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Flowering behavior and juvenile stage miR156/miR172 expression in annual and perennial Pooideae

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Abstract

Most plant seedlings undergo a juvenile stage of development, during which they are unresponsive to environmental signals that promote floral competence. The length of this phase is often extended in perennial versus annual species, whereby changes in the temporal expression patterns of two microRNAs, miR156 and miR172, and their targets, regulates the juvenile to adult transition and flowering in Brassicaceae species. Here I show that annual grasses of the subfamily Pooideae have shorter juvenile phases than their perennial counterparts, and that miR156/miR172 levels can explain developmental transitions in several Pooideae species as well as differences in flowering behaviour between growth habits. I also identified a novel profile for *Brachypodium sylvaticum* miR156 reduction in response to vernalization, perhaps involved in a yet undescribed floral pathway.

Acknowledgements

This work owes a great debt of gratitude to my main supervisor and mentor Siri Fjellheim. Thank you for your unwavering enthusiasm, constructive feedback, support and our many discussions throughout the process of this thesis, and in shaping the foundation of my research career over the last three years. Many thanks to my co-supervisor Martin Paliocha for help with statistics, and numerous hours spent supervising and teaching me laboratory techniques. Also, I thank Øyvind Jørgensen for technical assistance and help during the growth experiment. Lastly, great thanks to Jill Christine Preston, who has read my draft manuscripts critically. I have benefited immensely from her constructive suggestions and advice.

Ane Charlotte Hjertaas Ås, 4th December

Contents

Introduction

The ability to flower once versus several times over multiple growing seasons represents two distinct life history strategies in plants. Semelparous annual (hereafter annual) plants live fast and die young by completing their life cycle in a single year or season and are most often associated with high growth rates and large resource allocation into reproductive organs. This strategy allows for rapid development and subsequent escape from stressful conditions such as severe drought (Pitelka, 1977; Garnier, 1992; Garnier & Laurent, 1994; Li et al., 2004; Garnier et al., 2007; Franks, 2011). Iteroparous perennials (hereafter perennials), by contrast, persist and flower for many seasons, and are often termed "slow growers" with traits that accommodate persistence (e.g., high leaf and root density), defense and resistance to seasonal stressors (Roumet et al., 2006; Friedman, 2020). Moreover, studies have found that perennial plants exhibit a delay in flowering compared to their annual relatives (Primack, 1979; Hyun et al., 2017; Friedman, 2020; Fjellheim unpublished). Taken together, these observations suggest that annual plants can pass through developmental phases faster than perennials, making the duration of vegetative growth a candidate trait underlying the differences between annual and perennial growth strategies.

Newly germinated seedlings of many plant species start off in the juvenile phase of development, at which time they are unable to respond to signals that normally induce flowering. Only when individuals make the transition to the adult phase of vegetative development can they respond to permissive signals that will induce reproductive development in the form of inflorescences, flowers, and seeds (Poethig, 1990; Bäurle & Dean, 2006). Several regulatory pathways, such as those that respond to cold, daylength, developmental age, and hormones, have been identified as controlling plant transitions from germination to flowering (Teotia & Tang, 2014). The transition from the juvenile to adult vegetative phase, known as vegetative phase change, is both gradual and age-related and may last a few days or many years, depending on the species (Poethig, 1990; Bergonzi & Albani, 2011; Hyun et al., 2017). Two microRNAs, miR156 and miR172, have been identified as pivotal regulators of vegetative phase change as well as in competence to flower (Wang et al., 2011; Bergonzi et al., 2013; Hyun et al., 2017). MiRNAs are a class of endogenous, small non-coding RNAs. The mature miRNA, ~22 nucleotides in length, is processed from a hairpin-like structure of the primary transcript (pri-miRNA) and regulates gene expression at the post-transcriptional level by degrading mRNA or repressing translation of a target gene (Liu et al., 2017). MiRNA transcription is often under the direct control of

transcription factors (TFs) (Barh et al., 2015), many of the latter being affected transcriptionally and post-transcriptionally by various environmental factors.

The miR156 gene family is highly conserved in land plants (Axtell & Bowman, 2008) and members act as age-related timers by negatively regulating *SQUAMOSA PROMOTOR-BINDING PROTEIN-LIKE* (*SBP/SPL*) TFs involved in the juvenile to adult transition and flowering (Fig. 1; Wu & Poethig, 2006; Chuck et al., 2007; Wang et al., 2011; Bergonzi et al. 2013; Zhou et al. 2013; Wang & Wang, 2015). In leaves of the annual plant *Arabidopsis thaliana* (Brassicaceae), miR156 delays the transition to the adult phase, and in shoot meristems, it delays flowering (Hyun et al., 2017). As *A. thaliana* plants age and stored photosynthate increases, leaf miR156 levels decrease, resulting in de-repression of *SPL* genes (Yu et al., 2015; Hyun et al., 2017). Moreover, comparative studies of floral activator *SPL15* in *A. thaliana* and perennial relative *Arabis alpina* reveal diverse flowering pathways between growth habits (Hyun et al., 2019). *A. thaliana* responds to vernalization immediately after sowing (Chandler & Dean, 1994) and flowers independently of *SPL15* under long days through the photoperiodic pathway, whereas reduction in miR156, followed by an increase in SPL15, is required for flowering in short days (Hyun et al, 2019; Madrid et al., 2020). Competence to flower in *A. alpina* is, by contrast, acquired through an age-related sensitivity to vernalization, mediated by a decrease in miR156 levels (Bergonzi et al., 2013). When exposed to cold after six weeks of growth, transcriptional repression of miR156 enables the vernalization process to proceed in older meristems which in turn promotes flowering through SPL15 (Hyun et al., 2016; Hyun et al., 2019). These findings imply a role for SPL15 in determining the perennial strategy as its activity is restricted to older shoots of *A. alpina* (Hyun et al., 2019).

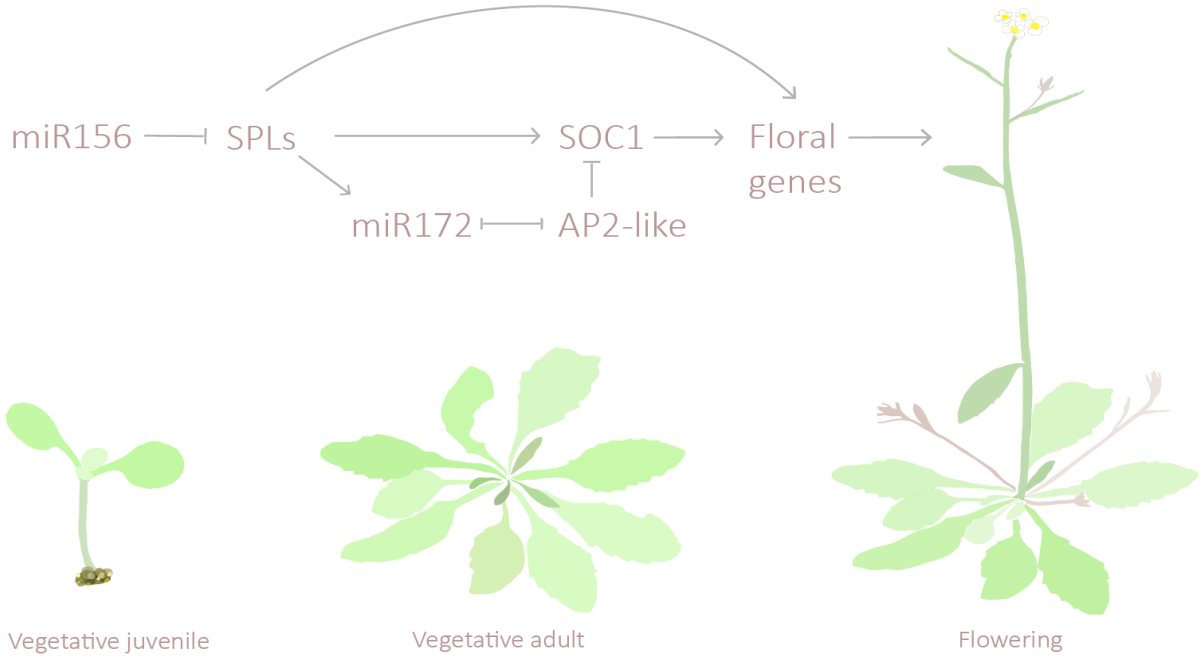


Figure 1. Age pathway in *Arabidopsis thaliana.*

The antagonistic age-dependent relationship between miR156 and miR172 negatively regulates floral repressors and positively regulates floral activators, respectively. In young seedlings, miR156 represses SPLs involved in developmental transitions. A decrease in miR156 with age mediates upregulation of miR172 through SPLs, whereas MiR172 negatively regulates flowering repressors AP2-like TFs, causing an upregulation of floral pathway integrator *SUPPRESSOR OF CONSTANS* (*SOC1*).

Opposite to the temporal pattern of miR156, the abundance of miR172 increases with age and as for miR156, the miR172 gene family is conserved in land plants (Axtell & Bowman, 2008). MiR172 promotes vegetative phase change and activates flowering through negative regulation of *APETALA 2* (*AP2*) and *AP2*-like TFs known to repress floral genes (Fig. 1; Aukerman & Sakai, 2003; Teotia & Tang, 2014). Overexpression of miR172 accelerates the transition to the adult vegetative phase and in *A. alpina* an *ap2*-like mutant flowers without vernalization, providing evidence for the central role of miR172 in the juvenile to adult transition and flowering (Bergonzi et al., 2013; Hyun et al., 2017). The inverse pattern of miR156 and miR172 is highly conserved in distantly related species and both genes have also been found to control vegetative phase change in trees (Wang et al., 2011), and the annual grasses *Zea mays* (maize, Panicoideae) (Chuck et al., 2007), *Oryza sativa* (rice, Oryzoideae) (Xie et al., 2012) and *Hordeum vulgare* (barley, Pooideae) (Tripathi et al., 2018). However, it is unknown whether

differences in the expression of known age pathway genes can explain growth habit transitions in grasses, similar to the case in Brassicaceae.

Grasses of the subfamily Pooideae are primarily north temperate, and similar to long-day temperate species *A. thaliana* and *A. alpina*, many Pooideae species flower in response to a cold treatment followed by long days (Andrés & Coupland, 2012; Fjellheim et al., 2014). In fact, responses to vernalization and long days have been characterized in a large set of Pooideae species and found to be major regulators of flowering throughout the subfamily (McKeown et al., 2016; Fjellheim & Preston, unpublished). Pooideae contain some of the world's most important annual grain crops, like wheat (*Triticum aestivum*), barley (*H. vulgare*), and oats (*Avena sativa*) along with perennial forage grasses, such as ryegrass (*Lolium perenne*) and timothy (*Phleum pratense*). Understanding the nature of annual and perennial strategies in grasses are therefore of much interest in terms of future sustainable food production. Species are also increasingly being targeted as sustainable biofuel crops that possess one or more perennial characteristics (FAO, 2016). A recent study on Pooideae growth habit evolution revealed a striking difference in the relative number of annual compared to perennial species in the cereal-containing 'core' Pooideae (37%) versus non-core lineages (3%), whereby a potential precursor trait for the evolution of annuality was identified at the base of the core Pooideae (Lindberg et al., 2020). Furthermore, growth traits were identified as strong candidates for the precursor trait, making a reduced juvenile phase in core versus non-core perennials a good candidate for the precursor trait. Here, I explore flowering time behaviour with respect to developmental age and gene expression in annual/perennial pairs of core Pooideae and perennial non-core Pooideae to test the non-exclusive hypotheses that (1) perennial species have a longer juvenile phase than their annual relatives, (2) the juvenile phase is extended in non-core versus core perennial Pooideae, (3) miR156 and miR172 are antagonistic regardless of growth habit, and (4) longer juvenile phases can be explained by prolonged miR156 expression.

Materials and methods

Species selection and plant growth conditions

Twelve core Pooideae species, organized into six core Pooideae annual-perennial species pairs and five non-core perennial species, were selected based on phylogenetic position, known or potential vernalization response and seed availability (Table 1). Seeds were acquired from the United States Department of Agriculture (USDA), Millennium Seed Bank (MSB), B & T World Seeds (B&T WS) and Graminor AS (Table 1). Seeds were sown in moist soil (Tjerbo P-jord, item number 11051/11081, Norway) and released from dormancy by different treatments due to species-specific requirements for germination (Table S1). After pre-germination treatments, species marked grey in Table 1 were transferred to growth chambers set to 20°C 16 h light/16°C 8 h dark, 65 ± 10% relative humidity with light intensity of 160 ± 10 µmol/m^2s-^{1} and red/far-red ratio at 2.5 ± 0.1. Light conditions were produced by incandescent light bulbs (NARVA A60 Classic Glühlampe E27, NARVA, Germany) and metal halide lamps (HQI-BT 400 W/D PRO, Osram GmbH, Germany). The remaining species (Table 1) were moved to an open greenhouse under the same temperature and daylength conditions. All seedlings were pricked out into individual 30 ml pots and fertilized every third week, except during vernalization.

To test for differences in the juvenile phase, plants were given 4, 8 or 12 weeks of growth prior to the vernalization treatment. Plants were acclimated and vernalized under short days (8 h light/ 16 h dark) in walk-in growth chambers for 8 weeks at 10°C, 8°C and 6°C (48 h at each temperature) and at 4°C for the remainder of the treatment. Light conditions during vernalization were produced by fluorescent lights (MASTER TL-D 58W/827, Philips, The Netherlands) with an intensity of 30 ± 10 µmol/m^2s-^{1}. Control plants were kept in similar walk-in chambers with equal light conditions under long days (16 h light/8 h dark) at 18°C. After vernalization, plants were maintained in an open greenhouse until flowering. Flowering was recorded every other day and calculated as days to heading (*DTH*) by subtracting extended juvenile growth periods between treatments from the date of germination. Differential growth between vernalized and control plants were corrected by adjusting *DTH* in vernalized plants. Assuming a linear relationship between growth and temperature (Baskerville & Emin, 1969), *DTH* in vernalized plants were temperature-adjusted as follows

$$DTHc = DTH - [L * \frac{(Tc - Tv)}{Tc}]$$

where *DTHc* is corrected days to heading, *L* the length of the vernalization period (56 days), *Tc* the temperature conditions for the control plants (18°C), and *Tv* the vernalization temperature (10°C for 2 days, 8°C for 2 days, 6°C for 2 days and 4°C for 50 days). *DTH* in vernalized plants where thus heat-unit corrected by 42.22 days. Species were scored as having a vernalization response if they flowered earlier with versus without vernalization, and the approximate length of the juvenile phase was determined based on flowering time after different pre-vernalization regrowth lengths. To account for differences in growth conditions within chambers, walk-in chambers and in the greenhouse, plants were systematically moved to a new position every other day.

Table 1. Species included in the growth experiment with those marked grey being targeted for gene expression analysis.

	Tribe	Habit	Species name	Seed source	Accession number	Country of origin
CORE POOIDEAE	Brachypodieae	Annual	*Brachypodium distachyon*	USDA	PI 170218	Turkey
		Perennial	*Brachypodium sylvaticum*	USDA	PI 269842	Tunisia
	Bromeae	Annual	*Bromus tectorum*	USDA	PI 204412	Turkey
		Perennial	*Bromus benekenii*	MSB	96920	UK
	Triticeae	Annual	*Hordeum vulgare*	USDA	PI 428488	Germany
		Perennial	*Hordeum bulbosum*	USDA	PI 240164	Italy
	Poeae	Perennial	*Hordeum jubatum*	Unknown	Unknown	Unknown
		Annual	*Briza maxima*	USDA	PI 257681	France
		Perennial	*Briza media*	USDA	PI 378956	France
		Annual	*Poa annua*	USDA	PI 442543	Belgium
		Perennial	*Poa supina*	USDA	PI 559922	Germany
		Annual	*Larmarckia aurea*	USDA	PI 378959	France
		Perennial	*Dactylis glomerata*	Graminor	54306	Norway
		Perennial	*Lolium perenne*	Graminor	Fagerlin	Norway
BASAL POOIDEAE	Meliceae	Perennial	*Melica nutans*	USDA	PI 442519	Belgium
	Stipeae	Perennial	*Stipa lagascae*	USDA	PI 250751	Iran
		Perennial	*Nassella leucotricha*	USDA	W6 24255	USA
		Perennial	*Piptochaetium napostaense*	USDA	PI 202062	Argentina
	Ampelodesmeae	Perennial	*Ampelodesmos mauretanicus*	B&T WS	62975	Unknown
	Nardeae	Perennial	*Nardus stricta*	B&T WS	4393	Unknown

Phylogenetic analysis

Phylogenetic analysis was performed to identify orthologs in study species. Based on a recent study of miR156 and miR172 in *H. vulgare* (Tripathi et al., 2018), *MiR156b* and *MiR172b* were chosen as candidate genes involved in the vegetative phase transition in Pooideae species. Sequences for *H. vulgare* obtained from Tripathi et al. (2018) were used to identify orthologs in the other study-species. Pre-microRNA sequences for *O. sativa* was obtained from the miRbase database (http://www.mirbase.org/). Genomic sequences for *B. sylvaticum* (B. sylvaticum v1.1) were identified using BLAST in Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html) and for *H. bulbosum*

genomic sequences were identified with nucleotide BLAST search in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments was performed using Mafft version 7 (https://mafft.cbrc.jp/alignment/server/index.html). Considering the secondary structure of microRNAs, the Q-INS-i setting was applied with further default settings. Phylogenetic trees were constructed using the maximum likelihood method with the smart model selection option (https://ngphylogeny.fr/).

Gene expression analysis

To test predictions of the hypothesis that miR156 and miR172 antagonistically specify the juvenile versus adult phases of vegetative phase growth, and that their expression will correlate with the length of the juvenile phase, species marked in grey in Table 1 were targeted for reverse transcriptase (RT)-quantitative (q)PCR. Pre-vernalized leaf tissue was sampled at day 7 (annuals only), 14 (perennials only), 28, 56, 84, and after vernalization at day 91, 119 and 147 three hours after dawn. Four replicates per accession at each time point were sampled, and leaf tissue was flash frozen in liquid nitrogen and stored at -80°C. Prior to RNA extraction, leaf tissue was disrupted and homogenized using a TissueLyser II Bead Mill Mixer (QIAGEN, Hilden, Germany). RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN Sample & Assay Technologies, Sweden) and trace quantities of DNA were removed using TURBO DNA-free Kit (Thermo Fisher Scientific, Waltham, MA, US), both following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using 1.0 µg of RNA with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). cDNA was stored at -20°C.

RT-qPCR

Genus- or species-specific primers for RT-qPCR were designed manually and two housekeeping genes, *UBIQUITIN 5 (UBQ5) and ELONGATION FACTOR 1a (Ef1a)* were chosen based on previous studies (Table S2) (Scoville et al., 2011; Woods et al., 2017; McKeown et al., 2017). Primer efficiency, ranging from 0.9 to 1.1 for all primer pairs, was determined using a 2-fold dilution series (Schmittgen & Livak, 2008) and amplicon identity for all genes were verified by sequencing. Gene expression was quantified with BIO-RAD CFX96 Touch Real-Time PCR Detection System using SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, US) with a total reaction volume of 10 µl per well. Quantification was performed with three or four biological replicates per time point and three technical replicates per biological replicate. Cycle quantification values (C_q) was corrected for primer efficiency and normalised against the geometric mean of the two house-keeping genes

(*Ef1a* and *UBQ5*). Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001) and the first sampling time point (day 7 or 14) was used as an internal reference.

Statistical testing

Statistical testing was performed in Microsoft Excel, version 2002 (https://www.microsoft.com/nb-no/microsoft-365/excel) and R v3.6.3 (R Core Team, 2020). Shapiro-Wilk's test supplied with a visual assessment of distribution curves was used to determine the distribution of flowering data (Shapiro & Wilk, 1965). Due to large deviations from normality, Mann-Whitney *u*-tests were performed to determine differences in *DTH* between two groups; its equivalent for multiple groups, Kruskal-Wallis *H* test followed by Dunn's test, was applied to determine differences between more than two groups (Mann & Whitney, 1947; Kruskal & Wallis, 1952; Dunn, 1964). Differential flowering was calculated for treatments with at least five flowering individuals. Gene expression data were normally distributed and tested with Welch's two-sample *t*-test (Welch, 1947).

Results

Evidence points to short juvenile phases explaining evolution of annuality in core Pooideae grasses

To test if the length of the juvenile phase affect flowering behaviour, study species (Table 1) were given three different pre-vernalization treatments: three months (hereafter long), two months (hereafter medium), and one month (hereafter short) of growth.

Within my annual-perennial species pairs, I predicted that perennials would take longer to become responsive to vernalization due to an extended juvenile period relative to their annual counterparts. In the case of genus *Hordeum*, annual *H. vulgare* showed a strong vernalization response regardless of treatments ($p < 0.001$). However, flowering was significantly faster with medium and long versus short treatment periods ($p = 0.010$) and marginally faster with long versus medium treatments ($p = 0.050$) (Fig. 2A). Low seed germination for perennial *H. bulbosum* meant that control and short treatments were not included, and thus vernalization responsiveness could not be tested. As predicted, *H. bulbosum* plants flowered later than those of *H. vulgare* within the same treatments, but the lack of a flowering difference between medium and long treatments for *H. bulbosum* cannot alone distinguish whether the juvenile phase is longer or shorter than 8-12 weeks (Fig. 2A).

In the case of *Brachypodium*, annual *B. distachyon* plants showed a strong vernalization response for all treatments ($p < 0.01$), but treatment period had no impact on flowering time, suggesting a juvenile phase of less than four weeks (Fig. 2B). Contrary to predictions, perennial *B. sylvaticum* only showed

a vernalization response for plants in the medium treatment group, again with treatment period having no impact on flowering time (Fig. 2B). Also, flowering time was not significantly different between *B. distachyon* and *B. sylvaticum* within treatments (Fig. 2B).

Interpretation of juvenile phase length in my other annual-perennial core Pooideae species pairs was limited by the fact that the perennials *Bromus benekenii, Dactylis glomerata, Poa supina*, and *Briza media* failed to flower within all treatments (Fig. 3). This suggests that they have a juvenile phase longer than 12 weeks, the vernalization period was too short and/or they could have been missing an additional cue critical for flowering. In the case of their annual counterparts, only *Bromus tectorum* had a vernalization response, and neither *B. tectorum* nor the other annuals flowered earlier with longer treatments (Fig. 3). Together with significantly earlier flowering of the annuals compared to their perennial relatives in each treatment, these data tentatively suggest the loss or reduction of the juvenile phase, and/or the loss of vernalization responsiveness for *Lamarckia aurea*, *Poa annua*, and *Briza maxima* in evolving annuality.

No difference in juvenile phase length between perennial core and non-core Pooideae

In addition to characterizing flowering behaviour in core Pooideae perennials, I determined DTH_C for several non-core Pooideae perennials. *Melica nutans* (Meliceae) had a strong vernalization response in terms of early flowering with medium and long treatments ($p < 0.001$) (Fig. 4A). Furthermore, plants given medium and long treatments were significantly faster flowering than those given the shorter treatment ($p < 0.001$), and there was a marginal difference in DTH_C between medium and long treatments ($p = 0.054$) (Fig. 4A). This suggests that the juvenile phase of *M. nutans* is 8 weeks or more. A vernalization response was also found for *Nassella leucotricha* (Stipeae), but this time in all treatment periods ($p < 0.001$) (Fig. 4B). Unexpectedly, *N. leucotricha* plants with the short treatment period flowered faster relative to those given medium and long treatments (Fig. 4B). This suggests that the juvenile phase is relatively short in this species. Similar treatments were set-up for an additional three non-core Pooideae, *Nardus stricta* (Nardeae), *Ampelodesmos mauritanicus*, and *Stipa lagascae* (Stipeae), but unfortunately none of the plants flowered before 300 days in any of the treatments.

To specifically test for later flowering in non-core versus core Pooideae perennials, I generated additional partial flowering datasets for the core Pooideae perennials *Lolium perenne* and *Hordeum jubatum*, and the non-core Pooideae perennial *Piptochaetium napostaense*, based on limited seed

availability (Fig. 5). Comparison of all core and non-core Pooideae perennials revealed no significant differences in DTH_C irrespective of treatment periods (Fig. 5).

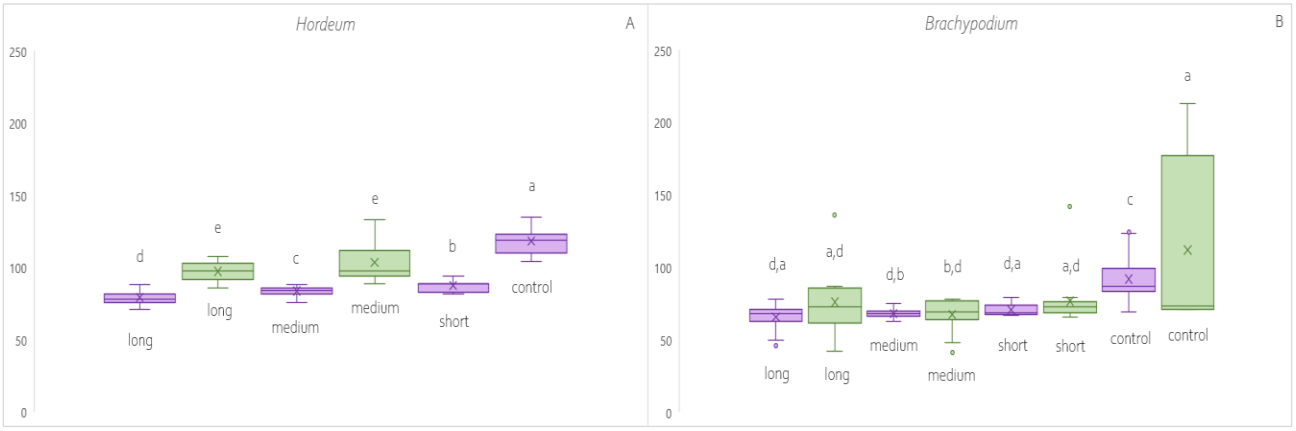


Figure 2. Flowering behaviour in *Hordeum* and *Brachypodium.* Purple = annuals, green = perennials. Different letters indicate a significant difference, $p < 0.05$. (Mann-Whitney *u*-test and Kruskal-Wallis *H* test supplied with Dunn's post hoc test).

A. *H vulgare*. Long, medium and short vs. control ($p = 0.0001$). Long vs. medium ($p = 0.05$), long vs. short ($p = 0.0001$) and medium vs. short ($p = 0.01$). *H. bulbosum.* Long vs. medium ($p = 0.7139$). *Hordeum.* Long ($p = 0.0001$). *Hordeum*. Medium ($p = 0.0095$).

B. *B. distachyon*. Long, medium and short vs. control ($p = 0.0001$, p = 0.0001, $p = 0.01$). Long vs. medium ($p = 0.7497$), long vs. short ($p = 0.2841$) and medium vs. short ($p = 0.4536$). *B. sylvaticum.* Long, medium and short vs. control ($p = 0.1497$, $p = 0.0240$, $p = 0.1951$). Long vs. medium ($p = 0.3689$), long vs. short ($p = 0.7974$) and medium vs. short ($p = 0.2146$). *Brachypodium.* Long ($p = 0.1224$). *Brachypodium*. Medium ($p = 0.4459$). *Brachypodium*. Short ($p = 0.3310$).

Figure 3. Flowering behaviour in core annual-perennial species pairs.

A-C. *Bromus tectorum* and *B. benekenii.* **D-F.** *Lamarckia aurea* and *Dactylis glomerata.* **G-I.** *Poa annua* and *P. supina*. **J-L.** *Briza maxima* and *B. media*. *Significance level.* *** $p < 0.001$ (Mann-Whitney *u*-test).

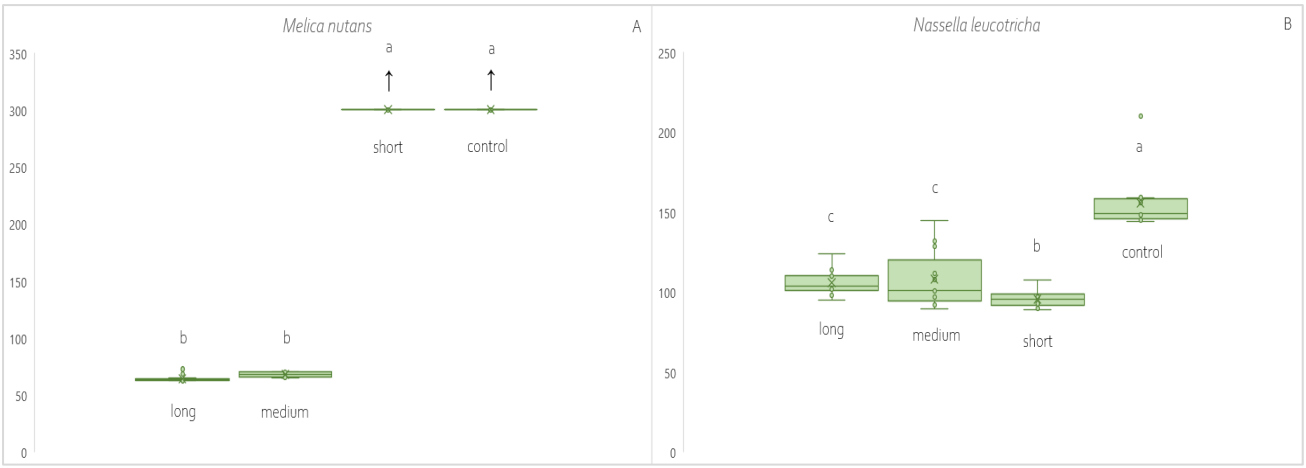


Figure 4. Flowering behaviour in non-core perennial species. Different letters indicate a significant difference, $p < 0.05$.

A. *Melica nutans*. Long and medium vs. short and control (p = 0.00001). Long vs. medium (p = 0.0540). **B.** *Nassella leucotricha*. Long, medium and short vs. control (p = 0.0001). Long vs. medium (p = 0.5727), long vs. short (0.0097) and medium vs. short (p = 0.0463).

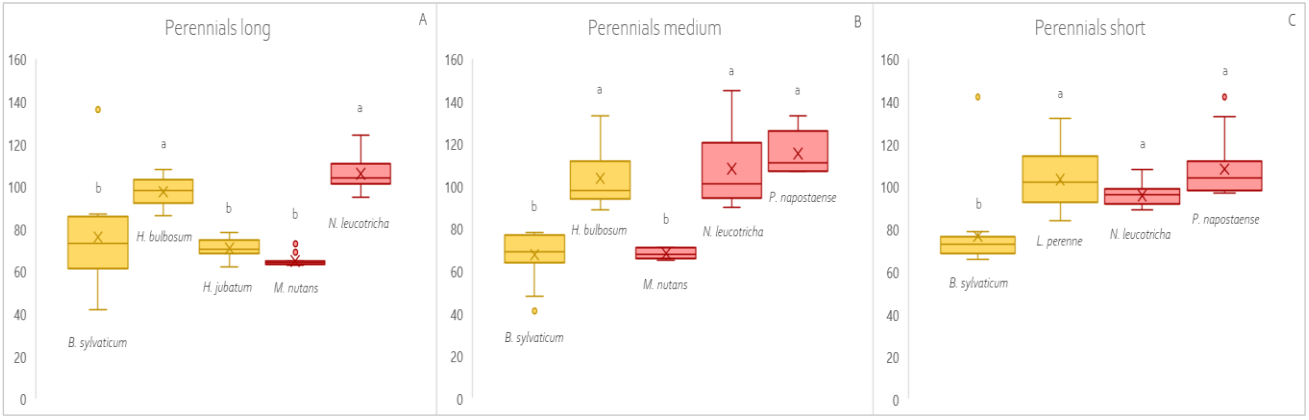


Figure 5. Flowering behaviour in core (yellow) and non-core (pink) perennial species. Different letters indicate a significant difference, $p < 0.05$.

A. Long. *Nassella leucotricha* vs. *Hordeum bulbosum* (NS) and *N. leucotricha* vs. *H. jubatum* and *Brachypodium sylvaticum* (p = 0.01). *Melica nutans* vs. *H. jubatum* and *B. sylvaticum* (NS) and *M. nutans* vs. *H. bulbosum* (p = 0.0001) **B.** Medium. *N. leucotricha* and *Piptochaetium napostaense* vs. *B. sylvaticum* (p = 0.001) and *N. leucotricha* and *P. napostaense* vs. *H. bulbosum* (NS). *M. nutans* vs. *H. bulbosum* (p = 0.0001) and *M. nutans* vs. *B. sylvaticum* (NS). **C.** Short. *N. leucotricha* and *P. napostaense* vs. *L. perenne* (NS) and *N. leucotricha* and *P. napostaense* vs. *B. sylvaticum* (p = 0.01) (Kruskal-Wallis H test supplied with Dunn's post hoc test).

Identification of *MiR156b* and *MiR172b* orthologs in study species

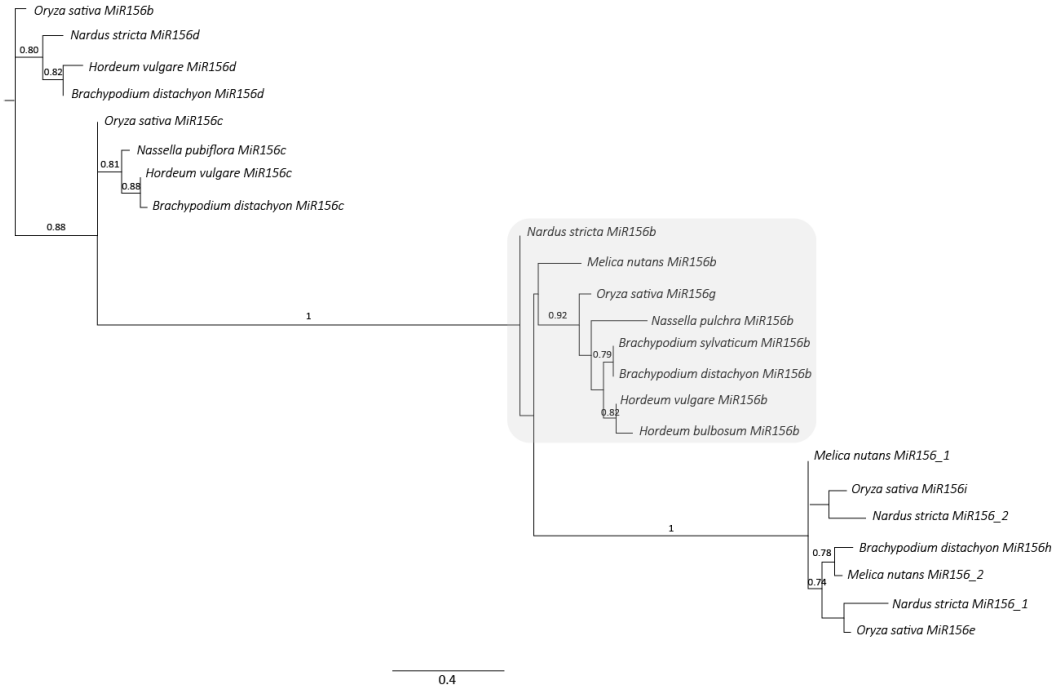


Figure 6. Maximum likelihood tree of miR156 members in Pooideae species with *O. sativa* (Oryzoideae) as an outgroup.

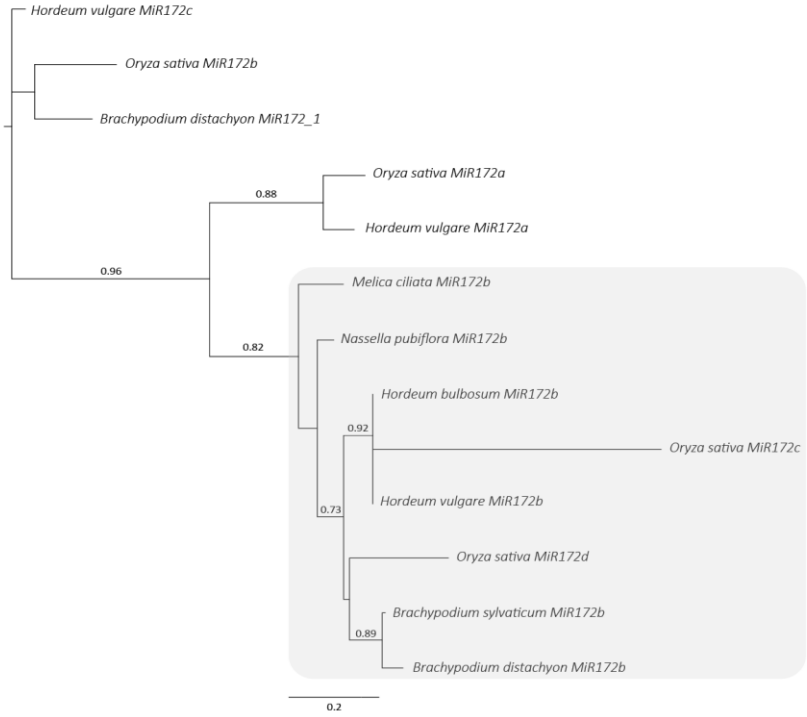


Figure 7. Maximum likelihood tree of miR172 members in Pooideae species with *O. sativa* (Oryzoideae) as an outgroup.

Gene expression in response to the length of the juvenile phase

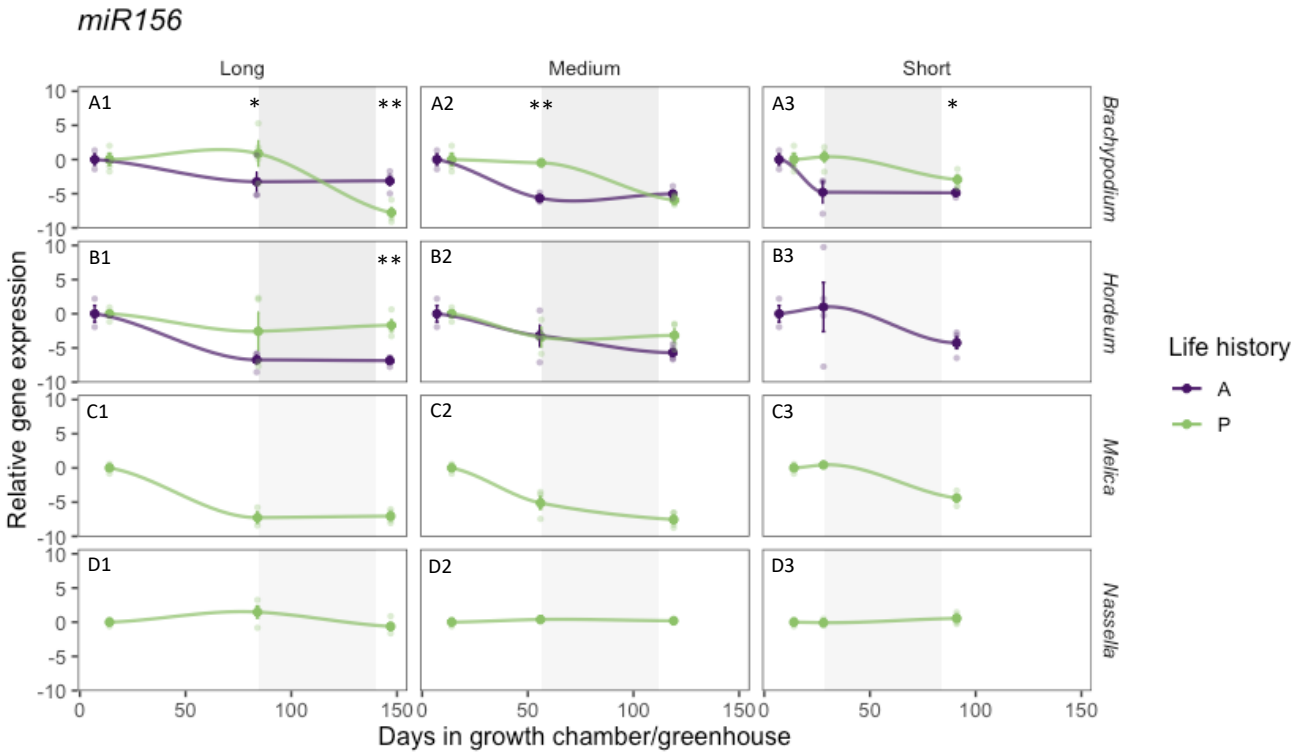
To test the hypotheses that miR156 and miR172 antagonistically control the juvenile to adult phase change across Pooideae, and that differences in the juvenile phase can be explained by miR156/miR172 expression, I examined transcript levels for these genes in two core Pooideae annual-perennial species pairs and two non-core Pooideae perennials (Fig. 8 and 9). As predicted, the overall picture for miRNA expression was decreasing and increasing levels of miR156 and miR172 over time, respectively. One exception was *N. leucotricha,* where miR156 expression is nearly unchanged at all time points (Fig. 8D).

In the case of *Hordeum*, the reduction of miR156 expression occurred before vernalization with a treatment period of 8 weeks, and was even more pronounced after 12 weeks of growth, particularly in annual *H. vulgare* (Fig. 8B). This pattern is consistent with a juvenile phase of 8 weeks or more in *H. vulgare*. Furthermore, a trend towards higher miR156 levels (p = 0.0711) in *H. bulbosum* after vernalization in the medium treatment (Fig. 8B), aligned well with later flowering in this species compared to *H. vulgare* (Fig. 2A). The reciprocal increase in miR172 expression followed a similar trajectory, whereby expression peaked by the medium treatment for both species (Fig. 9B). However, in this case, expression was significantly higher in *H. vulgare* than *H. bulbosum* by the end of the medium and long treatments (Fig. 9B).

Unlike *Hordeum*, the annual and perennial *Brachypodium* species showed no differences in flowering time either between species or treatment periods (Fig. 2B). Thus, a treatment and species effect on miR156 and miR172 expression was not predicted. Irrespective of the flowering time results, miR156 was significantly higher for perennial *B. sylvaticum* than *B. distachyon* at the medium and long treatment time points and after vernalization in the short treatment (Fig. 8A). Interestingly though, this trend was flipped after vernalization in the long treatment, with *B. distachyon* having higher levels of miR156 (Fig. 8A). For miR172, mean expression was already higher for both species at the short treatment time point (Fig. 9A), consistent with their short inferred juvenile phases (Fig. 2B). Furthermore, there were no differences in miR172 levels between species, except for higher expression in *B. distachyon* after vernalization in the short treatment that again did not correlate with flowering time (Fig. 2B; 9A).

As previously mentioned, miR156 expression in *N. leucotricha* was unaffected by time or treatment (Fig. 8D). On the other hand, miR172 expression did increase in long, medium and short treatments, (p = 0.001, p = 0.001 and p = 0.05, respectively) (Fig. 9D). For *M. nutans*, miR156 levels started to dip

at the medium treatment time point and continued this trend by 12 weeks treatment (Fig. 8C). The opposite trend was also seen for miR172 (Fig. 9C). Together, these data are consistent with an 8 week or longer juvenile phase for *M. nutans* (Fig. 4A).



*Coloured area indicates vernalization treatment.

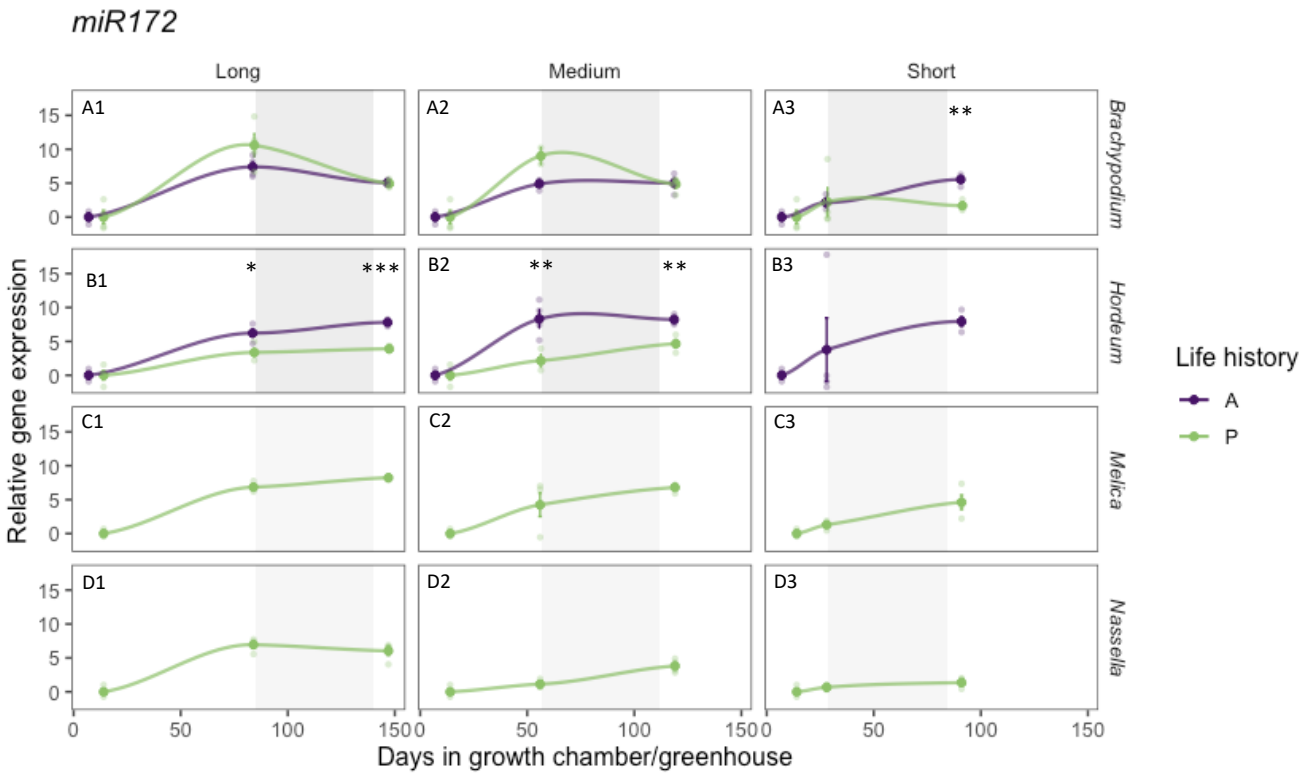
Figure 8. Relative expression levels of miR156 in *Brachypodium distachyon*, *B. sylvaticum*, *Hordeum vulgare*, *H. bulbosum*, *Melica nutans* and *Nassella leucotricha*. *Significance levels.* * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Welch's two-sample *t*-test).

A1. *Brachypodium* long. Significant difference in miR156 before and after vernalization. **A2.** Brachypodium medium. Significant difference before vernalization. **A3.** Brachypodium short. Significant difference after vernalization. **A1/A2/A3**. B. distachyon. MiR156 is mainly downregulated before vernalization and all levels are significantly different between the first and last sampling point. **A1.** Day 7 vs. 84 ($p = 0.0957$) and 7 vs. 147 ($p = 0.0323$). **A2.** Day 7 vs. 56 ($p = 0.0081$) and 7 vs. 119 ($p = 0.0111$). **A3.** Day 7 vs. 28 ($p = 0.0733$) and 7 vs. 91 ($p = 0.0114$). **A1/A2/A3.** *B.* sylvaticum. MiR156 is mainly downregulated between sampling points before and after vernalization. **A1.** Day 84 vs. 147 ($p = 0.0117$). **A2**. Day 56 vs. 119 (0.0015). **A3**. Day 28 vs. 91 ($p = 0.0156$).

B1. *Hordeum* long**.** Significant difference in miR156 after vernalization. **B2.** *Hordeum* medium. A trend towards higher miR156 levels for *H. bulbosum* after vernalization (0.0711). **B1/B2/B3.** *H. vulgare.* MiR156 is completely downregulated before vernalization at day 84 (long) and at low levels at day 56 (medium). Levels after vernalization are significantly higher in the short-treatment vs. long ($p = 0.045$), but not short vs. medium ($p = 0.2128$). **B2**. *H. bulbosum,* the only significant difference in miR156 levels is between day 14 and 119 ($P = 0.0396$).

C1. *M. nutans* long. MiR156 is downregulated before vernalization (0.01). **C2.** Medium. MiR156 is downregulated before vernalization ($p = 0.01$). **C3.** Short**.** MiR156 is downregulated during/after vernalization ($p = 0.01$) **C1/C2/C3.** MiR156 is significantly higher before vernalization at day 28 (**C3**) vs. 84 (**C1**) and 56 (**C2**) ($p = 0.0102$ and $p = 0.0087$), and after vernalization, day 91 (**C3**) vs. 147 (**C1**) and 119 (**C2**) ($p = 0.0076$ and $p = 0.0055$).

D1/D2/D3. *N. leucotricha*. No difference in expression levels within or between treatments.



*Coloured area indicates vernalization treatment.

Figure 9. Relative expression levels of miR172 in *Brachypodium distachyon*, *B. sylvaticum*, *Hordeum vulgare*, *H. bulbosum*, *Melica nutans* and *Nassella leucotricha*. *Significance levels*: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Welch's two-sample *t*-test).

A1. *Brachypodium* long. No significant difference. MiR172 is upregulated before vernalization ($p = 0.01$) and marginally downregulated during vernalization, *B. distachyon* ($p = 0.0577$) and *B. sylvaticum* ($p = 0.0470$). **A2.** *Brachypodium* medium. No significant difference. MiR172 is upregulated before vernalization, *B. distachyon* ($p = 0.01$) and *B. sylvaticum* ($p = 0.03$). **A3.** *Brachypodium* short. Significant difference in levels after vernalization**.** MiR172 levels for *B. sylvaticum* is lower with a short vs. long and medium juvenile phase ($p = 0.01$). MiR172 is upregulated before ($p = 0.01$) and during/after ($p = 0.02$) vernalization for *B. distachyon* and no significant difference is seen for *B. sylvaticum.*

B1. *Hordeum* long. Significant difference before and after vernalization. MiR172 is mainly upregulated before vernalization ($p = 0.05$). **B2.** *Hordeum* medium. Significant difference before and after vernalization. For H. vulgare miR172 is mainly upregulated before vernalization ($p = 0.01$) and in *H. bulbosum* both before and during/after ($p = 0.01$). **A3.** *Hordeum* short. MiR172 is significantly higher between first and last sampling point ($p = 0.001$).

C1. *M. nutans* Long. MiR172 is mainly upregulated before vernalization ($p = 0.001$). **C2.** Medium. MiR172 is upregulated before and during/after vernalization ($p = 0.001$). **C3**. Short. MiR172 is upregulated before and during/after vernalization ($p = 0.05$). **C1/C2/C3.** MiR172 levels are significantly lower after vernalization in the short versus long treatment ($p = 0.05$) and no difference is seen between medium and short.

D1. *N. leucotricha* Long. MiR172 is upregulated before vernalization (0.001). **D2.** Medium. MiR172 is mainly upregulated during/after vernalization ($p = 0.001$). **D3.** Short. MiR172 is upregulated before and during/after vernalization ($p = 0.05$). **D1/D2/D3**. MiR172 are higher after vernalization in the long-treatment vs. medium ($p = 0.05$) and short ($p = 0.01$) and higher in medium vs. short ($p = 0.01$).

Discussion

The juvenile mode of vegetative growth is recognized by the physiological age at which time plants are more or less insensitive to environmental signals that promote floral competence, such as vernalization. In Brassicaceae species, this physiological age is reached at the seed stage for annual *A. thaliana* (Michaels & Amasino, 1999), and only after 5-6 weeks of growth for perennial relatives *Cardamine flexuosa* and *A. alpina* (Zhou et al., 2013; Bergonzi et al., 2013; Park et al., 2017). The age-related responsiveness to cold is controlled by the miR156/SPL and miR172/AP2-like modules, whereby miR156 relates to an extended juvenile phase, and miR172 promote vegetative phase change and flowering (Hyun et al., 2016). Here, I have tested if core perennial species require a longer juvenile phase than their annual relatives, if non-core perennials have longer juvenile phases than core perennials, and whether miR156/miR172 levels can explain differences in developmental transitions and flowering time between growth habits in Pooideae grasses, similar to the case in Brassicaceae.

Annual Pooideae species have shorter juvenile phases than perennial counterparts

In my six annual/perennial species pairs, all annuals, except *B. distachyon* flowered faster than their perennial relatives in all treatments. Annual and perennial *Brachypodium* showed no differences in flowering time neither between species nor treatment periods. These findings agree with previous studies into the same *B. distachyon* line (Bd1-1). Bd1-1 responds to vernalization at the seed stage and flowers ~30 days after vernalization (Ream et al., 2014). Similarly, the average *DTH* for *B. distachyon* in my experiment is 28.4 (long), 28.9 (medium), and 33.5 (short) days when calculated as days after vernalization. Although less is known regarding flowering behaviour in *B. sylvaticum* line Ain-1, it has been shown that Ain-1 flowers after nine weeks when vernalized for two weeks at the seed stage (Steinwand et al., 2013). Taken together, these observations are inconsistent with juvenile phase length explaining growth habit differences in this genus.

As predicted, perennial *H. bulbosum* plants flowered later than those of annual *H. vulgare* within the same treatments. But unexpectedly, *H. vulgare* plants flowered faster with longer juvenile growth periods, whereas the lack of a flowering difference between medium and long treatments for *H. bulbosum* cannot alone distinguish whether the juvenile phase is longer or shorter than eight-twelve weeks. But as argued below, a long juvenile phase likely explains late flowering in *H. bulbosum* versus *H. vulgare* in the context of candidate gene expression levels.

For the remaining pairs, annuals flowered rapidly in all treatments, suggestive that they each have relatively short juvenile phases. In contrast, perennials exclusively failed to produce at least five flowering individuals in all treatments. One interpretation of the latter result is that my focal perennial species did not respond to vernalization because they have a long juvenile phase and were therefore incompetent to respond to these signals. Alternatively, the vernalization period was to short and/or they lacked an additional and required cue for flowering. Given the established roles of vernalization and photoperiod as major regulators of flowering in Pooideae species (Fjellheim et al., 2014; McKeown et al., 2016; Fjellheim & Preston, unpublished), combined with the fact that closely related annual species flowered, the latter seems unlikely. As for the vernalization treatment period being too short, this also seems unlikely since vernalization responsiveness have been characterized in a large set of Pooideae species following the same treatment period (Andersen et al., 2006; Schwartz, et al., 2010; Li et al., 2013; McKeown et al., 2016; Feng, et al., 2017). A study into natural *A. thaliana* populations, representing a variety of climatic conditions in Norway, also found that the vernalization response is more or less saturated after eight weeks of cold (Lewandowska-Sabat et al., 2012). These findings, together with evidence of floral competence in perennial core Pooideae species when juvenile growth exceeded four months (Diehn et al., 2018), suggests that the juvenile phase is longer than three months in the non-flowering perennials.

No difference in juvenile phase length between perennial core and non-core Pooideae

It was previously discovered that the elevated number of origins for annuality in core Pooideae could be explained, at least partially, by a precursor trait that evolved in the last common ancestor of the group (Lindberg et al., 2020). One candidate for this trait is a reduced juvenile phase in core versus non-core perennials. This hypothesis is only supported by a low proportion of flowering individuals (1/4) for *M. nutans* with a short juvenile phase, and my overall results reveal no consistency in phylogenetic relationships and flowering behaviour in response to juvenile phases in core versus non-core perennial species. It is, however, important to note that my experiment includes a small fraction of species relative to Pooideae species in total (*c*. 4000) and that large scale analyses are required to determine if this hypothesis holds up or not.

Antagonistic relationship between miR156 and miR172 in Pooideae

I found that miR156 and miR172 expression in Pooideae is similar to the Brassicaceae, whereby the former decreases, and the latter increases with age. Specifically, miR156 levels were higher in 7 (annual) or 14 (perennial) day old seedlings and progressively decreased with age in all species

except *N. leucotricha,* where levels remained at similar levels regardless of age. For miR172, the opposite was true for all species.

In addition to the antagonistic relationship between miR156/miR172, studies in Brassicaceae have identified two systems for the upregulation of miR172. In *A. thaliana* and *C. flexuosa*, miR156 influence miR172 levels through the activity of *SPL* genes regardless of vernalization (Wu et al., 2009; Zhou et al. 2013). By contrast, the expression profile of miR172 in *A. alpina* is not influenced by the age-related downregulation of miR156, and only upon exposure to cold is an increase seen (Bergonzi et al., 2013). All Pooideae species in my experiment showed upregulation of miR172 before vernalization, even for *M. nutans* with an obligate vernalization response, similar to *A. alpina*. These observations point to a direct link between miR156 and miR172 in Pooideae species, similar to the case in annual *A. thaliana,* short-lived perennial *C. flexuosa* (Zhou et al., 2013), and the annual grasses *Z. mays* (Chuck et al., 2007), *O. sativa* (Xie et al., 2012) and *H. vulgare* (Tripathi et al., 2018). Given the established roles of miR156 and miR172 in developmental transitions in Brassicaceae, it would be interesting to test if a direct link between the two genes can be explained by an age-dependent safety system to enable flowering under less favourable environmental conditions.

Mixed evidence for miR156/miR172 profiles explaining differences in developmental transitions and flowering time between growth habits in Pooideae

In *H. vulgare*, a complete downregulation of miR156 after twelve weeks of growth coincides with an earlier flowering phenotype compared to medium and short treatments. Due to a large spread in fold-values at week four, samples need to be retested. Still, given the pattern seen for miR156 in the long treatment, low levels at week eight probably coincide with earlier flowering in medium versus short treatment periods. Moreover, a delay in flowering for plants with a short versus long treatment correlates with higher miR156 levels after vernalization, further strengthening predictions of a link between miR156 levels and juvenile growth in *H. vulgare.* A similar profile for miR156 downregulation is also observed for *H. bulbosum*, but unlike *H. vulgare*, and in line with the flowering data, no difference is seen in expression levels between treatments. These results neither exclude nor confirm the involvement of *H. bulbosum* miR156 in phase change.

Finally, delayed flowering in *H. bulbosum* versus *H. vulgare* correlates with prolonged miR156 expression for plants given a long growth period. In the medium treatment, only a trend towards higher miR156 levels is seen. Nevertheless, my results point to a relationship for miR156 and juvenile

growth between growth habits as a medium treatment also correlates with delayed flowering in *H. vulgare*. To firmly establish a link between prolonged miR156 expression and an extended juvenile phase between growth habits in *Hordeum*, a relationship between miR156 levels and juvenile growth in *H. bulbosum* needs to be confirmed. It is also worth mentioning that miR172 levels are significantly higher in *H. vulgare* than *H. bulbosum* by the end of both treatments, suggesting that a juvenile phase over 12 weeks explains late flowering of the latter species after vernalization. Taken together, these observations suggest that miR156/miR172 levels can explain differences in developmental transitions between growth habits in *Hordeum*.

Given flowering data for *Brachypodium*, a treatment and species effect on gene expression was not expected. For *B. distachyon*, miR156 is mainly downregulated before vernalization, and as predicted, expression levels are not affected by treatment periods. In *B. sylvaticum*, 1/3 of the plants flowered before- and 1/4 during vernalization with a long treatment, making it difficult to connect flowering behaviour and miR156 levels as well as making comparisons with *B. distachyon*. Results from the long treatment are therefore excluded from further discussion in this section. *B. sylvaticum* plants given a medium treatment had significantly lower miR156 levels after vernalization than those with a short treatment, which cannot be explained by flowering behaviour. *B. sylvaticum* also had higher miR156 and lower miR172 levels than *B. distachyon* after vernalization with a short growth period, that again do not correlate with my flowering data. One explanation for this profile could be a larger contribution of other miR156/miR172 members at earlier developmental stages. It could also be that *B. sylvaticum,* to a larger extent, maintained vegetative growth under a short treatment period, perhaps leading to a shift in the balance in miR156/miR172 between growth habits.

Interestingly, the expression profile for *B. sylvaticum* differs from *B. distachyon* and *Hordeum* in that miR156 is mainly downregulated between sampling points before and after vernalization. Whether this profile results from a downregulation during vernalization or within the week after vernalization prior to sampling is unknown. In any case, my results implicate cold exposure in the reduction of *B. sylvaticum* miR156. An intriguing note in reference to this profile, is that only 50% of control plants flowered, in contrast to 100% for *B. distachyon*, suggesting that diversity between individuals in their vernalization response is coupled with differential downregulation of miR156. Also, in support of miR156 being downregulated during vernalization, is the fact that 1/4 of the plants given a long growth period flowered during the cold treatment. Temperature have been identified as regulating microRNAs in several species, such as *T. aestivum* (Xin et al., 2010), *B. distachyon* (Zhang et al., 2009),

A. thaliana (Zhou et al., 2008; Lee et al., 2010) and *Brassica campestris* (Ahmed et al., 2019), the mechanisms behind however, remains poorly understood. In *B. distachyon* and *A. thaliana* miR156 levels remain unchanged during cold stress (Zhou et al., 2008; Zhang et al., 2009), and in *A. alpina*, cold strongly delays, but do not entirely prevent miR156 reduction under long-term (60 weeks) cold exposure (Bergonzi et al., 2013). However, in *Brassica campestris,* heat stress causes both increasing and decreasing transcript levels in different miR156 genes, providing evidence for opposite temperature responses between miR156 members (Ahmed et al., 2019). As previously mentioned, an upregulation of miR172 during vernalization is involved in reproductive competence of *A. alpina* plants (Bergonzi et al., 2013). Moreover, a recent study into another microRNA, miR396, in perennial Pooideae, *Agrostis stolonifera*, show that overexpression of miR396 bypasses the requirement for vernalization, implicating yet another microRNA in the vernalization-mediated competence to flower (Yuan et al., 2020). In light of these findings, a novel mechanism by which floral competence is achieved through miR156 reduction during cold exposure seems plausible.

Non-core perennials *M. nutans* and *N. leucotricha* showed opposite flowering patterns in response to juvenile growth periods, whereby miR156/miR172 levels can explain flowering behaviour in *M. nutans*, but not in *N. leucotricha. M. nutans* plants flowered marginally faster with a long treatment versus a medium treatment period, whereas medium and long treatments resulted in significantly faster flowering individuals than for those in the short treatment group. These findings align well with miR156/miR172 expression levels. By the end of the long treatment, miR156 is completely downregulated and is at its lowest levels after a medium growth period. For plants with a short treatment, only 25% (versus 100% in the long treatment) of individuals flowered, and these with great delay in average *DTHc* compared to a long and medium juvenile phase (64.5, sd = 2.6 and 68, sd = 2.3 versus 106.3, sd = 12.1). Plants in the short treatment group also had significantly higher levels of miR156 than those given a long- and medium-treatment, both before and after vernalization. These findings suggest that *M. nutans* miR156 is involved in an age-dependent sensitivity to vernalization similar to the case in perennial Brassicaceae species *A. alpina* (Bergonzi et al., 2013) and *C. flexuosa* (Zhou et al., 2013). Finally, miR172 levels are significantly higher in plants with a long versus medium and short treatments, whereas no difference is seen between medium and short treatments, also suggesting that miR156 predominantly controls reproductive competence in *M. nutans*.

N. leucotricha is the only species in my experiment where an age-dependent decrease in miR156 levels is not seen. By contrast, miR172 levels are higher after vernalization for all treatments. Observations

from *A. thaliana* show that floral integrator, FLOWERING LOCUS T (FT), bypasses the requirement for SPL15 in flowering after vernalization under long-day conditions (Hyun et al., 2019). Whether a similar case is true for *N. leucotricha* or if unknown mechanisms are involved is not known. Still, given the conserved role of miR156 in distantly related species, the latter seems unlikely. Another possibility is that I have targeted a miR156 gene with a minor contribution in the transition from juvenile to adult. For example, *A. thaliana* has eight miR156 genes (a-h), whereby *MiR156a* and *MiR156c* mainly contribute to the pool of mature miR156 transcripts at the seedling stage (He et al., 2018). In support of this hypothesis are low loss rates for miR156 after gene duplication events (Zhang et al., 2015), likely leading to an increase in functional diversity (Maher et al., 2006), which is supported by findings of miR156 members being differentially expressed over developmental time in different organs and tissues (Jeong et al., 2011; Xu et al., 2018; Liu et al., 2020).

Concluding remarks

Studies into the miR156/SPL and miR172/AP2-like modules in three Brassicaceae species, *A. thaliana, C. flexuosa* and *A. alpina,* have identified diverse pathways involving the same genes/orthologs by which plants achieve reproductive competence. Here I show that the juvenile phase is extended in perennial versus annual Pooideae species, and that juvenile development and flowering behaviour in several of the study species, relates to the same inverse patterns for miR156/miR172, as seen in Brassicaceae. I also identified a novel profile for *Brachypodium sylvaticum* miR156 reduction in response to vernalization, as well as a probable link between miR156 and miR172 in all species examined, except *N. leucotricha*, pointing to diverse roles for miR156 and miR172 in determining not only differences between growths habits, but also in determining the perennial strategy. Taken together, my findings strengthen the predictions of juvenile growth as a candidate trait for understanding annual and perennial growth strategies, opens up for diverse roles of miR156/miR172 in explaining the perennial strategy, and provide a basis for further examinations into mechanisms underlying these opposite reproductive strategies in grasses.

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Supplementary information

Table S1. Species-specific treatments to release seeds from dormancy.

	Tribe	Species name	Scarification (Yes/no)	Stratification (days)	Warm treatment, ~30°C (days)
CORE POOIDEAE	Brachypodieae	*Brachypodium distachyon*	No	5 days	1 day
		Brachypodium sylvaticum	No	5 days	1 day
	Bromeae	*Bromus tectorum*	No	5 days	1 day
		Bromus benekenii	No	5 days	1 day
	Triticeae	*Hordeum vulgare*	No	5 days	1 day
		Hordeum bulbosum	No	5 days	1 day
		Hordeum jubatum	No	5 days	1 day
	Poeae	*Briza maxima*	No	5 days	1 day
		Briza media	No	5 days	1 day
		Poa annua	No	5 days	1 day
		Poa supina	No	5 days	1 day
		Larmarckia aurea	No	5 days	1 day
		Dactylis glomerata	No	5 days	1 day
		Lolium perenne	No	5 days	1 day
NON-CORE POOIDEAE	Meliceae	*Melica nutans*	No	5 days	1 day
	Stipeae	*Stipa lagascae*	No	5 days	1 day
		Nassella leucotricha	Yes	-	3 days
	Ampelodesmeae	*Ampelodesmos mauretanicus**	No	-	3 days
	Nardeae	*Nardus stricta*	No	5 days	1 day

* Seeds were not covered by soil/sand as they require light for germination.

Table S2. Primers used for RT-qPCR.

Species name	Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Brachypodium distachyon	*Bdi-MiR156b*	GGTGTCAGAACGGCTGACAGAAG	CAACAGCAGCTGCGCCTATC
Brachypodium sylvaticum	*Bsy-MiR156b*	GGTGTCAGAACGGCTGACAGAAG	CAACAGCAGCTGCGCCTATC
Hordeum vulgare	*Hvu-MiR156b*	GGTGTGAATGGCTGACAGAAGAG	GCAACAGCAGCTGCGTCTATC
Hordeum bulbosum	*Hbu-MiR156b*	GGTGTGAATGGCTGACAGAAGAG	GCAACAGCAGCTGCGTCTATC
Melica nutans	*Mnu-MiR156b*	GGCTAATCAAGGTGACAGAAGAGAG	GCCGCATCAGTTCTTTCATATATGAC
Nassella leucotricha	*Nle-MiR156b*	GCTGGCTGACAGAAGAGAGTGAG	GGTGCATGTGCCTCTTCCTTC
Brachypodium distachyon	*Bdi-MiR172b*	GCAGCACCACCAAGATTCACATC	GTTGCCGATGCAGCATCATCAAG
Brachypodium sylvaticum	*Bsy-MiR172b*	GCAGCACCACCAAGATTCACATC	GTTGCCGATGCAGCATCATCAAG
Hordeum vulgare	*Hvu-MiR172b*	GCAGCACCACCAAGATTCACATC	CCTGCTGATGCAGCATCATCAAG
Hordeum bulbosum	*Hbu-MiR172b*	GCAGCACCACCAAGATTCACATC	CCTGCTGATGCAGCATCATCAAG
Melica nutans	*Mnu-MiR172b*	GCAGGTGCAGCACCATTAAGATTC	GGAACCGATGGATCTCGATGATG
Nassella leucotricha	*Nle-MiR172b*	TGGAAGGAGAGGAAGGAGAA	TCACTTTCCAAGGACCTGATG
General	*Ef1α*	CCTTGCTTGAGGCTCTTGAC	GTTCCAATGCCACCAATCTT
General	*UBIQUITIN5*	AAGGAGTCAACCCTCCACCT	TCACCTTCTTGTGCTTGTGC

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