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Molecular genetic examination of *Circadian Clock Associated 1* gene (*CCA1*) in hexaploid wheat

Ádám D. Horváth¹, Tibor Kiss^{2*}, Balázs Kalapos¹, Ildikó Karsai^{1*} and András Cseh¹

Abstract

Background The circadian clock is one of the most crucial regulatory pathways controlling plant development and stress responses, with the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene playing a central role. While the regulatory mechanisms and structure of this gene have been well characterized in *Arabidopsis thaliana* L., only limited information is available for hexaploid wheat.

Results The daily expression of *TaCCA1*, measured as the combined transcript abundance across the three wheat subgenomes, showed a clear diurnal rhythm in all three winter wheat genotypes, and this rhythm was significantly modulated by ambient temperature. In the early-heading genotype, the peak expression estimated by the cosinor model was approximately threefold higher at 18 °C than at 25 °C ($p < 1 \times 10^{-15}$). We identified a substantially larger insertion in intron 3 of *CCA1* gene in barley, which is even longer in hexaploid wheat compared with the homologous region in *Arabidopsis*. Phylogenetic analysis of *CCA1* gene revealed a strong association between the subgroups and genomes of the studied species. An alternative isoform located in intron 6 of *LATE ELONGATED HYPOCOTYL* (*LHY*) gene in barley showed 95% similarity to a sequence found in intron 3 of *TaCCA1*. We also identified a low-temperature-responsive cis-regulatory motif in hexaploid wheat that was detected exclusively in the A subgenome and was highly conserved.

Conclusions The elucidated gene structure of *TaCCA1* provides additional insight into the molecular basis of ambient temperature-dependent circadian regulation in wheat.

Keywords *CCA1* gene, Daily expression patterns, Gene structural analysis, Phylogenetic analysis, SNP polymorphism, *Triticum aestivum* L.

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Background

As a result of global climate change, the frequency of the extreme weather events has significantly increased, which could threaten the productivity of the global agricultural system. Therefore, climate-smart farming is becoming an important method in breeding [1]. Re-engineering of the circadian rhythm in cereal species can support the sustainable crop production [1].

Despite the extensive characterization of circadian clock regulation in *Arabidopsis*, considerably less is known about the temperature-dependent regulation, structural variation, and alternative splicing of core circadian genes in hexaploid wheat. In particular, the daily expression dynamics and intron-based regulation of the *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*) gene remain poorly understood in cereals. The circadian rhythm is an endogenous regulatory mechanism (an autonomous oscillator) that enables plants to synchronize their internal biological processes with the changes in daily temperature and the light conditions of the external environment [2–4]. Moreover, it also participates in essential physiological processes, such as leaf movement, stomatal opening/closing, flowering, elongation of hypocotyl, photosynthesis, carbohydrate biosynthesis, and responses to biotic and abiotic stresses [5–12]. Significant interaction was described between the core circadian genes and the photoperiodic flowering and growth, which could determine the environmental adaptation of plants [13]. The regulatory mechanism of the plant circadian rhythm has already been explored extensively in *Arabidopsis thaliana* (L.). In this model plant, the circadian clock is organized into interconnected transcriptional feedback loops that coordinate daily gene expression with environmental cues. Among the core clock components, the Myb-related transcription factors *CCA1* and *LATE ELONGATED HYPOCOTYL* (*LHY*) show peak expression at dawn and play a central role in setting the phase and amplitude of circadian rhythms [14–16]. These morning-expressed genes repress evening-phased clock components, including *TIMING OF CAB EXPRESSION 1* (*TOC1*), thereby maintaining the daily oscillatory cycle [17–20]. Importantly, *CCA1* expression and function are sensitive to ambient temperature, and temperature-dependent regulation of *CCA1* and related clock genes has been linked to changes in gene expression amplitude and alternative splicing, highlighting intron structure as a potential regulatory element in circadian responses [21–30]. So far, the temperature-dependent isoforms of *CCA1* gene have been studied in *Brachypodium*, rice, barley, sugarcane, and maize. Both alternative isoforms, *CCA1 α* and *CCA1 β* , were identified [31] and examined to assess the effects of low ambient temperature on their expression [32]. A conserved intron retention event was identified in the intron 4

region of *CCA1 β* isoform in both *Brachypodium* and rice [33]. The increased expression level of the *CCA1 β* was significantly influenced by abiotic stress factors, such as high temperature and drought, as well as by biotic stress, including aphid infection [34, 35]. An alternative splicing isoform was identified in the intron 6 region of the *LHY* gene, in the ortholog of the *TaCCA1* gene, and in barley [36]. The correlation between the low ambient temperature and the alternative splicing events of core circadian genes (*LHY*, *PRR7*, *TOC1*, *PRR9*, *PRR5*, and *PRR3*) has been examined in *Arabidopsis* [37]. These interactions have also been discovered in sugarcane [38]. Three isoforms of the *ZmCCA1* gene (*ZmCCA1.1*, *ZmCCA1.2*, and *ZmCCA1.3*) were described in maize [39]. In addition to their response to low-temperature stress, the roles of circadian genes in regulating other abiotic stress mechanisms have also been investigated [40, 41]. The effects of various abiotic stress factors were studied in relation to one of the most important transcription factors of the abscisic acid regulatory pathway (*ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR3* (*ABF3*)), as well as the epistatic interactions between the *CCA1* and *LHY* genes [42].

However, in hexaploid wheat, the temperature-dependent regulation and structural features of *CCA1*, particularly those related to intron organization, remain largely unexplored. In general, it can be regarded that the circadian rhythm is essentially a temperature-compensating system: within a relatively wide temperature range, the daily rhythm of a given gene maintains a consistent periodicity, although the amplitude of expression may vary [43]. Temperature-dependent changes in *CCA1* expression amplitude have been reported in *Arabidopsis* and barley, indicating conserved thermal responsiveness of this core clock gene in plants [44, 45]. Furthermore, only a little information is available regarding structural variations in the *CCA1* gene in wheat, in related species, and in *Arabidopsis*.

Taken together, while circadian clock regulation has been extensively characterized in *Arabidopsis* and several homologous genes have been identified in wheat, only limited information is available on the ambient temperature-dependent regulation (above the vernalization level), structural diversity, and intron-based control of these genes in cereals. In this study we hypothesized that the *CCA1* gene plays a central role in regulating cereal development in response to environmental cues. Based on previous findings in *Arabidopsis* and other cereal species, we expected that (1) the *TaCCA1* gene would exhibit a robust daily expression rhythm with a morning peak under controlled long-day conditions, (2) ambient temperature would primarily modulate the amplitude rather than the phase (i.e. the timing of the daily expression peak) of *TaCCA1* expression in wheat genotypes,

and (3) conserved intron structure and intron-associated regulatory elements of the *CCA1* gene would be detectable among wheat, barley, and *Arabidopsis*, supporting a potential role of intron-based regulation in temperature-responsive circadian control. In addition, we aimed to characterize the allelic distribution of the *CCA1* gene in a genetically diverse winter wheat panel using KASP marker analysis. The allelic distribution of *TaCCA1* may reflect genetic diversity shaped by breeding and adaptation. Consequently, understanding these structural and regulatory variations could improve insights into cereal adaptability, productivity, and geographical distribution.

Materials and methods

The gene expression study of *CCA1* gene at different controlled ambient temperature levels

Plant materials

Three winter wheat cultivars with different plant development and genetic backgrounds ('Mv Toborzó' /AT1/, Hungary, 'Tommi' /AT3/, Germany, 'Charger' /AT20/, Great-Britain) were selected based on previous experiments [46, 47]. The genotypes represent the two allele types of the main photoperiod sensitivity gene (*PPD-D1*) in hexaploid wheat: one photoperiod-insensitive allele ('Mv Toborzó') and two photoperiod-sensitive alleles ('Tommi' and 'Charger'). The plant materials were obtained from the genebank of the HUN-REN Centre for Agricultural Research Institute (Martonvásár, Hungary).

Experimental conditions

The effect of two constant ambient temperature levels (an optimal 18 °C and a supra-optimal 25 °C) was studied under long photoperiod (16 h from 5:00 a.m. to 9:00 p.m.) with 240 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPDF) light intensity provided by metal halide light bulbs in two PGV 36 growth chambers (Controlled Environments Limited, Winnipeg, Canada) in the Phytotron facilities of HUN-REN Centre for Agricultural Research. The humidity was constantly 70%. Each technically identical PGV-36 growth chamber represented one temperature level, with all the other factors kept uniform between them throughout the experiment. Based on the methods of Kiss et al. [46], the plants were germinated in Jiffy pots (Jiffy® Zwijndrecht, Netherlands) at room temperature for one week. Following this, the vernalization treatment lasted for 60 days at 3 °C under short photoperiods (9 h light period/ 24 h) with 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (PPDF) low light intensity. The sampling methods and experimental parameters were based on the study of Kiss et al. [47]. After vernalization, plants at the 1–2 leaf stage were transplanted into pots measuring 12 cm in diameter and 18 cm in height. Before transferring the plants to the growth chambers, approximately 1.5 kg

of soil was filled into the pots with a 4:1 mixture of garden soil and sand. In order to study the effects of ambient temperature on the daily expression patterns of *TaCCA1*, temperature settings of 18 °C and 25 °C were applied in both chambers. In both treatments, leaf sampling started at Zeitgeber Time 1 (ZT1; 6:00 a.m.) with ZT0 defined as 5:00 a.m. (lights on). The last fully expanded leaves were collected from two-week-old (14 d) plants for two consecutive days (48 h) every three hours with three biological replications, and the process was finished at ZT1 of the 3rd day. Each biological replicate was pooled from the three plants from three separate pots, and they were immediately frozen in a 1.5 ml Eppendorf tube in liquid nitrogen. After this, they were stored in a – 80 °C freezer. A total of three biological replicates were used for each genotype (i.e., nine leaves per genotype at each sampling time). This design aimed to minimize potential physiological variation among individual plants. Each plant was sampled only once. Nighttime sampling was conducted under low-intensity green LED light (Philips, 1 W low-energy bulbs; Philips Electronics Ltd. Amsterdam, Netherlands).

Gene-expression studies

The gene-expression studies were carried out based on the methods described by Kiss et al. [47]. The total RNA was extracted by trizol-chloroform extraction with the Qiagen RNeasy Plant Mini Kit (Qiagen Ltd, Hilden, Germany). RNA extraction was performed using the QIAcube system (Qiagen Ltd, Hilden, Germany) with an additional DNase treatment step, following the manufacturer's instructions. The RNase-Free DNase Set (Qiagen, Hilden, Germany) was used to eliminate residual genomic DNA contamination. RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc. Waltham, USA) was applied for the cDNA transcription from 1.0 μg total RNA. The gene-specific primer pairs amplified the three homeoalleles (A, B, and D) of the *TaCCA1* gene in the exon 5 region (Table S1, Fig. S1).

Quantitative real-time PCR (qRT-PCR) reactions were carried out by three biological and two technical replications with SYBR-green methods on a Rotor-Gene Q equipment (Qiagen Ltd, Hilden, Germany). The expression level of the studied genes was measured by Rotor-Gene software. The relative concentration was calculated by the geometric average of three reference genes (*Actin*, β -*tubulin*, and *Ta30797*), based on the following formula [48]:

$$GA_{HKG} = \sqrt[3]{(\Delta Ct_{HKG1} \times \Delta Ct_{HKG2} \times \Delta Ct_{HKG3})}$$

$$RQ = \text{Ampl}^{GA_{HKG} - \Delta Ct_{TG}}$$

GA: Geometric average.

Ct: Cycling threshold.

HKG: Housekeeping genes (Reference genes).

RQ: Relative concentration.

TG: Target genes.

Ampl: Amplification coefficient [corresponds to the reaction efficiency of each sample (values of 2 = 100% efficiency)]

The stability of housekeeping genes (*Actin*, β -*tubulin*, and *Ta30797*) was evaluated based on mean Ct values and coefficients of variation (CV). The presence of a daily rhythm was tested using a linear cosinor model. The linear form of the cosinor model is expressed as:

$$Ct(t) = M + A \cdot \cos\left(\frac{2\pi t}{24}\right) + B \cdot \sin\left(\frac{2\pi t}{24}\right) + \varepsilon$$

where Ct(t) is the gene expression (Ct) at a given time t in hours, M is the mesor (mean Ct), A and B are the coefficients of the cosine and sine components, and ε represents the error term. This model was used to examine whether the expression of housekeeping genes exhibited a significant daily rhythm. Analyses were performed using linear mixed-effects models (lmer package, R; [49]), with gene and technical replicate included as random effects.

Among the tested candidate reference genes, *Actin* showed the lowest CV (1.54%), indicating the most stable expression across our experimental conditions, followed by β -*tubulin* (2.22%) and *Ta30797* (2.54%). Linear mixed-effects modeling including cosinor terms (cosine and sine of time) as fixed effects, with gene identity and biological replication as random effects, revealed that the tested reference genes did not display significant daily rhythmicity. These results confirm that the expression of *Actin*, β -*tubulin*, and *Ta30797* is stable over time, making them suitable reference genes for normalization in qRT-PCR experiments under the tested conditions.

Sequence collection and comparison of gene structures with in silico approaches

Potential interspecific differences in the *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*) gene sequence were examined using in silico approaches. The comparative analysis was performed using the Ensembl Plants online database (<https://plants.ensembl.org>). The protein-coding sequences (CDS) of the *CCA1* gene were identified in both diploid progenitors of bread wheat, that is, in *Triticum urartu* L. (A genome) and in *Aegilops tauschii* L. (D genome). Furthermore, the CDS sequences of the *CCA1* gene were also identified in the three homeologous genomes of hexaploid wheat (*Triticum aestivum* L.), as well as in their orthologous sequences in barley (*Hordeum vulgare* Morex 7 H subsp. *vulgare* L.) and

(*Arabidopsis thaliana* L.). A single genotype was examined for *Arabidopsis*, barley, and diploid progenitors, while in hexaploid wheat, 11 reference genotypes were analyzed for the A genome, 10 for the B genome, and 11 for the D genome (Table S2). In the present study, the gene structures of RefSeq v2.1 for Chinese Spring were identified and illustrated.

Phylogenetic analysis of the *CCA1* gene

The amino-acid sequences of the protein-coding regions of the *CCA1* gene were aligned by MUSCLE Multiple Sequence Alignment (MSA) algorithm in MEGA 11 software. The Maximum Likelihood (ML) method was applied for the construction of the phylogenetic tree [50] with the following parameters: 1000 replicates bootstrap value, model of Jones-Taylor-Thronton, Gamma Distributed Partial Deletion (JTT + G + Partial deletion). The pseudogenes were not used for the reconstruction of the phylogenetic tree [51].

Genotyping of a diverse group of 185 winter wheat varieties using KASP markers associated with the *CCA1* gene

The protein-coding sequences (CDS) of *CCA1* gene orthologs from wheat, barley, and *Arabidopsis* were retrieved from the Ensembl Plants database (<https://plants.ensembl.org>) (Table S2). These reference sequences were aligned by the MUSCLE Multiple Sequence Alignment (MSA) algorithm in MEGA 11 to identify the possible SNPs (Single Nucleotide Polymorphisms), which were visualized with the CLC Genomics Workbench 3.6.5 program. Two allelic-specified and one common primer sequence for Kompetitive Allele Specific Polymerase Chain Reaction (KASP) analysis were designed in the Polymarker online tool (<https://www.polymarker.info/>) (Table S3). The experimental identification of possible allelic variants of the *CCA1* gene was carried out using the KALGEN panel consisting of 185 winter wheat genotypes from the genebank of the HUN-REN Centre for Agricultural Research (Martonvásár, Hungary), which had previously been studied in detail by Horváth et al. [52]. Genomic DNA was extracted from young leaves (~100 mg) using the DNeasy Plant Mini Kit (Qiagen Ltd, Hilden, Germany), according to the manufacturer's protocol, and stored at -20 °C until use. The KASP Assays were prepared according to the instructions of 3CR Bioscience Ltd. (<https://3crbio.com/document/pace-2-0/>). Prior to analysis, the DNA samples were dried at 55 °C for one hour in a heat chamber. KASP assays were performed on 384-well plates using PACE 2.0 Genotyping Master Mix with a reaction volume of 5 µl per sample. The fluorescently labeled (FAM, HEX) samples were read by QuantStudio 5 Real Time PCR equipment (Applied Biosystems). The allelic distribution was analysed by

QuantStudio Design & Analysis Software v.1.5.2. The KASP marker segregating in the genetic panel was then built into the LD map consisting of 7273 polymorphic SNPs, that was published by Horváth et al. [52]. Genome Wide Association Study (GWAS) was then rerun on the plant developmental and morphological traits originated from a three-year field sown experiment (Horváth et al. 2023) applying the Compressed Mixed Linear Model (CMLM) in the GAPIT package [53] as referenced in Horváth et al. [52]. Here, the effect of the *CCA1* SNP was only taken into consideration.

The structural comparison of the intron 3 region between *Arabidopsis* and cereals

The structures of the exon-intron regions were compared using the CLC Genomics Workbench 3.6.5. program. The large insertion within the intron 3 region was examined by Basic Local Alignment Search Tool (BLAST) based in silico approaches [54] in the Ensembl Plant database. The intron region of the *CCA1* gene of a barley genotype ‘Morex’ was searched by BLAST against the Chinese Spring RefSeq v2.1 wheat genome to identify interspecific homology and detect potential repetitive sequences from barley within the corresponding homologous region in wheat. Figures 3 and 4 illustrates the comparison of insert lengths in intron 3 of *CCA1* gene between hexaploid wheat, its progenitor and the homologous region in barley was aligned by MUSCLE Multiple Sequence Alignment (MSA) algorithm in CLC Genomics Workbench 3.6.5. software.

Identification of an Alternative Splicing (AS) event in the intron 3 region of the *TaCCA1* gene

An alternative isoform in the intron 6 region of the *LHY* gene in barley was determined by Calixto et al. [36]. Based on this study, in silico analyses were performed on the intron 3 sequence of *CCA1* gene from *Triticum urartu* L., *Aegilops tauschii* L. and Chinese Spring to detect possible conserved AS events. The DNA sequences were aligned by the MUSCLE Multiple Sequence Alignment (MSA) algorithm implemented in MEGA 11 to identify polymorphisms among the isoforms. The intron sequence between the third exon and the alternative exon was submitted to the Plant CARE platform (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) to investigate the potential biological roles of the *Cys*-regulatory elements and the alternative isoform within the intron 3 of the *CCA1* gene in barley and in hexaploid wheat [55]. No experimental validation (e.g., splice-junction RT-PCR) was performed; therefore, all splicing events reported here are predicted. The intron 3 sequence between the alternative exon and fourth exon was also studied by MEME Suite 5.5.9 software (<https://meme-suite.org/meme/tools/meme>) to identify the poss

ible transposable elements and repeated motifs in barley and hexaploid wheat [56]. The following parameters were applied for the analysis: site distribution was set to Any Number of Repetitions (anr) and the Select the number of motifs were 3.

The further exon and intron sequences in the *CCA1* gene were aligned by the MUSCLE Multiple Sequence Alignment (MSA) algorithm performed in MEGA 11 to identify polymorphisms among cereals.

Primer design and sequencing

Three winter wheat cultivars were used in the current experiment (‘Mv Toborzó’ /AT1/, ‘Tommi’ /AT3/, ‘Charger’ /AT20/) for the determination of the *Cys*-regulatory elements and the alternative isoform in the intron 3 of the *CCA1* gene. Three A genome-specific primer pairs were designed by the Primer3 online tool (<https://primer3.ut.ee/>), which amplified three different lengths of the DNA sequences (Table S4). The PCR products were sequenced by the standard Sanger Sequencing services of the Microsynth Seqlab (<https://www.microsynth.com/home-ch.html>).

Statistical analysis

The variance components were estimated by the GenStat 18.0 (VSN International Ltd.) and the SPSS 23.0 (IBM Data Science Community, Thomas J. Watson Research Center, Yorktown Heights, NY, USA) software packages with a restricted maximum likelihood method (REML). Daily gene expression dynamics of the *CCA1* gene were analyzed using cosinor rhythmometry. Mean values $\pm$ SD and fitted cosinor curves were visualized with the R package ggplot2 [57].

Results

The daily expression patterns of the *TaCCA1* gene at different ambient temperature levels

An analysis of variance conducted on the complete dataset comprising three genotypes $\times$ two environments $\times$ eight daily time points $\times$ two days revealed that all four main factors were highly significant, though each explained a different proportion of the total variance. The *TaCCA1* was mostly influenced by the daily timing ($\sigma^2 = 62.9\%^{***}$) (Table S5). However, the ambient temperature also exerted significant effects on the gene function, both as a main factor ($\sigma^2 = 17.6\%^{***}$) and via the G $\times$ E interaction ($\sigma^2 = 11.3\%^{***}$) amounting to 28.9% of the total variance. The daily expression patterns of the *TaCCA1* gene were in good agreement with the expression of the homologous gene in *Arabidopsis*. In the early-heading cultivar (‘Mv Toborzó’) the peak expression of this gene nearly doubled at 9 a.m. (ZT4 and ZT28) under 18 °C compared to the late genotypes (‘Tommi’ and ‘Charger’). Under the same treatment, a smaller,

however still significantly elevated expression level was also observed at 6 p.m. (ZT13 and ZT37) in ‘Mv Toborzó’ and ‘Charger’ compared to the late heading variety ‘Tommi’ (Fig. 1). These results indicate that genotype and temperature significantly influence the daily average expression level of *TaCCA1*. The highest expression across the entire day was observed in ‘Mv Toborzó’ at 18 °C, while in the late-heading genotypes (‘Tommi’ and ‘Charger’), increased expression was restricted to the morning hours at 25 °C (Fig. 1). All in all, the significant genotypic effect was primarily due to the large, ambient temperature dependent difference in the amplitude of the photoperiod insensitive genotype ‘Mv Toborzó’. Cosinor analysis revealed temperature-dependent differences in rhythmic parameters across the examined genotypes (Fig. 1, Fig. S2). At 18 °C, all genotypes exhibited higher oscillation amplitudes compared to 25 °C. The reduction in amplitude at 25 °C was most pronounced in AT1 (amp_{18°C} = 7.8 vs. amp_{25°C} = 2.6), while AT3 and AT20 showed a more moderate decrease (AT3: 6.5 to 4.9; AT20: 5.9 to 3.2) (Fig. S2). Peak timing was relatively conserved between temperatures, with maxima occurring around 25.5–27 h at 18 °C and shifting slightly to approximately 30–30.5 h at 25 °C across genotypes.

The phylogenetic analysis of the *CCA1* gene among *Arabidopsis*, barley, and wheat

The phylogenetic tree of the *CCA1* gene was constructed to examine the possible evolutionary diversities between the homologous genes of *Arabidopsis* and two cereal species (barley and wheat) based on 36 amino acid sequences. Based on the phylogenetic analysis of *CCA1*, the genotypes clustered into five distinctly separated subgroups (Fig. 2). A strong association was observed between the subgroups and the genomes of the different species. The first cluster (highlighted in orange) corresponded to *Arabidopsis thaliana* L. 2G, the second (blue) to *Hordeum vulgare* ‘Morex’ 7 H, the third (red) to the 7 A genome of *Triticum aestivum* L., the fourth (green) to the 7B genome, and the fifth group (grey) to the 7D genome (Fig. 2). Among the two diploid wheat progenitors, *T. urartu* clustered with the red cluster and *Ae. tauschii* with the grey cluster, indicating the reliability of the method (Fig. 2). Although *T. urartu*, which carries the A genome, was dispersed within its group, *Ae. tauschii* showed close association with the reference genotypes. Interestingly, the cultivars ‘Lancer’ and ‘Kariaga’ formed a distinct subgroup within D genome cluster. Furthermore, within the B genome group (green), ‘Lancer’ also exhibited clear separation from the other reference genotypes (Fig. 2).

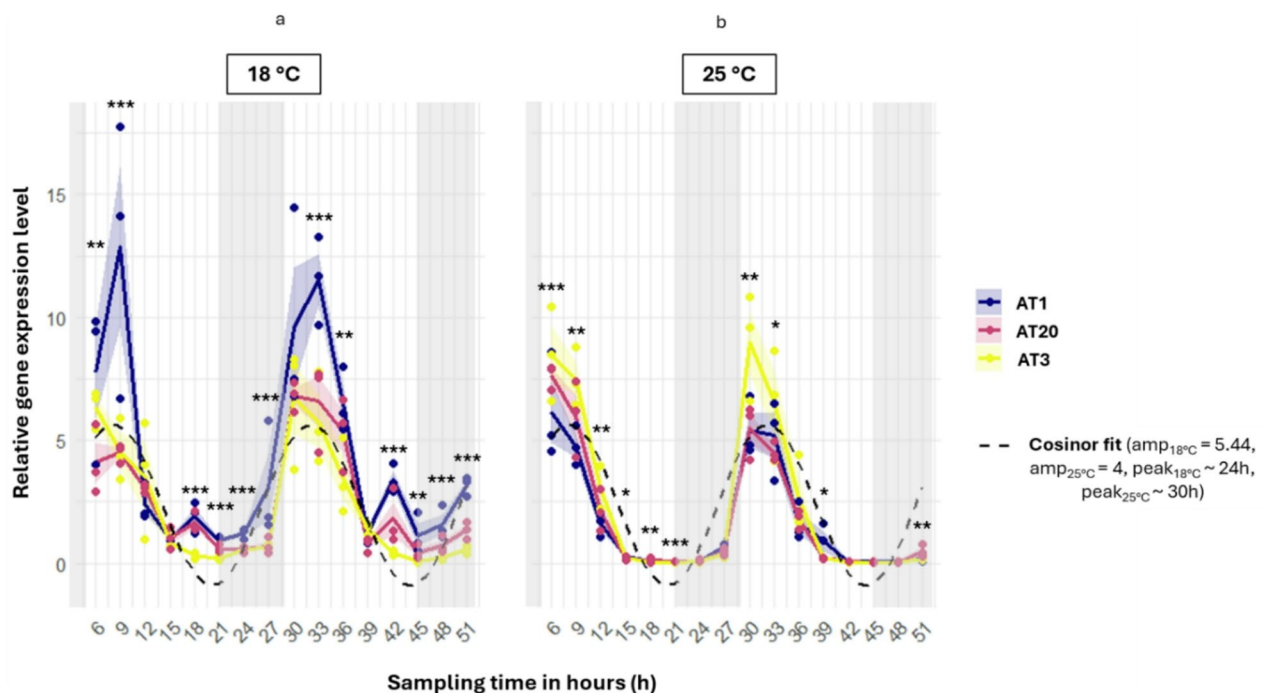


Fig. 1 The daily gene expression dynamics of *CCA1* normalized against the geometric means of three housekeeping genes in three winter wheat cultivars under two controlled environmental conditions: optimal 18 °C (a) and supra-optimal 25 °C (b). Data points represent individual measurements from three independent biological replicates; solid lines indicate mean expression values; shaded areas denote ±SE. Grey rectangles indicate the dark periods. *, **, *** denote significant differences at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ probability levels, respectively. AT1 = Mv Toborzó, AT3 = Tommi, and AT20 = Charger. Cosinor-fitted curves were generated from the average expression of all three cultivars, and the mean amplitude (amp) and peak time were calculated from these fits. Average AMP at 18 °C was 5.44 and at 25 °C 4.0, with mean peak times of 24 h and 30 h, respectively

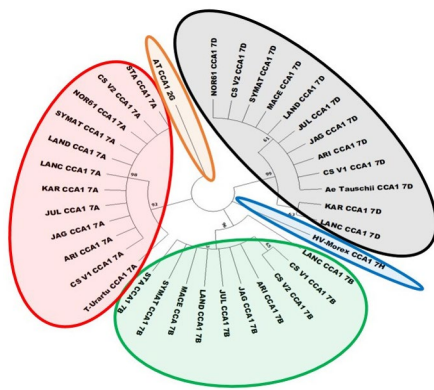


Fig. 2 Determination of the evolutionary diversities among the homologous genes of the CCA1 in *Arabidopsis* and two cereal species (*Hordeum vulgare* L. and *Triticum aestivum* L.). Abbreviations: CCA1=CIRCADIAN CLOCK ASSOCIATED 1, CS=Chinese Spring, STA=Stanley, Nor61=Norin 61, SYMAT=SY Mattis, LAND=Landmark, LANC=Lancer, KAR=Kariega, JUL=Julis, JAG=Jagger, ARI=Arinalfor, T. urartu=*Triticum urartu*, MACE=Mace, Ae. tauschii=*Aegilops tauschii*, AT=*Arabidopsis thaliana*, HV=*Hordeum vulgare*

The structural comparison of the protein-coding sequences of the CCA1 gene among wheat, barley, and *Arabidopsis*

For the structural comparison, the wheat homeolog sequences were aligned with the homologous intron 3 regions of barley and of *Arabidopsis* as well. The comparative analysis revealed a strong conservation among the protein-coding sequences of *AtCCA1*, *TaCCA1*, and *HvCCA1* genes in *Arabidopsis*, hexaploid wheat (*T. aestivum*, Chinese Spring 7 A, 7B, and 7D), and barley (*Hordeum vulgare*, Morex 7 H *supsp. vulgare*). In contrast, significant differences were observed in the non-coding regions of CCA1, most notably in the size of intron 3 (Fig. 3). A substantially larger insertion was determined within the intron 3 of the coding region in hexaploid wheat and barley compared to the homologue region in *Arabidopsis*. The length of this insertion was 1645 bp in barley, and even greater in hexaploid wheat: 3311 bp in genome A, 3428 bp in genome B, and 3303 bp in genome D, whereas the corresponding region in *Arabidopsis* measured only 479 bp (Figs. 3 and 4).

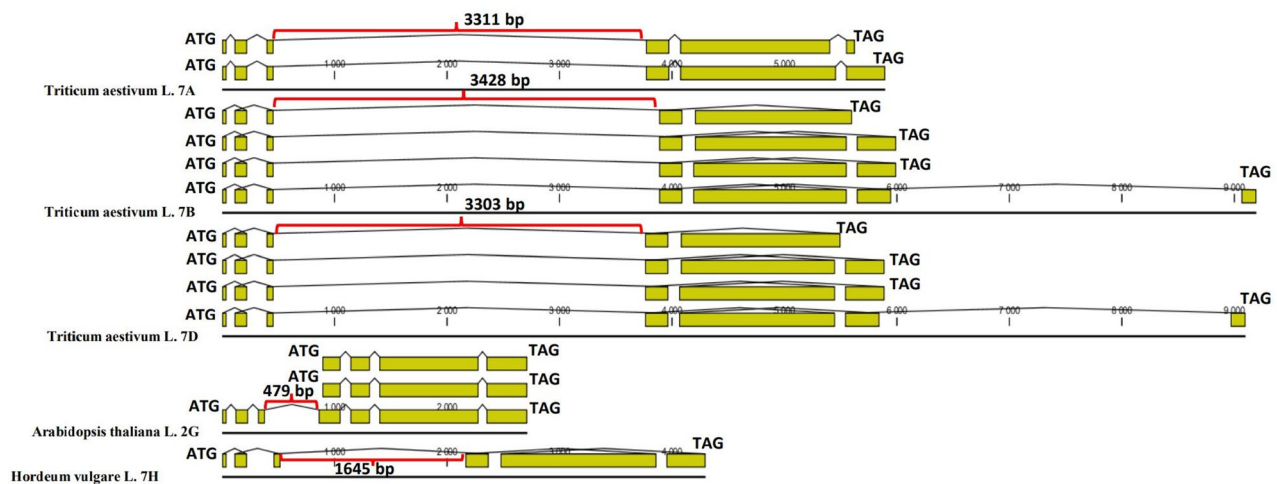


Fig. 3 The comparison of the different insert lengths in the intron 3 region of the CCA1 gene in hexaploid wheat, barley, and *Arabidopsis*. Abbreviations: ATG = start codon; TAG = stop codon; yellow rectangles = exon sequences; lines = intron sequences; thick line = scale (500 bp); red brackets = length of the intron 3 region. Genome-specific isoforms of the CCA1 gene in the IWGSC RefSeq v2.1 gene models for Chinese Spring are shown as separate gene structures for each genome

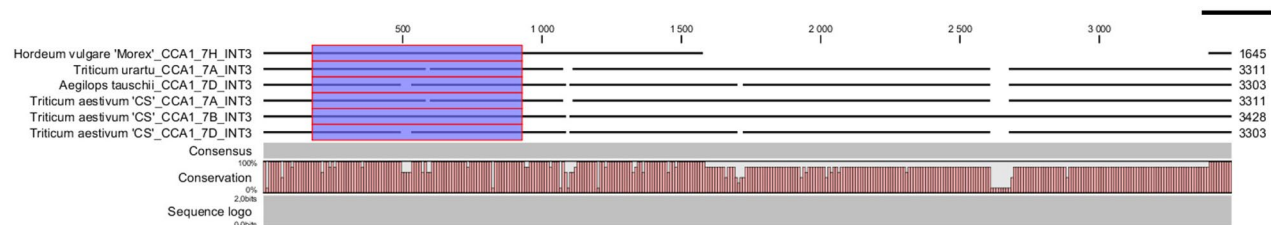


Fig. 4 Structural comparison of conserved Alternative Splicing (AS) events and insert lengths in the intron 3 region of CCA1 gene among hexaploid wheat, its diploid progenitors and barley. Abbreviations: CCA1=CIRCADIAN CLOCK ASSOCIATED 1, Int3=Intron 3, CS=Chinese Spring, lines=intron sequences, bold lines=scale (500 bp), purple box=isoform in the intron 3 sequences

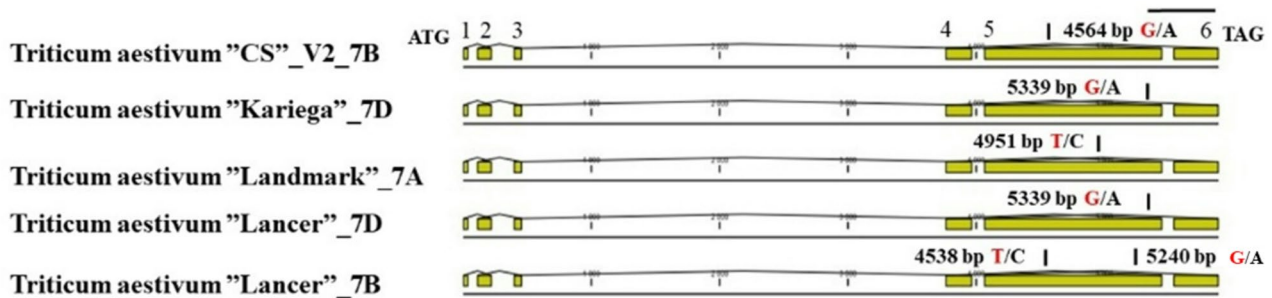


Fig. 5 SNP mutations in the exon 5 of the *CCA1* gene of wheat genotypes were investigated ('Chinese Spring' *TaCCA1* 7B, 'Kariega' *TaCCA1* 7D, 'Landmark' *TaCCA1* 7A 'Lancer' *TaCCA1* 7D and 7B). Abbreviations: ATG=Start codon, TAG=Stop codon, yellow rectangles=exon sequences, lines=intron sequences, thick line=scale (500bp), black vertical lines=position of SNP, SNP=Single Nucleotide Polymorphisms, G=Guanine, A=Adenine, T=Thymine, C=Cytosine, red letters=major allele, black letters=rare allele

Identification of the SNPs in the homeologous sequences of *HvCCA1*, *AtCCA1*, and *TaCCA1*

When we compared exon structures among 36 in silico wheat genotype sequences available in the Ensembl Plants database, we identified several single-nucleotide polymorphisms (SNPs) that distinguished the H, A, B and D genomes (Table S8). However, no polymorphisms were detected within any individual genome, with one exception. In exon 5 several SNPs were identified (Fig. 5). One nonfunctional mutation was found on chromosome 7A in 'Landmark' (GGC (Gly)) at position 4591 bp and on chromosome 7B in 'Lancer' (GCA (Ala)) at 5240 bp. A synonymous mutation was found in both 'Chinese Spring' (GAG (Glu)) at position 4564 bp and 'Lancer' (GCG (Ala)) at position 4538 bp on chromosome 7B. Additionally, both 'Kariega' and 'Lancer' shared the same functional mutation (AAC (Asn)) at position 5339 bp on chromosome 7D.

KASP assay analysis

The allelic frequency of the functional SNPs identified in the exon 5 of *TaCCA1* and its association with phenotyping traits were analyzed by Kompetitive Allele Specific Polymerase Chain Reaction (KASP) assay in a genetically diverse population of 185 wheat varieties (Table S6). Based on the results, the mutant allele types of the *TaCCA1* gene were found with low frequency in the examined wheat population. Based on the distribution of the MBD050 marker (7B), 184 genotypes carried the wild-type allele [G/G], in contrast, the mutant homozygous allele [A/A] was observed only in the 'Chinese Spring' variety. For the MBD051 marker (7B), 183 genotypes were homozygous wild-type [T/T], and 2 were heterozygous [T/C]; the homozygous mutant allele [C/C] was not detected in any sample. In the case of marker MBD043 (7D), 166 genotypes were homozygous for the wild-type allele [G/G], 12 were homozygous for the mutant allele [A/A], and 7 were heterozygous [G/A],

possibly due to a mutation in the primer binding site (Table S6).

The three winter wheat cultivars used in the *TaCCA1* gene expression study ('Mv Toborzo'/AT1, 'Tommi'/AT3, 'Charger'/AT20) all carried the wild-type homozygous allele variants for the three tested markers (Table S6). The MBD043 SNP marker was then included into the LD map of the KALGEN panel and association analyses were carried out on the phenotypic data of plant development under field conditions, the original phenotypic dataset of which having been published by Horváth et al. [52]. No significant effects could be detected at the *TaCCA1*-7D exon 5 specific SNP.

Identification of cis-acting elements and an alternative exon in the intron 3 region of the *TaCCA1* gene

The isoform of the *LHY* gene identified in barley [39] was also detected within the intron 3 of the *CCA1* gene in hexaploid wheat and its diploid progenitors (*Triticum urartu* L. and *Aegilops tauschii* L.) (Fig. S2). Three distinct insertion lengths were determined in the corresponding DNA region of the three hexaploid wheat genomes and their wild relatives when compared to the barley sequence. A 95% similarity was observed among the isoforms of cereal species. The average SNP density in the wheat sequences was 5% within the studied regions. Additionally, relative to barley, *Triticum urartu* differed by 39 SNPs, *Aegilops tauschii* by 41 SNPs, and the 7 A, 7B, and 7D genomes of hexaploid wheat by 41, 43, and 39 SNPs, respectively (Table S7). The sequences of the three *TaCCA1* homoeologs in hexaploid wheat differed from the barley *CCA1* homolog by an average of four SNPs. Moreover, the mean SNP density per 100 bp in the exon and intron regions of *CCA1* was 3.91 and 3.66, respectively (Table S8). The potential regulatory roles of the alternative exon were investigated by identifying cis-acting elements within the intron 3 region of cereals. Six cis-regulatory motifs were found in barley genome 7 H and hexaploid wheat genome 7 A, five in the genome

7B and four in the genome 7D, all located in the intron sequence upstream of the alternative isoform (Table S9). Based on their biological functions, these regulatory motifs were categorized into three groups: hormone-responsive elements, environmental adaptation elements, and core promoter components. In contrast, only two categories - hormone-responsive elements and core promoter components - were detected in the D genome of hexaploid wheat. The motif composition was identical (100%) between barley 7 H and wheat 7 A chromosome. Among the examined intron regions, core promoter components (CAAT-box, TATA-box) constituted the largest proportion: 50% in 7 H and 7 A, 60% in 7B, and 75% in 7D. Environmental adaptation elements (ARE- anaerobic induction, LTR- low- temperature responsive 7 H = 33.3%, 7 A = 33.3%, and 7B = 20%) were involved in the second group, while the third consisted of the hormone-responsive elements (TCA- salicylic acid responsiveness 7 H = 16.6%, 7 A = 16.6%, 7B = 20%, and 7D = 25%) (Table S9). For identifying the possible source of the large, ambient temperature dependent differences in the *CCA1* gene expressions of the three wheat cultivars, the intron 3 region of the *CCA1* gene was sequenced. However, 100% similarity between their DNA sequences and that of 'Chinese Spring' was found (Table S10).

To identify putative transposable elements (TEs) and repeated motifs, we used MEME Suite v5.5.9 to analyse the sequences in intron 3 of the *CCA1* gene between the alternative exon and exon 4 in barley and hexaploid wheat (Fig. S3). No known transposable elements were identified in the corresponding DNA sequences. The intron regions analysed were longer in hexaploid wheat than in barley, with insertions of 1,692 bp on 7 A, 1,796 bp on 7B and 1,692 bp on 7D. In addition, MEME Suite detected three classes of repeated motifs (blue, red and green boxes) with varying degrees of sequence similarity (Fig. S3); however, their functions are currently unknown. The three blue motifs were present in both barley and wheat. Relative to barley, the first blue motif differed by four SNPs on 7 A, four SNPs on 7B and three SNPs on 7D. The second blue motif differed by five SNPs on 7 A, five SNPs on 7B and four SNPs on 7D, whereas the third blue motif differed by two SNPs on 7 A, two SNPs on 7B and three SNPs on 7D. The red motif differed by 11 SNPs in each of the three wheat subgenomes. No polymorphism was detected in the green motif, which showed the highest level of conservation between the two cereals. This motif was located at the end of the sequences and, in wheat, also at the end of the inserted region (Fig. S3).

Because the intron 3 sequences and the analysed cis-elements were identical among the three cultivars, these variants cannot explain the cultivar-specific differences in expression as influenced by the ambient temperature above vernalizing level. This suggests that other

regulatory mechanisms likely underlie the genotype-dependent differences observed in *CCA1* expression patterns and warrant further investigation.

Discussion

Previous studies have highlighted the central role and strong conservation of expression of the *CCA1* gene in barley as is the case in *Arabidopsis*, and they found the gene function independent of the allele type of *PHOTOPERIOD-H1* (*PPD-H1*) gene [20, 58] & [59]. In barley, the *CCA1/LHY* homologs were also identified as being responsive to photoperiod and temperature changes, particularly to low temperature; however, their effect on flowering time has not yet been clarified [3, 60]. This study also identified a similar phenomenon in hexaploid wheat. By examining three winter wheat cultivars, we demonstrated that the *TaCCA1* gene exhibits a peak expression level at dawn, irrespective of genotype or ambient temperature. The timing of this peak within the 24-hour cycle was consistent, a finding that corresponded well with previously reported functions in other species; the peak value itself varied significantly depending on the ambient temperature (optimal or supra-optimal) and the wheat genotype. The early-heading variety (AT1) exhibited a notable increase in gene activity at 18 °C compared to the late-flowering genotypes [47]. Based on the cosinor-fitted parameters, elevated temperature primarily affected rhythm amplitude rather than phase. The consistent delay in peak timing at 25 °C suggests a modest temperature-dependent phase shift of the underlying rhythmic process, while the pronounced amplitude reduction, particularly in AT1 (photoperiod insensitive), indicates genotype-specific sensitivity of rhythm robustness to temperature. Together, these results imply that higher temperature dampens rhythmic strength without substantially disrupting temporal coordination, although the extent of attenuation differs among genotypes. This indicates that in addition to its central role in one of the core circadian regulatory loops, *CCA1* may participate in ambient temperature sensing not only in the lower ranges but also as a responsive element to higher temperatures and, through this, may directly contribute to the genetic regulation of plant development in cereals. In wheat, no published results are yet available regarding this phenomenon. Thus, we made attempts to clarify the possible regulative backgrounds of the ambient temperature dependent genotype-specific responses. For this purpose, structural differences in the *CCA1* gene were examined among its orthologs in wheat, barley, and *Arabidopsis* using in silico approaches. Structural similarities were identified in the exon structures of *CCA1* gene between two cereal species (*Hordeum vulgare* L. and *Triticum aestivum* L.). In contrast, substantial divergence was observed in the amino acid sequence of the

dicotyledonous *Arabidopsis* compared to the monocots. However, the *CCA1* gene homologues clustered according to their three respective genomes in the reference wheat genotypes.

Calixto et al. [61] reported a high degree of similarity between the exon-intron structures of core clock genes in barley and *Arabidopsis*. Peng et al. [28] determined also a high level of homology between the coding and non-coding regions of wheat and barley. In the present study, we detected considerable homology among the amino acid sequences of the *CCA1* gene in the examined monocot species (wheat, its progenitors, and barley), consistent with the findings of [59]. A high level of conservation was confirmed among the downloaded sequences of the exon regions of wheat genotypes (<https://plants.ensembl.org>). Within the exons, we identified only three functional SNPs, all located in the exon 5 region of the reference sequences. When examining the occurrences of these mutant SNPs in a modern winter wheat panel comprising 188 genotypes, they were found to be missing or represented by rare alleles. Based on our results, the mutant allele type of marker MBD043 (7D) was the most variable; however, even in this case, it was present only in 12 wheat genotypes (6.4% of the wheat panel), six of which were bred in Martonvásár, Hungary. The exact source of this rare allele in the Martonvásár breeding materials could not be identified, but it is likely related to the breeding practice of frequently incorporating exotic genotypes from diverse geographic origins in the development of new crosses [52, 62]. Lee et al. [63] reported that rare allele types in the exon region of the *OsCCA1* gene may contribute to the environmental adaptation in rice by fine-tuning flowering time. More recently, Gerhardt and Mehta [1] suggested that different types of the mutation in the circadian genes could contribute to the geographical expansion of the crops. In the three-year field experiment conducted at a single location in Martonvásár (Central Europe, Hungary) [52], we could not detect any significant effects of the *TaCCA1-7D* exon 5 SNP on the developmental or morphological traits of the modern winter wheat panel. The absence of significant associations could be attributed to several factors. In addition to being a rare allele, and therefore more difficult to identify, the complexity of field conditions—combined with the variability arising from the different allelic structures of major developmental genes—could easily mask any minor effects of *CCA1* [47]. Experiments performed under controlled conditions and/or across multiple locations representing broader environmental variation will be necessary to determine the extent to which the *CCA1* gene, as characterized by the exon 5 SNP, contributes to environmental adaptation in wheat. When comparing the structural composition of the *CCA1* gene between *Arabidopsis* and cereal species, we

identified significant divergence in the size of the homologous intron 3 sequences among *CCA1* gene orthologs in *Arabidopsis*, barley, and wheat, as previously published by Gong et al. [30]. A threefold increase in intron size was observed in barley, whereas our analysis revealed approximately a sevenfold increase in wheat. This intron size expansion detected in cereals is not unique to *CCA1*, it has also been described in several flowering-related genes [28, 64–66]. The increase in intron length has been proven to have significant impact on gene function in cereals via various processes, including epigenetic regulation, altered affinity of transcriptional regulatory complex binding sites, and the mechanisms of alternative splicing, among others [36, 37, 64, 66–68].

Calixto et al. [36] and James et al. [37] examined the regulation of intron-based transcription of *CCA1/LHY* genes in barley and *Arabidopsis*, and reported the presence of an alternative exon within intron 6 in barley. Based on our results, the sequence of an alternative exon in wheat, located within intron 3 and showing similarity to the barley counterpart, exhibited 95% identity between hexaploid wheat and its progenitors. Several cis-regulatory motifs were also identified by us in the intron 3 region of the *CCA1* gene, located between the third exon and the alternative exon. It should be noted that this alternative splicing event was predicted computationally and has not yet been experimentally validated in wheat RNA. Therefore, the potential regulatory role of this alternative exon in temperature-responsive circadian control remains putative. The largest proportion of these motifs (50%) corresponded to core promoter elements in the homeologous sequences of barley and hexaploid wheat (Table S9). The second most abundant motifs were associated with environmental adaptation functions on the 7H chromosome of barley and the 7A-specific copy of the *CCA1* gene, where they are likely to play important roles in abiotic stress responses. The LTR (low-temperature-responsive cis-motif) was identified only in genome A of hexaploid wheat and genome H of barley, suggesting that it may play a key role in regulating *CCA1* gene transcription in bread wheat under low-temperature conditions. This finding is consistent with the results of Ramírez-González et al. [69], who reported that approximately 30% of the homeologous genes in allohexaploid wheat exhibit distinct expression patterns across the A, B, and D genomes, likely due to epistatic effects. Our results suggest that the LTR cis-motif on genome A may exert a stronger influence on the temperature-dependent expression of the *CCA1* gene in hexaploid wheat. The same cis-motifs and alternative splicing sequence were detected in the intron 3 region of the *TaCCA1-7A* gene in three winter wheat genotypes ('Mv Toborzó', 'Tommi' and 'Charger'), and these sequences were found to be completely identical among the three cultivars. This

result suggests that the alternative splicing region is likely responsible only for the low-temperature response and, as such, may exhibit strong conservation among wheat genotypes. Therefore, the differences observed in *CCA1* gene expression patterns at 18 °C and 25 °C are likely regulated by another pathway. Since the intron-3 sequences and the tested cis-elements are identical among the three cultivars, these cannot explain the cultivar-specific differences. Alternative regulatory mechanisms may contribute, including variation in trans-acting upstream regulators, epigenetic modifications, and homeolog-specific expression. Future studies incorporating genome-wide expression analyses and epigenetic profiling will be required to elucidate these mechanisms.

Conclusion

The present study revealed substantial differences in the size of the homologous intron 3 sequences of the *CCA1* gene, showing more than a threefold increase in barley and nearly a sevenfold increase in wheat compared with *Arabidopsis*. Several cis-regulatory elements were identified in the intron 3 region of *CCA1* within the homeologous sequences of barley and hexaploid wheat, most of which corresponded to core promoter components. A low-temperature-responsive cis-motif was also identified in hexaploid wheat, detected exclusively on the A genome.

The presence of a large insertion, the high sequence conservation among alternative isoforms, and the occurrence of a highly conserved low-temperature responsive cis-motif in the A subgenome of hexaploid wheat and the H genome of barley collectively suggest a conserved regulatory role of *TaCCA1* in the low temperature dependent circadian sensing across cereal species. The data further suggest that the *CCA1*-associated regulation may differ between low-temperature and optimal or supra-optimal temperature ranges. Together, these findings provide additional insight into the molecular basis of temperature-dependent regulation of circadian genes.

While this study provides structural and expression-level evidence for temperature-associated regulation of *CCA1* gene in cereals, experimental validation of the predicted alternative splicing event and the regulatory activity of intron 3 cis-elements in wheat will further strengthen these conclusions. Because the intron 3 sequences and the analysed cis-elements were identical among the three cultivars, the genotype-specific expression differences observed at 18 °C and 25 °C likely involve additional layers of regulation, such as variation in trans-acting regulators, epigenetic modifications, and/or homeolog-specific expression. Future work should therefore confirm the predicted splice isoforms in wheat RNA and functionally test the low-temperature responsive motif and the intron 3 insertion using reporter assays and/or targeted mutagenesis under defined temperature regimes. Extending

genome-specific expression analyses to a broader set of genotypes and environments will help delineate how low versus optimal or supra-optimal temperatures reshape the *CCA1*-associated regulatory network.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-026-08219-0>.

Supplementary Material 1. Table S1. The generic primer sequences in the gene expression studies (qRT-PCR). Abbreviations: *CCA1* – *Circadian Clock-Associated 1*, *PGD* – *Phosphogluconate dehydrogenase*, F=Forward, R=Reverse. Table S2. The gene codes of the DNA sequences used for the in silico approaches of the *CCA1* gene in the studied plant species are listed below. Table S3. The list of primer sequences of the *CCA1* gene for KASP genotyping. The genome specific primers were designed by PolyMarker. Abbreviations: *CCA1* – *Circadian Clock-Associated 1*, F=FAM and V=VIC fluorescens dyes, C=Common primer, Tm=Melting temperature, red and green highlights: FAM and VIC fluorescens labeled sequences, the studied allele variants are indicated by red at the end of the primer sequences. Table S4. The list of A genome specific primer pairs used in the sequencing of the intron 3 of *TaCCA1* gene. The primers were designed by Primer3. Abbreviations: *CCA1* – *Circadian Clock-Associated 1*, F=Forward, R=Reverse, Tm=Melting temperature, bp=base pair. Table S5. The effects of the different factors on the variance components of the *CCA1* gene (σ^2 (%)) under two controlled environmental temperature values on 14-days-old winter wheat. The factor of Environment refers to the two ambient temperature levels 18 °C vs 25 °C. Abbreviations: The not-significant factors are indicated by ns (not significant). The asterisks denote the significance level. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Table S6. The allele distribution of the *CCA1* gene in a diverse wheat population. The description column indicates the genotypes in the gene expression study. Table S7. Sequence-based comparison of the alternative isoform of the *CCA1* gene among wheat its progenitors, and barley. Table S8. Sequence-based comparison of exon and intron regions of *CCA1* gene between wheat and barley. Table S9. Categorization of the different cis-regulating motifs and their proportion in the intron 3 sequence before the alternative exon in barley *HvCCA1-7H* and hexaploid wheat *TaCCA1-7A*, *TaCCA1-7B*, and *TaCCA1-7D* genes. Table S10. Comparison between the sequenced intron 3 region of the *CCA1* gene with cis-motifs and alternative isoform among Chinese Spring and the three examined hexaploid wheat genotypes. Abbreviations: CS=Chinese Spring, Yellow highlights=Set1F, Set2F primers, Red highlights=Set2R, Set3F primers, Green highlights=Set1R, Set3R, TATA-box is indicated by red letters, Low Temperature Response motif is indicated by blue letters, Bold letters=Sequence of the alternative exon, asterisk=conservation among the nucleotide positions. Fig. S1. The position of generic primer sequences in the structure of the *CCA1* gene. Abbreviations: ATG=Start codon, TAG=Stop codon, yellow rectangles=exon sequences, lines=intron sequences, thick line=scale (500 bp), position of the (F) Forward and (R) Reverse primers indicated by red rectangles. Fig. S2. The daily gene expression dynamics of *CCA1* in three winter wheat cultivars (AT1, a; AT3, b; and AT20, c) under two controlled environmental conditions: optimal 18 °C and supra-optimal 25 °C. Data points represent individual measurements from three independent biological replicates; solid lines indicate mean expression values; shaded areas denote $\pm$ SE. Grey rectangles indicate the dark periods. AT1 = Mv Toborzó, AT3 = Tommi, and AT20 = Charger. Cosinor-fitted curves were generated from the average expression values of each cultivar, and the mean amplitude (amp) and peak time were calculated from these fits. Fig. S3. (A) The positions and sequence-based comparison of the identified repeated motifs in the intron 3 sequence after the alternative exon among barley *HvCCA1-7H* and hexaploid wheat *TaCCA1-7A*, *TaCCA1-7B* and *TaCCA1-7D* genes. The sequences of *TaCCA1-7A* are shown beside the colored rectangles. (B) Determination of the evolutionary diversities among the three repeated motifs in corresponding intron 3 sequences of *HvCCA1-7H*, *TaCCA1-7A* genes. Abbreviations: *CCA1* – *Circadian Clock Associated*, colored rectangles=repeated motifs, lines=intron sequences, SNP=Single-Nucleotide Polymorphism, bp=base pair, red bold letters indicate the polymorphisms between wheat and barley, A=Adenine, G=Guanine, T=Thymine, C=Cytosine, W=wheat, B=barley.

Authors' contributions

IK, AC, ÁDH and TK: conceptualization, methodology, and writing—review and editing. ÁDH, TK, and BK: investigation. ÁDH, TK, AC, BK and IK: formal analysis. TK, AC and IK: supervision and funding acquisition. ÁDH, AC, TK and IK: visualization. ÁDH and TK: writing—original draft preparation. All authors read and approved the final manuscript.

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Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files. The gene sequence data (CCA1-INT3) reported in this article have been deposited in the NCBI data libraries under the GenBank accession numbers PX527344, PX527345, and PX527346.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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