Earliness per se 3 locus from wheat (*Triticum* L. sp.) and barley (*Hordeum vulgare* L.) disrupts circadian clock function

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1 Summary

In temperate grasses, such as wheat and barley, earliness *per se* is understood as the intrinsic difference in flowering time of fully vernalized plants grown under long day conditions.

One task of investigations was to confirm the position of the previously mapped *Eps3AL*^{m} locus (Hori et al. 2007; Shindo et al. 2002) and to narrow down the interval between the flanking markers. To this end, 110 einkorn wheat recombinant inbred lines were evaluated. The population had been obtained from a cross between an early flowering X-ray mutant KT3-5 (*Triticum monococcum* L.) of cultivated spring type and a wild type line KT1-1 of *Triticum boeoticum* Boiss. (winter type). In the current study, molecular marker mapping was performed resulting in a medium density genetic linkage map. QTL analyses of heading date, anthesis date, spikelet number and ear length showed the best linkage with markers located close to the telomeric region of 3AL. The highest LOD scores were 4.86, 4.18, 2.80 and 3.79 for heading date, anthesis date, spikelet number and ear length, respectively, with a threshold value equaling 2.1 (at p=0.05). One marker locus co-segregating with the LOD peak was found to be deleted in the mutant parent KT3-5. Thus, the *Eps-3A*^{m}*L* interval was delimited to <1cM with the successful usage of 14 new markers developed based on the colinearity with rice and *Brachypodium*.

Secondly, two einkorn wheat lines (*Triticum monococcum* L. x *T. boeoticum* Boiss.), RIL25 (early) and RIL71 (late) were selected from the RILWA1 population to generate a new F2 population for fine mapping of the *Eps-3A*^{m} locus. About 650 F2 individuals were screened for genetic recombinations and 4 new markers were added utilizing the physical map from barley

chromosome 3H (in collaboration with Dr. Nils Stein). This way, the locus could be delimited to approximately 350 kb and contained only two putative genes—*TmLUX* (*lux arrhythmo*, an evening clock element) and *TmPUMILIO* (*TmPUM*). Moreover, both genes were found to be deleted in the mutant parent of the RILWA1 population KT3-5 (*T. monococcum* L.) as well as in RIL25.

The deletion of *TmLUX* caused clock distortion and miss-expression of circadian clock-related genes. Both effects were detectable by using delayed fluorescence measurements as well as a time-course qRT-PCR experiment on key circadian clock genes; for example *TmTOC1* (*timing of CAB2 expression 1*), *TmPRR9* (*Pseudo response regulator 9*) and *TmLHY* (*late elongated hypocotyl*). On the other hand, sequences of the *TmPUMILIO* (RNA-binding protein) and *TmLUX* were subjected to screen a barley TILLING population of *cv.* "Barke", resulting in 34 mutations for *HvPUM* and 21 mutations for *HvLUX*, respectively. However, none of the mutants flowered earlier than *cv.* "Barke".

Moreover, it could be shown that the *early maturity 10* (*eam10*) locus on 3HL from a barley mutant Super Precoz 2H was in fact *Eps-3*. The DNA-binding domain (MYB) of the *HvLUX* re-sequenced from this genotype contained one unique amino acid substitution (serin to cystein). Importantly, missing function of the LUX protein in Super Precoz 2H could be verified by assaying the gene expression, indicating a similar circadian clock disruption like in the KT3-5 wheat mutant. Besides, mutants in both einkorn wheat and barley displayed similar phenotypes; photoperiod-insensitive early flowering and shortened spikes.

LUX was also re-sequenced in a collection of 96 diploid and tetraploid wheats revealing a single A-genome specific haplotype with a unique 21nt deletion found in the MYB domain. The Chinese cultivar Tsing Hua No. 599 from province Yunnan possessing this mutation was heading relatively early, thus supporting *LUX* as a sensible candidate for *Eps-3*. Time course qRT-PCR on this accession revealed up-regulation of *TtLUX* although without strong impact on the other clock genes analyzed.

Putative transgenic knock-down lines in barley were generated in collaboration with Dr. Götz Hensel. Time course qRT-PCR analysis on clock genes in two of them revealed expected down-regulation of *HvLUX* transcript and up-regulation of HvLUX target mRNAs. However, under long day conditions these lines did not flower early suggesting that severe clock distortion is a prerequisite for *earliness per se-3* trait.

2 List of abbreviations

BAC- **B**acterial **a**rtificial **c**hromosome

BES- **B**AC-**e**nd **s**equence

CAPS- **C**leaved **a**mplified **p**olymorphic **s**ite

cDNA- **C**omplementary **DNA,** usually derived from mRNA by RT

cM- **c**enti**M**organ

DNA- **D**eoxyribo**n**ucleic **a**cid

DF- **D**elayed **f**luorescence

GI- **Gi**gantea

LD – **L**ong **d**ay, usually 16h/8h, day/night

LBS- **L**UX **b**inding **s**ite

LHY- **L**ate elongated **hy**pocotyl

LUX- **LUX** ARRHYTHMO

MTP-**M**inimum **t**iling **p**ath

mRNA- **M**essenger **RNA**

PAV- **P**resence-**a**bsence **v**ariation

PCR- **P**olymerase **c**hain **r**eaction

PRR- **P**seudo **r**esponse **r**egulator

qPCR- **q**uantitative **PCR**

QTL- **Q**uantitative **T**rait **L**ocus

RAE- **R**elative **a**mplitude **e**rror

RIL- **R**ecombinant **i**nbred **l**ines

RNA- **R**ibo**n**ucleic **a**cid

RNAi- **R**NA **i**nterference

RT- **R**everse **t**ranscription

SD – **S**hort **d**ay, 8h/16h, day/night

SDW- **S**terile **d**istilled **w**ater

SEM- **S**tandard **e**rror of the **m**ean

SNP- **S**ingle **n**ucleotide **p**olymorphism

TILLING-**T**argeting **i**nduced **l**ocal **l**esions **i**n **g**enomes

TOC1- **T**iming **o**f **C**AB expression **1**

UBC- **Ub**iquitin-**c**onjugating enzyme

ZT- **Z**eitgeber **T**ime

3 Introduction

3.1 Wheat–a basic food source for humans

Among the most important food crops, in 2010 wheat production reached 651 million tons and only rice (672 m tons) and maize (844 m tons) production were larger, moving wheat to the third place (http://faostat.fao.org/). However, wheat (*Triticum sp.* L.) is unrivalled in its range of cultivation, from 67° N in Scandinavia and Russia to 45° S in Argentina, including elevated regions in tropics and sub-tropics (Feldman 1995). The 'Neolithic evolution', that took place ~10,000 years ago, was the time when people started agriculture instead of hunting and gathering of food. The earliest cultivated forms of wheat were diploid (2n=2x=14; genome AA; einkorn *T. monococcum* L.) and tetraploid (2n=4x=28; genome AABB; emmer *Triticum dicoccum* L.). Genetic analyses of their genomes revealed that they originated from southern-eastern part of Turkey (Dubcovsky and Dvorak 2007; Heun et al. 1997; Nesbitt 1998).

When hexaploid (2n=6x=42) bread wheat was "discovered" and selected by farmers, its cultivation spread to the Near East. It took place by about 9,000 years ago. The AA genomes of tetraploid and hexaploid wheats come from *T. urartu* L. (A^uA^u) and are clearly related to the A^mA^m genomes of wild and cultivated einkorn, while the D genome of hexaploid wheat is derived from *Aegilops tauschii*. On the contrary, the B genome of tetraploid and hexaploid wheats is probably derived from the S genome present in *Ae. speltoides* (Feldman 2001).

The importance of wheat as food supply is associated with several factors, such as high yield, presence of many forms adapted to different environments and unique properties of

dough formed from wheat flours (Shewry 2009). Currently, about 95% of the wheat grown worldwide is hexaploid bread wheat, with most of the remaining 5% being tetraploid durum wheat. Small amounts of other wheat species (einkorn, emmer, spelt) are still grown in some regions including Spain, Turkey, the Balkans and the Indian subcontinent (Shewry 2009). Nevertheless, diploid relatives of bread wheat–such as einkorn wheat–are a very valuable source for scientists because they can help to overcome the complications imposed by polyploidy, e.g. during positional cloning projects. The development of molecular markers and high-density genetic linkage maps are considerably simpler in diploid genomes. Such strategy was successfully applied to clone, for example vernalization genes *Vrn1* and *Vrn2* in *T. monococcum* (Yan et al. 2003; Yan et al. 2004).

3.2 Barley– a modern model crop

Apart from the obvious applications in animal feeding and brewing, barley (*Hordeum vulgare* L.) has become a modern model crop with constantly growing amount of resources, including extensive induced mutant collections. The barley physical map is now available giving the opportunity to quickly identify novel genes by map-based cloning. Moreover, allelic series of mutants constitute alternative and/or complementary approach towards unveiling the function of various important genes in cereal crops (Ramsay et al. 2011; The International Barley Genome Sequencing Consortium 2012; Zakhrabekova et al. 2012).

3.3 Flowering time as an important trait in cereal improvement

Plant domestication is the genetic modification of a wild species to create a new form of a plant altered to meet human needs (Doebley et al. 2006). From the beginning of breeding one

of the most important traits which were taken into consideration was flowering time. As it is understood, the time to flowering is a key adaptive trait of plants. Depending on the environment (longitude and latitude), it makes them able to flower at the optimum time for pollination, seed development and, in the case of wild plants, dispersal (Cockram et al. 2007). The so called "domestication syndrome" among different evidences also tells about high synchronization of flowering in cultivated plants in comparison to wild plants (Hammer 1984). This was mainly achieved by controlled crossing during breeding history; while discarding extremes, i.e. less valuable phenotypes.

Traits which are desirable from an agronomical point of view usually contribute for or co-segregate with features that are under negative selection pressure under wild conditions. This is the case, e.g. in maize, where polymorphisms at the *Dwarf8* gene, that are associated with early flowering also confer dwarfism (Thornsberry et al. 2001). Moreover, artificial selection leads to much stronger changes than naturally occur during evolution. This can only be achieved by choosing individuals that contain a single factor (locus) instead of multiple factors with small effects (Orr 2005). As a consequence, it might be observed that most of the variation is explained by a few major loci, while the minor loci are monomorphic in the cultivated germplasm.

It is well known that many natural mutations causing early flowering are associated with deleterious changes in flowering repressors. For example, complete deletion of *LATE FLOWERING* in *Pisum sativum* (Foucher et al. 2003) ; deletion of *VRN2* (a functional homologue of *FLC*- Flowering Locus C) in diploid wheat (*T. monococcum*) and barley (*H. vulgare*) (Yan et al.

2004); alteration of a key domain in the coding region of *Dwarf8* in maize (Thornsberry et al. 2001). Loss-of-function in the regulatory part of a flowering- promotive gene is also known: for instance, the independent natural deletions of the CArG box in *VRN1* in *T. monococcum* and in bread wheat (*T. aestivum*) (Yan et al. 2003).

3.4 Components of the flowering time determination

3.4.1 Photoperiodic response

Photoperiodicity is the length of day light favorable for a plant to flower. The general concept of the photoperiodic induction of flowering and the range of response types among plants was firstly established by scientists in the beginning of the twentieth century (Garner and Allard 1920). They divided plants into several types: short day (SD) plants which flower only below the critical day length and needs uninterrupted dark period; for example: Rice, Coffee, Strawberry, Tobacco and Maize; and long day (LD) plants which require a photoperiod with a certain number of light hours above a critical value, for instance: Clover, Oat and Ryegrass. Moreover they identified day neutral plants; e.g. Cucumbers, Roses and Tomatoes as well as some intermediates; i.e. long day facultative (Wheat, Barley, Pea and *Arabidopsis*) and short day facultative species (Cotton, Rice and Sugar cane).

3.4.2 Vernalization

Vernalization means long exposure to cold temperatures as a key signal to induce flowering. Plants displaying winter growth habit require a minimum period of 35 days for vernalization, i.e. all winter cereals and beets. Plants which do not need this treatment possess spring growth habit, i.e. bean, pea, spring varieties of cereals (Distelfeld et al. 2009).

3.4.3 Circadian clock

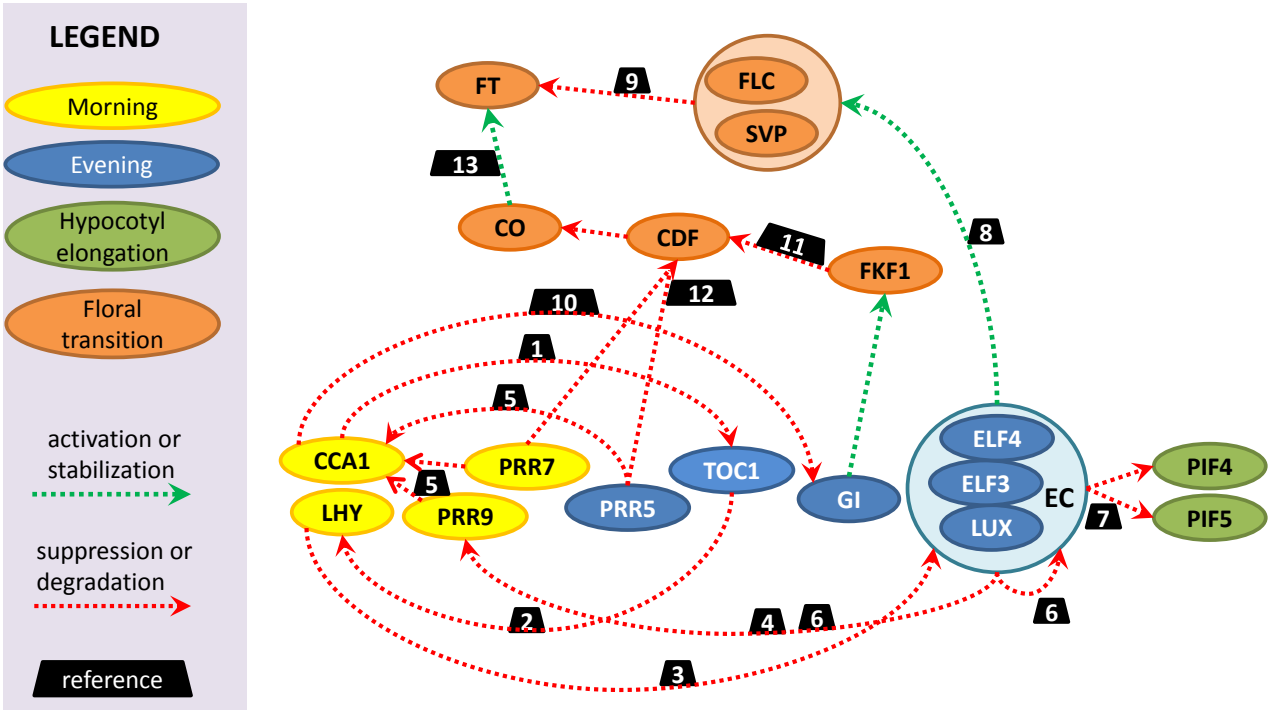

Figure 1 A simplified model of the circadian clock in *Arabidopsis thaliana* L. consisting of multiple interconnected feedback loops; each one regulates expression of the other. In the central loop two MYB transcription factors, *CCA1- circadian clock associated 1* and *LHY- late elongated hypocotyl*, act redundantly to repress *TOC1- timing of CAB2 expression 1* transcription in the morning by directly binding to its promoter (1) (Alabadi et al. 2001) . As the day progresses, expression of *LHY* and *CCA1* falls, allowing *TOC1* expression to rise. *TOC1* then closes the loop by direct suppression of *CCA1* and *LHY* (2) (Gendron et al. 2012; Huang et al. 2012; Pokhilko et al. 2012). Another loop involves *ELF3* (*early flowering 3*), that was shown to directly repress *PRR9* (*pseudo response regulator 9*), which then inhibits *CCA1/LHY* expression (3, 4, 5) (Dixon et al. 2011; Lu et al. 2012; Nakamichi et al. 2010). Similar action was proven for *LUX* (*lux arrhythmo*) but also including self-down-regulation (6) (Helfer et al. 2011). Moreover, *ELF3*, *LUX* and *ELF4* (*early flowering 4*) form a protein complex called evening complex (EC) that directly down-regulates *PIF4* and *PIF5* (*phytochrome interacting factor 4 and 5*) which are responsible for the hypocotyl growth arrest at dusk (7) (Nusinow et al. 2011). So far, there are evidences found for three possible pathways connecting the circadian clock to the floral transition event. (i) *ELF3* protein can promote accumulation of *SVP* (*short vegetative phase*) which is then necessary for the active binding of *flowering locus C* (*FLC*) to the *FT- flowering locus T* (florigen) chromatin (8,9) (Helliwell et al. 2006; Yoshida et al. 2009). (ii) every morning *CCA1* suppresses *GI* (*gigantea*) expression, however under long day *GI* can effectively stabilize *FKF1* (*F-box protein 1*) which later promotes degradation of *CDFs* (*cycling dof factors*) thus unblocking CO (*constans*) action (10,11) (Imaizumi et al. 2005; Nakamichi et al. 2007). (iii) *PRR9*, *PRR7* and *PRR5* down-regulate *CDFs* on the transcriptional level (12) (Nakamichi et al. 2007). During the absence of *CDFs*, *CO* promotes flowering by activating *FT* (13) (Yanovsky and Kay 2002)

The circadian clock refers to the endogenous rhythms in living organism with an approximate 24-hour period. The first evidence of this phenomenon was provided by d'Ortous de Mairan J.J. (1729); he noted 24-hour patterns in the movement of leaves from a heliotropic

plant, probably a *Mimosa* sp. The rhythmic movements continued even when the plants were kept in constant darkness (Pittendrigh 1993). The circadian clock regulates temperature and light sensing and influences most of the physiological processes; starting from gene expression towards metabolism, transport, growth and movements including photoperiodic flowering (Baudry and Kay 2008). A simplified model of the clock together with some output pathways in *Arabidopsis* is shown in Figure 1.

3.4.4 Thermo-sensitivity

Plants can perceive temperature differences as little as 1 degree (Kumar and Wigge 2010). As a result, large differences in flowering time can be observed while changing the temperature during growth. These differences are comparable with observations made after the switch from short to long day conditions. For instance, a mild increase in growth temperature, from 23°C to 27°C, is equally efficient in inducing flowering of *Arabidopsis* plants grown in 8-h short days and then transferred to 16-h long days (Balasubramanian et al. 2006). As a hypothetical explanation for this phenomenon authors proposed an altered function of gene products that participate in RNA splicing or contain RNA binding domains which may be a component of thermal response in plants. As a consequence, in a higher ambient temperature different forms of floral suppressors *flowering locus M (FLM)* and *MADS affecting flowering2 (MAF2)* were more abundant than in a lower temperature. However, the delayed flowering of wild accessions as a response to high ambient temperatures could be explained by the function of *flowering locus C* (*FLC*) which was found to be a potent suppressor of thermal flowering induction (Balasubramanian et al. 2006).

Many genes involved in thermo-sensitivity are circadian clock-controlled genes. One such example is *SPATULA* that inhibits vegetative growth under low ambient temperatures (Sidaway-Lee et al. 2010).

Recent research showed that a various set of genes can be involved in a high-temperature response mechanism in cereals (Hemming et al. 2012). The model proposed involves *HvFPF1-like3* (*Flowering promoting factor 1-like3)*, *HvFT1 (HvVrn3)* and *TaVER2* as promoting the generative development in such conditions, whereas *HvODDSOC2* (MADS-box protein) as being a counteracting partner (Hemming et al. 2012).

3.5 Molecular background of flowering in model plants

3.5.1 *Arabidopsis thaliana* L.

Arabidopsis thaliana L. is a small flowering plant that is widely used as a model organism in plant biology. *Arabidopsis* is a member of the mustard (*Brassicaceae*) family, which includes cultivated species such as oil seed rape (*Brassica napus* L.), cabbage (*B. oleracea* L.) and radish (*Raphanus sativus* L.). In the literature, *Arabidopsis* is described as a facultative long day plant-it flowers more rapidly under LDs, but will eventually flower under SDs (Gregory 1953). *Arabidopsis* offers important advantages for basic research in genetics and molecular biology among them are: known genomic sequence, large mutant collections, a rapid life cycle (about 6 weeks from germination to seed maturity) as well as established methods for genetic transformation utilizing *Agrobacterium tumefaciens* (http://www.arabidopsis.org/).

The underlying molecular mechanisms of flowering are very complex. It seems that the key signaling compounds in *Arabidopsis* are *FT*, also known as florigen, and *Flowering Locus C* (*FLC*)

which is the main suppressor of flowering. Both are directly involved in environmental sensing- *FT* is necessary for photoperiod response (Kardailsky et al. 1999; Kobayashi et al. 1999) whereas *FLC* plays the main role in the vernalization pathway (Koornneef et al. 1994; Lee et al. 1994). *FT* can be activated directly by *CONSTANS* (*CO*). Also *GIGANTEA* (*GI*) is a great promoter of flowering which acts negatively on *CO* repressors- *CYCLING DOF FACTORS* (*CDF*s) (Imaizumi 2009; Imaizumi et al. 2005; Sawa et al. 2007). GI is a circadian clock element which under long day conditions stabilizes F-BOX PROTEIN1 (FKF1). Then FKF1 causes CDF degradation, finally unblocking *CO* expression (Amasino 2010).

On the other hand *CO* activity is regulated by light receptors CRYPTOCHROME 1 (CRY1) and CRY2 as well as by PHYTOCHROME A (PHYA) and PHYTOCHROME B (PHYB). CRY1, CRY2 and PHYA stabilize CO protein while PHYB promotes its turnover (Valverde et al. 2004). Once FT is produced, it is transported to the shoot apical meristem (SAM) *via* phloem. Here flowering promotion requires binding of FT with transcription factor FD. Then the FT/FD complex activates *APETALA 1* (*AP1*) (Abe et al. 2005; Wigge et al. 2005) and other floral meristem-identity genes crucial for vegetative/generative phase transition; for instance *SOC1, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (Michaels et al. 2005; Yoo et al. 2005). Also another gene- *LEAFY* (*LFY*) can activate *AP1*, apart from its main function which is a differentiation of the SAM lateral cells into floral meristems (Liljegren et al. 1999).

The vernalization response at the molecular level mainly consists of different flowering suppressors. The probably most important and the first known element of this pathway is *FRIGIDA* (*FRI*); plants possessing a dysfunctional form of this gene do not require vernalization

(Johanson et al. 2000; Napp-Zinn 1987). However, the "centre" of vernalization requirement has already been mentioned—*FLC*. If over-expressed, it prevents flowering even during the absence of a functional *FRI* (Koornneef et al. 1994; Lee et al. 1994). Moreover, it was already proven that cold temperature rendered *FLC* of being insensitive to the activating effects of *FRI* (Michaels et al. 2004). The way *FLC* acts negatively on flowering is by repressing the expression of the floral activators *SOC1*, *FT* and *FD* by directly binding to them (Helliwell et al. 2006; Searle et al. 2006). On the other hand, FLC cannot work alone. As many MADS-box proteins it apparently needs another component to create a fully functional complex. In this case, it is a Short Vegetative Phase (SVP) protein which is expressed independently from vernalization treatment, unlike *FLC*. However, *SVP* activity is controlled during autonomous, thermo-sensory and gibberellin pathways (Li et al. 2008).

There are a few genes known which can switch off *FLC*: *Vernalization 2* (*VRN2*) (Gendall et al. 2001), *Vernalization Insensitive 3* (*VIN3*) (Sung and Amasino 2004) and *Vernalization 5* (*Vrn5*)/*Vin3-like 1* (*VIL1*) (Greb et al. 2007). Another internal control over flowering is contributed by micro RNAs (miRNA), which was proven of being separated from the *CO* pathway. miRNA-156 causes expression of miRNA 172 that inhibits *APETALA2*- like genes expression (*TOE1* and *TOE2*). Finally, the florigene- *FT* function is blocked (Jung et al. 2007).

There is only little understanding about the molecular background of thermo-sensing. Latest results showed that the mechanism is direct and acts on the nucleosomal level (Kumar and Wigge 2010). Scientists found that *Arabidopsis* early flowering mutant *arp6-10* was abnormally sensitive to ambient temperatures because it incorporated H2A.Z-containing nucleosomes into

the genome. H2A.Z is an alternative version of histones that makes the transcription of a nucleic acid wrapped over it highly depending on the temperature. The mutation that led to the nucleosomal enrichment of H2A.Z had very broad consequences; i.e. the higher the ambient temperature, flowering occurred earlier under short day conditions; similarly, faster elongation of hypocotyls and petiole growth were noticed (Kumar and Wigge 2010).

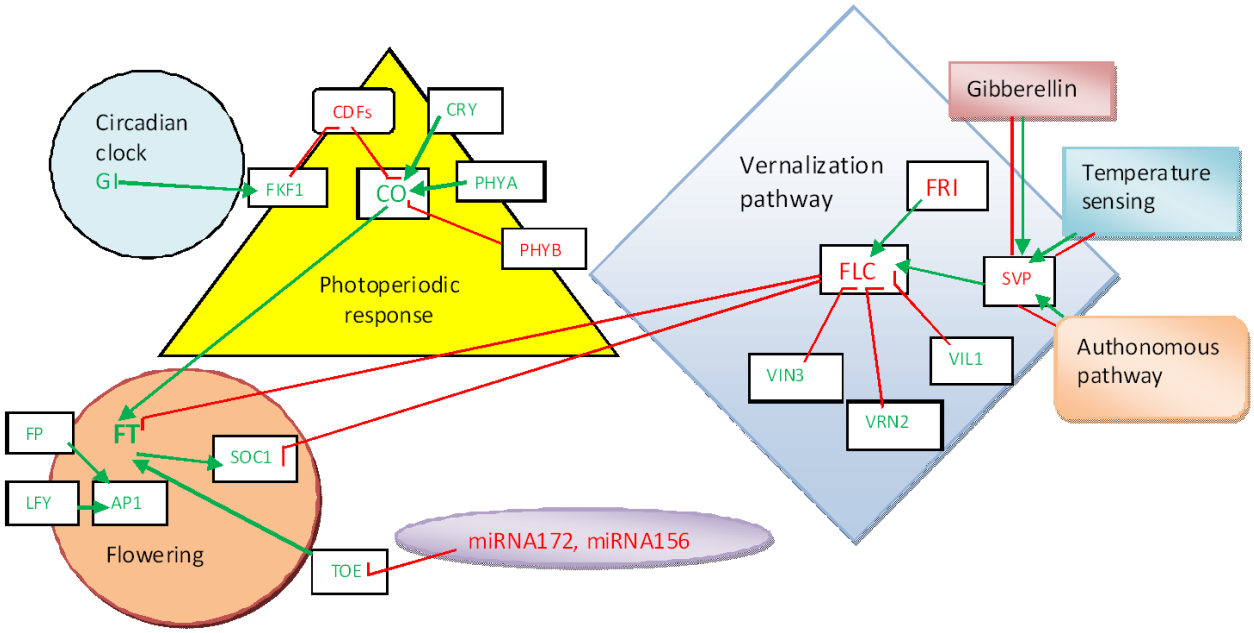

Figure 2 Flowering time regulatory pathways known in *Arabidopsis thaliana* L. simplified model, illustration to the text above. *Green font*: flowering promoter; *red font*: flowering suppressor; *green arrow*: activation or stabilization; *red line*: suppression or degradation.

3.5.2 *Oryza sativa* L.

Rice is a monocot species and its flowering is promoted under short day conditions; thus constituting an interesting subject for investigations in contrast to *Arabidopsis*. While being a major crop species, rice is also chosen for very broad spectrum of primary biology studies. Of a great advantage is an availability of the sequenced rice genome; due to a low genome

complexity the sequence appeared relatively soon (International Rice Genome Sequencing Project 2005).

The molecular mechanisms of flowering in rice are not as well understood as in *Arabidopsis*. Initially, 14 QTLs (*Hd1* till *Hd14*) were described that contributed to the flowering time regulation. Five of them, *Hd1*, *Hd2*, *Hd3*, *Hd5* and *Hd6*, were found to confer photoperiodic sensitivity (Lin et al. 2000; Yamamoto et al. 2000).

Despite the different photoperiodic requirements, many flowering-gene homologues between rice and *Arabidopsis* have already been described (see in a Table 1). However, a homolog of the *AtFLC* gene is still undiscovered (Cockram et al. 2007). One hypothesis explaining this lack says that it is related to the *FLC/MAF* group of MADS-box genes which evolved after the dicot/monocot divergence (Higgins et al. 2010).

Table 1 Genes involved in flowering regulation being cloned in rice end their orthologues from *Arabidopsis*

Gene name (rice)	Reference	Orthologue from *Arabidopsis*
OsHY1	(Izawa et al. 2000)	*HY1*
OsPHY	(Higgins et al. 2010)	*PHY/CRY*
OsFTa (Hd-3a)	(Kojima et al. 2002)	*FT*
OsFTb (Hd-3b)	(Monna et al. 2002)	*FT*
OsCO (Hd-1)	(Hayama et al. 2003; Yano et al. 2000)	*CO*
Ehd1	(Doi et al. 2004)	No orthologue present, B-type ARR
OsCKIIα (Hd6)	(Takahashi et al. 2001)	*CK2*
OsGI	(Izawa et al. 2002)	*GI*
Ghd7	(Xue et al. 2008)	No orthologue present, similar to *TaVRN2*

Under long day (LD) conditions expression of *OsCO* is enhanced but unlike in *Arabidopsis*, the orthologue of the At-*FT* gene is finally suppressed (Hayama et al. 2003). Under short day

conditions (SD), however, the function of *Hd-1* is the same as *CO* in *Arabidopsis*- i.e. the level of *OsFT* increases (Yano et al. 2000). In the rice genome, two paralogues of *FT* have already been found in closely linked positions. 'Type a' promotes flowering under SD conditions while 'type b' causes late flowering in the field and under the LD conditions (Monna et al. 2002). These facts confirmed that rice is adapted to short day conditions even at the molecular level.

Summing up: plants, like all living organisms, are basically programmed for reproduction; however, it is very important that the vegetative/generative phase transition has the right timing during development. That is why most of the flowering pathway genes delay flowering. The length of the delay depends on many environmental factors such as vernalization, photoperiod and temperature, which, as a result, make plants flower at the most proper time.

3.6 Inflorescence development in monocots

3.6.1 Spike initiation

According to Zadoks' growth scale, inflorescence development starts just after the vegetative/generative phase transition as soon as leaf development has ceased (Zadoks et al. 1974). In wheat, the first reproductive primordia of the immature spike develop into spikelets until the last spikelet (terminal spikelet) has been formed. The first stage of spike differentiation is called double ridge stage and it occurs shortly after the transition from a vegetative to a generative growth. Later on, after all the spikelet primordia are formed, initiation of subsequent floral elements can be observed starting from glumes and awns from the differentiating spikelet meristems up to stamens and anthers from the floral meristems. In

hexaploid wheat, the terminal spikelet is twisted by a 90 degrees angle in comparison to the rest of the spikelets (Kirby and Appleyard, 1984).

3.6.2 Heading and flowering

As soon as the spikelet number is determined the spike as well as the culm starts to elongate until awns appear above the flag leaf. However, "heading" continues until the complete spike has emerged. Then the plant starts to flower by shedding yellow anthers usually starting from the middle of each spike. In case of wheat, mature anthers are extruded from their florets (chasmogamy) but under stress conditions plants appear cleistogamous; however, barley plants are usually cleistogamous (Zadoks et al. 1974).

3.7 Molecular determinants of flowering in wheat and barley

3.7.1 Photoperiodic response

Flowering time loci conferring photoperiod sensitivity were localized on the short arm of group 2 chromosomes of bread wheat (*Ppd-1*) and barley (*Ppd-H1*) (Laurie et al. 1995; Law et al. 1978; Scarth and Law 1983; Welsh 1973). Furthermore, the underlying gene, *Pseudo-Response Regulator* (*PRR*), has been cloned and characterized in wheat (Beales et al. 2007) and barley (Turner et al. 2005), respectively. It was shown that the *Ppd-H1* gene was regulated by the circadian clock while being an ortholog of *PRR3* from *Arabidopsis* (Higgins et al. 2010; Turner et al. 2005). Barley plants possessing the recessive allele *ppd-H1* had lower expression of *CONSTANS*-like genes, which could explain their decreased sensitivity to the photoperiod. In fact, the floral transition was significantly delayed in these plants when grown under long day conditions (Turner et al. 2005). Different alleles at the photoperiodic response locus have been

already utilized in barley breeding. It was found that spring cultivars having such non-responsive allele (*ppd-H1*) gave higher yield in Western Europe due to elongated vegetative growth. On the other hand, cultivars bred in Israel contained a photoperiodic responsive allele at this locus (*Ppd-H1*) which caused early flowering. The latter used to be explained as an adaptation to escape from drought during dry and hot summer conditions (Lister et al. 2009; Wang et al. 2010).

3.7.2 Vernalization requirement

Vernalization requirement in wheat and barley is mainly encoded by three epistatic loci; *Vrn-1* (*Vrn-A1, -B1, -D1, -A^{m}1, H1*), *Vrn-2* and *Vrn-3*. The dominant *Vrn-1* genes are essential for spring growth habit (i.e. cold treatment is not required to induce flowering) and are up-regulated by *Vrn-3* (Yan et al. 2006). Whereas, *Vrn-2* confers winter growth habit and is down-regulated by the long cold treatment (Tranquilli and Dubcovsky 2000). The *Vrn-1* genes were mapped on the long arm of group 5 chromosomes and positionally cloned, revealing their similarity to *APETALA1 MADS* box transcription factor (Law et al. 1976; Yan et al. 2003). The second, *Vrn-H2*, was located on chromosome 4H of barley (Laurie et al. 1995) and its ortholog, *Vrn-A^{m}2*, on the 4A^m/5A^m translocated segment of einkorn wheat (Dubcovsky et al. 1998). *Vrn-A^{m}2* has been isolated and characterized as encoding a *CCT*-domain/*B-box* protein (Yan et al. 2004). Finally, the *Vrn-3* locus was identified being an ortholog of the *FLOWERING TIME LOCUS T* (*FT, florigen*) on the short arm of group 7 chromosomes in wheat and barley (Yan et al. 2006).

As in the case of photoperiodic responsive genes, also different vernalization requirement genes/alleles have been introduced to the cultivated germplasm of wheat and barley. It was

pointed out that loci *Vrn-H1* and *Vrn-H3* that included alleles coming from *Hordeum vulgare* ssp. *spontaneum* reduced the yield while *Vrn-H2* led to its increase. This makes the *Vrn-H2* locus promising for barley breeding (Karsai et al. 2006; Wang et al. 2010). Concerning wheat cultivars, it was found that earlier flowering plants used to give smaller yield but they could be preferred in regions with shorter vegetative season. Moreover, some allele combinations of *Vrn* loci were proposed which would have been considered during breeding in respect to different climatic conditions (Iqbal et al. 2007). Besides, a shortened vegetative growth phase resulted in increased grain protein content in bread wheat. So, earliness can sometimes be desirable considering kernel quality despite a lower yielding as a consequence (Herndl et al. 2008).

3.7.3 Earliness per se

Earliness per se (*eps*) is the third and least investigated factor influencing time to flowering in wheat and barley. Unlike photoperiodic and vernalization response, it is independent from environmental cues. *Eps* is often called earliness in the narrow sense, intrinsic earliness, or basic development rate (Flood and Halloran 1984; Hoogendoorn 1985; Kato and Yamagata 1988; Law and Worland 1997; Slafer 1996; Snape et al. 2001; Worland and Law 1986). Numerous *eps* loci have already been identified in barley (Laurie et al. 1995; Law and Worland 1997; Snape et al. 2001) and wheat (Hoogendoorn 1985; Millet 1987). Most importantly, meta-QTL analysis on heading time in bread wheat revealed that many QTLs co-located in chromosomal regions known to carry *eps* loci (Griffiths et al. 2009; Hanocq et al. 2007). So far there were only a few studies elucidating the molecular basis of *eps* loci (Comadran et al. 2012; Faricelli et al. 2010; Faure et al. 2012; Zakhrabekova et al. 2012). The earliest studies targeted the *Eps-A^{m}1* locus from einkorn wheat (Faricelli et al. 2010; Lewis et al. 2008). Phenotypic

analysis in lines carrying different *Eps-A^{m}1* alleles showed that *Eps-A^{m}1* significantly influenced kernel number per spike. This took place mainly through an altered duration of the vegetative and spike growth phases (Lewis et al. 2008). The single-seed-descent (SSD) lines carrying the late allele at *Eps-A^{m}1* headed around 61 days later and produced about 56% more spikelets per spike compared to those with the early *Eps-A^{m}1* allele (Lewis et al. 2008). Moreover, the phenotypic differences between the two allelic classes at *Eps-A^{m}1* were more pronounced when plants were grown in 16°C rather than 23°C, indicating that *Eps-A^{m}1* seemed to be thermo-sensitive. The authors concluded that *Eps-A^{m}1* could be utilized in breeding for specific climatic conditions, mainly considering the fine-tuning of heading time and yield potential (Lewis et al. 2008). Two later studies found a locus on barley chromosome 1H harboring a similar position like *Eps-A^{m}1*, namely *early maturity 8* (*eam8*) (Faure et al. 2012; Zakhrabekova et al. 2012). The mutated gene responsible for the *eam8* phenotype was similar to the *Arabidopsis* circadian clock element *ELF3* (*Early flowering 3*) (Faure et al. 2012; Zakhrabekova et al. 2012). Most recently cloned *eam6* locus from chromosome 2H was, in contrast to *eam8*, localized out of the circadian clock network and identified as being an ortholog of *Antirrhinum CENTRORADIALIS* (Comadran et al. 2012).

3.8 Modern genetic approaches in understanding important traits

3.8.1 The concepts of synteny and colinearity

Synteny refers to chromosomal segments or to gene loci in different organisms located on a chromosomal region originating from a common ancestor while colinearity is a conservation of the gene order within a chromosomal segment between different species (Keller and Feuillet 2000). Usually colinearity is shown as plotted loci coming from different species that can be

connected by common lines which do not cross each other, indicating a common order of their positions (Gale and Devos 1998; Moore et al. 1995). The concept of colinearity gives a great advantage while developing new molecular markers during map-based cloning approaches as well as in candidate gene approaches when sufficient genomic sequence information is lacking. However, recently made analyses using available genomic sequences of rice, sorghum, maize and *Brachypodium* species showed that there are a lot of exceptions from the conservation of colinearity and that one has to consider a lot of possible chromosomal rearrangements (Bowers et al. 2005; Devos 2005; Faris et al. 2008; Huo et al. 2009). Moreover, it is clear that the correlation between recombination frequency and gene content results in different abundance of colinearity distortions during evolution (Bowers et al. 2005; Ilic et al. 2003).

3.8.2 Marker types

Basic systematic of markers divides them into two types: phenotypic and molecular markers. Because the usage of the first type of markers is highly delimited, most of the genetic studies incorporate the latter group. However, because of the technical difficulties accompanied with elaborating protein markers; i.e. separation and dyeing, DNA markers are more commonly used. According to (Jones et al. 2009), several subgroups among molecular DNA markers can be distinguished:

- First-generation markers based on restriction fragment detection, such as restriction fragment length polymorphism (RFLP);

- Second- generation markers based on PCR which include plenty of techniques like cleavage amplification polymorphism (CAP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR) and so on;
- Third- generation markers based on DNA sequencing- for example different SNP assays which can be used in a highly automated mode;
- Genome scanning for expressed genes, for instance utilizing EST-derived SNPs;
- Markers using array technology like diversity array technology (DArT);
- Other, non-PCR marker systems such as single strand conformational polymorphism (SSCP) and temperature gradient gel electrophoresis (TGGE).

3.8.3 Genetic linkage mapping

Genetic linkage mapping uses the crossing-over (recombination events) occurring within linkage groups (chromosomes). It shows the relative recombination ratio between given loci and orders them in the way that reflects physical, linear order of DNA sequences. The smaller the distance between two loci on the map the less recombination occurs between them; so there is a positive correlation between these two (Jones et al. 1997). However, because of an unequal distribution of recombination frequency along the chromosome, distances on the genetic linkage map are highly deviant from the real, physical distances. Usually genetic linkage mapping requires dealing with large amount of data and there is a statistical tool which helps— the logarithm of odds (LOD). The LOD value is used to establish separate linkage groups and to order markers. In this case, the LOD threshold of 3 is usually applied, meaning that the probability of linkage between two distinct loci is 1000 times more likely than the probability of no linkage (Risch 1992). There are many population types that can be used for genetic linkage

mapping. Nevertheless, some of them like recombinant inbred lines (RIL) and doubled haploid (DH) have a substantial advantage over F2 and BC populations mainly due to their greater phenotype stability and also, in case of RIL—abundance of recombined loci (Jansen 2003).

3.8.4 QTL analysis

For traits which have a complex genetic constitution such as biomass, flowering time and level of fertility, special efforts have to be done to localize their causatives. The common property of these polygenic traits is their quantitative distribution in the population and continuous value scale. Genetic loci which contribute to the variation among these traits are commonly named quantitative trait loci (QTLs). Once the genetic map is generated, it is possible to associate the QTLs with particular markers and identify their locations (Jones et al. 1997). To detect QTL several methods can be used; such as single marker analyses (ANOVA, or more frequently linear regression) , simple interval mapping (SIM) and composite interval mapping (CIM) (Tanksley 1993). Linear regression allows for the estimation of the coefficient of determination (R^2) for marker/trait associations. The second method, simple interval mapping, is more advanced and analyzes intervals between adjacent pairs of linked markers instead of analyzing single markers (Lander and Botstein 1989). Finally, the last mentioned method—composite interval mapping—combines SIM with linear regression analysis and includes extra genetic markers in the statistical model in addition to an adjacent pair of linked markers for interval mapping. CIM allows for more effective and precise QTL mapping, particularly when linked QTLs are involved (Jansen 1993). To obtain the threshold LOD value of QTL for a trait of interest, permutation tests are usually performed (Churchill and Doerge 1994). In the end, LOD

graphs which are above the determined significance-threshold can be interpreted as regions containing loci significantly contributing to the trait.

3.8.5 Map-based cloning

Once QTLs or genes are localized on the genetic linkage map they can be further investigated. Usually the desired path to follow is map-based cloning which means isolation of a DNA sequence or interval that is functionally responsible for the investigated heritable trait. There are many examples in the literature of such approaches, for instance (Distelfeld et al. 2006; Feuillet et al. 2003; Huang et al. 2003; Simons et al. 2006; Yan et al. 2003; Yan et al. 2004). Usually map-based cloning requires the development of a large segregating population for high resolution mapping to delimit the gene/QTL interval. Moreover, the screening of a bacterial artificial chromosomes (BAC) genomic library has to be performed. The latter is necessary to anchor the fragmented genomic DNA to markers and characterize the QTL physically. The map-based cloning approach very often faces many difficulties such as low recombination frequency, lack of colinearity with model species that can really slow down the process. In case of temperate cereals, special efforts are currently undertaken to speed up such investigations in the future; i.e. international projects for wheat and barley whole genome sequencing, International Wheat Genome Sequencing Consortium (IWGSC) and International Barley Genome Sequencing Consortium (IBGSC), respectively.

4 Purpose of the study

4.1 Reasonability

Knowledge about *earliness per se* is very limited and the putative functions of genes contributing to this trait were completely unknown in cereals when this study was started. Hence a deeper elucidation of *eps* loci is very important from a scientific point of view. On the other hand, *eps* gene-based markers can become very valuable tools in marker-assisted selection/breeding. This is because they should enable breeders to fine tune the flowering time of future cultivars. Besides, the pleiotropic effect on kernel number and spike length can make some of the *eps* loci-based markers directly involved in yield optimization.

4.2 Aims

The main purpose of the study is to characterize an early-heading mutant in diploid einkorn wheat (*T. monococcum* L.), line KT3-5. It possesses a recessive single major *Eps* locus on the telomeric region of chromosome 3A long arm (*Eps-3A*^{m}). During the project, detailed phenotypic analyses and high-resolution genetic mapping of that mutant (line KT3-5) should be performed. This should enable to build a physical contig consisting of bacterial artificial chromosomes (BAC) clones to characterize the *Eps-3A*^{m} locus. In the next step, gene/-s which affect timing of transition from vegetative to generative phase of growth and regulate spikelet number shall be identified and isolated. Finally, expression pattern, tissue-specificity and function of candidate gene(s) should be investigated.

5 Materials and methods

5.1 RILWA1 population and einkorn wheat mutants evaluation

5.1.1 Plant material

In the present study, 110 diploid recombinant inbred einkorn wheat lines (F11) were initially analyzed (RILWA1 population). This population was derived from a cross between the early heading X-ray mutant KT3-5 (Shindo and Sasakuma 2001) of a cultivated form and a wild strain KT1-1 (*T. boeoticum* Boiss.). Subsequently, the RI lines were developed by SSD from F2. Additionally parental lines KT3-5, KT1-1, a collection of einkorn lines (see Table 2) and mutant donor line KT3-1 (*T. monococcum* L.) were evaluated. Seed samples of the used plant material were kindly donated by Genetic Resource Bank of Kihara Institute for Biological Research at Yokohama City University in Japan.

5.1.2 Phenotype evaluation

Seeds of the RILWA1 population as well as KT lines were sown into single 100 ml wells. After germination at room temperature, two weeks old seedlings were transferred to a cool room (15°C) for one week, followed by a vernalization treatment at 4-8°C for 8 weeks and a photoperiod of 10h/14h day/night. For acclimation after vernalization, plants were moved back to the cool room (15°C) for one week and transplanted to one liter pots. The experiment was designed that each line was represented by three replications of 3 to 4 plants growing in a one pot. After transplanting, the long day treatment (16h/8h) started at 16/20°C until harvesting. Each replication was manually randomized every week to avoid positional bias. To confirm the localization of QTL for *Eps-3A*^{m}, four traits that showed significant correlations in previous work

were analyzed (Hori et al. 2007). Heading time was scored when the visible part of awns on the main culm was about 1cm long. Anthesis time was recorded when anthers were extruded from approximately half of the florets per main culm spike. Final spikelet number and spike length were similarly scored on the main culm prior to harvest.

5.1.3 Marker development and genetic linkage map construction

Leaf samples were taken from each plant and pooled for every RI line, following DNA isolation according to the modified Doyle and Doyle method (Doyle and Doyle 1990). DNA concentrations were adjusted to approximately 30ng/µl. Primers for marker development were selected based on already mapped ESTs (Expressed Sequence Tags) (Dilbirligi et al. 2006; Sato et al. 2009) using Primer3 software (Rozen and Skaletsky 2000). Only ESTs mapped to the long arm of the 3A chromosome were chosen. Optimal melting temperature (Tm) of primers was defined as 60 or 65°C and their length as about 25 nucleotides. Colinearity with chromosome 2 from *Brachypodium* (www.brachybase.org) and chromosome 1 from rice (www.rice.plantbiology.msu.edu) was also used to develop additional markers following a modified procedure described in (Schnurbusch et al. 2007), (Initiative 2009; Project 2005). EST sequences used for primer design were selected at E^{-50} cut-off value and only when the similarity to the *Brachypodium* or rice coding sequence was at least 80%. PCR conditions were as follows: 94°C for 3 min, 8 cycles of 94°C for 1 min, 63 or 68°C for 1 min decreasing by 1°C per cycle and 72°C for 1 to 3 min. (depending on the length of the amplicon), then 40 cycles of 94°C for 1 min, 55 or 60°C for 1 min and 72°C for 1 to 3 min, and 72°C for 10 min. PCR products were run on a 2 % agarose gel to detect the presence/absence (loci with prefix PAV) or insertion/deletion (loci with prefix INDEL) type of polymorphism between DNA of parental lines

KT1-1 and KT3-5. The PCR products which did not show the above polymorphism were sequenced by the Sanger method using ABI 3730 XL technology. Obtained sequences were analyzed according to the presence of SNP (Single Nucleotide Polymorphism) as well as small insertions and deletions. Forward and reverse sequences were aligned using Bioedit software (Hall 1999). All identified SNP were verified manually and used to develop CAPS (Cleaved Amplified Polymorphic Sequence) (www.tools.neb.com/NEBcutter2) (Vincze et al. 2003) or dCAPS (derived CAPS) (www.helix.wustl.edu/dcaps/dcaps.html) (Neff et al. 2002) markers. Alternatively, in some of the cases, allele specific primers were designed (www.probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi; Rozen and Skaletsky 2000) which allowed for the dominant scoring (loci with prefix AS_PCR). During development of the latter assay, one additional mismatch per primer was introduced at the 2nd or 3rd position counting from the 3-prime end of the allele specific primer. However, in two cases AS_PCR primers could be multiplexed and used for the co-dominant scoring; marker loci TP_71_72 and TP_13_14 (TP= tetra-primed). Each of the 110 RI lines was genotyped using the polymorphic marker set. The obtained segregation data were analyzed using Join Map® 4 (Ooijen 2006). During the analysis, a linkage group was created at the LOD threshold equaling 3.0 and the maximum likelihood mapping algorithm was used to estimate marker distances.

5.1.4 QTL detection

QTLs were identified in the RILWA1 population by multiple-QTL models (MQM) using MapQTL® 5 software (Ooijen 2004). Threshold values at 95% significance level were calculated using permutation test to determine the presence of QTLs for the respective traits. Calculated LOD thresholds were 2.0 for heading time, anthesis time, spikelet number, and spike length.

Manual co-factor selection was based on two criteria; i) maximization of the LOD value for a given QTL and ii) reduction of the LOD values lying below the significance threshold. In this procedure, QTL detection was performed by using the interval mapping (IM) algorithm. During the next step, few marker loci placed outside of the estimated QTL region were selected as co-factors. The procedure was repeated a few times until the best combination of marker loci was found, matching the two criteria as described above.

5.1.5 Einkorn wheat mutants

It was important to verify whether the einkorn mutants (KT) were created by independent events or by mutagenesis of the already existing mutant KT3-5, or introgression of the mutated chromosomal segment. For this reason, the KT lines were genotyped with markers co-segregating with the highest LOD-scores for traits analyzed in the RILWA1 and with closest flanking markers. The lines which showed the mutant alleles around the locus were further analyzed by re-sequencing of three highly polymorphic markers from chromosome $3A^m$ to verify their genetic background, see Table 2.

Table 2 KT lines of einkorn wheat analyzed, see chapters 5.1.1 and 5.1.5 for the detailed description. Sixteen lines marked with an *asterisk* share a deletion at the *Eps-3A*^{m} locus and were selected for further analysis

No.	Accession No.	Description
1	KT001-002	*T. boeoticum ssp. boeoticum*
2	KT001-003	*T. boeoticum ssp. boeoticum*
3	KT002-001	*T. urartu variety nigrum*
4	KT003-002	*T. monococcum variety vulgare*
5	KT003-003	*T. monococcum variety flavescens*
6	KT003-004	*T. monococcum variety hornemanni*
7	KT003-038	*T. monococcum strain KUS 68*
8	KT003-039	*T. monococcum strain KUS 82*
9	KT003-006	*T. monococcum* strain vulgare spiral mutant
10	KT003-007	*T. monococcum* strain vulgare old rose (hetero) mutant
11	KT003-008	*T. monococcum* strain vulgare light green mutant
12	KT003-009	*T. monococcum* strain vulgare orange mutant
13	**KT003-010***	*T. monococcum* strain vulgare ej., non–glossy mutant

No.	Accession No.	Description
14	**KT003-011***	*T. monococcum* strain vulgare early, spiral mutant
15	**KT003-012***	*T. monococcum* strain vulgare early, green mutant
16	**KT003-013***	*T. monococcum* strain vulgare ej., glossy (stripe) mutant
17	**KT003-014***	*T. monococcum* strain vulgare albino mutant
18	**KT003-015***	*T. monococcum* strain vulgare pigmy mutant
19	**KT003-016***	*T. monococcum* strain vulgare pigmy, narrow leaf mutant
20	**KT003-017***	*T. monococcum* strain vulgare purple red mutant
21	KT003-019	*T. monococcum* strain vulgare nh. mutant
22	KT003-020	*T. monococcum* strain vulgare sg. mutant
23	KT003-021	*T. monococcum* strain vulgare moegi mutant
24	KT003-022	*T. monococcum* strain vulgare dwarf mutant
25	KT003-023	*T. monococcum* strain vulgare black glume mutant
26	KT003-024	*T. monococcum* strain vulgare branched spike mutant
27	KT003-025	*T. monococcum* strain vulgare soft spike mutant
28	KT003-026	*T. monococcum* strain vulgare wrapped glume mutant
29	**KT003-027***	*T. monococcum* strain vulgare rolled leaf mutant
30	**KT003-028***	*T. monococcum* strain vulgare rolled leaf, dwarf mutant
31	**KT003-029***	*T. monococcum* strain vulgare early, snith mutant
32	**KT003-030***	*T. monococcum* strain early Translocation aT1
33	**KT003-031***	*T. monococcum* strain early, male sg. Translocation aT1
34	KT003-032	*T. monococcum* strain Translocation aT2
35	**KT003-033***	*T. monococcum* strain Translocation aT5
36	**KT003-034***	*T. monococcum* strain Translocation mT6
37	**KT003-035***	*T. monococcum* strain Translocation aT7
38	KT003-036	*T. monococcum* strain Translocation mT9
39	KT003-037	*T. monococcum* strain haploid inducer

5.2 F2 population development

5.2.1 Parent selection

Quantitative distribution of the heading time and the other correlated traits in the RILWA1 population was an undesired condition for more detailed analysis. Considering the previous work by (Shindo et al. 2002), not only the major *Eps-3AL*^{m} locus was the causative but also the flowering time loci on chromosome 5A^m contributed to the observed distribution. Another disadvantage taken into account was the rachis brittleness trait coming from the wild parent KT1-1 of the RILWA1 population. Therefore, a few steps were undertaken to avoid segregation of the second *Eps-5A*^{m} locus, vernalization requirement and brittle rachis in the F2 population.

Rachis brittleness was examined manually and only non-brittle lines were further considered. Selection according to the vernalization requirement and *Eps-5A*^{m} locus was performed by PCR-CAPS analysis of the DNA. Therefore, two marker loci mapped to the chromosome 5A^{m} by Hori et al. (2007) were used for genotyping the RILWA1 population. First of them, t01301, coming from the 2.3 cM locus distal to the epistatic VRN2, and second, t03005 located only 0.7 cM far from the marker cdo1326a which co-localized with the LOD peak for the *Eps-5A*^{m} locus (Shindo et al. 2002). Importantly, the other two criteria, taken into account during the parent selection, were (1) contrasting alleles around the *Eps-3AL*^{m} locus and (2) at least one week difference in heading dates supported with a clear difference in the spike-related traits. Lines selected for the crossing were grown without vernalization treatment to ensure their desired spring growth habit.

5.2.2 Crossing

The first crossing approach during the experiment described in the chapter 5.1 failed. The probable reasons were (1) an inappropriately performed emasculation that led to the immediate drying of florets and (2) low number of crossed spikes. Only two selected lines–RILWA-25 and RILWA-71–were used for the later crossing experiments thus allowing for a larger scale performance. Totally 48 seeds of each of the two genotypes were sown into the 100-ml wells. Six seeds of each were planted the same day to confirm the one-week difference in heading dates. Remaining seeds were sown in groups of 16 or eight every two to three days to increase the chance of having plants ready for the crossing at the same time. The sowing of the RILWA-25 parent was significantly delayed by one week since it was predicted to flower earlier than RILWA-71 based on the previous experiment, see chapter 5.1. After four weeks,

seedlings were transplanted to the 1.1 liter pots (1 plant per pot). Heading date was scored as described before, chapter 5.1.2. During emasculation, a special care was taken not to damage the spikelets. The process was performed with two pairs of fine forceps; one for opening the floret and keeping the glumes away from each other and the second pair for anther removal. The emasculation was performed about eight days before the predicted anthesis (spikes were just fully emerged) and the pollination about one week later, when the stigmas at the carpel were well developed, usually at noon (in the middle of photoperiod). The successively pollinated florets could be easily recognized by tightly closed lemma and palea contrastingly to the unfertilized florets which always remained open. Totally, about 800 florets or potential seeds were crossed. However, only 36 seeds were obtained; many of which having low quality; i.e. abnormal shape and small size.

5.2.3 F1 genotyping

All the 36 seeds obtained were kept in the 4°C for one month to break the dormancy. The seeds were soaked in sterile distilled water (SDW) for a few hours at room temperature and kept separately in Eppendorf tubes supplied with a drop of SDW for a few days in 4°C to synchronize the germination. All the germinated seeds, in the number of 13, were sown in soil. The further procedure of leaf sampling and DNA extractions was performed like already described, chapter 5.1.3. All the plants were verified as being true hybrids by using five markers (CAPS_61_62, CAPS_131_132, CAPS_241_242, INDEL_271-272, PAV_295_296) spanning the *Eps3A*^{m} locus and being polymorphic between the parents RILWA-25 and RILWA-71. Restriction enzymes used for the digestion as well as conditions of PCR reactions and electrophoresis were as described before (chapter 5.1.3).

5.3 Evaluation of F2 and F3 populations

Seeds of the F2 population consisting of 658 individuals, as well as parental lines RILWA25 and RILWA71, were soaked in sterile distilled water (SDW) and placed on wet filter paper in Petri dishes. To synchronize germination, soaked seeds were kept in 4°C for two days and germinated at room temperature. Seedlings were transplanted into single 100ml wells and placed in a cool room (15°C) for two weeks, followed by a vernalization treatment at 4-8°C for three weeks and a photoperiod of 10h/14h day/night. For acclimation after vernalization, plants were moved back to the cool room (15°C) for two weeks and selected recombinant individuals plus 38 random plants were transplanted to 1.1 litre pots. The additional 38 plants were considered to create the opportunity for better analysis of the phenotypic variation and distribution. After transplanting, the long day treatment (16h/8h) started at 15°C/18°C until harvest. Each pot was manually randomized every three or four days to avoid positional bias. Heading time was scored when the visible part of awns on the main culm was about 1cm long. Anthesis time was recorded when anthers were extruded from approximately half of the florets per main culm spike. Final spikelet number and spike length were similarly scored on the main culm prior to harvest. 658 individuals were screened with flanking markers INDEL_271_272 and INDEL_201_202 giving size polymorphism, thus maximizing time- and cost-efficiency. Four new markers surrounding the locus were developed based on the sequence information gained from the physical contig95 in barley, see Table 3. Plants with a recombination event between the flanking markers were genotyped further with markers listed in Table 3 and Table 4.

Table 3 Additional marker loci developed during high-resolution mapping in F2 population along with the reference information

Marker	Primary source of the sequence	Rice gene	Brachypodium gene	Functional annotation[1)]
CAPS_321_322	BAC HVVMRXALLmA0519N21	Os01g0971000 (d)[2)]	Bradi2g61970 (d)	Not possible, 1[3)]
CAPS_325_326	BAC HVVMRXALLmA0519N21	Os01g0976300	Bradi2g62600	Heavy metal associated p., 2
CAPS_331_332	BAC HVVMRXALLmA0519N21	Os01g0976200	Bradi2g37470	Legumin-like, 4
PAV_261_262	Collinear region from rice	Os01g0971800	Bradi2g62070	LUX/PCL1-like, 5
CAPS_341_342	BAC HVVMRXALLeA0121L	Os05g0110000	Bradi2g04010	Ring-H2 zinc finger p., 7
CAPS_zt3_4	Collinear region from rice	Os01g0972200	Bradi2g04020	Zinc transporter, 8
CAPS_335_336	Collinear region from rice	Os01g0972800	Bradi2g62130	WRKY, 10

[1)] The functional annotation was based on the BLASTX search
[2)] (d) marks the possible duplicated ortholog or paralog when a more similar gene is already present in the einkorn wheat genome
[3)] The number after functional annotation corresponds to the gene number on Figure 8

Table 4 List of the markers used for genotyping F2 recombinant plants. Order presented reflects the real genetic order of mapped loci

Marker	Comment/enzyme	Forward primer (5'-3')	Reverse primer (5'-3')
INDEL_271_272	size polymorphism	CAAGGCTCTGCAGTACTTGACAGAG	GACTTCATCAAGCGTAAGCACATGTC
CAPS_321_322	*Hpy188I*	TTGCCGCTTAAGAATAAGCATCCTC	GTTTCCATCATAGGGGAAGTTTGTG
CAPS_325_326	*Hpy188I*	TTTCATTGTGCACCACCAACC	CAGCCGTGGTGCTCTTCTTCT
CAPS_331_332	*BamHI*	GCTACTCTGACTCCGCCAAGG	AGCCCGACCTCCTTCACCA
PAV_261_262	presence/absence	CTCTACGTCAAGCGGATGCAG	GTGGTAGTGCGGGTAGGAGGAG
PAV_295_296	presence/absence	CATACTGGTCTGTAGCAAGCAAGCA	AGCACGGCTCAGATAAAGGAGTTG
CAPS_341_342	*EcoNI*	CGAGAGCATGTCCGACTTC	ATATTCCCCAGTGTGTGATGC
CAPS_zt3_4	*BstUI*	GAGGCATGGAGGAACCTATGGAC	AACTTGAAGATGGGCTTGTCGAA
CAPS_335_336	*MspI*	CAGTGATGCAGGCGTGGAG	ATGGCCATGTCGATGTACGG
CAPS_311_312	*MvaI*	TCGTAATAAGATGTGCGGAGAGATG	CAATGAACCAACTGTTGCATTTTCA
TP_13_14	external primers	CAAGCCTGACCCAGTACTCAGG	CACAGCCTCCTCCCACAGTTC
TP_13_14	internal primers	ATGCGACTTCGGTTGATGCTC	ATTCACCACATTAGCAACGCA
AS_PCR_17_18	additional mismatch	ACATCTGAAGAGCCAAGTTGATCC	GCTATCACATACCGCAAGAGAATCTC
INDEL_201_202	size polymorphism	GACAAATGTAGTGTTCATGGGGATG	TTCCAGAGGATATGCCTTTGCACTTGG

5.4 Southern blot analysis

Total DNAs were extracted from four days old KT3-1 and KT3-5 seedlings according to the (Doyle and Doyle 1990). Ten micrograms of the DNAs were digested with four selected single

restriction enzymes: *XbaI*, *DraI*, *HindIII*, *BamHI* as well as in two additional combinations: *EcoRV*/*EcoRI* and *BsuRI*/*DraI*. The digestion reactions comprising of 400 µl each were performed overnight and the products were purified by using the UF plate (Qiagen) and diluted in 20 µl of TE buffer. Small aliquots of each reaction were examined by 1% agarose gel electrophoresis and in the case of incomplete digestion, the reactions were repeated but in a 30 µl volume. Digested total DNAs were fractionated on 0.75% agarose gel in TBE buffer. Electrophoresed DNAs were transferred to Hybond^{TM}XL membrane (Amersham Biosciences) using alkali conditions. In the case of *TmPumilio* (locus PAV_295_296), the following primers were used to amplify the probe: forward (F), 5'-CATACTGGTCTGTAGCAAGCAAGCA-3' and reverse (R), 5'-GTACGTGATCAAAATGGCAACCAC-3'. Whereas for the *TmLUX* probe (locus PAV_259_262): forward (F), 5'-GAGTTAGCCCGGCAGGTAACAAC-3' and reverse (R), 5'-ACAGAGCACACACTCTGCAACTCTC-3'. PCR conditions were as described in the previous chapter 5.1.3. Hybridization was carried out at 68°C for 24 h in a solution containing 5x SSPE, 5x Denhardt's reagent, 100 µg/ml ssDNA, along with the radioactively labeled probe. 25% formamide was used in the case of hybridization to the *TmLUX*. Finally, membranes were washed in 2x SSC–0.5% SDS at room temperature for 20 minutes, and then in 0.1x SSC –0.5% SDS at 68°C twice for 15 min.

5.5 Confirmation of the KT3-5 mutant background

To ensure that the KT3-1 line was the true donor of the KT3-5 mutant, 17 highly polymorphic markers, listed in the Appendix Table 3, were amplified on its genomic DNA and re-sequenced. DNA extraction, PCR conditions and re-sequencing followed the procedure already described for genotyping of the KT1-1 and KT3-5 lines, chapter 5.1.3.

5.6 Physical mapping of the *Eps3* locus in barley cv. Morex and MTP sequencing

Three markers CAPS_335_336, TP_13_14 and PAV_295_296 selected from the locus were used to screen three-dimensional pools of the barley BAC library (in collaboration with the group of Dr. Nils Stein). Additionally, BLAST searches were performed utilizing the existing sequence information; BAC-End Sequences and sequenced BACs (http://webblast.ipk-gatersleben.de/barley/viroblast.php). BACs were assembled by using the FPC software and the obtained contig was verified by the LTC software (in collaboration with Dr. Ruvini Ariyadasa). The latter one allowed for more precise Minimum Tiling Path (MTP) selection as well as getting rid of the problematic, misassembled BACs. The BAC sequencing (Dr. Axel Himmelbach) and shotgun 454-reads assembly (Burkhard Steuernagel) was generally performed like described elsewhere (Steuernagel et al. 2009). The only differences according to the aforementioned procedure were as follows; the FLX Titanium chemistry was used for the BAC sequencing and for the assembly–an updated version of Mira 3.2.1 software. The obtained sequences were annotated according to the presence of putative genes by using BLASTX and BLASTN in combination with the GENSCAN (http://argonaute.mit.edu/GENSCAN.html). The unique sequences found in the BACs were used for the new marker development following the procedure described in the chapter 5.1.3.

5.7 Physical mapping of the *Eps3* locus in wheat chromosome 3A cv. Chinese Spring

Physical mapping in bread wheat chromosome 3A was performed in collaboration with Prof. Bikram Gill and Dr. Sunish Sehgal from Kansas State University. FPC v 9.3 software was

used for the assembly at initial cut-off value equaling 1E-75. Markers PAV_259_260, PAV_295_296 and CAPS_321_322 were used to screen three-dimensional pools of the 3AL-specific BAC MTPs. All of them were mapped to the BACs assembled in a single contig (ctg1331). The MTP for the contig was initially selected based on the following criteria, 25-50% overlap between BACs and minimal amount of bands shared between the clones equaling 30. Further, the ctg1331 was re-fingerprinted and re-assembled at 1E-45 with 50% overlap and final assembly was performed at 1E-22 to ensure its correctness. MTP BACs from ctg1331 were single-colony picked, sequenced and annotated, following the same procedure like for barley MTP BACs (see chapter 5.6).

5.8 Circadian clock experiment –time-course RT-qPCR

5.8.1 Expression of clock genes in lines KT3-1 and KT3-5

Lines KT3-1 and KT3-5 were grown under controlled long day 16h/8h day/night at 18°C/23°C for four weeks in the incubator Heraeus Vötsch, type HPS 1500/S. During the next two days conditions were changed to 12h/12h at 18°C/23°C to synchronize the circadian clocks of the plants. In the last stage of the experiment, constant light and temperature (23°C) were set. Leaf samples for RNA extraction were taken every four hours starting from 3:00 am of the second day when conditions were 12h/12h at 18°C/23°C. Each sample was taken from an individual plant. Sampling was continued during subsequent three days of constant ambient conditions. Each leaf sample was immediately frozen in liquid nitrogen and kept in the -80°C freezer until the RNA was extracted. RNA extraction was performed by using PureLink^{TM}RNA MINI KIT (Invitrogen) in combination with TRIzol^{TM} Reagent (Invitrogen) as described in the protocol included to the kit. Quanti Tect Reverse Transcription Kit (Qiagen) was applied to synthesize

cDNA using 1µg of total purified RNA. qPCR reactions were performed on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) employing QuantiTect SYBR Green PCR KIT (Qiagen) at following thermal profile: 94°C for 15 min, 5 cycles of 94°C for 30 s, 65°C for 30 s decreasing by 1°C per cycle and 72°C for 30s, then 40 cycles of 94°C for 30 s, 60°C for 30s and 72°C for 30 s, and 72°C for 1 min. Primers used for cDNA quantification are listed in the Table 5. Constitutively expressed *TmUBC* (TC410705, ubiquitin-conjugating enzyme) was used as a normalization control. Products were electrophoresed on a 2% agarose gel to verify their uniformity and check for primer-dimer absence. Data were analyzed using relative quantification (ddCt) method in following software: Sequence Detection System ver. 2.2.2 (Applied Biosystems). One amplicon per each gene was sequenced to confirm the homology to the *Arabidopsis TOC1*, *LHY* and *LUX* by using the BLASTX.

Table 5 Primer sequences used for qPCR experiments

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size [nt]
TmTOC1, *HvTOC1*	CTTGATACCGACGACCACACATTC	AGCTCATACACCACCAACAATGCTC	150
TmLHY, HvLHY, TtLHY	CAAGGTCTTCTCCCTCTTTTTGCTC	GTTGACCTTGCTCCTGAGCTACTTG	150
TmLUX, TtLUX-A+B	ACAAGCGGTTCGTGGAGGTG	GACGTAGAGGCGGTACTTCTGGAG	137
TmUBC, HvUBC, TtUBC	AAGCAGCCAGAATGTACAGCGAGAAC	GGTACAGACCAGCAAAGCCAGAAATG	151
HvLUX	GGTGACCGAGTGGGAGACG	GCGGTGCACGTCCAGAAG	136
TmGI, HvGI, TtGI	TCCATGACAAAGTAGGTGGCTGA	CATGGTCCTGATGTTGAGTGGAG	135
TmPRR9, HvPRR9, TtPRR9	GTCTGGGTTCCTCCTACTCTCCAC	GCATTTGAAAACCATGCTAACTGC	106
TtLUX-A	GAGTTAGCCCGGCAGGTAACAAC	CCCTCCCCCGAAGTCAAAAC	199
TtLUX-B	GATTGGTGTTGCGAGGTTCG	CGTCTCCCACTCCGTCACC	131
TmPUMILIO	AGCGACTTCTCCACACTCCTCAG	AATATTTAACCACACGCCGCAAC	174

5.8.2 Expression of clock genes in *eam10*/Super Precoz 2H and cv Barke under LD conditions (20/17°C)

Seeds of eam10/Super Precoz 2H were soaked in SD water and kept in the cold (4-8°C) to synchronize germination. After one week, young seedlings were moved to room temperature for 2 days and planted in soil (100 ml wells). Plants were grown in the greenhouse at 20/17°C day/night under the photoperiod of 16h. Sampling was performed on 17 days old plants for one day every three hours (LD conditions). Leaf material for each sample was harvested from at least three plants (biological replicates) and immediately frozen in liquid nitrogen. RNA extraction, reverse transcription and qPCR were performed like in the previous experiment, chapter 5.8.1. Primers used for cDNA quantification are listed in the Table 5.

5.8.3 Expression of clock genes in various wheat and barley lines under constant light conditions

Seeds of wild type segregant in Golden Promise BG284_E11_38, putative knock-down lines BG284_E11_37, DH_BG284_E11_PP1 and BG353_1E15, *T. monococcum* KT3-1 and KT3-5, *T. turgidum* Tsing Hua no. 559 and Fo Shou Mai, *eam10* introgression line in Bowman and Barke were soaked in SD water and kept in the cold (4-8°C) to synchronize germination. After two days, young seedlings were moved to room temperature for two days and planted in soil (150 ml wells). Plants were grown in the greenhouse at 20°C/17°C day/night under a photoperiod of 16h for four weeks. In the actual phase of the experiment, plants were moved to the incubator Heraeus Vötsch, type HPS 1500/S. For the following two days conditions were changed to 12h/12h at 18°C/22°C to synchronize the circadian clocks of the plants. In the last stage of the experiment, constant light and temperature (22°C) were set for three days. Sampling was

performed on 31 days old plants starting at 6:00 am of the second day under constant light and temperature (at analogous time point during the experiment described in the chapter 5.8.1, mutant KT3-5 started being arrhythmic). Leaves were harvested every three hours (LL conditions) from at least three plants per time point per genotype (biological replicates) and immediately frozen in liquid nitrogen. RNA extraction, reverse transcription and qPCR were performed like in the previous experiment 5.8.1, primers used to amplify clock genes are listed in the Table 5. *TtLUX-A* and *TtLUX-B*, A and B genome specific primers, respectively, were developed initially by using the LUX sequences from following databases: http://www.wheatgenome.org/ and http://www.cshl.edu/genome/wheat. The genome-specificity was confirmed by amplification of the *TtLUX* from flow-sorted chromosome arms from cv. Chinese Spring. The 3AL, 3B and 3DL DNA samples were kindly donated by Dr. Hana Simkova from the Institute of Experimental Botany (Olomouc, Czech Republic).

5.9 Further analysis of candidate genes expression

5.9.1 Expression in various tissues

Root and shoot tissue samples were taken from five seedlings per genotype germinated on Petrie dishes. Mature leaves and immature spikes were harvested from several plants before heading. All the samples were taken between ZT=5 and ZT=6 (Zeitgeber Time), immediately frozen in liquid nitrogen and kept at -80°C until the RNA was extracted. Primers used for qRT-PCR of the *TmLUX* and *TmPUMILIO* are listed in the Table 5. *TmUBC* was used as a normalization control for both genes.

5.9.2 Cold-induced changes in the expression

Root and shoot tissue samples were taken from five seedlings per genotype, per time-point germinated on Petrie dishes and kept at room temperature for the “0” time-point, samples were taken at ZT=5 and ZT=9, or kept in the vernalization chamber (8°C, 10h/12h day/night) for three, eight and 15 days. Expression of *TmPUMILIO* was elevated upon cold treatment, this phenomenon has been already assigned to the *PUMILIO-like* genes during transcriptome profiling in barley (Greenup et al. 2011; Svensson et al. 2006).

5.10 Circadian clock experiment- delayed fluorescence measurement

The leaf samples were taken from four week-old plants, cut into pieces and floated on SDW containing 18mg/l fungicide Dithane poured into a 25 compartmental Petri dish. At dusk (10:00 pm, ZT=12) dishes were put into a Sanyo MIR-553 cooled incubator (Sanyo Gallenkamp, UK) and the DF was imaged using an ORCA-II-BT 1024 16-bit camera (Hamamatsu Photonics, Japan) cooled to -80°C. However, the “zero” time point was set at dawn next day (10:00 am, ZT=0). Leaf pieces were kept in the darkness intermitted every hour with a pulse of red/blue light (80µmol*m-2*s-1) lasting for one minute. Immediately after the pulsing, the picture was captured allowing for assaying the circadian clock output with one hour resolution. The whole process was automated by using the Wasabi software (Hamamatsu Photonics, Japan) for controlling the camera and the light source (light emitting diodes). The temperature during the experiments was kept constant at 22°C (experiment 1) or 17°C (experiment 2). The pictures were analyzed in the Metamorph 6.0 (Universal Imaging Corp., Downingtown, USA) and the numerical light intensities were extracted. Obtained data were normalized and de-trended using Excel (Microsoft). Regression equation of the polynomial trend line in the order of six was

used to subtract Y values (delayed fluorescence) thus removing the trend. The overall trend observed was decaying DF which could be explained by exhausting the nutrients in the cut leave tissues. The data could show a pattern of up to six peaks of the DF. The first peak of DF, out of six that were recorded, needed to be discarded as it was out of phase. This phenomenon has already been seen in most of the DF experiments and probably was caused by the period of time required by the photosynthetic apparatus to adapt to the new conditions (Dr. Peter Gould, personal communication, 2011). This led to the situation that a new phase of DF was set the next day and lasted until the end of the experiment. Data collected between hours 36 and 132 (normalized but not de-trended) were used to calculate periods corresponding to the four middle peaks of the delayed fluorescence. The periods at 95% confidence interval and relative amplitude errors (RAE) were calculated in BRASS by running fast Fourier transformed non-linear least-square analysis (Plautz et al. 1997). The last, sixth peak of the DF was out of consideration because it could not be detected in many leaf samples as they turned yellow by that time.

5.11 Mutant phenotypic analysis in the greenhouse under LD (18°C/15°C)

Twenty plants of each wild type KT3-1 and mutant KT3-5 were grown at 18°C/15°C and photoperiod of 16h/8h hours day/night. About half of the plants were used for apex dissections to determine double ridge stage appearance thus allowing for the calculation of the vegetative growth phase duration. For this reason, two plants of each genotype were dissected every three to seven days. The rest of the plants (seven of KT3-1 and ten of KT3-5) were phenotyped according to heading date and spikelet number as described for RILWA1 population (chapter 5.1.2) as well as leaf and tiller number.

5.12 Mutant phenotypic analysis in the incubator under SD

Ten plants of each wild type KT3-1 and mutant KT3-5 were grown in a controlled growth incubator Heraeus Vötsch, type HPS 1500/S. The temperature during the whole experiment was 22°C/18°C with a photoperiod of 8h/16h day/night. Heading date, spikelet number, spike length and tiller number were scored. The experiment was terminated after 214 days and the plants which did not head by this time were dissected to determine the developmental status of the main tiller apex.

5.13 Mutant phenotypic analysis in the incubator at low temperature (16°C)

Eighteen and 14 plants of wild type KT3-1 and mutant KT3-5, respectively, were grown in the incubator Heraeus Vötsch, type HPS 1500/S at 16°C/14°C with a photoperiod of 16h/8h day/night. Most of the plants were used for apex dissections to determine the duration of the vegetative phase until double ridge stage, double ridge stage to terminal spikelet and terminal spikelet stage to heading. For this reason, two plants of each genotype were dissected every three to seven days. The remaining plants (at least three of each KT3-1 and KT3-5) were phenotyped according to heading date and spikelet number as described for RILWA1 population (see chapter 5.1.2) as well as leaf and tiller number. Thermal time to heading was calculated by multiplying calendar days to heading by the average growth temperature and it was expressed in degree days (°Cd). Base temperature of 0°C was used as the temperatures during the experiment were constant and always above developmental base temperatures for wheat (Slafer and Savin 1991).

5.14 Mutant phenotypic analysis in the incubator at high temperature (26°C)

The procedure followed was almost identical with already described in the previous chapter 5.13. The only difference were the growing conditions in the incubator– the temperature was set to 26°C/24°C day/night (ten degrees higher) during the whole experiment. An online tool http://vassarstats.net/anova2u.html was used to perform two-way factorial analysis of variance.

5.15 Genetic transformation of barley cv. Golden Promise and T_0 plants analysis

Among the available techniques utilizing transgene technology for testing gene function, a constitutive knock-down of the target candidate gene with RNAi construct appeared to be the most reasonable choice. Barley cultivar Golden Promise was used for the experiments because of well established protocols for its efficient transformation (Hensel et al. 2008; Himmelbach et al. 2007). Suitable inserts for the RNAi construct were predicted based on the off-target analysis by the siRNA Scan (http://bioinfo2.noble.org/RNAiScan.htm) and SI-FI software (http://labtools.ipk-gatersleben.de/index.html). The inserts were amplified with the primers listed in the Table 6 using as a template Golden Promise or Chinese Spring genomic DNA.

Table 6 List of the primers used to amplify the inserts for the RNAi constructs. Extra nucleotides, written in *italics*, adapting the inserts to the restriction digestion were added to the 5'-ends of the primers

Transgenic lines	Forward primer (5'-3')	Reverse primer (5'-3')	Insert size [nt]
BG284, BG353/2	GGATGCTCTGCTCTCTGCTCATC	ATCCTAATTCCCTTGTTGGGCTTC	154
BG285	CACAAGCGGTTCGTGGAG	CTCCTGCTCATCAGTCAGTCAACAG	518
BG352	*GATATC*ACATGGTAATGGACAACACTCCTAC	*CTCGAG*AGTTGGACTTCCAATTCCACTGTA	290
BG353/1	*GATATC*GGATGCTCTGCTCTCTGCTCATC	*CTCGAG*ATCCTAATTCCCTTGTTGGGCTTC	389

PCR fragment was ligated with the pGEM®-T Easy Vector (Promega) and used for the transformation of the One Shot® TOP10 Competent Cells (Invitrogen) following the manufacturer's protocols. The positive clones were grown overnight and the plasmids were purified by the QIAprep Spin Miniprep Kit (Qiagen). Presence of the insert was verified by the restriction digestion with EcoRI (Fermentas). In the next few steps the insert and the pIPKTA38 vector were prepared for ligation. The insert containing plasmid was digested with BcuI (Fermentas), purified (MiniElute PCR Purification Kit, Qiagen) digested again with NotI (Fermentas), separated on a 1.5% agarose gel and purified (MiniElute Gel Extraction Kit, Qiagen). Whereas, the vector pIPKTA38 was digested with XbaI (Fermentas) leaving compatible ends with SpeI, following the NotI digestion and purification from the agarose gel like just described for the insert. For developing the BG353/1 lines, insert and pIPKTA38 vector were subjected to the double digestion with EcoRV and XhoI. Ligation reaction was performed by using the T4 ligase (Fermentas), 1X T4 buffer, 6µl of the insert eluate and 1µl of the vector. The construct was used for transformation of the One Shot® TOP10 Competent Cells (Invitrogen). The resulting clones were grown overnight and plasmids were purified (QIAprep Spin Miniprep Kit, Qiagen). Insert containing clones were selected by the double restriction digestion with HincII/PstI or EcoRV/XhoI (Fermentas). Positive clones were used for the clonase reaction with pIPKb007 (lines BG284 and BG285) or pIPKb027 vector (lines BG352 and BG353). *Agrobacterium*-mediated plant transformation was performed by Dr. Götz Hensel as described elsewhere (Hensel et al. 2008; Himmelbach et al. 2007).

T_0 regenerants were planted to 0.2 l pots and grown for 5 weeks under controlled conditions 10h/14h at 14°C/12°C day/night. Then potted to big 2.0 l pots and grown in the cold

room at 15°C for another 5 weeks. After this period the long day treatment (16h/8h) started at 15°C/18°C until harvest. Plants were tested for presence of the inverted repeats (transgene) using the PCR-based assay like described elsewhere (Himmelbach et al. 2007). The ploidy level was measured by Dr. Götz Hensel using a flow cytometer (Partec GmbH, Münster, Germany). Nuclei were stained with CyStain UV (Partec GmbH, Münster, Germany) according to the manufacturers' instructions.

Positive T_0 plants of lines BG284 and BG285 were additionally analyzed by RT-qPCR to verify the efficiency of candidate gene silencing. For this reason a primer pair *HvLUX* (see Table 5) was used following the procedure like described in the chapter 5.8.1. T_0 plants were phenotyped according to heading date (main culm spike), spikelet number and spike length (3 to 6 spikes including that of main culm). Selected lines BG284, BG285, BG352 and BG353 were used for doubled haploid (DH) production by Dr. Götz Hensel, following the published protocol (Coronado et al. 2005). After ploidy measurement, the still haploid plants were treated with the mitotic inhibitor colchicine (Luckett 1989; Takamura and Miyajima 1996). For this purpose, haploid plants which had developed at least two tillers were removed from the soil and the roots were carefully washed with tap water, cut back to 1 cm and immersed in aqueous colchicine solution 0.1% (v/v), 0.8% (v/v) dimethyl sulfoxide (DMSO), and 0.05% (v/v) Tween-20) at room temperature in dark for 5 h. After removal from the colchicine solution the roots were carefully rinsed with running tap water for a few minutes. Treated plants were transplanted in soil. After re-establishment, plants were vernalized in a cold room at 2°C and 8h day length for 6 weeks. Grains of doubled haploids were harvested at full maturity.

5.16 T1 plants analysis

Four best, having most effective gene silencing, diploid and early heading lines, two of each BG284 and BG285 were selected for further analysis. About 40 plants per line were grown in the greenhouse at 16°C/15°C with a photoperiod of 16h/8h day/night. All plants were genotyped according to the presence of inverted repeats like described in the previous chapter (5.15) and the ratio of transgene presence/absence was tested according to the expected 3:1 Mendelian segregation by using the Chi-squared test. Moreover, heading dates were recorded on the main culms of all plants. Spikelet number, seed number per spike and spike length for three average spikes were scored exclusively from the best lines; the ones having Mendelian segregation of the transgene and showing the earliest heading.

5.17 TILLING in barley cultivar BARKE

TILLING (Targeting induced local lesions in genomes) was performed in the population described elsewhere (Gottwald et al. 2009). The genomic DNAs were pooled eight-fold in two dimensions allowing quick identification of the putative mutants (Gottwald et al. 2009). The fragments of genes were amplified with primer pairs listed in the Table 7. Two additional primers, *HvPUMi* were used to re-sequence *HvPUMILIO* amplicon because of its large size (Table 7).

Table 7 List of primers used for TILLING the candidate genes in barley

Name	Forward primer (5'-3')	Reverse primer (5'-3')
HvLUX	GGTGACCGAGTGGGAGACG	ATCCTAATTCCCTTGTTGGGCTTC
HvPUM	CAGGCAATCAAAACAATGAGTCG	CGTACATTTGAAGGCTGAGTGCTAA
HvPUMi	ACCCACTCTATGCTCAATTTCTTCG	GCTTCTGTGACTGAACCAATGGA

The PCR thermal profile was as follows: 94°C for 3 min, 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, and 72°C for 10 min. Immediately after, the heteroduplex formation step was performed starting with denaturation for 10 min. at 99°C, then 23 cycles of 70°C for 20 s, 69.7°C for 20 s and 69.4°C for 20 s; decreasing at every step by 0.9°C per cycle. The obtained products were subjected to the standard procedure of *AdvanCE*™ TILLING kit. To describe it shortly, a premix was made containing a mismatch detecting CEL1 enzyme, namely dsDNA Cleavage Enzyme/T-Digest Buffer mixed in ratio 1:125. 2 µl of heteroduplexes were digested with 2 µl of the premix at 45°C for 30 min. The cleaved products were separated using the *AdvanCE*™ FS96 capillary electrophoresis system and results were analyzed in the *PRO Size*™ software. To distinguish the real putative mutation from the background products, sizes of two digested products after summing up were expected to give the size of the initial PCR product and only such instances were accepted. In case this criterion was not fulfilled, the original DNA pool was not considered as containing the putative mutant. All the possible mutations found were further verified by re-sequencing. For some instances only one dimension could be gathered; meaning that only one pool on the plate contained the putative mutant disabling the proper deconvolution leading to a single genotype. To prevent loss of potentially valuable mutants, all 8 individuals from such pools were sequenced. Detected and confirmed mutations were subsequently classified to one of the following types: silent (synonymous or localized in non-coding region), missense and nonsense. M3 families having nonsense mutations were directly selected for phenotyping. In case of missense mutations, alignments with homologous proteins from other plant species (Rice, *Brachypodium*, Maize, Sorghum, *Arabidopsis*) were analyzed by using BlastX. By this procedure, conserved and putatively essential amino acids

could be identified enabled selection of the amino acid substitutions that took place at such positions. M3 families having missense mutations were also selected for further analysis.

M3 families were grown in similar conditions as already described in chapter 5.1. The plants needed to be genotyped by re-sequencing the candidate genes due to the heterozygosity of the mutations detected in M2 plants. The DNA was extracted by using the modified Doyle and Doyle method (Doyle and Doyle 1987). Primer sequences and PCR conditions were as described for analysis of the M2 DNA; excluding the heteroduplex formation step. Heading dates were recorded for each plant when awns were visible.

5.18 Re-sequencing LUX in wheat collection

Forty one di-, 52 tetra- and three hexaploid wild and cultivated wheat accessions were selected by Dr. Benjamin Kilian for re-sequencing the LUX gene, Appendix Table 4. Main criteria for selection were to capture most of the genetic and phenotypic (flowering time) diversity in wheat. Genome specific primers were developed initially by using the LUX sequences from following databases: http://www.wheatgenome.org/ and http://www.cshl.edu/genome/wheat. Primers used for amplification and re-sequencing are listed in the Table 8. PCR and sequencing conditions were like in the previous experiment 5.1.3. To distinguish between a cool and warm climate, an average temperature was calculated from data for March and August from years between 1900 and 2009. The information was taken from the http://sdwebx.worldbank.org/climateportal as provided by the Climatic Research Unit (CRU) of University of East Anglia (UEA). Eighteen Celsius degrees were set as a threshold value, meaning

that locations having less than 18°C (average value from March and August) were classified as a cool climate and those having 18°C or more—as a warm climate.

Table 8 Primers used for amplification and re-sequencing of the *LUX* in a diverse wheat collection

Primer name	Genome amplified	Primer sequence
LUX_full_ORF_F	Tm/Tb/Tu/A[1]	GAGTTAGCCCGGCAGGTAACAAC
LUX_full_ORF_R	Tm/Tb/Tu/A	ACAGAGCACACACTCTGCAACTCTC
LUX_6X_3B_3D_F	A	ATCTGCGCTTTATTCCCTTTTC
LUX_6X_Universal_R	A	CGGGATGGATGGTTATCCTTA
PCL_orf_F1	B	TCCAATCCGTCCAATCCAATC
LUX_6X_3B_R	B	TTGACTGATCGACACAAACACAC
3D_LUX_pN2F	D	GGAGGCAGGGGAGGATATGG
LUX_6X_3D_R	D	TTGACTGATCGAGACACACACAG

[1]Genomes coming from Tm (*Triticum monococcum* L.), Tb (*Triticum boeoticum* L.), Tu (*Triticum urartu* L.) and genome A from tetraploid and hexaploid wheats could be amplified with the primers shown

5.19 *eam10* mutant

Seeds of the B4498 and B7490 were kindly donated by Dr. Mike Ambrose from the John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, and the seed of Super Precoz 2H and *eam10* introgression line in Bowman–by Dr. Harold E. Bockelman from the National Small Grains Collection (USDA). Seeds of following genotypes: Super Precoz 2H, B4498 (Malteria Heda, a donor line for B7490, MC20), B7490 (MC20, a donor line for Super Precoz 2H), Bowman and *eam10* introgression line in Bowman were soaked in SD water and kept in the cold (4-8°C) to synchronize germination. After two days, six young seedlings per genotype were moved to room temperature for two days and planted in soil (150 ml wells). Plants were grown in the greenhouse at 20°C/17°C day/night under a photoperiod of 16h and transplanted to the 1 l pots after three weeks. Growing conditions were the same like before the transplanting and the pots with plants were randomized weekly. Heading date, spikelet number and spike length were scored from the main culm of each plant prior to harvest.

6 Results

6.1 RILWA1 evaluation, high-density mapping and QTL analysis

6.1.1 Phenotype evaluation

T. monococcum mutant KT3-5, donor line KT3-1, and *T. boeoticum* KT1-1, headed 56±2.06 (± standard error of the mean, SEM), 93±2.50 and 66±2.21 days after the end of the vernalization treatment (d.a.v.), respectively (Table 9).

Table 9 Summarized are phenotypic results of heading time, anthesis time, spikelet number and spike length collected during the greenhouse experiment

Trait	KT1-1 (parent)			KT3-1 (donor of mutant KT3-5)			KT3-5 (parent/mutant)			M.a.c.[1)]	RILWA1 (population)		
	Mean[2)]	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.		Mean	Min.	Max.
Heading time [d.a.v.][3)]	66±2.21	60	74	93±2.50	90	95	56±2.06	48	67	-9	53	29	78
Anthesis time [d.a.v.]	78±1.91	74	83	102±2.50	99	104	68±	62	74	-9	66	46	94
Spikelet number	23±0.32	22	24	39±2,00	37	41	22±1.57	15	27	-4	22	8	36
Spike length [cm]	11±0.20	9.9	11	9±0.25	9	9.5	6±0.25	5	6.7	-1	7	4	11

[1)] M.a.c.-contribution of a mutant allele CAPS_285_286 to the variance of the trait in the RILWA1 population
[2)] Mean value plus/minus standard error of the mean (SEM)
[3)] Heading and anthesis time are expressed in days after the end of vernalization

Interestingly, the mean heading time score for the RIL population was eight days earlier than the average for the two parents (see Table 9). This could be explained by the distorted segregation ratios around the *Eps-3A*^{m} locus (see Figure 3), where the mutant allele giving earliness was more abundant; out of 110 lines, 73 carried the earlier allele.

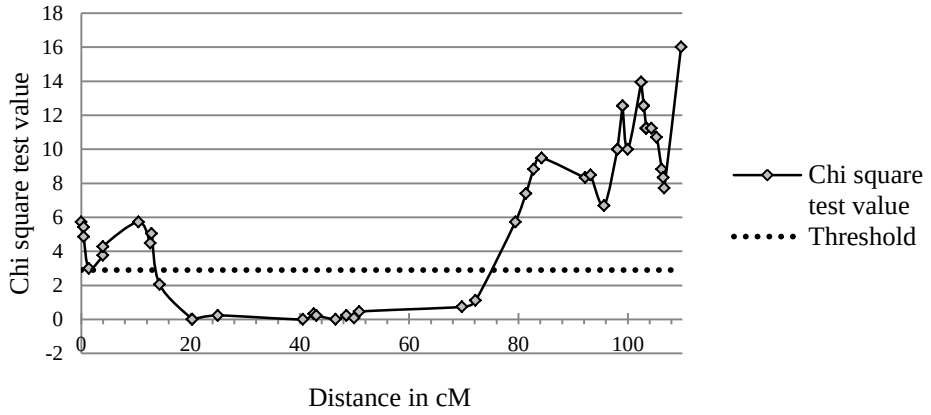

Figure 3 Chi square test values for the segregation of marker loci mapped to the 3AL chromosome according to the expected Mendelian ratio of 1:1. The *Eps-3A*^{m} locus was located at 102.9 cM and within the region where most of the marker loci were above the Chi squared test value. Such loci shall be considered to be significantly distorted ($p < 0.05$)

As already observed by (Shindo et al. 2002), variation of heading time within the RIL population (from 29 to 78 d.a.v.) was much greater than between the two mapping parents. This suggests transgressive segregation of the traits; as expected for this type of inheritance, the phenotypic frequency distribution for heading time was quantitative (see Figure 4).

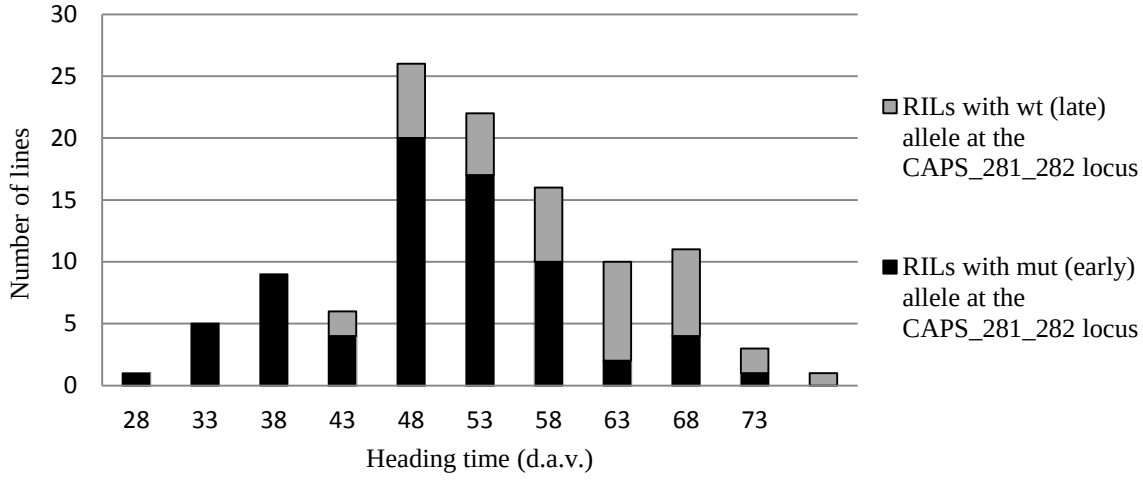

Figure 4 Phenotypic frequency distribution for heading time in the RILWA1 population consisting of 110 lines. *Black bars* represent lines carrying the early allele at *Eps3-A*^{m} locus whereas *grey bars* include lines with the late allele. Values are expressed in days after the end of vernalization (d.a.v.). Means from 10 plants per line were ranked for every 5 days. Lines were divided into two classes; wt and mut, according to the allele at the CAPS_281_282 locus.

All three developmental traits that were co-analyzed showed high correlations with heading time scores (see Table 10). The highest correlation was found between heading time and time to anthesis (+0.98), and the smallest between heading time and spike length (+0.76, Table 10).

Table 10 Phenotypic correlation matrix of the traits scored during the greenhouse experiment on the RILWA1 population consisting of 110 lines

Traits	Heading time	Anthesis time	Spikelet number
Anthesis time	0.98	-	-
Spikelet number	0.79	0.81	-
Spike length	0.76	0.77	0.89

6.1.2 Mapping and QTL analysis

Out of 158 designed primer pairs, 53 gave amplicons that showed polymorphism and 44 marker loci (19 CAPS, three dCAPS, eight INDEL, eight AS_PCR, two TP and four PAV) could be linked and ordered spanning 109.7 cM of the 3AL chromosome (see Appendix Table 1 and Appendix Table 2). Additionally five CAPS and two INDEL markers were selected and mapped as described in (Hori et al. 2007). From the total number of 51 marker loci, nine marker loci from the pericentromeric region of chromosome 3A showed a significant segregation distortion from the expected Mendelian ratio of 1:1 (χ^2=3.00-5.73, linkage group 0-12.9 cM); the allele coming from the KT1-1 line was more frequent. In the middle part of the long arm of 3AL (linkage group 14.3-72.1 cM), 13 marker loci segregated in the agreement with the 1:1 ratio (χ^2=0.00-2.06). However, the majority of marker loci (29) on the distal part of 3AL were significantly distorted with the mutant allele being more abundant (χ^2=5.73-16.01, linkage group 79.4-109.7 cM; see Appendix Table 1 and Figure 3). QTL analysis using the MQM algorithm revealed the highest LOD score values for heading time, anthesis time, spikelet number and spike length (5.96, 5.40,

3.33 and 5.10, respectively; see Table 11 and Figure 5) with a threshold value equalling 2.0 (at $p < 0.05$).

Table 11 Selected results from the *Eps-3A^m* QTL analysis on heading time, anthesis time, spikelet number and spike length obtained by using the multiple-QTL models (MQM) algorithm. Maximal LOD and explained variance values, flanking and linked marker loci as well as marker loci being selected as cofactors are shown. Respective traits were evaluated on the RIL population (RILWA1) consisting of 110 lines

Trait	Max. LOD score	Max. % explained variance	Marker loci matching max. LOD and max. % explained variance	LOD-1 interval	Markers chosen as cofactors
Heading time	5.96	21.6	CAPS_281_282; CAPS_zt4_zt5; PAV_295_296	CAPS_273_274—CAPS_289_290	CAPS_131_132; PAV_177_178
Anthesis time	5.4	19.9	CAPS_281_282; CAPS_zt4_zt5; PAV_295_296	CAPS_273_274—CAPS_289_290	CAPS_131_132; PAV_177_178
Spikelet number	3.33	12.9	CAPS_281_282; CAPS_zt4_zt5; PAV_295_296	PAV_185_186—CAPS_305_306	INDEL_111_112; AS_PCR_153_154; CAPS_139_140, INDEL_245_246, CAPS_131_132
Spike length	5.1	18.9	CAPS_281_282; CAPS_zt4_zt5; PAV_295_296	CAPS_273_274—CAPS_253_254	INDEL_243_244; PAV_177_178

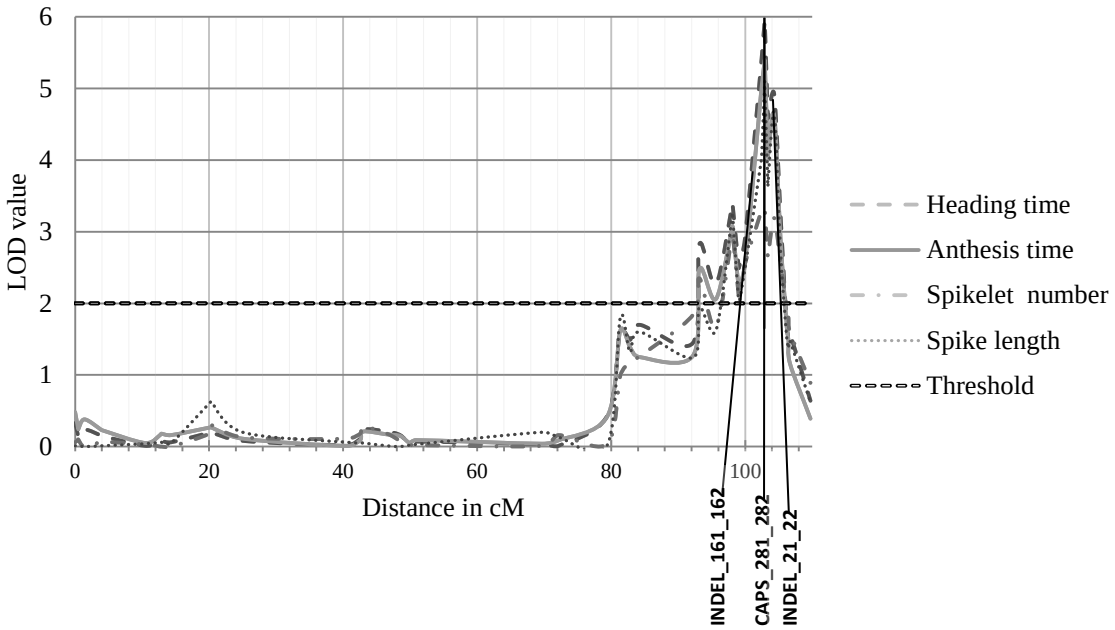

Figure 5 Mapping of the *Eps-3A^m* QTL to the distal part of chromosome 3A^{m}L. Marker locus CAPS_281_282 was associated with the top of QTL peak, whereas INDEL_161_162 and INDEL_21_22 flanked the locus within the 0.9 cM interval. . QTLs for heading time, anthesis time, spikelet number and spike length were localized by using the multiple-QTL models (MQM) algorithm. All traits were evaluated on the RIL population (RILWA1) consisting of 110 lines

Marker loci being selected as cofactors are listed in the Table 11. In total, three marker loci; CAPS_281_282, CAPS_zt4_zt5 and PAV_295_296 co-segregated with the highest LOD scores and explained the maximal percentage of variance for all traits. When looking at the selected 9 RILs with recombination around the *Eps-3A^m* locus, only one of them (RILWA1_15) with mutant alleles headed later than the earliest (RILWA1_75) with the wild type alleles (see Table 12). Nevertheless, the two closest recombinants were quite contrasting according to the all analyzed traits; RILWA1_32 with mutant alleles at *Eps-3A^m* headed 17 days earlier and developed two times smaller spikes than RILWA1_75 with wild type alleles (see Table 12).

Table 12 Selected phenotypic data collected from the RI lines showing recombination around the *Eps-3A^m* locus. Lines are classified into wt and mut according to the marker locus CAPS_281_282 co-segregating with the highest LOD values for heading time, anthesis time, spikelet number and spike length QTLs. Two highlighted lines had the closest recombination events found in the RILWA1 population.

RI line	HT[1] [d.a.v.]	AT[2] [d.a.v.]	SN[3]	SL[4] [cm]	*Eps-3A^m*	Recombination breakpoint between
RILWA1_75	*58±1.03*[5]	*69±0.63*	*23±0.37*	*10±0.29*	*wt*	*CAPS_281_282 (wt), INDEL_271_272(mut)*
RILWA1_110	61±1.24	72±0.96	30±0.47	10±0.23	wt	CAPS_315_316 (wt), INDEL_21_22 (mut)
RILWA1_18	74±0.22	89±0.48	36±0.5	10±0.17	wt	INDEL_271_272 (wt), CAPS_273_274 (mut)
RILWA1_32	*41±0.56*	*52±0.9*	*12±0.65*	*5±0.12*	*mut*	*INDEL_201_202 (wt,) CAPS_281_282 (mut)*
RILWA1_79	48±1.14	61±1.67	23±0.67	8±0.27	mut	INDEL_271_272 (mut), CAPS_273_274 (wt)
RILWA1_111	49±0.87	62±0.82	21±1.09	7±0.33	mut	INDEL_271_272 (mut), CAPS_273_274 (wt)
RILWA1_35	53±1.03	67±0.95	25±1.51	8±0.45	mut	INDEL_271_272 (mut), CAPS_273_274 (wt)
RILWA1_94	55±0.90	69±0.86	22±0.47	7±0.12	mut	INDEL_271_272 (mut), CAPS_273_274 (wt)
RILWA1_15	60±0.42	74±0.42	30±0.47	10±0.23	mut	CAPS_315_316 (mut), INDEL_21_22 (wt)

[1] Heading time (HT) expressed in the days after the end of vernalization [d.a.v.]
[2] Anthesis time (AT) expressed in the days after the end of vernalization [d.a.v.]
[3] Spikelet number (SN)
[4] Spike length
[5] Mean value plus/minus standard error of the mean (SEM)

6.2 Southern blot analysis and background of the KT3-5 mutant

In agreement with the PCR reactions, southern blot analysis revealed that the two genes, *TmLUX* and *TmPUM* were absent from the KT3-5 mutant genome while being present in the wild type donor line KT3-1 (see Figure 6). This strongly supports the hypothesis that the mutation caused by X-ray irradiation was a deletion which involved at least the two genes analyzed; *TmPUM* and *TmLUX*. Importantly, detected QTLs in the RILWA1 population led to the discovery of the mutation inherited from the KT3-5 parent.

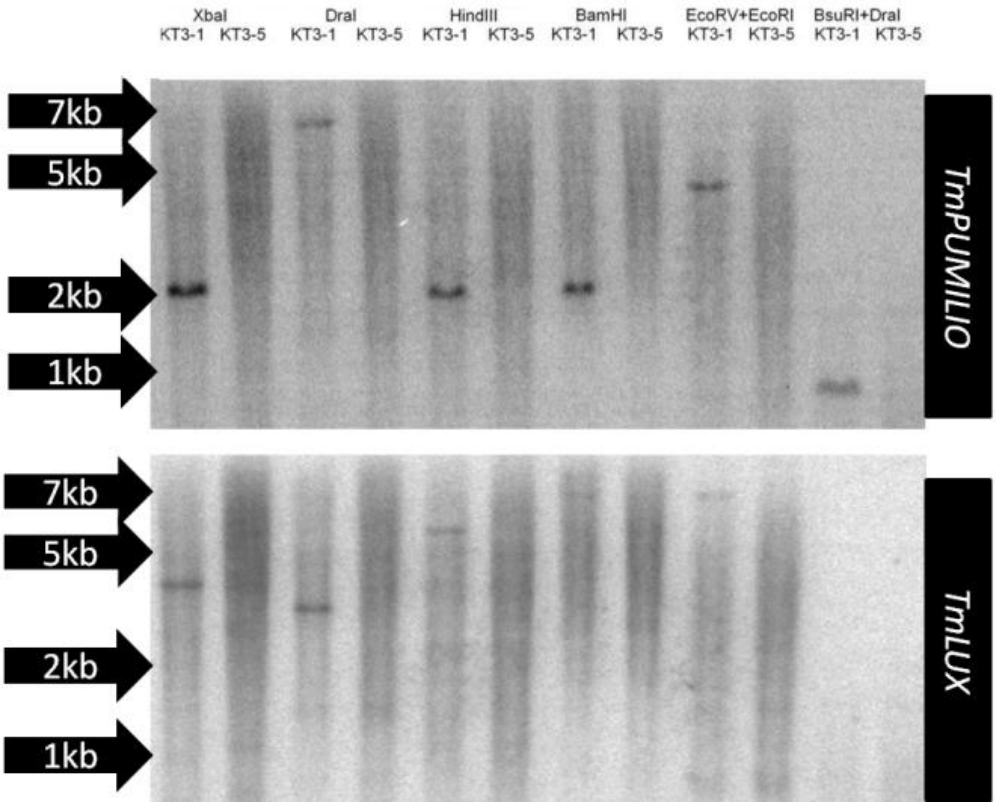

Figure 6 Results from southern blot hybridization showing deletion of the two genes, TmPUMILIO and TmLUX, co-segregating with the highest LOD values in the RILWA1 population. The experiment was performed according to the protocol described in the chapter 5.4. Shortly, DNA of KT3-1 and KT3-5 was digested with four restriction enzymes and two combinations of enzymes. Specific probes of *TmPUMILIO* and *TmLUX* were hybridized to the fractionated and blotted DNA products.

17 markers re-sequenced in lines KT3-1, KT3-5 and KT1-1 showed 0 polymorphisms between mutant KT3-5 and the assumed wild type donor KT3-1 in 14,700 bp. This strongly supports the aforementioned relationship between the KT3-5 and KT3-1

6.3 High-resolution and comparative mapping of the *Eps-3A^m* locus

The segregation ratio of the flanking marker INDEL_271_272 (187:150:321) in the F2 population was distorted in favor of the wild type allele with statistical significance at $p<=0.5$ ($\chi2=4.55$, $p=0.103$). This was contrasting to the segregation observed in the RILWA1 population, where mutant alleles at the *Eps-3AL^m* locus were more abundant. Two out of three markers (CAPS_335_336 and TP_13_14) used for the barley BAC library screening allowed for successful de-convolution and pointed BACs that had been already assembled within the single contig95 (see Table 3). One of the co-segregating genes (*TmLUX*) could be assigned to a given position on the BAC contig by BLAST analysis against the sequenced BES (BAC-End Sequences) as well as by using the already existing information about mapped markers. BLAST search revealed a very similar sequence to the 3'UTR of *TmLUX* present in the BES of BAC clone HVVMRXALLeA0370P14 (see Table 3). This BAC clone was also present in contig95, therefore the MTP for this contig was selected and sequenced (see Figure 8). F2 plants, in which *TmLUX* could be amplified headed later than those with the null allele, see Figure 7. Also, other traits scored (anthesis time, spikelet number and spike length) were similarly correlated.

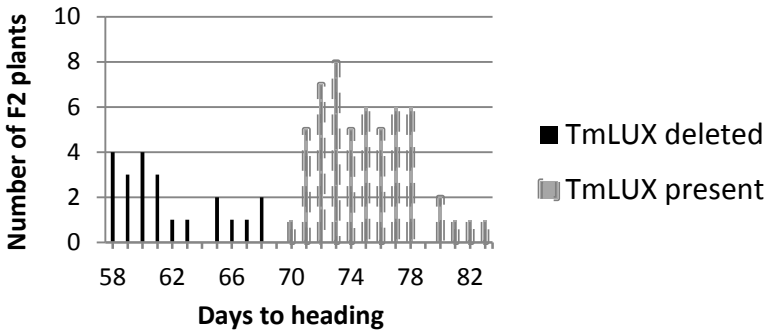

Figure 7 Phenotypic frequency distribution of heading time among the 38 F2 recombinants and 38 randomly selected individuals grouped according to the presence (*gray*)/absence (*black*) of the *TmLUX* gene. The grouping allowed for the best explanation of the variation in heading time, thus confirming *TmLUX* as a sensible candidate for the *Eps-3A*^{m} locus.

6.4 Comparison to the syntenic regions of barley chromosome 3H and wheat chromosome 3A

The locus aligned to the syntenic region on barley chromosome 3H had conserved order of markers, even those which could not be predicted based on Brachypodium –rice collinearity study or deduced from the barley genome zipper (Gawroński and Schnurbusch 2012; Mayer et al. 2011). A physical interval corresponding to the genetic interval between markers INDEL_271_272 and TP_13_14 spanned about 1.4 Mbp of DNA sequence and included 16 predicted genes. Later on, it was possible to delimit the interval to about 260 Kbp that contained only two genes by using the F2 recombinants, see Figure 8. However, six out of eight recombination events around the locus could not be precisely localized because of dominant scoring of the markers corresponding to the deleted genes. To resolve this, homozygous F3 plants were genotyped revealing that seven recombinations occurred on the proximal side of the locus and only one on the distal side (see Figure 8).

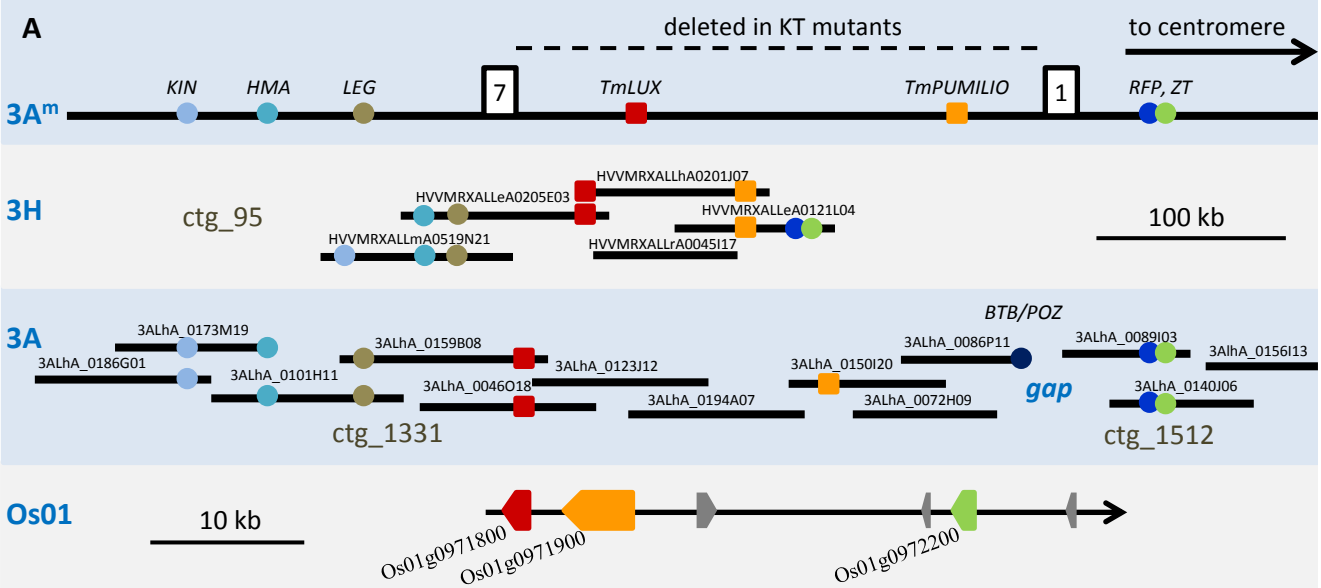

Figure 8 Comparative map-based cloning of the *Eps-3A*^{m} locus. (A) Genetically mapped *Eps-3A*^{m} locus (*3A*^{m}) was integrated with physical ctg_95 from barley chromosome 3H and two contigs, ctg_1331 and ctg_1512 from bread wheat chromosome 3A. Wheat contigs did not overlap as indicated by the *gap*. Only BAC addresses selected for the minimum tilling path (MTP) are shown. Putative genes (*color-coded*) annotated from the barley and wheat sequence could be mapped in F2 population of *T. monococcum* x *T. boeoticum* revealing higher synteny with barley. New recombinations found in the F2/F3 population (*rectangles filled with number of recombinations*) delimited the *Eps-3A*^{m} locus to only two genes: *TmLUX ARRHYTHMO* (*TmLUX, red*) and *TmPUMILIO* (*orange*). Moreover, both genes had been deleted from the genomes of the early heading *T. monococcum* KT mutants. Genes mapped proximally to the locus (*KIN*, *HMA* and *LEG*) did not have their putative orthologs in the collinear part of rice chromosome 1 (Os01) and the syntenic relationship was thus broken at this site. RFP, a putative ring finger protein and ZT, a putative zinc transporter flanked the *Eps-3A*^{m} locus from the distal site. A marker developed based on the putative transcription factor (*BTB/POZ*) annotated from the BAC 3ALhA_0086P11 could not be linked with the locus in einkorn wheat.

6.5 *Eps-3A*^{m} locus effect depends upon the temperature and creates a photoperiod-insensitive flowering time phenotype

Results from the SD experiment clearly indicated that the KT3-5 mutant missed the requirement for the long photoperiod to induce flowering, Table 13. The difference to the KT3-1 wild type could be estimated to be at least three months; however, KT3-1 did not undergo transition to the generative phase by the end of the experiment as verified by the apical meristem dissections. The difference in heading dates between KT3-1 and KT3-5 depended upon the SD/low temperature treatment during the vegetative phase of growth. After such

treatment the maximal difference was obtained, also according to the spikelet number, Table 9. However, the higher the temperatures during early development, the fewer spikelets were developed by both KT3-1 and KT3-5. In two experiments, when plants were grown all the time at 18°C/15°C and 26°C/24°C; day/night KT3-1 developed even smaller spikes than KT3-5. This would indicate that the *Eps-3A^m* locus can positively affect yield when plants are exposed to the high temperatures during the early development and there is a significant interaction between genotype and the temperature during spike development (Table 14).

Table 13 Resulting values from the mutant phenotypic analysis are shown. KT3-5 mutant was grown along with the donor line KT3-1 under various conditions described in the chapters 5.11 to 5.14 . Although the KT3-5 mutant always flowered earlier than KT3-1, difference in the spikelet number depended on the ambient temperature; KT3-1 developed more spikelets than KT3-5 at the low temperature but the situation was reversed at the high temperature. All values of KT3-1 and KT3-5 represent mean±SEM (standard error of the mean). *P* values were calculated by using the paired Student's t-test

Conditions	Trait	KT3-1	n	KT3-5	n	*p* value
LD 18°C/15°C, 16h/8h, day/night	Days to heading	93±1.32	7	67±0.63	9	$7.9*10^{-11}$
	Spikelet number	23±0.92	7	27±0.59	9	5.3*10-4
SD 22°C/18°C, 8h/16h, day/night	Days to heading	>214	10	168±2.14	10	Not applicable
	Spikelet number	No spikes	10	26±0.81	10	Not applicable
	Spike length [cm]	No spikes	10	6.82±0.22	10	Not applicable
	Tiller number	24±0.98	10	38.±1.49	10	$1.34*10^{-7}$
LD + cool 16°C/14°C, 16h/8h, day/night	Days to heading	86±0.52	7	67±0.23	8	$4.7*10^{-15}$
	Days to anthesis	103±0.56	7	82±0.34	8	$8.4*10^{-14}$
	Spikelet number	35±0.51	7	31±0.16	8	$8.3*10^{-8}$
	Spike length [cm]	8.56±0.07	7	8.3±0.14	8	0.14
	Tiller number	12±0.49	7	9±0.71	8	0.0052
	TKW* [g]	28.93	7	29.48	5	Not applicable
	KNP†	348±31	7	338±12	5	0.81
LD + warm 26°C/24°C, 16h/8h, day/night	Days to heading	100±0.99	7	80±0.62	7	$1.6*10^{-10}$
	Days to anthesis	108±1.47	7	88±0.76	7	$9.5*10^{-9}$
	Spikelet number	21±1.18	7	23±0.38	7	0.064
	Spike length [cm]	6.13±0.25	7	5.97±0.21	7	0.58
	Tiller number	17±3.63	7	16±1.65	7	0.75
	TKW*[g]	17.2	5	15.21	5	Not applicable
	KNP†	31±12	5	111.2±17	5	0.0047

*TKW = Thousand Kernel Weight, measured with 0.001 g precision

†KNP = Kernel Number per plant

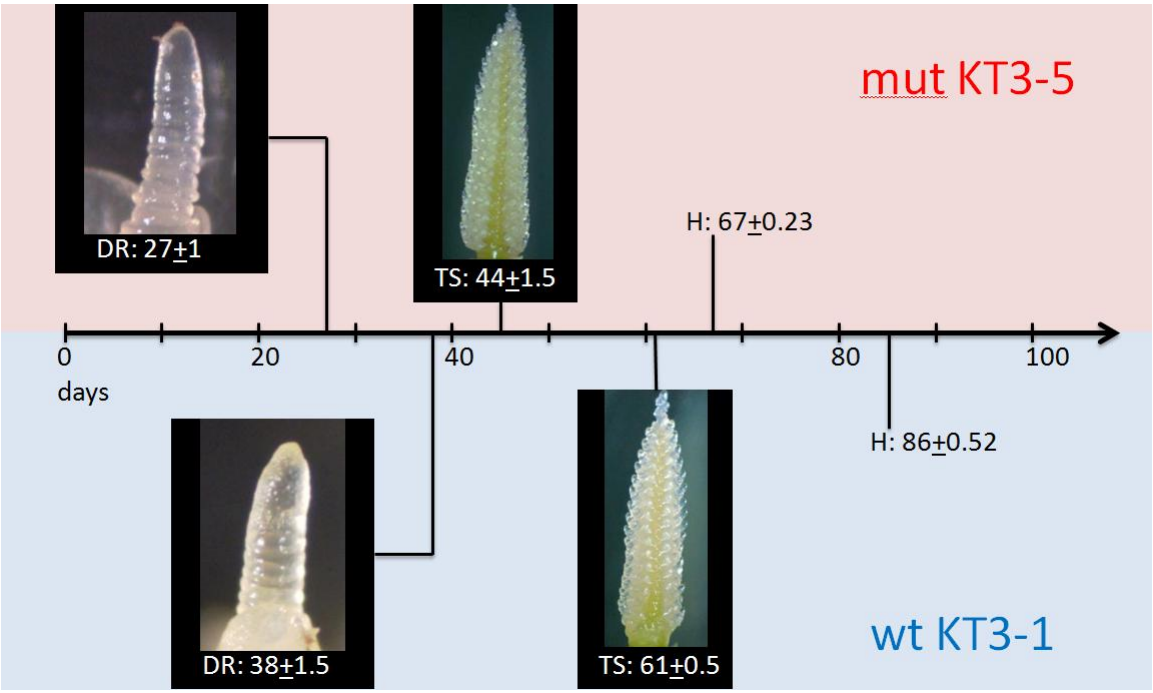

Figure 9 Mutant KT3-5 and wild type KT3-1 plants were grown under controlled environment at 16°C/14°C; 16h/8h day/night. Microscopic dissections of their apices were performed to obtain information about the duration of the vegetative phase, double ridge (DR) to terminal spikelet (TS) and terminal spikelet to heading (H) periods. Values represent mean±SEM (standard error of the mean). KT3-5 had shortened vegetative and DR to TS phases, whereas duration of TS to heading was similar to that of KT3-1

Several observations could be made by comparing the significance of the differences found between KT3-1 and KT3-5 grown under two different temperature regimes. When considering the whole period of plant development, no significant interaction between the genotype and temperature treatment was found, see Table 14. This was caused by the prolonged vegetative and shortened generative phases in both wild type and mutant plants grown at 25°C. However, when considering only the generative phase (from double ridge until heading) the interaction could be observed, mutant plants developed faster at 15°C than at 25°C in relation to the wild type plants, see Figure 10.

Table 14 Two-way factorial analysis of variance (ANOVAs) for time to heading, thermal time to heading, spikelet number, time from double ridge (DR) to heading and thermal time from double ridge to heading. Genotype: KT3-1 and KT3-5; temperatures (temp): 16°C/14°C and 26°C/24°C; 16h/8h day/night. Significant differences were shaded in *gray*

Trait						
			Time to heading			
	Source	SS	df	MS	F	P
Genotype		3299.3	1	3299.3	1447.55	<0.0001
Temp		2032.8	1	2032.8	891.88	<0.0001
Genotype*Temp		0	1	0	0	1
Error		59.26	26	2.28		
Total		4822.3	29			
			Thermal time to heading			
Source		SS	df	MS	F	P
Genotype		2867700.79	1	2867700.79	77.86	<0.0001
Temp		11516180.44	1	11516180.44	312.66	<0.0001
Genotype*Temp		0	1	0	0	1
Error		994500.4	27	36833.35		
Total		13995983.99	30			
			Spikelet number			
Source		SS	df	MS	F	P
Genotype		0.24	1	0.24	0.11	0.7428
Temp		807.87	1	807.87	378.33	<0.0001
Genotype*Temp		83.04	1	83.04	38.89	<0.0001
Error		55.52	26	2.14		
Total		946.67	29			
			Time from DR to heading			
Source		SS	df	MS	F	P
Genotype		1202.01	1	1202.01	527.38	<0.0001
Temp		883.05	1	883.05	387.43	<0.0001
Genotype*Temp		1613.98	1	1613.98	708.12	<0.0001
Error		59.26	26	2.28		
Total		3758.3	29			
			Thermal time from DR to heading			
Source		SS	df	MS	F	P
Genotype		412187.67	1	412187.67	333.09	<0.0001
Temp		142711.24	1	142711.24	115.33	<0.0001
Genotype*Temp		250147.18	1	250147.18	202.14	<0.0001
Error		32174.15	26	1237.47		
Total		837220.24	29			

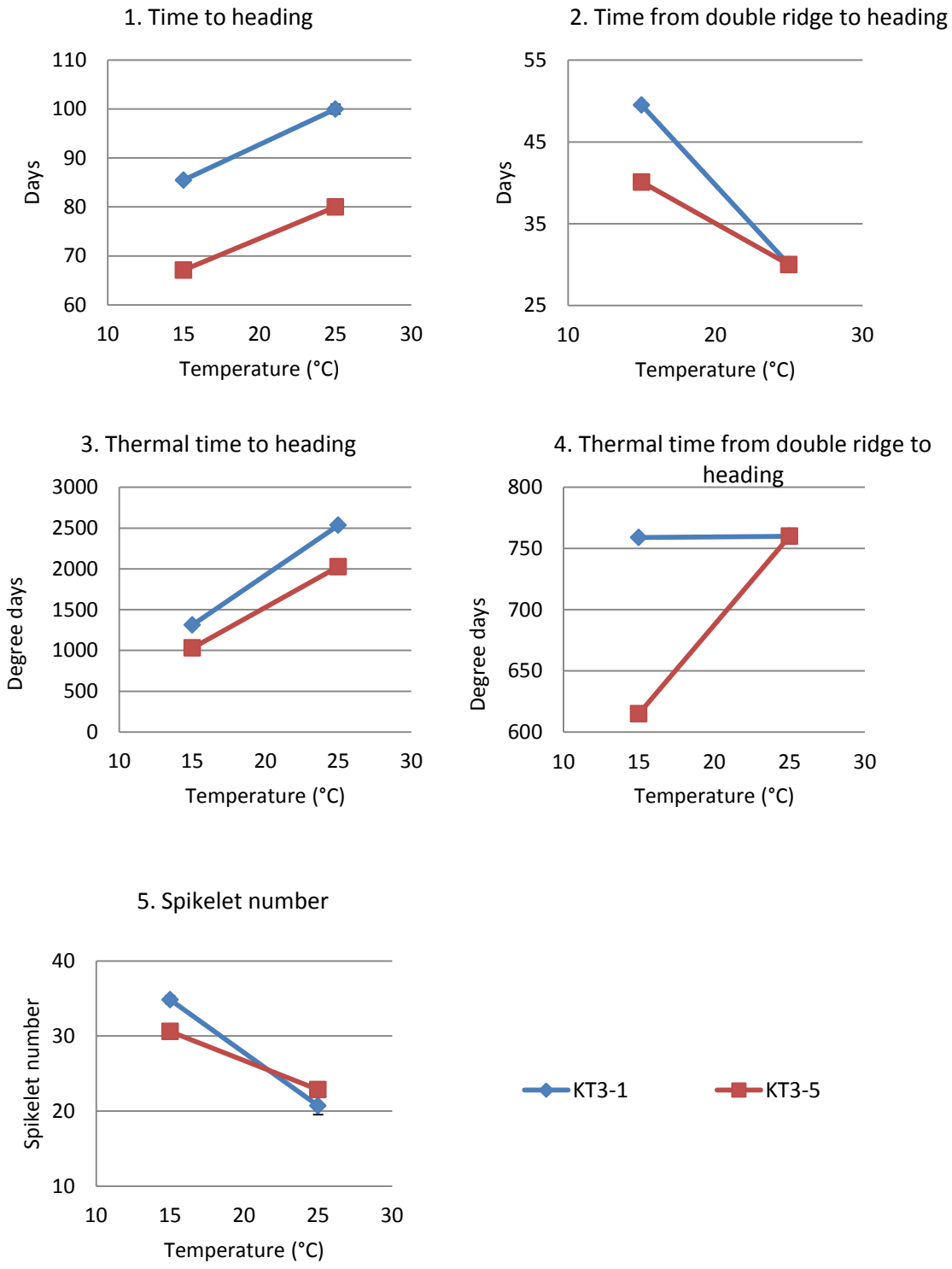

Figure 10 Phenotypic values from the analysis of KT3-1 and KT3-5 were plotted to show the interactions with temperature. Differences in slopes of the lines within a single plot indicate interaction. (Plot 1. and 3. show non-significant interaction, whereas plot 2., 4. and 5.– significant, see Table 14). Error bars indicate SEM

6.6 Initial expression analysis of *Eps3-A^m* candidate genes

Transcript level of *TmPUMILIO* was significantly lower in the mature leaves while being more abundant in all the other, developmentally active tissues analyzed, see Figure 11. This was consistent with the results obtained from the expression profiling of the other *PUMILIO* genes in wheat and barley (www.plexdb.org) as well as the findings in *Arabidopsis* (Abbasi et al. 2011). On the other hand, expression of *TmLUX* was similar in all the tissues, see Figure 12. Besides, expression of *TmPUMILIO* was significantly up-regulated by cold treatment, see Figure 13, suggesting a possible involvement in cold acclimation.

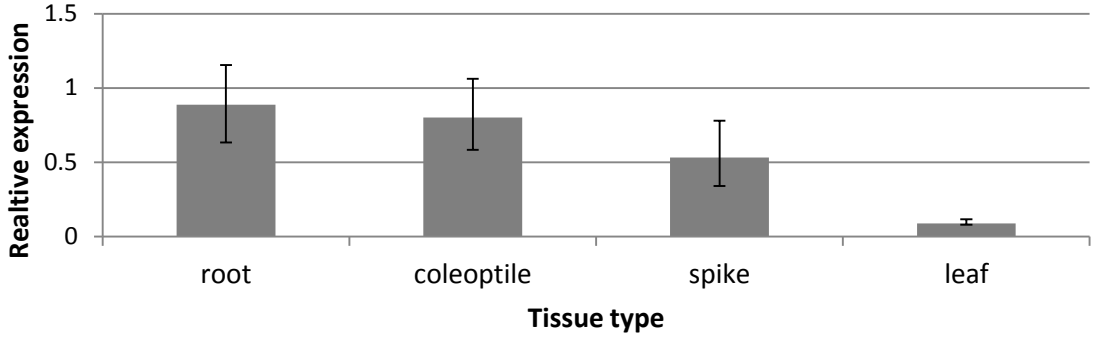

Figure 11 Relative transcript abundances of the *TmPUMILIO* in different tissues of the line KT3-1 show low level in the developed tissues (leaf) and higher in the active (root, coleoptile and spike). Error bars indicate SEM (standard error of the mean)

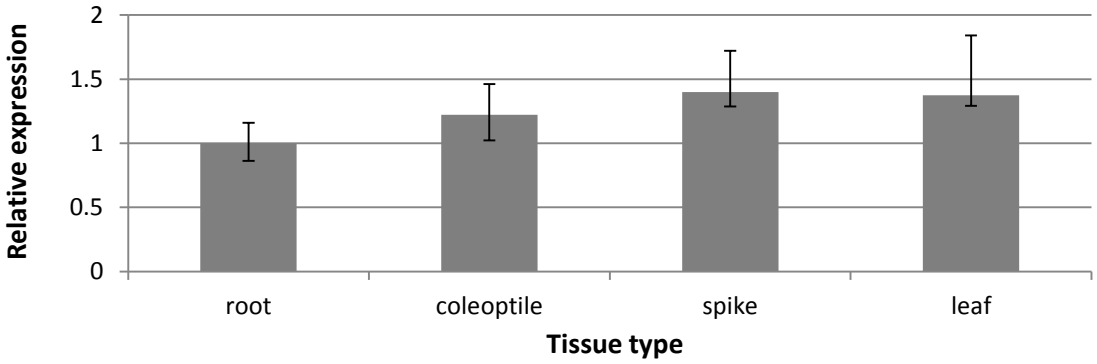

Figure 12 Relative transcript abundances of the *TmLUX* in different tissues of the line KT3-1. Unlike in the case of *TmPUMILIO*, no obvious differences were detected. Error bars indicate SEM (standard error of the mean)

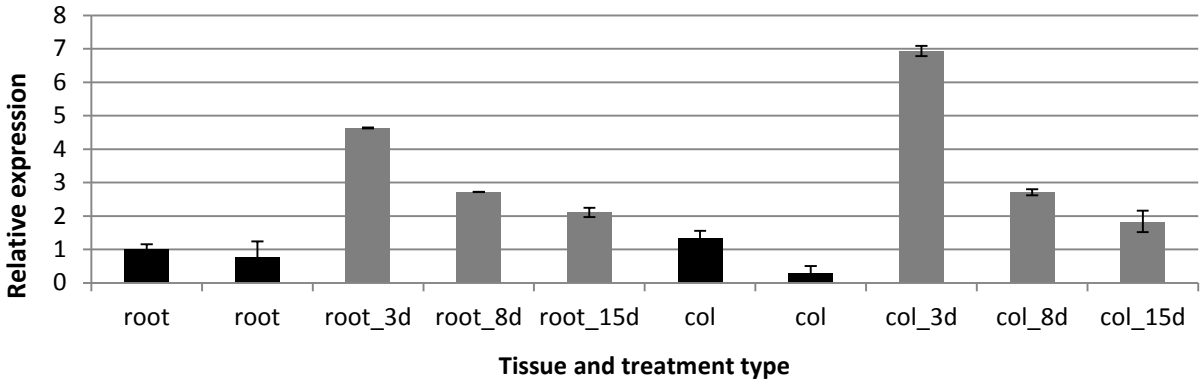

Figure 13 Relative transcript abundances of the *TmPUMILIO* gene in young roots (*root*) and coleoptiles (*col*) of the line KT3-1. *Black bars* indicate control treatment (room temperature), whereas *gray bars* show expression in the tissues cold-treated for 3, 8 and 15 days; respectively. *TmPUMILIO* shows elevated levels of expression upon the cold treatment. Error bars indicate SEM (standard error of the mean)

6.7 Circadian clock experiments

6.7.1 Time-course RT-qPCR on lines KT3-1 and KT3-5

Under constant light, transcript patterns of *TmLUX*, *TmLHY* and *TmTOC1* in wild type plants showed lower amplitude; see Figure 14, Figure 15 and Figure 16, respectively. Strong, dynamic changes observed in the expression of these genes during the first one and a half day were driven by the light/darkness environmental cue. In case of the *TmTOC1* expression, the rhythm was less robust in the mutant under the constant light condition; the additional peak was forming at ZT=21 of day three (see Figure 15, indicated with an arrow). However, the greatest differences could be observed for the expression of *TmLHY* as the mutant showed ceased levels of the *TmLHY* transcript along the whole constant light period, see Figure 16. The results obtained were similar to those gathered from the analysis of the knock-out LUX mutant in *Arabidopsis* (Hazen et al. 2005).

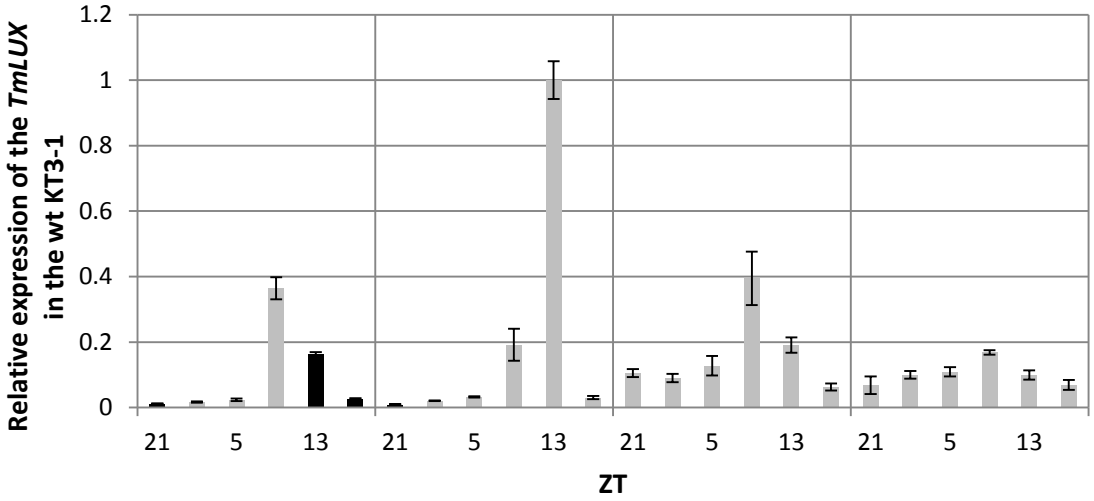

Figure 14 Relative expression of the *TmLUX* in KT3-1 measured during a time-course study. Peaks of expression occurred always in the evening between ZT=9 and ZT=13 (Zeitgeber Time, where"0" indicates beginning of the light phase). Only during the first night the lights were off (*black bars*), for the next three days constant light was set resulting in decreased amplitude of the diurnal oscillation. *Grey bars* indicate the time points when lights were on, whereas *black bars* –when lights were off. Error bars represent SEM (standard error of the mean)

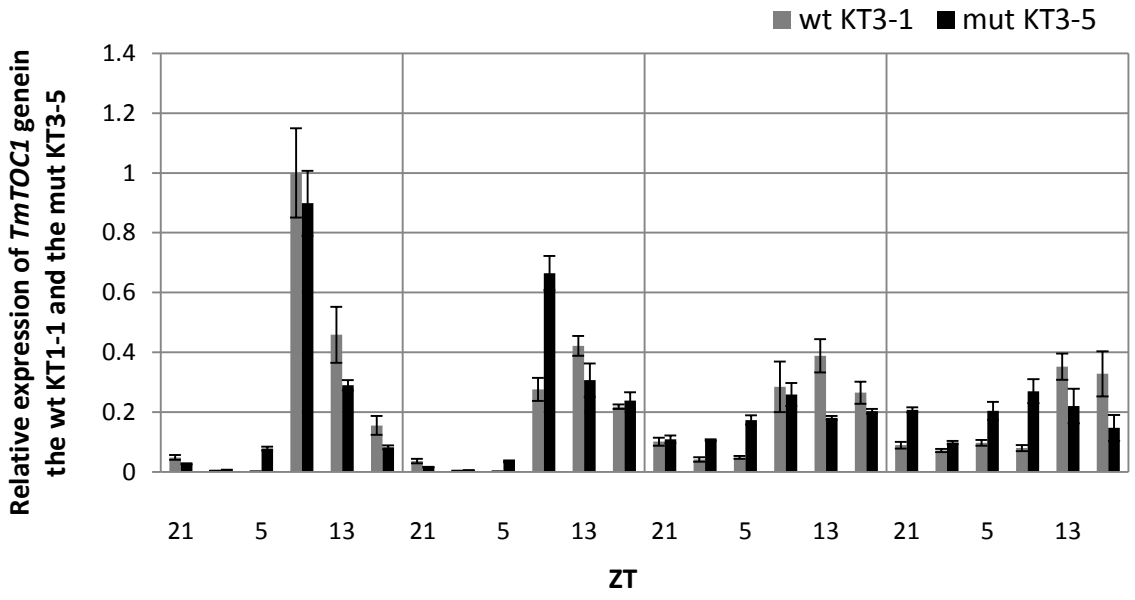

Figure 15 Relative expression of *TmTOC1* in KT3-1 (wt, *gray bars*) and KT3-5 (mut, *black bars*) measured during a time-course study. Peaks of expression occurred always in the evening between ZT=9 and ZT=13 (Zeitgeber Time, where"0" h indicates beginning of the light phase). Only during the first night the lights were off, for the next three days constant light was set resulting in decreased amplitude of the diurnal oscillation. Error bars represent SEM (standard error of the mean)

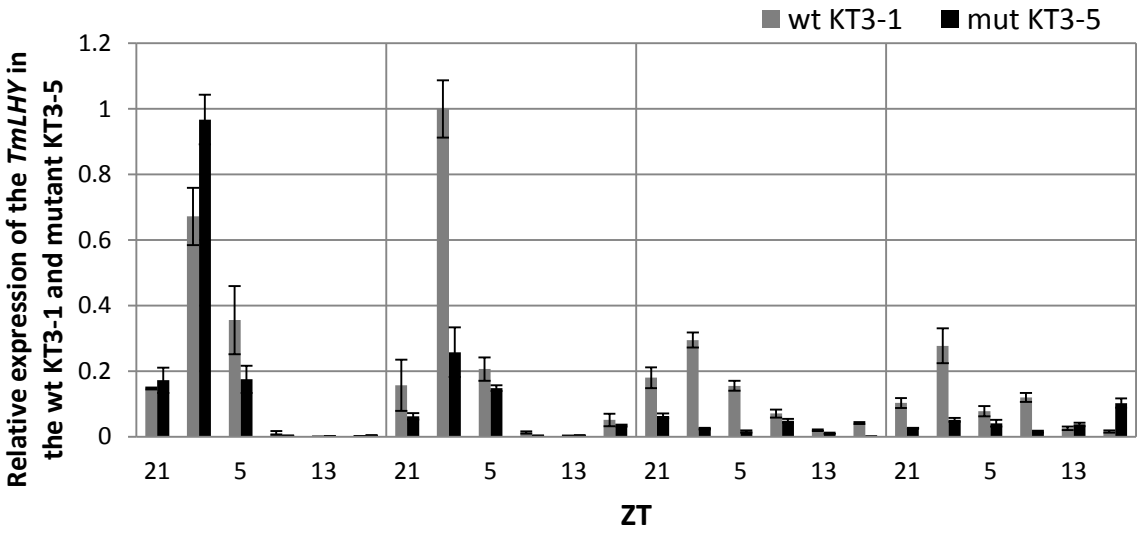

Figure 16 Relative expression of *TmLHY* in KT3-1 (wt, gray bars) and KT3-5 (mut, black bars) measured during a time-course study. Peaks of expression occurred always in the morning at ZT=1 (Zeitgeber Time, where"0" h indicates beginning of the light phase) Only during the first night the lights were off, for the next three days constant light was set resulting in decreased amplitude of the diurnal oscillation. Error bars represent SEM (standard error of the mean)

6.7.2 Measuring delayed fluorescence (DF) — the DF experiment

The measurement of delayed fluorescence (DF) performed in the current study proved to be a suitable tool for the examination of the circadian clock output. The period of DF oscillation in wild type KT3-1 plants was close to the expected 24h, see Table 15, Figure 17 and Figure 19. On the other hand, the oscillation of delayed fluorescence was clearly affected in the mutant line KT3-5; however, only at 22°C its period was significantly longer, 28.2h against 23.4h (at P=0.0001) and the rhythm ceased faster than in the wild type KT3-1 (see Table 15). In the aforementioned experiment about 18% more samples taken from KT3-1 where rhythmic than from KT3-5 (see Table 15). Also the number of DF peaks that could be clearly distinguished were five to six in case of wild type plants whereas only two up to four in case of mutant plants. However, at 17°C the differences were not so apparent and many mutant samples showed

regular peaks similar to wild type plants. In addition to this, at 22°C mutant plants (KT3-5) displayed also much more frequent phase shifts than at 17°C. Both observations could be well supported when comparing standard errors of the means (SEM) from two experiments. SEMs coming from the KT3-5 dataset at 22°C were much greater, especially when considering the second half of measurement, see Figure 17 and Figure 18. Results from the DF analysis indicated that the mutant phenotype depended upon the temperature and was more severe at 22°C than at 17°C (see Figure 17, Figure 18 and Figure 19). The experiment at 22°C was therefore repeated and the results obtained were similar, confirming that the KT3-5 mutant had indeed a distorted circadian clock.

Table 15 Selected results from the delayed fluorescence (DF) measurements performed on *T. monococcum* wild type KT3-1 and mutant KT3-5. Mutant samples, unlike the wild type, showed significant distortions in the DF oscillation when measured at 22°C. Percentage (%) of rhythmic regions, periods, and relative amplitude errors (RAE) were calculated in BRASS by running fast Fourier transformed non-linear least-square analysis (see chapter 5.10)

	17°C		**22°C**	
DF experiment				
Genotype	KT3-1, n=69	KT3-5, n=69	KT3-1, n=69	KT3-5, n=69
% of rhythmic regions	71	58	67	49
Period[1)]	25.85±0.36	27.03±0.68	23.44±0.51	28.19±1
RAE[1)]	0.32±0.04	0.42±0.02	0.38±0.02	0.45±0.03

[1)] Values represent partially weighted means ± partially weighted SEM

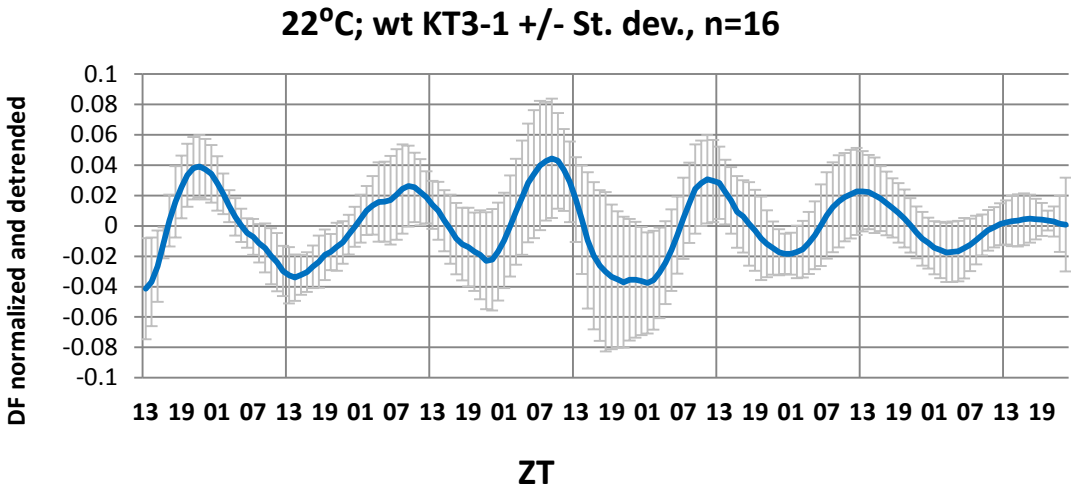

Figure 17 Oscillation of delayed fluorescence (DF) detected in wild type plants KT3-1 (*T. monococcum* L.). Leaf samples were kept under 22°C in the darkness interrupted every hour with a light pulse lasting for one minute. Error bars indicate standard deviations. For detailed description of the experimental procedure see chapter 5.10

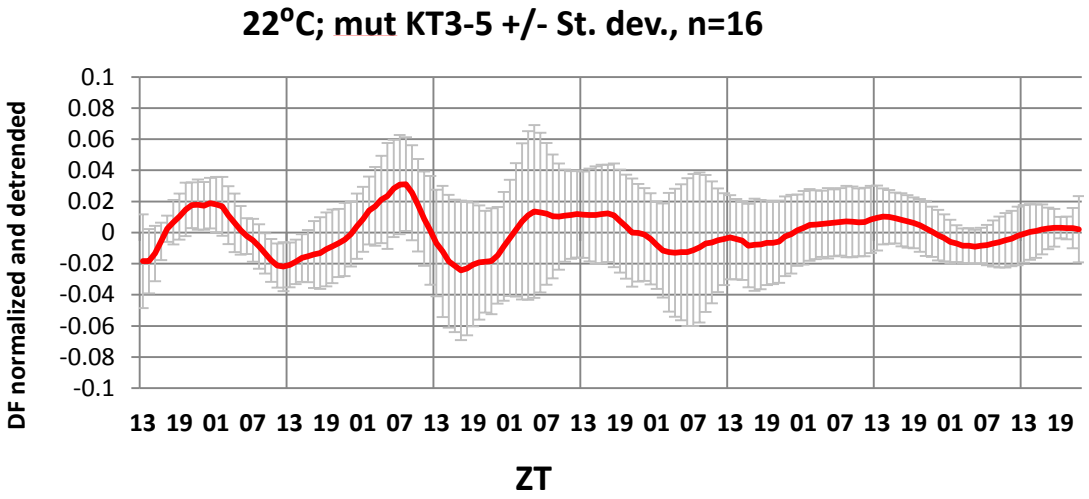

Figure 18 Oscillation of delayed fluorescence (DF) detected in mutant plants KT3-5 (*T. monococcum* L.) revealed a ceased rhythm from the third day of measurement. Leaf samples were kept under 22°C in the darkness interrupted every hour with a light pulse lasting for one minute. Error bars indicate standard deviations. For detailed description of the experimental procedure see chapter 5.10

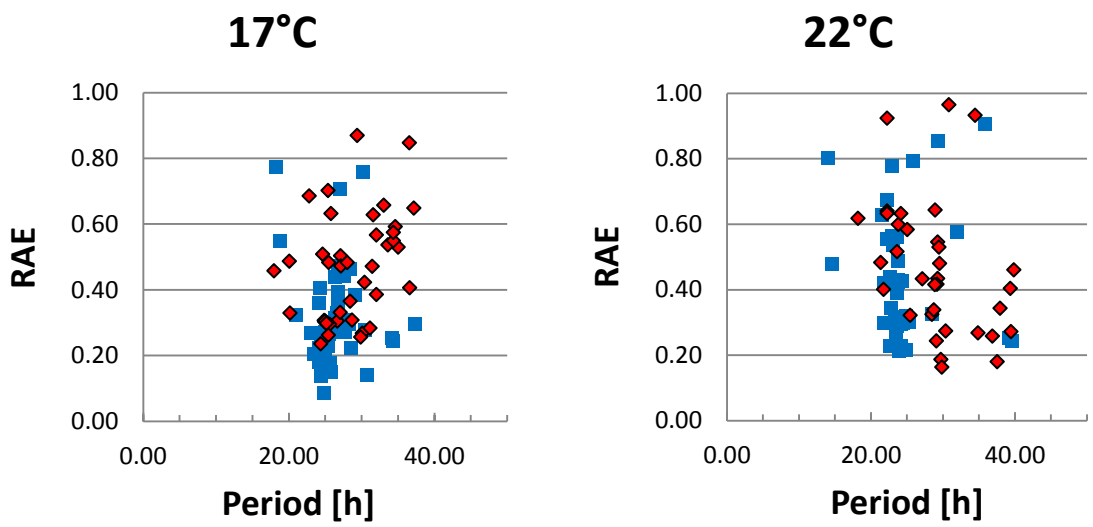

Figure 19 Relative amplitude errors of the delayed fluorescence (DF) oscillation are plotted against the periods generated from the BRASS software (see chapter 5.10). Periods were calculated for the DF from 36 to 132 hours. Each data point represents the time course measurement at a single region on the leaf from the *T. monococcum* wild type KT3-1 (*blue*) or mutant KT3-5 (*red*) plants. Experiments were performed in two different temperature regimes; 17°C (*left plot*) and 22°C (*right plot*). In both cases wild type samples were better clustered at the expected value of the period equaling 24h. At 17°C, independently from the genotype, samples showed mostly overlapping values of RAE and periods without any significant differences. Whereas at the higher temperature, mutant KT3-5 showed greater period lengthening than KT3-1, despite the higher RAE values found in both lines. These facts indicate that the temperature equaling 22°C was more useful to recognize a mutant clock phenotype

6.8 Einkorn wheat mutants analysis

Sixteen mutants in diploid wheat (KT), which showed similar null genotypes at the *Eps-3A*^{m} locus, were selected and listed in the Table 16. Genotyping of the flanking markers revealed that two of them; KT3-10 (haplotype 3A) and KT3-17 (haplotype 2) contained larger deletions affecting at least three and four genes (Table 16), respectively, whereas the remaining 14 mutants had the *Eps-3A*^{m} locus genotype identical with KT3-5 mutant used for mapping and population development. Out of 16 mutants, 15 showed earlier flowering (14 significantly at $P<0.05$) than the assumed donor line KT3-1, thus confirming the effect of the *Eps-3A*^{m} locus, see Table 16. Based on the re-sequencing of the three highly-polymorphic markers (see Table 16), the mutants could be grouped into four haplotypes indicating different genetic background

where the *Eps-3A*^{m} locus has been found. The other associated traits, spikelet number and spike length, showed the expected reduction in most of the KT lines; see Table 17. Lines KT3-10 and KT3-13 developed the smallest spikes with reduced spikelet number by 74.04% and 75.78%, respectively (Table 17).

Table 16 Marker haplotype analysis performed on 16 KT mutants at the *Eps-3A*^{m} locus. "A" indicates a haplotype identical with lines KT3-1 and KT3-3 (*T. monococcum* variet flavescens), "B"–KT3-39 (*T. monococcum* strain KUS 82), "C" –KT3-2 (*T. monococcum* variet vulgare). In the last four columns, "+" means presence of the PCR product, while "-"–absence. Two lines highlighted with the grey background had very likely independent deletion events at the *Eps-3A*^{m} locus as indicated by the absence of the flanking marker PCR products (CAPS_341_342 and CAPS_331_332). *P* values were calculated by the paired Student's t-test.

Marker haplotype	*eps* mutant	CAPS _23_24	CAPS _37_38	CAPS _131_132	CAPS _341_342	PAV _261_262 PAV _295_296	CAPS _331_332	Heading date difference according to KT3-1;	*p* value for heading date difference
1	KT003-013_mut	A	A	A	+	-	+	-45.17	$5.11*10^{-6}$
1	KT003-014_mut	A	A	A	+	-	+	-36	0.014
1	KT003-015_mut	A	A	A	+	-	+	-32.61	$1.05*10^{-5}$
1	KT003-016_mut	A	A	A	+	-	+	-32.17	$2.7*10^{-7}$
1	KT003-027_mut	A	A	A	+	-	+	-33	$5.87*10^{-6}$
1	KT003-028_mut	A	A	A	+	-	+	-30.42	0.0015
1	KT003-029_mut	A	A	A	+	-	+	-39	$5.28*10^{-5}$
1	KT003-030_mut	A	A	A	+	-	+	-32	$2.68*10^{-6}$
1	KT003-031_mut	A	A	A	+	-	+	-31.72	$4.03*10^{-7}$
1	KT003-033_mut	A	A	A	+	-	+	-25.5	0.11
1	KT003-034_mut	A	A	A	+	-	+	-31.17	$2.81*10^{-7}$
1	KT003-035_mut	A	A	A	+	-	+	-54.11	$1.6*10^{-7}$
2	KT003-017_mut	B	A	C	-	-	-	2.5	0.67
3A	KT003-010_mut	B	A	A	-	-	+	-60.25	$3.89*10^{-6}$
3B	KT003-011_mut	B	A	A	+	-	+	-52.5	$4.5*10^{-5}$
4	KT003-012_mut	A	A	C	+	-	+	-31.83	$1.47*10^{-6}$

Table 17 Phenotypic analysis performed on KT mutants having null alleles of PAV_261_262 and PAV_295_296 at the *Eps-3A*^{m} locus revealed consistently earlier heading as well as reduced spikelet number and ear length according to KT3-1. Phenotypic values of wild type line KT3-1 were set as 100%. *P* values were calculated by the paired Student's t-test.

***eps* mutant**	**Heading date difference according to KT3-1 [days];**	***p* value for heading date difference**	**Spikelet number difference [%] according to KT3-1**	***p* value for spikelet number difference**	**Spike length difference [%] according to KT3-1;**	***p* value for spike length difference**
KT003-010_mut	-60.25	$3.89*10^{-6}$	-74.04	$2.71*10^{-6}$	-62.03	$1.8*10^{-7}$
KT003-011_mut	-52.5	$4.5*10^{-5}$	-71.79	$3.23*10^{-7}$	-58.2	$2.13*10^{-7}$
KT003-012_mut	-31.83	$1.47*10^{-6}$	-32.05	$1.5*10^{-4}$	-23.6	0.0051
KT003-013_mut	-45.17	$5.11*10^{-6}$	-75.78	$1.24*10^{-5}$	-57.6	$2.52*10^{-6}$
KT003-014_mut	-36	0.014	-30.77	0.12	-22.16	0.24
KT003-015_mut	-32.61	$1.05*10^{-5}$	-53.99	0.0052	-41.02	0.0032
KT003-016_mut	-32.17	$2.7*10^{-7}$	-55.13	$1.9*10^{-4}$	-44.44	$6.51*10^{-5}$
KT003-017_mut	2.5	0.67	-56.41	0.099	-45.95	0.065
KT003-027_mut	-33	$5.87*10^{-6}$	-36.97	$7.9*10^{-4}$	-32.34	$5.9*10^{-4}$
KT003-028_mut	-30.42	0.0015	-37.39	$1.8*10^{-4}$	-27.57	$6.7*10^{-4}$
KT003-029_mut	-39	$5.28*10^{-5}$	-32.48	0.0016	-26.67	0.0018
KT003-030_mut	-32	$2.68*10^{-6}$	-26.5	$1.8*10^{-4}$	-15.14	$6.4*10^{-4}$
KT003-031_mut	-31.72	$4.03*10^{-7}$	-41.88	$3.2*10^{-4}$	-18.8	0.031
KT003-033_mut	-25.5	0.11	-35.9	0.15	5.95	0.42
KT003-034_mut	-31.16	$2.81*10^{-7}$	-49.57	$2.9*10^{-4}$	-41.26	$7.9*10^{-4}$
KT003-035_mut	-54.11	$1.6*10^{-7}$	-69.94	$6.35*10^{-10}$	- 47.21	$3.79*10^{-6}$

6.9 Transgenic lines analysis

Analyzed transgenic T0 plants showed high variation in heading dates (97-135 days) which did not coincide with the expected reduction in the expression of the target gene *HvLUX* (see Table 18 and Table 19). However, best four lines: BG284E10, BG284E11, BG285E01 and BG285E06 were selected to phenotypically test their T1 progeny. All four lines showed Mendelian segregation of the RNAi construct (3:1; presence:absence), see Table 20. However, line BG285E01 had at least two segregating copies of the construct inserted, each one missing either first or the second repeat. Phenotypic analysis of the T1 lines only confirmed the lacking effect of the transgene (see Table 21). The wild type Golden Promise plants were heading earlier than all the other lines analyzed. However, concerning the late heading of T1 segregants in which the RNAi construct was not detected ("null"), the cause for such unexpected difference could be different treatment of the wild type seeds. Therefore, reasonable was to compare also the "null" segregants with T1 positive plants, see Table 21. Such analysis revealed the expected trend of earlier heading of the positive plants in case of lines BG284E11 and BG285E06. However, the differences were statistically insignificant (at p=0.01) again confirming the missing influence of the RNAi construct in T1 lines analyzed.

Table 18 Results from the phenotypic analysis of T0 lines according to the heading time, spikelet number and spike length

T0 line	Days to heading	Spikelet number +/- SEM	Ear length [cm] +/- SEM
BG284E02	105	29.83±1.25	9.58±0.41
BG284E03	115	21.57±1.60	7.76±0.52
BG284E05	135	30±na	8.30±na
BG284E07	126	32±na	10±na
BG284E08	120	27.33±1.05	8.12±0.38
BG284E09	111	31.5±0.85	9.62±0.30
BG284E10	110	28.14±1.45	8.59±0.42
BG284E11	95	25.5±0.56	7.73±0.1
BG284E12	103	27.67±0.95	8.85±0.16
BG285E01	109	29.86±1.37	9.26±0.35

T0 line	Days to heading	Spikelet number +/- SEM	Ear length [cm] +/- SEM
BG285E02	116	30.33±1.86	9.27±0.41
BG285E03	109	27±1.53	8.20±0.51
BG285E06	97	27±1.63	8.16±0.48

Table 19 Results from the analysis of T0 lines according to the presence of an intact construct, ploidy level and percentage of silencing of the *HvLUX*

T0 line	Construct	Ploidy level	% of silencing before heading	% of silencing during heading
BG284E02	+	4x	9.13	40.66
BG284E03	+	4x	70.75	50.57
BG284E05	+	2x	4.39	9.47
BG284E07	+	4x	-	42.71
BG284E08	-	2x	-	63.65
BG284E09	-	2x	Set as 100% of *HvLUX* expression	
BG284E10	+	2x	-	48.77
BG284E11	+	2x	45.41	64.55
BG284E12	-	2x	-	52.49
BG285E01	+	2x	-	89.16
BG285E02	-	2x	-	68.20
BG285E03	+	2x	-	67.56
BG285E06	+	2x	17.25	55.51

Table 20 Results from the segregation of the inverted repeats from RNAi construct in selected T1 lines

T1 line	Chi-squared test for heading date segregation	*p*
BG284E10	0.03	0.86
BG284E11	0.01	0.93
BG285E01	1.56	0.21
BG285E06	0.01	0.93

Table 21 Results from heading time scoring of the selected T1 putative knock-down lines. No significant differences were found (at $p<0.05$)

T1 line	Heading date [Days from sowing to heading]	SEM	*p*
BG284E10	71.32	0.82	0.18
BG284E10 azygous	69.27	1.02	
BG284E11	65.10	0.82	0.21
BG284E11 azygous	67.20	1.33	
BG285E01	76.96	1.64	0.055
BG285E01 azygous	71.71	1.83	
BG285E06	67.90	1.00	0.76
BG285E06 azygous	68.50	1.27	
Golden Promise	62.63	0.96	

6.10 TILLING

Fragment spanning most of the 2nd, 3rd and 4th exons was selected to till *HvPUMILIO* (see Figure 20). This localization allowed for obtaining the best ratio between coding and non-coding sequence and most efficient usage of the TILLING capacity (Figure 20). In case of *HxLUX* the choice was simpler as it did not contain any intron (see Figure 22). Totally, 40 (33) and 39 (21) putative (confirmed) mutants were found in the TILLING population by screening with *HvPUMILIO* and *HvLUX* gene fragments, respectively (see Figure 21 and Figure 22). Importantly, one mutant 11266 in *HvPUMILIO* contained an SNP that introduced a premature stop codon, so it was expected to clearly answer whether the gene could still remain a candidate for the *Eps-3A*^{m} locus (Figure 21). The only plant that could germinate was heterozygous at the mutated nucleotide, therefore the next generation had to be phenotypically analyzed. However, M4 plants showed no effect of the detected mutation on flowering time, see Table 22 and Figure 23.

Two mutants, 11535 and 3775 found from a screening with the *HvLUX* amplicon contained amino acid substitutions within the MYB domain (Figure 22). However, based on the study on ARR10 (Hosoda et al. 2002) these amino acids were not essential for the nucleic acid binding, Figure 25. The only mutant in *HvLUX* which headed earlier than Barke, 4301, did not show association with the mutation at HvLUX (see Table 22). Apart from the aforementioned, 4 and 7 missense mutations exchanging conserved amino acids were found in *HvPUMILIO* and *HvLUX*, respectively (Figure 21 and Figure 22).

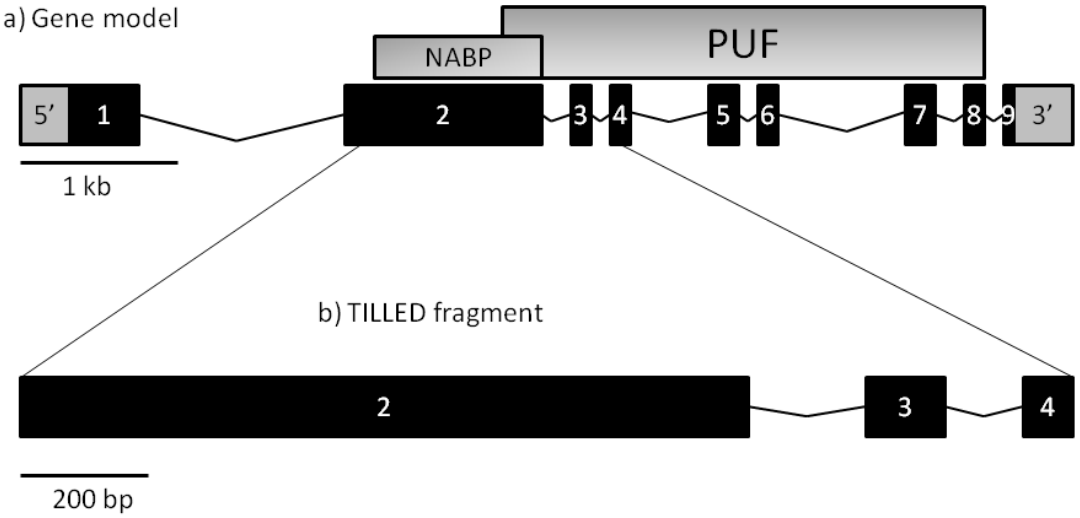

Figure 20 Gene model of *HvPUMILIO* (*a*) and a fragment selected for TILLING (*b*) are shown

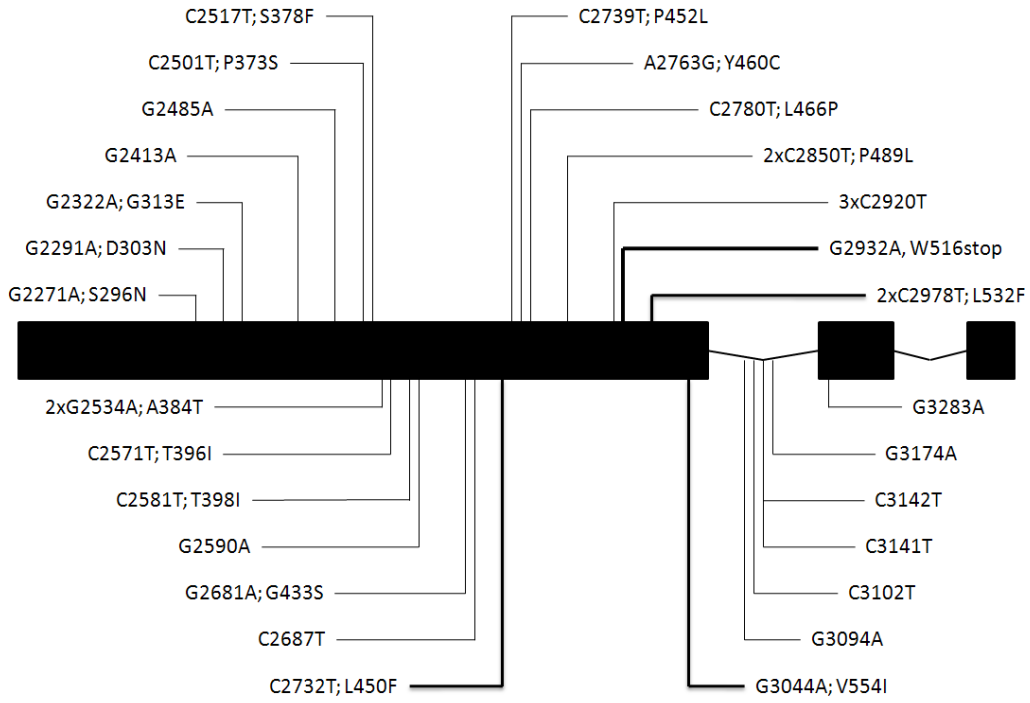

Figure 21 TILLING results of HvPUM. Thirty-three mutants were identified during the screening of the *cv* Barke population and confirmed by the Sanger sequencing. Fragment including most of the 2nd, 3rd and 4th exons of *HvPUMILIO* was analyzed. Numbers and letters indicate: wt nucleotide/position from the start codon including introns/mut nucleotide; wt AA/position from the start/mut AA. Mutations changing conserved amino acids are highlighted with the *bold font*

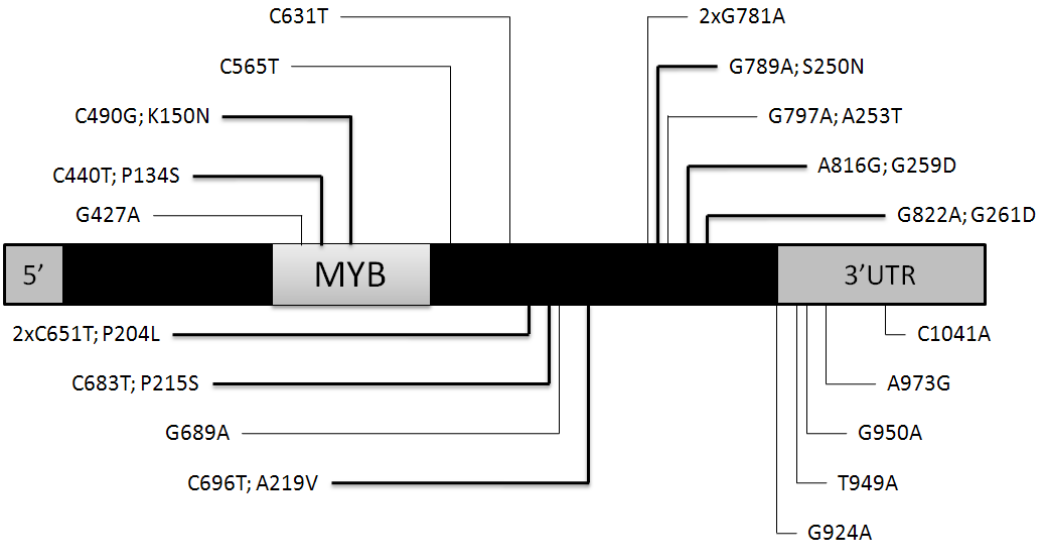

Figure 22 TILLING results of HvLUX. Twenty-one mutants were identified during the screening of the *cv* Barke population and confirmed by the Sanger sequencing. Fragment including most of the coding sequence of *HvLUX* was analyzed. Numbers and letters indicate: wt nucleotide/position from the start codon/mut nucleotide; wt AA/position from the start/mut AA. Mutations changing conserved amino acids are highlighted with the *bold font*

Table 22 Selected TILLING mutants were analyzed in M4 generation. None of them showed flowering time differences co-segregating with the mutation at *HvLUX* or *HvPUM*. Values represent means ± SEM (standard error of the mean)

Days to heading/M4				
TILLING mutants	Wild type/number of plants		Mutant/number of plants	
LUX_11535	-	0	56±2.56	10
LUX_3775*	58±4	10 (2)*	>74	1(0)*
LUX_4301	43±1.91	12	43±4.10	3
PUM_11266	48±1.94	13	51±0.5	3
Barke	44±1.38	6	-	0

*only two wild type plants from this family headed before 74 days after sowing

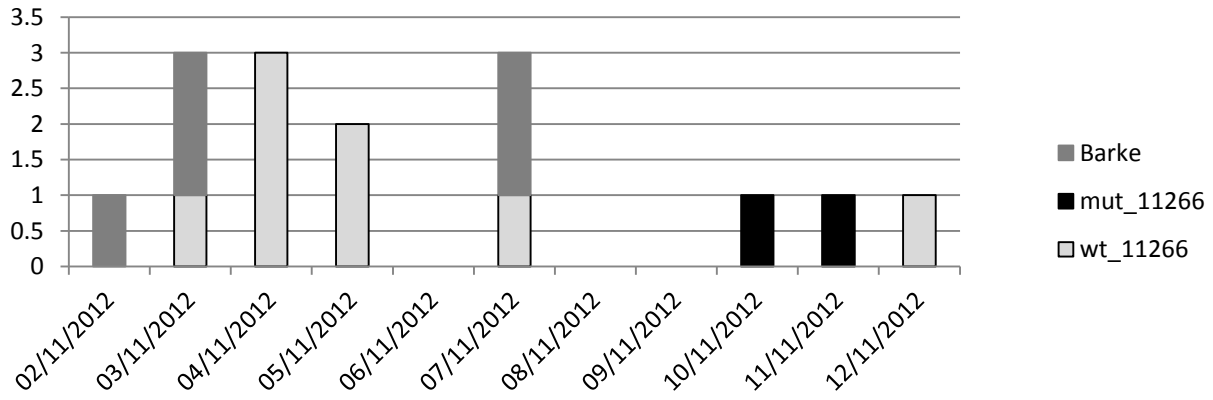

Figure 23 Distribution of heading dates in the M4 family of 11266 TILLING mutant. None of the plants harboring the premature stop codon (homozygous) at *HvPUM* showed early heading

6.11 *eam10* locus in barley

The mutant locus *eam10* present in a barley genotype "Super Precoz 2H" from Argentina has been mapped at a similar position to the *Eps-3A*^{m} (Borner et al. 2002; Gallagher et al. 1991). Moreover, it was found to give photoperiod-independent early flowering that makes it similar to the *Eps-3A*^{m} phenotype and function. Besides, the recently cloned *eam8* locus (an ortholog of *Elf3*) was found to be epistatic to *eam10* (Gallagher et al. 1991; Zakhrabekova et al. 2012). This would indicate that *eam10* and *eam8* from barley most likely interact or form a complex like LUX and ELF3 along with ELF4 in *Arabidopsis* (Nusinow et al. 2011).

Accessions PI 527381 (mutant line Super Precoz 2H) and GSHO 3421 (introgression line of *eam10* in Bowman) were found to contain one SNP A/T leading to an amino acid substitution at a highly conserved position within the MYB domain of HvLUX, Figure 24 and Figure 25. The change observed from serine (S, polar) to cysteine (C, hydrophobic) was expected to affect HvLUX function strongly or even mimic a knock-out mutation according to the ARR10 data published elsewhere (Hosoda et al. 2002). In the greenhouse, under the 16h photoperiod Super Precoz 2H headed six days earlier than its donor line B7490 (MC20, "mutante de cebada 20", $p<0.0001$) and ten days earlier than B4498 ("Malteria Heda", $p<0.000001$), a cultivar from which B7490 was derived. On the other hand, BM-NIL(*eam10*) was five days earlier than Bowman ($p<0.0001$).

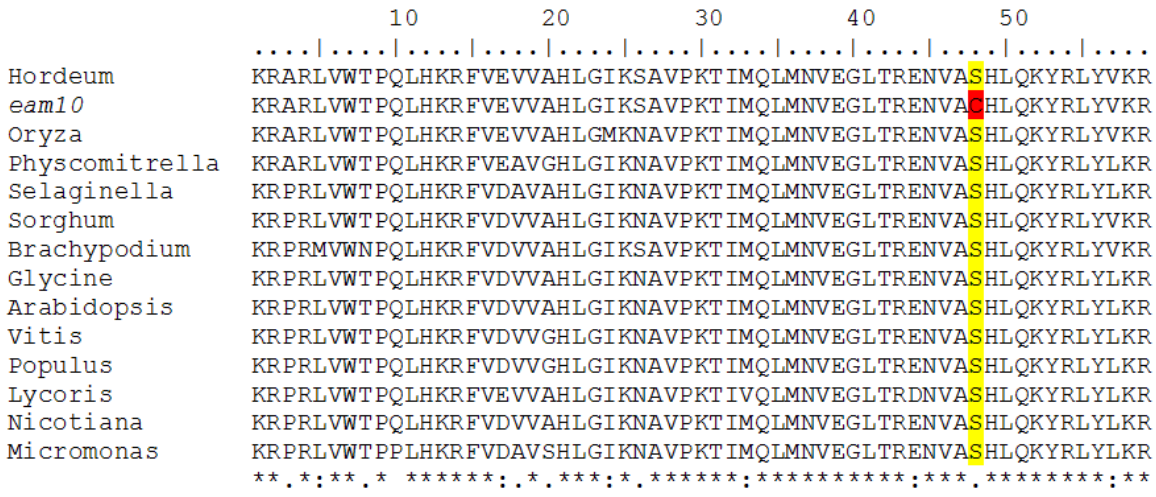

Figure 24 The MYB domain of LUX shows high conservation between plant species. Locus *eam10* mapped on barley chromosome 3H was a good candidate for *HvLUX* re-sequencing (Borner et al. 2002; Gallagher et al. 1991). This highly supports the hypothesis that the amino acid substitution (S to C) at the SHLQKY motif of *HvLUX* found in the *eam10* mutant indeed caused early heading of the genotypes analyzed; PI 527381, an *eam10* mutant line Super Precoz 2H and GSHO 3421, BM-NIL(*eam10*)

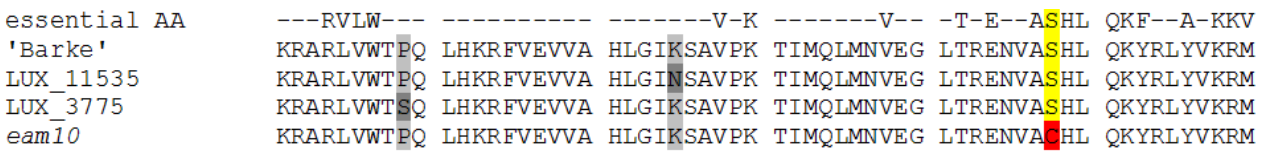

Figure 25 Only *eam10* mutant was affected at an essential amino acid (*red/yellow shading*) according to the previous report (Hosoda et al. 2002). Two other TILLING mutants in 'Barke', LUX_11535 and LUX_3775 were most probably not functional (*grey shading*)and LUX_11535 did not show early heading as expected (*grey*)

6.12 Time-course RT-qPCR on *eam10* mutant and cv. Barke

Time-course RT-qPCR study has been performed to verify whether the *eam10* mutant (Super Precoz 2H) had affected HvLUX function. Experimental results indicated the expected missing repressive action of HvLUX on the expression of *HvGI*, *HvTOC1* and *HvLUX* itself (see Figure 1 and Figure 26). In all the instances the evening genes were up-regulated in Super Precoz 2H compared to their expression in cv. Barke (Figure 26).

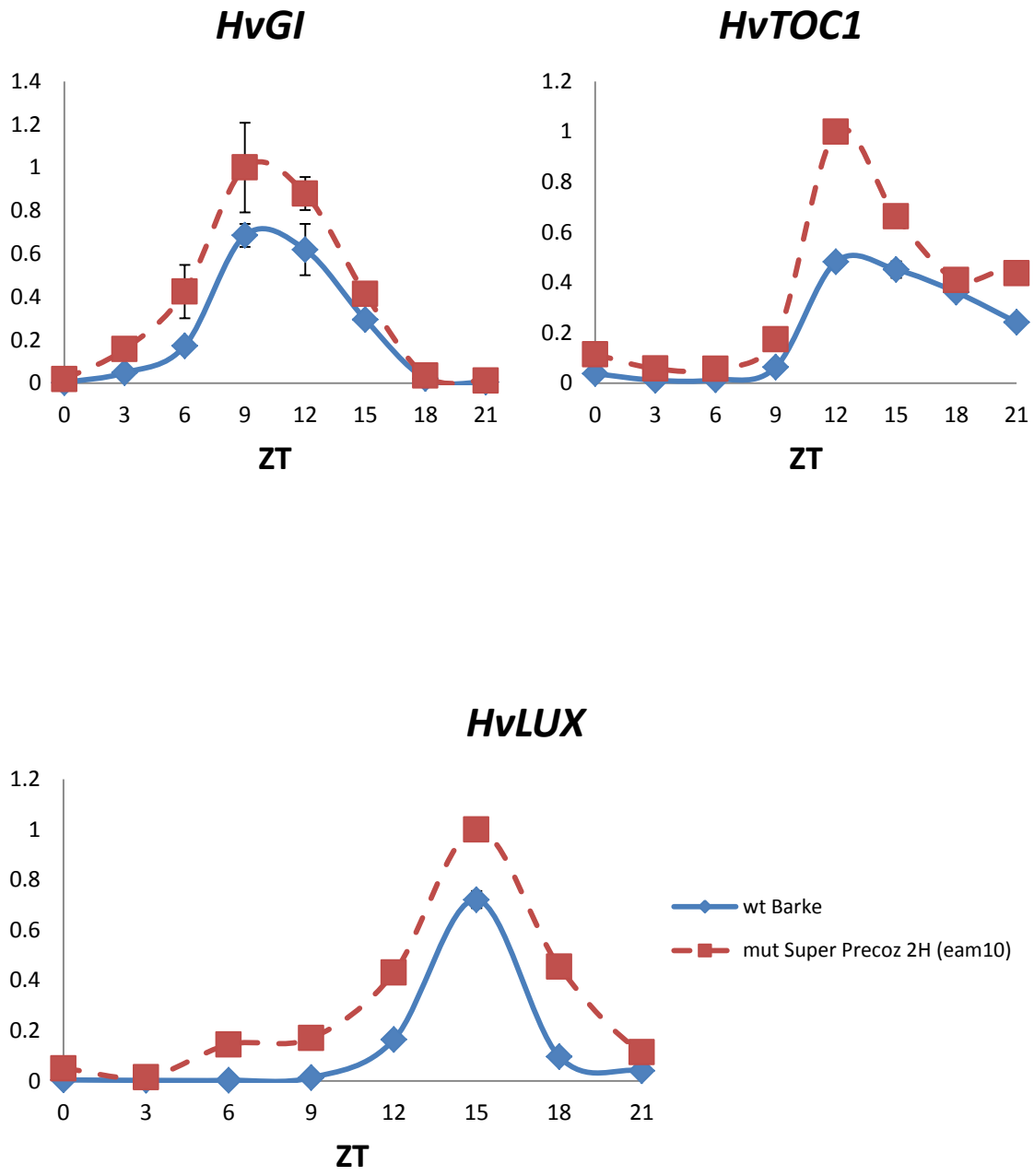

Figure 26 Relative transcript levels of selected circadian clock genes. *HvGI*, *HvTOC1* and *HvLUX* were measured from a one-day time-course RT-qPCR study on cv. Barke and *eam10* mutant (Super Precoz 2H). Plants were kept in the greenhouse at 20/17°C day/night under 16h photoperiod. For more detailed information about the experimental procedure see chapter 5.8.2. Error bars indicate standard error of the mean (SEM)

6.13 Time-course RT-qPCR on various wheat and barley genotypes

Under constant light and temperature *eam10* mutant introgression line in Bowman showed the most serious clock disruption from the genotypes analyzed. Both morning elements, *HvLHY*, *HvPRR9* (Figure 27) and evening elements, *HvGI*, *HvLUX* (Figure 27 and Figure 29) showed high degree of arrhythmia in their time-course transcript levels. Mutant KT3-5 in *T. monococcum* also displayed a severely dampened amplitude and/or arrhythmia in expression patterns of *TmLHY*, *TmPRR9*, *TmGI* and *TmTOC1* when compared with its wild type KT3-1, see Figure 28. KT3-5 did not express *TmLUX* as it had been found being deleted from its genome, see Figure 29 and Figure 6. The time course study allowed for a better analysis of the putative knock-down lines in Golden Promise (see chapter 5.15). Particularly, lines DH_BG284E11PP1 and BG_1E15 showed clear down-regulation of *HvLUX* when compared to the wild type segregant BG284E11_38, see Figure 27. However, the transcript patterns of *HvLHY*, *HvPRR9*, *HvGI* and *HvLUX* were not arrhythmic like in BM-NIL(*eam10*) (near isogenic line in Bowman carrying the *eam10* mutation) and KT3-5 mutants but affected in a quantitative manner instead (Figure 27). Transcript levels of *HvLHY* were only slightly up-regulated in DH_BG284E11PP1 and BG_1E15, whereas those of *HvPRR9* and *HvGI* were quite strongly up-regulated; see Figure 27. Analysis performed on the tetraploid *T. turgidum* cultivars revealed that the non-functional A-genome copy of *TtLUX* (*TtLUX-A*) (see chapter 6.14) could successfully be rescued by the B-genome homoeolog (*TtLUX-B*). Transcripts of *TtLUX-A* and *TtLUX-B* were significantly higher in the putative *TtLUX-A* mutant (cv. Tsing Hua no. 559) than in wild type *TtLUX-A/TtLUX-B* (cv. Fo Shou Mai) (Figure 30), whereas overall transcript patterns of *TtLHY*, *TtPRR9* and *TtGI* were very similar in both cultivars, see Figure 31. The indication of a phase-shift from ZT=12 (Fo Shou Mai)

to ZT=15 (Tsing hua no. 559) at the peak of *TtPRR9* and *TtGi* expression needs to be verified further (Figure 31).

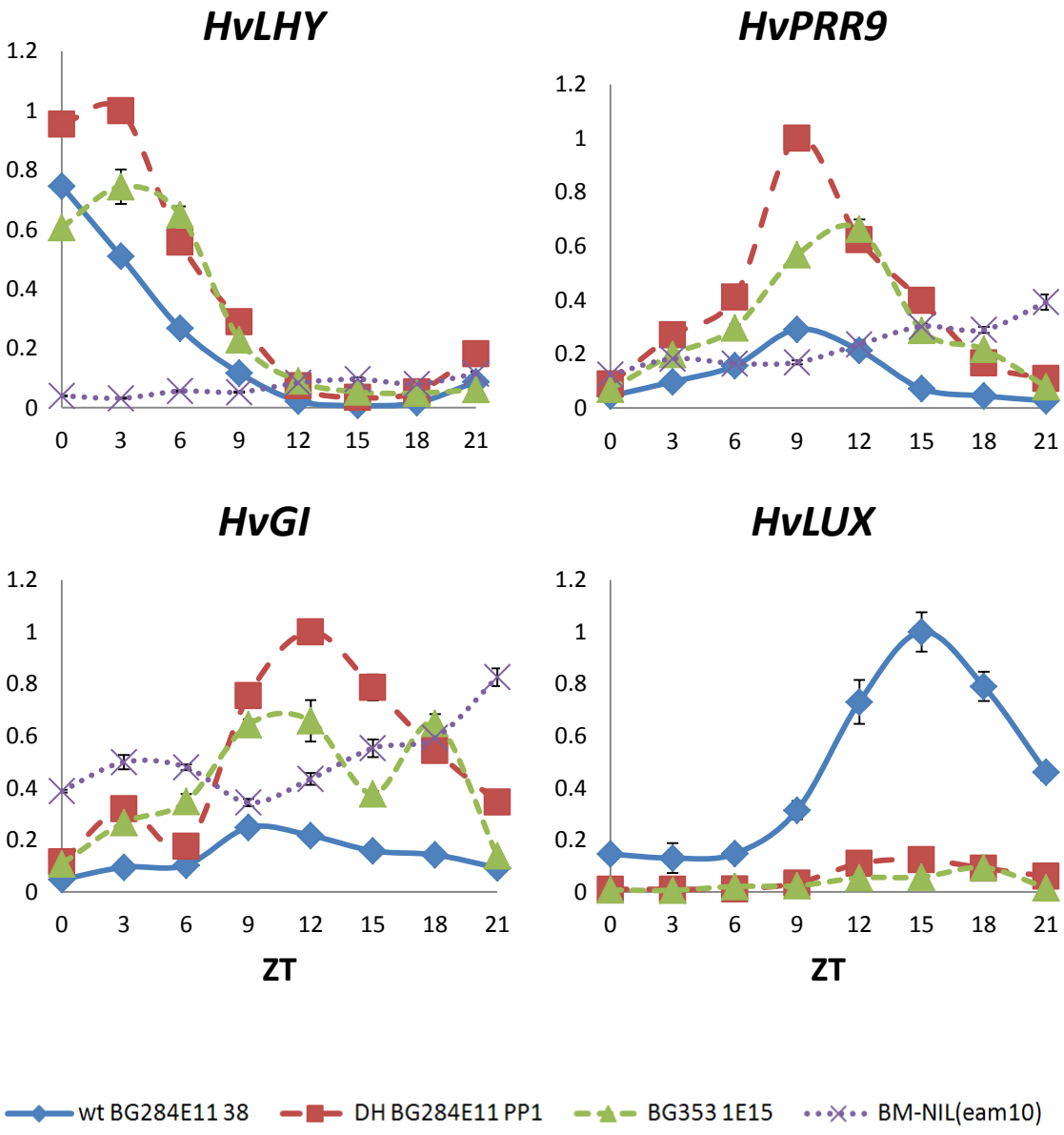

Figure 27 Relative transcript levels of selected circadian clock genes. *HvLHY*, *HvPRR9*, *HvGI* and *HvLUX* were measured from a one-day time-course RT-qPCR study on wt BG284E11 38 (Golden Promise), putative knock-down lines in Golden Promise DH BG284E11 PP1 and BG 1E15, and BM-NIL(*eam10*). Plants were kept under constant light and temperature, for more detailed information about the experimental procedures see chapter 5.8.3. Error bars indicate standard error of the mean (SEM)

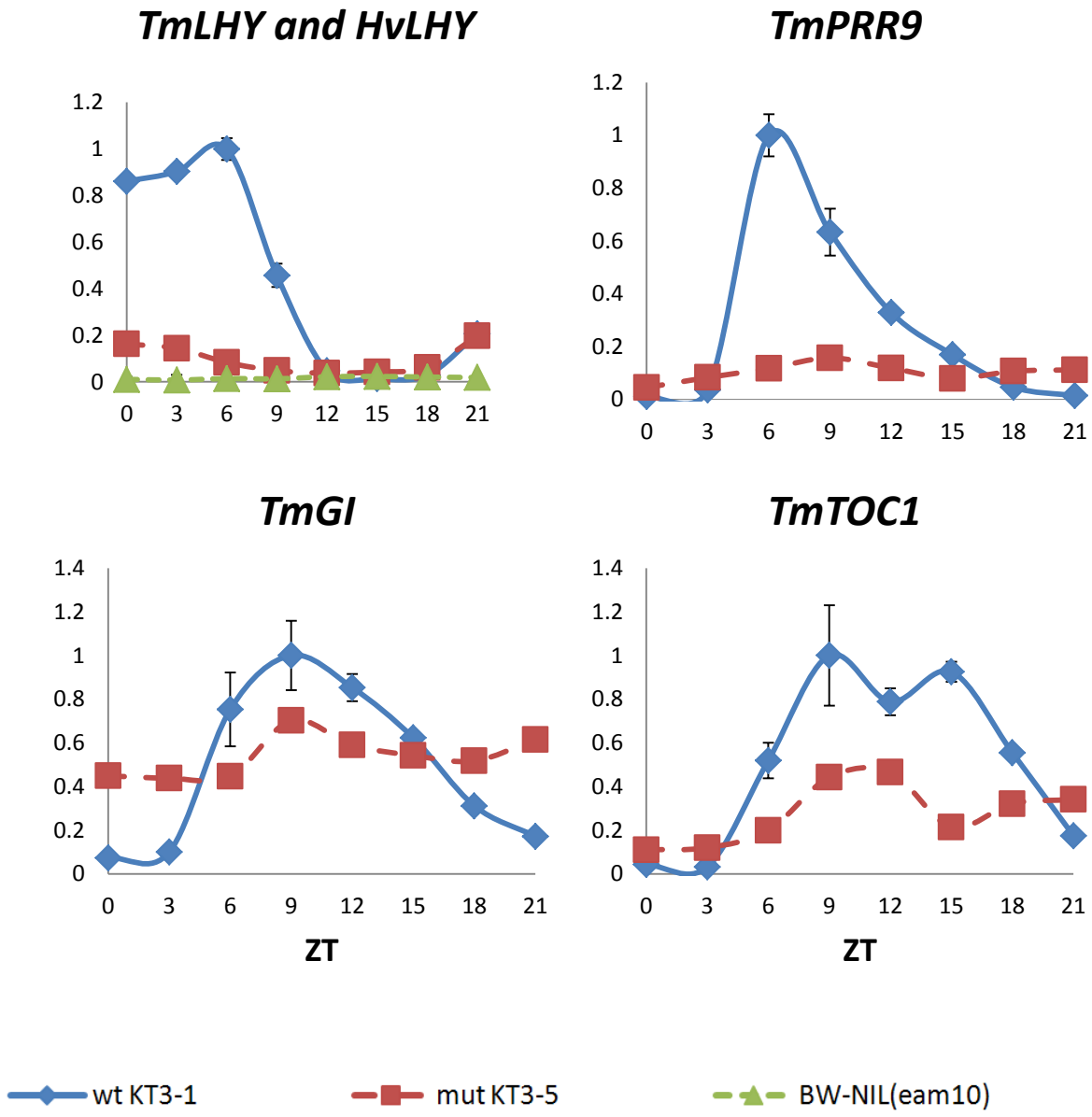

Figure 28 Relative transcript levels of selected circadian clock genes. *TmLHY*, *HvLHY*, *TmPRR9*, *TmGI* and *TmTOC1* were measured from a one-day time-course RT-qPCR study on wt KT3-1 (*Triticum monococcum* L.), mutant line KT3-5 (*T. monococcum* L.), and BM-NIL(*eam10*). Plants were kept under constant light and temperature, for more detailed information about the experimental procedures see chapter 5.8.3. Error bars indicate standard error of the mean (SEM)

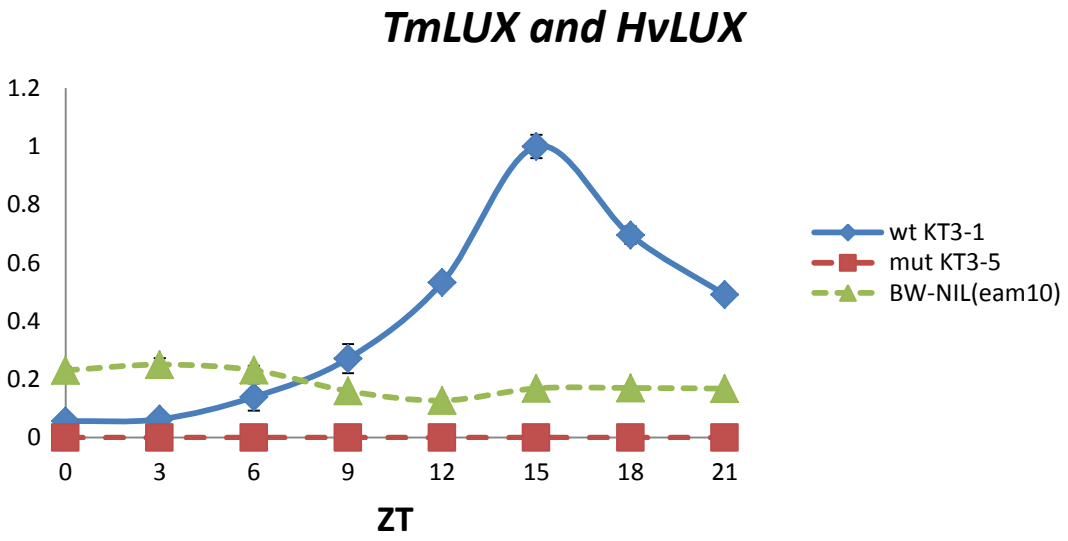

Figure 29 Relative transcript levels of *TmLUX* and *HvLUX* measured from a one-day time-course RT-qPCR study on wt KT3-1 (*Triticum monococcum* L.), mutant line KT3-5 (*T. monococcum* L.), and BM-NIL(*eam10*). Plants were kept under constant light and temperature, for more detailed information about the experimental procedures see chapter 5.8.3. Error bars indicate standard error of the mean (SEM)

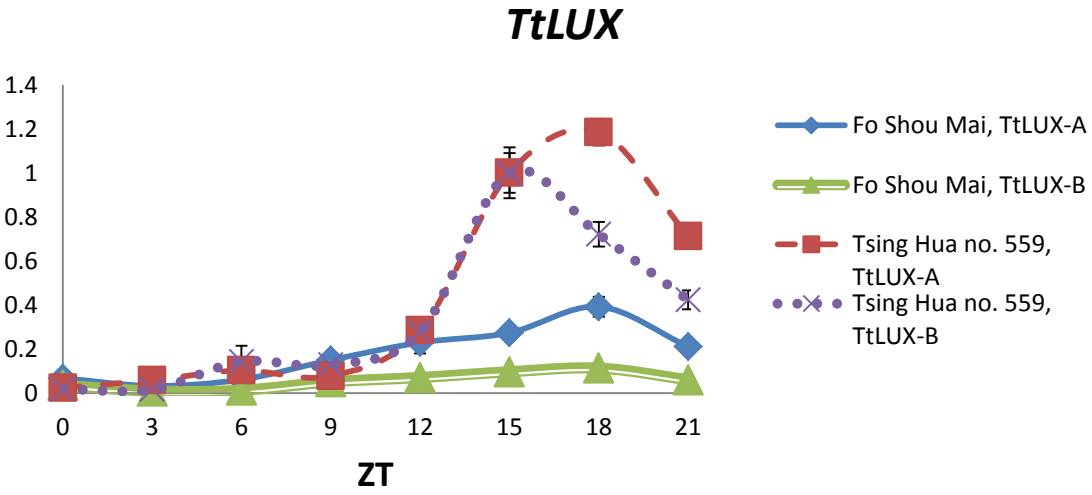

Figure 30 Relative transcript levels of *TtLUX-A* (A-genome copy of *LUX*) and *TtLUX-B* (B-genome copy of *LUX*) measured from a one-day time-course RT-qPCR study on cv. Fo Shou Mai (wild type, *Triticum turgidum* L.) and cultivar Tsing Hua no. 559 (mutant in A-genome LUX copy, *T. turgidum* L). Plants were kept under constant light and temperature, for more detailed information about the experimental procedures see chapter 5.8.3. Error bars indicate standard error of the mean (SEM)

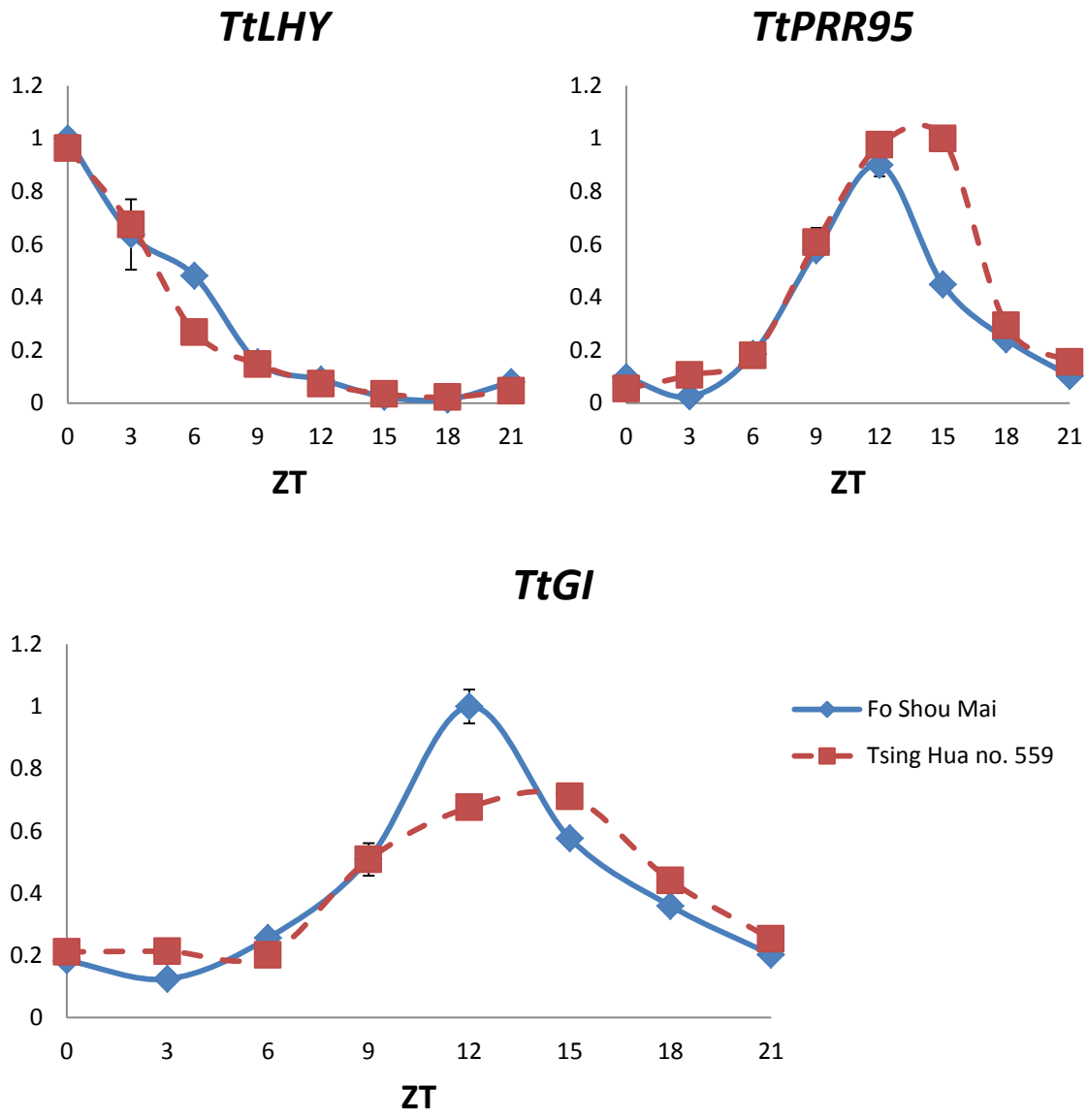

Figure 31 Relative transcript levels of selected circadian clock genes. *TtLHY*, *TtPRR9* and *TtGI* were measured from a one-day time-course RT-qPCR study on cv. Fo Shou Mai (wild type, *Triticum turgidum* L.) and cultivar Tsing Hua no. 559 (mutant in A-genome LUX copy, *T. turgidum* L). Plants were kept under constant light and temperature, for more detailed information about the experimental procedures see chapter 5.8.3. Error bars indicate standard error of the mean (SEM)

6.14 Re-sequencing of putative *LUX* homolog in the subset of 96 wheats

Ninety-six wild and cultivated wheat accessions were selected by Dr. Beniamin Kilian. The subset of genotypes covered most of the variation in flowering time. While focusing on A and B

genome copies of LUX which were most represented (Table 23), a higher number of haplotypes was found within the wild accessions, 18 *versus* 8 and 5 *versus* 4 for A and B copy, respectively, Table 24. Accessions were also grouped according to the climatic conditions at the site where had been collected, because of the discovery that KT3-5 mutant was thermo-sensitive (Table 14). This grouping revealed more variation present in the warmer climate within the A copy of *LUX*, 16 *versus* 12 (warm/cool) and one haplotype more in the B copy, 6 *versus* 7, Table 25. Mixed grouping showed that most haplotypes of the cultivated accessions came from the cooler climate (7 vs 4) whereas most haplotypes of the wild accessions were present in the warmer climate (14 vs 9), Table 26.

The major finding from the re-sequencing was a discovery of the new *LUX* allele containing seven-amino acid deletion in the MYB domain. Therefore the mutated A-genome homoeolog of the gene was considered to be non-functional and the cultivar harboring this mutation, 'Tsing Hua no. 559' was tested in the time-course RT-qPCR (see chapter 6.13). There was no correlation found between the heading dates and specific haplotypes of wheat *LUX* apart from the 'Tsing Hua no. 559', which headed among the early lines (headed on the 31st of May while the range for the total collection was from the 23rd of May to the 30th of June, data provided by Dr. Benjamin Kilian, see Figure 32).

Table 23 Results from re-sequencing of the wheat *LUX* in the collection of 96 accessions from Dr. Benjamin Kilian are shown

Wheat genome	Number of haplotypes found	Number of accessions analyzed
A	**21**	69
B	8	40
D	5	8
S	5	6

Table 24 Selected wheat accessions possessing A and B genomes grouped according to the cultivation status (wild or domesticated) to asses variation in the LUX sequence, expressed as the number of haplotypes

Number of accessions	Genome	Status	Number of haplotypes
47	A	Wild*	18
34	A	Domesticated**	8
10	B	wild	5
30	B	domesticated	4

*Wild = belonging to *Triticum boeoticum, T. dicoccoides* or *T. araraticum* species

** Domesticated = belonging to *Triticum monococcum, T. dicoccon*, *T. durum,T. aethiopicum*, *T. carthlicum*, *T. turanicum*, *T. turgidum* or *T. aestivum* species

Table 25 Selected wheat accessions possessing A and B genomes were grouped according to the climatic condition at the site of origin (cool or warm) to asses variation in the LUX sequence, expressed as the number of haplotypes

n	Genome	Climate	Number of haplotypes
44	A	cool*	12
37	A	warm**	16
20	B	cool	6
20	B	warm	7

* locations having less than 18°C (average value from March and August) were classified as a cool climate

**locations having 18°C or more—as a warm climate

Table 26 Selected wheat accessions possessing A genome were grouped according to the climatic condition at the site of origin (cool or warm) and cultivation status (wild or domesticated) to asses variation in the LUX sequence, expressed as the number of haplotypes

n	Genome	Climate and status	Number of haplotypes
22	A	cool, domesticated	7
12	A	warm, domesticated	4
22	A	cool, wild	9
25	A	warm, wild	14

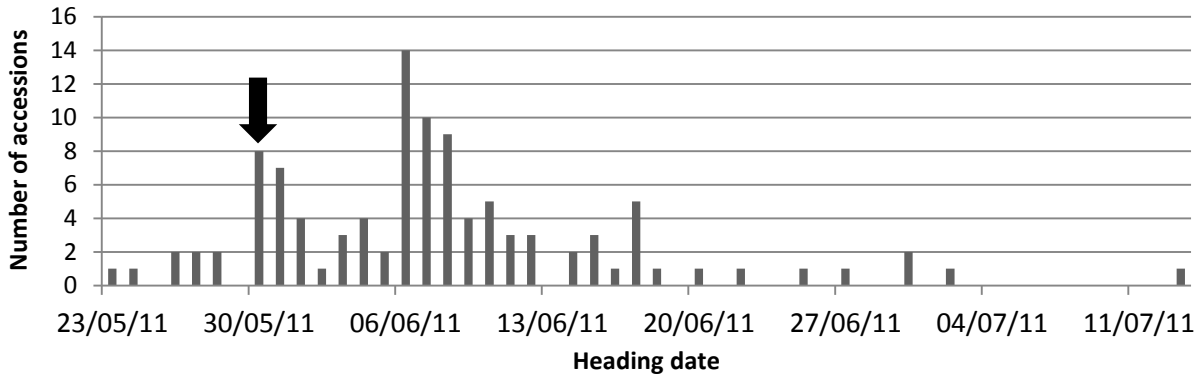

Figure 32 Heading date distribution for selected 96 wheat accessions grown at IPK field in 2011 is presented. Tsing Hua no. 559 containing a deletion in the *TtLUX-A* MYB domain was a relatively early heading genotype (indicated by the *black arrow*). Data were kindly provided by Dr. Benjamin Kilian

7 Discussion

7.1 Map-based cloning of the *Eps-3A^m* locus

7.1.1 QTL study, phenotypic evaluation of the RILWA1

The difference of earliness *per se* between the two mapping parents KT3-5 and KT1-1 was 10 days (p = 0.008), so 2.7 days greater than observed by Shindo et al. (2002). On the other hand, the average variation between KT3-5 (mutant) and KT3-1 (donor line of KT3-5) was 37 days (p = 0.000006); i.e. 5.2 days smaller compared to previous results under controlled conditions (Shindo and Sasakuma 2001). Nevertheless, the close similarities of the results reported in the previous studies allow the conclusion that photoperiodic and vernalization requirements were fulfilled in all experiments. The mutant allele at the *Eps-3A^m* locus was associated with earliness as well as involved in the reduction of spike size and spikelet number. This is in agreement with *eps* phenotypes previously described in the literature (Hoogendoorn 1985; Lewis et al. 2008; Millet 1987). Because the traits had quantitative phenotypic distributions and showed transgressive segregation, the possibility that another *eps* locus segregated in the RILWA1 population was also considered. From previous studies it was clear that such locus had already been mapped on the chromosome 5A^m, but with a rather small effect (LOD=4) (Shindo and Sasakuma 2001) However, the *Eps-5A^m* locus did not appear under field conditions; *Eps-3A^m* was the only locus and contributed 68.2% of the phenotypic variation in heading date under field conditions (Hori et al. 2007). In the current study, plants were evaluated only under greenhouse conditions; therefore the *Eps-5A^m* locus was monomorphically fixed in the newly developed F2 population in order to reduce its effect.

7.1.2 QTL study, molecular analysis of the *Eps-3A^m* locus in RILWA-1

Eps-3A^m was previously mapped by Shindo et al. (2002) using RFLP (Restriction Fragment Length Polymorphism) markers and further saturated with barley EST- based markers by Hori et al. (2007). However, the second article could show a very low polymorphism ratio between the mapping parents KT1-1 and KT3-5 equaling only 8.2%. Probably this was caused by the random approach in primer design that was used, rather than the low rate of polymorphism *per se* as discussed by the authors. Our strategy, targeting mainly intronic sequences for SNP discovery, revealed a much higher rate of polymorphism (33.5%). Moreover, the marker coverage around the *Eps-3A^m* was quite poor in the previous study. In the present work, 14 new markers were developed, suggesting that the syntenic region remained largely conserved between the grasses. Besides, three of the new marker loci were co-segregating with the LOD peak for heading time, anthesis time, spikelet number and spike length. Thus, the previous interval of 14.3 cM (Hori et al. 2007) between marker loci flanking *Eps-3A^m* was delimited to 0.9 cM.

It seems that using synteny with the available reference genomes of more than one species, rice and *Brachypodium*, was the most promising way for new marker development. A similar concept for the prediction of gene content and order in the barley genome has recently been published by (Mayer et al. 2011) and termed “genome zipper”. While looking for similarities between the suggested chromosome model of 3HL and the 3A^{m}L genetic map, all of the genes present in the syntenic regions of Os1 and Bd2 are predicted by the “genome zipper”. However, seven out of 51 (13.7%) genes which were localized according to the maps by Dilbirligi et al. (2006) and Sato et al. (2009) were missing in the “genome zipper”. In most of the cases this probably happened as the result of a broken colinearity with Os1 and Bd2.

Previously identified, possibly syntenic *Eps* loci were integrated to the low resolution maps based on RFLP or SSR (Simple Sequence Repeats) markers (Ali et al. 2011; Borner et al. 2002; Gallagher et al. 1991; Laurie et al. 1995; Miura et al. 1999; Miura and Worland 1994). Most importantly, the *eam10* locus from barley was shown in the current study being homologues to the *Eps-3A*^{m}. Moreover, in the work by (Marquez-Cedillo et al. 2001) there was a heading date QTL detected in the population Steptoe x Morex in a similar location to the *Eps-3A*^{m}. Assuming that the position of marker ABC172 on chromosome 3H was conserved (with PAV_177_178 as being derived from it), the QTL was associated with the proximal region on both maps. Also, the *Eps* locus on chromosome 3H detected by Laurie at al. (1995) was close to the telomeric region of this chromosome; however, there was no common marker in the current map to ensure syntenic relationship between the loci.

7.1.3 High-resolution mapping in the F2 population

By using the F2 population described, the *Eps-3A*^{m} locus could be delimited to only two putative genes. The above prediction was based on the comparative study with the physical contig from barley chromosome 3H spanning the complete locus and a partially overlapping contig from bread wheat chromosome 3A. The latter contig missed only flanking markers on the distal site of the locus. Map-based cloning projects usually lead to a few genes co-segregating with the phenotype; like in case of *Eps-A*^{m}*1* (Faricelli et al. 2010) and *Vrn2-A*^{m} (Yan et al. 2004). Low recombination frequency and polymorphism between the mapping parents were the main limitations preventing from narrowing down the loci to a single gene. However, in the current study the main obstacle was not the recombination frequency *per se* but a big deletion in line KT3-5 involving two genes, thus disabling any recombination to occur between

them. However, such big deletion affecting reproductive development has already been detected in the *maintained vegetative phase* (*mvp*) mutants in einkorn wheat (Distelfeld and Dubcovsky 2010). On the other hand, the polymorphism rate between RIL25 and RIL71 was substantially high, therefore allowing genetic mapping of all putative genes. Finally, the analysis performed on the F3 population confirmed the link between genotypes and phenotypes.

7.1.4 Candidate genes for the *Eps-3A^m* locus; *TmLUX ARRHYTHMO*

LUX ARRHYTHMO (LUX), known also as *PHYTOCLOCK 1 (PCL1),* is an element of the circadian clock which is well described in *Arabidopsis thaliana* L. (Hazen et al. 2005; Helfer et al. 2011; Nusinow et al. 2011; Onai and Ishiura 2005). It was shown that the LUX protein (MYB transcription factor) was essential for proper circadian clock rhythmicity under constant light conditions (Hazen et al. 2005; Onai and Ishiura 2005). This feature could also be seen in the current study while analyzing the expression of *TmTOC1* and *TmLHY* in the einkorn wheat mutant KT3-5 lacking *TmLUX*. In addition to that, the *eam10* mutant in barley displayed a similar arrhythmia of *HvLHY* expression. On the other hand, LUX seemed to be an important growth repressor that regulated elongation of the hypocotyl and flowering time (Hazen et al. 2005; Helfer et al. 2011; Nusinow et al. 2011).

A milestone discovery was the evident molecular connection found with EARLY FLOWERING 3 (ELF3) and EARLY FLOWERING 4 (Elf4) since mutants in these genes were associated with similar phenotypes like LUX (Doyle et al. 2002; Hicks et al. 2001; Zagotta et al. 1996). Particularly, LUX, Elf3 and Elf4 proteins formed a complex that acted together as an evening repressor of hypocotyl elongation (Nusinow et al. 2011). It is assumed that the hypocotyl and

cotyledon in dicots are analogous structures to the mesocotyl in monocots (Hoshikawa 1969). However, wheat and barley are exceptional monocots which do not elongate mesocotyl (Hoshikawa 1969), therefore this kind of morphological trait cannot be examined in case of the clock mutations present in these species. Recently, the cereal homolog of *Elf3* has been cloned by (Faure et al. 2012; Zakhrabekova et al. 2012) and the barley mutants affected in this gene showed very similar features to the *LUX* mutants described in the current work. Besides, it was shown that the recessive epistasis exists between ea_k (*eam8*/*Elf3*) and ea_{sp} (*eam*10/possibly *LUX*) (Gallagher et al. 1991). These facts support the hypothesis that also a cereal LUX forms a protein complex with Elf3.

Moreover, it was clear from the two independent studies that a *LUX* knock-out created a photo-insensitive flowering phenotype in *Arabidopsis* (Hazen et al. 2005; Onai and Ishiura 2005). This phenotypic feature could be similarly seen in the KT3-5 mutant when grown under SD conditions as well as in the *eam10* (Borner et al. 2002). Subsequent analysis of the protein binding microarrays revealed that the LUX directly regulated expression of the PSEUDO RESPONSE REGULATOR 9 (PRR9) by binding to its promoter (Helfer et al. 2011). This could explain the early flowering of the LUX mutants observed, since the PRR9 was involved in the CONSTANS (CO)-FLOWERING LOCUS T (FT) photoperiodic flowering pathway (Nakamichi et al. 2007). The model proposed was that PRR9, PRR7 and PRR5 acted negatively on the CYCLING DOF FACTOR1 (CDF1), which was proven to be a DNA-binding repressor of CONSTANS (Nakamichi et al. 2007). Moreover, *AtLUX* was considered to promote expression of the other important core clock morning elements, LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED1 (CCA1) by repressing the *PRR* genes, since mutants lacking LUX activity

showed down-regulation of *LHY* and *CCA1* (Hazen et al. 2005; Helfer et al. 2011) which has been similarly detected in the KT3-5 and *eam10* in the current study.

There was also a strong evidence that LUX was able to down-regulate itself as well as directly or indirectly another evening element– *TIMING OF CAB2 EXPRESSION 1* (*TOC1*) (Hazen et al. 2005; Helfer et al. 2011). Self down-regulation of the *TmLUX* could not be analyzed in the KT3-5 mutant because of the complete gene deletion. However, it was further verified in the *eam10* mutant in barley, as it clearly showed elevated levels of *HvLUX* mRNA compared to the cv. Barke. Whereas *TmTOC1* expression in KT3-5 was not so obviously changed like in *Arabidopsis* mutants studied before, thereby suggesting no direct interaction between *TmLUX* and *TmTOC1*. Nevertheless, all similarities found between the *LUX* mutant in *Arabidopsis* and KT3-5/*eam10* make the *TmLUX*/*HvLUX* a sensible candidate for the *Eps-3A*^{m}/*eam10* locus.

7.1.5 Candidate genes for the *Eps-3A*^{m} locus; *TmPUMILIO*

The knowledge about PUF proteins in plants is highly delimited. There are only a few works providing an insight into their molecular function in plants (Abbasi et al. 2010; Abbasi et al. 2011; Francischini and Quaggio 2009; Spassov and Jurecic 2003; Tam et al. 2010). So far, only one member of the family, PUM23, has been characterized in *Arabidopsis* to more extend (Abbasi et al. 2010). Authors of the study could show that the PUM23 was crucial for rRNA processing and the mutants lacking PUM23 activity displayed aberrant growth and morphology (Abbasi et al. 2010). It was known that PUF proteins could regulate mRNA stability and/or decay by direct binding to the 3'UTR region (Spassov and Jurecic 2003). They were mainly associated with meristematicaly active tissues and developing organs like young shoots, roots and

inflorescence (Abbasi et al. 2011). The fact brought some similarity to the well characterized animal models and human, where PUF proteins were involved in the regulation of stem cells activity (Crittenden et al. 2002; Forbes and Lehmann 1998; Moore et al. 2003).

While looking at the relevance to the current study, shortened vegetative growth of the KT3-5 mutant could be explained by the lack of action of *TmPUMILIO* in the shoot stem cell maintenance (Abbasi et al. 2011) However, PUF proteins constitute a medium sized (25) family in plants which members are considered to act redundantly, since most of the single knock-out mutants in *Arabidopsis* do not show any distinct phenotype (http://www.arabidopsis.org, personal communication from Dr. Andrew Cuming, 2011). In addition to this, the TILLING mutant in barley containing a premature stop codon at *HvPUM* did not flower early. These facts led to the conclusion that *TmPUMILIO* was probably less contributing to the phenotypic effect conferred by the *Eps-3A*^{m} locus.

7.2 *Eps-3A*^{m} locus affects the duration of early developmental phases

Development of KT3-5 mutant was further dissected into three phases–vegetative, double ridge to terminal spikelet and terminal spikelet to heading. The knowledge about the effect of *Eps-3A*^{m} on these phases could be utilized in crop breeding for specific climatic conditions. In case of *Eps-1A*^{m}, the mostly affected phases were vegetative and, with a little less effect, double ridge to terminal spikelet (Lewis et al. 2008). In the current study the situation was very similar; the differences in duration of the vegetative phase were more pronounced than those of double ridge (DR) to terminal spikelet (TS). Secondly, neither *Eps-1A*^{m} nor *Eps-3A*^{m} affected plant development between the terminal spikelet and heading. The shortened period of

spikelet initiation (DR to TS) in the mutant could be a consequence of a shorter vegetative phase and fewer divisions of the stem cells in the apex. An opposite scenario –i.e. shorter spikes caused by the reduced duration of spikelet initiation phase seems equally probable.

Importantly, the *Eps-3A*^{m} locus can have a positive impact on plant development when plants are exposed to high temperatures during vegetative growth because it shortens generation time with neutral or positive influence on the final spikelet number. In the current study no interaction was detected between locus and temperature when the whole period of plant growth was concerned, whereas such interaction had been detected for the *Eps-1A*^{m} locus (Lewis et al. 2008). This discrepancy between studies could be explained by different experimental designs–the authors studying *Eps-1A*^{m} vernalized plants for 8 weeks before starting the actual temperature treatment. Because both KT3-1 and KT3-5 are spring types, they were not vernalized in the current study. However, their development could be dissected into three phases and it could be shown that by removing the vegetative growth period, the significant interaction between genotype and temperature was also detected. But it similarly suggests that in the previous work by (Lewis et al. 2008) the vernalization treatment was crucial to detect the temperature-dependence and some plants (spring type) possessing the early allele of *Eps-1A*^{m} probably underwent the transition to the generative phase during the vernalization period.

7.3 Evidences supporting *LUX* as the candidate gene

7.3.1 Early mutants in einkorn wheat

Based on the results, *TmLUX* seems to be placed upstream of the *PRR* genes similar to *AtLUX*. Logically, the *LUX* mutant constitutes an alternative variant of the photoperiod insensitive phenotype that was well characterized in case of *ppd-1* mutants (Beales et al. 2007; Turner et al. 2005). All but two mutants in einkorn wheat affected at *Eps-3A*^{m} shared the same two-gene deletion. This fact led to the question whether *Eps-3A*^{m} had been introduced to these plants by crossing. Even if so, there was a strong indication that the events happened in different genetic backgrounds; although almost all were early flowering, there was still substantial variation in heading time ranging from 38 to 88 days after the end of vernalization. Moreover, the mutants showed a great spectrum of morphological abnormalities which could also indicate different genetic backgrounds and independent events during mutagenesis. Such phenomenon has also been described for mutations in *HvELF3* isolated from barley (*Mat-a*), where >40 mutant lines harbored identical four-nucleotide deletion (Zakhrabekova et al. 2012).

Some authors claim that different environmental stresses, including exposition to ionizing and non-ionizing radiation, could increase the activity of transposons (Capy et al. 2000; Walbot 1988, 1992). This way, by activating the same mechanism with X-ray radiation, similar or identical deletions could have occurred. Also, (Gottwald et al. 2009) found a functional SNP in the 'Barke' TILLING population (mutant 8408-1) leading to a six-rowed barley phenotype which was identical to already known mutants *hex-v28*, *cv* 'Foma' and *Int-d68*, *cv* 'Kristina', respectively. Similarly, TILLING analysis performed in the present study on *HvLUX* and *HvPUMILIO* revealed the same mutations at six different nucleotide positions which were

detected in two or more independent mutants. All these facts strongly supported the hypothesis that some regions in the DNA sequence might be more prone to be affected during various mutagenesis treatments.

7.3.2 Circadian clock disruption

It has been shown by using the delayed fluorescence measurement (DF) and confirmed by the time-course RT-qPCR that the mutant KT3-5 indeed had a distorted circadian clock. The current study showed that the DF measurement could be assessed to any species of interest with slight modifications in the protocol. Particularly, the constant darkness conditions interrupted every hour with light pulse were used instead of constant light as described elsewhere (Gould et al. 2009). The constant light conditions were found to be too severe even for the wild type to measure its DF oscillation for more than two days (data not presented). There is one question arising –why was any DF rhythm observed in the mutant? Based on results obtained in previous studies there are two possible explanations. One is that the DF measurement does not directly detect the nuclear circadian clock, but only its output in the chloroplast which can be, to some extent, independent (Gould et al. 2009). The second reason might be the experimental design, i.e. continuous light had more severe impact on the clock robustness than the darkness in case of *Arabidopsis LUX* mutant (Hazen et al. 2005). Nevertheless, the current work marks the first-step towards a possible usage of DF for large-scale application in crop species.

The most current model of the circadian clock tells that the Evening Complex (EC) acts on both *TOC1* and *PRR9* in *Arabidopsis* (Pokhilko et al. 2012). However, the two latter

synergistically down-regulate *CCA1*/*LHY* expression which could explain why plants having dysfunctional EC show greater disruption of the *LHY* rhythm in expression than of *TOC1*. The arrhythmia in *TmLHY* expression observed in the mutant KT3-5 was an additive effect of the changes in expression of both *TmTOC1* and *TmPRR9* (ortholog of PRR9). On the other hand, the *TmPRR9* is similarly strongly arrhythmic and this indicates that probably the whole morning loop is more affected than the evening loop. This can be interpreted in the way that other evening loop components are expressed in a similar phase like the EC and are to some extent degenerated in maintaining the rhythm of the central circadian clock oscillator. On the other hand, morning elements are antiphasic to the expression of the EC thus more depending on the proper evening loop expression.

7.3.3 *HvLUX* knock-down lines in barley

Analysis of T1 families of *HvLUX* knock-down lines in barley under LD conditions did not reveal any significant differences in heading time between putative knock-down lines and the wild type cv. Golden Promise or azygous plants. However, this was probably caused by the low effect of the knock-down on the clock since the arrhythmia was not detectable in expression patterns of clock genes under constant light. In this respect, the consequences of the auto-negative feedback loop that involves LUX should also be considered (Hazen et al. 2005; Onai and Ishiura 2005). Lower levels of *HvLUX* mRNA, and subsequently protein, will in fact cause increased transcription of *HvLUX*, thus compromising the effect of knocking-down the *HvLUX* transcript. However, the observed up-regulation of *HvPRR9* and *HvGI* should give some effect on heading time which might become more visible under the SD. First of all, both genes are considered to promote flowering in *Arabidopsis* (Imaizumi et al. 2005; Nakamichi et al. 2007).

Secondly, SD conditions allow for better separation of flowering time between wild type genotypes and mutants in any of the EC component (Doyle et al. 2002; Hazen et al. 2005; Hicks et al. 2001). Further experiments under SD shall be conducted to verify this hypothesis in barley.

7.3.4 Barley TILLING mutants

The mutation frequency obtained in the current study with on average 1 mutation per 0.48 Mb and 0.58 Mb in case of *HvPUMILIO* and *HvLUX*, respectively, was very similar to the average rate reported elsewhere; 1 mutation per 0.5 Mb (Gottwald et al. 2009). None of the M3 families' seed gave rise to a single plant showing the expected flowering phenotype. Also the M4 generation did not reveal any plants heading earlier than the wild type 'Barke' or wild type segregants. However, there was no knock-out mutation detected within the main candidate *HvLUX* and the premature-stop codon in *HvPUMILIO* expectedly did not lead to early heading. The latter shall be proposed as an indirect evidence that the *HvLUX* was a considerably better candidate for the *Eps-3A*^{m}/*eam10* locus.

7.3.5 *eam10* mutant

It was highly probable that the mutation of *eam10* present in the barley accession "Super Precoz 2H" from Argentina was in fact a mutated *LUX* gene. The assumption was based on the similar mapping position of *Eps-3A*^{m} and *eam10* (Borner et al. 2002; Gallagher et al. 1991; Gawronski and Schnurbusch 2012). Indeed, accessions numbered PI 527381 (mutant line Super Precoz 2H) and GSHO 3421 (introgression line of *eam10* in Bowman) were found to contain an amino acid substitution at a highly conserved position within the MYB domain of *HvLUX.*

Moreover, the change observed from serine (S, polar) to cysteine (C, hydrophobic) can be considered as disabling *eam10* to associate with the *LUX* binding site (LBS) (Helfer et al. 2011; Hosoda et al. 2002).

7.3.6 Re-sequencing of putative *LUX* orthologs/ homoeologs in a subset of 96 wheats

Allele mining in a diverse subset of 96 wheat accessions did not reveal any clear correlation between specific haplotypes of putative *LUX* orthologs/ homoeologs and heading date. This could suggest that cereal *LUX* may not be a major gene for flowering time determination in wheat. However, some trends in haplotype frequency versus climatic conditions were found which could support the hypothesis that wheat *LUX* is important for temperature adaptation. Consistent with the finding that at warmer temperatures KT3-5 mutant performed better than the donor wild type KT3-1, it would be expected to find higher frequency of mutations in warmer climates that could allow for appearance of some non-functional alleles. Such trend could be observed within the sequence of the A-genome copy of *LUX*. However, in the current study only one putatively functional mutation was found and a cultivar harboring this mutation, 'Tsing Hua no. 559' was classified as coming from the cooler climate, though the average temperature value was just below the threshold (17.5°C). Nucleic acid sequence and function of *LUX* seem to be generally conserved in the germplasm studied, underlining the importance of proper circadian clock function on the overall plant performance and breeding value. The only mutation found in the *TtLUX-A* of 'Tsing Hua no. 559' was not proven to be a cause of the relatively early heading of this cultivar, since the time-course expression of clock genes analyzed did not show any obvious abnormalities.

7.4 Importance of the circadian clock for plant adaptation

Proteins found to be involved in the core clock feedback loops (CCA1, ELF3, ELF4, LUX ARRHYTHMO, GIGANTEA) have in addition some other distinct functions in plant physiology and development (Hicks et al. 2001; Lu et al. 2012; Nusinow et al. 2011; Oliverio et al. 2007; Wang et al. 2011). These functions are usually defined as the clock input/output which are not crucial for generating the clock oscillations (de Montaigu et al. 2010). Several works suggested that having a robust clock gives a great adaptive advantage that exhibits itself by increased biomass production, disease resistance etc. (Ni et al. 2009; Song et al. 2011; Wang et al. 2011) However, the analysis performed could not separate the pure influence of the clock perturbations from the pleiotropic effects of the genes being involved also in the clock input/output. Neither analysis of the knock-out mutants nor over-expressing lines could provide the opportunity to do so; in such situation clock distortions (e.g. arrhythmia, lengthening or shortening of the period) were usually accompanied by physiological and morphological changes e.g. early/late flowering and long hypocotyl (Doyle et al. 2002; Hazen et al. 2005; Lu et al. 2012).

On the other hand, it should also be considered that both are probably equally important and permanently interconnected; meaning that a robust central oscillator perceives the environmental stimuli and releases the physiological response always on time, thus resulting in an increased performance of the plant. “Always on time” equals being well synchronized with the environmental fluctuations. This gives a valid explanation why the short/long period mutants displayed an advantage over the wild type plants only when placed in the conditions that matched their clock’s period; i.e. 20h day for short period, or 28h day for long period mutants (Dodd et al. 2005).

7.5 Summary and outlook

Genes characterized in the well-known model plant *Arabidopsis* cannot simply be considered as playing the same role in crop plants. Strong experimental support is required instead and this usually involves much more efforts to be spent. In the current study it was demonstrated that through joining the outputs from wheat and barley physical mapping projects, a similar amount of evidences could be collected in a reasonable time period.

In fact, the early flowering in wheat and barley mutant plants was caused by dysfunction of the *LUX ARRHYTHMO* ortholog from *Arabidopsis*, one of the main players in the circadian clock. Most of the higher organisms leaving on the Earth have developed this endogenous mechanism which has helped them to anticipate the environmental changes occurring every day. The circadian clock refers to the molecular machinery which sustains nearly 24h period in the activity dynamics of its every single element. This happens at the level of gene transcription, splicing, translation, protein modifications and turnover. The main feature of the circadian clock is the complexity of the network that makes it running. All types of interactions can be found here, but so far the overlapping negative feedback loops constitute the majority at the transcriptional level of regulation. Moreover, the right balance between redundant and universal functionality of the elements is responsible for the circadian clock robustness.

It was found that the mutated cereal *LUX* constituted the *earliness per se 3A*^{m} and *early maturity 10* loci on chromosomes 3A^{m} and 3H in einkorn wheat and barley; respectively. The conclusion was made after molecular-genetic analyses (map-based cloning) and re-sequencing of the putative gene ortholog in the latter species. The gene was deleted from einkorn wheat's

mutant genome, whereas in barley–an important amino acid substitution has been detected. The broad similarity between cereal and *Arabidopsis LUX* mutants was observed at the level of circadian clock distortion. First of all, ceased amplitude in transcript levels of selected clock genes was shown when plants were kept under constant light. Secondly, the delayed fluorescence measurements, reflecting the physiological state of chloroplasts, clearly indicated that the circadian clock of the einkorn wheat mutant (KT3-5) was distorted.

Results from this study pointed towards the conclusion that the putative cereal *LUX* homolog is a good candidate for the *eps3* trait. However, it will still remain an open question whether the KT3-5 mutant phenotype was caused by the *LUX* deletion alone. This could only be answered by performing a complementation test; although a missing transformation platform in einkorn wheat prevented from making such experiments.

Knowledge about flowering time determination in cereals is limited and the current study marks an important step in broadening it. The main advantage of knowing the *eps* loci is to enable fine-tuning of heading dates in modern cereal breeding. Further studies shall be conducted to discover novel alleles of cereal *LUX* and imply them in the selection for specific climatic conditions.

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9 Appendix

Appendix Table 1 Mapping results of segregating marker loci genotyped in the RILWA1 population. Orthologous genes from *Brachypodium* and rice as well as the EST markers that were used for primer design are shown (Dilbirligi et al. 2006; Hori et al. 2007). Loci predicted by the "genome zipper" are in italics on the grey background (Mayer et al. 2011). Loci being significantly distorted from the expected Mendelian ratio (at $p < 0.05$) have chi square values followed by an asterisk (*).

Nr.	Locus	Absolute map position (cM)	χ^2	*Brachypodium* gene[1]	Rice gene[2]	EST based marker	EST used for primer design (NCBI GenBank accession numbers)
1	*INDEL_111_112*	0.0	5.73*	Bradi2g41400.1	Os01g0566900	cdo920	CJ963739.1
2	*CAPS_155_156*	0.5	4.85*	Bradi2g44040.1	Os01g0625200	k06839	CJ682443.1
3	*CAPS_93a_94*	0.5	5.43*	Bradi2g44090.1	Os01g0626400	mwg582	CK194836.1
4	*dCAPS_99_100*	1.4	3*	Bradi2g47520.1	Os01g0699900	bcd927	CV781568.1
5	*AS_PCR_153_154*	4.0	4.28*	Bradi2g46520.1	Os01g0678600	k05129	CJ604366.1
6	*INDEL_105_106*	4.0	3.77*	Bradi2g46990.1	Os01g0689800	abg396	BQ169457.1, CJ870196.1
7	AS_PCR_149_150	10.5	5.73*	Bradi3g57450.1	Os03g0113700	k07096	CJ631750.1
8	*TP_71_72*	12.7	4.48*	Bradi2g51370.1	Os01g0772600	cdo281	CJ695432.1
9	*AS_PCR_147_148*	12.9	5.04*	Bradi2g51380.1	Os01g0772700	k02491	CJ809869.1
10	*AS_PCR_69_70*	14.3	2.06	Bradi2g51980.1	Os01g0784800	psr578	CJ561265.1
11	CAPS_251_252	20.3	0	Bradi2g54320.1	Os01g0836800	k08244	Primers as published in Hori et al. (2007)
12	*CAPS_143_144*	20.3	0	Bradi2g54920.1	Os01g0848200	k01229/cdo 113	CJ616075.1
13	*CAPS_139_140*	25.0	0.23	Bradi2g56320.1	Os01g0871200	k01163	CJ785585.1
14	*CAPS_61_62*	40.6	0	Bradi2g56810.1	Os01g0880200	bcd372	BJ268061.1, CJ945843.1
15	*dCAPS_57_58*	42.5	0.33	Bradi2g58190.1	Os01g0908700	bcd1555	BQ803448.1
16	*CAPS_249_250*	43.0	0.23	Bradi2g58250.1	Os01g0909100	k02004	Primers as published in Hori et al. (2007)
17	*CAPS_59_60*	46.6	0.01	Bradi2g58750.1	Os01g0917200	bcd515	BE500656.1, BG263254.1
18	*CAPS_45_46*	48.5	0.23	Bradi2g59210.1	Os01g0924000	abc161	CD930226.1
19	*CAPS_135_136*	49.9	0.08	Bradi2g59550.1	Os01g0929100	k01412	DR738416.1
20	*CAPS_37_38*	50.9	0.45	Bradi2g59480.1	Os01g0927600	bcd361	CJ688599.1, CJ688599.1
21	*INDEL_245_246*	69.7	0.74	Bradi2g104746	Os01g0940700	k06054	Primers as published in Hori et al. (2007)
22	CAPS_131_132	72.1	1.11	Bradi3g36580.1	Os11g0526200	k01427	CJ599687.1
23	*INDEL_243_244*	79.4	5.73*	Bradi2g61320.1	Os01g0958100	k00686	Primers as published in Hori et al. (2007)
24	CAPS_241_242	81.4	7.4*	Bradi5g02340.1	no	k08619	Primers as published in Hori et al. (2007)
25	*CAPS_239_240*	82.8	8.82*	Bradi2g61440.1	Os01g0960300	k04598	Primers as published in Hori et al. (2007)
26	*CAPS_121_122*	84.2	9.48*	Bradi2g61500.1	no	k08978	CJ945353.1
27	*AS_PCR_117_118*	92.2	8.33*	Bradi2g61840.1	Os01g0967100	k04421	CK209370.1
28	*PAV_185_186*	93.2	8.49*	Bradi2g61800.1	Os01g0966300	k07699_bags29d05	CV780148.1
29	*CAPS_209_210*	95.6	6.69*	Bradi2g61920.1	Os01g0968600	k05075	AV925255.1
30	AS_PCR_205_206	98.1	9.99*	Bradi1g20170.1	Os08g0110500	k07643	CJ676118.1
31	*INDEL_161_162*	98.1	9.99*	Bradi2g61950.2	Os01g0970400	GBS0879	CN011485.1
32	*CAPS_3_4*	99.0	12.56*	Bradi2g62010.1	no	k06868	Primers as published in Hori et al. (2007)
33	*dCAPS_191_192*	99.0	12.56*	Bradi2g62090.1	Os01g0971700	k00331	DR738808.1
34	*CAPS_273_274*	100.0	9.99*	Bradi2g61990.1	Os01g0971100	colinear gene	CJ890761.1

Nr.	Locus	Absolute map position (cM)	χ^2	*Brachypodium* gene[1]	Rice gene[2]	EST based marker	EST used for primer design (NCBI GenBank accession numbers)
35	*PAV_269b_270*	102.5	13.95*	Bradi2g61960.1	Os01g0970600	colinear gene	CK158853.1
36	*INDEL_271_272*	102.5	13.95*	Bradi2g61970.1	Os01g0970900	colinear gene	CJ870803.1, CJ661969.1
37	*PAV_295_296*	102.9	12.56*	no	Os01g0971900	colinear gene	BE516878.1
38	*CAPS_zt4_zt5*	102.9	12.56*	no	Os01g0972200	colinear gene	CJ871209.1
39	*CAPS_281_282*	102.9	12.56*	Bradi2g62130.1	Os01g0972800	colinear gene	CD935356.1
40	*TP_13_14*	103.4	11.24*	Bradi2g62150.2	Os01g0973000	colinear gene	CJ858329.1, CA708935.1
41	*CAPS_311_312*	103.4	11.24*	Bradi2g62170.1	no	colinear gene	CD057623.1
42	*AS_PCR_315_316*	103.4	11.24*	Bradi2g62250.1	no	colinear gene	CJ868407.1
43	*AS_PCR_17_18*	103.4	11.24*	Bradi2g62270.1	Os01g0973300	colinear gene	CJ688909.1
44	*CAPS_289_290*	103.4	11.24*	Bradi2g62290.1	no	colinear gene	GH727213.1
45	*INDEL_201_202*	103.4	11.24*	Bradi2g62280.1	Os01g0973400	k08293	CJ671375.1
46	*INDEL_21_22*	104.3	11.24*	Bradi2g62310.1	Os01g0973600	colinear gene	CV775997.1, CJ796395.1
47	*CAPS_253_254*	105.3	10.7*	Bradi2g62370.1	no	colinear gene	BG416120.1
48	CAPS_305_306	106.2	8.82*	no	no	k09203	CJ611238.1
49	*CAPS_23_24*	106.5	8.33*	Bradi2g62410.1	Os01g0974500	colinear gene	CJ777987.1
50	*CAPS_195_196*	106.7	7.72*	Bradi2g62470.1	no	k06412	CJ908234.1
51	PAV_177_178	109.7	16.01*	Bradi1g78770.1	no	abc 172	CA722154.1

[1] *Brachypodium distachyon* GBrowse v1.0 (www.brachybase.org)

[2] IRSGP/RAP build 5 (www.rapdb.dna.affrc.go.jp)

Appendix Table 2 List of the markers being polymorphic between mapping parents KT3-5 and KT1-1. Explanation of the abbreviations used for marker naming: INDEL, insertion/deletion polymorphism; CAPS, cleaved amplified polymorphic sequence; dCAPS, derived CAPS; AS_PCR, allele-specific primer; TP, multiplexed AS_PCR for co-dominant scoring; PAV, presence/absence polymorphism. Primers taken from Hori et al. (2007) are in italics and grey background.

Marker	Comment/enzyme	Forward primer (5'-3')	Reverse primer (5'-3')
AS_PCR_17_18	additional mismatch	ACATCTGAAGAGCCAAGTTGATCC	GCTATCACATACCGCAAGAGAATCTC
AS_PCR_69_70	additional mismatch	CACCATCATAAATTGACCTTTTTCC	ATATCACGCATGCTGCAATAGAAGT
AS_PCR_117_118	additional mismatch	CTGCTCTCTGCCTTGATTGTAGAAG	GCGAAACAAAAAAATGGAATCAAA
AS_PCR_147_148	additional mismatch	CTTCAGATGTTCCTTCAGCTGACTC	CGTAGAACCCTTACAAGCCAC
AS_PCR_149_150	additional mismatch	CCAGTGGTACCTACTTGTTCA	CACAAGATCAAACTCGCCAAGAAGT
AS_PCR_153_154	additional mismatch	TGACGCAATATCCAATTAATCACTC	GTCAAGAAGAGGGAGCGTCAGAAT
AS_PCR_205_206	external primers	CTGGAGCTGATGTCTGATCCTGTTA	CACTGTGGGAAGGAGATGCTCTATT
AS_PCR_205_206	internal primer	CGGGTTTATATCAAGTTGTGGCTCTAT	no
AS_PCR_315_316	additional mismatch	CATATGAAGGCGACAGCAAACAAG	GGAAGGAGGCGAAGTCACTAACTC
CAPS_3_4	*Eco88I (AvaI)*	*CATGACAGGACAGTGACGCT*	*CGACCGATGACTTTGAGGAT*
CAPS_zt4_zt5	*Hin6I*	AACTTGAAGATGGGCTTGTCGAA	TGGTGATCCTGCTGGTGTTCA
CAPS_23_24	*MboI*	CACCGCATCCTACCCTTCCTAC	GCACCATCACAATACCTTACCTTCAC
CAPS_37_38	*BclI*	TGATGCCACTGTTCAGAAGCCTATG	GGACCTCCTCCTTGGTGTAAATGC
CAPS_45_46	*Eco72I*	ACCATCCAGATGGCGCACAAG	GTGTGATCCTTGCCCATTCCAAC
CAPS_59_60	*MboI*	AACCTGCTGCTCACCACACAC	TGAGGATGCTGAAGATGACCTTG
CAPS_61_62	*BseJI*	CATGTCTGCTGCATTTCTGACACTTC	TCCAATCGGAAGTCTAAGCTTCTCC
CAPS_93a_94	*FspI*	GAGCTCAGCAACGACGACATGG	TGGATAAGGGCTGTTCTTGACG
CAPS_121_122	*Bsh1285I*	ACTACCTGAAGGATAACCACCACTG	GGAGGAAGTGCTCGTAGTTGTATCA
CAPS_131_132	*EcoRI*	GCTTAAGGTCATAAGCCAGAACCAG	GGGGAGATCCTGTCCTTCTTCAC
CAPS_135_136	*TaqI*	AGGAGGACCCCATCGACAAGTG	CGCAGAACATGACGATGAAGATAGC
CAPS_139_140	*HincII*	GAGGAAGAACTGAGACGGTCATACA	TCATTTCTGTGTGATATGTGGCAAG
CAPS_143_144	*MspI*	CTCTGCACCTAGACCAAAGCGAGTC	AAGGTTGATTATCCTGCAGCGTGTA
CAPS_155_156	*AlwNI*	GATGGAAAACAGAAAGTTTGCATTG	TTGGGTATTCTATCCACAAACGATG
CAPS_195_196	*Eco88I*	GGTCAAGTACGAGCTGGACAAGAAG	GCAGATGTAGGTGGCATAGAGATCC
CAPS_209_210	*TaqI*	GACTTGGCACAACATTACCAGAGAA	CAAGGTGTAAGCATGTCAAGTTTGG
CAPS_239_240	*Cfr42I*	*CATCCACATGCCTGAAACTG*	*TGACATGACGGTTCTCCTCA*
CAPS_241_242	*PvuII*	*TGAGATTGCTTAGCACGACG*	*TGGACTGCAAAGTTGACCAG*
CAPS_249_250	*Alw26I*	*GGCCAGCAGATTGCTTAGAC*	*ACTACGGGCAACAAAACTGG*
CAPS_251_252	*HaeIII*	*ACCGGCAACATTTGAAGCTA*	*GCTCTTCCGCCAGTTCTATG*
CAPS_253_254	*AvaII*	CCTACAACTGGGCCGTCTTCTT	GGACCAGCTGATGACCAATGAG
CAPS_273_274	*Eco130I*	TGCTCATCATGTAGGGGTTCACAT	GAGGAACACTGCTTTGACACCAGA
CAPS_281_282	*MspI*	CAACTGCTCGCCGTATGTGTTC	GATCATCGTGATGGACCAATTGAC
CAPS_289_290	*ApaI*	GTCATGGAACCTGAGGATGAAGGT	GAAGCTGTACACGTCGCTCTTCG
CAPS_305_306	*Bpu1102I*	CATGCACTGATCACATGGAGACTAC	ATCGACAACGACAACAACTCACATC
CAPS_311_312	*MvaI*	TCGTAATAAGATGTGCGGAGAGATG	CAATGAACCAACTGTTGCATTTTCA
dCAPS_191_192	*Eco88I*	AGGTTTCTCCTCTCCTCTCGCCCGA	CATCTCTCCCTCGTAGCAGTCGAT
dCAPS_57_58	*ApaLI*	ATATGTATCCTTCTTTTATTAATCTGAACTGTG	CCTCACAATGGCATCAACAGTC
dCAPS_99_100	*BamHI*	ACGTTTTGCCTGACTTATTTGGATC	CCGTGTATAGCGAGTATGTAGAAGAGC

INDEL_21_22	size polymorphism	CATGCTCGTCAGCGACTTCCTC	CTCTGCTTCAGGGACCATTTCG
INDEL_105_106	size polymorphism	GAGGAGAAGATGCTTGGTTTGGTG	CGAGCCCTTCTCTCCCAACAC
INDEL_111_112	size polymorphism	CCTTGTCTGAAATGATGATTCGAG	TCTGTATCCCTTGGTGTTCAAAAGA
INDEL_161_162	size polymorphism	CTGGACCTTCTGGTTCGACAAC	GCCACAACTGATGGTCCATTTAC
INDEL_201_202	size polymorphism	GACAAATGTAGTGTTCATGGGGATG	TTCCAGAGGATATGCCTTTGCACTTGG
INDEL_243_244	size polymorphism	*AAATGGCTTCAACAAATGCC*	*CGATAAGAAGGCACAGGCTC*
INDEL_245_246	size polymorphism	*TACACTTGGATGCCGCATTA*	*CAACGAGAACCAGAAGACCG*
INDEL_271_272	size polymorphism	CAAGGCTCTGCAGTACTTGACAGAG	GACTTCATCAAGCGTAAGCACATGTC
PAV_177_178	presence/absence	TAAACTTGTGGGTGAGAATCCAGCA	GTGAAGCTGGAAAATGCAAATCTCA
PAV_185_186	presence/absence	CTCCTCCACGGCAGCTTCTC	GAGCGTGTCGTAGCTGTAGACCAT
PAV_269b_270	presence/absence	CTTGTGCCAGATGGTTGAGAAGC	AGGTAGCCCGAGCGTTGAAGTT
PAV_295_296	presence/absence	CATACTGGTCTGTAGCAAGCAAGCA	AGCACGGCTCAGATAAAGGAGTTG
TP_13_14	external primers	CAAGCCTGACCCAGTACTCAGG	CACAGCCTCCTCCCACAGTTC
TP_13_14	internal primers	ATGCGACTTCGGTTGATGCTC	ATTCACCACATTAGCAACGCA
TP_71_72	external primers	GAGGCCTATTAGAATCGATGGTGCTC	ATGTGTTGATGAAGTCGGGCTTCTG
TP_71_72	internal primers	CCCATGAAACAGAGGTAACACCA	TGTGCAACCTATAAAGCCATGATCTAC

Appendix Table 3 Marker loci re-sequenced in lines KT1-1, KT3-1 and KT3-5 to confirm that the early heading mutant KT3-5 was derived from the line KT3-1

Marker Locus	Length	SNP, KT3-1 vs KT3-5	SNP, KT3-1 vs KT1-1	INDEL nt, KT3-1 vs KT1-1
TP_13_14	700	0	1	0
CAPS_23_24	800	0	15	0
CAPS_3_4'	300	0	2	0
CAPS_37_38	1400	0	6	0
AS_PCR_17_18	2000	0	73	39
CAPS_45_46	1400	0	3	0
CAPS_61_62	780	0	1	0
CAPS_135_136	600	0	1	0
CAPS_59_60	1240	0	2	0
CAPS_131_132	730	0	66	43
CAPS_93a_94	1300	0	2	0
dCAPS_99_100	800	0	1	0
AS_PCR_147_148	900	0	1	0
dCAPS_191_192	350	0	2	0
AS_PCR_205_206	350	0	1	0
CAPS_145_146	550	0	3	0
CAPS_209_210	500	0	23	5
Totally 17	14700	0	203	87

Appendix Table 4 Ninety-six wheat panel selected for re-sequencing of *LUX*

GENUS	SPECIES	GENOME	SOURCE	ACCESSION NO.
Triticum	*zhukovskyi*	GAA	DEU146	TRI 5416
Triticum	*zhukovskyi*	GAA	DEU146	TRI 7258
Triticum	*aethiopicum*	BA	DEU146	TRI 15024
Triticum	*aethiopicum*	BA	DEU146	TRI 15065
Triticum	*aethiopicum*	BA	DEU146	TRI 15432
Triticum	*carthlicum*	BA	DEU146	TRI 3426
Triticum	*carthlicum*	BA	DEU146	TRI 15127
Triticum	*carthlicum*	BA	USDA	PI 286070
Triticum	*dicoccon*	BA	DEU146	TRI 2024
Triticum	*dicoccon*	BA	DEU146	TRI 2214
Triticum	*dicoccon*	BA	DEU146	TRI 2884
Triticum	*dicoccon*	BA	DEU146	TRI 6141
Triticum	*dicoccon*	BA	DEU146	TRI 10318
Triticum	*dicoccon*	BA	DEU146	TRI 15033
Triticum	*dicoccon*	BA	DEU146	TRI 17738
Triticum	*dicoccon*	BA	ICARDA	IG 45091
Triticum	*durum*	BA	ICARDA	IG 99234
Triticum	*dicoccon*	BA	USDA	CItr 14621
Triticum	*dicoccon*	BA	USDA	PI 41025
Triticum	*dicoccon*	BA	USDA	PI 94671
Triticum	*dicoccon*	BA	USDA	PI 217637
Triticum	*durum*	BA	Kilian/MPIZ	DIC 175
Triticum	*ispahanicum*	BA	DEU146	TRI 6177
Triticum	*ispahanicum*	BA	DEU146	TRI 7260
Triticum	*karamyschevii*	BA	CZE122	01C0101162
Triticum	*polonicum*	BA	DEU146	TRI 3428
Triticum	*polonicum*	BA	DEU146	TRI 3478
Triticum	*militinae*	GA	CZE122	01C0202037
Triticum	*turanicum*	BA	USDA	PI 341414
Triticum	*turanicum*	BA	USDA	PI 349055
Triticum	*turgidum*	BA	USDA	PI 149812
Triticum	*turgidum*	BA	USDA	PI 502933
Aegilops	*tauschii*	D	Kyoto	2038
Aegilops	*tauschii*	D	Kyoto	2105
Aegilops	*tauschii*	D	Kyoto	2131
Aegilops	*tauschii*	D	Kyoto	2154
Aegilops	*tauschii*	D	Kyoto	2613
Aegilops	*tauschii*	D	Nelli	ARM 19

GENUS	SPECIES	GENOME	SOURCE	ACCESSION NO.
Aegilops	*tauschii*	D	USDA	PI 486275
Triticum	*urartu*	Au	Hako	2006-6-17-3-6
Triticum	*urartu*	Au	ICARDA	IG 45298
Triticum	*urartu*	Au	ICARDA	IG 109084
Triticum	*urartu*	Au	ICARDA	IG 140061
Triticum	*urartu*	Au	Kilian/MPIZ	ID 388 EP047-1
Triticum	*urartu*	Au	Kilian/MPIZ	ID 393 EP048-1
Triticum	*urartu*	Au	Kilian/MPIZ	ID 1395 SSD 2007+2009
Triticum	*urartu*	Au	-	ID 1442 EP038-EP039
Triticum	*urartu*	Au	Kilian/MPIZ	ID 1474 EP040-EP041
Triticum	*urartu*	Au	Kilian/MPIZ	ID 1503 EP042
Triticum	*urartu*	Au	Kilian/MPIZ	ID 1538 SSD 2007+2009
Triticum	*urartu*	Au	Kyoto	199- 3
Triticum	*urartu*	Au	-	TA 831
Triticum	*boeoticum*	Ab	ICARDA	IG 110785
Triticum	*boeoticum*	Ab	Kilian/MPIZ	ID 210 EP045
Triticum	*boeoticum*	Ab	Kilian/MPIZ	ID 379 EP046
Triticum	*boeoticum*	Ab	Kilian/MPIZ	ID 520 EP058-EP059
Triticum	*boeoticum*	Ab	Kilian/MPIZ	ID 597 EP062-EP063
Triticum	*boeoticum*	Ab	Kilian/MPIZ	ID 716 EP064-EP066
Triticum	*boeoticum*	Ab	Kilian/MPIZ	ID 752 EP067-EP072
Triticum	*boeoticum*	Ab	Kilian/MPIZ	ID 753 EP073-EP078
Triticum	*boeoticum*	Ab	Kilian/MPIZ	ID 758 EP079-EP086
Triticum	*boeoticum*	Ab	Kilian/MPIZ	ID 760 EP087-EP095
Triticum	*boeoticum*	Ab	Kilian/MPIZ	ID 771 EP097-EP100
Triticum	*boeoticum*	Ab	Kilian/MPIZ	ID 1089 EP003-EP004
Triticum	*boeoticum*	Ab	Kyoto	101-2
Triticum	*monococcum*	Am	Kilian/MPIZ	ID 1331 EP030-EP035
Triticum	*timopheevii*	GA	Kilian/MPIZ	TR 103 TP029-TP030
Triticum	*timopheevii*	GA	Kilian/MPIZ	TR 104 TP031-TP034
Triticum	*araraticum*	GA	DEU146	TRI 18478
Triticum	*araraticum*	GA	Hako	2006-6-20-2-2 D
Triticum	*araraticum*	GA	ICARDA	IG 116165
Triticum	*araraticum*	GA	Kilian/MPIZ	TR 1
Triticum	*araraticum*	GA	Kilian/MPIZ	TR 17 TP061-TP063
Triticum	*araraticum*	GA	Kilian/MPIZ	TR 52 SSD 2007+2009
Triticum	*araraticum*	GA	Kilian/MPIZ	TR 61 TP071
Triticum	*araraticum*	GA	Kilian/MPIZ	TR 69 TP072-TP076
Triticum	*araraticum*	GA	Kilian/MPIZ	TR 94 TP077-TP079
Triticum	*araraticum*	GA	Kilian/MPIZ	TR 122

GENUS	SPECIES	GENOME	SOURCE	ACCESSION NO.
Triticum	*dicoccoides*	BA	Kilian/MPIZ	DIC 19
Triticum	*dicoccoides*	BA	Kilian/MPIZ	DIC 44 TP011-TP020
Triticum	*dicoccoides*	BA	-	DIC 49
Triticum	*dicoccoides*	BA	Kilian/MPIZ	DIC 55 TP021-TP028
Triticum	*dicoccoides*	BA	Kilian/MPIZ	DIC 60
Triticum	*dicoccoides*	BA	Kilian/MPIZ	DIC 64
Triticum	*dicoccoides*	BA	Kilian/MPIZ	DIC 96
Triticum	*dicoccoides*	BA	Kilian/MPIZ	DIC 118 SSD 2007+2009
Triticum	*dicoccoides*	BA	Kilian/MPIZ	DIC 119 TP001-TP009
Triticum	*dicoccoides*	BA	Kilian/MPIZ	DIC 138
Triticum	*dicoccoides*	BA	Kilian/MPIZ	DIC 145
Triticum	*dicoccoides*	BA	Kilian/MPIZ	DIC 196 TP010
Aegilops	*speltoides*	S	Kilian/MPIZ	Spel-30/3
Aegilops	*speltoides*	S	Kilian/MPIZ	Spel-46/3
Aegilops	*speltoides*	S	Kilian/MPIZ	Spel-48/1
Aegilops	*speltoides*	S	Kilian/MPIZ	Spel-66/1-2
Aegilops	*speltoides*	S	Kilian/MPIZ	Spel-104/2
Triticum	*aestivum*	ABD	-	'Chinese spring'

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Curriculum vitae

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- 09.2009–12.2012: PhD student at the Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany; PhD thesis title "*Earliness per se 3* locus from wheat (*Triticum* sp. L) and barley (*Hordeum vulgare* L.) disrupts circadian clock function".

List of Publications

- Gawroński P, Schnurbusch T (2012) High-density mapping of the *earliness per se-3A^m* (*Eps-3A^m*) locus in diploid einkorn wheat and its relation to the syntenic regions in rice and *Brachypodium distachyon* L. Mol Breed 30:1097-1108
- Gawroński P, Ariyadasa R, Poursarebani N, Himmelbach A, Kilian B, Stein N, Steuernagel B, Hensel G, Kumlehn J, Sehgal SK, Gill BS, Gould P, Hall A, Schnurbusch T (2013) A distorted circadian clock causes early flowering and temperature-dependent variation in spike development in the *Eps-3A^m* mutant of einkorn wheat. *Accepted for publication in 'Genetics'*.

Talks and poster presentations at international conferences

- Oral presentation at the XX Plant & Animal Genome Conference in San Diego 15.01.2012 in frame of the "QTL cloning" WS: "Analysis of the *earliness per se 3A^m* Locus (*Eps-3^m*) in an Einkorn Wheat Mutant (*Triticum monococcum* L.) Revealed a Link to the Circadian Clock" https://pag.confex.com/pag/xx/webprogram/Paper1801.html
- Oral presentation at the conference "Biotechnology and plant breeding" in Radzików 10.09.2012: "Cloning of the *Earliness per se 3* (*Eps-3*) locus from wheat (*Triticum* sp. L) and barley (*Hordeum vulgare* L.) – important trait for short-season adaptation" http://www.ihar.edu.pl/en/img/143d3ef8.pdf
- Poster presentation at the XX Plant & Animal Genome Conference in San Diego 2012: "AdvanCE^{TM} FS96 Used for TILLING in Barley Allowed Cost Efficient and More Sensitive Mutant Detection" https://pag.confex.com/pag/xx/webprogram/Paper1848.html

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