Vernalization, gibberellic acid and photo period are important signals of yield formation in timothy (Phleum pratense)

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Timothy (Phleum pratense) is a widely grown perennial forage grass in the Nordic region. The canopy consists of three tiller types, of which the stem forming vegetative elongating (ELONG) tiller and generative (GEN) tillers contribute the most to dry matter yield. In this study, the regulation of tiller formation by vernalization, day length (DL) [12 h, short day length (SD); 16 h, long day length (LD)] and gibberellic acid (GA) was investigated in two timothy cultivars. Vernalization resulted in a shift of ELONG to GEN tillers. No vernalization was required for the development of ELONG tillers but SD strictly arrested stem elongation. Vernalization is an important regulator of tiller development but it seemed to be upstream regulated by DL. LD was essential for floral transition and could not be substituted by GA and/or vernalization treatments. Genotypic variation was found in the development of GEN tillers. The ability to produce GEN tillers was associated with significant upregulation of PpVRN3. PpVRN1 expression peaked at the time of vegetative/generative transition, and PpVRN3 after the transfer to LD, suggesting them to have similar functions with cereal vernalization genes. PpVRN1 alone was not sufficient to activate flowering, and upregulation of PpVRN3 possibly together with PpPpd1 was required. Although vernalization downregulated PpMADS10, this gene did not act as a clear flowering repressor. Our results show that flowering signals alter the tiller composition, so they have important effects on yield formation of timothy.

Introduction

Timothy (Phleum pratense) is a widely grown perennial forage grass in cold temperate regions in northern parts of Europe, North America and Asia (Stewart et al. 2011). It is more winter-hardy than other important grass species such as perennial ryegrass (Lolium perenne), and the nutritive value of the harvested biomass is good (Höglin et al. 2001, Kuoppala et al. 2008). In the Nordic region, timothy swards are harvested two to three times per year for silage in order to maximize both quality and quantity of yield (Höglin et al. 2005, Virkajärvi et al. 2012). In silage production, the entire canopy is harvested for feed, so the tiller density and the size of individual tillers determine the herbage biomass and dry matter (DM) yield. Three tiller types can be found

Abbreviations – DL, day length; DM, dry matter; ELONG, vegetative elongating tiller; GA, gibberellic acid; GEN, generative tiller; LD, long day length; PRR, pseudoresponse regulator; q-RT-PCR, quantitative real-time polymerase chain reaction; SD, short day length; VEG, vegetative tiller; VRN, VERNALIZATION gene.
in the timothy canopy. Vegetative tillers (VEGs) have a pseudostem and an apex producing only leaf primordia (Gustavsson 2011). In contrast, generative (GEN) tiller and so-called vegetative elongating (ELONG) tillers have true stems with nodes but the developmental stage of apices differs (Seppänen et al. 2010, Virkajärvi et al. 2012). In GEN tillers, the apices have developed to the generative stage (> A3) (Simon and Park 1981) whereas in ELONG tillers they remain vegetative. It is not known how the development of different tiller types is regulated. Typically the spring growth (first harvest), which has been exposed to vernalization during winter, consists mainly of GEN tillers whereas in the non-vernalized summer growth (second harvest), the majority is ELONG and VEG tillers (Seppänen et al. 2010, Virkajärvi et al. 2012). There is a positive correlation between the number of GEN tillers in the timothy canopy and DM yield, whereas the ELONG and VEG tillers are smaller and their number correlates negatively with DM yield. ELONG tillers are, however, important, as their proportion is notable affecting forage yield components and consequently DM production, the ability to transit from vegetative to generative growth is important. Flowering initiation is a multicomponent and multistep mechanism, and the most important regulators of flowering (Srikanth and Schmid 2011), and to our understanding canopy structure, in temperate grasses, are vernalization, photoperiod, gibberellins and endogenous signals. Most perennial grasses have a dual requirement (vernalization and long photoperiod) for flowering (Heide 1994). Timothy is a long day plant, so day length (DL) is an important regulator of flowering. The critical DL for flowering varies from 13 to 16.5 h in timothy cultivars of different origin, being longer in those adapted to higher latitudes (Heide 1982, Juntila 1985).

Vernalization requirement varies greatly between forage grass species, from 3 weeks (Poa sp.) to over 20 weeks (Festuca sp.) (Heide 1994). In earlier studies, timothy was identified as a species not requiring vernalization for flowering or without a vernalization response (Langer 1955, Heide 1994). We have shown earlier, however, that vernalization enhances flowering in timothy (vernalization response) and the studied cultivar ‘Ikki’ required vernalization for flowering (vernalization requirement) (Seppänen et al. 2010). Fiil et al. (2011) tested vernalization response of 38 timothy accessions from different origins and reported accelerated heading in all tested genotypes, incomplete heading in four and obligate requirement in one. Thus, a quantitative or qualitative vernalization requirement exists in this species.

In plants there are at least 27 different gibberellins [gibberellic acid (GA) and their derivatives (Evans 1999)]. Eight of these have been found to be endogenous in different parts of Lolium temulentum The role of GAs in flowering is species-specific and the effectiveness varies, GA3 being the most effective in many species, including L. temulentum (Evans et al. 1990). Production of GAs is regulated by photoperiod, and most often the level increases after transfer from short day length (SD) to long day length (LD) conditions. In several LD plants it has been shown that the LD requirement of flowering can be replaced with exogenous application of GAs (reviewed in Lang 1965, Zeevart 1983). In the LD plant L. temulentum, which does not require vernalization for flowering (Heide 1994), flowering was enabled in non-inductive SD conditions after GA treatment (Evans et al. 1990, King et al. 2001). In L. perenne, which requires double induction for flowering (both vernalization and long photoperiod), GA was able to replace the LD requirement only after vernalization (MacMillan et al. 2005).

The genetic pathways controlling flowering in monocots have been studied extensively and the complex interactions are starting to be understood. The genetic analysis of flowering via vernalization pathway in monocots, including wheat (Triticum aestivum) (Yan et al. 2003), barley (Hordeum vulgare) (Hemming et al. 2008, Sasani et al. 2009), perennial ryegrass (L. perenne) (Jensen et al. 2005) and Brachypodium distachyon (Higgins et al. 2010) has revealed three major VERNALIZATION (VRN) genes. VRN1 is held to be the primary target of the vernalization signaling in cereals and it is crucial for the transition of apices from vegetative to reproductive phase and eventually for flowering (Hemming et al. 2008, Sasani et al. 2009). VRN1 is similar to Arabidopsis thaliana APETALA1 (API), which is floral identity gene and controls the onset of flowering (Kaufmann et al. 2010). The different VRN1 alleles divide cereals into spring and winter types and determine the vernalization requirement (Hemming and Trevaskis 2011). Under long day conditions, VRN2 mainly acts as a repressor of VRN3, which itself is a strong promoter of flowering (Hemming et al. 2008). Sequence variations in VRN2, like those in VRN1, result in different degrees of vernalization requirement (Yan et al. 2004). The role of VRN3 (FLOWERING LOCUS T (FT) in Arabidopsis, Kardailsky et al. 1999) is an integrator of the vernalization and photoperiod pathways and it promotes the expression of VRN1 in the shoot apex (Yan et al. 2006). Ppd1 genes belong to the pseudoresponse regulator (PRR) gene family, and they have been identified in wheat (Whitechurch and Slafier 2002), Arabidopsis (Michael et al. 2003) and barley
(Turner et al. 2005). Ppd1 genes control photoperiod sensitivity and provide adaptive variation in photoperiod response (Distelfeld et al. 2009). These genes have great importance for adaptation to natural and agricultural environments in cereals (Turner et al. 2005).

Molecular studies in timothy have so far focused on abiotic stress responses and carbohydrate metabolism (Bertrand et al. 2003, Tamura et al. 2009). The flowering pathways are poorly studied and need to be dissected due to their strong effect on canopy architecture and consequently on DM yield and nutritive value. Understanding the process of tiller formation and regulation of flowering is important for breeding high yielding timothy cultivars for the future, when climate change alters growing conditions, especially by extending the growing period in the autumn, when the DL is shorter.

The aim of this study was to characterize on a molecular level how vernalization, photoperiod and GA regulate the tiller composition and flowering in timothy. Different flowering pathways were examined and expression of VRN and Ppd1 gene homologues were studied in two timothy cultivars.

Materials and methods

Plant material, growth conditions and vernalization treatments

Timothy cultivars ‘Iki’ and ‘Tuure’ were tested for different flowering signals in controlled conditions. ‘Iki’ is characterized as more a northern cultivar, which is suited to latitudes above 63°N, whereas ‘Tuure’ is suited to latitudes of 60–64°N. Timothy seedlings were grown in a greenhouse in SD conditions (20°C, 12 h DL) for 2 weeks in fertilized and limed peat (Kekkilä B2, Vantaa, Finland) with 10 plants in each 15-cm diameter pot (1.5 l). Pots were fertilized weekly (20 kg N ha⁻¹, Kastelulannoiute, Kekkilä).

In experiment 1, plants were transferred to vernalization conditions of 6/4°C (day/night) and 8 h DL in a growth chamber (Weiss Technik, Reiskirchen-Lindenstruth, Germany) for 2, 10, 12, 18 or 20 weeks, and in experiment 2, plants were vernalized for 10 weeks in the same conditions. In both experiments, non-vernalized plants were kept in the greenhouse in SD conditions, but were transferred to the LD greenhouse with the vernalized plants. In the growth chamber, pots were arranged in a completely randomized design with three replicates, and rotated once a week in order to minimize the effects of possible environmental variation within the chamber. In experiment 2, GA₃ (Invitrogen, Karlsruhe, Germany) was applied inside the uppermost leaf sheath with pipette at a dose of 25 μg in 10 μl of 20% ethanol, as described by MacMillan et al. (2005). Control plants were treated with 20% ethanol.

The height of the three individual plants from three replications was measured before vernalization and three times after vernalization at 1-week intervals. The formation of ELONG and GEN tillers was monitored weekly and the final number of each tiller type was calculated when growth had ceased. An ELONG tiller was defined as having a true stem with visible or palpable nodes without an inflorescence and a GEN tiller as having a true stem with inflorescence, either visible or still inside the flag leaf sheath. Leaf samples (pooled sample from three individual plants) were collected 0, 7 and 14 days after vernalization for molecular analyses. At the same time, three plants were dissected to determine the developmental stage of the apical meristem (Sweet et al. 1991).

Preparation, sequencing and annotation of cDNA libraries

Cultivar ‘Iki’ was used in greenhouse experiments, and plants were first grown for 2 weeks in SD and then treated with photoperiod (SD 12 h or LD 16 h), gibberellin (GA₄ or GA₅) or 10 weeks vernalization. RNA samples from leaves for cDNA libraries were collected at different times according to the treatment, with a time 0 for the whole experiment, then 3, 8, 12 or 14 weeks after initiation of the SD or LD treatment, 2, 8, 12, 24 and 48 h or 4 weeks after GA treatment and immediately or 1 week after the end of the vernalization treatment.

Total RNA (Trizol reagent, Invitrogen) was extracted and diluted in 20 μl H₂O. The quality of RNAs was confirmed with an RNA gel and samples of different collection times were pooled in equal quantity after extraction. PolyA mRNA was purified from 0.6 mg of total RNA using NucleoTrap Mini kit mRNA (Macherey-Nagel, Duren, Germany) followed by cDNA library synthesis by Super Script Double Stranded cDNA synthesis Kit (Invitrogen). Five micrograms of double-stranded cDNA was utilized for 454 sequencing. Seven timothy libraries were sequenced using the 454 GS FLX Titanium (Roche Applied Science, Basel, Switzerland) technology. For processing and analyzing of 454 sequencing reads, GS DE NOVO ASSEMBLER and GS REFERENCE MAPPER, version 2.6 (http://454.com/products/analysis-software/) were used. Homology searches against databases were done using BLAST (ftp://ftp.ncbi.nih.gov/blast/; Altschul et al. 1997).

Novel sequences were used in primer design for quantitative real-time polymerase chain reaction (q-RT-PCR) analysis (Table S1, Supporting information). The
putative VRN3 sequence had a homology with other Poaceae species, including *Triticum monococcum* FT (93%, AY705794), *L. perenne* FT (89%, FN993928) and *H. vulgare* VRN3 (91%, EU331872). The putative MADS10 had a homology with *L. perenne* MADS10 (91%, DQ110009), *Festuca arundinacea* VRT2 (79%, HM439237), *Brachypodium* VRT2 (78%, JN036648) and *Festuca pratensis* MADS16 (77%, GU574700). The putative PpD1 gene had a homology with *Brachypodium* PRR (83%, XM003562345) and *H. vulgare* PRR (86%, FJ515590).

**Gene expression analysis**

Total RNA was extracted from timothy leaves from both experiments using Trizol reagent (Invitrogen) according to the manufacturer’s instructions and diluted in 30 μl of water. cDNA synthesis was performed using the SuperScript III (Invitrogen) one-step cDNA kit, where 1 μg of total RNA was used per reaction, according to the manufacturer’s instructions.

q-RT-PCR analysis was carried out in a 96-well plate in a Roche Light Cycler 480 (Roche Applied Science), using the SYBR Green-based PCR assay. One reaction contained 0.5 μM forward and reverse primer (Table S1), 10 μl SYBR Green master mix (Roche), 3 μl water and 5 μl diluted cDNA. The PCR parameters were: an initial denaturation step at 95°C for 10 min, then 40 cycles of 10 s at 95°C, 20 s at 59°C and 30 s at 72°C. Melting curve analysis was included in the end of the program with 95°C for 5 s, 65°C for 1 min and continuous 97°C. Expression levels were calculated by the 2^{-ΔΔCt} method, where actin was used for normalization of the data, and results were shown as a fold change relative to a calibrator sample (‘Iki’ and ‘Tuure’ non-vernalized samples) (Livak and Schmittgen 2001).

**Statistical analyses**

Analysis of variance was performed using SAS PROC MIXED (version 9.2 SAS Institute, 1999).

**Results**

**Effects of vernalization duration on the tiller composition**

Vernalization released stem elongation in both studied cultivars (Fig. 1). After 3 weeks in the greenhouse, elongation was highest in plants vernalized for 18 weeks, after which the height slightly decreased. There were no differences between cultivars in their height growth after vernalization treatments (P = 0.36). The apices developed fastest in plants vernalized for 18 weeks, reaching stage 10 in 2 weeks after transfer to the greenhouse (Figs 2A, B and 5). Especially in ‘Iki’, the longer 20-week vernalization delayed development, so that after 2 weeks in the greenhouse the apices were at stage 4, compared with 8 in ‘Tuure’ (Figs 2A, B and 5). Thus, the height growth was linked with rapid vegetative/germinative transition of apices in both cultivars. The number of leaves in both studied cultivars increased with increasing vernalization duration and this trait did not differ between cultivars (data not shown).

Vernalization resulted in a dramatic change in the proportion of ELONG and GEN tillers in both cultivars (Fig. 2), and the analysis of variance showed highly
significant differences between vernalization treatments ($P < 0.001$). After 0–10 weeks of vernalization, over 90% of the stem-forming tillers were ELONG (Fig. 2) and no GEN tillers were detected (Fig. 2A, B). This indicates that the vernalization requirement was not yet saturated and development of GEN tillers was still inhibited. Vernalization longer than 10 weeks did, however, release the development of true stems, so they became ELONG (Figs 1 and 2). In plants vernalized for 18–20 weeks, the development of apices had clearly proceeded to the generative stage (Figs 2A, B and 5) and 60–70% of tillers became GEN in both cultivars (Fig. 2). The northern cultivar ‘Iki’ did not produce any flowering tillers without vernalization, but the more southern cultivar ‘Tuure’ had a few GEN tillers after all vernalization treatments (Fig. 2A, B). The proportions of GEN and ELONG tillers were significantly different between cultivars ($P = 0.014$, $P = 0.004$, respectively).

Effects of GA treatment in SD and LD on the tiller composition

SD was a strong inhibitor of stem elongation and flowering in both cultivars (Fig. 3A, C). In vernalized plants in SD, GA application was able to induce modest stem elongation (Fig. 3A, C) that did not, however, result in the development of ELONG or GEN tillers (data not shown). Vernalization combined with LD induced stem elongation and GA had an additional positive effect on the height growth (Fig. 3B, D). There was no significant difference between cultivars in stem elongation and response to the interaction of vernalization, DL and GA treatments ($P = 0.83$).

In SD, the development of ELONG and GEN tillers was arrested in both cultivars (data not shown). In LD, GA treatment increased the development of GEN tillers in ‘Tuure’ (Fig. 4B) whereas it did not have an effect on ‘Iki’, indicating cultivar differences in the sensitivity of non-vernalized plants to GA (Fig. 4A). Nevertheless, there was no significant difference in the number of ELONG ($P = 0.66$) or GEN tillers ($P = 0.094$) between cultivars. After vernalization, GA had an opposite effect by reducing the percentage of GEN tillers, especially in ‘Iki’ (Fig. 4A). The interaction between vernalization, cultivar and GA treatment had an effect on the formation of ELONG ($P = 0.046$) and GEN tillers ($P = 0.026$).

Effects of vernalization duration on the expression of PpVRN1, PpVRN3 and PpMADS10

Expression analyses showed that the $PpVRN1$ transcript ortholog started to accumulate in plants vernalized for 10–12 weeks, 1–2 weeks after their transfer to the greenhouse (Fig. 5A, B) and simultaneously with the induction of apex development from stage 1 to stage 2–3. The apices did not develop under vernalization conditions but remained at stage 1 in both cultivars. After 1 week in the greenhouse, all the apices of ‘Iki’ were still at the vegetative stage, but double ridges had started to form on the apices of plants vernalized for 18 and 20 weeks (Fig. 5A). The apices of plants vernalized for 18 and 20 weeks started to produce generative structures (stage 3–4) already after 1 week in the greenhouse, and after 2 weeks they had inflorescence structures (stage 7–9). In ‘Tuure’ the expression of $PpVRN1$ peaked after 10 weeks of vernalization, which was slightly earlier...
than in ‘Iki’. In both cultivars the highest \textit{PpVRN1} expression was detected before the vegetative/generative transition. In ‘Tuure’, a significant accumulation of \textit{PpVRN3} transcript was detected simultaneously with that of \textit{PpVRN1}, after 10 weeks of vernalization and 1 week of growth in the greenhouse (Fig. 5B). The expression level of both \textit{PpVRN1} and \textit{PpVRN3} was about twofold higher in ‘Tuure’ than in ‘Iki’. The fold increase in the relative expression of \textit{PpMADS10} (Fig. 5E, F) was much less than that of \textit{PpVRN1} or \textit{PpVRN3} (Fig. 5A–D), and the amount of \textit{PpMADS10} transcript did not correlate with the developmental stage of apices, e.g. decreased transcript levels were not observed after prolonged vernalization and the vegetative/generative transition.


In vernalized plants of cultivar ‘Iki’, GA treatment in SD caused a 400-fold increase in \textit{PpVRN1} and a fourfold increase in \textit{PpMADS10} transcript accumulation, but had no effect on \textit{PpVRN3} or \textit{PpPpd1} levels (Fig. 6A, C, E, G). No upregulation was observed in non-vernalized plants, indicating that vernalization increased the responsiveness of \textit{PpVRN1} and \textit{PpMADS10} to GA. Vernalization and growth in LD resulted in 1200- to 1300-fold higher expression of \textit{PpVRN1} and \textit{PpVRN3} and sixfold higher \textit{PpPpd1} levels than in non-vernalized plants (Fig. 6B, D). Simultaneously, the relative expression of \textit{PpMADS10} decreased threefold (Fig. 6F). Transcript levels of \textit{PpVRN1}, \textit{PpVRN3} and \textit{PpPpd1} were significantly reduced if vernalized plants were treated with GA before growth in LD (Fig. 6B, D, H). GA treatment also delayed the development as apices were at stage 7 in GA-treated plants and stage 9 in non-treated plants (Fig. 6B). Delayed inflorescence development was coincided with more pronounced stem elongation (Fig. 3), and taller pseudo stems.

**Discussion**

**Regulation of tiller composition by environmental signals**

The canopy structure of forage grasses determines the quality and quantity of yield (Parsons and Robson 1982) and thus the number of stems forming ELONG and GEN tillers is of great interest (Virkajärvi et al. 2012). Our results show that timothy has a vernalization response, which was seen as release of stem elongation, transition of tillers from ELONG to GEN, and accelerated vegetative/generative transition and flowering. LD but no vernalization was required for the development of ELONG tillers, indicating that processes related to stem elongation differed from those of flower initiation and were not necessarily under the control of vernalization pathways. Vernalization, however, resulted in accelerated vegetative/generative transition of apices and to a change in tiller composition of canopy as the proportion of GEN tillers increased. This indicates that a flowering blocker was removed and vernalization saturation, required for the transition to the generative phase, was reached. Similar results were obtained in our earlier greenhouse experiment, where vernalization resulted in a higher number of GEN tillers and faster flowering (Seppänén et al. 2010). It was also noticed that in the field, the first spring harvest consisted mainly of GEN tillers, due to vernalization saturation during winter (Seppänén et al. 2010). In greenhouse experiments, vernalization saturation was achieved in a northern cultivar of timothy after 10 weeks (Seppänén et al. 2010), in winter wheat after 6–7 weeks and in barley 4–5 weeks of vernalization (Mahfoozi et al. 2001). In temperate grasses (\textit{Poa, Festuca}) vernalization...
and Park (1981). The expression level is expressed as fold difference relative to transcript level of non-vernalized Iki plants. Black spots describe apex developmental stage in scale of Simon length and 0–2 weeks in a greenhouse (16 h day length). The expression levels are the averages of three biological replicates (Lang 1965). In this study, GA3 was used, as in earlier studies it has been shown to be effective in L. perenne Physiol. Plant. 2014 for most species, and GA1 and GA3 being effective between species, with GA1 and GA3 being effective for most species, and GA2, GA5 or GA7 for others (Lang 1965). In this study, GA3 was used, as in earlier studies it has been shown to be effective in L. perenne (MacMillan et al. 2005), which is the closest studied species to timothy. In LD, genotypic variation in the response to GA treatment was observed so that GA requirement ranges from 3 to 20 weeks (Heide 1994). It can be concluded that also in northern genotypes of timothy, vernalization is required for the development of GEN tillers.

SD is a strong inhibitor of flowering in timothy (Junttila 1985, Heide 1988). In the present study, we confirmed this, showing that LD is essential for the floral transition in timothy and cannot be substituted by GA3 application and/or vernalization treatment. Langer (1955) showed that enough time (>100 days) at sufficient DL can induce flowering in timothy. In agriculture, however, faster development of ELONG/GEN tillers is required, as the canopy is typically harvested 50–60 days after the onset of growing season (Virkajärvi et al. 2012). Gibberellins are well known for their promotion of stem elongation and internode elongation in the Poaceae (King 2012). Different GAs are active in elongation and flowering processes, and the most effective GA for flowering varies between species, with GA1 and GA3 being effective for most species, and GA2, GA5 or GA7 for others (Lang 1965). In this study, GA3 was used, as in earlier studies it has been shown to be effective in L. perenne (MacMillan et al. 2005), which is the closest studied species to timothy. In LD, genotypic variation in the response to GA treatment was observed so that GA increased the number of GEN tillers only in the more southern cultivar ‘Tuure’ that was able to produce GEN tillers without vernalization. Oka et al. (2001) reported that in the dicot Eustoma grandiflorum vernalization improved the sensitivity to gibberellin. This indicates that the vernalization requirement for flowering and responsiveness to GA could also be linked in timothy. The conversion of GAs to other forms is known to be regulated by DL, which may result in flowering of GA-treated plants at LD (Talon and Zeevart 1990; Evans 1999). The origin of the studied timothy cultivars differs, and thus also the critical DL for flowering varies (Heide 1994). The 16 h DL was chosen as it is typical of early and late summer in areas where timothy is commonly grown, but it may not have been long enough for the more northern cultivar used in this study.

Although timothy plants were not able to produce any true tillers in SD conditions, GA3 still had a positive effect on the height growth in both cultivars. Similarly Matthew et al. (2009) showed that GA treatment increased the height of individual tillers of L. perenne. Nevertheless, GA3 did not replace the need for LD for flowering in timothy as was shown in L. perenne (MacMillan et al. 2005). GA3 application was unable to release either stem elongation or flowering without vernalization in

Fig. 5. (A, B) PpVRN1, (C, D) PpVRN3 and (E, F) PpMADS10 transcript levels in cvs. ‘Iki’ and ‘Tuure’ leaves after 0–20 weeks vernalization (8 h day length) and 0–2 weeks in a greenhouse (16 h day length). The expression levels are the averages of three biological replicates ± SE. Relative expression is expressed as fold difference relative to transcript level of non-vernalized Iki plants. Black spots describe apex developmental stage in scale of Simon and Park (1981).
Fig. 6. (A, B) PpVRN1, (C, D) PpVRN3, (E, F) PpMADS10 and (G, H) PpPpd1a transcript levels in cv. ‘Iki’ leaves after 0 or 10 weeks vernalization (8 h day length) and 2 weeks in greenhouse (12 or 16 h day length) with GA3 (black bars) or control treatment (gray bars). The expression levels are the averages of three biological replicates ± SE. Relative quantification is expressed as fold difference relative to transcript level of non-vernalized plants.

L. perenne (MacMillan et al. 2005), but in timothy the response was determined by DL and genotype. In long day conditions, GA3-treated timothy plants had fewer flowering tillers than control plants. GA3 increased the height growth, which could mean that more energy was used for growth instead of flowering (Parsons and Robson 1982). In perennial grasses, carbohydrates can be stored in stems, and these reserves are greatest at the end of vegetative phase and are later used for flowering. Carbohydrate reserves are partitioned to different tissues depending on the growth status of the plant (Slewinski 2012). In this study, it seems that these reserves were used for excessive stem growth, leaving less energy for flowering. It is commonly known that GAs promote vegetative growth in perennials at the expense of reproductive development. For example in many horticultural woody species, GAs have been reported to inhibit flowering by promoting antagonistic vegetative growth (Wilkie et al. 2008). GA3 affected the transcription level of PpVRN1, PpVRN3 and partly PpPpd1a. In SD, the expression of PpVRN1 was higher in GA3-treated plants than in control plants, but it was not sufficient to release PpVRN3 expression and enable flowering. In Arabidopsis, GA was able to activate SOC1
expression, but not FT (VRN3) expression (Moon et al. 2003).

Our results show that vernalization requirement of timothy cultivars differed, so that the more southern ‘Tuure’ was able to produce a few flowering tillers without vernalization, whereas the northern ‘Iki’ did not. Similarly, Fiil et al. (2011) reported that northern timothy genotypes had a vernalization requirement, but some southern genotypes did not, in a range of germplasm originating from 38°N to 69°N. This and other earlier studies show that, most timothy genotypes seem to have vernalization response, but variation exists in its vernalization requirement (Seppänen et al. 2010, Fiil et al. 2011). As DL is an important regulator of flowering in timothy, the 16 h DL after vernalization might have been favorable for ‘Tuure’. ‘Iki’ has slow regrowth ability, in timothy, the 16 h DL after vernalization might have been favorable for ‘Tuure’. ‘Iki’ has slow regrowth ability, early growth cessation in autumn and consequently good over-wintering ability, so it is considered as adapted to more northern conditions (>63°N) than ‘Tuure’ (<64°N). For these reasons, we suspect that the critical DL for flowering may also be longer than 16 h in ‘Iki’.

After 20 weeks of vernalization, the number of flowering tillers decreased, which can mean that the plants were ‘over-vernalized’. This phenomenon has been observed at least in F. pratensis, in which some apices died during the prolonged vernalization (20 weeks) and the number of developing inflorescences decreased (Heide 1988). Similar results have also been reported in our earlier study with timothy (Seppänen et al. 2010).

Expression of PpVRN1, PpVRN3, PpMADS10 and PpPpD1a

In temperate cereals, variation in the expression of major vernalization genes (VRN1, VRN2 and VRN3) affects flowering (Distelfeld et al. 2009, Trevaskis et al. 2009). These vernalization genes interact strongly with each other, and even minor alteration in the transcript levels can have a significant effect on flowering. VRN7 of grasses is a complex gene, because it is expressed both in leaves and apex, and can have different roles in these tissues (Preston and Kellogg 2007). VRN3 is a universal flowering promoter, and to date in all investigated plant species, its orthologs promote flowering (Kobayashi and Weigel 2007). Distelfeld et al. (2009) presented a model that VRN3 has a central role in flowering, because it connects vernalization and photoperiod pathways via repression of VRN2 and promotion of Ppd1.

The expression of VRN3 in timothy has not been studied earlier. Here we report that the expression of PpVRN3 was associated with the expression of PpVRN1. The results here support our earlier observations, where the expression of PpVRN1 peaked at the time of vegetative/generative transition of apices (Seppänen et al. 2010). PpVRN1 induction was similar in both cultivars but the level of PpVRN3 was significantly higher in the southern ‘Tuure’. The higher expression of PpVRN3 was also linked to the development of apices and eventually to flowering. We hypothesize that the difference in critical DL between cultivars was seen in PpVRN3 expression levels because of the role of VRN3 in connecting vernalization and DL perception.

The essential role of DL as a flowering regulator in timothy was shown for the first time at gene expression level in this experiment. In SD, PpVRN1 was expressed at a low level, but PpVRN3 was not detected and no GEN tillers were produced. The reason for this could be the ability of VRN3 to upregulate VRN1 (Yan et al. 2006, Li and Dubcovsky 2008). In cereals, the expression levels of VRN1 under SD are significantly higher in vernalized plants than in non-vernalized plants (Distelfeld et al. 2009). This suggests that PpVRN1 is regulated by vernalization but without co-regulation of the other vernalization genes studied. The lack of expression of PpVRN3 in SD conditions may be attributed to its role in the regulation of the photoperiod pathway (Hemming and Trevaskis 2011). This has already been shown in barley, where the homolog of VRN3, HvFT, was expressed consistently at a very low level in SD (Turner et al. 2005). This supports our observation of the importance of PpVRN3 as an integrator of vernalization and photoperiod pathways, and its essential role in flowering also in timothy, which was now shown for the first time. In L. temulentum, the expression of LtMADS1 rapidly increased after LD exposure (Gocal et al. 2001), which was seen in timothy as well, where the expression of PpVRN3 increased significantly in LD conditions and allowed plants to produce GEN tillers in vernalized plants. In wheat (Dubcovsky et al. 2006.) and barley (Trevaskis et al. 2006) VRN1 transcript levels in non-vernalized plants were significantly higher in SD than in LD, because in SD VRN2 can repress VRN1 independently of its effect of VRN3 (Trevaskis et al. 2007a).

It was proposed that, LpMADS10 of L. perenne (similar to VRT2 in cereals, Kane et al. 2005) has an important role as a flowering repressor in the vernalization-induced regulation of flowering (Ciannamia et al. 2006). In our earlier study, the expression of a putative homolog of VRT2 was detected at low level in some timothy plants, but its significance was unclear (Seppänen et al. 2010). In this study, PpMADS10 was observed at a constant level in the vernalization-time experiment, but there was no clear connection with the flowering time data. In the GA-DL experiment, PpMADS10 was expressed similarly as
VRN2 in cereals, repressing flowering. In conclusion, the exact role of PpMADS10 in the flowering of timothy does seem not be as clear as it’s ortholog is in cereals, where it is a flowering inhibitor. Nevertheless, the role of VRT2 as a clear flowering blocker after vernalization and LD exposure in this study. It seems that there is an interaction between DL of GEN tillers after vernalization and LD exposure in this expression. This was also seen in the higher production LD conditions, and vernalization further increased Stoddard for checking the language.

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References


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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Species specific primers used in q-RT-PCR analysis.

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