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ORIGINAL ARTICLE

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Isolation and characterization of *PHYC* gene from *Stellaria longipes*: differential expression regulated by different red/far-red light ratios and photoperiods

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Abstract We have cloned and characterized the phytochrome C (*PHYC*) gene from *Stellaria longipes*. The *PHYC* gene is composed of a 110-bp 5′-untranslated leader sequence, a 3,342-bp coding region, and a 351-bp 3′-untranslated sequence. The *Stellaria PHYC* contains three long introns within the coding region at conserved locations as in most angiosperm *PHY* genes. DNA blot analysis indicates that the *Stellaria* genome contains a single copy of *PHYC*. *Stellaria* PHYC shares 60%, 58%, and 57% deduced amino acid identities with rice, *Sorghum*, and *Arabidopsis* PHYC, respectively. Phylogenetic analysis indicates that *Stellaria* PHYC is located in the dicot branch, but is divergent from *Arabidopsis* PHYC. The *Stellaria PHYC* is constitutively expressed in different plant organs, though the level of *PHYC* gene transcript in roots is slightly higher than in flowers, leaves, and stems. When 2-week old seedlings grown in the dark were exposed to constant white light, *PHYC* mRNA quickly accumulates within 1–12 h. When plants grown in darkness for 7 days were exposed to different red/far-red light (R/FR) ratios, the levels of *PHYC* mRNA at R/FR = 0.7 are much lower than under R/FR = 3.5. The levels of *PHYC* mRNA under short-day (SD) photoperiod are higher than under long-day (LD) photoperiod. Plants under SD conditions do not elongate, and are only about 1.7 cm tall at 19 days. In contrast, plants under LD conditions elongate with an average height of 21.2 cm at 19 days. The plants do not flower under SD conditions, but do so at 18–19 days under LD conditions. These results indicate that under SD conditions the high level of *PHYC* mRNA may inhibit stem elongation and flower initiation. In contrast, under LD conditions the high level of *PHYC* mRNA may promote stem elongation and flowering.

Keywords Functional and differential expression · Gene structure · Phytochrome gene · Phylogeny · *Stellaria longipes*

Abbreviations LDW: Long-day warm · PHY: Phytochrome gene · R/FR: Red/far-red light ratio · SDC: Short-day cold

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Introduction

Phytochrome genes have been identified in a broad range of species throughout the plant kingdom, from the algae and mosses to gymnosperms and angiosperms (Mathews and Sharrock 1997; Schneider-Poetsch et al. 1998; Clapham et al. 1999). In all plants examined, the phytochrome apoproteins are encoded by small, multigene families, and have been classified into four subfamilies: A, B/D, C/F, and E (Mathews et al. 1995; Mathews and Sharrock 1996, 1997; Pratt et al. 1997; Schneider-Poetsch et al. 1998; Schmidt and Schneider-Poetsch 2002). In *Arabidopsis*, the *PHY* family is composed of five members (*PHYA*, *PHYB*, *PHYC*, *PHYD*, and *PHYE*) and is classified into four subfamilies (A, B/D, C, E; Sharrock and Quail 1989; Clack et al. 1994). We recently identified the *PHY* family in *Stellaria*, which is composed of six members (four *PHYAs*, one *PHYB*, and one *PHYC*), but lacks the *PHYE* subfamily (Li and Chinnappa 2003). Although a large number of *PHY* DNA sequences have been reported in the databases, a significant fraction of these are either ESTs or partial sequences generated by PCR using primers to a conserved region of the sequences (Mathews et al. 1995; Mathews and Sharrock 1997; Pratt et al. 1997; Schneider-Poetsch et al. 1998). Relatively few full-length cDNA sequences have been reported. Fewer *PHY* gene

structures have been determined experimentally by comparison of DNA sequences to corresponding cDNAs (Basu et al. 2000).

The expression of the five *PHY* in *Arabidopsis* and tomato, as well as *PHYA* and *PHYB* in potato, has been described as uniform in different organs, at different developmental stages, and under different light conditions (Heyer and Gatz 1992a, 1992b; Clack et al. 1994; Hauser et al. 1997, 1998). Data from Northern blot analyses indicate that *PHYC* is less abundant compared to *PHYA* and *PHYB*, and is relatively unaffected by light in *Arabidopsis* and rice (Sharrock and Quail 1989; Clack et al. 1994; Basu et al. 2000).

The physiological functions of individual phytochrome species have been recently revealed by analysis of *PHY*-deficient mutants and overexpression studies of individual *PHY* genes. The results from analysis of *Arabidopsis phyA*-, *phyB*-, *phyD*-, or *phyE*-deficient mutants have revealed that individual members of the *PHY* family have different but partially overlapping functions in controlling plant responses (Whitelam and Devlin 1997; Devlin et al. 1998; Quail 2002). For example, *PHYA* plays a major role throughout the plant life cycle in sensing prolonged far-red (FR) light and in mediating very-low-fluence responses (Casal et al. 1997). *PHYB* and *PHYD* have highly overlapping roles in sensing the red/far-red light (R/FR) ratio and in mediating shade avoidance responses (Aukerman et al. 1997; Smith and Whitelam 1997; Devlin et al. 1999). *PHYE* seems to play a role in the regulation of shade-avoidance response in a manner partially conditionally redundant to *PHYB* (Devlin et al. 1998). *PHYE* is also involved in the control of seed germination (Hennig et al. 2002), and in the control of cotyledon expansion under constant red light (Franklin et al. 2003b). Although *PHYC*-deficient mutants have only recently been isolated, data from overexpression studies of *Arabidopsis PHYC* in both *Arabidopsis* (Qin et al. 1997) and tobacco (Halliday et al. 1997) suggest a possible specific role for *PHYC* in the control of *Arabidopsis* primary-leaf cell expansion and tobacco cotyledon expansion. A recent study with an *Arabidopsis PHYC*-deficient mutant has revealed a functional role for the *PHYC* gene throughout *Arabidopsis* photomorphogenesis (Franklin et al. 2003a; Monte et al. 2003). The *PHYC* gene may perform a significant role in the modulation of other photoreceptors, and *PHYA* and *PHYC* act together to regulate mature leaf morphology (Franklin et al. 2003a). *PHYC* function in seedling de-etiolation in constant red light, however, may require the presence of *PHYB* (Monte et al. 2003). In addition, the spectral characteristics of *PHYC* were strictly different from other *PHY* genes and the *PHYC* apoprotein chromophore adduct has undergone a strong dark reversion (Eichenberg et al. 2000). While most members of *PHY* genes are well characterized, information about the physiological function and expression pattern of *PHYC* is limited.

In addition, it is known that phytochromes also play an important role in adaptive plasticity in natural light environments in plants (Weinig 2002; Alokam et al. 2002). For example, *Stellaria longipes* has been used as a model system to understand the genetic regulation of phenotypic plasticity in various ecotypes (Emery et al. 1994; Kathiresan et al. 1998; Alokam et al. 2002). During the past two decades, we have studied the growth responses of several ecotypes under various light conditions (Macdonald et al. 1984; Emery et al. 1994; Alokam et al. 2002). We have recently determined the phytochrome gene family in the *S. longipes* complex (Li and Chinnappa 2003). In this study, we cloned the *PHYC* gene from *S. longipes* and identified its structure by comparison with the cDNA sequence. We also report characterization of light regulation, and patterns of expression of the *Stellaria PHYC* gene using semi-quantitative RT-PCR.

Materials and methods

Plant materials and growth conditions

Prairie genotypes of *S. longipes* were originally obtained from Kananaskis valley, Alberta, Canada. The plants were clonally propagated and maintained in 8 cm pots containing peat moss, sand and Terra green, a crushed baked clay growing medium (2:1:1), in the University of Calgary greenhouse. The plants used in this experiment were first transferred from the greenhouse into a short day cold (SDC) growth chamber (8 h/day photoperiod, 8°C day/4°C night) for a minimum of 90 days to simulate the winter cycle. (1) To study expression of *PHYC* in different organs, the plants were transferred from the SDC chamber to a long day warm (LDW) chamber (22°C day/18°C night, 16 h photoperiod) and maintained under LDW for 19 days until flowering. To obtain roots, the young ramets were planted in 8 cm pots containing sterile sand and maintained in a tray containing 1/2 liquid Murashige and Skoog basal medium under LDW for 14 days and then fresh roots were collected. (2) To study the effects of different R/FR ratios on the expression of *PHYC*, 2-week-old seedlings were used. Seeds were surface-sterilized for 10 min in 70% (v/v) alcohol and then for 10 min in 20% (v/v) bleach, rinsed at least five times with sterile water, and germinated in glass jars containing Murashige and Skoog basal agar medium (Sigma, St. Louis, Mo.) plus 2% (w/v) sucrose. The jars were kept in the dark at 4°C for 2 days, treated for 2 h with white light to induce germination, and then placed in complete darkness at 22°C for 14 days. After that, the jars were transferred to continuous white light at 22°C for 1–12 h. (3) To study the effects of different photoperiods on the expression of *PHYC* and stem elongation, the plants were transferred from SDC to a warm chamber (22°C day/18°C night) with 8 h/day (short day; SD) or 20 h/day (long day; LD) conditions. White light sources were provided by standard cool white fluorescent bulbs with a flux rate of 35 μmol $s^{-1}m^{-2}$. Red and far-red lighting was provided

by a light emitting diode (LED) system (Snap-Lite Solid State Lighting System for Plant Growth, Quantum Devices, Barneveld, Wis.). Fluency rates and spectral distribution of the light sources were recorded by the cosine-corrected remote probe of a calibrated LI-1800 Spectroradiometer (LI-COR, Lincoln, Neb.). The probe was held horizontally at the level of the shoot apices. Since *PHYC* gene expression showed a diurnal rhythm in *Arabidopsis* (Toth et al. 2001), we assumed a similar trend in *Stellaria*. Hence, all samples were collected between 10:00 am and 11:00 am, unless otherwise indicated.

Isolation of the *Stellaria PHYC* gene

A 3,237-bp *Stellaria PHYC* cDNA fragment was first cloned using degenerate primer PCR and 3′rapid amplification of cDNA ends (RACE; Li and Chinnappa 2003). Since this clone was missing a portion of exon 1, the sequence of the 5′ end of the *Stellaria PHYC* was obtained using a 5′RACE kit (Invitrogen, Gaithersburg, Md.). Total RNA was isolated from young ramets using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany). Two reverse *Stellaria PHYC* gene-specific primers, PhyCR1 and PhyCR3 (Table 1) near the 5′ end of the open reading frame (ORF) were designed based on the 3,237 bp *PHYC* cDNA sequence (Li and Chinnappa 2003). First-strand cDNAs were synthesized with primer PhyCR1 (Table 1). After synthesis of the first strand, the cDNAs were purified and tailed with dCTP, and then used directly in the 5′RACE PCR reaction. Primary PCR amplification reactions were achieved using gene-specific primer PhyCR1 and Abridged Anchor Primer (AAP, Invitrogen), and a secondary PCR was run using gene-specific primer PhyCR3 and Abridged Universal Amplification Primer (AUAP, Invitrogen) in a 50-µl reaction containing 1×PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 200 µM dNTP, 10 pmol each primer, and 2.5 U DNA polymerase (Invitrogen). The PCR program was 3 min at 94°C, 5 min at 72°C [at this point 0.5 µl 5 U/µl *Taq* DNA polymerase (Invitrogen) was added to each reaction] for 1 cycle; 0.5 min at 94°C, 1 min at 55°C, 2 min at 72°C for 35 cycles, and 10 min at 72°C for 1 cycle. RACE fragments were gel-purified using a QIAquick gel extraction kit (Qiagen), and then cloned into the pGEM-T Easy vector (Promega, Madison, Wis.) for sequencing.

To obtain the genomic sequence of the *Stellaria PHYC* gene, *Stellaria* genomic DNA was used as a template and amplified using PCR with three pairs of *PHYC*-gene-specific primers (PhyCF7/PhyCR7, QmCF/QmCR, and PhyCF6/PhyCR6, Table 1), which were designed based on the *Stellaria PHYC* cDNA sequence. PCR conditions were identical to those described above. Three fragments (1,790-bp, 2,351-bp, and 2,036-bp) were gel-purified, cloned and sequenced as described above. A 6-kb fragment was obtained by overlapping the three fragments using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

DNA sequencing and data analysis

DNA samples for sequencing were prepared with QIAprep Miniprep Kit (Qiagen) and sequenced in both directions with T7 and SP6 primers using an ABI Prism BigDye Sequencing kit (Applied Biosystems, Foster City, Calif.) at the University of Calgary DNA Sequencing Center. Analysis of the DNA sequences was carried out using the Advanced BLASTX search program (Altschul et al. 1990). Multiple sequence alignments were performed using CLUSTAL X version 1.8 (Jeanmougin et al. 1998). Characterization of the nucleotide sequences and conceptual translation of nucleotide sequences were made using DNAStrider 1.1 (Christian Marck, Department de Biologie Institut de Recherche Fondamentale, CEA, France). Phylogenetic analyses were performed with all full-length plant PHYC and PHYF protein sequences available in the databases (*Stellaria* PHYC AF544029, *Arabidopsis* PHYC X17343, *Oryza* PHYC AF141942, and *Solanum* PHYF AF178568). CLUSTAL X (Jeanmougin et al. 1998) was used to align the amino acid sequences. A neighbor-joining tree was created with NJplot software (Perriere and Gouy 1996) using these aligned amino acid sequences. *Arabidopsis* PHYA was the outgroup in this study.

Table 1 Primers used for the amplification of *Stellaria PHYC* and quantitative RT-PCR

Name	Sequence	Genes	Purpose	Direction	Positions
PhyCR1	5′CATTCTCACCTTATTCTTCAAGAAAAGAAACC3′	*PHYC*	5′RACE	Reverse	852–821
PhyCR3	5′CCTTATTCTTCAAGAAAAGAAACCGTGAAGCTTG3′	*PHYC*	5′RACE	Reverse	844–811
PhyCF7	5′GAGTGAATTTACGGAGAGAAATGTCG3′	*PHYC*	PCR	Forward	−20–6
PhyCR7	5′CACTATCATTTTCGACGTATCTGACATCG3′	*PHYC*	PCR	Reverse	1,770–1,742
QmCF	5′CGATGTCAGATACGTCGAAAATGATAGTG3′	*PHYC*	PCR and quantitative RT-PCR	Forward	1,742–1,770
QmCR	5′GCATGCGACCAACAGTGTACTCCTTGC3′	*PHYC*	PCR and quantitative RT-PCR	Reverse	4,092–4,066
PhyCF6	5′GCAAGGAGTACACTGTTGGTCGCATGC3′	*PHYC*	PCR	Forward	4,066–4,092
PhyCR6	5′GACTTCCAAGGAGCGGGCAAACATCG3′	*PHYC*	PCR	Reverse	6,101–6,076
23SF	5′GATGCGGACTACCTGCACCTGGACAG3′	*23S*	Quantitative RT-PCR	Forward	54–79
23SR	5′CCACTCGGCACCGTCGGATCACTAAGG3′	*23S*	Quantitative RT-PCR	Reverse	435–409

Identification of introns

Identification of introns was achieved by comparing the *Stellaria PHYC* genomic sequence with the cDNA sequence. The exact locations of the splice sites were found. These intron/exon designations were subsequently compared with those of *Arabidopsis* and rice *PHYC* genes.

Southern blot analysis

Genomic DNA was isolated from young ramets of *S. longipes* prairie ecotype, using the method of Doyle and Doyle (1987). Southern blot analysis was carried out with 10 μg/lane genomic DNA digested with *Dra*I, *Eco*RV, or *Hin*dIII. Blots were hybridized with a 660 bp fragment of the *Stellaria PHYC* gene (Fig. 2b). The 660 bp fragment was digested with *Bam*HI and *Eco*RI from p7B-PHYC-1 (Li and Chinnappa 2003), gel-purified (QIAquick gel extraction kit; Qiagen), and labeled with ^{32}P-dCTP using the Random Primers DNA Labeling System (Invitrogen) according to the manufacturer's instructions. The ^{32}P-labeled probe was purified with a Nick Column (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK) following the manufacturer's instructions. The blots were prehybridized at 42°C for 2–4 h in 50% deionized formamide, 5× SSC, 5× Denhardt's buffer, 0.5% (w/v) SDS, then hybridized with ^{32}P-labeled *PHYC* probe overnight with the same conditions and buffer as prehybridization, and washed twice in 2× SSC, 1% (w/v) SDS at room temperature for 10 min each, following by washing once in 0.5× SSC, 0.5% (w/v) SDS at 55°C for 30 min, and once in 0.1× SSC, 0.1% (w/v) SDS at 55°C for 10 min. The blots were exposed to XAR-5 film (Kodak X-Omat AR-5) using intensifying screens at −80°C for 3 weeks. The sizes of the hybridizing bands were estimated by comparing their mobility with λDNA/*Hin*dIII fragments (Invitrogen).

Semiquantitative RT-PCR

Total RNA (5 μg) treated with DNase I was annealed to an oligo (dT)-containing adapter primer (Invitrogen); first-strand cDNAs were then synthesized using Superscript II RT (Invitrogen) and used directly in RT-PCR. A pair of *Stellaria PHYC* gene-specific primers (QmCF and QmCR, Table 1), designed based on the *Stellaria PHYC* cDNA sequence, were used to amplify a 626-bp fragment of *Stellaria PHYC*. A 2,351-bp fragment of the genomic *PHYC* gene was amplified if total RNA was contaminated by genomic DNA. Both of these fragments can be distinguished from any contamination of genomic DNA by the fragment size. A pair of *Stellaria 23S* rRNA primers (23SF and 23SR, Table 1) were designed based on the *Stellaria 23S* rRNA gene cDNA sequence (GenBank accession number: AY251296), and used to amplify a 381-bp fragment as an internal standard. RT-PCR was carried out in a 50-μl reaction containing 1× PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 200 μM dNTP, 10 pmol each primer (QmCF and QmCR), 3 pmol each primer (23SF and 23SR) [except in the organ-specific expression experiment (10 pmol each primer used)], and 2.5 U *Taq* DNA polymerase (Invitrogen). A master mix was made excluding DNA polymerase and template cDNAs. From the master mix, an aliquot was added to 0.5 ml thin wall tubes, followed by addition of 2 μl 10× diluted first-strand cDNAs. We selected one sample in which the *PHYC* gene was expected to be the most abundant and used it to determine the PCR cycle number that gave a linear range of gene amplification. A cycle number in the linear range (26 cycles) was chosen for subsequent experiments. The amounts of the *23S* rRNA internal standard primers were determined in order to amplify both *PHYC* and *23S* at a similar level. PCR cycling conditions were 3 min at 94°C, 5 min at 72°C for 1 cycle; 0.5 min at 94°C, 1 min at 56°C, 1.5 min at 72°C for 21–28 cycles; and 10 min at 72°C for 1 cycle.

RT-PCR products (5 μl each) were separated on 1.7% (w/v) agarose gels, visualized by ethidium bromide staining under UV light, and quantified by quantitative image analysis of *PHYC* and *23S* rDNA bands using NIH Image 1.62 software (Research Services Branch of the National Institute of Mental Health, USA). *PHYC* gene transcript abundance levels were given as relative values normalized to *23S* mRNA levels that were the ratio of the optical density of *PHYC*/*23S* gene bands. The results shown were from at least two individual experiments.

Results

Identification and structure of the *Stellaria PHYC* gene

A 3,237-bp *Stellaria PHYC* cDNA was initially cloned using degenerate primer PCR and 3′RACE (Li and Chinnappa 2003). Since this clone was missing a portion of exon 1 (Fig. 1), the 5′ end of *Stellaria PHYC* sequence was obtained by 5′RACE. A 756-bp fragment, including PCR primers, was obtained by direct sequencing of the 5′RACE product. After overlapping the 756-bp fragment of the 5′end of *PHYC* cDNA with a 3,237-bp initial *PHYC* cDNA, a 3,803-bp full-length *PHYC* cDNA sequence was obtained, comprising a 110-bp 5′-untranslated leader sequence, a 3,342-bp coding region, and a 351-bp 3′-untranslated sequence (Fig. 1).

In order to obtain *PHYC* genomic sequence, three pairs of *PHYC* gene-specific primers (PhyCF7/PhyCR7, QmCF/QmCR, and PhyCF6/PhyCR6, Table 1) were designed based on the *Stellaria PHYC* cDNA sequence. A 1,790-bp fragment was amplified with PhyCF7/PhyCR7, a 2,351-bp fragment was amplified with QmCF/QmCR, and a 2,036-bp fragment was amplified with PhyCF6/PhyCR6 by PCR using *Stellaria* genomic DNA

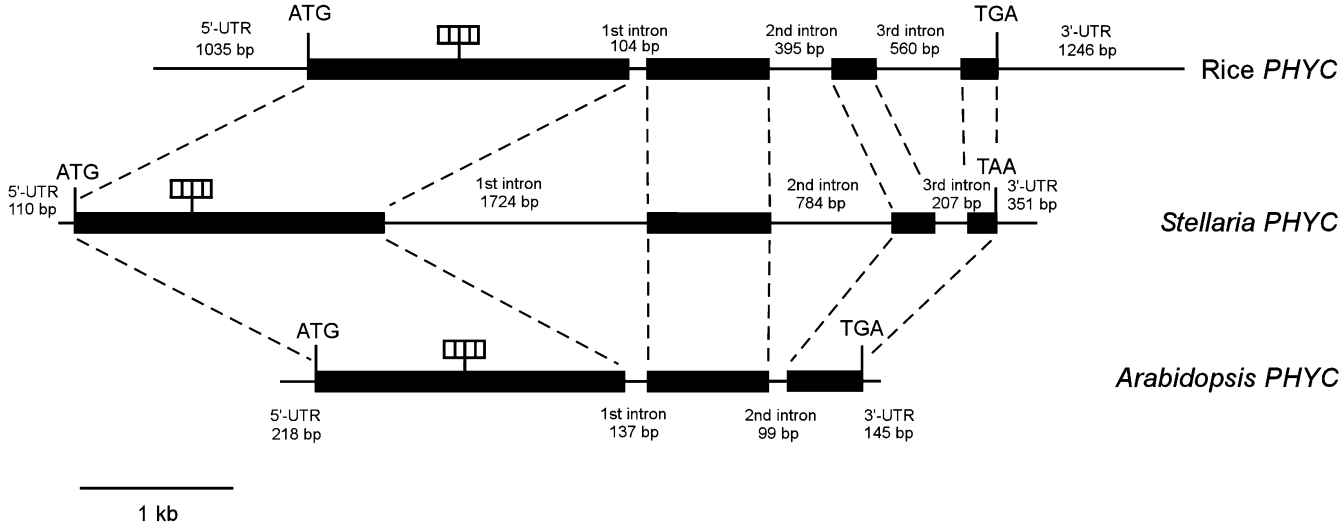


Fig. 1 A comparison of the *Stellaria*, rice and *Arabidopsis PHYC* gene structures over the protein-coding region. *Solid bars* Exons, *connecting lines* introns and untranslated portions of exons, *small hatched boxes* chromophore attachment sites. The number of nucleotides in each intron, and untranslated region is indicated

as template. A 6-kb genomic DNA fragment was obtained by overlapping these three fragments. Exon/intron boundaries were established by comparing the genomic sequence with the cDNA sequence. The physical organization of the *Stellaria PHYC* gene is similar to that of most other angiosperm phytochromes, with four exons of the coding region interrupted by three introns; an exception is *Arabidopsis PHYC* (Cowl et al. 1994), which lacks the third intron toward the 3′end of the coding region (Fig. 1). The *Stellaria PHYC* had three long introns of 1,724-bp, 784-bp and 207-bp in length, respectively (Fig. 1).

Southern blot analysis

S. longipes genomic DNA was digested with three different restriction enzymes and subjected to Southern

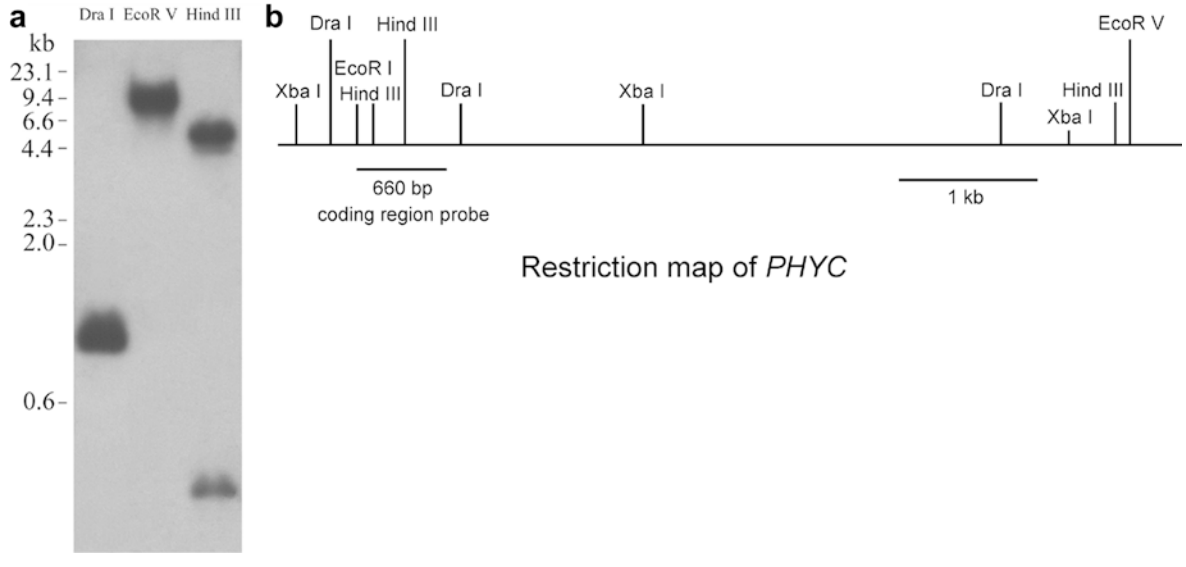
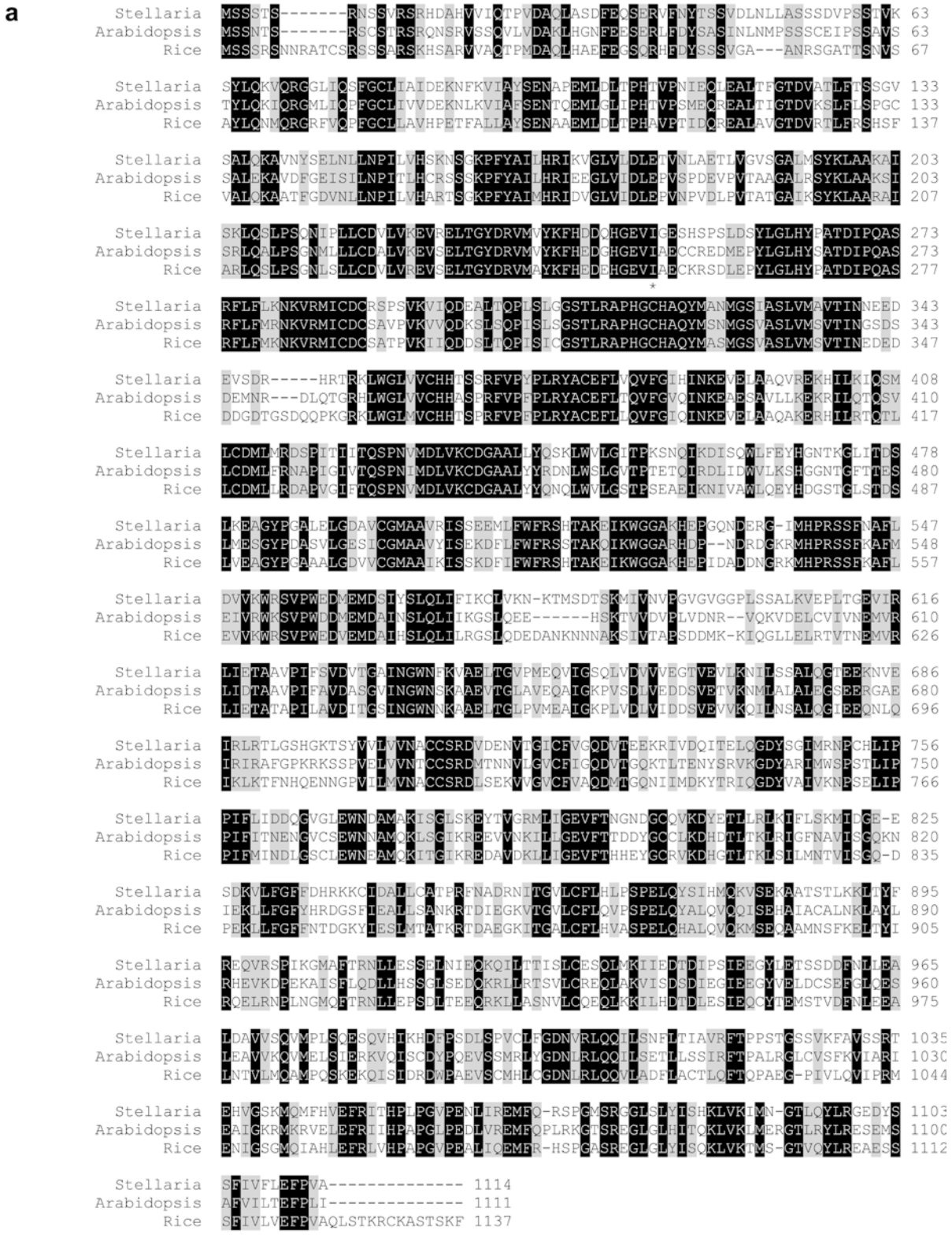


Fig. 2a,b Southern blot analysis and physical map of *Stellaria PHYC* gene. **a** Southern blot analysis. Total DNA (10 μg) from *Stellaria longipes* prairie ecotypes was digested with *Dra*I, *Eco*RV, or *Hin*dIII. Hybridization with an internal probe including the chromophore binding site was carried out under highly stringent conditions. The positions of λDNA/*Hin*dIII markers (kb) are shown on the left. **b** Restriction map of the *Stellaria PHYC* gene

▶

Fig. 3a,b *Stellaria* PHYC protein. **a** Alignment of the deduced amino acid sequences of the *Stellaria*, *Arabidopsis* and rice *PHYC* using CLUSTALX. Identical amino acids are highlighted in *black*, similar residues in *gray*. *Dashes* Deletions compared to the other genes, * predicted chromophore binding site (Cysteine). GenBank accession numbers: *Stellaria PHYC* (AF544029), *Arabidopsis PHYC* (X17343), rice *PHYC* (AF141942). **b** Multiple alignment of the histidine kinase-like domain (HKLD) of the *Stellaria* PHYC, PHYA1 (AF544027), PHYA2 (AY190013), *Arabidopsis* PHYC, rice PHYC, and *Sorghum* PHYC (U56731) proteins. * Histidine residue within HKLD domain present only in *Arabidopsis* PHYC and absent in all other PHY proteins


a
Stellaria
Arabidopsis
Rice
b
Stellaria PHYC 870
Arabidopsis PHYC 865
Rice PHYC 880
Sorghum PHYC 878
Stellaria PHYA1 876
Stellaria PHYA2 689

blot analysis (Fig. 2a). Using a coding region fragment of *Stellaria PHYC* as a probe (Fig. 2b), the homologous fragments showed strong hybridization signals. A single band was observed with *Dra*I or *Eco*RV digestion, and two bands were observed for *Hin*dIII digestion. Since *PHYC* has two *Hin*dIII recognition sites within the region targeted by the probe (Fig. 2b), digestion with *Hin*dIII should result in three bands. One of them should be 224-bp (Fig. 2a). However, we did not observe a third band corresponding to the 3′ *Hin*dIII fragment, presumably because either the region of overlap between the probe and the 3′ *Hin*dIII fragment (117-bp) is too short, or two *PHYC Hin*dIII fragments possibly comigrated. Since hybridization was carried out under highly stringent conditions, it is likely that only *PHYC* fragments were observed. The hybridization patterns indicate that the *PHYC* gene is present as a single copy in the *Stellaria* genome (Fig. 2a).

Stellaria PHYC protein

The polypeptide encoded by the *Stellaria PHYC* gene is predicted to be 1,114 amino acids in length (Fig. 3a). The nucleotide and amino acid sequence identities of the *Stellaria PHYC* compared with the individual members of the rice, *Sorghum*, *Arabidopsis*, and tomato *PHY* gene families have been analyzed by the alignment of complete *Stellaria PHYC* with other phytochromes (Table 2). The *Stellaria* PHYC shares 60%, 58% and 57% deduced amino acid identities with rice, *Sorghum*, and *Arabidopsis* PHYC, respectively. However, *Stellaria* PHYC and tomato PHYF share 62% identity (Table 2). The conserved residues are distributed throughout the polypeptide, but the region of highest conservation lies within the amino-terminal one-third of the protein (Fig. 3a). This portion of phytochrome has been suggested to be important for chromophore attachment to the apoprotein and for spectral integrity of the holophytochrome (Cherry et al. 1993; Clack et al. 1994). The chromophore-binding region is the same as in *Arabidopsis* and rice PHYC, which have a cysteine at position 318. In addition, the COOH-terminal domain of *Stellaria* PHYC has a copy of a sequence designated the histidine-kinase domain. The histidine residue of the canonical prokaryotic two-component sensor 'H-box' present in the majority of phytochromes (Quail 1997a, 1997b) is absent in *Stellaria* PHYC, PHYA1 and PHYA2 proteins (Li and Chinnappa 2003), as well as in rice and *sorghum* PHYC proteins (Fig. 3b; Basu et al. 2000).

Table 2 Identities (%) of *Stellaria PHYC* nucleotide and amino acid sequences with rice, *Sorghum* (monocot), *Arabidopsis*, tomato (dicot) *PHY* nucleotide and amino acid sequences

	Stellaria PHYC	
	Nucleotide	Amino acid
Rice *PHYA*	57	48
Rice *PHYB*	55	48
Rice *PHYC*	61	60
SorghumPHYA	57	49
SorghumPHYB	55	48
SorghumPHYC	61	58
Arabidopsis PHYA	57	51
Arabidopsis PHYB	57	49
Arabidopsis PHYC	62	57
Arabidopsis PHYD	56	48
Arabidopsis PHYE	55	45
Tomato *PHYA*	58	52
Tomato *PHYB1*	58	51
Tomato *PHYB2*	57	50
Tomato *PHYE*	57	45
Tomato *PHYF*	66	62

Phylogenetic analysis

Figure 4 presents the phylogenetic tree resulting from analysis of all full-length plant PHYC and PHYF amino acid sequences currently available in the databases. The tree indicates that all phytochromes examined are divided into two groups, monocots and dicots. *Stellaria* PHYC is located in the dicot branch as expected, but is relatively divergent from *Arabidopsis* PHYC.

PHYC gene expression

Stellaria PHYC gene expression has been determined in different organs, under different photoperiods, and at different R/FR ratios using semiquantitative RT-PCR. A 23S rRNA gene fragment was used as an internal control for RNA amount standardization. *PHYC* and *23S* gene-specific primers (QmCF/QmCR, 23SF/23SR; Table 1) were designed based on the *PHYC* and 23S cDNA sequences. They were initially tested with PCR and RT-PCR. A 2,351-bp *PHYC* fragment was amplified by PCR and a 627-bp *PHYC* fragment was amplified by RT-PCR with primer pair QmCF/QmCR. A 381-bp 23S fragment was amplified with primer pair 23SF/23SR by RT-PCR (data not shown). Both fragments (627-bp *PHYC* and 381-bp 23S) were amplified at the same time with the two primer pairs (QmCF/QmCR and 23SF/23SR; data not shown). If total RNAs were contaminated with genomic DNA, a 2,351-bp *PHYC* fragment would also be amplified by RT-PCR, in addition to the 627-bp *PHYC* fragment. Therefore, the primers for *PHYC* are not only gene specific, but can also distinguish the *PHYC* cDNA fragment from any contamination of genomic DNA by fragment size.

Figure 5 shows that *PHYC* is constitutively expressed in flowers, leaves, stems, and roots of mature *Stellaria* plants, but the level of *PHYC* mRNA in roots is slightly higher than in other organs. The relative transcript abundances of *PHYC* are 0.71 in roots, and 0.41–0.5 in flowers, leaves and stems (Fig. 5b).

To characterize the light stability of *Stellaria PHYC*, 2-week-old seedlings that were grown in the dark, and

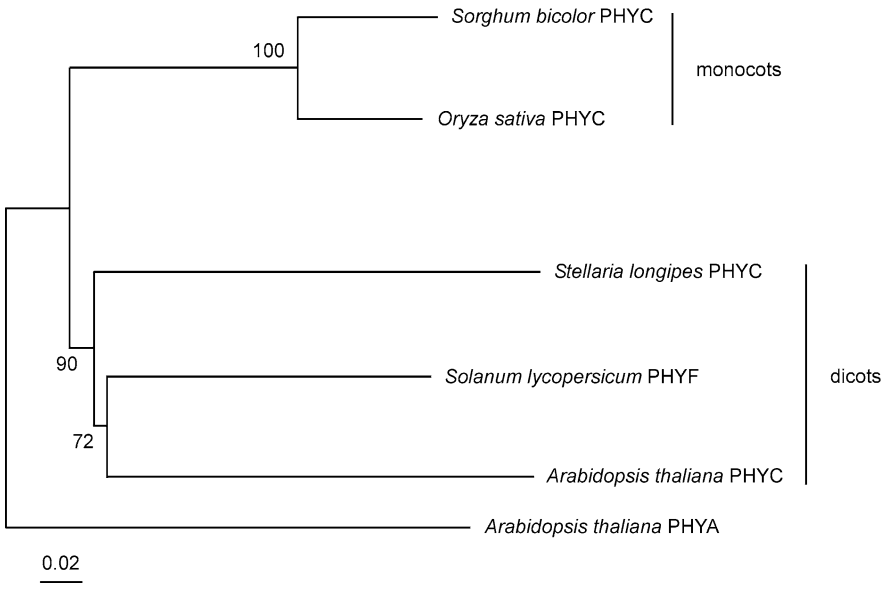


Fig. 4 A distance-based, neighbor-joining tree relating the complete *Stellaria* PHYC amino acid sequence to all known full-length plant phytochromes of PHYC/PHYF subfamily. Sequences were aligned with CLUSTALX 1.8 program. A neighbor-joining tree was constructed with NJplot. A bootstrap analysis (100 replicates) was performed with BOOTSTRAP. *Arabidopsis* PHYA was used as an outgroup. Sequences examined: *Arabidopsis* PHYA (X17341), PHYC (X17343), *Oryza* PHYC (AF141942), *Solanum* PHYF (AF178568), *Sorghum* PHYC (U56731), and *Stellaria* PHYC (AF544029)

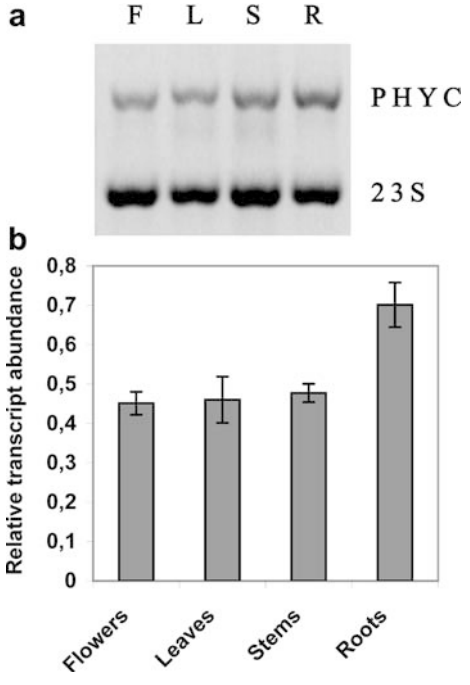


Fig. 5a,b Analysis of *PHYC* transcript levels in different organs. *Stellaria* plants grown in short-day cold (SDC) conditions for at least 90 days were transferred into a growth chamber with long-day warm (LDW) conditions (22°C day/18°C night, 16 h photoperiod) until reproductive stage, and then leaves (*L*), stems (*S*), and flowers (*F*) were collected. Roots (*R*) were obtained from the young remets planted in 8 cm pots containing sterile sand under LDW for 14 days. **a** RT-PCR analysis of *PHYC* expression; 5 µl RT-PCR product for each sample was separated on a 1.7% (w/v) agarose gel and visualized with ethidium bromide under UV light. **b** *PHYC* gene expression relative to *23S* control. *PHYC* transcript abundance levels are given as the ratio of optical density of *PHYC*/*23S* bands. *Error bars* Standard error of the mean. The results are from at least two individual experiments

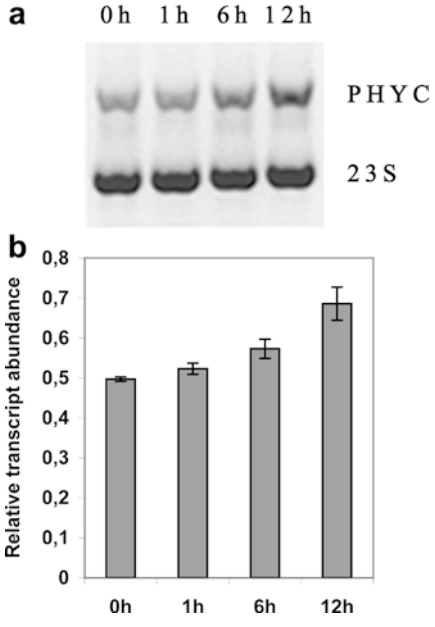


Fig. 6a,b *PHYC* gene expression patterns in seedlings. Seedlings were grown for 14 days in the dark (0 h), and then transferred to continuous white light for the times indicated (1 h, 6 h, 12 h). *PHYC* transcript levels were analyzed by semiquantitative RT-PCR. **a** RT-PCR analysis of *PHYC* expression; 5 µl RT-PCR product for each sample was separated on a 1.7% (w/v) agarose gel and visualized with ethidium bromide under UV light. **b** Graph of *PHYC* gene expression relative to *23S* control. Levels are given as the ratio of optical density of *PHYC*/*23S* bands. *Error bars* Standard error of the mean. The results are from at least two individual experiments

grown in the dark but irradiated with 1 h, 6 h or 12 h of continuous white light before harvest, were analyzed by semiquantitative RT-PCR. The levels of *PHYC* gene transcription increased slightly when *Stellaria* seedlings grown in complete darkness for 14 days (0 h) were exposed to continuous white light for 1 h, 6 h, or 12 h (Fig. 6a, b). The relative transcript abundance reached 0.7 at 12 h, which is about 1.5-fold higher than the control (0 h).

Plants grown in SDC were transferred to darkness for 7 days and then transferred to LDW with R/FR = 0.7 or 3.5. Dark-adapted plants showed the lowest level of *PHYC* mRNA (Fig. 7a–c). Transfer of the dark-adapted plants into R/FR = 0.7 or 3.5 increased *PHYC* gene transcript levels within 1 h of exposure. Maximum accumulation of *PHYC* transcript occurred after 9 h of exposure to R/FR = 0.7 (Fig. 7a, c), or 6 h of exposure to R/FR = 3.5 (Fig. 7b, c). An interesting observation is that the level of *PHYC* gene transcript mRNA under low R/FR ratio (0.7) was much higher than under high R/FR ratio (3.5) (Fig. 7c).

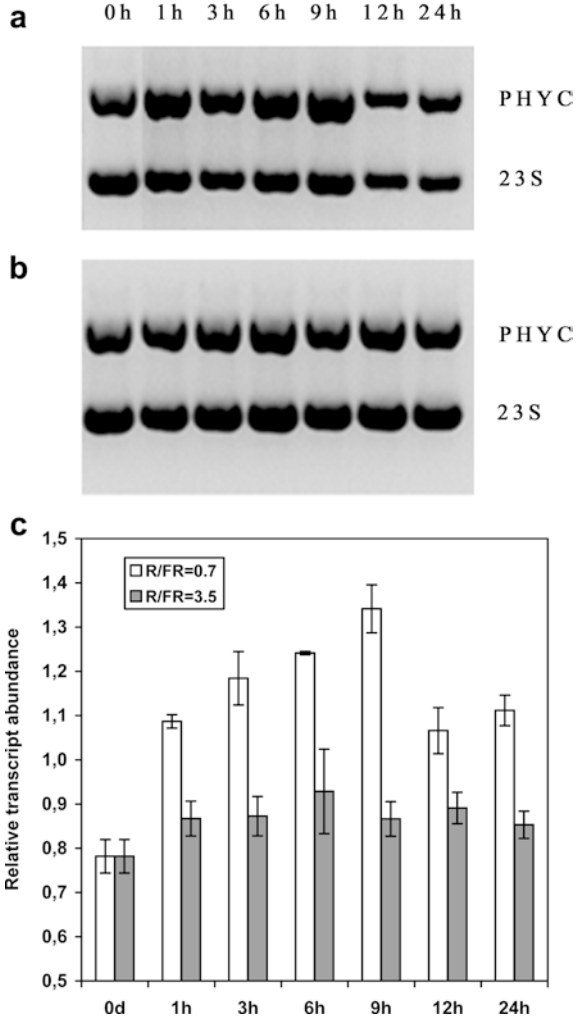


Fig. 7a–c *PHYC* transcript levels under varied red/far-red (R/FR) ratios. *Stellaria* plants grown under SDC conditions for at least 90 days and in the dark for 7 days (0 h), were transferred into two different chambers with R/FR = 0.7 or = 3.5 for 1–24 h. *PHYC* gene expression was analyzed by semiquantitative RT-PCR; 5 µl RT-PCR product for each sample was separated on a 1.7% (w/v) agarose gel, visualized with ethidium bromide staining under UV light, and analyzed with NIH Image analysis software (Research Services Branch of the National Institute of Mental Health, USA). **a** *PHYC* expression in R/FR = 0.7. **b** *PHYC* expression in R/FR = 3.5. **c** Graph of *PHYC* gene expression relative to *23S* control. The number is given as a ratio of optical density of *PHYC*/*23S* bands. *Error bars* Standard error of the mean. The results are from at least two individual experiments

The plants grown in SDC were transferred to short-day (8 h/day) and long-day (20 h/day) photoperiods, and were used to determine the effect of different photoperiods on *PHYC* gene expression. Under SD conditions, *PHYC* transcript levels were generally higher, except at 11 days (Fig. 8a, c), while under the LD conditions they remained at levels similar to those in SDC-grown plants except at 3 days (Fig. 8b, c). Maximum accumulation of *PHYC* transcript occurred at 15 days exposure to SD or LD conditions, which reached 1.11 and 0.85, respectively (Fig. 8c). The results of stem elongation under the two different photoperiods are shown in Fig. 8d. Plants grown under SD conditions did not elongate; however, plants grown under LD conditions did elongate. Average heights reached about 1.7 cm and 21.2 cm tall at 19 days under SD and LD conditions, respectively (Fig. 8d). In addition, we observed that plants did not flower (at 60 days) under SD conditions. In contrast, plants flowered at 18–19 days under LD conditions.

Discussion

PHY gene structure

All plant *PHY* genes thus far reported contain three introns located at relatively conserved regions within their coding regions, with the exception of the *Arabidopsis PHYC* which lacks the third intron (Cowl et al. 1994). Since rice, *Sorghum*, and other lower-plant *PHYC* genes have three introns, Basu et al. (2000) suggested that the absence of the *Arabidopsis* third intron is the result of a relatively recent evolutionary event after monocot/dicot divergence. However, our data indicates that *Stellaria PHYC* (dicot) also contains three introns at all conserved locations within the coding region, which is similar to rice *PHYC* (Fig. 1) and *Sorghum PHYC* (data not shown). Therefore, it is not clear as to when the *Arabidopsis PHYC* would have lost its third intron. In addition, the intron sizes between different *PHY* genes are significantly different. The first introns in the *Arabidopsis* and rice *PHYC* genes are only 137-bp and 104-bp in length, respectively. In contrast, the first intron of *Stellaria PHYC* is 1,724-bp in length (Fig. 1). The function of this long intron in *Stellaria PHYC* is currently unknown. A BLAST search using the 110-bp 5′-UTR of the *Stellaria PHYC* showed no match to the 5′-UTR of the *Arabidopsis*, rice, or *Sorghum PHYC* genes.

Stellaria PHYC protein

The deduced amino acid sequence of rice *PHYC* shares significantly higher identity with *Sorghum* PHYC (86%)

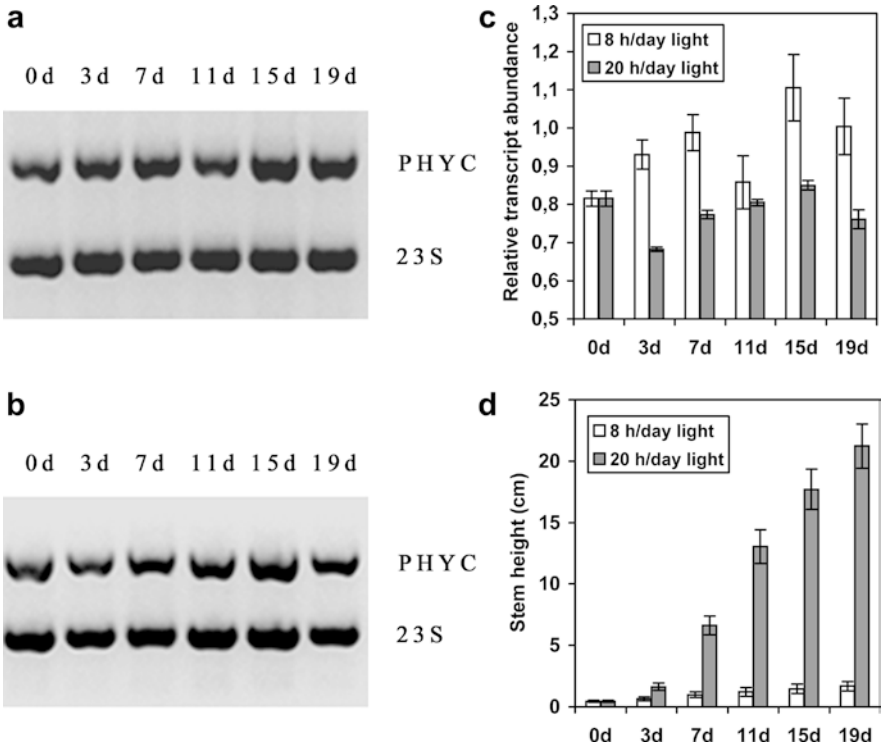


Fig. 8a–d *PHYC* gene expression and stem elongation under two different photoperiods. *Stellaria* plants grown under SDC conditions for at least 90 days (0 days) were transferred into two different growth chambers with 8 h/day (SD) or 16-h/day (LD) conditions for the indicated times. *PHYC* gene expression was analyzed by semiquantitative RT-PCR; 5 µl RT-PCR product for each sample was separated on a 1.7% (w/v) agarose gel, visualized with ethidium bromide under UV light, and analyzed with NIH Image analysis software. **a** *PHYC* gene expression under SD conditions. **b** *PHYC* gene expression under LD conditions. **c** Graph of *PHYC* gene expression relative to *23S* control. Levels are given as the ratio of optical density of *PHYC*/*23S* bands. *Error bars* Standard error of the mean. The results are from at least two individual experiments. **d** Stem elongation under SD and LD conditions. *Error bars* Standard error of the mean. The stem height results are from six individual plants

than with *Arabidopsis* PHYC (59%) (Basu et al. 2000). Since both *Stellaria* and *Arabidopsis* are dicots, the *Stellaria* PHYC should share a much higher identity with *Arabidopsis* PHYC than with rice or *Sorghum* PHYC. In fact, *Stellaria PHYC* shares lower identity (57%) with *Arabidopsis* PHYC than with *Sorghum* (58%), or rice *PHYC* (60%) (Table 2). This result indicates that the *Stellaria* PHYC has diverged more from *Arabidopsis* PHYC than from rice or *Sorghum* PHYC.

Comparison of the *Stellaria* PHYC amino acid sequence with other phytochromes indicates that it is similar to rice, *Sorghum*, and *Arabidopsis* PHYC protein. The *Stellaria* PHYC has a number of sequence features typical of conventional plant phytochromes (Quail 1997a, 1997b), including a C-terminal histidine-kinase-related domain (Fig. 3b). Although the histidine in the 'H-box' is present in the majority of eukaryotic phytochromes (Quail 1997b), e.g., rice and *Sorghum* PHYC, *Stellaria* PHYC does not have this histidine in the 'H-box'. *Stellaria* PHYA1 and PHYA2 (Li and Chinnappa 2003) also lack this histidine, in contrast to rice and *Arabidopsis* PHYA, which do contain this histidine residue (Fig. 3b). Thus, based on this analysis, the absence of the histidine in the 'H-box' is a feature not only of PHYC members (Basu et al. 2000), but also of some PHYA members.

PHYC gene phylogeny

The phytochromes in all plants examined are encoded by small, multigene families that have been classified into four subfamilies: *A*, *B*/*D*, *C*/*F*, and *E* (Mathews et al. 1995; Mathews and Sharrock 1996, 1997; Pratt et al. 1997). In the *Arabidopsis PHY* gene family, three diversification events have occurred: the *PHYA*/*C*-*PHYB*/*D*/*E* duplication near the origin of seed plants, and the *PHYA*-*PHYC* and *PHYB*/*D*-*PHYE* duplications near the origin of flowering plants (Mathews and Sharrock 1997). The *Stellaria PHY* gene family is composed of six members (four *PHYAs*, one *PHYB*, and one *PHYC*) that have been classified into three subfamilies: *PHYA*, *PHYB*, and *PHYC* (Li and Chinnappa 2003). Analysis of *PHYC* gene phylogeny indicates that the *Stellaria* PHYC branch is located at the middle of the rice/*Sorghum* PHYC branch (monocots) and *Arabidopsis* PHYC/tomato PHYF branch (dicots), though *Stellaria* PHYC belongs to the dicot group (Fig. 4). This is similar to the results we observed in the phylogenic analysis of *Stellaria PHYA* and *PHYB* (Li and Chinnappa 2003).

Stellaria PHYC expression

The *Stellaria PHYC* gene is expressed in different organs, but the level of *PHYC* mRNA in roots is slightly higher than in flowers, leaves and stems (Fig. 5a, b). These results are different from those reported for *PHYC* in *Arabidopsis* (Sharrock and Quail 1989; Clack et al. 1994). Based on analysis of Northern blots, the *Arabidopsis PHYC* mRNA has been found to be fairly uniformly distributed in roots, leaves, stems, and flowers of mature plants (Clack et al. 1994). This difference may be due to the different materials and methodologies used. In addition, mRNA levels increased slightly after seedlings were exposed to continuous white light for 1–12 h, reaching a relative level of 0.69 at 12 h (Fig. 6a, b), which was about 141% of that in the dark (data not shown).

Plants have evolved an endogenous circadian clock to allow the synchronization of internal events with daily changes in the external environment. For a circadian clock to function correctly, however, it must have not only a 24-h period but should also be entrained in the correct relationship to the local day/night cycle. Light signals at dawn and dusk are most important in entraining circadian clocks, though temperature cycles also contribute (Lumsden and Millar 1998). The entraining light signals are transduced by light input pathways, which are involved in at least two classes of photoreceptors, phytochromes and cryptochromes. However, the rhythmic expression of many genes becomes arrhythmic upon transfer to constant darkness, for example, adopting a constant level within two or three cycles. This could occur due to the effect of light on the clock via the input pathways, since many rhythmic processes are regulated by light, or because light affects the coupling between the clock and some target proteins (Hall et al. 2001). In tomato, following a light-to-dark transition, all five *PHY* transcripts exhibit modest increases during the subsequent 48 h. However, following a dark-to-light transition, *PHYA* and *PHYB2* transcripts exhibit a marked decline. *PHYF* exhibits a negligible response to the transition. *PHYB1* and *PHYE* undergo a transient increase during the first few hours, followed by a decline and, at least in the case of *PHYB*, a second transient increase (Hauser et al. 1998). In order to study the effect of different R/FR ratios on *PHYC* gene expression in *Stellaria*, we used 2-week-old seedlings grown in the dark as the initial material to minimize the effect of the rhythmic expression of the *PHYC*, followed by transfer to R/FR = 0.7 or 3.5 for 1–24 h (see Fig. 7a–c). The results indicate that patterns of *Stellaria PHYC* gene expression are different under various R/FR ratios. The level of *PHYC* mRNA under R/FR = 0.7 is much higher than under R/FR = 3.5. The peak period was 9 h for R/FR = 0.7 and 6 h for R/FR = 3.5 (Fig. 7a–c).

The pattern of *PHYC* gene expression under 8 h/day photoperiods is significantly different from 20 h/day (Fig. 8a–c). Under 8 h photoperiods, *PHYC* transcript levels were generally higher. However, under 20 h photoperiods *PHYC* transcript levels remained at levels similar to those in SDC-grown plants. The apparent inconsistencies in this data set are the *PHYC* transcript levels under 8 h photoperiods at 11 days and under 20 h photoperiods at 3 days (Fig. 8a–c). These results were from three replicated experiments. In each individual experiment, we observed similar results. In addition, we also determined the expression pattern of the *PHYC* gene under 16 h photoperiods. The patterns under 16 h photoperiods were identical to those under 20 h photoperiods (data not shown). The phenotype under 16 h photoperiods was identical to that under 20 h photoperiods, but was significantly different from that under 8 h photoperiods. However, although these observations were confirmed experimentally, it is not understood why the levels of *PHYC* mRNA were lower at 3 days under SD conditions or at 11 days under LD conditions. We also determined *PHYC* mRNA levels at 1 day, and 2 days after the plants were transferred from SDC to SD or LD conditions. Under LD conditions, the levels of *PHYC* mRNA decreased significantly at 1 days or 2 days. This result also confirms that the lowest level of *PHYC* mRNA occurred at 3 days. In contrast, under SD conditions, the levels of *PHYC* increased significantly at 1 day and 2 days (data not shown). Thus, we propose that the levels of *PHYC* mRNA between the 1st and 3rd day may play an important role in the control of stem elongation in *S. longipes*. During the period between 1 day and 3 days, the low or high levels of *PHYC* mRNAs may induce different physiological and developmental pathways.

Plants grown under an 8 h/day photoperiod did not elongate at all and were only about 1.7 cm tall at 19 days. In contrast, plants grown under a 20 h/day photoperiod elongated and had an average height of 21.2 cm at 19 days. In addition, plants did not flower under SD conditions, while plants flowered at 18–19 days under LD conditions. These results indicate that SD photoperiods may initially stimulate *PHYC* gene expression, and the subsequently higher levels of *PHYC* mRNA may inhibit stem elongation and flower initiation. If this hypothesis is correct, the question remains as to why there is a reduction of *PHYC* mRNA from 7 days to 19 days under a 20 h photoperiod, where elongation is induced. Recently, Monte et al. (2003) reported that *PHYC* is involved in photomorphogenesis throughout the life cycle of the plant, with a photosensory specificity similar to that of *PHYB*/*D*/*E* and with a complex pattern of differential crosstalk between *PHYA* and *PHYB* in the photoregulation of multiple developmental processes. In the *PHYA* background, for example, the *phyc* mutation led to late flowering under LD conditions compared with the wild type and *phya* mutants. The data suggested that *PHYC* has a positive effect on flowering under LD conditions in a manner redundant with *PHYA*. In contrast, under SD conditions, the *phyc* mutant exhibited early flowering compared with wild type, suggesting that *PHYC* has an inhibitory role in flowering induction. Thus, *PHYC* is

able to promote flowering under LD conditions, and inhibit initiation of flowering under SD conditions (Monte et al. 2003). Hence, we can assume that *Stellaria* plants did not flower under SD conditions due to the high levels of *PHYC* mRNA, which inhibits flower initiation under SD conditions. Under LD conditions, *Stellaria* plants that flower normally are likely to flower due to the high levels of *PHYC* during the initiation of flowering (~15 days) since *PHYC* is able to promote flowering under LD conditions. Hence, it is possible that *PHYC* may have functions in the control of stem elongation and in the control of floral initiation in *S. longipes* similar to those found in *Arabidopsis* studies (Franklin et al. 2003a; Monte et al. 2003).

Temperature is one of the most important factors regulating plant development (Blazquez et al. 2003; Halliday and Whitelam 2003; Halliday et al. 2003). Many plants, for example, have adopted a reproductive strategy that requires long periods of cold (1–10°C) to promote flowering. Halliday et al. (2003) demonstrated that ambient temperature is a significant modulator of photoreceptor action in the control of flowering. A modest reduction in growth temperature, from 22°C to 16°C, completely abolished the *phyB* mutant early-flowering phenotype frequently observed at higher temperature. Thus, small changes in ambient temperature can have a large impact on photoreceptor action (Halliday and Whitelam 2003). In addition, Blazquez et al. (2003) reported that the expression of some genes involved in the control of flowering is higher at 23°C than at 16°C, while others are lower at 23°C than at 16°C. In this study, we also observed that the levels of *PHYC* transcript increased markedly when the plants were transferred from SDC (8-h light/16-h dark, at 4°C; 0 days) to SDW (8-h light/16-h dark, at 22°C; 3 days) (Fig. 8c). The data suggests that *PHYC* gene expression is upregulated by temperature in *S. longipes*. The differences observed in the *PHYC* gene expression studies, however, are probably not due to the effects of temperature since the temperatures were identical, whereas R/FR ratios were different. Therefore, any differences in the levels of *PHYC* mRNA between R/FR = 0.7 and 3.5 (Fig. 7) or between 8 h/day and 20 h/day photoperiod (Fig. 8) were likely due to quality of light and photoperiod rather than temperature.

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