DOI: 10.1007/s10535-015-0568-0 BIOLOGIA PLANTARUM 60 (1): 95-104, 2016

BvPRR7 is a cold responsive gene with a clock function in beet

O. OMOLADE[1], A.E. MÜLLER[2], C. JUNG[1], and S. MELZER[1]*

Plant Breeding Institute, Christian-Albrechts-University of Kiel, Olshausenstr. 40, D-24098 Kiel, Germany[1]
Strube Research GmbH & Co. KG, D-38387 Söllingen, Germany[2]

Abstract

The life cycle of flowering plants is partially defined by environmental cues like day length and temperature. In the model plant *Arabidopsis thaliana* and temperate cereals, such as barley (*Hordeum vulgare*) and wheat (*Triticum* spp.), differences in life cycle control have been associated with a natural variation in *FLOWERING LOCUS C (FLC)* and *VERNALIZATION 1-3 (VRN1-3)*. In sugar beet (*Beta vulgaris* L.), variation in vernalization requirement and life cycle is determined by a major gene at the *B* locus. This gene has recently been identified as a pseudo-response regulator (*PRR*) gene *BOLTING TIME CONTROL 1* (*BTC1*). A second gene in beet with homology to *BTC1* and *ARABIDOPSIS PSEUDO RESPONSE REGULATOR 7* (*APRR7*) in *Arabidopsis* was identified and termed *Beta vulgaris PSEUDO RESPONSE REGULATOR 7* (*BvPRR7)*. We functionally characterized *BvPRR7* by transgenic analysis in *Arabidopsis* and expression profiling during development in beet. We show that *BvPRR7* was diurnally regulated and responded to cold. Constitutive expression of *BvPRR7* distorted diurnal rhythms and caused late flowering in *Arabidopsis* suggesting a conserved function of *BvPRR7* in clock regulation. Conceivably, the retention of a functional role of *BvPRR7* in clock regulation may have facilitated the evolution of a distinct role as major floral regulator of the second *PRR7* homolog in beet, *BTC1*.

Additional key words: *Arabidopsis thaliana*, *Beta vulgaris*, life cycle, pseudo response regulator.

Introduction

The circadian clock is ubiquitous internal time keeper in all organisms and has evolved as result of predictable earth's day and night cycles (Edgar *et al.* 2012). Consequently, to ensure maximum fitness and survival of plants, most physiological and developmental processes are under the control of the clock (Nagel and Kay 2012, Kinmonth-Schultz *et al.* 2013). One of the most dramatic changes in plant life history, which in part is clock controlled, is transition from vegetative to reproductive growth. Genetic studies have elucidated that the internal clock together with day length and temperature regulate at which time of the year plants flower (Andres and Coupland 2012).

The timely regulation of flowering during favorable conditions ensures a maximum of seed set in plants. In crops that are cultivated for vegetative storage organs, like sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) for sugar storing tap roots, flowering should not occur during the cultivation period. Sugar beet belongs to the *Amaranthaceae* family and has a genome of 714 - 758 Mbp in size (Arumuganathan *et al.* 1991) that was recently sequenced (Dohm *et al.* 2014). Breeding and cultivating sugar beet started in the 18th century and accounts for about one fifth of the world's annual sugar production today. Sugar beet is a biennial long day plant that usually develops a tap root during vegetative growth in the first year and starts reproductive growth in the second year after a certain period of cold during winter (vernalization). Since the development of an inflorescence is an energy consuming process, stored sugars in beet roots are remobilized during flowering and limit the yield of harvestable beets. Therefore, the time to flower is a critical process which affects the cultivation of sugar beet (Melzer *et al.* 2014).

Submitted 2 September 2014, *last revision* 2 May 2015, *accepted* 4 May 2015.
Abbreviations: Col-0 - ecotype Columbia; Mbp - mega base pairs; PLACE - plant *cis*-acting regulatory DNA elements; PCR - polymerase chain reaction; RT-qPCR - real time quantitative PCR; RLN - rosette leaf number; T1 - transgenic plant of the first generation; T2 - transgenic plant of the second generation.
Acknowledgements: The project was funded by the International Max Planck Research School (IMPRS), Plön, Germany. We thank Bettina Rohardt for technical assistance in the laboratory and Monika Bruisch for assistance in the greenhouse. Nadine Dally is acknowledged for critical discussions of results and Martina Blümel for careful revision of the manuscript. We thank also Dilan S.R. Patiranage and Shrikant Sharma for help with the figures.
* Corresponding author; fax: (+43) 1 8802566, e-mail: s.melzer@plantbreeding.uni-kiel.de

In many long day plants analyzed so far, *CONSTANS* (*CO*) is key regulator for a day length controlled flowering time. In *Arabidopsis*, an external coincidence model has been proposed that explains how transcriptional regulation of the *CO* gene and stability of the CO protein determine photoperiod controlled flowering (Sawa *et al.* 2008). Rhythmic *CO* expression peaks at the end of the light phase of the long day period and is regulated by the clock through *GIGANTEA* (*GI*) (Suarez-Lopez *et al.* 2001). *CO* directly activates the expression of *FLOWERING LOCUS T* (*FT*) and subsequently the FT protein is transported to apical meristems where flowering is induced (Turck *et al.* 2008). This CO-FT module seems to be conserved in many long day plants (Valverde 2011). In sugar beet, there is no known true *CO* ortholog (Chia *et al.* 2008). Recently, a B-box zinc finger transcription factor (*BvBBX19*) that belongs to the *CO* like family of genes has been identified as flowering time regulator. The *BvBBX19* is supposed to act together with the pseudo response regulator gene *BOLTING TIME CONTROL 1* (*BTC1*) (Dally *et al.* 2014) to control flowering. The *BTC1* shows the closest homology to the *Arabidopsis* clock associated gene *ARABIDOPSIS PSEUDO RESPONSE REGULATOR 7* (*APRR7*) (Pin *et al.* 2012). However, RNAi knock-down *BTC1* plants show no effect on the expression of beet clock genes. Instead, *BTC1* controls flowering by regulating two *FT* paralogs with antagonistic functions. Whereas *BvFT1* is a repressor of flowering and is down regulated by *BTC1*, *BvFT2* induces flowering and is positively controlled through *BTC1* (Pin *et al.* 2012). In temperate cereal crops like barley and wheat, *Photoperiod determinant* (*Ppd*) loci, *Ppd-H1* in barley and *Ppd-D1* and other paralogs in wheat, are the major photoperiod determinants (Turner *et al.* 2005, Beales *et al.* 2007). Like *BTC1*, these genes are also closely related to *APRR7*, but they are not involved in vernalization responses as *BTC1*. The *Ppd-H1* does not affect the expression of clock genes, but affects photoperiodic genes (Campoli *et al.* 2012). The *Arabidopsis* circadian clock is made up of three interlocking loops: the core central loop and the morning and evening loops (McClung 2010, Nagel and Kay 2012). Functionally, *APRR7* together with *APRR5* and *APRR9* belong to the morning loop (Locke *et al.* 2006, Zeilinger *et al.* 2006, Imaizumi 2010). Both *APRR7* and *APRR9* represses the expression of core clock genes *LATE ELONGATED HYPOCOTYL* (*LHY)* and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) (Farre *et al.* 2005). Together with *APRR1*, also named *TIMING OF CAB EXPRESSION 1* (*TOC1*), these genes constitute the central loop of the circadian clock (Nagel and Kay 2012). The third loop, referred to as the evening loop, is made up of *GI*, *ZEITLUPE*, and *APRR3* (Nagel and Kay 2012, Chen 2013). Compared to *APRR5* and *APRR9*, *APRR7* has only a subtle effect on flowering time (Yamamoto *et al.* 2003, Nakamichi *et al.* 2005) indicating that it is not a major flowering time gene in *Arabidopsis*.

Despite detailed knowledge on the molecular basis of the circadian clock in *Arabidopsis* and rice, relatively limited information is available from other plants. In beet, studies of the diurnal regulation of some selected clock genes have only been started recently (Pin *et al.* 2012). In light of a potential impact that a modulation of the circadian clock can have on breeding, further research in this area, by producing crops with altered growth periods as result of different flowering times (Jung and Müller 2009), would be beneficial.

The aim of this research was the characterization of the putative *APRR7* ortholog *BvPRR7* from sugar beet and determination if that gene is diurnally regulated in beet and if a *BvPRR7* transgene can distort diurnal rhythms in *Arabidopsis*.

Materials and methods

Plants: For *BvPRR7* expression studies, sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) plants from annual (seed code 001684) and biennial (seed code 940043) accessions were used. For flowering time experiments, the *Arabidopsis thaliana* L. *aprr7* mutant (SALK 030430) (http://signal.salk.edu/), which was described as *aprr7-11* (Yamamoto *et al.* 2003), and the Col-0 accession as reference were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, Ohio, USA). The mutation in the *aprr7-11* mutant was caused by multiple T-DNA insertions in exon 1 (Yamamoto *et al.* 2003). The T3 homozygous *Arabidopsis* lines constitutively expressing *BvPRR7* under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter in both the Col-0 accession and the *aprr7-11* mutant were named *BvPRR7*-OxCol-0 and *BvPRR7*-OxM1-7, respectively. For diurnal expression analysis in transgenic *Arabidopsis* seedlings over-expressing *BvPRR7*, two T3 transgenic lines (*BvPRR7*-OxCol-0 and *BvPRR7*-OxM5) were chosen and the *aprr7-11* mutant and wild type Col-0 accession were used as controls.

Isolation and characterization of *BvPRR7*: A 2.3 kb full-length cDNA sequence of *BvPRR7* was amplified from the annual beet accession by a two-step PCR. The first part was amplified with primers B620 and B621 and the second part with primers B622 and B623 (Table 2 Suppl.). Both amplicons have an overlap of 110 bp which contained a BglII restriction site. Each amplicon was inserted separately into a pGEM-T vector (*Promega*, Mannheim, Germany). Both fragments were fused together using an appropriate orientation of the insert *via* BglII and NcoI restriction. Using a second PCR amplification, SalI was introduced at both ends of the fragment which was subsequently ligated into the SalI site of the binary vector P7-35-OCS (*DNA Cloning*

Service, Hamburg, Germany). The binary vector with the BvPRR7 transgene under the control of the CaMV 35S promoter was transformed into *Agrobacterium tumefaciens* (strain GV3101, PMP90RK) (*DNA Cloning Service*) according to Höfgen and Willmitzer (1988). The Col-0 and aprr7-11 plants were transformed by floral dip (Clough and Bent 1998) and T1 seeds were collected. Per construct, 50 000 T1 seeds were sown and 30 putative transgenic plants were selected through glufosinate (1.7 g dm^{-3}; *Bayer*, Leverkusen, Germany) treatments and PCR analysis. The T2 plants were grown under long-day conditions (a 16-h photoperiod, a photon fluence rate of 150 µmol m^{-2} s^{-1}, day/night temperatures of 23/20 °C, and a relative humidity of 65 %) in a growth chamber. Homozygous T3 seeds were selected after glufosinate treatments.

Gene structure and phylogenetic analysis: The cDNA sequence of *BvPRR7* from the annual genotype of sugar beet was used in a *BLASTX* search against the published beet genome (*RefBeet 1.2*, http://bvseq.molgen.mpg.de/blast/) to identify the scaffold on which *BvPRR7* is located. To annotate the exon-intron structure, a pairwise alignment was performed between the identified scaffold and *BvPRR7* cDNA using *BLAST2* (http://blast.ncbi.nlm.nih.gov/Blast). The sequence 2 kb upstream of the transcription start site was considered as the potential promoter region. *Cis*-acting regulatory DNA elements were searched for within the selected 2 kb sequence by using *PLACE* (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

For phylogenetic study, all *Arabidopsis PRR* genes were downloaded from *TAIR* (http://www.arabidopsis.org/) and putative beet *PRR* homologs were identified through *BLASTX* against *RefBeet 1.2*. Domain search, annotation, and amino acid identity comparison were done using *SMART* (http://smart.embl-heidelberg.de/) and *CLC Main Workbench v. 5.5* (*CLC Bio*, Aarhus, Denmark), respectively. Multiple sequence alignment was done using *Clustal Omega* (http://www.ebi.ac.uk/Tools/msa/clustalo/). *PSEUDO RESPONSE REGULATOR* homologs from other plant species were also included. To construct a phylogenetic tree, the best protein evolutionary model was computed using both *ProtTest 2.4* (http://darwin.uvigo.es/software/prottest2_server.html) and *MEGA 6* (Tamura *et al.* 2011), and tree reconstruction with *MEGA 6* was done considering non-uniformity of evolutionary rates. Bootstrap values were calculated for 500 replications.

Flowering time measurements and growth conditions: The T3 homozygous lines were used for flowering time analysis. Seeds were stratified in darkness at 4 °C for 3 - 4 d and subsequently placed on soil. Two weeks after sowing, 12 plants per line were transplanted. The plants were grown in a climate chamber in both short-day conditions (an 8-h photoperiod and a temperature of 18 °C) or long-day conditions (a 16-h photoperiod and a temperature of 18 or 20 °C) under cool white fluorescent tubes providing a photon fluence rate of 260 µmol m^{-2} s^{-1} and at a 65 % relative humidity. For flowering time measurements, the rosette leaf number (RLN) was considered. Statistical tests were performed using the unpaired *t*-test (http://graphpad.com/quickcalcs/ttest1.cfm).

Expression analysis by RT-quantitative PCR: For *BvPRR7* cold induced and developmental expression analyses, seeds from annual and biennial sugar beet genotypes were sown in 9 cm pots and grown under long day conditions. Thereafter, different plant tissues from both beet genotypes were collected (at 6 h after the onset of light) at 9 different time points: 2 weeks after sowing, 1 d before the cold (4 °C) treatment, after 24 h of the cold treatment, 45 and 90 d after the beginning of the cold treatment, and 1 and 90 d after the cold treatment (Table 1 Suppl.). In all cases, three biological replicates per accession per nine different time points were harvested. In a separate experiment to analyze *BvPRR7* diurnal expression, both annual and biennial sugar beet seeds for three biological replicates per time point were grown under long day conditions. Young leaves were collected two weeks after sowing every three hours during a 24 h interval.

For diurnal expression analysis in *Arabidopsis* seedlings over-expressing *BvPRR7* either in the *aprr7-11* mutant background (*BvPRR7*-OxM5) or in the Col-0 background (*BvPRR7*-OxCol-0), the T3 transgenic lines were selected. The Col-0 plants and *aprr7-11* mutants were used as controls. Seeds from these selected samples were sown on soil and grown under long day for 14 d. Thereafter, five plants for each of three biological replicates per time point were sampled starting directly at ZT 0 at a 4 h interval for a 24 h interval. The harvested tissues were immediately frozen in liquid nitrogen and kept at -80 °C for further analyses.

Ribonucleic acid was extracted using a *PeqGOLD* plant RNA isolation kit (*Peqlab*, Erlangen, Germany) and treated with a DNase digestion kit (*Peqlab* or *Ambion*, Austin, USA). Concentration of all extracted RNAs was measured using a *NanoDrop 2000* spectrophotometer (*PeqLab*), and the integrity of the nucleic acids was analyzed on a 1.5 % (m/v) agarose gel. Either 0.5 µg (*Arabidopsis*) or 1 µg (beet) of total RNA was reverse-transcribed using a *First Strand* cDNA synthesis kit (*Fermentas*, St. Leon-Rot, Germany), and the synthesized cDNA was diluted 1:10 (cDNA:water). Thereafter, 2 mm^3 of the diluted cDNA was used in a PCR test using a housekeeping gene *ISOPENTENYL PYROPHOSPHATE 2* (*IPP2*) for *Arabidopsis* or *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*BvGAPDH*) for *BvPRR7* expression analysis in beet.

For all expression analyses, a *CFX96* real-time PCR machine (*Bio-Rad Laboratories*, Munich, Germany) was used for real time quantitative PCR (RT-qPCR). A reaction volume was 20 mm^3 and contained 10 µM each specific primer, 2 mm^3 of diluted reverse-transcribed products and 10 mm^3 *Platinum SYBR Green* qPCR

Supermix-UDG with *ROX* (*Invitrogen*, Darmstadt, Germany). All samples were analyzed with three biological replicates and in technical triplicates. Normalized expression values relative to the endogenous control gene (*BvGAPDH* or *AtIPP2*) were calculated using a modified formula from Pfaffl (2001), incorporating PCR efficiencies for both reference genes and target genes for all analyzed samples. All primers used are listed in Table 2 Suppl.

Results and discussion

The *APRR7* belongs to the *PRR3/PRR7* lineage whose primary role has been linked, together with those of other *PRR* genes, to circadian clock function. In beet there are two closely related genes, *BTC1* and *BvPRR7* which exhibit a similar grade of homology to the *Arabidopsis PRR7* gene. Pin *et al*. (2012) showed with transgenic knock down experiments that *BTC1* is a major regulator of flowering time, but expression analysis of clock genes did not reveal a mis-regulation of diurnal rhythms and thus may not be involved in the clock function. Therefore, we started to analyze whether *BvPRR7* might be a functional *APRR7* ortholog in sugar beet.

To functionally characterize *BvPRR7*, we analyzed the sequence of the gene and encoded protein and created a phylogenetic tree together with known *PRR7* homologs from other species. Therefore, a cDNA of 2.3 kb of *BvPRR7* was amplified with several primer combinations from an annual genotype, the fragments were sequenced and used with the reference sequence from a biennial genotype (Dohm *et al.* 2014) to annotate exon and intron boundaries. The *BvPRR7* gene model has nine exons with eight intervening introns (Fig. 3*A* Suppl.). In a 2 kb annotated promoter region, we found 13 GT-1 motifs (Fig. 1 Suppl.) as well as light (GT-CORE, SORLIP, and I-BOX) and cold regulatory consensus motifs (Fig. 1 Suppl.). We further compared the amino acid sequence between the two PRR7 homologs in beet (BvPRR7 and BTC1) with APRR7. The BTC1 had several small amino acid insertions compared to APRR7 and BvPRR7 whereas BvPRR7 had a large 32 amino acid insertion after a REC (receiver) domain and a truncated CCT domain due to a premature stop codon (Fig. 2 Suppl.). The BvPRR7 had an 81 % amino acid identity at the REC (domain), and 91 % at the truncated CCT domain to those of APRR7. An overall amino acid identity between BvPRR7 and APPR7 was slightly higher with 43.5 % than the 42.3 % between BTC1 and APRR7 (Fig. 3*B* Suppl.).

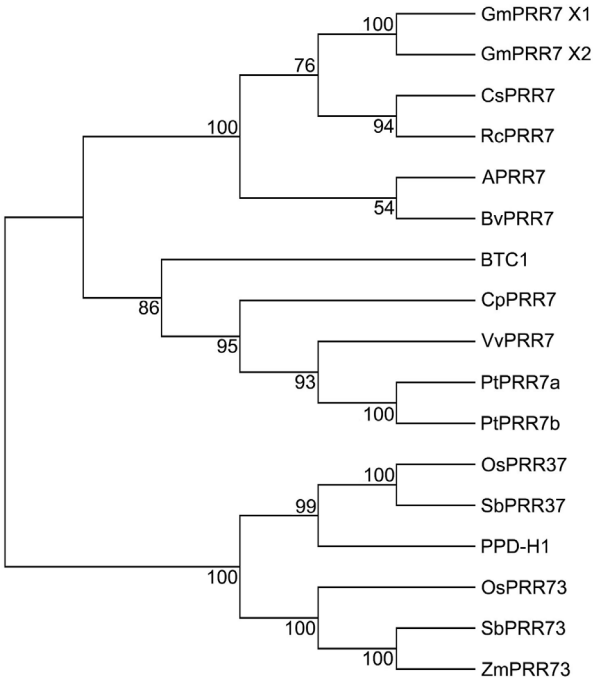

Fig. 1. The phylogenetic relationship of *Pseudo Response Regulator 7* (*PRR7*) homologs. Multiple protein alignments were done using *Clustal Omega*, and the tree was constructed using *MEGA 6* (considering a non-uniformity of evolutionary rates) protein model. Bootstrap values were calculated for 500 replications. Cp - *Carica papaya*, Vv - *Vitis vinifera*, Ppd-H1 - photoperiod-H1 from barley, Bv - *Beta vulgaris*, At - *Arabidopsis thaliana* showing the normal nomenclature for *APRR* genes; Gm - *Glycine max*, Cs - *Citrus sinensis*, Rc - *Ricinus communis*, Pt - *Populus tremula*, Os - *Oryza sativa*, Sb - *Sorghum bicolor*, Zm - *Zea mays*, BTC1 - Bolting Time Control 1 from *Beta vulgaris*. Bootstrap values [%] are shown at the nodes.

For a phylogenetic relationship among *PRR7* homologs in beet and some selected monocot and dicot species, the protein sequences were aligned, and a phylogenetic tree was constructed (Fig. 1). The rooted tree clearly separated into two monophyletic branches for monocots and dicots; with *Arabidopsis* APRR7 and beet BvPRR7 clustering together (Fig. 1). The tree suggests that the ancestral lineage that forms BTC1 existed prior to the common ancestral lineage of both BvPRR7 and APRR7 (Fig. 1 and Fig 4 Suppl.; a bootstrap value of 60 %). However, analyzing a relationship among family members of the *PRR* gene family across different selected species, the PRR lineage diverged into three clades *PRR3* and *7*, *PRR9* and *5*, and *TOC1* before the separation into the monophyletic classes of monocots and dicots (Fig. 4 Suppl.).

To determine expression of *BvPRR7*, tissues from both the annual and biennial beet genotypes were harvested at different developmental stages: whole seedlings (T1), leaves from vegetative plants, leaves from plants that flowered, as well as leaves, roots, stems, and flowers from plants that started to flower (Table 1 Suppl.). Transcription of *BvPRR7* was low in all tissues analyzed. In the leaf tissue of the vegetative plants and the root tissue of the flowering plants, the transcription was significantly higher in the biennial beet when

compared to the annual beet ($P < 0.05$). The same trend was visible in leaves at the bolting stage and in seedlings although not statistically significant (Fig. 2*A*). However, in tissues harvested at the flowering stage (leaves, stems, flowers), *BvPRR7* expression was somewhat higher in the annual samples.

An obligate requirement of cold and the need of subsequent long day for flowering are the two environmental factors which regulate reproductive development in sugar beet. In *Arabidopsis*, the clock genes *APRR7* and *APRR9* are involved in temperature responses (Salome and McClung 2005, Salome *et al.* 2010). Therefore, we analyzed whether *BvPRR7* responds to cold treatments. Leaf samples were collected at ZT 6 at different time points before, during, and after the cold treatment (T2 to T7, Table 1 Suppl.; Fig. 2*B*). Prior to vernalization (T2) and at day 1 at 4 °C (T3), the relative expression of *BvPRR7* was low (Fig. 2*B*) whereas after 45 d in the cold (T4), the transcription of *BvPRR7* increased about 13-fold

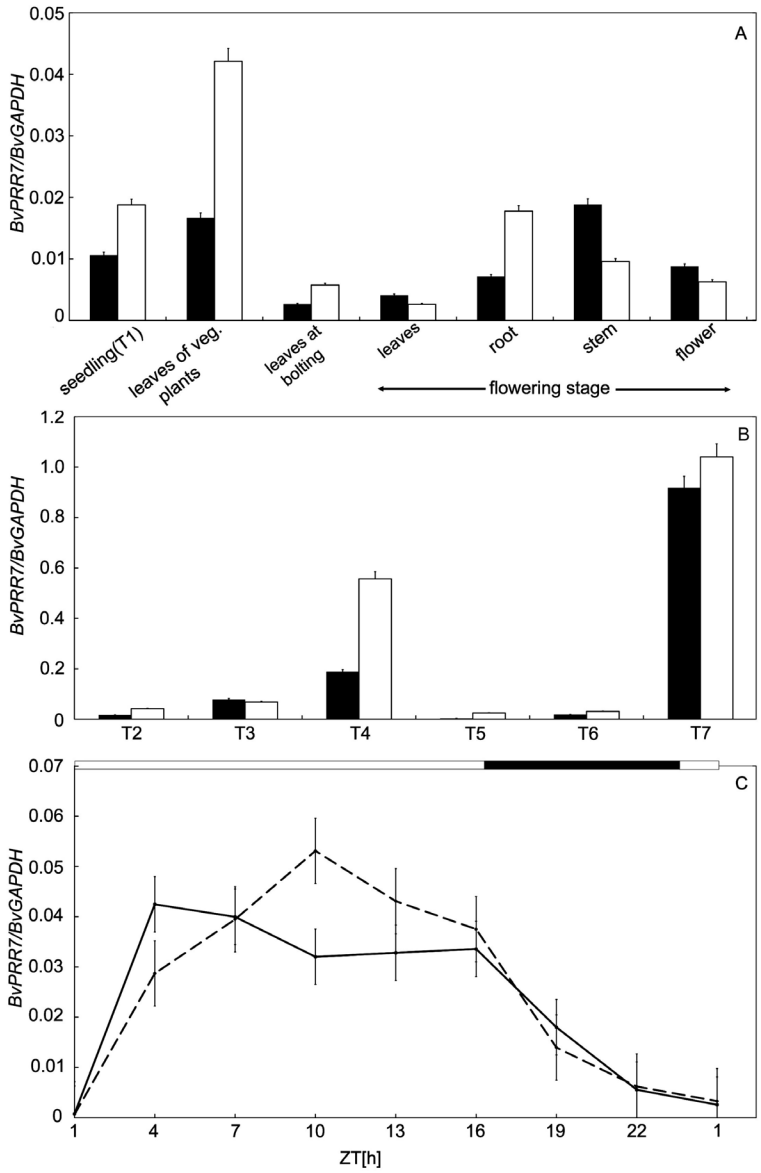

Fig. 2. Expression patterns of *BvPRR7* in annual and biennial beet accessions. *A* - Developmental expression profile of *BvPRR7* measured in different organs of annual (*black bars*) and biennial (*white bars*) plants. Samples taken for reproductive stage analysis were from plants which were initially vernalized at 4 °C, and thereafter transferred to 20 °C. *B* - Vernalization induced expression pattern of *BvPRR7* in annual (*black bars*) and biennial (*white bars*) plants. Leaf samples were harvested from plants grown for two weeks at 20 °C (T2), 24 h after transfer of plants to 4 °C (T3), 45 d at 4 °C (T4), 90 d at 4 °C, which marks the end of vernalization (T5), 24 h after return to 20 °C (T6), and 21 d at 20 °C (T7). *C* - Diurnal expression profile of *BvPRR7* in annual (a *dashed line*) and biennial (a *solid line*) plants after three weeks under a 16-h photoperiod. All analyses were done in three biological and three technical replicates per developmental stage per genotype. On top of the graph, light and dark phases are shown with an *open* and *black bar*, respectively.

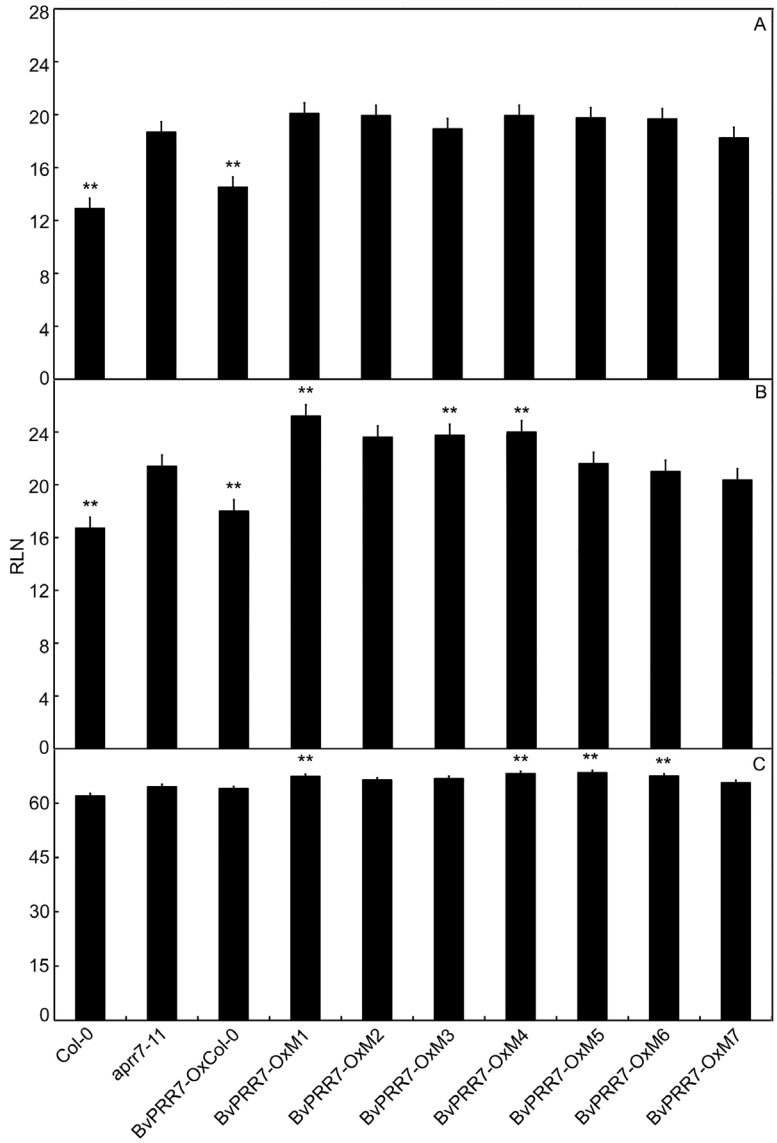

Fig. 3. Means of rosette leaf number (RLN) of selected T3 homozygous lines expressing *BvPRR7* under the constitutive CaMV 35S promoter in Col-0 (*BvPRR7*-OxCol-0) or an *aprr7-11* mutant (*BvPRR7*-OxM1-7). *A* - Plants grown under long day at 20 °C, *B* - plants grown under long day at 18 °C, *C* - plants grown under short day at 18 °C. Means ± SEs, $n = 9$ - 12. The *asterisks* indicate significant differences from Col-0 at $P \leq 0.05$.

in the annual and biennial types of beet and dropped after additional six weeks in the cold (T5). After return to long day at 20 °C (24 h; T6), the expression remained at the initial levels; it increased again dramatically after three weeks at 20 °C (T7; Fig. 2*B*).

Since the promoter of *BvPRR7* contains light regulatory elements and motifs sufficient for light induction, we analyzed whether *BvPRR7* transcript accumulation was altered in a diurnal light/dark cycle (16 h/8 h) at 20 °C. We found that *BvPRR7* expression oscillated diurnally (Fig. 2*C*). The peak of the expression in both genotypes occurred in the light phase at around ZT 4 and ZT 10. A decrease in expression in both genotypes coincided with the start of the night cycle at ZT 16 (Fig. 2*C*) and was similar to the expression of its homolog *APRR7* (Matsushika *et al.* 2000, Yamamoto *et al.* 2003, Ito *et al.* 2005, Nakamichi *et al.* 2005).

In *Arabidopsis*, Myb encoding transcription factors *CCA1/LHY* and *APRR1* (*TOC1*) form the core oscillator of the circadian clock and mutations in any of these genes affect multiple outputs (Wang and Tobin 1998, Nagel and Kay 2012). Previous experiments showed that *APRR7* and *CCA1* regulate each other's expression (Farre *et al.* 2005, Nakamichi *et al.* 2010). In addition, *APRR7* has a subtle effect on flowering time (Yamamoto *et al.* 2003). To confirm a conservation of function between *BvPRR7* and *APRR7*, we attempted to test the possibility of the *BvPRR7* protein to rescue the late flowering phenotype of the *aprr7-11* loss-of-function mutant. Therefore, we over-expressed *BvPRR7* under the control of the constitutive

35S CaMV promoter in both the Col-0 (*BvPRR7*-OxCol-0) background and the *aprr7-11* mutant background (lines *BvPRR7*-OxM1-7). The T3 homozygous lines were phenotyped under both short-day conditions and long-day conditions together with Col-0 and the *aprr7-11* mutant as controls. The RLN was used as measure of flowering time (Koornneef *et al.* 1991).

In all photoperiodic conditions tested, Col-0 flowered earlier than the *aprr7-11* mutant, and the effect was more pronounced under long-day conditions. Under long-day conditions at 20 °C, no differences were observed in the RLN between *BvPRR7* over-expressing lines in the *aprr7-11* mutant background (*BvPRR7*-OxM1-7) and the *aprr7-11* mutant control (Fig. 3*A*). Flowering was delayed at a lower temperature (18 °C), and four lines (*BvPRR7*-OxM1, 2, 3, 4) flowered later than the *aprr7-11* mutant (Fig. 3*B*). The same trend was visible under short-day conditions at 18 °C where four lines (*BvPRR7*-OxM1, 4, 5, 6) flowered later compared to the *aprr7-11* mutant although the difference in RLN between these plants was more subtle (Fig. 3*C*). In addition, the *BvPRR7* over-expressing line in the Col-0 background, *BvPRR7*-OxCol-0, flowered also a bit later, although statistically not significantly, than Col-0 under all photoperiodic conditions tested (Fig. 3*A*-*C*).

Furthermore, we analyzed the effect of the *BvPRR7* transgene on expression of *CCA1* under long days. We selected two T3 lines expressing *BvPRR7* either in the *aprr7-11* mutant (*BvPRR7*-OxM5) or in the Col-0 background (*BvPRR7*-OxCol-0) for expression analysis. We observed that the amount of *CCA1* mRNA was slightly lower in Col-0 compared to the *aprr7-11* mutant in the day and night cycle (Fig. 4) indicating a repressive function of *APRR7* on *CCA1* expression in the Col-0 plants. A stronger reduction in transcript levels of *CCA1* was observed in the transgenic lines over-expressing *BvPRR7*. In both transgenic lines, *BvPRR7*-OxCol-0 and *BvPRR7*-OxM5, the accumulation of transcripts was drastically reduced and the diurnal rhythm was almost abolished (Fig. 4).

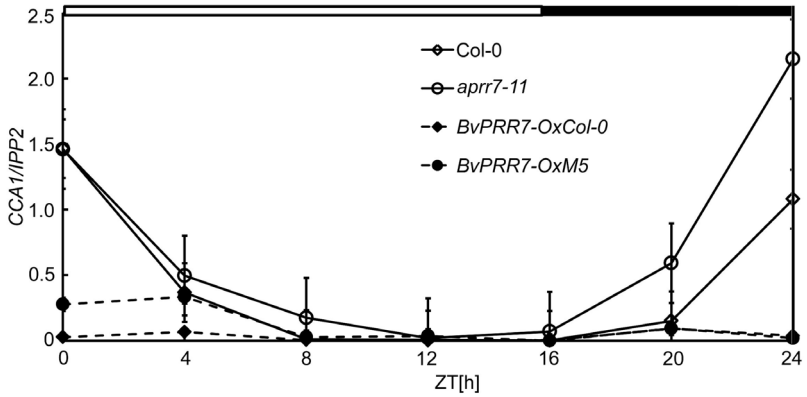

Fig. 4. Diurnal change of expression of the clock gene *AtCCA1* in response to *BvPRR7* over-expression in transgenic *Arabidopsis* seedlings. Two T3 transgenic lines (*BvPRR7*-OxCol-0 (*dashed line with black rhombi*) and *BvPRR7*-OxM5 (*dashed line with black circles*) expressing *BvPRR7* in Col-0 and *aprr7-11* backgrounds, in addition with an *aprr7-11* mutant (*solid line with open circles*) and Col-0 (*solid line with open rhombi*) as control were selected. The plants were grown under long day for 14 d and sampled in 4 h intervals. The expression value of *AtCCA1* is expressed relative to an endogenous gene *AtIPP2*. All reactions were performed in three biological replicates and in technical triplicates.

Sugar beet was established as sugar storing root crop only after the selection of biennial beet that did not flower in the first year as it is common among their wild relatives. Since flowering goes to the expense of root development and the amount of stored sucrose, roots are harvested after the end of the first growing period when the plants are still in their vegetative growth stage. Classical genetics localizes the bolting locus *B* as the main regulator for the decision of annual *versus* biennial life histories in beet (Abegg *et al.* 1936). It was shown that the *BTC1* gene at the bolting locus *B* encodes a PRR protein that is required for flowering in the first year of growth (Pin *et al.* 2012). The *BvPRR7* is closely related to *BTC1* and both belong to the *PRR3*/*PRR7* clade of PRR proteins (Pin *et al.* 2012). To understand functional difference between *BTC1* and *BvPRR7*, we characterized *BvPRR7* in beet and through a transgenic approach also in *Arabidopsis*. We found that *BvPRR7* was diurnally regulated and responded to the cold in the beet. In addition, our transgenic analyses show that *BvPRR7* was a clock gene and modulated the expression of *CCA1* in *Arabidopsis*. Finally, our results provide evidence for functional diversification of the *PRR7*-like genes in beet.

A phylogenetic relationship of PRR proteins from monocots and dicots revealed clustering BvPRR7 and APRR7, which may suggest a closer functional relationship of these two proteins than between BTC1 and APRR7 (Fig. 1 and Fig. 4 Suppl.). BvPRR7 and APRR7 also showed a slightly higher protein identity to each other than APRR7 to BTC1 (Fig. 3*B* Suppl.), suggesting an orthologous relationship of BvPRR7 and APRR7. Interestingly, BvPRR7 contained a shortened CCT domain (Fig. 2 Suppl.) that might still be able to mediate protein interactions but is not expected to have DNA binding properties due to lack of the C-terminal part of the domain. This is a prerequisite for all known

flowering time genes with a CCT domain, *e.g.*, CO (Wenkel *et al.* 2006), and the truncation might indicate that BvPRR7 does not have a major function in flowering time regulation.

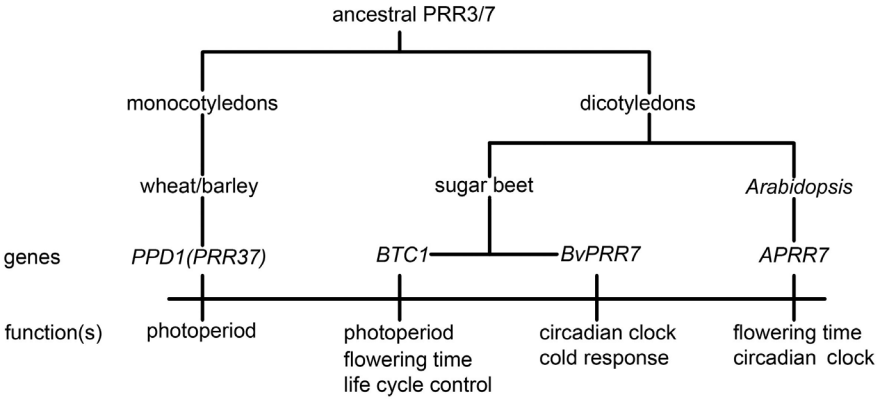

Fig. 5. Evolving functions of *PRR7* homologs in beet in relation to other species (for more information see Supplement).

Our expression analyses show that *BvPRR7* responded dramatically to a cold treatment (Fig. 2*B*). The observed increase in *BvPRR7* transcription was about 13-fold in comparison to the transcription before the cold treatment and was much higher than values for *BTC1* which showed only an increase of about 40 % in transcription after 40 d at 4 °C (Pin *et al.* 2012). The *APRR7* also responds to cold, but transcription declines immediately after transfer to lower temperatures, and it is then restored to the initial level (James *et al.* 2012). It has been shown previously that *APRR7* and other components of the circadian clock play a role in temperature compensation of the clock (Salome *et al*. 2010, Nagel *et al*. 2012). Furthermore, a link between the clock, sugar deficiency, and cold tolerance in *Arabidopsis* has also been observed (Cao *et al.* 2007). The observation that *BvPRR7* showed a much stronger response to lower temperatures than *APRR7* might indicate that *BvPRR7* could play a wider role in temperature compensation or other environmental temperature responses in beet.

Under long days, *aprr7* mutants of *Arabidopsis* show a subtle delay in flowering compared to the wild type Col-0 (Yamamoto *et al.* 2003, Nakamichi *et al.* 2005). Other *PRR7* homologs like *PPD-H1* in barley (Turner *et al.* 2005), *HEADING DATE 2* (*PRR37*) in rice (Yano and Sasaki 1997, Lin *et al.* 1998, Li *et al.* 2003), *MATURITY 1* (*Ma 1*) in *Sorghum* (Murphy *et al.* 2011, Yang *et al.* 2014) also affect flowering time depending on photoperiodic conditions. In an attempt to functionally complement *aprr7* mutants with the CaMV 35S promoter driven *BvPRR7* transgene, we analyzed if *BvPRR7* had an effect on flowering time of the mutant. However, over-expression of *BvPRR7* did not show a reversion of late bolting in the *aprr7-11* mutant. Surprisingly, some of the transformed mutants flowered even later than the untransformed control (Fig. 3*A*-*C*), which was more pronounced at 18 °C than at 20 °C (Fig. 3*A*). Over-expression of rice *OsPRR37* complements the late flowering phenotype of *aprr7-11*, but only when the transgene is under the control of the endogenous *Arabidopsis APRR7* promoter (Murakami *et al.* 2007). However, this was not shown for the *APRR7* gene itself. It has been reported earlier that in *Arabidopsis* the morning loop components of the clock, *CCA1*, *LHY*, *PRR7*, and *PRR9*, are not only involved in temperature compensation (Salome *et al.* 2010) but also regulate flowering time and other physiological and developmental processes (Ouyang *et al.* 1998, Wang and Tobin 1998, Mizoguchi *et al*. 2002, Dodd *et al.* 2005, Greenup *et al.* 2009). Disruption of the circadian rhythm due to constitutive expression of the clock gene *CCA1* has been shown to significantly delay flowering time in transgenic *Arabidopsis* plants (Wang and Tobin 1998). Similarly, we show here that the over-expression of *BvPRR7* in *Arabidopsis* affected the diurnal expression of the core clock gene *CCA1* (Fig. 4). Therefore, observed late flowering in the lines over-expressing *BvPRR7* might be a direct consequence of the disturbed rhythm of *CCA1*. A delayed flowering time is also seen in the transgenic line in the Col-0 background (*BvPRR7*-OxCol-0) under all conditions tested although subtle when compared to Col-0, and not statistically significant (Fig. 3). Farre and Kay (2007) also showed that *Arabidopsis* plants constitutively over-expressing *APRR7* have a severe loss of circadian and diurnal rhythms but have not reported any flowering time difference. Although it is premature to form a clear picture of a relationship between *BvCCA1* and *BvPRR7* in beet, it may be assumed that the core loop as described in *Arabidopsis* is also retained in beet. This notion is supported by evidence that the basic *CCA1*/*LHY* loop including components *PRR7* and *PRR9* was formed prior to the speciation of monocot and dicot species (McClung 2010, Takata *et al.* 2010, Karlgren *et al.* 2013).

Our data suggest that *BvPRR7* has retained core clock functions similar to its ortholog *APRR7*. Furthermore, our result together with the data of Pin *et al*. (2012) suggests that the *PRR7*-like genes in beet have evolved a different function (Fig. 5). *BTC1* is a major photoperiod response determinant in beet similar to most known *PRR37* genes in monocots, *e.g.*, *PPD-H1* in barley (Turner *et al.* 2005).

However, it has also adopted a new role in the vernalization response of beet (Pin *et al.* 2012). The *BTC1* regulates genes that are downstream of the circadian clock whereas obvious effects on circadian clock genes are not detected (Pin *et al.* 2012) indicating functions outside of the clock. By contrast, we show that *BvPRR7* not only exhibited a diurnal regulation in beet, but also disturbed the expression of the core clock gene *CCA1* if over-expressed in *Arabidopsis*. Finally, the observed drastic expression increase of *BvPRR7* in response to cold may suggest an additional unknown function for a *PRR7*-like gene in beet, which needs further assessment.

References

Abegg, F.A.: A genetic factor for the annual habit in beets and linkage relationship. - J. agr. Res. **53**: 493-511, 1936.

Andres, F., Coupland, G.: The genetic basis of flowering responses to seasonal cues. - Nat. Rev. Genet. **13**: 627-639, 2012.

Arumuganathan, K., Slattery, J.P., Tanksley, S.D., Earle, E.D.: Preparation and flow cytometric analysis of metaphase chromosomes of tomato. - Theor. appl. Genet. **82**: 101-111, 1991.

Beales, J., Turner, A., Griffiths, S., Snape, J.W., Laurie, D.A.: A pseudo-response regulator is misexpressed in the photoperiod insensitive *Ppd-D1a* mutant of wheat (*Triticum aestivum* L.). - Theor. appl. Genet. **115**: 721-733, 2007.

Campoli, C., Shtaya, M., Davis, S.J., Von Korff, M.: Expression conservation within the circadian clock of a monocot: natural variation at barley *Ppd-H1* affects circadian expression of flowering time genes, but not clock orthologs. - BMC Plant Biol. **12**: 97, 2012.

Cao, S.Q., Song, Y.Q., Su, L.: Freezing sensitivity in the *gigantea* mutant of *Arabidopsis* is associated with sugar deficiency. - Biol Plant **51**: 359-362, 2007.

Chen, Z.J.: Genomic and epigenetic insights into the molecular bases of heterosis. - Nat. Rev. Genet. **14**: 471-482, 2013.

Chia, T.Y., Muller, A., Jung, C., Mutasa-Gottgens, E.S.: Sugar beet contains a large *CONSTANS-LIKE* gene family including a *CO* homologue that is independent of the early-bolting (*B*) gene locus. - J. exp. Bot. **59**: 2735-2748, 2008.

Clough, S.J., Bent, A.F.: Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*.- Plant J. **16**: 735-743, 1998.

Dally, N., Xiao, K., Holtgrawe, D., Jung, C.: The *B2* flowering time locus of beet encodes a zinc finger transcription factor. - Proc. nat. Acad. Sci. USA **111**: 10365-10370, 2014.

Dodd, A.N., Salathia, N., Hall, A., Kevei, E., Toth, R., Nagy, F., Hibberd, J.M., Millar, A.J., Webb, A.A.: Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. - Science **309**: 630-633, 2005.

Dohm, J.C., Minoche, A.E., Holtgrawe, D., Capella-Gutierrez, S., Zakrzewski, F., Tafer, H., Rupp, O., Sorensen, T.R., Stracke, R., Reinhardt, R., Goesmann, A., Kraft, T., Schulz, B., Stadler, P.F., Schmidt, T., Gabaldon, T., Lehrach, H., Weisshaar, B., Himmelbauer, H.: The genome of the recently domesticated crop plant sugar beet (*Beta vulgaris*). - Nature **505**: 546-549, 2014.

Edgar, R.S., Green, E.W., Zhao, Y., Van Ooijen, G., Olmedo, M., Qin, X., Xu, Y., Pan, M., Valekunja, U.K., Feeney, K.A., Maywood, E.S., Hastings, M.H., Baliga, N.S., Merrow, M., Millar, A.J., Johnson, C.H., Kyriacou, C.P., O'Neill, J.S., Reddy, A.B.: Peroxiredoxins are conserved markers of circadian rhythms. - Nature **485**: 459-464, 2012.

Farre, E.M., Harmer, S.L., Harmon, F.G., Yanovsky, M.J., Kay, S.A.: Overlapping and distinct roles of *PRR7* and *PRR9* in the *Arabidopsis* circadian clock. - Curr. Biol. **15**: 47-54, 2005.

Farre, E.M., Kay, S.A.: PRR7 protein levels are regulated by light and the circadian clock in *Arabidopsis*. - Plant J. **52**: 548-560, 2007.

Greenup, A., Peacock, W.J., Dennis, E.S., Trevaskis, B.: The molecular biology of seasonal flowering-responses in *Arabidopsis* and the cereals.- Ann. Bot. **103**: 1165-1172, 2009.

Höfgen, R., Willmitzer, L.: Storage of competent cells for *Agrobacterium* transformation. - Nucl. Acids Res. **16**: 9877, 1988.

Imaizumi, T.: *Arabidopsis* circadian clock and photoperiodism: time to think about location. - Curr. Opin. Plant Biol. **13**: 83-89, 2010.

Ito, S., Nakamichi, N., Matsushika, A., Fujimori, T., Yamashino, T., Mizuno, T.: Molecular dissection of the promoter of the light-induced and circadian-controlled *APRR9* gene encoding a clock-associated component of *Arabidopsis thaliana*. - Biosci. Biotechnol. Biochem. **69**: 382-390, 2005.

James, A.B., Syed, N.H., Bordage, S., Marshall, J., Nimmo, G.A., Jenkins, G.I., Herzyk, P., Brown, J.W., Nimmo, H.G.: Alternative splicing mediates responses of the *Arabidopsis* circadian clock to temperature changes. - Plant Cell **24**: 961-981, 2012.

Jung, C., Müller, A.E.: Flowering time control and applications in plant breeding. - Trends Plant Sci **14**: 563-573, 2009.

Karlgren, A., Gyllenstrand, N., Kallman, T., Lagercrantz, U.: Conserved function of core clock proteins in the gymnosperm Norway spruce (*Picea abies* L. Karst). - PLoS ONE **8**: e60110, 2013.

Kinmonth-Schultz, H.A., Golembeski, G.S., Imaizumi, T.: Circadian clock-regulated physiological outputs: dynamic responses in nature. - Semin. cell. dev. Biol. **24**: 407-413, 2013.

Koornneef, M., Hanhart, C.J., Van der Veen, J.H.: A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*.- Mol. gen. Genet. **229**: 57-66, 1991.

Li, Z.K., Yu, S.B., Lafitte, H.R., Huang, N., Courtois, B., Hittalmani, S., Vijayakumar, C.H., Liu, G.F., Wang, G.C., Shashidhar, H.E., Zhuang, J.Y., Zheng, K.L., Singh, V.P., Sidhu, J.S., Srivantaneeyakul, S., Khush, G.S.: QTL × environment interactions in rice. I. heading date and plant height. - Theor. appl. Genet. **108**: 141-153, 2003.

Lin, S.Y., Sasaki, T., Yano, M.: Mapping quantitative trait loci controlling seed dormancy and heading date in rice, *Oryza sativa* L., using backcross inbred lines. - Theor. appl. Genet. **96**: 997-1003, 1998.

Locke, J.C., Kozma-Bognar, L., Gould, P.D., Feher, B., Kevei, E., Nagy, F., Turner, M.S., Hall, A., Millar, A.J.: Experimental validation of a predicted feedback loop in the multi-oscillator clock of *Arabidopsis thaliana*. - Mol. Syst. Biol. **2**: 59, 2006.

Matsushika, A., Makino, S., Kojima, M., Mizuno, T.: Circadian waves of expression of the *APRR1/TOC1* family of pseudo-response regulators in *Arabidopsis thaliana*: insight into the plant circadian clock.- Plant Cell Physiol. **41**: 1002-1012, 2000.

McClung, C.R.: A modern circadian clock in the common angiosperm ancestor of monocots and eudicots. - BMC Biol. **8**: 55, 2010.

Melzer, S., Müller, A.E., Jung, C.: Genetics and genomics of flowering time regulation in sugar beet. - In: Tuberosa, R., Graner, A., Frison, E. (ed.): Genomics of Plant Genetic Resources. Pp. 3-26. Springer, Dordrecht 2014.

Mizoguchi, T., Wheatley, K., Hanzawa, Y., Wright, L., Mizoguchi, M., Song, H.R., Carre, I.A., Coupland, G.: *LHY* and *CCA1* are partially redundant genes required to maintain circadian rhythms in *Arabidopsis*. - Dev. Cell **2**: 629-641, 2002.

Murakami, M., Tago, Y., Yamashino, T., Mizuno, T.: Characterization of the rice circadian clock-associated pseudo-response regulators in *Arabidopsis thaliana*. - Biosci. Biotechnol. Biochem. **71**: 1107-1010, 2007.

Murphy, R.L., Klein, R.R., Morishige, D.T., Brady, J.A., Rooney, W.L., Miller, F.R., Dugas, D.V., Klein, P.E., Mullet, J.E.: Coincident light and clock regulation of pseudoresponse regulator protein 37 (PRR37) controls photoperiodic flowering in sorghum. - Proc. nat. Acad. Sci. USA **108**: 16469-16474, 2011.

Nagel, D.H., Kay, S.A.: Complexity in the wiring and regulation of plant circadian networks. - Curr. Biol. **22**: R648-R657, 2012.

Nakamichi, N., Kiba, T., Henriques, R., Mizuno, T., Chua, N.H., Sakakibara, H.: *PSEUDO-RESPONSE REGULATORS 9*, *7*, and *5* are transcriptional repressors in the Arabidopsis circadian clock. - Plant Cell **22**: 594-605, 2010.

Nakamichi, N., Kita, M., Ito, S., Yamashino, T., Mizuno, T.: *PSEUDO-RESPONSE REGULATORS PRR9*, *PRR7* and *PRR5*, together play essential roles close to the circadian clock of *Arabidopsis thaliana*. - Plant Cell Physiol. **46**: 686-698, 2005.

Ouyang, Y., Andersson, C.R., Kondo, T., Golden, S.S., Johnson, C.H.: Resonating circadian clocks enhance fitness in cyanobacteria. - Proc. nat. Acad. Sci. USA **95**: 8660-8664, 1998.

Pfaffl, M.W.: A new mathematical model for relative quantification in real-time RT-PCR. - Nucl. Acids Res. **29**: e45, 2001.

Pin, P.A., Zhang, W., Vogt, S.H., Dally, N., Buttner, B., Schulze-Buxloh, G., Jelly, N.S., Chia, T.Y., Mutasa-Gottgens, E.S., Dohm, J.C., Himmelbauer, H., Weisshaar, B., Kraus, J., Gielen, J.J., Lommel, M., Weyens, G., Wahl, B., Schechert, A., Nilsson, O., Jung, C., Kraft, T., Müller, A.E.: The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. - Curr. Biol. **22**: 1095-1101, 2012.

Salome, P.A., McClung, C.R.: *PSEUDO-RESPONSE REGULATOR 7* and *9* are partially redundant genes essential for the temperature responsiveness of the *Arabidopsis* circadian clock. - Plant Cell **17**: 791-803, 2005.

Salome, P.A., Weigel, D., McClung, C.R.: The role of the *Arabidopsis* morning loop components *CCA1*, *LHY*, *PRR7*, and *PRR9* in temperature compensation. - Plant Cell **22**: 3650-3661, 2010.

Sawa, M., Kay, S.A., Imaizumi, T.: Photoperiodic flowering occurs under internal and external coincidence. - Plant Signal Behav. **3**: 269-271, 2008.

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., Coupland, G.: *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. - Nature **410**: 1116-1120, 2001.

Takata, N., Saito, S., Saito, C.T., Uemura, M.: Phylogenetic footprint of the plant clock system in angiosperms: evolutionary processes of pseudo-response regulators. - BMC Evol. Biol. **10**: 126, 2010.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S.: MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. - Mol. Biol. Evol. **28**: 2731-2739, 2011.

Turck, F., Fornara, F., Coupland, G.: Regulation and identity of florigen: *FLOWERING LOCUS T* moves center stage. - Annu. Rev. Plant Biol. **59**: 573-594, 2008.

Turner, A., Beales, J., Faure, S., Dunford, R.P., Laurie, D.A.: The pseudo-response regulator *Ppd-H1* provides adaptation to photoperiod in barley. - Science **310**: 1031-1034, 2005.

Valverde, F.: *CONSTANS* and the evolutionary origin of photoperiodic timing of flowering. - J. exp. Bot. **62**: 2453-2563, 2011.

Wang, Z.Y., Tobin, E.M.: Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. - Cell **93**: 1207-1217, 1998.

Wenkel, S., Turck, F., Singer, K., Gissot, L., Le Gourrierec, J., Samach, A., Coupland, G.: *CONSTANS* and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of *Arabidopsis*.- Plant Cell **18**: 2971-2984, 2006.

Yamamoto, Y., Sato, E., Shimizu, T., Nakamich, N., Sato, S., Kato, T., Tabata, S., Nagatani, A., Yamashino, T., Mizuno, T.: Comparative genetic studies on the *APRR5* and *APRR7* genes belonging to the *APRR1*/*TOC1* quintet implicated in circadian rhythm, control of flowering time, and early photomorphogenesis. - Plant Cell Physiol. **44**: 1119-1130, 2003.

Yang, S., Murphy, R.L., Morishige, D.T., Klein, P.E., Rooney, W.L., Mullet, J.E.: Sorghum phytochrome B inhibits flowering in long days by activating expression of *SbPRR37* and *SbGHD7*, repressors of *SbEHD1*, *SbCN8* and *SbCN12*. - PLoS ONE **9**: e105352, 2014.

Yano, M., Sasaki, T.: Genetic and molecular dissection of quantitative traits in rice. - Plant mol. Biol. **35**: 145-153, 1997.

Zeilinger, M.N., Farre, E.M., Taylor, S.R., Kay, S.A., Doyle, F.J.: A novel computational model of the circadian clock in *Arabidopsis* that incorporates *PRR7* and *PRR9*. - Mol. syst. Biol. **2**: 58, 2006.