Cellulose defects in the Arabidopsis secondary cell wall promote early chloroplast development

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SUMMARY

Lincomycin (LIN)-mediated inhibition of protein synthesis in chloroplasts prevents the greening of seedlings, represses the activity of photosynthesis-related genes in the nucleus, including LHCB1.2, and induces the phenylpropanoid pathway, resulting in the production of anthocyanins. In genomes uncoupled (gun) mutants, LHCB1.2 expression is maintained in the presence of LIN or other inhibitors of early chloroplast development. In a screen using concentrations of LIN lower than those employed to isolate gun mutants, we have identified happy on lincomycin (holi) mutants. Several holi mutants show an increased tolerance to LIN, exhibiting de-repressed LHCB1.2 expression and chlorophyll synthesis in seedlings. The mutations responsible were identified by whole-genome single-nucleotide polymorphism (SNP) mapping, and most were found to affect the phenylpropanoid pathway; however, LHCB1.2 expression does not appear to be directly regulated by phenylpropanoids, as indicated by the metabolic profiling of mutants. The most potent holi mutant is defective in a subunit of cellulose synthase encoded by IRREGULAR XYLEM 3, and comparative analysis of this and other cell-wall mutants establishes a link between secondary cell-wall integrity and early chloroplast development, possibly involving altered ABA metabolism or sensing.

Keywords: chloroplast, lincomycin, retrograde signaling, cell wall, phenylpropanoids.

INTRODUCTION

The vast majority of the several thousand proteins found in plastids are encoded by nuclear genes (Timmis et al., 2004). As endosymbiotic descendants of cyanobacteria, however, plastids still contain 80–230 genes, most of which are involved in essential plastid functions like energy production and plastid gene expression (PGE) (Ponce-Toledo et al., 2017). As a result, plastid multiprotein complexes (including the photosystems and ribosomes) consist of subunits encoded by both the nuclear and plastid genomes. This in turn accounts for the need for coordination of PGE and plastid gene expression (NGE). Thus, the nucleus influences activity in the plastids, including PGE, via ‘anterograde control/signaling’ (Stern et al., 2010), whereas plastids communicate their developmental and metabolic status to the nucleus via ‘retrograde signaling’, allowing the nucleus to adjust NGE appropriately (Kleine et al., 2009; Chi et al., 2013; Terry and Smith, 2013; Chan et al., 2016; Kleine and Leister, 2016). Plastid-derived retrograde signals can be divided into two classes: signals related to the operation of the plastid under changing environmental conditions (operational control) and signals triggered by changes in plastid and photosystem biogenesis (biogenic control) (Chan et al., 2016).

Forward-genetic screens have permitted the identification of components involved in retrograde signaling pathways (Kleine and Leister, 2016). They have exploited the fact that the expression of nuclear genes for plastid proteins like LHCB1.2 (a major light-harvesting chlorophyll a/b-binding protein) is reduced in seedlings exposed to inhibitors of PGE (e.g. lincomycin, LIN) or carotenoid biosynthesis (e.g. norflurazon, NF) (Oelmuller and Mohr, 1986; Oelmuller et al., 1986). The first mutant screen specifically designed to characterize components of biogenic plastid signaling was performed with Arabidopsis thaliana seedlings grown on NF (Susek et al., 1993), and identified mutant seedlings that continued to accumulate LHCB1.2 transcripts. Five different genomes uncoupled (gun)
mutants were initially isolated (Susek et al., 1993; Mochizuki et al., 2001). GUN1 codes for a nucleic-acid-binding chloroplast protein (Koussevitzky et al., 2007), whereas GUN2–GUN5 encode enzymes of the tetrapyrrole biosynthesis pathway (Mochizuki et al., 2001; Larkin et al., 2003).

Subsequent investigations of the gun mutants led to contradictory conclusions as to a putative plastid signaling function of the tetrapyrrole pathway intermediate Mg-protoporphyrin IX (Strand et al., 2003; Mochizuki et al., 2008; Moulin et al., 2008). To resolve these discrepancies, a gain-of-function screen based on activation tagging was conducted in the reporter line that was used in the original gun mutant screen (Woodson et al., 2011). This screen identified the gun6-1D mutant, which overexpresses ferrochelatase 1 (FC1), and prompted the proposal that the tetrapyrrole heme – specifically the fraction produced by FC1 – might function as a biogenic retrograde signal (Woodson et al., 2011; Terry and Smith, 2013). With the intention of identifying additional mutants with more subtle gun phenotypes than those detected in the original screen (Susek et al., 1993), a transgenic line in which the LHCBI.1 promoter was fused to the more sensitive reporter luciferase was used in a further screen (Ruckle et al., 2007), and mutants that exhibited a gun phenotype on NF were also tested on LIN-containing medium. As a result, four cryptochrome 1 (cry1) alleles and long hypocotyl 5 (hy5) were identified (Ruckle et al., 2007). More recently, overexpressors of GLK1 or GLK2 have been shown to behave like strong gun mutants when challenged with NF or LIN (Leister and Kleine, 2016; Martin et al., 2016).

Inhibitors like NF and LIN have numerous secondary effects, however: for example, the massive accumulation of anthocyanins (Cottage et al., 2010; Voigt et al., 2010). A modified version of the gun mutant screen was therefore designed, which used less NF and a lower light intensity, and resulted in fewer side effects, primarily by avoiding anthocyanin accumulation (Saini et al., 2011). Unlike the original gun mutants and wild-type (WT) plants, the happy on norflurazon (holi) mutants recovered in this screen remained green in the presence of (lower doses of) NF. The holi mutations were mapped to ClpR4, a nucleus-encoded subunit of the plastid-localized Clp protease complex, and to a putative chloroplast translation elongation factor, and thus are likely to interfere with PGE and plastid protein homeostasis (Saini et al., 2011).

Lincomycin and NF have similar effects on gun1, hy5 and cry1 (Ruckle et al., 2007) mutants, as well as on the GLK overexpressers (Leister and Kleine, 2016; Martin et al., 2016), but gun2, gun4 and gun5 mutants differ in their responses to these agents (Gray et al., 2003). This distinction suggests that NF and LIN trigger at least partially different signaling pathways.

In an effort to isolate additional gun mutants specifically for the LIN pathway(s), we screened an ethyl methanesulfonate (EMS)-mutagenized A. thaliana Col-0 population grown in the presence of a greater than four-fold lower dose of LIN than that used in the earlier screens. In this way, happy on lincomycin (holi) mutants that are able to green in the presence of LIN were identified. In a second screen with a fivefold lower NF concentration relative to that used in the original screens, we identified additional holi mutants. Characterization of these mutants suggested that: (i) there is no correlation between gun signaling and anthocyanin biosynthesis; and (ii) early chloroplast development is linked to cell-wall integrity.

RESULTS

Isolation of happy on lincomycin (holi) mutants

Seedlings grown on LIN experience severe photo-oxidative damage, plastid biogenesis is arrested at a proplastid-like stage, even under normal light conditions (Oelmuller and Mohr, 1986), and the expression of nuclear genes encoding chloroplast proteins is altered (Oelmuller et al., 1986). In screens intended to isolate mutants displaying the gun phenotype on LIN, a concentration of 220 μg ml⁻¹ LIN (high LIN) and light intensities of 100–125 μmol photons m⁻² sec⁻¹ were used (Koussevitzky et al., 2007; Ruckle et al., 2007). Under such conditions, 5-day-old A. thaliana Col-0 seedlings were retarded in growth, failed to green and accumulated appreciable concentrations of anthocyanins (Figure 1a). Using the same light intensity as in previous screens, we gradually reduced the LIN concentration until the coloration of the seedling population turned from purple (as a result of anthocyanin production) to greenish or white. Although some seedlings turned light green with a concentration of 25 μg ml⁻¹ LIN, growth on 50 μg ml⁻¹ LIN (low LIN) produced uniformly purple-colored seedlings (Figure 1a). The latter concentration was still sufficient to reduce the accumulation of the nucleus-encoded transcripts encoding the chloroplast proteins Lhcb1.2 and CA1 to 8.0% (high LIN, 1.5%) and 6% (high LIN, 1.0%), respectively, of the levels seen in MS-grown seedlings (Figure 1b). Thus, this low LIN dose still activates retrograde signaling. The gun1-1 mutant used as a control accumulated fewer anthocyanins than the WT when grown on high LIN, confirming a previous finding (Cottage et al., 2010). Approximately half of the gun1-1 seedlings grown on low LIN displayed whitish cotyledons, which were larger than those of the WT (Figure 1a).

Based on these observations, a screen was set up to identify mutants with an altered, visually discernible phenotype on low LIN. To this end, Col-0 seeds were mutagenized with EMS. The M1 plants were grown to maturity to produce M2 seeds, and ~20 000 4- to 5-day-old M2 seedlings were grown on low LIN and screened for alterations in the color or size of cotyledons. This led to the isolation of six holi mutants (Figure 2). The mutants holi1 and holi3
had yellowish cotyledons and did not accumulate anthocyanins. Cotyledons of the other mutants turned light green and accumulated anthocyanins to various levels. When grown on high levels of LIN, *holi2* displayed smaller cotyledons and hyperaccumulation of anthocyanins compared with all other mutants and the WT (Figure 2). A similar screen in which low LIN was replaced by low NF (1 μM) instead of the 5 μM NF used in the original *gun* mutant screen; Susek *et al.*, 1993) aimed to identify new *hon* mutants. This screen yielded *hon* mutants (*hon24, hon33* and *hon41*) with completely white cotyledons. On low LIN, the cotyledons of these *hon* mutants also appeared yellow-greenish, like those of the *holi* mutants (Figure 2). To confirm that the greenish color was caused by chlorophyll accumulation, autofluorescence was monitored after UV excitation of seedlings grown on low LIN (Figure 2). Col-0,
holi3 and hon24 seedlings displayed no autofluorescence, as expected when chlorophyll is absent; however, four mutants (holi2, holi4, holi5 and holi6) displayed marked levels of autofluorescence, whereas three others (holi1, hon33 and hon41) displayed weaker autofluorescence. Notably, none of the hon or holi mutants appeared greener on either high or low NF (Figure 2), in agreement with the absence of chlorophyll autofluorescence observed under these conditions.

Several holi mutants display a gun phenotype on low LIN

The chlorophyll-autofluorescence phenotype of some of the hon and holi mutants prompted us to test whether their continued plastid development despite growth on LIN was associated with altered signaling to the nucleus. To this end, RNA was prepared from 5-day-old Col-0, gun-1-1, and the various hon and holi mutant seedlings grown on low LIN, and subjected to Northern analysis to determine the steady-state levels of LHC1.2 mRNA. Following exposure to the low LIN concentration, the gun-1-1 mutant showed, as expected, higher LHC1.2 mRNA expression than the WT (Figure 3). Remarkably, LHC1.2 mRNA levels in the holi2, holi4, holi5 and holi6 mutants were comparable with, or even higher than, that of the gun-1-1 mutant. Under control conditions (without inhibitor treatment), however, LHC1.2 mRNA levels were already slightly elevated in the holi2, holi3, holi5 and holi6 mutants, which means that the gun phenotype on low LIN was relativized by around 1.7-fold (Figure 3). Grown on low NF, some of the holi mutants also displayed very weak gun phenotypes. But none of the identified hon and holi mutants behaved like a gun mutant when grown on high NF or high LIN (Figure 3).

The majority of Holi and Hon loci encode proteins involved in the flavonoid pathway

In order to explain the ability of some of the identified mutants to accumulate chlorophyll and maintain LHC1.2 transcript accumulation in the presence of low LIN, the M4 generation of the mutant plants was back-crossed to their parent Col-0, and seedlings displaying recessive and semi-dominant (in the case of holi2) mutant phenotypes were identified in the F2 generation. The underlying mutations were localized by next-generation sequencing (see Experimental procedures) and confirmed by Sanger sequencing. The holi1 and hon41 mutations turned out to be allelic, and both were mapped to the gene for the transcription factor MYB DOMAIN PROTEIN 75 (MYB75; PRODUCTION OF ANTHOCYANIN PIGMENT 1, PAP1). The C>T substitution at nucleotide (nt) 902 (relative to the start codon, as also in the following) in holi1 results in the replacement of an Arg by an Lys residue and permits some anthocyanin accumulation, whereas in hon41 a C>T substitution at nt 987 introduces a stop codon in the first exon (Figure 4), completely blocking anthocyanin accumulation (Figure 2).

Moreover, in holi3 a Trp codon is replaced by a stop in the gene for the transcription factor TRANSPARENT TESTA GLABRA 1 (TTG1, required for purple anthocyanin accumulation). The G>A substitution at nt 10 in hon24 and the C>T substitution at nt 987 in hon33 caused non-sense mutations in DIHYDROFLAVONOL 4-REDUCTASE (DFR; TRANSPARENT TESTA 3, TT3) and ANTHOCYANIDIN

Figure 3. LHC1.2 transcript levels found in WT, gun1-1, holi and hon mutant seedlings grown without inhibitor or in the presence of lincomycin (LIN) or norflurazon (NF). Seedlings were grown for 5 days under continuous light (100 μmol photons m⁻² sec⁻¹) on MS plates without inhibitor or supplemented with either LIN (50 or 220 μg ml⁻¹) or NF (1 or 5 μM). LHC1.2 mRNA levels were determined by Northern blot analyses. The methylene blue-stained blots served as loading controls (M.B.).
SYNTHASE (ANS; LEUCOANTHOCYANIDIN DIOXYGENASE, LDOX; TANNIN DEFICIENT SEED 4, TDS4; TT18), respectively. The mutation that most probably causes the **holi2** mutant phenotype is located at nt 4125, and replaces a Val by an Ile residue in **REDUCED EPIDERMAL FLUORESCENCE 4** (**REF4**; **MEDIATOR COMPLEX MED 5B**, **MED5B**; **MED33B**). The G>A substitution in **holi6** introduces a premature stop in exon 7 of **IRREGULAR XYEM 3** (**IRX3**; **CELLULOSE SYNTHASE 7**, **CESA7**; **MURUS 10**, **MUR10**). The mutations responsible for the **holi4** and **holi5** phenotypes could not be identified. With the exception of **IRX3**, all affected proteins have previously been shown to be involved in phenylpropanoid metabolism (Figure S1; Stout et al., 2008; Appelhagen et al., 2014). **MYB75** and **TTG1** are transcription factors, and **DFR** and **ANS** are enzymes that convert dihydroquercetin to leucocyanidin (**DFR**) and leucocyanidin to cyanidin, respectively (Figure S1). **REF4** is required for phenylpropanoid homeostasis and has been shown to interact directly with the conserved transcriptional coregulatory complex Mediator (Bonawitz et al., 2012).

**Disturbances in the phenylpropanoid pathway do not confer a** **gun** **phenotype**

Anthocyanins are produced via the flavonoid pathway, which is a branch of the general phenylpropanoid biosynthetic pathway (Tohge et al., 2005; Appelhagen et al., 2014). The **gun1-1** mutant clearly accumulates less anthocyanin than the WT when grown on both low and high LIN concentrations (Figure 1a), and it was noted previously that **gun2**, **gun4** and **gun5** mutants accumulate less anthocyanin than WT plants when grown on high NF (Voigt et al., 2010). Therefore, the growth of 5-day-old **gun1**, **gun4** and **gun5** mutant seedlings was also tested under our reduced inhibitor conditions (Figure S2). In the WT, **gun1-1** and **gun1-102** seedlings, anthocyanin accumulation was clearly discernible in seedlings grown on low and high NF in continuous white light; however, on high NF, the

**Figure 4.** Schematic representation of the positions of identified **HOLI** and **HON** mutation sites. Exons (black boxes), introns (black lines), and the 5’ and 3’ untranslated regions (UTRs; grey boxes) are shown. Numbers are given relative to the start codon ATG.

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stronger gun1 allele (gun1-102) displayed slightly less anthocyanin accumulation than the weaker allele (gun1-1) (Figure S2). In contrast, gun4-1 and gun5-1 accumulated less anthocyanin on both NF concentrations, whereas they accumulated WT levels of anthocyanins on high LIN. It has previously been speculated that plastid signals that require GUN2-GUN5 might stimulate anthocyanin biosynthesis, although anthocyanin content and LHCB1.2 mRNA accumulation in gun mutants are not strictly correlated (Voigt et al., 2010). As these authors considered only the accumulation of visible anthocyanins, reverse-phase ultra-performance liquid chromatography (UPLC) was used to profile the accumulation of phenylpropanoids in 5-day-old Col-0, gun1-1, gun2-1, gun4-1 and gun5-1 seedlings grown on MS in the absence or presence of high NF or high LIN. In the WT, high NF and high LIN caused approximately 2.0- and 1.5-fold increases in the total phenylpropanoid content, respectively (Figure S3; Table S1). Total phenylpropanoids were similarly boosted in the gun2-1, gun4-1 and gun5-1 mutants, but in gun1-1 they were approximately 1.5-fold induced after NF treatment and not induced at all by treatment with high LIN. A closer look at the accumulation of specific phenylpropanoid components revealed that the difference between gun1-1 and the WT is mainly attributable to a lack of induction of kaempferol derivatives in gun1-1 (Figure S3; Table S1). In particular, kaempferol 3-0-(6″-O(3-methoxy-4-hydroxy)glucoside) 7-O-rhamnolide (k3; see also Figure S1) was less effectively induced in all investigated gun mutants after NF treatment, as well as in gun1-1 after LIN treatment; none of the other detected compounds showed any consistent alteration in the gun mutants relative to the WT (Figure S3; Table S1). Levels of k3 are also reduced in the UDP-glucosyl transferase ugt78d1 ugt78d2 mutant (Yin et al., 2014). To definitively clarify whether disturbances in the phenylpropanoid pathway are linked to chloroplast development and/or a gun phenotype in the presence of inhibitors, 5-day-old ugt78d1 ugt78d2 mutants, together with mutants impaired in enzymatic steps of the general phenylpropanoid pathway (Figure S1), or regulatory factors of flavonoid biosynthesis and transporters involved in proanthocyanidin accumulation (Appelhagen et al., 2014), were first tested for chlorophyll autofluorescence on low LIN (Figure 5). Because cry1 mutants were previously identified as weak gun mutants on high LIN (Ruckle et al., 2007), the mutants cry1-304 and cry1-304 cry2-1, and the constitutive photomorphogenesis mutant cop1-4, were included as controls together with gun1-1. Chlorophyll autofluorescence could be detected in gun1-1, cry1-304, cry1-304 cry2-1, as well as in the cop1-4 mutant (Figure 5). All transport-related and regulation mutants showed some chlorophyll fluorescence, although this was restricted to the hypocotyl in ttg1-22, ttg2-5 and tt8-6 mutants. The biosynthesis mutants tt4-15, tt5-2, tt7-7, tt3-1 and tds4-2 showed the greenish fluorescence typical of kaempferol derivatives (Appelhagen et al., 2014) and only very weak or no chlorophyll autofluorescence (Figure 5). These mutants are defective in steps in the main pathway leading from chalcone synthase (tt4-15) to the conversion of leuocyanidin to cyanidin (tds4-2), which is the branch point for the production of anthocyanins and oxidized tannins (Figure S1). Thus, these mutants do not accumulate anthocyanins. The ban-5, tt15-4, tt6-2 and fis1-3 mutants displayed similar levels of chlorophyll fluorescence to the gun1-1 and cry1-304 mutants and the transport-related aha10-6 mutant. The tt10-8 mutant showed the strongest chloro-

When grown in the presence of low LIN or high NF, gun1-1, cry1-304 and cry1-304 cry2-1 seedlings accumulated LHCBI.2 in the presence of the inhibitors but, in accordance with Ruckle et al. (2007), the cop1-4 mutant did not (Figure 6a). It is noteworthy here that cry1 and cry1 cry2 seedlings grown on low LIN accumulated even higher levels of LHCBI.2 mRNA than gun1-1. The ugt78d1 ugt78d2 mutant (in which k3 is diminished) and the other phenylpropanoid mutants did not accumulate LHCBI.2 mRNA, with the sole exception of the tt10-8 mutant, which continued to express LHCBI.2 in the presence of low LIN but not in the presence of high NF (Figure 6b). TT10/LAC15 is similar to laccase-like polyphenol oxidases and is involved in lignin biosynthesis (Liang et al., 2006).

Taken together, these data imply that there is no direct link between phenylpropanoid accumulation and gun signaling.

A defect in the secondary cell wall promotes seedling greening

The holi6 mutant was among the identified mutants that displayed the strongest chlorophyll autofluorescence when grown on MS plates supplemented with low LIN (Figure 2). To confirm that the premature stop in IRX3 (CESA7) found in holi6 was responsible for this phenotype, two additional irx3 mutant alleles, irx3-2 (confirmation of the T-DNA insertion and a lack of the full-length transcript is shown in Figure S4) and irx3-4 (Brown et al., 2005), were grown on low LIN. Indeed, the cotyledons of irx3-2 and irx3-4 were visibly greener and displayed higher autofluorescence than the cotyledons of the WT (Figure 7a), which is reflective of a higher chlorophyll content (Figure 7b). Moreover, although attempts to determine the maximum quantum yield of photosystem II (Fv/Fm) with an Imaging PAM fluorometer were unsuccessful in WT grown on low LIN, this parameter could be measured in holi6, and irx3-2 and irx3-4 displayed even higher Fv/Fm values (Figure 7a).
IRX3/CESA7 is a member of the cellulose synthase (CESA) family. The CESA complexes required for the synthesis of primary and secondary cell walls differ in composition: IRX3, together with IRX1/CESA8 and IRX5/CESA4, is needed specifically for the synthesis of cellulose in the secondary cell wall, which also contains lignin (Meents et al., 2018; Polko and Kieber, 2019). CESA1/RADIIALLY SWOLLEN 1 (RWS1), CESA3 and CESA6-like proteins (CESA2, CESA5, CESA6 and CESA9) are involved in primary cell-wall synthesis (Meents et al., 2018; Polko and Kieber, 2019).

These findings raise the question of whether the holi phenotype might be caused by: (i) a general reduction in cellulose content in the secondary cell wall; (ii) reduced cellulose content in the primary cell wall; or (iii) a lack of hemicelluloses in the secondary cell wall. To clarify this issue, mutants with reduced cellulose content in the secondary cell wall (irx1-2, irx1-3 and irx5-4), together with a mutant with reduced cellulose content in the primary cell wall (rsw1-1; Williamson et al., 2001) and a mutant with reduced content of the hemicellulose xylan in the secondary cell wall (irx9-2; Bauer et al., 2006), were germinated on low LIN medium. The irx1 seedlings displayed comparably high autofluorescence to holi6 seedlings, whereas irx5-4 displayed weaker autofluorescence, and rsw1-1 and irx9-2 behaved like the WT (Figure 7c). Moreover, $F_{v}/F_{m}$ could not be detected in the additionally investigated mutants. Reduced cellulose production can affect growth and morphogenesis in various plant parts, as exemplified by the swollen roots of rsw1 mutants grown at 31°C (Arioli et al., 1998; Williamson et al., 2001). To test for any temperature dependency of the $F_{v}/F_{m}$ phenotype, rsw1-1 together with the other cell wall mutants was germinated at 31°C on control MS plates and on MS plates supplemented with low LIN. Cotyledons of the rsw1-1 mutant were smaller under both conditions, confirming the heat growth phenotype observed previously (Williamson et al., 2001), but, as in all other tested mutants, $F_{v}/F_{m}$ was WT-like (Figure S5a). Interestingly, both the greening and the elevated $F_{v}/F_{m}$ phenotypes were barely detectable under low LIN
conditions in hol6/irx3 and irx1 seedlings (Figure S5b), implying that the higher temperature overrides the capability of secondary cell wall mutants to green on LIN. Moreover, the potential greening capacity of rsw1-1 seedlings on LIN might be masked by the temperature sensitivity of this mutant.

Figure 6. Analysis of LHCB1.2 transcript levels of the wild type (WT), gun1, cry1, cry1 cry2, cop1-4 and mutants associated with phenylpropanoid biosynthesis grown in the presence of inhibitors. WT and the mutants described in the legend to Figure 6 were grown for 5 days under continuous light (100 μmol photons m⁻² sec⁻¹) on MS plates supplemented with (a) 50 μg ml⁻¹ lincomycin (LIN) or (b) 5 μM norflurazon (NF). LHCB1.2 mRNA levels were determined by Northern blot analyses. The methylene blue-stained blots served as loading controls (M.B.).

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In sum, it can be concluded that cellulose defects specifically in the secondary cell wall promote seedling greening, and that deactivation of IRX3 results in the strongest greening phenotype.

The *inx3* mutant behaves like a *gun* mutant on low LIN

A defect in IRX1 or IRX5, both of which are specific for the secondary cell wall CESA complex, results in the upregulation of ABA-responsive genes (Hernandez-Blanco et al., 2007). Moreover, *LHCB1.2* mRNA expression is higher when the *holi6* mutant is grown on low LIN, as well as in control conditions (Figure 3). To investigate the behavior of ABA-responsive genes and nuclear genes for chloroplast proteins on a transcriptome-wide level in *inx3* mutants, RNA-Seq analysis was performed on RNA isolated from 5-day-old WT and *inx3-2* seedlings grown in the absence of LIN (control) or on low LIN. Low LIN elicited substantial (more than twofold) changes in gene expression in the WT (915 up; 2313 down) and *inx3-2* (1139 up; 1978 down) seedlings (Figure 8a,b; Table S2). Of the genes up- and down-regulated in WT seedlings upon LIN treatment, 39 and 36%, respectively, were dependent on the presence of functional IRX3, namely those that were not more than twofold differentially expressed in *inx3-2*. Gene ontology (GO) analysis (Huang et al., 2009) of these IRX3-dependent genes identified an enrichment for the cellular component category ‘mitochondria’ among the 39% upregulated genes (Figure 8c), and in the categories ‘chloroplast stroma’, ‘thylakoid’, ‘chloroplast envelope’, ‘integral component of plasma membrane’ and ‘chloroplast’ among the 36% downregulated genes (Figure 8d). This showed that, in addition to *LHCB1.2*, other photosynthesis-related genes were de-repressed in *inx3* seedlings.

When grown under control conditions (without LIN), the transcriptome of the *inx3-2* mutant showed only moderate changes relative to the WT: the mRNA levels of 20 (41) or 46 (70) genes were significantly reduced or elevated (by more than 2.0- and 1.5-fold, respectively) (Figure 8a,b; Table S2). Analyses of the 1.5-fold changes revealed that in the downregulated gene set, only ‘chloroplast’ was significantly enriched in the cellular component category (CC), whereas the biological process (BP) categories ‘glucosinolate biosynthesis’, ‘leucine biosynthesis’ and ‘response to insect’ were more than 60-fold enriched (Figure 8e). In the upregulated gene set, the CC category ‘protein storage vacuole’ and several chloroplast-associated categories like...
plastoglobule’, ‘thylakoid’, ‘envelope’ and ‘stroma’ were enriched, and in the BP category ‘response to freezing’ and ‘glucosinolate catabolism’ were enriched approximately 40-fold, and ‘seed germination’, ‘photosynthesis’, ‘response to jasmonic acid’ and ‘response to abscisic acid’ were enriched approximately 10-fold, respectively (Figure 8e; Table 1). Among the abscisic acid (ABA)-responsive genes were the genes for the chloroplast-localized proteins COLD-REGULATED 15a (COR15a) and COR15b. Of note is also the slight (approximately 1.4-fold) but significant induction of genes encoding several Lhcb proteins and subunits of photosystems I and II (Figure 3; Tables S2 and S3), which was reflected in an approximately 1.5-fold higher chlorophyll content in 5-day-old holl6 and irx3 mutant seedlings (Figure 8f).

Taken together, these results suggest a role for the secondary cell wall in seedling greening, even under normal growth conditions. Moreover, a defect in IRX3 results in a weak gun phenotype on low LIN and leads to altered ABA metabolism or sensitivity.

DISCUSSION

Norflurazon is an inhibitor of phytoene desaturase and blocks carotenoid biosynthesis, whereas LIN binds to the 50S subunit of the plastid ribosome, thus inhibiting protein synthesis in the organelle. Treatment of seedling plants...
accumulation and suppresses the light-induced transcription of nuclear genes for photosynthesis, such as LHCBI.2. In all known gun mutants, LHCBI.2 expression is partly de-repressed in the presence of NF, but only a subset of gun mutants display this phenotype in the presence of LIN (Koussevitzky et al., 2007; Ruckle et al., 2007). We attempted to isolate further mutants that can better cope with LIN. High concentrations of LIN (220 µg ml⁻¹) were used in previous studies (Koussevitzky et al., 2007; Ruckle et al., 2007; Choy et al., 2008; Cottage et al., 2010; Sun et al., 2016), and we found that a greater than fourfold lower concentration (50 µg ml⁻¹) of LIN still repressed nucleus-encoded photosynthesis genes (Figure 1b). Moreover, the use of low LIN uncovered a clear phenotypical difference between gun1-1 and WT seedlings, as cotyledons were larger and anthocyanin accumulation was less pronounced in the gun1-1 mutant (Figure 1a). With the exception of the hon mutant screen, in which lower NF and light dosages were used (Saini et al., 2011), all previous gun mutant screens used reporter genes to identify mutants with de-repressed LHCBI expression (Kleine and Leister, 2016). In contrast, we attempted to isolate mutants based on visually discernible differences from the WT when grown on low LIN. Although the earlier hon mutant screen identified mutants that are affected in chloroplast protein homeostasis, our low-LIN screen led to the identification of ‘holi’ and additional ‘hon’ mutants for proteins involved in: (i) the flavonoid pathway; and (ii) secondary cell wall formation (Figure 4).

We used 5-day-old seedlings grown on MS supplemented with sucrose to investigate phenylpropanoid accumulation and parameters associated with chloroplast development, i.e. the maximum quantum yield of photosystem II, LHCBI.2 expression levels and greening. In 1992, it was found that several transcripts for enzymes of the flavonoid biosynthetic pathway reached a maximum in 3-day-old Arabidopsis seedlings grown in continuous light. The authors concluded that the peak anthocyanin content appeared to coincide with the maturation of chloroplasts, and the associated switch to photoautotrophic growth (Kubasek et al., 1992). Subsequent work showed that, in the presence of 2% sucrose, and in the absence of inhibitors, anthocyanin accumulation reaches a maximum in 5-day-old seedlings (Cottage et al., 2010). In addition to the presence of disaccharides, the induction of anthocyanins depends on a functional photosynthetic electron transport chain and on light (Jeong et al., 2010). Thus, the light-signaling mutants cry1 and hy5 exhibit significant inhibition of anthocyanin accumulation (Ahmad et al., 1995; Jeong et al., 2010). Notably, cry1 and hy5 mutants have been identified as gun mutants (Ruckle et al., 2007), and lower levels of anthocyanins were noted in the original set (gun1-gun5) in this present study and in other studies (Cottage et al., 2010; Voigt et al., 2010). The idea that changes in anthocyanin accumulation might trigger de-repression of LHCBI.2 in gun mutants has previously been rejected (Voigt et al., 2010); however, the consistent observation of lowered anthocyanin accumulation in mutants showing the gun phenotype prompted us to re-evaluate

Table 1 Differential expression of ABA-responsive genes, and genes involved in chloroplast biogenesis and light reactions in 5-day-old /x3-2 mutant seedlings, compared with Col-0

| Locus identifier | Fold change | Description | Gene symbol |
|------------------|-------------|-------------|-------------|
| Chloroplast      |             |             |             |
| AT2G20570        | 1.47        | GOLDEN2-LIKE 1 | GLK1        |
| AT1G61520        | 1.33        | PSI CHLOROPHYLL A/B BINDING PROTEIN 3 | LHC2A |
| AT3G47470        | 1.39        | PSI CHLOROPHYLL A/B BINDING PROTEIN A4 | LHC4A |
| AT1G29910        | 1.36        | PSI CHLOROPHYLL A/B BINDING PROTEIN 3 | LHC1A |
| AT2G34430        | 1.41        | PSI CHLOROPHYLL A/B BINDING PROTEIN B1 | LHC1B.2 |
| AT2G05100        | 1.51        | PSI CHLOROPHYLL A/B BINDING PROTEIN 2.1 | LHC2.1 |
| AT2G05070        | 1.63        | PSI CHLOROPHYLL A/B BINDING PROTEIN 2.2 | LHC2.2 |
| AT3G27690        | 1.53        | PSI CHLOROPHYLL A/B BINDING PROTEIN 2.3 | LHC2.3 |
| AT5G54270        | 1.47        | PSI CHLOROPHYLL A/B BINDING PROTEIN 3 | LHC3.1 |
| AT4G10340        | 1.33        | PSI CHLOROPHYLL A/B BINDING PROTEIN 5 | LHC5 |
| AT4G27440        | 1.32        | PSII CHLOROPHYLL A/B BINDING PROTEIN 5 | POR |
| AT4G28750        | 1.46        | PSI SUBUNIT E-1 | LHC2A-1 |
| AT1G62230        | 1.40        | PSI SUBUNIT H2 | LHC2A-2 |
| AT1G06380        | 1.40        | PSI SUBUNIT O | LHC2A-3 |
| ATCG00220        | 1.40        | PSI SUBUNIT M | LHC2A-4 |
| AT4G05180        | 1.36        | PSI SUBUNIT O-2 | LHC2A-5 |
| AT2G30570        | 1.33        | PSI SUBUNIT W | LHC2A-6 |
| AT1G67740        | 1.35        | PSI SUBUNIT Y | LHC2A-7 |
| ABA-responsive    |             |             |             |
| AT2G42540        | 3.44        | COLD-REGULATED 15A | COR15A |
| AT2G42530        | 3.17        | COLD REGULATED 15B | COR15B |
| AT1G29395        | 2.83        | COLD REGULATED 314 | COR131M1 |
| AT5G15970        | 1.98        | Stress-induced protein KIN2/COLD-REGULATED 6.6 | KIN2 |
| AT1G52400        | 2.19        | BETA GLUCOSIDASE 18 | BGLU18 |
| AT4G04020        | 1.61        | FIBRILLIN 1 | FBN1A |
| AT4G23800        | 2.74        | CORONATINE INDUCED 1 | COR3 |
| AT4G28520        | 4.52        | CRUCIFERIN 3 | CRU3 |
| AT5G25980        | 3.52        | GLUCOSIDE GLUCOHYDROLASE 2 | TGA2 |
| AT5G44120        | 4.67        | CRUCIFERIN A | CRA1 |

Seedlings were grown on MS without supplementation of LIN. Differential expression was determined with RNA-Seq analysis (Tables S2 and S3) and fold changes are represented. PS, photosystem.
this putative link. Because anthocyanins represent the only visibly perceptible products of the phenylpropanoid pathway, we performed reverse-phase UPLC to profile the accumulation of phenylpropanoids that absorb in the UV region (280 nm) of the spectrum (Figure S3; Table S1) and found that the kaempferol derivative k3 is less abundant in gun mutants after inhibitor treatment. A second approach using various mutants blocked at different steps in the phenylpropanoid pathway (Figures 6 and 7) strongly suggests that neither the abundance of k3 nor that of any other intermediate of the phenylpropanoid pathway is correlated with LHCBl.2 expression, however. Therefore, our data suggest that changes in phenylpropanoid levels cannot account for LHCBl.2 de-repression in inhibitor-treated gun mutants.

Of all the mutants identified here, holi6 displayed the strongest chlorophyll autofluorescence when grown in the presence of low LIN (Figures 2 and 8). HOL6 encodes the cellulose synthase subunit CESA7, also named IRX3, because in irx mutants the xylem collapses (Brown et al., 2005). Further results indicated that perturbation of cellulose formation specifically in the secondary cell wall leads to a happy-on-lincomycin phenotype (Figure 7). This may seem counterintuitive, but the weakening of the cell wall caused by defects in IRX1, IRX3 or IRX5 also confers enhanced resistance to some pathogens (Hernandez-Blanco et al., 2007; Miedes et al., 2014). In analogy to our findings that the rsw1-1 mutant with a defect in the primary cell wall is not able to green on LIN (Figure 7), susceptibility to these pathogens was not altered in mutants that affect the primary cell wall, like the cesal3 and rsw1 mutants (Hernandez-Blanco et al., 2007), although cesal3 mutants can be more resistant to other pathogens (Ellis et al., 2002). Could this mean that a weakened secondary cell wall might confer resistance to lincomycin? Presumably not, because the chlorophyll content of irx3 mutants is already higher than that of the WT under normal growth conditions (Figure 8). Moreover, the disease resistance phenotype of irx1, irx3 and irx5 mutants has been attributed in part to the constitutive activation of plant immune responses rather than to alterations in the passive wall barrier. In irx1-6 and irx5-5 plants a large number of ABA-regulated genes are constitutively upregulated (Hernandez-Blanco et al., 2007), which is in agreement with an increased accumulation of ABA in the irx1-6 mutant (Chen et al., 2005). Accordingly, we found that, in the irx3-2 mutant, ABA-responsive genes are upregulated under normal growth conditions (Figure 8). ABA has previously been shown to have an impact on LHCBl.2, plastid-encoded gene expression (Koussevitzky et al., 2007; Voigt et al., 2010; Yamburensko et al., 2013) and plastid differentiation (Rohde et al., 2000; Penfield et al., 2006; Kim et al., 2009). ABA seems to affect plastid differentiation in opposing ways. High concentrations suppress the expression of certain nucleus-encoded chloroplast proteins as well as plastid formation in etiolated and light-grown seedlings, and in seedlings grown in the presence of NF (Penfield et al., 2006; Koussevitzky et al., 2007), whereas lower concentrations stimulate these processes (Voigt et al., 2010; Kim et al., 2012). The tetrapyrrole biosynthesis proteins GUN4 and GUN5 (Voigt et al., 2010), the PPR protein GUN1 (Cottage et al., 2010) and GREENING AFTER EXTENDED DARKNESS 1 (GED1) (Choy et al., 2008) all enhance seedling development in the presence of ABA. Interestingly, the ged1 mutant was identified in a further attempt to isolate gun-like mutants (Gray et al., 2003; Choy et al., 2008); however, unlike holi6, ged1 is not a true gun mutant, because RBCS and LHCBl mRNA levels are already elevated in the absence of inhibitors, and ged1 shows only a very subtle gun phenotype upon treatment with NF or LIN (Choy et al., 2008).

The phenomenon of signaling from an altered cell wall to influence seedling photomorphogenesis in the dark has been recognized in the case of sugar- (Li et al., 2007) and zinc-responsive (Sinclair et al., 2017) growth and development. Our results suggest that defects in secondary cell walls also generate signals that modify nuclear gene expression and promote seedling greening, possibly via altered ABA metabolism or sensing.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

The mutant lines used in this study are listed in Table S4. The irx3-2 mutant was genotyped with the following primers: SAIL_885_D10_LP, 5′-AGGTTGGATCATGCAAGATG-3′; SAIL_885_D10_RP, 5′-CCAGCTGAATTCGAGATAC-3′, and LB, 5′-ATTTTGCCGATTTCGGAAC-3′. Surface-sterilized seeds were sown on Murashige and Skoog plates containing 0.8% (w/v) agar (pH 5.8), and stratified for at least 2 days at 4 °C. The growth medium contained 1% (w/v) sucrose, unless indicated otherwise. Seedlings were grown at 22 °C under continuous illumination (100 μmol photons m⁻² sec⁻¹) provided by white fluorescent lamps or at 31 °C under continuous illumination (80 μmol photons m⁻² sec⁻¹ provided by LEDs, which corresponds to 100 μmol photons m⁻² sec⁻¹ provided by white fluorescent lamps). For inhibitor experiments, MS medium was supplemented with the indicated concentration of lincomycin (Sigma-Aldrich, https://www.sigmaaldrich.com/united-kingdom.html) or norflurazon (Sigma-Aldrich).

EMS mutagenesis and whole-genome resequencing

Col-0 seeds were mutagenized using 0.2% (v/v) EMS (Sigma-Aldrich). The mutagenized M1 plants were grown in pools of 500 to produce the M2 generation of seeds. M2 plants were screened for holi or hon phenotypes. Segregating F2 populations were generated by backcrossing holi or hon mutants with the parental Col-0 line. To identify the causative mutations, positive pools of 50 plants each were selected based on their holi or hon mutant phenotype. DNA was extracted with the DNeasy Plant Mini kit (QIA-GEN, https://www.qiagen.com). Preparation of 250-bp insert DNA libraries and 150-bp paired-end sequencing was carried out at Novogene Biotech (https://en.novogene.com) on an Illumina HiSeq 2500 system (Illumina, https://www.illumina.com) with standard Illumina protocols. The sequencing depth was at least 7 G of raw data per sample, which corresponds to a more than 50-fold
subjected to the web application CandiSNP (Etherington and Durbin, 2009), which generates SNP density plots. The output list of CandiSNP was screened for non-synonymous amino acid changes and the hon/SNP was specific for the hon and hon mutants, the SNPs between each of the hon and hon mutants were compared with the SNPs of our Col-0 strain. The resulting hon- and hon-specific SNP lists were subjected to the web application CandiSNP (Etherington et al., 2014), which generates SNP density plots. The output list of CandiSNP was screened for non-synonymous amino acid changes and for the G/C to A/T transitions that were likely to be caused by EMS, with a special focus on the chromosome with the highest SNP density with an allele frequency of > 0.75.

Detection of chlorophyll autofluorescence

Chlorophyll autofluorescence of cotyledons was recorded with a Lumar V12 microscope equipped with the filter set Lumar 09 (no. 485009) connected to an AxioCam digital camera (Zeiss, https://www.zeiss.com).

Chlorophyll fluorescence measurements

Chlorophyll fluorescence was detected using an imaging Chi fluorometer (Imaging PAM, M-Series; Walz, https://www.walz.com) equipped with the computer-operated PAM control unit IMAGE-MAXI, as described previously (Xu et al., 2019).

Chlorophyll concentration measurements

For chlorophyll extraction, the cotyledons were blotted with filter paper to remove excess water, and hypocotyls were removed to ensure that only chlorophyll from the cotyledons was extracted. Briefly, 50-mg (fresh weight) cotyledon samples were ground and chlorophyll was extracted by adding 4 ml of 80% (v/v) acetone to each sample. The extract was centrifuged at 17 900 g for 10 min, and the pigments were quantified as described previously (Porra et al., 1989).

Determination of phenylpropanoid levels

Extraction, detection and analysis of phenylpropanoid contents was done as described in Appendix S1.

cDNA synthesis and quantitative RT-PCR analysis

Total RNA was extracted with the RNeasy Plant Mini kit (Qiagen) according to the manufacturer’s protocol, and 2 µg of the RNA was employed to synthesize cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, https://www.bio-rad.com). RT-qPCR analysis was performed on a Bio-Rad iQ5 real-time PCR instrument with the iQ SYBR Green Supermix (Bio-Rad). Each sample was quantified in triplicate and normalized using the pigments were quantified as described previously (Porra et al., 1989).

RNA gel-blot analysis

Total RNA was purified using the TRIzol reagent (Invitrogen, now ThermoFisher Scientific, https://www.thermofisher.com). To eliminate contaminating genomic DNA, RNA was treated with DNase I (New England Biolabs, https://www.neb.com). Total RNA (5 µg) was fractionated on a denaturing agarose gel, blotted onto a nylon membrane (Hybond-XL; GE Healthcare, https://www.chemilcore.com) and subsequently cross-linked by UV light. Hybridizations were performed at 65°C according to standard protocols. Details of these probes have been described previously (Kaczprzak et al., 2019).

RNA sequencing (RNA-Seq) and data analysis

Total RNA from plants was isolated using Trizol (Invitrogen, now ThermoFisher Scientific) and purified using Direct-zol™ RNA MiniPrep Plus columns (Zymo Research, https://www.zymoresearch.com) according to the manufacturer’s instructions. RNA integrity and quality were assessed with an Agilent 2100 Bioanalyzer (Agilent, https://www.agilent.com). Ribosomal RNA depletion, the generation of RNA-Seq libraries and 150-bp paired-end sequencing on an Illumina HiSeq 2500 system (Illumina) were conducted at Novogene Biotech with standard Illumina protocols. Three independent biological replicates were used per genotype.

RNA-Seq reads were analyzed on the Galaxy platform (Afgan et al., 2016), as described by Xu et al. (2019). Sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (Edgar et al., 2002) and are accessible through the GEO series accession number GSE130337.

Data analysis and statistical tests

One-way analysis of variance (ANOVA) was performed to determine statistical significances between genotypes (P < 0.05), followed by Tukey’s test for differences of group means at a 95% confidence interval using SPSS STATISTICS 17.0.

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CONFLICT OF INTEREST

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

Conceptualization: TK, DX Experiments: DX, RD, AG, H-P M, and TK Supervision: TK, DL, DX, RD, AG, H-P M, and TK Writing original draft: TK Writing review and editing: DL, DX, RD, AG, H-P M, and TK Supervision: TK Funding acquisition: TK and DL.

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DATA AVAILABILITY STATEMENT
RNA sequencing data have been deposited in the NCBI GEO (Edgar et al., 2002) and are accessible through the GEO series accession number GSE130337.

SUPPORTING INFORMATION
Additional Supporting Information may be found in the online version of this article.

Figure S1. Illustration of the phenylpropanoid and flavonoid pathways in Arabidopsis.

Figure S2. Phenotypes of gun mutants grown in the presence of lincomycin (LIN) or norfuraniz (NF).

Figure S3. Determination of phenylpropanoid contents of 5-day-old WT and gun mutant seedlings.

Figure S4. Confirmation of the iring3-2 T-DNA insertion mutant.

Figure S5. Phenotypes of WT, hol6 and mutants associated with cell-wall synthesis grown at 31°C on MS without or with low lincomycin (LIN).

Table S1. Detection of major phenylpropanoids in methanolic extracts from 5-day-old seedlings grown on MS plates without supplementation or supplemented with either 5 µM NF or 220 µg ml⁻¹ LIN.

Table S2. Genes with transcript levels that differed significantly from Col-0 in 5-day-old irx3-2 seedlings grown on MS plates without supplementation or supplemented with 50 µg ml⁻¹ LIN.

Table S3. Genes with transcript levels that differed significantly from Col-0 in 5-day-old irx3-2 seedlings grown on MS plates were sorted into different categories.

Table S4. Arabidopsis thaliana mutants used in this study.

Appendix S1. Supplemental materials and methods.

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