Evolutionary Relationships Among Barley and Arabidopsis Core Circadian Clock and Clock-Associated Genes

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Abstract The circadian clock regulates a multitude of plant developmental and metabolic processes. In crop species, it contributes significantly to plant performance and productivity and to the adaptation and geographical range over which crops can be grown. To understand the clock in barley and how it relates to the components in the Arabidopsis thaliana clock, we have performed a systematic analysis of core circadian clock and clock-associated genes in barley, Arabidopsis and another eight species including tomato, potato, a range of monocotyledonous species and the moss, Physcomitrella patens. We have identified orthologues and paralogues of Arabidopsis genes which are conserved in all species, monocot/dicot differences, species-specific differences and variation in gene copy number (e.g. gene duplications among the various species). We propose that the common ancestor of barley and Arabidopsis had two-thirds of the key clock components identified in Arabidopsis prior to the separation of the monocot/dicot groups. After this separation, multiple independent gene duplication events took place in both monocot and dicot ancestors.

Keywords Arabidopsis thaliana · Hordeum vulgare (barley) · Circadian clock · Reciprocal BLAST · Homologue

Introduction

Most living organisms optimise their day/night responses by measuring time and using this information to organize their physiology and morphology in anticipation of daily changes (Chen and McKnight 2007; Green et al. 2002; Okamura 2004). As sessile organisms, plants also rely on the circadian clock to optimise several physiological processes, such as expression of chlorophyll biosynthetic genes after dawn, to optimise chlorophyll content and carbon fixation (Dodd et al. 2005; Harmer et al. 2000; Haydon et al. 2013). The diversity of processes controlled by the circadian clock also reflects the number of genes under its control. Expression of about one-third of the Arabidopsis genome is regulated by the circadian clock (Covington et al. 2008). Only a relatively small number of genes establish and maintain the circadian rhythm of the clock. These core clock components are present in each cell and consist of a complex network of genes regulated by transcriptional feedback loops, post-transcriptional and post-translational modifications (Gallego and Virshup 2007; James et al. 2012; McClung 2014; Sanchez et al. 2010; Troein et al. 2009) (Fig. 1). The framework of the Arabidopsis circadian clock known as the interlocking-loop model comprises at least three interlocking gene expression feedback loops (Harmer 2010; Locke et al. 2006; Pokhilko et al. 2010; Zeilinger et al. 2006).

The central loop is formed by CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCHOTYL (LHY) and TIMING OF CHLOROPHYLL A/B BINDING
The circadian clock is composed of EARLY FLOWERING 3 (ELF3), ELF4, and LUX and it represses transcription of PRR9 (Chow et al. 2012). Interestingly, LUX represses its own expression (Helfer et al. 2011). Further post-translational regulation takes place in the evening, such as GI degradation by ELF3 (Yu et al. 2008) and F-box protein ZEITLUPE (ZTL) stabilisation by GI, allowing ZTL to control TOC1 protein degradation (Kim et al. 2007).

The circadian clock can be entrained by certain cues, for instance light (photoperiod) and temperature (Hotta et al. 2007), which is tightly linked to plant adaptation to specific environments (Michael et al. 2003). To address the impact of the clock in crop species, such as barley, one approach is to gain an understanding of key clock components and their interactions by examining how widely clock genes are conserved. Most information on plant circadian clocks is available for Arabidopsis (Nagel and Kay 2012; Nakamichi 2011). Translation of knowledge will not be straightforward due to differences in clock control between monocots and Arabidopsis, such as rhythmicity of growth (Matos et al. 2014; Poiré et al. 2010) and different versions of the clock operating in different parts of the plant (Endo et al. 2014; James et al. 2008). Understanding the evolutionary relationships among clock genes will aid the development of clock models for other species but it is important to note that the identification of barley homologous genes does not necessarily imply conserved clock function. To date, some clock genes have been identified in monocots such as Brachypodium distachyon (Higgins et al. 2010) and Zea mays (Wang et al. 2011) with most information on rice (Hayama et al. 2003; Higgins et al. 2010; Iwamoto et al. 2009; Murakami et al. 2007; Onai and Ishiura 2005; Shin et al. 2004). For barley, circadian rhythms have been observed at diverse levels including at transcript and protein abundance, and physiological processes (Lillo 2006; Martínez et al. 2003; Nagasaka et al. 2009; Valletian-Bindschedler et al. 1998). Diurnal and circadian expression analyses have been reported for HvLHY (HvCCA1), HvPPD-H1, HvPRR73, HvPRR59, HvPRR95, HvGI, HvTOC1, HvLUX and HvELF3 (Campoli et al. 2012b, 2013; Dunford et al. 2005; Faure et al. 2012; Higgins et al. 2010; Turner et al. 2005). Only three barley clock genes have been well characterised using mutant plants: Ppd-H1, ELF3 and LUX (Campoli et al. 2013; Faure et al. 2012; Turner et al. 2005; Zakhrabekova et al. 2012). The Ppd-H1/PRR37 allele is the major determinant of photoperiod response in barley and is the putative AtRRR7 orthologue (Turner et al. 2005). Mutations in the barley Ppd-H1/ PRR37 (PRR7) and ELF3 genes affect important traits, such as flowering time (Faure et al. 2012; Stracke et al. 2009; Turner et al. 2005; Zakhrabekova et al. 2012) and low-temperature tolerance (Fowler et al. 2001).
The availability of high-confidence barley gene sequences (Matsumoto et al. 2011; Mayer et al. 2012) now allows the identification of barley orthologues of clock and clock-associated genes. Here we have performed a systematic analysis of clock genes in ten different plant species and thereby identified the genomic sequences of 21 putative barley homologues of Arabidopsis core circadian clock genes and selected clock-associated genes and propose an evolutionary history for barley and Arabidopsis clock genes from a common ancestor.

Materials and Methods

Cross-Species Reciprocal BLAST

To identify plant orthologues of the Arabidopsis clock genes, systematic cross-species reciprocal BLAST searches were performed using default settings and gene sequences of ten different plant species: Arabidopsis, tomato, potato, barley, Brachypodium distachyon, sorghum, wheat, maize, rice and moss (Physcomitrella patens) (Table S1). First, a BLAST search (Altschul et al. 1990) was carried out using Arabidopsis gene sequences against various databases (Table S1) to identify putative orthologous sequences. Next, reciprocal BLAST analysis was performed using the top hit from all species against the Arabidopsis database. Subsequently, cross-species reciprocal BLAST analysis was performed using the top hit from all species against each species’ databases. When the top hit of a reciprocal BLAST successfully identified the original Arabidopsis sequence and the top hits from all other databases, these were taken as orthologues. Any additional hits with an E-value similar to the top hit were also subjected to reciprocal BLASTs. When the second/third/etc. best hits successfully identified the original Arabidopsis sequence and their orthologues in all other species, these were taken as paralogues.

However, when a reciprocal BLAST with the top hit identified a different Arabidopsis gene from the original candidate sequence, (1) the newly identified Arabidopsis gene(s) was used in cross-species reciprocal BLAST analysis; and (2) all gene family members of the new and original Arabidopsis candidate genes were also subjected to cross-species reciprocal BLASTs. Similarly, in this analysis with ‘additional’ Arabidopsis sequences, when the top hit of a cross-species BLAST reciprocally identified the top hit from another species, these were taken as orthologues. This analysis identified genes in Arabidopsis which were related to the initial candidate clock gene and their putative orthologues in other species. These cross-species reciprocal BLAST analyses of ‘additional’ Arabidopsis genes also considered any additional hits with E-value similar to the top hit, subjecting them to cross-species reciprocal BLASTs (as mentioned above). Overall, these analyses identified true orthologues and duplicated genes in the tested species.

Gene sequences and identifiers were taken from the databases described in Table S1. Schematic diagrams of genomic structures were initially made using the Exon–Intron Graphic Maker program (http://wormweb.org/exonintron). In some cases, the annotated exon/intron gene structures did not generate full length ORFs, when compared to homologous genes. Therefore, when necessary, re-annotation of genomic sequences was performed based on: (1) cDNA, EST and PUT (PlantGDB-assembled Unique Transcripts) data available for the related species; (2) the presence of GT and AG dinucleotides for intron boundaries (5’ and 3’ splice site, respectively); (3) ORF maintenance of each exon; and (4) the annotation of orthologous mRNA/protein sequences.

Phylogenetic Analysis

Nucleotide sequence alignments were performed such that they preserved the codon structure of putative coding sequences (CDS). For this, nucleotide alignments were based on the alignments of their deduced protein sequence using the ClustalW program (Larkin et al. 2007; Tamura et al. 2013). Gene tree estimation was performed using the neighbour-joining (NJ) method (Saitou and Nei 1987) available on MEGA6 software (Tamura et al. 2013). The moss P. patens was used as an outgroup for angiosperm species, and moss genes, when present, were used to root the phylogenetic trees. Statistical support for each branch on phylogenetic trees was generated from the bootstrap test (2,000 replicates; values shown when >50 %) (Felsenstein 1985). The evolutionary distances and branch lengths were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). Pseudogenes were not analysed in order to prevent poorly supported topologies on reconstruction of phylogeny from gene families, as suggested by Zimmer et al. (2007).

Results

Identification of Barley Core Clock and Clock-Associated Genes by Reciprocal BLAST

The Arabidopsis clock and clock-associated genes, including selected flowering-related genes: CCA1, LHY, TOC1 (PRR1), GI, ELF3, ELF4, PRR7, PRR3, PRR9, PRR5, LUX (PCL1), FKF1, ZTL, CHE (TCP21), GRP7 (CCR2), GRP8, CAB2, CO and FT were selected for a comparative approach to identify and confirm the genomic
sequences of related genes in barley. barley and Arabidopsis share a common ancestor but they have diverged considerably since their separation around 140 million years ago (Mya) (Chaw et al. 2004; Moore et al. 2007). Since orthology determination becomes more difficult when species are evolutionarily distant (Prosdocimi et al. 2009; Yu and Hinchcliffe 2011), additional species with whole genome sequence information from both dicot and monocot groups were included in the comparative analysis. These species were tomato, potato, moss (P. patens) and another five grasses: Brachypodium distachyon, sorghum, wheat, maize and rice (Table S1). The comparative approach comprised multiple cross-species reciprocal BLASTs (Altschul et al. 1990) as described in “Materials and methods”. These systematic analyses identified the range of species which contained true orthologues and a comprehensive list of the duplicated genes in the analysed species (Table 1, S2–S6). In a few cases, false duplicated genes, previously described in the literature, are described in Supplementary Note 1.

The Arabidopsis clock genes showed variation in their ability to identify true orthologues providing some information on the clock gene components in different species and their evolution. This is illustrated by considering genes with very different results from the analysis: LUX, LHY/CCA1 and ELF4. AtLUX identified true orthologues in all nine species analysed by cross-species reciprocal BLAST, including another parologue in Arabidopsis (AtBOA) and four gene copies in P. patens (Fig. 2a; Table S2). The latter species also has a number of particular features regarding its clock flowering-related genes where GI, FKFI, ZTL, CO and FT are present in all flowering plants but absent in P. patens (Tables S2, S4 and S6). At the other extreme is AtCCA1. This gene identified a gene in each of the nine species but it had no reciprocal hits with any species analysed. In fact, the reciprocal BLASTs all identified AtLHY

| Paralogues | Orthologues/Paralogues | Orthologues | Orthologues | Orthologues/Paralogues | Paralogues |
|------------|------------------------|-------------|-------------|------------------------|------------|
| AtCCA1 (At2g46830) | – | AtLHY (At1g01060) | HvLHY (MLOC_14118) | – | – |
| AtBOA (At5g59570) | – | AtLUX (At3g46640) | HvLUX (MLOC_37446) | – | – |
| EEC? | – | AtELF3 (At2g25930) | HvELF3 (MLOC_78552a) | – | – |
| – | – | AtGI (At1g22770) | HvGI (MLOC_70638b) | – | – |
| – | – | AtTOC1 (At5g61380) | HvTOC1 (MLOC_52387) | – | – |
| – | AtPRR5 (At5g24470) | – | – | HvPRR95 (MLOC_57021) | – |
| – | AtPRR9 (At2g46790) | – | – | HvPRR59 (MLOC_62596b) | – |
| – | – | AtPpd-H1 (MLOC_81154) | – | HvPRR73 (MLOC_12732) |
| AtLPK2 (At2g18915) | – | AtZTL (At5g57360) | – | HvZTLa (MLOC_44010) |
| – | – | AtFKF1 (At1g68050) | HvFKF1 (MLOC_53725) | – | – |
| – | AtGRP7 (At2g21660) | – | – | HvGRP7a (MLOC_17819b) |
| – | – | AtCO1 (MLOC_6921b) | HvCO1 (MLOC_59695b) | – | – |
| AtTSF (At4g02380) | At5g15840 (CO) | At5g15850 (COL1) | – | – |
| – | AtFT (At1g65480) | – | – | HvFT1 (MLOC_68576) |
| – | At2g40080 (ELF4) | At2g29950 (ELF4-like1) | – | HvFT2 (MLOC_10172b) |
| At1g17455 (ELF4-like4) | – | At2g06255 (ELF4-like3) | HvELF4-like3 (MLOC_70937) | – | HvELF4-likeA (MLOC_58590) |
| At1g72630 (ELF4-like2) | – | – | – | – | – |

a Determination of one-to-one gene orthologue/paralogue not defined
b MLOC represents partial sequence of the gene
instead of AtCCA1. When AtLHY was used, cross-species reciprocal BLASTs were successful with all ten species (Fig. 2b) suggesting that they contained true orthologues of AtLHY but no orthologues of AtCCA1. Therefore, barley and six other plants have a single LHY counterpart, whereas LHY gene duplications possibly occurred independently in maize, P. patens and Arabidopsis, the latter giving rise to AtCCA1.

Other genes, for example ELF4, only had cross-species reciprocal hits with dicot species suggesting that it is specific to dicots (Fig. 2c). In this analysis, the initial BLAST using the AtELF4 sequence identified sequences in monocots that did not identify AtELF4 reciprocally but instead identified AtELF4-like3. Using this gene and all known AtELF4 gene family members, orthologues and paralogues of ELF4-like3 genes in all species analysed were identified (Tables S5 and S6). Barley and wheat each have two genes in this family. Cross-species reciprocal BLAST using the single-exon genes AtCHE and AtCAB2 did not identify orthologues in any of the species analysed (Supplementary Note 2).

Genomic Structure of Barley and Arabidopsis Clock Genes

Having identified barley orthologues of clock genes, we were then able to examine the conservation of exon–intron organisation to gain further support for the relationships between orthologues. Genomic sequences of genes related to Arabidopsis clock genes were downloaded from the various plant databases for analysis and correctly annotated or re-annotated as necessary. The 21 genes which were (re)annotated are shown in Tables S2–S6.

The genomic structures of barley and Arabidopsis genes are generally well conserved in their exon/intron organisation (e.g. TOC1 in Fig. 3a). However, differences in the barley orthologues are mainly in the size of introns, which are generally much larger in barley, and in the UTR sequences. A clear example is the 5′ UTR of LHY in barley, which is considerably longer and has a complex multi-exon structure, while AtLHY only has two 5′ UTR introns (Fig. 3b). In the coding region, AtCCA1, AtLHY and HvLHY have a highly conserved gene structure, with the exception of one additional intron found in AtCCA1 and AtLHY (intron 5 or 6, respectively) when compared with HvLHY (Fig. 3b). The genomic structures of HvPRR37/Ppd-H1, GI and ELF3 have been analysed previously (Dunford et al. 2005; Turner et al. 2005; Zakhrabekova et al. 2012). An important consideration remains that the barley gene space is not complete (Mayer et al. 2012) and the extensive in silico analysis conducted here may still have missed possible orthologues or parts of genes (e.g. the 5′ UTRs of HvLHY and HvPRR95).

Phylogenetic Analyses of Clock Genes

To demonstrate and confirm the degree of relatedness of identified orthologous genes, phylogenetic trees were generated (Fig. 4a, b; Figs. S1–S3).

ZTL and FKF1 Orthologues

Gene members of the LOV (light, oxygen or voltage) blue light receptor subfamily, ZTL and FKF1, were identified in all flowering plants analysed (Fig. 4a). In Arabidopsis, FKF1 is functionally and evolutionary diverged from ZTL, which might have started sometime after euphylllophyte (ferns and seed plants) speciation (Suetsumu and Wada 2013). The ZTL gene has been duplicated in both the ancestor of monocots and in Arabidopsis. As a result,
monocots have \( \text{ZTL}a \) and \( \text{ZTL}b \) genes, while Arabidopsis has \( \text{ZTL} \) and the recent copy, \( \text{LPK2} \) (Lou et al. 2012). The exact relationships between both monocot \( \text{ZTL} \) genes and the dicot \( \text{ZTL} \) could not be determined, i.e. the true orthologue of \( \text{AtZTL} \) in monocots is either \( \text{ZTL}a \) or \( \text{ZTL}b \).

Monocots and dicots have maintained a single copy of \( \text{FKF1} \) except for maize, which has two copies due to a recent duplication.

**ELF4 and ELF4-like3 Orthologues**

The in silico analyses suggest two subgroups for the \( \text{ELF4} \)-like family: \( \text{ELF4} \), which includes \( \text{AtELF4} \)-like1 (Table S5), and \( \text{ELF4} \)-like2/3/4 (Table S6). \( \text{ELF4} \) family members are found only in dicot species and they are single-exon genes. \( \text{ELF4} \)-like2/3/4 family members are found in all plants analysed and most of them have a 5' UTR intron. Our analyses suggest that the ancestor of land plants contained one copy of the \( \text{ELF4} \)-like gene, most likely an orthologue of \( \text{AtELF4} \)-like3. This gene was duplicated in the ancestor of flowering plants, which then contained both \( \text{ELF4} \)-like3 and the new copy, \( \text{ELF4} \). Monocots lost the \( \text{ELF4} \) gene, while dicot species duplicated this gene multiple times (Fig. 4b). The \( \text{ELF4} \)-like3 gene was duplicated twice in monocots, but barley and wheat may have lost one of the copies. Dicots also had one or two duplication events from the \( \text{ELF4} \)-like3 gene and its subsequent copies.

**PRR Orthologues**

Most flowering plants analysed have five \( \text{PRR} \) genes. The \( \text{TOC1} \) gene is duplicated in maize and both \( \text{Solanum} \) species. \( \text{P. patens} \) has four \( \text{PRR} \)s, which are very closely related to the \( \text{PRR} \)s of angiosperms. It was not possible to determine \( \text{PRR} \) orthologues due to very complex results from BLAST and phylogenetic analysis (Fig. S1). The only evidence observed is that the ancestor of flowering plants had \( \text{TOC1} \), \( \text{PRR3/7} \) and \( \text{PRR9/5} \) genes. After the divergence of monocots and dicots, both ancestors independently duplicated \( \text{PRR3/7} \) and \( \text{PRR9/5} \) genes.

**LHY, LUX and GRP7 Orthologues**

Phylogenetic analyses confirmed true orthologues of \( \text{AtLHY} \) (Fig. S2a), \( \text{AtLUX} \) (Fig. S2b) and \( \text{AtGRP7} \) (Fig. S2c) in all species analysed. In particular, several paralogues of the single-intron \( \text{AtGRP7} \) gene were identified in all species analysed. In *silico* analyses suggest that the ancestor of land plants contained one copy of the \( \text{GRP7} \) gene. Two independent duplication events occurred within the \( \text{P. patens} \) branch, generating \( \text{PpGRP1} \), \( \text{PpGRP2} \) and \( \text{Pp1s136_70} \). The \( \text{GRP7} \) gene has undergone a series of independent duplications within dicots and once in monocots. In Arabidopsis, it is likely that this duplication gave rise to \( \text{AtGRP8} \), according to cross-species BLASTs. In
monocots, there are two copies of the GRP7 gene, which are hereafter called GRP7a and GRP7b. Rice has lost GRP7a and duplicated GRP7b. Wheat seems to be the only species with a third copy, TaGRP7c, but the predicted protein is around half the size of the other GRP7s in monocots and may therefore be a pseudogene or an error from sequencing and consensus sequence formation, and was eliminated from further analyses.

CO and FT Orthologues

Homologous members of the AtCO subfamily were identified in all flowering plants analysed, including barley (Fig. S3a). Protein alignment and BLAST analyses suggest that the ancestor of flowering plants contained one copy of a CO-related gene, which is the orthologue of AtCO or AtCOL1. Two independent duplication events have occurred within the Arabidopsis branch, which currently has AtCOL1, AtCO and AtCOL2. Monocots have one duplication event of the original CO-related gene, giving rise to both CO1 and CO2. Rice and maize have lost their CO2 gene copy. The exact relationship between both CO1 and CO2 genes in monocots and the dicot CO-related genes could not be determined, but homologues are clearly present. Similarly, the true orthologue of AtFT in monocots could not be determined, but at least two homologues (FT1 and FT2) are present in all monocots analysed (Fig. S3b). Rice in particular has two copies of the FT1 gene (OsFTL2 and OsFTL3).

ELF3 and GI Orthologues

Homologues of AtELF3 were identified in all species analysed. Paralogues were also observed and are probably due to a series of duplication events of the ELF3 gene. All in silico analyses suggest that the ancestor of land plants
contained one copy of the ELF3 gene. Two independent duplication events occurred within the P. patens branch, which has three homologues of ELF3. The original ELF3 gene was also duplicated in the ancestor of flowering plants, which then contained both the ELF3 gene and the new copy, ESSENCE OF ELF3 CONSENSUS (EEC) gene. However, this hypothesis for the origin of EEC has low support from phylogenetic analysis (59 % likelihood, Fig. S3c) and must be treated with care. Monocots have lost the support from phylogenetic analysis (59 % likelihood, Fig. S3c). Temperate grasses (Pooideae) lost the ELF3b gene, whereas rice lost ELF3a. Interestingly, the ELF3b copy present in the rice genome has undergone a recent duplication. The exact relationships between both ELF3 alleles in monocots and the dicot ELF3 could not be determined. Lastly, true orthologues of GI were identified and confirmed in all flowering plants analysed (Fig. S3d).

In summary, we have identified the genomic sequences of 21 putative barley homologues of Arabidopsis core circadian clock genes and selected associated genes and eliminated any similar unrelated sequences, i.e. sequences that are not descended from a common ancestral sequence. A single Arabidopsis true orthologue of the clock genes LHY, TOC1, GI, ELF3, LUX and FKF1 was identified in barley. Additionally, the ancestor of flowering plants possibly had a single copy of PRR37, PRR9/5, FT, CO/COL1, ZTL and GRP7 genes and after divergence of monocots and dicots both ancestors independently duplicated and maintained these genes. Orthologues of the AtCHE, AtELF4 and AtCAB2 gene families were not identified in barley or other monocot species.

Discussion

In Silico Identification of Clock Homologues

Putative homologues of Arabidopsis circadian clock genes were identified in tomato, potato, P. patens, Brachypodium, sorghum, wheat, maize, rice and barley (Tables S2–S6). Forty of those genes in monocots, including six in barley (HvZTLa, HvZTLb, HvGRP7b, HvELF4-like3, HvFKF1 and HvCABa), were hitherto unknown. Many genes were already known and had previously been used in simple analyses or, less commonly, a fully characterised study (see Tables S2–S6). The identification of previously described genes in various species confirmed that the in silico method used here is appropriate for identifying homologues, as well as confirming the identity of the previously described genes. Moreover, the comprehensive list of species with duplicated gene copies gives further confidence to the gene duplications identified in barley and has helped to identify some incorrect duplication events (Supplementary Note 1).

The identification of orthologous, paralogous and lost genes may provide information on the function of these genes and how they impact the growth habit of particular species. For example, CO and FT are key genes in the regulation of flowering time. AtCO is a member of a subfamily from Group Ia of the COL family (Griffiths et al. 2003; Valverde 2011). In silico analyses suggest Arabidopsis has three members from this subfamily, whereas barley has two: HvCO1 (Campoli et al. 2012a; Griffiths et al. 2003) and HvCO2 genes (Griffiths et al. 2003). Other monocots also have two gene copies, except rice [also suggested by Cockram et al. (2012)] and maize. These species require short day photoperiods to flower, while barley, wheat, Arabidopsis and potato, require long days. Therefore, the absence of the CO2 gene copy in rice and maize may have had a critical role in their domestication (Cockram et al. 2012; Miller et al. 2008). Similarly, the central component in mediating the onset of flowering, the FT gene, was present in the angiosperm ancestor and contributed to the evolution of flowering plants (Klintenäs et al. 2012; Pin and Nilsson 2012). AtFT is a member of the PHOSPHATIDYLETHANOLAMINE-BINDING PROTEIN (PEBP) FT-like family and it forms a subfamily with TWIN SISTER OF FT (TSF) (Faure et al. 2007; Kobayashi et al. 1999). Monocots have two members from this subfamily: FT1 and FT2 through duplication, but neither is an orthologue of AtTSF. The monocot FT1/FT2 duplication occurred after the divergence between the grasses and Arabidopsis. Therefore, this duplication is independent of the FT/TSF duplication in Arabidopsis, as suggested previously (Li and Dubcovsky 2008). Interestingly, FT copy number variation in cereals plays an important role in the regulation of plant flowering and development (Nitcher et al. 2013).

Dicot-Specific Clock Genes

Orthologues of four Arabidopsis genes from the initial candidate list were not identified in barley and most other plant species: ELF4, CAB2, CHE and ĈCA1. These are likely to be dicot- or Arabidopsis-specific genes. For ELF4, in particular, only members of the ELF4-like2/3/4 subclade have been found in monocots (Boxall et al. 2005; Higgins et al. 2010; Murakami et al. 2007). However, Kolmos et al. (2009) suggested that AtELF4 and AtELF4-like1 are the closest homologues of ELF4-like genes in monocots and that HvELF4-likeA fully complemented the elf4 loss-of-function phenotype in Arabidopsis, suggesting conserved functionality (Kolmos et al. 2009). It is noteworthy that some ELF4 family members were missing from most monocot species they analysed, which might have influenced the topology that suggested such homology. The lack of orthologues of the clock-associated genes AtCAB2 and AtCHE is discussed in Supplementary Note 2.
**CCA1**, along with **LHY**, plays an important role in the regulation of the circadian rhythm in Arabidopsis, but the presence of both counterparts in the genome of other plant species does not seem to be a common feature. Barley and six other plants analysed here have only one **LHY/CCA1** gene, and this suggestion is also confirmed in studies of barley (Campoli et al. 2012b), rice (Murakami et al. 2007) and *Brachypodium* (Higgins et al. 2010). This raises the question of whether most species contain an orthologue of **LHY** or **CCA1**? Some analyses indicate that **LHY**, as opposed to **CCA1**, is present in most plant species (Lou et al. 2012; Takata et al. 2009; Yon et al. 2012). For instance: (1) cross-species reciprocal BLAST is possible only for *AtLHY*, not *AtCCA1*; (2) *Solanum* species have only one gene, which is very similar in sequence to **LHY**; (3) **CHE**, the transcriptional repressor of **CCA1** is also an Arabidopsis-specific gene; (4) **CCA1** is a casein kinase II (CK2) target in Arabidopsis, whereas in rice the OsCK2 orthologue does not target OsCCA1, probably because OsCCA1 does not contain the correct amino acid for interaction, suggesting again that OsCCA1 is not a true orthologue of *AtCCA1* but of *AtLHY* (Ogiso et al. 2010) and (5) *AtLHY* and *HvLHY* have similar transcriptional and post-transcriptional responses to lower temperature transitions, as opposed to *AtCCA1* behaviour (Calixto et al., manuscript in preparation).

Therefore, most plant species do not contain **CCA1** and **LHY** but have only one gene, most probably **LHY**, which is necessary for maintenance of the circadian rhythm and plant survival. In Arabidopsis, where **LHY** has been duplicated, the gene copies have diverged such that both are important for the maintenance of the circadian rhythm.

**Evolution of Clock Genes**

Within angiosperms, in both monocots and dicots, a strong similarity exists among their clock components, architecture and functions (Song et al. 2010). To test for evolutionary homology of monocot and dicot clock genes, several investigations have used different approaches, such as phylogenetic analysis, studies of segmental duplication and functional gene assessments through gene expression studies and complementation tests (suggesting conserved biochemical function). For example, knockdown and overexpression of **LHY**, **ELF3** and **GI** genes from *Lemma gibba* plants indicated these genes are functionally conserved with Arabidopsis and rice genes (Sericawawa et al. 2008).

Here we propose a common evolutionary genetic history that gave rise to both barley and Arabidopsis clock genes from a common ancestor (Fig. 5). This hypothesis is based on robust in silico searches and phylogenetic analysis.

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**Fig. 5** Schematic diagram of the proposed evolutionary history of circadian clock components of barley, Arabidopsis and their putative common ancestor. Independent duplication events are represented by fine diagonal lines. The diagram at the bottom right is related to the main diagram and it refers to the numbers of genes from each group.
ancestor of land plants possessed orthologues of AtTOC1, AtPpRR7/3, AtPpRR9/5 and PpPRR1/2/3/4 in its genome, but only the PpPRR1/2/3/4 gene was maintained in the moss lineage, whereas angiosperms lost only the PpPRR1/2/3/4 orthologue (Satbhai et al. 2010).

Multiple independent clock gene duplications have occurred in both monocot and dicot ancestors, generating paralogues. Paralogues could be functionally equivalent to missing genes (e.g. ELF4-likeA) or deviate in terms of function/regulation. ELF3, ELF4-like3, FT, CO/COL1, GRP7, ZTL, PRR7 and PRR9/5 were independently duplicated and maintained in both monocots and dicots, which is an interesting example of convergent evolution. In the ancestor of moss, LHY/CCA1, ELF3, LUX, PpPRR1/2/3/4 and GRP7 were independently duplicated several times as supported by studies in diverse plant species, including barley (Campoli et al. 2012b; Cockram et al. 2012; Holm et al. 2010; McClung 2010; Satbhai et al. 2010). Convergent evolution also interfered with our phylogenetic analysis and the determination of one-to-one gene homology. For example, it is not certain which monocot gene, PRR95 or PRR59, is the orthologue of AtPRR9/5 (Takata et al. 2010).

A large proportion of gene duplication events has been generated by whole genome duplication (WGD) events (Paterson et al. 2010). The evolution of angiosperm genomes has been characterised by WGD events, typically accompanied by considerable gene loss (Paterson et al. 2010). However, plants have preferentially retained clock genes, which is consistent with the gene dosage hypothesis (Lou et al. 2012). This hypothesis predicts that genes encoding proteins engaged in dose-sensitive interactions, such as transcriptional or signalling networks, cannot be reduced back to single copies once all interacting partners are simultaneously duplicated in a WGD because the imbalance associated with this loss is likely to decrease fitness (Schnable et al. 2012). Additionally, paralogues could also deviate in terms of function or regulation. An example of sub-functionalisation is the PRR3 gene in Arabidopsis, which is expressed in the vasculature (Para et al. 2007), while other PRRs exhibit widespread expression. An excellent example of WGD coupled with retention of dose-sensitive duplicated clock genes has recently been reported for the evolution of Brassica rapa (Lou et al. 2012). In this work, it is suggested that such phenomena have permitted the evolution of increasingly complex circadian clock mechanisms (Lou et al. 2012). Clock complexity probably allowed for increased entrainment efficiency and temporal regulation of output pathways (Tauber et al. 2004), which has contributed to adaptation of plants to different environments. In summary, the availability of the barley gene space has allowed us to identify barley clock genes and propose their evolution in relation to the model plant Arabidopsis.

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Conflict of interest The authors declare that they have no conflict of interest.

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