Molecular and Functional Characterization of PEBP Genes in Barley Reveal the Diversification of Their Roles in Flowering^1[OA]

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Five barley (Hordeum vulgare) PEBP (for phosphatidylethanolamine-binding protein) genes were analyzed to clarify their functional roles in flowering using transgenic, expression, and quantitative trait locus analyses. Introduction of HvTFL1 and HvMFT1 into rice (Oryza sativa) plants did not result in any changes in flowering, suggesting that these two genes have functions distinct from flowering. Overexpression of HvFT1, HvFT2, and HvFT3 in rice resulted in early heading, indicating that these FT-like genes can act as promoters of the floral transition. HvFT1 transgenic plants showed the most robust flowering initiation. In barley, HvFT1 was expressed at the time of shoot meristem phase transition. These results suggest that HvFT1 is the key gene responsible for flowering in the barley FT-like gene family. HvFT2 transgenic plants also showed robust flowering initiation, but HvFT2 was expressed only under short-day (SD) conditions during the phase transition, suggesting that its role is limited to specific photoperiodic conditions in barley. Flowering activity in HvFT3 transgenic rice was not as strong and was modulated by the photoperiod. These results suggest that HvFT3 functions in flowering promotion but that its effect is indirect. HvFT3 expression was observed in Morex, a barley cultivar carrying a dominant allele of Ppd-H2, a major quantitative trait locus for flowering under SD conditions, although no expression was detected in Steptoe, a cultivar carrying Ppd-H2. HvFT3 was expressed in Morex under both long-day and SD conditions, although its expression was increased under SD conditions. HvFT3 was mapped to chromosome 1HL, the same chromosome that carries Ppd-H2. Genomic sequence analyses revealed that Morex possesses an intact HvFT3 gene, whereas most of this gene has been lost in Steptoe. These data strongly suggest that HvFT3 may be identical to Ppd-H2.

Floral transition (i.e. the change from a vegetative meristem to the reproductive stage) is a critical event in the life cycle of seed-propagated plants. Several pathways promote flowering, including vernalization, photoperiod, and autonomous and gibberellin pathways (Boss et al., 2004). In Arabidopsis (Arabidopsis thaliana), FLOWERING LOCUS T (FT), which encodes a protein with a unique phosphatidylethanolamine-binding protein (PEBP) domain (domain accession pfam01161), promotes flowering. FT plays a central role in the integration of flowering signals from the vernalization and photoperiod pathways (Kardailsky et al., 1999; Kobayashi et al., 1999). Arabidopsis is a long-day (LD) plant, and its flowering is induced under LD conditions. Under LD conditions, CONSTANS (CO), which is regulated by circadian clock factors such as GIGANTEA (GI) and which encodes a transcription factor with two B-box zinc fingers, induces FT expression, causing early flowering (Yanovsky and Kay, 2003). Recent studies indicate that the FT protein functions as a systemic signaling molecule from leaf to apex, a so-called “florigen” (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007). The FT protein interacts with the bZIP transcription factor FD at the apex and activates the floral meristem identity gene APETALA1 (Abe et al., 2005; Wigge et al., 2005).

Like the Arabidopsis FT gene, the rice (Oryza sativa) FT-like gene Hd3a was identified as a flowering-time quantitative trait locus (QTL), which promotes flowering under short-day (SD) conditions (Yano et al., 2001; Monna et al., 2002). Hd3a expression is regulated by Hd1, the rice ortholog of CO. However, the expression and regulation of Hd3a are completely opposite from those of FT in Arabidopsis. Hd3a is involved in the promotion of heading (the developmental stages responsible for the initial emergence of the inflorescence...
from the boot in grass plants) under SD conditions, and its expression is completely inhibited under LD conditions (Kojima et al., 2002; Hayama et al., 2003; Hayama and Coupland, 2004). This is consistent with the fact that rice is a SD plant that flowers earlier under SD conditions than LD conditions. These results obtained for Arabidopsis and rice show that FT expression is common to flowering induction in higher plants and that variation in the CO-FT interaction is a key difference between LD and SD flowering induction. Recently, in wheat (Triticum aestivum) and barley (Hordeum vulgare), a vernalization gene, VRN3, was shown to encode a homolog of Arabidopsis FT (Yan et al., 2006). Analyses of transgenic plants showed that VRN3 functions as a flowering promoter, like Arabidopsis FT and rice Hd3a. This finding indicates that the same type of genetic system controls the flowering process in wheat and barley.

Recent advances in plant biology have provided access to the complete genome sequences of flowering plant species, including those of two model organisms, Arabidopsis and rice (Arabidopsis Genome Initiative, 2000; International Rice Genome Sequencing Project, 2005). Using these sequence data, genome-wide searches have been carried out to discover all PEBP gene families present in plant genomes. In Arabidopsis, FT forms a small gene family with five other genes: TERMINAL FLOWER1 (TFL1), TWIN SISTER OF FT (TSF), BROTHER OF FT AND TFL1 (BFT), ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOGUE (ATC), and MOTHER OF FT AND TFL1 (MFT; Kardailsky et al., 1999; Kobayashi et al., 1999). Some of these genes are postulated to have important functions in the flowering pathway of Arabidopsis. On the other hand, Chardon and Damerval (2005) identified 19 PEBP genes, including the FT homolog Hd3a in the rice genome. Recently, Danilevskaya et al. (2008) identified 25 PEBP genes in maize (Zea mays). The number of PEBP family genes in cereal is three to four times greater than that in Arabidopsis, indicating that this family is much more complex in cereal plants than in Arabidopsis. This PEBP gene redundancy in cereals raises questions about the functional diversification that remain to be elucidated.

In barley, a study using EST database searches and bacterial artificial chromosome library screening identified at least five FT-like genes, HvFT1 to HvFT5 (Faure et al., 2007). This raised questions about the roles of these FT-like genes in the flowering process. Moreover, it is interesting to compare the functional roles of these FT-like genes between rice and barley, because barley is more closely related to rice than to Arabidopsis but is a LD plant like Arabidopsis. Although barley is classified as a LD plant, its photoperiod response is facultative (quantitative response). Therefore, flowering in barley is not only promoted by a suitable LD condition but can also occur even under an inappropriate SD condition, although it is delayed. In barley, two major photoperiod response genes, Ppd-H1 and Ppd-H2, are located on chromosomes 2HS and 1HL, respectively (Laurie, 1997). Ppd-H1 promotes flowering in response to increasing daylength under LD conditions, whereas Ppd-H2 affects flowering under SD conditions but has little effect under LD conditions. Recently, the Ppd-H1 gene was cloned and identified as a member of the pseudoresponse regulator family. This gene may modulate daylength induction of FT-like genes by controlling CO-like activity in barley (Turner et al., 2005).

In this study, we performed expression and transgenic studies to clarify the functional roles of three FT-like genes and two other PEBP genes with regard to the flowering time of barley using two cultivars with different photoperiod response behaviors and different genotype combinations for the major photoperiod-sensitive Ppd genes, Steptoe and Morex. Three FT-like genes had already been identified by Faure et al. (2007), and the other two were novel genes. Here, we demonstrate the functional differentiation between these genes in controlling flowering using rice plants overexpressing barley PEBP genes. Some of these genes carry out the same function for flowering as their orthologs in Arabidopsis and rice, whereas others do not function in flowering in the rice genetic background. We also show the expression profiles of barley FT-like genes under LD and SD conditions in relation to the photoperiod response genes, Ppd-H1 and Ppd-H2. Furthermore, we reveal the close relationship between one barley FT-like gene and Ppd-H2, a major photoperiod response QTL that promotes flowering under SD conditions (Laurie et al., 1995; Laurie, 1997).

RESULTS

Search for Barley PEBP Genes and Phylogenetic Analyses

To identify PEBP genes in barley, we performed an in silico search of an in-house sequence database of full-length barley cDNA libraries, which were constructed from the mixed cDNAs of various tissues of a Japanese two-row variety, Haruna-Nijio (T. Matsumoto, H. Kanamori, K. Kurita, T. Bito, A. Kikuta, K. Kamiya, M. Yamamoto, Y. Mukai, H. Ikawa, N. Fujii, H. Sakai, T. Itoh, K. Sato, and S. Nakamura, unpublished data). The protein sequence of HvFT1 was used as the query (accession no. DQ100327). Five entries were identified as PEBP genes by this TBLASTN search: NIASHv3142C18, NIASHv3064E22, NIASHv1003I22, NIASHv2071G09, and NIASHv3007O09. During the course of this study, Faure et al. (2007) published their results based on the same kind of survey for FT-like genes in barley. We compared our cDNA clones with their barley FT-like genes. Our three full-length cDNAs, NIASHv3142C18, NIASHv3064E22, and NIASHv1003I22, corresponded to their HvFT1, HvFT2, and HvFT3 genes, respectively. Our other two full-length cDNAs, NIASHv2071G09 and NIASHv3007O09, did not corre-
spond to any FT-like genes found by Faure et al. (2007). However, these cDNAs showed extensive similarity to Arabidopsis TFL1 and MFT, respectively. Therefore, we named these two cDNA clones (NIASHv2071G09 and NIASHv3007O09) HvTFL1 (accession no. AB447465) and HvMFT1 (accession no. AB447466), respectively.

Phylogenetic analysis of an amino acid sequence alignment of the PEBP domains from PEBP genes in barley, rice, and Arabidopsis (Chardon and Damerval, 2005; Faure et al., 2007; this study) revealed that the PEBP gene family contains three major clades. HvFT1, HvFT2, and HvFT3 belonged to the FT-like clade, whereas HvTFL1 and HvMFT1 were members, respectively, of the TFL1-like and MFT-like clades, as expected (Fig. 1; Supplemental Fig. S1). Protein sequence alignment also revealed an amino acid residue change from Tyr in barley FT-like proteins (HvFT1, HvFT2, and HvFT3) to His in HvTFL1 (Supplemental Fig. S1), which was a key residue for the functional difference between FT and TFL1 in Arabidopsis (Hanzawa et al., 2005).
The chromosomal position of HvFT3 is 6.7 cM distal from ABC307A and 8.4 cM proximal to cMWG706A on the long arm of chromosome 1H (Figs. 2 and 3), consistent with the results of Faure et al. (2007). As pointed out by Faure et al. (2007), it is interesting that HvFT3 maps to the same region as Ppd-H2, which is a major QTL for heading time under SD conditions in barley (Laurie et al., 1995; Laurie, 1997). Therefore, we conducted a QTL analysis for flowering time under SD conditions (12 h of light/12 h of dark) in a growth chamber at 20°C/17°C using the Steptoe/Morex DH population. Figure 3A shows the log of the odds (LOD) curve obtained for chromosome 1H using interval mapping for flowering time under SD conditions. One major peak for flowering time was detected in the region containing HvFT3. The LOD score for this peak was 11.3, which explained 30.4% of the phenotypic variation. We also identified a second QTL peak between markers ABC482 and ABG391 on the long arm of chromosome 5H. This peak demonstrated a LOD score of 4.13, explaining 13.1% of the phenotypic variation (Fig. 3B). Although HvTFL1 was located on the short arm of chromosome 5H (Fig. 2), there was no association between HvTFL1 and this minor QTL on 5H (Fig. 3B).

Overexpression of Barley PEBP Genes in Rice Plants

To investigate their possible functional roles, especially pertaining to flowering, five barley PEBP genes, HvFT1, HvFT2, HvFT3, HvTFL1, and HvMFT1, were introduced into rice plants under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Overexpression of HvTFL1 or HvMFT1 had no effects on flowering or other phenotypes in rice plants (Fig. 4B for flowering time; data not shown for other phenotypes). On the other hand, HvFT1 and HvFT2 transgenic rice plants displayed much earlier heading than control plants under both LD and SD conditions (Fig. 4). There were no differences in the heading times of HvFT1 and HvFT2 transgenic plants between LD and SD conditions. These results indicate that HvFT1 and HvFT2 function similarly as strong inducers of flowering in rice plants, whereas HvTFL1 and HvMFT1 demonstrated no obvious function for flowering.

HvFT3-overexpressing rice plants also showed earlier heading compared with control plants, as did HvFT1- and HvFT2-overexpressing plants (Fig. 4), suggesting that HvFT3 also plays a role in flowering. However, the effect of HvFT3 on heading time was weaker than those of HvFT1 and HvFT2. Interestingly, the heading time for HvFT3 transgenic rice plants differed between LD and SD conditions. Heading of HvFT3 transgenic rice plants under LD conditions was delayed by 14.7 d compared with heading under SD conditions (Fig. 4B). This difference was identical to that of the control plants (15.4 d). These results revealed that HvFT3 transgenic rice plants maintain the same flowering photoperiod response as control plants, suggesting that the role of HvFT3 in flowering is indirect and that flowering activity can be modulated by the photoperiod signals, in contrast to the cases of HvFT1 and HvFT2.

Expression Pattern of Barley FT-Like Genes during Different Developmental Stages

To determine whether FT-like gene expression is associated with the photoperiodic response in barley, quantitative reverse transcription (RT)-PCR was applied to two barley cultivars, Steptoe and Morex. Steptoe is a late-heading cultivar under field conditions (autumn sowing), whereas Morex exhibits early heading in the field. However, Morex heading is delayed under controlled LD conditions compared with Steptoe. Turner et al. (2005) revealed that Steptoe
possesses Ppd-H1, which promotes flowering under LD conditions, but Morex carries ppd-H1. Therefore, the heading delay of Morex under LD conditions can be explained by the difference in the Ppd-H1 genotype. In contrast, in the field and under controlled SD conditions, Morex undergoes heading earlier than Steptoe. As shown before, we identified a major QTL for SD heading on chromosome 1HS that may correspond to Ppd-H2; the positive and dominant allele for this QTL was derived from Morex. Therefore, we postulate that Steptoe and Morex carry ppd-H2 and Ppd-H2, respectively, which could explain the early heading of Morex under SD conditions.

To obtain an overall picture of barley FT-like gene expression, we compared expression patterns between Steptoe and Morex through different developmental stages under both photoperiodic conditions (LD and SD). Under LD conditions, the expression levels of HoFT1 and HoFT2 were higher in Steptoe than in Morex (Fig. 5, A and C). This result supports the hypothesis that Ppd-H1 expression in response to LD photoperiods in Steptoe induces HoFT1 and HoFT2.
expression and then promotes flowering under LD conditions. HvFT1 was expressed at an earlier stage (one-leaf stage, 1 week after sowing), whereas the expression of HvFT2 was detected later (after the four-leaf stage). Morex, which carries ppd-H1, showed extremely low HvFT1 and HvFT2 expression under LD conditions (Fig. 5, A and C), consistent with the late heading of Morex compared with Steptoe under LD conditions. These results suggest that HvFT1 plays a primary role in flowering induction in barley cultivars carrying Ppd-H1 under LD conditions.

Under SD conditions, Morex showed higher HvFT1 and HvFT2 expression than Steptoe, although their expression levels in Morex under SD conditions were lower than in Steptoe under LD conditions (Fig. 5, B and D). These results explain why Morex heads earlier than Steptoe under SD conditions. Under SD conditions, HvFT1 was expressed in Morex at an earlier stage (one-leaf stage), similar to Steptoe under LD conditions, indicating that HvFT1 plays an important role in flowering induction under SD conditions. These findings suggest that under SD conditions, Ppd-H2 induces the expression of HvFT1, equivalent to Ppd-H1 under LD conditions. This results in earlier heading, even under SD conditions. Moreover, we detected the expression of HvFT2 at the one-leaf stage, and its expression level was nearly the same as that of HvFT1 (Fig. 5, B and D), indicating that HvFT2 can cooperate with HvFT1 as a flowering inducer under SD conditions. However, unlike HvFT1, the expression of HvFT2 under SD conditions was observed not only in Morex but also in Steptoe. This suggests that Ppd-H2 has no effect on the expression of HvFT2 and that HvFT2 expression is regulated by other photoperiodic pathway(s).

The expression of HvFT3 was observed only in Morex under both LD and SD conditions (Fig. 5, E and F). We could not detect any HvFT3 expression in Steptoe under either LD or SD conditions. The HvFT3 expression level in Morex was several times higher under SD conditions than under LD conditions, although its expression was detected under both LD and SD conditions in Morex. Relatively high HvFT3 expression preceded the expression of HvFT1 and HvFT2; the levels decreased when HvFT1 and HvFT2 expression increased (Fig. 5, E and F).

On the other hand, HvTFL1 and HvMFT1 transcripts were not detected in leaves in either cultivar under either condition (data not shown). These results suggest that the two genes are not expressed in leaves and that they may not be associated with flowering initiation.

Diurnal Rhythmic Expression Patterns of HvFT1, HvFT2, and HvFT3

To investigate the relationship among the three barley FT-like genes, HvFT1, HvFT2, and HvFT3, their diurnal expression patterns were examined at the two-leaf and three-leaf stages, during which the shoot apical meristem (SAM) begins its transition from the vegetative to the reproductive phase, under LD and SD conditions, respectively. The experiment was performed using two barley cultivars, Steptoe and Morex, by quantitative RT-PCR.
In Morex, HvFT1 was transcribed beginning at dawn, and its expression peaked in the middle of the light phase under LD and SD conditions (Fig. 6, A and B). This diurnal oscillation of barley HvFT1 is nearly identical to that of the rice ortholog Hd3a under SD conditions (Kojima et al., 2002; Hayama et al., 2003). The expression of HvFT3 also demonstrated a diurnal oscillation in Morex under both photoperiod conditions (Fig. 6, E and F). However, the expression pattern of HvFT3 in Morex was different from that of HvFT1, which began to increase in the middle of the light phase, with expression peaking at dusk, early in the dark phase (Fig. 6, E and F). On the other hand, HvFT2 showed nearly the same pattern of diurnal oscillation under SD conditions as HvFT3, and it peaked at the beginning of the dark phase (Fig. 6D).

As discussed above (Fig. 5, B and F), transcripts of HvFT1 and HvFT3 in Steptoe were barely detectable under SD conditions during either the daytime or the nighttime (Fig. 6, B and F). However, the expression of HvFT2 in Steptoe exhibited a distinct diurnal oscillation, which was nearly the same as that observed in Morex (Fig. 6D).

**Genomic Structure of HvFT3 in Steptoe, Morex, and Their DH Lines**

To investigate the cause of the differences in HvFT3 expression between Steptoe and Morex shown in Figures 5 and 6, we determined the genomic structures of HvFT3 in both barley cultivars (Fig. 7A). We cloned a 1,966-bp region containing HvFT3 from Morex and...
identified the complete HvFT3 gene within this fragment (accession no. AB476614). The HvFT3 gene in Morex has four exons and three introns, a structure that is conserved across most of the FT-like genes in higher plants. However, we could not amplify the HvFT3 region from Steptoe using primer pairs HvFT3/F1 and HvFT3/R1 or HvFT3/F2 and HvFT3/R1, whereas these pairs worked when Morex was used as the template (Fig. 7B). Only the primer pair HvFT3/F3 and HvFT3/R1 produced a fragment from Steptoe (Fig. 7B). Therefore, we tried to isolate the HvFT3 region in Steptoe and finally obtained a 1,532-bp sequence from the HvFT3 region in Steptoe (Fig. 7A; accession no. AB476615). The Steptoe HvFT3 region contains only the 3′ portion of exon 4 (189 bp; Morex exon 4 was 245 bp), and it has lost an upstream region, including three exons (exons 1–3). Instead of the HvFT3 genomic region, an unknown sequence was
found upstream of the truncated exon 4 sequence (Fig. 7A). These sequence data indicate that Steptoe has no functional HvFT3 and that this locus is a null allele in Steptoe.

HvFT3 is postulated to be closely associated with Ppd-H2 (Fig. 3). Therefore, we analyzed the HvFT3 sequences from four DH lines (S/M-5, -32, -72, and -144) derived from the cross between Steptoe and Morex. The DH lines were segregated between Steptoe and Morex in addition to those from the parental cultivars (Table 1). All four DH lines carried the Steptoe genotype for markers ABC482 and ABG391, which are linked to the 5HL QTL for flowering time under SD conditions. However, the DH lines were segregated between Steptoe type (S/M-5 and -32) and Morex type (S/M-72 and -144) for markers ABC307A and cMWG706A, which lie adjacent to Ppd-H2. They were also segregated with regard to heading time, because S/M-72 and -144 displayed early heading under SD conditions (like Morex) and S/M-5 and -32 exhibited a late-heading phenotype (like Steptoe). The presence of an intact copy of the HvFT3 gene and its normal expression correlated with the segregation of the heading phenotype and the marker genotype (Fig. 7, B and C). Early-heading DH lines (S/M-72 and -144) possessed an intact HvFT3 gene and demonstrated similar expression to that of Morex, whereas late-heading and Steptoe-type genotype DH lines (S/M-5 and -32) carried no intact copies of HvFT3 and showed no expression of the corresponding transcript.

DISCUSSION

Compared with dicot plants, which have smaller gene families of approximately six to eight members, monocots possess large families of PEBP genes (e.g. 19 members in rice [Chardon and Damerval, 2005] and 25 members in maize [Danilevskaya et al., 2008]). The quantity of family members raises questions about the functional diversification and conservation of genes within the PEBP family in cereals.

In this study, we identified five PEBP genes in barley, three of which belong to the FT-like clade: HvFT1, HvFT2, and HvFT3. Two other genes, HvTFL1 and HvMFT1, were classified in the TFL1-like and MFT-like clades, respectively (Fig. 1). Each PEBP gene was subjected to further expression and transgenic analyses to reveal its functional role in flowering.

tfl1 mutants show early flowering and the promotion of terminal floral meristem formation in Arabidopsis (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). TFL1 encodes a protein with a PEBP domain (Bradley et al., 1997; Ohshima et al., 1997), and FT was later identified as a TFL1 homolog (Kardailsky et al., 1999; Kobayashi et al., 1999). In transgenic Arabidopsis plants that ectopically overexpress TFL1, both vegetative and reproductive phases are greatly extended (Ratcliffe et al., 1998). Amaya et al. (1999) overexpressed TFL1 and Antirrhinum CENTRORA-DIALIS (CEN) in tobacco (Nicotiana tabacum), which is a homolog of TFL1 (Bradley et al., 1996). Tobacco plants overexpressing CEN display an extended vegetative phase, whereas overexpression of TFL1 in tobacco plants does not significantly delay flowering time. This may reflect a divergence of the CEN and TFL1 proteins, or plants may possess a pathway for altering the phase change mechanism that involves TFL1-like proteins, and the pathway may differ between species. Two TFL1-like genes, RCN1 and RCN2, were detected in rice (Nakagawa et al., 2002). Overexpression of RCN1 and RCN2 in rice yielded similar phenotypes to overexpression of TFL1 in Arabidopsis (i.e. delayed flowering and altered panicle morphology). These results suggest that rice possesses the same type of molecular mechanism responsible for controlling the meristem phase transition as Arabidopsis.

In this study, we identified a barley TFL1-like gene, HvTFL1, which demonstrates the greatest similarity to rice RCN1 (Fig. 1). However, rice plants overexpressing HvTFL1 exhibited neither a flowering delay nor alteration of panicle morphology (data not shown). In

| Line     | Heading Date a | Genotype |
|----------|----------------|----------|
| Steptoe  | 104.8          | Ppd-H1   |
| Morex    | 69.4           | ppd-H1   |
| DH line  |                | 1HL QTL  |
|          |                | ABC307A  |
|          |                | cMWG706A |
| S/M-5    | 104.0          | M        |
| S/M-32   | 105.7          | M        |
| S/M-72   | 62.5           | M        |
| S/M-144  | 76.3           | M        |
|          | n.d.           | 5HS QTL  |
|          |                | ABC482   |
|          |                | ABG391   |
|          |                | S        |
|          |                | S        |
|          |                | S        |
|          |                | S        |
|          |                | S        |

Table 1. Heading date under SD conditions and genotypes of Ppd-H1 and DNA markers near SD-flowering QTLs

n.d., Not determined.

aDays to heading after sowing under SD conditions (12 h of light/12 h of dark). bAccording to the data of Turner et al. (2005).
addition, the HvTFL1 transcript was not detected in the leaves of any of the barley cultivars under any growth conditions, whereas RcN1 is expressed in leaves (Nakagawa et al., 2002). These results for HvTFL1 and RcN1 in transgenic rice are interesting and indicate the divergence of orthologous proteins and the differentiation of the flowering pathway between rice and barley, similar to the heterologous expression of CEN and TFL1 in tobacco (Amaya et al., 1999).

Similar observations were obtained from the study of HvMFT1, a homolog of Arabidopsis MFT. We overexpressed HvMFT1 in rice under the control of the CaMV 35S promoter. The HvMFT1-overexpressing plants showed no notable differences in flowering time or plant architecture when compared with the wild type (data not shown). Little is known about the role of MFT genes in other plants or even in Arabidopsis. Yoo et al. (2004) suggested that Arabidopsis MFT functions as a floral inducer but that it may act redundantly in determining flowering time based on observations using gain-of-function and loss-of-function alleles of MFT. However, we could not detect any induction of flowering when HvMFT1 was overexpressed in rice, suggesting that HvMFT1 does not function in flowering. Recently, Danilevskaya et al. (2008) revealed the seed-specific expression of two MFT-like genes in maize, ZCN9 and ZCN10. These two MFT-like genes are closely related to one of the MFT-like genes, OsMFT2, in rice. HvMFT1 may be orthologous to OsMFT2, as evidenced by sequence similarity and chromosome mapping (Figs. 1 and 2; Supplemental Fig. S1). HvMFT1 was mapped to the end of the short arm of barley chromosome 3H (Fig. 2), whereas OsMFT2 lies in the syntenous region of the short arm of rice chromosome 1. Expression analysis showed that HvMFT1 is not expressed, at least not in leaves. Together with the transgenic data and expression analysis obtained in this study and other reports, the data suggest that these cereal MFT-like genes function not in the flowering pathway but rather in the grain maturation pathways, as hypothesized by Chardon and Damerval (2005) and Danilevskaya et al. (2008).

We characterized three barley FT-like genes: HvFT1, HvFT2, and HvFT3. Transgenic rice plants possessing HvFT1 and HvFT2 demonstrated much earlier heading than control plants, regardless of the daylength (Fig. 4). The early-heading phenotypes of HvFT1 and HvFT2 transgenic rice plants were very similar to those of rice plants overexpressing Hd3a, the rice ortholog of FT (Kojima et al., 2002). These results indicate that HvFT1 and HvFT2 function similarly to rice Hd3a, a strong inducer of flowering in rice and probably also in barley. HvFT1 was mapped to the short arm of chromosome 7H (Fig. 2), which is syntenous with rice chromosome 6, which contains Hd3a. The results from gene-mapping and phylogenetic studies (Fig. 1) suggest that HvFT1 is an ortholog of rice Hd3a. The expression of HvFT1 in barley has been observed before SAM transition from the vegetative to the reproductive phase (i.e. the two-leaf and three-leaf stages under LD and SD conditions, respectively), although the expression level under LD conditions was higher than that under SD conditions (Fig. 5, A and B). On the other hand, HvFT1 expression seems to be regulated by the major photoperiod response genes, Ppd-H1 under LD conditions and Ppd-H2 under SD conditions, because very low expression was observed in cultivars possessing the recessive alleles of these Ppd genes, ppd-H1 (Morex) and ppd-H2 (Steptoe), under LD and SD conditions, respectively (Fig. 5, A and B). Taken together, the evidence indicates that HvFT1 plays a major role in flowering initiation in barley and that this role is regulated by the major response pathways during both photoperiod conditions.

HvFT2 is postulated to be orthologous to one of the rice FT-like genes, OsFTL1, based on phylogenetic data (Fig. 1) and gene-mapping data indicating that barley 3H, which contain HvFT2, is syntenous to rice chromosome 1, which contains OsFTL1 (Fig. 2). OsFTL1 is involved in floral promotion in rice, similar to Hd3a (Izawa et al., 2002). In wild-type rice, Hd3a mRNA expression was up-regulated under inductive SD conditions but not under LD conditions. Under SD conditions, Hd3a mRNA was diurnally expressed, exhibiting a peak before dawn. In contrast, OsFTL1 mRNA expression was detected under both LD and SD conditions, although mRNA levels were higher under SD conditions. These results indicate that these rice FT-like genes are expressed differentially in response to different photoperiods (Izawa et al., 2002). Transgenic analysis revealed that HvFT2 has a functional role during flowering initiation. However, the expression pattern of HvFT2 in barley differed from that of HvFT1. Under LD conditions, the expression of HvFT2 was delayed compared with HvFT1 and it was not detected at the two-leaf stage, during which SAM transitions from the vegetative to the reproductive phase (Fig. 5C). On the other hand, under SD conditions, HvFT2 expression was observed at the three-leaf stage in both Steptoe and Morex (Fig. 5D). However, HvFT2 expression alone does not appear to be sufficient to induce flowering under SD conditions. Steptoe, as well as Morex, shows HvFT2 expression under SD conditions, but Steptoe heading is greatly delayed compared with that of Morex. This may be due to the difference in HvFT1 expression between these two cultivars. These results suggest that HvFT2 functions as a floral activator specifically under SD conditions but that its function is supplementary to HvFT1 function. The results also suggest that HvFT2 participates in a separate pathway from the Ppd-H2-dependent photoperiod pathway, unlike HvFT1. barley has an adaptive mechanism to adjust flowering according to photoperiod changes using a combination of different FT-like genes.

Faure et al. (2007) postulated that HvFT3 is a good candidate gene for Ppd-H2, which affected flowering
time in a SD greenhouse experiment (10 h of light) and in an autumn-sown field experiment but was not detectable under LD conditions (Laurie et al., 1995). We identified this major QTL under SD conditions (12 h of light) using a DH population derived from a cross between Steptoe and Morex (Fig. 3).

By examining its overexpression in rice plants, HvFT3 was found to function as a flowering inducer, but its effect was weaker than that of HvFT1 or HvFT2 (Fig. 4). Unlike HvFT1 and HvFT2, heading of HvFT3 transgenic rice plants was sensitive to the photoperiod. The difference in heading time in HvFT3 transgenic rice plants under LD compared with SD conditions (14.7 d) was nearly identical to that of control plants (15.4 d; Fig. 4). These results clearly show that HvFT3 transgenic rice maintained the same photoperiodic response for flowering as control plants. Due to the weak flowering induction effect and the same level of photoperiod response as the control plant, we hypothesize that HvFT3 functions indirectly to promote flowering and that its activity can be modulated by photoperiod signals. This hypothesis is supported by the finding that HvFT1 and HvFT2 expression mirrors that of HvFT3 under SD conditions (Fig. 5).

HvFT3 transcripts in Morex, which carries Ppd-H2, were detected under both LD and SD conditions, but its expression levels were higher under SD than under LD conditions (Fig. 5, E and F). Especially high HvFT3 expression was observed in Morex at the early stage of development before the SAM transition under SD conditions, similar to HvFT1 (Fig. 5E). In contrast, Steptoe, a cultivar that contains Ppd-H2, demonstrated no expression of HvFT3 under any photoperiod conditions (Fig. 5, E and F) and very low expression of HvFT1 under SD conditions (Fig. 5B). The difference between Steptoe and Morex in flowering time under SD conditions is thought to be due to this difference in HvFT1 expression, which also suggests that HvFT3 expression facilitates the induction of HvFT1 expression. The expression patterns of HvFT3 and HvFT1 under SD conditions in Morex showed a clear diurnal rhythm but displayed their own oscillation phase (Fig. 6, B and F). Expression of HvFT3 begins during the middle of the light phase and peaks at dusk, whereas HvFT1 is expressed in the morning and peaks in the middle of the light phase. This phase difference is not inconsistent with the idea that HvFT3 expression participates in the induction of HvFT1 expression. Since analysis revealed that Morex had an intact and functional copy of HvFT3 but Steptoe had lost most of the gene, rendering it functionless (Fig. 7).

The heading behavior of HvFT3 transgenic rice, the chromosomal position of HvFT3, and the expression profile and structural analysis of HvFT3 using Ppd-H2 and Ppd-H2 cultivars strongly support the hypothesis that HvFT3 is a good candidate gene for Ppd-H2, which was reported by Faure et al. (2007). Further studies, including fine mapping and complementation tests with a functional HvFT3 gene, should be performed to verify this hypothesis.

In conclusion, this study of the barley PEBP genes reveals their presence and structure, which are well conserved among cereal plants, whereas their expression and function have diverged between rice and barley. To understand the molecular mechanism of flowering in a specific plant like barley, it is important to integrate the knowledge obtained from general studies that focus on model plants like Arabidopsis or rice with insights from specific studies of particular plants of interest.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Three varieties of barley (Hordeum vulgare ‘Steptoe’, ‘Morex’, and ‘Haruna-Nijo’) were used in this study. DH lines developed from the F1 cross between Steptoe and Morex were also used for gene mapping and QTL analysis (North American Barley Genome Mapping Project; Kleinhofs et al., 1993). The heading dates and genotypes of Ppd-H1 and DNA markers proximal to SD-flowering QTLs in Steptoe, Morex, and the DH lines used in this study are shown in Table I. Plants were grown in a growth chamber at 20°C ± 2°C (175 μmol m⁻² s⁻¹) under LD (6 h of light/8 h of dark) or SD (12 h of light/12 h of dark) conditions. For the expression study, leaves from first to last were harvested at the middle of the light period. For the diurnal expression study, the leaves of plants at the two- and three-leaf stages were harvested every 4 h for 2 d under LD and SD conditions, respectively.

**Phylogenetic Analysis of PEBP Genes**

Phylogenetic analysis using amino acid sequence alignment of the PEBP domain from PEBP genes in barley, rice (Oryza sativa), and Arabidopsis (Arabidopsis thaliana; Chardon and Damerval, 2005; Faure et al., 2007; this study) was conducted using ClustalX (http://www.qibmn.u-strasbg.fr/Bioinfo/ClustalX/Top.html; Thompson et al., 1997) and the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis for 1,000 replicates was performed to provide confidence estimates for the tree topologies using the neighbor-joining option in ClustalX. Results were displayed graphically using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

**Genetic Mapping of PEBP Genes**

HvFT1, HvFT2, HvFT3, HvTFL1, and HvMFT1 were mapped in the DH population from the F1 cross between Steptoe and Morex (North American Barley Genome Mapping Project; Kleinhofs et al., 1993). All genes except HvFT1 were mapped using the cleaved-amplified polymorphic sequence method. HvFT3 was mapped as a presence/absence polymorphism. The primers and experimental details are shown in Supplemental Table S1. Linkage map construction and QTL analyses were performed using MAPMAKER/EXP.

**Plasmid Construction and Rice Transformation**

Genomic sequences of HvFT1, HvFT3, and HvTFL1 and cDNA sequences of HvFT2 and HvMFT1 were amplified using Morex genomic DNA or Haruna-Nijo cDNA, respectively, as templates and specific gene primer pairs (Supplemental Table S2) and then cloned into the entry vector pKS221MCS (Wakasa et al., 2006). The LR reaction (Gateway System; Invitrogen) was carried out in order to transfer the inserts from the entry vector into the expression vector (pSTARH302GateA), which included the CaMV 35S promoter (H. Ichikawa, H. Nakamura, M. Hakata, Y. Nishizawa, and M. Kazikawa, unpublished data). The MCS from pKS221MCS was inserted into pSTARH302GateA to generate a control plasmid. The constructed plasmids were then introduced into Agrobacterium tumefaciens strain EHA101. Rice cv Nipponbare was used for Agrobacterium-mediated transformation (Toki, 1997). Plants regenerates from the transformed callus (R0) were selected on MS solid medium (Murashige and Skoog, 1962) containing 50 mg L⁻¹ hygromycin at 27°C under LD conditions at 40 μmol m⁻² s⁻¹. Regenerated plants were grown in a growth chamber or greenhouse for further experiments.
Growth Conditions for Transgenic Rice Plants

Twenty rice plants transformed with expression vectors containing HvFT1, HvFT2, HvMFT1, or a control vector (mock) were transplanted to soil in a growth chamber under SD conditions with a 28°C day and a 25°C night (9 h of light/15 h of dark; 270 μmol m⁻² s⁻¹) or under LD conditions in a greenhouse with a 28°C day and a 24°C night under natural light from the end of May until the end of August (approximately 14 h of light/10 h of dark). Twenty rice plants transformed with the expression vectors for HvFT1, HvFT3, HvTFL1, or the control vector (mock) were transplanted to soil in a growth chamber under LD conditions with a 28°C day and a 25°C night (13.5 h of light/10.5 h of dark; 270 μmol m⁻² s⁻¹) or in a greenhouse under SD conditions at 28°C under natural light from the beginning of August to the beginning of October (approximately 12.5 h of light/11.5 h of dark). The number of days from transplanting to the appearance of the first panicle was recorded.

Expression Analysis

Total RNA was extracted from leaves with the GenPure RNA Kit (Dojindo). 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). Real-time PCR was carried out using Mx3000P (Stratagene Products Division, Agilent Technologies) with Brilliant II SYBR Green PCR Master Mix (Stratagene) according to the manufacturer’s recommendations. A dilution series of pCR2.1-TOPO vectors (Invitrogen) containing the partial reaction solution using the TaKaRa RNA PCR kit (AMV) version 3.0 (Takara Bio). Light/15 h of dark; 270 μmol m⁻² s⁻¹ for the amplified with each specific primer pair (Supplemental Table S2). The value used to generate the standard curve. The barley luted series of pCR2.1-TOPO vectors (Invitrogen) containing the partial genomic and expression compendium of the expanded PEBP gene family in cereals. J Mol Evol 61: 579–590

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