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 HYOCOTYL (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 (Farre et al., 2005; Nakamichi et al., 2010). In the evening loop, GIGANTEA (GI) is repressed by TOC1,
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-na), 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 (Diaz 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 phenotype 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; Horii et al., 2007; Gawroński 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 Gawroński 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 (F13) 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 (RILWA; 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 DNasey 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
Early flowering wheat mutant lacking the *PCL1* gene

against the wheat genome database (CerealsDB: http://www.cerealsdb.uk.net/CerealsDB/Documents/DOC_CerealsDB.php; Brenchley 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 KAPA Taq 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 MAPMAKER/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

<table>
<thead>
<tr>
<th>Marker</th>
<th>Forward sequence (5′-3′)</th>
<th>Reverse sequence (5′-3′)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>GZ3243</td>
<td>CGGCTGTTCTACTCCTAGGTC</td>
<td>TGGTCAACTAAGAAGTGATGA</td>
<td>HpaII</td>
</tr>
<tr>
<td>GZ3248</td>
<td>TTCTCCACCGTCGAGACTTT</td>
<td>AGTCGGCTCACAATTAAGACT</td>
<td>–</td>
</tr>
<tr>
<td>GZ3250</td>
<td>TCATTTTTGCCATCAAAACAGGT</td>
<td>GGTACGAGATAATTGGAATT</td>
<td>TaqI</td>
</tr>
<tr>
<td>GZ3251</td>
<td>ATGCGCTGGGCGGCGAGAG</td>
<td>TCGAGCCGGTGAGCCAGCCAG</td>
<td>TaqI</td>
</tr>
<tr>
<td>GZ3252</td>
<td>ATCATTTGCCATGGGCGAGGA</td>
<td>GCGAGGCAACCGAAACACAG</td>
<td>–</td>
</tr>
<tr>
<td>GZ3255</td>
<td>TACAAAGGCCGCTTCATCCTCA</td>
<td>TAGACAGACTCCGCTTTGGT</td>
<td>AluI</td>
</tr>
<tr>
<td>GZ3256</td>
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<td>TGTCGGCAAACACAGCCAGG</td>
<td>MboI</td>
</tr>
<tr>
<td>GZ3259</td>
<td>TCTCGACTGCTGCTGCTGCTGCTG</td>
<td>TCTGTGATTTTTGAAACGGGG</td>
<td>–</td>
</tr>
<tr>
<td>GZ3260</td>
<td>GAGGGTGACGAGTGGGAGAGA</td>
<td>CCTCTCACTCAGCTCCCTGCT</td>
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</tr>
<tr>
<td>GZ3261</td>
<td>CAACGCATTGCATGCAGCCATC</td>
<td>GCTCAATTTCCTCGGACGC</td>
<td>–</td>
</tr>
<tr>
<td>GZ3262</td>
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<td>GGTACAGAGATTGGAATTG</td>
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<tr>
<td>GZ3263</td>
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<td>ACCGCGTATTGAAGAACGCGCTAC</td>
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<tr>
<td>GZ3267</td>
<td>GGTGCAAATGGGCGAGGA</td>
<td>GAGGTGTAAGTCGAGCAAGC</td>
<td>HpaII</td>
</tr>
<tr>
<td>GZ3287</td>
<td>ACCGCTGCCAGACACCTG</td>
<td>AGACGAGGGAGAGTAGAGG</td>
<td>TaqI</td>
</tr>
</tbody>
</table>

### Table 2. Primers used for gene expression analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence (5′-3′)</th>
<th>Reverse sequence (5′-3′)</th>
<th>Reference</th>
</tr>
</thead>
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<td><em>WPCL1</em></td>
<td>TCTCCCTCTACCCGCGCATAC</td>
<td>GGATGGATCATGGAAGCTAGTA</td>
<td>this study</td>
</tr>
<tr>
<td>GZ3261</td>
<td>GCCATTACATCTGGCCAGAG</td>
<td>CGCCTTGGCATCAGAAATAT</td>
<td>this study</td>
</tr>
<tr>
<td><em>TOC1</em></td>
<td>GAGGATAGCTGATCATGCTGCTG</td>
<td>GTCGCTGGCAGGCAAGAATAT</td>
<td>AK333193</td>
</tr>
<tr>
<td><em>LHY</em></td>
<td>AGGCGCGTGTGCGCAATGATT</td>
<td>TCTCCCTCCACATCAGTGGGTT</td>
<td>HQ222606</td>
</tr>
<tr>
<td><em>Ppd-1</em></td>
<td>GCCAGCTAGCTGTCTCAGCCA</td>
<td>GCATACGCAACATCAGTAGCAT</td>
<td>AB691782</td>
</tr>
<tr>
<td><em>WCO1</em></td>
<td>GAAACAGCTGCTGGCGGAGAG</td>
<td>TGTACGCTGCCTAGTTGCTT</td>
<td>Shimada et al., 2009</td>
</tr>
<tr>
<td><em>TuHd1</em></td>
<td>GCAATGACATGTCAGATCCATT</td>
<td>AGAAGCTTCCCAGTGCAGTT</td>
<td>AB094490</td>
</tr>
<tr>
<td><em>GI</em></td>
<td>TCTGTGATCTGGGAAGATGC</td>
<td>AAGCGTTCAGCTGCGAAAGC</td>
<td>Shaw et al., 2012</td>
</tr>
<tr>
<td><em>WFT</em></td>
<td>GGTACAGCTGCTGGCTCTGTT</td>
<td>CGAGCTGCTGGAAGAGC</td>
<td>Shaw et al., 2012</td>
</tr>
<tr>
<td><em>Actin</em></td>
<td>GGTGTTTTTTGCTGTGTCAGA</td>
<td>AATGAAGGGAAGGCTGGAAGAGA</td>
<td>Mizuno et al., 2010</td>
</tr>
</tbody>
</table>
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-
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 Genom-eZapper 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 LUXPCL1 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 generic 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
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).
Expression profiles of clock and flowering related genes in the WPCLI 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 (WPT) 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 WPCLI 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 previously mapped on the distal region of 3AL (Shindo et al., 2002; Horii et al., 2007; Gawroński 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/PCLI 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 WPCLI 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 WPCLI functions in the circadian clock like Arabidopsis LUX and that deletion of WPCLI 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 (Spassov 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
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 HcCCA1 and HcTOC1. 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; Diaz 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; Diaz 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 HcPpd-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 regulator of flowering, 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. monoccum, 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 acts as the repressor for heading and flowering as LUXPCL1 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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