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Divergence in red light responses associated with thermal reversion of *PHYTOCHROME B* between high- and low-latitude species

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Summary

- Phytochromes play a central role in mediating adaptive responses to light and temperature throughout plant life cycles. Despite evidence for adaptive importance of natural variation in phytochromes, little is known about molecular mechanisms that modulate physiological responses of phytochromes in nature.
- We show evolutionary divergence in physiological responses relevant to thermal stability of a physiologically active form of phytochrome (Pfr) between two sister species of Brassicaceae growing at different latitudes.
- The higher-latitude species (*Cardamine bellidifolia*; *Cb*) responded more strongly to light limited condition than its lower-latitude sister (*C. nipponica*; *Cn*). Moreover, *CbPHYB* conferred stronger responses to both light limited and warm conditions in the *phyB*-deficient mutant of *Arabidopsis thaliana* than *CnPHYB*: i.e., Pfr CbphyB was more stable in nuclei than CnphyB.
- Our findings suggest that fine-tuning Pfr stability is a fundamental mechanism for plants to optimise phytochrome-related traits in their evolution and adapt to spatially varying environments, and open a new avenue to understand molecular mechanisms that fine-tune phytochrome responses in nature.

Keywords: alpine plants; Brassicaceae; *Cardamine*; phytochrome; thermal reversion.

Introduction

Sensing ambient environments and subsequent physiological changes are crucial for survival and reproductive success of plants. Phytochromes, which are red- (R) and far-red (FR) light receptors, play a central role in physiological responses to light conditions throughout plant life cycles, including germination, seedling growth and flowering (Jiao *et al.*, 2007). Recently, phytochromes have also been identified as a thermal sensor as well (Jung *et al.*, 2016; Legris *et al.*, 2016), and therefore mediate physiological responses by integrating both light and temperature signals (Legris *et al.*, 2017; Casal & Questa, 2018). Seed plants have multiple phytochromes, of which three discrete classes of apoprotein-encoding genes (*PHYA–PHYC*) are conserved in angiosperms (Mathews, 2010). In the model plant *Arabidopsis thaliana*, five phytochromes (phyA–phyE) have been identified (Sharrock & Quail, 1989; Clack *et al.*, 1994). PhyA and phyB play prominent roles (Franklin & Quail, 2010), whereas phyC–phyE have redundant (Franklin *et al.*, 2003) but physiologically important roles in specific environments, including at low temperatures.

Molecular evolutionary analyses have detected a signature of divergent selection in *PHYA* of *Arabidopsis lyrata* (Toivainen *et al.*, 2014) and in *PHYE* of two alpine species, *Cardamine nipponica* (Ikeda *et al.*, 2009) and *Arcterica nana* (Ikeda & Setoguchi, 2010), of which plants growing at different latitudes have alleles encoding different amino acid sequences. In addition, amino acid replacements in PHYB2 of *Populus tremula* are associated with latitudinal variation in its bud set timing, which is an ecologically important trait (Ingvarsson *et al.*, 2006; Ingvarsson *et al.*, 2008). Although these studies did not demonstrate functional differences conferred by alternative alleles with amino acid changes, other studies of *A. thaliana* have shown that natural variation in amino acid sequences changes the light sensitivity of phytochromes; accessions from higher latitude had more light sensitive phytochromes [phyB (Filiault *et al.*, 2008) and phyC (Balasubramanian *et al.*, 2006)] than those from lower latitude. Furthermore, a recent study demonstrated that enhanced phyA activity in *Cardamine hirsuta* is responsible for its shade tolerance (Molina-Contreras *et al.*, 2019). Collectively, these studies suggest that modulating the light sensitivity of phytochromes as well as their physiological activity may be important in adaptation to spatially (in particular latitudinally) varying

environments.

Phytochromes are synthesized in physiological inactive form (Pr). Pr absorbs R light and changes its conformation to physiologically active one (Pfr), whereas Pfr absorbs FR light and returns to Pr. In addition, prolonged darkness and/or warmer conditions even in R light promotes reversion of Pfr to Pr [dark or thermal reversion (Legris *et al.*, 2016)]. Upon R light irradiation, phyB accumulates in the nucleus and forms nuclear bodies (NBs; Yamaguchi *et al.*, 1999; Chen *et al.*, 2003), which stabilize Pfr as well as its physiological activity even in prolonged darkness (Van Buskirk *et al.*, 2014). Given that amino acid changes can influence thermal stability of phyB Pfr (Kretsch *et al.*, 2000; Oka *et al.*, 2004; Oka *et al.*, 2008; Zhang *et al.*, 2013), fine-tuning of Pfr thermal stability might represent the evolutionary mechanism underlying divergent selection on phytochrome genes as well as the differential light sensitivity of phytochromes. However, there is neither evidence of natural variation in Pfr thermal stability (Legris *et al.*, 2017) nor evidence for evolutionary importance of fine-tuning Pfr stability (Enderle *et al.*, 2017). In this study, we assess evolutionary divergence in phytochrome-related phenotypes between two sister species of perennial herbs in Brassicaceae, *Cardamine bellidifolia* (*Cb*) and *Cardamine nipponica* (*Cn*) (Ikeda *et al.*, 2012); the former occurs in the circumarctic region as well as in the alpine regions of North America and East Asia and the latter is endemic to high mountains in the Japanese Archipelago (Fig. 1a). We show that these sister species growing at different latitude have phyB with different Pfr thermal stability; the lower-latitude *C. nipponica* has more thermal-sensitive phyB than the higher-latitude *C. bellidifolia*. Our findings suggest that fine-tuning Pfr thermal stability is one of molecular mechanisms for adapting to environments with different light and temperature conditions.

Materials and Methods

Seedling assays

Seeds of three accessions of each of *C. bellidifolia* and *C. nipponica* (Table S1), which were grown for five or more generations of selfing after initially collected from natural populations, were embedded on rockwool and irradiated with continuous light by 3-in-1 LED (ca. 70 μmol m^{-2} sec^{-1}; ca. 26, 14, 30 μmol m^{-2} sec^{-1} with peak wave length at 445, 520, 660 nm, respectively). After the root emerged, seedlings were grown in one of four light conditions for seven days at 22 ℃: cR, continuous R light with different intensity

(ca. 10, 30, and 150 μmol m^{-2} sec^{-1} with peak wave length at 660 nm); pR, 15 min R light pulses (ca. 150 μmol m^{-2} sec^{-1} with peak w ave length at 660 nm) every 12 hr darkness; pR+FR, pR followed by 15 min FR light pulses (ca. 100 μmol m^{-2} sec^{-1} with peak wave length at 730 nm) every 12 hr darkness; cD, continuous darkness (cD).

Seeds of *A. thaliana* were surface-sterilized and sown on 0.8% agar plates containing Murashige-Skoog (MS) medium without sucrose. In addition to transgenic plants overexpressing *PHYB* of *C. bellidifolia* and *C. nipponica* (see below), we also used Landsberg *erecta* (L*er*), phyB-5 mutation (Reed 1993), and transgenic *A. thaliana* expressing *PHYB* under the control of the authentic PHYB promoter (Bpro7 in Endo *et al.*, 2005) and under the 35S promoter (PBG in Yamaguchi *et al.*, 1999). The plates were kept in darkness at 4℃ for five days and then moved to cR (ca. 30 μmol m^{-2} sec^{-1} with peak wave length at 660 nm) at 22℃. After one day in cR, seedlings were grown in cR, pR at specific intervals of darkness (8, 12, or 16 hr), pR+FR or cD for five days at 16, 22, and 28℃.

Seedlings were photographed and their hypocotyl lengths were measured using ImageJ (https://imagej.nih.gov/ij/). The hypocotyl length was analyzed with a generalized linear mixed model (GLMM) using the glmer function from the package LME4 (Bates *et al.*, 2015) in R v3.2.3. For *Cardamine* seedlings, the effects of the light intensity (0, 10, 30, and 150 μmol m^{-2} sec^{-1}) or pR (pR, pR+FR and cD) and species (*C. bellidifolia* and *C. nipponica*) on hypocotyl length were examined by a model specifying independent accessions of each species [Ava, Den, and Yuk (*C. bellidifolia*); Dai, Kis, and Shi (*C. nipponica*)] as a random effect on the species. After optimization, Gamma distribution with log link and Gaussian distribution with log link was applied for the analyses of light intensity and pR, respectively. For *A. thaliana* transgenic seedlings, the effects of light (cR, cD and pR) or temperature (16, 22 and 28 ℃) and *PHYB* allele (*CbPHYB* and *CnPHYB*) on hypocotyl length were examined by a model specifying three replicate transgenic lines of each *PHYB* as a random effect. After optimization, Gamma distribution with log or inverse link was applied.

Sequence analyses of phytochrome genes

DNA of *C. bellidifolia* and *C. nipponica* were extracted from silica-dried leaves that were collected from the natural populations or extracted in the previous study (Ikeda *et al.*, 2008), and sequences of phytochrome genes were determined following the previous

study (Ikeda *et al.*, 2009). Together with the previously published sequences from 11 individuals (Ikeda *et al.*, 2009; Ikeda *et al.*, 2011), 16 individuals of each species were included in this study (Table S1). All new sequences of phytochrome genes were deposited in DDBJ (Table S1). After haplotype sequences of each gene were determined by Bayesian method implementing PHASE (Stephens & Donnelly, 2003), McDonald-Kreitman (MK) test was conducted to compare the number of synonymous and nonsynonymous substitutions of both fixed differences between the species and polymorphisms within species using DnaSP.

Molecular evolutionary analyses of *PHYB*

PHYB gene trees were estimated based on both amino acid and nucleotide sequences. In addition to *C. bellidifolia* and *C. nipponica*, 21 species of Brassicaceae were included in the analyses (Table S2). The nucleotide sequences were determined in the present study for three additional *Cardamine* species (*C. alpina*, C. *resedifolia*, and *C. glauca*) and coding DNA sequences (CDS) of *PHYB* were obtained from published genome data for the remaining species. Amino acid sequences were determined from the nucleotide sequences. After the amino acid and nucleotide sequences had been separately aligned using Clustal W (Edgar, 2004) implemented by MEGA7, the substitution model of amino acid (JTT+G+I) and nucleotide (HKY+G+I) sequences was selected based on Bayesian information criteria using Prottest v3.4 (Darriba *et al.*, 2011) and jModelTest v2.1 (Darriba *et al.*, 2012), respectively. Trees were estimated by maximum likelihood (ML) using PhyML (Guindon *et al.*, 2010) with the SPR tree search option and by Bayesian method using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Branch support was evaluated with 1,000 bootstrap resamplings for ML and posterior probability for Bayesian trees. Following a previous phylogenetic study (Kagale *et al.*, 2014), trees were rooted using *Aethionema arabicum* as the outgroup.

In addition, we analysed the ratio of nonsynonymous to synonymous substitutions ($\omega = d_N/d_S$) using CODEML in PAML4.7 (Yang, 2007). By assuming a branch model (Yang & Nielsen, 1998), where unique ω was applied for either *C. bellidifolia* or *C. nipponica*, the likelihood of the branch model was compared with a null model assuming a single ω (M0a). The nucleotide sequences corresponding to the most basal amino acid sequences in each of *C. bellidifolia* and *C. nipponica* were used for the analyses. Highly supported clades (bootstrap > 90 and posterior probability > 0.95 in either amino acid or nucleotide

177 trees) were applied for the initial topology for the analyses. The convergence of likelihood
178 and ω was evaluated by repeated analyses with different initial values (ω = 0, 1, and 10).
179 **Plasmid construction and plant transformation**
180 Total RNA of *C. bellidifolia* (Ava) and *C. nipponica* (Kis), accessions with the most
181 common sequence of PHYB in each species, was extracted from leaves of cultivated
182 plants using the RNeasy Kit (Qiagen, Hilden, Germany) and used for synthesizing cDNA.
183 The entire coding region of *CbPHYB* and *CnPHYB* was amplified from cDNA by PCR
184 using PrimeSTAR Max Premix (Takara BIO, Otsu, Japan) with a primer pair (Forward:
185 CACGGGGGACTCTAGAATGGTTTCCGGAGGCGGCGGA, Reverse:
186 TGCTCACCATGTCGACATATGGCATCATCAACATCATAT). After a transformation
187 vector P35S:P2G (Kong *et al.*, 2006), which cloned PHOT2-GFP into pPZP211/P_{35S}-*nos*T,
188 was cut by *Xba*I and *Sal*I to remove *PHOT2*, the amplified *CbPHYB* or *CnPHYB* was
189 inserted into the vector using InFusion (Clontech, Mountain View, CA, USA). The *phyB-*
190 *5* mutant (Accession, Landsberg *erecta*; Reed *et al.*, 1993) was transformed by
191 *Agrobacterium tumefaciens* mediated by the floral dip method (Clough & Bent, 1998),
192 and homozygous transgenic lines were selected following the previous procedure (Oka *et*
193 *al.*, 2004).
194 **Immunoblot assays of PHYB**
195 Total protein was extracted from ca. 35 mg seedling material of the transgenic plants with
196 *CbPHYB-GFP* or *CnPHYB-GFP* using extraction buffer (APRO Science, Tokushima,
197 Japan) and used for SDS-PAGE, protein blotting, and immunodetection following the
198 previous procedure (Oka *et al.*, 2004). Using anti-GFP monoclonal antibody (Nacalai
199 Tesque, Kyoto, Japan) and anti-Actin polyclonal antibody (Abcam, Cambridge, MA), the
200 relative level of PHYB-GFP to Actin was quantified with Clarity Western ECL Substrate
201 (Bio-Rad) using LAS-4000 (GE Healthcare). Three independent samples were used for
202 replicated measurements.
203 **Subcellular localization of *PHYB***
204 Seedlings of transgenic *A. thaliana* were grown on 0.8% agar plates containing MS
205 medium without sucrose in cR (ca. 10 μmol m^{-2} sec^{-1}) at 22 ℃. The green fluorescent
206 protein (GFP) in nuclei of hypocotyl epidermal cells in two-day old seedlings was
207 mounted on the stage under a dim green safelight and observed with a confocal laser
208 microscope (Olympus) before and after 12 hr darkness. Single scanned images of each

seedling immediately after preparation were used for the NB measurement. The size of NBs were measured using ImageJ. The effects of *PHYB* allele (*CbPHYB* and *CnPHYB*) and 12 hr darkness on NB sizes were examined by a generalized linear mixed model (GLMM) with three replicate transgenic lines of each *PHYB* as a random effect. Gamma distribution with log link was applied for the analysis.

In vitro assay of thermal reversion

In vitro thermal stability of Pfr was assessed using the N-terminal 651 amino acid fragment (N651) of CbPHYB and CnPHYB. The N651 of *C. bellidifolia* and *C. nipponica* were amplified from the above synthesized cDNA by PCR using PrimeSTAR Max Premix (Takara BIO) with a primer pair (Forward: GGCAAAGCACCCGGGATATGGCATCATCAACATCATAT, Reverse: CTTGGCAAAGCACCCTGCACCTAGCTCATCA) and were inserted into the pTYB2 vector (New England Biolabs, Beverly, MA) using InFusion (Clontech, Mountain View, CA). *Escherichia coli* strain BL21 (New England Biolabs) was transformed by the plasmids and cultured in 2 L of LB medium containing 50 μg mL^{-1} of carbenicillin for 20 hr at 28℃ until the cell density reached A_{600}= 0.8. Afterwards, 0.4 mM isopropyl-thio-B-D-galactoside was added, and the media were further cultured for 16 hr at 16℃. Proteins were extracted from lysed cells using sonication, purified with the IMPACT kit (New England BioLabs) and eluted by self-cleaved in the presence of 40 mM 1,4-dithiothreitol for two days at 4℃.

The purified protein was mixed with 5 μM phycocyanobillin (Oka *et al.* 2004) and used for measurement of absorption with a UV-1600PC spectrophotometer (Shimadzu, Kyoto, Japan) at 27℃. The baseline of each observation was recorded after irradiation with FR light for 5 min. After 3 min irradiation of R light, absorbance between 525 and 800 nm was repeatedly measured every 10 min for 60 min. Relative amounts of Pfr (Pfr/P_{total}) were calculated based on differences in the absorption peaks at red (648 nm) and far-red (709 nm) light, where the difference immediately after the R light irradiation was set as Pfr/P_{total} = 1. The effects of *PHYB* allele (CbPHYB and CnPHYB) and duration of darkness on Pfr/P_{total} were examined by a generalized linear mixed model (GLMM) with repeated measurement as random effects. After optimization, Gaussian

distribution with log link was applied for the analysis.

Results

Red light response differed between *Cardamine bellidifolia* and *C. nipponica*

Given that light sensitivity of phytochromes has been reflected in hypocotyl growth of seedlings under R light in *A. thaliana* (Maloof *et al.*, 2001; Balasubramanian *et al.*, 2006; Filiault *et al.*, 2008), we measured hypocotyl lengths of *C. bellidifolia* and *C. nipponica* grown in cR. Hypocotyl lengths differed significantly between the two species in weaker R light (10 and 30 μmol m^{-2} sec^{-1}) at 22℃ (Fig. 1b, c and Fig. S1; $P < 0.001$), whereas the two species had similar hypocotyl lengths in cD ($P = 0.66$) and strong R light (150 μmol m^{-2} sec^{-1}, $P = 0.24$), suggesting that *C. bellidifolia* and *C. nipponica* differ in their light sensitivity at R light limited conditions.

Pulse of R light at 12 hr intervals (pR) was much less effective than cR to inhibit the hypocotyl growth (Fig. 1b, d). Notably, the response to pR differed significantly between the two species at 22℃ [generalized linear mixed model (GLMM): Light × Species: $P < 0.0001$], with *C. nipponica* having longer hypocotyls in pR than *C. bellidifolia* (Fig. 1b, d: $P < 0.0001$). Pulse of FR light following pR diminished the repressive effects of hypocotyl growth in pR (pR vs. pR+FR: *Cb*, $P < 0.0001$; *Cn*, $P < 0.001$), resulting in similar hypocotyl lengths with cD (Fig. 1b, d, *Cb*, $P = 0.18$; *Cn*, $P = 0.28$). These observations suggest that the higher-latitude *C. bellidifolia* is more sensitive to R light and persist stronger phytochrome signals in darkness than the lower-latitude *C. nipponica.*

CnPHYB was exclusively divergent between the two species

One of possible explanation for the above observations is that Pfr in the higher-latitude *C. bellidifolia* was more stable than that in the lower-latitude *C. nipponica*. To explore whether phytochromes are responsible for the difference in seedling responses to R light, we investigated molecular evolution of four phytochrome genes (*PHYA–PHYC*, and *PHYE*) across range-wide samples of *C. bellidifolia* and *C. nipponica* (Fig. 1a, Table S1). Although all analysed genes exhibited similar level of divergence between species ($K = 0.0033–0.0040$; Table S2), *PHYB* and *PHYE* exhibited a non-neutral pattern of synonymous and nonsynonymous substitutions (Fig. 2a). *PHYB* showed the highest number of fixed amino acid differences between the two species, whereas *PHYE* showed excess of nonsynonymous polymorphisms within species. Notably, fixed nonsynonymous

substitutions between these species were exclusively found in *PHYB* (Fig. 2a).

To better understand the evolutionary history of *PHYB*, we constructed gene trees including 21 Brassicaceae species (Table S3) using both amino acid and nucleotide sequences. Consistent with previous phylogenetic inference (Ikeda *et al.*, 2012), both maximum likelihood and Bayesian trees resolved a reciprocally monophyletic relationship between *C. bellidifolia* and *C. nipponica* [Fig. 2b, S2; bootstrap values = 74 (amino acids) and 100 (nucleotides), posterior probabilities = 1.00 (both amino acids and nucleotides)]. The divergence of *CnPHYB* had higher ratio of nonsynonymous to synonymous substitutions ($\omega = 1.29$) than the remaining branches ($\omega = 0.07$, $P < 0.01$; Fig. 2b), whereas such accelerated evolution was not detected in the divergence of *CbPHYB* ($\omega = 0.32$, $P = 0.13$; Fig. 2b). Consistent with the higher evolutionary rate in *CnPHYB*, five of seven amino acid replacements were uniquely found in *C. nipponica* (Fig. 2c). In contrast, only one polymorphism (S495T) was shared among other species and one polymorphism (S594C) was uniquely found in *C. bellidifolia* (Fig. 2c and S3).

CbphyB was more sensitive to red light than CnphyB

As expected from thermal reversion of Pfr in darkness, pR with longer intervals of darkness resulted in longer hypocotyls in *A. thaliana* (Fig. S4). This hypocotyl response in pR was completely impaired in the *phyB*-deficient mutant, whereas these phenotypes in the mutants were strongly complemented in transgenics expressing *PHYB* of *A. thaliana* fused to GFP under control of either the *A. thaliana PHYB* promoter ($PHYB_{pro}$::*PHYB-GFP*) or the 35S promoter ($35S_{pro}$::*PHYB-GFP*; Fig. S4).

Transgenics overexpressing *CbPHYB* and *CnPHYB* showed little difference in hypocotyl length in cD at 22 ℃ (Fig. 3b; $P = 0.50$). In contrast, the response to the light differed between *CbPHYB* and *CnPHYB* (Fig. 3b). *CbPHYB* conferred significantly shorter hypocotyls in cR and pR than *CnPHYB* (Fig. 3c; $P < 0.001$ and $P < 0.0001$ for cR and pR, respectively), where responses to light conditions differed between *CbPHYB* and *CnPHYB* (GLMM: Light × *PHYB*: $P < 0.0001$). Notably, CnphyB Pfr was more stable in the transgenics than in *C. nipponica*, whose hypocotyls in pR have similar length as those in cD (Fig. 1), plausibly reflecting enhanced phyB Pfr activity due to overexpression. Immunoblot assays showed that there was no difference between CnphyB and CbphyB levels after 12 hr darkness (Fig. S5). Given that the polymorphisms in phyB barely influenced the sensitivity of antibody to detect GFP, degradation of phyB unlikely

explained the phenotypic difference in pR. In contrast, the difference in pR was diminished in pR+FR (Fig. 3; $P = 0.11$), resulting in hypocotyl length similar to with those in cD (*CbPHYB*, $P = 0.21$; *CnPHYB*, $P = 0.55$). Taken together, the hypocotyl response to pR could be attributed to the thermal stability of Pfr in darkness rather than protein levels. Furthermore, the size of nuclear bodies decreased more in CnphyB than CbphyB after 12 hr darkness (Fig. 3d, e; $P < 0.001$), directly supporting that CbphyB Pfr was more stable than CnphyB Pfr *in vivo*: CbphyB is extraordinarily stable compared to CnphyB, which was comparable to AtphyB (Van Buskirk *et al.*, 2014). However, the thermal stability of phyB Pfr *in vitro* was inconsistent with the observation *in vivo*: the CbphyB Pfr decreased more rapidly than CnphyB (Fig. S6; $P < 0.001$).

CbphyB is less sensitive to higher temperatures than CnphyB

Consistent with the faster thermal reversion of phyB in warmer conditions (Jung *et al.*, 2016; Legris *et al.*, 2016), phyB inhibited the hypocotyl elongation in *A. thaliana* more at lower temperatures in cR (Fig. S7). Transgenics overexpressing *CbPHYB* and *CnPHYB* showed little difference in hypocotyl length in cD (Fig. 4a; $P > 0.05$) as well as at 16℃ in cR (Fig. 4; $P = 0.47$). In contrast, the response to temperature significantly differed between *CbPHYB* and *CnPHYB* (Fig. 4; GLMM: Temperature × Light × *PHYB*: $P < 0.0001$). *CnPHYB* conferred longer hypocotyls than *CbPHYB* exclusively at higher temperatures in cR (Fig. 4; $P < 0.0001$ and $P < 0.0001$ for 22℃ and 28℃, respectively). In addition, *CnPHYB* conferred more sensitive responses to higher temperature than *CbPHYB* in pR (Fig. 4). Hence, as expected from the thermal stability, our results suggest that phyB in *C. bellidifolia* is less sensitive to high temperatures than phyB in *C. nipponica*.

Discussion

Light sensitivity of phytochromes is an important mechanism for plants to adapt to environments varying along latitude; plants growing in higher latitude have phytochromes with higher light sensitivity (Balasubramanian *et al.*, 2006; Filiault *et al.*, 2008). Together with the finding of difference in light sensitivity of phytochrome between two sister species growing at different latitude (Fig. 1), our study demonstrates that the lower-latitude *Cardamine nipponica* has phyB with lower light sensitivity probably due to less thermal stability of Pfr *in vivo* than the higher-latitude *C. bellidifolia* (Figs. 3, 4). Collectively, this study provides the first evidence of evolutionary divergence in Pfr

thermal stability of phyB between species or accessions, suggesting that fine-tuning Pfr stability is one of the molecular mechanisms in plants to modify light or temperature sensitivity of phytochromes and optimise phytochrome-related traits in nature. Given that higher thermal-stable Pfr confers shorter hypocotyls even in cR light (Fig. 4), Pfr stability may be associated with the previously observed latitudinal difference in light sensitivity of phytochromes in *A. thaliana* (Balasubramanian *et al.*, 2006; Filiault *et al.*, 2008). Although amino acid polymorphisms in the two *Cardamine* species were not shared among species in Brassicaceae (Fig. 2), fine-tuning Pfr stability might be a common mechanism underlying latitudinal adaptation.

Since *C. nipponica* grows under shorter photoperiods and higher temperature in summer (Fig. S8), it may frequently experience either higher temperatures in certain periods of days or larger diurnal temperature changes compared to *C. bellidifolia*, which grows in cooler temperature with less diurnal changes. Faster decline of phytochrome activity at higher temperatures might be beneficial under the temperature regime of *C. nipponica*, whose phytochrome activity was mostly diminished in 12 hr darkness (Fig. 1). Nevertheless, the present study exclusively focused on the hypocotyl response to R light in *C. bellidifolia* and *C. nipponica* (Fig. 1), and thus could clarify neither their difference in temperature responses nor the ecological importance of their difference in the Pfr stability. Given their genetic distinctiveness (Ikeda *et al.*, 2012), *C. bellidifolia* and *C. nipponica* may have numerous genetic differences in their physiological traits including light and temperature responses. Further studies are required to assess their ecological divergence as well as selective forces associated with the phyB Pfr stability.

It should be noted that our study did not fully elucidated molecular mechanisms that fine-tune Pfr stability. Since we investigated phytochrome responses in an identical genetic background, molecular properties of CbphyB and CnphyB could be responsible for the difference in hypocotyl responses of transgenics. Previous studies show that mutations on the PHY domain either weakened (G564A, S584F, A587T; Oka *et al.*, 2004; Oka *et al.*, 2008) or enhanced (G564E; Kretsch *et al.*, 2000) phyB Pfr stability consistently both *in vitro* and *in vivo*, and hence the three amino acid changes on the PHY domain (Fig. S3) might be responsible for the difference in Pfr stability between CbphyB and CnphyB. However, our *in vitro* assay did not support this hypothesis: i.e., *in vitro* Pfr stability of the N651 of CbphyB and CnphyB (Fig. S6) was discordant with Pfr stability *in vivo* (Fig.

3) as well as hypocotyl responses in the two *Cardamine* species (Fig. 1) and the transgenics (Fig. 3). Given that the C-terminal of phyB is essential for dimerization and accelerates thermal reversion (Burgie *et al.*, 2017), the present study may not be sufficient to evaluate *in vitro* thermal stability of full-length CbphyB and CnphyB. Furthermore, Pfr thermal stability *in vivo* is determined not only by phytochrome itself but also by interaction with chromophore (Burgie *et al.*, 2017) or other proteins, including PHOTOPERIODIC CONTROL OF HYPOCOTYL 1 (PCH1; Enderle *et al.*, 2017; Huang *et al.*, 2019), PCH1-LIKE (PCHL; Enderle *et al.*, 2017) and *Arabidopsis* response regulator 4 (ARR4; Sweere *et al.*, 2001). Addressing these issues is required for understanding molecular mechanisms that fine-tune phytochrome responses in nature.

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Author Contributions

H.I. designed the research and experiments, collected plant materials, performed all experiments and data analyses, interpreting data and wrote the draft of the manuscript; T.S. made transgenic *A. thaliana*; L.G. and C.B. collected plant materials; Y.O. and N.M.; A.N. designed experiments and interpreted data; all authors discussed and commented on the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Hypocotyl responses to various red light fluence rates in each accession of *Cardamine bellidifolia* and *Cardamine nipponica*.

Fig. S2 *PHYB* gene trees in Brassicaceae.

Fig. S3 Schematic image of phytochrome domain and alignment of amino acid sequences of CnPHYB and CbPHYB.

Fig. S4 Red light responses in transgenic *Arabidopsis thaliana*.

Fig. S5 Protein level of CbPHYB and CnPHYB in transgenic *Arabidopsis thaliana*.

Fig. S6 In vitro thermal stability of Pfr of CbPHYB and CnPHYB.

Fig. S7 Temperature dependent hypocotyl growth in transgenic *Arabidopsis thaliana*.

Fig. S8 Mean monthly temperature in localities of *Cardamine bellidifolia* and *Cardamine nipponica*.

Table S1 Information of samples used in this study (separate Excel file).

Table S2 Genetic diversity and divergence of phytochromes within and between *Cardamine bellidifolia* and *Cardamine nipponica*.

Table S3 Sources of outgroup sequences for phylogenetic analyses.

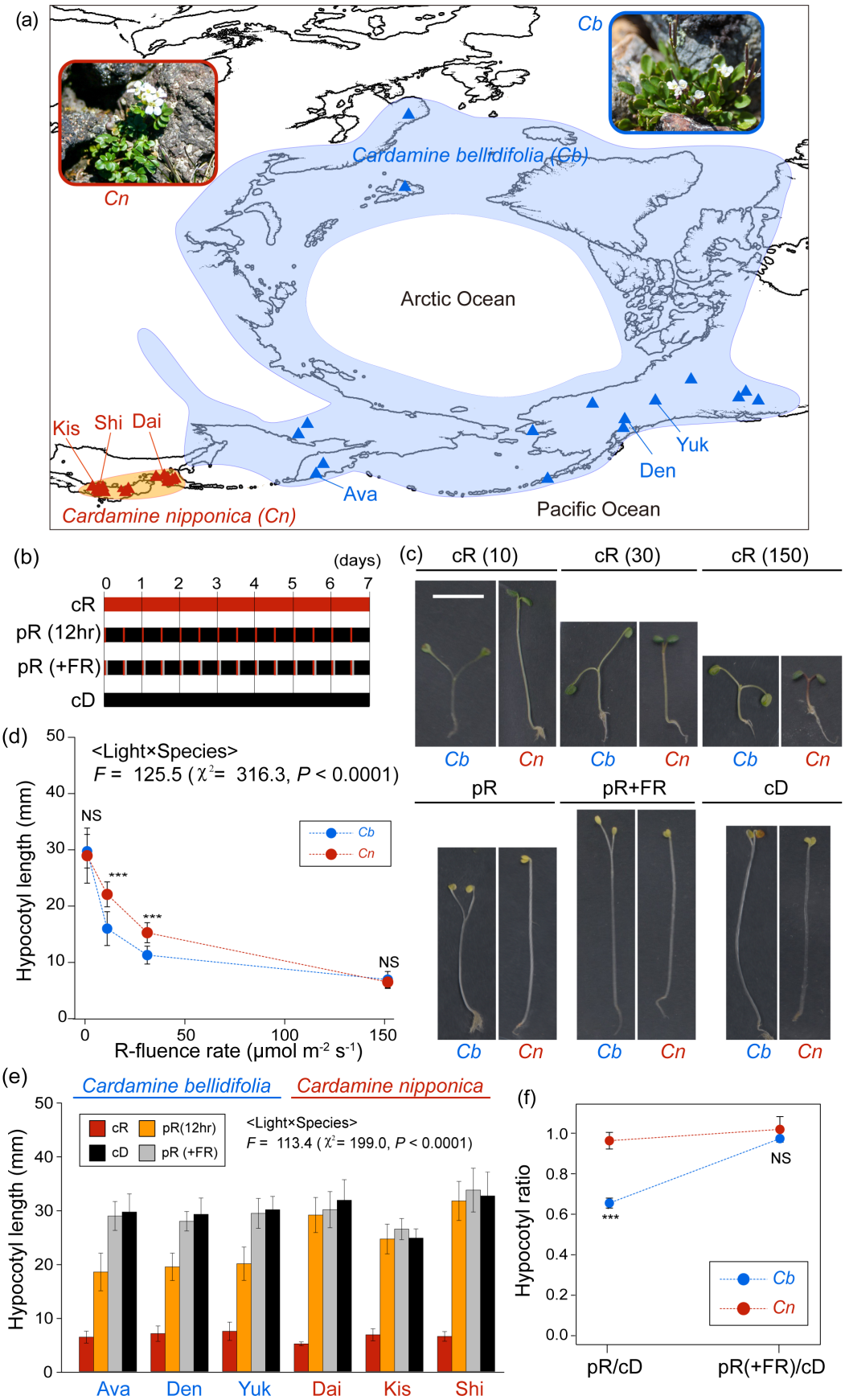
Figure legends

Figure 1. Red light sensitivity of *Cardamine bellidifolia* (*Cb*) and *Cardamine nipponica* (*Cn*). (a) Sampling localities (triangles) and entire geographic range of the higher-latitude species *Cb* (blue) and its lower-latitude sister species *Cn* (red). Localities of samples for seedling assays are indicated by three-letter abbreviations. (b) Light conditions for seedling assays. cR, continuous red light (R) light. pR, 15 min R pulses (ca. 150 μmol m^{-2} sec^{-1}) every 12 hr darkness. pR+FR, pR with 15 min far-red light pulse (ca. 100 μmol m^{-2} sec^{-1}) immediately after R pulses. cD, continuous darkness. Seedling assay was performed for seven days at 22 ℃. (c) Representative seedlings grown in the seedling assay. The number in parentheses after cR is R fluence rate (ca. 10, 30 and 150μmol m^{-2} sec^{-1}). Scale bar = 10 mm. (d) Hypocotyl growth responses to cR with different R fluence rates for seven days at 22 ℃. Data are the means ± SD ($n \geq 70$). *** and NS, significant ($P < 0.0001$) and nonsignificant result, respectively, evaluated by generalized linear mixed model. (e) Hypocotyl growth responses of each accession to cR (ca. 150 μmol m^{-2} sec^{-1}), pR, pR+FR and cD for seven days at 22 ℃. Data are the means ± SD ($n \geq 10$) (f) Relative hypocotyl length in pR and pR+FR compared to the length in cD. Data are the mean of mean ratios of each accession ± SD.

Figure 2. Molecular evolution of phytochrome genes of *Cardamine bellidifolia* and *Cardamine nipponica*. (a) Numbers of synonymous and nonsynonymous substitutions are shown for fixed differences between *C. bellidifolia* and *C. nipponica* (interspecific) and polymorphisms within both species (intraspecific). *, significant results of the McDonald-Kreitman test ($P < 0.05$). (b) Maximum likelihood tree of *PHYB* for the Brassicaceae based on amino acid (AA) sequences. ●, nodes supported both by AA and nucleotide (DNA) sequences (bootstrap > 90 and posterior probability > 0.95). ○, nodes supported solely by DNA sequence data. Evolutionary patterns Results of molecular evolution implemented by PAML are shown in the box. (c) Fixed differences in amino acid residues in *PHYB* between *C. bellidifolia* and *C. nipponica*. Amino acid residues of outgroups at each polymorphic site are also included.

Figure 3. Red light sensitivity conferred by *PHYB* of *Cardamine bellidifolia* (*Cb*) and *Cardamine nipponica* (*Cn*) in *Arabidopsis thaliana*. (a) Light conditions used for seedling assays. Seedlings were grown in continuous red light (cR; ca. 30 μmol m^{-2} sec^{-1}), 15 min red light pulses (ca. 30 μmol m^{-2} sec^{-1}) every 12 hr darkness (pR), 15 min far-red light pulses (ca. 100 μmol m^{-2} sec^{-1}) after pulse red [pR (+FR)] and continuous darkness (cD) for five days at 22℃. (b) Mean hypocotyl length (mm) of $35S_{pro}$::*PHYB-GFP* transgenic *A. thaliana* (*phyB-5* mutant) grown in cR, pR, pR (+FR) and cD ($n \geq 25$). Three independent lines of each transgenic line were analysed. Error bars indicate standard deviation. (c) Relative hypocotyl length in cR, pR and pR (+FR) compared to the length in cD. Blue and red line indicates transgenic lines with *CbPHYB* and *CnPHYB*, respectively. Data are the mean of mean ratios of each transgenic line ± SD. *** and NS, significant ($P < 0.001$) and nonsignificant result, respectively, evaluated by generalized linear mixed model. (d) Area of phyB nuclear bodies (NBs) in two-day old seedlings of transgenics before (0 hr) and after (12 hr) 12 hr darkness. The box represents interquartile, the bold black line in the box is the median, the whiskers indicate values greater than the interquartile excluding outliers, which are represented by circles. *CbPHYB* and *CnPHYB* includes three independent transgenic lines ($n \geq 25$ for each line). (e) Representative confocal images of GPF signals from NBs in nucleus of hypocotyl epidermal cells in two-day old seedlings . Scale bar = 2.0 μm.

Figure 4. Temperature dependent seedling growth conferred by *PHYB* of *Cardamine bellidifolia* (*Cb*) and *Cardamine nipponica* (*Cn*) *in Arabidopsis thaliana*. (a) Boxplot of hypocotyl length (mm) of $35S_{pro}$::*PHYB-GFP* transgenic *A. thaliana* (*phyB-5* mutant) grown at 16, 22, and 28℃ in continuous red light (cR; ca. 30 μmol m^{-2} sec^{-1}), 15 min red light pulses (ca. 30 μmol m^{-2} sec^{-1}) every 12 hr darkness (pR) and continuous dark (cD) for five days. The box represents interquartile, the bold black line in the box is the median, the whiskers indicate values greater than the interquartile excluding outliers, which are represented by circles. *CbPHYB* and *CnPHYB* includes three independent transgenic lines ($n \geq 25$ for each line). (b) Relative hypocotyl length in cR and pR compared to the length in cD. Data are the mean of mean ratios of each transgenic line ± SD. *** and NS, significant ($P < 0.001$) and nonsignificant result, respectively, evaluated by generalized linear mixed model.


(a)
Cb
Cn
Cardamine bellidifolia (Cb)
Arctic Ocean
Kis
Shi
Dai
Yuk
Den
Ava
Cardamine nipponica (Cn)
Pacific Ocean
(b)
(days)
0 1 2 3 4 5 6 7
cR
pR (12hr)
pR (+FR)
cD
(c)
cR (10)
cR (30)
cR (150)
Cb Cn Cb Cn Cb Cn
pR
pR+FR
cD
Cb Cn Cb Cn Cb Cn
(d)
<Light×Species>
F = 125.5 (χ²= 316.3, P < 0.0001)
Hypocotyl length (mm)
NS
***
***
NS
Cb
Cn
R-fluence rate (μmol m⁻² s⁻¹)
(e)
Cardamine bellidifolia
Cardamine nipponica
cR
pR(12hr)
cD
pR (+FR)
<Light×Species>
F = 113.4 (χ²= 199.0, P < 0.0001)
Hypocotyl length (mm)
Ava Den Yuk Dai Kis Shi
(f)
Hypocotyl ratio
NS
***
Cb
Cn
pR/cD
pR(+FR)/cD

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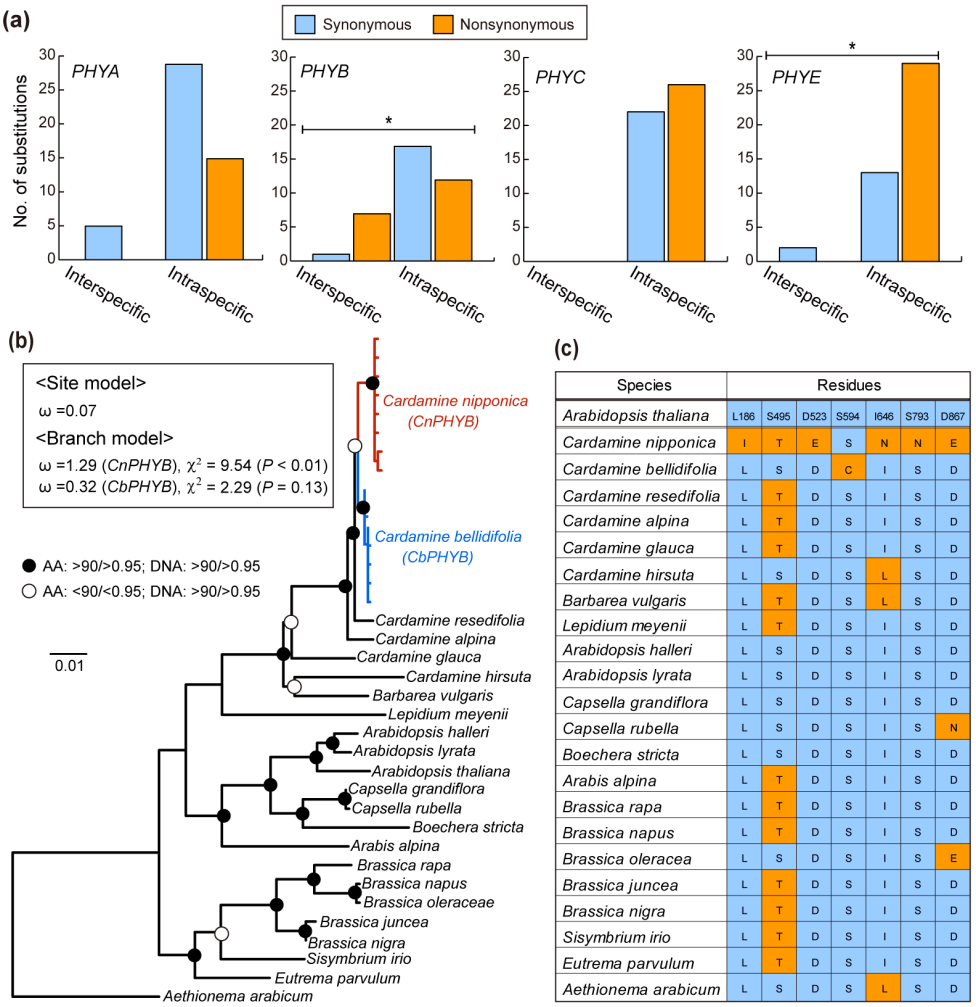

Species	Residues						
	L186	S495	D523	S594	I646	S793	D867
Arabidopsis thaliana	L	S	D	S	I	S	D
Cardamine nipponica	I	T	E	S	N	N	E
Cardamine bellidifolia	L	S	D	C	I	S	D
Cardamine resedifolia	L	T	D	S	I	S	D
Cardamine alpina	L	T	D	S	I	S	D
Cardamine glauca	L	T	D	S	I	S	D
Cardamine hirsuta	L	S	D	S	L	S	D
Barbarea vulgaris	L	T	D	S	L	S	D
Lepidium meyenii	L	T	D	S	I	S	D
Arabidopsis halleri	L	S	D	S	I	S	D
Arabidopsis lyrata	L	S	D	S	I	S	D
Capsella grandiflora	L	S	D	S	I	S	D
Capsella rubella	L	S	D	S	I	S	N
Boechera stricta	L	S	D	S	I	S	D
Arabis alpina	L	T	D	S	I	S	D
Brassica rapa	L	T	D	S	I	S	D
Brassica napus	L	T	D	S	I	S	D
Brassica oleracea	L	S	D	S	I	S	E
Brassica juncea	L	T	D	S	I	S	D
Brassica nigra	L	T	D	S	I	S	D
Sisymbrium irio	L	T	D	S	I	S	D
Eutrema parvulum	L	T	D	S	I	S	D
Aethionema arabicum	L	S	D	S	L	S	D

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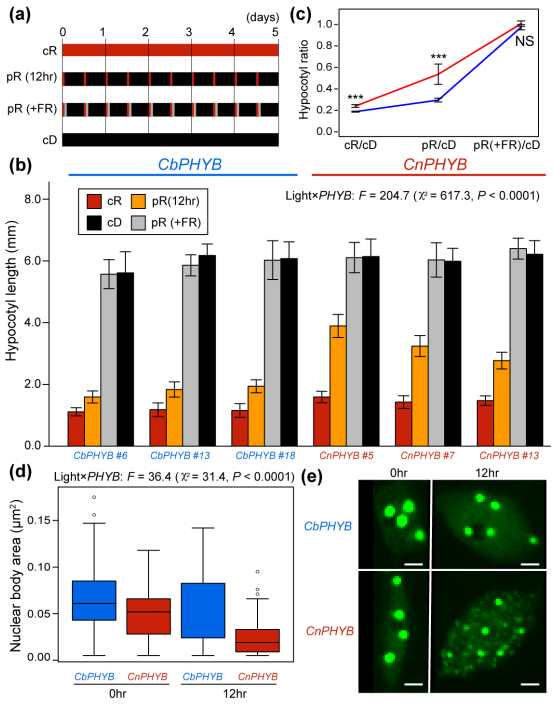

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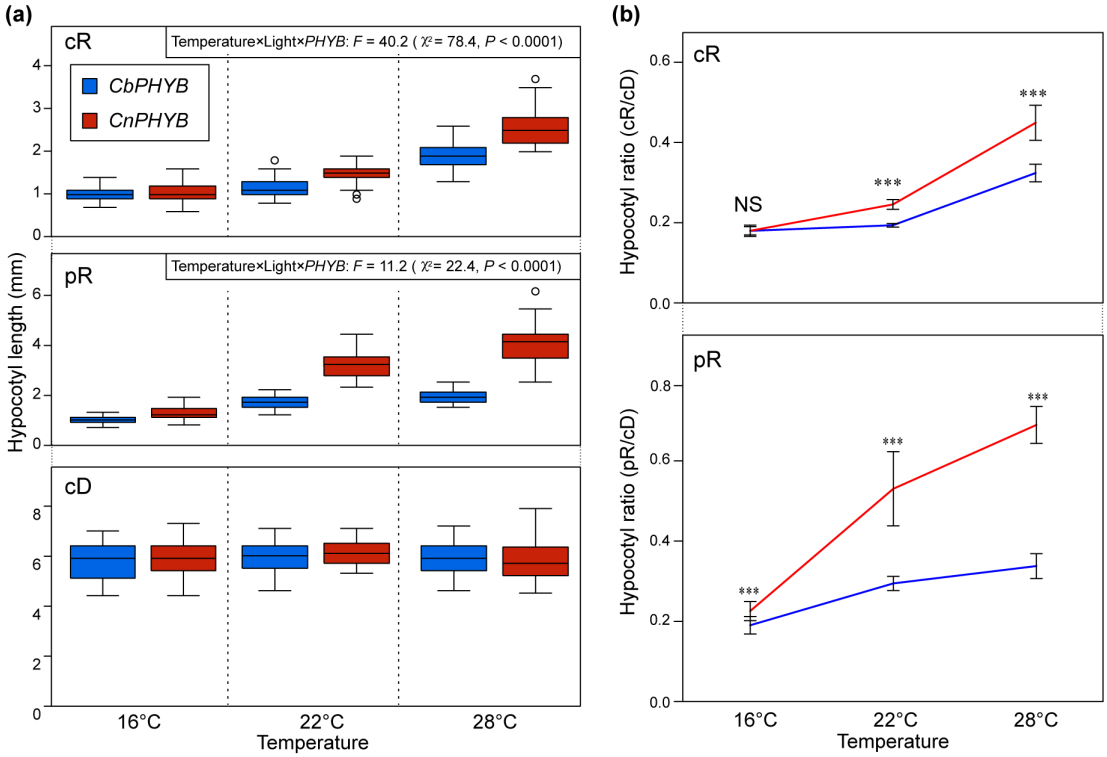

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