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Studies of genes potentially involved in the induction of flowering in *Festuca pratensis* (Huds.)

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CONTENT

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ABBREVIATIONS

CAPS – cleaved amplified polymorphic sequence
cDNA – complementary DNA
cds – coding sequence
InDel – insertion / deletion polymorphism
ORF – open reading frame
RT – reverse transcriptase
SNP – single nucleotide polymorphism
UTR – un-translated region
VR – vernalisation requirement

SAMMENDRAG

Mange planter i nordlige og kjølige områder krever en kuldeperiode (vernalisering) før blomstring kan starte. Ekspresjonen av noen gener som trolig er involvert i induksjon av blomstring i engsvingel (*Festuca pratensis*) ble undersøkt i denne oppgaven. Utgangspunktet var plantemateriale fra foreldrene og ti individer fra to ekstreme F2-populasjoner (en med høyt og en med lavt vernaliseringskrav) i vår kartleggingsfamilie. RNA fra vernaliserte og ikke vernaliserte planter ble isolert og cDNA syntetisert. cDNA fra disse individene ble brukt i RT-PCR for ekspresjonsanalyser og for noen av genene ble det gjort ytterligere undersøkelser.

VRN1 er en positiv regulator av blomstring i korn, og ekspresjonsanalysene i denne oppgaven viser at den trolig også induserer blomstring i engsvingel. *VRN1* var oppregulert av vernalisering i alle de testede plantene. Før vernalisering var uttrykk av genet i hovedsak funnet hos de plantene som kunne blomstre uten vernalisering, men ikke i de som hadde vernaliseringskrav.

Allel sammensetningen i F2 av en tilgjengelig *VRN1* CAPS markør hadde ingen klar sammenheng med vernaliseringskrav eller *VRN1* uttrykk. Dermed foreslås det at en positiv eller negativ regulator av *VRN1*, eller et annet område av *VRN1* regionen inneholder en polymorfisme mellom de to genotypene og dermed er ansvarlig for de observerte fenotypene. I korn er det blitt funnet variasjoner mellom genotyper med ulikt vernaliseringskrav i *VRN1* promoter sekvensen eller i genets første intron. Det foreslås at disse ulikhetene skaper variasjon i bindingsaffiniteten til transkripsjonsfaktorer som hemmer uttrykk av *VRN1*. I denne oppgaven ble de første 1000bp av *VRN1* intron 1 undersøkt, og det viste seg at denne regionen var svært lik i de to foreldrene (med forskjellig vernaliseringskrav). I sammenligning mellom *F. pratensis* og *Lolium perenne* i denne regionen ble det observert en stor insersjon/delesjon (indel) polymorfisme. Videre sekvensanalyser av disse to regionene er nødvendig for å fastslå om forskjellige *F. pratensis* genotyper har ulikheter som ligner de som er observert i korn.

VRN2 er en mulig repressor av VRN1. Det lyktes ikke å amplifisere *VRN2* i denne oppgaven.

VRT-2 er en mulig repressor av blomstring. I denne oppgaven vises tendenser som tyder på at ekspresjonen av dette genet avtar etter vernalisering i *F. pratensis* med vernaliseringskrav. Plantene som kan blomstre uten vernaliserings behandling viser stabil ekspresjon som er upåvirket av vernalisering.

PHYC og *RUBQ2* er gener som trolig er involvert i induksjon av blomstring. Disse genene har en litt uklar posisjon i blomsterinduksjons reaksjonssveien og resultatene i denne oppgaven ga heller ingen tydelige svar. Videre ekspresjonsanalyser er nødvendig for å trekke noen konklusjoner.

CONSTANS er en positiv regulator av blomstring i *Arabidopsis*. Genet ble sekvensert i denne oppgaven. Videre ekspresjonsanalyser er nødvendig for å si noe om CONSTANS sin rolle i vernaliserings indusert blomstring i F. pratensis.

ABSTRACT

Many plants in northern regions require a period of low temperatures and short days (called vernalisation) during the winter season to initiate the flowering process. In this experiment, some genes thought to be involved in vernalisation response and induction of flowering in meadow fescue (*Festuca pratensis*) has been investigated. The genes were chosen based on their possible contribution in the transition from vegetative to reproductive phase in cereals. The investigations were predominantly gene expression studies on vernalised and non-vernalised F2 and P plant material (from the mapping population of Alm et al. (2003)) from genotypes that are able to flower without vernalisation and genotypes with vernalisation requirement. These experiments were done using RT-PCR with cDNA from the different plant genotypes as templates. For some of the genes additional investigations were done.

VRN1, a positive regulator of flowering in cereals, was up regulated by vernalisation in *F. pratensis*. The results also showed a connection between *VRN1* expression and vernalisation requirement. Most vernalisation requiring plants expressed *VRN1* only after vernalisation. The ones that were able to flower without vernalisation had *VRN1* expression prior to vernalisation treatment also.

The F2 allele variation of the *VRN1* CAPS marker (Ergon et al. 2006) was not connected to *VRN1* expression or vernalisation requirement. This suggests that another gene, masking *VRN1* (a positive or negative regulator), or another part of the *VRN1* area contains a polymorphism between the two genotypes resulting in the respective different phenotypes. Genotypic differences have been identified in the *VRN1* promoter or intron 1 in cereals with differences in vernalisation requirement. These areas are suggested to contain binding sites for repressors of *VRN1*. The first 1kb of *F. pratensis VRN1* intron 1 was sequenced and shown to be identical in the two parents of our mapping population. One large indel polymorphism was found as a sequence difference between *F. pratensis* and *L. perenne* in this region.

VRN2 is thought to be a repressor of *VRN1* expression. *VRN2* was not successfully amplified in this experiment. But it is an interesting gene to study in this context, so designing of new primers for amplification is suggested.

VRT-2 is a putative repressor of flowering and has been investigated in wheat and barley. The very similar gene *MADS16* (studied in *L. perenne*) is probably the same gene. *VRT-2* expression has been shown to be low and stable in spring wheat and higher in non-vernalised winter wheat (Kane et al. 2005). Tendencies of the same expression pattern were observed in *F. pratensis* in this experiment. *MADS16* expression seemed to be down regulated by vernalisation in the plants that required vernalisation to flower. The plants that were able to flower without vernalisation had a *MADS16* expression un-affected by vernalisation. The degree of expression was similar in the plants that were able to flower without vernalisation and the vernalised plants with vernalisation requirement.

For *PHYC*, *RUBQ2* and *CONSTANS*, further investigation of expression is necessary to draw any conclusions concerning their role in vernalisation response and flower induction in *F. pratensis*. All three genes are suspected to be involved in induction of flowering in grasses. *PHYC* responds to photoperiod and *RUBQ2* is suspected to be up regulated by vernalisation, but their contribution in flower induction is not clear. Both genes seemed to be un-affected by vernalisation in *F. pratensis*, but *PHYC* seemed to be controlled by photoperiod. *CONSTANS* is a positive regulator of flowering in *Arabidopsis*.

INTRODUCTION

Meadow fescue

Meadow fescue (*Festuca pratensis* Huds.) is a diploid (2n=14) obligate outbreeding forage grass species. The genome size of this plant is approximately $1.83x10^8$ bp (1.9pg) per haploid genome (Seal, 1983). Meadow fescue grows under cool, moist conditions, and tolerates wet and occasionally flooded soils. Its tolerance to stress (cold in particular) makes it fit for cultivation in northern regions and after timothy (*Phleum pratense* L), *F. pratensis* is the most important forage grass in Norway. "Festuca" means grass or straw and "pratensis" means meadow. The plant has short creeping rhizomes and grows in clusters with rigid stems 0.3-1.0 meters tall (Feilberg, 2002). The leaves are 10-13 cm long, smooth under and usually rough above. The upright panicles are usually 7-25 cm long and often nodding at top. Flat, oval spikes (10-20 mm long) contain 6-12 individual flowers. Meadow fescue spikes and panicles are shown in figure 1.1.

Figure 1.1: **Meadow fescue**. The figure shows photos of the meadow fescue spikes (left) and panicles (right).

Meadow fescue belongs to the tribe Poeae, and is taxonomically closely related to ryegrass (*Lolium*).

Vernalisation

Vernalisation is the physiological process of induction of flowering by exposure to a cold period. Many plants in northern regions require a winter to start the transition from the vegetative to the reproductive state. For these plants, vernalisation either is absolutely

necessary for flowering or enhances the plant's ability to flower. The word vernalisation comes from the Latin word *vernum*, which means spring (Sung and Amasino, 2005). Cereals requiring vernalisation (called winter cereals) can be planted in the fall season, but flowering will not start until the cold period ends and the days get longer. This permits them to take full advantage of the favourable growing conditions in the spring.

Vernalisation is not to be confused with cold acclimation, the process making plants ready to survive exposure to cold temperatures. In temperate areas, like Norway, plants need a system to modify gene expression, membrane composition, metabolism and structural elements to withstand freezing temperatures encountered during winter. These alterations are induced by cold, non-freezing temperatures, and it is necessary for plants to become cold acclimated within a short period of time to survive sudden temperature changes. Vernalisation is a process that prevents flowering until the winter period is over. This provides protection of the temperature sensitive floral organs against the cold. It is important that this process does not start too early. Temperatures often fluctuate in the fall season, and it is critical for the plant that a short period of low temperatures followed by a warmer period is not sufficient for vernalisation (Sung and Amasino, 2005). So for vernalisation, low temperatures and short days over a longer period of time is necessary to indicate that the winter has passed. The optimal vernalisation temperature and number of weeks vary between different plants, but the temperatures needed are typically non-freezing temperatures below 10ºC. In many species vernalisation is not sufficient to induce flowering, but makes the plant competent to flower. Heide, (1994) has reviewed the control of flowering in grasses. Most temperate perennial grasses have a dual induction requirement for flowering. Primary induction is stimulated by low temperatures and/or short days (vernalisation). Secondary induction requires a transition to long days and is enhanced by higher temperatures. Both primary and secondary induction is necessary for flowering in plants with vernalisation requirement.

In some plants, only dividing cells (or cells in which DNA replication occurs) can become vernalised (Sung and Amasino, 2005). For these cells the competence to flower is mitotically stable, which means that the cells remember that winter has passed throughout the following season. This memory is lost during meiosis and the plants will require a new vernalisation period for flower induction in the next season.

In some cereal crop species (wheat, barley and oat) there are lines of genotypes requiring vernalisation (called winter lines) in addition to lines of genotypes with the ability to flower without vernalisation treatment (spring lines). In these species expression studies from the different lines can be compared to understand the genetics of induction of flowering by vernalisation.

Several genes are involved in vernalisation requirement and induction of flowering by vernalisation. These genes have been identified e.g. by mutants or by prior identification of chromosome regions responsible for this feature. These chromosomal regions are called QTL (Quantitative Trait Loci). Many agriculturally important characters are controlled by many genes and are known as quantitative traits. The regions within genomes that contain genes connected to a specific quantitative character are known as quantitative trait loci (e.g. vernalisation QTL) (Collard et al. 2005). QTL analysis has been used to identify chromosomal regions of genes controlling quantitative traits and to localise them on linkage maps (also known as QTL mapping).

The molecular control of vernalisation response has been studied in many plant species and particularly in the model plant *Arabidopsis thaliana* (Amasino, 2004, He and Amasino, 2005 and Henderson and Dean, 2004). In this plant, there are several signalling pathways involved in the induction of flowering, the long-day pathway, the vernalisation pathway and the autonomous pathway (reviewed by Putterill et al. 2004). Many of the genes involved in these pathways have been characterised and investigated. It is thought that the control of flowering in cereals and grasses involve similar gene pathways. Not as many genes have been characterised in those species, but Snape et al. (2001) reviews a number of genes controlling flowering time in wheat.

Some genes involved in vernalisation response and induction of flowering in cereals

Genes involved in induction of flowering have been sequenced and studied in a few members of the grass family, including wheat (Yan et al. 2003, 2004b), barley (vonZitzewitz et al. 2005) and perennial ryegrass (Martin et al. 2004, Jensen et al. 2005, Andersen et al. 2006 and Petersen et al. 2004, 2006). Models have been made (and some of them confirmed) suggesting

interactions between several genes and the mechanisms are thought to be similar in the different cereal species.

VRN1

The *VRN1* gene was identified and shown to be critical for flowering in hexaploid bread wheat (*Triticum aestivum*) and diploid wheat (*Triticum monococcum*) by Yan et al. (2003). *VRN1* have sequence similarity to the flower inducing *Arabidopsis* gene *APTALA1* (*AP1*). When discovered, *VRN1* was shown to be identical to other genes previously characterised and given several different names (e.g. *WAP1* by Murai et al. 2003 and Trevaskis et al. 2003 (wheat *AP1*), *TaVRT-1* by Danyluk et al. 2003 and *MADS1* by Petersen et al. 2004), and later *VRN1* became the most frequently used name.

VRN1 encodes a MADS box transcription factor that induces flowering. Yan et al. (2003) showed that *VRN1* is expressed only after vernalisation treatment in *T. monococcum* requiring vernalisation to flower, but in *T. monococcum* without vernalisation requirement *VRN1* is constitutively expressed. Jensen et al. (2005) identified *VRN1* in *L. perenne* (*LpVRN1*) on basis of sequence homology. The expression pattern of *LpVRN1* is similar to that of *TmVRN1* (Andersen et al. 2006 and Petersen et al. 2004, 2006).

VRN2

VRN2 encodes a transcription factor in the ZCCT family. The Z denotes a putative zinc finger domain located in the first exon, and the CCT domain in the second exon is similar to regions in *CONSTANS* (described below). Some other genes have also been shown to contain this CO, CO-like and TOC1 domain (CCT) (Yan et al. 2004b).

Yan et al. (2003, 2004b) proposed a model where the gene product of *VRN2* acts as a repressor of *VRN1* expression (repressing flowering). *VRN2* showed an opposite expression pattern of *VRN1*. The gene is transcribed only prior to vernalisation treatment in wheat plants requiring vernalisation. Vernalisation is thought to turn off transcription of *VRN2*, presumably allowing *VRN1* to be transcribed and flowering to be induced. If repression by VRN2 prohibits flowering, this repression is inactivated in spring lines (able to flower without vernalisation). The models for the mechanism of VRN2 inactivation in spring lines differ in different species. Mutations in the *VRN2* open reading frame (ORF) (possibly creating a non-

functional repressor protein) or deletions in the *VRN1* promoter area (possibly creating a non-functional repressor binding site) was observed as genetic differences in spring diploid wheat (*T. monococcum*) in comparison to winter plants (Yan et al. 2003). These *VRN2* ORF and *VRN1* promoter mutations were not observed in polyploid wheat (Yan et al. 2004a). However, large deletions in the first intron of *VRN1* in spring lines of barley, hexaploid wheat and tetraploid wheat have been observed and suggested to influence the VRN2 repressor binding affinity (Fu et al. 2005). In barley, vonZitzewitz et al. (2005) also found that the *VRN2* locus was present in winter genotypes, but deleted in spring lines.

Dubcovsky et al. (2006) showed a down-regulation of wheat *VRN2* in short day treatments. But in room temperature this *VRN2* down-regulation was not followed by up-regulation of *VRN1* until plants were transferred to long days again. This suggested an existence of another repressor of *VRN1* in addition to *VRN2*, repressing *VRN1* transcription until longer days appear. *VRT-2* (described below) has been suggested to be a *VRN1* repressor (Kane et al. 2005).

VRT-2

VRT-2 is regulated by vernalisation and photoperiod in wheat (Kane at al. 2005) and is located close to a vernalisation QTL in barley (Szucs et al. 2006). A very similar gene, called *MADS16* (DQ110011), has been sequenced in *L. perenne*. *MADS16* and *VRT-2* might be the same gene, but that has not been confirmed. *TaVRT-2* most likely encodes a member of the MADS-box family of transcription factors responsible for flower repression in several species (Kane et al. 2005). Kane et al. (2005) found the expression of *TaVRT-2* to be low and stable in spring wheat and higher in winter genotypes prior to vernalisation. The expression was down regulated after vernalisation in winter wheat, and *VRN1* started to accumulate. They also showed that *Ta*VRT-2 interacted with several transcription factors involved in flowering control and vernalisation response in wheat, including the gene products of *VRN1* and *VRN2*.

CONSTANS

CONSTANS (*CO*) is a gene known to be important in the initiation of flowering in response to changes in day length in *Arabidopsis* and has been extensively studied (Robson et al. 2001, Hayama and Coupland, 2004). Its analogue in rice, *Hd1*, has also been identified (Yano et al. 2000). *CO* is a transcription factor and a positive regulator of flowering.

Armstead et al. (2005) and Martin et al. (2004) cloned and characterised the gene *LpCO* from *L. perenne* suggesting it to be an *Hd1* orthologue. The expression of *LpCO* has been shown to increase during long days (compared to short days). The expression has also been shown to differ at different times of the day, it is regulated by the circadian clock (Martin et al. 2004).

In attempt to amplify and map *VRN2*, Andersen et al. (2006) mapped another CO-like gene, which turned out to be identical to the *LpCO* identified by Armstead et al. (2005) and Martin et al. (2004). Griffiths et al (2003) found 16 CO-like genes in the barley genome in their mapping studies. It is suggested that different CO-like genes also exists in other grasses.

The aims and scope of the present investigation

The aim of this study was to investigate some genes suspected to be involved in the induction of flowering in meadow fescue (*F. pratensis*). The genes were chosen on basis of their contribution in the induction of flowering in cereals and perennial ryegrass.

The mapping population of Alm et al. (2003) was the starting plant material. This population originated from a cross between the Norwegian genotype HF2/7 (♂) and the Yugoslavian genotype BF14/16 (♀) (P). From these parents an F1 and an F2 generation was generated. (F1 denotes the first generation arising from two parents (P). The generation arising from intercrossing the F1 generation is denoted F2, and so on.) The female P, BF14/16, require 12 weeks of vernalisation at 6ºC and 8h photoperiod to flower (resembling a winter cereal). The male P, HF2/7, can flower without vernalisation treatment, but the flowering is more powerful after vernalisation. HF2/7 resembles a spring cereal in its ability to flower without vernalisation. The difference is that vernalisation does not further enhance flowering in spring cereals.

One population requiring vernalisation and one population without vernalisation requirement from the F2 generation was compared in expression studies of the genes in question to search for differences between the two populations. Differences in gene expression in vernalised and non-vernalised plants and at three different times during the spring season was also investigated, as well as differences between the two parents.

The genes not already positioned on a chromosome map were attempted mapped. Differences contributing to possible *VRN1*-repressor binding affinity were also attempted localised and investigated.

The genes described above (*VRN1*, *VRN2*, *MADS16* and *CONSTANS*) and two additional genes (*PHYC* and *RUBQ2*) were investigated in this study. The genes *PHYC* and *RUBQ2* are thought to be involved in the induction of flowering. These suspicions are based on previous results obtained in our laboratory. *PHYC* is mapped on *F. pratensis* chromosome 4 (Henriksen, 2005), closely linked to *VRN1* in the QTL for vernalisation requirement. *RUBQ2* is one of the genes identified and collected in a library of genes being up regulated by vernalisation (Rudi et al. (*in prep*)).

Questions to be answered:

- Does *FpVRN1* have an expression pattern as expected for a positive regulator of flowering induced by vernalisation? (That is, up regulated by vernalisation and only expressed prior to the vernalisation treatment in the plants that are able to flower without vernalisation)
- Do *FpVRN2* and *FpMADS16* show expression patterns as expected for negative regulators of flowering? (That is, down regulated by vernalisation in the plants that require vernalisation and low or absent in the ones that are able to flower without vernalisation)
- Are there differences in *F. pratensis* between the parent requiring vernalisation and the parent that are able to flower without vernalisation in the *VRN1* promoter area or the first intron, indicating possible differences in repressor binding sites?
- Or are there differences between the two parents in the repressor (*FpVRN2* or *FpMADS16*) itself, creating possible differences in binding affinity?
- Does *FpCO* show an expression pattern as expected for a positive regulator of flowering? (That is, up regulated when flowering is initiated)
- Do *PHYC* and *RUBQ2* show expression patterns indicating that they are involved in vernalisation response and induction of flowering?
- Where on the *F. pratensis* chromosome map are *VRN2*, *MADS16* and *CONSTANS* found?

MATERIALS AND METHODS

The expression of some genes assumed to be involved in induction of flowering by vernalisation was investigated in this experiment. The plant material consisted of a family of meadow fescue (*F. pratensis*) genotypes and RNA was isolated from non-vernalised and vernalised plants. Complementary DNA (cDNA) was then synthesised from total RNA. The cDNA was used as template in RT-PCR for investigation of the expression pattern of genes that were suspected to be involved in the transition from the vegetative to the reproductive state.

Background and preparation of plant material

The starting point for this experiment was the *F. pratensis* mapping population of Alm et al. (2003), which resulted from a cross between the Norwegian genotype HF2/7 (♂) and the Yugoslavian genotype BF14/16 (♀).

Clonally maintained plants of the mapping population (F1 generation) had previously been characterized for vernalisation requirement (VR) (Ergon et al. 2006). The plants were given 0, 6, 9 or 12 weeks of vernalisation treatment at 6°C and 8 hours photoperiod, and then transferred to long photoperiod (16h) and warmer temperatures (18ºC to 24ºC, increasing through the spring season). The VR for each individual was determined according to the number of weeks of vernalisation shown to be required for flowering.
VR=1: Plants flowering after 0 weeks vernalisation treatment.
VR=2: Flowered after 6 weeks treatment, but not after 0 weeks treatment.
VR=3: Flowered after 9 weeks treatment, but not after 6 weeks treatment.
VR=4: Flowered after 12 weeks treatment, but not after 9 weeks treatment.
VR=5: Did not flower in any of the treatments.

Later, clonal plants of 15 F1 genotypes with VR=1 and 15 F1 genotypes with VR=4 were vernalised and allowed to flower, pollinate each other and set seeds in two separate chambers. Thus two extreme F2-populations were generated. F2 population 1 arose from F1 genotypes with VR=1 and F2 population 2 from F1 genotypes with VR=4.

Seeds were sown and approximately 22 individuals from each F2-population were tested for VR as described above. In the same experiment (experiment 1), some plants were used for sampling tissue material for gene expression analyses. Ramets consisting of 3 shoots each were placed in pots with a peat/soil mixture and grown in the greenhouse. The temperature was approximately 17°C (day) / 15°C (night) and 16h additional light was supplied with Osram HQI bulbs. After 3 weeks, plants to be vernalised were placed in a controlled environment chamber at 6°C with 8 hours photoperiod at approximately 70µmol $m^{-1}s^{-1}$ (Osram HQI bulbs) for 12 weeks. Tissue samples were taken from one pot per genotype and treatment after approximately 5 weeks in the greenhouse (for the non-vernalised plants) or at the end of the 12 weeks vernalisation treatment. The soil was rinsed off in water, the outer leaves were peeled off the individual tillers, and the 3-5 mm lower section of the stem bases were excised, frozen in liquid nitrogen and then kept at -80°C. A replicate experiment (experiment 2) was also performed. In this experiment an error was made setting the conditions for vernalisation. The temperature was still 6ºC, but the light and dark periods were switched, giving 8 hours darkness and 16 hours photoperiod. In experiment 2, samples from the non-vernalised plants were taken at two different time points (table 2.1).

An overview of the different experiments and treatments are given in table 2.1. Samples from the non-vernalised plants were taken at three different dates (experiment 1 in January and experiment 2 in March and May). This creates a series of plant samples throughout the spring season, from low temperatures and short days to higher temperatures and longer days (in the greenhouse).

Table 2.1: **Experiments and treatments performed on the *F. pratensis* plants.** The table shows the vernalisation treatments and sample dates in each experiment. Five genotypes from each F2 population in addition to the two parents were included in each treatment and sampled at the given dates. The first flowers appeared in March.

Experiment:	1		2		
Vernalisation treatment:	Non-vernalised	Vernalised 12 weeks	Non-vernalised	Non-vernalised	Vernalised 12 weeks
Sampling date:	14.01.05	01.01.05	16.03.05	29.05.05	28.05.05
Flower appearance:	Prior to flowering		Flowering initiated in population 1	All the plants in population 1	

				flowered	

Five genotypes with VR=1 from F2 population 1 (1-3, 1-4, 1-8, 1-12 and 1-14), and five genotypes with VR=4 from F2 population 2 (2-5, 2-6, 2-8, 2-11 and 2-18) were chosen for this experiment. Plant material from the five genotypes from each F2 population, which were exposed to the five different treatments/sample time points, in addition to one per treatment (and experiment) per grandparent (P) were available at -80ºC when I started the project.

RNA isolation

The plant material, ~50mg stem basis from each of the 59 F2 and P plants, was frozen on liquid nitrogen and crushed in eppendorf tubes containing small (5mm) stainless steel beads made of tungsten in a TissueLyser Mixer Mill Type MED MER 301 (Retsch, Haan, Germany). Total RNA was isolated using the Total RNA isolation TRIZOL reagent protocol (Invitrogen, CA, USA):

- Added 1ml TRIZOL reagent to the plant material, homogenised the samples and incubated for 5 min in room temperature.
- Added 200µl chloroform, shook for 15 sec and incubated in 2-3 min at room temperature.
- Centrifuged at 12,000 x g for 15 min at 4ºC.
- Transferred the aqueous phase to a clean tube and precipitated the RNA by adding 250µl isopropanol and 250µl salt precipitation solution (8M sodium citrate and 1.2M NaCl).
- Incubated for 10 min at room temperature and centrifuged in 10 min at 12,000 x g, 4ºC.
- Washed the RNA pellet in 1ml 75% ethanol and centrifuged for 5 min at 7,500 x g, 4ºC.
- Dried the RNA pellet and dissolved it in 20µl in RNase free DEPC (diethylpyrocarbonate)-treated H_2O at 60ºC for 10 min.

Determination of the RNA concentrations in the samples was done based on values given by measurement on a NanoDrop 300 N.A. spectrophotometer in two parallel sample dilutions.

The concentrations were confirmed by comparison to the fragment intensities in electrophoresis of the individual samples on an 1% agarose gel.

DNase treatment

The RNA samples were treated with DNase to make sure no genomic DNA was left in the samples. Ten µg total RNA from each sample was treated with the TORBU DNase Treatment and Removal Reagent system (Ambion, TX, USA) in the following protocol:

- A 50µl reaction containing the following ingredients:
  - 10µg RNA + DEPC treated H_2O to 44µl
  - 5µl TURBO DNase buffer
  - 1µl TURBO DNase

  Incubated at 37ºC for 30 min.
- Added 5µl resuspended DNase inactivation reagent and incubated for 2 min at room temperature.
- Centrifuged in 1.5 min at 10,000 x g and transferred the RNA to a clean tube.

cDNA synthesis

Complementary DNA, cDNA, was synthesised using reverse transcriptase (RT) in the kit SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Reverse transcriptase is an enzyme that translates RNA to DNA. This creates one DNA (cDNA) strand complementary to the RNA strand in a DNA-RNA hybrid. The RNA strand is then degraded by RNase H, leaving single stranded cDNA ready for PCR. In this case, total RNA was isolated and used as template for cDNA synthesis. The mRNA (which make up 1-5% of the total RNA) are the interesting fragments for the expression study.
Like polymerase, reverse transcriptase needs a primer to start the DNA synthesis. There are three alternative primer methods:

- Gene specific primers can be used to synthesise cDNA when the mRNA sequence is known. Primers like those used in PCR are used and only the fragment in question is synthesised.
- Oligo(dT) primers consists of a number of thymine residues. These primers bind the 3´ poly(A) tails of the mRNA and cDNA synthesis starts at the end of the mRNA. It may be difficult to get transcripts of the entire 5´ end of the

mRNA when oligo(dT) primers are used, but that depends on the length of the template.

- Random hexamers are (as given by the name) six and six nucleotides put together at random. These primers will bind at several locations in the total RNA and cDNA synthesis starts from there.

In this study random hexamers were used. Some of the PCR primers that were available were known to amplify the 5´end and part of the 5´UTR (5´un-translated region, a transcribed region preceding the protein sequence) of the respective genes.

The protocol was as follows:

- RNA/primer mixtures:
  - 8µl (1,6µg) total RNA
  - 1µl random hexamers (50ng/µl)
  - 1µl dNTP mix (10mM)
  - DEPC treated water to 10µl

  Incubated at 65ºC for 5 min, and then on ice for 1 min.
- Added a reaction mixture of the following components and amounts to each sample:
  - 2µl 10X RT buffer
  - 4µl 25mM $MgCl_2$
  - 2µl 0.1M DTT
  - 1µ RNaseOUT Recombinant Ribonuclease Inhibitor

  Incubated at 25ºC for 2 min.
- Added 1µl (50units) of SuperScript II RT and incubated at 25ºC for 2 min.
- Incubated at 42ºC for 50 min.
- Terminated the reactions at 70ºC for 15 min and chilled on ice.
- Added 1µl RNase H and incubated at 37ºC for 20 min.

The cDNA synthesis was done in 5 batches, each containing the 12 samples from one experiment/treatment/sample date (5 genotypes from population 1, 5 genotypes from population 2 and the 2 parents). In addition to these individual cDNA samples, cDNA was also synthesised from bulked samples. Equal amounts of RNA from the five genotypes from each population and treatment were combined and used as template in the synthesis of one cDNA sample representing all five genotypes. Bulk 1 consisted of population 1 in batch 1,

bulk 2 of population 2 in batch 1 and so on (see table 3.1). This gave ten bulked cDNA samples, one for each population in the five sampling time points, parents excluded.

cDNA synthesis was also done in a +RT-control by using the kit RNA, and a –RT-control by using a bulk of the 12 RNA samples in each cDNA synthesis batch of individual samples. The +RT control would, if necessary, show if the cDNA synthesis was successful. PCR using the –RT control (without reverse transcriptase added in the cDNA synthesis) as template would amplify fragments only if remains of DNA was present in the samples after the DNase treatment.

Developing PCR protocols

Polymerase chain reaction, PCR

Polymerase chain reaction is one of the most revolutionary methods in genetics The method gives amplification of specific DNA sequences by an enormous factor. For PCR, we need two oligonucleotide primers that flank the DNA sequence in question. The primers hybridise to opposite strands of denatured DNA and allow synthesis by DNA polymerase to begin. The principle of the PCR reaction is based on the fact that DNA molecules denaturates and anneals at different temperatures. These temperature changes occur in cycles to produce many copies of the DNA sequence. The first step is denaturation of double stranded DNA at 94ºC. Then the primers anneals to their complementary sequences at about 55ºC (depending on the primer base composition and length). Extension by the DNA polymerase from the two primers has an optimal temperature of 72ºC. This cycle is repeated 25 to 40 times to give exponential amplification of the desired DNA sequence. After 30 cycles, theoretically about 268 million copies is created. The DNA polymerase used in PCR is the thermo-stable Taq polymerase isolated from the bacteria *Thermus aquaticus*. This enzyme can survive the high temperatures and the temperature changes during the reaction.

The specificity of the PCR reaction depends on the specificity of the primers. The primers should be 17 to 30 nucleotides long. A GC content of about 50% is ideal. A lower GC content would lower the melting temperature. Sequences of long runs of a single nucleotide should be avoided and primers with significant secondary structures are undesirable. Complementarities

between the two primers will give primer dimers, which reduce the effectiveness of the amplification.

Before initiating the gene expression study on the cDNA, functional PCR protocols for the different genes were attempted developed using genomic DNA from the parents of the mapping population (BF16/16 and HF2/7) as template. Where genomic amplification was unsuccessful, amplification was attempted using cDNA as template. The ingredients, concentrations and amounts used in each PCR reaction are shown in table 2.2.

Table 2.2: **Standard ingredients used in the PCR reactions.** The table shows the components, amounts and concentrations used in each PCR reaction. Either 2µl DNA or 1µl cDNA was used as template.

Ingredient	**Final concentration**	**# µl in a 20µl reaction**
H_2O		To 20µl
10x PCR buffer for DyNAzymeII	1x	2µl
dNTP mix (10mM of each)	200µM	0,4µl
20µM Forward primer	0,4µM	0,4µl
20µM Revers primer	0,4µM	0,4µl
Taq polymerase, 2U/µl DyNAzymeII	0,05U/µl	1U (0,5µl)
DNA/cDNA template	1,5-5ng/µl	2µl/1µl

The PCR reactions were run on a Mastercycler ep (Eppendorf AG, Hamburg, Germany). The PCR program used was as follows (the number of cycles varies):

- 94°C 5 min

n cycles of:

- 94°C 30 sec, denaturation
- 55°C* 30 sec, annealing
- 72°C 30 sec**, extension

Final extension:

- 72°C 5 min

* The annealing temperature was adjusted to give optimal annealing conditions for the primers. Several temperatures were tried for each gene. In some cases it was necessary to lower the annealing temperature to get a satisfying amount of PCR product or to increase the temperature to eliminate undesirable PCR products.

**The extension time was prolonged to 45sec or 1min for the longer fragments (~500-1000bp).

To visualise the outcome of the PCR reactions the samples were run on agarose gels.

Gel electrophoresis

Gel electrophoresis is used to separate DNA fragments of different lengths as an analytical method or to purify specific DNA fragments. The gel is composed of agarose (used for fragments of 200bp to about 20kb) or polyacrylamide (used for smaller DNA fragments). An agarose gel is a complex network of polymeric molecules and the pore size depends on the buffer and the agarose concentration used. The gel containing the DNA sample is put in an electric field causing the negatively charged DNA to migrate towards the positive pole of the field. The speed of the DNA migration through the gel pores depends on the length of the fragments. Long fragments migrate slower (that means shorter) in the gel than short fragments. To decide the actual length of the different fragments comparison to molecular DNA ladders containing fragments of known sizes are used.

The gels were made of 1.5% or 2% agarose in 1xTAE-buffer with 1µl 10mg/ml ethidium bromide per 50ml gel. 2% agarose was used to visualise small fragments (<250bp) and to give better separation and more defined bands when necessary. 6x gel loading buffer (50% glycerol and bromphenolblue) was added to the PCR products before applied to the gel. The 50ml gels were run at 70V and the 250ml gels were run at 100V for 45 minutes. To view the gels a GEL Doc 2000 (BIO-RAD, CA, USA) with UV-light was used. The computer program Quantity One 4.3.1. Gel doc visualised the results. For identification of the fragment size, a 50bp or 1kb ladder (Invitrogen) with known fragment sizes and concentrations were used (figure 2.1).

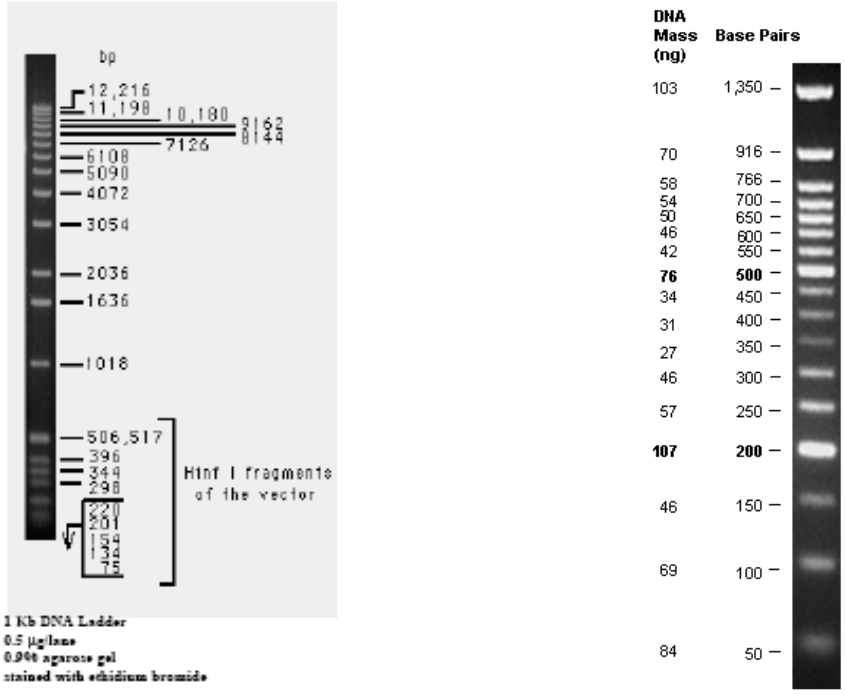

Figure 2.1: **The DNA ladders used.** The figure shows the 1kb DNA ladder (left) and the 50bp DNA ladder (right) used to decide the fragment lengths and concentrations in the agarose gels.

To verify that the gel fragments represented the desired genes, they were sequenced and compared with other sequences publicised in the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/).

Sequencing

There are different methods for identification of the base composition of a DNA sequence. One method often used is the dideoxy chain termination procedure. DNA polymerase has the ability to use 2',3'-dideoxynucleotides as substrates. Dideoxynucleotides (dideoxynucleoside triphosphate) lacks the 3' OH group and so, incorporation of this base instead of the usual 2'-deoxynucleotide terminates the elongation of the DNA molecule. Due to the fact that DNA polymerase randomly chooses a deoxy or a dideoxy base, presence of the four dideoxy bases together with the four deoxy bases in a PCR reaction produces DNA molecules of different lengths each ending with a dideoxy nucleotide. When this PCR product is run on a high-resolution gel, fragments differing in length of a single base can be separated. If the PCR is run in four separate tubes each containing all the four deoxy bases, but only one of the four dideoxy bases, the fragment lengths from each tube will give the position of the dideoxy base added in that tube respectively. Alternatively, dideoxy nucleotides labelled with fluorescence

can be used. In this procedure each of the four dideoxy bases is given a different colour, so the sequence reaction can be run using one tube (or two tubes, one with the forward primer and one with the reverse one). The PCR product will then contain DNA fragments of different lengths, each ending with a dideoxy nucleotide added the fluorescence colour of that specific base. This sample is run on a high-resolution gel in the sequence instrument and the fluorescent colour and the length of each fragment is read and memorised giving the complete sequence of the amplified fragment.

The fragments were cut out of the gels and purified using the QIAEX II Gel Extraction Kit (Qiagen, USA). The sequencing was done using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The amount of template necessary was dependent on the length of the sequences:

- 100-200bp 1-3ng
- 200-500bp 3-10ng
- 500-1000bp 5-20ng
- 1000-2000bp 10-40ng
- >2000bp 20-50ng

The concentrations of the fragments were estimated based on comparison to the DNA ladders. In addition to the template, the sequencing reactions contained the following components and amounts:

- Premix 2µl
- Primer 1,6µl (2mM primer solution)
- 5x seq. buffer 3µl
- dH_2O up to 20µl

The sequencing reactions were run at the same Mastercycler ep (Eppendorf AG) in the following program:

- 96°C – 1min

25 cycles of:

- 95°C – 10sec
- 50°C – 5sec
- 60°C – 4min

The Ethanol/EDTA/sodium acetate precipitation (BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems)) was used to precipitate the sequence products and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) visualised the sequences.

The sequences were edited in BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/page2.html) according to the quality of the curves in the sequencing diagram, and tested in a BLASTN or TBLASTX search to verify its identity. In a BLASTN search a nucleotide sequence is compared to all nucleotide sequences in the database. A TBLASTX search translates the nucleotide sequence and compares it to all translated nucleotide sequences in the database. The best protein sequence hits are shown. Alignments with available sequences or of the two parents were done using ClustalW (http://www.ebi.ac.uk/clustalw/).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR is a PCR reaction using cDNA as template. This method is said to be semi-quantitative because the expression of a gene can be quantified to a certain degree. The amount of mRNA of the gene of interest present, defines the amount of template for the PCR amplification, and is represented in the intensity of the fragment on the agarose gel. It can be seen if a fragment (representing a certain mRNA) is present or absent. Differences in fragment intensity between to fragments can also be seen, and the degree of difference can to a certain degree be calculated using the Gel doc computer program. But when the differences are small, they are hard to distinguish. And the band intensity may vary between gels, which makes comparison of different gels harder. This means that differences in expression of different genes or the same gene from different individuals can to a certain degree be seen using this method.

I had to make sure that the PCR reaction below saturation for the gene expression RT-PCR analyses to be meaningful. A PCR reaction gives an S-shaped curve of fragments after increasing number of cycles. After a given number of cycles (depending on the length of the fragment, amount of ingredients and the activity of the polymerase) additional fragments will not be amplified if the run continues. At this saturation point, it will be hard to distinguish differences in expression. The number of cycles should lie within the linear area of the PCR

S-curve. To be sure of this, at least two different PCR reactions with different numbers of cycles were run. If the agarose gels of one PCR set-up showed higher band intensity with increased n, the reaction with the lower n would consequently lie within the linear unsaturated area.

Designing the primers

Several genes are expected to be involved in the transition from vegetative to reproductive state in plants. Induction of flowering by vernalisation has been investigated in several cereals. Research has been done on wheat (*T. aestivum*, *T. monococcum*), barley (*Hordeum vulgare*) and ryegrass (*Lolium perenne*), among others. When reading this literature, some genes thought to be involved in this process were chosen to be investigated in *F. pratensis* in this study. The primers were in general either copies of primers used by others for the gene in question, or designed based on conserved regions of the gene in alignments with available sequences from other grass species. The primers were designed using the program PRIMER3 (Rozen and Skaletsky, 2000).

GAPDH

GAPDH is a constitutively expressed housekeeping gene and a frequently used control gene. The *GAPDH* primers used in this experiment were the same as the *GAPDH* primers used by Petersen et al. (2004). The sequences are shown in table 2.3.

ACTIN

ACTIN is also a housekeeping gene often used as control. The *ACTIN* primers are based on the *L. perenne ACTIN* sequence given by Heidi Rudi (*pers. comm.*), shown in table 2.3.

VRN1

The VRN1 primers are based on the *L. perenne VRN1* sequence. These primers were already available and known to amplify *VRN1* from *F. pratensis* (acc DQ108934). The primers are called lpVRN1_19for and lpVRN1_344rev and were used by Ergon et al. (2006), originally designed by Jensen et al. (2005). The sequences are shown in table 2.3.

VRN2

For *VRN2* five sets of primers were tried. All of the primers were based on the diploid wheat (*T. monococcum*) *VRN2* sequence from the article of Yan et al. (2004b), (acc. AY485644). Three of the primer sets were designed based on conserved regions of the Yan et al. (2004b) wheat *VRN2* sequence aligned to *VRN2* from other species (*Triticum turgidum* (AY485979), *H. vulgare* (AY485978, AY485977, Yan et al. 2004b). The last to primer sets were copies of primers used by Yan et al. (2004b). The sequences of the primer sets are shown in table 2.3.

MADS16

The first *MADS16* primers were designed based on the *L. perenne MADS16* sequence (DQ110011) in conserved regions in alignment with the wheat (*T. aestivum*) *TaVRT-2* (DQ022679, Kane et al. 2005) and the barley (*H. vulgare*) *HvVRT-2* (DQ201168, Szucs et al. 2006) sequences. These three sequences were very similar (about 90% identity) and are suggested to encode the same protein. To be sure of complete primer match in the expression studies, new (*F. pratensis*) *FpMADS16* primers were designed based on the sequence obtained using the (*L. perenne*) *LpMADS16* primers. The *FpMADS16* primers used in the RT-PCR are shown in table 2.3.

CONSTANS

The primers tried for amplification of *CONSTANS* are one of the sets used by Andersen et al. (2006) (vrn2_3_1F/vrn2_3_2R) to amplify a *CO*-like gene in *L. perenne*. The sequences are given in table 2.3.

PHYC

The *PHYC* primers were already available at the laboratory and were known to amplify *PHYC* from *F. pratensis* using 55ºC annealing temperature (Rudi et al. (*in prep*.), Henriksen, 2005). The sequences are shown in table 2.3.

RUBQ2

The primers used for amplification of *RUBQ2* were also available in the laboratory (Rudi et al. (*in prep*)). These primers had previously amplified three fragments from *F. pratensis* using genomic DNA as template (Henriksen, 2005). The sequences are shown in table 2.3.

Table 2.3: **Primer sequences for amplification using cDNA as template.** The table shows the sequences of the primers used or attempted in the RT-PCR expression studies. The annealing temperatures tried, the number of cycles and the expected lengths are shown.

Gene	For primer / Rev primer	Annealing temperature	Number of cycles (n)	Expected length cDNA
GAPDH	5′-CAGGACTGGAGAGGTGG-3′ / 5′-GGTACGACAACGAGTGAA-3′	55ºC	24, 21	380bp
ACTIN	5′-TCCTTCGTCTTGACCTTGCT-3′ / 5′-AAGCTGCTGGAATCCATGAG-3′	60ºC, 55ºC, 50ºC	40	305bp
VRN1	5′-TCTCCTCTTCTTCCCCACTG-3′ / 5′-CTACGAGTTCGCAACCGACT-3′	55ºC	30, 27, 26	320bp
VRN2	5′-ATGACTATTGACACAGAGATGATGG-3′ / 5′-GAGTCCAGAAAAGCTTACGCTGAG-3′ 5′-CAGCTGTGTGAGTACCAGTTCTTC-3′ / 5′-AAATAAGTCGGTTAGTGATTGATCG-3′ 5′-GACTATTGACACAGAGATGATGGTG-3′ / 5′-CTTCGCCCTATGATCCTAGTAAACT-3′ 5′-CCAACACATGGCTCACCTAGTG-3′ / 5′-ACCAACACTATTAGCAATGAAGCAA-3′ 5′-GCAATCATGACTATTGACACA-3′ / 5′-CATCATCTCCAGCTTCGCCC-3′	55ºC, 50ºC, 48ºC	40	Variable
MADS16	5′-GTGCGCGTTGAGGTTTCC-3′ / 5′-ATCATTCGTGCGAACTACGT-3′	55ºC	31, 28, 25	960bp
CONSTANS	5′-CGCGAAAATATCAGAAGCGG-3′ / 5′-GCGAACCTTAATTGATAAGGTA-3′	50ºC*	40	223bp
PHYC	5′-TATCTTGGCCTGCACTACCC-3′ / 5′-ATTTCAGGAACGGAAGAGCA-3′	55ºC	35, 30	1400bp
RUBQ2	5′-GCAGCTCCAACACAAAGACA-3′ / 5′-CAGGTGGAGGGTAGACTCCTT-3′	60ºC	35, 30	297bp

* For amplification of *CONSTANS* the PCR program used was the same as the one used by Andersen et al. (2006); 5min at 94ºC followed by 30 cycles of 20s at 94ºC, 20s at 50ºC and 60s at 65ºC, final extension 5min at 65ºC.

The RT-PCR was first done using the bulk cDNA samples as template. This was done as a preceding experiment to define expression differences and to decide which annealing temperature and number of cycles to use. Then this PCR protocol was used on the individual cDNA samples. The PCR amplifications of each gene from all the individual cDNA samples were done in the same PCR reaction and ran on the same gel to make the basis for the comparison as exact as possible.

MADS16 chromosome mapping attempt

A CAPS (cleaved amplified polymorphic sequence) marker is a polymorphism making a restriction enzyme target sequence present in one parent and absent in the other. Specific single genes of interest are PCR amplified. Digestion by the restriction enzyme whose target

sequence is present in one parent, gives different fragment patterns of the two parents on an agarose gel. The digestion is performed on a set of progenies to decide the allele variation. This marker can be mapped on the chromosome relative to other markers mapped in the same family.

The *FpMADS16* cDNA sequences of the grandparents (BF14/16 and HF2/7) were used in attempt to find a SNP (single nucleotide polymorphism) useful as a CAPS marker. The sequences were aligned using BioEdit and ClustalW.

MADS16 was only successfully amplified from cDNA. It was therefore suspected that the genomic sequence contained introns. Comparing the *FpMADS16* cDNA sequence to the genomic rice (*Oryza sativa*) chromosome 6 sequence (acc. AP008212) showed a possible exon/intron arrangement. New primers were designed amplifying intron 2 and 3:

- *FpMADS16*_F5: 5´-ATGAACAACTCGCTGAAGCA-3´
- *FpMADS16*_R5: 5´-TCATGCAACAGATCAGTGACC-3´

PCR using the primers F5/R5 was done in 40 cycles and 50ºC annealing temperature using genomic DNA from the two grandparents (P) as template. Submitting the sequences in NEBcutter v2.0 (http://tools.neb.com/NEBcutter2/index.php) showed all restriction enzymes digesting the sequence. Possible SNPs were localised and investigated on basis of their presence in any restriction enzyme recognition sequences.

VRN1 allele variation in F2

The CAPS marker used by Ergon et al. (2006) to map *VRN1* was used to determine the allele distribution of *VRN1* in F2. Genomic DNA was isolated from leaves of the 10 F2 plant genotypes from population 1 and 2. The cells were crushed using the same method as for the RNA isolation. DNA was isolated by the AquaPure Genomic DNA isolation kit (BIO-RAD) using 30 – 60mg plant leaves in the following protocol:

- Added 900µl cell lysis solution and incubated at 65ºC for 60 min, inverted the tubes 10 times after 30 and 60 min.
- Added 4.5µl RNase A solution, inverted tubes 25 times and incubated at 37ºC for 15 min.

- Added 300µl protein precipitation solution, vortexed for 20 sec and incubated on ice for 15 min.
- Centrifuged at max speed for 10 min and transferred the supernatant to tubes containing 900µ 100% isopropanol.
- Inverted tubes 50 times and centrifuged at max speed for 5 min.
- Washed the pellets in 900µl 70% ethanol and centrifuged at max speed for 5 min again.
- Dried the pellet and rehydrated the DNA in 50µl DNA hydration solution at 65ºC for 60 min

A 50µl PCR reaction in 40 cycles with an annealing temperature of 55ºC was done using 1µl DNA as template and the *VRN1* primers in table 2.3. 20µl PCR product was then digested with 5U BsmA1 and NEB buffer 3 in a 40µl reaction by incubation at 55ºC, 1.5h (New England Biolabs, MA, USA). Undigested and digested PCR product was visualised on 1.5% agarose gels and the allele compositions of the F2 individuals were decided compared to the digestion pattern of the grandparents (P).

VRN1 promoter region and intron1

The sequences of the *VRN1* promoter and the first intron from *L. perenne* were kindly provided by Andersen, Asp and Lübberstedt (Danish Institute of Agricultural Sciences, Research Centre Flakkebjerg). Based on these sequences and the part of *VRN1* 5´UTR and exon1 already sequenced by Ergon et al. (2006), new primers for amplification of about 1kb of both the promoter region and intron1 were designed. Two primer sets intended to amplify the promoter region, and two primer sets for intron1 were used in PCR on genomic DNA from BF14/16 and HF2/7 (P) with 40 cycles, 1min extension time and 55ºC and 48ºC annealing temperatures respectively:

Promoter:

- LpVRN1prom_F1: 5´-GAGTCCCAAACGGGAGAAG-3´
- LpVRN1_344rev*: 5´-CTACGAGTTCGCAACCGACT-3´
- LpVRN1prom_F2: 5´-ACCACCCCACTACTGTCAGC-3´
- FpVRN1prom_R1: 5´-GATTAGGGTTTGGGCTGCTC-3´

*LpVRN1_344rev is the same as the reverse primer used in the RT-PCR (table 2.3).

Intron1:

- FpVRN1exon1F1: 5′-GTCGGGCTCATCATCTTCTC-3′
- LpVRN1exon1R1: 5′-TCACCTAGGCATATCCAGCC-3′
- LpVRN1exon1R2: 5′-TACCCCATCTTAGCTTCCGA-3′

The forward primer was combined with both reverse primers.

The sequences were attempted aligned with the *L. perenne* sequences using ClustalW.

RESULTS AND DISCUSSION

The total RNA concentrations obtained from the RNA isolation and used in the calculation of template amount for the cDNA synthesis are shown in table I.B, appendix I.

The results from the RT-PCR expression studies, and the additional investigation done for some of the genes, are ordered gene by gene:

GAPDH

GAPDH (glyceraldehydephosphate dehydrogenase) was amplified from genomic DNA of the two *F. pratensis* parents (BF14/16 and HF2/7) of the mapping population of Alm et al. (2003), using an annealing temperature of 55ºC or 60°C, 40 cycles and the primers shown in table 2.3. PCR using both annealing temperatures amplified two fragments of about 750bp and 1000bp (gel photo shown in figure II.A, appendix II). Both fragment sequences had some similarity to *GAPDH* coding sequences (cds), however, the 1000bp fragment only aligned with known *GAPDH* sequences in the 50bp at the end of the sequence. The sequence of the 750bp fragment is shown in appendix III and the best BLASTN hit, a *T. aestivum* cytosolic glyceraldehyde-3-phosphate dehydrogenase (acc.AY319478), is shown in table 3.1.

Table 3.1: **Comparison of a genomic *F. pratensis GAPDH* sequence and a *GAPDH* cds sequence from *T. aestivum***. The table shows the areas of the 750bp *FpGAPDH* fragment that aligned with *T. aestivum GAPDH* (acc. AY319478)

750bp fragm.	*T. aestivum GAPDH*	E value*
nt 2 – 56	170 – 244	8e-16
nt 213 – 374	347 – 508	7e-44
nt 478 – 566	621 – 700	6e-29
nt 673 – 771	798 – 888	4e-33

*The E value states the expected numbers of hits by chance.

The distribution of aligned areas in table 3.1 indicated four exons in the 750bp fragment. This suggested an expected cDNA fragment length of 400bp.

Amplification of *GAPDH* from the 10 F2 cDNA bulk samples resulted in only one clear fragment of 370bp (suggesting the 1000bp fragment to be eliminated when cDNA was used as template). The sequence of this fragment (shown in appendix III) confirmed that this was a *GAPDH* gene. Alignment of the cDNA sequence with the genomic sequence gave the exon/intron arrangement shown in the genomic sequence in appendix III. This arrangement confirms the predicted exon positions from table 3.1 differing in a few bases only.

PCR using the bulked cDNA as template was done with 27 and 21 cycles and the agarose gel is shown in figure 3.1. The treatments of the different bulk samples are given in table 3.2.

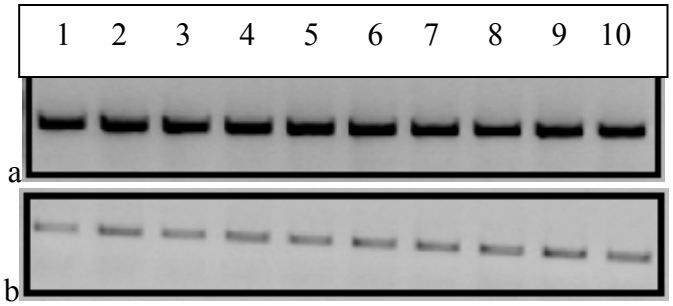

Figure 3.1: **PCR amplification of *GAPDH* from the bulked cDNA samples.** The figure shows the agarose gel of the PCR products of *GAPDH* using the bulked cDNA as template. The reactions were run with 27 (a) and 21 (b) cycles and the lane numbers refer to the cDNA bulk numbers given in table 3.2.

Table 3.2: **Treatment of the cDNA bulk samples.** The table gives information on the content and treatment of each bulk sample. The order of the samples is the same in the bulk gel figures.

Bulk:	1	2	3	4	5	6	7	8	9	10
Experiment:	1				2					
Vernalisation treatment:	Non-vernalised		Vernalised		Non-vernalised				Vernalised	
Population:	1	2	1	2	1	2	1	2	1	2
Month of sampling:	January				March		May			

GAPDH is a constitutively expressed gene frequently used as a control gene in gene expression studies (e.g. by Petersen et al. 2003). All the plant cells should contain the same amount of *GAPDH* mRNA when sampled. Variation in the band intensity would therefore represent differences in cDNA concentration. If present, these differences must be considered in analysing the expression of the rest of the genes.

Together with the *GAPDH* bulk cDNA PCR, PCR reactions using the –RT controls as templates were done. The –RT controls are bulks of the different batches used in cDNA synthesis without reverse transcriptase (RT) added. The PCR was done using the *GAPDH* primers, 55ºC annealing temperature, and 35 cycles. In addition to the –RT samples, cDNA bulk samples 1 and 2 were also used as template (as positive controls). The gel figure is shown in figure II.B, appendix II. The four first –RT samples show no fragments, but we can see one very weak fragment from the last –RT sample. This fragment is barely visual compared to the positive controls.

cDNA synthesis without RT added would not create any cDNA fragments. Fragments obtained by amplification using the –RT samples as template would indicate that genomic DNA still was present in the samples. The results confirm that all genomic DNA was eliminated during the DNase treatment. The weak fragment showing in amplification from the last –RT sample probably originated from some kind of contamination. The fragment is about the same size as the fragments amplified from cDNA. *GAPDH* amplified from genomic DNA would give larger fragments (750bp and 1000bp) as described above. Absence of these larger fragments in the RT-PCR expression studies is another verification of no genomic DNA present in the cDNA samples.

In figure 3.1 we see that the amplification of *GAPDH* in 27 cycles (figure 3.1.a) gives fragments of stronger intensity than amplification in 21 cycles (3.1.b), so 21 cycles gives a PCR reaction ending within the linear phase of increasing fragment amount. The intensity of the bands in figure 3.1.b is about the same in all the bulk samples. This means that the cDNA concentration seems to be similar. To verify this, the *GAPDH* RT-PCR was done with the same conditions in 21 cycles for all the individual F2 and P cDNA samples. The results are shown in figure 3.2.

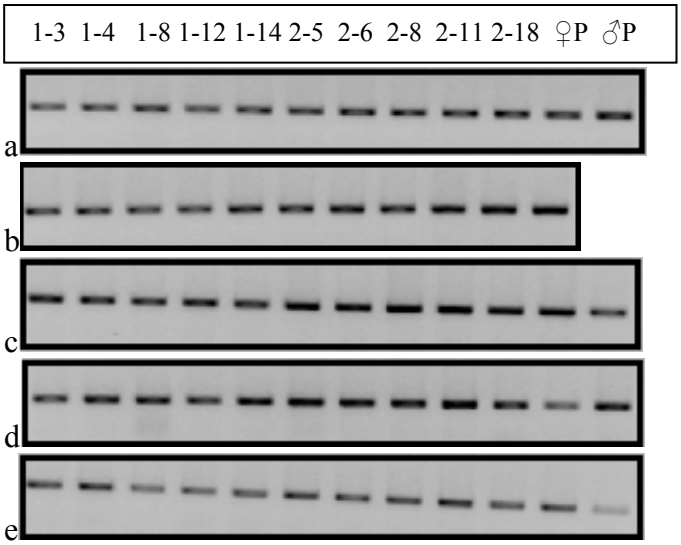

Figure 3.2: **PCR products of *GAPDH* amplified from the individual cDNA samples.** The panels all contain the five genotypes from each of the two F2 populations in addition to the female and the male P (BF14/16 and HF2/7 respectively), as given by the numbers explaining the content of each lane. The two different experiments of non-vernalised and vernalised plants, giving five different sampling times, are represented by the different panels (a – e) in the same order as in table 2.1.

The five first lanes of each panel in figure 3.2 contain the samples from population 1 and lanes six to ten contain population 2. The two last lanes (11 and 12) in 3.2.a, c, d and e and the last lane in 3.2.b contain the grandparents, BF14/16 and HF2/7 respectively. HF2/7 from the vernalised plants in experiment 1 (3.2.b) does not exist, the plant died.

The fragments in figure 3.2 show about the same intensity for all the samples. In the last lane in 3.2.e (vernalised HF2/7 from experiment 2) we see a band weaker than the others. This sample had a lower cDNA concentration than the others due to loss of plant material during the breaking of the cells. We also see some small intensity differences in the rest of the samples. This can be due to small cDNA concentration differences or small variations in the PCR setup e.g. pipette errors. In general the cDNA concentrations seemed to be equal.

ACTIN

Actin is the second constitutively expressed control gene. For this gene no successful PCR protocol was developed. The primer set based on the ryegrass *L. perenne* (sequence given in table 2.3) was tried with annealing temperatures of 60ºC, 55ºC and 50ºC (30 cycles) using

genomic DNA from the parents (BF14/16 and HF2/7) as templates. The same primer set was tried on the F2 bulk cDNA samples using 55ºC annealing. The primers did not seem to be working. Other primer sets could be tried, but since the *GAPDH* primers worked well, it was decided that one control gene was sufficient in this experiment.

VRN1

The *VRN1* primers shown in table 2.3 were known to amplify *VRN1* from *F. pratensis* P genomic DNA in one clear band. The first PCR was performed using 40 cycles, an annealing temperature of 55ºC and cDNA from experiment 1 (F2 and P, non-vernalised and vernalised samples) as templates. Surprisingly, three fragments were amplified from the non-vernalised plants. The sequences of the three fragments showed that the largest one (320bp) was *VRN1* (perfect match to DQ108934), the second fragment gave no useful sequence and the third one showed BLASTN alignments with chloroplast DNA. It was chosen to disregard the two unwanted fragments appearing in some of the PCR products, and investigate the expression of *VRN1*. The unwanted fragments may have taken up some of the primers in competition with the wanted fragments, but the expression pattern of *VRN1* was still clearly visual. The *VRN1* primers were tried on the 10 bulked F2 cDNA samples (table 3.2) using 33, 30, 27 and 24 cycles. The results (agarose gel) from the PCR using 30 and 27 cycles are shown in figure 3.3.

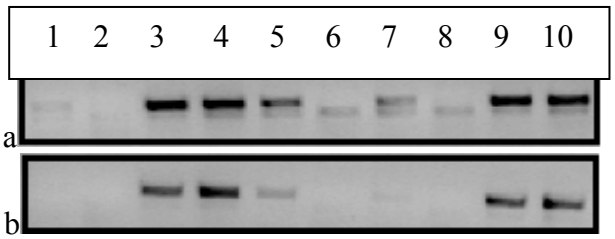

Figure 3.3: ***VRN1* PCR products using the bulk cDNA samples as template.** The reactions were run with 30 (a) and 27 (b) cycles. The lane numbers give the order of the samples and the content and treatment of each sample is shown in table 3.2.

The intensity of the fragments in the panels after 33 and 30 cycles indicated that the amplification of the non-vernalised samples were in the linear phase after 30 cycles (figure 3.3.a). Clear differences in *VRN1* expression between vernalised and non-vernalised plants

were observed. Lanes 1, 2, 5, 6, 7 and 8 represent the non-vernalised samples. In all these lanes we either see no expression or weaker expression than in the vernalised samples (lanes 3, 4, 9 and 10, the order and treatment of the bulk samples are shown in table 3.2). This expression pattern is consistent with the research of Yan et al. (2003) and Petersen et al. (2004) (among others), which proved that the *VRN1* expression was induced or enhanced by vernalisation in wheat, barley and ryegrass.

Clear differences were also seen in comparison of non-vernalised plants from population 1 and 2 in figure 3.3.a. The plants from population 1 (in lane 1, 5 and 7) do not require vernalisation to flower, while the plants from population 2 (in lane 2, 6 and 8) require vernalisation. No *VRN1* fragments were observed in the non-vernalised samples from population 2. The fragments appearing in these lanes are the smaller fragments with another sequence amplified due to similarity in the primer sites. In the non-vernalised population 1 samples we see a weak *VRN1* expression in lane 1 and stronger expression in lanes 5 and 7. *VRN1* expression only in the non-vernalised samples without vernalisation requirement resembles the situation in wheat and barley spring and winter types (Yan et al. 2003). Figure 3.3.a also shows more intense fragments in the vernalised samples of population 1 compared to the non-vernalised ones. Plants from population 1 respond to vernalisation with stronger *VRN1* expression, and this is consistent with the phenotype of these plants. They can flower prior to vernalisation treatment (but to a greater extent after vernalisation).

In table 3.2, the months for sampling of the non-vernalised plants are given (dates given in table 2.1). In addition to being used to repeat the results, the existence of three different non-vernalised sample sets taken at three different dates, can be used to investigate the expression pattern during the spring season. This comparison can be done in the non-vernalised plants from population 1, which show fragments at these times. We see a weak *VRN1* expression in lane 1, which represents the samples taken in January. These samples were taken prior to flowering. Existence of a fragment from these samples indicates that the plants started to prepare the flowering process already in January. The gene expression is initiated before the flowers can be seen. The *VRN1* expression is at its highest in lane 5, representing sampling in March. At this time, the flowering process was initiating. This suggests *VRN1* expression to be at its highest when flowering starts. Amplification from the samples taken in May is shown in lane 7. At this time, all the plants were flowering and it seems like the expression of *VRN1* is decreasing.

To repeat the results and to identify variation between the individual plants, the RT-PCR was done using all the individual F2 and P cDNA samples as templates. The PCR set-up was the same, using 30 cycles for the non-vernalised samples. The results (agarose gel) of the non-vernalised samples are shown in figure 3.4.

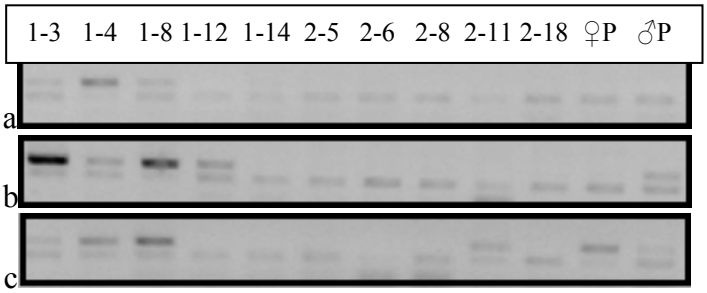

Figure 3.4: **PCR products of *VRN1* using the non-vernalised cDNA samples as templates.** The panels all contain the five genotypes from each of the two F2 populations in addition to the female and the male P (BF14/16 and HF2/7 respectively), as given by the numbers explaining the content of each lane. The panels contain the non-vernalised samples taken at three different dates; a: sampled in January, b: sampled in March and c: sampled in May.

The five first lanes of each panel in figure 3.4 show the samples from the five individuals in F2 population 1, lanes 6 to 10 show the samples from the five F2 population 2 plants and the two last lanes show the grandparents (P) (BF14/16 (♀) and HF2/7 (♂) respectively). These results confirm the results from the bulk samples. The four first lanes are the only F2 samples that show clear *VRN1* expression, and the three first show the fragment at all three dates. We only see expression in one of the population 2 samples, genotype 2-11 in panel c. The expression in population 1 is stronger in the samples taken in March (panel b), except for genotype 1-4, which is stronger in January. *VRN1* is not expressed in any of the grandparents in January (panel a). But the expression has started in HF2/7 in March (b). In May (c), the expression has decreased in HF2/7, but in BF14/16 the expression is stronger. BF14/16 requires vernalisation and HF2/7 is able to flower without vernalisation. We would expect no *VRN1* expression in non-vernalised BF14/16 based on the previous research (Yan et al. 2003, Petersen at al. 2004).

The results from the expression study of *VRN1* in the non-vernalised *F. pratensis* plants show a clear tendency of *VRN1* expression to be connected to plants having the ability to flower

without vernalisation. However, the *VRN1* fragment is amplified in one of the vernalisation requiring plants (2-11, panel c) and in the vernalisation requiring parent (♀ P, BF14/16, panel c), and missing in one of the plants without vernalisation requirement (1-14, all panels). This suggests that the *VRN1* expression pattern is not absolute. There might be other factors following *VRN1* in the flower induction pathway, masking the *VRN1* expression and still give the observed phenotypes.

Comparison between PCR products using 30 and 26 cycles in *VRN1* amplification from the individual vernalised cDNA samples showed that the use of 26 cycles gave a PCR reaction ending in the linear phase. The agarose gel from this amplification is shown in figure 3.5.

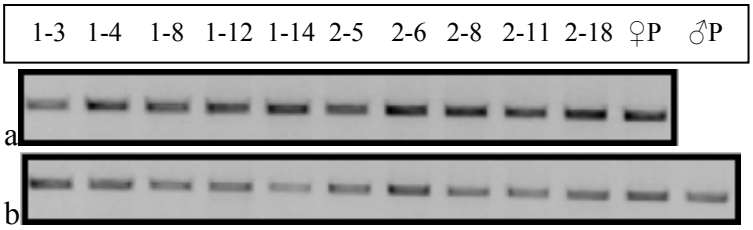

Figure 3.5: **Amplification of *VRN1* using 26 cycles and the individual vernalised cDNA samples as template.** The panels all contain the five genotypes from each of the two F2 populations in addition to the female and the male P (BF14/16 and HF2/7 respectively), as given by the numbers explaining the content in each lane. The vernalised samples from experiment 1 (a) and 2 (b) are shown.

The fragments in figure 3.5 confirm that the *VRN1* expression is up regulated by vernalisation in all the individual cDNA samples (compared to the fragments in figure 3.4). The samples in figure 3.4 and 3.5 were run in the same PCR reaction and on the same gel, and photographed using the same set-up in the Gel doc program. The *VRN1* expression level in the vernalised samples is about the same in all the genotypes. Comparison of experiment 1 and 2, figure 3.5.a and b respectively, show experiment 2 to have a generally weaker expression of *VRN1* than experiment 1. In the 12 weeks vernalisation treatment of 6ºC and 8 hours light period per day, an error was made in experiment 2. The photoperiod was set to 16 hours instead of 8 (the temperature still being 6ºC). Due to this error we can also draw some conclusions concerning the role of photoperiod in vernalisation induced gene expression. Weaker *VRN1* fragments in the samples exposed to longer days in the vernalisation treatment, suggests that short days increase the plants ability to flower further. In general, figure 3.5 shows that temperature is the most important parameter in induction of *VRN1* expression by vernalisation, since both

vernalised batches show stronger fragments than the non-vernalised ones. Adding a decrease in day length, results in an additional increase of expression.
The importance of short days for induction of flowering in vernalisation treatment is increasing when the temperature is increased (Heide et al. 1994).

VRN1 allele variation in F2

The CAPS marker used to map *VRN1* on *F. pratensis* chromosome 4 by Ergon et al. (2006) was used in this experiment to decide the *VRN1* allele variation in the F2 population. In the parents of the mapping population (P), BF14/16 is genotype ab and HF2/7 is genotype aa, where the b allele is digested by the restriction enzyme BsmA1. So in BF14/16 half of the PCR products are digested (giving three fragments) on the gel, while none of the fragments are digested in HF2/7 (giving one fragment). The agarose gel of the amplified and digested *VRN1* fragments from genomic DNA of the 10 F2 genotypes is shown in figure 3.6.

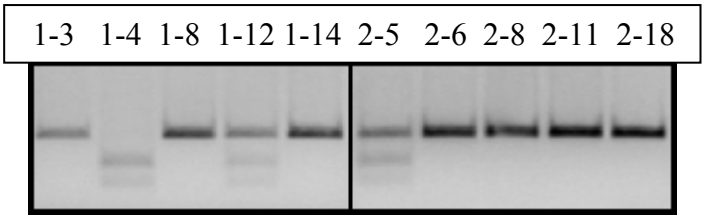

Figure 3.6: ***VRN1* amplified from the 10 F2 individuals and digested by BsmA1.** The figure shows the *VRN1* fragments digested with the enzyme used to map the gene (Ergon et al. 2006), for deciding the allele variation in F2. In this figure digested DNA extracted from the different genotypes in population 1 (left) and 2 (right) are shown.

In figure 3.6 we see the digested DNA extracted from the different genotypes in population 1 (left) and 2 (right). The genotypes shown are as follows: Population 1 (lanes 1 – 5): aa, bb, aa, ab, aa and population 2 (lanes 6 – 10): ab, aa, aa, aa, aa.

BF14/16 requires vernalisation to flower (resembles a winter genotype) and HF2/7 can flower without vernalisation (resembling a spring genotype). If the different *VRN1* alleles detected by this particular CAPS marker represent the functional variation creating the "winter" or "spring" genotypes, we would expect the "spring" F2 offspring (population 1) to resemble HF2/7 having an aa genotype (one fragment). The "winter" F2 offspring (population 2) would then be either ab or bb (three or two fragments respectively). The results given in figure 3.6

indicate that this CAPS marker does not detect the functional difference causing the variations in vernalisation requirement in our *F. pratensis* mapping population. No connection between the *VRN1* expression (figure 3.4 and 3.5) and the allelic constitution of this *VRN1* CAPS marker (figure 3.6) could be observed. This proposes the existence of another polymorphism responsible for variation in vernalisation requirement and *VRN1* expression.

One possible model is the existence of another difference in the *VRN1* gene that make one of the a alleles from this experiment different from the others. This variation must lie in another area of the gene, maybe in the promoter area or in an intron. The difference might create or eliminate a binding site for either an inducer or repressor (e.g. *VRN2*), which masks the expression of *VRN1* and is responsible for the observed differences in *VRN1* expression and vernalisation requirement. Alternatively, allelic variations in the repressor or inducer itself can be responsible for the genotypic variation. A combination of these two models could also exist.

VRN1 promoter region and intron1

Yan et al. (2003, 2004b) proposed a model that suggests the gene product of *VRN2* to be a repressor of *VRN1* expression. Allelic variations in the *VRN2* gene or in the VRN2 binding site might be responsible for the differences in vernalisation requirement. Absence of VRN2 repression would, according to the model, give constitutive expression of *VRN1*, which in turn makes the plants able to flower without vernalisation. Vernalisation is thought to down-regulate *VRN2* expression and make vernalised plants competent to flower. Yan et al. (2003) found variations in the promoter of *VRN1* in wheat between winter and spring genotypes. Variations between winter and spring genotypes have also been found in the first intron of wheat and barley *VRN1* (Fu et al. 2005). These variations might be associated with differences in VRN2 binding affinity. Based on these findings, it was chosen to investigate the *VRN1* promoter and intron1 sequences in the *F. pratensis* BF14/16 and HF2/7 to search for differences between the two genotypes. The *VRN1* promoter area is a large region to investigate, and so are intron1 (over 5000bp). J. R. Andersen, T. Asp and T. Lübberstedt (Danish Institute of Agricultural Sciences, Research Centre Flakkebjerg) in Denmark kindly provided 1500bp of the *VRN1* promoter sequence and the entire 5338bp of intron1 sequenced

from *L. perenne*. From primers based on these sequences about 1kb of the promoter region ending in exon1 and the first 1kb of intron1 was attempted amplified.

Andersen et al. (2005) showed a 325bp indel (insertion/deletion) polymorphism as a difference between a genotype requiring vernalisation and one being able to flower without vernalisation 775 – 1100bp upstream the open reading frame (ORF) of *VRN1* in *L. perenne*. Two primer sets for amplification of the promoter region in *F. pratensis* were tried and shown to amplify several fragments (gel photo shown in figure II.C, appendix II). I did not succeed in aligning any of the sequences with the promoter sequence from *L. perenne* and suspect them all to be something else, despite absence of hits in BLASTN searches. The promoter is a very variable region and might be very different, even in closely related species. But the reverse primers were designed within the sequence of *F. pratensis* exon1 sequenced in this experiment, so the fragment sequences were expected to align at least for the part in the end, being within exon1. New primer sets should be tried for amplification of this region.

Fu et al. (2005) have investigated differences in the first intron of *VRN1* between many wheat genotypes. To start the investigation of the *F. pratensis VRN1* intron1, the first 1065bp of this region was amplified and sequenced (gel photo shown in figure II.C, appendix II). Several fragments were obtained and sequenced, but alignment to the *L. perenne* sequence showed the largest of two fragments amplified by the primers FpVRN1exon1F1/ LpVRN1exon1R1 (shown in materials) to be the wanted one (sequence given in appendix III). Comparison of the two parents (BF14/16 and HF2/7) in this region revealed that they were very similar, except for some un-identified bases from the sequencing (ClustalW alignment shown in appendix III). But alignment of the *F. pratensis* and the *L. perenne* sequences showed some differences. This alignment is shown in figure 3.7.

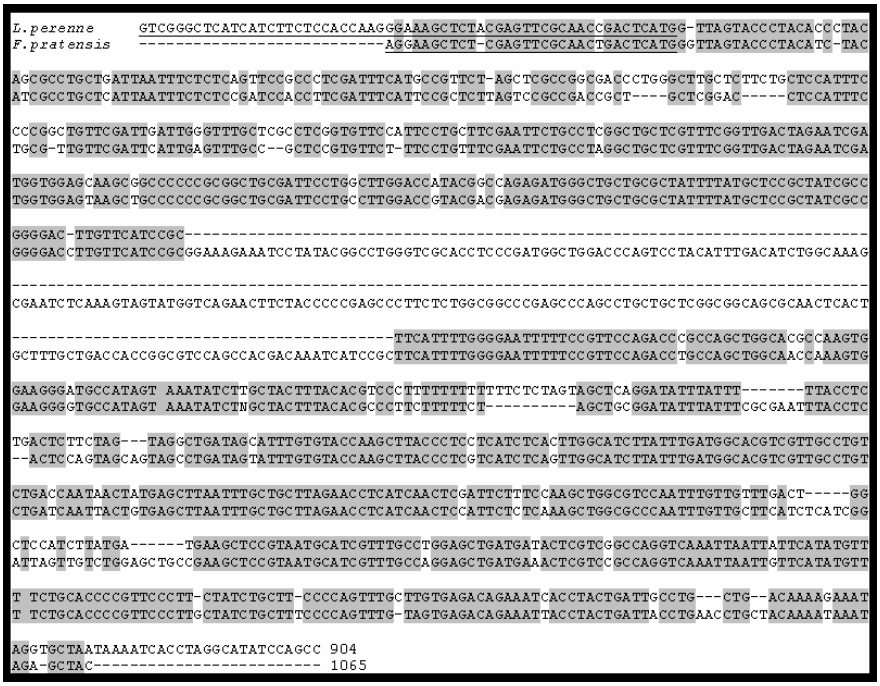

Figure 3.7: **Alignment of *L. perenne* and *F. pratensis VRN1* intron 1 (the first 1kb).** The perfectly aligned areas are marked grey and the underlined part is the end of exon1.

Figure 3.7 shows several differences between the two sequences; some nucleotide polymorphisms, several small and one large indel. Like the promoter, introns are also very variable areas.

Fu et al. (2005) found very large indels (over 1kb) in the wheat *VRN1* intron1 as variations between different genotypes. These indels were located further into the intron in what they called the critical region. It is thought that these differences might influence the binding of transcription factors, like VRN2, and manipulate the transcription of *VRN1*. To fully investigate this in *F. pratensis*, the whole intron1 must be sequenced. Even though the two parents are similar in this sequenced area, differences might be found further into their intron sequences.

To investigate possible binding sites for transcription factors in general, the sequence of the area in multiple individuals of the different phenotypes should be investigated for polymorphisms. Alternatively, binding studies or site-directed mutagenesis could be used.

VRN2

In this study I did not succeed in amplifying *VRN2* from *F. pratensis*. Five primer sets were tried, either copies of the ones used by Yan et al. (2004b) or designed based on the wheat *VRN2* sequenced by them (primer sequences shown in table 2.3). Annealing temperatures of 48ºC, 50ºC and 55ºC were tried with 40 cycles and both genomic DNA and cDNA bulk samples as template. Some of these amplifications gave fragments of wrong sizes and their sequences turned out either not to be *VRN2* or not to give useful sequences due to weak fragments on the gel (making the DNA concentration low in the purified gel fragment).

As previously mentioned, VRN2 is thought to be a repressor of *VRN1* expression (Yan et al. 2003, 2004b). *VRN2* is mapped in diploid wheat and barley, and relationships between chromosomal regions in wheat, barley and *F. pratensis* suggests that *VRN2* may be located in the QTL for vernalisation requirement on *F. pratensis* chromosome 4. *VRN2* is an interesting gene to study in this context. Chromosome mapping of the gene and searching for possible sequence differences between the genotypes in the two populations, in addition to expression analysis, are areas that could be investigated. Based on the model and the results of Yan et al. (2003, 2004b) in wheat, *VRN2* is expected to show an opposite expression pattern of *VRN1* in *F. pratensis*. The gene might be expressed prior to vernalisation in the plants requiring vernalisation, but not to the same degree in the ones able to flower without vernalisation.

For further investigation of this gene, it is suggested to try the use of CODEHOP primers in a touchdown PCR reaction. CODEHOP primers are primers being degenerated in half of the sequence (the half closest to the fragment to be amplified). Degenerated primers are useful in amplifications of genes based on sequences from more distant related species. They are based on the amino acid sequence rather than the nucleotide sequence of the gene. Due to the existence of several codons encoding the same amino acid, the nucleotide sequence of a gene might differ in different species, but still encode the same protein sequence. The different codons encoding one amino acid are often identical in the first or the two first bases, with variation in the last or the two last bases. Based on this knowledge Rose et al. (1998) invented the CODEHOP (COnsensus-DEgenerate Hybride Oligonucloetide Primer) strategy. This strategy involves designing primers fitting all possible nucleotide compositions encoding the amino acid in question in the degenerated area.

Touchdown PCR is a method invented by Don et al. (1991). This PCR reaction initiates with a high annealing temperature (e.g. 70ºC, or lower if no fragments are observed) and the

temperature is decreased by 1ºC per cycle in a number of cycles (e.g. 20). Further, a number of cycles (e.g. 20) run with a normal annealing temperature (e.g. 60ºC, depending on the nucleotide composition and length of the primers) are done to complete the reaction. In the first step, primer annealing is very specific, and it is thought that the primers only will bind at perfect primer match, amplifying the desired fragment only. In the second step additional amplification of these wanted fragments (available in higher concentrations) is done. This makes the PCR reaction more specific.
Morant et al. (2002) combined the CODEHOP and the touchdown method to amplify genes with differences in nucleotide sequence in different related species.

Designing CODEHOP primers and using the touchdown PCR programme might give successful amplification of *VRN2* from genomic DNA for chromosome mapping and studies of genotypic variation, or from cDNA for expression investigation.

MADS16

Several primer sets were designed for this gene on basis of the *L. perenne MADS16* sequence (gel photo shown in figure II.D, appendix II). From four combinations, all working with cDNA as template, the ones amplifying the largest fragment (960bp) were chosen for RT-PCR analysis of gene expression (sequences shown in table 2.3). The *FpMADS16* cDNA sequence (given in appendix III) aligned with *LpMADS16*, *TaVRT-2* and *HvVRT-2* in BLASTN search with E values of 0.0 and 1e-163 (for both *VRT-2*s) respectively. *FpMADS16* was amplified from the F2 cDNA bulk samples using 31, 28 and 25 cycles. The agarose gel of 31 and 28 cycles are shown in figure 3.8.

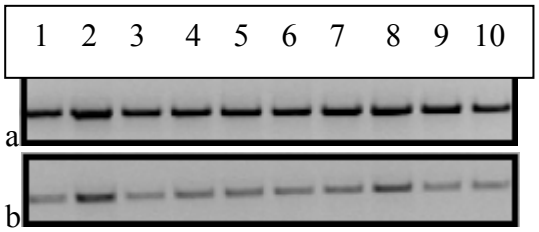

Figure 3.8: **RT-PCR of *MADS16* using the cDNA bulk samples as template.** The reactions were run with 31 (a) and 28 (b) cycles. The lane numbers gives the order of the samples and the content and treatment of the samples are shown in table 3.2.

The results of the *MADS16* expression study does not show as clear a pattern as the one seen for *VRN1*, but some tendencies can be seen. The discussion is based on the PCR reaction run with 28 cycles (figure 3.8.b), which gave weaker fragments than the one run with 30 cycles (and therefore is in the linear phase). The RT-PCR on the bulk cDNA samples using 28 and 25 cycles was done twice to confirm the results, both showing the same tendencies as seen in figure 3.8.b. As given in table 3.2, lanes 1 and 2, 5 and 6 and 7 and 8 represent population 1 and 2 at the three different dates in January, March and May respectively. Lanes 3 and 9 represents the vernalised plants from F2 population 1 and lanes 4 and 10 the vernalised F2 plants from population 2.

Stronger fragments in lane 2 than 1 and in lane 8 than 7 suggests a higher expression of *MADS16* in the non-vernalised plants of population 2 compared to the non-vernalised plants of population 1. Population 2 is vernalisation requiring. This suggests that *MADS16* is up regulated prior to vernalisation in plants that require vernalisation, compared to the genotypes that are able to flower without vernalisation (population 1).

MADS16 appeared to be down regulated by vernalisation in the plants that require vernalisation for flowering. This consumption was based on the appearance of weaker fragments in lane 4 compared to 2, and in lane 10 compared to 6 and 8. In the ones able to flower without vernalisation, the expression seems to be similar in the non-vernalised and the vernalised plants (lanes 1, 5 and 7 compared to 3 and 9 respectively).

In the previous investigations, this gene was called *VRT-2* when discovered in wheat and barley and *MADS16* in ryegrass. *F. pratensis* is more closely related to *L. perenne* than to wheat and barley, so it was chosen to call the gene *FpMADS16* in *F. pratensis*. The results from the *FpMADS16* expression experiment on the bulked cDNA samples are consistent with the results of Kane et al. (2005) from *TaVRT-2*. They showed the *TaVRT-2* expression to be low and stable in spring genotypes (able to flower without vernalisation), and stronger in non-vernalised winter genotypes (vernalisation requiring). These results are also expected if MADS16 is a repressor of flowering.

The expression of *MADS16* seems to be higher in population 2 in January (lane 2) and in May (lane 8) than in March (lane 6). This difference is hard to explain due to the need for vernalisation for flower induction in population 2. In population 1, the expression seems to be

similar at all the three dates, suggesting that *MADS16* is expressed at a similar rate before, during and after the initiation of flowering.

The agarose gel of the *MADS16* expression experiment on the individual F2 and P cDNA samples using 26 cycles are shown in figure 3.9.

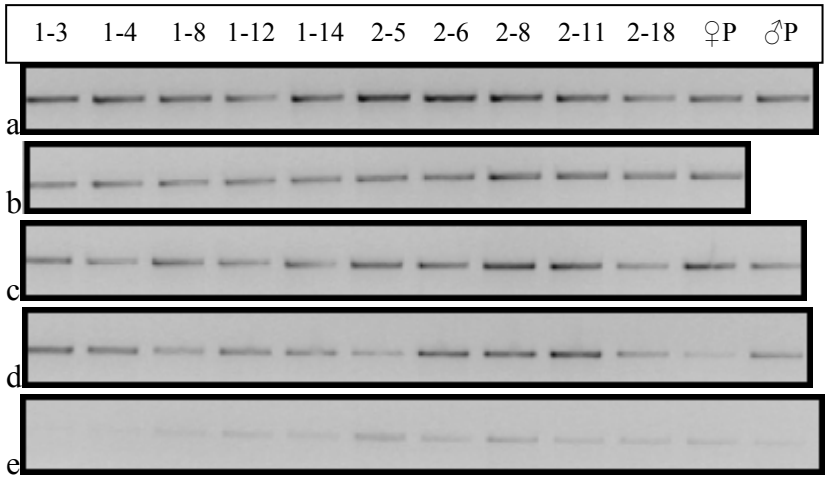

Figure 3.9: **PCR products of *MADS16* amplified from the individual cDNA samples using 26 cycles.** The panels all contain the five genotypes from each of the two F2 populations in addition to the female and the male P (BF14/16 and HF2/7 respectively), and the positions are given by the numbers explaining the content of each lane. The panels (a – e) represent the five different experiments/treatments in the same order as given in table 2.1.

The five first lanes of each panel in figure 3.9 contain the samples from population 1, and lanes six to ten contain population 2. In general, figure 3.9 pretty much confirms the results of the bulk samples in figure 3.8.b. We see a greater variation between the individual plants, but all in all, the non-vernalised individuals in population 2 have a stronger expression than the non-vernalised individuals in population 1 (panels a, c and d). In the panels from the amplification from the vernalised plants (panel b and e), the expression seems even in all the plants, but a bit higher in the ones from population 2.

The genotypes from population 1 had stable and low *MADS16* expression in both the non-vernalised and the vernalised plants (with the exception of the vernalised plants from experiment 2 (panel e) having an even weaker expression).

Comparing the two vernalised panels to each other, the one from experiment 2 in panel e shows weaker fragments in all the samples. These are the plants from the vernalisation treatment using longer days (16 hours, by mistake). This is a bit of a mystery and suggests that *MADS16* expression is down regulated in 12 weeks cold treatment with long photoperiods compared to shorter ones. *MADS16* is a putative flowering repressor, and so we would expect the expression to be weaker in the plants that were vernalised using short days than the ones vernalised in long days.

The two last lanes in 3.9.a, c, d and e and the last lane in 3.9.b contain the grandparents, BF14/16 and HF2/7 respectively. HF2/7 from batch 2 does not exist. There were no consistent difference between BF14/16 and HF2/7.

In the *MADS16* studies, we do not see as clear an expression pattern as seen in the *VRN1* studies. But we do see tendencies suggesting that the expression pattern is as suspected if *MADS16* is a repressor of flowering. These findings suggest that the *FpMADS16* from this investigation is the same gene as *VRT-2* and the expression pattern is similar to the *TaVRT-2* expression pattern shown by Kane et al. (2005). The results from the bulk cDNA samples are to a certain degree confirmed by the individual samples. Small inaccuracies in the RNA concentration might cause some of the individual samples to be over-represented in the bulk samples. This can be a source of error in the expression analysis of the bulk cDNA. To confirm the results, the RT-PCR on the individual samples could be done several times.

Alternatively, a maybe more time consuming and costly quantitative real-time PCR could be done. In real-time PCR, a fluorescent probe attached to the target sequence gives a change in dye signal when the primer is extended by polymerisation and meets the attached probe (invented by Livak et al. 1995). A real-time PCR instrument measures the change in fluorescence during the first amplification, which represents the number of template target sequences present. This is more accurate than measuring the amount of product after a fixed number of cycles.

FpMADS16 mapping attempt

In attempt to map *MADS16*, the cDNA sequences from the two parents were aligned and investigated for SNPs (single nucleotide polymorphisms) useful as CAPS markers.

Identifying the allele composition of individuals in the F1 generation would allow mapping of *MADS16* relative to the other markers mapped in the same population by Alm et al. (2003). One possible SNP was identified, but no restriction enzyme that included this nucleotide as a specific part of its recognition sequence was found. This SNP could, however, possibly be detected by another type of marker assay.

None of the primer sets that were designed for *MADS16* amplified the gene from genomic DNA. Different fragments were obtained in the PCR, but most of them were smaller than the cDNA fragments (see gel photo in figure II.D, appendix II). Amplification of the gene from genomic DNA should give fragments either bigger than the cDNA fragments (if the gene contained introns), or with the same size. Several fragments were sequenced and all shown to be something else. This proposed the suspicion of very large intron(s) present, which would make the amplification impossible.

The cDNA sequence contained only the exons of *MADS16*. If introns were present the probability of finding polymorphisms (CAPS markers) might be bigger in these regions, which usually are more variable. The *MADS16* cDNA sequence was compared to the best genomic hit in a BLASTN search (*Oryza sativa* chromosome 6, acc. AP008212) and several putative introns were found (shown in figure 3.10).

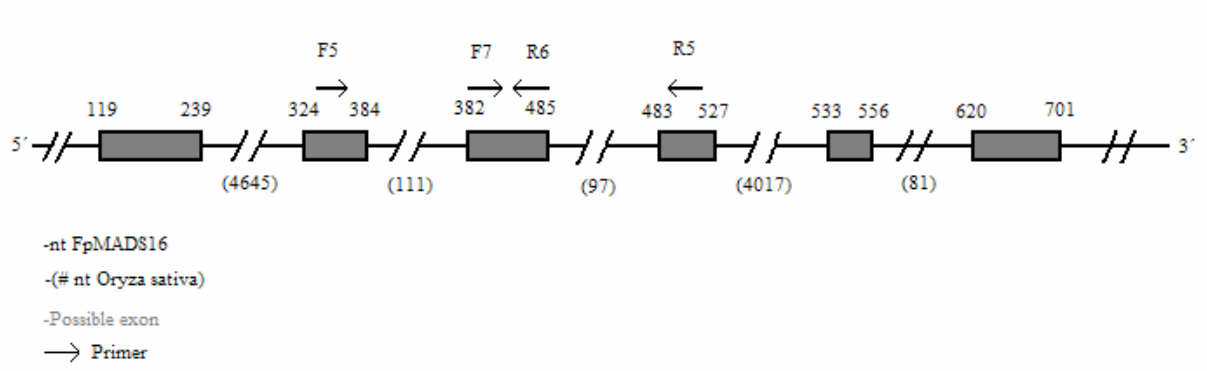

Figure 3.10: **Possible exon/intron arrangement in the *FpMADS16* gene.** The figure shows the possible location of introns in the *FpMADS16* cDNA sequence based on alignment to the genomic *Oryza sativa* sequence (acc. AP008212). The grey boxes indicate the putative exons and the nucleotide numbers in *FpMADS16* at the start and the end of the exons are shown above the boxes. The numbers under the line are the number of bases in the respective putative introns in *Oryza sativa*. The primers designed to amplify the smaller introns are shown as arrows.

The intron positions could differ in different species and there are many similar MADS-box genes, but it was chosen to design primers for amplification of the 111bp and 97bp introns shown in figure 3.10. Introns are very variable regions, so the intron sizes may differ greatly. But it was chosen to amplify two of the smaller introns in rice hoping that these introns were small also in *F. pratensis*.

Amplification of this new area of *FpMADS16* from BF14/16 and HF2/7 genomic DNA gave different fragments on the agarose gel (shown in figure II.E, appendix II). The F5/R6 and F7/R5 primer combinations amplifying intron 2 and 3 in figure 3.10 respectively, gave fragments differing in size between the two parents, and sequences of poor quality. But the F5/R5 combinations (sequences shown in materials) that amplified both introns gave strong fragments in both parents and sequences of good quality. Alignment of the genomic and cDNA sequences showed the introns to be located at almost the exact same position as predicted in figure 3.10. In appendix III, the genomic sequence obtained from the F5/R5 primers, together with the same area of the cDNA sequence is shown. The introns were 101bp and 87bp long. Unfortunately the genomic sequences were identical in the two parents, no SNPs were found.

The transcription factor *MADS16* may be located on *F. pratensis* chromosome 7. Kane et al. (2005) mapped the *TaVRT-2* gene to *T. aestivum* chromosome 7. From the research of Alm et al. (2003) we know that *T. aestivum* and *F. pratensis* chromosomes 7 are relatively similar. For further attempt of mapping this gene, it is suggested to try to amplify the other introns, or alternatively, a bigger part of the *FpMADS16* gene, and search for SNPs.

PHYC

In this experiment, the investigation of *PHYC* started and ended with the RT-PCR on the F2 cDNA bulk samples using the primers shown in table 2.3. Amplification was done using an annealing temperature of 55ºC and 35 and 30 cycles, agarose gel shown in figure 3.11. The fragments were identified as *PHYC* by sequence alignment to the gene already sequenced (Henriksen, 2005). The sequence is given in appendix III.

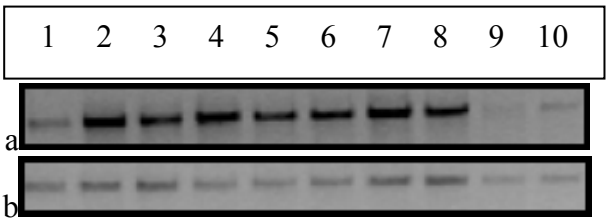

Figure 3.11: **RT-PCR of *PHYC* using the cDNA bulk samples as template.** The reactions were run with 33 (a) and 30 (b) cycles. The lane numbers give the order of the samples and the content and treatment of each sample is shown in table 3.2.

No clear pattern can be seen in figure 3.11. The figure shows the bulk cDNA samples arranged as shown in table 3.2. Some small differences can be seen in the fragment intensities of the unsaturated samples of figure 3.11.b. But no pattern can be seen in comparison of the two genotypes or the different treatments. The *PHYC* expression seems to be about the same in the two populations and un-affected by vernalisation. But the gene seems to be down regulated by vernalisation with long day (lanes 9 and 10) compared to all the other samples. These samples do not give saturated PCR reactions with 35 cycles. This indicates that *PHYC* is down regulated during vernalisation in longer days compared to short ones.

PHYC is the gene encoding phytochrome C, a photoreceptor induced by light signals to regulate plant growth. In general, phytochromes are plant pigments that come in two forms. Each form can convert to the other in response to exposure to red light of the proper wavelength. Phytochromes play an important role in controlling the initiation of flowering in short-day plants. This photoreceptor is activated or inactivated by light and dark periods. It is not known if this gene is part of the vernalisation response pathway. (Quail, 2002 reviews Photoreseptors). *PHYC*, previously amplified from genomic BF14/16 and HF2/7, is localised near *VRN1* on *F. pratensis* chromosome 4 (Henriksen, 2005). So it was thought that this gene might be involved in vernalisation response and induction of flowering.

I feel the need to do further investigation of this gene before any conclusions can be drawn. It looks like *PHYC* is expressed in about the same rate in both populations at all treatments and through the spring season. But the RT-PCR on the bulked cDNA shows an un-clear expression pattern and the results are not clearly confirmed by the two different numbers of cycles. Additional bulk RT-PCRs and RT-PCR on the individual samples should be done.

RUBQ2

RUBQ2 had previously been amplified from genomic DNA from the parents using the primers in table 2.3 together with several other fragments (Henriksen, 2005). PCR using 60ºC annealing temperature and the F2 cDNA bulk samples as template gave one clear fragment shown to be ubiquitin by alignment to the sequence already available (the sequence is given in appendix III). The agarose gel of the RT-PCR using the bulked cDNA as template and 35 and 30 cycles are shown in figure 3.12.

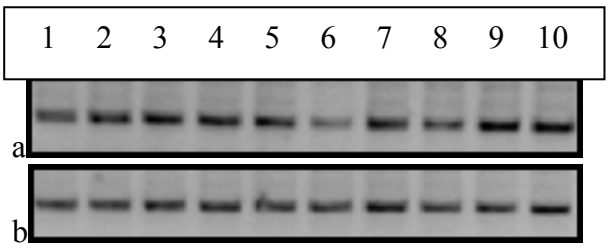

Figure 3.12: **RT-PCR of *RUBQ2* using the cDNA bulk samples as template.** The reactions were run with 35 (a) and 30 (b) cycles. The lane numbers gives the order of the samples and the content and treatment of the samples are shown in table 3.2.

In figure 3.12, we see about the same fragment intensity in all the samples (order given in table 3.2). *RUBQ2* expression does not seem to be affected either by vernalisation, vernalisation requirement or through the spring season.

RUBQ2 is a gene encoding ubiquitin. Ubiquitinylation (attachment of ubiquitin) is a post-translational modification of a protein marking it for destruction or alteration of its location or function. In surviving the cold and dark winter period, and in the transition to the warmer and lighter environment in the spring, a plant needs to turn on production of some proteins and turn off production of others. *RUBQ2* was expected to be up regulated by vernalisation based on previous studies (Rudi et al. (*in prep*)). Thus, we would expect the expression study to show stronger fragments in the vernalised samples than the non-vernalised ones. This suspicion is not confirmed by these results. The two panels in figure 3.12 do not show much difference in saturation. Differences in expression might be visual after a PCR using a smaller number of cycles. Additional RT-PCR studies on the bulk and individual samples are suggested to confirm these suspicions.

CONSTANS

CONSTANS (CO) was amplified from *F. pratensis* genomic DNA from the parents (BF14/16 and HF2/7) using the primers shown in table 2.3 and the same protocol as used by Andersen et al. (2006) (gel photo shown in figure II.F, appendix II). The sequence (given in appendix III) aligned with an E value of 1e-104 to all the three *LpCO* sequences of Andersen et al. (2006) (DQ202717), Armstead et al. (2005) (AJ833019) and Martin et al. (2004) (AY600919).

CONSTANS is a central gene in the flower induction pathway of Arabidopsis (Hayama and Coupland, 2004). The gene does also seem to play a role in the grass family (Armstead et al. 2005, Martin et al. 2004). The expression of this gene is induced by long days and it is affected by the circadian clock. Thus, the time of sampling must be considered when the expression pattern is analysed.

The investigation of *CONSTANS* was initiated at the end of my practical work in this experiment. RT-PCR on the bulk samples was tried, but the results were hard to read. It looks like there are some expression differences in the different vernalisation treatments. Due to weak fragments on the gel, the bulk RT-PCR was done twice, giving conflicting results. Additional PCRs are necessary, but due to time shortage this was not done in this experiment.

CONCLUSIONS

VRN1 expression is up regulated by vernalisation in all the *Festuca pratensis* plants tested in this assignment. Prior to vernalisation, differences in *VRN1* expression are strongly linked to vernalisation requirement. The gene is not expressed in plants that require vernalisation to flower, but expression is seen in plants that are able to flower without vernalisation. A few plants did not follow this expression pattern, but VRN1 is most likely a flowering inducer in *F. pratensis*.

The allele variation of the *VRN1* CAPS marker (Ergon et al. (2006)) in the ten F2 plants investigated in this study did not have any connection to vernalisation requirement or *VRN1* expression. Another polymorphism responsible for the genotypic variation in vernalisation requirement is suggested.

The first 1kb of *F. pratensis VRN1* intron 1 was sequenced in this assignment. The parent able to flower without vernalisation and the parent with vernalisation requirement are very similar in this region. One large indel polymorphism is present as a sequence difference between *F. pratensis* and *L.perenne* in the sequenced region of intron 1. The *VRN1* promoter was not successfully amplified. Further investigations by sequence comparison of the *VRN1* promoter and the rest of intron 1 in the two *F. pratensis* parents are suggested.

VRN2 was not successfully amplified in this study. Other primer sets are needed for studies of the expression of this gene.

About 1kb of the *FpMADS16* coding sequence plus the *FpMADS16* introns 2 and 3 was sequenced in this work. *MADS16* expression seems to be down regulated by vernalisation in *F. pratensis* plants that require vernalisation to flower. The plants that are able to flower without vernalisation have a stable *MADS16* expression un-affected by vernalisation. The degree of expression is similar in the plants that are able to flower without vernalisation and the vernalised plants with vernalisation requirement. Additional investigation is necessary to confirm that MADS16 is a repressor of flowering in *F. pratensis*.

CONSTANS was also sequenced from *F. pratensis* in this study. But further expression investigation is necessary to draw any conclusions concerning the role of this gene in the vernalisation induced flowering of meadow fescue.

F. pratensis PHYC and *RUBQ2* does not seem to be regulated by vernalisation based on the results obtained in this experiment. But *PHYC* seems to be controlled by photoperiod. Further investigation is suggested to confirm these results.

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