Phytochrome C Is A Key Factor Controlling Long-Day Flowering in Barley[1][W]

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The spring-type near isogenic line (NIL) of the winter-type barley (*Hordeum vulgare* ssp. *vulgare*) var. Hayakiso 2 (HK2) was developed by introducing *VERNALIZATION-H1* (*Vrn-H1*) for spring growth habit from the spring-type var. Indo Omugi. Contrary to expectations, the spring-type NIL flowered later than winter-type HK2. This phenotypic difference was controlled by a single gene, which cosegregated only with *phytochrome C* (*HvPhyC*) among three candidates around the *Vrn-H1* region (*Vrn-H1*, *HvPhyC*, and *CASEIN KINASE IIα*), indicating that *HvPhyC* was the most likely candidate gene. Compared with the late-flowering allele *HvPhyC-l* from the NIL, the early-flowering allele *HvPhyC-e* from HK2 had a single nucleotide polymorphism T1139C in exon 1, which caused a nonsynonymous amino acid substitution of phenylalanine at position 380 by serine in the functionally essential GAF (3′, 5′-cyclic-GMP phosphodiesterase, adenylate cyclase, formate hydrogen lyase activator protein) domain. Functional assay using a rice (*Oryza sativa*) *phyA phyC* double mutant line showed that both of the *HvPhyC* alleles are functional, but *HvPhyC-e* may have a hyperfunction. Expression analysis using NILs carrying *HvPhyC-e* and *HvPhyC-l* (NIL [*HvPhyC-e*] and NIL [*HvPhyC-l*], respectively) showed that *HvPhyC-e* up-regulated only the flowering promoter *FLOWERING LOCUS T1* by bypassing the circadian clock genes and flowering integrator *CONSTANS1* under a long photoperiod. Consistent with the up-regulation, NIL (*HvPhyC-e*) flowered earlier than NIL (*HvPhyC-l*) under long photoperiods. These results implied that *HvPhyC* is a key factor to control long-day flowering directly.

The flowering time of barley (*Hordeum vulgare* ssp. *vulgare*) is a complex character that is composed of three different subcharacters: earliness per se, vernalization requirement, and photoperiod sensitivity (Takahashi and Yasuda, 1970). The first character determines flowering time alone, while the latter two modify it in response to environmental signals including temperature and photoperiod. These are essential for successful seed set and maximizing yield by contributing greatly to adaptation to different climatic regions (Knüpffer et al., 2003; von Bothmer et al., 2003).

In winter-type barley, the vernalization requirement avoids frost injury by delaying flower induction until the extended period of cold temperature during winter satisfies the requirement. On the other hand, spring-type barley does not have such a requirement. The vernalization requirement is controlled by three major genes: *VERNALIZATION-H1* (*Vrn-H1*), *Vrn-H2*, and *Vrn-H3* (former *SPRING GROWTH HABIT2* [*Sgh2*], *Sgh1*, and *Sgh3*, respectively). *Vrn-H1* encodes a protein highly similar to Arabidopsis (*Arabidopsis thaliana*) *APETALA1* (*AP1*)/*FRUITFULL* (*FUL*) that determines meristem identity, flower organ formation, and flowering time (Yan et al., 2003). *Vrn-H2* encodes ZCCT protein with a putative zinc finger and a CCT (CONSTANS, CONSTANS-LIKE, and TIMING OF CHLOROPHYLL *A/B* BINDING PROTEIN EXPRESSION1) domain, which is expected to be involved in transcriptional regulation and is expressed under long photoperiods (Yan et al., 2004). *Vrn-H3*, which encodes an ortholog of Arabidopsis flowering promoter *FLOWERING LOCUS T* (*FT*) and long photoperiods up-regulate *Vrn3* expression (Turner et al., 2005; Yan et al., 2006). It was proposed that they form a feedback loop and interact to regulate their expression (Trevaskis et al., 2007; Distelfeld et al., 2009; Shimada et al., 2009).

Barley is a long-day plant in which photoperiod sensitivity delays flowering time under a short photoperiod compared with that under a long photoperiod. It is well known that photoperiod sensitivity greatly contributes to adaptation (Knüpffer et al., 2003). Two genes that influence photoperiod sensitivity are *PHOTOPERIOD-H1* (*Ppd-H1*) and *Ppd-H2* (Laurie et al., 1995). *Ppd-H1* controls flowering time under long photoperiods and encodes pseudoresponse regulator (PRR) whose ortholog is

[1] This work was supported in part by the Ministry of Agriculture, Forestry, and Fisheries of Japan (Integrated Research Project for Plant, Insect, and Animal Using Genome Technology grant no. GD–3005 to H.H. and Genomics for Agricultural Innovation grant no. FBW–1201 to H.N.).

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The author responsible for the distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Kenji Kato (kenkato@cc.okayama-u.ac.jp).

[W] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.113.222570

involved in circadian clock function in Arabidopsis (Turner et al., 2005). *Ppd-H2* controls flowering time under short photoperiods, and it encodes *HvFT3*, which belongs to the gene family of *FT* (Kikuchi et al., 2009).

In addition to the above-mentioned genes, novel gene resources for early flowering will be important to elucidate the genetic mechanism of the flowering time and future breeding programs. Recent comparative studies in genetic pathways for flowering revealed that temperate grass species share a similar gene set with dicot species Arabidopsis, especially for photoperiodic pathways, although it has been disclosed gradually that evolutionary distinct genes and pathways are associated with the photoperiodic pathways (Trevaskis et al., 2007; Higgins et al., 2010). These pathways include photoreceptors (phytochromes, cryptochromes, and phototropin) that perceive daily light/dark cycles, the circadian clock (*CIRCADIAN CLOCK ASSOCIATED1*, *TIMING OF CHLOROPHYLL A/B-BINDING PROTEIN EXPRESSION1*, *PRR*s [such as *Ppd-H1*], and *GIGANTEA* [*GI*; *HvGI* in barley]), which is entrained by the signals from photoreceptors, and downstream genes (*CONSTANS* [*CO*; *HvCO1* in barley], *FT* [*HvFT1* in barley], and *AP1/FUL* [*Vrn-H1*]), which integrate and transmit the signals from photoreceptors and the circadian clock. However, natural variation in many of such genes has not been characterized yet in barley.

Yasuda (1969) developed a series of near isogenic lines (NILs) carrying different spring alleles *Sgh2* (*Vrn-H1*) in a Japanese winter var. Hayakiso 2 (HK2) that had a winter allele *sgh2* (*vrn-H1*) originally. These NILs, compared with HK2, showed unexpectedly later flowering under autumn-sowing field conditions irrespective of their spring growth habit. Such behavior may be ascribable to the pleiotropic effect of *Vrn-H1* or an unknown flowering-time gene tightly linked with *Vrn-H1*. According to previous studies (Szücs et al., 2006; Kato et al., 2008), *Vrn-H1* is located closely to other two candidate genes for photoperiod sensitivity, *Phytochrome C* (*HvPhyC*) and *Casein Kinase II alpha* (*HvCK2α*). *HvPhyC* encodes the apoprotein of photoreceptor PHYC, which is involved in red/far-red light perception. *PhyC* orthologs in other species are also associated with flowering time: the rice (*Oryza sativa*) ortholog controls photoperiod sensitivity (Takano et al., 2005) and Arabidopsis alleles showed latitudinal cline, which suggested the association of photoperiod sensitivity (Balasubramanian et al., 2006). *HvCK2α* encodes the α subunit of CK2 protein. A rice flowering-time gene, *Heading date6*, which is considered to be an ortholog of *HvCK2α* by cross-referencing syntheny among barley, wheat (*Triticum aestivum*), and rice, controls photoperiod sensitivity (Takahashi et al., 2001; Ogiso et al., 2010; Kato et al., 2002, 2008).

In this study, we first conducted genetic and linkage analyses to identify a causal gene for early flowering in HK2. Secondly, the gene effect was evaluated by growing the NILs under different photoperiods. Third, functional assay using a rice transformation system was conducted to evaluate the functionality of the flowering-time gene. Finally, based on the results of expression analysis, the role of the flowering-time gene in the genetic pathways for flowering was discussed.

RESULTS

Genetic and Linkage Analyses

HK2 was crossed with a late-flowering NIL carrying the spring allele *Vrn-H1* from var. Indo Omugi (hereafter, NIL [*Vrn-H1*]). Frequency distribution for flowering time in the F2 population was continuous but trimodal, suggesting the segregation of early, intermediate, and late types caused by a single gene (Fig. 1). For accurate genotyping, a progeny test was conducted using F3 lines derived from F2 plants with flowering time within the overlap regions between different types. As a result, the F2 population was found to segregate 217 early, 446 intermediate, and 229 late types, which fit the

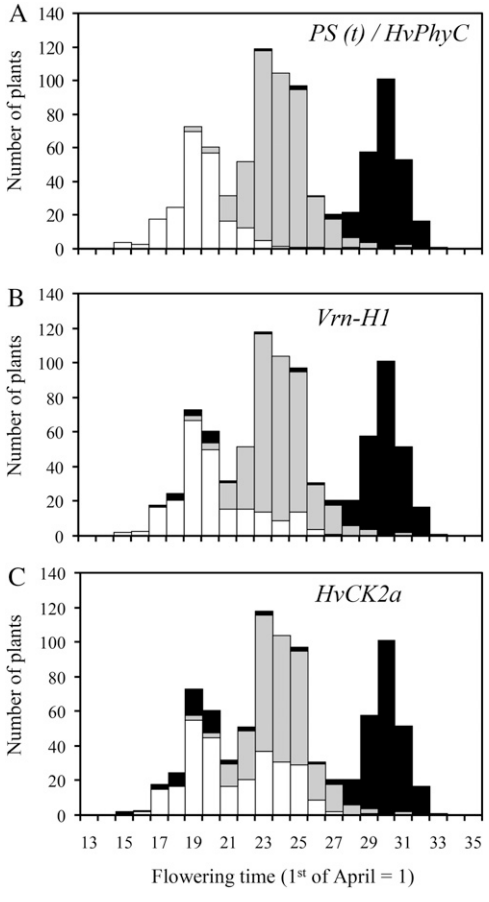

Figure 1. Frequency distribution of flowering time in the F2 population derived from a cross between HK2 and its NIL carrying the spring allele for *Vrn-H1* (NIL [*Vrn-H1*]) under field conditions. The 892 F2 plants could be classified into three types by the genotype of a causal gene for flowering time (*PS [t]*; A) as well as its candidate genes *HvPhyC* (A), *Vrn-H1* (B), and *HvCK2α* (C). White, gray, and black rectangles indicate homozygote for HK2 allele, heterozygote, and homozygote for NIL (*Vrn-H1*) allele.

ratio of 1:2:1 (χ^2 = 0.323, P = 0.851) for single gene segregation. Hereafter, the gene was designated tentatively as *PS* (*t*).

Linkage analysis using the same population showed that *HvPhyC* was linked by *Vrn-H1* and *HvCK2α* with genetic distances 1.5 and 3.1 centimorgans, respectively, and the gene order was estimated to be *Vrn-H1* – *HvPhyC* – *HvCK2α*. Among these three genes, *HvPhyC* cosegregated with *PS* (*t*) (Fig. 1A), strongly suggesting that it is the causal gene for *PS* (*t*). By contrast, several critical recombinations were observed between *Vrn-H1* and *PS* (*t*) and between *HvCK2α* and *PS* (*t*) (Fig. 1, B and C). From this result, *Vrn-H1* and *HvCK2α* could be ruled out as candidates. Hereafter, we designate the early-flowering (HK2) and late-flowering (NIL [*Vrn-H1*]) alleles for the flowering-time gene (*HvPhyC*) as *HvPhyC-e* and *HvPhyC-l*, respectively.

Sequence analysis of *HvPhyC* alleles revealed a single nucleotide polymorphism (SNP) in exon 1 (at the position 1,139 from the start codon) that causes nonsynonymous substitution at the C-terminal side of the GAF (3′, 5′-cyclic-GMP phosphodiesterase, adenylate cyclase, formate hydrogen lyase activator protein) domain (at position 380) in the deduced amino acid sequence (Fig. 2, A and B). The deduced amino acid residue from *HvPhyC-l* had Phe at this position, which was well conserved among several plant species (wheat, rice, sorghum [*Sorghum bicolor*], *Brachypodium distachyon*, and Arabidopsis), while that from *HvPhyC-e* had Ser, suggesting it to be a mutant allele (Fig. 2C).

Effect of *HvPhyC* on Flowering Time under Different Photoperiods

Each two independent NILs carrying *HvPhyC-e* and *HvPhyC-l* (four NILs) were selected out of the F4 progenies of the mapping population (Table I). All of these NILs have the same genotype for the other flowering-time genes, because the alleles from the NIL (*Vrn-H1*) were selected for *Vrn-H1* and *HvCK2α*, which segregated in the mapping population. Each two NILs carrying *HvPhyC-e* and *HvPhyC-l* were mixed together and designated as NIL (*HvPhyC-e*) and NIL (*HvPhyC-l*), respectively, because the same genotype NILs showed statistically the same flowering time. Under long (16-h) and extremely long (20-h) photoperiods, NIL (*HvPhyC-e*) flowered much earlier than NIL (*HvPhyC-l*) (22.5% and

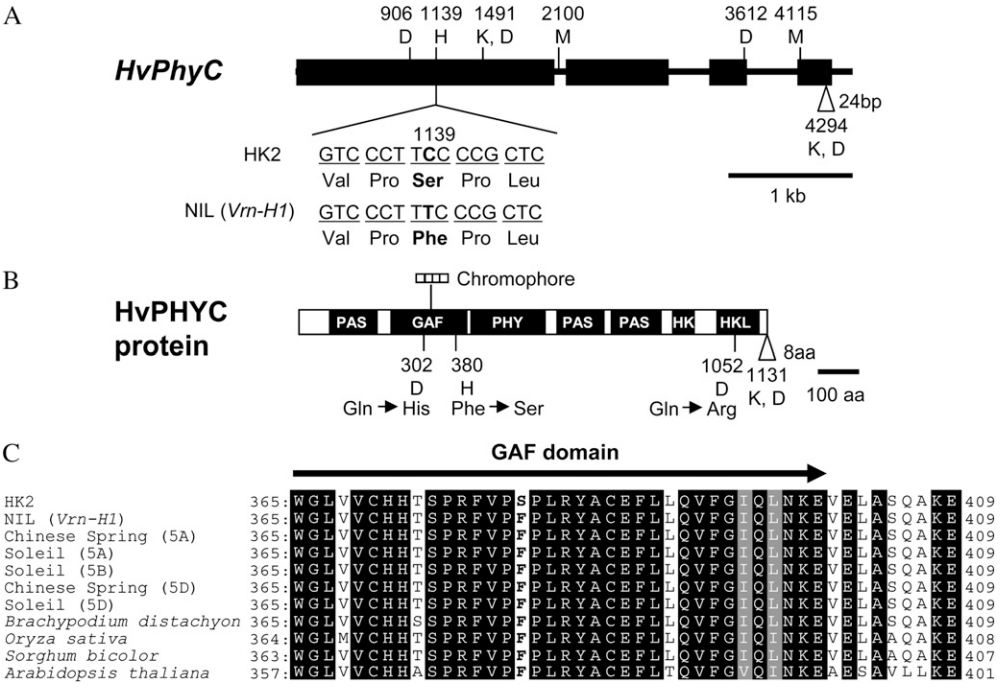

Figure 2. Structure of *HvPhyC* gene and its protein. A, *HvPhyC* gene sequences from HK2 and NIL (*Vrn-H1*) were compared with those from var. Morex (DQ238106), var. Dicktoo (DQ201145), and var. Kompolti Korai (DQ201146). Black boxes and horizontal lines between them indicate exons and introns, respectively. Vertical line indicates SNP when NIL (*Vrn-H1*) was compared with other varieties. White triangle indicates a 24-bp insertion. NIL (*Vrn-H1*), HK2, and Morex have this insertion, which results in an 8-amino acid insertion. Numbers and letters at polymorphic sites indicate the position and polymorphic varieties, respectively. H, M, D, and K indicate HK2, Morex, Dicktoo, and Kompolti Korai, respectively. B, Deduced amino acid sequences were compared based on the *HvPhyC* gene sequences. Black boxes indicate domains. Polymorphisms are shown in the same way as *HvPhyC* gene. HK, His kinase domain; HKL, His kinase-like ATPase domain. C, Deduced amino acid sequences around the C-terminal end of GAF domain were compared based on the genomic sequences of barley, wheat (GenBank: AY672995.1, AY673002.1, AY672998.1, AY672999.1, and AY673000.1), *B. distachyon* (Gbrowse: Bradi1g08400.1), rice (RAP-DB: Os03g0752100-01), and sorghum (GDB: Sb01g007850.1). The same and similar sequences are highlighted in black and gray, respectively.

Table I. *Genotype for flowering-time genes in HK2 and its NILs determined by diagnostic markers*

Variety/Line	Flowering Time	Photoperiod Sensitivity				Vernalization Requirement			
		HvPhyC	*HvCK2α*[a]	*Ppd-H1*[b]	*Ppd-H2*[c]	*Vrn-H1*[d]	*Vrn-H2*[d]	*Vrn-H3*[d]	Growth Habit
HK2	Early	*HvPhyC-e*	*H*	*Ppd-H1*	*Ppd-H2*	*vrn-H1*	*Vrn-H2*	*vrn-H3*	Winter
NIL (*Vrn-H1*)	Late	*HvPhyC-l*	*N*	*Ppd-H1*	*Ppd-H2*	*Vrn-H1*	*Vrn-H2*	*vrn-H3*	Spring
NIL (*HvPhyC-l*)	Late	*HvPhyC-l*	*N*	*Ppd-H1*	*Ppd-H2*	*Vrn-H1*	*Vrn-H2*	*vrn-H3*	Spring
NIL (*HvphyC-e*)	Early	*HvphyC-e*	*N*	*Ppd-H1*	*Ppd-H2*	*Vrn-H1*	*Vrn-H2*	*vrn-H3*	Spring

[a]H and N indicate the alleles from HK2 and NIL (*Vrn-H1*), respectively. [b]Recessive allele for *Ppd-H1* confers late flowering under long photoperiod. [c]Dominant allele for *Ppd-H2* confers early flowering under short photoperiod. [d]Dominant allele for *Vrn-H1* (identical with *HvVRN1-10* in Hemming et al. [2009]), recessive allele for *Vrn-H2*, and dominant allele for *Vrn-H3* confer spring growth habit.

24.4% reduction in days from sowing to flag leaf unfolding, respectively; Fig. 3). By contrast, no significant difference (only 4.3% reduction) was observed under a short (12-h) photoperiod. The result showed that *HvPhyC* controls photoperiod sensitivity under long photoperiods.

Functional Assay of *HvPhyC* Using Rice Transformation System

To evaluate the function of *HvPhyC-e* from HK2 and *HvPhyC-l* from NIL (*Vrn-H1*), we introduced these two alleles under the control of the *Cauliflower mosaic virus* 35S promoter into a rice *phyA phyC* double mutant line as the recipient with a genetic background of the Japanese var. Nipponbare, because the *phyA phyC* double mutant line flowers significantly earlier than the original var. Nipponbare under a natural (long) photoperiod (Takano et al., 2005).

The T1 control lines carrying the empty vector in Nipponbare and *phyA phyC* double mutant line flowered 59.6 and 45.6 d after sowing under a natural (long) photoperiod, respectively, confirming the effect of *PhyC* and *PhyA* genes on flowering time under a long photoperiod (Fig. 4).

One (no. 1-26) out of two T1 lines carrying *HvPhyC-l* (wild-type allele) segregated late-flowering (Nip mock-type) and early-flowering (*phyA phyC* mock-type) plants. All of the late-flowering plants expressed *HvPhyC-l* at a high level, while early-flowering plants expressed *HvPhyC-l* at a low level (Fig. 4). The other T1 line (no. 1-12) was composed of only early-flowering plants that expressed *HvPhyC-l* at a low level. Therefore, it was confirmed that high enough expression of *HvPhyC-l* can recover from the *phyA phyC* double mutant phenotype.

One (no. 17-14) out of four T1 lines carrying *HvPhyC-e* (mutant allele) segregated late-flowering plants, all of which expressed *HvPhyC-e* at a high level, while the others (nos. 17-1, 17-6, and 17-8) were composed of only early-flowering plants (Fig. 4). To our surprise, this result strongly suggested that the *HvPhyC-l* and *HvPhyC-e* alleles are both functional. However, even a lower expression level of *HvPhyC-e*, compared with *HvPhyC-l*, could recover from the *phyA phyC* double mutant phenotype, suggesting the hyperfunction of *HvPhyC-e*.

Expression Analysis of the Flowering Pathway Genes

There were no apparent differences in *HvPhyC* expression between NIL (*HvPhyC-e*) and NIL (*HvPhyC-l*), despite their allelic differences (Fig. 5A; Supplemental Fig. S1A). In both of the NILs, *HvPhyC* was expressed all day and seemed to show diurnal fluctuation under both long (16-h) and short (8-h) photoperiod conditions with the trend that it was up-regulated around dusk and down-regulated during the day. This result led to the hypothesis that the SNP (T1139C) in the GAF domain might affect its protein function rather than its own expression pattern and also the expression pattern of downstream genes interacting with HvPHYC protein.

Subsequently, we compared the expression of circadian clock-related genes *HvCK2α*, *HvGI*, and *Ppd-H1*, because the circadian clock is considered to interact with photoreceptors. Unexpectedly, two NILs showed similar expression patterns for all three genes (Fig. 5, B–D; Supplemental Fig. S1, B–D). *HvCK2α* was expressed all day and did not show apparent diurnal fluctuation under both long and short photoperiod conditions. *HvGI* and *Ppd-H1* were up-regulated during the day and down-regulated during the night under both long and short photoperiod conditions, consistent with previous studies (Dunford et al., 2005; Turner et al., 2005). Therefore, it was suggested that *HvPhyC* does not affect circadian clock-related genes but affects more downstream genes of both photoreceptors and the circadian clock.

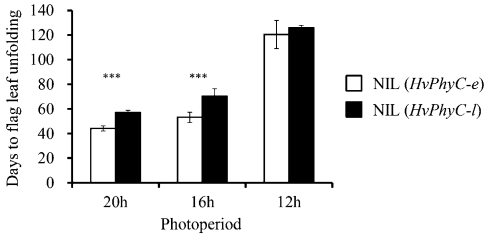

Figure 3. Photoperiodic response of the NILs carrying different *HvPhyC* alleles. Days from sowing to flag leaf unfolding (parallel with flowering time) of NIL (*HvPhyC-e*) and NIL (*HvPhyC-l*) were compared under three different (20-h, 16-h, and 8-h) photoperiod conditions. Triple asterisks indicate that the difference between the NILs is statistically significant ($P < 0.001$).

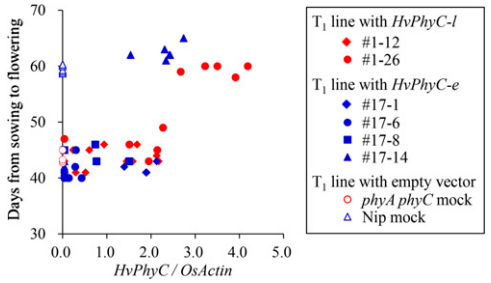

Figure 4. Functional assay of *HvPhyC* in rice by introducing different *HvPhyC* alleles (T1 generation). Mutant allele (*HvPhyC-e*; red-filled marks) and wild-type allele (*HvPhyC-l*; blue-filled marks) were introduced into rice *phyA phyC* double mutant lines with a var. Nipponbare genetic background via the *A. tumefaciens*-mediated transformation method. As the control, empty vector was introduced into *phyA phyC* double mutant (*phyA phyC* mock; open red circle) and var. Nipponbare (Nip mock; open blue triangle). All of the T1 lines were grown under natural (long) photoperiod conditions in a greenhouse. Expression level of *HvPhyC* was analyzed by semiquantitative RT-PCR using *OsActin* as the internal control.

HvCO1, which integrates signals from the circadian clock and photoreceptors, showed diurnal expression patterns under long and short photoperiod conditions (Fig. 5E; Supplemental Fig. S1E), consistent with previous studies (Turner et al., 2005; Campoli et al., 2012), although there were no clear differences between the two NILs. On the other hand, clear-cut differences were detected for *HvFT1* (Fig. 5F), which is expected to be the direct target of HvCO1 protein and acts as the flowering promoter "florigen" (*HvFT1* is also known as the vernalization requirement gene *Vrn-H3*). In the late-flowering NIL (*HvPhyC-l*), *HvFT1* showed a diurnal expression pattern that had a small peak early in the day and a large peak around dusk under long photoperiod conditions, which was consistent with Turner et al. (2005) and Campoli et al. (2012). In the early-flowering NIL (*HvPhyC-e*), *HvFT1* also showed diurnal expression, but small and large peaks appeared slightly later (by 4 h) than NIL (*HvPhyC-l*). Furthermore, NIL (*HvPhyC-e*) expression of *HvFT1* was considerably higher than in NIL (*HvPhyC-l*). This result was expected from several previous studies showing that the early-flowering line expressed *HvFT1* at a higher level (Turner et al., 2005; Campoli et al., 2012; Faure et al., 2012). Under short photoperiod conditions, *HvFT1* was not expressed in either NIL (Supplemental Fig. S1F), because the photoperiod was noninductive and NILs were still in the vegetative growth stage at the sampling time (15 d after sowing).

Another vernalization requirement gene *Vrn-H1* showed a diurnal expression pattern in both NILs under long and short photoperiod conditions (Fig. 5G; Supplemental Fig. S1G), which was consistent with Campoli et al. (2012). Under long photoperiod conditions, the expression level was slightly higher in NIL (*HvPhyC-e*) than in NIL (*HvPhyC-l*), while no such difference was observed under short photoperiod conditions. To confirm the difference under long photoperiod conditions, expression analysis with real-time reverse transcription (RT)-PCR was conducted (Supplemental Materials and Methods S1). As a result, a similar trend was observed as semiquantitative RT-PCR, but the differences were not significant at most of the time points (Supplemental Fig. S2). *Vrn-H2* locus deploys two family genes, *ZCCT-Ha* and *ZCCT-Hb*, both of which were expressed during the day and had two peaks early in the day and around dusk under long photoperiod conditions, while they were not expressed under short photoperiod conditions (Fig. 5, H and I; Supplemental Fig. S1, H and I). Their expression patterns were consistent with Trevaskis et al. (2006). There was no apparent difference in expression pattern between the NILs for both *ZCCT-H* genes.

We analyzed the expression of other photoperiodic response genes, *Ppd-H2* (*HvFT3*) and *HvCO9*, which control flowering time under short photoperiods (Kikuchi et al., 2009, 2012). There were no clear differences in either gene between the NILs (Fig. 5, J and K; Supplemental Fig. S1, J and K). *Ppd-H2* showed a diurnal expression pattern under short photoperiod conditions and much lower expression under long photoperiod conditions, which were consistent with a previous study (Kikuchi et al., 2009). *HvCO9* also showed a diurnal expression pattern under long and short photoperiod conditions and was up-regulated during the day. The expression level was higher under short than long photoperiod conditions. This was consistent with the observation by Kikuchi et al. (2012).

Epistatic Interaction between *HvPhyC* and *Vrn-H3*

Flowering times under a field condition were compared among HK2 NILs developed in this study and by Yasuda (1969). NILs carrying a winter allele *vrn-H3* flowered on April 27.4 on average, while those carrying a spring allele *Vrn-H3* flowered on April 16.3 on average (Table II). Within the group of NILs carrying *vrn-H3*, the NILs with *HvPhyC-e* flowered earlier than those with *HvPhyC-l* by 1 week. Contrarily, flowering times were not different between the NILs with *HvPhyC-e* and *HvPhyC-l*, all of which have *Vrn-H3*. The result strongly suggested that *Vrn-H3* is epistatic to *HvPhyC*.

Haplotype Analysis for *HvPhyC* and *Vrn-H1*

By using diagnostic markers (Supplemental Table S1), haplotype for *HvPhyC* and *Vrn-H1* was determined in Japanese varieties (Table III). Among 12 varieties, four had the same haplotype with HK2 that deploys *HvPhyC-e* and *vrn-H1* (identical with *HvVRN1* in Hemming et al. [2009]), while three had the same haplotype with NIL (*Vrn-H1*) that deploys *HvPhyC-l* and *Vrn-H1* (identical with *HvVRN1-10* in Hemming et al. [2009]). Other

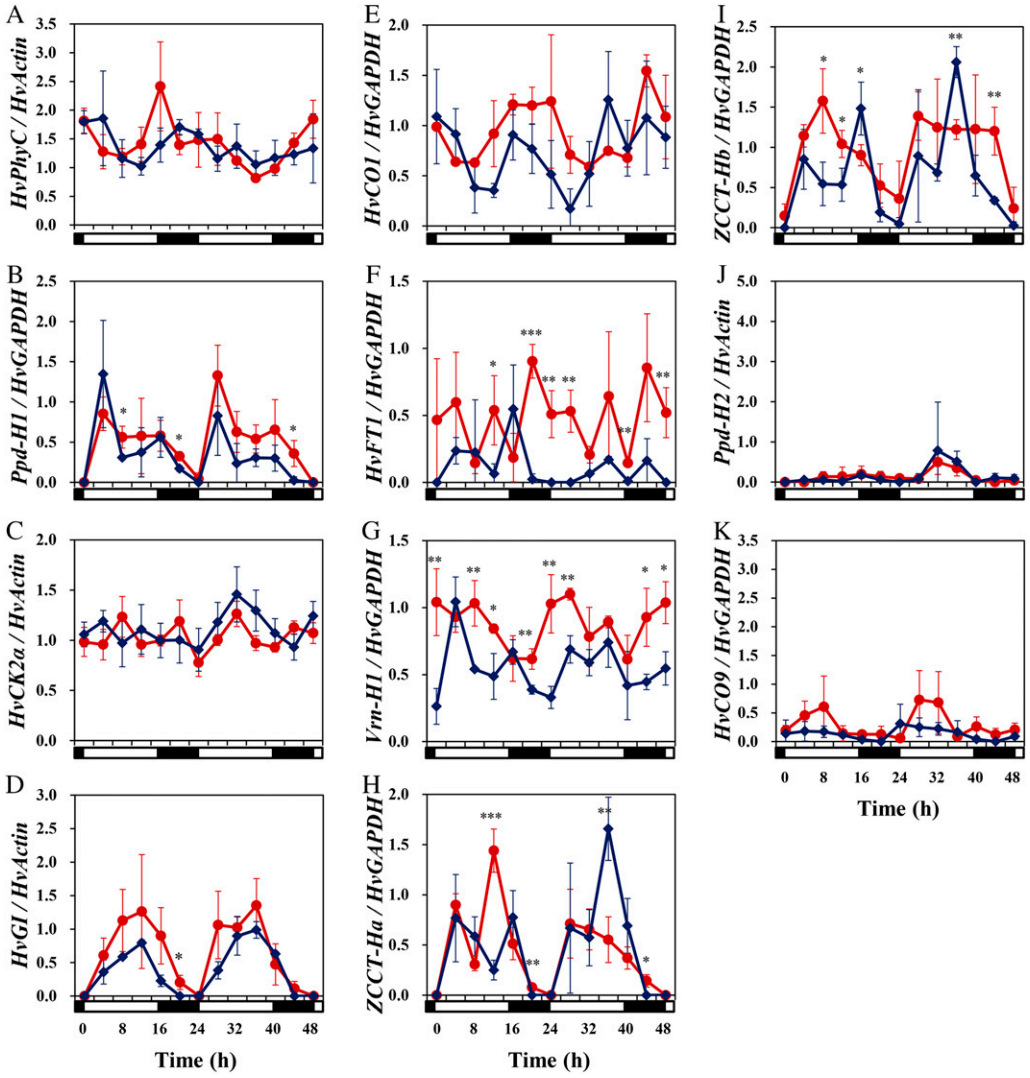

Figure 5. Expression pattern of flowering-time genes under a long (16-h) photoperiod. Red circles and blue diamonds indicate NIL (*HvPhyC-e*) and NIL (*HvPhyC-l*), respectively. Expression level is the average of band intensity obtained by semiquantitative RT-PCR from three biological replications. White and black horizontal bars below each graph indicate light and dark conditions, respectively. Vertical bar indicates SD. Asterisks above plots indicate that the expression level was significantly different between two lines when they were compared at the same time points. Single, double, and triple asterisks indicate significance at $P = 0.05$, 0.01, and 0.001, respectively.

five varieties carried a recombinant type that was comprised of *HvPhyC-l* and *vrn-H1*.

DISCUSSION

There have been several studies on phytochromes in model plant species such as rice and Arabidopsis using natural variations and mutants (Takano et al., 2005; Franklin and Quail, 2010). Such studies proved their importance in development and physiological responses, including the control of flowering time. By contrast, in barley, little is known about the effects of phytochromes, although it was suggested that one of the phytochrome genes, *HvPhyC*, might be involved in photoperiod sensitivity through the detection of a photoperiod sensitivity quantitative trait loci around the *HvPhyC* region (Szücs et al., 2006). In this study, we successfully identified the natural variation for *HvPhyC* that controlled flowering time under long photoperiods by affecting the expression of flowering-time genes downstream of *HvPhyC*.

In linkage analysis, the flowering-time gene *PS* (*t*) cosegregated only with *HvPhyC* among three candidate genes. Therefore, *HvPhyC* was considered to be the most likely candidate gene for the difference in flowering time between HK2 and NIL (*Vrn-H1*). According to Szücs et al. (2006), barley has a *HvPhyC* pseudogene in addition to the intact gene. They mapped the pseudogene at the same position as *Vrn-H1*, which is distinct from

Table II. *Flowering time of HK2 NILs carrying different alleles for HvPhyC, Vrn-H1, and Vrn-H3*

Line	Genotype[a]				Flowering Time ± SD[d]
	HvPhyC	*Vrn-H1*[b]	*Vrn-H3*	*HvCK2α*[c]	
HK2	*HvPhyC-e*	*vrn-H1*	*vrn-H3*	H	24.6 ± 1.1[p]
NIL (*HvPhyC-e*)	*HvPhyC-e*	*Vrn-H1*	*vrn-H3*	N	21.1 ± 1.3[q]
NIL (*HvPhyC-l*)	*HvPhyC-l*	*Vrn-H1*	*vrn-H3*	N	30.0 ± 0.9[r]
NIL (*Vrn-H1*)	*HvPhyC-l*	*Vrn-H1*	*vrn-H3*	N	33.8 ± 1.1[s]
NIL (*Vrn-H3*)	*HvPhyC-e*	*vrn-H1*	*Vrn-H3*	H	16.4 ± 1.8[t]
NIL (*Vrn-H1, Vrn-H3*)	*HvPhyC-l*	*Vrn-H1*	*Vrn-H3*	N	16.2 ± 1.3[t]

[a]All lines carry *Vrn-H2* (winter allele), *Ppd-H1*(early-flowering allele), and *Ppd-H2* (early-flowering allele) in common. [b]All the dominant alleles for *Vrn-H1* are identical with *HvVRN1-10* (Hemming et al., 2009). They were derived from the var. Indo Omugi except for the allele in NIL (*Vrn-H1*, *Vrn-H3*) that was derived from the Japanese var. Marumi 16. [c]H and N indicate the alleles from HK2 and NIL (*Vrn-H1*), respectively. [d]April 1 = 1. Values with different letters (p, q, r, s, or t) indicate significant difference ($P < 0.01$) by the Tukey test.

the intact *HvPhyC* by genetic mapping (Szücs et al., 2006). Furthermore, physical mapping located the pseudogene next to *Vrn-H1* (Yan et al., 2005; Szücs et al., 2006). Because of several recombinations between *PS* (*t*) and *Vrn-H1* in our linkage analysis, *PS* (*t*) was considered to be distinct from the pseudogene. Szücs et al. (2006) also showed that the pseudogene was separated into two segments (remnants) by a large (17-kb) insertion, including retroelements and miniature inverted-repeat transposable elements. Successful PCR amplification of the pseudogene in both HK2 and NIL (*Vrn-H1*) using the primers specific to the insertion and the genomic regions on opposite sides of the insertion across the pseudogene segments (Szücs et al., 2006) implied that both lines carry the pseudogene, which was nonfunctional (Supplemental Fig. S3). Therefore, it was concluded that the pseudogene is not the cause of the flowering-time difference between HK2 and NIL (*Vrn-H1*).

Another flowering-related gene *AGAMOUS-LIKE GENE1* (*AGLG1*), encoding a grass-specific *SEPALLATA*-like MADS-box protein, was expected to exist around *Vrn-H1* region because of the syntheny between barley and einkorn wheat (*Triticum monococcum*) genomes (Yan et al., 2005). Although little is known about *AGLG1* function in barley and wheat, a rice ortholog *PANICLE PHYTOMER2* (*PAP2*) has been characterized in detail (Gao et al., 2010; Kobayashi et al., 2010, 2012). PAP2 protein is involved in inflorescence meristem identity by forming protein complex with three *AP1/FUL*-like MADS-box proteins, OsMADS14, OsMADS15, and OsMADS18 (Kobayashi et al., 2012). Quadruple knockdown plants showed severely affected phenotype, including discordant transition from vegetative phase to reproductive phase that resulted in delayed flowering and impaired inflorescence development. In addition, *PAP2* alone functions as a positive regulator of spikelet meristem identity and a suppressor of the extra elongation of glumes (Gao et al., 2010; Kobayashi et al., 2010). Contrary to the quadruple knockdown plants, *pap2* single mutant showed milder phenotype, including increase in number of primary branch of rachis and elongation of sterile lemma and rudimentary glume, and did not affect flowering time (Gao et al., 2010; Kobayashi et al., 2010). From these results, *AGLG1* single mutation in barley was assumed to affect spike and spikelet morphology rather than flowering time. However, we did not find any differences in spike and spikelet morphology between HK2 and NIL (*Vrn-H1*). Therefore, *AGLG1* was ruled out from the candidate genes for *PS* (*t*).

By linkage analysis, the gene order was estimated to be *Vrn-H1* – *HvPhyC* – *HvCK2α*, which was supported by the barley EST map (CMap for Barley EST; http://map.lab.nig.ac.jp:8085/cmap; Sato et al., 2009) constructed using the double haploid (DH) population of a Japanese var. Haruna Nijo and the *Hordeum vulgare* ssp. *spontaneum* line H602. However, order *Vrn-H1* – *HvPhyC* – *HvCK2α* from proximal to distal on chromosome 5HL was inconsistent with previous reports. Szücs et al. (2006) mapped *HvPhyC* on the proximal side of *Vrn-H1* using the Dicktoo × Morex DH mapping population. *HvCK2α* was mapped to a more distal region on 5HL using the Steptoe × Morex DH population (Kato et al., 2008). Taken together, their results suggested that the order is *HvPhyC* – *Vrn-H1* – *HvCK2α* from proximal to distal on 5HL. This order

Table III. *Haplotypes for HvPhyC and Vrn-H1 in 12 Japanese varieties*

Variety	Haplotype		Growth Habit
	HvPhyC	*Vrn-H1*[a]	
Ishuku Shirazu	*HvPhyC-e*	*vrn-H1*	Spring
Ichibanboshi	*HvPhyC-e*	*vrn-H1*	Winter
Kawasaigoku	*HvPhyC-e*	*vrn-H1*	Spring
Haruna Nijo	*HvPhyC-e*	*vrn-H1*	Spring
Silky Snow	*HvPhyC-l*	*vrn-H1*	Winter
Kirarimochi	*HvPhyC-l*	*vrn-H1*	Spring
Sayakaze	*HvPhyC-l*	*vrn-H1*	Spring
Suzukaze	*HvPhyC-l*	*vrn-H1*	Spring
Shunrai	*HvPhyC-l*	*vrn-H1*	Spring
Hozoroi	*HvPhyC-l*	*Vrn-H1*	Spring
Marumi 16	*HvPhyC-l*	*Vrn-H1*	Spring
Kinai 5	*HvPhyC-l*	*Vrn-H1*	Spring

[a]*vrn-H1* and *Vrn-H1* are identical with *HvVRN1* and *HvVRN1-10* (Hemming et al., 2009), respectively.

is consistent with that in the corresponding regions of wheat (Kato et al., 2002; Yan et al., 2003), *B. distachyon* (Gbrowse; http://www.phytozome.net/cgi-bin/gbrowse/brachy/), rice (Rice Annotation Project Database; http://rapdb.dna.affrc.go.jp), and sorghum (*Sorghum bicolor* Genome Database; http://www.plantgdb.org/XGDB/phplib/resource.php?GDB=Sb), showing that this gene order is prevalent in grass species. Therefore, it was considered that the chromosomal rearrangement in this region might be shared in some barley varieties, including the Japanese var. HK2 and var. Haruna Nijo, the Taiwanese var. Indo Omugi, and *H. vulgare* ssp. *spontaneum* line H602.

A "supergene" is formed by a group of cosegregating genes whose allelic combinations (haplotypes) facilitate integrated control of adaptive phenotypes. "Supergenes" have been described in various species, e.g. *S* locus controlling heterostyly and self-incompatibility in *Primula* species (*Primula sinensis*; Mather, 1950) and *P* locus controlling wing color pattern in mimetic butterflies (Clarke et al., 1968; Brown and Benson, 1974; Nijhout, 2003). In a mimetic butterfly, *Heliconius numata*, chromosomal rearrangements (inversions) around the *P* locus prevented recombination within this supergene locus, and inversions that formed specific haplotypes were completely associated with corresponding wing color patterns (Joron et al., 2011). In this study, formation of a "supergene" was expected in chromosome 5HL, where four flowering-related genes (*Vrn-H1*, *HvPhyC*, *HvCK2α*, and *HvAGLG1*) are clustered and closely linked to each other. Especially for the inversion including the *Vrn-H1 – HvPhyC* region might played evolutionary and adaptive roles by establishing specific haplotypes (*vrn-H1/HvPhyC-e* [HK2 type] and *Vrn-H1/HvPhyC-l* [NIL {*Vrn-H1*} type]) that could control vernalization requirement and photoperiod sensitivity at the same time. Contrary to expectation, our preliminary analysis using Japanese varieties showed that there was a recombinant type (*vrn-H1/HvPhyC-l*) in addition to the above two types (Table III). To clarify the evolutionary and adaptive roles of *Vrn-H1* and *HvPhyC* as well as other flowering-related genes, worldwide landrace collection needs to be analyzed comprehensively.

To conduct functional assay of *HvPhyC*, we adopted the *Agrobacterium tumefaciens*-mediated transformation system of rice. The result showed that even the mutant allele (*HvPhyC-e*) is functional and may have a hyperfunction compared with the wild-type allele (*HvPhyC-l*). If this is the case, functional *PhyC* has a promoting effect in long-day plant barley under long photoperiod conditions, and conversely, it has a delaying effect in short-day plant rice under the same conditions. There is an another example in which orthologous genes affect flowering time conversely between long-day and short-day plants: in the long-day plant Arabidopsis, the functional *CO* promotes flowering under long photoperiod conditions, whereas the functional rice ortholog *Hd1* delays flowering under the same condition (Putterill et al., 1995; Yano et al., 2000). Rice is a reasonable model plant for functional assay of *HvPhyC* gene, because *phyC* null mutant lines are available in a var. Nipponbare genetic background (Takano et al., 2005), which facilitates transformation. On the other hand, transformation of barley is possible only in a 'Golden Promise' genetic background, and no *HvPhyC* null mutant genes are available so far. However, the best way to evaluate precise function of *HvPhyC* is complementation analysis introducing *HvPhyC-e* and *HvPhyC-l* under the control of native promoter into a *HvPhyC*-deficient mutant of barley (native genetic background), because it is unclear how the ectopic expression from viral promoter and how the differences of photoperiodic pathways between barley and rice affect *HvPhyC* gene function in rice (heterogeneous) genetic background.

Phytochrome protein deploys several domains: a GAF domain, a PHY (phytochrome-specific, GAF-related) domain, three PAS (*Drosophila period* protein, vertebrate aryl hydrocarbon receptor nuclear translocator protein, and *Drosophila single-minded* protein) domains, and two His kinase-related domains (Fig. 1B). The first half of this protein, including N-terminal PAS, GAF, and PHY domains, is considered to be crucial for light perception and signal transduction. And several missense mutations in this region caused a deficiency in signal transduction, chromophore incorporation and spectral integrity, and Pfr stabilization (Nagatani, 2010). In this study, an exon 1 SNP (T1139C) was found near the 3′ end of GAF domain containing a chromophore-binding site. This mutation resulted in nonsynonymous substitution at position 380 from nonpolar and hydrophobic Phe to polar and hydrophilic Ser. Because mutation at this position is unknown in plants including Arabidopsis, it remains unknown how the mutation affects the biochemical function of the protein. However, this amino acid residue is well conserved among many plant species (wheat, *B. distachyon*, rice, sorghum, and Arabidopsis), suggesting its importance for protein function (Fig. 1C). On the C-terminal side of the protein, both HK2 and NIL (*Vrn-H1*) had an 8-amino acid insertion, which was previously reported in var. Morex by Szücs et al. (2006; Fig. 1B). No such insertion was found in wheat, *B. distachyon*, rice, and sorghum PHYC proteins. However, this insertion is located close to the C-terminal end and outside the His kinase-like ATPase domain (Fig. 1B). In addition, the N-terminal side is rather important for light perception and signal transduction compared with the C-terminal side, although it is associated with dimerization (Nagatani, 2010). Therefore, the effect of the insertion on flowering time was considered to be small. Consistent with this idea, constitutive expression of both *HvPhyC* alleles in rice *phyA phyC* mutant could recover the wild-type phenotype, although the recovering levels were different (Fig. 4).

Expression analysis showed that SNP (T1139C) in *HvPhyC* did not affect the expression pattern of *HvPhyC* itself, circadian clock genes, and most of the downstream genes (Fig. 5, A–E and H–K; Supplemental Fig. S1, A–K). The only exception was *HvFT1* (*Vrn-H3*), which was up-regulated by the mutant allele *HvPhyC-e* under a long photoperiod (Fig. 5F). This was consistent with

physiological analysis in which *HvPhyC* affected flowering time only under long photoperiods. Therefore, *HvPhyC* was considered to bypass most of the genes that function in relatively upstream parts of the genetic pathways for flowering (e.g. circadian clock genes and *HvCO1*) and affect the gene (*HvFT1*) that functions in the most downstream part of the genetic pathways. Consistent with this, complete loss of three phytochromes in rice did not affect the expression of circadian clock genes and *CO*, while it affected the expression of *FT* (Izawa et al., 2002), although the sole effect of *PhyC* remains unknown. Furthermore, field experiment in this study also supported our idea (Table II). *HvPhyC-e*, compared with *HvPhyC-l*, had a promoting effect when coexisting with *vrn-H3*, while it did not have such effect when coexisting with *Vrn-H3*, which was derived from the Finnish var. Tammi. The Tammi allele for *Vrn-H3* was shown to include four copies of *HvFT1*, which is associated with earlier transcriptional up-regulation of themselves (Nitcher et al., 2013). Therefore, it was considered that the spring allele *Vrn-H3* (high expression of *HvFT1*) is epistatic to that of *HvPhyC-e* and *HvPhyC* functions in the same pathway as *Vrn-H3*.

Based on the feedback loop models (Trevaskis et al., 2007; Distelfeld et al., 2009; Shimada et al., 2009), up-regulation of *HvFT1* was expected to give rise to concordant up-regulation of *Vrn-H1* and down-regulation of *Vrn-H2*. Our result showed that *Vrn-H1* expression tended to be slightly higher in NIL (*HvPhyC-e*) than NIL (*HvPhyC-l*) (Fig. 5G; Supplemental Fig. S2). However, their differences were confirmed to be nonsignificant (Supplemental Fig. S2). This might be attributable to that both of the NILs carry the spring allele *Vrn-H1*. Because the spring allele *Vrn-H1* expresses at a high level even in young seedlings (Trevaskis et al., 2003; von Zitzewitz et al., 2005), further up-regulation of *Vrn-H1* by higher expression of *HvFT1* will be marginal even if it occurs. Consistent with this idea, the expression levels of *Vrn-H2* were not different between both NILs (Fig. 5, H and I). This might also be attributable to that expression of *Vrn-H2* was repressed by the spring allele *Vrn-H1* (Hemming et al., 2008) to a considerably low level where the differences were not detectable. Distelfeld and Dubcovsky (2010) suggested that lack of the entire *TaPhyC* gene down-regulated the *ZCCT* genes in the *maintained vegetative phase* mutant of diploid einkorn wheat. To disclose the effect of *HvPhyC* on *Vrn-H2* (perhaps through affecting *Vrn-H1*), a F2 population segregating for *HvPhyC* and *Vrn-H2* and carrying the winter allele *vrn-H1* needs to be developed.

Molecular genetic studies of Arabidopsis have disclosed the essential roles of phytochromes in transcriptional and posttranscriptional control of *CO*. *PHYB* is associated with posttranscriptional regulation of *CO* by degrading CO protein early in the day under a long photoperiod (Valverde et al., 2004). PHYB protein is known to form not only a homodimer, but also heterodimers with PHYC and PHYD proteins, while PHYC and PHYD proteins do not form a homodimer (Clack et al., 2009). If PHYB/PHYC heterodimer participates in CO protein stability, *PhyC* may affect flowering time through a *CO*-mediated photoperiodic pathway. Similarly, PHYC protein can participate in transcriptional regulation of *CO* by forming a heterodimer with PHYB. Clack et al. (2009) showed that PHYC protein, probably by forming a heterodimer with PHYB protein, can interact with Phytochrome Interacting Factor3 (PIF3), which is involved in signal transduction from phytochromes. Oda et al. (2004) showed that suppression of PIF3 resulted in up-regulation of *CO* and *FT* but did not affect the expression of circadian clock genes, including *LHY* (*LATE ELONGATED HYPOCOTYL*) and *CCA1* (*CIRCADIAN CLOCK ASSOCIATED1*), irrespective of possessing a possible binding sequence in their promoter. Our expression analysis suggests that the mutation (T1139C) in *HvPhyC* affects the posttranscriptional control of *CO* rather than the transcriptional control of *CO*, because *HvFT1* was up-regulated but *HvCO1* was not in NIL (*HvPhyC-e*). Taken together, our results indicate that *HvPhyC* is a key factor to control long-day flowering directly.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

NIL (*Vrn-H1*) was developed by backcrossing more than 10 times using the Japanese barley (*Hordeum vulgare* ssp. *vulgare*) var. HK2 as the recurrent parent and the Taiwanese var. Indo Omugi as the nonrecurrent parent (*Vrn-H1* donor; this NIL was originally developed by Shozo Yasuda who conducted backcrossings six times [Yasuda, 1969]. After publication, he conducted further backcrosses more than four times.).

The F2 population (918 individuals) and F3 lines (87 lines, 10–15 plants each) of HK2 × NIL (*Vrn-H1*) were subjected to genetic and linkage analyses for flowering-time gene. They were grown under natural conditions in the experimental field (the Faculty of Agriculture, Okayama University; 34° 41′N, 133° 55′E, 4 m above sea level). The sowing date of the F2 population was November 25, 2005, and those of F3 lines were December 20, 2006, December 21, 2007, and November 21, 2008. Flowering time was scored for each plant as the date when the first ear appeared from the sheath.

For evaluation of the gene effect on photoperiod sensitivity and the expression pattern of flowering-time genes, the NILs with different alleles for *HvPhyC* (Table I) were selected from F4 families using allele-specific DNA markers, as summarized in Supplemental Table S1. For the evaluation of photoperiod sensitivity, seeds of NIL (*HvPhyC-e*) and NIL (*HvPhyC-l*) were soaked in water at 4°C for 24 h and subsequently kept at 20°C for 24 h for germination. Germinated seeds were sown on the soil (1:1 mixture of field soil and bark compost). Each three plants per line (six plants for each genotype) were grown under short (12-h-light/12-h-dark), long (16-h-light/8-h-dark), and extremely long (20-h-light/4-h-dark) photoperiod conditions in growth chambers (LH-350SP; Nippon Medical and Chemical Instruments), where temperature was kept at 20°C. The light source was fluorescent lamps, and photon flux density was approximately 160 μmol m^{-2} s^{-1}. For expression analysis, the same lines were grown in the same way under short (8-h-light/16-h-dark) and long (16-h-light/8-h-dark) photoperiods. From 15 d after sowing, second and third leaves (three biological replicates for each genotype at every time point) were collected at 4-h intervals for 2 d.

For validation of the interaction between *HvPhyC* and *Vrn-H3*, flowering time of additional two HK2 NILs carrying *Vrn-H3* (Yasuda, 1969) as well as the NILs described above were evaluated under a natural condition. Five plants per each line were grown in the same field as described above. Sowing date was December 17, 2010.

Young seedlings of 12 Japanese varieties were grown for the *HvPhyC*/*Vrn-H1* haplotype analysis (Table III). Leaves of these varieties were collected for DNA extraction.

Functional Assay of *HvPhyC* Using Rice Transformation System

We adopted the rice (*Oryza sativa*) *phyA phyC* double mutant line as the recipient, instead of the *phyC* single mutant line, with a genetic background of the Japanese var. Nipponbare (Takano et al., 2005). This is because the phenotypic effect of the *PhyC* gene is prominent when *PhyA* is nonfunctional: the *phyA phyC* double mutant line flowers much earlier than the original var. Nipponbare and even earlier than the *phyC* single mutant line under a natural (long) photoperiod, while the *phyA* single mutant line flowers at the same time as the var. Nipponbare under the same conditions (Takano et al., 2005).

HvPhyC-e and *HvPhyC-l* cDNAs driven by the *Cauliflower mosaic virus* 35S promoter were introduced into the *phyA phyC* double mutant line via *Agrobacterium tumefaciens*-mediated transformation, as described by Kikuchi et al. (2009). Four T1 lines expressing *HvPhyC-e* and two T1 lines expressing *HvPhyC-l* developed from independent T0 plants were subjected to the analysis. T1 lines carrying the empty vector (mock) with the *phyA phyC* and Nipponbare variety background (*phyA phyC* mock and Nip mock, respectively) were used as the control. Their seeds were sown on July 26, 2012 in plastic pots, and plants were grown until flowering under a natural photoperiod in a greenhouse, as described by Kikuchi et al. (2009). Flag leaves were collected at 9 AM at the end of September for *HvPhyC* expression analysis.

DNA Extraction and Genotyping

Genomic DNA was extracted from each plant of the F2 population, F3 lines, their parents, NILs, and varieties following the cetyl trimethyl ammonium bromide method (Murray and Thompson, 1980).

For linkage analysis, PCR amplification was conducted using allele-specific DNA markers for *Vrn-H1*, *HvPhyC*, and *HvCK2α* (Supplemental Table S1). PCR products or digested PCR products were separated in agarose gels by electrophoresis. PCR products were visualized with ethidium bromide. A genetic map was constructed using MAPMAKER/EXP3.0 (Lander et al., 1987).

In addition to *Vrn-H1*, *HvPhyC*, and *HvCK2α*, other flowering-time genes, *Ppd-H1*, *Ppd-H2*, *Vrn-H2*, and *Vrn-H3*, were also genotyped for HK2, its NILs, and varieties using diagnostic markers (Supplemental Table S1).

Sequence Analysis

The *HvPhyC* gene region was amplified by long PCR using appropriate primers (Supplemental Table S2) and Phusion High-Fidelity DNA polymerase (Thermo Scientific) and cloned using the TOPO TA Cloning Kit (Invitrogen) following the manufacturers' instructions. Three clones per single PCR product were sequenced using a PRISM 3730 DNA Analyzer (Applied Biosystems).

Gene Expression Analysis

Young leaves (100 mg) of barley NILs and transgenic rice were frozen in liquid nitrogen and ground using a Multibeads shocker (Yasui apparatus). Total RNA was extracted from the ground leaf using Sepasol RNAI Super (Nacalai Tesque) and first-strand complementary DNA was synthesized using ReverTra Ace (TOYOBO) following manufacturers' instructions. Semiquantitative RT-PCR was conducted using specific primers for flowering-time genes and internal controls (Supplemental Table S3). PCR products were electrophoresed in agarose gels and stained by ethidium bromide. Band intensity was analyzed by Scion Image.

Sequence data from this article can be found in the GenBank database under the following accession numbers: *HvPhyC-e* (AB827939) and *HvPhyC-l* (AB827940).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression pattern of flowering time genes under a short (8-h) photoperiod.

Supplemental Figure S2. Expression pattern of *Vrn-H1* analyzed by real-time RT-PCR under a long (16-h) photoperiod.

Supplemental Figure S3. PCR amplification of *HvPhyC* pseudogene.

Supplemental Table S1. Primer sets for linkage analysis and diagnostic markers for flowering time genes.

Supplemental Table S2. Primer sets for sequence analysis of *HvPhyC*.

Supplemental Table S3. Primer sets for functional assay of *HvPhyC*, expression analysis of flowering-related genes, and amplification of *HvPhyC* pseudogene.

Supplemental Materials and Methods S1. Materials and methods for *Vrn-H1* expression analysis by real-time RT-PCR.

ACKNOWLEDGMENTS

We thank Shozo Yasuda (Institute of Plant Science and Resources, Okayama University) and Makoto Takano (National Institute of Agrobiological Sciences) for providing HK2 NILs and the rice *phyA phyC* double mutant line, respectively, and Koichiro Ushijima (Okayama University) for technical advice on the real-time RT-PCR analysis.

Received June 4, 2013; accepted September 2, 2013; published September 6, 2013.

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