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Genetic suppression of plant development and chloroplast biogenesis via the Snowy Cotyledon 3 and Phytochrome B pathways

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Abstract. Plant development is regulated by external and internal factors such as light and chloroplast development. A revertant of the *Arabidopsis thaliana* (L.) Heyhn. chloroplast biogenesis mutant *snowy cotyledon 3* (*sco3–1*) was isolated partially recovering the impaired chloroplast phenotype. The mutation was identified in the *Phytochrome B* (*PhyB*) gene and is a result of an amino acid change within the PAS repeat domain required for light-induced nuclear localisation. An independent *phyB-9* mutation was crossed into *sco3–1* mutants, resulting in the same partial reversion of *sco3–1*. Further analysis demonstrated that SCO3 and PhyB influence the greening process of seedlings and rosette leaves, embryogenesis, rosette formation and flowering. Interestingly, the functions of these proteins are interwoven in various ways, suggesting a complex genetic interaction. Whole-transcriptome profiling of *sco3–1phyB-9* indicated that a completely distinct set of genes was differentially regulated in the double mutant compared with the single *sco3–1* or *phyB-9* mutants. Thus, we hypothesise that *PhyB* and *SCO3* genetically suppress each other in plant and chloroplast development.

Additional keywords: *Arabidopsis thaliana*, chloroplast development, gene regulation phytochrome interacting factor 4.

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Introduction

Chloroplast development in plants is influenced by a variety of environmental cues such as light and abiotic stress. Although light is required to initiate chloroplast biogenesis (Pogson and Albrecht 2011), abiotic stress has been demonstrated to have a negative impact on chloroplast biogenesis and function (Meskauskiene *et al.* 2001; Dutta *et al.* 2009; Kim *et al.* 2009; Dalal and Tripathy 2012). Overall, plant development and chloroplast development are tightly connected as each process is affected by impairment in the other. For example, impaired or even just delayed chloroplast development affects flowering time, biomass production and seed set.

One primary environmental factor influencing seedling development is the perception of light by different photoreceptors such as the two major protein families, phytochromes and cryptochromes (Sullivan and Deng 2003). Whereas cryptochromes are involved in the perception of blue light, phytochromes are involved in red and far-red light-mediated signalling (Reed *et al.* 1993; Sullivan and Deng 2003). In *Arabidopsis thaliana* (L.) Heynh., five different phytochromes have been identified, with Phytochrome A and Phytochrome B (PhyB) being the most prominent in light-mediated signalling, as well as two cryptochromes, Cryptochrome 1 and Cryptochrome 2.

Phytochromes, upon perception of light, undergo structural changes from the inactive Phy in red light (Pr) form to the active Phy in far-red light (Pfr) form, which is subsequently transferred into the nucleus (Chen *et al.* 2005). Here, they are involved in regulating the activity of transcription factors (Castillon *et al.* 2007; Waters *et al.* 2009). In particular, one group of transcription factors was found to play an important role during seedling establishment and photomorphogenesis in regulating important genes that encode proteins for gibberellic acid biosynthesis and signalling, or chlorophyll biosynthesis (Castillon *et al.* 2007). This class of transcription factors has been termed phytochrome interacting factors (PIFs), for which at least five members have been described in *A. thaliana* (Castillon *et al.* 2007). Interestingly, interaction of PhyB with the different PIF proteins mostly results in their phosphorylation, which then targets the PIF proteins for ubiquitination-mediated protein degradation (Shin *et al.* 2009). Additionally, the developmental stage of the plant effects the functional interaction between PhyB and PIF (Shin *et al.* 2009;

Stephenson *et al.* 2009). The transcriptional networks of the PIF proteins can overlap; however, specific regulatory functions exist: for example, PIF1 has been described as functioning in germination and chlorophyll biosynthesis, and PIF3 in the greening of seedlings (Huq *et al.* 2004; Monte *et al.* 2004). Indeed, *phyB* mutants are pale green throughout the entire plant cycle due to impaired chloroplast development (Chory *et al.* 1989). In the absence of functional PhyB, the PIF transcription factors are not degraded and thus continue to repress transcription of the essential genes required for chloroplast development and function (Chory *et al.* 1989; Stephenson *et al.* 2009). Loss of PhyB function not only impairs chloroplast development but also affects other processes such as shade avoidance, water use efficiency and flowering time (Reed *et al.* 1993; Boccalandro *et al.* 2009).

Although chloroplast biogenesis differs between cotyledons and true leaves, some common regulators have been identified in the last decade (Bauer *et al.* 2001; Pogson and Albrecht 2011). These include proteins that affect gene transcription, protein translation, chloroplast protein import, thylakoid formation or regulation of photosynthesis (Bauer *et al.* 2001; Sakamoto *et al.* 2008; Kessler and Schnell 2009; Pogson and Albrecht 2011). We have identified a group of genes whose impact on chloroplast biogenesis is greater in cotyledons than leaves. These genes have been named *Snowy Cotyledon* (*SCO*) (Albrecht *et al.* 2006; Albrecht *et al.* 2008; Pogson and Albrecht 2011; Tanz *et al.* 2012; Albrecht-Borth *et al.* 2013). The *snowy cotyledon 3* (*sco3*) mutant was of particular interest, encoding a protein of unknown function that is not located in the chloroplasts but in the periphery of peroxisomes. Dysfunction of this protein impairs the fine structure of the cytoskeleton (Albrecht *et al.* 2010). In contrast, the other identified SCO proteins are required for chloroplast protein translation, as is the case for SCO1; chloroplast protein folding and targeting, as shown for the protein disulfide isomerase SCO2, or a likely novel protease activity, as is the case for SCO4 (Albrecht-Borth *et al.* 2013; Pogson and Albrecht 2011; Tanz *et al.* 2012). Although the effect of the *sco3* mutation on chloroplast development in cotyledons is apparent, only a few genes have been found to be misregulated in the *sco3* mutant (Albrecht *et al.* 2010).

Here we describe the identification of a revertant of *sco3–1* that is due to a mutation in the *PhyB* gene. We propose a mechanism of crosstalk possibly occurring between the phytochrome-mediated signalling pathway and SCO3-mediated processes, and investigate how they alter nuclear gene transcription to complement impaired chloroplast biogenesis.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh plants were grown either on sterile MS medium (Murashige and Skoog 1962) with 0.8% agar or on soil under normal long-day conditions (16 h light, 8 h dark) at 21°C. For sterile growth conditions, seeds were surface sterilised using 70% ethanol and washed three times with autoclaved bidistilled water before plating. After overnight stratification, the seeds were transferred to a room with a constant temperature and the described growth conditions.

Analyses of TILLING lines

The *SCO3* genomic sequence was submitted to the *A. thaliana* TILLING project (http://tilling.fhcrc.org/files/Welcome_to_ATP.html) to identify additional mutant alleles in *SCO3*. Seeds that should contain mutations according to the TILLING results were obtained from Arabidopsis Biological Research Centre (ABRC), sown on soil and monitored for the *sco3–1* typical phenotype. For each line, DNA was extracted as described by Albrecht *et al.* (2006) and the predicted genomic region of the mutation was amplified and subsequently sequenced to verify the mutation. In aim to outcross the *erecta* mutation in the parental Big Mamma line, the TILLING mutants were crossed several times into *A. thaliana* cv. Columbia (Col) and rescreened for a pale green phenotype. The segregation of the *sco3* mutation with the pale phenotype was analysed by resequencing the gene region.

Isolation of sco3–1 revertants

Approximately 25 000 seeds of *sco3–1* were mutagenised with ethyl methyl sulfonate (EMS) as described by Albrecht *et al.* (2006), and the M2 generation was plated on MS medium and screened for seedlings with an increased green cotyledon phenotype. Mutant lines identified in the M2 generation were rescreened in the M3 generation to verify the observed phenotype. Subsequently, they were backcrossed into *sco3–1* to eliminate most of the other mutations caused by ethyl methyl sulfonate and to identify the recessive or dominant nature of the second site mutation. One particular mutant with a long hypocotyl and greener cotyledons than *sco3–1* was identified in the obtained revertants. Because of its particularly *phyB*-like phenotype, the *PhyB* gene was amplified and sequenced to identify potential mutations in this gene. Since it turned out that the mutation in this mutant is dominant, we did not proceed to isolate the single *phyB-XD* mutation but introduced a recessive allele of *phyB* into *sco3–1*. The second mutant allele of *phyB*, *phyB-9*, was crossed into *sco3–1* to confirm the observed phenotype and mutation of the identified revertant.

Chlorophyll analysis

For chlorophyll quantification 30 mg of 7-day-old seedlings grown on MS medium under 16 h light conditions were harvested, frozen and ground in liquid nitrogen and resuspended in 80% acetone. The measurements were performed on a plate reader (Infinite M1000 PRO, TECAN, Switzerland) for optical density (OD)663 and OD645, and the calculations were executed as described by Albrecht *et al.* (2006).

Scanalyzer analysis

The analysis of the greenness in rosette leaves and the circumference of the rosettes in 3-week-old plants was performed using the Scanalyzer system (LemnaTec, Germany). For this analysis, plants were grown on trays of soil for 3 weeks (or longer for the analysis of flowering time) and were transferred under the camera of the Scanalyzer image capture system. Subsequent image analysis was performed according to the Scanalyzer manual (LemnaTec).

Microarray analyses and quantitative real-time PCR

Analysis of the differences in the gene expression profiles between Col, *sco3–1*, *phyB-9* and *sco3–1phyB-9* was performed using the Gene Chip *Arabidopsis* ATH1 genome arrays (Affymetrix, CA, USA) at the Ramaciotti Centre (Sydney, NSW, Australia) in biological triplicate. Total mRNA of 4-day-old seedlings grown under long day conditions (16 h light : 8 h dark) was isolated and the DNA was treated using the Spectrum Total RNA Kit (Sigma, St Louis, MO, USA). Quality check metrics were provided by the Ramaciotti Centre. The CEL files obtained were further analysed, including additional quality check analyses using the Partek Genomic Suite (http://www.partek.com/pgs, accessed 17 April 2015). Log-transformed (log base 2) data were analysed to detect differentially expressed genes between each line using a one-way ANOVA based on the method of moments (Eisenhart 1947) and Fisher's LSD contrast method for differential regulation between any two of the lines using the work-flow of the Partek Genomic Suite. Significantly misregulated genes had to be misregulated by at least twofold (up or down) using a Benjamini step-up adjusted *P*-value with false discovery rate of 0.05. Gene name and function were obtained from the The Arabidopsis Information Resource (TAIR) database (www.arabidopsis.org). Of particular interest were the genes that were specifically up- or downregulated in the *sco3–1phyB-9* double mutant. The microarray data produced and discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (Edgar *et al.* 2002) and are accessible via the Gene Expression Omnibus series accession number GSE67168 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67168).

For verification of the observed changes in gene expression in the microarray analysis, select genes of interest were analysed using quantitative real-time PCR. These genes were *Ferritin 1* (*FER1*, *At5g01600*), *Early Light-Induced Protein 1* (*ELIP1*, *At3g22840*), *Flowering Locus C* (*FLC, At5g10140*), *PIF4* (*At2g43010*) and *HEME2* (encoding uroporphyrinogen decarboxylase, *At2g40490*). For this, total mRNA of each of the four lines used in the microarray study was reverse transcribed using the Superscript III cDNA Synthesis Kit (Invitrogen, The Netherlands). Relative transcript abundance was quantified in Col, *sco3–1*, *phyB-9* and *sco3–1phyB-9* plants, using the SYBR Green JumpStart *Taq* Ready-Mix (Sigma-Aldrich, MO, USA). Three technical replicates, one for each of three biological replicates for each line, were performed using the Light Cycler 480 (Roche, Switzerland). The obtained data were analysed by relative quantification (quantitative cycle (Cq) values) using the *Protein Phosphatase 2A* gene (*At1g13320*) as a housekeeper control.

Gene Ontology annotation analysis was performed using the TAIR database and the subsequent data were analysed according to Narsai *et al.* (2007).

Generation of overexpression lines

The cDNA of *PIF4* (*At2g43010*) was cloned into the binary vector *pMDC85* under the control of the 35S promoter, transformed into Col and *sco3–1* plants, and selected on hygromycin. Since we did not obtain any transgenic plants for the *PIF4* overexpression construct in *sco3–1*, several independent transformed lines of Col containing the *PIF4* overexpression construct were crossed with *sco3–1*. The subsequent F2 generation was screened for plants harbouring both the PIF4 overexpression construct as well as being homozygous for *sco3–1*.

Results

Alellic SCO3 *point mutations demonstrate the involvement of* SCO3 *in chloroplast development at all stages of plant development*

Null alleles of SCO3 are lethal in the embryo and the *sco3–1* allele results in pale green cotyledons (Albrecht *et al.* 2010). Newly identified *sco3–5* and *sco3–6* alleles recovered from a TILLING screen resulted in both pale green cotyledons and true leaves (Fig. S1, available as Supplementary Material to this paper). Thus, SCO3 is essential for plastid development at all stages of a plant's life cycle, with the mutation's penetrance determining the degree of deleterious impacts at different developmental stages.

Mutations in the PhyB *gene rescue specific* sco3–1 *phenotypes and vice versa*

In order to determine how the peroxisomal-associated SCO3 protein impacts chloroplast biogenesis, a second-site mutagenesis was performed on 25 000 *sco3–1* seeds. The M2 population was screened for reversions of the *sco3–1* bleached cotyledon phenotype (i.e. plants that exhibited green cotyledons). Several of these mutants were identified and one of these had a long hypocotyl. Since it is known that *phyB* mutants exhibit a long-hypocotyl phenotype, the coding region of *PhyB* in this revertant was amplified, sequenced and shown to have a G to A mutation, which resulted in an amino acid change from alanine to threonine at Position 663 (A663T) (Fig. 1*a* and Fig. S2*b*). This amino acid is conserved in all phytochromes and is located at the beginning of the PAS repeat domain, which is required for nuclear localisation of the PhyB protein (Chen *et al.* 2005; Krall and Reed 2000). Since we observed a segregation of green and pale seedlings in the M2 generation, it is to be expected that the *phyB* mutation in this line is dominant. Subsequently, this revertant was termed *sco3–1phyB-XD*.

To determine the nature of the *phyB-XD* mutation, the *sco3–1phyB-XD* double mutant was crossed with *sco3–1*. In the F2 population, 813 seedlings were counted, where 615 (76%) were green and 198 (24%) were pale. This 3 : 1 segregation indicated that the *phyB-XD* mutation does indeed revert the *sco3–1* phenotype in a dominant manner. To further confirm the role of PhyB in the reversion, *sco3–1* was crossed with the recessive *phyB-9* allele with a premature stop codon (Fernandez *et al.* 2005) and the homozygous double mutant line was isolated in the F2 generation. Introducing the recessive *phyB-9* allele into the *sco3–1* mutant background also resulted in a partial greening of the pale cotyledons, as was observed in *sco3–1phyB-XD* (Fig. 1*b*). Comparison of the chlorophyll content of the double mutant with *sco3–1* and Col showed that *sco3–1phyB-9* has about twice as much chlorophyll as *sco3–1* in 7-day-old seedlings, whereas the initially identified *phyB-XD* allele almost tripled the amount of chlorophyll compared with *sco3–1* (Fig. 1*c*, Table 1). Thus mutations in the *PhyB* gene can partially rescue the pale green *sco3–1* phenotype.

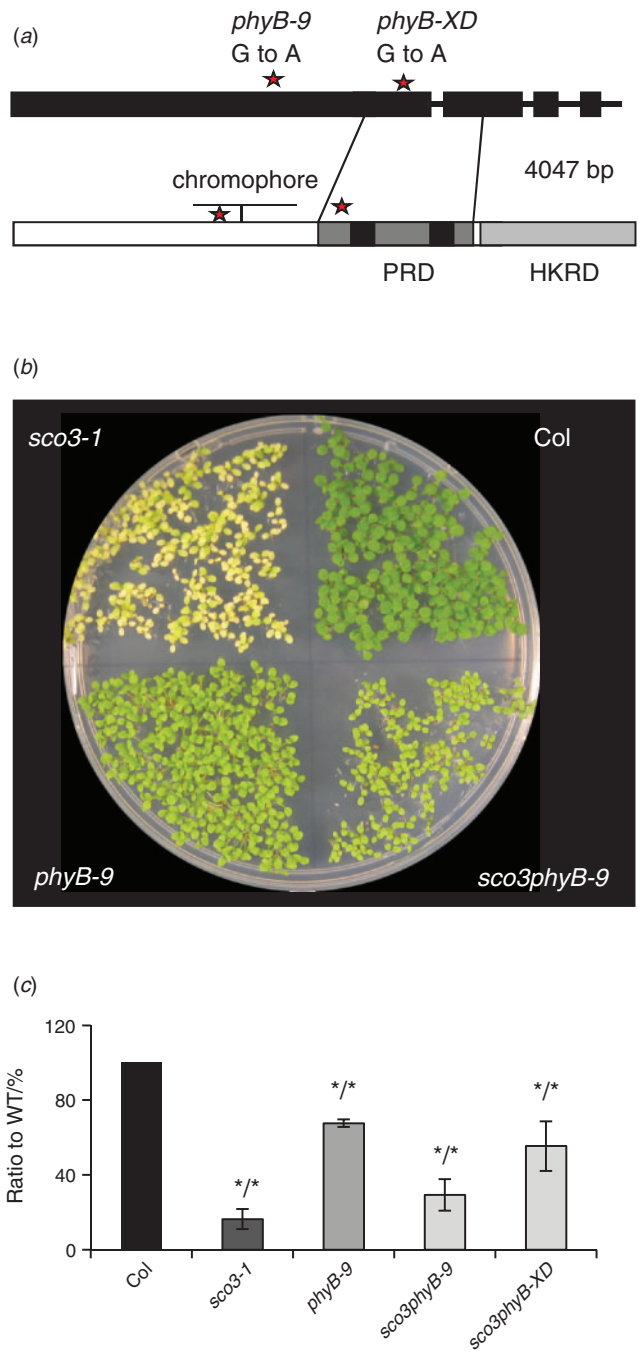

Fig. 1. *Phytochrome B* (*PhyB*) partially complements *Snowy Cotyledon 3* (*SCO3–1*) in *Arabidopsis thaliana*. (*a*) Location of the mutations for the different *phyB* mutant alleles on the genome (top) and in the different domains of the protein (bottom, according to Krall and Reed 2000). Asterisks indicate point mutations. PRD: PAS repeat domain, which is required for nuclear localisation of the PhyB protein (Chen *et al.* 2005; Krall and Reed 2000); HKRD, histidine-kinase related domain. (*b*) Phenotype of 7-day-old seedlings of the different mutant lines compared with the wild-type Col. (*c*) Chlorophyll content in 7-day-old seedlings of the different mutant lines compared with Col. Asterisks indicate a significant difference compared with Col or compared with *sco3–1* in the *t*-test at $P < 0.05$. WT, wild-type.

Similarly, although *phyB-9* mutant lines exhibited a pale true-leaf phenotype, quantification of the chlorophyll content of the leaves in 3-week-old plants showed that the introduction of the *sco3–1* mutation partially recovered the chloroplast development in true leaves (Fig. 2*a*, *b*, Table 1).

With respect to flowering time, combining the *phyB-9* and *sco3–1* mutations tended to enhance the early flowering phenotype normally observed in *phyB-9*. In other words, both the *phyB-9* mutant (9.5 ± 1.9 rosette leaves, 4 weeks) and *sco3–1phyB-9* double mutant (5 ± 0.8 rosette leaves, 3.5 weeks) started flowering earlier under long-day conditions compared with Col (12.3 ± 0.9 rosette leaves; 6 weeks) and *sco3–1* (>16 rosette leaves, 8–10 weeks) (Fig. 2*c*, Table 1). The rosette circumference of 3-week-old *sco3–1phyB-9* plants is smaller than that of *phyB-9* but bigger than that of the *sco3*–1 mutant plants, whereas *sco3–1phyB-XD* resembles wild-type plants in circumference and has wild-type green leaves with longer petioles (Fig. 2*d*, Table 1). With respect to leaf morphology, the introduction of the *sco3–1* mutation into *phyB-9* also led to enhanced defects: whereas both *sco3–1* and *phyB-9* were able to form large rosettes the double mutant had very few rosette leaves (Fig. 2*e*, Table 1).

Sco3–1 *does not alter the hypocotyl length of seedlings grown under red light*

An important function for PhyB is light-mediated photomorphogenesis under red light. Mutants impaired in *phyB* not only exhibit a longer hypocotyl under white light but are also insensitive to red light (Mira-Rodado *et al.* 2007). Therefore, an analysis was performed by germinating Col, *sco3–1*, *phyB-9* and the double mutant *sco3–1phyB-9* under increasing red light conditions. Whereas Col and *sco3–1* demonstrated a decrease in hypocotyl length with increased red light intensities, neither *phyB-9* nor *sco3–1phyB-9* responded to red light (Fig. S3). Thus the introduction of the *sco3–1* mutation into *phyB-9* does not influence the hypocotyl growth of the seedlings under red light.

SCO3 *influences post-germination chloroplast development via an embryogenesis-linked mechanism*

In a recent publication, it was shown that light during embryogenesis positively affects chloroplast development after germination in the *ex1ex2* double mutant (Lee *et al.* 2007). Since both of the single mutants, *sco3–1* and *phyB-9*, are impaired in chloroplast development in young seedlings, and PhyB is involved in light perception, a light-induced defect in chloroplast formation during embryogenesis cannot be excluded. To test this, every second silique of the main bolting stem was covered with aluminium foil for dark embryo maturation, whereas the other siliques were used as controls for embryo development in the light (Fig. 3*a*). Mature seeds were harvested at the same time, surface sterilised and sown on MS medium, and the chlorophyll content of 7-day-old seedlings was determined. Although there was no significant change in the *phyB-9* mutant, chlorophyll content was unexpectedly enhanced threefold in the *sco3–1* mutant and twofold in both double mutants, which suggests that the effect of the *sco3–1* mutation

Table 1. Comparison of phenotypes among the *Arabidopsis thaliana* strains Col, *sco3–1*, *phyB-9*, *sco3–1phyB-9*, and *sco3–1phyB-XD*
Greening is expressed as the ratio of dark-matured to light-matured seeds. Rosette circumference is expressed in relative units. *SCO3*, *Snowy Cotyledon 3*; *PhyB*, *Phytochrome B*

Genotype	Chlorophyll content in seedlings (*vs.* Col)	Chlorophyll content in rosettes (*vs.* Col)	Flowering time (weeks)	No. rosette leaves at flowering	Rosette circumference at 3 weeks	Greening of seedlings
Col	1	1	6	12.3 ± 0.9	2215 ± 194	1.3 ± 0.13
sco3–1	0.16 ± 0.05	0.97 ± 0.04	8–10	>16	598 ± 109	2.4 ± 0.19
phyB-9	0.67 ± 0.02	0.37 ± 0.04	4	9.5 ± 1.9	1852 ± 62	0.97 ± 0.15
sco3–1phyB-9	0.29 ± 0.08	0.71 ± 0.05	3.5	5 ± 0.8	1464 ± 254	1.84 ± 0.3
sco3–1phyB-XD	0.55 ± 0.13	0.97 ± 0.02	4–5	11 ± 1.2	2177 ± 702	1.9 ± 0.1
Genetic interaction	Suppressive	Suppressive	Additive	Additive	Suppressive	Suppressive

is mitigated by the absence of light during embryogenesis (Fig. 3*b*, Table 1).

A distinct set of genes is differentially regulated in sco3–1phyB-9 *compared with either* sco3–1 *or* phyB-9

In order to investigate the opposing impacts of *SCO3* and *PhyB* mutations in the greening of young seedlings, we measured the global transcript levels of nuclear-encoded genes in 4-day-old seedlings grown under long-day conditions. It is known that PhyB regulates the transcription of many nuclear-encoded genes (Sullivan and Deng 2003; Monte *et al.* 2004; Moon *et al.* 2008; Shin *et al.* 2009), whereas only a small number of transcripts are altered in young *sco3–1* seedlings grown under continuous light (Albrecht *et al.* 2010). We performed a new transcriptome analysis on 4-day-old seedlings using Affymetrix chips and compared the transcript levels of Col with those of the single and double mutants. As expected, many genes were downregulated (697) or upregulated (1223) in the *phyB-9* single mutant and only 50 genes were upregulated and 48 genes were downregulated in *sco3–1* (Fig. 4*a* and Table S3).

Interestingly, in the *sco3–1phyB-9* double mutant, 544 transcripts were downregulated and 1127 were upregulated. Volcano plots were made to compare the distribution of misregulated transcripts between the different genotypes (Fig. S4). From these plots, it is evident that many genes were uniquely misregulated in *sco3–1phyB-9* (green dots in Fig. S4*a*, *b* and Fig. 4*a*). Although some of the genes were specific for *sco3–1* (blue dots in Fig. S4*a*), introduction of the *phyB-9* mutation reverted the expression of 25 out of 48 downregulated genes and 21 out of 50 upregulated genes in *sco3–1*.There was also a considerable proportion of genes misexpressed in *phyB-9* (blue dots in Fig. S4*b*) that were not so in the double mutant (Fig. 4*a*, green dots in Fig. S4*b*). In addition, half of the genes misexpressed in the double mutant were unique to the double mutant compared with *phyB-9* and *sco3–1* (Fig. 4*a*, *b*, Table S2; genes misregulated in *sco3–1* are listed in Table S3). A question is whether the nonoverlapping genes between single and double mutants correspond to genes that narrowly miss significance criteria. The volcano plots do not support this hypothesis, as the distribution of 900 unique PhyB (blue dots) largely overlies the >20 000 unchanged transcripts (black dots) in the double mutant (Fig. S4*b*).

Analysis of the ontological (Gene Ontology) annotation of the genes that were uniquely up- or downregulated in the *sco3–1phyB-9* double mutant compared with the single mutants indicated an over-representation of plastid or nucleus-localised proteins compared with the whole genome distribution. Proteins of the cell wall and the extracellular compartment were over-represented in the downregulated genes in the double mutant. In the upregulated genes, an over-representation of proteins involved in abiotic and biotic stress responses were observed, whereas in both the up- and downregulated genes, protein metabolism was under-represented (Table S4). The latter seems to balance out the overproduction of proteins in *sco3–1*, which demonstrates the impairment in chloroplast development and thus results in greener seedlings. A similar observation has been made in revertants of the chloroplast development mutants *variegated*, where a reduction in chloroplast protein biosynthesis counterbalanced the deficiency in chloroplast protein degradation, resulting in the greening of the plants (Miura *et al.* 2007). However, the most pronounced is the expression of genes in both gene pools that are involved in abiotic and biotic stress responses, indicating that continuous stress was present in the double mutant.

As *sco3-1phyB-9* is a mutant with a partially recovered chloroplast development phenotype, we were interested to discover whether within the pool of genes uniquely up- or downregulated, there were genes that are known to be involved in or to affect chloroplast development. Indeed, when we looked at the list of genes that were upregulated in the double mutant and predicted to be localised in the chloroplast, many of the genes with known function were involved in the regulation of transcription or post-transcriptional processing, or protein translation such as the pentatricopeptide repeat (PPR) protein Early Chloroplast Biogenesis 2 or the RNA-processing protein High Chloroplast Fluorescence 152 (Meierhoff *et al.* 2003; Cao *et al.* 2011). Genes involved in chloroplast protein import are upregulated such as *Translocon at the outer envelope membrane of chloroplasts 55* (*TOC55*) or *Chloroplast import apparatus 2* (*CIA2*) and *CIA2-like*, both of which upregulate the transcription of the chloroplast import apparatus proteins TOC33 and TOC75 (Sun *et al.* 2009). Several genes were identified in the defective embryo screen, suggesting that an upregulation of these genes might benefit chloroplast development. In contrast, in the list of downregulated genes, only a few are with proteins of known functions such as the chlorophyll biosynthesis protein HEME2.

Interestingly, upon closer examination of the misregulated genes and their encoded protein functions, it was observed that

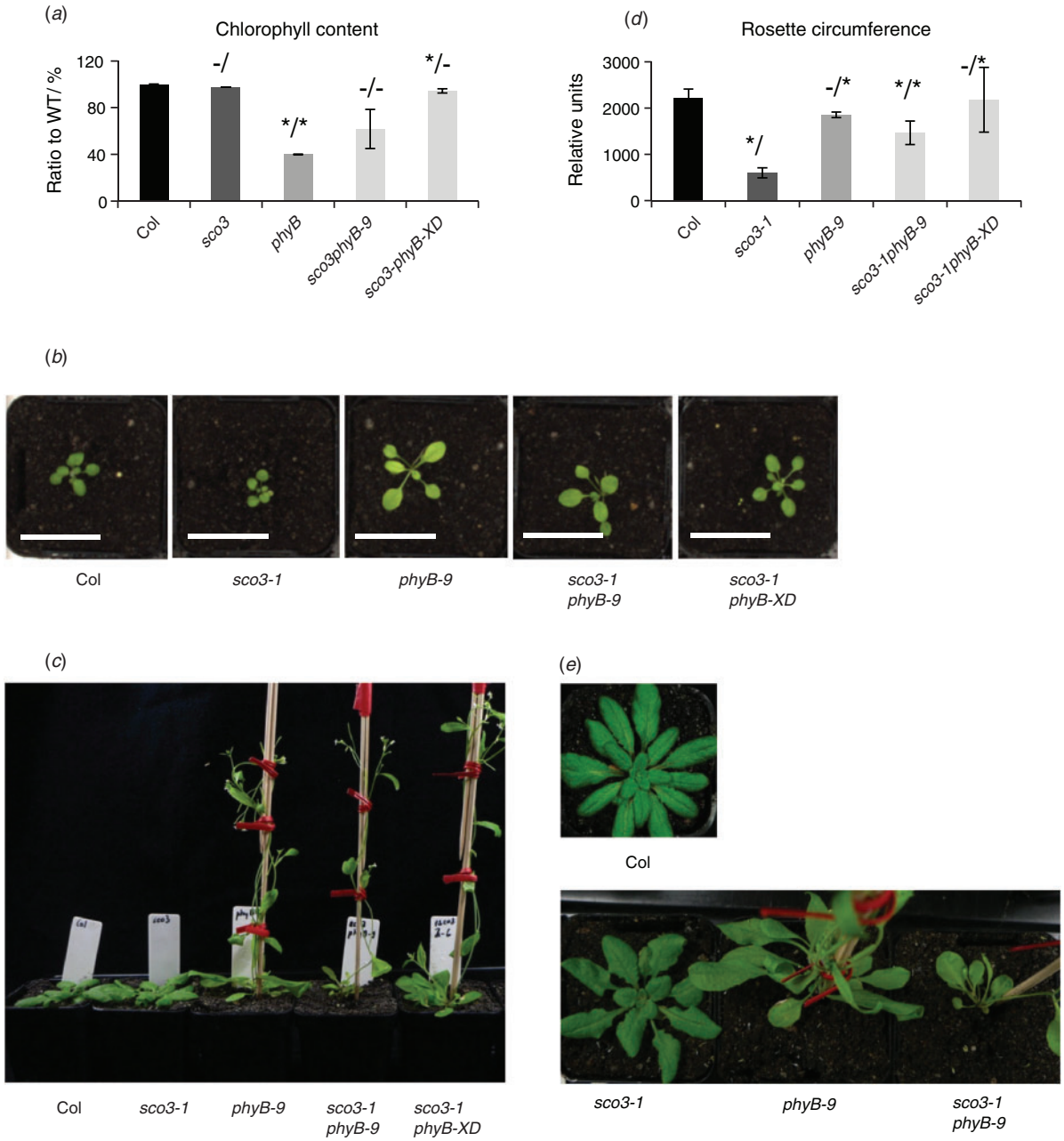

Fig. 2. Effect of the *snowy cotyledon 3* (*sco3–1*) mutation on the *phytochrome B* (*phyB*) phenotype in adult *Arabidopsis thaliana* plants. (*a*) Chlorophyll content of true leaves in 3-week-old plants compared with the wild-type (WT) Col. Asterisks indicate a significant difference compared with Col or *sco3–1* (*t*-test, $P < 0.05$). Error bars indicate the s.d. (*b*) Plant phenotype of 3-week-old plants at the time of chlorophyll and circumference measurements. Scale bar indicates 3 cm. (*c*) Early flowering of lines having the *phyB* mutations in 5-week-old plants. Rosette leaf numbers at flowering time are provided in the text. (*d*) Circumference analysis of 3-week-old plants. Asterisks indicate a significant difference compared with Col or *sco3–1* (*t*-test, $P < 0.05$). Error bars indicate the s.d. (*e*) Rosette phenotype of 5-week-old plants.

several gene families are over-represented in the list to a greater degree than expected, such as the upregulation of UDP glycosyltransferases involved in secondary metabolism (Ross *et al.* 2001), or the downregulation of proteins involved in cell wall metabolism such as the ketoacyl CoA synthases involved in long chain fatty acid elongation (Haslam and Kunst 2013), xyloglucan transferases and hydrolases (Rose *et al.* 2002), arabinogalactan proteins or extensins (Table S2).

To confirm the results obtained from the microarray data, quantitative real-time PCR on RNA extracted from the different lines was performed using cDNA-specific primers for selected genes that were either up- or downregulated in *sco3–1phyB-9* (Fig. 5*a*, *b*, Table S5). In summary, *FER1*, *Touch 4* (*TCH4*) and *ELIP1*were all upregulated, and *PIF4*, *HEME2* and *FLC* were all downregulated in *sco3–1phyB-9*. Except for *TCH4*, which was downregulated in the array, the

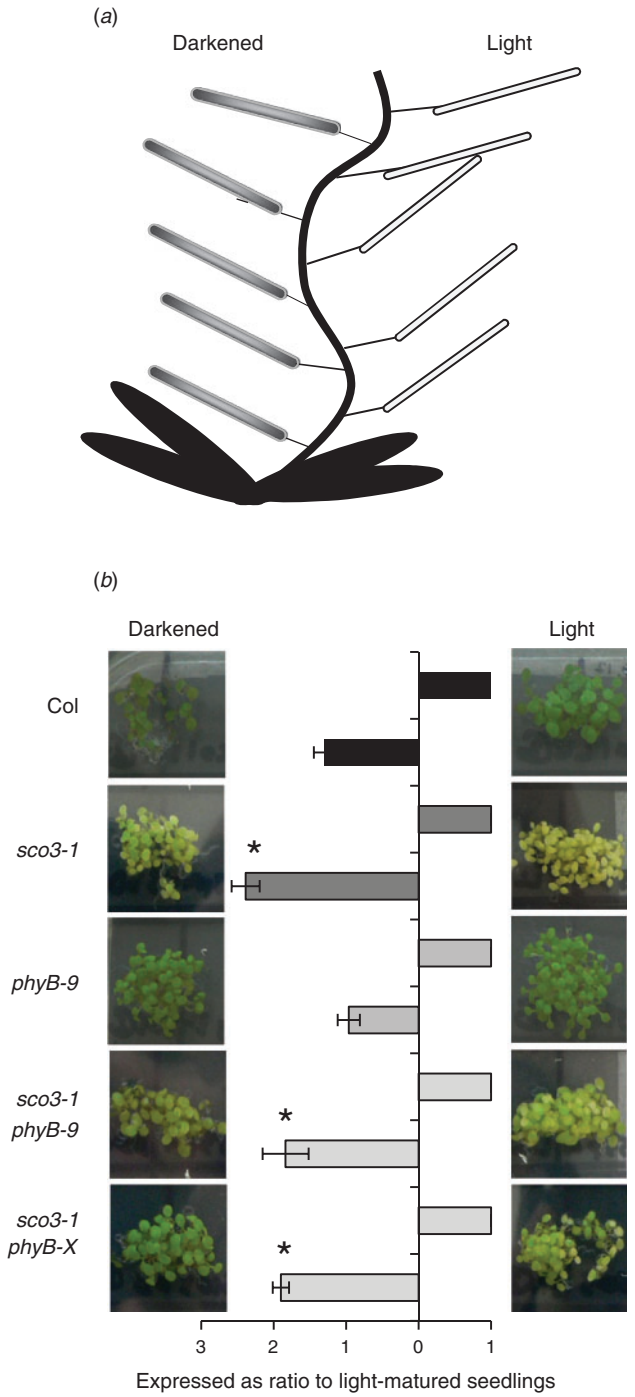

Fig. 3. Impact of embryogenesis on greening of *Arabidopsis thaliana* seedlings. (*a*) Schematic outline of covering the siliques with aluminium foil. (*b*) Phenotype and chlorophyll content in 7-day-old seedlings germinated from seeds matured in the dark compared with seeds matured in the light. Asterisks indicate a significant difference compared with Col (*t*-test, $P < 0.05$). Error bars indicate the s.d. *SCO3*, *Snowy Cotyledon 3*; *PhyB*, *Phytochrome B*.

data from the quantitative PCR confirmed (*FER1*, *FLC*) the array data or showed an enhanced up- or downregulation (*ELIP1*, *PIF4* and *HEME2*).

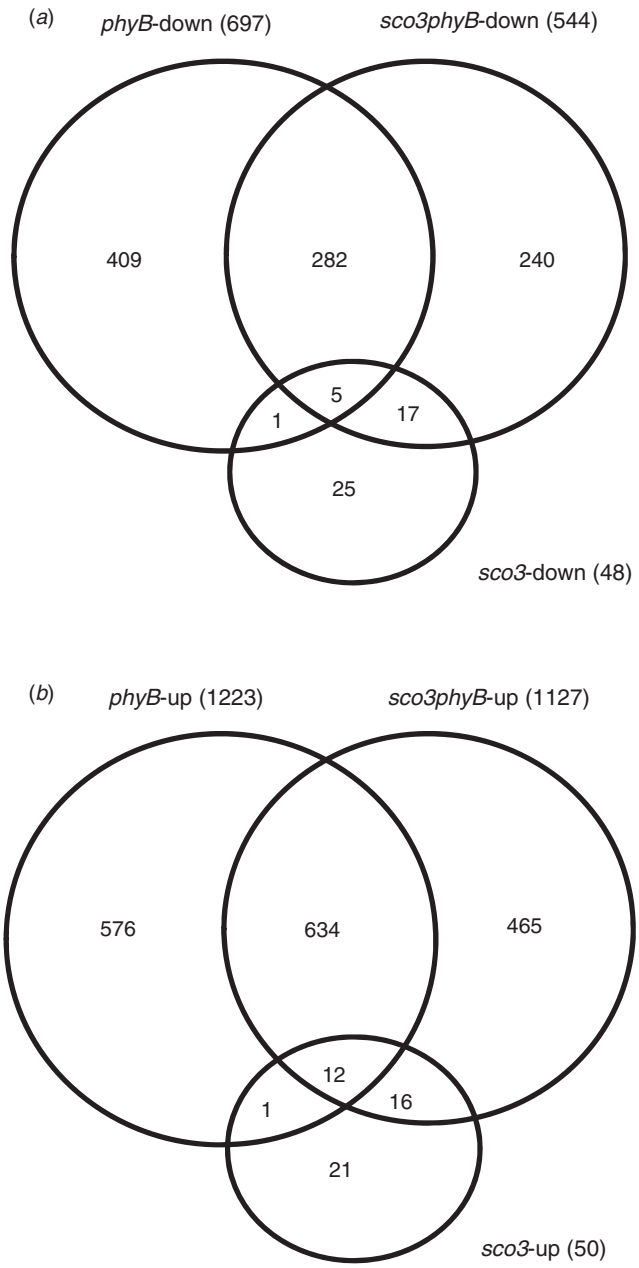

Fig. 4. Transcript analysis in 4-day-old *Arabidopsis thaliana* seedlings. (*a*) Venn diagram of downregulated genes compared with Col in *phyB-9*, *sco3–1* and *sco3–1 phyB-9*. (B) Venn Diagram of upregulated genes compared with Col in *phyB-9*, *sco3–1*, and *sco3–1 phyB-9*. *PhyB*, *Phytochrome B*; *SCO3*, *Snowy Cotyledon 3*.

Overexpression of PIF4 *is able to partially rescue the* sco3–1 *pale cotyledon phenotype*

Our previous transcript analysis on *sco3–1* revealed that very few transcription factors are downregulated, such as *Golden-like 1*, which encodes a protein involved in the regulation of many photosynthesis genes, and *PIF4* (Albrecht *et al.* 2010). PIF proteins interact with, are regulated by and regulate PhyB. Under continuous light, the transcript levels of *PIF4* in *sco3–1* seedlings was downregulated five fold and, in this analysis

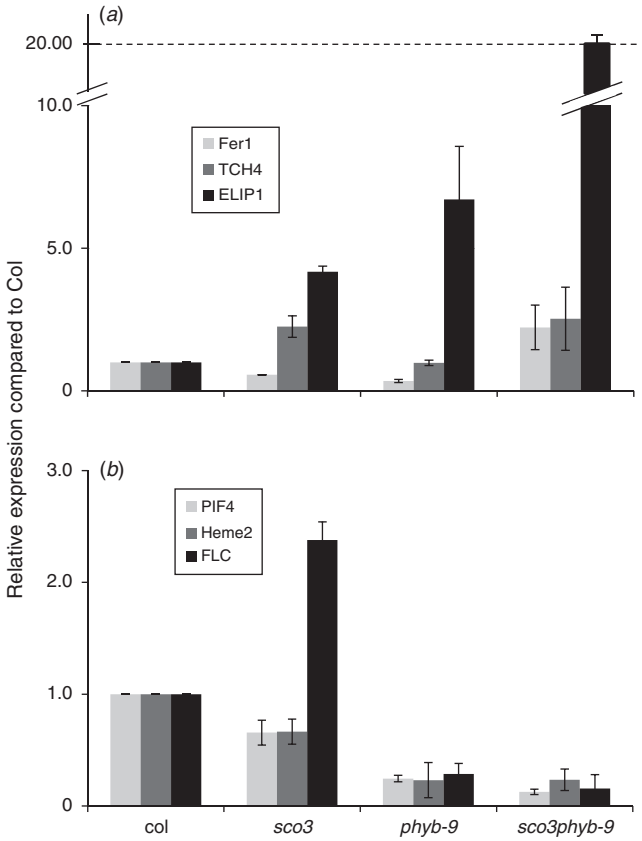

Fig. 5. Quantitative real-time PCR on selected *Arabidopsis thaliana* genes that were specifically (*a*) upregulated or (*b*) downregulated in the microarray analysis for *sco3–1 phyB-9*. *SCO3*, *Snowy Cotyledon 3*; *PhyB*, *Phytochrome B*; *Fer1*, *Ferritin 1*; *TCH4*, *Touch 4*; *ELIP1*, *Early Light-Induced Protein 1*; *PIF4*, *Phytochrome Interacting Factor 4*; *FLC*, *Flowering Locus C*.

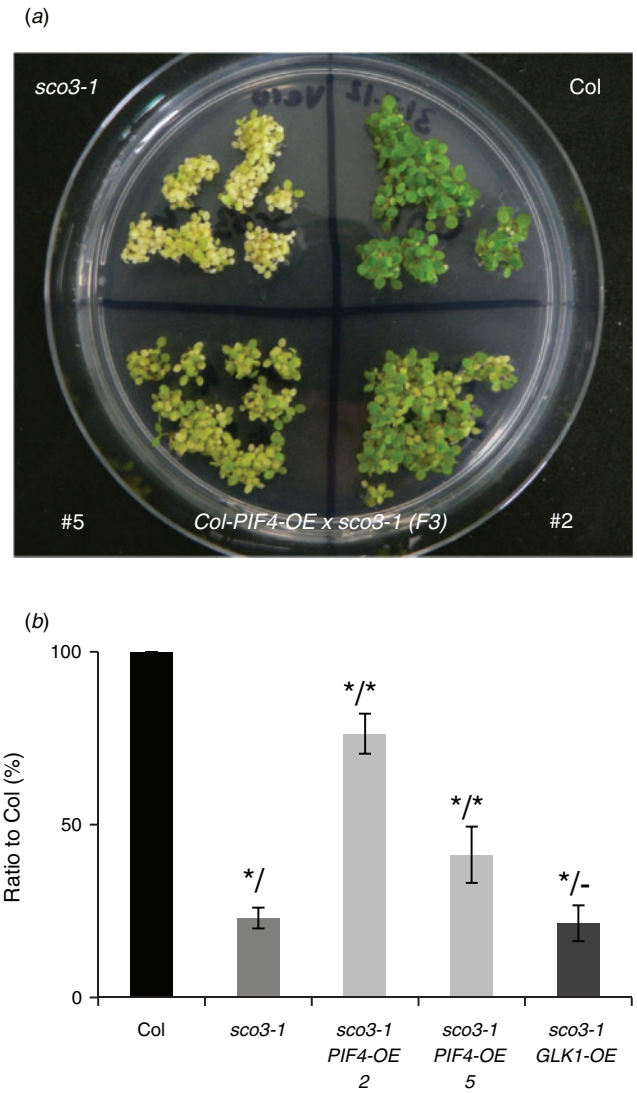

Fig. 6. Overexpression (OE) of *Phytochrome Interacting 4* (*PIF4*) partially complements *Snowy Cotyledon 3* (*SCO3–1*). (*a*) Phenotype of 7-day-old *Arabidopsis thaliana* seedlings of the different mutant lines compared with Col. (B) Chlorophyll content in 7 days old seedlings of the different mutant lines compared with Col. As a control, another transcription factor, *Golden-like 1*, was overexpressed in *sco3–1*. Asterisks indicate a significant difference compared with Col or *sco3–1* in the *t*-test at $P < 0.05$.

under long-day conditions, by –1.5 fold (Fig. 5) (Albrecht *et al.* 2010). Since PIF proteins are regulated by phytochrome proteins in a light-dependent manner (Castillon *et al.* 2007), we reasoned that overexpression of *PIF4* might complement *sco3–1*. Seedlings transformed with the *PIF4* overexpression construct resulted a similar greening of the cotyledons to that observed for the *sco3–1phyB* double mutants (Fig. 6*a*, *b*). Quantification of *PIF4* expression in these lines demonstrated an upregulation of almost twofold in transgenic lines compared with Col and fourfold compared with *sco3–1* (*PIF4* overexpression in *sco3–1* #2: 1.86 ± 0.68; *PIF4* overextression in *sco3–1* #5: 1.75 ± 0.9). Thus the PIF4 protein seems to affect the regulation of chloroplast development and overexpression improves chloroplast biogenesis in *sco3–1*. However, when one quantifies *PIF4* transcript levels in *sco3–1phyb-9*, in both the microarrays and quantitative PCR there is, in fact, a downregulation rather than the expected upregulation of *PIF4*. This result somewhat contradicts our observation of sion of pale cotyledon phenotype in *sco3–1* by overexpression of *PIF4*. This suggests a complex regulation that will require further investigation.

Discussion

In this study, we observed that the introduction of mutations into the *PhyB* gene in the *sco3–1* mutant background resulted in a partial recovery of the pale cotyledon phenotype in *sco3–1* but also demonstrated complex traits in phenotypes that can only be explained by the differential additive or suppressive genetic regulation summarised in Table 1, which will be discussed in more detail. These genetic networks reveal a complex interaction between the SCO3 and PHYB pathways to regulate plant development and chloroplast biogenesis.

SCO3 *and PhyB regulate a novel set of genes during seedling chloroplast biogenesis*

Loss of normal SCO3 protein function results in impaired development of chloroplasts from their progenitors, the proplastids, by affecting the fine structure of the cellular actin

cytoskeleton and impairing the adaptation of the plants to extreme CO_2 concentrations (Albrecht *et al.* 2010). The *sco3–1* mutant was reported to have no observable phenotype in the rosette leaves under normal growth conditions, whereas other newly identified mutations, *sco3–5* and *sco3–6*, resulted in a pale green adult leaf phenotype, indicating a role for SCO3 throughout plant development in photosynthetic tissues.

The loss of PhyB function in the *sco3–1* mutant resulted in a greening of the cotyledons, indicating improved chloroplast biogenesis (Fig. 1*b*). This might indicate that *SCO3* genetically suppresses *PhyB*. On the other hand, the combination of both mutations results in an intermediate phenotype, since both single mutants have deleterious mutations that result in pale embryonic leaves. Analysis of the transcriptome of the double mutant revealed that, in addition to an overlap of ~50% with up- or downregulated genes identified in either *phyB-9* or *sco3–1*, a completely distinct set of genes was differentially expressed in the double mutant (Fig. 4). The *phyB-9* mutant is a signalling mutant that impairs the transcription of many genes, such as photosynthesis genes, by interaction with the PIF transcription factors. This is reflected in our transcriptomic data, where almost 2000 transcripts were found to be differentially expressed in this mutant. On the other hand, the transcriptome analysis in *sco3–1* indicates that only a few genes were differentially expressed in the mutant compared with the wild-type, despite displaying impaired chloroplast biogenesis. *SCO3* can only indirectly influence the transcription of genes, as it is located in the peroxisome border. With the data from *sco3–1*, we can assume that even though chloroplast development is severely impaired in the single mutant, the majority of genes required for chloroplast development are being expressed but some other function for chloroplast development is impaired in the *sco3–1* mutant. The observations of the transcript regulation in the *sco3–1phyB-9* double mutant indicate that the observed changes of this subset of genes, which are differentially expressed in the double but not in the single mutants, result in an improvement in chloroplast development. Furthermore, the overexpression of one PhyB interacting factor, PIF4, in *sco3–1* resulted in a similar greening of the cotyledons to that observed in the *sco3–1phyB-9* mutant. This might indicate that PIF4, which regulates and is regulated by the function of PhyB (Leivar *et al.* 2008; Shin *et al.* 2009), is involved in integrating the regulatory pathways of SCO3 and PhyB. So far, no direct involvement in regulating chloroplast biogenesis has been demonstrated for PIF4, though its function in integrating diverse environmental signals has been discussed (Lucyshyn and Wigge 2009).

We investigated a set of genes that were differentially regulated in the double mutant and verified their expression in the single and double mutants. These genes are either involved in development, in cellular organisation and biogenesis (*TCH4*, *HEME2* and *FLC*) or in stress responses (*FER1*, *ELIP1*). *FER1* was demonstrated to be regulated by hydrogen peroxide in several stress analyses (op den Camp *et al.* 2003; Rossel *et al.* 2007; Gordon *et al.* 2012). In this study, *FER1* was observed to be downregulated in the single mutants; however, it was upregulated in the *sco3–1phyB-9* double mutant (Fig. 6*a*), suggestive of increased levels of or sensitivity to reactive oxygen species. *ELIP1* demonstrated an additive effect in gene regulation, as its transcript levels were four- to fivefold higher in the double mutant as they were in either of the single mutants, *sco3–1* and *phyB-9*. ELIP1 is located at the thylakoids and is involved in the greening of etiolated seedlings under continuous high light (Casazza *et al.* 2005). Indeed, a photoprotective function of ELIP1 has been identified as accumulating linearly with light intensity and correlates to the degree of photoprotection in PSII (Heddad *et al.* 2006), which could suggest a role for ELIP1 in partial complementation.

Loss of SCO3 *function dominates in the regulation of embryonic factors required for seedling chloroplast development*

The covering of siliques resulted in the recovery of the delayed chloroplast biogenesis in the *ex1ex2* double mutant, which is impaired in its ability to relay singlet oxygen-mediated signalling from the chloroplast to the nucleus (Kim *et al.* 2009). Darkening of the embryos interrupts some chloroplast functions such as photosynthesis and might affect not only gene transcription but also the assimilation of the different compounds normally produced in embryos exposed to light. Since PhyB is a major factor required for the perception of light and the subsequent regulation of many photosynthesis genes, we performed a similar experiment with our mutant lines to analyse the effect of darkness during embryogenesis on the greening process of the emerging seedlings. Darkening the embryos during maturation resulted in seedlings having increased chlorophyll levels except for the *phyB-9* mutant, indicating the importance of PhyB for light perception in the embryos (Fig. 3*b*, Table 1).

The greening effect of dark embryo maturation is enhanced in the lines containing the *sco3–1* mutation giving further evidence that SCO3 protein function is required for chloroplast development. What is curious is that the developing embryos of *sco3–1* are as green as those in the wild-type (Albrecht *et al.* 2010) but if they mature in the dark, the resulting seedlings are greener than if they mature in the light. This suggests an unknown embryonic factor will be required for chloroplast development in germinating seedlings. In their analysis on their double mutant *ex1ex2*, Kim *et al.* (2009) revealed that the influence of photosynthesis-derived singlet oxygen generated during embryogenesis has an impact on chloroplast development after germination. Since no singlet oxygen-specific genes are differentially regulated in the *sco3–1* mutant (as discussed in Albrecht *et al.* 2010) and there did not seem to be an overlap of genes differentially regulated between *ex1ex2* and *sco3–1phyB-9*, we assume that in *sco3–1* or *sco3–1phyB-9*, this type of signalling is not involved in the regulation of chloroplast development. Our analysis indicates that PhyB is involved in the formation of this yet unknown embryonic factor(s) as it is required for the increase of chlorophyll and thus improved chloroplast formation in the emerging seedlings.

Loss of PhyB function dominates in the regulation of flowering time

In addition to chloroplast development, SCO3 and PhyB also influence other aspects of plant development. PhyB regulates flowering time, since it is part of the signalling system entraining the circadian clock (Sullivan and Deng 2003). Loss of the PhyB signalling pathway results in early flowering plants, which is even

further enhanced by introducing the *sco3–1* mutation (Table 1). The mutation in *sco3–1* appears to result in delayed flowering and upregulation of *FLC*. That is, *FLC* is upregulated in *sco3–1* but downregulated in *phyB-9* and is even further upregulated in *sco3–1phyB-9*, which correlates with the observed flowering phenotype for each single and double mutant (Figs 2*a* and 6*b*). Indeed, a correlation between an abundance of mRNA and flowering time has been demonstrated by other groups, where a reduced amount of mRNA of the MADS-box transcription factor encoding gene *FLC* resulted in an earlier flowering of the plants (Swiezewski *et al.* 2007).

Interestingly, the formation of the typical morphology of a rosette in *A. thaliana* is dramatically impaired in the *sco3–1phyB-9* double mutant (Fig. 2, Table 1), which appears to be an additive genetic interaction, resulting in a phenotype that is more severe than the two single mutants. In the *phyB* mutant, a reduction of rosette leaves was observed, which was thought to be linked to the early flowering phenotype. This was only observed in the *sco3–1phyB-9* mutant and not in the *sco3–1phyB-XD* double mutant. Thus the inference is that this phenotype is influenced by the mutant allele, which, in *phyB-9*, is close to the binding region of the phytochromobilin chromophore; in *phyB-XD*, the mutation is within the PAS repeat domain close to the first PAS motif (Krall and Reed 2000). The latter was found to be required for nuclear relocalisation of the PhyB protein whereas phytochromobilin itself is necessary for the perception of light.

The analyses of the *sco3–1*, *phyB-9*, and *sco3–1phyB* lines revealed that a complex additive and suppresive genetic regulation of the two SCO3- and PhyB-dependent networks interferes in regulating different plant developmental stages (Table 1). The global expression data are consistent with this complexity and reveal similar proportions of coexpressed and uniquely expressed genes in *sco3–1phyB-9* compared with *phyB-9*. These analyses, while revealing novel aspects of the regulation of plant development, raise new questions. For example, what are the other factors in the integration of developmental process? What factors are generated during embryo development that influence chloroplast development in seedlings? Why is there a differential regulation between all the processes analysed during plant development?

SCO3 is an essential gene for embryogenesis and chloroplast development with an as yet unidentified function. We have shed new light on the role of SCO3 by identifying its novel but complex genetic interaction with PhyB and the canonical light-mediated transcriptional regulation pathway in plant development encompassing not only chloroplast development and the involvement of embryonic factors but also flowering time and rosette formation.

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