DOCTOR OF PHILOSOPHY

Alternative splicing in the regulation of the barley circadian clock

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Award date:
2013

Awarding institution:
University of Dundee

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A thesis submitted for the degree of Doctor of Philosophy in the College of Life Sciences in The University of Dundee

January 2014

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Acknowledgements

Firstly, I would like to thank my supervisor Professor John Brown for accepting me in his lab and for his prime guidance and support throughout my PhD. Secondly, I wish to greatly acknowledge the University of Dundee, the James Hutton Institute (JHI, Dundee) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) for the financial support and facilities to carry out my PhD.

I would also like to thank all of those within the JHI involved in this project, who gave me valuable guidance, support and advice. A special thanks to Professor Robbie Waugh and Dr Arnis Druka for providing the barley Bowman NILs; Dr Helena Oakey and Jim McNicol (BioSS, Dundee) for guidance on statistical analyses; Dr Gordon Simpson and Dr Edgar Huitema for support as members of my thesis committee; Dr David Marshall, Dr Frank Wright (BioSS, Dundee) and Professor Todd Barkman (Western Michigan University) for guidance on phylogenetic analysis; Dr Craig Simpson, Gillian Clark, Dominika, Nikoleta and other lab members (current and past) for their guidance, patience and technical support; Dr Christopher McClellan, Dr Clément Gravouil (SRUC, Edinburgh) and Philip Smith for helping with the proof-reading of this PhD thesis; Professor Maria Helena Goldman for her support as my CAPES tutor; and Dr Allan James and Gillian Nimmo (both from the University of Glasgow) for their advice on clock gene experiments.

The acknowledgement list would not be complete without the friends I made during my time here. Friends from all over the world, from different cultures and for all kinds of fun. We laughed together, worked together, climbed, and travelled to discover the world outside Dundee together. Thank you Alper, Benjamin (and his parents), Christopher, Eleni, Emily, Jaleh, Karolina, Matthieu, Monika, Nish, Orsi, Pedro,

Ross, Sabine, Samir, Sandra, Vinciane and many more... for making my time here easier, happier, more interesting and unforgettable!

Finally, many thanks to my family, for understanding and coping with the distance, for their positive encouragements and for always believing in me. Last but definitely not least, thanks *mon ange* Clément for always standing by me and for all the enjoyable moments we have together.

My PhD would not have been possible without the support from all of you, so

obrigada!

Declaration

I am the author of this PhD thesis and the results presented here are of investigations conducted by myself. Work other than my own have been consulted by me and is clearly identified with references to relevant researchers and their publications. I hereby declare that the work presented here is my own and has not been submitted in any form of any degree at this or other university.

Cristiane P. G. Calixto

I certify that Cristiane P. G. Calixto has fulfilled the conditions of the relevant Ordinance and Regulations of the University Court and is qualified to submit this thesis for the degree of Doctor of Philosophy.

Professor John W. S. Brown

College of Life Sciences

University of Dundee

Abstract

In the first year and a half of my PhD (October 2009 to March 2011) I studied the putative involvement of orphan snoRNAs in the regulation of alternative splicing (AS) in Arabidopsis. This project was very attractive but ran into serious and unforeseen problems with the genetic background of mutants used in the project. Despite deciding to terminate the project, it gave me more experience of molecular techniques and analysing RNA and expression. The novel work on AS in Arabidopsis clock genes coincided with availability of the barley genome sequence and the rest of my PhD was spent in examining AS in the circadian clock of barley.

Prior to this thesis, extensive alternative splicing (AS) was shown to regulate clock genes in Arabidopsis through dynamic changes in AS transcripts, some of which are temperature-dependent and altered levels of productive mRNAs through alternative splicing/Nonsense-Mediated Decay (AS/NMD). An objective of this thesis was to determine whether clock genes and their modes of regulation are conserved in other higher plants, such as barley. By use of a robust *in silico* analysis and nucleotide sequence of 27 *Arabidopsis* core clock/clock-associated genes, 21 barley genes were identified, 60% of which are true *Arabidopsis* orthologues. Most of the barley clock genes have a clear daily rhythm which is maintained in constant light conditions. Mutations of the barley clock genes *HvPPDH1* (orthologue of *AtPRR7*) and *HvELF3* strongly affect flowering time and have extended the geographic range where barley is grown. We show that both mutations affect expression of clock genes: the *Hvppdh1* mutation moderately affects expression levels and phase while the *Hvelf3* mutation causes arrhythmicity of most of these genes, which helps to explain the early flowering phenotype. Temperature-dependent AS was identified in some of the barley core clock orthologues. The focus of this part of the analysis was *HvLHY* and *HvPPDH1*. Although

specific AS events were poorly conserved, similar behaviour in terms of decreased functional mRNA was observed. This novel layer of fine clock control observed in two different species, a model plant and a crop species, might help our understanding of plant adaptation in different environments and ultimately may offer a new range of targets for plant improvement.

List of abbreviations

ABI	Applied Biosystems Incorporated (now Life Technologies)
Alt 3' ss	alternative 3' (acceptor) splice site
Alt 5' ss	alternative 5' (donor) splice site
ANOVA	Analysis of Variance
AS	alternative splicing
BEP	Bambusoideae, Pooideae and Ehrhartoideae subfamilies
BLAST	Basic Local Alignment Search Tool
bp	base pair
BP	Branch point adenosine
cDNA	complementary Deoxyribonucleic Acid
CDS	coding sequence
CrIn	cryptic intron
CTD	RNA pol II carboxyl terminal domain
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
eIF	eukaryotic Initiation Factor
EJC	Exon Junction Complex
ER	Endoplasmic Reticulum
eRF	eukaryotic release factor
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
EST	Expressed Sequence Tag
FAM	6-carboxyfluorescein
FS	fully spliced
g	gravitational force
Gb	gigabases
gDNA	genomic Deoxyribonucleic Acid
GS	growth stage
GTF	general transcription factor
HKG	housekeeping gene
hnRNP	heterogeneous nuclear ribonucleoprotein
HR	High Resolution
IPTG	isopropyl β-D-1-thiogalactopyranoside
IR	intron retention
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
LB	Luria-Bertani
LD	long day
LT	low-temperature
miRNA	micro Ribonucleic Acid
mRNA	messenger Ribonucleic Acid
mRNP	complex of mRNA and ribonucleoprotein particles
Mya	million years ago
ncRNA	non-coding Ribonucleic Acid
NIL	near isogenic line
NJ	neighbour-joining
NMD	Nonsense-Mediated Decay

nt	nucleotide
ORF	open reading frame
PCR	Polymerase Chain Reaction
PG	pseudogene
PIC	Pre-initiation complex
pre-mRNA	precursor messenger Ribonucleic Acid
PTC	premature termination codon
PTM	co- and post-translational modification
PUT	PlantGDB-assembled Unique Transcript
qPCR	quantitative PCR
RBP	RNA-binding proteins
RFU	Relative Fluorescent Unit
RNA pol	RNA polymerase
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid interference
RNase	ribonuclease
RNA-seq	RNA-sequence
RRM	RNA Recognition Motif
rRNA	ribosomal Ribonucleic Acid
RT	Reverse Transcription
SCN	suprachiasmatic nucleus
SD	short day
SDW	sterile distilled water
SEM	Standard Error of the Mean
snoRNA	small nucleolar Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
snRNA	small nuclear Ribonucleic Acid
ss	splice site
TBE	Tris-Borate-EDTA
tRNA	transfer Ribonucleic Acid
uAUG	upstream translation initiation codon
uORF	upstream open reading frame
UTR	untranslated region
WT	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Chapter 1. Introduction and review of literature

1.1 – Eukaryotic gene expression

Current classification of the living world divides all organisms into two superkingdoms: eukaryotes and prokaryotes (Sapp, 2005). This division is based on profound differences between these two groups, the main example being eukaryotes having a nuclear envelope, which separates transcription from translation, whereas prokaryotes have no such barrier, so transcription is coupled with translation (Philips, 2008). Interestingly, only nucleated organisms can exist in complex multicellular forms, exhibiting large inter-species differences in their morphology and behaviour (e.g. barley and humans). Such differences are also observed within tissues of the same organism (e.g. roots and flowers), even though the DNA sequence is the same in different tissues. The same exceptional complexity is not found in prokaryotes. Why then, are eukaryotes more intricate than prokaryotes? First, eukaryotes have bigger genomes with more complex sequences and organisation (Philips, 2008). Second, and most importantly, they are able to regulate gene expression in terms of time, space, quantity and 'quality'. Of course, regulation is not only a direct responses to environmental change, but also to actively control the gene expression programme to provide continuous and increasing vitality. Below is a brief description of eukaryote's rich cellular portfolios of gene expression control.

1.1.1 – Epigenetic regulation of expression

The definition of 'epigenetic' is highly debated (Bird, 2007; Pearson, 2008). The classical meaning defines it as "a change in the state of expression of a gene that does not

involve a mutation, but that is nevertheless inherited in the absence of the signal or event that initiated the change" (Pearson, 2008). In 2007, Bird suggested a much broader meaning, which is hereafter adopted: "epigenetics is the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states".

One of the best studied epigenetic modifications is the methylation of a cytosine residue, which is frequently followed by a guanine in the DNA molecule (known as CpG) (Bird, 2002; He *et al.*, 2011; Huidobro *et al.*, 2013). This epigenetic mark is involved in the regulation of several biological processes such as i) imprinting (a mechanism that controls parent-of-origin-specific gene expression); ii) X chromosome inactivation (Feil and Berger, 2007; Law and Jacobsen, 2010); iii) silencing of transposable elements, retroviruses and oncogenes (Stewart *et al.*, 1982; Zhang *et al.*, 2006; Huidobro *et al.*, 2013); and iv) temporal and spatial control of gene transcription (Bird, 2002; Zhang *et al.*, 2006).

Usually acting in concert with DNA methylation, histone modifications/remodelling are also important epigenetic marks for gene regulation. Negatively charged DNA molecules are associated with positively charged histones, which protect and compact the DNA, forming the chromatin complex. Tightly packed DNA is inaccessible to the transcriptional machinery, so this chromatin must be altered if gene transcription is to occur (Philips, 2008; Talbert and Henikoff, 2010). Chromatin unwinding is under strict control, being involved in the regulation of DNA repair (Groth *et al.*, 2007), DNA replication (Giri and Prasanth, 2012) and gene regulation (Skene and Henikoff, 2013).

There are two main ways to alter chromatin state to control expression of a particular gene: changing the position and type of histones present and/or modifying the histones themselves. As for the former, some histone subunits have evolved different variants, which have distinct properties in dictating DNA accessibility. Eukaryotes are able to selectively deposit or remove histone variants along particular regions of the DNA molecule (Deal and Henikoff, 2011; Skene and Henikoff, 2013). As for the latter, histone residues

can be subjected to acetylation, methylation, phosphorylation and ubiquitination, among others (Li *et al.*, 2007). These post-translational modifications loosen or tighten DNA-histone bonds and/or alter interactions with proteins that influence downstream processes in gene expression (Li *et al.*, 2007; Palazzo and Akef, 2012; Skene and Henikoff, 2013).

1.1.2 – Transcription

Gene expression genuinely starts with transcription. In simple terms, this process is the replication of information in the DNA into an RNA molecule. Up to 80% of all RNAs transcribed in a growing eukaryotic cell are ribosomal RNAs (rRNAs) (Paule and Lofquist, 1996). Such intense transcriptional activity in the nucleus is so prominent and organised that it forms a sub-compartment easily seen in microscopy, termed the nucleolus (Boisvert *et al.*, 2007). A specialised type of RNA polymerase (RNA pol), type I, solely transcribes these rRNAs (Nogi *et al.*, 1991). Separately, 5S rRNA subunits are transcribed elsewhere in the nucleus by a different type of polymerase, RNA pol III (Haeusler and Engelke, 2006). RNA pol III also transcribes transfer RNAs (tRNAs) and some short non-coding RNAs (ncRNAs) such as U6 small nuclear RNA (snRNA), H1 RNA (the RNA component of RNase P), among others (White, 2011). Last, but not least, RNA pol II is responsible for the synthesis of many types of ncRNAs, such as most snRNAs, microRNAs (miRNAs), small nucleolar RNAs (snoRNAs) and, most importantly, all messenger RNAs (mRNAs). Therefore, all protein-coding genes depend on RNA pol II activity for expression.

A closer look into the typical RNA pol II activity reveals a very intricate system. Transcription can be divided into a number of distinct steps and each of them is exploited to regulate gene transcription (Svejstrup, 2004). From selecting the transcriptional start site (Rojas-Duran and Gilbert, 2012) until the termination of transcription, there is tight regula-

tion (Proudfoot, 2011). If the transcriptional machinery/complex is correct at each step and repressors are not present, transcription goes to the next step (Singh, 1998; Svejstrup, 2004). Six major steps of the transcription process are detailed below.

1) Pre-initiation complex (PIC) assembly: Activators, co-activators and general transcription factors (GTFs) bind to the promoter, including the TATA box and transcriptional start site. RNA pol II is then positioned on the promoter to complete the PIC (Singh, 1998; Li *et al.*, 2007).

2) Open complex formation: 11–15 bp of DNA around the transcription start site is unwound and the single-stranded template is positioned in the RNA pol II cleft (Li *et al.*, 2007).

3) Initiation: Complementary ribonucleotides are bound to the single-stranded DNA template so the RNA polymerase can catalyse the first phosphodiester bond of the nascent transcript.

4) Promoter escape: This phase is critically important in the transition to the elongation phase, i.e. exiting (transcribing in the 5'→3' direction) the once 'attractive' promoter. The transcription complex becomes highly unstable from 2 bp until 15 bp of transcription, with a propensity to abort transcription. At this stage there is strong dependence on ATP, GTFs and downstream template to continue transcription (Dvir, 2002). Towards the end of promoter escape, RNA pol II is phosphorylated at its carboxyl terminal domain (CTD), losing contact with GTFs so it can proceed to the elongation phase (Li *et al.*, 2007).

5) Elongation: Elongation factors and RNA processing factors (discussed in the next Section, 1.1.3) are recruited to RNA pol II so that elongation can occur efficiently (Li *et al.*, 2007). At this step, there is also enhanced RNA pol II proof-reading activity (Libby and Gallant, 1991). It is noteworthy that chromatin structure still plays an important role at this step, controlling the speed of transcription (Svejstrup, 2004; Li *et al.*, 2007).

6) Termination: Although not much is known about RNA pol II termination, it is likely that reduction of RNA pol II elongation speed after a poly(A) site and lower stability of the RNA:DNA hybrid might contribute to destabilisation of the transcription complex and termination of transcription (Proudfoot *et al.*, 2002; Mischo and Proudfoot, 2013). An additional contribution is likely provided by a mechanism called the torpedo model, which occurs after poly(A) cleavage of the nascent mRNA and is acting in consort with RNA pol II-recruited poly(A) factors. In this model, the uncapped residual RNA still attached to the elongating polymerase is degraded by a 5'–3' exonuclease. Such exonuclease is in kinetic competition with the elongating RNA pol II and when they meet, this induces conformational changes in the polymerase to promote transcription termination (Proudfoot, 2011). Correct termination is important not only for the gene being transcribed, but also for downstream genes. After RNA pol II is released from the DNA template, it is recycled and participates in a new round of transcription (Svejstrup, 2004).

1.1.3 – Precursor messenger RNA (pre-mRNA) processing

The nascent (immature) pre-messenger RNA (pre-mRNA) from the elongation phase described above is processed into functional mRNA while and after it is transcribed, before it can leave the nucleus (Proudfoot *et al.*, 2002; Darnell, 2013). The majority of pre-mRNA processing is thought to be co-transcriptional. To facilitate co-transcriptional processing, most proteins and enzymes involved in RNA processing (e.g. capping, splicing and polyadenylation) also bind to the phosphorylated CTD of RNA pol II, so transcription influences a large part of mRNA processing (Darnell, 2013). There are four main pre-mRNA processing reactions:

1) Capping: After about 20–30 nucleotides have been synthesised on the nascent RNA, the 7-methylguanosine cap is linked to the first ribonucleotide of the transcript

(5’ end) (Proudfoot *et al.*, 2002). This three-step reaction forms an inverted 5'–5' triphosphate bridge and allows attachment of the cap binding complex. Capping is essential for protecting the mRNA against 5’-3’ exonucleases and for mRNA export through the nuclear pore complex (Proudfoot *et al.*, 2002; Shuman, 2002).

2) Splicing: The majority of eukaryotic genes contain introns. Therefore, in order to produce functional mRNAs, introns must be removed from the pre-mRNA and exons joined in a very precise way. This excision and choice of exons is highly controlled and can vary for several reasons (see Section 1.2.)

3) Editing: (pre-)mRNAs, mostly from animal cells and plant organelles, can undergo editing. RNA editing involves covalent modification, the substitution or insertion/deletion of a ribonucleotide residue in the RNA molecule (Gray, 2012). This process can affect translation, splicing or mRNA localisation (DeCerbo and Carmichael, 2005).

4) Polyadenylation: This process is divided into two major steps. First, the nascent mRNA is cleaved during RNA pol II elongation. Second, a poly(A) tail is synthesised at the free 3’ end. The cleavage reaction is catalysed by factors that recognise particular poly(A) signals in the nascent RNA (Darnell, 2013). The poly(A) *cis* signals are mostly conserved elements/motifs rich in A and U (Li and Hunt, 1997; Sherstnev *et al.*, 2012; Darnell, 2013). Eukaryotic genes frequently have more than one poly(A) signal/site, and polyadenylation is under tight regulation. Alternative polyadenylation can interfere with the mRNA coding sequence, its stability and translatability (Di Giammartino *et al.*, 2011; Proudfoot, 2011). After RNA cleavage and consequent release from RNA pol II, a poly(A) polymerase adds multiple adenines at the 3’ end of the mRNA. The length of the poly(A) tail is under regulation, and can also affect mRNA stability and translatability (Weill *et al.*, 2012). Lastly, the poly(A) tail bound by poly(A)-binding proteins is recognised by several

proteins, essential for the mRNA export through the nuclear pore complex (Proudfoot *et al.*, 2002).

1.1.4 - Nuclear export

The last level of gene expression control in the nucleus is the exit of an mRNA through the nuclear pore. From the beginning of transcription to the end of pre-mRNA processing, many proteins remain bound to the mRNA (Iglesias and Stutz, 2008). These proteins are seen as marks of correctly transcribed and processed mRNAs. Thereafter, these marker proteins recruit a number of other proteins which aid in the nuclear export (Palazzo and Akef, 2012). The final mRNA and accompanying proteins form the messenger ribonucleoprotein particle (mRNP) (Köhler and Hurt, 2007), which is then recognised by the nuclear export machinery, which initiates mRNA exit to the cytoplasm (Palazzo and Akef, 2012).

mRNA export is a tight quality control mechanism that keeps most products of spurious transcription and processing in the nucleus, where they are directed to be degraded (Fasken and Corbett, 2005; Palazzo and Akef, 2012). For instance, transcripts with defective poly(A) tails are degraded by the exosome complex (Fasken and Corbett, 2005). The degradation of mis-processed mRNAs is particularly important to avoid translation of aberrant transcripts, which could encode mutant or even toxic proteins (Fasken and Corbett, 2005). The yeast Mlp1p protein (homologous to human Tpr), for example, participates in a quality control step that prevents the export of intron-containing transcripts. This protein interacts with the hnRNP-like protein Nab2p, present on spliced mRNAs, allowing them to be exported (Galy *et al.*, 2004). Once arriving in the cytoplasmic environment, the mRNP, still bound to the nuclear export machinery, is remodelled (Iglesias and

Stutz, 2008). This prevents the mRNP from re-entering the nucleus and prepares the mRNA for translation (Köhler and Hurt, 2007; Palazzo and Akef, 2012).

1.1.5 – Translation

Translation is a well-characterised process where the ribosome decodes the information encoded by the mRNA to produce a linear polypeptide chain. There are three main reactions in translation:

1) Initiation: Soon after leaving the nucleus, the mRNP cap associates with eukaryotic Initiation Factors (eIFs). This association improves recruitment of the small 40S ribosomal subunit to the 5' end of the mRNA (Kozak, 2005; Horiguchi *et al.*, 2012). Additional eIFs bind to the initial complex, allowing the small ribosomal subunit to move along the RNA molecule in the 5′ to 3′ direction, which is known as *scanning*. It pauses at an appropriate sequence context that (usually) contains the first AUG (Kozak, 2005). This is followed by the dissociation of eIFs, allowing the large 60S ribosomal subunit to join with the small 40S subunit and form the 80S ribosome (Horiguchi *et al.*, 2012).

2) Elongation: This is when translation itself occurs. Surprisingly, few elongation factors are required for this task (Groppo and Richter, 2009). Throughout the elongation phase, the ribosome machinery continuously repeats the following cycle: 1) the aminoacyl-tRNA (tRNA charged with the appropriate amino acid) is selected via the triplet code, 2) the associated amino acid is attached to the growing polypeptide chain by formation of a peptide bond, catalysed by the ribosome and 3) the ribosome translocates to the next codon, faithfully maintaining the reading frame. The nascent polypeptide chain folds into its tertiary conformation while still bound to the translation machinery. Diverse proteins and molecular chaperones can aid protein folding (Groppo and Richter, 2009). Translation occurs mainly in the cytoplasm, except when an Endoplasmic Reticulum (ER) localisation

signal is present. In this case, the nascent polypeptide and the translation machinery are targeted to the ER (Brodsky and Skach, 2011).

3) Termination: When the reading frame in use by the translation machinery exposes a termination codon, the signal for translation to stop, a eukaryotic Release Factor (eRF) will bind to this codon, instead of a tRNA. This event initiates the release of the peptide chain, terminating translation. The ribosome is then recycled for new rounds of translation (Graille and Séraphin, 2012).

Gene regulatory mechanisms are also present during translation. First, ribosome translation can be prevented or greatly affected by: i) complex mRNA secondary structure (Mauger *et al.*, 2013); ii) miRNAs (Iwasaki and Tomari, 2009; Huntzinger and Izaurralde, 2011); iii) proteins/repressors (Gallie, 2002; Kong and Lasko, 2012); and iv) the presence of upstream AUG sequences (Kozak, 2005). Additionally, different surveillance pathways are able to detect and degrade mRNAs with faulty translation, for instance Non-Stop decay degrades mRNAs without stop codons (continuous ORF) (Vasudevan *et al.*, 2002). These surveillance mechanisms reduce accumulation of defective mRNAs and avoid the production of mutant or even toxic proteins (Graille and Séraphin, 2012).

1.1.6 – mRNA turnover/degradation

In addition to mRNA surveillance, described in other Sections (1.1.3, 1.1.4, 1.1.5 and 1.4.2) as degradation of defective mRNAs, functional mRNAs are also subjected to degradation. In fact, mRNA is an unstable molecule by nature, a situation aggravated by the presence of ribonucleases (RNases) in the cytoplasm (Pérez-Ortín *et al.*, 2012). Eukaryotes are able to regulate mRNA stability, therefore controlling gene expression levels. Such mRNA degradation control is particularly important in rapid responses to stress (Ambrosone *et al.*, 2012; Pérez-Ortín *et al.*, 2012).

The most common *trans*-acting factors controlling mRNA turnover are RNA-binding proteins (RBP) (Ambrosone *et al.*, 2012; Pérez-Ortín *et al.*, 2012) and miRNAs (Iwasaki and Tomari, 2009; Huntzinger and Izaurralde, 2011). These factors either protect the mRNA or target it for degradation (Belostotsky and Sieburth, 2009; Wu and Brewer, 2012).

1.1.7 – Protein modification

Co- and post-translational modifications (PTMs) covalently change the primary structure of nearly all proteins (Lothrop *et al.*, 2013). The most common PTMs are phosphorylation, glycosylation, acetylation, protein cleavage and ubiquitination, among many others (Khoury *et al.*, 2011). They change many aspects of a protein, such as structural conformation, enzymatic activity, sub-cellular localisation, interactions with substrates or binding partners (Kwon *et al.*, 2006) and lastly, rate of degradation (Matyskiela and Martin, 2013). Therefore, PTMs largely increase protein diversity and dynamics, including proteins involved in epigenetics, translation, pre-mRNA processing, mRNA nuclear export, mRNA stability and translation. Consequently, PTMs pervade and link the whole process of gene regulation in eukaryotes, with involvement from signal perception to cell response.

1.2 – Pre-mRNA splicing

Intron splicing (removal) is essential for the great majority of eukaryotic genes. For instance, 97% of protein-coding genes in humans have one or more introns. In plants the scenario is similar: 80% of *Arabidopsis thaliana* (Arabidopsis subsequently) and 75% of rice protein-coding genes have at least one intron (Alexandrov *et al.*, 2006).

Introns have been present since the earliest stages of eukaryotic evolution. There are two highly debated theories for the origin of these intervening sequences. Either introns appeared only in the eukaryote ancestor or they have always existed, since the beginning of life, before the eukaryote/prokaryote divergence (reviewed in Rogozin *et al.*, 2012). In the second case, prokaryotes saved more energy and space and produced proteins faster by losing the introns, whereas eukaryotes expanded their proteome and their control over gene expression by maintaining these introns. It is noteworthy that eukaryotes still maintain introns not only because of these advantages, but also because introns have become intrinsic to the cellular system, with a selective pressure that maintains them (Alexandrov *et al.*, 2006; Grzybowska, 2012). This pressure can be explained by a few factors, for instance nuclear export and mRNA surveillance control. These processes prevent transcripts that have not been spliced to stay in the cytoplasm. Regardless of the debate concerning intron evolution, there is no doubt that the existence of introns ultimately contributes to the complexity of eukaryotes.

Splicing introns from the primary transcript allows production of translatable mRNA. This process is catalysed by the (most?) complex ribonucleoprotein macromolecular machine, the spliceosome (Nilsen, 2003). Most of the splicing process occurs co-transcriptionally and can be influenced by RNA pol II activity and epigenetic state, among other factors (Darnell, 2013). In fact, the flexibility which pre-mRNA splicing provides in terms of very refined regulation of expression and increased protein complexity has contributed to the evolution of eukaryotes (Artamonova and Gelfand, 2007). Further information on this matter is presented in Sections 1.3, 1.4 and 1.5, while details of the canonical process through which introns are efficiently recognised and removed by the splicing machinery are described below.

1.2.1 – Components of the spliceosome machinery

Systematic analysis to elucidate the compositional information of the spliceosome has been performed in human and yeast spliceosomal complexes. Many orthologues of human splicing factors have been found in the Arabidopsis genome (Barta *et al.*, 2012), which suggests that the basic mechanism of intron removal is the same in all phyla. Overall, the spliceosome contains five snRNAs and around three hundred proteins (Jurica and Moore, 2003). Two types of spliceosome machinery coexist in the cell: the U2-type, which recognises and removes U2-type introns, and the less abundant U12-type, for the rare U12-type introns (Will and Lührmann, 2011).

The most common spliceosome, the U2-type, is composed of the U1, U2, U4, U5 and U6 uridine-rich snRNAs (Reddy *et al.*, 2012a). With the exception of U6, the spliceosomal snRNAs are transcribed by RNA pol II, which dictates their downstream maturation. These RNA pol II-specific primary snRNAs are exported to the cytoplasm for processing and re-imported to the nucleus, where they complete maturation. Maturation of the RNA pol III-specific U6 snRNA takes place solely in the nucleus (Kiss, 2004). The final mature spliceosomal snRNAs are assembled with specific proteins, forming the snRNP complexes. In addition to snRNPs, spliceosome activity also depends on the assembly of several other non-snRNP proteins such as the SR proteins (see Section 1.3.3) and DExH/D-box RNA ATPases/helicases (Long and Caceres, 2009; Staley and Woolford, 2009).

1.2.2 – Basic *cis*-acting elements

To carry out intron removal, the spliceosome must recognise the exact sites of the exon/intron and intron/exon boundaries, known as the 5' and 3' splice sites (ss), respectively (Figure 1.1). The pre-mRNA provides information to define these boundaries. Gen-

erally, the 5' ss has a conserved GU dinucleotide at the beginning of the intron, whereas the 3' ss has a conserved AG dinucleotide at the end of the intron (Reddy, 2007). Higher eukaryotes have additional poorly conserved intrinsic intron sequences downstream within the intron which help to define the splice sites. These are a branch site (containing a key adenine) and a polypyrimidine tract (a sequence enriched for uracil and cytosine) between the branch point and 3' ss (Reddy, 2007; De Conti *et al.*, 2013). The branch point is highly conserved in yeast introns and less well conserved in mammals and plants; the polypyrimidine tract is essential for splicing in mammals but less well conserved in plants (Simpson *et al.*, 2004).

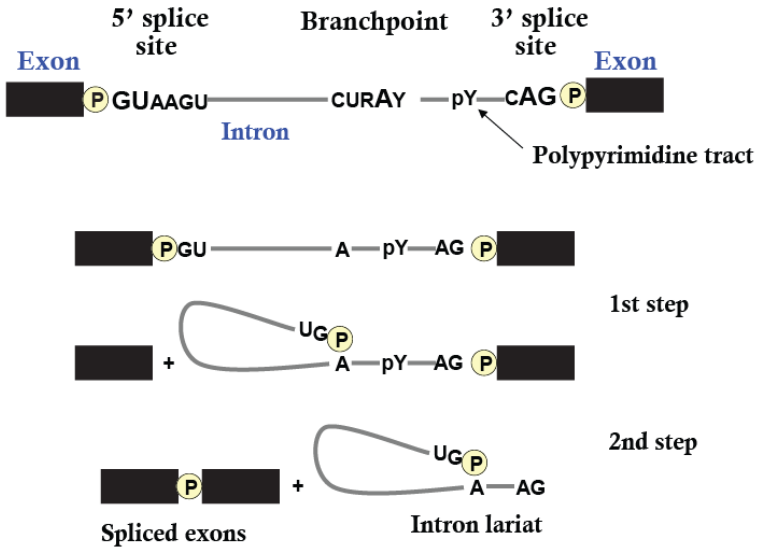


Figure 1.1: Schematic representation of the cis-acting splicing elements and the two-step mechanism of pre-mRNA splicing. Boxes and solid lines represent the exons and the intron, respectively. The branch point adenosine is indicated by the letter A in the CURAY branch site and the phosphate groups (p) at the 5′ and 3′ splice sites are also shown. Conserved sequences found at the 5′ and 3′ splice sites and branch site of U2-type pre-mRNA introns in eukaryotes are shown (Reddy, 2007). Y = pyrimidine and R = purine. The polypyrimidine tract is indicated by pY.

General characteristics of exons and introns also seem to affect splice site recognition. For instance, an intron or exon which is too small (< 50 nt) can interfere with their splicing because of splicing factors physically hindering each other and additional mechanisms are required for mini-exon and mini-intron splicing (De Conti *et al.*, 2013). In plants, introns must be at least 70 nt in length with high UA content (around 15% more than exons) for efficient splicing (Goodall and Filipowicz, 1989; Luehrsen *et al.*, 1994; Lorković *et al.*, 2000).

1.2.3 – Splicing cycle

The spliceosome assembly occurs in a stepwise manner, and can be grouped into two major steps (Figure 1.1 and Figure 1.2):

1) 5' ss cleavage/ligation: In the initial step, the U1 snRNP recognises and binds to the 5' ss through base-pairing, thus defining the exact exon/intron boundary. The human non-snRNP factor Sf1 (Bbp in yeast) interacts with the branch site, and the non-snRNP U2AF (Mud2 in yeast) associates with the polypyrimidine tract and the 3' ss. At this step, the spliceosome forms what is known as the E (early) complex (Zhang *et al.*, 2013b).

Subsequently, with the aid of DExD-H-box RNA ATPases/helicases, the U2 snRNP displaces Sf1 and interacts with the branch site through base-pairing. This forms what is known as the A (pre-spliceosome) complex (Figure 1.2) (Will and Lührmann, 2011). The pre-assembled U4/U6.U5 tri-snRNP is then recruited and integrated into the spliceosome. The conserved loop of U5 snRNA, in particular, base-pairs with exon sequences close to the 5' ss. The tri-snRNP integration forms the B (pre-catalytic spliceosome) complex (Will and Lührmann, 2011; Zhang *et al.*, 2013b).

Integration of additional non-snRNP proteins initiates major structural rearrangements: i) the 5' ss dissociates from the U1 snRNA and re-associates (base-pairs) with the U6 snRNA instead; ii) the U1 snRNP is removed from the complex; iii) the U4/U6 interaction is also disrupted followed by U4 removal; iv) the U2 snRNA forms new interactions with the U6 snRNA. These major rearrangements bring key sequences involved in the splicing reaction into close proximity and allow the spliceosome to become active (B^{act} complex, Figure 1.2) (Will and Lührmann, 2011). Subsequent catalytic activation of the spliceosome is achieved by the DEAH-box RNA helicase Prp2, which generates the B* complex.

The spliceosome is now able to perform the first step of splicing. It starts with a nucleophilic attack on the 5′ ss by the 2′ OH group of the branch point adenosine, present in the intron. This results in 5' ss cleavage and subsequent ligation of the 5′ end of the intron to the branch point adenosine, forming a lariat structure (Figure 1.1). After this first reaction, the spliceosome repositions the mRNA substrate to form the C complex (Will and Lührmann, 2011; Zhang *et al.*, 2013b).

2) 3' ss cleavage/ligation: The second catalytic reaction is performed by the C complex, soon after the first reaction. First, the 3′ OH group of the 5′ exon attacks the 3′ ss (Figure 1.1). This results in 3' ss cleavage and subsequent ligation of the 5′ and 3′ exons to form the mRNA (Figure 1.2). The spliceosome then undergoes post-catalytic rearrangements to release the intron lariat and the mRNA, and to dissociate the various spliceosomal components. Finally, the snRNPs are recycled for new rounds of splicing, and the intron lariat is debranched and degraded (Will and Lührmann, 2011; Zhang *et al.*, 2013b).

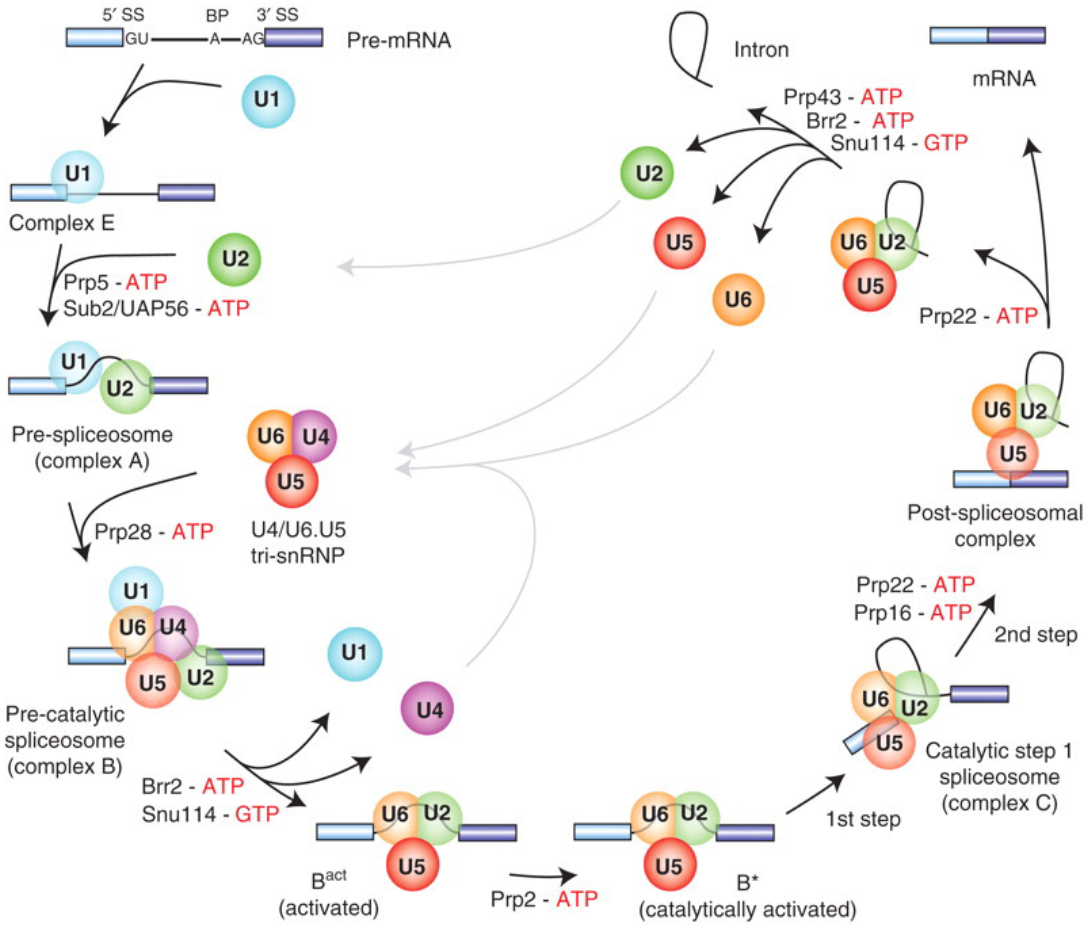


Figure 1.2: The splicing cycle (Figure from Will and Lührmann, 2011). Spliceosomal assembly complexes are named according to the metazoan nomenclature. Boxes and solid lines represent exons and introns, respectively. The branch point adenosine (BP) is indicated by the letter A.

1.3 – Alternative pre-mRNA splicing

Alternative pre-mRNA splicing is a variation of splice site usage among pre-mRNAs of the same gene. As a consequence, the same gene is able to produce different mRNAs, which can impact protein production. Hence, alternative splicing (AS) provides an excellent way to control gene expression.

AS events were uncovered soon after the discovery of pre-mRNA splicing in 1977 (Berget *et al.*, 1977), with studies of the adenovirus 2 gene (Chow *et al.*, 1977). Chow and

colleagues found that the adenovirus 2 gene was not only spliced but also alternatively spliced, giving rise to different mRNA isoforms (Chow *et al.*, 1977). After this initial discovery, additional studies, from individual gene analyses to genome-wide surveys, concluded that alternative splicing is a widespread phenomenon in higher eukaryotes (Kornblihtt *et al.*, 2013). For instance, 95% of human multi-exon genes are alternatively spliced (Pan *et al.*, 2008). In Arabidopsis, more than 61% of intron-containing genes are known to undergo AS, and this frequency is likely to increase, especially if more plant tissues and variable growth conditions are studied (Marquez *et al.*, 2012).

All in all, alternative splicing is essential and advantageous for eukaryotes (Artamonova and Gelfand, 2007; Mastrangelo *et al.*, 2012). Its control is considerably complex and to understand it, it is important to have a closer look at how splice site selection occurs. In this section, the mechanisms and evolution of AS are reviewed, whereas consequences and important advantages of AS are discussed in Section 1.4.

1.3.1 – Types of alternative splicing events

Exons that are always included and introns that are always removed from the final mRNA are considered constitutive/canonical (Black, 2003). Pre-mRNAs can be alternatively spliced through the selection of alternative splice sites to generate different mRNAs from the pre-mRNAs of the same gene. There are five main types of alternative splicing events (Figure 1.3):

A) Intron retention (Figure 1.3A). This splicing event refers to a 'failure' in removing an intron from the pre-mRNA.

B–C) Alternative splice-site usage. Exons and introns can also be lengthened or shortened by selection of different 5' (donor) or 3' (acceptor) splice sites (ss). For example, in a hypothetical exon (Figure 1.3B), the 5' splice site 'y' is most commonly used and de-

fines the 3' boundary of such exon. Usage of an alternative 5' ss that is 20 nt upstream within that exon (splice site 'x') removes 20 nt of it (Figure 1.3B). This also applies to alternative 3' splice site selection (Figure 1.3C).

D–E) Alternative exon (Figure 1.3D and Figure 1.3E). Alternative or cassette exons are either included or skipped (Figure 1.3D) in different transcripts. A relatively rare type of alternative splicing is mutually exclusive splicing (Figure 1.3E), which is the selection of only one exon from two or more adjacent cassette exons such that only one exon in the group is included at a time.

F) Cryptic intron (Figure 1.3F). A sequence unusually removed from within an exon is known as cryptic intron.

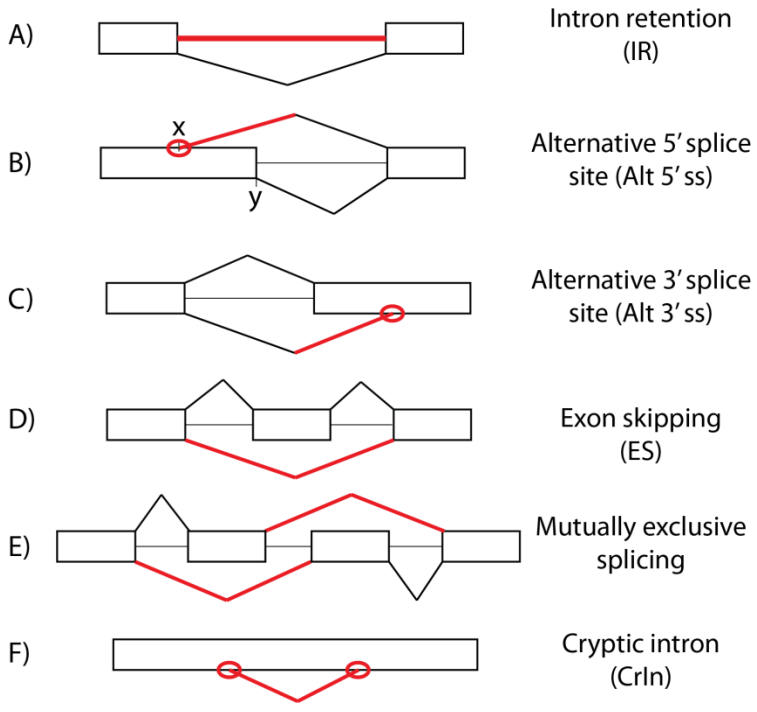


Figure 1.3: Types of AS events. Exons are open boxes and introns are straight fine black lines. Diagonal fine lines represent splicing events, where AS events are shown in red. A) Intron retention; B) and C) alternative 5' and 3' splice site usage, respectively; D) and E) represent two types of AS events using cassette exons; F) cryptic intron.

In cases when genes exhibit multiple alternative splicing sites within their sequences, these are generally used in a combinatorial manner to produce many different mRNAs (Black, 2003). In addition to these normal (*cis*-) splicing events, genes can undergo an unusual form of RNA splicing known as *trans*-splicing. This unusual mechanism, not necessarily carried out by the spliceosome, is responsible for processing different pre-mRNA molecules into one final mRNA (Bonen, 1993; Herai and Yamagishi, 2010). These hybrid mRNAs are common and essential in nematodes and trypanosomes (Lücke *et al.*, 1996; Blumenthal, 2012) but rare in plants and humans, where they may impact upon human health (Herai and Yamagishi, 2010).

1.3.2 – Additional *cis*-acting elements: splicing enhancers and silencers

Strong splice sites are those containing canonical *cis*-acting elements (mentioned in Section 1.2.2), where, for example, there is a high degree of complementarity between the 5' ss and U1 snRNA and the branch point and U2 snRNA. Weak splice sites contain sequence variations from the canonical *cis* elements, which result in less efficient recognition by the spliceosome and decreased splicing. Although strong or weak splice sites can be predicted by comparison to consensus sequences it is, however, generally not enough information to predict splicing activity *in vivo*. Additional *cis*- and *trans*-acting elements can collaborate to enhance or repress splicing of a particular splice site (Black, 2003).

The additional *cis* regulatory elements affecting splicing are termed according to their location (exon or intron) and function (enhancement or silencing usage of a site): exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic splicing silencers (ISSs) (Figure 1.4). These elements are diverse in sequence as they serve as binding sites for *trans*-acting factors, which then interact with the spliceosome. The exonic elements are embedded within nucleotides that also code for pro-

tein and affect splice sites over short distances. The intronic elements are usually present near the polypyrimidine tract, branch point or 5' splice site, and they can interfere with splice site selection over much longer distances compared to exonic elements (Black, 2003).

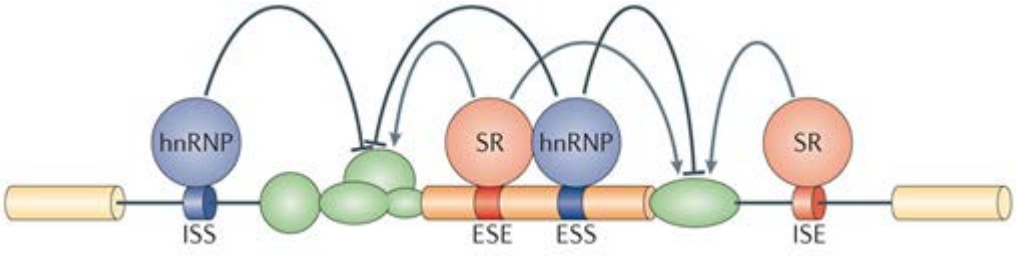


Figure 1.4: Simplified schematic diagram of some *cis*- and *trans*-acting regulatory sequences in the pre-mRNA (modified from Kornblihtt *et al.*, 2013). ISS, intronic splicing silencers; ESE, exonic splicing enhancer; ESS, exonic splicing silencers; ISE, intronic splicing enhancers. Two main families of trans-acting regulatory proteins are shown: SRs and hnRNPs (see Section 1.3.3). These regulatory proteins interact with components of the spliceosome (shown in green) either activating or inhibiting splicing.

Splicing enhancers and silencers, under the control and availability of *trans*-acting factors, are also essential for defining exons and introns. Yeast genes, for example, have very small introns and frequently have intron-defining sequences that enhance spliceosome assembly around the intron (Ellis *et al.*, 2008). In animals, large intron sequences (often tens of thousands of bp) separate relatively small exons. In this case, exon defining sequences can recruit splicing machinery components onto the splice sites around an exon, thus identifying the exon prior to interaction of these components across the intron and spliceosome formation (Reddy, 2007).

Overall, alternative splicing control through *cis*-acting factors is complex. Splicing enhancers and silencers are often found clustered to make a combination of regulatory se-

quences that mediate both positive and negative regulation (Black, 2003). To know if the splicing regulation will be positive or negative, it will depend on a series of factors such as the abundance and activity of the *trans*-acting population available (see below).

1.3.3 – Additional *trans*-acting splicing elements: regulatory proteins

Trans regulatory proteins bind to splicing enhancers and/or silencers to activate or inhibit the use of splice sites, respectively. They mostly include serine/arginine-rich proteins (SR) and heterogeneous nuclear ribonucleoproteins (hnRNP) (Schwartz *et al.*, 2008).

The SR proteins are involved in both constitutive and alternative splicing of pre-mRNAs. They also have additional roles in, for example, transcription, mRNA export and translation (Long and Caceres, 2009). These proteins have one or two conserved domain structure(s) at the N-terminus for interacting with specific (pre-)mRNA molecules, known as RNA Recognition Motifs (RRMs). At the C-terminus, they have a domain for protein interaction, which is variable in length and rich in serine/arginine dipeptide, termed the RS domain (Black, 2003). The RS domain can be highly phosphorylated, which controls protein localisation and activity. Other proteins containing RS-domains can also function in pre-mRNA splicing, but because they lack a conserved RRM domain they are referred to as SR-like or SR-related proteins (Long and Caceres, 2009).

Generally, SR proteins enhance splicing through binding to splicing enhancers (ESEs and ISEs) (Black, 2003). When SR proteins bind to ESEs, they mostly trigger spliceosome assembly and prevent exon skipping, thus providing a mechanism for defining exons. ISEs are also recognised by SR proteins, a process which plays a role in intron definition (Ellis *et al.*, 2008). A small subset of SR proteins can act as negative splicing regulators, for instance when binding to ISSs (Black, 2003; Long and Caceres, 2009).

The hnRNP proteins are also involved in both constitutive and alternative pre-mRNA splicing, with additional roles, for instance in telomere maintenance and mRNA export (He and Smith, 2009). They are a diverse group of regulators constituted of a number of proteins that bind RNAs via RNA binding domains which are usually RRM or K Homology (KH) domains, while protein interaction domains are normally repeats of Arg-Gly-Gly tripeptides (known as RGG boxes) but it can also contain auxiliary domains, such as a glycine-, acidic- or proline-rich domain (Busch and Hertel, 2012).

In most cases, hnRNP proteins inhibit splicing through binding to splicing silencers (ESS and ISS, Figure 1.4). The mechanisms by which hnRNPs repress splicing can differ between transcripts and proteins (Black, 2003). The hnRNPs can: i) interfere with spliceosome assembly; ii) interfere with splicing activation by preventing SR proteins from binding to adjacent ESEs; or iii) interact with other hnRNPs through dimerisation, which can create an isolating loop which keeps exons or splice sites away from the splicing apparatus. In a few examples, hnRNPs act as positive splicing regulators, depending on their positions relative to the regulated splice site (Black, 2003; Xue *et al.*, 2009; Busch and Hertel, 2012).

The polypyrimidine tract-binding (PTB) proteins are a class of hnRNP splicing factors, mainly involved in tissue-specific repression of exons, but also of polyadenylation and mRNA stability (Sawicka *et al.*, 2008). They contain four RRM domains that often bind to the polypyrimidine tract of regulated 3' splice sites. The mechanisms by which PTB proteins repress splicing can be by looping out exon sequences or competing against U2AF for binding to the polypyrimidine tract and thus, inhibiting splicing (Black, 2003). On rare occasions, PTB proteins can also enhance splicing, depending on their position relative to the target exons (Kafasla *et al.*, 2012).

In addition to the complex combination of *cis*-acting factors mentioned previously (Section 1.3.2), the intricate control of AS also depends on the combination of *trans*-acting

factors. First, many *trans*-acting factors can act as both enhancers and suppressors of splicing depending on their relative abundance, sequence and position of their target sites in the pre-mRNA (Black, 2003). Second, expression and activity states (regulated by PTMs) of many splicing regulators vary in different cells, tissues, developmental stages or under different conditions. Thus, the abundance and activity of splicing regulators affect splicing outcomes. Thirdly, the expression of many splicing factor genes are also under tight regulation, often by alternative splicing, pointing to networks of splicing factors and splicing regulation (Black, 2003).

1.3.4 – Epigenetic and transcriptional control of alternative splicing

Recently, a new dimension in the regulation of alternative splicing was revealed with the demonstration that splicing is not only regulated by splicing factors, but also by a more complex process involving epigenetics and transcription (Luco and Misteli, 2011; Kornblihtt *et al.*, 2013). As mentioned previously (Section 1.1.3), most splicing events occur co-transcriptionally, through a physical link between elongating RNA pol II and splicing factors. In such functional coupling, a key player is the RNA pol II CTD, as it is known to recruit splicing factors, as well as poly(A) cleavage factors, to the sites of transcription in a phosphorylation pattern-dependent manner (Gómez Acuña *et al.*, 2013). Moreover, RNA pol II is physically linked to the chromatin, which allows for a coupling between splicing, transcription and epigenetic regulation. Two main models explain how epigenetic chromatin marks and transcription can affect alternative splicing.

1) 'Kinetic model'. In this model, RNA pol II elongation rate can affect splicing. For example, a slow elongation rate or pausing of RNA pol II allows more time for a weak upstream exon to recruit the splicing machinery before the splicing sites of a stronger competing downstream exon are transcribed (Luco and Misteli, 2011). There are two main

ways in which the RNA pol II elongation rate is affected: by modifying the phosphorylation state of RNA pol II CTD and/or through chromatin remodelling and modification (Luco and Misteli, 2011). In the latter, histones can serve as a barrier, slowing down RNA pol II elongation rate (Hodges *et al.*, 2009). Interestingly, DNA sequences containing constitutive exons have significantly higher histone density than sequences coding for alternative exons, contributing to exon definition (Schwartz *et al.*, 2009). Additionally, constitutive and alternative exons have different histone modification marks, which can result in different chromatin unwinding patterns and consequently interfere with the accessibility and rate of RNA pol II, once again affecting exon definition (Luco and Misteli, 2011).

2) 'Recruitment model'. This model describes splicing factors as being recruited by either the RNA pol II CTD, an example being the SR protein Srsf3 (Das *et al.*, 2007), or through epigenetic information. In the latter, histone marks are recognised and bound by specific chromatin-binding proteins (adaptors), which in turn can influence the recruitment of splicing regulators to the nascent RNA, thus driving the splicing outcome (Luco and Misteli, 2011). The discovery of an 'adaptor complex' is based on analyses of several PTB-dependent genes. The best example is the work carried out by Luco and colleagues in human cells. They discovered that the chromatin-binding adaptor protein Mrg15 reads histone marks on different genes and recruits PTB proteins to bind ESSs on nascent pre-mRNAs, resulting in exclusion of the corresponding exon (Luco *et al.*, 2010).

To conclude, in addition to the complex combination of *cis*- and *trans*-acting splicing factors, epigenetics and transcription contribute to a new level of alternative splicing control. This new knowledge contributes to our understanding of the complexity in establishing and maintaining numerous alternative splicing programmes that are highly tissue-specific and developmentally regulated (Luco and Misteli, 2011).

1.3.5 – The splicing code

The 'splicing code' is the ultimate collection and integration of all information about regulation of different splicing events that can then be used to predict splicing outcomes in different tissues and developmental stages. Therefore, a splicing code must bring together information such as i) the strength of splice sites; ii) intron and exon size and presence of secondary structures; iii) splicing enhancers and silencers; iv) complex spatial and temporal combinations of active *trans*-acting splicing factors; v) spatial and temporal patterns of epigenetic marks and transcriptional effects on splicing.

Recent efforts to achieve a splicing code in mammals involved the analyses of alternatively spliced exons across different tissues and developmental stages, which allowed the characterisation of several splicing motifs and a splicing prediction with 65–90% accuracy (Barash *et al.*, 2010). The splicing code, however, is far from being deciphered. Emerging experimental and computational tools should be able to gather more information of the combinatorial features that control splicing, including relevant epigenetic and transcriptional data, to finally permit a more comprehensive and systematic analysis to decipher the code (Luco and Misteli, 2011). It is also important to carry out such studies in other organisms, such as plants (Reddy *et al.*, 2012b). Ultimately, the splicing code will allow for predictions of alternative splicing with high accuracy, as well as any effects from mutations within regulatory elements (Barash *et al.*, 2010).

1.3.6 – The evolution of alternative splicing

Before learning about the functions and roles of AS, it is essential to analyse why AS evolved. The evolution of AS is an area which is subject to several controversies mainly because many observations of specific evolutionary events are taken out of a more general context (Artamonova and Gelfand, 2007). For instance, point mutations in splice sites

can weaken them, increasing the chances for alternative splicing to occur. However, this situation is further complicated by *cis*- and *trans*-acting regulators, intron and exon size constraints, and epigenetics and transcriptional effects, which add extra layers of complexity to alternative splicing evolution (Artamonova and Gelfand, 2007).

Alternative splicing was present in the common ancestor of eukaryotes and it is currently prevalent in complex multicellular eukaryotes but rare in unicellular eukaryotes (Keren *et al.*, 2010), which suggests that AS is important in contributing to higher eukaryote complexity. This observation is corroborated by the considerable expansion of splicing regulatory proteins in higher eukaryotes, such as SR proteins and hnRNPs (Busch and Hertel, 2012). The functional relevance of widespread AS events in higher eukaryotes can be confirmed by conservation analysis. However, a closer look at AS conservation among eukaryotic species has not found high levels of conservation (Artamonova and Gelfand, 2007; Keren *et al.*, 2010). For instance, a large-scale comparison of AS between humans and mice suggests that alternative exons are less conserved than constitutive exons (Takeda *et al.*, 2008). Additionally, a wider analysis using multiple organs and different vertebrate species identified that the AS profiles of equivalent interspecies organs have diverged to the extent that the alternative transcripts are significantly more species-specific than organ-specific (Barbosa-Morais *et al.*, 2012).

The low conservation of AS events can be explained by different theories. A radical theory suggests that alternative regions are simply evolutionary intermediates that occur during transition to constitutive states, either by intron/exon loss or gain. However, evolution is rarely explained by a simple and dogmatic theory (Artamonova and Gelfand, 2007). Several lines of evidence suggest that AS is not a temporary remnant of evolution but rather an essential and actively regulated mechanism that provides a basis for selection to improve eukaryotic complexity. First, the rapid evolution of alternative splicing can be an advantage to speciation (Barbosa-Morais *et al.*, 2012). Additionally, the faster creation of

more AS isoforms provides more material for the selection of beneficial variants (Artamonova and Gelfand, 2007). Of course, faster creation is inevitably followed by rapid loss of AS isoforms, especially those that are not significantly beneficial to the species. This new and frequent generation of AS isoforms could expand the coding capacity of the genome. Moreover, new AS events could also generate isoforms that are removed from the cell by mRNA surveillance mechanisms, controlling mRNA levels. Taking all this advantages into account, cells have evolved mechanisms to control alternative splicing in a spatial and temporal manner, providing an extra level of gene expression control.

1.4 – Functions and roles of alternative splicing

Briefly, the primary consequence and function of AS is to affect protein expression. This can be achieved qualitatively and/or quantitatively. Regarding qualitative control, AS increases the possible number of protein isoforms, which can have different functions. Concerning quantitative control, AS can regulate protein expression levels by modifying how well an mRNA is translated and/or decreasing mRNA stability and targeting it for degradation (Black, 2003; Huang and Steitz, 2005; Stamm *et al.*, 2005; Reddy, 2007).

The ultimate roles of AS for eukaryotes are diverse and can range from opposite extremes such as causing disease to being used for gene therapy. AS can also be involved in stress responses and developmental control. It has a tremendous impact on eukaryotes and it contributes to their complexity, shaping their evolution (Rogozin *et al.*, 2012).

1.4.1 – Function of AS: increased proteome diversity

Proteome diversity is considerably increased by AS through the production of mRNA variants coding for proteins with different activity, localisation, stability and ability to interact with other proteins and substrates. In order to achieve this, AS usually occurs in

the coding sequence (CDS) and produces alternative mRNAs that are still translatable, functional and able to reach the cytoplasm. Therefore, such AS should not be random but tightly regulated (Stamm *et al.*, 2005).

Overall, AS tends to maintain core protein structure, generating subtle protein modifications (Stetefeld and Ruegg, 2005; Kelemen *et al.*, 2013). Additionally, these modifications tend to avoid disrupting sequences coding for protein domains; rather, they completely include or remove domains (Artamonova and Gelfand, 2007; Colantoni *et al.*, 2013). However, major changes can also occur, such as the extreme cases of 12 genes in *Drosophila melanogaster* that are able to code for two (or more) proteins with no overlapping residues, i.e. no coding exon is shared between the alternative mRNAs (Misra *et al.*, 2002).

Another fascinating example of increased protein diversity through AS comes from studies of the *down syndrome cell adhesion molecule* (*DSCAM*) gene in *D. melanogaster*, which codes for protein receptors located on the surface of neurons. Extensive protein diversity is crucial to nervous system development because it provides a unique signature that avoids self-crossing of axons (Celotto and Graveley, 2001; Venables *et al.*, 2012). *DSCAM* is able to provide this protein diversity by virtue of AS. This gene has the potential to produce up to 38,016 mRNA isoforms, each encoding a different protein (Schmucker *et al.*, 2000). This impressive number is nearly three times greater than the total number of protein coding genes in *D. melanogaster*! The gene structure of *DSCAM* is a key element in contributing to this stunning potential. It has 20 constitutive exons and groups of 12, 48, 33 and 2 mutually exclusive exons, allowing different combinations of exons in the final mRNA. In each mutually exclusive group of exons, competing secondary structures allow only one exon to be included at a time, which varies among tissues and developmental stages (Celotto and Graveley, 2001; Graveley, 2005; Hemani and Soller, 2012).

Protein sub-cellular localisation can also be modified by AS, which adds or removes mRNA regions coding for localisation signals. As a result, different protein isoforms can go to different cell compartments, where they may perform different activities. An example is the mammalian *lipin 1* gene, involved in lipid metabolism. *Lipin 1* is alternatively spliced and produces cytoplasmic and nuclear proteins, known as lipin 1α and lipin 1β, respectively (Kelemen *et al.*, 2013). Lipin 1α has a phosphatidate phosphatase activity, whereas nuclear lipin 1β co-activates transcription of genes that enhance lipid storage (Reue and Brindley, 2008). The *lipin 1β* mRNA isoform has an alternative exon (exon 6), absent on *lipin 1α*, that contributes to nuclear localisation of the protein. Since lipin 1β protein amplifies lipid storage signals, regulating splicing of exon 6 on *lipin 1* pre-mRNA is crucial to lipid metabolism. This exon is controlled by the splicing regulator SFRS10, which binds to the 5' ss of exon 6 and represses splicing. This results in exon 6 skipping and consequently lower levels of *lipin 1β* (Pihlajamäki *et al.*, 2011). Interestingly, SFRS10 is down-regulated in obese patients, leading to an increase of *lipin 1β* and a subsequent increase in lipid storage (Pihlajamäki *et al.*, 2011).

Finally, AS can change enzymatic activity and the best studied examples are protein kinases. Pre-mRNAs coding for these enzymes undergo AS that affect their active sites, resulting in generation of proteins that are inactive, constitutively active or have reduced activity (Kelemen *et al.*, 2013). Additionally, AS can modify protein sequences that decrease protein stability, making them more susceptible to degradation. For example, exon skipping in the *migration and invasion inhibitory protein* (*MIIP*) pre-mRNA changes the coding region of the C-terminus, which codes for a protein that is degraded soon after translation (Song *et al.*, 2005).

1.4.2 – Function of AS: regulation of mRNA and protein levels

In addition to increased proteome diversity, AS can fine-tune protein expression levels. This quantitative control can be exerted due to AS events in either the coding or untranslated regions of pre-mRNAs and hinders mRNA nuclear export, modifies mRNA translatability and stability. In eukaryotes, this control of expression is essential, being involved in several situations like stress and development (Staiger and Brown, 2013). Such AS is especially crucial when altered protein levels are needed despite opposing transcriptional behaviour (Dutertre *et al.*, 2011).

The AS effect on mRNA translation usually occurs through inclusion of *cis*-elements such as RNA secondary structures that increase or reduce translation efficiency (Jacobs *et al.*, 2012). One of the strongest effects on mRNA translatability is the removal or insertion of an upstream translation initiation codon (uAUG), which creates an upstream open reading frame (uORF) in the 5' untranslated region (UTR). According to the ribosome scanning model, uAUGs interfere with or prevents translation initiation of downstream AUGs (Kozak, 2005). A genome-wide analysis in *Saccharomyces cerevisiae* showed that transcripts with uORFs have significantly lower levels of their corresponding proteins than transcripts without uORFs (Yun *et al.*, 2012). Regulation of the inclusion of uORFs through AS is crucial to regulating many eukaryotic mRNAs. Essentially, uORFs prevent harmful overproduction of proteins with regulatory functions (Kochetov *et al.*, 2008). For example, in mammalian spermatogenic cells, almost all genes are overexpressed, which would cause deleterious phenotypes if they were all efficiently translated (Kleene, 2001; Kochetov *et al.*, 2008). To avoid protein overexpression, pre-mRNAs of these genes undergo AS that retain uAUGs (uORFs), which does not occur in somatic cells (Kleene, 2001). This AS control reduces mRNA translation to healthy levels, relieving the consequences of protein overexpression.

Alternative uAUGs/uORFs can increase proteome diversity. In this case, uAUGs in the same reading frame increase the number of translation start sites. Alternative translational start sites create protein isoforms that differ at their N-terminus, which can have an impact on the function of the protein (Kochetov *et al.*, 2008). Additionally, in some cases the protein translated from a uORF can be functional, thereby further increasing the diversity of proteins from such genes (Calvo *et al.*, 2009).

However, uORFs can also hinder translation of the functional ORF completely or even cause the ribosome to stall on the mRNA. These mRNAs are considered 'non-functional' because they cannot be translated into functional proteins. They are easily recognised and degraded by complexes such as the No-Go Decay, in the case of mRNAs with stalled ribosomes, or Nonsense Mediate Decay (NMD) pathway, a system that degrades most 'non-functional' mRNAs (McGlincy and Smith, 2008; Harigaya and Parker, 2010).

The most abundant class of 'non-functional' mRNAs which are subjected to degradation through the NMD process are those containing premature termination codons (PTCs) in the CDS. Such PTCs can arise from mutations in the DNA, errors of transcription or splicing, RNA editing, or regulated AS. PTC-containing mRNAs can code for truncated and potentially harmful proteins and are therefore mostly removed from the cell. The precise and brilliant mechanism by which the NMD machinery discriminates between functional mRNAs and most uORF- and PTC-containing mRNAs varies among eukaryotes, but some of the core components are conserved (Conti and Izaurralde, 2005; Nyikó *et al.*, 2013). Higher eukaryotes have proteins bound to exon–exon junctions after splicing, known collectively as the Exon Junction Complex (EJC). These EJCs are normally removed by the first translating ribosome (Chang *et al.*, 2007). In PTC-containing mRNAs, EJCs > 50–55 nt downstream of the PTC enhance the activation of NMD (McGlincy and Smith, 2008; Nyikó *et al.*, 2013). In this EJC-dependent NMD pathway, up-frameshift (UPF) proteins UPF2 and UPF3 are part of the EJC and interact with the ribosome which

is paused at the PTC and recruits translation termination factors. UPF1 is then recruited as the first step in degradation of the transcript (Chang *et al.*, 2007). In yeast, the NMD protein UPF1 also promotes proteolysis of the nascent polypeptide, ensuring that the unsafe protein fragment does not interfere in cellular processes (Kuroha *et al.*, 2009).

Functional mRNAs are usually not recognised by the NMD machinery because their stop codons are frequently present in the last exon (Nagy and Maquat, 1998). A second mechanism to trigger NMD is now recognised in a wide range of species, which is NMD due to long 3' UTRs (Kalyna *et al.*, 2012). In normal mRNAs, the ribosome reaches the authentic translation stop codon where it recruits the translation release factors and an interaction occurs between the ribosome, eRF and poly(A) binding proteins (PABP) bound to the poly(A) region. This interaction is the signal to tell the ribosome that it has reached the end of the mRNA and to release the mRNA and polypeptide chain. If a PTC occurs in the transcript, depending on where this occurs, the interaction with PABP proteins will be prevented or impaired (e.g. by an unusually long 3' UTR), leading to recruitment of UPF proteins and NMD (Rebbapragada and Lykke-Andersen, 2009). Additionally, an mRNA that contains uORF can also be subject to NMD (Nyikó *et al.*, 2009). The ribosome can begin translation at a uAUG and when it reaches the uORF stop codon it can trigger NMD by the long 3' UTR or EJC-dependent mechanism. Finally, functional or alternatively spliced mRNAs with 3' UTR introns can also be degraded by NMD if the splice site junction/EJC is > 55 nt from the authentic stop codon (McGlincy and Smith, 2008).

Alternative splicing can generate PTC-containing transcripts which are targeted by NMD. This process has been termed Regulated Unproductive Splicing and Translation (RUST) (Lareau *et al.*, 2007). One example is the SR and hnRNP proteins that can regulate their own splicing so as to produce mRNAs targeted for NMD (McGlincy and Smith, 2008). This negative feedback control allows for crucial homeostatic or oscillatory levels of these splicing factors (example in Section 1.8.1) (McGlincy and Smith, 2008).

There are also ways by which AS can regulate mRNAs. For example, AS can regulate the presence of miRNA-binding sites on the mRNA, consequently affecting mRNA stability (Kelemen et al., 2013). Additionally, AS can affect mRNA sub-cellular localisation, targeting it to different compartments, depending on the stimulus (Kelemen *et al.*, 2013).

In summary, transcript and protein levels can be altered co- or post-transcriptionally through AS. This is most often achieved by inclusion of uORFs or PTCs in the mRNA, which typically affects mRNA translation and stability, respectively. Regarding mRNA stability, NMD is the most common mechanism that degrades PTC-containing transcripts, avoiding production of proteins with partially disrupted domains, which could act as dominant negatives. Overall, quantitative control of gene expression through AS is crucial to physiological and developmental processes. Therefore, several mRNAs previously regarded as 'non-functional' or having 'splicing errors' actually have a key role, for their regulated synthesis helps to control protein levels and, consequently, cell responses.

1.4.3 –Alternative splicing in diseases

Pre-mRNA splicing must occur in a very precise and controlled way if a gene is to be expressed correctly. In humans, most erroneously spliced transcripts are removed by the cell but some remain, and can constitute either the cause or consequence of diseases (Tazi *et al.*, 2009). Disease-related AS events are mostly caused by point mutations and deletions, but they also occur when there are changes in the cell microenvironment such as low pH or hypoxia (Kelemen *et al.*, 2013). For example, AS of Cyr61, coding for a matricellular protein with proangiogenic capabilities, is induced under hypoxia, which has potential biological consequences in breast cancer progression (Hirschfeld *et al.*, 2009). Interestingly, of

all the mutations causing diseases in humans, 50% affect *cis*- and *trans*-acting splicing elements, affecting AS regulation (Wang and Cooper, 2007). Aberrant alternative transcripts are more likely to cause diseases in tissues with a complex proteome, for instance neurons, muscles and cells of the immune system (Barta and Schümperli, 2010).

In general, there are two main types of mutations that cause disease through effect on the production of specific proteins. Firstly, some mutations introduce PTCs or affect *cis* splicing signals, which can be either splice sites (15% of diseases) or binding sites for splicing factors, altering splicing patterns. Examples of diseases caused by such mutations are β-thalassemias and Duchenne muscular dystrophy (Tazi *et al.*, 2009). The second main group of mutations are mutations which affect splicing factor genes themselves. Such mutations affect the abundance or activity of splicing factors which can affect the splicing/AS of many downstream genes. Examples of diseases caused by such mutations are retinitis pigmentosa (Wilkie *et al.*, 2008), frontotemporal lobar dementias and spinal muscular atrophy (Tazi *et al.*, 2009). In addition, some mutations affect intricate regulation of specific genes. One such example is Prader-Willi syndrome where AS likely causing a disease involves a snoRNA. The prevalent role of snoRNA is to guide rRNA processing but during evolution some of them lost complementary sequence to rRNAs and developed complementarity to mRNAs instead (Kishore and Stamm, 2006; Bazeley *et al.*, 2008; Ender *et al.*, 2008; Saraiya and Wang, 2008). The human snoRNA *HBII52* has complementarity to exon 5 of the brain-specific *serotonin 2C receptor* (*5HT2CR*) gene. This interaction, at the pre-mRNA level, represses an internal 3' ss of exon 5, which would otherwise be used to produce a non-functional receptor (Kishore and Stamm, 2006). Therefore, the presence of *HBII52* snoRNA is essential for expression of functional 5HT2CR. In Prader-Willi syndrome patients, most snoRNAs from the *HBII* family are not present due to a deletion, in-

cluding *HBII52*. As a consequence, no functional receptor is expressed, which ultimately causes the clinical features seen in patients with Prader–Willi syndrome (Tazi *et al.*, 2009).

The involvement of AS in cancer is greatly studied. A large number of splicing alterations occur in tumourigenesis and despite most of them resulting from the malignant phenotype, some AS events are required for cancer formation and development (Tazi *et al.*, 2009). In fact, all steps of cancer progression depend on AS, including cell cycle control, survival, and metastasis (David and Manley, 2010). An interesting example is the AS of apoptotic genes in cancer cells. AS of these genes often results in isoforms with opposing roles that either promote or prevent cell death (Schwerk and Schulze-Osthoff, 2005). This phenomenon was initially observed in the *b-cell lymphoma x* (*BCLX*) gene in humans. The *BCLX* pre-mRNA can be alternatively spliced into *BCLX long* ($BCLX_L$) or *BCLX short* ($BCLX_S$) transcripts, coding for proteins with anti- and pro-apoptotic activities, respectively. The long isoform is obtained when the alternative 5' ss of intron 2 is used, lengthening exon 2 to encode an additional 63 amino acids (Boise *et al.*, 1993). AS in cancer cells increases production of the long isoform, subsequently increasing cell survival. This can be used as a therapeutic target, as artificially synthesised oligonucleotides that bind to the alternative 5' ss used in $BCLX_L$ blocks splicing cleavage at this site (David and Manley, 2010). As a consequence, splicing of the upstream 5' ss (used in the $BCLX_S$ transcripts) is enhanced, inducing apoptosis (Mercatante *et al.*, 2002).

Individual and genome-wide studies of disease-related AS have assisted in deciphering the splicing code. This knowledge allows the development of splicing-based therapeutic approaches. These therapies can be achieved by using small-molecules, antisense oligonucleotides, or introducing trans-splicing events that reverse splicing of target genes or create new splicing patterns to treat genetic disorders and alleviate symptoms (Wang and

Cooper, 2007). Therefore, AS is not only the cause or consequence of many diseases, but also a solution.

1.4.4 – Alternative splicing in developmental control

AS has a crucial role in the development of multicellular eukaryotes. In fact, almost all genes involved in metazoan development are alternatively spliced (Lopez, 1998). These alternatively spliced forms are expressed at particular times and in specific tissues, and they either have significant differences in function that control major developmental decisions or provide subtle but selectively advantageous fine-tuning roles in developmental control (Lopez, 1998).

AS controls genes in eukaryote development from before birth/germination until after death (Kelemen *et al.*, 2013), but the majority of AS changes occur when there is a pronounced change in morphology, for instance the transition from embryo to larva (Irimia *et al.*, 2009). In humans, AS is especially crucial to genes that contribute to neuronal differentiation (Grabowski, 2011) and testis development (Yeo *et al.*, 2004), but it also plays a significant role in the epithelial-to-mesenchymal transition (Biamonti *et al.*, 2012) and heart development (Xu *et al.*, 2005), among many others.

The best example of developmental control through AS comes from studies of the sex determination pathway in *D. melanogaster*. In female flies, this pathway begins with the presence of two X chromosomes that confer early expression of the active, full-length sex-lethal (Sxl) protein (Black, 2003; Venables *et al.*, 2012). Sxl is a transcription factor and splicing regulator, which also regulates its own splicing in a positive feedback loop. It binds near the alternative exon 3 of the *SXL* pre-mRNA, which contains a premature stop codon. This binding leads to exon 3 skipping, which allows the full-length Sxl protein to be expressed (Black, 2003). Subsequently, active Sxl represses genes of male organ develop-

ment and activates genes dictating female organ development. Conversely, males are not able to produce early active Sxl nor skip exon 3 of the same gene, so that only truncated and inactive Sxl protein is present (Black, 2003; Venables *et al.*, 2012). In the absence of active Sxl, the gene expression cascade for male development is initiated. The continuation of the sex determination pathway in both males and females has more examples of gene AS control, for instances *transformer* and *doublesex* (Black, 2003; Venables *et al.*, 2012).

1.5 – Alternative splicing in plants

Although current AS knowledge indicates that AS is essential for higher eukaryotes in general, most of this knowledge comes from studies in metazoans. In fact, plant AS was considered rare until a decade ago (Reddy, 2007). Recent work confirms that it is also common and essential in plant gene expression, generating complex transcriptome and proteome diversity (Reddy, 2007; Syed *et al.*, 2012). However, the magnitude of the latter role in the plant genome remains to be uncovered (Severing *et al.*, 2009; Severing *et al.*, 2011). The recently discovered high frequency of AS in Arabidopsis (Marquez *et al.*, 2012) has also been described in other plant species, including crops (Staiger and Brown, 2013).

1.5.1 – Characteristics of AS in plants compared to animals

A number of AS differences are observed between plants and animals and the largest difference is related to the frequency of different AS types (Table 1.1). The most common type of AS event in plants is intron retention, which is rare in animals. In contrast, alternative exons are relatively rare in plants, but the most common type of AS in animals. Accordingly, intron-definition is significantly more common in plants (Xiao *et al.*, 2007), whereas exon-definition is more prevalent in animals (McGuire *et al.*, 2008), thus elucidating their preference for type of AS. The evolutionary reason for these differences are poorly

understood but may reflect both differences in gene structure and technical consequences. For example, the majority of plant introns are relatively short (e.g. 80% of Arabidopsis introns are between 80 and 120 nt) and when RNA is extracted for cDNA synthesis, transcripts which have not been completely spliced might enter EST databases as cases involving IRs. In animals, where many introns are thousands of nt in length, such transcripts are not captured. In addition, it is reasonably clear that alternative exons allow for greater protein diversity than intron retention, like the example of alternative exons in the *DSCAM* gene (detailed in Section 1.4.1) (McGuire *et al.*, 2008). In plants, greater protein diversity is easily achieved through whole or partial genome duplications, which are not common in animals, as well as gene duplications and therefore reduce the need for a high frequency of alternative exons in plants to increase protein diversity (Kim *et al.*, 2008a).

Table 1.1: Extent of alternative splicing in plants and animals.

Organism	AS	Intron retention [c]	Alternative exon [c]	Alt. 3' and/or 5' ss [c]	Adds PTC [c]	Reference
Arabidopsis	61% [a]	40%	3%	31%	50%	(Reddy, 2007; Marquez *et al.*, 2012)
Rice	48% [b]	54%	14%	33%	48%	(Reddy, 2007; Lu *et al.*, 2010a; Zhang *et al.*, 2010)
Human	95% [a]	5%	58%	37%	46%	(Reddy, 2007; Pan *et al.*, 2008; Saltzman *et al.*, 2008)
Mouse	57% [b]	2%	16%	8%	43%	(Pan *et al.*, 2006; Chacko and Ranganathan, 2009)

[a] Approximate percentage relative to total number of intron-containing genes.

[b] Approximate percentage relative to total number of genes.

[c] Approximate percentage relative to total number of AS events.

Additionally, different *cis*- and *trans*-acting elements are likely to be involved in AS variation between plants and animals. Some possible reasons are: i) plant introns are richer in uracil (U) and are significantly smaller when compared to vertebrates (Reddy, 2007; Rogozin *et al.*, 2012); ii) the polypyrimidine tract is a strong splicing signal in animals but

intermediate in plants (Schwartz *et al.*, 2008); and iii) plants have a higher diversity of splicing-related proteins compared to metazoans (Reddy and Shad, 2011). The presence of more than one splicing-related homologue in the Arabidopsis genome is due to several duplication events. These close protein homologues might therefore have acquired different regulatory splicing activities in evolution. This occurrence is particularly evident among the SR proteins genes (Barta *et al.*, 2012). For example, while there is a single ASF splicing factor in humans, Arabidopsis has four members of the SF2/ASF-like splicing factor family, with distinct expression patterns and activities (Barta *et al.*, 2012).

There are also several AS similarities between plants and animals. For example, the frequency of 5' (GU) and 3' (AG) ss usage and their consensus sequences are relatively similar among higher eukaryotes (Reddy, 2007; McGuire *et al.*, 2008). Additionally, alternative 3' ss are used approximately two-fold more often than alternative 5' ss in most plants and animals (Marquez *et al.*, 2012; Walters *et al.*, 2013). Thirdly, plants and animal genes have more AS events in the 5' UTR than in the 3' UTR, which can affect mRNA transport, translation and stability (Reddy, 2007). Another example of AS similarity is regarding PTC-containing mRNAs. Lastly, about 50% of alternative splicing events in both plants and animals introduce PTCs, which potentially target the mRNA to NMD or increase protein diversity (Section 1.4.2) (Reddy, 2007).

1.5.2 – Function of AS in plants

AS was first described in plants many years ago but over the last 5 or so years there has been an explosion of studies demonstrating the widespread natures of AS with involvement in many aspects of physiology, development and stress responses (Reddy, 2007; Syed *et al.*, 2012; Staiger and Brown, 2013). The first AS event identified in plants was in the *RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE* (*RUBISCO*) *ACTI-*

VASE gene of spinach and Arabidopsis (Werneke *et al.*, 1989). AS of *RUBISCO ACTIVASE* pre-mRNA generates two mRNA isoforms that code for active protein isoforms differing only at the carboxyl terminus. This difference allows only the large isoform to be redox regulated (Zhang *et al.*, 2002).

DNA mutations affecting AS can contribute to plant adaptation and evolution and crop improvement. One well-known example is in the *WAXY* (*WX*) gene in rice, which encodes a granule-bound starch synthase that increases amylose content in the endosperm (Larkin and Park, 1999). Higher levels of amylose interfere with starch hydrolysis and impair rice palatability, but have beneficial effects on human health because they prevent excessive sugar release (Reed *et al.*, 2013). Some rice cultivars have a functional WX protein and consequently high levels of amylose, whereas other rice cultivars have a mutation that reduces the level of functional WX proteins, decreasing amylose content. This mutation is a G-to-T polymorphism at the 5' ss of intron 1 in the *WX* gene that reduces splicing efficiency of this intron (Cai *et al.*, 1998; Hirano *et al.*, 1998). As a result, splicing errors occur, including retention of intron 1, which introduces uORFs that reduce mRNA stability and translatability (Cai *et al.*, 1998; Isshiki *et al.*, 1998; Larkin and Park, 1999). Therefore, a single-nucleotide polymorphism in the *WX* gene allows for AS events that reduce WX protein levels, having a significant impact on rice grain traits.

As sessile organisms, plants are strongly influenced by environmental changes in ambient light, temperature, water and salt content in the soil, among other abiotic stress factors. AS plays an important role in plant resistance to abiotic stress. For example, low temperature induces AS in many genes (Staiger and Brown, 2013), including the *INDETERMINATE DOMAIN 14* (*IDD14*) gene, a transcription factor in Arabidopsis. *IDD14* undergoes AS that potentially produce two protein isoforms: the functional IDD14α that activates starch degradation, and IDD14β, which is expressed only in cold conditions (4 °C)

and is a competitive inhibitor of the former (Seo *et al.*, 2011). During cold nights, Arabidopsis plants expressing both transcripts have lower rates of starch degradation and accumulate biomass. Contrastingly, during warmer nights (23 °C) plants do not express enough IDD14β protein to inhibit active IDD14α, degrade starch during the night and fail to accumulate biomass (Seo *et al.*, 2011).

In addition to plant resistance to abiotic stress, AS plays an important role in plant defence responses against pathogens. There is evidence of AS in several defence genes and most research progress comes from studies of *RESISTANCE* (*R*) genes (Gassmann, 2008). *R* genes are responsible for recognising pathogen avirulence factors and triggering defence responses (Staiger *et al.*, 2013). Most *R* genes encode a nucleotide binding site (NBS) involved in signalling and a leucine-rich repeat (LRR) domain that controls protein–protein interactions (Marone *et al.*, 2013). A well-known example of an *R* gene regulated by AS is the tobacco *N* gene, which is responsible for resistance against the viral pathogen tobacco mosaic virus (TMV). In addition to NBS-LRR domains, the N protein has a Toll/interleukin 1 receptor (TIR) domain involved in signal transduction (O'Neill, 2000). The *N* gene produces two transcripts by means of AS, a short (N_S) transcript that is more prevalent in healthy plants and encodes the full-length protein, and a long (N_L) transcript that predominates 4–8 h after TMV infection and contains an alternative exon normally within intron 3 (Dinesh-Kumar and Baker, 1999). This alternative exon shifts the protein ORF, resulting in a truncated protein that lacks 13 of the 14 LRR domains (Reddy, 2007). It is not known yet if N_L is translated or not, but the AS that produces it is required to confer complete resistance to TMV. Therefore, AS of the tobacco *N* gene is essential to disease resistance because it regulates the correct ratio of N_S and N_L at the right time (Dinesh-Kumar and Baker, 1999; Takabatake *et al.*, 2006).

There are several cases of AS affecting protein localisation in plants. One example is the *BWMK1* gene in rice, a member of the MITOGEN-ACTIVATED PROTEIN KINASES (MAPK) family. *OsBWMK1* pre-mRNA is alternatively spliced and produces transcripts that code for proteins with different sub-cellular localisations (Koo *et al.*, 2007). The nuclear OsBWMK1 protein isoform is normally expressed at low levels but its expression is induced under stress conditions. Moreover, defence signalling-related molecules induce translocation of cytoplasmic OsBWMK1 isoforms to the nucleus (Koo *et al.*, 2007). In the nucleus, OsBWMK1 proteins mediate defence responses by activating expression of defence genes (Koo *et al.*, 2009).

AS controls spatial and temporal expression of numerous genes involved in plant development (Iida *et al.*, 2004; Staiger and Brown, 2013). In Arabidopsis, a recent tissue-specific AS isoform was described for the *YUCCA4* gene, which codes for a flavin-dependent monooxygenase involved in auxin biosynthesis (Kriechbaumer *et al.*, 2012). *YUCCA4* is expressed in all plant tissues and the fully spliced mRNA codes for a cytoplasmic protein. In flowers, however, a different AS event occurs, which produces mRNAs coding for an additional C-terminal transmembrane domain (TMD) that enables the translocation of the alternative YUCCA4 protein to the Endoplasmic Reticulum membrane (Kriechbaumer *et al.*, 2012). Auxin is a major growth hormone in plants and compartmentalisation of auxin biosynthesis, by means of tissue-specific *YUCCA4* tissue-specific AS is crucial to plant development (Kriechbaumer *et al.*, 2012). The above examples illustrate some of the processes in which AS is involved and some of the functional consequences of AS. For more comprehensive collection of AS in plants see Staiger and Brown (2013).

1.6 – The circadian clock

Living organisms have had to cope with light/dark and warm/cold cycles every day for the past 3.5–4 billion years (Nisbet and Sleep, 2001). The rhythm of these changes, explained by the Earth's rotation, allowed the development of a mechanism that anticipates such changes. This mechanism, known as the circadian clock (from Latin '*circa diem*', meaning: approximately a day), can organise the physiology and behaviour of most organisms to optimise their fitness during both day and night (Green *et al.*, 2002; Okamura, 2004; Chen and McKnight, 2007).

In eukaryotes, the circadian clock regulates diverse processes, from the cell cycle to locomotor activity (Stoleru *et al.*, 2004b; Chen and McKnight, 2007; Dibner *et al.*, 2010), and is so fundamental that disruptions in clock rhythm can cause cancer and various other disorders (Dodd *et al.*, 2005; Dubrovsky *et al.*, 2010; Maury *et al.*, 2010; Shi *et al.*, 2013). The diversity of processes controlled by the circadian clock further reflects the number of genes under its control. Numerous genes have their expression clock-regulated (Hazen *et al.*, 2009; Hughes *et al.*, 2012), but only a few genes actually establish and maintain the circadian clock. The core components of the circadian clock consist of a complex network of genes, which are known as clock genes. They are mainly regulated by regulatory feedback loops at the transcriptional, epigenetic, translational, post-translational, metabolic, and the co- and post-transcriptional levels (Gallego and Virshup, 2007; Sanchez *et al.*, 2011; Hughes *et al.*, 2012).

Circadian rhythm is generated endogenously and the expression of most clock genes is not constant, but oscillates in a rhythmic fashion, generating a rhythm with a period of approximately 24 hours. This 24-hour period often persists even when the organisms are subjected to special environmental conditions such as when seasons change or non-oscillating (free-running) conditions (McClung, 2006). In these cases, clock genes modify

their expression peak time (phase) and level (amplitude) (Figure 1.5), without severely disturbing the period, to synchronise (match) their clock to that of the external cue, a process called entrainment. Clock entrainment is optimised by the regulation of input genes that sense environmental time cues (Zeitgebers). Common examples of zeitgebers are light, temperature, DNA damage and food supply (Ashmore and Sehgal, 2003; Millar, 2004; James *et al.*, 2008; Oklejewicz *et al.*, 2008; Challet, 2013). Clock input genes modify the expression of core clock genes, resetting the clock to the new condition so that metabolic output processes (e.g. gene expression, protein modification and changes in metabolite levels) are adjusted accordingly. Therefore, the clock always ensures that the organism profits optimally from environmental changes. More details of the clock mechanism, including its evolution and the role of AS in clock regulation, are given below.

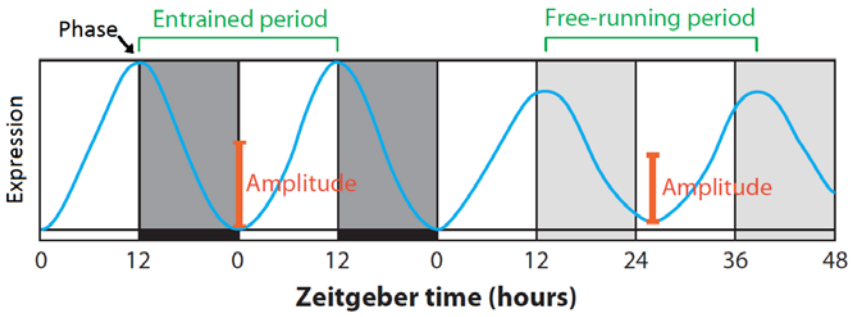


Figure 1.5: Putative clock gene expression in light/dark cycles (entraining conditions) and constant light (free-running conditions) (Figure modified from Harmer, 2009). Phase is the time of the day when the gene expression reaches its maximum (expression peak). Amplitude is half of the peak-to-trough distance. Period is the time to complete one full cycle.

1.6.1 – Evolution of the circadian clock

Around 2.5–5 billion years ago, extreme levels of ultraviolet irradiation were a serious problem for primitive marine organisms and avoiding it was critical to survival. A cyclic 'escape from light' mechanism was essential and it might have driven the origin and

evolution of a 'protoclock' during the Precambrian era (Pittendrigh, 1965; Tauber *et al.*, 2004). Protection from sunlight was probably achieved by vertical migrations to lower ocean depths and/or activation of light-sensing enzymes that repair UV-induced DNA damage, such as photolyases (Tauber *et al.*, 2004; Uchida *et al.*, 2010). Additionally, it was probably crucial to carry out certain steps of the cell cycle, mainly DNA synthesis, only during the night (Hut and Beersma, 2011).

Two other factors might have contributed to the development and evolution of the circadian clock: energy supply and oxygen levels. Regarding the former, photosynthetic organisms developed a rhythmic energy-storage behaviour during the day, later used during the night, allowing for increased fitness (Hut and Beersma, 2011). As for the latter, with increasing oxygen levels, certain organisms, like yeast, developed both oxidative and reductive respiratory cycles, which required cyclic DNA defence mechanisms against oxidative damage (Tauber *et al.*, 2004).

Therefore, organisms developed different circadian metabolic rhythms that favoured their fitness during day and night. The circadian clock presumably originated, through a succession of small evolutionary steps, because it efficiently organised these chaotic combination of daily behaviours (Tauber *et al.*, 2004). The centralised clock control of most metabolic processes together with the clock's ability to measure time, through a self-sustained oscillation, have ultimately allowed for anticipation (planning) of environmental changes (Hut and Beersma, 2011).

The low conservation of clock proteins among plants, fungi, metazoans, and some prokaryotes suggests it originated independently in the ancestors of each one of these lineages (Young and Kay, 2001). Despite multiple clock origins, all organisms have adopted an autoregulatory genetic network that maintains a self-sustained clock oscillation. This network can be rather simple or extremely complex. Higher eukaryotes, in particular, have

a highly complex clock regulation and function, driven by gene duplication events (Tauber *et al.*, 2004).

1.6.2 – The cyanobacterial clock

In the unicellular world, the most interesting organisms for studies of the clock mechanism are cyanobacteria. They are among the oldest organisms on Earth and have not changed much since their origin, possibly representing the only direct link with the ancient world (Tauber *et al.*, 2004). The cyanobacteria *Synechococcus elongatus* is the prokaryotic model system for clock studies and has provided great insights into the molecular function and components of the circadian clock (Mackey *et al.*, 2011). The rather simple core clock of *Synechococcus* is composed of three proteins: KaiA, KaiB, and KaiC (Ishiura *et al.*, 1998). KaiC, the oldest member (Tauber *et al.*, 2004), has ATPase, autophosphatase and autokinase activities (Dong *et al.*, 2010). Autophosphorylation of KaiC is slow and temperature-compensated, and it occurs upon stimulation by KaiA, but KaiC spontaneously dephosphorylates when bound to KaiB (Villarreal *et al.*, 2013). KaiC transcription, translation, and post-translational modifications are cyclic, repeating every 24 hours both *in vivo*. Remarkably, KaiC post-translational modifications are also cyclic *in vitro* (Nakajima *et al.*, 2005; Kitayama *et al.*, 2008). KaiABC interactions ultimately mediate rhythmic and topological changes in the status of the cyanobacterial chromosome, regulating the cell cycle and transcription of all genes in *S. elongatus* (Woelfle and Johnson, 2006; Dong *et al.*, 2010).

1.6.3 – The *Neurospora crassa* clock

The filamentous fungus *Neurospora crassa* has an extensively analysed clock system (Lakin-Thomas *et al.*, 2011). It serves as an attractive model due to a visible manifestation

of its internal clock: rhythmic production of alternating zones of mycelia and conidia every 24 hours, forming a linear pattern down a glass tube (Figure 1.6) (Baker *et al.*, 2012). This growth pattern, controlled by the circadian clock, allows for simple characterisation of core clock mutants and identification of clock components.

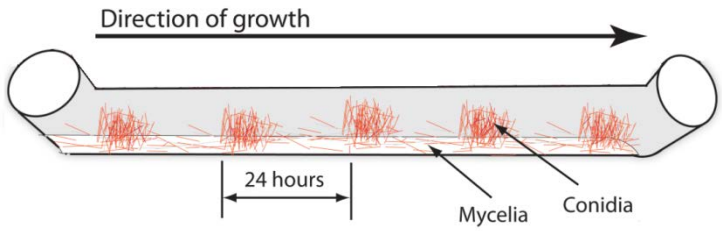


Figure 1.6: *Neurospora* growth assay for clock studies (Figure modified from Baker *et al.*, 2012). Glass tubes containing growth media are inoculated at one end and mycelia grow down the tube in a linear fashion. Every 24 hours a developmental switch occurs: mycelia growth alternates with the production of asexual conidia, a process controlled by the circadian clock.

The first core clock gene discovered in *Neurospora* was *frequency* (*FRQ*) (Gardner and Feldman, 1980; Baker *et al.*, 2012). Transcription of *FRQ* is induced by the white collar complex (WCC), which is formed by the transcription factors white collar 1 (WC1) and white collar 2 (WC2) (Crosthwaite *et al.*, 1997; Baker *et al.*, 2012). Upon translation, FRQ is stabilised by forming homodimers and binding to FRQ-interacting RNA helicase (FRH) (Dunlap *et al.*, 2007). This complex enters the nucleus, allowing FRQ to promote WCC phosphorylation, which results in WCC removal from the *FRQ* promoter. *FRQ* transcription, consequently, is decreased (Baker *et al.*, 2012). At a later time, FRQ becomes hyperphosphorylated, which induces FRQ ubiquitination and degradation (He *et al.*, 2003). The cycle then reinitiates.

Regulated AS of *FRQ* is extremely important in the function of the *Neurospora* circadian clock. Temperature regulates AS of an intron located in the 5' UTR, which con-

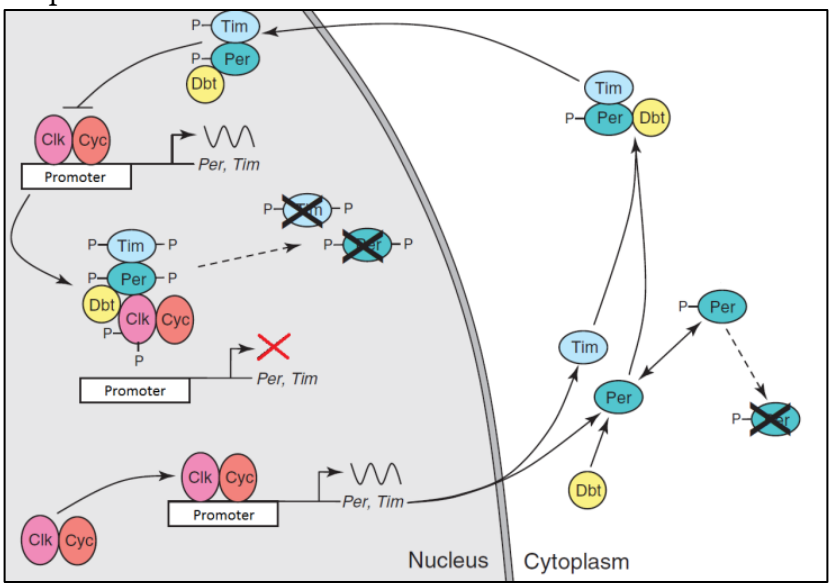
tains an in-frame uORF that gives rise to a longer isoform (FRQ_L) (Colot *et al.*, 2005). This regulation is important for fine-tuning the circadian mechanism because it allows robust oscillations under a wide range of temperatures. Furthermore, there is significant conservation of this AS event within the Sordariaceae family (including *Neurospora*), confirming the importance of AS for the proper regulation of the circadian clock (Colot *et al.*, 2005; Sanchez *et al.*, 2011).

1.6.4 – The *Drosophila melanogaster* clock

The *D. melanogaster* molecular clock is one of the best understood clock mechanisms. Studies of this model have provided several ground-breaking discoveries. For example, the first study of a clock mutant was for the *period* (*PER*) gene in *D. melanogaster* and it led to the expansion of clock genetics research (Konopka and Benzer, 1971; Zhang and Kay, 2010). Moreover, further analyses of the same gene allowed for the first identification of AS in a clock gene, described below (Majercak *et al.*, 1999; Sanchez *et al.*, 2011).

The *D. melanogaster* clock has two interlocked feedback loops, which function in the brain and a variety of peripheral tissues (Benito *et al.*, 2007). The *PER* gene operates at the core of a *PER/ TIM* loop (Figure 1.7A). During the light period, PER is phosphorylated by the double-time (DBT) kinase, which targets PER for degradation. During the night, phosphorylated PER is stabilised by forming a complex with timeless (TIM) (Yu and Hardin, 2006). Increased DBT-PER-TIM levels allows for TIM phosphorylation and entry of the DBT-PER-TIM complex into the nucleus. Subsequently, this complex removes transcription factors, mainly clock (CLK) and cycle (CYC) proteins, from *per* and *tim* promoters, repressing their transcription. At dawn, the photoreceptor cryptochrome (CRY) binds to TIM, resulting in their degradation, followed by degradation of PER (Hardin, 2005; Yu and Hardin, 2006).

A) *PER/TIM* loop



B) *CLK* loop

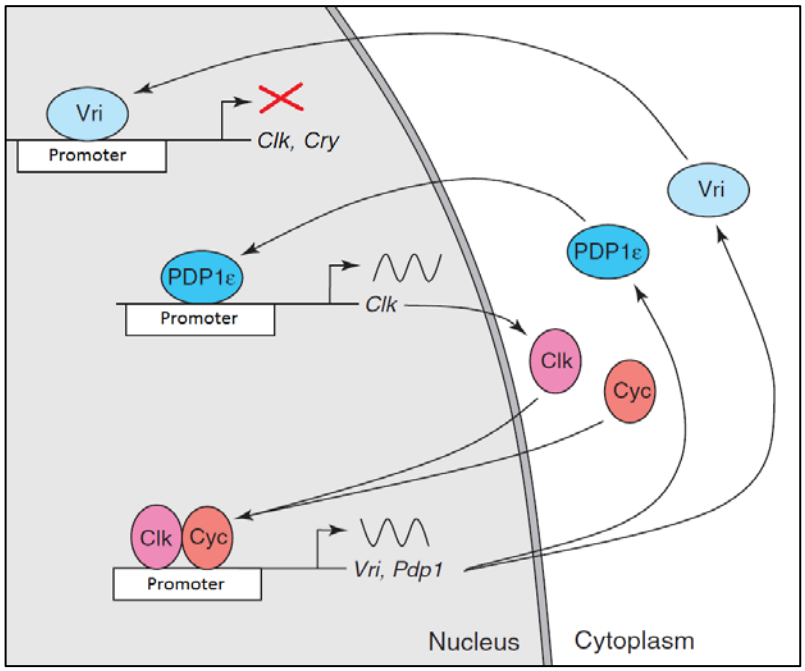


Figure 1.7: Simplified diagrams of the *D. melanogaster* clock (Figure modified from Hardin, 2005). A) *PER/TIM* feedback loop. P- represents protein phosphorylation; dashed lines and black X's mean protein degradation. B) *CLK* loop. In both diagrams, solid and dashed lines represent sequential steps in the feedback loops, which can be regulated by additional proteins that are not shown; wavy lines show mRNA expression, whereas red X's shows absence of their transcription.

The second feedback loop, known as the *CLK* loop (Figure 1.7B), is closely interlocked with the *PER/TIM* loop. CLK-CYC clusters activate the expression of *vrille* (*VRI*) and *par domain protein 1ε* (*PDP1ε*) (Yu and Hardin, 2006). As VRI accumulates in the nucleus during the mid to late day, it represses *CLK* and *CRY* transcription. Accumulation of PDP1ε near the end of the night, when VRI levels decline, allows it to bind *CLK* and *CRY* promoters and activate their expression, which closes the loop (Yu and Hardin, 2006).

Both *CLK* and *TIM* undergo AS at their 5′ UTRs, but their consequences are still unknown (Hughes *et al.*, 2012). The best example of AS in the *D. melanogaster* clock occurs in the *PER* gene. It produces two mRNA isoforms, one that retains a 3' UTR intron and another one that removes it (Majercak *et al.*, 1999). This AS is regulated by temperature, photoperiod, and other clock genes, affecting *PER* mRNA and protein levels. Changes in PER oscillations, by virtue of AS, improve seasonal adjustments in locomotor behaviour (Majercak *et al.*, 1999; Sanchez *et al.*, 2011). Interestingly, *PER* loss-of-function affects AS of several output genes, suggesting a key involvement of this circadian gene in regulating AS in *D. melanogaster* (Hughes *et al.*, 2012).

1.6.5 – The mammalian clock

The master mammalian circadian clock is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. It resets the internal time of organisms with the external light/dark cycles, thus synchronising the physiology and behaviour with the environment (Barclay *et al.*, 2012). Additional and different circadian clocks are present in the majority of peripheral tissues, e.g. liver clock (van den Pol and Dudek, 1993; Reppert and Weaver, 2001). Close interaction must exist between different central and peripheral clocks to maintain robust circadian rhythms of physiology and metabolism (Barclay *et al.*, 2012).

Mammalian circadian clock genes are generally conserved with those of *D. melanogaster* (Yu and Hardin, 2006). However, the mammalian clock is more complex and most genes are different and/or present in multiple versions. For example, while *D. melanogaster* has one *CLK* gene, mammals have both *CLK* and *neuronal PAS domain protein 2* (*NPAS2*) genes carrying out overlapping functions (Crumbley *et al.*, 2010). Additionally, mammals have two *CYC* genes, known as *brain muscle ARNT (aryl hydrocarbon receptor nuclear translocator)-like protein 1* and *2* (*BMAL1* and *BMAL2*), three *PER* genes, and two *CRYs*, which are the most important co-regulators of *PERs* other than *TIM* (Wijnen and Young, 2006). The function of *D. melanogaster* VRI and PDP1ε are carried out by different proteins in mammals: the nuclear receptor proteins REV-ERBα and RORα, respectively (Wijnen and Young, 2006; Yu and Hardin, 2006).

Regulation of mammalian core clock and output genes also involves AS. Several splice variants of *BMAL1* and *BMAL2* have been identified in human, mouse, and rat, but the functional consequences of these AS events are not fully understood (Schoenhard *et al.*, 2002). In particular, AS of *BMAL2* pre-mRNA can modify the N-terminal region of its encoded protein, increasing protein diversity, or even cause frameshifts that introduce PTC. *BMAL2* splice variants differ among tissues, possibly allowing different tissues to regulate CLK:BMAL2 heterodimer function (Schoenhard *et al.*, 2002). Recent studies in rats and mice revealed that the circadian clock further regulates AS of output genes in spatial- and temporal-dependent manners (McGlincy *et al.*, 2012; Shapiro-Reznik *et al.*, 2012). Together, AS studies of core clock and output genes add a novel dimension to the regulation of mammalian AS and clock genes.

1.7 – The plant (Arabidopsis) clock

As sessile organisms, plants rely on the circadian clock to match several physiological processes, such as leaf movement, immune responses and stomatal aperture according to the appropriate time of day (Harmer, 2009; Pruneda-Paz and Kay, 2010; Wang *et al.*, 2011a). As an example, just before dawn, there is a peak in the expression of transcripts encoding for products that protect the plant from ultraviolet radiation, which could be a great advantage to protect the cell from photo-damage during the impending daylight (Harmer *et al.*, 2000; Hotta *et al.*, 2007). Additionally, the expression peak of chlorophyll biosynthetic genes is organised to occur four hours after dawn by the circadian clock (Harmer *et al.*, 2000), optimising chlorophyll content and carbon fixation during the day (Dodd *et al.*, 2005). Defence responses are also stronger when the plant is most likely to be attacked (Wang *et al.*, 2011a; Goodspeed *et al.*, 2012). For example, plants synchronised with the feeding behaviour of herbivores are less susceptible to herbivore attack than out-of-phase plants (Goodspeed *et al.*, 2012).

1.7.1 - The framework of the Arabidopsis clock

All plant cells presumably contain a circadian clock, but this clock is not identical in all organs. The master plant circadian clock is located in the shoots, which parallels to the mammalian master clock SCN. The plant analogue of mammalian peripheral clocks, such as liver clock, is the root clock. Only a restricted set of genes are rhythmic in roots, where photosynthesis-related signals from the shoot can reset the root clock, but the reverse does not occur (James *et al.*, 2008). The general framework of the Arabidopsis clock, mostly based on experiments using whole seedlings, is comprised of several interlocking gene expression feedback loops (Figure 1.8) (Locke *et al.*, 2006; Zeilinger *et al.*, 2006; Harmer, 2010; Pokhilko *et al.*, 2010). The central loop is formed by *CIRCADIAN CLOCK*

ASSOCIATED 1 (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*) and *TIMING OF CHLOROPHYLL A/B BINDING PROTEIN 1* (*TOC1* - also known as *PSEUDO RESPONSE REGULATOR 1*, *PRR1*) (Alabadí *et al.*, 2001). CCA1 and LHY are closely related and partially redundant myeloblastosis (MYB) transcription factors that accumulate at dawn and bind to the promoter region of *TOC1*, inhibiting its expression. Recent studies suggest TOC1 is also responsible for reducing *CCA1* and *LHY* expression (Gendron *et al.*, 2012; Huang *et al.*, 2012; Pokhilko *et al.*, 2012).

During the morning, CCA1 and LHY play parallel roles in the central loop, further fine-tuning the clock. These genes induce expression of the transcriptional repressors PSEUDO RESPONSE REGULATOR 7 and 9 (PRR7 and PRR9), which along with PSEUDO RESPONSE REGULATOR 5 (PRR5) inhibit expression of *CCA1* and *LHY* (Locke *et al.*, 2006; Zeilinger *et al.*, 2006; Nakamichi *et al.*, 2010). This molecular link between *CCA1/LHY* and *PRR7/9/5* during the morning constitutes a second feedback loop called the 'morning loop'. Further regulatory clock control is carried out by CCA1 and LHY through transcriptional inhibition of *EARLY FLOWERING 3* and *4* (*ELF3* and *ELF4*), *LUX ARRHYTHMO* (*LUX*, also known as *PHYTOCLOCK 1*, *PCL1*), and *GIGANTEA* (*GI*) genes (Nagel and Kay, 2012). In the 'evening loop', TOC1 represses expression of *PRR5*, *PRR7*, *PRR9*, *LUX*, *GI*, and *ELF4* (Gendron *et al.*, 2012; Huang *et al.*, 2012). An important component of the evening loop is the Evening Complex (EC). The EC is composed of ELF3, ELF4, and LUX and it represses transcription of *PRR9* (Chow *et al.*, 2012). Interestingly, LUX represses its own expression (Helfer *et al.*, 2011). Further post-translational regulation takes place in the evening, such as GI degradation by ELF3 (Yu *et al.*, 2008) and F-box protein ZEITLUPE (ZTL) stabilisation by GI, allowing ZTL to control TOC1 protein degradation (Kim *et al.*, 2007).

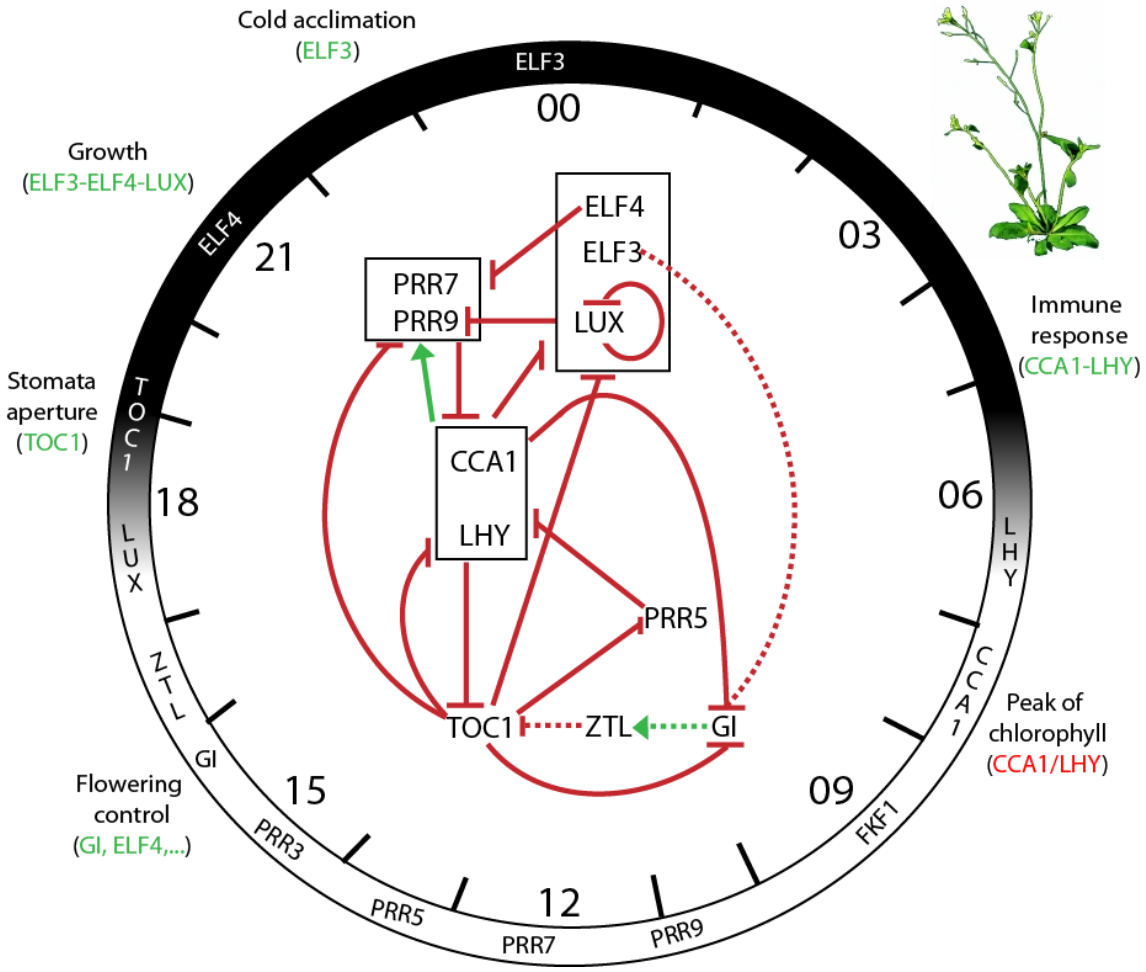


Figure 1.8: Simplified schematic diagram of the 24-hour Arabidopsis clock. Feedback loops of the core clock genes are represented in the centre. Full lines represent transcriptional feedback loops, whereas dashed lines represent post-translational regulations. Green lines are for activation, while red lines are for repression. For simplicity, the PRR3 component was not included in the above regulatory network. Expression peaks of clock genes are represented at different times of the day (Nakamichi, 2011). Several physiological processes, indicated on the periphery of the clock, are regulated by clock genes (in brackets), allowing them to occur at the right time of the day.

In addition to self-regulation, core clock genes regulate expression of output genes which are involved in several physiological processes (Figure 1.8). About one third of the genome expression in Arabidopsis is controlled by clock genes (Covington *et al.*, 2008). Some examples are: i) CCA1 and LHY repress transcription of chlorophyll-related genes (Lu *et al.*, 2009) and optimise immune responses (Zhang *et al.*, 2013a); ii) GI and ELF4, among other clock genes, regulate flowering time (Kim *et al.*, 2012); iii) the EC complex is crucial to controlling hypocotyl growth (Nusinow *et al.*, 2011); iv) TOC1 protects the plant against drought partially through regulation of stomata aperture (Legnaioli *et al.*, 2009); and v) PRR7 regulates several genes involved in abiotic stress responses (Liu *et al.*, 2013).

1.7.2 – Plant clock entrainment

All transcriptional feedback loops occur within 24 h in the Arabidopsis clock. However, in constant conditions, the circadian rhythm in Arabidopsis takes 22 to 29 h to complete, depending on ecotype (Michael *et al.*, 2003). The endogenous circadian rhythm is synchronised with the 24-h geophysical period through clock entrainment, which refines the phase of the clock after every cycle, adjusting it to a period close to 24 h (Hotta *et al.*, 2007). Therefore, in addition to anticipation of light and temperature changes, the circadian clock also senses these cues through multiple pathways. Temperature cycles are able to entrain the clock in constant light, however, there are significant differences between light and temperature entrainment. A warm–cold temperature cycle in etiolated seedlings is only able to entrain a small proportion of the total transcripts entrained by light–dark cycles (Wigge, 2013). Adding to the complexity, plant responses to light and thermal-cycles are intimately connected and highly context dependent.

Clock entrainment from both light and temperature cues affects diverse physiological processes, for example the transition from vegetative to reproductive phases (Hayama

et al., 2003; Cao *et al.*, 2005b; Mizoguchi *et al.*, 2005; Kim *et al.*, 2008b; Más and Yanovsky, 2009). Some plants have an efficient light and temperature resetting mechanism, so they are better adapted to fluctuating photoperiods and temperatures, typical of seasons in high latitudes (Pittendrigh and Takamura, 1989; Flood *et al.*, 2000; Turner *et al.*, 2005; Hotta *et al.*, 2007; Abbo *et al.*, 2009). Other plants do not respond to photoperiod and can be susceptible to cold, decreasing fitness significantly when grown in regions or seasons beyond their normal conditions (Turner *et al.*, 2005; Cober and Morrison, 2010). Therefore, photoperiod and temperature responsiveness provides a selective advantage, raising the question of which plant receptors transduce the light and temperature signals to the clock and how the clock responds to the signals in order to control the overall biology of the plant.

For light signalling, photoreceptors such as phytochromes and cryptochromes are the molecules responsible for transferring information on light phasing to the plant circadian clock and changing the phase and period of clock gene expression (Hotta *et al.*, 2007; Kim *et al.*, 2007; Wenden *et al.*, 2011b). Direct light input on the expression of core clock genes also occurs. For example, ZTL interactions with GI, and consequent stabilisation of ZTL, is strongly enhanced by blue light (Kim *et al.*, 2007). *CCA1* post-transcriptional and *LHY* translational regulation are also directly affected by light (Kim *et al.*, 2003; Yakir *et al.*, 2007). Upon light entrainment, a number of circadian-regulated output processes are reorganised at the molecular level, generating the appropriate biological responses (Harmer *et al.*, 2000; Hotta *et al.*, 2007). As an example, shaded plants perceive decreased light through photoreceptors, which in turn affect expression of circadian genes (e.g. *PRR5*), leading to a series of gene activations responsible for stem and petiole elongation to maximise light incidence (Salter *et al.*, 2003; Takase *et al.*, 2013).

The link between the circadian clock and temperature is also of great interest. Cold temperatures affect the biochemical properties of most enzymes, including those involved

in the circadian clock. Slower enzymes could slow down the pace of the circadian rhythm and affect anticipation responses. However, plants can entrain the temperature cue in the clock to compensate for changes in reaction rates, and thus maintain the period (Hotta *et al.*, 2007) (see Section 5.1). In Arabidopsis, this 'temperature compensation' is poorly understood but it probably involves *CCA1*, *LHY*, *GI*, *PRR7*, *PRR9*, and *ELF3* (Salomé and McClung, 2005; Gould *et al.*, 2006; Salomé *et al.*, 2010; Thines and Harmon, 2010; Boikoglou *et al.*, 2011). More recently, it was discovered that temperature entrainment and compensation could involve temperature-dependent alternative splicing of some clock genes (James *et al.*, 2012a, 2012b, see below).

1.8 – Cross-talk between AS and the Arabidopsis clock

AS affects many plant processes and functions and is a common co- and post-transcriptional regulatory mechanism. The circadian clock also controls diverse genes and processes in plants. Therefore, it is not surprising that cross-talk exists between AS and the circadian clock in plants. Recently, numerous cases of clock control over AS, and AS regulation of clock genes have been identified in Arabidopsis. This cross-talk, summarised below, controls many aspects of the overall biology of the plant, being crucial for efficient adjustment of biological responses to daily environmental changes.

1.8.1 – Clock control over AS

A two-day time course analysis of Arabidopsis plants under free-running conditions and using genome tiling arrays detected almost 500 introns having rhythmic expression (Hazen *et al.*, 2009). About 43% occurred in genes that also showed oscillating expression, but in 57% of the cases, the rhythmic intron occurred in a gene with arrhythmic expression, suggesting specific circadian control of AS. One explanation for this phenomenon is

that the clock regulates key splicing factors, resulting in oscillating expression and a subsequent rhythmic effect on AS processes. A recent analysis has shown that the mRNA levels of 83 out of 426 splicing-related Arabidopsis genes are regulated by the circadian clock, constituting possible candidates that mediate circadian regulation of AS (Perez-Santángelo *et al.*, 2013).

The best studied examples of clock-regulated splicing factors are the hnRNP-like proteins *GLYCINE RICH RNA-BINDING PROTEIN 7* and *8* (*GRP7* and *GRP8*). These RNA-binding proteins regulate splicing of several genes (Streitner *et al.*, 2012), and they also negatively autoregulate and reciprocally cross-regulate their splicing (Schöning *et al.*, 2008). The proteins bind to their pre-mRNAs and promote the use of an alternative 5' ss that causes part of an intron to be included in the mRNA transcript and introduces a PTC. As a result, these alternative transcripts are subjected to degradation through the NMD process, affecting their oscillatory expression.

1.8.2 – AS regulation of clock genes

Extensive AS has been identified in core clock genes. Genome-wide identification of rhythmic introns, mentioned previously (Section 1.8.1), detected circadian AS of clock genes such as intron 2 of *ELF3*, which creates a PTC (Hazen *et al.*, 2009). This rhythmic AS event could produce a truncated protein relatively similar to one expressed in the early flowering and arrhythmic *elf3-1* mutant (Hicks *et al.*, 2001), suggesting crucial involvement of this AS in flowering and clock control.

Alternatively spliced products have been identified in Arabidopsis clock genes in response to temperature (Filichkin *et al.*, 2010; James *et al.*, 2012a). In addition to altered transcript levels, temperature variation reduces or increases abundance of about 15 AS events from different clock genes (*LHY*, *CCA1*, *PRR9*, *PRR7*, *PRR5*, *PRR3* and *TOC1*). The

majority of these events introduce uORFs or PTCs, which induce NMD and reduce levels of functional protein. In some cases, AS events reach up to 10–50% of the total transcripts or are virtually absent in some conditions (James *et al.*, 2012a). These temperature-dependent AS events may play a key role in clock temperature compensation.

For *LHY*, in particular, low temperature reduces the total transcript and protein levels. This reduction is due to the increase of AS transcripts which retain a uORF-containing intron and/or include a PTC-containing alternative exon (E5a) at the expense of fully spliced mRNAs. It is noteworthy that reduced *LHY* mRNAs occur while transcription rate remains the same (i.e. promoter strength is not affected). In this case, low *LHY* expression is probably a result of the post-transcriptional control that generates NMD-degraded mRNAs (James *et al.*, 2012a).

Interestingly, *LHY* and *CCA1* which are highly related, partially redundant genes, show completely different AS behaviour under low temperatures. Total transcript and protein levels of *CCA1* have a transient increase, while alternative retention of intron 4 decreases at low temperatures (Filichkin *et al.*, 2010; James *et al.*, 2012a). Interestingly, AS of intron 4 of *CCA1* is observed in different plant species (*Brachypodium*, *Oryza*, and *Populus*), pointing to a functional importance (Filichkin *et al.*, 2010). Similarly, the partially redundant *PRR7* and *PRR9* genes have different AS behaviours under different temperatures, implying functional differences between them (James *et al.*, 2012a).

1.8.3 – Regulators of clock AS

The plant AS regulatory network controls core clock functions. Very little is known about the factors which regulate AS in clock genes. Recent studies have found that the circadian-regulated type II PROTEIN ARGININE METHYLTRANSFERASE 5 (PRMT5) protein, also known as SHK1 KINASE BINDING PROTEIN 1 (SKB1), regulates AS of

several genes (Deng *et al.*, 2010; Hong *et al.*, 2010a). PRMT5 targets *PRR9*, which is essential for the normal operation of the Arabidopsis clock (Sanchez *et al.*, 2010). The mechanism by which PRMT5 controls splicing is still unknown, but it could be through histone or spliceosomal protein methylation (Sm proteins of snRNPs), which affect 5' splice site recognition (Sanchez *et al.*, 2010; Perez-Santángelo *et al.*, 2013). Of the four different *PRR9* mRNA isoforms, only one leads to production of the full-length protein, whereas the other three contain PTCs (e.g. intron 3 retention), which controls levels of *PRR9* functional mRNAs. One AS event, in particular, is an alt 5' ss and it is an NMD target, controlling *PRR9* mRNA levels. In the *prmt5* mutant, there are increased levels of NMD-targeted *PRR9* mRNAs and a strong reduction of the mRNA encoding the full-length protein, thus confirming PRMT5 involvement in the correct splicing of *PRR9* (Sanchez *et al.*, 2010). Interestingly, loss-of-function of the *D. melanogaster PRMT5* homologue, *DART5*, alters splicing of *PER* and several clock-associated genes, resulting in disrupted locomotor activity. Therefore, a conserved role of *PRMT5* in the AS regulation of clock genes confirms the importance of AS and the circadian clock (Sanchez *et al.*, 2010).

Another putative regulator of AS in clock genes is the *SNW/SKI-INTERACTING PROTEIN* (*SKIP*) gene in Arabidopsis. *SKIP* codes for a splicing factor that physically interacts with SR45 to control splicing of *PRR7*, *PRR9*, and possibly other clock pre-mRNAs (Wang *et al.*, 2012). In the *skip-1* mutant, unproductive AS variants of *PRR7* and *PRR9* increase, lengthening the circadian period in a temperature-dependent manner. AS of *PRR7* and *PRR9*, among others, are important for proper temperature compensation of the Arabidopsis circadian clock (James *et al.*, 2012a). Therefore, the temperature-related SKIP phenotype and its control over *PRR7* and *PRR9* AS suggests *SKIP* is an essential input gene for the clock temperature compensation mechanism (James *et al.*, 2012a; Wang *et al.*, 2012). Similarly, a mutation within the Arabidopsis gene *RBP SPLICEOSOMAL TIME-*

KEEPER LOCUS1 (*STIPL1*) induces a long period (Jones *et al.*, 2012). STIPL1 is a homolog of the human spliceosomal protein TFP11 (Ntr1p in yeast) involved in spliceosome disassembly. The *stipl1* mutation reduces the splicing efficiency of several introns and alters the accumulation of circadian-associated transcripts including increased levels of the intron 3 retained variant of *PRR9* (Jones *et al.*, 2012; Staiger and Brown, 2013).

1.9 – Knowledge translation from Arabidopsis to barley

Since 1941, Arabidopsis has been used a model plant, accumulating a rich collection of genetic information, that exceeds any other plant species (Srikanth and Schmid, 2011). However, understanding biological processes in other species, in particular crops of high economic interest, is of utmost importance and can be enhanced by transferring knowledge from model species. For example, the knowledge transferred from one species to another helps to confirm the importance of certain processes in the overall biology of the plant. In addition, this knowledge may offer new targets for optimisation of crop yield and quality. Therefore, it becomes an attractive strategy to translate knowledge from Arabidopsis to crops with the proviso that evolutionary distance is likely to be reflected in differences between species (Spannagl *et al.*, 2011). An interesting example of crop improvement through knowledge translation was discussed by Silverstone and Sun (2000). The discovery of how gibberellin-related genes function in Arabidopsis and the identification of orthologous genes in wheat helped improve certain characteristics of wheat such as grain yield and growth statures (Silverstone and Sun, 2000). Additional studies targeted genes that confer tolerance to various abiotic stresses in crops, for instance, drought stress (Dubouzet *et al.*, 2003; Zhang *et al.*, 2008; Spannagl *et al.*, 2011).

The recently established link between AS and the circadian clock in Arabidopsis (Section 1.8) has not been examined to any extent in crop plants, although individual

events in specific genes have been recognised (e.g. I4R in *CCA1*)(Filichkin *et al.*, 2010). Therefore, taking similar approaches, the translation of knowledge from Arabidopsis is a practical way to expand AS and clock information in crops. However, in order to carry out AS studies in a crop, it is necessary to know the group of clock gene homologues, their gene structures and, the precise location and size of introns, as well as the presence of gene duplications in the genome. Therefore, access to genomic data of the candidate crop species is essential. DNA sequence information from several plant genomes are publicly available and could be used for AS studies, for instance rice (Ouyang *et al.*, 2007), potato (Consortium *et al.*, 2011), and barley (Consortium, 2012a).

1.9.1 – Barley (*Hordeum vulgare* L.)

Barley, a member of the grass (Poaceae) family, has an important role in human history. Its use dates back thousands of years, before the development of agriculture and it is one of the earliest cultivated crops, alongside wheat (Puruggannan and Fuller, 2009). Upon domestication, cultivated barley diversified into several elite varieties with worldwide use. Around 75% of barley production is used for animal feed, and it is also the best-suited grain for malting. Malt is a key element in the production of beer and whisky (Newton *et al.*, 2011). Barley represents the fourth most abundant cereal in the world, behind wheat, rice and maize (FAOSTAT, 2011).

1.9.1.1 – Barley genetics and AS

Barley is considered a model for plant genetic research, especially within the Triticeae tribe, which includes bread and durum wheats, barley and rye. Barley is a diploid species, unlike its wheat relatives, which are tetraploid or hexaploid, and it is therefore

more amenable for genetics studies. Additionally, barley is a self-fertilising crop, which facilitates reproduction in glasshouses by eliminating the need for pollinators.

A large collection of barley germplasm containing diverse cultivars and mutants provides an extensive resource for gene discovery and breeding programmes for crop improvement (Druka *et al.*, 2011). To advance gene discovery and genome-assisted crop improvement, a barley genome gene space was recently published and provides access to the majority of barley genes in a highly structured physical and genetic framework (see Section 3.1.3) (Consortium, 2012a). Moreover, this work has identified extensive post-transcriptional control over barley genes. In particular, extensive transcriptomic NGS analysis has revealed that 73% of intron-containing genes undergo AS, mostly in a spatial and temporal manner. Interestingly, around 20% of alternative transcripts contained a PTC (Consortium, 2012a). Despite such evidence of vast AS in barley, there are few well-characterised examples of AS (Halterman *et al.*, 2003), limiting a deeper understanding of connections between barley AS and their functional biological roles (Consortium, 2012a).

1.9.1.2 – Barley clock genes and flowering

Barley shows a circadian rhythm at diverse levels such as at the transcript, protein and physiological levels (Vallelian-Bindschedler *et al.*, 1998; Martínez *et al.*, 2003; Lillo, 2006; Nagasaka *et al.*, 2009). However, the molecular mechanism underlying the establishment of these rhythms, i.e. the barley clock, is only beginning to be addressed. Only a few putative barley clock genes have been identified and characterised well, such as *GI*, *PPDH1, ELF3* and *LUX* (Dunford *et al.*, 2005; Turner *et al.*, 2005; Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012; Campoli *et al.*, 2013). Transcriptional analyses have also been described for *LHY* (*CCA1*), *PRR73*, *PRR59*, and *PRR95* (Higgins *et al.*, 2010; Campoli *et al.*, 2012b; Faure *et al.*, 2012).

Flowering is a crucial developmental stage, and it is tightly regulated to optimise reproductive success, which for the purposes of agricultural interest means seed production or yield (further discussed in Chapter 4). Barley cultivars are divided into two major groups depending on their flowering phenotype: namely winter and spring. Winter barley plants have an efficient light response mechanism and/or promote early flowering, so they are better suited to short growing seasons that are typical of high latitudes. Conversely, spring barley varieties do not respond as well to photoperiod and are late flowering compared to winter barley. As a result, spring barley plants have higher yield when grown in long growing seasons (Flood *et al.*, 2000; Turner *et al.*, 2005).

Several genes control flowering time in plants, including several clock genes (detailed information in Chapter 4). Natural and induced mutations in some barley clock genes have considerably affected flowering time and extended the geographic range where barley is grown (Turner *et al.*, 2005; Jones *et al.*, 2008; Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012). Additionally, there is evidence that the barley clock contributes to cold resistance (discussed in Chapter 5) (Hemming *et al.*, 2008). Therefore, the circadian clock has a huge impact on barley traits and raises questions about its function and the molecular basis which controls plant processes.

1.10 – Objective and aims of this work

The recent realisation that AS could be an important regulator of clock gene expression and function, particularly in response to low temperature, and the recent generation of the barley 'gene-ome', provided an ideal basis to undertake an analysis of the barley clock and the impact of AS in this system. To address this overall objective, my key aims were:

1) To identify barley putative homologues of core circadian clock genes and selected output genes using bioinformatic analysis;

2) To analyse core clock gene expression and clock outputs in circadian time courses in light/dark and free-running light/light experiments to establish circadian behaviour and provide evidence that they are, in fact, clock-related genes.

3) To examine the phenotypic effect of mutations in two core clock genes, *ELF3* and *PPDH1*, and whether they affect the expression of other clock-related genes, thus providing information that contributes to better understanding of the clock functions.

4) To define the AS events in barley clock genes and use this information to establish an RT-PCR panel for expression and AS studies of barley clock and clock-associated genes; and

5) To perform temperature transition experiments to identify difference in expression and splicing of core clock genes and presence of productive/non-productive transcripts, using the RT-PCR panel developed.

Chapter 2. General materials and methods

Unless stated otherwise, all basic molecular biology procedures, for example, agarose gel electrophoresis of DNA and DNA digestion with restriction enzymes, were carried out according to Sambrook *et al.* (1989). Methods specific to each Results chapter are presented in the corresponding Chapter.

2.1 – Plant growth conditions

2.1.1 – Plant material

Seed of all of the genetic lines used in this study were kindly provided by Professor Robbie Waugh and Dr. Arnis Druka, from the James Hutton Institute, Dundee, UK. It consisted of three barley lines:

- the two-rowed wild-type spring barley cultivar Bowman (PI 483237) (Franckowiak *et al.*, 1985). This line contains the naturally occurring monofactorial recessive *Hvprr37_ppdh1* gene.
- the Bowman near isogenic line (NIL) *eam1.d* (line ID BW281) (Druka *et al.*, 2011) is homozygous for the monofactorial dominant *HvPRR37_PPDH1* gene from KT1031 (a winter barley from Bulgaria, GSHO 1568). This is a BC8 line (8^{th} backcross with Bowman) with a small gene interval, < 1 cM, introgressed on chromosome 2H.
- the Bowman NIL *eam8.w* (line ID BW290) (Druka *et al.*, 2011) is homozygous for the monofactorial recessive *early heading k/ Hvelf3* gene from Early Russian (natural-

ly occurring variant, Clho 13839) (Franckowiak and Lundqvist, 2007). This is a BC6 line with 1.5 cM introgressed on chromosome 1H.

2.1.2 – Growth cabinet: environment and treatment

An environmentally controlled cabinet was used, the Microclima 1000 model (Snijders Scientific, The Netherlands). The cabinet had 0.91 m^2 of floor area and 1.2 m of growth height. Illumination of 400 μmol m^{-2} s^{-1} was provided by 12 daylight fluorescent tubes and 3 red/far red fluorescent tubes.

Barley plants were grown in cereal compost (Appendix A1) and watered frequently (daily or every other day if the soil was still moist). Humidity was kept at 70%. Artificial daylight did not include dimming typical of sunrise or sunset. No crop protection treatment was applied.

2.2 – RNA extraction

First, frozen tissue was ground, using mortars and pestles (sterilised and RNase-free) chilled with liquid nitrogen, until a fine talc-like powder was produced. RNA from up to 100 mg of ground tissue was extracted with the RNAeasy Plant MiniKit (QIAGEN, UK) following manufacturer's instructions. RNA preparations were DNase-treated (on columns) to remove DNA using the RNase-free DNase (QIAGEN, UK), again following the manufacturer's instructions. Each RNA sample was quantified and quality assessed by measuring sample absorbance at 230, 260 and 280 nm using a Nanodrop® 1000 spectrophotometer (Thermo Fisher Scientific, USA). Samples with a 260/280 ratio outside the range of 1.7 – 2.3 and/or a 260/230 ratio outside the range of 1.4 – 2.8 were cleaned using phenol extraction (see 2.2.1 below).

To detect any possible genomic DNA contamination, each RNA sample was subjected to PCR amplification, using primers for *HvPRR59* (Table 2.1), as described in Section 2.4. DNA-free RNA samples were stored at -80 °C. Any samples with persistent genomic DNA contamination were subjected to an additional DNase treatment (Section 2.2.2).

2.2.1 – RNA clean-up using phenol extraction

RNA samples were diluted to 200 µL using DEPC-treated water (Appendix A2). The same volume (200 µL) of phenol solution saturated with 0.1 M citrate buffer, pH 4.3 (Sigma-Aldrich, UK), was added and the solutions were vortexed. Subsequently, samples were centrifuged at 21,100 × *g* at 4 °C for 2 min (Heraeus Fresco 21, Thermo Fisher Scientific, USA). The aqueous phases were transferred to fresh RNase-free microcentrifuge tubes (1.5 mL, Ambion®, Life Technologies), where 200 µL of a 24:1 mixture of chloroform (Thermo Fisher Scientific, USA) and isoamyl alcohol (AnalaR NORMAPUR®, VWR International, Belgium) was added and vortexed. Samples were centrifuged at 21,100 × *g* at 4 °C for 2 min. The aqueous phases were transferred to fresh RNase-free microcentrifuge tubes, where 5 µg of RNase-free glycogen (UltraPure™, Invitrogen, UK) was added. Subsequently, 18 µL 3M sodium acetate (pH 5.5, DEPC-treated) (Appendices A2 and A3.1) and 500 µL ethanol absolute (Sigma-Aldrich, UK) were added. Samples were placed in a freezer (-20 °C) overnight. The following day, samples were centrifuged at 21,100 × *g* at 4 °C for 30 min. After centrifugation, the supernatant was removed from each sample with a glass Pasteur pipette (VWR International). Lastly, pellets were resuspended in 50 µL RNase-free water. RNA was quantified and quality assessed by measuring absorbance, as mentioned previously (Section 2.2).

2.2.2 – Additional DNase treatment

RNA samples were diluted to 76 µL using DEPC-treated water. The following compounds from Promega (UK) were added to the RNA samples: 1 µL RNasin® Ribonuclease Inhibitor (40 U), 10 µL RQ1 10X Reaction Buffer, 10 µL RQ1 DNase, 1 µL DTT. Solutions were incubated in a heating block (Grant, UK) at 37 °C for 30 min. RNA samples were then cleaned up using phenol extraction, as described above (Section 2.2.1).

2.3 – DNA preparation

2.3.1 – Complementary DNA (cDNA) synthesis

cDNA was synthesised from 4 µg of total RNA (20 µL) using the Sprint RT Complete – Double PrePrimed kit (Clontech Laboratories, Takara Bio Company, USA). In this kit, both oligo $(dT)_{18}$ and random hexamer primers are present. Reverse transcriptase reactions were incubated in a thermal cycler (2720 Thermal Cycler, Applied Biosystems®, Life Technologies, USA) for 1 h at 42 °C, followed by 10 min at 70 °C to terminate the reaction. Subsequently, cDNA samples were diluted by adding 120 µL sterile distilled water (SDW) and stored at -20 °C.

2.3.2 – Genomic DNA (gDNA) preparation

Leaf material (0.5 cm^2) was ground in a microcentrifuge tube with a small plastic pestle for about 15 seconds. Subsequently, 400 µL of gDNA extraction buffer (Appendix A4) was added. Samples were vortexed for 5 seconds and centrifuged at 20,800 × *g* for 1 min (Microcentrifuge 5417C, Eppendorf, Germany). Supernatants (approximately 300 µL) were transferred to a fresh microcentrifuge tube, where 300 µL absolute isopropanol (AnalaR NORMAPUR®, VWR International, Belgium) was added and mixed. After

2 min, samples were centrifuged at 20,800 × *g* for 5 minutes to pellet the DNA. Supernatants were removed and pellets were dried by leaving tubes open for a few minutes. Then, 500 µL 10 mM Tris buffer (pH 7.5) was added to dissolve the DNA by gentle shaking (not vortexed).

2.4 – Polymerase Chain Reaction (PCR)

2.4.1 – Primer design

Gene-specific primers were designed using the program PrimerQuest from IDT (http://eu.idtdna.com/PrimerQuest/Home/Index) to amplify between 150–1200 bp of genomic sequence. The following parameters were used: melting temperature, 59 °C to 65 °C; oligo length, 17 nt to 30 nt; GC content, 35% to 65%. Maximum self-complementarity allowed was 4 nt and maximum primer dimer length was 5 nt. Suggested primer pairs were aligned to paralogous barley genes using the ClustalW program (Larkin et al., 2007; Tamura et al., 2011). No 3' end complementarity to any paralogous gene was allowed. This analysis was performed to ensure gene specificity of the primer pair. If a primer suggestion from PrimerQuest was not possible, alternative gene-specific primers were manually designed and quality assessed using the NetPrimer program (www.premierbiosoft.com/netprimer/). In this alternative procedure, more relaxed parameters were used: melting temperature 50 °C to 70 °C; GC content: 30 to 70%.

2.4.2 – Primers used in expression and alternative splicing analysis

For each gene, one primer pair was designed to measure expression and AS. A record of primers used can be seen in Table 2.1 and Appendix A5.

Table 2.1: List of barley gene-specific primers and their sequences used for expression and AS analyses. Primer name includes directions: Fw is Forward, whereas Rv is Reverse.

Genes	Primer name	Sequence (5' → 3')
LHY	HvLHY-Ex6Fw	AACAAAGCGACCAGTTGTCAGCAC
	HvLHY-Ex8Rv	TGAGCATGGCTTCTGATTTGCACG
TOC1	HvPRR1-Ex3Fw	TCTGTTGTTGTCAAGTGCTTGCGG
	HvPRR1-Ex6Rv	CAATGAGCTGTTTGCTGGAGTGCT
PPDH1	HvPpdH1-Ex1fw	GGAGTTCCTACACAGGAAGACCAT
	HvPpdH1-Ex4Rv	TCGTCGTTGCGATCATTGCTGTTG
PRR59	HvPRR59-Ex7Fw	TGGACTGTGAGCGGTGAAACTGAT
	HvPRR59-3UTRv	TCACCTGGGAAGGGAGGTATCAAA
PRR95	HvPRR95-Ex6FwB	GCTGAACAACTCTGGCAACAGCAA
	HvPRR95-3UTRv	TTCAAAGGACACACCAAATCGGCG
PRR73	HvPRR73-Ex3Fw	TTAACTGAGGTCGCCATGCCTTGT
	HvPRR73-Ex5Rv	TCATCATGACTGCCGCCACTGTTT
LUX	HvLUXFw	GGCTCCTTCCCGTCCTTCC
	HvLUXRv	TTGACGTAGAGGCGGTACTTCTGGA
ELF3	HvELF3-Ex2Fw	TCTCCAGATGATGTTGTCGGTGCT
	HvELF3-Ex4Rv	TGAGATGGTGAAGCCTGGTTTCCT
ZTLa	HvZTLa-5UTRFw	AGGAGGAGGGATAGGAGGCG
	HvZTLa-Ex2Rv	AGGGGTCAGATGCAACCTGT
ZTLb	HvZTLb-5UTRFw	TAGCGAGCGAGCGATGGGATA
	HvZTLb-Ex2RvB	GGGCTTTAGTTGCAGACTCG
FKF1	HvFKF1-Ex1Fw	CGGGGCTGGGGATGGCAGGC
	HvFKF1-Ex2Rv	ATCGGTTGTTTACTGGAGCT
GI	HvGI-Ex14Fw	TCGTGCTACAGATGGGATGCTTGT
	HvGI-Ex16Rv	GTCCACTGTTCAAGATGTCACGGA
CABa	HvCABa-5UTRFw	AACACACGACACACCTCTAGCCTT
	HvCABa-3UTRv	TGTGCAGTTCACTGTAGTCGCCTT
GRP7a	HvGRP7a-Ex1Fw	GGGTTTAGCGGCGGGGAGAAGGAT
	HvGRP7a-3UTRv	AACGGTAACAAGTAGCACGGAACG
GRP7b	HvGRP7b-Ex1Fw	GAGAAGGAGAGGAGGAGATCCATG
	HvGRP7b-3UTRv	CGCGATCAAAAACACCACAGGACC
CO1	HvCO1-Ex1Fw	TGTGAGAAGGCCACATCAGAGTGG
	HvCO1-Ex2Rv	ATGTCTGGTCCACTTCCATTTCCGC
CO2	HvCO2-Ex1FwB	GAGCAGCAAGGGAGCAATTA
	HvCO2-3UTRv	TACGGTGCCCATCATTCCATGTGT
ELF4-like3	HvELF4-like3Fw	TCAACAAGAGTTTGGAGAGGGA
	HvELF4-like3Rv	ATGCTAGGAAACAGCAGGCACCAT
ELF4-likeA	HvELF4likeAFw	ATATCCATCTTCCTCCCGCACGCA
	HvELF4likeARv	ATGGTCTGCCTCCTCTACTGTGT
FT1	HvFT1-5UTRFw	ACAGCTTACATTGCTGCTCTCTGC
	HvFT1-3UTRv	TGGATAATTTGGTGACTTGGCGGC
FT2	HvFT2-5UTRFw	ACGCCTTCATTTCATCACACACCG
	HvFT2-Ex4Rv	ACTACAAGGAGTCGATCATCACAT

2.4.3 – High Resolution (HR) RT-PCR

HR-PCR analysis, usually and hereafter referred to as HR RT-PCR, is based on Simpson *et al.* (2008). For each primer pair, the forward primer was labelled with

6-carboxyfluorescein (FAM). Each HR RT-PCR reaction was run in individual 0.2 mL wells of a 96-well PCR plate (Thermo Fisher Scientific, UK) and usually contained 3 μL of cDNA (template), 20 μM of each primer, 2 μL of 10 × PCR reaction buffer with $MgCl_2$ (Roche, UK), 0.25 μL Taq DNA Polymerase (5 U/μL, Roche, UK), 1 μL dNTP (5 mM of each dNTP, Invitrogen, Life Technologies, USA) and SDW up to a final volume of 20 μL. The general PCR programme included an initial 2 min step at 94 °C, followed by 20–28 cycles of: 1) 15 sec at 94 °C; 2) 15 sec at 50 °C; and 3) 1 min and 30 sec at 70 °C (Simpson *et al.*, 2008). At the end of cycling, a final extension step of 5 min at 70 °C was performed. For all experiments with a primer pair, negative controls containing RNA template and one positive control containing gDNA were subjected to the same procedure to exclude any possible contamination or detect PCR inhibitors. Subsequently, 1 μL of the resultant PCR was mixed with 8.5 μL of Hi-Di™ formamide (Applied Biosystems, USA) and 0.05 μL of GeneScan™ 500 or 1200 LIZ® standard (Applied Biosystems®, Life Technologies, UK) and loaded on the ABI 3730 automatic DNA sequencer (Applied Biosystems®, Life Technologies, UK). PCR products were accurately sized, to single base pair resolution, and quantified in Relative Fluorescent Units (RFU), which are the peak areas from spectral data (proportional to amount of the product present), calculated by GeneMapper software (version 3.7, Applied Biosystems®, Life Technologies, USA). To achieve meaningful comparisons of transcript data, errors arising from unequal quantities of starting RNA were removed by normalising clock transcript RFU to the mean RFU values of fully spliced *HvUBC21* and *HvPP2AA2* transcripts (Equation 1).

$$\frac{RFU^{clock}}{(RFU^{UBC}+RFU^{PP2AA2})/2}$$

Equation 1: Normalisation procedure for each HR RT-PCR. RFU: Relative Fluorescent Units of a transcript calculated by the GeneMapper software. Clock: transcript of a candidate clock gene. UBC: fully spliced *HvUBC21* transcript. PP2AA2: fully spliced *HvPP2AA2* transcript.

Chapter 3. *In silico* identification of barley homologues of Arabidopsis genes involved in the circadian clock

3.1 – Introduction

3.1.1 – Gene discovery

Gene discovery is important to understanding biology and it forms the basis of molecular genetics. There are several methods and tools to discover new genes and determine their function. Forward genetics, for example, seeks to identify the gene responsible for a phenotype whereas reverse genetics starts with a putative gene and seeks to identify its function.

The first gene to be sequenced and identified codes for the bacteriophage MS2 coat protein (Jou *et al.*, 1972). Since then, an overwhelming number of genes have been characterised and sequenced with the greatest impact coming from sequencing of whole genomes of prokaryotes and complex higher eukaryotes. In fact, since the establishment of massively parallel sequencing technologies it is no longer difficult to produce whole genome sequences and transcriptome data, providing a massive outpouring of gene and gene expression information. Nevertheless, it remains a challenge to identify genes within the billions of nucleotides. The use of *in silico* analyses can greatly improve the detection of functional and undefined genes, facilitating gene discovery (Yu and Hinchcliffe, 2011).

Common and useful *in silico* tools for gene identification include linkage analysis (studies of segregating genetic markers), association mapping (for instance, examination of single-nucleotide polymorphisms), and phenotype mining (for instance, experiments using the RNA interference method). Moreover, there is an emerging, and possibly compulsory,

research field that further facilitates gene discovery, 'comparative studies' (Yu and Hinchcliffe, 2011; Yoon *et al.*, 2012). In a comparative multi-genomic approach, candidate genes with a crucial cellular function are compared (associated) across different genomes, allowing for identification and prediction of function in other species. This is especially useful in newly sequenced genomes or when original data are comparably sparse, as in the case of uncharacterised human diseases (Prosdocimi *et al.*, 2009; Yu and Hinchcliffe, 2011).

3.1.2 – Comparative studies

Comparative studies have rigorous guidelines in order to produce the correct interpretation of results and avoid personal bias (Harvey and Pagel, 1991). First, input data must have an evolutionary relationship, i.e. a common ancestor, to justify similarities. Second, evolved entities must have a tree-like structure of relationships where nodes represent common ancestry. This makes phylogenetic trees essential for any robust comparative analysis (Prosdocimi *et al.*, 2009).

Phylogenetic studies frequently use terms to explain evolutionary relationships that are also used in genome comparisons for gene discovery (Li *et al.*, 2003). Homologues refer to two major types of evolutionary relationships: orthologues and paralogues. The former refers to genes evolved from a common ancestor by speciation, and the latter term applies to genes that are related by duplication events (Fitch, 2000). A true orthologue is usually under selective pressure to maintain function whereas paralogues accumulate mutations that tend to alter the gene function. Conservation among orthologues facilitates their identification in comparative studies. Gene copies that accumulate mutations which eventually disrupt gene function are considered pseudogenes. It is noteworthy that the precise definition of 'pseudogene' is highly debated (Balakirev and Ayala, 2003). The classical meaning

defines it as a non-functional gene, with no expression or regulation (Pearson, 2008). In *in silico* analyses, pseudogenes are easily detected by the loss of whole regions of genes or the presence of PTCs or frameshift mutations that disrupt expression of a functional full-length protein.

3.1.3 – A sequence-enriched barley genomic framework

An integrated and ordered physical, genetic and functional sequence database for barley was recently released by the International Barley Genome Sequencing Consortium (2012a). Barley is a diploid species (see Section 1.9.1) with a large haploid genome of about 5.1 gigabases (Gb). The physical map developed by the Barley Consortium has 4.98 Gb of genome sequence from barley cultivar Morex (>50-fold haploid genome coverage). The gene-enriched genomic nucleotide information was contained within 9265 whole-genome shotgun sequence contigs. Alignment of the genomic contigs against deep RNA sequence data (1.67 billion RNA-seq reads) obtained from eight stages of barley development as well as 28,592 barley full-length cDNAs (Matsumoto *et al.*, 2011) identified more than 79 thousand transcript clusters. Of those, 26,159 were 'high-confidence' genes with homology support to at least one plant reference genome (sorghum, rice, *Brachypodium* and Arabidopsis).

The availability of the barley genomic framework allows the discovery of barley genomic sequences using bioinformatic analysis. As mentioned previously (Sections 1.7–1.9), the molecular components of the plant clock have been extensively identified in Arabidopsis and rice (Murakami *et al.*, 2007), whereas the corresponding pathways in barley are beginning to be studied. *In silico* gene discovery approaches have been carried out for rice (Murakami *et al.*, 2007), soybean (Quecini *et al.*, 2007) and canola (*Brassica rapa*) (Lou *et al.*, 2012). The economic benefits of manipulating clock genes in barley and the facilitation of

in silico approaches have motivated the discovery and confirmation of the molecular mechanism of the barley clock described in this Chapter. As a result, we have identified the genomic sequences of 21 putative barley homologues of Arabidopsis core circadian clock genes and selected clock-associated genes.

3.2 – Materials and methods

3.2.1 – Cross-species reciprocal BLAST

To identify plant orthologues of the Arabidopsis clock genes, cross-species reciprocal BLAST searches were performed using default settings. First, a BLAST search (Altschul *et al.*, 1990) was carried out using Arabidopsis gene sequences against various databases (Table 3.1) to identify putative orthologous sequences in plants (Figure 3.1A). Next, reciprocal BLAST analysis was performed using the top hit from all species against the Arabidopsis database (Figure 3.1B). Subsequently, cross-species reciprocal BLAST analysis was performed using the top hit from all species against each species' databases (Figure 3.1C). When the top hit of a reciprocal BLAST successfully identified the original Arabidopsis sequence and the top hits from all other databases, these were taken as orthologues. Any additional hits with an E-value similar to the top hit were subjected to reciprocal BLASTs as well. When the second/third/etc. best hits successfully identified the original Arabidopsis sequence and their orthologues in all other species, these were taken as paralogues.

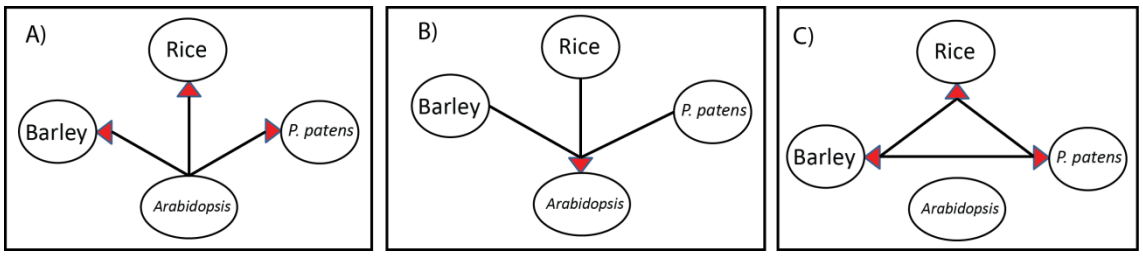


Figure 3.1: Diagram of a cross-species reciprocal BLAST analysis. For simplicity, only four plant databases are shown. Red arrows indicate the direction of BLAST analysis, i.e. a sequence from one database was used to identify orthologues in the database of another species. A) Arabidopsis gene sequences were used in BLAST searches against various databases. B) Reciprocal BLAST analysis of sequences from all species against the Arabidopsis database. C) cross-species reciprocal BLAST within all other species.

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

Table 3.1: The database resources of 10 plant genome sequences analysed in this work.

Species	Common name	Label on genes	Genomic database	Annotation Version [a]
Arabidopsis thaliana	thale cress	At	The Arabidopsis Information Resource (www.arabidopsis.org) (Lamesch *et al.*, 2011)	TAIR10
Brachypodium distachyon	purple false brome	Bd	The Munich Information Center for Protein Sequences (http://mips.helmholtz-muenchen.de/plant/) (Initiative, 2010)	MIPS/JGI Bradi release 1.2
Hordeum vulgare **cultivar Morex**	barley	Hv	Barley Morex assembly3 (Consortium, 2012a). (http://penguin/ngs/cereals_blast_page.html)	N/A
Oryza sativa **ssp.** ***japonica***	rice	Os	MSU Rice Genome Annotation Project Database and Resource (http://rice.plantbiology.msu.edu) (Ouyang *et al.*, 2007)	MSU RGAP Release 7.0
Physcomitrella patens **ssp.** ***patens***	moss	Pp	Phytozome (http://www.phytozome.net/) (Rensing *et al.*, 2008)	JGI v1.6
Solanum lycopersicum	tomato	Sl	The International Tomato Genome Sequencing Consortium (http://solgenomics.net). (Consortium, 2012b)	ITAG Release 2.3
Solanum tuberosum **Group** ***Phureja DM1-3 516 R44***	potato	St	Potato Genome Sequencing Consortium (http://www.potatogenome.net) (Consortium *et al.*, 2011)	PGSC_DM_v3.4_gene or PGSC_DM_v3_scaffolds *
Sorghum bicolor	sorghum	Sb	The Munich Information Center for Protein Sequences (http://mips.helmholtz-muenchen.de/plant/) (Paterson *et al.*, 2009)	MIPS/JGI Sbi release 1.4
Triticum aestivum	bread wheat	Ta	TaGDB (http://www.plantgdb.org/TaGDB/)	GenBank v175 or **P**lantGDB-assembled **u**nique **t**ranscripts (PUTs) v163b
Zea mays **ssp.** ***mays***	maize	Zm	The Phytozome (http://www.phytozome.net/). Line Mo17. Unpublished data produced by the Maize Genome Sequencing Project.	Zmb73 v2 release 5b.60,

a When necessary, (re)annotation of genomic sequences was performed (detailed in Section 3.2.2).

* A few genes of potato have not been annotated but their sequence is present in the DM scaffold data version 3.

N/A: Not available.

3.2.2 – Miscellaneous bioinformatic analyses

Gene sequences and names/identifiers were taken from the databases described in Table 3.1. Schematic diagrams of genomic structures were initially made using the Exon-Intron Graphic Maker program (Bhatla, 2009). In some cases, the annotated exon/intron gene structures did not generate full-length ORFs, when compared to homologous genes. Therefore, when necessary, re-annotation of genomic sequences was performed based on:

1) cDNA, EST and PUT (PlantGDB-assembled Unique Transcripts) data available for the related species;

2) Presence of GT and AG dinucleotides for intron boundaries (5' and 3' ss, respectively);

3) ORF maintenance of each exon;

4) Annotation of homologous mRNA/protein sequences.

Nucleotide sequence alignments were performed and they preserved codon structure of putative coding sequences (CDS). For this, nucleotide alignments were based on the alignments of their deduced protein sequence using the ClustalW program (Larkin *et al.*, 2007; Tamura *et al.*, 2011).

3.2.3 – Phylogenetic analysis

Gene tree estimation was performed using the neighbour-joining (NJ) method (Saitou and Nei, 1987) available on MEGA software version 5.05 (www.megasoftware.net) (Tamura *et al.*, 2011). The moss *Physcomitrella patens* was considered the outgroup of angiosperm species and were used to place the root of phylogenetic trees. Statistical support for each branch on phylogenetic trees was generated from the bootstrap test (2000 replicates; values shown when > 50%) (Felsenstein, 1985). The evolutionary distances and branch lengths were computed using the Maximum Composite Like-

lihood method (Tamura *et al.*, 2004). Pseudogenes were not analysed in order to prevent poorly-supported topologies on reconstruction of phylogeny from gene families (as suggested by Zimmer *et al.*, 2007).

3.3 – Results

Arabidopsis clock-related genes were selected for a comparative approach to identify and confirm the core clock genes and some clock-associated genes in barley. When adopting a comparative analysis, special attention should be given to the evolutionary distance between the species selected because orthology determination becomes more difficult when species are evolutionarily distant (Prosdocimi *et al.*, 2009; Yu and Hinchcliffe, 2011). Barley and Arabidopsis share a common ancestor, their separation occurred more than 140 million years ago (Mya) and they have diversified considerably since then (Chaw *et al.*, 2004; Moore *et al.*, 2007). To resolve this issue, additional species with whole genome sequence information from both dicot and monocot groups were included in the comparative analysis. These species were: tomato, potato, moss (*P. patens*) and another five grasses: *Brachypodium distachyon*, sorghum, bread wheat, maize and rice (Table 3.1).

The comparative approach adopted comprised of initial cross-species reciprocal BLASTs, followed by genomic structure and phylogenetic analyses. At least 165 homologues were identified in all species mentioned. In order to avoid repetitive description of results, a brief summary from the BLAST and genomic analyses are found in Sections 3.3.1 and 3.3.2, respectively. Further detailed information from each gene, after the final phylogeny studies, are presented in Section 3.3.3.

3.3.1 – BLAST searches for core clock and clock-associated genes

To identify barley orthologues of core clock and some clock-associated genes as defined in Arabidopsis, a robust *in silico* analysis was initially performed. It comprised of cross-species reciprocal BLASTs using databases from nine different species (Table 3.1). Initially, all core clock and some clock-associated candidate sequences from Arabidopsis were used in BLAST searches against various databases. Selection of Arabidopsis clock-associated candidate sequences was based on their role in AS regulation or plant flowering. The candidate genes analysed were *CCA1*, *LHY*, *TOC1* (*PRR1*), *GI*, *ELF3*, *ELF4*, *PRR7*, *PRR3*, *PRR9*, *PRR5*, *LUX* (*PCL1*), *FKF1*, *ZTL*, *CHE* (*TCP21*), *GRP7* (*CCR2*), *GRP8*, *CAB2, CONSTANS* (*CO*) and *FT*. Subsequently, cross-species reciprocal BLAST analysis was performed using best hit sequences from all species against all other species' databases, including Arabidopsis. In the end, these systematic analyses identified the range of species which contained true orthologues and a comprehensive list of the duplicated genes in the analysed species (Table 3.2 –Table 3.6).

The different Arabidopsis clock genes showed variation in their ability to identify true orthologues, which provided some information on the clock gene components in different species and their evolution. This is illustrated by considering two extremes of analysis of clock gene orthologues: *LUX* and *CCA1*. *AtLUX* identified true orthologues in all nine species analysed by cross-species reciprocal BLAST, including another paralogue in Arabidopsis (*AtBOA*) and four gene copies in *P. patens* (Table 3.2). The latter species has a number of particular features regarding its clock genes. The clock and flowering-related genes *GI*, *FKFI*, *ZTL*, *CO*, and *FT* are present in all flowering plants but absent in *P. patens* (Table 3.2, Table 3.4 and Table 3.6). At the other extreme is *AtCCA1*. This gene identified a gene in each of the nine species. However, it had no reciprocal hits with any species analysed. In fact, the reciprocal BLAST identified *AtLHY* instead. When this sequence was

used, cross-species reciprocal BLASTs were successful with all ten species showing that these species contained true orthologues of *AtLHY* but no orthologues of *AtCCA1* (Figure 3.2A).

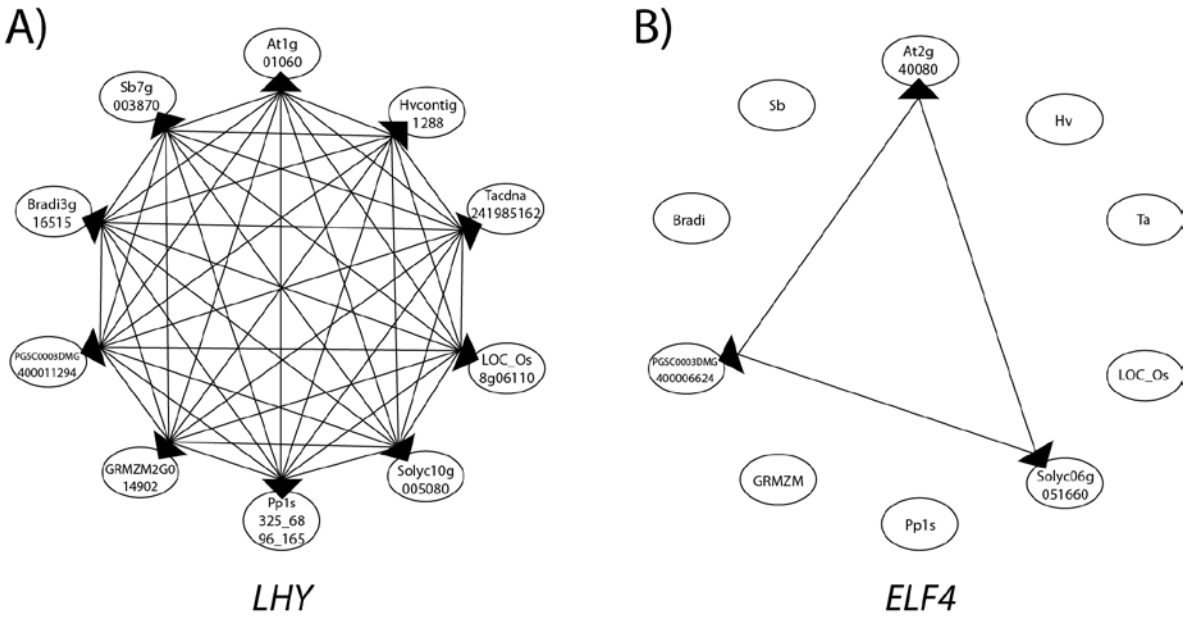


Figure 3.2: Cross-species reciprocal BLAST diagram of A) *LHY* and B) *ELF4* genes. Arrows indicate direction of BLAST analysis, i.e. a sequence from one database was used to identify orthologous sequences in the database of another species.

Other genes, for instance *ELF4*, only had cross-species reciprocal hits with dicot species suggesting that it is specific to dicots (Figure 3.2B and Table 3.5). In this analysis, the initial BLAST using the *AtELF4* sequence identified sequences in monocots that did not identify *AtELF4* reciprocally. Subsequently, a newly identified Arabidopsis gene from this reciprocal BLAST was used in cross-species reciprocal BLAST analysis, as well as all known *AtELF4* gene family members. This analysis identified orthologues and paralogues of *ELF4-like3* genes in all species analysed (Table 3.6). Barley and bread wheat have two genes in this family.

Table 3.2: Homologues of *LHY* and *CCA1*, *LUX*, *ELF3*, and *GI* in different land plant species. Work that has previously described these genes is shown for each species and genes, except Arabidopsis.

	LHY* and *CCA1	***LUX***	***ELF3***	***GI***
Arabidopsis thaliana	At1g01060 (*LHY*) At2g46830 (*CCA1*)	At3g46640 (LUX) At5g59570 (BOA)	At2g25930	At1g22770
Zea mays	GRMZM2G014902 (*ZmCCA1*) (Wang *et al.*, 2011b) GRMZM2G474769 (*ZmLHY*) [a] (Hayes *et al.*, 2010)	GRMZM2G067702 (Khan *et al.*, 2010)	GRMZM2G045275 (*ZmELF3b*) AC233870 (*ZmELF3*)	GRMZM2G107101 (*GI1A*) GRMZM5G844173 (*GI1B*) (Hayes *et al.*, 2010)
Brachypodium distachyon	Bradi3g16515 (Higgins *et al.*, 2010)	Bradi2g62067 (Campoli *et al.*, 2013)	Bradi2g14290 (Higgins *et al.*, 2010)	Bradi2g05226 (*BdGI*) (Hong *et al.*, 2010b)
Sorghum bicolor	Sb7g003870 (Murphy *et al.*, 2011)	Sb03g047330 (Campoli *et al.*, 2013)	Sb09g030700 (*ELF3*) (Zakhrabekova *et al.*, 2012) Sb03g025560 (*ELF3b*) (Higgins *et al.*, 2010)	Sb03g003650 (*GI*) (Bhosale *et al.*, 2012)
Oryza sativa	LOC_Os8g06110 (Murakami *et al.*, 2007)	LOC_Os01g74020 (Murakami *et al.*, 2007)	LOC_Os01g38530 (*OsEF3*) (Fu *et al.*, 2009) LOC_Os06g05060 (*EF7*) (Murakami *et al.*, 2007; Saito *et al.*, 2012)	LOC_Os01g08700 (Hayama *et al.*, 2002b)
Hordeum vulgare	Hvcontig_51288/1567295 (*HvCCA1* on Faure *et al.*, 2012) [a]	Hvcontig_2548416 (*HvLUX1* on Campoli *et al.*, 2013)	Hvcontig_80895/67536 (Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012)	Hvcontig_58270/1580005 (Dunford *et al.*, 2005)
Triticum aestivum	TAcdna_241985162 (Campoli *et al.*, 2012b)	Ta_PUT0106334 [c]	TAcdna_118767202 (*TaELF3*) * (Faure *et al.*, 2012) TAcdna_241985055 (*TaELF3b*) *	TAcdna_33333146 (*GI1)* (Zhao *et al.*, 2005) * TAcdna_50593493 (*GI2*) * TAcdna_50593495 (*GI3*) *
Solanum tuberosum	PGSC0003DMG400011294 [a]	PGSC0003DMG400002144 (Campoli *et al.*, 2013)	PGSC0003DMG400013826 (*ELF3a*) PGSC0003DMG400029303 (*ELF3b*)	PGSC0003DMS000000006 (*GI1*) [b] PGSC0003DMG400018791 (*GI2*) [a]
S. lycopersicum	Solyc10g005080	Solyc06g005680 (Campoli *et al.*, 2013)	Solyc08g065870 (*ELF3a*) Solyc12g095900 (*ELF3b*)	Soly04g071990 (*GI1*) Soly12g056650 (*GI2*)
P. patens **(Holm *et al.*, 2010)**	Pp1s325_68 (*PpCCA1a*) Pp1s96_165 (*PpCCA1b*) (Okada *et al.*, 2009a; Okada *et al.*, 2009b)	Pp1s27_359 Pp1s104_175 Pp1s29_23 Pp1s29_32	Pp1s86_214 Pp1s11_285 Pp1s87_90	**None**

* Alleles, sequenced in hexaploid bread wheat, are a putative homoeoallelic series on the same group of chromosomes.
a: re-annotated; b: annotated; c: partial sequence.

Table 3.3: Homologues of the pseudo-response regulator genes *TOC1*, *PRR5(9)* and *PRR9(5)*, *PRR3(7)* and *PRR7(3)* in different land plant species. Work that has previously described these genes is shown for each species and genes, except Arabidopsis.

	TOC1	***PRR5(9)* and *PRR9(5)***	***PRR3(7)* and *PRR7(3)***
Arabidopsis thaliana	At5g61380	At5g24470 (*PRR5*) At2g46790 (*PRR9*)	At5g60100 (*PRR3*) At5g02810 (*PRR7*)
Zea mays	GRMZM2G020081 (*TOC1*) (Wang *et al.*, 2011b) GRMZM2G148453 [a] (*TOC1b*) (Hayes *et al.*, 2010)	GRMZM2G135446 (*PRR59*) (Hayes *et al.*, 2010) GRMZM2G179024 (*PRR95*) (Campoli et al., 2012b)	GRMZM2G095727 (*PRR73*) (Hayes *et al.*, 2010) GRMZM2G033962 (*PRR37*) (Hayes *et al.*, 2010) [a] GRMZM2G005732 (PG) [a]
Brachypodium distachyon **(Higgins et al., 2010)**	Bradi3g48880	Bradi4g24967 (*PRR59*) Bradi4g36077 (*PRR95*)	Bradi1g65910 (*PRR73*) Bradi1g16490 (*PRR37*)
Sorghum bicolor **(Takata et al., 2010)**	Sb4g026190 (Murphy *et al.*, 2011)	Sb5g003660 (*PRR59*) Sb2g030870 (*PRR95*) [a]	Sb1g038820 (*PRR73*) Sb6g014570 (*PRR37*) (Murphy *et al.*, 2011)
Oryza sativa **(Murakami et al., 2007)**	LOC_Os2g40510	LOC_Os11g05930 (*PRR59*) LOC_Os9g36220 (*PRR95*)	LOC_Os3g17570 (*PRR73*) LOC_Os7g49460 (*PRR37*)
Hordeum vulgare **(Campoli et al., 2012b)**	Hvcontig_37494 (*HvTOC1*) (Faure *et al.*, 2012)	Hvcontig_46739 (*PRR59*) Hvcontig_41351 (*PRR95*) (Higgins *et al.*, 2010)	Hvcontig_1563982 (PRR73) (Higgins *et al.*, 2010) Hvcontig_94710 (*PPDH1/PRR37*) (Turner *et al.*, 2005; Jones *et al.*, 2008)
Triticum aestivum	TAcdna_241985932 (Campoli et al., 2012b)	Ta_PUT2939165448 (*PRR59*) [c] TAcdna_241983556 (*PRR95*) (Campoli *et al.*, 2012b)	Ta_PUT18538 (*PRR73*) [c] *PPDD1*, *PPDB1*, *PPDA1* (*PRR37*s) (Beales *et al.*, 2007) *
Solanum tuberosum	PGSC0003DMG400033048 (*TOC1*) [a] PGSC0003DMG400019518 (*TOC1-like*)	PGSC0003DMG400000584 (*PRR5*) PGSC0003DMG402011297 (*PRR9*)	PGSC0003DMS000000129 (*PRR3*) [b] PGSC0003DMS000000068 (*PRR7*) [a]
S. lycopersicum	Solyc06g069690 (*TOC1*) Solyc03g115770 (*TOC1-like*)	Solyc03g081240 (*PRR5*) Solyc10g005030 (*PRR9*)	Solyc04g049670/80 (*PRR3*) [a] Solyc10g086000 (*PRR7*)
P. patens	Pp1s412_23 (*PpPRR1*), Pp1s81_131 (*PpPRR2*), Pp1s412_35 (*PpPRR3*), Pp1s81_144 (*PpPRR4*) (Holm *et al.*, 2010; Satbhai *et al.*, 2010)		

* Three alleles, sequenced in hexaploid bread wheat, are a homoeoallelic series on the group 2 chromosomes. Sequences retrieved from literature (Beales *et al.*, 2007).
a: re-annotated; b: annotated; c: partial sequence; PG: pseudo-gene.
Phylogenetic tree is shown in Figure 3.6.

Table 3.4: Homologues of *ZTL*, *FKF1*, *GRP7* and *GRP8* genes in different land plant species.

	ZTL	***FKF1***	***GRP7* and *GRP8***
Arabidopsis thaliana	At5g57360 (*ZTL*) At2g18915 (*LKP2*)	At1g68050	At2g21660 (*GRP7*) At4g39260 (*GRP8*)
Zea mays	GRMZM2G113244 (*ZmZTLa*) GRMZM2G147800 (*ZmZTLb*) GRMZM2G115914 (PG)	GRMZM2G106363 (*ZmFKF1a*) GRMZM2G107945 (*ZmFKF1b*) (Hayes *et al.*, 2010)	GRMZM2G165901 (*GRP7a*) GRMZM2G080603 (*GRP7b*)
Brachypodium distachyon	Bradi1g33610 (*BdZTLa*) Bradi3g04040 (*BdZTLb*) (Higgins *et al.*, 2010)	Bradi4g16630 (Higgins *et al.*, 2010)	Bradi1g12787 (*GPR7a*) Bradi4g00940 (*GRP7b*)
Sorghum bicolor	Sb10g028340 (*SbZTLa*) Sb04g003660 (*SbZTLb*)	Sb05g021030	Sb08g022740 (*GRP7a*) Sb01g012300 (*GRP7c*)
Oryza sativa	LOC_Os02g05700 (*OsFBO08/ZTL1*) LOC_Os06g47890 (*OsFBO09/ZTL2*) (Murakami *et al.*, 2007)	LOC_Os11g34460 (Murakami *et al.*, 2007; Higgins *et al.*, 2010)	LOC_Os03g46770 (*GRP3*) LOC_Os12g43600 (*GRP6*) (Kim *et al.*, 2010)
Hordeum vulgare	Hvcontig_273830 (*HvZTLa*) Hvcontig_158755 (*HvZTLb*)	Hvcontig_38586	Hvcontig_1578172 (*GRP7a*) [a] (Campoli et al., 2012b) Hvcontig_43832/46175 (*GRP7b*) [a]
Triticum aestivum	TAcdna_241984947 (*TaZTLa*) Ta_PUT43520 (*TaZTLb*) [c]	TAcdna_118767204	Tacdna_241988564 (*GRP7a*) and Tacdna_114145393 (*GRP7a'*) Tacdna_974604 (*GRP7b*) and Tacdna_241988180 (*GRP7b'*)
Solanum tuberosum	PGSC0003DMS000000971 [b]	PGSC0003DMG400019971	PGSC0003DMG400000708 (*GRP7*) PGSC0003DMG400033902 (*GRP7-like1*) PGSC0003DMG400033903 (*GRP7-like2*)
S. lycopersicum	Solyc07g017750 [a]	Solyc01g005300 [a]	Solyc01g109660 (*GRP7*) Solyc10g051380 (*GRP7-like1*) Solyc10g051390 (*GRP7-like2*)
P. patens	**None** (Holm *et al.*, 2010)	**None** (Holm *et al.*, 2010)	Pp1s42_251 (*GRP1*), Pp1s123_58 (*GRP2)* (Nomata *et al.*, 2004) Pp1s136_70

a: re-annotated; b: annotated; c: partial sequence; PG: pseudo-genes.

Table 3.5: Dicot-specific homologues of *ELF4* and *EEC* in three dicotyledonous plant species and the Arabidopsis-specific *CHE* and *TSF*.

	ELF4	***EEC***	***CHE***	***TSF***
Arabidopsis thaliana	At2g40080 (*ELF4*) At2g29950 (*ELF4-like1*)	At3g21320	At5g08330	At4g20370
Solanum tuberosum	PGSC0003DMG400006624 (*ELF4*) PGSC0003DMG400001221 (*ELF4-like6*) PGSC0003DMG400030357 (*ELF4-like5*)	PGSC0003DMG400004837	**None**	**None**
S. lycopersicum	Solyc06g051660 (*ELF4*) Solyc11g028200 (*ELF4-like5a*) Solyc06g076960 (*ELF4-like5b*) scf7180001945491 [PG]	Solyc06g062480	**None**	**None**

PG: Tomato pseudogene: sequence present in the Tomato WGS Alternate Scaffolds cabog1.00.

Table 3.6: Homologues of *CO*, *FT* and *ELF4-like* genes in different land plant species.

	CO	***FT***	***ELF4-like2/3/4***
Arabidopsis thaliana	At5g15840 (*CO*) At5g15850 (*COL1*) At3g02380 (*COL2*)	At1g65480	At2g06255 (*ELF4-like3*) At1g17455 (*ELF4-like4*) At1g72630 (*ELF4-like2*)
Zea mays	GRMZM2G405368 (*CONZ1*) (Miller *et al.*, 2008) *ZmCO2* [PG]	GRMZM2G373928 (*ZNC14*) GRMZM2G051338 (*ZCN15*) (Danilevskaya *et al.*, 2008)	GRMZM5G877647 (*ELF4-like3*) GRMZM2G382774 (*ELF4-likeB1*) GRMZM2G359322 (*ELF4-likeB2*) GRMZM2G025646 (*ELF4*) (Zhang, 2011)
Brachypodium distachyon	Bradi1g43670 (*CO1*) Bradi3g56260 (*CO2*) (Higgins *et al.*, 2010)	Bradi2g07070 (*FTL1*) Bradi1g48830 (*FTL2*) (Higgins *et al.*, 2010)	Bradi4g13227 (*ELF4-like3*) Bradi4g29580 (*ELF4-likeA*) Bradi1g60090 (*ELF4-likeB*)
Sorghum bicolor	Sb10g010050 (*CO1*) (Murphy *et al.*, 2011) Sb04g029180 (*CO2*) [c]	Sb10g003940 (*FTL2*) (Murphy *et al.*, 2011) Sb03g001700 (*FTL1*)	Sb05g025110 (*ELF4-like3*) Sb02g023990 (*ELF4-likeA*) Sb01g032750 (*ELF4-likeB*)
Oryza sativa	LOC_Os06g16370 (*HD1* or *OsA*) (Yano *et al.*, 2000; Cockram *et al.*, 2012)	LOC_Os06g06300 (*FTL3_RFT1*) LOC_Os06g06320 (*FTL2_ Hd3a*) LOC_Os01g11940 (*FTL1*) (Faure *et al.*, 2007)	LOC_Os11g40610 (*ELF4-like3*) LOC_Os03g29680 (*ELF4-likeB*) LOC_Os08g27860 (*ELF4-likeA1*) LOC_Os08g27870 (*ELF4-likeA2*)
Hordeum vulgare	Hvcontig_138334 (*CO1*) (Campoli *et al.*, 2012a) Hvcontig_6805 (*CO2*) (Griffiths *et al.*, 2003)	Hvcontig_54983 (*FT1* or *VRN-H3*) (Yan *et al.*, 2006) Hvcontig_1558556/136243 (*FT2*) (Faure *et al.*, 2007)	Hvcontig_42805 (*ELF4-likeA*) (Kolmos et al., 2009) Hvcontig_58806 (*ELF4-like3*)
Triticum aestivum	Tacdna_169807975 (*WCO1*) (Shimada *et al.*, 2009) Tacdna_36789816 (*HD1-3*) *, GenBank_AB094488 (*HD1-2*)* [c] and Tacdna_36789805 (*HD1-1*)* (Nemoto *et al.*, 2003)	Tacdna_169807973 (*FT1a*)*, Tacdna_40644759 (*FT1b*) [a] * and Tacdna_56694631 (*FT1c*) * Tacdna_ 32128602 (*FT2*) (Yan *et al.*, 2006)	TaPUT_145474 (*ELF4-like3*) [a] TaPUT_3048165449 (*ELF4-likeA*)
Solanum tuberosum	PGSC0003DMG402010056 (*COL1*) PGSC0003DMG401010056 (*COLa*) (González-Schain *et al.*, 2012)	PGSC0003DMG400023365 (*SP6A*) (Initiative, 2010; Consortium *et al.*, 2011) PGSC0003DMB000000142 (*FT_SP3D*) (Navarro *et al.*, 2011) PGSC0003DMB00512 (*SP5Ga* and *SP5Gb*) [b]	PGSC0003DMG400002144 (*ELF4-like3*) PGSC0003DMG400009846 (*ELF4-like8*) PGSC0003DMG400011596 (*ELF4-like7*)
S. lycopersicum	Solyc02g089540 (*TCOL2*) Solyc02g089520 (*TCOL3*) (Ben-Naim *et al.*, 2006)	Solyc05g055660 (*SP6A*), Solyc03g063100 (*FT_SP3D*) Solyc05g053850 (*SP5G*) (Carmel-Goren *et al.*, 2003)	Solyc07g041340 (*ELF4-like3*) Solyc12g049290 (*ELF4-like7*)
P. patens	None	**None** (Hedman *et al.*, 2009; Karlgren *et al.*, 2011)	Pp1s180_31 (*ELF4-like3*)

* Three alleles, sequenced in hexaploid bread wheat, are derived from three homoeologous genomes.
a: partial sequence; b: annotated; c: re-annotated; PG: pseudogene.

3.3.2 – Definition of genomic structure of clock genes

From the cross-species reciprocal BLAST data, genomic sequences of genes related to Arabidopsis clock genes were downloaded from the various plant databases for analysis. In some cases, genome annotation is incomplete. Therefore, all genomic sequences selected were controlled for quality by comparing gene structure (number of exons), open reading frames (ORFs) and exon-intron junctions with available EST data. On this basis, gene sequences were correctly annotated or re-annotated, giving a final set of genomic sequences for each clock gene in each species (data not shown). The 21 genes which were (re)annotated are shown in Table 3.2, Table 3.3, Table 3.4, and Table 3.6. One example of re-annotation occurred for the potato *LHY* gene. Approximately 96% of *StLHY* genomic structure was not annotated in the potato database (shown in grey in Figure 3.3). Manual re-annotation of *StLHY* was based on the annotation from the orthologous sequence, Solyc10g005080 (*SlLHY*), while carefully observing the presence of GT and AG dinucleotides for intron boundaries and ORF maintenance.

Detailed analysis of genomic segments uncovered a peculiar aspect regarding the *LHY* and *CCA1* genes in the dicot species analysed. The genomic segment where *LHY* is located in the tomato and potato genomes is close to the *PRR9* gene locus (Figure 3.3). Close to *AtPRR9* on chromosome 2 is *AtCCA1*, whereas *AtLHY* is on a different chromosome (chromosome 1). The genomic segment where *AtLHY* is located has no clear co-linearity with tomato or potato, or with the segment of chromosome 2 where *AtCCA1* is located (data not shown). This suggests that selective pressure to maintain *LHY* in Arabidopsis occurred on the duplicated gene copy (on chromosome 1), while the copy on chromosome 2, next to *PRR9*, accumulated mutations and is now known as *AtCCA1*.

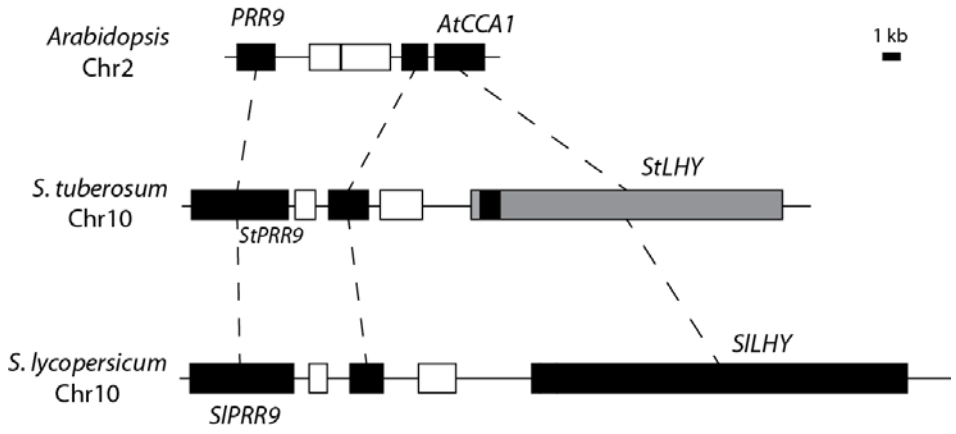


Figure 3.3: Co-linear segments of Arabidopsis chromosome 2, and potato and tomato chromosomes 10, containing *PRR9* and *CCA1/LHY*. Boxes represent whole genes (exon/intron structure is not illustrated) and their colours refer to sequence conservation: black and grey mean conserved sequences, connected by dotted lines, whereas white are non-conserved genes. In particular, grey also indicates non-annotated sequence in its database.

Gene annotation within the barley genome gene space was hitherto not available. Therefore, all barley genomic sequences retrieved were manually annotated. Annotation was carried out by comparing genomic sequences with available transcript data for barley and, in a few cases, bread wheat and *Brachypodium*. In this manner, a final set of genomic structures for all barley clock orthologues and paralogues was defined.

It is worth mentioning a particular complication of some barley clock genes and their genomic sequences. The genomic sequences of *HvLHY*, *HvGI*, *HvELF3*, *HvGRP7b* and *HvFT2* are found on two different contigs in the barley database for each gene (Table 3.2, Table 3.4 and Table 3.6). Additionally, the 5' end sequence of *HvPRR95* is not present in the barley database. To identify the missing sequence linking both contigs and the 5' end sequence of *HvPRR95*, a search for genomic and transcript sequences was carried out in the GenBank database. These analyses allowed the complete characterisation of the genomic sequence of *HvELF3*, *HvGRP7b* and *HvFT2*. Unfortunately, the sequence of intron 2

from *HvLHY* and the 5' end of *HvPRR95* were not retrieved due to their unavailability in barley and GenBank databases.

In comparison with Arabidopsis, the genomic structures of barley genes are considerably conserved in regards to their exon/intron organisation, including the single exon genes *LUX* and *CAB*. However, differences also occur. The gene structures of barley orthologues differ from those of Arabidopsis mainly in the size of introns, which are generally larger in barley, and in the UTR sequences. A clear example is the 5' UTR of *LHY* in barley, which is considerably longer and has a complex multi-exon structure, while *AtLHY* only has two small 5' UTR introns. However, the unavailability of a complete *HvLHY* intron 2 sequence, present in the 5' UTR, compromises robust comparison with the 5' UTR of *AtLHY*. Therefore, further assumptions regarding *HvLHY* 5' UTR must be treated carefully. More information and figures of genomic structures from barley and Arabidopsis clock genes are found in Chapter 5.

3.3.3 – Phylogeny of clock genes

To demonstrate and confirm the degree of relatedness of identified orthologous genes, phylogenetic trees were generated using the neighbour-joining method (Saitou and Nei, 1987) with MEGA software version 5.05 (Tamura *et al.*, 2011). For true gene homology, phylogenetic trees should reflect evolution of the species analysed (Figure 3.4). This is observed for the majority of cases, confirming that the sequences identified are homologues of the Arabidopsis genes. However, a few rice genes do not display the expected topology, and they are represented in grey on the respective gene trees. Detailed analyses for each gene are described below:

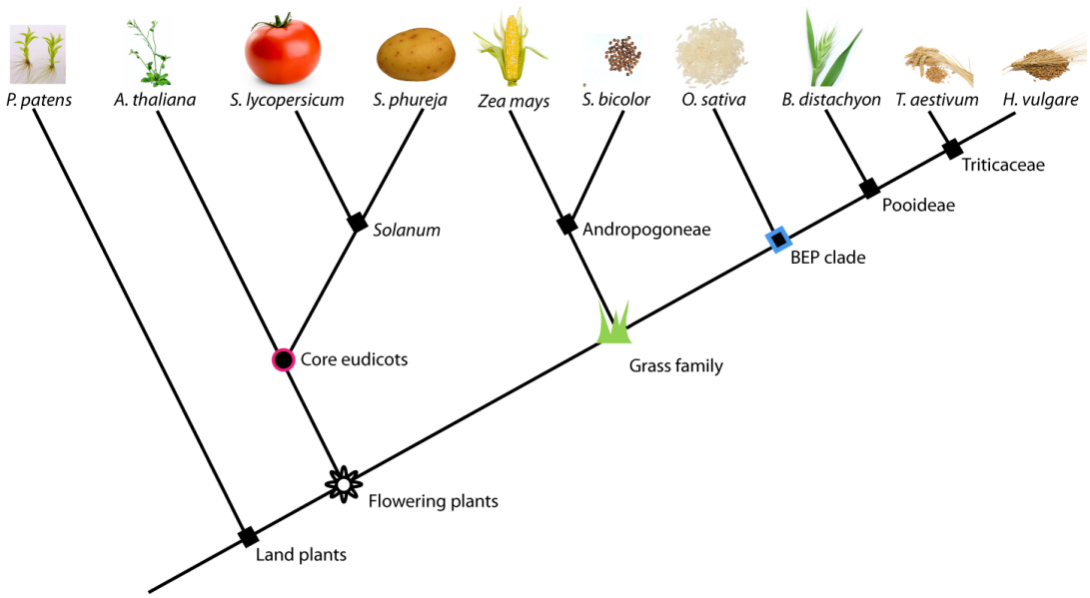


Figure 3.4: Expected tree of life of the 10 land plant species used in this study. Labelling of each node was based on the lowest rank of the common ancestor (according to Benson *et al.*, 2009). Blue square: BEP clade; red circle: core eudicots; green grass: grass family; flower: flowering plants.

- *LHY* and *CCA1* (Table 3.2 and Figure 3.5A). True orthologues of *AtLHY* were identified in all species analysed. Barley and six other plants have a single *LHY* counterpart, whereas *LHY* gene duplications possibly occurred independently in maize, *P. patens* and Arabidopsis, which gave rise to *AtCCA1*.
- *LUX* (Table 3.2 and Figure 3.5B). True orthologues of the *AtLUX* gene were identified in all species analysed, including four copies in *P. patens*. In this case, complex results from BLAST and phylogenetic analysis made the identification of the true *P. patens* orthologue difficult. Arabidopsis has a recently duplicated *LUX* paralogue, known as *AtBOA*.
- *ZTL* and *FKF1* (Table 3.4 and Figure 3.5C). Gene members of the LOV (light, oxygen or voltage) blue light receptor subfamily were identified in all flowering plants analysed and mostly have a single exon. *In silico* analyses suggest that the ancestor of

flowering plants carried both *ZTL* and *FKF1* genes. A single copy of *FKF1* is maintained for both monocots and dicots, except for maize, which has two copies due to a recent duplication. The *ZTL* gene has been duplicated in both the ancestor of monocots and in Arabidopsis. As a result, monocots have *ZTLa* and *ZTLb* genes, while Arabidopsis has *ZTL* and the recent copy, *LPK2*. The exact relationships between both monocot *ZTL* genes and the dicot *ZTL* could not be determined, i.e. the true orthologue of *AtZTL* in monocots is either *ZTLa* or *ZTLb*.

- *PRRs* (Table 3.3 and Figure 3.6). Most flowering plants analysed have five *PRR* genes. The *TOC1* gene is duplicated in maize and both *Solanum* species. *P. patens* has four *PRRs*, which are very closely related to the *PRRs* of angiosperms. It was not possible to determine *PRR* orthologues due to very complex results from BLAST and phylogenetic analysis. The only evidence observed is that the ancestor of flowering plants had *TOC1*, *PRR3/7* and *PRR9/5* genes. After the divergence of monocots and dicots, both ancestors independently duplicated *PRR3/7* and *PRR9/5* genes. The monocot paralogous genes *PRR73* and *PRR37* are highly similar to the protein and gene structure of *AtPRR7*, as opposed to *AtPRR3*. However, this similarity could not be observed on the *PRR* gene tree (Figure 3.6).

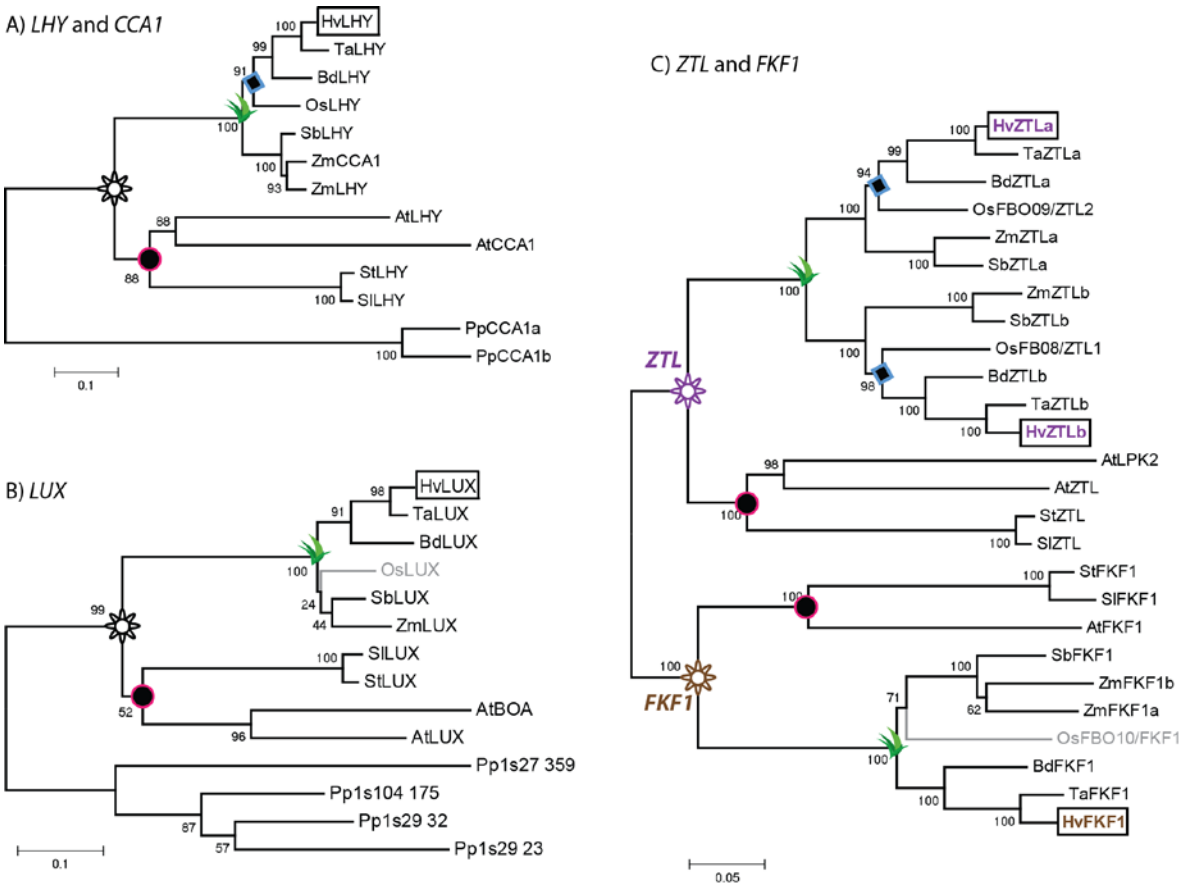


Figure 3.5: Phylogenetic trees of *LHY*, *CCA1*, *LUX*, *ZTL* and *FKF1* genes. A) *LHY* and *CCA1* tree. B) Phylogenetic tree of *LUX* genes. Due to the lack of complete CDS data for the *TaLUX* gene, the partial related CDS from PUT0106334 was used to represent bread wheat *LUX*. C) Phylogenetic tree of *ZTL* and *FKF1* genes. Due to the lack of complete sequence information for the *TaZTLb* gene, the partial bread wheat *ZTLb* CDS from PUT43520 was used to represent bread wheat. Since *P. patens* does not contain a true orthologue of *ZTL* or *FKF1*, the root was placed on the *FKF1* family branch. Evolutionary distances are presented in number of base substitutions per site. In constructing the trees, all gaps and missing data were eliminated from sequence alignments. Genes that do not follow expected topology are found in grey. Labelling of each node was based on Figure 3.4. Barley genes are boxed.

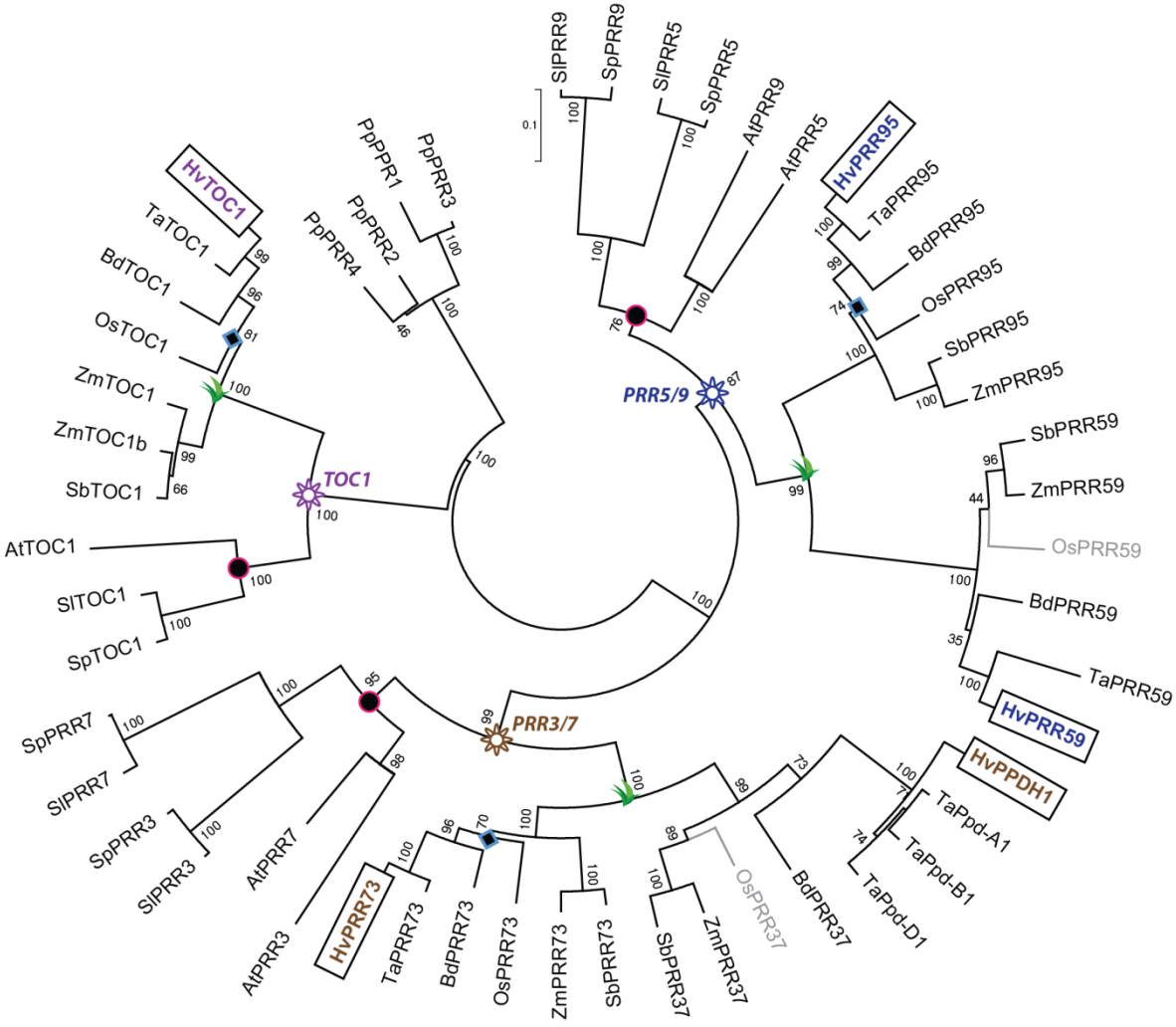


Figure 3.6: Phylogenetic tree of *PRR* genes identified by cross-species reciprocal BLAST. For simplicity, *TOC1-like* sequences from tomato and potato were not included in the analysis. Alignment is based on putative mRNA sequences. Due to the lack of complete CDS data for the *TaPRR73* and *TaPRR59* genes, the partial related cDNAs from PUT18538 and PUT2939165448, respectively, were used to represent these bread wheat branches. The evolutionary distances are presented in number of base substitutions per site. In constructing the tree, all gaps and missing data were eliminated from the sequence alignment. Genes that do not follow expected topology are found in grey. Labelling of each node was based on Figure 3.4. Barley genes are boxed.

- *CO* (Table 3.6 and Figure 3.7A). Homologous members of the *AtCO* subfamily were identified in all flowering plants analysed, including barley. Protein alignment and BLAST analyses suggest that the ancestor of flowering plants contained one copy of a *CO*-related gene, which is the orthologue of *AtCO* or *AtCOL1*. Results from cross-species reciprocal BLAST analysis were very complex and made the identification of true *CO*-related orthologues very difficult. Two independent duplication events have occurred within the Arabidopsis branch, which currently has *AtCOL1*, *AtCO* and *AtCOL2*. Monocots have one duplication event of the original *CO*-related gene, giving rise to both *CO1* and *CO2*. Rice and maize have lost their *CO2* gene copy. The exact relationship between both *CO1* and *CO2* genes in monocots and the dicot *CO*-related genes could not be determined, but homologues are clearly present. Exon numbers for homologous genes commonly varied between one and two among the species analysed (Figure 5.6).
- *FT* (Table 3.6 and Figure 3.7B). The true orthologue of *AtFT* in monocots could not be determined, but at least two homologues (*FT1* and *FT2*) are present in all monocots analysed. Rice in particular has two copies of the *FT1* gene (*OsFTL2* and *OsFTL3*). Exon numbers for homologous genes varied between three and four among the species analysed.
- *ELF3*(*-like*) (Table 3.2 and Figure 3.7C). Homologues of *AtELF3* were identified in all species analysed. Paralogues were also observed and are probably due to a series of duplication events of the *ELF3* gene. All *in silico* analyses suggest that the ancestor of land plants contained one copy of the *ELF3* gene. Two independent duplication events occurred within the *P. patens* branch, which has three homologues of *ELF3*. The original *ELF3* gene was also duplicated in the ancestor of flowering plants, which then contained both the *ELF3* gene and the new copy, *ESSENCE OF ELF3 CONSENSUS* (*EEC*)

gene. However, this hypothesis for the origin of *EEC* has low support from phylogenetic analysis (59% likelihood, Figure 3.7) and must be treated with care. Monocots have lost the *EEC* gene and duplicated *ELF3*, creating the *ELF3a* and *ELF3b* genes. Temperate grasses (Pooideae) lost the *ELF3b* gene, whereas rice lost *ELF3a*. Interestingly, the *ELF3b* copy present in the rice genome has undergone a recent event of duplication. The exact relationships between both *ELF3* alleles in monocots and the dicot *ELF3* could not be determined, i.e. the true orthologue of *AtELF3* was not identified but homologues are clearly present.

- *GI* (Table 3.2 and Figure 3.7D). True orthologues of *GI* were identified in all flowering plants analysed. One duplication event occurred in both maize and the *Solanum* ancestor.

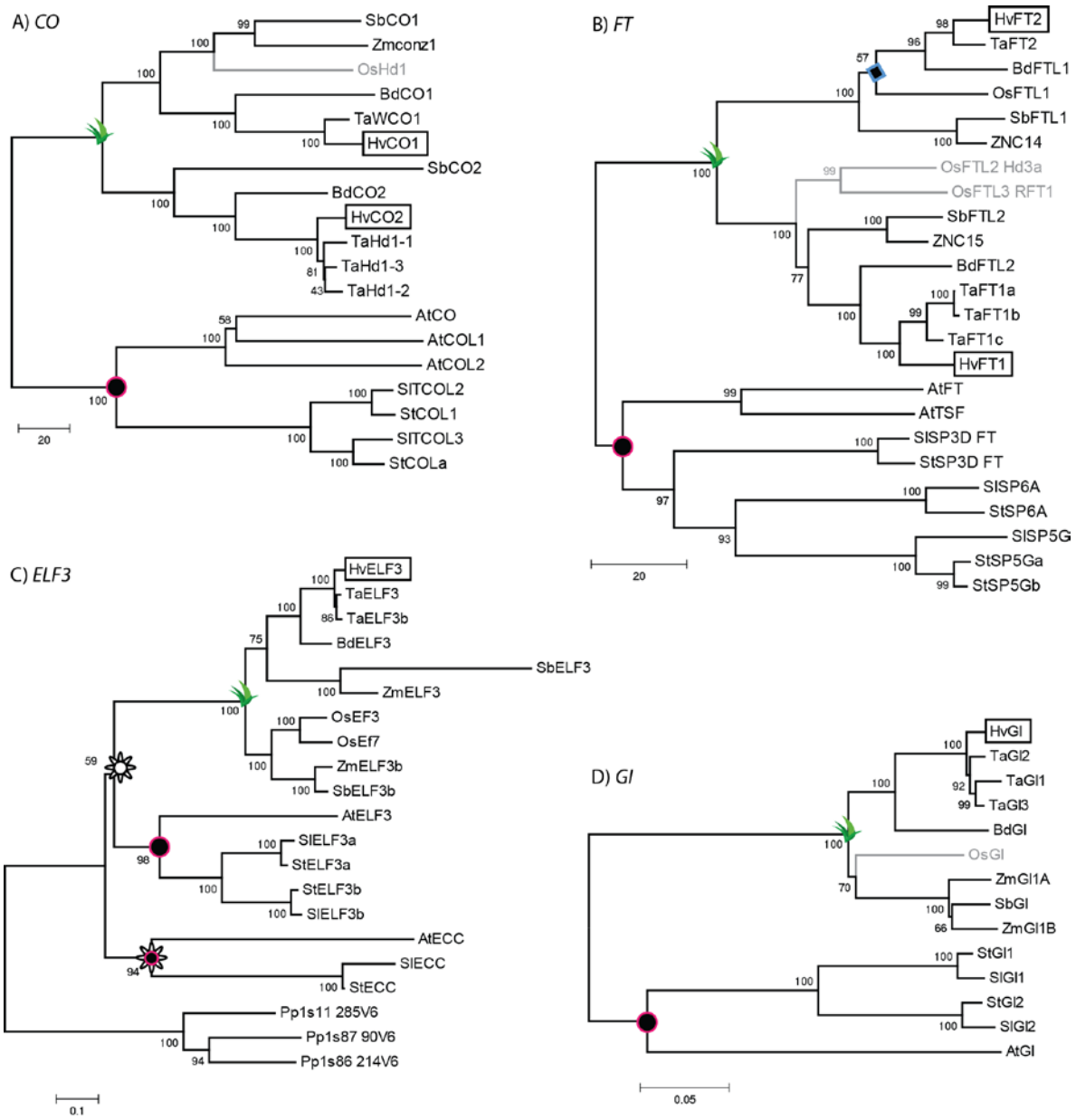


Figure 3.7: Phylogenetic trees of *CO*, *FT*, *ELF3* and *GI* genes. A) Subfamily of *COL* genes from Group Ia, which includes *AtCO*. B) Genes of the *FT* subgroup. C) *ELF3* and *EEC* genes. In constructing the tree, all gaps and missing data were eliminated from the *CO*, *FT* and *ELF3* sequence alignments. D) *GI* genes. In constructing the tree, all gaps and missing data were eliminated from each pairwise sequence alignment. *P. patens* does not contain a true orthologue of *FT*, *CO*, or *GI* and therefore the root was placed on the dicot *FT*, *CO*, and *GI* branches, respectively. The evolutionary distances are presented in number of base differences per site. Genes that do not follow expected topology are found in grey. Labelling of each node was based on Figure 3.4. Barley genes are boxed.

- *ELF4*(*-like*) (Table 3.5, Table 3.6 and Figure 3.8A). Gene orthologues of *AtELF4* could not be identified in any monocot species analysed, including barley. The whole set of *in silico* analyses suggests two subgroups for the *ELF4-like* family: *ELF4*, which includes *AtELF4* and *AtELF4-like1* (Table 3.5), and *ELF4-like2/3/4* (Table 3.6). *ELF4* family members are found only in dicot species and they are single exon genes. On the other hand, *ELF4-like2/3/4* family members are found in all plants analysed and most of them have a 5' UTR intron. Analysis at the protein sequence level suggests that the ancestor of land plants contained one copy of the *ELF4-like* gene, most likely an orthologue of *AtELF4-like3*. This gene was duplicated in the ancestor of flowering plants, which then contained both *ELF4-like3* and the new copy, *ELF4*. Monocots lost the *ELF4* gene while dicot species duplicated this gene multiple times. The *ELF4-like3* gene was duplicated twice in monocots, but barley and bread wheat may have lost one of the copies. Dicots also had one or two duplication events from the *ELF4-like3* gene and its subsequent copies. In summary, orthologues of *AtELF4* were identified only in dicot species (tomato and potato). On the other hand, *AtELF4-like3* orthologues are present in all land plant species.
- *GRP7* and *GRP8* (Table 3.4 and Figure 3.8B). Single-intron homologues of *AtGRP7* were identified in all species analysed. *In silico* analyses suggest that the ancestor of land plants contained one copy of the *GRP7* gene. Two independent duplication events occurred within the *P. patens* branch, generating *PpGRP1*, *PpGRP2* and Pp1s136_70. The *GRP7* gene has undergone a series of independent duplications within dicots and once in monocots. In Arabidopsis, it is likely that this duplication gave rise to *AtGRP8*, according to cross-species BLASTs. In monocots, there are two copies of the *GRP7* gene, which are hereafter called *GRP7a* and *GRP7b*. Rice has lost *GRP7a* and duplicated *GRP7b*. Strangely, bread wheat seems to be the only species with a

third copy, *TaGRP7c*, according to sequence analysis of PUT19138 (data not shown). The putative GRP7c protein encoded by this contig is around half the size of the other GRPs in monocots. Due to this unique feature, *GRP7c* might be a pseudogene or an error from sequencing and alignment procedures, and it was eliminated from further analyses.

- *CHE* (Table 3.5). Cross-species reciprocal BLAST using the single exon gene *AtCHE* could not identify any orthologue in any species analysed. Another Arabidopsis gene, At5g23280 (*AtTCP7*), was identified by reciprocal BLAST. Subsequently, the newly identified *AtTCP7* was used in cross-species reciprocal BLAST. This analysis confirmed *AtTCP7* orthologues in all species analysed, as well as several paralogues (data not shown). *AtCHE* is likely one of its paralogues, present only in Arabidopsis (of all species analysed here). Barley gene members of the *TCP7/CHE* subfamily were discarded from further analysis for a number of reasons. Firstly, AtCHE is a transcriptional repressor of *AtCCA1*. It is likely that both genes are not present in monocots (discussed in Section 3.4.3). Secondly, there is hitherto no evidence of any involvement of *AtTCP7* in clock regulation. Lastly, *HvTCP7* is a single-exon gene, and is therefore unlikely to have AS.
- *CAB2* (data not shown). Orthologues of *AtCAB2* could not be identified in any species analysed. This gene belongs to a large family of single exon genes and other gene members are found in dicots and monocots: Arabidopsis has 5 genes that belong to this family, while *Brachypodium* has 4 genes, maize has 6 genes, sorghum has 5 genes and rice has 3 genes (Plaza, 2011). Barley and bread wheat have at least 17 gene members (data not shown). Complex results from cross-species reciprocal BLAST analysis made the identification of a true *CAB* orthologue very difficult.

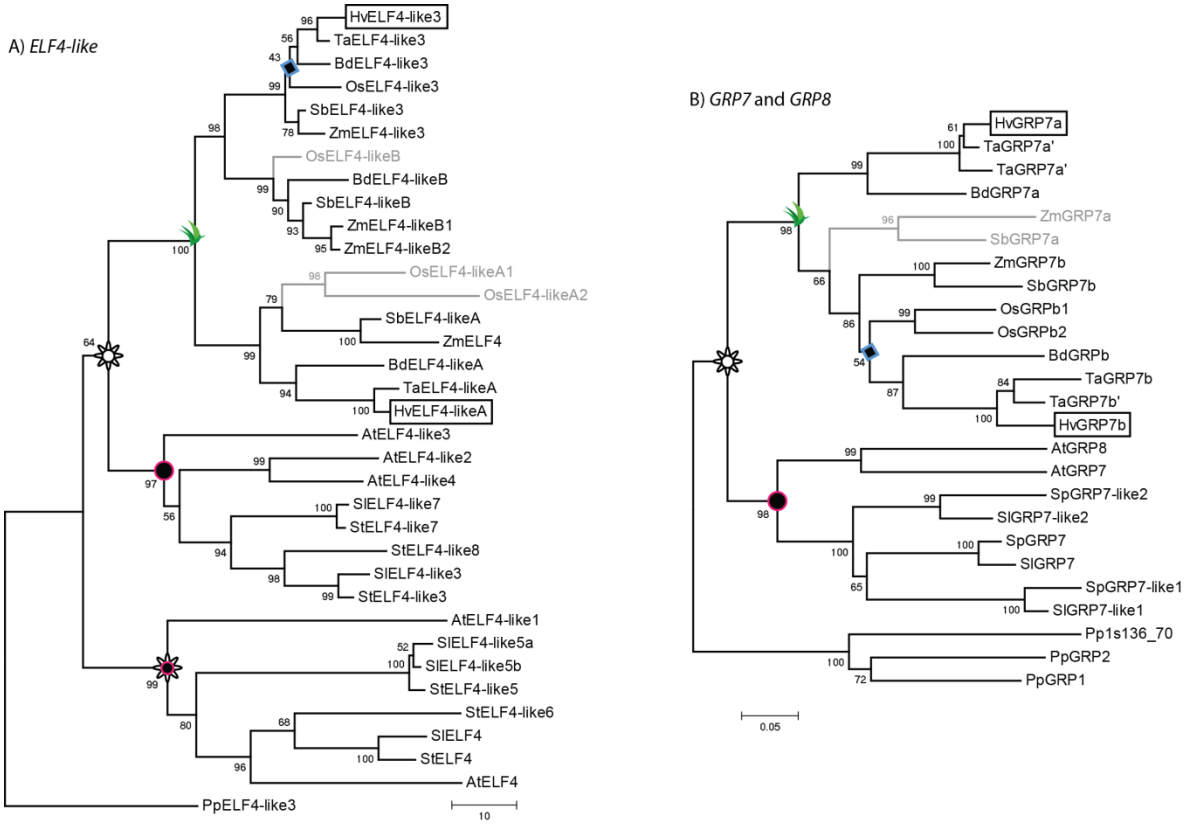


Figure 3.8: Phylogenetic trees of *ELF4*-related, *GRP7* and *GRP8* genes. A) Genes of the *ELF4-like* family. Due to the lack of complete CDS data for the *TaELF3-like3* the partial related cDNA from PUT145474 was used to represent this bread wheat branch. In constructing the tree, all gaps and missing data were eliminated from the sequence alignment. B) *GRP* genes. In constructing the tree, all gaps and missing data were deleted from each pairwise sequence alignment. Evolutionary distances are presented in number of base differences per site. Genes that do not follow expected topology are shown in grey. Labelling of each node was based on Figure 3.4. Barley genes are boxed.

3.3.4 – Reference genes

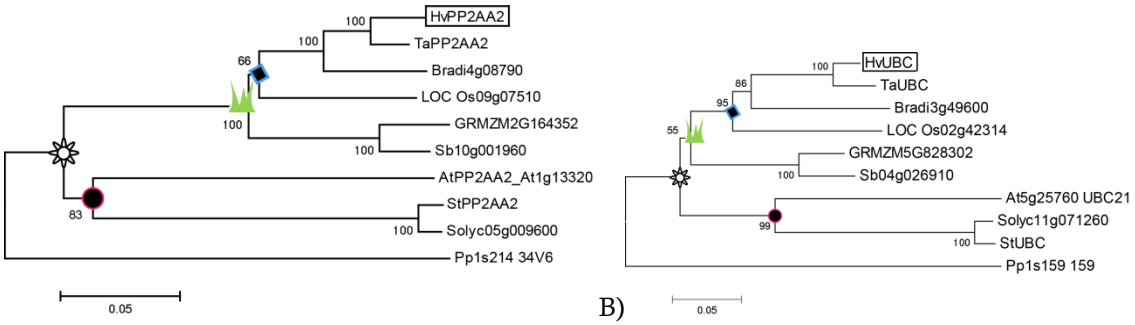
In order to analyse the expression profiles of all clock genes (Chapter 4) reference genes which have constant expression levels are required for normalising transcript levels. Thus, reference genes were identified from the barley genome gene space (Table 3.7). Identification was carried out using a set of robust *in silico* analyses that was comprised of cross-species reciprocal BLASTs, followed by genomic structure and phylogenetic analyses. Initially, two Arabidopsis candidate sequences were used in BLAST searches against various databases. These candidates were: *PROTEIN PHOSPHATASE 2AA3* (*AtPP2AA3*) and *POLYUBIQUITIN 21* (*AtUBC21*).

PP2AA3 is present only in Arabidopsis. Nonetheless, *PP2AA2*, another member of the *PP2A* family, is present in all land plants, according to cross-species reciprocal BLASTs (Figure 3.9A). Therefore, *HvPP2AA2* was used as a control gene in barley studies. True orthologues of *UBC21* are present in all land plants and their sequences are highly conserved and reconstructed the expected phylogeny (Figure 3.9B). The expression of *HvPP2AA2* and *HvUBC21* was analysed under a wide range of developmental stages and environmental conditions and they have shown very stable expression levels (see Section 3.4.4)

Table 3.7: Homologues of *AtPP2AA2* and *AtUBC21* genes in different land plant species.

	PP2AA2	***UBC21***
Arabidopsis thaliana	AT3G25800	At5g25760
Zea mays	GRMZM2G164352 (*PP2AA2*) GRMZM2G122135 (*PP2AA2*")	GRMZM5G828302 (*UBC21a*) GRMZM2G161545 (*UBC21b*)
Brachypodium distachyon	Bradi4g08790 (*PP2AA2*) Bradi4g08720 (*PP2AA2*")	Bradi3g49600 (*UBC21*) Bradi4g13871 (*UBC21b*)
Sorghum bicolor	Sb10g001960 (*PP2AA2*)	Sb04g026910 (*UBC21*) Sb09g007410 (*UBCa*)
Oryza sativa	LOC_Os09g07510	LOC_Os02g42314
Hordeum vulgare	Hvcontig_126673	Hvcontig_159435/46087 Hvcontig_137760 [PG]
Triticum aestivum	Tacdna_241984800 (*PP2AA2*) Tacdna_32129024 (*PP2AA2"*) [a]	Tacdna_241987785 [a]
Solanum tuberosum	PGSC0003DMG400012971	PGSC0003DMG402018585
S. lycopersicum	Solyc05g009600	Solyc11g071260
P. patens	Pp1s214_34 Pp1s197_114 Pp1s117_137	Pp1s159_159

a: re-annotated. PG: Pseudogene with no expression data or detectable ORF.



A) B)

Figure 3.9: Phylogenetic tree of A) *PP2AA2* and B) *UBC21* orthologous genes. Symbols on branch nodes represent common ancestors from Figure 3.4. Evolutionary distances are in number of base substitutions per site. Barley genes are boxed.

3.4 – Discussion

3.4.1 – *In silico* identification of clock homologues

Genomic sequences of 21 putative barley homologues of Arabidopsis core circadian clock genes and selected associated genes were identified. This analysis was carried out carefully in order to identify homologous genes and to eliminate any similar unrelated se-

quences, i.e. sequences that are not descended from a common ancestral sequence (Hall, 2008). For this, cross-species reciprocal BLAST searches were carried out on databases of tomato, potato, moss (*P. patens*) and six other grasses: *Brachypodium*, sorghum, bread wheat, maize, rice and barley. This procedure had already been used in other studies, for instance on searches for clock genes in rice (Murakami *et al.*, 2007) and a more extensive identification of all homologous genes (in the whole genome) among photosynthetic eukaryotes (Zimmer *et al.*, 2007). This method has proven to be a highly efficient method for detection of orthologues. Parallel linkage analysis could not be performed because of rare synteny between dicots and monocots, which reduces the number of orthologues detectable by positional conservation. This phenomenon is probably due to the frequent rearrangements, translocations and gene losses that occurred along the evolution and speciation of dicots and monocots (Spannagl *et al.*, 2011).

Subsequently, sequences retrieved from BLAST searches were used in phylogenetic analysis as a test for homology, as it is expected that homologous clusters should reconstruct the tree of life. However, in this study most of the annotated CDSs of putative clock genes did not initially retrieve the expected topology and manual annotation and re-annotation was necessary to obtain the correct phylogeny. Similar mis-annotation issues have previously been described for eukaryotes, bacteria and archaeal genome databases (Veloso *et al.*, 2005; Mariotti and Guigó, 2010). These errors may be caused by automatic gene finding programs that do not recognise uncommon or unique gene features, for instance, long introns.

Even after (re)annotation, some rice genes did not follow the expected topology (in grey in respective gene trees), where they frequently formed groups with sorghum and maize. This phenomenon could have occurred with rice sequences for a few reasons. First, rice (re)annotations might not represent a real full-length transcript. Second, other gene members from the same family are missing, which could cause poorly supported topolo-

gies upon reconstruction of phylogeny. Additionally, the alignment and tree construction methods were not quite appropriate, for instance, NJ is a distance-based method and the use of partial sequences from mis-annotations can result in incongruent results. Also, some species have variable evolutionary rates, which might interfere with the phylogenetic reconstruction (Felsenstein, 1978; Mushegian *et al.*, 1998). Lastly, rice, sorghum and maize have been under a similar selective pressure during domestication, suggesting a convergent evolution of their genes, as previously reported (Paterson *et al.*, 1995; Hu *et al.*, 2003). Regardless of the reason for these rice genes presenting an unexpected topology, they do not interfere with the confirmation that the barley sequences identified are homologues of the Arabidopsis clock-related genes.

Finally, putative homologues of Arabidopsis circadian clock genes were identified in tomato, potato, *P. patens*, *Brachypodium*, sorghum, bread wheat, maize, rice and barley. 40 genes in monocots, including six in barley, were hitherto unknown: for instance *HvZTLa*, *HvZTLb*, *HvGRP7b*, *HvELF4-like3*, *HvFKF1* and *HvCABa*. Most of the genes identified were already known and had previously been used in simple analyses or, less commonly, a fully characterised study. References for these genes are found in Table 3.2–Table 3.6. The identification of previously described genes confirms that the *in silico* method adopted is appropriate for identifying homologues, as well as confirming the identity of the previously described genes. Moreover, the comprehensive list of species with duplicated gene copies is good evidence confirming the number of gene copies in barley. This information, along with the genomic structure of barley genes, is essential for AS studies. Of the 21 genomic sequences identified in barley, thirteen were available or described previously: *LHY* (*CCA1*), five *PRRs*, *LUX*, *CO1*, *CO2*, *FT1*, *FT2*, *GI* and *ELF3* (discussed in the next Section). *HvLHY*, in particular, was not correctly annotated and manual re-annotation was performed.

An important consideration remains that the barley gene space is not complete (Consortium, 2012a). It is therefore possible that the extensive *in silico* analysis conducted here may still have missed possible orthologues or parts of genes. This is clearly the case for the sequences from intron 2 of *HvLHY* and the 5' end of *HvPRR95*. Some barley introns are relatively large with a high proportion of repetitive DNA sequence, which can be difficult to identify and analyse. Therefore, it is likely that technical constrains have caused parts of the genomic sequences of *HvLHY* and *HvPRR95* to be missed. This also helps to explain why some barley genes are found in more than one genomic contig. To identify the missing sequence linking both contigs, a search for additional genomic and transcript sequences was carried out. Similarly, to overcome the absence of whole gene sequences from the barley gene space, transcriptome data were also analysed, but retrieved only sequences already present in the barley gene space. Further and specific discussion of each clock gene, including detailed information regarding the barley clock genes are found below. The overall conclusion and proposed evolutionary history of clock genes are found in Section 6.1 and Figure 6.1.

3.4.2 – Barley core clock and clock-associated genes

HvGI is present as a single copy in the barley genome and it is the true orthologue of *AtGI*, also confirmed elsewhere (Dunford *et al.*, 2005; Campoli *et al.*, 2012b). Another single copy gene in barley is *HvELF3*, which is the homologue of *AtELF3.* Recently, *HvELF3* characterisation was concomitantly published by two independent labs (Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012). In fact, this gene is present as a single copy in Pooideae species but in the work of Yang *et al*. (2013) it was suggested that *Brachypodium*, which belongs to Pooideae (Figure 3.4), has two *ELF3* homologues. Curiously, this information is not present on their phylogenetic tree, which shows one single *BdELF3* (Bradi2g14290), the one

identified in cross-species reciprocal BLASTs (Table 3.2). In the work of Yang *et al.* (2013) no further information is provided regarding the second *ELF3* gene in *Brachypodium*. Therefore, the determination of *ELF3* gene copies in Pooideae could not be clarified.

One of the most important barley clock genes, characterised 8 years ago, is *HvPPDH1* (Turner *et al.*, 2005). This major determinant of barley photoperiod response belongs to the *PRR* gene family, which has another four members in flowering plants, confirmed by *in silico* analysis. The *PRR73* member is a paralogue of *PRR37* (*HvPPDH1)* in monocots (*PRR3* and *PRR7* in dicots), whereas the *TOC1* member is a single copy gene in most species. The monocot genes *PRR59* and *PRR95* (*PRR5* and *PRR9* in dicots) are the other two members. The exact relationships of *PRR3/7* and *PRR5/9* genes between monocots and dicots could not be determined on the basis of sequence divergence (and in the absence of functional information), i.e. it is difficult to distinguish the true ancestral orthologue from paralogues. Similar problems occur for both copies of *ZTLs*, *GRP7s*, *COs* and *ELF3s* in monocots. It is likely that during monocot and dicot speciation both ancestors independently duplicated and maintained most of these genes for different reasons, and it is now difficult to trace back the oldest allele (true orthologue). This hypothesis is also supported by other studies in diverse plant species, including barley (Higgins *et al.*, 2010; Satbhai *et al.*, 2010; Campoli *et al.*, 2012b; Cockram *et al.*, 2012). Despite these difficulties, a few observations can be made regarding the evolution of these genes. The monocot paralogous genes *PRR73* and *PRR37* are more similar to *AtPRR7* as opposed to *AtPRR3*, which is also confirmed by other studies (Turner *et al.*, 2005; Higgins *et al.*, 2010). Regarding *ZTL*, the duplication in Arabidopsis generated *LPK2* which is probably the newest allele. This hypothesis is confirmed by recent work, which suggests that *AtLPK2* arose from the α duplication event in the Brassicaceae lineage and thus confirms *AtZTL* as

the oldest allele (Lou *et al.*, 2012). *AtELF3* and *AtGRP7* might also be the oldest allele, as opposed to *AtEEC* and *AtGRP8*, respectively.

For *ZTL* in particular, there is a peculiar study which found a *ZTL*-type gene in barley, called *HvDRF*, involved in disease resistance (Dagdas *et al.*, 2009). The sequence available for this gene on GenBank (FJ913271) codes for a protein highly similar to *AtZTL* and *TaZTLa*. Unexpectedly, phylogenetic analysis demonstrates that *HvDRF* forms a sister branch with all monocots rather than *TaZTLa* (data not shown), which is incongruent with phylogeny. Moreover, cross-species reciprocal BLASTs using this sequence did not retrieve any orthologue in any species. Additionally, the barley genomic sequence for this gene is not available in any database. Therefore, the evolutionary history of *HvDRF* and its relation with *HvZTLa* and *HvZTLb* could not be determined.

Another member of the LOV (light, oxygen or voltage) blue light receptor subfamily, besides *ZTL*, is *FKF1*. Both members are functionally and evolutionary diverged, which might have started sometime after euphyllophyte (ferns and seed plants) speciation (Suetsugu and Wada, 2013). In contrast to *ZTL*, most monocots and dicots maintained a single copy of *FKF1*, also confirmed elsewhere (Murakami *et al.*, 2007; Higgins *et al.*, 2010), including the hitherto unknown single orthologue in barley.

The MYB-transcription factors *LHY* and *CCA1* play an important role in the regulation of the circadian rhythm in Arabidopsis. However, barley and another six plants analysed have only one counterpart, most likely the *LHY* gene. Detailed discussion of *LHY/CCA1* is found below (Section 3.4.3), which describes dicot-specific genes.

A single homologue of the Arabidopsis *LUX* is present in monocots, also suggested elsewhere (Murakami *et al.*, 2007; Higgins *et al.*, 2010; Khan *et al.*, 2010), while Arabidopsis has duplicated this gene (*AtBOA*, At5g59570). Regarding *HvLUX* in particular, there is a conflicting study where two *LUX* genes were found in barley, called *HvLUX1* and

HvLUX2 (Campoli *et al.*, 2013). In fact, they identified at least two *LUX* genes in other monocot species and they propose that gene duplications occurred independently in the evolution of monocots (*LUX1* and *LUX2*) and Arabidopsis (*LUX* and *BOA*). The sequence available for *HvLUX1* on GenBank (BAJ88719) was identical to *HvLUX* identified by cross-species reciprocal BLAST analysis using *AtLUX* (At3g46640) (Figure 3.5B and Table 3.2). On the other hand, reciprocal BLASTX using *HvLUX2* cDNA against the Arabidopsis protein database identified different genes. The top 3 hits were the transcription factors At3g10760, At2g40970 and At5g050090, which all identify *HvLUX2* on GenBank. Moreover, phylogenetic analysis of the *LUX* superfamily on the Plaza database showed separate clusters of *LUX* genes from land plants, indicating that their common ancestor had both *LUX1* and *LUX2* genes. In this case, *LUX2* in Arabidopsis was duplicated twice and it is now the three transcription factors mentioned above (At3g10760, At2g40970 and At5g050090). Therefore, it is likely that *HvLUX2* belongs to a different subfamily of *LUX*-related genes, which have evolutionary diverged from *HvLUX1* since speciation in land plants. This theory explains the extensive distance between *LUX1* and *LUX2* clades observed on the phylogeny of Campoli *et al.* (2013), as well as their incongruences with phylogeny at the level of order and families.

Homologues of the clock-output gene *AtCO*, which is involved in flowering control, were identified. *AtCO*, it is a member of a subfamily from Group Ia of the COL family (Griffiths *et al.*, 2003; Valverde, 2011). *In silico* analyses suggest Arabidopsis has three members from this subfamily whereas barley has two: *HvCO1* (Griffiths *et al.*, 2003; Campoli *et al.*, 2012a) and *HvCO2* genes (Griffiths *et al.*, 2003). Other monocots also have both gene copies, except rice (also suggested by Cockram *et al.*, 2012) and maize. These species require short day photoperiods to flower while barley, wheat, Arabidopsis, and potato, require long days. Therefore, the absence of the *CO2* gene copy in rice and maize

probably had a critical role in their domestication (Miller *et al.*, 2008; Cockram *et al.*, 2012). Also, *FT* copy number variation plays an important role in the regulation of plant flowering and development (Nitcher *et al.*, 2013).

The central component in mediating the onset of flowering, the *FT* gene, has homologues in all flowering plants analysed. Additional phylogenetic studies confirm that a *FT* gene was present in the angiosperm ancestor and contributed to the evolution of flowering plants (Klintenäs *et al.*, 2012; Pin and Nilsson, 2012). *AtFT* is a member of the phosphatidylethanolamine-binding protein (PEBP) *FT*-like family and it forms a subfamily with *TWIN SISTER OF FT* (*TSF*) (Kobayashi *et al.*, 1999; Faure *et al.*, 2007). Barley has two members from this subfamily: *HvFT1* (Yan *et al.*, 2006) and *HvFT2* (Faure *et al.*, 2007). Other monocots also have both gene copies and rice has a tandem duplication of the *FT1* copy (Chardon and Damerval, 2005). The monocot *FT1/FT2* duplication occurred after the divergence between the monocots and dicots. Therefore, this duplication is independent of the *FT/ TSF* duplication in Arabidopsis, as suggested previously (Li and Dubcovsky, 2008).

3.4.3 – Dicot-specific clock genes

Orthologues of four Arabidopsis genes from the initial candidate list were not identified in barley and most other plant species: *ELF4*, *CAB2*, *CHE* and *CCA1*.

3.4.3.1 – *ELF4*

ELF4 is likely to be a dicot-specific gene in that it could not be identified in moss or monocot species, but only in dicots (Arabidopsis, tomato and potato). In fact, only members of the *ELF4-like2/3/4* clade are found in monocots. This hypothesis is also supported

by the Plaza database (Plaza, 2011) and other studies (Boxall *et al.*, 2005; Murakami *et al.*, 2007; Higgins *et al.*, 2010). However, in the work of Kolmos et al. (2009), a Bayesian consensus tree suggested that *AtELF4* and *AtELF4-like1* are the closest homologues of *ELF4-like* genes in monocots. It is noteworthy that in their study, some *ELF4* family members were missing from most monocot species analysed, which might have created the poorly-supported topology that suggested such homology.

3.4.3.2 – *CAB2*

CAB2 belongs to a large family of light-harvesting chlorophyll a/b-binding proteins (CAB) whose evolution has been highly debated (Dittami *et al.*, 2010). Nevertheless, several observations suggest that *CAB2* is not present in monocots. Cross-species reciprocal BLASTs did not identify *CAB2* orthologues in any other plant species. *AtCAB2* is likely to be a tandem duplication of *AtCAB1*, which occurred during the evolution of the Arabidopsis ancestor (Plaza, 2011). Therefore, each taxon, moss, monocots and dicots, might have evolved their own *CAB* genes independently during their evolution (Green, 2001; Umate, 2010).

CAB genes have proven roles in clock functions and barley has more than 17 family members. In order to reduce the number of barley *CAB* genes for further analysis, a careful selection took place. *HvCABa* was chosen because it is the closest to Bradi4g07380 on the phylogenetic tree (data not shown). Bradi4g07380 is the top BLAST hit when any of the five Arabidopsis *CAB* sequences are used. In addition, *HvCABa* is represented in the array feature baak26h09, which was identified as being differentially expressed in the clock mutant *eam8* (*elf3* loss-of-function), when compared with WT (Faure et al 2012). HvCABa is a hitherto uncharacterised paralogue of the barley *CAB* family. Other *HvCAB* members have

been identified and analysed elsewhere (Beator *et al.*, 1992; Campoli *et al.*, 2012b; Faure *et al.*, 2012).

3.4.3.3 – *CHE*

CHE (*TCP21*) was not identified in any other species, according to cross-species reciprocal BLAST analysis, and is present only in Arabidopsis (of all species analysed). In fact, *CHE* has a recent duplication (~50 Mya, from the α duplication event) from *AtTCP7* (At5g23280) in Arabidopsis (Navaud *et al.*, 2007) and thus, it is not expected to be present in the other plant species analysed in this study. Higgins *et al.* (2010) describes a *CHE* gene in *Brachypodium*, Bradi3g60350 (*BdCHE*), but cross-species reciprocal BLAST disproves this inference. *AtTCP7* belongs to an angiosperm-specific subfamily of *TCP*s (Navaud *et al.*, 2007) and is likely to be the real orthologue of *BdCHE*. This suggestion is also supported by information in the Plaza database (Plaza, 2011).

3.4.3.4 – *CCA1*

CCA1, along with *LHY*, plays an important role in the regulation of the circadian rhythm in Arabidopsis, but the presence of both counterparts in the genome of other plant species does not seem to be common. Barley and six other plants analysed have only one *LHY/CCA1* gene, and this suggestion is also confirmed in studies of barley (Campoli *et al.*, 2012b), rice (Murakami *et al.*, 2007) and *Brachypodium* (Higgins *et al.*, 2010). This raises the question of which gene most species contain an orthologue to: *LHY* or *CCA1*? Some analyses indicate that *LHY*, as opposed to *CCA1*, is present in most plant species (Takata *et al.*, 2009; Lou *et al.*, 2012; Yon *et al.*, 2012). For instance: 1) cross-species reciprocal BLAST is possible only for *AtLHY*, not *AtCCA1*; 2) *Solanum* species have only one gene, which is

very similar in sequence to *LHY* (Figure 3.5); 3) *CHE*, the transcriptional repressor of *CCA1* is also an Arabidopsis-specific gene (described above); 4) *CCA1* is a casein kinase II (CK2) target in Arabidopsis, whereas in rice the OsCK2 orthologue does not target *OsCCA1*, probably because *OsCCA1* does not contain the correct amino acid for interaction, suggesting again that *OsCCA1* is not a true orthologue of *AtCCA1* (Ogiso *et al.*, 2010).

Therefore, it can be suggested that for most plant species, both counterparts of *CCA1* and *LHY* are not present and only one, possibly *LHY*, is necessary for maintenance of the circadian rhythm and plant survival. In some cases, a species has duplicated their *LHY* gene copy, which likely occurred during the α or β duplication event in Arabidopsis (Lou *et al.*, 2012). It is important to consider that when the *LHY* gene duplication occurred, possibly both copies shared their roles and they are now both important for the maintenance of the circadian rhythm in species where duplication occurred.

3.4.4 – Reference genes

Previous studies on expression of clock genes in barley have used *GAPDH* (Dunford *et al.*, 2005), *actin* (Hemming *et al.*, 2008) and 18S rRNA (Turner *et al.*, 2005) for normalising clock transcript levels. The reason why those genes were chosen is not well explained, but may be due to the fact that those genes are housekeeping genes (HKG), thought to have stable expression. However, it has been shown that HKG do not necessarily have stable expression and choosing them to normalise other genes can compromise expression analysis (Czechowski *et al.*, 2005; Patel and Jain, 2011). In order to carefully choose barley reference genes for this study it was decided to base the choice on recent studies developed in barley and other species (Czechowski *et al.*, 2005; Patel and Jain, 2011; Dekkers *et al.*, 2012). The expression of a large set of genes from a wide range of developmental stages and environmental conditions was analysed and the genes with the

most stable expression were identified. For instance, genes from the *PP2A* and *UBC* families have been shown to be considerably more stable in at least nine different species, including barley, under various conditions. Additionally, in the work of James *et al.* (2012), one member of each family (*PP2AA3* and *UBC21*, respectively) was chosen as reference gene for clock transcript analyses. Barley homologues of these genes, *HvPP2AA2* and *HvUBC21*, were identified and used as control in clock expression studies.

Microarray data of *PP2AA2* genes in barley, Arabidopsis, tomato, maize, and wheat show stable expression, suggesting it is a good candidate for normalising transcript levels (Czechowski *et al.*, 2005; Patel and Jain, 2011; Dekkers *et al.*, 2012). Therefore, *HvPP2AA2* was used as a control gene in barley expression studies. In order to make these studies more robust, the *HvUBC21* gene was used as a second control. Regarding the barley microarray data available on PLEXdb, there is no information for *HvUBC21* because this gene is not present on the Barley1 Affymetrix® Chip.

Chapter 4. Functional and expression analysis of putative barley clock genes

4.1 – Introduction

As mentioned previously (Section 1.7), the molecular components of the Arabidopsis clock are able to generate circadian rhythms during both day and night and over seasons through complex autoregulatory interlocking positive and negative feedback loops. These components are mostly transcription factors, but there are also components involved in protein degradation and stability, among others (Kim *et al.*, 2007; Perales and Más, 2007; Gendron *et al.*, 2012). Clock genes have an endogenous rhythmic expression, maintained even in free-running conditions (McClung, 2006).

Input and, mostly, output genes from the core clock regulate various physiological processes such as photosynthesis, hormone production, stress responses, organ movements and transition to flowering (Harmer, 2009). These processes have agronomic importance, and their regulators, clock genes, are key elements in crop improvement programmes. Time to flowering, in particular, is one of the most important breeding targets.

4.1.1 – Timing of flowering

In all flowering plants, the major physiological change from vegetative growth to reproductive development is rarely reversible. The correct timing of this event has adaptive value and plants have evolved a complex regulatory system to control it. Flowering time regulation is carried out by a complex system that integrates external cues (for instance,

light and temperature) and endogenous signals, which vary with climate and species. Regarding endogenous signals, there are four main genetic pathways controlling flowering: the gibberellin, photoperiod, autonomous and vernalisation pathways.

The gibberellin signalling pathway is tightly regulated by developmental and environmental cues, and it assists with normal growth of almost all plant organs, including flowering initiation in some species, such as Arabidopsis (Blázquez *et al.*, 1998). In these species, gibberellins are probably important to allow flowering only at the appropriate growth stage for a given environmental condition (Mutasa-Göttgens and Hedden, 2009).

The photoperiod pathway refers to regulation of flowering in response to day length and involves the circadian clock (further discussed below in Section 4.1.2). In the autonomous (or constitutive) pathway, components of the pathway trigger plant flowering by preventing expression of the flowering repressor *FLOWERING LOCUS C* (*FLC*). FLC inhibits the expression of flowering genes that would otherwise be activated by the photoperiod and gibberellin pathways (Simpson, 2004). The autonomous pathway itself, however, is independent of day length or gibberellic acid, therefore the name 'autonomous' (Srikanth and Schmid, 2011).

The vernalisation pathway has evolved in plants exposed to seasonal cold environments (Preston and Sandve, 2013). Upon chilling, genes from this pathway activate or accelerate the plant's ability to flower by gradually repressing *FLC* (Chouard, 1960; Mouradov *et al.*, 2002).

4.1.2 – Photoperiod pathway

The further from the equator, the more day length varies between summer and winter. Plants have evolved a mechanism that ensures that reproduction occurs in the best-suited season by sensing day length and coupling this information to the circadian clock.

This allows the clock to efficiently control the appropriate flowering time. Clock control over photoperiodic flowering has been studied in great detail in Arabidopsis, whose flowering is induced in long day (LD) conditions. The components of the photoperiod pathway responsible for perceiving light, i.e. daylight measurements, are input, core clock and output genes, which result in synchronisation of clock function with the external photoperiod and subsequent flowering control. Similar to the way that light controls clock function, there is also evidence of clock control over light signal transduction, increasing the complexity of the photoperiod pathway (Tóth *et al.*, 2001). For example, to maintain self-sustained oscillations, the light signal is uncoupled (removed) from the clock by the *AtELF3* gene. The evening-expressed AtELF3 gates light input to the clock, for instance by suppressing *CAB2* mRNA expression and PHYB protein levels (McWatters *et al.*, 2000; Liu *et al.*, 2001). Ultimately, ELF3 controls photoperiodic flowering and shade avoidance mechanisms (further information about *elf3* mutants in Section 4.1.3) (Yu *et al.*, 2008; Yoshida *et al.*, 2009; Jiménez-Gómez *et al.*, 2010; Thines and Harmon, 2010).

In Arabidopsis, the genetic network that initiates photoperiodic flowering occurs through expression of *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) in leaves (

Figure 4.1) (An *et al.*, 2004). During light under LD conditions, FKF1 interacts with GI and promotes degradation of CYCLING DOF FACTORS (CDFs), which are *CO* repressors. Meanwhile, PRR9, PRR7 and PRR5 repress mRNA accumulation of *CDF*s (Nakamichi *et al.*, 2007; Liu *et al.*, 2013). These result in daytime *CO* transcription and translation. In daytime conditions, the CO protein is stabilised by several components, including FKF1 (Song *et al.*, 2012), which induces *FT* and, consequently, flowering (Valverde *et al.*, 2004; Sawa *et al.*, 2007; Srikanth and Schmid, 2011). Additionally, GI directly activates the expression of *FT* (Sawa and Kay, 2011).

In short day (SD) conditions, the photoperiod pathway behaves differently. Under these conditions, FKF1 is mostly present during the early night, when there is no blue light to allow interaction with GI and degradation of CDFs. As a result, *CO* transcription cannot occur during the daytime and occurs at night time. In the dark, the CO protein is degraded by the proteasome. Without CO, *FT* and flowering are not induced (Valverde *et al.*, 2004; Sawa *et al.*, 2007; Srikanth and Schmid, 2011). An alternative pathway to the classical GI-CO pathway is ELF3-mediated repression of *FT* expression through accumulation of the flowering repressor SHORT VEGETATIVE PHASE (SVP) (Yoshida et al., 2009).

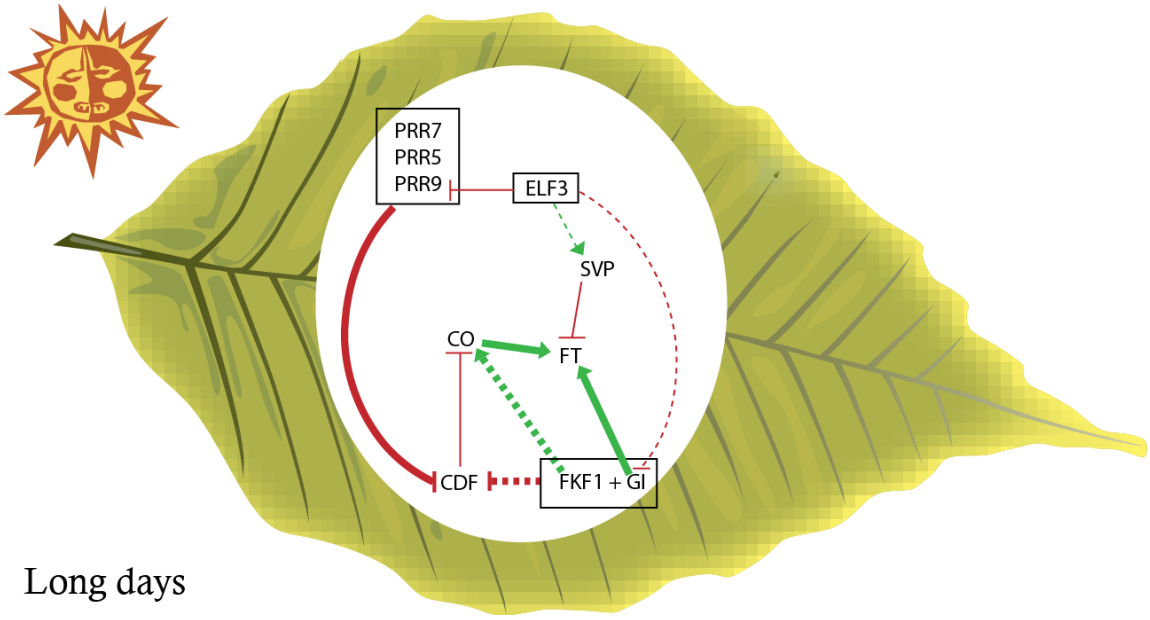


Figure 4.1: A schematic and simplified diagram of the photoperiod pathway in Arabidopsis leaves under LD conditions. Full lines represent transcriptional regulation, whereas dashed lines represent regulation at the protein level. Green lines are for activation, while red lines are for repression. Thick lines represent increased control under LD conditions, while thin lines occur mostly under SD conditions. Clock-related genes are highlighted with a box.

4.1.3 – Barley clock mutants

Mutations in clock genes affect the circadian system and consequently cause aberrant regulation of the photoperiodic timing of flowering. These mutations have allowed the identification of many clock components in Arabidopsis (forward genetics) and are particularly important in crop species, contributing to increased diversity of flowering time. This diversity has an impact on yield and has been exploited to enable cultivation of crops in environments with extreme photoperiods, as well as efficient use of the growing season and the practice of crop rotation (Turner *et al.*, 2005; Murphy *et al.*, 2011; Zakhrabekova *et al.*, 2012). Naturally-occurring and induced mutations in clock genes have been key factors to the success and spread of agriculture, including barley. There are three well-characterised *early maturity* alleles of barley clock genes: *eam1*, *eam8* and *eam10*. Regarding the latter, the candidate gene underlying this locus in barley is the orthologue of *AtLUX* (Campoli *et al.*, 2013). Barley *eam10* mutants are loss-of-function mutations of *HvLUX*, which results in accelerated flowering under long and short days (Campoli *et al.*, 2013). Below, we will concentrate on the *eam1* and *eam8* plants.

Barley varieties are generally classified as winter or spring types. Winter (fall-sown) barley varieties carry the *eam1* allele and are early flowering under LD and late flowering under SD, which is the wild-type phenotype and that which barley ancestors exhibited (Jones *et al.*, 2008). Spring (spring-sown) barley varieties have lower responsiveness to photoperiodic changes, flowering later than winter barley under LD, but no difference is observed under SD (Turner *et al.*, 2005). This reduced responsiveness to LD photoperiodic flowering of spring barley is caused by the presence of the *Hvppdh1* (*Hvprr37*) mutant allele, and it is advantageous in long growing seasons. In this case, late flowering allows an extended time in the vegetative growing stage and biomass accumulation, which consequently supports higher yields.

In fact, *eam1* plants carry the functional *HvPPDH1* (*HvPRR37*) gene, which is the major determinant of photoperiod response in barley and is the homologue of Arabidopsis *PRR7* (Turner *et al.*, 2005). *AtPRR7* undergoes AS in response to temperature, which suggests its involvement in temperature compensation mechanisms (Salomé and McClung, 2005; Gould *et al.*, 2006; Salomé *et al.*, 2010; James *et al.*, 2012a). The mutation of *Hvppdh1* which explains the spring barley flowering phenotype under LD is not precisely known because no robust biochemical or functional analysis was hitherto performed (e.g. complementation tests), but there are some hypotheses formulated by sequence-based polymorphism studies. The causal *Hvppdh1* mutation might lie either in the conserved 'constans, constans-like and TOC1' (CCT) domain (involved in protein interactions and nuclear localisation) (Turner *et al.*, 2005) or the more recently suggested SNP48 (Figure 5.8) (Jones *et al.*, 2008). The *Hvppdh1* alleles are likely to cause a reduction in gene function, as opposed to complete loss-of-function or a gain-of-function because: i) they are recessive alleles; ii) the ORF is maintained; iii) photoperiod response is reduced under LD, not eliminated; iv) photoperiod response is similar to *PPDH1* under SD.

The *eam8* mutants are knock-out mutants of the *HvELF3* and are early flowering due to an effect on photoperiod sensitivity (Zakhrabekova *et al.*, 2012). The *HvELF3* gene is a homologue of *AtELF3* and controls the mRNA levels of most core clock genes (Dixon *et al.*, 2011). It is involved in diverse mechanisms from temperature response to flowering time (Yu *et al.*, 2008; Yoshida *et al.*, 2009; Jiménez-Gómez *et al.*, 2010; Thines and Harmon, 2010). In Arabidopsis and barley, mutations in the *ELF3* gene disrupt plant sensitivity to photoperiod and these mutants flower considerably early, even in non-inductive SDs (Zagotta *et al.*, 1996; Fu *et al.*, 2009; Faure *et al.*, 2012; Saito *et al.*, 2012; Yang *et al.*, 2013; Zakhrabekova *et al.*, 2012). Early flowering mutants are commercially important for

environments with short growing seasons, like Scandinavia and Iceland (Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012).

Because *ppdh1* and *elf3* mutations affect photoperiod response and sensitivity to induce flowering and both are in core clock genes, we studied the influence of these mutations on the clock and expression of other clock genes to gain a better understanding of clock functions. First, the influences of the *ppdh1* and *elf3* mutations on core clock genes were analysed at the phenotypic level, which confirmed their roles in barley biology as described previously (Turner *et al.*, 2005; Faure *et al.*, 2012). Second, the 21 barley homologues of the Arabidopsis core clock and selected associated genes identified *in silico* (Chapter 3) were examined in light/dark and free-running conditions to confirm that they were clock genes. Lastly, we examined the effect of *ppdh1* and *elf3* mutations on clock gene expression.

4.2 – Materials and methods

4.2.1 – Plant growth conditions

Barley plants (see 2.1.1) grown in the glasshouse were placed in plastic cylinder pots (20 cm height × 16 cm top diameter) filled with cereal compost (Appendix A1). Growing conditions were 16 h daylight at approximately 20 °C and 8 h dark at approximately 16 °C (see Appendix A6). Plants were grown for two to three months, until seeds were set and ready for harvest. For short days, plants were grown in a controlled environment (see 2.1.2). The light regime of SD was 8 h light and 16 h dark per day and temperature was maintained at 20 °C. SD plants were grown in medium square plastic pots (10 cm^3).

4.2.2 – Phenotyping

Plant phenotyping was carried out according to the Zadoks code, which is a decimal code for the growth stages (GS) of cereals (Zadoks *et al.*, 1974; Lancashire *et al.*, 1991). Flowering time was calculated from the day plants were transferred to soil until the day they reached GS49. At this stage, the first awns are visible protruding from the flag leaf. Additionally, the lengths of flag leaf -1 and spike were measured at GS59 (Figure 4.2A). Data collection was carried out on the main stem, not side shoots. The final number of grains harvested per plant was also counted. Phenotyping was carried out for both long (LD) and short days (SD). For LD, 4 plants were grown in a glasshouse environment, whereas for SD, 10 plants were grown in the controlled environment of a growth cabinet. To compare phenotypes from different barley lines, sets of data were analysed using an Analysis of Variance (ANOVA). The associated *P* values calculated in the ANOVA and Mann-Whitney U tests were two-tailed and a significance level of 5% ($\alpha = 0.05$) was used. GenStat (15th Edition version 15.1.0.9425, VSN International LTD) was used for all statistical analysis.

Figure 4.2: Schematic representation of cereals at GS59 and GS11 of the Zadoks code. A) Inflorescence at GS59, when the lengths of flag leaf -1 and spike were measured (drawing modified from Rawson and Macpherson, 2010). B) Seedlings at GS11 were harvested in light/dark and free-running experiments. At this stage, seedlings have one leaf (L) unfolded and a second leaf present, not yet fully expanded (drawing modified from Dow, 2010).

4.2.3 – Plant material for light/dark and free-running conditions (constant light)

Grains from each line were first germinated in the dark at 4 °C on sterile water-soaked filter papers and transferred to small square plastic pots (5 cm^3) filled with cereal compost (Appendix A1). Each pot contained 5 grains from the same line. Plants were grown in a controlled environment growth cabinet at 20 °C with 16 h light (400 μmol m^{-2} s^{-1}) and 8 h dark (LD treatments). Temperature and humidity were monitored with a data logger (EL-USP-2 from Lascar Electronics) that confirmed stable conditions at around 20 °C and 70%, respectively (data not shown). No crop protection treatment was applied. To analyse plants at the same stage of development, the majority of the plants were harvested at GS11 of the Zadoks code, when the first leaf is unfolded (Figure 4.2B) (Zadoks *et al.*, 1974; Lancashire *et al.*, 1991). Plants which were not at this stage at the start of harvest were discarded. Plants were harvested at approximately 2 h 30 min intervals for a total of 48 hours from the start of an initial dark period (ZT0). For harvesting under dark conditions, samples were quickly collected in low, indirect light. After LD sampling, barley plants were subjected to continuous light (20 °C, LL treatments). Sampling started at ZT0 of continuous light, during the subjective night, and continued at approximately 2 h 30 min intervals for a total of 69.5 hours (Figure 4.3). A minimum of two biological replicates were taken per time point, each comprising one or two leaves from at least four independent plants from one pot. Additionally, to avoid interference in the experiment coming from a possible microclimate within the growth cabinet, pots with different barley lines and replicates were placed in a randomised array. Plants for each time point were also previously selected/determined in a random fashion from the array (Appendix B3). Furthermore, order of sampling at each time point (9 samples) was random for the barley lines and replicates and it was performed in less than 10 min. Leaf material for each time-point, line and

replicate were collected and rapidly frozen in liquid nitrogen and stored at -80 °C until isolation of RNA.

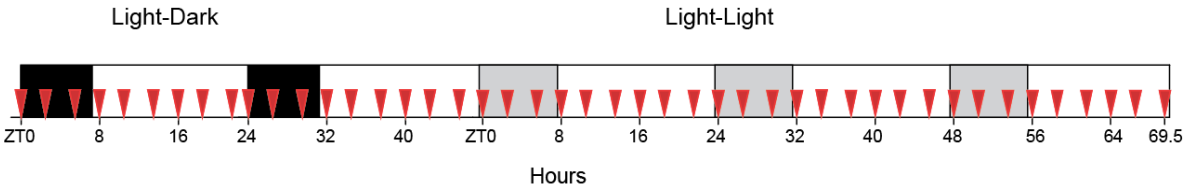


Figure 4.3: Sampling regime of light/dark and constant light experiments. Red arrows represent time points when sampling occurred. Black boxes are for dark, white boxes are for light and grey boxes represent subjective dark. ZT0 points are indicated at the start of sampling and the start of free-running conditions.

4.2.4 – Statistical tests on gene expression data

Expression data were obtained from HR RT-PCR experiments, which involved an initial RT reaction, carried out according to instructions in Section 2.3.1 and used RNA samples (extracted according to Section 2.2) obtained from experiments described in Section 4.2.3. Subsequently, PCR reactions and product analyses were carried out according to instructions in Section 2.4.3. Once quantification of the PCR products had been achieved, the normalised expression data were plotted using SigmaPlot software version 12.0 and edited using Adobe® Illustrator® CS5 software version 15.0.2. Data plotted on the graphs were mean expression values of biological replicates calculated using Microsoft Excel 2010. For long-day (LD) conditions, expression data over the first two days of light/dark were considered as replicates of a 24-hour day. Error bars are Standard Error of the Mean (SEM) calculated from four (LD) or two (LL) biological replicates from a pool of four plants in each replicate.

Confirmation of rhythmic transcript expression was carried out using the Biological Rhythms Analysis Software System (BRASS, available at www.amillar.org), running fast Fourier transform nonlinear least-squares (FFT-NLLS) estimation. FFT-NLLS analysis estimated the relative amplitude error (RAE, a measure of goodness of fit to a theoretical sine wave), which was taken as an objective measure of rhythmic transcript expression. Lines with RAE values were considered as significantly rhythmic. The number of tested biological replicates is n=2. These were analysed within the circadian range of 15–35h with a 90% confidence limit.

To compare expression values from different barley lines, sets of data were first tested for normality using the Shapiro-Wilk test for Normality. Normally distributed data sets were analysed using an Analysis of Variance (ANOVA) from the GenStat software. Biological replicate and time point (when relevant) were defined as random effects in the ANOVA in order to remove the residual errors resulting from these factors and giving more appropriate testing for differences between barley lines. For LD data sets deviating substantially from a normal distribution ($p < 0.001$), comparison analyses were performed with the non-parametric Mann-Whitney U test using the GenStat statistical software. The associated *P* values calculated in the ANOVA and Mann-Whitney U tests are two-tailed and a significance level of 5% ($\alpha = 0.05$) was used. GenStat (15^{th} Edition version 15.1.0.9425, VSN International LTD) was used for all statistical analysis.

4.3 – Results

The 21 barley homologues of the Arabidopsis core clock and selected associated genes identified *in silico* were tested for circadian expression. Firstly, the influence of the two mutations in the core clock genes *HvPPDH1* and *HvELF3* were analysed at the phenotypic level, which allowed confirmation of their roles in the overall biology of the barley

plant. Secondly, the expression behaviour of the putative barley clock genes was analysed in light/dark and free-running conditions, which suggested functional conservation of these genes. Lastly, the effect of *ppdh1* and *elf3* mutations on clock gene expression was examined.

Three barley lines were selected for the experiments described in this work: Bowman (*ppdh1*), *eam1.d* and *eam8.w*. Bowman (*ppdh1*) was selected for two main reasons: 1) its genome is one of the lines undergoing genome sequencing and 2) the availability of two Bowman NILs containing different alleles of clock genes: *eam1.d* and *eam8.w* (Druka *et al.*, 2011). Bowman contains the naturally occurring recessive *Hvprr37* (*ppdh1*) gene. The functional *HvPRR37* (*HvPPDH1*) allele is found in the *eam1.d* Bowman NIL. It is noteworthy that this line is a BC8 line (8th backcross with Bowman) with a small gene interval, < 1 cM, introgressed on chromosome 2H. This introgressed segment contains not only the *PPDH1* allele but also additional sequence from KT1031 barley cultivar, which was introgressed because of its close proximity to the *PPDH1* locus (Professor Robbie Waugh, personal communication). Genes contained on this additional sequence could potentially interfere with our studies but, to date, there is no evidence for this region influencing phenotype and was therefore not taken into account in the analyses carried out here. Nevertheless, this line is the only one carrying the functional *PPDH1* allele in the Bowman background and is, therefore, the effective 'wild type' (WT) for clock studies. A functional clock is an important starting premise to investigate mutation in clock genes (such as *ppdh1*), as well as for comparisons to results from clock research in the model plant Arabidopsis (which are mostly reported for WT plants). For this reason, *eam1.d* Bowman NIL hereafter represents the WT and was used in comparisons to Bowman (*ppdh1*) and *eam8.w*. The *eam8.w* mutant carries a loss-of-function mutation of the *ELF3* gene, which is a single point mutation that introduces a PTC in exon 2 of *HvELF3* (Figure 4.4). It is noteworthy

that this line has 1.5 cM sequence from Early Russian barley cultivar introgressed into chromosome 1H. As *eam8.w* is in the Bowman (*ppdh1*) background, it also carries the *Hvppdh1* allele and therefore represents a double mutant when compared to Bowman (*ppdh1*) and the effective wild-type (*eam1.d*). For simplicity, the *eam8.w* NIL is hereafter referred to as the *Hvelf3* mutant.

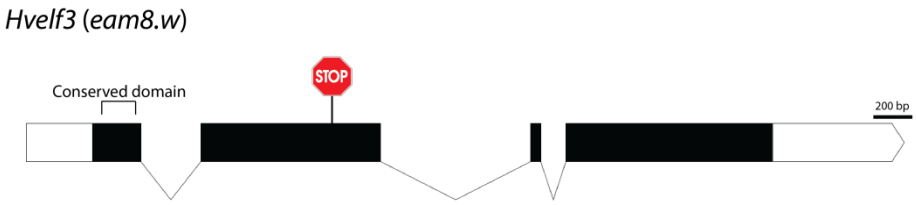


Figure 4.4: Genomic structure of *Hvelf3* in the *eam8.w* Bowman (*ppdh1*) NIL. The causal *Hvelf3* mutation is a PTC in the coding region (black boxes) (Zakhrabekova *et al.*, 2012), which is represented by a 'STOP' sign in the diagram.

4.3.1 – Phenotype of barley clock mutants

An initial group of seeds from the three barley lines, kindly provided by Dr Arnis Druka, were individually and directly sown into pots for bulking seed in the glasshouse (LD conditions). This procedure ensured a high enough number of seed sets from each line for the expression analyses. At the same time, these lines were phenotyped. For this approach, a small number of biological replicates and physiological traits were analysed because plants carrying these mutations have been extensively characterised previously (Campoli *et al.*, 2012b; Faure *et al.*, 2012). Therefore, the phenotyping approach adopted here was mainly to confirm the genetic identity of these barley lines. Moreover, the number of replicates used was enough to confirm significant differences of phenotypes between these lines.

Significant morphological differences existed between Bowman (*ppdh1*), wild type and the *Hvelf3* mutant under LD conditions. Both NILs had smaller leaves and flowered considerably early, especially WT (*eam1.d*), when compared to Bowman (*ppdh1*) (Figure 4.5A and B). A similar difference in phenotype was observed for spike length, which was also reflected in the number of grains per plant (Figure 4.5C). In fact, many traits, including height, number of tillers and stem diameter were smaller in WT and *Hvelf3* plants when compared to Bowman (*ppdh1*) (Appendix B1). This suggests a general reduction in biomass for these early flowering plants. However, a unique exception was observed for awn length, which was similar among the tested barley lines (Appendix B2). Grain size was similar among the three barley lines (data not shown), but studies of this trait would require more robust analysis. Nevertheless, despite the small length of spikes and reduced number of grains in early flowering plants, the similar size of other flowering structures suggests that their inflorescence development was not affected by the early flowering phenotype and all structures were correctly formed.

To analyse phenotypes of plants in short day (SD) conditions, ten plants of each line (Bowman, WT, and *Hvelf3*) were first germinated in sterile water-soaked filter papers and transferred to soil in a controlled environment growth cabinet under SD conditions. Cabinet conditions were monitored with a data logger (EL-USP-2 from Lascar Electronics) that confirmed stable temperature and humidity, at around 20 °C and 70% respectively (data not shown). The most striking difference observed was the flowering time of the *Hvelf3* mutants. These plants flowered around 100 days earlier than Bowman (*ppdh1*) and WT plants. Additionally, *Hvelf3* plants showed significantly smaller leaves and spikes, and reduced number of grains per plant when compared to Bowman (*ppdh1*) (Figure 4.6). In contrast, the WT plants flowered at the same time as Bowman (*ppdh1*), with similar leaf and spike lengths. There was a tendency for WT plants to generate fewer grains per plant

when compared to Bowman (*ppdh1*), but this was not statistically significant, probably due to high variation of grain production per plant in both WT and Bowman (*ppdh1*).

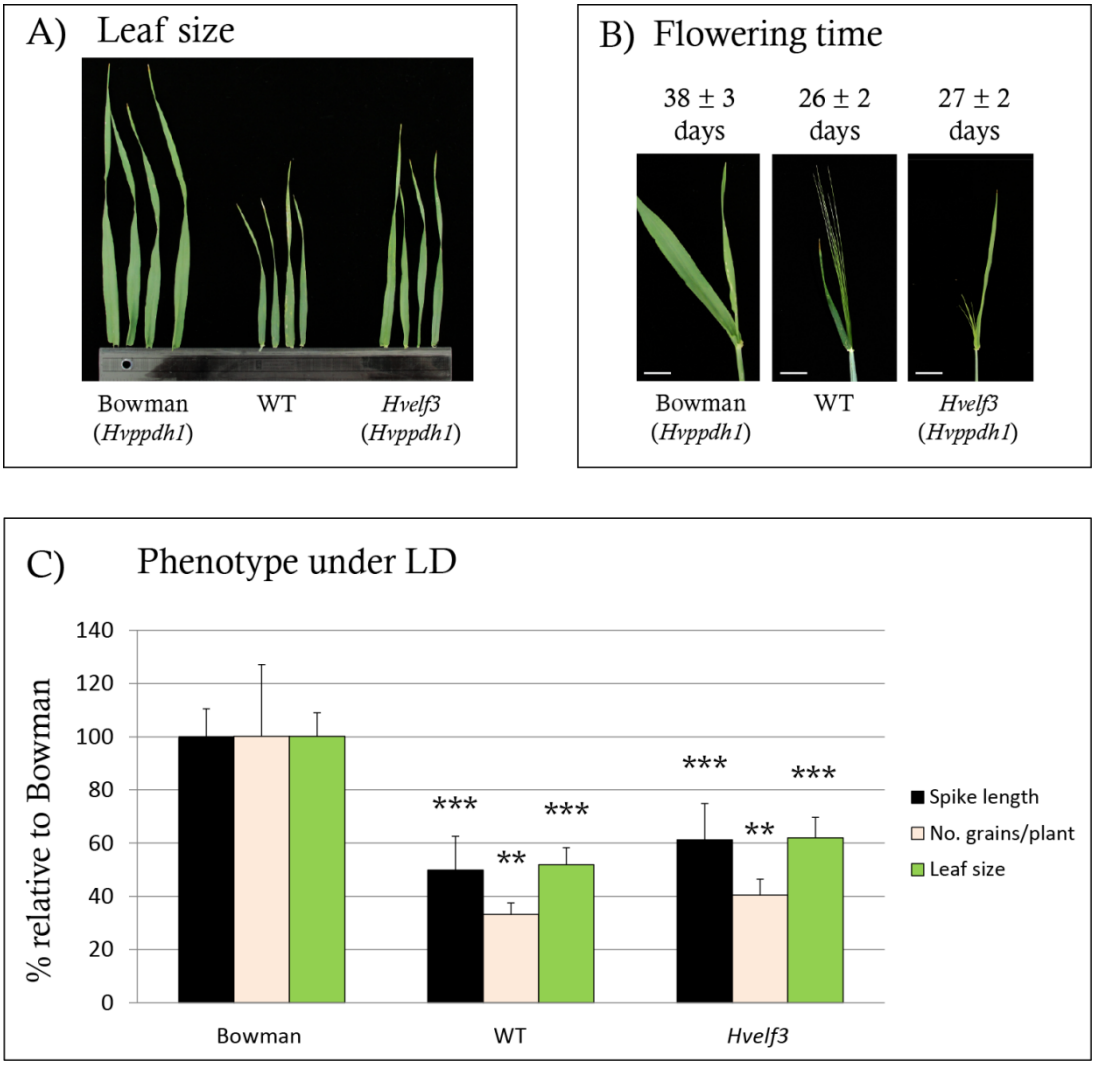


Figure 4.5: Phenotypes of Bowman (*ppdh1*), WT and *Hvelf3* plants in LD. A) Leaf size phenotype. Photograph taken of detached leaves from 28-day old plants (n=4). The 31-cm ruler at the bottom is to show scale. B) Flowering time phenotype. Photograph taken of inflorescences from 28-day old plants (n=1). White bar at the bottom represents a 3 cm scale. Variation in days to flowering among plants are represented by plus-minus sign (±). C) Plotted data of spike and leaf length, and number of grains per plant (n=4). Mean values and standard deviations (error bars) shown are relative to Bowman (*ppdh1*). *P* values represent statistical comparisons of Bowman (*ppdh1*) to equivalent values of WT and *Hvelf3*. **, $P < 0.01$; ***, $P < 0.001$.

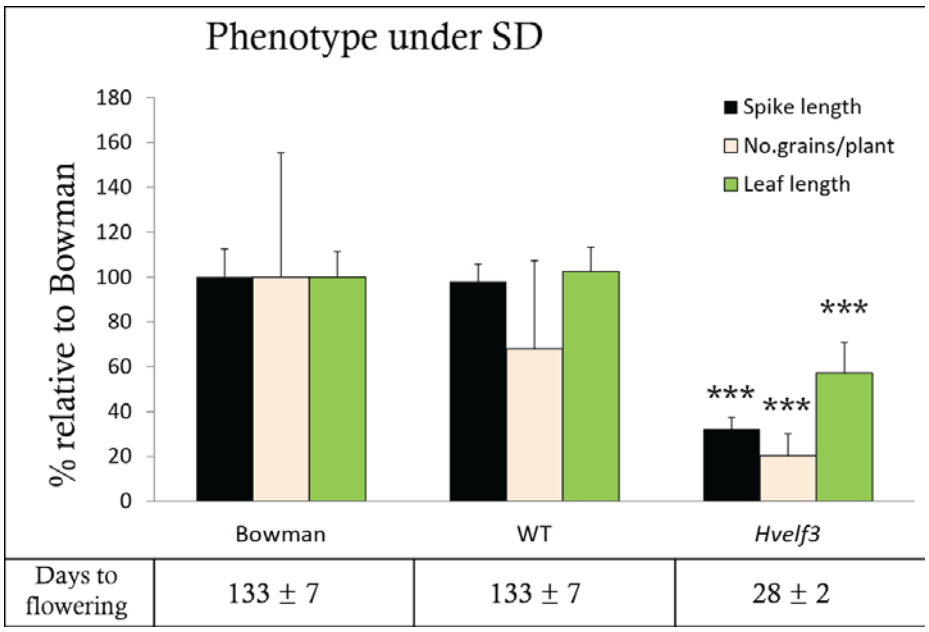


Figure 4.6: Phenotype of Bowman (*ppdh1*), WT and *Hvelf3* plants under SD conditions (n=10). Plotted data of spike and leaf length, and number of grains per plant. Mean values (in %) and standard deviations (error bars) shown are relative to Bowman (*ppdh1*). *P* values represent statistical comparisons of Bowman (*ppdh1*) to equivalent values of WT and *Hvelf3*. ***, $P < 0.001$. Flowering times are shown in days, below each barley line. Variation in days to flowering among plants are represented by plus-minus sign (±).

In summary, allelic differences in the barley clock genes found in Bowman (*ppdh1*), WT and *Hvelf3* plants have affected their response to photoperiodic flowering (Table 4.1). The *ppdh1* allele interfered with the flowering time only in LD, delaying flowering by around 12 days when compared to WT plants. In contrast, the *Hvelf3* mutation (*elf3*/*ppdh1* double mutant) caused insensitivity to photoperiod and plants flowered considerably early under SD and LD. In LD, in particular, the *Hvelf3* mutation rescued the late flowering phenotype caused by the *ppdh1* allele. Moreover, biomass and yield were greatly affected in early flowering plants under the conditions analysed.

Table 4.1: Barley plants and alleles present, along with flowering phenotypes. PP: photoperiod. NIL allele in Bowman (*ppdh1*) background is shown in red.

Barley plant	Putative *PRR7* allele	*ELF3* allele	Phenotype for LD	Phenotype for SD
Bowman	*ppdh1*	*HvELF3*	***Late flowering***	Late flowering
WT (*eam1.d*)	*PPDH1* (PP responsive)	*HvELF3*	Early flowering	Late flowering
***Hvelf3* (*eam8.w*)**	*ppdh1*	*Hvelf3-eam8.w* (PP insensitive)	Early flowering	***Early flowering***

4.3.2 – Expression analysis of barley circadian clock genes

The expression patterns of putative barley genes identified and confirmed *in silico* (Chapter 3) were analysed. It is expected that clock genes have an endogenous rhythmic expression, maintained even in free-running conditions (McClung, 2006). However, mutations in core clock genes might influence the expression of other clock genes. Therefore, the expression patterns of the 21 barley clock genes were analysed in Bowman (*ppdh1*) and the two NILs (WT and *Hvelf3*). These plants (Appendix B4) were grown under 16 h light, 8 h dark conditions and then under constant light (Figure 4.3). cDNA templates were prepared from two biological replicates of 45 different time points throughout two day/night cycles and almost 70 h of constant light. A total of 272 samples were used for systematic HR RT-PCR expression analysis using a pair of gene-specific primers for each clock gene (Table 2.1). Expression data were plotted and peaks of expression determined and whether expression was rhythmic or arrhythmic. The data are summarised in Table 4.2 and examples of the expression plots are shown for a small number of genes with the remaining plots presented in Appendix B5. In addition, the results for each gene are described below. No expression data were obtained for five genes, from the initial list of 21, despite multiple attempts to design gene-specific primers that give reproducible results. These genes are *HvGRP7a*, *HvGRP7b*, *HvELF4-like3*, *HvFT2*, and *HvZTLb*.

Table 4.2: Approximate expression peak or, in a few cases, trough of barley clock genes in different lines and conditions.

	WT		***Hvelf3***		**Bowman (*ppdh1*)**	
	LD	LL	LD	LL	LD	LL
LHY	2.5/5.5 h after dawn ▼	8 h after subj. dawn ▼	2.5/5.5 h after dawn ▼	Ar	2.5/5.5 h after dawn ▼	8 h after subj. dawn ▼
CABa	Day	22 h period ▼	Trough 2.5 h before dusk	Ar	Day ▼	Ar
FKF1	5.5/2.5 h before dusk ▼	Subj. dusk ▼	5.5/2.5 h before dusk ▼	5.5 h before subj. dusk	5.5/2.5 h before dusk	2.5 h before subj. dusk ▼
GI	5.5 h before dusk ▼	Around subjective dusk ▼	2.5 after dawn & at dusk	Ar	5.5 h before dusk ▼	Around subjective dusk
TOC1	2.5 h before dusk ▼	28 h period ▼	2.5 h before dusk ▼	Ar	2.5 h before dusk ▼	2.5 h before dusk ▼
ELF3	2.5 h after dusk	Ar	NMD	Ar	Ar	Ar
ELF4-likeA	2.5 h before dusk	Ar	2.5 h before dusk	Ar	2.5 h before dusk	Ar
LUX	2.5 h before dusk	2.5 h before subj. dusk ▼	Trough at 2.5 h after dawn	Ar	2.5 h before dusk	28h period ▼
ZTLa	Ar	Ar	Ar	Ar	Ar	Ar
Ppd-H1	Day	16 h period	Day	8 h before subj. dusk	Day ▼	8 h before subj. dusk
PRR73	Day ▼	16 h period	Day	8 h before subj. dusk	Day ▼	8 h before subj. dusk
PRR95	5.5 h before dusk ▼	5.5 h before subj. dusk ▼	5.5 h before dusk ▼	30 h period ▼	5.5 h before dusk ▼	5.5 h before subj. dusk ▼
PRR59	5.5 h before dusk ▼	5.5 h before subj. dusk	Trough at 2.5 h after dawn	Ar	5.5 h before dusk ▼	5.5 h before subj. dusk
CO1	2.5 h before dusk ▼	16 h period	Dusk ▼	Ar	Dusk ▼	Ar
CO2	2.5 h before dusk ▼	16 h period	Dusk ▼	Ar	2.5 h before dusk ▼	Ar
FT1	2.5 h before dusk	6h before subjective dusk	2.5 h after dusk	Ar	2.5 h after dawn	28 h period ▼

Day: expression over a large part of the day, i.e. 2.5 h after dawn until dusk. Ar, arrhythmic. NMD, mRNA likely degraded by NMD. ▼, Rhythmic 24-h (unless stated otherwise) expression profile confirmed by BRASS.

It is noteworthy that expression graphs generated from expression analyses have different scales for different reasons. First, barley clock-related genes are expressed in different levels. For example, *CABa* mRNAs were very abundant (5 times the control levels), while *ELF4-likeA* were found in very low levels (15% of the control levels). However, these differences are relative as they were minimised by measuring the abundance of FS transcripts after 20 to 28 cycles of amplification, depending on the abundance from different clock genes. Second, different primers may have different amplification efficiencies, explained, for example, by the GC content and size of primers or the regions being amplified.

- ***LHY*** (Figure 4.7A,B). *HvLHY* was the only clock gene expressed exclusively in the morning, with a peak of expression between 2.5 h and 5 h after dawn for WT, Bowman (*ppdh1*) and *Hvelf3* plants under normal conditions. There was a significant difference in *LHY* expression between WT and the clock mutants (Bowman and *Hvelf3*). On one hand, the peak of Bowman (*ppdh1*) *LHY* was higher than that of WT plants for more than 5 h, returning to similar levels in the evening and night. On the other hand, *LHY* levels of *Hvelf3* plants were half of those found in WT plants during the day. Interestingly, *Hvelf3* plants had significantly higher levels of *LHY* during the night, when compared to WT plants. In constant light, *LHY* had lower amplitude and maintained a rhythmic expression in WT and Bowman (*ppdh1*) plants, with the peak of expression delayed by at least 3 hours. In *Hvelf3* plants, *LHY* expression was considerably lower and effectively lost rhythmicity on the second day under constant light.
- ***CABa*** (Figure 4.7C,D). For both WT and Bowman (*ppdh1*) plants, *HvCABa* had a broad expression peak in light/dark conditions, spanning the greater part of the day: between 2.5 h after dawn and before dusk. In *Hvelf3* plants, *HvCABa* expression decreased towards the end of the day (at around 2.5 h before dusk) but then increased in the night, but no clear single peak was observed. In constant light conditions, WT

plants maintained rhythmic expression with a 22 h period. In Bowman (*ppdh1*) and *Hvelf3* plants, although expression increased and decreased, it was sporadic with no continuous rhythm or consistent difference for *HvCABa* expression among these barley lines analysed.

- ***FKF1*** (Figure 4.7E,F). *HvFKF1* had a peak of expression between 5.5 h and 2.5 h before dusk in WT, Bowman (*ppdh1*) and *Hvelf3* plants. However, daily activation of expression of *FKF1* occurred earlier in Bowman (*ppdh1*) and *Hvelf3* plants when compared to WT plants. When *FKF1* expression profiles of these clock mutants were manually delayed by 1.5 h in the Adobe Illustrator® program, they matched the WT profile (Appendix B6) such that *FKF1* expression in the mutants is advanced by 1.5 h. In the first day under constant light conditions, the three barley lines had different *FKF1* expression behaviour, suggesting differential control over this gene during clock entrainment. The Bowman mutant in days 1 and 2 of LL looks much like the WT in LD – with the expression peaking later in the WT and earlier in *Hvelf3*.

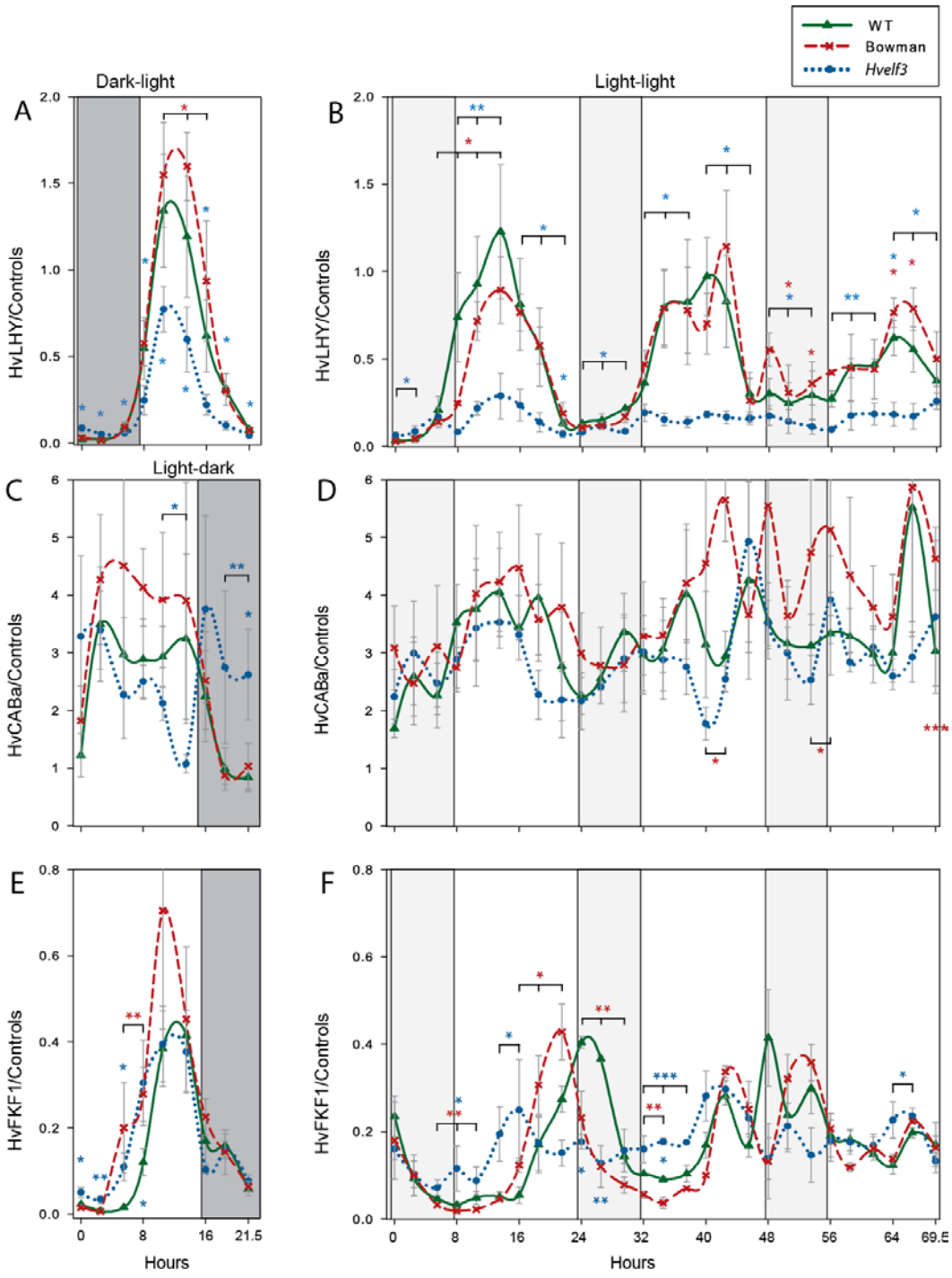


Figure 4.7: Transcript levels of *HvLHY*, *HvCABa* and *HvFKF1* from WT, Bowman (*ppdh1*) and *Hvelf3* plants. A, dark/light conditions are shown for better representation of the start of expression of *LHY* at the end of the night. Dark and light periods are shown with dark grey and white backgrounds, respectively. C and E represent light/dark conditions. B, D and F represent transfer to continuous light. Subjective nights are on light grey backgrounds. Error bars: SEM. *P* values represent statistical comparisons of WT against each mutant (colour coded). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

- ***GI*** (Figure 4.8A,B). In WT and Bowman (*ppdh1*) plants, *HvGI* was rhythmic in light/dark cycles, with a peak of expression at around 5.5 h before dusk. There was no significant difference in *HvGI* expression levels between WT and Bowman (*ppdh1*). In *Hvelf3* plants, *HvGI* expression peaked at 2.5 h after dawn and at dusk, having higher expression in the morning and at night, when compared to WT. In constant light, *HvGI* had lower amplitude and maintained a rhythmic expression in WT and Bowman (*ppdh1*) plants, with the peak of expression delayed by at least 2 hours. In *Hvelf3* plants, *HvGI* expression showed no clear rhythm under constant light conditions but has higher levels of expression.
- ***TOC1*** (Figure 4.8C,D). *HvTOC1* was rhythmic in light/dark cycles with a peak of expression at around 2.5 h before dusk for the three barley lines analysed. Rhythmic expression was maintained in constant light conditions for WT and Bowman (*ppdh1*) plants. There was no significant difference in *HvTOC1* expression levels between WT and Bowman (*ppdh1*). In *Hvelf3* plants, *HvTOC1* is arrhythmic and had significantly higher expression levels during the morning and subjective morning.
- ***ELF3*** (Figure 4.8E,F). This gene was probably rhythmic in WT barley, with a peak at 2.5 h after dusk. In Bowman (*ppdh1*) plants, *HvELF3* was arrhythmic, and it was significantly expressed at higher levels in the morning, compared to WT. In contrast, *Hvelf3* mutants express the *Hvelf3* gene 50% less of when compared to WT. This reduction is likely due to the nonsense mutation in the *Hvelf3* gene which targets the transcripts to NMD. No rhythmicity was observed for *ELF3* in constant light conditions for the three barley lines analysed.

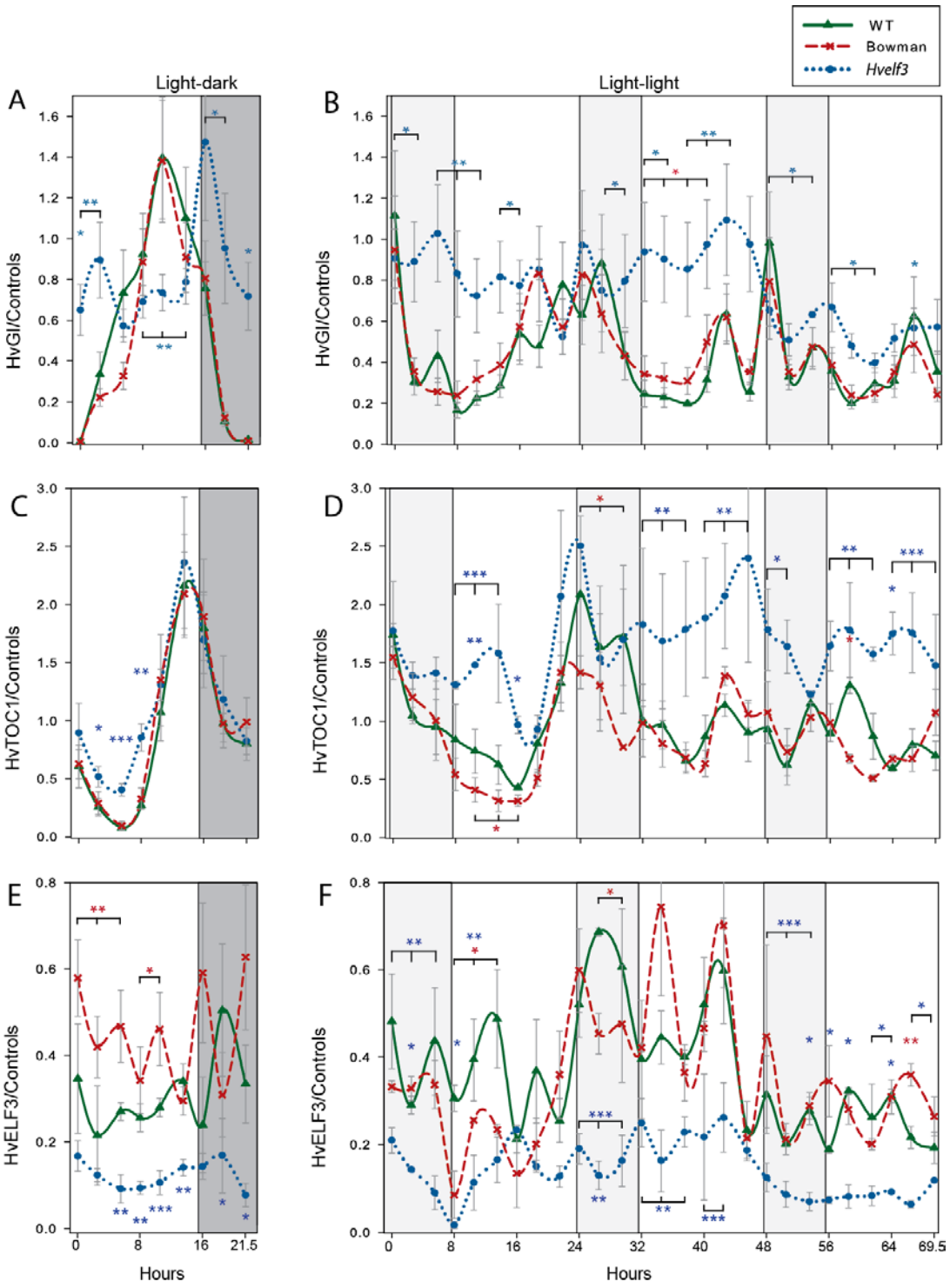


Figure 4.8: Transcript levels of *HvGI*, *HvTOC1* and *HvELF3* from WT, Bowman (*ppdh1*) and *Hvelf3* plants. A, C and E represent light/dark conditions. Light and dark periods are shown with white and dark grey backgrounds, respectively. B, D and F represent transfer to continuous light. Subjective nights are on light grey backgrounds. Error bars: SEM. *P* values represent statistical comparisons of WT against each mutant (colour coded). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

- ***ELF4-likeA*** (Appendix B5). This gene probably had a rhythmic expression in barley, with a peak at 2.5 h before dusk and a secondary peak at dawn. No rhythm was observed in constant light conditions. No significant or marked difference was observed in the expression of *ELF4-likeA* in the three barley lines analysed.
- ***LUX*** (Appendix B5). *HvLUX* was rhythmic in light/dark cycles with an abrupt peak of expression at 2.5 h before dusk for WT and Bowman (*ppdh1*). There was no significant difference in the *HvLUX* expression levels between WT and Bowman (*ppdh1*). In *Hvelf3* plants, this peak was significantly reduced and an increased expression of *HvLUX* was seen in the morning. In constant light, *Hvelf3* plants were arrhythmic, while WT and Bowman (*ppdh1*) plants continued to be rhythmic. Interestingly, on the first subjective night and morning, WT plants had significantly higher expression levels of *HvLUX* when compared to Bowman (*ppdh1*).
- ***ZTLa*** (Appendix B5). In barley, *HvZTLa* was arrhythmic. In Bowman (*ppdh1*) and *Hvelf3* plants, *HvZTLa* was expressed at higher levels in the morning, when compared to WT plants. In constant light conditions, there were no consistent differences in *HvZTLa* expression levels between WT, Bowman (*ppdh1*) and *Hvelf3*.
- ***PPDH1 (PRR37)*** (Figure 4.9A,B). *HvPPDH1* (WT) and *Hvppdh1* (Bowman) alleles had a broad expression peak in light/dark conditions, spanning the greater part of the day: between 2.5 h after dawn and at dusk. Bowman (*ppdh1*) and *Hvelf3* plants had significantly higher levels of *Hvppdh1*, when compared to WT, which continues in constant light. *Hvelf3* mutants, in particular, had higher *Hvppdh1* expression at most time points, particularly during the morning and night. Expression of *Hvppdh1* in Bowman (*ppdh1*) and *Hvelf3* (also *ppdh1*) plants might continue to be rhythmic in free running conditions, whereas WT plants could have a shorter period, around 16 h (although not confirmed by BRASS).

- ***PRR73*** (Figure 4.9C,D). In WT and Bowman (*ppdh1*) plants, *HvPRR73* had a broad expression peak in light/dark conditions, spanning the greater part of the day: between 2.5 h after dawn and declining before dusk. Bowman (*ppdh1*) and *Hvelf3* plants had significantly higher levels of *HvPRR73* when compared to WT. *Hvelf3* mutants, in particular, had higher *HvPRR73* expression at most time points, particularly during the morning and night, which was less evident in continuous light. Expression of *HvPRR73* in Bowman (*ppdh1*) and *Hvelf3* plants might continue to be rhythmic in free running conditions, whereas WT plants could have a shorter period, around 16 h (although not confirmed by BRASS).

Interestingly, both *HvPPDH1* and *HvPRR73* had very similar expression profiles in LD and LL, suggesting their transcriptional control is similar. However in LD, *HvPRR73* expression was reduced at night and in the morning, while expression of the paralogue *HvPPDH1* was reduced only between 2.5 h before dawn and at dawn.

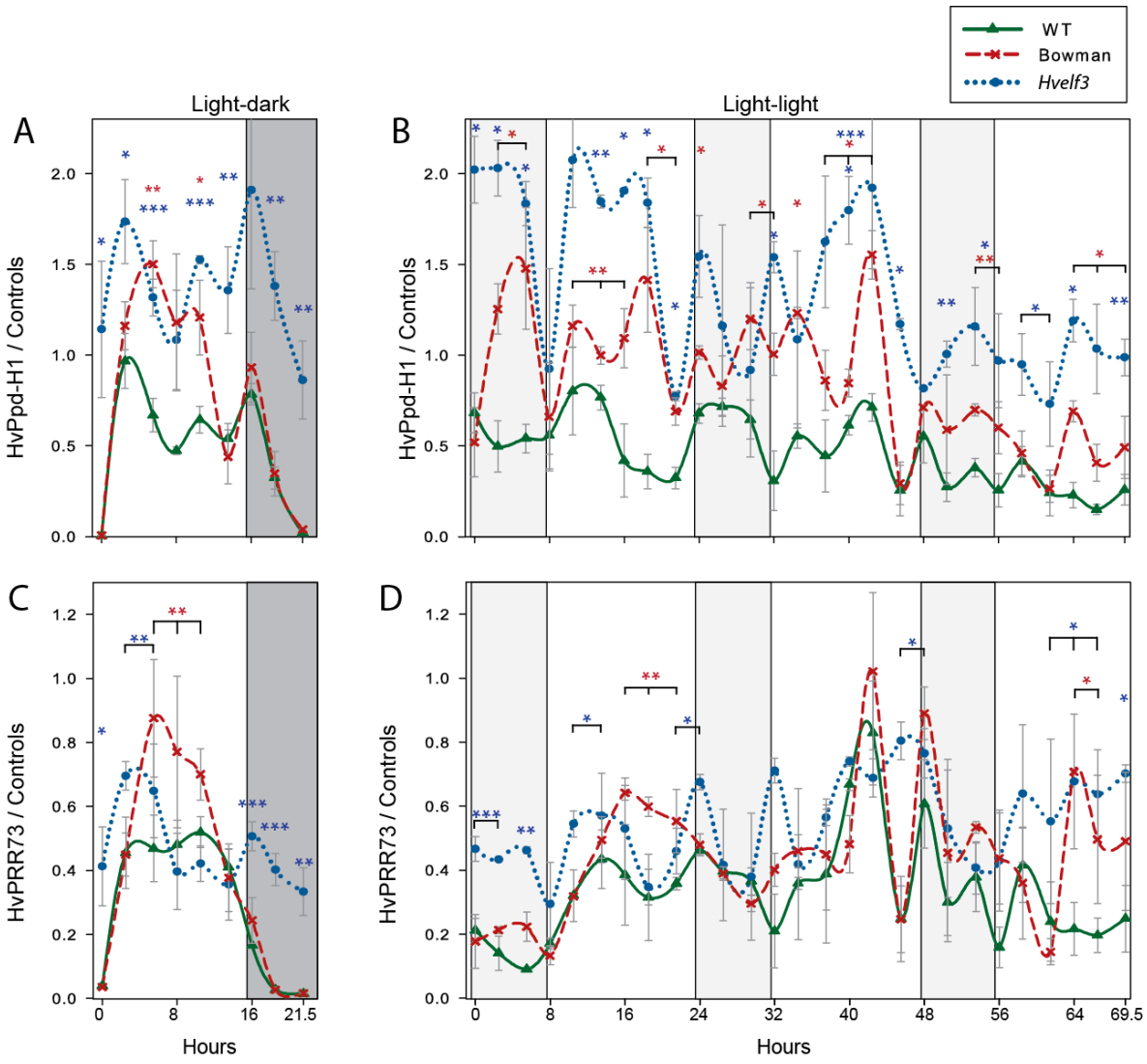


Figure 4.9: Transcript levels of *HvPPDH1* and *HvPRR73* genes from WT, Bowman (*ppdh1*) and *Hvelf3* plants. A and C represent light/dark conditions. Light and dark periods are shown with white and dark grey backgrounds, respectively. B and D represent transfer to continuous light. Subjective nights are on light grey backgrounds. Error bars: SEM. *P* values represent statistical comparisons of WT against each mutant (colour coded). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

- ***PRR95*** (Figure 4.10A,B). In the three barley lines analysed, *HvPRR95* was rhythmic with an expression peak at 5.5 h before dusk. Bowman (*ppdh1*) plants had significantly higher transcript levels of this gene in the afternoon when compared to WT. In *Hvelf3* plants, *HvPRR95* was highly expressed in the morning and during evening and night when compared to WT plants. In free running conditions, *HvPRR95* was rhythmic for the three barley lines analysed. *Hvelf3* mutants, in particular, showed a 30 h period of expression.
- ***PRR59*** (Figure 4.10C,D). In WT plants, *HvPRR59* had a broad expression peak in light/dark cycles, spanning the greater part of the evening: between 5.5 h before dusk and at dusk. Daily activation of expression of *HvPRR59* occurred around 1 h earlier in Bowman (*ppdh1*) when compared to WT plants (Appendix B6). Expression of this gene was probably rhythmic in free running conditions for WT and Bowman (*ppdh1*) plants. In *Hvelf3* mutants, *HvPRR59* lost its peak in the evening and became highly expressed in the morning when compared to WT plants. This behaviour continued for the first two days in constant light.

Both *HvPRR95* and *HvPRR59* had very similar expression profiles in LD and LL, suggesting their transcriptional control is alike. However, in LD *HvPRR95* expression was reduced in the night and morning, while expression of the paralogue *HvPRR59* was reduced only after dawn. Interestingly, such similarity with subtle expression differences was also observed between paralogues *HvPRR37* and *HvPRR73*, suggesting both pair of genes conserved part of their transcriptional control after gene duplication event.

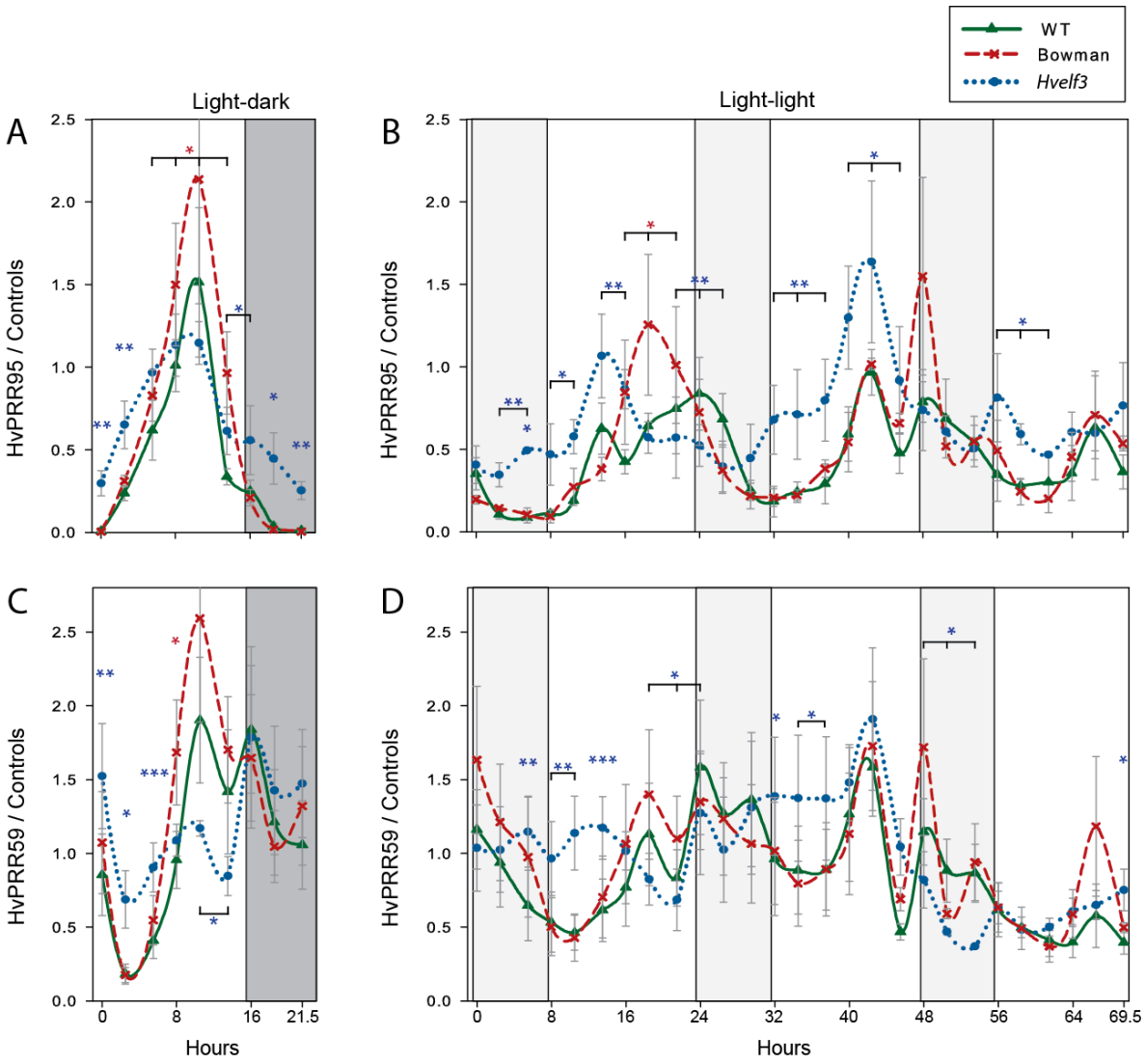


Figure 4.10: Transcript levels of *HvPRR95* and *HvPRR59* genes from WT, Bowman (*ppdh1*) and *Hvelf3* plants. A and C represent light/dark conditions. Light and dark periods are shown with white and dark grey backgrounds, respectively. B and D represent transfer to continuous light. Subjective nights are on light grey backgrounds. Error bars: SEM. *P* values represent statistical comparisons of WT against each mutant (colour coded). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

4.3.2.1 – Flowering activators *CO* and *FT*

- ***CO1*** (Figure 4.11A,B). *HvCO1* was an evening-expressed gene in WT plants. Bowman (*ppdh1*) and *Hvelf3* mutants had a *CO1* expression peak at dusk, which was delayed by circa 2.5 h when compared to WT. Bowman (*ppdh1*) in particular had a delayed increase of *HvCO1* expression, when compared to WT. In free running conditions, Bowman (*ppdh1*) and *Hvelf3* plants had 50% less *CO1* transcripts when compared to WT plants. No clear 24-h rhythmic expression period was observed in free running conditions for the three barley lines analysed. However, it might be possible that WT plants have a shorter period of 16 h, which is very similar to the *PPDH1* expression rhythm (although not confirmed by BRASS).
- ***CO2*** (Figure 4.11C,D). Interestingly, fully spliced transcripts, antisense spliced transcripts and unspliced mRNAs were detected from the *HvCO2* locus. These were detected and confirmed using the approach detailed in Chapter 5. In order to analyse only fully spliced transcripts, expression data from other mRNA isoforms were eliminated from the analysis. This elimination was possible when using the HR RT-PCR system that distinguishes PCR products based on size. Subsequently, only fully spliced *HvCO2* mRNAs were taken into account. This gene was expressed in the evening in WT plants. In Bowman (*ppdh1*) plants, *CO2* expression was significantly reduced and was induced much later in the evening when compared to WT plants. The same delay in *HvCO2* expression was observed in *Hvelf3* plants, as well as faster reduction in transcript levels during the night. In constant light conditions, both Bowman (*ppdh1*) and *Hvelf3* plants had significantly lower *HvCO2* transcript levels when compared to WT, and these plants were probably arrhythmic for this gene. It might be possible that WT plants have a shorter period of 16 h, which is very similar to the *PPDH1* and *CO1* expression rhythm (although not confirmed by BRASS).

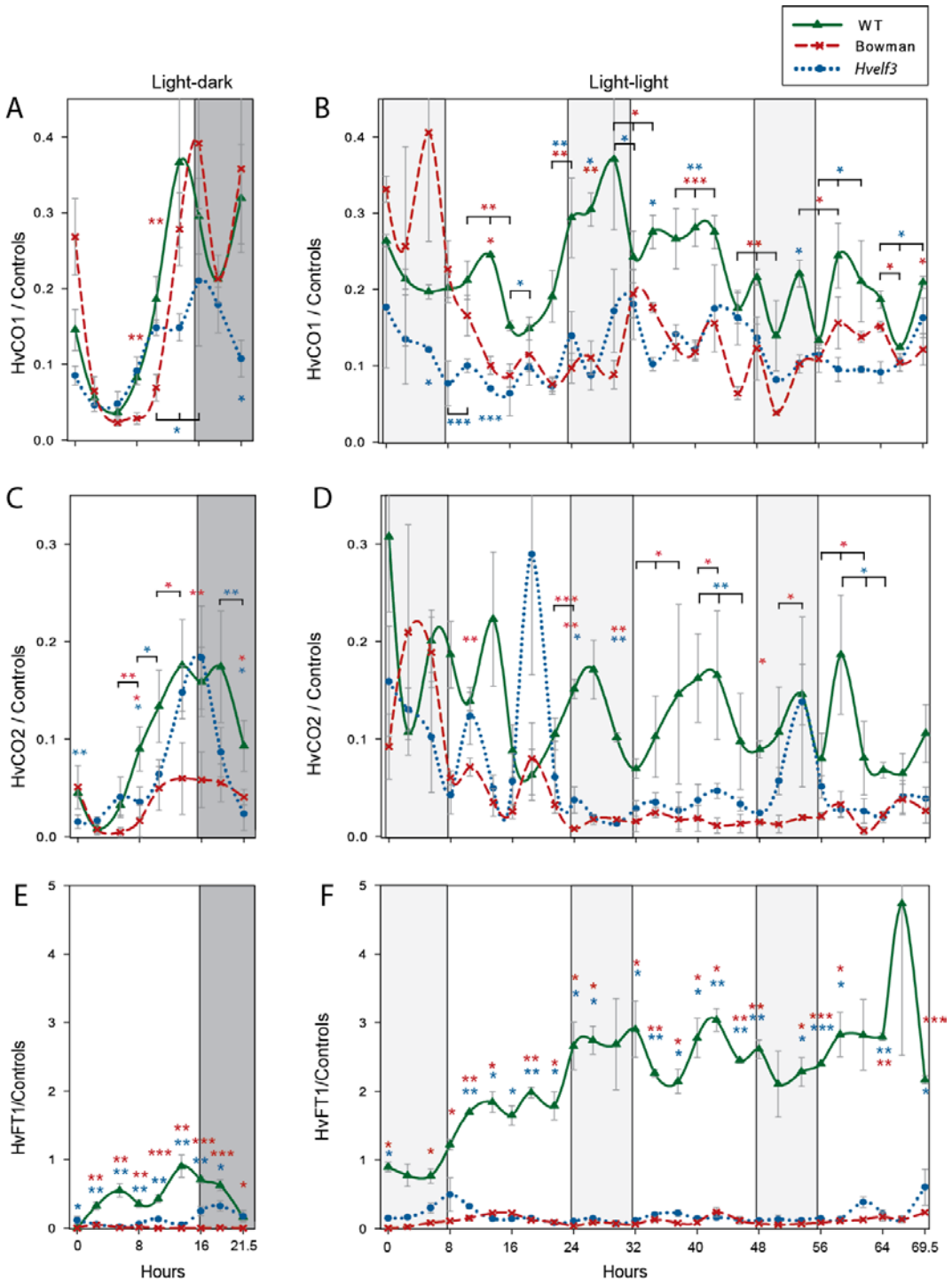


Figure 4.11: Transcript levels of *HvCO1*, *HvCO2* and *HvFT1* genes from WT, Bowman (*ppdh1*) and *Hvelf3* plants. A, C and E represent light/dark conditions. Light and dark periods are shown with white and dark grey backgrounds, respectively. B, D and F represent transfer to continuous light. Subjective nights are light grey backgrounds. Error bars: SEM. *P* values represent statistical comparisons of WT against each mutant (colour coded). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

- ***FT1*** (Figure 4.11E,F). *HvFT1* is an evening-expressed gene in WT plants, with a secondary expression peak in the morning. Rhythmic *HvFT1* expression was probably maintained in free running conditions in WT plants (although not confirmed by BRASS). Bowman (*ppdh1*) and *Hvelf3* mutants had very reduced *FT1* expression when compared to WT. *FT1* expression in Bowman (*ppdh1*) was morning-specific, while in *Hvelf3* plants, it was delayed by circa 5.5 h when compared to WT plants (Appendix B6). In free running conditions, *Hvelf3* mutants had no clear rhythm.

4.4 – Discussion

Functional conservation of Arabidopsis clock homologues has been demonstrated in many plants (Boxall *et al.*, 2005; Murakami *et al.*, 2007; McClung, 2013), from simple monocotyledonous plants such as *Lemna* (Miwa *et al.*, 2006; Serikawa *et al.*, 2008), to more complex models, like perennial trees (Ibáñez *et al.*, 2010; Kozarewa *et al.*, 2010) and crops, for instance soybean (Quecini *et al.*, 2007). Here, the putative barley clock genes identified *in silico* have demonstrated some shared behaviour with the Arabidopsis homologues, suggesting certain conservation of clock mechanisms. Conservation of phenotype and expression patterns of barley clock homologues, further discussed below, supports the suggestion that the barley clock genes identified *in silico* function in the circadian clock.

4.4.1 – Growth conditions and clock experiments

A 16 h light and 8 h dark photoperiod was chosen for the LD analyses. However, to define this photoperiod as an appropriate condition of a LD, a few facts were taken into consideration. The major phenotypic difference among the three barley lines analysed in clock experiments was flowering time. Regarding WT in particular, daylight length between 16 and 20 hours per 24 hour period causes small differences to flowering time, i.e.

WT plants reach their maximum response to flowering from 16 hours of day-light (Faure *et al.*, 2012). Also, many barley cultivars have their maximum yield when grown with 16-hour day lengths, when compared to 20 and 24 hours (Borthwick *et al.*, 1941). Therefore, 16 hours of light and 8 hours of dark was chosen for LD experiments. An opposite photoperiod was used for SD conditions, 16 hours of dark and 8 hours of light, which efficiently delays flowering in WT plants when compared to other photoperiods (Figure 4.5, Figure 4.6, and Faure *et al.*, 2012).

In clock experiments, plants were analysed at the same stage of development, GS11 of the Zadoks code (Figure 4.2B) (Zadoks *et al.*, 1974; Lancashire *et al.*, 1991). This stage was chosen for a few reasons. First, it avoided the juvenile phase of barley seedlings, during which plants do not respond to photoperiods (Roberts *et al.*, 1988; Ellis *et al.*, 1989). Second, after the juvenile phase, clock mutants and WT desynchronise their stages of development. This is caused by early-flowering plants having a significantly higher development rate than late-flowering plants. Comparing plants in different developmental stages was avoided because it could produce contrasting results. Also, early-flowering plants become insensitive to photoperiod once they reach anthesis (GS60), and their analyses should also be avoided (Boyd *et al.*, 2003). Therefore, the GS11 stage was preferable for the clock experiments carried out here. Also related to the harvesting procedure, samples at GS11 were quickly harvested at each time point. Quick sampling is important because intervals as small as thirty min can result in differential expression analysis between samples (Hsu and Harmer, 2012).

4.4.2 – Morphological effects of mutations on barley genes *PPDH1* and *ELF3*

Allelic variations in the barley clock gene *PPDH1* affect flowering time. The Bowman NIL (*eam1.d*) carrying the dominant *PPDH1* allele activates flowering in LD. Con-

versely, plants carrying the recessive *ppdh1* allele (naturally occurring in the Bowman cultivar) have lower photoperiod responsiveness and take longer to flower under LD. Interestingly, no significant morphological or developmental differences were observed for *PPDH1* allelic variants when plants were grown under SD conditions. These results are consistent with previous findings (Turner *et al.*, 2005; Faure *et al.*, 2012).

The nucleotide difference between *HvPPDH1* and *Hvppdh1* alleles that explains the reduced photoperiod responsiveness of Bowman (*ppdh1*) is not known, but it might lie in the CCT domain (Turner *et al.*, 2005) or in SNP48 (Jones *et al.*, 2008). Other species also have mutations within homologous *PPDH1* genes. In wheat, a 2 kb deletion in the promoter region of *TaPPDH1a* leads to photoperiod insensitivity (Beales *et al.*, 2007). Plants carrying the *Tappdh1a* allele flower early in SD and LD. In sorghum, allelic variants of the *SbPRR37* gene affect flowering control. In this SD flowering plant, loss-of-function *Sbprr37* alleles reduce photoperiod sensitivity and plants flower earlier in LD. The early-flowering phenotype of mutations in the *prr37* (*ppdh1*) genes of wheat and sorghum contrast with the late flowering phenotype of *Hvppdh1* in barley, probably because they have different mutations or because their clock functions are to some degree different. In Arabidopsis, the knock-out *Atprr7* mutant, generated by a T-DNA insertion, was a late flowering plant in LD conditions (Yamamoto *et al.*, 2003; Nakamichi *et al.*, 2005), similar to barley *ppdh1*. This suggests a functional conservation between *HvPPDH1* and its *PRR7* homologue in Arabidopsis, as suggested by *in silico* analysis (Figure 3.6 and Turner *et al.*, 2005).

Regarding the *ELF3* gene in barley, Bowman (*ppdh1*) plants carrying the loss-of-function *Hvelf3* allele (*eam8.w*) became early flowering in both LD and SD conditions, when compared to Bowman (*ppdh1*). This NIL mutant has previously been characterised for LD, also showing an early-flowering phenotype (Zakhrabekova *et al.*, 2012). Moreover, different loss-of-function alleles of *Hvelf3* also generate early flowering in LD (16 h or 18 h

light) and SD (10 h light), regardless of their *PPDH1* allele (Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012). In Arabidopsis, *elf3* knock-out mutants are also early-flowering plants in SD when compared to WT plants (Zagotta *et al.*, 1996; Yu *et al.*, 2008). The conservation of phenotypes of barley and Arabidopsis *elf3* plants suggests a functional conservation of *ELF3* in both species to repress flowering, as suggested by *in silico* analysis. However, the two paralogous rice *ELF3* genes are involved in flowering activation: *OsEF7* and, to a lesser degree, *OsEF3*. In this SD flowering plant, gene silencing and a T-DNA insertion targeting the *OsEF7* (*OsELF3-1*) gene delay flowering in LD and SD when compared to wild-type plants (Zhao *et al.*, 2012).

In addition to flowering time effects, allelic variations of *PPDH1* and *ELF3* were related to changes in size and/or numbers of many plant organs, including the number of grains. In other words, early flowering of WT (*PPDH1*) and *Hvelf3* plants was strongly correlated with small leaves and low yield. Plant yield is dependent upon biomass. Reduction in plant biomass can be caused by a decrease in photosynthesis and/or increased energy allocation to flowering at the expense of new vegetative structures. Regarding the former, no analysis of photosynthetic rate has been performed, except for studies of *HvCABa* expression. When compared to WT plants grown in LD, late-flowering Bowman (*ppdh1*) plants seemed to have higher mRNA levels of this chlorophyll-related gene, which could explain the bigger leaves of Bowman (*ppdh1*). However, this difference in expression of *HvCABa* was not statistically significant. Nevertheless, there is increasing evidence for circadian clock control over photosynthesis (Dodd *et al.*, 2005; Noordally *et al.*, 2013), and further work will clarify any direct or indirect effects from *ppdh1* and *elf3* mutations on photosynthesis and, consequently, biomass.

An alternative explanation for the reduced size of leaves in early flowering plants, mentioned previously, is the limitation of resources to carry out both flowering and vegeta-

tive growth. Most grasses lose meristems when flowering occurs, resulting in decreased biomass accumulation and, ultimately, plant death after reproduction (Lord, 1998). Therefore, it is very likely that early flowering itself is involved in the reduced sizes and numbers of different plant organs in barley carrying the *HvPPDH1* and *Hvelf3* alleles. Namely, small leaves and small yield might be a result of short vegetative growth period caused by the early activation of flowering by *PPDH1* (in LD) and *elf3* (in LD and SD). This hypothesis has also been suggested in other plants and for other genes. Over-expression of the *OsMADS45* gene in rice reduces heading date, which causes a reduction in plant height, biomass and the number of spikelets per panicle (Wang *et al.*, 2013). Another study in rice revealed that enhanced expression of the flowering time regulator *GHD7* under LD conditions delays heading and increases both plant height and panicle size (Xue *et al.*, 2008).

4.4.3 – Expression of barley clock genes

Expression profiles of 16 putative barley clock genes were analysed in the three barley lines using the HR RT-PCR approach and a putative framework of the barley clock in WT plants is summarised in Figure 4.12. It is noteworthy that the HR RT-PCR approach has been validated previously for mRNA quantitation, being in good agreement with data from qPCR (James *et al.*, 2012a). All barley clock genes, except *HvZTLa*, were rhythmic under light/dark conditions, and most were rhythmic under free-running conditions, similar to those in Arabidopsis. This indicated that the mechanisms controlling clock gene expression are, at this level, conserved between these species. In the *Hvppdh1* mutant (Bowman), most clock genes were expressed rhythmically, and some have a different expression peak (phase) and/or level, when compared to WT. Regarding the *Hvelf3* mutant (Bowman NIL *eam8.w*), circadian rhythms of most clock genes were significantly altered and were arrhythmic in free-running conditions.

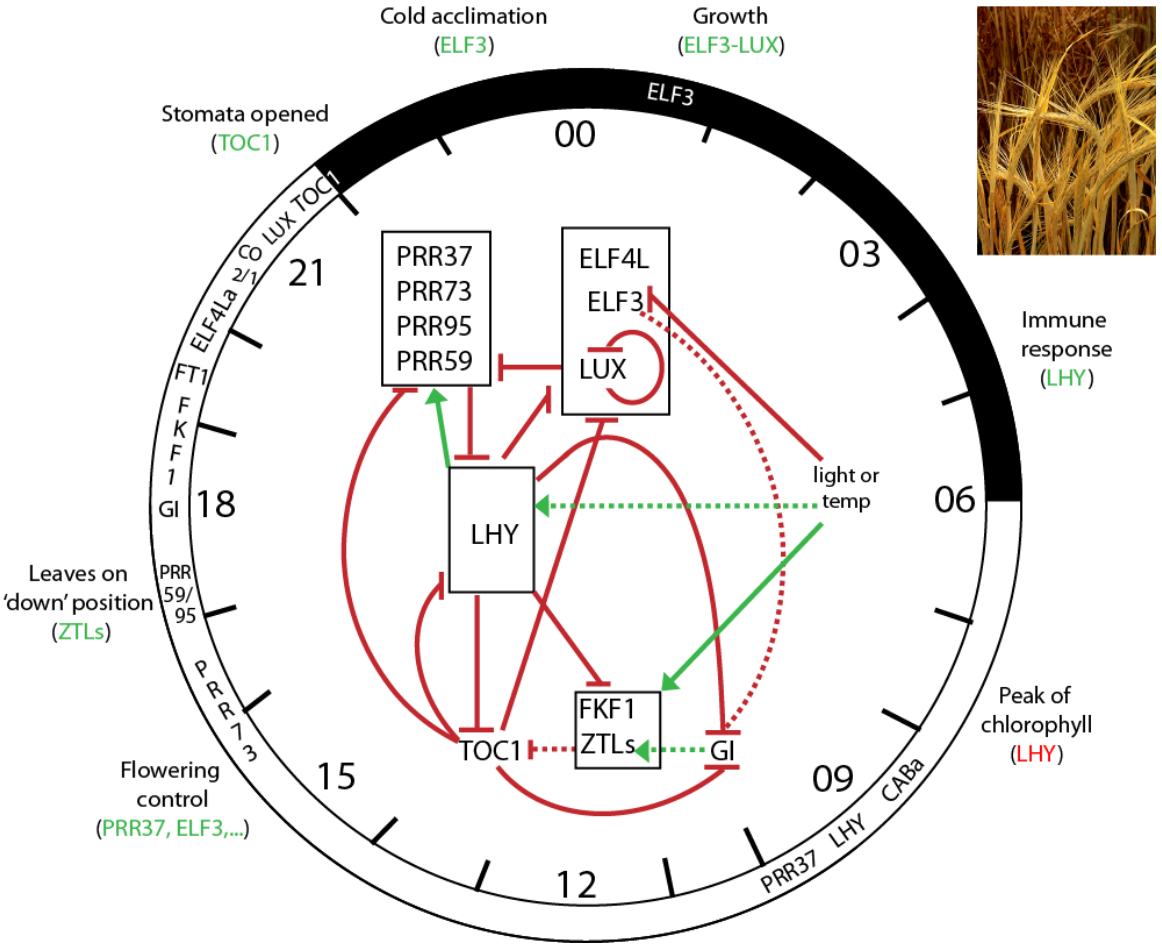


Figure 4.12: Proposed schematic diagram of the barley circadian clock system superimposed on the model from Arabidopsis. Feedback loops of the core clock genes are represented in the centre. Full lines represent transcriptional feedback loops, whereas dashed lines represent post-translational regulations. Green lines are for activation, while red lines are for repression. For simplicity, the *CABa* component was not included in the above regulatory network. Expression peaks of clock genes are represented at different times of the day. Several physiological processes written on the periphery of the clock are possibly regulated by clock genes (in brackets), allowing them to occur at the right time of the day.

Before discussing the expression data obtained here, a few facts must be taken into consideration. To begin with, expression of genes without a clear rhythm must be treated with caution. It is now known that after 48 hours in LL, leaves develop diversified waves

of clock gene expression, observed only at the cellular level (Wenden *et al.*, 2012). By using the whole leaf, any alteration in expression in single cells would be averaged out and consequently, some examples of arrhythmia could be 'false negatives', rather than complete loss of rhythm. Additionally, it is not possible to strictly compare expression results with those published previously. First, comparative analysis of barley Bowman (*ppdh1*) and *Hvelf3* (also carrying the *ppdh1* allele) mutants with other species must be treated carefully because the causal mutation of *Hvppdh1* is not accurately known (see Sections 4.1.3 and 4.4.2), which makes it difficult to perform a confident comparative analysis. Additionally, the experimental protocols for plant growth and sampling published previously were mostly different from the ones used here, for instance, the use of LD versus SD conditions, vegetative versus flowering growth stages, leaves versus whole plant samples. Nevertheless, general comparisons are made and discussed cautiously.

Mutations in barley clock components affect the circadian system and flowering time, which contributed to crop domestication and provided a template for elucidating the barley photoperiod pathway. However, it is certainly a very difficult task to uncover the overall operational mechanisms of photoperiodic flowering in barley from the experimental results obtained here. The molecular clock (in the photoperiod pathway) operates through complex autoregulatory feedback loops at different levels: transcriptional, post-translational, spatial, temporal and quantitative, among others. Therefore, it is very ambitious to interpret the underlying biochemical function of a barley clock component when removing it from this complex integrated network. For example, the early flowering *Hvelf3* loss-of-function mutant has higher levels of the flowering activator *HvGI* during the night (Figure 4.8), even though its transcriptional night-repressor, *HvTOC1* is also highly expressed at night. Such incongruences exist because the direct regulation of *GI* by TOC1 was analysed separately from the integrated clock picture that involves additional regula-

tors of *GI*. To facilitate the task of a comprehensive analysis, differential equations have been generated and applied in a number of organisms, including Arabidopsis (Locke *et al.*, 2006). These models have proven useful in uncovering the general mechanism of circadian clocks, as well as providing a robust approach with which to interpret experimental results (Akman *et al.*, 2012; Pokhilko *et al.*, 2012). As yet, a computational model of the barley circadian clock is not available to analyse the expression results obtained here and relate these with the flowering phenotype. Nevertheless, further discussion of each clock gene and their orthologues from other species are found below, while flowering-related clock genes and their interactions and relationship with the flowering phenotype are discussed in Section 4.4.4.

4.4.3.1 – *LHY*

The morning-exclusive *HvLHY* is rhythmic in light/dark and continuous light conditions. Similar expression for *LHY* genes has been observed in Arabidopsis (Alabadí *et al.*, 2001), rice (Murakami *et al.*, 2007), maize (Hayes *et al.*, 2010), sorghum (Murphy *et al.*, 2011), and barley (Campoli *et al.*, 2012b; Faure *et al.*, 2012) confirming that there is functional conservation between *HvLHY* and its orthologues in other species, as suggested by *in silico* analysis. The data similarity with previously described barley *LHY* (Campoli *et al.*, 2012b; Faure *et al.*, 2012) also confirms that the experimental procedure carried out was appropriate to study clock genes.

In the barley clock mutants carrying the alleles *ppdh1* (Bowman) and *elf3/ppdh1* (double mutant named *Hvelf3*), there is an influence on *LHY* expression profiles. When compared to WT plants, *HvLHY* expression is significantly higher in Bowman (*ppdh1*) plants during three time points of the day. This effect was rejected or overlooked in the work of Campoli *et al.* (2012b), probably because they analysed only one biological repli-

cate, as opposed to the four replicates used here. In Arabidopsis, *prr7* mutants have unaffected expression of *AtLHY*, but *AtLHY* levels are considerably higher in *prr7/prr9* and *prr7/prr5* double mutants, as well as *prr7/prr5/prr9* triple mutants (Farré *et al.*, 2005; Nakamichi *et al.*, 2005). As mentioned in the general introduction (Section 1.7), PRR7 is the transcriptional repressor of *CCA1* and *LHY*. Therefore, *PRR7/PPDH1* loss-of-function (as in the case of *Atprr7*) or 'reduced-function' (as in the case of barley *Hvppdh1*) mutations can affect the rhythmic 'shape' of *LHY/CCA1* expression (Nakamichi *et al.*, 2010). This suggests that *AtPRR7* and *HvPPDH1* have a conserved function. In constant light, *LHY* has lower amplitude and maintains a rhythmic expression in Bowman (*ppdh1*) plants, confirming that the barley clock in this line is functional, consistent with other reports (Campoli *et al.*, 2012b; Faure *et al.*, 2012). In Arabidopsis*, prr7* mutants exhibit modest *CCA1* and *LHY* period lengthening after a few days in free running conditions (from former photocycles and thermocycles), having an important function in clock entrainment to light and temperature (Salomé and McClung, 2005). Such observation was not detected in our work due to only examining three days of free running conditions.

Regarding the barley *Hvelf3* mutant, *LHY* expression is lower than WT plants during the day but higher during early night, which is consistent with previous work in barley (Faure *et al.*, 2012). In Arabidopsis and rice, *elf3* knock-out plants also express lower levels of *LHY/CCA1* in light/dark conditions (Dixon *et al.*, 2011; Yang *et al.*, 2013). In constant light, *elf3* plants become arrhythmic for *LHY/CCA1* expression in barley, rice (Zhao *et al.*, 2012; Yang *et al.*, 2013) and Arabidopsis (Wenden *et al.*, 2011a; Herrero *et al.*, 2012). Faure *et al.* (2012) suggested that *LHY* is still rhythmic in *Hvelf3* mutants growing in constant light conditions, but they have analysed only two days of continuous light, whereas *HvLHY* arrhythmicity becomes obvious on the third day (Figure 4.7).

4.4.3.2 – *CAB*

HvCABa is rhythmically expressed during a large part of the day, similar to the Arabidopsis homologue *AtCAB2* (Hicks *et al.*, 1996). In *Hvelf3* barley plants, *HvCABa* is also expressed during the night, which was not observed in WT or Bowman (*ppdh1*) plants. This aberrant expression of *HvCABa* has been previously noticed in a microarray analysis using early night samples of another *Hvelf3* mutant (Igri NIL *eam8_ea8.k*) and WT (Igri cultivar) (Faure *et al.*, 2012). In constant light conditions, *HvCABa* maintains a rhythmic 22 h period in WT plants, whereas Bowman and *Hvelf3* become arrhythmic. The Arabidopsis homologue *AtCAB2* was rhythmic in constant light conditions, except in *elf3* mutants (Hicks *et al.*, 1996), which is similar to the results obtained in this study. Additional *HvCAB* genes have been analysed in barley (Campoli *et al.*, 2012b; Faure *et al.*, 2012), but they are not part of the *CAB1/2/3* subfamily, to which *AtCAB2* belongs.

4.4.3.3 – *FKF1*

Barley *FKF1* is an evening-expressed gene, similar to the expression of its homologues in maize (Hayes *et al.*, 2010), rice (Murakami *et al.*, 2007) and Arabidopsis (Imaizumi *et al.*, 2003). Interestingly, daily activation of expression of *HvFKF1* occurs earlier in Bowman (*ppdh1*) and *Hvelf3* plants when compared to WT, which is similar to *FKF1* expression in Arabidopsis *elf3* mutants (Kim *et al.*, 2005). This earlier *FKF1* activation also occurs during Bowman (*ppdh1*) and *Hvelf3* entrainment in constant light conditions. In WT barley, Bowman (*ppdh1*) and *Hvelf3*, *FKF1* expression is still rhythmic in constant light, similar to WT Arabidopsis (Nelson *et al.*, 2000) and WT rice (Murakami *et al.*, 2007).

4.4.3.4 – *ELF4-like*

In the three barley lines analysed, *HvELF4-likeA* probably has a daily bimodal cycling pattern (dawn and before dusk), but this rhythm does not continue in continuous light conditions. The *AtELF4* gene from the *ELF4* family, to which the *ELF4-like2/3/4* subfamily belongs, peaks before dusk (LD) or during early night (SD) (Doyle *et al.*, 2002). Expression of homologues from the *ELF4-like2/3/4* subfamily from other species has not, to my knowledge, been described so far. However, *HvELF4-likeA* can fully complement the *elf4* loss-of-function phenotype in Arabidopsis, suggesting conserved function (Kolmos *et al.*, 2009).

4.4.3.5 – *LUX*

HvLUX is rhythmically expressed near the end of the light period in LD. However, in SD it is expressed during the dark period (Campoli *et al.*, 2013). In Arabidopsis plants grown in 12 h light and 12 h dark cycles, *AtLUX* is expressed around dusk (Helfer et al., 2011). In constant light, WT and Bowman (*ppdh1*) plants maintained rhythmicity, as observed in WT Arabidopsis (Helfer et al., 2011). In the work of Campoli *et al.* (2013), Bowman (*ppdh1*) plants transferred from SD to LL have a significant reduction in the expression of *HvLUX*, not observed here for LD-LL transitions or in Arabidopsis plants (Helfer et al., 2011). Higher expression levels of *HvLUX* in WT plants on the first subjective night and morning when compared to Bowman (*ppdh1*) suggests different mechanisms for clock entrainment in these two plants. In *Hvelf3* plants, *HvLUX* did not have a clear expression peak and an increased expression was observed in the morning when compared to WT plants.

4.4.3.6 – *ZTL*

ZTL protein levels oscillate over a day, but their transcript levels are not oscillatory in Arabidopsis (Schultz *et al.*, 2001; Kim *et al.*, 2007) or rice (Murakami *et al.*, 2007). Similarly, barley *HvZTLa* mRNAs were constitutively expressed throughout day and night. In rice, transcript levels of *OsZTL1* under LDs were largely increased in *ef7* mutants (Yang *et al.*, 2013), which was not seen for *Hvelf3* plants here.

4.4.3.7 – *TOC1*

HvTOC1 is rhythmically expressed in the evening of light/dark cycles and subjective evenings for up to two days in constant light for the three barley lines analysed. This expression behaviour is similar to the expression of its homologues in maize (Hayes *et al.*, 2010), sorghum (Murphy *et al.*, 2011), rice (Murakami *et al.*, 2007; Zhao *et al.*, 2012) and barley (Campoli *et al.*, 2012b; Faure *et al.*, 2012). In constant light, *HvTOC1* maintains rhythmic expression in WT and Bowman plants. In rice, WT and *Osef7* plants are also rhythmic for *TOC1* (Zhao *et al.*, 2012). In Arabidopsis WT and *prr7* plants, *TOC1* evening expression is maintained (Farré et al., 2005). These *TOC1* results in rice and Arabidopsis are similar to those observed in barley, confirming their orthology. In *Hvelf3* plants, *HvTOC1* has significantly higher expression levels during morning and subjective morning, also observed in barley (Faure *et al.*, 2012), Arabidopsis (Dixon *et al.*, 2011) and rice (Yang *et al.*, 2013).

4.4.3.8 – *PPDH1* and *PRR73*

In barley, *HvPPDH1*(*PRR37*)/*Hvppdh1* and *HvPRR73*, are co-expressed during the greater part of the day and they reach very high levels by mid-day, similar to previous find-

ings in barley (Campoli *et al.*, 2012b; Faure *et al.*, 2012; Campoli *et al.*, 2013), rice (Murakami *et al.*, 2003; Zhao *et al.*, 2012), and maize (Hayes *et al.*, 2010). In Arabidopsis, the orthologous gene *PRR7* is expressed around mid-day, before the evening-expressed paralogue *PRR3* (Matsushika *et al.*, 2000). This differential expression between both paralogues was not observed in monocots, but similar expression behaviours of *PRR7* and *PRR37/73* around mid-day suggests some functional conservation and possibly orthology of these genes. Bowman (*ppdh1*) and *Hvelf3* plants had significantly higher expression levels of *Hvppdh1*, despite the intrinsic mutation, when compared to the WT *HvPPDH1* allele, which continued in constant light. This is in agreement with an earlier report (Faure *et al.*; 2012).

4.4.3.9 – *PRR59* and *PRR95*

In barley, *HvPRR59* and *HvPRR95* are co-expressed in the afternoon, after expression peaks of *HvPPDH1* and *HvPRR73*. These expression patterns are similar to previous findings in barley (Campoli *et al.*, 2012b; Campoli *et al.*, 2013) and rice (Murakami *et al.*, 2003). In Arabidopsis, the homologous gene *PRR9* is expressed in the morning, while *PRR5* is expressed in the afternoon (Matsushika et al., 2000). This differential expression between both paralogues was not observed in monocots, but similar expression behaviours of *PRR5* and *PRR95/59* in the afternoon suggests functional conservation and, possibly, orthology of these genes.

Arabidopsis *elf3* mutants display high levels of *PRR9* and *PRR7* in light/dark and constant light conditions (Kolmos *et al.*, 2011). Bowman (*ppdh1*) and *Hvelf3* plants have significantly higher levels of *PRR37/73/95/59* transcripts when compared to WT. A similar expression increase was observed for *OsPRR59* in rice *ef7* mutants under LDs (Yang *et*

al., 2013). These results suggest conserved function of homologous *ELF3* genes in repressing *PRR7*/*PRR9* in Arabidopsis and *PRR* genes in monocots.

4.4.4 – Photoperiodic flowering in barley

4.4.4.1 – *ELF3*

WT barley probably has a rhythmic expression of *HvELF3* during the night of LD, also observed in SD (Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012). In Arabidopsis, *ELF3* is expressed at night (12 h light and 12 h dark) (Dixon *et al.*, 2011), similar to rice *OsEF3* (Zhao *et al.*, 2012). Additionally, rice paralogue *OsEF7* is expressed at dawn in both LD and SD conditions (Zhao *et al.*, 2012). These night-related expression patterns of *ELF3* homologues suggests that there is functional conservation of this gene among different species.

In Bowman (*ppdh1*) plants, *HvELF3* is expressed at significantly higher levels in the morning, when compared to WT. Since *ELF3* is a flowering repressor, higher levels of this gene in the morning of Bowman (*ppdh1*) plants might explain their late-flowering phenotype. *Hvelf3* mutants have lower levels of the *elf3* transcript when compared to WT. A similar reduction was also observed in the same *Hvelf3* NIL growing in SD conditions (Faure *et al.*, 2012). This reduction probably occurs due to the *elf3* mutation that generates PTC-containing transcripts, which are likely degraded by NMD. This loss-of-function in the *ELF3* gene is the cause of the early flowering phenotype (discussed previously in Section 4.4.2) (Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012).

Unexpectedly, no clear rhythmicity was observed for the *HvELF3* gene in constant light conditions for the three barley lines analysed. In previous reports, *HvELF3* maintained rhythm in Bowman (*ppdh1*) plants transferred from SD to constant light conditions

(Faure *et al.*, 2012) as well as in Arabidopsis (Hicks *et al.*, 2001). These incongruent results are probably due to different experimental procedures adopted here and by other labs, and may be clarified when additional technical replicates are analysed here.

4.4.4.2 – *GI*

HvGI is rhythmic in WT and Bowman (*ppdh1*) under light/dark and continuous light conditions. This expression profile is similar to the expression profiles of its homologues in maize (Hayes *et al.*, 2010), sorghum (Murphy *et al.*, 2011), rice (Hayama *et al.*, 2002a), Arabidopsis (Park *et al.*, 1999) and barley (Dunford *et al.*, 2005; Turner *et al.*, 2005; Campoli *et al.*, 2012b; Faure *et al.*, 2012), confirming that there is functional conservation between *HvGI* and its orthologues in other species.

Plants with both *PPDH1* and *ppdh1* alleles have identical *GI* mRNA levels, but nothing is known about their GI protein levels. ELF3 negatively regulates GI protein levels in Arabidopsis (Yu *et al.*, 2008). Bowman (*ppdh1*) has higher levels of *ELF3* mRNA during the morning, mentioned above, which if translated might reduce HvGI protein levels. A putative reduction of a flowering activator gene like GI might contribute to the Bowman (*ppdh1*) late-flowering phenotype.

HvGI in *Hvelf3* plants is expressed throughout the day, including early in the morning and late night. This *GI* expression behaviour has been described for *elf3* mutants in barley (Faure *et al.*, 2012), Arabidopsis (Kim *et al.*, 2005; Dixon *et al.*, 2011), and rice (Hayama *et al.,* 2002). In this case, higher expression levels of a flowering activator gene such as *GI* might explain the early flowering phenotype of *Hvelf3* mutants (Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012).

4.4.4.3 – *CO*

HvCO1 and *HvCO2* are both expressed before and after dusk in WT plants, which is consistent with previously published data for barley (Turner *et al.*, 2005; Campoli *et al.*, 2012a; Campoli *et al.*, 2012b). Similarly, Arabidopsis *CO* is also expressed before and after dusk in LD, resulting in flowering (Sawa et al., 2007). Expression of *CO1* and *CO2* in Bowman (*ppdh1*) was delayed and reached maximum levels at dusk. Interestingly, expression of Arabidopsis *CO* under non-inductive SD conditions also peaks at/after dusk, which delays flowering. Therefore, a significant reduction of *CO1* and *CO2* in Bowman (*ppdh1*) plants during the day might help explain their late flowering phenotype. Moreover, since AtPRR7 indirectly activates expression of *CO* (Nakamichi *et al.*, 2007), it was expected that the reduced function of the *Hvppdh1* (PRR7 homologue) allele in Bowman would fail to efficiently activate expression of the homologous *HvCOs*, therefore causing the late flowering phenotype of Bowman (*ppdh1*). These results suggest the *PPDH1*/*PRR37* gene controls expression of *CO1* and *CO2*, consistent with other reports in barley and sorghum (Murphy *et al.*, 2011; Turner *et al.*, 2005; Faure *et al.*, 2012). This hypothesis is further supported by the putative similar expression patterns of *HvPPDH1*, *HvCO1* and *HvCO2* in free-running conditions that supports their linked regulation, which is not seen in plants carrying the *Hvppdh1* allele. However, Campoli *et al.* (2012b) partially rejected this hypothesis because the expression difference of *HvCO1*/*CO2* between plants carrying *ppdh1* or *PPDH1* differed only in LL, not LD. This is probably due to the difference in the experimental protocols adopted here and by Campoli *et al.* (2012b). For instance, one-week old plants in the Bowman (*ppdh1*) background were analysed here in four replicates per time point using the HR RT-PCR approach, whereas in the work of Campoli *et al.* two-week old plants in the Scarlett background, containing different *PPDH1* alleles, were analysed in one replicate per time point using the qPCR approach.

In Arabidopsis *elf3* mutants, *CO* expression had a moderate rise in LD conditions (Kim *et al.*, 2005). Contrastingly, *Hvelf3* mutants carrying the *ppdh1* allele had low and delayed expression of *HvCO1* and *HvCO2*, respectively. This might be due to the *ppdh1* allele, which fails to activate expression of *HvCO*s efficiently. These results suggest that expression of *HvCO*s in the light is not required for the early-flowering phenotype of *Hvelf3* mutants, also suggested by Faure *et al.* (2012). It is possible that *Hvelf3* early flowering occurs via an alternative pathway to the classical GI-*CO* pathway. ELF3 possibly regulates *FT* via stimulation of SHORT VEGETATIVE PHASE (SVP) protein accumulation, a flowering repressor (Yoshida *et al.*, 2009). In this case, loss-of-function *Hvelf3* mutants might have decreased levels of SVP protein, which permits *FT* expression.

Regarding *HvCO2* in particular, antisense and unspliced transcripts generated from the *HvCO2* locus were detected. These additional transcripts do not code for the functional CO2 protein. Previously published data of *HvCO2* expression did not account for these transcripts, and they show unclear and probably inaccurate *HvCO2* rhythms in light/dark cycles (Turner *et al.*, 2005; Campoli *et al.*, 2012a; Campoli *et al.*, 2012b). Here, only transcriptional data from the fully spliced *HvCO2* transcript was used, which revealed a clear and robust *HvCO2* rhythm. Interestingly, *AtCOL1* is within a potential natural antisense gene that is also present in spliced and unspliced versions (At5g15845). The same was not observed for the paralogous genes *AtCO* and *AtCOL2*. Therefore, this similarity of *HvCO2* and *AtCOL1* suggests conserved regulatory mechanisms of transcription. However, *CO* paralogues *COL1* and *COL2* do not interfere with the photoperiodic flowering pathway, suggesting that the function of *CO* was not conserved by its paralogues during evolution (Ledger *et al.*, 2001).

Regarding continuous light conditions, no clear 24-hour rhythmic expression was observed in free-running conditions for *HvCO1* and *HvCO2* in the three barley lines ana-

lysed. However, it might be possible that WT plants have a shorter period (16 h) for these genes. In the work of Campoli *et al.* (2012b), the *HvCO* genes have a 24 h rhythm in WT, but not Bowman (*ppdh1*), plants under free-running conditions. Conversely, in the work of Faure *et al.*, (2012), *HvCO1* was rhythmic in Bowman (*ppdh1*) plants transferred from SD to LL conditions. These conflicting results illustrate once again the difficulties in detecting rhythmic expression of clock-related genes in barley plants, as well as the diversity of results coming from different experimental procedures that compromise appropriate comparisons and data interpretation.

4.4.4.4 – *FT*

In LD, *HvFT1* expression peaks at dusk, also observed in different barley cultivars (Turner *et al.*, 2005; Campoli *et al.* 2012a). In Arabidopsis, *FT* also has an expression peak in LD at around dusk (Turck *et al.*, 2008), suggesting that there is a conservation in the regulation of barley *HvFT1* and its Arabidopsis homologue. In Bowman (*ppdh1*) plants, *HvFT1* expression is barely detectable, similar to other barley cultivars carrying *ppdh1* alleles, which is in accordance with the late flowering phenotype (Turner *et al.*, 2005; Campoli *et al.* 2012a; Faure *et al.*, 2012).

In Arabidopsis *elf3* mutants, *FT* expression was greatly elevated in LD conditions (Kim *et al.*, 2005). Surprisingly, the first leaf of one-week old *Hvelf3* mutants have very reduced *FT1* expression when compared to WT. It was previously shown that the second youngest leaves of two-week old *Hvelf3* mutants have increased expression of *HvFT1* in SD, which is related to their early-flowering phenotype (Faure *et al.*, 2012). Therefore, it is possible that the different experimental procedures of both studies helps to explain these contrasting results.

In summary, genetic analysis of circadian behaviour using the HR RT-PCR approach suggests that the circadian expression profiles of the putative barley clock-related genes are similar with those of their Arabidopsis counterparts. Effects of *ppdh1* and *elf3/ppdh1* mutations resemble *prr7* and *elf3*, respectively, loss-of-function mutations in Arabidopsis. However, some genes are not present in barley (e.g. *CCA1*) and there is variation in the expression profiles of some clock genes (e.g. *HvLHY* is expressed later in the morning, while Arabidopsis *LHY* is expressed at dawn). Also, there is some variation in the effects of mutated *Hvppdh1* and *Hvelf3/Hvppdh1* when compared to Arabidopsis *prr7* and *elf3*, respectively. Such variations might be due to the fact that monocots and dicots have evolved their clocks independently (see Chapter 6).

Chapter 5. Alternative splicing regulation of barley clock-related genes

5.1 – Introduction

The circadian clock controls many processes and regulation of more than one third of genes in Arabidopsis (Covington *et al.*, 2008). Regulation of the circadian clock is complex, involving interlocked transcription feedback loops, control of protein phosphorylation and degradation, and chromatin remodelling (see Section 1.7). Recently, alternative splicing (AS) was shown to be another mechanism by which clock functions could be controlled. Extensive AS was shown to regulate Arabidopsis clock gene expression through dynamic changes in alternative transcript levels, some of which altered the level of productive mRNA through AS/NMD of alternatively spliced transcripts (see Section 1.8). Interestingly, AS regulation of the Arabidopsis, *Drosophila* and *N. crassa* clocks has been shown to be temperature dependent. In fact, temperature greatly affects the overall biology of the plant and this is an area of great interest (McClung and Davis, 2010).

5.1.1 – Temperature effects on plants

Other than the various aspects of light, temperature is probably the other most important external cue that affects the overall biology of the plant because plants need a conducive temperature to survive and propagate (Srikanth and Schmid, 2011). Plants have evolved mechanisms to endure temperatures extremes from -30 °C in arctic winter to almost 50 °C in African deserts (McClung and Davis, 2010). Furthermore, individual plants may be subjected to ambient temperatures that vary in the short term by more than 30 °C

as a result of the vagaries of radiation input caused by clouds, wind speed and angle of incidence of sunlight (Helliker and Richter, 2008; McClung and Davis, 2010). Despite these daily, latitudinal and seasonal variations of temperature, most plant cellular and physiological processes (for instance, hormone signalling, flowering time, circadian clock and photosynthesis) have an optimal functional temperature and plants will work to maintain leaf temperatures near that optimum value. Firstly, plants attempt to modulate their endogenous temperature from ambient temperatures through morphological and physiological modifications, for example controlling water loss and losing heat through convection and reflectance (Helliker and Richter, 2008). Secondly, cellular processes have a functional plasticity so they can acclimate their temperature optimum to the ambient temperature (temperature acclimation) (Yamori *et al.*, 2013).

The thermal perception system(s) responsible for transducing temperature information to the plant cellular processes is one of the great unknowns in plant science. Two events have been hypothesised as the primary temperature sensing events: alteration of membrane fluidity and elevation of cytosolic free calcium levels ($[Ca^{2+}]_{cyt}$) (Penfield, 2008; McClung and Davis, 2010). These are probably associated and are the earliest events that occur upon temperature variation. Ca^{2+} is a secondary messenger used in the transduction of numerous signal and is essential for initial response and subsequent temperature acclimation in plants (Hepler, 2005; McClung and Davis, 2010). Some responses to temperature may be initiated by Ca^{2+} signalling events, others initiated by the direct physical effects of temperature on protein conformation and enzyme activity. Additionally, sensing and signal transduction of non-stressful ambient temperature changes are partially different to sensing extreme temperature changes (temperature stress) (McClung and Davis, 2010). Several plant thermometers are hypothesised which might form the plant temperature signalling network (Penfield, 2008; McClung and Davis, 2010). Upon temperature perception, plants respond to temperature changes through many genetic, physiological and de-

velopmental processes such as major chromatin reconfiguration, massive transcriptional changes, increases in levels of cryoprotectants and heat-shock proteins, alterations in membrane composition, changes in the hormone levels and flowering control (Penfield, 2008; McClung and Davis, 2010).

The predicted consequences of global climate change include increased fluctuation of temperature with greater extremes and a trend to warmer mean temperatures. It is estimated that around one-third of all European plant species may become extinct by 2080 in response to predicted climate change (Thuiller *et al.*, 2005). In addition to profound consequences for wild species, agricultural plant communities and their yield are likely to be affected by global climate change (McClung and Davis, 2010). To understand and possibly cope with the consequences of such change, much more about the basic biology of the plant and how plants sense and respond to temperature must be learned.

5.1.2 – Clock temperature entrainment and compensation

Two major influences of temperature on the circadian clock are entrainment and compensation. In the former, temperature differences as small as 4 °C between subjective night and day can entrain the Arabidopsis clock (McClung *et al.*, 2002). Clock entrainment to environmental temperature changes occurs through altered gene expression (Gould *et al.*, 2006; Bieniawska *et al.*, 2008; James *et al.*, 2012a) and at least three clock components are essential for this process: *PRR7*, *PRR9* and *ELF3* (Salomé and McClung, 2005; Thines and Harmon, 2010). Arabidopsis *elf3* mutants are unable to entrain to thermocycles in darkness (Thines and Harmon, 2010), whereas *prr7/prr9* double mutants are unable to entrain to thermocycles of 22°C/12°C, but are able to respond to cycles of 28°C/22°C (Wigge, 2013). Upon temperature entrainment, the endogenous clock rhythm is synchronised with external cues and a number of circadian-regulated output processes are reorgan-

ised at the molecular level, generating the appropriate biological responses according to temperature condition. Adding to the complexity of temperature entrainment and output control, the clock can gate the low-temperature (LT) expression of the cold tolerance *C-REPEAT/DRE BINDING FACTOR* genes during the evening and night and modulates Ca^{2+} signals (Fowler *et al.*, 2005; Dodd *et al.*, 2006; Keily *et al.*, 2013).

Another critical behaviour of the clock in regards to temperature is the process of temperature compensation. In this process, the plant circadian clock is able to robustly perform an approximately daily cycle across a wide range of temperature regimes (for instance, between 4 °C and 28 °C in Arabidopsis), beyond which the clock stalls and transcript levels of many clock genes become arrhythmic (Ramos *et al.*, 2005; Gould *et al.*, 2006; Bieniawska *et al.*, 2008; Ibañez *et al.*, 2008; Penfield, 2008). The Arabidopsis genes *CCA1*, *LHY*, *PRR7*, *PRR9* and *GI* have an important role in this process (Gould *et al.*, 2006; Salomé *et al.*, 2010). Analyses of transcript levels of core clock components within a physically relevant temperature range showed that *LHY* expression increases with temperature, and this appears to be compensated for by increases in *TOC1* and *GI* (Wigge, 2013). In an Arabidopsis *prr7/prr9* double mutant background, excessive *LHY* and *CCA1* gene expression causes a significant temperature over-compensation phenotype, with circadian period dramatically lengthening at warmer temperatures (Salomé *et al.*, 2010). Interestingly, reduction in *LHY* and *CCA1* activity is sufficient to restore the wild-type clock behaviour in this background (Salomé *et al.*, 2010). Furthermore, recent evidence suggests that temperature entrainment and compensation involve temperature-dependent alternative splicing of some clock genes. For example, upon cold treatment, the expression levels of functional *REVEILLE8* (*RVE8*) transcripts, encoding a MYB-like transcription factor, are decreased and this is probably due to an increase in non-functional AS isoforms at the expense of

functional mRNAs (James *et al.*, 2012b). For more information on temperature-dependent alternative splicing of clock genes, please refer to Section 1.8.

Prediction and appropriate responses to temperature changes by the plant circadian clock provides a fitness benefit to plants, which makes this area of interest in agriculture. In barley, the correct molecular functioning of the barley clock in different temperatures improves agronomic traits. In winter barley, for example, photoperiod sensitivity (as well as vernalisation requirement) is an important adaptive feature that prevents flowering during winter. Preventing flowering subsequently provides efficient LT tolerance through induction of LT genes (Fowler *et al.*, 2001). However, future efforts are still required to understand how the barley clock remains functional within a wide range of temperatures. The close link of the circadian clock and AS in Arabidopsis raised the question as to whether AS might be involved in the operation of the barley circadian clock or its response to altered temperature conditions. Therefore, we investigated the expression levels and AS of barley circadian clock genes in response to low temperature. Numerous AS events occur in barley clock genes and particularly for *LHY* and *PPDH1*, many are temperature sensitive, potentially having a major role in regulation of gene expression. Collectively, the data suggests that AS is a novel mechanism involved in operation and regulation of the barley circadian clock.

5.2 – Materials and methods

5.2.1 – AS discovery in barley clock genes

5.2.1.1 – Search for AS in transcript databases

BLAST searches were performed using barley clock genomic sequences identified previously (Chapter 3) against GenBank and barley RNA-seq (Consortium, 2012a) tran-

script databases to identify AS isoforms. For the RNA-seq data set, AS discovery analyses were carried out using Tablet program version 1.7.0_21 (Milne *et al.*, 2013), which is a sequence visualisation tool that aligns barley genomic contigs against transcript reads produced from several barley developmental stages (Consortium, 2012a).

5.2.1.2 – RT-PCR and cloning of AS transcripts

To detect novel AS events and determine the sequences of unknown alternatively spliced isoforms, systematic RT-PCR and cloning of amplified products was carried out. These procedures, described below, followed the relevant manufacturers' instructions. RT reactions were carried out according to the protocol in Section 2.3.1 on RNA samples (extracted according to Section 2.2) from experiments described in 4.2.3, 5.2.2 and Appendix A7.1. This was followed by PCR reactions to amplify the alternatively spliced products (Appendix A7.2). PCR samples containing the putative AS products were purified using the MinElute® PCR purification kit (QIAGEN, Germany). The resulting purified PCR products were ligated into the pGEM-T easy vector (Promega, USA), and heat-shock transformation of the ligation reactions was performed using high-efficiency JM109 competent cells (Promega, USA). Transformation cultures were plated onto Luria-Bertani (LB, Appendix A8) solid agar plates supplemented with ampicillin (50 μg/mL, Appendix A9), IPTG (25 μg/mL) and X-Gal (25 μg/mL) and incubated overnight at 37 °C. Screening of transformants for inserts of interest was performed by blue-white selection screening of colonies from indicator plates (Appendix A10) and followed by colony PCR (Appendix A7.2). Around 50 white and light blue colonies per transformation were used in PCR screening with gene-specific primers. PCR samples were run on a 1.5% agarose (UltraPure™, Invitrogen, Life Technologies, USA), 1 x TBE (Appendix A3.6) gel to detect bands of the expected (FS) and unexpected sizes (AS isoforms). This enabled the identifi-

cation of colonies with plasmids containing cloned AS isoforms with inserts of variant sizes. Selected colonies were grown further by inoculating in 5 mL liquid LB medium supplemented with ampicillin and cultured overnight at 37°C with constant agitation. Overnight cultures were used in plasmid preparations utilising the QIAprep® Spin miniprep kit (QIAGEN, Germany). The identity of the purified plasmids and size of the insert was confirmed by EcoRI (Promega, UK) enzyme digestions, followed by analyses by electrophoresis using 1.5% agarose, 1 x TBE gels. Plasmids with unique inserts were Sanger sequenced (service provided by the Sequencing facility at the James Hutton Institute, UK) using both M13F and M13R primers. Where inserts were > 500 bp, one or two internal gene-specific primers were also used to ensure sequencing over the full length of the cloned product. Analysis of the sequence was conducted using Sequencher software version 4.9 (Gene Codes Corporation), which allowed trimming and cleaning of the sequences before alignment on to genomic contigs to identify where splicing had occurred and which splice sites had been used.

5.2.2 – Analyses of AS in regulating mRNA expression of clock genes in response to temperature changes

To observe the responsiveness of clock gene expression and AS to changes in temperature, grains from three barley lines (Section 2.1.1) were germinated in the dark at 4 °C on sterile water-soaked filter papers and transferred to small square plastic pots (5 cm^3) filled with cereal compost (Appendix A1). Each pot contained 4 grains from the same line. Plants were grown in a controlled-environment growth cabinet at 20 °C with LD treatments for seven days. No crop protection treatment was applied. Leaf samples of one-week old plants (GS11) were collected at 2.5 h after dawn. At dusk, 15.5 h after the first harvesting, the temperature of the cabinet was reduced to 4 °C, and this temperature was

maintained, along with LD condition, for four days. Additional leaf samples were harvested at 2.5 h after dawn on days 1, 2 and 4 after transfer to 4 °C (Figure 5.1). At dawn of the 12th day, the temperature was increased from 4 °C to 20 °C. Another two sampling points took place at 2.5 h after dawn on days 1 and 2 after adjustment to 20 °C. Humidity and temperature were monitored with a data logger (EL-USP-2 from Lascar Electronics) that confirmed relative humidity at around 70% and efficient temperature transitions during the experiment (Appendix B7.1). The cabinet took less than 90 min to decrease the temperature from 20 °C to 4 °C and vice-versa.

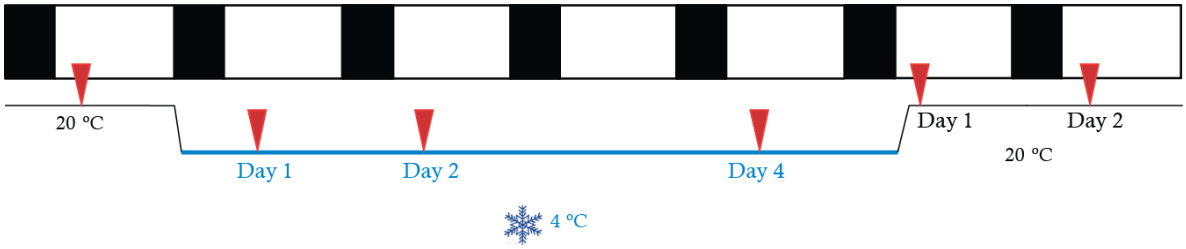


Figure 5.1: Sampling and temperature regime used in analyses of AS in regulating mRNA expression of clock genes. Red arrows represent time points when sampling occurred (2.5 h after dawn). Black boxes are for dark, white boxes are for light.

It is noteworthy that over the total duration of the experiment, plants were kept in the same growth cabinet to eliminate the potential effects of minor changes in light intensity and quality on gene expression. A minimum of three biological replicates were taken per time point, each comprising of one or two leaves from at least three independent plants from one pot. Additionally, pots were placed in a random array. Plants for each time point were also selected in a random fashion from the array (data not shown). Leaf material was pooled, rapidly frozen in liquid nitrogen and stored at -80 °C.

To compare AS data against FS values, the peak areas of RT-PCR products were normalised to control gene values (Equation 1 in Chapter 2) and analysed using ANOVA.

The associated *P* values calculated in the ANOVA were two-tailed, and a significance level of 5% ($\alpha = 0.05$) was used. GenStat (15th Edition version 15.1.0.9425, VSN International LTD) was used for all statistical analyses.

5.3 – Results

5.3.1 – Identification of AS isoforms of barley clock genes

Recent reports of AS in Arabidopsis clock genes (Hazen *et al.*, 2009; Filichkin *et al.*, 2010; Sanchez *et al.*, 2010; James *et al.*, 2012a) have motivated a systematic identification of AS isoforms of barley core clock genes. Firstly, Arabidopsis clock homologues were identified and confirmed in barley using an *in silico* approach (Chapter 3). This approach identified both genomic and cDNA sequences of barley clock genes, which were used to annotate and determine gene structure (intron/exon) and develop gene models for AS studies. Secondly, barley clock homologues were confirmed as having rhythmic mRNA expression profiles typical of clock genes, using the HR RT-PCR system and phenotyping two barley clock mutants (Chapter 4). Finally, from this list of barley clock homologues, the intron-containing genes *HvFT1, HvFT2, HvLHY*, *HvCO1*, *HvCO2, HvTOC1*, *HvGI, HvELF3, HvGRP7a, HvGRP7b, HvPRR37*, *HvPRR73*, *HvPRR59* and *HvPRR95* were selected for AS analyses in barley.

AS events in selected barley clock genes were identified using two different approaches. Firstly, publicly available ESTs, cDNA and RNA-seq data were aligned on to barley genomic sequences and, secondly, systematic RT-PCR was carried out to detect alternative transcripts. For the former, most AS information was obtained from the extensive barley RNA-seq data, which provided independent confirmation of the existence of AS events in barley clock genes, such as *LHY* Alt 3' ss I1 (Appendix B8). For systematic RT-PCRs, plants were grown in environmentally controlled cabinets, maintaining constant

temperatures and photoperiods (LD at 20 °C and SD at 4 °C; Appendix A7.1). Leaves of one-week old seedlings were harvested at different time points over a 24 h period. RT-PCR was performed on total RNA extracted from a mixture of plant material. The primers used for AS discovery for the subset of genes chosen are shown in Table 2.1 and Appendix A5. Alternative transcripts were cloned and sequenced (Sanger sequencing). In some cases, the identity of a novel event was predicted (especially for IR events) based on expected product size, intron sequence and presence of potential alternative splice sites in amplified regions. This initial identification of AS events in barley clock genes allowed the establishment of an RT-PCR panel for studies of barley clock and clock-associated genes.

Before learning about the different AS events in the barley clock, it is important to bear in mind the possible effects of different AS events in gene expression. The primary function of AS is to affect protein expression either quantitatively and/or qualitatively. In order to achieve the latter, AS usually occurs in the CDS, maintaining the ORF to produce alternative mRNAs that are still translatable, functional and able to reach the cytoplasm. Another possibility is if the AS occurs at the 5' UTR and adds an in-frame uAUG sequence, which modifies the N-terminus coding region. However, uORFs which are not in-frame with the functional ORF can, depending on their length and position, affect translation, trigger NMD (for example when a uORF overlaps the main start codon; Kalyna *et al.*, 2012) or produce micro-proteins. Additionally, when the AS event interrupts the functional ORF and introduces a PTC (e.g. IR event), the mRNA can either: 1) become an NMD target; 2) be unable to leave the nucleus (predicted for many IR-containing transcripts; Kalyna *et al.*, 2012); 3) be translated into a truncated protein.

In all, 55 novel AS events were detected for the set of eleven clock genes (Table 5.1–5.4). These AS events generated up to 69 total transcripts, including normally spliced transcripts, but it is important to consider the transcript diversity of the alternatively spliced clock genes in terms of how the 55 different AS events are distributed among dif-

ferent transcripts. The AS events in each gene can be mutually exclusive, independent, or interact such that one splicing event affects whether another occurs. For example, *HvPPDH1* has two significant mutually exclusive events, Alt 5' ss I6 that adds 45 nt or 50 nt, both of which generated mRNAs with different outcomes: increase in proteome diversity or inclusion of a PTC, respectively. These events cannot occur in the same transcript. Alternatively, events can be independent, particularly within different regions of the pre-mRNA. Furthermore, AS transcript isoforms have also been identified containing combinations of the individual AS events and this may occur over the whole pre-mRNA.

In WT plants grown under LD conditions and 20 °C, abundance analyses of alternative transcripts undergoing one or two of these AS events found 35 transcripts were very low in abundance (< 1% of the total transcripts), 13 were between 1 and 2% of transcripts, twelve were between 2 and 10%, and nine were between 10 and 50% (including one *PPDH1* FS isoform, Figure 5.18). Many of the AS events were IR events (29/55), of which, 20 were found in low abundance in all conditions analysed here (< 2% of total transcripts) and probably represent partially spliced or unspliced transcripts. No alternative transcript isoform were detected for *HvFT1, HvFT2* or *HvCO1*. It should be noted that low abundance AS events detected in the plant growth conditions here may be abundant under other environmental conditions not tested in this work.

Table 5.1: Information of AS events in barley *LHY* detected by RT-PCR experiments. Transcript abundance information is relative to WT plants grown under LD and 20 °C.

Gene	Primer pair	Primer position	Product size (bp)	AS event name	Sequencing	AS event	Transcript abundance
LHY	HvLHY-Ex3Fw	5' UTR	278	FS	NGS, Sanger	FS	****
	HvLHY-Ex6Rv		268	Alt 3' ss E4	Sanger	Alt 3' ss E4 (-10 nt)	**
			294	Alt 3' ss I2	NGS, Sanger	Alt3' ss I2 (+16 nt)	*
			358	Alt 3' ss I1	NGS, Sanger	Alt 3' ss I1 (+80 nt)	**
			393	I2R	NGS, Sanger	Intron 2 retained	*
			440	I1R	Sanger	Intron 1 retained	-
			555	I1R + I2R	Sanger	Introns 1 and 2 retained	-
			666	I3R	Sanger	Intron 3 retained	-
			746	Alt 3' ss I1+ I3R	Predicted	Alt 3' ss I1 (+80 nt) and I3 retained	-
			781	I2R + I3R	Sanger	Introns 2 and 3 retained	-
			943	I1R, I2R + I3R	Sanger	Introns 1, 2 and 3 retained, unspliced	-
	HvLHY-Ex6Fw	MYB-coding domain	255	FS	NGS, Sanger	FS	****
	HvLHY-Ex8Rv		235	Alt 5' ss E4	NGS	Alt5' ss E4 (-20 nt)	-
			325	I4R	NGS	Intron 4 retained	*
			490	I5R	Predicted	Intron 5 retained	-
			560	I4R + I5R	Predicted	Introns 4 and 5 retained, unspliced	-
	HvLHY-Ex8Fw	Span long intron 6	188	FS	NGS, Sanger	FS	****
	HvLHY-Ex9Rv		938	Alt E6a	NGS, Sanger	Alt E6a from intron 6 (+756 nt)	-
			1833	I6R	Unknown	Intron 6 retained	-
	HvLHY-Ex10FwB	C-terminus coding region	916	FS	NGS	FS	****
	HvLHY-3UTRv		1005	I8R	NGS	Introns 8 retained, unspliced	**

- Not visible or transcript level < 1% of total transcripts; * Transcript level between 1 and 2% of total transcripts; ** Transcript level between 2 and 10% of total transcripts; *** Transcript level between 10 and 50% of total transcripts; **** Transcript level equal or greater than 50% of total transcripts.

Table 5.2: Information of AS events in barley *GI, TOC1* and *ELF3* detected by RT-PCR experiments. Transcript abundance information is relative to WT plants grown under LD and 20 °C.

Gene	Primer pair	Primer position	Product size (bp)	AS event name	Sequencing	AS event	Transcript abundance
GI	HvGI-5UTRFw	5' UTR and	391	FS	NGS, Sanger	FS	****
	HvGI-Ex4Rv	N-terminus	251	E2S	NGS	E2 skipping	**
		coding region	263	E2S + Alt 3' ss I2	NGS, Sanger	E2 skipping and Alt 3' ss I2 (+12 nt)	***
			395	alt 5' ss E2 + alt 3' ss I2	NGS	alt 5' ss E2 (-8 nt) and alt 3' ss I2 (+12 nt)	**
			403	alt 3' ss I2	NGS	Alt 3' ss I2 (+12 nt)	***
			540	I2R	NGS, Sanger	Intron 2 retained	**
	HvGI-Ex12FwB	Exons 12 - 14	402	FS	NGS, Sanger	FS	****
	HvGI-Ex14Rv		549	I13R	NGS	Intron 13 retained	-
	HvGI-Ex14Fw	Exons 14 - 16	248	FS	NGS, Sanger	FS	****
	HvGI-Ex16Rv		336	I15R	NGS	Intron 15 retained	-
			407	I14R	NGS	Intron 14 retained	-
			495	I14R+I15R	Predicted	Introns 14 and 15 retained, unspliced	-
TOC1	HvPRR1-5UTRFwB	Exons 1 - 3	678	FS	NGS, Sanger	FS	****
	HvPRR1-Ex3Rv		389	alt 5' ss I1 + alt 3' ss E2	Sanger	alt 5' ss I1 (-189 nt) and alt 3' ss E2 (-100 nt)	**
			392	CrIn.E1 (-313)	Sanger	Cryptic intron in E1 (-313 nt)	**
			568	Unknown	Unknown	Unknown	*
			619	CrIn.E1 (-221) + I1R	Sanger	Cryptic intron in E1 (-221 nt) and I1R	*
			840	I1R	Sanger	Intron 1 retained	-
	HvPRR1-Ex3Fw	Exons 3 - 6	463	FS	NGS, Sanger	FS	****
	HvPRR1-Ex6Rv		297	E4S	NGS, Sanger	E4 skipping	-
			593	I3R	NGS, Sanger	Intron 3 retained	*
			793	I3R+I4R	NGS	Introns 3 and 4 retained, unspliced	-
ELF3	HvELF3-Ex2Fw	Exons 2 - 4	338	FS	NGS, Sanger	FS	****
	HvELF3-Ex4Rv		468	I3R	NGS, Sanger	Intron 3 retained	*

- Not visible or transcript level < 1% of total transcripts; * Transcript level between 1 and 2% of total transcripts; ** Transcript level between 2 and 10% of total transcripts; *** Transcript level between 10 and 50% of total transcripts; **** Transcript level equal or greater than 50% of total transcripts.

Table 5.3: Information of AS events in barley *CO2*, *PRR59, PRR95* and *PRR37* detected by RT-PCR experiments. Transcript abundance information is relative to WT plants grown under LD and 20 °C.

Gene	Primer pair	Primer position	Product size (bp)	AS event name	Sequencing	AS event	Transcript abundance
CO2	HvCO2-Ex1FwB	C-terminus	509	FS	NGS, Sanger	FS	****
	HvCO2-3UTRv	coding region	579	Alt 3' ss	Sanger	Alt 3' ss (+68 nt, PTC)	***
			1004	CrIn	Sanger	Cryptic intron antisense strand	*
			1094	IR	NGS, Sanger	Intron retained, unspliced	***
PRR59	HvPRR59-Ex3Fw	Exons 3 - 5	309	FS	NGS, Sanger	FS	****
	HvPRR59-Ex5RvB		611	I4R	NGS	Intron 4 retained	-
			614	I3R	NGS	Intron 3 retained	-
	HvPRR59-Ex5Fw	Exons 5 - 7	673	FS	NGS, Sanger	FS	****
	HvPRR59-Ex7RvB		601	CrIn E6	NGS	Cryptic intron E6 (-72 nt, same	-
			866	I3R	NGS	Intron 7 retained	-
	HvPRR59-Ex7Fw	C-terminus	344	FS	NGS, Sanger	FS	****
	HvPRR59-3UTRv	coding region	470	I7R	NGS	Intron 7 retained	*
PRR95	HvPRR95-5UTRFw	N-terminus	610	FS	NGS, Sanger	FS	****
	HvPRR95-Ex4Rv	coding region	704	I2R	NGS	Intron 2 retained	-
	HvPRR95-Ex4Fw	Exons 4 - 6	612	FS	NGS, Sanger	FS	****
	HvPRR95-Ex6Rv		330	Unknown	Unknown	Unknown	-
			842	I4R	Predicted	Intron 4 retained	-
			932	I5R	Predicted	Intron 5 retained	-
			1171	I4R+I5R	Predicted	Introns 4 and 5 retained	-
	HvPRR95-Ex6FwB	C-terminus	363	FS	NGS, Sanger	FS	****
	HvPRR95-3UTRv	coding region	450	I6R	NGS	Intron 6 retained	-
PRR37	HvPpdH1-5UTRfw	5' UTR and	250	FS	NGS, Sanger	FS	****
	HvPpdH1-Ex1Rv	coding region	194	Unknown	Unknown	Unknown	**
			230	Unknown	Unknown	Unknown	*
			246	Alt 3' ss E2	NGS	Alt 3' ss E2 (-4 nt)	-
			362	I1R	NGS, Sanger	Intron 1 retained	***

- Not visible or transcript level < 1% of total transcripts; * Transcript level between 1 and 2% of total transcripts; ** Transcript level between 2 and 10% of total transcripts; *** Transcript level between 10 and 50% of total transcripts; **** Transcript level equal or greater than 50% of total transcripts.

Table 5.4: Information of AS events in barley *PRR37* and *PRR73* detected by RT-PCR experiments. Transcript abundance information is relative to WT plants grown under LD and 20 °C.

Gene	Primer pair	Primer position	Product size (bp)	AS event name	Sequencing	AS event	Transcript abundance
PRR37	HvPpdH1-Ex4Fw	Exons 3 - 5	411	FS	NGS, Sanger	FS	***
	HvPpdH1-Ex6Rv		405	alt 3' ss E6	NGS, Sanger	alt 3' ss E6 (-6 nt)	***
			450	alt 5' ss I6 (+45) + alt 3' ss E6	Sanger	alt 5' ss I6 (+ 45 nt) and alt 3' ss E6 (-6 nt)	***
			456	alt 5' ss I6 (+45)	NGS, Sanger	alt 5' ss I6 (+ 45 nt)	***
			461	alt 5' ss I6 (+50)	NGS, Sanger	alt 5' ss I6 (+ 50 nt), adds PTC	**
			547	alt 3' ss E6 + I6R	Sanger	alt 3' ss E6 (-6 nt) and I6R	*
			553	I6R	Sanger	Intron 6 retained	-
	HvPpdH1-Ex6fwB	C-terminus	902	FS	NGS, Sanger	FS	****
	HvPpdH1-3UTRv	coding region	653	CrIn E8	Sanger	Cryptic Intron E8 (-249 nt), in frame	*
			984	I7R	NGS, Sanger	Intron 7 retained	**
PRR73	HvPRR73-5UTRFw HvPRR73-Ex3Rv	5' UTR and N-terminus coding region	606	FS	NGS, Sanger	FS	****
			599	alt 3' ss E2	Predicted	alt 3' ss E2 (-7 nt), uORF decreased from 36 to 11 aa.	-
			861	alt 5' ss I1 + alt E1a	NGS	alt 5' ss Intron 1 and Alt E1a (+255 nt)	-
	HvPRR73-Ex3Fw	Exons 3 - 5	320	FS	NGS, Sanger	FS	****
	HvPRR73-Ex5Rv		439	I4R	Predicted	Intron 4 retained	-
			465	I5R	NGS	Intron 5 retained	-
			584	I4R+I5R	Predicted	Introns 4 and 5 retained	-
	HvPRR73-Ex5Fw	Exons 5 and 7	549	FS	NGS, Sanger	FS	****
	HvPRR73-Ex7Rv		632	I6R	NGS, Sanger	Intron 6 retained	*
	HvPRR73-Ex7Fw	Exons 7 and 8	319	FS	NGS, Sanger	FS	****
	HvPRR73-Ex8RvB		404	I7R	NGS	Intron 7 retained	**

- Not visible or transcript level < 1% of total transcripts; * Transcript level between 1 and 2% of total transcripts; ** Transcript level between 2 and 10% of total transcripts; *** Transcript level between 10 and 50% of total transcripts; **** Transcript level equal or greater than 50% of total transcripts.

- ***HvLHY***. The LHY gene is associated with two contigs. One contig contains the entire coding sequence, while the other appears to contain part of the 5' UTR. The two contigs are not as yet connected in genomic databases. At least 20 AS events were observed in barley *LHY*. However, AS events in contig_51288 (data not shown), containing part of the 5' UTR of *LHY* (2 exons at 5' end), should be treated with caution because the sequence linking both genomic contigs for *LHY* is missing from genomic databases. To avoid misinterpretation of results, contig _51288 is not considered in further AS analyses and discussion. This leaves ten AS events in barley *LHY* from contig_1567295 (Figure 5.2). *HvLHY* has six AS events within the 5' UTR, four of which add upstream AUGs/ORFs: alternative 3' ss in intron 3 (adds one uORF) and retention of introns 1, 2 and 3 (adding 2, 3 and 7 uORFs, respectively). All uORFs code for small peptides (< 32 amino acids) with no homologues in Arabidopsis and terminate before the functional *LHY* ORF (Appendix B7.2). In the coding region, four AS events add PTCs: alternative 5' ss in exon 4 (removes 20 nt, which changes the ORF), intron 4 retention, alternative exon 6a and intron 8 retention. In light/dark and LL conditions, these alternative *LHY* transcripts are found in low abundance and fully spliced transcripts represent more than 90% of total transcripts.

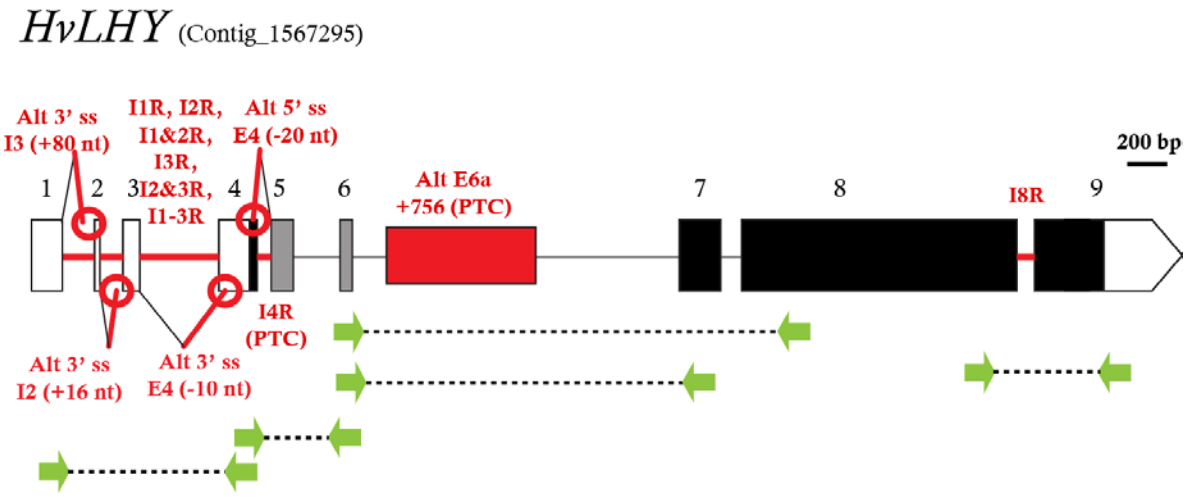


Figure 5.2: Genomic structure and AS events of *HvLHY*. Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except MYB domain-encoding exons, which are shaded grey. AS events are represented in red. Alt, alternative; ss, splice site; I, intron; R, retention; E, exon. The approximate positions of most primer pairs used in barley AS studies are shown by green arrows joined by a dotted line.

- ***HvGI.*** *HvGI* contain ten exons that are constitutively spliced and six exons that show AS. Three AS events occurred in the 5' UTR (exons 2 and 3), and the other three are intron retention events within the coding region, which add PTCs at I13R, I14R and I15R (Figure 5.3). AS events in the 5' UTR involve skipping exon 2, which removes three uORFs, and retaining intron 2, which adds four uORFs. All of these uORFs code for small peptides (< 26 amino acids) that terminate before the AUG of the functional *GI* ORF. Such uORF-containing *HvGI* mRNAs may be translated less efficiently or are less stable, when compared to uORF-free transcripts. No homologues or conserved AS involved in the formation of uORFs was found in Arabidopsis. In light/dark and LL conditions (20 °C), *GI* transcripts with alternative 5' UTR are rhythmic (Appendix B9.1) and uORF-containing transcripts represent 75% of total transcripts. Intron retention (IR) events were found in low abundance (< 1% of total transcripts) for the same conditions.

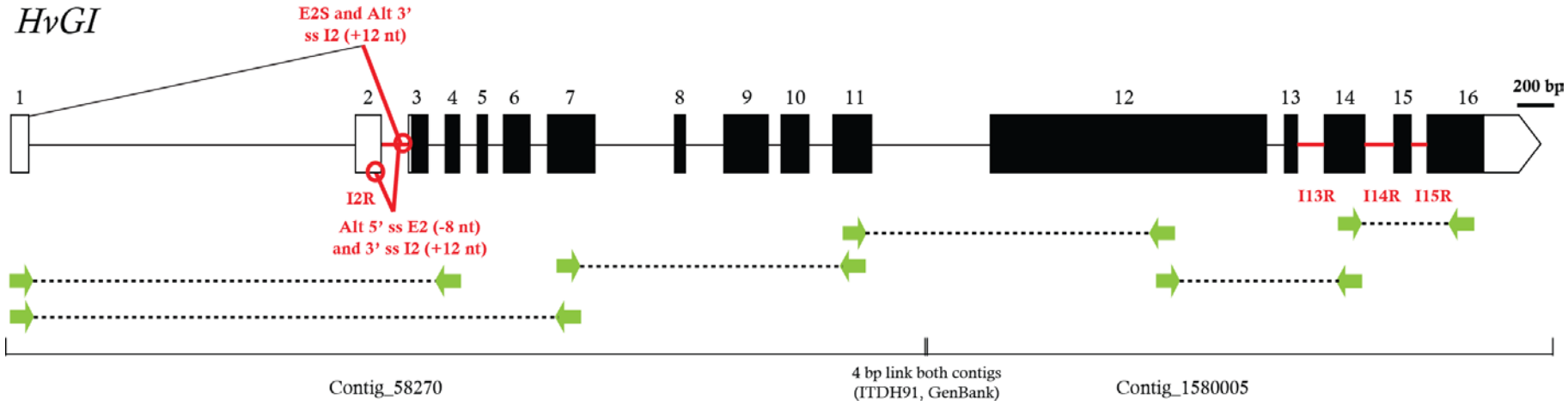


Figure 5.3: Genomic structure and AS events of *GI* barley core clock gene. Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes. AS events are represented in red. Alt, alternative; ss, splice site; I, intron; R, retention; E, exon; S, skipping. The approximate positions of most primer pairs used in AS studies are shown by green arrows joined by a dotted line.

- ***HvTOC1.*** *HvTOC1* contains six exons and has at least seven AS events (Figure 5.4). Three AS events are IRs in the coding region (I1R, I3R and I3R+I4R), which add PTCs. Another AS event skips exon 4, removing 168 nt of the protein coding sequence which leads to an in-frame removal of 56 amino acids immediately after the PRR domain. Two cryptic introns and both alternative 5' and 3' ss were identified at the 5' end. These remove the functional AUG, which may lead to the use of a downstream in-frame AUG and may encode for a protein with a partial PRR domain. When plants were grown in light/dark and LL 20 °C conditions, *HvTOC1* AS events represented less than 10% of total transcripts.

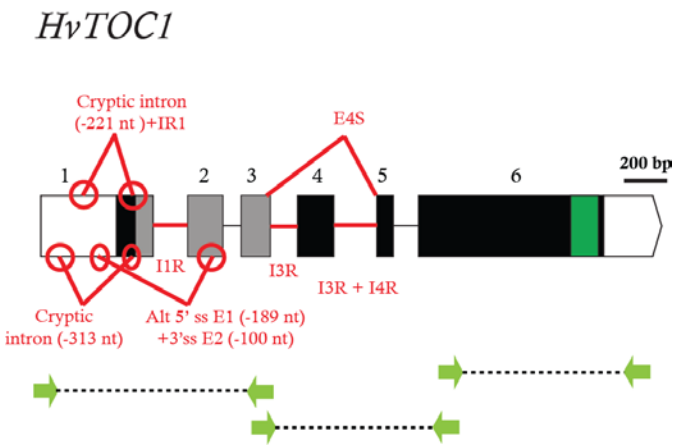


Figure 5.4: Genomic structure and AS events of *HvTOC1*. Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except domain-encoding exons, which are shaded grey (PRR domain) or green (CT domain). AS events are represented in red. Alt, alternative; ss, splice site; I, intron; R, retention; E, exon; S, skipping. The approximate positions of most primer pairs used in barley AS studies are shown by green arrows joined by a dotted line.

- ***HvELF3.*** *HvELF3* contains four exons and AS analyses identified retention of intron 3, which disrupts the coding region by adding a PTC (Figure 5.5). In light/dark and LL 20 °C conditions, this AS event represents less than 2% of total transcripts.

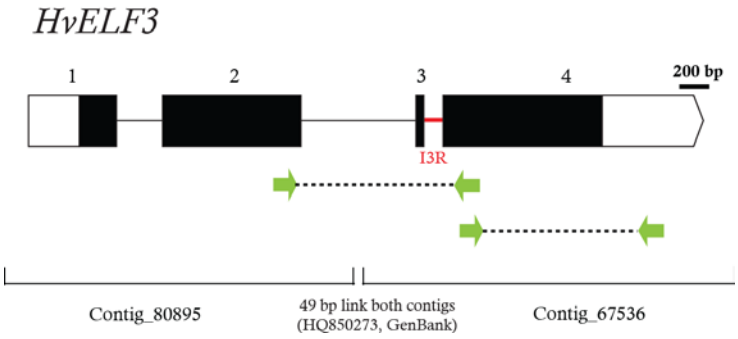


Figure 5.5: Genomic structure and AS event of *ELF3* barley core clock gene. Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes. AS event is represented in red. I, intron; R, retention. The approximate positions of two primer pairs used in AS studies are shown by green arrows joined by a dotted line.

- ***HvGRP7a/b.*** Both single intron genes undergo IR events, according to RNA-seq data (data not shown). However, RT-PCR experiments with these genes were not successful and so were not confirmed.
- ***HvCO2.*** Three AS events were observed using *HvCO2* specific primers in RT-PCR experiments. This single intron gene shows evidence of IR, an alt 3' ss and cryptic intron (CrIn) removal (Figure 5.6). However, it is likely that unspliced (IR) and CrIn transcript isoforms are generated from a gene on the opposite strand, also seen for *AtCOL1*. First, the CrIn has canonical splice sites (GT-AG) if spliced from the complementary transcript but non-canonical splice sites if spliced from the sense (*HvCO2*) transcript (GT-TC). Second, the expression of both IR and CrIn are rhythmically opposite to the *HvCO2* transcripts (FS and alt 3' ss) in light/dark conditions (Appendix B9.2). TBLASTX analysis suggests that this barley antisense gene is similar to the *longevity assurance* (At1g13580) gene in Arabidopsis (similarity of 71% at the protein level). However, this BLAST search is not reciprocal, suggesting they are not orthologues.

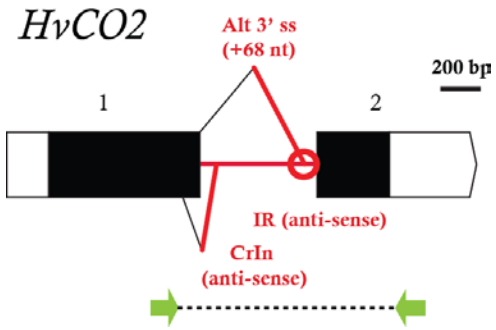


Figure 5.6: Genomic structure and AS events in *HvCO2* locus. Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes. AS events are represented in red. Alt, alternative; ss, splice site; I, intron; R, retention; CrIn, cryptic intron. The approximate positions of the primer pair used in AS studies is shown by green arrows joined by a dotted line.

- ***HvPRR95* and *HvPRR59*.** Both of these genes have a highly conserved gene structure, with the exception of an additional exon (exon 5) in the coding region of *HvPRR59* (Figure 5.7). In each gene, five AS events were identified and four (*HvPRR59*) or all five (*HvPRR95*) are IR events in the coding region, which add PTCs. Retention of both of the 3' most introns, I7R (*HvPRR59*) and I6R (*HvPRR95*) is conserved between these paralogues. I7R from *HvPRR59* is not susceptible to NMD degradation because intron inclusion maintains the reading frame and may increase protein diversity. I6R from *HvPRR95* contains a PTC which is present in the last exon and, therefore, might not be targeted to NMD, coding for a protein lacking a functional CT domain. In light/dark and LL conditions (20 °C), all alternative transcripts identified are found in low abundance (< 2% of total transcripts). AS events in the 5' UTR could not be comprehensively assessed due to technical problems with the PCR and cloning procedure.

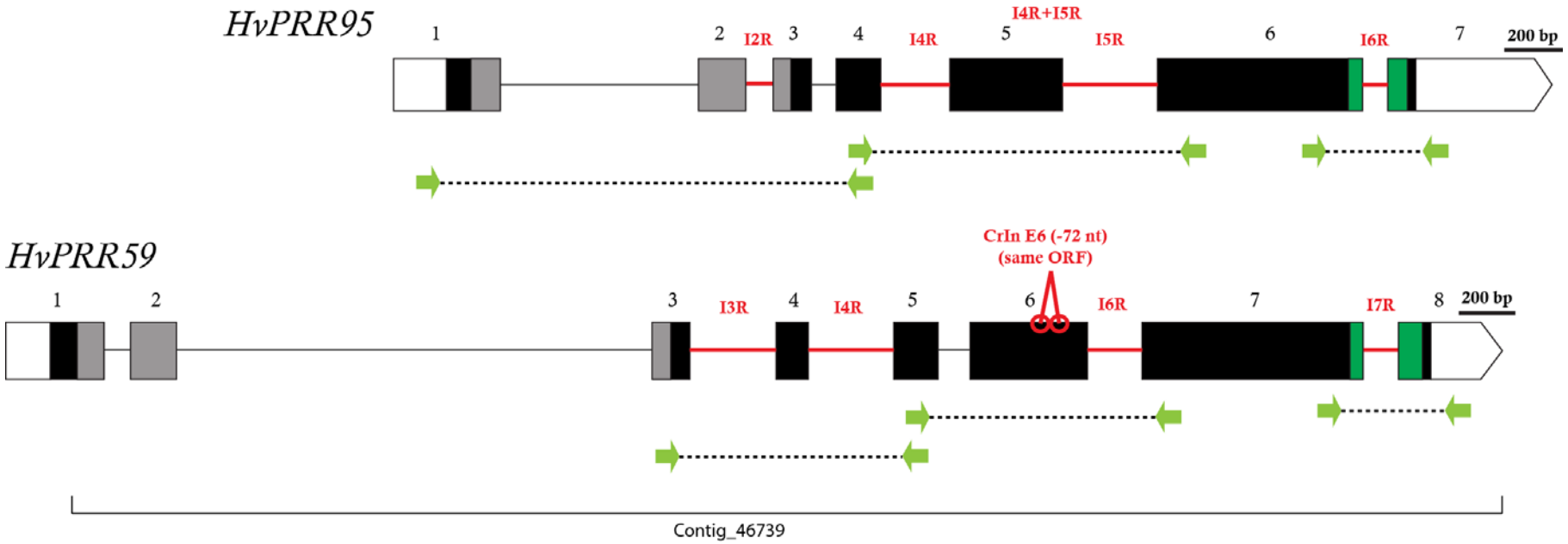


Figure 5.7: Genomic structure and AS events of *HvPRR95* and *HvPRR59*. Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except domain-encoding exons, which are shaded grey (PRR domain) or green (CT domain). AS events are represented in red. I, intron; R, retention; CrIn, cryptic intron; E, exon. The approximate positions of most primer pairs used in barley AS studies are shown by green arrows joined by a dotted line.

- ***HvPRR37* and *HvPRR73***. Alignment of *PRR37* and *PRR73* shows a very similar gene model structure. The eight exons covering the protein coding region show identical intron positions. However, the introns vary in sequence and length and this may help explain the contrasting alternative splicing events. Of all AS events identified (nine and seven for *PRR37* and *PRR73*, respectively) only two were conserved, in introns 6 and 7 (I6R and I7R) (Figure 5.8). Five AS events in *PRR73* are IR events in the coding region that add PTCs. In the 5' UTR of *PRR73*, two events occur and may affect mRNA translation. First, an alternative (alt) 5' ss that removes seven nucleotides of exon 2 (E2) shortened the length of the uORF from 36 to 11 amino acids, and second, an alt 5' ss in intron 1 and the addition of an alternative exon (E1a) add 255 nt to the 5' UTR sequence (with no effect on uORFs) (Figure 5.8). In the 5' UTR of *PRR37*, two AS events were observed. First, a CrIn event was observed in about 20% of the reads from the RNA-seq data, while the other 80% reads retained the CrIn. Removal of the CrIn results in the loss of an uORF coding for 48 amino acids, which may affect translation of the functional ORF. Second, I1R changes the N-terminus coding region and creates a uORF which codes for 45 amino acids that overlapped the functional ORF by 35 nt. As a result, the I1R event probably affects mRNA stability, e.g. transcript targeted to NMD (Figure 5.9) (Kalyna *et al.*, 2012). BLAST analyses revealed no putative *PRR37* uORF Arabidopsis homologues. In the *PRR37* coding region, seven AS events were detected, including four alternative ss, one CrIn in E8 and two IR described earlier (I6R and I7R) (Figure 5.8). The CrIn in E8 and two alternative splice sites increase the number of *PRR37* protein-coding mRNA isoforms by removing 249 nt (83 amino acids) from E8 and by removing 6 nt and/or adding 45 nt to exon 6 (involving 2 and 15 amino acids, respectively).

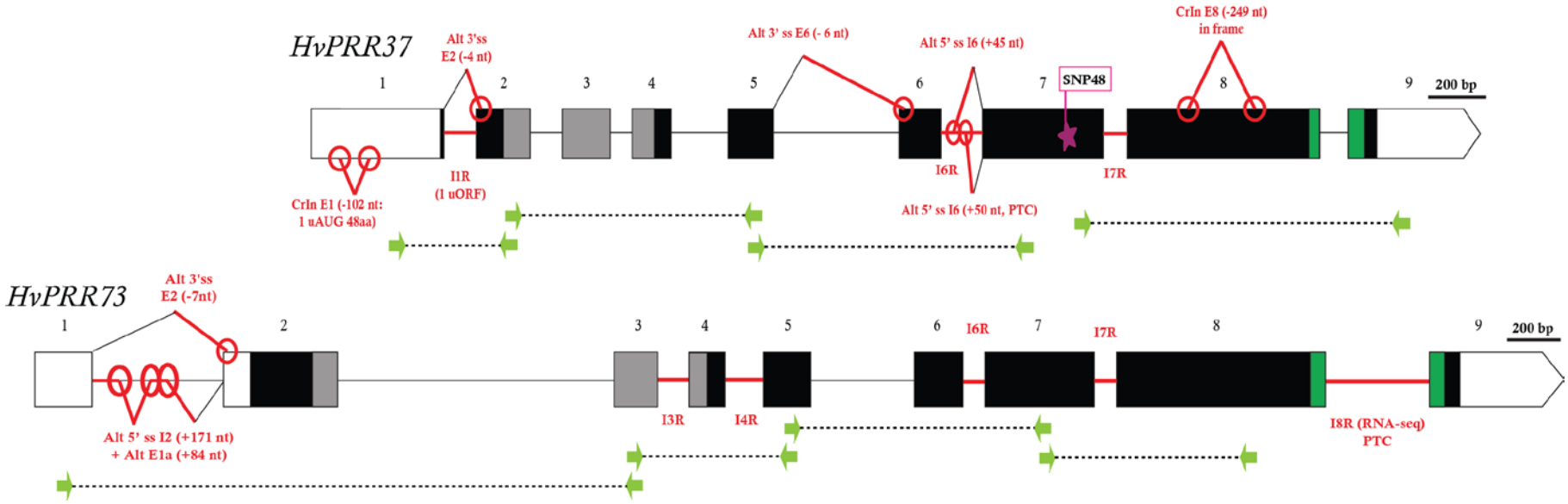


Figure 5.8: Genomic structure and AS events of *HvPRR37* and *HvPRR73* barley core clock genes. Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except domain-encoding exons, which are shaded grey (PRR domain) or green (CT domain). AS events are represented in red. Alt, alternative; ss, splice site; I, intron; R, retention; E, exon. The approximate positions of most primer pairs used in barley AS studies are shown by green arrows joined by a dotted line. SNP48 of *HvPRR37* is indicated.

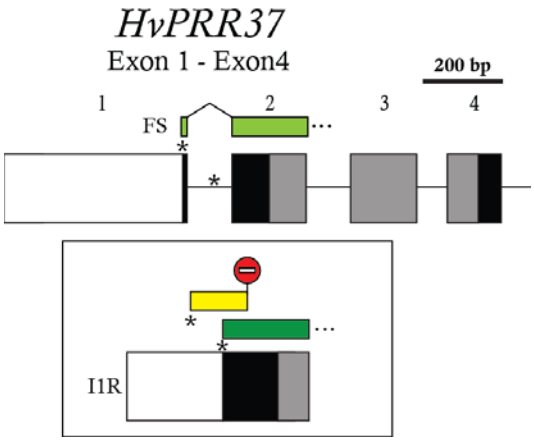


Figure 5.9: *HvPRR37* (*HvPPDH1*) genomic structure of 5' terminus-coding region and focus on I1R event. Exons are numbered on the genomic structure; 5' UTRs are open boxes; coding sequences are dark boxes, except PRR-encoding region, which are shaded grey; introns are straight fine black lines. Splicing events are thin diagonal lines. I1R event is shown below genomic structure. *, AUG. Stop sign, PTC. Putative uORF is yellow box, whereas functional ORF is green box (different green shades for different isoforms).

Another two alternative splice sites in *HvPRR37* add PTCs by either removing four nucleotides of E2 and changing the ORF, encoding a different polypeptide with 107 amino acids, or by adding 50 nt to exon 6 (Figure 5.8). Both of these PTC-containing AS transcripts may undergo NMD. In light/dark and LL conditions (20 °C), alternative *PRR37* and *PRR73* transcripts are found in low abundance (< 10% of total transcripts), except alt 3' ss E6 (-6 nt) and Alt 5' ss I6 (+45 nt) in *PRR37*, which are more abundant than the predicted FS transcript (see Section 5.3.3.2). A BLASTn search on GenBank monocot EST database using Exons 5–7 of *HvPRR37* identified transcripts with the equivalent 5' ss I6 (+45 nt) event, except for two cases. FS transcripts were detected in *Leymus* (Triticeae) species (Kaur *et al.*, 2008) and the equivalent 5' ss E6 (-6 nt) was seen in *Oryza* species (Jantasuriyarat *et al.*, 2005; Yang *et al.*, 2010). These results suggest conservation of both AS event in the BEP clade (where *Brachypodium*, *Oryza* and Triticeae species are grouped).

5.3.2 – Arabidopsis vs. barley AS in plant core clock genes

In barley and Arabidopsis plants, abundance analyses of alternative transcripts found several similarities. First, most transcripts (> 50%) were in low abundance (< 2% of the total transcripts) in both species. Second, many of the AS events were IR events (> 50%), of which mostly were found in low abundance in both barley and Arabidopsis. These low abundant IR events probably represent partially or unspliced spliced transcripts in the RNA samples.

5.3.2.1 – *LHY* and *CCA1*

AS studies of Arabidopsis clock genes described twelve putative AS events for *LHY*, whereas its paralogue *CCA1* probably undergoes eleven AS events (James *et al.*, 2012a). At least ten AS events were observed here in barley *LHY*. Comparison of the gene structure and AS events of *AtCCA1*, *AtLHY* and *HvLHY* homologues identified their similarities and differences (Appendix B10.1). These genes have a highly conserved gene structure, with the exception of one additional intron found in *AtCCA1* and *AtLHY* (intron 5 or 6, respectively) when compared with *HvLHY*. Both *LHY* orthologues have several AS events in their 5' UTR. *HvLHY* had four exons contained within the 5' UTR sequences. However, another contig sequence (contig_51288) was discovered that may also contain part of the 5' UTR of *HvLHY*. This could potentially add a further two exons to the 5' end of the 5' UTR sequence. Due to the complexity of the *HvLHY* 5' UTR and low conservation between the 5' UTR of *HvLHY* and its Arabidopsis homologues it was difficult to determine whether there is any conservation of AS between the two species (data not shown). In the coding region of *AtLHY*, *AtCCA1* and *HvLHY*, the MYB domain is encoded by two small exons. The three homologues have intron retention events at or near the MYB-coding region, which introduce PTCs. Furthermore, the long intron 5 or 6 of *AtLHY*

and *HvLHY*, respectively, contains a PTC-containing alternative exon (E5a and E6a for *AtLHY* and *HvLHY*, respectively). The sizes of these alternative exons are significantly different but both PTC-containing alternative transcripts are unable to code for a functional LHY protein and the Arabidopsis E5a-containing transcript, in particular, is degraded by NMD (James *et al.*, 2012a).

5.3.2.2 – *GI*

The genomic structure of the *GI* orthologues is highly conserved, except for two additional introns (introns 2 and 3) in the 5' end of *HvGI* when compared with *AtGI* (Appendix B10.2). Both *GI* orthologues have only a small number of AS events. *AtGI* has three putative AS events (James *et al.*, 2012a), whereas its barley orthologue undergoes six AS events. Two intron retention events in the 3' coding region are conserved between both orthologues (introns 12 and 13 of *AtGI* and introns 14 and 15 of *HvGI*), which introduce PTCs. However, these IR events are of low abundance in both barley and Arabidopsis (James *et al.*, 2012a). *HvGI* has three additional AS events in the 5' UTR which are abundant, and two of them result in uORFs.

5.3.2.3 – *TOC1*

The genomic structure of Arabidopsis and barley *TOC1* orthologues are conserved. *AtTOC1* has eight putative AS events (James *et al.*, 2012a), whereas its barley orthologue undergoes seven AS events (Appendix B10.3). I1R, I3R and I3R+I4R events disrupt the coding region of mRNAs from both orthologues. Furthermore, an exon skipping (ES) event was identified for exons 5 and 4 in Arabidopsis and barley, respectively. Both IR and ES events are of low abundance in barley and Arabidopsis plants grown at 20 °C (James *et*

al., 2012a). Interestingly, levels of *AtTOC1* transcripts retaining I4 transiently increase during cold acclimation (James *et al.*, 2012a). *HvTOC1* has three additional AS events involving the 5' end of the transcript, not observed in Arabidopsis, which are present in a moderate amount (transcript levels between 2 and 10% of total transcripts). These AS events remove the functional AUG such that use of a downstream in-frame AUG potentially could generate an N-terminal truncated form of the protein without a functional PRR domain. Although it is not possible to predict how the function of a PRR protein would be affected in the absence of a PRR domain, truncated proteins can have functions and may act as dominant negatives.

5.3.2.4 – *PRR5(9)/PRR9(5)*

Six cases of AS have been described in *PRR5* of Arabidopsis (James *et al.*, 2012a), while its paralogue *PRR9* has five AS events. Both barley homologues, *PRR59* and *PRR95*, undergo five AS events. Comparison of gene structure and AS events of *AtPRR5*, *AtPRR9*, *HvPRR59* and *HvPRR95* homologues show that these genes have a highly conserved gene structure in the PRR (3 exons) and CT (2 exons) domain-coding exons (Appendix B10.4). Both Arabidopsis paralogues have several AS events in their N-terminal coding regions, but it was not possible to establish their conservation in barley. The C-terminal coding regions in *AtPRR9* and both barley homologues have conserved IR events which introduce PTCs. These PTC-containing alternative transcripts are unable to code for the CT domain in Arabidopsis or barley (James *et al.*, 2012a). Interestingly, I2R (*HvPRR95*) and I3R (*HvPRR59*) events are similar to the cold-dependent I3R AS events in *PRR9* (James *et al.*, 2012a), which is also affected in *prmt5* and *stipl1* mutants (Sanchez *et al.*, 2010; Jones *et al.*, 2012).

5.3.2.5 – *PRR3(7)/PRR7(3)*

Arabidopsis *PRR3* and its paralogue *PRR7* undergo five AS events (James *et al.*, 2012a). Barley homologues *PRR37* and *PRR73* undergo nine and seven AS events, respectively. Comparison of gene structures and AS events of *AtPRR3*, *AtPRR7*, *HvPRR37* and *HvPRR73* homologues identified their similarities and differences (Appendix B10.5). *AtPRR3* has 11 exons, while *AtPRR7*, *HvPRR37* and *HvPRR73* have very conserved gene structure with 9 exons, confirming the orthology, as suggested by *in silico* and mRNA expression analyses. Furthermore, *AtPRR7* and *HvPRR37* have a CrIn in E8 that removes 84 nt or 249 nt, respectively, both maintaining the ORF. Interestingly, *AtPRR3* and *HvPRR73* undergo two similar AS events, alt 5' ss E2 and I4R, even though they are not orthologues. Regarding the alt 5' ss E2 (-7 nt) event, it has opposite results in both species: in barley the length of a uORF is reduced from 36 to 11 amino acids, while in Arabidopsis it increases a uORF from nine to 29 amino acids.

5.3.3 – Effect of low temperature on expression and AS of barley clock genes

The Arabidopsis clock genes mostly regulated by low temperature AS are *AtLHY* and *AtPRR7* (James *et al.*, 2012a). Having defined the AS events of clock genes in barley, it was investigated whether transient or longer term low temperature conditions influence AS of *HvLHY*, *HvPRR37* and *HvPRR73*. For this, the HR RT-PCR system was used to examine the relative levels of fully-spliced (FS) versus transcripts containing different AS events identified for these genes (see above). Leaf samples of three barley lines grown in LD conditions were collected at 2.5 h after dawn in different temperature conditions. This time point was chosen because it is when *HvLHY*, *HvPRR37* and *PRR73* have their expression peaks (identified previously in Chapter 4). However, results obtained from these analyses must be interpreted carefully. Clock genes have a rhythmic expression, which might be al-

tered as temperature changes, i.e. phase (time of the expression peak) might be shifted as part of clock temperature entrainment and compensation. In this case, upon temperature changes, 2.5 h after dawn may not be representative of the expression peak any more.

Plants were grown for one week at 20 °C and sampled. Subsequently, plants were subjected to 4 °C for four days. Plants analysed on Day 4 at 4 °C were considered as acclimated to cold. After four days in the cold, the temperature was switched back to 20 °C, as before the cold treatment. Unlike the temperature reduction regime, which was initiated at dusk, temperature increase took place at dawn to avoid any conflict between clock-entraining light/dark and warm/cool cycles. This approach tested whether AS events identified were temperature-dependent and reversible rather than age-dependent and permanent. Plant material was collected at six time points: 1) 20 °C (Day 7); 2) Day 1 at 4 °C; 3) Day 2 at 4 °C; 4) Day 4 at 4 °C; 5) Day 1 at 20 °C; 6) Day 2 at 20 °C. The primer strategies and representative HR RT-PCR panel electropherograms produced by the ABI 3730 genotyping software are shown in Table 5.1–5.4, Figure 5.11 and Figure 5.13. The abundance of several transcripts was measured after 20 to 28 cycles of amplification. Changes in transcript levels were observed and ten important temperature-related AS events in *HvLHY* and *HvPRR37* were detected. These were either virtually absent at 20 °C and only visible during cold treatment (or vice-versa) or increased to between 10 and 50% of total transcripts during at least one phase of the temperature transitions or acclimations. Furthermore, expression and AS changes were restored upon return to 20 °C conditions, i.e. the opposite was seen during a temperature increase.

The design of the cold experiment mentioned above was carried out on the basis that the barley clock is a homeostatic mechanism that maintains its 24-h period despite rapid temperature switches. Hence, monitoring AS during the transitions between temperatures would be valuable to understand the role of splicing in the clock temperature entrainment process, while monitoring AS steady states would potentially give information

about splicing in temperature compensation mechanisms of the clock. However, to test whether sudden cold stress could change splicing patterns by inactivating or lowering efficiency of splicing machinery, introns from control genes *HvUBC21* and *HvPPDAA2* were also analysed. This was particularly important because several cold-dependent AS events in *LHY* that were observed transiently upon cooling are IR events. For both control genes, the relative abundance of transcripts containing IR events was not affected by cooling (data not shown). In addition, in the clock genes themselves many introns are as efficiently removed at 4 °C as at 20 °C. Therefore, transient AS events seen upon cooling are not caused by a non-specific decline in the splicing activity under these conditions, but rather, represent subtle and specific modulation of mRNA diversity and expression levels.

5.3.3.1 – *HvLHY*

The effects of temperature changes on *HvLHY* expression and splicing patterns were tested in three barley lines using four different HR RT-PCR primer pairs (Table 5.1), which spanned most of the gene sequence. This approach allowed for a quantitative analysis in which total *LHY* transcript was assessed as the sum of all isoforms detected with these primers (which were considered as technical replicates). Some changes in total *LHY* transcript levels were clearly observed during temperature transition experiments (Figure 5.10). First, *Hvelf3* plants grown at 20 °C have significantly lower levels of *LHY* when compared to WT and Bowman (*ppdh1*) plants ($P < 0.002$). These results confirm the expression analysis described in Chapter 4. Second, at 4 °C (Days 1, 2 and 4) and Day 1 at 20 °C, no significant difference was observed in *LHY* levels among the three barley lines analysed, which suggests that under low temperature conditions *ppdh1* and *elf3* mutations have no effect on *LHY* expression. Third, *LHY* mRNA levels in WT and Bowman (*ppdh1*) plants decreased from 100% at 20 °C to 52–55% on Day 1 at 4 °C, whereas *Hvelf3* plants

had increased levels of around 126% in a similar comparison. Lastly, on Day 2 at 20 °C, *LHY* transcript levels in WT and Bowman (*ppdh1*) increased to four to six times over the 4 °C (Day 4) levels. The *LHY* levels on Day 1 at 20 °C were low and probably reflected that this time point represents effectively one hour after switching to 20 °C from 4 °C, showing that recovery of transcript levels takes longer than 2.5 h. In contrast, *Hvelf3* plants maintained the same expression level in a similar comparison.

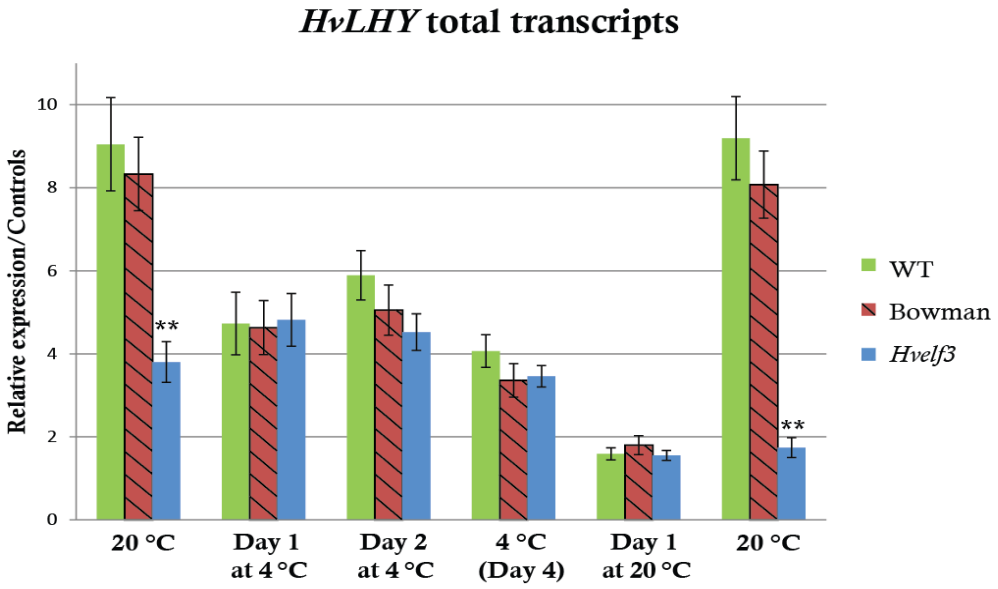


Figure 5.10: Total *LHY* transcript levels in the morning (2.5 h after dawn) under six conditions: 20 °C (Day 7), Day 1 at 4 °C, Day 2 at 4 °C, 4 °C (Day 4), Day 1 at 20 °C and 20 °C (Day 2). Results obtained with four different HR RT-PCR primer pairs (Table 5.1) were considered as technical replicates. Error bars: SEM from three biological replicates. Comparison are of *LHY* levels between *Hvelf3*, WT and Bowman (*ppdh1*). $P < 0.002$, represented by **.

In addition to affecting expression levels, transient and long-term temperature changes alter *LHY* splicing. Using HR RT-PCR primers spanning the 5'UTR, a significant but transient retention of *LHY* introns 1, 2 and 3 (adding 2, 3 and 7 uORFs, respectively) was detected on the first day during the transition from 20 °C to 4 °C (Figure 5.11, Figure 5.12 and Appendix B7.2), which roughly returned to original levels on the following days.

The relative abundance (proportion of total *LHY* transcripts) of alt 3' ss I1 (adding 1 uORF) also followed the same behaviour as IRs. These uORF-containing transcripts represent around 8% of total transcript levels at 20 °C, but on Day 1 at 4 °C they were significantly different ($P < 0.001$), representing around 38.5% of total *LHY* transcripts. These results are virtually identical for the three barley lines analysed (data not shown), indicating that the *ppdh1* and *elf3* mutations have no effect on *LHY* splicing. The levels of two isoforms with alternative splice sites (alt 3' ss E4 and alt 3' ss I2) are low in all conditions analysed (< 3% of total transcript) and show different behaviour from IRs and alt 3' ss I1. Alt 3' ss E4 and I2 decreased on Day 1 at 4 °C and maintain low levels at 4 °C. These two 'alt ss' isoforms are not included in Figure 5.12 or further analysis because they may represent inherent splicing variation in this region of the transcript. This is suggested for the following reasons: First, they are related to the abundance of FS transcripts, i.e. their level decreases when *LHY* FS levels decrease, and vice-versa. Second, they represent less than 3% of total transcript levels in all conditions analysed. Third, they have no known or predicted effect on the potential functions of the transcripts (such as altering uORFs). Lastly, there is no conservation of alt 3' ss E4 or alt 3' ss I2 in *AtLHY* and *AtCCA1* homologues.

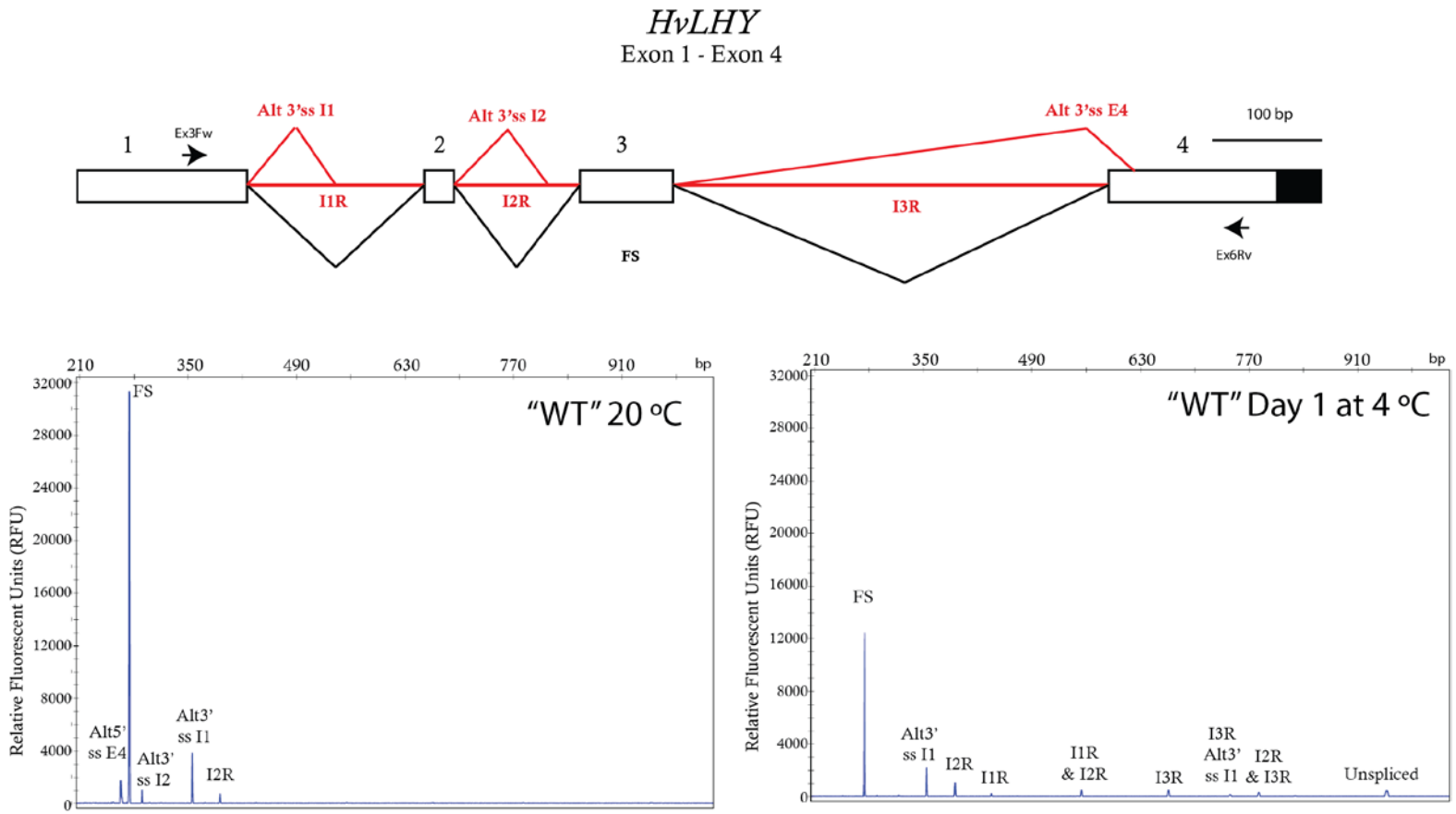
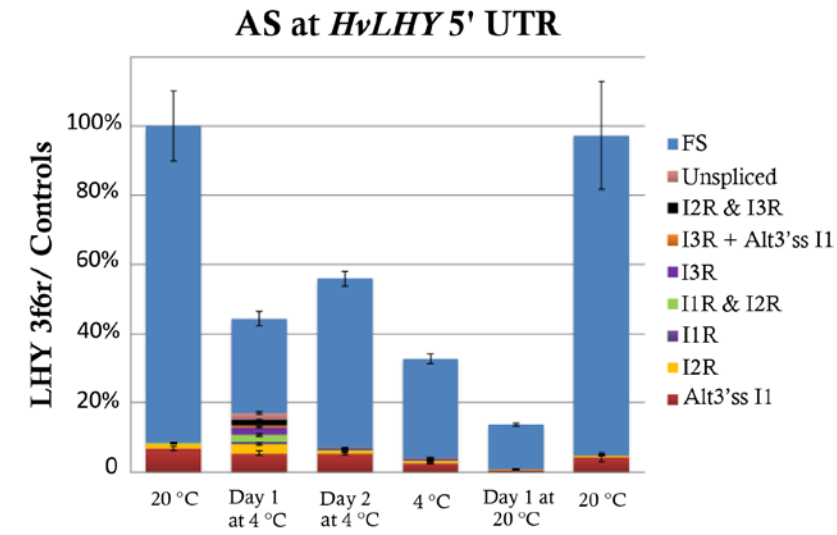


Figure 5.11: HR RT-PCR analysis of AS in the 5' UTR of *HvLHY*. Exons are numbered on the genomic structure; 5' and 3' UTRs are open boxes; coding sequences are dark boxes. Diagonal lines represent splicing events. AS events are shown in red. Approximate positions of primers are shown by arrowheads. Representative electropherograms of spectral data collected during the sample run and produced by GeneMapper® show the size of detected peaks corresponding to RT-PCR products from *LHY* FS and AS transcripts. Individual *LHY* transcript isoforms are labelled.



% of total transcript level

FS	87	61	86	86	93	93
AS (uAUG)	8.5	**38.5**	12	12	5	5

Figure 5.12: Relative abundance of *LHY* FS and AS transcripts in the 5' UTR region containing additional uORFs, AS events I1R, I2R, I3R and alt3' ss I1. WT plants were assessed using HR RT-PCR primers spanning the 5' UTR (Table 5.1). Error bars are SEM of three biological replicates. Significantly different AS/FS ratios when compared with 20 °C data are shown in red ($P < 0.001$).

HR RT-PCR analysis of the region encoding the *LHY* MYB domain (Figure 5.13) and the C-terminus coding region show a striking retention of *LHY* intron 4 (I4R) and 8 (I8R) during the transition from 20 °C to 4 °C (Figure 5.14A and B). I4R ($P < 0.001$) and I8R ($P < 0.01$) significantly increased in both absolute terms and as a proportion of total *LHY* transcripts, but they decreased considerably over the following days. I4R changed from around 1% of total transcripts at 20 °C to 40% during the first day after reduction in temperature and 14% after acclimation to 4 °C. I8R changed from around 10% of total transcripts at 20 °C to 37% during the first day at 4 °C and 9% after acclimation to 4 °C. These results were nearly identical for the three barley lines analysed (data not shown), confirming once again that *ppdh1* and *elf3* mutations have no effect on *LHY* splicing. As

mentioned previously (Section 5.3.1), the retention of *LHY* intron 4 and 8 create PTCs. Analyses of NMD of transcripts with retained introns in Arabidopsis suggest that they are not degraded by the NMD pathway (Kalyna *et al.*, 2012). This may be explained if such transcripts, for example, do not leave the nucleus. Transcripts with retained introns have not yet been shown to be insensitive to NMD in barley and it is not currently possible to predict the effects of I4R or I8R on translatability or stability of such mRNAs. However, it is clear that I4R mRNAs cannot code for the full-length LHY protein. Were these transcripts to be translated, they would encode an N-terminal truncated protein with no MYB domain (I4R) or a protein with an altered C-terminus (I8R), which could interfere with LHY protein function. Three additional transcript isoforms changes were observed at very low levels around the MYB-encoding exons: alt 5' ss E4, I5R and unspliced (I4R and I5R, 3.5% of total transcripts on Day 1 at 4 °C). Alt 5' ss E4 and I5R, in particular, were not included in Figure 5.14A or in further analyses because they represent less than 0.5% of total transcript levels in all conditions analysed.

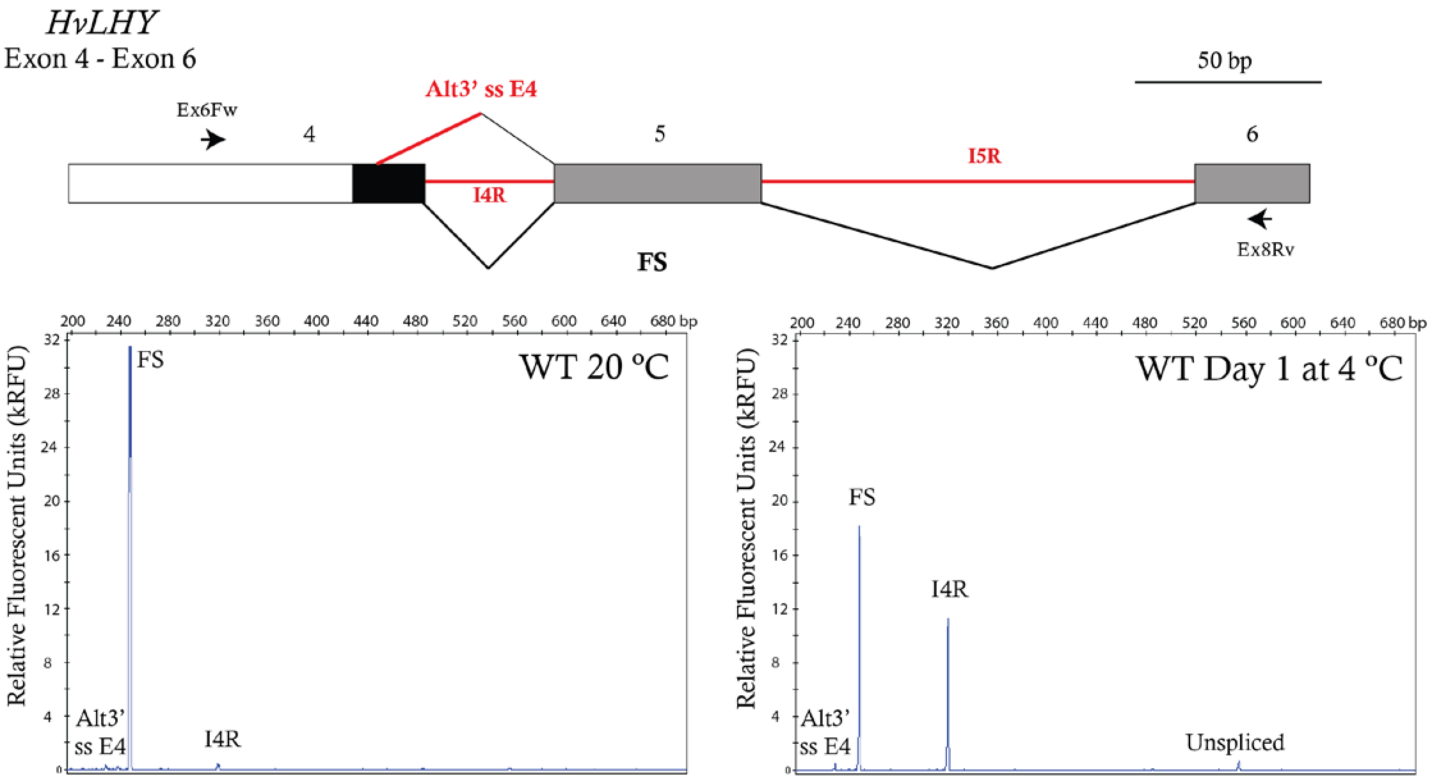


Figure 5.13: HR RT-PCR analysis of AS in the MYB-coding domain region of *HvLHY*. Exons are numbered on the genomic structure; 5' UTR is the open box; coding sequences are dark boxes, except MYB-encoding exons, which are shaded grey. Diagonal lines represent splicing events. AS events are shown in red. Approximate positions of primers are shown by arrowheads. Representative electropherograms of spectral data collected during the sample run and produced by GeneMapper® show the size of detected peaks corresponding to RT-PCR products from *LHY* FS and AS transcripts. Individual *LHY* transcript isoforms are labelled.

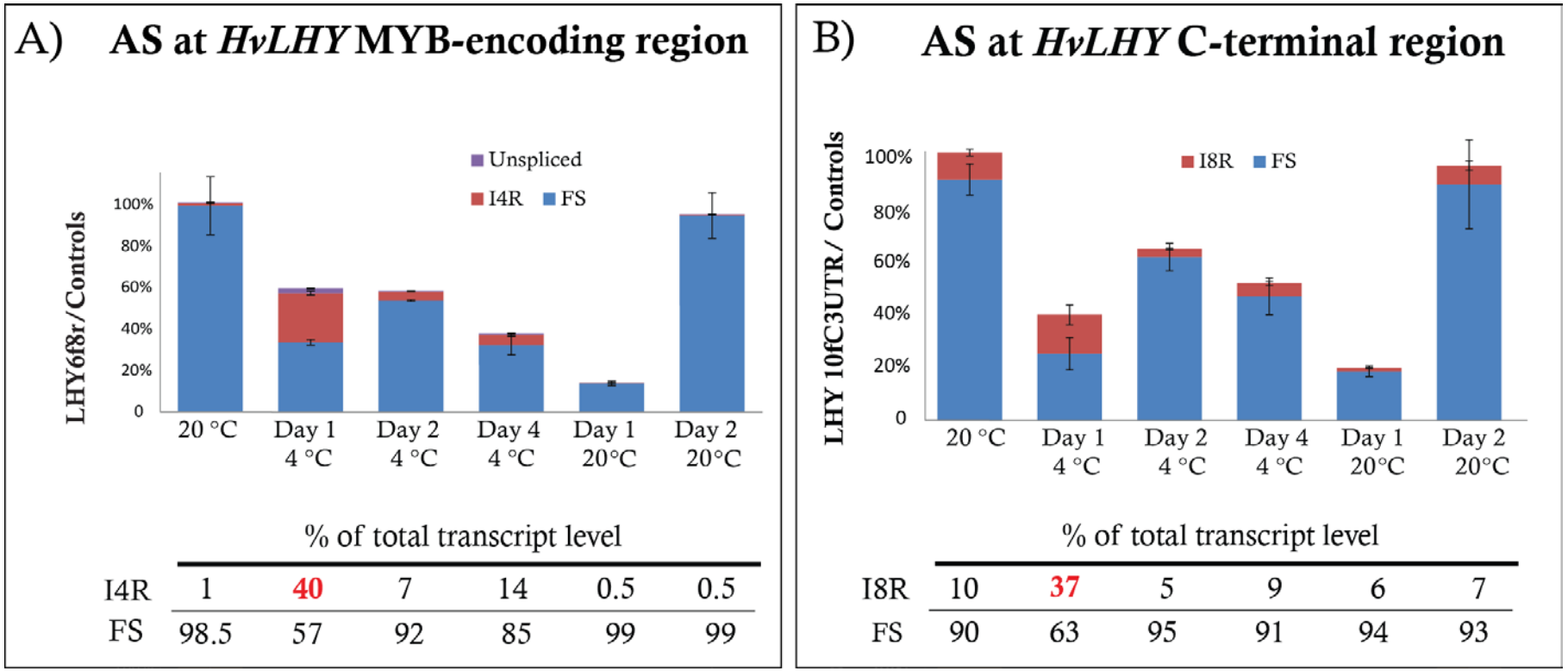


Figure 5.14: Relative abundances of *LHY* FS and AS transcripts. WT plants were assessed using HR RT-PCR primers (Appendix B7.1) spanning A) the MYB-encoding region and B) the C-terminal coding region. Error bars are SEM of three biological replicates. Significantly different AS/FS ratio compared with 20 °C data is shown in red (I4R: $P < 0.001$; I8R: $P < 0.01$).

In Arabidopsis, an important low temperature-dependent AS event is the inclusion of an alternative exon (E5a), in the long intron 5 (James *et al.*, 2012a). The equivalent intron in *HvLHY* is intron 6. At 20 °C, single products representing the fully spliced transcript (FS) were observed using HR RT-PCR primers spanning *LHY* exons 6 and 7 (Appendix B7.3). WT, Bowman (*ppdh1*) and *Hvelf3* plants acclimated to 4 °C expressed very low levels (< 0.7% of total transcripts) of an AS transcript with a cold-specific alternative exon E6a (Appendix B7.3). To obtain a clearer indication of when alternative E6a is included, an RT-PCR experiment was carried out using primers located in E6 (forward) and E6a (reverse) (Figure 5.15). The proportion of *LHY* transcripts specifically containing E6a increased upon cooling to 4 °C and continued to be detectable while the plants were maintained at 4 °C, but decreased when plants were returned to 20 °C. Analysis suggests that there is some retention of intron 6, as well as E6a inclusion, but this information could not be confirmed due to technical constrains in amplifying and cloning transcripts with this long intron (1,657 bp). E6a- and putative I6-containing transcripts are probably non-functional due to the presence of PTCs.

Collectively, the data indicate extensive AS of *LHY* transcripts, the levels of which can change transiently or show gradual accumulation upon transfer to lower temperatures. At low temperatures, the overall levels of *LHY* transcripts decrease and cold-dependent AS isoforms contribute to a further reduction in *LHY* mRNA levels, which is likely to lead to a decrease in functional LHY protein.

Figure 5.15: Cold-dependent *LHY* AS E6a transcripts detected by RT-PCR using primers located in E6 (forward) and E6a (reverse). WT samples were harvested in the morning (2.5 h after dawn) in six temperature conditions: 20 °C (Day 7), Day 1 at 4 °C, Day 2 at 4 °C, 4 °C (Day 4), Day 1 at 20 °C and 20 °C (Day 2). Amplification of UBC (*HvUBC21*) and PP2A (*HvPP2AA2*) served as reference genes. Three biological replicates were analysed. 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except E6a, which is shaded yellow. 812, unspliced product; 640, E6a-containing product; -, negative control (RNA template).

5.3.3.2 – *HvPPDH1* (*HvPRR37*)

Since Arabidopsis *PRR7* undergoes extensive cold-dependent AS and has been implicated in responses to temperature (Salomé and McClung, 2005; James *et al.*, 2012), the effect of cold on expression and splicing of barley *HvPRR37* and *HvPRR73* were evaluated. *PRR37* and *PRR73* are paralogues and show similar expression behaviours (Chapter 4). However, they respond differently to a reduction in temperature from 20 °C to 4 °C.

The effects of temperature changes on *HvPPDH1* expression and splicing patterns were tested in the three barley lines using four different HR RT-PCR primer pairs, which spanned most of the gene sequence (Figure 5.8). Primer pairs spanning the C-terminus re-

gion did not efficiently amplify *PPDH1* transcripts and no expression data were obtained. Total *PPDH1* transcript levels were calculated as the sum of all isoforms detected with three different primer pairs (which were considered as technical replicates). Some changes in total *PPDH1* transcript levels were clearly observed (Figure 5.16). First, at 4 °C (Days 1, 2 and 4) and Day 1 at 20 °C, *Hvelf3* plants had significantly higher levels of *ppdh1* when compared to WT *PPDH1* expression, which suggests that under low temperature conditions, the *elf3* mutation has an effect on *ppdh1* expression, which is similar to the effect under 20 °C conditions, as mentioned in Chapter 4. In contrast, when plants are acclimated to cold conditions, Bowman (*ppdh1*) plants have significantly ($P < 0.05$) lower levels of *ppdh1* when compared to WT *PPDH1* allele. This suggests that different *PPDH1* alleles have different behaviours upon cold acclimation, which was also observed for 20 °C conditions (Chapter 4). This may reflect differential stability of the *PPDH1* and *ppdh1* alleles, but is difficult to predict, as the mutation in *ppdh1* is not known. Additionally, *PPDH1* mRNA levels in WT and Bowman (*ppdh1*) plants decreased from 100% at 20 °C down to 45% and 75%, respectively, on Day 1 at 4 °C and 13% on Day 4 at 4 °C, whereas *Hvelf3* plants maintained the same levels on Day 1 at 4 °C and decreased down to 50% on Day 4 at 4 °C. Lastly, on Day 2 at 20 °C, *PPDH1* transcript levels in the three analysed barley lines increased around four-fold when compared to Day 1 at 20 °C (effectively 1 hour at 20 °C after the former 4 °C conditions), and roughly returned to original levels, as detected before the cold treatment.

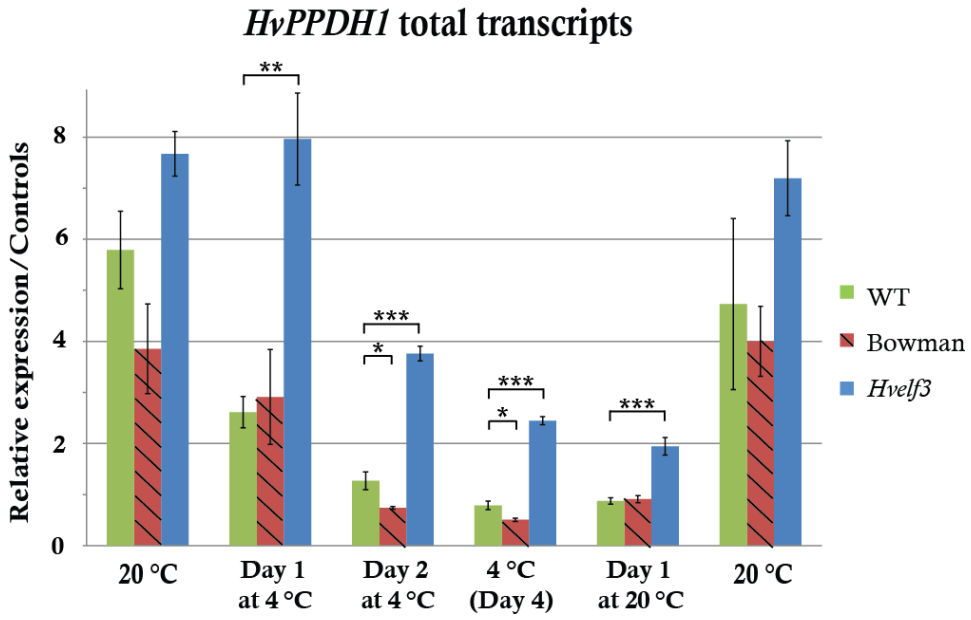


Figure 5.16: Total *PPDH1* transcript levels in the morning (2.5 h after dawn) under six conditions: 20 °C (Day 7), Day 1 at 4 °C, Day 2 at 4 °C, 4 °C (Day 4), Day 1 at 20 °C and 20 °C (Day 2). Results obtained with three different HR RT-PCR primer pairs (Appendix B7.3 and B7.4) were considered as technical replicates. Error bars: SEM from 3 biological replicates. *P* values represent statistical comparisons of data from WT against each mutant. *, $P < 0.05$; **, $P \leq 0.005$; ***, $P < 0.001$.

Exposure of plants to cold temperatures alters *PPDH1* splicing, according to HR RT-PCR analyses using primers spanning *PPDH1* exons 5 and 7. There are seven different mRNA isoforms generated from this region, detectable at 20 °C. Of these, three contain PTCs and therefore are likely to be non-functional: I6R, I6R + alt 3' ss E6 (-6 nt) and alt 5' ss I6 (+50 nt). The other four are all mRNA isoforms coding for slightly different proteins and are due to transcripts containing four different combinations of two alternative splicing events: an alt 3' ss E6 which adds or removes 6 nt (two codons for two amino acids) and an alt 5' ss in I6 which adds or removes 45 nt (codons for 15 amino acids). The four transcripts represent between 18 and 32% each and together a more than 90% of the total at 20 °C (Figure 5.17). No enzymatic domain has been described for this region in the PPDH1

protein so it is not possible to predict any impact on the function of these AS events and potentially they represent functional isoforms with or without subtle differences.

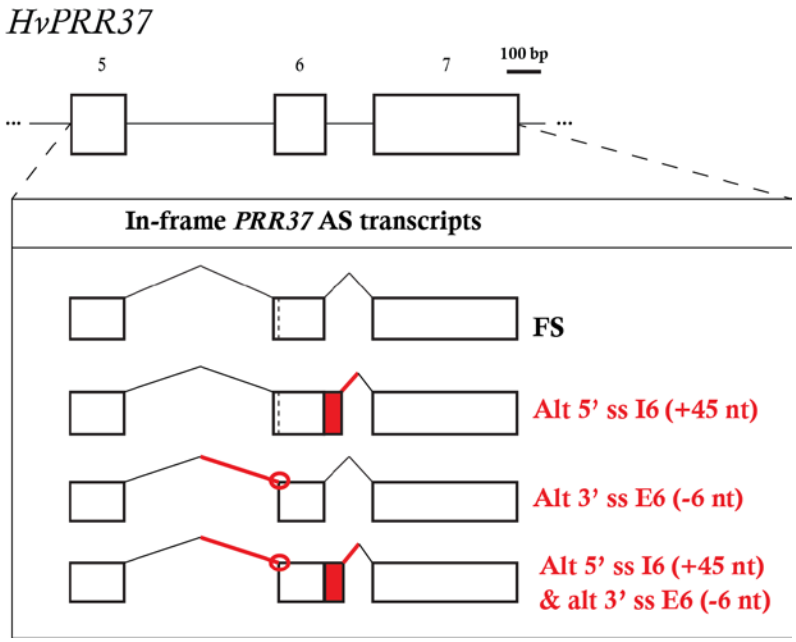
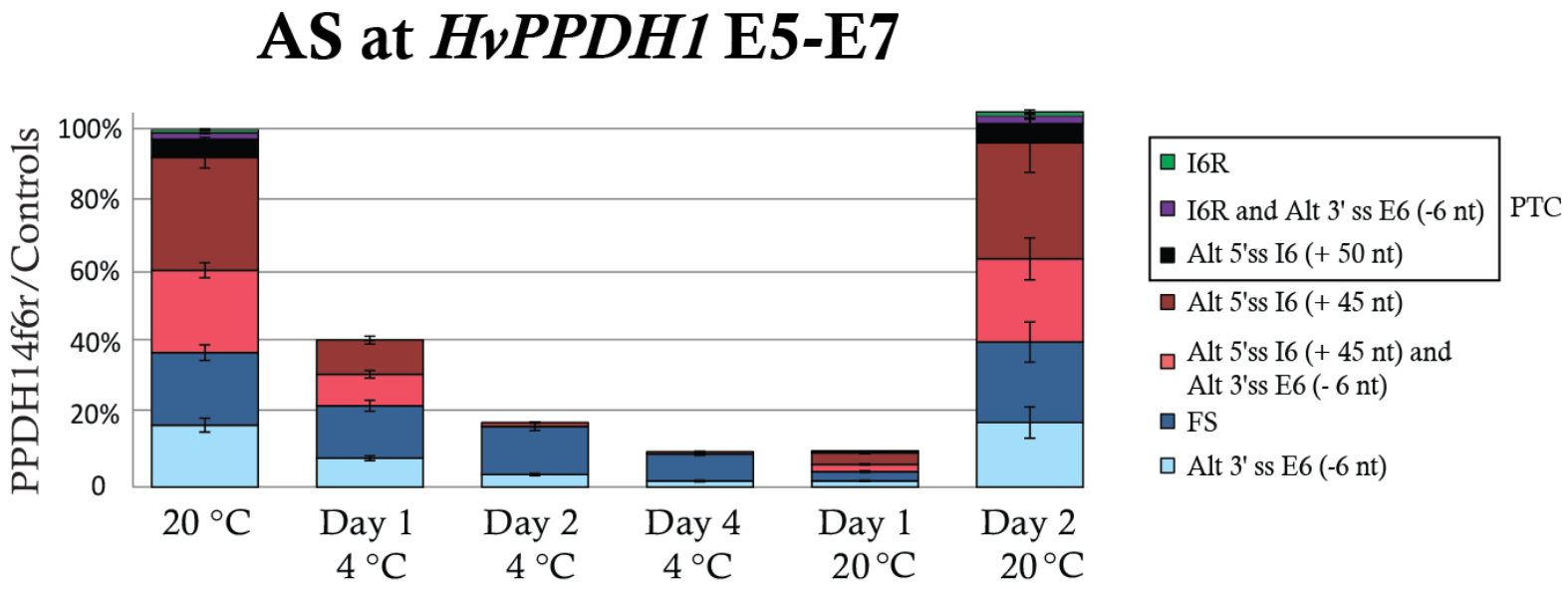


Figure 5.17: *HvPRR37* genomic structure and in-frame transcript isoforms from exons 5, 6 and 7. Exons are numbered on the genomic structure; coding sequences are white boxes; introns are straight black lines. Diagonal lines represent splicing events. AS events are shown in red.

The gene model in the barley database and proposed by Turner *et al.*, (2005) defines the alt 5' ss I6 (+45 nt) transcript as the FS version. Here, we suggest that this splicing event is an alternative event because it is greatly reduced in low temperature conditions (less than 6% of total transcripts). We propose instead that the transcript with removal of the 45 nt is the FS version as it is the most abundant at 4 °C. Of the seven different AS events in *HvPPDH1*, four were considered temperature-dependent: alt 5' ss I6 adding 45 nt or 50 nt, alt 3' ss E6 (-6 nt) and I5R (Figure 5.18) and different transcripts were detected undergoing one or a combination of two of these AS events. Transcripts involving the two alternative splice sites (removing 6 nt of E6 and/or adding 45 nt from I6) were all signifi-

cantly reduced in both relative and absolute terms upon cold acclimation. In particular, the two in frame transcripts which contained alt 5' ss I6 (+45 nt) were greatly reduced from around 54.7% of total transcripts at 20 °C to 5.7% after acclimation to 4 °C. As a result, FS transcripts changed from around 20.3% of total transcripts at 20 °C to 77% after acclimation to 4 °C. Interestingly, on Day 1 at 20 °C, which is effectively 1 hour at 20 °C from former 4 °C condition, alt 5' ss I6 (+45 nt) event returned to original levels detected before the cold treatment, suggesting an immediate response to increase in temperature. This shows that the presence of products containing alt 5' ss I6 (+45 nt) is very clearly related to temperature rather than simply to the abundance of the transcript. Additionally, transient and longer term changes in *PPDH1* expression and AS pattern can be detected irrespective of the direction of temperature change.

The rapid response of alt 5' ss I6 (+45 nt) to temperature increase may reflect features of I6 itself, for example, secondary structure. However, no strong secondary structure was detected within the I6 sequence using RNAfold software (http://rna.tbi.univie.ac.at) and other factors may be involved in the regulation of this AS event. Two additional AS events, Alt 5' ss I6 adding 50 nt and I5R, add PTCs and cannot code for full-length PPDH1 protein. These PTC-containing transcripts represent around 7.7% of total transcripts at 20 °C and become undetectable upon transfer and acclimation to 4 °C. The data for *Hvelf3* mutant, which expresses *Hvppdh1* at higher levels than WT (*PPDH1*) and Bowman (*ppdh1*), show that the presence of these PTC-containing products is clearly related to temperature rather than simply to the reduced levels of *HvPRR37* transcripts at 4 °C (Day 1) (Appendix B11.1). This relationship is highlighted by the fact that the splicing of intron 5 is more efficient at 4 °C than 20 °C, which might not be expected if the spliceosome was adversely affected at the lower temperature.



% of total transcript level

FS	20.3	35.6	**74.3**	**77**	25.5	21.5
AS (PTC)	7.7	**0**	**0**	**0**	5.6	8.2
Alt 5'SS I6 (+ 45 nt)	54.7	44.7	**6.2**	**5.7**	52	53.2

Figure 5.18: Relative abundances of *PPDH1* FS and AS transcripts. WT plants were assessed using HR RT-PCR primers spanning exons 5 and 7 (Appendix B7.4). Error bars are SEM of three biological replicates. Significantly different AS/FS ratios when compared with 20 °C data are shown in red ($P < 0.001$).

Using HR RT-PCR primers spanning the 5'UTR or *PPDH1* exons 2 through 5, no cold-dependent AS events were detected (Appendix B11.2). In different temperatures analysed here, retention of *PPDH1* intron 1 (adding 1 uORFs) was related to the abundance of FS transcripts. I1R analyses for WT, Bowman (*ppdh1*) and *Hvelf3* plants were very similar (data not shown), indicating the *ppdh1* and *elf3* mutations may have no effect on *PPDH1* splicing. When using primers spanning *PPDH1* exons 2 and 5, only fully spliced transcripts (FS) were detected.

In summary, the data indicate extensive AS of *PPDH1* transcripts, the extent of which changes both immediately on transfer to and following acclimation to lower temperatures. Temperature-dependent AS clearly contributes to the diversity of PPDH1 isoforms and to control of *PPDH1* mRNA levels but it is not possible to predict whether the in frame isoforms are functionally different. Thus, temperature has a major effect on the AS of *PPDH1* transcripts, which is not affected by mutations in the clock genes *PPDH1* and *ELF3*.

5.3.3.3 – *HvPRR73*

Effects of temperature changes in *HvPRR73* expression and splicing patterns were tested in the three barley lines using four different HR RT-PCR primer pairs, which spanned most of the gene sequence (Figure 5.8). Quantitative analysis of total *PRR73* transcript levels was calculated as mentioned for *PRR37* above (Section 5.3.2.2). *PRR73* mRNA levels in WT and Bowman (*ppdh1*) plants decreased from 100% at 20 °C down to around 65% on Day 1 at 4 °C, whereas *Hvelf3* plants maintained the same levels in a similar comparison. In fact, on Day 1 at 4 °C, *Hvelf3* plants had significantly higher levels of *PRR73* when compared to WT and Bowman (*ppdh1*), which suggests that on transition to colder conditions, the *elf3* mutation has an effect on *PRR73* expression. Upon cold acclima-

tion, the three barley lines analysed return to original expression levels (detected at 20 °C), suggesting that cold conditions affect *PRR73* expression during temperature transitions rather than temperature acclimation.

Analysis of AS by HR RT-PCR, using primers spanning most of the *PRR73* gene sequence did not detect any highly significant cold-dependent AS events (Appendix B10.2). In different temperatures analysed here, retention of *PRR73* introns 3, 4, 5, 6 and 7 and alt 3' ss E2 were related to the abundance of FS transcripts. AS analyses for WT, Bowman (*ppdh1*) and *Hvelf3* plants were very similar (data not shown), indicating *ppdh1* and *elf3* mutations have no effect on *PRR73* splicing. Therefore, *PPDH1* and *PRR73* behave very differently in their AS response to low temperature.

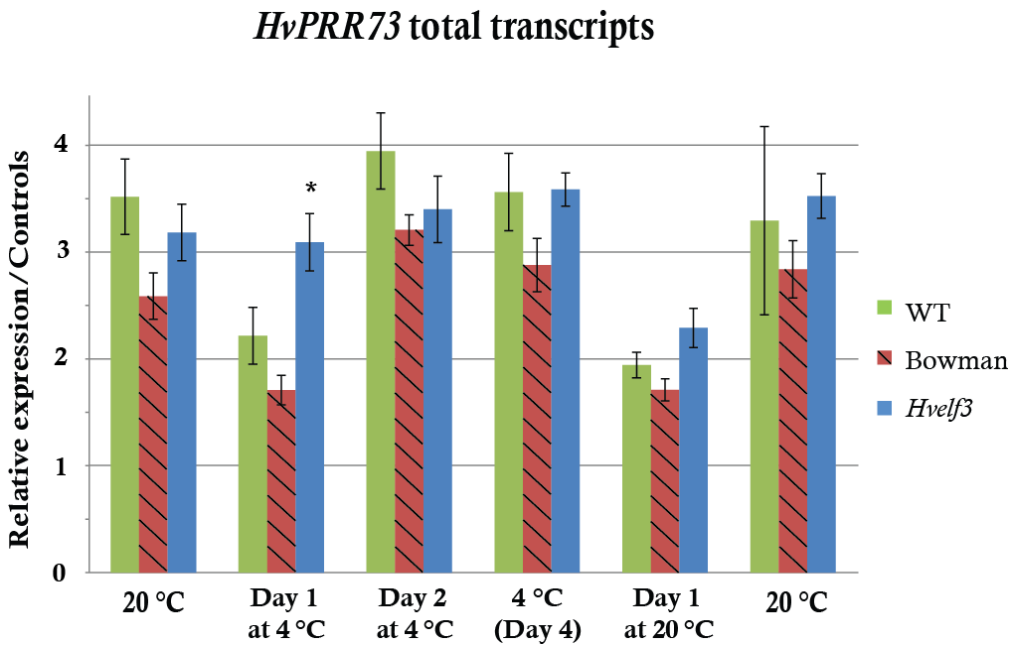


Figure 5.19: Total *PRR73* transcript levels in the morning (2.5 h after dawn) under six conditions: 20 °C (Day 7), Day 1 at 4 °C, Day 2 at 4 °C, 4 °C (Day 4), Day 1 at 20 °C and 20 °C (Day 2). Results obtained with four different HR RT-PCR primer pairs (Appendix B7.4) were considered as technical replicates. Error bars: SEM from 3 biological replicates. *P* values represent statistical comparison of data from WT, Bowman (*ppdh1*) and *Hvelf3* plants. *, $P < 0.02$.

5.4 – Discussion

As well as identifying the gene structure and confirming cyclic expression of barley clock components (Chapters 3 and 4), it is also important to study the role of the clock genes and how the clock integrates its timing information into signalling pathways, such as cold response. Such understanding of the clock function would give further evidence that it offers competitive advantages in a changing world. Temperature is an important external cue that entrains plant clocks to local time and environmental conditions, contributing to increased fitness. This work provides initial evidence that temperature-dependent AS has a role in the operation and function of the barley circadian clock. We have identified 55 AS events in barley clock-related genes at a system-wide level, ten of which are significantly modulated by temperature and analysed in different clock mutant backgrounds. These analyses also allowed a direct comparison to Arabidopsis clock AS data, and it was possible to identify conservation of some AS events or of cold-induced AS behaviour (e.g. increased non-functional isoforms at the expense of functional mRNAs) even if specific AS events are not conserved.

Under the conditions used here, more than half of the alternative transcripts are not abundant (< 1% of total transcripts), and most of them were IR events. Low abundance of transcripts with IR events most probably represent partially spliced transcripts in RNA samples, or they may be abundant under other environmental conditions or tissues not tested in this work (our analysis is also based on RNA extracted from whole shoots).

5.4.1 – Effect of temperature regulation on the expression of clock genes

Analyses of total *HvLHY* transcript levels in WT barley plants show a decreased expression from 100% at 20 °C to 44–65% at 4 °C (Days 1, 2 and 4). In Arabidopsis, temperature affects *AtCCA1* and *AtLHY* expression. When temperature was reduced from 20 to

4°C, *CCA1* transcript abundance was higher or similar to that of 20 °C conditions at dawn. By contrast, decreasing temperature causes a marked reduction in the expression peak of *LHY* (Section 5.4.2) (Bieniawska *et al.*, 2008; James *et al.*, 2012a). The similar *AtLHY* and *HvLHY* responses to lower temperature, as opposed to *AtCCA1* behaviour, further support their orthology, as suggested by *in silico* analyses (Chapter 3).

In Arabidopsis, temperature affects expression of paralogues *AtPRR7* and *AtPRR3* (James *et al.*, 2012a). When temperature is reduced from 20 to 4°C, the *AtPRR7* expression peak decreases from 100% at 20 °C to 80% at 4 °C (Day 4). Temperature reduction causes a marked decrease in the *PRR3* expression peak from 100% at 20 °C to 35% at 4 °C (Day 4). Analyses of total transcript levels of the barley homologues *HvPPDH1* and *HvPRR73* carried out here confirm that although they show similar expression behaviour in light and dark conditions at 20 °C, cold acclimation affected their expression in different ways. *HvPPDH1* expression gradually decreased from 100% at 20 °C to 13% at 4 °C (Day 4), whereas *PRR73* expression has a slight decrease on Day 1 at 4 °C but increased on Day 2 at 4 °C so that its expression is virtually identical between plants acclimated to 20 °C or 4 °C (Day 4) conditions. These results suggest that at the level of expression control upon cold acclimation *HvPPDH1*, as opposed to *HvPRR73*, is similar to *AtPRR7* and *AtPRR3*.

Mutation in the barley clock gene *ELF3* affects clock entrainment. *Hvelf3* plants are non-responsive to cold temperature transition, as seen in *HvLHY*, *HvPDH1* and *HvPRR73* expression behaviours when plants are transferred from 20 °C to 4 °C. Upon cold acclimation, significant differences were observed in *PPDH1* levels among the three barley lines analysed while *LHY* and *PRR73* expression were unaffected by the *elf3* allele. This suggests that in cold acclimated plants, ELF3 strongly regulates *PPDH1* but not *LHY* or *PRR73* expression. In Arabidopsis, *elf3* mutant seedlings are also unable to entrain to thermocycles. For instance, *PRR7* expression in this mutant shows no significant high-temperature induc-

tion (Thines and Harmon, 2010). Therefore, results obtained here for *elf3* mutants confirm previous suggestions (Thines and Harmon, 2010) that the *ELF3* clock component is involved in temperature transition responses in plants.

The *PPDH1* mutation has no effect on the cold responses of the clock genes *LHY* and *PRR73*. However, significantly different expression between WT *PPDH1* and Bowman *ppdh1* on Day 2 and 4 at 4 °C (Figure 5.16) might suggest they have different clock temperature entrainment and compensation behaviours. Arabidopsis *prr7* mutants exhibit modest *CCA1, LHY* and *TOC1* period lengthening under free-running conditions, failing to correctly entrain light and temperature cues (Salomé and McClung, 2005). Therefore, it is likely that the *PPDH1* and *PRR7* components are involved in clock temperature responses.

5.4.2 – AS regulation of *LHY* and *PPDH1* in response to temperature

Temperature regulation of AS occurs in clock genes of *Drosophila* (see Section 1.6.4) and *N. crassa* (Colot *et al.*, 2005). Additionally, AS of Arabidopsis clock genes *CCA1*, *LHY*, *TOC1*, *PRR3* and *PRR7* is strongly affected when plants are transitioned or acclimated to lower temperatures (James *et al.*, 2012a). Having defined the AS events of clock genes in barley, this work has also shown that transient or long term low temperature conditions influence AS of *HvLHY* and *HvPRR37*, which are orthologues of the Arabidopsis genes mentioned above. In discussing the various AS events in clock genes and their response to low temperature (below), it is important to note that most introns are spliced efficiently such that the AS behaviour is not caused by a transient reduction in general splicing activity on cooling. Of all AS events described for the *AtCCA1*, *AtLHY* and *HvLHY* homologues, some are significantly temperature dependent. Both *LHY* orthologues have AS events in their 5' UTR that add uORFs, which increase under cold conditions. In Arabidopsis *LHY*, this AS event is I1R (James *et al.*, 2012a), while in barley eight different transcript isoforms

are observed (Figure 5.12). However, as mentioned previously, it is not possible to identify a conservation of specific AS events in the 5' UTR. Nevertheless, these 5' UTR AS isoforms increase considerably on Day 1 at 4 °C, representing 50% (*AtLHY*) (James *et al.*, 2012a) and 38.5% (*HvLHY*) of total transcripts.

Regarding the coding region at or near the MYB domain of *AtLHY*, *AtCCA1* and *HvLHY*, there are temperature-dependent AS events, but these do not always have a conserved position within the gene structure or similar temperature responses among the homologues. For example, I4R of *AtCCA1*, which occurs immediately after the MYB-encoding region, decreases in cold conditions (Filichkin *et al.*, 2010; James *et al.*, 2012a). In contrast, I4R of *HvLHY*, which is present immediately before the MYB-encoding region, has a strong and transient increase in low temperature conditions. In an example where there appears to be some conservation, both PTC-containing alternative exons E5a and E6a of *AtLHY* and *HvLHY*, respectively (occurring in the long intron immediately after the MYB-encoding region), are included in plants acclimated to cold. However, in plants acclimated to cold, *AtLHY* E5a is present at much higher levels (20% of total transcripts) when compared to *HvLHY* E6a (0.7% of total transcripts).

The function of AS events in *LHY* regulation is particularly clear in Arabidopsis. *AtLHY* transcripts and protein levels are reduced upon cooling, while the *LHY* promoter strength is not affected on Day 1 at 4 °C. Therefore the mechanism by which *LHY* expression is reduced is not through transcriptional regulation. Interestingly, the proportion of *LHY* AS transcripts that are non-functional (which mostly contain I1 and E5a) increased, while functional mRNA and LHY protein levels decrease (James *et al.*, 2012a). These transcripts are NMD-sensitive, which explains the reduced *LHY* transcript levels upon cooling. In summary, *AtLHY* expression is regulated in the cold post-transcriptionally by AS events that produce non-functional transcripts that are degraded by NMD (James *et al.*, 2012a). In

barley, I4R and I8R increase substantially at 4 °C and are unable to code for full-length LHY protein. The effects of AS in the 5' UTR and whether they are also NMD-sensitive is currently unknown.

Arabidopsis *PRR7* and the barley orthologue *HvPPDH1* (*HvPRR37*) undergo extensive cold-dependent AS splicing (James *et al.*, 2012a ; this study) but the AS events are not conserved. The abundance of AS *PRR7* transcripts in which exon 4 is skipped (E4S) or intron 3 is retained (both are mutually exclusive events) are substantially but transiently increased on Day 1 at 4 °C, reaching more than 50% of total transcripts. These transcripts introduce PTCs and E4S, in particular, is targeted for NMD (James *et al.*, 2012a). In contrast, two barley alternative PTC-containing *PPDH1* transcripts (alt 5' ss I6 adding 50 nt and I5R) are reduced from 7% of total transcripts at 20 °C to barely detectable levels on Day 1 at 4 °C. Furthermore, *HvPPDH1* had three abundant AS transcripts with one or both of two in frame AS events: alt 5' ss I6 adding 45 nt (coding for 15 amino acids) and alt 3' ss E6 (-6 nt, coding for 2 amino acids). All are significantly reduced upon cold acclimation, and in particular, transcripts with alt 5' ss I6 (+ 45 nt) is reduced almost ten times from 20 °C to 4 °C (Day 4), which affects the abundance of protein-coding *PPDH1* isoforms. Although is not possible to predict whether the presence or absence of these 15 amino acids affects *PPDH1* function, there is an overall reduction in PPDH1 transcripts. Therefore, *AtPRR7* AS events have a large effect on reducing the abundance of functional *PRR7* in the first day after transition to cold conditions, whereas *HvPPDH1* AS has increased PPDH1 protein diversity at ambient temperatures, which is greatly reduced at 4 °C. The barley *HvPPDH1* paralogue, *HvPRR73*, had no significant AS events, while the Arabidopsis *AtPRR7* paralogue, *AtPRR3*, shows three AS events with modest effects on *PRR3* gene expression (James *et al.*, 2012a).

5.4.3 – AS regulation of the barley clock

Recently, AS has been suggested to play a much more widespread, complex and quantitatively important role in the plant clock than previously thought (James *et al.*, 2012a). Studies in barley carried out here and in Arabidopsis (Filichkin *et al.*, 2010; James *et al.*, 2012a; Seo *et al.*, 2012) suggest that AS of clock genes is sensitive to temperature in the normal ambient range (between 20 °C and 4 °C), and plays a significant role in the entrainment of the plant circadian clock to temperature. This is shown in barley by the marked transient and long-term accumulation of uORF-containing and non-productive *LHY* transcripts and decrease in putative *PPDH1* protein-coding isoforms following cold treatment. These results were obtained by using the HR RT-PCR approach that analyses dynamic changes in the ratio of FS and alternatively spliced transcripts during temperature changes. This HR system is an efficient approach that compares AS events to the total level of mRNAs from many different samples (Simpson *et al.*, 2008; James *et al.*, 2012a).

The pattern of accumulation of *HvLHY* and *HvPPDH1* AS transcripts varies, and similar variation has been described for Arabidopsis clock genes (James *et al.*, 2012a). As mentioned above for *HvLHY*, some events become abundant only on Day 1 at 4 °C but are barely detectable in plants acclimated to 20 °C or 4 °C. Other events, such as the alt 5' ss I6 (+ 45 nt) in *HvPPDH1*, change when plants become acclimated to one temperature after being shifted from another, while others, such as those in *HvPRR73* and *HvGI*, do not change in their relative abundance. Since some barley AS events comprise at least 10 to 70% of total transcripts, under some conditions, they are likely to be of physiological importance. In fact, since alternative transcripts can represent more than 30% of the total for some genes, conclusions based on measurements of total transcript by traditional microarrays or qPCR must be treated with caution (James *et al.*, 2012a). Such abundant AS events in barley clock genes involved IR and alternative splice sites, which are also found in Ara-

bidopsis clock genes (James *et al.*, 2012a). In Arabidopsis, the majority of AS events introduce PTCs, leading to non-productive mRNAs (James *et al.*, 2012a). In barley clock genes, there are AS events introducing PTCs, as well as alteration of uORF (which may affect translation or stability of the mRNA) and an increase in the mRNA protein-coding capacity (e.g. *PRR37* in-frame alternative mRNAs).

uORF- and PTC-containing transcripts can trigger NMD, as has been described for Arabidopsis clock gene *LHY* and *PRR7* (James *et al.*, 2012a) or can be immune to NMD, which is the case for most IR transcripts (Kalyna *et al.*, 2012). Although a great deal of progress has been made on characterising the NMD system in Arabidopsis (Kalyna *et al.*, 2012) little is known about how NMD function in barley (Gadjieva *et al.*, 2004). Nevertheless, the factors which determine the fate of different plant uORF- and PTC-containing transcripts are complex and, particularly for uORFs, poorly understood and further research is still needed to define this system. However, some rules have been established on the impact of plant uORFs and PTCs based on the behaviour of a small number of Arabidopsis genes or artificial constructs (Kalyna *et al.*, 2012). For example, the NMD machinery has different efficiency rates in degrading uORFs and PTC-containing transcripts depending on the features of the target.

Transcripts with long 3' UTRs (> 350 nt) or with PTCs with downstream splice junctions or with uORFs (coding for more than 30 amino acids or overlapping functional ORF) are likely to be targets of NMD in Arabidopsis and they can be generated by AS events that add PTC/uAUG. With regard to the detection and fate of the barley AS transcripts identified here, some contain classic NMD features (PTC before splice junctions), some are intron retentions (presumably immune to NMD) and others contain uORFs whose function is difficult to predict. In many cases, the PTC-containing transcripts are in low abundance at 20 °C and 4 °C. If such transcripts are efficiently degraded by NMD,

they may, in fact, represent a significant proportion of transcripts which are under-represented. In addition, transcripts containing introns or intron fragments are likely recognized as aberrant prior to nuclear export by virtue of proteins binding to the UA-rich intron sequences and therefore do not connect with the NMD machinery (Kim *et al.*, 2009). This may explain the high abundance of IR events detected in *HvLHY* (e.g. I4R) upon transition to cold. If we assume that intron retaining transcripts in barley are not degraded by the NMD pathway, the fate of cold-dependent non-functional IR AS transcripts (e.g. *HvLHY* containing I4 and *PRR37* containing I1), may be different from other PTC-containing transcripts but, ultimately, they will all affect protein levels. Additional efforts (e.g. analysis of barley *UPF* mutants or cycloheximide treatments) are necessary for comprehensive identification of all AS events in the barley circadian clock genes.

It is also possible that PTC-containing transcripts can produce truncated proteins, as suggested for Arabidopsis *CCA1* (Seo *et al.*, 2012). However, this paper suggests the production of a C-terminal peptide which would require the ribosome to ignore the authentic translation start AUG and many other AUGs or reinitiate translation (e.g. through an internal ribosome entry site), which is unlikely (Meijer and Thomas, 2002; Kozak, 2005). Lastly, alternative protein isoforms are particularly interesting for LHY and PPDH1, since they can form homo- and hetero-dimers with different protein isoforms (Ito *et al.*, 2003). The different combinations are likely to have different properties, such as activity and substrates, and it will be interesting to investigate the relative functions of the abundant in frame isoforms of PPDH1. Overall, the data presented here cast additional light on the operation of the barley circadian clock and strongly suggest that AS provides one mechanism through which the barley clock is regulated. Transient and long-term AS events have been observed during shifts from 20 to 4 °C. However, the extent and duration of temperature changes required to trigger changes in AS need to be determined.

Chapter 6. General discussion and future work

It is an interesting time in the study of plant clocks. A great deal of progress has been made over the last 20 years in understanding Arabidopsis clock architecture and function. In recent years, some of this knowledge has been extended to other plant species such as rice, moss, barley and canola (Murakami *et al.*, 2007; Holm *et al.*, 2010; Faure *et al.*, 2012; Lou *et al.*, 2012). In particular, characterisation of the circadian clock in crop species advances the understanding of clock control over many phenotypic traits, which is especially important in light of global climate change. The work reported here is an example of how studies on different concepts in plant genetics and physiology in Arabidopsis can facilitate knowledge translation to barley, a crop plant of high economic value in Scotland. Here, I have identified 21 clock-related homologues, determined their expression profiles in diurnal and free-running conditions and examined the effects of mutations in *HvPPDH1* and *HvELF3* genes. Additionally, I have found evidence, for the first time, that AS has a role in the operation and functions of the barley circadian clock, which ultimately may offer new understanding of traits in barley, such as earliness, providing a new information for barley improvement.

6.1 – Evolution of clock genes

"Nothing in biology makes sense except in the light of evolution" (Dobzhansky, 1973). This concept, alongside the 'artificial' selection of crop species since domestication, motivates studies of the evolution of clock genes. Within angiosperms, in both monocots and dicots, a strong similarity exists among their clock components, architecture, and func-

tions (Song *et al.*, 2010). Evolutionary theory dictates that if those similarities were due to a process of descent, with modifications, from common ancestors then these clock systems have evolutionary homology (Prosdocimi *et al.*, 2009). To test this, several investigations have used different approaches, such as phylogenetic analysis, studies of segmental duplication and functional gene assessments through gene expression studies and complementation tests (suggesting conserved biochemical function). For example, knockdown and overexpression of *LHY*, *ELF3* and *GI* genes from *Lemna gibba* plants indicated these genes are functionally conserved with Arabidopsis and rice genes (Serikawa *et al.*, 2008).

Here we propose a common evolutionary genetic history that gave rise to both barley and Arabidopsis clock genes from a common ancestor (Figure 6.1). This hypothesis is based on experiments described in Chapters 3–5 (robust *in silico* searches, phylogenetic analysis, gene expression studies under light/dark, LL, and different temperature conditions and AS research of different barley clock genes), combined with careful comparisons with related investigations published elsewhere. Homologues of the core clock components *LHY*, *TOC1*, *PRR7* (*PRR37* in monocots), *PRR9/5*, *GI*, *LUX*, *ELF3*, *FKF1* and *ZTL* and the clock-related genes *ELF4-like3*, *COL1*, *FT* and *GRP7* were present in the common ancestor of monocots and dicots. Therefore, about 60% of barley clock genes are true orthologues of the Arabidopsis clock genes. *TOC1*, *FKF1*, *LUX* and *GI* remain as a single copy for most monocots and dicots. One exception is *AtBOA*, a duplicated copy of *AtLUX*. Of the core Arabidopsis clock genes, *CCA1*, *CHE* and *ELF4* are absent in barley. *ELF4*, in particular, was present in the ancestor but has been lost in monocots. This particular observation raises the question of a similar situation occurring in dicots, i.e. a clock component present in monocots that does not exist in dicots. In this case, our analysis would not have identified such components because it depends on previous knowledge from Arabidopsis. Therefore, it is important to bear in mind that, although the approach carried out here is systematic,

additional efforts (e.g. forward genetics) are necessary for comprehensive identification of all circadian clock components in barley.

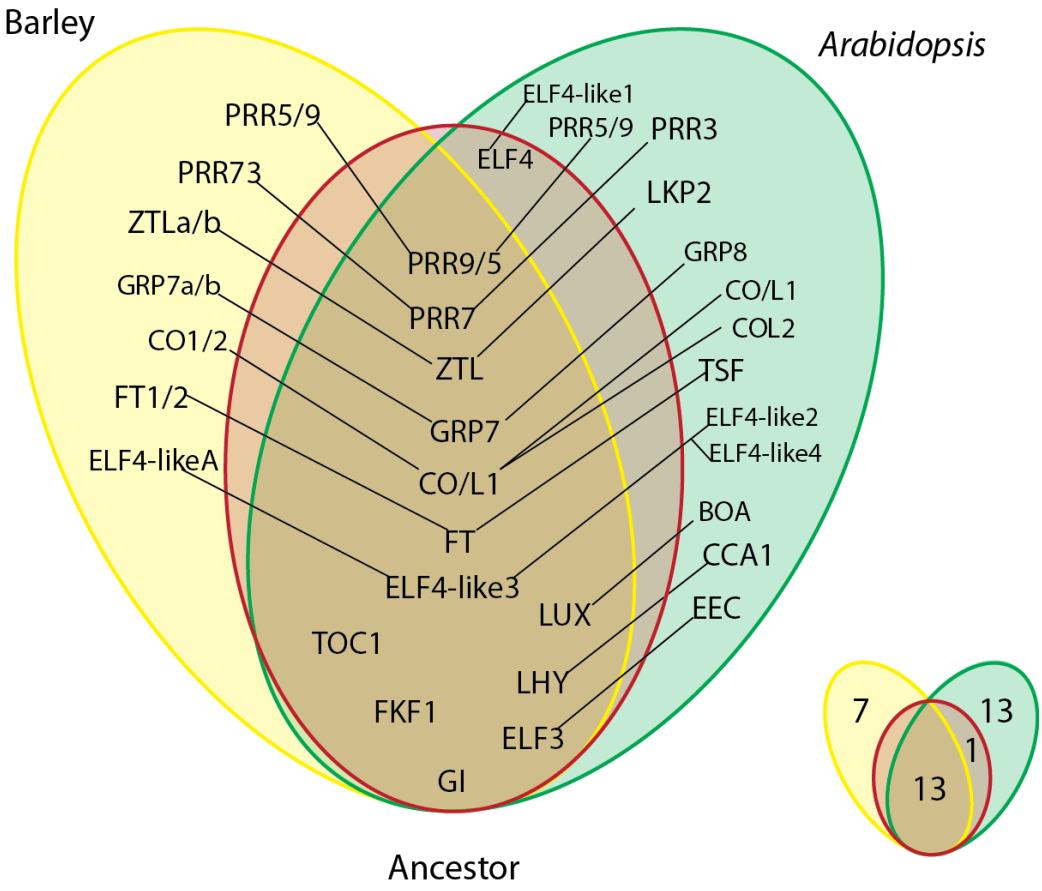


Figure 6.1: Schematic diagram of the proposed evolutionary history of circadian clock components of barley (yellow background), Arabidopsis (green background) and their putative common ancestor (red background). Independent duplication events are represented by fine diagonal lines. Diagram at the bottom right is related to the main diagram and it refers to the numbers of genes from each group.

An additional interesting observation relating to clock genes in angiosperms is the occurrence of multiple independent gene duplication events in both monocot and dicot ancestors, generating paralogues. Paralogues could be functionally equivalent to missing genes (e.g. *ELF4-likeA* commented in Section 4.4.3.4) or deviate in terms of func-

tion/regulation. *ELF3*, *ELF4-like3*, *FT*, *CO/COL1*, *GRP7*, *ZTL*, *PRR7* and *PRR9/5* were independently duplicated and maintained in both monocots and dicots, which is an interesting example of convergent evolution. One exception of such maintenance which is worth mentioning is that the ancestor of Pooideae species (*Brachypodium* and Triticeae, Chapter 3) lost the duplicated copy of *ELF3*. Convergent evolution also interfered with our phylogenetic analysis and the detection of one-to-one gene homology. For example it remains unknown which monocot gene, *PRR95* or *PRR59*, is the monocot orthologue of *PRR9/5* and vice-versa.

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

poral regulation/anticipation of output pathways (Tauber *et al.*, 2004), which has contributed to adaptation of plants to different environments.

6.2 – The framework of plant circadian clocks

Circadian clock components and their function between monocots and dicots started to diverge around 140 million years ago (Chaw *et al.*, 2004; Moore *et al.*, 2007). Additionally, certain species such as crops have been under 'artificial' selection during plant domestication and breeding, which raises the question of whether crops have evolved clock systems similar to Arabidopsis and how critical their circadian clock component are for cultivation. As mentioned above, homologues of most Arabidopsis clock genes exist in monocots, although not all components are present, for example, *CCA1*. Recent studies of the function of some clock genes in monocots have provided us with some clues to help understand the general roles of plant circadian clocks (Song *et al.*, 2010).

The circadian clock is so fundamental to most organisms that disruptions in clock rhythm can cause major disadvantages, such as cancer and reduced fitness (Dodd *et al.*, 2005; Dubrovsky *et al.*, 2010; Maury *et al.*, 2010; Shi *et al.*, 2013). In contrast, mutations in clock genes have been selected for during plant domestication and breeding because of the phenotype which they generate. For example, mutations in barley *PPDH1* and *ELF3* affect flowering time (Chapter 4) and have extended the geographic range where barley is grown (Turner *et al.*, 2005; Zakhrabekova *et al.*, 2012). Winter barley plants, the ancestral phenotype, require vernalisation and show strong promotion of flowering in response to LDs. In contrast, spring barley plants (*ppdh1* reduced function) lack vernalisation requirements and respond less to photoperiod, taking longer to flower under long-day condition (LD), which is an advantage in long growing seasons, as in Western Europe and much of North America. Such reduced response to photoperiod allows spring-sown plants to extend the period

of vegetative growth and produce more biomass, and consequently, higher yields (Turner *et al.*, 2005). In another example, loss of *HvELF3* function disrupts the barley clock but provides advantages in other circumstances. Northern environments have short growing seasons, and plants with early flowering behaviour, such as *Hvelf3* plants, are better adapted to these conditions. Additionally, these environments have long day lengths, which can exceed 20 h light in one day. *Hvelf3* plants have reduced gating of certain physiological processes, such as the expression of *HvCABa* (Chapter 4), which could potentially increase light harvesting and photosynthesis and increase biomass accumulation under very long days. Therefore, these analyses of barley *elf3* mutants show that in some circumstances, there are benefits from the severe loss of circadian clock function (Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012). This idea is corroborated by a parallel study on reindeer (*Rangifer tarandus*) from Scandinavia, which do not show circadian organization of behaviour under constant conditions (van Oort *et al.*, 2005). This 'arrhythmic' behaviour was caused by reduced rhythmicity of two key clock genes (*Bma1* and *Per2*) (Lu *et al.*, 2010b). Such results lead to the conclusion that both plants and animals from high latitudes are better adapted by reducing their dependency on the rhythms of the circadian clock. This raises the question of what other circadian clock mutations can improve crop yield even further before plant performance is compromised? This will be an interesting area for future research (Trevaskis, 2012).

Additional studies suggest that monocot crops have evolved clock systems that are different from Arabidopsis in some respects. For example, *PRR7* has a moderate contribution to the determination of flowering time in Arabidopsis and was not significantly associated with flowering time in natural populations (Ehrenreich *et al.*, 2009; Brachi *et al.*, 2010). In contrast, the monocot orthologue *PRR37* is the major determinant of photoperiodic flowering (Turner *et al.*, 2005; Murphy *et al.*, 2011). Additionally, *GI* knockdowns in

L. gibba almost completely abolish the circadian rhythm, whereas Arabidopsis *gi* loss-of-function mutants have a much milder effect on the clock. Also, activation of expression of homologous *FT-like* genes by day length (photoperiodic response) is reversed in long-day plants compared with short-day plants (Andrés and Coupland, 2012). Therefore, some homologues of Arabidopsis clock-related genes have (slightly) distinct roles in monocots.

Nevertheless, most clock orthologues identified in monocots and dicots have a strong similarity in circadian behaviour, also evidenced by the work carried out here. Analysis of circadian behaviour using the HR RT-PCR approach suggests that the circadian expression profiles of barley clock-related genes are largely similar to those of their Arabidopsis counterparts. Similar conclusions were obtained with expression analysis in rice (Murakami *et al.*, 2007). Furthermore, in their work, heterologous overexpression of both *OsTOC1* and *OsZTL* in Arabidopsis caused a similar arrhythmic clock phenotype to the overexpression of *AtTOC1* and *AtZTL*, respectively (Murakami *et al.*, 2007). Moreover, *OsPRR1* and *OsPRR37* rescue the clock phenotypes of *toc1* and *prr7* mutants in Arabidopsis, respectively (Murakami *et al.*, 2007). Even the protein regulation of LHY is likely conserved in rice, as OsLHY protein also accumulates under light (Ogiso *et al.*, 2010). Similar heterologous transfection analysis in *L. gibba* with overexpression and RNAi constructs of *LHY*s and *ELF3* revealed that these genes have functions similar to their Arabidopsis counterparts (Serikawa *et al.*, 2008). Additionally, the expression profiles of *CO* homologues are similar in dicots and monocots, including barley (Chapter 4), suggesting that the molecular mechanisms of transcriptional regulation of homologous *CO*s may also be highly conserved among plants (Song *et al.*, 2010). Thus, the results obtained from studies using several monocots suggest that both dicots and monocots possess, not surprisingly, molecular circadian clocks with many similarities.

An interesting question will be whether and how well the current Arabidopsis clock computational model will be applicable to barley. Data like that generated here will be important in addressing this. As yet, there is no computational model for the barley clock, but in an attempt to pull our observations together, we propose a framework of the barley clock (Figure 6.2). This hypothesis is superimposed on the knowledge of the Arabidopsis circadian clock and is based on experiments described in Chapters 3–5 combined with careful comparisons with related investigations published elsewhere. In summary, our current understanding of the circadian clock suggests considerable conservation in many plant species. However, the function of clock genes and the molecular architecture of clocks in barley and other plants other than Arabidopsis are just beginning to be understood. Further work is needed to truly unravel the molecular clock systems in all plants.

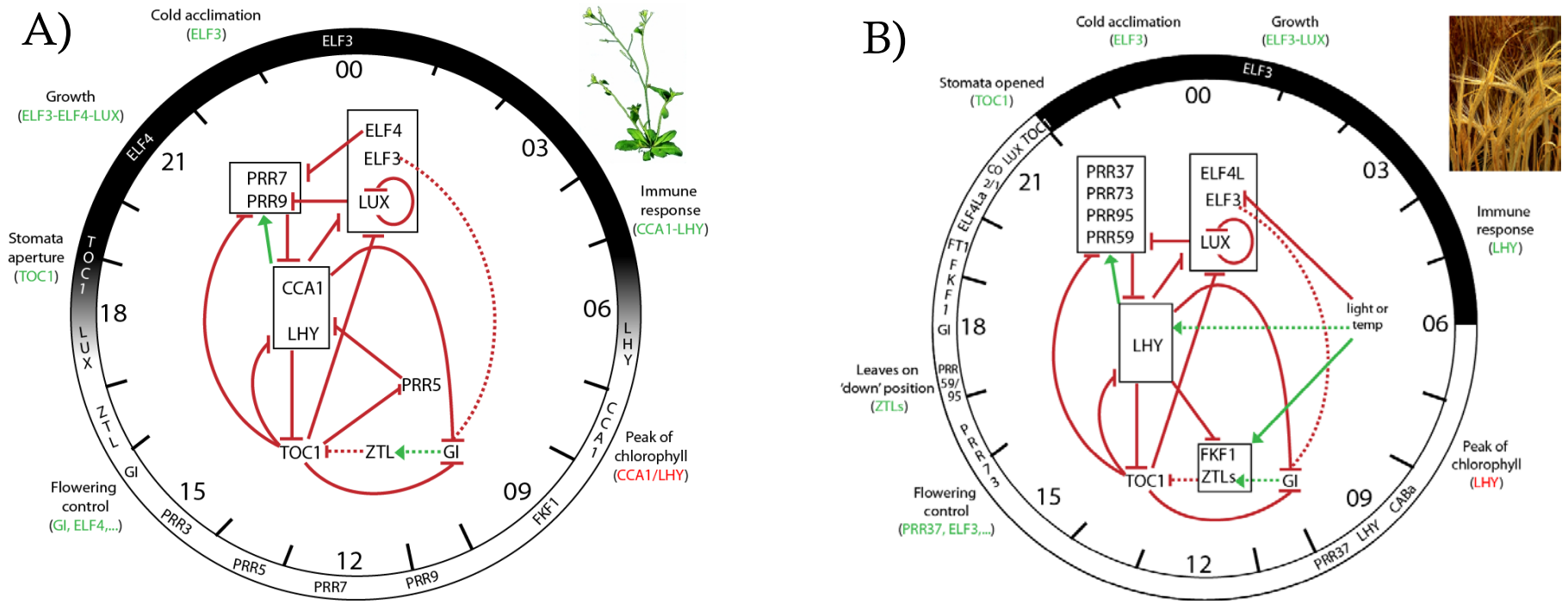


Figure 6.2: Schematic diagram of the 24-hour clock in A) Arabidopsis (12 h light and 12 h dark, natural conditions) and B) barley (LDs). Feedback loops of the core clock genes are represented in the centre. Full lines represent transcriptional feedback loops, whereas dashed lines represent post-translational regulation. Green lines are for activation, while red lines are for repression. For simplicity, the AtPRR3 and AtFKF1 components were not included in the Arabidopsis regulatory network. Expression peaks of clock genes are represented at different times of the day (information taken from Nakamichi, 2011, for Arabidopsis and Chapter 4 for barley). Several physiological processes, indicated on the periphery of the clock, are regulated by clock genes (in brackets), allowing them to occur at the correct time of day.

Phenotypic studies and genetic analyses of circadian behaviour and cold responses of plant clock genes, including the work described here, were mostly carried out under controlled environmental conditions in growth chambers. However, plants have evolved to survive in habitats with more complex environmental conditions. In nature, for example, there is constant variation in temperature and day length, gradual light changes between day and night, as well as differences in light intensity and humidity over a day, not to mention biotic and abiotic stresses. Therefore, the artificial conditions used in most laboratory experiments provide a less authentic interpretation of the adaptive significance of the results and are likely to produce data incongruent with those of natural environments. For example, field experiments carried out with Arabidopsis mutants impaired in different signalling pathways demonstrated that their flowering time behaved differently from expected under laboratory conditions, mostly because it depended strongly on germination time (Donohue *et al.*, 2005; Wilczek *et al.*, 2009; Andrés and Coupland, 2012). Furthermore, analyses of nearly 20,000 Arabidopsis plants in such field conditions using a genome-wide association (GWA) study combined with traditional linkage mapping detected the genetic bases underlying natural variation in flowering time. This study suggested that *TWIN SISTER OF FT* (*TSF*) and *COL1* contribute more strongly to flowering time variation than could be predicted from experiments under greenhouse conditions (Brachi *et al.*, 2010). More recently, clock studies in *Drosophila* also revealed that several key laboratory-based assumptions about *Drosophila* circadian behaviour are not supported by natural observations. Under laboratory light/dark conditions, flies show crepuscular locomotor activity in anticipation to light on and off signals (Helfrich-Förster, 2001). This bimodal activity has helped define the morning (M) and evening (E) oscillators in the *Drosophila* brain (Stoleru *et al.*, 2004a). However, analyses of fly locomotor rhythms in the wild have revealed they are diurnal rather than crepuscular and pointed to a third major locomotor component in

addition to M and E, termed 'A' (afternoon) (Vanin *et al.*, 2012). This A locomotor component is not observed in artificial laboratory conditions and characterises an afternoon 'siesta'. This 'siesta' was associated with temperature-sensitive splicing of the *per* 3' UTR (mentioned in Section 1.6.4). In natural conditions, however, levels of *per* 3' UTR splicing have no significant association to locomotor behaviour or time of the day, arguing against a significant role for *per* 3' UTR splicing in this natural phenotype (Vanin *et al.*, 2012). Interestingly, these natural conditions phenotypes can be observed in the laboratory by using realistic temperature and light cycle simulations. Therefore, results of phenotypic and genetic studies of barley clock genes, including those obtained here, should be confirmed under simulated natural conditions or field conditions in order to clarify the underlying molecular functions of the plant clock under natural states.

6.3 – Why temperature-dependent alternative splicing (AS) of plant clock genes?

As sessile organisms, plants depend on the perception and appropriate response to external cues, both for their survival and for the regulation of key developmental events. The ability to cope with extreme temperature conditions is particularly interesting in light of the global climate change that is to affect both agricultural and non-agricultural plant communities (Thuiller *et al.*, 2005; McClung and Davis, 2010). However, there is little knowledge on the mechanisms of how plants sense and adapt to temperature variations. There is no doubt that plants rely on temperature to alter metabolic rates but they also actively respond to it (Penfield, 2008). Additionally, predicting changes in temperature provides increased fitness to plants, and the circadian clock is required for this process (McClung and Davis, 2010). Several sources of evidence confirm that a mutual interaction between temperature and the clock are important. For example, the clock is involved in

gating the low-temperature (LT) induction of downstream 'cold-response' genes, thus conferring rhythmic cold responses (Fowler *et al.*, 2005). Furthermore, it has been shown that *GI* mRNAs expression is elevated at low temperatures, and that *gi* mutants are defective in freezing tolerance (Cao *et al.*, 2005a). Studies in barley suggest that the expression of LT-induced genes is optimised under SD conditions through regulation by the photoperiod pathway (Fowler *et al.*, 2001; Mahfoozi *et al.*, 2006). Two additional examples of essential temperature and clock interactions are temperature entrainment (resetting the clock to temperature changes) and temperature compensation (ensuring robust clock rhythmicity) (Section 5.1.2). A key aspect common to all situations mentioned above is that the temperature must be perceived by the clock, but the identity of this initial mechanism, also known as the plant thermometer, remains unknown. It is likely that there are several plant thermometers transducing temperature signals to the circadian clock. Calcium oscillations, as well as phytochromes themselves, may integrate temperature and circadian information (Hotta *et al.*, 2007; Penfield, 2008). Increasing evidence suggests that temperature-associated AS is functionally important in the clock (Chapter 5 and James *et al.*, 2012a). Whether AS is another putative plant thermometer, or is downstream of it, is a subject for further research. However, given the increasing association of AS to abiotic responses (Kazan, 2003; Iida *et al.*, 2004; Duque, 2011; James *et al.*, 2012b; Mastrangelo *et al.*, 2012), it is likely that temperature-dependent AS is integrating temperature sensing with downstream processes (James *et al.*, 2012b). In that event, AS in the clock may help to synchronise temperature cues with downstream processes in both temperature entrainment and temperature compensation mechanisms (James *et al.*, 2012a; James *et al.*, 2012b).

AS is the major mechanism generating structural and functional diversity of gene products in higher eukaryotes. In plants, AS promotes plant genome plasticity and versatility through regulation of the expression of key multi-exon genes, such as clock genes

(James *et al.*, 2012a; Mastrangelo *et al.*, 2012). The functional meaning of AS in response to environmental changes has started to be defined for many clock genes. AS of clock genes mostly modulates the levels of functional and translatable mRNAs (e.g. reduced *LHY* levels upon transition to cold observed here and by James *et al.*, 2012a). Additionally, here we report AS events increasing protein-coding capacity of the core clock gene *PPDH1* (Chapter 5). It is noteworthy that it is not clear if splicing variants occur in the same cell or tissue types, and further work is necessary to clarify this issue. Also, although many of the temperature-dependent AS events in clock genes have been analysed at extreme temperatures, changes as small as 4°C also have a significant effect on a range of AS events in Arabidopsis (James *et al.*, 2012a; Staiger and Brown, 2013). Plants can experience a multitude of temperature changes in 24 h period. We know that AS of clock genes can respond dynamically (and with different dynamics) but do not know how multiple cues are integrated into clock rhythm and downstream processes. Some AS regulation in core clock genes is conserved between barley and Arabidopsis. Nevertheless, non-conserved events indicate that AS likely represents an evolutionary strategy that rapidly increases genome plasticity and develops new clock gene functions, in addition to other mechanisms like gene duplication (Mastrangelo *et al.*, 2012).

The changes in clock AS may reflect features of the pre-mRNAs themselves (e.g. secondary structure) or temperature-dependent changes in levels and activity of *trans*-acting splicing factors or other factors (James *et al.*, 2012a). Our knowledge of the splicing factors that control alternative splicing of circadian clock genes in a temperature-sensitive manner is very poor and only a few studies have uncovered such links. Wang *et al.* (2012) suggest the involvement of a splicing factor, named SKIP, in regulating the alternative splicing of circadian clock genes in a temperature-sensitive manner. Additionally, the *PRMT5* and *STIPL1* genes in Arabidopsis are involved in the correct splicing of *PRR9* (Sanchez *et al.*,

2010; Jones *et al.*, 2012), and examination of all PRMT5 and STIPL1 targets will be needed to assess whether they modulate any other AS events found in plant clocks or whether they are involved in the modulation of AS during temperature changes. Knowledge of splicing factors and their binding sites would be invaluable in deciphering the regulation of LT AS and allow an assessment of variation in AS in different lines.

AS may fine-tune expression of genes in response to changing environmental cues. Splicing and AS depends on sequence elements in the pre-mRNA and the interaction of *trans*-acting factors which bind to these *cis*-elements to define where the spliceosome assembles. Mutation and natural variation in gene sequences can affect splice site choice or efficiency of splicing providing expression variation which can affect fitness. The widespread nature of AS and the effect that subtle sequence changes can have in gene expression illustrates how AS is likely to have contributed significantly to plant adaptation. A future challenge will be to link genomic SNPs to integrated transcription/AS data or protein isoform production to phenotypic variation. Quantitative RNA-seq combined with genome-wide association mapping may identify key functional AS variation. The identification of clock gene regulation through AS in plants, combined with future experiments in natural conditions, may be used as candidates for plant improvement, which can be achieved by, for example, selecting genotypes with superior performance under adverse environmental conditions.

6.4 – Conclusions

- Genomic sequences of 21 putative barley homologues of Arabidopsis core circadian clock genes and selected associated genes were identified. 60% of barley clock genes are true orthologues of Arabidopsis clock genes. Paralogues were generated by multiple independent gene duplication events in both monocot and dicot ancestors.

- Circadian behaviour under light/dark and free-running conditions suggests that the circadian expression profiles of the putative barley clock-related genes are largely similar to those of other plants.
- Allelic differences in the barley *PPDH1* and *ELF3* clock genes affect plant responses to photoperiodic flowering and, consequently, biomass and yield. The *ppdh1* allele delays flowering under LD. In contrast, the *Hvelf3/ppdh1* double mutant is insensitive to photoperiod and plants flower considerably earlier under SD and LD.
- Mutations in barley clock genes *PPDH1* and *ELF3* affect the expression of other clock-related genes under light/dark and free-running conditions; this effect was also observed with mutations in homologues in other plant species. The *elf3* loss-of-function mutation, in particular, has severe effects on rhythmicity and expression levels of other clock-related genes.
- More than 55 different AS events were detected for a set of eleven barley clock genes. This initial identification of AS events allowed the establishment of an RT-PCR panel for studies of barley clock and clock-associated genes.
- AS plays a significant role in the responses of the *HvLHY* and *HvPPDH1* to changes in temperature and is thus an additional mechanism involved in the operation and regulation of the barley clock.

6.5 – Future work

The work carried out here has raised many important questions and new ideas. There are also some questions that were not fully answered during the work and these need further investigation. For example, it would be interesting to assess the AS profile of additional clock genes (e.g. *HvTOC1*, *HvPRR95* and *HvPRR59*) under LT conditions. Below are more key questions and further experiments:

- To identify the functional differences between the putative HvPPDH1 and Hvppdh1 proteins, including the causal *ppdh1* 'reduced-function' mutation, that affects photoperiod response, it will be necessary to carry out biochemical analysis and complementation tests. It will be also interesting to investigate the relative functions of the abundant in-frame isoforms of PPDH1 (reported in Section 5.3.2.2).
- It is important to determine whether PTC-containing alternative clock transcripts in barley are targeted for NMD. In the absence of barley *upf* mutants, it will be necessary to perform alternative experiments that prevent mRNA decay through the NMD pathway, such as cycloheximide (CHX) treatments. We are currently carrying out experiments to study the levels of *HvLHY* transcripts under LT conditions in CHX treated leaves.
- An important aspect of the analysis of AS in AtLHY was the demonstration that the LHY promoter strength was not affected by a night at 4 °C such that the decrease in AtLHY transcripts is most likely due to AS/NMD. Production of promoter::luciferase transgenics of barley clock and output genes would be very useful in clock and AS studies.
- Plants with both *PPDH1* and *ppdh1* alleles have identical *GI* mRNA levels but nothing is known about GI protein levels, which could affect expression of the *CO* genes. Furthermore, in the absence of antibodies for all of the barley clock proteins, we are currently only able to extrapolate effects on protein levels from the relative abundance of the AS isoforms. Therefore, it would be important to carry out analysis of barley clock genes at the protein level. In fact, I am currently performing Western blot analysis to detect HvLHY levels in LT transition and acclimation conditions. This will reveal whether AS in *HvLHY* contributes to further decrease in protein levels. However, improving proteomics approaches may be one way to address dynamic expression of clock genes.

- The Nordgen gene bank contains more than 1000 uncharacterised earliness (early flowering) barley mutants. We would predict that some of these mutants are in other clock genes. With our current knowledge of the genomic sequence of 21 barley clock-related genes, we could characterise those early mutants simply by re-sequencing their 21 clock-related genes. This will identify which genes contain mutations in the lines and will identify allelic variants for each gene. Additionally, expression data of clock genes in different mutants could ultimately be integrated to generate a mathematical model for the barley clock.
- Circadian behaviour and cold-dependent AS studies of barley clock genes should be carried out under field conditions in order to confirm the adaptive value of the results obtained here. Additionally, to further contribute to our understanding of such adaptation, it would be interesting to carry out expression and AS analyses of barley clock genes (using the HR RT-PCR developed here) in locally adapted landraces and cultivars from across geographical clines. This will allow the establishment of a relationship between variation in expression and AS of clock genes and different barley genotypes. Ultimately, this information will allow us to determine which combinations of different clock alleles contribute to improved niche adaptation to specific conditions.
- The work carried out here can be easily translated to wheat. Therefore, we have facilitated putative further AS analyses in polyploid wheat, where there are homeologous gene copies under differential control.

Chapter 7. References

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Appendix A. Material and Methods

A1 - Cereal Compost

225 L	Levingtons M2
225 L	Perlite
0.5 kg	Osmocote mini
2.5 kg	Celcote

Levingtons M2 is a medium grade and strength peat mixture. Perlite is used as a soil amendment because it prevents soil compaction. Osmocote mini contains fertiliser (16% N + 8% P_2O_5 + 11% K_2O) and micronutrients (2% MgO, 0.02% B, 0.05% Cu, 0.4%Fe, 0.06% Mn, 0.02% Mo and 0.015% Zu). Celcote is a compost additive that increases water holding capacity.

A2 - Diethyl pyro carbonate (DEPC)-treated solutions

To treat solutions with DEPC, 0.1% DEPC (Sigma-Aldrich, UK) was added and mixed. Solutions were incubated at room temperature overnight. On the following day, solutions were autoclaved (high pressure steam at 121°C for 30 min).

A3 - Stock solutions

For pH measurements, a Kent EIL 7020 pH meter was used. After preparation, all solutions were autoclaved and stored at room temperature.

A3.1 3 M Sodium acetate (NaOAc) pH 5.5

40.82 g	NaOAc (AnalaR NORMAPUR®, VWR International, Belgium)
50 mL	Sterile distilled water (SDW)

Compounds were dissolved and pH was adjusted to 5.5 using glacial acetic acid (>10 mL). SDW was added to a final volume of 100 mL.

A3.2 1 M Tris-HCl pH 7.5

121.14 g	Tris (Bio-Rad Laboratories, USA)
600 mL	SDW

Compounds were dissolved and pH was adjusted to 7.5 using 2.5 N HCl. Final volume was adjusted to 1 L with SDW.

A3.3 5 M NaCl

29.22 g	NaCl (AnalaR NORMAPUR®, VWR International, Belgium)
100 mL	SDW

Compounds were mixed by stirring until salt was dissolved.

A3.4 500 mM Ethylenediaminetetraacetic acid (EDTA) pH 8.0

186.1 g Na_2.EDTA.$2H_2O$ (Sigma-Aldrich, UK)

600 mL SDW

Compounds were dissolved and pH was adjusted to 8.0 using NaOH pellets (AnalaR NORMAPUR®, VWR International, Belgium). Final volume was adjusted to 1 L with SDW.

A3.5 10 % Sodium dodecyl sulphate (SDS)

50 g SDS (Sigma-Aldrich, UK)

400 mL SDW

Compounds were dissolved by stirring on a magnetic heating block (Magnetic Stirrer SM1, Stuart Scientific, UK) at 70 °C until SDS was dissolved. Final volume was adjusted to 500 mL with SDW.

A3.6 Tris-Borate-EDTA (TBE) Buffer (10X)

108 g	Tris
55.0 g	Orthoboric acid (AnalaR NORMAPUR®, Belgium)
40 mL	0.5 M EDTA (pH 8.0)
900 mL	SDW

Compounds were mixed until salts were dissolved. Final volume was adjusted to 1 L with SDW. All electrophoresis gels were stained with ethidium bromide (0.5 µg/mL final concentration) and observed under a UV transilluminator (G:Box, Syngene, UK).

A4 - gDNA extraction buffer

40 mL	1 M Tris-HCl pH 7.5
10 mL	5 M NaCl
10 mL	500 mM EDTA pH 8
10 mL	10 % SDS

Mix all solutions and make up to 200 mL using SDW.

A5 - Gene-specific primers used in clock and AS analyses

Table A5.1: Gene-specific primers of barley reference genes used in clock and AS analyses and their sequences.

Genes	Primer name	Sequence (5' → 3')
PP2AA2	HvPP2AA2-Ex2Fw	CCGCATTGGTGCACAGATGAAAGA
	HvPP2AA2-Ex3Rv	CCAAGATTTGATGCAGCTGCCCTT
UBC21	HvUBC-Ex3Fw	AGTACAAGGAGGTGCAGCGAGAAA
	HvUBC-Ex4Rv	CTGCTCGGGAATTGAGAATGCAAG

Table A5.2: List of barley clock gene-specific primers used in clock and AS analyses and their sequences.

Genes	Primer name	Sequence (5' → 3')
LHY	HvLHY-Ex3Fw	GCAAAGTACTGGAATTTCTGAGCCC
	HvLHY-Ex8Fw	CGTGCAAATCAGAAGCCATGCTCA
	LHY-Ex10FwC	GCTAACGACAAGACACAGCAAGCA
	HvLHY-Ex6Rv	GTGCTGACAACTGGTCGCTTTGTT
	LHY-Ex9Rv	TTGGAACTTCTCTGGTGGGTGTCT
	HvLHY-Ex8aRv	AGGACAAGCAGCAAAGAACAGAA
	LHY-3UTRv	GGGCTCAGAAATTCCAGTACTTTGC
TOC1	HvPRR1-5UTRFwB	GCCGGACTGCGACGGGCGG
	HvPRR1-Ex1Fw	ATGTGCTTCGCCTCCTCTGTAACT
	HvPRR1-Ex6Fw	CAAGCACTCCAGCAAACAGCTCAT
	HvPRR1-Ex1Rv	AGTTACAGAGGAGGCGAAGCACAT
	HvPRR1-Ex3Rv	CCGCAAGCACTTGACAACAACAGA
	HvPRR1-Ex6Rv	CAATGAGCTGTTTGCTGGAGTGCT
	HvPRR1-3UTRv	GGGTTGAGGATGTATTGTGTGAGC
ZTLb	HvZTLb-Ex2RvA	TTGGAGTTGGTCGGAGTGTG
ELF3	HvELF3-Ex4Fw	AGGAAACCAGGCTTCACCATCTCA
	HvELF3UTRv	TTTCTTTCTTTCTGCCGGCCAACC
PP2AA2	HvPP2AA2-Ex2Fw	CCGCATTGGTGCACAGATGAAAGA
	HvPP2AA2-Ex3Rv	CCAAGATTTGATGCAGCTGCCCTT
UBC21	HvUBC-Ex3Fw	AGTACAAGGAGGTGCAGCGAGAAA
	HvUBC-Ex4Rv	CTGCTCGGGAATTGAGAATGCAAG
PPDH1	HvPpdH1-5UTRfw	TTCCAGGAGACGATTCATTCCGCT
	HvPpdH1-Ex4Fw	CAACAGCAATGATCGCAACGACGA
	HvPpdH1-Ex6fwB	TGCCATATCTTGAGCTGAGCCTGA
	HvPpdH1-Ex7Fw	TCCAGTGTTGTCAATCCTTCGGGT
	HvPpdH1-Ex1Rv	GGATGGTCTTCCTGTGTAGGAACT
	HvPpdH1-Ex6Rv	AAGTCAGCAGCTCGAACAATTGGC
	HvPpdH1-Ex7Rv	ACCCGAAGGATTGACAACACTGGA
	HvPpdH1-3UTRv	TATAGCTAGGTGCGTGGCGGGA
GI	HvGI-5UTRFw	TTCGCCTCTGCCTCAGCCTC
	HvGI-Ex7Fw	ACACCTCTGACCAGGCTACAACAA
	HvGI-Ex11Fw	ACTGCAATGTCTATGAGGGCTGGT
	HvGI-Ex12FwB	AACAGCACCAGCTCATCGGGTAAT
	HvGI-Ex4Rv	CGCTGTCAGATGTGAACTGACCAA
	HvGI-Ex7Rv	TTGTTGTAGCCTGGTCAGAGGTGT
	HvGI-Ex11Rv	AGACATTGCAGTTCCAATCGCACG
	HvGI-Ex12RvB	ATTACCCGATGAGCTGGTGCTGTT
	HvGI-Ex14Rv	ACAAGCATCCCATCTGTAGCACGA
	HvGI-3UTRv	AGACTGGGAATGGAAAGCCATCCT
PRR59	HvPRR59-Ex3Fw	CACGGTGCTAAAGTGCATGCAGAA
	HvPRR59-Ex5Fw	TAATGTGGCAAGCTCACCCAGAGA
	HvPRR59-Ex5RvB	TCTCTGGGTGAGCTTGCCACATTA
	HvPRR59-Ex6Rv	ACGTTGCTGCTTCCCTAAGTTGAC
	HvPRR59-Ex7RvB	CAAAGGAGCCTGAGCTTGCATTGT
PRR95	HvPRR95-5UTRFw	TGGTGGCTACTTGATCTGCGAGT
	HvPRR95-Ex4Fw	AAGAGAATGTCGCAGAACAGCACG
	HvPRR95-Ex4Rv	CGTGCTGTTCTGCGACATTCTCTT
	HvPRR95-Ex6Rv	GAGCCGCCCAATTTCCATTCTTGT
PRR73	HvPRR73-5UTRFw	AAACATCCACGGCTACTTTCACGC
	HvPRR73-Ex5Fw	AGTGTTCCAAACCAAAGACTGGTG
	HvPRR73-Ex7Fw	ACACAGAATCGGAACAGGCTGCTA
	HvPRR73-Ex8Fw	TCCAGCATCACCATCATGGACACT
	HvPRR73-Ex3Rv	CAAGGCATGGCGACCTCAGTTAAT
	HvPRR73-Ex7Rv	TAGCAGCCTGTTCCGATTCTGTGT
	HvPRR73-Ex8RvB	TCCATCTTGCAGGTGGAGTTCGTT
	HvPRR73-3UTRv	TGGCTACTGGCTTGCTCCTTCTTA
FKF1	HvFKF1-Ex2Fw	ATTCCTGCTTGACTTGACGAA
	HvFKF1-Ex2RvB	TAACTCTACATGGGACGTCG

A6 - Glasshouse: environment and treatment

Supplementary lighting units were activated when light intensity was below 200 watts per square meter, which was provided by using Philips SGR 140 with 400w SONT agro bulbs. Shading, activated when light intensity was above 450 watts per square meter, was carried out with internal, horizontal Ludvig Svensson environmental screens. Temperature control was carried out by wall-mounted variable speed fans and heating was available from hot water piping. Overall cubicle environment was controlled by a Van Vliet Automation 'Synopta' computer system.

Soil containing growing barley plants was watered frequently (daily or every other day) until seeds were set and plants were left to dry. Plants grown for bulking seeds that were not being phenotyped or used in clock experiments received liquid feeding (1:1:1 N:P:K) every two weeks. Plants were equally sprayed with fungicides and insecticides, when needed, for disease control. Fungicides were Opus®Steam, Land Gold and Intercept, whereas insecticides were Conserve®SC and Savona Insecticidal Soap.

A7 - Experiments to detect novel AS events

A7.1 Growth conditions

To detect novel AS events, grains from three barley lines were germinated for 1 week in the dark at 4 °C on sterile water-soaked filter papers and transferred to medium square plastic pots (10 cm^3) filled with cereal compost (Appendix A1). Each pot contained 4 grains from the same line. Plants were grown in two different environments: glasshouse (20 °C with LD treatment) or growth cabinet (4 °C with SD treatment). No crop protection treatment was applied. Harvesting of plants was carried out at GS12 (Zadoks *et al.*, 1974) with 4 h intervals for a total of 24 hours. Sampling started at 1 hour after the start of the dark period (Figure A7.1). For collections under dark conditions, samples were quickly collected in low, indirect light. The second leaves of two independent plants, grown in different pots, was taken for each time point. Leaf material was pooled and rapidly frozen in liquid nitrogen and stored at -80 °C.

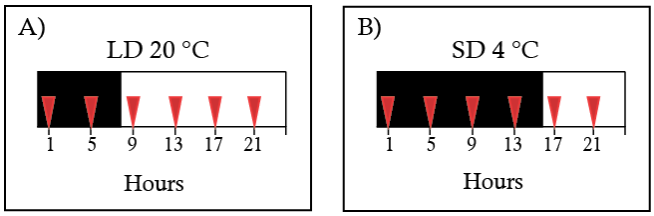

Figure A7.1: Sampling regime for AS discovery experiments. Red arrows represent time points when sampling occurred. Black boxes are for dark, white boxes are for light. Sampling started one hour after the dark period.

A7.2 PCR conditions

Each PCR was carried out in individual 0.2 mL polypropylene PCR tubes (Axygen®, Corning®, USA) and contained 3 µL cDNA template, 20 µM of each primer, 4 µL

of 5 × GoTaq® reaction buffer (Promega, USA), 0.2 µL GoTaq® DNA Polymerase (5 U/µL, Promega, USA), 1 µL dNTP (5 mM of each dNTP, Invitrogen, Life Technologies, USA) and SDW up to a final volume of 20 µL. The general PCR programme included an initial 2 min step at 94 °C, followed by 25 cycles of: 1) 15 sec at 94 °C; 2) 15 sec at 50 °C; and 3) 1 min and 30 sec at 72 °C. At the end of cycling, a final extension step of 10 min at 72 °C was performed. For all experiments with a primer pair, negative controls containing RNA template and one positive control containing gDNA were subjected to the same procedure to exclude any possible contamination or to detect PCR inhibitors. Once amplification of the required PCR product had been achieved, 10 µL of the final PCR product was run on a 1.5% agarose, 1 x TBE gel to detect bands of the expected (FS) and unexpected sizes (AS isoforms).

A8 - Luria-Bertani (LB) liquid broth and solid agar

10 g	Select peptone (Sigma-Aldrich, UK)
5 g	Select yeast extract (Sigma-Aldrich, UK)
10 g	NaCl
900 mL	SDW

All components were mixed. For solid LB agar, 15 g of Select agar (Sigma-Aldrich, UK) was added. pH was adjusted to 7.5 with NaOH pellets. Final volume was adjusted to 1 L with SDW and the solution was autoclaved and stored at room temperature.

A9 - Ampicillin

Stock solution (200 mg/mL): 1.0 g ampicillin (Sigma-Aldrich, UK) was dissolved in 5 mL SDW. After solubilisation, the antibiotic solution was filter-sterilised via a 0.2 µm membrane (Nalgene®, Thermo Fisher Scientific, USA) and stored in the freezer (-20 °C). In LB growth medium (100 µg/mL): 50 µL ampicillin (stock solution) was diluted in 100 mL LB medium (liquid broth or solid agar).

A10 - Blue-white screening

Colour screening of recombinant clones is possible due to a vector-based molecular cloning technique involving the enzyme β-galactosidase. In the presence of X-gal substrate and IPTG de-repressor, cells transformed with the empty vector grow into blue colonies. The blue colour is a visual indication that the α-peptide (present in the pGEM-T easy vector) of the β-galactosidase is expressed. Cells transformed with plasmids where the insert interrupts the coding sequence of the α-peptide produce white, and sometimes light-blue, colonies.

Chapter 8. Appendix B. Results

B1 - Barley clock mutants

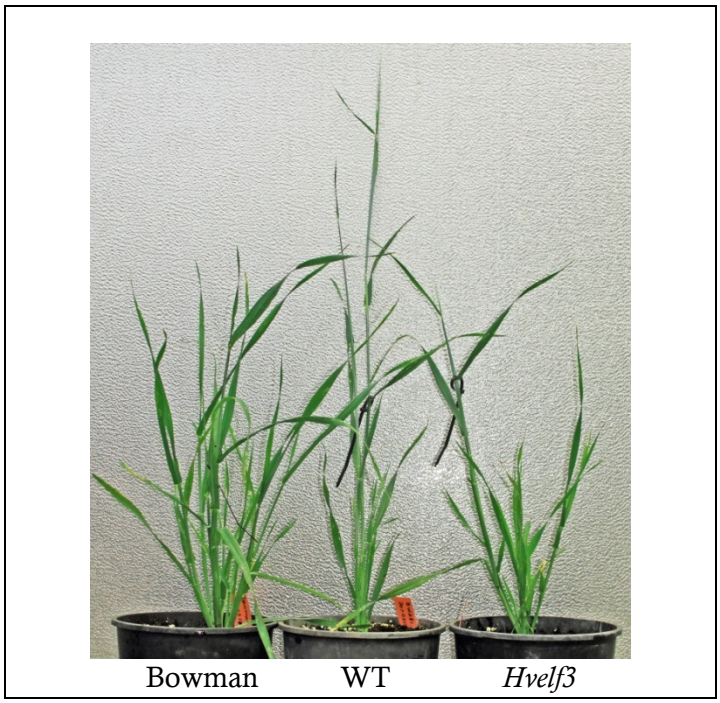

Figure B1: Bowman, WT and *Hvelf3* 28-day old plants grown in LD conditions (n=1).

B2 - Awn length of barley clock mutants

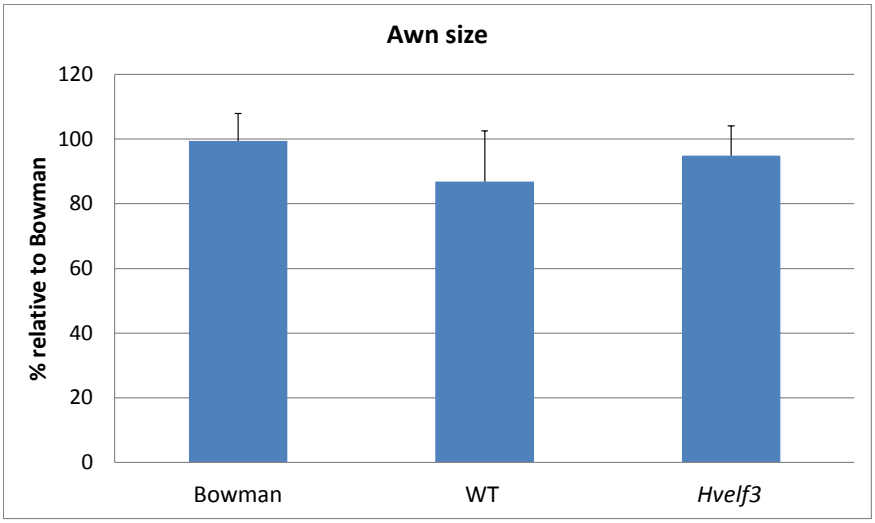

Figure B2: Awn sizes of Bowman, WT and *Hvelf3* plants in LDs. Plotted data represent mean values (n=4) and standard deviations (error bars) relative to Bowman. No significant differences between Bowman or WT or *Hvelf3* lines were observed.

B3 - Array design for clock experiments

Table B3: Random array design of barley samples grown for clock experiments. Randomisation was performed using Microsoft Excel 2000 and manual editing.

8w LD1 T6	1d LD1 T3	1d LL2 T7	1d LD2 T6	8w LD1 T5	1d LL3 T9	1d LD2 T2	1d LL3 T3	8w LD2 T0	1d LL1 T6	B LL3 T3	8w LL2 T3	8w LL3 T4	8w LL2 T4	B LD2 T2	8w LD2 T9	8w LL3 T3	B LD1 T2	B LD1 T6	8w LD2 T6	B LL3 T9	1d LL1 T3	B LD2 T4	1d LL3 T0	1d LD1 T6	8w LD1 T8	8w LD1 T6
8w LD2 T5	8w LD1 T0	8w LL1 T8	1d LD1 T9	B LL1 T9	1d LL2 T6	B LD2 T4	1d LD2 T3	8w LD2 T5	B LL1 T7	8w LL1 T3	B LD2 T7	8w LD1 T4	8w LL2 T7	B LL3 T0	B LL2 T6	B LD1 T0	8w LL2 T5	1d LL1 T4	B LL3 T6	8w LD1 T3	B LL1 T3	B LL3 T4	B LL2 T4	8w LL3 T6	1d LD2 T3	8w LL2 T5
1d LD1 T0	B LD2 T0	B LL2 T5	8w LD2 T3	B LL2 T4	8w LD1 T8	1d LL1 T2	B LL1 T0	1d LL1 T0	1d LL3 T0	1d LD1 T9	8w LL3 T5	B LD1 T9	1d LL1 T5	8w LL3 T9	B LL2 T5	B LL1 T5	1d LL2 T3	B LD2 T3	1d LL1 T9	1d LL3 T8	8w LL1 T9	8w LL2 T2	1d LL2 T4	B LD1 T7	8w LL1 T4	B LD1 T3
8w LD1 T3	B LD2 T7	1d LL2 T3	B LD2 T0	1d LL1 T9	B LL1 T2	1d LL3 T5	8w LD2 T0	B LL2 T8	8w LL3 T5	8w LL3 T7	B LL1 T6	1d LL1 T3	B LL1 T0	1d LL2 T5	B LD2 T9	1d LD1 T8	1d LL3 T8	1d LL2 T6	1d LD1 T0	1d LL1 T0	1d LD1 T4	B LD1 T7	1d LL2 T8	8w LL2 T0	1d LD2 T5	B LD2 T2
B LL2 T0	8w LD1 T2	1d LL2 T0	B LL3 T8	8w LD1 T0	1d LL2 T8	8w LL3 T6	B LD2 T8	1d LL2 T9	8w LL1 T6	8w LL3 T4	8w LL3 T8	B LL3 T8	8w LD2 T8	B LL2 T2	B LD2 T6	B LL1 T8	B LL2 T9	B LL1 T2	B LL3 T2	1d LD2 T2	1d LD2 T8	1d LL3 T4	8w LL1 T7	B LD1 T5	1d LL2 T2	8w LD1 T2

8w LL1 T8	1d LL1 T4	B LD1 T9	B LL2 T3	1d LD1 T4	B LL3 T9	8w LL3 T2	8w LL1 T0	1d LL3 T6	8w LL2 T8	B LL1 T5	8w LL2 T7	B LL1 T9	1d LL3 T5	B LD1 T8	8w LD1 T9	1d LL2 T2	8w LD1 T7	B LD2 T6	B LL1 T4	B LL3 T6	8w LL2 T9	B LL1 T8	1d LL1 T8	8w LL3 T2	1d LL2 T9	1d LD2 T0
B LL2 T7	B LL2 T2	1d LL2 T5	1d LL3 T2	1d LD2 T4	1d LD2 T5	1d LL3 T4	8w LD2 T8	8w LL3 T3	1d LL1 T6	8w LL1 T3	B LD2 T8	B LD2 T9	B LL3 T4	1d LD1 T8	1d LD1 T6	B LD2 T5	8w LL1 T9	8w LL1 T7	B LD1 T5	8w LL3 T0	8w LD2 T2	8w LL3 T0	B LL2 T8	1d LD1 T7	1d LD1 T5	8w LL2 T6
1d LL2 T4	8w LL1 T4	8w LD1 T7	1d LL3 T7	8w LD T4	B LD T0	B LD2 T3	1d LL T3	B LL3 T5	8w LL1 T6	B LL3 T7	8w LL3 T9	1d LD2 T6	8w LD1 T9	B LL2 T6	B LL3 T7	B LL3 T3	B LL2 T7	8w LL3 T8	1d LL3 T6	B LL1 T4	1d LD2 T4	1d LD1 T2	1d LL3 T2	B LL3 T5	B LD1 T3	1d LD2 T7
B LD2 T5	B LL1 T7	B LL1 T6	8w LD2 T7	1d LD1 T2	1d LD1 T3	1d LD2 T7	B LD1 T2	8w LL2 T4	1d LL2 T0	1d LD1 T7	1d LL1 T8	8w LD2 T7	B LL3 T2	8w LL3 T7	B LL1 T3	1d LL1 T7	8w LL2 T3	B LL2 T0	8w LD2 T2	8w LL1 T2	1d LD2 T9	8w LD2 T6	1d LD1 T5	B LL3 T0	1d LD2 T9	1d LL1 T7
1d LL1 T2	8w LL2 T6	B LD1 T4	B LL2 T3	8w LD T4	B LD1 T6	8w LL1 T2	8w LD2 T4	B LL2 T9	1d LL3 T9	8w LL2 T9	1d LL1 T5	B LD1 T8	B LD1 T4	8w LD2 T3	1d LD2 T0	8w LL1 T0	8w LD2 T9	8w LL1 T5	1d LD2 T8	8w LL2 T8	8w LD1 T5	1d LL3 T7	8w LL2 T0	8w LL2 T2	1d LL2 T7	8w LL1 T5

1d: eam1.d NIL; **8w**: eam8.w NIL; **B**: Bowman. LD: long day; LL: light-light. T: time point.

B4 - Barley plants from clock experiments

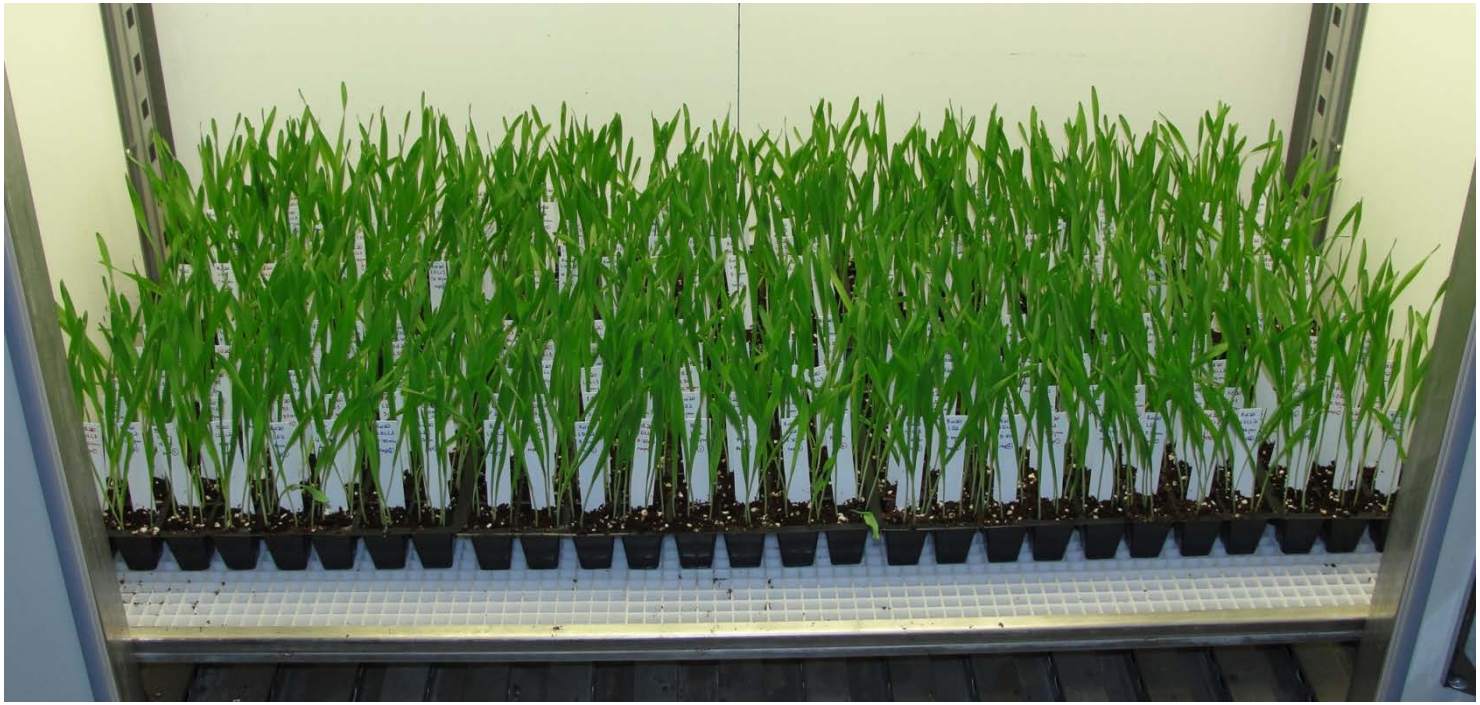

Figure B4: Barley plants at stage 11 used in clock experiments. Photograph was taken on day 1 of sampling (LD1).

B5 - Clock gene expression

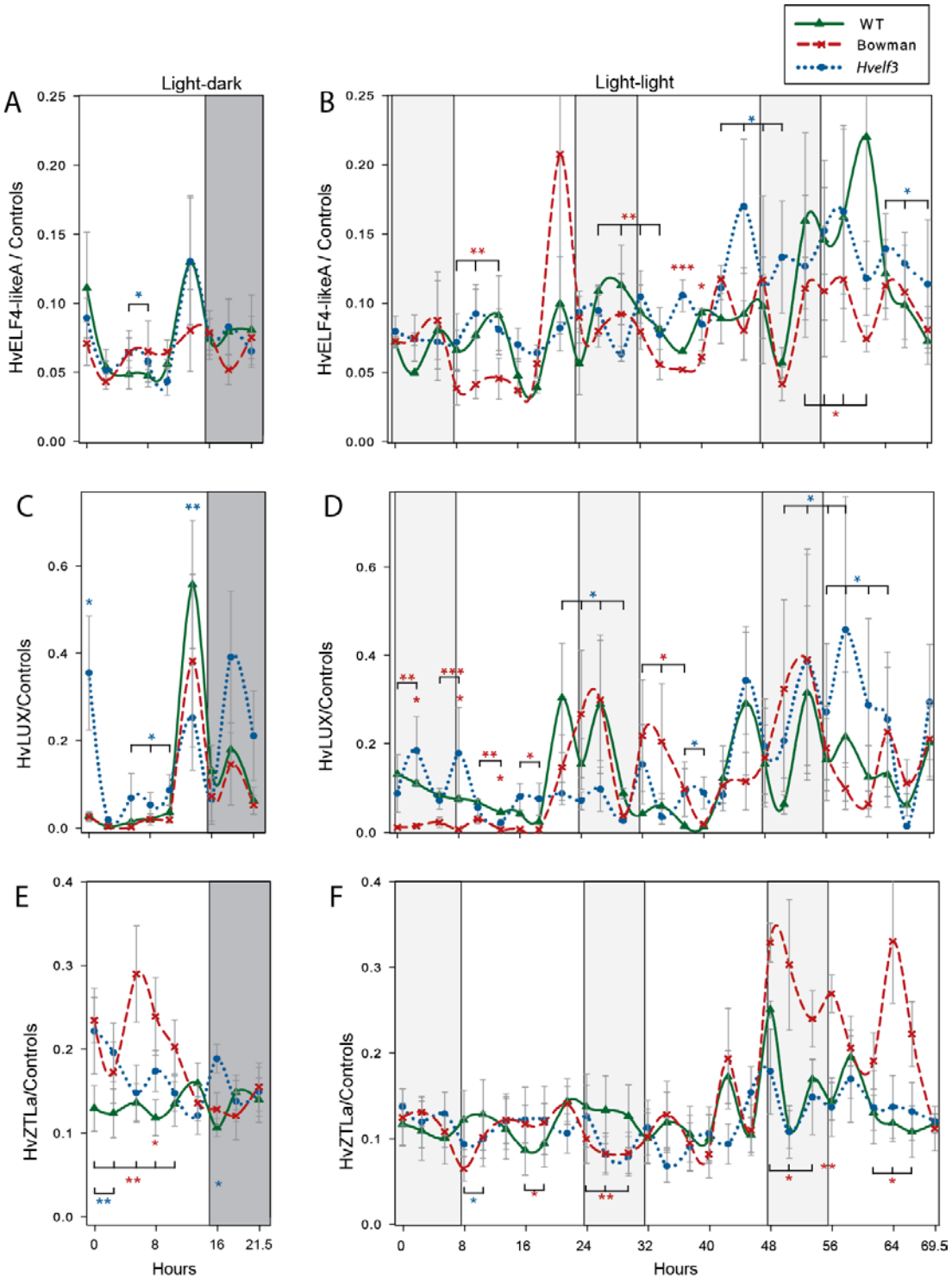

Figure B5: Transcript levels of *HvELF4-likeA*, *HvLUX* and *HvZTLa* from WT, Bowman (*ppdh1*) and *Hvelf3* plants. A, C and E represent light/dark conditions. Light and dark periods are shown with white and dark grey backgrounds, respectively. B, D and F represent transfer to continuous light. Subjective nights are on light grey backgrounds. Error bars: SEM. P values represent statistical comparisons of WT against each mutant (colour coded). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

B6 - Phase differences in clock gene expression

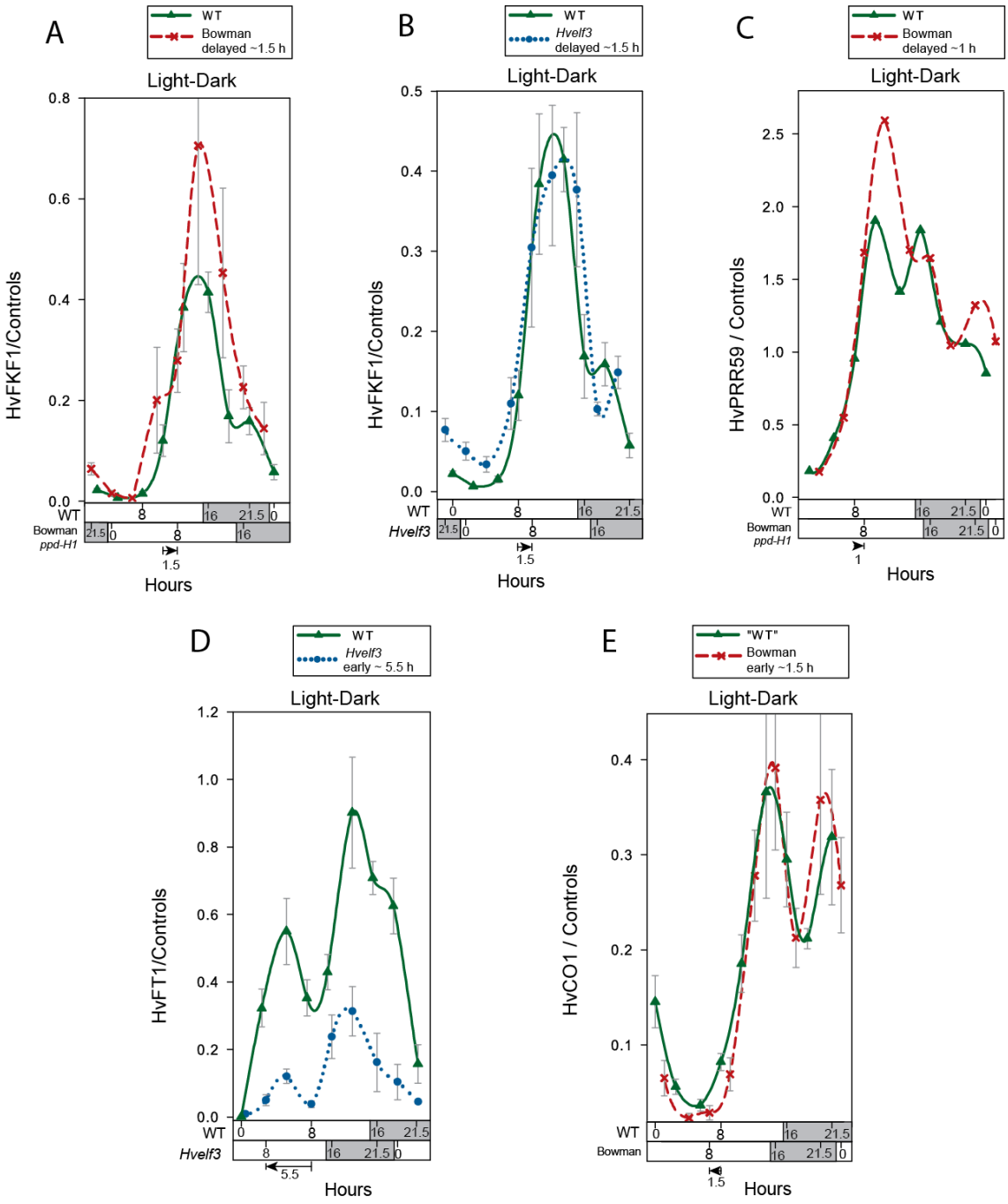

Figure B6: Shifted analysis of *HvFKF1*, *HvPRR59*, *HvFT1* and *HvCO1* transcript levels in Bowman and *Hvelf3* plants, compared to WT. A) and B) Expression data of *HvFKF1* from WT and Bowman (*ppdh1*) or *Hvelf3* plants. Time points were plotted differently for the two plants analysed: expression of Bowman and *Hvelf3* plants was shifted 1.5 h later. C) Expression data comparison of *HvPRR59* from WT and Bowman plants shifted 1 h later. D) Expression data comparison of *HvFT1* from WT and *Hvelf3* plants shifted 5.5 h earlier. E) Expression data of *HvCO1* from WT and Bowman (*ppdh1*) plants. Time points were plotted differently for the two plants analysed: expression of Bowman plants was shifted 1.5 h earlier. Error bars: SEM.

B7 - Temperature-dependent AS of barley clock genes

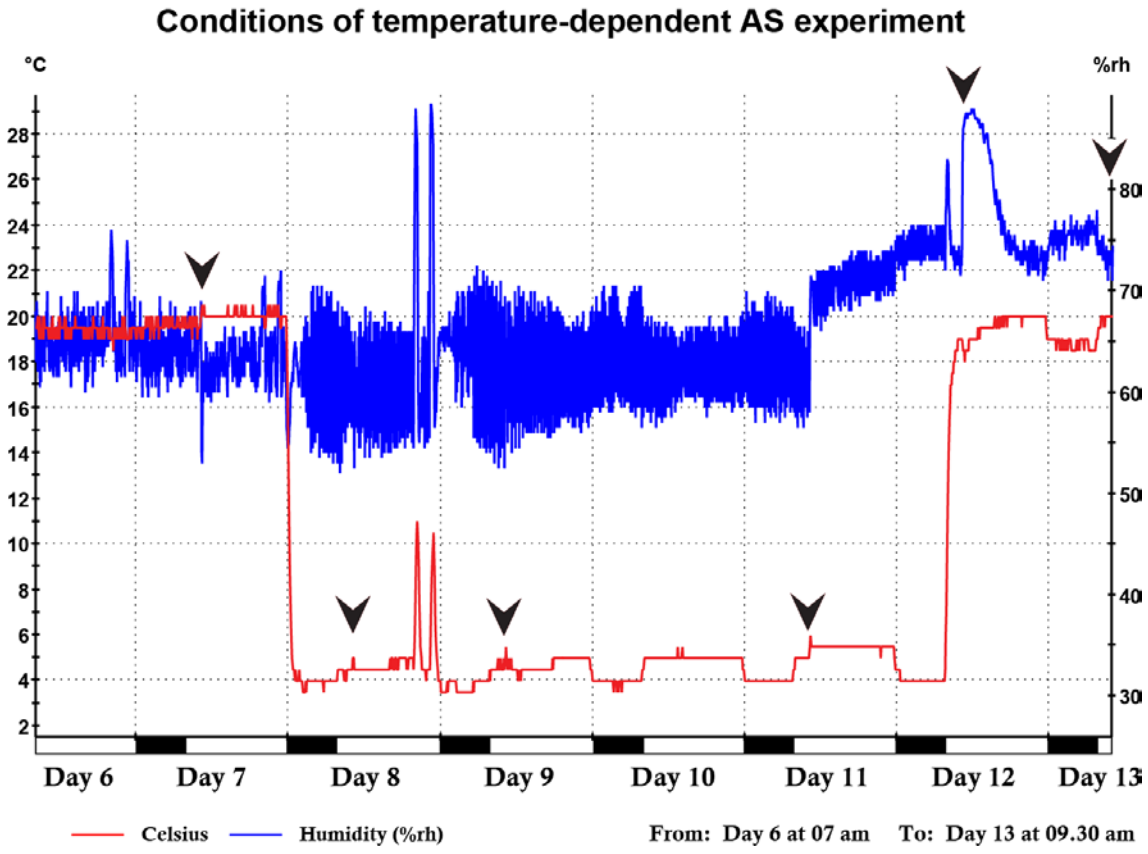

Figure B7.1: Temperature (red line) and humidity (blue line) information measured inside growth cabinet over harvesting period (Day 7 to 13) of temperature-dependent AS experiment. Black boxes, below the graph, are for dark, white boxes are for light. Black arrows indicate time points when sampling occurred.

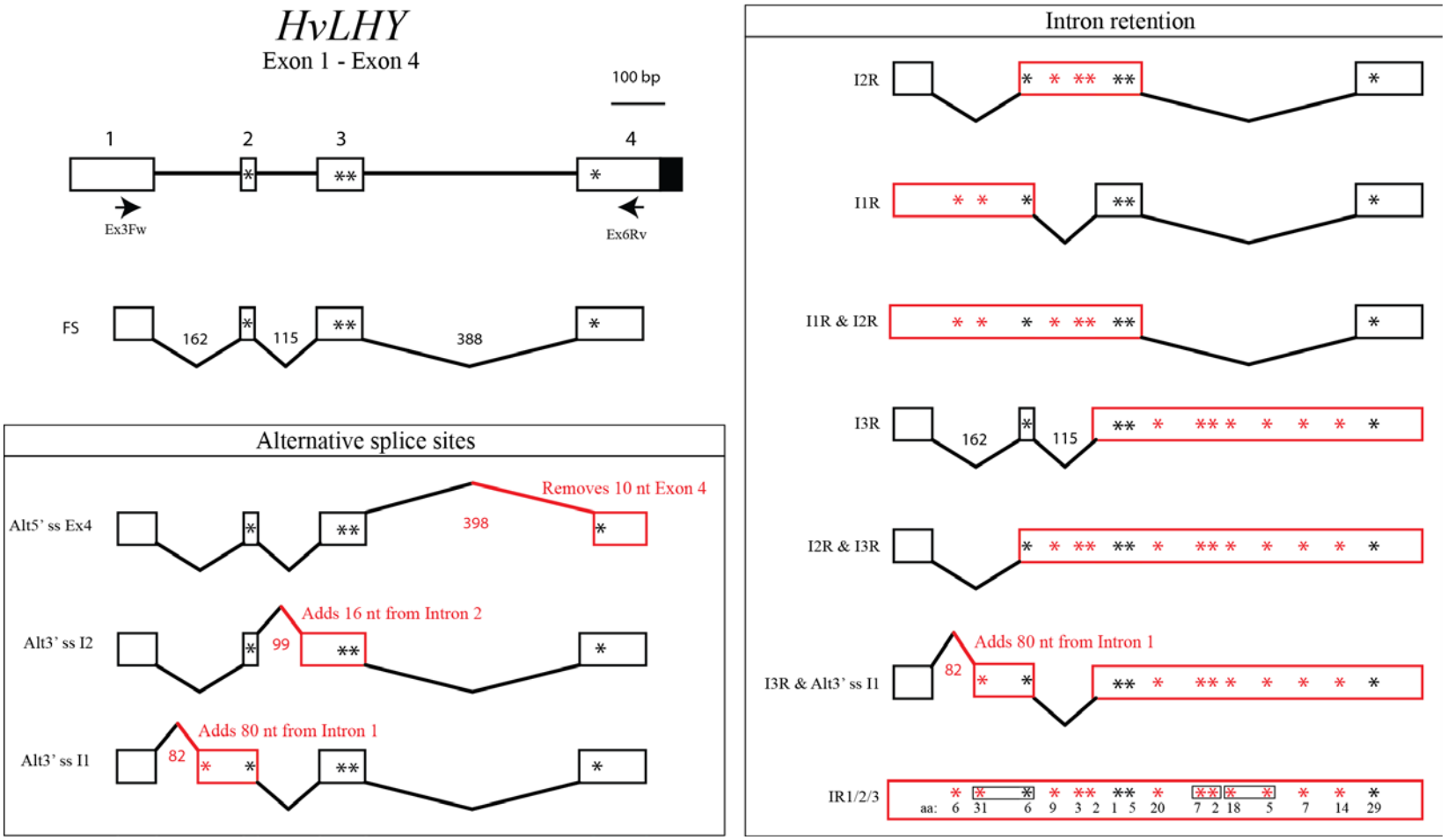

Figure B7.2: *HvLHY* genomic structure of 5' UTR, FS and AS transcript isoforms. RT-PCR analysis used primers spanning exons 1 and 4 (approximate primer positions shown by arrowheads). Exons are numbered on the genomic structure; 5' and 3' UTRs are open boxes; coding sequences are dark boxes; introns are straight black lines. Diagonal lines represent splicing events, where putative intron sizes are listed nearby in most cases. AS events are shown in red. *, uAUG. Putative CDS length, in amino acid (aa), are shown below the IR1/2/3 transcript; in frame uAUGs are connected by a small box.

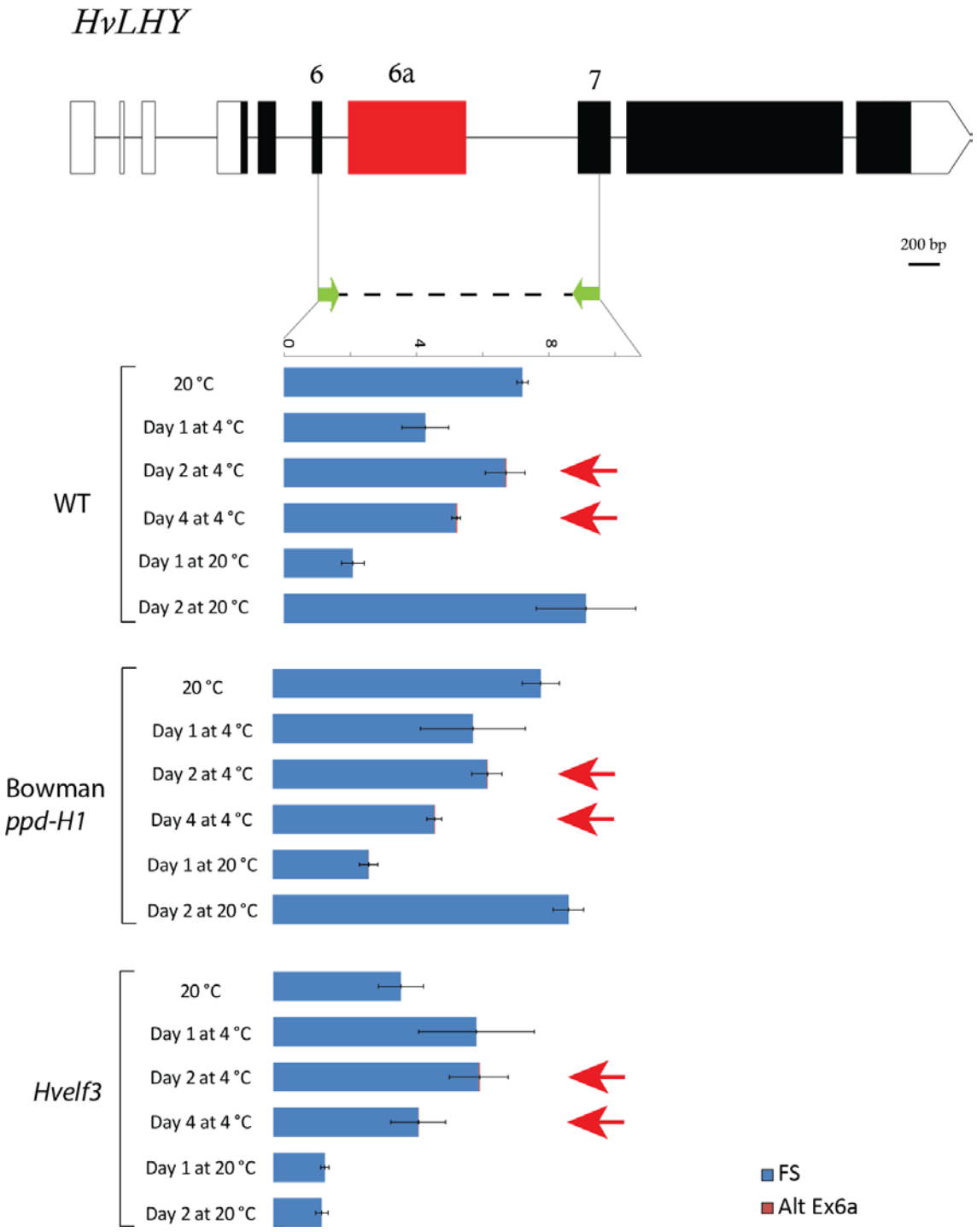

Figure B7.3: Relative abundances of *LHY* FS and AS transcripts containing E6a. The latter, in particular, is very low abundant (< 0.7% of total transcripts) and is present on samples pointed by red arrow heads. Plants were assessed using HR RT-PCR primers spanning Exons 6 and 7 (green arrow heads). Error bars are SEM of three biological replicates. Exons 6, 6a and 7 are labelled; 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except E6a, which is shaded red.

B8 - Analysis of publicly available transcript data for AS discovery

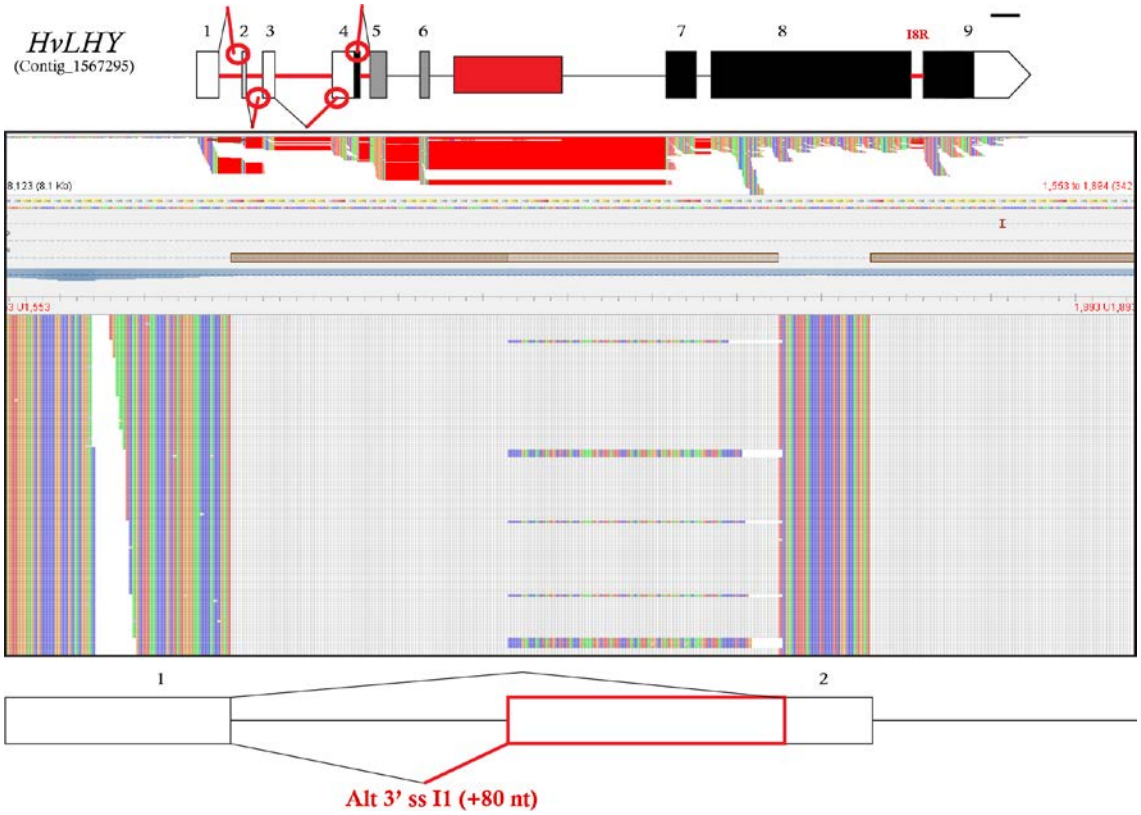

Figure B8: Evidence of an alternative 3' ss event in *HvLHY* (contig_1667295) intron 1, adding 80 nt. Similar AS discovery analyses were carried out using the Tablet program, which aligned barley genomic contigs against transcript data (reads) (Consortium, 2012). Print screen of the analysis is shown in the middle of the figure, which is further clarified with diagrams of the genomic structure and AS events (shown in red) above and below the print screen (zoom of intron 1 and exon 2). Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except MYB-encoding exons, which are shaded grey. Alt, alternative; ss, splice site; I, intron. Small black line on the top right of the gene structure represents a scale of 200 bases.

B9 - Rhythmic AS in barley clock genes

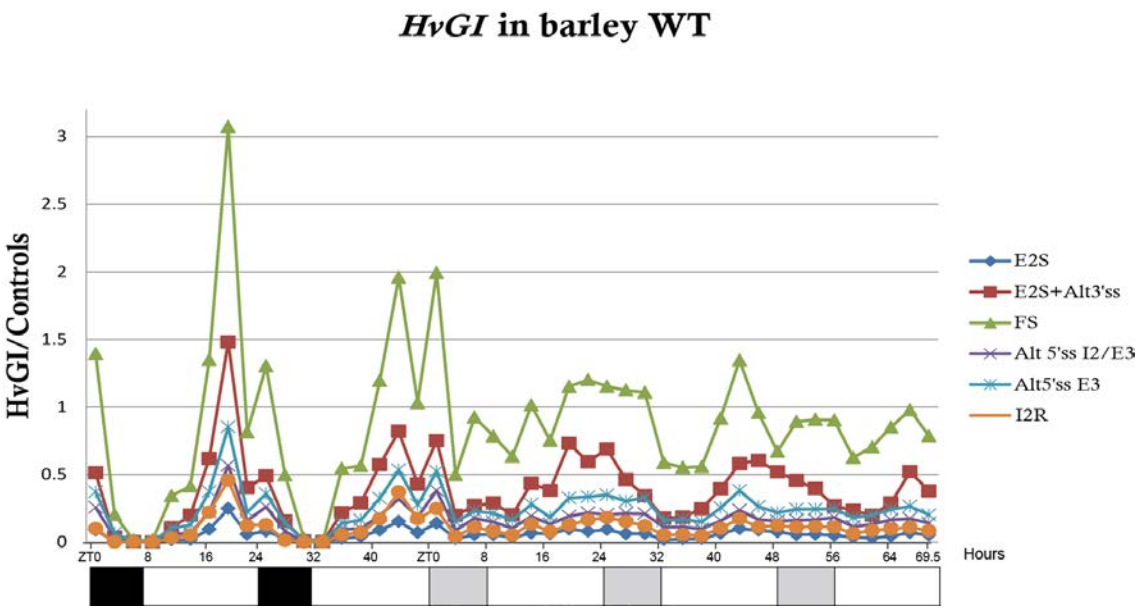

Figure B9.1: Rhythmic AS in 5' UTR of *HvGI*. Levels of *HvGI* alternative transcripts in WT plants under light-dark and light-light conditions. Expression data were obtained through HR RT-PCR experiments carried out with primers HvGI-5UTRFw (FAM) and HvGI-Ex4Rv. Dark boxes represent dark and white boxes are for light. Subjective nights are light grey backgrounds. Each time point shows data for one biological replicate. Two ZT0 are indicated, at the start of sampling and the beginning of effective free-running condition. E, exon; S, skipping; Alt, alternative; ss, splice site; I, intron; R, retention.

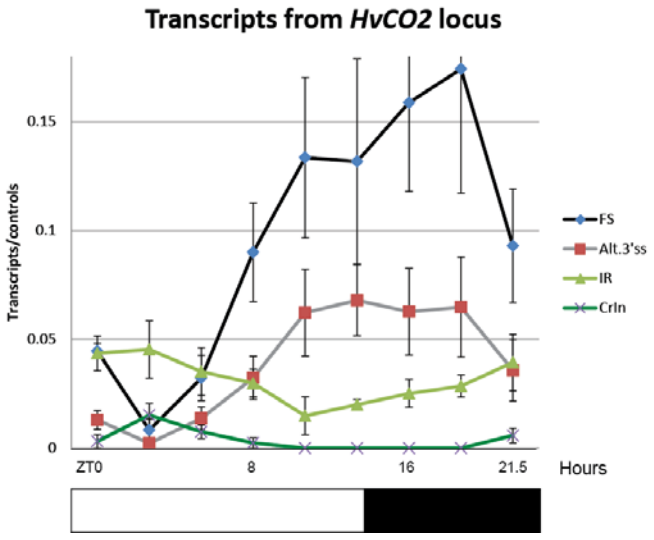

Figure B9.2: Rhythmic AS of the *HvCO2* locus. Levels of *HvCO2* alternative transcripts were analysed by HR RT-PCR in WT plants under light-dark conditions. Dark box represents dark and white box is for light. Each time point shows mean data of four biological replicates. Error bars: SEM. ZT0 indicated the start of sampling. Alt, alternative; ss, splice site; IR, intron retention; CrIn, cryptic intron.

B10 - Genomic structure and AS events of core clock homologues in Arabidopsis and barley

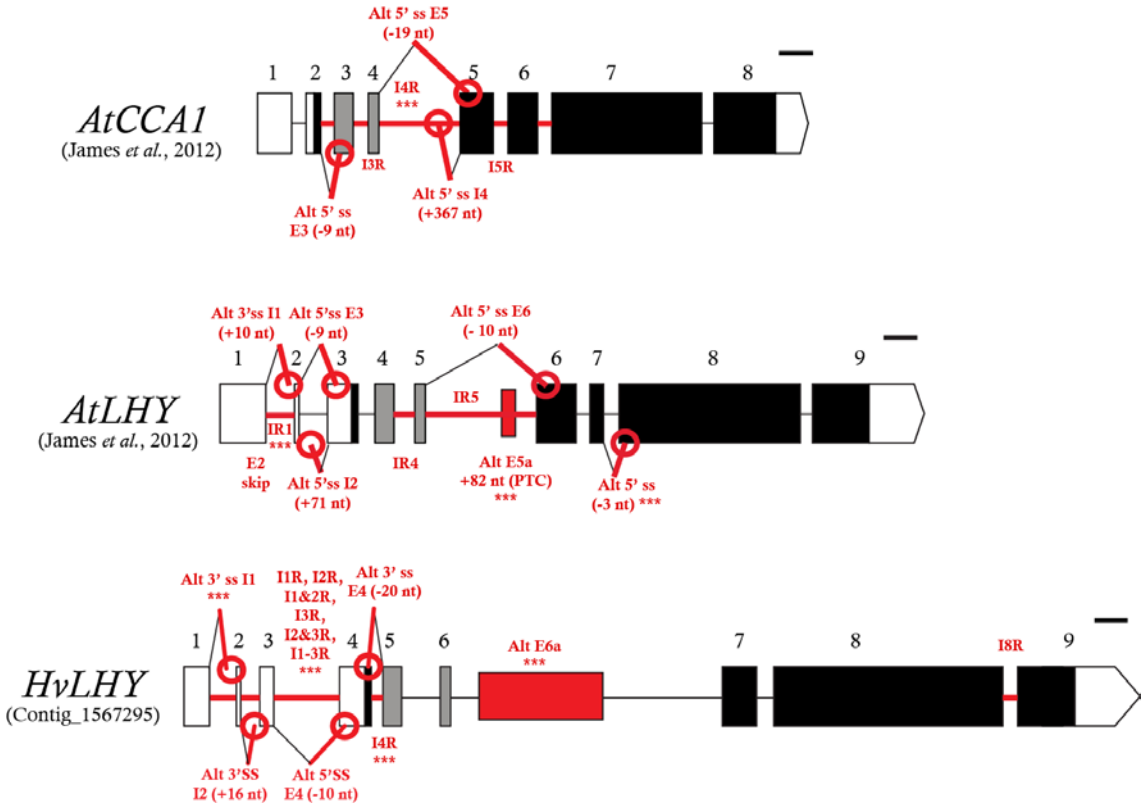

Figure B10.1: Genomic structure and AS events of *LHY* homologues in Arabidopsis and barley. Arabidopsis data are taken from James *et al.* (2012). Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except MYB-encoding exons, which are shaded grey. AS events of Arabidopsis (James *et al.*, 2012) and barley genes significantly associated with temperature-dependent AS are labelled with asterisks. AS events are represented in red. Alt, alternative; ss, splice site; I, intron; R, retention; E, exon. The approximate positions of primer pairs used in barley AS studies are shown by green arrows joined by a dotted line. Small black line on the top right of each gene structures represents a scale of 200 bases.

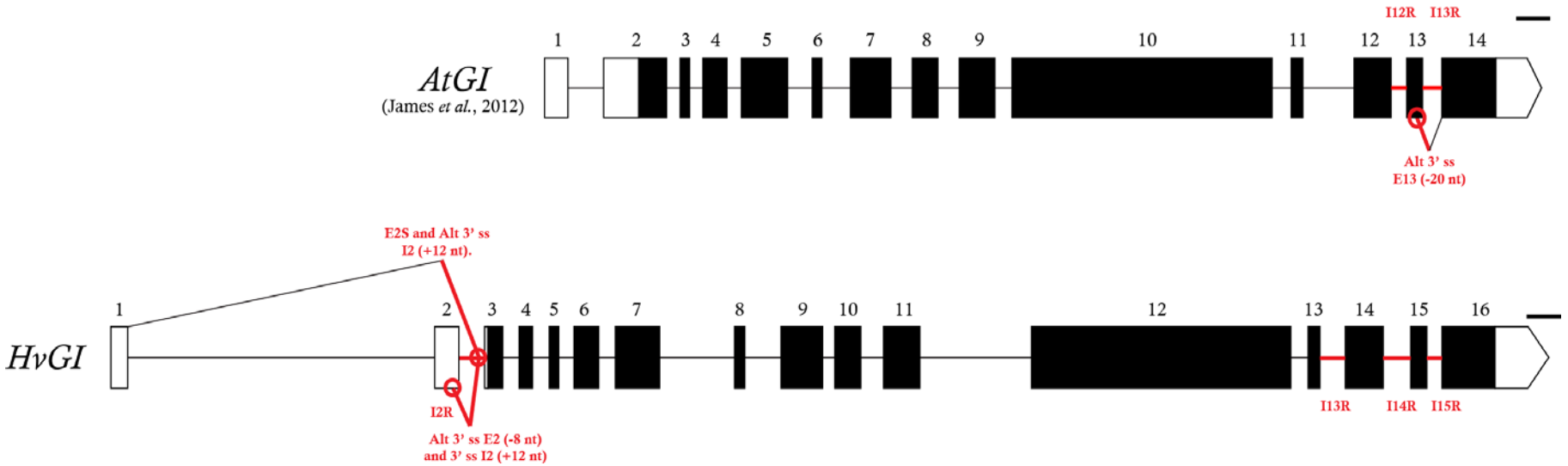

Figure B10.2: Genomic structure and AS events of *GI* orthologues in Arabidopsis and barley. Arabidopsis data are taken from James *et al.* (2012). Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes. AS events are represented in red. Alt, alternative; ss, splice site; I, intron; R, retention; E, exon. Small black lines on the top right of each gene structures represent a scale of 200 bases.

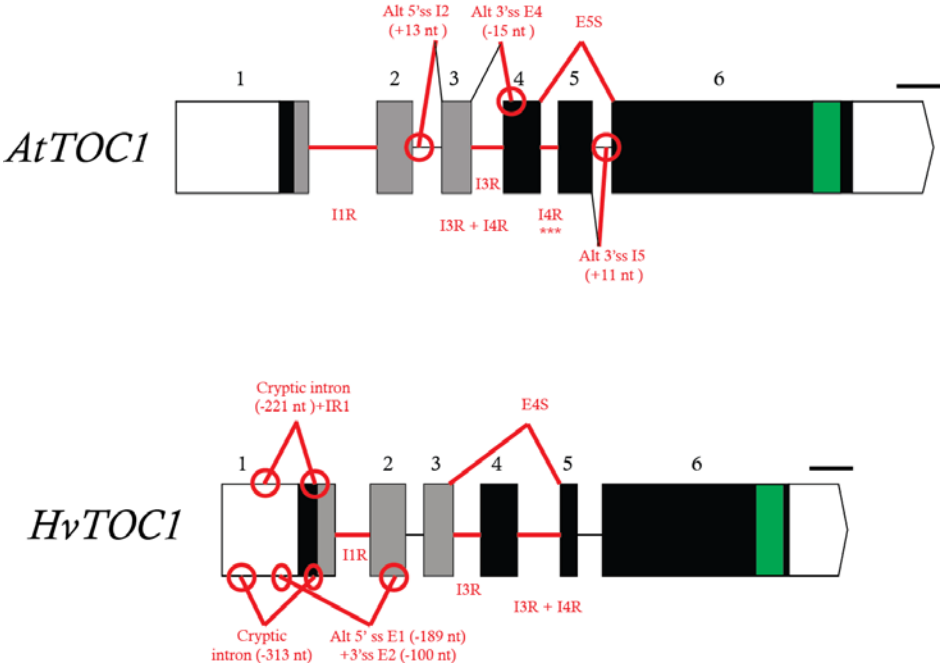

Figure B10.3: Genomic structure and AS events of *TOC1* orthologues in Arabidopsis and barley. Arabidopsis data are taken from James *et al.* (2012). Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except domain-encoding exons, which are shaded grey (PRR domain) or green (CT domain). AS events are represented in red and the ones in Arabidopsis significantly associated with temperature (James *et al.*, 2012) are labelled with asterisks. Alt, alternative; ss, splice site; I, intron; R, retention; E, exon. Small black lines on the top right of each gene structures represent a scale of 200 bases.

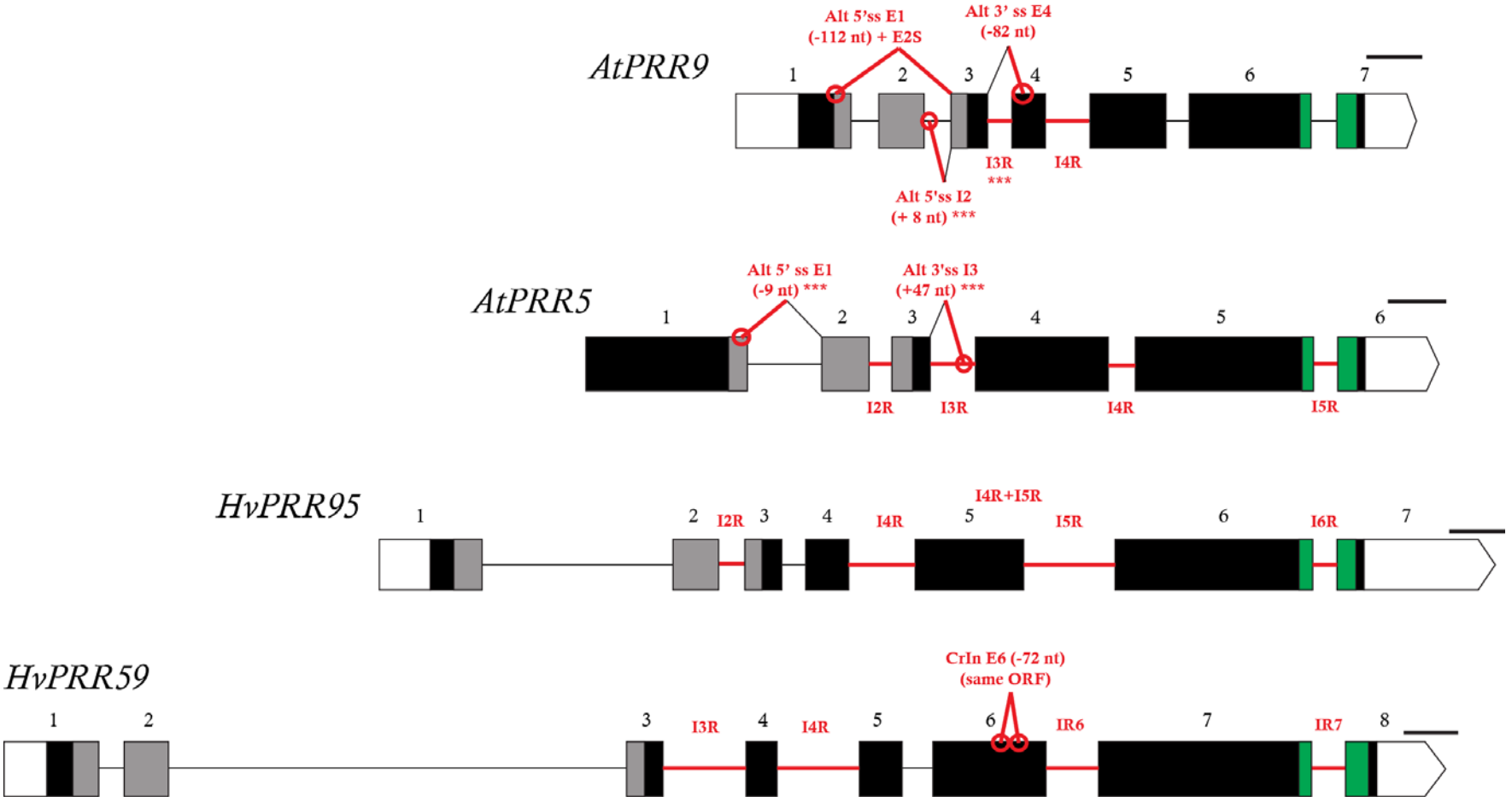

Figure B10.4: Genomic structure and AS events of *PRR5(9)/9(5)* orthologues in Arabidopsis and barley. Arabidopsis data are taken from James *et al.* (2012). Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except domain-encoding exons, which are shaded grey (PRR domain) or green (CT domain). AS events are represented in red and the ones in Arabidopsis significantly associated with temperature (James *et al.*, 2012) are labelled with asterisks. Alt, alternative; ss, splice site; I, intron; R, retention; E, exon; ES, exon skipping; CrIn, cryptic intron. Small black lines on the top right of each gene structures represent a scale of 200 bases.

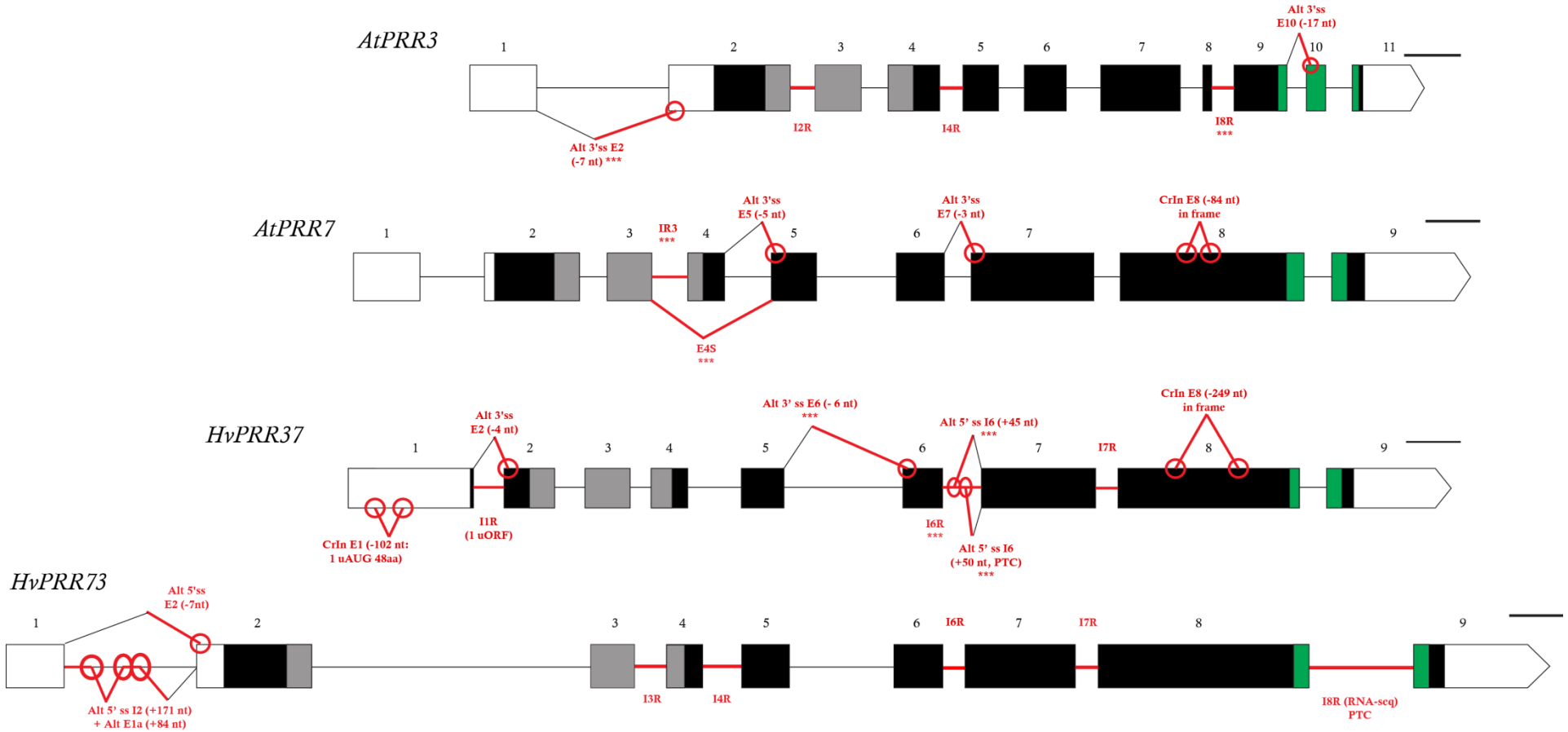

Figure B10.5: Genomic structure and AS events of *PRR3(7)/7(3)* orthologues in Arabidopsis and barley. Arabidopsis data are taken from James *et al.* (2012) and TAIR database. Exons are numbered; 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except domain-encoding exons, which are shaded grey (PRR domain) or green (CT domain). AS events of Arabidopsis (James et al., 2012) and barley genes significantly associated with temperature-dependent AS are labelled with asterisks. AS events are represented in red. Alt, alternative; ss, splice site; I, intron; R, retention; E, exon; ES, exon skipping; CrIn, cryptic intron. Small black lines on the top right of each gene structures represent a scale of 200 bases.

B11 - AS analyses of *HvPPDH1*

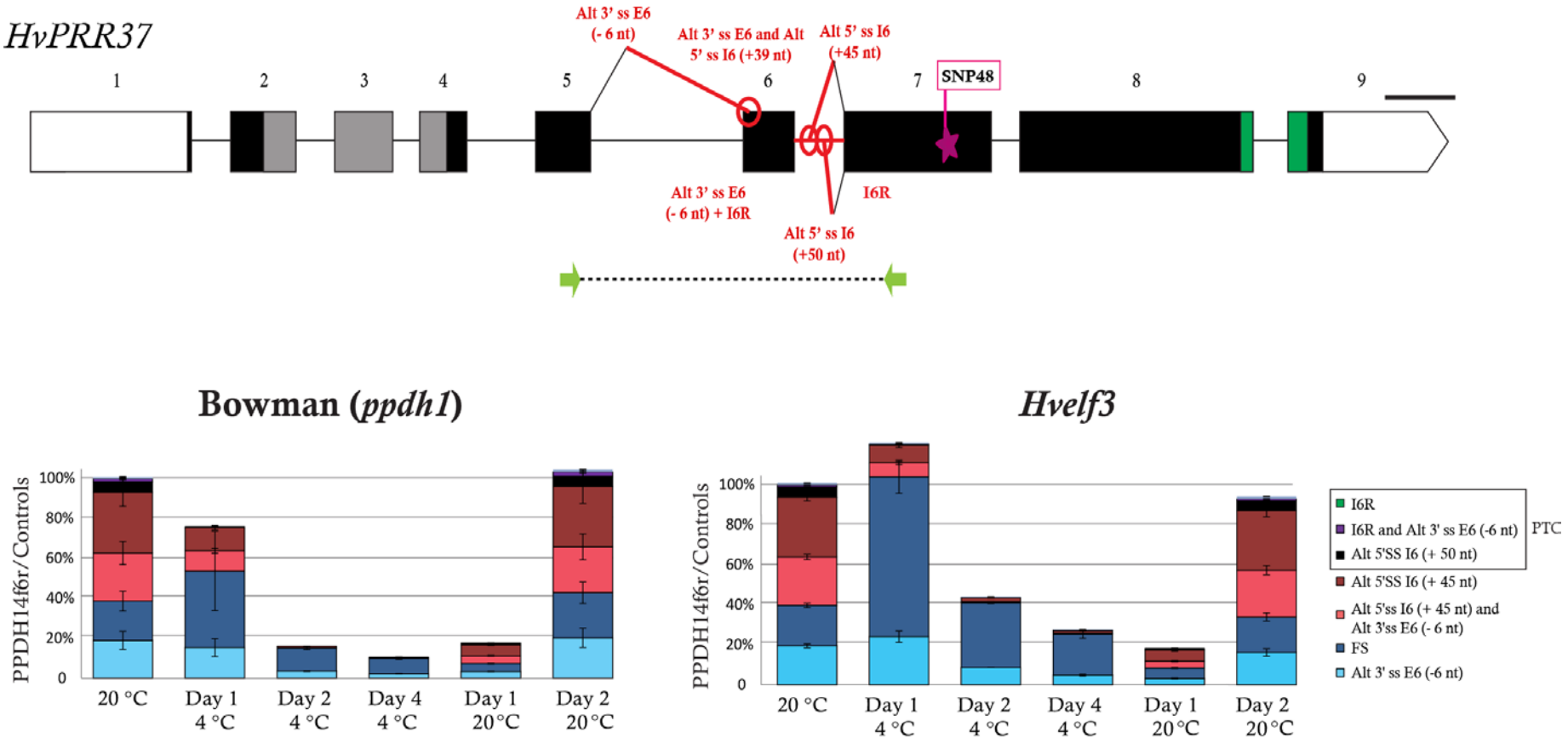

Figure B11.1: Relative abundances of *PPDH1* FS and AS transcripts. Bowman (*ppdh1*) and *Hvelf3* plants were assessed using HR RT-PCR primers spanning exons 5 and 7 (Appendix B7.4). Error bars are SEM of three biological replicates.

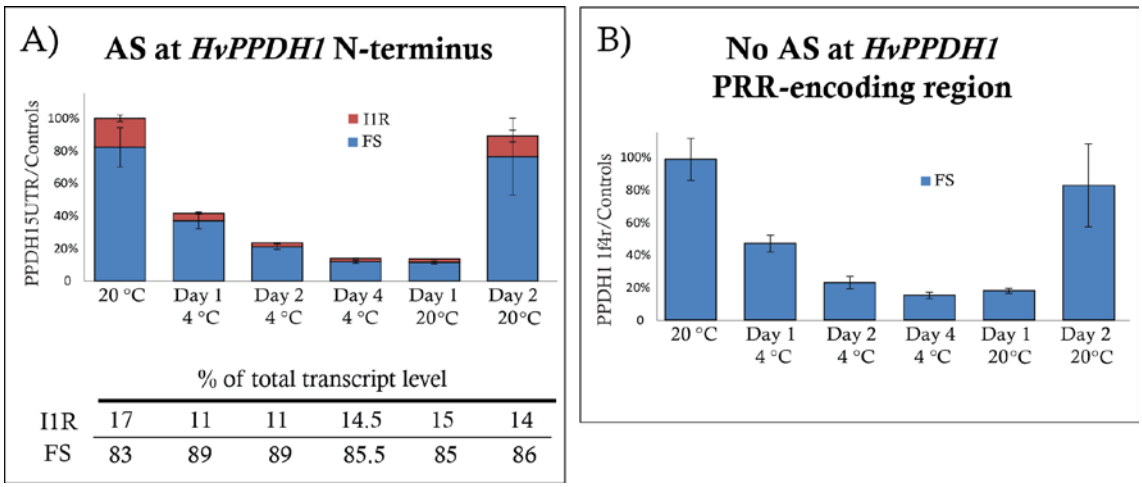

Figure B11.2: Relative abundance of *PPDH1* FS and AS transcripts containing I1. WT plants were assessed using HR RT-PCR primers spanning the A) 5' UTR and B) Exons 2 through 5. Error bars are SEM of three biological replicates. I1R/FS ratios compared with 20 °C data were not highly significantly different (P values > 0.004).

B12 - AS analyses of *HvPRR73*

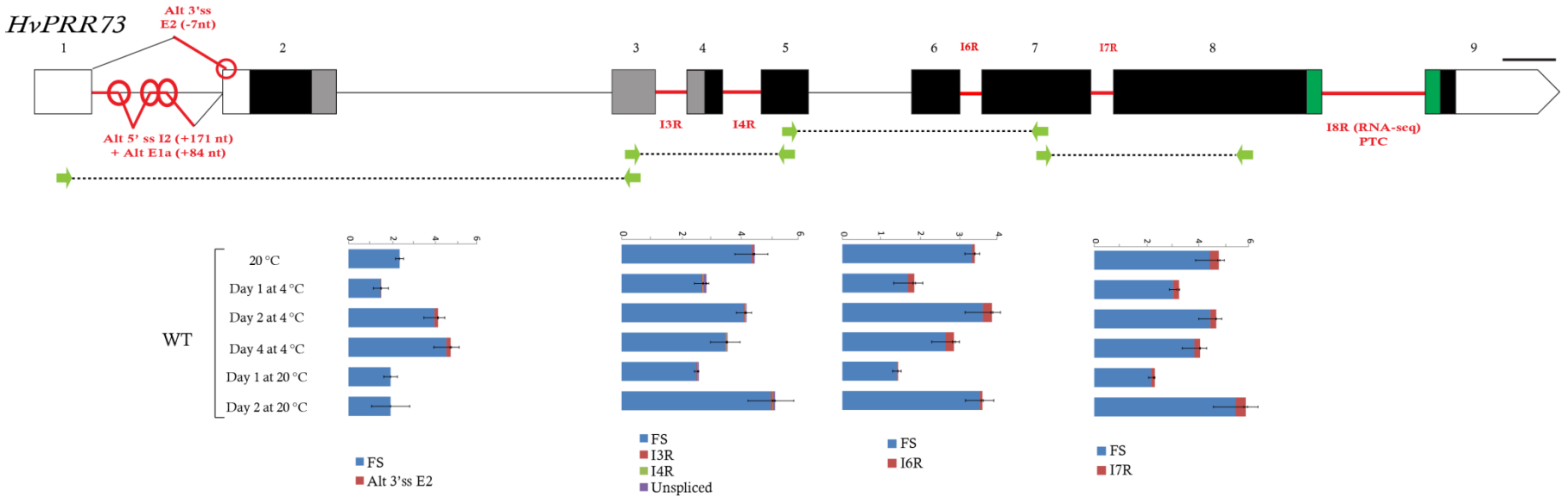

Figure B12: Relative abundance of *PRR73* FS and AS transcripts. Plants were assessed using HR RT-PCR primers spanning most of the gene sequence. Error bars are SEM of three biological replicates. Exons are labelled; 5' and 3' UTRs are open boxes; coding sequences are dark boxes, except domain-encoding exons, which are shaded grey (PRR domain) or green (CT domain). AS events are represented in red. Alt, alternative; ss, splice site; I, intron; R, retention; E, exon; ES, exon skipping; CrIn, cryptic intron. Small black lines on the top right of each gene structures represent a scale of 200 bases. The approximate positions of four primer pairs used in HR RT-PCR studies are shown by green arrows joined by a dotted line and correspondent results are shown below each dotted line.