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## Abbreviations

ABA	abscisic acid
AFP	anti-freeze protein
AP1	apetala1
bp	base pair
CAL	cauliflower
CA	cold acclimation
CAMTA	calmodulin binding transcription activator
CBF	C-repeat binding factor
CCA1	circadian clock-associated 1
cDNA	complementary deoxyribonucleic acid
COR	cold regulated
CRT/DRE	dehydration responsive element
CT	cycle threshold
CCT	comparative cycle threshold
DHNs	dehydrins
DEPC	diethylpyrocarbonate
DREB1	dehydration responsive element binding1
DRE	dehydration-responsive element
1-FFT	fructan-fructan 1-fructosyltransferase
1-SST	sucrose-sucrose 1-fructosyltransferase
6G-FFT	fructan-fructan 6G-fructosyltransferase
FLC	flowering locus C
FRI	frigida
FT	freezing tolerance
FT	flowering locus T
FTs	fructosyltransferase
FUL	fruitful
EF1A	elongation factor 1 alpha
FDL2	flowering locus d-like 2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

h	hour
HOS1	high expression of osmotically responsive genes1
LT	low temperature
6-SFT	sucrose: fructan 6-fructosyltransferase
ICE	inducer of CBF Expression
LEA	late embryogenesis-abundant
LFY	leafy
LHY	late elongated hypocotyl
PCR	polymerase chain reaction
PI	primary induction
PHD	plant homeodomain
PPD-H1	Photoperiod-H1
PPFD	photosynthetic photon flux density
qRT-PCR	quantitative reverse transcription PCR
PRR7	pseudo-response regulator 7
PSII	photosystem II
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase PCR
SI	secondary induction
SOC1	suppressor of overexpression of co 1
SVP	short vegetative phase
SUMO	small ubiquitin-related modifier
TFL1	Terminal flower 1
$\Delta\Delta$ CT	delta-delta CT
<i>VIN3</i>	<i>VERNALIZATION INSENSITIVE 3</i>
<i>VRN1</i>	<i>VERNALIZATION 1</i>
<i>VRN2</i>	<i>VERNALIZATION 2</i>
<i>VRN3</i>	<i>VERNALIZATION 3</i>

## Abstract

Forage grasses like perennial ryegrass (*Lolium perenne* L.) and Festulolium are extensively cultivated worldwide and constitute primary feed for ruminants due to their high nutrient value, high palatability and digestibility. Perennial ryegrass usually needs vernalization treatment (prolonged exposure to low temperatures) to initiate flowering. Vernalization requirement is particularly important for preventing flower development during winter and protect the environmentally sensitive floral organs. Understanding the control of flower initiation and flowering time in diverse plant species gives us insights into the ancestral control of flowering time and the evolution of alternative mechanisms in different plant lineages. Vernalization requirement of perennial ryegrass is often determined using controlled climate conditions. In order to study natural adaptation during autumn and winter, gene expression can also be studied by sampling under field conditions. Under natural field conditions, climate variations are much greater since temperature, light intensity and photoperiod are not controlled. In this thesis, investigations were undertaken, therefore, to determine gene expression of genes involved in vernalization in perennial ryegrass under field conditions. Gene expression patterns at different time points of plant growth throughout the winter were detected for the vernalization genes *VRN1*, *VRN2*, and *VIN3* using qRT-PCR. Plants were established, cold hardened at 2-6 °C for 2 weeks and continue their life cycle with temperature fluctuations ranging from -10 °C to +6 °C. The relative gene expression of the three target genes was measured in two genotypes from the Norwegian cultivar 'Fagerlin', i.e. genotype 201 (flower without vernalization) and genotype 204 (require vernalization to flower). The results indicate that there is a differential gene expression pattern for vernalization genes. Increased transcript levels for *VRN1* and *VIN3* genes in response to cold stress were observed during the mid-December to mid-January (-6 to -10) °C period for both genotypes, whereas *VRN2* expression is low and was not affected by vernalization in both genotypes. The expression levels of *VIN3* were correlated with *VRN1* with similar expression patterns. Under controlled conditions, both genotypes underwent two types of treatments, i.e. the first treatment with vernalization followed by cold hardening, de-hardening, and re-hardening, and the second treatment was conducted same as first treatment but without vernalization. The results show that both *VRN1* and *VIN3* were upregulated during cold hardening and downregulated during the de-acclimation period. We also investigated the gene expression patterns of *VRN1*, *VIN3*, *fructan* and *dehydrins* in two genotypes of Festulolium, i.e. genotype 266 (flower without

vernalization) and genotype 329 (require vernalization to flower) at controlled conditions with two treatments as mentioned before. The expression pattern of *VRN1* in *Festulolium* with the vernalization treatment followed by cold hardening, de-hardening, and re-hardening showed increased expression level in cold hardening and re-hardening for 329 genotype. The relative expression of *VIN3* gene was upregulated without the vernalization treatment followed by cold hardening, de-hardening, and re-hardening. The fructan gene expression was observed in vernalization treated conditions for both 329 (de-hardening treatment) & 266 genotypes (de-hardening & re-hardening) of *Festulolium*. *Dehydrins* also activated by exposure of cold temperature and increased transcript level was observed in cold hardening and re-hardening conditions for 329 genotype of *Festulolium*. Further, we validated the gene expression data by comparing to the RNA transcriptome data of all these genes.

## Sammendrag

Engvekster som flerårig raigras (*Lolium perenne* L.) og raisvingel (*Festulolium*) dyrkes over store områder i verden og utgjør et svært viktig grunnlag for grovfôr til drøvtyggere pga. sine gode egenskaper som høy næringsverdi, god fordøyelighet og smaklighet. Flerårig raigras trenger vanligvis vernalisering (en forlenget periode med lav temperatur) for å initiere blomstring. Et krav til vernalisering er spesielt viktig for å hindre for tidlig reproduktiv utvikling i løpet av vinter/tidlig vår noe som er skadelig for reproduktive organer som er sensitive for lav temperatur. Kunnskap om hvordan blomsterinduksjon og blomstringstid styres og kontrolleres i ulike plantearter gir oss innsikt i opprinnelse og evolusjon av disse prosessene i ulike planteslekter. Vernaliseringskrav hos genotyper av flerårig raigras bestemmes som regel ved forsøk i kontrollert klima (vekstroom). For å studere naturlig tilpasning gjennom høst og vinter kan man bl.a. gjøre studier av genekspressjon basert på sampling av plantemateriale som er dyrket under naturlige forhold i felt. Under naturlige forhold i felt er variasjonen i klimaforholdene mye større enn i kontrollert klima siden temperatur, lysintensitet og daglengde ikke kontrolleres. I denne masteroppgaven er det derfor utført forsøk med formål å undersøke ekspressjon av gener som er involvert i vernaliseringsprosessen under naturlige forhold i felt. Ved bruk av qRT-PCR ble ekspressjon av vernaliseringsgenene *VRN1*, *VRN2*, og *VIN3* studert i prøver tatt til ulike tidspunkter gjennom vinteren fra planter i felt. Plantene ble etablert i veksthus, kuldeherdet ved 2-6 °C i 2 uker og var utsatt for temperaturer mellom -10 °C og +6 °C utendørs. Relativ genekspressjon av de tre genene ble undersøkt i to genotyper fra den norske sorten 'Fagerlin' av flerårig raigras, dvs. genotype 201 som ikke trenger vernalisering (V-) for å blomstre og genotype 204 som trenger vernalisering (V+). Resultatene viser at det er klare forskjeller i genekspressjonsmønstre mellom de tre genene og mellom genotypene. Økt transkripsjon for *VRN1* og *VIN3* som en reaksjon på kulde ble observert hos begge genotypene i perioden fra midt i desember til midt i januar hvor lufttemperaturen varierte fra -6 til -10 °C, mens ekspressjon av *VRN2* var lav i begge genotypene og ikke påvirket av lav temperatur. Ekspressjon av *VIN3* var korrelert med *VRN1* og hadde det samme ekspressjonsmønstret. Under kontrollerte klimaforhold ble ekspressjon hos de to genotypene undersøkt i to forsøk, ett med vernalisering etterfulgt av kuldeherding, avherding og reherding, og ett tilsvarende hvor vernalisering ikke var inkludert. Resultatene viste at både *VRN1* og *VIN3* ble

oppregulert ved kuldeherding og nedregulert ved avherding. Vi har også undersøkt genekspressionsmønstre av *VRN1*, *VIN3*, *fructan* og *dehydrins* i to genotyper av *Festulolium*, dvs. genotype 266 (som ikke trenger vernalisering (V-)) og genotype 329 (som trenger vernalisering (V+).) ved kontrollerte forhold med to behandlinger som nevnt før. Uttrykket mønster av *VRN1* i *Festulolium* med vernalisering behandling etterfulgt av kuldeherding, avherding, og reherding vis induert transkripsjon nivå i kulden herdende og re-herding for 329 genotype. *VIN3* vise oppregulert karakternivå uten vernalisering behandling. Nivået av *fructan* genekspressjon ble observert i vernalisering behandlede betingelser for både 329 (avherding) og 266 genotyper (avherding og reherding) av *Festulolium*. Dehydrins også aktiveres ved eksponering av kald temperatur og induert transkripsjon ble observert i kuldt herding og re-herding betingelser for 329 genotype av *Festulolium*. Videre vi validert genuttrykk data ved å sammenligne til RNA transkriptomet data av alle disse genene.

## 1. Introduction

### 1.1 Perennial ryegrass and Festulolium

Perennial ryegrass (*Lolium perenne* L.) is an important temperate forage grass due to its pastoral production globally, with its rapid establishment rate, fast growing and a good persistency against grazing. Moreover, perennial ryegrass is used as a highly nutritious livestock feed in this region. However, Forage cultivation is one of the essential prerequisites to secure animal production with its self-sufficient production. Therefore, development of new forage varieties with higher productivity, forage quality, tolerance to summer and winter stress should be given high priority for its sustainable environmental and economic development (Yamada et al. 2005).

The agronomists, evolutionists and plant breeders have long interest for the genus *Festuca* and its closely related genus *Lolium*. These species are widely studied among the non-cereal grasses for the nourishment, persistency, seed productivity and stabilization of soils. Moreover, these grasses have good herbage feeding value. Intergeneric hybridization between closely related *Lolium* and *Festuca* species are being used by plant breeders to widen the gene pool. It gives opportunity to the plant breeder to combine high quality traits with adaptation to a range of environmental stresses like drought, cold and pathogens (Humphreys et al. 2003; Humphreys et al. 2006). Hybridization between these species brings an abundant richness of genetic variability and high potential for genetic exchange. Consequently, it gives opportunity for production of new hybrid varieties with the combinations of new characters that are economically viable and suited to modern grassland farming (Thomas et al. 2003). *L. perenne* is better for its grazing, nutritive values and good regrowth capacity whereas *F. pratensis* is highly persistent and winter hardy (Humphreys et al. 1998). *Lolium* and *Festuca* species hybridize naturally and these intergeneric crosses lead to combinations of disease resistance and tolerance to abiotic stresses such as cold and drought from *Festuca* with the improved palatability and high forage quality of *L. perenne* (Thomas & Humphreys 1991). A number of *Lolium* X *Festuca* hybrids (*Festulolium*) have been developed as recent temperate forage grasses (Humphreys et al. 2003). Uses of *Festulolium* hybrids is increasing all over the world due to their high quality agronomic characteristics (Kopecký et al. 2006).

Large acreages of forage grasses, including *Lolium perenne* L. and *Festulolium*, are grown worldwide for ruminants. However, cold stress occur regularly in northern Europe, which is considerably limiting the forage production and affecting plant persistency throughout the winter

season. As the result of winter damages, a lot of research has been initiated trying to find suitable genotypes and cultivars with good freezing tolerance and winter survival. Most of the studies of freezing tolerance have focused on temperate regions where it is considered a major limiting factor for forage production.

## 1.2 Vernalization

The synchronization of flowering time with ambient environmental conditions secures the reproductive success of a plant and it plays a major role in fertilization and seed production. Initiation of flowering in a plant is a crucial part to fulfill its life cycle. Flowering time is highly responsive to seasonal cues. The forage grasses evolved with a signal perception and transduction that senses prolonged exposure to cold during autumn and winter. By this process, known as vernalization, the plants become competent to flower through the translation of these environmental cues (Schmitz & Amasino 2007). Vernalization plays an important role to suppress the expression of genes that encode repressors of flowering in plants. In addition, plants require seasonal cues before flower initiation.

When crops are environmentally adapted to different latitudes and diverse cropping seasons, genetic variation is required for vernalization and photoperiod response. In temperate regions, most of the grasses including perennial ryegrass (*Lolium perenne* L.,  $2n=2x=14$ ) require vernalization treatment, followed by long days to induce flowering, i.e. increase in photoperiod (Heide 1994). Vernalization and photoperiod treatments are required to ensure flowering and seed production through the adjustment to the ambient conditions in spring and summer. However, for forage grasses, delay or even suppression of flowering is related to high forage quality and can be an important breeding goal (Jensen et al. 2004), contrary to the breeding aim of high yield of grass seed production. The identification and determination of expression patterns of genes regulating flowering time in *Lolium perenne* is thus of great interest (Andersen et al. 2006).

Most of the perennial grass species have a dual induction requirement for flowering. A primary induction (PI), which is obtained by low temperature (vernalization) or SD (short days), and a secondary induction (SI) which requires alteration to long days and is accelerated by moderately higher temperatures (Heide 1994). Both the primary and secondary induction requirement vary substantially among perennial ryegrass ecotypes and genotypes. In accordance with this, the requirements for the PI and SI increase with increasing latitude of origin of the germplasm (Aamlid

et al. 2000). The molecular control of the vernalization response has been studied intensively in different plant species, especially in *Arabidopsis* (Amasino 2004; Putterill et al. 2004). Through the genetic analysis in *Arabidopsis*, *FLOWERING LOCUS C (FLC)* has been identified as a potent repressor of flowering. In the absence of an active *FRI* allele, overexpressed *FLC* from a heterologous promoter is enough to delay flowering of the plants (Michaels & Amasino 1999). Initiation of flowering in plants by vernalization through the down regulation of the protein FLC, involves histone methylation at the *FLC* locus (Bastow et al. 2004).

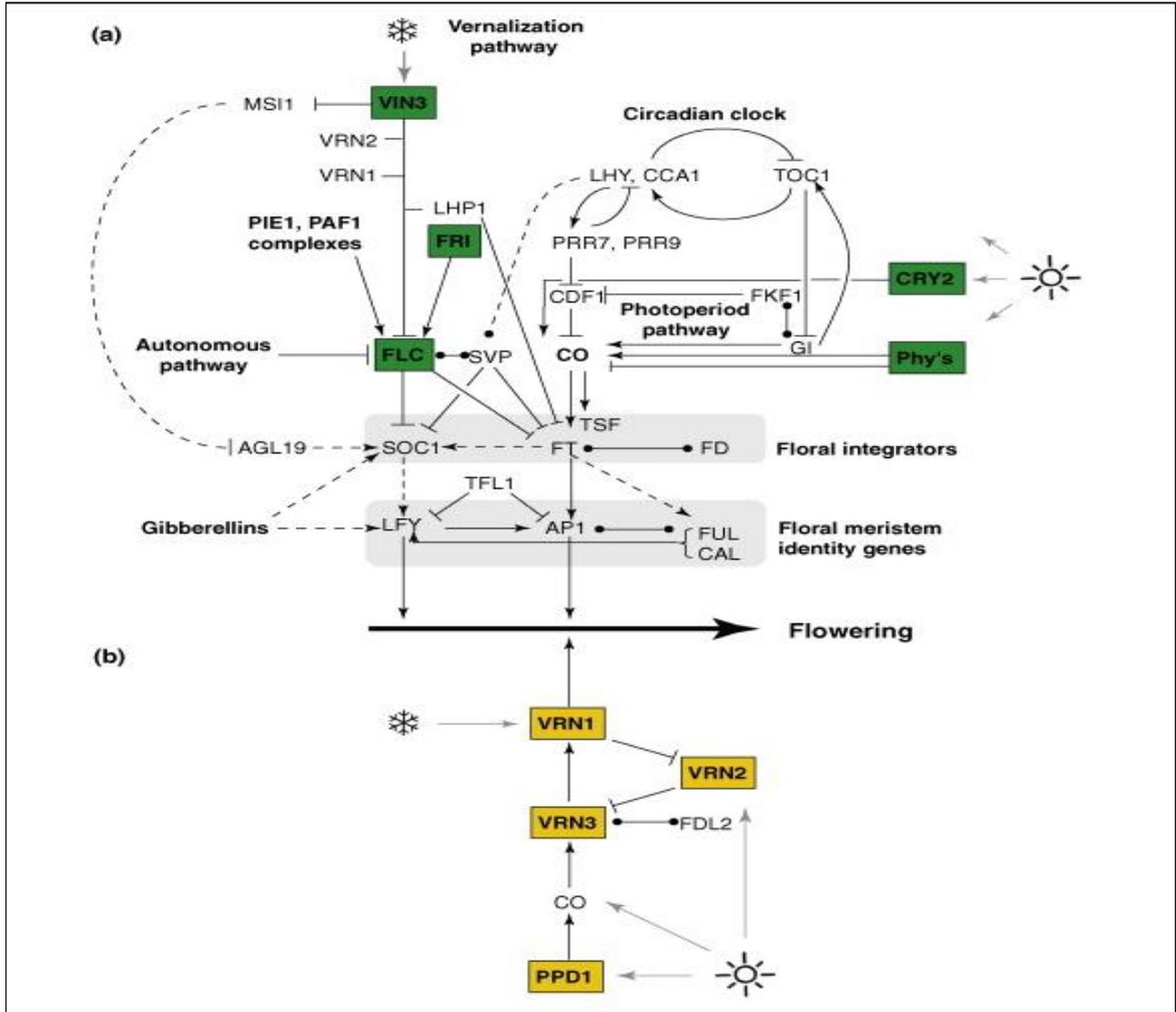
### **1.2.1 Divergence and conservation of vernalization pathways**

All plants go through diverse developmental transitions during their life cycle. Environmental and developmental cues stimulate the plants to alter vegetative stage to reproductive stage. The success of reproduction of a plant relies on synchronization of flowering time with ambient environmental conditions to enhance fertilization and seed development. To make sure that flowering takes place in spring or the summer season, plants growing in temperate climates have evolved a signal sensing and transduction pathway; prolonged periods of cold during winter turn this environmental cue into an improved capability to flowering (Schmitz & Amasino 2007).

Studies on *Arabidopsis* has described the epigenetic behaviour of the underlying processes and demonstrated at the molecular level how vernalization allow the plants to respond to the seasonal stimulus (day length) by removing block to flowering. *FLC*, one of the main genes in *Arabidopsis* that maintain the vernalization requirement and response, is a MADS-box transcription factor that suppress the floral transition (Schmitz & Amasino 2007). The abundance of both positive and negative regulators closely controls the expression of *FLC* (**Figure 1a**). Johanson et al. (2000) stated that “allelic variation at the *FRIGIDA (FRI)* locus is a major definitive of natural variation for flowering time in *Arabidopsis*. Dominant alleles of *FRI* confer late flowering that is reversed to earliness by vernalization”.

The prime activator of *FLC* is *FRI (FRIGIDA)*; responsible for much of the natural variation in vernalization need amid *Arabidopsis* accessions. *FRI* loci that carry mutations result in early flowering in *Arabidopsis* (Gazzani et al. 2003; Johanson et al. 2000; Le Corre et al. 2002; Shindo et al. 2005). The autonomous pathway comprises an association of factors related to epigenetic regulation that repress the *FLC* where *FRI* and *FRI*-like genes is dominant, to a great extent

controlled by a set of negative regulators of *FLC* (Michaels 2009; Simpson 2004), even though they can be overcome by vernalization (Schmitz & Amasino 2007).



**Figure 1.** Schematic illustration of flowering time control in *Arabidopsis* (a) and cereals (b). Exogenous cold and light signals are indicated by symbols (Jung & Müller 2009).

*VIN3* (*VERNALIZATION INSENSITIVE 3*) is the key component of the vernalization pathway and it is induced and gradually up-regulated after prolonged periods of cold exposure. *VIN3* is associated with the commencement of alteration of the *FLC* chromatin structure (Sung & Amasino

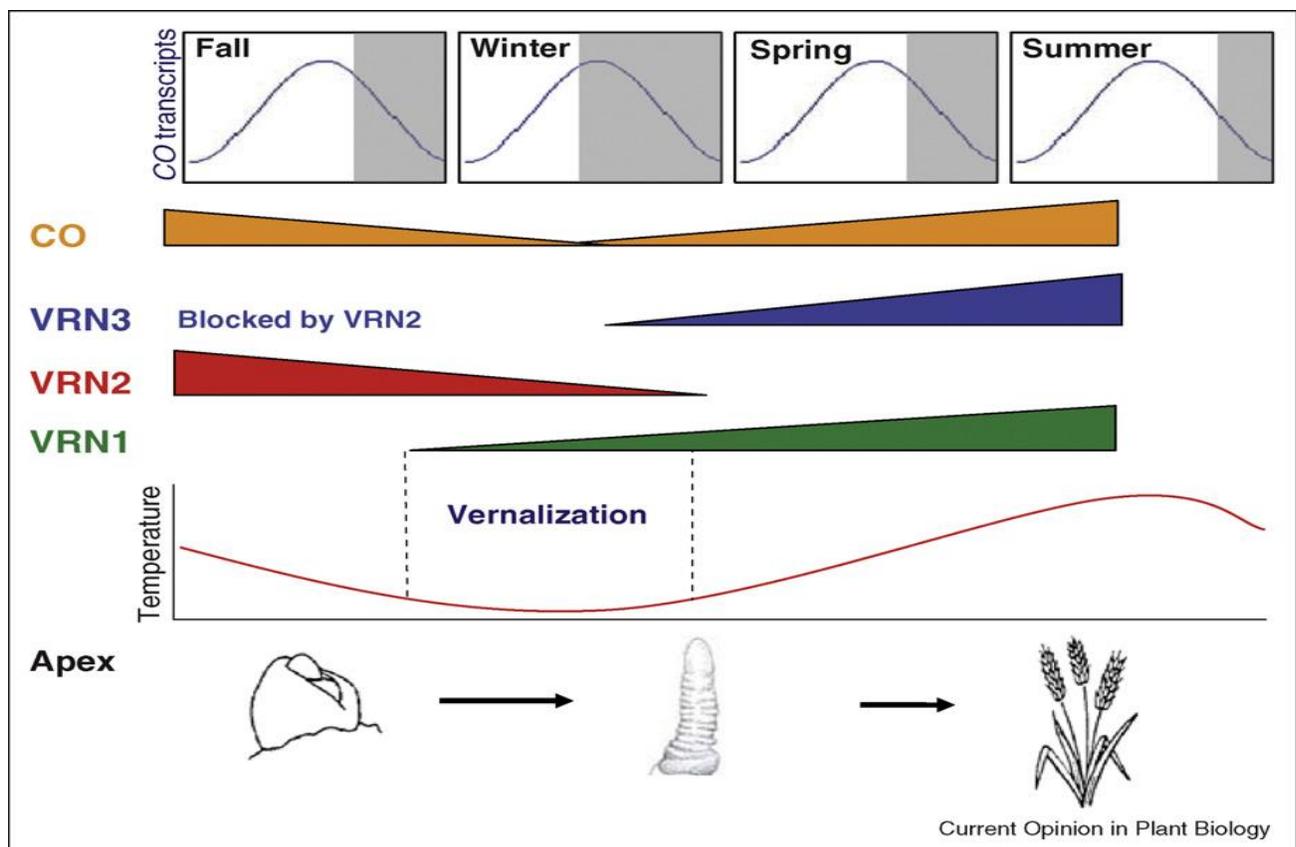
2004a). In the succeeding generation, reinstatement of *FLC* expression is a key step around the time of early embryogenesis (Choi et al. 2009; Sheldon et al. 2008), thereby making sure to regenerate the requirement for vernalization. Tadege et al. (2001) discovered that *FLC* encodes a dosage dependent repressor that is responsible for flowering in *Arabidopsis*. *FLC*-like expressed sequences were identified in *Brassica napus* and expression of each of the sequences in *Arabidopsis* delayed flowering notably ranging from 3 weeks to more than 7 months.

In monocot plant species, *FLC* and *FLC*-like genes have recently been detected; they belong to a major MADS-box gene family (Ruelens et al. 2013). In temperate cereals, the requirement for vernalization and its effects has been widely studied and are well understood (Colasanti & Coneva 2009; Distelfeld et al. 2009; Greenup et al. 2009; Trevaskis et al. 2007) (**Figure 1b**). The key genes for vernalization (*VRN1*, *VRN2* and *VRN3*) have been identified in bread wheat (*Triticum aestivium* L.) and these genes are controlling the transition from vegetative to reproductive phase. These key regulators are not only homologous to the vernalization genes in *Arabidopsis* but also to other *FLC*-like genes. Interestingly, the components of the regulatory pathway are homologous with other floral regulatory genes in *Arabidopsis*. In short, expression of the MADS-box transcription factor *VRN1* is crucial for floral transition (Shitsukawa et al. 2007). This MADS -box transcription factor share its homology with the floral meristem identity genes, i.e. *APETALAI (API)*, *FRUITFULL (FUL)* and *CAULIFLOWER (CAL)* in *Arabidopsis* and are up-regulated gradually by vernalization (Yan et al. 2003).

### **1.2.2 Genes regulating vernalization**

Genetic studies of vernalization response in bread wheat found that major vernalization response (*VRN*) loci govern the flowering and maturity times, and it has been mapped to the middle of the long arms of chromosomes 5 (Barrett et al. 2002; Dubcovsky et al. 1998; Galiba et al. 1995; Iwaki et al. 2002). Since bread wheat is hexaploid with three sub-genomes, three distinct *VRN* loci are present and control the vernalization response. *VRN1* genes, which are solely responsible for the flowering and maturity times, are located on chromosomes 5AL, 5BL and 5DL (Preston & Kellogg 2008; Trevaskis et al. 2003). The first vernalization genes (*VRN-1*) were cloned by map-based cloning techniques (Yan et al. 2003). After that, *VRN2* and *VRN3* genes were also cloned by map-based cloning in wheat and barley (Yan et al. 2004; Yan et al. 2006). *VRN1* gene expression is regulated by vernalization and by development, but daylength is not affecting its expression. In

many winter wheat varieties, *VRN1* is induced by cold temperature and represses the expression of *VRN2*; this is affecting the growth habit of wheat (Trevaskis et al. 2006). Depending on the expression pattern, and the characteristics of the protein encoded by *VRN2*, it has been confirmed that *VRN2* plays an important role in blocking the flowering in long-days by repressing *FLOWERING LOCUS T (FT)*. The *VRN3* gene is homologous to the *Arabidopsis FT* gene (Faure et al. 2007; Yan et al. 2006), and it exhibits full expression if its dominant allele is present. The fully expressed *VRN3* gene accelerate flowering and omit the vernalization requirement (Yan et al. 2006). Hence, the nature of growth habits and vernalization requirement of cereal crops are predominantly governed by three vernalization genes (*VRN1*, *VRN2* and *VRN3*) (Figure 2).



**Figure 2.** Regulation of vernalization genes by environmental alteration during the growing season in winter cereals (Distelfeld et al. 2009).

### **1.2.2.1 Role of *VERNALIZATION 1 (VRN1)***

*VRN1* is MADS-box transcription factor. This wheat gene is orthologues to the *Arabidopsis* meristem identity genes *APETALA1* and *FRUITFUL* (Loukoianov et al. 2005). The induction of *VERNALIZATION 1* gene expression is determined by cold exposure, initially expressed at low levels. *VRN1* functions as repressor of the expression of *VRN2* (Trevaskis et al. 2006). The expression of the *VRN1* gene depends very much on the length of the vernalization treatment (Yan et al. 2003), which is affecting the timing of flowering quantitatively.

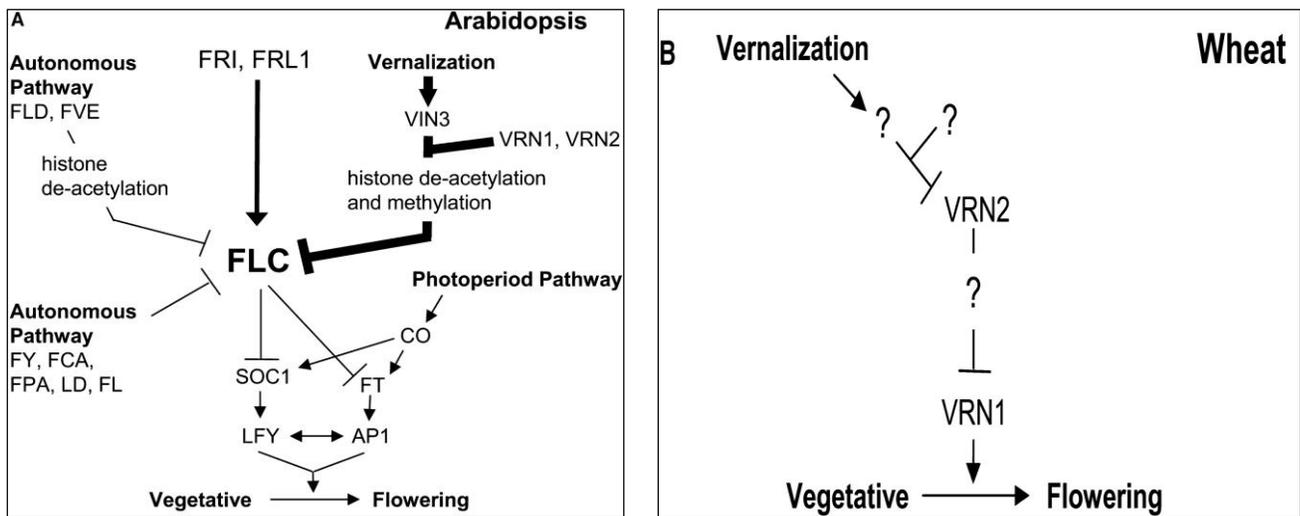
### **1.2.2.2 Role of *VERNALIZATION 2 (VRN2)***

*VRN2* function as floral repressor until the plants vernalized. *VRN2* encodes a CCT domain protein with a putative zinc-finger motif which mediates the DNA binding (Yan et al. 2004). *VRN2* is expressed in long days but is down-regulated in short days (Trevaskis et al. 2006). The key role of *VRN2* is to hinder the flowering in long day conditions by repressing *FT* and this happen due to the interaction between the *FT* gene sequence and *VRN2* protein directly or, indirect interaction with PPD-H1; a component of the photoperiod pathway.

The vernalization of plants in long days leads to suppression of *VRN2* expression whilst *VRN1* expression level increases. So based on reciprocal expression patterns, it has been realized that low temperature repress *VRN2* gene expression but it gives scope for increased *VRN1* expression (Yan et al. 2004). Trevaskis et al. 2006 stated that vernalization response is mediated through *VRN1*, whereas *ZCCTa* and *ZCCTb* (*VRN2*, which has been mapped to a chromosome region containing *ZCCT* zinc finger transcription factor genes) respond to day length cues to repress flowering under long day condition in non-vernalized plants

### 1.2.2.3 VERNALIZATION INSENSITIVE 3 (VIN3)

Bond et al. (2009a) stated that “To promote flowering in *Arabidopsis thaliana*, *VIN3* is needed for the vernalization-mediated epigenetic repression of *FLOWERING LOCUS C (FLC)* and it is quantitatively induced in response to low temperatures”. Interestingly, *VIN3* is identified as an upstream gene in *Arabidopsis* but this gene is not present in wheat (**Figure 3**). *VIN3* encodes a homeodomain finger containing protein (PHD) which accumulates after cold exposure at a sufficient level to trigger vernalization (Sung & Amasino 2004a).



**Figure 3.** Flowering pathways in a) *Arabidopsis* and b) *Wheat*, the thicker lines indicate the *FLC* regulation and diagram for wheat shows the absence of *VIN3* genes (Amasino 2004).

The study of *VRN1* and *VRN2* has revealed an interesting feature of the vernalization mechanism. During the vernalization, *FLOWERING LOCUS C (FLC)* is repressed but repressed state of *FLC* is not stably maintained in *VRN1* and *VRN2* upon return to warm conditions. On the other hand, *VIN3* is responsible for the initial repression of *FLC* during cold exposure but repression of *FLC* never occurs under extended cold conditions.

The recent model of vernalization response suggests that *VIN3* is expressed by minimizing acetylation of histones in domains of the *FLC* gene with the result that *FLC* is repressed. Later, histone methylation occur by *VRN1* and *VRN2* that ensure that *FLC* is kept in a repressed and stable condition after the plants are returned to warm conditions (Sung & Amasino 2004a) and until the chromatin is reset throughout the meiosis.

### **1.3 Cold acclimation and its effects on plants**

Freezing temperatures have significant impacts on plant production and plants demonstrate variation in the ability to survive freezing. As opposed to plants grown in tropical and subtropical regions where plants are damaged by slightly freezing temperature, plants grown in temperate regions shows varying degrees of tolerance to survive. As winter approaches, cold stress is unfavorable for plant growth and development, which is significantly restricting the spatial allocation of agricultural production. A wide range of studies indicates that cell membrane of the plants is the initial site of freezing injury and damaged cell membrane turn to severe dehydration. In addition, other stresses impede on the expression of full genetic potential and metabolic processes of plants. Cold acclimation is a complicated process that leads to alteration in lipid composition in the plant cell membrane, increased concentration of proline and sugars, and augmented soluble protein (Thomashow 1999).

The process of cold acclimation is complex but it is controlled by physiological, biochemical and molecular changes. Xin and Browse (2000) stated that “Although many biochemical and gene-expression changes occur during cold acclimation, few have been unequivocally demonstrated to contribute to the development of freezing tolerance.”. The physiological, biochemical and molecular changes has wider effects on plant growth and water balance. These changes modify the cell wall composition, lipid unsaturation or solute accumulation, result in increased antioxidant production, and induce the expression of cold regulated genes to protect the plant from freeze-induced injury (Thomashow 1999; Xin & Browse 2000). These changes in gene expression boost the plants to increase freezing tolerance and enable the plant to survive winter conditions. Moreover, genes involved in physiological, biochemical and molecular changes regulating cold acclimation signal transduction pathways enable the plant to endure cold stress conditions.

#### **1.3.1 Genes regulating cold acclimation**

Studies show that many genes induced by cold exposure act to keep the membranes intact against freezing injury. Moreover, a transcription factor, C-repeat-/DRE-Binding Factor1 (*CBF1*) that controls expression of cold-regulated genes (*COR*) which activate freezing tolerance, is expressed rapidly and transiently after sensing cold (Thomashow 1999). Some of the cold-induced genes keep up high expression levels during cold acclimation whilst others are express shortly (Tang et al. 2005; Xiong & Fei 2006). The cold induced genes have been divided into two major groups: a)

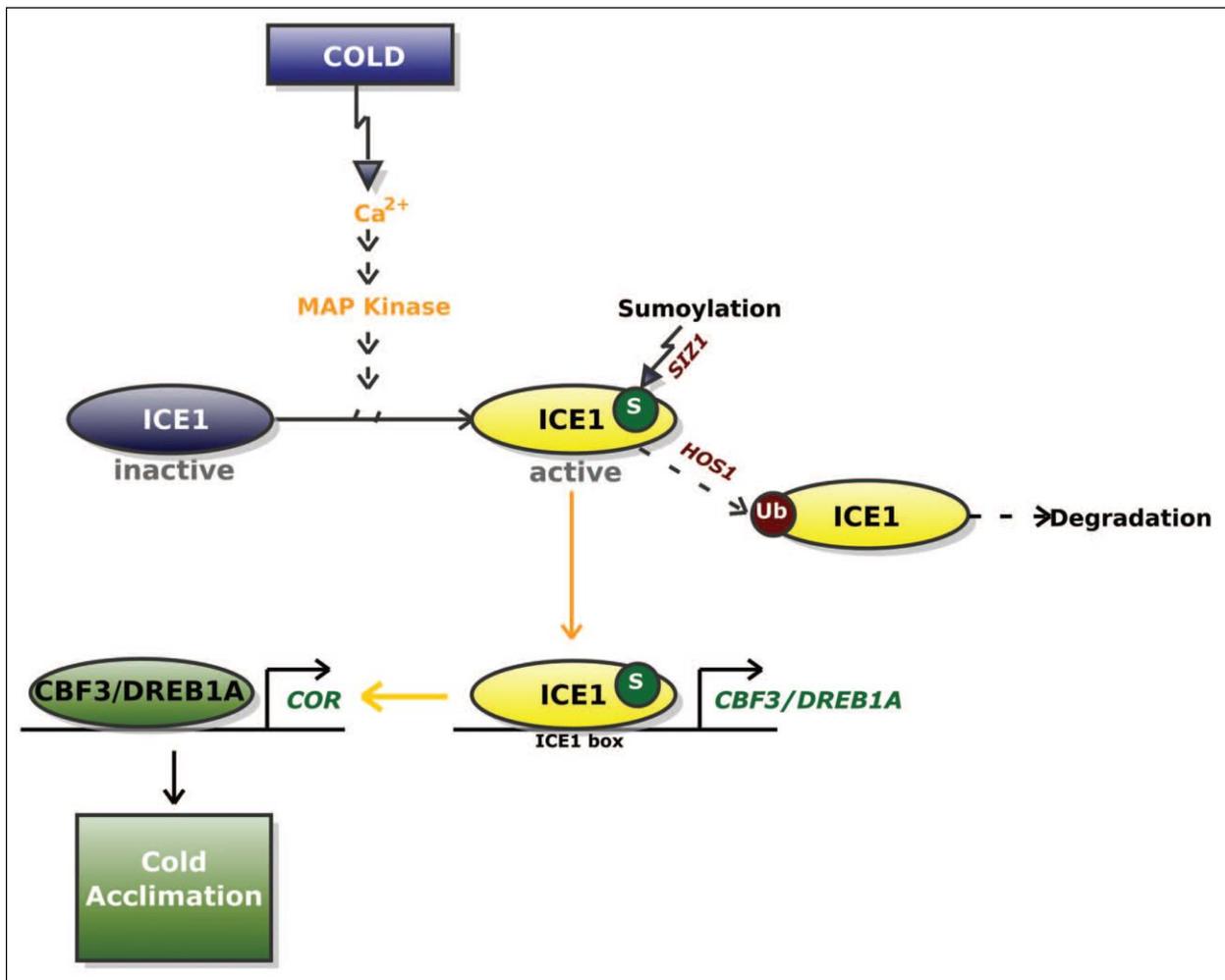
genes that encode proteins for regulation of gene expression and signal transduction involving transcription factors that controls expression of cold-regulated genes, e.g. protein kinases, proteinases, and the well-studied *CBF* gene family (Nanjo et al. 1999; Thomashow 1999); b) genes that encode the (*DRE*) dehydration-responsive element, COR proteins and AFP (antifreeze proteins) (Seki et al. 2001; Seki et al. 2002; Tang et al. 2005). Acute cellular dehydration occurs in plant tissues by ice formation, which causes quickest freezing and damage the cellular membranes (Atıcı & Nalbantoğlu 2003; Griffith et al. 1997; Thomashow 2001). Griffith et al. (1997) discovered that of antifreeze proteins genes may originate from pathogenesis-associated proteins while keeping up their same catalytic activities; which may increase both disease resistance and freezing tolerance in overwintering plants. Another important point is that the expression of antifreeze protein genes may limit the mechanical stress occurring during ice formation in plant tissues (Atıcı & Nalbantoğlu 2003).

### **1.3.2 Cold Acclimation pathways and Freezing Tolerance in Plants**

Low temperatures have an effect on plant adaptation such as plant growth, freezing tolerance and abiotic stress tolerance (Lissarre et al. 2010). Through the process of cold acclimation, acclimated plants can survive throughout the winter. During the period of low temperature, the plants biochemical, metabolic and physiological status is altered; changes are controlled by low temperature at the gene expression level in most cases. Under natural field conditions, cold acclimation takes place in the fall before low non-freezing temperatures. Freezing tolerance lost at the temperature when temperatures return above the freezing point. Freezing tolerance is controlled by multiple genes and its integrated programme enable the plants to overwinter.

Due to ice formation in plant tissues exposed to low temperature, cytoplasmic water is lost and accumulates as extracellular ice. Cells of freeze tolerant plants gather low molecular weight molecules (sugars, glycinebetaine and proline) avoiding the loss of cytoplasmic water. This is happening at low temperature involving expression of genes encoding enzymes important for biosynthesis. A recent advance in understanding cold acclimation in *Arabidopsis* was the discovery of the C-repeat/dehydration-responsive element binding factor (*CBF*) cold-response pathway. The *CBF/DREB1* operates the expression of cold response genes and freezing tolerance (Gilmour et al. 2000; Gilmour et al. 2004). In *Arabidopsis*, *INDUCER OF CBF EXPRESSION1 (ICE1)*, a MYC-type transcription factor, controls the *CBF/DREB1* pathway (**Figure 4**). The overexpression

of *ICE1* augments freezing tolerance; on the other hand *ICE1* mutations reduce the freezing and chilling tolerance through blocking the expression of *CBF3* (Chinnusamy et al. 2003). Chinnusamy et al. (2003) also indicates that *ICE1* is an upstream transcription factor which controls the transcription of *CBF* genes in the cold stress condition; *ICE1* instigates the *CBFs* to express only under the condition of cold stress and other essential things are required to decrease the expression of downstream genes by *ICE1* mutation. Besides the cold, ABA treatment increases the *CBF* protein levels and it is sufficient for induction of cold regulated genes; by the induction of abscisic acid (ABA), cold-inducible *CBF1-3* transcript activates the C-repeat element (CRT; dehydration-responsive) (Knight et al. 2004), it means that *ICE1* can control the ABA-induced expression of *CBF3/DREB1A*.



**Figure 4.** The cold acclimation pathway, which involves *ICE1* and *CBF3/DREB1A* in *Arabidopsis* during low temperature condition (Lissarre et al. 2010).

Sumoylation and ubiquitination are post-translational modifications that regulate cold signalling pathways. It has been proposed that high expression of the osmotically responsive gene1 (*HOS1*), a RING-type ubiquitin E3 ligase acting as a negative regulator for cold responses (early flowering), mediates the ubiquitination of *ICE1*. Induction *HOS1* gene mediates the degradation of *ICE1* (**Figure 4**) and also sensitivity to freezing stress increases due to the overexpression of this gene (Dong et al. 2006). Sumoylation is mediated by the small ubiquitin-related modifier (SUMO) E3 ligase SIZ1. SIZ1-dependent sumoylation of *ICE1* mediates the *ICE1* stability and activity that act as a positive regulator for *CBF3/DREB1A* dependent cold signalling, cold-inducible genes and freezing tolerance. In addition, sumoylated *ICE1* inactivates MYB15 which negatively regulates *CBF3/DREB1A*, freezing tolerance and cold-inducible genes (Miura & Hasegawa 2008). So it implies that both of the post-translational modifications are functionally competitive. The gene transcripts MYB15 is up-regulated by freezing stress and interacts with *ICE1*. MYB15 binds to MYB recognition *cis*-elements in the promoters of *CBF* genes, the mutant plants from MYB enhances the cold tolerance and expression of *CBF* genes whereas overexpression of MYB15 decreases the cold tolerance in plants. In addition *ICE1* has a negative effect on the regulation of expression of MYB15 (Agarwal et al. 2006). The zinc finger transcription factor gene (*ZAT12*) function as a down-regulator of expression of *CBF/DREB1s* genes, which indicates a negative role in cold acclimation. In addition, expression of *ZAT12* constitutively in *Arabidopsis* increases the cold tolerance (Vogel et al. 2005).

Doherty et al. (2009) found that “members of the calmodulin binding transcription activator (CAMTA) family of transcription factors bind to the CM2 motif, that CAMTA3 is a positive regulator of *CBF2* expression, and that double *camta1 camta3* mutant plants are impaired in freezing tolerance. These results establish a role for CAMTA proteins in cold acclimation and provide a possible point of integrating low-temperature calcium and calmodulin signaling with cold-regulated gene expression”.

## **1.4 The role of fructans and dehydrin genes in freezing tolerance**

### **1.4.1 Fructans**

Fructans are non-structural storage carbohydrates and are synthesized from sucrose. Fructans are composed of branched or linear polymers of fructose attached to sucrose through glycosidic bonds of various linkage types, stored in the vacuole, and may serve functions other than carbon storage.

Fructans are present in the vegetative tissue of many economically important plants such as wheat, barley, temperate forage grasses (Poaceae family such as *Lolium*, *Festuca*) and are also present in approximately 15% of flowering species (Hendry 1993). Due to the role of fructans as reserve carbohydrates, there are several physiological advantages for plants: i) to adjust the plants to environmental stress conditions (drought and cold stress), ii) plant development, iii) assimilate partitioning etc. Fructan-accumulating plants are perennials, their growth are restricted to drought or low temperatures conditions (Pollock & Jones 1979). Fructan is primarily stored in stems and found in leaves and roots of grass species. On the other hand, it has been observed that excised leaves of four species (oat, wheat, barley and timothy) accumulated a distinct and species-specific pattern of fructans (Cairns & Ashton 1993).

In order to understand the role of fructans in adaptation to cold stress conditions; studying fructan biosynthesis under low temperature is important. Fructan is synthesized by the collective action of multiple fructosyltransferases (FTs). Many FTs enzymes are involved in the synthesis of five different classes of fructans in plants I) inulin, II) inulin neoseris, III) levan, IV) levan neoseris and V) mixed levan. These five classes of fructans are synthesized by the four fructosyltransferases (FTs) enzymatic activities. The initial step in fructan synthesis catalyzes the transfer of a fructosyl residue from sucrose to another sucrose molecule resulting in the formation of the trisaccharide 1-kestose by sucrose-sucrose 1-fructosyltransferase (1-SST). Further elongation of the fructan chain is catalyzed by the involvement of fructan-fructan 1-fructosyltransferase (1-FFT), sucrose-fructan 6-fructosyltransferase (6-SFT), and fructan-fructan 6G-fructosyltransferase (6G-FFT) (Vijn & Smeeckens 1999). It has been reported that 6-SFT gene expression changes and accumulation of fructan in leaf bases is caused by exposures to low temperatures (Del Viso et al. 2009). Other studies also revealed that fructosyltransferase (FTs) gene expression is regulated in winter wheat by low temperature during time of cold hardening (Kawakami & Yoshida 2002). Winter cereals increase their freezing tolerance from autumn to winter through the cold hardening. In winter wheat, the cold acclimation process is not only associated with increasing the cellular contents of mono- and disaccharides such as sucrose, but also with increases in the fructan content (Yoshida et al. 1998). The changes of FTs gene expression is mainly controlled at the transcriptional level which has been detected in chicory (Van Laere & Van den Ende 2002). Similarly, other studies in cool season grasses revealed that the transcriptional level (mRNA) of 6-SFT gene increases in plants in response to low temperature treatment (Wei & Chatterton 2001; Wei et al. 2002). To

understand better the relationship between freezing tolerance and fructan levels of plants, transgenic plants have been used in experiments. A tobacco cultivar was transformed to accumulate fructan by expressing the SacB gene coding for fructan polymerase from *Bacillus subtilis* (Konstantinova et al. 2002) and transgenic perennial ryegrass plants expressing 6-*SFT* genes (Hisano et al. 2004) both demonstrated that transgenic plants accumulate increased levels of fructan compared to non-transgenic plants as well as increased levels of freezing tolerance. Chalmers et al. (2005) suggested that fructan accumulation is important for perennial ryegrass to obtain freezing tolerance, in which fructans are the main storage carbohydrates.

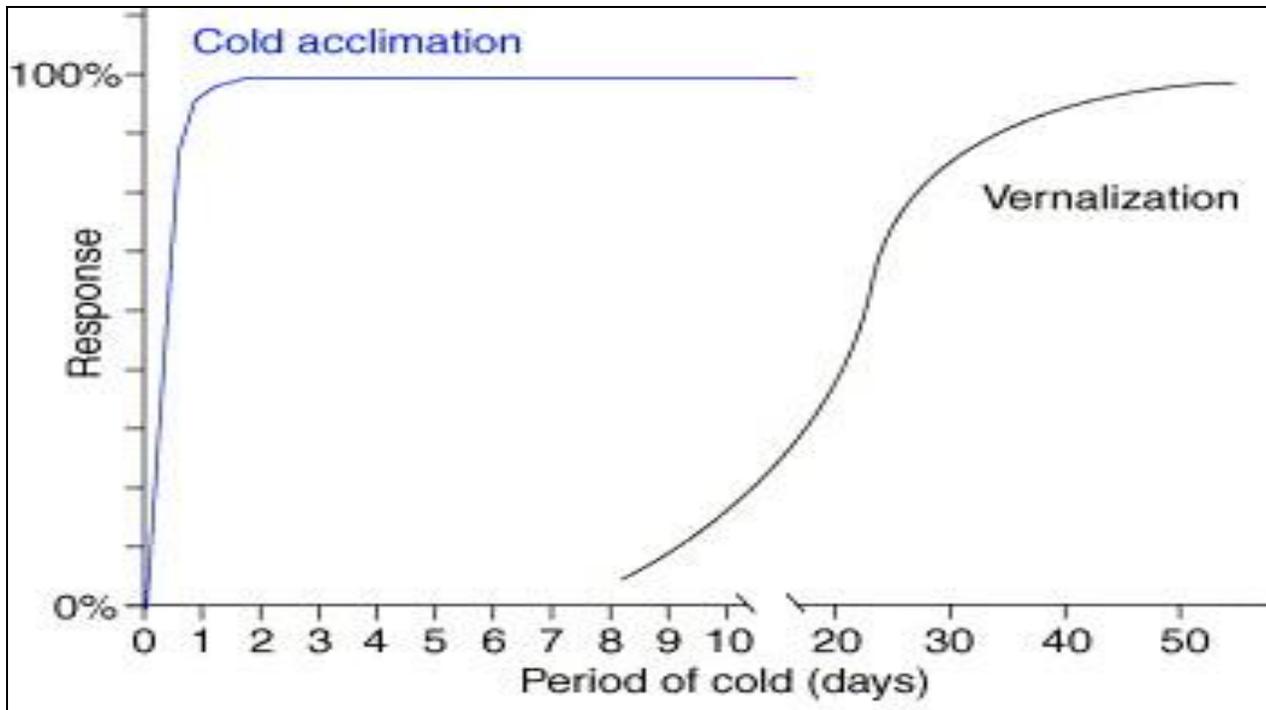
### **1.4.2 Dehydrins**

Plants respond to stressful conditions through morphological, physiological and metabolic processes. A number of proteins that is associated with dehydration, i.e. drought, low temperature, and salinity, accumulates in plants in response to environmental stimuli (Close 1996). Among the induced proteins are dehydrins (DHNs) which belong to group 2 of LEA (late embryogenesis abundant) proteins known as highly hydrophilic proteins. DHNs are induced by a reduced water potential under certain environmental conditions (Ingram & Bartels 1996; Rorat 2006). These proteins are associated with intracellular molecules by hydrophobic interactions and dehydrins are capable to protect the functions of intracellular molecules by inhibiting their coagulation during environmental stress condition through maintaining the structural integrity (Close 1997). Ismail et al. (1999) reported that dehydrin gene was involved in chilling tolerance in cowpea and without cold acclimation, transgenic tobacco expressing dehydrins shows more cold tolerance than wild type plants (Kaye et al. 1998). Thus, production of dehydrin in plants is supposed to be major strategy to acquire tolerance to freezing stress.

### **1.5 Interaction between frost tolerance and vernalization**

Cold acclimation and vernalization have a common requirement for exposure to non-freezing low temperatures and this suggests a potential relationship between these two processes (Galiba et al. 2011). During the winter season, vernalization keep perennials and bi-annuals in a vegetative state but it maintains the cold tolerance. Cold acclimation and vernalization need non-freezing low temperatures to continue metabolic activity in plants. On the other hand, metabolic activity would

be interrupted by freezing temperature. Contrary to vernalization, cold acclimation can be attained within a short period of time (Lee & Amasino 1995; Thomashow 1999) (**Figure 5**).



**Figure 5.** Time period of the cold acclimation and vernalization responses in *Arabidopsis thaliana* (Sung & Amasino 2004b), the acquisition of cold tolerance occurs within days whereas vernalization requires several weeks of cold exposure

The immediate establishment of cold acclimation is beneficial for the plants because they need to protect themselves from freezing rapidly, even if cold period prevail for short-term (for instance, late autumn). This is contrary to the vernalization where prolonged periods of cold is important to ensure that the plants are adapted to a complete winter but not responsive during the autumn when temperature fluctuations occur. Most of the temperate plant species will tolerate extracellular ice formation in their vegetative tissues when they are cold acclimated. Winter habit plants (wheat, oat, barley etc.) in temperate climates are responsive to vernalization, which prevents the plants from premature induction of flowering before the disastrous of freezing stress, and it may occur when winter just has passed. As a result, alleles of the genes for induction of vernalization need to ensures that the plant overwinter in the vegetative phase. Fowler et al. (1996) proposed that plants start to acclimate quickly at low temperature when expose to a constant 4 °C and 16-h day length

and then cold acclimation commenced to decline. When plants turn from vegetative to reproductive stage; the freezing tolerance starts to decline considerably. Major agricultural crops (rice, maize, cotton, tomato and soybean) are weak to cold acclimate and chilling sensitive also. In addition, they are incapable of tolerating ice formation in their tissue. For all that, the earlier exposure of chilling-sensitive crops to suboptimal low temperature shows the lowered chilling damage (Anderson et al. 1994). Another important observation is that activation of vernalization down-regulates the low temperature tolerance genes but vernalization saturation does not result in a "switching off" of the low-temperature tolerance genes, still plants are partially able to reacclimate followed by deacclimation after vernalization saturation (Fowler et al. 1996). Rife and Zeinali (2003) also found that after vernalization saturation winter rapeseed (*Brassica napus* L. var. *oleifera f. biennis*) is fully able to restore the low temperature tolerance following the deacclimation.

## **1.6 Gene expression studies**

Quantification of gene expression levels is a vital step for functional exploration of a candidate gene. It is especially necessary to compare the gene expression patterns under particular growth conditions. Northern blot hybridization and reverse transcription (RT)-mediated PCR (RT-PCR) is commonly used for evaluating the gene expression levels, and both of these techniques are used for quantification of expression levels of a small set of genes. Nowadays, qRT-PCR (quantitative reverse transcription (RT)-mediated PCR) is the preferred method for measuring gene expression levels in diverse samples using a limited number of candidate genes. It provides sensitive and exact quantification of gene transcript levels; it even provides correct quantification for genes with low transcript levels (Bustin et al. 2005; Bustin 2005; Nolan et al. 2006). Nevertheless, during sample preparation, procedural work and data analysis, experimental deviations or error might occur which makes the quantification of gene transcripts untrustworthy. In order to avoid such experimental deviations or errors, normalization of the quantitative real time polymerase chain reaction (qRT-PCR) data is important. To normalize the data, suitable internal reference genes (example, GAPDH, actin, EF 1A) are commonly used and the ideal internal reference genes shows the expression at constant level in all plant tissues under different growth conditions. In addition, their expression levels should not be affected by exogenous applied growth hormones and environmental changes.

## **Aims of the study**

Perennial ryegrass (*Lolium perenne* L.) and *Festulolium* are important temperate forage grasses but these grasses are not well adapted at higher latitudes and are sensitive to abiotic stresses. Obtaining information about the regulation of flowering in perennial ryegrass and *Festulolium* will help to improve the adaptation of these grasses to specific environmental conditions. Therefore, the aims of this study were to: i) study expression patterns of vernalization genes in two genotypes (201 and 204) of *Lolium perenne* with differing vernalization requirements under natural field conditions using qRT-PCR; ii) validate gene expression estimated in the VARCLIM project using RNA sequencing of the two perennial ryegrass genotypes and two *Festulolium* genotypes (266 and 329) grown under controlled climate conditions; and iii) compare the vernalization response between natural and artificial conditions in *Lolium perenne*.

## 2. Materials and Methods

### 2.1 Plant material and growth conditions

Experiments were performed using clones of two genotypes (201, 204) from the Norwegian *L. perenne* cv. 'Fagerlin' differing in vernalization requirements, i.e. genotype 201 flowered without vernalization and genotype 204 need vernalization to flower. Each genotype was cloned, from which approx. 3 ramets (tillers) were transplanted into pots filled with standard soil media for grass. A total number of 100 pots were cloned from the two genotypes (201, 204) and was used to quantify the expression levels of vernalization genes in the two genotypes under natural field conditions using qRT-PCR. The plants were grown in a controlled growth room with growth conditions of 18/20 °C day/night temperature, 18 h photoperiod at a light intensity of 220-240  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for four weeks provided by cool white fluorescent lamps and bulbs for 4 weeks until they had 2 - 4 axillary shoots.



a) Newly cloned plants inside the greenhouse



b) Plants covered with soil outdoor under natural conditions



c) Plants under snow cover



d) Plants taken inside after digging up

**Figure 6.** Plant conditions inside the greenhouse and natural conditions throughout the autumn and winter (Photo by Kamrul Islam)

Four weeks old ramets were placed outside in 17<sup>th</sup> October, 2013 and exposed to cold weather for cold acclimation. Subsequently, all of the pots were transferred to natural field conditions and covered with sand.

## 2.2 Tissue sampling

Leaf and crown tissues with three biological replicates were collected for RNA extraction every two weeks at the same time throughout winter (November to March) from plants in the field, with temperature fluctuations ranging from -10 °C to +6 °C (**Table 1**). About 1.5 inches leaf and 3 mm crown tissues were collected from the 3 tillers per pot and immediately transferred to liquid nitrogen. The samples were stored at -80 °C until used for total RNA isolation.

**Table 1. Sampling time points and temperature recordings**

SL No	Sampling date	Specimens	Temperature °C
1	06.11.13	Leaf and crown tissues	4
2	20.11.13	Leaf and crown tissues	-5

3	4.12.13	Leaf and crown tissues	4
4	18.12.13	Leaf and crown tissues	-6
5	02.01.14	Leaf and crown tissues	-4
6	15.01.14	Leaf and crown tissues	-10
7	30.01.14	Leaf and crown tissues	-3.8
8	13.2.14	Leaf and crown tissues	2.2
9	27.2.14	Leaf and crown tissues	3.4
10	13.3.14	Leaf and crown tissues	3.7

### 2.3 Total RNA extraction and first strand cDNA synthesis

The frozen leaf samples were crushed independently in liquid nitrogen with pestle and mortar and the powdered sample stored at -80 °C until used for total RNA isolation. Total RNA was isolated from 50-100 mg frozen ground samples using TRIZOL Reagent according to the manufacturer's specifications (Appendix 1). Thus, total RNA was eluted from the 30 µl from RNase-free water. The mRNA concentration and purity was determined using the Nano drop (Nano drop Technologies, Wilmington, DE, USA). Each sample was measured twice and an average value determined. RNA samples eluted with water were stored at -80°C before used for first strand cDNA synthesis.

Ten ng of messenger RNA (mRNA) of each sample was reverse transcribed to generate the single-stranded cDNA using the Superscript® VILO™ cDNA Synthesis Kit (Invitrogen) in a total reaction volume of 20 µl. All cDNA samples were diluted to 100 fold with DEPC-treated water prior to use in qPCR.

## 2.4 Primer design

Specific primers for qRT-PCR were designed using the Primer3 software (<http://primer3.ut.ee/>). Primers were designed for amplification of 100 to 200 bp fragments in the 3' end of the coding sequence of the gene of interest. Primers were synthesized by Invitrogen (see **Table 2**).

**Table 2. Sequences of forward and reverse primers used to amplify vernalization genes from RNA samples collected under natural conditions**

Primer name	Primer sequence 5' to 3'	Primer Tm (°C)
LpVRN1-F	GCAAGCGGATCGAGAACAAGAT	71
LpVRN1-R	TCTCGTGCGCCTTCTTGAG	70
LpVRN2-F	CTCAATGCCAGCAGTAATGGTG	71
LpVRN2-R	GAGGGCGGGGAAGGAG	70
LpVin3-F	CCAGATGGCATCCCAAAGT	68
LpVin3-R	CCTCAGAGCTACCGGACTGG	74
LpGAPDH-F	TGGTGCCAAGAAGGTCATCAT	62
LpGAPDH-R	GACCATCAACAGTCTTGG	62

## 2.5 qRT-PCR conditions & expression data analysis

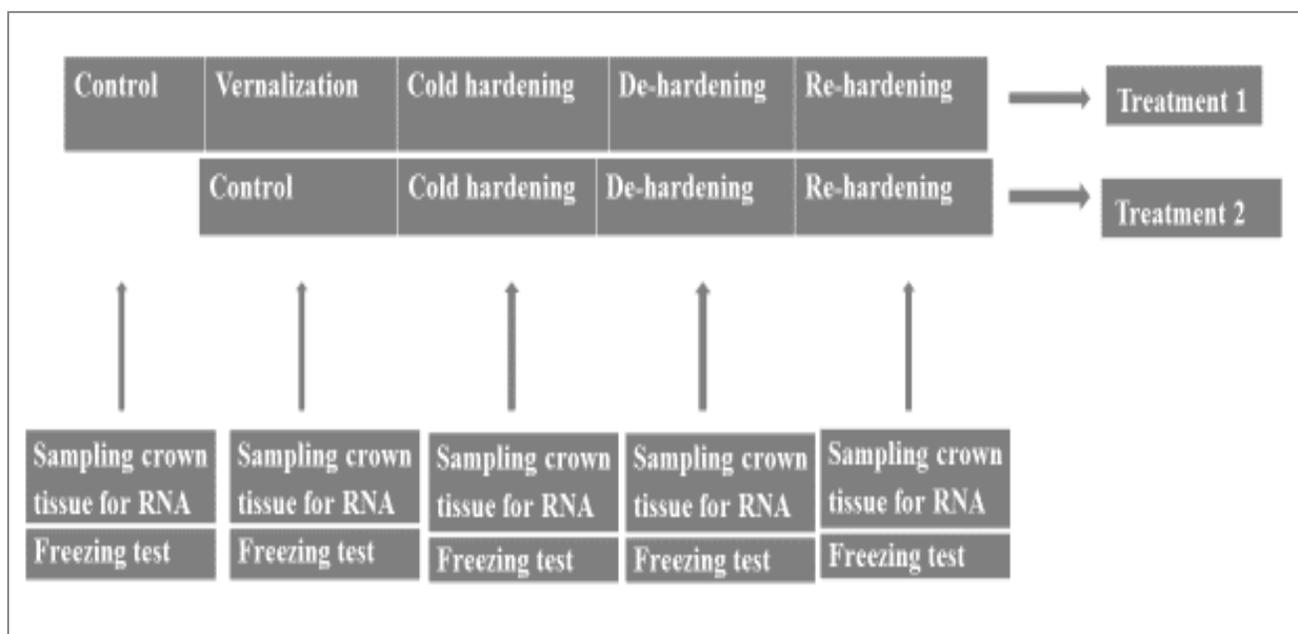
qRT-PCR was performed in 96-Fast optical plates with 7500 Fast Real-time instrument using 1 X SYBR Green PCR Master Mix (Life technologies, Invitrogen, catalog number 4309155). The reaction mixture contained 2 µl of cDNA, 10 µl of SYBR Green, 0.5 µl of each of the forward and reverse primers (10 µM) and 7µL of DEPC treated water in a final volume of 20 µl. The following reaction conditions were applied: 2 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 62 °C (annealing). The melting curve was used to confirm that there were no primer dimers and verified the amplicon specificity. All of the samples were run with 3 technical replicates and there were no template control (NTC) included in every run to detect the probable DNA contamination. The changes in expression of the target genes *VRN1*, *VRN2*, and *VIN3* normalized

to the housekeeping genes GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were observed for plants kept under natural conditions throughout the winter season. The threshold cycle (Ct) values were generated from the real time PCR instrument.. To analyze gene expression data and quantify the relative changes in gene expression the  $2^{-\Delta\Delta CT}$  method was used. The  $\Delta\Delta CT$  is calculated by the equation  $\Delta\Delta CT = (CT \text{ of target} - CT \text{ of GAPDH})_{\text{time x}} - (CT \text{ of target} - CT \text{ of GAPDH})_{\text{calibrator sample (time zero)}}$ . The mean CT values of biological replicates for both the target and housekeeping genes were determined at each time point and used in the above equation. We calculated the fold change in gene expression at each time point and relative to the expression at time zero using the  $2^{-\Delta\Delta CT}$  equation (Livak & Schmittgen 2001).

## **2.6 Validation of expression levels of genes identified in the VARCLIM project by RNA sequencing**

### **2.6.1 Plant material and growth condition**

Two genotypes (204,201) of *Lolium perenne* (cv. 'Fagerlin') and two genotypes (329,266) from *Festulolium* (Norwegian breeding population) was used throughout this study (**Figure 7**). These genotypes have differential vernalization requirements, i.e. 204 and 329 need vernalization to flower while 201 and 266 flower without vernalization. Plants were propagated vegetatively from mature tiller in pots containing standard soil media for grasses in the greenhouse. The plants were grown in a controlled growth room with growth conditions of 18/20 °C day/night temperature, 18 h photoperiod at a light intensity of 220-240  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for four weeks provided by cool white fluorescent lamps and bulbs for 4 weeks until they had 2 - 4 axillary shoots. After that, plants were transferred to controlled conditions at 8 h photoperiod and a light intensity 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  was maintained at Særheim.



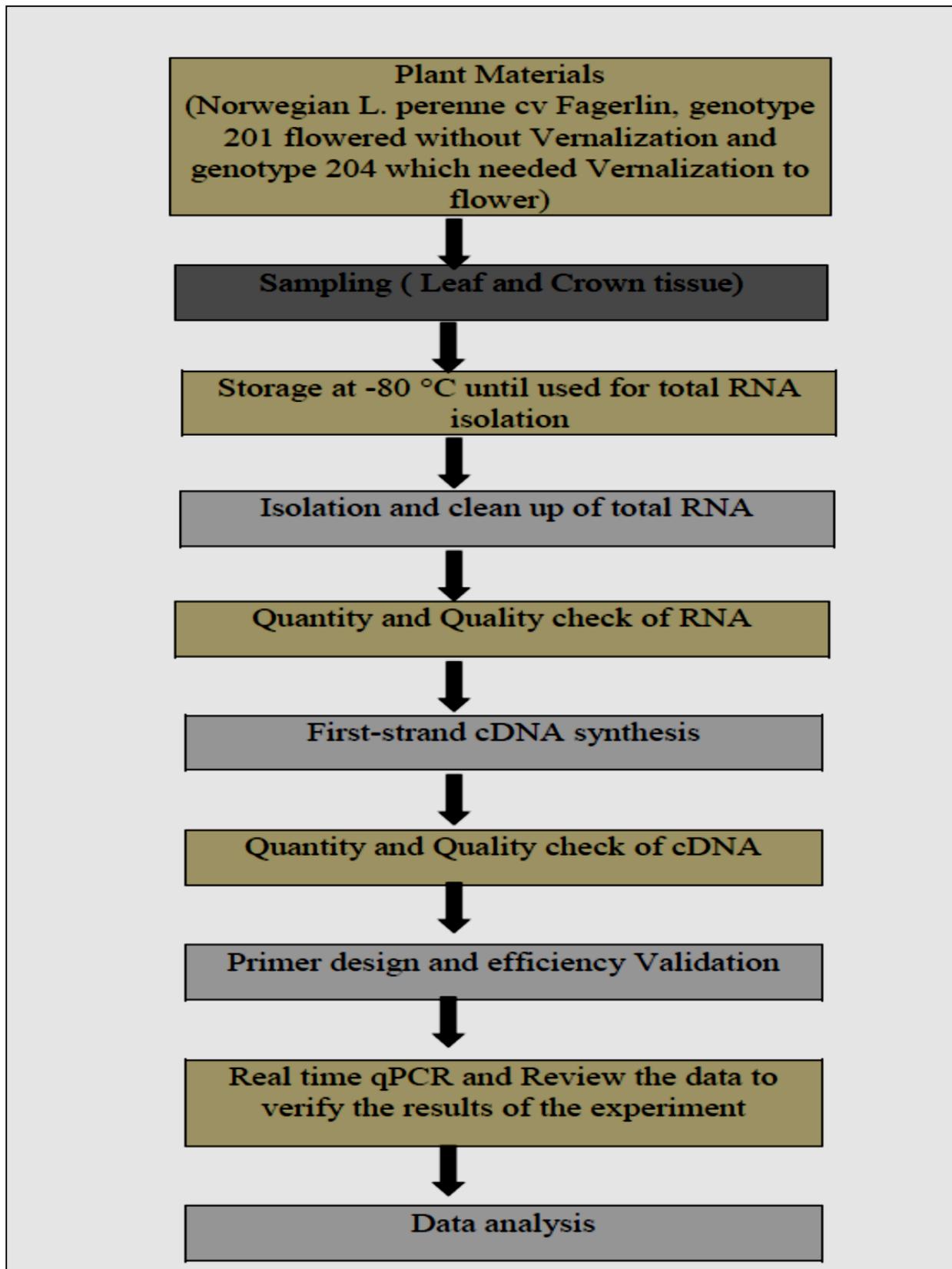
**Figure 7. RNA extraction at critical time points**

### 2.6.2 Validation of RNA-seq data by quantitative real-time RT-PCR (qRT-PCR)

RNA preparation and cDNA synthesis were conducted as described above. The primer sets for each transcript were designed using Primer3 software (<http://primer3.ut.ee/>) where transcripts used as input (see **Table 3**). Determining the primer efficiency, a pool of cDNA from 108 samples was used to perform the qRT-PCR reactions. All primer amplification efficiencies were almost 95%. qRT-PCR was performed in 96-Well optical plates with 7500 Fast Real-time instrument using 1 X SYBR Green PCR Master Mix (Life technologies, Invitrogen), and starting quantity was estimated from critical thresholds using the standard curve method. Reference gene EF1A were used in the case of validation of 6 genes identified in the VARCLIM project by Kovi et al. (unpublished) using the RNA sequencing approach for the 4 genotypes (210 and 204 of *Lolium*; 266 and 329 of *Festulolium*). Data for each sample were calculated in relation to the reference gene EF1A using  $2^{-\Delta\Delta CT}$  method (Livak & Schmittgen 2001). All of the samples were tested in triplicate, and the experiments were performed on three biological replicates.

**Table 3. List of primers used to validate the genes**

<b>Primer name</b>	<b>Primer sequence 5' to 3'</b>	<b>Primer Tm (°C)</b>
LpVRN1_F	GCAAGCGGATCGAGAACAAGAT	71
LpVRN1_R	TCTCGTGCGCCTTCTTGAG	70
LpVin3_F	CCAGATGGCATCCCAAAGT	68
LpVin3_R	CCTCAGAGCTACCGGACTGG	74
DH_266_F	TCCAACGACCAAGTGAGCTA	68
DH_266_R	CCCGCTACCTTTGCAGAATA	68
Fruct_266_F	CTCACCGAAAAGGTCTCACC	70
Fruct_266_R	ACGACGAGTCACGGTCAAC	70
EF1A_F	CCTTGCTTGAGGCTCTTGAC	58.8
EF1A_R	GTTCCAATGCCACCAATCTT	56.6



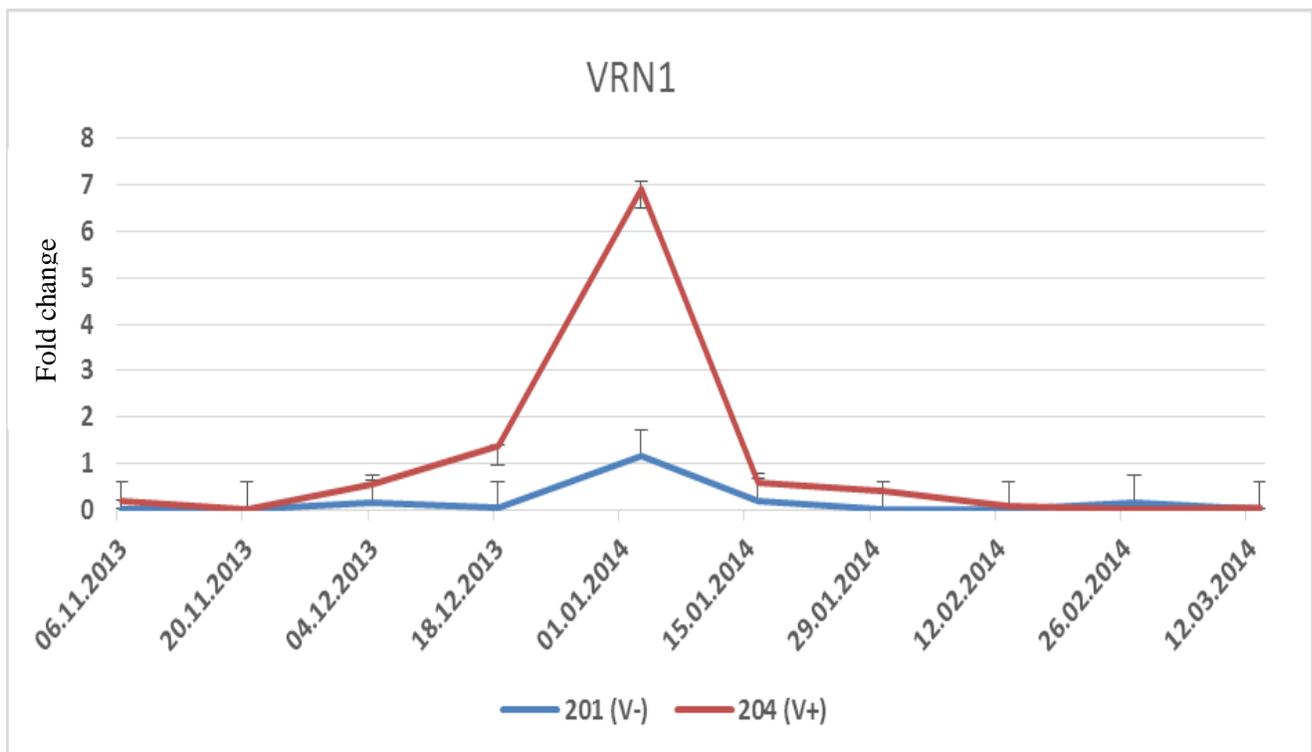
**Figure 8.** Flow chart for gene expression studies using qRT-PCR

### 3. Results

#### 3.1 Differentiation in vernalization requirement for flowering under natural field conditions

A Quantitative Reverse-Transcriptase Polymerase Chain Reaction assay (Figure 8) was used to compare the expression levels of the *VRN1*, *VRN2* and *VIN3* genes between two genotypes from the Norwegian *L. perenne* cv 'Fagerlin' differing in vernalization requirements, i.e. genotype 201 flower without vernalization (V-) and genotype 204 need vernalization to flower (V+) (Figure 6). In this section, I present expression data obtained from samples collected from the two genotypes under field conditions during the entire winter season.

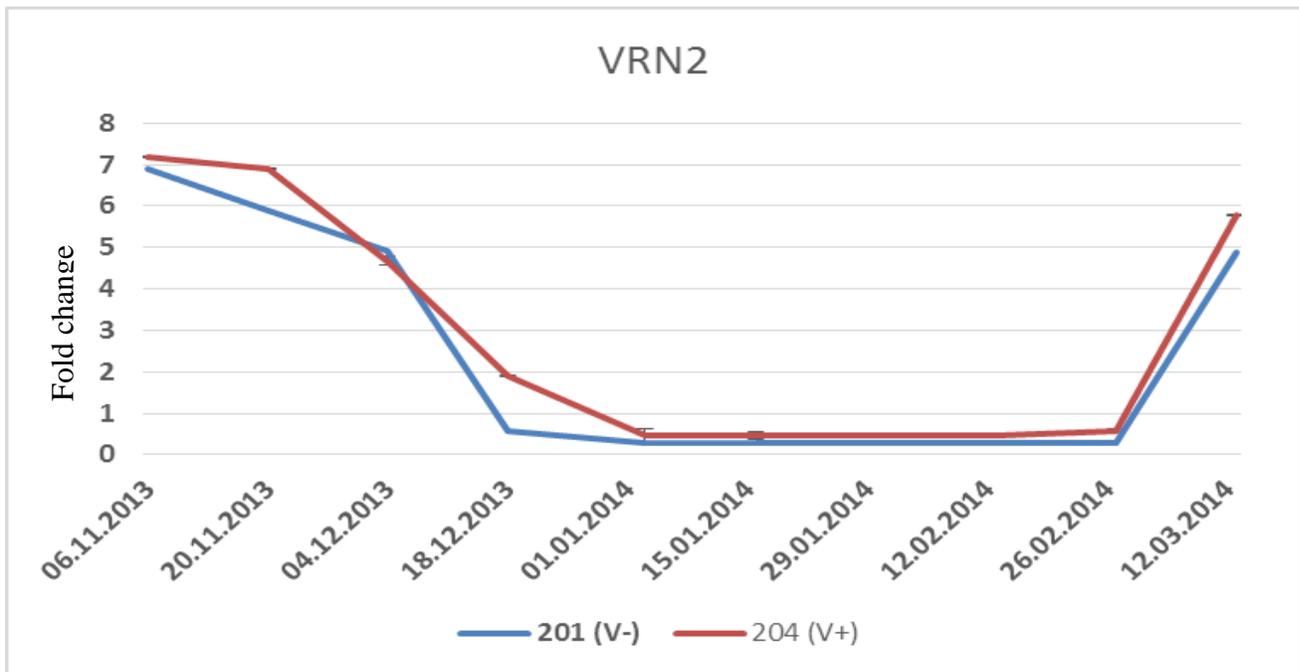
##### 3.1.1 VERNALIZATION 1



**Figure 9.** Expression patterns of *VERNALIZATION1* (*VRN1*) gene in *Lolium perenne* at natural field conditions. The X-axis represents the time points of sample collections and the Y-axis represents the fold change in expression of respective genotypes. Error bars represent the standard error of the means calculated from two biological replicates.

*VERNALIZATION 1 (VRN1)* is the key vernalization gene in grasses controlling the flowering pattern. *VRN1* is initially expressed at low levels and significantly more induced by vernalization during the mid-December to mid-January (-6 to -10 °C) in the V+ (204 genotype) which required vernalization compared to the V- (201 genotype) which does not require vernalization. The relative expression increased from 1 to 7 fold in the 204 genotype in mid-January. It was evident that *VRN1* start to increase in mid-December and peaked but decreased after mid-January. The highest expression (1 fold increase) of *VRN1* was detected in 201 genotype during the period of mid-December to mid –January but after that same as in the 204 genotype.

### 3.1.2 *VERNALIZATION 2*

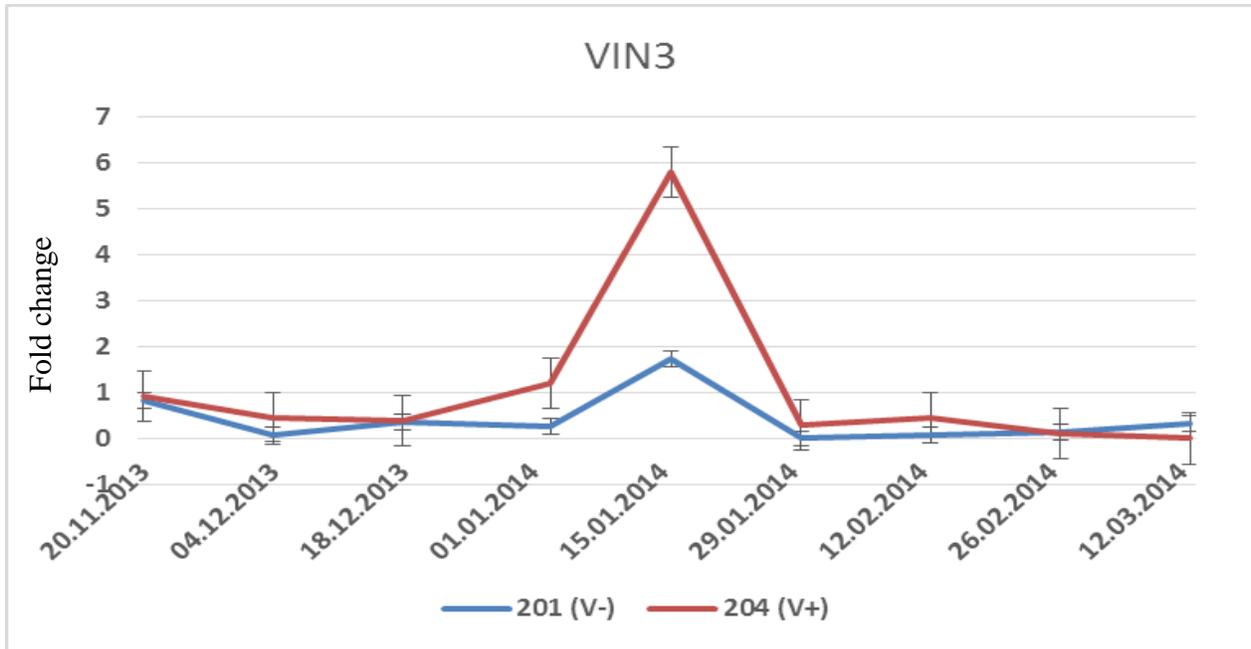


**Figure 10.** Expression patterns of *VERNALIZATION2 (VRN2)* gene in *Lolium perenne* at natural field conditions. The X-axis represents the time points of sample collections and the Y-axis represents the fold change in expression of respective genotypes. Error bars represent the standard error of the means calculated from two biological replicates

*VERNALIZATION 2 (VRN2)* gene is a floral repressor that delays flowering until plants are vernalized. The expression drops quickly in both genotypes in the period Nov-Dec during

vernalization, and is very low in mid-January when the expression for *VRNI* is peaking in the V+ genotype. During mid-December to February, temperature is so low and vernalization is not going on. The vernalization is happening in Nov-Dec and the expression is reduced substantially.

### 3.1.3 *VERNALIZATION INSENSITIVE 3*



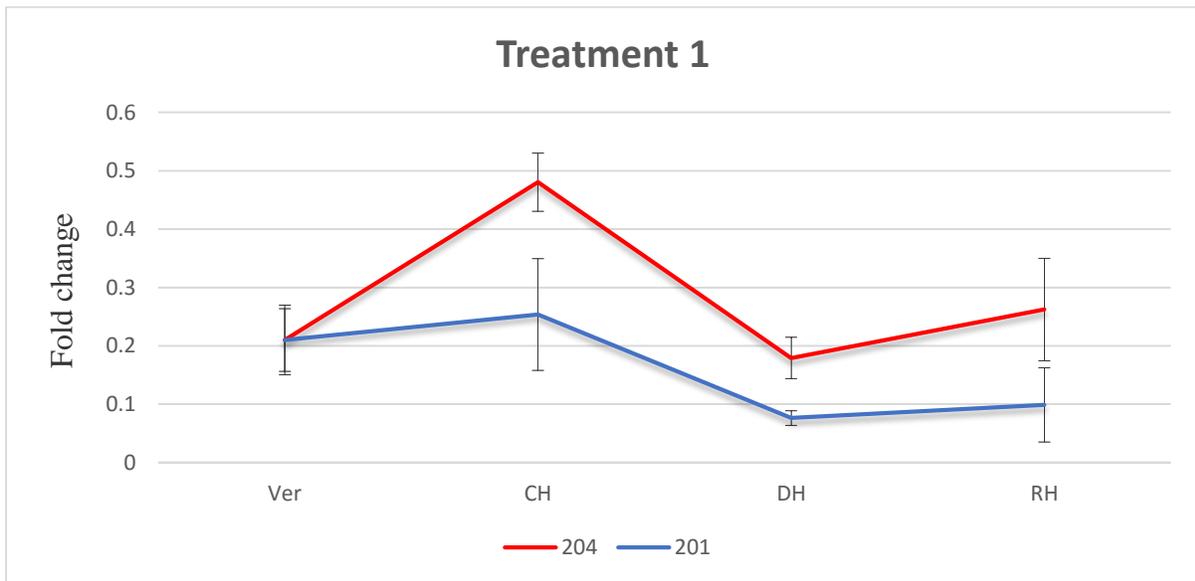
**Figure 11.** Expression patterns of *VERNALIZATION INSENSITIVE (VIN3)* gene in *Lolium perenne* at natural field conditions. The X-axis represents the time points of sample collections and the Y-axis represents the fold change in expression of the respective genotypes. Error bars represent the standard error of the means calculated from two biological replicates

*VERNALIZATION INSENSITIVE 3 (VIN3)* is the most up-stream gene in the *Arabidopsis* vernalization mediated flowering pathway. Upon exposure to low temperature, mRNA expression pattern of *VIN3* gene was observed using the quantitative reverse-transcriptase polymerase chain reaction. In the beginning, slight differential expression pattern was confirmed, it was not significantly different among the genotypes but strong expression (constitutively expressed) was observed during mid-December to mid-January (-6 to -10 °C) in the V+ (204 genotype), although there is a small increase in expression in the V- genotype also. The expression pattern of *VIN3* is almost similar to *VRNI*. In case of the V+ genotype, the transcript level increase from 1 to 6-fold

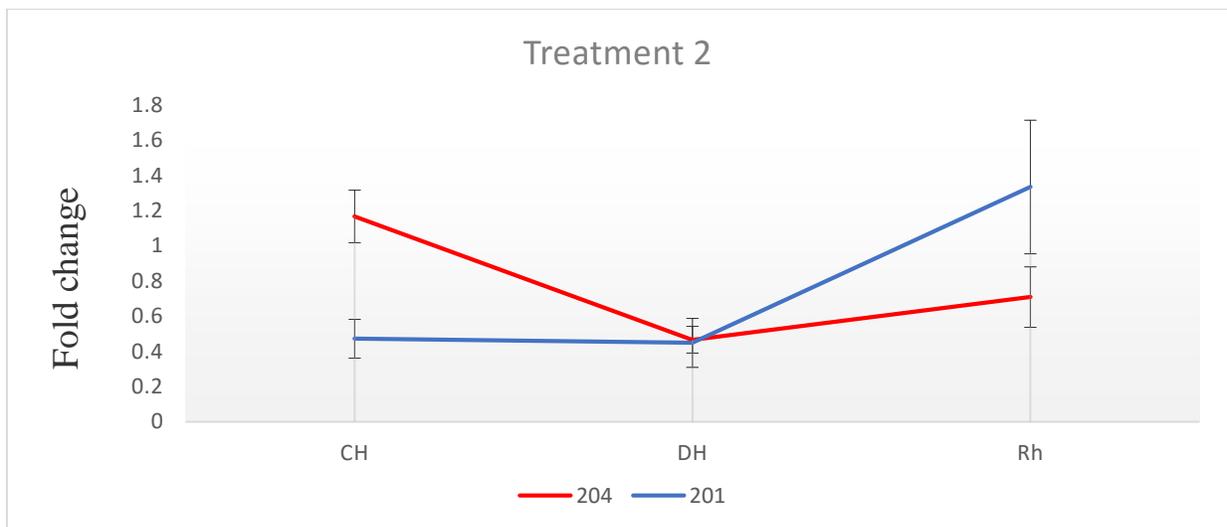
while it increased up to 2 fold for the V- genotype. Expression of *VIN3* increased rapidly in mid-December but showed a slower decrease in activity during mid-January and returned to basal expression levels after that.

### 3.2 Analysis of gene expression under vernalization and non-vernalization conditions followed by cold hardening, de-hardening and re-hardening in *Lolium perenne*

#### 3.2.1 VERNALIZATION 1



a) Vernalization followed by cold hardening (CH), de-hardening (DH), and re-hardening (RH)



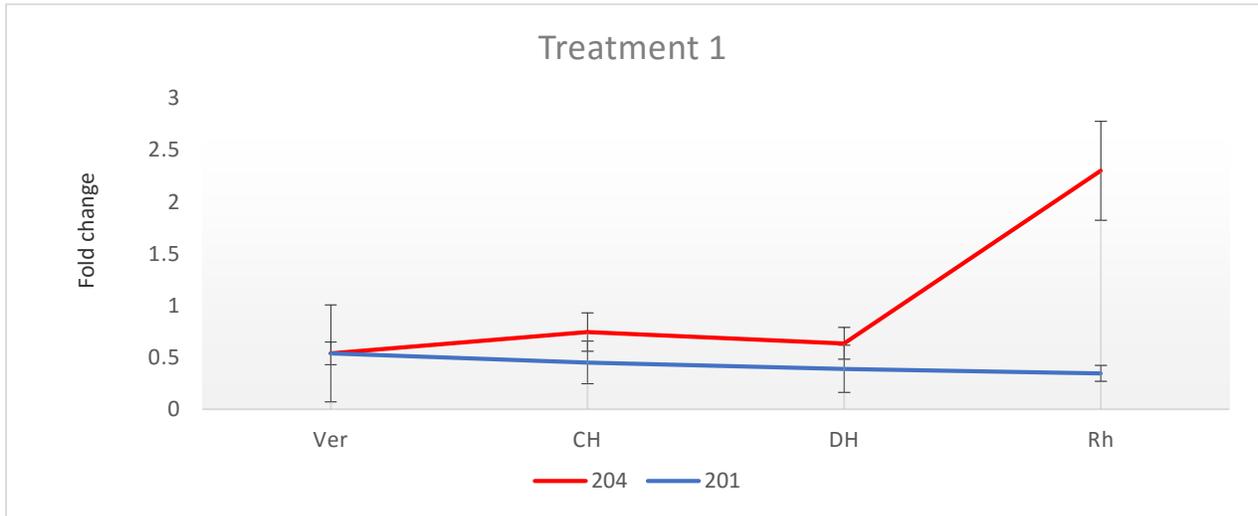
b) Without vernalization followed by cold hardening, de-hardening, and re-hardening

**Figure 12.** The expression pattern of *VRN1* in *Lolium perenne* at controlled conditions. The results shown are normalized with respect to the level of the housekeeping gene EF1A for each genotype and treatment. The X-axis represents sampling after cold hardening, de-hardening, and re-hardening as indicated, and the Y-axis represents the fold change in expression of respective genotypes. Error bars represent the standard error of the means calculated from three biological replicates.

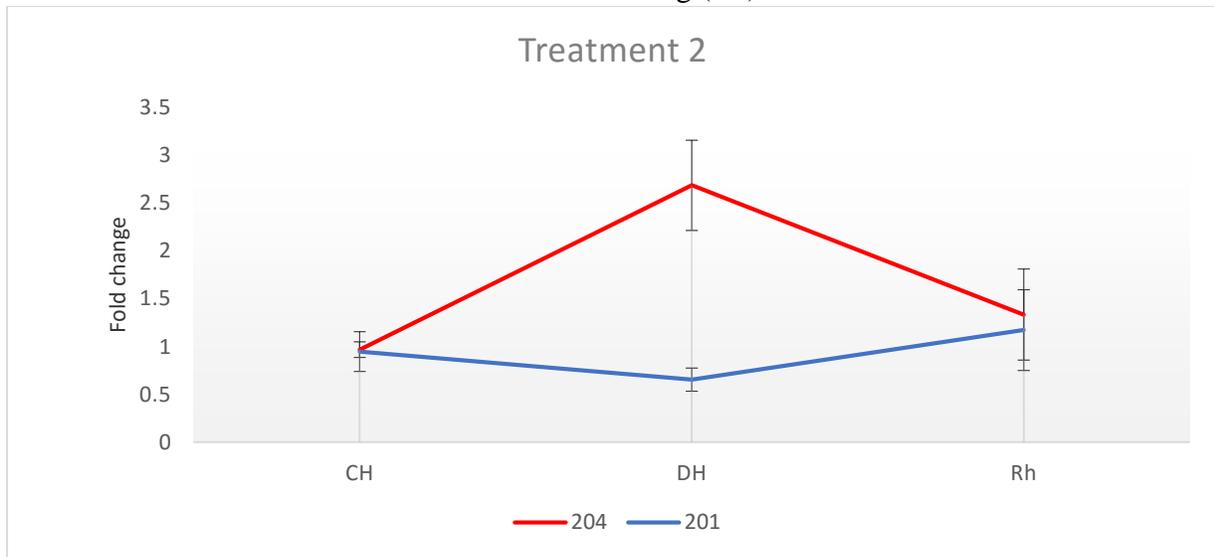
In treatment 1 (Vernalization followed by cold hardening, de-hardening and re-hardening), vernalized plants of the 204 genotype (V+) of *Lolium perenne* at 6 °C with 8h photoperiod at a light intensity of 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  shows upward trend while the *VRN1* gene expression of the 201 genotype (V-) is relatively decreased (**Figure 12a**). During the 3 weeks acclimation of plants at 2 °C, *VRN1* is significantly induced in the V+ genotype compared to the V- genotype. The relative expression of *VRN1* increases from 0.2 fold to 0.5-fold in the V+ (204) genotype. After cold acclimation, plants of both genotypes were treated with 10 days de-hardening at 10 °C. De-hardening leads to a similar decreased expression of *VRN1* in both genotypes, while *VRN1* expression increased again in both genotypes during the period of re-hardening where plants were maintained at 2 °C for 3 weeks. However, the expression of *VRN1* is relatively lower after re-hardening compare to the situation after cold hardening.

In treatment 2 (no vernalization before cold hardening) *VRN1* shows higher expression in the V+ (204) genotype (1.2 fold) relative to the V- (201) genotype (0.5 fold) after cold hardening (**Figure 12b**). After de-hardening, the expression was drastically reduced in the V+ genotype while it remained constant for the V- genotype. Following re-hardening, the highest expression was detected in V- genotype while slight changes of expression was observed in the V+ genotype.

### 3.2.2 VERNALIZATION INSENSITIVE 3



a) Vernalization (Ver) followed by cold hardening (CH), de-hardening (DH) and re-hardening (Rh)



b) Without vernalization followed by cold hardening (CH), de-hardening (DH) and re-hardening (Rh)

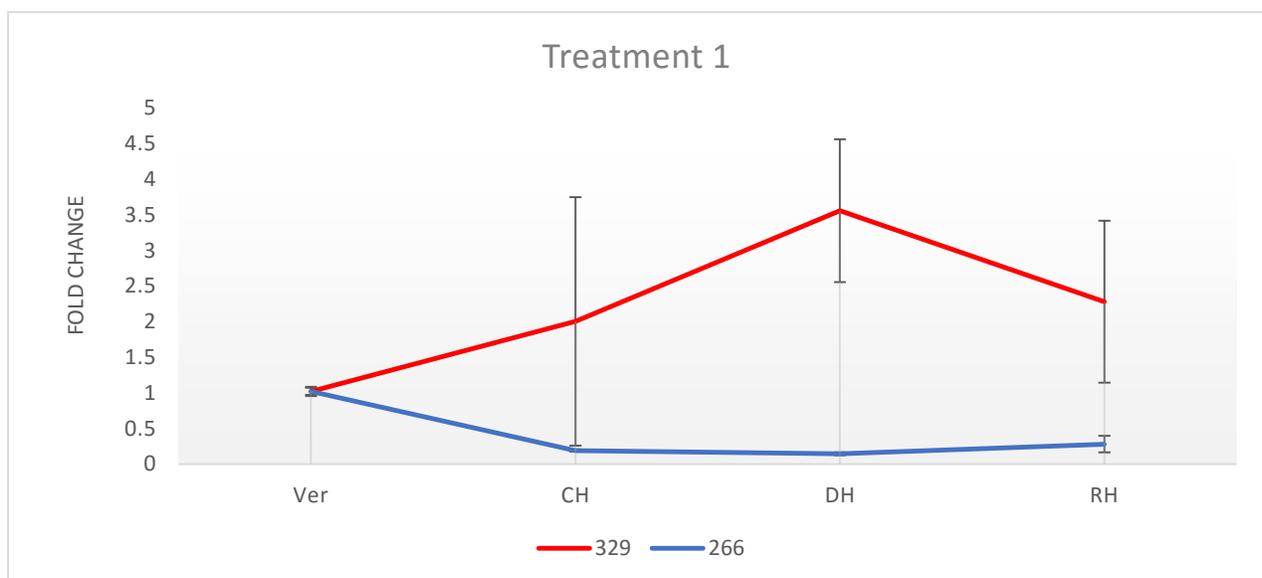
**Figure 13.** The expression pattern of *VIN3* in *Lolium perenne* at controlled conditions. The results shown are normalized with respect to the level of the housekeeping gene *EF1A* for each genotype and treatment. The X-axis represents cold hardening, de-hardening, and re-hardening as indicated, and the Y-axis represents the fold change in expression of respective genotypes. Error bars represent the standard error of the means calculated from three biological replicates

*VERNALIZATION INSENSITIVE 3 (VIN3)* is the repressor of *FLOWERING LOCUS C (FLC)* in *Arabidopsis thaliana* that is quantitatively induced in response to cold temperatures. In treatment 1 with vernalization, the relative expression of *VIN3* remained constant during all conditions (vernalization, cold hardening, de-hardening and re-hardening) in the V- (201) genotype (**Figure 13a**). On the other hand, the expression pattern of *VIN3* in the V+ (204) genotype was similar to the V- (201) genotype until after de-hardening but increased sharply (0.5 to above 2 fold) following re-hardening.

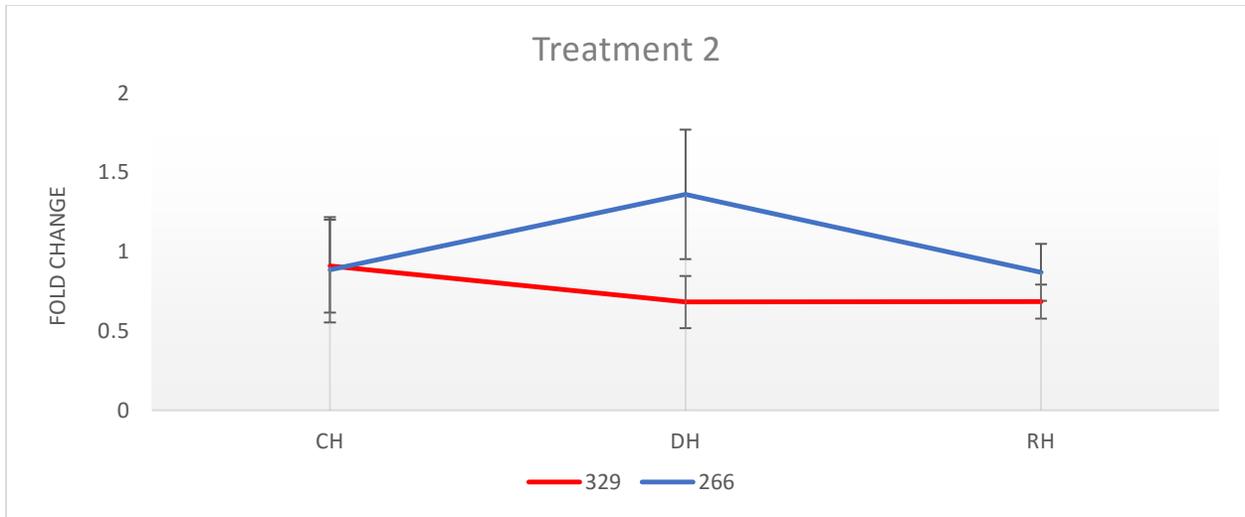
In treatment 2 (without vernalization), the relative of expression of *VIN3* was the same for both genotypes following cold hardening (**Figure 13b**). After de-hardening, however, the expression increase significantly in the V+ (204) genotype (1 to 2.5 fold) while the expression was relatively unaffected in the V- (201) genotype. After re-hardening, the expression level was similar in the two genotypes; reduced in the V+ (204) genotype and slightly induced in the V- (20) genotype.

### 3.3 Analysis of gene expression under vernalization and non-vernalization conditions followed by cold hardening, de-hardening and re-hardening in *Festulolium*.

#### 3.3.1 VERNALIZATION 1



a) Vernalization (Ver) followed by cold hardening (CH), de-hardening (DH) and re-hardening (Rh)



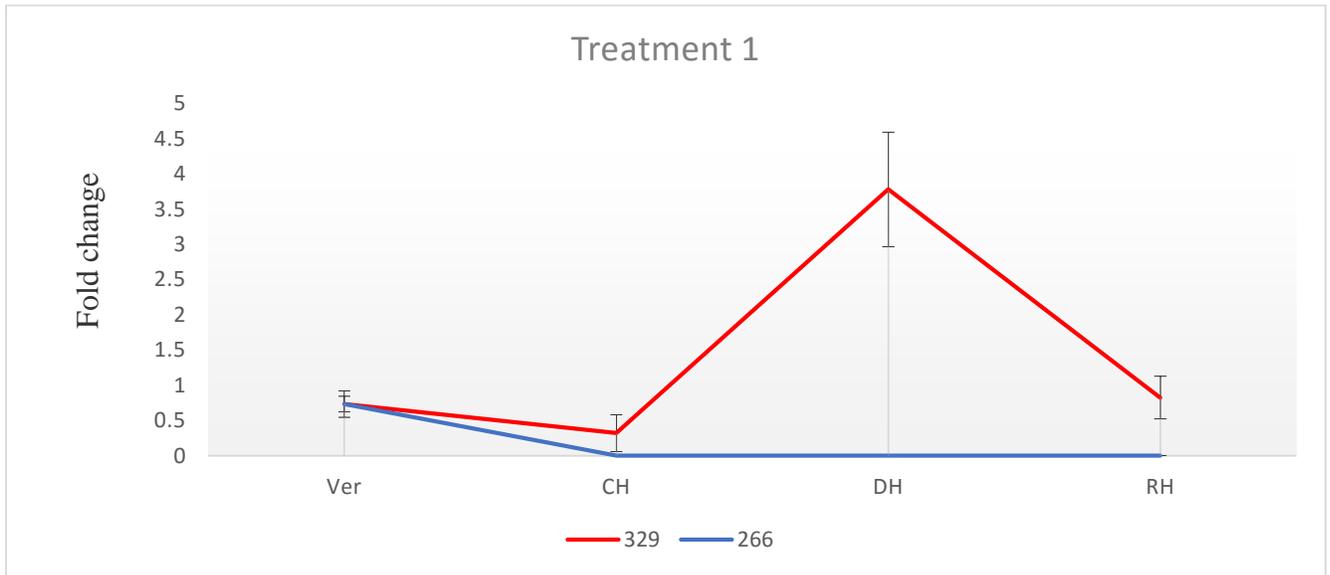
b) Without vernalization followed by cold hardening (CH), de-hardening (DH) and re-hardening (Rh)

**Figure 14.** The expression pattern of *VRNI* in *Festulolium* at controlled conditions. The results shown are normalized with respect to the level of the housekeeping gene *EF1A* for each genotype and treatment. The X-axis represents the cold hardening, de-hardening, re-hardening as indicated and the Y-axis represents the fold change in expression of respective genotypes. Error bars represent the standard error of the means calculated from three biological replicates

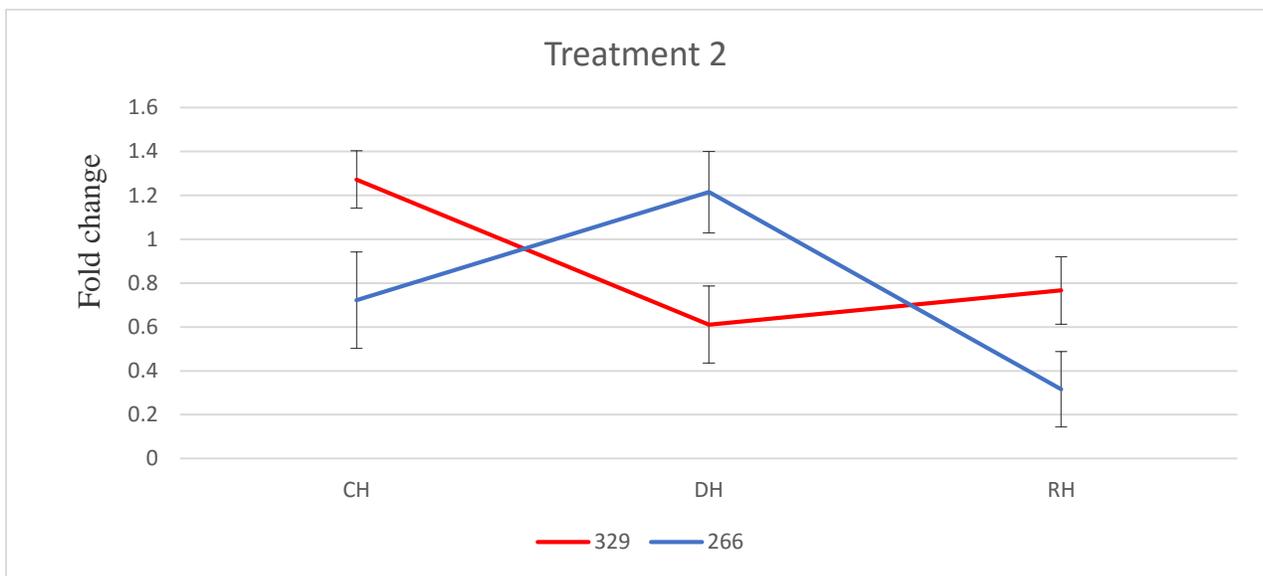
In treatment 1 with vernalization, It can be clearly seen that after the vernalization treatment (treatment 1), the *VRNI* expression levels was similar for both genotypes, genotype 329 which require vernalization to flower (V+) and genotype 266 which flower without vernalization (V-) (**Figure 14a**). *VRNI* expression is induced during cold hardening in the V+ (329) genotype (1 to 2 fold) while expression in the V- (266) genotype decreased and remained more or less constant during the subsequent treatments. Expression in the V+ genotype was strongly affected; it increased during de-hardening and decreased during re-hardening.

In treatment 2 without vernalization, the relative expression of *VRNI* is similar for both genotypes after cold hardening (**Figure 14b**). During de-hardening the expression level increased in the V- genotype (1 to 1.5 fold) while it was slightly reduced in the V+ genotype and remained constant during re-hardening. Re-hardening reduced the expression of *VRNI* in the V- (266) genotype.

### 3.3.2 VERNALIZATION INSENSITIVE 3



a) Vernalization (Ver) followed by cold hardening (CH), de-hardening (DH) and re-hardening (RH).



b) Without vernalization (Ver) followed by cold hardening (CH), de-hardening (DH) and re-hardening (RH).

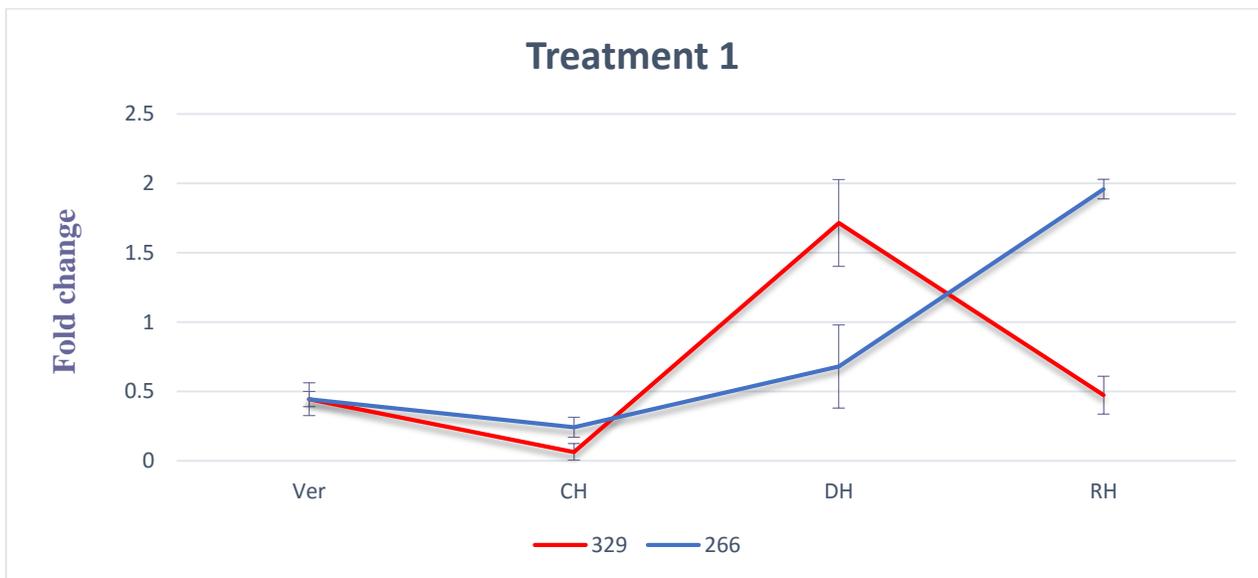
**Figure 15.** The expression pattern of *VIN3* in *Festulolium* at controlled conditions. The results shown are normalized with respect to the level of the housekeeping gene *EF1A* for each genotype and treatment. The X-axis represents the cold hardening, de-hardening, re-hardening as indicated

and the Y-axis represents the fold change in expression of respective genotypes. Error bars represent the standard error of the means calculated from three biological replicates

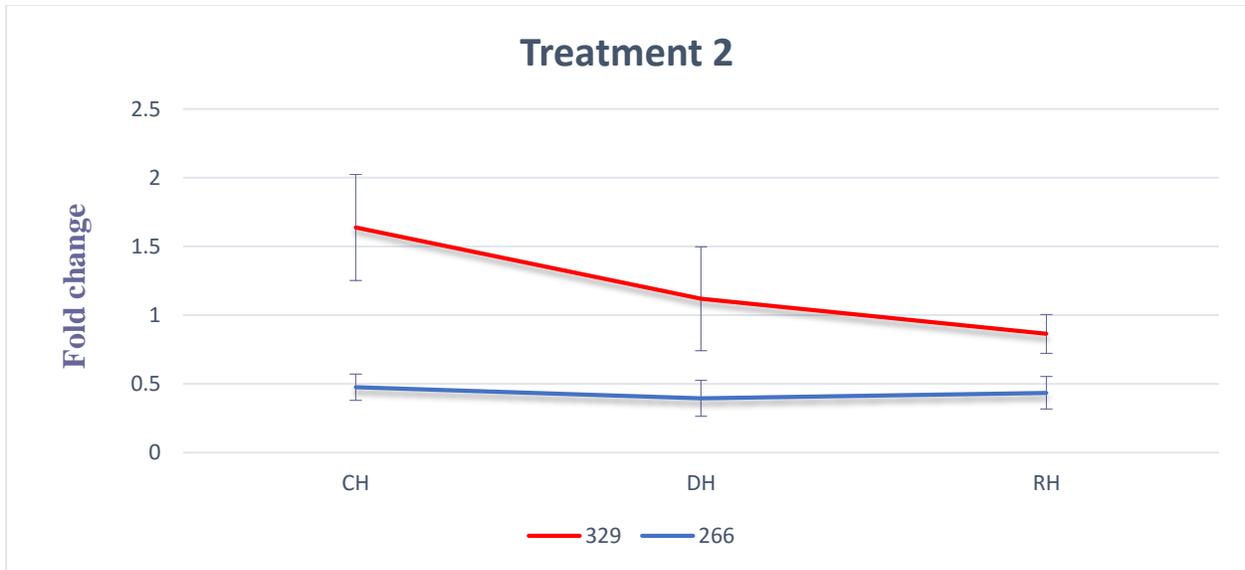
In treatment 1, qRT-PCR did not detect any significant differences in *VIN3* expression among the genotypes after the vernalization treatment (**Figure 15a**). The expression of *VIN3* was very low in both genotypes after cold hardening. However, differences of expression was observed in de-hardening treatment for both genotypes. During de-hardening, *VIN3* expression increased sharply (0.5 fold to 3.5 fold) and reached the peak level in the V+ (329) genotype while it dropped off during re-hardening. Contrary to this, no expression was apparent during de-hardening and re-hardening for the V- (266) genotype.

In treatment 2 without vernalization, differential expression levels were observed for the genotypes after cold hardening; upregulated in the V+ (329) and downregulated in the V- (266) genotype (**Figure 15b**). During de-hardening and re-hardening the genotypes displayed opposite expression patterns of the *VIN3* gene; increased expression during de-hardening and reduced expression during re-hardening in the V- (266) genotype, while expression was completely opposite in the V+ (329) genotype.

### 3.3.3 Fructan



a) Vernalization (Ver) followed by cold hardening (CH), de-hardening (DH) and re-hardening (RH).



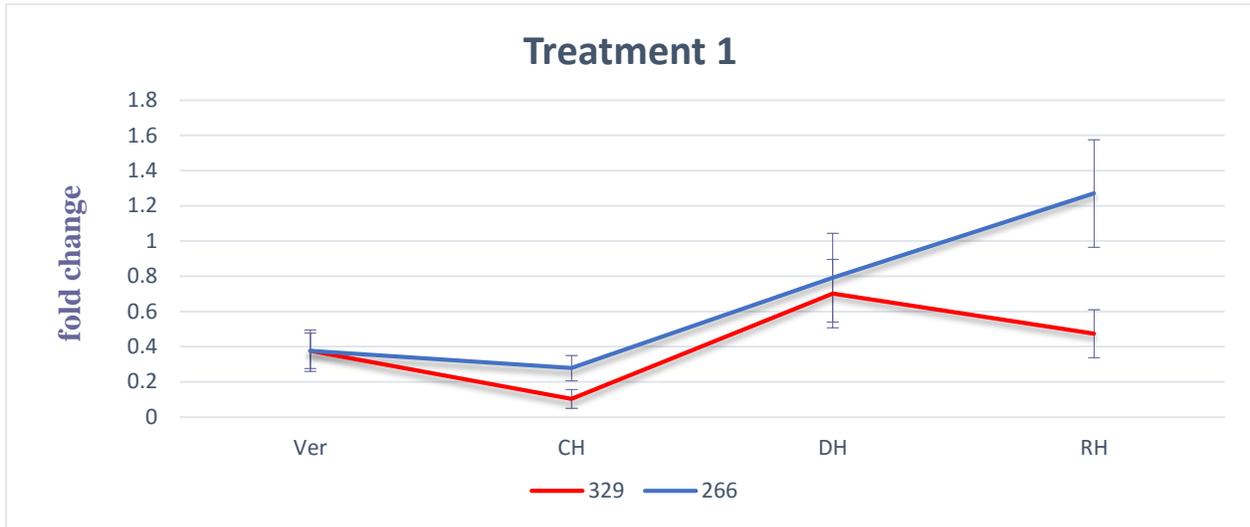
**b)** Without vernalization (Ver) followed by cold hardening (CH), de-hardening (DH) and re-hardening (RH).

**Figure 16.** The expression pattern of *Fructan* in *Festulolium* at controlled conditions. The results shown are normalized with respect to the level of the housekeeping gene EF1A for each genotype and treatment. The X-axis represents the cold hardening, de-hardening, re-hardening as indicated and the Y-axis represents the fold change in expression of respective genotypes. Error bars represent the standard error of the means calculated from three biological replicates

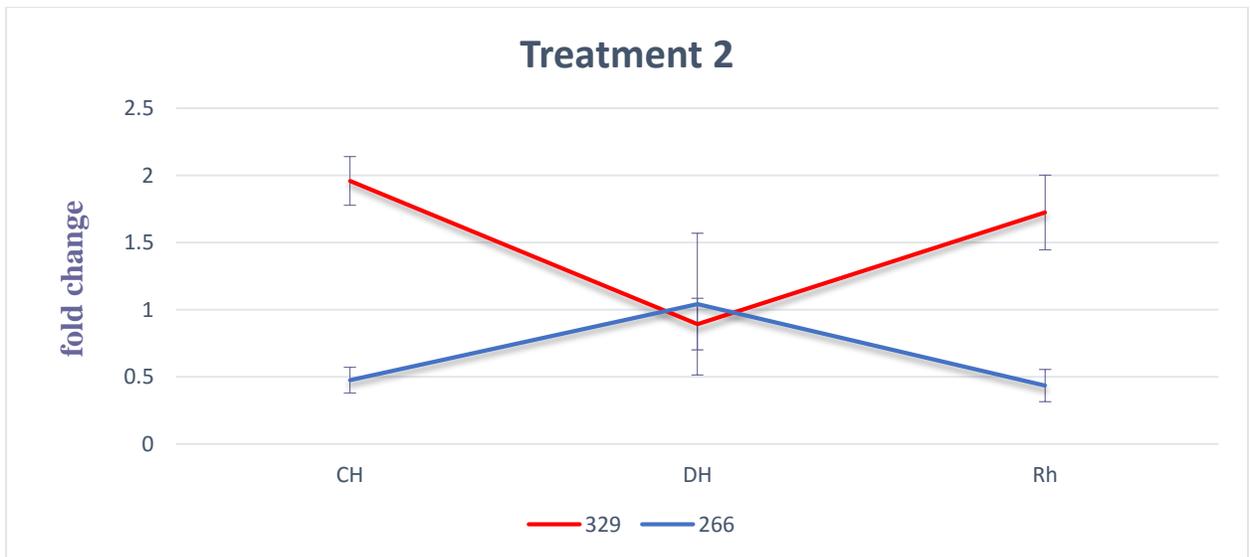
After the vernalization treatment (Treatment 1), fructan gene expression was similar for both genotypes, and no apparent differences was noticed after cold hardening (**Figure 16a**). Expression increased during de-hardening treatment for both genotypes, more in the V+ (329) than in the V- (266) genotype. Expression dropped sharply in the V+ genotype during re-hardening while it increased substantially in the V- (266) genotype.

In treatment 2 without vernalization, differential expression between the genotypes was apparent after cold hardening, about 1.6 fold change for the V+ (329) and 0.5 fold change for the V- (266) genotype (**Figure 16b**). The expression level remained constant during the subsequent treatments in the V- (266) genotype, while it dropped off for the V+ (329) genotype during both de-hardening and re-hardening.

### 3.3.4 Dehydrins



a) Vernalization (Ver) followed by cold hardening (CH), de-hardening (DH) and re-hardening (RH).



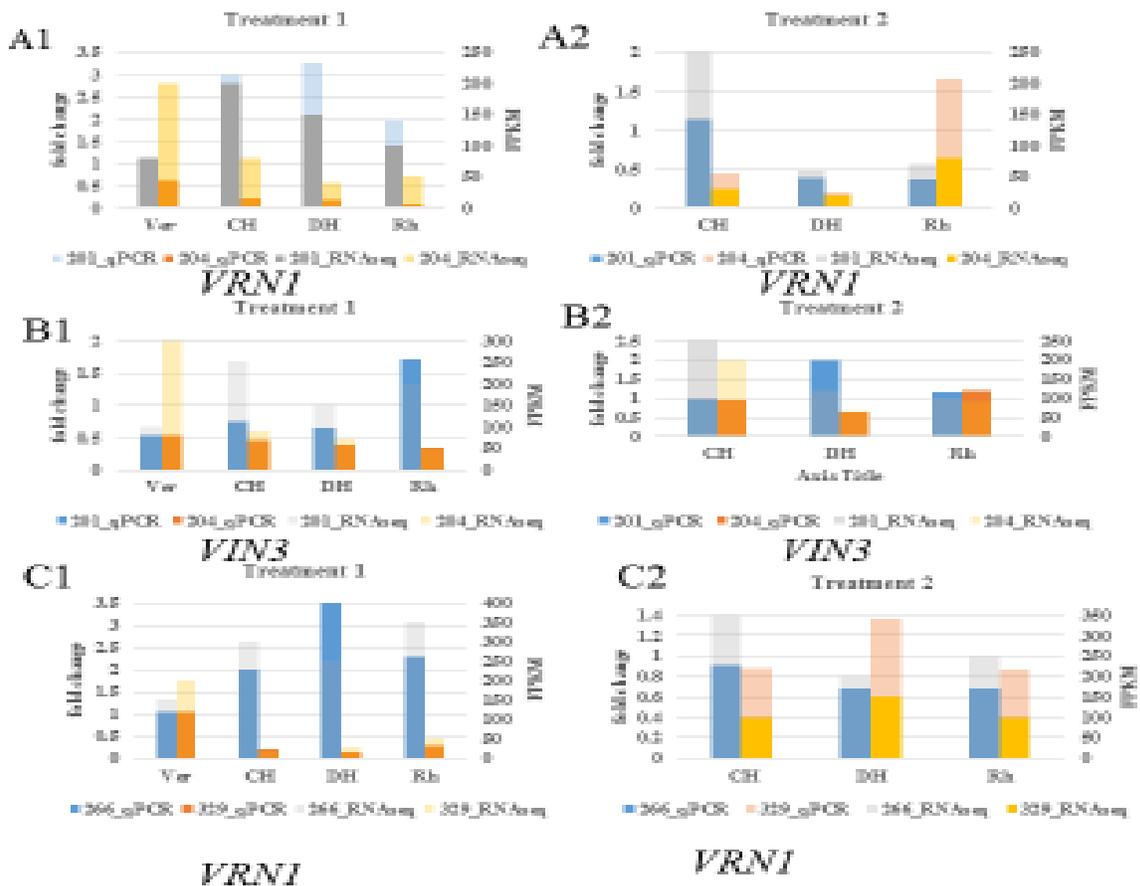
b) Without vernalization (Ver) followed by cold hardening (CH), de-hardening (DH) and re-hardening (RH).

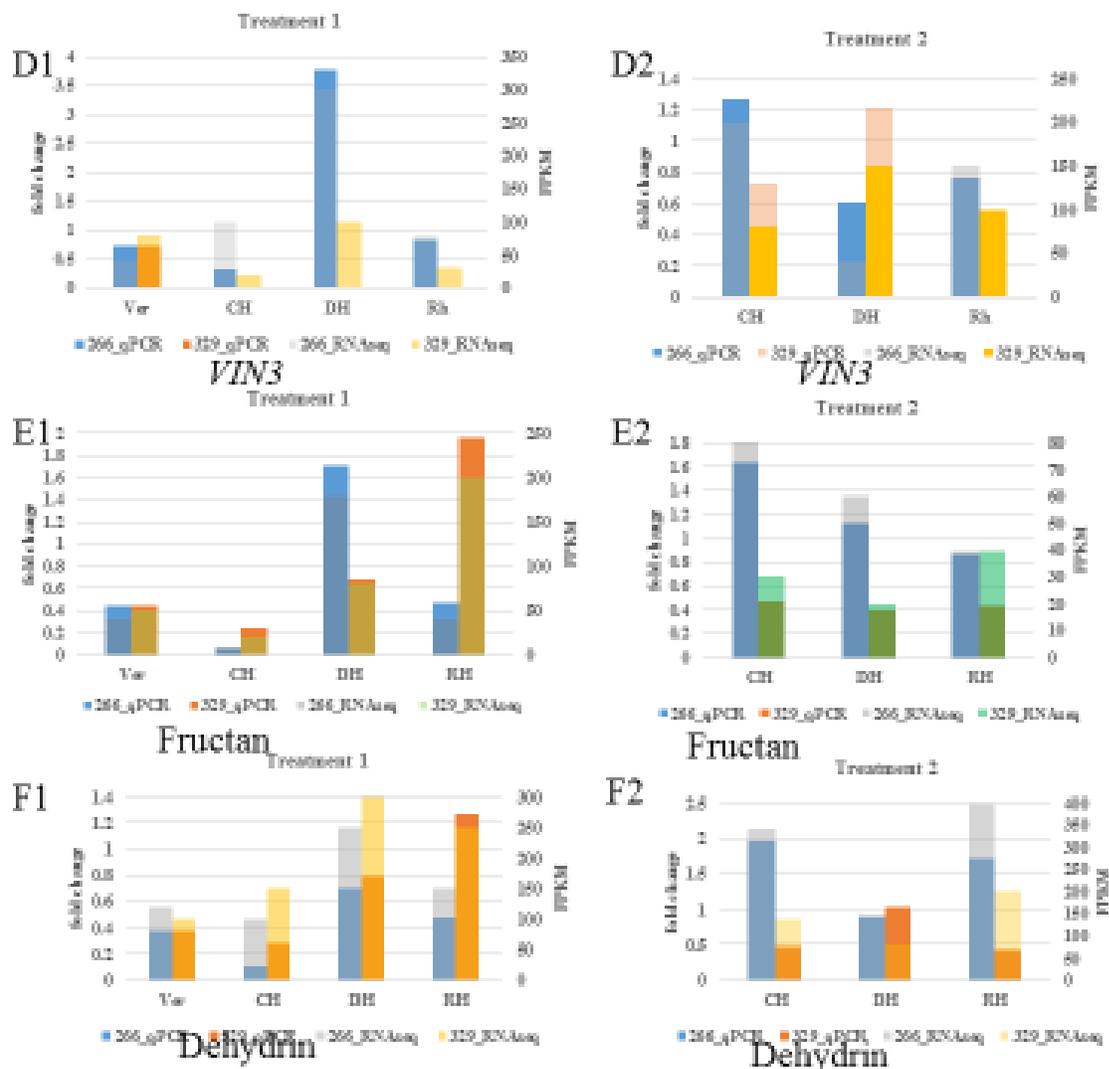
**Figure 17.** The expression pattern of *Dehydrin* in *Festulolium* at controlled conditions. The results shown are normalized with respect to the level of the housekeeping gene EF1A for each genotype and treatment. The X-axis represents the cold hardening, de-hardening, re-hardening as indicated and the Y-axis represents the fold change in expression of respective genotypes. Error bars represent the standard error of the means calculated from three biological replicates

After vernalization, the expression of Dehydrins was similar in the two genotypes, while cold hardening reduced the expression in both genotypes, most in the V+ (329) genotype (**Figure 17a**). Expression increased during de-hardening in both genotypes and continued to increase during re-hardening in the V- (266) genotype while it decreased again in the V+ (329) genotype.

In treatment 2 without vernalization, Dehydrins expression was activated by cold hardening for both genotypes, but up-regulated much more in the V+ (329) than in the V- (266) genotype (**Figure 17b**). De-hardening reduced expression in the V+ genotype and increased expression in the V- genotype, ending at the same levels, while re-hardening induced expression in the V+ (329) genotype again, reaching the same level as after cold hardening, and reduced expression in the V- (266) genotype back to the same level as after cold hardening.

### 3.4 Validation of expression levels of genes identified in the VARCLIM project by RNA sequencing





**Figure 18.** Validation of gene expression

RNA transcriptome data were used for the all genes. All the genes are almost correlated to the RNA transcriptome data.

## 4. Discussion

Two important mechanisms, cold acclimation (cold hardening) and vernalization requirement have evolved by plants to cope with low-temperature stress (Fowler, et al. 1996). Cold acclimation allows the plant to protect its physiological function and cell structure during the time of low temperature (freezing temperature). On the other hand, responsiveness of plants towards vernalization protects the forage grasses from the hazard of entering into cold-sensitive reproductive growth stage until the risk of low temperature has passed. Flowering is an indispensable part of the life cycle of plants, but it is a complex process that evolved numerous genes activation and repression engaged in different biochemical and molecular pathways. To survive diverse environmental stresses, plants have developed survival mechanisms. In this study, it was possible to cold acclimate the perennial ryegrass to withstand  $-10^{\circ}\text{C}$  and even lower temperatures. qRT-PCR was used to analyze gene expression of perennial ryegrass and *Festulolium* genotypes during cold hardening, de-hardening and re-hardening. Upon cold exposure, the expression of vernalization genes was either induced or repressed. Expression of most genes returned to normal levels when temperature reach to warm condition. Byun et al. (2014) found that expression of many cold responsive genes was slightly induced during re-hardening, but the expression of these genes remains at low levels during re-hardening compared to during cold hardening and dynamic changes of gene expression were observed during cold hardening, de-hardening and re-hardening. Rife and Zeinali (2003) working with winter oilseed rape cultivars observed that all the lines were able to re-harden to the same level as before the de-hardening treatment.

*Lolium perenne* is found throughout most of the temperate regions of the world. A close examination of grasses in natural cold weather conditions indicates that they are tolerant to freezing stress. The fitness of survival in freezing temperatures varies greatly in plants. For instance, plants from tropical regions does not have the capacity to survive even the slightest freeze (Thomashow 1998). To carry out the present study, plants were grown in natural field conditions, although field studies are rare. In order to exhibit the influences of environmental conditions on the plants, field studies were maintained. In natural condition, variations are much greater; the temperature, light intensity and photoperiod are not controlled and growth conditions are not favourable for reproduction. Few of the authors acknowledge that experiments in natural conditions may have

different results due to variable field conditions compared to controlled conditions. Thus, determination of plants winter survival capacity under controlled condition is important because that can provide valuable information, but understanding how plants are adapted to field conditions has a greater practical significance. However, natural environmental conditions has been used in a few research works, e.g. to study fitness-related quantitative traits, reproductive timing and flowering time in *Arabidopsis* (Brachi et al. 2010; Malmberg et al. 2005; Weinig et al. 2002).

#### **4.1 Variation of gene expression in vernalization requirement for flowering at natural field conditions**

The relationship between vernalization requirements and freezing tolerance has been reported in many studies. Markowski and Rapacz (1994) found significant correlations between vernalization requirements and frost resistance of winter rape lines derived from double haploids. Rapacz and Markowski (1999) found a strong relationship between vernalization requirement and both freezing tolerance and field survival in high erucic acid European winter oilseed rape cultivars. The completion of vernalization is supposed to lower the low temperature tolerance observed in winter cereals but a strong vernalization requirement, i.e. long periods of vernalization are needed, is responsible for retarding the transition to the reproductive growth stage (Fowler, et al. 1996). Rife and Zeinali (2003) reported that rapeseed plants may survive cold weather under natural conditions effectively before reaching vernalization saturation compared to after the vernalization requirement has been met. Prášil et al. (2005) reported that loss of both freezing tolerance and their ability to re-induce significant frost tolerance occur gradually in winter wheat due to the short photoperiod and low temperature following warm periods in winter. It has been suggested that maximum freezing tolerance obtained under short day field conditions occur about one month after vernalization and reduction of frost tolerance occurring later indicate that reproductive development has started. Furthermore, vernalization alone was not enough to induce the plants into generative induction or initiate reduction of freezing tolerance (Bergjord et al. 2009). Fowler et al. (1996) demonstrated that cereal crops retained freezing tolerance partially following exposure to warm temperatures after vernalization saturation, indicating that vernalization saturation does not result in a "switching off" of the low-temperature tolerance genes. The photoperiod and vernalization responses are considered as primary regulator of the transition

from vegetative to reproductive growth stage and this process has many scientific as well as practical associations.

By examining the effects of vernalization, cold hardening responses and day lengths, we decided to monitor the mRNA expression pattern of vernalization related genes (*VRN1*, *VRN2* and *VIN3*). *VERNALIZATION 1* (*VRN1*) is the key vernalization gene in grasses controlling flowering pattern. *VRN1* was initially expressed at low levels and significantly induced by vernalization during the mid-December to mid-January (1-fold to 7-fold changes) in the V+ (204) perennial ryegrass genotype which require vernalization to flower compared to the V- (201) genotype (1-fold change) which does not require vernalization (**Figure 9**). *VERNALIZATION 2* (*VRN2*) gene encodes a nuclear-localized zinc finger protein and act as a floral repressor that delays flowering until plants are vernalized. *VRN2* expression was low and unaffected by vernalization during the period of mid-December to February in both genotypes (**Figure 10**). Vernalization has strongest effect on *VRN1*, *VRN2* and in *VIN3*; in addition day length has the greatest effect on the expression of these vernalization responsive genes. It has been suggested that *VRN1* transcripts start to accumulate after the 24 hours onset of cold and expression of *VRN1* induced at temperatures between 15 and -2 °C, but maximal response observed between 2 and -2 °C (Oliver et al. 2013). Loukoianov et al. (2005) observed a negative relationship between the *VRN1* and *VRN2* genes in young isogenic lines of hexaploid wheat and hypothesized that upregulated *VRN1* transcripts down-regulates the *VRN2*. Results from the *VRN1* and *VRN2* confirmed the validity of this hypothesized. According to Chen and Dubcovsky (2012), *VRN1* acts as promoter to change the phase (transition from apical meristem to the reproductive phase) in grasses and plays a central role in flowering by down-regulating and maintaining the low transcripts level of the flowering repressor *VRN2*. Expression of *VRN1* is critical for flower initiation in early developmental stages and reduction of *VRN1* expression level delays the transition of the apex to reproductive phase while it increases the leaf numbers and delay the heading time (Loukoianov et al. 2005). Our studies reveal that *VRN2* expression does not occur when *VRN1* is expressed. Under long days conditions, *VRN2* blocks the flowering before vernalization by down-regulating the *VRN3*, overexpression of *VRN3* overcomes *VRN2* repression and induce *VRN1* expression and also start flower initiation (Li et al. 2011). The vernalization gene *VRN3* is a cereal homologue of the *Arabidopsis* *FLOWERING LOCUS T* (*FT*) and induction of *FT* in the leaves promotes flowering (Yan et al. 2006). Dhillon et al. (2010) suggested that *VRN1* is needed to initiate the regulatory cascade that down-regulate the genes in

cold acclimation pathways but long days regulate additional genes required for down-regulation of cold regulated genes. Short day length down-regulate the *VRN2* but do not up-regulate *VRN1* which are known as a meristem identity gene until long day prevail, whereas this is in contrast to up-regulation of *VRN1* followed by *VRN2* down-regulation through vernalization (Dubcovsky et al. 2006). *VRN2* reduces the levels of floral repressor FLC in response to vernalization especially after the cold treatment instead of maintaining low levels but FLC expression in plants returns to normal levels when ambient temperatures prevail (Gendall et al. 2001) and inhibit the flowering in plants in their first growing season (Sung & Amasino 2004b). During the vernalization, *VERNALIZATION INSENSITIVE 3(VIN3)* is induced and the PHD protein *VIN3* accumulates but start to decline rapidly when the cold period has passed (Sung & Amasino 2004a; Wood et al. 2006). In our experiment, it was observed that *VIN3* is significantly induced during the period of low temperature but returned to normal level when weather started to warm (**Figure 11**). On the other hand, induction of *VIN3* is associated with extended levels of histone H3 and histone H4 acetylation in response to prolonged cold treatment (Jean Finnegan et al. 2005). Histone H3 acetylation, which is important for *VIN3* induction, increased rapidly at the transcription start site during the short-time cold exposure (Bond et al. 2009b). *VIN3* is the most up-stream gene in the *Arabidopsis* vernalization mediated flowering pathway. According to Bond et al. (2011), no connections exist between the cold acclimation pathways and vernalization. However, other studies showed a correlation between *VRN1*, key vernalization genes and cold acclimation controlled by *CBF*, *COR* and *LEA* levels in diploid and hexaploid wheat, describing different *VRN1* alleles causing differences in freezing tolerance. In the present study, the expression levels of *VIN3* was correlated with *VRN1* with similar expression patterns.

## **4.2 Comparison of gene expression between natural and artificial conditions in *Lolium perenne***

It is well established that exposure of temperate grasses to low temperature ensure plants to induce freezing tolerance and survive entire winter seasons. Contrary, warm temperature reduces the freezing tolerance in plants. The acquisition of freezing tolerance is complicated, including many metabolic changes that may come into play at different phases of plant development. According to Gusta et al. (2009), plants can obtain freezing tolerance partially in artificial (controlled) climate conditions, because it is difficult to reproduce the complex environmental conditions similar to natural conditions when plants are grown in controlled climates. There are meaningful differences

between natural and artificial conditions as regards acclimation of plants. Artificially (growth chamber) cold acclimated plants may respond differently compared with acclimation in natural conditions. Dhanaraj et al. (2007) reported that transcripts were upregulated in control conditions than under natural field conditions. We compared the changes in gene expression of vernalization genes under field and growth room conditions. Under natural conditions, remarkable changes in *VRNI* gene expression were observed (**Figure 9**). *VRNI* was significantly induced by vernalization (cold) during mid-December to mid-January (-6 to -10 °C) in the V+ (204) genotype which require vernalization compared with the V- (201) genotype which does not require vernalization. The *VRNI* gene is expressed in leaves and crown tissues around the time of transition from vegetative to reproductive development. *VRNI* expression was initially repressed until plants were exposed to low temperature. Sasani et al. (2009) also reported that expression of *VRNI* was induced by a prolonged cold treatment with downregulation of *VRN2* in leaves. Again, *VRNI* starts to decrease when plants are exposed to warm temperatures. In controlled conditions, treatment 1 (**Figure 12a**) shows that *VRNI* is induced in both genotypes (204, 201) during the period of cold hardening. After cold hardening, de-hardened plants shows down-regulation of gene expression. Again, higher rate of *VRNI* expression was observed for both genotypes during the period of re-hardening where plants were maintained at 2 °C for 3 weeks but the expression of *VRNI* is relatively lower after re-hardening compare with after cold hardening. In treatment 2 (**Figure 12b**), differential gene expression was observed after cold hardening and the V- (201) genotype shows higher rate of gene expression. After cold hardening, the relative expression was drastically reduced for the V+ (204) genotype during the period of de-hardening while expression of the gene was constant in the V- (201) genotype. During re-hardening, the highest expression of *VRNI* was noticed in the V- (201) genotype while slight changes in gene expression was observed in the V+ (204) genotype. In conclusion, *VRNI* expression is up-regulated during cold hardening and down-regulated during de-hardening. De-hardening limits the effectiveness of cold hardening by reducing the amount of available energy (Rapacz 1998). It has been observed that many cold responsive genes are moderately induce during re-hardening and remains at comparatively low levels comparison to the expression during the period of the first cold hardening (Byun et al. 2014). Zuther et al. (2015) also stated that “Correlations between freezing tolerance and the expression levels of COR genes and the content of glucose, fructose and sucrose, as well as many correlations among transcript and solute levels, that were highly significant in cold acclimated plants, were lost during

deacclimation''. After the completion of vernalization, low temperature tolerance start to decrease gradually. Vernalization is solely responsible for cold tolerance observed in winter cereals and crops were maintained at temperatures for long periods in the optimum range to achieve low temperature acclimation (Fowler et al. 1996). The relative gene expression of *VRNI* was higher in natural conditions comparison to plants grown in the control conditions (greenhouse). This observation reveals that expression level of *VRNI* was subjected to light intensity and day length. The level of *VRNI* expression is positively associated with vernalization and transition from the vegetative stage to the reproductive stage is connected with lowered freezing tolerance in winter cereals. On the other hand, *VRNI* is constitutively upregulated in vernalization required spring habit genotypes (Danyluk et al. 2003).

In this study, we have characterized *VIN3* gene expression patterns in the 204 and 201 genotypes under natural field conditions (**Figure 11**) and compared these with changes of *VIN3* gene expression patterns in control conditions. Under natural field conditions, initially slight differential expression patterns were observed, differences were not significant but highest expression was observed during mid-December to mid-January (-6 to -10°C) in the V+ (204) genotype, although there is a small increase in the V- (201) genotype. This expression pattern is almost similar to that observed for *VRNI*. Expression of *VIN3* increased rapidly in mid-December but showed a slower decrease in activity during mid-January and returned to basal expression levels after that. *VIN3* start to upregulate after exposure to cold. Two independent treatments were conducted for analyzing the *VIN3* gene expression under controlled climate conditions (**Figure 13**). In both treatments, we observed contrasting gene expression in the V+ and V- genotypes. In treatment 1 with vernalization, no significant changes was observed in expression level of the *VIN3* genes in the V- (201) genotype. However, mRNA expression pattern of *VIN3* in the V+ (204) genotype is similar to the V- (201) genotype until de-hardening but strong expression level was shown during the period of re-hardening. Histone H3 acetylation at the transcription start site increase rapidly after short-term cold exposure, indicating that it is important for *VIN3* induction. During prolonged cold exposure, subsequent alteration in histone H3 and H4 acetylation takes place following continued *VIN3* transcription (Bond et al. 2009). *VIN3* is the most up-stream gene in the vernalization pathway, which is mediated by a PHD finger protein and induced in response to cold exposure. Expression of *VIN3* is repressed to basal levels in contact with warmer conditions (Sung & Amasino 2004b). During the period of vernalization, the induction of *VIN3* take place at the site

of cold perception and *FLOWERING LOCUS C (FLC)* is repressed. Interestingly, *VIN3* act as a master of vernalization-specific regulation. According to Sung and Amasino (2004a), *VIN3* is involved in modification of the *FLC* chromatin structure. *VRN1* and *VRN2* are also required for silencing of *FLC*.

### **4.3 Differences of *VRN1* and *VIN3* gene expression between *Lolium perenne* and *Festulolium* in controlled conditions**

We observed that *VRN1* gene expression was up-regulated in the *Lolium perenne* V+ (204) genotype during cold hardening and re-hardening, but down-regulated during de-hardening (**Figure 12a**). In treatment 2, V+ (204) genotype and V- (201) genotypes showed gene expression in cold hardening and re-hardening condition (**Figure 12b**). In contrast, the *VRN1* gene showed differential gene expression in *Festulolium*. In treatment 1 the V+ (329) genotype showed increased gene expression during cold hardening and de-hardening but started to decline during the period of re-hardening. There were no changes in gene expression for the V- (266) genotype (**Figure 14a**). In the case of treatment 2, the V- genotype displayed up-regulated gene expression during de-hardening while the V+ genotype showed decreased gene expression in all treatments (**Figure 14b**).

*VIN3* gene showed upward gene expression in the *Lolium perenne* V+ (204) genotype during re-hardening for the vernalization treated plants (treatment 1) followed by cold hardening, re-hardening and de-hardening (**Figure 13a**). In treatment 2, mRNA expression pattern of *VIN3* was strongly induced during the period of de-hardening in the V+ (204) genotype while in the V- (201) genotype it was slightly induced during re-hardening (**Figure 13b**). In the case of *Festulolium*, the V+ (329) genotype showed strong gene expression after de-hardening in treatment 1, similar as the V+ *Lolium* genotype in treatment 2, while expression in the V- (266) genotype was totally repressed (**Figure 15a**). In treatment 2, the V+ (329) genotype showed up-regulated gene expression after cold hardening and slightly induced expression after re-hardening but reduced expression during de-hardening. The V- (266) genotype showed a reverse gene expression pattern, gene expression was only strongly induced during the period of de-hardening.

#### 4.4 Gene expression of Fructan and Dehydrins

Fructans are the major storage carbohydrate in grasses and a number of fructosyltransferases (FTs) act as catalyzers to metabolize these. The data presented show that transcripts of fructan266 start to increase after the onset of cold hardening and peaked during the period of de-hardening in both *Festulolium* genotypes grown in controlled conditions with vernalization (treatment 1). The level of fructan266 expression for 329 genotypes (+ vernalization) returned to normal levels during re-hardening in the V+ (329) genotype but elevated expression for was maintained during re-hardening in the V- (266) genotype (**Figure 16a**). The initial transcriptional response of fructan266 to cold acclimation (treatment 2) was slightly stronger than compared to other treatments in the V+ (329) genotype. However, there were no apparent changes of gene expression observed in the V- (266) genotype (**Figure 16b**). Hisano et al. (2008) found that mRNA expression levels of the fructan-like genes *prft1* and *prft2* increased gradually upon cold exposure. On the other hand, 1-SST and 6G-FFT increased initially but decreased in contact with warm temperature. Repeatedly, 1-SST and 6G-FFT genes increase again during a longer period of low temperature treatment. Tamura et al. (2014) reported that fructan exohydrolase (Pp6-FEH1) gene transcript rapidly increased in timothy when temperature was below 0°C. Induction of Pp6-FEH1 gene expression was also observed in seedlings under -3°C.

Dehydrin gene expression is linked with plant response to stress like dehydration. Dehydrin proteins protect the plant cells from fluctuating temperature or water deficit. In our experiment, we studied the dehydrin gene 'DH266' and it showed differential gene expression under different treatments. In treatment 1, the transcripts of Dehydrin start to accumulate after cold hardening treatment and maintained the elevated level until re-hardening for V- (266) genotype while the level of DH266 expression was up-regulated until de-hardening and started to decrease during the period of re-hardening in the V+ (329) genotype (**Figure 17a**). In treatment 2, transcripts level increased initially after cold hardening but decreased during de-hardening and increased during re-hardening in the V+ (329) genotype. Genotype V- (266) showed a completely opposite gene expression pattern in this treatment (**Figure 17b**). Rorat (2006) reported that dehydrin gene transcripts accumulate in late embryogenesis and mostly in all vegetative tissues under optimum growth conditions. DHN gene started to accumulate in contact with stress that leads the plants to cellular dehydration, e.g. freezing temperature, drought and salinity. Similarly, Puhakainen et al.

(2004) also mentioned that DHN genes play roles in obtaining freezing tolerance in plants and suggested that this tolerance could be achieved partly due to their defensive membranes system or cryoprotection of the plasma membrane (Danyluk et al. 1998).

We validated the gene expression data by comparing to the RNA transcriptome data of all these genes (**Figure 18**). All the genes are almost correlated to the RNA transcriptome data.

## 5. CONCLUSIONS and FUTURE PROSPECTS

- The results of this study indicate that there is a differential gene expression induced between the genotypes of *Lolium perenne* at different time points during cold acclimation, de-hardening and re-hardening.
- Our study reveal that vernalization responsive genes are significantly induced by vernalization (cold) during the mid-December to mid-January in a *Lolium* genotype (204) the require vernalization (V+) to flower under natural field conditions.
- Under artificial climate conditions (controlled growth rooms), expression of these vernalization responsive genes are also up-regulated during the period of low temperature in the V+ (204) genotype.
- In addition, there were interesting differences between natural and artificial conditions as regards the gene expression responses to low temperature. Artificially cold acclimated plants may respond differently to plants acclimated in natural conditions.
- The vernalization responsive genes (*VRN1*, *VRN2* and *VIN3*) demonstrated large variation in gene expression during the different temperature treatments. In further studies, other vernalization related genes should be studied.
- To localize the vernalization gene expression, particularly transition from vegetative stage to reproductive stage, RNA *in situ* hybridization experiments should be performed.

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## **Appendix 1: Isolation of Total RNA from Plant Cells**

### **Procedure:**

#### **a) Homogenizing samples**

1. Add 1 mL TRIzol® Reagent per 50–100 mg of tissue sample.
2. When preparing samples with high content of fat, proteins, polysaccharides, or extracellular material (e.g., muscle, fat tissue, or tuberous plant material), an additional isolation step may be required to remove insoluble material from the samples.
  - ✓ Following homogenization, centrifuge the sample at  $12,000 \times g$  for 10 minutes at 4°C.
  - ✓ Remove and discard the fatty layer.
  - ✓ Transfer the cleared supernatant to a new tube.
3. Proceed to Phase separation, or store the homogenized sample. Homogenized samples can be stored at room temperature for several hours, or at –60 to –70°C for at least one month.

#### **b) Phase separation**

1. Incubate the homogenized sample for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex.
2. Add 0.2 mL of chloroform per 1 mL of TRIzol® Reagent used for homogenization. Cap the tube securely.
3. Shake tube vigorously by hand for 15 seconds.
4. Incubate for 2–3 minutes at room temperature.
5. Centrifuge the sample at  $12,000 \times g$  for 15 minutes at 4°C.

Note: The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is ~50% of the total volume.

6. Remove the aqueous phase of the sample by angling the tube at 45° and pipetting the solution out. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase.
7. Place the aqueous phase into a new tube and proceed to the RNA Isolation Procedure.

#### **c) RNA Isolation Procedure**

Always use the appropriate precautions to avoid RNase contamination when preparing and handling RNA.

### **RNA precipitation**

1. 2. Add 0.5 mL of 100% isopropanol to the aqueous phase, per 1 mL of TRIzol® Reagent used for homogenization.
2. Incubate at room temperature for 10 minutes.
3. Centrifuge at  $12,000 \times g$  for 10 minutes at  $4^{\circ}\text{C}$ .
4. Note: The RNA is often invisible prior to centrifugation, and forms a gel-like pellet on the side and bottom of the tube.
5. Proceed to RNA wash.

### **d) RNA wash**

1. Remove the supernatant from the tube, leaving only the RNA pellet.
2. Wash the pellet, with 1 mL of 75% ethanol per 1 mL of TRIzol® Reagent used in the initial homogenization.

Note: The RNA can be stored in 75% ethanol at least 1 year at  $-20^{\circ}\text{C}$ , or at least 1 week at  $4^{\circ}\text{C}$ .

3. Vortex the sample briefly, then centrifuge the tube at  $7500 \times g$  for 5 minutes at  $4^{\circ}\text{C}$ . Discard the wash.
4. Vacuum or air dry the RNA pellet for 5–10 minutes. Do not dry the pellet by vacuum centrifuge.  
Note: Do not allow the RNA to dry completely, because the pellet can lose solubility. Partially dissolved RNA samples have an A260/280 ratio  $<1.6$ .
5. Proceed to RNA resuspension.

### **e) RNA resuspension**

1. Resuspend the RNA pellet in RNase-free water or 0.5% SDS solution (20–50  $\mu\text{L}$ ) by passing the solution up and down several times through a pipette tip.

Note: Do not dissolve the RNA in 0.5% SDS if it is to be used in subsequent enzymatic reactions.

2. Incubate in a water bath or heat block set at  $55\text{--}60^{\circ}\text{C}$  for 10–15 minutes.
3. Proceed to downstream application, or store at  $-70^{\circ}\text{C}$ .

## **Appendix 2: cDNA Synthesis Protocol**

The following protocol has been optimized for generating first-strand cDNA using the SuperScriptR VILO™ cDNA Synthesis Kit.

**Procedure:**

1. For a single reaction, combine the following components in a tube on ice.
  - ✓ 4  $\mu$ l of 5X VILO™ Reaction Mix
  - ✓ 2  $\mu$ l of 10X Super ScriptR Enzyme Mix
  - ✓ 10  $\mu$ g of total RNA (template)
  - ✓ 4  $\mu$ l of DEPC-treated water
2. Gently mix tube contents and incubate at 25°C for 10 minutes
3. Incubate tube at 42°C for 60 minutes.
4. Terminate the reaction at 85°C at 5 minutes.
5. Store the cDNA at -20°C until use.

**Appendix 3: qPCR****Procedure:**

1. Set up the reaction on ice
2. Prepare master mix containing the components (table 1. below without template)
3. Add 18  $\mu$ l of master mix in to 96 well-PCR plate and add 2  $\mu$ l of cDNA template of the gene of interest.
4. Prepare no-template control (NTC) reactions to test for DNA contamination of the enzyme/primer mixes.
5. Cap or seal each PCR plate, and gently mix. Make sure that all components are at the bottom of the tube/plate; centrifuge briefly at 1500 rpm for 2 min.
6. Place reactions in a real-time instrument programmed as described in methods
7. Collect data and analyze results.

Master mix components	Stock concentration	Final Concentration	Amount per reaction ( $\mu$ l)
Water			7.0 $\mu$ l
SYBR green			10.0 $\mu$ l
Primer-F	10 $\mu$ M	250nM	0.5 $\mu$ l
Primer-R	10 $\mu$ M	250nM	0.5 $\mu$ l
Total			18 $\mu$ l
		+	

Template			2.0 $\mu$ l
Total			20 $\mu$ l



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