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ORIGINAL PAPER

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# The GRAS protein SCL13 is a positive regulator of phytochrome-dependent red light signaling, but can also modulate phytochrome A responses

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**Abstract** Phytochrome photoreceptors enable plants to perceive divergent light signals leading to adaptive changes in response to differing environmental conditions. However, the mechanism of light signal transduction is not fully understood. Here we report the identification of a new signaling intermediate from *Arabidopsis thaliana*, Scarecrow-like (SCL)13, which serves as a positive regulator of continuous red light signals downstream of phytochrome B (phyB). *SCL13* antisense lines exhibit reduced sensitivity towards red light, but only a distinct subset of phyB-mediated responses is affected, indicating that SCL13 executes its major role in hypocotyl elongation during de-etiolation. Genetic evidence suggests that SCL13 is also needed to modulate phytochrome A (phyA) signal transduction in a phyB-independent way. The SCL13 protein is localized in the cytoplasm, but can also be detected in the nucleus. Overexpression of both a nuclear and cytoplasmic localized SCL13 protein leads to a hypersensitive phenotype under red light indicating that SCL13 is biologically active in both compartments. SCL13 is a member of the plant-specific GRAS protein family, which is involved in various different developmental and signaling pathways. A previously identified phytochrome A signaling intermediate, PAT1, belongs to the same subbranch of GRAS proteins as SCL13. Although both proteins are involved in phytochrome signaling, each is specific for a different light condition and regulates a different subset of responses.

**Keywords** Phytochrome · phyB · Light · Signal transduction · GRAS-proteins · *Arabidopsis thaliana*

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## Introduction

The possibility to perceive and to respond to changing environmental conditions is an absolute requirement for the survival of phototrophic organisms. In order to detect ambient light conditions and to adapt to it, plants make use of several classes of photoreceptors and corresponding downstream signaling pathways (for a recent review, see Chen et al. 2004). One of the best studied photoreceptor families are the phytochromes (Smith 1999; Quail 2002; Schäfer and Bowler 2002; Gyula et al. 2003). These receptors sense the red:far-red ratio in the surrounding light environment and allow plants to adjust their growth and development accordingly. The *Arabidopsis thaliana* genome encodes five phytochrome (phyA–E) proteins (Clack et al. 1994). They are present as dimeric chromoproteins with a linear tetrapyrrol chromophore attached and are capable of photoreversible conformational changes between the red-light (Pr) and the far-red light (Pfr) absorbing form (Quail 1997; Nagy and Schäfer 2002). Upon light perception the Pfr form of phytochromes, which is considered the active form, has been shown to migrate into the nucleus (Nagy and Schäfer 2000; Kircher et al. 2002; Nagatani 2004). The ensuing signaling cascade leads to a modulation of gene expression triggering downstream events. Whereas phyA predominates in the etiolated tissues as it is light labile, phyB–E are light stable and found mainly in green tissues (Quail 1997). Studies of mutants lacking functional photoreceptors have established that the five Arabidopsis phytochromes have different, but also partially redundant and overlapping functions. These photoreceptors are needed for many responses during plant development

including seed germination, de-etiolation, internode elongation and flowering time (Sullivan and Deng 2003). PhyA is necessary for the de-etiolation process under very low (VLFR) and under high fluences (HIR) of far-red (FR) light and is the primary sensor of FR light (Furuya and Schäfer 1996; Smith 1999). PhyB, and to a lesser extent phyC, D and E are responsible for red (R) light responses under low fluences (LFR) and characterized by their red/far-red reversible induction responses. PhyB (and to minor part phyD and E) is important for the adaption of plants to changing R:FR ratios, caused for example by light reflected from neighboring plants or shading from canopies (Smith 2000; Aukerman et al. 1997; Devlin et al. 1998, 1999; Hennig et al. 2002; Franklin et al. 2003a, b; Monte et al. 2003). The shade-avoidance syndrome is a phenomenon of adaptive growth to such conditions evoking survival responses such as rapid elongation to evade competing vegetation and acceleration of flowering (Smith and Whitelam 1997; Morelli and Ruberti 2002; Franklin and Whitelam 2005). Mutants lacking phyB have an apparent phenotype during the entire life-cycle resembling plants with the shade-avoidance syndrome: in the seedling stage the hypocotyl is elongated under white and red light; in the adult stage the plants are pale and spindly, with long petioles and flower early (Reed et al. 1993; Devlin et al. 1996).

Genetic screens and yeast two-hybrid assays have suggested how the red light-triggered phytochrome signal may be transmitted. Many proteins that have been identified as signaling intermediates are transcription factors: basic helix-loop-helix proteins such as PIF1/PIL5, PIF3, PIF4, PIF5/PIL6 and PIL1, most of which can directly interact with phytochromes (for a review see Duek and Fankhauser 2005); the leucine zipper proteins HY5 and HYH (Oyama et al. 1997; Holm et al. 2002); homeobox proteins such as ATHB2, which is involved in the shade-avoidance response (Steindler et al. 1999); MYB factors such as CCA1 and LHY and transcription factors with DOF domains such as COG1 and OBP3 (Wang et al. 1997; Schaffer et al. 1998; Park et al. 2003; Ward et al. 2005). Furthermore, several response/pseudo-response regulators such as ARR4, TOC1/APRR1, APRR3 and APRR9 are also implicated in these processes (Strayer et al. 2000; Sweere et al. 2001; Nakamichi et al. 2005). Several of the intermediates that transduce red light signals also act downstream of other photoreceptors such as phyA or cryptochrome (e.g., PKS1, COG1, OBP3, FyPP, PIF3, NDPK2, HY5, HYH; Fankhauser et al. 1999; Ward et al. 2005; Kim et al. 2002; Zhu et al. 2000; Choi et al. 1999; Oyama et al. 1997; Holm et al. 2002). Other phyB signaling intermediates are known to regulate hormone signaling pathways. For example, RED1, HY5, NDPK2 and SHY2/IAA3 modulate the auxin signaling pathway, whereas ARR4 is involved in cytokinin responses (Hoecker et al. 2004; Tian et al. 2002; Fankhauser 2002; Choi et al. 2005). In addition, many Arabidopsis mutants that exhibit defects in photomorphogenesis during de-etiolation in response to red light are also affected in circadian clock function (Fankhauser and Staiger 2002; Millar 2003). These include *elf3* (Reed et al. 2000), *gigantea* (*gi*; Huq et al. 2000), *srr1* (Staiger et al. 2003), *ztl/ado/lkp* (Somers et al. 2000) and *toc1* (Mas et al. 2003).

We have previously reported the isolation of the Arabidopsis mutant *phytochrome A signal transduction (pat)1-1* that is specifically disrupted in the phyA signaling pathway (Bolle et al. 2000). PAT1 is a member of the plant-specific GRAS protein family that regulates diverse aspects of plant development (Bolle 2004). The family name is derived from the first three members to be cloned, *G*AI, *RGA* and *S*CR (Pysh et al. 1999). Some GRAS proteins are involved in developmental processes such as meristem formation and maintenance (e.g., LS/LAS, HAM; Schumacher et al. 1999; Greb et al. 2003; Stuurman et al. 2002) or radial patterning (e.g., SCR, SHR; Di Laurenzio et al. 1996; Helariutta et al. 2000), whilst others are involved in signal transduction pathways. Besides PAT1, which is involved in phyA-specific signaling, members of the DELLA protein subbranch (GAI, RGA, RGL1-3) are negative regulators of the gibberellin signal transduction (Peng et al. 1997; Silverstone et al. 1998). The GRAS protein family is relatively large with at least 33 identified ORFs in the Arabidopsis genome (Bolle 2004; Tian et al. 2004). Sequence alignment and phylogenetic analysis reveal several subfamilies. Four proteins in Arabidopsis show high similarity to PAT1, namely SCL1, SCL5, SCL13 and SCL21. Because of these similarities we reasoned that perhaps all proteins of the PAT1 branch may be involved in light signaling pathways.

Here we report the physiological and cell biological analysis of Scarecrow-like (SCL)13, a member of the PAT1 subbranch. We show that SCL13 is indeed important for light signal transduction, with a specificity for red light.

## Materials and methods

### Plant material and growth conditions

All lines used in this study are in the *A. thaliana* Columbia background (Col-0). Seeds were surface-sterilized for 10 min in 30% (v/v) commercial bleach with the addition of 0.05% Triton X-100, rinsed at least three times and sown on Petri dishes (11 cm diameter) containing half-strength Murashige and Skoog basal medium (Sigma, St. Louis) and 0.8% (w/v) agar. To select transgenic lines the medium was supplemented with kanamycin or Basta and 3% sucrose. Unless used for germination assays, plates with sterilized seeds were stored at 4°C for 3 days and germination was induced by 2 h of white light followed by 22 h of darkness at 21°C. After this treatment plates were transferred into the appropriate light conditions. Light intensities were determined with spectroradiometers (W, B and R light: model Li-1800, LiCor, Lincoln, NE; FR light: model SKP200 with a sensor for 730 nm,

Skye Instruments, UK). The blue, red and FR light sources were generated by LED using diodes with a maximum at 469, 660 or 740 nm, respectively (Quantum Devices, Barneveld and PVP GmbH, Willich).

## Physiological measurements

For fluence response experiments seedlings were grown under appropriate light conditions for 4 days. All lines were analyzed in parallel. The experiments were repeated at least three times and each time a minimum of 50 seedlings were analyzed. Hypocotyl and petiole lengths and cotyledon sizes were documented using a digital camera (Coolpix 700, Nikon, Tokyo) and measured with the NIH Image software (ImageJ, National Institutes of Health, Bethesda, MD, USA). Data derived from hypocotyl length assays were subjected to statistical analysis included in Microsoft Office Excel 2003 using ANalysis Of VAriance (ANOVA)-tests to verify hypotheses about differences between two mean values. Differences were assumed to be insignificant when the *P* values associated with these tests exceeded 0.05.

Germination assays were performed according to Shinomura et al. (1996). Briefly, seeds were sterilized and plated on half-strength MS medium without sucrose. After plating, the seeds were pulsed with FR light (1 μmol $m^{-2}$ $s^{-1}$) for 10 min and transferred to D. To test for red light responsiveness seeds were illuminated again after 3 h with R light (5 μmol $m^{-2}$ $s^{-1}$) for 10 min. To test for FR light responsiveness, a FR light pulse (1 μmol $m^{-2}$ $s^{-1}$) was given after 48 h. After the appropriate light pulse the seedlings were kept in darkness for 6 days and germination was scored positive as soon as the radicle was visible. Germination efficiency was normalized for seeds that could germinate without the second light pulse and seeds that did not germinate under W light.

To test for the EOD FR light response, seedlings were sown on 1× MS agar-plates containing 1% sucrose and kept under short-day conditions (8 h light, 16 h dark). One plate was subjected to an additional 10-min-long FR light pulse (1 μmol $m^{-2}$ $s^{-1}$) just before the dark period, whereas the control plate was not. After 7 days hypocotyl lengths were measured.

For chlorophyll measurements triplicates of 20 seedlings each were extracted with 1 ml of 80% acetone overnight in the dark. The OD of the solution was determined at 647 and 663 nm, and the chlorophyll content was calculated (μg/μl chlorophyll = $18.71 \times OD_{647} + 7.15 \times OD_{663}$).

For determination of flowering time, lines were grown at 22°C under long (16 h light, 8 h dark) or short (8 h light, 16 h dark) day conditions. Plants were considered to be flowering upon emergence of a visible floral bud.

## Transgenic plants

To generate *SCL13* antisense plants, a portion of the *SCL13* cDNA was amplified with *Xba*I-*Kpn*I adaptors at the 5′- and 3′-end (5′-GCTCTAGAATGGAAGCCACAG-TCAAAATATTC-3′; 5′-GGTACCTCATTCTGACCCTCCATTTC-3′). The resulting PCR fragment was cloned into the appropriate sites of a binary vector (van der Krol and Chua 1991) in the reverse orientation under the control of a 35S CaMV promoter. For overexpression, full-length *SCL13* cDNA was amplified and cloned into a binary vector containing a basta-resistance gene (Kost et al. 1998). To generate the *SCL13-promoter-GUS* fusions, the *SCL13* promoter fragments were amplified and cloned into the pENTR/D-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA). For directed cloning a 5′-CACC-extension was added to the forward primers. Two DNA fragments containing the SCL13 5′ upstream sequences were used. The 5′ end of both fragments was located at 2,514 bp upstream of the ATG start codon (5′-CACCGTCTGTCTCTTCTCTGGTAC-3′). One fragment had its 3′ end located at the beginning of the 5′-UTR, 882 bp upstream of the ATG start codon leading to the promoter fragment lacking the 5′-UTR and the intron therein (5′-GCTGAAGAAATTTTGTTGAATGGG-3′), whereas the 3′ end of the other fragment was located 117 bp downstream of the ATG generating the *SCL13-promoter-5′-UTR* construct (5′-CCAGCAATACACTACACAGCTC-3′;). The 5′ end of the third construct, which contained only the 5′-UTR with the intron sequence began at the predicted transcription start site, 908 bp upstream of the ATG (5′- CACCTCCC-ATTCAACAAAATTTCTTCAG-3′) and the 3′ end was located 117 bp downstream of the ATG. With the help of the LR Clonase (Invitrogen) the fragments were combined into the Gateway-adapted vector pKGWFS7 (Karimi et al. 2002), which contained the coding sequence of GFP and GUS downstream of the insertion site.

NES and NLS elements with the amino acid sequence "DTAGLLEKLTVEE" and "APKKKRKV", respectively, were cloned with *Kpn*I/*Not*I adaptors into the pENTR4 vector (Invitrogen). The NES sequence is derived from the Arabidopsis Ran-binding proteins (Kim and Roux 2003), whereas the NLS sequence originates from the large T antigen of Simian virus 40 (Benditt et al. 1989). The coding sequence of *SCL13* was cloned in frame into the *Sal*I/*Kpn*I sites creating a N-terminal fusion to the tag. With the help of the LR Clonase (Invitrogen) the fragments were combined into the Gateway-adapted vector pKGWFS7, to which a 35S promoter fragment had been added 5′ of the insertion site (Karimi et al. 2002). All constructs were verified by restriction analysis and sequencing.

Constructs were transformed into *A. thaliana* plants via the *Agrobacterium tumefaciens* floral dip method (Clough and Bent 1998). Transformants were selected on kanamycin or Basta-containing media, respectively, self-fertilized and homozygous progeny was selected.

## Generation of SCL13-ASxphyA and SCL13-ASxphyB lines

Because of the co-segregation of the *SCL13* antisense construct with the kanamycin resistance marker, kana-

mycin selection was used to isolate F1 seedlings after crossing *phyA* and *phyB* mutants with the two *SCL13* antisense lines, which showed the strongest reductions in RNA levels. Seedlings were selfed and for the *SCL13-ASxphyA* cross homozygous *phyA* mutant-like seedlings were selected from the F2 population under FR light, whereas for the *SCL13-ASxphyB* cross homozygous *phyB* mutant-like seedlings were selected under red light. These lines were then plated on kanamycin to select for the transgene. The reduction of *SCL13* mRNA was confirmed via RT-PCR.

### RNA analysis

For the analysis of *SCL13* transcript levels a reverse transcription reaction was performed with total RNA extracted from WT and *SCL13* antisense lines. An oligo$(dT)_{18}$ primer was hybridized to 1 µg of total RNA. Reverse transcription was performed with the Omniscript RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. After the RT reaction, 1 µl of the cDNA was used for the PCR reaction with gene-specific primers for *SCL13* (5′-CTCCCATTCAACAAAATTT CTTCA-3′, 5′-CCAGCAATACACTACACAGCTC-3′). To be able to discriminate between genomic DNA and cDNA amplification products the 5′-forward primer was located 5′ of the intron in the *SCL13* leader, which also prevented amplifying the antisense construct. PCR reactions were stopped after 25 and 30 cycles and the reaction mixtures analyzed on agarose gels. Each PCR was repeated for three times. Amplification of 18S cDNA (20 cycles) was used as normalization controls (primers: 18S rRNA-f 5′-GCTCAAAGCAAGCCTA-CGCTCTGG-3′, 18S rRNA-r 5′-GGACGGTATCTGATCGTCTTCGA GCC-3′).

To determine the 5′ transcription start site a cDNA library, which was generated by fusion of linkers to the 5′- and 3′-ends of cDNAs (Marathon cDNA Amplification Kit, BD Biosciences Clontech, San Jose, CA, USA), was used for rapid amplification of cDNA ends (RACE) according to the manufacturer's instructions. Because of discrepancies with the published Arabidopsis sequence, both the cDNA and the genomic DNA were resequenced and the correct sequences deposited at the Arabidopsis database.

### Cellular and subcellular localization

The *SCL13* open reading frame was amplified by PCR from cDNA using primers containing restrictions sites for *Xba*I and *Kpn*I and inserted into the pGFP vector (Kost et al. 1998) generating an *SCL13-GFP* fusion driven by the *35S CaMV* promoter. Onion epidermis cells were bombarded with this construct using a helium biolistic gun and incubated in darkness for 12 h. To test for possible effects of light, the cells were subsequently incubated for 3 h under either R or WL. A *35S-GFP* construct was used as a control. To visualize GFP fluorescence cells were examined using an Axioskop microscope (Carl Zeiss).

For GUS staining transgenic seedlings were incubated for 3–24 h in 0.1 M phosphate buffer, pH 7.0, containing 0.1% Triton X-100 (v/v), 10 mM EDTA, 0.5 mM ferrocyanide, 0.5 mM ferricyanide and 0.125 mM X-Gluc (Roth, Karlsruhe). Chlorophyll was removed with 70% ethanol and the blue staining analyzed with a microscope.

### Sequence analysis

Alignment of sequences was performed with the ClustalW program (DNAStar, MegAlign 6.1). The phylogenetic tree was generated with the help of the PHYLIP program 3.6 (http://www.evolution.genetics.washington.edu/phylip.html) using SEQBOOT for bootstrapping (100), PROTDIST and NEIGHBOR analysis. The phylogenetic tree was produced with DRAWTREE.

### Analysis of microarray data

The original data published by the AtGenExpress consortium (http://www.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm) was evaluated according to the analysis performed at NASC (http://www.affymetrix.arabidopsis.info). Values not marked with P were discharged. A mean value was generated from the triplicates and standard deviation was calculated. Furthermore, the data were compared using the Genevestigator program (Zimmermann et al. 2004).

## Results

### SCL13 is part of the PAT1-branch of the GRAS proteins

Phylogenetic tree analysis showed that the *A. thaliana* SCL13 (AtGRAS-24; At4g17230) protein is a member of the PAT1-branch of the GRAS protein family (Bolle 2004; Tian et al. 2004, Fig. 1a). PAT1 was previously isolated as a component of the phytochrome A signal transduction pathway (Bolle et al. 2000). Members of the PAT1-branch have an overall identity to other GRAS proteins of 15–26%, whereas within this group the identity is increased to between 37 and 66%. Nevertheless, the signature motifs described for GRAS proteins are present in all members of the PAT1-branch. *SCL13* encodes a putative protein of 529 amino acid residues, with a more variable N-terminal and a conserved C-terminal domain compared to other members of this branch. The conserved C-terminal part of the protein contains the signature motifs defined for GRAS proteins, namely two leucine-rich (LR) domains (for SCL13: LRI: L-$(X)_{13}$-L-$(X)_7$-L-$(X)_3$-L-$(X)_{10}$-L-$(X)_9$-L-$(X)_6$-L; LRII: L-(X)-L-$(X)_4$-L-$(X)_2$-L-$(X)_{24}$-L-(X)-L-$(X)_{13}$-L-$(X)_{16}$-L-L-(X)-L-$(X)_3$-L) flanking a conserved domain around the amino acid residues V/I HIID (Fig. 1b). Furthermore, the motifs PFYRE, RVER, SAW and a putative tyrosine phosphorylation site, [R]-X(2)-[E]-X(3)-Y (Patschinsky et al. 1982), are highly conserved. Besides these motifs, whose func-

tional implications are still unknown, no other functional domains could be determined.

As more sequences from plant species other than Arabidopsis became available, we searched for potential homologs of *SCL13* in other organisms. Two cDNA clones, one from lotus (*Lotus japonicus, LjSCL13*; TM0367.12 50320899-50319265; http://www.kazusa.or.jp) and one from poplar (*Populus trichocarpa, PtSCL13*; LG_XVI, 236419-238053; genome.jgi-psf.org) have higher sequence similarity to SCL13 than to any other member of the GRAS protein family (54 and 59% identity, respectively, Fig. 1). Surprisingly, although rice and maize have more GRAS proteins than Arabidopsis, no SCL13 homolog could be identified, even though there are at least five rice and seven maize GRAS proteins in the PAT1 branch (Tian et al. 2004; Lim et al. 2005).

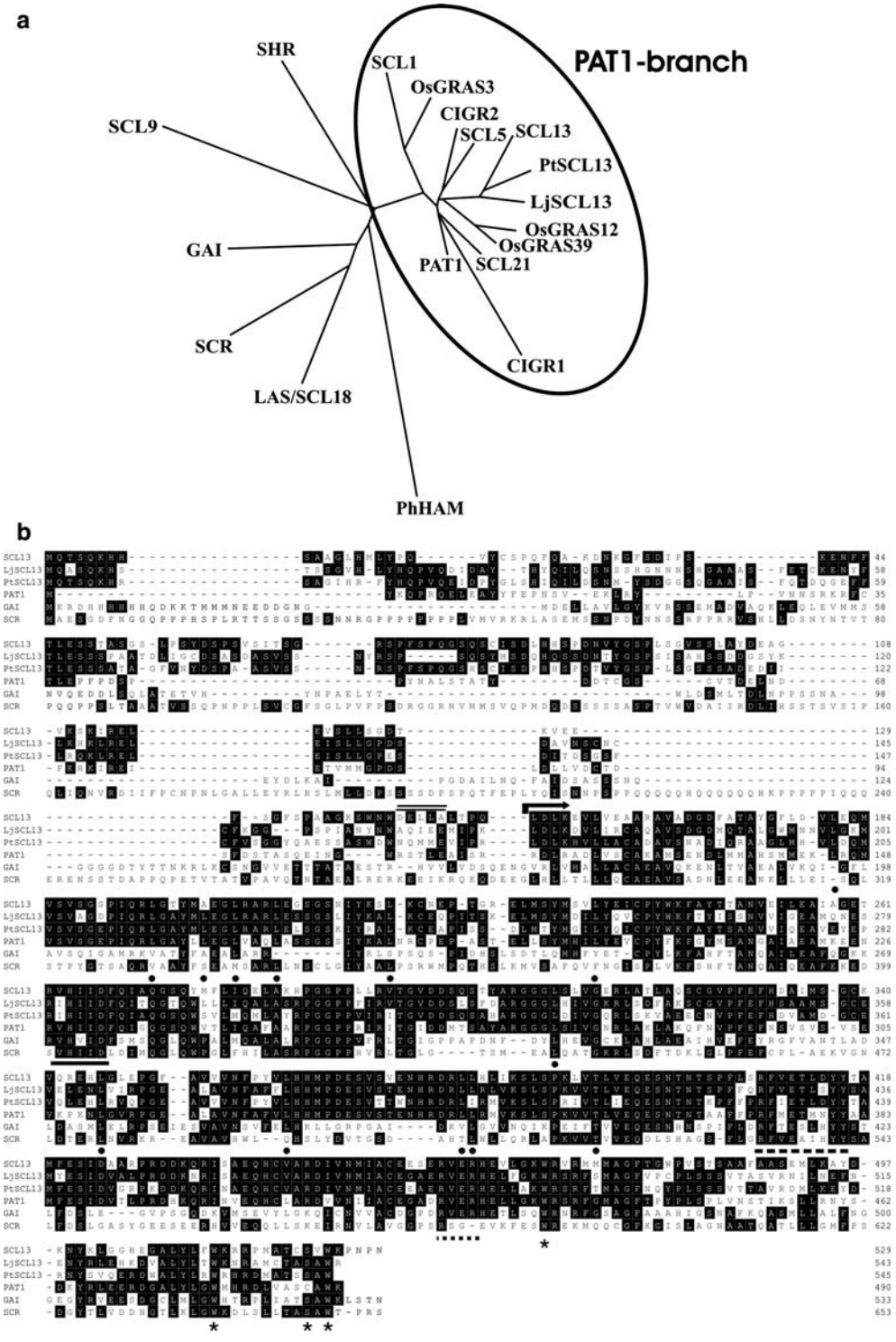


**Fig. 1** SCL13 is a member of the PAT1-branch of the GRAS protein family. **a** Neighbor-joining tree of the GRAS protein family. Representatives of all major branches of the GRAS family are shown (GAI, SCR, LAS/SCL18, SCL9, SHR, PhHAM). All Arabidopsis proteins in the PAT1 cluster and all rice homologs of the PAT1 branch are indicated (Os3, 12 and 39, CIGR1, CIGR2) along with the closest homologs of SCL13 in poplar (*Populus trichocarpa*; PtSCL13; LG_XVI 236419-238053) and lotus (*Lotus japonicus*; LjSCL13; TMO367.12). The tree was generated with the PHYLIP program. **b** Alignment of the amino acid sequences of SCL13 with LjSCL13, PtSCL13, PAT1, GAI and SCR. Conserved sequences are *shaded*. Gaps introduced to facilitate alignment of conserved residues are indicated as *dashes* in the sequence. Leucine residues in the leucine-rich domains are indicated with a *black circle*. Conserved motives such as the VHIID domain (*solid*), the putative tyrosine phosphorylation site (*dashed*) and the RVER motif (*dotted*) are *underlined*. *Asterisks* indicate the conserved amino acids of the SAW motif. The cryptic DELLA-sequence in SCL13 is marked with a *double line* above. The *arrow* marks the beginning of the conserved C-terminal part of the protein

We used the RACE technique to isolate full-length Arabidopsis *SCL13* cDNAs. Sequence analysis revealed an unusually large intron of 750 bp in the 5′-UTR. By contrast, the coding region is not interrupted by any intron, a feature common to most GRAS protein encoding transcripts in Arabidopsis. Furthermore, we found a misannotation of the *A. thaliana* sequencing project owing to two frameshifts caused by missing nucleotides. The difference of our cDNA sequence compared to the sequences released by the Arabidopsis sequencing project has been confirmed on the cDNA and genomic levels.

## Generation of transgenic lines with reduced SCL13 function

Insertion mutants for *SCL13* are presently not available for this locus. To address the question if SCL13 is involved in light signaling, we generated several antisense lines for *SCL13* to decrease its mRNA accumulation and to determine a partial loss-of-function phenotype.

Several independent antisense lines were used in this study. Overexpression of the antisense RNA was confirmed by Northern blot analysis and the reduction of the endogenous *SCL13* mRNA was determined by semi-quantitative RT-PCR (Fig. 2a). The reduction of *SCL13* mRNA varied between 20 and 90%, but none of the transgenic lines showed a complete loss of expression. Both seedlings under red light and adult plants showed a similar reduction of the mRNA levels. To verify that only the targeted mRNA was reduced and not other related *SCL* transcripts, the Northern blots were re-hybridized with probes specific for the closest-related family members (SCL1, SCL5 and PAT1). The expression levels of the tested *SCL* genes tested were not affected (data not shown).

## Inhibition of hypocotyl elongation under red light conditions is specifically impaired in *SCL13* antisense lines

Two independent antisense lines, *SCL13-AS1* and *SCL13-AS2*, which showed the strongest reduction in *SCL13* expression, were used for physiological analysis. The hypocotyl elongation of transgenic *SCL13* antisense lines were analyzed under all major light regimes. We found a reduced inhibition of hypocotyl elongation compared to wild type (WT) when transgenic seedlings were grown under continuous red (R) light for 4 days. These differences were statistically significant for both lines ($P < 0.01$). This result suggests the involvement of SCL13 in the phytochrome B, C, D or E signaling pathways (Fig. 2b, c). In addition, a marginally elongated hypocotyl could be observed under FR light conditions, which proved not to be statistically significant at the 95% confidence level. When grown under blue (B) or white (W) light seedlings of the antisense lines were indistinguishable from WT, indicating that SCL13 is specific for red light signaling and not a general regulator of light responsiveness. The antisense lines also had normal growth responses in darkness (D) establishing that the phenotype is light-dependent (Fig. 2b). Fluence rate response curves with different intensities of R light were used to quantitatively characterize the sensitivity toward R. Our results confirmed that *SCL13* antisense lines are taller than WT seedlings at all fluence rates tested, therefore showing reduced sensitivity to red light, but not as strongly as in a *phyB* mutant (Fig. 2c). The hypocotyl length of the *SCL13-AS3* line, which exhibits a higher residual accumulation for *SCL13* mRNA as *SCL13-AS1* and *2*, is more sensitive to red light under higher fluence rates. Therefore, we were able to correlate the reduction of *SCL13* mRNA with the severity of the phenotype. These findings indicate that SCL13 plays a role in seedling de-etiolation processes under prolonged red light.

## SCL13 regulates a specific subset of red light-dependent responses

The involvement of SCL13 in the red light signaling pathway prompted us to further analyze other phytochrome-related phenotypes in these lines. Because phytochrome B (phyB) is the major red light photoreceptor in Arabidopsis, we tested *SCL13* antisense lines mainly for typical phyB-mediated responses besides hypocotyl elongation.

The germination efficiency of seeds that have been imbibed for 3 h and then illuminated with a 10-min R light pulse is reduced in a *phyB* mutant compared to the induction with a W light pulse (Shinomura et al. 1996). In the *SCL13* antisense lines the germination rate was not as low as in *phyB* mutants, suggesting that germination efficiency is not or only marginally affected by a reduction of SCL13 (Fig. 3a). As a control phyA-dependent germination of seeds imbibed for 48 h and illuminated with a 10 min FR light pulse was tested. All lines, besides *phyA*, were able to germinate efficiently.

Cotyledon expansion is controlled by continuous red light. To test if SCL13 has any effect on this response we measured the cotyledon size of transgenic *SCL13* antisense lines and WT under higher R light fluence rates (1 μmol $m^{-2}$ $s^{-1}$). *PhyB* seedlings have very small and only partially unfolded cotyledons under these conditions, but the *SCL13* antisense lines were not affected in their size and the cotyledons unfolded similar to WT (Fig. 3b). Also under non-saturating red light conditions (0.1 μmol $m^{-2}$ $s^{-1}$) the size of the cotyledons did not vary significantly from WT. It seems that the effect of red light is stronger on hypocotyl elongation than on cotyledon expansion.

A saturating pulse of FR light given at the end of the day induces enhanced hypocotyl elongation in Arabidopsis (Robson et al. 1993; Aukerman et al. 1997; Franklin and Whitelam 2005). This end-of-day far-red (EOD FR) response is greatly diminished in *phyB, phyD* and *phyE* mutants and is a way to assess shade-avoidance responses and to determine how plants can react to changing R:FR ratios. We analyzed the hypocotyl elongation in short-day environment with or without an

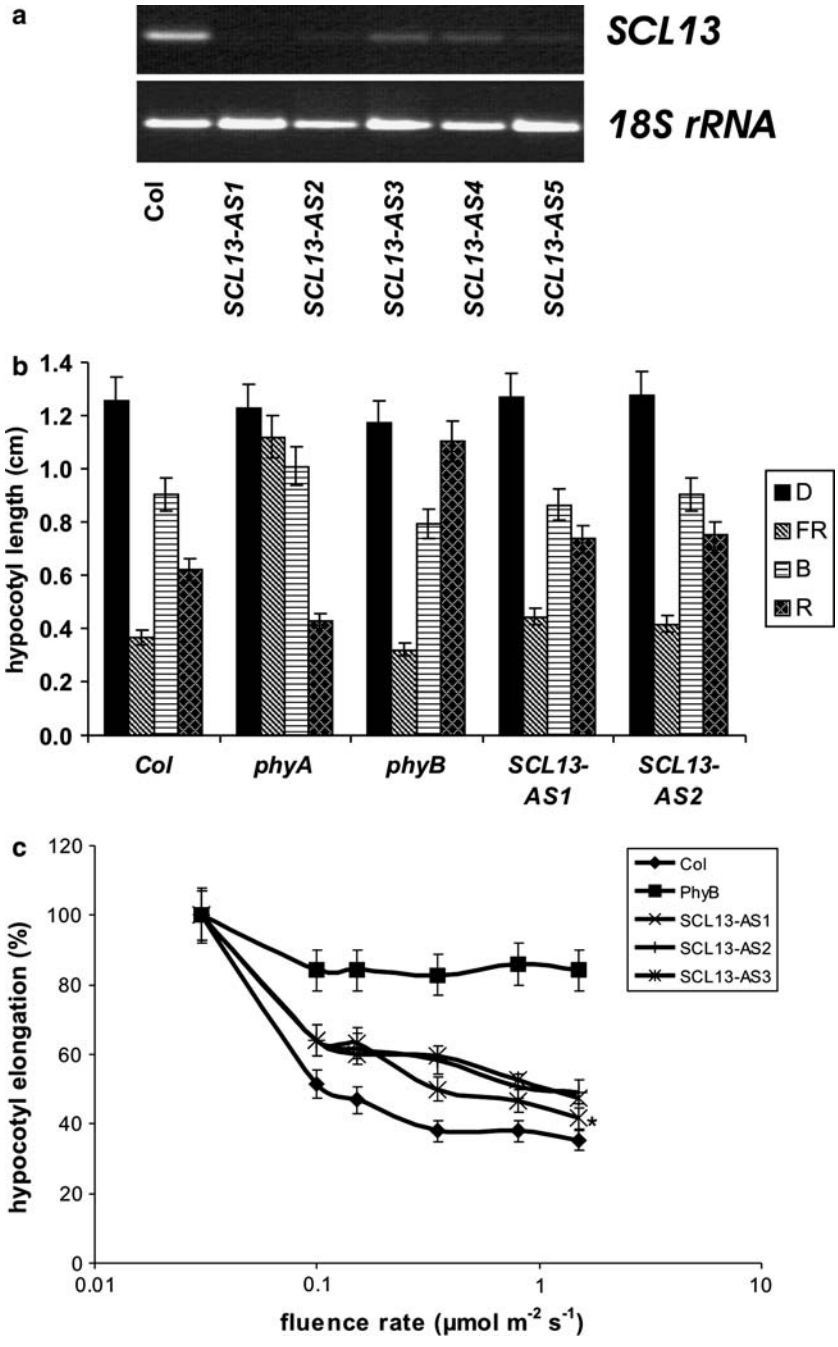


**Fig. 2** Transgenic *SCL13* antisense lines show a reduction in the endogenous *SCL13* mRNA and a reduced inhibition of hypocotyl elongation under red light. **a** RT-PCR for *SCL13* expression levels was performed on total RNA from 4-day-old plants grown under continuous red light (0.5 µmol $m^{-2}$ $s^{-1}$). The expression levels in five *SCL13* antisense lines are shown in comparison to WT (Col). Control PCR using primers amplifying the 18S rRNA gene showed that equal amounts of reverse transcribed RNA were used. **b** Hypocotyl length of 4-day-old seedlings grown in darkness (*D*), or under continuous far-red (*FR*; 0.5 µmol $m^{-2}$ $s^{-1}$), red (*R*; 1 µmol $m^{-2}$ $s^{-1}$) and blue (*B*; 5 µmol $m^{-2}$ $s^{-1}$) light. **c** Fluence rate response curve for hypocotyl elongation under red light of WT (Col), *phyB* null mutant and *SCL13-AS1, 2* and *3*. Hypocotyl length in *D* was considered 100%. More than 20 seedlings were used per replicate ($n = 3$). The *asterisk* indicates an insignificant difference from the wild type by Anova test ($P > 0.05$), all other values were significantly different from WT ($P < 0.01$). *Error bars* = standard deviation

additional FR light pulse at the end of each day. The *SCL13* antisense lines behaved similar as WT being twofold longer with an additional FR light pulse, whereas a *phyB* mutant showed equally elongated hypocotyls under both conditions (Fig. 3c).

Consistent with their role in shade avoidance, *phyB* mutants have elongated petioles and hypocotyls also under W light conditions. This effect has often been described as "constitutive shade-avoidance response" (for review see Franklin and Whitelam 2005). No elongation of petiole and hypocotyl could be detected in *SCL13* antisense lines compared to WT. Even under dim white light, which leads to a weak stimulation of all photoreceptors, no significant difference to WT could be observed (Fig. 3d).

To determine whether *SCL13* antisense lines can accumulate chlorophyll to the same extent as WT, we examined 7-day-old seedlings grown under W light conditions. Unlike *phyB* the antisense lines like WT seedlings displayed no reduction in greening and chlorophyll accumulation. Furthermore, we assessed the accumulation of chlorophyll in etiolated seedlings, exposed to 24 h of R light. In the *phyB* mutant this leads to reduced chlorophyll accumulation whereas in the WT and *SCL13* antisense lines chlorophyll accumulation levels were comparable.

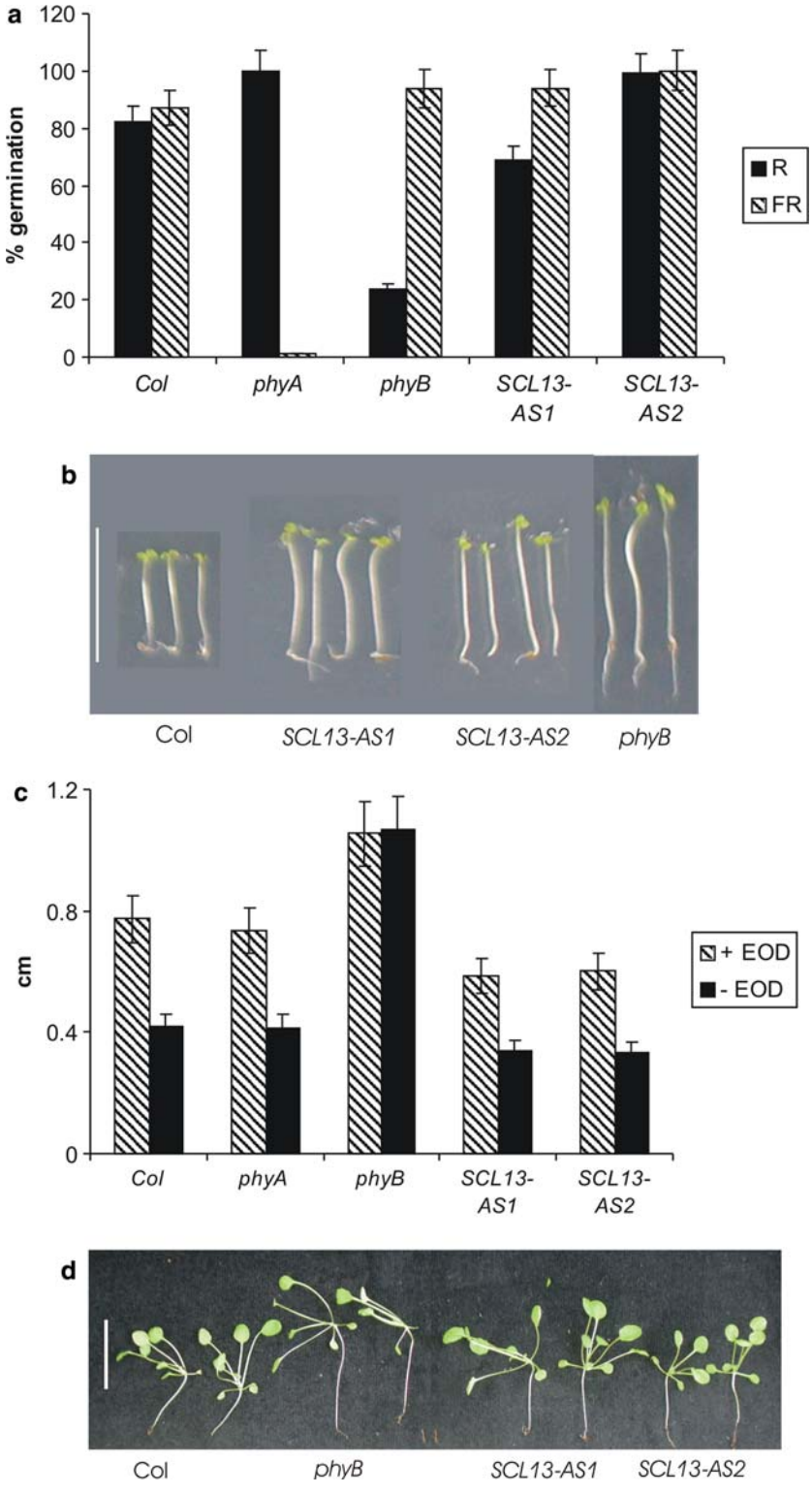


**Fig. 3** Physiological analysis of the *SCL13* antisense lines. **a** Germination assays of WT (Col), *SCL13-AS1* and *SCL13-AS2*, *phyA* and *phyB* mutants. Seeds were treated with a FR light pulse after sterilization. Treated seeds were irradiated with a 10-min R light pulse (5 µmol $m^{-2}$ $s^{-1}$; *R*) after 3 h or a 10-min FR light pulse (1 µmol $m^{-2}$ $s^{-1}$; *FR*) after 48 h. Germination was assessed after 7 days in darkness. More than 80 seedlings were used per replicate ($n = 3$). **b** Phenotype of *SCL13-AS1* and *SCL13-AS2* after 4 days under continuous *R* light conditions (1 µmol $m^{-2}$ $s^{-1}$) in comparison with *phyB* and WT (Col). *Bar* is 1 cm. **c** Effect of EOD-FR treatments on hypocotyl elongation in *SCL13-AS* lines. Seedlings were kept for 7 days under short-day conditions in white light. One population (+EOD) was treated with a 10-min FR light pulse (1 µmol $m^{-2}$ $s^{-1}$) before the dark period, whereas the other was not (-EOD). More than 30 seedlings were used per replicate ($n = 3$). *Error bars* = standard deviation. **d** Phenotype of *SCL13-AS1* and *SCL13-AS2* after 9 days in low white light conditions (long day) in comparison with *phyB* and WT (Col). *Bar* is 1 cm

Previous studies have shown that *phyA* mutants have a delayed onset of flowering whereas *phyB* mutants flower earlier (Reed et al. 1993). Indeed, *SCL13* antisense lines flowered about 3 days earlier than WT under long-day conditions, but not as early as a *phyB* mutant (about 4–6 days; Table 1). Also under short-day conditions it can be observed that *SCL13* antisense lines flowered earlier then WT. In this case SCL13 antisense lines flowered around 25 days earlier than WT whereas *phyB* flowered around 36 days earlier. However, no effect could be detected on the development and height of the inflorescence stem.

To investigate the molecular phenotype of *SCL13* antisense lines we performed Northern blot analysis on

**Table 1** SCL13-AS lines flower earlier under long-day (LD) and short-day (SD) conditions

| | Flowering time under LD | Flowering time under SD |
|---|---|---|
| Col | 22.0 ± 2 | 52.3 ± 8 |
| *phyB* | 16.8 ± 2 | 15.8 ± 5 |
| *SCL13-AS* | 19.0 ± 2 | 27.3 ± 6 |

Days to bolting under LD and SD conditions for WT (Col), *phyB* and *SCL13-AS* lines

several selected marker genes regulated by phytochromes including genes coding for the major light-harvesting chlorophyll protein of thylakoid membranes (*CAB*), the chalcone synthase (*CHS*) and the small subunit of Rubisco (*RBCS*). However, we did not find any significant difference between WT and the transgenic lines in response to red light (data not shown). Taken together, these data suggest that reduced levels of SCL13 affect only a very specific subset of physiological responses.

Besides the flowering phenotype no striking influence of SCL13 was observed on any aspect of a plant's adult phenotype compared to WT. We tested the lines for phenotypes which have been described in other *GRAS* mutants. No effect on root development, gravitropism, branching and height could be observed. Germination was assayed on paclobutrazol, an inhibitor of GA biosynthesis, to see if SCL13 has any function in GA signaling, especially that a cryptic "DELLA" motif (albeit with different flanking regions) could be identified within the N-terminal part of the protein (Fig. 1b). Nevertheless, germination was inhibited as strongly as in WT.

## Phytochrome B is needed for SCL13 action

As the *SCL13* antisense lines have a hyposensitive phenotype under continuous red light, we crossed the antisense lines into *phyA* and *phyB* null mutants to analyze whether phyA and phyB are required for the phenotype of these antisense lines.

Figure 4a shows that the *SCL13-ASxphyA* line is still less sensitive to red light compared with *phyA*, suggesting that either phyA is not required or only plays a minor role in the phenotype of the *SCL13* antisense phenotype under red light. Furthermore, germination after a R light pulse and the EOD-FR response were similar in WT and the *SCL13-ASxphyA* lines. The hypocotyl length of the *SCL13-ASxphyB* line was statistically indistinguishable from the monogenic *phyB* mutant seedling when grown in R light conditions ($P > 0.05$) and the EOD-FR response was reduced similar to the *phyB* mutant itself. The effects of the loss-of-function of SCL13 in *phyB* mutants under red light seems to have no additive effect. These data indicate that hypocotyl responsiveness to continuous red light is fully dependent on phyB.

## SCL13 modulates phyA signaling

Interestingly, under FR light conditions, the *SCL13-ASxphyB* lines showed an increase in hypocotyl elongation compared to WT ($P < 0.05$; Fig. 4b, c). This suggests that in the absence of phyB, SCL13 can act downstream of phyA or at least modulate phyA signals. Cross-talks between phyA and phyB signaling have been reported previously (Cerdan et al. 1999; Hennig et al. 1999, 2001; Cerdan and Chory 2003).

In a *SCL13* antisense line in a *phyA* background the *phyA* phenotype dominated under FR light. This was not only true for the hypocotyl elongation but also for germination efficiency under FR light. This result suggests that SCL13 is not necessary for phyA signaling, whereas it can modulate hypocotyl length under FR light in a phyB-independent way, as phyB is not activated by FR light. Together, these data indicate that SCL13 might be able to antagonize phyA function in continuous FR.

## *SCL13* gene expression is upregulated in green tissues

To test for the expression pattern of *SCL13* under different conditions, we performed digital Northern analysis using the data generated by microarrays, especially by AtGenExpress (Schmid et al. 2005). Expression profiles at various developmental stages have been generated using the Affymetrix ATH1 full genome chip. These data were analyzed and, if possible, compared to those generated by the 8k Affymetrix-GeneChip. In 7-day-old seedlings *SCL13* expression is highest in the green tissues (cotyledons and young leaves), whereas in the shoot apex and the hypocotyl less expression can be detected. Additionally, *SCL13* is also expressed in roots (Fig. 5a). In adult plants, 21-day-old or older, high *SCL13* transcript levels can be observed in leaves, with higher levels in younger rosette leaves. Within a leaf, the distal region shows higher *SCL13* mRNA levels than the proximal region or the petiole (Fig. 5b). In addition, expression is elevated in senescing leaves. As in young seedlings, less *SCL13* mRNA can be detected in shoots, stem and apical meristems even after transition to flowering. Furthermore, high *SCL13* mRNA levels can be observed in petals and sepals of flowers, which were tested at different developmental stages (Fig. 5b). In later stages, such as in siliques and during seed formation, no elevated levels could be seen. This indicates that SCL13 plays a role not only in the seedling stage but also throughout the lifetime of Arabidopsis. This notion is confirmed by our observation that the *SCL13* antisense lines flower earlier than WT.

In order to detect the promoter activity of the *SCL13* gene *in vivo* transgenic lines were generated that carried different *SCL13-promoter-GUS* constructs. The expression of a *SCL13-promoter-5′-UTR-GUS* fusion in transgenic lines confirmed the data derived from microarray analysis, as younger leaves showed stronger GUS staining compared to adult leaves (Fig. 6). As in the microarray, GUS activity was strongest in cotyledons and roots.

As the 5′-UTR of the *SCL13* transcript includes a 750 nt-long intron, we wanted to investigate a possible role of this intron for expression. The 5′-UTR including the intron on its own was not able to induce any GUS

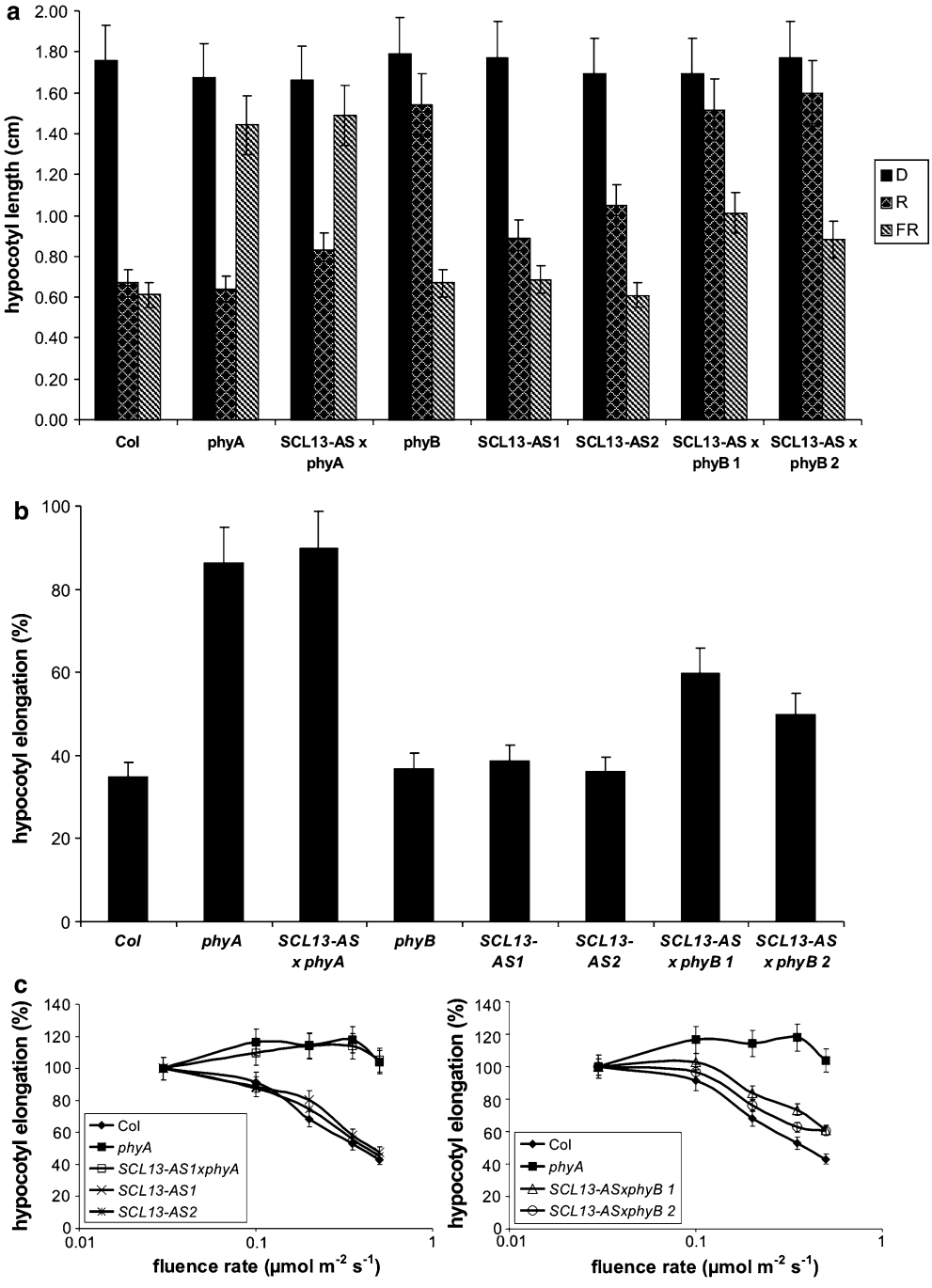


**Fig. 4** Analysis of hypocotyl elongation of photoreceptor mutants with reduced levels of *SCL13* mRNA. **a** Hypocotyl length of 4-day-old seedlings that were grown in darkness (*D*), under continuous red (*R*; 1 µmol $m^{-2}$ $s^{-1}$) or FR (0.35 µmol $m^{-2}$ $s^{-1}$) light. The lines *SCL13-AS1xphyA* and *SCL13-AS2xphyB* were compared to the respective single mutants and WT (Col). **b** Relative hypocotyl elongation under FR light. Hypocotyl length was normalized to that in darkness, which was considered 100%. **c** Fluence rate response curve for hypocotyl elongation under FR light of WT (Col), *phyA, SCL13-AS1* and *2*, *SCL13-AS1xphyA* and *SCL13-AS2xphyB1, 2*. More than 20 seedlings were used per replicate ($n = 3$). *Error bars* = standard deviation

activity, suggesting that no alternative transcription start sites are available within this region. A promoter construct that lacked the 5′-UTR and the intron generated the same spatial distribution of *GUS* expression, although at a weaker level, indicating that enhancing elements could be located within the 5′-UTR or the intron.

Evaluation of microarray data and semiquantitative RT-PCR experiments detected no changes in *SCL13* expression under different light conditions compared to darkness suggesting that *SCL13* is not induced or repressed under R or FR light (Fig. 5c, data not shown). Only pulses of UV-B light induced *SCL13* expression, but this is probably due to a stress response as *SCL13* is also induced by some other stresses, such as osmotic or salt stress or after treatment with methyl jasmonate (Fig. 5d). When seedlings carrying the *SCL13-promoter-5′-UTR-GUS* construct were grown under different light qualities the overall GUS activity did not change, but its distribution varied. Nearly no GUS activity was detected in the hypocotyl under W light conditions, but when seedlings were grown under darkness or red light conditions some GUS staining in the hypocotyl can be detected (Fig. 6). Furthermore, seedlings grown for 4 days either on 300 mM Mannitol or 100 mM $NaCl_2$

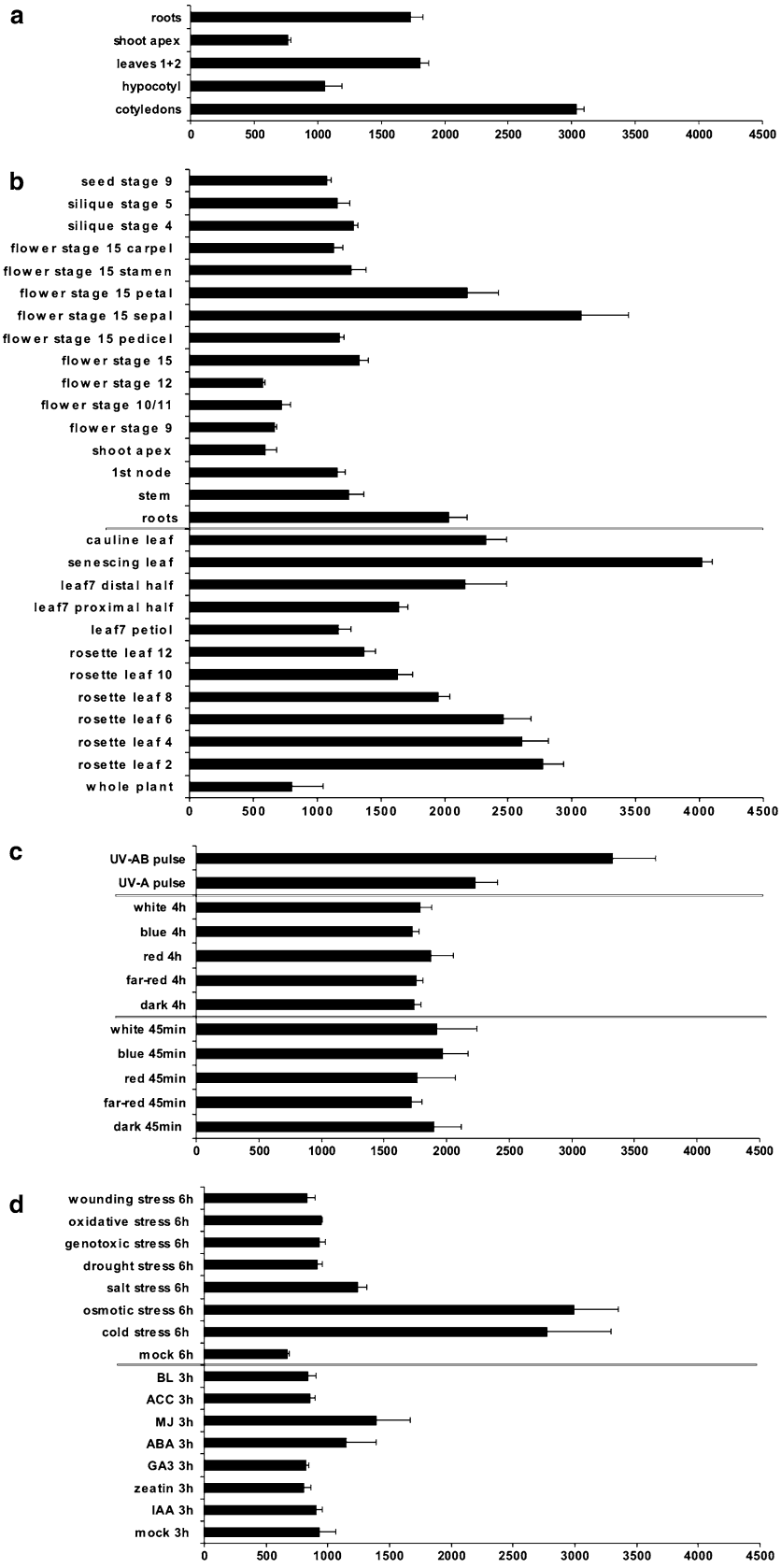


**Fig. 5** Expression profiling of *SCL13*. **a** Expression of *SCL13* in different tissues of 7-day-old seedlings (Schmid et al. 2005). **b** Expression of *SCL13* in different tissues of adult plants. Developmental stages are described in Schmid et al. (2005). **c** Expression of *SCL13* after 45 min or 4 h of continuous W, R, B and FR light compared to the expression in D and after a pulse of UV-AB or UV-A light (light treatments are described under http://www.arabidopsis.org/servlets/TairObject?type = expression_set&id = 1007966126, data provided by Kretsch et al., unpublished data). Panel GUS activity in Arabidopsis transformed with a *SCL13-promoter-5′-UTR -GUS* construct observed in 4-day-old seedlings under continuous W or R light or under darkness (D). **d** Expression of *SCL13* after treatment with hormones for 3 h or stresses for 6 h compared to mock treatment (Goda et al. 2004). Data are derived from the AtGenExpress 24k microarray expression profiling experiments. Mean values of replicas were generated. *Error bars* = standard deviation

showed stronger GUS staining patterns in the roots than seedlings on medium without stressors, confirming that salt and osmotic stress indeed induce the gene expression of *SCL13*. On the other hand, we could not observe any growth inhibition of *SCL13* antisense lines grown under these conditions which differed from WT.

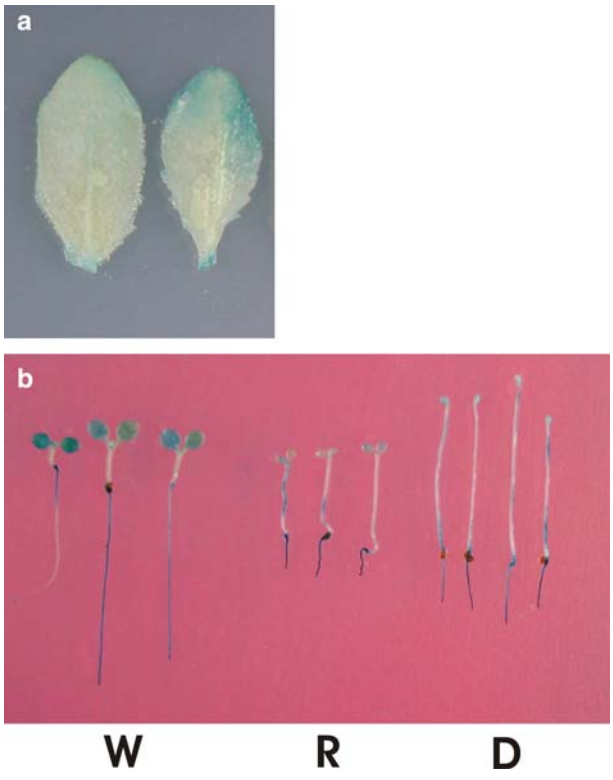


**Fig. 6** Expression analysis of *SCL13*. GUS activity in Arabidopsis lines transformed with a *SCL13-promoter-5′-UTR-GUS* construct observed **a** in leaves of 20-day-old plants grown under greenhouse conditions, **b** in 4-day-old seedlings under continuous *W* or *R* light or under darkness (*D* )

### Subcellular localization of SCL13

No potential nuclear localization signals (NLS) were found within the predicted amino acid sequence of SCL13. To investigate the subcellular localization of SCL13 we fused the gene encoding the green fluorescent protein (GFP) to the 3′-end of *SCL13*. The fusion gene was placed under the control of a *35S CaMV* promoter and the construct transiently expressed in onion epidermal cells. GFP fluorescence could be detected in the cytoplasm but also in the nucleus (Fig. 7b). The subcellular localization of SCL13-GFP did not change in response to D, R, FR or W light. These results provide evidence that although the SCL13 protein lacks a known NLS, it could still be transported into the nucleus due to an yet unidentified NLS or with the help of interacting proteins. Nevertheless, as we were not able to test this construct in complementation analysis for functionality, the results have to be treated with caution.

To determine in which compartment SCL13 is biologically active a conserved NLS was fused to the N-Terminus of the SCL13 protein. This leads to an exclusive nuclear localization of the protein to the nucleus, suggesting that the SCL13 protein is not actively exported from the nucleus. If the NLS was substituted for a nuclear exclusion signal (NES), the protein localized predominantly in the cytoplasm. These results could be visualized in transient assays in onion epidermis cells with the help of a C-terminally fused GFP protein (Fig. 7c, d), but also in transgenic lines expressing the construct with the help of an additionally fused GUS protein (Fig. 7e–h). Only few transgenic lines displayed high GUS activity and those lines expressing the NLS-containing constructs were always weaker in staining. The tissue specific expression pattern was sometimes spotted, but appeared in principle similar to that in the transgenic lines carrying the *SCL13-promoter-5′-UTR-GUS* fusion. In all cases analyzed the sub-cellular localization followed the respective tag (Fig. 8).

Lines that expressed either the NLS- or the NES-containing fusion protein at high levels were chosen for phenotypic characterizations under R and FR light. A consequence of overexpressing the nuclear and cytoplasmatically localized SCL13 protein at high levels is a shorter hypocotyl under R light ($P < 0.05$), whereas no effect could be seen under FR light (Fig. 7i). This suggests that under red light conditions SCL13 is actively involved in signaling both in the cytoplasm and the nucleus. The weaker GUS activity observed in NLS-SCL13 overexpressing lines could be due to a faster turnover of the protein in the nucleus. Furthermore, the results underline the findings that SCL13 is important for the red light signaling as no hypersensitive effect could be measured under darkness and FR light conditions.

## Discussion

### SCL13 is involved in red light signaling

Several lines of evidence show that SCL13 functions as a positive regulatory component of the red light signaling pathway, predominantly dependent on phyB. First, the reduction of *SCL13* mRNA in antisense lines specifically decreased the inhibition of hypocotyl elongation under red light conditions. The observation that the antisense lines had mainly WT phenotype under other wavelengths of light (FR, B, W) and in darkness established that the phenotype is light dependent and specific for red light. Second, a *phyB* null allele was epistatic to the reduction of *SCL13* mRNA under red light conditions. Third, *SCL13* appears to be expressed normally in *phyB* mutants (Tepperman et al. 2004). The alternative possibility that the red light specific phenotype is an indirect consequence of phyB reduction appears unlikely as only very specific phyB responses are impaired by reduced *SCL13* expression and the effect is not as severe as that of the *phyB* mutant. Furthermore, several lines of evidence suggest that the observed phenotype is not a general effect of cell elongation under low fluences of light. The effect could be observed also under non-saturating conditions of R, but not of FR light (Figs. 2c, 4c). Besides, no elongation of the hypocotyl and petioles

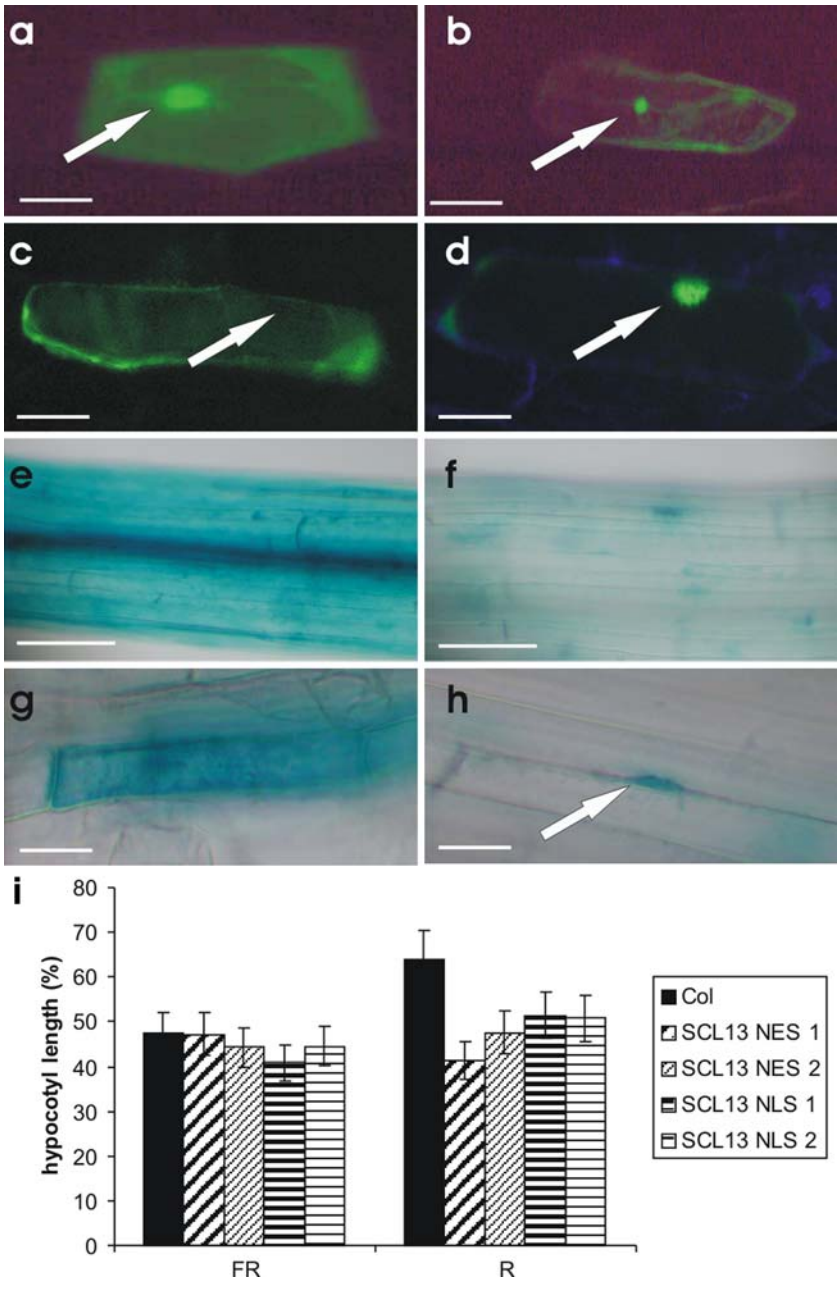


**Fig. 7** Subcellular localization of SCL13. Onion epidermal cells were bombarded either with **a** *35S-GFP*, **b** *35S-SCL13-GFP*, **c** *35S-NES-SCL13-GFP* or **d** *35S-NLS-SCL13-GFP* and visualized using a fluorescence microscope. In vivo localization of **e, g** *35S-NES-SCL13-GUS* or **f, h** *35S-NLS-SCL13-GUS* in transgenic Arabidopsis lines. *Bar* = 50 μm (**a**–**d**, **g**, **h**) and 500 μm (**e**, **f**). *Arrows* mark the position of the nuclei. **i** Hypocotyl elongation of 4-day-old seedlings grown under continuous far-red (*FR*; 0.4 μmol $m^{-2}$ $s^{-1}$) or red (*R*; 1 μmol $m^{-2}$ $s^{-1}$) light. Hypocotyl lengths in D were also measured and considered 100%. More than 20 seedlings were used per replicate ($n = 3$). *Error bars* = standard deviation

compared to WT could be observed under dim white light conditions (Fig. 3d).

## SCL13 regulates only a specific subset of red light dependent responses

The data presented here demonstrate that the reduction of *SCL13* mRNA leads to a highly specific defect in the seedling de-etiolation process and to a lesser extent in the adult stage. We could not detect any changes in the many well-described phyB-dependent responses such as germination under red light, cotyledon expansion, EOD-FR response, petiole elongation, chlorophyll accumulation and gene expression. The main phenotype that could be observed was the sensitivity of the hypocotyl elongation toward red light, and the earlier onset of flowering under short- and long-day conditions.

Compared to the phenotypes of *phyB*/*C*/*D*/*E* null mutants and other known intermediates of red light signaling, SCL13 is the only one to our knowledge in which the loss-of-function specifically affects hypocotyl length and not cotyledon expansion.

## Is SCL13 positioned downstream of phyB, C, D or E?

In *A. thaliana* three phytochromes have been shown to be involved in red light signaling besides phyB, namely phyC, D and E. As phyB is the predominant photoreceptor in red light, mutants with defects in phyB show the most drastic phenotype. Besides having an elongated

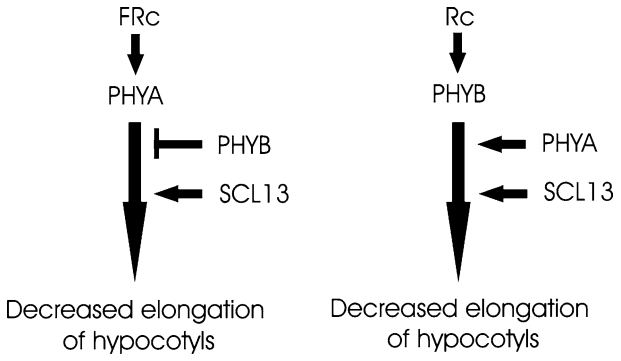


**Fig. 8** The proposed mechanism for the function of SCL13 under continuous FR and R light (FRc, Rc). Under FR-light conditions phyB represses hypocotyl elongation, whereas SCL13 plays a positive role. This can only manifest itself in the absence of phyB. Under R light conditions both SCL13 inhibits hypocotyl elongation and this is also promoted by phyA

hypocotyl and smaller cotyledons under continuos red and white light, they are slightly pale because of reduced chlorophyll accumulation. They also have longer petioles, flower earlier and do no longer respond to an EOD FR pulse (Reed et al. 1993).

Besides phyB, phyC operates as a weak red light sensor, and could perform a significant role in the modulation of other photoreceptors. When grown under constant red light, *phyC* mutant seedlings exhibited a partial loss of sensitivity, observable as longer hypocotyls and smaller cotyledons than those seen in the WT and an earlier flowering time. PhyD and E work together with phyB in a functionally redundant manner as they are involved in shade avoidance (Aukerman et al. 1997; Devlin et al. 1998, 1999; Franklin et al. 2003a, b). Furthermore, phyE is also known to modulate flowering time (Halliday and Whitelam 2003). As the photosensory specifities of these phytochromes are all very similar to phyB we cannot exclude that SCL13 can also act downstream of phyC and E or modulate their responses. Judging by the physiological analysis it is highly unlikely that SCL13 acts downstream of phyD, as this phytochrome has a role in shade avoidance responses, which are not affected in *SCL13* antisense lines.

## SCL13 modulates phyA signaling

If SCL13 is specific for red light signaling, its reduction should not have any additive effects in a *phyA* mutant background. Analysis of the *SCL13ASxphyA* line showed that this is the case under red and FR light conditions. Unexpectedly, the *SCL13-ASxphyB* lines, which behaved as a *phyB* mutant under red light conditions, displayed an additional phenotype under FR light. Previous reports have established a complex pattern of cross-talks between phyA and phyB (and other photoreceptors) in the photoregulation of multiple developmental processes (Casal 1996; Canton and Quail 1999; Casal et al. 2000; Hennig et al. 2001). PhyA has a synergistic effect with phyB under high irradiance conditions, whereas under very low fluences the effect is inhibitory (Neff and Chory 1998; Hennig et al. 1999). Overexpression of phyB leads to a decreased inhibition of hypocotyl elongation under FR light, and consequently, an increased inhibition of hypocotyl elongation can be observed in *phyB* mutants (Sakamoto and Nagatani 1996; Wagner et al. 1996; Hennig et al. 1999). Activation of phyB signaling appears to be unnecessary for this modulation of phyA-mediated high irradiance responses because FR light, which does not induce signaling via phyB, is sufficient for the effect. Furthermore a point mutation in phyB (*phyB-4*) can still inhibit the phyA-dependent HIR, whereas it is unable to induce a signaling cascade. It was speculated that phyA and phyB might compete for the same signaling partner in the cytoplasm leading to a negative interaction (Hennig et al. 2001).

The phenotype of the *SCL13-ASxphyB* line under FR light appears similar to that of a phyB overexpressing line as the phyA-dependent HIR is decreased. If SCL13 is the factor phyA and phyB are competing for, we expect an elongated hypocotyl under FR light if phyB is overexpressed, as SCL13 is no longer available for phyA signaling. On the other hand, the phenotype of the *SCL13* antisense lines should be similar to the *SCL13-ASxphyB* lines as in both cases SCL13 cannot act in the FR light signal transduction. But this is not the case, as the phyB mutant with reduced levels of *SCL13* mRNA displays an additive phenotype under FR light. Unless residual SCL13 can account for this discrepancy, SCL13 is probably not the factor postulated by Hennig et al. (2001). The most straightforward hypothesis to explain the phenotype is to assume that SCL13 is a positive regulator of phyA signaling counteracting phyB inhibition (Abb. 8). In the *SCL13-ASxphyB* lines hypocotyls are longer compared to the *SCL13* antisense line as the inhibitory effect of phyB is removed. The modulation of SCL13 of the phyA signal transduction could take place in the cytoplasm or the nucleus in a phyB-independent way. PhyB is not present in its active form under these conditions as FR light is not able to activate phyB, which therefore remains in the cytoplasm.

## SCL13 could play a biological role in the cytoplasm and the nucleus

From the phenotype of the *SCL13* antisense lines we conclude that SCL13 acts as a positive regulator of red light signal transduction. GFP localization experiments have suggested that SCL13 is localized in the cytoplasm and nucleus. The constitutive expression of *SCL13* indicates that SCL13 has to be already present under non-induced conditions so that a quick response can take place after illumination and activation of the phytochrome. SCL13 could interact with phyB to transport the latter to the nucleus upon illumination. Another possibility is that SCL13 is a signaling intermediate, which amplifies the red light signal either in the cytoplasm or in the nucleus. The fact that lines overexpressing an NLS-SCL13-GFP-GUS fusion protein in the nucleus as well

as lines overexpressing the cytosolic form of SCL13 have a hypersensitive reaction under red light in their hypocotyl elongation suggests that SCL13 can perform its role in both compartments.

Not many intermediates in phyB signaling have been identified so far. One reason may be that functional redundancy renders it difficult to identify mutants impaired in red light signaling. On the other hand, phyB can directly interact with transcription factors, especially basic helix-loop-helix proteins (bHLH) such as PIF3 (Ni et al. 1998). This direct regulation of the transcription machinery by the photoreceptor suggests a short signal transduction chain. Most of the proteins that have been identified as intermediates of the phyB signal transduction, such as bHLH proteins (PIF3, 4, PIL1,6), GI, ELF3 and ELF4 are localized in the nucleus (Liu et al. 2001; Fowler et al. 1999; Huq et al. 2000; Doyle et al. 2002; Khanna et al. 2003). However, *SRR1* codes for a nuclear/cytoplasmic protein (Staiger et al. 2003) and RED1 encodes a cytoplasmatic cytochrome P450 (Hoecker et al. 2004). This suggests that the red-light signal transduction is at least modulated in the cytoplasm. Further experiments will be necessary to determine how SCL13 executes its function.

## Expression pattern of *SCL13*

The *SCL13* mRNA is not regulated in a light-dependent fashion, at least under the conditions used in the microarray experiments. In the expression profiling experiment of Tepperman et al. (2001, 2004) and Wang et al. (2002). *SCL13* mRNA levels were not regulated by neither phyA nor phyB. Experiments with transgenic lines carrying the *SCL13-promoter-5′-UTR-GUS* fusion showed that the overall GUS activity is similar under different light conditions, but that the tissue-specific expression varied. In W light grown seedlings GUS activity was mainly restricted to the cotyledons and the root, whereas hardly any activity was detectable in hypocotyls. By contrast, seedlings grown in darkness or in red light, which had elongated hypocotyls compared to WT, showed also GUS staining in hypocotyls. It looks as if *SCL13* is predominantly expressed in the active elongation zones of a hypocotyl. This observation is consistent with data from the de-etiolation assays, where SCL13 plays a role in hypocotyl elongation. Differences between microarray data and the GUS expression patterns could arise from the fact that GUS assays were performed on 4-day-old seedlings rather than 7-day-old seedlings used for the microarray assays or from differences in fluence rates. Microarray analysis revealed that *SCL13* mRNA is induced in all green tissues and the root based on microarray studies. This implies a function of the protein during the entire life cycle of a plant—similar to phyB (Sharrock and Clack 2002).

No effects of cytokinins (zeatin), auxin (IAA), ethylene (ACC), gibberellin (GA3) and brassinosteroids (BR) on the *SCL13* mRNA levels could be detected. No visible phenotype was seen when seeds were germinated on paclobutrazol, an inhibitor of Gibberellin biosynthesis. Nonetheless, compared to mock treatment of seedlings, methyl jasmonate (MJ) and to a lesser extent abscisic acid (ABA) induced some expression (Fig. 5). Similar effects as with MJ could be observed under UV-B-light, osmotic and cold stress, suggesting that *SCL13* is induced under different abiotic stresses. This fact was confirmed by the increased GUS staining of roots on mannitol- or salt-containing media.

Interestingly, based on microarray data, the addition of cyclohexamide, an inhibitor of protein biosynthesis, increased the steady-state level of *SCL13* strongly, suggesting the existence of a repressor of SCL13 with a fast turnover. Degradation of mRNA provides a powerful tool for controlling gene expression during different developmental stages or under certain environmental conditions. For fast responses of proteins, which play important regulatory roles, rapid decay of mRNAs ensures the desensitization of the system. By adding cordycepin, a modified nucleotide that acts as a polyadenylation inhibitor, the rate of decay of specific mRNAs was evaluated by Gutierrez et al. (2002) using microarrays. In this study it was demonstrated that *SCL13* encodes an extremely unstable mRNA.

It should be emphasized that mRNA levels, however, do not necessarily reflect the presence of an active protein in the different developmental stages. Protein degradation can affect protein levels and deplete active protein from the system, therefore having a strong impact on signaling (Schwechheimer and Schwager 2004). One line of evidence that SCL13 might be tightly regulated at the protein level is the fact that overexpression of *SCL13* in transgenic lines did not produce any observable phenotype albeit the overexpressed mRNA can be visualized (data not shown). Only in lines overexpressing *SCL13* fused to NLS or NES to a very high extent did we detect some reduction in hypocotyl elongation (Fig. 7i). The additional amino acid extensions could help to stabilize the protein in these lines.

## GRAS proteins and light signaling

GRAS proteins have been shown to be involved in many different developmental processes (Bolle 2004). So far, only two proteins (PAT1 and SCL13), both members of the PAT1 subbranch of the GRAS protein family, have been shown to play roles in light signal transduction (Bolle et al. 2000). The PAT1 subbranch has several members in Arabidopsis, but in other plant species, proteins can also be identified that cluster to this subbranch. The high conservation of homologous proteins to SCL13 from poplar and lotus indicates that these proteins could act in a similar fashion as SCL13. At least 50 GRAS proteins have been described in rice but none of them is closely related to SCL13, although several of them cluster to the PAT1 branch. This suggests that SCL13 might be more important for dicots then for monocots.

Both SCL13 and PAT1 share all the conserved motifs of GRAS proteins. The most variable part of these

proteins is the N-terminus, which could encode the specificity for the different signaling cascades. GRAS proteins have been suggested to act as transcription factors. Indeed, in yeast one-hybrid assays SCL13 was found to display some transactivation capacity (Rothmeier and C. Bolle, unpublished data). As PAT1 and SCL13 are also localized in the cytoplasm, this would mean that in order to activate the transcription machinery they have to migrate to the nucleus. As both PAT1 and SCL13 possess no conserved NLS, future research should focus on the mechanisms by which these proteins are transferred into the nucleus for light signaling.

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