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Regulation of flowering and canopy structure in timothy (*Phleum pratense* L.)

DOCTORAL THESIS

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ACADEMIC DISSERTATION

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CONTRIBUTIONS

The contributions of the authors of the original publications associated with this thesis are presented in the following table:

	I	II	III
Planning the experiment	MS, VJ, KP, PV	VJ, MS	VJ, MS
Conducting the experiment	VJ, KP	VJ	VJ
Data analyses	VJ, MS, KP	VJ, JT	VJ
Providing material, setting up analysis	JA, AF, AS	JT	
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ABBREVIATIONS

BOR	Boreal Plant Breeding Ltd accession
DL	daylength
DM	dry matter
ELONG	vegetative elongating tiller
GA	gibberellic acid
GEN	generative tiller
q-RT-PCR	quantitative real time polymerase chain reaction
LD	long day
NDF	neutral detergent fibre
PP	photoperiod
SD	short day
VEG	vegetative tiller
VRN	vernalization gene

ABSTRACT

Timothy (*Phleum pratense* L.) is one of the most important forage grass species grown at high latitudes, mainly because of its good winter tolerance and relatively good feeding value. Its harvested biomass is mainly used as silage. Its sward canopy structure, as well as the development of individual tillers, determines the quantity and quality of the yield. Nonetheless, processes behind the transition to flowering, as well as the formation of stems and the connection with forage quality have not been studied in detail for timothy.

Seven experiments were conducted to explore the effect of vernalization, photoperiod and gibberellin (GA_3) treatments on stem elongation, flowering and canopy structure in different cultivars or accessions of timothy. The vernalization response (accelerated flowering) or requirement of the accessions was also reported. In addition, the expression of key regulator genes, *VRN1* and *VRN3*, as well as the flowering repressors *VRN2* and *MADS10* were studied, and the connection between these and flowering induction and stem formation was revealed.

Results showed that photoperiod is the most important regulator of flowering in timothy and that short photoperiod is a strong flowering repressor. In addition, vernalization response was reported in most of the tested accessions, which was seen as faster flowering. Tested accessions were grouped, based on their vernalization response, into groups having obligate vernalization requirement, intermediate response or no response. However, if the photoperiod was long enough, even northern accessions were able to flower without vernalization. Thus, a long-enough period of vernalization or photoperiod removed the differences between accessions in their responses. It was found that the application of GA_3 could not replace the LD requirement for flowering. It was hypothesized that GA_3-dependent response to flowering in timothy is regulated by photoperiod and genotype.

Results showed that the requirement for flowering and stem elongation vary, and stem elongation could take place without flowering induction. If vernalization and/ or photoperiod were long enough there was a shift from elongating (ELONG) to generative (GEN) tillers. Vernalization released the height growth of tillers, instead of flowering, especially in northern accessions, whereas in southern accessions vernalization was not required either for stem elongation or flowering. More ELONG and GEN tillers were produced after vernalization conditions, whereas at SD conditions more vegetative (VEG) tillers were produced. Moreover, in southern accessions the development of GEN lateral tillers was more synchronized than in northern accessions.

Final leaf number (FLN) has been used in cereals to describe the vernalization saturation, i.e. the time required at vernalization conditions for flowering initiation. In timothy FLN did not function as an indicator of vernalization saturation, because in most of the studied accessions FLN remained unchanged. It is suggested that this is the cause of different growth strategies between forage grasses and cereals. Flowering is also associated with decreased digestibility of grass stems. Our results showed, however, that flowering induction was not required for the development of the lignified sclerenchyma ring in developing stems, but rather lignin accumulation was as a result of stem elongation and requirements for mechanical support.

At molecular level, novel vernalization-related partial cDNAs were identified through sequencing. Both *PpVRN1* and *PpVRN3* homologs induced the transition to reproductive development, but *PpVRN3* was reported to be required for successful flowering in timothy. These results are in agreement with results obtained for other monocots and they support the theory of universal flowering-promoting system between species. The expression of the putative repressor homolog, *PpMADS10*, was connected to the developmental stage of the apex, so that higher expression and vegetative stage were observed simultaneously. The genetic differences were also seen at molecular level, probably indicating different photoperiod requirements and supporting the result of different requirements for flowering between accessions.

Results obtained in work reported in this thesis shed new light on the regulation of flowering and canopy structure in timothy. It is concluded that large variation exists among timothy accessions in their responses to vernalization and photoperiod. This information can be utilized in breeding for high-yielding new cultivars for different growing conditions at high latitudes and for different harvesting strategies.

1 INTRODUCTION

Timothy (*Phleum pratense* L.) is a widely used perennial forage grass species. It is particularly important in high latitude areas of Europe (55- 65° N latitude) mainly because of its greater winter hardiness, compared with most other temperate grasses, and its relatively high nutritive value. Forage yield comprises the total of above-ground harvested biomass, so the formation of tillers and leaves, and the phenological status of individual tillers, are of great interest. However, research focusing on stem formation and flowering of timothy has been very limited. The regulation of canopy structure is a complex process and it is affected by several exo- and endogenous factors. In addition, management practices during the growing season affect the herbage accumulation and the nutritive value of the herbage dry matter (DM) yield. Therefore their effect, and in particular the timing of the harvest, is of great interest. In this study the effect of different environmental cues, vernalization and photoperiod, on the regulation of flowering and stem formation have been explored, as well as the connection of the vernalization and photoperiod genes to the regulation. Information on the regulation of flowering and stem formation is especially important in terms of efforts to increase the yield potential of high quality biomass that can be used for silage, as well as in the adaptation of forage production to future climate change.

1.1 Timothy as a forage grass species

Timothy belongs to family of *Poaceae*, and subfamily *Pooideae*, which includes some of the world´s most important cereal crops, such as wheat, barley, oats, and forage grass species, such as genus *Lolium* and *Festuca* (Hartley 1973). The *Phleum* genus includes 15 annual and perennial species, and timothy is the most commonly cultivated species in the genus (Joachimiak 2005; Stewart et al. 2011). Timothy occurs naturally in northern latitudes, including Europe, North America and Asia, and it is also cultivated for agricultural purposes (Stewart et al. 2011). In some areas timothy is the one of the few forage grass species that is possible to use in managed grasslands, due to it having substantially lower winter damage and better yield stability than the other main forage grass species, such as perennial ryegrass (*Lolium perenne* L.) (Casler and Kallenbach 2007). In addition, new timothy cultivars have been bred especially for the extreme harsh growing conditions of high latitudes (Helgadóttir and Kristjánsdóttir 2006). The geographical distribution of timothy extends over a large area, and there is substantial genotypic variation between timothy populations. In total, some 7000 timothy accessions have been collected in European gene banks (eurisco.ipk-gatersleben.de/, accessed 2.2.2015), and there are other gene banks, e.g. in USA, that store more than 550 accessions (www.ars-grin.gov/, accessed 23.5.2015). Most of the cultivated timothy accessions have a hexaploid set of chromosomes (2n=6x=42), whereas wild populations have different polyploids, from diploid to octoploids (Cenci et al. 1984; Cai et al. 2003).

Timothy is typically used as a component in seed mixtures with other forage grass species and/or leguminous plants, and is well suited for growth in mixtures, e.g. with red clover (Charlton and Stewart 2000; Rinne and Nykänen 2000). Timothy is used mainly because of its adaptation to the harsh winter conditions (LT_{50} >-21 °C; Andrews and Gudleifsson, 1983), persistency (Østrem et al. 2013), and its relatively high nutritive value (Kuoppala et al.

2008). The negative characteristics of timothy include drought sensitivity due it having a shallow root system (Virkajärvi et al. 2003; Jonaviciene et al. 2012) and relatively slow regrowth after cutting (Larsen and Marum 2006). Most timothy-based swards are mown and harvested for silage or hay. Forage nutritive value decreases rapidly during the period of spring growth, which is typically used for first-cut silage, whereas for dry hay the harvest window is wider (Hay and Walker 1992). Timothy does not tolerate frequent cutting (Virkajärvi et al. 2003), and it is more suited to a two-cut system rather than systems with three or more cuts, due to its slow regrowth (Nissinen and Hakkola 1994). However, most of the new timothy cultivars have been bred for three-cut systems.

1.2 Canopy structure

Forage yield comprises the total above-ground harvested biomass, and thus the canopy structure of grasses has vital role in the development of the sward and its consequences for yield. Sward characteristics that affect the canopy structure include the total herbage mass, canopy height, tiller density, tiller morphology and leaf-to-stem ratio. When studying the grass population in detail, the basic production unit is a tiller. The most commonly used grass species in temperate areas [cocksfoot (*Dactylis glomerata),* perennial ryegrass, timothy] have a tufted growth habit with several daughter tillers growing from axillary buds. Tiller formation normally starts when the leaf above the tiller is expanded fully, and further new tillers are produced from the axil of the third-youngest leaf buds (Ryle 1964). It has been shown in several studies that in perennial leys the tiller density remains at a constant level and it is supposed that over the course of one year each tiller is replaced by one daughter tiller (Hay and Walker 1992). However, during the growing season tiller density varies substantially, as new tillers are produced continuously and vegetative tillers, especially, might have a relatively short lifespan (Hay and Walker 1992; Virkajärvi et al. 2003).

After receiving the required environmental signals (temperature and/or photoperiod) in spring, a tiller will become reproductive; this will lead to termination of the production of new leaves, and allow stem elongation and development of reproductive structures in the apex. The unique characteristic of grasses is that they can still produce vegetative daughter tillers even though the mother tiller has become generative (Hay and Walker 1992). However, the vegetative daughter tillers frequently die during stem elongation, because assimilates are transferred to the flowering main shoots (Colvill and Marshall 1984). In addition, generative tillers can use solar radiation more effectively than vegetative tillers, because the leaves of generative tillers are higher in the canopy, whereas the leaves of vegetative tillers are in shade (Parsons et al. 1988). The grass canopy is in a dynamic state, because simultaneously new leaves and tillers are produced and senescence naturally during the growing season (Hay and Walker 1992). Grass growth begins typically in spring from leaves. New leaves are produced from apical meristem, and the energy for the first leaves (one to three) is stored in the crown (Hay and Walker 1992). For the leaves that follow, energy is produced by the photosynthesis of the older leaves. The capacity of leaves for photosynthesis is highest when they have become fully expanded. The first leaves die during summer, when their capacity for carbohydrate production has declined (Hay and Walker 1992). For example, a perennial ryegrass tiller can have only three green leaves at

one time; when the fourth is emerging, the first is senescencing simultaneously, and in total it produces around ten leaves during its lifetime (Davies 1974).

Timothy is a bunchgrass species, and the average height of the canopy at maturity is around 100 cm (Moser and Jennings 2007). Most grass species have only vegetative (VEG) and generative (GEN) tillers, whereas timothy has a third tiller type, called vegetative elongating tiller (ELONG) (Figure 1) (Charlton and Stewart 2000; Höglind et al. 2005, Pakarinen et al. 2008; Virkajärvi et al. 2010). These ELONG tillers have a true stem with several palpable nodes, but they do not produce an inflorescence, and the apices remain at the vegetative stage, and thus are capable of constantly producing new leaf primordias (Gustavsson 2011; Virkajärvi et al. 2012). Timothy is able to flower in the first summer after sowing without any vernalization treatment, and it can also produce flowering tillers after the first cut, in regrowths for second and third harvests (Gustavsson 2011; Virkajärvi et al. 2012). Most of the studies that have reported observations of the growth process in forage grasses have been conducted with perennial ryegrass, because of its importance globally. However, it has been shown that not all the results of these studies can be generalized to apply to all forage grass species. For example, Virkajärvi and Järvenranta (2001) challenged the “three leaves rule” which has been used for perennial ryegrass. They concluded that timothy has a distinct growth pattern, and the number of leaves is generally higher than in perennial ryegrass, and the number of leaves is already defined early in the growing season (Virkajärvi and Järvenranta 2001). Moreover, Ryle (1964) reported that timothy starts to produce tillers until five leaves or more are produced above the node, and only one to three tillers are visible when around 10 leaves are formed.

Figure 1. Three tiller types found in timothy canopy: (A) generative (GEN), (B) vegetative elongating (ELONG) and (C) vegetative (VEG). Note the leafiness of ELONG tillers compared to GEN tillers in the upper parts of the stems. Photographs: Perttu Virkajärvi, LUKE.

Forage harvested for use as silage for animal production should have a high DM yield per hectare as well as good nutritive value and digestibility. Organic matter digestibility (g kg^{-1} DM) is by far the most important factor determining the nutritive value of forages (Huhtanen 1998). Digestibility is often expressed as D-value and it is affected by the fibre content (NDF; neutral detergent fibre, Van Soest 1967) which represents the cell wall fraction of DM (Huhtanen 1998). The harvested herbage mass varies greatly depending on the species present, the harvest time and sward management (Rinne et al. 1999), and in Finland it is in the range 1000 to 10 000 kg DM ha^{-1} (LUKE 2015). In silage-based feeding systems a D-value around 680-700 g kg^{-1} is desirable for ruminants, and the fibre content should be around 500-600 g NDF kg DM^{-1} (Rinne et al. 1999). The cell wall composition of grass stems has a significant effect on the nutritive value and digestibility. When a stem starts to mature it will accumulate lignin, and a lignified sclerenchyma ring is developed (Stafford 1962; Jung et al. 2012). The digestibility of cell walls is reduced because lignin prevents the polysaccharides from being digested by rumenal enzymes. This negative connection between lignin content and digestibility has been shown in several studies (e.g. Casler et al. 2008).

Stem elongation and flowering increases the biomass accumulation of forage grasses but it is associated with rapid decline in nutritive quality (Robson et al. 1988), and thus the harvest of the primary growth should be timed carefully to avoid declines in forage quality. The D-value of stems decreases rapidly (ca. 8 g kg^{-1} DM d^{-1}), whereas in leaves the rate is slower (ca. 1 g kg^{-1} DM d^{-1}) (Rinne and Nykänen 2000). In addition, the proportion of the canopy that consists of leaves will decrease (from 38 % to 23 % over 16 days), whereas the proportion that consists of stems will increase (from 58 % to 67 % in 16 days) as the primary cut is delayed (Rinne and Nykänen 2000). Thus, because of the high proportion of stems in the primary growth, relative to the proportion that consists of leaves and flower heads (Rinne and Nykänen, 2000; Nissinen et al. 2010; Virkajärvi et al. 2012), the nutritive quality of the whole of the primary growth will decrease rapidly, and therefore the timing of the harvest is important to ensure high feed value for the silage crop. In addition, the leaf-stem ratio also affects the digestibility. Earlier studies focused on the feeding quality of the whole forage canopy (e.g. Rinne and Nykänen 2000), whereas recent studies have provided additional information at the level of the tiller type in different silage cuts (Virkajärvi et al. 2010, Virkajärvi et al. 2012). Results have shown that ELONG tillers are lighter than GEN tillers, and they contribute less to the total DM yield than do GEN tillers, but more than VEG tillers (Virkajärvi et al. 2010, Virkajärvi et al. 2012). In addition, ELONG tillers may have high D-value in both first and second cuts (Pakarinen et al. 2008, Virkajärvi et al. 2012). It has been shown that individual grass tillers do not respond similarly to environmental cues, and thus the morphological developmental of tillers should be used to assess the nutritive value of forage, rather than phenological peaks (Rossignol et al. 2013). The grass canopy comprises tillers of different ages and phenological growth stages and therefore, the proportion of different tiller types has an effect on the quantity and quality of the yield (Gustavsson 2011).

1.3 The regulation of flowering and canopy structure by environmental factors and gibberellins

Flowering is a crucial event for most plant species, ensuring their survival and maximizing fertility (reviewed in King and Heide 2009). The timing of flowering and phenological development has important implications for forage production and utilization, because delayed harvest can negatively affect the forage quality, particularly through decreased digestibility. The processes that regulate the transition from vegetative growth to reproductive flowering in forage grass species have been studied intensively during past decades (Heide 1994; Andersen et al. 2006; Fjellheim et al. 2014). Flowering is regulated by environmental cues (i.e. temperature, daylength and available nutrients), and by the plant´s autonomous regulation, such as plant size, age and GA (Srikanth and Smith 2011). Forage grass species can be classified according to their vernalization and daylength requirements as being double-induction, short-day or long-day-requiring species (Heide 1994). Even closely related species (e.g. timothy and *Phleum alpinum*) may differ greatly in their requirements for flowering, and in the next chapters these aspects are described in more detail. Flowering time can be defined as 'heading date' (HD), which expresses the number of days the plant requires to start produce inflorescences.

1.3.1 Photoperiod

Most of the perennial forage grass species that are grown at high latitudes, e.g. *Lolium* and *Festuca ssp.*, require double induction for flowering initiation (Heide 1994). The primary induction is gained most often in form of vernalization during winter and/or short-day conditions (Heide 1994). The secondary induction occurs when temperature starts to rise and daylength increases in spring (Heide 1994). It has been shown in several different grass species, e.g. *Bromus inermis,* cocksfoot and *Phleum alpinum*, that the primary induction alone is not enough to induce flowering, but secondary induction is also required (Heide 1984; Heide 1987; Heide 1990).

In addition, there are few temperate grass species that can flower only if induced by long-day conditions alone; these include timothy (Langer 1955; Heide 1982; Junttila 1985), wood bluegrass (*Poa nemoralis)* (Heide 1986), *Phippsia algida* (Heide 1992) and annual darnel (*Lolium temulentum)* (Cooper and Calder 1964). In other words, these species do not have a winter requirement for flowering, and they are able to flower even in the year of sowing (Heide 1994). The critical photoperiod (i.e. the amount of daylength that the plant requires for flowering) depends on the origin of the plant species or population. Thus, 10-h DL is enough for many Mediterranean species, whereas some species or populations from higher latitudes require more than 16-h DL (Heide 1994). The number of effective LD cycles for flowering also varies between species and cultivars, and ranges from one 24-h DL cycle (*Phippsia algida*; Heide 1992) to several days (*Poa nemoralis*; Heide 1986). However, the low number of LD cycles often results in a low number of flowering plants, and more cycles are required for complete flowering, as shown in studies conducted with timothy and *Poa nemoralis* (Ryle and Langer 1963; Evans and King 1985; Heide 1986; Heide 1992).

Flowering initiation in timothy, as measured under controlled growth conditions, has been studied by few researchers. The earliest studies in the 1960s focused on flowering initiation in different photoperiods and under different temperatures, and key findings suggested that shorter DL and cooler temperatures increase the number of inflorescences (Ryle and Langer 1963). Over the following two decades more detailed studies were carried out to reveal the exact critical photoperiod for flowering in European timothy cultivars (Heide 1982; Junttila 1985; Hay and Pedersen 1986). Heide (1982) tested a total of six British and Norwegian cultivars, and observed that the critical photoperiod varied between 14 and 16 hours. In addition, Heide (1982) showed that the number of flowering plants in different photoperiods was also dependent on the growing temperature, so that more flowering plants were produced under conditions of cooler temperatures. In addition, there was a negative correlation between the number of flowering plants and total stem number, but a positive correlation between the number of flowering plants and stem height (Heide 1982). The geographic origin of the cultivar did not always explain the critical daylength for flowering, since flowering induction occurred at same DL in southern Norwegian and British cultivars (1982). Junttila (1985) used four Norwegian cultivars, and defined the critical photoperiod for flowering to be between 13 and 16,5 hours. Hay and Pedersen (1986) compared only extreme DLs (8 and 24 h), and they observed that southern cultivars were able to produce more flowering tillers under continuous light compared to northern cultivar. However, it should be noted that commercial grass cultivars are often bred by crosses between northern and southern parental lines.

Most of the earlier studies on DM accumulation in timothy focused on the nutritive value of the entire canopy (e.g. Rinne et al. 1997; Brink et al. 2007), and aspects such as the formation of tillers or their nutritive value have not been studied in detail. Studies on the canopy development and forage quality conducted under controlled growing conditions have shown that dry matter accumulation increases under longer photoperiods (Heide 1982; Heide et al. 1985; Hay and Pedersen 1986). Moreover, the total number of tillers per plant, as well as the number of leaves, have been shown to be higher under SD conditions. This is probably due to the allocation of energy to vegetative growth instead of to generative growth (Heide 1982; Hay and Pedersen 1986). The inductive effect of long photoperiods on stem elongation and height growth is well documented (e.g. Heide 1982; Hay and Pedersen 1986). However, it seems that the photoperiodic response to growth might also depend on the geographic origin of genotype (Hay 1990; Bertrand et al. 2008).

1.3.2 Vernalization

Most of the forage grass species that are grown at high latitudes require vernalization as a primary induction for inflorescence initiation (Heide 1994). Vernalization is defined as a prolonged period of low temperature to induce the apical meristem to start producing flowering primordia (Chouard 1960). Most of the *Poaceae* species originating from temperate regions require vernalization during winter to allow flowering in spring when the climatic environment is most favourable (Hemming and Trevaskis 2011). Vernalization not only prevents plants from flowering during winter at low temperatures, but it also accelerates flowering in spring before the onset of high temperatures in summer (Hemming and Trevaskis 2011). The effect of vernalization on plants can be classified as vernalization requirement and/or response. Winter cereals require vernalization conditions before they

can initiate flowering in spring, whereas spring types of cereals do not (Fowler et al. 1996) (i.e., for winter-type plants, vernalization is required for flowering, and they cannot produce inflorescences without vernalization). Vernalization response has been explained as the differences in flowering time between non-vernalized and vernalized plants, as vernalization always accelerates flowering (Szücs et al. 2007). In addition, Szücs et al. (2007) identified a third concept: vernalization sensitivity, which was defined as a delay in flowering time due to lack of vernalization. Vernalization requirement has been characterized at the molecular level quite well in the case of cereals (Figure 2) (Trevaskis et al. 2007a; Dennis and Peacock 2009).

Heide (1994) reported that the vernalization requirement varies between grass species (e.g. *Poa alpina, Poa pratensis, Bromus inermis, Dactylis glomerata, Phleum alpinum, Festuca vivipara, Festuca pratensis, Festuca rubra, Phalaris arundinaceae, Lolium perenne*), and that it also varies between cultivars based, for example, on their geographic origin, so that southern types require shorter vernalization than northern ones. For example, arctic-alpine *Poa* species require only four to 10 weeks vernalization (Heide 1980; Heide 1989), whereas the most-northern *Festuca pratensis* genotypes can require up to 16-20 weeks vernalization (Heide 1988). Furthermore, the cultivars of one species can have distinct requirements; e.g. in perennial ryegrass a Mediterranean variety flowered without vernalization whereas Northern European varieties required nine weeks vernalization (Aamlid et al. 2000). Thus, even species that have a northern origin may have relatively distinct requirements for vernalization. It is also of interest that the alpine timothy (*Phleum alpinum*) differs from cultivated hexaploid timothy in having a dual-induction requirement for flowering (Heide 1990). Vernalization has not been studied extensively in timothy, because for a long time it was classified as a species only with obligate long-day requirement, and with no need for winter treatment (Langer 1955; Heide 1982; Junttila 1985). However, response to vernalization was also observed in some early studies as shortened time for flowering (Langer 1955; Knievel and Smith 1970), and a more recent study by Fiil et al. (2011) showed that timothy has a vernalization response which is affected by the geographic origin of the genotype.

Timothy is noted as having good winter hardiness, especially due its good tolerance to freezing, which is typically gained through cold acclimation (hardening). The first stages of cold acclimation typically start in the autumn when temperatures drop to between 0°C and 10°C, and the second phase occurs when temperatures drop below 0°C (Tumanov 1940). The length of the cold acclimation period required to attain adequate freezing tolerance depends on the individual plant species, and it ranges from days to months. For example, for two *Agrostis* species six weeks of cold acclimation was enough to reach an LT_{50} value of -14,6 °C (Espevig et al. 2011). In most plant species vernalization and cold hardening are connected, so that fully vernalized plants are not able to harden as fast as non-vernalized plants (Fowler et al. 1999; Fowler and Limin 2004). Although timothy does not require vernalization, the cold hardening ability may be similarly connected to plant growth and development (Rapacz 2002; Kalberer et al. 2006). The vernalization saturation and initiation to flowering in cereals is connected to the lower freezing tolerance and higher expression of *VRN1* (*VERNALIZATION 1*) and decline in *COR* (*COLD-REDULATED*) genes (Fowler et al. 2001; Danyluk et al. 2003; Kosová et al. 2008). Several studies have assumed that *VRN1* has a clear role in the down-regulation of cold acclimation of cereals in spring (e.g. Limin and Fowler

2006), but a more recent study by Dhillon et al. (2010) suggested that additional genes are needed for repressing the *COR* genes.

1.3.3 Gibberellins

Gibberellins (GAs) are large group of plant hormones, and generally they promote cell division and elongation, and are related to normal growth of plants (Mutasa- Göttgens and Hedden 2009). In total, there are over 130 gibberellins in plants, fungi and bacteria (Yamaguchi 2008). However, only around 30 are biologically active and these include the most important GAs for plants: GA_1, GA_3, GA_4, GA_5, GA_6 and GA_7 (Yamaguchi 2008). It is assumed that all higher plants have at least one bioactive gibberellin, but probably several occur in different concentrations in different tissues (Mutasa- Göttgens and Hedden 2009). Different gibberellins have different roles in plants, and the most effective gibberellin varies between species and growth status of plants (Evans 1999; King et al. 2003). In *L. temulentum*, GA_5 and GA_6 were more effective gibberellins for flowering than GA_1 and GA_4, and furthermore, the levels of GA_5 and GA_6 doubled after LD exposure (King et al. 2001; King et al. 2003). Gibberellins GA_1 and GA_4 are detected in leaves before flowering, and the levels of these are increased in apices after several days of flowering (Gocal et al. 1999; King et al. 2001). Thomas & Hedden (2006) suggested that increases of GA_1 and GA_4 are required for the promotion of transition to flowering, but MacMillan et al. (2005) proposed that GA_4 is active for general growth but not for reproductive growth. In sugar beet, GA_4 promoted bolt initiation under short-day conditions (Mutasa-Göttgens et al. 2010). King et al. (2003) hypothesized that GA_5 and/or GA_6 were LD-induced flowering promoters, and that furthermore, the role of GA_5 was strengthened in flowering, but not in stem elongation or in other growth processes (MacMillan et al. 2005).

GA_3 is the most intensively studied gibberellin in plants. It is well known that GA_3 activates stem elongation, e.g. in *Poa pratensis* and *Bromus inermis* (Heide et al. 1998), and that is activates flowering in several plant species, including *Myosotis alpestris* (Michiniewicz and Lang 1962) *L. temulentum* (Evans et al. 1990; King et al. 2001), *L. perenne* (MacMillan et al. 2005), and *Arabidopsis* (Eriksson et al. 2006). It has been also shown that GA_3 can replace the requirement for LD for flowering in *L. perenne* (MacMillan et al. 2005), but not vernalization requirement.

1.4 Genetic regulation of flowering in monocots

Several flowering-related genes are conserved between plant species (Higgins et al. 2010), and therefore model species of *Arabidopsis* and *Poaceae* are used as examples to describe flowering pathways in timothy in the following paragraphs.

The induction of flowering is crucial for plant species to ensure reproductive success and also their possible adaptation to new environments. The molecular regulation of flowering is a complex system, and it includes at least five different pathways. These are environmental-responded pathways of: 1) photoperiod and 2) temperature (vernalization); and endogenous pathways: 3) gibberellin, 4) autonomous and 5) aging pathways (Srikanth and Schmid 2011). These pathways have been studied extensively in the model species

Arabidopsis, and several other plant species including cereals and forage grasses (reviewed by, e.g., Jarillo and Piñeiro 2011; Srikanth and Schmid 2011). Some of the genes are conserved between species, but not all (Higgins et al. 2010). The outlines of the complex regulation system via photoperiod and vernalization pathways in cereals and *Arabidopsis* are illustrated in Figure 2. All these pathways include numerous different genes; however, several studies have also shown that these pathways are not clearly separated from each other, and crosstalk between pathways is evident (Lee and Lee 2010). In the present study, the main focus is on the photoperiod and vernalization pathways, and in addition the interaction of GA with vernalization and PP were studied. The following subchapters provide further information relating to these pathways.

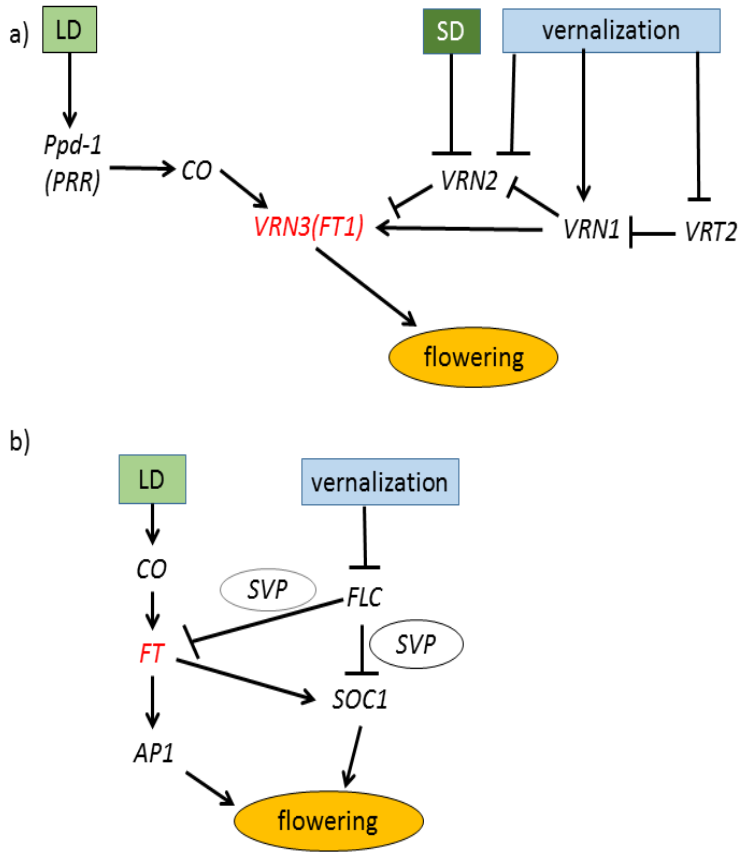


Figure 2. Simplified photoperiod- and vernalization-induced pathways in: a) cereals and b) in *Arabidopsis*. Arrows show promoting effects and T-bars repressing effects. In cereals *VRN3/FT1*and *FT* in *Arabidopsis* (marked with red) are flowering integrator genes which integrate the vernalization and photoperiod pathways. Pathways are modified using the proposed models from Trevaskis et al. 2007a; Higgins et al. 2010; Deng et al. 2015.

In *Arabidopsis FLC* (*FLOWERING LOCUS C*) is the key gene controlling flowering as a strong repressor, mainly inhibiting the integrator genes *FT (FLOWERING LOCUS T), SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS)* and *FD* (Helliwell et al. 2006; Searle et al.

2006) (Figure 2). Vernalization represses the transcription of *FLC*, and the levels stay low after the warmer temperatures (Sheldon et al. 2000). Also, *SVP* (*SHORT VEGETATIVE PHASE*) is floral repressor gene, and it has been shown that *FLC* and *SVP* interact (Lee et al. 2007). Under long-day conditions FT activates transcription of *SOC1* directly, and eventually *LFY* via *SOC1* (Figure 2; Moon et al. 2003; Wigge et al. 2005). At the final stages different pathways are combined and meristem identity genes *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*) (Wagner et al. 1999; Ferrandiz et al. 2000) are expressed. These genes regulate the transition of the vegetative growth to reproductive growth when favourable growing conditions (photoperiod and/or temperature) occur in spring, finally allowing plants to flower.

In cereals (Danyluk et al. 2003; Trevaskis et al. 2003, Yan et al. 2003) and related species (Gocal et al. 2001, Andersen et al. 2006, Ergon et al. 2013) *VRN1 (VERNALIZATION1)* -like genes control the vernalization response (Figure 2), and are similar to *Arabidopsis AP1, CAL* and *FUL* genes. *VRN1* is a promoter of flowering, and its transcript starts to accumulate simultaneously with vernalization, but higher expression is required for the transition to flowering (Yan et al. 2003; Sasani et al. 2009). In addition, the transcripts of *VRN1* also stay relatively high after vernalization, and in the apices it possibly induces reproductive development, and in leaves it is needed for the response of flowering under long-day conditions (Trevaskis et al. 2006; Sasani et al. 2009). Moreover, allelic studies of the *VRN1* in cereals have revealed that insertions and deletions in the promoter and first intron of *VRN1* result in spring and winter types (Yan et al. 2004a; Fu et al. 2005; Pidal et al. 2009). Spring types have constantly higher expression of *VRN1* whereas winter types require a sufficient length of vernalization for the expression (Fu et al. 2005). It is not yet entirely understood how *VRN1* interacts with other vernalization-related genes, but several models have been suggested and these are presented in following paragraphs.

The flowering-promoting molecular mechanisms seem to be conserved in *Arabidopsis* and cereals. For example, *FT/VRN3*-like genes and *CONSTANS* (*CO*) seem to have quite similar regulation systems after vernalization and photoperiod (Figure 2). *FT1/VRN3* is supposed to be a universal flowering promoter, and it has been found in several species including wheat (Yan et al. 2006), *Brachypodium* (Higgins et al. 2010), and perennial ryegrass (Skøt et al. 2011). In *Arabidopsis* under LD conditions FT is transcribed in leaves and further transported to the apex for promoting flowering (Corbesier et al. 2007). In cereals *FT1/VRN3* is expressed at a very low level before vernalization, regardless of the photoperiod, but under long-day conditions in vernalized plants it is induced allowing the transition to reproductive growth (Yan et al. 2006). Hence, the expression of *VRN1* is required for the activation of *FT1/VRN3* and further flowering (Figure 2). A recent study by Deng et al. (2015) confirmed this result, reporting *VRN3* to be the direct target of *VRN1*. It has been suggested also that *VRN3* is an integration point of vernalization and photoperiod pathways in several species (Kim et al. 2009). This suggestion was also confirmed by Deng et al. (2015). In conclusion, it seems that *VRN3/FT1* activation can lead directly to flowering, but the elevated expression of *VRN1* is also required (Figure 2; Deng et al. 2015).

A potential repressor of flowering in cereals is *VRN2*, which is typically expressed before vernalization in long-day conditions, and *VRN1* down-regulates it during vernalization (Yan et al. 2004b; Sasani et al. 2009). Another potential flowering repressor is *ODDSOC2*, which is suggested to be repressed by *VRN1* at normal growing temperatures in barley cultivars

which flower without vernalization (Greenup et al. 2009). A recent study supported these models, revealing direct binding of *VRN1* to the promoter of *VRN2* and *ODDSOC2* (Deng et al. 2015). It is surprising that, to date, no *FLC* orthologues have been found in monocots. However, several genes have been identified which have some repressive functions in the vernalization-regulated pathway. The first of putative repressing genes to be found in cereals was *VRN2* (Yan et al. 2004b), which was described genetically to repress flowering, similar to *FLC* in *Arabidopsis*, and *ODDSOC2* (Greenup et al. 2009). As there seems to be a lack of *FLC* homologues in monocots, the *SVP/AGL24* group of genes have been considered as potential repressors. *SVP*-like genes in monocots, which have been identified to date, include *TaVRT-2* in wheat (Kane et al. 2005), *BM1* in barley (Trevaskis et al. 2007b), *Bd21* in *Brachypodium* (Higgins et al. 2010), *LpMADS10* in *Lolium perenne* (Petersen et al. 2006), and *FpMADS10* and *FpMADS16* in *Festuca pratensis* (Ergon et al. 2013). It was supposed that *TaVRT-2* is down regulated by vernalization and further interacted with *VRN2* to repress *VRN1* (Kane et al. 2005), but a study by Trevaskis et al. (2007b) challenged this observation because *VRT2* was not down-regulated by cold. Further, they reported that *BM1* gene could have a role in meristem identity in barley (Trevaskis et al. 2007b). In contradiction of this, Petersen et al. (2006) identified the *SVP*-like homolog, *LpMADS10*, the expression of which was down-regulated by vernalization, but it did not have an effect on the flowering time of the tested plants. In the forage grass species *Festuca pratensis*, two *SVP*-like genes, *FpMADS10* and *FpMADS16*, were found, but the expression was not related to vernalization and the expression was constant under different conditions (Ergon et al. 2013). In conclusion, there are conflicting findings on the expression of *SVP*-like genes in the regulation of flowering in several monocot species, and further studies are needed.

PRR/PpD1 (*Pseudo- response regulators/ PHOTOPERIOD1*) genes have a crucial role in flowering through the photoperiod-regulated pathway, because they activate *FT/VRN3* only under long-day conditions, when growing conditions are favourable for plants (Turner et al. 2005). These genes have a crucial role in cereals especially in the adaptation to environmental conditions through photoperiodic sensitivity and variation (Turner et al. 2005, Distelfeld et al. 2009). There are photoperiodic-insensitive genotypes, which have either deletions in the *PpD1* promoter or differences in the copy number, and they are able to flower under short-day conditions also (Beales et al. 2007; Diáz et al. 2012).

2 OBJECTIVES OF THE STUDY

Environmental and endogenous regulation of the flowering and canopy structure in timothy has not been studied in detail previously. These factors have vital effects on the quantity and quality of forage yield. Therefore, this study explored the effect of vernalization, photoperiod and GA_3 treatments on the formation of different tiller types and on flowering in timothy. The physiology of plants and also the molecular regulation of flowering were studied. In addition, the responses to these factors were studied in different timothy cultivars or accessions to reveal the genetic variation in these traits which can be used for the adaptation to changing climate.

The specific objectives of the study were:

1. To investigate the regulation of flowering and canopy structure by the environmental and endogenous regulators of vernalization, photoperiod and GA_3, as well as the link between flower induction and stem lignification.

2. To examine the role of the most important vernalization and photoperiod-related genes on the phenological development.

3. To explore the genetic variation in timothy in growth characteristics and response to environmental and endogenous flowering signals, and translation of these differences to agronomic parameters.

3 MATERIAL AND METHODS

The materials and methods used in experiments reported in this thesis are described in this section only in outline. More detailed descriptions of materials used and methods are presented in the publications I-III as listed in Table 1.

3.1 Plant material, greenhouse and field experiments

The experiments were conducted mainly as a greenhouse and growth chamber experiments in Viikki, Helsinki, Finland (60° 13’ N, 25° 10’ E) during years 2008-2013, and as a one field experiment in Maaninka MTT Agrifood Research Station, Finland (63 °10’ N, 27 ° 18’ E) during years 2007-2008. A total of 13 different timothy cultivars or accessions were used in the experiments (Table 1).

3.2 Growth conditions and treatments

For greenhouse experiments plants were first grown in 12-h DL as a seed propagated (papers I and II) or clonal material (III) in fertilized peat (White 420 W, Kekkilä Oy, Vantaa, Finland), and after that transferred to different vernalization and daylength conditions (I, II, III). In paper II, gibberellin GA_3 (Invitrogen, Karlsruhe, Germany) was pipetted into the inside of the uppermost leaf sheath at a dose of 25 µg in 10 µl of 20 % ethanol, as described in MacMillan et al. (2005). Control plants were treated similarly with 20 % ethanol.

3.3 Measurements and analyses

3.3.1 Physiological measurements

Several physiological measurements were performed to reveal the growth of plants. In all experiments (I, II, III) the number of flowering tillers was measured, and also for some experiments the number of different tiller types (II, III) was monitored. In addition, height (II, III), final leaf number (FLN) (III) and days to heading (HD) (III) were measured. Details are given in Table 1. In all experiments the developmental stage of the apices was defined after different growth conditions using the scale of Sweet et al. (1991).

3.3.2 Freezing test

In paper I the freezing tolerance of timothy plants was analysed using regrowth tests. Plants were exposed to freezing temperatures between -1 °C and -25°C in glycogen path, and the regrowth ability after freezing test was monitored in greenhouse conditions (I).

3.3.3 Anatomical studies

In field experiment (I) true stem samples were collected and stored in FAA (formatin- acetic acid- alcohol) solution before sample dehydration in ethanol series and paraffin

embeddition. Paraffin from cross-sections was removed using Histo-Clear (National Diagnostic, UK), and samples were rehydrated in ethanol series. Finally, samples were stained with safranin-alcian stain, and mounted in Histomount (National Diagnostic, UK). Cross sections were examined with a microscope and photographed.

3.3.4 Preparation, sequencing and annotation of cDNA libraries

Samples for cDNA libraries were collected from greenhouse experiments (details in paper II). Total RNA (Trizol reagent, Invitrogen, Karlsruhe, Germany) was extracted and PolyA mRNA was purified using NucleoTrap Mini kit mRNA (Macherey-Nagel, Germany) following cDNA library synthesis by Super Script Double Stranded cDNA synthesis Kit (Invitrogen, Karlsruhe, Germany). In total, five micrograms of double-stranded cDNA was used for 454 sequencing, and libraries were sequenced using the 454 GS FLX Titanium (Roche Applied Science, Basel, Switzerland). GS De Novo Assembler and GS Reference Mapper, version 2.6 (http://454.com/products/analysis-software/) were used for processing and analysing of 454 sequencing reads. Finally, BLAST (ftp://ftp.ncbi.nih.gov/blast/; Altschul et al., 1997) was used to homology searches. Novel sequences having homologies to related species were used in primer design for q-RT-PCR analyses (II, III).

3.3.5 q-RT-PCR analyses

q-RT-PCR analyses were carried out in a 96-well plate system using the SYBG Green-based PCR assay, in Paper I ABI7000 (Applied Biosystems, Foster City CA, USA) and in Papers II and III, Roche Light Cycler 480 (Roche Applied Science, Basel, Switzerland). Expression levels of *VRN1* and *VRN2* were analysed using Q-gene software tool (Muller et al. 2002) (I), and expression levels of *PpVRN1*, *PpVRN3*, *PpMADS10* and *PpPpD1* calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) (II, III). In all q-RT-PCR analyses actin was used as a reference gene.

3.4. Statistical analyses

The data from greenhouse experiments were analysed using SAS (version 9.2 SAS Institute, 1999) or PASW/SPSS versions 18-21 (SPSS Corp., Chicago, USA). All greenhouse experiments were carried out using completely randomized designs, and four to six replications were used. ANOVA analysis was used, and in addition some of the data were also analysed using Tukey´s test to explore the differences between different groups.

Table 1. Timothy cultivars/ accessions treatments/ growing conditions, measurements and analyses used in the original publications I-III.

Paper no.	Exp. no.	Treatments	Cultivars/ accessions	Origin of the accession	Measurements	Analyses
I	1	Vernalization 0-20 wks + 16 h DL	Iki	Finland, N, above 63° N	Number of flowering plants, tillers, leaves	Freezing test + regrowth test q-RT-PCR (*VRN1, VRN2*)
	2	Field exp.	Tammisto II	Finland, N	Dissection of apices	Anatomical studies, q-RT-PCR
II	1	Vernalization 0-20 wks + 16 h DL	Iki	Finland, N, above 63° N	Height	q-RT-PCR
			Tuure	Finland, N, 60-64 ° N	Number of ELONG, GEN tillers	(*PpVRN1, PpVRN3, PpMADS10*)
	2	Vernalization 0 or 10 wks + GA3 12 or 16 H DL	Iki	Finland, N, above 63° N	Height	q-RT-PCR
			Tuure	Finland, N, 60-64 ° N	Number of ELONG, GEN tillers	(*PpVRN1, PpVRN3*,
					Dissection of apices	*PpMADS10, PpPD1*)
	3		Iki	Finland, N, above 63° N	none	cDNA library
III	1	Vernalization 0-15 weeks + 16 H DL	BOR S	Czech, extreme S	Height	q-RT-PCR
			BOR N	Finland, extreme N	Number of VEG, ELONG, GEN	(*PpVRN1, PpVRN3*,
			BOR 1, Donatello	Finland, N	(main+ lateral tillers)	*PpMADS10*)
			Grindstad	Norway, S	HD	
			Närekumpu	Finland, N	FLN	
			Karasjok	Norway, N	Dissection of apices	
			Saltum	Denmark, S		
			Turkey	Turkey, S		
			6116	Italy, S		
			51998	England, S		
	2	Vernalization 0, 10 or 15 weeks + 12, 16 or 20 H DL	BOR S	Czech, S	Height, dissection of apices	q-RT-PCR
			BOR N	Finland, N	Number of VEG, ELONG, GEN	(*PpVRN1, PpVRN3*,
			BOR 1	S	(main+ lateral tillers)	*PpMADS10*)
			Grindstad	Norway, S	HD, FLN	

4 RESULTS AND DISCUSSION

4.1. Environmental regulation of growth in timothy

4.1.1 Photoperiod is a key factor regulating flowering of timothy

Timothy is widely distributed in grasslands in high latitudes of Europe, and it has adapted to harsh northern growing conditions. It is described as a plant species that has obligate long-day requirement for flowering (Langer 1955) and thus, the effect of photoperiod on flowering has been the subject of relatively many studies in the past. Here, the effect of photoperiod on flowering and stem elongation in different timothy accessions was studied in two separate experiments. In the first experiment, day length periods of 12 and 16 hour were compared to reveal the role of photoperiod in the flowering in two timothy cultivars 'Iki' and 'Tuure' (II). SD photoperiod (12 h) was a strong flowering inhibitor, and neither of the tested cultivars was able to produce flowering tillers in SD. In addition, any stem elongation was not observed in 12-h DL (II). More diverse combinations of experimental photoperiods and vernalization treatments were selected to reveal genetic diversity among accessions in the critical daylength response (III). Day lengths of 12, 16 and 20 hours were used to compare the formation of different tiller types in four selected accessions of different geographic origin (III). The results of paper II were confirmed in paper III for the inhibitor effect of SD in the flowering, but the tillers of BOR1 were able to elongate, which could indicate a shorter critical DL for stem elongation (III). Several earlier studies have also reported the inhibitor effect of 12-h DL, or less, for flowering in European timothy cultivars (Heide 1982; Junttila 1985; Hay and Pedersen 1986). Thus, it seems that the critical photoperiod for flowering might be different to that of stem elongation in some timothy accessions.

In general, the critical photoperiod is shorter for southern cultivars compared to northern ones (Heide 1982; Junttila 1985). Here, in the second experiment, 16 h-DL was long enough to initiate flowering in both of the studied cultivars that were of relatively northern origin (II). For the third study, more distinct accessions were used to reveal the genetic differences in the photoperiod response (III). The 16 h-DL without vernalization treatment was not long enough to promote flowering in the accession BOR N; however, all other accessions were able to flower in similar conditions (III). In studies with Norwegian and British timothy cultivars the requirement for inductive photoperiod varied between 13 and 16,5 hours when tested under 12 to 24 h (Heide 1982) and under 8 to 16 h (Junttila 1985). Throughout the studies the critical photoperiod was longer for northern cultivars compared to southern (14-16, 5 h vs. 13-15 h, Junttila 1985; 16 h vs. 14 h, Heide 1982). Furthermore, the results of the third experiment revealed that 20 hours DL was a long enough period to induce flowering without vernalization treatment in the extreme northern accession, BOR N (III). Based on these observations the critical photoperiod for BOR N probably would be between 16 h- and 20-h. Also, the HD was reached in a shorter period in 20 h-DL compared to16 h-DL in all tested accessions, and genetic differences in HD were reduced by increasing DL (III). Taken together, the essential role of long photoperiod in the transition to flowering in timothy remains justified. In addition, the results obtained in this study provide new

information on the response to different length of photoperiods in accessions that are of distinct geographic origin.

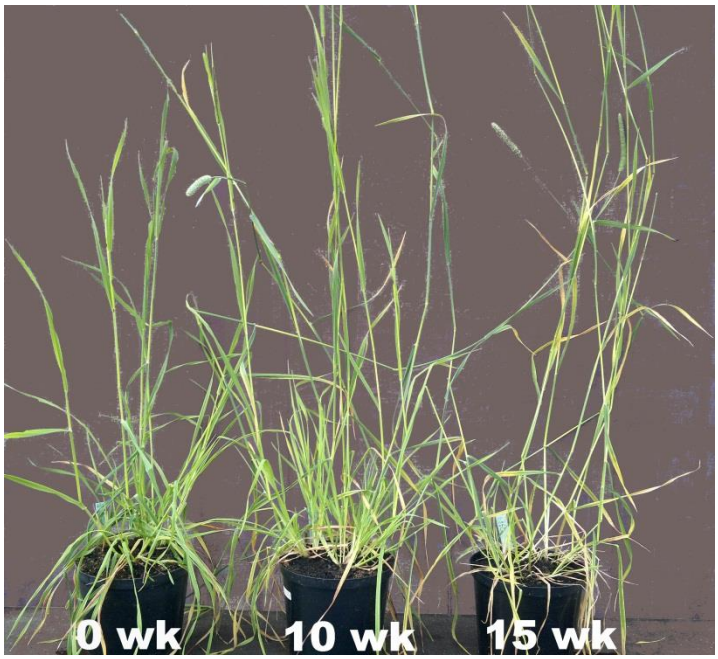
4.1.2 Vernalization enhances flowering of timothy

Timothy is defined as a plant species that has no vernalization requirement for flowering (Langer 1955; Knievel and Smith 1970). However, updated information on the role of vernalization in the flowering of timothy has been rather limited and is also contradictory. To reveal the exact role of vernalization in timothy, several greenhouse experiments were performed (I, II, III). The first experiment showed that vernalization enhances flowering in cultivar 'Iki' (I). These results clearly demonstrated that the northern cultivar at least has vernalization response, and the most inducible vernalization duration for flowering was around 10 weeks (I). The second experiment was conducted using two cultivars, and vernalization requirement for 'Iki' was reported, whereas the more southern 'Tuure' flowered without vernalization in 16-h DL but also had vernalization response (II). Also, Fiil et al. (2011) showed clear vernalization response for some timothy accessions after 15 weeks vernalization, and even obligate vernalization requirement. Based on the study of Fiil et al. (2011) a new series of experiments was established, which studied partly the same timothy accessions but using 0 to 15 weeks vernalization durations at 16-h DL (III). It should be noted that in our experiments, extreme southern (BOR S) and northern (BOR N) breeding lines were used, and these were not included in the study of Fiil et al. (2011). The tested accessions were grouped based on their vernalization response or requirement, and large variation existed among accessions (Table 3). The most southern accessions (BOR S and Saltum) had no vernalization response as the heading date remained unchanged after different experimental conditions (III) (Table 3, Figure 3). Northern accessions (BOR N, Karasjok and Närekumpu) showed the opposite response, requiring a minimum of two to 10 weeks vernalization (III) (Table 3, Figure 3). The results are in accordance with our earlier studies (I, II). In addition, a group of plants representing an intermediate flowering response was formed (III). These plants were able to flower without vernalization, but flowering was accelerated in the presence of vernalization (III). Overall, a faster flowering response was reported in vernalized plants, where northern accessions showed strongest response (III) (Table 3), as shown also by Fiil et al. (2011). Thus, it can be concluded that there is wide variation in flowering time among timothy accessions in their response to vernalization.

In cereals, spring cultivars are able to flower rapidly without vernalization, whereas winter types require vernalization (Fowler et al. 1996; Danyluk et al. 2003). The results obtained here suggest that also in timothy some kind of spring and winter types might exist. However, the genetic differences between accessions diminished after a sufficient period of vernalization or photoperiod treatments. In addition, timothy seems to have a critical sensitivity to photoperiod, which has not been reported in *Arabidopsis* ecotypes (Grillo et al. 2013), but has, to some degree, in *Brachypodium* (Ream et al. 2014). Tanhuanpää and Manninen (2012) reported timothy to be genetically a very polymorphic species, and that genetic variation exists mainly within accessions. In addition, a recent study of Fjellheim et al. (2015) reported that Nordic accessions of timothy especially are genetically diverse. The

results here indicate that there is enough variation in timothy accessions for breeding new cultivars that can be optimized for the growing conditions of high latitudes.

a)



b)

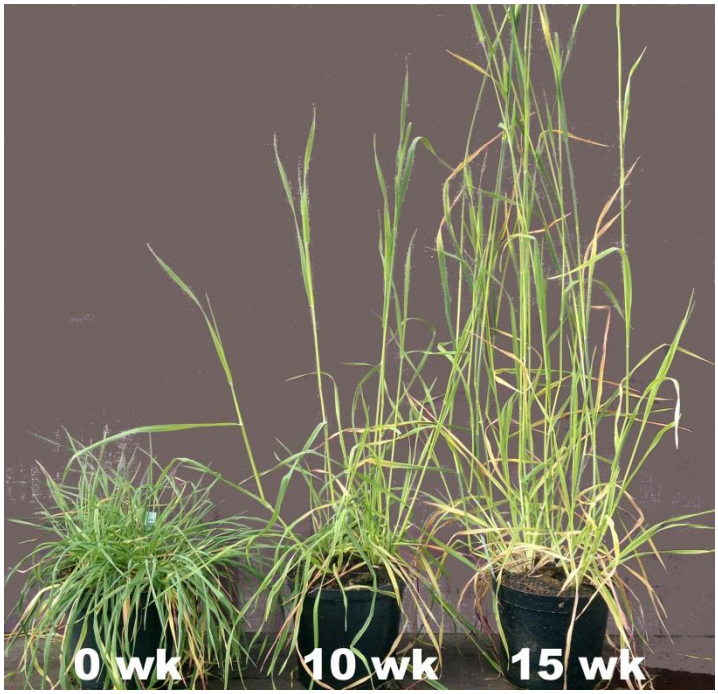


Figure 3. Effect of vernalization on the flowering and canopy structure in a) BOR S and b) BOR N. Plants were vernalized for 0, 10 or 15 weeks at +4°C, and grown in greenhouse at 16-h DL for 12 weeks.

Table 3. Summary of the effect of vernalization and photoperiod on the flowering of timothy cultivars/accessions in conducted experiments.

Paper no.	Exp. no.	Treatments	Cultivars/ accession	Vernaliz. time (wks) where first flowering tillers observed at 16-h DL	mean of HD at these conditions	Inductive photoperiod for flowering without vernalization	Repressive conditions for flowering
I	1	Vernalization 0-20 weeks + 16 h DL	Iki	10	not tested	not tested	vernalization <10 weeks
II	1	Vernalization 0-20 weeks + 16 h DL	Iki	12	not tested	not tested	vernalization <12 weeks
			Tuure	0	not tested	not tested	not any
	2	Vernalization 0 or 10 weeks + 12 or 16-h DL	Iki	10	not tested	not available	12-h PP, vernalization <10
			Tuure	0	not tested	16 h	12-h PP
III	1	Vernalization 0-15 weeks + 16 h DL	BOR S	0	36	not tested	not any
			Saltum	0	44	not tested	not any
			Turkey	0	82	not tested	not any
			Närekumpu	2	168	not tested	vernalization <2 weeks
			6116	0	151	not tested	not any
			BOR 1	0	119	not tested	not any
			Donatello	0	155	not tested	not any
			Karasjok	10	125	not tested	vernalization <10 weeks
			51998	10	122	not tested	vernalization <10 weeks
			Grindstad	0	187	not tested	not any
			BOR N	10	132	not tested	vernalization <10 weeks
	2	Vernalization 0, 10 or 15 weeks + 12, 16 or 20 h DL	BOR S	0	34	16 h	12-h PP
			BOR 1	0	94	16 h	12-h PP
			Grindstad	0	37	16 h	12-h PP
			BOR N	10	37	20 h	12-h PP, vern.<10 weeks at 16-h PP

HD= heading date

At high latitudes winters can be extremely long, and even exceed six months. Most grass species require a maximum four to five months of vernalization as a primary induction (Heide 1994). Hence, occasionally plants will experience a vernalization period that is too long, referred to here as 'over-vernalization'. This phenomenon is not well studied and there is very little corresponding literature available. For winter cereals it has been described as a phenomenon whereby plants that have already fulfilled their vernalization requirement remain at vernalization conditions for too long, and thus development will be delayed (McMaster 2005). Our results indicate that over-vernalization also affects the number of flowering tillers in timothy (I, II).The highest number of flowering tillers was gained after 10 to 12 weeks vernalization in cultivar 'Iki', whereas the number was reduced after 18 and 20 weeks treatment (I). In contrast to these findings, in the second experiment most of the flowering tillers were produced in plants vernalized for 18 and 20 weeks in the more southern cultivar 'Tuure' (II). According to Heide (1988), in *Festuca pratensis* the continuation of vernalization conditions over too long a period resulted in death of apices and reduced number of developing inflorescences. The third experiment was conducted using a maximum 15 weeks vernalization treatment (III), and no over-vernalization reaction was observed. These contradictory results could be explained partly by the timing of the greenhouse experiments, because temperature conditions are difficult to control during summer months, and temperatures that are too high have a negative effect on inflorescence development (Heide 1994). The first experiment (I) was conducted during hot summer months (May to August), whereas later experiments (II, III) were conducted during the cool months of winter and spring. The results obtained here give an overview of the effect of vernalization on the initiation of flowering in timothy, and it seems that, in all the experiments conducted, vernalization accelerated flowering in all the tested accessions, except the extreme southern accessions, which flowered constantly after different vernalization conditions. For the extreme northern accessions, vernalization was needed for proper flowering initiation if the photoperiod was less than 20-h.

The link between freezing tolerance and vernalization was studied in plants vernalized for 0 to 20 weeks (I). The maximal freezing tolerance (expressed as Lt_{50} value), -20 °C, was obtained in plants vernalized for ten weeks, whereas in plants vernalized for 18 or 20 weeks only, a temperature of -15 °C was tolerated (I). Flowering was also enhanced in plants that were cold tested and vernalized for 20 weeks, because more flowering tillers were observed in plants with freezing temperatures compared to plants that were transferred directly to the greenhouse (I). It seems that the transition to reproductive stages in timothy is not very sensitive to freezing temperatures. In cereals transition from vegetative to generative growth and vernalization saturation have been shown to have negative effects on the freezing tolerance (Danyluk et al. 2003). However, timothy is probably well adapted to northern growing conditions and it is not as sensitive to freezing as cereals. In addition, grasses have more buds which can survive winter and growth can start in spring. Winter rye (*Secale cereale*) can also continue growing while it is still cold acclimating (Griffith and McIntyre 1993). To sum up, plant responses to vernalization, freezing tolerance and flowering are not always highly conclusive.

4.1.3 The effect of photoperiod and vernalization on yield components of timothy

For forage yield the entire above-ground biomass is harvested, and thus the canopy structure affects both the harvested DM production and also the nutritional value of the DM. Therefore an understanding of the canopy structure is an important topic in research. The components of forage yield include the number of the tillers per unit area of the sward, and also the DM weight of individual tillers. The nutritive value of harvested DM is also affected by the leaf to stem ratio. In this work, the canopy structure was studied after applying different flowering inductive conditions (I, II, III). Our results show for the first time in timothy, that flowering and stem elongation have distinct critical vernalization and photoperiod requirements. It was observed that the release of height growth after vernalization requires adequate daylength (II, III). Nonetheless, the third experiment showed that BOR1 was able to produce ELONG tillers with a true stem in SD (III) also. Moreover, genetic differences were seen in the vernalization response: in the case of northern accessions vernalization accelerated height growth, but this was not observed in the southern accessions (III). However, the 16-h DL period was too short for the extreme-northern BOR N for proper height growth response, whereas 20-h was long enough (III). In previous research, Nordheim-Viken et al. (2009) reported that the northern cultivar 'Engmo' had taller plants and faster daily growth rate than the southern cultivar 'Grindstad'. In contrast, in accessions 51998 (England) and 6116 (Italy), stem elongation was arrested and was not affected by vernalization (III). These plants remained short, which may indicate an adaptation to grazing. Induction of height growth by vernalization indicates adaptation of northern ecotypes to the conditions of the local climate, as the growth of non-vernalized plants would harm cold acclimation and winter survival. Strong vernalization response in northern accessions may also relate to short growing season in the Boreal hemisphere, which requires a rapid rate of development to ensure successful seed set. Without vernalization the northern accessions were unable to flower, but they continued to produce more tillers and leaves thereby forming denser canopies (III, Figure 3). Similar observations have been done, for example in certain *Brachypodium* accessions (Ream et al. 2014). The restrictive role of short daylength for stem elongation is a well-studied phenomenon (King and Evans 2003). Furthermore, several studies have shown that in shorter photoperiods more lateral tillers are produced (Heide 1982; Hay and Heide 1983; Hay and Pedersen 1986). Plants will remain at the vegetative stage, and only leaf and vegetative primordia are produced.

The results of the first experiment showed that stem formation and development of the lignified sclerenchyma ring is not directly connected to apex development (I). Rather, development of the sclerenchyma ring is linked to stem height and need for mechanical support. This hypothesis was later supported by Kärkönen et al. (2014), where *in vitro* organic matter digestibility of timothy was found to be correlated negatively with tiller height. Moreover, the lack of vernalization resulted in more VEG tillers and generally shorter tillers, especially in northern accessions, and further, that the height growth was released simultaneously with flowering initiation (III). In addition, the formation of different tiller types in both main and lateral tillers of 11 tested accessions was studied (III). Vernalization increased the proportion of ELONG and GEN tillers in both main and lateral tillers. This observation of shift from ELONG to GEN tillers due to vernalization was also seen in the research reported in Paper II. Vernalization saturation progressed stepwise so that first the

main tillers were saturated, and then later the lateral tillers (III). The agronomic consequence for this is that the southern accessions are more synchronized and the rapid development of main and lateral tillers results in faster DM accumulation and decrease in the feeding value of the forage, compared to northern accessions. It can be concluded that grass species are generally well adapted to different growing conditions. For example, in the drier Mediterranean region, at 35-45^{o} latitude, grasses grow more efficiently during winter, but at higher latitudes grasses have a relatively short growing season, which they must exploit well in order to achieve maximum dry matter yield and ensure reproduction (Hay 1990).

The concept of final leaf number (FLN) for determining the vernalization saturation in winter cereals has been in use for decades (Berry et al. 1980; Frank and Bauer 1995; Mahfoozi et al. 2001). In short, the FLN is higher in plants which have remained for a long time at vegetative stage, whereas in generative plants the FLN is lower (Mahfoozi et al. 2001). To our knowledge, the work reported in Paper III was the first instance of FLN being used to determine the vernalization saturation in forage grasses. The results showed that in most of the accessions FLN was at constant level after different experimental conditions, and it was not related to the vernalization saturation of the plant (III). The only exceptions were the northern accessions Närekumpu and Karasjok, for which FLN decreased with the vernalization saturation (III). In general, it seems that FLN is not a suitable tool to evaluate the vernalization saturation in timothy, and this probably also applies to other forage grasses. For example, tillers of *Lolium perenne* typically have only three living leaves at any one point in time, and thus its leaf number is stable regardless of the growing conditions (Davies 1974). The yield formation and growth habit of forage grasses and cereals differ, and thus the importance of grasses to produce more tillers instead of leaves might be the main reason for the unsuitability of FLN in this context.

Seed of timothy is included in perennial forage mixtures, and even in Nordic conditions it can grow for up to five years in the same field (Virkajärvi et al. 2015). Perennials grown at high latitudes must have different growth and flowering strategies compared to annuals, in order that they may tolerate several growth cycles in harsh conditions. It is a typical situation that not all apices will develop to produce generative apices, and part of the meristem is saved for the growth in following seasons (Turck and Coupland 2013). Both the perennial growth between years and timing of the vegetative and generative growth cycles are aspects that have not been well studied. In perennial *Arabis alpina*, Wang et al. (2009) revealed that *PEP1* is a key gene contributing to perennial traits including the inhibition of flowering duration, regulation of the transition to flowering in some tillers, and controlling the responses to winter temperatures. *PEP1* was found to be an ortholog to *FLC* in *Arabidopsis*; however, the transcription and chromatin modification patterns differed between these genes (Wang et al. 2009). In all experiments the apices of BOR S developed more rapidly than the apices of BOR N, especially in the shorter DLs (III). This might suggest the existence of a more perennial growth habit in BOR N, because its apices remained vegetative more often than those of BOR S. Fjellheim et al. (2014) suggested that the repressive role of *VRN2*-like genes in perennial grasses might be more complex due the need to inhibit flowering during the long PP in summer and maintain vegetative growth in autumn. The detailed understanding of the role of perenniality in the growth of perennial grasses, and timothy, requires extensive studies, at both the molecular and physiological

level. In addition, the possible ability of timothy to "remember" winter, similar to that reported for some *Brachypodium* accessions (Woods et al. 2014) is of great interest.

4.1.4 The role of GA_3 in the regulation of flowering

Several studies have shown that different gibberellins have an important role in the growth of plants, especially in stem elongation and flowering (Mutasa-Göttgens and Hedden 2009). In temperate forage grass species the effect of GAs has been studied mainly in the *Lolium* genus, and it was shown that GA_3 can substitute the requirement for LD for flower initiation in vernalized *L. perenne* plants, but not in non-vernalized plants (MacMillan et al. 2005). The effect of GA_3 application in plants grown after and under different vernalization and photoperiod conditions was studied in two timothy cultivars (II). It was seen, for the first time that in timothy, that the application of GA_3 could not replace the LD requirement for flowering (II) that had been shown for *L. perenne* (MacMillan et al. 2005). Similar observations were made in the study by Heide et al. (1987; 1998), where GA_3 inhibited primary induction in *Poa pratensis* under short-day conditions. They proposed that high levels of GA_3 from the beginning of the SD conditions may leave the plants more susceptible to SD, and inhibit the reproductive growth (Heide et al. 1987). In contrast, Evans et al. (1990) suggested that DL (SD or single LD) does not have an effect on the response of GAs, but even only one LD can change the proportions of different GAs in plants. Further, in SD conditions, none of the tested plants produced flowering tillers, but GA_3 application led to stem elongation (II). Similar observations of the inhibitor role of GA_3 in flowering have been made in *L. temulentum* (Evans et al. 1990; King et al. 2003), *Poa pratensis* (Heide et al. 1998), and *L. perenne* (Matthew et al. 2009). In contrast, MacMillan et al. (2005) proposed that in both *L. temulentum* and *L. perenne* enhanced stem elongation is not strictly required for enhanced flowering. Evans et al. (1990) and King et al. (2003) concluded that GA_3 is more effective for stem elongation, whereas GA_5 or GA_6 are more effective for flowering.

In addition, 10 weeks vernalization together with GA_3 application resulted in taller plants compared with the treatments without vernalization and/or GA_3, but the same effect was not seen in the number of GEN tillers (II). This might be due to carbohydrate partitioning to excessive height growth, instead of to flowering. Moreover, it seems that in species requiring double induction for flowering initiation, GA application alone cannot replace the LD requirement, and that vernalization is also required (MacMillan et al. 2005). In obligatory LD plants such as *L. temulentum* GA can independently act as a LD floral stimulus (King et al. 2001). In addition, in the case of timothy, there seem to be genetic differences in the response of GA_3. The more southern cultivar 'Tuure' had more flowering tillers after GA_3 application compared to more northern 'Iki' (II). It is assumed that growth strategies and the ability to produce flowering tillers vary between cultivars. Taken together, it seems that the GA_3 dependent response to flowering in timothy is regulated by photoperiod and genotype, whereas in some other grasses the length of vernalization has the vital role. Moreover, the exact roles of specific GAs on the growth processes and flowering initiation are complex, and have large difference even between close relative grass species.

4.2 Identification of photoperiod and vernalization-related homologs in timothy

During the past two decades, molecular information on the different *Poaceae* species gained through sequencing cDNA libraries has exploded (e.g. Peng and Lapitan 2005; Vogel et al. 2006). The molecular information on timothy has been limited, and the conducted studies have focused mainly on different stress responses, sugar metabolism and nutritive value (e.g. Bertrand et al. 2003; Tamura et al. 2009; Tanaka et al. 2013). Identification of the genes which control formation of different tillers, and eventually the canopy structure, would help to understand the molecular regulation of growth processes. To enable more detailed molecular studies, deep sequencing of timothy cDNA libraries of different flowering pathways (vernalization, photoperiod, GA_4 and GA_5) was conducted. Sequencing results from seven timothy libraries generated a total of 1 390 295 reads with an average length of 261 bp (II). Assembling all of the reads using the 454 Newbler Assembler produced 70083 contigs and 169447 singletons (II). The average length of an assembled contig was 311 bp, and the average of a large contig was 853 bp (II). The quality of cDNA libraries was satisfactory, measured as read lengths, nucleotide distribution and quality scores.

BLASTX analysis revealed several putative homologs of *Poaceae* and forage grass genes, including those for vernalization, sugar metabolism and cold regulated. Novel sequences were further used to design species-specific primers for quantitative real time PCR.

4.3 The molecular regulation of flowering in timothy

4.3.1 The role of *PpVRN1* and *PpVRN3* in the transition to flowering

One of the first genes found to control vernalization-induced flowering in *Poaceae* species was *VRN1* (Danyluk et al. 2003; Trevaskis et al. 2003; Yan et al. 2003). It is well known that it promotes flowering, probably in co-operation with other flowering promoters (Distelfeld et al. 2009; Trevaskis 2010). It was for this reason that the experiment studying the expression of *VRN1* in timothy and the connection to flowering and canopy structure was conducted (I). The experiment was planned using existing information on the *VRN1* homologs in related species, and primers were designed based on this information (I). The study was successful, and the potential *VRN1* homolog was found in timothy by sequencing of the PCR product. The expression patterns of this were studied by q-RT-PCR in both greenhouse- and field-grown plants, and results showed that the elevated expression was connected to the developmental stage of the apex and transition to flowering (I). Moreover, it was noted that the lignification of the stem was not related to the apex development, and further, the expression of *VRN1* and transition to flowering (I). In part, the expression pattern of *VRN1* was similar to cereals, but it was not as consistent throughout the samples, especially in the field experiment (I). These conflicting observations might be the result of the differences between the growth habits of timothy and cereals, which were also seen earlier in our studies, e.g. in form of FLN. In the first experiment it was seen that *VRN1* was only expressed after vernalization (I), which has been seen also in cereals, and it may suggest a low-temperature requirement for the expression of *VRN1* (e.g. Trevaskis et al. 2006; Sasani et al. 2009). This observation of cold-induced expressions was confirmed in the next

experiments, in which the transcript accumulation of *VRN1* after different environmental conditions was studied in detail (II, III). The first experiment had already shown the possibility that *VRN1* is not sufficient by itself for the transition to the reproductive stage, and therefore other vernalization genes were studied in the experiments that followed. In other research, Ergon et al. (2013) suggested that the role of *VRN1* may not be as important in the transition to flowering in outcrossing grasses as it is in cereals.

The two experiments that followed were conducted using the sequence information gained through cDNA libraries, and primers were designed also for identification of the putative homolog of *VRN3/FT* (II). *VRN3* has been shown to be the universal flowering promoter in several plant species, and its role in flowering seems to be vital as a combiner of the vernalization and photoperiod pathways (Kobayashi and Weigel 2007; Distelfeld et al. 2009). The results of the experiments reported in the second paper showed that in SD conditions *PpVRN1* was expressed at low level, and GA_3 application increased slightly the transcript accumulation compared to the control treatment (II). However, the elevated level of *PpVRN1* was not able to induce flowering in SD, and *PpVRN3* was significantly up-regulated under LD conditions allowing flowering (II). Nonetheless, the expression of *PpVRN3* was always linked with the elevated expression of *PpVRN1* (II, III). Moreover, the transcript accumulation of *PpVRN1* was associated with the apex development and *PpVRN3* expression peaked after the transfer to LD and was absent under SD conditions (II, III). Vernalization saturation was always seen once the expression levels of *PpVRN1* and *PpVRN3* were the highest (I, II, III). A recent study by Deng et al. (2015) has revealed that *VRN3/FT* is the key target of *VRN1* and it may also have the final role in the transition to flowering in cereals. These results showed for the first time for timothy, the importance of *PpVRN1* and *PpVRN3* in the regulation of flowering. The crucial role of photoperiod in the transition to flowering was also proven at molecular level, and the role of *PpVRN3* as an integrator of photoperiod and vernalization pathways, which has been suggested in several studies conducted with other monocots (Kobayashi and Weigel 2007; Distelfeld et al. 2009; Ream et al. 2014; Deng et al. 2015).

Genotypical differences in the expression levels of *PpVRN3* were seen in two of the conducted studies, showing that southern accessions had higher transcript accumulation and the transcript was also present in non-vernalized plants (II, III). Moreover, higher expression of *PpVRN3* was connected to rapid apex development when vernalization saturation was achieved (III). Cereal experiments have shown that *VRN3* is connected to photoperiodic sensitivity so that spring cultivars have higher expression of *VRN3/FT1* (Hemming et al. 2008; Diallo et al. 2012; Nava et al. 2012). In addition, results obtained in these experiments allow speculation that southern accessions of timothy may possibly have the ability to bypass vernalization response and flower constantly, regardless of vernalization. Yan et al. (2006) and Faure et al. (2007) have reported that cereals which have dominant alleles of *VRN1* and *FT/VRN3* can possibly bypass the vernalization requirement.

4.3.2 The more complicated repressor system

The putative flowering repression system in timothy was first studied by designing primers using the existing sequence data from closely related species including barley and einkorn wheat (*Triticum monococcum*) *VRN2* homologs (I). Based on earlier studies (Trevaskis et al.

2007a; Hemming et al. 2008; Sasani et al. 2009), we expected that the expression of *VRN2* would decrease, while expression of *VRN1* would increase. Moreover, it was expected that longer cold treatments should result in greater down- regulation of *VRN2* (in barley; Sasani et al. 2009). However, the results from the first experiment were not in line with the results obtained from cereals, and the link to vernalization-induced flowering remained open. The expression pattern of *VRN2* was not connected to the vernalization saturation, but higher levels were seen in the greenhouse experiment after the transfer to long-day conditions, and also in the field in the primary growth (I). However, in samples collected from non-vernalized plants during regrowth, expression of *VRN2* remained unchanged between samples and it was relatively low.

After the sequencing of the cDNA library, more information was available for designing new primers for the potential repressor homologs (II). The sequence BLAST comparisons revealed highest homology to the *L. perenne LpMADS10* gene, which has been shown to be a putative flowering repressor in perennial ryegrass belonging to *SVP*-like genes sub- group (Ciannamea et al. 2006). The expression of *PpMADS10* after different vernalization and photoperiod conditions was studied in four separate experiments (II, III). Results obtained were quite different between experiments, the expression pattern of *PpMADS10* was related to the duration of vernalization treatment (II, GA- experiment), but in other experiments no clear connection existed (II, III). However, the expression levels of *PpMADS10* were always connected to the developmental stage of the apex, so that higher expression and vegetative apex were linked (II, III). The first studies identifying *SVP*-like genes in cereals suggested that wheat *TaVRT2* has a role in the low-temperature response together with *VRN2* to down-regulate *VRN1* (Kane et al. 2005). Trevaskis et al. (2007b) and Sasani et al. (2009) showed that the expression of *VRT2* was slightly increased after vernalization treatments, but it was not directly down-regulated by cold. Thus, the proposed role of *VRT2* as a direct suppressor of *VRN1* gene was challenged. The role of other putative members of *SVP*-likes genes in repression in several monocots including *L. perenne* (Petersen et al. 2006), *Brachypodium* (Higgins et al. 2010) and *F. pratensis* (Ergon et al. 2013) has also been questioned. Petersen et al. (2006) proposed that *LpMADS10* could not possibly be a true ortholog of *SVP*-like genes, whereas Higgins et al. (2010) placed the *VRT2* homolog of *Brachypodium* in two locations on the regulation system: as a repressor of *VRN1* and as a meristem identity gene. Results from *F. pratensis* were quite similar to our results, because expression of two potential *VRT2* homologs, *FpMADS10* and *FpMADS16,* were constant after different experimental conditions and were not related to vernalization (Ergon et al. 2013). They proposed that the down-regulation of *VRT2* homologs observed in some studies could be due to transition to reproductive rather than vernalization conditions (Ergon et al. 2013).

The third experiment showed possible genetic differences in the transcript accumulation of *PpMADS10* (III) of two extreme accessions, BOR and BOR N. Differences were seen especially in conditions where accessions responded differently to vernalization, e.g. short vernalization and growing at 16-h DL in the greenhouse, where BOR S was able flower and BOR N was not (III). In addition, under favorable growing conditions, where both accessions were able to flower, no differences in the expression of *PpMADS10* were seen (III). It was hypothesized that the variation of *PpMADS10* between timothy accessions can explain the differences of flowering time, similarly to the differences of *FLC* in flowering of *Arabidopsis* ecotypes (Grillo et al. 2013). Thus, it seems that *PpMADS10* has also role in the regulation of

the flowering system in timothy, but the exact role and importance remain open to further investigation.

4.3.3 The role of *PpPpD1* on flowering

Photoperiod genes (*PpD1/PRR*) also have an important role in the flowering of cereals and forage grasses through the photoperiod pathway. Here, the expression of *PpPpD1* was studied after 0 or 10 weeks vernalization and growth at 12-h or 16-h DL (II). Higher expression was observed plants grown at LD conditions, and vernalization also increased the transcript accumulation (II). It was proposed that the higher expression of *PpPPD1* at LD together with vernalization might be result of the positive interaction between DL and vernalization. Turner et al. (2005) have reported that *PpD1* controls the sensitivity to photoperiod, so that in sensitive cultivars expression of *PpD1* will up-regulate *VRN3/FT* under LD conditions and allow faster flowering. However, in our study only one cultivar was studied, and thus results on the genetic differences cannot be drawn. It should be noted that, at high latitudes in most cases, the photoperiod is always long enough during the growing season to fulfill the requirement for photoperiod (Marshall et al. 1989). Therefore, under the conditions of higher latitudes, *PpD1*-like genes might not have as important role in determining flowering time.

5 CONLUSIONS

The results of this study showed that, in timothy, apex development and lignification of the stem were not connected. The lignified sclerenchyma ring that formed was instead supposed to be required for mechanical support of the stem.

There seemed to be special photoperiod sensitivity in timothy for flowering, and the short photoperiod was a strong repressor of flowering. Even accessions that have obligatory vernalization requirements were able to flower if the photoperiod was long enough, and no vernalization was needed. Vernalization enhanced flowering in most of the timothy accessions that were tested, and it was required for flowering only for northern accessions if photoperiod was too short. Timothy accessions could be grouped on the basis of their vernalization response: accessions with vernalization requirement/ strong vernalization response, intermediate and no vernalization response.

GA_3 could not substitute for the LD requirement for flowering, as previously reported in some other grass species, but it accelerated stem elongation and had a negative effect on the number of GEN tillers in vernalized plants. Moreover, under SD conditions the expression of *PpVRN1* was higher in vernalized-plus-GA_3-treated plants compared to control plants. However, the *PpVRN3* transcript was not accumulated under these conditions, and thus flowering was not induced.

Under conditions that were unfavourable for flowering, ELONG tillers were, nevertheless, able to be produced. In addition, in some accessions stem elongation occurred under SD conditions where flowering was absent. The shift from ELONG to GEN tillers was seen if the vernalization period was long enough. Vernalization increases the number of ELONG and GEN tillers, whereas at SD photoperiod more VEG tillers are produced. In cereals, final leaf number has been used successfully to show vernalization saturation, but in timothy this was a poor indicator, as the number of leaves remained unchanged. The production of GEN lateral tillers was better synchronized in the studied southern accession compared to northern accessions.

Both *PpVRN1* and *PpVRN3* induced the transition to reproductive development, but *PpVRN3* was required for successful flowering in timothy. The genetic differences were also seen at the molecular level, probably indicating different photoperiod requirements. Moreover, it was seen that the transcript accumulation of *PpVRN3* in the southern accession was high, even in non-vernalized plants, whereas in northern accessions vernalization was required for higher expression and further for flowering. The role of putative repressor homolog, *PpMADS10*, remains open for further investigation in timothy, but it was hypothesized that it could have role in the regulation of flowering.

There were genetic differences in responses to vernalization and photoperiod, but these differences disappeared after a long enough period of time. There are reported to be different types of timothy accessions, which partly show similar responses to vernalization and photoperiod as spring and winter cereals, e.g. the studied BOR S could be characterized as a spring-type that does not have a vernalization requirement. The results obtained in this study have provided new information on the regulation of flowering and tiller formation in timothy. These results could be utilized in the future for the breeding new high-yielding timothy cultivars adapted to the conditions of high latitudes.

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