehydratase; CAO, Chl a oxygenase; CBR, chlorophyll b reductase; ChlS, chlorophyll synthase; CPO, coproporphyrinogen III oxidase; DVR, divinyl protochlorophyllide reductase; FeCh, Fe chelatase; FLU, flourescent; GluRS, glutamyl-tRNA synthetase; GluTR, glutamyl-tRNA reductase; GluTRBP, GluTR binding protein; GSAT, glutamate-1-semialdehyde aminotransferase; HBS, hydroxymethylbilane synthase; HCAR, 7-hydroxymethyl chlorophyll a reductase; HO, heme oxygenase; MgCh, Mg chelatase; MTF, Mg protoporphyrin IX methyltransferase; PBS, phytochromobilin synthase; POR, light dependent NADPH-protochlorophyllide oxidoreductase; PPOX, protoporphyrinogen IX oxidase; UROD, uroporphyrinogen III decarboxylase; UROM, uroporphyrinogen III methyltransferase; UROS, uroporphyrinogen III synthase.
LHCB studies found increased levels of LHCB transcripts in dark-grown *C. reinhardtii* cultures fed with Mg porphyrin, as well as in *Chlamydomonas* *CHLH* and *CHLD* (Figure 1) deletion mutant strains where Mg porphyrin had accumulated, which underlines the correlation between Mg porphyrin-mediated signaling and NGE (Kropat et al., 1997; La Rocca et al., 2001; Vasileuskaya et al., 2004). The accumulation of MgProtoME in dark-incubated cress seedlings through inhibition by thujaplicin resulted in elevated LHCB mRNA content that was likely related to a metabolic response resembling light-induced control of nuclear genes for photosynthesis and plastid development (Oster et al., 1996). Last, inactivation of Mg chelatase, Mg protoporphyrin IX (MgProto) methyltransferase, and Chl synthase (Figure 1) resulted in altered expression of multiple nuclear-encoded genes in the tetrapyrrole biosynthesis in transgenic tobacco plants (Papenbrock et al., 2000b; Alawady and Grimm, 2005; Shalygo et al., 2009).

A mutant screen of *Arabidopsis* seedlings treated with norfluorazon (NF), an inhibitor of carotenoid biosynthesis, identified components of retrograde signaling contributing to un-coupling of LHCB expression from chloroplast development (Suske et al., 1993). Four out of five genomes uncoupled (gun) mutants were reported to encode proteins involved in tetrapyrrole biosynthesis (Figure 1). GUN2 and GUN3, encoding the heme oxygenase and the phycobobilin synthase (Mochizuki et al., 2001), are allelic to hy1 and hy2, respectively. The *Arabidopsis* *hy* mutants have been initially identified in mutant screens for deficits in light signaling (Chory et al., 1989). These mutants are phytochrome-deficient due to impaired synthesis of the chlorophyll phytochromobiline. GUN4 encodes a regulator of Mg chelatase activity (Larkin et al., 2003; Peter and Grimm, 2009) and GUN5 encodes the CHLH subunit of Mg chelatase (Mochizuki et al., 2001). GUN1 encodes a plastid-localized protein of the pentatricopeptide-repeat protein family that acts independently of tetrapyrrole biosynthesis (Koussovitzky et al., 2007). Accumulation of MgProto was compared from wild-type, *gun*5 and *gun*2 and it has been proposed that due to its prevalence MgProto likely acts as a signaling molecule for the expression of *LHCB1* and other nuclear-encoded genes (Strand et al., 2003). However, in more recent studies, no clear correlation was shown between steady-state levels of MgProto and LHCB expression in NF-treated *Arabidopsis* seedlings. It was suggested that phenotypical differences between *gun* and wild-type seedlings, including LHCB expression, may be attributed to ROS-based retrograde signaling (Mochizuki et al., 2008; Moulin et al., 2008). In addition, transgenic plants overexpressing FCI encoding ferrochelatase I (Figure 1) show *gun*-like altered expression of photosynthesis-associated nuclear genes (PhAANGs) highlighting heme as a potential signaling molecule in plastid-to-nucleus communication (Woodson et al., 2011).

Taken together, this evidence indicates that tetrapyrrole intermediates, such as Mg porphyrins, and endproducts, such as heme, are potential emitters of plastid-derived retrograde signals generated from tetrapyrrole metabolism. However, steady-state levels of tetrapyrrole metabolites and endproducts are also usually correlated with the synthesis of 5-aminolevulinic acid (ALA, Figure 1), which is the rate-limiting step of tetrapyrrole biosynthesis (Papenbrock et al., 2000a; Alawady and Grimm, 2005; Shalygo et al., 2009; Peter et al., 2010).

In cyanobacteria, algae and plants ALA is synthesized in a three-step reaction including activation of glutamate by binding to a plastid tRNA<sup>ALA</sup> followed by a reduction of glutamate to glutamate-1-semialdehyde catalyzed by glutamyl-tRNA reductase (GluTR) and a final transamination mediated by glutamate-1-semialdehyde aminotransferase (GSA; Figure 1). Following synthesis, ALA is further processed in the branched pathway leading to the tetrapyrrole end products Chl, heme, siroheme, and phytochromobilin (Figure 1; Papenbrock and Grimm, 2001; Tanaka and Tanaka, 2007).

5-Aminolevulinic acid synthesis is controlled at the transcriptional and posttranslational level by changes in environment (e.g., light, temperature) and endogenous stimuli, such as hormonal and metabolic feedback regulation. Furthermore, several feedback-control mechanisms affect ALA biosynthesis on a posttranslational level. The negative regulator FLU binds to GluTR and mediates the suppression of ALA synthesis in the dark (Meskauskiene et al., 2001; Meskauskiene and Apel, 2002), most likely by interaction with protoclorophyllide oxidoreductase (POR) and in response to immediate accumulating Pchlide (Richter et al., 2010; Kaus et al., 2012). For example, the *Arabidopsis* *flu* mutant does not down-regulate GluTR activity, and accumulates Pchlide in darkness which subsequently generates singlet oxygen upon light exposure (Op den Camp et al., 2003).

Our aim was to examine the strong regulatory impact of ALA synthesis on tetrapyrrole biosynthesis, including its potential role in retrograde signal-mediated NGE derived from tetrapyrrole metabolism. We show that reduced ALA synthesis in *gun*4-1 mutants, and inhibition of ALA synthesis by gabaculine (GAB) in wild-type *Arabidopsis* seedlings, lead to elevated transcript levels of photosynthetic genes if treated with NF. Taken together, these data support the idea that ALA-mediated retrograde signaling may modify NGE in *Arabidopsis* plants.

**MATERIALS AND METHODS**

**PLANT GROWTH CONDITIONS**

The *hy1* and *hy2* mutants of *Arabidopsis* *thaliana* L. originated from the ecotype Landsberg erecta (Ler-0) background (Chory et al., 1989), and *gun*1-1 and *gun*4-1 mutants (Col-0 background) were initially described in Suske et al. (1993) and Larkin et al. (2003), respectively. For analysis of etiolated, de-etiolated, and 7-day-old wild-type and mutant seedlings, seeds were surface-sterilized and plated onto 0.5× MS medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose 0.88% (w/v) agar. After vernalization at 4°C for 2 days, plates and pots were transferred to a growth chamber with light intensity of 100–120 μmol photons m<sup>−2</sup> s<sup>−1</sup> at 22–23°C under a 12-h light/12-h dark regime. Etiolated seedlings were exposed to light for 2 h to synchronize germination before they were dark-incubated for 3 days. Ten micromolars of GAB (5-aminocyclohexa-1,3-diene-1-carboxylic acid) or 100 μM ALA or 1 μM NF were supplied to the MS medium as indicated in the results.

**DETERMINATION OF ALA**

5-Aminolevulinic acid content was measured using the method from Mauzerall and Granick (1956). Seedlings or leaves (100–200 mg fresh weight) were harvested 1 h after the start of

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illumination and incubated in a buffer containing 50 mM Tris–HCl (pH 7.2) and 40 mM levulinate for 3 h at 22–23°C and 100 μmol photons m⁻² s⁻¹. Following the incubation, plant material was dried, weighed, and frozen in liquid nitrogen. Frozen samples were homogenized, resuspended in 20 mM K-phosphate buffer (pH 6.8) and centrifuged for 10 min at 16,000 × g. Four hundred microliters of the supernatant were mixed with 100 μl ethyl acetate and boiled for 10 min at 100°C. Samples were mixed with 500 μl modified Ehrlich’s reagent (373 ml acetic acid, 90 ml 70% v/v perchloric acid, 1.55 g HgCl₂, 9.10 g 4-dimethylaminobenzaldehyde diluted to 500 ml with H₂O) and centrifuged for 5 min at 16,000 × g. Absorption was measured at 526, 553, and 720 nm and the ALA content of the samples was calculated using a standard curve generated by commercial ALA (Sigma–Aldrich Inc.).

DETERMINATION OF THE PCHLIDE CONTENT

Extraction of Pchlide was performed according to the protocol of Koski and Smith (1948). Etiolated Arabidopsis WT and mutant seedlings (100–300 mg fresh weight) were treated with steam for 2 min, ground in liquid nitrogen, and extracted three times in alkaline acetone (9 vol 100% acetone: 1 vol 0.1 N NH₄OH). After centrifugation at 16,000 × g for 10 min the supernatants were collected and Pchlide content was determined by fluorescence spectroscopy. Extracts were excited at 433 nm and fluorescence emission was recorded at 632–633 nm. For calibration, a Pchlide standard was extracted from 7-day-old etiolated barley leaves (Koski and Smith, 1948) and quantified using the Pchlide extinction coefficient in diethyl ether at 623 nm of 35,600 M⁻¹ cm⁻¹ (Dawson et al., 1986).

PIGMENT ANALYSIS

Chlorophyll and carotenoids were extracted from 100 mg (fresh weight) of seedlings or leaf material with 80% (v/v) acetone containing 10 μM potassium hydroxide. Supernatants of three extraction steps were combined and analyzed spectrophotometrically (Lichtenhaller, 1987; Czarnecki et al., 2011b).

QUANTITATIVE REAL-TIME PCR

Total RNA of seedlings was extracted using the Invisorb Spin Plant RNA Mini Kit (Invitek, Germany). A cDNA was reversely transcribed using 2 μg total RNA, oligo-dT₁₈ Primer and RevertAid™-M-MulV Reverse Transcriptase (Fermentas) according to the manufacturer’s instructions. Real-time PCR was performed using a StepOnePlus and SYBR Green PCR Master Mix (Applied Biosystems) containing ROX as internal control. For all PCRs the following cycling conditions were used: 10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 60°C. Primers used are given in Table S1 in Supplementary Material. Calculation of expression levels in relation to WT and mutant, microarray signal intensities were calculated from raw data by using robust multi-array average (RMA) expression measure (Irizarry et al., 2003). Subsequently, pairwise comparisons between all wild-type replicates and all GAB-treated replicates or all gun4-1 replicates were performed to tag differentially expressed genes in treated or mutant seedlings. For this purpose, the LIMMA package (Smyth, 2004) in R/Bioconductor (Gentleman et al., 2004) was used with an empirical Bayes linear modeling approach. The obtained P-values for multiple testing were corrected according to Benjamini and Yekutieli (2001). Genes were considered to be significantly differentially expressed if their respective P-value was ≤0.05 and the log-fold change was either <log(2/3) or >log(3/2).

Heatmaps

A set of these differentially expressed genes was clustered according to their log-fold change using a k-means clustering approach in R (Hartigan and Wong, 1978; Ihaka and Gentleman, 1996). The expression of each gene in each cluster was depicted using a heatmap implemented in the R stats package. The scaling was based on rows, thus left at default, while the colors were set to an interpolation of blue and yellow. The genes being clustered in a Venn diagram were depicted alike.

Gene ontology-enrichment analysis

Gene ontology (GO)-enrichment analysis was performed using different algorithms: (i) a customized program was used for calculating over-represented GO terms (Ashburner et al., 2000) in a given set of genes. GO files (released 2010/05) were downloaded from the GO website¹, and GO annotations for Arabidopsis from the TAIR website² (release TAIR9). The subsequent calculation was based on hypergeometric distribution. The resulting P-value was corrected using the Bonferroni correction. GO terms with a probability of ≤0.05 were accepted as significant, whereas greater values lead to a rejection of a specific GO term. (ii) The program BiNGO 2.44 with Cytoscape 2.7 (Maere et al., 2005) was used together with the Amigo website³. A correction for false discovery rates was determined with BiNGO software using the method of Benjamini and Hochberg (1995).

Motif discovery

For motif discovery we used FIRE, Finding Informative Regulatory Elements (Elemento et al., 2007). Based on the concept of

¹http://www.kfb-regensburg.de
²http://www.geneontology.org
³http://www.arabidopsis.org
⁴http://amigo.geneontology.org
mutual information, FIRE calculates putative cis elements in a set of genes using a database of promoter elements. Further, FIRE scores simple motif definitions in the form of k-mers, searching each combination for under- or over-representation in a defined set of genes. It is possible to define a set of genes using a continuous FIRE approach or using a pre-clustered set as discrete approach. For our analysis, we used a 2-kb upstream promoter annotation based on TAIR9. We further used FIRE in a continuous analysis with a standard robustness index threshold of 6, and with 3–10 k-mers.

**STATISTICAL NOTE**

All experiments were performed independently three to six times. In order to test the significant differences between calculated values, equality of variances was tested by a F-test followed by Student’s t-test using a P-value of <0.05 as the threshold for significant difference.

**RESULTS**

**ARABIDOPSIS GUN MUTANTS SHARE REDUCED ALA SYNTHESIS RATES**

Inactivation of the expression of Mg chelatase, MgProto methyltransferase, and Chl synthase, enzymes in the Chl-synthesizing branch, results in diminished ALA-forming capacity (Papenbrock et al., 2000b; Alawady and Grimm, 2005; Shalygo et al., 2009). Since all but one known Arabidopsis gun mutants are also affected in genes related to tetrapyrrole biosynthesis (Nott et al., 2006), we collectively tested the Arabidopsis gun1-1, hy1 (allelic to gun2), hy2 (allelic to gun3), and gun4-1 mutants for their in vivo ALA synthesis rate. Analysis of 7-day-old hy1, hy2, and gun4-1 mutant seedlings revealed a severe reduction in ALA synthesis rate compared to their corresponding wild-types Ler-0 and Col-0 (Figure 2); ALA formation was also reduced in gun1-1 seedlings. As a consequence of decreased ALA formation, the contents of Chl (a + b) and carotenoids were significantly reduced in 7-day-old hy1, hy2, gun1-1, and gun4-1 seedlings compared with wild-type seedlings (Table 1).

**THE GUN PHENOTYPE COINCIDES WITH THE MODULATION OF ALA SYNTHESIS RATES**

Application of 10 µM GAB was reported to diminish Chl accumulation by approximately 50% in de-etiolating barley leaves (Hill et al., 1985; Nair et al., 1990) based on the inhibition of GSAT activity (Grimm et al., 1991), though not affecting the GtuTR and GSAT abundance (Demko et al., 2010). In Arabidopsis application of 10 µM GAB resulted in an 80% reduction of Chl a and b contents in treated seedlings compared to untreated, de-etiolating seedlings illuminated for 6 h as well as 6-day-old green seedlings (Figure S1A in Supplementary Material). The phenotype of the GAB-treated plants with low pigment contents was similar to the visible phenotype of the Arabidopsis gun4-1 mutant (Figure S1B in Supplementary Material). Since gun mutants and GAB-treated seedlings share the reduced ALA synthesis rate, we were interested to prove whether Arabidopsis wild-type seedlings with GAB-inhibited ALA synthesis modulate expression of marker genes for plastid signaling similarly to gun mutants.

In parallel to GAB inhibition of ALA biosynthesis, we intended to modify reversely the endogenous pool of ALA by additional supply of ALA to seedlings. It was previously reported that feeding of etiolated seedlings with increasing amounts of ALA correlates with synchronously increasing levels of non-photoconvertible Pchlide (Armstrong et al., 1995). In order to raise the intracellular ALA pool without photo-oxidative risks we performed preliminary experiments to select the appropriate amounts of ALA. Application of ALA to etiolated Arabidopsis seedlings caused increasing Pchlide levels when young seedlings were fed with ALA at amounts greater than 50 µM. Application of 100 µM ALA resulted in four-time higher Pchlide levels in etiolated seedlings in the analyzed time period compared to untreated seedlings (Figure S2 in Supplementary Material; 1.0 ± 0.3 vs. 4.5 ± 1.3 pmol mg fw⁻¹). Light-grown seedlings fed with 100 µM ALA did not show visible photo-bleaching at the given light exposure (Figure S3 in Supplementary Material), though the addition of ALA at concentrations

**Table 1** | Pigment content of 7-day-old Arabidopsis hy1, hy2, gun1-1, and gun4-1 mutants and the corresponding wild-type seedlings Ler-0 and Col-0.

| Line  | Chl (a + b) (ng mg fw⁻¹) | Carotenoids (ng mg fw⁻¹) |
|-------|--------------------------|--------------------------|
| Ler-0 | 172 ± 29                 | 42 ± 5                   |
| hy1  | 102 ± 9*                 | 32 ± 3                   |
| hy2  | 68 ± 15*                 | 22 ± 6*                  |
| Col-0 | 207 ± 19                 | 54 ± 5                   |
| gun1-1| 114 ± 19*                | 36 ± 7*                  |
| gun4-1| 64 ± 7*                  | 22 ± 2*                  |

Given are means ± SD of three independent extractions. * Indicates a significant difference from the wild-type (P < 0.05).
greater than 250 µM generated a visible stress phenotype in illuminated Arabidopsis seedlings (at 100 µmol photons m⁻² s⁻¹) that was likely due to elevated levels of photo-reactive tetrapyrrole intermediates.

Then, wild-type Col-0, gun1-1 and gun4-1 seedlings were subjected to 10 µM GAB or 100 µM ALA during germination and were incubated with NF to replicate experimental conditions that were applied to study the gun phenotype. Control and chemically treated wild-type and gun mutants were harvested after 3-day-growth in darkness, 3 days in darkness followed by 1 day light exposure, or after 6 days growth in photoperiodic light (12 h, 100–120 µmol photons m⁻² s⁻¹). The transcription levels of two photosynthesis genes, AtLHCB1.2 and AtRBCS, were determined by qRT-PCR in these samples (Figures 3 and 4).

The analysis of the transcription levels of AtLHCB1.2 and AtRBCS in wild-type Col-0, gun1-1 and gun4-1 seedlings subjected to 10 µM GAB during germination and incubated with NF revealed the following results: (i) Inhibition of carotenoid biosynthesis by NF treatment drastically lowered the transcript level in light-exposed, but not in etiolated seedlings (Figures 3 and 4). (ii) The AtLHCB1.2 and AtRBCS expression of NF-treated and light-exposed gun1-1 and gun4-1 seedlings was de-repressed relative to that of wild-type Col-0 and confirmed the expected gun phenotype in our experimental approaches (Figures 3 and 4). (iii) Inhibition of ALA synthesis by GAB did not affect the expression of AtLHCB1.2 and AtRBCS in etiolated, greening, or green seedlings, with the exception of slightly reduced expression levels of both genes in the wild-type Col-0 and gun1-1 after 1 day of de-etiolation (Figures 3B, E). Six-day-old, green gun1-1 seedlings showed elevated AtLHCB1.2 expression in response to GAB (Figure 3C). (iv) GAB supply of NF-incubated Arabidopsis seedlings resulted in elevated AtRBCS and AtLHCB1.2 expression levels compared to seedlings treated solely with NF. This counteracting effect of NF plus GAB vs. NF alone on elevated transcript contents was observed in the wild-type Col-0, but also in gun1-1 and gun4-1 in both 1 day illuminated (Figures 3B, E) and 6-day-old green seedlings (Figures 3C, F).

Inhibition of ALA synthesis by GAB in NF-treated wild-type Col-0 led to the increased accumulation of both AtLHCB1.2 and AtRBCS at rates that resembled the gun phenotype. The effects of GAB treatment were even more enhanced in the NF-treated gun1-1 mutant, indicating additive acting mechanisms on NGE in gun1-1. However, in comparison with the wild-type Col-0, the modulation of AtLHCB1.2 and AtRBCS transcript accumulation was less pronounced when GAB was added to the NF-treated gun4-1 mutant (Figure 3).

In contrast to the results observed when GAB was added to inhibit ALA synthesis, ALA addition caused decreasing AtLHCB1.2 and AtRBCS transcript levels in Arabidopsis wild-type Col-0 seedlings (Figures 4B, E). Since reduced AtLHCB1.2 and AtRBCS transcript contents were also determined in etiolated seedlings, it can be excluded that photo-oxidative inhibition mainly caused the changes of NGE of photosynthetic genes (see also phenotypes displayed in Figure S3 in Supplementary Material). It is concluded that supply of low and non-phototoxic acting amounts of ALA in NF-treated and light-exposed seedlings abrogates the gun phenotype. Thus, regulatory consequences of the ALA synthesis rate and the endogenous ALA pool on NGE can be studied in both experimental approaches without the confounding side effects.

GLOBAL RESPONSE OF NGE TO INHIBITION OF ALA SYNTHESIS

After etiolated Arabidopsis Col-0 seedlings were illuminated, Chl contents increased from zero to 31 ± 3 ng Chl a and 12 ± 4 ng Chl b mg⁻¹fw⁻¹ within 6 h of illumination (Figure S4 in Supplementary Material). After 48 h, the contents of Chl a and b were elevated to 157 ± 17 and 44 ± 6 ng mg⁻¹fw⁻¹, respectively. Carotenoid contents increased from 11 ± 1 in etiolated seedlings to 64 ± 6 ng mg⁻¹fw⁻¹ during 48 h of de- etiolation (Figure S4 in Supplementary Material). Since ALA synthesis is highly regulated and determines the amount of metabolites that are introduced into the branched metabolic pathway of tetrapyrrole biosynthesis, it is likely that the capacity of ALA synthesis has a strong impact on chloroplast biogenesis.

In continuation of our studies on the regulatory effect of ALA synthesis on NGE, we compared the transcriptome of developing Arabidopsis seedlings in response to inhibited ALA synthesis. An Affymetrix GeneChip (ATH1) microarray analysis was performed with RNA from 6 h light-exposed etiolated Arabidopsis seedlings incubated with or without 10 µM GAB. Moreover, in comparison to wild-type control we analyzed NGE of the 6 h light-exposed de-etiolation gun4-1 mutants. The time point for the comparative transcriptome analysis was chosen since initial amounts of Chl accumulated upon illumination of etiolated seedlings (Figure S4 in Supplementary Materials). We decided on gun4-1, because the mutant shows posttranslational reduction of ALA synthesis without reduced expression of the ALA-forming enzymes (Peter and Grimm, 2009). We searched for early responsive genes upon inhibited ALA biosynthesis to minimize interference of pleiotropic effects of ALA deficiency (e.g., reduced photosynthetic capacity). Differentially expressed genes were identified in the transcriptome analysis with a P-value of ≤0.05 and a log-fold change of <log(2/3) or >log(3/2), respectively, compared to untreated Arabidopsis wild-type Col-0 seedlings (Figure 5; Data Sheet 2 in Supplementary Material). Comparison of the transcript profiles revealed that GAB treatment of developing Arabidopsis wild-type Col-0 seedlings resulted in the differential regulation of 325 genes representing approximately 1.5% of the entire Arabidopsis genome. Inhibited ALA synthesis resulted in the repression of 158 genes and the up-regulation of 167 genes. Compared to wild-type Col-0, gun4-1 displayed 1536 mis-regulated genes (7.2% of the total ATH1 genome) at the same selected time point of 6 h of
de-etiolation, whereas 993 and 543 genes showed a down- and upregulated expression, respectively (Figure 5; Data Sheet 2 in Supplementary Material). However, a high number of genes were collectively down-regulated (119 genes) and up-regulated (88 genes) in GAB-treated and gun1-1 seedlings (Figure 5B; Data Sheet 2 in Supplementary Material). The overlapping genes represented 12.5% of the differentially expressed genes (equals 207 combined genes out of the total amount of 1654 differentially regulated genes...
observed in both microarrays). Apart from two exceptions, all genes were congruently found to be either down- or up-regulated in both sets of seedlings with lower ALA synthesis (GAB-treated and the gun4-1 seedlings) when compared with control seedlings (Data Sheet 2 in Supplementary Material). Tables 2 and 3 present the transcripts of the common set of genes in GAB-treated and
gun4-1 seedlings in comparison to untreated wild-type seedlings with the greatest response to inhibition of ALA synthesis based on fold repression (<0.5, Table 2) and fold induction (≥2, Table 3).

Transcriptomic comparisons showed that a high number of genes commonly affected by reduced ALA synthesis in GAB-treated Arabidopsis seedlings were also found in gun4-1 mutants. Nearly 75% of the genes down-regulated in GAB-treated Arabidopsis seedlings were also down-regulated in gun4-1. These genes represent 12% of the 993 genes down-regulated in gun4-1 (Figure 5B). Similarly, 53% of the genes up-regulated in GAB-treated seedlings were also up-regulated in gun4-1. These 88 genes represented 16% of the genes up-regulated in gun4-1. The results indicate a high number of identical genes identified in both transcriptomic comparisons, which are commonly affected by reduced ALA synthesis in GAB-treated Arabidopsis seedlings and gun4-1. This set of identical early responsive genes in both transcriptome analyses supports the view that besides the impaired Mg chelatase activity, gun4-1 is also significantly compromised in ALA biosynthesis. Due to additional effects of GUN4 deficiency on other physiological processes, the transcriptome of gun4-1 displayed a broader range of modulated genes.

Analysis of GO categories revealed an overrepresentation of transcripts encoding proteins involved in nuclear DNA replication within the cluster of genes being down-regulated in both...
Table 2 | Listed are genes that showed a significantly ($P \leq 0.05$) decreased transcript abundance after 6 h of de-etiolation in response to inhibited ALA formation (GAB) and in the gun4-1 mutant.

| AGI number       | GAB vs. Col-0 fold change | GAB vs. Col-0 $P$-value | gun4-1 vs. Col-0 fold change | gun4-1 vs. Col-0 $P$-value | SUBA localization          | Description                                                                 | qRT-PCR performed (see Table S2 in Supplementary Material) |
|------------------|----------------------------|-------------------------|------------------------------|-----------------------------|----------------------------|-----------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|
| At5g08030        | 0.247                      | 0.000                   | 0.249                        | 0.000                       | Mito, Cp, ER, Nuc, Ex, Vac | Gyerocephosphoryl diester phosphodiesterase                                   | Yes                                                                                                           |
| At1g13650        | 0.273                      | 0.000                   | 0.381                        | 0.000                       | Mito, Perox                 | Unknown protein                                                             | Yes                                                                                                           |
| At1g220570       | 0.300                      | 0.000                   | 0.351                        | 0.000                       | Cyt                        | GPRII (GBF'S Pro-rich region-interacting factor 1)                          | Yes                                                                                                           |
| At5g46690        | 0.345                      | 0.000                   | 0.344                        | 0.000                       | Nuc                        | Beta HLH protein 71 (bHLH071)                                              | Yes                                                                                                           |
| At1g61800        | 0.347                      | 0.000                   | 0.486                        | 0.000                       | Cyt, Mito, Ex, PM           | Glucose-6-phosphate transmembrane transporter (GPT2)                        | Yes                                                                                                           |
| At3g26960        | 0.372                      | 0.000                   | 0.288                        | 0.000                       | Ex, ER, Nuc                | Unknown protein                                                             | Yes                                                                                                           |
| At5g55450        | 0.376                      | 0.005                   | 0.278                        | 0.000                       | Ex, ER                     | Protease inhibitor                                                          | Yes                                                                                                           |
| At4g04610        | 0.385                      | 0.019                   | 0.166                        | 0.000                       | Cyt                        | APS reductase 1 (APR1)                                                     | Yes                                                                                                           |
| At4g28250        | 0.387                      | 0.003                   | 0.182                        | 0.000                       | Ex, ER, Vac                | AT3XPB3 (Expansin B3)                                                      | Yes                                                                                                           |
| At2g07550        | 0.407                      | 0.000                   | 0.176                        | 0.000                       | Ex                         | AT3XPB1 (Expansin B1)                                                      | Yes                                                                                                           |
| At3g1820         | 0.414                      | 0.000                   | 0.344                        | 0.000                       | Cyt, Ex                    | Aspartyl protease family protein                                           | Yes                                                                                                           |
| At3g47380        | 0.431                      | 0.005                   | 0.465                        | 0.002                       | Ex, Mito, ER, Nuc           | Invertase/pectin methylesterase inhibitor                                   | Yes                                                                                                           |
| At1g56650        | 0.439                      | 0.000                   | 0.351                        | 0.000                       | Nuc                        | RAP1 (production of anthocyanin in pigment 1); DNA-binding/transcription factor | Yes                                                                                                           |
| At5g45650        | 0.443                      | 0.001                   | 0.356                        | 0.000                       | Mito, ER, Ex               | Subtilase family protein                                                   | Yes                                                                                                           |
| At1g24020        | 0.457                      | 0.017                   | 0.160                        | 0.000                       | Cyt, Perox                 | MLP423 (MLP-like protein 423)                                             | Yes                                                                                                           |
| At3g59670        | 0.459                      | 0.000                   | 0.528                        | 0.000                       | Nuc                        | Unknown protein                                                             | Yes                                                                                                           |
| At1g71270        | 0.460                      | 0.000                   | 0.645                        | 0.005                       | Mito, Nuc, Cp              | CDT1A (homolog of yeast CDT1 A); cyclin-dependent protein kinase            | Yes                                                                                                           |
| At4g11480        | 0.468                      | 0.014                   | 0.271                        | 0.000                       | Ex, ER, Nuc, Vac           | Protein kinase family protein                                              | Yes                                                                                                           |
| At4g34588        | 0.474                      | 0.003                   | 0.428                        | 0.000                       | No data                    | CuORF2 (conserved peptide upstream open reading frame 2)                    | Yes                                                                                                           |
| At4g34590        | 0.474                      | 0.003                   | 0.428                        | 0.000                       | Nuc                        | GBF51 (G-BOX BINDING FACTOR 5)                                             | Yes                                                                                                           |
| At5g05690        | 0.477                      | 0.014                   | 0.170                        | 0.000                       | ER, Ex                     | Protease inhibitor (seed storage/lipid transfer protein (LTP) family protein) | Yes                                                                                                           |
| At4g28680        | 0.480                      | 0.001                   | 0.432                        | 0.000                       | Cyt, Mito, ER              | Putative tyrosine decarboxylase                                           | Yes                                                                                                           |
| At3g05600        | 0.481                      | 0.005                   | 0.459                        | 0.001                       | Cyt, Perox, Cyt            | Putative epoxide hydrolase                                                 | Yes                                                                                                           |
| At4g39510        | 0.485                      | 0.001                   | 0.369                        | 0.000                       | Mito, ER, Cyt, Ex, Nuc     | CYP96A12; electron carrier/heme binding/iron ion binding/monoxygenase/oxygen binding | Yes                                                                                                           |
| At1g66940        | 0.493                      | 0.002                   | 0.262                        | 0.000                       | Ex, ER, Cyt, Ex            | Protein kinase-related                                                     | Yes                                                                                                           |
| At4g38950        | 0.500                      | 0.004                   | 0.393                        | 0.000                       | Nuc                        | Kinesin motor family protein                                               | Yes                                                                                                           |

In contrast to the results shown in Figure 5, there are only transcripts listed with a fold change ≤ 0.5. Given are the fold changes, corresponding $P$-values, the putative subcellular localizations using the SUBA database (Heazlewood et al., 2007) and gene annotations based on the TAIR9 release of the Arabidopsis genome. (Cp, chloroplasts; Cyt, cytosol; ER, endoplasmic reticulum; Ex, extracellular; Mito, mitochondria; Nuc, nucleus; Perox, peroxisome; PM, plasma membrane; Vac, vacuole).
Table 3 | Listed are genes that showed a significantly ($P \leq 0.05$) increased transcript abundance after 6 h of de-etiolation in response to inhibited ALA formation (GAB) and in the *gun4-1* mutant.

| AGI number | GAB vs. Col-0 fold change | GAB vs. Col-0 $P$-value | *gun4-1* vs. Col-0 fold change | *gun4-1* vs. Col-0 $P$-value | SUBA localization | Description | qRT-PCR performed (see Table S2 in Supplementary Material) |
|------------|--------------------------|-------------------------|-------------------------------|-----------------------------|--------------------|-------------|------------------------------------------------------|
| At1g05560  | 4.010                    | 0.000                   | 2.057                         | 0.000                       | Cyt, Mito, Cyt     | UDP-glucose:4-aminobenzoate acylglycosyltransferase (UGT75B1) | Yes |
| At1g22890  | 3.708                    | 0.000                   | 6.331                         | 0.000                       | ER, Nuc, Cp       | Unknown protein | TX1; antitumor/multidrug efflux pump/multidrug transporter/transporter | Yes |
| At2g04040  | 3.267                    | 0.001                   | 3.156                         | 0.000                       | PM, Ex, Vac       | TX1; antitumor/multidrug efflux pump/multidrug transporter/transporter | Yes |
| At3g2060   | 2.838                    | 0.000                   | 3.278                         | 0.000                       | Ex, Vac           | Receptor protein kinase-related | Yes |
| At2g19800  | 2.838                    | 0.000                   | 2.686                         | 0.000                       | Cyt               | MIOX2 (Myo-inositol oxygenase 2) | Yes |
| At5g16980  | 2.773                    | 0.000                   | 1.635                         | 0.011                       | Cyt, Mito, Cp     | Putative NADP-dependent oxidoreductase | Yes |
| At3g20340  | 2.728                    | 0.000                   | 1.903                         | 0.001                       | Nuc               | Down-regulated under photo-oxidative stress. | Yes |
| At5g43450  | 2.722                    | 0.000                   | 3.317                         | 0.000                       | Cyt, Nuc          | Putative 2-oxoglutarate-dependent dioxygenase | Yes |
| At3g47340  | 2.680                    | 0.000                   | 5.921                         | 0.000                       | Cyt, Cp           | ASIN1 (glutamine-dependent asparagine synthase 1) | Yes |
| At1g05680  | 2.591                    | 0.005                   | 2.247                         | 0.003                       | Mito, Cyt, Cp     | UDP-glucuronosyl/UDP-glucosyl transferase | Yes |
| At5g16970  | 2.590                    | 0.000                   | 1.791                         | 0.000                       |                   | ATAER (2-alkenal reductase) | Yes |
| At3g28730  | 2.455                    | 0.001                   | 2.472                         | 0.000                       | Mito, ER, Ex, PM  | CYP81D1; electron carrier/heme binding/protein ion binding/moonooxygenase/oxygen binding | Yes |
| At3g20270  | 2.445                    | 0.011                   | 2.036                         | 0.012                       | Cp, Cyt, Ex       | Lipid binding serum glycoprotein in family protein | Yes |
| At2g17880  | 2.423                    | 0.001                   | 3.176                         | 0.000                       | Cyt, Mito, Nuc    | Putative DNAJ heat shock protein | Yes |
| At1g35140  | 2.362                    | 0.000                   | 12.734                        | 0.000                       | Ex                | Phosphate induced 1 (PHI-1) | Yes |
| At4g31870  | 2.340                    | 0.002                   | 2.257                         | 0.001                       | Cyt, Mito, Perox, Nuc | ATGPX7 (glutathione peroxidase 7) | Yes |
| At1g08630  | 2.307                    | 0.001                   | 8.617                         | 0.000                       | Cyt, Nuc, Cp      | THA1 (threonine aldolase 1) | Yes |
| At5g7560   | 2.218                    | 0.000                   | 6.492                         | 0.000                       | Ex, Mito, Er      | TCH4 (touch 4); glycolysis, acting on glycose bonds/xyloglucan:xyloglucosyl transferase | Yes |
| At5g06860  | 2.187                    | 0.001                   | 1.775                         | 0.001                       | Ex                | PGP1 (polygalacturonase inhibiting protein 1) | Yes |
| At2g29340  | 2.143                    | 0.001                   | 2.036                         | 0.000                       | Cyt, Ex, ER       | Short-chain dehydrogenase/reductase (SDR) family protein | Yes |
| At5g17300  | 2.069                    | 0.001                   | 2.115                         | 0.000                       | Nuc               | Myb family transcription factor | Yes |
| At4g15260  | 2.044                    | 0.000                   | 2.059                         | 0.000                       | Cyt, Cp           | UDP-glucuronosyl/UDP-glucosyl transferase | Yes |
| At5g32020  | 2.042                    | 0.005                   | 2.472                         | 0.000                       | PM                | ATBCB (blue-copper binding protein) | Yes |
| At5g14470  | 2.010                    | 0.001                   | 6.425                         | 0.000                       | Cyt, Nuc          | GHMP kinase-related | Yes |
| At3g15760  | 2.005                    | 0.001                   | 2.027                         | 0.000                       | Ex, Mito, Nuc, Cp | Unknown protein | Yes |

In contrast to the results shown in Figure 5, there are only transcripts listed with a fold change $\geq 2.0$. Given are the fold changes, corresponding P-values, the putative subcellular localizations using the SUBA database (Heazlewood et al., 2007) and gene annotations based on the 9.0 release of the Arabidopsis genome. (CP), chloroplasts; Cyt, cytosol; ER, endoplasmic reticulum; Ex, extracellular; Mito, mitochondria; Nuc, nucleus; Perox, peroxisome; PM, plasma membrane; Vac, vacuole.)
GAB-treated Arabidopsis wild-type Col-0 and gun4-1 seedlings (Figure 6A). Within the cluster of up-regulated genes, neither cellular components nor functions were overrepresented. Genes encoding proteins responding to sucrose stimulus, oxidative stress, and the absence of light were overrepresented within the biological process category, whereas transcripts involved in posttranslational modification of proteins (disulfide bonds and glycosyl groups) were overrepresented in the molecular function category (Figure 6B). GO analyses extended to all differentially expressed transcripts additionally revealed that reduction of ALA synthesis in GAB-treated seedlings and in gun4-1 mutants caused a reduced abundance of transcripts for sulfur metabolism (sulfate assimilation, sulfate reduction, and adenylyl-sulfate reductase activity) at an early stage of development (Figure S5A in Supplementary Material). The significantly over-represented GO categories additionally identified in up-regulated transcripts were more diverse and include transcripts related to the circadian rhythm, redox homeostasis, auxin signaling, and tocopherol biosynthesis (Figure S5B in Supplementary Material).

It is remarkable that within the small set of 207 combined genes that differentially respond to two different mechanisms of inhibited ALA synthesis (Figure 5B), no genes were found which are involved in Chl biosynthesis and photosynthesis (Tables 2 and 3; Data Sheet 2 in Supplementary Material). We compared microarray data with transcripts levels obtained by qRT-PCR of two genes involved in ALA synthesis, AtHEMA1 and AtGluTRBP (Czarnecki et al., 2011a) and other marker genes for chloroplast functions, e.g., AtTOC159, AtELIP1, AtELIP2, and AtLHCB1.2 in 6 h de-etiolated GAB-treated wild-type and gun4-1 seedlings. In both sets of seedlings the expression levels of these genes were not significantly altered in response to inhibition of ALA formation (Figure 7; Table S2 in Supplementary Material), except a 30–50% stimulated expression of AtHEMA1 and AtTOC159 in gun4-1 compared to the control transcript levels of wild-type seedlings.

Screening of genomic sequences upstream of the transcription initiation region of differentially expressed genes in GAB-treated or gun4-1 Arabidopsis seedlings using FIRE analysis did not reveal an over-representation of a promoter motif in GAB-treated Arabidopsis seedlings. However, an I-box motif (5′-CTTATCC-3′) was overrepresented in three clusters representing transcripts with enhanced expression in gun4-1 seedlings (Figure 8; Data Sheet 3 in Supplementary Material). Consistently, over-represented GO categories were identified within genes forming clusters with over-represented promoter motifs. The I-box motif found in clusters C1–C3 formed by genes over-expressed in gun4-1 seedlings seems to be a promoter element of genes involved in sucrose and light stress, but also secondary metabolic systems, e.g., cell wall biosynthesis and defense reactions (Table 4).

Quality and robustness of expression data obtained by microarray analysis were validated by independent expression analysis of selected genes via qRT-PCR. Therefore, 36 genes up- or down-regulated in both gun4-1 and GAB-treated wild-type seedlings were selected either by reason of the putative chloroplast localization of their encoded proteins, their function as transcription factor, or the strong alterations in the transcript abundance determined by the microarray analysis in comparison to control (Table S2 in Supplementary Material). Quantitative RT-PCR expression analysis was performed with de-etiolated seedlings after 6 and 24 h of illumination. The data of the global transcriptome analysis were confirmed in approximately 67% of the performed qRT-PCR reactions. Moreover, the majority of transcripts being mis-regulated in GAB-treated wild-type and gun4-1 seedlings after 6 h of de- etiolation were also mis-regulated after 24 h of light exposure.
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AtHEMA1 AtTOC159 AtELIP1 AtELIP2 AtLHCB1.2

rel. expression [2] (qRT-PCR data)

AtHEMA1 AtTOC159 AtELIP1 AtELIP2 AtLHCB1.2

rel. expression [fold change] (microarray data)

microarray results

qRT-PCR

AtGluTRBP

Col-0 + 10 µM GAB
gun4-1

microarray results

qRT-PCR

-∆∆ Ct

**

* Indicates a significant difference from the untreated Arabidopsis wild-type Col-0 (P-value of <0.05).

FIGURE 7 | Comparison of expression data obtained by microarray analysis and qRT-PCR. RNA from GAB-treated Arabidopsis wild-type seedlings and gun4-1 mutant seedlings was extracted 6 h after light exposure of 3-day-old etiolated seedlings. In addition to microarray analysis, transcript abundance of a defined set of genes was determined by quantitative real-time PCR and calculated in relation to AtSAND expression compared to untreated Arabidopsis wild-type Col-0 seedlings \([2−\Delta\Delta Ct]\). qRT-PCR data are shown as average ± SD of three biological replicates.

FIGURE 8 | Identification of informative promoter sequence motifs of regulated transcripts in gun4-1 mutant seedlings compared to untreated Arabidopsis wild-type Col-0 seedlings. Columns on the left correspond to groups of genes clustered according to the similarity in their expression pattern [log (fold change)] within the microarray experiments. Clusters highlighted in red (C1, C2, and C3) show a significant over-representation of the specified motif. The sequence on the right represents the optimized motif and its name based on JASPAR, TRANSFAC, or PLACE. Names and descriptions of transcripts forming clusters C1–C3 were given in Data Sheet 3 in Supplementary Material.

(Figure 9; Table S2 in Supplementary Material). This confirmation revealed a robust data set created by microarray analysis of transcriptional changes in response to reduced ALA synthesis.

DISCUSSION

COMPROMISED ALA BIOSYNTHESIS IS A TRAIT OF GUN MUTANTS

By using GAB to reduce ALA formation we proved a role of ALA-synthesizing activity in plastid-to-nucleus communication and modulation of NGE. Transcript accumulation of LHCB1.2 has been previously examined in NF-treated Arabidopsis seedlings to identify mutants affected in retrograde signaling (Susek et al., 1993). Four out of five initially identified gun mutants are perturbed in tetrapyrrole biosynthesis. We found that reduced ALA synthesis rates are a common metabolic trait of gun1-1, hy1 (allelic to gun2), hy2 (allelic to gun3), and gun4-1 mutants (Figure 2). A lower ALA-synthesizing capacity has been previously reported for gun4-1 (Peter and Grimm, 2009), and other transgenic lines with reduced expression of CHLH, CHLI, CHLM, and CHLG encoding either one of the three different Mg chelatase subunits, Mgproto methyltransferase, or Chl synthase, respectively (Figure 1; Papenbrock et al., 2000a,b; Alawady and Grimm, 2005; Shalygo et al., 2009). Mutants with deficiency in the synthesis of Mg chelatase subunits are considered to be phenotypically equivalent to the gun5 mutant.

We added GAB to Arabidopsis wild-type seedlings to simulate reductions in ALA synthesis and changes in NGE of the gun
Table 4 | Cluster C1–C3 (Figure 8) showing significant over-represented specific motifs in mis-regulated genes of *gun4-1* mutants analyzed for overrepresentation of gene ontology (GO) categories.

| Cluster | Domain (C, F, P) | P-value | GO category | Description |
|---------|-----------------|---------|--------------|-------------|
| C1      | F               | 0.014   | GO:0016491   | Oxidoreductase activity |
|         | P               | 0.004   | GO:0009743   | Response to carbohydrate stimulus |
|         |                 | 0.032   | GO:0010200   | Response to chitin |
| C2      | C               | 0.037   | GO:0044464   | Cell part |
|         | F               | 0.047   | GO:0005199   | Structural constituent of cell wall |
|         |                 | 0.036   | GO:0016762   | Xyloglucan:xyloglucosyl transferase activity |
|         | P               | 0.023   | GO:0050832   | Defense response to fungus |
|         |                 | 0.006   | GO:0009646   | Response to absence of light |
|         |                 | 0.026   | GO:0010200   | Response to chitin |
|         |                 | 0.005   | GO:0009744   | Response to sucrose stimulus |
| C3      | C               | 0.037   | GO:0016491   | Oxidoreductase activity |
|         | F               | 0.047   | GO:0005199   | Structural constituent of cell wall |
|         |                 | 0.036   | GO:0016762   | Xyloglucan:xyloglucosyl transferase activity |
|         | P               | 0.023   | GO:0050832   | Defense response to fungus |
|         |                 | 0.006   | GO:0009646   | Response to absence of light |
|         |                 | 0.026   | GO:0010200   | Response to chitin |
|         |                 | 0.005   | GO:0009744   | Response to sucrose stimulus |

The genes forming the respective clusters (Data Sheet 3 in Supplementary Material) were analyzed for overrepresented GO categories (P ≤ 0.05). Intermediate GO categories are omitted. C, cellular component; F, molecular function; P, biological process.

Additional ALA Supply and Accumulation of Photosynthetic Genes

Since reduced ALA synthesis was shown to mimic a *gun* phenotype in wild-type seedlings, we then added low amounts of ALA to germinating *Arabidopsis gun1-1* and *gun4-1* seedlings to test if elevated ALA amounts suppress the *gun* phenotype. Additional supply of low amounts of ALA decreased the transcript levels of *LHCBI.2* and *RBCS* in both etiolated *gun* mutants and wild-type seedlings. ALA feeding potentiated the NF-induced decrease of transcript contents of photosynthetic genes in greening and green wild-type and *gun1-1* seedlings. In contrast, elevated ALA pools in *gun4-1* resulted in less pronounced changes of NGE compared to both wild-type Col-0 and *gun1-1*, indicating that *gun4-1* is less sensitive to exogenously applied ALA (Figure 4).

It was previously reported that ALA feeding of *gun* mutants mimics the *gun* phenotype in *Arabidopsis* wild-type seedlings and results in elevated transcript levels of the photosynthetic genes *LHCBI.2* and *RBCS* compared to the NF treatment without GAB addition (Figure 3). GAB addition had similar effects on NF-treated *gun1-1* and *gun4-1* seedlings (Figure 3). In contrast to NF-treated and light-exposed seedlings, the combined addition of NF and GAB did not influence the transcript levels of *LHCBI.2* and *RBCS* in etiolated *gun*-mutant and wild-type seedlings. The lack of an effect of GAB addition on NGE in etiolated seedlings is likely explained by a minimal ALA synthesis rate caused by FLU-mediated inactivation of GluTR (Meskauskiene et al., 2001; Meskauskiene and Apel, 2002; Richter et al., 2010; Kauss et al., 2012).

As feeding of *Arabidopsis* wild-type, *gun1-1* and *gun4-1* mutants with exogenous ALA represses the *gun* phenotype under NF treatment (Figure 4), we suggest that the observed changes in expression of *LHCBI.2* and *RBCS* are either due to modification of ALA biosynthesis rates or the ALA pool. This is consistent with the observed changes in the accumulation of these mRNAs in response to applied NF and GAB (Figure 3). Considering the opposite effects of ALA supply and inhibition of ALA synthesis, a direct impact of ALA biosynthesis or the ALA steady-state level is proposed.

It is remarkable that transcript accumulation of the *LHCBI.2* and *RBCS* in the NF-treated *gun4-1* mutant showed a lower sensitivity to the addition of GAB and ALA. This favors the idea of GUN4 being a component of the mechanism translating the state of plastid ALA synthesis into a nuclear response. A role of GUN4 in posttranslational regulation of ALA formation has been previously proposed (Peter and Grimm, 2009). It is hypothesized that GUN4 stimulates ALA synthesis in light. This is supported by observations that *gun4* knock-out mutants cannot survive photoperiodic growth conditions and do not accumulate Chl under these conditions, but slowly green and grow under continuous dim light.

However, although we clearly show that GABA treatment mimics the *gun* phenotype in *Arabidopsis* wild-type seedlings and excess ALA represses the *gun* phenotype of NF-treated *gun* mutants, it should be kept in mind that NF generally is a very deleterious herbicide leading to a bleaching leaf phenotype initially caused by inhibition pytoene desaturase, an enzyme involved in carotenoid biosynthesis (Breitenbach et al., 2002). It was previously suggested that retrograde signals emitted by chloroplast destruction under NF treatment are heavily influenced by production of a complex ROS pattern that likely acts as a modulator of NGE itself (Op den Camp et al., 2003; Apel and Hirt, 2004; Kim et al., 2008; Moulin et al., 2008).
Considering recently developed ideas that retrograde signaling is not necessarily the result of single and distinct emitted signal molecules but rather the result of continuously sensed metabolic and genetic activities in the organelles (Pogson et al., 2008; Kleine et al., 2009; Pfannschmidt, 2010), changes in NGE provoked by NF-induced inhibition of chloroplast development may not reflect signaling pathways under non-destructive or non-lethal conditions. In this context, it is an important observation that 6-day-old low light-exposed gun mutants do not display significantly increased LHCBI.2 expression under NF treatment (Voigt et al., 2010). Moreover, the authors also found that under NF treatment LHCBI.2 expression was not increased in several Arabidopsis T-DNA insertion mutants affected in genes of tetrapyrrole biosynthesis. In consequence, the appropriateness of PhANGs (e.g., LHCBI.2, RBCS, CA) may put into question as primary target genes of plastid signaling.

INHIBITION OF ALA BIOSYNTHESIS INDUCES CHANGES IN THE ARABIDOPSIS TRANSCRIPTOME

We intended to examine the hypothesis that reduced ALA formation instantaneously releases a retrograde signal and performed a transcriptome analysis to explore rapid changes in transcript profiles in response to lower ALA biosynthesis before other regulatory mechanisms of successive changes in tetrapyrrole biosynthesis and chloroplast biogenesis proceed (e.g., photosynthesis, redox poise, ROS). The microarray analysis with GAB-treated
seedlings and gun4-1 seedlings revealed a common set of significantly up- and down-regulated genes in response to reduced ALA synthesis (Figure 5; Tables 2 and 3). In addition to previously proposed signaling derived from tetrapyrroles (Mochizuki et al., 2001; Strand et al., 2003; Woodson et al., 2011), this list of deregulated genes indicates the potential of ALA synthesis-derived retrograde signaling.

Kobayashi et al. (2009) opened the view on the interrelation between tetrapyrrole biosynthesis, function of tetrapyrrole intermediates, and the cell cycle process involved in DNA replication. The authors found a strong interdependence of plastid and mitochondrial DNA replication prior to nuclear DNA replication in the primitive red alga Cyanidioschyzon merolae. The coordination of nuclear DNA replication followed after the organellar replication was regulated through tetrapyrrole signaling contributing to organelle-nucleus communication. In this context it is remarkable to identify groups of over-represented GO categories among genes deregulated in seedlings with reduced ALA-synthesizing capacity that are involved in DNA replication and nuclear chromatin organization (Figure 6A). Moreover, the regulatory dependency of Mg porphyrins and plastid function of GUN1, a pentatricopeptide-repe at protein with the potential to bind nucleic acids implicates a regulatory link between tetrapyrrole biosynthesis and control of genes for DNA and RNA metabolism (Koussevitzky et al., 2007).

**WHAT COMMENDS ALA SYNTHESIS TO BE A SIGNAL EMITTER FOR NGE?**

Plastid signals have been proposed to convert multiple light signaling pathways controlling PhANGs (Ruckle et al., 2007). The over-representation of target genes reacting to abiotic stimuli and absence of light indicate a role of reduced ALA synthesis for modulation of light signaling networks (Figure 6B). It is conceivable that the metabolic pathway of Chl biosynthesis contributes to intracellular communication between chloroplast and nucleus in response to environmental and biological cues, such as abiotic stress and sucrose status to adjust the significant energy-converting processes of photosynthesis. In this context, the cis-element I-Box was found to be overrepresented in promoters of deregulated genes in gun4-1 (Figure 8). On the other hand, among deregulated genes carrying an I-box in the promoter region genes responding to sucrose and light stress are overrepresented (Table 4). This finding is in agreement to previous results of I-box-binding factors that are regulated by light, sugar sensing, and the circadian clock (Donald and Cashmore, 1990; Borello et al., 1993).

The compilation of up- and down-regulated genes identified in the transcriptomes of GAB-treated seedlings as well as gun4-1 knock-down seedlings revealed a significant and representative set of identically regulated genes indicating that modified ALA biosynthesis and/or the ALA pool in plastids have an impact on NGE at early stages of plant development. Since the time point chosen in our comparative transcriptome analysis corresponded to the first detectable accumulation of Chl (Figure S4 in Supplementary Material) upon illumination of etiolated seedlings, it may be assumed that the described changes in NGE are primary effects in response to inhibited ALA formation. Moreover, typical marker genes for photosynthesis and chloroplast biogenesis are missing among these genes identified in both experimental approaches (Figure 7).

**FIGURE 10 | Model summarizing control mechanisms that act on and are emitted from ALA biosynthesis.** The contribution to plastid-derived retrograde signaling is highlighted. ALA formation is posttranslationally controlled by several known feedback mechanisms of the Chl and Mg branch of the pathway (e.g., heme, Mg chelatase assembly, GUN4) also reflecting the impact of the gun2-gun5 mutations on ALA synthesis. Intermediates of the tetrapyrrole biosynthetic pathway e.g., Mg Protoporphyrin IX were discussed to be involved in signaling pathways and likely act via reactive oxygen species (ROS)-mediated changes in nuclear gene expression (NGE). Gabaculine (GAB) directly inhibits ALA formation resulting in changes of NGE at very early stages of chloroplast development. Norflurazon (NF) inhibits tetrapyrrole biosynthesis indirectly.
It can be concluded that our experimental setup allows an insight in the primary impact of modulated ALA biosynthesis on NGE. Consequentially, compromised tetrapyrrole biosynthesis will induce numerous regulatory adaptations to long-term perturbation of ALA biosynthesis, including modification of PhaNGs expression and additional protective mechanisms against photo-oxidative damage (Op den Camp et al., 2003). This holds true for mutants with impaired tetrapyrrole biosynthesis, such as several gun mutants, which experience a reduced ALA synthesis followed by extenuated Chl contents. When NF-treated seedlings are short of carotenoids, Chl-binding proteins cannot be stabilized, free Chl accumulates and causes severe photo-oxidative damage in wild-type seedlings. It is suggested that reduced metabolic flow in the tetrapyrrole biosynthetic pathway will modify the potential risk of photo-oxidative stress. This is in agreement with biochemical and genetic investigations of NF-treated gun mutants which point to an integrative plastid signaling converging at least signals derived from tetrapyrrole biosynthesis, organellar gene expression, and abscisic acid signaling (Voigt et al., 2010).

We propose an extended concept of tetrapyrrole-mediated retrograde signaling that moves an ALA synthesis-dependent mechanism controlling NGE into focus (Figure 10). This model integrates changes in NGE of many mutants affected in tetrapyrrole biosynthesis (Mochizuki et al., 2001; Larkin et al., 2003; Woodson et al., 2011) and takes into account the deleterious properties of intermediates of the Mg branch of tetrapyrrole biosynthesis in plastid-to-nucleus communication (Mochizuki et al., 2008; Moulin et al., 2008; Kleine et al., 2009). This ALA-dependent signaling occurs apart from GUN1-mediated-signaling (Koussevitzky et al., 2007) since regulatory impact of feeding with ALA or GAB on LHCBl.2 and RBCS expression acts additively to the gun1-1 phenotype (Figures 3 and 4). Since we observed that ALA feeding did not suppress expression of LHCBl.2 and RBCS in gun4-1, a function of GUN4 in the retrograde signaling pathway from ALA biosynthesis is suggested (Figure 4). Because multiple endogenous and environmental factors control the rate-limiting ALA synthesis and, thus, control the metabolic flow of tetrapyrrole intermediates for Chl and heme synthesis, retrograde signals emitted at the level of ALA formation are proposed to adjust the transcriptional machinery in the nucleus.

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AUTHOR NOTE

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Plant_Physiology/10.3389/fpls.2012.00236/abstract

Table S1 | PCR Primers to amplify gene specific probes used for quantitative real-time PCR. Primers for AtLHCB1.2, and AtRBCS were taken from Mochizuki et al. (2008).

Table S2 | Verification of microarray results via qRT-PCR. RNA from GAB-treated Arabidopsis wild-type seedlings and gun1-1 mutant seedings was extracted 6 and 24 h, respectively, after light exposure. Transcript abundance of a defined set of genes was determined by quantitative real-time PCR and calculated as relative expression (2^(-ΔΔCt)) compared to untreated Arabidopsis wild-type Col-0 seedlings. qRT-PCR data are given as average ± SD of three biological replicates. Listed are relative expressions obtained by microarray experiments (sampling 6 h after light exposure) as well as qRT-PCR in order to compare relative expressions in both experimental approaches. Relative expressions of qRT-PCR data were calculated using AIsAND (for 6 h values) and AIsACT2 (for 24 h values) as reference. Bold: Relative transcript abundance in GAB-treated or gun1-1 seedlings (determined by qRT-PCR) significantly differs from the untreated Arabidopsis wild-type Col-0 (P < 0.05).

Figure S1 | Pigment content (A) and phenotype (B) of Arabidopsis wild-type Col-0 seedlings, Col-0 seedlings treated with 10 µM gabaculine (GAB), and gun4-1 mutant seedlings 6 h after de-etiolation or after 6 days growing on MS medium (control) or MS medium ± 10 µM GAB. The contents of Chl a, Chl b, and Car of the untreated controls were 0.7 ± 0.2, 0.4 ± 0.2, and 2.6 ± 0.6 mg mg^{-1} Chl after deetiolation and 172 ± 20, 44 ± 3, and 57 ± 5 mg mg^{-1} Car for 6 days old seedlings, respectively.

Figure S2 | Phenotypes of etiolated Arabidopsis Col-0 seedlings grown in liquid MS medium containing increasing concentrations of ALA. Surface sterilized seedlings were germinated and grown for 3 days in darkness under continuous shaking. Above an ALA concentration of 50 µM the medium seedlings over-accumulate Pchlide.

Figure S3 | Phenotypes of Arabidopsis wild-type Col-0, gun1-1, and gun4-1 treated with combinations of 1 µM norflurazon (NF), 10 µM gabaculine (GAB), and 100 µM ALA, respectively. Seedlings were grown on 0.5 MS medium supplemented with 1% (w/v) sucrose containing the indicated chemicals for 6 days under photoperiodic growth light (12 h light/12 h dark) at 100–120 µmol photons m^{-2} s^{-1}.

Figure S4 | Pigment accumulation during de-etiolation of 3-day-old etiolated Arabidopsis Col-0 seedlings. Given are means ± SD of three independent extractions.

Figure S5 | Overrepresentation of gene ontology (GO) categories in genes showing decreased (A) and increased (B) transcript abundance in GAB-treated Arabidopsis wild-type Col-0 and/or gun4-1 mutants 6 h after illumination of etiolated seedlings. All 1032 genes being down-regulated (A) and 622 genes being up-regulated (B) in both GAB treated and gun4-1seedlings, were analyzed for overrepresented GO categories (P-value < 0.05). Dotted lines indicate intermediate categories that are not shown. Calculation of overrepresented GO categories based on BINGO 2.44.
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ALA contributes to retrograde signaling

Czarnecki et al. (2012) investigated the role of ALA in retrograde signaling. They found that ALA contributes to the regulation of nuclear gene expression in plants. The study highlights the importance of ALA in the integration of metabolic and developmental pathways, particularly in the context of chlorophyll synthesis.

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