Genome-wide circadian gating of a cold temperature response in bread wheat

Calum A. Graham[1,2], Pirita Paajanen[1], Keith J. Edwards[2], Antony N. Dodd[1,3]

1. Department of Cell and Developmental Biology, John Innes Centre, Norwich Research Park, Norwich NR4 7RU, UK.

2. School of Biological Sciences, University of Bristol, Bristol Life Sciences Building, 24 Tyndall Avenue, Bristol BS8 1TQ, UK.

3. Corresponding author, antony.dodd@jic.ac.uk

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Abstract

Circadian rhythms coordinate the responses of organisms to their daily fluctuating environments, by establishing a temporal program of gene expression. This schedules aspects of metabolism, physiology, development and behaviour according to the time of day. Circadian regulation in plants is extremely pervasive, and is important because it underpins both productivity and seasonal reproduction. Circadian regulation extends to control of environmental responses through a regulatory mechanism known as circadian gating, whereby the circadian clock regulates the response to environmental cues such that the magnitude of response varies according to the time of day. Here, we show that there is genome-wide circadian gating of responses to cold temperatures in plants. By using bread wheat as an experimental model, we establish that circadian gating is crucial to the programs of gene expression that underlie the environmental responses of a crop of major socioeconomic importance. Furthermore, we identify that circadian gating of cold temperature responses are distributed unevenly across the three wheat subgenomes, which might reflect the geographical origins of the ancestors of modern wheat.

Teaser

There is genome-wide circadian gating of a response to low temperatures in a crop of major socioeconomic importance.

Introduction

Circadian rhythms are biological cycles with a period of about 24 h that persist in the absence of environmental cues, and establish a temporal structure within biological systems. A feature of circadian rhythms is that they determine the time of day (phase) when specific processes occur (*1, 2*). The process of entrainment aligns the phase of the circadian clock with the phase of the environment and, in turn, the circadian clock extensively influences the phasing of gene expression, metabolism and physiology (*3*). One way that this occurs in

plants is through clock controlled gene promoters, which temporally coordinate transcript accumulation according to the time of day (*1, 4-8*). The regulation of cellular processes by circadian clocks is important for crop performance, and understanding circadian regulation in crops forms an important part of the strategic design of climate change resilient crops (*9-12*).

Fluctuating spring temperatures and unpredictable cold periods caused by climate instability represent a threat to cereal crops worldwide (*13-15*). During multiple stages of development, wheat is susceptible to low temperatures (*16-18*), including during seedling establishment (*19, 20*). Low temperatures trigger genome wide responses in wheat that can reduce photosynthetic capacity, cause photoinhibition, and consequently reduce yield (*18, 21-25*). Therefore, wheat represents an excellent model to understand the processes that underlie responses of plants to low temperature conditions, because this has a direct impact upon crop yield.

One way that the circadian oscillator influences responses to environmental cues, such as temperature fluctuations, is through circadian gating. Circadian gating is the process whereby the circadian clock regulates the magnitude of a response to the environment, such that the response is restricted to certain times of day, or varies according to the time of day (*26, 27*). In plants, there are widespread examples of the circadian gating of responses to high and low temperature cues (*9, 28-36*), with temporal gating also occurring in cultivated rice fields and wild plant populations (*37, 38*). We do not know whether circadian gating of cold temperature responses occur at a genome-wide scale. This remains an important open question, because the pervasive nature of circadian gating of temperature responses makes it a core part of understanding how plants- including crops- respond to their fluctuating environments.

In hexaploid bread wheat, around 30% of the transcriptome is regulated by the circadian clock (*39*). Circadian clock gene loci such as *TaPpd* (orthologous to Arabidopsis *PRR3* or *PRR7*) determine agriculturally-important photoperiod responses of domesticated wheat (*40-*

43). The complexity of this signal integration is increased in polyploid crops such as wheat by the presence of multiple gene paralogs or homoeologs that can have differential patterns of circadian regulation (*39, 44*). In hexaploid wheat, over 50% of genes (approximately 50,000 genes) occur as sets of three gene homoeologs termed triads (*45, 46*), providing an informative genomic structure to study environmentally-responsive gene expression within polyploid genomes.

We investigated the involvement of circadian regulation in the genome-wide responses of hexaploid bread wheat to low temperatures. Given that the cold-responsive CBF pathway is gated by the circadian clock in Arabidopsis (*28*) and a third of wheat genes are circadian-regulated (*39*), we reasoned that there might be a pervasive influence of the circadian clock upon cold temperature responses. Using wheat as a model to investigate this question also provides insights into a species of vast socioeconomic importance. It also provides insights into the integration of circadian and environmental cues in polyploids. Taken together, our data identify a major role for circadian regulation in shaping the cold-responsive transcriptome in plants.

Results

The time of day affects the number of genes that respond to cold temperature conditions

We wished to discover whether there is circadian gating of responses to temperature cues in hexaploid bread wheat (*Triticum aestivum)*. We used low temperature treatments as an experimental model, because circadian gating of responses to cold has been detected for certain genes in Arabidopsis (*28, 47, 48*). Spring wheat varieties are more cold-sensitive than winter wheat (*49, 50*), and are exposed to low spring temperatures during seedling establishment. Therefore, we selected for experimentation a cold-responsive hexaploid spring wheat cultivar (Cadenza) that has no vernalization requirement, some low temperature tolerance, and good genome sequencing coverage and germplasm resources.

To test for the occurrence of circadian gating, short cold treatments were applied to separate batches of wheat seedlings under free running (constant) conditions (Fig. 1A). This comprised identical three-hour cold treatments ($4^{o}C$), at six sequential timepoints, following transfer to free running conditions (Fig. 1A). After each treatment, tissue was harvested for RNA isolation from the second leaf of control temperature and cold-treated seedlings, with each seedling sampled once only (Fig. 1A). Transcript count values (isoforms) were condensed to the gene level, so transcript abundance changes described here refer to changes at the gene level.

The number of differentially expressed genes (DEGs) in response to the cold treatment was different at each at each timepoint (log fold change > 0.5, false discovery rate < 0.05; Fig. 1B). This indicates that the time of subjective day determines the cold-induced transcriptome in wheat (Fig. 1B). The greatest number of genes were differentially expressed in response to cold at zeitgeber time (ZT) 48 (ZT48), with 4350 and 3403 genes significantly up or down-regulated, respectively (Fig. 1C). Two-thirds fewer genes were differentially expressed in response to a cold treatment at ZT28 or ZT44 (Fig. 1C), despite a similar total number of transcripts detected at these timepoints (Table S1). There was a large difference in the direction of change of DEGs at ZT36 and ZT40, with the number of up-regulated genes comparable to those at ZT32 and ZT48, but a smaller number of down-regulated genes that was comparable to the number at ZT28 and ZT44 (Fig. 1C). These data suggest the wheat transcriptome is more responsive to cold around the middle of the subjective day (ZT32) and towards the end of the subjective night (ZT48) (Fig. 1C).

There were fewer cold-downregulated than cold-upregulated genes at each timepoint, whereas the proportion of downregulated genes that were unique to each timepoint was consistently greater than the proportion upregulated genes that were unique to each timepoint (Fig. 1D). This suggests that genes downregulated in response to cold are especially influenced by the time of day. Interestingly, the proportion of cold-responsive genes that were unique to each timepoint corresponded to the total number DEGs at the

timepoint (Fig. 1C), suggesting that there is a relationship between the total number of cold-responsive genes and the number of genes that are cold-responsive at specific times of day.

The nature and cold-sensitivity of cold-responsive genes fluctuates across the 24 h cycle

We wished to understand whether the composition of the cold-responsive transcriptome changes over the 24 h cycle. To achieve this, we compared the identity of the genes that were cold responsive at each timepoint. Four of the largest six groups of DEGs were cold-induced at specific timepoints, so more genes responded to cold at specific (unique) times than were cold responsive at all timepoints (Fig. S1A). Furthermore, the top four sets of transcripts that were downregulated by cold were unique to single timepoints (Fig. S1B). Therefore, under free running conditions, the set of genes that responds to cold changes across the day. Some transcripts were cold responsive at single timepoints, some across part of the 24 h cycle, and some were cold-responsive regardless of the time (Fig. S1A, B).

Because the set of cold responsive genes changes across the 24 h cycle (Fig. 1D; Fig. S1A, B), we reasoned that there might be circadian gating of the responses to cold of the wheat transcriptome. There were a variety of responses to the time course of cold treatments, with cluster analysis revealing a large proportion of cold-responsive genes to have temporally-fluctuating response profiles (Fig. 1E). For example, certain genes are upregulated by cold in the subjective day and down-regulated by cold during the subjective night (Groups 5 and 6; Fig. 1E), whilst other genes are downregulated by cold during the subjective day, and upregulated or have little response to cold during the subjective night (Group 3; Fig. 1E). This indicates that the magnitude of transcriptional responses to cold in wheat can vary across a 24 hour cycle, suggesting that the circadian clock gates the transcriptional response to cold of a variety of genes.

The responsiveness of signalling genes can be gated by the circadian clock

To assess how the time of day affects cold-sensitivity of key signalling components, four genes that are known to be circadian regulated either in Arabidopsis or wheat were studied

as examples. The hexaploid nature of the bread wheat genome means that many transcripts are present as triads, with gene homoeologs on the A, B and D subgenomes. Therefore, all three homoeologs were considered to obtain a representative view of how a signalling component responds to a stimulus.

C-repeat binding factors (CBFs) are well-described cold responsive transcription factors (*51, 52*), with up to 25 CBFs present in wheat (*53*). Certain *CBF* transcripts have a circadian rhythm in barley and wheat (*39, 54*), and in Arabidopsis the cold-induction of CBFs is gated by the circadian clock (*28*). Our data indicate that circadian gating of *CBF* cold-responsiveness is conserved in wheat and can extend across homoeolog triads, such as for *TaCBFIVc-B14*, which is a cold-responsive *CBF* ortholog with high homology to *AtCBF2* (Fig. 2A, B, C) (*24, 53*).

The B-BOX CONTAINING PROTEINs (BBXs) participate in responses to fluctuating environmental conditions (*55*), such as the circadian modulation of *AtBBX17* transcript responses to heat (*34, 56*). Transcripts from the *TaBBX17 7ABD* triad, which has highest homology to *AtBBX17*, share with Arabidopsis rhythmic accumulation under control temperature conditions (Fig. 2D, E, F). The response to cold varied across the triad, with cold induced upregulation of *TaBBX17 7A* at the start of the subjective day, compared to abundance of *7B* and *7D* at the end of the subjective day (Fig. 2D, E, F). This example suggests that features of circadian gating are not always conserved across triads.

In Arabidopsis, the circadian clock regulates photosynthesis and starch metabolism, with these thought to provide a selective advantage to plants (*57-59*). A range of related Arabidopsis transcripts encoding starch degradation enzymes are circadian regulated, but this is not always conserved in wheat, where certain orthologs of β-amylase (BAM, e.g. *BAM3*) have a circadian rhythm, whereas *BAM1* does not (*39*). Under our conditions, *TaBAM1* triad and *TaBAM3* triad were cold-responsive during the subjective day (Fig. 2G-L). *TaBAM1* was not responsive to cold during the subjective night, whereas *TaBAM3* was (Fig.

2G-L). The cold-induced relative abundance of *TaBAM3 1B* and *TaBAM3 1D* was approximately ten-fold greater than the corresponding homoeologs of *TaBAM1* (Fig. 2H, I, K, L), supporting the notion of a role for BAM3 in cold conditions.

Circadian gating of transcriptional responses to cold is extensive, and not restricted to specific times of day

We considered circadian gating to be defined as a 24 h fluctuation in the sensitivity of a transcript to a stimulus, such that the magnitude to an identical stimulus oscillates with a 24 h cycle. Given that our data identified that different subsets of transcripts are cold-responsive at different times of day (Fig. 1B, D, E), we reasoned that there might be widespread circadian gating of the cold-responsive transcriptome. To investigate this, we obtained a measure of the cold-sensitivity of each transcript, at each timepoint, by calculating the difference between the mean CPM of the transcript between control and cold temperature conditions (Fig. 3A). This provided a measure of the response to cold, at each timepoint, that we termed ∆CPM (Fig. 3A). Using this measure, a 24 h fluctuation in the sensitivity of any given transcript to the cold treatment will manifest as an oscillation in ∆CPM (Fig. 3A, lower panel). We tested whether there was a circadian rhythm in this cold-sensitivity profile (meta2d analysis for rhythmicity) (Wu *et al.*, 2016). Circadian rhythms of cold sensitivity (∆CPM) were detected for 4388 transcripts, which we filtered to include only transcripts that were differentially expressed in response to cold at least once across the time course. This yielded 1677 transcripts that we consider to undergo circadian gating of their responses to cold in wheat (Fig. 3B).

We found that this set of transcripts divides into three groups: circadian gated cold-inductions (where mean[∆normalisedCPM] > 0.5), circadian gated cold-reductions (mean[∆normalisedCPM] < -0.5), and cold induced phase delays (mean[∆normalisedCPM] between -0.5 and 0.5) (Fig. 3A). In the case of phase delays, transcripts are cold-induced at certain times of day and cold-repressed at other times (Fig. 3A). Within these cold response

types, we calculated the estimated time of maximum responsiveness (ETMR) using phase estimates from meta2d (Fig. 3C). This approach allowed us to estimate ETMR that fell between sampling timepoints, thereby overcoming some limitations of the sampling frequency. For circadian gated reductions (Fig. 3A), the time of maximum cold-responsiveness was the trough of the oscillation.

The greatest number of transcripts with a rhythmic response to cold were those whose phase was delayed by the cold treatment (Fig. 3C, D). Furthermore, over 500 transcripts had circadian gating of their induction by cold (Fig. 3C, D). Within these transcripts, the most common ETMR was between 16 h and 20 h (Fig. 3D), with a large proportion of the transcripts also phasing between 4 h and 8 h. The largest proportion of cold-repressed transcripts had greatest cold-sensitivity during the subjective night (Fig. 3D).

This indicates that there is transcriptome-wide circadian gating of the cold-responsive transcriptome in bread wheat. These responses are not restricted to a particular direction of change (upregulation *vs*. downregulation), and different transcripts have maximum cold-responsiveness at different times of day. Our analysis might under-estimate the number of circadian gated cold-responsive genes, because a longer and higher resolution time course would provide greater statistical power.

Circadian gating of cold responses have a subtle subgenome bias

The wheat subgenomes arose from hybridisation of three diploid ancestors, resulting in a circadian oscillator that likely incorporates more components than diploid species. No specific subgenome is favoured across the circadian regulated transcriptome of wheat (*39*), although circadian regulated paralogs have differential expression patterns in other crops (*44*). Furthermore, environmentally-responsive gene triads do not always have expression that is balanced across the subgenomes (*46*). Therefore, we wished to determine whether there was subgenome bias in the circadian gating of transcriptomic responses to cold.

The relative proportion of transcripts originating from A, B and D subgenomes remained relatively constant during the day for circadian gated inductions and phase delay (30 - 40%; Fig. 4A). However, during the subjective night, the largest proportion of circadian gated cold induced transcripts derived from the D subgenome (ETMR > 12 h) (Fig. 4A). This was restricted to cold-induced transcripts, and did not occur for transcripts with a phase delay. The number of genes with circadian-gated repression by cold was rather small, so the proportional subgenome contribution to cold-repressed transcripts is noisy (Fig. S2).

Over 60% of circadian-gated cold induced transcripts with a ETMR before 16 h can be assigned to a triad, with a smaller proportion for transcripts with ETMR over 16 h (Fig. 4B). Approximately 10 % of transcripts with ETMR < 4 h, 4-8 h, 12-16 h and 16-20 h were detected as complete triads of three homoeologs, but no complete triads were present in the ETMR groups 12-16 h and > 20 h (Fig. 4B). Only single transcripts from triads had circadian gated responses to cold in the 8-12 h ETMR group, suggesting differential behaviour within triads (Fig. 4A, Fig. 4B). Furthermore, it appears that the time of maximum responsiveness of some triads is spread across multiple phases, and homeolog-dependent (Fig. 4B). This suggests there is sometimes circadian gating bias within triads, with only certain homoeologs responding to the cold stimulus at particular times of day.

The cold-responsiveness of each homoeolog to the overall cold responsiveness of each triad was assessed (*46*). The relative contribution of each subgenome to the cold-responsiveness of the triad determined the position of the triad within a ternary plot (example in Fig. 4C). In general, no subgenome obviously contributed more or less than the other subgenomes (Fig. 4D). Many triads had dynamic responses to cold, in that the contribution from each subgenome to total transcript level changed after the cold treatment (Fig. S3). The variation in the contribution of each subgenome to triad cold responsiveness, in each timing group, suggests that there is no bias towards a particular subgenome despite individual gene homeologs occupying different EMTR groups.

Genes associated with light responses can be circadian gated to the subjective day

Gene ontology-term analysis identified multiple biological processes associated with responses to light as over-represented in transcripts that had a maximum cold induction during the subjective day (ETMR 0-12 h). Processes such as “cellular responses to blue light”, “cellular responses to UV-A” and “anthocyanin containing compound biosynthesis” were enriched in multiple subjective day ETMR groups (Fig. S3A; adjusted $p < 0.001$). Enrichment of terms such as “photoprotection” and “stress-activated MAPK cascade” suggests the cold stimulus elicited a broad response when delivered in the subjective day, at a time when light and low temperatures may be expected simultaneously.

There was a clear split between the GO-terms that were enriched in subjective day ETMR groups and subjective night ETMR groups. “Cold acclimation” is shared across two subjective night groups (Fig. S4A; adjusted $p < 0.001$), suggesting that night-time low temperatures could have a key role in adapting wheat to cold seasonal weather. Also enriched are terms associated with DNA transcription, which can be an indication of preparation for a large shift in gene expression (Fig. S4A; adjusted $p < 0.001$). “Photosystem II” associated processes were enriched in subjective night ETMR group 16 – 20 h, which might relate to unexpected exposure to continuous light conditions (Fig. S4A; adjusted $p < 0.001$). Whilst GO-term analysis interpretation requires caution, it is possible that restriction of the cold response of certain processes to specific times of day provides an advantage to wheat by ensuring that energy demanding cold-responsive processes are not induced at inappropriate times of day (*60*).

Cold treatments delay the phase of certain circadian clock components

We noticed that a variety of transcripts had a phase delay in response to the cold treatment (e.g. Fig. 5A, B). We wished to systematically identify the set of transcripts that had these phase delays. We developed an analytical method that used the ΔCPM to consider the relationship between the transcript levels at adjacent timepoints (Fig. 5C; see methods for

details). Over three-quarters of the 882 transcripts that have a phase delay and rhythmic ∆CPM have a delay ratio of < 1, supporting the interpretation that the phase of the cold CPM profile for these transcripts is delayed relative to the control (Fig. 5D). A similar proportion of the 3849 transcripts with phase delay with rhythmic control and cold CPM profiles had a delay ratio of < 1, suggesting a temporal delay in the profile of these transcripts also (Fig. 5E). A possible explanation is that the cold treatment maintained a relatively constant level of these transcripts for the duration of the treatment, resulting in a pause in the rhythm of the transcript level that is detected as a time-delay. Only 47% of transcripts with circadian gated induction responses had delay ratios < 1, suggesting around half of the transcripts with a gated response to cold did not have a phase delay.

The GO-term “circadian rhythm” was enriched within the genes that had a phase delay in response to cold (adjusted $p < 0.001$; Fig. S5). This suggests that the cold treatment might delay the phase of certain circadian oscillator components. All homoeologs of six triads of circadian clock genes (*39*), including morning phased *TaLHY* and evening phased *TaGI*, had a delay ratio less than 1 (Fig. 5F). Therefore, the cold treatment delayed the oscillation in the expression of these triads (Fig. 5F). Homoeologs in the triads *TaELF3*, *TaELF4*-L and *TaTOC1* had a mixture of delay ratios, indicating that the cold treatment did not equally affect the cycling of homoeologs of all circadian clock transcripts (Fig. 5F).

Cold treatments did not have a consistent impact upon all circadian oscillator components. For example, cold had little effect upon expression of the *TaLUX-Lb* and *TaLUX/BOA* triads, which had lower mean ∆CPM before adjustment in all three homoeologs (Fig. 5F). Therefore, cold does not appear to affect all circadian clock transcripts equally, as exemplified by *TaGI* having a phase delay, whereas *TaLUX/BOA* does not (Fig. 5G). This supports the idea that cold has an unbalanced effect upon circadian clock components, reflecting a complex relationship between the circadian clock and cold.

Discussion

A dual effect of cold on both the clock and clock-controlled genes

Here, we have identified for the first time extensive genome-wide circadian gating of the response to a cold temperature treatment in bread wheat. This demonstrates that the circadian gating of responses to cold of single genes (*28*) extends across the entire genome, and supports reports of the circadian gating of responses to high temperature stress in Arabidopsis (*32, 61*). Given that the circadian clock gates the responses of certain genes to cold in Arabidopsis, and the considerable phylogenetic distance between wheat and Arabidopsis, we reason that the circadian gating of low temperature responses might be conserved across the angiosperms.

Our results demonstrate the importance of circadian regulation in shaping transcriptomic responses to short-term cold. The diversity of temporal responses to cold suggests considerable complexity in the integration of low temperature and circadian signals (Fig. 1E, Fig. 3). The presence of a circadian rhythm for a transcript under control temperature conditions does not mean that its cold-responsiveness is gated by the circadian clock, because some circadian regulated transcripts are not cold-responsive (Fig. 6A; Fig. S6A). Likewise, some transcripts are cold-responsive, but this is independent from circadian regulation because these transcripts are cold-induced by a similar magnitude, irrespective of time of day (Fig. 6B; Fig. S6B). In one form of circadian gating, the circadian clock restricts the magnitude of response to the stimulus to certain times, and the transcript is completely unresponsive to cold at other times (Fig. 6C, Fig. S6C). This type of circadian gating is bidirectional, because suppression of transcript levels by cold can also be circadian-gated (Fig. 3C). In another form of circadian gating, a circadian modulation of sensitivity occurs across the day, such that a response to cold always occurs, but the response is greater at certain times (e.g. *TaBAM3*; Fig. 3J-L). The phase of maximum cold responsiveness of some transcripts is sometimes aligned with the phase of the underlying circadian rhythm

under control temperature conditions, but in other cases (e.g. *TaBAM3*) the phase was misaligned (Fig. 3J-L; Fig. 6D; Fig. S6D).

Short cold treatments affect the dynamics of certain wheat circadian clock components, by delaying the phase of their oscillation for approximately the duration of the cold treatment (Fig. 6E). This phase delay pervades hundreds of other circadian-regulated cold-responsive transcripts (Fig. 6F). It is possible that the cold-induced delay in the circadian oscillator causes the delay in the response of downstream circadian-regulated transcripts. Interestingly, these phase delays occur for some circadian clock genes (e.g. *TaPRR73* and *TaGI*) but not others (e.g. *LUX* orthologues) (Fig. 5F). One potential explanation for this bifurcating response is that the stability of certain transcripts is insensitive to cold, so the transcript level continues to change under low temperature conditions. Another explanation could be that different cell types in the leaves of wheat have differential temperature responses which, combined a predominance of different circadian clock components in different cell types (*62*), causes bifurcated dynamics of distinct circadian clock components in response to cold. Finally, an open question is of whether the hexaploid wheat genome might support partial decoupling of subgenome-derived circadian oscillators in single cells, although uneven temperature responses have also been reported across the Arabidopsis circadian oscillator (*63*). In future, it will be informative to know whether this phase shift in response to cold can act as a zeitgeber for the wheat circadian clock, in a manner reminiscent of the phase shifts caused in Arabidopsis by moderate temperature reductions (*64*). It will also be informative to understand conserved and differing aspects of these responses in winter wheat, given its different vernalization and photoperiod requirements compared with spring wheat varieties such as Cadenza. Taken together, these results suggest that fluctuations in environmental temperature might cause a dynamic adjustment of circadian phase in cultivated wheat (*65*).

Our data suggest two broad processes underlying the dynamics of circadian regulated and cold-responsive transcripts in spring wheat. In the first case, low temperature delays the

phase of the circadian oscillator, maintaining the abundance of circadian-regulated gene targets. In the second case, the circadian oscillator constrains or amplifies the influence of a cold treatment on a gene, with that temporal input having a phase that is aligned with or different from the underlying rhythm of expression of the transcript. This is well demonstrated by the cold responsiveness of *TaBAM3*, for which the phase of the maximum responsiveness is not defined by the phase of the transcript under control conditions (Fig. 2J-L). This suggests that for some genes, separate circadian signalling components might regulate underlying rhythms and cold responsiveness.

Potential cold induction bias towards D subgenome during subjective night

Genes that had circadian gated inductions by cold, with greatest cold responsiveness during the subjective day, had a balanced contribution of transcripts from each subgenome. In contrast, for transcripts with maximum cold induction phased to the subjective night, the greatest proportion of transcript derived from the D subgenome (Fig. 4A). However, when comparing the relative contributions from homoeologs that are present as triads, there did not seem to be a bias towards the D subgenome (Fig. 4E). Therefore, the greater proportion of transcripts from the D subgenome could arise from genes that are present as diads or singletons. This might relate to the origins of the D subgenome donor, *Aegilops tauschii*. The D subgenome hybridizations are thought to involve *A. tauschii* derived from mountainous regions near the Caspian Sea (*66, 67*), so perhaps the greater cold induction from the D subgenome during the subjective night relates to adaptation of *A. tauschii* to relatively cold conditions.

Relationship between the circadian gating of the cold-responsive transcriptome and circadian clock outputs

Rhythms in transcript abundance do not always lead to rhythms in protein abundance or activity (*68-71*), although there is circadian gating of the high temperature-responsive translatome (*56*). In many cases, the abundance of proteins encoded by rhythmic transcripts

remains constant (*71-73*), whilst in some cases (e.g. BAM3 in Arabidopsis) the protein abundance tracks the transcript abundance (*73*). Circadian regulation of protein turnover occurs in eukaryotes (*74, 75*), and it is thought that circadian regulation of gene expression might maintain proteostasis in the presence of rhythmic protein degradation (*76*). This might explain why rhythms in protein abundance are less prevalent than rhythms of transcript abundance.

This idea could be relevant to interpreting the physiological significance of circadian gating in wheat. For approximately half of the genes that have a circadian gated response to cold, the phase of maximum cold sensitivity was similar to the phase of the rhythm at control temperatures (delay ratio < 1; Fig. 6C). This likely increases the availability of mRNA at certain times, perhaps in anticipation of a greater rate of protein turnover (*77*). This idea is exemplified by the phasing to the subjective day of the cold induction of genes having light signalling GO terms (Fig. S3). We reason that the regulatory network leads to increased transcript levels for these components in anticipation of cold- and light-induced oxidative damage. Conversely, transcripts that are down regulated and circadian gated could encode proteins that are stabilised in cold or contribute to processes that are not preferred in cold conditions. Alternatively, down regulated genes may represent repressive regulators like many components of the circadian clock (*78*), so a reduction in their abundance could lift repression of cold response mechanisms.

Conclusions

We identified that the circadian clock plays a key role in shaping the cold responsive transcriptome through the process of circadian gating. This establishes that circadian regulation is central to the environmental responses of a major cereal crop. Our study supports the notion that circadian gating is a widespread regulatory mechanism found across plant life, from peach trees and grape vines (*79, 80*) to Arabidopsis (*34*), and here in bread wheat. Our findings open opportunities to understand the contributions of circadian

regulation to wheat crop performance, and could underpin biotechnological developments that optimize the resilience of cereals to an increasingly unpredictable climate.

Materials and methods

Plant materials and growth conditions

Seeds of *Triticum aestivum* cv. Cadenza (donated by James Simmonds, John Innes Centre) were stratified on damp filter paper for 3 days at 4°C before germination at 22°C in darkness for two days. Seedlings were cultivated in 24-cell trays for 12 days on a bespoke cereal cultivation mixture (65% peat, 25% loam, 10% horticultural grit, 3 kg m^{-3} dolomitic limestone, 1.3 kg m^{-3} PG Mix fertiliser (Yara), 3 kg m^{-3} osmocote extract) under cycles of 12 h light and 12 h darkness, at approximately 200 µmol m^{-2} s^{-1} of white light at 22°C.

Circadian time course sampling

14 day old wheat seedlings at Zadok stage GS1.2 were transferred to constant light for 24 h before experimentation commenced, to ensure data were free from transitory effects caused by the final dawn (Fig. S7A). After 25 h of constant conditions (ZT 25), three individuals were transferred to 4°C with identical light conditions for 3 h, whilst control plants remained at 22 ^{o}C. After the 3 h cold treatment, 3 cm of tissue from the second leaf was collected from each control and treated plant (Fig. S7A), immediately frozen in liquid N_2, and stored at -80°C until processing. Six such treatments were conducted, beginning at ZT 25, ZT 29, ZT 33, ZT 37, ZT 41 and ZT 45, and ending at ZT 28, ZT 32, ZT 36, ZT 40, ZT 44 and ZT 48, respectively (Fig. 1A). Each set of samples used a different batch of plants.

RNA sequencing data collection, QC and read mapping

RNA was extracted using Macherey-Nagel NucleoSpin RNA Mini Kit with on-column DNase treatment, according to manufacturer's instructions. RNA was stored at -80°C in nuclease-free water. RNA integrity was assessed using an Agilent 2100 Bioanalyzer System at the John Innes Centre, and again by our sequencing partner Novogene (Cambridge, UK). All samples had RIN scores > 6 and concentrations > 50 ng RNA µl^{-1}, thereby passing quality control. Libraries were constructed by Novogene using a mRNA polyA enrichment library

protocol involving fragmentation, cDNA synthesis, end repair, A-tailing, adapter ligation, size selection, amplification and purification. Libraries were verified on a Bioanalyzer before pooling for sequencing. After verification, 150bp paired-end sequencing was performed on the Illumina NovaSeq 6000 platform to generate a minimum of 15 GB data per sample, generating 675.5 GB total data with an average read depth of 62.5 million reads per sample. All samples had ≥ 98.5 % clean reads suitable for alignment.

RNA sequencing analysis

Transcript quantification was conducted using the RNA-seq pseudoaligner kallisto v0.44.0 (Bray et al., 2016) using 31 bp k-mers and default parameters. The index file was constructed from the high confidence CDS sequence from the International Wheat Genome Sequencing Consortium (IWGSC) *T. aestivum* cv. Chinese Spring RefSeqv1.1 (*45*).

Differential gene expression analysis

Kallisto transcript count data were condensed to the gene level using R v2022.07.1 (*81*). The Bioconductor (*82*) packages edgeR (*83-85*) and Limma (*86*) were used to identify differentially expressed genes between cold-treated and control seedlings at each timepoint. Counts per million (CPM) were determined by edgeR and low-expressed transcripts filtered out of the data (edgeR function filterByExpr, which uses the filtering strategy described in Chen *et al.* 2016) (Dataset S1, Dataset S2). This filtering strategy retains genes with a CPM value above *k* in *n* samples, where *k* is determined from the set minimum count value and sample size, and *n* determined from the smallest group size within the design matrix (*85*). Around 68.5% of transcripts remained after filtering (Dataset S3). Empirical Bayes moderation was performed for predictions of gene-wise variability (*87*) and a correction for multiple testing (Benjamini-Hochberg method) was used to obtain the false discovery rate (FDR) of differentially expressed genes. Differentially expressed genes were defined as those with an adjusted p-value < 0.05 and logFC > 0.5 (50% change).

Data processing and visualization

CPM and logFC values were assembled using base R and the tidyverse package dplyr (*88, 89*). Graphs were produced using ggplot2 (*90*) and radar charts with fmsb function radarchart (*91*). Upset plots (Fig. S1) were created with UpSetR (*92*) and GO-terms presented using code adapted from (*39, 93*). The sensitivity heatmap was produced using mean normalised CPM values determined by dividing the average CPM at any given timepoint by the mean CPM for each gene across the entire time course under both temperature conditions. The heatmap was created with ComplexHeatmap, using the Ward D.2 method for clustering, with k = 12 (*94, 95*).

Identification of genes having a circadian gated response to cold

We used the difference between the mean control transcript level and mean cold-treated transcript level (ΔCPM) at each timepoint as a measure of the sensitivity of gene expression to cold. To test for rhythmicity of cold sensitivity, the ΔCPM profiles were analysed using the MetaCycle v1.2.0 function meta2d (*96*). The minimum and maximum permitted period lengths were 20 h and 28 h, respectively (*97*). We considered meta2d to be especially suitable for this analysis because it integrates period, phase and p-values from three algorithms, reducing the limitations of individual algorithms, and is appropriate for analysis of a relatively small number of time-points (*97*). A rhythmicity cut-off of meta2d p-value < 0.05 was used. In general, time-series close to this p-value threshold had approximately 24 h rhythmicity (Fig. S8). As an additional filter, only genes with rhythmic ΔCPM that were also differentially expressed at least once in the time-series were considered to be circadian gated. Only transcripts with average CPM > 1 under control temperature conditions or cold temperature conditions, across the time-series, were included in the analysis to eliminate low-expressed genes with large relative variation between replicates due to the low counts. The difference between mean normalised CPM values (Δmean-normCPM) was used to assign transcripts to three directional response groups: circadian-gated inductions by cold

(Δmean-normCPM > 0.5), cold induced phase delay (-0.5 < Δmean-normCPM < 0.5) and circadian gated reductions by cold (Δmean-normCPM > -0.5).

The estimated time of maximum cold responsiveness (ETMR) refers to the time of the peak ΔCPM. This was estimated from the phase measured using meta2d analysis, which provides the peak phase of the time-series data. For the ETMR for circadian gated cold reductions, the ETMR was determined using meta2d phase estimates for the inverse ΔCPM values, because they represent the phase of the trough. This allowed a total of 18 directional ETMR groups to be defined (Fig. 3C). Extra filters were applied to ETMR groups < 4 h and > 20 h for inductions and phase delays, to remove 135 trend anomalies that had greater ΔCPM at the opposite timepoint (+ 12 h) to the meta2d phase estimate.

Gene ontology-term analysis

GO-term enrichment analysis for biological processes was performed using TopGo (*98*) for each cold-induction ETMR group. The group sizes were sufficiently large for this approach (n>20). GO-terms were retrieved from the IWGSC RefSeqv1.0 annotation and transferred to annotation v1.1 using the method described by (*99*). Enrichment analysis was performed on gene lists through comparison against a gene universe of 75251 genes, which comprised all genes expressed in our dataset that had high quality annotation and could be associated with a GO term (nodeSize = 10, algorithm = weight01) (*98*). WeightedFisher values were adjusted for multiple testing using the Benjamini-Hochberg correction method to generate adjusted p-values, filtered stringently for adjusted p < 0.001. The same pipeline was used for genes with CPM profiles that were temporally delayed by cold, but did not have rhythmic ΔCPM (delay ratio <0.5).

Investigation of the contribution of wheat subgenomes to triad responsiveness

Subgenome occupancy was determined using information within the gene identifier (e.g. TraesCS**1A**02G123456). Genes were organised into triads with a 1:1:1 correspondence using the triad numbers in the High Confidence Triad table from (*46*)

(https://github.com/Uauy-Lab/ WheatHomoeologExpression/Data/data/TablesForExploration/HCTriads.csv). Triads were considered to be part of a circadian gated induction ETMR group if any homoeolog from the triad was categorised into the group. Individual homeologs of triads were sometimes within different ETMR groups (Fig. 4D). An average ∆CPM value for the ETMR for each gene was determined as:

$$\Delta CPM_{ETMR} = \frac{\Delta CPM_{ZTlower} + \Delta CPM_{ZTupper}}{2}$$

where ZTlower is the ZT timepoint corresponding to the lower time limit of the ETMR group and ZTupper is the ZT timepoint corresponding to the upper time limit of the ETMR group, e.g for ETMR group 4 – 8 h, ZTlower = ZT 28 and ZTupper = ZT 32. The relative contribution of each homoeolog to the triad responsiveness for the ETMR was determined as:

$$Relative\ Contribution\ [x] = \frac{\Delta CPM_{ETMR}[x]}{\Delta CPM_{ETMR}[x] + \Delta CPM_{ETMR}[y] + \Delta CPM_{ETMR}[z]}$$

where x is the subgenome of the homoeolog being investigated, and y and z are the remaining two subgenomes. This used a method similar to Ramirez-Gonzalez et al. (*46*). The relative contributions were determined and visualized in R using ggtern (*100*).

Identifying temporal delays between cold-treated and control CPM profiles

We wished to identify the set of transcripts that had a phase delay in response to the cold treatments. To achieve this, transcripts with rhythmicity at control temperatures and in response to 3 h cold treatments were identified using the ARSER algorithm in Metacycle (using meta3d) (*96, 101*). Genes previously defined as having a circadian gated upregulation or downregulation in response to cold were removed from this group to prevent double-counting of individual genes. Adjusted ∆CPM (Adj.∆CPM) values were were determined for timepoints ZT28-44 for each gene as:

$$Adj.\Delta CPM_{ZTx} = Cold[CPM_{ZTx+4}] - Control[CPM_{ZTx}]$$

Where ZTx is the is sampling timepoint. A time course mean ∆CPM for each gene was determined as:

$$Mean.\Delta CPM = \frac{|\Delta CPM_{ZT28}| + |\Delta CPM_{ZT32}| + |\Delta CPM_{ZT36}| + |\Delta CPM_{ZT40}| + |\Delta CPM_{ZT44}|}{5}$$

And a mean for adjusted ∆CPM for each gene was determined as:

$$Mean.Adj.\Delta CPM = \frac{|Adj.\Delta CPM_{ZT28}| + |Adj.\Delta CPM_{ZT32}| + |Adj.\Delta CPM_{ZT36}| + |Adj.\Delta CPM_{ZT40}| + |Adj.\Delta CPM_{ZT44}|}{5}$$

A delay ratio value was determined for each gene in this group using mean ∆CPM and mean adjusted ∆CPM values as:

$$Delay\ ratio = \frac{Mean.Adj.\Delta CPM}{Mean.\Delta CPM}$$

Delay ratios less than 1 indicate a reduced mean ∆CPM following adjustment. Gene identifiers for putative wheat circadian clock genes were obtained from (*39*), and delay ratios determined for each.

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Author contributions

Conceptualization: CAG, KJE, AND.

Investigation: CAG, PP.

Analysis and visualization: CAG, PP.

Writing: CAG, PP, KJE, AND.

Competing interests

Authors declare that they have no competing interests.

Data availability

Raw sequencing data are deposited as European Nucleotide Archive dataset PRJEB56923. Processed data are provided as supplemental information within the manuscript.

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Figure legends

Figure 1. Different sets of transcripts respond to cold at different times of day in bread wheat. (A) *Triticum aestivum* cv. Cadenza seedlings were entrained under 12:12 light/dark cycles before transfer to free-running conditions. Under free running conditions, tissue was collected after 3 h cold treatments, with six such treatments given over 24 h of free running conditions. **(B)** Differentially expressed genes after a 3 h cold treatments given at six timepoints (log fold change > 0.5, FDR < 0.05). Yellow and grey shading indicate subjective day and night, respectively. **(C)** The number of differentially expressed genes (DEGs) at each timepoint. **(D)** The proportion of DEGs that only responded to cold at single timepoints. **(E)** Summary of the difference between mean normalised control and cold transcript abundance of DEGs across the time course. Orange and teal colours indicate normalized change in cold-induced and cold-repressed transcripts, respectively.

Figure 2. Circadian gating of the cold response of selected wheat triads. (A-C) *TaCBFVc-B14*; triad no. 15598. **(D-F)** *TaBBX17*; triad no 9634. **(G-I)** *TaBAM1*; triad no 15540. **(J-L)** *TaBAM3*; triad no 15539 (*46*). Yellow/grey shading = subjective day/night. Solid lines are mean (N = 3 biological replicates; blue/green shading = ± s.e.m). Asterisks indicate times when the transcript was differentially expressed between the control and cold temperature conditions (LogFC >0.5, FDR < 0.05). CPM (counts per million) determined by edgeR (*83*).

Figure 3. The temporal structure of the cold-responsive transcriptome. (A) We defined circadian gating of the response of genes to cold as a 24 h cycle of sensitivity of the response of the gene to identical cold treatments. To identify such genes, we calculated the difference between the mean control and cold-treated CPM (termed ∆CPM). Circadian gating of a cold response manifests as a 24 h oscillation of ∆CPM, which captures cold induction, repression, or a phase delay. **(B)** The number of significantly cold-responsive transcripts (LogFC >0.5, FDR <0.05) that have a rhythmic ∆CPM profile across the time

course (meta2d, $p < 0.05$). This represents a set of wheat transcripts with a circadian gated response to cold. **(C)** Transcripts with circadian gating of their response to cold, organized according to their estimated time of maximum peak cold responsiveness (ETMR). Direction of response (induction, reduction, phase delay) was based on the average difference between mean normalised control and cold-treated CPM to normalize differences in ΔCPM profile amplitude across the transcriptome. The direction was formalized as circadian-gated inductions (Δmean.normCPM > 0.5), cold induced phase delay (-0.5 < Δmean.normCPM < 0.5) and circadian gated reductions (Δmean.normCPM > -0.5). ETMR was derived from meta2d phase estimates. Yellow/grey shading = subjective day/night. Pink lines indicate mean Δmean.normCPM in each directional ETMR group. **(D)** The number of genes within each directional cold ETMR group. 1533 genes were included, after filtering to remove ΔCPM profiles from ETMR groups < 4 h and > 20 h having peak CPM at an opposing timepoint from the ETMR. Darker colours indicate greater numbers of transcripts.

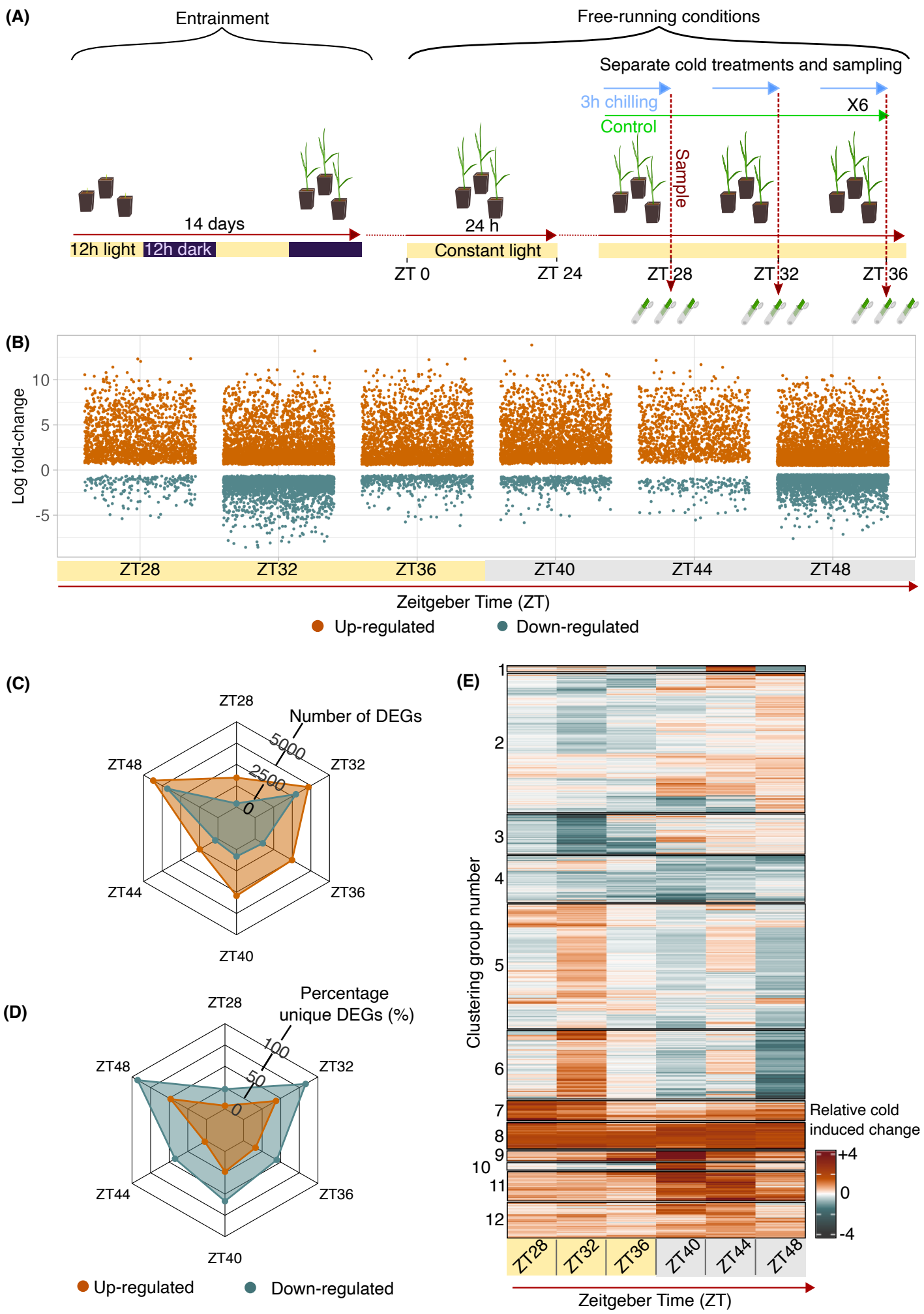
Figure 4. Subgenome organization of the circadian gating of the cold-responsive transcriptome. (A) The number and proportion of transcripts from each subgenome that have a circadian gated response to cold, divided according to the time of maximum cold responsiveness. Large proportional differences between subgenomes generally occur when total number of transcripts is small. (**B)** The proportion of transcripts that have a circadian gated response to cold that belong to an annotated triad (*46*). Not all homeologs within any given triad are gated, and this is indicated by the bar colour. (**C)** Example ternary plot showing the relative contribution of each homoeolog within a triad to the combined transcript accumulation from the triad (*46, 100*). The position of each point on the plot reflects the proportion of total contribution from the three homeologs within the triad (subgenome A, yellow axis; subgenome B; green axis, subgenome D, blue axis). A point in the centre of the plot represents a triad where all three homoeologs are equally cold-responsive. Points towards corners (shaded areas) represent triads where the majority of the cold response derives from a specific homoeolog. (**D)** The relative contributions of homoeologs to the cold

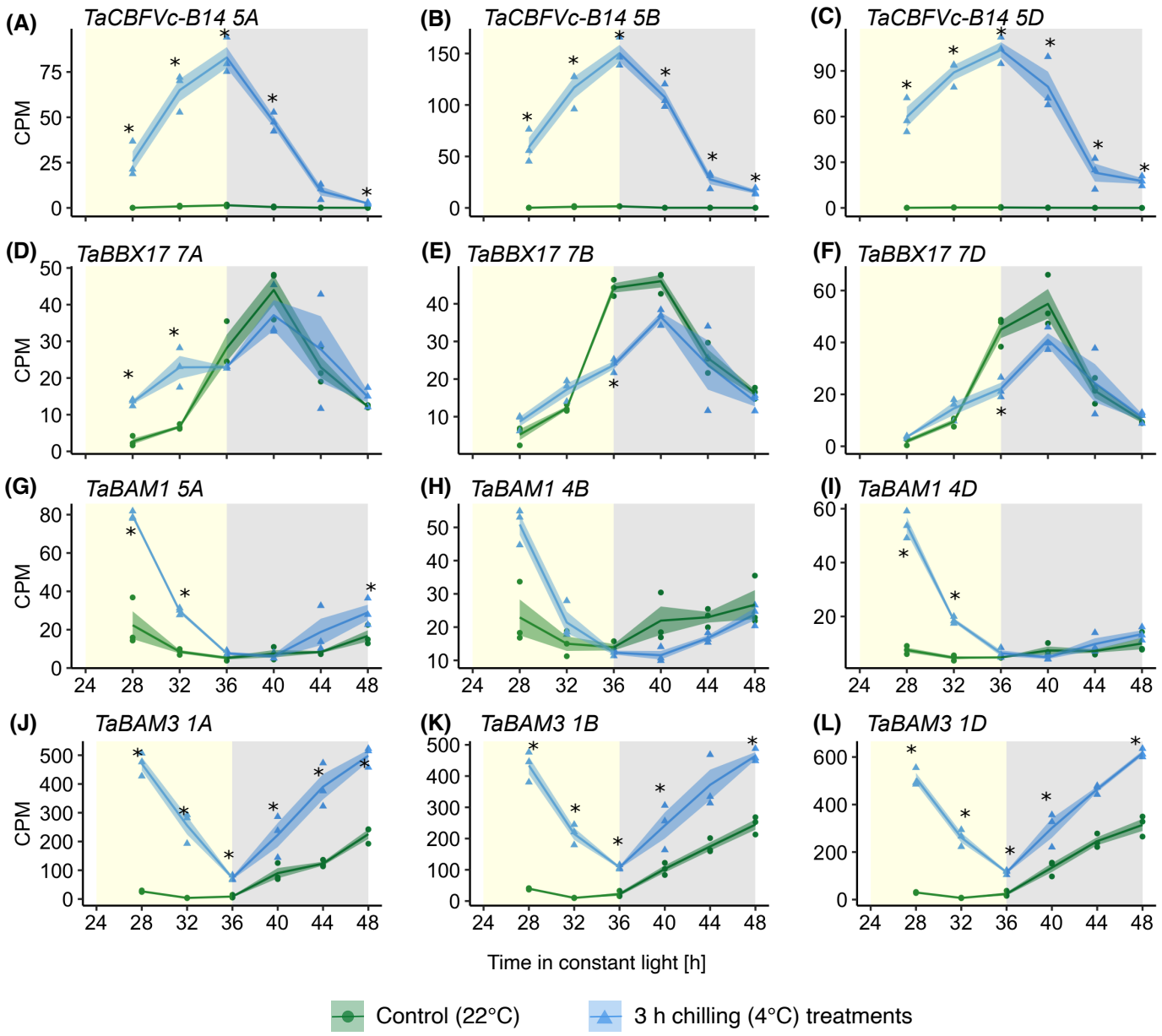
responsiveness of each triad, organized according to the time of maximum cold responsiveness. Each triad contains at least one homoeolog that is within the ETMR group. Therefore, certain triads are present in only one ETMR (orange points), some in two (dark pink) and some in three (light).

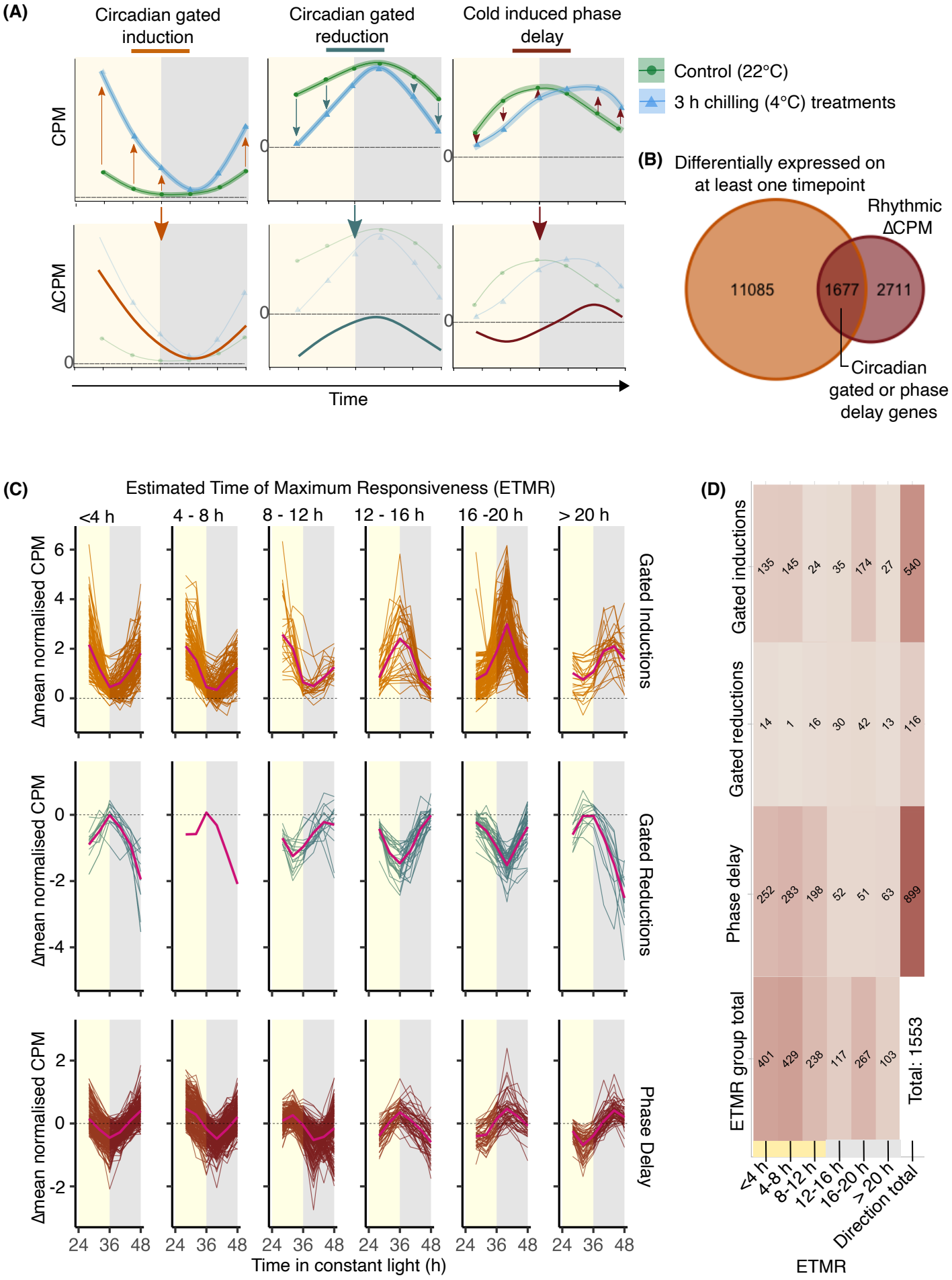
Figure 5. Cold temperature delays the phase of a set of rhythmic transcripts. (A) Dynamics of a phase delay group transcript (*TraesCS4A02G091700*), whereby the oscillation in cold treated plants (blue) is similar to control temperature conditions (green), but delayed in time. The difference between cold and control treated transcript abundance (∆CPM) is shown in dark red. **(B)** Dynamics of a rhythmic transcript (*TraesCS7A02G416300*; meta3d: ARS p <0.05) that has a phase delay and a more linear ∆CPM profile. **(C)** Artificial data including a time delay. ∆CPM is the difference between the control and cold CPM at each timepoint (dark red arrows), and adjusted ∆CPM is the difference between the control temperature CPM at one timepoint and the cold-treated CPM at the next timepoint (blue arrows). **(D)** a delay ratio value was calculated from the average adjusted ∆CPM as a proportion of the original average ∆CPM for phase delay genes, and **(E)** all transcripts with rhythmic control and cold-treated CPM profiles (meta3d: ARS p <0.05). In **(D, E)** The larger circle represents 100% of these transcripts, the middle circle represents transcripts with a delay ratio <1, and the smaller circle transcripts with a delay ratio < 0.5. Delay ratios < 1 suggest a phase delay. **(F)** Delay ratios of eleven circadian clock gene triads (Rees *et al.*, 2022) suggest phase delays in the majority. (**G)** Transcript dynamics of phase-delayed circadian clock gene *TaGI 3A* (*TraesCS3A02G116300*) and no-delay *TaLUX/BOA 3A* (*TraesCS3A02G526600*). Solid lines are mean (N = 3). Blue/green shading= ± s.e.m. Red line represents ∆CPM. Yellow/grey shading = subjective day/night.

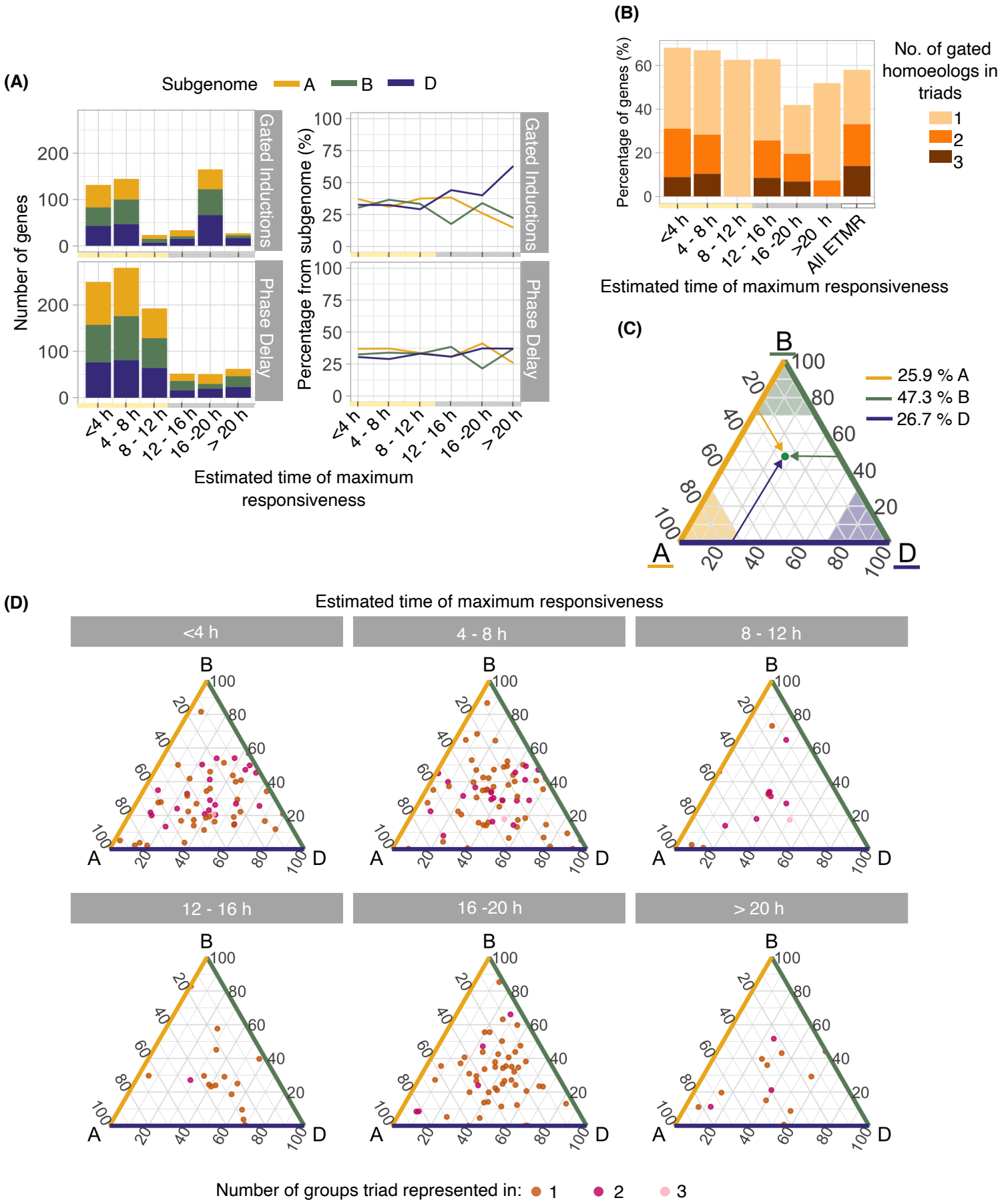
Figure 6. Features of the circadian gating of cold temperature responses. The interaction between the circadian clock and cold produces a diversity of transcript response profiles across the wheat transcriptome. Diagrams conceptualize how the circadian clock (red clocks) and cold (blue arrows) can affect underlying control-temperature transcript

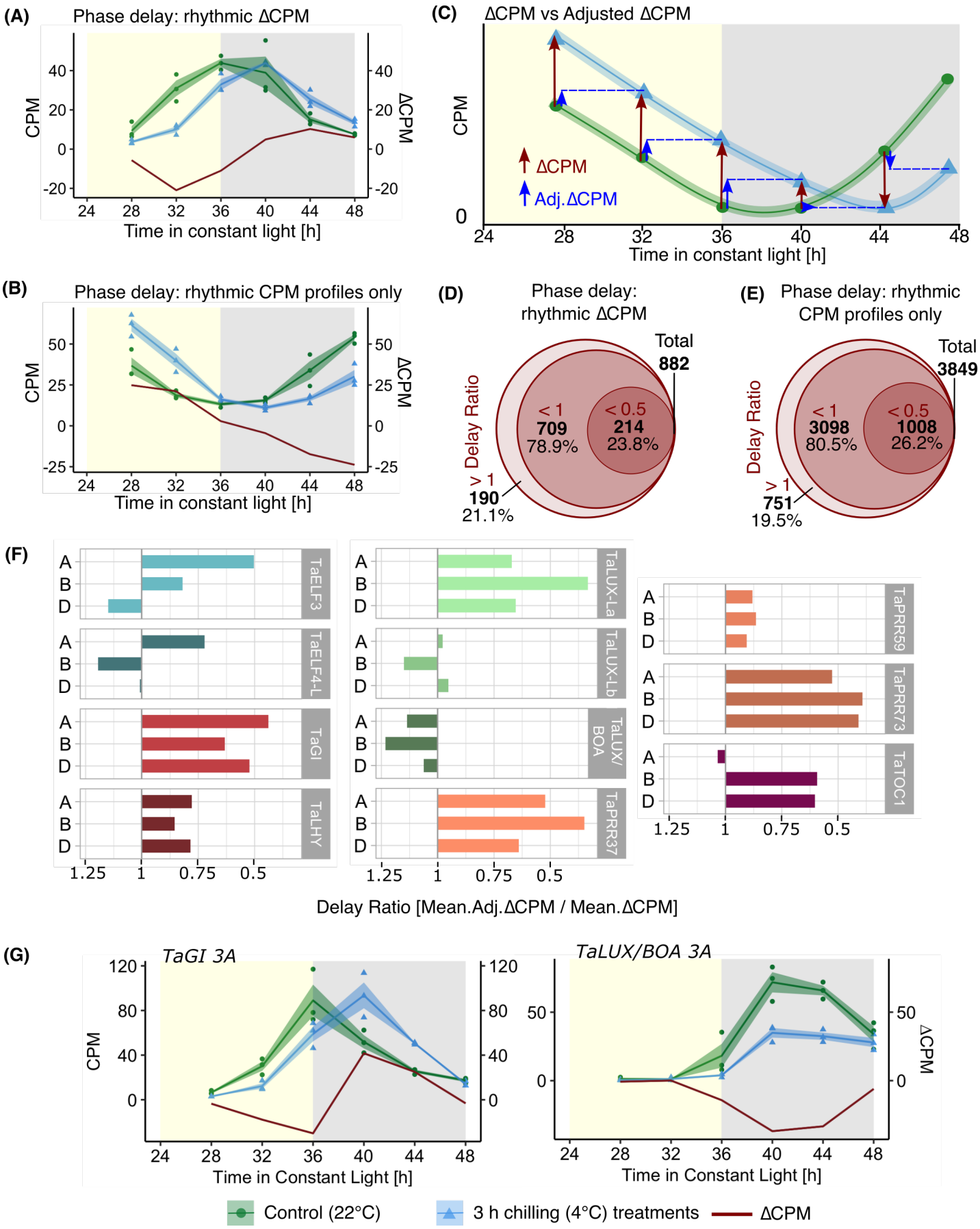
levels (dotted black line), producing a diversity of cold-responsive transcript profiles (orange line). **(A)** certain transcripts can be regulated by the circadian clock but transcript levels are unaltered by cold (e.g. *TraesCS5D02G447500* and *TraesCS1D02G372000*). **(B)** transcripts can respond equally to cold, independent of the time of subjective day (e.g. *TraesCS2D02G284400* and *TraesCS3B02G287400*). **(C)** the cold-responsiveness of transcripts can be gated by the circadian clock, resulting in a response to cold at specific times of day (e.g. *TraesCS3D02G325300)*. **(D)** the circadian clock gates the response to cold of the transcript, with the gate having a different phase from the phase of the underlying circadian rhythm of the transcript (e.g. *TaBAM3*). **(E)** cold affects the circadian oscillator directly (cold blue clock), stabilizing oscillator transcript levels for the duration of cold, thereby holding the level of clock transcripts. This delays the phase (e.g. *TaGI* 3A, *TraesCS5B02G271300*). **(F)** the cold-responsiveness of a transcript is restricted to a particular phase, and cold also delays the phase of the circadian clock. In combination, this upregulates and delays the accumulation of the transcript (e.g. *TraesCS1A02G289800* and *TraesCS1B02G095000*).

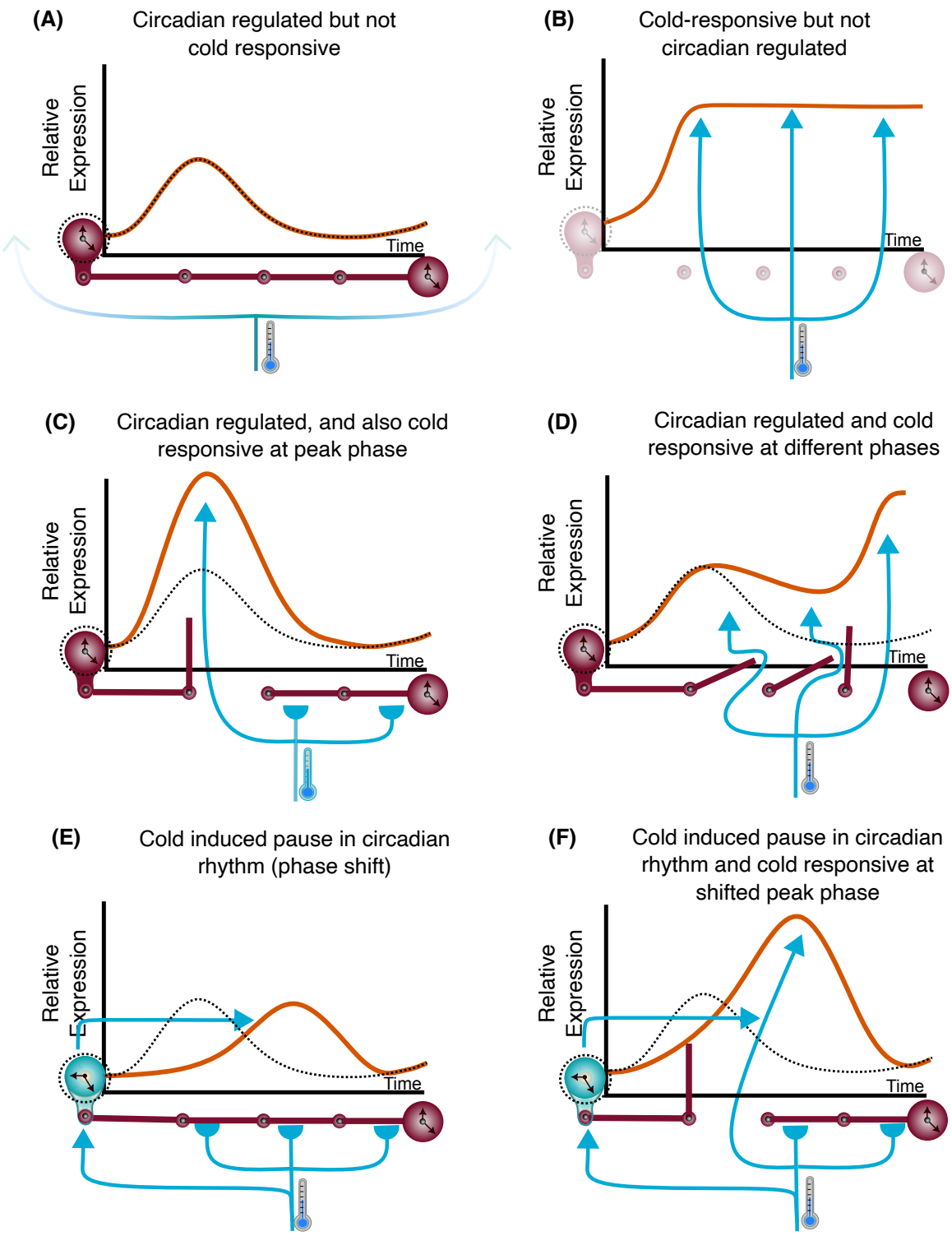

(A)
Entrainment
Free-running conditions
Separate cold treatments and sampling
3h chilling
Control
X6
Sample
14 days
12h light
12h dark
24 h
Constant light
ZT 0
ZT 24
ZT 28
ZT 32
ZT 36
(B)
Log fold-change
10
5
0
-5
ZT28
ZT32
ZT36
ZT40
ZT44
ZT48
Zeitgeber Time (ZT)
Up-regulated
Down-regulated
(C)
Number of DEGs
5000
2500
0
ZT28
ZT32
ZT36
ZT40
ZT44
ZT48
(D)
Percentage unique DEGs (%)
100
50
0
ZT28
ZT32
ZT36
ZT40
ZT44
ZT48
Up-regulated
Down-regulated
(E)
Clustering group number
1
2
3
4
5
6
7
8
9
10
11
12
Relative cold induced change
+4
0
-4
ZT28
ZT32
ZT36
ZT40
ZT44
ZT48
Zeitgeber Time (ZT)


(A) TaCBFVc-B14 5A
(B) TaCBFVc-B14 5B
(C) TaCBFVc-B14 5D
(D) TaBBX17 7A
(E) TaBBX17 7B
(F) TaBBX17 7D
(G) TaBAM1 5A
(H) TaBAM1 4B
(I) TaBAM1 4D
(J) TaBAM3 1A
(K) TaBAM3 1B
(L) TaBAM3 1D
CPM
Time in constant light [h]
Control (22°C)
3 h chilling (4°C) treatments


(A)
Circadian gated induction
Circadian gated reduction
Cold induced phase delay
Control (22°C)
3 h chilling (4°C) treatments
CPM
ΔCPM
Time
(B)
Differentially expressed on at least one timepoint
Rhythmic ΔCPM
11085
1677
2711
Circadian gated or phase delay genes
(C)
Estimated Time of Maximum Responsiveness (ETMR)
<4 h
4 - 8 h
8 - 12 h
12 - 16 h
16 -20 h
> 20 h
Δmean normalised CPM
Gated Inductions
Gated Reductions
Phase Delay
Time in constant light (h)
(D)
Gated inductions
Gated reductions
Phase delay
ETMR group total
135 145 24 35 174 27 540
14 1 16 30 42 13 116
252 283 198 52 51 63 899
401 429 238 117 267 103
Total: 1553
<4 h
4-8 h
8-12 h
12-16 h
16-20 h
> 20 h
Direction total
ETMR


(A)
Subgenome A B D
Number of genes
Percentage from subgenome (%)
Gated Inductions
Phase Delay
<4 h
4 - 8 h
8 - 12 h
12 - 16 h
16 -20 h
> 20 h
Estimated time of maximum responsiveness
(B)
Percentage of genes (%)
No. of gated homoeologs in triads
1
2
3
All ETMR
Estimated time of maximum responsiveness
(C)
25.9 % A
47.3 % B
26.7 % D
(D)
Estimated time of maximum responsiveness
Number of groups triad represented in: 1 2 3


(A)
Phase delay: rhythmic ΔCPM
CPM
ΔCPM
Time in constant light [h]
(B)
Phase delay: rhythmic CPM profiles only
CPM
ΔCPM
Time in constant light [h]
(C)
ΔCPM vs Adjusted ΔCPM
CPM
ΔCPM
Adj.ΔCPM
Time in constant light [h]
(D)
Phase delay: rhythmic ΔCPM
Total
882
Delay Ratio
< 1
709
78.9%
< 0.5
214
23.8%
> 1
190
21.1%
(E)
Phase delay: rhythmic CPM profiles only
Total
3849
Delay Ratio
< 1
3098
80.5%
< 0.5
1008
26.2%
> 1
751
19.5%
(F)
TaELF3
TaELF4-L
TaGI
TaLHY
TaLUX-La
TaLUX-Lb
TaLUX/BOA
TaPRR37
TaPRR59
TaPRR73
TaTOC1
Delay Ratio [Mean.Adj.ΔCPM / Mean.ΔCPM]
(G)
TaGI 3A
TaLUX/BOA 3A
CPM
ΔCPM
Time in Constant Light [h]
Control (22°C)
3 h chilling (4°C) treatments
ΔCPM


(A)
Circadian regulated but not cold responsive
Relative Expression
Time
(B)
Cold-responsive but not circadian regulated
Relative Expression
Time
(C)
Circadian regulated, and also cold responsive at peak phase
Relative Expression
Time
(D)
Circadian regulated and cold responsive at different phases
Relative Expression
Time
(E)
Cold induced pause in circadian rhythm (phase shift)
Relative Expression
Time
(F)
Cold induced pause in circadian rhythm and cold responsive at shifted peak phase
Relative Expression
Time