

A wheat homologue of *PHYTOCLOCK 1* is a candidate gene conferring the early heading phenotype to einkorn wheat

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An X-ray mutant showing an early flowering phenotype has been identified in einkorn wheat (*Triticum monococcum* L.), for which a major QTL for heading time was previously mapped in the telomeric region on the long arm of chromosome 3A. Recent advances in Triticeae genomics revealed that the gene order in this region is highly conserved between wheat and barley. Thus, we adopted a hypothetical gene order in barley, the so-called GenomeZipper, to develop DNA markers for fine mapping the target gene in wheat. We identified three genes tightly linked to the early heading phenotype. PCR analysis revealed that early-flowering is associated with the deletion of two genes in the mutant. Of the two deleted genes, one is an ortholog of the *LUX ARRHYTHMO* (*LUX*)/*PHYTOCLOCK 1* (*PCL1*) gene found in *Arabidopsis*, which regulates the circadian clock and flowering time. We found distorted expression patterns of two clock genes (*TOC1* and *LHY*) in the einkorn *pcl1* deletion mutant as was reported for the *Arabidopsis lux* mutant. Transcript accumulation levels of photoperiod-response related genes, a photoperiod sensitivity gene (*Ppd-1*) and two wheat *CONSTANS*-like genes (*WCO1* and *TaHd1*), were significantly higher in the einkorn wheat mutant. In addition, transcripts of the wheat florigen gene (*WFT*) accumulated temporally under short-day conditions in the einkorn wheat mutant. These results suggest that deletion of *WPCL1* leads to abnormally higher expression of *Ppd-1*, resulting in the accumulation of *WFT* transcripts that triggers flowering even under short-day conditions. Our observations from gene mapping, gene deletions, and expression levels of flowering related genes strongly suggest that *WPCL1* is the most likely candidate gene for controlling the early flowering phenotype in the einkorn wheat mutant.

Key words: circadian clock, einkorn wheat, flowering, *Ppd-1*, *PHYTOCLOCK 1*

INTRODUCTION

The timing of flowering is one of the most important traits for local adaptation and is controlled by environmental cues such as temperature and day-length (Izawa, 2007; Andrés and Coupland, 2012). *Arabidopsis thaliana* is a long-day (LD) plant species, whose photoperiod response is well characterized (McClung, 2011; Andrés and Coupland, 2012). The vast majority of defective mutations in circadian clock genes affect flowering time, suggesting that the circadian clock is tightly associated with photoperiodic control of flowering time (summarized in Niwa et al., 2009). In *Arabidopsis*, the circadian clock

is comprised of three main feedback loops: the morning, central, and evening loops (McClung, 2011). In the central loop, two morning-expressed Myb transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) act as negative regulators of evening-expressed *TIMING OF CAB EXPRESSION* (*TOC1*) (Wang and Tobin, 1998; Schaffer et al., 1998; Strayer et al., 2000). In combination with other clock genes such as *LUX ARRHYTHMO*/PHYTOCLOCK 1 (*LUX*/*PCL1*), accumulated TOC1 proteins positively regulate the expression of CCA1 and LHY for the next loop (Alabadi et al., 2001). In the morning loop, three PSEUDO-RESPONSE REGULATORS (PRRs), PRR5, PRR7, and PRR9, act as repressors of CCA1 and LHY (Farré et al., 2005; Nakamichi et al., 2010). In the evening loop, *GIGANTEA* (*GI*) is repressed by TOC1,

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whereas GI promotes *TOC1* expression. Outputs from the circadian clock control expression of the *CONSTANS* (*CO*) gene, an activator of florigen gene *FLOWERING LOCUS T* (*FT*) expression in *Arabidopsis* (Imaizumi and Kay, 2006). The circadian clock and photoperiod genes in *Arabidopsis* are well conserved in other LD plants such as the temperate cereals, wheat and barley (Higgins et al., 2010). Thus, information accumulated from *Arabidopsis* research helps promote enhanced understanding of the circadian clock and photoperiod response in temperate cereals.

In temperate cereals, the timing of heading and therefore flowering is generally controlled by three factors: i.e., vernalization requirement, photoperiod sensitivity and narrow-sense earliness (earliness *per se*) (Worland and Snape, 2001). In wheat and barley, photoperiod (day length) sensitivity is controlled by the Photoperiod-1 (*Ppd-1*) gene that encodes a protein with sequence similarity to *Arabidopsis* PRR7 (Turner et al., 2005; Beales et al., 2007). A semi-dominant allele of *Ppd-1* (*Ppd-1a*), which causes early flowering under both LD and SD conditions, was identified in all tested accessions of common wheat (Beales et al., 2007; Wilhelm et al., 2009; Shaw et al., 2012). Common wheat has three homoeologous loci, *Ppd-A1a*, *Ppd-B1a*, and *Ppd-D1a* in the A, B, and D genomes, respectively (Laurie, 1997). Deletions in the promoter regions of *Ppd-A1* (1027 bp or 1117 bp) and *Ppd-D1* (2089 bp) were associated with photoperiod insensitivity (Beales et al., 2007; Wilhelm et al., 2009). Photoperiod insensitive alleles at *Ppd-A1a* and *Ppd-D1a* share a common 886 bp region whose functional importance was proposed. In contrast, there have not been any identified mutations associated with photoperiod insensitivity in the *Ppd-B1a* gene (Díaz et al., 2012). Photoperiod insensitivity by structurally intact *Ppd-1Ba* alleles is presumed to be due to increased copy number and altered expression. Recently, Nishida et al. (2013) reported novel mutations found in the 5' upstream regions of *Ppd-A1* and *Ppd-B1*. Wheat plants with at least one semi-dominant *Ppd-1a* allele were associated with an increased expression level of wheat *FT*, which well accounts for the early flowering (Shaw et al., 2012). Also in the vernalization pathway, the *FT* gene plays an important role in the transition from vegetative to reproductive phases (Yan et al., 2006). A vernalization requirement is determined by the *VRN1*, *VRN2* and *VRN3* genes in wheat and barley (Takahashi and Yasuda, 1971; Murai et al., 2003; Yan et al., 2003, 2004, 2006). Wheat *FT* (*WFT*) is identical to the *VRN3* gene, and its overexpression in winter wheat results in a spring growth habit (Yan et al., 2006; Li and Dubcovsky, 2008). Since *WFT* functions as an integrator of the vernalization and photoperiod pathways (Hemming et al., 2008), heading time is strongly associated with *WFT* expression levels in wheat.

An X-ray mutant line showing an early heading pheno-

type was identified in einkorn wheat, *Triticum monococcum* L. (Yamashita, 1952; Shindo and Sasakuma, 2001). QTL analysis using recombinant inbred lines (RILs) derived from a cross between the early mutant and *T. boeoticum* Boiss. indicated a single major QTL for early heading in field conditions that mapped to the telomeric region of the long arm of chromosome 3A (Shindo et al., 2002; Hori et al., 2007; Gawronski and Schnurbusch, 2012). The QTL for narrow-sense earliness was also mapped to this region by QTL analysis using the same RILs (Shindo et al., 2002). Recently, advances in barley and wheat genomics allow fine mapping using the synteny among grass species (Brenchley et al., 2012; Mayer et al., 2012). The gene order within this region is highly conserved among grasses (Mayer et al., 2011). Based on the synteny among grasses, a hypothetical gene order (GenomeZipper) was established in barley (Mayer et al., 2011) that let us develop markers to the narrow target region in wheat (Iehisa et al., 2012). Using this strategy, we have developed genic markers in the 3AL region and have re-evaluated the genotype-phenotype association for early flowering (Mizuno et al., in preparation). A similar line of experiments was independently carried out by Gawronski and Schnurbusch (2012) showing that the causal gene could be mapped within a 0.9 cM interval. Here, we report identification of the candidate gene for early heading by synteny-based mapping. Moreover, the expression of clock genes and clock output genes was analyzed to support the hypothesis that the putative circadian clock gene is involved in the mechanism of early flowering. Our findings indicate that deletion of the clock gene is the most likely cause of early flowering, and that the clock gene has an important role in the upstream regulation of the heading.

MATERIALS AND METHODS

Plant materials *T. monococcum* L. accession KT3-5 is an X-ray mutant of KT3-1 (Shindo and Sasakuma, 2001). Ninety three recombinant inbred lines (F_{11}) that were derived from a cross between cultivated einkorn wheat, *T. monococcum* L. (KT3-5), and wild einkorn wheat, *T. boeoticum* Boiss. (KT1-1), were used for mapping and phenotypic evaluation (RILWA1; Shindo et al., 2002). These lines were provided by the National BioResource Project-Wheat, Japan.

Marker development and fine mapping Total DNA was extracted from the leaves of RILWA using a DNeasy Plant Mini Kit (Qiagen, Germany). We developed gene-based markers in the target region on 3AL according to the barley hypothetical gene order (GenomeZipper; <http://mips.helmholtz-muenchen.de/plant/barley/gz/index.jsp>; Mayer et al., 2011). The barley, rice, or *Brachypodium* gene sequences were used as the query for blastn search

against the wheat genome database (CerealsDB: http://www.cerealsdb.uk.net/CerealsDB/Documents/DOC_CerealsDB.php; Brechley et al., 2012) that contains assemblies of 454 survey sequences with 5X genome equivalents. Based on the blastn results, PCR primers were designed to amplify the region including introns and/or untranslated regions (UTR) (Table 1). To detect the polymorphisms between accessions KT3-5 and KT1-1, PCR and PCR-RFLP analyses were performed. Thirty-two cycles of PCR were performed using KAPATaq Extra (KAPA BIOSYSTEMS, USA) and following reaction conditions: 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. The PCR products without polymorphisms between KT1-1 and KT3-5 were digested by five 4-bp-cutting restriction enzymes (Table 1). The polymorphic markers were integrated into the linkage map (Shindo et al., 2002; Hori et al., 2007). The genetic map was constructed using MAP-MAKER/EXP version 3.0b software (Lander et al., 1987), and map distances were calculated using the Kosambi

function (Kosambi, 1944). The genotype and heading time data of RILWA1 were published by Hori et al. (2007).

Isolation and phylogenetic analysis of the *WPCL1* gene A contig sequence with similarity to *PCL1* was identified by a blastn search against the CerealsDB. Based on the contig sequence, we designed primer pairs to amplify the entire *PCL1* coding sequences in einkorn wheat: 5'-TCTAT CCACC ATGTG CAAG AAT-3' and 5'-TCCTC CTCTC GTCAG CTACA-3'. Amplification conditions were as described above. Amplified PCR fragments were cloned using a TOPO TA Cloning kit (Invitrogen, USA) according to the manufacturer's instructions. Nucleotide sequences were determined with BigDye Terminator version 3.1 (Applied Biosystems, USA) using an Applied Biosystems 3730xl DNA Analyzer. Nucleotide sequences and their predicted amino acid sequences were analyzed by GENETYX-MAC version 12.00 software (Whitehead Institute for Biomedical Research, USA).

Table 1. Markers, their primers and restriction enzymes used for linkage map construction

Marker	Forward sequence (5'-3')	Reverse sequence (5'-3')	Restriction enzyme
GZ3243	CGGCTGCTTCATCCTAGGTC	TGCTGCAACTAAGAGTGATGA	<i>HpaII</i>
GZ3248	TTCTCCACCGTCGAGGACTT	AGTCGGCTCCAACATTCAACT	–
GZ3250	TCATTTTGGATCATCAAAGCAAGT	GGTAACGAGATATTGAATTG	<i>TaqI</i>
GZ3251	ATCGCCATGGGGCCAGAG	TCACGACGCGGTAGACCAG	<i>TaqI</i>
GZ3252	ATCATGGTGCAATGGCCAGA	GCAGGCAACCACGAAAACAG	–
GZ3255	TACAACGGCGCTTCATCTCA	TAGACAGACTCCGCCTTGTT	<i>AluI</i>
GZ3256	ATGTAGGAGGCAGTTGTCCG	TGTGGGCAAACCTAAGACCAGG	<i>MboI</i>
GZ3259	TCTGCAGTTCATGGCCTCTG	TCTGCTGATTTTGTAAAGCAGGT	–
GZ3260	GAGGGTGACGGAGTGGGAGA	CTTCACTTCAGCTCCCCTTGT	–
GZ3261	CAACGCATCACATGCCCATC	GCTCAATTTCTTCGGGCAGC	–
GZ3262	TGGAGAGATTTAGTTGTGTT	GGTAACGAGATATTGAATTG	<i>TaqI</i>
GZ3263	TCCACCAGGACAAGGCCTTC	ACGGCATTGTAAGAAAGCCTTCAG	<i>HhaI</i>
GZ3267	GGTCGAAATGGGCCACGA	GAGGATTGATGTGGCAAGC	<i>HpaII</i>
GZ3287	ACCCGTGGCAGACCTG	AGACGACGGAGGAGTAGAGC	<i>TaqI</i>

Table 2. Primers used for gene expression analyses

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Reference
<i>WPCL1</i>	TCTCCTCCTACCCGCACTAC	GGATGGATCATGGACTGACTGA	this study
<i>GZ3261</i>	GCCATTACATCTGCCACAGG	CGCCTTGCCATCGGAAATAA	this study
<i>TOC1</i>	GAGGATAGCTGAGTCATCTGC	GTCGGTCGCACCAGAGAAAT	AK333193
<i>LHY</i>	ACGGCAGTTGCTCAGAAGTT	TCCTTCCCCACATCAATGGAA	HQ222606
<i>Ppd-1</i>	GCCTTGTCTGCTACTGCCC	GCATAGCACAATCAGCAGTACG	AB691782
<i>WCO1</i>	GCACCACTTGTAGGGGCAGA	TTGATCCTTGGCCGTGCTT	Shimada et al., 2009
<i>TaHd1</i>	GCAATGACATATCTTTCTCATCAA	AGAGCTCCATGGCTTCACT	AB094490
<i>GI</i>	TCTGGATTGCTCGAGATGAC	AAGCTTCACCGTCGACAA	Shaw et al., 2012
<i>WFT</i>	GGTACAACCTGGTGCCTCGTT	CGAGCTGCTGGAAGAGC	Shaw et al., 2012
<i>Actin</i>	GGCTGGTTTTGCTGGTGACGAAT	AATGAAGGAAGGCTGGAAGAGGA	Mizuno et al., 2010.

Amino acid sequences of PCL1 proteins and their homologous GARP proteins were aligned by ClustalW in MEGA5 (Tamura et al., 2011). Accession numbers of the proteins used in phylogenetic analysis are indicated in Fig. 4. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) in the MEGA5 package. The nucleotide sequences of *T. boeoticum* and *T. monococcum* *PCL1* genes have been deposited in the DDBJ database under the accession numbers AB773825 and AB773826, respectively.

Quantitative reverse-transcriptase (RT)-PCR
KT3-1 and KT3-5 plants were grown at 23°C under SD (9 h light and 15 h dark) conditions. Two-week-old leaves

were sampled every 3 h for a 24 h period. Total RNA was isolated using an RNeasy Plant Mini kit (Qiagen, Germany) with DNaseI treatment. First-strand cDNA was synthesized from 1 µg of the RNA sample in a 20 µL reaction solution with oligo-dT primers using a ReverTra Ace -α- kit (TOYOBO, Japan). Primers used for quantitative RT-PCR are shown in Table 2. The wheat *TOC1* sequence was not annotated in the NCBI database. Instead, barley *PRR1/TOC1* (accession number; JN603243) was used as the query sequence for a blastn search against a wheat EST database (<http://www.shigen.nig.ac.jp/wheat/komugi/>). The blastn search provided a full length cDNA sequence (AK333193) homologous to barley *PRR1*. Based on the cDNA sequences, primers were designed for quan-

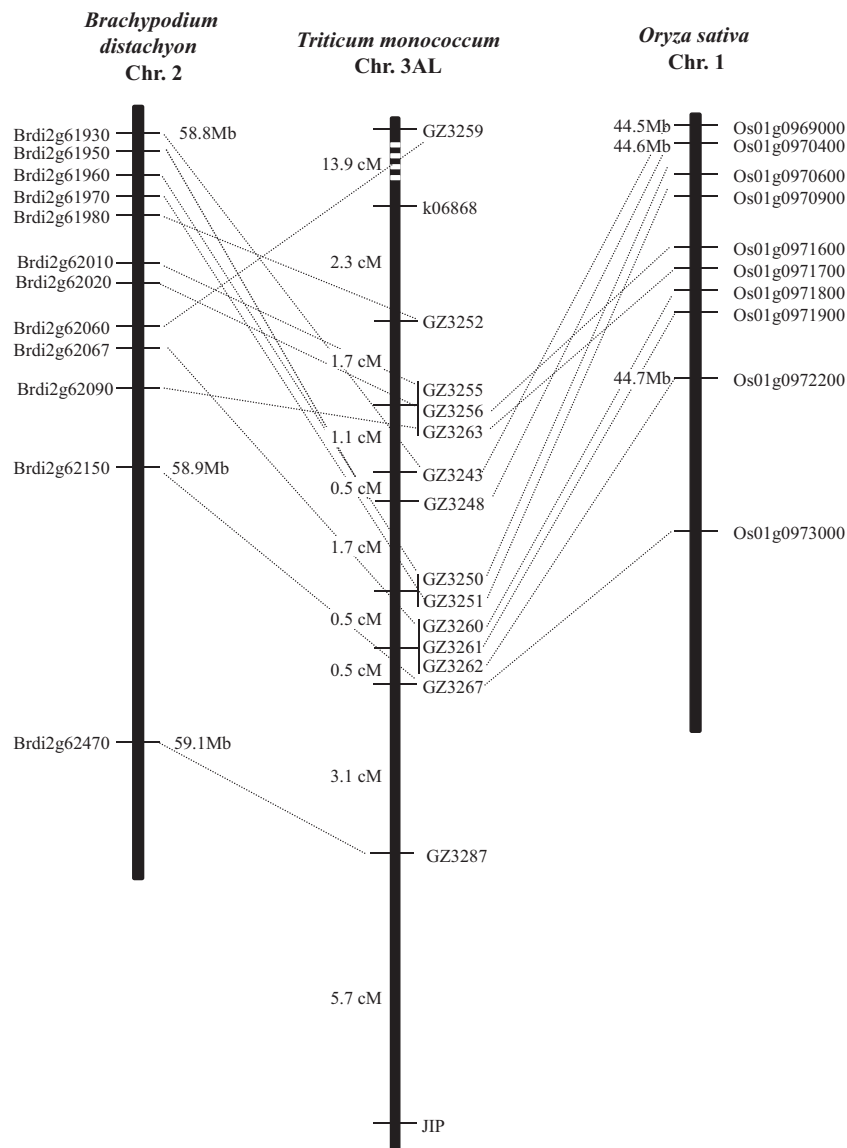


Fig. 1. Linkage map of the region between markers k068068 and JIP on the long arm of chromosome 3A of einkorn wheat. Gene order of einkorn wheat 3AL was compared to the corresponding chromosomal regions on chromosome 1 of rice and chromosome 2 of *Brachypodium distachyon*. Rice and *Brachypodium* genes homologous to wheat markers are connected by dashed lines.

titative RT-PCR of wheat *TOC1*. Relative transcript accumulation levels were examined using a LightCycler Nano System with a FastStart Essential DNA Green Master kit (Roche Diagnostics, Germany). Quantitative RT-PCR was performed according to the manufacturer's protocol. The *Actin* gene (AB181991) was used as the internal control (Mizuno et al., 2010).

RESULTS

Fine mapping and identification of the candidate genes The QTL for heading time was previously found between the k06868 and JIP markers (Hori et al., 2007). The LOD score at the peak of the QTL was 21.5 and the explained phenotypic variance was 68.2%. We developed markers between k06868 and JIP by referring to GenomeZipper and CerealsDB. Based on the homologous contig sequences, primers were designed within introns or UTRs to maximize the chance of detecting polymorphisms. We detected polymorphisms between KT3-5 and KT1-1 in five markers (GZ3248, GZ3252, GZ3259, GZ3260 and GZ3261) without any restriction digestion. To obtain the polymorphisms of the other markers, PCR-RFLP analysis was then performed using five 4-bp-cutting restriction enzymes. PCR-RFLP analysis yielded nine polymorphic markers. In total, 14 polymorphic markers were mapped to the target region on the 3AL chromosome arm (Fig. 1). Comparisons of gene order between our map and those of other grass species showed that, overall, the gene orders in this chromosomal region are highly conserved with some exceptions (GZ3252, GZ3255, GZ3256, GZ3259 and GZ3263). The genetic distance between GZ3252 and GZ3287 were 9.1 cM, corresponding to about 300 kb in physical distance in the *Brachypodium distachyon* and *Oryza sativa* genomes.

Next, we searched for flowering-related genes previously reported within the mapped region based on genomic information from *Brachypodium* and rice. We found that Bradi2g62067 and Os01g0971800 were homologous to a flowering related-gene in *Arabidopsis* named *LUX ARRHYTHMO* (*LUX*) /*PHYTOCLOCK 1* (*PCL1*). The gene corresponds to the GZ3260 marker in einkorn wheat (Fig. 1), which exhibited dominant inheritance (present in KT1-1, and absent in KT3-5) in the RIL population. *Arabidopsis LUX* encodes a Myb transcription factor belonging to the GARP family, and the *Arabidopsis lux* mutant has an aberrant circadian rhythm and flowers early especially in SD conditions (Hazen et al., 2005; Onai and Ishiura, 2005). To test whether the genotype of the wheat *LUX/PCL1* homolog is associated with the early heading phenotype, we selected seven RILs with recombination sites in the vicinity of the GZ3260 locus. Of the seven recombinants, four RILs with the KT3-5 allele on the GZ3260 locus showed an early heading phenotype (Fig. 2A). In contrast, three RILs with the KT1-

1 allele exhibited the late heading phenotype. Next, we compared the heading time of two subpopulations grouped according to their genotypes at the GZ3260 loci in the RIL population (88 RILs were compared because the data for heading time was missing in five RILs). Of the 88 RILs, 61 RILs with the KT3-5 allele flowered significantly earlier than 27 RILs with the KT1-1 allele (*t*-test, $p < 0.001$) (Fig. 2B). Two genic markers, GZ3260 and GZ3261, cosegregated in the RIL population and are dominant markers present in KT1-1 and absent in KT3-5 (Fig. 3). The rice counterparts are 10 kb apart. We presumed the two marker genes were deleted by X-ray irradiation through the course of mutagenesis (Yamashita, 1952). We confirmed this hypothesis by PCR analysis of KT3-1, a parental line of KT3-5, using multiple primer pairs designed from the GZ3252, GZ3260, GZ3261, and GZ3262 sequences. The wild type accession, KT3-1, produced amplicons with all tested primer combinations, indicating that these sequences are present in the wild type. PCR products of the GZ3252 and GZ3262 loci were found in all lines (Fig. 3). By contrast, no PCR products were obtained with any tested primer combination (three in GZ3260 and two in GZ3261) for the GZ3260 and

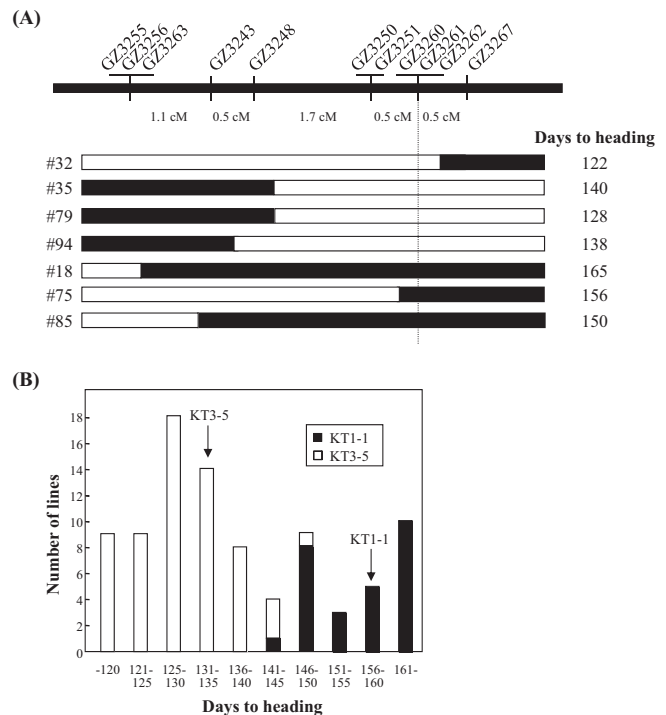


Fig. 2. Comparison of heading time between RILs with KT1-1 and KT3-5 alleles at the six loci surrounding the GZ3260 locus. (A) Graphical genotypes and days to heading of seven RILs with recombination near the GZ3260 locus. Black and white boxes indicate the KT1-1 and KT3-5 alleles, respectively. (B) Frequency distribution of days to heading in the 88 RILs. Black and white boxes indicate the RILs with KT1-1 or KT3-5 alleles at the GZ3260 locus. The heading time data of RILs and their parents is according to Hori et al. (2007).

GZ3261 sequences in the mutant KT3-5, indicating that the chromosomal region including GZ3260 and GZ3261 was deleted. The rice homologue of GZ3261 and GZ3262 are Os01g0971900 and Os01g0972200 (Fig. 1) that encode a putative Pumilio family RNA-binding protein and a

Zinc transporter 2 (ZRT/IRT-like protein 2), respectively, whose functions in flowering have not been elucidated.

Isolation and expression pattern of *WPCL1* We isolated the genomic sequence carrying the GZ3260 marker in einkorn wheat by PCR amplification with primers designed based on the contig sequence found in CerealsDB. The *GZ3260* gene consisted of a single exon, and the deduced amino acid sequence was 285 residues in length in *T. boeoticum* strain KT1-1 and *T. monococcum* strain KT3-1. There were only two non-synonymous mutations between KT1-1 and KT3-1. A phylogenetic tree showed that GZ3260 belonged to the same cluster as rice PCL1 and *Arabidopsis* LUX, separate from the other GARP-type Myb transcription factors (Fig. 4). Thus, we named the GZ3260 gene *WPCL1*. We studied the expression patterns of *WPCL1* in a time-course experiment under SD and LD conditions by quantitative RT-PCR. Expression analysis revealed that expression of the *WPCL1* gene peaked in the evening and had a diurnal expression pattern under LD and SD conditions as reported for *Arabidopsis* (Hazen et al., 2005). By comparison, the other gene deleted in KT3-5, *GZ3261*, did not have a clear diurnal expression pattern in either the SD or LD conditions (Fig. 5).

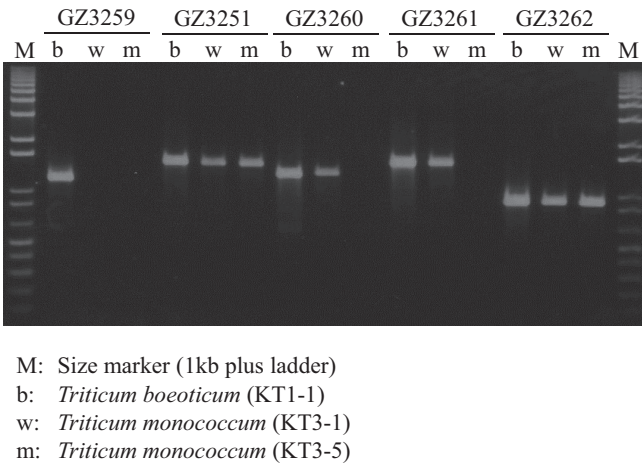


Fig. 3. An example of PCR analysis using the markers mapped surrounding the GZ3260 locus. PCR amplification of GZ3260 and GZ3261 markers were not detected in the early mutant (KT3-5). The 1 kb plus DNA ladder (Invitrogen, USA) was used as a molecular marker (denoted “M” on the top).

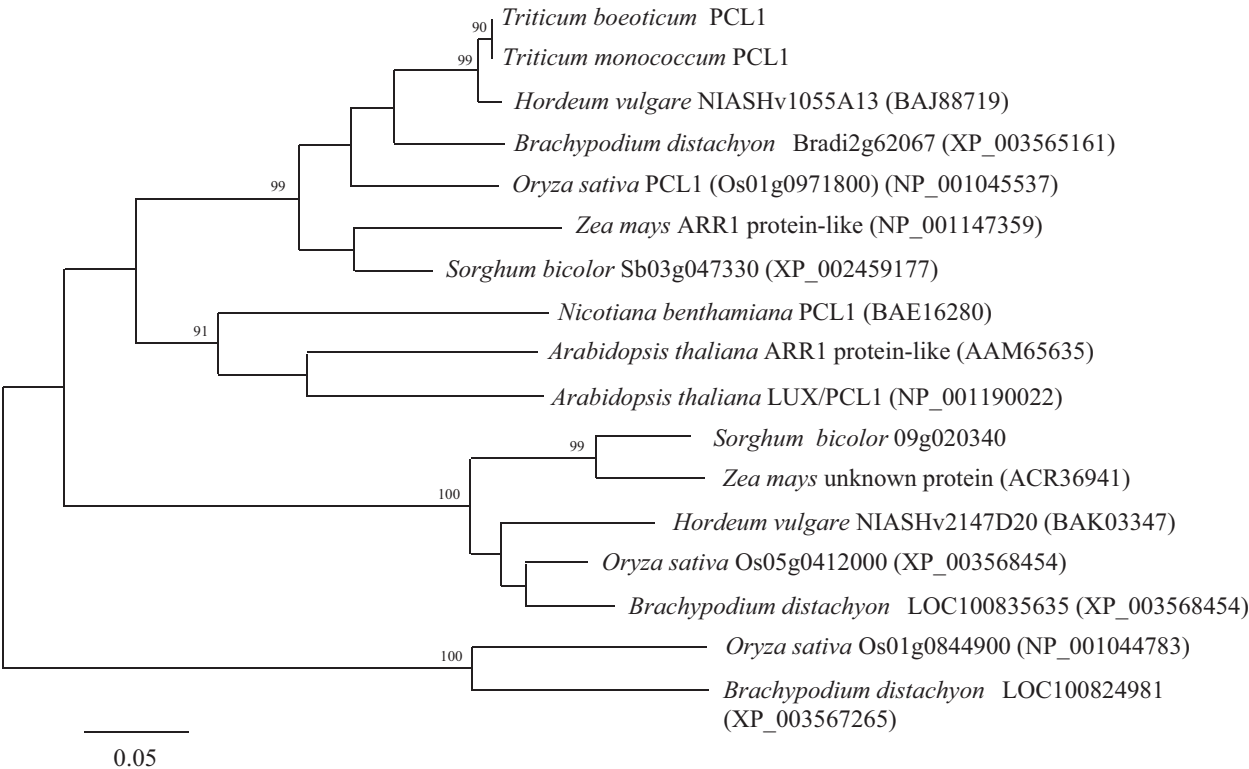


Fig. 4. A phylogenetic tree based on the amino acid sequences of LUX/PCL1 proteins and their homologous GARP proteins. The tree was constructed using the neighbor-joining method. Bootstrap values (percentage) based on 1000 replicates are shown on the branches. Species names, protein names (if available), and accession numbers of the proteins used in phylogenetic analysis are indicated to the right of branches.

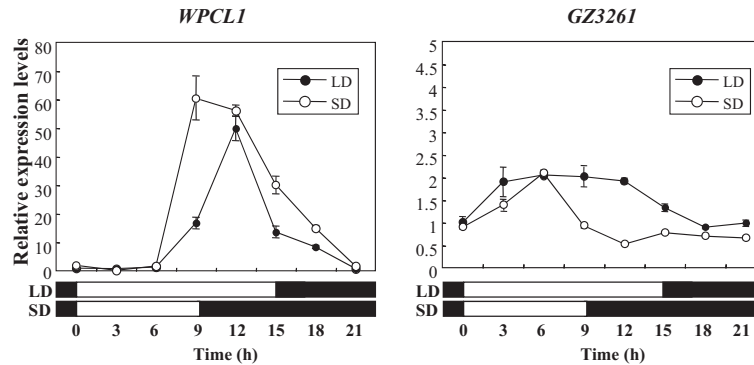


Fig. 5. Gene expression profiles of *WPCL1* and *GZ3261* genes during long-day (LD) and short-day (SD) conditions. White and black boxes indicate light and dark periods, respectively. The 2-week-old seedlings were sampled at 3 h intervals over 24 h. Means $\pm$ standard deviations were calculated from data in three repeated experiments. The *Actin* gene (AB181991) was used as an internal control.

Expression profiles of clock and flowering related genes in the *WPCL1* deletion mutant under SD conditions

The circadian clock genes of the *Arabidopsis lux* mutant have aberrant expression patterns (Hazen et al., 2005). In wild-type einkorn wheat (KT3-1), the expression level of wheat *TOC1* peaked at the beginning of night as reported for *Arabidopsis* (Hazen et al., 2005) and barley (Faure et al., 2012) (Fig. 6). In the mutant (KT3-5), transcript accumulation levels of *TOC1* and *GIGANTEA* (*GI*) began to increase earlier than in KT3-1. Expression of wheat *LHY* in KT3-5 was significantly lower at the peak of expression (beginning of the day) and the circadian expression rhythm seemed to be disordered. Next, we examined the expression pattern of the clock output genes. The transcript accumulation patterns of *Ppd-1* in KT3-5 and KT3-1 were different; KT3-5 had constitutively higher expression than KT3-1. Both lines exhibited a diurnal expression pattern; however, the peak expression levels in KT3-5 proceeded those of KT3-1 by 3 h. In wheat, two *CONSTANS*-like genes, *WCO1* and *TaHd1*, were identified that are downstream of *Ppd-1* in the flowering signaling network. (Nemoto et al., 2003; Shimada et al., 2009). In KT3-5, *WCO1* and *TaHd1* were significantly up-regulated compared with their expression in KT3-1. Unlike *WCO1*, *TaHd1* was highly repressed with no detectable diurnal expression pattern in KT3-1. Expression levels of the wheat florigen gene (*WFT*) were repressed in wild type KT3-1 in SD conditions. Expression of *WFT* in KT3-5 exhibited a single peak 3 h after the end of the day period.

DISCUSSION

The deletion of *WPCL1* is strongly associated with the early heading phenotype The X-ray mutant (KT3-5) of einkorn wheat had an early flowering phenotype (Shindo and Sasakuma, 2001). A QTL was previ-

ously mapped on the distal region of 3AL (Shindo et al., 2002; Hori et al., 2007; Gawronski and Schnurbusch, 2012). Our fine mapping revealed three candidate genes tightly linked with the early heading phenotype (Figs. 1 and 2). Of the three genes, two genes were deleted in the X-ray mutant KT3-5 (Fig. 3). One gene is an ortholog of *Arabidopsis LUX/PCL1* and the other gene encodes a Pumilio family RNA-binding protein. *Arabidopsis lux* mutants display an early flowering phenotype especially in SD conditions (Hazen et al., 2005). In the *Arabidopsis lux* mutant, *LHY* and *CCA1* were repressed, whereas *TOC1* was activated, indicating that *LUX* is involved in the regulation of the circadian clock. Our expression analysis showed that in the einkorn *WPCL1* deletion mutant *LHY* was repressed and *TOC1* was up-regulated when the transcript levels were compared to wild type at their peaks of expression (Fig. 6). This observation is consistent with what has been reported for the *Arabidopsis lux* mutant (Hazen et al., 2005). These findings strongly suggest that *WPCL1* functions in the circadian clock like *Arabidopsis LUX* and that deletion of *WPCL1* results in defective circadian rhythms. Another deleted gene, *GZ3261*, is homologous to a Pumilio RNA-binding protein that is evolutionarily conserved from yeast to mammals and plants (Spasov and Jurecic, 2002). Pumilio proteins bind to specific sequences in the 3' UTR of target mRNAs and function as translational repressors during development and differentiation (Wickens et al., 2002; de Moor et al., 2005). In *Arabidopsis*, several Pumilio RNA-binding protein genes (*APUM1*, *APUM2*, *APUM5* and *APUM6*) showed higher expression in shoot meristems, suggesting that these genes are required for active cell division (Abbasi et al., 2011). Indeed, the *apum23* mutant had shorter roots and a slower growth phenotype (Abbasi et al., 2010). In wheat, the Pumilio RNA-binding protein gene did not show a diurnal expression pattern in either the SD or LD conditions (Fig. 5). Therefore, we

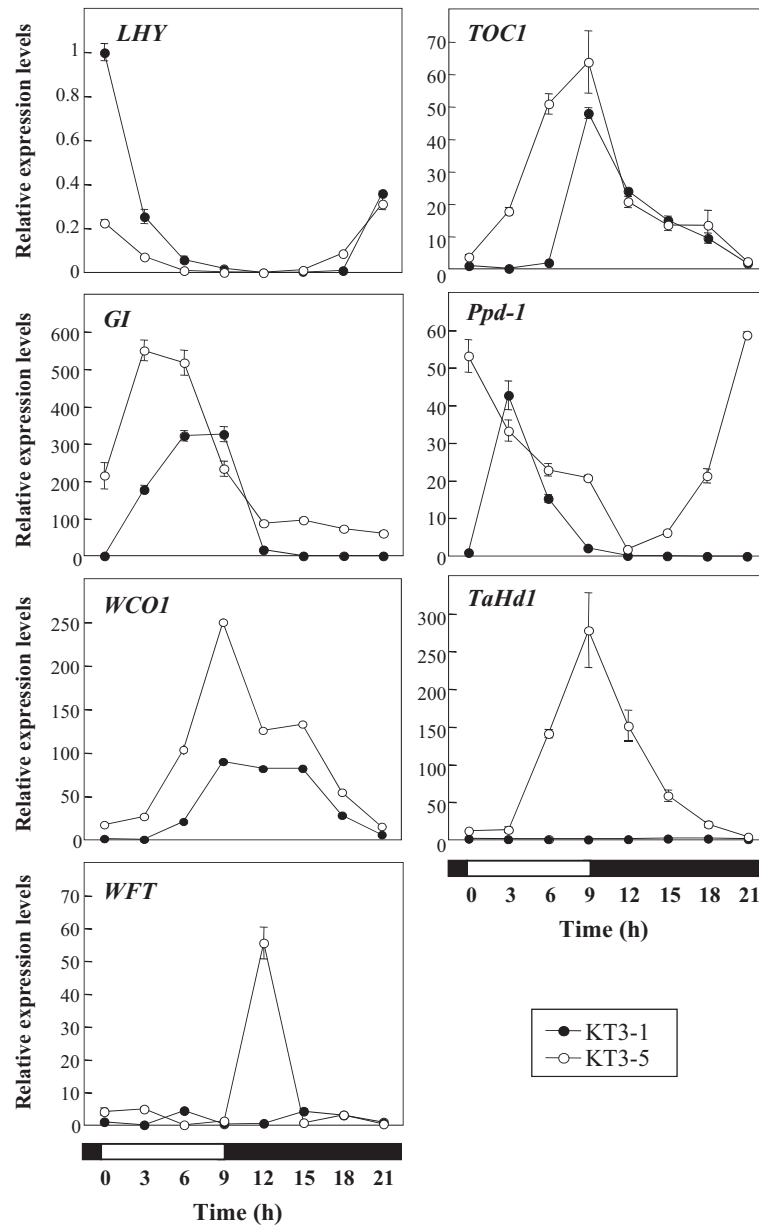


Fig. 6. Gene expression profiles of circadian clock and photoperiod response genes during SD condition. The transcript accumulation levels of *TIMING OF CAB EXPRESSION* (*TOC1*: AK333193), *LATE ELONGATED HYPOCOTYL* (*LHY*: HQ222606), *GIGANTEA* (*GI*: AF543844), *Photoperiod-1* (*Ppd-1*: AB691782), the wheat *CONSTANS*-like genes (*WCO1*: AB361064, and *TaHd1*: AB094490) and the wheat florigen gene (*WFT*: DQ890163) were compared between KT3-1 and KT3-5. White and black boxes indicate light and dark periods, respectively. The 2-week-old seedlings were sampled at 3 h intervals over 24 h. Means $\pm$ standard deviations were calculated from data in three repeated experiments. The *Actin* gene (AB181991) was used as an internal control.

presume that deletion of the Pumilio RNA-binding protein gene might not be associated with the early flowering phenotype in the einkorn wheat mutant, and that *WPCL1* is the most plausible candidate gene conferring the early heading phenotype to the einkorn wheat mutant KT3-5.

Recently, the barley *eam8* (an ortholog of *Arabidopsis* *ELF3*) mutant was reported to head early under both LD and SD conditions (Faure et al., 2012). The *eam8*

mutant also had altered expression of clock genes such as *HvCCA1* and *HvTOC1*. In *Arabidopsis*, LUX, ELF3 and ELF4 constitute the ELF4-ELF3-LUX complex, sometimes referred to as the “evening complex” (Nusinow et al., 2011). The genes encoding components of the evening complex shared similar diurnal expression patterns and their mutants had early flowering phenotypes (Hicks et al., 2001; Doyle et al., 2002; Hazen et al., 2005).

WPCL1 had a diurnal expression pattern similar to barley *ELF3* as well as *Arabidopsis* *LUX* and *ELF3* (Fig. 5). In addition, repression of *CCA1* or *LHY* and up-regulation of *TOC1*, with respect to their expression peaks, were commonly observed in the early flowering mutant KT3-5 and the barley *eam8* mutant. These findings also support the hypothesis that *WPCL1* is the causal gene for early heading in the early mutant.

WPCL1 regulates clock output genes and heading time In wheat and barley, the *Ppd-1* gene has been considered to be the main regulator of photoperiod sensitivity (Laurie, 1997). Wheat and barley *Ppd-1* show sequence similarity to *Arabidopsis* *PRR7* (Turner et al., 2005; Beales et al., 2007; Díaz et al., 2012), suggesting that *Ppd-1* functions in a similar manner in the circadian clock and acts upstream of *FT* and *CO* (Shimada et al., 2009). Wheat cultivars with a semi-dominant allele of *Ppd-1* (*Ppd-1a*) showed the mis-expression of *Ppd-1* that resulted in photoperiod insensitivity in wheat (Beales et al., 2007; Wilhelm et al., 2009; Díaz et al., 2012; Shaw et al., 2012). Our quantitative RT-PCR results revealed that transcript accumulation levels of *Ppd-1* were consistently higher and peaked earlier in the early mutant KT3-5 than in the wild-type KT3-1 (Fig. 6). Up-regulation of *Ppd-H1* was also observed in the barley *eam8* mutant, suggesting that barley *ELF3* affects the circadian clock by regulating *PRR* genes including *HvPpd-1* (Faure et al., 2012). In *Arabidopsis*, *LUX* protein binds to the *PRR9* promoter to repress transcription, whereas *LUX* did not bind to the promoter of *PRR7*, a homolog of *Ppd-1* (Helfer et al., 2011). Although it remains unclear whether *WPCL1* directly controls *Ppd-1* expression, our study revealed that deletion of *WPCL1* altered *Ppd-1* expression patterns in both the expression level and in the timing of peak expression. The abnormal expression of *Ppd-1* is likely to cause early heading in the einkorn wheat mutant.

In wheat, the altered expression of *Ppd-1* is associated with elevated expression of wheat *FT* (*WFT*), leading to early heading (Shaw et al., 2012). *FT* expression is promoted by *CO*, a key gene in the photoperiod pathway, in *Arabidopsis* (Putterill et al., 1995). In wheat, two *CO*-like genes, *WCO1* and *TaHd1*, were identified that are downstream of *Ppd-1* in the flowering signaling network (Nemoto et al., 2003; Shimada et al., 2009). *WCO1* negatively regulates the expression of *WFT* and *TaHd1* positively regulates the expression of *WFT*. In the einkorn wheat mutant, expression of *WCO1* and *TaHd1* were up-regulated under SD conditions (Fig. 6). In wild-type einkorn wheat, *WCO1* showed a diurnal expression pattern, whereas *TaHd1* did not. Unlike the SD conditions, *TaHd1*, as well as *WCO1*, transcripts were abundantly accumulated in wild-type einkorn wheat during the dark period under LD conditions (data not shown). This

result indicates that *TaHd1* functions in the photoperiod response under LD conditions in *T. monococcum*, although its expression is repressed under SD conditions. Compared to wild-type, *TaHd1* was highly up-regulated during the day under SD conditions. *CO* proteins are degraded in the dark and function only during light periods (Valverde et al., 2004). Moreover, *TaHd1* activates *WFT* under SD conditions in wheat, unlike *WCO1* (Kitagawa et al., 2012). Therefore, abundantly accumulated *TaHd1* transcripts during day may promote *WFT* expression under SD conditions in the early mutant. Alternatively, as suggested in previous studies (Shaw et al., 2012; Faure et al., 2012), increased expression of *CO* genes is not necessarily associated with the induction of *FT* in wheat and barley. If this is the case, *WFT* might be up-regulated independently of *WCO1* and *TaHd1* expression levels in einkorn wheat mutant. Regardless, the up-regulation of *WFT* under SD conditions can explain the early heading phenotype in the einkorn wheat mutant KT3-5.

Our results strongly suggest that a wheat *LUX* homolog *WPCL1* has crucial roles in regulation of heading time. We could indicate that *WPCL1* acted as the repressor for heading and flowering as *LUX/PCL1* did in *Arabidopsis*. Since our mapping was based on synteny among Gramineae species, we cannot rule out the possibility that other genes may be present within the critical chromosomal region spanning the deleted chromatin in the early mutant. Thus, it remains somewhat uncertain whether the deletion of *WPCL1* is the cause of the early-flowering phenotype. To test our hypothesis, further experiments such as knock-down and/or transformation of the most plausible candidate gene (*WPCL1*) should be conducted in near future. The control of heading time is one of the most important agronomic characters in wheat breeding. In this report, we have demonstrated the association of *WPCL1* deletion and the early flowering phenotype, and we propose that the *WPCL1* gene will be a good marker for finding early-heading wheat accessions. The flowering signal network in monocot species is similar to but different from that in the dicot model species *Arabidopsis* (Greenup et al., 2009; Higgins et al., 2010; Song et al., 2010). Our finding that *WPCL1* affects expression of *Ppd-1* will help understanding the mechanism of early heading in LD flowering monocot species.

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