HvLUX1 is a candidate gene underlying the early maturity 10 locus in barley: phylogeny, diversity, and interactions with the circadian clock and photoperiodic pathways

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Summary

- Photoperiodic flowering is a major factor determining crop performance and is controlled by interactions between environmental signals and the circadian clock. We proposed HvLUX1, an ortholog of the Arabidopsis circadian gene LUX ARRHYTHMO, as a candidate underlying the early maturity 10 (eam10) locus in barley (Hordeum vulgare L.).
- The link between eam10 and HvLUX1 was discovered using high-throughput sequencing of enriched libraries and segregation analysis. We conducted functional, phylogenetic, and diversity studies of eam10 and HvLUX1 to understand the genetic control of photoperiod response in barley and to characterize the evolution of LUX-like genes within barley and across monocots and eudicots.
- We demonstrate that eam10 causes circadian defects and interacts with the photoperiod response gene Ppd-H1 to accelerate flowering under long and short days. The results of phylogenetic and diversity analyses indicate that HvLUX1 was under purifying selection, duplicated at the base of the grass clade, and diverged independently of LUX-like genes in other plant lineages.
- Taken together, these findings contribute to improved understanding of the barley circadian clock, its interaction with the photoperiod pathway, and evolution of circadian systems in barley and across monocots and eudicots.

Introduction

Barley (Hordeum vulgare L.) is an important crop cultivated in a wide range of environments and has recently emerged as a model species for Triticeae as a result of its diploid nature and abundance of genomic resources (International Barley Sequencing Consortium (IBSC), 2012). Genetic variation in photoperiod response was crucial for the successful expansion of barley cultivation from its origin in the Fertile Crescent to northern latitudes (Turner et al., 2005; Jones et al., 2008). Barley is a facultative long-day plant; long days (LDs) promote flowering in spring, while short days (SDs) delay reproductive development. Flowering under LDs is controlled by the major photoperiod response gene PHOTOPERIOD 1 (Ppd-H1) (Turner et al., 2005). Ppd-H1 is homologous to PSEUDO-RESPONSE REGULATOR (PRR) genes implicated in the circadian clock of the model species Arabidopsis thaliana (hereafter Arabidopsis, Turner et al., 2005). The dominant allele of Ppd-H1 is prevalent in the wild barley progenitor H. vulgare spp. spontaneum and in Mediterranean cultivated barley genotypes and accelerates flowering under LDs, as an adaptation to short growing seasons. A natural mutation in the conserved CCT domain of Ppd-H1 causes a reduced response to LDs and was selected for adaptation to long growing seasons (Turner et al., 2005; von Korff et al., 2006, 2010; Jones et al., 2008; Wang et al., 2010). Under LD conditions, Ppd-H1 up-regulates HvFT1, which is the barley counterpart of the Arabidopsis ‘florigen’ FLOWERING LOCUS T (FT). Delayed flowering in genotypes with a mutated ppd-H1 allele was associated with reduced expression of HvFT1 (Turner et al., 2005; Campoli et al., 2012a).

Barley early maturity mutants (eam) with a reduced or no response to photoperiod have been isolated and used in Swedish, Australian, and South American breeding programs to adapt cultivars to short growing seasons (Laurie et al., 1995; Lundqvist, 2009; Zakhрабекова et al., 2012). These eam mutants have been introgressed into the spring barley cv Bowman (Druka et al., 2011) and represent a valuable resource to decipher the genetic control of photoperiod response in the model crop barley. Recently, HvELF3, a homolog of the Arabidopsis circadian clock regulator EARLY FLOWERING 3 (ELF3), was identified as a gene underlying the eam8 quantitative trait locus (QTL), which causes a day-neutral early flowering phenotype (Faure et al., 2012; Zakhрабекова et al., 2012). Interestingly, the mutation in HvELF3 caused an up-regulation of Ppd-H1 and the downstream HvFT1 under noninductive SD conditions (Faure et al., 2012). In addition, the Hvelf3 mutants were severely compromised in...
the expression of clock oscillator and output genes. This study suggested that circadian clock homologs play an important role in the control of flowering in barley. Circadian clocks synchronize biological processes with the diurnal cycle, using molecular mechanisms that include interlocked transcriptional feedback loops. In Arabidopsis, the circadian clock is composed of three negative feedback loops: the inhibition of evening complex (EC) genes ELF3, EARLY FLOWERING 4 (ELF4), and LUX ARRHYTHMO (LUX, also known as PHYTOCLOCK1) by the rise of CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) late at night; the inhibition of PRR genes by the EC early at night; and the inhibition of LHYCCA1 by TIMING OF CAB EXPRESSION1 (TOC1) in the morning (Kolmos et al., 2009; Huang et al., 2012; Nagel & Kay, 2012; Pokhilko et al., 2012). Furthermore, the evening-expressed GIGANTEA (GI) protein was modeled as a negative regulator of the EC, which in turn inhibits TOC1 expression (Herrero et al., 2012; Pokhilko et al., 2012).

Campoli et al. (2012b) have shown that circadian clock genes are structurally conserved between barley and Arabidopsis, and their circadian expression patterns suggested conserved functions. However, phylogenetic analyses revealed that duplications/deletions of clock genes occurred throughout the evolution of eudicots and monocots. For example, the ancient three PRR clades expanded independently in monocots and eudicots, supposedly as a result of paleoduplications (Takata et al., 2010; Campoli et al., 2012b). In monocots, these events gave rise to two paralogous pairs of PRR genes, termed PRR3/PRR73 and PRR59/95. In this context, it is noteworthy that PRR37 orthologs in monocots, PPD1 in barley and wheat (Turner et al., 2005; Beales et al., 2007) and SbPRR37 in sorghum (Sorghum bicolor) (Murphy et al., 2011), are major determinants of photoperiod sensitivity and flowering time, while natural variation in PRR genes in Arabidopsis did not have a strong effect on flowering time (Ehrenreich et al., 2009). These results indicated that the molecular function of genes participating in the circadian clock and photoperiod response diverged and specialized in a lineage-specific manner. Because of the prominent role of photoperiod insensitivity in breeding, it is essential to identify the genetic components controlling this pathway in temperate cereals.

We present the characterization of the barley eam10 mutation, which causes an early-flowering phenotype under both SDs and LDs. eam10 was described as an X-ray-induced mutation in Super Precox (2H) of unknown parentage (Favret & Ryan, 1966; Gallagher et al., 1991; Gallagher & Franckowiak, 1997) and was mapped onto chromosome 3HL (Börner et al., 2002). Using a high-throughput sequencing approach, we identified the candidate gene underlying the eam10 locus in barley as HvLUX1, a barley ortholog of the Arabidopsis circadian clock regulator LUX. We demonstrated that eam10 caused circadian defects and interacted with the major barley photoperiod response gene Ppd-H1 to accelerate flowering under LD and SD conditions. Based on the results of phylogenetic and diversity analyses, we conclude that HvLUX1 was under purifying selection, duplicated at the base of the grass clade, and diverged independently of the LUX-like genes in other plant lineages.

Materials and Methods

Plant material and growth conditions

Flowering time (days to emergence of the main spike awns) of the barley spring cv Bowman and three Bowman-derived introgression lines Bowman(Ppd-H1), Bowman(eam10), and Bowman(Ppd-H1+eam10) (kindly provided by R. Waugh, James Hutton Institute, and by David Laurie, John Innes Centre, UK) was recorded for 15-18 plants per genotype. To score flowering, plants were grown in soil in a glasshouse under SDs (8 h light, 20°C: 16 h dark, 18°C) and LDs (16 h light, 20°C: 8 h dark, 18°C). To investigate expression levels of HvFT1, plants were sampled 2 h before light-off after 14 and 28 d under LDs and SDs, respectively. Diurnal and circadian expression of core clock and flowering time genes was tested under SD and free-running conditions in Bowman and Bowman(eam10). Plants were grown in soil in a controlled-environment growth chamber (CEGC). After 21 d under SDs, leaf material was harvested every 2 h for a total of 24 h from the start of the light period (time point T0). Night samples (T10 to T22) were collected in the dark. After SDs, plants were released into continuous light and constant temperature (20°C: LL) and sampled every 2 h for 48 h, starting after 8 h of continuous light (T8). Two biological replicates, comprising the second youngest leaves of three independent plants, were analyzed. All samples were immediately frozen in liquid nitrogen and stored at −80°C until processed.

Meristem development was scored in a separate experiment with Bowman and Bowman(eam10) grown in soil in a CEGC. The main stem of three plants per genotype was dissected starting 7 d after germination every 2–3 d under LDs and every 3–4 d under SDs until flowering. After day 79 under SDs the experiment was stopped when the shoot apical meristems of the remaining plants had died. Meristem development was scored following the Waddington scale (Waddington et al., 1983). To generate developmental series for the analysis of HvFT1 expression in the leaf and to link it to specific meristem stages, two biological replicates were harvested 2 h before light-off at every developmental stage under both photoperiods.

To investigate natural diversity of a candidate gene, a set of 88 wild (H. vulgare ssp. spontaneum and H. agriocriton) and cultivated barley genotypes was selected from germplasm collections of the Max Planck Institute of Plant Breeding Research in Cologne, Germany (Badri et al., 2000) and the Barley 1K collection (Hüblner et al., 2009; Supporting Information, Table S2).

Gene expression analysis

Gene expression was analyzed in leaf samples harvested from Bowman, Bowman(Ppd-H1), Bowman(eam10), and Bowman(Ppd-H1+eam10) grown under LDs and SDs, from a developmental series of leaf samples from Bowman and Bowman(eam10), and from diurnal and circadian sampling of Bowman and Bowman(eam10) leaves. Total RNA extraction, cDNA synthesis, and quantitative reverse transcription polymerase chain reactions (qRT-PCRs) using gene-specific primers were
performed as explained in Campoli et al. (2012b). Expression of HvLUX1 was analyzed using the following primer combinations: LUX_1077F 5′-AATTCAGTCCACGGATGCTC-3′ and LUX_1289R 5′-CTTCACCTCGATCCTCCCTT-3′.

Identification and characterization of a candidate gene underlying eam10

To identify a candidate gene for the eam10 QTL, we sequenced a set of flowering-related genes from the genotypes Bowman and Bowman(eam10) using high-throughput sequencing of multiplexed enriched libraries. A sample of pooled barcoded TruSeq libraries was prepared following the standard Illumina protocol with modifications (Methods S1). The pooled library was enriched for flowering-related genes using the SureSelect target enrichment system (Agilent Technologies, Böblingen, Germany) according to the manufacturer’s recommendations with the following modification. To alleviate biased PCR amplification of GC-rich regions, the post-enrichment amplification of the library was performed using the ‘long denaturation’ Phusion (Thermo Scientific, St Leon-Rot, Germany) protocol (Aird et al., 2011). The enriched library was paired-end-sequenced on a HiSeq 2000 machine (Illumina, San Diego, CA, USA).

The genes for targeted enrichment were selected by following two approaches. First, known flowering-related barley genes and gene families were directly extracted from NCBI GenBank (Table S3a). Second, additional barley genes were selected based on nomenclature and annotation of Brachypodium genes as determined by the Blastx search in HarvEST assembly 35 (http://harvest.ucr.edu) (Table S3b). The nomenclature and annotation of Brachypodium genes were as reported by Higgins et al. (2010). Selected barley genes were used as a template to design a library of baits for the solution-based target enrichment approach and as a reference target genome for mapping in subsequent bioinformatics analyses.

Read datasets were preprocessed using a set of filters (adapter trimming, quality and length, sequencing artefacts, contamination) using the Galaxy server at Wageningen University, the Netherlands (http://galaxy.wur.nl; Methods S2) and mapped to the reference target genome as a single-end dataset using BWA (Li & Durbin, 2009a). Reads mapped to several locations and PCR duplicates were removed using ‘view’ and ‘rmdup’ functions of SAMtools 0.1.18 (Li et al., 2009b). Mean depth of coverage was estimated using BAMstats 1.25 (http://bamstats.sourceforge.net). Polymorphisms were discovered using ‘IndelRealigner’ and ‘UnifiedGenotyper’ algorithms of the GATK 2.1.3 package (DePristo et al., 2011). A set of stringent ‘hard’ filters were applied to the raw single nucleotide polymorphism (SNP) set to select reliable SNPs (Methods S3).

Genes polymorphic between Bowman and Bowman(eam10) were located on barley linkage groups using GenomeZipper (Mayer et al., 2011). The location of the eam10 QTL on the barley consensus genetic map (OPA123-2008 consensus; Close et al., 2009) was extracted from the GrainGenesCMap browser (http://wheat.pw.usda.gov/cgi-bin/cmap). The position of the MYB domain on HvLUX1 was determined using InterProScan (Quevillon et al., 2005).

Segregation analysis

A set of 1002 BC1,F2 plants derived from backcrossing Bowman (eam10) to Bowman were grown in the field in Cologne in 2012 (spring sowing). A total of 215 (21%) plants were scored as early flowering and seeds were harvested. Early-flowering BC1,F2,3 lines were sown in the glasshouse under LDs. Leaf samples were harvested and flowering time was scored. Barley genomic DNA was extracted using the BioSprint 96 kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations and quantified using Quant-iT™ microplate reader (BioTek, Bad Friedrichshall, Germany). A full-length coding sequence (CDS) of HvLUX1 was amplified using specific primers LUX_135F-T3 5′-aatatccttacac taagggTGCGCGGTAAGTGGATTC-3′ LUX_1108R-T7 5′-aatacagcactaragggGAGCAGAGGAGGACATCCCC-3′ with T3 and T7 overhangs for sequencing (in lower case). PCR reactions (0.5× HF buffer, 0.16 μM dNTPs, 0.5 μM primers, 0.8 U Phusion Hi-Fi polymerase (Thermo Scientific), 100 ng DNA) were incubated in the PTC DNA Engine thermocycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 95°C for 3 min; 30 cycles of 95°C for 20 s, 60°C for 30 s, 72°C for 1 min; 72°C for 5 min. PCR fragments were purified using the QIAquick PCR purification kit following the manufacturer’s recommendations and Sanger-sequenced. The sequences were assembled using SeqMan software (DNASTAR Lasergene® 8 Core Suite, Madison, WI, USA).

Multiple alignment and phylogeny reconstruction

A barley cDNA, HvLUX2 homologous to HvLUX1 was extracted from NCBI GenBank using Blastn search in the nr/nt database and, because of the misannotation of the LUX2 cDNA in GenBank, it was manually translated. HvLUX1 and HvLUX2 proteins were further used as NCBI Blastp queries to extract homologs from other plant species (E-value cutoff 10⁻³³; last search on 22 November 2012). In addition, LUX-like protein from S. bicolor (Sb03 g039610) was retrieved from the Gramene database (http://www.gramene.org/Sorghum_bicolor). One representative per taxa was kept in case several identical sequences were retrieved. The nomenclature and accession numbers of analyzed LUX-like sequences (species – genotype [database accession number]) are as follows: A. thaliana: At1 – Columbia (LUX1, NP_001190022; NOX1a, NP_200765), At2 – Wassilewskija/Landsberg erecta (NOX1b, AAM65365); A. lyrata: Al – MN47 (LUX1, XP_002875796; NOX1, XP_002864640); B. distachyon: Bd – Bd21 (LUX1, XP_003565161; LUX2, XP_003567265); Glycine max: Gm – Williams 82 (LUX1, XP_003539607; LUX2, XP_0035379777); Lycoris longituba: Lt – A17 (LUX1, XP_003606714); Nicotiana benthamiana: Nb – unknown (LUX1, BA16280); H. vulgare ssp. vulgaris Haruna
Nigo (LUX1, BAJ88719; LUX2, AK356714 manually translated); *Hyacinthus orientalis*: Ho – Delft Blue (LUX1, AAS21003); *Oryza sativa ssp. indica*: Osi – 93-11 (LUX1, EAY77400; LUX2, EEC71789); *O. sativa ssp. japonica*: Osj1 – Zhonghua11 (LUX1a, AAS90600); Osj2 – Nipponbare (LUX1b, NP_001045537; LUX2, NP_001044783); *Physcomitrella patens*: Pp – Gransden2004 (LUX1, XP_001757337; LUX2, XP_001768612; LUX3, XP_001757677; LUX4, XP_001757676); *Ricinus communis*: Re – Hale (LUX1, XP_002520534); *Selaginella moellendorffii*: Sm – Plants Delights Nursery (LUX1, XP_002962201); *Solatum lycopersicum*: Sl – unknown (LUX1, BAIE16281); *S. tuberosum*: St – unknown (LUX1, BAE16282); *S. bicolor*: Sb – BTx623 (LUX1, XP_002459177; LUX2, Sb03 g039610); *Vitis vinifera*: Vv – PN40024 (LUX1, XP_002283159); *Zea mays*: Zm – mixed genotype (LUX1a, NP_001143908; LUX1b, NP_001147359; LUX2, DAA56868). Protein sequences were aligned using MAFFT v.6.851b with the E-INS-i algorithm (Katoh & Toh, 2008). Visually unaligned regions were cropped from the alignment using BioEdit 7.0.9.0 software (Hall, 1999). The alignment was converted from fasta to Phylip4 format using ReadSeq 2.1.30 tool implemented at EBI (http://www.ebi.ac.uk/cgi-bin/readseq.cgi). A best-scoring maximum likelihood tree and pairwise distances were calculated using the fast bootstrapping algorithm of the RAXML 7.3.5 with the GAMMAJTT model and ‘autoMRE’ parameter determining an optimal number of bootstrap replicates (Stamatakis et al., 2008). The best-fitting amino-acid substitution model (JTT) was estimated using ProteinModelSelection Perl script (http://www.exelixis-lab.org/software/ProteinModelSelection.pl). The tree was edited in Dendroscope 3 software (Huson & Scornavacca, 2012). Microsynteny analysis at the *LUX* loci in *B. distachyon*, *O. sativa ssp. japonica*, and *S. bicolor* was performed using CoGe Gevo script (Lyons & Freeling, 2008) with the default BlastZ parameters except for the score threshold 6000 (Methods S4). The sequence logo of the SHAQKYF motif was generated using WebLogo (Crooks et al., 2004).

Analysis of *HvLUX1* natural diversity

A CDS of *HvLUX1* was amplified and sequenced as indicated in the ‘Segregation analysis’ section (GenBank accession nos. KC668259–KC668274). The sequences were assembled using SeqMan software (DNASTAR Lasergene® 8 Core Suite) and polymorphic haplotypes (sequence variants) were discerned manually based on the revealed polymorphisms. The Median Joining haplotype network was constructed using SplitsTree4 with default parameters (Huson & Bryant, 2006). Indels were encoded as a nucleotide substitution to include them in the analysis as an informative character.

Statistical analysis

Significant differences in flowering time among Bowman, Bowman(*eam10*), and Bowman(*eam10* + *Ppd-H1*) grown under SDs or LDs were calculated using paired t-test with a 95% confidence level (P<0.05). Significant differences in meristem development and gene expression between Bowman and Bowman(*eam10*) were calculated using a general linear model in the SAS software 9.1.3 (SAS Institute, 2009) with the factors genotype, time point, biological replicate, and first-order interaction effects. Significant differences (P<0.05) between least-squares means (LSmeans) of the genotype × time interactions were calculated using a Tukey–Kramer adjustment for multiple comparisons.

Results

The *eam10* mutant is early flowering under SD and LD conditions

In this study, we analyzed the effect of *eam10* on photope-riod-dependent flowering in barley and its interaction with natural variation in the major barley photoperiod response gene *Ppd-H1*. Flowering time was scored in the spring cv Bowman(*ppd-H1*) and in three derived introgression lines Bowman(*eam10*), Bowman(*Ppd-H1*), and Bowman(*Ppd-H1* + *eam10*) under LDs and SDs (Fig. 1a). Under LDs, Bowman(*Ppd-H1* + *eam10*) flowered first at 27 d after sowing (DAS), followed by Bowman(*Ppd-H1*) at 31 DAS, Bowman(*eam10*) at 42 DAS, and Bowman at 46 DAS. Under SDs, Bowman and Bowman(*Ppd-H1*) did not flower until 90 DAS when the experiment was terminated. By contrast, Bowman(*eam10*) and Bowman(*Ppd-H1* + *eam10*) flowered under SDs at 76 and 35 DAS, respectively. Thus, *eam10* accelerated flowering under both LDs and SDs. The combination of *Ppd-H1* and *eam10* resulted in the earliest flowering phenotype irrespective of the photoperiod. Interestingly, variation at *Ppd-H1* caused a significant difference in flowering time in the background of *eam10* under SDs. Taken together, these findings suggest that *Ppd-H1* interacts epistatically with *eam10* to accelerate flowering under both LD and SD conditions.

To determine the effect of *eam10* on the development of the shoot apical meristem (SAM), we dissected meristems of Bowman and Bowman(*eam10*) plants starting 1 wk after germination until heading and scored morphological changes of the SAM based on the Waddington developmental scale (Fig. 2a; Waddington et al., 1983). Under LDs and SDs, the SAM of Bowman(*eam10*) developed significantly faster than that of Bowman (Table S1). Significant genetic differences at single time points of dissection were only detected after Waddington stages 8 and 4 in LD and SD conditions, respectively (Fig. 2a). Under SDs, the most prominent differences between genotypes were observed in the distance between the base and the meristem (stem elongation), which was significantly larger in Bowman(*eam10*) than in Bowman at most time points (Fig. 2a). Therefore, *eam10* primarily affected stem elongation and had marginal effects on SAM development.

In barley, expression of *HvFT1* correlates with flowering time (Turner et al., 2005). To test whether differences in the expression of *HvFT1* could explain the observed flowering-time
phenotypes, we measured expression of HvFT1 in the different Bowman introgression lines under LDs and SDs (Fig. 1b). After 2 wk under LDs, HvFT1 was expressed only in lines with the dominant Ppd-H1 allele, Bowman(Ppd-H1) and Bowman(Ppd-H1+eam10). After 4 wk under SDs, HvFT1 expression was detected in Bowman(Ppd-H1+eam10), but not in the other genotypes. To identify expression differences between Bowman and Bowman(eam10) during plant development, we analyzed HvFT1 expression in leaf samples from plants dissected for meristem scoring under LDs and SDs (Fig. 2b). Under LDs, HvFT1 expression was higher in Bowman(eam10) than in Bowman at the double-ridge stage until early stem elongation (Waddington stages 2–6; 9 and 24 DAS). Under SDs, HvFT1 expression was detected in Bowman(eam10) after the beginning of stem elongation (beyond Waddington stage 3; 79 DAS), but not in Bowman. Thus, the eam10 mutation caused an up-regulation of HvFT1 and accelerated flowering time under both LDs and SDs. The dominant Ppd-H1 allele in the background of eam10 caused a stronger up-regulation of HvFT1 than the recessive ppd-h1 allele under both LD and SD conditions (Fig. 1b).

eam10 affects the expression of circadian clock homologs

Previous studies have shown that mutations in circadian clock genes caused earliness and day-neutrality in Arabidopsis and barley plants (Davis, 2002; Hazen et al., 2005; Onai & Ishiura, 2005; Faure et al., 2012; Zakhrabekova et al., 2012). To investigate whether the eam10 mutation led to a disruption of clock rhythmicity, we studied diurnal (under SDs) and circadian (IL) expression of barley clock homologs, HvCCA1, Ppd-H1 (HvPRR37), HvPRR73, HvPRR59, HvPRR95, HvPRR1, HvGI, and HvLUX1 in Bowman and Bowman(eam10) (Fig. 3).

Under SDs, Bowman(eam10) showed reduced expression of HvCCA1 and HvPRR73 compared with Bowman. Conversely, expression levels of Ppd-H1 and HvPRR1 were higher in Bowman(eam10) than in Bowman during the day, while the night peak of expression of HvLUX1 and HvPRR73 was higher in Bowman(eam10) than in Bowman. Expression differences observed under SDs were even more pronounced under LL. The strongest differences between Bowman and the eam10 mutant were detected in the expression of HvCCA1 and Ppd-H1 under LL. Under these conditions, in Bowman(eam10), circadian amplitude of HvCCA1 expression was significantly reduced during subjective days, while Ppd-H1 was significantly up-regulated in Bowman(eam10) compared with Bowman at all time points. In contrast to Ppd-H1, expression of its homologs HvPRR73, HvPRR59, and HvPRR95 was not strongly affected by the eam10 mutation. The evening-expressed genes HvPRR1 and HvGI were up-regulated in Bowman(eam10) compared with Bowman. Taken together, these results indicate that eam10 alters the expression of barley homologs of Arabidopsis clock genes, in particular the expression of Ppd-H1 and HvCCA1.

In Arabidopsis, the expression of genes implicated in the regulation of photosynthesis and photoperiod-dependent flowering is under circadian control. We therefore tested whether eam10 changed the diurnal and circadian expression of the following genes: the photoperiod response genes HvCO1 and HvFT1; the chlorophyll A/B binding protein gene HvCABIII from the photosynthetic pathway; and HvCCR2, encoding the barley ortholog of the GYLCINE-RICH RNA-BINDING PROTEIN 7 (GRP7/CRR2), characterized as a slave (nonself-sustaining) oscillator (Schöning & Staiger, 2005; Fig. S1). Under SDs, HvCO1 did not show differences in expression, and HvFT1 expression was not detected in any of the two genotypes at 21 DAS. Under LL, HvCO1 showed a small but significant up-regulation in Bowman(eam10) at some time points during the subjective night. Bowman(eam10) exhibited a strong up-regulation of HvFT1 during the subjective day compared with Bowman. In Bowman(eam10), HvCABIII was up-regulated at nights and subjective nights, while the peak expression of HvCCR2 was reduced under SDs. The eam10 mutation thus affected the expression of clock output genes.
Identification of barley Hvlux1 as a candidate gene underlying eam10

To identify candidate genes underlying the eam10 QTL, we investigated polymorphism of 133 flowering-related barley genes in Bowman and Bowman(eam10) using high-throughput sequencing of libraries enriched for flowering-related genes. In total, we found five SNPs between Bowman and Bowman(eam10) (Fig. S2). These SNPs resided within three flowering-related HarvEST unigenes, 16001, 19636, and 22370, homologous to the Arabidopsis genes CONSTITUTIVE PHOTO-MORPHOGENIC 1 (COP1), LUX, and ABA-RESPONSIVE ELEMENT BINDING PROTEIN 2 (ABF4), respectively.

As reported by Druka et al. (2011), Bowman(eam10) carries introgressions on four chromosomes, 3H (single polymorphic marker), 4H (c. 11 cM), 6H (c. 15 cM), and 7H (single polymorphic marker). Using the GenomeZipper (Mayer et al., 2009), we mapped all three barley genes polymorphic between Bowman...
and Bowman(eam10) within the introgressed regions. The barley LUX-like gene designated as HvLUX1 was located on the linkage group 3H (Hv3) at the position of POPA marker 2_1500 (171.64 cM), whereas the two other barley genes were mapped on the linkage group 6H both in the region from 85.16 to 91.79 cM. Among the three genes, only HvLUX1 colocated with the eam10 QTL, mapped on the distal end of the long arm of chromosome 3H below the marker ABC166 (155.85 cM; Börner et al., 2002) (Fig. S2).

The Harvest unigene 19636 comprised only a partial coding sequence of HvLUX1. Therefore, we extracted its homolog Hv.20312, a full-length unigene, from NCBI GenBank (AK357505). Similarly to the Arabidopsis LUX gene (Hazen et al., 2005; Onai & Ishiura, 2005) characterized as a transcription factor (TF), the intronless HvLUX1 gene encodes an MYB-domain-containing SHAQKYF GARP family protein of 274 amino-acid residues (Fig. 4a). An evening element (EE, AAATATCT) characteristic of clock genes expressed in the evening (e.g. Arabidopsis TOC1 and LUX; Alabadí et al., 2001; Hazen et al., 2005) was found 208 bp upstream of the start codon. In addition to the EE, the promoter region of HvLUX1 comprised two conserved LUX binding site motifs (GATACG and GATTCG) required for LUX autoregulation in Arabidopsis (Helfer et al., 2011).

Database search identified 36 LUX-like proteins from 19 plant species. Multiple alignment of these proteins revealed two highly

**Fig. 3** Expression patterns of main circadian clock genes in barley (Hordeum vulgare). Expression of HvCCA1, HvLUX1, Ppd-H1 (HvPRR37), HvPRR73, HvPRR59, HvPRR95, HvPRR1, and HvGI in Bowman (black lines) and Bowman(eam10) plants (red lines) under short-day and continuous-light conditions. White, black and grey bars indicate days, nights and subjective nights, respectively. Values represent averages of two biological and two technical replicates of expression values relative to HvActin ± SD. Significant differences in gene expression are indicated: *, P < 0.05.
conserved regions corresponding to the MYB and as yet uncharacterized domains (Fig. S3). The family-defining SHAQKYF motif (SHLQKY(R/Q) in the case of LUX-like genes) was conserved across all LUX-like sequences, thus indicating that the integrity of this motif may be critical for the function of LUX-like TFs. The mutation identified in the LUX-like gene from Bowman(eam10) changed the first amino-acid residue in this conserved motif (Fig. 4b; GenBank accession no. KC668258). Therefore, we suggested that this could lead to a disruption of LUX function and tentatively assigned HvLUX1 as a candidate gene underlying eam10.

To corroborate the link between the HvLUX1 mutation and the early flowering, we conducted a segregation analysis using 1002 BC1F2 lines derived from backcrossing Bowman(eam10) to Bowman. Based on sequencing analysis of early-flowering BC1F2,5 lines, we confirmed that the HvLUX1 mutation cosegregated with the earliness phenotype.

Since the region of the eam10 QTL, spanning c. 17 cM, might contain alternative candidate genes, explaining the observed phenotypes and physically linked to HvLUX1, we searched this region for homologs of clock and flowering genes. A barley homolog of Arabidopsis response regulator (ARR) clock gene (Salomé et al., 2006) was found c. 16 cM above HvLUX1 and excluded as an alternative candidate based on the sequence analysis (Notes S1; Tables S4, S5).

**Natural diversity of HvLUX1**

Natural variation at flowering time loci in barley has been proposed to be adaptive (Jones et al., 2008; Comadran et al., 2012). To investigate natural variation in HvLUX1, we sequenced the full-length gene from 88 diverse wild and cultivated barley genotypes from various geographic origins. A haplotype analysis identified 16 HvLUX1 haplotypes (Fig. 5a) distinguished by seven SNPs and six indels. The nonsynonymous SNP(545) and six in-frame indels resulted in minor changes in the amino-acid composition of HvLUX1, although none of them led to a protein truncation. The majority of the investigated genotypes, 52 samples, carried a ‘haplotype 1’ of HvLUX1. Diversity of HvLUX1 was much higher in wild than in cultivated genotypes. We identified 16 haplotypes in 52 wild barley accessions, while 35 out of 36 cultivated accessions comprised a ‘haplotype 1’. In our study, mutations in HvLUX1 did not associate with particular subspecies or place of origin (Fig. 5b; Table S2). The HvLUX1 allele was not found in the natural population, apparently because of its artificial origin, but the pattern of its variation clearly suggests that it originated from haplotype 1.

**Phylogenetic framework of LUX-like genes in plants**

The maximum-likelihood analysis of the LUX homologs extracted by the Blast search defined a LUX-family clade comprising Arabidopsis LUX along with the 38 LUX-like proteins from 7 monocots, 10 dicots, the spikemoss S. moellendorffii, and the moss P. patens. To infer phylogenetic relationships within the LUX family, we recalculated the phylogeny, including only members of the LUX-family clade (Fig. 6). The LUX-like proteins from the angiosperms fell into two monophyletic clades of dicots and monocots. Within these clades, distinct subclades and branches at the level of orders and families could be recognized. The topology of the Brassicales and Poales clades was indicative of independent duplications of the LUX-like loci in these taxa. In the Arabidopsis genus, such duplication apparently preceded speciation (LUX and NOX/BROTHER OF LUX genes; Hazen et al., 2005; Dai et al., 2011), whereas in monocots we tentatively ascribed it to a deeper phylogenetic level, the common ancestor.
of the Poaceae species (duplicates assigned as LUX1 and LUX2).
It is remarkable that the LUX outparalogs (sensu Sonnhammer & Koonin, 2002) in Poaceae significantly diverged after duplication as manifested by the distance between the LUX1 and LUX2 clades (ML distance 1.22). By contrast, the divergence between the Arabidopsis LUX-like outparalogs remained relatively low (ML distance 0.42).

To investigate in detail the ancestral LUX duplication in the Poaceae, we analyzed microsynteny of the regions comprising LUX-like loci in Brachypodium, barley, rice, and sorghum (Fig. 7). This analysis revealed a ‘fuzzy’ homology of a c. 40 kbp fragment of the barley physical map (IBSC, 2012), carrying the HvLUX1 locus, with the corresponding genomic regions of the other Poaceae species. Apparently, the order of the contigs in this region of the barley physical map awaits further refinement. Nevertheless, we could locate HvLUX1 on the barley chromosome 3H (Hv3).

The LUX genes resided in syntenic regions of the chromosomes Bd2, Hv3, Os1, and Sb3 of Brachypodium, barley, rice, and sorghum, respectively, which have been shown to be orthologous (Mayer et al., 2011). It is noteworthy that a c. 10 kbp fragment comprising BdLUX1 was inverted, while the immediately adjacent orthologous gene pairs were in a collinear order. The LUX2 gene was found on the same chromosomes in the inverted fragment located c. 5.2–6.8 mbp upstream of the LUX1 locus in

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**Fig. 5** HvLUX1 haplotypes in wild and cultivated barley (Hordeum vulgare). (a) Sixteen polymorphic haplotypes of HvLUX1. Insertions: INS1, GGAGGAGGA; INS2, ACAGCAACA; INS3, ACA; INS4, ACAGCAACAACA; INS5, GCC; deletion: DEL1, GTC. A nonsynonymous single nucleotide polymorphism (SNP) is highlighted in red. (b) Median-joining network of 16 HvLUX1 haplotypes. Numbers at the nodes indicate the number of genotypes carrying the corresponding haplotype (out of 88 accessions). The haplotype frequency is also reflected in the relative size of a node. The color of a node corresponds to the different species: blue, Hordeum vulgare ssp. vulgare; red, H. vulgare ssp. spontaneum; green, H. agriocrithon. The mutated X-ray-derived Hvlux1 allele from Bowman is labeled with an asterisk.
all the three grass species. Several genes in the vicinity of LUX loci were unique to either rice or Brachypodium. This implies that gene gain and loss events occurred at these loci after speciation.

Discussion

Bowman(eam10) is a circadian clock mutant

In this study, we describe the barley mutant locus eam10, which accelerates flowering under LDs and, in addition, allows the plant to flower and mature under SDs. eam10 has been isolated and described by Favret & Ryan (1966), when early cultivars had become an important goal in barley breeding programs and a large number of additional eam mutants were isolated in the Swedish breeding programs (Lundqvist, 2009). Recently, mutations underlying eam8 have been located in HvELF3 (Faure et al., 2012; Zakhrabekova et al., 2012). These mutations resulted in the disrupted expression rhythmicity of barley homologs of Arabidopsis clock genes such as HvCCA1, HvPPR1 and HvGI, the up-regulation of genes involved in the photoperiodic pathway (e.g. Ppd-H1) and the acceleration of flowering independent of photoperiod. Similar effects of mutations in ELF3-like genes on the rhythmicity of clock genes and photoperiodic reactions have been reported in Arabidopsis, rice, pea (Pisum sativum), and lentils (Lens culinaris) (Zagotta et al., 1996; Saito et al., 2012; Undurraga et al., 2012; Weller et al., 2012; Zhao et al., 2012). In Bowman(eam10), diurnal and circadian expression patterns of clock components such as HvCCA1, HvPPR95, and HvGI dampened under LL conditions, thus indicating that the circadian rhythms in Bowman(eam10) were compromised. In both eam8 and eam10 mutants, disruption of circadian rhythmicity, as attested by the change in expression patterns of clock genes, accelerated flowering irrespective of day length. As shown for Bowman(eam8) (Faure et al., 2012), early flowering of Bowman(eam10) was associated with an upregulation of HvFT1 under SDs (Fig. 2b). Thus, mutations in eam8 and eam10 caused an induction of the long-day photoperiod pathway under noninductive SD conditions. Therefore, eam8 and eam10 might be part of the mechanism modulating light signal transduction from receptors to downstream components of the photoperiod pathway as has been shown for the EC genes in Arabidopsis (McWatters et al., 2000; Carre, 2002).

Rate of stem elongation was significantly accelerated in eam10 mutants, whereas the timing of meristem development closely followed that of Bowman, especially at the earlier stages. In Arabidopsis, abnormally elongated hypocotyls are characteristic of a dysfunctional circadian clock, and molecular pathways underlying this phenomenon have been outlined (Niwa et al., 2009; Nusinow et al., 2011). It is tempting to speculate that while organs abnormally elongated at the early developmental stages of circadian-defective barley and Arabidopsis plants are clearly different, underlying molecular mechanisms might be similar.

eam10 regulates expression of Ppd-H1

Measurements of circadian-regulated gene expression suggested that the circadian defects in Bowman(eam10) were weak under SD conditions and significantly enhanced under LL in the

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**Fig. 6** Maximum-likelihood phylogeny of LUX-like proteins in 19 plant species. Bootstrap values (%) are shown at the nodes. Clock pictograms mark Arabidopsis paralogs, LUX and NOX implicated in regulation of the circadian clock (Dai et al., 2011; Helfer et al., 2011). The LUX-like protein encoded by the gene described in this study is marked by a red star pictogram. Scale bar, 0.1 amino-acid substitutions per site. Colour of the branches indicates angiosperm orders. Protein nomenclature is as described in the Materials and Methods section.
absence of external cues. Light and temperature signals could thus drive rhythms in Bowman(eam10). However, under LD and SD conditions when external cues were present, eam10 had significant effects on flowering time, apparently through a direct or indirect interaction with Ppd-H1. eam10 mutant lines with the wildtype Ppd-H1 allele flowered earlier than those with the

Fig. 7 Microcollinearity in the vicinity of duplicated LUX-like loci in Poaceae species. Comparison of the Sorghum bicolor chromosome 3 (Sb3), the Oryza sativa ssp. japonica chromosome 1 (Os1), and the Brachypodium distachyon chromosome 2 (Bd2) was based on the microsynteny between corresponding regions on the physical maps of these species (Sb, JGI map v1.4; Os, MSU Rice Genome Annotation v7; Bd, JGI v1) as revealed by the CoGe GEvo script (http://genomevolution.org/CoGe/GEvo.pl). Location of the Hordeum vulgare ssp. vulgare LUX1 locus (HvLUX1, as POPA marker 2_1500) is shown on the consensus genetic map (as found in the barley GenomeZipper); location of HvLUX2 could not be established. Orthologs are connected by horizontal solid lines, outparalogs by dashed lines. LUX-like genes (SbLUX1, Sb03 g047330; Osj2LUX1b, LOC_Os01 g7420; BdLUX1, Bradi2 g62070; SbLUX2, Sb03 g039610; Osj2LUX2, LOC_Os01 g62660; BdLUX2, Bradi2 g54790) are highlighted in red. Scale bar, 10 kbp.
recessive *ppd-H1* allele under LDs and SDs. This difference was most pronounced under SDs. Thus, variation at *Ppd-H1* in the background of *eam10*, affected flowering under SDs, while previously it has been postulated that *Ppd-H1* acts exclusively under LDs (Turner et al., 2005). The interaction between *eam10* and *Ppd-H1* was further supported by a significant up-regulation of *Ppd-H1* expression in Bowman(*eam10*) compared with Bowman under both SDs and LLs. These results suggested that *Eam10* acts as a repressor of *Ppd-H1*. Early flowering of lines carrying *Ppd-H1* and *eam10* correlated with an up-regulation of *HvFT1* under SDs (Fig. 1b), presumably caused by the up-regulation or differences in the diurnal expression pattern of *Ppd-H1* in the *eam10* background.

**HvLUX1** is a candidate gene underlying *eam10*

The identification of a mutation in an extremely conserved region of *HvLUX1* strongly suggested *Hvlux1* as a gene underlying the *eam10* locus. The segregation analysis, the flowering and expression phenotypes, and the screening of genes in the vicinity of *eam10* solidified the link between *Hvlux1* and *eam10*, despite the possible shortcomings of using introgression lines carrying multiple genes transferred from donor germplasm. The mechanistic role of *Hvlux1* in the *eam10* flowering and expression phenotypes is yet to be discovered.

It has been shown in Arabidopsis that LUX and ELF3 together with ELF4 form the so-called ‘evening’ complex and have the same transcriptional targets (Kolmos et al., 2011; Nusinow et al., 2011; Herrero et al., 2012). Both LUX and ELF3 physically associate with the promoter of *PRR* to repress its transcription (Dixon et al., 2011; Herrero et al., 2012). Up-regulation of *Ppd-H1* in *eam10* and *eam8* mutants (Faure et al., 2012) suggested that *Hvlux1* and *HvELF3* act in the same pathway, and that their transcriptional targets are partly conserved between Arabidopsis and barley. Interestingly, the repressive function of *eam10* was only seen for *HvPRR37*, while expression of its paralogs *HvPrr73* and *HvPrr59/95* was not strongly affected. By contrast, EC genes in Arabidopsis control expression of *PRR7* and *PRR9* (Kolmos et al., 2009; Herrero et al., 2012). Differences in the transcriptional targets of barley *Eam8*/*Eam10* and Arabidopsis ELF3 suggest a different clock construction in barley and Arabidopsis. Bowman(*eam10*) was characterized by a downstream regulation of *HvCCA1* and an up-regulation of *HvPrr1*, *HvGI*, and *HvLUX*. This is consistent with the role of Arabidopsis LUX, which activates CCA1 and represses TOC1 (PRR1), GI, and LUX itself (Hazén et al., 2005; Onai & Ishiura, 2005). Up-regulation of *Hvlux1* in *eam10* and the presence of two conserved LUX binding site motifs (GATACG and GATTCG) in the promoter region of *Hvlux1* suggested that *Hvlux1* controls its own expression, as seen in Arabidopsis (Helfer et al., 2011).

**LUX** duplicated independently in Poaceae and Arabidopsis

Interspecific comparison of clock genes within phylogenetic frameworks may facilitate integration of knowledge and also pinpoint dissimilar patterns of gene evolution, when direct comparisons between homologous genes from distant plant lineages should be made with greater caution. Phylogeny-supported comparison of flowering genes in grasses and Arabidopsis has identified several cases when direct orthology between members of gene families could not be established (e.g. *CDF1*, *TOE1*, *TEM*, *HAP* genes; Higgins et al., 2010). In the case of clock genes, such information is extremely scarce. It has been suggested that, in monocots and eudicots, genome duplication events led to the independent duplication and diversification of the *PRR* gene family (Murakami et al., 2007; Takata et al., 2010; Campoli et al., 2012b).

In our study, *LUX*-like genes could be traced down to mosses and apparently represent an ancient branch of the GARP transcription factor family. We found duplication of *LUX* genes in the ancestor of Poaceae (*LUX1* and *LUX2*) independent of the duplication in Arabidopsis (*LUX* and *NOX*). While *LUX* paralogs in Arabidopsis retained partially redundant functions (Dai et al., 2011) and remained phylogenetically close, *LUX* paralogs in grasses significantly diverged before grass species radiation. This suggests that evolutionary fate of individual members of clock gene families might be different, especially in distant plant lineages.

Microsynteny around *LUX* loci in Brachypodium, rice, and sorghum revealed ancestral duplication, which is consistent with the phylogeny. However, we could not resolve whether this duplication originated from the ancient whole-genome duplication event or local translocation. Several gene loss/gain and micro-inversion events occurred independently in the genomes of the three grass species, while both *LUX* loci remained intact. These data implicate selection for retention of *LUX* loci in grass genomes, corroborating previous findings, which suggested preferential retention of duplicated clock genes in *Brassica rapa* (Lou et al., 2012).

**HvLUX1** is under purifying selection

Structured allelic variation at several flowering- and clock-related loci has been proposed as a signature of plant adaptation to either natural or agricultural environments (Izawa, 2007; Clotault et al., 2012; Weller et al., 2012; Anwer & Davis, 2013). Allelic variation at three flowering loci, *Ppd-H1*, *HvCEN*, and *HvELF3*, associated with the geographical distribution of wild and cultivated barley habitats has been suggested to be adaptive (Jones et al., 2008; Comadran et al., 2012; Faure et al., 2012; Zakhraibekova et al., 2012).

We found that *HvLUX1* diversity is high (16 haplotypes in 88 accesses). Surprisingly, only one haplotype was prevalent in cultivars and landraces from diverse geographic origins; the diversity of *HvLUX1* was predominantly confined to wild barley (16 haplotypes in 49 accesses). Remarkably, all in-frame indels identified in several *HvLUX1* haplotypes resulted from variation in a number of microsatellite repeats. Functional and adaptive variation in the number of tandem amino-acid repeats have been demonstrated in flowering and clock genes, such as *ELF3* in Arabidopsis and *FCA* in endemic Hawaiian mint (*Stenogyne rugosa*) (Lindqvist et al., 2007; Undurraga et al., 2012). Whether
variation in HvLUX1 is functional warrants further investigation. The absence of frameshifts and predominance of a single haplotype indicate that HvLUX1 was under purifying selection. While barley plants with nonfunctional Hvelf3 might have selective advantage in certain environments, integrity of HvLUX1 seems to be under functional constraints.

Conclusion

Isolation and characterization of Hvlux1 advance our understanding of the photoperiod response pathway in temperate cereals. The mutation in HvLUX1 is linked to ear10, which caused early flowering independent of the photoperiod. HvLUX1 was associated with similar, but weaker, molecular and flowering phenotypes than Hvelf3. It thus represents an interesting resource for modulating photoperiod response and ultimately adaptation in barley. Revealed patterns of diversification of duplicated LUX loci and their natural variation offer some insight into a number of questions regarding the differential fate of duplicated genes in different plant lineages and the impact of the circadian clock components on adaptation in natural populations.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Expression patterns of clock output genes.

Fig. S2 Location of genes and SNPs distinguishing between Bowman and Bowman(eam10) on barley linkage groups.

Fig. S3 Multiple alignment of LUX-like protein sequences from 19 plant species.

Table S1 ANOVA for meristem development and gene expression differences

Table S2 Accessions used for the resequencing of HvLUX1

Table S3 Barley flowering-related genes selected for targeted enrichment

Table S4 PCR primers specific for the barley ARR-like gene

Table S5 Results of the gene ontology analysis of Brachypodium genes surrounding the \(LUX\) locus

Methods S1 Preparation of TruSeq libraries.

Methods S2 Read processing workflow using the Galaxy server (http://galaxy.wur.nl).

Methods S3 Polymorphism filtering workflow.

Methods S4 Permanent web links to the detailed results of the synteny analysis in the vicinity of \(LUX\) genes in three Poaceae species.

Notes S1 Sequencing of the barley ARR-like gene.

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