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Identification of the causal mutation in early heading mutant of bread wheat (Triticumaestivum L.) using MutMap approach

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Abstract

In bread wheat (*Triticum aestivum* L.), fine-tuning the heading time is essential to maximize grain yield. *Photoperiod-1* (*Ppd-1*) and *VERNALIZATION 1* (*Vrn-1*) are major genes affecting photoperiod sensitivity and vernalization requirements, respectively. These genes have predominantly governed heading timing. However, *Ppd-1* and *Vrn-1* significantly impact heading dates, necessitating another gene that can slightly modify heading dates for fine-tuning. In this study, we developed an early heading mutant from the ethyl methanesulfonate-mutagenized population of the Japanese winter wheat cultivar “Kitahonami.” MutMap analysis identified a nonsense mutation in the clock component gene *Wheat PHYTOCLOCK 1/LUX ARRHYTHMO* (*WPCL-D1*) as the probable SNP responsible for the early heading mutant on chromosome 3D. Segregation analysis using F_2 and F_3 populations confirmed that plants carrying the *wpcl-D1* allele headed significantly earlier than those with the functional *WPCL-D1*. The early heading mutant exhibited increased expression levels of *Ppd-1* and circadian clock genes, such as *WPCL1* and *LATE ELONGATED HYPOCOTYL* (*LHY*). Notably, the transcript accumulation levels of *Ppd-A1* and *Ppd-D1* were influenced by the copy number of the functional *WPCL1* gene. These results suggest that a loss-of-function mutation in *WPCL-D1* is the causal mutation for the early heading phenotype. Adjusting the functional copy number of *WPCL1* will be beneficial in fine-tuning of heading dates.

Introduction

The optimal heading and subsequent flowering time in bread wheat (*Triticum aestivum* L.) is a critical trait for ensuring stable grain production. Fine-tuning heading/floweirng timing is essential to mitigate potential yield reductions caused by climate change and maximize grain yield (Snowdon et al. 2020; Sheehan and Bentley 2021). In wheat, heading/flowering is primarily controlled by three main pathways: vernalization, photoperiod, and earliness *per se* (*Eps*) (Kato and Shunji 1991; Hyles et al. 2020; Fernández-Calleja et al. 2021; Cao et al. 2021). Vernalization is mainly regulated by specific genes, including *VERNALIZATION 1* (*Vrn-1*), *VERNALIZATION 2* (*Vrn-2*), and *Wheat FLOWERING LOCUS T* (*WFT*) (Yan et al. 2003; Distelfeld et al. 2009; Shimada et al. 2009; Chen and Dubcovsky 2012). These genes determine the need for prolonged exposure to cold conditions. A dominant allele of *Vrn-1* or the presence of recessive loss-of-function alleles of *Vrn-2* eliminates the vernalization requirement, resulting in a spring growth habit (Yan et al. 2004; Hyles et al. 2020). In addition to vernalization, long-day (LD) conditions accelerate wheat heading/flowering.

Photoperiod sensitivity is primarily controlled by *Photoperiod-1* (*Ppd-1*) in wheat (Beales et al. 2007; Seki et al. 2011; Shaw et al. 2012; Seki et al. 2013; Shaw et al. 2013). *Ppd-1* acts as a positive regulator of *WFT* and exhibits two types of alleles with distinct photoperiodic sensitivity. Photoperiod-sensitive alleles (*Ppd-A1b*, *Ppd-B1b*, and *Ppd-D1b*) exhibit a diurnal expression pattern, promoting heading under LD conditions (Shaw et al. 2013; Shi et al. 2019). In contrast, photoperiod-insensitive alleles (*Ppd-A1a*, *Ppd-B1a*, and *Ppd-D1a*) display irregular expression rhythms and increase *WFT* gene expression, resulting in early heading and flowering (Shaw et al. 2012; Shi et al. 2019; Hyles et al. 2020). The photoperiod insensitivity of *Ppd-A1a* and *Ppd-D1a* is attributed to a deletion in their promoter region, which contains

predicted transcription factor binding sites (Beales et al. 2007; Nishida et al. 2013; Kiseleva et al. 2017). In the case of *Ppd-B1a*, either promoter region insertion or increased copy number variation confers photoperiod insensitivity (Díaz et al. 2012; Nishida et al. 2013). Therefore, this region appears to be crucial for regulating *Ppd-1*.

Numerous *Eps* genes in wheat have been identified as quantitative trait loci (Snape et al. 2001; Griffiths et al. 2009; Zikhali et al. 2014). However, only a few *Eps* genes have been cloned, and their functions in heading/flowering are not fully understood. Among the identified *Eps* genes in bread wheat, *LUX ARRHYTHMO/PHYTOCLOCK1* (*LUX/PCL1*) and *EARLY FLOWERING 3* (*ELF3*), are orthologues to circadian clock components in *Arabidopsis* (Bendix et al. 2015; Hyles et al. 2020; Mizuno et al. 2016; Zikhali et al. 2016). The circadian clock operates as a 24-h rhythm with multiple feedback loops (McClung 2011; Bendix et al. 2015; Robertson McClung 2021) that respond to light and temperature, regulating plant growth and ultimately controlling flowering time (Robertson Mcclung 2021; Maeda and Nakamichi 2022).

LUX/PCL1, a key gene in the circadian clock, is one of the *Eps* genes identified in wheat and barley (Mizuno et al. 2012; Campoli et al. 2013). In *Arabidopsis thaliana*, *LUX/PCL1* expresses in the evening, forming the Evening Complex (EC) alongside other clock components, *ELF3* and *ELF4* (Onai and Ishiura 2005; Helfer et al. 2011; Nusinow et al. 2011; Huang and Nusinow 2016). Within the 24-h cycle, EC represses various clock genes, including *TIMING OF CAB EXPRESSION 1* (*TOC1*), *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*), *PRR9*, and *GIGANTEA* (*GI*) (McClung 2011; Bendix et al. 2015; Robertson Mcclung 2021). Additionally, EC itself represses the expression of *LUX/PCL1* near dawn (Huang and Nusinow 2016). A loss-of-function mutation in any of the EC components results in altered expression of clock genes and an early heading phenotype (Campoli et al. 2013; Gawroński et al. 2014; Zikhali et al. 2016; Nishiura et al. 2018). In einkorn wheat (*Triticum monococcum* L.), Mizuno et al. (2012) identified *Wheat LUX/PCL1* (*WPCL1*) as a candidate gene for the earliness *per se Eps-3A*^{m} locus that is responsible for early heading in the mutant "KT3-5." Loss-of-function mutation of *WPCL1* is associated with the extra early heading trait in a bread wheat variety, "Chogokuwase" (Mizuno et al. 2016). Both "KT3-5" and "Chogokuwase" exhibit abnormal expression patterns of circadian clock genes and higher expression of *Ppd-1* and *WFT* (Mizuno et al. 2012; Mizuno et al. 2016). An early heading phenotype and disruption of the expression patterns of circadian clock genes were also reported in the *ELF3* mutant (Alvarez et al. 2016; Zikhali et al. 2016). These altered expression patterns were observed in *A. thaliana lux/pcl1* mutant or *elf3* mutant (Onai and Ishiura 2005; Thines and Harmon 2010). Consequently, it is suggested that EC shares conserved functions with *A. thaliana* and wheat concerning its effects on flowering and heading.

Significant changes in heading time occur when using different genotypes of the major genes *Vrn1* and *Ppd-1*, making precise adjustment of heading time challenging (Mizuno et al. 2022). To overcome such limitation, manipulation of the *Eps* pathway is focused. The genes within the *Eps* pathway are associated with minor variations in heading/flowering times beyond vernalization and photoperiod requirements. The limited impact of the *Eps* pathway on heading/flowering time presents an opportunity for fine-tuning (Snape et al. 2001; Hyles et al. 2020). Mutants displaying early heading/flowering have been identified in

einkorn wheat (Mizuno et al. 2012; Gawroński et al. 2014; Nishiura et al. 2018; Hashimoto et al. 2021). Deletion of circadian clock genes such as *WPCL1* and *WHEAT WD REPEAT 1* (*WWDR1*) has been identified as the causative gene for these mutations. However, their heading time was approximately one month earlier than that of the original variety (Mizuno et al. 2012; Nishiura et al. 2018; Hashimoto et al. 2021), resulting that they did not exhibit a desirable phenotype. In this research, we mutagenized a Japanese elite bread wheat cultivar, "Kitahonami," with ethyl methanesulfonate (EMS) and generated an early heading mutant, "E15-1069." The mutant heads 2–5 days earlier than the wild-type, making it a valuable material for fine-tuning the heading time. This study aims to identify the causal genes for the early heading phenotype of the mutant "E15-1069." We employed bulked segregant analysis with short-read genome sequencing, known as the MutMap approach. MutMap detected a loss-of-function mutation in the circadian clock gene *WPCL-D1*. The expression analysis showed that some circadian clock genes and photoperiod sensitivity genes were altered in this mutant. Our results suggest that *WPCL1* would be a valuable target for controlling heading time in wheat breeding.

Materials and methods

Plant materials and growth condition

To generate mutants, seeds of the Japanese elite winter wheat cultivar "Kitahonami" were treated with 0.50%, 0.70%, or 0.75% EMS. "Kitahonami" was released from the Kitami Agricultural Experiment Station, Hokkaido, and is adapted to the northern area of Japan (Yanagisawa et al. 2007). A mutant showing beneficial agronomic traits could be used to improve "Kitahonami" and other wheat cultivars in Japan (Komura et al. 2022). To obtain an early heading mutant, the heading date of M_2 mutants was evaluated in the experimental field of the Institute of Crop Science, NARO (Tsukuba, Japan; lat. 36.1 N and long. 140.6 E.), under natural field conditions in the cropping season 2014–2015. Three lines that headed earlier than "Kitahonami" were selected from 1,600 M_2 mutants. One of them, named "E15-1069," showed the early heading phenotype stably in subsequent generations and was used for analyses in this study.

For the bulked-segregant analysis and the segregation analysis of "E15-1069," F_2 and F_3 populations were generated from a cross between "E15-1069" and "Kitahonami." For measurement of heading time, "Kitahonami," "E15-1069," and their F_2 progenies were sown on November 8, 2017, and F_3 progenies and their parents were sown on October 24, 2018, at the experimental field of Institute of Crop Science, NARO. The date when the tip of the first spike appeared was recorded as the heading date for the two seasons.

For the gene expression analysis, "Kitahonami," "E15-1069," and "Chihokukomugi" were grown at 26°C under short-day (SD) conditions (9 h light/15 h dark) for 2 weeks. "Chihokukomugi" is also a Japanese winter wheat cultivar and has been developed at Kitami Agricultural Experiment Station (Ozeki et al. 1987). Since "Chihokukomugi" has the same alleles in *Vrn-1* and *Ppd-1* homoeoloci as "Kitahonami" (Mizuno et al. 2022), it was used as a control for the gene expression analysis.

Examination of agronomic and quality traits

Agronomic traits of the mutant “E15-1069” were examined in the two cropping seasons, 2016–2017 and 2017–2018, in the experimental field of the Kitami Agricultural Experiment Station in Kitami (Hokkaido, Japan; lat. 43.7 N and long. 143.7 E.). The heading stage was recorded when the tip of the spike emerged from half of the tillers. The maturity stage was recorded when the grains had a 40% moisture content. Measurements were taken for culm length, spike length, and number of spikes per square meter in each experimental plot. Grain samples harvested from field trials were subjected to measure grain yield per ha, 1,000-grain weight, grain protein content, flour yield, and flour color values as described in Ishikawa et al. (2020) and Komura et al (2022). The grain quality survey data was obtained only for the 2016–2017 season.

Genome sequencing of mutants

For genome sequencing, thirteen individuals showing mutant traits were selected from 664 F_3 progenies generated by a cross between “Kitahonami” and “E15-1069.” Total DNA was extracted from the leaves of selected F_3 individuals, “Kitahonami” and “E15-1069” using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and DNA libraries were constructed using the TruSeq DNA library Preparation Kit (Illumina, San Diego, CA, USA). Genome sequencing was performed on Illumina NovaSeq by paired-end 150-bp.

Quality control, alignment, and SNP calling

The obtained short reads were checked by FASTQC ver. 0.11.7 (Andrews 2010) and filtered by Trimmomatic version 0.33 (Bolger et al. 2014) with an average minimum Phred quality score per four bp of less than 20 and a length of less than 40 bp. The filtered reads were aligned to the reference genome of bread wheat cv. “Chinese Spring” (CS) version 1.0 (IWGSC 2018) using BWA version 0.7.17 (Li and Durbin 2009) with default options. To conduct bulked-segregant analysis based on MutMap (Abe et al. 2012; Sugihara et al. 2022), each sequence data of thirteen F_3 individuals was merged using SAMtools version 1.9 (Li et al. 2009). The average number of aligned reads to the reference sequence, and the coverage of reads were estimated using BBmap version 37.77 (Bushnell 2014). SNP and indel calling were conducted using BCFtools version 1.9 (Li 2011). Annotations of SNPs and indels were conducted using SnpEff version 4.3t (Cingolani et al. 2012).

Detection of candidate mutations

MutMap analysis (Abe et al. 2012; Sugihara et al. 2022) was performed to identify the causal genes of early heading mutants. We filtered out low-quality SNPs that did not satisfy the following criteria: 1) Mapping qualities of SNPs were more than or equal to 40, 2) The SNP detection site was homozygous in the wild-type, 3) The number of reads aligned to the SNP detection site was more than or equal to five, 4) The frequency of SNPs in bulked samples was higher than or equal to 0.3.

Sanger sequencing of WPCL-D1

Total DNA was extracted from leaves of “Kitahonami” and “E15-1069” using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For amplification of *WPCL-D1*, a specific primer set listed in Table S1 was

used. Primers were designed using EnsemblPlants (Andrew et al. 2022) and Benchling (https://benchling.com/academic [Accessed 21 Dec 2021]). PCR was conducted using *ExTaq* polymerase (Takara Bio, Kusatsu, Japan) under the following conditions: Pre-denaturing at 98°C for 2 min, 35 cycles of denaturation at 98°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, followed by post-extension at 72°C for 1 min. The sequencing reaction of the purified PCR products was conducted using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) was used for sequencing. The DNA sequences of *WPCL-D1* were deposited in the DDBJ database under the accession numbers LC782576 (Kitahonami) and LC782577 (E15-1069).

Genotyping of WPCL-D1

"Kitahonami," "E15-1069," and their 137 F_2 and 664 F_3 progenies were used for segregation analysis. The genotype of the F_2 population was estimated from the phenotypes of the F_3 plants. We used a specific primer set for the investigation of the *WPCL-D1* genotype (Table S1). The total DNAs used for the segregation analysis were extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). PCR was conducted using Quick-Taq HS DyeMix (Toyobo, Osaka, Japan) under the following conditions: Pre-denaturing at 98°C for 2 min, 40 cycles of denaturation at 98°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 1 min, followed by post-extension at 68°C for 1 min. The PCR product was digested with restriction enzyme BsaHI (NEW ENGLAND Biolabs, Ipswich, MA, USA) and electrophoresed in a 2% agarose gel. The alleles of *Vrn-1*, *Ppd-1*, and *WPCL1* homoeoloci in "Kitahonami" and "Chihokukomugi" had been determined by Mizuno et al. (2022).

Estimation of WPCL1 gene structure

Total RNA was extracted from the leaves of "Kitahonami" and "E15-1069" using RNeasy Plant Mini kit (Qiagen, Hilden, Germany). RNA-seq libraries were constructed using TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA, USA), and sequencing was performed on Illumina NovaSeq6000 by paired-end 100-bp. The obtained short reads were checked by FASTQC ver. 0.11.7 (Andrews 2010) and filtered by Trimmomatic version 0.33 (Bolger et al. 2014) with an average minimum Phred quality score per four bp of less than 20 and a length of less than 40 bp. The filtered reads were aligned to the reference genome of CS version 1.0 (IWGSC 2018) using HISAT2 version 2.1.0 (Kim et al. 2019) with default options. Alignment reads with mapping quality of less than 40 were removed using SAMtools version 1.9 (Li et al. 2009). The gene structures of *WPCL-B1* and *WPCL-D1* were estimated from the reads alignments using Integrative Genomics Viewer (Robinson et al. 2011).

Gene expression analysis

Total RNA was extracted from leaves of two-week-old seedlings in three biological replicates using Maxwell RSC Plant RNA Kit (Promega, Madison, WI, USA) with Maxwell® RSC instrument (Promega, Madison, WI, USA) according to the manufacturer's protocol. cDNA was synthesized from 2.5 µg of RNA sample in an 80 µL reaction solution using ReverTra Ace (Toyobo, Osaka, Japan) and an oligo$(dT)_{20}$

primer. A real-time PCR was performed using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) with THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The relative expression levels were calculated as the average of three biological replicates and three technical replicates for each biological replicate using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The *Cell Division Control Protein* (*CDCP)* gene was used as an internal control (Paolacci et al. 2009). Primers used for gene expression analysis are shown in Figure S1, and the sequences are listed in Table S2.

Construction of protein structure

The protein 3D structure was constructed using a fully automated protein structure homology-modeling server, SWISS-MODEL (Waterhouse et al. 2018). For the modeling, the amino acid sequence of WPCL-D1 in CS (IWGSC 2018) and *A. thaliana* LUX/PCL1 (Silva et al. 2020) were used as a target sequence and a template, respectively.

Results

Characterization of early heading mutant of Japanese winter wheat cultivar "Kitahonami"

We identified an early heading mutant, designated as "E15-1069," in the EMS mutagenized population of the Japanese elite winter wheat cultivar "Kitahonami." The mutant exhibited heading approximately five days earlier than "Kitahonami" in Tsukuba (Fig. 1), while in Kitami, Hokkaido, it displayed a heading stage two days earlier than that of "Kitahonami" (Table 1). In addition, the agronomic and quality characteristics of the mutant were compared with those of "Kitahonami." Given the earlier heading stage of the mutant "E15-1069", its maturity stage also occurred earlier than that of "Kitahonami" (Table 1). The mutant had slightly shorter culm and the spike lengths compared to those of "Kitahonami," along with a significant reduction in the number of spikes, resulting in decreased grain yield (kg/ha). Additionally, the grain protein content was slightly increased in the mutant, accompanied by discoloration of flour color, indicated by a decrease in L* value and an increase in a* value (Table 1).

Table 1
Agronomic and quality characteristics of “Kitahonami” and “E15-1069”

	2016–2017		2017–2018	
	Kitahonami[1]	E15-1069	Kitahonami	E15-1069
Agronomic characteristics				
Heading stage (date)[2]	June 9, 2017	June 7, 2017	June 7, 2018	June 5, 2018
Maturity stage (date)[2]	July 25, 2017	July 24, 2017	July 30, 2018	July 28, 2018
Culm length (cm)	75	74	79	75
Spike length (cm)	8.4	8.2	8.7	8.1
Spike number per m^2	523	495	820	650
Grain yield (kg/ha)	7470	6380	8160	7280
1000-grain weight (g)	41.5	41.4	42.8	40.6
Quality characteristics				
Grain protein content (%)	11.2	12.7	-	-
Flour ash content (%)	1.01	1.05	-	-
Flour yield	71.0	70.2	-	-
Flour color L*	87.34	86.36	-	-
a*	−0.94	−0.69	-	-
b*	15.43	14.79	-	-

[1] Data of “Kitahonami” for 2016–2017 season was reproduced according to Komura et al. (2022) with modifications.

[2] Sowing dates were September 22, 2016 and September 25, 2017.

To estimate the number of genes responsible for the early heading trait, we measured the days to heading of F_2 and F_3 populations generated by crossing “Kitahonami” and “E15-1069” in Tsukuba. In the 2017–2018 cropping season, the mutant and “Kitahonami” had heading days of 167 and 171, respectively, while the 137 F_2 individuals showed a distribution between 167 and 176 days (Fig. 1). To clarify the segregation of the heading trait in the F_2 population, we grew 3–5 F_3 plants for each F_2 plant and estimated the F_2 individuals' genotypes based on the F_3 plants' phenotypes in the subsequent 2018–2019 season. The mutant and “Kitahonami” displayed heading days of 182–183 and 187–188, respectively, while the F_3 plants exhibited a distribution between 181 and 191 days. Consequently, we

classified F_3 individuals into “mutant-type” with an earlier heading than 184 days and “wild-type” with a heading later than 186 days. This F_3 result was compared with the phenotype data of corresponding F_2 individuals. Among the 118 F_2 plants with clear segregation in F_3 individuals, 30 were homozygous for “wild-type,” 55 were heterozygous, and 33 were homozygous for “mutant-type.” The segregation rate of the F_2 population adhered to the 1:2:1 segregation ratio ($\chi^2 = 0.69$, $P = 0.71$), indicating that the early heading is a recessive trait caused by a mutation occurring in a single locus.

Identification of WPCL-D1 as a candidate gene causing early heading

To identify the causal gene responsible for the early heading mutant “E15-1069,” we conducted a MutMap analysis (Abe et al. 2012; Sugihara et al. 2022). Thirteen individuals showing the early heading phenotype were selected from the 664 F_3 progenies from a cross between “Kitahonami” and “E15-1069,” and then whole-genome resequencing of the selected F_3 individuals was performed. The resulting reads of the selected individuals were merged into the F_3 mutant bulk. The sequencing reads of “Kitahonami” from our previous study (Komura et al. 2022) were also used. Following quality control, we obtained 2.7 billion and 1.4 billion short reads for “Kitahonami” and the F_3 mutant bulk, respectively (Table 2). These reads were aligned to the CS reference genome version 1.0 (IWGSC 2018). After removing low-quality alignment reads, the average depth was estimated to be 14.97 for “Kitahonami” and 14.45 for the F_3 mutant bulk, with both samples covering more than 94% of the CS reference genome. We identified a total of 132,324 SNPs between “Kitahonami” and the F_3 mutant bulk (Table S3), with 90.25% of these SNPs being G/C to A/T transitions, indicating that most of these SNPs were introduced through EMS mutagenesis. Of these SNPs, 1,680 were located within exons, and 75 were predicted to have deleterious effects, such as nonsense mutations or start codon losses (Table S3).

Table 2
Summary of alignments and coverages of genome sequencing of “Kitahonami” and bulked sample of F_3 mutant type plants

Name	Total filtered reads[1]	Aligned reads after removal of low-quality reads[2]	Reference bases covered (%)	Average depth-of-coverage
Kitahonami[3]	2,724,430,844 (93.63%)	2,323,699,205 (85.29%)	94.32	14.97
Mutant type F_3 bulk	1,478,929,060 (94.99%)	1,420,173,940 (96.03%)	94.26	14.45

[1] An average base quality score per 4 bp ≥ 20

The filtered reads rate = Total filtered reads / Total reads × 100

[2] The filtered reads rate = Aligned reads after removal of low-quality reads / Total filtered reads × 100

[3] “Kitahonami” sequencing data were reproduced according to Komura et al. (2022).

MutMap analysis indicated that the moving average of the SNP-index exceeded the 99% confidence interval in the region of 605 to 610 Mbp on chromosome 3D (Fig. 2), suggesting that the causal gene resides in this region. Among the 53 SNPs with SNP-index exceeding the 99% confidence interval in this region, two were located within gene sequences. One of them was positioned at the 387th position from the start codon in TraesCS3D01G529200, annotated as a “Pentatricopeptide repeat-containing protein” (IWGSC 2018), resulting in a synonymous mutation of the 129th glutamic acid. The other SNP was at the 411th position from the start codon within exon 1 of TraesCS3D01G531900, annotated as a “Two-component response regulator with the function of a Myb-like DNA-binding domain” (IWGSC 2018). This SNP caused a nonsense mutation at the 137th residue of tryptophan within the Myb domain (Fig. 3a). This gene is known as the clock gene *WPCL-D1* (Mizuno et al. 2016). The wild-type *WPCL-D1* consists of 281 amino acids, whereas “E15-1069” carries a truncated protein with 136 amino acids, lacking the Myb domain (Fig. 3ab). Consequently, the *WPCL-D1* allele in “E15-1069” was assumed to be nonfunctional. Given previous observations in wheat, *A. thaliana*, and barley that mutations in *LUX/PCL1* cause early heading and flowering (Onai and Ishiura 2005; Mizuno et al. 2012; Campoli et al. 2013), *WPCL-D1* was assumed to be the gene responsible for the early heading phenotype of “E15-1069.”

To confirm the relationship between the nonsense mutation in *WPCL-D1* and the early heading trait, we conducted a segregation analysis involving 137 F_2 and 648 F_3 plants. The genotypes of *WPCL-D1* in F_2 and F_3 populations were determined using a cleaved amplified polymorphic sequence (CAPS) marker for the two *WPCL-D1* alleles (Fig. 3c). In both F_2 and F_3 populations, homozygous plants for the mutant allele displayed significantly earlier heading than homozygous plants for the wild-type allele and heterozygous

plants (Fig. 3d). This result supported the conclusion that a loss-of-function mutation in *WPCL-D1* was responsible for the early heading trait in “E15-1069.”

Gene expression analysis of clock and photoperiod sensitivity genes

WPCL1 is a clock gene that regulates the circadian clock in plants. In the *wpcl1* null mutants of bread wheat and einkorn wheat, expression patterns of circadian clock genes and photoperiod sensitivity genes under their control were disrupted (Gawroński et al. 2014; Mizuno et al. 2016). To investigate the impact of *WPCL-D1* loss-of-function on plant heading, we examined gene expression patterns of circadian clock genes, *WPCL1*, *TaTOC1*, and *LATE ELONGATED HYPOCOTYL* (*TaLHY*) (Zhang et al. 2015; Zhao et al. 2016) and photoperiod sensitivity genes, *Ppd-1*, under SD conditions using quantitative RT-PCR. In *Arabidopsis*, a reciprocal regulation between LHY and TOC1 functions as the core of the circadian clock (McClung 2011; Bendix et al. 2015). Since “Kitahonami” carries a loss-of-function *wpcl-B1* allele, while “E15-1069” possesses only the functional *WPCL-A1* (Table 3), we included the bread wheat cultivar “Chihokukomugi” in the analysis, which has three functional *WPCL1* homoeologues, to compare the effect of the copy number of functional *WPCL1* on gene expression. The alleles of the major genes, *Ppd-1* and *Vrn-1*, in these three varieties are common: a photoperiod insensitive *Ppd-A1a* allele, photoperiod sensitive *Ppd-B1_Hapl-I* and *Ppd-D1_Hapl-II* alleles, and three recessive *vrn-1* alleles (Table 3; Mizuno et al. 2022).

Table 3
Vrn-1, *Ppd-1* and *WPCL1* alleles in “Kitahonami”, “E15-1069” and “Chihokukomugi"

Strain	***Vrn-1***[1]	***Ppd-1***[1]	***WPCL1***[2]
Kitahonami	*vrn-A1/ vrn-B1/ vrn-D1*	*Ppd-A1a/Ppd-B1_Hapl-I /Ppd-D1_Hapl-II*	*WPCL-A1/wpcl-B1/WPCL-D1*
E15-1069	*vrn-A1/ vrn-B1/ vrn-D1*	*Ppd-A1a/Ppd-B1_Hapl-I /Ppd-D1_Hapl-II*	*WPCL-A /wpcl-B1/wpcl-D1*
Chihokukomugi	*vrn-A1/ vrn-B1/ vrn-D1*	*Ppd-A1a/Ppd-B1_Hapl-I /Ppd-D1_Hapl-II*	*WPCL-A1/WPCL-B1/WPCL-D1*

[1] Alleles determined according to Mizuno et al. (2022).

[2] Alleles determined according to Mizuno et al. (2016).

The expression level of *WPCL-A1* did not exhibit significant differences among the three tested plants (Fig. 4). In contrast, the expression level of *WPCL-D1* was notably reduced in “E15-1069” at 9 h after dawn (Zeitgeber time, ZT9). The expression level of *WPCL-B1* in “Chihokukomugi” remained consistently lower than in the other plants. Additionally, the expression level of *TaLHY* at ZT0 was significantly higher in “E15-1069” than in the other two plants. *TaTOC1* showed that transcript accumulation peaked in “E15-

1069" at ZT9, which was 3 h earlier than that in "Kitahonami" and "Chihokukomugi." Significantly higher transcript accumulation level of *Ppd-A1* and *Ppd-D1* was observed in "E15-1069" at ZT21 and ZT6, respectively, than those in the other plants, whereas *Ppd-B1* also displayed a non-significantly higher expression level in "E15-1069" than in the other plants. The accumulation level of *Ppd-A1* and *Ppd-D1* genes appeared to be dependent on the copy number of the functional *WPCL1* gene: "E15-1069" with only one copy, exhibited the highest level of transcript accumulation, while "Chihokukomugi," with three copies, had the lowest level. These results revealed that the expression patterns of circadian clock and photoperiod sensitivity genes were altered in "E15-1069," with particular sensitivity of *Ppd-A1* and *Ppd-D1* transcript accumulation to the copy number of functional *WPCL1* genes.

Discussion

WPCL-D1, a candidate gene responsible for the early heading phenotype in "E15-1069"

In this study, we mutagenized the Japanese winter wheat cultivar "Kitahonami" with EMS, resulting in the early heading mutant "E15-1069." Through whole-genome resequencing and bulked segregant analysis based on MutMap (Abe et al. 2012; Sugihara et al. 2022), we detected a nonsense mutation in *WPCL-D1* on chromosome 3D in "E15-1069" (Figs. 2 and 3a). This nonsense mutation was located in the Myb domain of *WPCL-D1*, leading to a truncated protein (Fig. 3b). The evolutionary conservation of the *WPCL1* Myb domain in plant species such as *A. thaliana*, barley, and wheat suggests that *WPCL-D1* in "E15-1069" is a loss-of-function allele (Onai and Ishiura 2005; Mizuno et al. 2012; Campoli et al. 2013; Mizuno et al. 2016). In F_2 and F_3 populations of "Kitahonami" and "E15-1069," the nonsense mutation in *WPCL-D1* was significantly associated with days to heading (Fig. 3d). In *A. thaliana, LUX/PCL1* is a circadian clock gene and constitutes the evening complex (EC) along with other clock genes like, *ELF3* and *ELF4* (Nusinow et al. 2011). The EC regulates plant flowering, growth, and thermomorphogenesis by interacting with other clock components (Huang and Nusinow 2016; Zhang et al. 2021). Mutants of *A. thaliana*, barley, einkorn wheat, and bread wheat that lack the functional *LUX/ PCL1* gene exhibited arrhythmic expression patterns of circadian clock genes, resulting in early flowering and heading traits (Helfer et al. 2011; Mizuno et al. 2012; Campoli et al. 2013; Mizuno et al. 2016). Our results in "E15-1069" are consistent with these findings (Figs. 3 and 4). Therefore, the loss-of-function mutation in *WPCL-D1* is the most likely cause of the early heading phenotype in the mutant "E15-1069."

Dosage effect of WPCL1 on gene expression and heading time

The *wpcl1* null mutants of einkorn wheat and bread wheat exhibited abnormal expression patterns of circadian clock and photoperiod sensitivity genes (Mizuno et al. 2012; Mizuno et al. 2016). In "E15-1069," the expression patterns of these genes, including *TaTOC1*, *TaLHY* and *Ppd-1*, deviated from those in "Kitahonami" (Fig. 4). However, these changes in expression patterns do not align with those observed in previous studies of the *wpcl1* null mutants. For instance, the expression of *TaLHY* is reduced at dawn in *wpcl1* null mutants (Mizuno et al. 2012; Gawroński et al. 2014; Mizuno et al. 2016); in contrast, "E15-1069" exhibited enhanced *TaLHY* expression at the same time. Similarly, the bread wheat *wpcl1* null

mutant and barley *hvpcl1* mutant showed high accumulation levels of *LUX/PCL1* truncated transcripts near dusk (Campoli et al. 2013; Mizuno et al. 2016). However, the accumulation level of *WPCL-D1* transcript was reduced in “E15-1069” at ZT9 (Fig. 4). The plant circadian clock comprises multiple feedback loops (McClung 2011; Bendix et al. 2015). The EC, which includes LUX/PCL1, ELF3, and ELF4, participates in regulating other clock gene expressions (Huang and Nusinow 2016). As “E15-1069” possesses a functional *WPCL-A1* allele, disturbance of the feedback loop by *wpcl-B1* and *wpcl-D1* would be partially compensated by *WPCL-A1*. However, the altered gene expression pattern in “E15-1069” differs from that in “Kitahonami” and previously reported null mutants. Moreover, differences in gene expression patterns are also observed between “Kitahonami” and “Chihokukomugi”, with slight differences in *TaTOC1* gene expression (Fig. 4). These observations suggest that functional homoeologues do not fully compensate for the expression levels of the clock genes, and are also influenced by the gene dosage of functional *WPCL1*. Additionally, while previous studies using einkorn wheat *wpcl1* mutants showed heading 20–30 days earlier than the wild-type (Mizuno et al. 2012; Gawroński et al. 2014), “E15-1069” was headed around 5 days earlier than “Kitahonami.” In addition, the heading date of “Chihokukomugi” was about 4 days later than that of “Kitahonami” (Mizuno et al. 2022). The difference in the extent of early heading between the einkorn mutant and “E15-1069” supports the hypothesis that *WPCL1* controls heading time in a dosage-sensitive manner. The accumulation pattern of *Ppd-1* transcripts may reflect this suggestion. The transcript accumulation level at the peak of *Ppd-1* gene expressions depends on the copy number of functional *WPCL1* (Fig. 4). As our hypothesis was made on the basis of an indirect comparison between two different cultivars, further studies are needed to explore the relationship between heading time and the gene dosage effect of circadian clock genes using near-isogenic lines or genome editing lines.

The difference in the days to heading between “Kitahonami” and “Chihokukomugi” coincides with that between “E15-1069” and “Kitahonami.” These results imply that the heading time can be fine-tuned by altering the copy number of functional *WPCL1*. This feature is advantageous in polyploid wheat improvement because the loss-of-function of *WPCL1* in diploid wheat induces substantial changes in heading time. Recently, another circadian clock gene, *TaELF-B3*, a homoeologue of *TaELF3*, demonstrated that its deleted allele also leads to earlier heading by approximately four days compared to the wild-type allele (Mizuno et al. 2023). This finding indicated that *TaELF-B3* has contributed to the fine-tuning of flowering time in wheat breeding. Both *WPCL1* and *TaELF3* constitute EC (Alvarez et al. 2023). Thus, manipulating clock genes, particularly EC components, offers the opportunity to create a wide range of flowering times for local adaptation.

Regulation of Ppd-1 gene expression by WPCL1

Wheat *Ppd-1* is the homologous gene of *A. thaliana PRR7* and *PRR3* and primarily regulates photoperiod sensitivity (Beales et al. 2007; Zhang et al. 2016). In *A. thaliana*, LUX/PCL1 binds to the LUX binding site in the promoter region of *PRR9* and represses its expression (Helfer et al. 2011). Therefore, *WPCL1* is hypothesized to function as a negative regulator of *Ppd-1* by binding to its promoter region and repressing *Ppd-1* expression (Mizuno et al. 2016). In the wheat *wpcl1* null strain, up-regulation of *Ppd-1*

was observed only in the photoperiod sensitive allele (*Ppd-A1b*), and not in the photoperiod insensitive allele (*Ppd-D1_Hapl-I*) (Mizuno et al. 2016). Photoperiod insensitive alleles, *Ppd-A1a* and *Ppd-D1_Hapl-I*, carry 1 kbp and 2 kbp deletions, respectively, both of which have deletions in the 5' UTR region (Beales et al. 2007; Nishida et al. 2013). These deletions contain LUX binding site motifs (GATACG or GATTCG) discovered in *A. thaliana* (Helfer et al. 2011). Thus, the photoperiod insensitive allele is assumed to be released from regulation by *WPCL1.* However, in our study, increased expression levels of *Ppd-1* were observed in both the photoperiod-sensitive allele (*Ppd-D1_Hapl-II*) and the photoperiod insensitive allele (*Ppd-A1a*). These results suggest that *WPCL1* also regulates the expression of *Ppd-1* using elements other than the LUX binding site motifs in the deletion region. These additional elements may include the G-box motif or the LUX binding site motif located further upstream of the deletion regions (Helfer et al. 2011; Ezer et al. 2017), or regulation may occur indirectly through other pathways.

Prospects for utilizing the "E15-1069" in breeding

As the mutant "E15-1069" originates from the Japanese elite cultivar "Kitahonami," it is readily available as a breeding material for fine-tuning heading time. However, it is worth noting that "E15-1069" exhibited a lower yield compared to "Kitahonami" (Table 1). This reduction in grain yield is attributed to a decreased spike number. Since the nonsense mutation of *WPCL-D1* only leads to a few days of early heading, the yield decrease likely arises from factors other than a shortened vegetative growth period due to the early transition from the vegetative to reproductive phase. In rice (*Oryza sativa* L.), a circadian clock gene, *OsCCA1*, a homologue of *TaLHY*, negatively regulates tiller number via strigolactone signaling and sugar sensing (Wang et al. 2020). "E15-1069" exhibited higher *TaLHY* expression at ZT0 than "Kitahonami" (Fig. 4), suggesting that the increased expression of *TaLHY* in "E15-1069" may have resulted in reduced tillering. Additionally, previous studies have shown that heading/flowering-associated genes like *Vrn-1*, *Ppd-1*, and *FT* influence tiller number in wheat (Kato et al. 2000; Dyck et al. 2004; Dixon et al. 2018; Liu et al. 2019). While the heading/flowering control system appears to be related to tiller number, further research is needed to understand it for more efficient breeding using "E15-1069."

Exploiting the dosage effect of circadian clock genes such as *WPCL1* offers a new breeding material, as described above. Although practical use of these resources requires verification, the combination of clock gene alleles has the potential to create diversity in the genetic control of heading/flowering time. Moreover, these genes enable precise adjustment of heading/flowering time, which was previously challenging by modifying *Vrn-1* and *Ppd-1*. Accurate control of heading/flowering time allows for the expansion of suitable land for wheat cultivation, contributing to increased wheat production.

Declarations

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Author contributions

YO, HH, and FK generated EMS induced mutants and selected the early heading mutant. HJ and FK evaluated the phenotype of the mutant. SK and KY analyzed genome sequencing data. FK generated genome sequencing data. SK performed genotyping and gene expression analysis. SK, KY, ST, and FK designed experiments and genome sequencing analyses. SK, KY, and FK wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Data availability

The DNA sequences of *WPCL-D1* were deposited in the DDBJ database under the following accession numbers: LC782576 (Kitahonami) and LC782577 (E15-1069).

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Figures

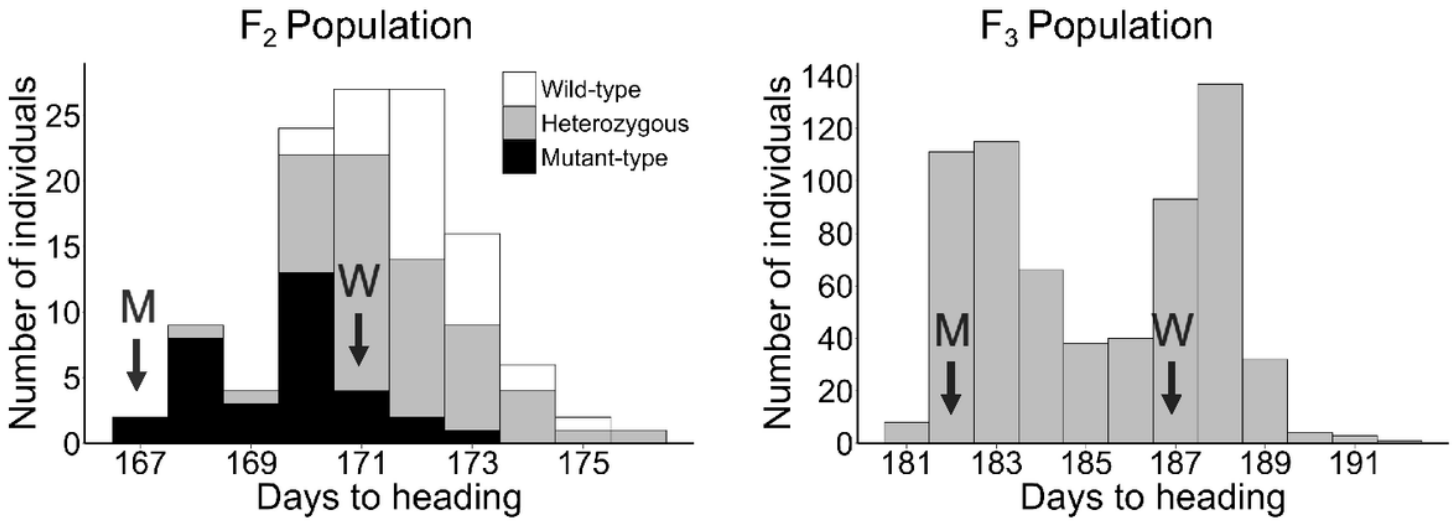

Figure 1

Histograms of heading dates of F_2 (left) and F_3 (right) individuals of a cross between the wild-type "Kitahonami" and early heading mutant "E15-1069." The genotypes of F_2 plants were estimated by the phenotype of corresponding F_3 plants and their segregations. W and M indicate heading dates of the wild-type and the early heading mutant, respectively.

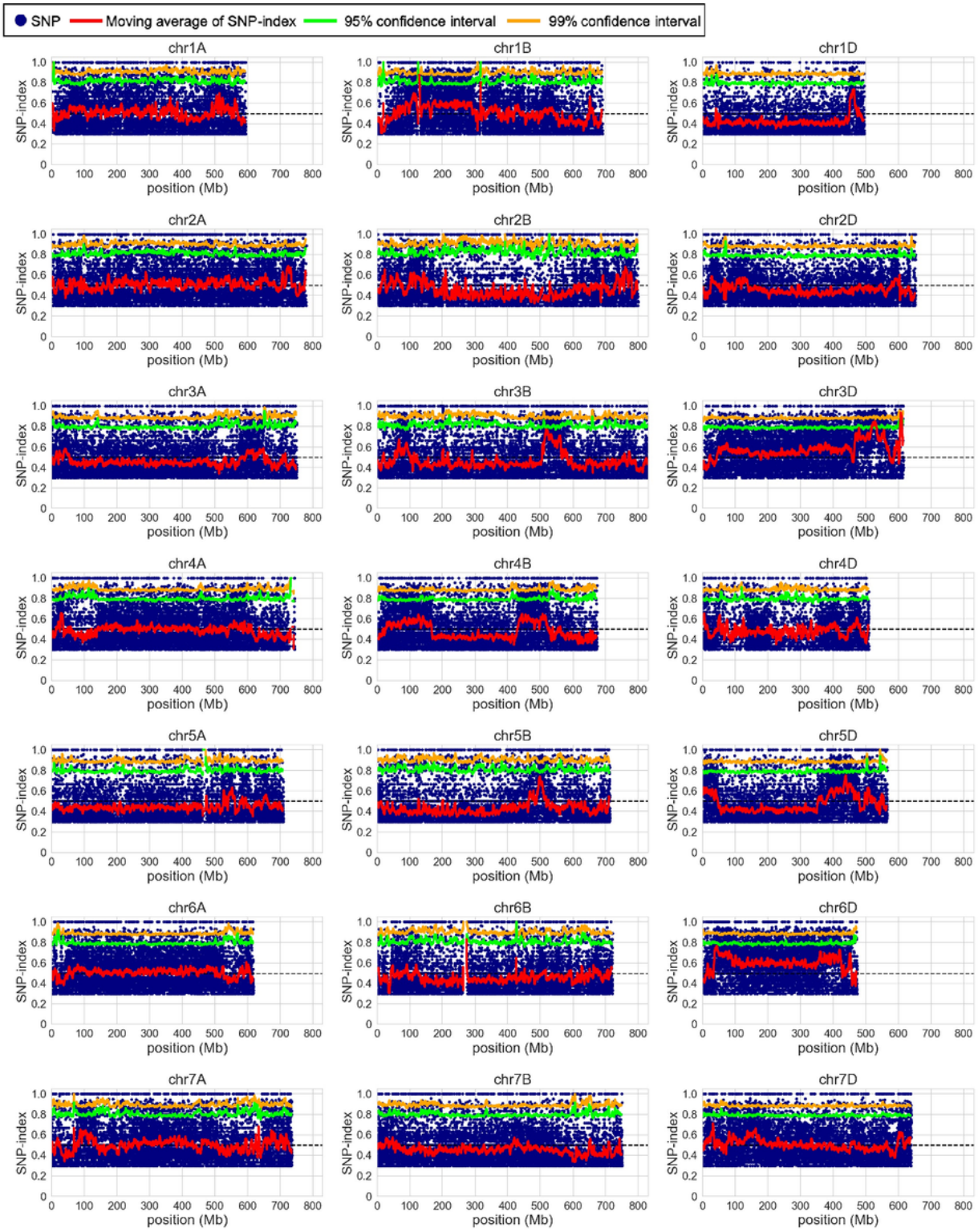

Figure 2

Distribution of SNP-index of the early heading mutant “E15-1069” F_3 mutant bulk across all chromosomes. Blue plots indicate SNP-index at each position of SNPs. The moving average of SNP-index is shown in the red line. Green line and orange line show 95% confidence interval and 99% confidence interval, respectively. The window size was 4 Mbp with 200 kbp step size.

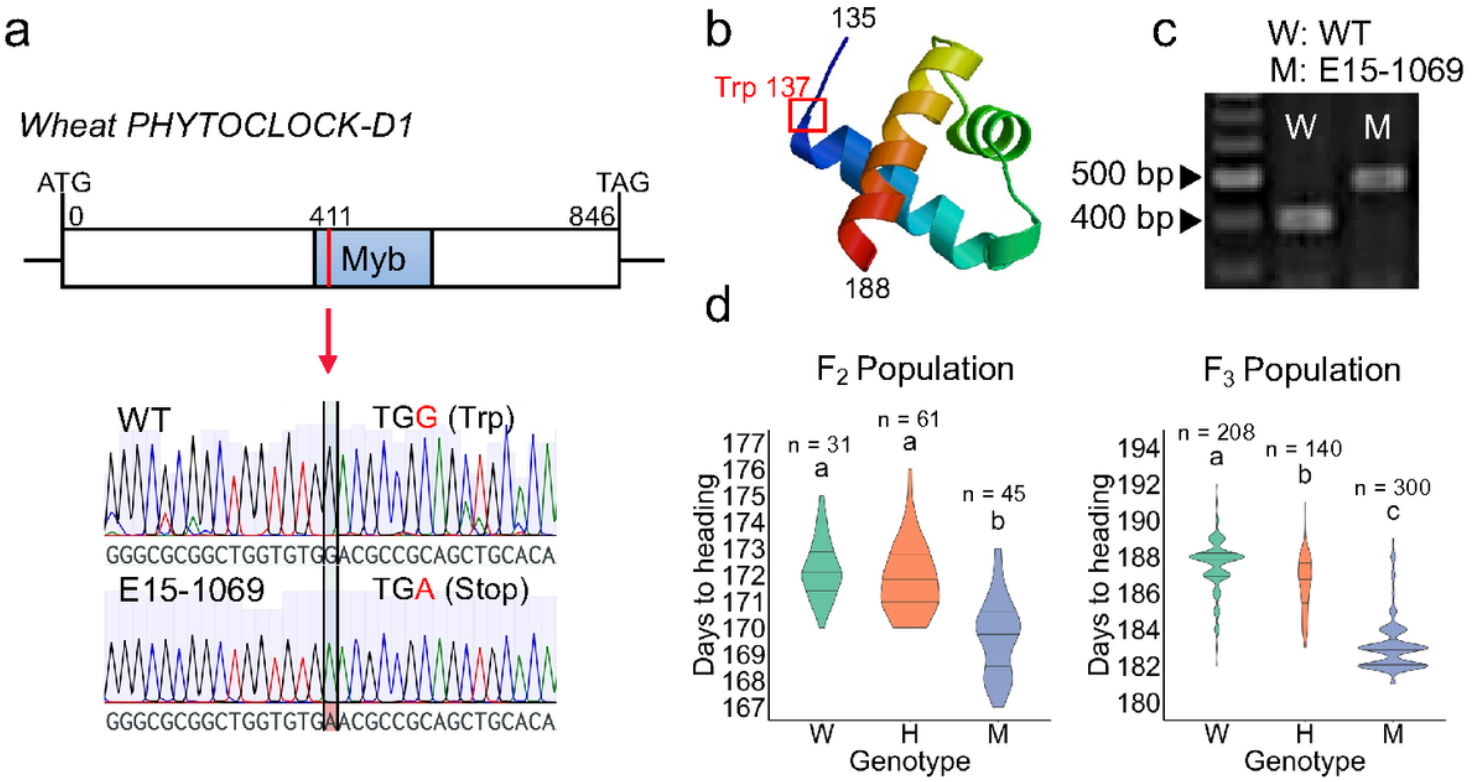

Figure 3

Identification of loss-of-function mutation in *Wheat PHYTOCLOCK-D1* (*WPCL-D1*) (a) Schematic diagram showing *WPCL-D1* and confirmation of mutation by sanger sequence. A red line in the gene model indicates the nonsense mutation site. *WPCL-D1* is composed of one exon. (b) Protein structure model of WPCL1 Myb domain. The position of tryptophan at 137th is surrounded in red. Protein structure model was made from 135th to 188th amino acid. (c) The results of PCR amplification of CAPS markers of *WPCL-D1*. W and M indicate the wild-type and the mutant "E15-1069", respectively. (d) Heading date investigation of F_2 and F_3 segregation populations of the wild-type × the mutant "E15-1069." Violin plot of days to heading for each genotype. W, H, and M are homozygous alleles of the wild-type, heterozygous alleles of the wild-type, and the mutant homozygous alleles of the mutant, respectively. Different letters designate significant differences between the means in Tukey-Kramer test ($p < 0.05$).

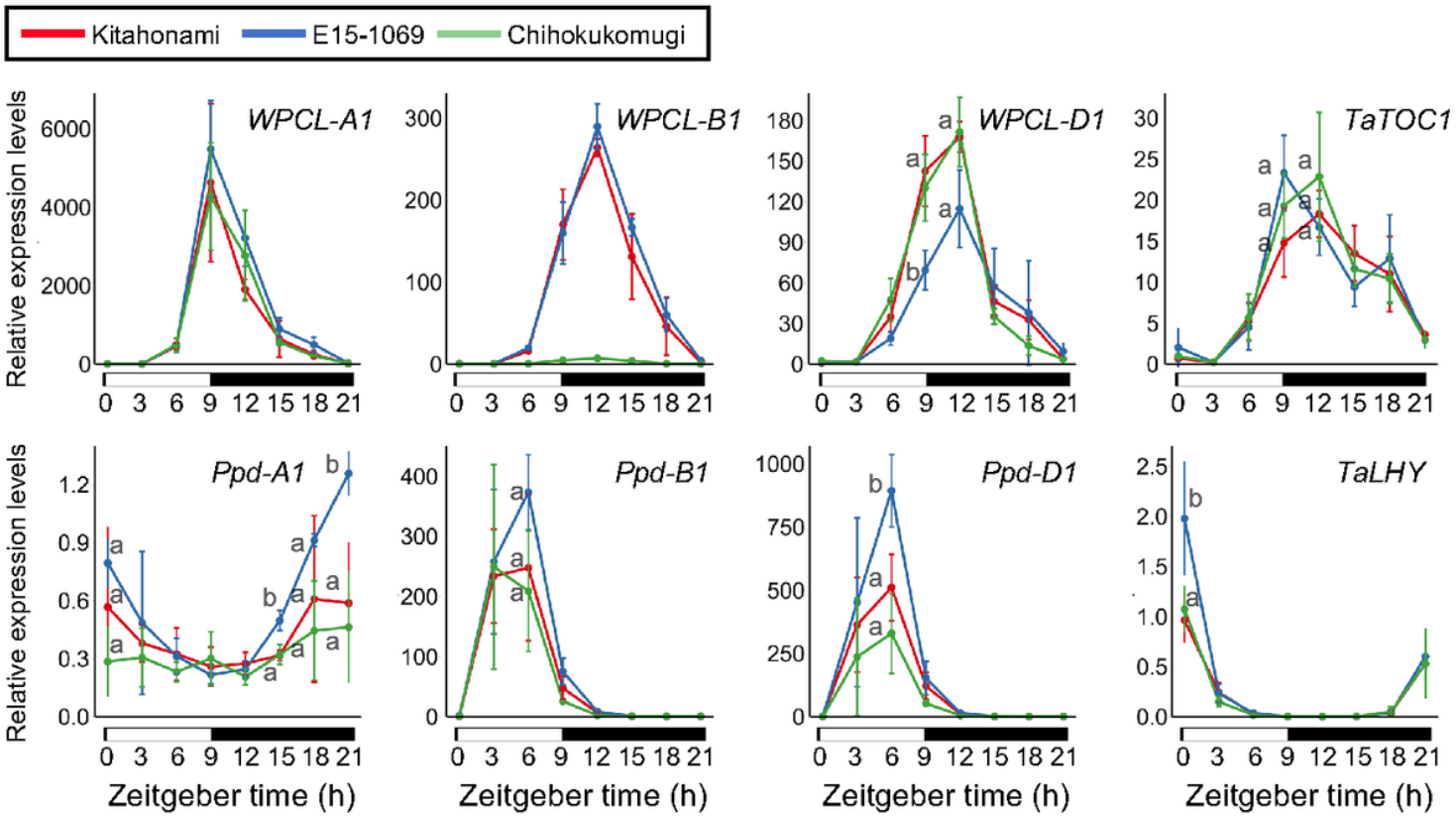

Figure 4

Gene expression patterns of clock component genes and photoperiod sensitivity genes in the wild-type “Kitahonami,” the early heading mutant “E15-1069” and “Chihokukomugi” under short-day condition. Red, blue, and green lines indicate “Kitahonami,” “E15-1069,” and “Chihokukomugi,” respectively. Three independent two-week-old plants were sampled every 3 h over 24 h. Open and black boxes indicate light and dark periods, respectively. Means ± SD were calculated from data obtained in three biological replicates and three technical replicates for each biological replicate. *Cell Division Control Protein* (Paolacci et al. 2009) gene was used as an internal control. Different letters designate significant differences between the means in Tukey-Kramer test ($p < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile1.pdf
- Supplemetanryfile2.xlsx