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Research Paper

An early-flowering einkorn wheat mutant with deletions of *PHYTOCLOCK 1/LUX ARRHYTHMO* and *VERNALIZATION 2* exhibits a high level of *VERNALIZATION 1* expression induced by vernalization

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ABSTRACT

Using heavy-ion beam mutagenesis of *Triticum monococcum* strain KU104-1, we identified a mutant that shows extra early-flowering; it was named *extra early-flowering 3 (exe3)*. Here, we carried out expression analyses of clock-related genes, clock downstream genes and photoperiod pathway genes, and found that the clock component gene *PHYTOCLOCK 1/LUX ARRHYTHMO* (*PCL1/LUX*) was not expressed in *exe3* mutant plants. A PCR analysis of DNA markers indicated that the *exe3* mutant had a deletion of wheat *PCL1/LUX* (*WPCL1*), and that the *WPCL1* deletion was correlated with the mutant phenotype in the segregation line. We confirmed that the original strain KU104-1 carried a mutation that produced a null allele of a flowering repressor gene *VERNALIZATION 2* (*VRN2*). As a result, the *exe3* mutant has both *WPCL1* and *VRN2* loss-of-function mutations. Analysis of plant development in a growth chamber showed that vernalization treatment accelerated flowering time in the *exe3* mutant under short day (SD) as well as long day (LD) conditions, and the early-flowering phenotype was correlated with the earlier up-regulation of *VRN1*. The deletion of *WPCL1* affects the SD-specific expression patterns of some clock-related genes, clock downstream genes and photoperiod pathway genes, suggesting that the *exe3* mutant causes a disordered SD response. The present study indicates that *VRN1* expression is associated with the biological clock and the *VRN1* up-regulation is not influenced by the presence or absence of *VRN2*.

1. Introduction

The early-flowering or early-heading phenotype in bread wheat (*Triticum aestivum*) cultivars is important as it can produce an early harvest; this characteristic is particularly beneficial in East Asia as it allows harvesting to occur before the onset of the rainy season. In autumn-sown wheat cultivars grown in central to southwestern Japan, reduced photoperiod sensitivity is the major determinant of earliness (Tanio et al., 2005). The photoperiod response is controlled by three major genes, *Photoperiod-A1* (*Ppd-A1*), *Ppd-B1* and *Ppd-D1*, which are located on the chromosome 2 homoeologs (Scarth and Law, 1984). The barley (*Hordeum vulgare*) *Ppd-1* ortholog, *Ppd-H1*, has been cloned and identified as a member of the *PSEUDO-RESPONSE REGULATOR* (*PRR*) family of *Arabidopsis* (Turner et al., 2005). PRR proteins contain a receiver-like/pseudo-receiver domain at their N-terminal end and a CCT [CONSTANS (CO), CO-like and TIMING OF CAB EXPRESSION 1 (TOC1)] domain near their C-terminus (Mizuno and Nakamichi, 2005). The CCT motif is a plant-specific and widespread motif, and may be important in regulating the expression of flowering control genes including the *CO*-like family (Griffiths et al., 2003) and of the vernalization gene of temperate cereals *VERNALIZATION 2* (*VRN2*) (Yan et al., 2004a). In *Arabidopsis*, the PRR family consists of five members, PRR1/TOC1, PRR3, PRR5, PRR7, and PRR9, and functions as a clock oscillator that is central to circadian rhythms (Mizuno and Nakamichi, 2005).

Abbreviations: exe, extra early flowering; PCL1/LUX, PHYTOCLOCK 1/LUX ARRHYTHMO; WPCL1, wheat PCL1/LUX; VRN1, VERNALIZATION 1; Ppd1, photoperiod 1; PRR, PSEUDO-RESPONSE REGULATOR; CO, CONSTANS; TOC1, TIMING OF CAB EXPRESSION 1; CCT, [CONSTANS (CO) CO-like and TIMING OF CAB EXPRESSION 1 (TOC1)]; FT, FLOWERING LOCUS T; LHY, LATE ELONGATED HYPOCOTYL; CCA1, CIRCADIAN CLOCK ASSOCIATED 1; ELF3, EARLY FLOWERING 3; FKF1, [FLAVIN-BINDING KELCH REPEAT F-BOX 1]; GI, GIGANTEA; CDFs, CYCLING DOF FACTORs; AP1/FUL-like, APETALA1/FRUITFULL-like; PIF4, PHYTOCHROME-INTERACTING FACTOR 4; eam10, early maturity 10; CRT, [CBF1/DREB1b a C-repeat]; DRE, drought-responsive element

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Ppd-H1 shows highest homology to *PRR7* among the Arabidopsis *PRR* genes (Turner et al., 2005), suggesting that *Ppd-1* is an ortholog of *PRR7* and functions as a clock component. Beales et al. (2007) identified the orthologs of wheat A, B and D genomes, and reported that the photoperiod-insensitive *Ppd-D1a* allele is associated with a 2089-bp deletion upstream of the coding region. Compared with photoperiod-sensitive *Ppd-D1b*, *Ppd-D1a* is misexpressed and this is associated with altered expression of the florigenic gene *FLOWERING LOCUS T* (*FT*). Genomic sequence analyses revealed that photoperiod-insensitive *Ppd-A1a* and *Ppd-B1a* alleles were associated with a 1085-bp deletion and 308-bp insertion in the 5′ upstream region, respectively (Nishida et al., 2013). Copy number variations (CNVs) are also present at the *Ppd-B1a* alleles and these cause increased basal gene expression levels (Díaz et al., 2012). The photoperiod-insensitive alleles of *Ppd-1* have been used for wheat breeding programs for early-heading varieties in several countries, including Japan (Seki et al., 2011, 2013). In addition, *Ppd-1* is associated with yield traits such as spikelet numbers (Worland et al., 1998). Recently, Boden et al. (2015) reported that *Ppd-1* plays a key role in inflorescence architecture.

In *Arabidopsis*, the gene network controlling the circadian clock involves a series of transcriptional and post-transcriptional feedback loops that create gene expression rhythms (reviewed in Bendix et al., 2015; Johansson and Staiger, 2015). Two transcription factors, LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), are expressed and active at dawn. During the morning, LHY and CCA1 repress the evening expressing genes, *PHYTOCLOCK 1/LUX ARRHYTHMO* (*PCL1/LUX*), *EARLY FLOWERING 3* (*ELF3*) and *ELF4*. PCL1/LUX, ELF3 and ELF4 are expressed from evening until midnight, and repress the *PRR* family (*PRR5*, *PRR7* and *PRR9*). The *PRRs* then repress *LHY* and *CCA1*. Another important negative feedback loop is involved in TIMING OF CAB EXPRESSION 1 (TOC1)/PRR1. *TOC1/PRR1* expression is suppressed by LHY and CCA1, and TOC1 represses *LHY* and *CCA1* during the course of the day.

The photoperiod pathway that is mainly composed of *FKF1* (*FLAVIN-BINDING, KELCH REPEAT, F-BOX 1*), *GI* (*GIGANTEA*), *CDFs* (*CYCLING DOF FACTORs*), and *CO* (*CONSTANS*) is downstream of the circadian clock genes described above (reviewed in Song et al., 2015). During long day (LD) conditions, the expression peaks of the clock-regulated genes *FKF1* and *GI* are in the afternoon. The FKF1 protein absorbs blue light and interacts with GI. The FKF1-GI complex degrades CDF proteins (CDF1, 2, 3, and 5), which are negative regulators of *CO*. The CO protein induces florigenic *FT* expression, leading to flowering (Song et al., 2015).

To understand the molecular mechanism of flowering in wheat, we constructed a large-scale mutant panel in diploid einkorn wheat (*T. monococcum*) using heavy-ion beam mutagenesis (Murai et al., 2013). Einkorn wheat seeds were exposed to a heavy-ion beam and then sown in the field. Selfed seeds from each spike of M_1 plants were used to generate M_2 lines. Every year over the past 15 years, we have obtained approximately 1000 M_2 lines and have built up a mutant panel with 10,000 M_2 lines. This mutant panel is being systematically screened for mutations affecting reproductive growth, and especially for flowering-time mutants. From the large scale mutant panel, we have identified four extra early-flowering mutants, named *extra early-flowering1* (*exe1*), *exe2*, *exe3*, and *exe4* (Nishiura et al., 2014). The four *exe* mutants fall into two groups, namely Type I (moderately extra early-flowering type: *exe1* and *exe3*) and Type II (extremely extra early-flowering type: *exe2* and *exe4*). Analysis of plant development in a growth chamber showed that the speed of leaf emergence is accelerated in the *exe* mutants compared to wild-type (WT) plants. Overall, the speed of leaf emergence is faster in Type II than Type I plants. Type I plants show reduced photoperiodic sensitivity, and Type II plants show a distorted photoperiod response. Analysis of *VERNALIZATION 1* (*VRN1*), a flowering promoter gene, shows that it is more highly expressed in seedlings at early developmental stages in Type II mutants than Type I mutants. These findings indicate that the difference in earliness between Type I and Type II mutants is associated with the level of *VRN1* expression. *VRN1* is an activator of *FT* (Shimada et al., 2009), and encodes an APETALA1/FRUITFULL-like (AP1/FUL-like) MADS-box transcription factor that is up-regulated by vernalization (Yan et al., 2003; Murai et al., 2003; Trevaskis et al., 2013; Danyluk et al., 2003).

In this study, we demonstrate that the Type I *exe3* mutant has a deletion of a clock component gene *PCL1/LUX*, abbreviated to *Wheat PCL1* (*WPCL1*), which was previously identified by Mizuno et al. (2012). We also confirm that the original strain KU104-1 carries a natural mutation of a null allele of the *VRN2* gene. Therefore, the *exe3* mutant has loss-of-function mutations of both *WPCL1* and *VRN2*. The present study indicates that the up-regulation of *VRN1* expression after vernalization is induced in the absence of expression in the clock component gene *WPCL1*; however, *VRN1* up-regulation is not influenced by the presence or absence of *VRN2*.

2. Materials and methods

2.1. Plant materials

Wild-type (WT) diploid einkorn wheat (*Triticum monococcum*) strain KU104-1 and two extra early-flowering mutants, *extra early-flowering* (*exe*) *1* and *exe3* were used in the experiments. The *exe1* and *exe3* mutations were generated by heavy-ion beam irradiation in our previous study (Nishiura et al., 2014), and plants of the M_3 or M_4 generations of *exe1* and *exe3* mutants were used in the experiments. The M_2 plants in the segregation line with the *exe3* phenotype were used for genomic DNA isolation. Expression analyses were performed using WT and *exe3* mutant plants. Wild diploid species *Triticum boeoticum* strain KT1-1 was used as the control genotype for *VRN2*, repressor of flowering.

2.2. Field experiments

The WT and *exe* mutant plants were sown in the middle of October in an experimental field at Fukui Prefectural University. The heading dates of each line were scored in the season 2013/2014 and 2014/2015. In the 2013/2014 season, newly unfolding leaves were sampled from WT and *exe3* mutant plants at the end of April to examine the expression patterns of clock-related genes, clock downstream genes and photoperiod pathway genes.

2.3. Growth chamber experiments

To examine the effects of vernalization and photoperiod, we screened the number of days from 2-leaf unfolding to flag-leaf unfolding (D2f) in vernalized and non-vernalized plants of WT and *exe3* mutant plants kept in a growth chamber under long day (LD; 16 h light/8 h dark) conditions or short day (SD; 10 h light/14 h dark) conditions at 20 °C (light intensity ~100 $\mu E\,m^{-2}\,s^{-1}$). Vernalization treatment was performed in a growth chamber under short day (SD; 10 h light/14 h dark) conditions at 5 °C for 2 weeks or 6 weeks. At the 3-leaf stage, leaves were sampled and gene expression analyses for *VRN1* and *WFT* were performed.

For gene expression analysis at different plant growth stages, non-vernalized WT and *exe3* mutant plants were grown under LD (16 h light/8 h dark) or SD (10 h light/14 h dark) conditions at 20 °C (light intensity ~100 $\mu E\,m^{-2}\,s^{-1}$). Newly unfolding leaves were sampled from seedlings at the 1-leaf stage and 3- to 6-leaf stages. In this study, leaf stages were defined using the criterion that one leaf stage lasted from initial unfolding to the unfolding of the next leaf, thus, for example, the 1-leaf stage was assumed to last from the unfolding of the first leaf to the unfolding of the second leaf.