Molecular genetics of the floral response in Xerophyta humilis

Marleen Myers B.Sc., M.Sc.

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Abstract

Desiccation tolerant or resurrection plants have the ability to dry to equilibrium with the ambient air and recover when rehydrated. *Xerophyta humilis* is a monocotyledonous resurrection plant that loses chlorophyll during dehydration. For X. *humilis* to survive in their natural habitat, correct timing of flowering is essential. Environmental factors such as temperature and photoperiod play an important role in floral induction in several plant species. In this study environmental cues and molecular genetics involved in the regulation of vernalisation and photoperiod are analysed. It was found that flowering was promoted by a prolonged cool period followed by transfer to long-day (LD) photoperiods with high light. Results show that in the complex floral pathway of *X. humilis* cold and high light play important roles. A change in the photoperiod or desiccation only did not initiate flowering. A homologue of *VRN2*, a floral repressor in *Triticum monococcum* was isolated by using PCR and RACE. The full length sequence predicted a CCT domain and two B-boxes, similar to the floral pathway gene *CONSTANS*, a floral activator in Arabidopsis thaliana and so the homologue was termed *XhCO*. Bioinformatic analyses for *cis* acting motifs of a 1.1 kb 5' region identified light, circadian and stress responsiveness. *XhCO* expression was monitored by semi-quantitative RT PCR and showed a diurnal rhythm with a peak in the middle of the day and a larger peak in the night, which coincides with the expression pattern of *VRN2*. Expression appears to be light dependent, with more transcript accumulating in leaves of plants grown in low light compared to ones grown in high light under LD. In short-days (SD) the expression was of similar levels in low and high light and the peaks shifted slightly compared to LD. These results together suggest *XhCO* has a similar function to *VRN2*.

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Dedication

To Anna Myers, who is full of beautiful smiles.

Chapter 1. Introduction

1.1 Resurrection plants

Desiccation tolerance, the ability to dry to equilibrium with the ambient air and recover when rehydrated, is not exceptional in mosses, ferns, seeds, spores and pollen of higher plants (Alpert, 2006). In vegetative tissues of higher plants however, this phenomenon is rare and only few plants have the unique ability to revive from an air dry state (Gaff, 1971; Farrant, 2007). The rehydration of some of these plants can give the appearance of reviving an apparently dead plant; therefore they are often referred to as 'resurrection plants' (Gaff, 1971). Desiccation tolerant plants are found on all continents, except Antarctica and mostly grow in South Africa, West Australia and East South America (Gaff 1971, 1977; Gaff and Latz, 1978). Their habitats are mostly on rocks in shallow soil, which dries out frequently throughout the year. Desiccation sensitive plants cannot populate these habitats, so competition is absent for the desiccation tolerant ones. Several monocotyledonous and dicotyledonous species have been identified within the angiosperms; no gymnosperms have been reported. Some resurrection plants lose their chlorophyll during dehydration (poikilochlorophyllous) and others retain their chlorophyll (homoichlorophyllous) when dehydrating. When chlorophyll is lost, rehydration of the plant takes several days as recovery of the metabolic activity needs chloroplasts and photosynthetic pigments to be reconstituted (Sherwin and Farrant, 1996).

Xerophyta humilis is a monocotyledonous poikilochlorophyllous resurrection plant that is able to survive desiccation to 5% relative water content (RWC). *X. humilis* is an indigenous Southern

African plant, where it experiences dry cold winters and warm wet summers. To survive the dry winters, the plants have to dry down and stay dehydrated until enough water is available for rehydration. During this process, the damage of turgour loss is minimised and macromolecules such as proteins and nucleic acids stay functional. Mechanisms whereby this might be achieved are outlined in Farrant (2007). When the plants are rehydrated, oxidative damage is minimised by the plant and repair mechanisms are activated. *X. humilis* takes approximately nine days to dehydrate (Farrant *et al.*, 1999) and three to four days to rehydrate. The drying and rehydration rate is important for recovery, as when this happens too fast, the plant does not survive (Farrant *et al.*, 1999; Sherwin and Farrant, 1996).

The wet period needs to be long enough for *X. humilis* plants to rehydrate, photosynthesise and grow before drying occurs again. An even longer period is needed when the plant is ready to reproduce and flowering has to be initiated. Other environmental cues, such as exposure to cold and light intensity could also play an important role in the flowering and reproduction process. In this study flowering of *X. humilis* was analysed by studying the environmental cues and the molecular genetics of the flowering process. Molecular genetics has been studied during the dehydration – rehydration process in *Sporobolus stapfianus*, a desiccation tolerant grass (Ngoc Le *et al.,* 2007) and in *X. humilis* (Colett *et al.*, 2004). No genes involved in flowering were identified and only up or down regulation of the genes was analysed.

1.2 Flowering in model species

The transition from the vegetative state into reproductive state is a major decision that needs accurate timing. Correct timing is essential for plants to survive and to maximize reproductive success. Environmental cues, such as day length and temperature play an important role in floral transition. Ecophysiology dictates that plants flower in the right season to allow for pollination and seed production, which is also important for farmers to harvest crops in the right season. The flower industry is also interested in the maximal production of flowers in different seasons.

The genetics of flowering has been studied thoroughly in the model plant *Arabidopsis thaliana*. *A. thaliana* is a facultative long day plant, which means that long day photoperiods promote, but are not essential for flowering (Laibach, 1951). *A. thaliana* is a rosette plant which flowers with relatively few leaves (6-10) in long-days, but eventually flowers in non-promotive short-days with many more leaves produced before bolting (25-70) depending on the accession or ecotype. Some biennial *A. thaliana* accessions isolated from Northerly latitudes also require an extended period of cold, a vernalising period, in order to be made competent to flower in long-days (Napp-Zink, 1987).

Photoperiod

Multiple pathways regulate the floral transition (see Figure 1.1) and interaction of these pathways is necessary for correct floral timing. In *A. thaliana* the photoperiod accelerates flowering when days are lengthened. Light is perceived by five phytochromes (PHY) (A through

E) and two cryptochromes (CRY) (1 and 2) (reviewed in Lin, 2000). Different light quality affects the floral transition in different ways: blue (440 nm) and far red (735 nm) promote flowering through PHYA and CRY1 and 2 (Weller *et al.*, 2001; Platten *et al.*, 2005; Guo *et al.*, 1998), whereas red light (660nm) inhibits flowering through PHYB and D (Aukerman *et* al., 1997; Halliday *et al.*, 1994).

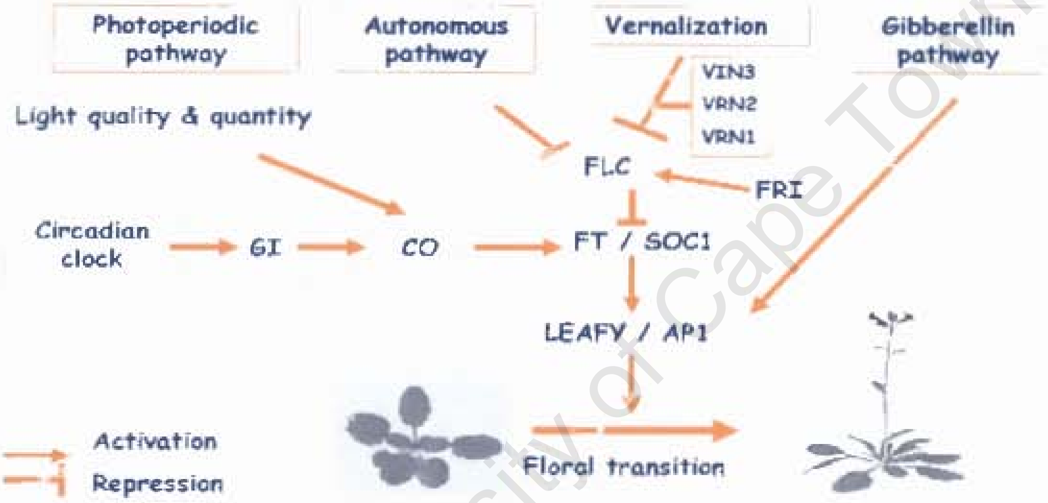


Figure 1.1. Modified from Mouradov *et al.*, 2002. An overview of the flowering pathway in *A. thaliana*. The photoperiodic pathway activates CONSTANS (CO), which activates the floral integrators FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1). FRIGIDA (FRI) activates FLOWERING LOCUC C (FLC), which is repressed by vernalisation through VERNALIZATION1 (VRN1), VRN2 and VERNALIZATION INSENSITIVE 3 (VIN3). The autonomous pathway represses FLC when FRI is inactive. FLC represses the floral integrators. The gibberellin pathway activates flowering.

The photoperiod regulates the activity of *CONSTANS* (*CO*) (Valverde *et al.*, 2004), which is also regulated by the circadian clock (Suárez-López *et al.*, 2001). In plants the circadian clock modulates a wide variety of biological and biochemical events, such as stomatal and organ movement, photosynthesis and induction of flowering (reviewed in Hotta *et al.*, 2007). Environmental cues, such as light and temperature entrain the clock, which coordinates a circadian rhythm. The circadian clock runs with a period close to 24 hours, even in absence of environmental cues, and maintains a relatively constant period. In *A. thaliana* the clock can be divided into three parts: the central oscillator, a set of genes that maintains the circadian rhythm; input pathways, which carry environmental information to entrain the central oscillator and output pathways, which regulate physiological processes (see figure 1.2).

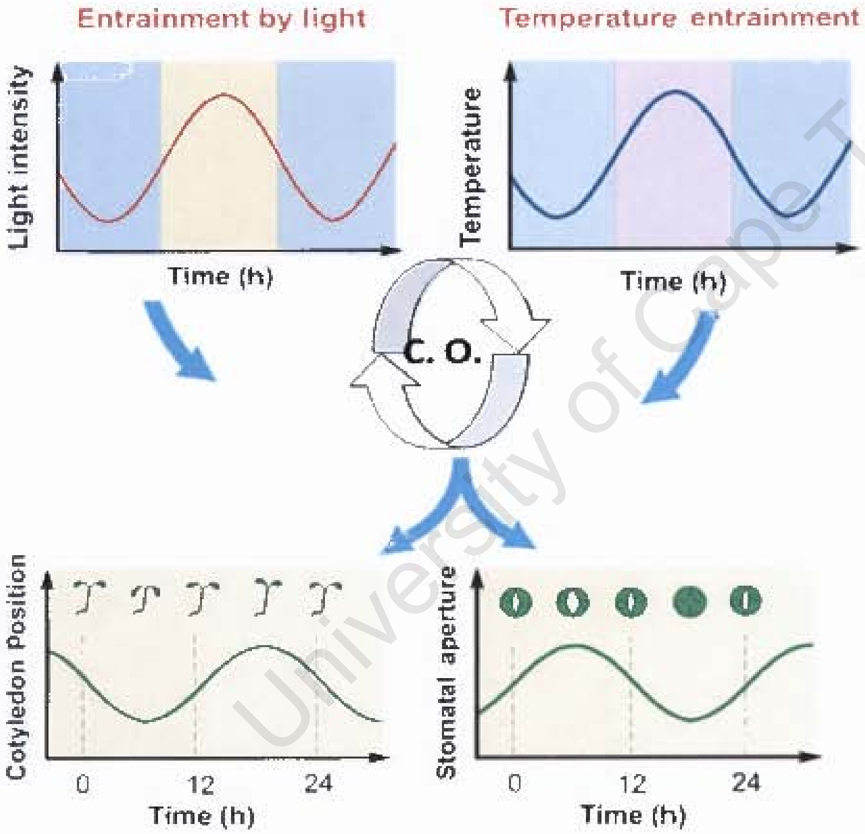


Figure 1.2. Modified from Hotta *et al.* (2007). Circadian rhythm in *Arabidopsis thaliana*. Environmental cues entrain the core oscillator (C.O.), which regulates a range of physiological outputs and maintains these rhythms in an appropriate phase relationship with the entraining environmental cues. Arrows indicate a positive (inductive) relationship between components. Rhythms in cotyledon movement and stomatal opening are illustrated as examples of differently phased circadian outputs.

The clock controls daily rhythms in gene expression such as *CO* (Figure 1.3). CO is a zinc finger transcription factor (Puterill *et al.,* 1995) and activates flowering by regulating the floral integrators FT and SOC1 (Samach *et al.*, 2000). *FT* encodes a small protein that acts as a long distance signal to induce flowering (Corbesier *et al.*, 2007). SOC1 is a MADS box transcription factor that promotes the transition to reproductive apex development (Lee *et al.*, 2000).

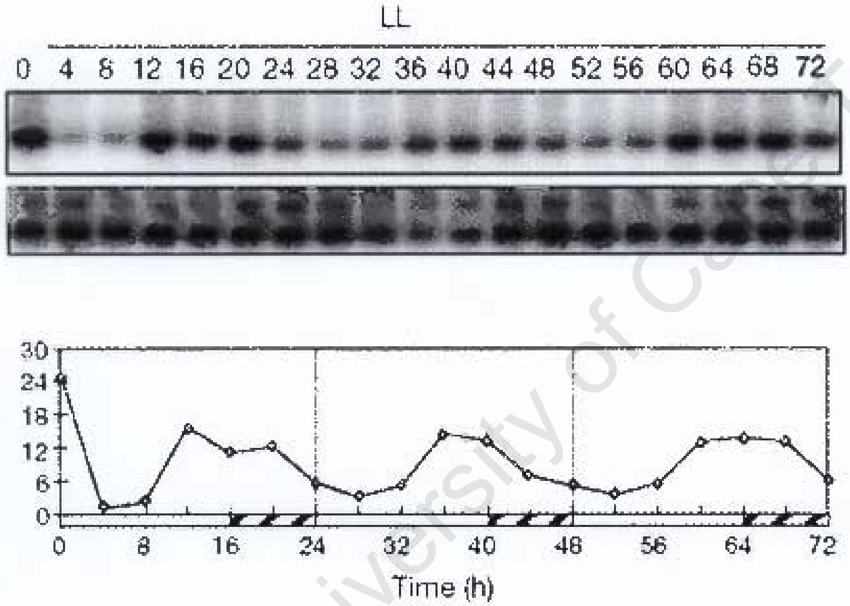


Figure 1.3. From Suárez-López *et al.* (2001). CO expression in wild type-plants. Top: CO mRNA abundance in plants entrained in LD and transferred to LL (constant light) Samples were collected at the times shown after dawn (time 0). Bottom: Quantifcation of CO mRNA from the experiment above. Representative result of three independent experiments in LL.

CO mutants flower later in long days (LD), but not in short days (SD) (Puterill *et al.,* 1995). Over expression of *CO* results in early flowering compared to wild type (Onouchi *et al.*, 2000). CO mRNA accumulates at the end and beginning of the photoperiod in LD, whereas under SD accumulation only occurs in darkness (Suárez-López *et al.*, 2001). CO protein is stabilized by blue and far red light through CRY2 and PhyA (Valverde *et al.*, 2004). CO contains two highly conserved domains, an N-terminal Zinc finger region that resembles a B-box domain and a C-terminal CCT (CO, CO-like, Timing of CAB expression 1) domain (Robson *et al.*, 2001). GI (GIGANTEA) activates flowering through CO and FT and has an effect on circadian rhythms (Mizoguchi *et al.*, 2005).

Flowering time in cereals, sometimes also referred to as heading date, is similarly influenced by the photoperiod. Components of the flowering pathways of the LD plant *A. thaliana* are conserved with the flowering pathway in SD plants, such as rice (*Oryza sativa*). *OsGI* is a homologue of *AtGI* and has a very similar expression pattern under LD and SD (Hayama *et al.*, 2002). *Hd1 (Heading date 1)* is a *CO* orthologue in rice, which encodes a protein with a zinc finger domain (Yano *et al.*, 2000). *Hd1* promotes heading under SD conditions and inhibits heading under LD conditions. *Hd1* upregulates *Hd3a*, which is a rice orthologue of *FT* (Kojima *et al.*, 2002). Kojama *et al.* show that the mRNA levels of *Hd1* and *Hd3a* oscillate during the day and night. However, no experiments were done during constant dark and constant light, so no conclusions can be drawn if the expression is regulated by the circadian clock (circadian) and/or by the photoperiod (diurnal). The protein encoded by *Hd3a* moves from the leaf to the shoot apical meristem and induces heading in rice (Tamaki et al., 2007), which is conserved with FT protein in *A. thaliana*. Differences in the flowering pathway in SD plants versus LD plants are necessary to flower in the right conditions. *Hd3* for example, is induced under SD, whereas *FT* is induced under LD. OsGI suppresses flowering, which is opposite to *Arabidopsis*, where GI promotes flowering (Hayama et al, 2003).

GA pathway

Hormones such as Gibberellic acids (GA), auxins, cytokinins and brassinosteriods have been associated with flowering time control. GA, which is studied the most, promotes flowering when applied to *A. thaliana.* GA mutants however, are dwarfed and delayed in flowering or fail flowering all together (for review see Schwechheimer, 2008). Mutants that block GA signaling (*gai*) or biosynthesis (*ga1-3*) delay flowering, particularly in SD (Wilson *et al*, 2002). In SD, when the LD flowering pathway is not active, the GA pathway is the major flowering pathway and prevents flowering when it is not functional. GA mutations also have an effect on other plant growth aspects such as plant growth development, stem elongation, germination and floral development.

Autonomous and vernalisation pathways

The autonomous pathway promotes flowering by repressing FLC (Flowering Locus C) and therefore autonomous pathway mutants (*fca, fy, fpa, ld, fld*) are late flowering due to elevated levels of FLC (Koorneef *et al.,* 1991). FLC is a MADS box transcription factor that represses floral transition, which is repressed by vernalisation, a prolonged exposure to cold (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Vernalisation is required for many plant species to ensure they stay vegetative in winter and flower in the right season. In biennial accessions of *A. thaliana* FRIGIDA (FRI) is dominant of the autonomous pathway and upregulates FLC. Vernalisation turns FLC off, so flowering can occur after winter (Johnson *et al.*, 2000). Annual accessions are rapid cycling types that have either a non functional *fri* allele or a weak *fri* allele.

Therefore the autonomous pathway regulates flowering in these accessions (Gazzani *et al.*, 2003). The vernalisation genes *VERNALIZATION 1 (VRN1)*, *VRN2* and *VERNALIATION INSENSITIVE 3 (VIN3)* are involved in repressing *FLC* by modifying *FLC* chromatin. VRN2 is required for maintenance of FLC transcriptional repression (Levy *et al.*, 2002; Gendall *et al.*, 2001; Sung and Amasino, 2004).

In barley and wheat, also monocots like rice, flowering is induced in LD. Winter varieties of barley and wheat require vernalisation to flower, whereas spring varieties are able to flower without exposure to a cold temperature. Three genes have been identified that are involved in vernalisation in wheat and barley: *VRN1*, *VRN2* and *VRN3* (Takahashi and Yasuda, 1971; Yan *et al.*, 2004; Yan *et al.*, 2006). VRN1 is a MADS box transcription factor and is similar to *A. thaliana APETALA1* (*AP1*) which is a floral meristem identity gene (Yan *et al.*, 2003). *VRN1* is induced by vernalisation. The length of vernalisation exposure has a direct effect on *VRN1* expression levels (Danyluk et al., 2003; Trevaskis *et al.*, 2003, Yan *et al.*, 2003). *VRN1* is dominant in spring varieties and represses *VRN2*. *VRN2* is dominant in winter varieties and is a floral repressor. *VRN2* contains a zinc finger domain and a CCT domain (von Zitzewitz et al., 2005; Yan et al., 2004). Diurnal expression of *VRN2* has been observed under LD, but expression is absent under SD (in winter) (Trevaskis *et al.*, 2006; Dubcovsky *et al.*, 2006). VRN2 modulates the quantitative levels of VRN3, which is a *FT* homologue. *VRN3* is induced by *PPD-H1* (*PHOTOPERIOD1*), a pseudo-response regulator gene and induces flowering in LD. *PPD-H1* encodes a protein with a pseudo receiver domain, which is involved in signal transduction and a CCT domain. *PPD-H1* has a diurnal expression pattern (Yan *et al.*, 2006).

Flowering in *Xerophyta humilis*

Although large parts of the flowering pathway are conserved in SD and LD plants, monocots and dicots, annuals and biennials evolved their own flowering pathway to maximise survival. FLC for example is an important floral repressor in *A. thaliana*, a dicot, however no FLC homologues have been identified in monocots. In cereals, VRN2 acts as a floral repressor and is upregulated by VRN1. Polymorphisms in the promoter or the first intron of *VRN1* are found in spring varieties, which do not require vernalisation. Regions in the first intron are required for transcriptional repression, like in FLC. Possibly VRN1 and VRN2 together provide a similar vernalisation mechanism to FLC in *A. thaliana*.

Xerophyta humilis (Baker) Dur and Schinz is a monocotyledonous plant and it is not known whether it is photoperiod responsive. Also, *X. humilis* is a perennial, which gives the possibility of a different flowering pathway to the discussed model plants. The aim of this study is to identify environmental cues and genes involved in the regulation of vernalisation and the photoperiod. Environmental cues such as photoperiod and temperature were analysed and the response to desiccation.

To identify homologues to known genes in model species, several techniques can be used. A well known method is to design degenerate primers to conserved regions of the gene of interest and these primers are amplified to cDNA or genomic DNA of the non model species. If a PCR product of correct size is amplified, it can be purified, cloned into a vector and sequenced. The sequence will reveal weather the correct product was amplified. Another technique to identify homologues is to screen a BAC library containing the genomic DNA or cDNA with a probe that anneals to the gene of interest. Once the library is made, colonies with possibly the right gene need to be screened, which takes time and is costly. When using this method, it is important to know when the gene of interest is expressed and in which tissue, so the correct tissue is harvested at the correct time. As this information was unavailable this method was not used in the current study.

Once part of a gene of interest is identified, there are several ways to 'walk' the genome and identify 5' and 3' regions of this particular gene. RACE (random amplification of cDNA) is possibly the easiest for both upstream and downstream regions. 3'RACE uses the polyA-tail as a starting point for the primer to anneal. The 5' region does not contain this sequence and a small sequence of the same oligo is added to the 5'end for 5'RACE (Sambrook and Russel, 2006). This sequence can then be used as an annealing point for a primer. TAIL-PCR (Thermal asymmetric interlaced – PCR) is another technique to identify 5' and 3' regions of a gene of interest (Liu and Whittier, 1995). The advantage of this technique is that genomic DNA can be used as template and therefore introns and promoter regions can be identified. This is an easy and cost effective method, like RACE. It is also possible to digest DNA with a restriction enzyme and then amplify an upstream or downstream product with a specific primer and a primer that anneals to the restriction site. To avoid unspecific priming a vectorette (Arnold and Hodgson, 1991) or splinkerette (Devon *et al.,* 1995) can be used. This technique is very similar to TAIL-PCR; however a digest of the DNA is required. In this study RACE and TAIL-PCR were combined to identify the full sequence of the gene of interest, including the polyA-tail, an intron and a promoter region.

Different techniques have been developed to monitor gene expression. Real Time – PCR is currently the most utilized method. Apart from the cost and the preparation required for optimisation of the reaction, this is an easy, accurate and effective method. Real Time – PCR is a quantitative method; copies of DNA are immediately measured after each cycle, usually by a fluorescent dye. Semi-quantitative RT PCR measures the copies of DNA by measuring UV intensity once the PCR product is separated on an agarose gel and stained with ethidium bromide. Although this method is less accurate, it is more cost effective. Other possible techniques are Northern blotting, which requires radioactive labelling of the RNA once blotted on a membrane or Digoxigenin (DIG; Roche, Germany) labelling. As Northern blots are non-amplifying, low abundant mRNA is difficult to visualise. Therefore semi-quantitative RT PCR was used for this study.

Chapter 2. Materials and Methods

2.1 Flowering response of *Xerophyta humilis* to different environmental conditions

Xerophyta humilis plants were collected from Pilanesberg National Park and Baraklo National Park, South Africa. The plants were maintained in a glasshouse at University of Cape Town under ambient conditions as described in Dace *et al.*, 1998. The effects of different environmental factors namely photoperiod, hydration status and duration of desiccation, light intensity and temperature on flowering of *X. humulis* were analysed as outlined below.

Hydration status and duration of desiccation

It had been reported that *X. humulis* plants that had been maintained in a desiccated state, at a relative water content (RWC) of >10 %, for extended periods of more than six to eight weeks flowered upon rehydration (Jill Farrant, Department of Molecular and Cell Biology, University of Cape Town, personal communication). In order to systematically investigate this phenomenon, *X. humilis* were acclimatised for eight weeks in a phytotron. The phytotron that was used is a custom made facility, which temperature can be controlled and is equipped with 250 W incandescent light bulbs and metal halide canopies (SunMaster Cool Deluxe), providing light intensities in the range of 1000 µmol m^2s^{-1}. The following conditions were set: a 14 hour photoperiod with an average light intensity of 380 µmol m^2s^{-1} (incandescent light for one hour, both incandescent and high pressure halide lamps for 12 hours, incandescent light for one hour), and a daytime temperature of 27 °C and 20 °C during the night period. Drying was initiated by withholding water. The water content was measured at full turgour and the relative water content (RWC) was calculated according to the following equations, where AWC is the Absolute water content:

$$AWC = \frac{\text{wet mass} - \text{dry mass}}{\text{dry mass}} \qquad \%RWC = \frac{\text{AWC}}{\text{AWC @ full turgor}} \text{ X } 100\%$$

To measure the AWC plants were watered after dark and samples from non vascular mesophyll tissue were collected 13 hours later in the dark, before photosynthesis had started. Samples were placed in Eppendorf tubes in an airtight bag and weighed on a balance. The samples were then dried over silica gel at 70 °C for 48 hours. After drying, samples were cooled in a desiccator for approximately 10 minutes before weighing. Samples for RWC were collected in the light.

When the plants reached an RWC <10 %, the plants were kept dry for a period of three to nine weeks in the conditions mentioned above. After three, four, five, six, seven and eight weeks plants were rehydrated by resuming soil watering under the same environmental conditions and the flowering response was monitored. Wet controls were watered every other day, or more frequently as required, for the whole duration of the experiment.

Photoperiod, light intensity, temperature and hydration status

It was observed that *X. humulis* plants moved in winter from the glasshouse to the warm conditions of the Phytotron tended to flower. In order to systematically test the effects of changing temperature, photoperiod and light intensity, the following set of experiments (summarised in Tables 2.1 and 2.2) were carried out.

X. humilis plants were acclimatised for one week in a 12 hr light: 12 hr dark (12:12) photoperiod at an average light intensity of 70 µmol m^{2}s^{-1} at a temperature between 26 and 27 °C. All experiments were carried out with cool white fluorescent bulbs. Plants (wet controls) were transferred to an incubator at 12 °C, which had an average light intensity between 25-30 µmol m^{2}s^{-1} and a 12:12 photoperiod for two and six weeks, or kept in a 12:12 photoperiod at an average light intensity of 70 µmol m^{2}s^{-1} for two or six weeks at 26-27 27 °C. Plants were then transferred to a photoperiod of 16 hours light and 8 hours dark (long-days; LD) or 8 hours light and 16 hours dark (short-days; SD) with a light intensity between 25-30 µmol m^{2}s^{-1} at 26-27 °C. To include rehydration stress in the experiment, water was withheld from some plants. When they reached a RWC of <10 % the plants were transferred to the cold incubator. After two, four or six weeks these plants were rehydrated in either LD or SD conditions. Temperature controls were kept at a light intensity of 70 µmol m^{2}s^{-1} at 26-27 °C, dehydrated as before and moved into LD and SD after two, four and six weeks.

Table 2.1. Experimental conditions employed in order to test the flowering response of *X. humilis* to day length, temperature and hydration status. 1212 - 12 hr light and 12 hr dark; SD - 8 hr light and 16 hr dark; LD -16 hr light and 8 hour dark. Low light in a 12:12 photoperiod was 70 µmol m^{2}s^{-1} at 26-27 °C and 25 µmol m^{2}s^{-1} at 12 °C. SD and LD low light was 25-30 µmol m^{2}s^{-1}.

12:12 photoperiod, low light, 26-27 °C		12:12 photoperiod, low light		SD/LD photoperiod, low light, 26-27 °C
Wet plants (controls)	➔	• 26 °C 2 and 6 weeks • 12 °C 2 and 6 weeks	➔	SD or LD (dry plants rehydrated)
Dry plants (RWC < 10 %)		• 26 °C 2,4,6 weeks • 12 °C 2,4,6 weeks		

The effect of light intensity was also tested in similar experiments. It was noticed plants were in an unhealthy state at 12 °C, therefore the cold incubator was set at 14 °C. *X. humilis* were acclimatised for one week in a 12:12 hour photoperiod at an average light intensity of 70 µmol m^2s^{-1} at 26-27 °C. Light sources were all cool white fluorescent. Plants were desiccated in a LD photoperiod at an average light intensity of 30 µmol m^2s^{-1} at 26-27 °C. When the plants reached a RWC <10 %, they were moved into an incubator at 14 °C (cold), which had an average light intensity of 25-30 µmol m^2s^{-1} (low light) with a LD photoperiod. After three weeks in the cool incubator the plants were rehydrated in a LD photoperiod at an average light intensity of 400 µmol m^2s^{-1} (high light) at 26-27 °C (warm). Temperature controls were kept at low light conditions at 26-27 °C after drying down for three weeks and moved into the same warm high light conditions. Wet controls were transferred from the acclimatising conditions (see above) into warm or cold low light conditions and were then transferred after three weeks into warm high light conditions (see Table 2.2).

Table 2.2. Experimental conditions employed in order to test the flowering response of *X. humulis* to light intensity and day length, temperature and hydration status. 12:12 - 12 hr light and 12 hr dark with low light = 70 µmol m^2s^{-1}; LD - 16 hr light and 8 hour; SD - 8 hr light and 16 hr dark; low light = 25-30 µmol m^2s^{-1}, high light = 400 µmol m^2s^{-1}.

12:12 photoperiod		Condition 1		Condition 2
Low light 26 °C	➔	LD low light 14 °C wet LD low light 26-27 °C wet LD low light 14 °C dry LD low light 26-27 °C dry	➔	LD Photoperiod (high light) 26-27 °C
		SD low light 14 °C wet SD low light 26-27 °C wet SD low light 14 °C dry SD low light 26-27 °C dry		

The above experiment was repeated replacing LD in condition one (see Table 2.2) with SD. Plants (wet controls) were transferred from the cool incubator with a SD photoperiod into high light warm conditions with a LD photoperiod with the light intensities as above. Temperature controls were kept at a SD photoperiod at low light conditions at 26-27 °C and then moved in high light conditions (condition 2 Table 2.2). For the short day experiment, plants were also dehydrated as described above and rehydrated after three weeks in the high light LD conditions.

In this experiment dehydration occurred at warm temperatures (26-27 °C) before moving the plants into the cool incubator. In order to investigate if dehydration at cold temperatures would influence initiation of flowering the following experiment was done: plants were acclimatised for one week in a 12: 12 hr photoperiod at an average light intensity of 70 µmol m^2s^{-1} (cool white fluorescent) at a temperature between 26 and 27 °C. Plants were then transferred into the cool incubator at 14 °C, which had an average light intensity of 25-30 µmol m^2s^{-1} with a SD photoperiod. Drying was initiated by withholding water. Three weeks after plants reached a RWC <10%, they were transferred into the high light conditions mentioned above.

2.2. Molecular genetics of flowering

DNA extraction

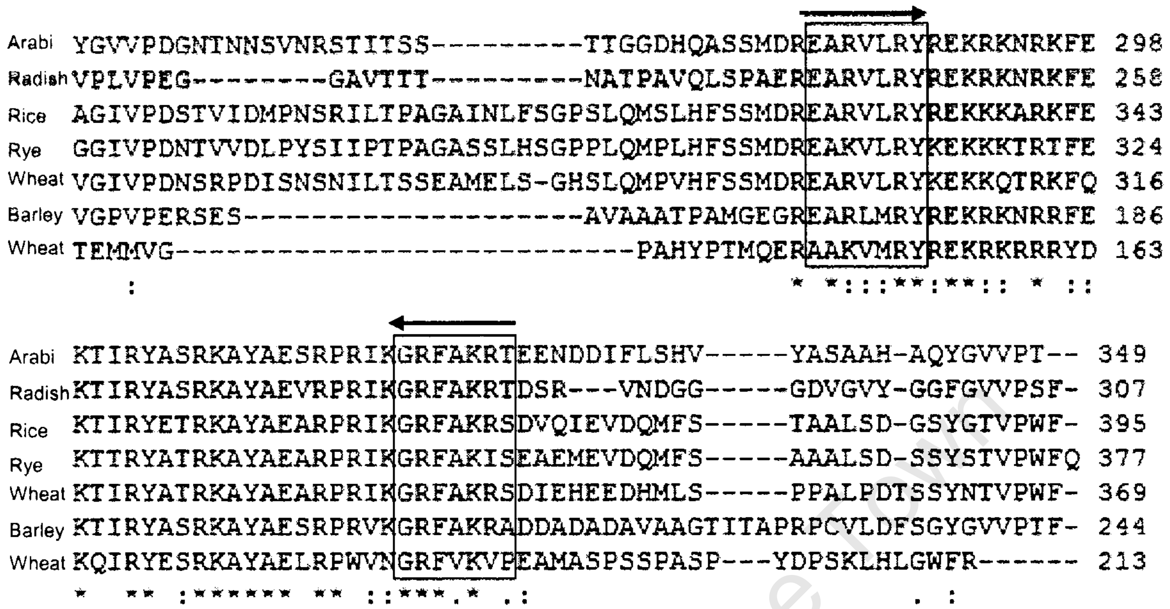
DNA extraction was based on the method of Dellaporta *et al.* (1983) with the following alterations: 1 g *X. humilis* leaf tissue was ground in liquid nitrogen and the fine powder was added to 15 ml extraction buffer (0.1 M Tris, 0.05 M EDTA, 0.5 M NaCl, 10 mM β-mercaptoethanol, 1 % (w/v) Polyvinylpyrrolidone (PVP) in a 50 ml Beckman tube (Catalogue number 357003). 2 ml 10 % (w/v) Sodium dodecyl sulphate (SDS) was added, mixed thoroughly and incubated at 37 °C for 5 minutes. 5 ml 5 M potassium acetate was added, vortexed and incubated on ice for 20 minutes. The sample was centrifuged for 20 minutes at 4 °C at 6500 *g* (9000 rpm) using a JA-20 rotor in a Beckman centrifuge. The supernatant was filtered through cheese cloth into a clean Beckman tube containing 10 ml Isopropanol and placed at -20 °C for 30 minutes. The new tube was centrifuged for 20 minutes as before. The pellet was dissolved in 700 µl 0.05 M Tris/0.01M EDTA and incubated overnight at -20 °C. The DNA solution was transferred to a 1.5 ml Eppendorf tube and 0.4 µl of 100 mg ml^{-1} RNaseA was added and incubated at 37 °C for 30 minutes. Insoluble material was removed by centrifuging for 10 minutes at 15000 *g* and discarding the pellet. 500 µl Isopropanol, 75 µl 3 M sodium acetate was added to the supernatant and this was centrifuged for three minutes at 15000 *g*. The pellet was washed with 500 µl 80% Ethanol, centrifuged for three minutes at 15000 *g*, dried at 37 °C for 10 minutes and dissolved in 100 µl sterile water.

RNA extraction

RNA was extracted according to Azevedo *et al.* (2003) from *X. humilis* with optimisations by Mariette Smart (Department of Molecular and Cell Biology, University of Cape Town; personal communication). Extraction buffer (100 mM Tris pH 8.0, 2 % (w/v) cetyltrimethyl ammonium bromide (CTAB), 30 mM EDTA, 2 M NaCl, 2 % (w/v) polyvinylpolypyrrolidone (PVPP), 0.05 % (w/v) Spermidine, 2 % (v/v) β-mercaptoethanol, 0.3 mg ml^{-1} Proteinase K) was pre-heated in a 1.5 ml Eppendorf tube at 42 °C for 10 minutes. Two to three leaves of *X. humilis* were ground in liquid nitrogen to a fine powder and this was added to 1 ml extraction buffer and vortexed thoroughly. The sample was incubated for 90 minutes, with vortexing two to three times during the incubation. The sample was then centrifuged for 15 minutes at approximately 13000 *g* at 4 °C and the supernatant was transferred to a clean Eppendorf tube. An equal volume of 24:1 chloroform: isoamyl alcohol was added to the supernatant and the mixture was vortexed thoroughly. This was centrifuged for 15 minutes at approximately 13000 *g* at 4 °C. The chloroform: isoamyl alcohol extraction was repeated and a quarter volume of 10 M LiCl was added to the supernatant. The RNA was precipitated at -20 °C overnight. After 25 minutes centrifuging at 13000 *g* at 4 °C the pellet was washed by vortexing in 250 µl of 70 % (v/v) ethanol. The pellet was centrifuged for 25 minutes at 13000 *g* at 4 °C and dried at 37 °C in a heating block for approximately 10 minutes. The pellet was dissolved in 30 µl water. RNA quality was analysed by gel electrophoresis and the quantity was estimated by measuring the absorbance at 260 nm using a ND-1000 NanoDrop® spectrophotometer (NanoDrop Technologies, Wilmington USA)

Gene identification

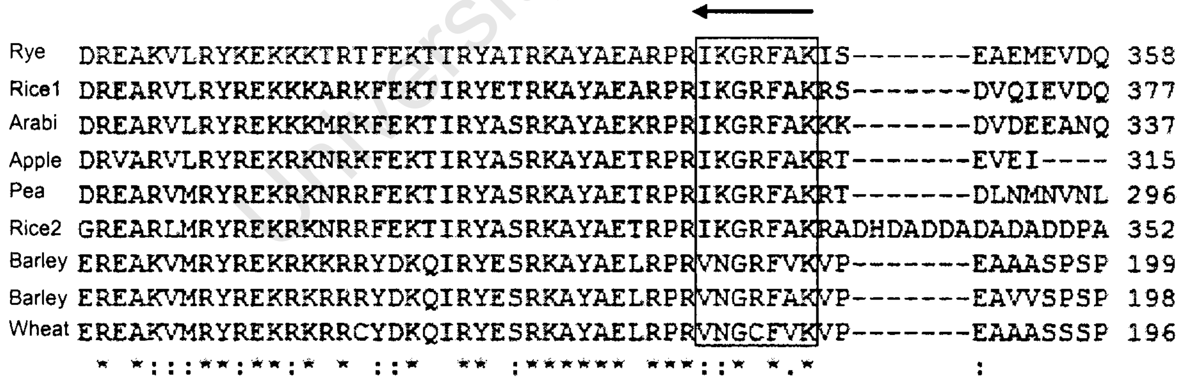
Primers were designed to amplify a homologue of *VRN2* (also called *ZCCT1*) from wheat (accession number AAS5848), which is a dominant repressor of flowering that is down regulated by vernalisation (Yan *et al.*, 2004). ZCCT1 contains a CCT (CO, CO-like, TIMING OF CAB EXPRESSION 1) domain (Strayer *et al.,* 2000), which has similarities with CO and CO-like proteins of Arabidopsis and rice. Primers were designed to this conserved CCT domain (Figure 2.1).



```
Arabi  YGVVPDGNTNNSVNRSTITSS---------TTGGDHQASSMDREARVLRYREKRKNRKFE 298
Radish VPLVPEG--------GAVTTT---------NATPAVQLSPAEREARVLRYREKRKNRKFE 258
Rice   AGIVPDSTVIDMPNSRILTPAGAINLFSGPSLQMSLHFSSMDREARVLRYREKKKARKFE 343
Rye    GGIVPDNTVVDLPYSIIPTPAGASSLHSGPPLQMPLHFSSMDREAKVLRYKEKKKTRTFE 324
Wheat  VGIVPDNSRPDISNSNILTSSEAMELS-GHSLQMPVHFSSMDREARVLRYKEKKQTRKFQ 316
Barley VGPVPERSES--------------------AVAAATPAMGEGREARLMRYREKRKNRRFE 186
Wheat  TEMMVG---------------------------PAHYPTMQERAAKVMRYREKRKRRRYD 163
          :                                      * *:::**:**:: * ::

Arabi  KTIRYASRKAYAESRPRIKGRFAKRTEENDDIFLSHV-----YASAAH-AQYGVVPT-- 349
Radish KTIRYASRKAYAEVRPRIKGRFAKRTDSR---VNDGG-----GDVGVY-GGFGVVPSF- 307
Rice   KTIRYETRKAYAEARPRIKGRFAKRSDVQIEVDQMFS-----TAALSD-GSYGTVPWF- 395
Rye    KTTRYATRKAYAEARPRIKGRFAKISEAEMEVDQMFS-----AAALSD-SSYSTVPWFQ 377
Wheat  KTIRYATRKAYAEARPRIKGRFAKRSDIEHEEDHMLS-----PPALPDTSSYNTVPWF- 369
Barley KTIRYASRKAYAESRPRVKGRFAKRADDADADAVAAGTITAPRPCVLDFSGYGVVPIF- 244
Wheat  KQIRYESRKAYAELRPWVNGRFVKVPEAMASPSSPASP---YDPSKLHLGWFR------ 213
       *  ** :****** ** ::***.* .:                      . :
```

Figure 2.1. Alignment of CCT domains of CO-like and ZCCT proteins. Arabi: *Arabidopsis thaliana* CO-like, Radish: *Raphanus sativus* CO-like, Rice: *Oryza sativa* Hd1, Rye: *Lolium perenne* CO-like, Wheat: *Triticum aestivum* Hd1-like, Barley: *Hordeum vulgare* CO-like, Wheat: *Triticum monococcum* ZCCT1. The forward arrow indicates the forward primer and the reverse arrow the reverse primer. Proteins 3 and 7 were reverse translated into nucleotides and degenerate primers were designed according to the nucleotide sequences.



```
Rye    DREAKVLRYKEKKKTRTFEKTTRYATRKAYAEARPRIKGRFAKIS-------EAEMEVDQ 358
Rice1  DREARVLRYREKKKARKFEKTIRYETRKAYAEARPRIKGRFAKRS-------DVQIEVDQ 377
Arabi  DREARVLRYREKKKMRKFEKTIRYASRKAYAEKRPRIKGRFAKKK-------DVDEEANQ 337
Apple  DRVARVLRYREKRKNRKFEKTIRYASRKAYAETRPRIKGRFAKRT-------EVEI---- 315
Pea    DREARVMRYREKRKNRRFEKTIRYASRKAYAETRPRIKGRFAKRT-------DLNMNVNL 296
Rice2  GREARLMRYREKRKNRRFEKTIRYASRKAYAETRPRIKGRFAKRADHDADDADADADDPA 352
Barley EREAKVMRYREKRKKRRYDKQIRYESRKAYAELRPRVNGRFVKVP-------EAAASPSP 199
Barley EREAKVMRYREKRKRRRYDKQIRYESRKAYAELRPRVNGRFAKVP-------EAVVSPSP 198
Wheat  EREAKVMRYREKRKRRCYDKQIRYESRKAYAELRPRVNGCFVKVP-------EAAASSSP 196
        * *:::**:**:* * ::*  ** :****** ***::* *.*         :
```

Figure 2.2. Alignment of CCT domains of ZCCT and CO-like proteins. Only part of the CCT domain is shown. Rye: *Lolium perenne* CO-like, Rice1: *Oryza sativa* Hd1, Arabi: *Arabidopsis thaliana* CO, Apple: *Malus domestica* CO-like, Pea: *Pisum sativa* CO-like, Rice2: *Oryza sativa* CO-like, Barley: *Hordeum vulgare* ZCCT-Hb, Barley: *Hordeum vulgare* ZCCT-Ha, Wheat: *Triticum monocuccum* ZCCT1. The reverse arrow indicates the reverse primer. Proteins 2 and 9 were reverse translated into nucleotides and a degenerate primer was designed according to the nucleotide sequence.

To confirm the sequence, a second reverse primer was designed, in order to carry out nested PCR (Figure 2.2). All oligonucleotides were synthesised by Synthetic DNA Laboratory, University of Cape Town, South Africa.

PCR amplification of CCT-like sequences from *X. humulis* cDNA was carried out using VRNF 5'GAGGC(C/G)A(A/G)GGTG(C/A)T(G/C)AGGTA3' as a forward and VRNR 5'G(G/A)T(C/A)(C/T)CTTG(A/G)C(AG)AA(A/G)CGGCC3' as a reverse primer. The PCR reaction contained 3 µl 3-fold diluted cDNA, 0.3 mM dNTPs, 1.5 mM $MgCl_2$, 0.8 µM VRNF, 0.8 µM VRNR, 5 µl 5 x GoTaq® Flexi buffer (Promega, Madison WI), 0.75 units GoTaq® polymerase (Promega, Madison WI) in a total volume of 25 µl. PCR cycling of 95 °C 5 minutes one cycle, 35 cycles of 95 °C 30 seconds, 55 °C 30 seconds, 72 °C 30 seconds, hold at 72 °C for 5 minutes was used with the Geneamp® PCR system 2700 (Applied Biosystems). This PCR was repeated with the primers VRNF and VRNref 5' CCT G(G/A)C(G/A)AA(G/A)C(G/A)GGC(A/C)TTGA3' under the same PCR conditions. PCR reactions were analysed by electrophoresis through 1 % (w/v) agarose gels in 1X TAE (0.04M Tris Acetate 0.001M EDTA). Both primer sets amplified a product of approximately 300 bp, which was gel purified with QIAGEN gel extraction kit (QIAGEN, GmbH, Germany) according to the manufacturer's instructions. The purified PCR product was ligated into the pGEMTeasy vector (Promega, Madison, WI), which was used to transform chemically competent DH5α *Escherichia coli* cells. 5 µl of the ligation reaction was added to an aliquot of 200 µl $CaCl_2$ competent *E. coli* cells on ice. After 30 minutes on ice the cells were heat shocked at 42 °C for 90 seconds. The cells were placed on ice for 2 minutes and 800 µl of room temperature Luria Bertani (LB) broth was added. The cells were incubated at 37 °C with agitating for one hour. Cells were plated on LB agar plates containing 60 µg ml^{-1} ampicillin. Colonies were analysed for plasmid with insert by digesting the plasmid with *Eco*RI (Roche, Basel, Switzerland). The correct plasmids were extracted and purified using a Peqlab I miniprep kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) and sequenced using Big Dye terminator technology (Macrogen Sequencing, Seoul, Korea).

Rapid amplification of cDNA ends (RACE)

The 3' end of a potential VRN2 homologue was isolated according to Protocol 8.10: Amplification of 3' cDNA (3'RACE) (Sambrook and Russel, 2006) with some modifications. Two reactions were set up, with the following primers: VRNF (see above section 'gene identification') and $(dT)_{17}$ Adapter-primer 5' GACTCGAGTCGACATCGA$(T)_{17}$ (Sambrook and Russel, 2006) for the first reaction and 3'RACE 5'CAAGGATCAAGGGAAGGTTCGCA3' and $(dT)_{17}$ for the second reaction. The PCR reaction contained 2 µl 2-fold diluted cDNA, 0.4 mM dNTPs, 2 mM $MgCl_2$, 0.64 µM VRNF or 3'RACE, 0.64 µM $(dT)_{17}$-Adapter or 0.64 µM $(dT)_{17}$, 10 µl 5 x GoTaq® Flexi buffer (Promega, Madison WI), 1.5 units GoTaq® polymerase (Promega, Madison WI) in a total volume of 50 µl. The PCR conditions are given in Table 2.3.

Table 2.3 Cycle conditions for 3'RACE using the Geneamp® PCR system 2700 (Applied Biosystems).

VRNF and $(dT)_{17}$Adapter-Primer		3'RACE and $(dT)_{17}$	
Number of Cycles	PCR conditions	Number of Cycles	PCR conditions
1	94 °C 5 minutes 55 °C 2 minutes 72 °C 10 minutes	1	94 °C 5 minutes 56 °C 2 minutes 72 °C 10 minutes
35	94 °C 40 seconds	35	94 °C 40 seconds

	55 °C 45 seconds 72 °C 1.5 minutes		56 °C 1.5 minutes 72 °C 1.5 minutes
1	94 °C 40 seconds 55 °C 1 minute 72 °C 15 minutes	1	94 °C 40 seconds 56 °C 1 minute 72 °C 10 minutes

PCR reactions were analysed by electrophoresis through 1 % (w/v) agarose gels in 1X TAE and products of 500 bp (VRNF/(dT)$_{17}$Adapter-Primer) and 400 bp (3'RACE/(dT)$_{17}$) were excised, eluted form the gel using Peqlab I miniprep kit (Biotechnologie GmbH Erlangen, Germany) and cloned into the pGEMTeasy vector (Promega, Madison, WI) as described before (see section 'gene identification') and sequenced. Six and eight colonies respectively from each reaction were sequenced.

Table 2.4 Cycle conditions for 5'RACE using the Geneamp® PCR system 2700 (Applied Biosystems).

Number of Cycles	PCR conditions
5	94 °C 30 seconds 72 °C 3minutes
5	94 °C 30 seconds 70 °C 30 seconds 72 °C 3 minutes
25	94 °C 40 seconds 68 °C 30 seconds 72 °C 3 minutes

The 5' end of the potential *VRN2* homologue was isolated with the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, USA). The BD SMART™ RACE user manual was followed for cDNA synthesis and RACE. The BD Advantage™ PCR kit was used for the PCR reaction. The PCR reaction contained 0.4 µM Gene specific primer 2 (GSP2) 5'GCGATAACCGTGCAGCAGCGTCGGCAGGT3', 2.5 µl 11-fold diluted cDNA, 0.2 mM dNTPs, 5 µl 10X UPM (Universal Primer Mix), 5 µl 10X BD Advantage 2 PCR Buffer, 1 µl 50X BD Advantage 2 Polymerase Mix in a total volume of 50 µl. The PCR conditions are given in Table 2.4.

PCR reactions were analysed by electrophoresis through 1 % (w/v) agarose gels in 1X TAE and a 1.2 kb PCR product was excised and gel eluted using Peqlab I miniprep kit (Biotechnologie GmbH Erlangen, Germany) and cloned into the pGEMTeasy vector (Promega, Madison, WI) as described previously (see section 'gene identification'). Six different colonies were sequenced. The sequence revealed a CONSTANS-like sequence, containing two B-boxes (an N-terminal zinc-finger region) and a CCT domain.

TAIL (Thermal Asymmetric Interlaced) - PCR

The promoter region and the intron of the now potential *CO* homologue were isolated combining the protocols of Liu and Whittier, 1995 and Michiels *et al.* 2003. Four arbitrary degenerate (AD) primers were used (Liu and Whittier, 1995) and three gene specific primers, GSP2 (sequence see section '5'RACE'), GSPrev 5'GCGAACCCTCCCTTGATC3' and NesRev 5'TTTCGGCGTAAGCTTTGC3'. The primary PCR reaction contained 100 ng genomic DNA, an AD primer (4.8 µM AD1or AD2, 2.4 µM AD3 or AD4), 0.4 µM GSP2, 0.3 mM dNTPs, 2 mM $MgCl_2$, 5 µl 5 x GoTaq® Flexi buffer, 0.75 units GoTaq® polymerase in a total volume of 25 µl. The secondary PCR reaction contained 2 µl 20-fold dilution of the primary reaction, the same AD primer as used in the primary reaction (3 µM AD1 or AD2, 1.6 µM AD3 or AD4), 0.4 µM GSPrev, 0.3 mM dNTPs, 2 mM $MgCl_2$, 5 µl 5 x GoTaq® Flexi buffer (Promega, Madison WI), 0.75 units GoTaq® polymerase (Promega, Madison WI) in a total volume of 25 µl. The tertiary PCR reaction contained 2 µl 20-fold dilution of the secondary reaction, an AD primer (0.8 µM, the same as used in previous cycles), 0.4 µM NesRev, 0.3 mM dNTPs, 2 mM $MgCl_2$, 5 µl 5 x GoTaq® Flexi buffer(Promega, Madison WI), 0.75 units GoTaq® polymerase (Promega, Madison WI) in a total volume of 25 µl. The PCR conditions are given in Table 2.5.

Table 2.5. PCR conditions for TAIL-PCR

Reaction	Number of cycles	PCR Conditions
Primary	1	93 °C 1 min, 95 °C 1min
	5	94 °C 30 s, 62 °C, 72 °C 2.5 min
	1	94 °C 30 s, 25 °C ramping to 72 °C over 3 min, 72 °C 2.5 min
	15	94 °C 20 s, 68 °C 3.5 min, 94 °C 20 s, 68 °C 3.5 min, 94°C 30 s, 44 °C 1 min, 72 °C 2.5 min
	1	72 °C 5 min
Secondary	12	94 °C 20 s, 56 °C 1 min, 72 °C 2 min, 94 °C 20 s, 56 °C 1 min, 72 °C 2 min, 94 °C 20 s, 44 °C 1 min, 72 °C 2 min
	1	72 °C 5 min
Tertiary	30	94 °C 30 s, 44 °C 30 s, 72 °C 2.5 min
	1	72 °C 5 min

PCR reactions were analysed by electrophoresis through 1 % (w/v) agarose gels in 1X TAE and a 600 and an 800 bp tertiary PCR product were excised from the gel, eluted using Peqlab I miniprep kit (Biotechnologie GmbH Erlangen, Germany) and cloned into the pGEMTeasy vector

(Promega, Madison WI) as described previously (see section 'gene identification'). Four colonies were selected for sequencing. All the sequencing results together suggests a *CO/CO*-like homologue in *X. humilis* was found and from now this sequence will be referred to as *XhCO* (*Xerophyta humilis CONSTANS*).

XhCO **gene expression analysis**

Sample collection

The expression profiles of *XhCO* under the different conditions used in the flowering experiments were analysed. The environmental conditions are summarised in Table 2.2. Samples were collected from plants in environmental condition one after 20 to 21 days. Plants were then moved to environmental condition 2 and after 9 to 10 days samples were collected from the same plants (see Table 2.2).

X. humilis leaf samples were collected every four hours over a 24 hour period, starting at 10am and ending at 10 am the following day. Samples taken during the dark phase were harvested using a green safe light (LEE filter 090 Dark yellow Green; LEE filters, Hampshire England). Dead or brown tips of the leaves were cut off and healthy leaves were taken when possible. Samples were flash frozen in liquid nitrogen and stored at -80 ˚C until RNA was extracted.

Samples were also collected from plants dehydrated in the cool incubator in a SD photoperiod and rehydrated in high light warm conditions (condition 2, Table 2.2). Samples were collected in the high light conditions only, ten days after being transferred into condition 2.

The influence of light was studied by transferring *X. humilis* plants at dusk after five days from a 12:12 hr photoperiod with a light intensity of 70 µmol m^2s^-1 at 26-27 °C into constant light (with a light intensity of 70 µmol m^2s^-1 at 26-27 °C) or constant dark. Samples were collected in constant light and constant dark, using a green safe light in constant dark, every four hours over a 28 hour period starting at 10 am (2 hours after previous dusk) and ending at 2 pm the following day.

RNA of the individual samples was extracted according to the method above and cDNA of each RNA sample was synthesised with ImpromtII RT Kit (Promega, Madison WI) according to the manufacturer's instructions. cDNA was synthesised with oligo $d(T)_{17}$ from the kit, using 1 µg of total RNA.

Semi-quantitive RT-PCR analysis of *XhCO*

The expression of *XhCO* was analysed under different environmental conditions (see Table 2.2) with semi-quantitative RT-PCR. cDNA of the samples collected (see section 'sample collection' above) was used as template for the PCR reaction. *XhCO* was amplified with the intron spanning primers to allow discrimination between transcript and genomic copies: RTF 5'GTGGCTGACAGCGTCGTTCC3' and RTR 5'GTCGACCTCGGCGTCGACCT3'. The PCR product was normalised against 18S RNA. The primers for 18S RNA, Xv18SF 5'CAGGCGCGCAAATTACCCAATCC3' and Xv18SR 5'CCTACCGTCCCGTCCCAAGGTC3' were designed by Revel Iyer, (Department of Molecular and Cell Biology, University of Cape Town).

The PCR reaction contained 2.5 µl three-fold diluted cDNA, 0.33 µM Forward primer (RTF or Xv18SF), 0.33 µM Reverse primer (RTR or Xv18SR), 0.27 mM dNTPs, 1.5 mM $MgCl_2$, 6 µl 5 x GoTaq® Flexi buffer (Promega, Madison WI), 1 unit GoTaq® polymerase (Promega, Madison WI) in a total volume of 30 µl. A PCR cycle of 95°C 3 minutes, 24 cycles of 95 °C 30 seconds, 59 °C 45 seconds, 72 °C 45 seconds, hold at 72 °C for 5 minutes was used with the Geneamp® PCR system 2700 (Applied Biosystems). The PCR product was electrophoresed through a 1.5% (w/v) agarose gel and stained in 500 µl/ 500ml ethidium bromide for 20 minutes. The gel was de-stained in distilled water for 10 minutes and visualised under UV light using a Gel Doc XR™ (Biorad, Hercules, USA). The intensity of the ethidium bromide staining was measured with Quantity One 4.5.2 software. (Biorad, Hercules, USA). Relative levels of XhCO to 18s transcripts were plotted against time.

Chapter 3. Results

3.1 Flowering response of *Xerophyta humilis* to different environmental conditions

Hydration status and duration of desiccation

In the natural environment, *X. humilis* grows in habitats where they experience cold, dry winters and warm wet summers, during which they produce flowers. However, it had been reported that *X. humulis* plants that had been maintained in a desiccated state, at a relative water content (RWC) of >10 %, for extended periods of more than six to eight weeks flowered upon rehydration (Jill Farrant, Department of Molecular and Cell Biology, University of Cape Town, personal communication). Therefore the effect of the duration of desiccation on flowering of *X. humilis* plants was investigated in further detail. Observation of flowering of *X. humilis* during rehydration, after different dehydration treatments was carried out as detailed in section 'hydration status and duration of desiccation' in chapter two. Plants were dehydrated and rehydrated under LD at 27 °C, day temperature and 20 °C night temperature. Rehydrated plants were monitored for a period of six weeks following resumption of watering, and the wet controls were monitored for the entire duration of the experiment.

Flowers were observed after three weeks in control 1 and six weeks in control 2 (Table 3.1). Flowers were also observed in trays from which the plants had been desiccated for five and six weeks. As the size of tray and thus number of plants varied, the number of per area (flowers dm^{-2}) is also given. Although flowering was initiated after five and six weeks of dehydration, flowers only appeared three and six weeks respectively after rehydration. Flowering was also observed in the wet controls after three and six weeks. Possibly the plants that were used for the experiment were suffering from other stress factors, which initiated flowering, such as the environment of the phytotron. We concluded flowering was not initiated in response to desiccation only and that more environmental cues needed to be investigated.

Table 3.1. Number of flowers observed following different desiccation and rehydration treatments. Plants were desiccated for three, four, five, six, seven or eight weeks and then rehydrated.

Plant condition	Number of flowers	Flowers dm^{-2}
Wet Control 1	2	0.15
Wet Control 2	3	0.22
Desiccated 3 w	0	0
Desiccated 4 w	0	0
Desiccated 5 w	3	0.36
Desiccated 6 w	2	0.25
Desiccated 7 w	0	0
Desiccated 8 w	0	0

Photoperiod, light intensity, temperature and hydration status

Some plants, such as certain ecotypes of *Arabidopsis thaliana* and winter wheats (*Triticum monocuccum*) require exposure to cold in order to flower (for review see Henderson and Dean, 2004 and Trevaskis *et al.* 2007). This requirement of cold exposure is called vernalisation. It is possible that *X. humilis* also needs vernalisation to initiate flowering. When it was observed that *X. humulis* plants tended to flower when they were moved from the winter conditions in the glasshouse to warm conditions in the phytotron, the influence of exposure to extended periods of cold temperature was included in the environmental experiments. The conditions of photoperiod and temperature investigated are summarised in Table 2.1. No flowers were observed under these conditions, which suggest another environmental cue is necessary to initiate flowering in *X. humulis*. As these experiments were all carried out at relatively low light intensity and light quantity can affect flowering (Thomas, 2006) the effect of light intensity was tested in a similar experiment (outlined in Table 2.2).

Of the treatments summarised in Table 2.2, only plants that were transferred from LD wet at 14 °C into to high light conditions at 27 °C caused flowers to appear after approximately 10 days in the high light conditions. Desiccated plants that were rehydrated upon transfer from LD 27°C and SD 14°C into the high light conditions also flowered, but this was only after three weeks in the high light conditions. It is possible the plants flowered because of stress caused by the high light. Tray 1B did not flower as expected, possibly because the plants in this tray had just flowered a few months prior to this experiment (see Figure 3.1).

Table 3.2. Number of flowers formed after different environmental treatments in low light (25 µmol m^{-2} s^{-1} at 14 °C or 30 µmol m^{-2} s^{-1} at 22 °C) followed by transfer to long days of high light (400 µmol m^{-2} s^{-1}) at 27 °C. SD: 8 hr light and 16 hr dark; LD: 16 hr light and 8 hr dark.

Condition	Tray No.	No. flowers	No. Flowers dm^{-2}	Condition	Tray No.	No. Flowers	No. Flowers dm^{-2}
LD wet 14°C	1A	5	0.38	SD wet 14°C	5A	0	0
	1B	0	0		5B	0	0
LD wet 22°C	2A	0	0	SD wet 22°C	6A	0	0
	2B	0	0		6B	0	0
LD dry 14°C	3A	0	0	SD dry 14°C	7A	3	0.55
	3B	0	0		7B	0	0
LD dry 22°C	4A	0	0	SD dry 22°C	8A	0	0
	4B	3	0.95		8B	0	0

In order to confirm the floral response of *X. humilis* when transferred from LD low light at 14°C to LD high light 27°C, this part of the experiment was repeated with plants that had not previously been used in any other experiments. Also these plants were recently removed from their original environment. Three biological repeats were analysed, two of which flowered upon transfer to the high light conditions (Table 3.3).

Table 3.3. Confirmation of floral induction in *X. humulis* in response to LD cold low light transferred into LD warm high light treatment (see table 3.2, 1A and 1B).

condition	Tray No.	No. flowers	No. Flowers dm^{-2}
LD wet 14°C	7	0	0
	8	11	0.83
	9	3	0.4

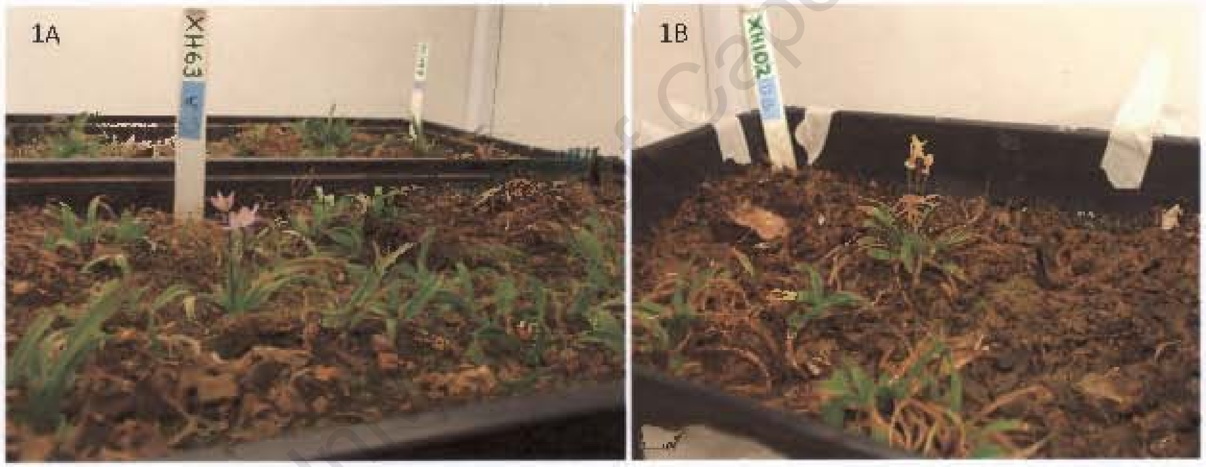


Figure 3.1. *X. humilis* under high light LD photoperiod at 27°C. Tray 1A shows three flowers; tray 1B shows three dry flowers from a previous experiment.

Both trays eight and nine produced flowers 9-12 days after rehydration in the high light condition. It is possible that tray seven did not flower as expected as the tray was much smaller than the others and therefore experienced slightly different soil rehydration conditions. That is, there was less soil to withhold water and it is possible that there was not sufficient water available for the plants to flower. Also there are fewer plants in the smaller tray and since not all plants in a tray flower (even in the large tray) (see Figure 3.1 and 3.2), it is possible that by chance none of the plants were ready to flower in the small tray.

Figure 3.2. *X. humilis* under high light LD photoperiod at 27°C. A total of 11 flowers were observed in tray 8 (left) and three flowers in tray 9 (right).

X. humilis flower in the warm wet season after a cold and dry winter season, it was therefore expected for desiccated plants that were transferred from SD at 14 °C into LD high light at 27 °C, to flower, as this simulates natural environmental conditions. However, dry plants exposed to cold, which mimics the natural winter conditions and transferred into warm high light conditions, which mimics summer conditions did not flower. It is likely that as these plants were transferred into the cold when already dry, the plants did not sense the cold. *X. humilis* is a poikilochlorophyllous plant, thus it loses chlorophyll during dehydration. This is a protection mechanism against oxidative stress, caused by dehydration (Sherwin and Farrant 1996; 1998). When the plants are desiccated it is no longer possible for photosynthesis to take place, which is possibly why plants cannot 'sense' the cold. Therefore an experiment was carried out where plants were dried down in the cool incubator (instead of drying down in warm conditions as was done in the previous experiment) with a SD photoperiod at 14 °C at a low light intensity 25 µmol m^2s^{-1}. Flowers appeared after 8-12 days after transferring into high light conditions in one of the three trays that were observed. This confirms that plants dried in the cold under SD, do indeed flower when transferred to long warm days of high light intensity. It is unclear why they other two trays did not flower, although it is possible the plants suffered when drying down in the cool incubator and did not fully recover when rehydrated.

3.2 Molecular genetics of flowering

Gene identification

The flowering pathway in *Arabidopsis* involves many different proteins. One of the key proteins involved is Flowering Locus C (FLC), which is a MADS-box transcriptional regulator. FLC represses flowering, which is down regulated by vernalisation (Sheldon *et al.*, 1999, Michaels and Amasino, 1999). Although other components of the photoperiodic pathway from *Arabidopsis* have been found in monocots, *FLC* genes have only been identified in dicots. *VRN1* and *VRN2* have been identified as vernalisation genes in cereals (Dubcovsky *et al.* 1998) and show strong epistatic interactions (Tranquilli and Dubcovsky, 2000). *VRN1* is dominant in spring wheat and promotes early flowering, whereas VRN2 is dominant in winter wheat (Tranquillli and Dubcovsky, 2000), represses flowering and is down regulated by vernalisation (Yan *et al.*, 2004). *TaVRN1 and TaVRN2* are different from the *AtVRN1* and *AtVRN2* genes of *Arabidopsis*. In fact *TaVRN1* is similar to *Arabidopsis APETALA1* (*AP1*) which is a floral meristem identity gene, and TaVRN2 has similarities to AtCONSTANS (CO) and CO-like proteins (Yan *et al.*, 2004) which are involved in photoperiodic flowering (Putterill *et al.*,1995). Two genes have been identified at the wheat *VRN2* locus, *TaZCCT1* and *TaZCCT2*. It was found that *ZCCT1*, which has a Zinc finger domain and CCT domain is *VRN2* (Yan *et al.*, 2004). Just as there is no *FLC* homologue in cereals, no *VRN2* homologue exists in *Arabdopsis*. This could indicate a different vernalisation pathway in monocots compared to the dicots.

Primers were designed to amplify a *VRN2* homologue in *X. humilis* (see Figure 2.1 and 2.2), which amplified in the conserved CCT domain. A 350 bp sequence was amplified, which was used to identify the 5' end and the 3' end. When the full sequence was identified using RACE-PCR and TAIL PCR, the DNA sequence from start till stop codon was translated into amino acids *in silico*. This revealed two B-boxes and a CCT domain (see Figure 3.3)

Figure 3.3 Conserved domains in *XhCO* sequence, translated *in silico* into amino acids (www.ncbi.nlm.nih.gov/blast). Red bar: CCT motif. This short motif is found in a number of plant proteins. It is rich in basic amino acids and has been called a CCT motif after CO, COL and TOC1. The CCT motif is about 45 amino acids long and contains a putative nuclear localisation signal within the second half of the CCT motif. *toc1* mutants have been identified in this region. Blue bars: B-boxes, which are zinc-finger regions at the N-terminal.

In animals B-boxes show protein-protein interaction and the CCT domain has a role in nuclear localization and protein-protein interaction (Robson *et al.*, 2001). Homology was shown with CO and CO-like proteins, mainly of plant species, which indicates that a CO homologue has been isolated (See table 3.4). Similarity is highest in the CCT domain.

Table 3.4. Similarities of XhCO amino acid sequence with database entries. E is the Expectation value. The lower the E value, the more significant the score (See BLAST information). Values have been listed from highest alignment score with XHCO to lowest score with XhCO.

Accession No	Protein	Species	Score	E value
AAC99310	Constans-like protein 2	*Malus x domestica* (Apple)	244	5e-63
AAX47173	Constans-like b	*Pisum sativum* (Pea)	244	5e-63
ABR57243	Constans-like 4	*Picea abies* (conifer)	243	9e-63
AAL99264	Constans- like CO5	*Hordeum vulgare* (barley)	239	1e-61
AAM62947	Constans-like 8	*Arabidopsis thaliana*	238	3e-61
NP_197088	Constans	*Arabidopsis thaliana*	211	3e-53

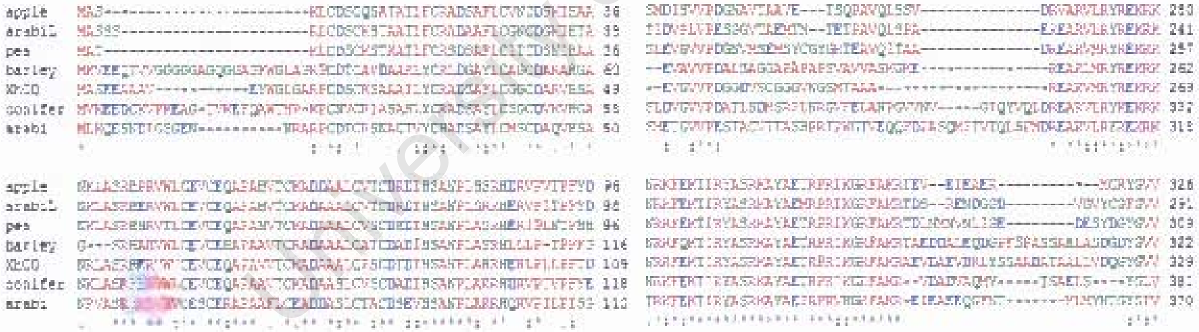


Figure 3.4. Alignment of sequences of Table 3.4. Only conserved areas are shown. Asterisk (*) shows conserved amino acids. Apple: *Malus x domestica* (AAC99310), ArabiL: *Arabidopsis thaliana* (AAM62947); Pea: *Pisum sativum* (AAX47173); Barley: *Hordeum vulgare* (AAL99264); XhCO: *Xerophyta humilis*; Conifer: *Picea abies* (ABR57243); Arabi: *Arabidopsis thaliana* (NP_197088)

The sequencing results of RACE and TAIL-PCR were combined by searching for the overlapping regions using the Clustal global alignment tool at: www.ebi.ac.uk /clustalw/. The sequence of the *XhCO* locus is given in figure 3. An alignment of XhCO and sequences outlined in Table 3.4 are given in Figure 3.4.

1	CAACGGCTGAGACGAAGATGGATGGCGATGAAGTGATTCGGTGAAGATCGGCGGCGAT
59	GACGTCCATCACAATCAGCACAGGTCTCGAGAA***ATTG***TATCAATGTTTCTCCGATTAT
117	TTGACCTATTTTAAACCATTTTTTTTTGTAAAACTTCTAACAAAAAAGTTGTACATTT
175	**GATG**CAATGTATAACTTTGTAGTTGAAAGTTTTTGACAT***ATTG***TCTGGCATTTTTTGG
233	AAAAAAATTTATCTCAAGGTTATAT***ATTG***CAT***CAAAT***CTAAAACTTTACAGTTGAAAG
291	TCTTTCCAAAAAATATCAAACGACGTCCCAAAG***ATTG***ATCAAAATTTTGATCATTTAT
349	TTTTTTCATATTGTTTGATACTTTTTTTGAAGAACTTTCAACTGAAAAGTTGAATATT
407	TGATGCAATCTATAACTTTGTAGTTGAAAAGTTTTTACAAAAGTA***CCAAT***AATATGTC
465	ACAAAGATGATTTAAAACTTTAATTAAAATATCTCTTTCGCATATCATTTTGTATTTT
523	*TTGAA*AAAAACTTT***C***AACTA***CAATT***TTACAGATTTTGATGTAATTTACAACTTTATAG
581	TTGAAGTTTTTCTCAAAAAGTACTTAAAAATAGCTCAAATAAGCAAACGAAAATTTTC
639	TGCCGTTTTTCGAGATTATGATGACGGCTGAGATCGCCTTCACGCCA[illegible]CAG
697	TAATTGGATAAAGAAGTTGGTATAATGCCAAG***ATTG***TA***ATTG***TTGGAAAGAATACGGG
755	***CAAAT***ACCTAGCAACATCAAAACGCGTTTTTAAACAGTACATGAGGGGCTGAGCTGGT
813	AGGAGAAAAAAGGTGAGGTTTGATATGGAACCCGAATATC***ATTTG***GGGGGCTGATACG
871	GCATTTCCCCAACTTTATAACCAATTAATTATGAAGTCAGAACGTAACTGACTTATCC
929	TAATTGACTGATCCAGTAGAAAACAAGTAATTAAAAAGAATAATTAATAATATTTTAG
987	ATTCACGTGCCACGTCAATCGCGACTCACCATCTTATGTTCTGGTACTGATCCTTTTA
1045	**GATAGCCTTG**ATGCCGATACAGCCTCTTGCTACAACCAGACATCTCC*ATTG*CTAACCA
1103	GCAGAGAGCTTATCATTTATCAAAGAAGGAGAAGAAGAAGTAGAAGAGTGATGGCTAG
1161	CGAGGAGGCTGCCGGCACTGGAGTACTGGGGACTGGGGCTCGGCCATGCGACTCATGC
1219	AAGTCGGCGGCGGCGATTCTCTACTGCCGTGCCGACTCGGCGTTCCTCTGCGGCGGTT
1277	GCGATGCCCGTGTCCACTCCGCCAACCGACTTGCTTCCCGCCATGAACGTGTCTGGGT
1335	ATGCGAGGTGTGCGAGCAAGCTCCGGCGGTGGTGACCTGCAAGGCAGACGCCGCCGCC
1393	CTCTGCCCCTCCTGCGACACCGACATCCACTCTGCCAACCCCCTCGCCCACCGCCACG
1451	AGCCCCTCCCTCTTCTTCCCTTCACCGATCCCCCACTGAAACCCTCGCATCACCACCA
1509	TGATGAAATCCTTGTCGTTGACCACAATAGCAACAACCAGTTAGATGACGAGAACGAT
1567	GACGCCGAGGGGGAGGCTGCGCCGTGGTTGCTCCCCCCTGCATCGGCCAGTAATCCTC
1625	ATAAAGGTGTGGATGTAATGGAGGCGCCGGAGGTGAAAGAGACGGCGGCGGCTCAGTT
1683	TTATTTTCACGATATAGATCCTTACCTTGATCTAGATTTTGTTTCTCACCACGCCGTG
1741	GCTGACAGCCGTCGTTCCCGTCGCCCGAAAACCAGTTCTAGCCGCGCCCGTCCACTGTC
1799	CTCCGTTCGCCTCCTATGCCTCCATGAGCCACAGGGTAAGTATTCATACCTACGAAA
1857	GTATAACTATGAAATTCGTATAATTACAAATATCCGTTGCTAGAATTTTTTTCACTTT
1915	TTCTTGAAAGGAATTTATAGCTCGTAGAATATATTTAGAACTATATTTTCTATAACTA
1973	AGCATTAGTCTGAA[illegible]TGTCCG[illegible]ATAT[illegible]GGCATGTTATGACTTTCAT[illegible]
2031	TAGAAGTGACATAGTATGTACCTGTTAAGAACGTTATAAGTTATATAATTATATTGTC
2089	ATTGATCGTGATCGATGACTGCACGCAGATGTCGTCGTCGGAGGTGGGGCTGGTGCCT
2147	GACGGCAGGGGACCTGTCGTCGGCGGAGGAGTGAATGGATCGATGACGGCAGCACCGA
2205	GAGACGCCGAGGGTGATGAGGTACAGGGAGAAGAGGAAGAACAGAAGGTTCGAGAAGAC
2263	GATACGCTATGCTTCGGCGCAAAGCTTACGCCGAAACGCGGCCAAGGATCAAGGGAAGG

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2321    TTCGCAAAGCGGGCGGAGGTCGACGCCGAGGTCGACCGGCTCTACTCCTCGGCCGCGG
2379    ACGCCACGGCGGCGCTGCTTGTCGACCAAGGTTATGGCGTCGTTCCAACCTTCTGAAG
2437    TATTTTACTACATATGTATATATGCGCAAGGCATATATAACTAAAAAGTACTCACCTG
2495    CCGACGCTGCTGCACGGTTATCGCCGGCGAACCTCGCCGTCGACGGCCGTAATTGTTT
2553    AATGTCAGGTTTTTGCAATGCTGTACTATTAATATTTTTCTTCTTACTCGATTTCCTG
2611    CTGCTTCTGCTGTATATTTATTGCTATTGAGAATGGACTCAAGTAAAGCTCTTCTTTT
2669    TTTAAAAAAAAAAAAAAAAAA
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Figure 3.5. Nucleotide sequence of *XhCO.* The start codon (ATG), the intron and the stop codon (TGA) are inverted (white text with black background). The sequences highlighted in the 5' upstream region are listed in table 3.6

TAIL -PCR revealed not only the intron in the sequence; also part of the upstream region was identified. It is possible that the promoter or part of the promoter has been identified of *XhCO.* To analyse the promoter region, the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was searched for conserved *cis*-acting motifs in the 5' upstream region and in the rest of the gene. Several motifs involved in light response were found in the promoter region. Also motifs involved in stress responsiveness, circadian control and promoter elements were found. Table 3.5 lists the different motifs and Figure 3.4 indicates the position of the motif within the XhCO gene locus.

Table 3.5 Motifs in the promoter region of *XhCO* of July the 9th 2008.

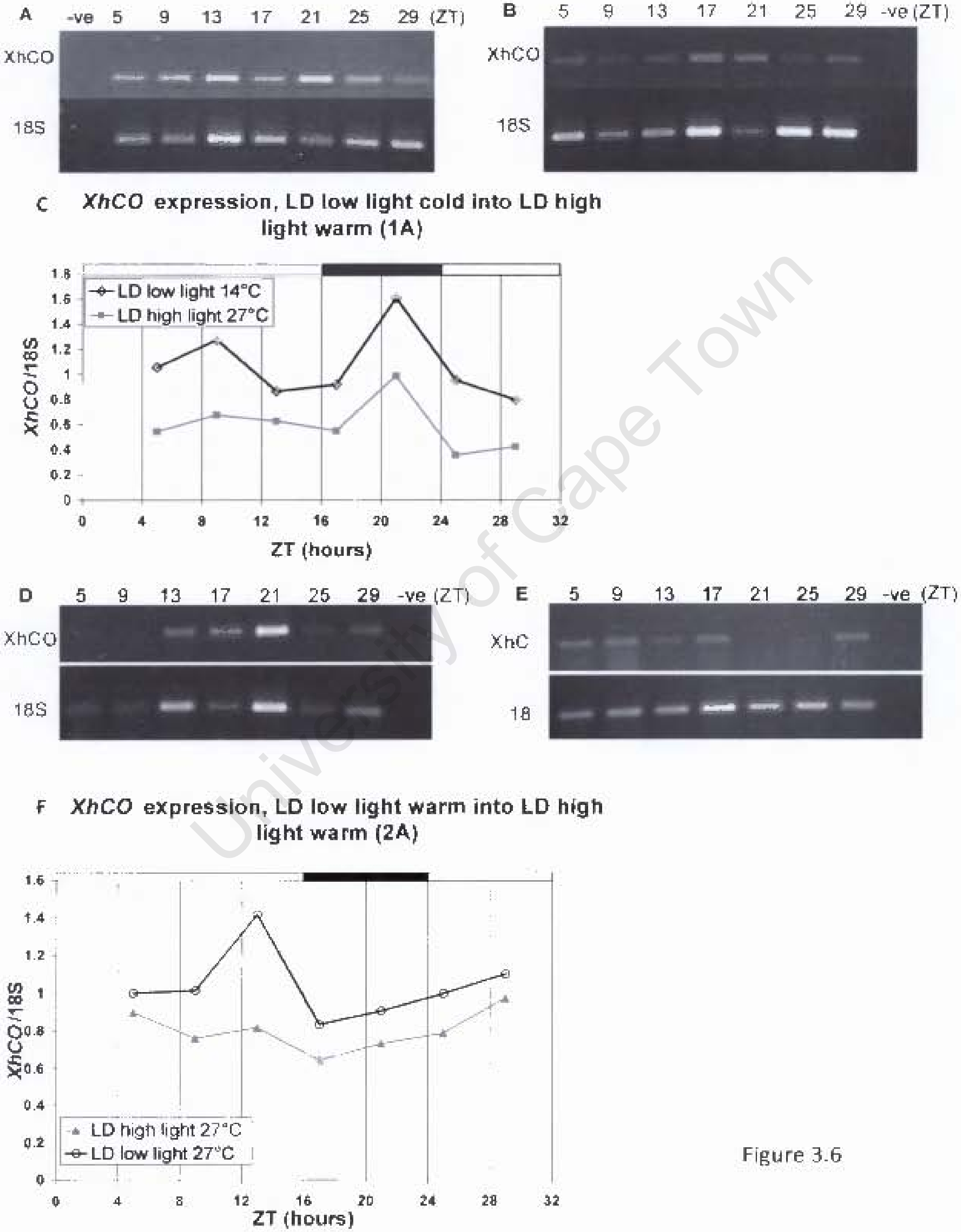
Motif found	Strand	Position	Sequence	Function
3-AF1 binding site	-	491	AAGAGATATTT	light responsive element
AE-box	-	102	AGAAACAT	part of a module for light response
Box 4	+	894/971	ATTAAT	part of a conserved DNA module involved in light responsiveness
Box I	-	522	TTTCAAA	light responsive element
G-box	+	990	CACGTG	cis-acting regulatory element involved in light responsiveness
GA-motif	+	467	AAAGATGA	part of a light responsive element
GAG-motif	-	687/985	GGAGATG	part of a light responsive element
As-2-box	+	111	GATAatGA	involved in shoot-specific expression and light responsiveness

			TG	
HSE	+/-	199/528	AAAAACTTTC	cis-acting element involved in heat stress responsiveness
MBS	+/-	280/915	(C/T)AACTG	MYB binding site involved in drought-inducibility
TC-rich repeats	+	347	ATTTTCTTCA	cis-acting element involved in defense and stress responsiveness
circadian	-	1045	CAANNNNATC	cis-acting regulatory element involved in circadian control
CAAT	+/-	N/A	CAAT	common cis-acting element in promoter and enhancer regions

In *Arabidopsis*, flowering is promoted by light coinciding with AtCO under LD, but not under SD conditions (Valverde *et al.*, 2004) and is modulated by the circadian clock (Suárez-López *et al.*, 2001). The environmental cue experiments shows that light intensity plays an important factor in flowering (see section 'photoperiod, light intensity, temperature and hydration status'). Also the upstream region of *XhCO* is involved in light and stress responsiveness and circadian control (Table 3.5). These results, together with the identification of conserved domains for light regulation in the 5' upstream sequence of *XhCO* (Figure 3.4), supports *XhCO* function as a *CO* or *CO-like* gene. It is possible XhCO is regulated by photoperiod, like CO in *Arabidopsis*. To confirm the influence of light on *XhCO* expression, expression patterns were studied in *X. humilis* plants.

XhCO gene expression analysis

Expression of *XhCO* was analysed with semi-quantitative RT-PCR under the environmental conditions summarised in Table 2.2. Figure 3.6 shows the expression patterns of *XhCO* analysed in plants that were transferred from low light LD conditions into high light LD conditions. A small peak occurs at nine hours after dawn (ZT9) and a larger peak occurs at ZT21 hours in both low light and high light conditions (Figure 3.6 C). Flowering was initiated under these conditions.



C *XhCO* expression, LD low light cold into LD high light warm (1A)

F *XhCO* expression, LD low light warm into LD high light warm (2A)

Figure 3.6

Figure 3.6. Expression pattern analyses of *XhCO* in *X. humilis*. A: Diurnal expression pattern of *XhCO* that had experienced long days of 25-30 μmol m^{-2} s^{-1} at 14 °C for 21 days followed by (B) transfer to long days of 400 μmol m^{-2} s^{-1} at 26-27 °C for 10 days. This treatment caused plants to flower. A representative of two technical repeats and four biological repeats is shown. C: quantification of A (open diamonds) and B (closed squares). D: Diurnal expression pattern of *XhCO* in plants that had experienced long days of 25-30 μmol m^{-2} s^{-1} at 26-27 °C for 21 days followed by (E) transfer to long days of 400 μmol m^{-2} s^{-1} at 26-27 °C for 10 days. This treatment did not cause plants to flower. A representative of two technical repeats is shown. F: quantification of D (open circles) and E (closed triangles). XhCO RNA was analysed by semi-quantitative RT-PCR and values are shown after normalising to 18S rRNA levels. Open and shaded bars represent light and dark respectively.

When plants were transferred from LD warm low light into LD warm high light (Figure 3.6 D) and flowering did not occur, the second peak was lost and the first peak was shifted. Also expression in high light dampens the oscillation. *XhCO* expression appears to be higher in low light conditions than in the high light conditions (Figure 3.6 C and D).

When plants were transferred from SD cold conditions into LD high light conditions, a peak at ZT9 hours and ZT21 hours was observed in the high light conditions (Figure 3.7 C). This treatment did not cause plants to flower, however the pattern in the LD high light conditions are similar to the expression pattern in Figure 3.6 C, where plants were also exposed to cold, but then under LD, which did cause plants to flower.

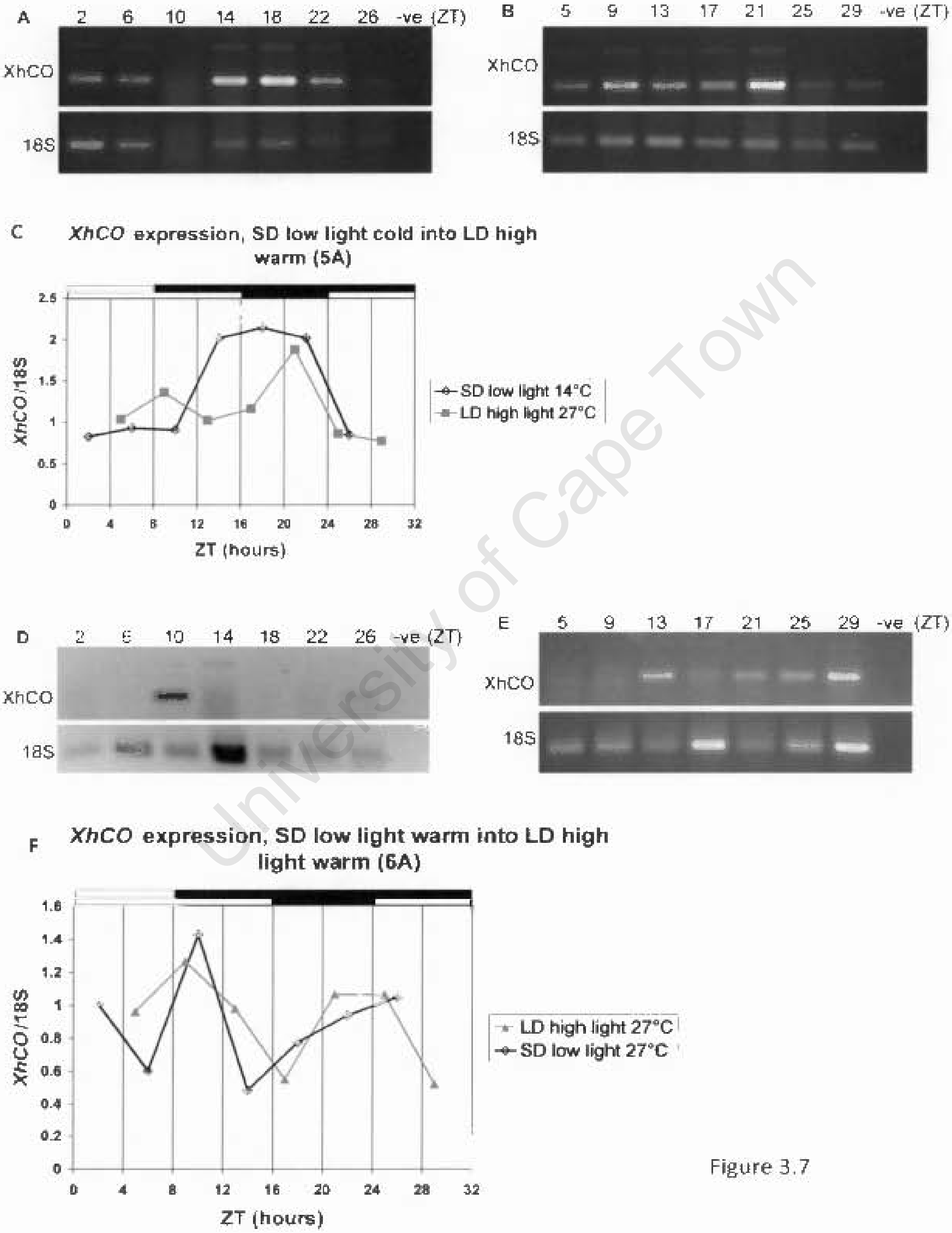

A
2
6
10
14
18
22
26
-ve (ZT)
XhCO
18S
B
5
9
13
17
21
25
29
-ve (ZT)
XhCO
18S
C
XhCO expression, SD low light cold into LD high warm (5A)
XhCO/18S
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
28
32
ZT (hours)
SD low light 14°C
LD high light 27°C
D
2
6
10
14
18
22
26
-ve (ZT)
XhCO
18S
E
5
9
13
17
21
25
29
-ve (ZT)
XhCO
18S
F
XhCO expression, SD low light warm into LD high light warm (6A)
XhCO/18S
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
4
8
12
16
20
24
28
32
ZT (hours)
LD high light 27°C
SD low light 27°C

Figure 3.7

Figure 3.7. Expression pattern analyses of *XhCO* in *X. humilis*. A: diurnal expression pattern of plants that had experienced short days of 25-30 µmol m^{-2} s^{-1} at 14 °C for 21 days followed by transfer (B) into long days of 400 µmol m^{-2} s^{-1} at 26-27 °C for 10 days. This treatment did not cause plants to flower. C: quantification of A (open diamonds) and B (squares). Representative of two technical repeats is shown. D: diurnal expression pattern of plants that had experienced short days of 25-30 µmol m^{-2} s^{-1} at 26-27 °C for 21 days followed by (E) transfer into long days of 400 µmol m^{-2} s^{-1} at 26-27 °C for 10 days. This treatment did not cause plants to flower. F: quantification of D (open diamonds) and E (triangles). A representative of two technical repeats is shown. *XhCO* RNA was analysed by semi-quantitative RT-PCR and values are shown after normalising to 18S rRNA levels. Open and shaded bars represent light and dark respectively.

The expression pattern under SD low light condition had one large peak at ZT21 hours under cold conditions (Figure 3.7 C) and a peak at ZT9 under warm conditions (Figure 3.7 F). In both SD treatments the expression patterns of *XhCO* are similar in low light SD and high light LD (Figure 3.7 C and F), whereas the expression was higher in LD low light (Figure 3.6 C and F).

As the plants did not flower as expected in the SD experiment, which is represented in Figure 3.6, plants were dried down in the cold which is presented in figure 3.8. This treatment did cause plants to flower and expression patterns were analysed of the rehydrated plants. The expression pattern of *XhCO* is similar to LD high light conditions when plants were transferred from LD cold low light (see Figure 3.6 C), which also caused flowering. A clear peak occurs at 21 hours, while the smaller peak seems to have shifted to ZT13 hours.

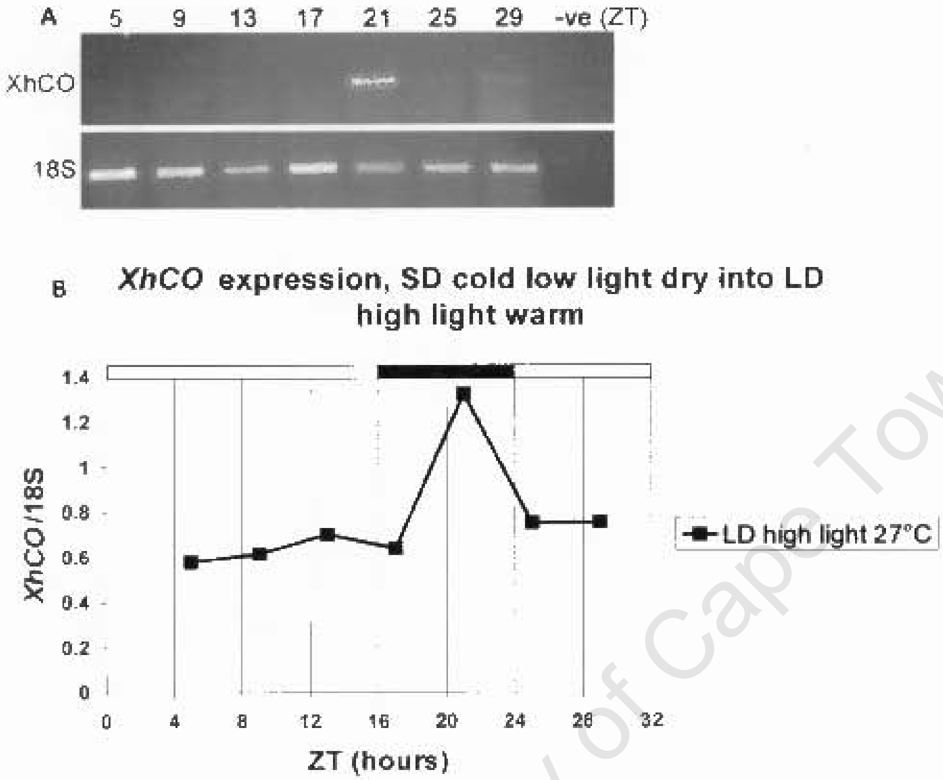


Figure 3.8. Diurnal expression pattern analyses of *XhCO* in *X. humilis*. Expression pattern of plants that had experienced short days of 25-30 µmol m^{-2} s^{-1} at 14 °C for 21 days and dried down in these conditions followed by transfer to and rehydration in long days of 400 µmol m^{-2} s^{-1} at 26-27 °C for 10 days (closed squares). This treatment caused plants to flower. A representative of two technical repeats is shown. *XhCO* RNA was analysed by semi-quantitative RT-PCR and values are shown after normalising to 18S rRNA levels. Open and shaded bars represent light and dark respectively.

In order to determine if XhCO is circadian or diurnal regulated, the expression pattern was analysed in constant light and constant dark (Figure 3.9). The oscillation dampens in both constant light and constant dark, however in constant dark the oscillation dampens faster than in constant light. This further indicates that light affects *XhCO* expression, but it seems that the diurnal oscillation of XhCO is not driven by a circadian rhythm.

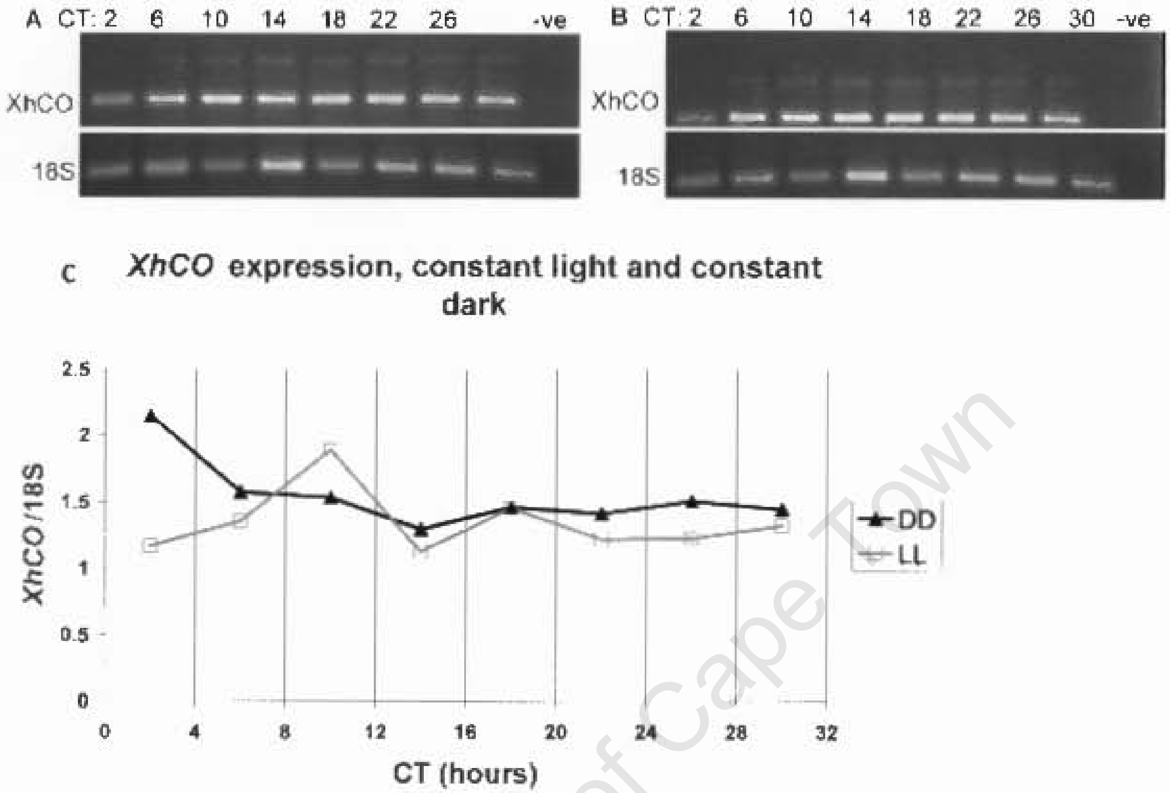


Figure 3.9. Expression pattern analyses of *XhCO* in *X. humilis* in constant condtions. Expression pattern of plants that had experienced a 12:12 photoperiod of 70 µmol m^{-2} s^{-1} at 26-27 °C for five days followed by constant light of 70 µmol m^{-2} s^{-1} at 26-27 °C for 10 days (open squares) or constant dark (triangles). A representative of two technical repeats is shown.

Chapter 4. Discussion and conclusion

4.1 Flowering response of *Xerophyta humilis* to different environmental conditions

X. humilis is a desiccation tolerant plant and thus has the ability to revive from an air dry state. To survive the dry winters in the natural environment it is necessary that the transition from vegetative state into reproductive state is timed very accurately. In *Arabidopsis thaliana* the floral timing is regulated and modulated through the interactions of multiple flowering pathways (see Fig 1.1). Environmental factors such as temperature and photoperiod play an important role in the floral transition. It is not known which environmental factors are involved in the floral transition in *X. humilis*. In fact, most research into resurrection plants focuses on the dehydration mechanism, with some studies investigating rehydration. Correct timing of the floral transition is also part of the survival mechanism, however, no research has been done on regulation of flowering in resurrection plants.

Firstly we were interested in which environmental cues induce flowering in *X. humilis*. It had been observed that *X. humilis* plants flowered upon rehydration, after being in a desiccated state for a period of six weeks or longer (Jill Farrant, Department of Molecular and Cell Biology, University of Cape Town, personal communication). Therefore the flowering response to the hydration status and desiccation duration was investigated. From these experiments it was concluded that flowering was not initiated by desiccation only. Although flowers were formed by plants that had endured five and six weeks of dehydration, they only appeared three and six weeks respectively upon rehydration, which was similar to control plants. This indicates that a different cue must have caused flowering. This cue could have been the bright lights in the phytotron, as later experiments showed that high light plays a role in the floral pathway in *X. humilis*. As we were interested in which cues initiate flowering, other environmental cues were also investigated.

From the environmental experiments shown in Table 2.2 and 3.1 it was concluded that a combination of cues is necessary for *X .humilis* to flower. These experiments also show that changing the length of the day does not initiate flowering on its own. Combining photoperiod with cold and desiccating plants also did not initiate flowering. This shows that the flowering pathway in *X.humilis* is a complicated network, and it is likely that several pathways are responsible for floral induction.

The experiments discussed above were carried out under low light (light intensity between 25-30 µmol m^2s^{-1}) and did not result in flowering, whereas when plants were moved into high light (average light intensity of 400 µmol m^2s^{-1}) conditions, flowering did occur (See table 2.2 and 3.2 and Figure 3.1 and 3.2). This shows that light quantity plays an important role in the flowering pathway of *X. humilis*. Surprisingly plants moved from LD into LD flowered, whereas plants moved from SD needed to be desiccated in the cold in order to flower in LD. These and the results from the first experiment show that changes in the photoperiod alone cannot induce flowering. Possibly *X. humilis* is a day-neutral plant and flowering is not induced by either LD in response to photoperiod changes. It is also possible *X. humilis* is a LD plant, but other cues such as cold are required to make the plants competent to flower when favourable conditions return, similar to biennial accessions of *A. thaliana*, *Triticum monococcum* (wheat) and *Beta vulgaris* (sugar beet). Interestingly, these results show that it is not necessary for the plants to desiccate to be able to produce flowers, as plants transferred from cold low light LD conditions into warm high light LD conditions flowered. This shows that in the complex flowering pathway of *X. humilis* exposure to cold is an important factor.

When plants were desiccated under low light conditions prior to incubation in cold, they did not flower upon rehydration in the high light conditions. However, when plants were dried in the cold incubator, plants did flower upon rehydration. This was expected as in the wild *X. humilis* plants flower after they have experienced a cold and dry winter. *X. humilis* loses chlorophyll during dehydration, therefore photosynthesis ceases when the plants are desiccated. Photosynthesis is possibly necessary to sense low temperatures. Under low temperatures the rates of enzymatic reactions involved in C, N and S reduction are more reduced than photochemical and photophysical processes (reviewed in Ensminger *et al.,* 2006). This could indicate that theses processes are necessary for sensing low temperatures and therefore *X. humilis* plants cannot sense cold when desiccated.

The above results show that both cold and high light are necessary to induce flowering in *X. humilis*. Soimato *et al.*, 2008 compared genes that were up regulated in cold and light and genes that are up regulated in cold and dark and they showed that low temperature enhanced the expression of several genes. The cold/light induced genes included transcription factors, from which the largest group was zinc finger family transcription factors including *CONSTANS.* They conclude that light is a necessity for induction of these transcription factors. In *X. humilis* plants high light could be a necessity to induce transcription factors involved in the floral pathway.

4.2 Molecular genetics of flowering

Flowering is controlled by a large set of genes acting together in a complex network which is influenced by the environment and various internal signals. Some of these genes have a conserved sequence and function in several plant species. The gene *CONSTANS* (*CO*) for example has been found in many species. *CO* has been identified in monocots like *Oryza sativa* (rice) (Hayama *et al.,* 2002), dicots like *Arabidopsis thaliana* (Putterill *et al.* 1995) and *Pisum sativa* (pea) (Hecht *et al.*, 2005) and even in gymnosperm trees such as *Pinus sylvestris* (pine) (Pyhajarvi *et al*., 2007). Other genes of the flowering pathway are unique to a specific group of plants. *FLOWERING LOCUS C (FLC)* for example is a floral repressor in *A. thaliana* and so far has not been identified in monocots. FLC is repressed by vernalisation and is central to the control of both the vernalisation and autonomous pathways. Because it was observed that *X. humilis* plants flowered when moved from a cold glass house to a warm phytotron, our first interest was to find a *FLC* homologue, which could be involved in vernalisation. However, no homologue was found. It is possible *X. humilis* has a different vernalisation mechanisms to *A. thaliana*, like in the monocots wheat and barley. Therefore I attempted to isolate a *VRN2* homologue, which represses flowering in winter varieties of wheat and barley (Yan *et al*., 2004).

The homologue was amplified from *X. humilis* cDNA with PCR (polymerase chain reaction), using primers homologous to the gene of interest, cloned and sequenced. This means that if a homologue was not found, it does not exclude the possibility of *X. humilis* containing this gene. The primers are designed to conserved areas of the gene of interest and because degenerate primers were used, it is possible to pick up the wrong or no product, because of differences in the sequence between the gene of interest in the model species and in *X. humilis*. A different method to identify a homologue would be to screen a BAC library from *X. humilis*, by using a probe of the gene of interest and screen the colonies that the probe anneals to. For this method a library of the genome is required, which is a time consuming process. At present, only cDNA libraries fom *X. humilis* are available (Collett *et al.,* 2003) and as it was unknown when the gene of interest could be expressed the existing libraries could not be used.

To identify the full length of the gene of interest several techniques were used. Because cDNA has a polyA-tail at the 3' end, it is relatively easy to identify this region with 3'RACE (rapid amplification of cDNA). Therefore an existing protocol (Sambrook and Russel, 2006) of 3'RACE could be used with small adjustments. However, the 5' end does not contain an easy start point for a primer to anneal to and a short sequence needs to be added to the 5' end. Unfortunately, because of the GC and CC rich sequence in the *X. humilis* homologue, the existing protocol in Sambrook and Russel, 2006 could not be used to identify the 5' end. Therefore, a 5'RACE kit (BD Biosciences Clontech, USA) was used, which is more costly but a lot less time consuming. The enzyme provided with the kit to synthesize cDNA, adds several dC residues when it reaches the end of mRNA. A specific primer is provided that anneals to the tail of the first strand of cDNA and serves as an extended template for BD powerscript Reverse Transcriptase, which is used to amplify the 5' end of the DNA. Because several steps are no longer necessary, using this kit is more efficient than standard protocols. The enzyme provided for the RACE PCR is very efficient, which makes the PCR easier to carry out. When the same RACE PCR reaction was done with a similar but different enzyme, no product was amplified (data not shown). TAIL- PCR (thermal asymmetric interlaced PCR; Liu and Whittier, 1995) is another technique to identify either the 3' end or the 5' end of the gene of interest. The advantage of this technique is that genomic DNA can be used as template, and therefore introns and other non-coding sequences can be identified. To identify upstream and downstream regions it is also possible to digest the genomic DNA with a restriction enzyme, which is not a frequent cutter. A primer can then be used to anneal to the restriction site and a specific primer to anneal to the gene of interest. To avoid unspecific priming a vectorette (Arnold and Hodgson, 1991) or splinkerette (Devon *et al.,* 1995) can be used. TAIL-PCR however was more convenient to identify the unknown sequences, as the primer sequences of Lui and Whittier, 1995 could be used. A combination of RACE and TAIL-PCR was used to identify the full-length cDNA and the full-length genomic DNA sequence including an intron and part of the promoter region.

Translation of the full length sequence into protein *in sillico* revealed two B-boxes and a CCT domain, which indicates a CO or CO-like homologue was identified. When the translated protein was analysed with the BLAST web tool, it showed homology with CO and CO-like proteins, which fits in with previous in silico results. A search in the PlantCARE database for *cis* acting motifs of the 5' upstream region showed that this region is involved in light and stress responsiveness and also circadian control. These results suggest that the isolated homologue from *X.humilis* is not a *VRN2*, but a *CO* homologue and it was termed *XhCO.* This also coincides with the fact CO is conserved in a great variety of plant species.

Expression of *XhCO* was analysed by semi-quantitative RT PCR. This method measures the gene expression by converting the RNA into cDNA with reverse transcriptase (RT) and then amplifying this cDNA with PCR. The PCR product can then be detected on an ethidium bromide stained agarose gel under UV light. Real- Time PCR, a more costly method, is quantitative, whereas it measures the amplification of cDNA immediately, usually with a fluorescent dye. Northern blot hybridisation and detection with radioactive probes are also used for the quantification of transcript levels. Although the technique is very sensitive, it is not able to detect transcripts of low abundance. As *XhCO* is a homologue of *A. thaliana CONSTANS,* which is expressed at a very low abundance, semi-quantitative RT PCR was used to monitor its expression levels. Firstly a radioactive probe was used to detect the cDNA amplification (data not shown), however it was found that ethidium bromide staining was sensitive enough for detecting transcript levels at a very low copy number. Care was taken to ensure that the number of amplification cycles used fell within the linear range of amplification, and that the assay was indeed quantitative by analysing the linearity of signal as template was increased. The *XhCO* primers amplified an intron-spanning sequence, to allow for distinction of amplification from genomic DNA versus cDNA. *XhCO* expression was normalised to 18S RNA as this was the only 'housekeeping' gene sequence available. A $d(T)_{17}$ primer was used to synthesise the cDNA of the different samples, which gives the possibility that no cDNA was synthesised from 18S RNA, unless the $d(T)_{17}$ primer has a sequence to anneal to. The 18S RNA from sequence *X. humilis* does show polyA parts that give possibility for a primer to anneal to (accession number EF418586; see appendix A). To confirm that the d(T)$_{17}$ primer can anneal to the 18S RNA sequence, a PCR reaction was carried out with the synthesised cDNA and a primer set of d(T)$_{17}$ and a 18S RNA specific primer. Then a nested PCR was done with the diluted PCR product. This resulted in a PCR product of expected size, which indicates it is possible to amplify 18S RNA from the cDNA samples. This does not exclude the possibility of the primers annealing to genomic DNA that is possibly contaminating the cDNA sample. A housekeeping gene like *Actin* with intron spanning primers would have been a better candidate to normalise the gene expression against. Unfortunately the PCR reaction for the *Actin* gene or other house keeping genes using primers derived from *A. thaliana* sequence could not be optimised. To confirm if the primer set for 18S RNA would amplify to any DNA contamination in the samples, a PCR reaction was done with RNA only of the samples, before cDNA was synthesised. The template was diluted in the same way the cDNA template was diluted. As the primers cannot anneal to the RNA, any product would have to come from DNA. This resulted in a PCR product that was not visible to the eye, which means the DNA in the cDNA samples, if present was diluted enough to ignore 18S amplification from gDNA data not shown.

In *A. thaliana CO* expression peaks at the end and beginning of the photoperiod in LD (Figure 4.1), whereas under SD accumulation only occurs in darkness (Suárez-López *et al.*, 2001). CO activates flowering by regulating FT and SOC1, which are floral integrators, and is circadian regulated (see figure 1.3).

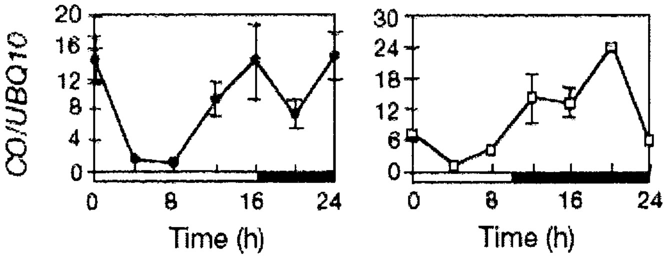


Figure 4.1. From Suárez-López *et al.*, 2001. *CO* expression in wildtype plants. Left shows expression in LD, right in SD.

Interestingly, the expression pattern of *XhCO* also shows two peaks (see Figure 3.6 C and 3.6 F); however, these peaks are at seven hours before dark and five hours after dark, which is different to the *CO* mRNA expression pattern. Trevaskis et al., (2006) showed that in LD *VRN2* mRNA has a small peak just after dawn, eight hours before dark and just before dark (See Figure 4.2) and is absent or very little expressed in SD. These peaks in LD are similar to the *XhCO* peaks, which indicates *XhCO* could be a floral repressor like *VRN2* in barley and wheat rather than a floral activator like *CO* in *A. thaliana*.

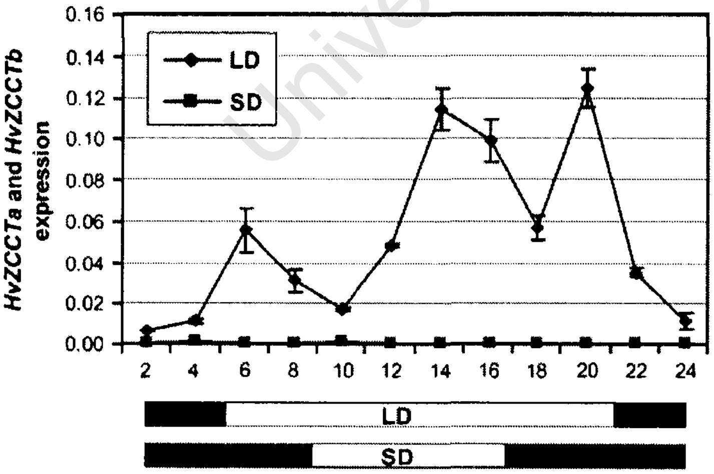


Figure 4.2. From Trevaskis *et al.* (2006). Expression of *HvZCCTa* and *HvZCCTb* shows diurnal rhythm in plants grown in LD. *ZCCTa* and *b* are also known as *VRN2.*

In LD *XhCO* expression is higher in low light conditions than in the high light conditions which induced flowering (see Figure 3.5 C and 3.5 F). This shows that *XhCO* mRNA is accumulated in low light and is light dependent. It seems that *XhCO* is repressed when plants are flowering, which supports the hypothesis that *XhCO* is a floral repressor.

Expression of *XhCO* in LD cold low light and LD warm low light has a similar pattern, but flowering did not occur without cold exposure. This confirms that exposure to low temperature is important for floral transition in *X. humilis*. It is interesting that the second peak in the high light conditions (Figure 3.5 F) is absent. It is possible this second peak is important for floral induction. These results show that *X. humilis* operates a complicated floral pathway, which is probably maintained by several pathways. The vernalisation could be linked to other pathways, therefore without vernalisation the plants are unable to flower.

A different expression pattern of *XhCO* was observed in plants under SD (Figure 3.7 C and F). Under these conditions the plants did not flower. A similar amount of mRNA accumulated under high light and low light, unlike in LD conditions (Figure 3.6 C and F). This could mean that *XhCO* is not repressed in high light conditions and therefore flowering did not take place. More *XhCO* mRNA seems to be accumulated under SD than in LD.

When plants were dried down in the cold under SD and transferred into high light where floral transition was initiated, the same peaks of *XhCO* were observed in high light as when plants that were moved from LD cold low light conditions (see figure 3.6 C and 3.7 B) into high light, which also caused plants to flower. A similar amount of mRNA was observed in the leaves of plants that were transferred from these SD conditions (Figure 3.7 B) to plants that were transferred from LD (Figure 3.6 C) conditions, which could mean that *XhCO* is also repressed in the leaves of plants that are desiccated in low temperatures .

Figure 3.8 C shows that oscillation dampens in constant dark and constant light. The oscillation seems to dampen faster in constant dark than in constant light. This shows that the diurnal regulation of *XhCO* is not driven by the circadian clock, unlike *CO* in *A. thaliana*. These results together show that although *XhCO* has a similar sequence to *CO* in *Arabidopsis*, containing two B-boxes and a CCT domain, the function is possibly a floral repressor, like VRN2 in wheat and barley.

Although the effects on floral induction of only a few environmental factors have been observed, from the above experiments it can be concluded that light quantity and vernalisation are important factors in the induction of flowering of *X. humilis*. This is conserved with the flowering pathway in model plants, such as *A. thaliana*. It has also been revealed that *XhCO* is a *CO* homologue, but possibly with a similar function to *VRN2* in barley and wheat. To confirm the function of XhCO, further experiments need to be done, such as a complementation study of *A. thaliana co*-null mutant and analyse the effects of overexpression of *XhCO* in wildtype *A. thaliana* plants. If XhCO is able to accelerate flowering when overexpressed, or rescue the late flowering phenotype of the co-null mutant, it is possible to conclude a similar function to *AtCO.* Also, a 1.1 kb of the upstream region has been revealed, thus promoter studies could be carried out, which could reveal more of the function of *XhCO.*

References

Arnold C, Hodgson IJ. 1991. Vectorette PCR: a novel approach to genomic walking. PCR methods applied. 1 (1): 39-40

Alpert P. 2006. Constraints of tolerance: why are desiccation-tolerant organisms so small or rare? Journal of Experimental Biology 209: 1575-1584

Aukerman MJ, Hirschfeld M, Wester L, Weaver M, Clack T, Amasino RM, Sharrock RA. 1997. A Deletion in the PHYD Gene of the Arabidopsis Wassilewkija Ecotype Defines a Role for Phytochrome D in Red/Far-Red Light Sensing. The Plant Cell 9:1317-1326

Azevedo H, Lino-Neto T, Tavares RM. 2003. An Improved Method of High-Quality RNA Isolation from Needles of Adult Maritime Pine Trees. Plant Molecular Biology Reporter 21: 333-338

Blast information: http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html. Last accessed 05-10-2007

Collett H, Butowt R, Smith J, Farrant J, Illing N. 2004. Photosynthetic genes are differentially transcribed during the dehydration-rehydration cycle in the resurrection plant, Xerophyta humilis. Journal of Experimental Botany 54 (392): 2593-2595

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G. 2007. FT Protein Movement Contributes to Long-Distance Signalling in Floral Induction of *Arabidopsis*. Science 316: 1030-1033

Dace H, Sherwin HW, Illing N, Farrant JM. 1998. Use of Metabolic Inhibitors to elucidate Mechanisms of Recovery from Desiccation Stress in the Resurrection Plant *Xerophyta humilis.* Plant Growth Regulation 24: 171-17

Danyluk J, Kane NA, Breton G, Limin AE, Fowler DB, Sarhan F. 2003. TaVRT-1, a Putative Transcription Factor Associated with Vegetative to Reproductive Transition in Cereals. Plant Physiology 132: 1849-1860

Dellaporte SL, Wood J, Hicks JB. 1983. A plant DNA minipreparation: version II. Plant Molecular Biology Reporter 1: 19-21

Devon RS, Porteous DJ, Brookes AJ. 1995. Splinkerettes improved vectorettes for greater efficiency in PCR walking. Nucleic Acids Research 23 (9): 1644-1645

Dubcovsky J, Lijavetzky D, Appendino L, Tranquili G. 1998. Comparative RFLP Mapping of Triticum monococcum Genes Controlling Vernalization Requirement. Theoretical and Applied Genetics 97: 968-975

Dubcovsky J, Loukojanov A, Fu D, Valarik M, Sanchez A, Yan L. 2006. Effect of Photoperiod on the Regulation of Wheat Vernalization Genes *VRN1* and *VRN2*. Plant Molecular Biology 30: 469-480

Ensminger I, Busch F, Huner NPA. 2006. Photostasis and Cold Acclimation: Sensing Low Temperature through Photosynthesis. Physiologia Plantarum 126: 28-44

Farrant JM, Cooper K, Kruger LA, Sherwin HW. 1999. The Effect of Drying Rate on the Survival of Three Desiccation-tolerant Angiosperm Species. Annals of Botany 84: 371-379

Farrant JM. 2007. Mechanisms of Desiccation Tolerance in Angiosperm Resurrection Plants. In: Jenks MA, Wood AJ, eds. Plant Desiccation Tolerance. Wallingford, UK: CAB International

Gaff DF. 1971. Desiccation-tolerant Flowering Plants in Southern Africa. Science 174: 1033-1034

Gaff DF. 1977. Desiccation Tolerant Vascular Plants of Southern Africa. Oecologia 31, 95-109

Gaff DF, Latz PK. 1978. The Occurrence of Resurrection Plants in the Australian Flora. Australian Journal of Botany 26: 485-492

Gazzani S, Gendall AR, Lister C, Dean C. 2003. Analysis of the Molecular Basis of Flowering Time Variation in Arabidopsis Accessions. Plant Physiology 132: 1107-1114

Gendall AR, Levy YY, Wilson A, Dean C. 2001. The Vernalization 2 Gene Mediates the Epigenetic Regulation of Vernalization in Arabidopsis. Cell 107: 525-535

Guo H, Yang H, Mockler TC, Lin C. 1998. Regulation of Flowering Time by Arabidopsis Photoreceptors. Science 279: 1360-1363

Halliday KJ, Koornneef M, Whitelam GC. 1994. Phytochrome B and at Least One Other Response of Arabidopsis thaliana to Low Red/Far Red Ratio. Plant Physiology 104: 1311-1315

Hayama R, Izawa T, Shimamoto K. 2002. Isolation of Rice Genes possibly involved in the Photoperiodic Control of Flowering by a Fluorescent Differential Display Method. Plant Cell Physiology 43: 494-504

Hayama R, Yokoi S, Tamaki S, Yano M, Shimamoto K. 2003. Adaptation of Photoperiodic Control Pathways produces Short-Day Flowering in Rice. Nature 422: 719-722

Hecht V, Foucher F, Ferrándiz C, Macknight R, Navarro C, Morin J, Vardy ME, Ellis N, Beltrán JP, Rameau C, Weller JL . 2005. Conservation of Arabidopsis Flowering Genes in Model Legumes. Plant Physiology 137: 1420-1434

Henderson IR, Dean C. 2004. Control of Arabidopsis Flowering: The Chill Before The Bloom. Development 131 (16): 3829-38

Hotta CT, Gardner ML, Hubbard KE, Jin Baek S, Dalchau N, Suhita D, Dodd AN, Webb AAR. 2007. Modulation of Environmental Responses of Plants by Circadian Clocks. Plant, Cell and Environment 30: 333-439

Johanson U, West J, Lister C, Michaels S, Amasino R, Dean C. 2000. Molecular Analysis of *FRIGIDA*, a Mahor Determent of Natural Variation in *Arabidopsis* Flowering Time. Science 290: 344-347

Kojima S, Takahashi Y, Kobayashi Y, Monna L, Sasaki T, Araki T, Yano M. 2002. *Hd3a*, a Rice Ortholog of the *Arabdopsis FT* Gene, Promotes Transition to Flowering Downstream of Hd1 under Short-Day Conditions. Plant Cell Physiology 43 (10): 1096-1105

Koornneef M, Hanhart CJ, Van der Veen JH. 1991. A Genetic and Physiological Analysis of Late Flowering Mutants in *Arabidopsis Thaliana*. Molecular and General Genetics 229: 57-66

Laibach F. 1951. Uber sommer und winteranuelle Rasse von *Arabidopsis thaliana* (L.) Heyn. Ein Betrag zur Atiologie der Blumenbildung. Beitr. Biol Pflanz 28: 173-210

Lee H, Suh SS, Park E, Cho E, Ahn JH, Kim S, Lee JS, Kwon YM, Lee I. 2000. The AGAMOUS-LIKE 20 MADS Domain Protein integrates Floral Inductive Pathways in *Arabidopsis*. Genes and Development 14: 2366-2376

Levy YY, Mesange S, Mylne JS Gendall A, Dean C. 2002. Multiple Roles of Arabidopsis VRN1 in Vernalization and Flowering Time Control. Science 297: 243-246

Lin C. 2000. Photoreceptors and Regulation of Flowering Time. Plant Physiology 132: 39-50

Liu Y and Whittier R. 1995. Thermal Asymmetric Interlaced PCR: Automatable Amplification and Sequencing of Insert End Fragments from PI and YAC Clones for Chromosome Walking. Genomics 25: 674-681

Michaels SD, Amasino RM. 1999. Flowering Locus C Encodes a Novel MADS Domain Protein that acts as a Repressor of Flowering. Plant Cell 11: 949-956

Michiels A, Tucker M, van den Ende W, van Laere A. 2003. Chromosomal Walking of Flanking Regions From Short Known Sequences in GC-Rich Plant Genomic DNA. Plant Molecular Biology Reporter 21: 295-302

Mizoguchi T, Wright L, Fujiwara S, Cremer F, Lee K, Onouchi H, Mouradoy A, Fowler S, Kamada H, Putterill J, Coupland G. 2005. Distinct Roles of GIGANTEA in Promoting Flowering and Regulating Circadian Rhythms in Arabidopsis. The Plant Cell 17: 2255-2270

Mouradov A, Cremer F, Coupland G. 2002. Control of Flowering Time: Interacting Pathways as a Basis for Diversity. The Plant Cell 14(supplement): s111-s130

Napp-Zinn K. 1987. Vernalization. Environmental and genetic regulation. In: JG Atherton, ed, Manipulation of Flowering. Butterworths, London, pp 123-132

Ngoc Le T, Blomstedt CK, Kuang J, Tenlen J, Gaff DF, Hamill JD, Neale D. 2007. Desiccation – tolerant Specific Gene Expression in Leaf Tissue of The Resurrection Plant *Sporobolus stafianus*. Functional Plant Biology 34: 589-600

Onouchi H, Igeño IM, Périlleux C, Graves K, Coupland G. 2000. Mutagenesis of Plants Overexpressing CONSTANS Demonstrates Novel Interactions among Arabidopsis Flowering-Time Genes. The Plant Cell 12: 885-900

Platten JD, Foo E, Elliott RC, Hecht V, Reid JB, Weller JL. 2005. Cryptochrome 1 Contributes to Blue-Light Sensing in Pea. Plant Physiology 139: 1472-1482

Putterill J, Robson F, Lee K, Simon R, Coupland G. 1995. The CONSTANS Gene of Arabidopsis Promotes Flowering and Encodes a Protein Showing Similarities to Zinc Finger Transcription Factors. Cell 80: 847-857

Pyhajarvi T, Garcia-Gil MR, Knurr T, Mikkonen M, Wachowiak W, Savolainen O. 2007. Demographic History has influenced Nucleotide Diversity in Pinus sylvestris Populations. Genetics 177: 1713-1724

Robson F, Costa MMR, Hepworth SR, Vizir I, Piñeiro M, Reeves PH, Putterill J, Coupland G. 2001. Functional Importance of Conserved Domains in The Flowering-Time gene CONSTANS Demonstrated by Analysis of Mutant Alleles and Trangenic Plants. The Plant Journal 28 (6): 619-631

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF, Coupland G. 2000. Distinct Roles of CONSTANS Target Genes in Reproductive Development of *Arabidopsis*. Science 288: 1613-1616

Sambrook J, Russel DW. 2006. The Condensed Protocols, From: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schwechheimer C. 2008. Understanding Gibberellic Acid Signaling – Are we there yet? Current Opinion in Plant Biology 11 (1): 9-15

Sheldon CC, Burn JE, Perez PP, Metzger J, Edwards JA, Peacock WJ, Dennis ES. 1999. The FLF MADS box gene: a Repressor of Flowering in Arabidopsis Regulated by Vernalization and Methylation. Plant Cell 11: 445-458

Sherwin HW, Farrant JM. 1996. Differences in Rehydration of Three Desiccation –tolerant Angiosperm Species. Annals of Botany 78: 703-710

Sherwin HW, Farrant JM. 1998. Protection mechanisms against excess light in the resurrection plants Craterostigma wilmsii and Xerophyta viscosa. Plant Growth Regulation 24: 203-210.

Soitamo AJ, Piippo M, Allahverdiyea Y, Battchikova N, Aro E. 2008. Light has a Specific Role in Modulating *Arabidopsis* Gene Expression at Low Temperature. BMC Plant Biology 8: 13

Strayer C, Oyama T, Schultz TF, Raman R, Somers DE, Mas P, Panda S, Kreps JA, Kay SA. 2000. Cloning of the Arabidopsis Clock Gene *TOC1*, an Autoregulatory Response Regulator Homolog. Science 289: 768-771

Suárez-López P, Wheatley K, Robson HO, Valverde F, Coupland G. 2001. *CONSTANS* Mediates Between The Circadian Clock and The Control of Flowering in *Arabidopsis*. Nature 410: 1116-1120

Sung S, Amasino RM. 2004. Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. Nature 427: 159-164

Takahashi R, Yasuda S. 1971. Genetics of Earliness and Growth habit in Barley. In: Barley Genetics II (Proceedings of the Second International Barley Genetics Symposium). Seattle: Washington State University Press pp 388-408

Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K. 2007. Hd3a Protein is a Mobile Flowering Signal in Rice. Science 316: 1033-1036

Thomas B. 2006. Light Signals and Flowering. Journal of Experimental Botany 57: 3387-3393

Tranquilli G and Dubcovsky J. 2000. Epistatic Interaction Between Vernalization Genes *Vrn-*A^m*1* and *Vrn-*A^m*2* in Diploid Wheat. The Journal of Heredity 91 (4): 304-306

Trevaskis B, Bagnall DJ, Ellis MH, Peacock J, Dennis ES. 2003. MADS Box Genes control Vernalizaion-induced Flowering in Cereals. PNAS 100: 13099-13104

Trevaskis B, Hemming MN, Peacock WJ, Dennis ES. 2006. HvVRN2 Responds to Daylength, whereas HvVRN1 is Regulated by Vernalization and Developmental Status. Plant Physiology 140: 1397-1405

Trevaskis B, Hemming MN, Dennis ES, Peacock J. 2007. The Molecular Basis of Vernalization-Induced Flowering in Cereals. Trends in Plant Science 12 (8): 352-357

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G. 2004. Photoreceptor Regulation of CONSTANS Protein in Photoperiodic Flowering. Science 303: 1003-1006

von Zitzewitz J, Szucs P, Dubcovsky J, Yan L, Francia E, Pecchioni N, Casa A, Chen THH, Hayes PM, Skinnen JS. 2005. Molecular and Structural Characterization of Barley Vernalization Genes. Plant Molecular Biology 59: 449-467

Weller JL, Beauchamp N, Huub L, Kerckhoffs LHJ, Platten JD, Reid JB. 2001. Interaction of Phytochromes A and Bin the Control of De-etiolation and Flowering in Pea. The Plant Journal 26 (3): 283-294

Wilson RN, Heckman JW, Somerville CR. 1992. Gibberellin Is Required for Flowering in Arabidopsis thaliana under Short Days. Plant Physiology 100 (1): 403-408

Yan L, Loukojanov A, Tranquillli G, Helguera M, Fahima T, Dubcovsky J. 2003. Positional Cloning of the Wheat Vernalization Gene VRN1. PNAS 100: 3263-6268

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dubcovsky J. 2004. The Wheat *VRN2* Gene Is a Flowering Repressor Down-Regulated by Vernalization. Science 303: 1640-1644

Yan L, Fu D, Li C, Blechi A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Yasuda S, Dubcovsky J. 2006. The Wheat and Barley Vernalization Gene *VRN3* is an Orthologue of *FT*. PNAS (51) 103: 19581-19586

Yano M, Katayose Y, Ashikari M, Yamanouchi U, Monna L, Fuse T, Baba T, Yamamoto K, Umehara Y, Nagamura Y, Sasaki T. 2000. *Hd1*, a Major Photoperiod Sensitivity Quantitative Trait Locus in Rice, Is Closely Related to the Arabidopsis Flowering Time Gene *CONSTANS*. The Plant Cell 12: 2473-2484

Appendix A

EF418586: Xerophyta humilis 18S ribosomal RNA gene, partial sequence

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TGATGTACCGGCTACCACAATCCCAAGGGAAGCCAACCAGGCGCGCAAATTACCCAATCCCTGACCCGGG
GAGGTTAGTGACAATTAAATGACATACCGGGCTCAATCACTCCTGGTAATTGGAATGAGTACAATCTGAA
ATCCCTTAACGAGGATCCATTGGAGGGCCAATTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAG
CGTACATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTCGGACCTTGGGACGGGACGGTAGGTCCGCCCAC
CAGGGTGTGCACCGCCGGCCTCGTCCCTTCTGCCGGCGATGCGCTCCTGGCCTTAACTGGCCGGGTCGTGC
CTCCGGCGCCGTTACTTTGAAGAAATTAGAGTGCTCAAAGCAAGCCTACGCTCTGTATACATTAGCATGG
GATAACATCATAGGATTCCGGTCCTATTGTGTTGGCCTTCGGGATCGGAGTAATGATTAACAGGGACAGT
CGGGGGCATTCGTATTTCATAGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACCACTGCGAAAGC
ATTTGCCAAGGATGTTTTCATTGATCAAGAACGAAAGTTGGGGGCTCGAAGACGATCAGATACCGTCCTA
GTCTCAACCATAAACGATGCCGACCAGGGATCGGCGGATGTTGCTTTTAGGACACCGCCGGCACCTTATG
AGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGAC
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The forward primer is high lighted in yellow, the reverse primer in blue. Poly-A sequences for the $d(T)_{17}$ primer to anneal to is are highlighted in grey. It is possible longer poly-A sequences are present downstream of the shown sequence, as this is only a partial sequence. The full sequence was not available.