

Ectopic expression of a phytochrome B gene from Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) in *Arabidopsis thaliana* promotes seedling de-etiolation, dwarfing in mature plants, and delayed flowering

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Abstract Phytochrome B (phyB) is an essential red light receptor that predominantly mediates seedling de-etiolation, shade-avoidance response, and flowering time. In this study, we isolate a full-length cDNA of *PHYB*, designated *BrPHYB*, from Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*), and we find that BrphyB protein has high amino acid sequence similarity and the closest evolutionary relationship to *Arabidopsis thaliana* phyB (i.e., AtphyB). Quantitative reverse transcription (RT)-PCR results indicate that the *BrPHYB* gene is ubiquitously expressed in different tissues under all light conditions. Constitutive expression

of the *BrPHYB* gene in *A. thaliana* significantly enhances seedling de-etiolation under red- and white-light conditions, and causes dwarf stature in mature plants. Unexpectedly, overexpression of BrPHYB in transgenic *A. thaliana* resulted in reduced expression of gibberellins biosynthesis genes and delayed flowering under short-day conditions, whereas AtPHYB overexpression caused enhanced expression of FLOWERING LOCUS T and earlier flowering. Our results suggest that BrphyB might play an important role in regulating the development of Chinese cabbage. BrphyB and AtphyB have conserved functions during de-etiolation and vegetative plant growth and divergent functions in the regulation of flowering time.

Mei-Fang Song, Shu Zhang and Pei Hou have contributed equally to this work.

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Introduction

Light is a major environmental signal that affects multiple plant developmental processes, including seed germination, photomorphogenesis, leaf expansion, stem elongation, phototropism, stomata and chloroplast movement, shade avoidance, circadian rhythms, and flowering time (Deng and Quail 1999; Wang and Deng 2003; Jiao et al. 2007; Li et al. 2011). Plants have evolved a variety of photoreceptors to monitor light changes in their environments (Wang 2005; Mathews 2005, 2006; Mathews and McBreen 2008). In *Arabidopsis thaliana*, five distinct phytochromes, designated phytochrome A (phyA) to phyE, have been characterized as red (R)/far-red (FR) reversible photoreceptors (Sharrock and Quail 1989; Clack et al. 1994). PhyA is the only light labile or type I phytochrome in *A. thaliana*, and

phyB-E are light stable or type II phytochromes (Quail et al. 1973; Clough and Vierstra 1997; Quail 1997; Hirschfeld et al. 1998).

The *A. thaliana* phyB apoprotein, which contains a GAF domain, two Per-Arnt-Sim (PAS) domains, a His Kinase A domain (HisKA), and a HATPase_c domain, promotes seed germination and seedling de-etiolation in response to R light and inhibits shade-avoidance responses (Koornneef et al. 1980; Nagatani et al. 1991; Somers et al. 1991; Reed et al. 1993; Koornneef et al. 1995; Krall and Reed 2000; Tepperman et al. 2004). Several mutants deficient in *PHYB* have been found in crop species, such as rice (*Oryza sativa* L.), maize (*Zea mays* L.), pea (*Pisum sativum* L.), sorghum [*Sorghum bicolor* (L.) Moench], tomato (*Solanum lycopersicum* L.), soybean (*Glycine max* L.), and barley (*Hordeum vulgare* L.) (Weller and Reid 1993; Childs et al. 1997; Weller et al. 1997; Lazarova et al. 1998; Hanumappa et al. 1999; Takano et al. 2005; Sheehan et al. 2007; Wu et al. 2011). These previous studies indicated that *phyB* mutants of sorghum and rice, and the *phyB1/phyB2* double mutant of maize, display the constitutive shade-avoidance syndrome (SAS) symptoms, which include increased plant height, increased internode length, reduced tiller number, and early flowering (Pao and Morgan 1986a, b; Childs et al. 1992, 1997; Sheehan et al. 2007).

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is a widely cultivated and economically important vegetable crop in Asia. The main objectives of Chinese cabbage breeding have been to increase yield, enhance resistance to environmental stresses, improve nutritional quality, and modify flowering time, by means of genetic manipulation. Although the *phyB* mutant of *B. rapa* has been found (Devlin et al. 1992, 1997), the molecular characteristics have not been well described. In this study, we isolated a *PHYB* homolog (designated as *BrPHYB*) from Chinese cabbage (inbred line “G90E16”) and analyzed its expression profile and functional roles in transgenic *A. thaliana* plants. Our results suggest that *BrPHYB* plays an important role in modulating the development of Chinese cabbage and that *BrPHYB* overexpression significantly enhances seedling de-etiolation and mature plant dwarfing. The functions of *BrphyB* and *AtphyB* in de-etiolation and dwarfing are conserved, but their regulation of flowering time differs markedly.

Materials and methods

Plant materials and growth conditions

The Chinese cabbage inbred line “G90E16” (Zhengzhou City, Henan Province, People’s Republic of China) was grown in a greenhouse under a 16-h-light/8-h-dark

(long-day conditions, LD) photoperiod or an 8-h-light/16-h-dark (short-day conditions, SD) photoperiod at 22 °C. The *phyB-9* mutant (Reed et al. 1993; McNellis et al. 1994) and the *AtPHYB-GFP* transgenic line (Zheng et al. 2013) are of the *A. thaliana* Columbia (Col-0) ecotype. *A. thaliana* seeds were surface-sterilized and sown in a Murashige & Skoog (MS) medium (pH 5.8 and 0.9 % w/v agar). The seeds were incubated at 4 °C for 3 days, exposed to white (W) light for 3 h to stimulate germination, and then cultured for 4 days at 22 °C under different light and photoperiodic conditions. FR, R, blue (B), and W light treatments for seedlings were as described previously (Yang et al. 2005; Zheng et al. 2013). FR, R, and B light conditions were imposed using light-emitting diode (LED) light sources (model E-30LEDL3; Percival Scientific, Perry, IA, USA), with irradiance fluence rates of approximately 2.0, 30.0, and 50.0 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively, unless otherwise indicated (model ILT1400A with sensor model SEL-033/F/W; International Light Technologies, Peabody, MA, USA). White light was supplied using cool-white fluorescent lamps with an irradiance fluence rate of approximately 100.0 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Hypocotyl lengths were measured to an accuracy of 0.1 mm using the ImageJ software (<http://imagej.nih.gov/ij/>).

Isolation of the full-length cDNA, sequence analysis, plant transformation and selection of transgenic plants

Total RNA was extracted from the second true leaves of the inbred line “G90E16” using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and converted into first-strand cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). A full-length *BrPHYB* cDNA was isolated by PCR using the gene-specific forward and reverse primers 5'-ATGGTTTCCGGAGTCG-3' and 5'-CTATGGCATCATCAACATCATGTC-3'. The PCR products were inserted into the pEASY-T1 cloning vector (TransGen Biotech, Beijing, China) and sequenced by Beijing AuGCT DNA-SYN Biotechnology Co., Ltd. (Beijing, China).

The *BrPHYB* coding sequence was subsequently cloned into the binary expression vector *pJIM19-Myc* with a CaMV-35S promoter driving constitutive expression, a c-myc epitope tag, and a hygromycin resistance gene (*hpt*) as a selectable marker (Yang et al. 2005). The *pJIM19-Myc-BrPHYB* binary construct was electroporated into the *Agrobacterium* strain *GV3101* and then introduced into the wild-type (WT) *A. thaliana* (Columbia ecotype, Col-0) and the *phyB-9* mutant via a floral dip method (Clough and Bent 1998). Transgenic plants were selected on germination plates containing 25 $\mu\text{g/mL}$ hygromycin for *Myc-BrPHYB*. We selected about 40 T₁ lines and allowed them to produce T₂ seeds. Phenotypic analysis was conducted on T₂ plants with single T-DNA insertion and then confirmed

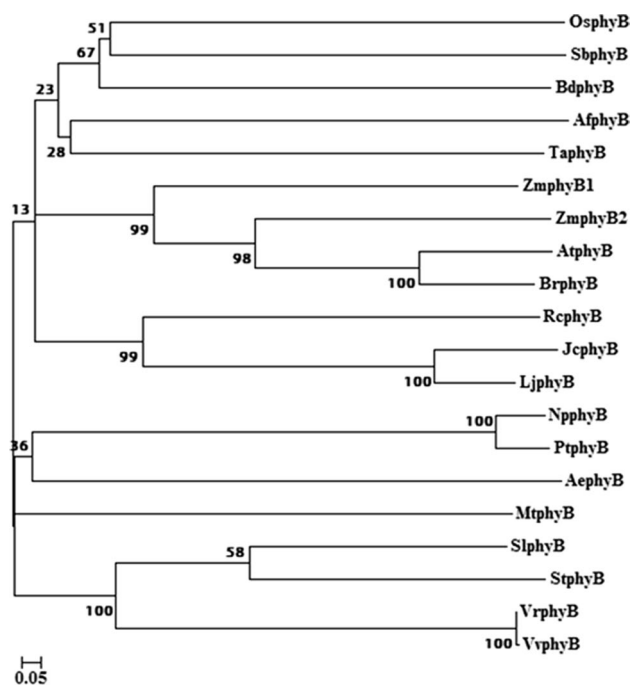


Fig. 1 Phylogenetic analysis of amino acid sequences of BrphyB and other related phyB. A neighbor-joining (Saitou and Nei 1987) tree was constructed using MEGA version 6.0 (<http://www.megasoftware.net/>; Tamura et al. 2013) based on the full-length amino acid sequences. Bootstrap analysis with 1000 replicates was performed to obtain confidence levels for the branches of the tree (Felsenstein 1985). The bar represents 0.05 amino acid substitutions per site in the primary structure. PhyB sequences and selected species (GenBank or UniProtKB/Swiss-Prot accession number in parenthesis) are as follows: *Amphicarpaea edgeworthii* phyB (AephyB, AEA41043), *Aquilegia formosa* phyB (AfphyB, ACV87354), *Arabidopsis thaliana* phyB (AtphyB, NP_179469), *Brachypodium distachyon* phyB (BdphyB, XP_003558068), *Brassica rapa* phyB (BrphyB, AGW45010), *Glycine max* phyB1 (GmphyB1, NP_001240097), *Glycine max* phyB2 (GmphyB2, ACE79199), *Jatropha curcas* phyB (JcphyB, KDP27911), *Lotus japonicus* phyB (LjphyB, BAF44083), *Medicago truncatula* phyB (MtphyB, XP_003594734), *Nicotiana glauca* phyB (NpphyB, CAA74992), *Oryza sativa* L. ssp. *japonica* phyB (OsphyB, Q10MG9), *Populus trichocarpa* phyB (PtphyB, XP_002312330), *Ricinus communis* phyB (RcphyB, XP_002519230), *Sorghum bicolor* phyB (SbphyB, XP_002467973), *Stellaria longipes* phyB (SlphyB, AAQ11872), *Solanum tuberosum* phyB (StphyB, NP_001274786), *Triticum aestivum* phyB (TaphyB, AAX76779), *Vitis riparia* phyB (VrphyB, ACC60970), *Vitis vinifera* phyB (VvphyB, XP_002278263), *Zea mays* phyB1 (ZmphyB1, DAA45039), and *Zea mays* phyB2 (ZmphyB2, NP_001168077). See also Figure S1

in the T₃ generation. Most experiments involved homozygous T₃ or T₄ plants.

Quantitative RT-PCR

Quantitative RT-PCR assays for *BrPHYB*, *BrACT1* (*BrACT2*), *CO*, *FT*, *SOC1*, *GA3OX1*, *GA20OX1*, *AtACTIN2* (*ACT2*) were performed using the gene-specific primers

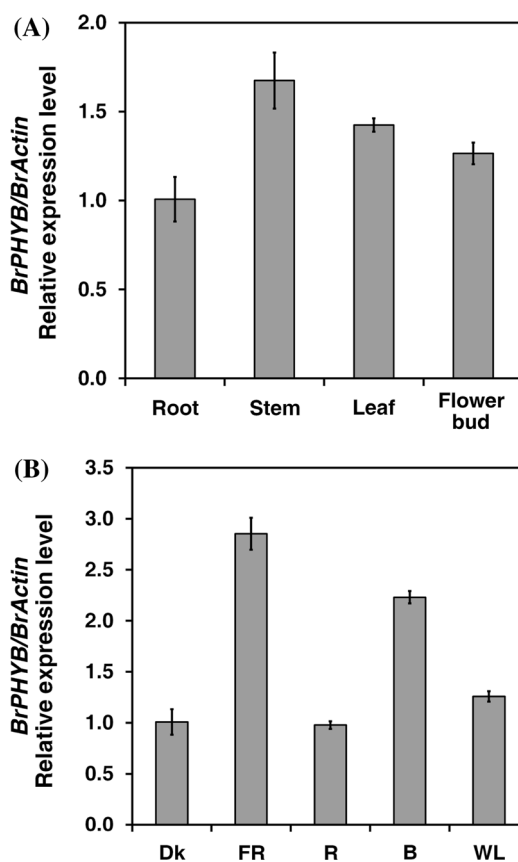


Fig. 2 Tissue-specific and light-regulated expression of *BrPHYB* gene in Chinese cabbage. **a** Tissue-specific expression of *BrPHYB* was analyzed by quantitative reverse-transcription (RT)-PCR in roots, stems, leaves, and flower buds. Seedlings of Chinese cabbage (inbred line “G90E16”) were grown under long-day (LD, 16-h-light/8-h-dark) conditions for 60 days. Each column shows the mean relative expression of *BrPHYB/BrActin* of three biological repeats. Error bars indicate the standard deviation. **b** Light-regulated expression of *BrPHYB*. Seedlings were grown in the dark (Dk), far-red light (FR), red light (R), blue light (B), or white light (WL) for 4 days

(Table S1) and an ABI StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR Premix ExTaq polymerase (Takara, Shiga, Japan). For tissue-specific expression of *BrPHYB*, plants of Chinese cabbage (inbred line “G90E16”) were grown under long-day (LD, 16-h-light/8-h-dark) conditions for 60 days. For the expression levels of regulatory genes related to flowering time and GA biosynthesis in transgenic *Myc-BrPHYB* or *AtPHYB-GFP* lines, the seedlings were grown under short-day (SD, 8-h-light/16-h-dark) for 12 days. The thermal cycle used was 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. Changes in the threshold crossing (Ct) were used to calculate relative levels of each mRNA using the formula $2^{-\Delta Ct}$. Relative expression was determined after normalization against the reference gene *BrACT* (Fig. 2) or *AtACT2* (Figs. 4b, 6a–e).

Expression data were analyzed using the StepOne software (ABI, Applied Biosystems). Each column represented the mean relative expression of three biological repeats; error bars indicated the SE. The primers for quantitative RT-PCR are listed in the Supplemental Data (Table S1).

Flowering time

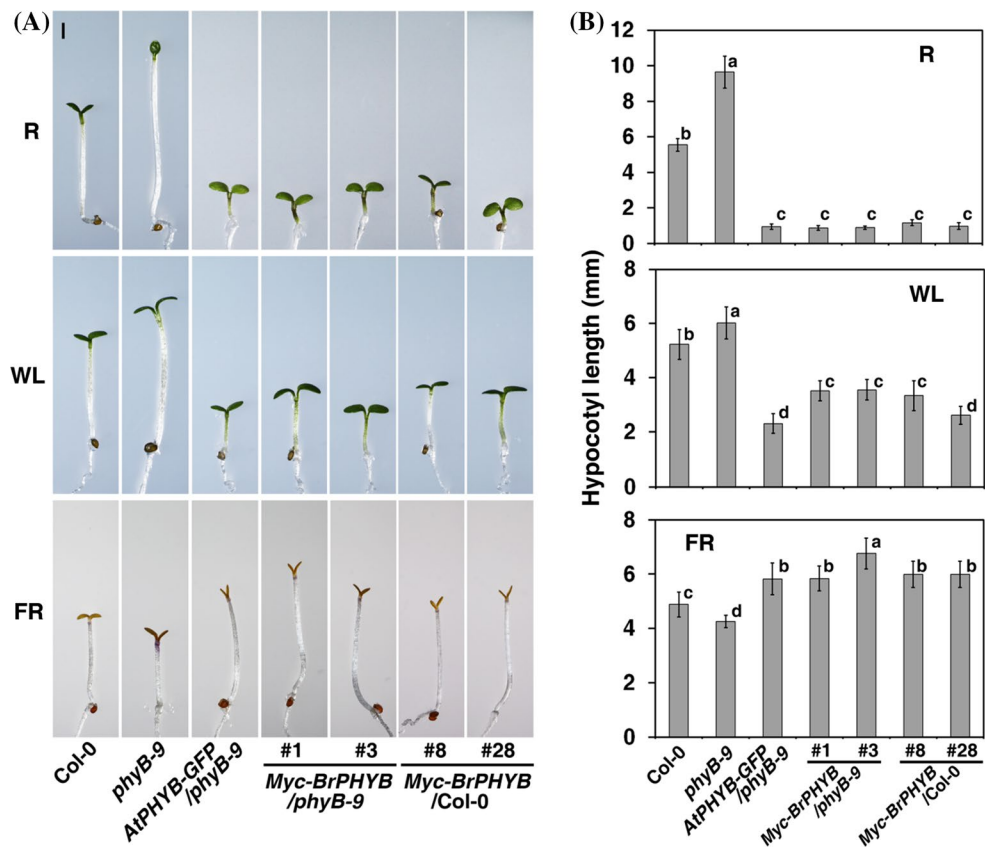
To study flowering time, Col-0 WT, *phyB-9* mutant (Reed et al. 1993; McNellis et al. 1994), *AtPHYB-GFP* and *Myc-BrPHYB* transgenic plants were grown in a greenhouse under LD or SD photoperiod conditions at 22 °C. The number of rosette leaves formed by the primary meristem and the number of days to flowering were recorded. Means were calculated over at least 20 plants per line.

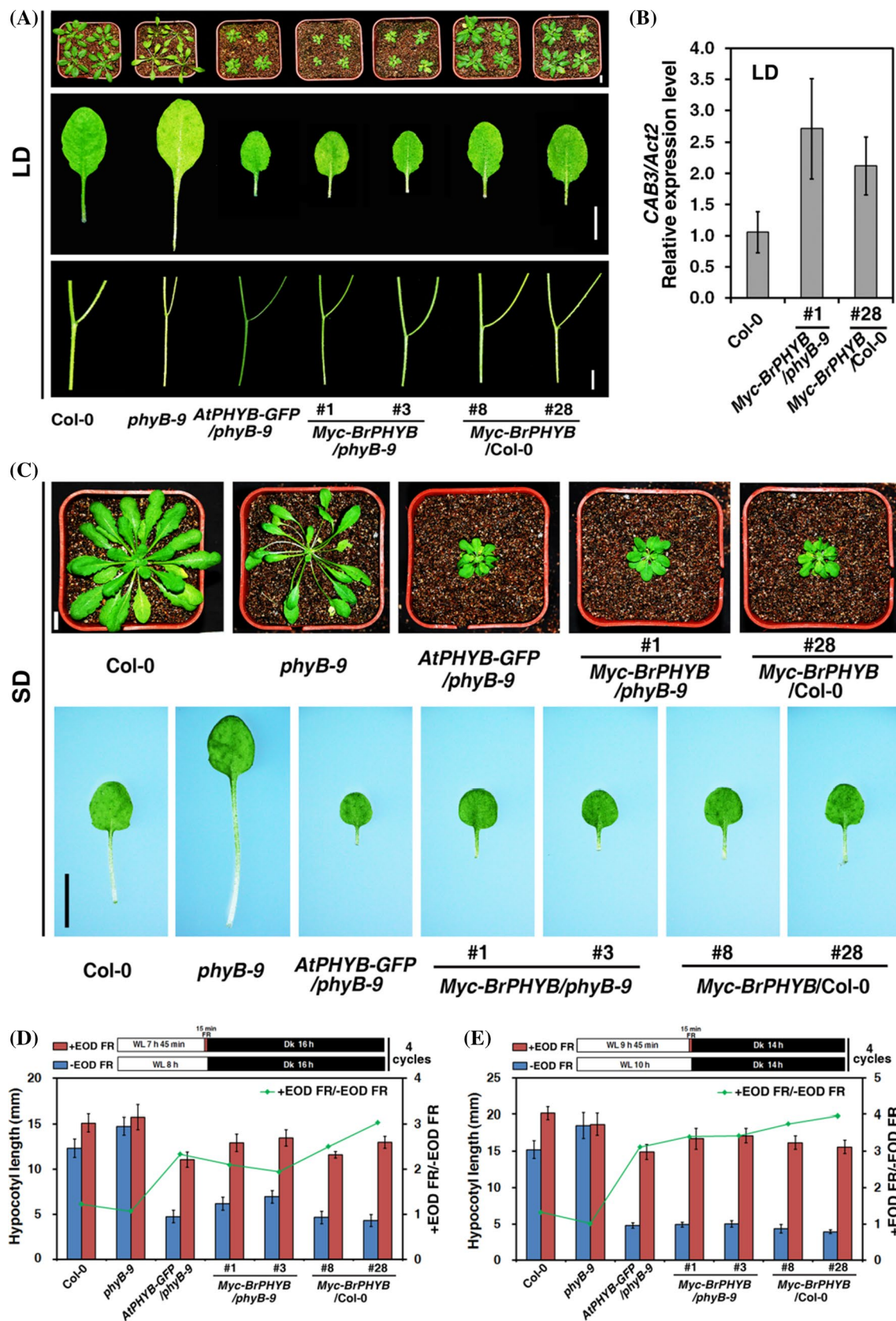
Immunoblot analysis

For immunoblots of plant extracts, the total protein fractions were analyzed as described previously (Zheng et al. 2013). The specific anti-phyB peptide antibody was raised in rabbits and affinity-purified against the peptide EQAQSS-GTKSLRPR (both of AtphyB and BrphyB, amino acids

Fig. 4 Phenotypes of mature plants under long- and short-day conditions and the end-of-day far-red (EOD-FR) response of transgenic *Myc-BrPHYB* plants. **a** Seedling phenotypes, morphology of the third leaf, and the angle between the main inflorescence and the lateral branch, of mature Col-0, *phyB-9*, *AtPHYB-GFP/phyB-9*, *Myc-BrPHYB/phyB-9* (lines #1 and #3), and *Myc-BrPHYB/Col-0* (lines #8 and #28) plants under long-day (LD, 16-h-light/8-h-dark) conditions for 28, 28, and 45 days, respectively. Bar = 1 cm. **b** Quantitative reverse transcription (RT)-PCR analysis of *CAB3* transcript levels in Col-0, *Myc-BrPHYB/phyB-9* (line #1), and *Myc-BrPHYB/Col-0* (line #28) plants under LD conditions for 14 days. Quantitative reverse transcription (RT)-PCR analysis was performed as in Fig. 2. **c** Seedling phenotypes of the mature Col-0, *phyB-9*, *AtPHYB-GFP/phyB-9*, *Myc-BrPHYB/phyB-9* (line #1), and *Myc-BrPHYB/Col-0* (line #28) grown under short-day (SD, 8-h-light/16-h-dark) conditions for 60 days. Morphology of the third leaf from Col-0, *phyB-9*, *AtPHYB-GFP/phyB-9*, *Myc-BrPHYB/phyB-9* (lines #1 and #3), and *Myc-BrPHYB/Col-0* (lines #8 and #28) plants grown under SD conditions for 30 days. Bar 1 cm. **d** EOD-FR of Col-0, *phyB-9*, *AtPHYB-GFP/phyB-9*, *Myc-BrPHYB/phyB-9* (lines #1 and #3), and *Myc-BrPHYB/Col-0* (lines #8 and #28). Seedlings were grown in four cycles of SD photoperiod (-EOD-FR), or in the same photoperiod with a 15-min FR pulse before being transferred into the dark (+EOD-FR). The white-light intensity was 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For measurement of hypocotyl lengths, the average of 30 seedlings was computed. Error bars indicate standard deviations. **e** The treatment of EOD-FR was as in (d) with the exception of the photoperiod (10-h-light/14-h-dark) and white light intensity (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$)

Fig. 3 Morphology (a) and hypocotyl lengths (b) of seedlings for *Myc-BrPHYB* overexpression lines under different light conditions. Seedlings of Col-0, *phyB-9*, *AtPHYB-GFP/phyB-9*, *Myc-BrPHYB/phyB-9* (lines #1 and #3), and *Myc-BrPHYB/Col-0* (lines #8 and #28) were grown under red light (R), white light (WL), and far-red light (FR) for 4 days. The #1 or #28 lines were used as representative lines for *Myc-BrPHYB/phyB-9* or *Myc-BrPHYB/Col-0*, respectively, unless otherwise indicated. For measurement of hypocotyl lengths, the average of 30 seedlings was computed. Error bars indicate standard deviations. The same lower case letter indicates no significant difference ($p < 0.05$) between two lines according to *F* test in an analysis of variance (ANOVA), while different lower case letters indicate a significant difference. See also Figure S2





27-40) (Hang-Zhou Hua-An Biotechnology, Hangzhou, China) (Zheng et al. 2013). Myc-BrphyB and AtphyB-GFP proteins were detected with a monoclonal anti-myc antibody (clone 9E10; Santa Cruz Biotechnology, Dallas, USA) and an anti-GFP polyclonal antibody (P30010; Shanghai Abmart Biotech, Shanghai, China), respectively. Proteins were visualized by incubating specimens with goat anti-mouse or rabbit secondary antibodies conjugated to alkaline phosphatase (1:5000; Sigma-Aldrich, St. Louis, USA) in the presence of 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium as substrates.

Results

Isolation of *BrPHYB* from Chinese cabbage

A full-length cDNA encoding the phyB protein with high sequence identity to *AtPHYB*, designated *BrPHYB*, was isolated from a Chinese cabbage inbred line “G90E16” using specific primers (Table S1). *BrPHYB* encoded a putative polypeptide of 1170 amino acids with a calculated molecular mass of 128.8 kDa and an isoelectric point of 5.73 (Accession Number KC342233). In addition, BrphyB contains a GAF domain, two PAS domains, and a histidine kinase-related domain (including a HisKA and a HATPase_c domain), all of which exist in almost all phyB proteins (<http://smart.embl-heidelberg.de/>) that have been cloned (Wu and Lagarias 2000; Bae and Choi 2008). The deduced amino acid sequence of BrphyB and known sequences of various plant phyB holoproteins were aligned and used to generate a phylogenetic tree (Fig. 1). On the basis of their structures, all of the phyB proteins were classified into two major groups that corresponded with whether the source plants were monocotyledonous or dicotyledonous species. As expected, BrphyB had the highest sequence similarity (94 %) to AtphyB and belonged to a dicotyledonous species group (Supp. Fig. 1). This result indicates that BrphyB may have diverged less from AtphyB than from other dicotyledonous and monocotyledonous species, and interspecific divergence in the phyB sequence appears to largely reflect phylogenetic relationships (Abdurakhmonov et al. 2010; Wu et al. 2011).

Tissue- and light-specific expression of *BrPHYB*

To determine whether *BrPHYB* expression displayed tissue specificity and was regulated by light, its expression was examined by quantitative RT-PCR. *BrPHYB* was constitutively expressed in all of the tissues examined, although *BrPHYB* gene transcript levels were slightly

higher in stems than in roots, leaves, and flower buds (Fig. 2a). When seedlings were grown under FR, R, B, and W light conditions, transcript levels were slightly higher in the seedlings grown under FR and B light conditions compared with seedlings grown in the dark; however, expression levels in the seedlings grown under R and W light conditions were the same as in the seedlings grown in the dark (Fig. 2b).

Overexpression of *BrPHYB* inhibits hypocotyl elongation under R and W light conditions, but slightly promotes hypocotyl elongation under FR light

In *A. thaliana*, hypocotyl elongation is an important parameter for detecting the extent of photomorphogenic development (Wang and Deng 2003; Bae and Choi 2008). *BrPHYB* function was explored by overexpressing it in both the WT and the *phyB-9* mutant backgrounds. The effect of *BrPHYB* in responses to the dark, or FR, R, B, and W light conditions were determined using *Myc-BrPHYB/phyB-9* (*BrPHYB* overexpressed in the *phyB-9* background) and *Myc-BrPHYB/Col-0* (*BrPHYB* overexpressed in the WT background), compared with *AtPHYB-GFP/phyB-9* [transgenic *A. thaliana* *PHYB* fused with green fluorescent protein (GFP) in the *phyB-9* mutant background; Zheng et al. 2013]. Previous studies showed that phyB is the primary photoreceptor mediating de-etiolation in response to R or W light (Reed et al. 1993). We first observed the phenotypes of transgenic *BrPHYB*-overexpression (*Myc-BrPHYB*) lines under R or W light conditions. Under R or W light, *phyB* mutant seedlings developed longer hypocotyls than the WT seedlings (Fig. 3), but the seedlings overexpressing *BrPHYB*, regardless of their transgenic background, exhibited shortened hypocotyl elongation and expanded cotyledons consistent with the phenotype of *AtPHYB-GFP/phyB-9* seedlings (Fig. 3). This result demonstrates that, like AtphyB, BrphyB mediates seedling de-etiolation and plays a major role in controlling hypocotyl elongation under R and W light conditions.

Previous studies have shown that overexpression of *PHYB* represses seedling de-etiolation under FR light (Wagner et al. 1996a, b; Short 1999; Hennig et al. 2001; Zheng et al. 2013a). Under FR light, *phyB-9* seedlings displayed less hypocotyl elongation than the Col-0 seedlings, whereas all the *PHYB*-overexpression (*PHYB-OE*) lines, including *AtPHYB-GFP/phyB-9*, *Myc-BrPHYB/phyB-9*, and *Myc-BrPHYB/Col-0*, clearly promoted hypocotyl elongation (Fig. 3). We also noted that all *PHYB* overexpression lines had smaller cotyledons less cotyledon unfolding than the WT (Fig. 3a). When the transgenic *PHYB* lines were grown in the dark and under B light, no distinguishable differences were found compared with the WT (Supp. Fig. 2).

Overexpression of *Myc-BrPHYB* causes dwarfing in mature plants with small leaves and short petioles

Overexpression of *PHYB* leads to a short hypocotyl phenotype in transgenic *A. thaliana* seedlings (Wagner et al. 1991; Yamaguchi 1999; Zheng et al. 2013). Under LD conditions, the *phyB* mutant seedlings were much taller than Col-0 seedlings, while the *Myc-BrPHYB* and *AtPHYB-GFP* overexpression lines showed a dwarf stature, with shortened petioles and small, flattened leaves (Fig. 4a, c). Since the chlorophyll A/B-binding protein gene (*CAB*) expression can serve as an indicator of seedling de-etiolation (Hall et al. 2002), we compared *CAB3* expression of *Myc-BrPHYB* lines with that of the WT. The *CAB3* transcript levels were considerably higher in transgenic plants, regardless of their background, than in Col-0 plants (Fig. 4b). The dwarf phenotype was also exhibited under SD conditions, indicating that photoperiod did not affect dwarf phenotypes (Fig. 4c).

It was reported previously that *phyB* mutants display a constitutive shade avoidance syndrome, with long hypocotyls, long petioles and early flowering (Reed et al. 1993). In addition, expression of *AtPHYB* in transgenic potato (*Solanum tuberosum* L.) shows semi-dwarfism, decreased apical dominance, a higher number of smaller and thicker leaves, and increased pigmentation; the transgenic seedlings reduce the extent of the shade avoidance syndrome (Thiele et al. 1999). To further investigate the function of

BrphyB in the shade avoidance response, we imposed an end-of-day far-red (EOD-FR) treatment (Fankhauser and Casal 2004). The *phyB-9* mutant displayed a considerably reduced EOD-FR response (Fig. 4d, e), while the *AtPHYB-GFP* or *Myc-BrPHYB* transgenic lines showed a greater +EOD-FR/–EOD-FR ratio of hypocotyl elongation than the WT or the *phyB-9* mutant (Fig. 4d, e), implicating either *BrphyB* or *AtphyB* in this response.

Myc-BrPHYB delays flowering under short-day conditions but not long-day conditions

Phytochromes are photoreceptors that are critical for the regulation of floral induction (Thomas and Vince-Prue 1997; Lin 2000; Quail 2002). *Arabidopsis thaliana phyB* mutants flower earlier than the WT (Goto et al. 1991; Reed et al. 1993; Bagnall et al. 1995; Koornneef et al. 1995). To understand *BrPHYB* functions on flowering time under LD and SD conditions, rosette leaf number and days to flowering were measured in those *A. thaliana* transgenic lines, respectively. Under LD conditions, consistent with previous results, *phyB-9* plants had fewer rosette leaves than Col-0 plants and reduced days to flowering, indicating that *phyB-9* plants flowered somewhat earlier than Col-0 plants; nevertheless, *AtPHYB-GFP/phyB-9* plants flowered slightly later than Col-0 plants (Fig. 5a, b). *Myc-BrPHYB* plants rescued the flowering time phenotype of the *phyB-9* mutant plants, but were not different when compared with Col-0

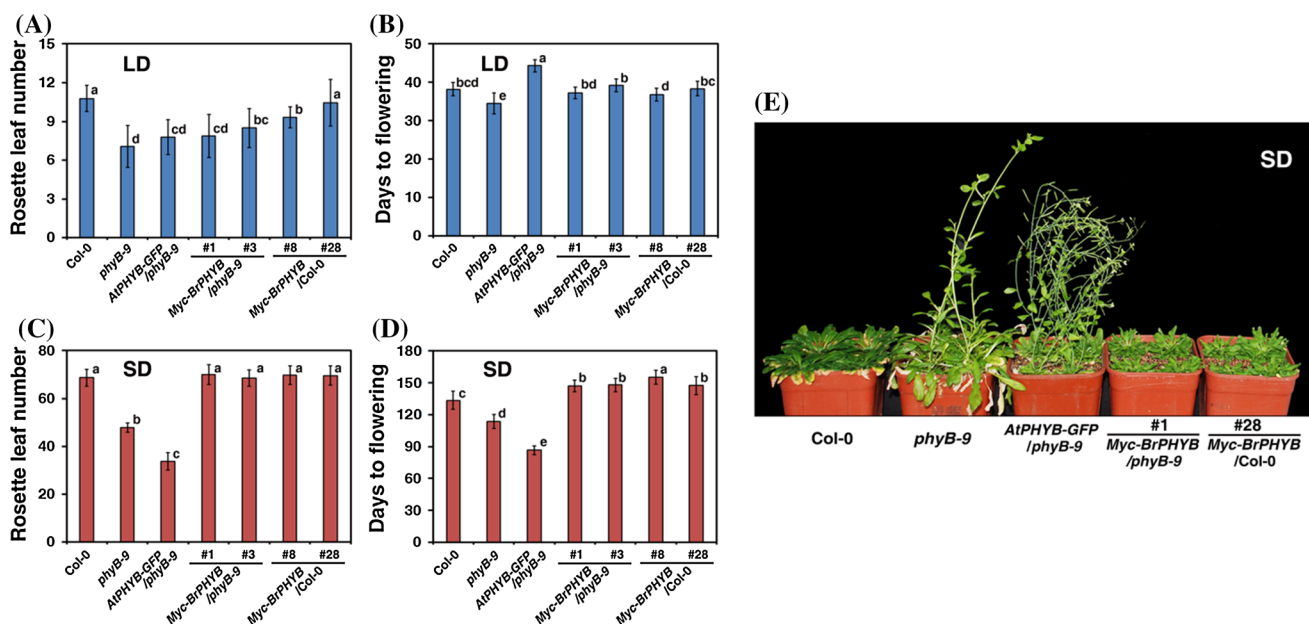


Fig. 5 Overexpression of *Myc-BrPHYB* delays flowering under short-day conditions, but not under long-day conditions. Rosette leaf number (a, c) and days to flowering (b, d) under long-day (LD) conditions (a, b) and short-day (SD) conditions (c, d). Error bars denote standard deviations; $n = 20$ plants. F tests of the significance

of differences in rosette leaf number (a, c) and days to flowering (b, d) were performed as in Fig. 3b. e Flowering phenotypes of Col-0, *phyB-9*, *AtPHYB-GFP/phyB-9*, *Myc-BrPHYB/phyB-9* (line #1), and *Myc-BrPHYB/Col-0* (line #28) plants under SD conditions for 112 day

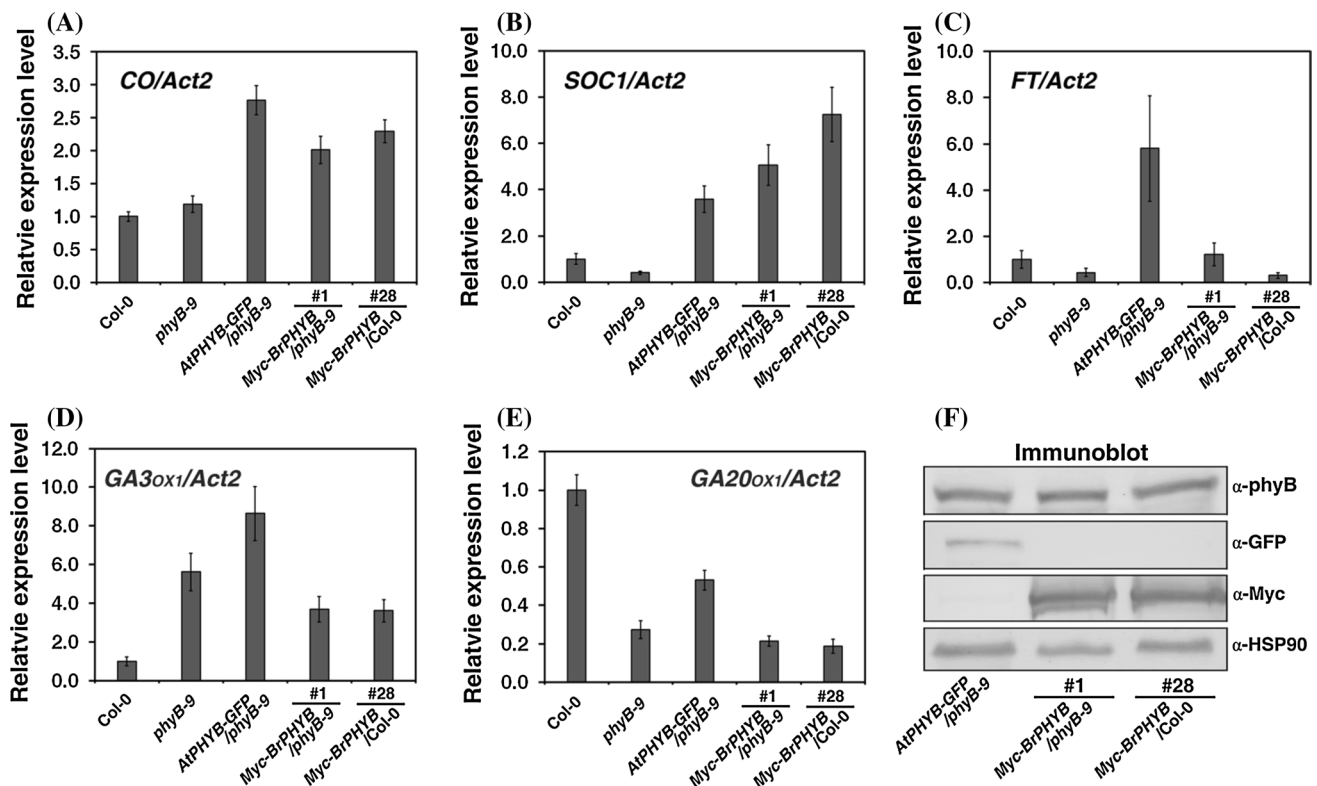


Fig. 6 Effect of *BrPHYB* overexpression on the expression levels of regulatory genes related to flowering time and GA biosynthesis. *CONSTANS* (*CO*; **a**), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*; **b**), *FLOWERING LOCUS T* (*FT*; **c**), *GIBBERELLIN 3-OXIDASE 1* (*GA3OX1*; **d**), and *GIBBERELLIN 20-OXIDASE 1* (*GA20OX1*; **e**) transcript levels, respectively, in 12-day-old Col-0, *phyB-9*, *AtPHYB-GFP/phyB-9*, *Myc-BrPHYB/phyB-9*, and *Myc-BrPHYB/Col-0* seedlings grown under short-day

(SD, 8-h-light/16-h-dark) conditions. The samples were harvested at the end of 8-h-light exposure on the 12th day. Quantitative reverse-transcription (RT)-PCR assay was performed as in Fig. 2. Each column shows the mean relative expression levels of three biological repeats. Error bars indicate standard deviations. **f** PhyB protein levels in *AtPHYB-GFP/phyB-9*, *Myc-BrPHYB/phyB-9*, and *Myc-BrPHYB/Col-0* plants under SD conditions. An anti-HSP90 immunoblot is shown below, indicating approximately equal loading

plants under LD (Fig. 5a, b). Under SD conditions, *phyB-9* plants had fewer rosette leaves than Col-0 plants and fewer days to flowering (Fig. 5c–e). Interestingly, the *AtPHYB-GFP/phyB-9* plants flowered earlier than Col-0 and *phyB-9* plants, while *Myc-BrPHYB* plants flowered much later than *phyB-9* and a little later than Col-0 (Fig. 5c–e). All these results indicated that ectopic expression of *BrPHYB* only rescued the phenotype of the *phyB-9* mutant. Our results indicate that *AtPHYB* and *BrPHYB* are functionally divergent because of different species and also with regard to the transition from the vegetative to the reproductive development of cruciferous plants.

Overexpression of *Myc-BrPHYB* suppresses gibberellin synthesis under SD conditions

Both photoperiod and gibberellin (GA) were shown to be involved in regulating flowering time (Langridge 1957; Wilson et al. 1992). To better understand the difference between *BrPHYB* and *AtPHYB* in the regulation

of flowering time under SD conditions, we determined the expression levels of genes related to flowering time and GA biosynthesis. The transcript abundances of flowering time genes, such as *CONSTANS* (*CO*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), were upregulated to a considerably higher level in all *AtPHYB-GFP* and *Myc-BrPHYB* transgenic lines rather than their transgenic backgrounds, Col-0 or *phyB-9* (Fig. 6a, b). However, higher level of *FLOWERING LOCUS T* (*FT*) was found in *AtPHYB-GFP/phyB-9* plants (Fig. 6c). In contrast to *CO* and *SOC1*, two GA synthesis-related genes, *GA3OX1* and *GA20OX1*, were repressed in most *AtPHYB-GFP* and *Myc-BrPHYB* transgenic lines (Fig. 6e, f). These results suggest that *BrPHYB* might regulate flowering time mainly through GA synthesis.

Because *phyB* protein abundances may affect flowering time, we also compared *phyB* protein levels in *AtPHYB-GFP/phyB-9*, *Myc-BrPHYB/phyB-9*, and *Myc-BrPHYB/Col-0* plants (Fig. 6d). *PhyB* levels were comparable in all of

the transgenic plants, suggesting that differential flowering times may be caused by differences in the activities of phyB proteins from different species, not their protein abundance.

Discussion

PhyB plays important roles in mediating cellular and light signaling events (Wagner et al. 1996a, b; Zhu et al. 2000; Zheng et al. 2013). Although *phyB* mutants have been found in Chinese cabbage, only their phenotypes were described previously (Devlin et al. 1997). To better understand the roles of *BrPHYB* in light signaling at the molecular level, we isolated and characterized the *BrPHYB* gene from Chinese cabbage. Comparison of the BrphyB amino acid sequence with other known phyB proteins showed that BrphyB had the highest homology with AtphyB. In addition, BrphyB contains GAF, PAS (two), HisKA, and HATPase_c domains, which are characteristic of the AtphyB protein (Fig. 1; Supp. Fig. 1). We examined *BrPHYB* expression profiles in Chinese cabbage and found that *BrPHYB* was expressed in all tissues and under all light conditions tested (Fig. 2a, b), suggesting that *BrPHYB* is likely to be involved in multiple developmental processes under all light conditions.

Different species share fundamental developmental processes and often have highly conserved orthologous genes that regulate these processes (Sussex 1989; Coen and Nugent 1994; Ma 1994; Manak and Scott 1994). The photomorphogenesis and mature plant dwarfing phenotypes in the *Myc-BrPHYB* transgenic lines were similar to those of *AtPHYB-GFP* transgenic plants in both the seedling and mature stages (Fig. 3; Supp. Fig. 2). These phenotypes were also observed in *A. thaliana*, potato, tobacco (*Nicotiana tabacum* L.), and soybean (Wagner et al. 1991; McCormac et al. 1992; Wester et al. 1994; Halliday et al. 1997; Wu et al. 2011), indicating that phyB functions are conserved among species. Under FR light, the *PHYB-OE* lines (*Myc-BrPHYB/phyB-9*, *Myc-BrPHYB/Col-0*, *AtPHYB-GFP/phyB-9*, *GmPHYB/phyB-9*, and *GmPHYB/Col-0*) had longer hypocotyls than the WT, while *phyB-9* showed shorter hypocotyls than Col-0 (Wagner et al. 1996a, b; Short 1999; Hennig et al. 2001; Wu et al. 2011; Zheng et al. 2013). These results demonstrate that phyB is a negative regulator of seedling de-etiolation under FR light. Recently the relationship between phyB and SPA1 has been described, indicating that phyB represses photomorphogenesis by promoting nuclear accumulation of SPA1 under FR light (Zheng et al. 2013). These results indicate that BrphyB may function in response to FR light through a mechanism similar to that of AtphyB.

Photoperiod is one of the most important environmental factors affecting flowering time (Piñeiro and Coupland

1998; Lin 2000). *Arabidopsis thaliana* and Chinese cabbage are LD plants, in which flowering is accelerated under LD conditions and delayed under SD conditions (Lin 2000). Photoreceptors, including phytochromes and cryptochromes, monitor and sense photoperiodic change and can enter the nucleus to regulate flowering time (Lin 2000). Under SD conditions, *AtPHYB* overexpression promotes flowering, but *BrPHYB* overexpression rescues only the early flowering phenotype of the *phyB-9* mutant, indicating that the role of phyB in floral initiation is complex and is integrated with the activities of other photoreceptors under SD conditions (Piñeiro and Coupland 1998; Lin 2000; see also Fig. 5). Mutations of genes such as *CO*, *FT*, and *GIGANTEA* (*GI*) cause late flowering under LD conditions, but do not delay flowering under SD conditions when compared with the wild type (Moon et al. 2003; Endo et al. 2005). Our results showed that transcription abundances of *CO* and *SOC1* were upregulated in the *Myc-BrPHYB* lines compared to both Col-0 and *phyB-9* (Fig. 6a, b). Experiments involving the application of exogenous GA (Langridge 1957; Chandler and Dean 1994) and GA signaling mutants (Wilson et al. 1992; Jacobsen and Olszewski 1993) indicate that GA accelerates flowering under SD conditions. Our results demonstrated that GA synthesis-related genes (e.g., *GA3OX1* and *GA20OX1*) were downregulated in the *Myc-BrPHYB* transgenic lines (Fig. 6d, e) compared with *phyB-9* and *AtPHYB-GFP/phyB-9* plants. These results indicate that *BrphyB* might regulate flowering time by different accumulation of GA. The earlier flowering and the higher transcript levels of *CO*, *FT*, and *SOC1* in the *AtPHYB-GFP* transgenic lines, compared with the wild type (Figs. 5c, d, 6a, b), suggests that *AtPHYB* promotes early flowering by activating the photoperiodic pathway under SD conditions.

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