Freezing Tolerance and expression of candidate genes in Timothy (phleum pratens l.).

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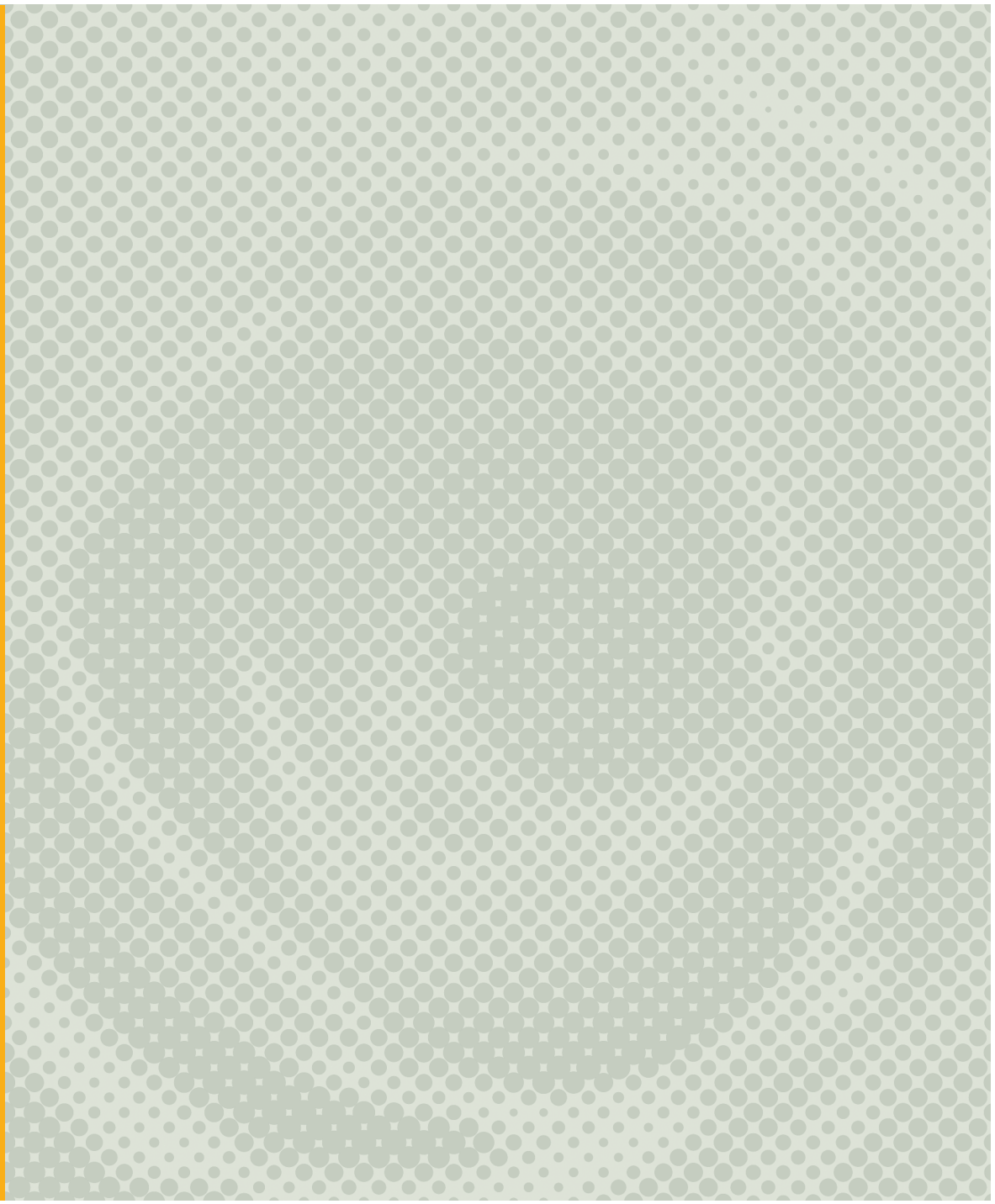

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Fig. Flow chart of the master thesis Work (green House and lab work)

Abbreviations

ABA	Abscisic acid
AFP	Anti freeze protein
bp	base pair
CBF	C-repeat binding factor
CA	Cold acclimation
cDNA	Copy deoxyribonucleic Acid
COR	cold regulated
CRT/DRE	Dehydration responsive element
CT	Cycle Threshold
CCT	comparative cycle threshold
d/n	day/ night
d	day
DREB1	Dehydration responsive element binding1
EST	Express sequence tags
1-FFT	sucrose : sucrose1-fructosyltransferase
FT	Freezing tolerance
h	hour
HFT	High-freezing tolerant
LFT	Low-freezing tolerant
LT	Low temperature
6-SFT	Sucrose: fructan 6-fructosyltransferase
ICE	Inducer of CBF Expression
IRI	Ice recrstilization inhibition
LEA	late embryogenesis-abundant
NCA	Non cold acclimated
PCR	Polymerase chain reaction
RT-PCR	Reverse-transcriptase -PCR
Pp	Phleum pratense
PSII	Photosystem II
ROS	Reactive Oxygen Species
RNA	Ribonucleic acid
WH	Winter hardness
ΔΔCT	Delta-Delta CT

Abstract

Low temperature is the major abiotic stress limiting the productivity and the geographical distribution of many important crops, including forage grasses. To gain a better understanding of low temperature stress responses in timothy (*Phleum pratense* L.), we investigated variation in freezing tolerance among 30 genotypes of timothy and gene expression of two frost tolerant candidate genes, sucrose: fructan 6-fructosyltransferase (*6-SFT)* and *QM* in response to low temperature treatment. Plant materials were established, cold acclimated at 1°C for two weeks, and freezing tolerance was assayed at -10 °C for 24 h by visual scoring of regrowth after 2 weeks using a scale ranging from 0 (completely dead) to 9 (re-growth without damage). An average survival score was determined and high and low frost tolerant genotypes were identified. The relative gene expression of the two candidate genes was measured for two high and two low frost tolerant genotypes using the comparative cycle threshold (CCT) method. The results indicate that there is significant variation among some of the genotypes for freezing tolerance. Increased transcript levels for both candidate genes in response to cold stress were observed in all the genotypes except for *6-SFT* in the high frost tolerant genotype AP31 (from Russia) which was unaffected or slightly down-regulated at all time points studied. Although not conclusively shown, the reason for this result could be lack of primer binding due to sequence variation in the binding sites. Generally, an increase in transcript levels was observed for both genes until day 7 after commencement of cold acclimation and declined thereafter. Variation for freezing tolerance exists among genotypes of timothy that can be utilized for improvement for frost tolerance in this species. Both genes studied might possibly be good candidate genes for further investigations and selection for freezing tolerance of timothy.

Sammendrag

Lav temperatur er den viktigste abiotiske stressfaktoren som begrenser produktiviteten og geografisk utbredelse av mange kulturplanter, inkludert engvekster. For å få en bedre forståelse stressresponser på lave temperaturer i timotei (*Phleum pratense* L.) undersøkte vi variasjonen i frosttoleranse blant 30 genotyper av timotei og genuttrykket til to kandidatgener for frosttoleranse, sukrose:fructan 6-fructosyltransferase (*6-SFT*) og *QM* som respons på lav temperatur. Plantematerialer ble etablert, kuldeakklimatisert ved 1 ^{0}C i to uker, og frosttoleranse ble estimert ved en testtemperatur på -10 °C i 24 timer. Genotypene ble visuelt bedømt for overlevelse etter gjenvekst i 2 uker på en skala fra 0 (fullstendig død) til 9 (ikke skade), og gjennomsnittlig overlevelse ble brukt til å identifisere genotyper med høy og lav frost-toleranse. Den relative genuttrykket av de to kandidatgenene ble målt for to genotyper med høy frost-toleranse og to genotyper med lav frost-toleranse ved hjelp av CCT-metoden (sammenlignende syklus-terskel metode). Resultatene tyder på at det er betydelig variasjon i frost-toleranse blant noen av genotypene. Økte genuttrykk av kandidatgenene som respons på kulde ble observert i alle genotypene unntatt for 6-SFT i genotypen AP31 fra Russland som var blant de mest frost-tolerante genotypene i testen. I denne genotypen var 6-SFT enten uendret eller svakt nedregulert ved alle tidspunkter. Uten at det ble fastslått er årsaken sannsynligvis manglende primerbinding pga. sekvensvariasjon i bindingssetene for primerne i denne genotypen. Generelt ble det observert en økning i transkripsjonsnivåene for begge genene fram til dag 7 etter oppstart av kuldeakklimatiseringen og deretter en reduksjon. Variasjon i frosttoleranse eksisterer blant genotyper av timotei og denne variasjonen kan utnyttes til forbedring av frosttoleranse i denne arten. Begge genene som er studert her kan muligens være gode kandidatgener for videre undersøkelser av og seleksjon for frosttoleranse i timotei.

1. Introduction

1.1 Timothy (*Phleum pratense* L.)

Timothy (*Phleum pratense* L.) which belongs to the Poaceae family (grasses) is the most widely cultivated grass species in the genus *Phleum.* It is claimed to be native to Eurasia, currently widely distributed throughout the cool temperate regions of the world; cultivated as far north as above the Arctic Circle (Reed, 1976). Cultivated timothy is hexaploid (2n=6x=42) with a large genome size. It is referred to as *P. pratense* subsp. *bertolonii* (Stewart et al., 2008). Timothy grass is a major cool-season, short-lived perennial bunchgrass, that reaches about 1 m of height at the heading stage. It can tolerate annual mean temperatures of 4.4 to 18.6 °C, frost, diseases and demonstrates large variation in winter hardiness (Duke, 1978).

In the EU, timothy ranks third in the certified seed trade of forage grasses, after perennial (*Lolium perenne* L.) and Italian ryegrass (*Lolium multiflorum* L.) (Tamaki et al., 2010). Timothy has high nutritive quality and is among the most winter-hardy perennial forage grass species. Hence, it is used in temperate grassland regions where the winter climate is severe (Tamura and Yamada, 2007). Availability of cultivars with good adaptation to cool and humid northern climates makes timothy a widely cultivated forage grass species in the northern part of the Nordic countries. Timothy grass covers 60-70% of meadow production in Finland, and in Sweden and Norway about 50% of the seed production acreage of forage grass is covered with this species. Contribution of milk and meat production of total agricultural income counts 48, 30, 64 and 17 % of in Finland, Sweden, Norway and Denmark, which could not be possible without high quality forages (http://www.nordictimothy.net/index_files/page0002.html).

Fig. 1. Timothy plants at Bardujord, Troms, 69 °N (Photo: Odd A. Rognli)

Large amounts of forage, including from timothy, are produced for ruminants from the large areas of grassland all over the world. However, serious winter damages, which significantly

lower forage yields, occur regularly in the Nordic region. These winter damages initiated many investigations into finding genotypes and cultivars with good adaptation to such limiting winter environments. Among the cool-season forage grasses, timothy has the longest history of formal breeding activity in both Europe and North America (Casler et al., 2001). As research reports show, long-term natural selection under localized stressful conditions creates a considerable amount of genetic variation within timothy cultivars (Rognli, 1988; Cenci, 1980). Such genetic variation between and within crop plant cultivars, or between crop species and their wild relatives, can be used to improve the yield and other economically important traits of crop plants. In forage grasses, a lot of research has been conducted in order to understand the genetic and molecular control of important traits that cause variation in tolerance to abiotic stresses (Sandve et al., 2011). Studies of low temperature stress tolerance have been the focus especially in the cool temperate regions where it is major factor limiting plant productivity.

1.2 Low temperature impact on plant growth and productivity

Major abiotic stress conditions like cold, drought and salinity usually influence plant growth. These stressors, due to their widespread occurrence, limit spatial distribution of plant and cause significant economic losses in agriculture.

Low temperature, the predominant problem on the northern hemisphere, is also a major limitation of plant growth in other parts of the world. Cold stress, similar to drought and salinity, affect the water relations of a plant at the cellular level, as well as the whole plant level, causing damages and adaptation reactions (Beck et al., 2007). According to Beck, stress due to low-temperature is one of the most critical environmental factors for plants and is responsible for economically important crop losses worldwide. In temperate climate areas, cold stress imposes a considerable negative effect on crop productivity, quality, survival, and geographical distribution of plants. This results in significant crop losses, which could be utilized for food and feeds (Xin and Browse, 2000). The intensity of these loses varies with the year and the location depending on the stress type and level, responsiveness and tolerance capacity of the plant species (Khan, 1976; Humphreys, 1989).

1.2.1 Low temperature induced damage

As sessile organisms, plants cannot escape the effects of low winter temperatures like men by warming a room, wearing gloves and over coating jackets. Facing the challenges of winter temperature expected to plants and their only chance is to struggle for survival to the extent they can. However, low temperature stress outside of the optimum range of plant tolerance can cause serious damages at the physiological, cellular, and molecular level, which is expressed by various phenotypic symptoms on plants. At the whole plant level, it causes poor germination, yellowing of leaves, stunted seedlings, reduced leaf expansion, wilting, and, at most extreme, leads to death of tissue. Low temperature stress limits water uptake, utilization of inputs (fertilize) and reduce expression of the full genetic potential of plants by direct inhibition of metabolic reactions. Many low temperature related factors contribute to winter damage of plants. For example, inadequate hardening due to late emergence in autumn or a sudden drop in temperature and long periods of cold-induced desiccation (Gusta et al., 1997a), prolonged periods of low sub-zero temperatures, in particular, mid-winter temperatures below -15ºC result in the rapid loss of winter hardiness (Gusta et al., 1997b), and alternate freezing and thawing, which causes increased injury from ice crystal growth with each freeze (Olien, 1969) have been reported.

Disruption of cellular membranes, particularly the plasma membrane is the most common damage to cells that cause freezing injury in plants. Many studies report that dehydration and rehydration of cells during freeze-thaw cycles results in membrane destabilization and loss of selective permeability (Levitt, 1980; Steponkus, 1984). It is possible that tolerance to freezing injury is correlated with tolerance of membrane to dehydration-induced damage. When plants are exposed to temperatures below zero this may cause ice formation in the extracellular space (apoplast) where the solute concentration is lower than in the cytosol. Formation of ice causes water deficit in the apoplast that leads to water influx from cells with dehydration of the cells as a result. If excessive liquid water is drawn as a result of growing ice crystals, the effects of dehydration might be to the extent osmotically unresponsive, protein and other constituents precipitate and death of cells occurs (Jiffrey and Nilson, 2007). Multiple forms of membrane damage occur as a consequence of freeze induced cellular dehydration and production of

reactive oxygen species. Notable damages observed to occur are expansion-induced-lysis (cells expansion and rupture as result of growing ice crystal), lamellar-to-hexagonal-II phase transitions, fracture jump lesions, and membrane rigidity (Steponkus, 1984). Cell membrane damage, cell rupture and protein degradation were also reported (Mckersie et al., 1997).

Extreme low temperature affects stability and conformation of both RNA and proteins. Consequently, loss of function and degradation of various proteins have also been reported to occur when plants are under low temperature (Shun et al., 2006). Degradation of proteins involved in photosynthesis such as the Rubisco large subunit, which plays a vital role in CO_2 fixation in C3 plants, Rubisco activase, and PSII oxygen involving complex protein, and ATP synthase alpha chain, glycogen phosphorylase and nucleoside diphosphate kinase1 has also been reported (Shun et al., 2006; Nijat et al., 2004). How can plants protect themselves/reduce damages from freezing temperatures during winter?

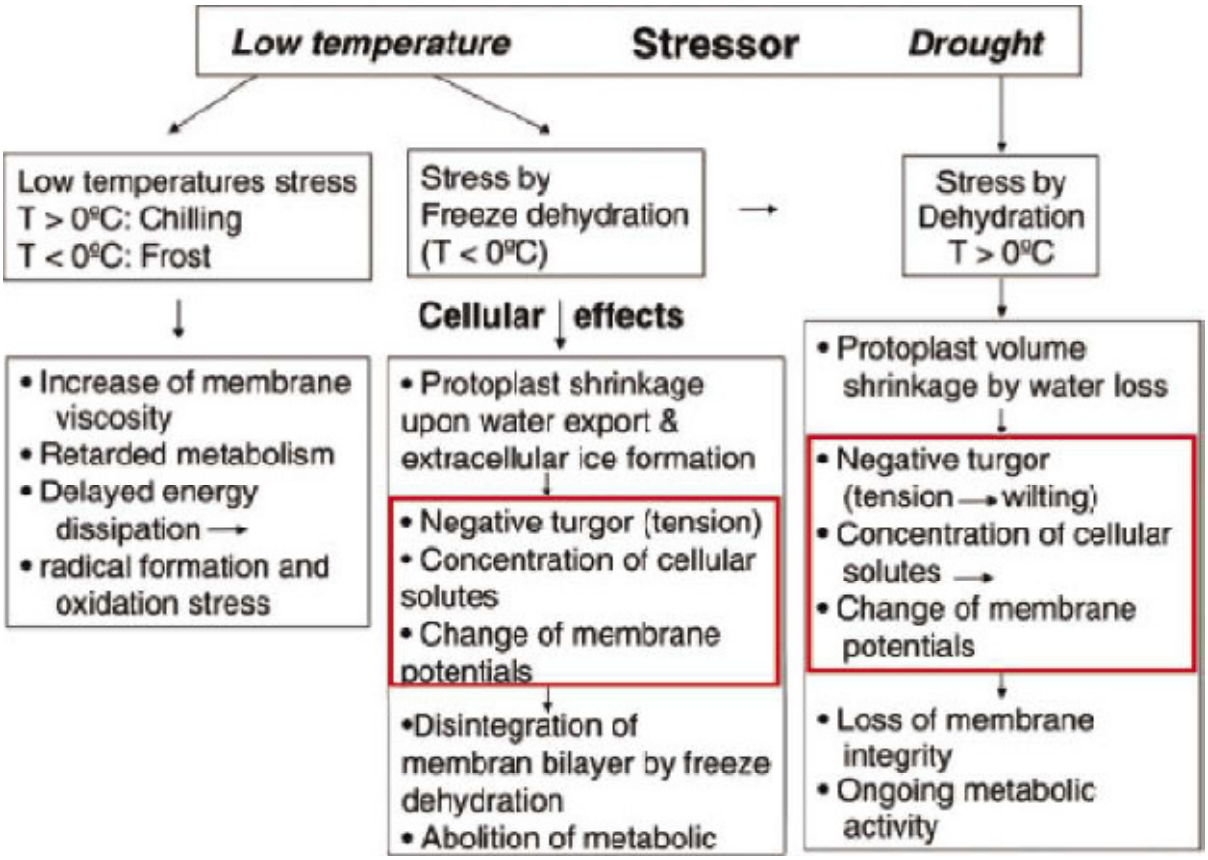


Fig. 2. Effect of low temperature stress on plants at the cellular level (from Beck et al., 2007)

1.2.2 Cold acclimation: as a preparation for winter survival

Under natural conditions, the freezing tolerance of plants varies and dramatically increases upon exposure to low temperature (cold acclimation or hardening) during the early winter/autumn. Many cold acclimation (CA) studies have confirmed that low temperature triggering CA responses and signal cascades are involved in the initial steps of freezing tolerance. Various topics including factors triggering cold acclimation, cold sensing mechanisms and signal transduction pathways, mechanisms underlying differences in freezing tolerance between cold acclimated and non-acclimated plants, genes involved in cold tolerance and how they contribute to freezing tolerance are some of the topics commonly addressed in cold acclimation studies. Much information has been obtained from these studies and has already been applied for improving freezing tolerance of crop plant in ways that would be impossible by traditional plant breeding approaches (Gilmour et al., 1998).

During winter it is common to observe grasses covered with a pile of snow and it may be difficult for us to imagine that they could recover and continue their lives during spring. However, during spring soon after the snow melts away, we start to see a green grass cover on grassland areas. How could they recover? Most temperate plants are well prepared to tolerate freezing during early winter. To survive and sustain their life cycle, many temperate plants evolved and developed mechanisms of adaptation to this freezing temperature. Forage grasses use an array of mechanisms to survive freezing injury. Avoidance of exposure to low freezing temperature by accumulating solutes during CA and tolerance is achieved by preventing ice formation when temperatures decline below zero are well documented mechanisms of freezing tolerance (Jiffrey and Nilson, 2007). These mechanisms of adaptation can be enhanced by the process of cold acclimation. The major role of cold acclimation is the stabilization of cell membranes against freeze-induced injury. Low temperature above zero and good light conditions prior to winter season promote cold hardening in grasses resulting in increased tolerance to freezing and ice encasement (Tronsmo, 1984; Gudleifsson and Larsen, 1993). Changes happen and the contribution of early winter natural hardening of plants to their freezing tolerance has been thoroughly studied using experiments in controlled green house

environments. Studies of CA biochemistry indicate that plant cells undergo biochemical, molecular, physical and structural rearrangement in their macromolecules to tolerate freezing-induced cellular damage during winter. Some of the most frequently described metabolites that contribute to freezing tolerance are soluble sugars, proline, increased levels of antioxidants, the modulation of activities of various enzymes, the production of dehydrins, osmotins, antifreeze proteins (AFPs), and chaperones (stabilize and maintain correct conformation of proteins and RNAs) (Thomashow, 1990; Griffith et al., 2005; Shinozaki and Yamaguchi, 2007; Sandve et al., 2008). How can increased concentration of intracellular solutes trigger freezing tolerance? The suggested mechanism are i) a lower intracellular solute potential will decrease the extent of cellular dehydration at **s**ubfreezing temperatures and ii) various solutes minimize membrane destabilization during dehydration (Steponkus et al., 1993; Yancey, 2005).

One of the key roles of CA induced cellular metabolites is to stabilize cell membranes against freezing injury during the winter period. According to Steponkus et al. (1993), cold acclimation increases freezing tolerance in rye and other plants by preventing expansion-induced-lyses and the formation of hexagonal II phase lipids in the membranes. According to Steponkus and Daniel (1989), such stabilization of membranes is as a result of osmotic adjustment and the accumulation of solutes such as sucrose and proline.

1.3 Plant winter hardiness

Winter hardiness (WH) is the ability of plants to survive from all interacting factors, i.e. low temperature, low light intensity, short photoperiod, frost, ice encasement, low temperature favoured fungi and soil heaving that impede on normal growth during winter (Tronsmo, 1993; Larsen, 1994). Hence, the level of winter survival of plants is determined by the severity of these interacting factors. As winter hardiness is an important fitness component of grasses in temperate climates, it calls for through investigations and understanding of the genetic basis of these component traits for efficient breeding of winter hardy cultivars.

Several environmental factors contribute to the risk of winter injury in perennial forage grasses. According to McKersie and Meclean (1980), suitable warm and humid growth conditions during fall interfere with the hardening process and hence reduce accumulation of stored metabolites. Fluctuation of temperatures above and below the freezing point and prolonged exposure to temperature above zero during winter reduces cold hardiness and increase the risk of winter damage in forage grasses. A collection of local populations of timothy was made in the northernmost part of Norway in 1972-73. The collected populations were multiplied by seed and tested to study if there was variation for important traits and to identify ecotypes with good winter hardiness. Insignificant variation in winter hardiness was observed during the first test (Schjelderup, 1982). Later, further selections was made among promising populations (high dry matter yield and good general performance) and tested for variation in WH. The results showed significant differences between populations (Schjelderup and Aastveit, 1994).

Low temperature is the main factor that affects winter hardiness of plants. Therefore, in this thesis I focus on this factor as it is the most limiting one particularly in temperate climate regions of the world. Due to their winter survival capacity developed through adaptation, cool season grasses are generally said to be tolerant to low freezing temperatures compared to more susceptible warm season grasses. However, there still exists wide variation for freezing tolerance even within the same cultivars. The ability of these plants to withstand freezing temperatures varies with plant species, genotype, and geographical location (Guy, 1990; Thomashow, 1998).

Several freezing tolerance experiments have been carried out on different grasses under natural and controlled conditions in order to evaluate the inherent capacity of grasses to tolerate winter conditions. According to Pulli et al. (1996), frost tolerance is one of the main traits for determining levels of winter hardiness. Larsen and Tronsmo (1991) demonstrated freezing tolerance of timothy at two locations, Ås in the South East and Bodø in Northern Norway, and observed a steady increase in freezing tolerance of timothy during early autumns (mid August to early November). Cool season grasses are more tolerant to freezing temperature than legumes (Ouellet, 1976). Larsen (1994) suggested using the freezing tolerance test as overall indicator of wintering ability for gramineous plants. Plant tolerance to low freezing temperatures develops after sensing cold from the environment.

Hence, how plant sense low temperature stress, and how the plants react to secure survival are briefly highlighted in the next paragraph.

1.4 Cold sensing mechanism and changes in stress tolerance factors during cold acclimation

Plants may not have complex tissues and a nervous system, but they still "feel" and communicate with their environment. Like any other organism, plants have their natural ways of sensing environmental conditions and defend themselves from harsh conditions that threat their survival. Signal transduction pathways and changes that happen within plant cells during CA have been addressed in many studies. Even though several defence mechanisms have been proposed, changes in membrane fluidity is thought to be one of the most important ways in which plant cells can sense temperatures changes. Lipid membranes surrounding the cells are rigidified when plants are stressed by low temperature (Alonso et al., 1997). Cold stress induced membrane rigdification is manifested by reduced membrane fluidity, cytoskeleton rearrangement, change in protein conformation, and induction of stretch sensitive calcium channel to let Ca^{2+} influx to cytosol which trigger expression of cold induced genes and acclimation (Levitt, 1980; Sangwan, 2001). To confirm this, Sangwan et al. (2001) and Orvar et al. (2000) chemically rigidified membranes by treatment with dimethylsulfoxide and observed the consequent expression of COR genes in alfalfa protoplast. This shows that the plasma membrane is most probably the primary sensor of cold stress. In alfalfa membrane fluidity has been reported to acts as a thermo sensor (Orvar et al., 2000). As stated before, low temperature affects membrane fluidity, and protein structural stability and flexibility. This may lead to problems of protein interactions and failure in gene regulation. In general the regulatory circuits that happen during the stress period include stress sensors, signalling pathways comprising a network of protein-protein reactions, transcription factors and promoters, and finally the output proteins or metabolites (Bartels and Sunkar, 2005).

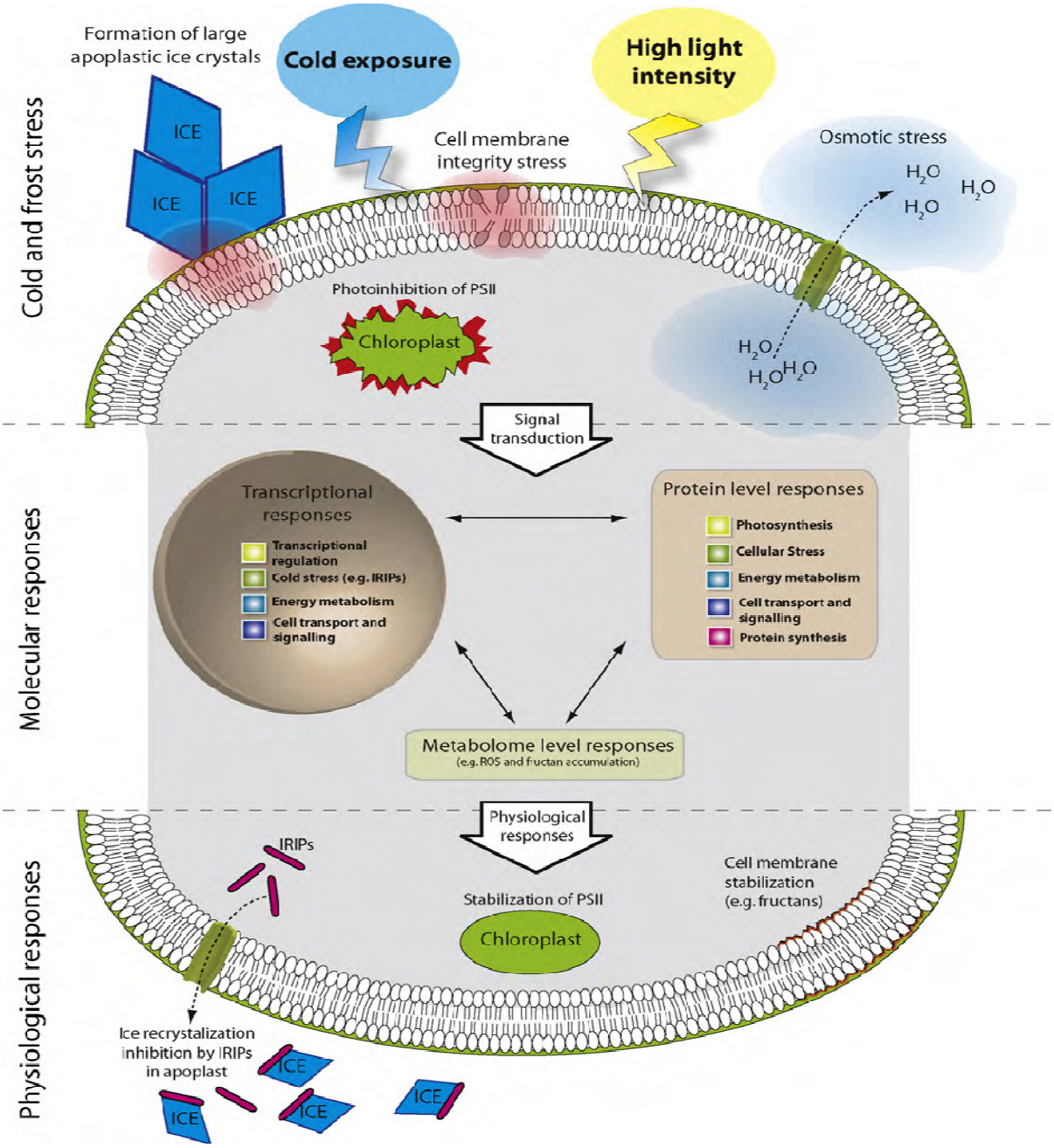


Fig. 3. Signal transduction pathway during cold acclimation of plants (from Sandve et al. 2011)

Cold acclimation is associated with many changes that may happen from cellular to whole organism level. According to Kaye and Guy (1995), many physiological and metabolic processes are involved in plants cold adaptation. As a result of altered expression patterns of a specific set of genes, changes at the molecular, biochemical, and physiological level may occur (Stefanowska, 2002; Thomashow, 1999). Changes in the membrane structure and composition, accumulation of protecting compounds like carbohydrates, absiscic acid (ABA), free amino acids, polyamines and antifreeze proteins in apoplastic regions have also been documented (Janda et al., 2003). Modifications in enzyme complement like more enzyme production to maintain adequate activity, increase in the activation state of the enzyme during cold stress has also been reported (Guy et al., 1992). Several changes that happens at the macromolecular process such as synthesis and degradation of proteins, change in membrane structure and function have also been observed (Uemura and Steponkus, 1994; Guy et al., 1985). Other change know to be involved includes, changes in lipid composition and the accumulation of compatible solutes with cryoprotective properties (Thomashow, 1999), increased level of sugars, soluble protein content and the appearance of new isozymes, and striking increase in total amino acid content (Elisabetta et al., 2006). Increase in amino acid pools in response to a cold shock treatment was also reported in *Arabidopsis* metabolomic profiling analyses (Kaplan et al., 2004). These compatible osmolytes (amino acids and derivatives, polyols (glycerol), sugars, methylamines and methylsulfonium compounds) are reported to act both by avoiding cell dehydration through their contribution to osmotic adjustment (turgor maintenance), and by stabilizing the quaternary structure of proteins and membranes (Yancey, 2005). In particular, proline and trehalose appear to bind to head groups of membrane phospholipids, in effect replacing water molecules. Thus, they can stabilize membranes during cell shrinkage (Storey and Storey, 1996; Rudolph and Crowe, 1985).

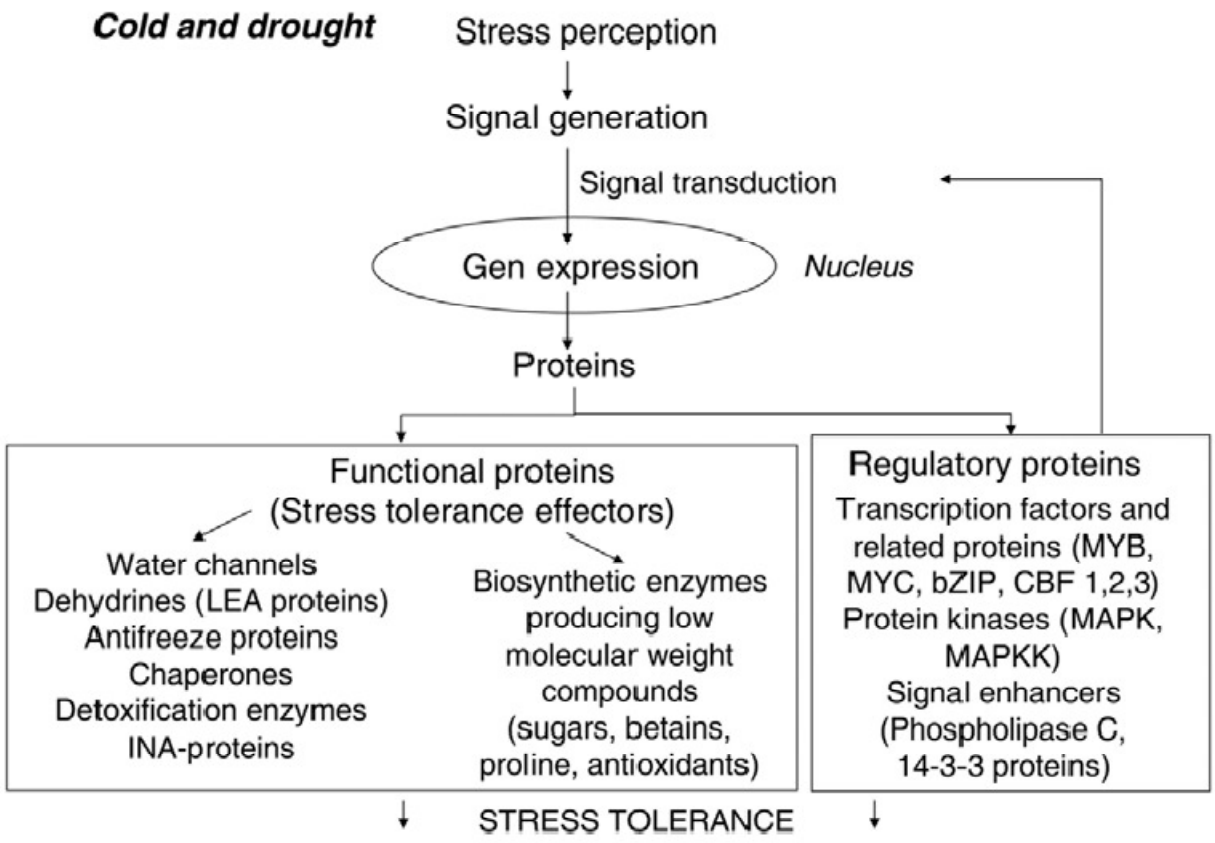


Fig. 4. Different stress tolerance factors produced in adaptive responses of a plant to cold and drought stress (from Beck et al., 2007)

1.5 Cold regulated genes and role in freezing tolerance

In higher organisms with complex development patterns, genes tend to be controlled by the interaction of multiple positive factors. When plant are exposed to low temperature, many genes are witched on and off (Fowler and Thomashow, 2002). This means there is a change in gene expression of the cell depending on the type of signal they receive and the cell requirement. Guy et al. (1985) first conceived this idea of cold induced changes in gene expression. Later, it received attention by many researchers and they characterized and identified the function of cold induced genes using different molecular biology and molecular genetics techniques. Many of the genes that respond to low temperature stresses have been identified and described at the transcriptional level, and their gene products are thought to function in stress tolerance (Thomashow, 1999; Bartels and Sunkar, 2005).

During CA many cold responsive genes are known to be up-regulated or down-regulated (Fowler and Thomashow, 2002). These low temperature initiated/induced genes are collectively called cold-regulated genes (COR) (Thomashow, 1999). COR genes are induced stepwise i.e. genes involved in C-repeat binding factors (CBFs) and those involved in signal transduction are induced during the earlier phase of CA whereas genes that function in cellular metabolism and protection against freezing damage are induced later (Lee et al., 2005). Following cold stress, the expression of c-repeat binding factors/dehydration responsive element binding1 (CBF/DREB1b) genes was found to be induced rapidly and the transcripts accumulated within 15 min of exposure to low temperature in Arabidopsis (Joaquın et al., 1999; Gilmour et al., 1998). Constitutively expressed inducer of CBF expression1 (*ICE1*) genes which are located upstream of CBF3 gene promoters induces the transcription of CBF3 genes (Chinnusamy et al., 2003) and consequently CBF gene products are produced. CBFs play important roles in transcriptional, post-transcriptional, and post-translational regulation of COR genes whose expression influences the freezing tolerance of plants.

Hundreds of COR genes are induced during the CA process. Different studies have documented a variable number of COR genes induced during CA, depending on plant species, plant tissue and laboratory conditions used in the investigation. For example, a gene expression study in *Arabidopsis* during a 7-day experiment identified 306 genes as being cold responsive. Out of these, the transcripts of 218 genes increased while the transcripts of 88 genes decreased (Fowler and Thomashow, 2002). Lee et al. (2005) indentified 939 COR genes in *Arabidopsis* out of which 655 and 284 were up- and down-regulated, respectively. Another report showed that 2735 gene were cold regulated in barley (Svensson et al., 2006).

Studies show that a large number of low temperature induced genes encode key metabolic enzymes, late embryogenesis-abundant (LEA) proteins, detoxification enzymes, chaperones, protein kinases, and transcription factors (Thomashow, 1999; Bartels and Sunkar, 2005) that potentially increase freezing tolerance. During CA several genes are expressed but not all of them are involved in freezing tolerance (Thomashow, 1999). Genes induced during CA have two major functions; (i) to produce important metabolic proteins that function to protect plant cells from stress, and (ii) regulation of genes involved in receiving stress signals from the

environment (Fowler and Thomashow, 2002). To mention few of the genes, *Arabidopsis* cold induced *FAD8* gene encodes a fatty acid desaturase that might contribute to freezing tolerance by altering the lipid composition (Gibson et al., 1994), a spinach heat-schock protein (hsp70) and *Brassica napus* hsp90 genes encoding a molecular chaperones which was reported to stabilize proteins against freeze-induced denaturation and thereby contributes to freezing tolerance (Anderson et al., 1994; Krishna et al., 1995). Expression of CRT/DRE regulated *Arabidopsis* CBF genes increased freezing tolerance in canola plants (Jaglo et al., 2001). Expression of a wheat gene (*COR15a*) was reported to contribute to freezing tolerance by stabilizing membranes (Artus et al., 1996). Anti-freezing proteins (AFP) that decrease the temperature at which ice is formed by binding to the surface of ice nuclei and inhibiting ice crystal growth have been isolated from plants (Antikainen and Griffith 1997). Some of the COR genes increase anti-oxidative mechanism, sugar levels in intercellular spaces and molecular chaperones that protect the plants against cold induced stress (Mckersie et al., 1997). Even though the role of CBFs as transcriptional regulators has been experimentally verified only in a few major crops, it has been widely studied using the model plant *Arabidopsis*. This model plant has been extensively studied in order to understand the physiological, biochemical and molecular basis of plant freezing tolerance. Reports indicate that the CRT/DRE element binding CBF/DREB1 transcription factors play a major role in cold sensing by regulating the expression of cold-responsive genes in an ABA-independent manner (Lee et al., 2005; Thomashow et al., 2001; Stockinger et al., 1997).

1.6 Candidate genes

Hundreds of candidate genes induced during cold stress determine frost tolerance of forage grasses. A few studies has been conducted in the forage grasses meadow fescue (*Festuca pratensis* Huds.) and perennial ryegrass (*Lolium perenne* L.) aiming to identify genes involved in minimizing the damaging effects of cold stress and many candidate genes have been identified, functionally characterized and related to freezing tolerance (Wei et al., 2002; Rudi et al., 2011). Since few DNA sequences from timothy exist in databases there is little information about candidate genes in this species. In this study we were interested in two candidate genes, sucrose: fructan 6-fructosyltransferase (*6-SFT*) and *QM*, which are well characterized in other grass species and confirmed to be involved in frost tolerance.

1.6.1 The role of the 6-SFT gene and fructans in freezing tolerance

In vascular plants acclimation to freezing temperatures is always accompanied by an accumulation of low- and high-molecular-weight compounds (Levitt, 1980). Fructans are a common structurally diverse class of oligo- and polysaccharides based on fructose. It has been estimated that 15% of plant species accumulate fructans instead of starch as their main carbohydrate reserves (Hendry, 1987; Hendry, 1993). Many important angiosperm plant species accumulate fructans, most of them can be found in the prominent plant orders Poales (grasses), Asterales and Liliales. Fructans are found in species like cereals, such as wheat and barley; vegetables such as chicory, onion and lettuce, and forage grasses such as perennial ryegrass and *Festuca* species (Hendry, 1993). Fructans are found in the leaves of many cool-season grass species. Compared to other grasses such as wheat and barley, timothy predominantly accumulates simple levans with a high degree of polymerization (Cairns and Eashton, 1993).

Fructans are synthesized by the collective action of fructosltransferase (FTs). Many FTs enzymes are involved in synthesis of the various fructan types in plants, i.e. inulin, levan, graminan, inulin neoseries, and levan neoseries. Linkage between adjacent fructose units, the presence of branches and the position of the sugar residue distinguish fructans. In the first step, the enzyme, sucrose: sucrose 1-fructosyltransferase (*1-SST*) catalyzes the initial fructosyl transfer between two sucrose molecules and further elongation of the chain is catalyzed by the involvement of other FTs (*1-FFT*, *6G-FFT* and *6-SFT*, etc.). For example in wheat and barley, *6-SFT* is capable of synthesizing all of the fructan species currently known to occur in these cereals (Bancal et al., 1992; Bancal et al., 1991). The relative activity of the different enzymes involved in fructan metabolism have a profound influence on the specific fructan mixture accumulated in a given grass species. It has been reported that *6-SFT* is an enzyme catalyzing the formation and extension of β-2, 6-linked fructans typical of grasses. Fructan encoding *6-SFT* homologues have also been identified in various plants such as *L. perenne* and *L. temulentum* (Hisano et al., 2008; del Viso et al., 2009) and *P. secunda* (big bluegrass), for which *6-SFT* was suggested as the major enzyme involved in fructan biosynthesis (Wei et al., 2002). In all of these grasses, *6-SFT* enzymes were reported to be involved in fructan biosynthesis.

Different fructan types have different roles and the fructan types that accumulate in plant species are also different. As it has been reported, a mixture of fructan types accumulates in monocotyledonous and inulin series type in dicotyledonous plant species (Van den and Laere, 2007). Maleux and Ende (2007) reported that fructans of the graminan and levan type have important roles as short-term reserves and as long-term storage compounds to enable winter survival in grasses. Many studies have recognized fructans as protective agents against abiotic stresses by their stabilization of membranes. Yoshida et al. (2007) demonstrated that graminan- and levan type fructans accumulate during the period of CA of wheat, and it was associated with freezing tolerance and over-wintering ability. Correlation between fructan accumulation and plant tolerance to cold and drought stress have been confirmed through biochemical and physiological studies (De Roover et al., 2000; Hincha et al., 2002). It has also been suggested that fructans might protect plants against freezing/drought stresses (Valluru and Ende, 2008). Studies of transgenic plant involving fructan metabolism further support the association of fructan and stress tolerance (Kawakami et al., 2008; Li et al., 2007). Increased amount of carbohydrate is a major metabolic response during CA (Cook et al., 2004) and this can affect freezing tolerance through aiding photosynthetic acclimation (Strand et al., 2002) and cell membrane interaction and stabilization (Hincha et al., 2000). Damel et al. (1978) in their studies on model membranes demonstrated that fructans interact with lipid membranes in a strong and specific way and the incorporation of fructan into lipid bi-layer of cell membranes increases membrane stability and improve freezing tolerance. The same effect has been reported in transgenic *Lolium perenne* (Hisano et al., 2004). Transgenic *Lolium* had high indigenous fructan levels and increased membrane stability compared to its wild type. Reports indicate that graminan- and levan type fructans accumulate during the period of cold acclimation in wheat and this accumulation has been associated with freezing tolerance and with over-wintering ability of the plant (Yoshida et al., 2007). Rutten and Santarius (1992) studied the relationship between frost tolerance and sugar concentration in various bryophytes. Their study showed that the variation in freezing tolerance of most species roughly coincided with the relative sugar concentration of the tissue. This is not always the case as some studies have shown that the increased concentration of carbohydrate cannot guarantee increase in FT. It was reported that ontogenetic variation in the frost tolerance of leaves of *Plagiomnium* species cannot be attributed to differences in the cellular levels of sucrose, glucose and fructose (Rutten and Santarius, 1992).

1.6.2 QM gene

The *QM* gene was identified from humans as putative suppressor of Wilm's tumour. This suggestion of the functional role of the gene was based on subtractive cDNA/RNA hybridization between a tumorigenic Wilms' tumor cell line and a non-tumorigenic microcell hybrid cell (Dowdy et al., 1991). They observed higher expression levels of the *QM* gene in non-tumorigenic Wilms' microcell hybrid cells than in the tumorigenic parental cell line. Since then its homologues have been identified and cloned from several species including members of higher vertebrates, and the plant and fungal kingdoms. Sequence comparisons indicate that the homologues of *QM* are highly conserved among the eukaryotes. *QM* homologues has been identified in many plant species including meadow fescue, tomato, tea, *Caragana jubat*a, pineapple, and rice (Rudi et al., 2011; Chen et al., 2006; Singh et al., 2009; Bhardwaj et al., 2010; Xio et al., 2002). Expression pattern and the suggested function of *QM* gene homologues in the above plants were reported to be different in different tissue and under different conditions (Rudi et al., 2011; Chen et al., 2006; Anderson et al., 1994; Xio et al., 2002; Singh et al., 2009). Low temperature induced expression study of the *QM* gene in rice leaves showed that it is up-regulated after 6 h and decreased back to normal after 24 h of cold acclimation. No influence of cold acclimation was observed in root tissue treated under the same conditions (Xio et al., 2002). The expression of the *QM* gene was up-regulated in some of the above mentioned studies during exposure to cold and confirmed that it is involved directly or indirectly in tolerance against freezing damage. The expression level varies not only with tissue considered but also the type of environmental cues. Singh et al. (2009) examined expression of camellia sinensis *QM* gene (*CcQM*) in active Vs dormant growth period and in response to stresses of (drought, ABA and gibberelic acid). They observed higher expression of CcQM gene in actively growing apical buds, which give a clue that the genes has important role in growth and development of the plant. The same is observed in response to gibberellic acid, i.e. its treatment increased the transcript. In response to drought, which exert similar stress on plant as cold stress the *CsQM* gene was down-regulated. Rudi et al. (2011) observed higher expression of *QM* gene in leaves than the in crown and stem tissues of meadow fescue. In yeast it was reported that deletion of the *QM* homologue *GRC5/QSR1* was lethal. The mutation in *GRC5/QSR1* resulted in a defect in protein synthesis, growth and cell division arrest, abnormalities of the actin cytoskeleton and mitochondrial respiration (Tron et al., 1995).

Aims of the study

Timothy has paramount importance as a forage grass and timothy cultivars with wide adaptation to winter conditions exist. Obtaining information about sequence variation and expression of candidate genes for freezing tolerance could be beneficial for further improvement of freezing tolerance and increase productivity by improving winter-hardiness. Therefore, the aims of this study were; (i) to check variation in freezing tolerance among timothy genotypes, (ii) to obtain information on the expression patterns of two candidate genes (*6-SFT* and *QM*) in leaves of high-frost tolerant (HFT) and low-frost tolerant (LFT) genotypes, and (iii) to study the relationship between expression levels and development of freezing tolerance. In order to achieve these goals we performed freezing tolerance test in a controlled environment for selection of HFT and LFT genotypes, and we measured relative expression levels of the candidate genes using real-time quantitative PCR (rt-qPCR).

2. Materials and methods

2.1 Plant materials and growth conditions

Freezing tolerance assays was conducted using one genotype from each of 30 accessions of *Phleum pratense* L. originally assembled from different gene banks as part of the project 'Phenotypic and genotypic characterisation of genetic resources of Nordic timothy (*Phleum pratense* L.)' funded by the Nordic Joint Committee for Agricultural Research (NKJ). Identity and geographic origin of the accessions is presented in Table 1, and the geographic origin is plotted on the map in (Fig. 6.). Each genotype was cloned into 6 ramets/tillers and transplanted into pots filled with standard soil media for grasses. The pots were kept on rectangular flats with plastic inserts each capable of accommodating one ramet from each of all accessions. Six such rectangular flats containing ramets from all genotypes thus represented six replications. The pots were completely randomized within flats. The plants were grown in a controlled growth room with growth conditions of 19/16 °C day/night temperature, 18 h photoperiod at a light intensity of 120 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps and bulbs for 4 weeks until they had 2 - 4 axilliary shoots.

Table. 1. Designation and information about origin of timothy accessions used in the study.

Clone number	Number on the map	Genbank	Accession number	Country of origin	Locality
AP01	7596	NGB	NGB7596	Norway	01-6-48-12
AP02	4053	NGB	NGB4053	Denmark	SR Saltum MH0202
AP03	1332	NGB	NGB1332	Sweden	Klubbsjö SH0301
AP04	1118	NGB	NGB1118	Finland	Yliollitervon ME0201
AP05	4140	NGB	NGB4140	Iceland	Korpa
AP06	17198	NGB	NGB17198	Norway	
AP07	14403	NGB	NGB14403	Finland	
AP08	722	NGB	NGB722	Sweden	
AP09	1	RICP	14G2400116	Czech Republic	
AP10	2	RICP	14G2400152	Slovakia	
AP11	4	RCAT	RCAT41183	Hungary	Csesznek
AP12	5	IGER	ABY-BD 3199	Russia	Sakhalin Region
AP13	7	IGER	ABY-BD 3267	Romania	Radauti
AP17	12	Kew	147408	Canada	Alberta, Yellowhead
AP23	19	Kew	51998	England	West Sussex
AP24	22	Bulgaria	2007-PHLPR_1A7E0001	Bulgaria	Pamporovo
AP25	23	GRIN	199262	Greece	
AP26	25	GRIN	204480	Turkey	
AP27	26	GRIN	204909	Turkey	
AP28	27	GRIN	210426	Greece	
AP29	28	GRIN	251595	Yugoslavia	
AP30	29	GRIN	251670	Serbia	
AP31	30	GRIN	315489	USSR	Pavlovsk
AP33	32	GRIN	319080	Spain	
AP34	34	GRIN	325461	Russia	Stavropol region. 10 km north of Teberda reservation, along main road.
AP35	35	GRIN	345665	USSR	St. Petersburg
AP36	37	GRIN	371957	Bulgaria	
AP37	38	GRIN	381926	France	Angers
AP38	41	GRIN	539034	Russia	2 km NW of Kamlak, Altai Mountains, Siberia
AP39	42	GRIN	539037	Russia	Along river bottom, 4 km N of Gorno-Altysk, Altai Mountains, Siberia

NGB=Nordic gene bank; RICP=Research Institute of Crop Production, Czech Republic; RCAT= Institute for Agrobotany, Tápiószele, Hungary; IGER=Institute of Grassland and Environmental Research, Aberystwyth, Wales; Kew= Royal Botanical Garden, Kew, England; GRIN= Germplasm Resource Information Network, USA.

Fig. 5. Map locations of accessions used in this study (Fjellheim S., unpublished).

2.2 Cold acclimation

The 4 weeks old ramets were first pre-acclimated at 12/6°C day/night temperature in 12 h photoperiod using a light intensity of 120 µmol m^{-2} s^{-1} for one week, and then cold acclimated for two weeks at 1°C in 18 h photoperiod using the same light intensity as during pre-acclimation.

Fig. 6. Timothy plants during cold acclimation (Photo by Dereje H. Buko)

2.3 Freezing tolerance test

2.3.1 Screening experiment to detect LT_{50}

In order to detect the temperature that discriminated best between genotypes, i.e. the LT_{50} temperature, a screening experiment was conducted with different freezing temperatures. Cold acclimated clones were frozen at temperatures of -10, -12, -14, -16, and -18 ^{0}C for a period of 24 h, thawed at 2 °C for 12 h, cut back to approximately 3 cm height, and transferred back to the normal growth conditions (growth condtions before cold acclimation). After 2 weeks, the regrowth of the plants was determined by visual scoring using a scale ranging from 0 (completely dead) to 9 (re-growth without damage). Based on average survival scores of the genotypes -10 ^{0}C was chosen as an appropriate temperature for testing LT_{50}.

2.3.2 Freezing experiment

Cloning, growth and acclimation conditions were the same for the screening and the freezing experiment. Cold acclimated plants were subjected to freezing stress in chambers that was stabilized at 0 °C and then the temperature was lowered to -2 °C at a cooling rate of 1 ^{0}C per hour and held there for 12 hours. The temperature was then lowered at the same cooling rate to the predetermined freezing temperature (-10 °C). The cold acclimated plants were frozen at this temperature for 24 hours. Then the temperature was raised to -1 °C by 1 °C per hour, held there

for 12 hours then raised to 0 °C and held there for 12 hours and finally raised to 2 °C. The frozen ramets were thawed at 2 °C for 12 h, cut back approximately to 3 cm height, and transferred back to the normal growing conditions. Two and three weeks after the transfer, surviving plant's regrowth capacity were scored visually using a scale ranging from 0 (completely dead) to 9 (re-growth without damage) (Larsen, 1978). The visual scoring was based on evaluation of characters like maintenance of turgidity, resumption of growth, green leaf resumption, and regrowth of axilliary shoots. Mean scores of six biological replicates were calculated for each genotype and the freezing tolerance of the genotypes were compared.

2.4 Total RNA extraction

Green and healthy leaves were sampled in triplicate from each genotype before cold acclimation (0 h) and during cold acclimation at 4 h, 1 day, 4, 7, and 14 days after transfer to 1°C. The samples were stored at -80 °C until used for total RNA isolation. Total RNA was isolated from 100 mg frozen leaves using the RNeasy plant Mini, RNA isolation kit (Qiagen, Norway) according to the manufacturer's specifications (Appendix 1). On column DNase digestion was performed with 80 µl DNase I incubation mix added directly to the RNeasy column membrane (RNeasy Mini Handbook, Qiagen). Finally, total RNA was eluted from the RNeasy Mini columns with 50 µl of RNase-free water. The RNeasy Mini Elute kit (Qiagen, Norway) was used for clean-up (Appendix 2). The concentration and purity of total RNA was measured using the Nanodrop (Nanodrop Technologies, Wilmington, DE, USA). Each sample was measured twice. To determine the integrity of the RNA we used the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) (Appendix 3).

2. 5 Primer and probe design

The Primer express® v3.0 software (Applied Biosystems, USA) was used to design three primer pairs and probes (see Appendix 4 for sequence information). The *Phleum pratense* 6-SFT mRNA complete coding sequence (AB436697.1) (Appendix 5) from the NBCI database was used to make the primers (Pp_6SFT_F, Pp_6SFT_R) and probe (Pp_6SFT-Probe). A consensus sequence from contig 862 of a QM homolog from timothy identified using 454 sequencing of a timothy EST library (unpublished data) was used to design the primer pairs

Pp_QM_F and Pp_QM_R and the Pp_QM-Probe. GAPDH was used as an internal control gene and contig 5582, a GAPDH homolog from timothy identified using 454 sequencing of a timothy EST library was used to design the primer pairs Pp_GADPH_F and Pp_GADPH_R and the probe Pp_GADPH_Probe. The sequences of the timothy contigs 862 and 5582 were blasted and aligned against the *Nicotiana benthamiana QM* gene (FJ824852.1) and the *Festuca pratensis* Huds. GAPDH sequence (DQ991044.1). Sequences with high E-values were chosen.

2.6 Real Time qPCR

The EXPRESS two-step qRT-PCR universal kit (Invitrogen) with the superscript VILO cDNA synthesis kit (Invitrogen) was used for quantitative RT-PCR of the target genes according to the manufacture's protocol (Appendix 6 & Appendix 7). Synthesis of cDNA was conducted by using 2.5 µg of total RNA template in 20 µl total reaction volume. 2 µl of the cDNA synthesis was used in each real-time qPCR reaction. A master mixture containing all of the other ingredients was pipetted into individual tubes of a Fast Optical 96 well plate with barcode (see master mix from Appendix 7, Table A2). The fast cycling program (developed using the AB7500 in fast mode) with some modification of duration of elongation was used to amplify the transcripts. Target amplification consisted of 95 °C for 20 sec, 40 cycles of 95 °C for 3 sec (denaturation) and 60 °C for 35 sec (annealing). The samples were subjected to 7500 Fast Real-Time instrument. Taq Man® gene expression assays were used to detect the amplified products. The change in expression of the target genes (*6-SFT* and *QM*) normalized to the internal control gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was monitored over 14 days exposure to low temperature. Cycle Threshold (CT) data were generated from the real time PCR instrument. Since the amplification efficiency was close to 100% for both genes (Appendix 8), we used the equation ΔΔCT = (CT of target – CT of GAPDH) time x - (CT of target – CT of GAPDH) calibrator sample (time zero). (Time x is the different time points after CA). The mean CT values of biological replicates for both the target and internal control genes were determined at each time point and used as raw data 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 $2^{-\Delta\Delta CT}$ equation (Livak & Schmittgen, 2001).

2.7 PCR

PCR reactions were performed in order to amplify specific fragments of the target genes and use these for sequencing. We used a reaction mix of 5 µl of 10x buffer (PCR buffer), 2µl of cDNA, 2µl (10µM) of each primer, 1 µl dNTPs (10 µM), 0.5-µl Taq DNA polymerase (New England Biolabs Inc.) and filled with dH_20 (37.5µl) to a total reaction volume of 50 µl. The components were mixed and spun down. Then the reaction mix was run on a PCR machine (Master cycler **ep**gradient, Eppendrof) using the following cycling program: 94°C for 3 minute, then 40 cycles of 94 °C for 3 s, 59 °C for 30 s, and 72 °C for 1 min, and a final elongation at 72 °C for 7 min. QIAquick PCR purification kit was used to purify the PCR product (Appendix 9). The purified PCR products were used for sequencing.

2.8 DNA sequencing

The **BigDye® Terminator** v3.1 **Cycle Sequencing Kit** (Applied Biosystems) was used for sequencing. The reaction components were 2 µl of Big dye sequencing terminator, 3 µl of 5x BigDye sequencing buffer, 4 µl primer (1pmol/µl), 10 µl of Milli Q water and 1 µl of template (PCR product) with a total volume of 20 µl. We used the following PCR program: 96 °C for 30 s (denaturation), then 25 cycles of 50 °C for 15 s (annealing) and elongation at 60 °C for 4 min. The sequencing products were purified and precipitated as follows. We added 2 µl EDTA, 2 µl NaAc, and 52 µl 96 % EtOH to the 20 µl product from the sequencing reaction in an Eppendrof tube and incubated this at room temperature for 15 min. This was centrifuged at 14000 rpm for 30 min, the supernatant was discarded and the pellet was washed with 70 µl of 70 % EtOH and centrifuged at 14000 rpm for 15 min. The EtOH was carefully removed from the tube and the pellet was dried at room temperature in a laminar flow cabinet. Afterwards the samples were stored at -20 °C and run on the ABI PRISM 3100 DNA Sequencer (Perkin Elmer Biosystems) at the Department of Chemistry, Biotechnology and Food Science at UMB. Sequence data were edited using the Sequencher 4.10 programs (Gene Codes Corporation, Ann Arbor, MI, USA). Edited sequences were used. Two pairs of sequencing primers were designed using the Primer 3 software (Steve and Helen, 2000) (see primer sequences Appendix 10).

3. Result

3.1 Screening experiment to detect LT_{50} temperature

Appropriate freezing temperature for the freezing test was determined by freezing ramets of each genotype at a series of subzero, i.e. -10, -12, -14, -16, and -18 temperatures.

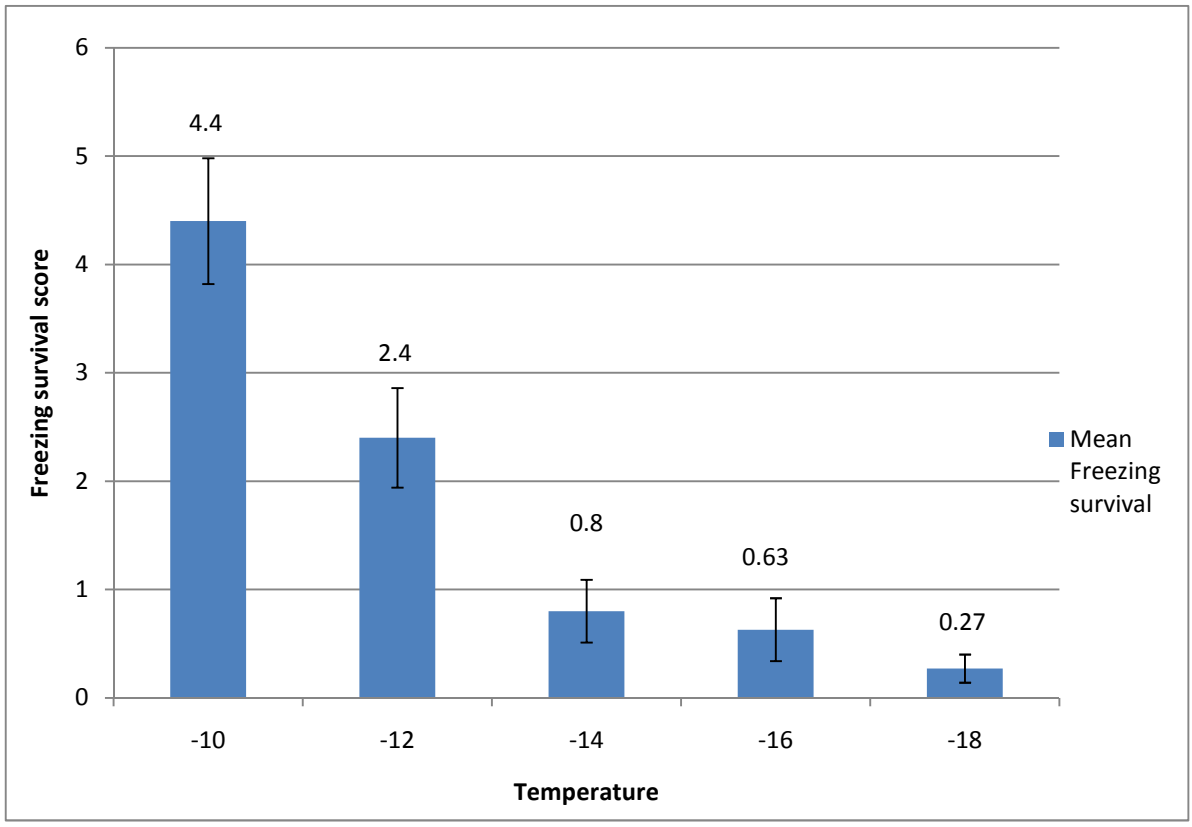


Fig. 7. Average regrowth of timothy genotypes after exposure to different freezing temperatures.

It was observed that the aboveground tissues of almost all ramets wilted and died within the first few days after transfer back to the normal growth temperature conditions after being exposed to -14, -16 and -18 ^{0}C. The results show that 64, 69 and 91 % of all ramets exposed to -14, -16 and -18 ^{0}C died, respectively. The average freezing survival scores of the ramets exposed to -10, -12, -14, -16, -18 were 4.4 ± 0.58, 2,4 ± 0.46, 0.8 ± 0.29, 0.63 ± 0.29 and 0.27 ± 0.13 respectively. The average survival scores showed that -10 ^{0}C was the appropriate temperature to use for testing LT_{50} of this sample of genotypes. Visual scoring of regrowth of each ramet was done using a scale from 0 (completely injured and dead) to 9 (no damage). The phenotypes of the plants corresponding to different stages of the scale are presented in Fig. 8.

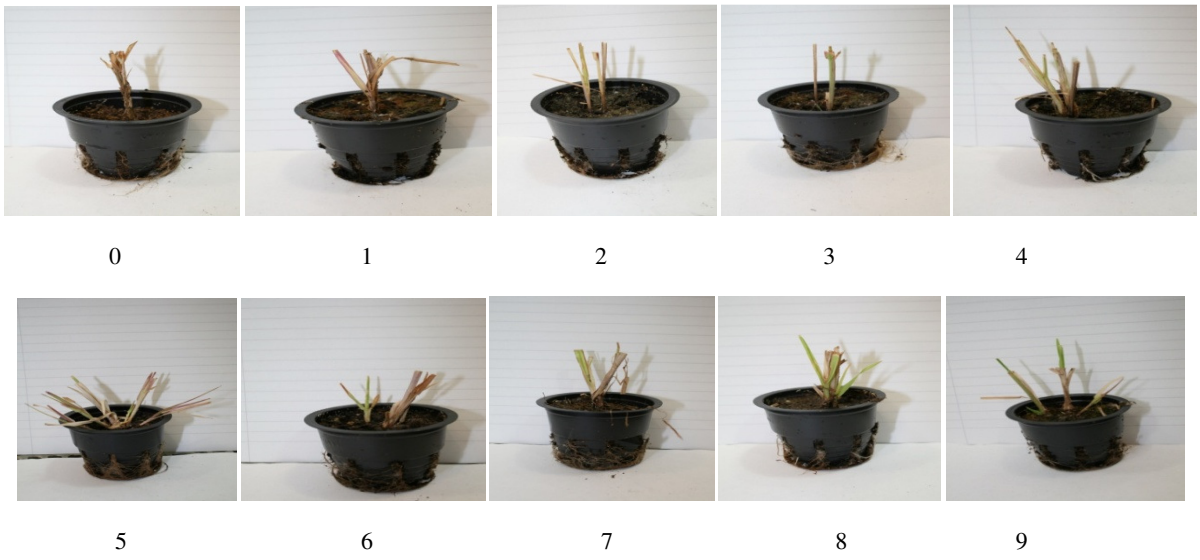


Fig. 8. Scale used in the visual scoring of regrowth of ramets of timothy genotypes.

3. 2 The freezing experiment

3.2.1 Variation in freezing tolerance among accessions of *Phleum pratense* L.

Average survival scores of the genotypes clearly showed that significant variation in freezing tolerance exist among the genotypes. Based on the regrowth scores after two weeks our results showed that variation in survival ranges from nearly complete injury with an average score of 0.17 ± 0.16 for genotype AP24 (from Bulgaria) to 6.6 ± 0.2 for genotype AP7 (from Finland) (Table 2, Fig. 9.). The mean scores decreased to 0 (completely dead) for AP24 and to 5.8 ± 0.4 for AP7 when evaluation was done after 3 weeks of recovery from freezing (data not shown). AP7 and AP31 are significantly different in their freezing tolerance compared to genotype AP25, AP2, AP11, AP13. Genotypes AP7, AP31, AP38, AP26, and AP17, have relatively high freezing tolerance compared to other genotypes (Table. 2 Fig. 9). Based on the survival score, AP13, AP29, AP23, AP11, AP30, AP34 and AP24 are more likely low frost tolerant genotypes.

Table 2. Summary of genotypes and mean freezing tolerance comparison (mean ± SEM)

Genotype	**Mean freezing survival score**
AP7	6.60 ± 0.20^{a}
AP31	5.33 ± 0.49^{ab}
AP38	5.17 ± 1.14^{ab}
AP 26	5.00 ± 0.53^{ab}
AP17	5.00 ± 1.35^{abc}
AP10	4.83 ± 1.27^{abcd}
AP8	4.83 ± 0.89^{abcd}
AP3	4.50 ± 1.35^{abcde}
AP27	4.17 ± 1.00^{abcde}
AP1	4.17 ± 0.81^{abcde}
AP6	3.83 ± 1.22^{bcde}
AP35	3.80 ± 0.73^{bcde}
AP5	3.67 ± 0.61^{bcde}
AP37	3.00 ± 0.77^{bcdef}
AP28	$3,00 \pm 0.77^{bcdef}$
AP9	3.00 ± 0.53^{bcdef}
AP25	2.83 ± 1.18^{cdef}
AP2	2.60 ± 0.89^{cdefg}
AP36	2.50 ± 1.14^{defg}
AP33	2.33 ± 0.73^{efg}
AP13	2.33 ± 1.00^{efg}
AP29	2.17 ± 1.27^{efg}
AP23	0.83 ± 0.8^{fg}
AP11	0.83 ± 0.41^{fg}
AP30	0.67 ± 0.65^{fg}
AP34	0.17 ± 0.16^{g}
AP24	0.17 ± 0.16^{g}

*Means that do not share the same letter are significantly different

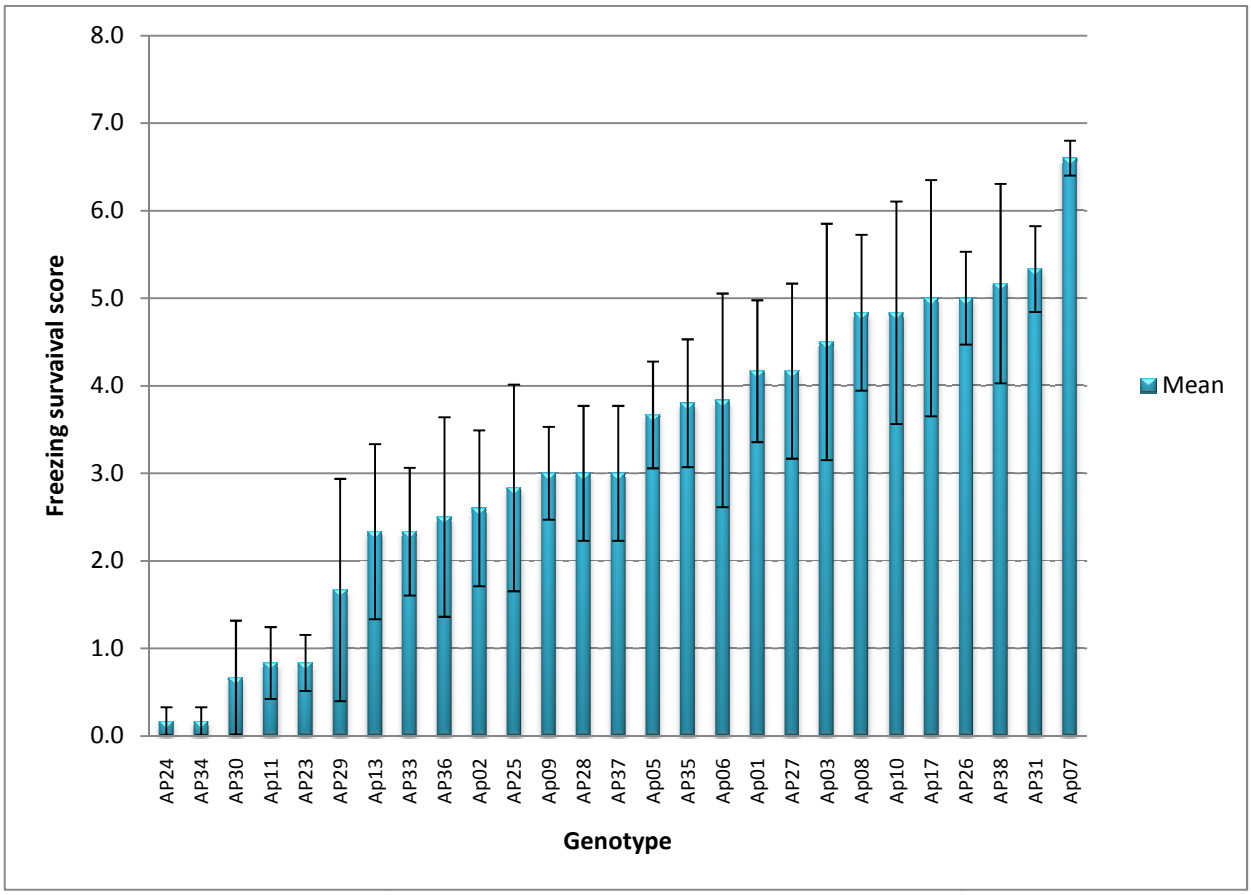
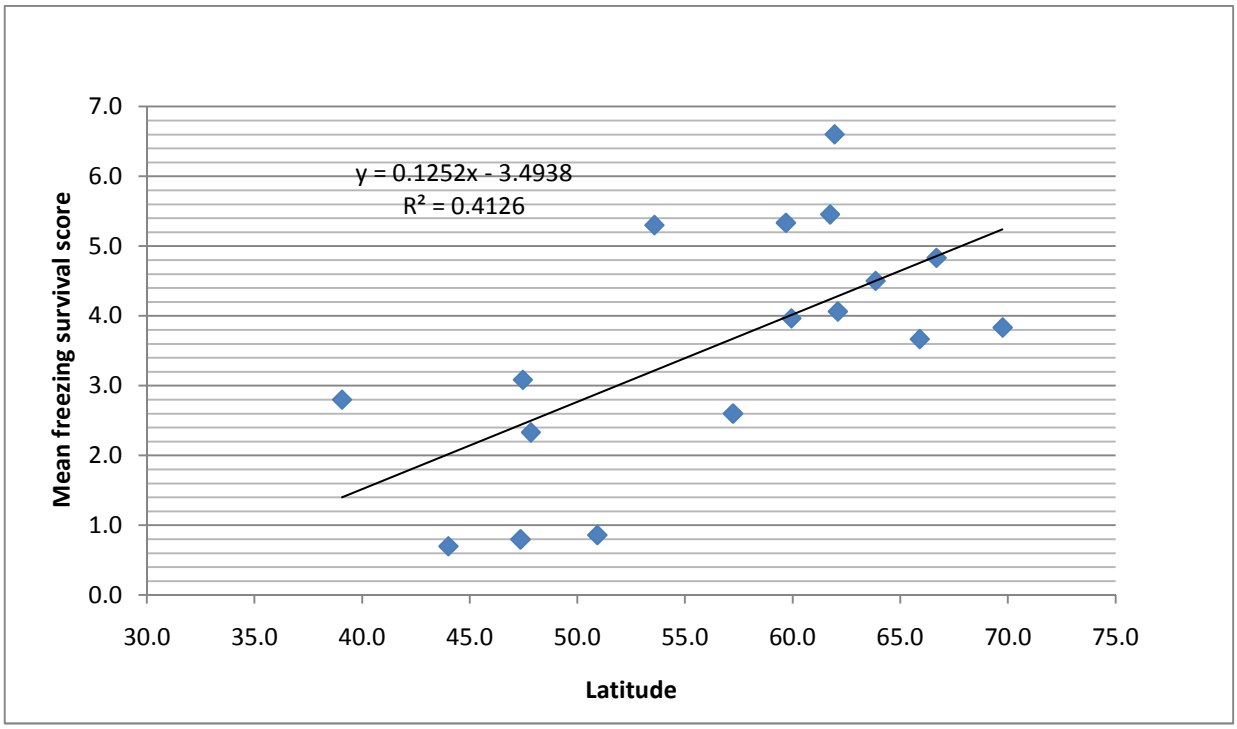


Fig. 9. Survival scores of clones of 30 accessions of *Phleum pratense* L. Ramets were grown for 4 weeks in the greenhouse at 19/16 °C day/night, 18 h photoperiod at a light intensity of 120 µmol m^{-2} s^{-1}, then cold acclimated at 1 °C for 2 weeks at 12/6 h day/night photoperiod, and frozen at -10 °C for 24 h. Average scores of survival (regrowth) were recorded 2 weeks after the ramets were transferred to normal growing conditions**.**

3.2.2 Relation between freezing tolerance and latitude of origin of genotypes

In our study we tried to find out whether the freezing tolerance of the genotypes are correlated with latitude of origin of the genotypes. The regression line in Fig. 10A (for genotypes of known location) indicates the latitude of origin and freezing survival scores of the genotypes show relatively good correlation. As might be expected, genotypes originating from higher latitudes have better freezing tolerance than genotypes from lower latitudes. However, latitude of origin only explains about 41% of the variation in freezing tolerance so some genotypes from higher latitudes does not have higher frost tolerance than genotypes from lower latitudes.



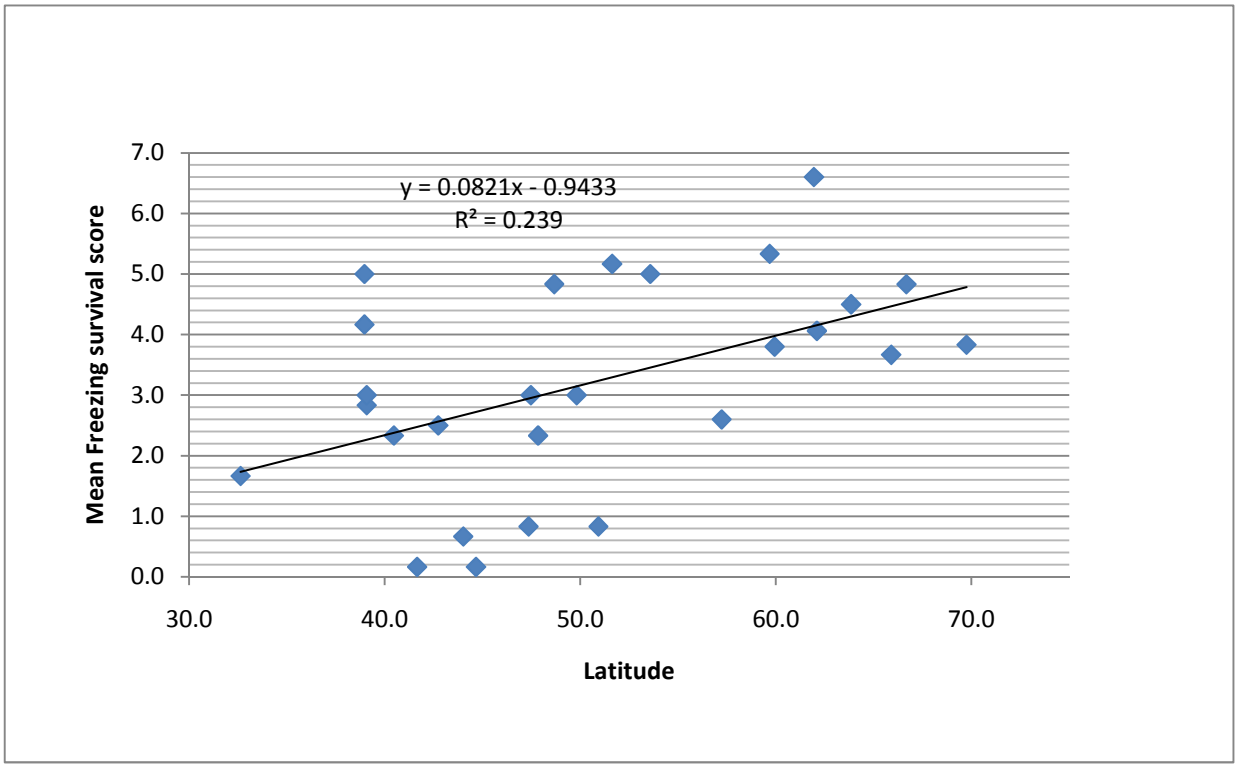


Fig. 10. Regression of mean freezing survival scores on latitude of origin for accessions of *Phleum pratense* L. Data are from cold acclimated plants assayed for freezing tolerance at –10°C and evaluated 2 weeks after regrowth. **(A)** top diagram includes accessions of known locations within country of origin (AP1, AP2, AP3, AP5, AP6, AP7 AP8, AP11, AP13, AP17, AP23, AP24, AP25, AP31, AP35, AP37 and AP38) and **(B)** bottom diagram represents all accessions used in the study including those accessions whose exact location within country is not known (Table 1).

3. 4 Gene expression profiles of target genes

3.4.1 *Phleum pratense* 6-SFT gene (Pp6-SFT)

In order to study freezing tolerance of timothy genotypes at the molecular level we investigated gene expression profiles of the two candidate genes *6-SFT* and *QM* in leaves at various time points after cold acclimation. As presented in (Fig. 11), the result showed that *6-SFT* gene transcript level is changed in response to low temperatures for all the four genotypes. Both up-regulation and down-regulation of the transcript was observed for the different genotype studied. The relative expression level of the *6-SFT* genes were 33.68 and 14.42 fold for genotype AP11 and AP7 respectively compared to non-acclimated controls. These results were attained at time course of 7 days after CA. Whereas the relative higher expression for genotype AP13 (13.42 fold) was attained at 4d after CA. For genotype AP7 and AP11, AP13, it seems overall the expression of *6-SFT* increase after CA to a top level around 7 days and then decrease again. An interesting observation is that it seems there is gradual increase in the ability of the plants to express transcript up on exposure to low temperature. Special for genotype AP31, we observed slight down-regulation of the expression of *6-SFT* genes in cold acclimated sample at all time point after CA compared to expression in NA sample (Fig. 11). The transcripts of this genotype are decreased right after cold acclimation has started. Expression study after 4hr of CA showed that there is 0.78-fold reduction in transcript level for this genotype. The reduction in the expression level remained between 1 and 0.4-fold throughout the whole study period of 14 days.

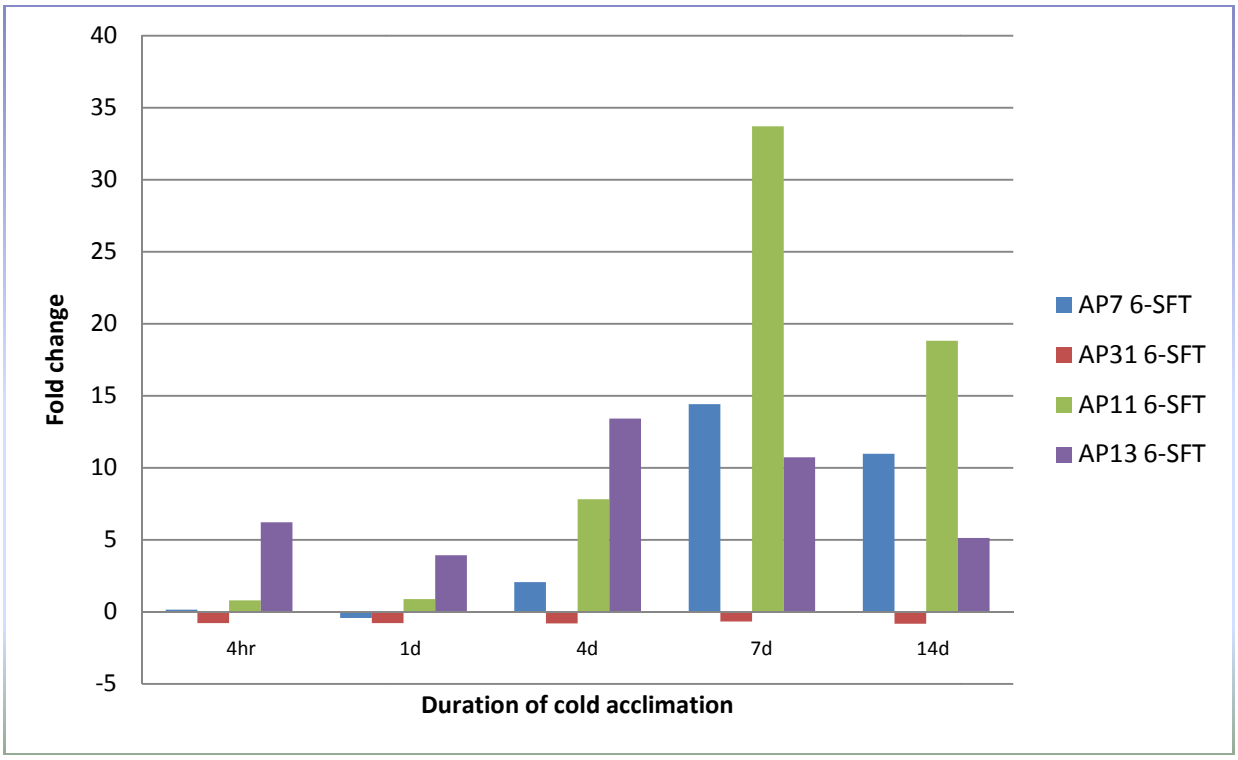


Fig.11. Expression of the *6-SFT* gene in leaves of four timothy (*Phleum pratense* L.) genotypes with contrasting freezing tolerance, i.e. high-frost tolerance genotypes (AP7 and AP31) and low-frost tolerance genotypes (AP11 and AP13). Real time RT-PCR analysis of mRNA isolated from leaf tissues of plants cold treated for 4 h, 1day, and 4, 7 and 14 days. The transcript levels were assessed relative to non-acclimated (NA) plants of the same genotype by the comparative CT method. The X-axis represents different durations of cold acclimation, and the Y-axis represents relative fold change in expression compared to non-acclimated (NA) controls of the respective genotypes.

3.4.2 *Phleum pratense* QM gene (PpQM)

The Pp*QM* gene expression is up-regulated during CA in the leaves of all of four genotypes, however, the fold changes vary between the different genotypes (Fig. 12). Except for genotype AP31 our results indicate that there is a uniform trend in the expression levels of the target gene, i.e. the expression increases until day seven of CA and decreases thereafter. The relative expression is highest at 7 days of CA for genotypes AP7, AP11 and AP13 with expression levels of 6.73, 3.16 and 2.67 fold changes, respectively. For genotype AP31 the expression was highest at the 4'th day of CA (2.16 fold change) and decreased thereafter. Compared to the rest of the genotypes, expression of the *QM* gene of HFT genotype AP7 was relatively low during the first four days of the CA period and peaked at day 7. This was in contrast to the HFT genotype AP31 for which the expression was lowest at day 7.

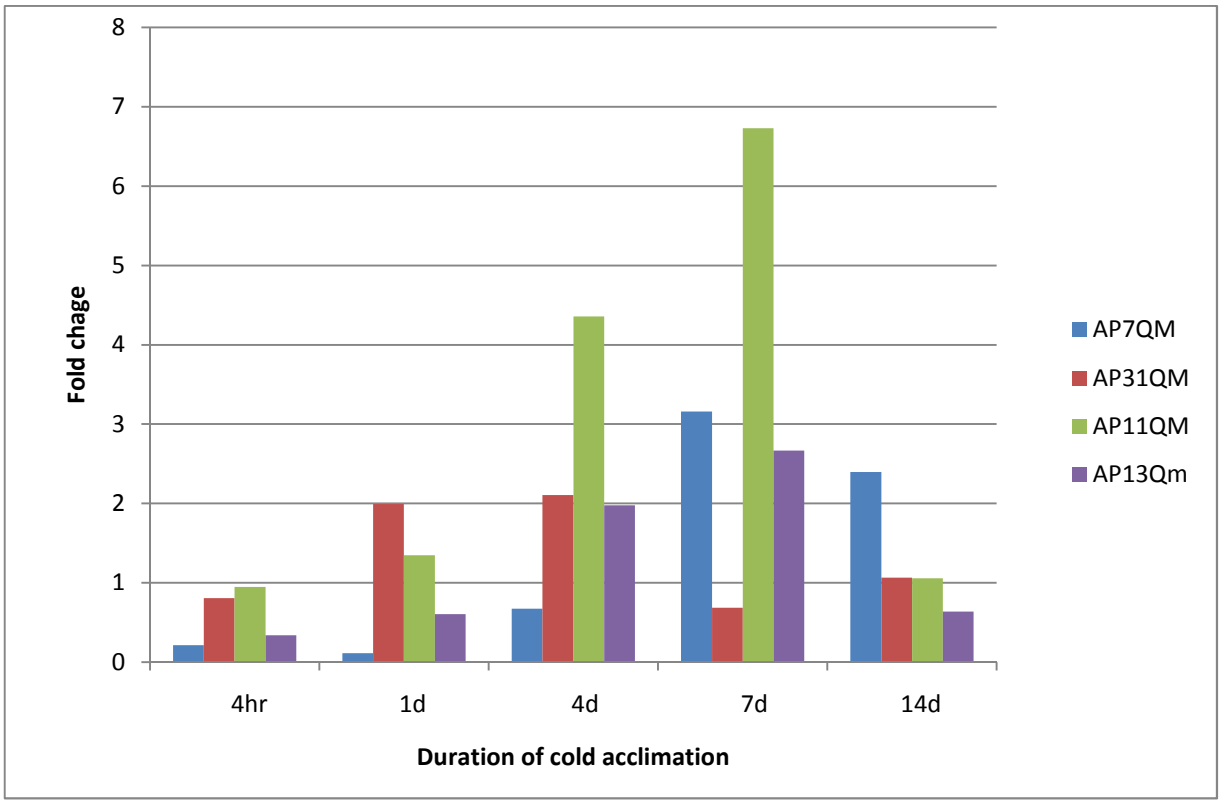


Fig. 12. Expression of the *PpQM* gene in leaves of four timothy (*Phleum pratense* L.) genotypes with contrasting freezing tolerance, i.e. high-frost tolerance genotypes (AP7 and AP31) and low-frost tolerance genotypes (AP11 and AP13). Real time RT-PCR analysis of mRNA isolated from leaf tissues of plants cold treated for 4 h, 1day, and 4, 7 and 14 days. The transcript levels were assessed relative to non-acclimated (NA) plants of the same genotype by the comparative CT method. The X-axis represents different durations cold acclimation, and the Y-axis represents relative fold change in expression compared to non-acclimated (NA) controls of the respective genotypes.

3.5 Sequencing of primer and probe binding sites in the target genes

In order to check for variations in the primer/probe binding sites in the target genes (*6-SFT* and *QM*) we sequenced the binding sites in the different genotypes. This was done in order to understand why the expression of the *6-SFT* gene was very low or slightly down-regulated in genotype AP31.

New primers were made outside the primer/probe binding sites and PCR was performed on cDNAs as templates (Fig. 13). The resulting PCR products of approximately 300 bp were sequenced. The PCR products of *6-SFT* did not give a good sequence for any of the genotypes and for AP31 no PCR product was obtained at all. For the *QM* gene we obtained good sequences from all genotypes.

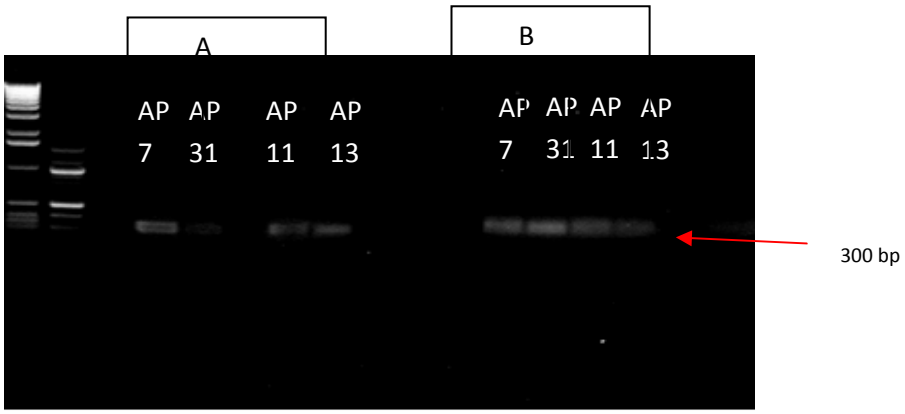


Fig. 13. PCR products from amplification of cDNAs of *Pp6-SFT* (A) and *PpQM* (B) of different timothy genotypes. 20 µl of PCR product was loaded in each lane.

We aligned the *QM* gene sequences from the different genotypes to check if there were any differences in the sequences and binding sites for primers and probes. Our results indicate that there was no variation among genotypes.

4. Discussion

4.1 Variation in freezing tolerance among accessions of timothy

Considerable variation in freezing tolerance was observed between the various accessions of timothy. It has been reported that freezing tolerance in plants varies across latitudes and climates (Xin and Browse, 2000). This variation in tolerance to freezing temperatures might be due to various physiological and biochemical changes that happen during cold acclimation that might be different in different genotypes. Such changes can have noticeable effects on freezing tolerance by enabling plants to withstand low temperatures several degrees lower than normal conditions during the growth season (Zhen and Ungerer, 2008). Even though the mechanism underlying freezing tolerance (FT) in non-acclimated plant is not well understood yet, frost tolerance of cold-acclimated plant involves a wide array of metabolic changes governed by extensive re-programming at the level of gene expression (Thomashow, 1999). Increased freezing tolerance of plants after cold acclimation is associated with many metabolic changes (Cook et al., 2004; Kaplan et al., 2004). According to Levitt (1980), plants respond to low temperature at the whole-organism level (reductions or delays in growth and reproduction) and determine the level of tolerance.

As it was observed from our data (Fig. 7) many plant wilted and died upon exposure to low freezing temperatures during the screening test. In this test, most of plants treated at temperatures below -10 ^{0}C died. A large variation in freezing tolerance between accessions of timothy was observed during the freezing experiment (Fig. 9). Genotypes AP7 (from Finland), AP31 (from Pavlovsk, USSR) and AP38 (from Siberia, Russia) were among the most freezing tolerant genotypes based on average regrowth score, whereas genotypes AP24 (from Paporovo, Bulgaria), AP34 (Stavropol, Russia), AP30 (from Serbia), AP23 (from West Sussex, England) and AP11 (from Csesznek, Hungary) had low freezing tolerance according to our results. These differences in FT are most likely reflecting the different areas of origin of the genotypes where they experience different selection pressures for freezing tolerance. According to Cenci (1980) and Rognli (1988), long-term natural selection under localized stressful conditions creates a

considerable amount of genetic variation among and within timothy cultivars. Schjelderup et al. (1994) found significant genetic variations for winter hardiness even within six timothy populations originating from the northern regions of Norway. Frost tolerance is a highly dynamic trait changing with fluctuations in the ambient temperature and other environmental stimuli (Eagles, 1994). According to Larsen (1994) freezing tolerance in cultivars of timothy varies across locations. He found similar frost tolerance in both cvs. Grindstad and Engmo grown under continental weather conditions where mid-winter temperatures normally are low and stable, while under coastal weather conditions, where temperatures are more variable, cv. Engmo had the highest level of frost tolerance.

Zhen and Ungerer (2008) also observed considerable FT variation among accessions of *Arabidopsis thaliana* and suggested that differences among accessions in their CA-capacity and in their intrinsic physiology contribute to variation in FT. Low temperature tolerance of genotypes of the same species of different origin is expected to vary. This is because they experience different forces of natural selection that might result in slight differences in their physiology, genetic makeup and capacity to cold acclimate. It is also clear that there might be differences in their levels of specific metabolites that protect cells against freezing damage. Since the timothy genotypes used in our study were originally from a wide range of geographic origin this could be one of the reasons for the observed variation in FT. It seems like there is a relationship between latitude of origin and freezing tolerance of the timothy accessions (Fig. 10). As the regression shows the freezing tolerance is increasing with increasing latitude of origin. A stronger relationship was observed between freezing tolerance and latitude of origin among accessions of known origin. There is a slight deviation from this conclusion for a few accessions (AP26 and AP27 from Turkey) since they showed good freezing tolerance being originally from relatively low latitudes (Fig. 10B). Freezing tolerance of genotypes from the Nordic countries generally showed good relationships with latitude and had better average survival scores than others. This result agrees with the observation in *Arabidopsis* demonstrating a positive relationship between latitude of origin and levels of freezing tolerance (Zhen and Ungerer, 2008). They concluded that natural selection has a role in shaping the variation in freezing tolerance of *Arabidopsis thaliana.* Differences in day length requirements may also affect growth and freezing tolerance among ecotypes of different latitudinal origin. According to Davik et al. (2006), the long days and cool nights at northern latitudes appear,

for example, to increase the production of aromatic compounds compared with the plants of the same species grown in the south. Tessadori et al. (2009) examined variation in chromatin compaction measured in leaf mesophyll cells of 21 diverse accessions of *Arabidopsis thaliana* of different geographical origins and from different habitats, and confirmed latitudinal genetic adaptation in plants. They obtain a positive correlation between the latitude of origin and chromatin compaction and conclude that the level of compaction appeared to be dependent on light intensity. During CA many plants accumulate different metabolites and the amount and type of metabolites could be influenced by the particular gene(s) expressed and the level(s) of gene expression. This will in turn be governed by the type of signal perceived by plant from its environment and the extent of the temperature stress.

4. 2 Characterization of gene expression of candidate genes

Changes in expression of cold induced genes during cold acclimation (CA) has been reported in many plant species (Thomashow, 1990; Guy et al., 1992). In the present investigation we studied relative accumulation of two candidate genes, *Pp6-SFT* and *PpQM* after different durations of CA of timothy genotypes compared to non-acclimated plants. The expression pattern of candidate genes in leaves showed that there are detectable differences between genotypes in gene transcript accumulation in response to low temperature treatment and exposure time.

4.2.1 Expression of the *PpQM* gene in response to CA

The expression of the *PpQM* gene was up-regulated after CA in leaves in all of the four timothy genotypes with slight variation in the relative level of expression (Fig. 11). Similar results were obtained by Rudi et al. (2011) showing that the *QM* gene in *F. pratensis* genotypes was upregulated following cold acclimation. In *Festuca pratensis* the relative expression of the *FpQM* gene gradually increased and was highest after 1 week in stems and attained peak levels in leaves after 2 weeks. This indicates that expression of a given gene vary in different parts of the plant. We also observed slight degree of variation among genotypes in the relative expression level but we did not study expression pattern across different part of the plant. Our results indicate that there is a general increase in the relative expression until day seven and then a decrease in expression in all four genotypes. We observed variation in

expression among genotypes at each time point after start of CA. Genotype AP11 had a much higher expression than the other genotypes at its peak point of expression.

This shows that each genotype might need different cold acclimation periods to attain a state of cold acclimation. Except for AP31 which showed peak expression at day 4 after start of CA, highest expression was obtained after 7 days of CA for the other genotypes (AP11, AP7 and AP13). Common to all the genotypes is the commencement of expression at 4 h but the levels of expression vary between genotypes. This result is comparable with other studies of the *QM* expression pattern. In *Caragana jubat*a expression was observed during the early period of CA and remained high to the last time-point studied (48 h) (Bhardwaj et al., 2010). Length of exposure to cold temperature has considerable effect on the amount of transcript accumulation.

4.2.2 Expression of the *Pp6-SFT* gene in response to CA

The current study showed that for genotypes AP7, AP11 and AP13 the *Pp6-SFT* gene showed an oscillating expression; it increased during the first 4 h of CA, was down-regulated after 1 day, then increased again until day 7, and decreased thereafter (Fig. 10). It seems that there is a gradual increase in *Pp6-SFT* expression on exposure to low temperature until day 7. There is also variation in transcript accumulation with time among the different genotypes which indicate differential response of genotypes to CA. We also observed variation in the amount of transcript at each time point for particular genotypes which might indicate that plant transcript accumulation increases starting from the initial exposure to low temperature to the time point when the genotype reached a cold acclimated state. This pattern was also seen for the *PpFT1* gene, a SFT timothy *6-SFT* homolog, which gradually increased up to 3 days of cold acclimation with concomitant increase in fructan accumulation after 7 days (Tamura et al., 2009). This indicates that this time point might represent a time of exposure required for accumulation of transcripts and metabolites required to maintain the cold acclimated state. This result also agrees with the *Bp6-SFT* expression pattern detected in *Bromus pictus*, for which the transcript accumulation increased gradually till day 7 and declined thereafter (del Viso et al., 2009). A similar time-course of accumulation was reported for wheatgrass (*A. cristatum*) (Wei et al., 2000). In big bluegrass (*Poa secunda*) on the other hand, *6-SFT* transcripts were observed as soon as 2 h after commencement of CA and reached a maximum after 2 days but high levels were found at day 7.

Similar to our result, the signal began to decline at day 8 and they observed small amounts of transcripts at day 15.

In contrast to the other three genotypes, the *6-SFT* transcript from genotype AP31 was down-regulated (or unaffected) at all time-points of CA. The decline was observed after 4 h of CA and continued at nearly the same relative expression throughout the CA period (14 days). This might be due to variation in the sequence of the of 6-SFT gene where we have designed our primers and probe for real-time PCR and therefore no binding has occurred. It could also be mutations in other parts of the gene i.e. in the promoter region that binds transcription factors and cis- or transacting proteins. We tried to investigate this by sequencing the part of the gene covering the primers and probe binding area using cDNA from the AP31 genotype; however, we did not obtain any good sequences from the *6-SFT* gene.

5. Candidate gene transcript accumulation and freezing tolerance among the different genotypes

In the current study we showed a direct correlation of high transcript accumulation during CA and high freezing tolerance phenotype of genotype AP7 from Finland. This was true for both candidate genes studied. Genotype AP13 (from Romania) showed relatively low freezing tolerance compared to AP7 and transcript levels of both genes were also lower than that of AP7 especially at day 7 when the expression was expected to be highest for HFT genotypes. Based on a comparison of these genotypes, it thus seems likely that expression of the two candidate genes is partly responsible for the difference in freezing tolerance. However, we observed high transcript accumulation of candidate genes and low freezing tolerance in the case of genotype AP11 from Hungary. Despite the fact that this genotype is among the LFT genotypes with very low survival, the transcript accumulation in response to CA was the highest of all genotypes particularly after 7 days. This is intriguing and indicates that there is genotype dependent genetic regulation of genes/alleles involved in freezing tolerance which might have been selected during adaptation of timothy genotypes in diverse climates. The low freezing tolerance of genotype AP11 might be caused by differential expression of other genes affecting freezing tolerance than the two candidate genes investigated herein.

In the case of AP31 (from Pavlovsk, USSR), which has the highest freezing tolerance of the genotypes studied, the *6-SFT* gene expression could unfortunately not be properly determined due to lack of proper primer/probe binding. The *QM* gene expression was also lower in this genotype compared to the other HFT genotype (AP7) especially at day 7 after start of CA. Rudi et. al. (2011) also reported that up-regulation of a *QM* homologue was associated with differences in frost tolerance in *F. pratensis* genotypes. From another study of the relationship between expression of *QM*, accumulation of proline and stress tolerance in yeast, it was revealed that the tomato *QM (LeQM*) gene was expressed in yeast and protected *Saccharomyces cerevisiae* cells against oxidative stress (Chen et al., 2006). *LeQM* gene expression enabled the yeast cells to produce higher amount of proline that lower intercellular ROS level. We did not study the accumulation of the *6-SFT* enzyme or fructan in connection with *6-SFT* transcript accumulation, however, in other cool season grasses it has been reported that the concomitant accumulation of *6-SFT* transcript, 6-SFT enzyme and fructans in various treated leaves suggest that *6-SFT* is a major enzyme responsible for fructan biosynthesis (Wei et al., 2002). They also highlighted that in most cases sucrose and *6-SFT* transcript accumulation occur simultaneously, which may indicate that *6-SFT* is also sucrose regulated. Several studies with transgenic plants involving fructan metabolism have confirmed that there is an association between fructan accumulation and stress tolerance (Li et al., 2007; Kawakami et al., 2008).

6. Conclusions

Current strategies for improving agricultural productivity mainly focus on improving crop adaptation to conditions such as low temperature and drought. In order to do this the natural variation that exists in natural population needs to be exploited to improve agriculturally important traits. In the present study we found significant variation among some timothy genotypes in freezing tolerance. The variation in freezing tolerance that we have observed can contribute to further improvement of freezing tolerance through breeding. Our study revealed that expression of two candidate genes, the *6SFT* and *QM* genes, increase during cold acclimation and are among some candidate genes that may explain why timothy is most widely cultivated in the northern part of Europe where there is extreme cold temperatures. We also demonstrated that there is variability both between genes and genotypes in expression levels,

and also the timing of gene expression, which is not related in a simple fashion to freezing tolerance. However, the work we have conducted may contribute to the understanding of freezing tolerance mechanism, particularly in grasses, and improve crop performance in cold climate regions of the globe where distribution of species is limited due to low temperature conditions.

Future work

The present results does not permit us to conclude about the variation in freezing tolerance between genotypes based on variation in transcript accumulation of just two candidate genes. As regards the *6-SFT* gene, the relation between *6-SFT* transcript levels, *6-SFT* enzyme and fructan accumulation should be studied at the same time. This would generate more useful data which can be used to compare and explain the probable reasons for the observed differences between genotypes in their freezing tolerance. Also expression of many more candidates genes should be followed in a number of genotypes with diverse freezing tolerances.

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Appendixes

Materials used in the study

Chemicals	producer
1-kb ladder	
Agarose Gel	Invitrogen
B-merchapto ethanol	Sigma
dNTPS	
EDTA	Sigma
Ethidium bromide	sigma
Ethanol	Kemetyl
Liquid Nitrogen	
NaAc	
Solutions	
10 x buffers (PCR buffer)	**New** England BioLabs $_{inc}$
RNA Extraction Buffers	Qiagen
Buffer RLT	”
Buffer RPA	”
Buffer RW1	”
cDNA purification Buffers	Qiagen
Buffer PE	”
Buffer EB	”
Buffer PN	”
Buffer EB	”
1X TAE buffer	
DEPC treated water	Invitrogen
dH_2O	
MiliQ water	Milipore
RNA free water	Qiagen
Enzymes	
RNase Away	Qiagen

Taq DNA polymerase	NEW ENGLAND BioLabs inc
DNase I	Qiagen
Dyes	
Rox reference dyes	Invitrogen
Kits	
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
EXPRESS two-step qRT-PCR universal kit	Invitrogen
RNA plant Mini Kit	Qiagen
RNeasy Min Elute kit	Qiagen
RNA 6000 Nano reagent and supplies	Agilent Technology
QIAquick PCR Purification Kit	Qiagen
Equipments	
7500 Fast Real-Time pcr instrument	Applied Biosystems
ABI PRISM 3100 DNA Sequencer	Perkin Elmer Biosystems
Agarose gel electrophoresis equipment	Pharmacia
Agilent 2100 Bioanalyzer	Agilent technologies
Collection tubes	Qiagen
Eppendorf tubes	Qiagen
Fast Optical 96 well plate with barcode(0.1 ml)	Applied Biosystems
Freezer (-20)	BOSCH
Freezer (-80)	SANYO
Freezer	
Gel-DOC 2000	BIO-RAD
Laminar flow cabinet	
Master Cycler (ep gradient)	Eppendof
Microcentrifuge	Qiagen
Microwave	
Micromax Centrifuge	IEC
Minispine	Eppendorf
NanoDrop® ND 1000	NanoDrop® Technologies
QIAshredder	Qiagen
Heater	

Rotar mixer	HATI
RNeasy spin column	Qiagen
RNeasy MinElute spin column	Qiagen
Refrigerator (-4)	Bosh
PCR tubes	ISO/AXYGEN
Thermal cycler	Applied Biosystems
Software	
BioEdit sequence alignment editor	Share Ware NCSU
BLASTn	NCBI(www.ncbi.nlm.nib.gov)
Excel	Microsoft
Minitab	
Mega	NCBI(www.ncbi.nlm.nib.gov)
Sequencher 4.10	Gene Codes Corporation
Primer express software	Applied Biosystems

Appendix 1: Isolation of Total RNA from Plant Cells

Procedure:

1. Add 10 µl β-ME per 1 ml Buffer RLT to be used
2. Determine the amount of plant material. Do not use more than 100 mg.
   Immediately place the weighed tissue in liquid nitrogen, and grind thoroughly with mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.
3. Add 450 µl Buffer RLT to a maximum of 100 mg tissue powder. Vortex vigorously.
4. Incubate for 1–3 min at 56°C to disrupt the tissue.
5. Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Carefully transfer the supernatant of the flow-through to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. Use only this supernatant in subsequent steps.
6. Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.

7. Transfer the sample (usually 650 µl), including any precipitate that may have formed, to an RNeasy spin column (pink) placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at 10,000 rpm. Discard the flow-through.

Optional On-Column DNase Digestion with the RNase-Free DNase Set

Do the following steps to eliminate genomic DNA:

a. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 10,000 rpm to wash the spin column membrane. Discard the flow-through.

b. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.

c. Add 80 µl of DNase I incubation mix directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min.

d. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 10,000 rpm. Discard the flow-through

8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 10,000 rpm to wash the spin column membrane. Discard the flow-through.
9. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 10,000 rpm to wash the spin column membrane.
10. Carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
11. Place the RNeasy spin column in a new 2 ml collection tube and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.
12. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 10,000 rpm to elute the RNA.
13. Repeat step 11 using another 50 µl RNasefree water.

Appendix 2: RNA cleanup protocol

Procedure

1. Adjust the sample to a volume of 100 µl with RNase-free water. Add 350 µl Buffer RLT, and mix well.
2. Add 250 µl of 96–100% ethanol to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to the next step.
3. Transfer the sample (700 µl) to an RNeasy MinElute spin column placed in a 2 collection tube. Close the lid gently, and centrifuge for 15 s at 10,000 rpm. Discard the flow-through. For samples > 700 µl, transfer the remaining sample and repeat the centrifugation. Discard the flow-through.
4. Place the RNeasy MinElute spin column in a new 2 ml collection tube. Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at 10,000 rpm to wash the spin column membrane. Discard the flow-through.
5. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at 10,000 rpm to wash the spin column membrane. Discard the flow-through and collection tube.
6. Place the RNeasy MinElute spin column in a new 2 ml collection tube.
7. Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.
8. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube. Add 14 µl of RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

Appendix 3: RNA integrity checks using Agilent RNA 6000 Nano Assay

Procedure:

A. Loading the Gel-Dye Mix

1. Allow the gel-dye mix to equilibrate to room temperature for 30 min before use and protect the gel-dye mix from light during this time.

2. Take a new RNA Nano chip out of its sealed bag.
3. Place the chip on the chip priming station.
4. Pipette 9.0 µl of the gel-dye mix at the bottom o the well marked G and dispenses the gel-dye mix.
5. Set the timer to 30 seconds, make sure that the plunger is positioned at 1 ml and then close the chip priming station. The lock of the latch will click when the Priming Station is closed correctly.
6. Press the plunger of the syringe down until it is held by the clip.
7. Wait for exactly 30 seconds and then release the plunger with the clip release mechanism.
8. Visually inspect that the plunger moves back at least to the 0.3 ml mark.
9. Wait for 5 seconds, and then slowly pull back the plunger to the 1 ml position.
10. Open the chip priming station.
11. Pipette 9.0 µl of the gel-dye mix in each of the wells marked G

B. Loading the RNA 6000 Nano Marker

1. Pipette 5 µl of the RNA 6000 Nano marker (green ?) into the Well marked with the ladder symbol and each of the 12 sample well.

C. Loading the Ladder and Samples

1. Before use, thaw ladder aliquots and keep them on ice (avoid extensive warming upon thawing process)
2. To minimize secondary structure, heat denature (70 °C, 2 minutes) the samples before loading on the chip.
3. Pipette 1 µl of the RNA ladder into the well marked with the ladder symbol.
4. Pipette 1 µl of each sample into each of the 12 sample wells.
5. Set the timer to 60 seconds.
6. Place the chip horizontally in the adapter of the IKA vortex mixer and make sure not to damage the buldge that fixes the chip during vortexing.

7. Vortex for 60 seconds at 2400 rpm.

8. Insert the chip in the Agilent 2100 bioanalyzer. Make sure that the run is started within 5 minutes.

Appendix 4:

Table A1. Primer and probe sequence used for qPCR

Name	Sequence 5`-3	Tm	Length	% GC
Pp_6SFT_F	CGGGCCGGGTTCCAT	60	15	73
Pp_6SFT_R	GACCGTTGGGATCGCTCAT	59	19	58
Pp_6SFT-Probe	CAGACGGAGAAGAAC	70	15	53
Pp_GADPH_F	CATCTCGCGTCGTCACAGTT	58	20	55
Pp_GADPH_R	CCGTTGATTCCGATCTTAATCTTG	60	24	42
Pp_GADPH_Probe	CACTCGCGCCATGG	68	14	71
Pp_QM_F	CGAGGTGCTATCGCCAGA TC	59	20	60
Pp_QM_R	CCACGGCAGTACCTGGACTT	59	20	60
Pp_QM-Probe	AGAACAAGCCATACCC	70	16	50

Appendix 5:

a. Sequence used to search QM and GAPDH gene homologs in timothy 454 sequences to design primer for real time PCR

GenBank	Sequence, Festuca pratensis GAPDH (GAPDH) gene, partial cds
DQ991044.1	**Sequence** ATCATTCCCAGCAGCACTGGAGCTGCCAAGGTTAGTATAATTCAGCAGCACCGAAGTGTGTACAGTGTAA ATATGGCATCTGTGTCTATCTGACAGAATTGTATGCTTATGCATTGTGGTTCTCACTGATGTGTTATCCT TTATATTGATCTTGTTCTGACTTGTTAATCTTTCAACAGGCTGTTGGCAAGGTGCTCCCAGTCCTTAACG GAAAGTTGACAGGAATGGCCTTCCGTGTCCCAACTGTTGACGTTTCTGTTGTTGATCTGACCGTTAGACT TGAGAAGGCTGCCACCTATGACCAGATCAAGGCTGCGATCAAGTAAGTAATGCTACCATATAGGTGGTTT GTATATGGTTCTGTATATTGTTGCAGGCTTTAGTGTGTTTGCATAACATAGATGGTTTGCTTTGGATGAT TACTGATCTGCAACATCCTTTCAGGGAGGAGTCTGAGGGTAAGCTCAAGGGCATTTTGGGTTACGTCGAT GAGGACCTTGTTTCCACCGACTTCCAGGGTGACAGCAGGTATTTGTCGTTGCTGCTTTCACTGTGCAACA TAATGATTATCTGAATCATCTTAGTTAGCACATACAGACCTGCTTACAAGGCATCTGAATTCACCATTAA
FJ824852.1	>gi|254212160|gb|FJ824852.1| Nicotiana benthamiana QM mRNA, complete cds
	Sequence TGTTCCTCAAGCCGCAGACCAATCGCAGAGAGAATTAGCCATGGGGAGAAGACCTGCAAGATGTTATCGC CAGATTAAGAACAAACCTTATCCAAAATCACGGTTTTGCCGTGGTGTCCCAGATCCAAAGATCAGGATCT ATGATGTGGGTATGAAGAAAAAGGGAGTTGATGAATTTCCTTTCTGTGTGCACTTGGTCAGTTGGGAGAA GGAGAATGTTTCAAGTGAGGCACTTGAAGCTGCTCGTATTGCGTGCAACAAGTACATGACCAAGTCCGCT GGAAAGGATGCTTTCCACCTCAGGGTTAGGGTACATCCCTTCCATGTTTTGCGAATTAACAAGATGTTGT CATGTGCTGGGGCTGATCGGCTCCAAACTGGTATGAGGGGAGCTTTTGGTAAGCCACAGGGAGTCTGTGC TCGTGTTGCTATTGGTCAGGTTCTTCTCTCTGTTCGCTGCAAAGATGGTAATGCTAACCATGCTCAAGAG GCACTGCGCCGTGCAAAGTTCAAGTTCCCTGGTCGACAAAAGATTATTGTCAGCAGGAAGTGGGGGTTCA CTAAGTTCAGCCGTACTGATTATCTGAAATACAAGTCAGAGAATCGTATTGTTCCAGATGGTGTCAATGC CAAGCTTCTCGGTTGTCATGGCCGACTTGCTGCACGTCAACCTGGAAGAGCTTTTTTGGAAGCAGTGGGG AATTGAAGTTGCGAACTTACCAAACTGAACCTTTAGTTGTCTACTTCTGTTGAATGAAATTTTGTGTCCT GATCATCGATCTACTTTTGAACTATTACTTGAGTTACTGCTTTGAACATTTTAGTTTACTGGTAAAGATT GGCTTGTTTTAGTTGGTTGAAAAAAAAAAAAA

b. BLASTIN result; selected sequence (contigs) with good e-value

Gene	Sequences producing significant alignments:	Score (Bits)	Length	E Value
GAPDH	Consensus from Contig5582 Average coverage: 7, 72	105		7e-023
QM	Consensus from Contig862 Average coverage: 5,51	545	872	3e-155

c. Sequences against which primers were designed

>gi 862 From 454 Sequencing	A. Timothy 862 QM gene sequence for designing the primer pair and probe for PpQM
	Sequence GACCTGCGAGGTGCTATCGCCAGATCAAGAACAAGCCATACCCCAAGTCCAGGTACTGCCGTGGTGT CCCTGATCCCAAGATCAGGATCTACGATGTCGGCATGAAGAAGAAGGGTGTGGATGAGTT CTCTCACTGTGTTCACCTTGTCTCATGGGAGAAGGAGAACGTCTCCAGTGAGGCTCTTGAG GCTGCCCGTATTGCTTGCAACAAGTACATGACCAAGCACGCAGGAAAGGATGCCTTCCACC TCAGGGTCCGGGTTCACCCATTCCATGTGCTGCGCATCAACAAGATGCTTTCGTGTGCTGG GGCTGATAGGC
AB436697.1 From NCBI	B. | Phleum pratense 6-SFT mRNA sequence, used to design primer pair and probe for Pp 6-SFT
	Sequence CACCCATCCAGCCGAGTCTACTAAAAGAAATTCCTTGGAACTCGGCTGCGAGTAGGGAATTTCGGCA ATGGCGCCGCCCCAGGCCATTGCTAACGGCGCGCCGGCACCGCTGCCGTACGCGTACGCACGGCTG CCCTCCTCCGGTGACGAGAAGCAGGACCAGAGCAAGAGCGGCGGCGCGAGGTACTGCCGTGCGTG CGTCGCCGGGGTGGCGGCGCTCCTCATCGTGGCCGGCGCGCTCGCCGGAGCCAGGGTGGGTCTGGG GGGCATCTACGACGACGCGGACGCCTTCGCGTGGAACAACTCCATGCTGCAGTGGCAGCGGGCCGG GTTCCATTTCCAGACGGAGAAGAACTTCATGAGCGATCCCAACGGTCCGGTGTACTATCGTGGATATT ACCACCTCTTCTACCAGTACAACATGAAGGGCGTCGTGTGGGACGACGGTATTGTGTGGGGCCACGT TGTGTCCCGGGACCTAGTCCATTGGCGCCACCTCCCGATTGCCATGGTGCCCGACCACTGGTATGACA GCATGGGCGTCTTATCGGGCTCCATCACCGTGCTACAAAACGGTTCGCTCGTCATGATCTACACCGGG GTCTTCTCCAAGACCACCGACAGGAGTGGCATGATGGAGGTCCAGTGCCTCGCTGTCCCCGCCGACC CCAACGATCCCCTCCTCCGTAGCTGGACCAAGCACCCCGCCAACCCTGTCCTTGTTCATCCGCCGGGG ATCAAGGACATGGACTTCAGAGACCCTACCACTGCCTGGTTCGACGAGTCCGACTCCACGTACCGCA CTGTCATTGGTACCAAGGATGACCATCACGGCAGCCATGCCGGCTTTGCCATGGTGTATAAGACCAA GGATTTCCTTAGCTTCCAGCGCATCCCGGGCATCTTGCACAGCGTCGAGCATACCGGCATGTGGGAG TGCATGGACTTCTACCCCGTTGGCGGGGGAGACAACTCATCATCGGAGGTGTTGTATGTGATAAAGG CGAGCATGGACGATGAACGGCATGACTACTATGCGTTAGGGATGTACGATGCAGCTGCAAACACGT GGACACCATTGGACCAGGAGCTGGACTTGGGGATCGGGCTGAGGTACGACTGGGGCAAGCTCTATG CGTCCACGACGTTCTATGATCCAGCAAAGCGGCGGCGCGTGATGCTGGGGTACGTCGGGGAGACCG ACTCTAGGCGGTCCGACGAAGCCAAGGGATGGGCCTCAATCCAGTCGATTCCAAGGACAGTGGCAC TGGACGAGAAGACCCGGACGAACCTCCTCCTATGGCCGGTGGAGGAGATCGAGACCCTCCGCCTCA ACGCCACCGAGTTCAATGACATCAACATCGACACCGGCTCCGTCTTCCACCTCCCCATCCGCCAAGGC AATCAGCTCGACATCGAGGCCTCCTTCCGCCTCGACGCTTCCGCCGTGGCCGCCATCAACGAGGCCG ACGTCGGCTACAACTGCAGCAGCAGCGGCGGCGCTGCCACCCGTGGCGCGCTCGGCCCCTTCGGCCT GCTTGTCCTTGCCGCCGAGGGCATTGGCGAGCAGACGGCGGTGTACTTCTACGTGTCTAGGGGCCTT GATGGGGGCCTCCGGACCAGCTTCTGCAACGATGAGTTGCGGTCATCGTGGGCCAGGGACGTGACG

	AAGCGGGTGGTCGGCAGCACGGTGCCTGTGCTCAACGGCGAGACATTGTCGATGAGGGTGCTCGTG GACCACTCCATCGTGCAGAGCTTCGCGATGGGCGGGAGGGTCACAGCGACGTCGCGGGTGTACCCG ACGGAGGCTATCTATGCGGCGGCTGGGGTGTACCTGTTCAACAACGCAACCAACGCCAGCGTGACG GCCGAGAGGATCATCGTGCACGAGATGGACTCTATCGACAACAACCAAATCTTCTTGATCGACGATTT GTAGCTTAAGTGAGCTATATTGGCATGGTAATGAGTGTGTGGTCATCTACATAAGTTTTATCTAGCGT TCGCTCTGTTGGCGGCTTACTCTCTATCTTGGGTGCAGATGGAGATGGAGTAATAGCTACTATATATG TATGCTTAATTATTGTCTTCTCTTTGTTTGTATGTCGAGTTTTCCTCTTCGCGAGGCGGTCAAGGGTTT GCAAATAAAGAAATCCATATTTGTTGTTAAAAAAAAAAAAAAAAA
>gi 5582 From 454 Sequencing	C. Housekeeping gene; *Pp-GADPH-5582*, used to design primer pair and probe for Pp-GAPDH
	Sequence GGGGAACCGCTTCCAGACACTTCTTCCAATCCCATCCCGTCCCCCAGCGAACCTCCCGTCTCCATCCAT CTCGCGTCGTCACAGTTCCACTCGCGCCATGGGCAAGATTAAGATCGGAATCAACGGTTTCGGAAGG ATCGGGAGGCTCGTCGCCAGGGTCGCCCTCCAGAGCGACGATGTCGAGCTCGTCGCCGTCAACGAC CCCTTCATCACCACCGAGTACATGACCTACATGTTCAAGTATGACTCCGTGCACGGCCACTGGAAGCA CAGTGACATCAAGCTCAAGGACAACAAGACTCTCCTCTTTGGCGAGAAGGCCGTCACTGTCTTCGGC GTCAGGAACCCTGAGGAAATCCCATGGGCTGAGGCCGGCGCCGACTATGTTGTGGAGTCCACTGGT GTCTTCACTGACAAGGACAAGGCTGCTGCTCACTTGAAGGGTGGTGCCAAGAAGGTGGTCATCTCAG CCCCCAGCAAAGATGCCCCTATGTTTGTTGTTGGTGTTAATGAGGACAAGTACACCTCCGACGTTGAC ATTGTCTCAAACGCTAGCTGCACCACCAACTGTCTTGCTCCCCTAGCCAAGATCATTAATGACAACTTT GGTATTGTTGAGGGTCTGATGACCACTGTTCATTCCATCACTGCCACCCAGAAGACCGTCGACGGTCC CTCGAGCAAGGACTGGAGAGGTGGGAGGGCTGCCAGCTTCAACATCATTCCAAGCAGCACTGGCGC TGCCAAGGCTGTTGGTAAGGTTCTTCCTGAGTTGAACGGCAAGCTTACCGGTATGTCATTCCGGGTTC CCACTGTGGATGTGTCAGTTGTTGATCTCACTGTTAGAATCGAGAAGGCAACTCATGTCCAACTAAGC ATGAACTCGGCGTATGACGACGCCATCCACATTTATTCACTGCATAGACAAAGAAGGCTCGTCTCCGA CTCGCCCAGAAGAAAGCGAAACACTCTACTGAGTCTTGGCCATGTGGCGGATCAGGTCAACAACACG GTTGCTGTAACCCCACTCGTTGTCGTACCACGAGACGAGCTTCACGAAGTTGTCGTTAAGAGCAATTC CAGCCTTGGCGTCGAAGATGCTCGACCTGCTGTCACCAATGAAGTCAGTCGAGACCAAATCTTCCTCA ACGTAACCCATGATTCCCTTGAGGTTTCCCTCAGATGCAGCCTTGATAGCCTTCTTGATGTCCTCATAT GATGCAGCCTTCTCAATTCTAACAGTGAGATCAACAACTGACACATCCACAGTGGGAACCCGGAATG ACATACCGGTAAGCTTGCCGTTCAACTCAGGAAGAACCTTACCAACAGCCTTGGCAGCGCCAGTGCT GCTTGGAATGATGTTGAAGCTGGCAGCCCTCCCACCTCTCCAGTCCTTGCTCGAGGGACCGTCGACG GTCTTCTGGGTGGCAGTGATGGAATGAACAGTGGTCATCAGACCCTCAACAATACCAAAGTTGTCAT TAATGATCTTGGCTAGGGGAGCAAGACAGTTGGTGGTGCAGCTAGCGTTTGAGACAATGTCAACGTC GGAGGTGTACTTGTCCTCATTAACACCAACAACAAACATAGGGGCATCTTTGCTGGGGGCTGAGATG ACCACCTTCTTGGCACCACCCTTCAAGTGAGCAGCAGCCTTGTCCTTGTCAGTGAAGACACCAGTGGA CTCCACAACATAGTCGGCACCAGCCTCAGCCCATGGGATTTCCTCAGGGTTCCTGACGCCGAAGACA GTGACGGCCTTCTCGCCAAAGAGGAGAGTCTTGTTGTCCTTGAGCTTGATGTCACTGTGCTTCCAGTG GCCGTGCACGGAGTCATACTTGAACATGTAGGTCATGTACTCGGTGGTGATGAAGGGGTCGTTGAC GGCGACGAGCTCAACATCGTCGCTCTGGAGGGCGACCCTGGCGACGAGCCTCCCGATCCTTCCGAAA CCGTTGATTCCGATCTTAATCTTGCCCATGGCGCGAGTGGAACTGTGACGACGCGAGAT

Appendix 6: 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 µl of 5X VILO™ Reaction Mix
    - 2 µl of 10X Super ScriptR Enzyme Mix
    - 2.5 µg of total RNA (template)
    - 20 µl 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 7: qPCR

Procedure

1. Set up the reaction on ice
2. Prepare master mix containing the components (table 1. below without template)
3. Add 18 µl of master mix in to 96 well-PCR plate and add 2 µl 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.

Table A2. Reaction mix protocol for the validation experiment and real time qPCR experiment

Concentration	Master mix components	Amount per reaction (µl)
	Express qPCR super mix Universal	10.00
10 µM (final 200nM)	probe	0.40
10 µM (final 500nM)	primer -F	1.00
10 µM (final 500nM)	primer -R	1.00
	Rox reference dye (25µM)	0.04
	DEPC-treated water	5.56
	Total mix	18.00
	+ template cDNA	2.00
	Total rxn volume	20.00

Appendix 8: Real-time PCR amplification efficiencies and linearity

Real-time PCR efficiencies for target genes were calculated from log concentrations and CT value data obtained from a light cycler PCR machine. We calculated the corresponding real-time PCR efficiency (*E*) of the targets according to the equation: $E = 10^{[-1/\text{slope}]}-1$. Our results showed that the real-time PCR efficiency values obtained is inside the acceptable range for a good reaction, which is 100% $\pm$ 10. The slope for a 10-fold dilution series of template cDNA for the target 6SFT gene is -3.60 with efficiency (%) of 89.57, -3.51 for the QM gene with E = 92.7 and with slope = -3.46, and E = 94.5 for the house keeping gene (Fig. A1).

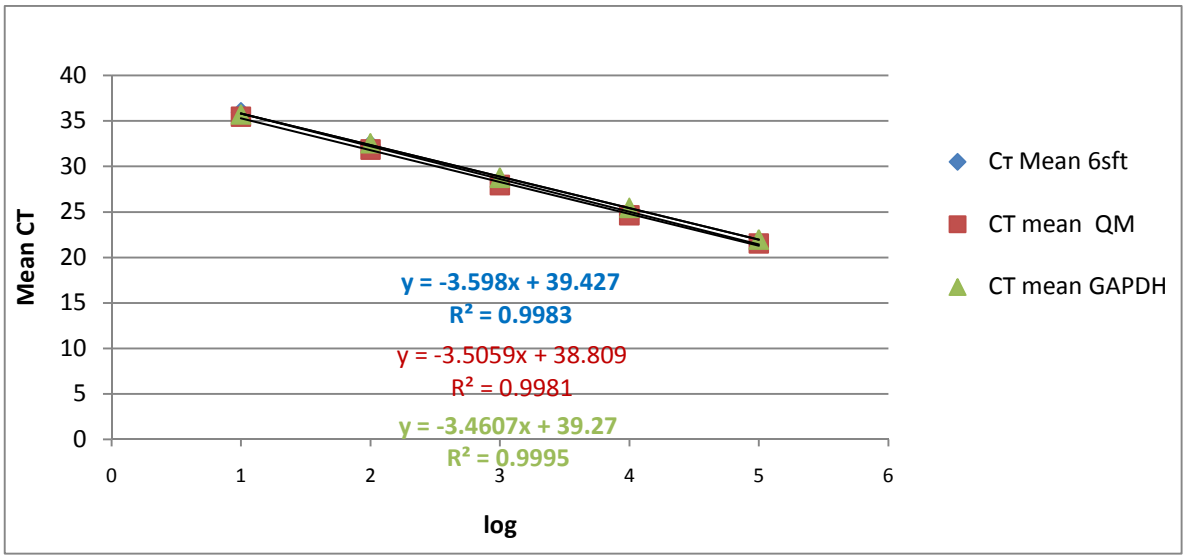


Fig. A1. A plot of CT versus log cDNA concentrations constructed to validate the $2^{-\Delta\Delta CT}$ method. 5-log dilution range of 10 fold serial dilutions of target PCR products, each subjected to real time PCR amplification. Efficiency values were measured using the CT slope method, effiencies calculated from slope of standard curve ($E=10^{(-1/slope)} - 1$).

Appendix: 9 QIAquick PCR Purification Kit Protocol using a micro centrifuge

Procedure

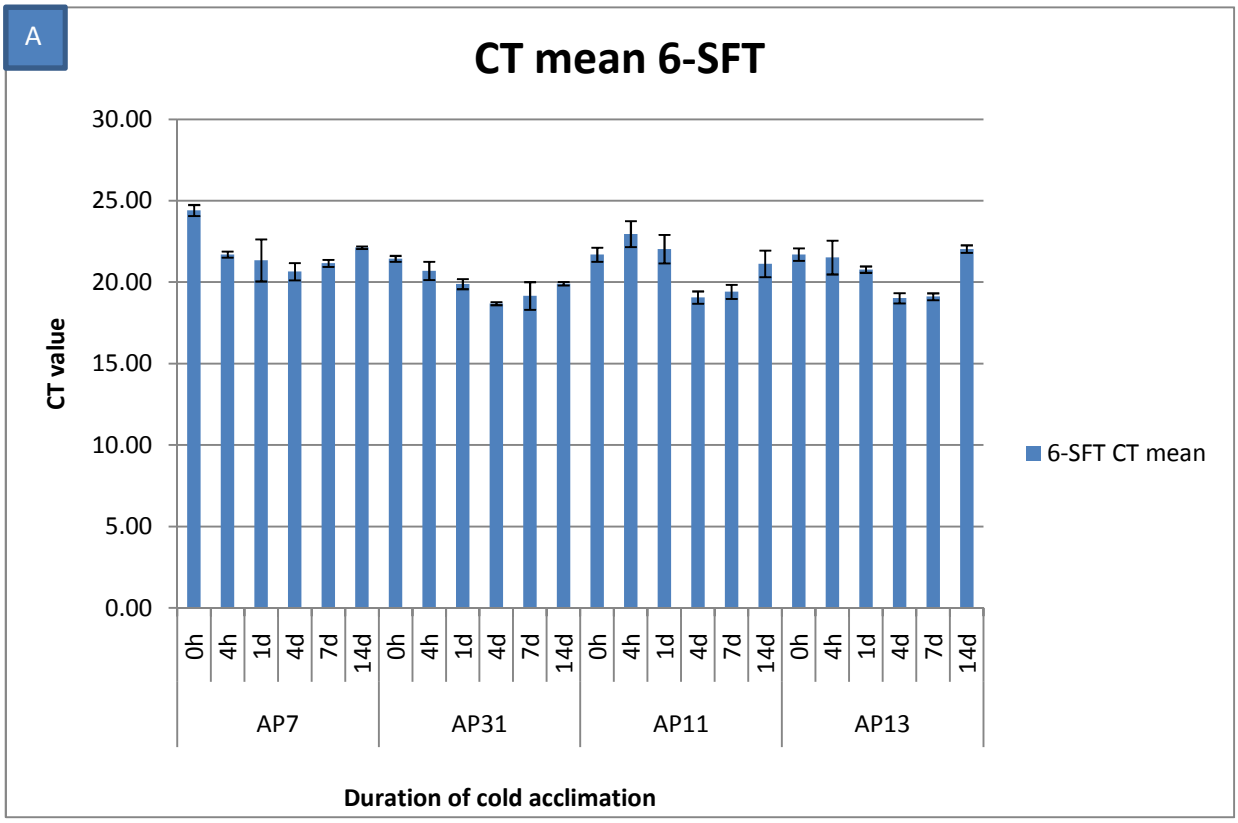
1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix.
2. Place a QIAquick spin column in a 2 ml collection tube.
3. Apply the sample to the QIAquick column (to bind DNA) and centrifuge for 60s.
4. Discard flow-through. Place the QIAquick column back into the same tube.
5. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 60s
6. Discard flow-through and place the QIAquick column back in the same tube and centrifuge the column for an additional 1 min.

   Important: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation
7. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
8. Add 30 µl Buffer EB to the center of the QIAquick membrane and centrifuge the column for 1 min (to elute DNA).

Appendix 10:
Table A3. Sequencing primers

Name	Sequence 5`-3	Tm	Length	% GC
Pp_6SFT_ LEFT	GACGAGAAGCAGGACCAGAG	60.14	20	60
Pp_6SFT_ RIGHT	CCCATGCTGTCATACCAGTG	59.98	20	55
Pp_QM_ LEFT	CTGCGAGGTGCTATCGCC	63.06	18	66.67
Pp_QM_ RIGHT	ATTCCGGTCTGGAGCCTATC	60.43	20	55

Appendix 10: Summary of CT data of the target genes (6-SFT and QM)



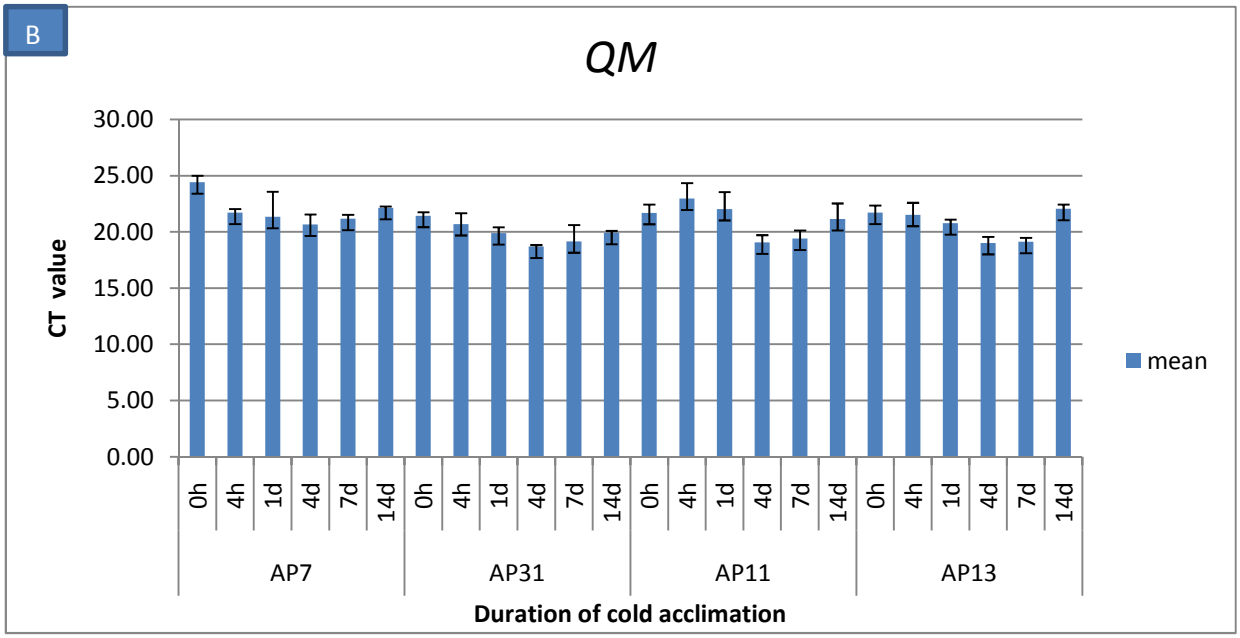


Fig. A2. A and B represent CT means of the 6-SFT and QM genes from which the expression levels were calculated. The variation and standared between CT data was checked before the fold change was calculated. Y-axis represents the average CT values, and the x-axis the time points and genotypes studied.

Appendix 11

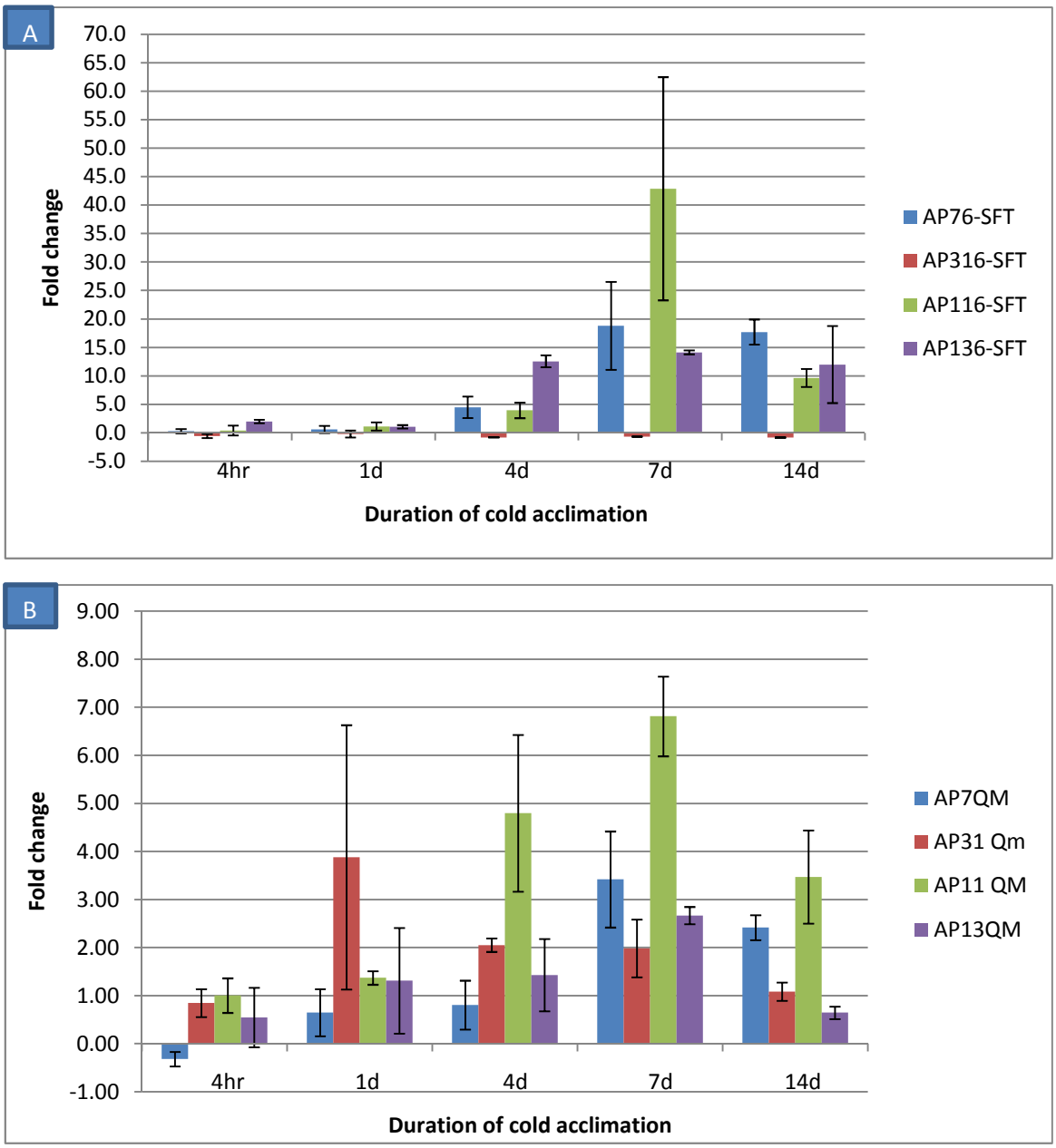


Fig.A3. Expression of the *6-SFT* gene (A) and the *QM* gene (B) in leaves of four timothy (*Phleum pratense* L.) genotypes with contrasting freezing tolerance, i.e. high-frost tolerance genotypes (AP7 and AP31) and low-frost tolerance genotypes(AP11 and AP13). Real time RT-PCR analysis of mRNA isolated from leaf tissues of plants cold treated for 4 h, 1day, and 4, 7 and 14 days. The transcript levels were assessed relative to non-acclimated (NA) plants of the same genotype by the comparative CT method. Fold change was calculated from each CT data of the individual biological replicate and average fold change was determined. The X-axis represents different durations of cold acclimation, and the Y-axis represents relative fold change in expression compared to non-acclimated (NA) controls of the respective genotypes.

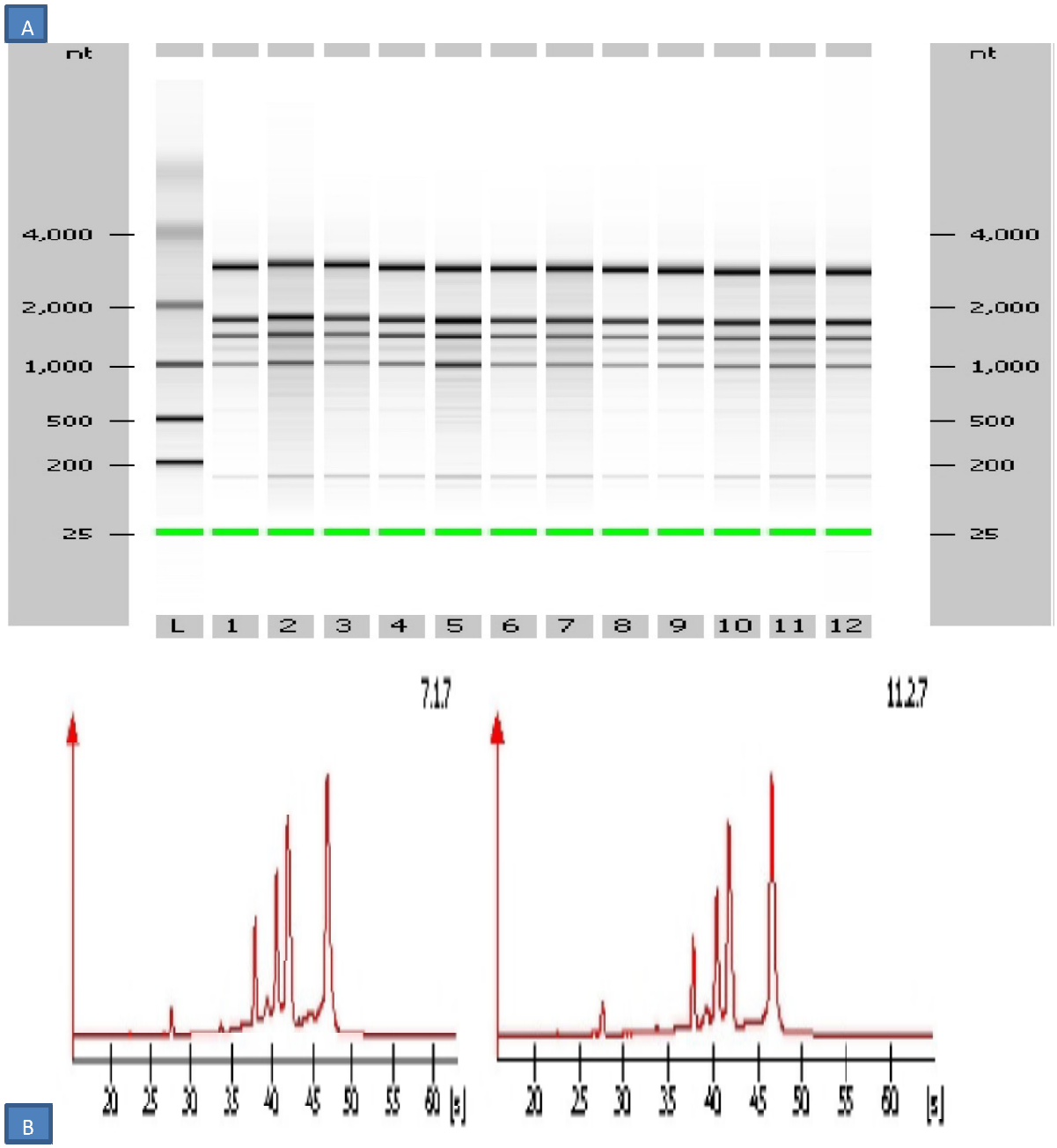


Fig. A4 B.Electrophoresis File Run Summary of RNA samples extracted from leaves of timothy sampled 7 days after start of cold acclimation. The first digit stands for genotype, the second for biological replicate, the third time after start of CA, e.g. 7.1.7 is 7 = genotype AP7, 1 = biological replicate 1, 7 = sample taken after 7 days of cold acclimation.

Fig A4 A: lane no. - 1, 2 & 3= biological replicate (BR) of AP7
- 4, 5 & 6 = biological replicate (BR) of AP31
- 7, 8 & 9= biological replicate (BR) of AP11
-10, 11 & 12= biological replicate (BR) of AP13

Table A4. Data used in the regression of mean survival scores on latitude of origin of the timothy genotypes.

Genotypes	Latitude of origin	Mean survival score
AP29	32.60	1.70
AP27	39.00	4.20
AP26	39.00	5.00
AP25	39.10	2.80
AP28	39.10	3.00
AP33	40.50	2.30
AP24	41.70	0.20
AP36	42.70	2.50
AP30	44.00	0.70
AP34	44.70	0.20
AP11	47.40	0.80
AP37	47.50	3.00
AP13	47.80	2.30
AP10	48.70	4.80
AP09	49.80	3.00
AP23	50.90	0.80
AP38	51.60	5.20
AP17	53.60	5.00
AP02	57.20	2.60
AP31	59.70	5.30
AP35	59.90	3.80
AP07	61.95	6.60
AP01	62.10	4.06
AP03	63.85	4.50
AP05	65.90	3.67
AP08	66.68	4.83
AP06	69.75	3.83