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RESEARCH PAPER

The differential expression of *HvCO9*, a member of the *CONSTANS*-like gene family, contributes to the control of flowering under short-day conditions in barley

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Abstract

***HvCO9* was characterized to elucidate the barley flowering control mechanisms and to investigate the functional diversification of the barley *CONSTANS*-like (*CO*-like) genes in flowering. *HvCO9* was located on the same chromosome, 1HL, as *Ppd-H2* (*HvFT3*), which is a positive regulator of short-day (SD) flowering. A phylogenetic analysis showed that *HvCO9* was located on the same branch of the *CO*-like gene tree as rice *Ghd7* and the barley and wheat *VRN2* genes, which are all negative regulators of flowering. High level *HvCO9* expressions were observed under SD conditions, whereas its expression levels were quite low under long-day (LD) conditions. *HvCO9* expression correlated with *HvFT1* and *HvFT2* expression under SD conditions, although no clear effect of *HvCO9* on *HvFT3* expression, or vice versa, under SD conditions was observed. The over-expression of *HvCO9* in rice plants produced a remarkable delay in flowering. In transgenic rice, the expression levels of the flowering-related *Ehd1* gene, which is a target gene of *Ghd7*, and its downstream genes were suppressed, causing a delay in flowering. These results suggest that *HvCO9* may act as a negative regulator of flowering under non-inductive SD conditions in barley; this activity is similar to that of rice *Ghd7* under non-inductive LD conditions, but the functional targets of these genes may be different. Our results indicate that barley has developed its own pathways to control flowering by using homologous genes with modifications for the timing of expression. Further, it is hypothesized that each pathway may target different genes after gene duplication or species diversification.**

Key words: Barley, *CO*-like genes, flowering, gene expression, *HvCO9*, negative regulator, photoperiod.

Introduction

Flowering is a crucial developmental phase in the life cycle of seed-propagated plants. A regulatory mechanism that responds to environmental changes (such as day length and temperature) provides a sophisticated control for flowering and ensures that flowering occurs under the most appropriate conditions to maximize seed production and reproductive success.

The molecular mechanisms that control the regulation of flowering have been extensively studied using model plants, such as *Arabidopsis thaliana* and rice (*Oryza sativa* L.). *Arabidopsis* is a long-day (LD) plant: LD plants flower when the days become longer than a critical day length (Thomas and Vince-Prue, 1997). In *Arabidopsis*, the LD-photoperiodic signals that are mediated by *CONSTANS* (*CO*), which encodes a zinc-finger transcriptional activator, induce the transcription of *FLOWERING LOCUS T* (*FT*) which encodes a mobile flowering signal, called florigen, to promote flowering (Suárez-López *et al.*, 2001; Imaizumi and Kay, 2006). The *CO-FT* pathway is conserved in rice,

a short-day (SD) plant that flowers when the days become shorter than a crucial length (Thomas and Vince-Prue, 1997). In rice, *Hd1*, an orthologue of *CO*, induces the transcription of *Hd3a*, which is an orthologue of *FT* (Yano *et al.*, 2000; Hayama *et al.*, 2003; Hayama and Coupland, 2004), to promote flowering under SD conditions. It is interesting to note that photoperiodic pathways containing homologous genes are involved in the regulation of flowering in both *Arabidopsis* and rice; these pathways are conserved across the differentiation of dicots/monocots, even though these two species show different responses to environmental signals, such as LD and SD flowering induction, respectively.

Although the major components of the regulatory flowering pathways are conserved among distantly related plants, each plant species has evolved its own unique mechanisms to induce flowering under optimal conditions. Recently, the functional differentiation of the barley PEBP genes, including *FT*- and *TFL1* (*TERMINAL FLOWER 1*)-like genes, was reported in flowering regulation (Kikuchi *et al.*, 2009). Some of these genes perform the same function in flowering as their orthologues in *Arabidopsis* and rice; for others, however, no evidence was found to support their involvement in flowering. *HvFT1* is an integrator of the flowering pathway under both LD and SD conditions, whereas the role of *HvFT2* is specifically limited to SD conditions. *HvFT3* functions indirectly to promote the expression of *HvFT1*, which results in flowering under non-inductive (SD) conditions. *HvFT3* is also a good candidate gene for *Ppd-H2*, which is a major barley photoperiod-sensitive gene that promotes flowering under SD conditions. These findings suggest that barley has an adaptive mechanism that adjusts flowering according to photoperiodic changes using a combination of different *FT*-like genes.

CO is a zinc-finger protein that is necessary for inducing *FT* expression and is a member of a *CO*-like gene family in the genomes of higher plants (the family has 17 members in *Arabidopsis*, 16 members in rice, and nine members in barley; Griffiths *et al.*, 2003). This redundancy of the *CO*-like genes indicates a possible functional diversification in the regulation of flowering, which is similar to that of the *FT*-like genes. In fact, although rice *Hd1*, an orthologue of *CO*, promotes flowering under SD conditions, another rice *CO*-like gene, *Ghd7* (named *OsI*), acts as a floral repressor under non-inductive (LD) conditions and suppresses the transcription of *Ehd1*, a floral activator of multiple flowering signals (Doi *et al.*, 2004; Komiya *et al.*, 2008; Xue *et al.*, 2008). In addition to the nine *CO*-like genes in barley (Griffiths *et al.*, 2003), *VRN-H2* (Yan *et al.*, 2004; Trevaskis *et al.*, 2006) is also included in the *CO*-like gene family (Greenup *et al.*, 2009). *VRN-H2* is a floral repressor in the vernalization response in barley, and this locus consists of three homologous *CO*-like genes, *HvZCCT-Ha*, *HvZCCT-Hb*, and *HvZCCT-Hc* (Dubcovsky *et al.*, 2005; Karsai *et al.*, 2005).

In this study, the expression pattern of one of the *CO*-like genes in barley, *HvCO9*, was analysed under various photoperiodic conditions, and transgenic rice plants that over-expressed this gene were characterized to reveal its functional roles in flowering. The expression pattern of *HvCO9* was compared with that of *HvCO1*, a barley orthologue of *Hd1* that promotes flowering (Turner *et al.*, 2005), under different genetic backgrounds with regard to the major photoperiodic response genes, *Ppd-H1* and *Ppd-H2* (Laurie *et al.*, 1995) to reveal the functional divergence of these two *CO*-like genes. Furthermore, the functional and evolutionary relationships between *HvCO9*, rice *Ghd7*, and barley *VRN-H2*, which form a distinct clade within the *CO*-like gene family of cereal plants are discussed (Griffiths *et al.*, 2003; Yan *et al.*, 2004; Dubcovsky *et al.*, 2005). The expression pattern of *HvCO9* was compared with those of rice *Ghd7* and barley *VRN-H2* and the diversification of their possible functional target genes is discussed.

Materials and methods

Plant materials and growth conditions

Two cultivars of barley (*Hordeum vulgare*), cv. Steptoe and cv. Morex, were used in this study. The doubled haploid (DH) lines that were developed from the F_1 cross between Steptoe and Morex have also been used for gene mapping and expression studies (North American Barley Genome Mapping Project; Kleinhofs *et al.*, 1993). The DH lines that were used for the expression analyses of *HvCO9* and the *FT*-like genes are summarized in Table 1. The plants were grown in a growth chamber at 20±2 °C (175 μmol m^{-2} s^{-1}) under LD (16/8 h light/dark) or SD (12/12 h light/dark) conditions. For the expression analysis, the leaves were harvested in their order of appearance in the middle of the light period. For the diurnal expression analysis, the leaves of plants that were at the two- and three-leaf stages were harvested every 4 h for 2 d under LD and SD conditions.

Isolation of HvCO9

Partial sequences of *HvCO9* had been reported previously under the accession numbers AY082965 (Griffiths *et al.*, 2003) and FJ767842 and FJ 767852 (Cockram *et al.*, 2010). To isolate the entire coding region of *HvCO9*, total RNA was extracted from the leaves of cv. Morex using the RNeasy Plant Mini Kit (Qiagen,

Table 1. The DH lines that were used to identify the relationship between *HvCO9* and the *FT*-like genes under SD conditions

DH line	Genotypes[a]			
	VRN-H1	Ppd-H1	Ppd-H2 (HvFT3)	HvCO9
S/M-130	S	M	M	M
S/M-144	M	S	M	M
S/M-32	M	M	S	M
S/M-136	S	M	M	S
S/M-72	M	S	M	S
S/M-148	M	M	S	S
S/M-5	S	S	S	S
Steptoe	S (*VRN1-4*[b])	S (*Ppd-H1*)	S (*ppd-H2*)	S
Morex	M (*VRN1-1*[b])	M (*ppd-H1*)	M (*Ppd-H2*)	M

[a] S indicates the Steptoe-type genotype and M indicates the Morex-type genotype.
[b] Based on the classification by Hemming *et al.* (2009); and both genotypes are of the early-heading type without vernalization.

Germany). To isolate the downstream region of the coding region, the 3′ RACE System for the Rapid Amplification of cDNA Ends (Invitrogen, CA, USA) was used. The primary PCR was performed using a gene-specific primer, HvCO9-F1, and a universal amplification primer (UAP) and was followed by a secondary PCR using the HvCO9-F2 and UAP primer pair.

The first-strand cDNA was synthesized using the TaKaRa RNA PCR Kit (AMV) version 3.0 (Takara Bio, Japan). To isolate the region that was further upstream of part of the coding region, an *in silico* search was performed of a sequence database that contained full-length barley cDNA libraries that were constructed from the mixed cDNAs of various tissues of the Japanese two-row cultivar, Haruna-Nijo (Matsumoto *et al.*, 2011). One entry, NIASHv1066B04, was identified and, using this information, forward primers were designed to isolate the entire coding region of *HvCO9*. Lastly, the genomic or cDNA sequences that covered the entire coding region of *HvCO9* were amplified using Morex genomic DNA or cDNA as the templates and the HvCO9-F3 and HvCO9-R1 and the HvCO9-F4 and HvCO9-R2 primer pairs for the nested PCR technique. To clone the promoter region of *HvCO9*, the Morex genome sequence database in the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Germany (http://webblast.ipk-gatersleben.de/barley/indext.php; Mayer *et al.*, 2011) was screened using our *HvCO9* sequence as a query. The promoter region was amplified by PCR using a primer pair that consisted of HvCO9P-F and HvCO9-R. The descriptions of the primers that were used in this study are summarized in Table 2.

The sequence data for *HvCO9* that were obtained in this study were deposited in the DDBJ database under accession numbers AB592331 (cv. Steptoe) and AB592332 (cv. Morex).

Table 2. Sequences of the primers that were used in this study for the isolation, genetic mapping, plasmid construction, and quantitative RT-PCR of *HvCO9* and the expression analysis of the transgenic rice plants

Primer name	Sequence (5′–3′)	Reference
For 3′ RACE		
HvCO9-F1	AAGCTGATGCGGTACAAAGAGA	This study
HvCO9-F2	GTACAAAGAGAAGCGGAAGAGG	This study
For isolation of full-length cDNA and genome		
HvCO9-F3	AAATCGGCCATCACGTGGGGC	This study
HvCO9-R1	GGCAGCCTCCTACGGCAGCAT	This study
HvCO9-F4	TCACGTGGGGCAAGCTGATG	This study
HvCO9-R2	GCACTACGTAGCGTGCGCGTGT	This study
HvCO9P-F	ATGGCAATCCCTACTCCTTACAT	Contig1008434[a]
HvCO9P-R	AGGCAGGAGCAGTCCTCAGA	Contig1008434[a]
For mapping of *HvCO9*		
HvCO9-F5	AGCTGATGCGGTACAAAGAGAAGC	This study
HvCO9-R3	ACCCGACCAAGAAATGATCC	This study
For plasmid construction		
HvCO9-F4	TCACGTGGGGCAAGCTGATG	This study
HvCO9-R3	ACCCGACCAAGAAATGATCC	This study
For quantitative RT-PCR		
HvCO9-F1	AAGCTGATGCGGTACAAAGAGA	This study
HvCO9-R4	GAACCACCCGAGGTCGAG	This study
HvCO1-F	GGGGCAGAGCAGGCTGCCTC	AF490468
HvCO1-R	TGGCTTCTCTCTCCTTGGAGC	AF490468
HvFT1-F	ATCTCCACTGGTTGGTGACAGA	DQ898520
HvFT1-R	TTGTAGAGCTCGGCAAAGTCC	DQ898520
HvFT2-F	CCTTCTACACCCTGGTGATGGT	DQ297407
HvFT2-R	CCCTCTGGCAGTTGAAGTAGAC	DQ297407
HvFT3-F	GGTTGTGGCTCATGTTATGC	DQ411319
HvFT3-R	CTACTCCCCTTGAGAACTTTC	DQ411319
Actin-F1	GCCGTGCTTTCCCTCTATG	AY145451
Actin -R1	GCTTCTCCTTGATGTCCCTTA	AY145451
For expression analysis of transgenic rice plants		
HvCO9-F1	AAGCTGATGCGGTACAAAGAGA	This study
HvCO9-R4	GAACCACCCGAGGTCGAG	This study
Ehd1-F	TCTGAAGTGCAGCTACAAGGTTAC	AB092507
Ehd1-R	TTTCCAACCATGTTATTGTTCTTG	AB092507
Hd1-F	TGAGTACTTTGATCTTGTCGGGTA	AB041838
Hd1-R	TATCACCGTGCTGTCTGGTACTAT	AB041838
Hd3a-F	AGCTAGCAGCTGCAGCTAGTAAGC	AB052944
Hd3a -R	TGCAGCAGATCGATCGGGATCATC	AB052944
RAP1B-F	CCAGTAATCACAAGTTGCAACCT	AB041020
RAP1B -R	TGCCTCTGAATAACAGATGTTTCAA	AB041020
Actin-F2	GCCGTGCTCTCCCTGTATG	AY145451
Actin -R2	GCTTCTCCTTGATGTCCCTTA	AY145451

[a] Contig name from the Morex genome sequence database in the IPK (Meyer *et al.*, 2011).

Genetic mapping of HvCO9

HvCO9 was mapped in the DH population from the F_1 cross between Steptoe and Morex (North American Barley Genome Mapping Project; Kleinhofs *et al.*, 1993) using the cleaved-amplified polymorphic sequence method. The primers are shown in Table 2. The *Msl*I digestion produced fragment sizes of 413 bp for Steptoe, and 304 bp and 109 bp for Morex.

Phylogenetic analysis of the CO*-like gene group*

A phylogenetic analysis of the amino acid sequence alignment of the CCT (CONSTANS, CO-like, and TOC1) domains of the *CO*-like genes from barley, wheat, rice (*Oryza sativa*), and *Arabidopsis* (Griffiths *et al.*, 2003; this study) was conducted using ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html; Thompson *et al.*, 1997) and the Neighbor–Joining method (Saitou and Nei, 1987). A bootstrap analysis for 1000 replicates was performed to provide confidence estimates for the tree topologies using the Neighbor–Joining option in ClustalX. The results were compiled graphically using NJplot (http://pbil.univ-lyon1.fr/software/njplot.html).

Rice transformation and growth conditions for transgenic rice plants

The genomic sequence of *HvCO9* was amplified using Morex genomic DNA as a template and gene-specific primers (Table 2), and the amplified fragment was then cloned into the pSTARH302-GateA expression vector under the control of the cauliflower mosaic virus (CaMV) 35S promoter (H Ichikawa, H Nakamura, M Hakata, Y Nishizawa, M Kajikawa, unpublished data). The constructed plasmids were used in the *Agrobacterium*-mediated transformation of the rice plants (Kikuchi *et al.*, 2009).

Fifteen plants (T_0 generation) that were transformed with the expression vector containing *HvCO9* or a control vector (mock) were transplanted into soil under two types of SD conditions. The first SD condition (SD1) was in a growth chamber at 28 °C during the day and 25 °C at night (9/15 h light/dark; 270 μmol m^{-2} s^{-1}). The second SD condition (SD2) was in a greenhouse at 28 °C during the day and 24 °C at night under natural light from the middle of September until the end of December (approximately 10.5/13.5 h light/dark). The *HvCO9*-overexpressing rice plants (line 9) or mock plants of the T_1 generations (plants from the seeds of regenerated plants) were sown in a growth chamber under SD conditions at 28 °C during the day and at 25 °C at night (9/15 h light/dark; 270 μmol m^{-2} s^{-1}) or under LD conditions in a greenhouse at 28 °C during the day and 24 °C at night under natural light from the middle of March until the end of July (approximately 14/10 h light/dark). The number of days that elapsed between the date of transplanting (T_0 generations) or sowing (T_1 generations) and the appearance of the first panicle was recorded.

Expression analysis

Total RNA was extracted from the leaves using the Get Pure RNA Kit (Dojindo, Japan). The first-strand cDNA was synthesized from 1 μg of each RNA sample in a 20 μl reaction solution using the TaKaRa RNA PCR Kit (AMV) version 3.0 (Takara Bio). For the expression analysis of the flowering-related genes, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using gene specific primer pairs for each gene (Table 2). Real-time PCR was carried out using an Mx3000P (Stratagene Products Division, Agilent Technologies, CA, USA) with Brilliant II SYBR Green QPCR Master Mix (Stratagene) according to the manufacturer's recommendations. A dilution series of the pCR2.1-TOPO vectors (Invitrogen) containing partial fragments of *HvCO9* or *HvActin* and the pTA2 vector (TOYOBO, Japan) that contained a partial fragment of *HvCO1* were used to generate the standard curves. The *HvCO9* and *HvCO1* transcripts were amplified with specific primer pairs (Table 2). The values for *HvCO9* and *HvCO1* were normalized to *HvActin* as an internal standard. The real-time PCR results that are presented reflect the results of two independent experiments.

Database analysis for micro-colinearity in the HvCO9 and VRN-H2 regions among members of the grass family

The following sequence resources were used to examine the micro-colinearity among the members of the grass family: (i) the sequence data for the rice genome (RAP-DB, http://rapdb.dna.affrc.go.jp/), the *Brachypodium* and sorghum genomes (Plant DB provided at the MIPS plant genomics group in the German Research Center for Environmental Health, http://mips.helmholtz-muenchen.de/plant/) and the maize genome (MaizeGDB, http://www.maizegdb.org/) and (ii) mapped DNA marker data (GrainGenes; http://wheat.pw.usda.gov/GG2/index.shtml) (Close *et al.*, 2009; Thiel *et al.*, 2009).

Results

Isolation and characterization of the HvCO9 *gene*

The partial sequences of *HvCO9* have previously been reported under the accession numbers, AY082965 (Griffiths *et al.*, 2003) and FJ767842 and FJ 767852 (Cockram *et al.*, 2010). Based on these data, the genomic sequences that covered the entire coding region of *HvCO9* from two barley cultivars, Steptoe and Morex, were determined. The former is a late-heading cultivar under field conditions and has the genotype, *Ppd-H1*/*ppd-H2*, whereas the latter is an early-heading cultivar that contains the genotype, *ppd-H1*/*Ppd-H2*. The genomic sequences of *HvCO9* revealed that this gene contains two exons of 489 bp and 399 bp and a single intron (Fig. 1). *HvCO9* encodes a protein of 295 amino acids that, similar to other *CO*-like proteins, contains a CCT domain near its carboxy terminus. The gene structures and the deduced amino acid sequences of *HvCO9* were well conserved between the two cultivars, except for two amino acid changes, which are noted in Fig. 1. These results indicate that both Steptoe and Morex contain an intact and functional copy of *HvCO9*. the Sequences located up to 1.6 kb upstream of the initiation codon of *HvCO9* were also obtained from both cultivars; a sequence comparison of the possible promoter regions between Steptoe and

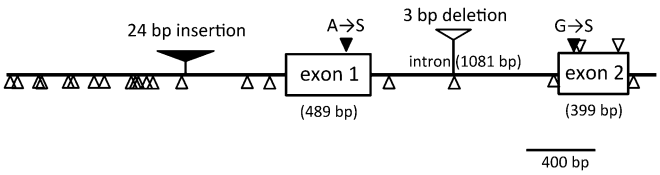

Fig. 1. Gene structure of *HvCO9* in the barley cultivar Steptoe. The boxes indicate exons. The nucleotide changes that were observed in the Morex cultivar are indicated by the filled and open triangles with and without the amino acid changes, respectively. The filled and open triangles that contain a vertical bar indicate the position of insertions or deletions, respectively, that were found in the Morex *HvCO9* gene.

Morex revealed the presence of one insertion/deletion of 24 bp in size and several SNPs (Fig. 1).

Relationship between HvCO9 and related genes

Using a doubled haploid (DH) population that was derived from the cross between Steptoe and Morex, *HvCO9* was mapped to a position that was 9.9 cM proximal to ABC160 and 2.2 cM distal from Glb1 on chromosome 1HL (see Supplementary Fig. S1at *JXB* online); this is the same chromosome arm on which *Ppd-H2* is located, which is nearly consistent with previous mapping studies (Griffiths *et al.*, 2003). Barley chromosome 1H displays partial synteny to segments of rice chromosomes 5 and 10 (Close *et al.*, 2009; Thiel *et al.*, 2009). A phylogenetic analysis of the amino acid sequence alignment of the CCT domains of the *CO*-like genes from barley, wheat, rice, and *Arabidopsis* (Griffiths *et al.*, 2003; Karsai *et al.*, 2005; Szücs *et al.*, 2007; this study) revealed that *HvCO9* is the most closely related to *OsH* (Os10g0560400), which is one of the rice *CO*-like genes, and that these two genes comprised a distinct clade (see Supplementary Fig. S2at *JXB* online). These results clearly indicate that *HvCO9* is the barley orthologue of *OsH*. This clade includes other cereal *CO*-like genes, which include *OsI* (*Ghd7*) from rice and three *HvZCCT* genes from the *VRN-H2* locus from barley, all of which play repressive roles in flowering (Xue *et al.*, 2008; Greenup *et al.*, 2009). It would be interesting to investigate the functional roles of *HvCO9* and *OsH* in flowering and compare them with *OsI* and *VRN-H2*.

The evolutionary relationships between *HvCO9* and *VRN-H2* was examined to clarify the functional similarities and differences between them. Cockram *et al.* (2010) revealed that a segment of barley chromosome 1H is duplicated on chromosome 4H and this duplication is conserved in all of the grass family members that have been examined to date. *VRN-H2* is located on this collinear duplicated region of the long arm of chromosome 4H (Laurie *et al.*, 1995), and this locus contains three *CO*-like genes, *HvZCCT-Ha*, *HvZCCT-Hb*, and *HvZCCT-Hc* (Dubcovsky *et al.*, 2005; Karsai *et al.*, 2005). However, no orthologues of the *HvZCCT*s were identified in these duplicated regions in other grass species, with the exception of wheat (see Supplementary Fig. S3 at *JXB* online).

Expression of the barley CO*-like genes,* HvCO9 *and* HvCO1*, during development*

To determine whether *HvCO9* expression is associated with the photoperiodic flowering response in barley, a quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using RNA samples extracted from the leaves that were harvested in the middle of the light period from the Steptoe and Morex barley cultivars and the expression of *HvCO9* was compared with *HvCO1*. In barley, the photoperiodic control of flowering depends on two major genes, *Ppd-H1* and *Ppd-H2*, which promote flowering under LD and SD conditions, respectively. Recently, *Ppd-H1* and *Ppd-H2* have been identified as members of the pseudoresponse regulator family and the *FT*-like gene family, respectively (Turner *et al.*, 2005; Faure *et al.*, 2007; Kikuchi *et al.*, 2009). Steptoe carries *Ppd-H1*/*ppd-H2* and flowers later than Morex under field conditions (autumn sowing) but earlier than Morex under LD conditions. By contrast, Morex has the *ppd-H1*/*Ppd-H2* genotype and flowers early under field conditions.

In both Steptoe and Morex, the expression levels of *HvCO9* were higher under SD conditions than under LD conditions (Fig. 2A, B), which suggests that the function of *HvCO9* is more significant under SD conditions. Varietal differences were also observed for the expression of *HvCO9*: the expression level of *HvCO9* was higher in Steptoe than in Morex (Fig. 2A, B). Similarly, Steptoe displayed a higher level of *HvCO1* expression than Morex under LD conditions (Fig. 2C); under SD conditions, however, the level of *HvCO1* expression was quite low in both cultivars (Fig. 2D). These results support the hypothesis that the expression of *Ppd-H1* under LD conditions in Steptoe increases *HvCO1* expression and, thus, promotes flowering. These results are consistent with a previous report (Turner *et al.*, 2005) that showed that the expression of *HvCO1* was reduced in *ppd-H1* plants.

Diurnal oscillation of HvCO9 *and* HvCO1 *expression levels in barley*

To investigate the relationship between *HvCO9* and *HvCO1* in flowering, their diurnal expression patterns were examined by qRT-PCR at the two- and three-leaf stages (during which the shoot apical meristem changes from a vegetative to a reproductive phase in an early flowering variety) under LD and SD conditions. The *HvCO9* transcript began to increase in the dark phase and peaked in the early morning under both of the photoperiodic conditions (Fig. 3A, B). This diurnal expression pattern was the same as that of rice *Ghd7*, although the expression of *Ghd7* was much higher under LD conditions than under SD conditions (Xue *et al.*, 2008; Itoh *et al.*, 2010). By contrast, the accumulation of the *HvCO1* mRNA was observed at the end of the light phase under LD conditions and at the early dark phase under SD conditions (Fig. 3C, D); nearly identical patterns have been observed for rice *Hd1* and wheat *WCO1* (Kojima *et al.*, 2002; Shimada *et al.*, 2009). No significant differences in the diurnal expression patterns of *HvCO9* and *HvCO1* were observed between Steptoe and Morex, although there were differences in the levels of gene expression between these two cultivars (Fig. 3).

The expression of and their relationship between HvCO9 *and* Ppd-H2 *under SD conditions*

The expression analysis of *HvCO9* revealed that *HvCO9* expression was under photoperiodic control; therefore, this gene may function preferentially under SD conditions, as mentioned above. Conversely, *Ppd-H2* (*HvFT3*) has been shown to be a positive regulator of flowering under SD conditions (Kikuchi *et al.*, 2009). To identify the

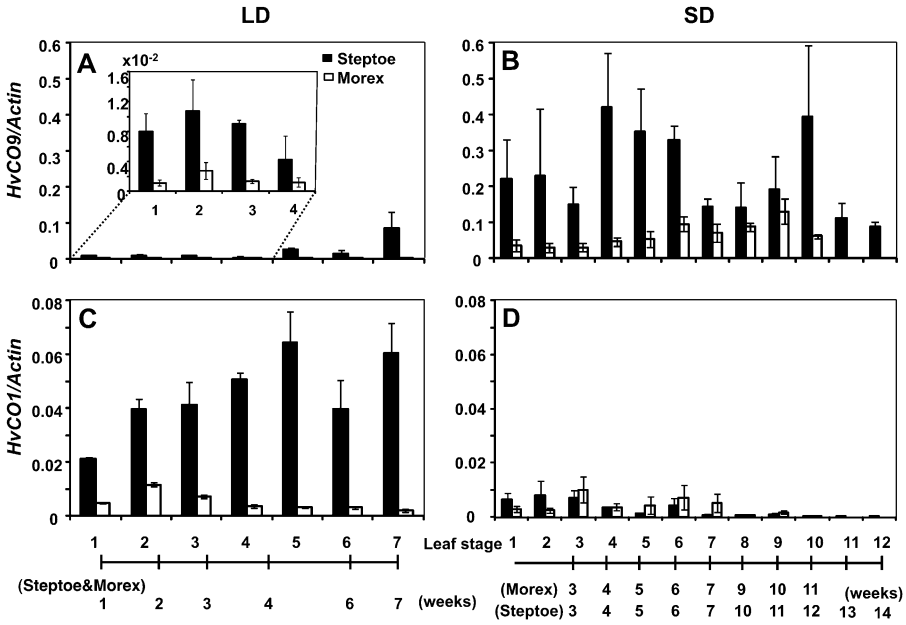

Fig. 2. Expression of the barley *CO*-like genes, *HvCO9* (A, B) and *HvCO1* (C, D), in Steptoe (*Ppd-H1/ppd-H2*) and Morex (*ppd-H1/Ppd-H2*) at each leaf stage under LD conditions (16/8 h light/dark) and SD conditions (12/12 h light/dark), respectively. The inset shows an enlarged graph of the earlier stages. The black bars indicate Steptoe, and the white bars indicate Morex. Each mRNA sample was quantified relative to the *HvActin* mRNA. The data were standardized with two independent experiments (means ±SE). The numbers at the bottom indicate each developmental stage as a leaf stage, and the time scales show the number of weeks after sowing.

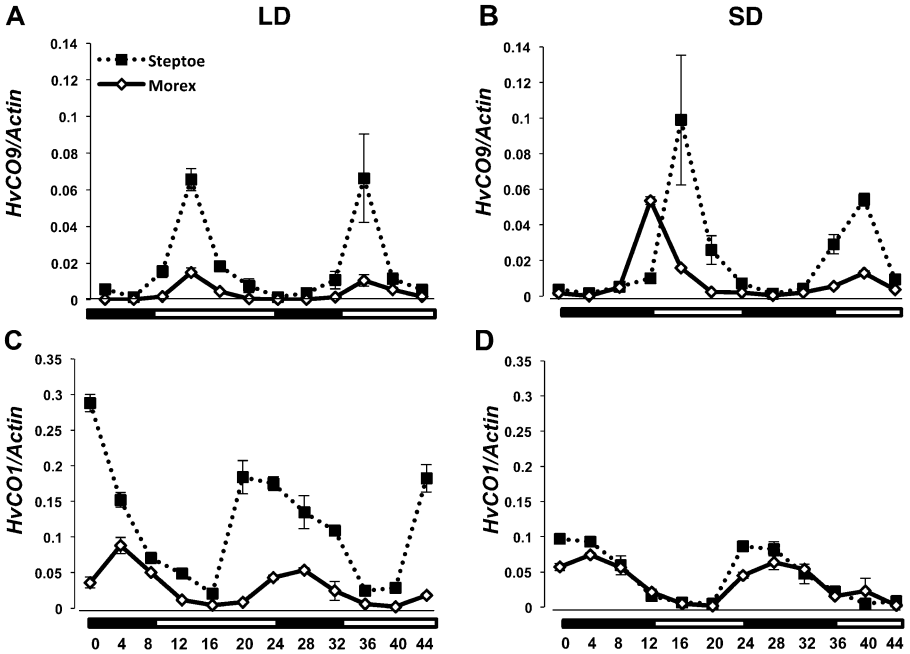

Fig. 3. Diurnal expression of the *CO*-like genes, *HvCO9* (A, B) and *HvCO1* (C, D), in Steptoe (*Ppd-H1/ppd-H2*) and Morex (*ppd-H1/Ppd-H2*) under LD and SD conditions, respectively. The dotted lines with black squares represent Steptoe and the solid lines with white diamonds represent Morex. The leaves were harvested from the plants at 4 h intervals during the two-leaf and three-leaf stages for 2 d. The vertical axis shows the relative mRNA levels of the *CO*-like genes that were normalized to *HvActin*. The mean quantified values ±SE for two independent experiments are shown. The white and black bars at the bottom indicate the light and dark periods, respectively.

relationship between *HvCO9* and *Ppd-H2* under SD conditions, the expression pattern of *HvCO9* was investigated by qRT-PCR using the DH lines, which contained different genotype combinations of *HvCO9* and *Ppd-H2* and were derived from the F_1 cross between Steptoe and Morex (Table 1).

All of the DH lines with the Morex-type *HvCO9* (hereafter designated as *HvCO9m*) that were investigated in

this study showed a low level of *HvCO9* expression; this level was the same as that of Morex and was irrespective of the *Ppd-H2* genotype (Fig. 4A). By contrast, the DH lines that contained the Steptoe-type *HvCO9* (hereafter designated as *HvCO9s*) showed slightly different expression levels than the DH lines that contained *HvCO9m* (Fig. 4B). Two *HvCO9s* DH lines that contained *Ppd-H2* showed an *HvCO9* expression level that was intermediate between that of Morex and Steptoe in the early growth phase; thereafter, the expression level decreased to that of Morex throughout the rest of development. The DH line, S/M-5 (*HvCO9s* and *ppd-H2*), exhibited a high level of *HvCO9* expression, which was at the same level as Steptoe in the early growth phase. However, the other DH line, S/M-148, which contained the same genotype combination (*HvCO9s* and *ppd-H2*) as S/M-5, showed a low *HvCO9* expression level that was the same as that in Morex (Fig. 4B).

The effect of the *HvCO9* genotype on the expression of three *FT*-like genes, *HvFT1*, *HvFT2*, and *HvFT3* (*Ppd-H2*), was also investigated under SD conditions (Fig. 5). No expression of *HvFT3* was detected in the DH lines with a Steptoe-type *HvFT3* (hereafter designated as *HvFT3s*)

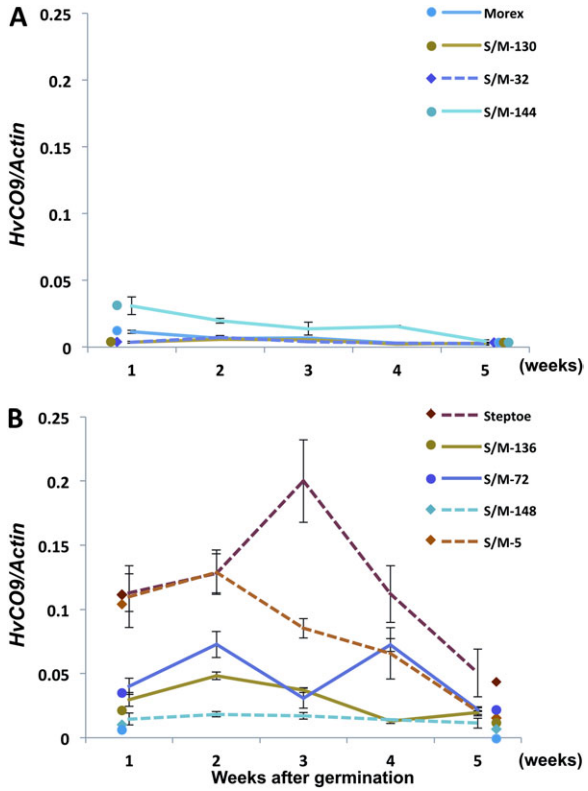

Fig. 4. Expression of *HvCO9* in the DH lines with different combinations of two genes, *HvCO9* and *Ppd-H2* (*HvFT3*), and their parental cultivars, Steptoe and Morex, under SD conditions. (A) The expression patterns of *HvCO9* in the DH lines that contained *HvCO9m*. (B) The expression patterns of *HvCO9* in the DH lines that contained *HvCO9s*. The solid lines indicate the DH line that contained *Ppd-H2* and the dotted lines indicate the DH line that contained *ppd-H2*. Each mRNA was quantified relative to the *HvActin* mRNA. The data were standardized using two independent experiments (means ± SE).

(Fig. 5E). This observation is consistent with our previously published data, in which Steptoe had lost most of the *HvFT3* gene, rendering it functionless (Kikuchi *et al.*, 2009). The expression of *HvFT3* was observed in the DH lines that contained the Morex-type *HvFT3* (hereafter designated as *HvFT3m*) although there was no obvious difference in the *HvFT3* expression patterns between the DH lines that contained *HvCO9s* (dotted lines) or *HvCO9m* (solid lines) (Fig. 5F). For the expression levels of *HvFT1* and *HvFT2*, correlations with the *HvCO9* genotype were observed in the DH lines that contained *HvFT3s*; the expression levels of these two *FT*-like genes in the DH line that contained *HvCO9m* (S/M-32) were higher than those in the DH line that contained *HvCO9s* (Fig. 5A, C). In the DH lines that contained *HvFT3m*, there were no clear differences in the *HvFT1* and *HvFT2* expression patterns between the DH lines that contained *HvCO9s* (dotted lines) or *HvCO9m* (solid lines) (Fig. 5B, D).

This observation led to the following hypothesis: *Ppd-H2* (*HvFT3*) and *HvCO9* are not directly connected in the flowering pathway under SD conditions, but under limited conditions, they interact indirectly with other associated factor(s) or interact directly with each other. However, other *FT*-like genes, such as *HvFT1* and *HvFT2*, are probably located downstream of *HvCO9,* and it is possible that *HvCO9* affects their expression levels directly or indirectly.

Over-expression of HvCO9 *in rice plants*

To investigate the function of *HvCO9* in flowering, *HvCO9*, which was under the control of the CaMV 35S promoter, was introduced into rice plants. The T_0 generation of the resulting transgenic rice showed a late flowering phenotype under SD conditions compared with the control plants (Fig. 6A, B). The T_1 generation of *HvCO9*-over-expressing line 9 also exhibited significantly late flowering under SD conditions. Late flowering was also observed under LD conditions, but the difference between the transgenic and the control plants was smaller than under the SD conditions (Fig. 6C).

The expression profiles of several rice genes that are related to flowering, including *Ehd1*, *Hd1*, *Hd3a*, and *RAP1B*, were examined by RT-PCR using two *HvCO9*-overexpressing lines (lines 9 and 10) and two lines of control plants (mock, lines 4 and 5) under LD conditions. The days to heading for the control plants, lines 4 and 5, and the transgenic plants, lines 9 and 10, were 30, 46, 85, and 62 d, respectively. *Ehd1*, *Hd3a*, and *RAP1B* were not expressed at detectable levels in the transgenic lines (Fig. 6D), which indicated that the heading delay of the *HvCO9*-overexpressing plants may be due to the suppression of *Ehd1* and its downstream genes. The expression profile that was observed in the *HvCO9*-overexpressing plants was the same as that of *Ghd7*, which acts as an LD-specific floral repressor in rice (Xue *et al.*, 2008).

Discussion

The *CONSTANS-FLOWERING LOCUS T* (*CO-FT*) interaction is a key pathway in the photoperiodic regulation

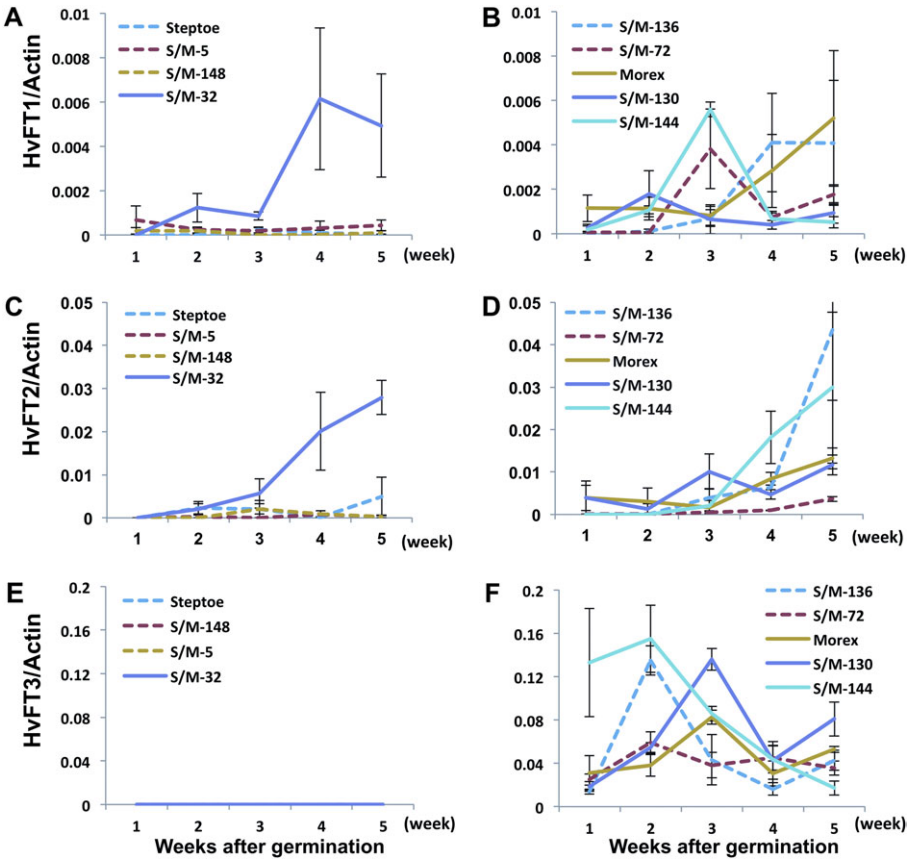

Fig. 5. Expression of *HvFT1* (A, B), *HvFT2* (C, D), and *HvFT3 (Ppd-H2)* (E, F) in the DH lines with different combinations of two genes, *HvCO9* and *Ppd-H2* (*HvFT3*) and their parental cultivars, Steptoe and Morex. (A, C, E) The expression patterns in the DH lines that contained *ppd-H2*. (B, D, F) The expression patterns in the DH lines that contained *Ppd-H2*. The solid lines indicate the DH line that contained *HvCO9m* and the dotted lines indicate the DH line that contained *HvCO9s*. Each mRNA was quantified relative to the *HvActin* mRNA. The data were standardized using two independent experiments (means ±SE).

of flowering and is conserved among distantly related plants (Greenup *et al.*, 2009; Yanovsky and Kay, 2003).

In this study, the focus was on *HvCO9*, a barley *CO*-like gene, rather than *HvCO1*, in order to clarify the functional role of the *CO*-like gene family in barley. The previous phylogenetic analyses revealed that *HvCO9* was located in a subgroup with two related rice genes, *OsH* and *OsI* (*Ghd7*), and Triticeae *VRN2*, which is a floral repressor of the vernalization response (Griffiths *et al.*, 2003; Yan *et al.*, 2004; Hemming *et al.*, 2008; Greenup *et al.*, 2009). However, this subfamily does not have an *Arabidopsis* counterpart; in other words, it is a grass species-specific *CO*-like subfamily. Furthermore, two genes in this subfamily, rice *Ghd7* and Triticeae *VRN2*, have been identified as floral repressors (Xue *et al.*, 2008; Greenup *et al.*, 2009). Our analysis using transgenic *HvCO9*-over-expressing rice plants suggests that *HvCO9* has the same function as *Ghd7*, namely, the negative regulation of flowering (Fig. 6). It is interesting that grass species have developed systems for flowering repression that are different from those of *Arabidopsis*, which contains its own *CO*-like gene subfamily.

Our transgenic studies revealed the possible regulatory targets of *HvCO9* in the photoperiodic gene pathway. The over-expression of *HvCO9* in rice under LD conditions apparently suppresses the expression of *Ehd1* and such downstream genes as *Hd3a*, a rice *FT* orthologue, but it did not affect *Hd1* expression (Fig. 6D). This observation is similar to the case of *Ghd7*, which has been shown to suppress *Ehd1* specifically under LD conditions (Xue *et al.*, 2008). From these results, there is speculation that a possible function of *HvCO9* in barley is the suppression of the function of an *Ehd1*-like gene and that *HvCO9* acts as a floral suppressor. However, a fundamental issue with this hypothesis is that *Ehd1* is unique to rice, and to date, no obvious orthologous genes have been identified in *Arabidopsis* or temperate cereals, including barley (Doi *et al.*, 2004; Greenup *et al.*, 2009; Higgins *et al.*, 2010). It will be of great interest to identify the target gene of *HvCO9* in barley and to determine how the pathway containing *HvCO9* functions for the regulation of flowering in barley.

To characterize the function of *HvCO9* further, an extensive expression analyses was conducted of *HvCO9*. The co-ordinated expression analyses of *HvCO9* and *HvCO1* revealed that these two *CO*-like genes displayed contrasting expression patterns. *HvCO9* was highly expressed under SD conditions and showed very low expression under LD conditions (Fig. 2A, B). Under SD conditions, Morex, a cultivar that contains *Ppd-H2*, showed a lower expression level than Steptoe, a cultivar that

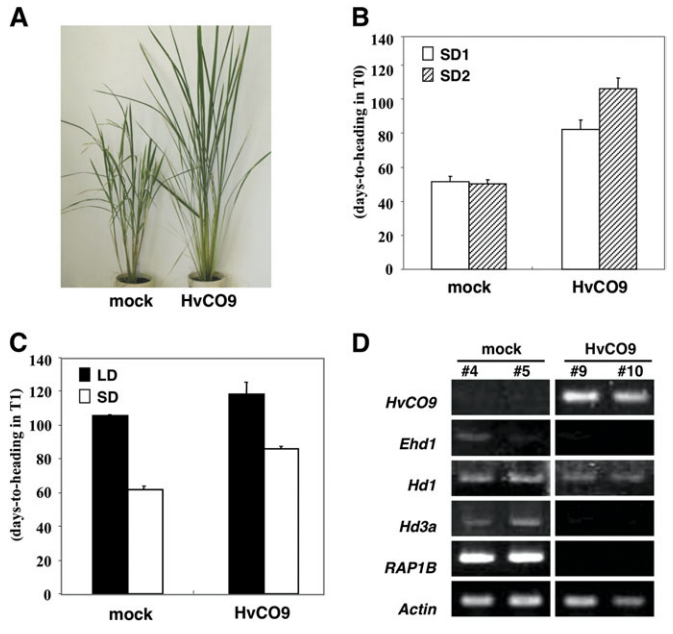

Fig. 6. The phenotype (A), flowering time (B, C), and expression pattern of flowering-related genes (D) in transgenic rice plants that over-expressed *HvCO9*. (A) The photographs show a transgenic plant (HvCO9) and a control plant (mock) at the heading stage under SD conditions. (B) The average number of days to heading ±SE of 15 transgenic T_0 plants under two SD conditions; the white bars and bars with diagonal lines indicate the SD1 and SD2 conditions, respectively. Both of the conditions are described in the Materials and methods. (C) The average number of days to heading ±SE of the T_1 generations of transgenic line 9 under LD (black bars) and SD conditions (white bars). (D) The expression of flowering-related genes in the transgenic rice plants and mock controls. The leaves were harvested 5 weeks after sowing under LD conditions. Mock indicates the transgenic plant that contained only the vector construct (negative control).

contains *ppd-H2* (Fig. 2B). By contrast, high *HvCO1* expression levels were observed under LD conditions, and the expression levels were extremely low under SD conditions (Fig. 2C, D). Under LD conditions, Steptoe (with *Ppd-H1*) showed a higher expression of *HvCO1* than Morex (with *ppd-H1*) (Fig. 2C). The diurnal expression patterns of *HvCO9* and *HvCO1* were conserved under both LD and SD conditions, and these diurnal expression patterns were similar in the two cultivars, although the expression levels were different (Fig. 3). The analysis of *HvCO9* and *HvCO1* expression using two barley cultivars that contain different combinations of photoperiod-sensitive genes suggests that these two *CO*-like genes act in different photoperiodic response signalling pathways: *HvCO9* acts in the SD signalling pathway, and *HvCO1* acts in the LD pathway. In addition, these two genes have opposite effects on flowering: *HvCO9* acts as a repressor, and *HvCO1* acts as an inducer.

If *HvCO9* is situated in the SD signalling pathway, then it would be interesting to investigate the relationship between *HvCO9* and *Ppd-H2*, which is the SD signal mediator that promotes flowering. Our previous study (Kikuchi *et al.*, 2009) revealed that the Morex cultivar contains an active form of *Ppd-H2* (*HvFT3m*) that promotes flowering under SD conditions, and it also revealed that *ppd-H2* in Steptoe is a truncated form of *HvFT3* (*HvFT3s*) and does not produce a functional protein. Using the DH lines with different genotype combinations of *HvCO9* and *Ppd-H2*, the influence of the *Ppd-H2* genotype on the expression of *HvCO9* was investigated (Fig. 4). The impact of *Ppd-H2* on the expression of *HvCO9* differed with respect to the genotype of *HvCO9*. All of the DH lines that contained *HvCO9m* showed a low level of expression, regardless of the *Ppd-H2* genotype (Fig. 4A). However, the *HvCO9* expression in the DH lines that contained *HvCO9s* was affected by the *Ppd-H2* genotype, in which a higher expression under *ppd-H2* and a lower expression under *Ppd-H2* were observed (Fig. 4B). These results indicate that the regulation of *HvCO9* expression differs by cultivar and that a clear hierarchical relationship does not exist between *HvCO9* and *Ppd-H2*. One large insertion/deletion and several SNPs were identified in the possible promoter region (up to 1.6 kb upstream of the *HvCO9* initiation codon) between Steptoe and Morex (Fig. 1). Database searches (PlantPAN, http://plantpan.mbc.nctu.edu.tw/index.php; Chang *et al.*, 2008) using the upstream sequence of Morex revealed that the 24 bp insertion/deletion region contained a GAMYB or Opaque-2 motif, both of which are described as *cis*-regulatory elements for seed development. These sequence variations may cause differences in expression, although the functional roles of these sequence variations in the regulation of *HvCO9* expression could not be specified.

VRN-H2 (*HvZCCTs*) is the gene that is most closely related to *HvCO9*. Both *HvCO9* and *VRN-H2* have a common function as a repressor of flowering, that is, they act as floral repressors. *VRN-H2* expression is clearly regulated in a daylength-dependent manner in the vernalization-responsive accessions: it is highly expressed in the leaves under LD conditions, but the expression is very low or absent under SD conditions (Trevaskis *et al.*, 2006). This finding is completely opposite to the photoperiodic pattern of *HvCO9* expression (Fig. 2). The over-expression of *VRN-H2* in transgenic barley plants delays flowering by 4 weeks, compared with the controls (Hemming *et al.*, 2008). *HvFT1* expression was significantly lower in the transgenic plants than in the control plants, but the expression levels of *VRN-H1*, a key gene for floral induction in the vernalization pathway, did not differ between the plants that were over-expressing *VRN-H2* and the control lines (Hemming *et al.*, 2008). The effects on flowering by the over-expression of both *HvCO9* and *VRN-H2* were nearly same, although the plant materials that were used in the transgenic studies were different: rice was used for *HvCO9*, and barley was used for *VRN-H2*. These results suggest that these two genes encode proteins that share a common function in repressing flowering and that their expression is regulated by day length, although the response to the daylength condition is opposite for the two genes.

VRN2 is located on barley chromosome 4H and the wheat homoeologous chromosome group 5 (Laurie *et al.*,

1995; Dubcovsky *et al.*, 1998). The surrounding regions of *VRN-H2* originated from the duplication of the *HvCO9* region on barley 1H (see Supplementary Figs S1 and S2 at *JXB* online), and this duplication has been observed in many species of grass, suggesting that the duplication occurred in the common ancestor to all grass species (Cockram *et al.*, 2010; see Supplementary Fig. S4 at *JXB* online). However, the *VRN2* gene is specific to the Triticeae, and no *VRN2* homologues were identified in other species. Cockram *et al.* (2010) suggested that several deletions of the *VRN2* locus have occurred during the evolution of the grass family, but it is more likely that the *VRN2* locus was created from the targeted duplication of *HvCO9* to the homologous region after the divergence of Triticeae. If so, the diversification of the regulation of gene expression in response to different daylengths is estimated to occur in a short time after the gene duplication.

Based on the results for the relationship between *HvCO9* and *VRN-H2*, it can be hypothesized that the functional targets of these genes may be the same. As shown in Fig. 6, the over-expression of *HvCO9* in rice clearly suppresses rice *Ehd1*, which is a key activator for multiple flowering signal pathways; however, no orthologueous gene has been found in the barley genome thus far (Greenup *et al.*, 2009; Higgins *et al.*, 2010). Conversely, the target gene of *VRN-H2* has been postulated to be *HvFT1* (by a transgenic study) (Hemming *et al.*, 2008) or *HvFT3* (by an extensive expression analysis) (Casao *et al.*, 2011). These data led us to postulate that the target gene of *HvCO9* may be an *FT*-like gene, as has been found for *VRN-H2*. Our previous study has revealed that two *FT*-like genes, *HvFT1* and *HvFT2*, function together in floral induction under SD conditions (Kikuchi *et al.*, 2009). Therefore, the influence of *HvCO9* on the expression of these *FT*-like genes in barley was investigated (Fig. 5). Because *Ppd-H2* had a strong effect under SD conditions on the regulation of flowering-related genes which included *FT*-like genes, the DH lines that contained *ppd-H2* (*HvFT3s*) were used for the expression analyses of the *FT*-like genes to avoid the influence of *Ppd-H2*. As shown in Fig. 5A and C, the expression levels of *HvFT1* and *HvFT2* were correlated with the *HvCO9* genotypes, which suggests that the possible targets of *HvCO9* under SD conditions are *FT*-like genes rather than an *Ehd1*-homologue, a case that is similar to *VRN-H2* under LD conditions. As a limited number of lines were used for our expression analysis in the present study, it will be interesting to investigate the relationship between the genotypes and the gene expression levels in future studies using additional lines or different populations.

Gene duplication and any ensuing functional diversification are thought to be the major driving forces for each plant species to construct its own unique mechanisms for flowering. In addition to the case of the *CO*-like genes in barley that are reported in the present study, an example in rice has previously been published. Rice has two related *FT*-like genes, *Hd3a* and *RFT1*, which were derived from a local gene duplication after the diversification of rice from the other cereal species (Komiya *et al.*, 2008, 2009). Both of the *FT*-like genes function as major floral activators, but *Hd3a* is specific to SD conditions, whereas *RFT1* is LD-specific. These results suggest that, although each plant species is dependent on commonly conserved functions, they have evolved their own unique mechanisms by integrating specific factors with a duplicated origin in their individual system.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1.Chromosomal location of *HvCO9* and the comparative mapping of the *HvCO9* region of barley chromosome 1H with rice chromosomes 5 and 10.

Supplementary Fig. S2. Phylogenetic tree of the *CO*-like proteins from barley, wheat, rice, and *Arabidopsis*.

Supplementary Fig. S3. (a–d) Micro-collinearity among the Poaceae at the *VRN2* locus and the *OsH* region.

Supplementary Fig. S4. Putative evolutionary history of the duplicated region, including the orthologueues of *HvCO9* in the grass family.

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