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RESEARCH PAPER

The *Arabidopsis* plastid-signalling mutant *gun1* (*genomes uncoupled1*) shows altered sensitivity to sucrose and abscisic acid and alterations in early seedling development

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Abstract

Developing seedlings of the *Arabidopsis gun1* (*genomes uncoupled1*) mutant, which is defective in retrograde plastid-to-nucleus signalling, show several previously unrecognized mutant phenotypes. *gun1* seedlings accumulated less anthocyanin than wild-type seedlings when grown in the presence of 2% (w/v) sucrose, due to lower amounts of transcripts of early anthocyanin biosynthesis genes in *gun1*. Norflurazon and lincomycin, which induce retrograde signalling, further decreased the anthocyanin content of sucrose-treated seedlings, and altered the temporal pattern of anthocyanin accumulation. Lincomycin treatment altered the spatial pattern of sucrose-induced anthocyanin accumulation, suggesting that plastids provide information for the regulation of anthocyanin biosynthesis in *Arabidopsis* seedlings. The temporal pattern of accumulation of *LHCB1* transcripts differed between wild-type and *gun1* seedlings, and *gun1* seedlings were more sensitive to sucrose suppression of *LHCB1* transcript accumulation than wild-type seedlings. Growth and development of *gun1* seedlings was more sensitive to exogenous 2% sucrose than wild-type seedlings and, in the presence of lincomycin, cotyledon expansion was enhanced in *gun1* seedlings compared to the wild type. *gun1* seedlings were more sensitive than wild-type seedlings to the inhibition of seedling growth and development by abscisic acid. These observations clearly implicate GUN1 and plastid signalling in the regulation of seedling development and anthocyanin biosynthesis, and indicate a complex interplay between sucrose and plastid signalling pathways.

Key words: Abscisic acid, anthocyanin, chloroplast, *LHCB1*, lincomycin, norflurazon, plastid signalling, retrograde signalling, sucrose, sugar signalling.

Introduction

Chloroplasts are believed to have evolved from a cyanobacterium-like prokaryote that formed an endosymbiotic relationship with a protoeukaryote, followed by the large-scale relocation of genes from the endosymbiont genome to the nuclear genome of the host (Bonen and Doolittle, 1975; Abdallah *et al.*, 2000; Martin *et al.*, 2002). In *Arabidopsis thaliana* the nuclear genome now encodes more than 90% of all chloroplast-located proteins (Abdallah *et al.*, 2000; Martin *et al.*, 2002) and only ~80 protein-coding genes remain in the chloroplast genome (Sugiura, 1992). However, chloroplasts retain some control over the expression of many of these nuclear genes, including those that encode proteins that function in photosynthesis (Gray *et al.*, 2003; Nott *et al.*, 2006). Evidence for retrograde chloroplast-to-nucleus signalling was initially obtained from mutant plants defective in

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plastid protein synthesis (Bradbeer *et al.*, 1979) or carotenoid synthesis (Mayfield and Taylor, 1984; Batschauer *et al.*, 1986; Giuliano and Scolnik, 1988). The subsequent use of inhibitor treatments of wild-type plants provided evidence for chloroplast-to-nucleus signalling in a wider range of plant species (Oelmüller and Mohr, 1986; Simpson *et al.*, 1986; Stockhaus *et al.*, 1987; Sagar *et al.*, 1988; Susek *et al.*, 1993). Inhibition of carotenoid biosynthesis, for example with norflurazon (Breitenbach *et al.*, 2001), or of plastid gene expression, with chloramphenicol, lincomycin, erythromycin or tagetitoxin (Oelmüller *et al.*, 1986; Rapp and Mullett, 1991; Gray *et al.*, 1995; Sullivan and Gray, 1999), resulted in reduced accumulation of transcripts of nuclear genes encoding photosynthesis-related proteins. This was shown to be due to decreased transcription of the nuclear genes, by the use of nuclear run-on assays (Batschauer *et al.*, 1986; Burgess and Taylor, 1988; Ernst and Schefbeck, 1988; Sagar *et al.*, 1988) or transgenic plants containing promoter–reporter gene constructs (Simpson *et al.*, 1986; Stockhaus *et al.*, 1987; Bolle *et al.*, 1994; Gray *et al.*, 1995). Various other treatments that affect the redox status of photosynthetic components or generate reactive oxygen species have been shown to affect the expression of nuclear genes (Escoubas *et al.*, 1995; Oswald *et al.*, 2001; Fey *et al.*, 2005; Piippo *et al.*, 2006). Plastid-to-nucleus signalling has been extensively reviewed in the last few years (Nott *et al.*, 2006; Piñas Fernández and Strand, 2008; Pogson *et al.*, 2008; Woodson and Chory, 2008; Kleine *et al.*, 2009).

The isolation of *Arabidopsis genomes uncoupled* (*gun*) mutants, which express nuclear genes encoding chloroplast-located proteins in the presence of norflurazon (Susek *et al.*, 1993), has allowed the identification of retrograde signalling-related components. The original mutant screen identified five different *gun* loci that expressed the *uidA* reporter gene under the control of the *LHCB1.2* promoter in the presence of norflurazon (Susek *et al.*, 1993). Double mutant analyses placed *gun2*, *gun3*, *gun4*, and *gun5* in the same chloroplast signalling pathway, whereas *gun1* appeared to be distinct from this group (Vinti *et al.*, 2000; Mochizuki *et al.*, 2001). *gun2* and *gun3* were shown to be allelic to the long hypocotyl mutants *hy1* and *hy2*, respectively, with *GUN2 (HY1)* encoding haem oxygenase and *GUN3 (HY2)* encoding phytochromobilin synthase (Davis *et al.*, 1999; Muramoto *et al.*, 1999; Kohchi *et al.*, 2001). *GUN4* encodes a tetrapyrrole-binding protein that has a role in the activation of Mg-chelatase (Larkin *et al.*, 2003) and *GUN5* encodes the H-subunit of Mg-chelatase (Mochizuki *et al.*, 2001). The chloroplast signalling mutants *gun2–gun5* all affect proteins with a role in tetrapyrrole biosynthesis and it was suggested that Mg-protoporphyrin IX (Mg-Proto IX) was the plastid-derived signal that modulates nuclear gene expression (Strand *et al.*, 2003). However, recent analyses have failed to confirm any relationship between the accumulation of Mg-Proto IX and repression of nuclear gene expression (Mochizuki *et al.*, 2008; Moulin *et al.*, 2008).

GUN1 appears to have a role in plastid signalling distinct from that mediated by the tetrapyrrole biosynthesis proteins, as indicated by double mutant analyses (Vinti *et al.*, 2000; Mochizuki *et al.*, 2001) and by microarray experiments, which showed differences in the genes affected by the *gun1* and *gun2–gun5* mutations (Strand *et al.*, 2003). In addition, treatment with the plastid protein synthesis inhibitor lincomycin led to a loss of photosynthesis-related nuclear gene expression in seedlings of the wild type and *gun2–gun5* but not of *gun1* (Gray *et al.*, 2003). The *gun1* mutant was also shown to be defective in signalling in response to high-light treatment (Koussevitzky *et al.*, 2007), and it was suggested that GUN1 acts to integrate signals produced in response to norflurazon, lincomycin, and high-light treatments. *GUN1* encodes a plastid nucleoid-associated protein containing 10 copies of the PPR (pentatricopeptide repeat) motif and an SMR (small MutS-related) domain near the C-terminus (Koussevitzky *et al.*, 2007; Cottage *et al.*, 2008). The GUN1 SMR domain was shown to bind DNA (Koussevitzky *et al.*, 2007) and PPR motifs are found in a large number of mitochondrial and plastid proteins involved in RNA processing (Schmitz-Linneweber and Small, 2008), but the mechanism of action of the GUN1 protein is currently unknown.

The AP2-like transcription factor ABI4 has been proposed to act downstream of GUN1 in the plastid-signalling pathway (Koussevitzky *et al.*, 2007). Many photosynthesis-related nuclear genes controlled by plastid signals contain sequences resembling ABA-response elements in their promoters (Koussevitzky *et al.*, 2007). The *abi4* mutant was shown to have a weak *gun* phenotype, and over-expression of *ABI4* was able to suppress the *gun1* phenotype (Koussevitzky *et al.*, 2007). Previously, the *abi4* mutant *sun6* (*sucrose-uncoupled6*) had indicated an interaction between plastid-derived redox signals and sucrose-regulated gene expression (Oswald *et al.*, 2001). SUN6 had also been implicated in sucrose repression of phytochrome A signal transduction pathways (Dijkwel *et al.*, 1997), indicating interplay of sucrose, light, and plastid signalling pathways.

gun1 mutant plants display no obvious physiological or morphological abnormalities and are indistinguishable from wild type when grown under a variety of conditions (Susek *et al.*, 1993). However, closer examination revealed that a significant number of *gun1* seedlings failed to de-etiolate when transferred from dark to light (Susek *et al.*, 1993; Mochizuki *et al.*, 1996) or produced variegated seedlings when grown in continuous light for 6 d (Ruckle *et al.*, 2007). The isolation of *cryptochrome1* (*cry1*) mutants from a screen for new *gun* mutants (Ruckle *et al.*, 2007) provided further evidence for cross-talk between plastid and light signalling networks, and it was shown that *gun1cry1* double mutants produced a higher proportion of variegated seedlings than *gun1* mutants. Experiments examining the effect of different light qualities and quantities on single and double mutants of *gun1*, *cry1*, and *hy5* grown in the presence of lincomycin suggested that plastid signals play important roles in both chloroplast biogenesis and photomorphogenesis (Ruckle *et al.*, 2007; Ruckle and Larkin, 2009). GUN1-dependent plastid signals repressed cotyledon expansion in low fluence blue light, and stimulated

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hypocotyl extension in blue or white light, particularly in a *hy5* background (Ruckle and Larkin, 2009). The *gun1* mutants also accumulated less anthocyanin in lincomycin-treated seedlings in blue light (Ruckle and Larkin, 2009).

During our characterization of *gun* mutants (Cottage *et al.*, 2008), several subtle sucrose-dependent phenotypic differences were observed between *gun1-1* and its parental Col-0, particularly during the early stages of seedling development. Seedlings of *gun1-1* accumulated less anthocyanin than wild-type seedlings when grown in the presence of either norflurazon or lincomycin on medium containing 2% sucrose. Similar observations were made with the *gun1-100* mutant (Cottage *et al.*, 2008) and its parental Ws, although anthocyanin accumulation was considerably lower in Ws compared to Col-0. Anthocyanin accumulation in *Arabidopsis* seedlings is induced by sucrose in a distinct temporal and spatial fashion and there are known to be clear differences between ecotypes (Tsukaya *et al.*, 1991; Kubasek *et al.*, 1992; Aukerman *et al.*, 1997; Mita *et al.*, 1997; Ohto *et al.*, 2001; Teng *et al.*, 2005; Solfanelli *et al.*, 2006). This paper describes our observations on anthocyanin and *LHCB1* transcript accumulation in *gun1-1* and wild-type Col-0 seedlings, leading to the conclusion that the absence of functional GUN1 results in alterations in early seedling development and altered sensitivity to sucrose and ABA. This provides additional evidence for the complex interplay of sucrose, light, and plastid signalling pathways in plant development.

Materials and methods

Plant materials and growth conditions

gun1-1 seed was obtained from J Chory (Plant Biology Laboratory and Howard Hughes Medical Institute, The Salk Institute, La Jolla, CA 92037, USA). Col-0 seed was obtained from the European Arabidopsis Stock Centre (NASC Stock code: N1092). Standard growth medium contained 0.5× Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Duchefa Biochemie, Haarlem, The Netherlands) and 1% (w/v) agar. Where stated, medium additionally contained 2% sucrose, 58 mM sorbitol (Melford laboratories, Suffolk, UK), 5 μM norflurazon (Sandoz Agro Inc, Des Plaines, IL), 0.5 mM lincomycin (Duchefa, Haarlem, The Netherlands) or ABA (Sigma) in the range of 0.5–25 μM. Prior to plating, seeds were surface-sterilized by washing in 70% ethanol, followed by 10% sodium hypochlorite solution, available chlorine >8% (Fisher Scientific, Leicester, LE11 5RG, UK) and then rinsed with sterile H_2O. Plated seeds were stratified for 48 h at 4 °C in the dark. Plates were then incubated at 22 °C in continuous light (120 μmol m^{-2} s^{-1}) in an Infors growth cabinet (Infors, Bottmingen, Switzerland, CH-4103) fitted with Gro-Lux fluorescent lights.

Anthocyanin extraction

Twenty-five seedlings were ground with a plastic pestle in a 1.5 ml microcentrifuge tube in 300 μl of 1% HCl in methanol with 100 mg of quartz sand (Sigma). The samples were diluted with 200 μl H_2O and centrifuged for 3 min at 14 000 *g* in a bench-top microfuge (Microcentaur-MSE). The supernatant was recovered, 500 μl chloroform added, and the samples vortexed and centrifuged for 2 min at 14 000 *g*. The upper aqueous phase was removed to a clean tube and 300 μl of 1% HCl in methanol and 200 μl H_2O added. Absorbance at 657 nm and 530 nm was measured with a Perkin Elmer lambda 9 UV/VIS spectrophotometer. Anthocyanin content was calculated from A_{530} corrected for the background A_{657}. Five replicates were analysed for each treatment and averages and standard errors calculated. All experiments were repeated at least three times.

RNA extraction

RNA was extracted from 500 mg of frozen whole seedlings using Tripure (Roche), in accordance with the manufacturer's instructions. The RNA pellet obtained was resuspended in 200 μl RNase-free H_2O, an equal volume of 4 M LiCl was added and incubated overnight at 4 °C. The RNA pellet was collected by centrifugation at 14 000 *g* for 15 min at 4 °C and resuspended in 250 μl RNase-free H_2O. Citrate-buffered phenol pH 4.3:chloroform:isoamyl alcohol (24:24:1, by vol; 250 μl per sample) was added, the sample vortexed and centrifuged at 14 000 *g* for 5 min at 4 °C. The upper aqueous phase was transferred to a fresh tube, an equal volume of isopropanol:3 M potassium acetate (25:1, v:v) was added and the sample incubated at –20 °C for 1 h. Samples were centrifuged at 14 000 *g* for 20 min and the RNA pellet washed with 1 ml 70% ethanol. Ethanol was removed and the pellet allowed to air dry. Pellets were then resuspended in 15 μl RNase-free H_2O.

First-strand synthesis and RT-PCR

For first-strand synthesis, 5 μg of total RNA were added to 0.5 μg poly-dT primer (1 μg μl^{-1}) (Roche Applied Science, Lewes, East Sussex, UK, BN7 1LG) and the volume made up to 12 μl with RNase-free H_2O. Samples were then incubated at 70 °C for 5 min, followed by 4° C for 5 min. To each tube, 20 U RNasin (Promega), dNTPs to a final concentration of 0.5 mM, 4 μl 5× reaction buffer (as supplied), 50 U Bioscript reverse transcription enzyme (Bioline) and RNase-free H_2O, to a final volume of 20 μl, were added. Tubes were incubated at 42 °C for 60 min, followed by 70 °C for 10 min.

The products of first-strand synthesis (5 μl of a 1/10 dilution) were used as template in a standard PCR reaction containing 5 μl 10× NH_4 reaction buffer (Bioline), 2 μl 50 mM $MgCl_2$ (Bioline), 2 μl 10 μM dNTP mix, 0.1 μl BioTaq DNA polymerase (Bioline), 2 μl each 5' and 3' primers (10 μM stock concentration) made up to 50 μl with H_2O. All PCR reactions included 5' and 3' primers for *ACT7* or *UBQ10* (Table 1). The following PCR conditions were used: initial denaturation, 94 °C for 5 min, followed by 94 °C for 30 s, 55–60 °C for 30 s (Table 1) and 72 °C for 30 s, for 24–32 cycles and a final extension period at 72 °C for 5 min. PCR products were visualized by electrophoresis on 1.5% agarose gels containing ethidium bromide and quantified using ImageQuant software (Molecular Dynamics). The ratio of the band intensity of the gene of interest to that of the *ACT7* control was calculated. For *LHCB1* transcript analysis this was done in triplicate; averages and standard errors were calculated. All experiments were repeated a minimum of four times (five times in total).

Seedling development assay

Seeds were surface-sterilized and sown on plates containing 1% agar, as described in 'Plant material and growth conditions'. Seedlings (three replicates of 100 seedlings) were scored at 16–24 h time points throughout development. The percentage of seedlings having reached each growth stage was calculated as well as the averages and standard errors. Growth stages were defined as follows: 0.1, seed imbibition; 0.5, radicle emergence; 0.7, hypocotyl and cotyledon emergence; 1, cotyledons fully open (Boyes *et al.*, 2001). All experiments were repeated a minimum of four times (five times in total).

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Table 1. Primers used for RT-PCR

Details of the 5′ and 3′ primers used for RT-PCR to quantify transcripts of the *LHCB1* and anthocyanin biosynthesis genes. *ACT7* or *UBQ*10 were used as standards, and primers were included in duplex PCR reactions with primers for the gene of interest. The table shows the annealing temperature, number of cycles, and the expected size of the PCR product for each pair of primers.

Gene	Accession number	5′ primer	3′ primer	PCR product size (bp)	Annealing temperature (°C)	Number of cycles	Number of *ACT7* cycles
ACT7	At5g09810	TACAACGAGCTTCGTGTTGC	GAATCTCTCAGCTCCGATGG	360	55–60	28–33	28–33
UBQ10	At4g05320	CCACCCTTCATCTTGTTCTC	GACGAAGATCTGCATACCTCC	306	58	29	29
LHCB1	At1g29910	GAGGAAGACTGTTGCCAAGC	CCTGCGACTCTGTAGCCTTC	664	60	24	28
PAL	At2g37040	TGTGAAGGTGGAGCTATCGG	GGAAAATCCTTGGAGGAGAG	246	55	29	29
CHS	At5g13930	GACATGCCTGGTGCTGACTA	TTCCATACTCGCTCAACACG	604	60	28	28
CHI	At3g55120	CATGTAGACTCCGTCACGTT	TTGTTCTTCATCATTAGCTGAG	626	55	32	28
F3H	At3g51240	TGCCGTCAGATCGTTGAGGC	GGCCACCGTGGTAGTCTCT	323	60	28	28
DFR	At5g42800	CCGGAACCGTTAATGTAGAAGA	CTCCGATATGGATTGGTACGAT	872	55	33	33
LDOX	At4g22870	TCGATCTAAAGAACATCGAG	GAACCATGTTGTGTAGAATG	591	56	34	30

Results

gun1 *seedlings accumulate less anthocyanin than wild-type seedlings*

To confirm our preliminary observation that *gun1* seedlings accumulated less anthocyanin than wild-type seedlings, seedlings were grown in the presence or absence of 2% sucrose, 0.5 mM lincomycin or 5 μM norflurazon in continuous light for 4 d following stratification. Different treatments produced marked differences in the anthocyanin content of the seedlings (Fig. 1). The anthocyanin content was quantified spectrophotometrically following extraction in acidic methanol. The anthocyanin content of seedlings grown in the absence of sucrose was fairly low and there were only small differences between *gun1* and wild-type seedlings. However, in the presence of sucrose there was a marked induction of anthocyanin accumulation in both wild-type and *gun1* seedlings in all treatments. The anthocyanin content was 18–25-fold higher in wild-type and *gun1* seedlings grown on 2% sucrose, compared with those grown in the absence of sucrose, although the *gun1* seedlings accumulated about 20% less anthocyanin than wild-type seedlings. A similar pattern was observed with seedlings grown in the presence of norflurazon or lincomycin, although the differences between the wild-type and *gun1* seedlings were more easily visible to the naked eye, due to the absence of chlorophyll (Fig. 1). The *gun1* seedlings accumulated only ~50% of the amount of anthocyanins in wild-type seedlings grown on sucrose and norflurazon or lincomycin. These results suggest that sucrose-induced anthocyanin accumulation is perturbed in *gun1* seedlings.

The location of the accumulated anthocyanins in the presence of norflurazon was similar in wild-type and *gun1* seedlings, and included the entire abaxial and adaxial epidermal layers of the cotyledons and the upper hypocotyl (Fig. 1). By contrast, the pattern of anthocyanin accumulation in the presence of sucrose and lincomycin was different in *gun1* compared with the wild type (Figs 1, 2). In the wild type, anthocyanin pigmentation was present in the epidermal layers of the entire abaxial surface and the edges of the adaxial surface of the cotyledons, and in the upper hypocotyls, as described by Kubasek *et al.* (1992); in *gun1* seedlings, anthocyanins were restricted to the edges of both the abaxial and adaxial surfaces of the cotyledons, with a greater proportion of the pigment in the upper hypocotyl (Fig. 2).

A clear developmental difference was observed between 4-d-old wild-type and *gun1* seedlings grown on medium containing lincomycin, with or without sucrose. All wild-type seedlings had unexpanded hypocotyls and cotyledons whereas all *gun1* seedlings had extended hypocotyls and expanded cotyledons (Fig. 2). The maximum width of cotyledons from wild-type and *gun1* seedlings grown in the presence of lincomycin was found to differ by ~50%; wild-type cotyledons had an average width of 1.05±0.03 mm whereas *gun1* cotyledons had an average width of 1.55±0.04 mm (Fig. 2).

Transcripts of 'early' anthocyanin biosynthesis genes are less abundant in gun1 *seedlings*

To examine the effect of the *gun1* mutation on the expression of genes encoding enzymes of the anthocyanin biosynthesis pathway, PCR was carried out on the products of reverse transcription of RNA extracted from wild-type and *gun1* seedlings grown on medium containing 2% sucrose for 4 d under continuous illumination (Fig. 3). Genes encoding anthocyanin biosynthesis enzymes have been shown to fall into two groups showing distinct temporal expression patterns (Kubasek *et al.*, 1992; Pelletier *et al.*, 1999). The first group contains genes that are expressed 'early' in response to light and includes genes encoding phenylalanine ammonia-lyase 1 (*PAL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), and flavanone 3-hydroxylase (*F3H*). The second group contains genes that are expressed 'late' in response to light and includes the genes encoding dihydroflavonol reductase (*DFR*), and leucoanthocyanidin dioxygenase (*LDOX*) (Kubasek *et al.*, 1992; Pelletier *et al.*, 1999). Most of these genes are members of small gene families, but transcriptome profiling has identified the sucrose-responsive genes as At2g37040 (*PAL*), At5g13930 (*CHS*), At3g55120 (*CHI*), At3g51240 (*F3H*), At5g42800 (*DFR*), and At4g22870

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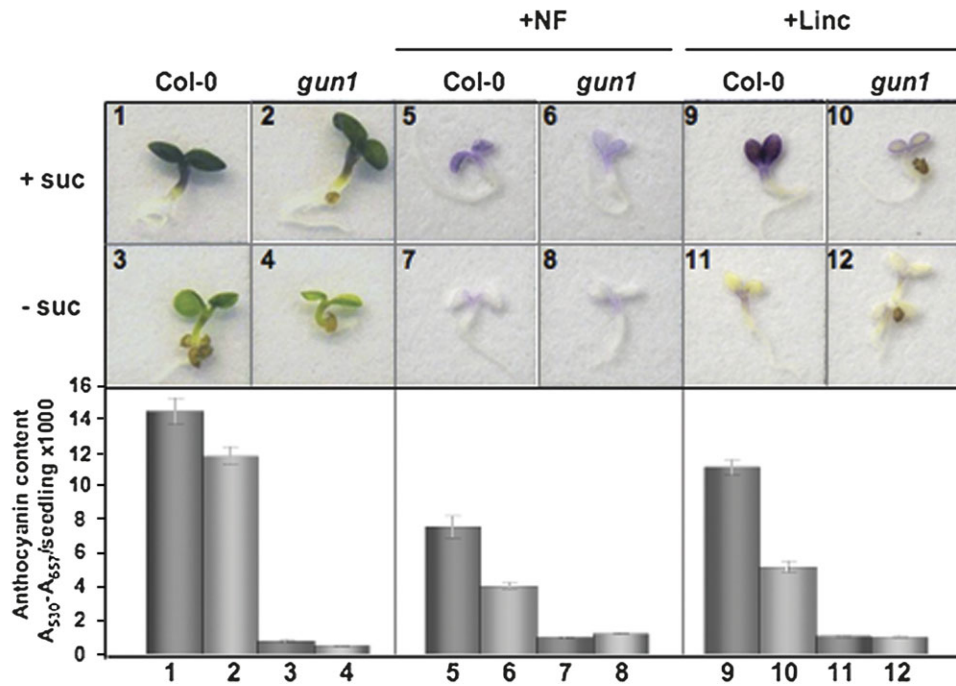

Fig. 1. Effect of sucrose, norflurazon, and lincomycin on anthocyanin accumulation in wild-type and *gun1* seedlings. The upper part of the figure shows 1 cm × 1 cm images of representative wild-type (Col-0) and *gun1* seedlings grown for 4 d on 0.5× MS-agar medium, ±2% sucrose (suc), ±0.5 mM lincomycin (Linc), or ±5 μM norflurazon (NF). The top row of images shows seedlings grown in the presence of sucrose (+suc), with the bottom row showing seedlings grown in the absence of sucrose (–suc). The lower part of the figure shows the anthocyanin content of the seedlings, with the bars numbered corresponding to the numbered images above. Anthocyanins were extracted from five replicate samples of 25 seedlings from each treatment by homogenization in acidified methanol. The anthocyanin content was determined from the absorbance of the extract at 530 nm and 657 nm, and expressed as $1000 \times A_{530} - A_{637}$ per seedling. The results are shown as mean ±standard errors for each set of five replicates. The experiment was repeated four times with essentially identical results.

(*LDOX)* (Solfanelli *et al.*, 2006). Gene-specific PCR primers were designed for each of these genes (listed in Table 1) and the relative transcript abundance in wild-type and *gun1* seedlings was determined by reference to transcripts from *ACT7* or *UBQ10*, which did not differ between wild-type and *gun1* (Fig. 3). *gun1* seedlings contained fewer transcripts of the early anthocyanin biosynthesis genes than wild-type seedlings. *PAL* transcripts were undetectable by RT-PCR in *gun1* seedlings, whereas transcripts of *CHS*, *CHI*, and *F3H* were present at 30%, 60%, and 45% of wild-type transcript amounts, respectively (Fig. 3). By contrast, transcripts of the late anthocyanin biosynthesis genes *DFR* and *LDOX* were slightly more abundant or unchanged in *gun1* seedlings (Fig. 3). The *gun1* mutation therefore results in decreased transcript abundance of genes encoding enzymes that function early in the anthocyanin biosynthesis pathway, and this may contribute to the lower amounts of anthocyanin accumulated in *gun1* seedlings.

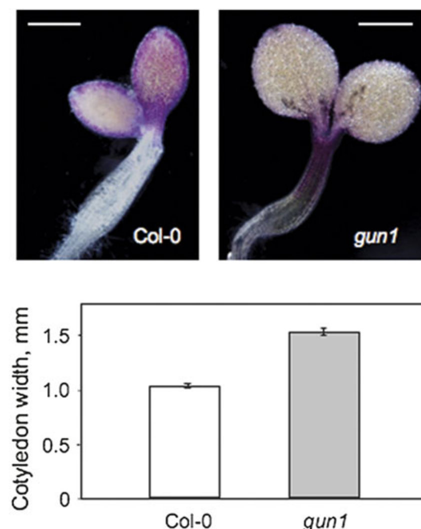

Fig. 2. Cotyledons of wild-type and *gun1* seedlings grown in the presence of sucrose and lincomycin. The figure shows representative 4-d-old wild-type (Col-0) and *gun1* seedlings grown on 0.5× MS-agar medium containing 2% sucrose and 0.5 mM lincomycin. Size bar=1 mm. The lower part of the figure shows the mean width ±standard error of the mean for 100 4-d-old seedlings.

Norflurazon and lincomycin disrupt sucrose-induced anthocyanin accumulation

Sucrose-induced anthocyanin accumulation follows a distinct temporal pattern, with anthocyanin content reaching a maximum 5 d after germination and subsequently declining (Kubasek *et al.*, 1992). The peak of anthocyanin

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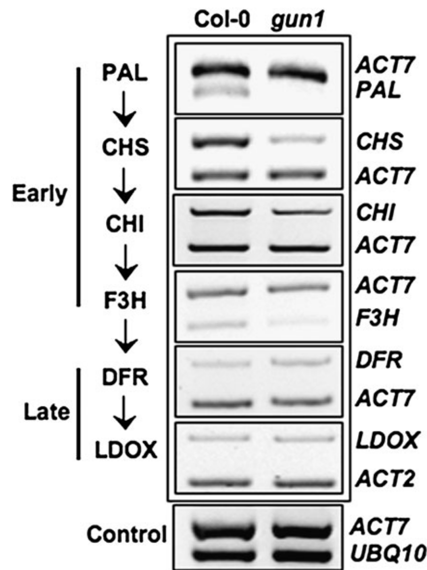

Fig. 3. Transcripts of genes encoding anthocyanin biosynthesis enzymes. RT-PCRs were performed on RNA extracted from 4-d-old wild-type (Col-0) and *gun1* seedlings grown on 0.5× MS-agar medium containing 2% sucrose. 'Early' anthocyanin biosynthesis genes: *PAL*, phenylalanine ammonia-lyase 1 (At2g37040); *CHS*, chalcone synthase (At5g13930); *CHI*, chalcone isomerase (At3g55120), *F3H*, flavanone 3-hydroxylase (At3g51240). 'Late' anthocyanin biosynthesis genes: *DFR*, dihydroflavonol reductase (At5g42800); *LDOX*, leucoanthocyanidin dioxygenase (At4g22880). Controls: *ACT7*, actin2/7 (At5g09810); *UBQ10* ubiquitin 10 (At4g05320). The primers used are listed in Table 1.

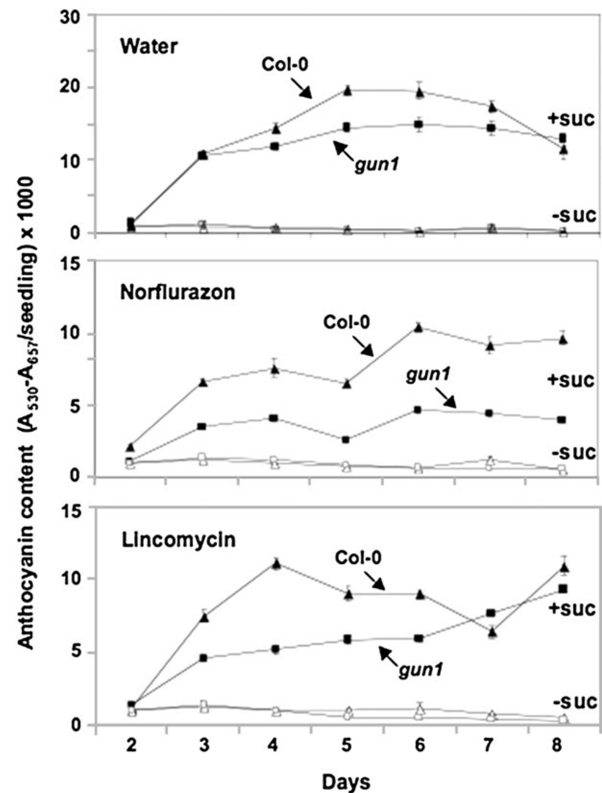

Fig. 4. Time-course of anthocyanin accumulation in wild-type and *gun1* seedlings. Anthocyanins were extracted in acidified methanol from five replicate samples of 25 seedlings of wild-type (Col-0) and *gun1* seedlings grown on 0.5× MS-agar medium, ±2% sucrose (suc), ±5 μM norflurazon or ±0.5 mM lincomycin. Seedlings were harvested daily 2–8 d after germination. Anthocyanin content was determined from the absorbance of the extract at 530 nm and 657 nm, and the results expressed as 1000×A_{530}–A_{637} per seedling. The results are shown as mean ±standard errors for each set of five replicates. The experiment was repeated four times with essentially identical results.

content appears to coincide with the maturation of chloroplasts and the associated switch to photoautotrophic growth (Kubasek *et al.*, 1992). To examine the effect of the *gun1* mutation on the temporal pattern of anthocyanin accumulation, the anthocyanin content of whole seedlings was measured over the period 2–8 d after germination (Fig. 4). In the presence of 2% sucrose, and in the absence of inhibitors, a typical anthocyanin accumulation curve reaching a maximum at day 5 was observed for wild-type seedlings (Fig. 4, top panel). In *gun1* seedlings, anthocyanin accumulation reached a maximum at day 6 and the subsequent decline in anthocyanin content was much less pronounced than in wild-type seedlings (Fig. 4, top panel). From day 3 onwards, *gun1* seedlings accumulated less anthocyanin than the wild-type; the greatest difference in anthocyanin content (26%) was observed on day 5. This suggests that the *gun1* mutation has perturbed the normal temporal regulation of anthocyanin accumulation during early seedling development.

In the presence of norflurazon and sucrose, *gun1* seedlings accumulated less anthocyanin than wild-type seedlings at each time point (Fig. 4, middle panel). The greatest difference in anthocyanin content between wild-type and *gun1* seedlings was on day 6, when *gun1* contained only 55% of the anthocyanin present in wild-type seedlings. The anthocyanin accumulation curves generated from seedlings grown on norflurazon and sucrose differed from those generated from seedlings grown on sucrose alone. In the presence of norflurazon an initial peak of anthocyanin was observed on day 4, followed by a drop on day 5 and a further peak on day 6, in both wild-type and *gun1* seedlings. This double peak contrasted with the single peak seen on sucrose alone (Fig. 4, compare top and middle panels).

In the presence of lincomycin and sucrose, the anthocyanin content of wild-type seedlings reached a maximum on day 4, 1 day earlier than seedlings grown on sucrose alone (Fig. 4, bottom panel). The anthocyanin content subsequently declined after day 4 in wild-type seedlings, and a second increase in anthocyanin was observed on day 8, coinciding with the emergence of the first true leaves. On day 8, anthocyanins were present in these leaves but not in the cotyledons. True leaf emergence was seen in Col-0 and *gun1* at day 7–8 regardless of treatment. Anthocyanins were obvious in these leaves on media containing both sucrose and norflurazon or lincomycin, although greater amounts were seen with sucrose and lincomycin (Fig. 1). The time-course clearly indicates that lincomycin treatment perturbs

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the accumulation of anthocyanin in wild-type seedlings. The temporal pattern of anthocyanin accumulation in *gun1* seedlings grown on lincomycin and sucrose was completely different from that of wild-type seedlings (Fig. 4, bottom panel). The anthocyanin content continued to rise over the 2–8-d sampling period, without showing a distinct peak (Fig. 4, bottom panel). Up to day 6, *gun1* seedlings contained less anthocyanin than wild-type seedlings; the greatest difference in anthocyanin content occurred on day 4 when *gun1* contained only 47% of wild-type amounts.

These experiments indicate that the functional state of plastids, affected by treatment with norflurazon or lincomycin, influences the temporal pattern of anthocyanin accumulation in wild-type and *gun1* seedlings. However, the effects of norflurazon are different from those of lincomycin suggesting that the treatments do not operate through identical plastid signalling pathways.

Sucrose-induced anthocyanin accumulation is not an osmotic effect

To determine if the differences in anthocyanin accumulation in wild-type and *gun1* seedlings were due to an osmotic effect, seedlings were grown on equimolar concentrations of sorbitol (58 mM) or sucrose (2%=58 mM) for 4 d and the anthocyanin content was assayed after extraction with acidic methanol. Sorbitol failed to induce anthocyanin accumulation in either wild-type or *gun1* seedlings (Fig. 5). The anthocyanin contents of seedlings grown on sorbitol were 3.6% and 1.9% of those grown on sucrose in wild type and *gun1*, respectively. Sucrose-induced anthocyanin accumulation cannot therefore be regarded as an osmotic effect.

Sucrose repression of LHCB1 *transcription is disrupted in* gun1 *seedlings*

The presence of sucrose in growth media represses the expression of photosynthesis-related nuclear genes (Pego *et al.*, 2000; Rolland *et al.*, 2006). To determine whether *gun1* showed any difference to the wild type in the effect of sucrose on photosynthesis-related nuclear gene expression, the abundance of transcripts of *LHCB1* genes in 4-d-old seedlings was examined by PCR (Fig. 6). In the absence of added sucrose, *LHCB1* transcripts were about 15% higher in *gun1* than in the wild type. However, growth in the presence of sucrose had a greater repressive effect on *LHCB1* transcripts in *gun1* than in wild-type seedlings. *LHCB1* transcript abundance in *gun1* seedlings grown in the presence of sucrose was decreased to only 4% of that in *gun1* seedlings grown in the absence of sucrose, whereas wild-type seedlings contained 25% of the *LHCB1* transcripts. This suggests that the repressive action of sucrose on *LHCB1* expression is enhanced in the absence of GUN1 function, i.e. the *gun1* mutant is more sensitive to sucrose effects. Growth of seedlings in the presence of lincomycin and sucrose confirmed that the *gun1* seedlings retained the *genomes uncoupled* phenotype; *gun1* seedlings contained about 20-fold more *LHCB1* transcripts than wild-type seedlings. In wild-type seedlings, lincomycin in the presence of sucrose further repressed *LHCB1* expression, whereas in *gun1* seedlings *LHCB1* genes were partially released from sucrose repression by lincomycin (Fig. 6).

Because the *gun1* mutation altered the temporal accumulation of anthocyanin in the presence of sucrose (Fig. 4), the time-course of *LHCB1* transcript accumulation was examined in 3–8-d-old *gun1* and wild-type seedlings grown in the presence or absence of sucrose and lincomycin (Fig. 7). Three replicate samples of RNA were analysed at each time point, and the whole experiment was repeated four times. Reproducible differences in the patterns of *LHCB1* transcript abundance were observed between *gun1* and wild-type seedlings, although the rapid decrease in *LHCB1* transcript abundance in *gun1*, seen on day 6 in the absence of sucrose in Fig. 7, was observed to occur on day 5 in some experiments. In the absence of sucrose, wild-type seedlings

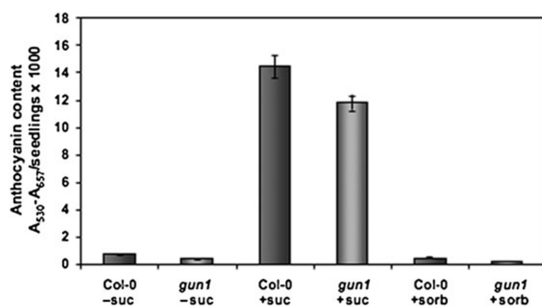

Fig. 5. Anthocyanin content of wild-type and *gun1* seedlings grown in the presence of sucrose or sorbitol. Anthocyanins were extracted from five replicate samples of 25 seedlings of wild-type (Col-0) and *gun1* seedlings grown for 4 d on 0.5× MS-agar medium, ±2% sucrose (suc) or 58 mM sorbitol (+sorb). Anthocyanin content was determined from the absorbance of the extract at 530 nm and 657 nm, and the results expressed as $1000 \times A_{530}$–A_{637} per seedling. The results are shown as mean ±standard errors for each set of five replicates.

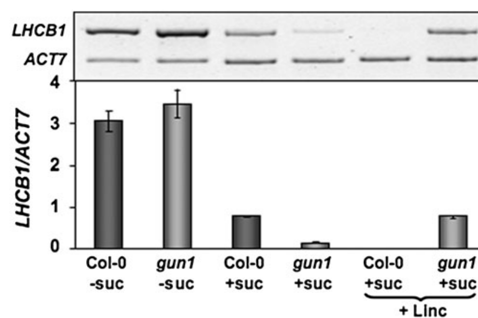

Fig. 6. Effect of sucrose and lincomycin on *LHCB1* transcripts in wild-type and *gun1* seedlings. RT-PCRs were performed on three replicate samples of RNA extracted from 4-d-old wild-type (Col-0) and *gun1* seedlings grown on 0.5× MS-agar medium, ±2% sucrose (suc) and ±0.5 mM lincomycin (Linc). The PCR products are shown in the upper part of the figure. The amounts of *LHCB1* PCR product normalized to those of *ACT7* are shown in the lower part of the figure. Three independent RT-PCRs were analysed for each treatment.

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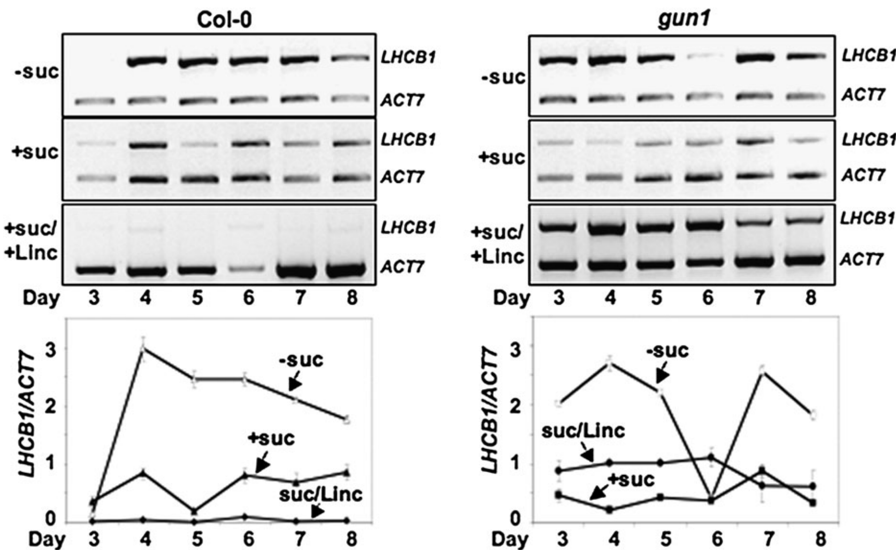

Fig. 7. Time-course of *LHCB1* transcript abundance in wild-type and *gun1* seedlings. RT-PCRs were performed on three replicate samples of RNA extracted from 3–8-d-old wild-type (Col-0) and *gun1-1* seedlings grown on 0.5× MS-agar medium, ±2% sucrose (suc), and ±0.5 mM lincomycin (Linc). The PCR products are shown in the upper part of the figure. The amounts of *LHCB1* PCR product normalized to those of *ACT7* are shown in the lower part of the figure. The ratios are expressed as mean ±standard error for the three independent RT-PCRs analysed for each treatment.

contained very low amounts of *LHCB1* transcripts on day 3, and showed maximal *LHCB1* transcripts on day 4, followed by a slow decline over the next 4 d (Fig. 7, left panel). In *gun1* seedlings in the absence of sucrose, *LHCB1* transcripts reached a maximum on day 4 but, unlike wild-type seedlings, *LHCB1* transcripts were already present at a high level on day 3. Transcripts then declined sharply, followed by an increase on day 7 (Fig. 7 right panel). Sucrose repressed *LHCB1* in both *gun1* and wild-type seedlings. There were reproducible fluctuations in the amounts of *LHCB1* transcripts in both sets of seedlings over the period day 4 to day 8, with the pattern in *gun1* seedlings appearing to be a mirror image of the pattern in wild-type seedlings. This indicates that the temporal pattern of *LHCB1* regulation by sucrose is perturbed in *gun1* seedlings.

Lincomycin completely repressed *LHCB1* in wild-type seedlings grown in the presence of sucrose throughout the time course (Fig. 7, left panel). There were much higher amounts of *LHCB1* transcripts in *gun1* seedlings grown in the presence of lincomycin (Fig. 7 right panel), as expected for a *gun* mutant. In comparison to seedlings grown in the presence of only sucrose, *gun1* seedlings showed increased amounts of *LHCB1* transcripts, whereas wild-type seedlings showed decreased amounts of *LHCB1* transcripts, throughout the time-course (Fig. 7). These results demonstrate that the patterns of *LHCB1* transcript accumulation are altered in *gun1* seedlings.

Early seedling development is altered in gun1 *seedlings*

The results presented above demonstrate that different temporal patterns of anthocyanin and *LHCB1* transcript accumulation occur in *gun1* seedlings in comparison to wild-type seedlings, possibly as a consequence of differences in early seedling development. To investigate possible differences in seedling development between *gun1* and wild-type seedlings, three replicates of 100 seeds of *gun1* and wild type were sown on MS-agar medium with or without 2% sucrose and the growth stage of each seedling was monitored every 16–24 h, using the convention introduced by Boyes *et al.* (2001). Growth experiments were repeated a further four times with reproducible results. Growth stages were defined as: 0.1, seed imbibition; 0.5, radicle emergence; 0.7, hypocotyl and cotyledon emergence; 1.0, cotyledons fully open (Boyes *et al.*, 2001). Representative seeds/seedlings at each growth stage are shown in the upper panel of Fig. 8. Seedling development was perturbed in *gun1* compared to wild type, particularly in the presence of sucrose. With seedlings grown in the presence of sucrose for 54 h, 82% of wild-type seedlings showed hypocotyl and cotyledon emergence (stage 0.7) whereas only 54% of *gun1* seedlings had reached this stage (Fig. 8). Sucrose is known to inhibit early seedling development in *Arabidopsis* (reviewed in Gibson, 2003), and sucrose was observed to perturb *gun1* seedling development at each growth stage (Fig. 8). However, the effect of sucrose on seedling development was greater with *gun1* than with wild-type seedlings. Sucrose effects were visible at the radicle emergence stage with *gun1*, whereas with wild-type seedlings effects were not observed until cotyledon opening (Fig. 8). However, the developmental delay observed in *gun1* seedlings at 54 h (Fig. 8) was transient and after 96 h *gun1* and wild-type seedlings both had open expanded cotyledons whether they had been grown in the presence or absence of sucrose (data not shown).

gun1 *seedling development is hypersensitive to ABA*

Screens for mutants showing alterations in sucrose effects on seedling development have largely yielded sugar-insensitive

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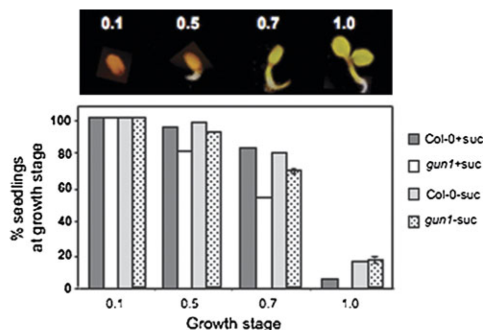

Fig. 8. Effect of sucrose on development of wild-type and *gun1* seedlings. Upper panel: growth stages as defined by Boyes *et al.* (2001); 0.1, seed imbibition; 0.5, radicle emergence; 0.7, hypocotyl and cotyledon emergence; 1.0, cotyledons fully open. Lower panel: percentage of seedlings having reached each growth stage 54 h after transfer of 4 °C dark-stratified seeds to continuous illumination (120 μmol m^{-2} s^{-1}) at 22 °C. Wild-type (Col-0) and *gun1-1* seedlings were grown on 0.5× MS-agar medium ±2% sucrose (suc). Three replicate samples of 100 seedlings were examined and the results presented as means ±standard errors.

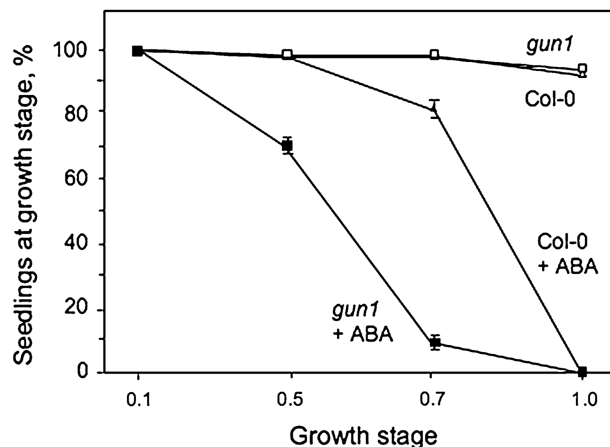

Fig. 9. Effect of ABA on development of wild-type and *gun1* seedlings. Percentage of 9-d-old seedlings having reached the growth stages defined by Boyes *et al.* (2001); 0.1, seed imbibition; 0.5, radicle emergence; 0.7, hypocotyl and cotyledon emergence; 1.0, cotyledons fully open. Three replicate samples of 100 wild-type (Col-0) and *gun1* seeds were sown on 0.5× MS-agar medium ±0.5 μM ABA, stratified for 48 h in the dark at 4 °C and then transferred to continuous illumination (120 μmol m^{-2} s^{-1}) at 22 °C. The number of seedlings reaching, or passing, each growth stage was counted 9 d after transfer to light at 22 °C. Results are expressed as mean ±standard error for three replicate samples.

mutants, many of which are also ABA insensitive, establishing links between sugar and ABA signalling. Far fewer sugar-hypersensitive mutants have been identified (reviewed in Leon and Sheen, 2003), and few of these are also hypersensitive to ABA. To examine the possible effect of ABA on seed germination and seedling development, *gun1* and wild-type seeds were sown on a range of ABA concentrations (0–25 μM) and seedling growth monitored over 21 d. The greatest difference in seedling development between wild-type and *gun1* seedlings was seen in 9-d-old seedlings grown on 0.5 μM ABA; only 11% of *gun1* seedlings had reached the stage of cotyledon emergence whereas 80% of wild-type seedlings had reached this stage (Fig. 9). *gun1* can therefore be considered to have an ABA-hypersensitive phenotype.

Discussion

gun1 seedlings show altered developmental phenotypes

We have demonstrated that *gun1* seedlings show several previously unrecognized mutant phenotypes indicating a complex interaction between sucrose and plastid signalling pathways affecting seedling development. *gun1* seedling development is hypersensitive to sucrose and ABA, and *gun1* seedlings show differences in the effects of sucrose and plastid inhibitors on the accumulation of anthocyanins and *LHCB1* transcripts in comparison to wild-type seedlings. Originally *gun1* mutants were described as 'remarkably normal under most growth conditions', with only a subtle de-etiolation phenotypic difference between *gun1* and wild-type seedlings (Susek *et al.*, 1993; Mochizuki *et al.*, 1996). More recently, however, Ruckle and Larkin (2009) have uncovered more phenotypic differences while examining interactions of blue-light and plastid signals and their effects on photomorphogenesis. They observed differences in hypocotyl elongation, cotyledon expansion, epidermal cell development, and anthocyanin accumulation between *gun1* and wild-type seedlings grown under low fluence blue light (Ruckle and Larkin, 2009). Together, these observations implicate GUN1 in a range of developmental processes in seedlings and indicate a complex set of interactions among sucrose, light, and plastid signalling pathways.

Although GUN1 has been identified as a plastid nucleoid-associated PPR protein with a C-terminal SMR (small MutS-related) domain (Koussevitsky *et al.*, 2007; Cottage *et al.*, 2008), the structure of the protein has given few clues to its mechanism of action in plastid retrograde signalling. From an analysis of effects on nuclear gene expression in *gun1* seedlings and plants, GUN1 has been implicated in signalling pathways involving tetrapyrrole intermediates (Koussevitsky *et al.*, 2007), plastid gene expression (Susek *et al.*, 1993; Gray *et al.*, 2003) and photosynthesis, as affected by excess-light treatment (Koussevitsky *et al.*, 2007), and it has been suggested that GUN1 integrates signals from all of these sources (Koussevitsky *et al.*, 2007). From the data presented here, and by Ruckle and Larkin (2009), it is clear that GUN1-dependent signalling affects more plant processes than just the expression of nuclear genes related to chloroplast biogenesis. The differences in hypocotyl elongation, cotyledon expansion and epidermal cell development in *gun1* seedlings treated with lincomycin

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indicate the involvement of GUN1 in signalling the functional state of the chloroplasts. The absence of functional chloroplasts is known to affect epidermal and palisade cell differentiation in several plants (Reiter *et al.*, 1994; Chatterjee *et al.*, 1996; Keddie *et al.*, 1996; Aluru *et al.*, 2001) and it therefore seems probable that GUN1 provides the major route for signalling chloroplast dysfunction.

Functional chloroplasts and GUN1 *are necessary for full sucrose-induced anthocyanin accumulation*

It is thought that anthocyanin production evolved as a result of intense UV radiation when plants first colonized the land (Bell and Charlwood, 1980). Anthocyanins are likely to be most useful as UV protectants when cotyledons expand and before chloroplasts are fully developed. It would appear that cotyledon expansion and anthocyanin accumulation are co-ordinately regulated; thus, when a seedling first perceives light, triggering cotyledon expansion and chloroplast development, the plant simultaneously begins to synthesize anthocyanins. The addition of sucrose to the growth medium results in the up-regulation of anthocyanin biosynthesis via a sucrose-dependent glucose-independent pathway (Teng *et al.*, 2005). Genes encoding anthocyanin biosynthesis enzymes fall into two groups and the members of each group have been shown to be co-regulated. The first group comprises genes that are expressed 'early' in response to light and includes *PAL*, *CHS*, *CHI*, and *F3H*. The second group contains genes that are expressed 'late' in response to light and comprises *DFR* and *LDOX* (Kubasek *et al.*, 1992; Pelletier *et al.*, 1999). 'Early' anthocyanin biosynthesis gene transcript abundance was lower in *gun1* than in wild-type seedlings for all four genes examined (*PAL*, *CHS*, *CHI*, and *F3H*) (Fig. 3). In contrast, wild-type and *gun1* seedlings had similar amounts of transcripts of both 'late' anthocyanin genes (*DFR* and *LDOX*) (Fig. 3). The 'early' anthocyanin biosynthesis genes are co-regulated by MYB transcription factors, including MYB11, MYB12, and MYB111 (Mehrtens *et al.*, 2005; Strache *et al.*, 2007), whereas a transcriptional complex of a different set of MYB proteins with the WD40 protein TTG1 and basic helix-loop-helix (bHLH) transcription factors is required for the activation of the 'late' genes (Zimmerman *et al.*, 2004). It therefore appears that the MYB-regulated expression of the 'early' genes, but not the MYB/TTG1/bHLH-regulated expression of the 'late' genes, is influenced by the functional state of GUN1.

Norflurazon and lincomycin inhibited sucrose-induced anthocyanin accumulation in wild-type seedlings and, to a greater extent, in *gun1*. This observation suggests that functional chloroplasts and GUN1 are both, in some way, necessary to achieve full sucrose-induced anthocyanin accumulation. However, the mechanisms by which this is achieved are not clear. If negative signals from dysfunctional chloroplasts are transmitted exclusively via GUN1, then these signals should be abrogated in *gun1* mutants. However, the effects of norflurazon and lincomycin on sucrose-induced anthocyanin accumulation are greater in *gun1* than in wild-type seedlings. This suggests the possibility that dysfunctional chloroplasts and GUN1 operate independently, at least under certain circumstances. The interaction with the sugar-signalling pathway is also not clear. It is possible that part of the sugar-signalling pathway required for the induction of anthocyanin biosynthesis operates via GUN1, i.e. there is a chloroplast-located component in a sugar-signalling pathway upstream of GUN1. Alternatively, GUN1 and dysfunctional chloroplasts may provide signals that directly influence anthocyanin accumulation, independently of signals from a separate cytosolic sugar-signalling pathway. However, it is also possible that the GUN1 and dysfunctional chloroplasts regulate the cytosolic sugar-signalling pathway, providing additional fine control of sucrose-induced anthocyanin synthesis. Further work is needed to unravel the complexities of the interplay between sucrose and plastid signalling pathways regulating anthocyanin accumulation.

Temporal nuclear photosynthesis gene expression is differentially affected by sucrose in wild-type and gun1 *seedlings*

Nuclear and chloroplast photosynthesis gene expression are repressed by the addition of sugars to the external medium (reviewed in Pego *et al.*, 2000 and Rolland *et al.*, 2006). In our experiments, sucrose and lincomycin had an additive effect in repressing *LHCB1* expression in wild-type seedlings, whereas lincomycin partially released *LHCB1* from sucrose repression in *gun1* seedlings (Figs 6, 7). This provides further evidence for the interaction of signalling pathways from sucrose and dysfunctional chloroplasts. The concept that retrograde signalling may interact with sugar signalling is not new. McCormac and Terry (2004) showed that *gun5* seedlings do not express nuclear photosynthesis genes in the presence of norflurazon unless sucrose is provided in the growth medium. Koussevitzky *et al.* (2007) showed that 7% glucose significantly reduced *LHCB* expression when applied to 3-d-old wild-type seedlings, but not to *gun1* seedlings. This suggests that the sugar sensitivity of *gun1* seedlings changes during development; seed germination and early seedling development are hypersensitive to sucrose, whereas older *gun1* seedlings are insensitive to exogenous sugars. These changes in sugar sensitivity of *LHCB* expression seem to parallel changes in sensitivity of *LHCB* expression to inhibitors of plastid protein synthesis. Inhibitors such as chloramphenicol and lincomycin decrease nuclear gene expression when applied to young (0–3-d-old) seedlings, but have no effect on nuclear gene expression when applied to older (>3-d-old) seedlings (Oelmüller *et al.*, 1986; Gray *et al.*, 1995). The timing of the changes in sensitivity to sugars and to plastid protein synthesis inhibitors seems to be similar to the switch to phototrophic growth.

In developing seedlings, sucrose is mobilized from cotyledon lipid reserves, but lipid mobilization is retarded in the presence of exogenous sugars (To *et al.*, 2002). In the absence of exogenous sugars the switch to phototrophic growth

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occurs in 2–3-d-old seedlings (Falk *et al.*, 1998), but in the presence of sucrose metabolic switching is delayed. Moreover, the effects of sucrose on lipid mobilization are not seen if sucrose is applied after 3 d (To *et al.*, 2002). In our experiments, *LHCB1* transcripts increased dramatically in wild-type seedlings on day 4, and may be indicative of the switch to photosynthetic competence. *LHCB1* transcript abundance remained fairly constant after day 4 in wild-type seedlings. However, the expression profile was completely different in *gun1* seedlings; *LHCB1* transcripts were already present at a high level from day 3 and, in contrast to wild-type, declined rapidly after day 4 (Fig. 7). *LHCB1* transcripts were much higher (>10-fold) in *gun1* seedlings, compared with wild-type seedlings, grown in the presence of sucrose and lincomycin, and there was little variation in transcript abundance over the 3–8-d time-course. This identifies GUN1 as necessary for correct *LHCB1* expression during early seedling development, in response to sucrose and plastid signals.

gun1 seedling development is hypersensitive to sucrose and ABA

We have shown that early development of *gun1* seedlings is hypersensitive to sucrose and ABA, compared with wild-type seedlings. This suggests that the GUN1 signalling pathway influences seedling responses to sucrose and ABA in wild-type seedlings. Links between sugar and ABA signalling were first uncovered when sugar developmental arrest screens identified several mutants allelic to ABA synthesis (*aba*) and ABA insensitive (*abi)* mutants (reviewed in Leon and Sheen, 2003). One of these mutants, *abi4*, was shown to contain a lesion in the APETELA 2 type transcription factor ABI4 (Finkelstein *et al.*, 1998) and is now strongly implicated in plastid-regulated photosynthesis gene expression (Oswald *et al.*, 2001; Koussevitzky *et al.*, 2007). ABI4 has been shown to regulate *RBCS* gene expression in response to sucrose and ABA, via an S-box motif in association with the light-responsive G-box element (Acevedo-Hernandez *et al.*, 2005). Furthermore, regulation of nuclear photosynthesis gene expression by ABI4 has been shown to be coupled to the mobilization of lipid reserves in the developing embryo (Penfield *et al.*, 2006).

abi4 mutants have sucrose- and ABA-insensitive phenotypes, and indeed most sugar screens have identified sugar-insensitive mutants, many of which are also ABA insensitive. Far fewer sugar-hypersensitive mutants have been identified (reviewed in Leon and Sheen, 2003), and few of these are also hypersensitive to ABA. Two of the few sucrose- and ABA-hypersensitive mutants, *lba1* and *prl1*, have defects in starch synthesis (Yoine *et al.*, 2006*a*, *b*) and sucrose regulation (Németh *et al.*, 1998), respectively. The *prl1* mutation enhances Snf1-related protein kinase (SnRK) activity, which leads to a loss of sucrose repression of target genes (Németh *et al.*, 1998). *gun1* does not appear to be a mutant in starch synthesis, as iodine staining revealed no obvious differences between *gun1* and wild type (data not shown); it is, however, clearly disrupted in sucrose sensing or signalling.

In conclusion, GUN1 has been shown to function in the regulation of photosynthesis-related nuclear gene expression, cotyledon opening, and anthocyanin biosynthesis, all of which may be linked to its role in plastid retrograde signalling. In addition, the GUN1 signalling pathway has been shown to be responsive to sucrose and ABA. The pleiotropic responses of *gun1* seedlings to various stimuli indicate that the GUN1 signalling pathway is intricately interlaced with other signalling pathways, and may be responsible for optimizing the switch to photoautotrophic growth, depending on available lipid reserves, light conditions, and chloroplast development.

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