Evolution of adaptations to temperate climate in the grass subfamily Pooideae

Evolusjon av tilpasninger til temperert klima i gras-underfamilien Pooideae

Philosophiae Doctor (PhD) Thesis

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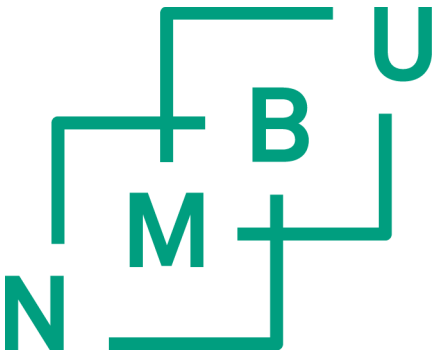

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Marian Moss, September 2016

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*Contributed equally

II. Schubert* M, Grønvold* L, Sandve SR, Hvidsten TR, Fjellheim S. **Evolution of cold adaptation in temperate grasses (Pooideae).** (Manuscript)

*Contributed equally

III. McKeown M, Schubert M, Marcussen T, Fjellheim S, Preston JC. 2016. **Evidence for an early origin of vernalization responsiveness in temperate Pooideae grasses**. *Plant Physiology* 172:416-426.

IV. McKeown* M, Schubert* M, Fjellheim S, Preston JC. **Evolution of the miR5200-FLOWERING LOCUS T flowering time regulon in the temperate grass subfamily Pooideae**. (Manuscript)

*Contributed equally

Contributions of the candidate

Paper I: The candidate contributed to the design of the experiment, provided research material, conducted growth experiments, collected leaf material and isolated RNA, assembled *de novo* transcriptomes, helped to design the bioinformatic pipeline and to conduct comparative analyses, contributed to the positive selection analysis, performed the phylogenetic analyses and wrote the manuscript.

Paper II: The candidate contributed to the design of the experiment, provided research material, conducted growth experiments, collected leaf material and isolated RNA, assembled *de novo* transcriptomes, helped to design the bioinformatic pipeline, performed the phylogenetic analyses and wrote the manuscript.

Paper III: The candidate provided research material, performed positive selection tests and contributed to the writing of the manuscript.

Paper IV: The candidate conceived the original research plan, provided research material identified *de novo* pre-miRNA sequences, performed growth experiments, isolated RNA and synthesized cDNA, analyzed the data and wrote the manuscript.

Summary

Temperate biomes are characterized by strong temperature and photoperiod seasonality and frequently occurring frost. Temperate plants have evolved to overcome challenges like cold stress, prolonged winters and short growing seasons. Through cold acclimation, which is a period of cold, non-freezing temperatures, plants increase their frost tolerance. To secure successful reproduction, many temperate plants synchronize flowering with favorable conditions of the short growing season by a two-step induction process. First, the exposure to long, cold periods, i.e. vernalization, increases the competency of many temperate plants to flower and prevents early flowering in autumn. Second, long days of late spring and early summer will promote the transition of shoot apices from vegetative to reproductive stages and subsequent inflorescence development. These mechanism are regulated by complex genetic pathways which are partly interlaced with each other.

Among the Poaceae (grass family), the subfamily Pooideae (temperate grasses) is the most important temperate lineage, because it includes the majority of cereals and grass crops cultivated in temperate regions, e.g. wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and perennial ryegrass (*Lolium perenne*). Cold adaptation, vernalization response and photoperiod-dependent flowering has been studied extensively in these cultivated species. However, little is known about the evolutionary history of temperate adaptations in Pooideae and the biome shift, during which the Pooideae shifted their range from the tropical biomes of their ancestors to extant temperate biomes.

In this PhD project we investigated the evolutionary history of temperate adaptions in the Pooideae subfamily and possible consequences for the biome shift. We focused our research on Pooideae lineages that had diverged early in the evolution of the Pooideae and that have not been studied in regard of their temperate adaptations. We tested if well-studied genetic mechanisms regulating cold response, vernalization and photoperiod-dependent flowering in core Pooideae species were conserved in species of early-diverging lineages. We used an array of experiments and analyses, ranging from classical growth experiments and quantitative real-time PCR to phylogenetic reconstruction and comparative transcriptomics. We found that a

fraction of the genetic basis underlying temperate adaptation evolved early in the Pooideae subfamily. However, we also found that most of the investigated genes and genetic pathways had undergone extensive lineage-specific evolution, possibly promoted by the successive climate cooling starting around 50 million years ago.

Sammendrag

Tempererte biomer karakteriseres av sterk sesongvariasjon i temperatur og daglengde samt regelmessig forekommende frostperioder. Tempererte planter har evolvert til å møte utfordringer som kuldestress, lange vintre og korte vekstsesonger. Ved hjelp av kuldeakklimering - en kald, men frostfri periode - kan tempererte planter øke frosttoleransen. For å sikre reproduksjonssuksess synkroniserer planter blomstringstiden med gunstige forhold i vekstsesongen. I den korte vekstperioden i tempererte områder går plantene gjennom en to-trinns induseringsprossess. Plantene blir blomstringskompetente ved å gjennomgå en lang og kald periode, dvs. vernalisering. Dette vil forhindre at plantene blomstrer om høsten. Etterpå induserer de lange dagene i seint vår og tidlig sommer apikalmeristemets overgang fra vegetative til reproduktive fasen og fremmer utviklingen av blomsterstanden. Disse mekanismene er regulert av komplekse, genetiske signalveier som er delvis tilknyttet hverandre.

I Poaceae (gras familien) er underfamilien Pooideae den viktigste tempererte underfamilien da den inneholder flertallet av kultiverte korn- og fôrvekster i tempererte strøk, som for eksempel hvete (*Triticum aestivum*), bygg (*Hordeum vulgare*) og flerårig raigras (*Lolium perenne*). Derfor er kuldetilpassing, vernaliseringsrespons og blomstring i respons til ulike daglengder grundig studert i disse kulturvekstene. Vi vet imidlertid lite om den evolusjonære historien av tilpasninger til temperert klima i den tidlige evolusjonen i Pooideae og hvordan Pooideae endret sin utbredelse fra de tropiske strøkene forfedrene befant seg i til den tempererte utbredelsen vi ser i dag.

I dette doktorgradsprosjektet har vi undersøkt evolusjonshistorien til grasunderfamilien Pooideae og evolusjonære tilpasninger til temperert klima. Forskning er fokusert på Pooideae linjer som divergerte tidlig i Pooideaes evolusjon og som ikke før er studert med fokus på tempererte tilpassinger. Vi har testet om kjente genetiske mekanismer som regulerer kuldetilpassing, vernaliseringsrespons og blomstring i forhold til ulike daglengder i kultiverte Pooideae arter er konservert i arter fra tidlig divergerte linjer. Vi har brukt en rekke eksperimenter og analysemetoder, fra klassiske vekstekseperimenter og kvantitativ PCR til fylogenetisk

rekonstruksjon og komparativ transkriptomik. Funnene våre viser at kun en liten del av det genetiske grunnlaget som kontrollerer tilpasning til temperert klima evolverte tidlig i underfamilien Pooideae. De fleste av de undersøkte gener og genetiske signalveier evolverte videre i spesifikke linjer, muligens indusert av både gradvise og plutselige klimaendringer i de siste 50 million år.

1 Introduction

1.1 Grasses – a diverse plant family

The Poaceae (grass family) are one of the largest and most diverse angiosperm families in the world. Its nearly 12.000 species are represented in virtually every habitat around the globe, and are able to survive in extreme hot, cold, dry or wet environments. The vast majority of Poaceae species is distributed within nine lineages, i.e. subfamilies, that are split into two clades (Fig. 1). The subfamilies Panicoideae, Aristidoideae, Chloridoideae, Micrairoideae, Arundinoideae, and Danthonioideae belong to the PACMAD clade, while subfamilies Bambusoideae (bamboo subfamily), Oryzoideae (rice subfamily), and Pooideae (temperate grasses) belong to the BOP clade (Soreng et al. 2015). The most recent common ancestor of the Poaceae is thought to have been adapted to tropical forest ecosystems (Bouchenak-Khelladi et al. 2010). Most grasses that are adapted to warm and dry conditions belong to the PACMAD clade. The diverse BOP clade consists of lineages adapted to open or closed habitats and tropical or temperate biomes. Most of the cold tolerant grasses are found in subfamilies Danthonioideae (PACMAD) and Pooideae (BOP), of which the Pooideae are the dominant grasses in Northern temperate biomes (Hartley 1973).

With its roughly 4200 species the Pooideae is the largest subfamily in Poaceae. It consists of 14 tribes (Soreng et al. 2015), of which we define the tribes Brachyelytreae, Nardeae, Lygeeae, Phaenospermateae, Brylkinieae, Meliceae, Ampelodesmeae, Stipeae and Diarrheneae as the early diverging lineages. The tribe Brachypodieae contains the model grass *Brachypodium distachyon* (Draper et al. 2001) and is sister clade to the core Pooideae (Fig. 1). The core Pooideae comprise the tribes Littledaleeae, Poeae, Bromeae and Triticeae (Davis and Soreng 1993) and harbor most of the extant Pooideae species and all economically important crops of temperate regions; for example the cereal crops wheat (*Triticum aestivum*) or barley (*Hordeum vulgare*) and fodder- and turfgrasses like perennial ryegrass (*Lolium perenne*) or meadow fescue (*Festuca pratensis*). In contrast to tropical and subtropical relatives, temperate grasses cope with strong seasonality and

cold stress, including frost. The next two sections will describe mechanisms that evolved in the Pooideae as adaptations to temperate biomes.

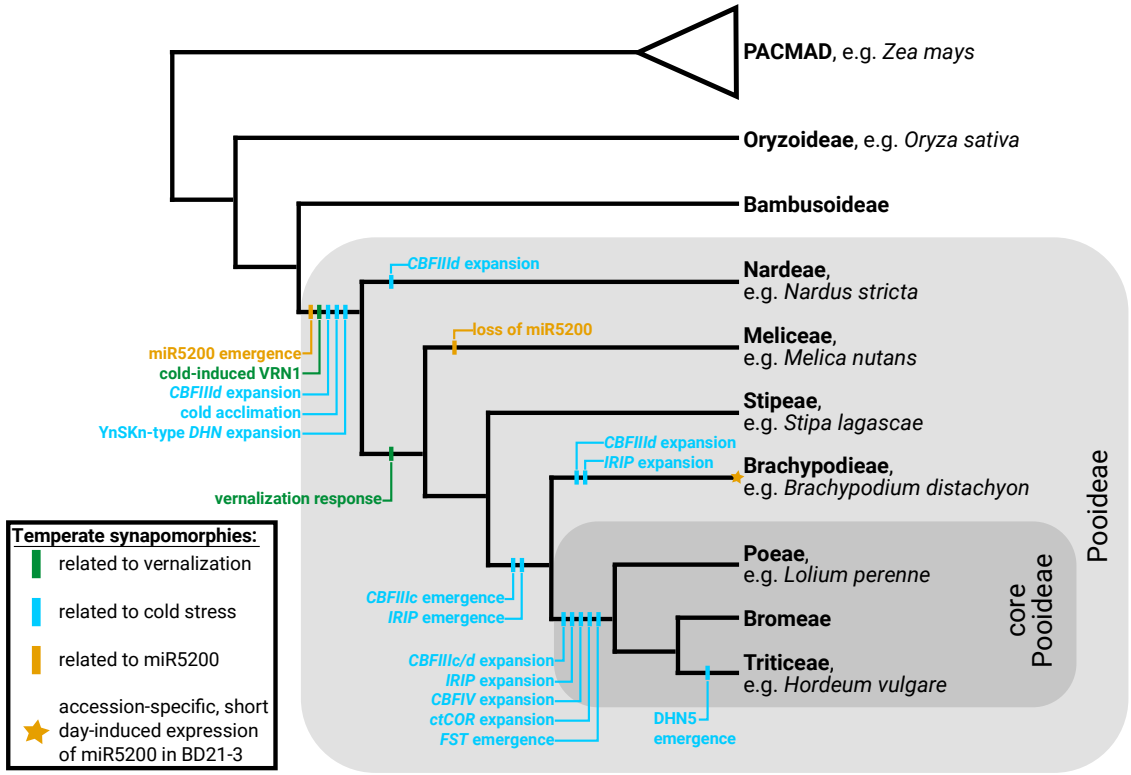

Figure 1: Schematic Pooideae phylogeny with temperate synapomorphies. Pooideae phylogeny is displayed for three of the core Pooideae tribes (Triticeae, Bromeae and Poeae), their sister tribe Brachypodieae and three of the early diverging tribes Stipeae, Meliceae and Nardeae. Furthermore, the two remaining subfamilies of the BOP clade (Bambusoideae and Oryzoideae) and the PACMAD clade are displayed. Topology is in accordance with Soreng et al. (2015). Temperate synapomorphies identified in this PhD project are marked as colored boxes on the phylogenetic tree.

1.2 Coping with cold temperature and frost

Frost and chilling, i.e. low, non-freezing temperatures, impose major stresses on plants in temperate biomes (reviewed by Sandve et al. 2011; Preston and Sandve 2013). Ice formation during freezing conditions restricts the availability of soluble water and increases the osmotic potential between plant cells and the environment. Osmotic stress can be intensified by growing ice crystals that damage plasma membranes. Already at chilling temperatures plasma membranes tend to become more rigid and are prone to

rupture. Furthermore, low temperature decreases the metabolic turnover rate of the photosynthetic machinery, leading to accumulation of reactive oxygen species (ROS) which can damage membranes and inhibit photosynthesis (Crosatti et al. 2013).

In order to prepare for winter frost, core Pooideae species are able to cold acclimate, i.e. increase their frost tolerance after experiencing long periods with low, non-freezing temperatures (Thomashow 1999, Galiba 2009, Sandve et al. 2011). Cold acclimation processes are well-studied in species of the core Pooideae, due to their economic importance. Some of the most-investigated proteins involved in cold acclimation include C-repeat binding factors (CBF). The *CBF* genes were previously characterized as “master-switches” of cold regulation and cold acclimation (Sarhan et al. 1998; Thomashow 1999; Tondelli et al. 2011), but are also involved in various other kinds of stress response (Agarwal et al. 2006; Akhtar et al. 2012). Especially two groups of *CBF* genes – *CBFIII* and *CBFIV* – are important for cold acclimation in the Pooideae and are restricted to that subfamily (Badawi et al. 2007; Li et al. 2012). Several studies have shown that an accumulation of *CBF* genes correlates with an increase in freezing tolerance (Galiba et al. 2009; Knox et al. 2010; Li et al. 2011; Jeknić et al. 2014).

Genes that protect plants from dehydration stress were previously described as dehydrins (Close 1997). Dehydrins are a family of hydrophilic proteins that share a common, lysine-rich sequence, called “K-segment” (Koag et al. 2003; Koag et al. 2009). These segments are known to interact with membranes and proteins (Koag et al. 2009) and their amphipathic structure is assumed to protect against dehydration and might act as cryprotectant (Close 1997; Danyluk et al. 1998; Houde et al. 2004). Dehydrin genes are expressed in response to abiotic stress, particularly osmotic stress (Rorat 2006; Kosová et al. 2014). In core Pooideae species, dehydrins seem to play an important role in cold acclimation and short term frost response (Olave-Concha et al. 2004; Kosová et al. 2007).

Already during chilling conditions chloroplasts experience stress because reduced CO_2 fixation rates lead to an overexcitation of the photosystems and an accumulation of reactive oxygen species (ROS) (Crosatti et al. 2013). ROS accumulation leads to photodamage through the inhibition of protein synthesis and might damage membranes

through lipid peroxidation (Murata et al. 2007; Crosatti et al. 2013). The expression of the two chloroplast targeting, cold induced (COR) genes *WCS19* and *COR14*, was found to be linearly correlated with frost tolerance in several core Pooideae species (Crosatti 1999; Tsvetanov et al. 2000; Crosatti et al. 2013).

Core Pooideae species avoid cellular freezing damage by synthesizing ice-recyrstallization inhibition proteins (IRIPs) during cold periods (Griffith and Ewart 1995; Antikainen and Griffith 1997; Sidebottom et al. 2000; Tremblay et al. 2005). Those proteins will bind to the edges of microscopic ice grains and inhibit them from growing to bigger ice crystals that would damage cells and plant tissue (Griffith and Yaish 2004). The synthesis of IRIPs during chilling conditions increases the frost tolerance of cold acclimated plants (Antikainen and Griffith 1997; John et al. 2009; Kumble et al. 2008; Zhang et al. 2010).

The degree of frost tolerance positively correlates with the accumulation of fructans and fructosyltransferases (FSTs) (Hisano et al. 2004; Livingston et al. 2009; Tamura et al. 2014). Fructans are the major carbohydrate storage in core Pooideae species, and FST, responsible for fructan biosynthesis, are induced by cold (Hisano et al. 2004; Sandve et al. 2008; Sandve et al. 2011). It is not exactly clear how fructans and FSTs increase frost tolerance, but Hincha et al. (2000) suggested that fructans affect membrane stability, which decreases during low temperatures. Apart from their involvement in cold acclimation, *FST* genes are also induced during short-term cold periods and are involved in the direct response to cold stress.

1.3 Regulation of flowering in temperate biomes

In temperate biomes, seasonal differences in temperature are an important environmental cue, influencing the life cycle of plants and determining when conditions for the reproductive phase are favorable. Being able to time flowering with the short growing season, i.e. summer, increases the chances of a successful reproduction by, for example, reducing the risk of frost damage to floral tissue. In core Pooideae species, timing of flowering is regulated by a two-step mechanism (reviewed by Fjellheim et al. 2014). First, a prolonged exposure to cold temperatures, i.e. winter, increases the

competency of plants to flower. This mechanism is referred to as vernalization. Second, during late spring and early summer long days will induce flowering in competent plants.

Angiosperms sense environmental cues in their leaves and when conditions are favorable, they send flowering promoting signals to the shoot apex to induce the transition from vegetative to reproductive stage. In the molecular pathway regulating flowering, *Flowering locus T* (*FT*) has been identified as the main floral integrator of environmental cues (reviewed by Turck et al. 2008; Amasino 2010) and this function seems to be conserved throughout angiosperms, including Pooideae grasses (Higgins et al. 2010; Shrestha et al. 2014).

Flowering in response to vernalization

Vernalization responsive species are known from several angiosperm families (Ream et al. 2013). Because most angiosperm families diverged before the global climate cooled around 40 Million years ago (Mya), it is likely that vernalization response originated independently in those lineages. Molecular mechanisms regulating the vernalization response have been studied in several plant groups (reviewed by Preston and Sandve 2013, Shrestha et al. 2014). Although *FT* plays a central role in all investigated plants, the regulatory pathways are quite different. At the core of the genetic network that regulates vernalization in temperate cereals (Fig. 2) are three genes: *VERNALIZATION1* (*VRN1)*, *VRN2* and *VRN3* (synonymous to *FT*) (reviewed by Fjellheim et al. 2013). During autumn, the expression of *VRN3/FT* in vernalization-reponsive core Pooideae plants is repressed by high levels of *VRN2* (Sasani et al. 2009). This mechanism prevents premature flowering during the long days of early autumn. Prolonged cold periods during winter and early spring induce the expression of *VRN1* (Distelfeld et al. 2009; Trevaskis 2010) in leaves and the shot apex (Fig. 2). In leaves, the MADS-box transcription factor VRN1 binds directly to the promoter of *VRN2* (Deng et al. 2015) and inhibits its expression during and after vernalization (Fig. 2). Low levels of *VRN2* alleviate the repression of *VRN3*. Expression of *VRN3* and inflorescence development is however not induced until days become long enough in late spring (Fig. 2; Sasani et al. 2009). It has been shown that cold induction of *VRN1* is mediated by epigenetic signals

that reduce repressing histone modifications at the *VRN1* locus during winter (Oliver et al. 2009).

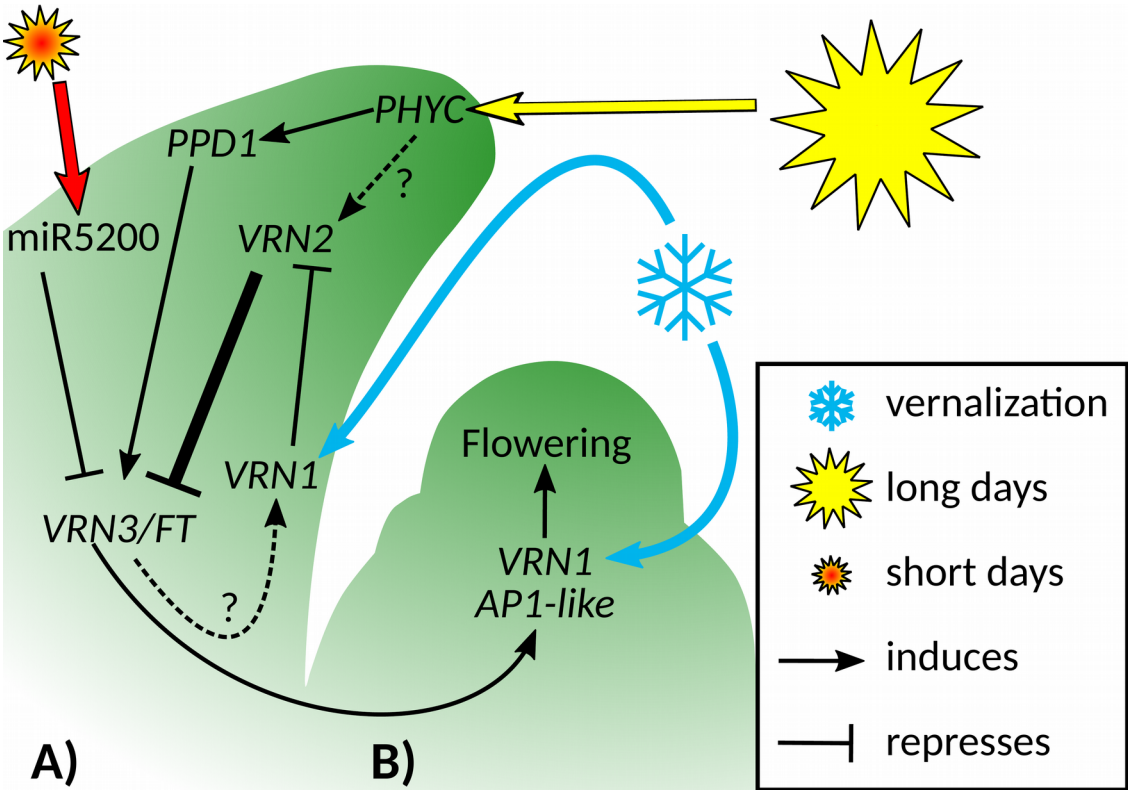

Figure 2: Flowering pathway in cereals. Schematic overview of the molecular pathways involved in the regulation of flowering in temperate cereals (*H. vulgare* and *T. aestivum*) according to Wu et al. 2013, Chen et al. 2014 and Fjellheim et al. 2014: a) leaf; b) shoot apex.

In the vernalization-responsive accessions of the model grass *B. distachyon*, the roles of *VRN1* and *VRN3* in the regulation of vernalization seem to be conserved. Vernalization induces the transcription of *VRN1* (Colton-Gagnon et al. 2014) and during long days, *VRN3* levels increase faster in vernalized plants compared to non-vernalized plants (Ream et al. 2014). However, unlike the core Pooideae species, decreased expression patterns of the flowering-repressor gene *VRN2* during vernalization is not observed in *B. distachyon* and other vernalization-responsive species of early-diverging Pooideae lineages (Ream et al. 2014; Woods et al. 2016). Additionally, Woods et al. (2016) did not confirm the repressive function of the VRN1 transcription factor upon

VRN2 expression. Those findings suggest that vernalization response and the function of *VRN1* and *VRN3* is conserved in the Pooideae subfamily, but *VRN2* was first in the core Pooideae co-opted into the vernalization pathway.

Flowering in response to daylength

Long days induce flowering in core Pooideae species (King et al 2006; Greenup et al. 2009, Sasani et al. 2009) and *B. distachyon* (Ream et al. 2014), which distinguishes them from related short day-requiring grasses like *Oryza sativa* (rice [Hayama et al. 2003]). In *H. vulgare and T. aestivum* the gene *PHOTOPERIOD1* (*PPD1*) seems to be the main integrator of photoperiodic signals (Fig. 2). It is expressed under long days and promotes flowering by inducing *VRN3/FT* expression (Turner et al. 2005; Higgins et al. 2010). The protein VRN3/FT is able to form heterodimers with FLOWERING LOCUS D-like (FDL) proteins that bind to the *VRN1* promoter and thus might form a positive feedback-loop after vernalization and in long days (Li and Dubcovsky 2008). Accumulation of the MADS-box transcription factor VRN1 in the shoot apex promotes the transition from vegetative to reproductive stage (Danyluk et al. 2003; Murai et al. 2003). Recently *PHYTOCHROME C* (*PHYC*) was identified as part of the photoperiodic flowering pathway (Fig. 2) mediating long day induced activation of *PPD1* expression (Distelfeld and Dubcovsky 2010; Chen et al. 2014). There was also evidence that *PHYC* up-regulates *VRN2* during long days (Fig. 2), which would infer that the *VRN2*-mediated repression of *FT* is epistatic in relation to *PPD1*-mediated activation.

In a recent study, Wu et al. (2013) investigated the function of the newly discovered microRNA miR5200 (previously identified as miR2032 [Wei et al. 2009]) in *B. distachyon* accession Bd21-3, and found that it represses flowering under short days (Fig. 2). MiR5200 targets the two *FT*-like *B. distachyon* genes and negatively regulates their expression. Additionally, overexpression and knock-down of the microRNA gene delayed and accelerated flowering, respectively. Northern blots of core Pooideae species grown under different daylengths showed an accumulation of miR5200 under short-day (Wu et al. 2013). Based on these findings and the fact that miR5200 is not conserved outside the Pooideae, Wu et al. (2013) proposed that differential expression of miR5200 under different daylengths regulates photoperiod-dependent flowering in Pooideae.

1.4 Biome shift and adaptive evolution

In this thesis the concept of a biome is used as a term for “broad vegetation types defined by climate, life-form and ecophysiology” (Crisp et al. 2009, page 754) which goes back to the work of Woodward et al. (2004). The term temperate is used here to describe climates that correspond to the warm-temperate and snow climates defined by the Köppen-Geiger climate classification, including cool steppe climates (updated by Kottek et al. 2006). Thus, temperate biomes are characterized by strong, annual temperature seasonality and frequently-occurring frosts.

The Poaceae family likely originated the last ~80-50 Mya (Kellogg 2001; Christin et al 2014; Magallón et al. 2015) when global climate was warm (Zachos et al. 2001; Mudelsee et al. 2014) and tropical biomes more abundant and expanded than today (Fine and Ree 2006; Greenwood et al. 2010; Harrington et al. 2012; Pross et al. 2012). During the early Eocene, subtropical and tropical biomes like rainforests were abundant and even reached the Arctic and Antarctic (Pross et al. 2012; Greenwood et al. 2010). In the late Eocene, climate gradually cooled and temperate biomes expanded at the expense of tropical biomes (Potts and Behrensmeyer et al. 1992; Kerkhoff et al. 2014). This expansion was intensified by a climate supercooling during the Eocene-Oligocene (E-O) transition, around 34 Mya (Zachos et al. 2001, Eldrett et al. 2009). The E-O transition likely affected the distribution range of many plant taxa, and most of the extant temperate angiosperm lineages emerged from tropical ancestors during the last 34 million years (Kerkhoff et al. 2014; Meseguer et al. 2015). Sandve and Fjellheim (2010) hypothesized that the super cooling during the E-O transition affected the evolutionary history of the Pooideae subfamily by increasing selection pressure for improved cold adaptation.

Shifts to temperate biomes are thought to be rare (Donoghue 2008) and in the Poaceae, cold tolerance is predominantly restricted to two subfamilies. In the PACMAD clade only lineages of the Danthonioideae subfamily managed to evolve cold tolerance (Humphreys and Linder 2014). Among the members of the BOP clade (Fig. 1), who share a tropical ancestor that was distributed in Africa (Bouchenak-Khelladi et al. 2010), the Pooideae are the only subfamily that managed the shift to temperate biomes

(Edwards and Smith 2010). Given the modern distribution of the Pooideae (Hartley 1973) and the lack of entirely tropical Pooideae lineages, it is most parsimonious to assume that an early ancestor of the Pooideae was adapted to temperate conditions. Results from several studies support this assumption. Edwards and Smith (2010) inferred that the shift to temperate biomes occurred at the base of the Pooideae phylogeny. Woods et al. (2016) presented evidence suggesting that vernalization response evolved early in the Pooideae subfamily. Finally, genes involved in cold stress response have been under positive selection in early Pooideae lineages (Vigeland et al. 2013). The complex mechanisms regulating cold response and flowering time in core Pooideae suggest that the Pooideae's shift to temperate conditions must have been accompanied by major adaptive changes. It is however still unclear, when particular adaptive changes emerged and which genetic mechanisms contributed to their evolution.

Studying adaptive traits can contribute to the understanding of the evolutionary mechanisms that facilitated biome shifts. The reconstruction of the independent evolution of C4-photosynthesis in several PACMAD grasses for instance, indicated that the potential to evolve C4-photosynthesis was influenced by the anatomical traits and the genetic constitution of the PACMAD ancestor (reviewed by Christin and Osborne 2014; Christin et al. 2015). Several, independent C4-lineages preferentially co-opted the same genes into the C4 pathways, suggesting that suitable, pre-existing genes might have facilitated the evolution of C4-photosynthesis (Christin et al. 2013).

Molecular mechanisms underlying the Pooideae's temperate adaptation have mainly been studied in economically important plants of the core Pooideae (described above). Despite the model grass *B. distachyon* recently becoming subject of comparative studies (Li et al. 2012; Colton-Gagnon et al. 2014; Ream et al. 2014; Woods et al. 2016), we still lack knowledge about cold adaptation and flowering time regulation in the early-diverging Pooideae lineages. Knowledge about the evolutionary history of temperate adaptations would allow us to reconstruct how the Pooideae adapted to environmental conditions of the temperate biomes. Despite this knowledge gap, three studies provided some insights into evolutionary mechanisms involved. Sandve and Fjellheim (2010) found evidence that gene family expansion played a role in the

evolution of cold adaptation genes in core Pooideae. Sandve et al. (2008) and Li et al. (2012) showed that the ice-binding domain of IRIPs emerged and multiplied in an ancestor of Brachypodieae and core Pooideae, possibly as response to increased cold stress. Additionally, multiple gene duplications contributed to a diversified cold adaptation (Sandve et al. 2008; Li et al. 2012). Whether similar genetic mechanisms exist in early-diverging Pooideae lineages is unknown. Also, the extent to which core Pooideae, Brachypodieae and early-diverging lineages share temperate adaptations is not clear.

Being able to date evolutionary events in order to correlate them with paleoclimatic conditions is another important aspect of the reconstruction of adaptive evolution. Due to the lack of fossils, molecular dating of Pooideae evolution is challenging and several authors suggested a time between 41.4 and 53 Mya for the divergence of Pooideae and Oryzoideae (Bremer 2002; Gaut 2002; Strömberg 2005; Sandve et al. 2008; Bouchenak-Khelladi et al. 2010; Vicentini et al. 2008; Christin et al. 2014), others even up to ~75 Mya (Prasad et al. 2011; Christin et al. 2014). Macrofossils assigned to the Pooideae tribe Stipeae are dated to 35 Mya (Manchester 2001), which sets the minimum age for this lineage. In a recent attempt to date the divergence of the core Pooideae and the Brachypodieae tribe, an average divergence time of 44.4 Mya was estimated using three chloroplast loci (Marcussen et al. 2014). Those results suggest that the Pooideae subfamily emerged during the warm climate of the early to mid-Eocene. In the light of this assumption, it appears questionable if the Pooideae MRCA had to cope with similar climatic conditions as extant Pooideae species cope with today. Like the ancestor of the C4-grasses however, the Pooideae MRCA might have influenced the evolutionary potential of its descendants to adapt to temperate conditions. Pre-existing traits might have been instrumental in the initial colonization of the newly emerging, temperate biomes.

1.5 Hypotheses and objectives

The biogeographic history renders the Pooideae subfamily an excellent system to study evolutionary aspects of biomes shifts – from tropical to temperate – in a large plant

group and over a long time period. The shared origin of the extant Pooideae lineages suggests that at least a fraction of their adaptation to temperate biomes dates back to a common source and is conserved throughout the subfamily. If such conserved adaptations exist, they might have been instrumental in the shift from tropical to temperate biomes. This hypothesis formed the starting point for the design of this PhD project. Accordingly, all experiments were set up in a comparable manner, to test if adaptive features – mostly known from core Pooideae – are conserved in other Pooideae lineages. A central role was played by the Pooideae lineages that diverged between the Pooideae MRCA and the 'Brachypodieae-core Pooideae' clade, i.e. the early-diverging lineages (Fig. 2). We previously had no knowledge about the cold response and flowering time regulation in early-diverging Pooideae lineages and started to fill this knowledge gap with the experiments conducted in this PhD project.

The motivation for this project was to gain insights into the early adaptive evolution of Pooideae subfamily, to i) increase our understanding of the importance of various adaptations for the Pooideae's diversification into temperate biomes, ii) where in the phylogeny those adaptations evolved and iii) which evolutionary processes might have played a role during their biome shift.

The objectives of this PhD thesis were to:

1) Compare expression patterns in response to cold between a variety of Pooideae species, to determine the fraction of conserved, cold-responsive genes in five Pooideae species (**Paper I**).

2) Reconstruct the phylogenetic history of genes known to be involved in cold adaptation in core Pooideae to test if they are conserved in early-diverging Pooideae lineages (**Paper II**).

3) Determine if species from early-diverging lineages respond to vernalization and if *VRN1* and *VRN3* are induced during and after vernalization, respectively, in order to test if vernalization response is conserved in the Pooideae (**Paper III**).

4) Compare expression patterns of *miR5200* in Pooideae plants during short day to test if microRNA-mediated repression of *FT* expression could have evolved early in Pooideae (**Paper IV**).

2 Summary of material and methods

During this PhD project we conducted three experiments to investigate the evolutionary history of genes and traits, connected with the Pooideae's adaptation to temperate conditions.

In the first experiment (described in **Paper I** and **II**), we subjected Pooideae plants (core Pooideae *H. vulgare*, model grass *B. distachyon* and species of early-diverging lineages *Stipa lagascae* [tribe Stipeae], *Melica nutans* [tribe Meliceae] and *Nardus stricta* [tribe Nardeae]) to cold conditions and recorded gene expression after exposure to short- and long-term cold. Using RNAseq, we produced *de novo* transcriptomes for five Pooideae species. To test to what extent cold-responsive genes are shared among Pooideae, we compared the gene expression in response to cold between the five species applying a novel analysis pipeline to identify orthologous genes (**Paper I**). Cold acclimation plays a central role in the core Pooideae's adaptation to cold, but it is not known if cold acclimation also exists in other Pooideae lineages. Therefore, we tested if species from several early-diverging Pooideae lineages exhibit improved frost tolerance after cold acclimation (**Paper II**). Additionally, we reconstructed the evolutionary history of well-studied, cold-responsive gene families, applying phylogenetic methods on *de novo* transcriptome data.

In the second experiment, we investigated the evolution of vernalization in the Pooideae subfamily (**Paper III**). We tested if early-diverging Pooideae species exhibited accelerated flowering in response to vernalization and identified genes involved in vernalization. Using qRT-PCR, we tested if well-studied expression patterns of *VRN1* and *FT* were conserved in vernalized plants of early-diverging lineages compared to non-vernalized plants. Using ancestral state reconstruction, we tested the hypothesis that vernalization and cold-induced expression pattern of *VRN1* evolved early in the evolution of the Pooideae.

In the third experiment, we tested the recent hypothesis that a newly discovered microRNA (miRNA5200 [Wu et al. 2013]) is involved in regulation of the Pooideae's

flowering by suppressing *FT* under short days (**Paper IV**). Using the transcriptome data produced in the first experiment, we identified species from early-diverging lineages that contained miR5200 and performed classical growth experiments under short- and long-day conditions. Conducting qRT-PCR we tested if miR5200 and *FT* were differentially expressed.

3 Main findings and discussion

3.1 A shared potential to evolve temperate adaptations

Several of our results provide evidence for conserved temperate traits in the Pooideae subfamily. All species investigated in Paper I shared a small, but statistically significant number of cold-responsive genes that exhibited conserved, cold-responsive expression patterns. A closer investigation revealed that most of those genes were induced by short term cold and many of them are known to respond to osmotic stress in other angiosperms. Thus it is likely that this type of stress response already evolved in an ancestor of the angiosperms. The two genes *DHN8* and *DHN13*, known to be important in cold response in core Pooideae species, were conserved and possessed the same expression patterns in all investigated species of Paper I and II. Like most of the genes with conserved cold response identified in Paper I, *DHN8* and *DHN13* were induced by short term cold. One of them, *DHN8*, is known to be involved in osmotic stress outside the Pooideae (Lee et al. 2005; Badicean et al. 2012). In total, six out of the ten investigated genes in Paper II possessed cold induced homologs in early-diverging lineages, suggesting that they already were present in the MRCA. We also provide evidence that vernalization-induced up-regulation of *VRN1* and subsequent long day induction of *VRN3* likely evolved in the MRCA (Paper III).

These findings support the hypothesis that early Pooideae species shared the evolutionary potential to evolve adaptations to temperate climates. For example, short term cold-induced genes involved in osmotic stress response and regulation of photosynthesis (see gene ontology (GO) enrichment tests, Paper I) seem to have played an important role as early adaptations to cold. These type of genes might have evolved

from originally drought-responsive genes and they must have been beneficial for early Pooideae species in response to osmotic stress during sudden frost spells or to adjust the photosynthetic machinery during chilling.

Although not all tested Pooideae species responded to vernalization, *VRN1* was most likely cold-induced in the Pooideae MRCA (Paper II), suggesting that a rapid transition from vegetative to reproductive state during a prolonged period of cold was beneficial for the Pooideae MRCA. Winters did not necessarily have to be very cold to induce this transition, since vernalization is already effective at mild temperatures up to ~12°C (Robertson et al. 1996; Ream et al. 2014). This result hints to the possibility that the Pooideae MRCA might have experienced seasonal variations in temperature.

Factors that promote or impede biome shifts of plant lineages have been discussed by several authors (Wiens et al. 2004; Donoghue 2005; Crisp et al. 2009; Crisp and Cook 2012; Edwards and Donoghue 2013; Donoghue and Edwards 2014), who also underlined the important role that pre-existing traits and adaptive potential play in the facilitation of biome shifts. For instance, it has been shown that adaptations of the Antarctic fellfield flora were established by refining pre-existing stress responses rather than evolving novel adaptations (Block et al. 2009). I already mentioned studies by Christin et al. (2013, 2015) showing how pre-existing genetic and anatomic traits enabled the evolution of C4-photosynthesis in grasses of the PACMAD clade. In a recent study, Yeaman et al. (2016) could show how the potential of two distantly related conifer species to evolve cold adaptations was genetically constrained and restricted to certain genes. Furthermore, although not involved in biome shifts, the evolution of adaptive traits in certain legume lineages could be traced back to pre-existing evolutionary “precursors” (Marazzi et al. 2012).

Shifts to temperate biomes are rare (Donoghue 2008), because many lineages are thought to lack the evolutionary potential or opportunity (Donoghue and Edwards 2014) to adapt to temperate climatic conditions. Apparently, the Pooideae subfamily was able to perform this shift, but it is not clear which role the few conserved traits that have been described here played in the Pooideae’s shift to temperate biomes. We hypothesize that they likely provided a selective advantage for early Pooideae species during the first

encounters with temperate conditions. A handful of cold responsive genes might have offered enough protection against irregularly occurring stresses to assure survival during an early phase of temperate colonization. Although the Pooideae MRCA might not have been a temperate grass itself, it likely equipped its descendants well for their future journey into temperate biomes.

3.2 Evidence for recent, lineage-specific adaptations

Results from Paper I revealed large fractions of lineage-specific, cold-responsive genes, which indicate that most of the genes involved in cold response evolved after the main lineages had diverged from each other. This assumption is further supported by a lack of phylogenetic signal in regulatory similarities. In theory, closely related species with similar climate adaptations are more similar in gene regulation than species that are more distantly related. For example, gene expression of *B. distachyon* and *H. vulgare* is expected to be significantly more similar than gene expression of *H. vulgare* and *M. nutans*. However, we did not find such patterns among cold-responsive genes. Most of the genes investigated in Paper II also exhibited differences between lineages, caused by altered expression patterns and changed gene copy numbers.

Although cold acclimation seems to be part of the cold adaptation in all species investigated in Paper II, it is unlikely that this trait evolved in a common ancestor. If this would be the case, we would expect that genes, essential for cold acclimation in core Pooideae, were conserved. But we did not identify homologous genes for the *DHN5*, *FST* or *IRIP* family in early-diverging lineages, nor were homologous genes from the *CBFIV* family induced by long-term cold. Therefore, we hypothesize that genetic networks regulating cold acclimation mostly evolved independently in Pooideae lineages, with one exception – the *ctCOR* family.

Cold acclimation is a compound trait consisting of a highly complex interplay between a large amount of genes (Thomashow 2010). At the core of cold acclimation processes lies an interaction between light and temperature pathways (Gray et al. 1997; Hüner et al. 2013), whereby the chloroplast is often sensor of cold signals and target of early acclimation (Crosatti et al. 2013). *Chloroplast-targeted COR* genes are well-

known for their involvement in the cold acclimation of the chloroplast. Interestingly, we found that homologs of the *ctCOR* family were induced by long term cold in all but one species of early-diverging lineages (Paper II). Based on this finding, we hypothesize that *ctCOR*-mediated cold acclimation of the chloroplast was the first step in the Pooideae subfamily towards a more complex regulation of cold acclimation. However, also the *ctCOR* family exhibits signs of lineage specific evolution, since there has been at least one duplication events that lead to two *ctCOR* paralogs in the core Pooideae.

Recent insights in the flowering time regulation of *B. distachyon* and species from early-diverging lineages suggests a similar evolutionary scenario for vernalization response. We have learned that cold regulated *VRN1* likely existed in the Pooideae MRCA (Paper III). But the function of *VRN2* to suppress *FT* and ultimately flowering in long days before winter first evolved in the core Pooideae (Ream et al. 2014; Woods et al. 2016). Hence, the core mechanism of vernalization response, a rapid transition of the shoot apex into a reproductive state during cold, might have been the first stage in the evolution of vernalization responsiveness. *VRN2* was then later co-opted into the vernalization pathway of the core Pooideae to further fine-tune flowering regulation.

Fine-tuning of flowering regulation is also mediated by miR5200-induced repression of *FT* in *B. distachyon* control accession Bd21-3 (Paper IV). Although we found the miR5200 sequence to be conserved throughout the Pooideae, we observed short day-induced expression exclusively in Bd21-3. MiR5200 might also be involved in flowering regulation in the core Pooideae, but we could not identify short day-induced expression in *H. vulgare*. Previously reported short day-induced accumulation of miR5200 in core Pooideae might therefore stem from a daylength-sensitive microRNA maturing process.

Taken together, these findings suggest that the major Pooideae lineages, i.e. tribes, evolved large parts of their adaptations to temperate biomes after they had diverged from each other. Many mechanisms underlying cold adaptation and flowering regulation are known in core Pooideae and *B. distachyon*. It is plausible that similar mechanisms also regulate the cold response and flowering in species of the early-diverging lineages, but further research is needed to reveal them.

3.3 Expansion of cold responsive gene families

Beside the utilization and refinement of existing traits, plant lineages evolve novel traits during biome shifts and ongoing research attempts to identify evolutionary mechanisms that lead to such adaptive innovation. The expansion of gene families (Lespinet et al. 2002, Flagel and Wendel 2009) is one mechanism that seems to have been important for cold adaptation in Pooideae. Sandve and Fjellheim (2010) suggested that gene family expansion in the *FST*, *IRIP*, *CBFIIIc/d* and *CBFIV* gene families contributed to the cold adaptation of the Pooideae. Our results showed that expansion of the *CBFIIIc* and *CBFIV* gene family could not have contributed to cold adaptation of the entire Pooideae subfamily, because they are specific for the core Pooideae lineage. Interestingly, there is evidence supporting the importance of *CBFIIId* expansion for cold adaptation. In three of the investigated Pooideae lineages, i.e. Nardeae, Brachypodieae and core Pooideae, we identified independent expansion events of the *CBFIIId* family (Paper II) that were dated around or shortly after the E-O transition. Members of the CBF family are important transcription factors involved in mediating stress response (Akhtar et al. 2012). There is evidence that the expansion of transcription factor families is an important feature of Pooideae cold adaptation. Lee et al. (2013) showed that in *Deschampsia antarctica* – one of the only two angiosperms in Antarctica – stress-related transcription factor families were significantly expanded when compared to other Pooideae species. Also the stress-responsive *NAC* transcription factor family has been shown to be expanded in *B. distachyon* (Zhu et al. 2015).

We furthermore identified Pooideae-specific gene family expansions of the Y_nSK_n-type *DHN* gene family and lineage-specific expansions of *ctCOR* and *IRIP* gene family (Paper II), the latter confirming results from Li et al. (2012). Also results from Paper I indicated that gene family expansion was important in the Pooideae's cold adaptation. Many, previously identified cold responsive transcripts (Greenup et al. 2009) have high rates of duplications, creating complex gene family structures (Paper I). Hence, our results emphasize the importance of gene family expansion for the evolution of cold adaptation. These findings support previous studies, like for instance Hanada et al. (2008), who showed that gene families expanded by tandem duplications tended to be

involved in stress response and environmental stimuli. A recent study investigating cold adaptation in two independent conifer species (Yeaman et al. 2016) found that genes involved in cold adaptation tended to be duplicated.

Evolution of new protein motifs as well as motif multiplication seem to have played a role in the Pooideae's cold adaptation. Sandve et al. (2008) and Li et al. (2012) suggested that *IRIP* and *FST* gene families emerged from newly evolved protein motifs which is supported by our data (Paper II). Results from Paper II indicate that also the *DHN5* gene family might have evolved through motif multiplication. Genes in this family code for K_n-type dehydrins that exclusively consist of several K-segments – the characteristic protein motif omnipresent in all dehydrins (Close 1997). Here we show that the *DHN5* gene family is limited to the Triticeae tribe, suggesting *DHN5* in *H. vulgare* and its ortholog *WCS19* in *T. aestivum* evolved, putatively by motif multiplication of existing K-segments, after the Triticeae had diverged from the other core Pooidea tribes.

In addition to gene family expansion and the evolution of novel protein motifs, adaptive novelties may also arise through changes in *cis*-regulatory elements (reviewed by Wittkopp and Kalay, 2012), especially by creating new spatial and/or temporal expression patterns of existing genes (Holloway et al. 2007). In *H. vulgare*, repression of *VRN1* expression before winter is mediated by *cis*-regulatory elements in its intron and promoter region (reviewed by Trevaskis, 2010). Because the cold-induced expression pattern of *VRN1* is conserved in the Pooideae (Paper III), a next step in the investigation of its co-option history will be to test if the same *cis*-regulatory elements are involved in *VRN1* repression in *B. distachyon* and early-diverging Pooideae species. Interestingly, Zou et al. (2009) found that *cis*-regulatory elements of genes responding to environmental stimuli in *Arabidopsis thaliana* were inherited asymmetrically during gene duplication events. This means that one of the duplicated genes loss a bigger fraction of *cis*-regulatory elements than its paralog. This mechanism is assumed to provide new sources for adaptive novelties, since the gene that retained less of the regulatory element has an increased evolutionary potential to gain a new response. The *CBFIV* gene family is an interesting candidate to test the described scenario. In Paper II

we found that core Pooideae species had up to four copies with diverse expression patterns that likely evolved through multiple duplications of an ancestral copy.

3.4 Connection between vernalization and cold acclimation evolved in the core Pooideae

As described above, complex cold acclimation pathways in Pooideae result from an interplay of temperature and light which evolved independently in differnet lineages. Additionally, it has been suggested that cold acclimation is regulated by photoperiod and vernalization pathways in *T. aestivum* (Fowler et al. 2001; Limin and Fowler 2006). Several studies found that plants from temperate cereals were less tolerant to frost when grown under long day as opposed to plants grown under short day (Limin and Fowler 2006, Stockinger et al. 2007, Dhillon et al. 2010). Similarly, vernalization and *VRN1* expression reduced frost tolerance. Galiba et al. (2010) hypothesized that temperate cereals had evolved a way to use the environmental cues affecting *VRN1* expression to down-regulate genes involved in cold acclimation. This hypothesis was based on the findings that *CBF, DHN* and *ctCOR* genes are down-regulated by *VRN1* (Dhillon et al. 2010; Galiba et al. 2009).

The Pooideae-specific *CBF* genes form three distinct gene families (*CBFIIIc, CBFIIId* and *CBFIV* [Badawi et al. 2007]) as confirmed by results in Paper II. We could show that all the *CBF* genes down-regulated by *VRN1* (Dhillon et al. 2010; Galiba et al. 2009) exclusively belong to the *CBFIV* family that first expanded in the core Pooideae. The only *DHN* gene that is known to be down-regulated by *VRN1* is *DHN5*, which we showed is Triticeae-specific (Paper II). From the two *ctCOR* genes, *COR14*, but not *WCS19* was down-regulated by *VRN1* (Dhillon et al. 2010). Since a core Pooideae-specific duplication gave rise to those two genes (Paper II), the *COR14-VRN1* connection might have evolved after this duplication.

Based on these findings, we hypothesize that the complex interplay of cold acclimation and vernalization pathways, known from core Pooideae species and possibly including the photoperiod pathway (Chen et al. 2014), evolved independently from early-diverging lineages and Brachypodieae. Hence, the integration of several

regulatory pathways might be a recent adaptation which emphasizes the importance of a cross-talk between adaptive mechanisms to trade off their consequence for the plant. Further support for this scenario stems from evidence that *VRN2* was first co-opted into the vernalization pathway in the core Pooideae to tightly regulate time of flowering (Woods et al. 2016). It is possible that these three pathways are also interconnected in early-diverging lineages and Brachypodieae, but this interconnection likely evolved recently and independent form other lineages. If this idea holds true and all Pooideae lineages faced similar environmental conditions during the biome shift, we would expect to find that cold acclimation and photoperiod pathways of early-diverging Pooideae lineages and Brachypodieae are interconnected with the vernalization pathway.

3.5 Was the Pooideae MRCA a long day plant?

Core Pooideae species are known long day plants, i.e. the development of their inflorescences is accelerated by long days (Dubcovsky et al. 2006; Trevaskis et al. 2006, Schwartz et al. 2010). Since the Pooideae share a common, most likely tropical, ancestor with *O. sativa* (rice, [Bouchenak-Khelladi et al. 2010]) which is a tropical, short day plant (Hayama et al. 2003), long day-induced flowering seems to be a Pooideae-specific adaptation to the conditions in temperate biomes. Supporting this assumption, it was shown that short days can compensate a missing vernalization period in vernalization-responsive accessions of core Pooideae species and *B. distachyon* (Dubcovsky et al. 2006; Trevaskis et al. 2006, Schwartz et al. 2010).

It is not known how species of early-diverging lineages respond to different daylengths, but it is possible that the Pooideae MRCA was a long day plant. We did not specifically test if flowering in species of early-diverging lineages was significantly accelerated by long days compared to short days. However, parts of our experiments speak against the scenario that long day-induced flowering evolved in the Pooideae MRCA. Flowering was in fact induced in long days in some of the vernalized plants of early-diverging lineages (Paper III), but it is not clear if those plants flowered primarily in response to vernalization. Species from the earliest diverging lineages, i.e.

Brachyelytrum aristotum, *Lygeum spartum* and *N. stricta*, did not flower during the whole experiment, even when vernalized, thus contradicting the idea of long day-induced flowering in those species. Although, miR5200 was conserved in *N. stricta* and *S. lagascae*, short day-induced repression of *FT* (which might hint at a long day-induced flowering) was not conserved and *M. nutans* had completely lost miR5200. It is plausible that miR5200 is involved in the regulation of *FT*-expression due to its complementary sequence, but it needs to be investigated further how miR5200 responds to environmental cues in *N. stricta* and *S. lagascae*. It is still unclear if long day-induced flowering and its underlying, regulatory mechanisms are conserved in the Pooideae subfamily, but based on our anecdotal observations it seems now less likely that the Pooideae MRCA was a long day plant.

3.6 Did the E-O transition shape adaptive evolution of Pooideae?

Sandve and Fjellheim (2010) hypothesized that the drop in temperature during the E-O transition, which led to mass extinctions (Ivany et al. 2000), increased selection pressure for improved cold tolerance in Pooideae. The study used calibrated gene trees to show that gene families involved in cold response expanded during the E-O transition. Such analyses, however, are very sensitive to correctly placed calibration points and newly available data push our estimates for the core Pooideae-specific expansion of *IRIP* and *CBFIV* (Paper II) back in time relative to the results from Sandve and Fjellheim (2010). Additionally, we could show that the expansion of the *CBFIIId* lineages might have happened at the base of the Pooideae phylogeny. Interestingly, three expansions of the *CBFIIId* family in independent lineages and one core Pooideae-specific duplication event in the *ctCOR* family correlated with the E-O transition. Only the expansion of the *FST* family exhibited similar time estimates as the results presented by Sandve and Fjellheim (2010).

In addition to causing mass extinctions, sudden climatic changes can drive evolution of novel adaptations (Nevo 2011). As the climatic impulses of the E-O transition led to the expansion of temperate biomes (Potts and Behrensmeyer 1992) and

many extant temperate plant lineages first emerged after the E-O transition (Kerkhoff et al. 2014, Meseguer et al. 2015), it is plausible that also the evolutionary history of the Pooideae subfamily was influenced the E-O transition. The cumulative evidence of recent estimates for Pooideae divergence time (Christin et al. 2014; Marcussen et al. 2014, Paper II and III in this thesis) indicate that at least some features important for temperate adaptation in Pooideae are older than the E-O transition. Hence, it is very likely that the major Pooideae lineages, i.e. tribes (Soreng et al. 2015), experienced independent selection pressure introduced by the E-O transition.

It remains unclear which cold adaptations evolved in each of the Pooideae lineages during the gradual climate cooling in the mid to late Eocene, and which evolved as a response to the supercooling at the E-O transition. Finally, the rapid climatic oscillations during the past few million years (Zachos et al. 2001) might have dramatically influenced the evolution of temperate organisms (Hewitt 1996, Hewitt 2004). Therefore, we would expect to find adaptive traits and adjustments of regulatory networks that evolved in the Pooideae during this time period and that thus are specific to lineages that emerged recently.

4 Concluding remarks and future perspectives

In conclusion, we found evidence for conserved ortholog family structure and cold-regulation of genes and traits, that are important elements of vernalization and cold response and that might have been instrumental in the early colonization of temperate biomes. However, our work suggests that the majority of molecular cold-responses that likely represent adaptations to temperate climate evolved independently and did not exist in the Pooideae MRCA. Our results further support previously presented hypothesis suggesting that expansion of gene families and evolution of novel protein motifs were involved in cold adaptation. We also found evidence that the E-O transition played a role in shaping the evolution of cold adaptation in several Pooideae lineages. Although flowering in core Pooideae and Brachypodieae is induced under long days

(Dubcovsky et al. 2006; Trevaskis et al. 2006, Schwartz et al. 2010), and in both clades miR5200 seems to be involved in suppressing flowering under short days (Wu et al. 2013; Paper IV in this thesis), it is still unclear if the Pooideae MRCA was a long day plant. Finally, we hypothesize that the integration of several molecular pathways, regulating vernalization, cold acclimation and photoperiodism is a recent innovation which at least evolved in the MRCA of the core Pooideae, but possibly in other lineages as well.

The knowledge of evolutionary changes accompanying biome shifts in plants not only contributes to the concept of how phenotypic variation evolves in response to the environmental changes, but also leads to a better understanding of the complex genetics underlying adaptive traits. The Pooideae subfamily harbors major crops of temperate regions and expanded knowledge of their evolutionary history will be beneficial for plant breeding. Major advances in breeding techniques (Al-Khayri et al. 2015) and targeted genomic editing (Morrell et al. 2012; Bortesi and Fischer 2015; Kumar and Jain 2015) offer precise methods to produce well-adapted crops (Hartung and Schiemann 2014), but many traits, important from a breeding perspective, are regulated by complex molecular networks involving several genes. Insights into the evolution of such networks enable breeders to more precisely target genes to generate desirable traits. Therefore, utilizing the knowledge from evolutionary studies becomes increasingly important for plant breeding (Morrell et al. 2012; Lenser and Theißen 2013). The results presented in this project, emphasize the adaptive differences between lineages but also indicate common “core” traits that likely formed the corner stone of the Pooideae’s ecological success.

To date many genes involved in vernalization and cold tolerance have been identified in Pooideae. Although new genes and their functions continue to be discovered (Kippes et al. 2015), future research will also focus on the evolution of gene expression and the origin of expressional diversity. Concerning the Pooideae, comparative studies will strive to investigate the conservation of cis-regulatory elements of the *VRN1* gene in early-diverging Pooideae. As *VRN2* does clearly not possess a conserved role in vernalization in early-diverging lineages (Woods et al. 2016), and it

will be important to investigate its function in early-diverging lineages and compare *VRN2* to its homolog in *O. sativa, Grain number, plant height, and heading date7* (*Ghd7*). The identification of changes in expression patterns across several Pooideae lineages (Paper II) offers an excellent basis to study the evolution of gene regulation in cold responsive gene families. Furthermore, the lineage-specific, cold-responsive genes identified in Paper I are promising candidates to investigate and compare to genes that got co-opted into cold adaptation by several lineages.

Like cold tolerance and vernalization response, long day-induced flowering is an important adaptation for temperate plants. Although its regulation involves many molecular pathways in Pooideae species (Fjellheim et al. 2014), the genes *PHYC* and *PPD1* seem to be the main determinants of long day-induced flowering (Chen et al. 2014). To elucidate the evolutionary history of long day-induced flowering in Pooideae, ongoing and future research will investigate flowering response to different daylengths in early-diverging lineages and will test the conservation of *PHYC* and *PPD1*. This will contribute to more complete picture of early Pooideae evolution and clarify the importance of long day-induced flowering for the temperate biome shift.

Divergence times of Poaceae and particular Pooideae lineages are still under debate and our analyses demonstrate how important a robustly dated Pooideae phylogeny is for the reconstruction of the evolutionary history of gene families. Especially the uncertainty introduced by phytolith data and the discrepancy between chloroplast and nuclear markers (Christin et al. 2014) calls for improved, dated phylogenies with higher resolution. Although more detailed models of the paleoclimate in temperate biomes would be beneficial, the incorporation of more, internal calibration points derived from taxonomical unambiguous macrofossil data to strengthen the estimates of Pooideae divergence times should be the focus of future studies.

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Paper I

Comparative genomics reveal lineage-specific cold response evolution in Pooideae

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Abstract

The subfamily Pooideae is one of the few grass lineages that adapted to temperate biomes. The temperate biomes are characterized by strong seasonality and frequently occurring frost. Genes involved in the cold adaptation of Pooideae are well-studied in economically important species like *Triticum aestivum* (wheat), *Hordeum vulgare* (barely) and *Lolium perenne* (perennial ryegrass), but little is known about the molecular basis for cold adaptation of non-cultivated species. The geographical distribution of its nearly 4200 species suggests that adaptation to cold is a shared feature among Pooideae species. To test this hypothesize we performed classical growth experiments under cold and warm conditions and compared the cold response of five Pooideae species. Beside *H. vulgare*, we included the model grass *Brachypodium distachyon* and three species of early-diverging lineages in our experiments and used *de novo* comparative transcriptomics to investigate differential gene expression in response to cold. We found that the five species share a small, but significant number of cold-responsive genes. Most of the genes involved in cold response however, evolved within each lineage, indicating that a large fraction of cold adaptation evolved after the main Pooideae lineages had diverged from each other. These results speak against the hypothesis that the most recent common ancestor (MRCA) of the Pooideae was a cold adapted grass. However, it is likely that the genes inherited from the MRCA increased the potential of the Pooideae to evolve cold adaptation.

Introduction

The temperate grass flora is dominated by members of the subfamily Pooideae (Hartley 1973), and the most extreme cold environments are inhabited by Pooideae species (John et al. 2009). The ancestors of this group were, however, most likely adapted to tropical or subtropical climates (Bouchenak-Khelladi et al. 2010; Strömberg 2011). Many Pooideae species experience cold winters (Figure 1B) and although a recent study inferred adaptation to cooler environments at the base of the Pooideae phylogeny (Edwards and Smith 2010), it is still not known whether the Pooideae's most recent common ancestor (MRCA) already was adapted to cold stress, or if adaptation to cold evolved independently in specific Pooideae lineages.

Pooideae is a large subfamily comprising ~4200 species (Soreng et al. 2015), amongst them economically important species such as *Triticum aestivum* (wheat) and *Hordeum vulgare* (barley). Given the commercial importance of this group, various aspects of adaptation to temperate climate such as flowering time, cold acclimation, frost and chilling tolerance have been the object of numerous studies (reviewed by Tsvetanov, Atanassov, and Nakamura 2000; Galiba et al. 2009; Thomashow 2010; Kosová, Vítámvás, and Prášil 2011; Sandve et al. 2011; Tondelli et al. 2011; Crosatti et al. 2013; Preston and Sandve 2013). These studies are, however, confined to a handful of species in the species rich, monophyletic clade "core Pooideae" (Davis and Soreng 1993) and recently also to its sister clade, containing the model grass *Brachypodium distachyon* (Li et al. 2012; Colton-Gagnon et al. 2014). It is thus unknown how adaptation to temperate climate evolved in earlier-diverging Pooideae lineages to promote the success of this subfamily in temperate biomes.

Environmental stress is an assumed strong evolutionary force (Sandve and Fjellheim 2010; Nevo 2011), and the colonization of temperate biomes by Pooideae was likely accompanied by adaptation to cold conditions. A MRCA already adopted to cold (the ancestral hypothesis) offers a plausible basis for the ecological success of the Pooideae subfamily in the northern temperate regions (Hartley 1973). However, paleoclimatic reconstructions infer a generally warm climate and a limited abundance of temperate biomes during the time of Pooideae emergence, around 50 million years ago (Mya) (Zachos et al. 2001; Eberle and Greenwood 2011; Pross et al. 2012; Schubert et al. 2012; Mudelsee et al. 2014), which renders the acquisition of cold adaptation by the Pooideae MRCA unlikely.

It was not before ca. 34 Mya, during the Eocene-Oligocene (E-O) transition (Mudelsee et al. 2014), that the global climates suddenly began to cool (Figure 1A [Eldrett et al. 2009; Hren et al. 2013]). Indeed, climate cooling at the E-O transition coincided with the emergence of many temperate plant lineages (Kerkhoff et al. 2014) and also increased the selection pressure for improved cold tolerance in Pooideae (Sandve and Fjellheim 2010; Vigeland et al. 2013). If the E-O cooling event has been the major evolutionary driving force for cold adaptation in Pooideae grasses, those findings lend support for lineage-specific evolution of cold adaptation (the lineage specific hypothesis), as all major Pooideae lineages had already emerged during the time of the E-O transition (Bouchenak-Khelladi et al. 2010; Marcussen et al. 2014). During the parallel evolution of environmental adaptations in independent lineages same genes and gene families tend to be recruited (Christin et al. 2013; Preston and Sandve 2013; Christin et al. 2015). In case of several independent origins of cold adaptation, Pooideae species might have co-opted same genes into cold response pathways

A restricted number of plant lineages successfully transitioned into temperate biomes, emphasizing the difficulties in evolving the coordinated set of physiological changes needed to withstand low temperatures (Donoghue 2008). During freezing, plants above all need to maintain the integrity of cell membranes to avoid osmotic stress (Thomashow 1999). This is achieved through cold acclimation; a period of extended, non-freezing cold triggered by the gradually lower temperature and daylength in the autumn. Cold acclimation is a highly complex process comprising a suite of physiological changes and different molecular pathways resulting in an increase of sugar content in cells, change in lipid composition of membranes and synthesis of anti-freeze proteins (Janská et al. 2010; Preston and Sandve 2013). Also, low non-freezing temperatures may affect plant cells negatively by decreasing metabolic turnover rates, inhibiting the photosynthetic machinery and decreasing stability of biomolecules (e.g. lipid membranes) (Sandve et al. 2011; Crosatti et al. 2013). Several studies have used transcriptomics to compare cold stress response in disparate plants. However, these studies have focused on closely related taxa or varieties within model species (Carvallo et al. 2011; Zhang et al. 2012; Colton-Gagnon et al. 2014; Abeynayake et al. 2015; Lindlöf et al. 2015; Y.-W. Yang et al. 2015) and were therefore unable to investigate evolutionary mechanisms underlying adaptation to cold climates of entire clades.

Here, we use *de novo* comparative transcriptomics across the Pooideae phylogeny to study the evolution of cold adaptation in Pooideae. Transcriptomes of three non-model species *Nardus stricta*, *Stipa lagascae* and *Melica nutans* – which belong to early-diverging lineages – the model grass *Brachypodium distachyon* and the core Pooideae species *H. vulgare* (barley), were used to investigate if molecular responses to

cold are conserved in the Pooideae subfamily or if adaptation to cold evolved separately in different Pooideae lineages.

We found that in the investigated species only a small number of genes share a cold response, which suggests that their common ancestor only possessed few and possibly preliminary cold response mechanisms. Literature studies of genes that are cold responsive across all investigated species suggest that they belong to ancient pathways responding to abiotic stress. Beyond this conserved set of genes, many of the cold responses seem to have evolved lineage-specifically, indicating that a major part of the Pooideae's cold response evolved after the major Pooideae lineages had diverged from each other. Evidence for parallel co-option of cold response genes, suggests a shared adaptive potential of the Pooideae species.

Materials and methods

Plant material, sampling and sequencing

From five Pooideae species seeds were collected in nature: *Nardus stricta* (collected in Romania, [46.69098, 22.58302], July 2012), *Melica nutans*, (collected in Germany, [50.70708, 11.23838], June 2012); or acquired from germplasm collections: *Stipa lagascae* (PI 250751, U.S. Department of Agriculture (USDA) National Plant Germplasm System (NPGS) via Germplasm Resources Information Network [GRIN]), *Brachypodium distachyon* (line 'Bd1-1', W6 46201, USDA-NPGS via GRIN) and *Hordeum vulgare* (line 'Igri', provided by Prof. Åsmund Bjørnstad, Department of Plant Sciences, Norwegian University of Life Sciences). Seeds were germinated and initially grown in greenhouse at a neutral day length (12 hours of light), 17°C and a minimum artificial light intensity of 150 µmol/m2s. Depending on species, one to eight week old plants were randomized and distributed to two cold/vernalization chambers with short day (8 hours of light), 6°C and a light intensity of 50 µmol/m2s. Leaf material for RNA isolation was collected i) in the afternoon (8 hours of light) before (D0) and 8 hours after (D1) cold treatment and ii) in the morning (at lights on) before (W0), 4 weeks after (W4) and 9 weeks after (W9) cold treatment (Figure 1D). For each time point, 5 flash frozen leaves were individually homogenized using a TissueLyser (Qiagen Retsch) and total RNA was isolated (from each leaf) using RNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. Purity and integrity of total RNA extracts was determined using a NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Scientific) and 2100 Bioanalyzer (Agilent), respectively. RNA extracts were delivered to the Norwegian Sequencing Centre, where strand-specific cDNA libraries were prepared and

paired-end sequenced on an Illumina HiSeq 2000 system. Raw reads are available at the European Nucleotide Archive (ENA, http://www.ebi.ac.uk/ena) with accession number XXXXXXX.

Transcriptome assembly and ortholog inference

Illumina TruSeq adapters were removed from the raw reads and bad quality bases trimmed using a sliding window implemented in Trimmomatic v0.32 (Bolger et al. 2014). Read quality was controlled using fastqc v0.11.2. For each species, transcripts were assembled *de novo* with Trinity v2.0.6 (Grabherr et al. 2011) (strand specific option, otherwise default parameters) using reads from all samples. Coding sequences (CDS) in the transcripts were identified using TransDecoder rel16JAN2014 (Haas et al. 2013). In cases where Trinity reported multiple isoforms, only the longest CDS was used. Orthologous groups (OGs) were identified in the *de novo* transcriptomes together with public reference transcriptomes of *H. vulgare*, *B. distachyon*, *O. sativa*, *Z. mays*, *S. bicolor* (Table S1) and *L. perenne* (GenBank TSA accession GAYX01000000) using OrthoMCL v2.0.9 (Li et al. 2003).

High confidence orthogroup filtering

The protein sequences within each OG were aligned using mafft v7.130 (Katoh and Standley 2013) and converted to codon alignments using pal2nal v14 (Suyama et al. 2006). Gene trees were then constructed from the codon alignments using Phangorn v1.99.14 (Schliep 2011) (maximum likelihood GTR+I+G). Trees with apparent duplication events before the most recent common ancestor of the included species were split into several trees. This was done by identifying in-group (pooideae) and out-group (*Z. mays*, *S. bicolor* and *O. sativa*) clades in each tree, then splitting the trees so that each resulting sub-tree contain a single out-group and in-group clade. Further, the trees were filtered such that: all species in the tree each formed a clade (i.e. no out-paralogs); *B. distachyon* and *H. vulgare* formed a clade; and at least three of the five *de novo* species were included. The resulting split and filtered trees defined the HCOGs.

Species tree

Codon alignments for all OGs (after splitting the trees, see above) that had a single ortholog in each of our five pooideae and *O.sativa* (the other reference species were removed) were used to infer dated gene trees with BEAST v1.7.5 (Drummond et al. 2007). Prior estimate for the *Oryza*-Pooideae and *Brachypodium-Hordeum* divergence time was set to 53 Mya (Christin et al. 2014) and 44.4 Mya (Marcussen et al. 2014). A species tree was estimated based on the most common topologies of the gene trees and

internal node ages were estimated by taking the mean of the corresponding node ages of all the gene trees.

Differential gene expression

The reads were aligned to the corresponding *de novo* transcriptomes using bowtie v1.1.2 (Langmead et al. 2009), and read counts were calculated with RSEM v1.2.9 (Li et al. 2011). In HCOGs containing paralogs, the read counts of the paralogs were summed for each species. In HCOGs with missing orthologs, the read count of the missing ortholog was set to zero. The resulting read counts for all samples were then placed in a single multispecies expression matrix. Differential expression was calculated with DESeq2 v1.6.3 (Love et al. 2014) using a model combining the species factor and timepoint factor (timepoint W4/9 as a single level). For each species, two contrasts were tested: the difference between D0 and D1 (short-term response); and the difference between W0 and W4/9 (long-term response). *B. distachyon* lacked the W9 samples and long-term response was calculated as the difference between W0 and W4. *S. lagascae* lacked the W0 sample and long-term response was therefore calculated as the difference between D0 and W4/9.

Comparison with known cold responsive genes

A set of *H. vulgare* genes independently identified as cold responsive were acquired from supplementary table S10 in Greenup et al. 2011. These “Greenup genes” have been found to be responsive to cold in three independent experiments with Plexdb accessions BB64 (Svensson et al. 2006), BB81 (no publication) and BB94 (Greenup et al. 2011). In order to map the probesets of the Affymetrix Barley1 GeneChip microarray, which was used in these studies, to our sequences, we blasted (blastx) the probeset contigs of the Greenup genes against all protein sequences in all our OGs. Each contig was assigned to the OG with the best match against the *H. vulgare* reference. If several contigs were assigned to the same OG, only the contig with best hit was used. Because very few of the Greenup genes mapped to the HCOGs, we opted to compare against all OGs. For OGs containing paralogs, only the paralog with best match against the corresponding contig was used. For each species, DESeq2 was used to identify all short-term response DEGs for all sequences that belong to an OG. The log2 fold change values from the *H. vulgare* Dicktoo chill experiment included in the table from Greenup et al. 2011 were compared to the differential expression results from our species (Figure 3). Cold response similarity between our data and the Greenup genes was tested by counting the number of genes that were differentially expressed in the same direction and comparing against a null distribution generated by 100 000 random sets of genes.

Gene ontology tests

GO annotations for *B. distachyon* were downloaded from Ensembl Plants Biomart and assigned to the corresponding HCOG. The TopGO v2.18.0 (Alexa and Rahnenfuhrer 2010) R package was used to calculate fisher test P-values for the Biological Process gene ontology for each set of branch-specific DEGs (see Figure 4) using all annotated HCOGs as background. The GO was limited to only include the subset defined in the "plant slim" (http://www.geneontology.org/ontology/subsets/goslim_plant.obo) and only GO terms that got a P-value < 0.05 in at least one test was included in the figure.

Positive selection tests

Positive selection was calculated for each of the HCOGs using the branch-site model in codeml, part of the PAML v4.7 (Yang 2007). Since some HCOGs had missing species and gene trees with topologies different than the species tree, they were filtered based on the following criteria: Firstly, the tested branch had to be an inner branch also in the gene tree (i.e. at least two species present under the branch). Secondly, the species under and outside the branch in the gene tree had to be the same as in the species tree or a subset thereof. Lastly, the first species to split off under the branch had to be the same in the gene tree as in the species tree (for the ES branch, either *S. lagascae* or *M. nutans* was allowed).

Results

We sampled leaf material in five Pooideae before and after subjecting them to a drop in temperature and short days (Figure 1C). RNA-sequencing (RNA-Seq) was then used to reveal the presence and abundance of transcripts in the samples.

De novo transcriptome assembly and ortholog detection

The transcriptome of each species was assembled *de novo* resulting in 146k-282k contigs, of which 68k-118k were identified as containing coding sequences (CDS, Table 1). Ortholog groups (OGs) were inferred with OrthoMCL using the protein sequences from the *de novo* assemblies, the reference genomes of *H. vulgare* and *B. distachyon*, the outgroup references of *Oryza sativa*, *Sorghum bicolor* and *Zea mays*, and the reference of the core pooid, *Lolium perenne*. The *de novo* species were represented in 24k-33k of the OGs (Table 1).

Gene trees were generated for each OG and a set of high confidence OGs (HCOGs) was identified by filtering based on the topology of the gene trees (see Methods for

details). This resulted in 8633 HCOGs containing at least three of the five *de novo* species (Table 1).

De novo assembly followed by ortholog detection resulted in a high number of monophyletic paralogs (i.e. in-paralogs) in each of our *de novo* species, which did not correspond to the number of paralogs in the reference genomes of *H. vulgare* and *B. distachyon*. This apparent overestimation of in-paralogs is most likely the result of the *de novo* procedure assembling alleles or alternative transcript isoforms into separate contigs. We also observed some cases where the number of paralogs were under-estimated compared to references, which may be due to low expression of these paralogs or the assembler collapsing actual paralogs into single contigs. Since the *de novo* assembly procedure did not reliably differentiate paralogs, we opted to represent each species in each HCOG by a single read-count value equal to the sum of the expression of all assembled transcripts (i.e. in-paralogs). By using the sum of in-paralog read counts and setting counts for missing orthologs to zero, all the expression data could be represented in a single cross species expression matrix with HCOGs as rows and samples as columns.

Dated species tree

Dated gene trees were generated using prior knowledge about the divergence times of *Oryza*-Pooideae (Christin et al. 2014) and *Brachypodium-Hordeum* (Marcussen et al. 2014). A dated species tree was then estimated based on gene tree topologies and the mean of their estimated divergence times (Figure 1A). In the most common gene tree topology *S. lagascae* or *M. nutans* form a monophyletic clade, but topologies where either *S. lagascae* or *M. nutans* diverge first were also common (Figure S1). The *S. lagascae* and *M. nutans* branches were collapsed in the species tree due to the uncertainty of their topology. Earlier studies report contradicting results regarding the placement of the *Stipa* and *Melica* lineages in the Pooideae phylogeny (Soreng et al. 2007; Bouchenak-Khelladi et al. 2010; Grass Phylogeny Working Group II 2012; Hochbach et al. 2015; Soreng et al. 2015).

All species show a general similarity in the cold response

To investigate broad scale expression patterns in cold response, we clustered all samples (including replicates) using neighbor joining of Manhattan distances after centering the expression of each gene per species (i.e. subtracting the mean expression for each species across samples, Figure 2A). The centering allowed us to focus on the differential effect of the treatments and resulted in a tree with replicates, and then time points, clustering together. An exception is time points W4 and W9, which tend to cluster

together and by species, showing that responses after 4 and 9 weeks are very similar. The fact that time points mostly cluster together before species indicates a common response to cold across species. There is also a clear effect of the diurnal rhythm, with time points sampled in the morning (W0, W4 and W9) forming one cluster and time points sampled in the afternoon (D0 and D1) forming another.

Differentially expressed genes are mostly lineage-specific

Genes with a significant change in expression level (FDR adjusted p-value < 0.05 and >2-fold change) from before cold treatment to 8 hours (short term response) or 4-9 weeks (long-term response, W4 and W9 merged) after cold treatment, were defined as cold responsive (Figure 1C). These differentially expressed genes (DEGs) were mostly lineage specific, with relatively low overlap between species (Figure 2B), indicating that each species has mostly individual response to cold. Although low, the overlaps between any pair of species were still higher than expected by random (Fisher´s exact test $p < 0.05$, Figure 2C), suggesting some level of conserved cold response. Also, when comparing the fold change in one species with another there was a significant correlation between any pair of species (Figure 2D). However, both the DEG overlaps and fold change correlations did not agree with the expected phylogeny, that is, the more closely related species did not show a more similar cold response than those between more distant species.

Conserved cold response genes include known abiotic stress gene

Genes that share the same response in all five species are potentially functionally important as it indicates that their response to cold has been conserved throughout the evolution of Pooideae. Table 2 lists the 16 genes that had the same type of cold response (short- or long-term) in the same direction (up or down) in all five species. Note that this is a subset of the 83 genes in Figure 2B, which include any type of response in all species. Several of the listed genes belonging to families known to be involved in cold stress or other abiotic stress responses in other plants. The most common type of response is short-term up regulated, indicating that stress response, as opposed to long-term acclimation response, is more conserved.

Verifying cold response genes with previous studies

We compared the cold response in our data to a compilation of *H. vulgare* genes shown to be responsive to low temperature in several previous microarray studies, subsequently referred to as the Greenup genes (Greenup et al. 2011, table S10). We were able to map 33 of the 55 genes in the list to unique OGs, of which 11 were HCOGs. Since there was

so few HCOGs associated with the Greenup genes, we tested differential expression using all transcripts in all OGs (including in-paralogs), and then used the differential expression result for the transcript with the best sequence match to the microarray probe sequences. We found that the Greenup genes had a similar cold response in our data set, i.e. a significant number of the genes were differentially expressed in the same direction (compared to randomly selected genes, $p < 0.05$. Figure 3); although to a substantially less degree in the other species compared to *H. vulgare*. This shows that cold response is reproducible across different experiment setups and to some degree generalize to other Pooideae.

Gene ontology analysis

We identified branch-specific DEGs, i.e. genes that are exclusively differentially expressed in all species in a clade, and tested for enrichment of biological process gene ontology (GO) annotations (Figure 4). Such branch-specific DEGs indicate that the response evolved in the branch leading to that clade. For the pooideae base (PB) DEGs, i.e. genes that are differentially expressed in all our species, we found enrichment of genes annotated with response to abiotic stimulus, photosynthesis and metabolism. When dividing the genes into those that were exclusively up- or down-regulated, there was some indication of up-regulation of signal transduction (two pseudo response regulators and diacylglycerol kinase 2 (DGK2)) and abiotic stimulus (Gigantea, LEA-14, DnaJ and DGK2), while the down-regulated genes were mostly related to photosynthesis and metabolism. For the early split (ES) DEGs, i.e. genes that are exclusively differentially expressed in all species except *N. stricta*, there was no enrichment in the up-regulated genes, but down-regulated genes were again enriched for GO annotations related to metabolism and photosynthesis, somewhat similar to the PB DEGs.

Positive selection

For each HCOG, we tested for positive selection at each of the internal branches of the species tree. The tests were only performed on the branches where the gene tree topology was compatible with the species tree (see methods). 16-18% of the HCOGs showed significant signs of positive selection ($P<0.05$) depending on the branch (Figure 5). Next we tested for overrepresentation of positive selection among the branch-specific DEGs (Figure 5). There is a tendency that gain of cold response is associated with positive selection, at the ES and LS branches ($P=0.077$ and $P=0.072$ respectively, hypergeometric test).

Discussion

The ecological success of the Pooideae subfamily in the northern temperate regions must have critically relied on adaptation to colder temperatures. However, it is unclear how this adaptation evolved within Pooideae. To investigate this, we applied RNA-seq to identify short- and long-term cold responsive genes in five Pooideae species ranging from early-diverging lineages to core Pooideae. Since three of the species lacked reference genomes, we employed a *de novo* assembly pipeline to reconstruct the transcriptomes and showed that this pipeline was able to identify most of a set of *H. vulgare* genes previously identified as cold responsive (Figure 3). In order to compare the five transcriptomes, we compiled a set of 8633 high confidence ortholog groups with resolved gene tree topologies. Gene expression clustering based on these ortholog groups arranged samples according to replicates, then time points and finally species, indicating that cold response is the primary signal in the data and confirming the soundness of the approach (Figure 2A).

Lineage specific adaptations to cold climates

A substantial portion of the individual Pooideae transcriptomes responded to cold (1000-3000 genes), however, only a small number of genes respond to cold in all the investigated species (83 genes, Figure 2B). Even fewer genes responded to cold in the same way in all species (e.g. short-term up-regulation, 16 genes, Table 2) and these conserved genes primarily included general abiotic stress genes, which are clearly not representative of all the different molecular pathways constituting a fully operational cold response program. Our results were based on high confidence ortholog groups that excluded complex families with duplication events shared by two or more species. Since many of the previously described *H. vulgare* cold-responsive genes belonged to such complex families, we could have underestimated the number of conserved cold responsive genes. However, we specifically investigated the regulation of these genes using all ortholog groups, and again found that few genes displayed conserved cold response across all species (Figure 3), thus confirming our conclusion that cold response in Pooideae is largely lineage-specific. Taken together, our findings indicate that the most recent common ancestor of the Pooideae possessed no, or only a limited, response to cold, and, consequently, that our data appears more consistent with the lineage specific hypothesis of Pooideae cold adaptation than the ancestral hypothesis.

The drastic cold stress during the E-O transition was likely an important cause for the evolution of cold adaptation in the Pooideae. Previous studies have shown that many of the extant temperate plant lineages have emerged since the E-O transition (Kerkhoff

et al. 2014) and that the expansion of well-known cold responsive gene families in Pooideae coincided with this transition (Sandve and Fjellheim 2010; Li et al. 2012). From the dated phylogeny (Figure 1A) it is clear that all major Pooidaee lineages, including the core Pooideae, had emerged by the late Eocene. Hence, the five lineages studied here experienced the E-O transition as individual lineages (Figure 1C). Furthermore, we found that closely related species did not share a higher fraction of cold responsive genes than more distantly related species (no phylogenetic pattern, Figure 2BC). The observation that the five Pooideae lineages emerged during a relatively warm period before the E-O transition, and the finding that these species harbor high numbers of lineage-specific cold responsive genes with no phylogenetic pattern, together suggest that most cold responses in Pooideae lineages evolved in parallel the last 40 M years. During this period, temperatures were constantly decreasing and major climatic events took place, such as the cooling during the E-O transition and the rapid climatic oscillations during the past few million years (Zachos et al. 2001).

Our results suggest that the Pooideae lineages evolve cold response in parallel using, to a large degree, unrelated genes. This implies that different genes can be co-opted into the functional cold response of the Pooideae. It is worth noticing, however, that although we observed many lineage-specific cold response genes, all species pairs displayed a statistically significantly overlap in cold responsive genes (Figure 2C) and a statistically significant correlation in cold response across all HCOGs (Figure 2D). This most likely reflects the fact that some genes code for proteins with biochemical functions more suited to be recruited for cold response than others (Christin et al. 2013; Preston and Sandve 2013; Christin et al. 2015), and that different species thus have ended up co-opting orthologous genes into their cold response program more often than expected by chance.

An adaptive potential in the Pooideae ancestor

Multiple independent origins of cold adaptation raise the question whether connecting traits exists in the evolutionary history of the Pooideae that can explain why the entire Pooideae lineages were able to shift to temperate biomes. The transcripts that were cold responsive across all focal species (Table 2) represent genes that might have gained cold responsiveness in the Pooideae most recent common ancestor and contributed to increase the potential of Pooideae lineages to adapt to cold temperate climate. Several of these conserved genes are known to be involved in abiotic stresses in other plants such as drought or other osmotic stress, which share some physiological effects experienced during freezing. Co-option of such genes into a cold-responsive pathway might have been the key to acquire cold tolerance. In fact, other studies have implied that drought

tolerance might have facilitated the shift to temperate biome (Kellogg 2001; Schardl et al. 2008; Sandve and Fjellheim 2010). Interestingly, most of the conserved genes are short-term cold responsive (Table 2) and strengthen the hypothesis that existing stress genes might have been the first to be co-opted into the cold response program. Another observation is that three of the conserved cold responsive genes (GIGANTEA, PRR95 and AtPRR3-like) are associated with the circadian clock that is known to be affected by cold (Bieniawska et al. 2008; Nakamichi et al. 2010; Johansson et al. 2015). This might suggest that clock genes have had an important function in the Pooideae cold adaptation, for example by acting as a signal for initiating the cold defense. More generally, transcripts involved in photosynthesis and response to abiotic stimuli were significantly enriched among the genes with cold response in all species (Figure 4). An expanded stress responsiveness towards cold stress and the ability to down-regulate the photosynthetic machinery during cold temperatures to prevent photoinhibition might have existed in the early evolution of Pooideae. In conclusion, the conserved stress response genes discussed here may have given a fitness advantage for the Pooideae ancestor in the newly emerging environment with incidents of mild frost allowing time to evolve the more complex physiological adaptations required to endure the temperate climate with strong seasonality and cold winters that emerged following the E-O transition (Eldrett et al. 2009). Consistent with this, Schubert et al. (unpublished) showed that the fructan synthesis and ice recrystallization inhibition protein gene families known to be involved in cold acclimation in core Pooideae species (Sandve et al. 2011) evolved around the E-O split, whereas also earlier evolving Pooideae species show capacity to cold acclimate.

Evolution of coding and regulatory sequences

The molecular mechanisms behind adaptive evolution are still poorly understood, although it is now indisputably established that novel gene regulation plays a crucial role (Romero et al. 2012). The evolution of gene regulation proceeds by altering non-coding regulatory sequences in the genome, such as (cis-) regulatory elements (Wittkopp and Kalay 2012), and has the potential to evolve faster than protein sequence and function. The high number of lineage-specific cold response genes observed in this study is thus most consistent with the recruitment of genes with existing cold tolerance functions by means of regulatory evolution. However, previous studies have also pointed to the evolution of coding sequences (Vigeland et al. 2013) as underlying the acquisition of cold tolerance in Pooideae. To investigate possible coding evolution, we tested for the enrichment of positive selection among branch-specific cold responsive genes (Figure 4). This indicate a tendency for positive selection in genes gaining cold response in a period of gradual cooling preceding the E-O event. Thus, we see evidence

of both coding and regulatory evolution playing a role in cold adaptation in Pooideae, and that these processes may have interacted. Finally, gene family expansion has previously been implied in cold adaptation in Pooideae (Sandve and Fjellheim 2010; Li et al. 2012). As previously discussed, the conservative filtering of ortholog groups employed in this study removed complex gene families containing duplication events shared by two or more species. Interestingly, out of the 33 previously described *H. vulgare* cold responsive genes (Figure 3), as many as 22 failed the high confidence ortholog group filtering, the main reason being that they belong to gene families with high rate of duplications. This observation thus confirms that duplication events as a relatively common feature of cold adaptation.

Conclusion

Here we investigated the cold response of five Pooideae species ranging from early-diverging lineages to core Pooideae species to elucidate evolution of adaptation to cold temperate biomes. We primarily observed lineage-specific cold response that seems to have evolved chiefly after the *B. distachyon* lineage and the core Pooideae diverged, possible initiated by the drastic temperature drop during the E-O transition. However, we do also see signs of conserved response that potentially represents a shared potential for cold adaptation that explain the success of Pooideae in temperate biomes. This includes several general stress genes with conserved short-term response to cold as well as the conserved ability to down-regulate the photosynthetic machinery during cold temperatures. Taken together, our observations are consistent with a scenario where many of the biochemical functions needed for cold response was present in the Pooideae common ancestor, and where different Pooideae lineages have assembled different overlapping subsets of these genes into fully functional cold response programs through the relatively rapid process of regulatory evolution.

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Tables

Table 1: Summary statistics from transcriptome assembly, coding sequence detection and ortholog group inference.

	H. vulgare	*B. distachyon*	*M. nutans*	*S. lagascae*	*N. stricta*
Number of samples	10	13	21	7	8
Samples per timepoint (W0,D0,D1,W4,W9)	1,1,3,1,4	1,4,4,4,0	5,4,4,4,4	0,1,1,1,4	1,1,1,1,4
Total number of reads	165M	218M	296M	96M	147M
Assembled contigs (excluding isoforms)	146240	149821	263503	282259	229298
Coding transcripts	67963	67532	99208	117979	96748
Number of orthogroups with at least one coding transcript	25228	23869	29514	33371	30789
Number of high confidence orthogroups (total 8633) with at least one transcript	8021	7875	7771	7438	7824

Table 2: HCOGs with conserved cold response in all five Pooideae species (S = short-term response, L = long-term response. ↗ = up regulated, ↘ = down regulated). Annotations were inferred from literature using orthologs.

Bd ortholog	Description and relation to stress response	response
Bradi2g39230	**HyperOSmolality-gated CA2+ permeable channel (OSCA)** - Stress-activated calcium channels (Yuan et al. 2014) that are highly conserved in eukaryotes (Hou et al. 2014). In Oryza sativa OSCA genes are differentially expressed in response to osmotic stress (Li et al. 2015).	S↗
Bradi2g06830	**Calcium-binding EF-hand containing calcium exchange channel (EF-CAX)** - Calcium ions are important mediators of abiotic stress in plants (Day et al. 2002; Bose et al. 2011). Expression of calcium binding proteins correlates with exposure to cold stress in several plants, e.g. Arabidopsis thaliana (Thomashow 1999), Musa × paradisiaca (Q.-S. Yang et al. 2015) and Hordeum vulgare (Greenup et al. 2011).	S↗
Bradi2g05226	**GIGANTEA** - Promotes flower development in plants (Andrés and Coupland 2012). In Arabidopsis thaliana involved in CBF-independent freezing tolerance (Cao et al. 2005; Xie et al. 2015), and responsive to cold in Zea mays (Sobkowiak et al. 2014). Also part of the circadian clock.	S↗
Bradi4g24967	**Arabidopsis Pseudo-Response Regulator 3-like (AtPRR3-like)** - AtPRR3 is a member of the circadian clock quintet AtPRR1/TOC1 (Murakami-Kojima 2002; Murakami 2004). No association to stress response found in literature. However, AtPRR3-like might be closer related to AtPRR5/9 than to AtPRR3	S↗
Bradi2g09060	**Triacylglycerol lipase, alpha/beta-Hydrolase superfamily** - Studies in Arabidopsis thaliana (Wang et al. 2011) and Ipomoea batatas (Liu et al. 2014) suggest that genes with alpha/beta-Hydrolase domains respond to osmotic stress. In Triticum monococcum atriacylglycerol lipase was induced by pathogen stress (Guan et al. 2015).	S↗
Bradi2g07480	**Late-Embryogenesis-Abundant protein 14 (LEA-14)** - Responsive to drought, salt and cold stress in Arabidopsis thaliana (Kimura et al. 2003; Singh et al. 2005), Betula pubescens (Rinne et al. 1998) and Brachypodium distachyon (Gagné-Bourque et al. 2015).	S↗
Bradi1g04150	**SNAC1-like / NAC transcription factor 67** - NAC transcription factors mediate abiotic stress responses. Osmotic stress increases the expression of SNAC1 in Oryza sativa (Nakashima et al. 2012), NAC68 in Musa ×	S↗

	paradisiaca (Negi et al. 2015; Q.-S. Yang et al. 2015) and NAC67 Triticum aestivum (Mao et al. 2014).	
Bradi4g36077	**Pseudo-Response Regulator 95 (PRR95)** - Homologous to conserved circadian clock gene AtPRR5/9 (Murakami 2003; Campoli et al. 2012). AtPRR5 gene is cold regulated in Arabidopsis thaliana (Lee et al. 2005) and PRR95 is cold responsive in Zea mays (Sobkowiak et al. 2014).	S↗
Bradi2g43040	**DnaJ chaperon protein** - DnaJ co-chaperons are vital in stress response and has been found to be involved in maintenance of photosystem II under chilling stress and enhances drought tolerance in tomato (Kong et al. 2014; Wang et al. 2014)	S+L ↗
Bradi3g33080	**Glycogenin GlucuronosylTransferase (GGT)** - GGT belongs to the GT8 protein family (Yin et al. 2011). In Oryza sativa OsGGT transcripts are induced in submerged plants and respond to various abiotic stresses except cold (Qi et al. 2005; Uddin et al. 2012).	L↗
Bradi1g04500	**Major facilitator superfamily transporter** - Association to stress response unknown	L↗
Bradi3g14080	**Glycosyl transferase** - Association to stress response unknown	L↗
Bradi1g35357	**Uncharacterized membrane protein** - Association to stress response unknown	S↗
Bradi2g48850	**Uncharacterized protein** - Association to stress response unknown	S↗
Bradi1g33690	**Uncharacterized protein** - Association to stress response unknown	S↗
Bradi1g07120	**Putative S-adenosyl-L-methionine-dependent methyltransferase** - Association to stress response unknown	L↘

Table 2 continued

Figures

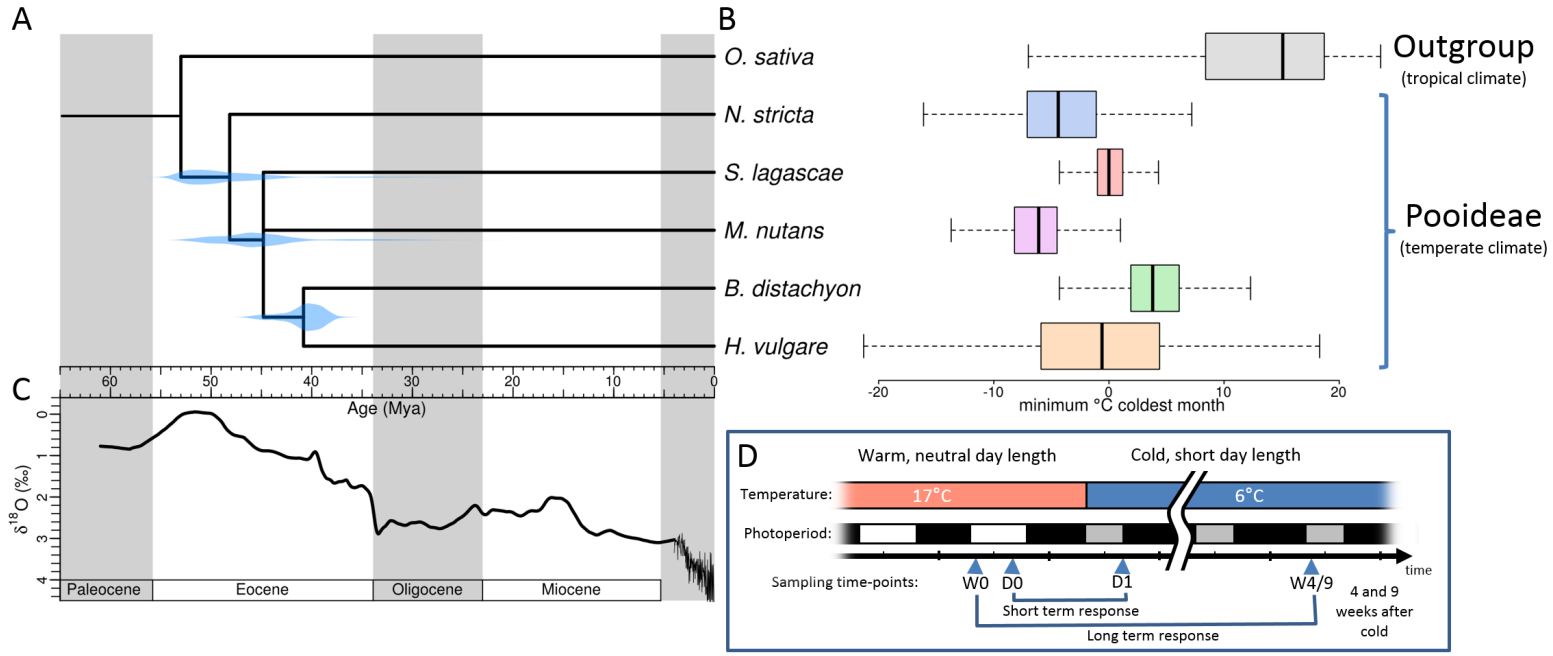

Figure 1: A) Dated phylogenetic tree of the Pooideae species included in this study with *O. sativa* as out group species. Estimated species phylogeny was inferred from 3914 dated gene trees. Distribution of gene-tree node ages shown in blue. **B**) Minimum temperature of the coldest month (Bioclim variable 6, 2.5 km^2 resolution) in each species distribution based on WorldClim v1.4 dataset (Hijmans et al. 2005). **C**) Oxygen isotope ratio as a proxy for historic global temperature (Zachos et al. 2001; Mudelsee et al. 2014) **D**) Cold response experiment design. Plants from five species of Pooideae were subjected to a drop in temperature and shorter days to induce cold response. Leaf material was sampled on the day before the onset of cold (W0 and D0), once 8 hours after cold (D1) and two times after 4 and 9 weeks (W4/W9). Short-term response was identified by contrasting gene expression in time points D0 and D1, while long-term response was identified contrasting W0 and W4/W9.

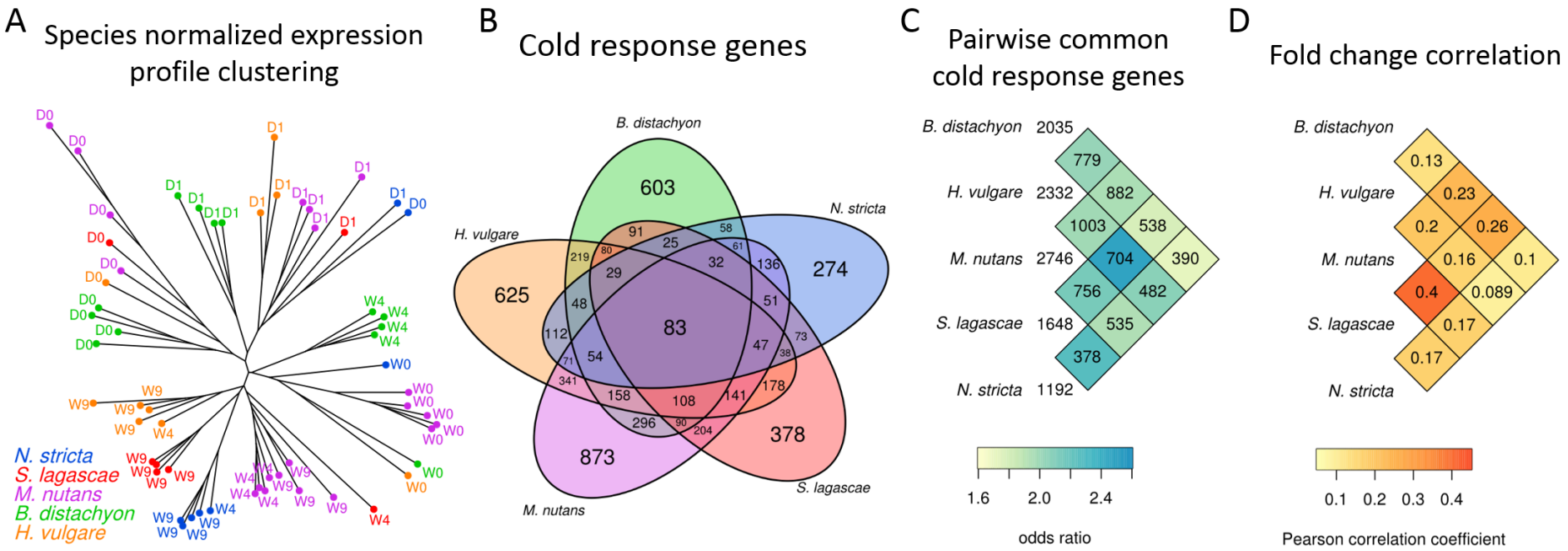

Figure 2: A) Expression profile clustering tree generated by neighbor-joining of distances given as sum of log fold change between all highly expressed genes after subtracting the mean expression per species. Each tip corresponds to one sample. **B**) Number of differentially expressed genes (DEGs) in each species and overlap between species (p.adj. < 0.05, |fc| > 2, in either short-term or long-term response). **C**) Number of DEGs per species and number of overlapping DEGs between pairs of species with associated odds ratios. **D**) Correlation between short-term cold response log fold change in each species.

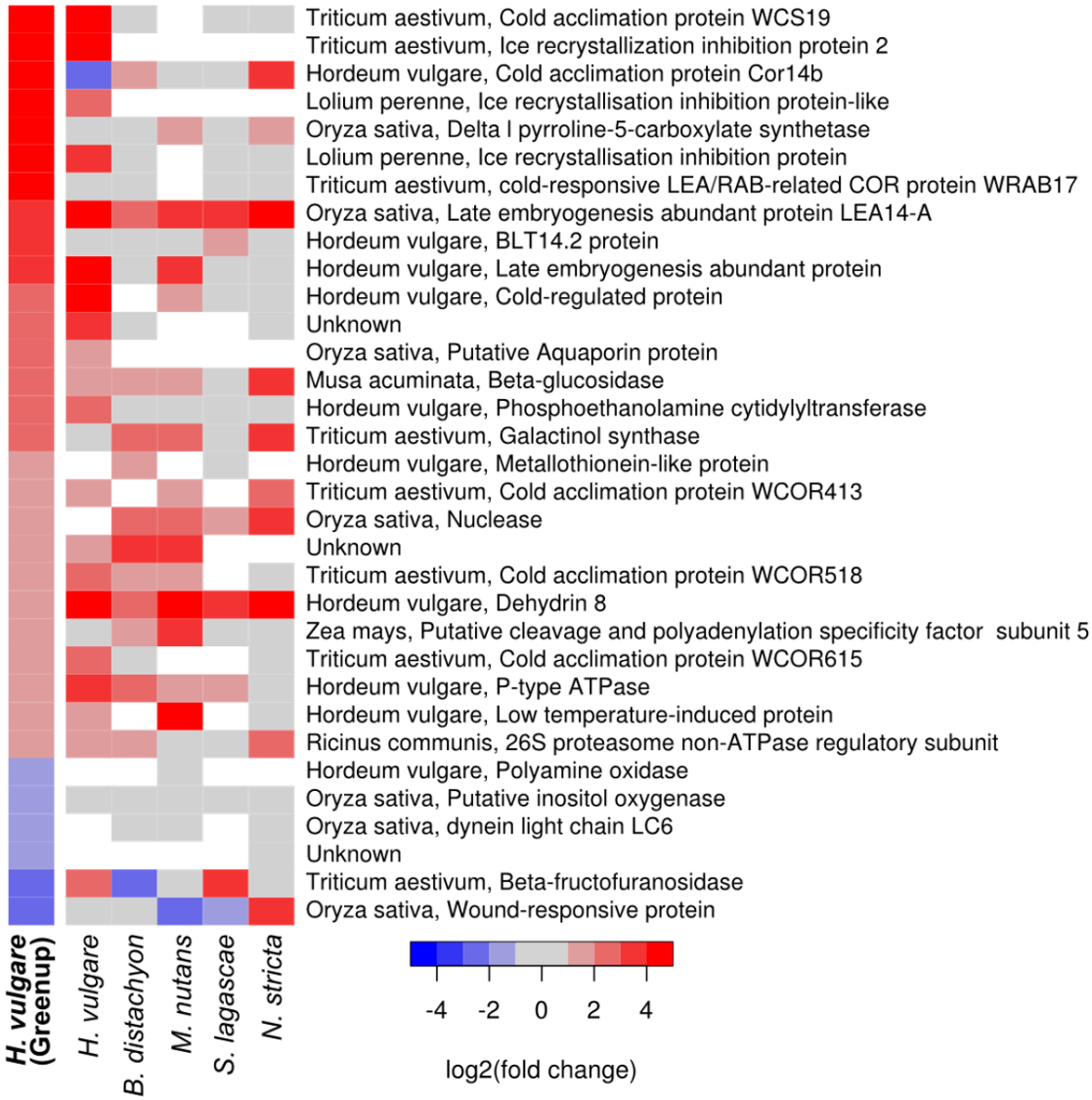

Figure 3: A reference set of *H. vulgare* genes independently shown to respond to cold in another studie (Greenup et al. 2011) is compared to our data using short-term log fold change values. White cells represent missing orthologs.

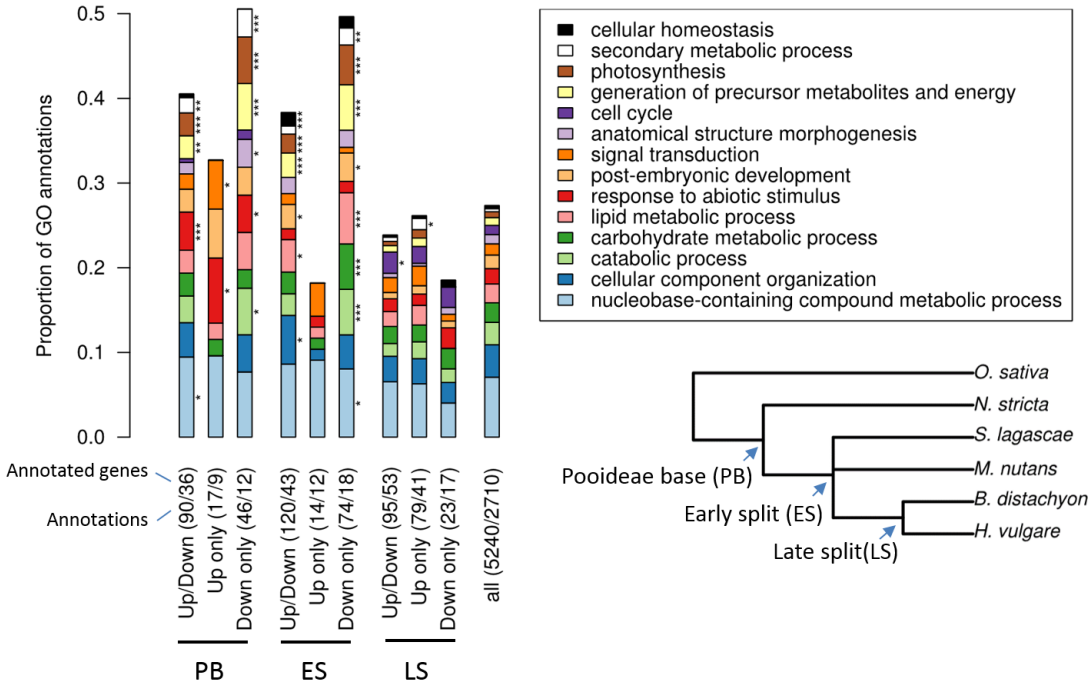

Figure 4: Gene ontology enrichment analysis of the set of genes that are differentially expressed in all species (PB), differentially expressed only in species after *N. stricta* diverged (ES) or differentially expressed only in *B. distachyon* and *H. vulgare* (LS). A GO annotation is called enriched if it makes up a significantly higher proportion in a set of genes than expected by chance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, Fisher's exact test). Since each gene can have several GO annotations, or no annotation at all, the number of genes with annotations and total number of annotations for each set of genes is specified in the plot.

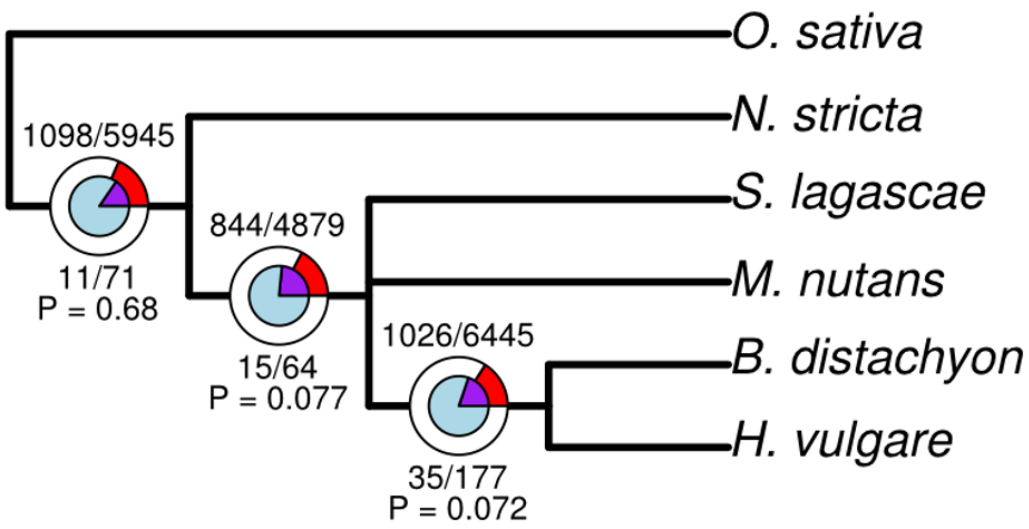

Figure 5: Positive selection at different branches in Pooideae phylogeny. The circles at each branch represents the HCOGs that were tested for positive selection at the corresponding branch. The outer circle represents the tested HCOGs that are not branch-specific DEGs, where the red pie-slice is the proportion of those with significant positive selection ($P < 0.05$). The inner blue circle represents the branch-specific DEGs, i.e. genes that are cold responsive exclusively in the species in this respective clade, where the purple pie-slice represents the proportion with positive selection. The P-value indicate the r overrepresentation of positive selection among the branch-specific DEGs (hypergeometric test).

Supplementary material

Table S1: Reference genomes source URLs (last accessed July 2015)

Species	Sequence type	Source URL:
B. distachyon	Protein	ftp://ftpmips.helmholtz-muenchen.de/plants/brachypodium/v1.2/brachypodium_1.2_Protein_representative.fa
	CDS	ftp://ftpmips.helmholtz-muenchen.de/plants/brachypodium/v1.2/brachypodium_1.2_CDS.fa
H. vulgare	Protein	ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/genes/barley_HighConf_genes_MIPS_23Mar12_ProteinSeq.fa
	CDS	ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/genes/barley_HighConf_genes_MIPS_23Mar12_CDSSeq.fa
S. bicolor	Protein	ftp://ftpmips.helmholtz-muenchen.de/plants/sorghum/sorghum1.4Proteins.fa
	CDS	ftp://ftpmips.helmholtz-muenchen.de/plants/sorghum/sorghum1.4CDS.fa
O. sativa	Protein	ftp://ftpmips.helmholtz-muenchen.de/plants/rice/rap2Protein.fa
	CDS	ftp://ftpmips.helmholtz-muenchen.de/plants/rice/rap2BestGuessCds.fa
Z. mays	Protein	ftp://ftp.maizesequence.org/pub/maize/release-5b/working-set/ZmB73_5a_WGS_translations.fasta.gz
	CDS	ftp://ftp.maizesequence.org/pub/maize/release-5b/working-set/ZmB73_5a_WGS_cds.fasta.gz

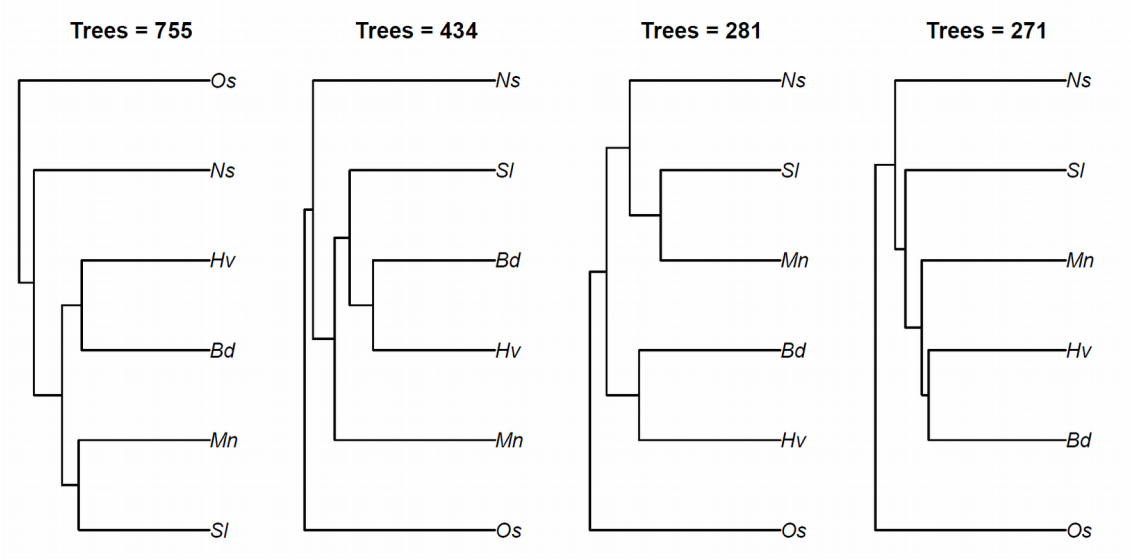

Figure S1: The four most common gene tree topologies out of 3914 gene trees.

Paper II

Evolution of cold adaptation in temperate grasses (Pooideae)

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Abstract

In the past 50 million years climate cooling has triggered the expansion of temperate biomes. During this period, many extant plant lineages in temperate biomes evolved from tropical ancestors and adapted to seasonality and cool conditions. Among the Poaceae (grass family), one of the subfamilies that successfully shifted from tropical to temperate biomes is the Pooideae (temperate grasses). Subfamily Pooideae contains some of the most important crops cultivated in the temperate regions including wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). Due to the need of well-adapted cultivars, extensive research has produced a large body of knowledge about the mechanisms underlying cold adaptation in cultivated Pooideae species. Especially cold acclimation, a process which increases the frost tolerance during a period of non-freezing cold, plays an important role. Because cold adaptation is largely unexplored in lineages that diverged early in the evolution of the Pooideae, little is known about the evolutionary history of cold adaptation in the Pooideae. Here we test if several species of early-diverging lineages exhibit increased frost tolerance after a period of cold acclimation. We further investigate the conservation of five well-studied gene families that are known to be involved in the cold adaptation of Pooideae crop species. Our results indicate that cold acclimation exists in early-diverging lineages and that many of the investigated gene families likely existed in the most recent common ancestor of the Pooideae. However, most of the gene families show signs of lineage-specific evolution and support the hypothesis that gene family expansion is an important mechanism in adaptive evolution.

Introduction

The temperate biomes emerged following the global cooling throughout the Eocene and the transition from Eocene to Oligocene (E-O), *ca.* 34 million years ago (Mya) (Potts and Behrensmeyer 1992; Donoghue 2008; Stickley et al. 2009; Kerkhoff et al. 2014; Mudelsee et al. 2014). In plants, biome shifts are rare and restricted to a small number of lineages (Crisp et al. 2009) and only a few, ancestrally tropical angiosperm lineages have adapted to temperate biomes (Judd et al. 1994; Wiens and Donoghue 2004). Successful colonizers of these biomes faced increased temperature seasonality, prolonged periods of cold, frost and short growing seasons (Zachos et al. 2001, Eldrett et al. 2009, Mudelsee et al. 2014).

Among the grasses (Poaceae), the temperate biomes are dominated by members of the Pooideae subfamily and in Northern temperate biomes 90% of the grass flora belong to this subfamily (Hartley 1973; Clayton 1981). Since their emergence in the late Paleocene or early Eocene (Bouchenak-Khelladi et al. 2010, Christin et al. 2014), the Pooideae grass subfamily successfully shifted their distribution from the tropical/subtropical biomes of their ancestors (Edwards and Smith 2010; Strömberg 2011) to temperate biomes. Except for a few hundred species in early-diverging Pooideae tribes most of the *ca.* 4200 Pooideae species belong to the species-rich 'core Pooideae' lineage (*sensu* Davis and Soreng 1993; Soreng and Davis 1998) and its sister tribe Brachypodieae, which contains the model grass *Brachypodium distachyon* (Soreng et al. 2015). Because core Pooideae contain economically important species like wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) as well as several forage grasses like ryegrass (*Lolium perenne*), the need for well-adapted cultivars has provided an extensive body of knowledge about molecular mechanisms underlying adaptation to temperate environments (Thomashow 1999; Sandve et al. 2008; Galiba et al. 2009; Sandve et al. 2011, Preston and Sandve 2013; Fjellheim et al. 2014). Key molecular responses to low temperatures in Pooideae species may represent adaptations to the new climatic regime whose emergence coincided with the early evolution of the Pooideae subfamily.

Plants in temperate biomes regularly experience incidents of cold shock as well as periods of prolonged frost and they respond with a suite of physiological and biochemical adaptations controlled by complex regulatory programs. The main challenge of exposure to frost is maintaining the integrity of the cellular membranes to avoid dehydration (Pearce et al. 2001). This is accomplished by adjusting the lipid composition of membranes to increase membrane stability (Uemura et al. 1995; Danyluk et al. 1998;) and to accumulate sugars and anti-freeze proteins (Griffith and Yaish 2004; Sandve et al. 2011). Additionally, accumulation of reactive oxygen species (ROS) damages lipid membranes and increases protein degradation (Murata et al. 2007; Crosatti et al. 2013) to which plants react by synthesizing proteins that decrease ROS-mediated stress (Crosatti et al. 2013). Through the process of cold acclimation – a period of cold, but non-freezing temperatures – some plants can increase their tolerance to frost to prepare for prolonged freezing during winter (Thomashow 1999; Thomashow 2010).

Five of the best studied cold-stress related gene families coding for C-repeat binding factors (CBF), dehydrins (DHN), chloroplast-targeted cold-regulated (ctCOR), ice recrystallization inhibition proteins (IRIP) and fructosyl transferases (FST). All of these are known to be induced by cold and play important roles during different stages of the cold-stress response in core Pooideae (*CBF*: Badawi et al. 2007; Li et al. 2012. *DHN*: Olave-Concha et al. 2004; Rorat 2006; Kosová et al. 2007, 2014. *ctCOR*: Gray et al. 1997; Crosatti et al. 1999, 2013; Tsvetanov et al. 2000. *IRIP*: Antikainen and Griffith 1997; Hisano et al. 2004; Kumble et al. 2008; Sandve et al. 2008; John et al. 2009; Zhang et al. 2010; Sandve et al. 2011. *FST*: Hisano et al. 2004; Tamura et al. 2014). The CBFs are transcription factors and function as “master-switches” of cold regulation and cold acclimation (Sarhan et al. 1998; Thomashow 1999) and are involved in various kinds of stress response (Agarwal et al. 2006; Akhtar et al. 2012). Two groups of *CBF* genes – *CBFIII* and *CBFIV* – are especially important for cold acclimation in Pooideae and are restricted to this subfamily (Badawi et al. 2007, Li et al. 2012). The *DHN* gene family encodes hydrophilic proteins that share a lysine-rich sequence, the “K-segment”, which interacts with membranes (Koag et al. 2003; Koag et al. 2009) and protect against dehydration-related cellular stress and possibly also acts as cryoprotectant (Close 1997;

Danyluk et al. 1998; Houde et al. 2004). ctCOR proteins (e.g. COR14 and WCS19 [Gray et al. 1997; Crosatti et al. 1999; Tsvetanov et al. 2000]) are thought to alleviated damage from cold-induced overexcitation of the photosystems and accumulation of reactive oxygen species (ROS) (Crosatti et al. 2013). IRIPs are proteins that bind to the edges of microscopic intracellular ice grains and restrict the growth of larger ice crystals (Griffith and Ewart 1995; Antikainen and Griffith 1997; Sidebottom et al. 2000; Griffith and Yaish 2004; Tremblay et al. 2005; Sandve et al. 2011). Lastly, frost tolerance correlates with the accumulation of fructans which are synthesized by *FST* genes (Hisano et al. 2004; Tamura et al. 2014). Fructans are the major carbohydrate storage in model Pooideae species (Chalmers et al. 2005), but they have also been shown to improve membrane stability during freezing stress (Hincha et al. 2000). Interestingly, Li et al. (2012) identified cold responsive *IRIP* and *CBFIII* homologs in the model grass *Brachypodium distachyon*, which is sister to the core Pooideae lineage, while homologous *CBFIV* and *FST* genes were absent.

It is unknown when and how the Pooideae subfamily adapted to temperate biomes. Our knowledge about cold adaptation in Pooideae mostly stems from studying a handful of species in the core Pooideae-Brachypodieae clade. The knowledge about key genes involved in cold response pathways of the core Pooideae provides however, an excellent basis to investigate the importance of those genes for the biome shift of the Pooideae. A prerequisite for a successful shift lies in lineage's evolutionary potential, which is controlled by the genetic toolkit of its ancestor (Edwards and Donoghue 2013; Christin et al. 2015). Here, we test the hypothesis that the key cold response gene families of the core Pooideae existed in the most recent common ancestor (MRCA) of the Pooideae and are involved in cold response pathways also in early-diverging lineages. If the MRCA was already cold tolerant, those genes were likely part of its cold response and we expect to find a high degree of conservation and same expression patterns among the investigated species. Alternatively, if cold adaptations evolved independently in several Pooideae lineages those genes might have been recruited in parallel and we expect a lower degree of conservation and a more diverse expression pattern.

We combine a classical growth experiment with comparative transcriptomics and phylogenetic reconstruction of gene families to test if cold acclimation and expression of key genes in core Pooideae cold-response pathways are conserved across *B. distachyon* and the three early-diverging Pooideae species *Nardus stricta*, *Melica nutans* and *Stipa lagascae*. All species increase their freeze tolerance with cold acclimation, but only two out of the ten studied gene families exhibited completely conserved cold response across Pooideae. Nevertheless, we suggest that gene families *DHN*, *ctCOR*, *CBFIIId* and *CBFIV* were instrumental in the Pooideae's shift to temperate biomes. The Pooideae MRCA might not have been cold tolerant, but those gene families were likely part of its genetic toolkit that steered the evolutionary trajectory of its descendants towards cold tolerance.

It is unclear when and how the Pooideae subfamily adapted to temperate biomes. Our knowledge about cold adaptation in Pooideae mostly stems from studying a handful of species in the core Pooideae-*Brachypodieae* clade, because molecular responses to low temperatures have not been investigated in early-diverging Pooideae lineages. The knowledge about key genes involved in cold response pathways of the core Pooideae provides however, an excellent basis to investigate the importance of those genes for the biome shift of the Pooideae. One prerequisite for a successful shift lies in lineage's evolutionary potential, which is influenced by the genetic toolkit of its ancestor (Edwards and Donoghue 2013; Christin et al. 2015). For instance, it has been shown that the C3 ancestor of C4 grass lineages possessed certain genetic traits that affected the C4-evolvability in those lineages (Christin and Osborne 2013; Christin et al. 2015). Furthermore, it seems that some ancestral gene families were suitable to evolve C4-functions and were therefore more likely to be recruited into C4-photosynthesis than other gene families (Christin, Boxall et al. 2013). Here, we test the hypothesis that the key cold response gene families of the core Pooideae existed in the most recent common ancestor (MRCA) of the Pooideae and are involved in cold response pathways also in early-diverging lineages. If the MRCA was already cold tolerant, those genes were likely part of its cold response and we expect to find a high degree of conservation and same expression patterns among the investigated species. Alternatively, if cold adaptations evolved independently in several Pooideae lineages those genes might have

been recruited in parallel and we expect a lower degree of conservation and a more diverse expression pattern.

We combine comparative transcriptomics with phylogenetic reconstruction of gene families to test if expression of key in core Pooideae cold-response pathways are conserved across *B. distachyon* and the three species *Nardus stricta*, *Melica nutans* and *Stipa lagascae*, which belong to early-diverging lineages. Because frost tolerance is an important adaptation in temperate biomes, we test if cold acclimation increases frost tolerance in various *Brachypodium* species and the species from early-diverging lineages. Although only two out of the ten studied gene families exhibited completely conserved cold response across Pooideae, we suggest that gene families *DHN*, *ctCOR*, *CBFIIId* and *CBFIV* existed in the Pooideae ancestor and were instrumental in the Pooideae's shift to temperate biomes. The Pooideae MRCA was probably not cold tolerant, but those gene families were likely part of its genetic toolkit and steered the evolutionary trajectory of its descendants closer towards cold tolerance.

Material and methods

Plant material and RNA sampling

From five Pooideae species seeds were collected in nature: *Nardus stricta* (collected in Romania, [46.69098, 22.58302], July 2012), *Melica nutans*, (collected in Germany, [50.70708, 11.23838], June 2012); or acquired from germplasm collections: *Stipa lagascae* (PI 250751, U.S. Department of Agriculture [USDA] National Plant Germplasm System [NPGS] via Germplasm Resources Information Network [GRIN]), *Brachypodium distachyon* (line 'Bd1-1', W6 46201, USDA-NPGS via GRIN) and *Hordeum vulgare* (cultivar 'Igri', provided by Prof. Åsmund Bjørnstad, Department of Plant Sciences, Norwegian University of Life Sciences, Ås NO-1432, Norway). Seeds were germinated and initially grown in greenhouse at a neutral day length (12 hours of light), 17°C and a minimum light intensity of 150 µmol m^{-2} s^{-1}, complemented with artificial light when necessary. Depending on species, one to eight week old plants were randomly distributed to two growth chambers and subjected to cold treatment (6°C) under short days (8 hours of light) and a light intensity of 50 µmol m^{-2} s^{-1}. Leaf material

for RNA isolation was collected i) in the afternoon (8 hours of light) before (D0) and 8 hours after (D1) cold treatment and ii) in the morning (at lights on) before (W0), 4 weeks after (W4) and 9 weeks after (W9) cold treatment.

For each time point, 5 flash-frozen leaves were individually homogenized using a TissueLyser (Qiagen Retsch, Haan, Germany) and total RNA was isolated (from each leaf) using RNeasy Plant Mini Kit (Qiagen Inc., Germany), following the manufacturer's instructions. Purity and integrity of total RNA extracts was determined using a NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), respectively. Pooled and individual total RNA extracts were delivered to the Norwegian Sequencing Centre (NSC), Centre for Evolutionary and Ecological Synthesis (CEES), Department of Biology, University of Oslo, Norway, where strand-specific cDNA libraries were prepared and paired end sequenced on a HiSeq 2000-system (Illumina, San Diego, CA, USA).

Data processing workflow

Illumina TruSeq adapters were removed from the raw reads and bad quality bases trimmed using a sliding window implemented in Trimmomatic v.0.32 (Bolger et al. 2014). Read quality was controlled using fastqc v0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). For each species, transcripts were *de novo* assembled with Trinity v2.0.6 (Grabherr et al. 2011, strand specific option, otherwise default parameters) using reads from all samples. Coding sequences (CDS) in the *de novo* transcripts were identified using TransDecoder rel16JAN2014 (Haas et al. 2013). In cases where Trinity reported multiple isoforms, only the longest CDS was used. CDS of each gene defined by Trinity are hereafter called 'transcripts'. Ortholog groups were identified with OrthoMCL v2.0.9 (Li et al. 2003) using all *de novo* transcriptomes together with reference transcriptomes for *H. vulgare*, *B. distachyon*, *O. sativa*, *Sorghum bicolor* (all from: ftp://ftpmips.helmholtz-muenchen.de/plants/), *Zea mays* (www.maizesequence.org) and *L. perenne* (GenBank accession GAYX01000000).

To test differential expression, RNAseq reads were aligned to corresponding *de novo* transcriptomes using bowtie v1.1.2 (Langmead et al. 2009), and read counts were calculated with RSEM v1.2.9 (Li et al. 2011). When a (gene) transcript identified by Trinity had several isoforms, read counts of the isoforms were summed up. Differential expression was calculated with DESeq2 v1.6.3 (Love et al. 2014). Two contrasts were tested: the difference between D0 and D1 (short-term cold response); and the difference between W0 and W4/9 (long-term cold response, W4 and W9 were treated as same timepoint). *B. distachyon* lacked samples for W9 and long-term cold response was calculated as the difference between W0 and W4. *S. lagascae* lacked the W0 sample and long-term cold response was therefore calculated as the difference between D0 and W4/9. Genes with an FDR corrected p-value < 0.05 are referred to as cold responsive genes in this article.

Identification of candidate genes

For five well described cold response gene families (*C-repeat binding factor* genes (*CBFIII* and *CBFIV*), *dehydrin* genes (*DHN*), *chloroplast targeting cold-regulated* genes (*ctCOR*), *ice-recrystallization inhibition protein* genes (*IRIP*) and *fructosyltransferase* genes (*FST*)) we extracted translated aminoacid (AA) reference sequences of *H. vulgare* from GenBank [*REF,* supplementary table]. Candidate transcripts were identified by performing protein BLAST searches against AA sequences produced in the OrthoMCL pipeline (maximum bitscore > 90, e-value > 1E-021). When at least one transcript met the criteria, all transcripts of the respective ortholog group were defined as candidate transcripts. Transcripts not part of an ortholog group were included as well (maximum bitscore > 110, e-value > 1E-31). MUSCLE v.3.8.31 (Edgar 2004) was used to create multiple alignments by aligning all candidate transcripts with *H. vulgare* reference CDS and best CDS matches for *Triticum sp., Lolium sp., Oryza sp., Sorghum sp.* and *Zea sp.* acquired from GenBank. To ensure proper tree root resolution, further outgroups were included where necessary. Alignments were manually trimmed and optimized using AliView v1.7.1 sequence editor (Larsson 2014). Transcripts were excluded from subsequent analyses when cross contamination was suspected, i.e. very low total expression and sequence nearly identical to highly expressed contamination source.

Gene tree reconstruction and calibration

For each multiple alignment, the best model of nucleotide substitution (either HKY + Γ, or GTR + Γ) was chosen based on estimations of jModelTest v2.1.7 (Darriba et al. 2012). Using BEAST v1.8.2 (Drummond et al. 2012), we estimated gene trees under an uncorrelated lognormal relaxed clock model with the mean substitution rate uniformly distributed between 0 and 0.06 and a Yule tree prior (birth only) for 100 Million MCMC generations while model parameters were logged every 100000 generations. Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/) was used to assure that all parameters had an effective sample size (ESS) above 200. Ten percent of all trees were discarded (burn-in). The remaining trees were concatenated to the maximum clade credibility tree by TreeAnnotator v2.3.0 (Drummond and Rambaut 2012) and visually adjusted using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Posterior probabilities equal to or greater than 0.8 were considered to be a significant node support. During BEAST analyses, two normally distributed node age priors were used to calibrate gene trees. Prior for *H. vulgare* and *B. distachyon* divergence was set to 44.4 My (3.53 standard deviation) according to estimates from Marcussen et al. (2014) and priors for *O. sativa* and *B. distachyon* divergence was set to 53 Mya (3.6 standard deviation) according to estimates from Christin et al. (2014). To ensure correct rooting of the gene trees, we constrained Pooideae transcripts to form a monophyletic clade with their closest *O. sativa* sequence.

Cold acclimation

The species were tested for ability to acclimate to increase frost tolerance. For this experiment, we used the *H. vulgare* (cultivar ‘Sonja’, provided by Professor Åsmund Bjørnstad, Department of Plant Sciences, Norwegian University of Life Sciences, Ås NO-1432, Norway). In addition to the focal taxa we included *L. perenne* (cultivar ‘Fagerlin’, provided by Dr. Kovi, Department of Plant Sciences, Norwegian University of Life Sciences, Ås NO-1432, Norway), as well as perennial representatives for the Triticeae and Brachypodieae tribes (collected in Norway, October 2015, *Elymus repens* [59.66111, 10.89194], *B. pinnatum* [59.71861, 10.59333] and *B. sylvaticum* [59.68697, 10.61012]). Plants were raised in a green house at 20°C under natural day light before

acclimation. Each individual was divided into four clones, one for each treatment and control. The plants were acclimated at 4°C and short (8h) days for three weeks. Control conditions were short days and 20°C. The light intensity was 50 µmol m^{-2} s^{-1}. At the end of the cold acclimation period, plants were subjected to freezing at three different temperatures (-4, -8 and -12°C) following Alm et al. (2011). For each temperature we used 15 acclimated and 15 non-acclimated individuals per species. Additional 15 individuals per species were kept at control conditions. After freezing, plants were cut down to approximately 3 cm and grown at 20°C under long days in a greenhouse with natural light conditions. Two and three weeks after the plants were moved into 20°C and long days they were assessed for regeneration ability and scored from 0 (dead) to 9 (growth without damage). Differences between acclimated and non-acclimated individuals within each species were tested with a one-tailed *t* test in R (R Core Team 2016) using the 'stats' package.

Results

Freezing tests

Freezing tests revealed that cold acclimation, i.e. the improvement of freezing tolerance through exposure to cold non-freezing temperatures, exists in species of early-diverging lineages. All plants from early-diverging lineages clearly exhibited a higher survival rate at -4 and -8 °C compared to non-acclimated plants (Fig. 1), although the *t* test was not significant at $p<0.05$ for *S. lagascae* and *N. stricta*. Interestingly, non-acclimated plants of early-diverging Pooideae species performed better at -4 °C than non-acclimated *Brachypodium* species and *H. vulgare*, comparable with the survival rates of non-acclimated, perennial core Pooideae species, i.e. *L. perenne* and *Elymus repens*.

***CBFIIIc/d* and *CBFIV* gene family**

We reconstructed two monophyletic, Pooideae-specific *CBFIII* clades (Fig. 2). In the *CBFIIIc* clade, only transcripts from *H. vulgare* were present but none of them were differentially expressed. The *CBFIIId* clade contained numerous transcripts from all study species. With the exception of a transcript-rich *N. stricta* clade that was sister to

the remaining *CBFIIId* homologs and a monophyletic clade that contained all *CBFIIId* transcripts and homologs from *B. distachyon*, the *CBFIIId* clade did not clearly follow the Pooideae's phylogeny. All but two transcripts were differentially expressed and either induced in short-term cold (*N. stricta* and *M. nutans*), long-term cold (*B. distachyon*), or in both cold treatments (*H. vulgare* and *S. lagascae*).

The CBFIV gene tree reconstructed the four known *CBFIV* clades (Fig. 3) but no transcripts from *N. stricta* or *B. distachyon* were present. However, two transcripts from *S. lagascae* and *M. nutans* formed a monophyletic clade together with the *CBFIV* homologs, both of which were cold-regulated. In accordance with the fact that there are no known *CBFIVb* homologs in *H. vulgare*, we only identified cold induced *H. vulgare* transcripts in the remaining three *CBFIV* clades. Interestingly, the two early-diverging Pooideae transcripts related to the *CBFIV* homologs were induced by short-term cold.

Dehydrin genes

Dehydrin (*DHN*) genes are well studied in *H. vulgare* and to date there are 13 known dehydrin homologs. Structurally they can be grouped into four distinct types based on the presence of amino acid segments (Y, K and S): SK_3-type (Hv*DHN8*), KS-type (Hv*DHN13*), K_9-type (Hv*DHN5*) and Y_nSK_n-type dehydrins (the 10 remaining Hv*DHN*-genes) (Kosová et al. 2007). Because these four groups are likely to represent phylogenetically distinct clades (Karami et al. 2013), we reconstructed individual gene trees for each group.

Short-term cold treatment induced *DHN8* transcripts were present in all investigated Pooideae species, two in *B. distachyon*, while the other species possessed one homolog (Fig. S3). The literature contains no reports of *DHN8* homologs species of the *Poeae* tribe, e.g. *L. perenne*, and no homologous sequences have been deposited to GenBank (confirmed with blast searches). Contrary to this expectation, we identified one *L. perenne* transcript that clearly formed a sister relationship with the *Triticeae*'s *DHN8* clade.

We observed similar results for the *DHN13* gene lineage (Fig S4). Transcripts were identified for all investigated species which formed a well supported monophyletic clade. For *B. distachyon* and *M. nutans*, we identified two *DHN13* transcripts.

Beside two short- and long-term cold induced *DHN5* transcripts in *H. vulgare*, we did not identify *DHN5* related transcripts in any of the other investigated species, which is in accordance with the fact that K_n-type dehydrins have previously only been described in *Triticeae*.

The remaining dehydrins identified in barley (*HvDHN1*, *HvDHN2*, *HvDHN3*, *HvDHN4*, *HvDHN6*, *HvDHN7*, *HvDHN9*, *HvDHN10*, *HvDHN11* and *HvDHN12*) belong to the Y_nSK_n-type and they formed six distinct clades (only four shown in Fig. 4). All identified transcripts belong to four clades (*HvDHN1-2*, *HvDHN9-12*, *HvDHN3-4-7* and *DHN10)*, which are specific to Pooideae (Fig. 4), but form a Poaceae-specific clade with homologs from *O. sativa*, *Z. mays* and *S. bicolor*. Three of the Pooideae-specific clades contained transcripts from early-diverging Pooideae species, with *DHN1-2* being specific to the core group and *B. distachyon*. While all transcripts of *N. stricta* and *M. nutans* were induced by short- and long-term cold treatment, *DHN* transcripts of *H. vulgare*, *B. distachyon* and *S. lagascae* were not differentially expressed.

The last two Y_nSK_n-type clades – containing *HvDHN6* and *HvDHN11* – were clearly less related to the other Y_nSK_n-type *DHN* homologs, because they were nested outside the monophyletic, Poaceae-specific Y_nSK_n-type clade (data not shown). *HvDHN11* formed a monophyletic clade with *O. sativa RAB25* and *B. distachyon RAB25*-like. We did not identify any cold-induces transcripts in those two clades, which is expected given that *HvDHN6* and *HvDHN11* are known to be expressed in embryos and caryopses only (Choi and Close 2000; Tommasini et al. 2008).

Chloroplast cold regulated genes

The reconstruction of the *ctCOR* genes resulted in two previously reported (Crosatti et al. 2013), sister clades, containing *WCS19* and *COR14* (Fig. 5), but their sister relationship was not well supported. The two clades were specific to core Pooideae but

nested within a monophyletic, Pooideae-specific clade, containing transcripts from *B. distachyon*, *M. nutans* and *S. lagascae*, which were most similar to *WCS19*. This *ctCOR* clade was sister to an unsupported clade containing homologous *ctCOR*-like transcripts.

While transcripts of *H. vulgare*, *B. distachyon*, *M. nutans* and *S. lagascae* in the *ctCOR* clade are induced by cold, only *S. lagascae* possessed a cold induced *ctCOR*-like transcript. Interestingly, one of the two *ctCOR*-like transcripts of *N. stricta* was induced by short- and long-term cold.

Ice-recrystallization inhibition protein genes

We identified five *H. vulgare* and four *B. distachyon* transcripts, which were nested within a monophyletic *IRIP* clade (Fig. S2). Two of those putative *IRIP* transcripts were truncated and did not contain any IRI motifs, but they were differentially expressed. Within the *IRIP* clade, *B. distachyon* transcripts were solely placed in a monophyletic clade together with sequences from *L. perenne*. We did not find any *IRIP* or *IRIP*-like transcripts in *M. nutans* and *N. stricta*, but identified one *IRIP*-like transcript in *S. lagasceae* and one in *H. vulgare*. These two *IRIP*-like transcripts shared more sequence similarity with *LRR-PSR2* outgroup genes than *IRIP* genes and were placed between the *LRR-PSR2* outgroup and *IRIP* clade.

Expression profiles showed that all but one putative *IRIP* transcript in *B. distachyon*, and all transcripts in *H. vulgare*, were induced during cold. The *IRIP*-like transcripts of *H. vulgare* were induced by cold, while the one transcript of *S. lagascae* was not differentially expressed.

Fructosyltransferase genes

No *FST* transcripts were found in early-diverging Pooideae species (Fig. S1). The *FST* tree contained *1-SST* and *6-SFT* transcripts for *H. vulgare*, but no *1-FFT* transcript. *6G-FFT* genes are only known from species of the *Poeae* tribe and, according to our expectations, we did not find any *6G-FFT* transcripts in the *H. vulgare* transcriptome. Our analyses showed that the *FST* genes formed a monophyletic clade that is sister to a core Pooideae specific clade containing *vacuolar invertase*-like (*vaINV*-like) genes.

Together with two *vaINV*-like genes from *B. distachyon* and *M. nutans*, the two described clades formed a sister clade to a Pooideae specific *vaINV3* clade.

Although the two *H. vulgare FST* transcripts were induced by short- and long-term cold, none of the *vaINV*-like transcripts were differentially expressed. As shown in another study (Li et al. 2012), *vaINV3* transcripts from *B. distachyon* and *H. vulgare* were induced by short-term cold treatment. Interestingly the *vaINV3* transcript from *N. stricta* was not differentially expressed, but contained a fructosyltransferase motif (data not shown).

Few cold responsive genes are conserved in Pooideae

Only 2/10 (i.e. *DHN8* and *DHN13*) of the well described genes involved in cold-stress in core Pooideae, were found to have be conserved completely in cold-response in early-diverging Pooideae lineages (Fig 6). The remaining gene families (8/10) either exhibited different cold responsive expression (Y_nSK_n-type dehydrins, Fig. 4) and showed signs of gene family expansion, (*CBFIIId*, Fig. 2; *CBFIV*, Fig. 3; *ctCOR* Fig. 5) or were specific to certain lineages (*IRIP*, Fig. S2; *CBFIIIc*, Fig. 2; *FST*, Fig. S1; *DHN5*, not shown). However, 6/10 of the gene families exhibited cold induced transcripts in early-diverging lineages.

Discussion

Cold responsive gene families existed in the Pooideae MRCA

To what extent Pooideae species share a common molecular adaptation to cold (the ancestral hypothesis), or alternatively, if cold adaptation in Pooideae has evolved independently (the lineage specific hypothesis) is yet unknown. Regardless of whether the Pooideae MRCA was cold tolerant or not, our results indicate that the MRCA was in possession of a genetic toolkit, which was inherited by its descendants and likely affected the Pooideae's potential to evolve cold adaptation. Of the ten gene families known to be involved in molecular responses to cold stress in core Pooideae species, six (i.e. *DHN8*, *DHN13*, Y_nSK_n-type *DHN*s, *CBFIIId*, *ctCOR* and *CBFIV*) were likely inherited from the Pooideae MRCA as we identified respective homologs in early-

diverging Pooideae lineages. Interestingly, each of those six gene families possess cold-induced transcripts in early-diverging lineages and might have therefore been cold-induced in the MRCA. No homologs were identified in early-diverging lineages for the remaining four gene families (*CBFIIIc*, *IRIP*, *FST* and *DHN5*). Hence, those likely evolved after the early lineages had diverged. Among those six gene families only the *DHN8* (Fig. S3) and *DHN13* (Fig. S4) gene families exhibited the same expression pattern in all species, in addition to highly conserved nucleotide sequences and copy number. These findings suggest that the cold-responsive function of *DHN8* and *DHN13* has been conserved since the MRCA. Because all other gene families showed signs of lineage specific evolution, homologs of these gene families likely gained various functions in different lineages, highlighting the prevalence of lineage specific evolution during cold adaptation.

Pooideae lineages evolved specific cold adaptation by expanding gene families

Our results indicate that gene family expansion was an important mode of adaptation to cold stress in the Pooideae subfamily. This may have lead to functional specialization or novel functions of various gene copies. Other studies showed that stress related gene families tend to expand via tandem duplications (Hanada et al. 2008), which may lead to lineage-specific expansion of the gene family (Lespinet et al. 2002). Six gene families expanded in different lineages, putatively by various duplication events. The Y_nSK_n-type *DHN* family (Fig. 4) expanded into at least three distinct clades early in the Pooideae history, possibly in the MRCA. Another Y_nSK_n-type *DHN* clade (*HvDHN1-2*) seems to have emerged in the Brachypodieae and core Pooideae, and might represent a specific cold adaptation for this group. It is hard to assess where in the Pooideae phylogeny *CBFIIId* gene family (Fig. 2) began expanding, but it is apparent that there were at least three independent expansions in the Nardeae tribe, Brachypodieae tribe and the core Pooideae. Except of a *IRIP* gene family expansion in the Brachypodieae (Fig. S2), all other gene family expansions (*CBFIIIc* [Fig. 2], *CBFIV* [Fig. 3], *ctCOR* [Fig.5], *FST* [Fig. S1] and *IRIP* [Fig. S2]) were identified at the stem of the core Pooideae. Our results corroborate findings from Sandve and Fjellheim (2010), who identified an

increase of gene copy number in four (*CBFIIIc/d*, *CBFIV*, *IRIP* and *FST*) of those gene families as an evolutionary force of cold climate adaptation of Pooideae species.

Cold acclimation evolved independently in different lineages

Cold acclimation is not restricted to core Pooideae and *B. distachyon*, but is also part of the cold adaptation in early-diverging Pooideae lineages (Fig. 1). In core Pooideae, the gene families *DHN5*, *FST*, *IRIP*, *CBFIV* and *CBFIIId* are known to improve freezing tolerance during cold acclimation (Choi et al. 2002; Vágújfalvi et al. 2003; Badawi et al. 2007; Knox et al. 2008; Zhang et al. 2010; Livingston et al. 2009; Knox et al. 2010; Soltesz et al. 2013; Jeknić et al. 2014; Todorovska et al. 2014; Marozsán-Tóth et al. 2015), and long-term cold induced expression patterns of these gene families in our samples of *H. vulgare* are in line with this research. However, such expression patterns and some of the gene families are not conserved throughout the Pooideae. Hence, cold acclimation evolved independently in different Pooideae lineages.

For the *H. vulgare* cold acclimation gene *DHN5* and its ortholog in *T. aestivum* (*WCS120*) (Kosová et al. 2012), no homologs were identified outside the Triticeae tribe, e.g. in *L. perenne*. Thus, it seems likely that this gene evolved within the Triticeae lineage of the core Pooideae. Two of the gene families whose function in cold acclimation is well described are the *FST* and *IRIP* gene families (reviewed by Sandve et al. 2011), but neither of these exist in species of early-diverging lineages, and the FST gene family is exclusive for the core Pooideae. Hence, these gene families represent more recently evolved functions of the cold acclimation response in Brachypodieae and core Pooideae. *Brachypodium distachyon* possesses long-term cold induced transcripts for the *IRIP* (Fig. S2) and also *CBFIIId* (Fig. 2) gene families. Both gene families expanded specifically in the *B. distachyon* lineage and we therefore argue that their function in cold acclimation evolved independently from the core Pooideae.

As opposed to core Pooideae and *B. distachyon*, *CBFIIId* (Fig. 2) and *CBFIV* (Fig. 3) homologs in early-diverging lineages are short-term cold induced (except one long-term cold induced *CBFIIId* transcript in *S. lagascae*), which suggests that they are not involved in cold acclimation processes. The core Pooideae-specific expansion of *CBFIV*

further suggests that this gene family was first co-opted into cold acclimation pathways in core Pooidae.

A gene family that might be involved in cold acclimation exclusively in early-diverging lineages is the Y_nSK_n-type *DHN* gene family (Fig. 4). While Y_nSK_n-type *DHN* homologs are known to be expressed during frost in core Pooideae – which is in line with our findings for *H. vulgare* – transcripts from *M. nutans* and *N. stricta* are induced by long-term cold.

The only gene family whose function in cold acclimation might be conserved throughout the Pooideae is the *ctCOR* gene family (Fig. 5). Except for *S. lagascae*, all investigated species exhibited long-term cold induced transcripts. In *T. aestivum* and *H. vulgare,* expression of *ctCOR* genes during cold acclimation is nearly linearly correlated with the freezing tolerance (reviewed by Crosatti et al. 2013). The putative duplication events, which happened once after the divergence of *N. stricta* and a second time in the core Pooideae, point however towards an independent co-option of *ctCOR* homologs in different lineages.

Our estimates placed gene family expansions of the *FST*, *IRIP*, *ctCOR* and *CBFIIId* families in the period of the E-O transition (Fig. 6). Most interestingly the *CBFIIId* expansions in the lineage of *N. stricta* and *B. distachyon* happened independently during the same time period. Those findings partly confirm analyses run by Sandve and Fjellheim (2010), who suggested that cold responsive gene families expanded due to increased selection pressure for improved cold adaptation during the E-O transition. In contrast to Sandve and Fjellheim (2010), we were unable to correlate initial core Pooideae-specific expansions of the *CBFIII* and *CBFIV* families with the E-O transition. This might be due to unresolved gene phylogenies (*CBFIIId*), or deviating dating estimates (*CBFIIIc*, *CBFIV*). In the case of *FST* and *IRIP* gene families the increased cold stress during the E-O transition might have led to the evolution of novel protein domains in the core Pooideae and Brachypodieae (Sandve et al. 2008; Li et al. 2012). It remains to be tested if similar novelties evolved in other lineages. Based on our findings, there is evidence that the E-O transition affected the molecular evolution of cold adaptive mechanisms in all investigated Pooideae lineages. By the time of the E-O

transition, all major Pooideae lineages had already diverged (Marcussen et al. 2014; Grønvold et al. unpublished) and this supports the indications in our data that cold adaptation largely evolved separately in different Pooideae lineages.

Drought tolerance – an evolutionary basis for cold tolerance?

Parts of the early cold tolerance in Pooideae might have been derived from ancestral drought tolerance. Cold stress may cause dehydration due to decreased membrane stability as well as lead to accumulation of ROS (Kratsch and Wise 2000; Murata et al. 2007; Crosatti 2013), similar to stresses occurring during drought (Mahajan and Tutej 2005). Following this, several authors have speculated about the importance of drought tolerance for the early evolution of the Pooideae (Kellogg 2001; Schardl et al. 2008; Vigeland et al. 2013).

Due to their molecular functions in membrane stabilizing and ROS scavenging, ancestrally drought-responsive genes were suitable candidates for cold responsive pathways when ancestral Pooideae species faced temperate conditions. It has been shown that genes with suitable, pre-existing molecular functions seem to be recruited into certain molecular pathways preferentially (True and Carroll 2003; Christin, Osborne et al. 2013; Christin et al. 2015). Some of our findings lend support to this scenario. Firstly, early-diverging lineages possess cold-induced transcripts of *CBFIIIc* (Fig. 2) and *CBFIV* (Fig. 3) and many *CBF* genes are known to be involved in drought tolerance in several angiosperms (Agarwal et al. 2006; Akhtar et al. 2012). Secondly, *ctCOR* (Fig. 5) transcripts are cold induced in all species. The *ctCOR* genes *COR14* and *WCS19* are thought to be involved ROS-mediated stress (Crosatti et al. 2013), which is both beneficial during drought and cold stress. Thirdly, *DHN8* (Fig. S3) is an ancestral drought-responsive gene, that gained a function in cold tolerance. Orthologs of *DHN8* are involved in the protection of plasma membrane during cold (Yang et al. 2014) and drought stress (Danyluk et al. 1998; Houde et al. 2004), due to a putative ROS scavenging function (Kumar et al. 2014). Since its drought responsiveness seems to be conserved outside the Pooideae (Lee et al. 2005; Badicean et al. 2012), it is likely that *DHN8* gained its cold responsiveness first in the Pooideae MRCA.

Conclusion

Taken together, our results provide valuable insights in the early adaptive evolution of Pooideae and contribute to the understanding how plants evolve cold adaptation. We found signs of conserved cold response that likely existed in the Pooideae MRCA and increased the Pooideae's potential to evolve cold tolerance. The conserved fraction of cold response might have enabled early Pooideae members to survive the first encounters with temperate conditions, making subsequent cold adaptation possible. Additionally, we observed a trend of lineage specific evolution, which led to increased gene family complexity, particularly within the core Pooideae lineage (Fig. 6). Hereby gene family expansion might have played an important role.

Due to the scarce fossil record, divergence times of early-diverging Pooideae lineages are still under dispute and so is the stem age of the Pooideae. In the absence of reliable fossil calibration points our results contribute to a better understanding of climatic changes, that influenced molecular evolution of certain gene families in Pooideae subfamily. However, more reliable calibration points and a resolved genome-level phylogeny of the Pooideae subfamily will help us to improve the dating of the evolutionary history of cold adaptation. This will enable us to confidently correlate paleoclimatic events, like the E-O transition, with molecular innovations in order to reconstruct the colonization of temperate biomes by the Pooideae subfamily. And lastly, higher resolution, i.e. higher phylogenetic coverage, of cold adaptation evolution in the core Pooideae lineage will be valuable to understand the molecular traits that might have contributed to their putative rapid radiation, species richness and expansion into extreme habitats.

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Figures

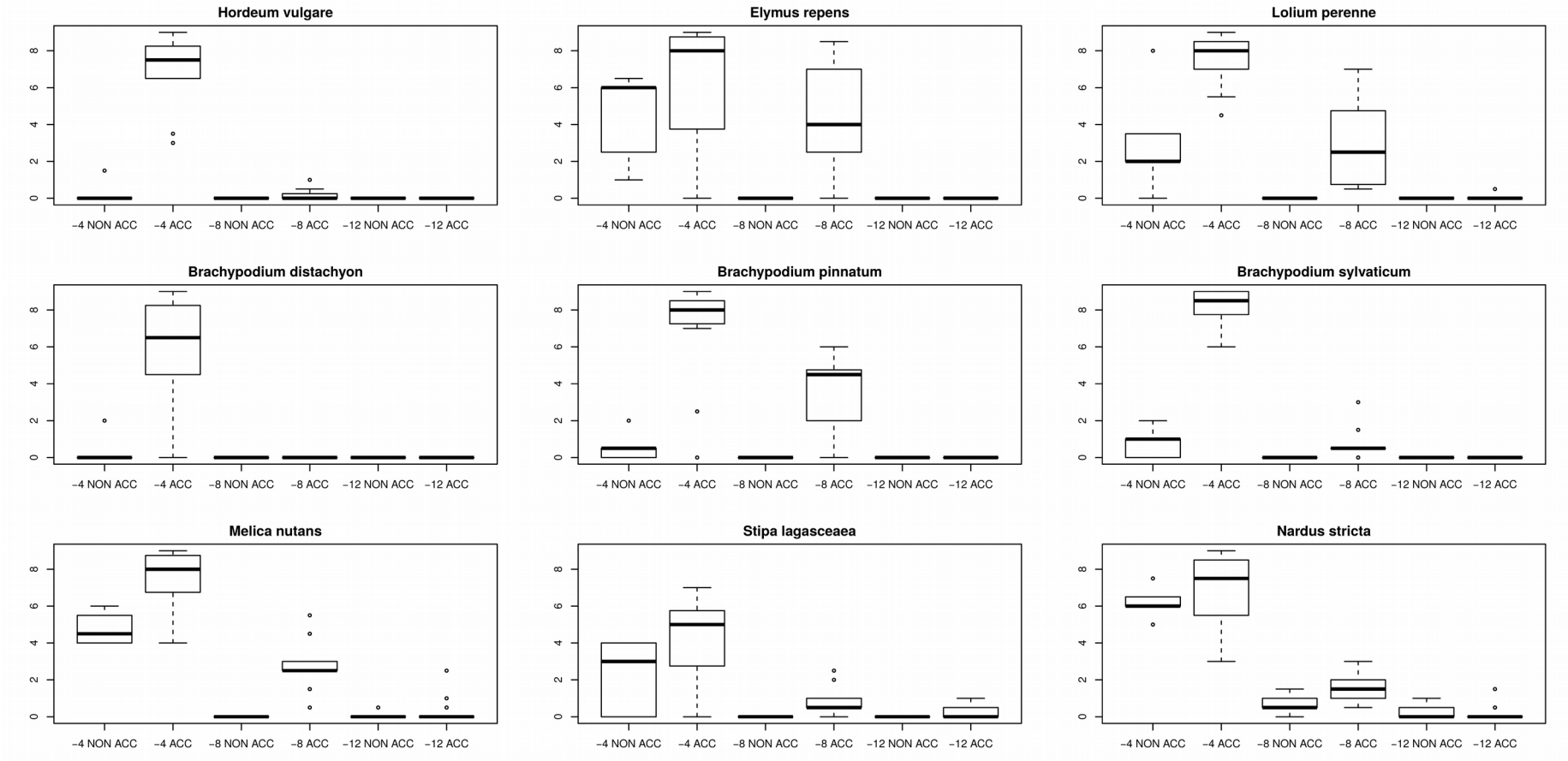

Figure 1: Frost tolerance in response to cold acclimation. Survival rates of nine acclimated and non-acclimated Pooideae species in response to three freezing temperatures (-4°C, -8°C and -12°C). Significant differences between treatments are indicated by asterisks.

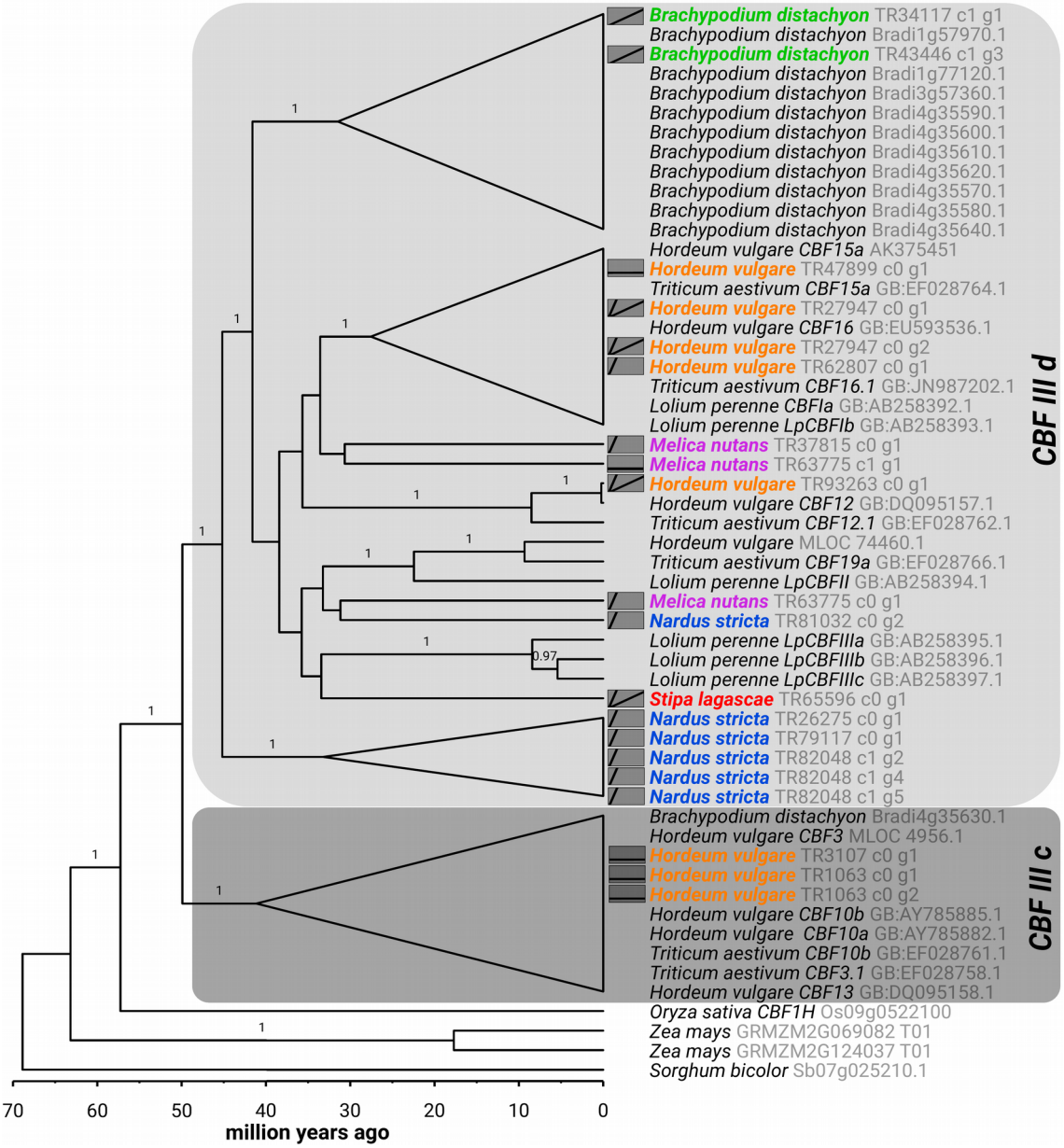

Figure 2: Time calibrated phylogeny for the Pooideae *CBFIIIc/d* gene family. The phylogeny was estimated with BEAST v1.8.2 using a HKY+Γ and an uncorrelated, lognormal distributed molecular clock model. Significant posterior probabilities are shown as branch labels. *CBFIIIc* and *CBFIIId* form to distinct clades.

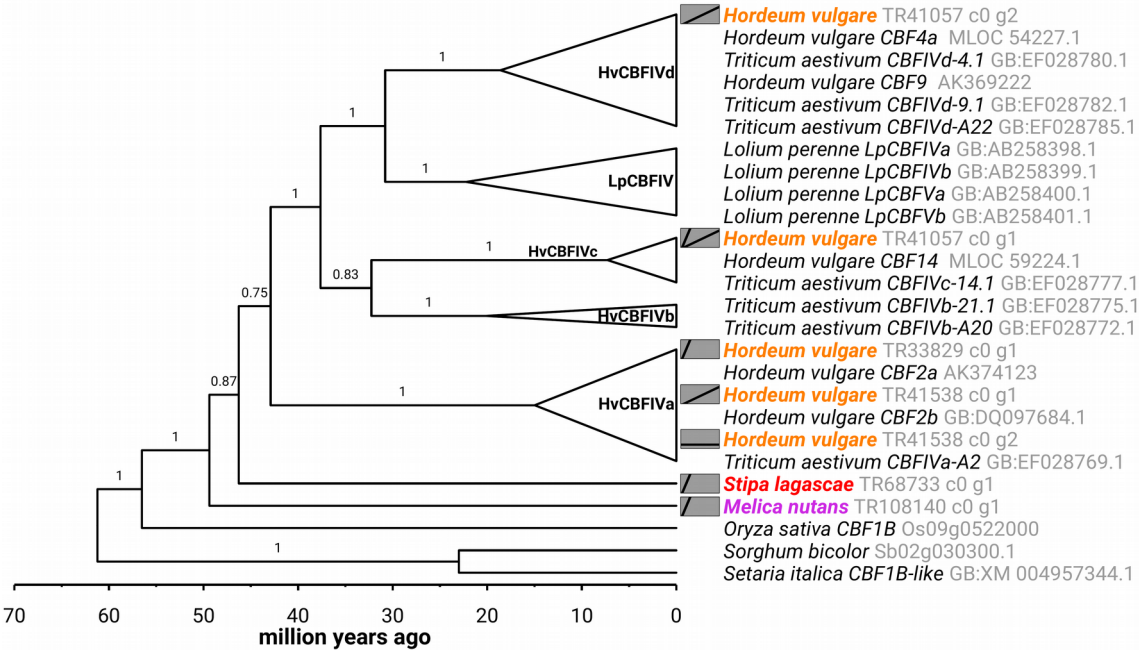

Figure 3: Time calibrated phylogeny for the Pooideae *CBFIV* gene family. The phylogeny was estimated with BEAST v1.8.2 using a HKY+Γ and an uncorrelated, lognormal distributed molecular clock model. Significant posterior probabilities are shown as branch labels

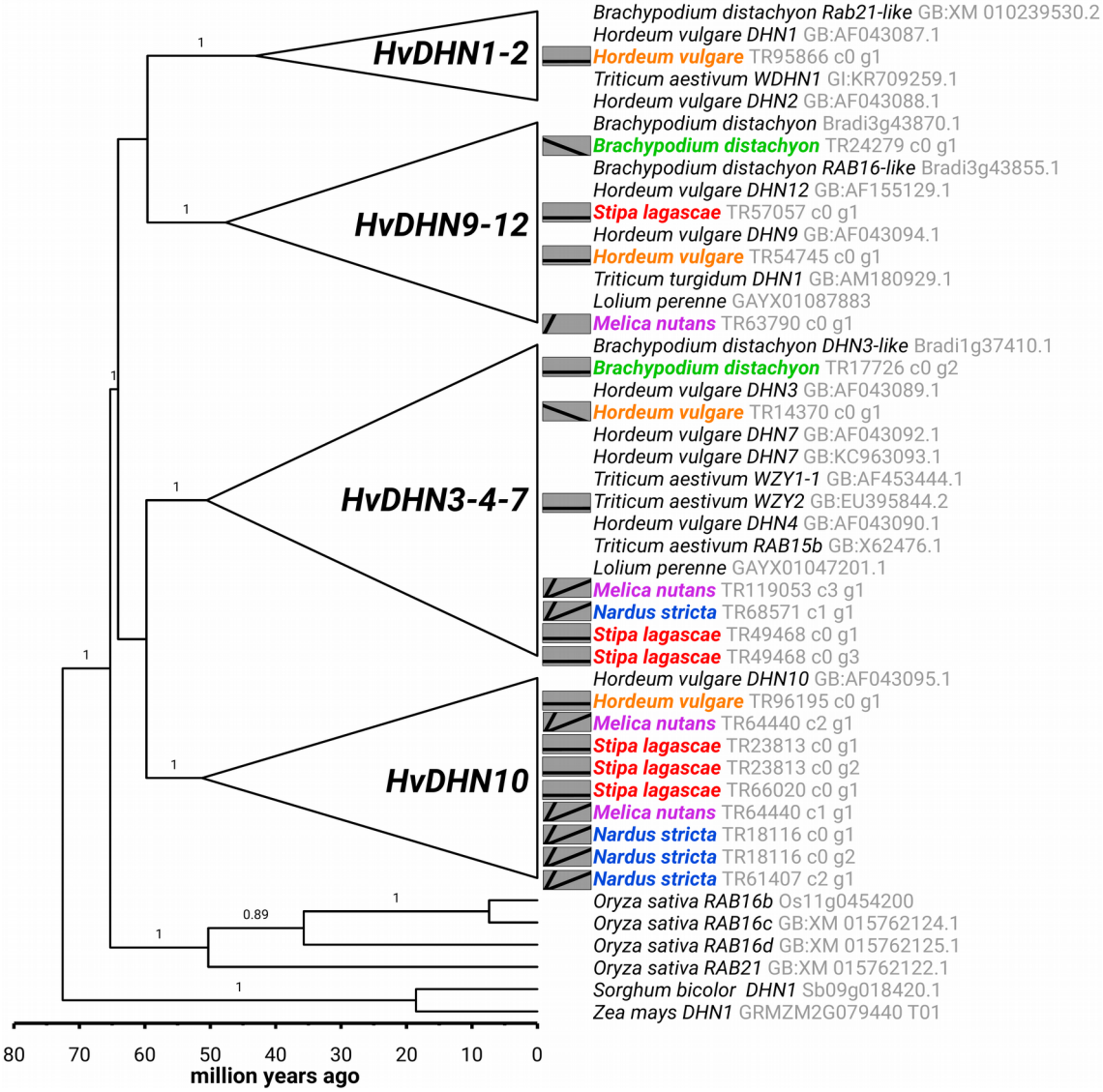

Figure 4: Time calibrated phylogeny for the Pooideae Y_nSK_n-type *DHN* gene family. The phylogeny was estimated with BEAST v1.8.2 using a GTR+Γ and an uncorrelated, lognormal distributed molecular clock model. Significant posterior probabilities are shown as branch labels

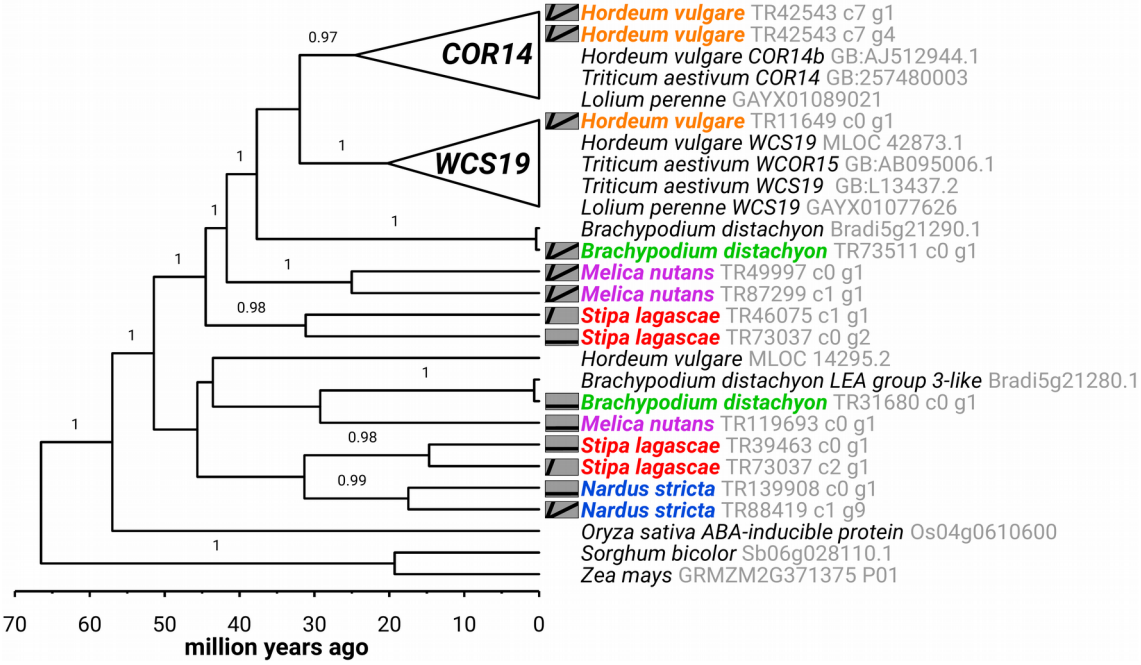

Figure 5: Time calibrated phylogeny for the Pooideae *ctCOR* gene family. The phylogeny was estimated with BEAST v1.8.2 using a GTR+Γ and an uncorrelated, lognormal distributed molecular clock model. Significant posterior probabilities are shown as branch labels.

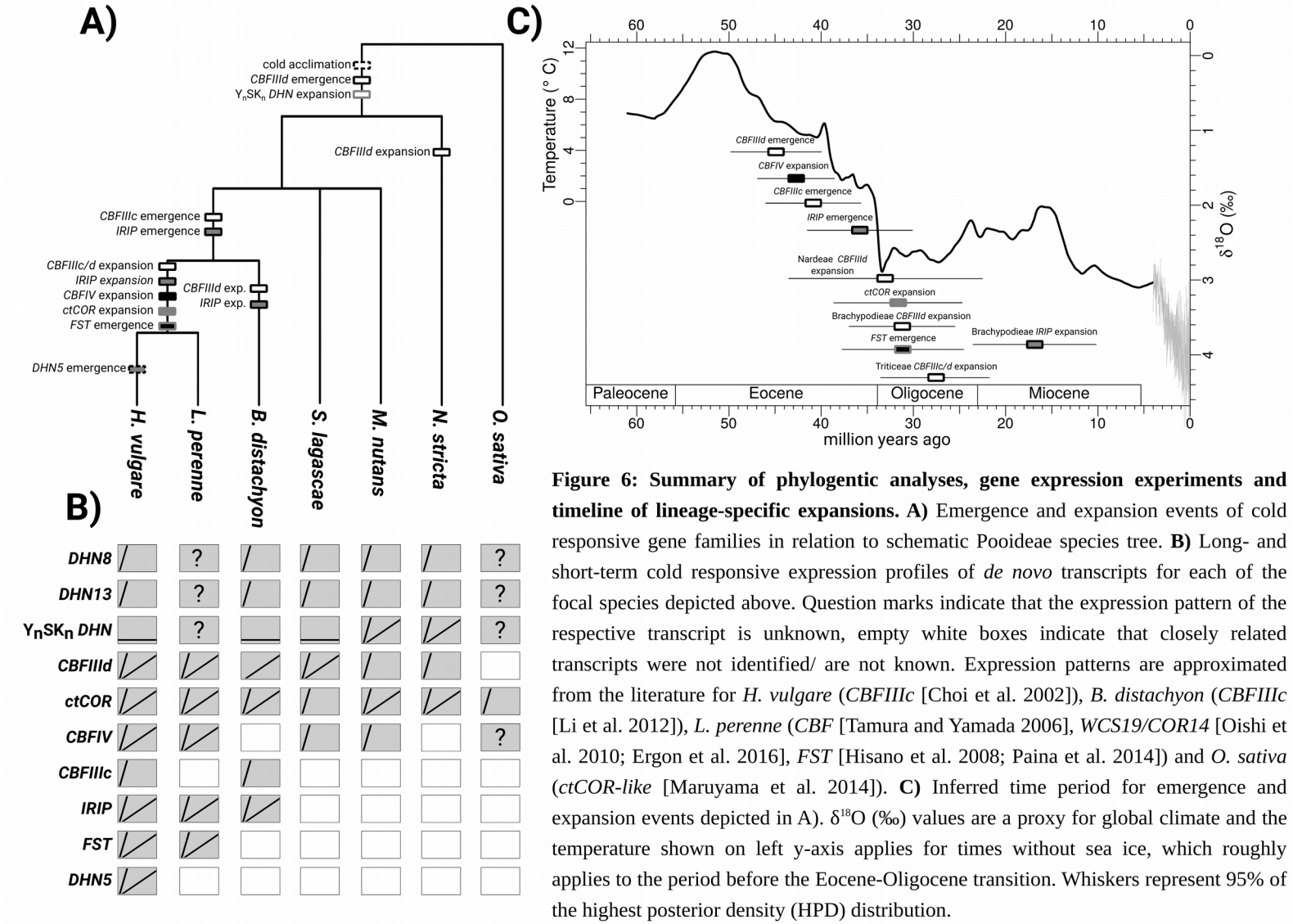

Figure 6: Summary of phylogentic analyses, gene expression experiments and timeline of lineage-specific expansions. A) Emergence and expansion events of cold responsive gene families in relation to schematic Pooideae species tree. **B)** Long- and short-term cold responsive expression profiles of *de novo* transcripts for each of the focal species depicted above. Question marks indicate that the expression pattern of the respective transcript is unknown, empty white boxes indicate that closely related transcripts were not identified/ are not known. Expression patterns are approximated from the literature for *H. vulgare* (*CBFIIIc* [Choi et al. 2002]), *B. distachyon* (*CBFIIIc* [Li et al. 2012]), *L. perenne* (*CBF* [Tamura and Yamada 2006], *WCS19/COR14* [Oishi et al. 2010; Ergon et al. 2016], *FST* [Hisano et al. 2008; Paina et al. 2014]) and *O. sativa* (*ctCOR-like* [Maruyama et al. 2014]). **C)** Inferred time period for emergence and expansion events depicted in A). $\delta^{18}O$ (‰) values are a proxy for global climate and the temperature shown on left y-axis applies for times without sea ice, which roughly applies to the period before the Eocene-Oligocene transition. Whiskers represent 95% of the highest posterior density (HPD) distribution.

Supplementary material

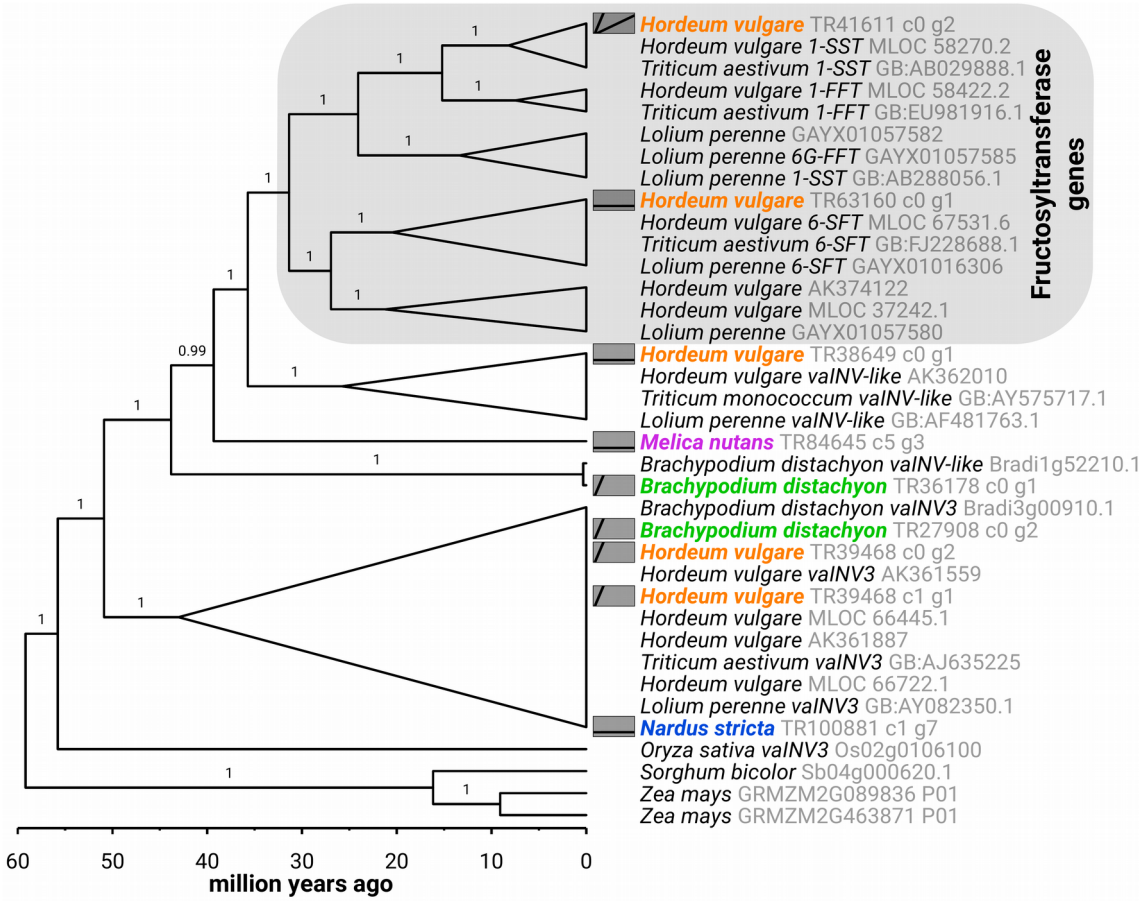

Figure S1: Time calibrated phylogeny for the Pooideae *FST* gene family. The phylogeny was estimated with BEAST v1.8.2 using a GTR+Γ and an uncorrelated, lognormal distributed molecular clock model. Significant posterior probabilities are shown as branch labels.

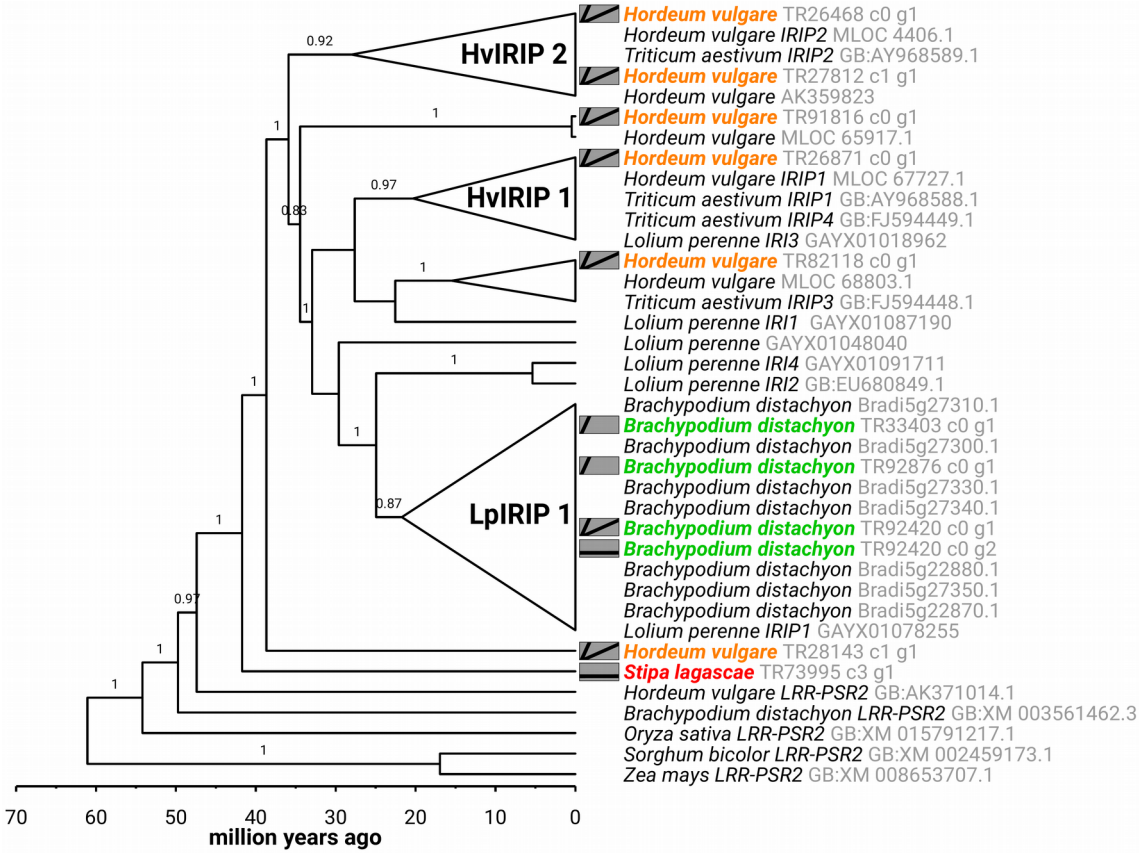

Figure S2: Time calibrated phylogeny for the Pooideae *IRIP* gene family. The phylogeny was estimated with BEAST v1.8.2 using a GTR+Γ and an uncorrelated, lognormal distributed molecular clock model. Significant posterior probabilities are shown as branch labels.

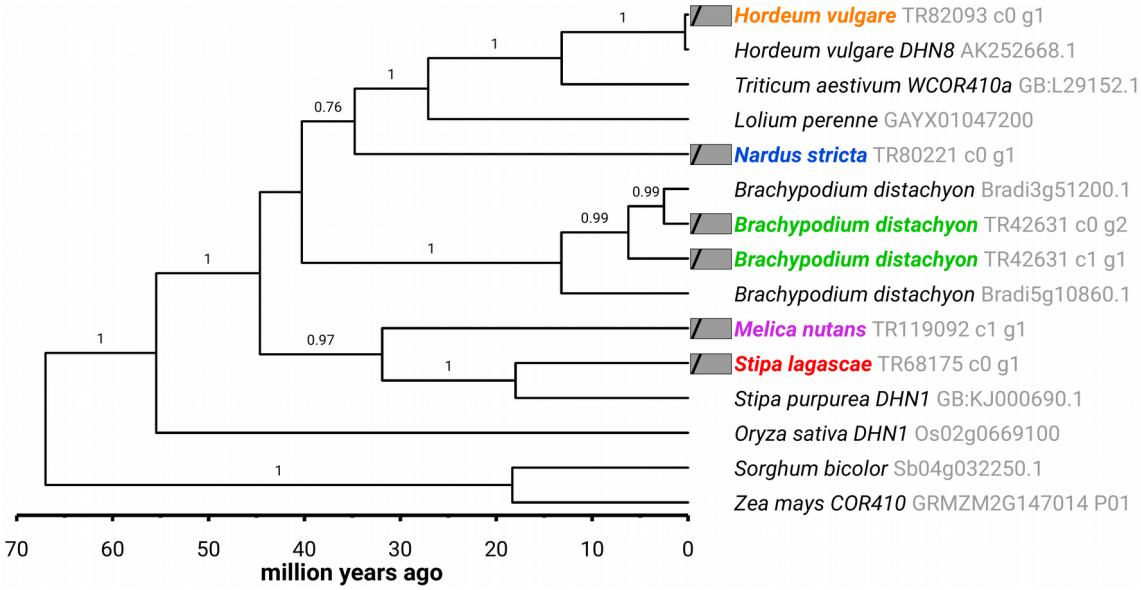

Figure S3: Time calibrated phylogeny for the Pooideae *DHN8* gene family. The phylogeny was estimated with BEAST v1.8.2 using a GTR+Γ and an uncorrelated, lognormal distributed molecular clock model. Significant posterior probabilities are shown as branch labels.

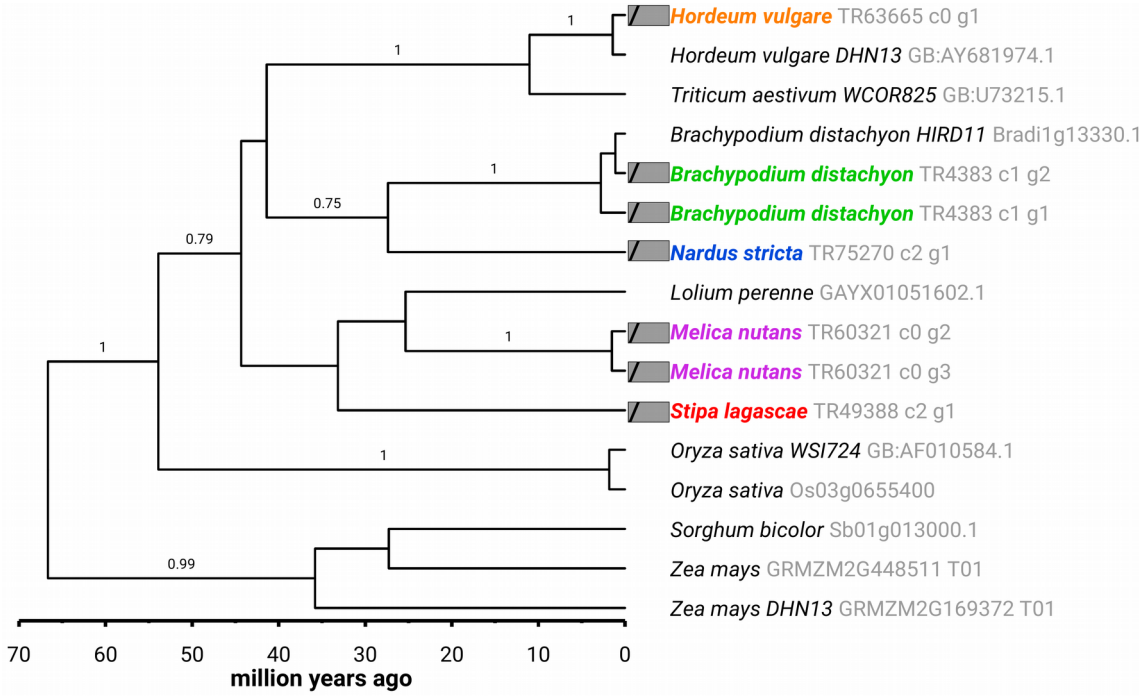

Figure S4: Time calibrated phylogeny for the Pooideae *DHN13* gene family. The phylogeny was estimated with BEAST v1.8.2 using a GTR+Γ and an uncorrelated, lognormal distributed molecular clock model. Significant posterior probabilities are shown as branch labels.

Paper III

Evidence for an Early Origin of Vernalization Responsiveness in Temperate Pooideae Grasses[1][OPEN]

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The ability of plants to match their reproductive output with favorable environmental conditions has major consequences both for lifetime fitness and geographic patterns of diversity. In temperate ecosystems, some plant species have evolved the ability to use winter nonfreezing cold (vernalization) as a cue to ready them for spring flowering. However, it is unknown how important the evolution of vernalization responsiveness has been for the colonization and subsequent diversification of taxa within the northern and southern temperate zones. Grasses of subfamily Pooideae, including several important crops, such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and oats (*Avena sativa*), predominate in the northern temperate zone, and it is hypothesized that their radiation was facilitated by the early evolution of vernalization responsiveness. Predictions of this early origin hypothesis are that a response to vernalization is widespread within the subfamily and that the genetic basis of this trait is conserved. To test these predictions, we determined and reconstructed vernalization responsiveness across Pooideae and compared expression of wheat vernalization gene orthologs *VERNALIZATION1* (*VRN1*) and *VRN3* in phylogenetically representative taxa under cold and control conditions. Our results demonstrate that vernalization responsive Pooideae species are widespread, suggesting that this trait evolved early in the lineage and that at least part of the vernalization gene network is conserved throughout the subfamily. These results are consistent with the hypothesis that the evolution of vernalization responsiveness was important for the initial transition of Pooideae out of the tropics and into the temperate zone.

The ability of plants to match reproductive output with favorable environmental conditions is a key component of individual fitness and likely contributes to current distributional patterns of biodiversity (Rathcke and Lacey, 1985; Chuine and Beaubien, 2008; Matsuoka et al., 2008). A key aspect of reproductive output is flowering time, a major developmental transition that is controlled by exogenous and endogenous factors, including temperature seasonality in temperate plants (Bäurle and Dean, 2006; Dalchau et al., 2010; McClung and Davis, 2010; Tsuji et al., 2011). In "winter" annuals and perennials, the flowering transition is induced by an extended period of cold that results in a physiological switch allowing plants to flower when secondarily induced by specific day lengths and/or warm temperatures. Rapid flowering following exposure to cold temperatures is termed vernalization responsiveness (Chouard, 1960) and is postulated to be an evolutionary strategy to ready temperate plants for spring flowering (Wollenberg and Amasino, 2012; Preston and Sandve, 2013; Fjellheim et al., 2014). However, in regions experiencing little cold or temperature seasonality, a response to vernalization can substantially delay flowering, resulting in reduced fitness. The latter mismatch between seasonal cues and flowering has been postulated to be one of the negative impacts of global warming on plant fitness (Franks et al., 2007; Cook et al., 2012). Thus, understanding the relative lability and genetic architecture underlying gains/losses of vernalization responsiveness will facilitate our understanding of local adaptation and plant responses to climate change.

An exemplar lineage in which to study the evolution of vernalization responsiveness is the ecologically diverse grass family (Poaceae), which originated 51 to 82 million years ago, probably in tropical forests (Kellogg, 2001; Prasad et al., 2011; Christin and Osborne, 2013; Christin et al., 2014; Magallón et al., 2015). Despite its tropical origins, a few grass clades have successfully diversified in temperate environments (Edwards et al., 2010; Edwards and Smith, 2010; Humphreys and Linder, 2013). In particular, the diversification of Pooideae grasses in the northern hemisphere coincides with a period of major global cooling that peaked during the Eocene-Oligocene boundary 34 to 33.5 million years ago (Zachos et al., 2001; Mannion et al., 2014). During this time, it is hypothesized that Pooideae became more cold tolerant (Sandve et al., 2008, 2011; Sandve and Fjellheim,

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J.C.P. conceived the original research plan; all authors conducted experiments; M.M., M.S., and J.C.P. analyzed the data; S.F. and J.C.P. provided research materials; M.M. and J.C.P. wrote the article with contributions by all the authors.

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2010; Preston and Sandve, 2013; Vigeland et al., 2013) and evolved a response to vernalization (Preston and Kellogg, 2008; Ream et al., 2012). Indeed, vernalization responsiveness has been identified in several so-called core group Pooideae (Davis and Soreng, 1993), such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oats (*Avena sativa*), and ryegrass (*Lolium perenne*), and a species sister to core Pooideae, *Brachypodium distachyon* (Brachypodieae; Takahashi and Yasuda, 1971; Heide, 1994; Trevaskis et al., 2003; Yan et al., 2003; Preston and Kellogg, 2008; Greenup et al., 2011; Ream et al., 2014). However, it is unknown whether vernalization responsiveness evolved at the base of the entire Pooideae, concomitant with the tropical to temperate zone transition.

The genetic basis of vernalization responsiveness has been extensively studied in a few economically important core Pooideae species (Ream et al., 2012), revealing two key epistatic vernalization genes, *VERNALIZATION1* (*VRN1*; = *FRUITFULL1* [*FUL1*]) and *VRN2*, and their downstream floral integrator *VRN3* (= *FLOWERING LOCUS T* [*FT*]; Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; Amasino, 2004; Preston and Kellogg, 2006, 2008; Distelfeld et al., 2009; Greenup et al., 2009; Ream et al., 2012). In winter wheat and barley, cold nonfreezing temperatures of the fall/winter cause an increase in leaf and shoot apical meristem transcription of the flowering promoter *VRN1*, leading to repression of the floral repressor *VRN2* and the consequent derepression of *VRN3* (Yan et al., 2003; Yan et al., 2004a; Dubcovsky et al., 2006; Trevaskis et al., 2007; Preston and Kellogg, 2008; Distelfeld et al., 2009). The activation of *VRN1* expression during cold occurs in response to histone modifications, resulting in the achievement of flowering competency, which can be visualized by the transition of the shoot apical meristem to the double-ridge stage (Preston and Kellogg, 2008; Oliver et al., 2009, 2013; Trevaskis, 2010). Once competent, vernalized plants are able to respond to inductive warm temperatures and long days of spring, resulting in the up-regulation of *VRN3* in leaves, and *VRN1* in shoot apices, followed by a rapid transition to flowering.

Allelic variation in *VRN1*, *VRN2*, and *VRN3* has been shown to correlate with population differences in cold-mediated flowering in barley, wheat, ryegrass, and *B. distachyon* (Danyluk et al., 2003; Yan et al., 2003, 2004a, 2004b, 2006; Fu et al., 2005; von Zitzewitz et al., 2005; Szűcs et al., 2007; Kippes et al., 2015). For example, disruption of the regulatory site in the first intron or CArG-like motif of the wheat and barley *VRN1* promoter results in constitutive expression of *VRN1*, leading to early flowering in the absence of cold (Yan et al., 2003, 2004b; von Zitzewitz et al., 2005; Cockram et al., 2007; Pidal et al., 2009). The extent to which Pooideae species outside the core group plus Brachypodieae are responsive to vernalization is unclear (but see Woods et al., 2016). Indeed, although evidence suggests the late incorporation of *VRN2* into the vernalization network at the base of core Pooideae (Woods et al., 2016), it remains to be determined whether other components of this gene network trace back earlier to the ancestor of Pooideae.

Here, we test predictions of the hypothesis that *VRN1*-mediated vernalization responsiveness evolved early in the diversification of Pooideae. Although we find no evidence of positive selection along branches leading to the Pooideae or core Pooideae *VRN1* lineages, our data demonstrate that *VRN1* and/or *VRN3* expression is directly (*VRN1*) or indirectly (*VRN3*) upregulated by cold in several phylogenetically widespread species and that this correlates with the reconstructed early origin of vernalization-controlled flowering. Taken together, these results are consistent with recruitment of flowering time genes into the vernalization network at or around the base of Pooideae.

RESULTS

Identification of Vernalization-Responsive Species

In order to determine if species across Pooideae are responsive to vernalization, temperature adjusted days to flowering, tiller number at flowering, and leaf number at flowering were measured and compared between vernalized and unvernalized plants of phylogenetically representative species. Adjusted days to flowering takes into account the difference in accumulated heat units between warm and cold conditions during the 6-week vernalization period (33.6 d) and attempts to correct for the potential negative impact of reduced temperature on growth (Kirby et al., 1989; Baloch et al., 2003). Of the 61 focal taxa that germinated under growth chamber conditions (Supplemental Table S1), 40 had at least three flowering individuals in one or both treatments, including 10 core and 30 noncore Pooideae (Fig. 1). Within the flowering core Pooideae that were not previously known to be responsive to vernalization (i.e. excluding the controls barley, wheat, *Festuca pratensis*, and ryegrass) five out of six (83%) flowered significantly earlier with versus without vernalization, whereas this ratio (removing the two *B. distachyon* controls) was only 13 out of 28 (46%) for taxa outside the core group (Fig. 2). Vernalization-responsive species also showed reduced tiller number at flowering with versus without cold treatment, except for *Achnatherum calamagrostis*, *Duthiea brachypodium*, *Hesperostipa spartea*, *Macrochloa tenacissima*, *Nassella pubiflora*, and *Piptatherum aequiglume* (Supplemental Fig. S1). However, in the case of *H. spartea* and *M. tenacissima*, total number of leaves at flowering was significantly ($P < 0.05$) lower with versus without vernalization (Supplemental Fig. S2).

Evolutionary History of *VRN1*- and *VRN3*-Like Genes

Bayesian and maximum likelihood (ML) analyses support two major clades within the grass *VRN1*/*FUL2* gene tree, consistent with gene duplication at the base of Poaceae (Preston and Kellogg, 2006; Fig. 2A). The *VRN1* clade is strongly supported by a Bayesian posterior probability (PP) value of 1.0 and a ML bootstrap

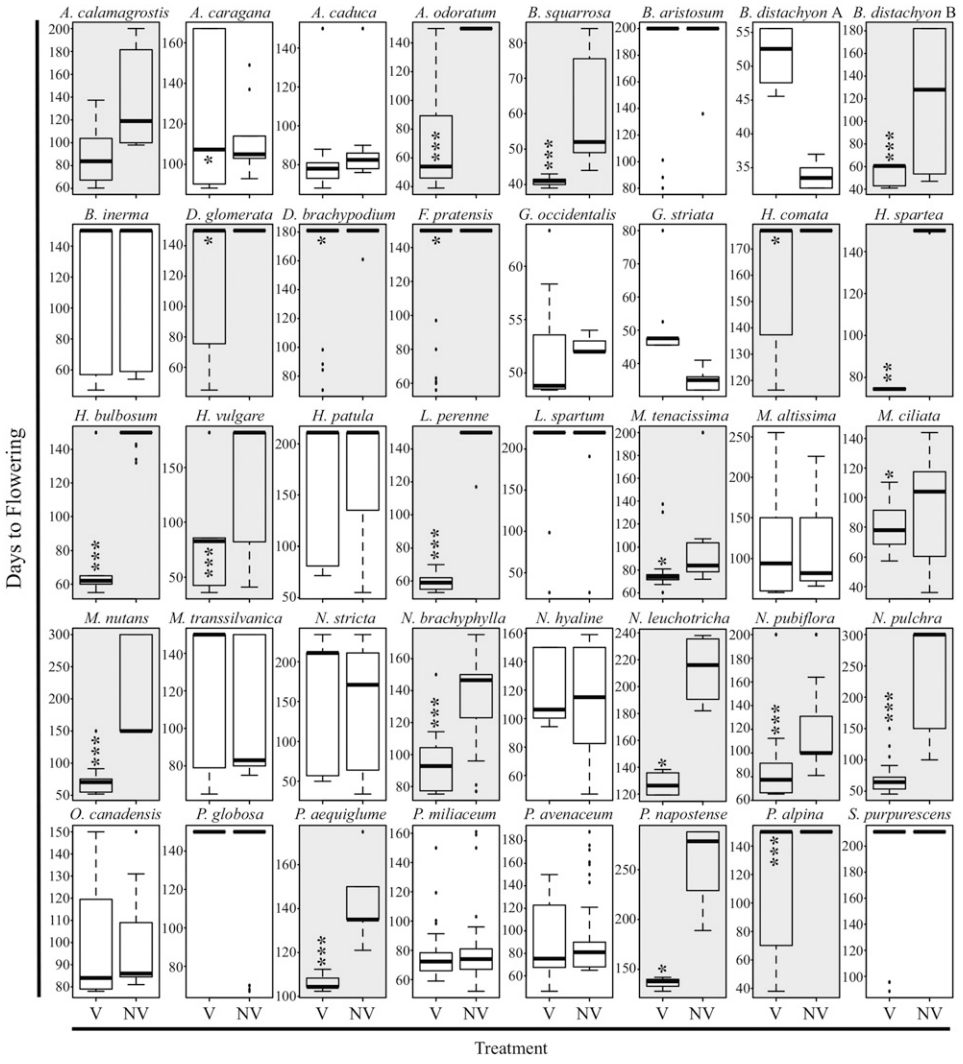

Figure 1. Box plots of days to flowering under vernalization (V) and control (NV) conditions. Days to flowering are adjusted by heat units relative to control plants. Data shown are from two to three experimental replicates for species that had at least three flowering individuals per replicate. Days to flowering for nonflowering individuals was scored as the number of days at which experiments were terminated, i.e. between 150 and 300 d. Gray boxes denote responsive taxa; white boxes unresponsive taxa. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

support value (MLB) of 98%, whereas the *FUL2* clade is strongly supported by a PP value of 1.0 and a MLB value of 97%. Within each clade, topologies largely track relationships found in the Poaceae species tree (Grass Phylogeny Working Group II, 2012). One major exception in the *VRN1* clade is the position of Ehrhartoideae sequences (e.g. rice [*Oryza sativa*] *OsMADS14*) sister to all other sequences from other major grass subfamilies. Within the well-supported Pooideae clades (1.0 PP and 92% MLB for *VRN1*; 0.99 PP for *FUL2*), relationships adhere to the tribal-level species tree, except for the position of *Nardus stricta VRN1* and *FUL2*. Similar to the *VRN1/FUL2* tree, Bayesian and ML analyses suggest a gene duplication event at or before the base of grasses, giving rise to two major *VRN3/FT* clades (Fig. 2B). The *VRN3* clade is strongly supported by PP and MLB values of 1.0 and 100%, respectively, whereas the *VRN3*-like clade is supported by PP values of 1.0 and MLB values of 76%. Tribal-level relationships within Pooideae *VRN3* tree are weakly supported.

To test for evidence of positive selection that might indicate novel protein-binding domains within Pooideae *VRN1* genes, PAML analyses were conducted by comparing two competing branch-site models. The likelihood ratio test estimated that it was not significantly likely that positive selection occurred on the branch leading to Pooideae ($\omega = 1.88$; $P = 0.81$; Fig. 2) nor on the branch leading to the core Pooideae ($\omega = 1.00$; $P = 1.00$; green box in Fig. 2). This suggests that *VRN1* evolved under purifying or neutral selection along these branches.

VRN1 and *VRN3* Gene Expression in Response to Vernalization

In order to test predictions that *VRN* genes will increase in expression during (*VRN1*) and shortly following (*VRN3*) vernalization, *VRN1* and *VRN3* expression profiles were determined for six phylogenetically representative species outside core Pooideae. As predicted, *VRN1* transcript levels increased significantly in the vernalization requiring species *Nassella pulchra* (Stipeae; $P < 0.001$) and *Melica nutans* (Meliceae; $P < 0.001$)

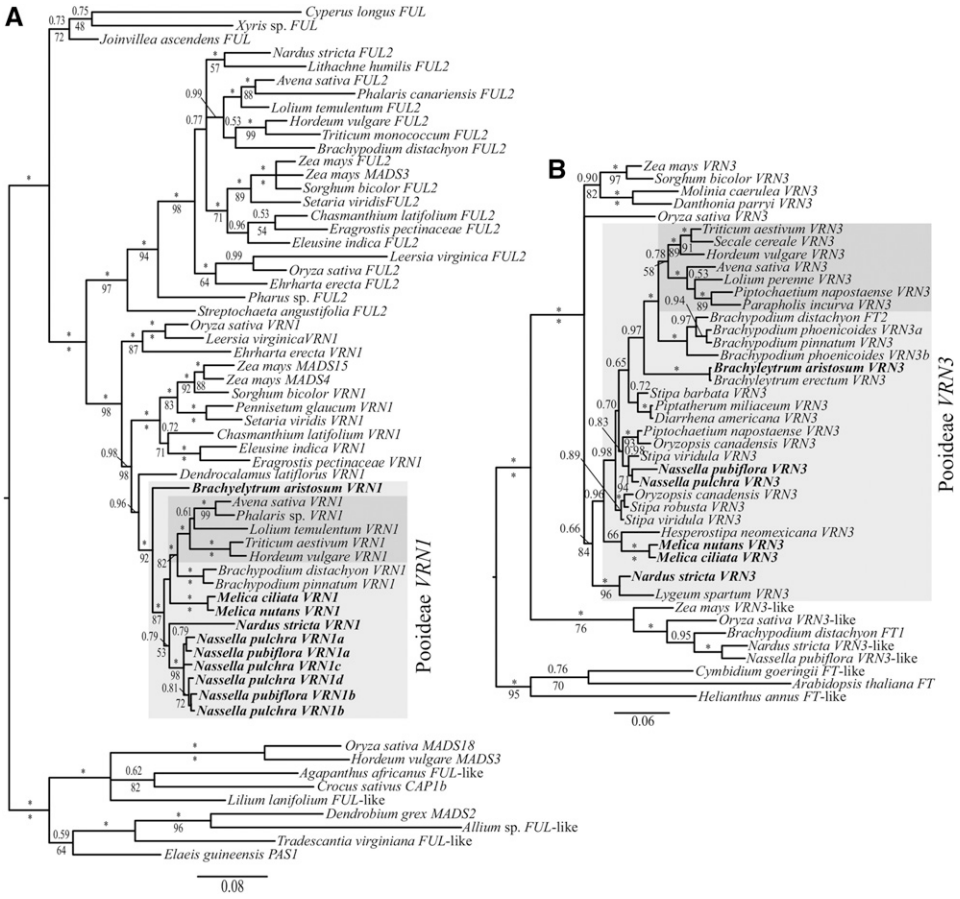

Figure 2. *VRN1* (A) and *VRN3* (B) Bayesian 50% majority rule tree with PPs > 0.5 (above branches) and MLB support values > 50% of 1,000 bootstrap replicates (below branches). Bars indicate substitutions per site. Focal genes are indicated in bold, focal clades are denoted with gray boxes, and focal core Pooideae genes are indicated with dark-gray boxes. Asterisks denote PP values of 1.0 and MLB values of 100%.

during vernalization (Fig. 3), at which stage apical meristems of both species had clearly transitioned to flowering (Fig. 4, B and G). Furthermore, there was a significant interaction between time point and treatment (*N. pulchra*, $P < 0.001$; *M. nutans*, $P < 0.01$), with *VRN1* leaf mRNA transcripts increasing more sharply from pretreatment to "4wk" or "6wk" cold time points compared with pretreatment to "4wk" or "6wk" warm time points (Fig. 3; Supplemental Table S2). The inability of warm-treated *N. pulchra* and *M. nutans* to up-regulate *VRN1* expression correlated with the delay in shoot apical meristem development relative to vernalized plants (Fig. 4, B versus C, and F versus G).

In contrast to their close relatives, leaf *VRN1* expression in weakly vernalization responsive *N. pubiflora* and *Melica ciliata* did not increase with vernalization (Fig. 3) and showed no difference in response between warm and cold treatments (interaction term in Supplemental Table S2), and both species transitioned to the double ridge stage of flowering faster (in terms of uncorrected days) in warm versus cold treatments (Fig. 4, A and D versus E). However, in the distantly related nonresponsive species *N. stricta* (Nardeae) and *Brachyelytrum aristosum* (Brachyelytreae), *VRN1* was significantly upregulated during vernalization (*N. stricta*, $P < 0.001$; *B. aristosum*, $P < 0.001$), and relative transcript levels showed a strong time point by treatment interaction (*N. stricta*, $P < 0.05$; *B. aristosum*, $P < 0.05$; Fig. 3). This lack of correlation between flowering time and *VRN1* expression in *N. stricta* and *B. aristosum* is suggestive of independent losses of vernalization responsiveness through a *VRN1*-independent mechanism, as has been observed in spring cereals. Alternatively, vernalization responsiveness evolved after the *VRN1-VRN3* regulon was in place, after the divergence of Brachyelytreae and Nardeae from the rest of Pooideae.

In species where *VRN1* was upregulated during cold, we predicted that *VRN3* transcription would rapidly increase postcold. In line with this prediction, *N. pulchra*, *M. nutans*, and *B. aristosum* showed a significant time point by treatment interaction for *VRN3* expression (Supplemental Table S2). Transcript levels were significantly higher postvernalization relative to prevernalization in *N. pulchra* and *B. aristosum* (Fig. 3; Supplemental Table S2). Furthermore, *VRN3* expression in *M. nutans* at the "post" time point was significantly higher in cold than warm ($P < 0.001$), despite only marginal up-regulation from pre- to postvernalization time points (Fig. 3). In the case of *N. stricta*, although there was a trend toward increased *VRN3* expression following cold (Fig. 3), this was not significant (Supplemental Table S2).

Although *VRN1* transcript levels were not significantly upregulated by cold in *N. pubiflora* or *M. ciliata*,

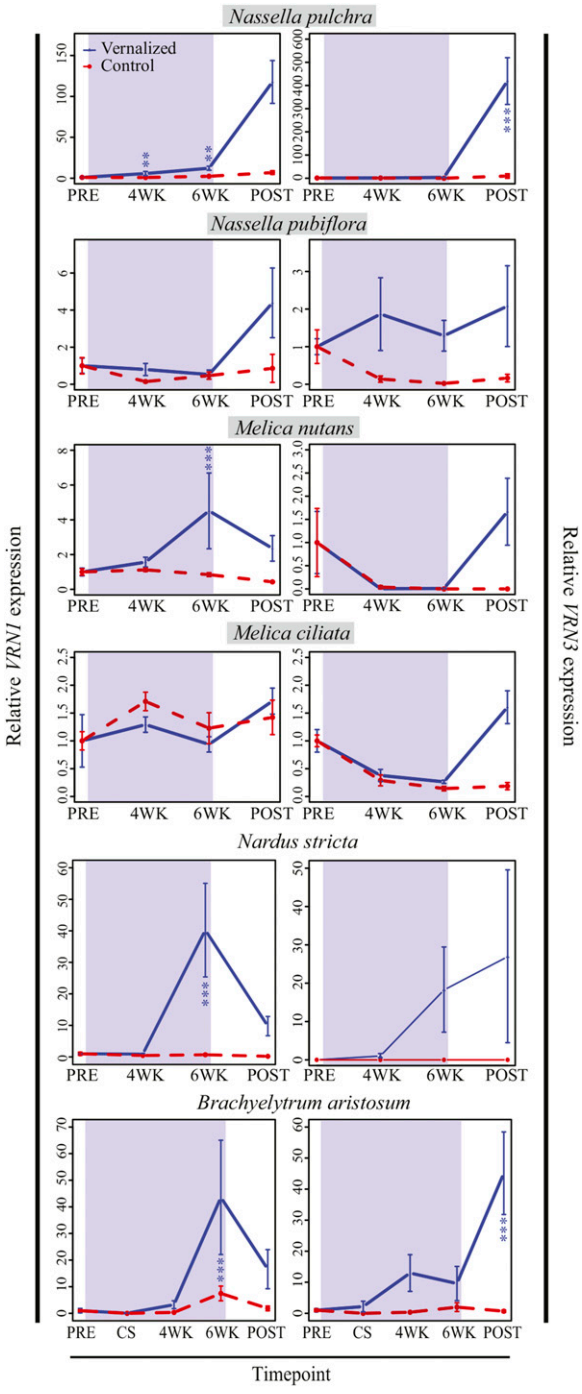

Figure 3. Relative *VRN1* and *VRN3* expression in vernalization responsive (gray boxed taxa) and unresponsive noncore Pooideae species. Blue background indicates the timing of cold treatment when relevant; color-coded asterisks indicate level of significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$) for differences between samples collected after 6 weeks cold (blue) or warm (red; *VRN1* only) or posttreatment cold (blue) or warm (red; *VRN3* only) versus pretreatment samples. Error bars indicate SE. Data shown represents one to three experimental replicates with three to four biological replicates each. PRE, after 4 weeks at 20°C; CS, 1 d cold shock at 4°C (cold treatment only); 4WK, 4 weeks at 4 or 20°C; 6WK, 6 weeks at 4 or 20°C; POST, 2 weeks after treatment at 20°C.

VRN3 expression showed a significant time point by treatment interaction for both species ($P < 0.05$ and $P < 0.01$, respectively), with higher mean transcript levels in vernalization versus control conditions (Supplemental Table S2). However, the fact that both species had transitioned to flowering well before the posttreatment time point (56 d or earlier) under warm conditions (Fig. 4, A and E) suggested that the peak of *VRN3* expression in warm conditions might have been missed. To test this interpretation, follow-up experiments were run, with RNA samples taken after only 1, 2, and 3 weeks of treatment (Supplemental Fig. S3; Supplemental Table S3). As expected, *N. pubiflora VRN3* expression significantly increased in warm, but not cold treatment (Supplemental Table S2; Supplemental Fig. S3). Interestingly, in *M. ciliata*, *VRN3* expression did not significantly differ between cold and warm treatments, as transcript levels in both treatments steadily decreased across the seven week experiment (Supplemental Table S3; Supplemental Fig. S3) and did not correlate with the transitional apical meristem morphology (Fig. 4, D and E).

Ancestral State Reconstruction of Vernalization-Mediated Flowering and *VRN1* Expression

To formally test the hypothesis that vernalization responsiveness in both flowering and *VRN1* expression evolved at the base of Pooideae, we carried out ancestral trait reconstruction using a rooted chloroplast phylogeny that included all focal taxa (Fig. 5). Bayesian MCMC analyses supported a one-rate/symmetrical model of evolution with an early origin of vernalization responsiveness in flowering, at least in the ancestor of Duthieae-Meliceae-Stipeae-Diarrheneae-Brachypodieae-core Pooideae (PP = 0.83 ± 0.09). The ancestor of this group plus Nardeae was also reconstructed as vernalization responsive in flowering, but with less support (PP = 0.67 ± 0.07), whereas the ancestral state of all Pooideae was largely equivocal (PP = 0.55 ± 0.05; Fig. 5). Although most other ancestors of major clades in Pooideae were reconstructed as either vernalization responsive (e.g. Brachypodieae-core Poodieae, PP = 0.72 ± 0.12) or equivocal, the ancestor of Meliceae was estimated to flower without a response to vernalization (PP = 0.90 ± 0.07; Fig. 5). According to this scenario, the ancestor of Meliceae lost a flowering response to vernalization, meaning that vernalization responsiveness in *Melica* is derived within Pooideae. A similar reconstruction of gene expression strongly supported the ancestor of Pooideae *VRN1* (PP = 0.90 ± 0.12) and that of most other major clades as being responsive to vernalization (Fig. 5). The exception again was the ancestor of Meliceae, although in this case the ancestral state was equivocal (PP = 0.58 ± 0.06 for nonresponsiveness).

DISCUSSION

Vernalization responsiveness has evolved multiple times independently within the angiosperms and is

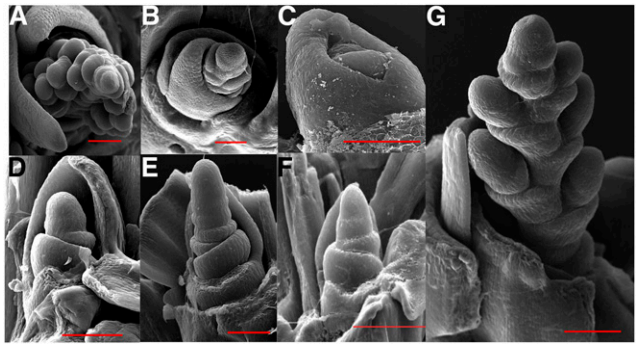

Figure 4. Shoot apical meristem (SAM) developmental transition in *Nassella* and *Melica* with and without vernalization treatment. A, *N. pubiflora* SAM has transitioned to an inflorescence meristem after 56 d (4 week time point) in the warm (control) treatment. B, *N. pulchra* SAM has transitioned to a floral meristem after 70 d (6 week time point) in the cold treatment. C, *N. pulchra* vegetative SAM after 84 d (post time point) in the warm treatment. D, *M. ciliata* SAM after 70 d in the cold treatment. E, *M. ciliata* SAM elongating and transitioning to a floral meristem after 70 d in the warm treatment. F, *M. nutans* SAM remains in the vegetative state after 84 d in the warm treatment. G, *M. nutans* SAM has transitioned to an inflorescence meristem after 84 d in the cold treatment. Bar = 100 μm.

hypothesized to have played a key role in repeated plant diversification within the cold temperate zone (Ream et al., 2012; Preston and Sandve, 2013; Preston et al., 2016; Woods et al., 2016). One prediction of this hypothesis is the early origin of a vernalization-induced flowering gene network in predominantly temperate clades, facilitating their niche transition. To test this prediction, we generated flowering data for representative taxa of cool-season Pooideae grasses and reconstructed vernalization responsiveness across the subfamily. Additionally, we leveraged genetic data on economically important crop species (Takahashi and Yasuda, 1971; Heide, 1994; Trevaskis et al., 2003; Yan et al., 2003; Preston and Kellogg, 2008; Greenup et al., 2011; Ream et al., 2014) to determine if orthologs of the vernalization gene *VRN1*, and the floral integrator *VRN3*, are cold responsive across the diversity of Pooideae. Our findings demonstrate that vernalization responsiveness is widespread in Pooideae and probably evolved early in the subfamily. Moreover, the increase in *VRN1* transcription and that of its positively correlated target *VRN3* in response to cold is reconstructed as ancestral to all Pooideae. Potential caveats to our study are the lack of population-level sampling and restrictions to the number of flowering accessions we were able to work with. However, we attempted to control for bias toward vernalization responsive or nonresponsive taxa by selecting accessions from across the Pooideae phylogeny and those that span the northern (e.g. *Nassella viridula*) to southern temperate (e.g. *Nassella hyaline*), to highland tropical (e.g. *Nassella brachyphylla*) zones. Taken together, our data support the early evolution of vernalization responsiveness in Pooideae. This could have coincided with a major niche shift from the tropics to the temperate zone.

It was previously demonstrated in wheat and barley that loss of *VRN1* function occurs readily via mutations in cis-regulatory sequences within the promoter and first intron (Yan et al., 2003, 2004b; von Zitzewitz et al., 2005; Cockram et al., 2007; Pidal et al., 2009; Kippes et al., 2015). Furthermore, loss of the *VRN3* repressor VRN2 and mutations in cis-regulatory regions of the VRN3 promoter and first intron have been implicated in transitions from winter to spring flowering phenotypes (Yan et al., 2004b, 2006; Trevaskis et al., 2007; Kippes et al., 2016). Although not supported by the ancestral trait reconstruction analysis, which favored a symmetric model of evolution, these functional data together suggest that vernalization responsiveness is easier to lose than gain, lending further weight to the early origin hypothesis for the evolution of vernalization responsiveness in Pooideae. To our knowledge, no members of Bambusoideae have been explicitly tested for a response to vernalization. Regardless, we assume it unlikely that the ancestor of Bambusoideae and Pooideae used cold as a flowering cue. This is largely based on the tropical reconstruction of the Bambusoideae-Ehrhartoideae-Pooideae clade and the largely unpredictable timing of flowering in many bamboos (Nadguada et al., 1990; Edwards and Smith, 2010; Guerreiro 2014; Veller et al., 2015). Furthermore, although some highland rice (Ehrhartoideae) varieties are chilling tolerant (Chawade et al., 2013), there are no examples of vernalization-responsive rice populations.

In addition to supporting an early origin of vernalization responsiveness in Pooideae, our data suggest multiple independent losses, and at least one derived gain, of this trait. Based on our reconstruction, vernalization responsiveness in flowering has been lost at least once in the Duthieae and Stipeae, similar to multiple losses documented in cereal cultivars of the core Pooideae (Jensen et al., 2005; Kippes et al., 2016) and Brachypodieae (Higgins et al., 2010). Since cold-activated *VRN1* expression is present in the non-vernalization responsive sister tribes to most Pooideae (i.e. Brachyleytreae and Nardeae), we suggest that cold regulation of *VRN1* evolved at the base of Pooideae but was inadequate to induce flowering in its early inception, as suggested by *N. stricta VRN3* expression. Indeed, in addition to the vernalization response, cold-regulated *VRN1* expression has been implicated in regulating the cold acclimation response, through a negative interaction with dehydration responsive element-binding/C-repeat Binding Factor (DREB1/CBF) genes (Dhillon et al., 2010; Deng et al., 2015). If this interaction is present in *B. aristosum* and *N. stricta*, it might explain the initial evolution of cold responsiveness in *VRN1*. Alternatively, if vernalization responsive flowering is actually ancestral to Pooideae, it might be that it was lost independently in Brachyleytreae and Nardeae due to a dampening of VRN1-mediated *VRN3* regulation or the evolution of a parallel flowering pathway.

Another example of an inferred loss of vernalization responsiveness is in the ancestor of Meliceae. If this

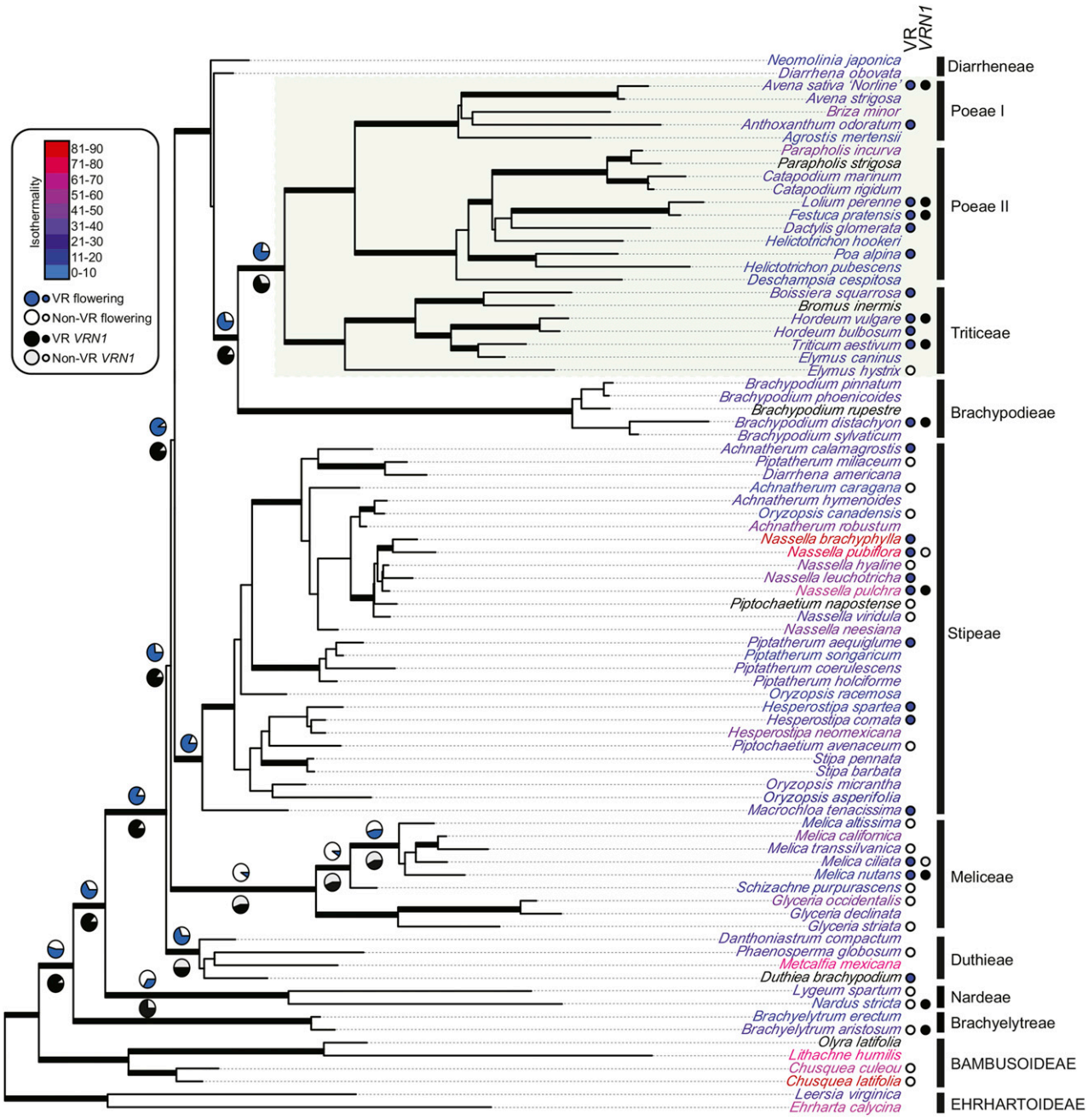

Figure 5. Best Pooideae Bayesian tree topology based on partitioned *matK* and *ndhF* sequences. Thick black branches denote > 0.95 Bayesian posterior probability. Green box defines core Pooideae. Taxon label colors show average isothermality (ratio of diurnal to seasonal temperature variation) based on Worldclim bio3 data extracted from GBIF locality points. Vernalization-responsive (VR) flowering and *VRN1* expression are indicated at branch tips where available. Probabilities of responsiveness are shown as pie charts at selected branches of interest.

scenario holds, we would hypothesize one or more derived origins for vernalization responsiveness in *Melica* using a conserved or derived *VRN1-VRN3* pathway, as observed in *M. nutans*. One prediction of the derived *VRN1-VRN3* pathway hypothesis is novel *VRN1* cis-regulatory mutations, or *VRN1* regulatory modules, in *M. nutans* relative to other vernalization responsive Pooideae species such as *B. distachyon* or *N. pulchra*. It would also be of interest to determine whether temperate grasses outside Pooideae flower in response to vernalization, such as *Danthonia californica* (Danthoniodeae) and *Themeda triandra* (Panicoideae), and if these independent origins involved the repeated cooption of *VRN1*.

Despite evidence for an early origin of vernalization responsiveness in Pooideae (this study), data suggest modifications to the underlying genetic pathway, at least at the base of the core Pooideae (Woods et al., 2016). In barley, VRN1 directly binds to the promoter of the flowering repressor *VRN2*, resulting in the down-regulation of *VRN2* during winter cold (Deng et al., 2015). However, although it likely functions as a flowering repressor across grasses (Yan et al., 2004a; Weng et al., 2014; Nemoto et al., 2016; Woods et al., 2016), noncore Pooideae *VRN2* transcripts are not down-regulated in response to cold or *VRN1* up-regulation (Woods et al., 2016). The co-option of *VRN2* into the vernalization network might therefore have occurred through changes in the DNA-binding domain of VRN1 or through novel cis-regulatory changes in the *VRN2* promoter.

In the case of *VRN1*, further steps should be taken to understand the underlying mechanism for its regulation by cold. Were discrete cis-regulatory changes needed in order to accomplish both inhibition of *VRN1* during warm conditions and induction during prolonged cold? Did protein-coding changes occur in upstream regulators of the *VRN1* locus? Is the epigenetic cold regulation of *VRN1* conserved across Pooideae? Understanding the conservation of these mechanisms will help elucidate the complex evolution that has facilitated the emergence of seasonally driven flowering.

MATERIALS AND METHODS

Growth Conditions and Experimental Design

Controlled growth chamber experiments were performed to determine absence or presence of vernalization responsiveness in 79 Pooideae species. Species were selected to represent all major tribes and geographic localities of the subfamily, as outlined in Supplemental Table S3. For each experimental replicate conducted at the University of Vermont (UVM), 40 to 100 seeds of each accession were germinated on 1% agar plates in the dark, planted in soil, and randomly assigned to either a vernalization or control treatment, both of which were conducted under long days (16 h light:8 h dark). Seedlings in the vernalization treatment were initially exposed to four weeks of 20°C, followed by 6 weeks of 4°C, and then 2 weeks at 20°C. Seedlings assigned to the control treatment were maintained at 20°C for 12 weeks. Plants in both treatments were then moved to a 20 to 22°C greenhouse until flowering, death, or termination of the experiment. Experiments were replicated after switching chambers at the University of Vermont or by running similar experiments twice at the Norwegian University of Life Sciences (NMBU). In the latter case, control temperatures were set to 18°C instead of 20°C, and the period of vernalization was increased to 8 weeks under 8-h short days. Since the basal Pooideae *Brachyelytrum aristosum* and *Nardus stricta* failed to flower within 2 years of seed germination, several fruiting plants were collected from a single population in Vermont (*B. aristosum*) and Norway (*N. stricta*) and grown in a long-day greenhouse at 18 to 22°C for 6 months. Plants were then randomly assigned to a treatment, and flowering time experiments were conducted as previously described.

Follow-up growth chamber experiments with *Melica ciliata* and *Nassella pubiflora* were performed as previously described with two replicates, except that after seedlings were initially exposed to 4 weeks at 20°C they spent only 3 weeks with or without vernalization. Rather than subtracting vernalization time completely from days to flowering (e.g. Woods et al., 2016), heading was calculated in temperature/light-adjusted days (Kirby et al., 1989; Baloch et al., 2003), i.e. total days to flowering minus 33.6 (UVM) or 55.2 (NMBU) days in the vernalization treatment. This correction was calculated by assuming that the control plants (18–20°C) accumulated 4.5 to 5 times more heat units than the vernalized individuals (4°C) across the 42 to 56 d of vernalization, based on a baseline of growth succession at 0°C (Baloch et al., 2003), and that short-day vernalized plants (NMBU only) received half as much light units. However, since some plants experience nonlinear growth rates in response to temperature, we also counted number of leaves and tillers at flowering and made comparisons between treatments. Days to flowering, leaf number at flowering, and tiller number at flowering were calculated when the first inflorescence overtopped the flag leaf.

RNA Extraction and cDNA Synthesis

In order to test predictions for *VRN1* and *VRN3* expression in response to vernalization across the Pooideae phylogeny, obligately and facultatively responsive pairs were chosen from the two largest noncore Pooideae tribes (*Melica nutans* and *M. ciliata* in Meliceae, and *Nassella pulchra* and *N. pubiflora* in Stipeae, respectively). Further species were also chosen to represent the Pooideae lineage sister to remaining Pooideae (*N. stricta* in Nardeae and *B. aristosum* in Brachyelytreae). During the course of each growth chamber experiment (see above), RNA was extracted from the youngest expanded leaf for each of four randomly selected individuals from each focal species per treatment and experimental replicate without repeated measures. Time points for tissue collection were after 4 weeks at 20°C (4-week pretreatment), 4 weeks at 20°C followed by 4 weeks cold or warm (8 weeks total), 4 weeks at 20°C followed by 6 weeks cold or warm (10 weeks total), and 2 weeks at 20°C after the previous 6 weeks cold or warm (12 weeks total, posttreatment). Time points for tissue collection in the *M. ciliata* and *N. pubiflora* follow-up experiment were after 2 weeks at 20°C (2 weeks pretreatment), 4 weeks at 20°C (4 weeks pretreatment), followed by 1 (5 weeks total), 2 (6 weeks total), and 3 weeks (7 weeks total) of 4°C vernalization or 20°C control conditions. Leaf tissue was frozen in liquid nitrogen and RNA was extracted using TRI Reagent (Ambion) followed by DNase treatment with TURBO DNA-free DNase (Ambion) according to the manufacturer's instructions. cDNA was synthesized using 0.5 μg of RNA in an iScript cDNA synthesis reaction (Bio-Rad).

Gene Isolation, Cloning, and Phylogenetic Analyses

To determine their evolutionary history, *VRN1*- and *VRN3*-like genes were amplified from RNA-derived cDNA synthesized from vernalized *Parapholis incurva*, *Brachypodium pinnatum*, *Brachypodium phoenicoides*, *Stipa barbata*, *Stipa robusta*, *N. pulchra*, *N. pubiflora*, *Nassella viridula*, *Piptochaetium napostense*, *Piptatherum miliaceum*, *Oryzopsis canadensis*, *Hesperostipa neomexicana*, *M. nutans*, *M. ciliata*, *Diarrhena americana*, *N. stricta*, *Lygeum spartum*, *Brachyelytrum erectum*, and *B. aristosum*. Each amplicon was cloned and sequenced using degenerate PCR primers designed on previously available grass sequences (Supplemental Table S4). Cloning was done using the pGEM-T kit (Promega) and 8 to 10 clones were sequenced per amplicon using the T7 forward primer by Beckman Coulter Genomics. Newly generated nucleotide sequences were initially aligned with *VRN1*- and *VRN3*-like genes downloaded from GenBank using MAFFT (Katoh and Standley, 2013), followed by manual adjustment in Mesquite (Maddison and Maddison, 2011). Bayesian analyses were done using MrBayes 3.2.2 in XSEDE (Miller et al., 2010) on the Cipres Science Gateway server using 10 million generations, with 25% of saved trees discarded as burn-in. The *VRN1* and *VRN3* analyses were run under the GTR + Γ and GTR + I + Γ models of evolution, respectively, based on results of MrModeltest 2.3 (Nylander, 2004). ML analyses were done using RAxML-HPC BlackBox on the Cipres Science Gateway (Miller et al., 2010).

Ancestral State Reconstructions for Vernalization Traits

A Pooideae phylogeny was estimated using a partitioned dataset of the chloroplast markers *matK* and *ndhF*. Gene sequences were obtained from GenBank or were generated with gene-specific primers as previously described (Liang and Hilu, 1996; Davis and Soreng, 2007). Nucleotides were initially aligned using MAFFT (Katoh and Standley, 2013), followed by manual alignment, and subjected to Bayesian analyses in MrBayes on the Cipres Science Gateway (Miller et al., 2010). To obtain support for tree topologies, Bayesian posterior probabilities were generated by running MrBayes for 10 million replicates with two independent runs, four chains, and 25% of trees discarded as burn-in.

A Bayesian (MCMC) approach was used to reconstruct the evolutionary history of discrete vernalization response traits (flowering and *VRN1* expression) across Pooideae using the Multistate function in BayesTraits V2 (Pagel et al., 2004; Pagel and Meade, 2006). As outgroups, the two bamboo species *Chusquea culeou* and *Chusquea latifolia* were coded as nonresponsive to vernalization in flowering based on previous studies (Guerreiro, 2014). To account for phylogenetic uncertainty, 200 rooted trees with branch lengths were selected

from two independent runs of Bayesian post-burn-in trees and used to assess different models of character evolution. Comparison of marginal likelihoods based on stepping stone estimation identified a one-rate/symmetrical, rather than a two-rate/asymmetrical, model as a better fit for reconstruction of both traits. MCMC analyses were carried out using 10 million generations, sampling every 1,000th tree, with a burn-in of 25%. Means of the posterior probability distributions were calculated for ancestral state estimates at specific nodes based on the Bayesian majority-rule consensus tree.

Georeference data for each species were downloaded from the Global Biodiversity Information Facility (www.gbif.org; GBIF Secretariat), purged of duplicate samples, and used to extract isothermality (bio3; ratio of diurnal to yearly temperature variation) and minimum temperature of the coldest month (bio6) downloaded from Worldclim (http://www.worldclim.org/; Hijmans et al., 2005) with the raster package in R version 3.1.2. Bioclim variables were averaged across all unique locality points for each species.

Tests for Positive Selection

To estimate the ratio (ω) between nonsynonymous (d_N) and synonymous (d_S) substitution rates of protein-coding *VRN1* sequences, we used the *codeml* branch-site model (Zhang et al., 2005) in PAML (Yang, 2007). *Codeml* calculates the likelihood of the data (*VRN1* alignment and gene tree) under two competing models. The null model allows codons to evolve under either purifying ($0 < \omega < 1$) or neutral ($\omega = 1$) selection along all branches. The alternative model differs by allowing codons on specified branches to evolve under positive selection ($\omega > 1$). To ensure convergence of the likelihoods, tests for positive selection were run four times independently using different ω-seeds (0.5, 1, 1.5, and 2). A likelihood ratio test implemented in PAML was used to test if ω values were significantly higher under the alternative model.

qRT-PCR

Gene-specific qRT-PCR primers were designed in Primer3 (Rozen and Skaletsky, 2000) based on results of phylogenetic analyses (Supplemental Table S4). For each primer pair, amplification efficiencies were determined using a dilution series (Scoville et al., 2011), and amplicon identity was confirmed by sequencing. Following correction for primer efficiency, target gene critical threshold values were normalized against the geomean of two housekeeping genes, *UBIQUITIN5* (*UBQ5*) and *ELONGATION FACTOR1a* (*EF1α*; Supplemental Table S4), as previously described (Scoville et al., 2011). To minimize plate effects, full 96-well plates were arrayed per primer set containing all samples per species using the Fast SYBR Green Master Mix (Applied Biosystems) on a StepOne real-time PCR machine (Life Technologies). Three technical replicates were averaged per biological replicate, and two to three experimental replicates each with three biological replicates were assayed per time point/treatment.

Statistical Analyses

To determine whether to classify a species as vernalization responsive or unresponsive, a one-tailed *t* test was performed using R version 3.1.2 comparing corrected days to flowering, leaf number at flowering, and tiller number at flowering for plants subjected to warm versus cold treatments. We classified a species as vernalization responsive if it flowered in significantly less time, with significantly less leaves or tillers ($P < 0.05$) with versus without vernalization.

To test for the fixed effects of temperature, time point, and their interaction on gene expression, linear mixed effects models were employed in R version 3.1.2 (R Development Core Team, 2008) using the multcomp (Hothorn et al., 2008) and nlme (Pinheiro et al., 2016) packages, treating replicate and time as random effects where applicable. To reduce heteroscedasticity, data that had no bearing on a priori predictions (posttreatment for *VRN1*; 4 weeks and 6 weeks for *VRN3*) were excluded from analyses; remaining data were subjected to log transformation as required to increase normality. When interaction terms were significant for *VRN1*, pairwise contrasts were conducted on the difference in expression in vernalization versus control treatments between 4 week "pre" and 8 or 10 week time points. When no interaction between temperature and time point was found, models were rerun without an interaction term, and pairwise comparisons were conducted only across time points. The same statistical test for the interaction of time point and treatment was conducted on data from the *M. ciliata* and *N. pubiflora* follow-up experiment. When interaction terms were significant for *VRN3*, contrasts were conducted on the difference in expression between vernalization and control treatments from the 2- and 4-week pretreatment time points to the 1-, 2-, and 3-week vernalization and control time points.

Scanning Electron Microscopy

Approximately four shoot apical meristems were dissected at each RNA extraction time point, fixed in formalin acetic acid (50% ethanol, 5% glacial acetic acid, and 10% of 37% formaldehyde) solution for 8 to 12 h, and progressively transitioned from 50 to 100% ethanol over a 3-h time period before critical point drying. Samples were placed on stubs and further dissection was conducted before sputter coating with gold particles. Imaging was done using a JEOL 6060 scanning electron microscope using an accelerating voltage of 25 kV.

Accession Numbers

New *VRN1* and *VRN3* gene sequences were deposited in GenBank under accession numbers KX588675-KX588711, *matK* under KX601224-KX601244, and *ndhF* under KX601245-KX601267. Aligned matrices are available on request.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Box plots of tiller number at flowering under vernalization and control conditions.

Supplemental Figure S2. Box plots of total leaf number at flowering under vernalization and control conditions for a subset of plants grown at the University of VT.

Supplemental Figure S3. *VRN3* expression during three weeks of vernalization in *Melica ciliata* and *Nassella pubiflora*.

Supplemental Table S1. Accessions used for vernalization experiments.

Supplemental Table S2. Summary statistics for linear mixed effects models calculating the influence of time point and temperature on *VRN1* and *VRN3* expression.

Supplemental Table S3. Summary statistics for linear mixed effects models calculating the influence of early time point and temperature on *VRN3* expression.

Supplemental Table S4. Primers used for *VRN1* and *VRN3* cloning and qRT-PCR.

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Paper IV

Evolution of the *miR5200-FLOWERING LOCUS T* flowering time regulon in the temperate grass subfamily Pooideae

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Abstract

Flowering time is a carefully regulated trait controlled primarily through the action of the central genetic regulator, *FLOWERING LOCUS T* (*FT*). Recently it was demonstrated that a microRNA, miR5200, targets the end of the second exon of *FT* under short-day photoperiods in the grass subfamily Pooideae, thus preventing *FT* transcripts from reaching threshold levels under non-inductive conditions. Pooideae are an interesting group in that they rapidly diversified from the tropics into the northern temperate region during a major global cooling event spanning the Eocene-Oligocene transition. We hypothesize that miR5200 photoperiod-sensitive regulation of Pooideae flowering time networks assisted their transition into northern seasonal environments. Here, we test predictions derived from this hypothesis that miR5200, originally found in bread wheat and later identified in *Brachypodium distachyon*, 1) was present in the genome of the Pooideae common ancestor, 2) is transcriptionally regulated by photoperiod, and 3) is negatively correlated with *FT* transcript abundance, indicative of miR5200 regulating FT. Our results demonstrate that miR5200 did evolve early in Pooideae, but only acquired photoperiod-regulated transcription within the *Brachypodium* lineage. Based on expression profiles and previous data, we posit that the progenitor of miR5200 was co-regulated with *FT* by an unknown mechanism.

Abbreviations: FT, FLOWERING LOCUS T; LD, long day, SD, short day

Introduction

Flowering time is critical to plant survival and reproductive success, and is tightly regulated by the integration of several exogenous and endogenous signals, including photoperiod, temperature, and developmental age (Lang 1952; Lacey 1986; Bernier and Périlleux 2005; Andrés and Couplan 2012; Song et al. 2015). One of the most important promoters of the floral transition is *FLOWERING LOCUS T* (*FT*), whose small protein product acts as a 'florigen', moving from leaves through the phloem to the shoot apex (Corbesier et al. 2007; Turck et al. 2008). Although the role of FT as the central florigen is highly conserved across many flowering plant groups (Turck et al. 2008), recent work suggests that many points of regulation have evolved in response to abiotic signals such as photoperiod (Greenup et al. 2009; Wu et al. 2013) and vernalization (Yan et al. 2006; Fjellheim et al. 2014). Studies examining the evolutionary history of *FT* regulation in response to the environment are critical to inform our understanding of how plant genetic architecture may be modified in response to current and future climatic changes.

Recently it was demonstrated that transcription of *FT*-like genes *FTL1* and *FTL2* (*Triticum aestivum* [tribe Triticeae, wheat] *FT* ortholog) in the long-day (LD) temperate grass *Brachypodium distachyon* (tribe Brachypodieae) (Schwartz et al. 2010) are negatively regulated by the microRNA miR5200 (previously known as miR2032) in a photoperiod-sensitive manner (Wei et al. 2009; Wu et al. 2013). On chromosome one in *B. distachyon*, two potential genes encode miR5200 transcripts, and are thus named *MIR5200a* and *MIR5200b* (Wu et al. 2013). Another miRNA, with a one-nucleotide difference to miR5200a, has also been identified, and named miR5200c (Zhang et al. 2009; Wu et al. 2013). Suggesting evolutionary conservation, *MIR5200a* and *MIR5200b* are syntenic in *Triticum monococcum* (tribe Triticeae, einkorn wheat) and *B. distachyon* (Abrouk et al. 2012; Lucas and Budak 2012; Wu et al. 2013).

Biosynthesis of mature plant microRNAs from their initial transcripts occurs through various processing steps. In general, trimming of the 3'- and 5'-ends of the primary miRNA transcript (pri-miRNA) produces the precursor miRNA (pre-miRNA) comprising a complementary stem region and a loop. Excision of the double-stranded

miRNA duplex, consisting of miRNA/miRNA* (where miRNA* is complementary to the mature miRNA), is mediated by Dicer-like1 (DCL1). The mature miRNA molecule binds to ARGONAUT (AGO), which together with other proteins forms the miRNA-induced silencing complex (miRISC) that is able to cleave mRNA transcripts complementary to the miRNA (Voinnet 2009). It is becoming clear that the abundance of mature miRNAs can be regulated at several points in the maturation process. These points of regulation may be condition specific (Meng et al. 2011; Kai and Pasquinelli 2010). Processes that affect the final abundance of mature miRNA range from pri- and pre-miRNA base editing to miRISC loading, and AGO protein complex binding (Winter et al. 2009; Meng et al. 2011). One recent hypothesis proposes that several different AGO proteins control the stability of mature miRNAs and thus may determine miRNA abundance in the cell (Winter et al. 2009).

FTL1 and *FTL2* from *B. distachyon* possess sequences complementary to the mature form of miR5200a and miR5200b, suggesting that both miRNAs target *FT*-like transcripts for degradation (Wu et al. 2013). Supporting this hypothesis, overexpression and knock-down of *B. distachyon* Bd21-3 miR5200 previously resulted in delayed and accelerated flowering times, respectively, with concurrent decreases or increases in *FT* expression (Wu et al. 2013). Similar to *FTL1* and *FTL2*, *B. distachyon* miR5200a and miR5200b displayed diurnal expression patterns, peaking four hours before dawn (Wu et al. 2013). MiR5200a and miR5200b transcript abundance was also relatively high under four and eight hour short day (SD) conditions, but nearly undetectable under 16- and 20-hour LD photoperiods (Wu et al. 2013). When plants grown in LDs were moved to SDs, miR5200a/b expression increased after seven days, while *FTL* expression steadily decreased (Wu et al. 2013). Interestingly, repressive histone marks (H3K27me3) were enriched surrounding hairpin structure regions of *MIR5200* in LDs, while promoting histone marks (H3K4me3) were present in SDs, matching expression changes seen in these conditions (Wu et al. 2013). Together, these data support a role for the regulation of miR5200a and miR5200b transcription in the SD repression of FT-regulated flowering in *B. distachyon* Bd21-3.

Triticum aestivum and *B. distachyon* are members of the Pooideae subfamily of grasses that originated and quickly transitioned from the tropics into the northern

temperate region about 50-35 million years ago (Gaut 2002; Christin et al. 2008; Vincentini et al. 2008; Bouchenak-Khelladi et al. 2010), during a gradual cooling period at the middle and end of the Eocene (Zachos et al. 2001; Stickley et al. 2009; Mannion et al. 2014). The subfamily, which comprises over 4,200 species (Soreng et al. 2015), is thus hypothesized to have evolved several seasonal adaptations, such as vernalization that prevents precocious flowering during the seasonal low winter temperatures, while triggering flowering under the inductive LD photoperiods of summer (Preston and Sandve 2013; Fjellheim et al. 2014; Ream et al. 2013; McKeown et al 2016; Woods et al. 2016). LD flowering is crucial for temperate plants because it enables them to efficiently exploit the short growing season. In the core Pooideae, which is the species-rich sister clade to the Brachypodieae and includes all temperate cereals and forage grasses (e.g. *T. aestivum*, *Avena sativa* [tribe Poeae, oats], and *Lolium perenne* [tribe Poeae, perennial ryegrass]) (Saarela et al. 2015), photoperiod and vernalization networks are entwined, and are often regulated by overlapping mechanisms (Ream et al. 2014). Despite this, miR5200 expression does not appear to be influenced by vernalization in both vernalization responsive and non-responsive *B. distachyon* accessions (Wu et al. 2013), suggesting that an independent photoperiod-sensitive module has evolved to regulate *FT*.

Homologs of *B. distachyon* miR5200 have been found in several core Pooideae species, but are lacking from the fully sequenced genomes of rice (*Oryza sativa*, Ehrhartoideae), maize (*Zea mays*, Panicoideae), sorghum (*Sorghum bicolor*, Panicoideae), and *Arabidopsis thaliana* (Brassicaceae) (Wu et al. 2013). Based on this observation, and given the potential importance of the miR5200-FT regulon in repressing temperate winter flowering, we hypothesize that miR5200 appeared *de novo* at the base of Pooideae, and was quickly integrated into the photoperiod pathway to negatively regulate *FT* transcription during the non-inductive SD photoperiods of winter. Specifically, we hypothesize that 1) miR5200 evolved at the base of Pooideae, 2) SD-induced miR5200 expression is conserved across Pooideae, and, 3) the negative correlation between *FT* and miR5200 transcript abundance is conserved across Pooideae, suggesting a prominent role for transcriptional regulation of miR5200 in mediating *FT* expression patterns. To test these hypotheses, we isolated miR5200

transcripts from phylogenetically representative Pooideae species, and compared the expression profiles of miR5200- and *FT*-like genes under different photoperiods. Our results support an origin of miR5200 prior to the diversification of Pooideae, but suggest that control of miR5200 transcription by photoperiod evolved independently in the *B. distachyon* Bd21-3 accession.

Materials and Methods

Plant growth and experimental design

Five phylogenetically diverse species were chosen for the study: *Nardus stricta* (tribe Nardeae, collected in Romania, [46.69098, 22.58302], July 2012), *Stipa lagascae* (tribe Stipeae, PI 250751, Western Regional Plant Introduction Station), *Melica nutans*, (tribe Meliceae, collected in Germany, [50.70708, 11.23838], June 2012), *B. distachyon* (line Bd21, tribe Brachypodieae, W6 36678, Western Regional Plant Introduction Station), and *Hordeum vulgare* (tribe Triticeae, barley) 'Morex' (USDA GRIN NPGS, 35761). The first three species belong to lineages outside of the *Brachypodieae*-core Pooideae clade, referred to as early-diverging lineages (Fig. 1A). As a control we included the specific *B. distachyon* accession Bd21-3 (W6 39233, Western Regional Plant Introduction Station) used in Wu et al. (2013) (personal communication Long Mao).

Seeds of *N. stricta* were germinated on 1% agar plates for two weeks, before being planted in soil and randomly assigned to one of four 17-20°C growth rooms at the Centre for Plant Research in Controlled Climate, Norwegian University of Life Sciences NMBU, in Ås, Norway. *S. lagascae* and Bd21 seeds were placed directly in soil at the start of the experiment and divided into treatments as described for *N. stricta*. Two replicates of the experiment occurred simultaneously with two treatment chambers and two control chambers. Initially, all chambers were set to 16-hour light: 8-hour dark LD photoperiodic conditions for three weeks. Subsequently, the two treatment chambers were set to an 8-hour light: 16-hour dark SD photoperiod regime for two weeks, followed by LDs until flowering. The two control chambers were set to LDs for the entire experiment, and all chamber temperatures cycled from 20°C during the day to 17°C at night. The longest fully expanded leaf was sampled for RNA from four

independent individuals for each species at three weeks post planting (set to day 0) and after one week in treatment conditions (day 7), times identical to those reported in Wu et al. (2013). To assess the influence of time on gene expression, leaves were sampled at one additional time point to Wu et al. (2013): two weeks into each treatment (day 14). Sampling occurred after the initial three weeks in LDs (day 0) and after one and two weeks (day 7 and 14, respectively) in SDs with the respective time point taken in the control chambers. After the treatments, plants were transferred to LD and days to heading were recorded.

Experiments with plants from the control accession *B. distachyon* Bd21-3 and *H. vulgare* were conducted at the University of Vermont (UVM). For Bd21-3 we used the same experimental design and leaf harvest time as above, except that experiments were replicated in time with chamber swapping, rather than simultaneously. For *H. vulgare*, seeds were planted in pots and assigned to either a SD or LD chamber, where they remained for the duration of the experiment. *H. vulgare* leaf material was harvested for RNA from five individuals per treatment prior to the reproductive transition (marked by the double-ridge stage), when plants were 8-11 cm tall, six days post-germination for both treatments. All chambers were held at a constant temperature of 20°C. To assure that the sampled *H. vulgare* and *B. distachyon* Bd21-3 plants had not transitioned to the reproductive stage, we examined the shoot apical meristem (SAM) at each timepoint.

RNA extraction and cDNA synthesis

All leaf material for RNA extraction was sampled two hours post dawn as performed in Wu et al. (2013). Leaf samples collected at NMBU were extracted using RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. RNA from leaves collected at UVM was extracted using TRI Reagent (Ambion) followed by DNase treatment with TURBO DNA-free DNase (Ambion) according to the manufacturer's instructions. cDNA was synthesized using 1.0 µg of RNA in an iScript cDNA synthesis reaction (BioRad).

Transcriptome query and gene expression analyses

To test whether miR5200 evolved early in Pooideae and is thus present in early-diverging lineages, we conducted nucleotide BLAST searches in transcriptomic datasets

assembled with Trinity v.2.0.6 (Grabherr et al. 2011) and collected under both 12 h neutral days and 8 h SD photoperiods for *N. stricta*, *S. lagascae*, *M. nutans*, and *H. vulgare* (Grønvold and Schubert et al. unpublished). Additionally, we queried the newly available moso bamboo (*Phyllostachys edulis*) genome (Peng et al. 2013) using the same BLAST algorithm as for the other focal species. Primers for *H. vulgare*, *N. stricta* and *S. lagascae* were generated using Primer3 (Rozen and Skaletsky 2000), based on an identical match to the mature miR5200 sequences (primers listed in Table S2). Primers were not designed for *M. nutans*, as no sequence with similar identity to miR5200 was found. *FT* primers for *H. vulgare* and *N. stricta* were based on sequences published previously (Yan et al. 2006; McKeown et al. 2016). *FT* primers for *S. lagascae* were designed based on orthologous sequences found in the transcriptome of *S. lagascae*. To discount potential target mRNAs lost to cleavage, at least one set of *FT*-primer spanned the putative cut-site of miR5200. Primers used for *B. distachyon* expression analyses were identical to those used in Wu et al. (2013). Primer efficiencies were calculated and qPCR was performed as previously described using two housekeeping genes, *UBIQUITIN 5* (*UBQ5*) and *ELONGATION FACTOR 1a* (*EF1α)* (Scoville et al. 2011; Woods et al. 2016). Three biological replicates per time point were used in each of two experimental replicates that were run for *B. distachyon* Bd21, *N. stricta*, and *S. lagascae*. Five biological replicates per time point were used in each of two experimental replicates that were run for *H. vulgare*. Six to eight biological replicates per time point were used in each of two experimental replicates that were run for control *B. distachyon* Bd21-3 plants.

Statistical analyses

Single-tailed bonferroni-corrected t-tests between LD and SD at 7- and 14-day timepoints, and between ‘pre’ and 7-day timepoints in SDs were calculated using R version 3.1.2 (R development core team 2008). *Hordeum vulgare* data were analysed using a one-tailed t-test in R to compare expression in SDs and LDs. Correlation between *FT* and *miR5200* expression was analysed using the Pearson’s product-moment correlation in R (R development core team 2008).

Results

Identification of miR5200 in species of early-diverging Pooideae lineages

Transcriptome-wide nucleotide BLAST searches on *de novo* pooled assemblies from leaf tissue resulted in matches of mature and pre-miR5200 transcript sequences in *N. stricta*, *S. lagascae*, and *H. vulgare* (Fig. 1). No putative matches for miR5200 were identified in the moso bamboo (*Phyllostachys edulis*) genome or the *M. nutans* transcriptome. Close complementation was observed between the putative miR5200 sequences and the end of the second exon of *FT*, with five mismatches in *N. stricta*, four mismatches in *H. vulgare*, and two mismatches in *S. lagascae* (Fig. 1B). There were four mismatches between *FTL1* and miR5200a/b and two mismatches between *FTL2* and miR5200 a/b in *B. distachyon* (Bd21 and Bd21-3 accessions were identical in sequence) (Fig. 1B).

Flowering and expression of miR5200 and FT in response to photoperiod

Nardus stricta and *S. lagascae* plants were not induced to flower under our treatment conditions prior to termination of the experiment around 120 days. In contrast, *B. distachyon* Bd21 plants flowered after an average of 27.2 days (sd=2.6) after LD-treatment and 40.9 days (sd=4.3) after SD-treatment, and Bd21-3 flowered after an average of 66.4 days (sd=10.9) after LD-treatment and failed to flower prior to termination (around 100 days) of the experiment after SD-treatment. In the case of *H. vulgare* 'Morex', SAMs had transitioned to flowering in both LD and SD treated plants by day 15. However, heading was earlier under LD versus SD conditions.

Bonferroni-corrected statistical comparisons after seven days in SD or LD treatment indicated no significant treatment difference in *N. stricta* miR5200, *S. lagascae* miR5200, or *B. distachyon* Bd21 miR5200b transcript levels (Fig. 2; Table 1). Likewise, there was no significant difference in miR5200 expression between LD- and SD-treated *H. vulgare* 'Morex' (one tailed t-test, t = 0.20, df = 15.2, P = 0.424) (Fig. 3A). In contrast, miR5200a/b transcript levels in our control accession *B. distachyon* Bd21-3, and Bd21 miR5200a were significantly upregulated in SDs compared to LDs after seven days in treatment (Table 1). Comparison of expression between the “pre”

and seven-day time points in SDs showed no significant differences for any miR5200 tested, with the exception of *B. distachyon* Bd21-3 miR5200a (Fig. 2C; Table 1). In the case of *FT*, expression was significantly higher in six-day *H. vulgare* (Fig. 3A) and 14-day *N. stricta* LD versus SD treated plants. However, *FT* expression did not significantly differ between LD and SD treatments (Fig. S1) of Bd21-3, Bd21, or *S. lagascae*. The lack of *FT* upregulation corresponds with the fact that Bd21-3, Bd21, and *S. lagascae* SAMs had not transitioned to the reproductive stage by day seven (Bd21-3) or 14 of the experiment (data not shown). This is critical because miR5200 is predicted to negatively regulate the transition to flowering via FT during the vegetative stage.

Correlation between miR5200 and FT expression

To determine whether *FT* and miR5200 transcripts have an antagonistic relationship, regardless of photoperiodic regulation, we examined the correlation between *FT* and miR5200 transcript levels under SD and LD photoperiods (Fig. 3B and 4). In contrast to our prediction of a negative correlation, we found that miR5200 expression was positively correlated with *FT* expression in *N. stricta* and *S. lagascae* under both SDs and LDs (Table 2). In each case, results were similar whether *FT* primer pairs spanned the putative miR5200 cut site or a region within a different exon (Table 2, Fig. 4). Likewise, in Bd21 there was a significant positive correlation between miR5200a/b and *FTL2*, the ortholog of *FT* in *T. aestivum* and *H. vulgare*, whereas we did not observe any correlation between Bd21 miR5200a/b and *FTL1*, the latter of which is located on a separate chromosome to the miRNAs (Table 2). In each case, results were similar whether *FT* primer pairs spanned the putative miR5200 cut site or a region within a different exon (Table 2, Fig. 4). We did not observe this same positive correlation pattern in *H. vulgare*, nor in our control accession *B. distachyon* Bd21-3 (Table 2).

Discussion

miR5200 evolved early in Pooideae but SD-induced expression is not conserved

The upregulation of miR5200 under SD photoperiods to negatively regulate *FT* transcription in *B. distachyon* (Wu et al. 2013) suggests a mechanism by which Pooideae might have suppressed SD flowering during its initial diversification in the

temperate zone. Consistent with this hypothesis, our data demonstrate that miR5200 evolved somewhere between the diversification of subfamily Bambusoideae and the early-evolving Pooideae *N. stricta*, and that it is largely conserved throughout Pooideae. In contrast, although combined pri- and mature miR5200s are upregulated under SDs in Bd21-3, attendant with decreased repressive histone marks under LDs (Wu et al. 2013), we found no evidence of similar SD-induced miR5200 transcription in Bd21, *H. vulgare*, *S. lagascae*, or *N. stricta*. Collectively, these data suggest that SD-induced miR5200 transcription evolved relatively recently in the *Brachypodium* lineage and likely did not contribute to diversification of Pooideae into northern temperate regions (Fig. 5). However, we cannot discount the possibility that miR5200 maturation, rather than transcription, is the target of photoperiod regulation across Pooideae, in which case we would expect the mature miR5200 to increase under SD relative to LD conditions.

A role for photoperiod-dependent regulation of miR5200 maturation?

If miR5200 negatively regulates *FT* expression (Jones-Rhoades and Bartel 2004; Kawashima et al. 2009) then we would expect to see a negative correlation between the two genes at the level of transcription, as observed for Bd21-3 pri-miR5200a/b and *FTL1/2* expression in Wu et al. (2013). Following this, the lack of correlation between Bd21-3 pri-miR5200a/b and *FTL1* as well as pri-miR5200a and *FTL2* expression is consistent with miR5200 regulating *FT* transcript abundance, thus confirming the findings of Wu et al. (2013). However, we failed to observe a consistent negative correlation between pri-miR5200 and *FT* transcript abundance under either photoperiod in our focal taxa. In the case of *H. vulgare*, although the abundance of mature miR5200 was previously found to increase under SDs relative to LDs (Wu et al. 2013), we found that transcription of the pri-miR5200 was photoperiod-insensitive. These data suggest that the difference in mature miR5200 abundance in *H. vulgare* stems from daylength mediated post-transcriptional regulation (Voinnet 2009) of the miR5200 transcripts.

An increasing amount of work demonstrates that the abundance of mature miRNA is regulated post-transcriptionally, in addition to regulation at the level of transcription (Obernosterer et al. 2006; Thomson et al. 2006; Wulczyn et al. 2007; Winter et al. 2009). For example, abundance of miR172, a miRNA involved in the regulation of photoperiodic-dependent flowering in *Arabidopsis thaliana*, was shown to be regulated

during the maturation process (Jung et al. 2007). Due to lack of data on developmental stage in our experiment, results from *N. stricta, S. lagascae* and Bd21 neither confirm nor exclude regulation of miR5200 maturation, as maturation may depend on developmental stage in addition to photoperiod. We have in other studies failed to induce flowering in *N. stricta* and *S. lagascae* (Fjellheim et al. unpublished data), hence, the right developmental window may well have been missed. Future studies investigating the abundance of mature miR5200 under SD and LD photoperiods will be required to test whether regulation of miR5200 maturation is confined to *H. vulgare*, or can be traced further back in the Pooideae phylogeny.

Opposite to our predictions, and with the exception of genes in *H. vulgare* and the control accession Bd21-3, we identified a strong positive correlation between expression of miR5200 and *FT* in our focal taxa. These unexpected results might be partly explained by the physical location of interacting genes within the genome. Specifically, since *MIR5200a* and *MIR5200b*, and *FTL2* are co-localized (ca. 31 kb and 3 kb apart from *FTL2*, respectively) on *B. distachyon* chromosome 1, potentially through inverted duplication of the target gene *FT* (see the mechanistic review in Allen et al. 2004; Rajagopalan et al. 2006; Fahlgren et al. 2007, 2010), we hypothesize co-localization as a foundation for enabling co-transcription of these loci (Wu et al. 2013).

Model for miR5200 evolution

To summarize, the combination of our data with that from Wu et al. (2013) support the evolutionary scenario where miR5200 evolved through an inverted partial duplication of *FT* at the base of Pooideae, possibly resulting in common transcriptional regulation by cis or trans elements. Evidence for transcriptional regulation of miR5200 by photoperiod is limited to a single cultivar of *B. distachyon*. However, the fact that mature miR5200 levels in *H. vulgare* are higher under SDs versus LDs, suggests that post-transcriptional regulation of pri-miR5200 by photoperiod evolved within the core Pooideae, or earlier during the transition of Pooideae from tropical to temperate environments. Future work examining pri-miR5200 expression and mature miR5200 abundance in several other Pooideae species across a wider developmental time frame will be paramount to uncover the patterns of miR5200 evolution.

The positive correlation would be evident if we failed to capture the developmental window where regulation of miR5200 maturation is active in *N. stricta*, *S. lagascae* and Bd21. Likewise, the expected negative relationship would be evident in samples with functional, mature miR5200, i.e. in *H. vulgare* (photoperiod-dependent maturation) and Bd21-3 (SD-induced transcription). Examination of the genomic position of miR5200 relative to *FT* in early-diverging Pooideae lineages will lend further insight into the evolution of coordinated expression between these loci.

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Tables

Table 1. Single-tailed bonferroni corrected t-test statistics for a significant increase of miR5200 under SD versus LD conditions after seven or 14 days. *Brachypodium distachyon* accessions Bd21 and Bd21-3 show miR5200a and miR5200b expression, respectively. T-statistic is listed with degrees of freedom in subscript. NA indicates test was not made due to an altered experimental design. Parentheses around the *H. vulgare* statistic indicate that the timepoint was taken after 6 days. * $P < 0.05$, ** $P < 0.005$

Species/Timepoint	Pre – 7day SD	7-day	14-day
Bd21 (a/b)	$-0.416_{15.5}$/$-0.793_{11.4}$	$2.91_{9.36}$**/$1.56_{7.54}$	$0.139_{9.84}$/$-0.078_{9.98}$
Bd21-3 (a/b)	2.23_{7}*/$1.12_{22.5}$	2.23_{7}*/$1.95_{16.2}$*	NA
N. stricta	$1.30_{8.45}$	$-1.13_{6.62}$	$-1.07_{9.09}$
S. lagascae	$-1.20_{9.41}$	$0.375_{8.94}$	$0.977_{7.62}$
H. vulgare	NA	($0.195_{15.22}$)	NA

Table 2. Statistical results testing correlations between miR5200 and *FT* expression. Columns indicate correlation coefficient (*r)*, t-statistic with degrees freedom indicated as subscript (t_{df}), P-value for Pearson's Product Moment Correlation test (*P*), and adjusted R-squared value (*Adj. R*). **FT* primer sets that amplify transcripts spanning microRNA target sites. Significant P-values ($P < 0.05$) are indicated in bold. NA indicates that the test was not performed due to lack of detectable gene expression.

Species/Statistic	*r*	t_{df}	*P*	*Adj.* R_s
Long days				
Bd213miR5200a/FTL1*	NA	NA	NA	NA
Bd213miR5200a/FTL2*	NA	NA	NA	NA
Bd213miR5200b/FTL1*	0.603	2.62_{12}	**0.023**	0.031
Bd213miR5200b/FTL2*	0.191	0.952_{24}	0.351	-0.004
Bd21miR5200a/FTL1*	-0.280	-1.24_{18}	0.232	0.027
Bd21miR5200a/FTL2*	0.834	7.10_{22}	**4.02e-07**	0.682
Bd21miR5200b/FTL1*	-0.282	-1.25_{18}	0.229	0.028
Bd21miR5200b/FTL2*	0.973	19.9_{22}	**1.55e-15**	0.945
NsmiR5200/FT	0.978	21.8_{22}	**2.22e-16**	0.954
NsmiR5200/FT*	0.984	26.2_{22}	**2.20e-16**	0.968
SlmiR5200/FT	0.456	2.40_{22}	**0.025**	0.172
SlmiR5200/FT*	0.908	10.2_{22}	**9.14e-10**	0.816
HvmiR5200 – FT*	0.549	1.86_{8}	0.100	0.215
Short days				
Bd213miR5200a/FTL1*	0.599	1.83_{6}	0.117	0.252
Bd213miR5200a/FTL2*	0.260	0.659_{6}	0.534	-0.088
Bd213miR5200b/FTL1*	-0.114	-0.282_{6}	0.787	-0.151
Bd213miR5200b/FTL2*	0.212	0.687_{10}	0.508	-0.050
Bd21miR5200a/FTL1*	-0.470	-1.30_{6}	0.240	0.091
Bd21miR5200a/FTL2*	0.912	7.02_{10}	**3.65e-05**	0.814
Bd21miR5200b/FTL1*	-0.490	-1.37_{6}	0.218	0.113
Bd21miR5200b/FTL2*	0.996	36.8_{10}	**5.27e-12**	0.992
NsmiR5200/FT	0.957	10.5_{10}	**1.04e-06**	0.908
NsmiR5200/FT^	0.912	7.05_{10}	**3.50e-05**	0.816
SlmiR5200 – FT	0.791	4.10_{10}	**0.002**	0.589
SlmiR5200 – FT^	0.907	6.80_{10}	**4.73e-05**	0.805
HvmiR5200 – FT*	-0.134	-0.382_{8}	0.7125	-0.105

Figures

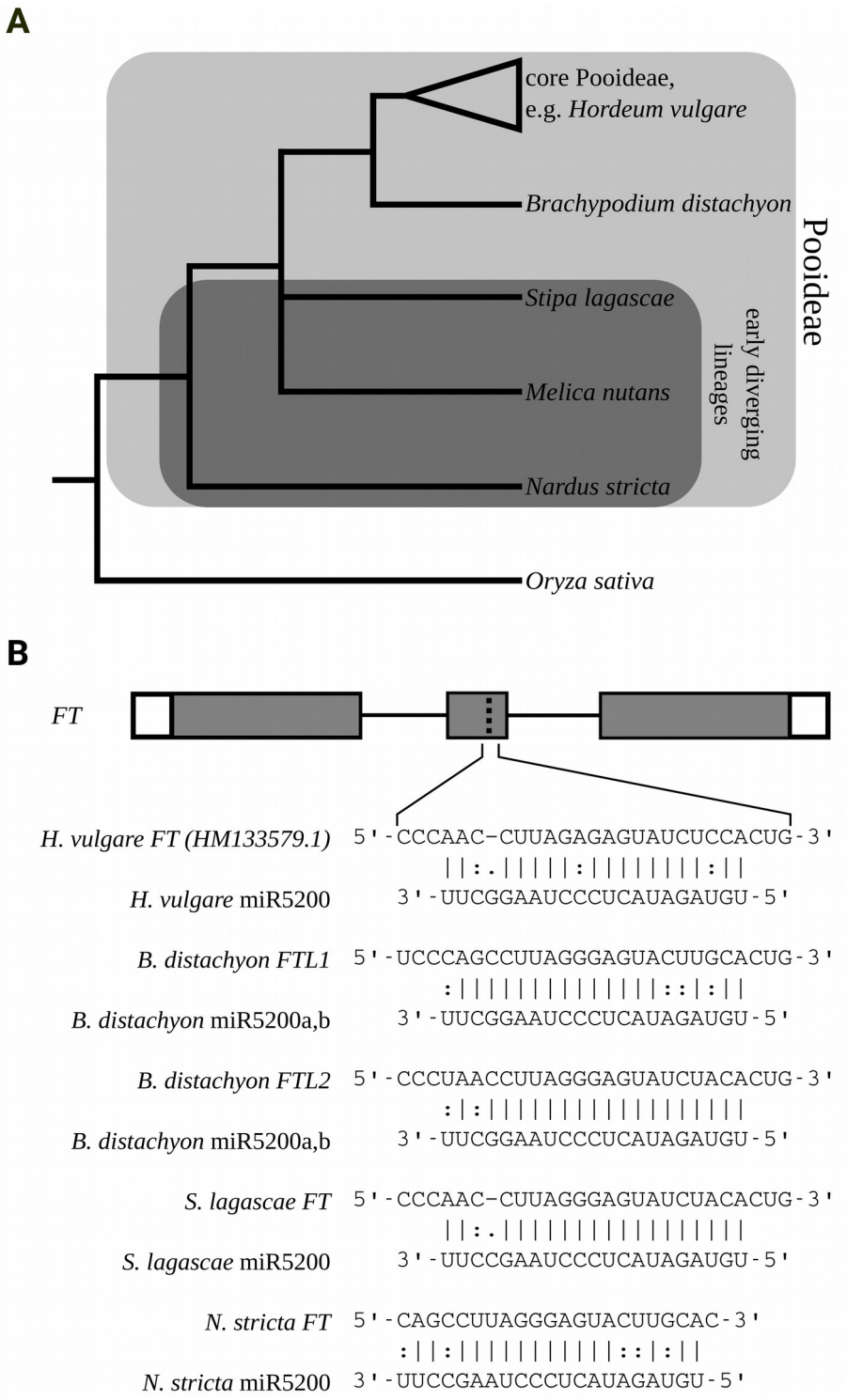

Figure 1. Simplified phylogeny of Pooideae based on Grass Phylogeny Working Group II (A) and alignment of the miR5200 and corresponding *FT* target sequence (B) in *H. vulgare*, *B. distachyon*, *S. lagascae*, and *N. stricta*. Gray boxes represent exon structure, with lines between representing introns. White box represents 5' and 3' UTRs.

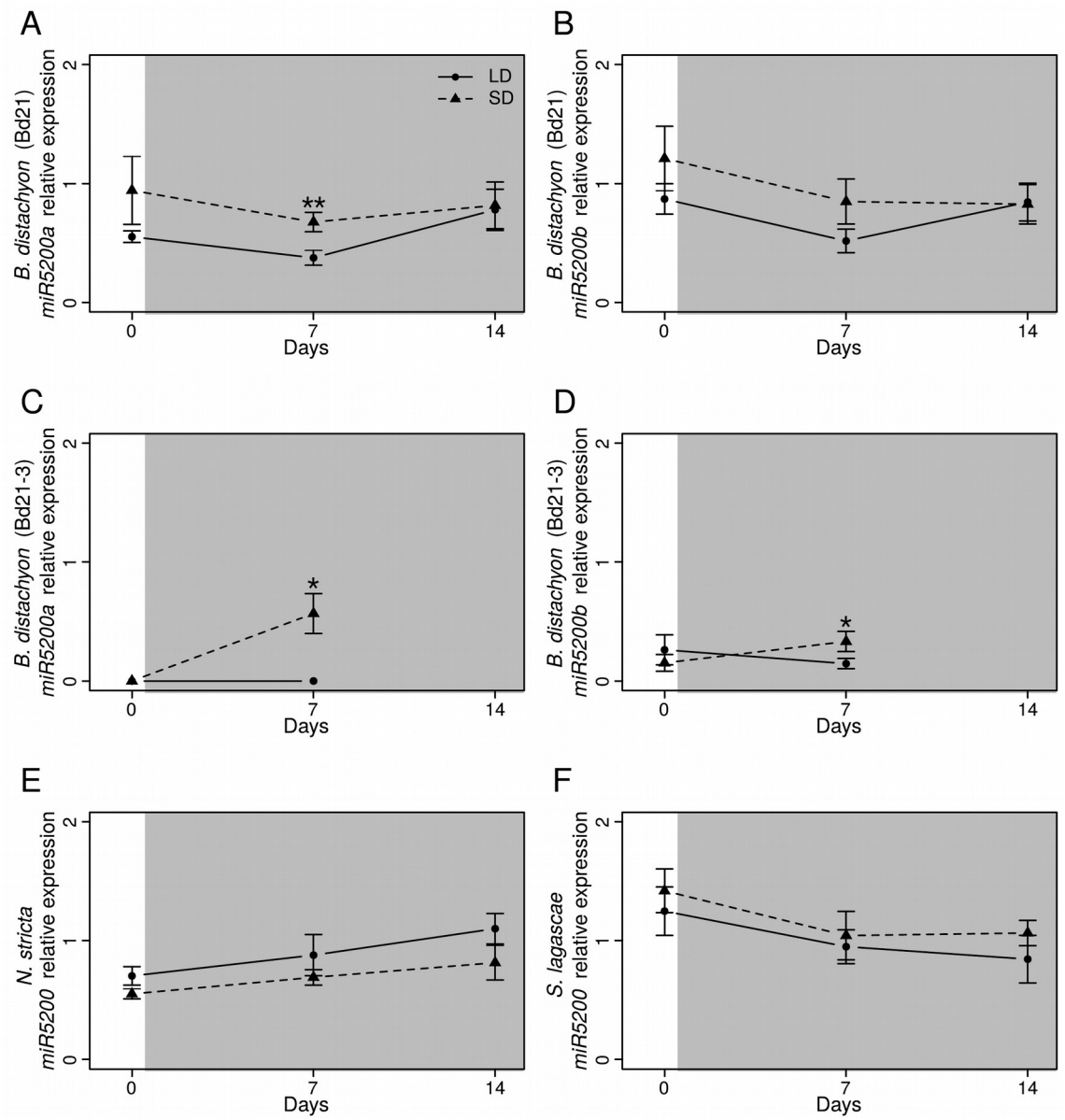

Figure 2. Expression of miR5200 relative to *UBQ5* and *EF1α* for (A) *B. distachyon* Bd21 miR5200a, (B) *B. distachyon* Bd21 miR5200b, (C) *B. distachyon* Bd21-3 miR5200a, (D) *B. distachyon* Bd21-3 miR5200b, (E) *N. stricta* miR5200, and (F) *S. lagascae* miR5200. Gray boxes indicate times during the experiment when plants assigned to the SD treatment experienced the SD photoperiod. Error bars indicate standard error for two experimental replicates with three biological replicates per experiment. $P < 0.05$, *, $P < 0.01$, **.

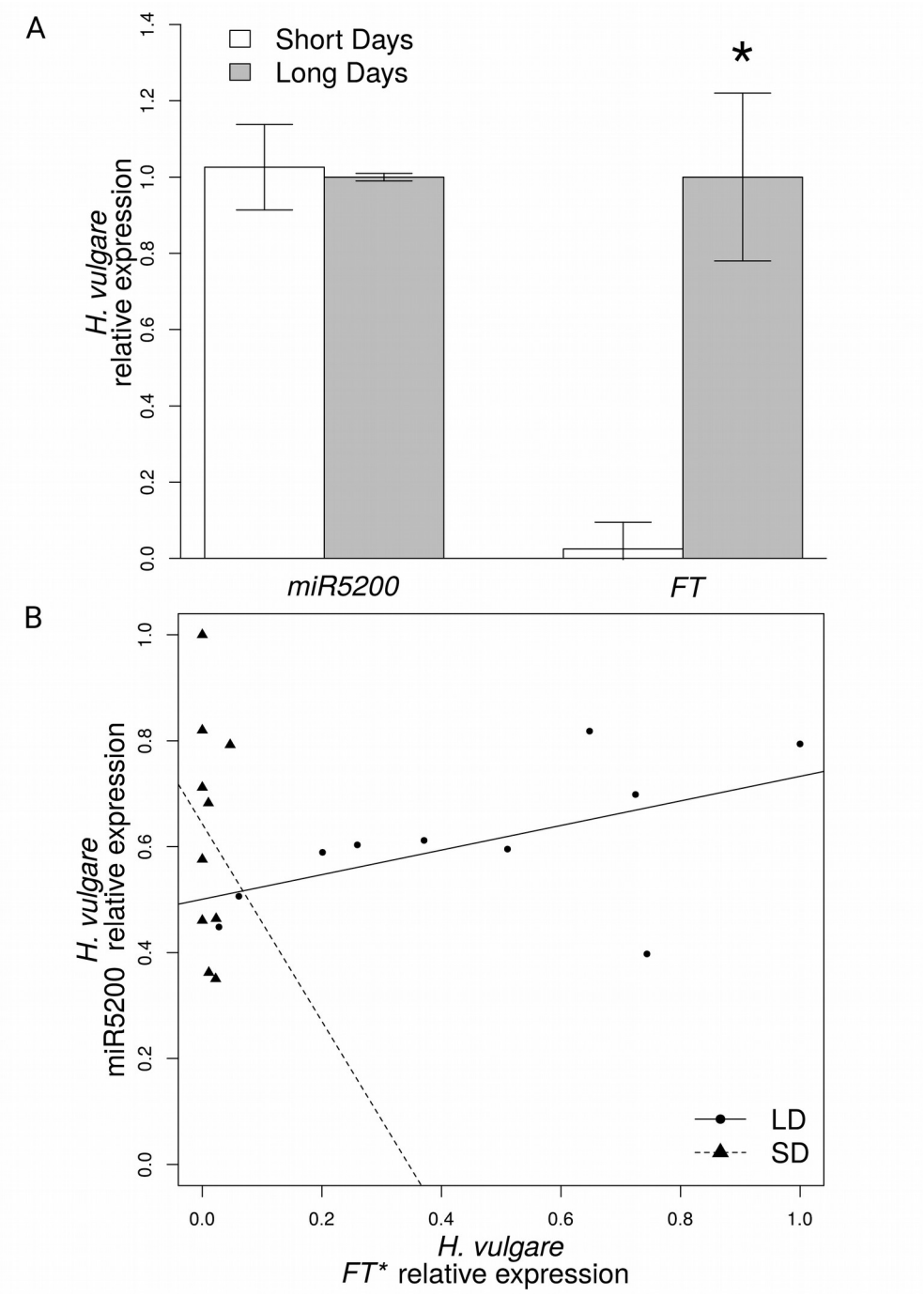

Figure 3. Relative expression of *H. vulgare* 'Morex' miR5200 and *FT* in SDs and LDs. (A) Expression at six days post-germination. Error bars indicate standard error for five individuals. White bars indicate SD; gray bars indicate LD. Significant P-values ($P < 0.05$) are indicated with asterisks. (B) Correlational scatterplot of *H. vulgare* FT and miR5200 under SD and LD photoperiods with projected best-fit line. Triangle indicates individual sampled under SD, circle indicates under LD.

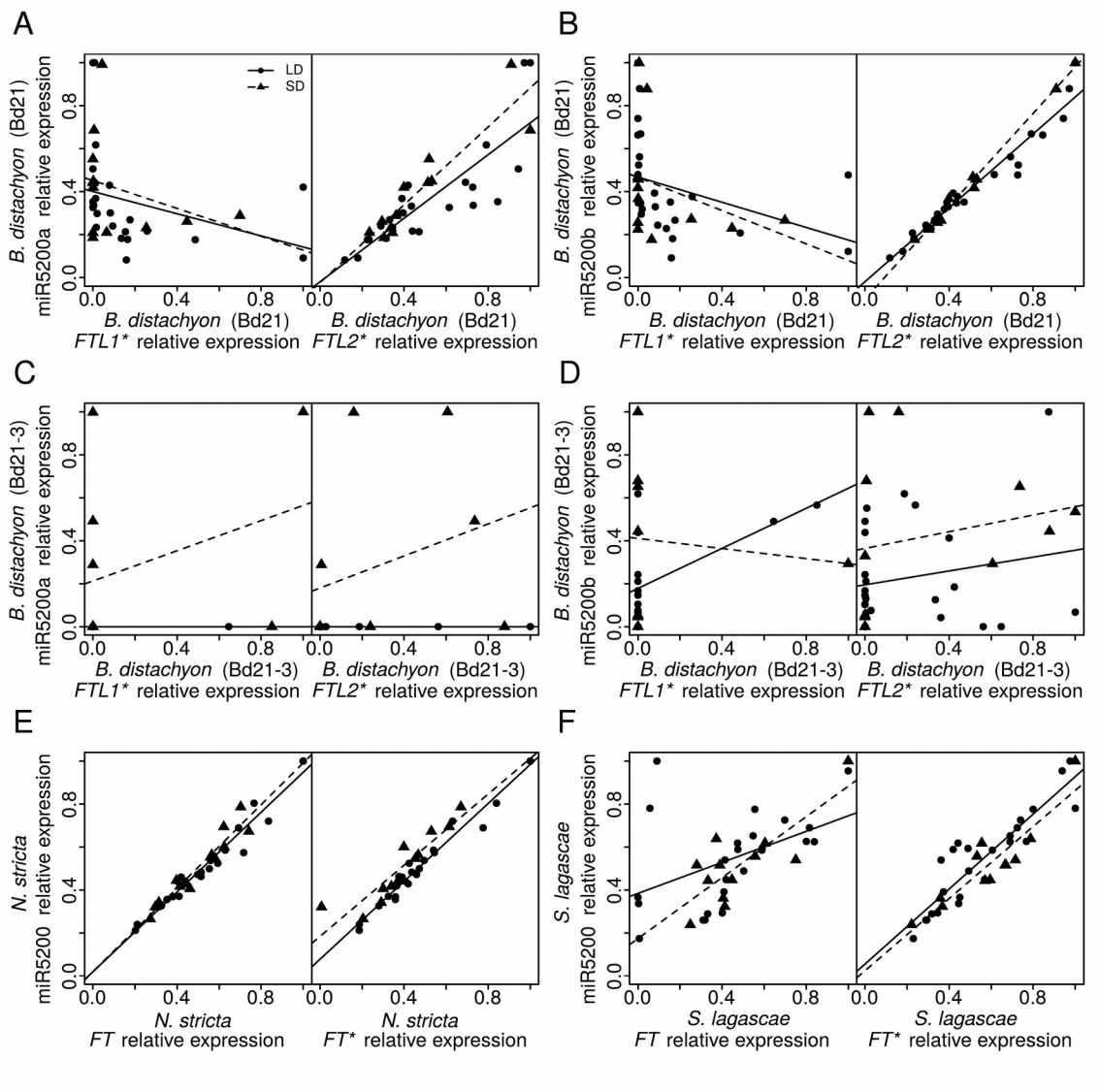

Figure 4. Correlational scatterplots between miR5200 and *FT* transcriptional levels in Pooideae species. (A) *B. distachyon* Bd21 miR5200a is significantly positively correlated with *FTL2*, but not *FTL1* expression. (B) *B. distachyon* Bd21 miR5200b is significantly positively correlated with *FTL2*, but not *FTL1* expression. (C) *B. distachyon* Bd21-3 miR5200a is not correlated with *FTL1* or *FTL2* expression. (D) *B. distachyon* Bd21-3 miR5200b is not correlated with *FTL1* or *FTL2* expression. (E, F) miR5200 expression in *N. stricta* (E) and *S. lagascae* (F) is significantly positively correlated with *FT* expression, whether primers used spans the microRNA cut site (denoted by asterisk), or amplify a region within a separate exon. Triangle indicates individual sampled under SD, circle indicates under LD.

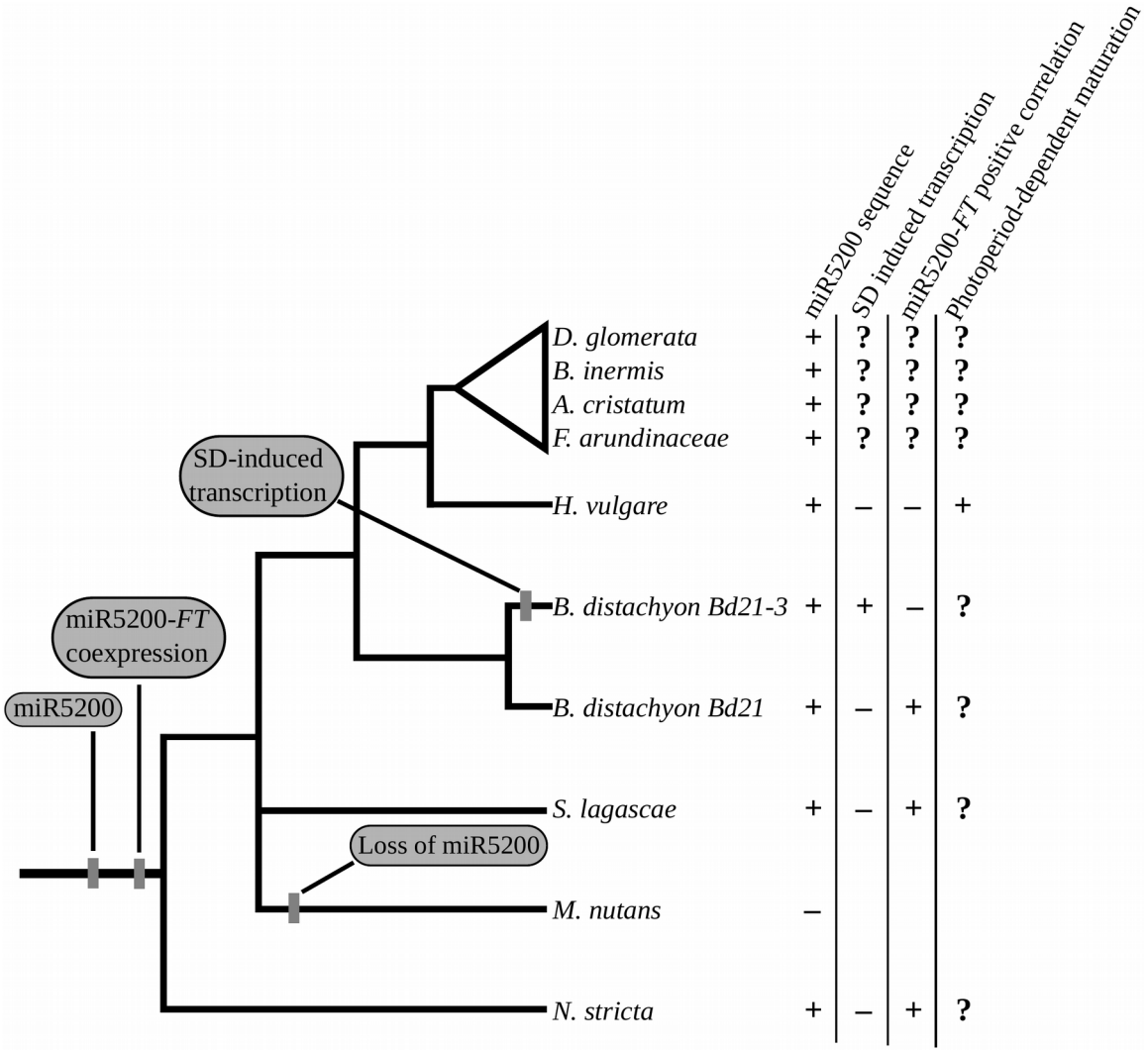

Figure 5. Summary model of miR5200 evolution in Pooideae based on the present study and Wu et al. (2013). '+' denotes presence, '–' absence, and '?' ambiguity. Where miR5200 is absent (*M. nutans*), fields were left empty. Gray boxes with black borders indicate putative gains or losses of certain features. Except of the relationship of *M. nutans* and *S. lagascae* which is disputed, the schematic Pooideae phylogeny is in accordance with a recently published study (Soreng et al. 2015).

Supplemental material

Table S1. Single tailed t-test statistics for *FT* expression under LD photoperiods after seven or 14 day timepoints. T-statistic is listed with degrees of freedom in subscript. **FT* primer sets that amplify transcripts that span microRNA target sites. *H. vulgare* timepoint was taken after six days instead of seven. NA indicates that the test was not performed due to absence of data. Significant P-values ($P < 0.05$) are indicated in bold.

Species	7-day	14-day
Bd21 FTL1	$1.06_{7.17}$	$0.959_{5.09}$
Bd21 FTL2	$-1.53_{9.69}$	0.122_{10}
Bd21-3 FTL1	$0.35_{14.0}$	NA[1]
Bd21-3 FTL2	$-0.4_{19.17}$	NA[1]
N. stricta FT	$1.36_{6.71}$	$1.4_{9.41}$
*N. stricta FT**	$1.32_{6.05}$	**$1.94_{9.43}$**
S. lagascae FT	$-0.357_{9.65}$	$-2.08_{8.36}$
*S. lagascae FT**	$-0.255_{9.02}$	$-0.963_{6.72}$
*H. vulgare FT**	**$4.33_{9.04}$**	NA

Table S2. Primers used for qRT-PCR

qRT-PCR primer	Species	Target	Sequence (5’ to 3’)
Lolium_Ef1α_f	General	*Ef1α*	CCTTGCTTGAGGCTC TTGAC
Lolium_Ef1α_R	General	*Ef1α*	GTTCCAATGCCACCA ATCTT
Lolium_UBQ5_f	General	*UBIQUITIN 5*	AAGGAGTCAACCCTCCACCT
Lolium_UBQ5_R	General	*UBIQUITIN 5*	TCACCTTCTTGTGCTTGTGC
Bd_miR5200a_f	*B. distachyon*	*miR5200*	AAAAGATGACTTCTTAATAG CAAAACCG
Bd_miR5200a_r	*B. distachyon*	*miR5200*	TTCTCAAATGTCCACAACAA TCCAATAC
Bd_miR5200b_f	*B. distachyon*	*miR5200*	GAGCAAAATACTCAAAGATC AACCAGAA
Bd_miR5200b_r	*B. distachyon*	*miR5200*	GCAATTCCAATTAAGTATAG TCACAACG
Bd_FTL1_f	*B. distachyon*	*FTL1*	CCCCGACATGCGCACCTTCT ACA
Bd_FTL1_r	*B. distachyon*	*FTL1*	TCCAGACTCCCTTTGGCAGT TGAAGTAA
Bd_FTL2_f	*B. distachyon*	*FTL2 (VRN3)*	TGGTAGACCCAGATGCTCCTA
Bd_FTL2_r	*B. distachyon*	*FTL2 (VRN3)*	TCTCGTAGCACATCACCTCCT
Nard_VRN3_763_f (internal)	*N. stricta*	*VRN3*	ATCTGCTACGAGAACCCAAGG
Nard_VRN3_886_r (internal)	*N. stricta*	*VRN3*	GTCCCTGGTGATGAAGTTCTG
Nard_VRN3_1124_f (spans cut site)	*N. stricta*	*VRN3*	TGATGGTAGACCCAGATGCTC
Nard_VRN3_1872_r (spans cut site)	*N. stricta*	*VRN3*	TCGTAGCAGATCACCTCTTGC
Nard_miR5200_2607_f	*N. stricta*	*miR5200*	TCGCACTAATCAAAACCTTGG
Nard_miR5200_2715_r	*N. stricta*	*miR5200*	AGGAGCAGTTCTTCGTTCCTC
StiLag_miR5200_6099_f	*S.*	*miR5200*	CAGTTAGGCCTTTGGTGTGAC

	lagascae		
StiLag_miR5200_6203_r	*S. lagascae*	*miR5200*	AAGATCACTGTTGGGCTTTCA
StiLag_VRN3_1796_f (internal)	*S. lagascae*	*VRN3*	GAGGTGATGTGCTACGAAGC
StiLag_VRN3_1928_r (internal)	*S. lagascae*	*VRN3*	CGAAGTCCTTGGTGTTGAAGT
StiLag_VRN3_743_f (spans cut site)	*S. lagascae*	*VRN3*	TGATGGTAGACCCAGATGCTC
StiLag_VRN3_1771_r (spans cut site)	*S. lagascae*	*VRN3*	GCTCTCGTAGCACATCACCTC
HvFT1_f	*H. vulgare*	*FT1 (VRN3)*	ATCTCCACTGGTTGGTGACAGA (From Yan et al. 2006)
HvFT1_r	*H. vulgare*	*FT1 (VRN3)*	TTGTAGAGCTCGGCAAAGTCC (From Yan et al. 2006)
Hv_miR5200_3532_f	*H. vulgare*	*miR5200*	ACGTTCGACCTGAATCCACTAT
Hv_miR5200_3662_r	*H. vulgare*	*miR5200*	GGCTGAAAGTACCCAAATTGAC

Table S2 continued

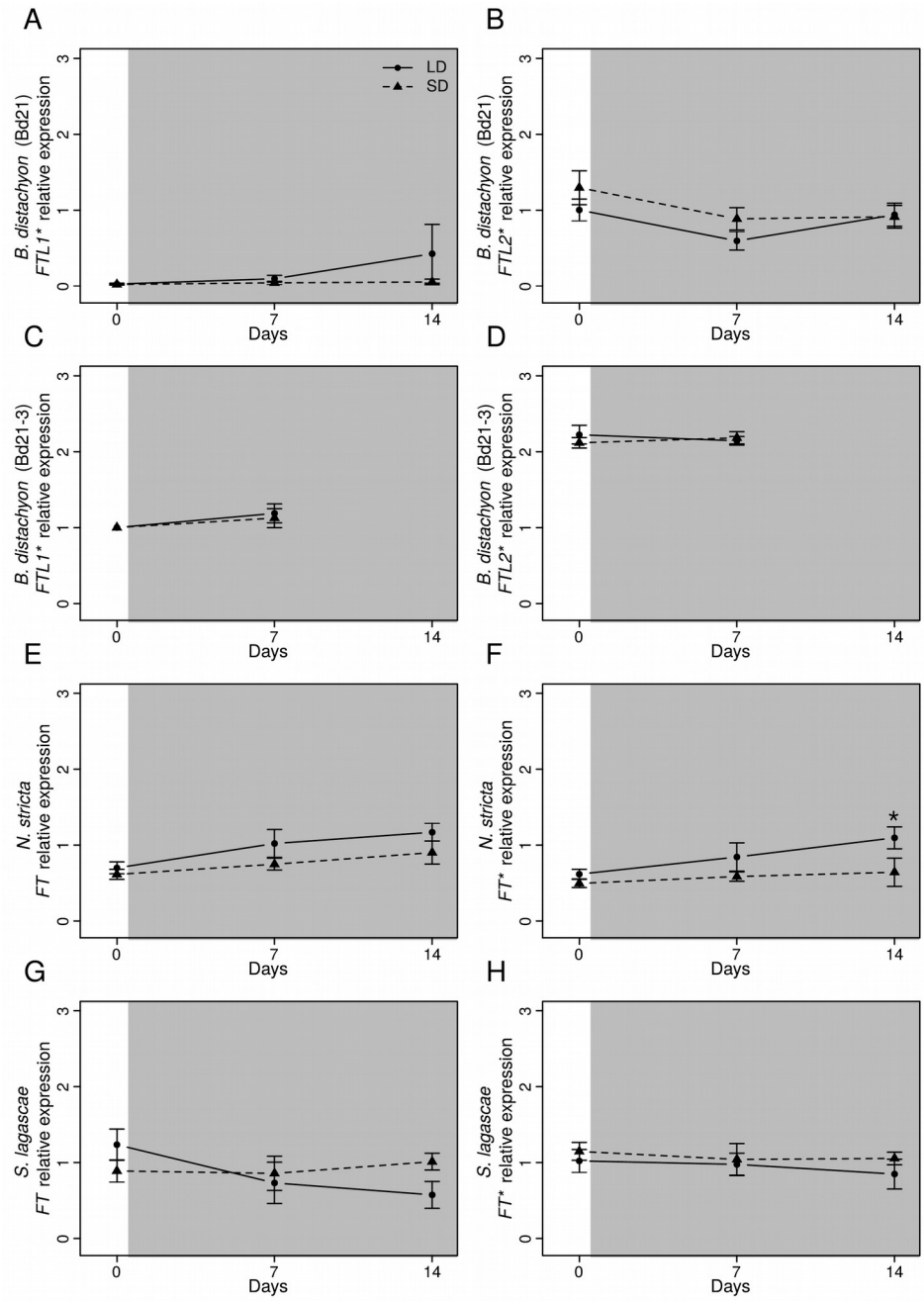

Figure S1. Expression of *FT* relative to *UBQ5* and *EF1α* in tested Pooideae species. Asterisks after *FT* in (F) and (H) indicate that primers span the miRNA cut site. Gray boxes indicate times when SD treatment plants experienced SDs. Error bars indicate standard error for two experimental replicates with three biological replicates each. Circles with solid lines represent LD treatment; triangles with dashed lines represent SD treatment. Significant P-value ($P < 0.05$) for the primer set spanning the miRNA cut site in *N. stricta* is indicated with asterisk at the 14-day timepoint.