early maturity 7 promotes early flowering by controlling the light input into the circadian clock in barley

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Abstract
Breeding for variation in photoperiod response is crucial to adapt crop plants to various environments. Plants measure changes in day length by the circadian clock, an endogenous timekeeper that allows plants to anticipate changes in diurnal and seasonal light–dark cycles. Here, we describe the early maturity 7 (eam7) locus in barley (Hordeum vulgare), which interacts with PHOTOPERIOD 1 (Ppd-H1) to cause early flowering under non-inductive short days. We identify LIGHT-REGULATED WD 1 (LWD1) as a putative candidate to underlie the eam7 locus in barley as supported by genetic mapping and CRISPR-Cas9-generated lwd1 mutants. Mutations in eam7 cause a significant phase advance and a misregulation of core clock and clock output genes under diurnal conditions. Early flowering was linked to an upregulation of Ppd-H1 during the night and consequent induction of the florigen FLOWERING LOCUS T1 under short days. We propose that EAM7 controls photoperiodic flowering in barley by controlling the light input into the clock and diurnal expression patterns of the major photoperiod response gene Ppd-H1.

Introduction
Flowering time substantially impacts crop yield and is thus a vital breeding target to produce new varieties better adapted to diverse and changing climatic conditions (Cockram et al. 2007). Climate models predict an increase in global temperatures and extreme weather events. Breeding for early maturing varieties is among the most effective strategies to improve adaptation to short growing seasons with terminal stress such as heat and drought events (Tewolde et al. 2006; He et al. 2022). Identification and characterization of alleles conferring early maturity greatly support these efforts.

The temperate crop barley (Hordeum vulgare) is among the most widely grown cereals with superior adaptation to marginal stress-prone agricultural lands (von Korff et al. 2008). Like most temperate cereals, barley is a quantitative long-day (LD) species that accelerates reproductive development with increasing photoperiods. In contrast, short photoperiods delay or even impair floral development (Digel et al. 2015). PHOTOPERIOD 1 (Ppd-H1), orthologous to PSEUDO-RESPONSE-REGULATOR (PRR) genes in Arabidopsis (Arabidopsis thaliana), has been identified as the central gene in photoperiodic flowering in barley (Laurie et al. 1995). Natural variation at Ppd-H1 determines variation in reproductive development under LD. The wild-type Ppd-H1 allele, prevalent in wild and winter barley, induces rapid floral development in response to LD (Turner et al. 2005; Jones et al. 2008). A single amino acid change in the conserved CCT (CONSTANS, CO-like, and TOC1) domain of Ppd-H1
delays flowering. This allele was selected and is prevalent in spring barley varieties in central and northern Europe (Jones et al. 2008). Ppd-H1 initiates flowering by inducing the expression of FLOWERING LOCUS T1 (FT1) in the leaves (Turner et al. 2005). FT1 is orthologous to florigen FT in Arabidopsis and Hd3a in rice (Oryza sativa), which move as proteins from the leaf to the shoot apical meristem and induce the transition from vegetative to reproductive growth (Kardailsky et al. 1999; Corbesier et al. 2007; Tamaki et al. 2007). In barley, increased FT1 expression in the leaf correlates with early flowering and the upregulation of floral inducers such as MADS-box genes VERNALIZATION 1 (VRN1), BARLEY MADS-BOX 3 (BM3), and BM8 in the leaf and meristem (Turner et al. 2005; Digel et al. 2015).

Differences in photoperiod are perceived by photoreceptors, which transmit this information to the circadian clock. The circadian clock is an endogenous timekeeper that allows organisms to anticipate seasonal and daily changes in light–dark rhythms. The core circadian clock is largely conserved across eudicots and monocots (Song et al. 2010). It comprises 3 interlocking, negative feedback loops of transcriptional repressors that are expressed sequentially during a 24-h period and repress previous and subsequent clock components. Transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) are expressed first in the morning, followed by PRR9, 7, and 5 during the progressing day, and PRR1 at dusk. CCA1/LHY represses the expression of PRR genes, which, in turn, suppress CCA1/LHY. During the night, the evening complex (EC) genes EARLY FLOWERING 3 (ELF3), EARLY FLOWERING 4 (ELF4), and LUX ARRHYTHMO (LUX) are expressed, repressing PRR genes and CCA1/LHY (Farré et al. 2005; Kikis et al. 2005; Huang et al. 2012; Mizuno et al. 2014). In Arabidopsis, the clock controls the diurnal expression pattern of the central photoperiod response gene CONSTANS (CO), resulting in CO expression peaking at the end of the light period in LD, but in the dark under SD (Suárez-López et al. 2001). The coincidence of CO expression with the light period is necessary for protein stabilization and the expression of the florigen FT under LD (Suárez-López et al. 2001; Valverde et al. 2004; Sawa et al. 2007; Jang et al. 2008). By contrast, in temperate monocots, the length of the night, rather than the length of the day, is critical for the perception of inductive photoperiods (Pearce et al. 2017; Gao et al. 2019). The length of the night is measured by PHYTOCHROME B (PHYB) and PHYTOCHROME C (PHYC), which are necessary for the light activation of Ppd-H1, and mutations in either of these genes result in the downregulation of Ppd-H1 and very late flowering (Chen et al. 2014; Kipps et al. 2020). In addition, PHYB and PHYC control the light-induced degradation of ELF3 (Gao et al. 2019; Alvarez et al. 2023). ELF3, presumably together with EC members LUX1 and ELF4, binds to the promoter of Ppd-H1 during the night to repress its expression (Gao et al. 2019; Andrade et al. 2022; Alvarez et al. 2023).

Mutations conferring photoperiod-independent early flowering, so-called early maturity (eam) loci, have been used in Scandinavian breeding programs since the 1960s to enable a geographic range extension of barley cultivation to areas with short growing seasons (Lundqvist 2009). Several genes that underlie eam loci have been identified in the last decade: eam5 was identified as a gain-of-function mutation in PHYC, eam8 as a knock-out mutation in ELF3, and eam10 as a single amino acid exchange in LUX1 (Faure et al. 2012; Zakhrabekova et al. 2012; Campoli et al. 2013; Pankin et al. 2014). Plants carrying eam mutations are characterized by reduced photoperiod sensitivity and accelerated flowering in inductive LD and non-inductive short days (SDs). While clock mutants are characterized by loss of circadian transcriptome oscillations and severely perturbed clock functions (Müller et al. 2016, 2020), they have been instrumental in expanding the cultivation of many crops to new geographic regions with altered annual patterns of temperature and photoperiod (McClung 2021). It is thus interesting to identify new genes and alleles and decipher the molecular networks important for photoperiod response in crops.

Here, we describe the early maturity 7 (eam7) locus, originally identified as a natural mutation in the line Atsel derived from the cultivar Atlas, that causes early flowering under non-inductive SD conditions (Stracke and Börner 1998). We identify LIGHT-REGULATED WD 1 (LWD1) as a putative candidate to underlie the EAM7 locus in barley as supported by genetic mapping and allelism tests with CRISPR-Cas9-generated lwd1 mutants. We demonstrate that eam7 interacts with Ppd-H1 to promote flowering under non-inductive photoperiods and affects plant architecture, spike development, spike fertility, and grain set. We propose that EAM7 controls photoperiodic flowering in barley by repressing Ppd-H1 at night, possibly mediated through ELF3. Mutations in EAM7 cause a significant phase advance and a downregulation of core clock and clock output genes under diurnal conditions. We thus suggest that EAM7/LWD1 controls photoperiod response by modifying the light entrainment of the clock and clock gene expression.

Results

eam7 accelerates reproductive development under short-day conditions

We investigated the effects of eam7 and Ppd-H1 on flowering time under long and short photoperiods. For this purpose, the spring cultivar Bowman (BW) and 3 derived introgression lines were cultivated under controlled conditions under long-day (LD, 16 h light/8 h dark, 20 °C/16 °C) or short-day (SD, 8 h light/16 h dark, 20 °C/16 °C) conditions to score flowering time. BW carries a natural mutation in the CCT domain of Ppd-H1 that delays flowering time under LD conditions (Turner et al. 2005). The derived introgression line BW(Ppd-H1) carries a wild-type Ppd-H1 allele and is early flowering under LD (Druka et al. 2011). In addition, we used BW(eam7), an introgression line with the eam7g mutation that causes early maturity under LD and SD (Stracke and Börner 1998; Druka et al. 2011). We crossed BW(Ppd-H1) and
BW(eam7) to generate a line with a wild-type Ppd-H1 allele and the mutation at eam7, which we termed BW(Ppd-H1, eam7).

Under LD, both introgression lines with a wild-type Ppd-H1 allele, BW(Ppd-H1) and BW(Ppd-H1, eam7), flowered 27 and 26 days after emergence (DAE), respectively, and therefore significantly earlier than BW and BW(eam7), which flowered 41 DAE (Fig. 1A). No significant differences in time to flowering were observed between BW and BW(eam7) and between BW (Ppd-H1) and BW(Ppd-H1, eam7) under LD. Consequently, Ppd-H1 but not eam7 controlled time to flowering under LD. Under SD, BW(eam7) flowered 90 DAE and thus significantly earlier than BW and BW(Ppd-H1), which flowered on average 98 and 101 DAE, respectively (Fig. 1B). However, 10% of BW and 22% of BW(Ppd-H1) plants had not flowered until the experiment was stopped at 125 d. BW(Ppd-H1, eam7) exhibited the fastest development and flowered 38 DAE and thus 52 d earlier than BW(eam7), indicating that eam7 and Ppd-H1 interacted to accelerate flowering under SD.

We then analyzed which stages of reproductive development were affected by variation at EAM7 and Ppd-H1. The main shoot apices (MSAs) of plants grown under LD and SD were dissected over development and scored according to the scale by Waddington et al. (1983). The Waddington scale rates the development based on the carpel of the most advanced floret of the spike. MSA development can be categorized into vegetative growth (W1.0 to W2.0), in which leaf primordia are initiated, early reproductive growth (W2.0 to W3.5) with the initiation of spikelet meristems (SM), and late reproductive growth and floral development until anthesis and pollination (W3.5 to W10.0).

Under LD, BW(Ppd-H1, eam7) transitioned to reproductive growth (W2.0) 6 DAE and thus 2 d earlier than BW(Ppd-H1) and BW (eam7) and 3 d earlier than BW (Fig. 1D). While BW(Ppd-H1, eam7) developed faster than BW(Ppd-H1) during early reproductive growth, their development synchronized during floral organ growth, and both genotypes reached pollination (W10.0) 34 DAE. In contrast, BW(eam7) and BW developed similarly during early reproductive growth but BW(eam7) development accelerated after carpel initiation (W4.5), and plants reached pollination 3 d earlier than BW (Fig. 1D). Under SD, the MSA of BW(Ppd-H1, eam7) transitioned to reproductive growth 9 DAE and thus 11 d earlier compared to the other 3 genotypes (Fig. 1E). BW(Ppd-H1, eam7) plants displayed a linear reproductive development under SD so that pollination (W10.0) occurred 41 DAE and thus only 7 d later compared to LD (Fig. 1C). By contrast, BW, BW(Ppd-H1), and BW(eam7) showed a substantial delay in floral development after carpel initiation (W4.5). However, floral development was still faster in BW(eam7) compared to BW and BW(Ppd-H1), and pollination occurred at 98 DAE, compared to 111 DAE in BW and BW(Ppd-H1) (Fig. 1E).

While we cannot exclude the possibility that BW(Ppd-H1), BW(eam7), and BW(Ppd-H1, eam7) carry additional variation for photoperiod response in the introgressed regions, our results suggested that Ppd-H1 interacted with eam7 to control photoperiod response. Under LD, eam7 accelerated spikelet initiation in the background of Ppd-H1 and floral growth in the background of Ppd-H1. Under SD, eam7 strongly accelerated all stages of reproductive development in the background of the wild-type Ppd-H1 allele. In contrast, it only accelerated floral organ growth by a few days in the background of the mutated Ppd-H1 allele.

Next, we investigated the effects of variation at EAM7 and Ppd-H1 on inflorescence architecture by scoring the initiation of SM and the number of florets and grains on the main spike. Under LD, BW produced the highest number of florets and grains per main spike, followed by BW(eam7), BW(Ppd-H1), and BW(Ppd-H1, eam7) (Supplemental Fig. S2, A to C, E). This result was associated with a longer duration of SM initiation and a higher number of total SM initiated in BW (Supplemental Fig. S1A, Supplemental Table S1). Spike fertility, the number of grains per florets on the spike, was close to 75% for BW, BW(Ppd-H1), and BW(eam7) in contrast to BW(Ppd-H1, eam7) with reduced fertility of only 25% (Fig. 2D). The low number of grains per spike in BW(Ppd-H1, eam7) was thus caused by a reduced number of initiated SM and reduced floret fertility. Under SD, BW(Ppd-H1, eam7) still initiated significantly less SM compared to the other genotypes, spike fertility, however, was relatively higher in BW(eam7) and BW(Ppd-H1, eam7) compared to BW(Ppd-H1) and BW (Fig. 2F; Supplemental Fig. S1B, Supplemental Table S1). The effects of Ppd-H1 and EAM7 on inflorescence architecture and floret fertility thus differed between photoperiods (Fig. 2, F, G to J).

In addition, we determined the effects of EAM7 on plant height, tiller number, and number of leaves on the main culm under LD and SD. Under LD, variation in shoot architecture was mainly affected by Ppd-H1, as BW(Ppd-H1) and BW(Ppd-H1, eam7) transitioned to reproductive growth earlier and thus produced fewer leaves on the main culm, fewer tillers and grew less tall compared to BW and BW(eam7) (Supplemental Fig. S2, A to C). Similarly, under SD, the fast-developing BW(Ppd-H1, eam7) plants produced significantly fewer leaves and tillers and were characterized by shorter plant height compared to the other 3 genotypes with a slower reproductive development (Supplemental Fig. S2, D to J). We also scored flag leaf size under SD and found that flag leaf length and width were reduced in BW(Ppd-H1, eam7) and BW(eam7) compared to BW and BW(Ppd-H1) (Supplemental Fig. S2, K to M).

In conclusion, under LD, Ppd-H1 had a significant effect on developmental timing, while variation at EAM7 only had minor effects on floral growth and the timing of pollination. Ppd-H1 strongly affected the rate and duration of SM initiation and floret and grain number, further modulated by variation at EAM7. Under SD conditions, eam7 in the background of Ppd-H1 strongly accelerated reproductive development and caused near day-length neutrality and early flowering independent of the photoperiod. EAM7 interacted
Figure 1. Effects of eam7 and Ppd-H1 on plant reproductive development. **A and B)** Flowering time of spring cultivar Bowman (BW) and introgression lines BW(eam7), BW(Ppd-H1), and BW(Ppd-H1, eam7) was scored in days after emergence (DAE) under long days (LDs) (A) and short days (SDs) (B). Genotypes carrying the EAM7 allele (BW and BW(Ppd-H1)) are shown in light gray, and genotypes carrying the eam7 allele (BW(eam7) and BW(Ppd-H1, eam7)) in dark gray. Each dot represents the mean of 5 to 42 plants, error bars indicate the standard deviation of the mean. Significance levels were determined by one-way ANOVA and subsequent Tukey’s test, \( P \leq 0.05, n = 5 \) to 42 plants. Numbers below the graph in (B) indicate the number of plants that flowered in EAM7 genotypes BW and BW(Ppd-H1) until the end of the experiment (125 DAE) compared to the total number of plants scored. **C)** Inflorescences of BW, BW(eam7), BW(Ppd-H1), and BW(Ppd-H1, eam7) plants grown under short days. The main culm was dissected 6, 16, 23, and 40 DAE. White scale bars indicate 100 μm, and gray scale bars indicate 500 μm. **D and E)** Main shoot apex (MSA) development under long-day (D) and short-day (E) conditions according to the scale by Waddington et al. (1983) by DAE. Dot sizes indicate the number of plants per data point (1 to 4), and gray areas show a 95% confidence interval of a polynomial regression (Loess smooth line). Horizontal lines indicate the start of spikelet initiation (W2.0) and the start of floral development (W3.5).
with Ppd-H1 to control SM initiation and floret fertility under SD. In the background of a mutated Ppd-H1 allele, eam7 did not affect SM initiation but still affected floret and grain number, and spike fertility. Early flowering decreased plant height, the number of leaves and tillers, and flag leaf size under LD and SD.

The expression pattern of circadian clock genes is altered in eam7 plants under SD

Day-length neutrality and early flowering under SD conditions have been associated with genetic variation in circadian clock genes and phytochromes in cereal crops (Faure et al. 2012; Campoli et al. 2013; Pankin et al. 2014; Müller et al. 2016).
and BW (every 1 to 2 wk between 20 and 60 DAE at ZT9 (1 h after time points during development). We sampled leaf material in the photoperiod response pathway of barley (eam7)). We could also not link differences in expression to controlled misexpression of clock genes, together with functional variation in Ppd-H1, were linked to differences in the diurnal expression of Ppd-H1 and CO1, and the upregulation of FT1 in BW(Ppd-H1, eam7) under SD conditions.

The expression patterns of PHYB, PHYC, and clock genes were strongly altered in BW(eam7) and BW(Ppd-H1, eam7) compared to BW and BW(Ppd-H1) (Fig. 3). At the same time, expression patterns of phytochromes and clock genes did not differ between BW and BW(Ppd-H1) and between BW(eam7) and BW (Ppd-H1, eam7), suggesting that eam7, but not Ppd-H1 had a major impact on the diurnal expression patterns. PHYB/C and ELF3 were significantly downregulated at their expression peaks in BW(eam7) and BW(Ppd-H1, eam7) compared to BW and BW(Ppd-H1) (Fig. 3, A to C). Furthermore, expression peaks of the evening-expressed clock genes LUX1, PRR59, GI, PRR1, and LHY were advanced by 2 to 4 h and strongly reduced in BW(eam7) and BW(Ppd-H1, eam7) (Fig. 3, D, G, I to K). The night-time expression of Ppd-H1, PRR73, PRR59, and PRR95 was released in BW(eam7) and BW(Ppd-H1, eam7), which resulted in high transcript levels in the night and morning (Fig. 3, E to H). This effect was particularly prominent for Ppd-H1.

Since eam7 caused the diurnal misregulation of circadian genes and the central photoperiod response gene Ppd-H1, we further tested the expression of central floral activators in the photoperiod response pathway of barley (Campoli et al. 2012). CO1 expression occurred only during the dark period in BW and BW(Ppd-H1) but was advanced by 4 to 6 h and peaked at the end of the light period in BW(eam7) and BW(Ppd-H1, eam7) (Fig. 3L). CO2 transcripts could not be detected at any time point during the day in any of the genotypes. FT1, typically only expressed under LD, showed detectable, but low levels of expression in BW(Ppd-H1, eam7), while no transcripts could be detected in the other 3 genotypes (Fig. 3M). FT1 expression under SD was thus controlled by eam7 together with allelic variation at Ppd-H1. The upregulation of FT1 in BW(Ppd-H1, eam7) is linked to significantly higher transcript levels of the MADS-box gene and floral inducer VRN1 compared to BW, BW(eam7), and BW(Ppd-H1) (Fig. 3N).

Because FT1 was only expressed in BW(Ppd-H1, eam7), but also BW(eam7) flowered earlier under SD, we tested the expression of FT1 and its homologs FT2 and FT3 at later time points during development. We sampled leaf material every 1 to 2 wk between 20 and 60 DAE at ZT9 (1 h after dusk) in BW and the introgression lines and tested FT1, FT2, FT3, and VRN1 transcript levels. However, we could not detect any FT1 transcripts during development in BW(eam7). We could also not link differences in expression levels to the expression of BW(eam7) under SD compared to BW (Supplemental Fig. S3, A to D).

In conclusion, clock genes and phytochromes displayed marked alterations in diurnal expression patterns. This suggests that the gene underlying eam7 is either a component of the circadian clock or is involved in the light-driven entrainment of the barley circadian clock. The eam7-controlled misexpression of clock genes, together with functional variation in Ppd-H1, were linked to differences in the diurnal expression of Ppd-H1 and CO1, and the upregulation of FT1 in BW(Ppd-H1, eam7) under SD conditions.

Biparental mapping identifies LIGHT-REGULATED WD 1 (LWD1) as a candidate gene for eam7

The recessive eam7 mutation was mapped to the centromeric region of chromosome 6H (Stracke and Börner 1998). For the identification and characterization of eam7, the mutant locus eam7g was backcrossed several times to Bowman to generate the introgression lines BW287 (BC1) and BW288 (BC3, BW(eam7)) (Druka et al. 2011). Both lines are early flowering under SD and were thus proposed to carry the same eam7g mutation (Franczowiak and Lundqvist 2015). Genotyping of BW(eam7) (BW288) with the 1,536 SNP array identified an introgression of 151.1 cm (BOPA 2_0886 to BOPA 1_1261) on chromosome 6H as the likely location of the causative eam7 mutation (Druka et al. 2011). We genotyped BW(eam7) with the 50k SNP array to confirm the large introgression on 6H, which could be separated into 2 individual introgressions (Fig. 4A; Supplemental Table S2, Supplemental Data Set 1). The first introgression (6H-1) is spanning 54.75 cm/380.6 Mbp (position 22 to 382.8 Mbp, Mores V3, Mascher et al. 2021) and contains 802 polymorphic SNPs compared to the recipient parent BW and 2,329 high-confidence (HC) genes. The second introgression could be excluded as a likely location of the eam7 locus since it is located on the long arm of chromosome 6 (Supplemental Table S2).

To confirm and narrow down the eam7 location on 6H, we scored 423 F2 plants from a cross between BW(eam7) and BW(Ppd-H1) for flowering time under SD and genotyped the population for selected SNPs in the introgressed region on 6H (Supplemental Tables S3 and S4). The population showed a bimodal distribution in flowering time, with 89 plants flowering between 36 and 59 d and thus significantly earlier than the 334 remaining lines, which flowered between 77 and 128 d or did not flower until the end of the experiment. We could confirm that all F2 plants that exhibited early flowering under SD carried a homozygous introgression on 6H and were either homozygous or heterozygous for the wild-type Ppd-H1 allele on 2H (Supplemental Data Set 2). The observed segregation ratio corresponded to the expected ratio of 133 (χ² = 1.459, P = 0.2271), assuming a recessive eam7 mutation and a dominant Ppd-H1 wild-type allele. We could reduce the area of introgression 6H-1 to 2.98 cm/286 Mbp (position 96.64 to 382.8 Mbp) containing 1,084 HC genes (introgression 6H-1-reduced, Fig. 4A; Supplemental Table S2). These included 10 genes homologous to Arabidopsis genes with functions in the circadian clock (Arabidopsis 11 annotation, Cheng et al. 2017) (Supplemental Table S5, Supplemental Data Set 3).
Figure 3. Gene expression pattern of circadian clock genes under SD conditions. Relative expression of PHYB, PHYC, ELF3, LUX1, Ppd-H1, PRR73, PRR95, PRR95, GL, PRR1, LHY, CO1, FT1, and VRN1 in Bowman (gray), BW(Ppd-H1) (blue), BW(eam7) (pink), and BW(Ppd-H1, eam7) (yellow) under short-day conditions. Plants were sampled every 2 h from Zeitgeber Time (ZT) 0 to 22. White bars indicate day and black bars indicate night. Each value represents the mean of 3 independent biological replicates, each consisting of 2 pooled plants. Error bars indicate the standard deviation of the mean; significant differences are indicated by asterisks (*P ≤ 0.05) comparing BW and BW(Ppd-H1) to BW(eam7) and BW(Ppd-H1, eam7) (A to L, N) or BW, BW(Ppd-H1), and BW(eam7) to BW(Ppd-H1, eam7) (M) with Student’s t-test, n = 3.
Figure 4. Mapping of eam7 and identification of LWD1 as a candidate gene. A) Overview of the eam7 introgression in parent Bowman (BW), based on 50k illumina Infinium iSelect SNP array. SNPs were mapped to Morex V3 (Mascher et al. 2021). Horizontal gray bars indicate SNPs that do not differ in BW and BW(eam7), while pink bars show SNPs polymorphic in BW(eam7) compared to BW. The vertical black line represents the significant marker interval determined by biparental mapping and its flanking markers (M-13 and M-PRR1), and the red arrow indicates the predicted centromere position (256 Mb, Mascher et al. 2021). Black arrows show the approximate position of candidate genes. B) Overview of putative eam7 candidates related to the term “circadian” within the significant marker interval. C) Schematic overview of the LWD1 gene. LWD1 is a single-exon gene with 5 W40 repeat domains. The eam7 allele has a 32 bp deletion at the end of the coding sequence from position 1,219 to 1,250 (relative to the start codon). The stop codon is indicated in bold. D) Flowering time under short days of F2 plants of a biparental mapping population, segregating for eam7 and Ppd-H1. This shows 100% co-segregation of the 32 bp lwd1 deletion (lwd1 (del)) with early flowering in the presence of a homozygous (white) or heterozygous (light gray) wild-type Ppd-H1 allele. Plants with other allele combinations (homozygous for the mutated Ppd-H1 allele, heterozygous for lwd1(del), or no deletion in lwd1) were grouped together (dark gray). Arrows indicate the approximate mean flowering time of parents BW(Ppd-H1, eam7) (37.8 ± 0.9 d), BW(eam7) (95.6 ± 5.7 d), BW (99.5 ± 6.4 d), and BW(Ppd-H1) (106.1 ± 9.7 d). Fifty-seven of 423 plants did not flower until the end of the experiment (130 DAE).

These candidates included 4 genes which are all associated with early flowering in barley or Arabidopsis: the clock gene PRR1 (HORVU.MOREXr3.6HG0595250), blue light receptor CRYPTOCHROME 1a (CRY1a, HORVU.MOREXr3.6HG0587680), GLYCIN-RICH RNA-BINDING-PROTEIN 1 (GR-RBP1, HORVU. MOREXr3.6HG0592840), and HORVU.MOREXr3.6HG0583670 with high protein sequence identity (79% and 76%) to Arabidopsis LIGHT-REGULATED WD1 (LWD1) and LWD2 (Fig. 4B; Supplemental Fig. S4). Due to the higher sequence similarity to LWD1, we termed the gene HvLWD1.
We Sanger sequenced these candidate genes in BW and the derived introgression lines BW(Ppd-H1), BW(eam7), and BW(Ppd-H1, eam7), the original eam7.g mutant Atsel and its parent, Atlas and the derived introgression donor GSHOS79 which was introgressed into Bowman carrying the eam7 mutation (Druka et al. 2011). CRY1a and GR-RBP1 did not show any SNPs within the coding sequence between BW and BW(eam7) and were therefore excluded as candidates for eam7. For PRR1, we detected 2 non-synonymous SNPs (T215A and S434P) between BW(eam7) and BW (Supplemental Table S5). These SNPs were also present in eam7 genotypes GSHOS79 and Atsel, but likewise in the parent Atlas, which does not carry the eam7 allele (Supplemental Fig. S5A). In addition, genotyping PRR1 in the F2 population revealed one recombinant plant, which was with 104 DAE late flowering but carried the SNP haplotype from the introgressed segment (S434P, marker M-PRR1, Supplemental Table S3). We, therefore, excluded PRR1 as a candidate for eam7. For LWD1, we identified a 32 bp deletion (position 1,219 to 1,252) in BW(eam7) and BW(Ppd-H1, eam7) but not in BW and BW(Ppd-H1) (Fig. 4C; Supplemental Table S5). This deletion is not within a conserved domain but shortens the protein length from 415 to 410 aa. The deletion was also present in eam7 genotypes GSHO 579 and Atsel, but not in Atlas (Supplemental Fig. S5B). The complete co-segregation of LWD1 with the early flowering phenotype was confirmed by genotyping the F2 population for the 32 bp deletion in LWD1 (Fig. 4D; Supplemental Table S3). Sanger sequencing revealed that this deletion, however, was not present in the eam7 introgression line BW287, suggesting that the causative mutations for early flowering under SD differed between BW(eam7) and BW287.

In summary, the genetic mapping reduced the introgression on chromosome 6H and revealed LWD1 as a putative candidate gene for eam7.

CRISPR-generated mutants confirm LWD1 as the gene underlying the eam7 locus

To confirm LWD1 as the gene underlying the eam7 locus, we generated lwd1 mutant plants using CRISPR-Cas9. GP-fast (spring cultivar Golden Promise with a dominant Ppd-H1 introgressed from Igr, Gol et al. 2021) was transformed with 2 different constructs. These targeted either the start or the end of the CDS of LWD1 to create either a complete knockout or a mutation similar to the 32 bp deletion present in BW(eam7). From 16 individual mutation events, 3 homozygous M2 lines were chosen for further experiments: lwd1-26 and lwd1-390, with mutations early in the coding sequence and a total protein length of 26 and 390 aa, respectively, and lwd1-402, with modifications close to the C terminus and a total protein length of 402 aa (Supplemental Fig. S6).

Homozygous M2 and GP-fast plants were grown under SD conditions to test whether the mutant plants were early flowering under SD as observed for eam7. All mutant plants flowered early in comparison to GP-fast: lwd1-26 flowered 45 DAE, followed by lwd1-402 with 51 DAE and lwd1-390 with 67 DAE as compared to GP-fast, which flowered 104 DAE (3 plants) or had not flowered (20 plants) when the experiment was terminated (Fig. 5A). lwd1 shoots and spikes were shorter than in GP-fast, similar to the differences in plant height and spike length between BW(Ppd-H1, eam7) and BW (Fig. 5, B and C). While lwd1 plants produced fewer florets than GP-fast, grain set and spike fertility significantly increased (Supplemental Fig. S7).

We then tested if the lwd1 mutant lines were also altered in phytochrome and clock gene expression as observed for the eam7 genotypes. We grew GP-fast and lwd1 mutant plants under SD for 2 wk and sampled them in 4 h intervals for a complete light–dark cycle. We could confirm that lwd1 mutant plants were characterized by altered diurnal transcript patterns for phytochromes, clock, and flowering time genes comparable to those observed in eam7 plants. The expression of phytochromes and ELF3 showed a strong downregulation in the 3 lwd1 mutant lines compared to GP-fast, as seen in eam7 genotypes compared to BW (Fig. 5, D to F). The peak expression of LUX1, PRR1, and LWD1 itself was reduced and advanced by 4 h in the lwd1 mutants compared to GP-fast, similar to eam7 plants (Fig. 5, G to I; Supplemental Fig. S3E). Ppd-H1 transcription was de-repressed in lwd1 mutants at night, which increased Ppd-H1 expression levels in the night and morning (Fig. 5I). In lwd1 mutants, CO1 expression peaked earlier and at the end of the light phase (Fig. 5K). All lwd1 lines showed low FT1 expression levels, whereas no FT1 transcripts could be detected for GP-fast (Fig. 5L).

To confirm that lwd1 and eam7 are allelic, complementation experiments were performed by crossing homozygous lwd1-26, lwd1-390, and lwd1-402 in the background of GP-fast with homozygous BW(Ppd-H1, eam7). Since eam7 and all 3 lwd1 mutations are recessive, we expected that the resulting F1 plants would display the mutant phenotype if eam7 and lwd1 are allelic. As controls, we also crossed the lwd1 mutants with the parental backgrounds GP-fast and BW(Ppd-H1), which are homozygous for the wild-type EAM7 allele.

All plants carrying at least one functional copy of LWD1 flowered late under SD, between 98 and 114 DAE, or did not flower until the end of the experiment at 125 DAE (Fig. 6A). A larger proportion of GP-fast plants failed to flower compared to BW(Ppd-H1), suggesting that additional minor variation for flowering time under SD segregated between GP-fast and BW. All F1 offspring plants from the 3 lwd1 BW(Ppd-H1, eam7) crosses flowered early under SD (Fig. 6; Supplemental Fig. S8). The F1 hybrids flowered on average 15 d earlier than the homozygous lwd1 mutants in the GP-fast background, likely because BW carried additional variation for early flowering compared to GP-fast as observed in the control crosses. However, as only the lwd1 BW(Ppd-H1, eam7) crosses flowered early under SD and did not show any abortion of the main spike, these results suggested that lwd1 and eam7 are allelic.
In summary, lwd1 mutants phenocopied Bowman eam7 plants. They were early flowering under SD, and the diurnal expression pattern of phytochromes, circadian clock, and flowering genes was altered. Complementation tests suggested that eam7 and lwd1 are allelic.

Natural variation of LWD1
LWD1 belongs to the WD40-repeat proteins, which are conserved across eukaryotes and are involved in highly diverse processes, including flowering and floral development (van Nocker and Ludwig 2003). In Arabidopsis, 2 functionally redundant LWD proteins, LWD1 and LWD2, control photo-period response and flowering time under LD and SD (Wu et al. 2008). Barley and other Triticaceae carry 2 paralogous genes, WD40-1, homologous to LWD1 and LWD2, and WD40-2 (HORVU.MOREX.r3.6HG0604400), homologous to Arabidopsis TRANSPARENT TESTA GLABRA 1 (TTG1) (Strygina and Khlestkina 2019). Comparison of LWD1 protein
sequences across barley, bread wheat (Triticum aestivum) and emmer wheat (Triticum didococcum), Brachypodium distachyon, rice, sorghum (Sorghum bicolor), and maize (Zea mays) demonstrated that the amino acid sequences of LWD1 are highly conserved in grasses (Supplemental Fig. S9, A and B). Furthermore, alignment of the last 15 amino acids from HvLWD1 with 281 homologous sequences from plant WD proteins showed that the protein terminus deleted in eam7 is highly conserved even though located outside the WD repeats (Supplemental Fig. S9C).

We examined natural variation in the coding sequence of LWD1 by exploiting publicly available exome resequencing data from extensive collections of diverse barley germplasm (Russell et al. 2016; Bustos-Korts et al. 2019). Among the 670 investigated barley accessions, we identified 9 haplotypes in a haplotype network analysis (Supplemental Fig. S10, Supplemental Data Set 4). The 2 major haplotypes (I and II) comprised 97.8% of the analyzed accessions and were the only ones identified in elite barley cultivars. Haplotype I included LWD1 from the reference cultivar Morex and haplotype II carried a synonymous SNP. Seven additional minor haplotypes (III-IX) were identified in wild and landrace germplasm. Of these, only haplotypes VIII and IX carried nonsynonymous changes in LWD1 and these were observed in only 4 and 2 genotypes, respectively. Their effect on protein function was assessed using the Sorting Intolerant from Tolerant (SIFT) algorithm (Sim et al. 2012), which predicted tolerated effects on the protein function for both amino acid substitutions (Supplemental Table S6).

In summary, natural variation in the coding sequence of LWD1 was low, and only a few landraces and wild barley accessions showed variation in LWD1 with no or predicted low-effect changes in the LWD1 protein. While we cannot exclude that natural variation in the regulatory sequences existed in the diverse germplasm set, this result suggested that LWD1 is functionally conserved and under strong selection.

**Discussion**

**LWD1 is a candidate for the eam7 locus**

We identified LIGHT-REGULATED WD 1 (LWD1) as a putative candidate gene underlying the eam7 locus. We demonstrate that eam7 in the background of a wild-type Ppd-H1 allele causes rapid flowering and near day-length neutrality under SD.

Stracke and Börner (1998) mapped eam7 close to the centromere on the short arm of chromosome 6H, and Druka et al. (2011) identified an introgression of 151.1 cM on 6H in the background of Bowman as the likely location of the causative eam7 mutation. We narrowed down the genomic location of eam7 to 2.98 cM using a biparental mapping population of a cross between the eam7 introgression line BW(eam7) and the wild-type Ppd-H1 introgression line BW(Ppd-H1). Within this mapping interval with more than 1,000 genes, we identified 4 putative candidate genes with functions in flowering time and circadian regulation based on the current barley reference (Mascher et al. 2021). We would like to point out that we might have excluded genes absent in the reference genome, and genes with unknown new functions in photoperiod control with this approach. Based on genetic mapping and sequencing of the 4 candidate genes, we revealed LWD1 with a 32 bp deletion in the C terminus of the gene as a promising candidate gene underlying the early flowering phenotype under SD (Fig. 4). To confirm LWD1 as a candidate for eam7, we used CRISPR-Cas9 to generate different lwd1 mutants in the genotype GP-fast carrying a wild-type Ppd-H1 allele. All analyzed homozygous lwd1 mutants flowered significantly earlier than the wild-type under SD conditions, irrespective of the size of the protein.
truncation (Fig. 5), confirming that LWD1 controls flowering under SD in barley. In addition, all 3 lwd1 mutants displayed the same changes in diurnal expression patterns of core clock genes and clock output genes as observed for eam7 compared to the wild-type genotypes. Finally, we generated F1 hybrids between BW( Ppd-H1, eam7) and the lwd1 mutant lines to show that lwd1 did not complement eam7, suggesting that both mutations are allelic. The strong effects of C-terminal protein truncations in eam7 and lwd1-402 and the high degree of conservation of the C-terminal amino acids in WD proteins across different taxa indicated that the terminal part of the LWD1 protein is crucial for its function. However, fine-mapping of eam7 down to the 32 bp deletion would provide the ultimate evidence that this C-terminal truncation is causative for the eam7 phenotype.

**EAM7 is important for photoperiod sensing in barley**

We demonstrated that eam7 in the background of a wild-type Ppd-H1 allele causes rapid flowering and near day-length neutrality under SD. We thus concluded that eam7 is important for photoperiod perception in barley. This effect is reminiscent of the early maturity mutants eam5, eam8, and eam10, which are also early flowering under non-inductive photoperiods and were identified as mutant alleles of PHYC and the core clock components ELF3 and LUX1, respectively (Faure et al. 2012; Campoli et al. 2013; Pankin et al. 2014).

In eam7 plants, allelic variation at the major photoperiod response gene Ppd-H1 strongly affected flowering time under SD; eam7 plants with a wild-type Ppd-H1 allele flowered more than 50 d earlier than those with a mutated Ppd-H1 allele (Fig. 1). Furthermore, eam7 plants were characterized by a strong upregulation of Ppd-H1 in the night and the morning, which was linked to the expression of FT1 and early flowering under SD (Fig. 3). We thus propose that eam7 is an upstream transcriptional regulator of Ppd-H1. The upregulation of Ppd-H1 in the night has already been associated with FT1 expression under SD and day-neutral flowering in eam5, eam8, and eam10 and photoperiod insensitive wheat lines (Faure et al. 2012; Shaw et al. 2012; Campoli et al. 2013; Pankin et al. 2014). eam7 thus likely alters photoperiodic flowering by controlling the diurnal expression pattern of Ppd-H1. However, in contrast to eam8 (elf3), which causes day-neutral flowering irrespective of allelic variation at Ppd-H1, eam7 has only a minor effect on flowering time in the background of a mutated Ppd-H1 allele. Early flowering under SD in eam7 mutants was thus dependent on the presence of the wild-type Ppd-H1 allele and the night-time upregulation of Ppd-H1.

It has been demonstrated that the repression of Ppd-H1 at night is controlled by ELF3 (EAM8) and LUX1 (EAM10) in barley and wheat (Faure et al. 2012; Campoli et al. 2013; Alvarez et al. 2023). In Arabidopsis, ELF3 and LUX interact to bind the promoter of the Ppd-H1 ortholog PRR7 to repress the expression during the night (Mizuno et al. 2014), and this function is likely conserved in barley and wheat (Faure et al. 2012; Campoli et al. 2013; Alvarez et al. 2023). ELF3 expression was strongly downregulated in eam7 plants suggesting that LWD1 controls Ppd-H1 expression through ELF3 (Fig. 3). However, EAM7 might also directly regulate Ppd-H1 expression, since in Arabidopsis the paralogs LWD1 and LWD2 directly bind to the promoters of PRR genes (Wang et al. 2011). In contrast to EAM7 in barley, Arabidopsis LWD1 and LWD2 act as positive regulators of PRR expression (Wang et al. 2011). In addition to ELF3, we also observed the downregulation of PHYC and PHYB in eam7 plants. PHYB and PHYC are necessary for the light activation of Ppd-H1 and act as upstream repressors of ELF3 in barley, wheat, and Brachypodium (Chen et al. 2014; Pankin et al. 2014; Alvarez et al. 2023; Woods et al. 2023). EAM7 might therefore affect flowering time by modifying the expression of phytochromes and ELF3 and, thus, the light input into the photoperiod pathway.

In Arabidopsis thaliana, LWD1 and LWD2 regulate photoperiodic flowering by advancing the expression phase of core clock and clock output genes under light/dark conditions (Wu et al. 2008). The early flowering phenotype of the Arabidopsis lwd1lwd2 double mutant was attributed to a phase shift of the clock target and central photoperiod response gene CONSTANS (CO) and a consequent increase in FT expression. Similarly, in eam7 mutants, the expression phase of evening-expressed clock genes PRR59/95 and GI shifted approximately 4 h forward, suggesting that LWD1 controls the expression phase of the central oscillator genes in barley (Fig. 3). This phase shift was associated with a forward shift and day-time expression of CO1 under SD in the eam7 mutants (Fig. 3). In Arabidopsis, the coincidence of CO expression with the light period is crucial for stabilizing the protein and expressing the florigen FT (Sawa et al. 2007; Jang et al. 2008). Similar to the Arabidopsis lwd1lwd2 double mutants, the eam7 mutants with an altered diurnal expression of CO1 were characterized by FT1 expression in non-inductive photoperiods. However, in barley and wheat, CO1 mainly acts as a weak heading time repressor and accelerates flowering only in the absence of Ppd-H1 (Shaw et al. 2020). Furthermore, night-break experiments have revealed that the length of the night and not of the light period is critical for the perception of inductive photoperiods in monocots (Pearce et al. 2017; Gao et al. 2019). Furthermore, BW(eam7) also displayed a shift in CO1 phase expression into the day as observed in BW(Ppd-H1, eam7), but no FT1 expression and only a minor acceleration in flowering under SD. Night-breaks during long nights cause the rapid up-regulation of Ppd-H1, followed by FT1 expression and flowering (Pearce et al. 2017). Similarly, Ppd-H1 is up-regulated during a long night in eam7 and lwd1 mutants but strongly downregulated in wild-type plants. These parallels in the molecular and flowering phenotype between night-break experiments and eam7/lwd1 mutants suggested that eam7 alters photoperiod sensing or downstream signaling. Further, the night-time de-repression of Ppd-H1 either by night-breaks or mutations in upstream regulators such as eam7 might thus be relevant for early flowering under SDs
(Fae et al. 2012; Campoli et al. 2013; Pankin et al. 2014; Pearce et al. 2017). Nevertheless, the advance in phase expression of clock and clock output genes was comparable between barley eam7 mutants and Arabidopsis lwd1 lwd2 double mutants, and the function of LWD1 in controlling the light entrainment of the clock, is thus likely conserved across these taxa.

In summary, we have successfully identified LWD1 as a promising candidate to underlie the eam7 locus. We propose that LWD1 functions as an upstream activator of the night-time repressor ELF3 in the light entrainment pathway of the barley circadian clock. eam7 plants were early flowering in non-inductive photoperiods due to the reduced activation of ELF3, the upregulation of Ppd-H1 during the night, and consecutive FT1 expression under SD. LWD1 is an interesting target to modulate photoperiod sensitivity to breed for barley cultivars adapted to short growing seasons.

Materials and methods

Plant material
Barley (Hordeum vulgare) spring cultivar Bowman (BW) with a mutated Ppd-H1 allele and 3 Bowman-derived introgression lines were used in this study. The introgression line BW281 (GSHO 1872) carries a wild-type Ppd-H1 allele introgressed from winter barley KT1031 (GSHO 1568) (Druka et al. 2011). BW288 (GSHO 2068, NGB 20572) is an introgression line with the eam7g mutation introgressed from Club Mariout/6*California Mariout (GSHO579, Gallagher et al. 1991) in Bowman by 3 rounds of backcrossing (Druka et al. 2011). We termed these introgression lines BW(Ppd-H1) and BW(eam7). We crossed BW(Ppd-H1) and BW(eam7) to generate a line with a wild-type Ppd-H1 allele and the mutation at eam7g. The F2 generation of this cross was grown under SD to identify early flowering plants homozygous for the wild-type Ppd-H1 allele and the eam7 introgression. The Ppd-H1 allele was determined by using the CAPS marker designed by Turner et al. (2005) (Supplemental Table S4), and the presence of the eam7 introgression was genotyped based on the 32 bp deletion in LWD1 and a CAPS marker placed within the introgression (M-eam7, Supplemental Table S4). An early flowering plant homozygous for eam7 and Ppd-H1 was used for single seed propagation by selfing the plants twice until used for experiments. We termed this genotype BW(Ppd-H1, eam7).

The F2 generation of the cross between BW(Ppd-H1) and BW(eam7) was used for segregation analysis and mapping of eam7. Furthermore, the eam7 introgression donor GSHO579 and the original eam7g mutant Atsel (Clho 6250) and its genetic background Atlas (PI 539108) were included to sequence potential candidate genes. We also included BW287 (NGB 20571), which was generated by backcrossing Club Mariout/6*California Mariout with eam7g into Bowman by 2 cycles of crossing and was thus reported to be allelic to BW(eam7) (Druka et al. 2011).

The introgression line GP-fast, spring cultivar Golden Promise with a dominant Ppd-H1 allele introgressed from winter cultivar Igri (Gol et al. 2021), was transformed to generate mutants in LIGHT-REGULATED WD 1 (LWD1, HORVU.MOREX.r3.6H0583670) using CRISPR-Cas9. Three independent homozygous M2 lines, lwd1-26, lwd1-390, and lwd1-402, were selected for functional analyses and crossing to BW(Ppd-H1, eam7). lwd1-26 has 2 deletions within the CDS (--C21, --C23) and one single insertion (+T114) that lead to a frameshift and premature stop codon, reducing the protein from 415 (WT) to 26 amino acids (aa). lwd1-390 has 2 deletions (66 bp, position 15 to 80 and 9 bp, position 109 to 117) that are in frame and lead to the deletion of amino acids 6 to 27 and 37 to 39, reducing the protein size to 390 aa. lwd1-402 has a 39 bp deletion (position 1,198 to 1,238) that reduces protein length to 402 aa.

Plant phenotyping and growth conditions
BW and the derived introgression lines BW(Ppd-H1), BW(eam7), and BW(Ppd-H1, eam7) were cultivated under controlled growth conditions for phenotyping and gene expression analyses. All plants were grown in soil (Einheitserde ED73, Einheitserde Werkverband e.V., with 7% sand and 4 g/L Osmocote Exact Hi.End 3 to 4 m, 4th generation, ICL Group Ltd.) and were stratified for 4 d in 4 °C before moving them to controlled growth conditions.

For phenotyping, plants were grown in plant growth chambers under long-day (LD, 16 h light, 20 °C, photosynthetically active radiation (PAR) ~250 μmol m−2 s−1; 8 h dark, 16 °C) and short-day (SD, 8 h light, 20 °C, PAR ~250 μmol m−2 s−1; 16 h dark, 16 °C) conditions in QuickPot E 24/10 trays (HerkulPlast Kubern GmbH). Flowering was scored in DAE as the period between emergence from soil and reaching Zadoks stage 49 when the awns exited the leaf sheath (Zadoks et al. 1974). Plant height, leaf number on the first emerging shoot (main culm), and tiller number were scored at flowering. Plant height was scored as the distance from soil to the flag leaf ligule of the main culm. Leaf number was scored on the main culm, and tiller number as all tillers emerging after the main culm. The length and width of the flag leaf on the main culm were measured as the leaf blade length (from the ligule to the leaf tip) and the maximum width of the blade under SD. Spike length, floret number, and grain number of the main culm were scored at maturity. Fertility was calculated as the percentage of florets on the mature spike that developed into grains. The experiment was stopped 125 DAE, and all plants that had not flowered up to this point were scored as “not flowering”.

MSA development of all 4 genotypes was monitored and quantified based on the scale by Waddington et al. (1983). Once or twice a week, the development of the MSA of the main stem of 4 randomly chosen plants per genotype was dissected. MSA development was documented using the stereo microscope Nikon SMZ18 with a Nikon DS-Fi2 camera and was analyzed with the NIS-Elements Software (version 5.2.1.03, Nikon Instruments Europe BV). Under LD, plants were dissected every 2 to 10 d starting from 5 DAE until pollination (W10.0). Under SD, plants were dissected every 6 to
8 d between 6 and 47 DAE and every 4 to 13 d from 48 DAE until pollination. The number of developing SM, including those that had initiated floret meristems (FM) or developed into florets, was determined on the main inflorescence. The R package segmented (version 1.6-2, Muggeo 2008) was used to calculate broken-line regressions for SM initiation and FM abortion by plotting the number of SM against the Waddington stage. One breakpoint was calculated automatically to separate initiation from abortion and was set as the maximum SM number stage. The corresponding maximum SM number was calculated using linear regression calculated with segmented.

The *lwd1* mutant and wild-type plants were grown in controlled plant growth chambers under SD in QuickPot 96 T trays (HerkuPlast Kubern GmbH) until maturity. Flowering, plant height, leaf and tiller number, leaf size, and yield parameter were scored as described above. Plants that did not flower until 130 DAE were scored as “not flowering”.

Gene expression analysis

Gene expression analysis was performed in 2 independent experiments in Bowman and derived introgression lines BW(Ppd-H1), BW(eam7), and BW(Ppd-H1, eam7) and in the CRISPR-Cas9-generated mutants *lwd1-*6, *lwd1-*390, *lwd1-*402, and wild-type GP-fast.

Plants were sown in QuickPot 96 T trays (HerkuPlast Kubern GmbH) and transferred to plant growth chambers after 4 d of stratification at 4 °C. Plants were cultivated under SD conditions until 14 DAE. BW and derived introgression lines were sampled every 2 h, the *lwd1* mutants and wild-type GP-fast every 4 h over a complete light/dark cycle of 24 h (light from ZT0-8, dark from ZT8-24). For each replicate, the middle sections of the youngest, fully elongated leaf of 2 plants were pooled. Three biological replicates were sampled, frozen immediately in liquid nitrogen, and stored at −80 °C until further analysis. In addition, the middle sections of the youngest, fully elongated leaf of the same genotypes were sampled at ZT9 once a week for 6 wk, starting at 19 DAE. These leaf samples were used to monitor *Actin* (Thermo Fisher Scientific) according to the manufacturer’s instructions. cDNA was synthesized on 2 g of total RNA (Thermo Fisher Scientific) according to the manufacturer’s instructions. RNA was resuspended in 60 µL of diethyl dicarbonate-treated water at 4 °C overnight. The remaining DNA was removed by subsequent DNAse I treatment (Thermo Fisher Scientific) according to the manufacturer’s instructions. cDNA was synthesized on 2 µg of total RNA using ProtoScript II First Strand cDNA Synthesis Kit (NEB) following the manufacturer’s instructions. Gene expression levels were determined by RT-qPCR in a LightCycler 480 (Roche) using gene-specific primers (Supplemental Table S7). The reaction was performed using 4 µL of cDNA, 5 µL of 2X Luna qPCR Master Mix (NEB), 0.02 mM of forward and reverse primer, and 0.75 µL of water with the amplification conditions 95 °C for 5 min, 40 cycles of 95 °C (10 s), 60 °C (10 s) and 72 °C (10 s). Non-template controls were added to each plate, and dissociation analysis was performed at the end of each run to confirm the specificity of the reaction. Starting amounts for transcript levels were calculated based on the titration curve for each target gene using the LightCycler 480 Software (Roche; version 1.5.1.62). Two technical replicates were used and averaged in analyses for each biological replicate. The expression of *Actin* was used as a reference to calculate the relative gene expression of the target genes.

Identification of a candidate gene underlying *eam7*

DNA was extracted from BW(eam7) leaf using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer’s instructions, and plants were genotyped with the 50k Illumina Infinium iSelect SNP Array (Bayer et al. 2017). The *eam7* introgression area was visualized by comparing the BW(eam7) allele to the Bowman allele for each SNP. SNPs were plotted against their respective position within the Morex V3 genome assembly in Megabase pairs (Mb, Mascher et al. 2021). Ambiguous (including heterozygous) and failed SNPs were removed. The *eam7* introgression was defined as the region between the flanking SNPs carrying the Bowman allele.

For segregation analyses, the Bowman introgression lines BW(eam7) and BW(Ppd-H1) were crossed, and 423 F2 plants together with 9 plants each of BW, BW(eam7), and BW(Ppd-H1) were sown in QuickPot E24/10 trays. After 4 d of stratification at 4 °C, plants were cultivated under SD conditions, and DNA was extracted using the KingFisher Flex (Thermo Fisher Scientific) and the BioSprint 96 DNA Plant Kit (QIAGEN). Flowering time was scored as described above. Plants that did not flower until the end of the experiment (130 DAE) were scored as “not flowering”.

Based on the introgression area determined with the 50k SNP array, several CAPS markers were designed on chromosome 6 with indCAPS and Primer3Plus to localize *eam7* (Untergasser et al. 2007; Hodgens et al. 2017). In addition, the CAPS marker designed by Turner et al. (2005) was used to determine whether the plants carried a wild-type or mutated *Ppd-H1* allele. Co-segregation of early flowering with a 32 bp deletion in *LWD1* was tested by amplifying the surrounding area with the primer pair *lwd1-del* and comparing fragment sizes on an agarose gel. All used CAPS markers and primers are listed in Supplemental Table S4.

*eam7* candidate genes *CRY1a*, *GR-RBP1*, *LWD1*, and *PRR1* were amplified in Bowman and the derived introgression lines BW(Ppd-H1), BW(eam7), BW287 and BW(Ppd-H1, eam7), in GSHO 579 and the original *eam7* mutant *Atsel* and its parent Atlas. The full genomic sequence was Sanger sequenced to identify mutations. Primers used for amplification and Sanger sequencing can be found in Supplemental Table S8.

Generating *lwd1* mutants using CRISPR-Cas9

To confirm *LWD1* as a candidate gene for *eam7*, *lwd1* mutants were generated using CRISPR-Cas9. The vector system by Kumar et al. (2018) was used to design transformation vectors targeting *LWD1*. Two approaches were used:
Approach 1 included 2 guide RNAs (gRNAs) targeting the start of the CDS of LWD1 (CDS position 7 and 97). The second approach targeted the end of the coding sequence (CDS position 1,182 and 1,222, Supplemental Table S9) to generate mutations comparable to BW(eam7). gRNAs were designed using RGEN Tools Cas-Designer (Ba et al. 2014; Park et al. 2015). Cloning was performed according to the protocol by Kumar et al. (2018): The single gRNA strands were hybridized and cloned into the shuttle vectors pMGE625 or pMGE627 by a BpiI cut/ligation reaction. A second cut/ligation reaction (Bsal) was used to transfer the gRNA transformation units (TUs) to the recipient vector pMGE599. The final vectors were used to transform GP-fast via embryo transformation according to the protocol by Hensel et al. (2009). Successful insertion of the transformation vector into the genome was tested by PCR (primer Hyg-156 and Hyg-047, Supplemental Table S8) on M0 plants. M2 plants were genotyped for mutations by amplifying the entire genomic sequence of LWD1 (primer LWD1_72us_F and LWD1_21ds_R) and subsequent Sanger sequencing (Supplemental Table S8). Three lines that showed different mutations were selected for further experiments and were termed lwd1-26, lwd1-390, and lwd1-402.

Allelism tests
Allelism tests were performed by generating F1 crosses of the mutant lines lwd1-26, lwd1-390, and lwd1-402 with BW(Ppd-H1, eam7). As controls, plants were crossed with BW(Ppd-H1) and GP-fast. Five to ten plants per cross of the resulting F1 generation were grown in 7 × 7 × 7.5 cm pots with the parental plants under SD conditions in plant growth chambers, and flowering time was scored as described above. The experiment was terminated after 125 DAE and all plants that did not flower were scored as “not flowering”. DNA was extracted from all F1 and parent plants using the KingFisher Flex (Thermo Fisher Scientific) and the BioSprint 96 DNA Plant Kit (QIAGEN). The complete LWD1 CDS was amplified by PCR and sequenced using Sanger sequencing with the same primers described above (Supplemental Table S8).

Natural haplotype
A haplotype network analysis was conducted based on combined exome resequencing data from Russell et al. (2016) and the WHEALBI collection (Bustos-Korts et al. 2019). This combined set includes 213 cultivars, 303 landraces, 111 wild barley accessions (H. vulgare ssp. spontaneum), one H. agricriothion accession, and 42 H. vulgare ssp. vulgare accessions with unassigned breeding history (referred to as “unknown”). The haplotype network was constructed as described by Walla et al. (2020).

Putative orthologs of HvLWD1 in other grasses were identified in the Ensembl Plants database (Bolser et al. 2016). The multiple protein sequence alignment was performed using CLUSTAL Omega (1.2.4) (Madeira et al. 2022), and conserved domains were identified with NCBI conserved domains (Marchler-Bauer et al. 2017). Sequence conservation analysis was performed as described in Pankin et al. (2014). The last 15 amino acids from HvLWD1 were used to extract 281 sequences of proteins annotated as WD proteins from plants using NCBI Blastp (e-value cutoff: 0.05). Sequences were mapped using MAFFT v7 (“auto” method, Katoh et al. 2019), and the sequence logo was visualized with WebLogo 3 (Crooks et al. 2004).

Statistical analyses
All statistical tests were performed using R Studio (RStudio Team 2022). A 2-tailed, paired Student’s t-test (function t.test from the package rstatix, v0.7.2) was used to determine the significance between 2 group means, with a P-value cutoff at ≤ 0.05. Significance between more than 2 groups was determined using a one-way ANOVA (function aov) and a subsequent Tukey test (function HSD.test from package agricolae, v1.3-5), P-value cutoff at ≤ 0.05. Polynomial regressions (Loess smooth line) were calculated with a 95% confidence interval.

Accession numbers
Accession numbers of genes described in the text can be found in Supplemental Table S10.

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Author contributions
Gesa H. and M.v.K. conceived and designed the experiments. Gesa H. conducted plant phenotypic analyses, generated BW(Ppd-H1, eam7) crosses, designed CAPS markers, cloned CRISPR-Cas9 transformation vectors, did sequence analyses, and analyzed the data. A.W. performed the haplotype analysis. T.R. and Gesa H. performed RT-qPCR experiments and genotyping of the mapping population. G.B. and Götz H. transformed plants and regenerated lwd1 mutants. R.S. generated allelic lwd1 crosses. Gesa H. wrote the manuscript with the help of A.W. and M.v.K.

Supplemental data
The following materials are available in the online version of this article.

**Supplemental Figure S1.** Effect of eam7 on spikelet meristem (SM) number.

**Supplemental Figure S2.** Effects of eam7 and Ppd-H1 on plant architecture under LD and SD.

**Supplemental Figure S3.** Gene expression pattern of FT-like genes, VRN1 over development, and diurnal expression of LWD1 under SD conditions.
Supplemental Figure S4. Amino acid sequence comparison of HvLWD1 with AtLWD1 and AtLWD2.

Supplemental Figure S5. Nucleotide alignment of eam7 candidate genes.

Supplemental Figure S6. Protein sequence alignment of wild-type, eam7, and lwd1 mutants.

Supplemental Figure S7. Effect of lwd1 mutations on spike morphology under SD.

Supplemental Figure S8. Allelism tests of lwd1 and eam7 mutants.

Supplemental Figure S9. Sequence comparison of barley LWD1 orthologs in grasses.

Supplemental Figure S10. Median-joining network of 9 LWD1 haplotypes identified in a diverse collection of 670 barley accessions.

Supplemental Table S1. Spikelet meristem number on MSA of plants grown under LD and SD.

Supplemental Table S2. Introggression areas on chromosome 6 in BW(eam7).

Supplemental Table S3. Mapping of eam7 in a segregating F2 population.

Supplemental Table S4. CAPS and PCR marker used to map the position of eam7.

Supplemental Table S5. Candidate genes for eam7.

Supplemental Table S6. SIFT results for LWD1 haplotypes.

Supplemental Table S7. RT-qPCR primer used in this study.

Supplemental Table S8. PCR primer used in this study.

Supplemental Table S9. Guide RNAs for CRISPR-Cas9 used in this study.

Supplemental Table S10. NCBI GenBank accession numbers of genes.

Supplemental Data Set 1. SNP data from the 50k Illumina iSelect SNP Array.

Supplemental Data Set 2. Flowering time and results of genotyping of biparental mapping population in short-day conditions.

Supplemental Data Set 3. High-confidence genes (Morex V3) within the introgression “6H-1-reduced”.

Supplemental Data Set 4. List of accessions used for haplotype analysis and the respective haplotype.

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Conflict of interest statement. None declared.

Data availability
The data underlying this article are available in the article and in its online supplementary material.

References
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