THE INFLUENCE OF COLD TOLERANCE AND FLOWERING TIME GENES ON PERENNIAL WHEAT HABIT

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Abstract

Wheat (*Triticum aestivum* L.) is either a spring or winter annual, completing its life cycle in one growing season. Perennial plants grow over multiple years, alternating between vegetative and reproductive phases. Perennial wheat would be a valuable innovation in agriculture with economic and environmental advantages. To develop perennial wheat, it is often crossed with a close perennial relative, wheatgrass (*Thinopyrum* spp.). However, these perennial wheat lines are only weakly perennial, generally dying before the second growing season. We hypothesize that perennial habit is dependent on cycling control between the vegetative and reproductive phases, involving altered gene expression and the development of sufficient cold hardiness to successfully overwinter. To test this hypothesis, expression of cold tolerance and flowering genes were examined and quantified in perennial wheat, annual wheat and wheatgrass lines grown under varying photoperiods and temperatures.

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AP1 – APETALA1
CBF12 – C-REPEAT BINDING FACTOR12
CBF14 – C-REPEAT BINDING FACTOR14
CO/CO2 – CONSTANS/CONSTANS2
CONTIG5 - Hexose transporter gene
COR14 – COLD REGULATED14
CSD – Cold short day
ELF1a – ELONGATION FACTOR1a
FD – FLOWERING LOCUS D
FDL2 – FLOWERING LOCUS D-LIKE2
FLC – FLOWERING LOCUS C
FRI – FRIGIDA
FT – FLOWERING LOCUS T
FUL – FRUITFUL
LD – Long day
LFY – LEAFY
Log2 – Logarithm base 2 transformation
NF-Y – NUCLEAR FACTOR-Y
PCR – Polymerase chain reaction
PEP1 – PERPETUAL FLOWERING1
PHYC – PHYTOCHROMEC
PPD1 – PHOTOPERIOD1
PSCR – Post sexual cycle regrowth
qPCR – Quantitative reverse transcription polymerase chain reaction
SAM – Shoot apical meristem
SD – Short day
SOC1 – SUPPRESSOR OF OVER EXPRESSION OF CONSTANS1
SPL15 – SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15
SVP – SHORT VEGETATIVE PHASE
TFL1 – TERMINAL FLOWER1
VRN1 – VERNALIZATION1
VRN2 – VERNALIZATION2
VRN3 – VERNALIZATION3

CHAPTER ONE: FLOWERING AND COLD TOLERANCE IN ANNUAL AND PERENNIAL PLANTS

1.1 Literature Review

1.1.1 Annual and perennial life cycles

Plants go through several developmental transitions throughout their life cycle. Once a seed germinates, it begins the juvenile vegetative phase (Bäurle and Dean 2006). After the juvenile phase, plants transition to an adult vegetative phase at which point they can respond to signals from genes involved early on in the flowering control pathway, allowing them to begin the reproductive phase at the onset of flowering (Bäurle and Dean 2006). Wheat (*Triticum aestivum* L.) is either a spring or a winter type annual. Spring annuals complete their life cycle in one growing season, while winter annuals grow over portions of two seasons (Acquaah 2009). As depicted in Figure 1.1., spring wheat is seeded in the spring and after germination begins to grow vegetatively (Acquaah 2009, Friedman and Rubin 2015). The plant initiates the reproductive phase at flowering and is harvested once senescence has occurred in the fall (Acquaah 2009, Friedman and Rubin 2015). Winter wheat is planted in the fall and the seedling begins vegetative growth (Figure 1.1) (Acquaah 2009). Winter wheat must be able to induce cold tolerance to survive the winter (Fowler 2008). The plant meristems remain dormant over the winter, not resuming vegetative growth until the spring (Rohde and Bhalerao 2007, Acquaah 2009). The reproductive phase is initiated upon flowering and the plant is harvested in the summer, thus completing the winter wheat life cycle (Acquaah 2009). Perennial plants

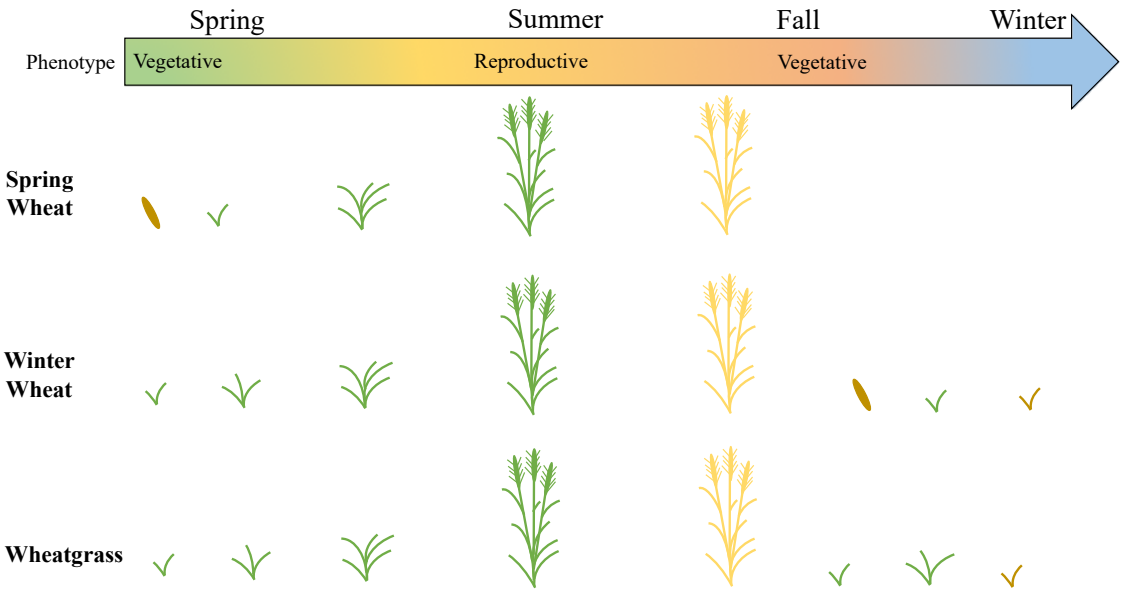


Figure 1.1. Life cycle of annual spring and winter wheats and perennial wheatgrass. Spring wheat is seeded in the spring and harvested in the fall, while winter wheat is seeded in the fall, remains dormant throughout the winter, and resumes growth in the spring. Perennial wheatgrass can grow over multiple years as it alternates between vegetative and reproductive phases.

such as wheatgrass (*Thinopyrum* spp.), a close relative of wheat, are able to grow over multiple years (Acquaah 2009). Annual plants are monocarps, so their shoot apical meristems (SAMs) all become reproductive at the same time, while perennials are polycarps with some of their SAMs remaining vegetative, while others become reproductive (Wang et al. 2009). In the fall, perennials transition to the vegetative phase and become cold tolerant to survive the winter, resume vegetative growth in the spring and begin the reproductive phase at flowering (Figure 1.1) (Moore et al. 1991, Wang et al. 2009, Friedman and Rubin 2015). For most the crops, the plants are harvested after senescence (Friedman and Rubin 2015). By responding to temperature and daylength cues in the fall, perennials are able to transition back to the vegetative state at which point they undergo cold acclimation to prepare for the winter (Thomashow 2001, Andrés and Coupland 2012, Friedman and Rubin 2015). Wheatgrass frequently has some vegetative post-harvest regrowth in the fall, while these leaves often die during the winter, the plant meristems remain dormant until the spring (Rohde and Bhalerao 2007, Murphy et al. 2010). In the spring, the plant resumes growth and is able to repeat its cycle (Friedman and Rubin 2015). To be considered a perennial in temperate climates, a plant must be able to regulate its cycling between vegetative and reproductive phases and be able to sufficiently cold acclimate to its environmental conditions to survive the winter (Andrés and Coupland 2012, Friedman and Rubin 2015, Hayes et al. 2018).

1.1.2 Annual vs. perennial wheat

Annual wheat is Canada's largest crop, and an industry that is worth over 6 billion dollars in 2018 (AAFC 2019). In 2018 over 31 million metric tons of wheat was produced

across Canada, with 24.4 million acres of wheat being harvested (Statistics Canada 2018a, Statistics Canada 2018b). The largest number of harvested acres were in Saskatchewan with 12.2 million acres, Alberta with 7.4 million acres and Manitoba with 2.9 million acres (Statistics Canada 2018c). Canada also ranks high on a global scale and in 2017 came in 7th for world wheat production behind China, India, Russia, USA, France and Australia (FAO 2017). Over 23.5 million tons of spring wheat, 2.5 million tons of winter wheat, and 5.7 million tons of durum wheat were produced in Canada in 2018 (AAFC 2019).

Despite wheat production being an important industry, annual crops have economic and environmental inefficiencies. Planting of annual crops can be expensive as farmers have to pay yearly for the seed, fertilizer, fuel, labour costs and equipment maintenance (Bell et al. 2008, Pimentel et al. 2012). Because perennial crops would have reduced input costs from seeding once every three or four years, the development of perennial crops have the potential to be an environmentally and economically sustainable alternative to annual crops (Glover et al. 2010). According to Cassman et al. (2002), 18-49% of nitrogen fertilizer is utilized by annuals crops, with the rest being lost by runoff and leaching to groundwater. Based on a study by Jungers et al. (2019), perennial crops fertilized with a high nitrogen fertilizer treatment of 160 kg/ha, experienced nitrogen-nitrate leaching that was 89-99% lower than in maize, suggesting a decreased need for fertilizer application of perennials. The decreased need for fertilizer is due to continuous ground cover that could reduce nitrogen loss due to runoff (Glover et al. 2010, Pimentel et al. 2012). As well, continuous ground cover out-competes weeds, reducing need for herbicides (Glover et al. 2010, Pimentel et al. 2012). Tilling of annuals in crop rotation

systems can cause soil erosion, decreasing the amount of water and nutrients retained in soil, while deep root systems and continuous groundcover of perennials could reduce erosion, prevent water loss, and increase organic matter in the soil (Cox et al. 2006, Cox et al. 2010, Glover et al. 2010, Pimentel et al. 2012). According to a study by Gantzer et al. (1990) that collected data for 100 years, annual crops had 50 times more soil erosion than perennial crops, indicating perennials are better at preventing loss of top soil. Annual crops can also cause habitat loss and decreased biodiversity, while the continuous groundcover of perennial crops, especially perennial grasses, provide improved habitat for wildlife (Glover et al. 2010, Pimentel et al. 2012). Annual crops store less carbon than perennial crops, while perennials contribute to reduced carbon dioxide in the atmosphere due to their root systems sequestering carbon in the soil (Pimentel et al. 2012). Perennial grains also have the potential to be profitable for mixed farming, by allowing livestock to graze post-harvest (Bell et al. 2008).

1.1.3 Development of perennial wheat

Because of the potential economic and environmental benefits, development of perennial wheat has been attempted since the 1920's (Wagoner and Schaeffer 1990). Two methods have been utilized for developing perennial wheat: domestication and wide hybridization (Cox et al. 2006). Domestication involves selecting a close perennial relative of wheat with desirable traits, such as perennial habit, high yield and synchronized growth (Wagoner and Schaeffer 1990). Intermediate wheatgrass (*Thinopyrum intermedium* L.) is most frequently used for domestication because it has many desirable traits including: perennial growth, synchronized growth, suitable seed

size, and shatter resistance (Wagoner 1990). A domesticated intermediate wheatgrass called Kernza®, developed by The Land Institute USA, is currently available to farmers (Pimentel et al. 2012, DeHaan et al. 2013, DeHaan and Ismail 2017).

Wide hybridization involves crossing annual wheat with *Thinopyrum* spp. (Cox et al. 2006). Wide hybridization to develop perennial wheat was first done in the 1920's by crossing annual wheat with perennial forage grasses or intermediate wheatgrass, producing semi-fertile hybrids (Tsitsin and Lubimova 1959, Wagoner and Schaeffer 1990). Since then, hybridization has been a common approach for developing perennial grain crops (Pimentel et al. 2012). For example, perennial cereal rye was developed by crossing annual rye (*Secale cereal* L.) with perennial mountain rye (*Secale strictum* ssp. *strictum*) (Oram 1996, Frederiksen and Petersen 1998, Acharya et al. 2004). The development of several other perennial crops has been attempted as well, such as perennial barley by crossing annual barley (*Hordeum vulgare* L.) with its perennial relative *Hordeum bulbosum* L., and perennial maize by crossing annual maize (*Zea mays* L.) with perennial *Zea perennis* and *Zea diploperennis* (Wagoner and Schaeffer 1990). However, these crosses did not result in successful hybrids and current research is more focused on perennial wheat and rye (Wagoner and Schaeffer 1990, Hayes et al. 2018).

Currently, hexaploid annual wheat (containing genomes AABBDD, 2n=42) is the most common wheat parent for hybridization. Both spring and winter type cultivars are used for hybridization (Hayes et al. 2018). However, tetraploid durum wheat (*Triticum turgidum* var. *durum*) (containing genomes AABB, 2n=28) is sometimes used as a parent (Hayes et al. 2018). The wheat parent is often crossed with wheatgrass species decaploid

Thinopyrum ponticum (2n=70), containing genomes JJJJJJJ^{s}J^{s}J^{s}J^{s}, diploid *Thinopyrum elongatum* (2n=14), containing genomes E^{e}E^{e}, and hexaploid *Thinopyrum intermedium* (2n=42) , containing genomes JJJ^{s}J^{s}SS to produce perennial wheat lines (Fedak and Han 2005, Hayes et al. 2012, Gazza et al. 2016).

1.1.4 Challenges with current perennial wheat lines

A number of studies have been conducted on current perennial wheat lines, and several problems have been identified: plants are meiotically unstable, have low yield, are unable to survive the winter in temperate climates, and do not seem to revert back to the vegetative state, which all indicate that they are weak perennials (Bell et al. 2008, Hayes et al. 2012, Hayes et al. 2018). Most perennial wheat lines are partial amphiploids, containing a full set of chromosomes from hexaploid wheat (2n=42) and a partial set of chromosomes from the wheatgrass parent (Fedak and Han 2005). Perennial wheat lines generally have a chromosome number of 2n=56, which helps to contribute to meiotic stability, indicating that 14 chromosomes have to be donated from the wheatgrass parent, either decaploid *Thinopyrum ponticum* (2n=70), diploid *Thinopyrum elongatum* (2n=14) or hexaploid *Thinopyrum intermedium* (2n=42) (Gazza et al. 2016). In the case of the donor parent being *Thinopyrum elongatum*, a full amphiploid is produced (Hayes et al. 2012). When tetraploid durum wheat is the parent (2n=28), generally 28 chromosomes are donated from wheat, with 14 (*Th. elongatum*) or 28 chromosomes (*Th. ponticum* and *Th. intermedium*) donated from the wheatgrass parent, for a total chromosome number of 42 or 56 chromosomes respectively (Hayes et al. 2018). In the case of durum wheat

crossed with *Thinopyrum elongatum* (2n=14), a full amphiploid is produced (2n=42) (Hayes et al. 2018).

Although most perennial wheat lines with a hexaploid wheat parent have 56 chromosomes, some crosses do not produce 56 chromosomes and even have chromosome variation within the same line (Fedak and Han 2005, Hayes et al. 2012). Crosses that result in 56 chromosomes have been shown to produce fertile seeds, have robust growth, and have potential for post sexual cycle regrowth (PSCR) in the fall (Fedak and Han 2005, Hayes et al. 2012, Hayes et al. 2018). Crosses with tetraploid durum wheat, resulting in 42 chromosomes, also seemed to grow well in milder climates and have a relatively high yield compared to other perennial wheat lines (Hayes et al. 2018). It is suggested that the best performing plants in terms of fertility, yield, and PSCR, contain a full set of hexaploid (AABBDD) or tetraploid (AABB) chromosomes from wheat and a one whole genome donated from wheatgrass (Hayes et al. 2018).

Perennial wheat lines have been shown to have low yields, which likely occurs due to chromosome instability, but other factors such as the plant allocating resources towards regrowth and winter survival may also have an impact (Jaikumar et al. 2012, Hayes et al. 2018). In a study by Jaikumar et al. (2012), perennial wheat lines had an average yield that was 48% of annual wheat. Similar studies show that perennial wheat lines yield poorly and have small grain size (Hayes et al. 2012, Hayes et al. 2018). Yield is at its highest during the plant's first year, but decreases in subsequent years (Hayes et al. 2012, Hayes et al. 2018). An Australian study discovered that on profitable land with good soil conditions, perennial wheat would need to yield 85-135% higher than annual

wheat in order to be as profitable (Bell et al. 2008). Although perennial wheat yields would need to be much higher, perennial grains often have a premium associated with their price, that could allow them to potentially have a lower yield than annual wheat (Ryan et al. 2018). However, current yields suggest that current perennial wheat lines do not have a high enough yield for farmers to choose perennials over more profitable annual wheat (Bell et al. 2008).

In order for a plant to be considered a perennial it must be able to survive the winter and alternate between the vegetative and reproductive phases throughout its life by responding to environmental cues synchronized with seasonal changes, such as temperature and photoperiod (Bäurle and Dean 2006, Acquaah 2009). However, the most critical problems with current perennial wheat lines is that they have poor winter survival and persistence, which may be due to the plant failing to induce cold tolerance post reproduction, or the plant having insufficient flowering control, thus remaining fixed in the reproductive phase and not responding to temperature or daylength (Hayes et al. 2012, Gazza et al. 2016, Hayes et al. 2018). Winter survival and persistence is influenced by both the environment, including climate and agronomic practices, and the genetics of the plant (Murphy et al. 2010, Hayes et al. 2018). In general, lines that have spring wheat as one of the parents had poor winter survival (Murphy et al. 2010). A study by Hayes et al. (2018) revealed that when perennial wheat lines were grown across nine countries, plants were generally better adapted to countries with warmer climates, and had higher winter survival, larger grain size and higher yield. *Thinopyrum intermedium* crosses were better at surviving in colder climates, while *Thinopyrum ponticum* and *Thinopyrum elongatum* crosses were suitable for warmer climates (Hayes et al. 2018). Regardless of the wheat

parent, either hexaploid or tetraploid, perennial wheat lines seemed to survive better in warmer climates, although some crosses with tetraploid durum had somewhat better winter survival and longevity (Hayes et al. 2018). Despite this, plants still had poor winter survival rates and Hayes et al. (2018) reported that no plants lived longer than 3 years. Similar studies also showed low winter survival of perennial wheat lines, with most plants dying during the first winter (Murphy et al. 2010, Hayes et al. 2012).

Besides poor survival, perennial wheat lines were observed to perpetually flower in the field during their first year, without reverting back to a vegetative state after harvest (Hayes et al. 2018). Numerous studies have looked at the PSCR of perennial plants, which refers to the regrowth of crown meristems followed by a second cycle of tiller growth after senescence and harvest, indicating that the plant is growing in the vegetative state (Murphy et al. 2010). Hayes et al. (2012) reported that 107/176 (61%) perennial wheat lines displayed some PSCR, while other studies of other lines have also found relatively low values for PSCR (Murphy et al. 2010, Gazza et al. 2016). Plants that remain in the vegetative state during the fall and winter will likely survive and have improved persistence, so perennial lines that are able to grow vegetatively each fall need to be identified (Hayes et al. 2018).

1.1.5 Flowering control

The transition of vegetative to reproductive growth is dependent on flowering control. Some species have several pathways that are involved in flowering including autonomous, gibberellic acid, or environmental signals (Michaels and Amasino 1999).

Two main environmental pathways that are essential to determining flowering time are: vernalization, which is exposure to a cold period that induces a flowering response, and photoperiod, which is a daylength associated flowering response (Shimada et al. 2009). In grasses, these two flowering pathways are often called primary and secondary induction respectively (Heide 1994, Jensen et al. 2005). In order to ensure flowering at the correct time to prevent winter damage of reproductive tissue in temperate climates, plants can detect variations in photoperiod (Greenup et al. 2009). For example, many plants that grow in temperate climates begin to flower as daylength increases, so that flowering occurs in the spring and summer (Greenup et al. 2009). These are called long day (LD) plants, and they will only flower after the daylength is above a certain threshold (Turck et al. 2008, Andrés and Coupland 2012). Some examples of LD plants include most accessions of *Arabidopsis thaliana*, wheat and barley (Greenup et al. 2009). Plants, such as maize and rice (*Oryza sativa* L.), can also be classified as short day (SD), when flowering occurs after the daylength is below a certain threshold, or day neutral plants, like tomato (*Solanum lycopersicum* L.), that have no response to photoperiod (Turck et al. 2008, Andrés and Coupland 2012). The flowering of perennials and winter type cultivars also responds to cold temperature. To reduce risk to reproductive tissues by restricting flowering to spring, plants will only flower after exposure to a sufficiently long cold period (vernalization) (Greenup et al. 2009). The flowering control pathways have been relatively well described in *Arabidopsis* and annual cereals (Cockram et al. 2007). However, the flowering time pathway is not as well described in perennials.

1.1.6 Flowering pathway in Arabidopsis

Most accessions of *Arabidopsis* are LD plants, preferring to flower under a long day photoperiod (Turck et al. 2008). The circadian clock of *Arabidopsis* is light sensitive and can determine if the plant is being exposed to an appropriate photoperiod in order to induce flowering (Imaizumi and Kay 2006). In the photoperiodic flowering pathway of *Arabidopsis, GIGANTEA* (*GI*) is regulated by the circadian clock and binds to *CONSTANS* (*CO*), causing *CO* to have the highest levels of expression 16 h after dawn (Suárez-López et al. 2001) (Figure 1.2). The upregulation of *CO* under LDs causes the upregulation of *FLOWERING LOCUS T* (*FT*), the *Arabidopsis* florigen, a signal that moves from the leaf to the shoot apex, thus initiating flowering (Corbesier et al. 2007, Turck et al. 2008) (Figure 1.2 B). Because the detection of photoperiod occurs in the leaf, *FT* is first upregulated in the leaf, but is transported through the phloem to the SAM, where the floral transition occurs (Corbesier et al. 2007). Once transported to the SAM, *FT* interacts with transcription factor *FLOWERING LOCUS D* (*FD*), forming a heterodimer that upregulates meristem identity genes *APETALA1* (*AP1*), *FRUITFUL* (*FUL*), *LEAFY* (*LFY*), and *SUPPRESSOR OF OVER EXPRESSION OF CONSTANS1* (*SOC1*), to stimulate flowering at the shoot apex (Ferrándiz et al. 2000, Turck et al. 2008, Kaufmann et al. 2010) (Figure 1.2 B). *TERMINAL FLOWER1* (*TFL1*) a shoot identity gene, is expressed in the SAM and is thought to be induced by *CO* (Wang et al. 2011, Wickland and Hanzawa 2015). *TFL1* is a floral repressor and opposes *FT*, preventing a vegetative to reproductive phase change in the SAM (Hanano and Goto 2011) (Figure 1.2 A). *TFL1* forms a complex with *FD* and causes the downregulation of meristem identity

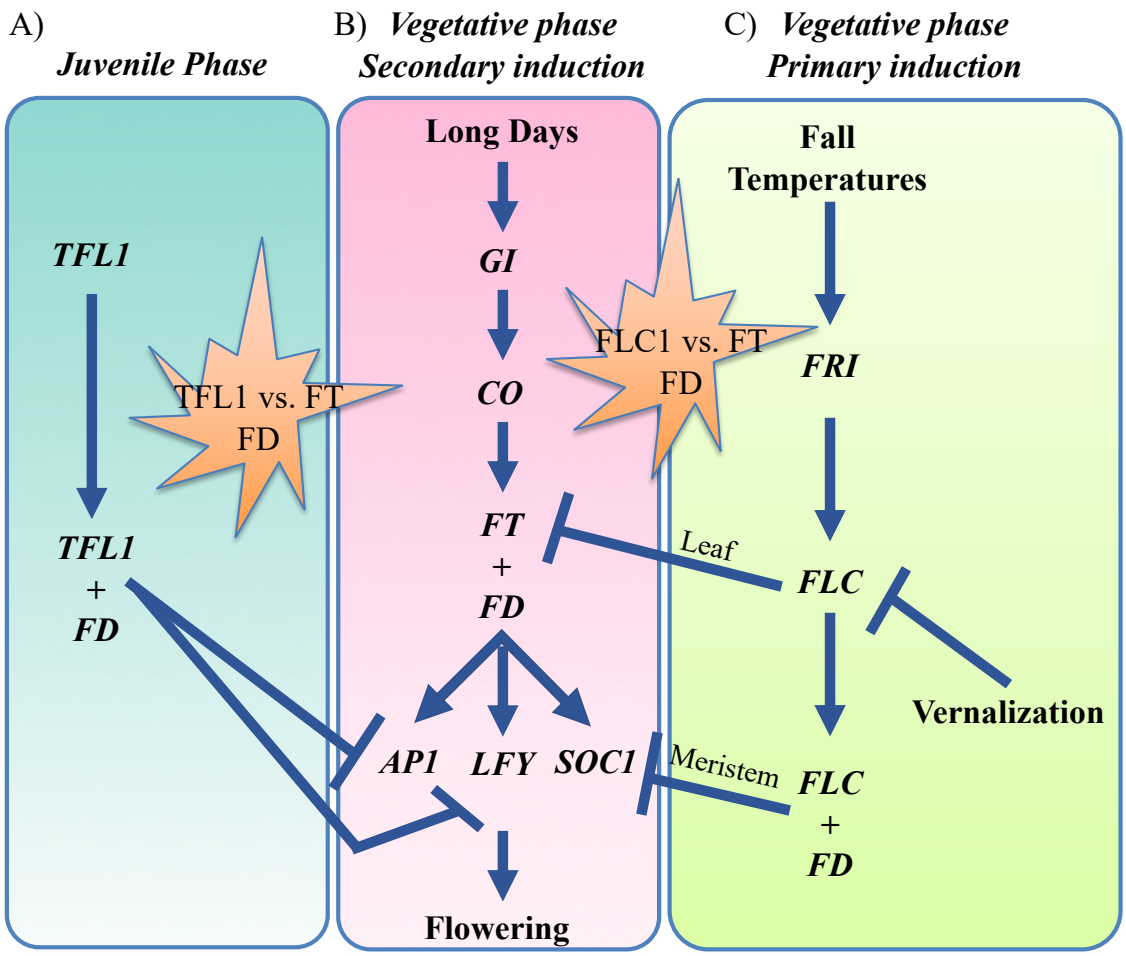


Figure 1.2 Flowering time pathway in *Arabidopsis* for (A) juvenile phase, (B) vegetative phase secondary induction and (C) vegetative phase primary induction. Orange stars represent genes that outcompete one another to form a complex with *FD*. Integrated ideas from Greenup et al. (2009) and Andrés and Coupland (2012).

genes *AP1* and *LFY,* thus preventing flowering (Hanano and Goto 2011, Wickland and Hanzawa 2015) (Figure 1.2 A).

Winter type *Arabidopsis* accessions display a flowering response to vernalization, in order to prevent flowering in the fall, and promote flowering under LDs in the spring. Winter accessions have dominant alleles at two loci, *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*) (Bloomer and Dean 2017). Exposure to fall temperatures causes the upregulation of *FRI*, thus upregulating *FLC*, a floral repressor that codes for a MADS-box (*MCM1, AGAMOUS, DEFICIENS, SRF*) transcription factor (Michaels and Amasino 1999) (Figure 1.2 C). *FLC* can act in both the leaf and the SAM to prevent flowering (Searle et al. 2006). In the leaf, the upregulation of *FLC* causes the downregulation of *FT*, therefore preventing *FT* from signaling to SAM genes downstream in the pathway and delaying flowering (Searle et al. 2006) (Figure 1.2 C). In the SAM, *FLC* binds to *FD* and *SOC1*, thus preventing *FT* from binding to and upregulating these genes (Searle et al. 2006) (Figure 1.2 B, C). When *FLC* is expressed in both the leaf and the SAM, flowering is more repressed than if *FLC* was only expressed in one tissue type (Searle et al. 2006). *SHORT VEGETATIVE PHASE* (*SVP*), is another gene that encodes a MADS-box transcription factor, and also negatively regulates flowering (Li et al. 2008). Unlike *FLC*, *SVP* is not regulated through the vernalization pathway, rather it responds to indicators from the autonomous and gibberellic acid pathways. However, *FLC* and *SVP* are thought to work together to bind on the promoter region of *SOC1* at the SAM, thus preventing floral initiation (Li et al. 2008). After exposure to a vernalization period *FLC* expression is decreased, causing *FT* to be upregulated under LDs, promoting flowering (Michaels and Amasino 1999).

1.1.7 Flowering pathway in annual cereals

The photoperiodic flowering pathway in cereal crops has been shown to have similarities to *Arabidopsis* (Shimada et al. 2009). Wheat and barley are LD plants and most spring type cultivars have a flowering response when exposed to long photoperiods (Turner et al. 2005). Plants have three gene families that code for phytochromes, red and far-red light receptors, which are involved in the LD flowering pathway (Chen et al. 2014). One of these phytochromes, *PHYTOCHROMEC* (*PHYC*), causes the activation of *PHOTOPERIOD1* (*PPD1*), which is expressed in a diurnal rhythm under LDs (Chen et al. 2014). The upregulation of *PPD1*, causes the upregulation of *VERNALIZATION3* (*VRN3*), a gene shown to be orthologous to the *Arabidopsis* florigen *FT*, leading to flowering (Chen et al. 2014) (Figure 1.3 B). Similar to *Arabidopsis*, cereal crops have *GI*-like and *CONSTANS2* (*CO2*), a *CO*-like gene, as well as expression that follows a similar gene pathway, with *GI* and *CO2* being upregulated by *PPD1* and the circadian clock, leading to *VRN3* expression and subsequent flowering (Shimada et al. 2009) (Figure 1.3 B). Barley lines that have a non-functional *PPD1* gene have been shown to have reduced *CO2* expression, downregulated *VRN3*, and weak flowering under LDs (Turner et al. 2005). Mutating *PPD1* in wheat has been shown to alter *PPD1* expression from its usual diurnal rhythm and cause the plant to flower under SDs rather than LDs (Beales et al. 2007). This suggests that *PPD1* expression, mediated by LDs, is essential for the upregulation of *VRN3* and subsequent flowering in cereals (Shimada et al. 2009) (Figure 1.3 B). The *TFL1* gene has also been identified in cereals, and is thought to have a similar function as in *Arabidopsis* (Chardon and Damerval 2005). *TFL1* is expressed in the SAM and is suggested to be a floral repressor that regulates meristem identity genes and

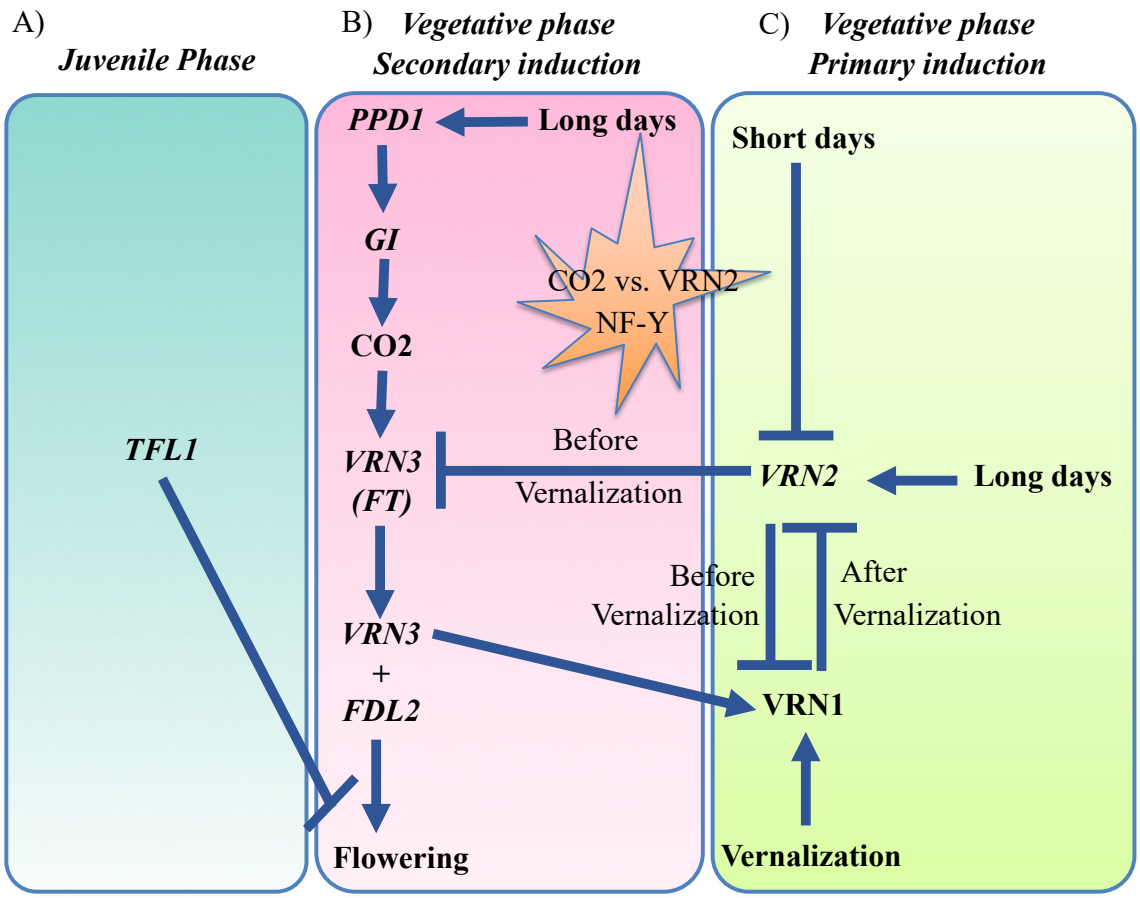


Figure 1.3 Flowering time pathway in annual cereal crops for (A) juvenile phase, (B) vegetative phase secondary induction and (C) vegetative phase primary induction. The orange star represents genes that outcompete one another to form a complex with NF-Y proteins. Integrated ideas from Cockram et al. (2007), Greenup et al. (2009) and Andrés and Coupland (2012).

maintains vegetative growth, although it is not as well understood as *TFL1* in *Arabidopsis* (Jensen et al. 2001) (Figure 1.3 A).

Similar to *Arabidopsis*, winter cereals also have a vernalization flowering response that promotes flowering after being exposed to an extended cold period. A gene with a similar sequence to the meristem identity gene *AP1* in *Arabidopsis*, called *VERNALIZATION1* (*VRN1*) was identified in wheat and barley (Yan et al. 2003). Like *AP1*, *VRN1* codes for a MADS-box transcription factor and is responsible for the transition between vegetative and reproductive growth (Yan et al. 2003, Distelfeld et al. 2009a). Exposure to a cold period causes *VRN1* to be upregulated in cereal leaves and SAMs (Yan et al. 2003). The upregulation of *VRN1* causes the upregulation of *VRN3*, leading to flowering (Yan et al. 2006) (Figure 1.3 C). A floral repressor encoded by a zinc-finger-CCT transcription factor called *VERNALIZATION2* (*VRN2*), was also identified in wheat and barley (Yan et al. 2004). Although *Arabidopsis* floral repressor *FLC* is part of a different family of transcription factors than *VRN2*, both act to repress *FT/VRN3* (Yan et al. 2004). *VRN2* is upregulated prior to vernalization, thus causing *VRN1* and *VRN3* to be downregulated, preventing flowering (Yan et al. 2004) (Figure 1.3 C). Vernalization suppresses *VRN2*, causing *VRN1* expression to increase and upregulate *VRN3* triggering the plant to flower (Shimada et al. 2009) (Figure 1.3 C). For some winter cereal cultivars, vernalization can be replaced by exposure to SDs (Dubcovsky et al. 2006). In this case, *VRN2* is downregulated when exposed to SDs (Dubcovsky et al. 2006). Because *VRN2* is downregulated by exposure to cold in the vernalization pathway and by SDs, it links the vernalization and photoperiod pathways (Dubcovsky et al. 2006). Under LDs, facilitated by *CO2* and *PPD1*, *VRN3* is also able to upregulate *VRN1* (Yan et

al. 2006, Distelfeld et al. 2009a) (Figure 1.3 B). *VRN3* increases in the leaf under LDs and is transported to the SAM where *VRN3* forms a complex with *FLOWERING LOCUS D-LIKE2* (*FDL2*), a transcription factor similar to *FD* in *Arabidopsis* (Li and Dubcovsky 2008). The *VRN3/FDL2* complex binds to the *VRN1* promoter, causing it to become upregulated (Li and Dubcovsky 2008) (Figure 1.3 B). The expression of *VRN3* and *VRN1* under LDs causes the downregulation of *VRN2,* thus promoting flowering (Yan et al. 2006). Transcription factor *NUCLEAR FACTOR-Y* (*NF-Y*) also connects the photoperiod and vernalization pathway by interacting with *VRN2* and *CO2* (Li et al. 2011). *VRN2* and *CO2* compete to interact with NF-Y proteins. If a *VRN2*/NF-Y complex is formed, *VRN2* expression increases, thus preventing flowering (Li et al. 2011) (Figure 1.3 C). Under LDs *CO2* is upregulated, leading to the formation of a *CO2*/NF-Y complex, upregulating *VRN3* and leading to flowering (Li et al. 2011) (Figure 1.3 B).

1.1.8 Flowering pathway in perennials

Although the flowering time pathway has been relatively well described in annual cereals, it has yet to be characterized in perennials (Cockram et al. 2007). However, some research into these pathways has been conducted on the perennial forage crop timothy (*Phleum pretense* L.) (Seppänen et al. 2010, Jokela et al. 2015), on perennial ryegrass (*Lolium perenne* L.) (Jensen et al. 2001), and on a close perennial relative of *Arabidopsis*, *Arabis alpina* (Wang et al. 2009, Hyun et al. 2019). As in cereal crops, vernalization and photoperiod are important for flowering in perennial grasses (Seppänen et al. 2010). Similar to cereals, it has been shown that perennial grass accessions can have both spring and winter types, therefore spring varieties do not require vernalization (Jokela et al.

2015). Perennials have also displayed different gene regulation than annuals at their SAMs, as perennials are polycarps (Wang et al. 2009).

Many of the flowering time genes are thought to be functionally homologous between annual cereals and perennial grasses, however these genes have not been well studied in grasses. *VRN1* has been identified in timothy and perennial ryegrass and *VRN3* has been identified in timothy (Ciannamea et al. 2006, Seppänen et al. 2010, Jokela et al. 2014). Similarly to cereals, *VRN1* has been shown to be upregulated at the transition from vegetative to reproductive growth and during vernalization (Seppänen et al. 2010) (Figure 1.4 C). As in cereals, *VRN3* in timothy has been proven to connect photoperiod and vernalization, being upregulated by the presence of vernalization and *VRN1*, as well as a long photoperiod (Jokela et al. 2015) (Figure 1.4 B, C). The upregulation of *VRN3* is essential to flowering in timothy, suggesting functional homology to *VRN3* in cereals (Jokela et al. 2015). In contrast, the function of *VRN2* in timothy is unknown, and it is unclear if it functions as a floral repressor as it does in cereals (Seppänen et al. 2010, Jokela et al. 2015) (Figure 1.4 C). In *Brachypodium distachyon* it has been shown that *VRN2* is upregulated during the cold and most cultivars have lost the SD vernalization ability as *Brachypodium* does not respond to SDs during vernalization like most annual cereals (Woods et al. 2019). While in cereals *VRN2* is downregulated in cold temperatures and SDs, suggesting that *VRN2* may function differently in grasses than in cereals (Woods et al. 2019). Most perennial grasses flower with exposure to LDs, however some grasses have variations that allow them to flower under SDs (Woods et al. 2019). It is suggested that *PPD1* may also be analogous to cereals because it is also

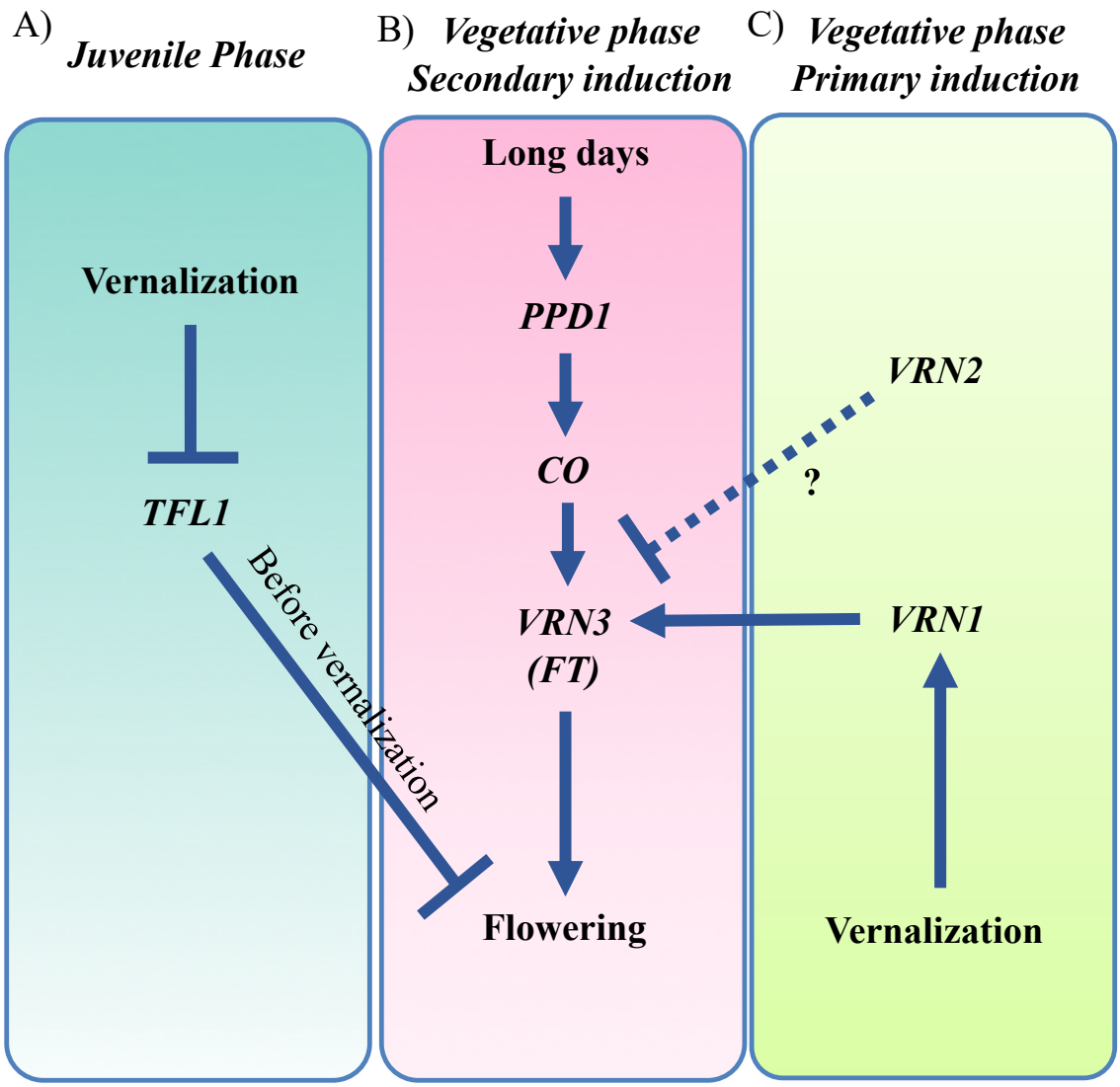


Figure 1.4 Flowering time pathway in perennial grasses for (A) juvenile phase, (B) vegetative phase secondary induction and (C) vegetative phase primary induction.

highly expressed when exposed to long photoperiods (Jokela et al. 2014) (Figure 1.4 B). A *CO*-like gene was discovered in perennial ryegrass, and is functionally homologous to *CO* in *Arabidopsis*, in that it responds LDs, and is expressed in a diurnal rhythm (Martin et al. 2004) (Figure 1.4 B).

Although the function of *VRN2* is unknown in timothy, *PERPETUAL FLOWERING1* (*PEP1*), a gene similar to floral repressor *FLC* and cereal floral repressor *VRN2*, has been studied in *A. alpina* (Wang et al. 2009). While *FLC* represses flowering until the plant is vernalized, *PEP1* stops flowering before and after vernalization to prevent some SAMs from transitioning to the reproductive phase and producing flowers (Wang et al. 2009). This allows some SAMs to remain vegetative in order for *A. alpina* to transition back to the vegetative phase after flowering, thus allowing the plant to maintain polycarpy (Wang et al. 2009). Although *PEP1* in *A. alpina* helps to support a perennial life cycle, it is not completely responsible for it and other factors and genes are likely involved (Wang et al. 2009). A second system for maintaining some SAMs in a vegetative state involves microRNA156 (miR156) (Hyun et al. 2019). The miRNA156 pathway is age dependent in that miR156 appears in young SAMs, keeping them vegetative when exposed to vernalization (Hyun et al. 2019). PEP1 and miR156 systems both affect *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15* (*SPL15*), a floral promoter. *PEP1* binds to *SPL15* DNA, while miR156 targets *SPL15* mRNA (Hyun et al. 2019) *SPL15* expression increases during vernalization in the SAM of 6 week old plants, while exposure to vernalization does not cause *SPL15* to be upregulated in 2 week old plants (Hyun et al. 2019). This suggests that *SPL15* is responsible for flowering in response to age during vernalization, with older

SAMs becoming reproductive and eventually flowering while *PEP1* is repressed (Hyun et al. 2019).

TFL1, identified in perennial ryegrass and in *A. alpina,* is thought to have a similar function to *TFL1* in cereals (Jensen et al. 2001, Wang et al. 2011). *TFL1* in perennial ryegrass is a floral repressor and is expressed in the meristem, suggesting that it is responsible for controlling meristem identity genes (Jensen et al. 2001). *TFL1* is upregulated during vegetative growth, and over the winter *TFL1* expression decreases, causing floral promoters to be upregulated (Jensen et al. 2001) (Figure 1.4 A). This suggests that low *TFL1* expression levels in perennial ryegrass and *A. alpina* are connected to a short juvenile period, indicating that *TFL1* might have a role in the perennial life cycle (Jensen et al. 2001, Wang et al. 2011).

Poplar (*Populus* sp.) has a similar flowering time pathway to *Arabidopsis* and several photoperiodic pathway genes have been discovered (Triozzi et al. 2018). Both *GI* and *CO* like genes have been identified in poplar and were shown to be regulated by the photoperiod (Ding et al. 2018). *FT*-like genes have also been discovered in poplar and in apple (*Malus pumila* L.) and are functionally similar to *FT* in *Arabidopsis* and *VRN3* in annual cereals and perennial grasses (Tränkner et al. 2010, Ding et al. 2018). In apple the overexpression of *FT* induced flowering, suggesting that it is the floral promoter (Tränkner et al. 2010). When *FT* is upregulated in the spring in poplar it leads to bud formation and reproductive growth, while the downregulation of *FT* during the fall causes reproductive growth to stop (Ding et al. 2018). In *Arabidopsis* and cereals, the upregulation of *GI* causes the upregulation of *CO*, leading to the upregulation of

FT/VRN3 and eventual flowering (Ding et al. 2018). However, in poplar *CO* does not seem to have as important of a role in the upregulation of *FT*, and *GI* likely regulates *FT* in a pathway that does not include *CO* (Ding et al. 2018, Triozzi et al. 2018). Exposure to vernalization to induce flowering has not been shown in trees, however trees in temperate climates do need to be exposed to chilling in order to release winter dormancy (Brunner and Nilsson 2004). It is thought that there may be floral repressors acting in the winter during the dormancy period, however genes similar to *FLC* or *VRN2* have not been discovered in poplar (Brunner and Nilsson 2004).

1.1.9 Cold tolerance

In winter type cultivars and perennials cold tolerance is essential for the plant to survive the winter. If a plant is not cold tolerant, freezing can occur where ice is formed in the extracellular area between the xylem and phloem (Rihan et al. 2017). The ice will expand to other extracellular spaces and will draw water away from surrounding cells, causing dehydration and potential membrane damage (Preston and Sandve 2013, Rihan et al. 2017). In order to prevent this, winter cultivars and perennials undergo cold acclimation, and increase low temperature tolerance, which occurs when a plant is exposed to low but non-freezing temperatures (Thomashow 2001). A plant's ability to cold acclimate greatly improves its cold tolerance and ability to prevent freezing damage and survive the winter (Thomashow 2010). Cold acclimation occurs quickly in *Arabidopsis*, within days of cold exposure, while cold exposure in wheat is relatively quick but is a more cumulative process that can take place over several weeks based on threshold induction temperatures (Sung and Amasino 2004a, Fowler 2008). This quick

cold acclimation is essential for the plant to protect itself from freezing damage, especially in the event of an unexpected onset of cold temperatures (Sung and Amasino 2004a). The cold tolerance pathway has been extensively studied in *Arabidopsis*, is relatively known in cereals, but is not well understood perennial grasses.

1.1.10 Cold tolerance in Arabidopsis

Winter accessions of *Arabidopsis*, which have the ability to cold acclimate to prevent freezing, have been widely studied for cold tolerance (Thomashow 2010). Calcium is proposed to start a signaling cascade when a plant is exposed to stress, such as cold (Knight et al. 1991). As low temperatures increase, Ca^{2+} levels in the plant increase, leading to the formation of a calcium-calmodulin complex that binds to the promoter region of *CBF/DREB1* (*C-REPEAT BINDING FACTOR/DEYDRATION-RESPONSIVE ELEMENT-BINDING1*) genes (Thomashow 2001). *CBF*s encode transcription factors belonging to the AP2/EREBP (APETALA2 and ethylene-responsive element binding proteins) family of plant transcription factors, which have been shown to be involved in cold tolerance. Three *CBF* genes have been identified in *Arabidopsis*, *CBF1*, *CBF2* and *CBF3*, also known as *DREB1b, DREB1c* and *DREB1a* (Stockinger et al. 1997, Gilmour et al. 1998, Liu et al. 1998). *CBF* transcripts rapidly increase when *Arabidopsis* is exposed to cold temperatures, and *CBF* levels have been shown to increase after just 15 minutes of exposure to cold (Gilmour et al. 1998). After the upregulation of *CBF* genes by cold temperatures, *COR* (*COLD REGULATED*) genes become upregulated within a few hours (Gilmour et al. 1998). *CBFs* bind to the CRT/DRE (C-repeat/dehydration responsive element) located in the regulatory regions of *COR* genes in order to induce their

expression, leading to cold tolerance (Gilmour et al. 1998, Steponkus et al. 1998). *ICE1* (*INDUCER OF CBF EXPRESSION1*), has also been shown to upregulate *CBF* genes (Chinnusamy et al. 2003). *ICE1* is upregulated when plants are exposed to cold temperatures and codes for a transcription factor that binds to the promoter region of *CBF3,* causing it to become upregulated, leading to the upregulation of *COR* genes (Chinnusamy et al. 2003) (Figure 1.5 A). It is suggested that *ICE1* expression is facilitated by *SIZ1* and *HOS1* (*HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1*). *HOS1* is expressed in warm temperatures and represses *ICE1* expression, but is degraded upon exposure to cold temperatures, while SIZ1 mediates the upregulation of *ICE1* under cold temperatures, causing the upregulation of *CBF*s, followed by the expression of *COR* genes leading to cold tolerance (Chinnusamy et al. 2007) (Figure 1.5 A).

Photoperiod also influences the cold acclimation pathway of *Arabidopsis. CBF* genes have very low expression levels on warm days, but expression has been shown to be influenced by the circadian clock, with the highest level of *CBF* transcripts occuring 8 hours after dawn (Lee and Thomashow 2012). Under LD conditions *PHYB* along with *PIF4* and *PIF7*, two transcription factors involved with the circadian clock, cause *CBF* genes to be downregulated (Thomashow 2010) (Figure 1.5 B). SDs cause *PHYB*, *PIF4* and *PIF7* to be downregulated, leading to the upregulation of *CBF* and *COR* genes (Lee and Thomashow 2012) (Figure 1.5 B). It was also shown that *Arabidopsis* plants exposed to SDs had 3-5 times more expression of *CBF* genes at 8 h after dawn than plants grown under LDs (Lee and Thomashow 2012). This suggests that most winter type *Arabidopsis* are able to cold acclimate more efficiently under SDs than LDs

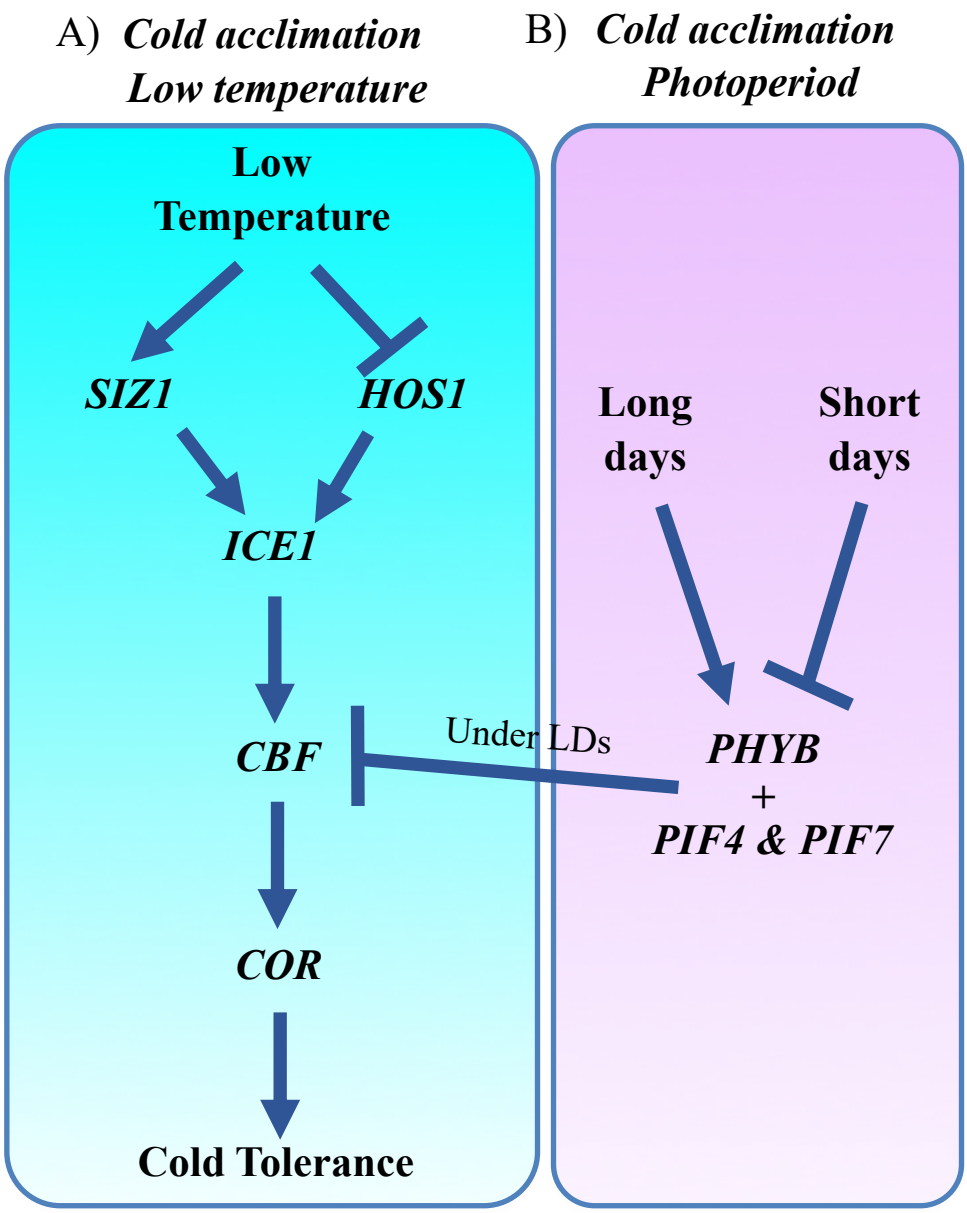


Figure 1.5 Cold tolerance pathway in *Arabidopsis* for (A) low temperature and (B) photoperiodic cold acclimation. Integrated ideas from Thomashow (2001) and Preston and Sandve (2013).

(Lee and Thomashow 2012). This may have evolved so that under SDs (during the fall) the plant can prepare for the upcoming winter, and in LDs (during the summer) the plant does not have to waste energy and nutrients to cold acclimate when it is not necessary (Lee and Thomashow 2012).

In *Arabidopsis* there is a connection between flowering time and cold tolerance. Flowering time can be influenced by surrounding temperature. Flowering occurs under warm temperatures but is repressed by cold temperatures (Seo et al. 2009). *SVP*, a floral repressor, is able to sense cool temperatures and along with *FLC*, binds to *SOC1* in order to prevent the upregulation of *FT*, the floral promoter (Li et al. 2008). During cool temperatures and vegetative growth, *CBF* genes become upregulated causing *SOC1,* a meristem identity gene, and *FT* to become downregulated and prevent flowering (Seo et al. 2009) (Figure 1.6 B, D). However, in warm temperatures *SOC1* is upregulated, inhibiting the expression of *CBF* genes, causing the plant to lose its cold tolerance and induce flowering (Seo et al. 2009) (Figure 1.6 B, D). This suggests that cold tolerance and flowering time genes are connected (Seo et al. 2009). While transient cold temperatures, like in the fall, prevent flowering from occurring over the winter, vernalization speeds up the flowering process (Sung and Amasino 2004a). Like cold acclimation, vernalization also causes the upregulation of *CBF* and *COR* genes (Seo et al. 2009). However, vernalization causes the downregulation of *FLC*, but the plant must be exposed to at least 30-40 days of cool temperatures for this to occur (Sung and Amasino 2004b) (Figure 1.6 C). *VERNALIZATION INSENSITIVE3* (*VIN3*) is upregulated by exposure to a long cold period (Sung and Amasino 2004b). The upregulation of *VIN3* causes the downregulation of *FLC*, therefore promoting flowering (Sung and Amasino 2004b).

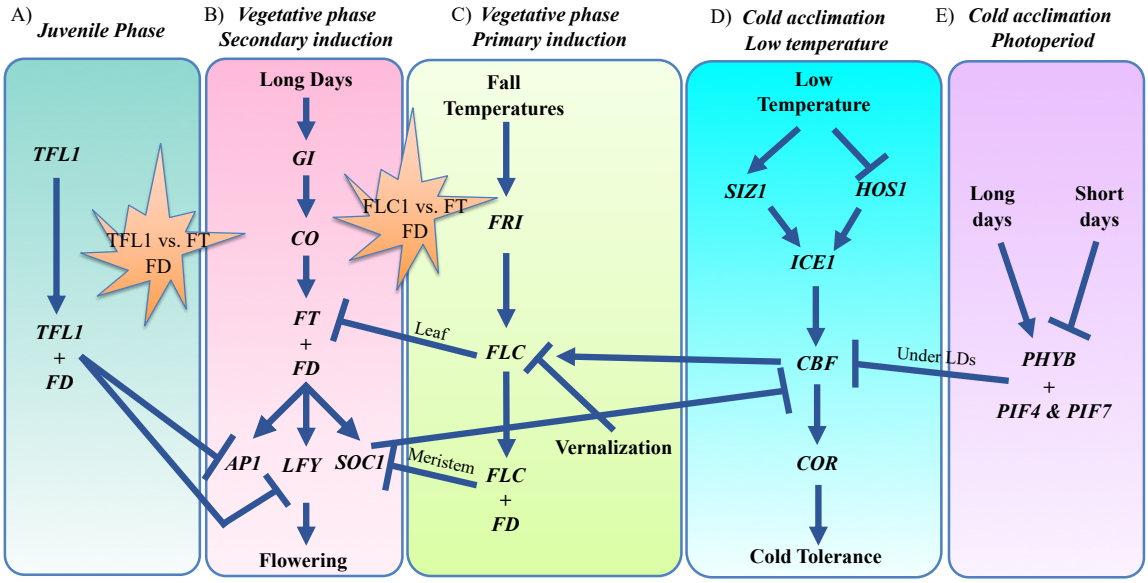


Figure 1.6 Connection between flowering time and cold tolerance in *Arabidopsis* for (A) juvenile phase, (B) vegetative phase secondary induction, (C) vegetative phase primary induction, (D) low temperature cold acclimation and (E) photoperiodic cold acclimation. Orange stars represent genes that outcompete one another to form a complex with *FD*. Integrated ideas from Thomashow (2001), Greenup et al. (2009), Andrés and Coupland (2012) and Preston and Sandve (2013).

1.1.11 Cold tolerance in annual cereals

In winter cereal crops, cold tolerance is essential to survive the winter. According to Fowler (2008) the most cold tolerant cereal crop is rye followed by wheat and barley. Rye likely is more cold tolerant because its threshold induction temperature, the temperature at which cold tolerance is induced, is higher than wheat and barley's (Fowler 2008). Rye's induction temperature is about 18°C, while wheat and barley is around 10°C (Fowler 2008). Unlike *Arabidopsis,* which only has three *CBF* genes, a large number of *CBF*s have been discovered in cereal crops (Campoli et al. 2009). In wheat, 37 *CBF* genes have been found, while 20 have been identified in barley (Skinner et al. 2006, Campoli et al. 2009). Although cereal crops have more *CBF* genes than *Arabidopsis*, they belong to the same family of transcription factors and are functionally similar to *Arabidopsis CBF*s in that they are rapidly upregulated when exposed to cold temperatures and induce *COR* genes (Figure 1.7) (Miller et al. 2006, Campoli et al. 2009). An *ICE1*-like gene has been discovered in wheat and barley and is thought to have a similar function as *ICE1* in *Arabidopsis* as *ICE1* in cereals has been shown to cause the upregulation of *CBF* genes, followed by *COR* genes leading to cold tolerance (Figure 1.7) (Skinner et al. 2006, Badawi et al. 2008). A subset of important cold tolerance genes including *COR14, CBF12* and *CBF14* have been identified in wheat and barley (Rapacz et al. 2008, Galiba et al. 2009, Novák et al. 2015, Erath et al. 2017). *COR14*, which is upregulated by *CBF* genes and low temperatures, encodes a chloroplast-targeted protein that protects the plant from photodamage due to light exposure after freezing (Rapacz et al. 2008, Galiba et al. 2009). *CBF12* and *CBF14* transcription factors are more sensitive

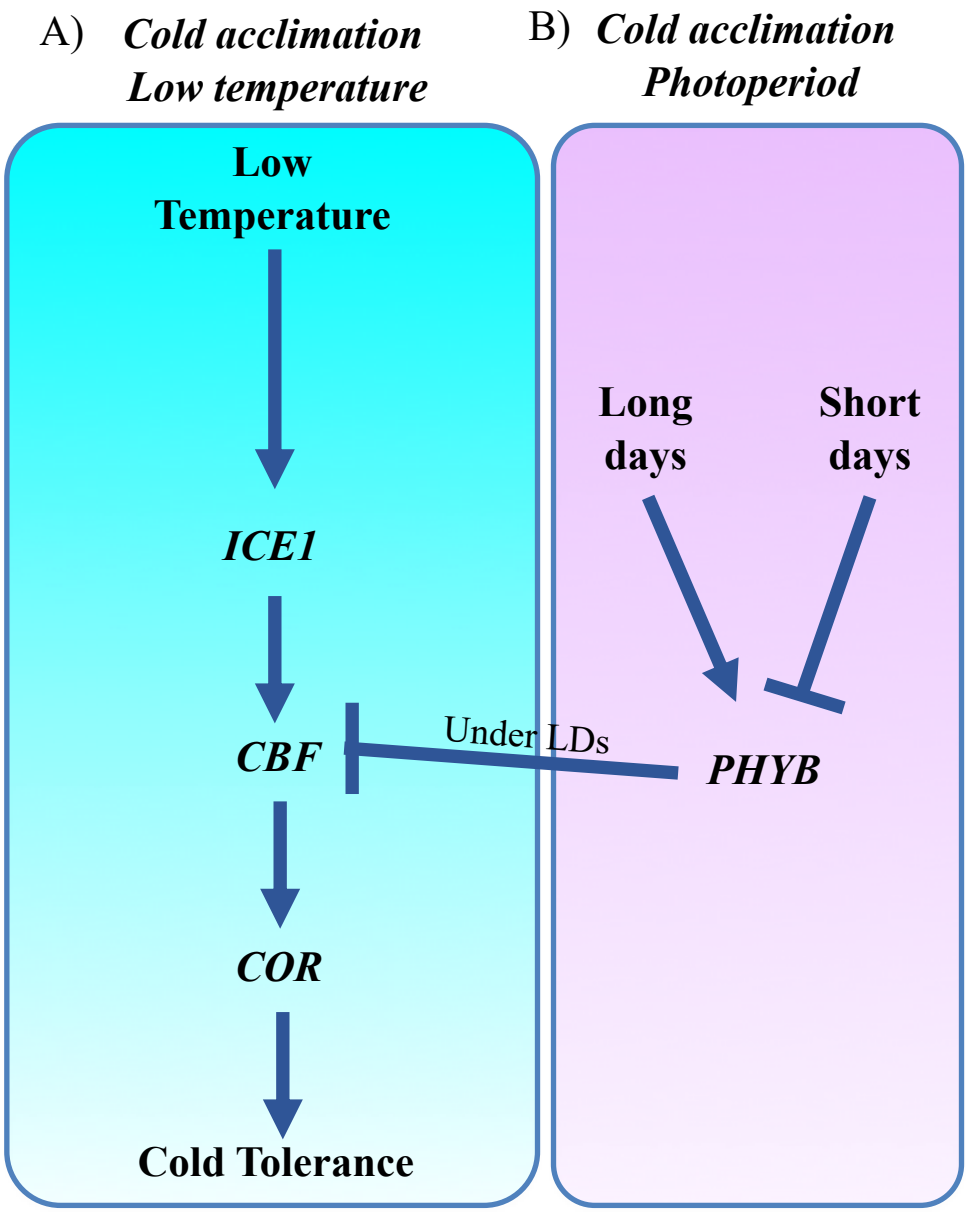


Figure 1.7 Cold tolerance pathway in annual cereal crops for (A) low temperature and (B) photoperiodic cold acclimation. Integrated ideas from Preston and Sandve (2013).

to threshold induction temperatures than other *CBF*s, becoming upregulated around 15°C in wheat and barley (Campoli et al. 2009, Novák et al. 2015).

Similar to *Arabidopsis*, the relationship between *CBF* and *COR* genes with vernalization could indicate a connection between cold tolerance and flowering time in cereals (Galiba et al. 2009). Alonso-Peral et al. (2011) showed that *VRN1* has binding sites in its promoter for *CBF* and *ICE* genes, suggesting that *VRN1* regulates their transcription. During the fall when the plant is in a vegetative stage, *VRN1* is downregulated by *VRN2*, while *CBF* and *COR* genes become upregulated when exposed to cool temperatures (Dhillon et al. 2010) (Figure 1.8 C, D). Cereals are only able to cold acclimate during the vegetative state, so a transition to the reproductive phase decreases the capability of cereals to cold acclimate (Limin and Fowler 2006). When the vernalization requirement is fulfilled in late winter or early spring *VRN1* expression increases and *CBF* and *COR* genes become downregulated, causing the plant to lose its cold tolerance (Dhillon et al. 2010) (Figure 1.8 C, D). This transition to the reproductive phase occurs at the same time that *VRN1* is being upregulated, providing further evidence that *VRN1* negatively regulates cold tolerance genes (Limin and Fowler 2006, Dhillon et al. 2010).

Photoperiod and temperature act together to influence the developmental stage and cold acclimation pathway in annual cereals (Mahfoozi et al. 2001). Cereal crops maintain vegetative growth and their cold acclimating ability more efficiently under SDs and cool temperatures (Limin and Fowler 2006). According to Limin and Fowler (2006), wheat exposed to SDs was able to tolerate temperatures 8.5°C colder than wheat grown

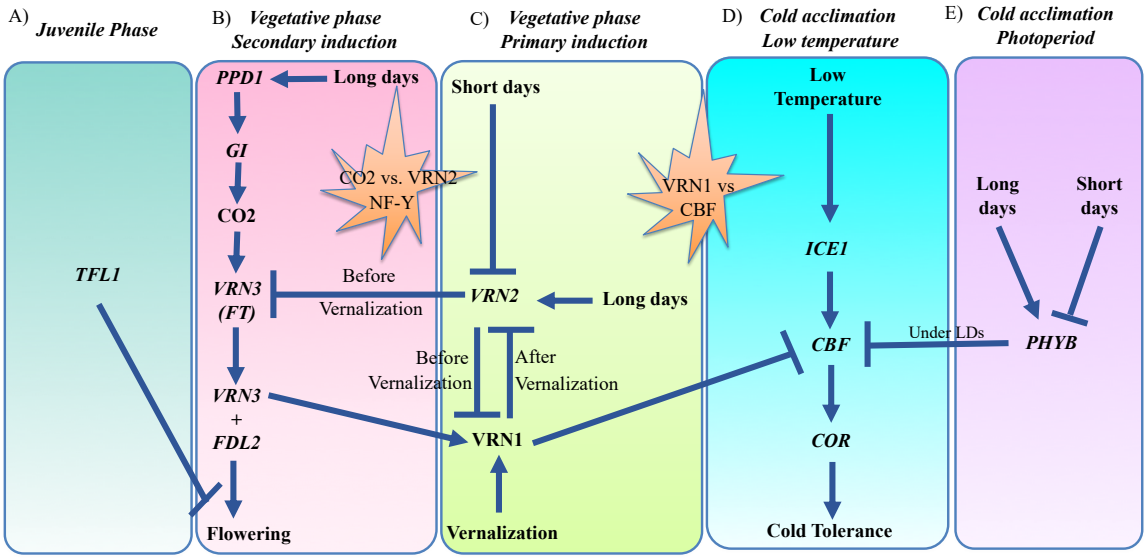


Figure 1.8 Connection between flowering time and cold tolerance in annual cereal crops for (A) juvenile phase, (B) vegetative phase secondary induction, (C) vegetative phase primary induction, (D) low temperature cold acclimation and (E) photoperiodic cold acclimation. Orange stars represent genes that outcompete one another to form a complex with NF-Y proteins and the vegetative vs. reproductive antagonism of *VRN1* and *CBF* genes. Integrated ideas from Cockram et al. (2007), Greenup et al. (2009), Andrés and Coupland (2012) and Preston and Sandve (2013).

under LDs, suggesting cereals are able to better withstand cold temperatures and induce cold tolerance when exposed to SDs. As in *Arabidopsis*, exposure of wheat and barley to SDs causes the downregulation of *PHYB*, leading to the upregulation of *CBF* genes (Novák et al. 2015) (Figures 1.7 B, 1.8 E). According to Novák et al. (2015) in the fall under SDs, there is a lower red to far-red white light ratio, while LDs during the summer have a higher red to far-red white light ratio. The lower red to far-red white light ratio under SDs in the fall causes *PHYB* to be downregulated and *PHYA* to be upregulated leading to an increase in *CBF14* expression in winter wheat and barley (Novák et al. 2015). SDs may also inhibit the expression of *VRN1* and *VRN3*, causing the plant to remain vegetative and preventing the positive feedback loop between *VRN1* and *VRN3* (Danyluk et al. 2003, Yan et al. 2006, Dhillon et al. 2010).

1.1.12 Cold tolerance in perennials

Cold tolerance genes have been relatively well studied in *Arabidopsis* and are somewhat understood in annual cereals, however identification and regulation of genes involved in cold tolerance in perennial grasses is largely unknown. Although much of the cold tolerance pathway in perennials is not identified, it is thought to be similar to cereals (Sandve et al. 2011, Wingler 2014). The *CBF* pathway is assumed to be conserved or at least partially conserved in perennial grasses, and several *CBF* genes have been identified in perennial ryegrass (Xiong and Fei 2006, Tamura and Yamada 2007, Zhao and Bughrara 2008). *CBF*s in perennial ryegrass have been shown to be rapidly upregulated after exposure to a cold period, similar to *CBF*s in *Arabidopsis* and cereal crops (Tamura and Yamada 2007) (Figure 1.9 A). A *COR* gene in perennial ryegrass was induced by a

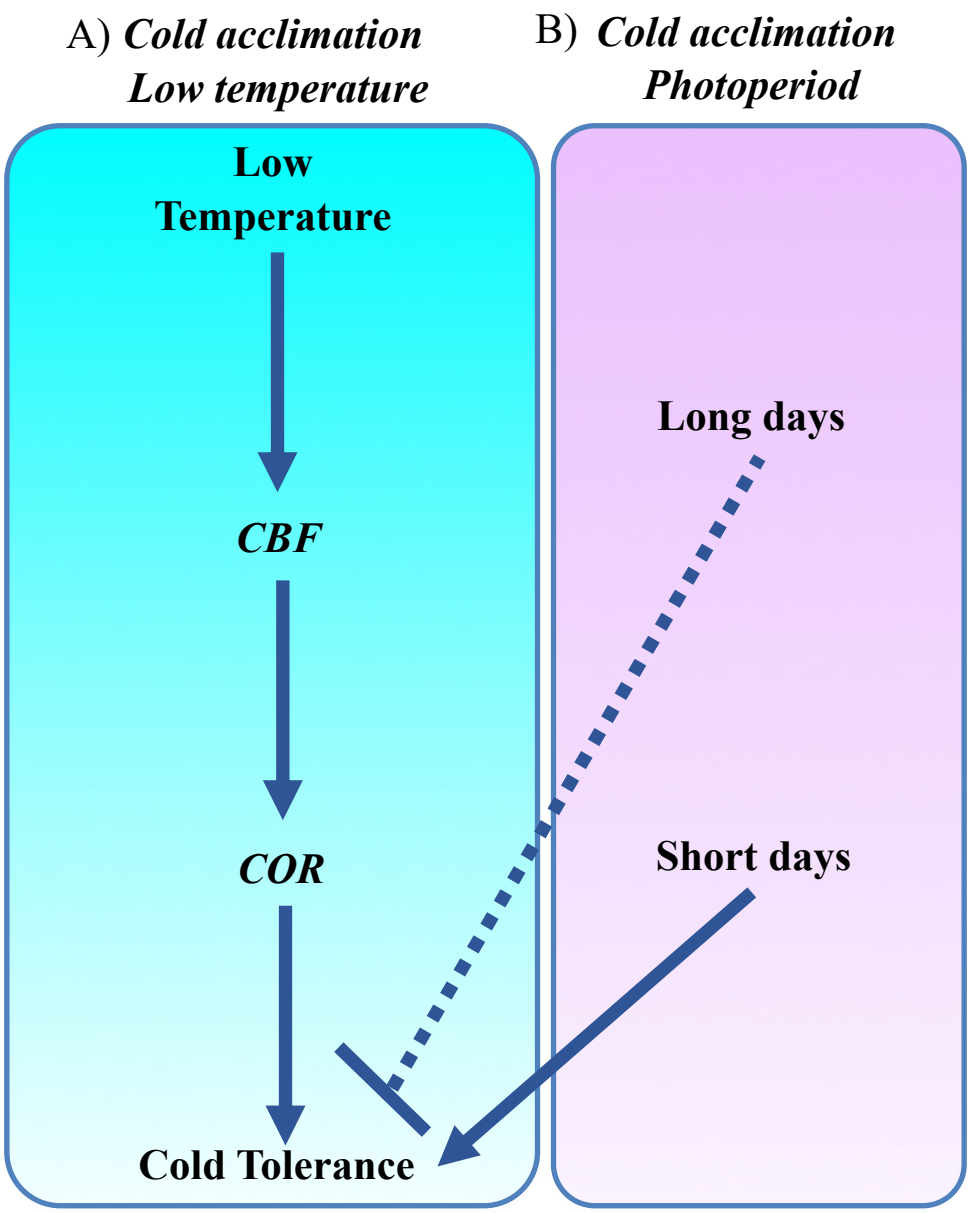


Figure 1.9 Cold tolerance pathway in perennial grasses for (A) low temperature and (B) photoperiodic cold acclimation.

CBF gene under cold temperatures (Zhao and Bughrara 2008) (Figure 1.9 A). This suggests that *CBF* and *COR* genes follow the same expression pattern and likely have the same function in perennial grasses as they do in *Arabidopsis* and cereal crops, althoughlittle is known about specific cold tolerance genes (Zhao and Bughrara 2008, Sandve et al. 2011, Wingler 2014).

In perennials, the connection between cold tolerance and vernalization genes is not clear, although their expression has been shown to be linked in meadow fescue (*Festuca pratensis* L.) (Ergon et al. 2016). During vernalization under SDs, *VRN1* expression increased while *COR14* was expressed at a constant level, but under LDs, *COR* and *CBF* genes were downregulated (Ergon et al. 2016). This could suggest that in meadow fescue *VRN1* can cause the downregulation of cold tolerance genes towards the end of the vernalization period, and as photoperiod increases (Kovi et al. 2016) (Figure 1.10 C, D).

Photoperiod influences the cold acclimation pathway of perennials, similar to that of *Arabidopsis* and cereals. SD exposure causes perennials to remain vegetative and stop growth (Malyshev et al. 2014). Perennial ryegrass, timothy, white clover (*Trifolium repens L.*) and false oat grass (*Arrhenatherum elatius* L.) all were able to cold acclimate more efficiently under SDs than LDs, displaying increased regrowth and survival (Junttila et al. 1990, Malyshev et al. 2014, Dalmannsdottir et al. 2017) (Figure 1.9 B). This suggests that the response to cold is influenced by photoperiod in perennials, however more studies are needed to better understand this connection (Malyshev et al. 2014).

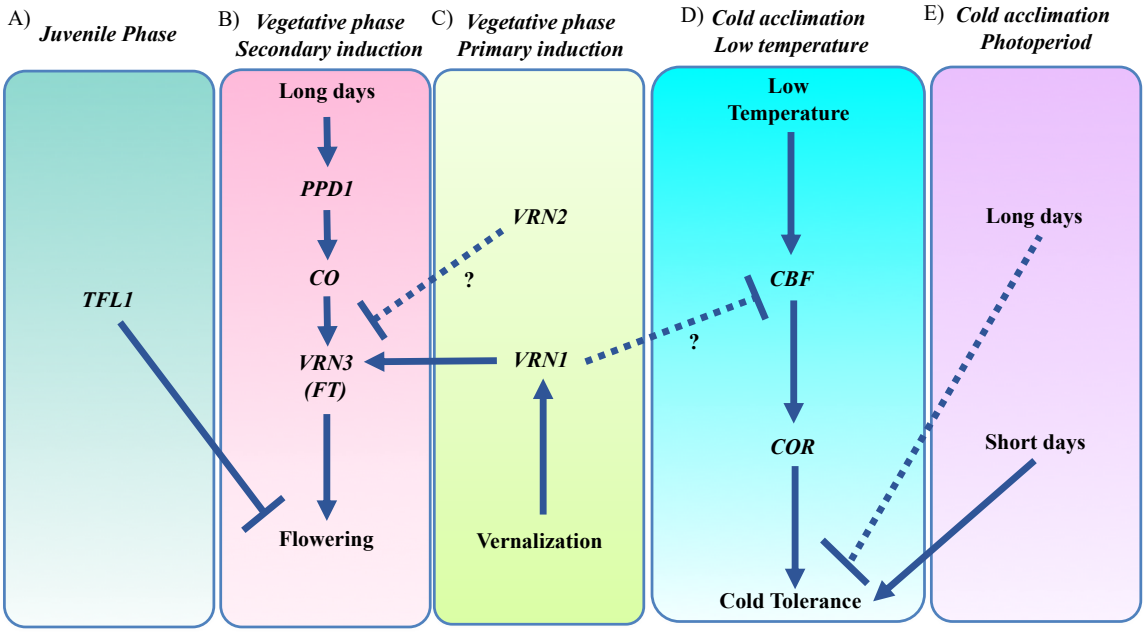


Figure 1.10 Connection between flowering time and cold tolerance in perennial grasses for (A) juvenile phase, (B) vegetative phase secondary induction, (C) vegetative phase primary induction, (D) low temperature cold acclimation and (E) photoperiodic cold acclimation.

Cold tolerance in temperate trees is better understood than in perennial grasses and can provide insight to perennial cold acclimation. Like herbaceous perennials, trees are able to repeatedly cold acclimate each fall to prepare for winter (Vitasse et al. 2014). Tree cold acclimation occurs in the fall and the tree remains dormant over the winter and deacclimates, or loses its cold tolerance, in the spring (Vitasse et al. 2014). Acclimation, dormancy and deacclimation are influenced by changes in temperature and photoperiod (Vitasse et al. 2014). Similar to *Arabidopsi*s, as temperature decreases, and trees are exposed to SDs, trees stop growth and become dormant, where the tree cold hardens causing cells to be able to survive exposure to cold temperatures (Welling and Palva 2006, Charrier and Améglio 2011, Vitasse et al. 2014). In the spring, trees begin to lose cold tolerance which is enhanced by LDs and warmer temperatures (Charrier and Améglio 2011, Vitasse et al. 2014).

Poplar, apple, and peach (*Prunus persica* L.), have been shown to have a similar cold acclimation pathway to *Arabidopsis*, *ICE* and *CBF*-like and genes have been discovered (Wisniewski et al. 2018). *CBF* genes have been shown to be upregulated when exposed to cold temperatures, as well as in a diurnal rhythm regulated by the circadian clock (Artlip et al. 2013, Wisniewski et al. 2018). Unlike in *Arabidopsis*, in which *CBF*s are upregulated only early on following cold exposure, some tree *CBF*s remain upregulated for a long period of time (Wisniewski et al. 2018).

1.1.13 Objectives

Current perennial wheat lines do not appear to revert back to the vegetative state in the fall and have poor winter survival rates, suggesting that they are weak perennials (Hayes et al. 2018). It has been suggested that the flowering time and cold tolerance pathways of annual cereals are conserved in perennial grasses, however applying this information to perennial crops may not be appropriate as they display a different life cycle (Seppänen et al. 2010, Sandve et al. 2011, Jokela et al. 2014, Wingler 2014). Determining the life cycle and persistence of current perennial wheat lines would be a valuable innovation in agriculture. Understanding flowering time and cold tolerance in perennials could allow breeders and researchers to select the best existing lines for cultivation based on their gene expression pattern and growth habit or develop new varieties of perennial cereals.

For this research project, several experiments were conducted involving flowering time and cold tolerance of annual wheat, wheatgrass and perennial wheat lines. First, chromosomes of perennial wheat lines were counted to ensure that all lines used for the project had 56 chromosomes, a number that was determined to help contribute to meiotic stability and a perennial life cycle (Hayes et al. 2012). Next, wheat, wheatgrass and perennial wheat plants were grown without being exposed to a vernalization period in order to see if they could flower without vernalization, providing insight to if they behave as a winter or spring type cultivar and if they were appropriate lines to use for the study. Next, leaf and apical meristem tissue was sampled throughout different stages of development during the first vegetative and sexual cycle for wheat, wheatgrass and

perennial wheat lines. qPCR was performed to analyze gene expression of flowering time genes *VRN1*, *VRN2*, *VRN3*, *PPD1* and *TFL1* to compare annual wheat, wheatgrass, and perennial wheat lines. At the end of their first life cycle, when plants were considered to be in the PSCR stage, one wheatgrass and one perennial wheat line were exposed to three different photoperiods and temperatures, long day (LD), short day (SD), and cold and short day (CD) in order to simulate three different fall growing conditions for eight weeks. A qPCR experiment was conducted using the same genes of interest to analyze gene expression to determine if the perennial wheat line will respond similarly to the wheatgrass line to a change in growing conditions.

The first cold tolerance experiment involved sequencing genes *CBF12*, *CBF14* and *COR14* in two species of wheatgrass, *Thinopyrum ponticum* and *Thinopyrum elongatum*. Sequencing results were compared to wheat to determine if wheatgrass sequences were similar or different to wheat. Next, a freezing test was conducted on wheat, wheatgrass and perennial wheat lines to determine the LT_{50} of the plants. Expression levels of cold tolerance genes *CBF12*, *CBF14* and *COR14* were analyzed by qPCR in leaf and apical meristem tissue of wheat, wheatgrass and perennial wheat throughout different stages in their first vegetative and sexual cycle to develop a baseline for cold tolerance gene expression in perennial wheat. Next, one wheatgrass and one perennial wheat line were exposed to three different photoperiods and temperatures, long day (LD), short day (SD), and cold and short day (CD) for eight weeks at the end of their first life cycle, in what is considered to be PSCR. Gene expression of *CBF12*, *CBF14* and *COR14* were analyzed in leaf and apical meristem tissue by qPCR to determine if the

wheatgrass and perennial wheat line will have a similar response to a change in growing conditions.

CHAPTER TWO: EXPRESSION OF FLOWERING TIME GENES IN WHEAT, WHEATGRASS AND PERENNIAL WHEAT

2.1 Introduction

2.1.1 Development and challenges of perennial wheat

The development of perennial wheat by wide hybridization methods of crossing wheat (*Triticum* spp.) with a close perennial relative, wheatgrass (*Thinopyrum* spp.), has been attempted since the 1920's due to its potential economic and environmental benefits (Wagoner and Schaeffer 1990). Perennial plants are defined as plants that are able to flower repeatedly over multiple years. In temperate climates they must be able to survive freezing winter conditions and alternate between vegetative and reproductive growth in response to particular environmental cues and at specific stages throughout their life (Acquaah 2009, Andrés and Coupland 2012). Perennial wheat development has the potential to reduce input costs, decrease the need for fertilizer and herbicide, reduce soil erosion, and decrease carbon dioxide in the atmosphere through carbon sequestering due to deep root systems compared to annuals (Gantzer et al. 1990, Bell et al. 2008, Glover et al. 2010, Pimentel et al. 2012). In general, perennial wheat development involves crossing hexaploid wheat (*Triticum aestivum* L., 2n=42) containing genomes AABBDD, or tetraploid durum wheat (*Triticum turgidum* var. *durum*, 2n=28) containing genomes AABB, with either decaploid *Thinopyrum ponticum* (2n=70), containing genomes $JJJJJJJ^sJ^sJ^s$, diploid *Thinopyrum elongatum* (2n=14), containing genomes E^eE^e, or hexaploid *Thinopyrum intermedium* (2n=42) , containing genomes JJJ^sJ^sSS, although

there are some exceptions (Fedak and Han 2005, Hayes et al. 2012, Gazza et al. 2016). Current perennial wheat lines have several challenges (Fedak and Han 2005, Hayes et al. 2012, Gazza et al. 2016). The most important problems identified in current perennial wheat lines are that plants are meiotically unstable, do not seem to revert back to the vegetative state and thus are unable to survive cold winters with freezing conditions, which all indicate that they are weak perennials (Bell et al. 2008, Hayes et al. 2012, Hayes et al. 2018). An indicator of perennial habit in perennial wheat lines is post sexual cycle regrowth (PSCR), which is defined as the regrowth of crown meristems followed by tiller growth after senescence and harvest in the fall (Murphy et al. 2010).

Perennial wheat lines that contain a full set of hexaploid (AABBDD) or tetraploid (AABB) chromosomes from wheat and a partial set from wheatgrass that contains one complete genome, are generally more meiotically stable, at a minimum are partially fertile, produce viable seeds, have robust growth, and have potential for PSCR in the fall (Hayes et al. 2018). In the case of hexaploid wheat being the parent, perennial wheat lines usually have a full set of chromosomes donated from wheat (2n=42) and a partial set of chromosomes that contains one complete genome donated from the wheatgrass parent *Th. ponticum* or intermediate wheatgrass, resulting in a perennial wheat line with 56 chromosomes (Fedak and Han 2005). For example, perennial wheat line Agrotana has a chromosome constitution of: 4"J + 4"J^s + 20" wheat (Fedak and Han 2005). In the case of diploid *Th. elongatum* being the wheatgrass parent, a full set of chromosomes is donated, resulting in a perennial wheat line also with 56 chromosomes (Fedak and Han 2005). However, some crosses do not result in lines with 56 chromosomes and even have chromosome variation within the same line (Fedak and Han 2005, Hayes et al. 2012).

Lines that that do not have 56 chromosomes have been shown to be meiotically unstable, are less likely to be fertile, produce non-viable seeds and have less PSCR than lines that produce 56 chromosomes (Fedak and Han 2005, Murphy et al. 2010, Hayes et al. 2012, Hayes et al. 2018).

Current perennial wheat lines have poor winter survival and persistence, which could be caused by the plant having insufficient flowering control, not responding to seasonal cues (changes to temperature or daylength) and remaining fixed in the reproductive phase (Hayes et al. 2012, Gazza et al. 2016, Hayes et al. 2018). Several studies have shown that most perennial wheat lines have poor survival and often die during their first winter (Murphy et al. 2010, Hayes et al. 2012, Hayes et al. 2018). Hayes et al. (2018) also observed perennial wheat lines perpetually flowering in the field during their first year, without reverting back to a vegetative state after harvest, thus not displaying PSCR. A study of PSCR in perennial wheat lines indicated that only 61% of plants had some PSCR or potential for PSCR (Hayes et al. 2012). Plants would likely have improved persistence and survival if the plant was able to remain in the vegetative state throughout the fall and winter, as winter damage would be reduced and resources would not be allocated towards reproduction, so it is important that these lines are identified (Greenup et al. 2009, Hayes et al. 2018).

2.1.2 Flowering Control

The ability of a plant to cycle between vegetative and reproductive growth at specific times throughout plant development is dependent on flowering control and

determines perennial or annual habit. Vernalization, the exposure to a long cold period that induces a flowering response, and photoperiod induction, a daylength flowering response, are important to determining flowering time (Shimada et al. 2009). Plants can either have a long day (LD) photoperiod response, where flowering occurs as daylength increases in the spring and summer, a short day (SD) photoperiod response, where flowering occurs as daylength shortens, or day neutral plants, where there is no response to photoperiod (Turck et al. 2008, Andrés and Coupland 2012). Adaptations such as reducing winter damage in cold climates, are influenced by photoperiod and vernalization (Greenup et al. 2009). Many winter type cultivars and perennials respond to cold temperature changes and vernalization. However, some perennials such as most timothy (*Phleum pratense*) cultivars, do not require vernalization, although vernalization causes flowering to be enhanced (Seppänen et al. 2010). Exposure to a long vernalization (cold) period will cue the plant to begin flowering once the risk of frost is over (Greenup et al. 2009). Flowering control has been well described in *Arabidopsis thaliana* and a database of flowering time genes, called FLOR-ID has been developed (Bouché et al. 2016). The flowering of annual cereals has also been relatively well described, however not much is known about the control of flowering in perennials.

2.1.3 Flowering pathway in Arabidopsis

Most accessions of *Arabidopsis* flower under LDs (Turck et al. 2008). Under LDs *GIGANTEA* (*GI*) is regulated by the circadian clock and causes the upregulation of *CONSTANS* (*CO*) (Suárez-López et al. 2001). *CO* expression under LDs causes the upregulation of *FLOWERING LOCUS T* (*FT*), the *Arabidopsis* florigen in the leaf,

inducing flowering (Corbesier et al. 2007). Once *FT* upregulation occurs in the leaf, it is then transported via phloem to the shoot apical meristem (SAM) where the reproductive transition occurs (Corbesier et al. 2007). In the SAM, FT binds with FLOWERING LOCUS D (FD) to form a heterodimer, and the heterodimer upregulates meristem identity genes like *APETALA1* (*AP1*), *FRUITFUL* (*FUL*), *LEAFY* (*LFY*), and *SUPPRESSOR OF OVER EXPRESSION OF CONSTANS1* (*SOC1*), which promote the formation of reproductive shoots, leading to flowering (Ferrándiz et al. 2000, Turck et al. 2008, Kaufmann et al. 2010). *TERMINAL FLOWER1* (*TFL1*) is expressed in the SAM and induced by *CO*. *TFL1* acts as a floral repressor by outcompeting *FT* to form a complex with *FD*, causing the repression of *AP1* and *LFY*, therefore preventing flowering (Hanano and Goto 2011, Wickland and Hanzawa 2015).

To promote flowering in the spring, winter type *Arabidopsis* exhibits a flowering response to vernalization. *FRIGIDA* (*FRI*) is upregulated when exposed to fall temperatures, and in turn causes the upregulation of *FLOWERING LOCUS C* (*FLC*), a MADS-box (*MCM1, AGAMOUS, DEFICIENS, SRF*) transcription factor that acts as a floral repressor (Michaels and Amasino 1999). In the leaf, *FLC* causes FT to be downregulated, inhibiting it from binding to FD and signaling to meristem identity gene *SOC1* expressed in the SAM (Searle et al. 2006). *FLC* can also bind to and repress the expression of *SOC1*, thus preventing flowering (Searle et al. 2006). Another floral repressor is *SHORT VEGETATIVE PHASE* (*SVP*), which encodes a MADS-box transcription factor (Li et al. 2008). *SVP* is not regulated through the vernalization pathway, but the SVP protein does work with FLC to bind to the promoter region of *SOC1* in the SAM and prevent flowering (Li et al. 2008). Exposure to a vernalization

period causes *FLC* to be downregulated, leading the upregulation of *FT*, initiating flowering (Michaels and Amasino 1999).

2.1.4 Flowering pathway in annual cereals

The flowering pathway of cereal crops has many similarities to *Arabidopsis*. Like *Arabidopsis*, wheat and barley are mostly LD plants that flower when exposed to long photoperiods (Turner et al. 2005). *Brachypodium distachyon*, a model grass that is related to wheat and barley, also has a similar flowering pathway to *Arabidopsis* and cereal crops (Ream et al. 2014). *PHYTOCHROMEC* (*PHYC*), a red and far-red light receptor, causes the upregulation of *PHOTOPERIOD1* (*PPD1*), which is expressed under LDs in a diurnal rhythm (Chen et al. 2014). The upregulation of *PPD1* causes the upregulation of the *VERNALIZATION3* (*VRN3*), a gene orthologous to *FT* in *Arabidopsis* and *Brachypodium*. (Chen et al. 2014, Ream et al. 2014). *PPD1,* along with the circadian clock, was also shown to cause the upregulation of *GI* and *CONSTANS2* (*CO2*), a *CO*-like gene, causing the upregulation of *VRN3* leading to flowering (Shimada et al. 2009). *TFL1* in cereals and *Brachypodium* is thought to be similar to *TFL1* in *Arabidopsis*, as it acts as a floral repressor that regulates meristem identity genes to prevent flowering (Jensen et al. 2001, Chardon and Damerval 2005, Higgins et al. 2010).

Winter cereals, including wheat and barley, display a vernalization flowering response similar to that of *Arabidopsis*. *VERNALIZATION1* (*VRN1*), a gene similar to *AP1* in *Arabidopsis*, codes for a MADS-box transcription factor and becomes upregulated in leaves and SAMs when exposed to a vernalization period (Yan et al. 2003, Distelfeld et

al. 2009a). In wheat, barley and *Brachypodium*, the upregulation of *VRN1* causes the upregulation of *VRN3*, initiating flowering (Yan et al. 2006, Ream et al. 2014). *VERNALIZATION2* (*VRN2*) is a floral repressor found in wheat and barley, but not in *Brachypodium*. Although *VRN2* is part of a different family of transcription factors, it has a similar function to *FLC* in *Arabidopsis* (Yan et al. 2004, Higgins et al. 2010). Before vernalization, *VRN2* is upregulated, leading to the downregulation of *VRN1* and *VRN3*, preventing flowering (Yan et al. 2004). Exposure to vernalization causes the downregulation of *VRN2* leading to the upregulation of *VRN1* and subsequently *VRN3* which together promote flowering (Shimada et al. 2009). *VRN3* is also able to upregulate *VRN1* under LDs when *CO2* and *PPD1* are upregulated (Yan et al. 2006, Distelfeld et al. 2009a). Under LDs, *VRN3* becomes upregulated in the leaf and is transported to the SAM where it binds with *FLOWERING LOCUS D-LIKE2* (*FDL2*), a transcription factor similar to *FD* in *Arabidopsis,* to form a complex. (Li and Dubcovsky 2008). The *VRN3/FDL2* complex then binds to the promoter region of *VRN1*, thus upregulating it (Li and Dubcovsky 2008). The upregulation of *VRN3* and *VRN1* then causes the downregulation of *VRN2*, leading to flowering (Yan et al. 2006). *NUCLEAR FACTOR-Y* (*NF-Y*), a transcription factor, can interact with *VRN2* or *CO2*, which compete to bind with NF-Y proteins to either repress or promote flowering (Li et al. 2011). If *VRN2* binds with NF-Y proteins, flowering is repressed (Li et al. 2011). If *CO2* is upregulated and binds to NF-Y proteins, then *VRN3* becomes upregulated leading to flowering (Li et al. 2011).

Previous studies indicate that most winter cereal cultivars require vernalization and LDs in order to flower, while most spring cultivars rely on only daylength to induce

flowering (Cockram et al. 2007, Greenup et al. 2009, Kamran et al. 2014). *VRN1* orthologues in wheat include 3 genes called *Vrn1-A1*, *Vrn1-B1* and *Vrn-D1* which map to chromosome 5A, 5B and 5D (Kamran et al. 2014). *VRN2* has two orthologous genes, *Vrn-A2* and *Vrn-B2,* located on chromosome 5A and 5B, however *Vrn-A2* is not functional (Kamran et al. 2014). Spring wheat with one or more dominant *VRN1* alleles will alleviate the requirement of a cold period to flower (Fu et al. 2005, Kamran et al. 2014). The dominant *Vrn1-A1* allele is the strongest, causing the plant to be totally insensitive to vernalization while dominant *Vrn1-B1* and *Vrn1-D1* alleles cause the plant to be partially insensitive (Zhang et al. 2008). Winter wheat has dominant *VRN2* alleles, while *VRN1* is recessive, thus causing the plant to require vernalization (Yan et al. 2004, Distelfeld et al. 2009b, Kamran et al. 2014).

2.1.5 Flowering pathway in perennials

The flowering time pathway has been relatively well described in annual plants including cereals, however little is known about flowering in perennials (Cockram et al. 2007, Galiba et al. 2009). Some insight comes from research carried out on timothy (*Phleum pretense* L.), perennial ryegrass (*Lolium perenne* L.) and *Arabis alpina*, a close perennial relative of *Arabidopsis* (Jensen et al. 2001, Wang et al. 2009, Seppänen et al. 2010, Jokela et al. 2015). *Brachypodium sylvaticum*, a perennial relative of *Brachypodium distachyon,* is also a candidate model for perennials, however little research has been done on flowering time genes (Steinwand et al. 2013). The two main flowering pathways, photoperiod and vernalization, are also important for flowering in perennial plants (Seppänen et al. 2010). The SAMs of perennials have been shown to

have different gene regulation than annuals. The SAMs of annual plants all become reproductive at once, known as monocarpy, while perennials have some SAMs that remain vegetative as others shift to reproductive, known as polycarpy (Wang et al. 2009).

The flowering pathway of perennial grasses is thought to be similar to that in cereals, however flowering time genes have not been extensively studied in perennial grasses. *VRN1* was identified in timothy and perennial ryegrass and is upregulated during vernalization and as the plant begins the reproductive phase (Seppänen et al. 2010). *VRN3* was identified in timothy and has been shown to have a homologous function to *VRN3* in cereals as it is upregulated by *VRN1* and vernalization as well as LDs, leading to flowering (Jokela et al. 2015). *PPD1* was shown to be upregulated when exposed to LDs, suggesting that it may have the same function as *PPD1* in annual cereals (Jokela et al. 2014). A gene with sequence similarity to *VRN2* in annual cereals was discovered in timothy, but it is not known if it functions like *FLC* in *Arabidopsis* and *VRN2* in annual cereals as a floral repressor (Seppänen et al. 2010, Jokela et al. 2015). Although the function of *VRN2* is unknown, studies on *PERPETUAL FLOWERING1* (*PEP1*), a similar gene to *Arabidopsis FLC*, have been performed in *A. alpina* (Wang et al. 2009). A second system for maintaining vegetative SAMs was discovered involving microRNA156 (miR156) which targets *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15* (*SPL15*), a floral promoter (Hyun et al. 2019). *SPL15* causes older SAMs to become reproductive and also promotes flowering when *PEP1* is downregulated (Hyun et al. 2019). *PEP1* prevents flowering before and after vernalization so that some SAMs remain vegetative (Wang et al. 2009). *TFL1* was identified in perennial ryegrass by sequence similarity to *TFL1* in *Arabidopsis,* and was shown to be expressed in the meristem and

function as a floral repressor (Jensen et al. 2001). *TFL1* in perennial ryegrass was upregulated during vegetative growth and downregulated as floral promoters were upregulated, indicating that *TFL1* has a similar role in perennial grasses as it does in cereals (Jensen et al. 2001). In *A. alpina*, low expression of *TFL1* was associated with a short juvenile period, suggesting that *TFL1* could have a role in the perennial lifecycle (Wang et al. 2011).

2.1.6 Objectives

Understanding the life cycle and persistence of perennial wheat would be an important advancement in agriculture. Current perennial wheat lines do not appear to revert back to the vegetative state in the fall, and have poor survival rates after the first year of growth, suggesting that they are weak perennials (Hayes et al. 2018). Presently, not much is known about control of flowering time in perennial wheat plants and most of the information about flowering comes from annual cereal crops, which does not allow a complete understanding of the perennial life cycle. Knowledge of control of flowering time in perennials could allow breeders and researchers to select the best existing varieties for cultivation based on their growth habit and gene expression pattern or develop new varieties of perennial cereals. It has been suggested that the flowering time pathway of annual cereals is similar in perennial grasses, however recent studies suggest that some of the flowering traits may be different and further exploration in this area is required to understand the basic mechanism of perennial habit in grasses (Seppänen et al. 2010, Sandve et al. 2011, Jokela et al. 2014, Wingler 2014).

Perennial wheat lines 235a, OK7211542, Agrotana, and 11955, derived from crosses between annual wheat and *Thinopyrum ponticum* or *Thinopyrum elongatum*, were analyzed for chromosome number and flowering time gene expression and compared to expression in wheat cultivars Norstar and Chinese Spring, and wheatgrass cultivars *Th. ponticum* and *Th. elongatum* (Table 2.1).

1. Examine the ploidy of perennial wheat lines used in this study. Ensure lines have 56 chromosomes.

2a. Evaluate expression of flowering time genes *VRN1*, *VRN2*, *VRN3*, *PPD1* and *TFL1* in leaf and apical meristem tissue at different stages of development during the first vegetative and sexual lifecycle of wheat, wheatgrass and perennial wheat lines.

2b. Evaluate expression of the same flowering time genes in leaf and apical meristem tissue of plants considered to be in the PSCR stage.

Table 2.1 Plant types, accessions and ploidy levels grown for flowering experiments.

Plant Type	Cultivar/Accession	Pedigree	Expected Growth Habit	Ploidy	Reference
Wheat	(1) Norstar (2) Chinese Spring	-	(1) Winter (2) Spring	(1) 2n=6x=42 (2) 2n=6x=42	Grant 1980 CFIA 2013
Wheatgrass	*(1) Thinopyrum ponticum* (PI206624) *(2) Thinopyrum elongatum* (PI531718)	-	(1) Perennial (2) Perennial	(1) 2n=10x=70 (2) 2n=2x=14	GRIN 2007a GRIN 2007b
Perennial Wheat	(1) 235a (2) OK7211542 (3) Agrotana (4) 11955	(1) Madsen//Chinese Spring/PI531718 (2) Wheat -*Thinopyrum ponticum* partial amphiploid (3) Wheat-*Thinopyrum ponticum* partial amphiploid (4) Wheat-*Thinopyrum ponticum* partial amphiploid	(1) Unknown (2) Unknown (3) Unknown (4) Unknown	(1) Full Amphiploid 2n=8x=56 (2) Partial Amphiploid 2n=8x=56 (3) Partial Amphiploid 2n=8x=56 (4) Partial Amphiploid 2n=8x=56	Hayes et al. 2012

2.1.7 Hypothesis

Perennial wheat lines will have similar gene expression to perennial wheatgrass and annual wheat during the first sexual cycle, but as temperature and daylength changes, perennial wheat lines will not be able to completely respond to these cues, thus remaining fixed in the reproductive phase and being unable to transition to the complete vegetative phase after the first sexual cycle.

2.2 Materials and Methods

2.2.1 Plant materials and planting

Two accessions of wheatgrass (PI206624, *Thinopyrum ponticum* and PI531718, *Thinopyrum elongatum*), two wheat cultivars (winter wheat Norstar and spring wheat Chinese Spring) and four lines of perennial wheat (235a, OK7211542, Agrotana, and 11955) were used for flowering time experiments (Table 2.1) (Grant 1980, GRIN 2007a, b, Hayes et al. 2012, CFIA 2013). Three biological replicates of each plant type were grown. The seeds were germinated for at least one week on damp filter paper at room temperature and then planted in 4x8 Rootrainers™ (Rootrainers International, Canada). The potting mix used was based on a peat blend developed by Boodley and Sheldrake (1972) and was made by mixing 1 bale of sphagnum peat moss (108 L), 2 bags of vermiculite (18.6 kg/bag), Turface MVP (22.7 kg), fertilizer mix (1500 g of 18-6-12 Osmocote, 1200 g of monocalcium-dicalcium phosphate, 1000 g of calcium carbonate powder, 15 g of 13.2% chelated iron, and 7 g of 14% chelated zinc), and water (9-23 L). Plants were grown in a 16/8 h day/night growth chamber at 20ºC/16ºC day/night with a light intensity of 340 μmol/m^2/s and watered once daily. At Zadoks stage Z22 (Zadoks et al. 1974), plants were moved to a 4ºC room to vernalize for 8 weeks (wheat and perennial wheat) or 10 weeks (wheatgrass) with 8/16 h day/night and a light intensity of 575 μmol/m^2/s. After vernalization, the plants were moved back into the 16/8 h day/night growth chamber at 20ºC/16ºC day/night.

2.2.2 Chromosome counting – Preparing the root tips

Seeds for perennial wheat lines 235a, OK7211542, Agrotana, and 11955 and annual wheat Norstar, were germinated as described in section 2.2.1 for approximately one week, until the radicle emerged. The root tip was cut to about 1 cm and placed in a 10 ml test tube containing 6 ml of H_2O. The root tips were incubated at 4ºC for two days. After incubation, the root tips were fixed by immersing them in 3:1 ethanol: glacial acetic acid (99.7%) and placed at -20ºC where they were stored prior to chromosome counting.

2.2.3 Chromosome counting – Microscopy

Root tips were soaked in 1 M HCl in a 60ºC water bath for 7 minutes. After blotting excess liquid, the root tips were stained with a 1% acetocarmine solution (1 L of 45% glacial acetic acid mixed with 10 g carmine powder). A cover slip was then placed over the root and repeatedly tapped with a pencil eraser until the root tissue was transparent and completely squashed. The slide was warmed briefly on a hot plate and the root was further squashed with a cork. The slides were viewed using a Nikon Eclipse Ci microscope (Nikon, Canada). For each wheat line, chromosomes were counted from roots of 10 plants with two cells per plant. Pictures were taken with a Leica Microsystems DM6000B microscope and attached camera Leica Microsystems DFC310 FX (Leica Microsystems Inc., Canada).

2.2.4 Flowering without vernalization

Five plants of each cultivar including, PI206624, PI531718, Norstar, Chinese Spring, 235a, OK7211542, Agrotana, and 11955, as well as extra spring wheat cultivars AAC Awesome, Bhishaj, AC Meena and AC Nanda (Sadasivaiah et al. 2004, Randhawa et al. 2011, CFIA 2013, 2018), were germinated as described in section 2.2.1 and planted in 1 L pots. Plants were grown for 5 months without vernalization (20ºC/16ºC day/night with a light intensity of 340 μmol/m^2/s). Plants were then examined for flowering and photos were taken.

2.2.5 Sampling of plant tissue for gene expression analysis during the first vegetative and sexual cycle

Seeds were germinated, seedlings were planted and grown as described above in section 2.2.1. Three biological replicates of leaf and apical meristem tissue were sampled at Zadoks stages: Z22 (tillering), Z22 at 6 h, 24 h, 2 w and 8 w after vernalization, Z31 (first node visible), Z39 (flag leaf), Z47 (booting), Z65 (anthesis) and Z85 (soft dough) (Zadoks et al. 1974).

2.2.6 Preparation of cDNA

Total RNA was isolated from leaves and apical meristem tissue using the RNeasy Plant Mini Kit (QIAGEN, Canada). RNA quality was checked using the Bioanalyzer instrument (Agilent, USA) or QIAxcel (QIAGEN), followed by an off-column DNA

digestion using the DNase I Amplification Grade Kit (ThermoFisher Scientific Inc., Canada). A standard PCR using primers *ELF1a* or *CONTIG5* (primer sequences can be found in Table 2.2) was conducted on the DNase treated RNA samples to ensure that they were DNA free. The PCR product was run on a 1% agarose gel using gel electrophoresis. In some cases, a qPCR using the QuantiTect SYBR Green PCR Kit (ThermoFisher Scientific Inc.) was performed on the DNase treated RNA samples instead of a standard PCR. For one reaction, 5 µL of SYBR Green, 0.6 µL each of 10 µM *ELF1a* or *CONTIG5* forward and reverse primers, 1.3 µL of H_2O, and 2.5 µL of $^1/_{10}$ diluted RNA sample, were mixed for a total reaction of 10 µL. The following program was used to amplify the samples on the ABI QuantStudio 6 Flex qPCR System: 1 cycle at 50ºC for 2 min, 1 cycle at 95ºC for 3 min, 40 cycles at 95ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 30 sec (ThermoFisher Scientific Inc.). cDNA was synthesized from the purified RNA, following the protocol from the SuperScript III Reverse Transcriptase kit to use in qPCR (ThermoFisher Scientific Inc.).

2.2.7 Quantitative Polymerase Chain Reaction (qPCR) of the first vegetative and sexual cycle

Primers for *VRN1, VRN2, VRN3, PPD1* and *TFL* were previously designed and tested based on the *Thinopyrum sp.* and wheat sequence of these flowering time genes (Table 2.2) (Pahari et al. 2015). Housekeeping genes, *CONTIG5* and *ELF1a,* were used to calculate the relative expression of the genes of interest (Table 2.2). Three biological and technical replicates of each sample were utilized. The qPCR experiment was completed using QuantiTect SYBR Green PCR Kit (QIAGEN). For one reaction 5 µL of SYBR

Table 2.2 Flowering time primer sets selected for qPCR to amplify genes of interest.

Gene of Interest	qPCR Primer	Tm (°C)	Expected Size of qPCR Product (bp)	Accession Number (GenBank)
VRN1	Forward: TGA AGC GGA TCG AGA ACA AGA Reverse: CAT GAC TCG GTG GAG AAC TCG	60	163	LC052274.1
VRN2	Forward: GCA GCG AAG GTG ATG AGG TAT Reverse: CTT CGG GTA CCT TGA CAA AGC	60	133	AY485967.1
VRN3	Forward: AGA TGC TCC AAG TCC AAG CG Reverse: AGG GCT CTC GTA GCA CAT CA	60	118	MH264458.1
PPD1	Forward: GAG GTC TGC AAG GAC ATC CC Reverse: ACA TGC GCC CAA AGG TTC TT	60	143	MH264490.1
TFL1	Forward: CTA AGG GAG CAC CTT CAC TGG Reverse: TTG GGC TTT GGG CTC TCA TA	54	98	MF805804.1
ELF1a	Forward: GGT GAT GCT GGC ATA GTG AA Reverse: GAT GAC ACC AAC AGC CAC AG	60	125	M90077.1
CONTIG5	Forward: CTG CAG TGC GTG CAT ATT TT Reverse: AAC AAG AAC GAT GCC GAG TT	60	141	CK155621.1

green, 0.6 μL each of 10 μM forward and reverse primer,1.3 μL of H_2O, and 2.5 μL of $^1/_{10}$ diluted cDNA template, were mixed for a total reaction of 10 μL. The epMotion 5070 pipetted the qPCR reactions into 384 well plates (Eppendorf, Canada). The ABI QuantStudio 6 Flex qPCR System was used to amplify samples using the following program: 1 cycle at 50ºC for 2 min, 1 cycle at 95ºC for 3 min, 40 cycles at 95ºC for 30 sec, 60ºC/54ºC (depending on the primer) for 30 sec, and 72ºC for 30 sec (ThermoFisher Scientific Inc.).

2.2.8 Data analysis

qPCR results were analyzed using the Relative Expression Software Tool (REST) program (Pfaffl et al. 2002). REST removes variation by comparing CT values of target genes across housekeeping genes to normalize the data (Pfaffl et al. 2002). Gene expression at different stages is shown relative to expression at Z22. Significant values ($p < 0.05$) compared to Z22 were analyzed by an ANOVA (Pfaffl et al. 2002). To more clearly represent the data on a graph, the $\log_2$ of the expression value was used. Stages of development were also directly compared to each other using SAS software, the Glimmix Procedure, in order to determine if there were significant differences analyzed by an ANOVA ($p < 0.05$) between stages of development.

2.2.9 Sampling and preparation of plant tissue for gene expression analysis during the end of the first life cycle and beginning of the second life cycle

One accession of wheatgrass, PI206624 (*Thinopyrum ponticum*), perennial wheat line 235a and winter wheat variety Norstar were grown according to section 2.2.1, until they reached the end of their first sexual cycle at stage Z85. At Z85, pants were separated into three different growing conditions for eight weeks to simulate different fall temperatures and photoperiods: Long day (LD) conditions (16/8 hours day/night at 20ºC), short day (SD) conditions (10/14 hours day/night at 20ºC), and cold and short day (CSD) conditions (10/14 day/night at 4/2ºC day/night). At Z85 LD and SD plants were trimmed short once and CSD plants were trimmed twice to fit under the lights in the vernalization room. Three replicates of young leaf and apical meristem tissue was collected for Z85 at 0 w, 2 w, 4 w, 6 w and 8 w from the plants while under these growing conditions. After 8 w of exposure to LDs, SDs, or CSDs, plants were moved back into normal growing conditions (16/8 hours day/night at 20ºC) and photos were taken of the plants at Z39 of their second cycle.

2.2.10 Quantitative Polymerase Chain Reaction (qPCR) of LD, SD and CSD grown lines

The same protocols for RNA extraction and qPCR techniques that were used during the first life cycle, were utilized to carry out qPCR for the sexual cycle on the five flowering time genes of interest: *VRN1, VRN2, VRN3, PPD1* and *TFL1* (Table 2.2). Analysis of qPCR results was conducted following the same procedure as the first sexual cycle, by using the REST program (Pfaffl et al. 2002).

2.3 Results

2.3.1 Chromosome counting

Chromosomes of the four perennial wheat lines 235a, OK7211542, Agrotana and 11955 were counted to verify that they had 56 chromosomes as described in the literature (Hayes et al. 2012). All lines had hexaploid wheat as one parent. As the wheatgrass parent, 235a had *Thinopyrum elongatum* (2n=14), while the other three lines had *Thinopyrum ponticum* (2n=70) (Table 2.3). All perennial wheat lines had a chromosome number of 56 (Table 2.3).

2.3.2 Flowering without vernalization

Wheatgrass accessions PI206624 and PI531718, wheat cultivars Norstar, Chinese Spring, AAC Awesome, Bhishaj, AC Meena and AC Nanda, and perennial wheat lines 235a, OK7211542, Agrotana and 11955 were grown without being vernalized for 5 months and assessed for flowering. Pictures were taken 3 months after seeding for all cultivars except PI531718. Wheat cultivars Chinese Spring, AAC Awesome, Bhishaj, AC Meena and AC Nanda all flowered (Z65) at about 3 months after seeding (Figure 2.1). None of the perennial wheat lines, Norstar or PI206624 flowered. Wheatgrass PI531718 grew much more slowly than the other cultivars and flowered at about 5 months after seeding (Figure 2.1).

Table 2.3 Chromosome numbers in root tips of four perennial wheat lines 235a, OK7211542, Agrotana and 1195.

Name	Pedigree	Donor Wheatgrass Species	Root Tips Counted (2 cells/plant)	Chromosome Number
235a	Madsen//Chinese Spring/PI531718	*Thinopyrum elongatum* (2n=14)	10	56
OK7211542	Wheat-*Thinopyrum ponticum* partial amphiploid	*Thinopyrum ponticum* (2n=70)	10	56
Agrotana	Wheat-*Thinopyrum ponticum* partial amphiploid	*Thinopyrum ponticum* (2n=70)	10	56
11955	Wheat-*Thinopyrum ponticum* partial amphiploid	*Thinopyrum ponticum* (2n=70)	10	56

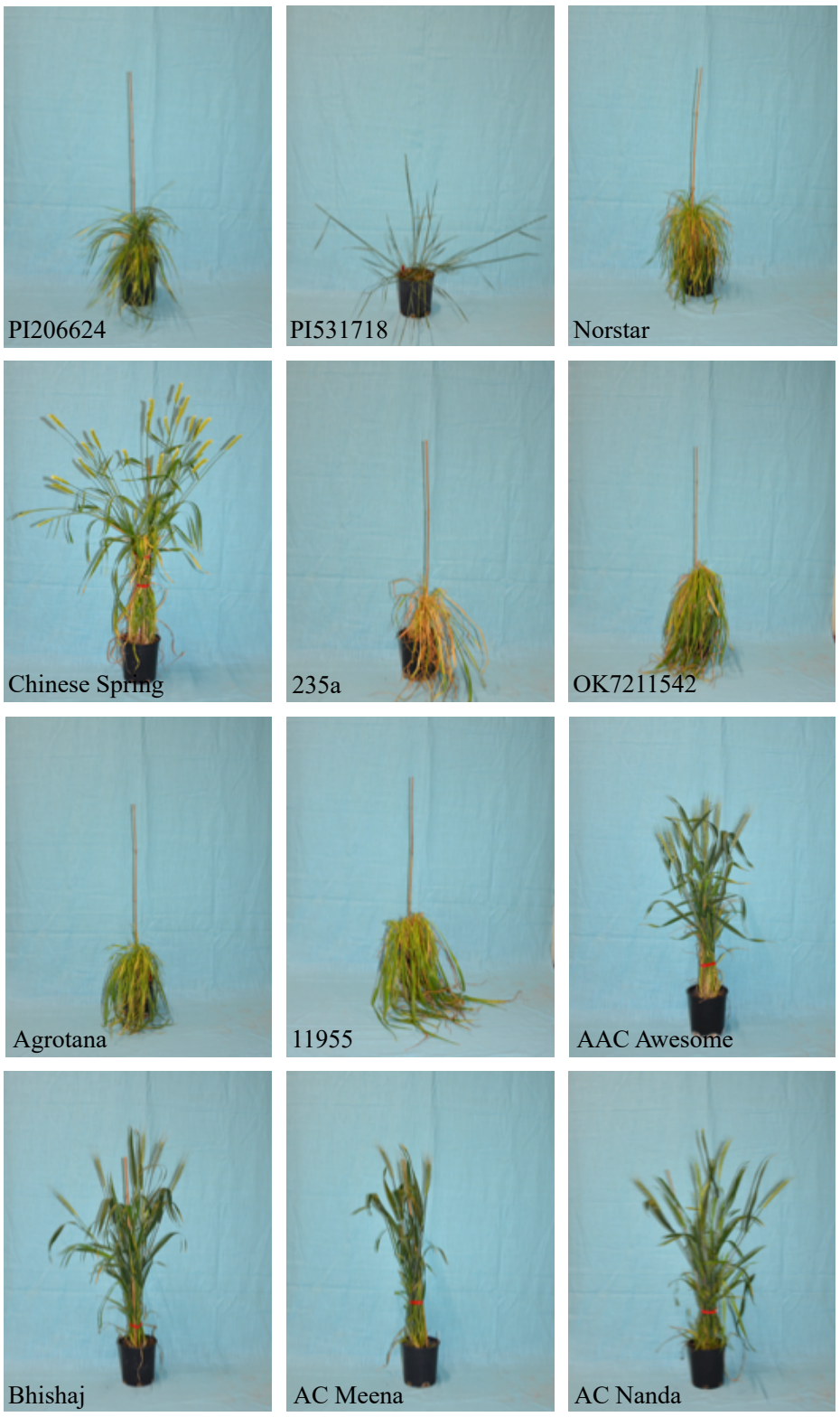


Figure 2.1 Photographs of wheatgrass, wheat and perennial wheat lines grown without vernalization. Cultivar names are in the bottom left-hand corner. All pictures were taken 3 months after seeding except for PI531718, which is at 5 months.

2.3.3 Flowering time gene expression analysis using qPCR during the first vegetative and sexual cycle

The log2 expression of flowering time genes *VRN1*, *VRN2*, *VRN3*, *PPD1* and *TFL1* in leaf and apical meristem tissues was graphed over Zadoks stages of development for the first sexual cycle of perennial wheatgrass accessions PI206624, PI531718, wheat cultivars Norstar and Chinese Spring, and perennial wheat lines 235a, OK7211542, Agrotana and 11955. Sampling stages were Z22 (tillering), Z22 at 6 h, 24 h, 2 w and 8/10 w after vernalization, Z31 (first node visible), Z39 (flag leaf), Z47 (booting), Z65 (anthesis) and Z85 (soft dough) (Zadoks et al. 1974). Gene expression at different stages is shown relative to expression at Z22 which is represented by zero (Figures 2.2 – 2.6). Significant values ($p < 0.05$) compared to Z22 were analyzed by ANOVA. Standard error is only indicated on bar graphs (Appendices 1 – 5).

VRN2 in cereals is a floral repressor that is upregulated in leaf tissue prior to vernalization thus causing *VRN1* and *VRN3* to be downregulated, preventing flowering (Yan et al. 2004). *VRN2* in leaf tissue has significant upregulation for most cultivars at Z22 6 h and 24 h and starts to become downregulated at Z22 2 w (Figure 2.2 A). *VRN2* expression at Z22 2w is significantly downregulated compared to Z22 24 h in all lines except 11955 (Appendix 6). *VRN2* expression remains downregulated for all lines until Z31, and after that remains downregulated for most lines, throughout the rest of the first cycle (Figure 2.2 A). For several lines at late stages, *VRN2* expression was too low to be detected by REST (represented as ND, Not Detected on the graph). In leaf tissue, *VRN2*

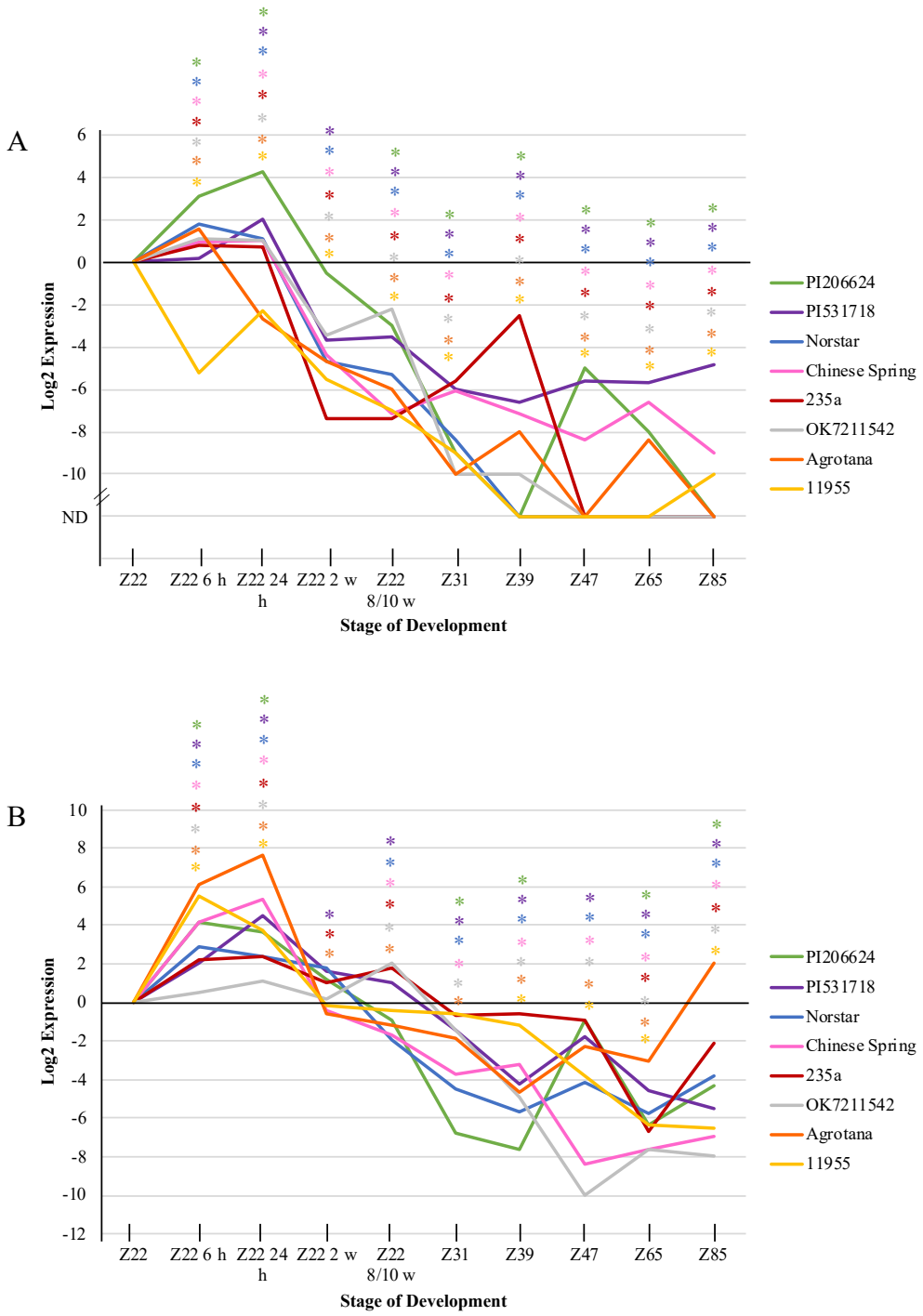


Figure 2.2 Log2 gene expression trends of *VRN2* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend. ND (Not Detected) indicates that REST was unable to detect gene expression.

was the most downregulated in 235a, OK7211542, Agrotana, 11955 and PI206624 and was not detected towards the end of the first sexual cycle (Figure 2.2 A). Although *VRN2* is mainly expressed in leaf tissue, expression in meristem tissue follows a similar trend (Figure 2.2 B) (Yan et al. 2004). *VRN2* is significantly upregulated early on during vernalization and starts to become downregulated at Z22 2 w (Figure 2.2 B). *VRN2* expression at Z22 2w is significantly downregulated compared to Z22 24 h in all lines except Norstar and 11955 (Appendix 7). *VRN2* becomes more downregulated until the end of the first sexual cycle, and at Z85 has a slight increase in expression for most lines (Figure 2.2 B). *VRN2* in meristem tissue did not have as low of expression as *VRN2* in leaf tissue near the end of the first sexual cycle, as it was detected by REST at all stages of development (Figure 2.2 A, B).

In both annual cereals and perennial grasses, *VRN*1 has been shown to be upregulated at the vegetative to reproductive transition during vernalization in both leaf and meristem tissue, and the upregulation of *VRN1* leads to the upregulation of *VRN3*, thus promoting flowering (Yan et al. 2006, Seppänen et al. 2010). In leaf tissue of all lines in this study *VRN1* starts to become upregulated during vernalization and expression increases until Z31 and remains around the same level for the rest of the first sexual cycle (Figure 2.3 A). Expression of *VRN1* at Z85 is significantly downregulated compared to Z65 in PI206624 and 235a (Figure 2.3 A, Appendix 8). In general, Norstar, PI531718, Chinese Spring and OK7211542 have the highest expression levels, while PI206624, 235a, Agrotana and 11955 appear have slightly lower expression levels with a similar expression pattern, although this was not tested statistically (Figure 2.3 A). Meristem tissue shows a similar expression pattern, with *VRN1* being upregulated during

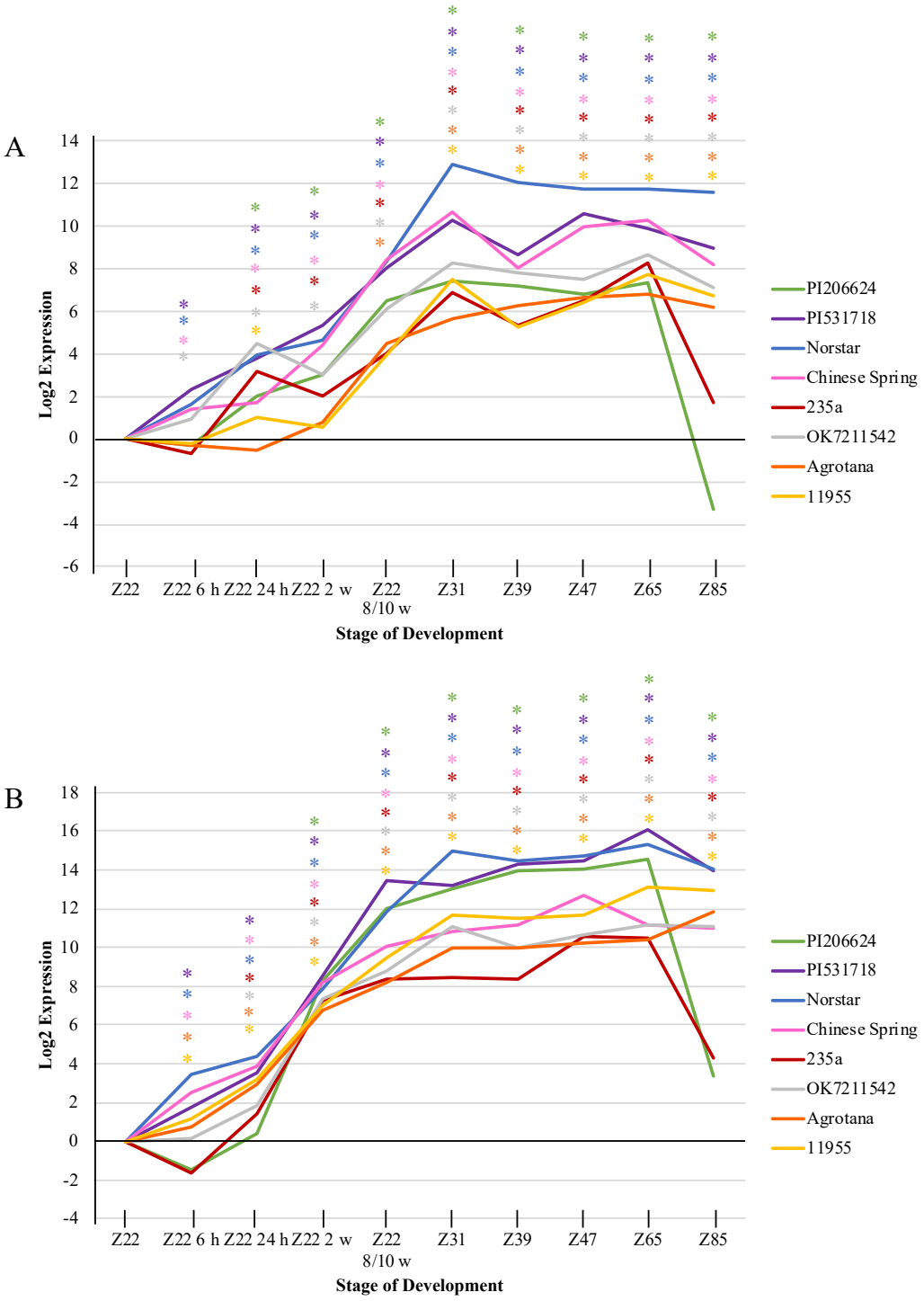


Figure 2.3 Log2 gene expression trends of *VRN1* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

vernalization and remaining upregulated throughout the first sexual cycle (Figure 2.3 B). As in leaf tissue, PI206624 and 235a in meristem tissue have a significant downregulation of *VRN1* at stage Z85 compared to Z65 (Figure 2.3 B, Appendix 9). In meristem tissue Norstar, PI206624 and PI531718 appear to have slightly higher expression than the other lines, although the expression pattern is similar (Figure 2.3 B). Norstar and PI531718 also were more highly expressed in leaf tissue, while 235a and Agrotana had lower expression in both tissue types (Figure 2.3 A, B).

VRN3 is a floral promoter in both cereals and perennial grasses and is upregulated by both *VRN1* and LDs (Yan et al. 2006, Shimada et al. 2009, Jokela et al. 2015). Previous studies have shown that *VRN3* is expressed in the leaf and is then transported to the meristem, where it is also expressed (Li and Dubcovsky 2008). In leaf tissue *VRN3* has low expression at the beginning of vernalization begins to increase in expression at Z22 8/10 w, just after *VRN1* starts to become upregulated in most lines (Figure 2.4 A). At stage Z31, *VRN3* becomes highly upregulated for all lines and remains upregulated throughout the rest of the first sexual cycle (Figure 2.4 A). At Z85 there is a significant decrease in *VRN3* expression compared to Z65 for PI531718 and 235a (Figure 2.4 A, Appendix 10). In general, *VRN3* is the most highly upregulated in Norstar, Chinese Spring, 235a and PI206624, and has the lowest expression in Agrotana and 11955 (Figure 2.4 A). *VRN3* expression in meristem tissue displays a similar pattern to *VRN3* expression in leaf tissue (Figure 2.4 B). *VRN3* starts to become significantly upregulated for most lines around Z22, just after *VRN1* starts to be upregulated (Figures 2.4 B). At Z31 *VRN3* becomes highly significantly upregulated compared to Z22 and Z22 8/10 w, remaining

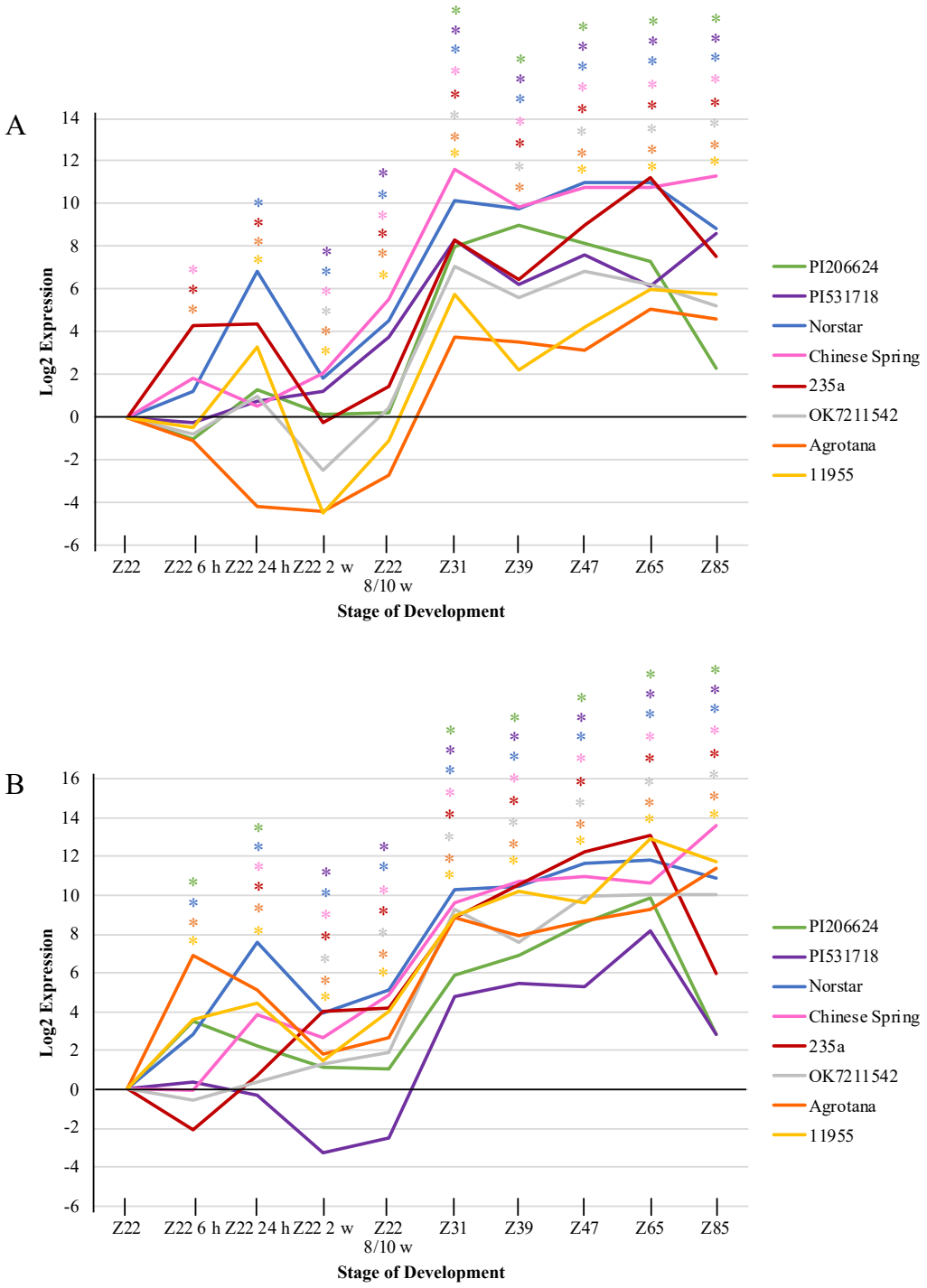


Figure 2.4 Log2 gene expression trends of *VRN3* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

upregulated until the end of the first sexual cycle (Figure 2.4 B, Appendix 11). At Z85 there is a decrease in *VRN3* expression for PI206624, PI531718 and 235a (Figure 2.4 B). Similar to leaf tissue, Norstar, Chinese Spring and 235a seem to be slightly more upregulated than other lines, however Agrotana and 11955 have higher relative expression than they do in leaf tissue (Figure 2.4 A, B).

PPD1 in cereals is upregulated under LDs, mainly in leaf tissue, and along with *VRN1*, causes the upregulation of *VRN3,* thus leading to flowering (Yan et al. 2006, Kitagawa et al. 2012, Chen et al. 2014). In the lines we studied, *PPD1* in leaf tissue is significantly downregulated at Z22 2 w and V8/10 w during vernalization (Figure 2.5 A). Post-vernalization at stage Z31, *PPD1* expression levels are similar to Z22 expression for most lines (Figure 2.5 A). For the rest of the first sexual cycle *PPD1* remains around the same expression level, with most lines having no significant difference in expression between stages (Figure 2.5, Appendix 12). *PPD1* then begins to decrease for almost all cultivars at Z85 (Figure 2.5 A). In leaf tissue, *PPD1* expression is generally the highest in PI206624 and Norstar and the lowest in Chinese Spring and 235a (Figure 2.5 A). *PPD1* in meristem tissue becomes significantly downregulated for most lines during vernalization at stage Z22 2 w and V8/10 w (Figure 2.5 B). Post vernalization, *PPD1* remains slightly upregulated in most lines, with most lines having no significant difference in expression between stages for the rest of the first sexual cycle until Z85, where expression levels begin to decrease for most cultivars (Figure 2.5 B, Appendix 13). *PPD1* in meristem tissue is more upregulated post vernalization than in leaf tissue (Figure 2.5 A, B).

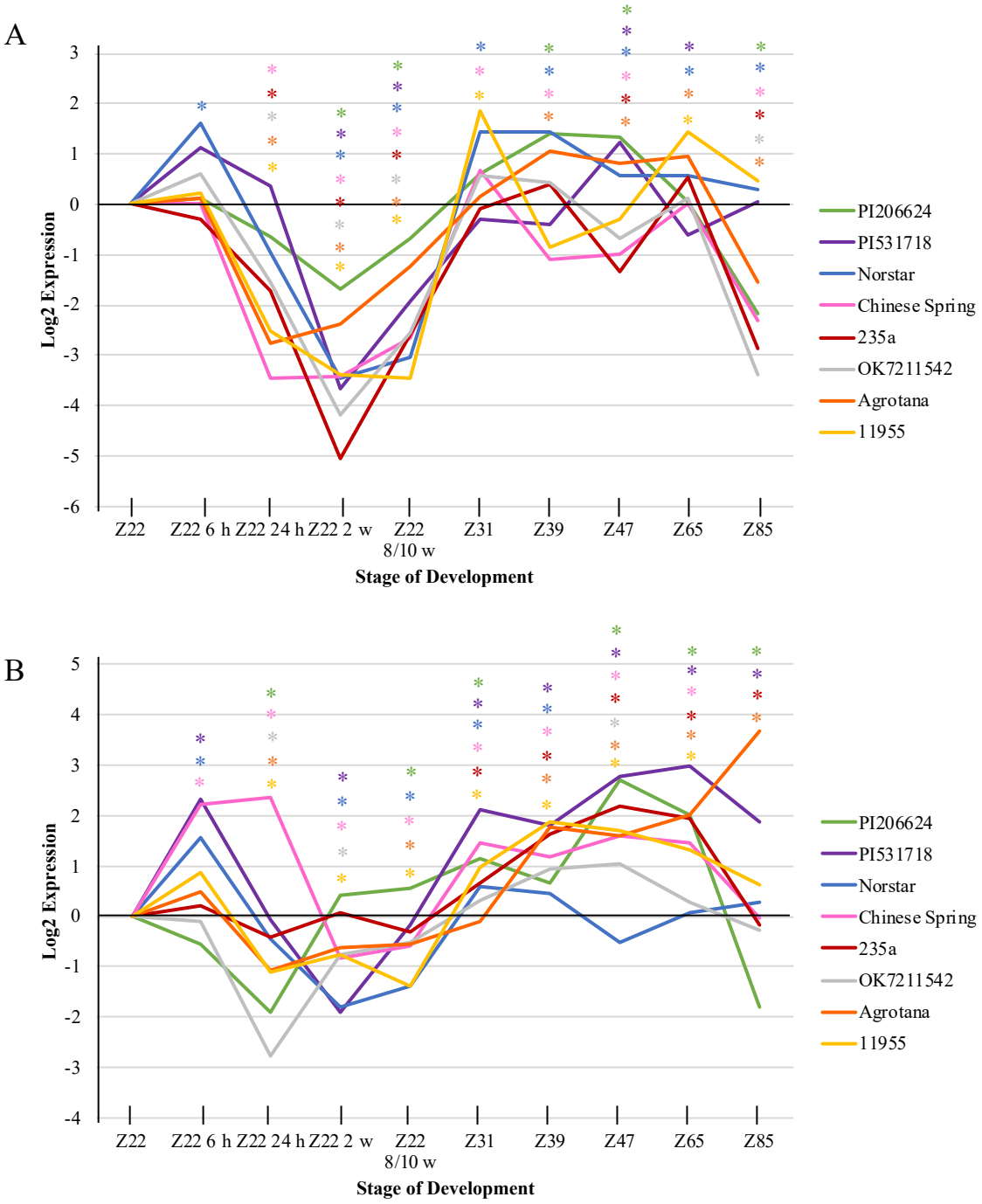


Figure 2.5 Log2 gene expression trends of *PPD1* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

In annual cereals and perennial grasses, *TFL1* is a floral repressor that is upregulated during vegetative growth in meristem tissue, thus preventing the expression of floral meristem identity genes (Jensen et al. 2001). *TFL1* in leaf tissue has significant upregulation for half of plant lines at Z22 24 h and 2 w (Figure 2.6 A). For the rest of the first sexual cycle, *TFL1* has similar expression compared to stage Z22 (Figure 2.6 A). *TFL1* in Chinese Spring becomes downregulated during Z22 6 h and 24 h, and *TFL1* has a spike in expression at Z22 8/10 w in Norstar that is significant compared to Z22 2w (Figure 2.6 A, Appendix 14), however these cultivars have large error bars for these specific stages (Appendix 5). *TFL1* in meristem tissue has low but significant expression relative to Z22 gene expression for most of the first sexual lifecycle, expression begins to slightly increase around Z31 until Z65. *TFL1* is more upregulated throughout these stages in wheatgrass and perennial wheat lines, while it has lower expression in Norstar and Chinese Spring (Figure 2.6 B). By Z85 *TFL1* expression was significantly downregulated compared to Z65 in PI206624, PI531718 and 235a (Appendix 15).

2.3.4 Flowering time gene expression of plants exposed to varying photoperiods and temperatures

Wheatgrass accession PI206624, winter wheat Norstar, and perennial wheat 235a were grown under LD conditions until stage Z85. At Z85 PI206624 and 235a were separated into LD, SD and CSD growing conditions for eight weeks. Log2 gene relative expression was compared to expression at Z22 because PI206624 had new tillers with relatively young tissue (around Z22). 235a plants grew very few new tillers and had older tissue sampled that was closer to Z85. Either Z22 or Z85 could have been used for

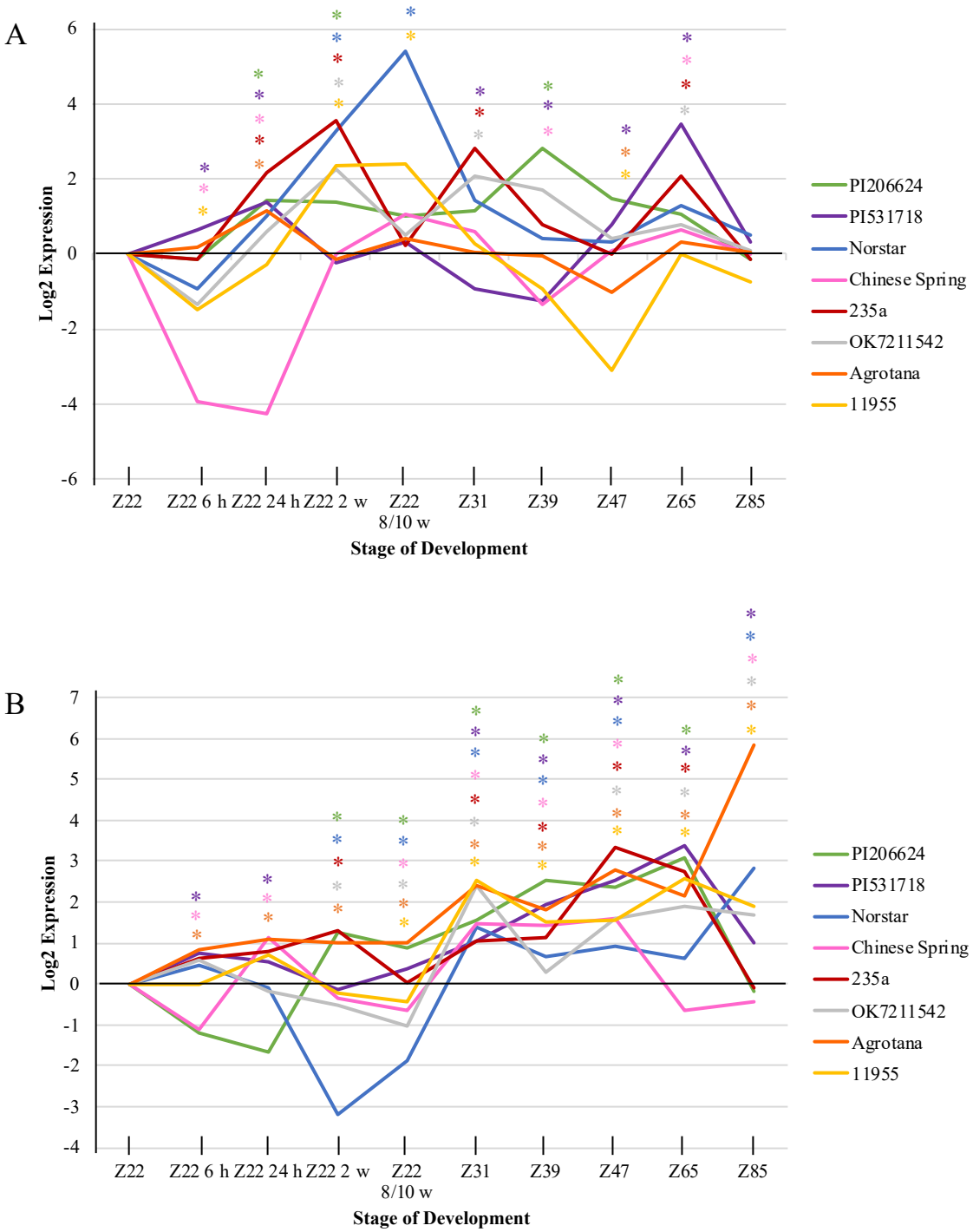


Figure 2.6 Log2 gene expression trends of *TFL1* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

comparison, however Z22 was chosen as PI206624, the known perennial, had tissue at that stage of development and it allowed for comparison to gene expression during the first vegetative and sexual cycle. Log2 gene expression relative to expression at Z22, which was represented as zero, of flowering time genes *VRN1*, *VRN2*, *VRN3*, *PPD1* and *TFL1* were plotted for leaf and meristem tissue at different stages of plant development. Expression levels at each stage were tested for significance using ANOVA ($p<0.05$) compared to Z22. Bar graphs with standard errors are presented in Appendices 16 – 20.

In cereals, *VRN2* is a floral repressor in leaf tissue that causes the downregulation of *VRN1* and *VRN3* prior to vernalization, thus repressing flowering (Yan et al. 2004). *VRN2* in leaf tissue is downregulated for PI206624 and 235a in LD, SD and CSD growing conditions compared to Z22 (Figure 2.7 A). *VRN2* in both PI206624 and 235a under LDs is the least downregulated and has higher expression from the Z85 W2 – W8 sampling period than stage Z85 (Figure 2.7 A). *VRN2* is more downregulated in PI206624 under SDs than LDs or CSDs. In 235a, *VRN2* is not detectable (ND) under SDs and CSDs (Figure 2.7 A). *VRN2* is much more downregulated in 235a for all three growing conditions than in PI206624 (Figure 2.7 A). *VRN2* expression in meristem tissue follows a similar trend, but as *VRN2* is mainly expressed in leaf tissue it is not as downregulated in meristem tissue (Figure 2.7 B) (Yan et al. 2004). *VRN2* is significantly downregulated for CSDs for Z85 W2 –W8 and has low expression compared to Z22 in SDs and LDs (Figure 2.7 B). In both leaf and meristem tissue when the reference is Z85 instead of Z22, a similar expression pattern of *VRN2* can be seen (Appendix 21).

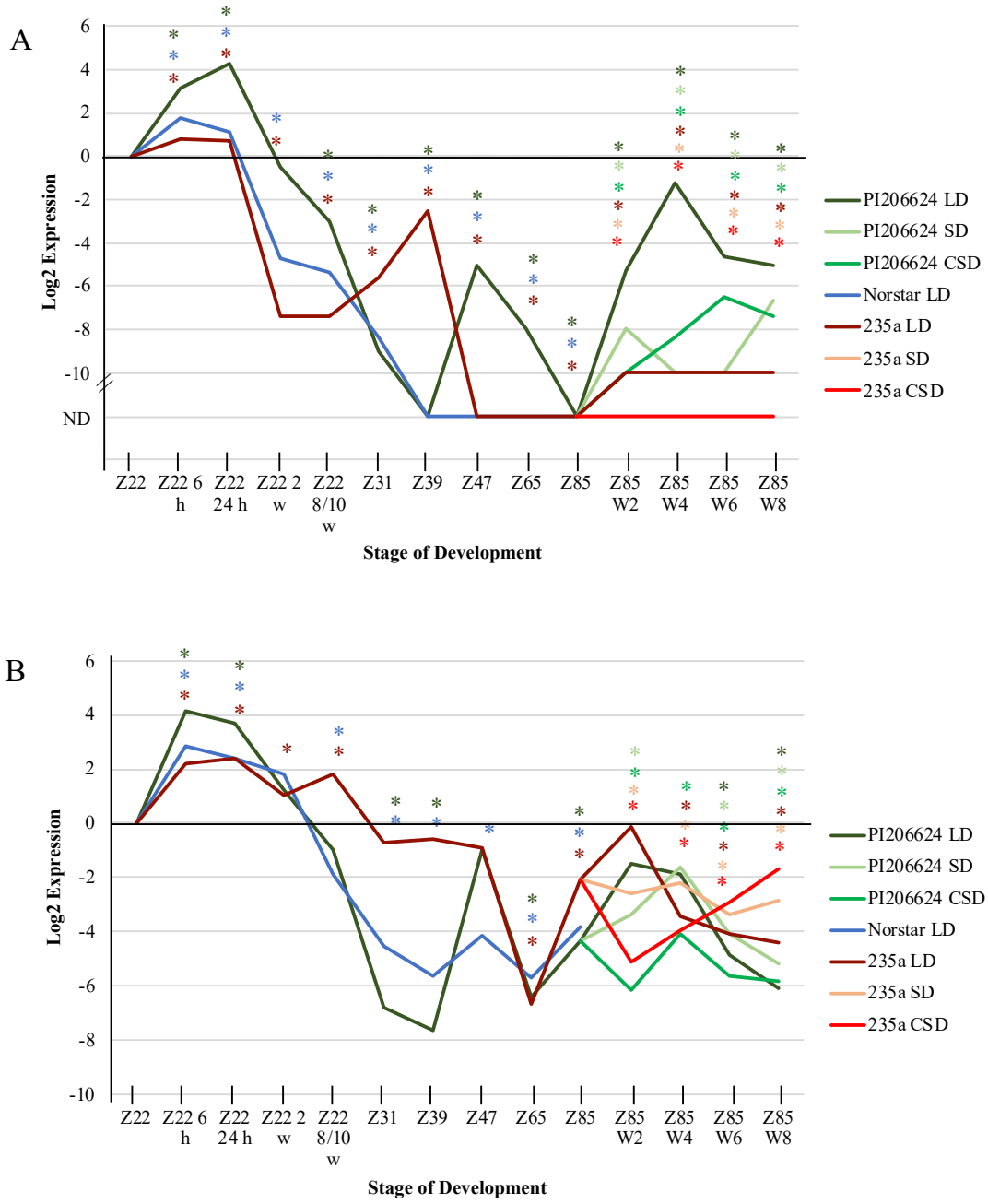


Figure 2.7 Log2 gene expression trends of *VRN2* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend. ND (Not Detected) indicates that REST was unable to detect gene expression.

VRN1 in perennial grasses and cereals is upregulated in the leaf and meristem by vernalization as the plant transitions from the vegetative to reproductive phase, leading to the upregulation of *VRN3* and subsequent flowering (Yan et al. 2006, Seppänen et al. 2010). At Z85 *VRN1* expression in PI206624 and 235a sharply decreases but becomes significantly upregulated from Z85 W2 – W8 under CSDs (Figure 2.8 A). In plants exposed to LDs and SDs *VRN1* is not as upregulated as in CSDs, however under both LDs and SDs *VRN1* is upregulated compared to stage Z22 in 235a, while it is downregulated or has low expression levels for PI206624 (Figure 2.8 A). *VRN1* is more highly expressed in 235a than in PI206624 under all three growing conditions. (Figure 2.8 A). Meristem tissue has a similar expression pattern in PI206624 and 235a, with *VRN1* expression decreasing at Z85 and becoming significantly upregulated under CSDs (Figure 2.8 B). *VRN1* is significantly upregulated in LDs for 235a, and significantly upregulated for PI206624 at Z85 W6, while plants grown under SDs have the lowest expression of *VRN1* in both PI206624 and 235a (Figure 2.8 B). A similar trend of *VRN1* expression in leaf and meristem tissue is shown when Z85 is used as the reference instead of Z22 (Appendix 22).

In annual cereals and perennial grasses, *VRN3* is a floral promoter that is upregulated by *VRN1* and LDs (Yan et al. 2006, Shimada et al. 2009, Jokela et al. 2015). At Z85 *VRN3* expression decreases in PI206624 and 235a (Figure 2.9 A). After the plants are exposed to the different photoperiods and temperatures, *VRN3* is upregulated under LDs in both PI206624 and 235a (Figure 2.9 A). *VRN3* is downregulated under SDs for both PI206624 and 235a and is downregulated under CSDs for PI206624 and upregulated for 235a (Figure 2.9 A) Compared to 235a, PI206624 *VRN3* expression is generally more

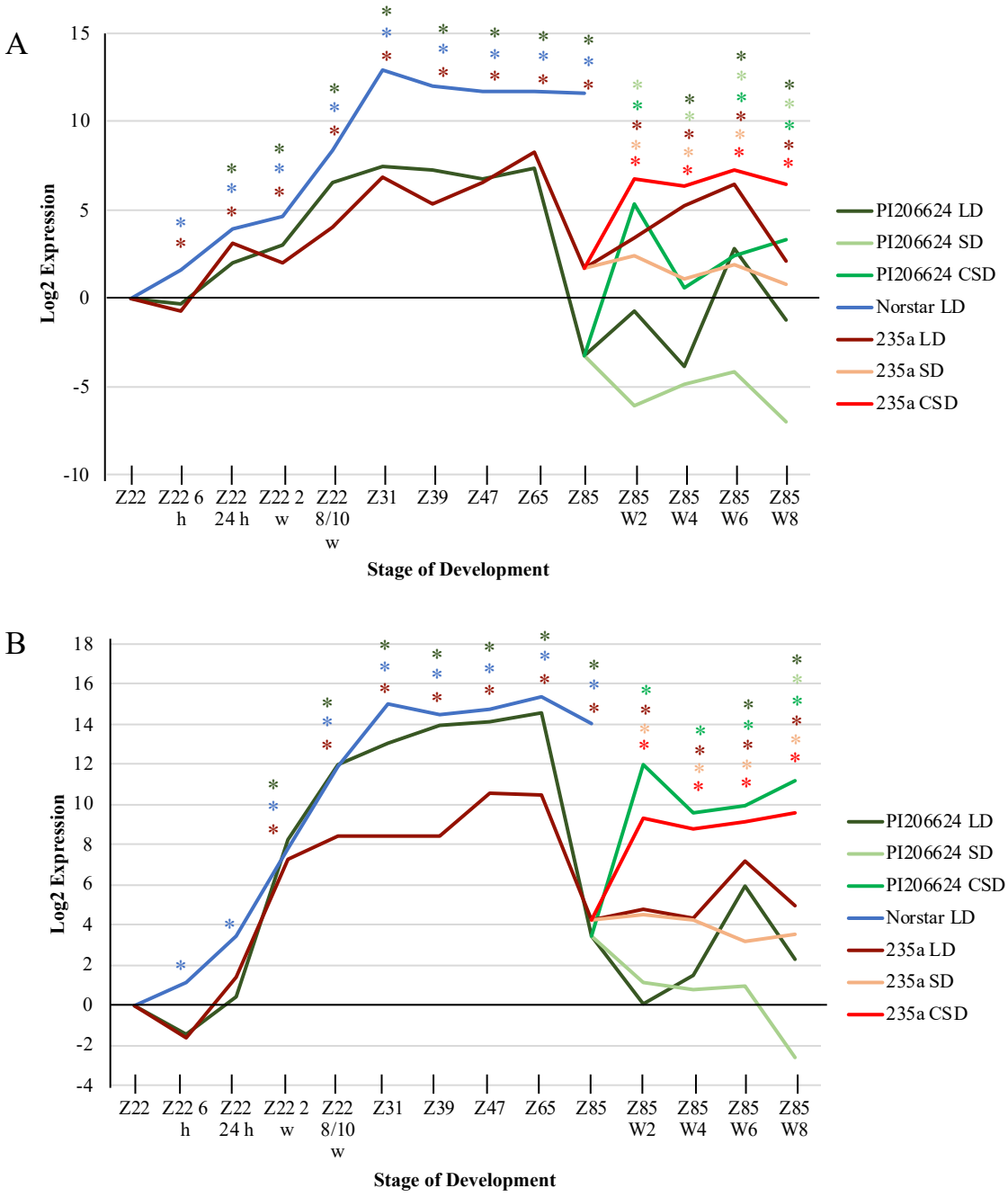


Figure 2.8 Log2 gene expression trends of *VRN1* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

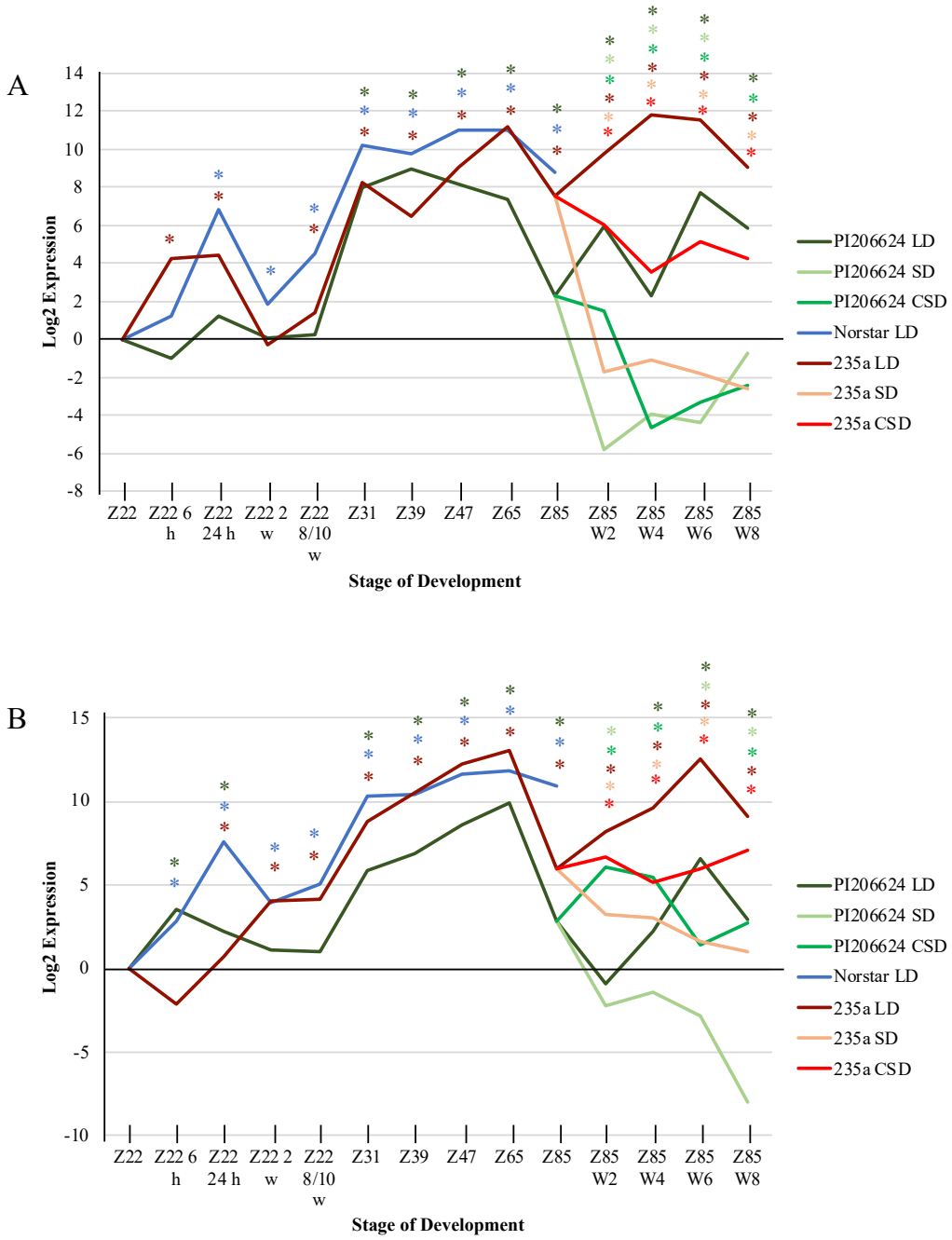


Figure 2.9 Log2 gene expression trends of *VRN3* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

downregulated after stage Z85 (Figure 2.9 A). Meristem tissue displays a *VRN3* expression pattern similar to leaf tissue. At Z85 *VRN3* meristematic tissue taken from plants exposed to LDs is upregulated in both PI206624 and 235a (Figure 2.9 B). In PI206624 *VRN3* is also upregulated under CSDs while it is downregulated under SDs. 235a has lower *VRN3* expression levels under CSDs and SDs than under LDs (Figure 2.9 B). Comparing all stages of development to Z85 rather than Z22 results in a similar expression pattern of *VRN3* in leaf and meristem tissue (Appendix 23).

PPD1 in cereals becomes upregulated in leaves by LDs and leads to the upregulation of *VRN3* and subsequent flowering (Kitagawa et al. 2012). From stages Z85 2 – 8 w *PPD1* in leaves under LDs remains most similar to expression at Z22 for both PI206624 and 235a, while expression under SDs and CSDs become more downregulated relative to Z22 (Figure 2.10 A). Meristem tissue of PI206624 and 235a exposed to LDs has *PPD1* expression similar to that of Z22, while expression of *PPD1* in SDs and CSDs is significantly downregulated compared to stage Z22 (Figure 2.10 B). A similar trend of *PPD1* expression can be seen when Z85 is used as the reference instead of Z22 (Appendix 24).

TFL1 in cereals and perennial grasses is a floral repressor that prevents the expression of floral meristem genes during vegetative growth (Jensen et al. 2001). As *TFL1* is mainly expressed in meristem tissue, expression in leaf tissue was more difficult to detect by qPCR (Jensen et al. 2001). The plants that were exposed to different

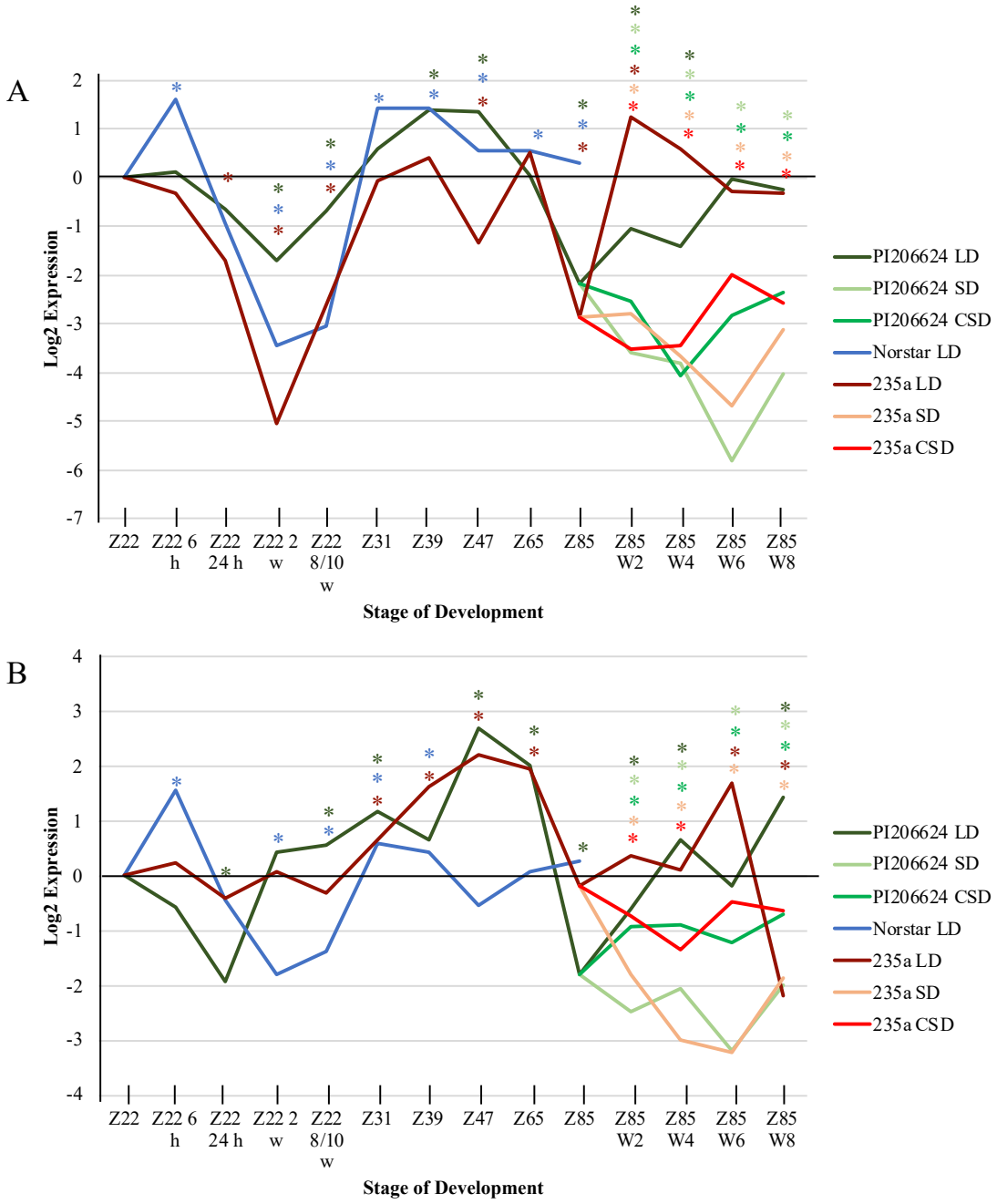


Figure 2.10 Log2 gene expression trends of *PPD1* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend

temperatures and photoperiods had the highest leaf *TFL1* expression in LDs and CSDs for PI206624 and in LDs for 235a (Figure 2.11 A). Expression of *TFL1* was downregulated SDs for both lines, while *TFL1* expression in CSDs was downregulated in 235a (Figure 2.11 A). In meristem tissue under LDs, *TFL1* is slightly upregulated in PI206624 and has low expression in 235a relative to Z22 (Figure 2.11 B) *TFL1* under CSDs was significantly downregulated for PI206624 and 235a, while under SDs *TFL1* was the most downregulated in both lines (Figures 2.11 B). *TFL1* is expressed in a similar trend when Z85 is used as a reference rather than Z22 (Appendix 25).

Although PI206624 and 235a plants were only sampled until Z85 W8 for gene expression analysis, plants continued growing into their second life cycle. Photos of PI206624 and 235a plants grown under LDs, SDs and CSDs were taken at Z39 of the second cycle to show phenotypic differences between lines and growing conditions (Figure 2.12). PI206624 had more vegetative leaves throughout the second life cycle in all three growing conditions, while 235a appeared to remain more reproductive (Figure 2.12). CSD plants of both PI206624 and 235a also looked more vigorous compared to LD and SD plants throughout the second life cycle.

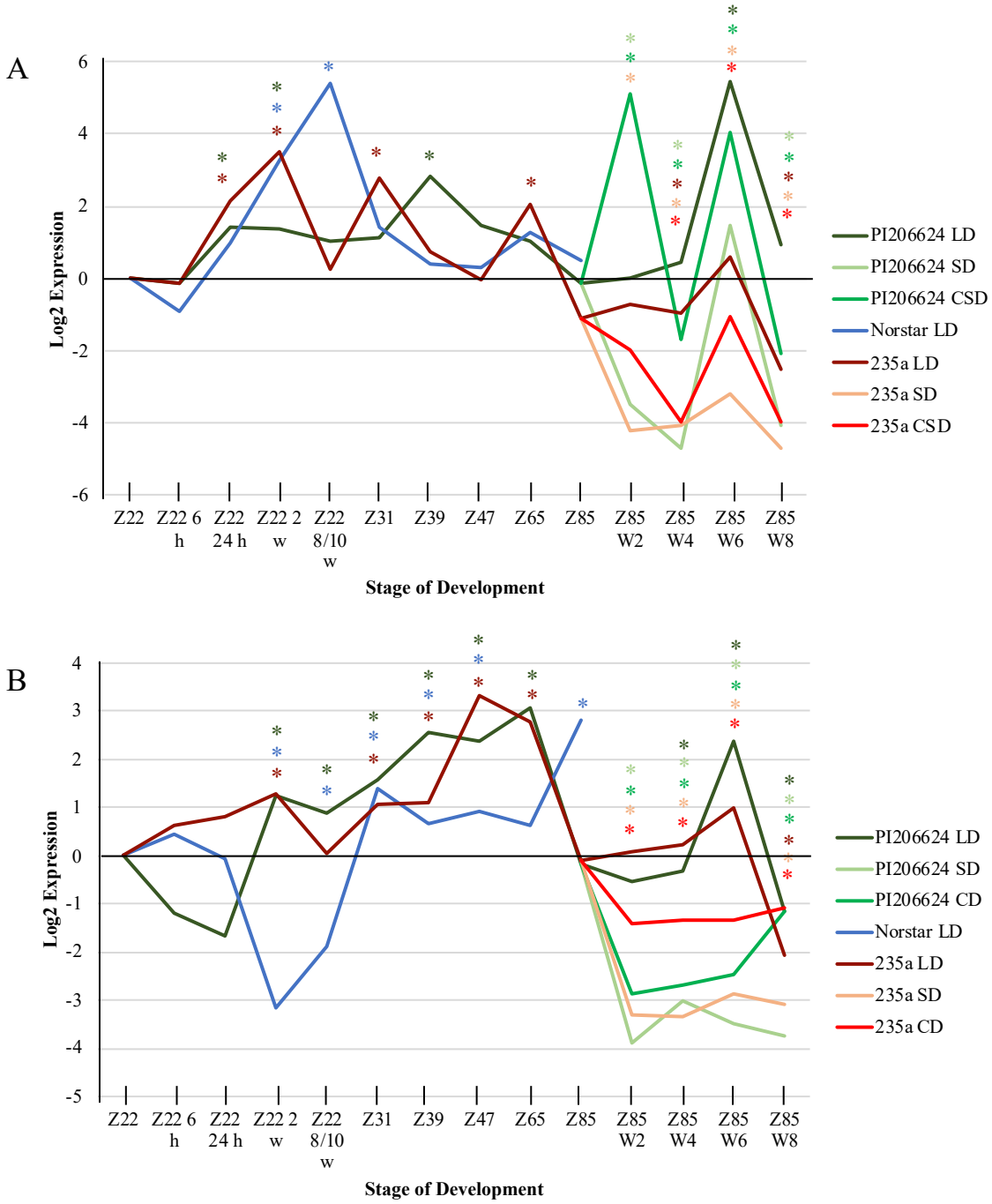


Figure 2.11 Log2 gene expression trends of *TFL1* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

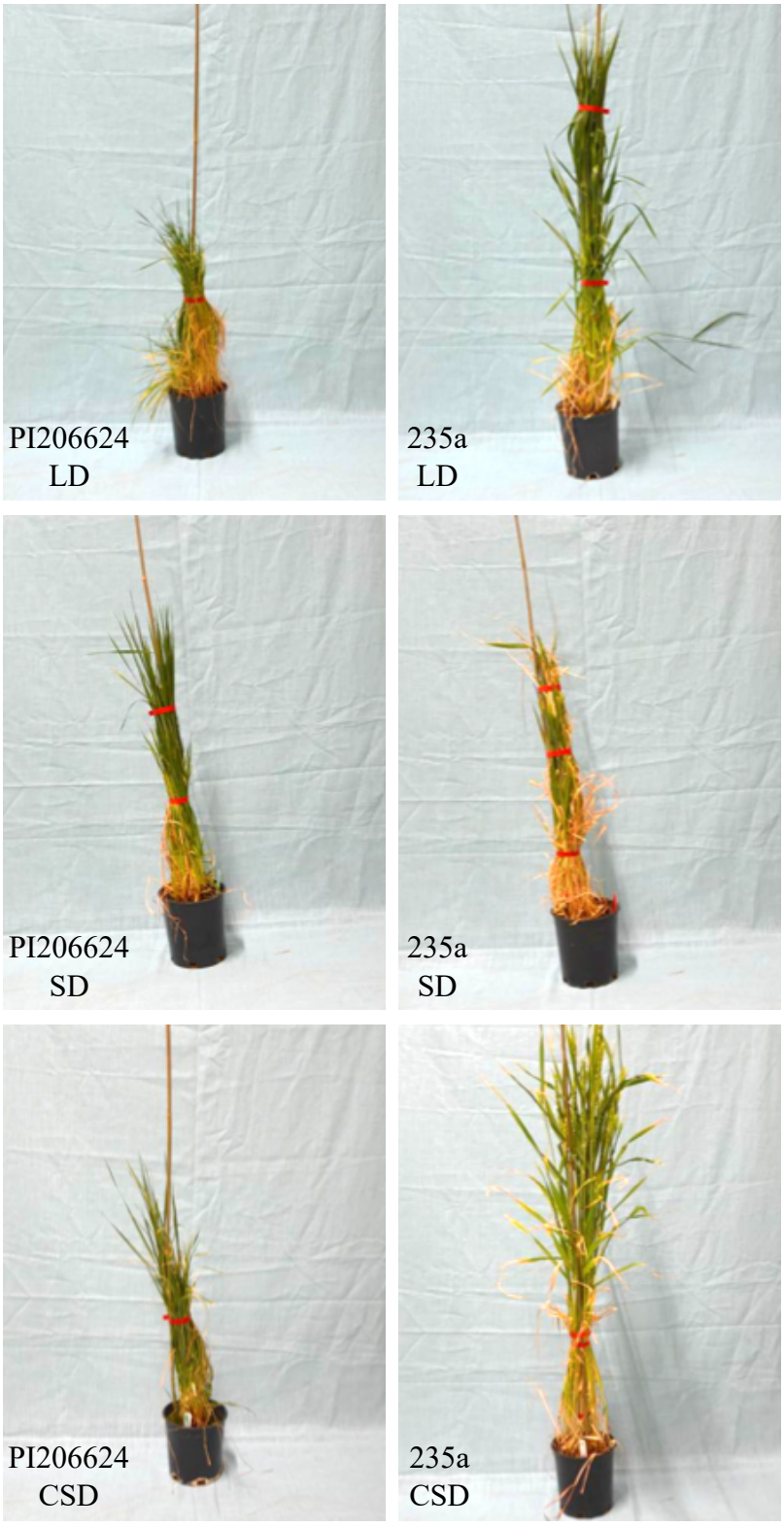


Figure 2.12 Wheatgrass line P1206624 and perennial wheat line 235a photographed at Z39 of the second sexual cycle, following exposure to either long days (LD), short days (SD) or cold temperatures and short days (CSD).

2.4 Discussion

2.4.1 Chromosome counting

Several studies involving meiotic stability relating to chromosome number of perennial wheat lines have been conducted (Fedak and Han 2005, Hayes et al. 2012, Hayes et al. 2018). It has been suggested that perennial wheat lines have the best performance in terms of yield, PSCR and fertility when they have a set of chromosomes from hexaploid (AABBDD) or tetraploid (AABB) wheat and a partial set from wheatgrass (Hayes et al. 2018). If hexaploid wheat is the parent, as for the perennial wheat lines used in this study, a chromosome number of 56 typically results (Hayes et al. 2012). However, because perennial wheat lines are often meiotically unstable, chromosome variation can occur even within the same line (Fedak and Han 2005).

The current study examined chromosome numbers in perennial wheat lines 235a, OK7211542, Agrotana and 11955 to ensure that they had a stable number of chromosomes. All lines have hexaploid wheat as a parent (2n=42) and 235a has *Thinopyrum elongatum* (2n=14) as the wheatgrass parent, while OK7211542, Agrotana and 11955 have *Thinopyrum ponticum* (2n=70) as the wheatgrass parent. Results confirmed that all perennial wheat lines had 56 chromosomes, which was expected from previous studies (Fedak and Han 2005, Hayes et al. 2012). This indicates that 235a is a full amphiploid, containing a full set of chromosomes from both parents, while OK7211542, Agrotana and 11955 are partial amphiploids (Hayes et al. 2018). The results ensure that the perennial wheat lines used for further experiments in this study have a

chromosome number that helps contribute to meiotic stability and likely will have better, yield, PSCR and stability for qPCR experiments in this study compared to other perennial wheat lines used in research (Hayes et al. 2012, Hayes et al. 2018).

2.4.2 Flowering without vernalization

Wheatgrass lines PI206624 and PI531718, wheat cultivars Norstar, Chinese Spring, AAC Awesome, Bhishaj, AC Meena and AC Nanda, and perennial wheat lines 235a, OK7211542, Agrotana and 11955 were grown without being vernalized to see if plants were able to flower. Previous studies indicate that most winter cultivars require vernalization and LDs in order to flower, while most spring cultivars only rely on daylength to induce flowering (Cockram et al. 2007, Greenup et al. 2009, Kamran et al. 2014). As expected, spring wheat cultivars Chinese Spring, AAC Awesome, Bhishaj, AC Meena and AC Nanda, which require only LDs for flowering, all flowered without being vernalized (Cockram et al. 2007). As expected, winter wheat Norstar did not flower, as winter cultivars require vernalization (Kamran et al. 2014). Wheatgrass line PI531718 also flowered without vernalization. As PI531718 is a perennial it was thought that it would require vernalization, like wheatgrass line PI206624, in order to flower, so this result was surprising. This suggests that PI531718 may only be partially insensitive to vernalization and flowered due to photoperiodic pathway (Zhang et al. 2008). It was anticipated that 235a would have a spring habit based on its parentage (Chinese Spring x PI531718 crossed to Madsen winter wheat), however, 235a did not flower without vernalization, suggesting that it takes on the recessive winter traits of Madsen (Allan et al. 1989). OK7211542, Agrotana and 11955 also did not flower. In summary all four

perennial wheat lines of interest behaved like winter cultivars and therefore required vernalization to flower, while wheatgrass cultivar PI531718 unexpectedly flowered without vernalization.

2.4.3 Wheat, wheatgrass and perennial wheat have comparable flowering time gene expression during the first vegetative and sexual cycle

Flowering time gene expression of *VRN1*, *VRN2*, *VRN3*, *PPD1* and *TFL1* was compared in wheatgrass lines PI206624 and PI531718, wheat cultivars Norstar, Chinese Spring and perennial wheat lines 235a, OK7211542, Agrotana and 11955 through the first vegetative and flowering cycle. Similar expression patterns were observed in all lines at early stages of development, suggesting that wheat, wheatgrass and perennial wheat have comparable flowering time gene expression in that developmental timeframe. The function of *VRN2* has been previously unknown in perennial grasses, while in annual cereals *VRN2* function as a floral repressor that is upregulated in leaf tissue prior to vernalization, causing *VRN1* and *VRN3* to be downregulated (Yan et al. 2004, Seppänen et al. 2010, Jokela et al. 2015). Results indicate that *VRN2* expression is similar in all lines and decreases during vernalization coincident with increased expression of *VRN1* and *VRN3*. This suggests that as in annual cereal crops, *VRN2* likely has a similar function as a floral repressor in wheatgrass and perennial wheat (Figure 2.13).

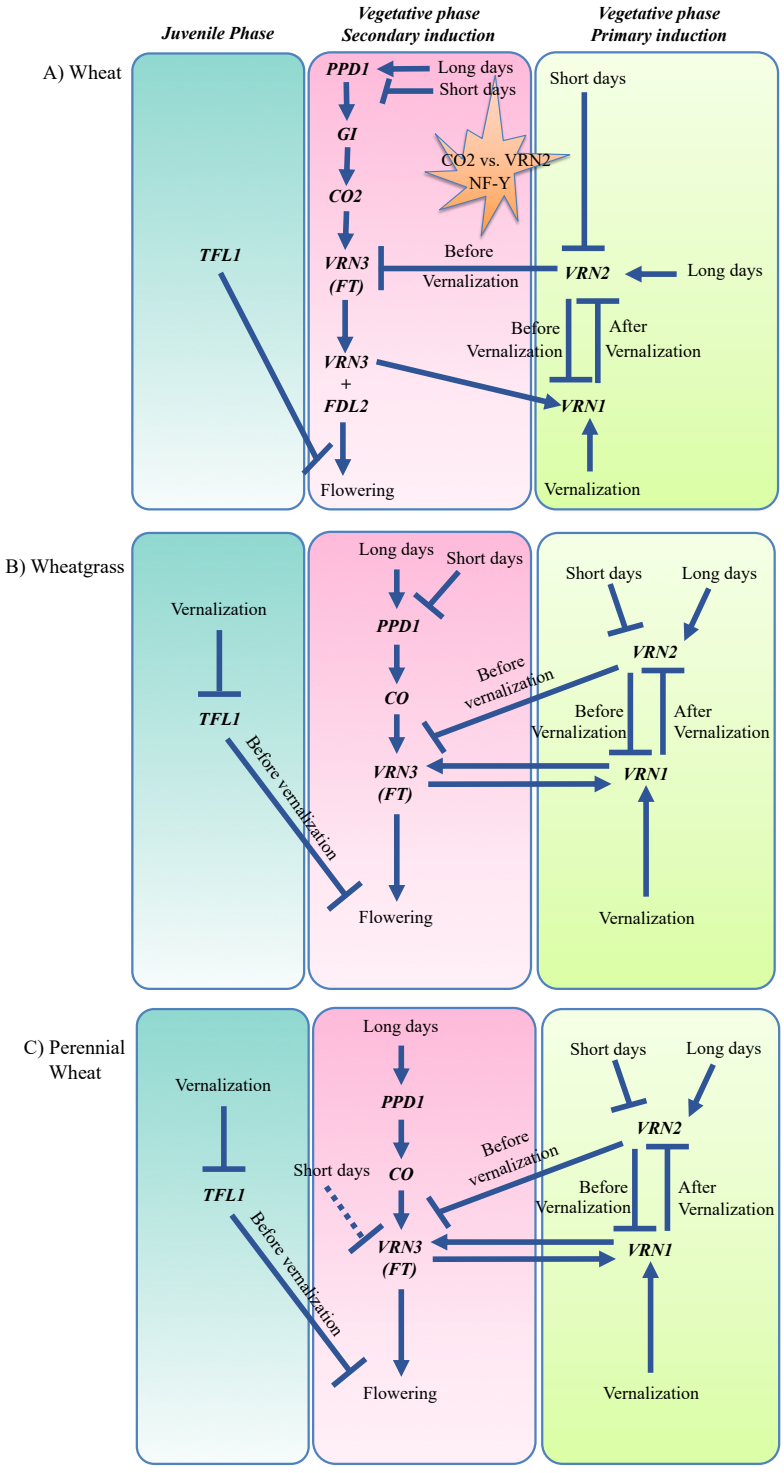


Figure 2.13 Updated flowering time pathway in (A) cereal, (B) wheatgrass and (C) perennial wheat. Dotted line represents SDs that are not as efficient at downregulating *VRN3*.

2.4.4 Perennial wheat line 235a has similar expression to perennial wheatgrass lines during the first vegetative and sexual cycle

Perennial wheat line 235a had the most similar expression of flowering time genes, especially *VRN1* and *VRN3,* to the wheatgrass lines. At stage Z85 in leaf and meristem tissue of 235a and both wheatgrass lines expression of *VRN1* and *VRN3* decreases. None of the perennial wheat lines show a comparable decrease in *VRN1* and *VRN3* expression. In perennials, this decrease in *VRN1* and *VRN3* at the end of flowering is expected, as perennials should begin to transition from the reproductive to the vegetative state in the fall to prepare for winter (Moore et al. 1991, Wang et al. 2009, Friedman and Rubin 2015). Since 235a is the only perennial wheat line that follows the expression pattern typical of perennial cycles it may be more efficient at transitioning to the vegetative state in the fall than other perennial wheat lines. Our results are consistent with previous field trials where 235a has also performed well in terms of persistence, PSCR and yield compared to other perennial wheat lines, suggesting it may be one of the better current perennial wheat lines (Hayes et al. 2012, Gazza et al. 2016, Hayes et al. 2018).

2.4.5 Expression of flowering time gene expression of plants exposed to varying photoperiods and temperatures

Expression of flowering time genes in general seems to indicate that PI206624 and 235a plants grown under LDs were likely in the reproductive phase during PSCR. *PPD1* expression under LDs was similar to its expression at Z22, consistent with previous

results in annual and perennial cereal crops. *PPD1* in leaf tissue of cereals and perennial grasses has been shown to be expressed when exposed to LDs (Kitagawa et al. 2012, Chen et al. 2014, Jokela et al. 2014). Floral promoters *VRN1* and *VRN3* were upregulated in PI206624 and 235a. In agreement with the results, LDs cause the upregulation of *PPD1* in cereals, which leads to the upregulation of *VRN3* (Shimada et al. 2009, Kitagawa et al. 2012, Chen et al. 2014). LD exposure also directly induces *VRN1*, causing *VRN1* and *VRN3* to interact in a positive feedback loop (Danyluk et al. 2003, Yan et al. 2006, Shimada et al. 2009, Jokela et al. 2015). The results of the study would be consistent with either mechanism. Floral repressor *VRN2* was downregulated, and *TFL1* had an expression level similar to the Z22 comparison stage. Low, or downregulation of these two floral repressors as *VRN1* and *VRN3* are upregulated, suggests that PI206624 and 235a grown in LD conditions are likely in the reproductive phase as *VRN2* and *TFL1* are only expressed when the plant is vegetative (Jensen et al. 2001, Yan et al. 2004).

Under short photoperiods in the fall, perennial plants begin to transition to the vegetative phase from the reproductive phase in order to prepare for winter (Moore et al. 1991, Wang et al. 2009, Friedman and Rubin 2015). In SD conditions *PPD1* is downregulated in both PI206624 and 235a which is consistent with the finding that *PPD1* requires LDs for upregulation (Chen et al. 2014). Under SD conditions, PI206624 and 235a are likely at least partially in the vegetative phase as *VRN1* and *VRN3* are downregulated (Yan et al. 2003, Yan et al. 2006, Seppänen et al. 2010, Jokela et al. 2014). Surprisingly, floral repressors *VRN2* and *TFL1* are downregulated or not detected whereas upregulation of *VRN2* and *TFL1* would be expected if plants are in the vegetative stage (Yan et al. 2003, Yan et al. 2006). Similarly, Seppänen et al. (2010) was unable to

detect *VRN2* in re-growing timothy tillers after harvest. Because timothy is a hexaploid and *VRN2* may have allelic variations, the result was difficult to interpret (Seppänen et al. 2010). *TFL1* is upregulated during vegetative growth in the fall (in perennial ryegrass, *A. alpina* and fruit trees such as apple and pear) and is required to prevent flowering and to keep the plant in the juvenile phase (Jensen et al. 2001, Kotoda et al. 2006, Wang et al. 2011, Freiman et al. 2012). The fact that *TFL1* and *VRN2* are downregulated or not detected in SDs for both PI206624 and 235a may indicate that the plants are not completely in the vegetative phase.

In CSD conditions the expression of flowering genes suggest that PI206624 and 235a plants are at least partially in the reproductive phase. *PPD1* was downregulated in both PI206624 and 235a following the expected pattern of *PPD1* being downregulated when exposed to a short photoperiod (Beales et al. 2007, Chen et al. 2014, Jokela et al. 2014). *VRN1* is upregulated under CSDs, suggesting that the plants may have fulfilled their vernalization requirement by the eight week exposure to both cooler temperatures and a short photoperiod, and have begun reproductive growth (Yan et al. 2003, Dhillon et al. 2010, Seppänen et al. 2010, Ergon et al. 2016). *VRN3* expression also increases in both tissue types of 235a and in meristem tissue of PI206624, suggesting that the increase in *VRN1* expression caused *VRN3* expression to increase, as found in cereals (Yan et al. 2006, Jokela et al. 2015). Floral repressor *VRN2* was downregulated or not detected and *TFL1* was downregulated, suggesting that the plants are not in the vegetative state (Jensen et al. 2001, Yan et al. 2003, Yan et al. 2006, Seppänen et al. 2010, Jokela et al. 2014). Since *VRN1* and *VRN3* are upregulated during CSDs in PI206624 and 235a, it suggests that the plants are at least partially in the reproductive phase and likely were able to fulfill

their vernalization requirement, thus causing floral repressors *VRN2* and *TFL1* to be downregulated (Jensen et al. 2001, Yan et al. 2003). This result is somewhat unexpected, as CSD grown plants were exposed to short photoperiods, which have been shown to cause floral promoters to be downregulated and cause the plant to begin to transition to the vegetative phase (Yan et al. 2003, Yan et al. 2006, Seppänen et al. 2010, Jokela et al. 2014). Plants may have remained partially in the reproductive phase because the light intensity of the plants in the vernalization room exposed to cold days was higher (575 μmol/m^2/s) than the plants exposed to SDs (340 μmol/m^2/s). In a study by Karsai et al. (2008), barley variety Dicktoo had a significant delay in flowering when exposed to lower light intensities between 210 and 290 μmol/m^2/s, while it flowered more quickly when exposed to a higher light intensity of 500 μmol/m^2/s. This could suggest that the higher light intensity in the CSD treatment caused the plants to begin reproductive growth. Plants may also not have gene expression levels in the PSCR stage return to the levels that they were expressed at Z22. As no studies have been conducted on this, expression levels of flowering time genes during PSCR and the second life cycle are unknown.

Z22 was chosen as a reference point to assess relative expression of flowering time genes. The phenotype of the known perennial wheatgrass PI206624 appeared closest to the Z22 stage of development, suggesting comparisons of gene expression to that stage were appropriate. Z85 could have been chosen as a reference point because 235a had phenotypic characteristics more similar to Z85. However, using Z85 as a reference resulted in similar gene expression trends for all flowering time genes in both PI206624 and 235a, suggesting that whether Z22 or Z85 was used as a reference, expression trends would remain the same.

2.4.6 Perennial wheat line 235a may not be as efficient at responding to photoperiod and temperature cues as wheatgrass PI206624

Expression of flowering time genes *VRN1*, *VRN2*, *VRN3*, *PPD1* and *TFL1* in plants PI206624 and 235a exposed to different photoperiods and temperatures display some similarities, as well as some important differences. Expression of flowering time genes *VRN2*, *PPD1* and *TFL1* are relatively similar between PI206624 and 235a under LDs, SDs and CSDs, while important differences are observed in *VRN1* and *VRN3* expression levels, where 235a had higher relative expression levels of these genes than PI206624 under SD and CSD conditions. Under short photoperiods in the fall, perennial plants begin to transition to the vegetative phase from the reproductive phase in order to prepare for winter (Moore et al. 1991, Wang et al. 2009, Friedman and Rubin 2015). This vegetative phase change in the fall allows perennials to prevent freezing in the reproductive state and to cold acclimate to their environmental conditions to survive the winter (Andrés and Coupland 2012, Friedman and Rubin 2015, Hayes et al. 2018) As a result, expression of *VRN1* and *VRN3* is expected to be downregulated when plants are exposed to SDs and CSDs (Yan et al. 2003, Distelfeld et al. 2009a, Seppänen et al. 2010, Malyshev et al. 2014). This is the case for PI206624, and although 235a had a decrease in *VRN1* and *VRN3* expression when exposed to a short photoperiod, its expression of *VRN1* and *VRN3* was much higher than in PI206624 (Figure 2.13 C). Although 235a seemed to perform better than other perennial wheat lines, the high expression of *VRN1* and *VRN3* under CSDs and SDs still suggest that 235a may not be as efficient at responding to photoperiod and temperature cues and therefore likely is not able to transition to the vegetative phase as completely as perennial PI206624.

Although PI206624 and 235a plants were only sampled until Z85 W8 for gene expression analysis, plants continued growing into their second life cycle. Photos of PI206624 and 235a plants grown under LDs, SDs and CSDs taken at Z39 of the second cycle reveal phenotypic differences between lines and growing conditions. PI206624 plants had more vegetative leaves throughout the second life cycle in all three growing conditions, while 235a plants remained more reproductive. CSD plants of both lines also looked more vigorous throughout the second life cycle. It is likely that the cooler temperature slowed growth under CSDs compared to LD and SD conditions. Alternatively, the CSD plants were cut short several times in order to fit under the lights in the vernalization room, allowing them to develop new tillers, while LD and SD plants were only trimmed once. The phenotypic results corresponded with gene expression and reversion to vegetative growth.

2.4.7 Summary

The similar expression of flowering time genes *VRN1, VRN2, VRN3, PPD1* and *TFL1* during the first vegetative and sexual cycle suggest that wheat, wheatgrass and perennial wheat share similar mechanisms driving flowering time. Results suggest that *VRN2* likely functions as a floral repressor in wheatgrass and perennial wheat as it does in cereal crops during the first vegetative and sexual cycle. During the first vegetative and sexual cycle, 235a was the only perennial wheat line that followed the expression pattern typical of perennial cycles, suggesting that it may be more effective at transitioning to the vegetative state in the fall than other perennial wheat lines. This result is in agreement with previous field trials where 235a also performed well in terms of persistence, PSCR

and yield compared to other perennial wheat lines, indicating it may be one of the better current perennial wheat lines (Hayes et al. 2012, Gazza et al. 2016, Hayes et al. 2018). Since gene expression and field trial results are consistent, this suggests that gene expression experiments may be an alternative, or first step to identifying potential lines to grow in field trials. Gene expression results from PI206624 and 235a plants exposed to varying photoperiods and temperatures during the PSCR using Z85 as a reference resulted in similar gene expression trends for all flowering time genes in both PI206624 and 235a, suggesting that regardless if Z22 or Z85 was used as a reference, expression trends are the same. Plants exposed to LD conditions are likely in the reproductive phase based on genes tested, SD exposed plants are at least partially in the vegetative phase, and unexpectedly CSD exposed plants are likely partially reproductive. Differences in expression level of flowering time genes *VRN1* and *VRN3* were observed between PI206624 and 235a, where 235a had higher relative expression levels of these genes than PI206624 under SD and CSD conditions. These expression patterns support the hypothesis that compared to the perennial wheat grass (PI206624), line 235a is responding less efficiently to photoperiod and temperature and therefore is not able to transition as completely to the vegetative phase.

CHAPTER THREE: EXPRESSION OF COLD TOLERANCE GENES IN WHEAT, WHEATGRASS AND PERENNIAL WHEAT

3.1 Introduction

3.1.1 Development and challenges of perennial wheat

Perennial wheat development has been investigated since the 1920's due to potential economic and environmental benefits such as reduced input costs, decreased need for fertilizer and herbicide, reduced soil erosion, and improved sequestration of carbon dioxide from the atmosphere (Gantzer et al. 1990, Wagoner and Schaeffer 1990, Bell et al. 2008, Glover et al. 2010, Pimentel et al. 2012). Wide hybridization is currently a common method for developing perennial wheat (Pimentel et al. 2012). Recent hybridization methods involve crossing hexaploid wheat (*Triticum aestivum* L.) or tetraploid wheat (*Triticum turgidum*) with wheatgrass species such as *Th. ponticum, Th. elongatum* or *Th. intermedium* to produce perennial wheat lines (Fedak and Han 2005, Gazza et al. 2016). However, current perennial wheat lines have several challenges such as poor winter survival and weak perennial growth habits (Bell et al. 2008, Hayes et al. 2012, Hayes et al. 2018).

In relation to cold tolerance, the main challenge of current perennial wheat lines is that they have poor winter survival and persistence (Hayes et al. 2018). Plant survival over the winter is affected by both genetics and the environment (Murphy et al. 2010). According to a study by Hayes et al. (2018), perennial wheat lines that had spring wheat

as one of the parents had poor winter survival, while lines that had a winter wheat parent had better survival, likely due to that fact that winter wheat has the ability to better cold acclimate. Hayes et al. (2018) also showed that climate had an influence on perennial wheat persistence, with *Th. intermedium* crosses more suitable for colder climates and *Th. ponticum* and *Th. elongatum* crosses persisting better in warmer climates. However, most perennial wheat lines had low winter survival rates, with no plants living longer than 3 years (Hayes et al. 2018). Several other studies have also revealed poor winter survival rates, with most plants dying during the first winter, suggesting that the plants may not be able to induce a high or at least sufficient level of cold tolerance post sexual cycle reproduction (Murphy et al. 2010, Hayes et al. 2012). Perennial wheat lines that are able to cold acclimate need to be identified, as these plants are likely to have improved winter survival.

3.1.2 Cold tolerance

A sufficient level of cold tolerance is essential for winter type cereal cultivars and perennials for the plant to survive the winter. When plants are exposed to freezing temperatures ice can form in the extracellular area between the xylem and phloem tissue and cells causing cellular dehydration from the water being drawn away from surrounding cells (Preston and Sandve 2013, Rihan et al. 2017). The ice will expand to other extracellular spaces and will draw water away from surrounding cells, causing dehydration and potential membrane damage (Preston and Sandve 2013, Rihan et al. 2017). In order to prevent this from occurring, winter cultivars and perennial grasses undergo cold acclimation (Thomashow 2001). Cold acclimation, or low temperature

tolerance takes place when a plant is exposed to low, non-freezing temperatures (Thomashow 2001). Cold acclimation induces cold tolerance, prevents freezing damage, and increases overwintering survival (Thomashow 2010). Evidence suggests that cold tolerance can only be acquired by the plant during the vegetative phase, while the ability to cold acclimate is lost once the plant begins the reproductive phase (Mahfoozi et al. 2001). Cold acclimation happens rapidly, with cold tolerance genes being upregulated within a few hours of being exposed to a specific induction temperature (Fowler 2008). However, cold acclimation is cumulative and most plants do not completely cold acclimate until the temperature is below the induction temperature (Fowler 2008). The cold tolerance pathway is relatively well understood in *Arabidopsis* and annual cereals, while little is known about cold tolerance in perennials.

3.1.3 Cold tolerance in Arabidopsis

Winter cultivars of *Arabidopsis*, that have the ability to cold acclimate to prevent freezing, have been widely studied for cold tolerance (Thomashow 2010). Ca^{2+} levels increase under low temperatures, causing a calcium-calmodulin complex to bind to the promoter region of *CBF* (*C-REPEAT BINDING FACTORS*) genes (Thomashow 2001). Three *Arabidopsis CBF* genes have been identified, *CBF1*, *CBF2* and *CBF3*, which are members of the AP2/EREBP (APETALA2 and ethylene-responsive element binding proteins) transcription factor family (Stockinger et al. 1997, Gilmour et al. 1998, Liu et al. 1998, Thomashow 2001). Once exposed to cold temperatures, *CBF* expression quickly increases followed by the upregulation of *COR* (*COLD REGULATED*) genes leading to cold tolerance (Gilmour et al. 1998). As *CBF* and *COR* genes are upregulated quickly, an

increase in freezing tolerance can occur within a day, however cold tolerance typically increases over time (Wanner and Junttila 1999). *ICE1* (*INDUCER OF CBF EXPRESSION1*) can also be upregulated under cold temperatures, with its expression being regulated by *SIZ1* and *HOS1* (*HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1*). Under warmer temperatures *HOS1* downregulates *ICE1*, while under cooler temperatures the SIZ1 protein causes the upregulation of *ICE1*, followed by the upregulation of *CBF*s and *CO*R genes leading to cold tolerance (Chinnusamy et al. 2003, Chinnusamy et al. 2007).

Photoperiod has been shown to influence the *Arabidopsis* cold acclimation pathway. *Arabidopsis* plants exposed to SDs (short days) were shown to have higher *CBF* expression than plants grown under LDs. The mechanism of this is that under LDs (long days), *PHYB* (*PHYTOCHROMEB*) and transcription factors *PIF4* and *PIF7* cause *CBF*s to be downregulated (Thomashow 2010). In SDs, the opposite occurs with *PHYB*, *PIF4* and *PIF7* being downregulated, allowing *CBF* and *COR* genes to be upregulated (Lee and Thomashow 2012). This control framework allows winter type *Arabidopsis* cultivars to cold acclimate more efficiently when exposed to SDs than LDs (Lee and Thomashow 2012).

Flowering time and cold tolerance genes have also been shown to interact in *Arabidopsis. FLOWERING LOCUS C* (*FLC*) expression increases in cool temperatures, to prevent the plant from flowering, but as the temperature increases *SOC1* (*SUPPRESSOR OF OVER EXPRESSION OF CONSTANS*) becomes upregulated, leading to the downregulation of *CBF* expression and flowering to occur (Seo et al. 2009). This

downregulation of *CBF* genes shows that cold acclimation is lost once the plant begins the reproductive phase as *SOC1* is upregulated (Seo et al. 2009). As in cold acclimation, vernalization can also cause *CBF* and *COR* genes to be upregulated, however vernalization causes *FLC* to be downregulated after 30-40 days of cold exposure (Sung and Amasino 2004b, Seo et al. 2009). Exposure to a long vernalization period causes *VERNALIZATION INSENSITIVE3* (*VIN3*) to be upregulated, therefore downregulating *FLC* and promoting flowering (Sung and Amasino 2004b).

3.1.4 Cold tolerance in annual cereals

Winter cereal crops are able to cold acclimate to prevent freezing, allowing winter survival (Fowler 2008). While *Arabidopsis* has only three *CBF* genes, 37 *CBF* genes have been identified in wheat and 20 have been found in barley (Skinner et al. 2006, Campoli et al. 2009). However, *CBF* genes in the three species function in a similar way (Campoli et al. 2009). Cereal *CBF* genes are rapidly upregulated when plants are exposed to cold temperatures (Campoli et al. 2009). The proteins they encode contain an *AP2* DNA binding domain which allows binding to the CRT/DRE (C-repeat/dehydration responsive element) motif located in the regulatory regions of the *COR* genes (Campoli et al. 2009). The resulting *COR* induction leads to cold tolerance (Campoli et al. 2009). *ICE1* in cereals causes the upregulation of *CBF* genes under cold temperatures, leading to the upregulation of *COR* genes, causing the plant to become cold tolerant (Skinner et al. 2006, Badawi et al. 2008). Important cold tolerance genes *CBF12*, *CBF14* and *COR14* have been identified in wheat and barley (Rapacz et al. 2008, Galiba et al. 2009, Novák et al. 2015, Erath et al. 2017). *CBF12* and *CBF14* become upregulated around 15°C and are

therefore more sensitive to threshold induction temperatures than other *CBF*s which usually become upregulated only at colder temperatures (Campoli et al. 2009, Novák et al. 2015). *COR14* codes for a chloroplast-targeted protein that protects the plant from photodamage due to light exposure after freezing and is upregulated by *CBF* genes (Rapacz et al. 2008, Galiba et al. 2009).

CBF and *COR* genes have been shown to be connected to flowering time genes in cereals (Galiba et al. 2009). When plants are in the vegetative state in the fall, cold temperatures induce *CBF* and *COR* while *VRN1* (*VERNALIZATION1*) is downregulated by *VRN2* (*VERNALIZATION2*) (Dhillon et al. 2010). In late winter or early spring when the vernalization requirement has been fulfilled, *VRN1* is upregulated whereas *CBF* and *COR* expression decreases, causing the plant to lose its cold tolerance (Dhillon et al. 2010). It has been suggested that *VRN1* negatively regulates cold tolerance genes, as an increase in *VRN1* expression causes cold tolerance genes to become downregulated. (Limin and Fowler 2006, Dhillon et al. 2010).

Temperature and photoperiod also act together in the cereal cold tolerance pathway (Mahfoozi et al. 2001). SD exposure causes *PHYB* to be downregulated leading to the upregulation of *CBF*s in wheat and barley (Novák et al. 2015). Cereals grown under SDs have also been shown to be able to tolerate temperatures 8.5°C colder than those grown under LDs, implying that cereals are able to induce cold tolerance more efficiently in SDs (Limin and Fowler 2006). Exposure to SDs will also repress *VRN1* and *VRN3* (*VERNALIZATION3)* expression, preventing the positive feedback loop between

these genes and causing the plant to remain vegetative (Danyluk et al. 2003, Yan et al. 2006, Dhillon et al. 2010).

3.1.5 Cold tolerance in perennials

Identity and regulation of cold tolerance genes in perennial grasses is largely unknown, although it is believed to be similar to the cold tolerance pathway in cereals (Sandve et al. 2011, Wingler 2014). Several *CBF* genes have been identified in perennial ryegrass (*Lolium perenne* L.), and they have been shown to become quickly upregulated after cold exposure leading to the upregulation of *COR* genes (Xiong and Fei 2006, Tamura and Yamada 2007, Zhao and Bughrara 2008). This indicates that the cold tolerance pathway in cool season grasses is likely conserved, however little is known about specific cold tolerance genes (Sandve et al. 2011, Wingler 2014). Cold tolerance genes and *VRN1* have been shown to interact in meadow fescue (*Festuca pratensis* L.) in that *VRN1* was shown to potentially cause the downregulation of cold tolerance genes as photoperiod increased towards the end of the vernalization period (Ergon et al. 2016, Kovi et al. 2016).

Photoperiod also influences the cold acclimation of perennial grasses. Under SD exposure, perennial ryegrass (*Lolium perenne*), timothy (*Phleum pretense*), white clover (*Trifolium repens L.*) and false oat grass (*Arrhenatherum elatius* L.) were able to cold acclimate more efficiently than under LDs, displaying increased regrowth and survival (Junttila et al. 1990, Malyshev et al. 2014, Dalmannsdottir et al. 2017).

3.1.6 Objectives

Understanding the cold acclimation ability and the relationship with the life cycle of perennial wheat would be an important innovation in agriculture. Perennial wheat lines currently have poor persistence, and generally die after the first year of growth, suggesting that they are weak perennials (Hayes et al. 2018). Currently little is known about cold tolerance in perennial grasses, however, the pathways are thought to be conserved between annual and perennial cool season grasses. Therefore, most of the information about cold tolerance in cool season perennial grasses is based on annual cereal crops (Sandve et al. 2011, Wingler 2014). In fact, aspects of the pathway are thought to be different (Sandve et al. 2011, Wingler 2014). Understanding cold tolerance in perennials could allow researchers to better understand cold tolerance in existing varieties and develop new varieties of perennial cereals with improved cold tolerance and possibly better persistence. This study compared cold tolerance gene expression in two species of wheatgrass *Th. ponticum* and *Th. elongatum*, two wheat cultivars Norstar and Chinese Spring, and perennial wheat lines 235a, OK7211542, Agrotana, and 11955 derived from crosses between annual wheat and *Th. ponticum* or *Th. elongatum* (Table 3.1) (Grant 1980, GRIN 2007a, b, Hayes et al. 2012, CFIA 2013).

1. Establish sequences of cold tolerance genes *CBF12*, *CBF14* and *COR14* in two species of wheatgrass *Th. ponticum* and *Th. elongatum*.
2. Determine LT_{50} values of wheat lines (Norstar and Chinese Spring), wheatgrass cultivars (*Th. ponticum* and *Th. elongatum*), and perennial wheat lines (235a, OK7211542, Agrotana, and 11955).

Table 3.1 Plant types, accessions, growth habit and ploidy levels grown for cold tolerance experiments.

Plant Type	Cultivar/Accession	Pedigree	Expected Growth Habit	Ploidy	Reference
Wheat	(1) Norstar (2) Chinese Spring	-	(1) Winter (2) Spring	(1) 2n=6x=42 (2) 2n=6x=42	Grant 1980 CFIA 2013
Wheatgrass	*(1) Thinopyrum ponticum* (PI206624) *(2) Thinopyrum elongatum* (PI531718)	-	(1) Perennial (2) Perennial	(1) 2n=10x=70 (2) 2n=2x=14	GRIN 2007a GRIN 2007b
Perennial Wheat	(1) 235a (2) OK7211542 (3) Agrotana (4) 11955	(1) Madsen//Chinese Spring/PI531718 (2) Wheat -*Thinopyrum ponticum* partial amphiploid (3) Wheat-*Thinopyrum ponticum* partial amphiploid (4) Wheat-*Thinopyrum ponticum* partial amphiploid	(1) Unknown (2) Unknown (3) Unknown (4) Unknown	(1) Full Amphiploid 2n=8x=56 (2) Partial Amphiploid 2n=8x=56 (3) Partial Amphiploid 2n=8x=56 (4) Partial Amphiploid 2n=8x=56	Hayes et al. 2012

3a. Evaluate expression of cold tolerance genes *CBF12*, *CBF14* and *COR14* in leaf and apical meristem tissues at different stages of development during the first vegetative and sexual life cycle of wheat, wheatgrass and perennial wheat.

3b. Evaluate expression of the same cold tolerance genes in leaf and apical meristem tissue of plants considered to be in the PSCR stage.

3.1.7 Hypothesis

Perennial wheat lines will have similar cold tolerance gene expression to perennial wheatgrass and annual wheat during the first sexual cycle, but as temperature and daylength changes, perennial wheat lines will not be able to completely respond to these changes and will not be able to upregulate cold tolerance genes in a similar way to perennial wheatgrass species *Th. ponticum*.

3.2 Materials and Methods

3.2.1 Plant materials and planting

Eight plant lines, including two varieties of wheat (spring wheat Chinese Spring and winter wheat Norstar), two accessions of wheatgrass (PI206624 (*Thinopyrum ponticum*) and PI531718 (*Thinopyrum elongatum*)), and four lines of perennial wheat (235a, OK7211542, Agrotana, and 11955) were used for cold tolerance experiments (Table 3.1) (Grant 1980, GRIN 2007a, b, Hayes et al. 2012, CFIA 2013). Perennial wheat lines 235a, OK7211542, Agrotana, and 11955 were chosen as they had good PSCR, winter survival and yield performance in previous studies (Hayes et al. 2012, Hayes et al. 2018). Three biological replicates of each of the eight lines were grown. The seeds were germinated on damp filter paper placed in a 150 mm x 15 mm petri dish at room temperature in darkness and watered twice a week. After one week, seedlings were planted in 4x8 Rootrainers™ (Rootrainers International, Canada). The potting mix used was blended by the greenhouse staff at the Lethbridge Research and Development Centre and was based on a peat-based mix developed by Boodley and Sheldrake (1972), including 1 bale of sphagnum peat moss (108 L), 2 bags of vermiculite (18.6 kg/bag), a bag of Turface MVP (22.7 kg), fertilizer mix (1500 g of 18-6-12 Osmocote, 1200 g of monocalcium-dicalcium phosphate, 1000 g of calcium carbonate powder, 15 g of 13.2% chelated iron, and 7 g of 14% chelated zinc), and water (9-23 L). Plants were grown in a 16/8 h day/night growth chamber at 20ºC/16ºC day/night at a light intensity of 340 µmol/m^2/s and watered once daily. The Zadoks scale, developed to describe the growth stages of cereals, was used to assign developmental stages to the plants. At Zadoks stage Z22, plants were moved to a 4ºC room to vernalize for 8 weeks (wheat and perennial wheat) or 10 weeks (wheatgrass)

with 8/16 h day/night photoperiod under a light intensity of 575 μmol/m^2/s. After vernalization, the plants were moved back into the 16/8 h day/night growth chamber at 20ºC/16ºC day/night.

3.2.2 Sampling and preparation of wheatgrass tissue for cloning and sequencing

Wheatgrass seedling accessions PI206624 (*Th. ponticum*) and PI531718 (*Th. elongatum*) were used for the cloning and sequencing experiment. Plants were germinated, planted and grown as described above in section 3.2.1. At Zadoks stage Z14, plants were moved to a 4ºC growth chamber for vernalization with 10 h of light and 14 h of dark and a light intensity of 575 μmol/m^2/s (Zadoks et al. 1974). The sampling protocol of wheatgrass was based on a similar experiment (Campoli et al. 2009). Three replicates of leaf and crown meristem tissues were collected from the plants at 0, 6 and 24 h after the start of the vernalization treatment.

3.2.3 Preparation of cDNA

Total RNA was extracted from frozen tissue following the RNeasy Plant Mini Kit protocol (QIAGEN, Canada). The quality of the RNA was checked using a Bioanalyzer (Agilent, USA) or a QIAxcel (QIAGEN, Canada), followed by an off-column DNA digestion using the DNase I Amplification Grade Kit (ThermoFisher Scientific Inc., Canada). A standard PCR, using HotStar Taq DNA polymerase (QIAGEN, Canada), was conducted on the RNA samples using housekeeping gene primers for *ELONGATION FACTOR1a* (*ELF1a)* or *CONTIG5* (Table 3.2). One reaction was made by mixing 5 μL of

Table 3.2 Cold tolerance primer sets selected for qPCR to amplify cDNA from genes of interest.

Gene of Interest	qPCR Primer	Tm (°C)	Expected Size of qPCR Product (bp)	Accession Number
CBF12	Forward: TAG TAA CGG CCG ATG GGT GT Reverse: CTC GGC GGT GAC GTG C	60	85	HG530926.1
CBF14	Forward: CCA AGG ATC TGG GCG AGA AG Reverse: TTT GCT CAC ATC CTC GAC CG	60	104	HG530930.1
COR14	Forward: CCA AGG ATC TGG GCG AGA AG Reverse: TTT GCT CAC ATC CTC GAC CG	60	116	FJ6052070.1
ELF1a	Forward: GGT GAT GCT GGC ATA GTG AA Reverse: GAT GAC ACC AAC AGC CAC AG	60	125	M90077.1
CONTIG5	Forward: CTG CAG TGC GTG CAT ATT TT Reverse: AAC AAG AAC GAT GCC GAG TT	60	141	CK155621.1

H_2O, 2.5 µL Q Solution, 2 µL 10x buffer, 2 µL dNTP, 2µL each of forward and reverse primer, 0.15 µL HotStar Taq and 2 µL of $^1/_{10}$ diluted template. PCR was followed by gel electrophoresis, using a 1% agarose gel and 1x sodium borate buffer, to determine that samples were free of DNA. Sometimes qPCR using the QuantiTect SYBR Green PCR Kit, was performed on the DNase treated RNA samples instead of a standard PCR to increase the number of samples that could be run at once. For one reaction, 5 µL of SYBR Green, 0.6 µL each of *ELF1a* or *CONTIG5* forward and reverse primer, 1.3 µL of H_2O, and 2.5 µL of $^1/_{10}$ diluted RNA sample, were mixed for a total reaction of 10 µL. The following program was used to amplify the samples on an ABI QuantStudio 6 Flex qPCR System: 1 cycle at 50ºC for 2 min, 1 cycle at 95ºC for 3 min, 40 cycles at 95ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 30 sec (ThermoFisher Scientific Inc., Canada). Once the samples were determined to have no DNA contamination, cDNA was synthesized from the purified RNA to use in PCR according to the supplier's instructions in the SuperScript III Reverse Transcriptase kit (ThermoFisher Scientific Inc., Canada).

3.2.4 PCR primer design

PCR primer pairs were developed to evaluate cold tolerance genes of interest, *CBF12*, *CBF14* and *COR14* (Table 3.3). The primers were created by using known sequences from close relatives of wheatgrass including wheat, barley, and rye (Table 3.3). Sequences were obtained from NCBI and aligned using Geneious in order to determine a consensus sequence (Kearse et al. 2012). The primers were located about 20 bases before

Table 3.3 Cold tolerance primer sets developed based on a consensus sequence of close relatives of wheatgrass, selected to PCR amplify genes of interest for sequencing.

Gene of Interest	Primer	Tm (°C)	Expected size of gene in wheat (bp)	Accession number of sequences used for consensus (GenBank)
CBF12	Forward: CCT CCT CCA GTC AAC TAG TCA AG Reverse: TTG ACC GGA GTC CCT CGG	62	738	Wheat: HG530926.1 Barley: DQ095157.1 Rye: HQ730767.1
CBF14	Forward 1: ATG GAC GCC GCT GAT GCC G Reverse 1: TTA GTC GAA CAA GTA GCT CC Forward 2: CGC AGC AGC TAA ACA CGC TAA Reverse 2: ACT CAA GTA CAA ATG CGC CCT C	56 58	629 646	Wheat: HG530930.1 Barley: DQ095159.1 Rye: HQ730768.1
COR14	Forward 1: ATG GCT TCT TCT TCC GTG CT Reverse 1: TCA TTT GCT CAC ATC CTC GAC Forward 2: ATC CAT CGG CGC CAG ACT AC Reverse 2: ACG TGA CCC ACA AAA GAC ACT C	56 58	423 520	Wheat: FJ6052070.1 Barley: AK359732 .1 Rye: AF491839.1

and after the start and stop codon respectively, and their sequences were chosen based on the conserved bases in the Geneious Pairwise/Multiple Alignment (Kearse et al. 2012).

3.2.5 Cloning and sequencing

A standard PCR protocol using HotStar Taq DNA polymerase (QIAGEN, Canada) as described in section 3.2.3, was used to amplify the coding region of cold tolerance genes, *CBF12, CBF14* and *COR14,* using the gene specific primers developed above, from a pool of wheatgrass cDNA (QIAGEN). Once amplified, the PCR products were separated using gel electrophoresis on a 1% agarose gel and 1x TAE buffer, and viewed under a Blue Light LED Transilluminator (BLook) (GeneDireX, Inc., USA). DNA bands of the expected size for each gene of interest (between 420-750 base pairs) were extracted and purified using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel Inc., Canada). Purified DNA was inserted into the pDrive Cloning Vector (QIAGEN) using the QIAGEN PCR Cloning Kit (QIAGEN). A ligation mixture containing 2.5 µL of 2X buffer, 0.5 µL of pDrive Vector and 2.0 µL of the insert was made for each ligation reaction with a 3:1 target insert:vector ratio. The ligation reaction was incubated for 2 h at 4°C. The pDrive vector containing the coding region of interest was transformed into *E. coli* DH5α chemically competent cells (ThermoFisher Scientific Inc., Canada). DH5α cells were grown on LB agar plates containing 100 µM ampicillin and 100 µM X-gal. Using blue/white colony selection, single white colonies were chosen to be added to a standard PCR mixture, using TopTaq DNA polymerase (QIAGEN), for colony PCR to amplify the insert in the pDrive vector in the presence of vector specific primers M13 forward primer (TGT AAA ACG ACG GCC AGT) and M13 reverse primer

(CAG GAA ACA GCT ATG AC). A single PCR reaction contained: 20.5 µL of H_2O, 2.5 µL 10x buffer, 1 µL dNTP, 0.5 µL each of forward and reverse primer, 0.15 µL TopTaq, and one white colony (QIAGEN). Positive colonies were selected based on the expected size of the product by comparing against a 100 bp plus DNA ladder in a 1% agarose gel (ThermoFisher Scientific Inc., Canada). Positive colonies were grown overnight at 37°C in 5 mL of LB containing 100 µM of ampicillin in a 15 mL tube, with agitation at 210 rpm. Plasmid DNA was extracted from the successful transformants using the QIAprep Spin Miniprep Kit (QIAGEN) and sent for sequencing at Genome Quebec (Genome Quebec 2018). The sequenced cold tolerance genes in wheatgrass were compared to cold tolerance nucleotide and amino acid sequences in wheat, barley and rye using Geneious 8.9.1 Pairwise/Multiple Alignment to confirm the sequence was from the targeted gene (Kearse et al. 2012).

3.2.6 Plant materials for freezing test

Seeds of PI206624, PI531718, Norstar, Chinese Spring, 235a, OK7211542, Agrotana, and 11955 were germinated as described in section 3.2.1 (Table 3.1). Seedlings were planted in every second book of a 5x12 Rootrainers™, with books in between being empty, resulting in 20 plants/ Rootrainer™. For one replicate, six Rootrainers™ were planted for each line (one Rootrainer™ for each of the six temperatures tested, for a total of 20 plants per temperature point). A total of three replicates were tested. Plants were grown in a 16/8 h day/night growth chamber at 20º C/16 ºC day/night with a light intensity of 340 µmol/m^2/s and watered once daily.

3.2.7 Freezing test

At the 1.5 leaf stage plants in Rootrainers™ were moved to the vernalization room (8/16 h day/night at 4 ºC and a light intensity of 575 µmol/m^2/s for 3 w). Plants in Rootrainers™ were watered evenly the day before the start of cold tolerance testing. Twenty plants in the Rootrainer™ for each line were left at 4 ºC to be used as a control. The other plants were moved to a freezing chamber set at -3 ºC with no light to acclimate for one day. The following day the temperature was dropped -2 ºC/h until the temperature points for this experiment (-3 ºC, -7 ºC, -11 ºC, -15 ºC and -19 ºC) were reached. Once plants reached the desired temperature point, they were removed and placed back in the 4 ºC vernalization room with no direct light to thaw for 24 h. After thawing, plants were moved to the greenhouse, with regular LD conditions (16/8 h day/night at 20ºC/16ºC day/night) and a light intensity of 230 µmol/m^2/s, to grow for 3 weeks until evaluation.

3.2.8 Survival ratings and data analysis

After 3 weeks of growth, plants were evaluated based on a survival rating scale (Table 3.4). Plants were rated from 1 - 4 based on how much freezing damage occurred (Table 3.4). Survival ratings were individually analyzed using Proc Probit in SAS (Statistical Analysis System) to determine the LT_{50} value.

Table 3.4 Survival rating scale used for freezing test.

Rating	Phenotype
4	Re-growth similar to control plants.
3.5	Plant is healthy with slight stunting observed compared to the control. Tillering is observed.
3	Stunting observed with some leaves senescing; however, the plant is growing and tillers are observed.
2.5	Freezing injury has caused senescence of all leaves. New leaves and some tillers are growing and visibly emerging from the stem.
2	Severe freezing injury. Very slight regrowth initiated from the crown with leaf tips just visible.
1.5	Plant is dead with no visible regrowth; however, senescence was slow. Mostly yellow with some green visible on senescing tissue.
1	Plant is dead with no visible regrowth. No green tissue observed, plant is completely yellow or brown.

3.2.9 Sampling and preparation of plant tissue for gene expression analysis during the first vegetative and sexual cycle

PI206624, PI531718, Norstar, Chinese Spring, 235a, OK7211542, Agrotana, and 11955 plants (Table 3.2) were grown according to section 3.2.1. Leaf and apical meristem tissue from was sampled from three biological replicates of each line at growth stages: Z22 (tillering), Z22 at 6 h, 24 h, 2 w and 8 w or 10 w after the start of the vernalization treatment, Z31 (first node visible), Z39 (flag leaf), Z47 (booting), Z65 (anthesis) and Z85 (soft dough) (Zadoks et al. 1974). RNA was extracted and prepared for cDNA synthesis in the same manner as described in section 3.2.3.

3.2.10 Quantitative Polymerase Chain Reaction (qPCR) of first vegetative and sexual cycle

PCR primers for *CBF12, CBF14* and *COR14* were designed and tested based on *Thinopyrum sp.* sequences determined in section 3.2.4, as well as known sequences in wheat (Table 3.2). Two housekeeping genes, *CONTIG5* and *ELF1a,* were also used to calculate the relative expression of the genes of interest in comparison to the expression of the housekeeping genes. Three technical and three biological replicates of each sample were utilized. The qPCR experiment was completed using the QuantiTect SYBR Green PCR Kit (ThermoFisher Scientific Inc., Canada). A single qPCR reaction was made by mixing 5 μL of SYBR Green, 0.6 μL each of forward and reverse primer,1.3 μL of H_2O, and 2.5 μL of $^1/_{10}$ diluted cDNA template, for a total reaction of 10 μL. All qPCR reaction components were pipetted into 384 well plates using a liquid handler (epMotion 5070

Eppendorf, Canada). The reactions were processed in the ABI QuantStudio 6 Flex qPCR System for 1 cycle at 50ºC for 2 min, 1 cycle at 95ºC for 3 min, 40 cycles at 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s (ThermoFisher Scientific Inc., Canada).

3.2.11 Data analysis

The Relative Expression Software Tool (REST) program, was used to analyze qPCR results (Pfaffl et al. 2002). REST compares Cycle Threshold (Ct) values of target genes across housekeeping genes and normalizes the data to remove variation among samples (Pfaffl et al. 2002). Gene expression at different stages is shown relative to expression at Z22. Significant values ($p < 0.05$) compared to Z22 were analyzed by an ANOVA (Pfaffl et al. 2002). The standard log2 of the expression value was used, in order to better represent the data on a graph. Stages of development were also directly compared to each other using SAS software, the Glimmix Procedure, in order to determine if there were significant differences analyzed by an ANOVA ($p < 0.05$) between stages of development.

3.2.12 Sampling and preparation of plant tissue for gene expression analysis during the end of the first life cycle and beginning of the second life cycle

One accession of wheatgrass, PI206624 (*Th. ponticum*) and the perennial wheat line 235a were grown as in section 2.2.1, until near the end of their first lifecycle at Zadoks stage Z85 (Zadoks et al. 1974). Once the plants reached developmental stage Z85, they

were separated into three different growing conditions to simulate different fall temperature growing conditions. LD conditions had 16/8 h day/night at 20ºC with a light intensity of 340 µmol/m^2/s , SD conditions had 10/14 h day/night at 20ºC with a light intensity of 340 µmol/m^2/s, while cold and short day (CSD) conditions had 10/14 h day/night at 4/2ºC day/night with a light intensity of 575 µmol/m^2/s. Plants were kept under these conditions for eight weeks, during which three biological replicates of leaf and apical meristem tissue were collected for Z85 at 0 w, 2 w, 4 w, 6 w and 8 w. Winter wheat cultivar Norstar was also grown as a control to match the perennial lines at their stages of development.

3.2.13 Quantitative Polymerase Chain Reaction (qPCR) of LD, SD and CSD grown lines

The same RNA extraction, cold tolerance qPCR primers (Table 3.2), and qPCR techniques described above were utilized to carry out qPCR for the three cold tolerance genes of interest, *CBF12, CBF14* and *COR14*, on the samples exposed to different growing conditions at the end of their first life cycle and beginning of their second cycle. qPCR results were analyzed as described in section 3.2.8.

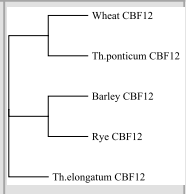
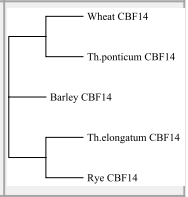
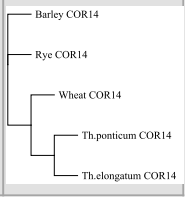
3.3 Results

3.3.1 Gene Sequencing

Cold tolerance genes *CBF12*, *CBF14* and *COR14* were sequenced in wheatgrass accessions PI206624 (*Th. ponticum*) and PI531718 (*Th. elongatum*) (Table 3.5). The *CBF12* sequence in wheat has a length of 738 bp (GenBank: HG530926.1) while the length in PI206624 and PI531718 is 726 bp and 732 bp respectively, indicating two indels (Table 3.5). Although the size of the *CBF12* gene is different in wheat and wheatgrass, the cladogram indicates that the wheatgrass sequences are still closely related to wheat and the sequences are 96% and 94% similar to wheat in *Th. ponticum* and *Th. elongatum* respectively (Table 3.5). The alignment of the wheatgrass sequences with wheat and other cereal crops show that the wheatgrass *CBF12* sequences are conserved, with some SNPs and indels (Appendix 26). *CBF12* in wheat has a conserved APETALA2 (AP2) domain that binds to an 11 base pair GCC box of the target DNA. This domain is from the AP2/EREBP family of transcription factors, which have various roles in plant development and stress response, such as cold tolerance (Campoli et al. 2009). This domain was also conserved in the *CBF12* sequences of both wheatgrass accessions.

The *CBF14* sequence for *Th. ponticum* was 99% similar to wheat, while the *Th. elongatum* sequence was 97% similar to wheat (Table 3.5). Both wheatgrass *CBF14* sequences had a size of 645 bp, the same as the wheat sequence (GenBank HG530930.1) (Table 3.5). The cladogram indicates that the *Th. ponticum CBF14* sequence was more

Table 3.5 Gene sequencing results of cold tolerance genes *CBF12*, *CBF14* and *COR14* in wheatgrass accessions *Thinopyrum ponticum* and *Thinopyrum elongatum* compared to wheat sequences.

Genes	Size of gene in wheat (bp)	Wheatgrass	Size of gene in wheatgrass (bp)	% Similarity to wheat	Major indels with regards to wheat	Cladogram
CBF12	738	*Thinopyrum ponticum* *Thinopyrum elongatum*	726 732	96% 94%	Conserved with few SNPs and indels and conserved AP2 domain	
CBF14	645	*Thinopyrum ponticum* *Thinopyrum elongatum*	645 645	99% 97%	Conserved with few SNPs and conserved AP2 domain	
COR14	423	*Thinopyrum ponticum* *Thinopyrum elongatum*	420 420	94% 95%	Conserved with few SNPs and indels	

closely related to wheat, while the *Th. elongatum* sequence was more closely related to rye, however both of these sequences were highly conserved when aligned with the wheat sequence, and only had a few SNPs (Table 3.5, Appendix 27). Like *CBF12*, the wheat *CBF14* gene has a conserved AP2 (APETALA2) domain that binds to an 11 base pair GCC box, and this is also conserved in both wheatgrass sequences (Campoli et al. 2009).

The *COR14* sequence in PI206624 and PI531718 had a 94% and 95% similarity to the *COR14* wheat sequence (GenBank: FJ6052070.1) respectively, and the cladogram indicates that the two wheatgrass sequences are closely related to the wheat sequence (Table 3.5). The size of the *COR14* gene in both wheatgrasses was 420 bp, while *COR14* was 423 bp in wheat, indicating one indel. A Geneious Muscle alignment (Kearse et al. 2012) of wheat *COR14* and other cereal crops with the two accessions of wheatgrass showed a few SNPs, however the gene was highly conserved between wheat and wheatgrass (Appendix 28).

3.3.2 Freezing Test and LT_{50}

The LT_{50} of wheatgrass accessions PI206624 and PI531718, wheat cultivars Norstar and Chinese Spring, and perennial wheat lines 235a, OK7211542, Agrotana and 11955 was calculated using SAS. All LT_{50} values fell between a range of -14.8 °C to -24.1 °C (Table 3.6). Norstar survived at the lowest temperature, with an LT_{50} at -24.1 °C while PI206624 had the poorest survival, with an LT_{50} at -14.8 °C (Table 3.6). Norstar had the highest average survival rating, 2.26, at exposure to -19 °C, indicating that the plants had

Table 3.6 LT_{50} and survival ratings of wheatgrass, wheat and perennial wheat lines.

Cultivar	Average Rating						LT_{50} (°C)
	4 °C	-3 °C	-7 °C	-11 °C	-15 °C	-19°C	
PI206624	4.00	4.00	3.95	3.33	1.64	1.00	-14.8
PI531718	4.00	3.97	3.86	3.03	2.13	1.03	-15.6
Norstar	4.00	4.00	3.78	3.50	2.98	2.26	-24.1
Chinese Spring	4.00	4.00	3.77	3.15	1.79	1.03	-14.9
235a	4.00	3.98	3.69	3.39	2.13	1.02	-15.3
OK7211542	4.00	3.99	3.95	3.54	1.99	1.03	-15.4
Agrotana	4.00	3.99	3.75	3.12	1.64	1.12	-14.9
11955	4.00	4.00	3.96	3.32	1.89	1.20	-15.6

severe freezing injury, but some regrowth with just the tips of the leaves visible from the meristem (Table 3.6). PI206624 had the lowest survival ratings of 1.00 at -19 °C, indicating all the plants were dead (Table 3.6). All other plants except Norstar had ratings between 1.00-1.20, indicating that most plants were dead (Table 3.6). Norstar had the highest plant survival, with 17 of 20 plants surviving at -19 °C, while PI206624 had zero of 20 plants survive (Figure 3.1). All other lines had poor survival at -19 °C reflected by their LT_{50} values (Figure 3.1, Table 3.6).

3.3.3 Cold tolerance gene expression

qPCR results of cold tolerance genes *CBF12*, *CBF14* and *COR14* were analyzed with REST (Pfaffl et al. 2002), and the Log2 of the expression value was graphed over various Zadoks stages of development for the first sexual cycle of wheatgrass accessions PI206624, PI531718, wheat cultivars Norstar and Chinese Spring, and perennial wheat lines 235a, OK7211542, Agrotana and 11955. To facilitate comparisons across growth stages for each wheat, wheatgrass or perennial wheat line, gene expression at Z22 was used to normalize the data for subsequent growth stages. Line graphs for leaf and meristem tissue were generated to show significant relationships which have a p-value less than 0.05. To ease the graphical presentation of the data, standard error was visualized using bar graphs (Appendices 29 - 31).

CBF genes in winter cereals and perennial grasses are rapidly upregulated after cold exposure and cause the upregulation of *COR* genes, leading to cold tolerance (Xiong and Fei 2006, Campoli et al. 2009, Galiba et al. 2009). *CBF* genes have been shown to be

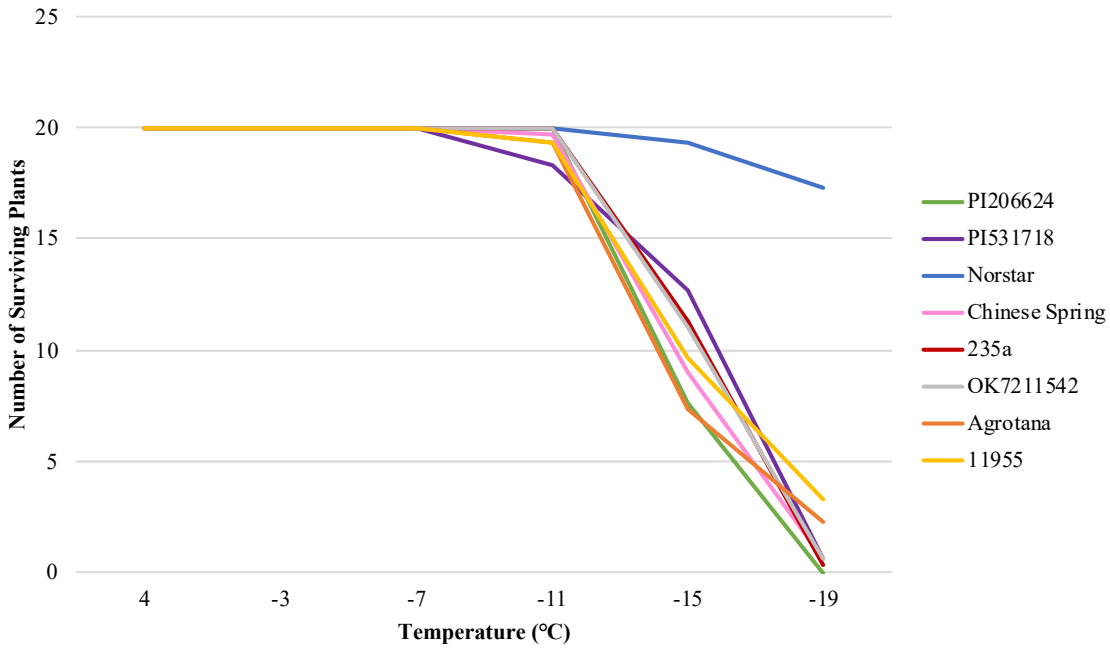


Figure 3.1 Number of surviving wheatgrass, wheat and perennial wheat lines exposed to varying temperatures. (n=20)

upregulated in both leaves and meristems of cereal crops, although studies are most commonly conducted on leaf tissue (Campoli et al. 2009, Galiba et al. 2009). Generally, in leaf tissue, *CBF12* is upregulated early on during the vernalization period at stages Z22 6 h and Z22 24 h with most values being significant (Figure 3.2 A). Expression decreases at Z22 2 w and remains at the about the same level for the rest of the first sexual cycle (Figures 3.2 A). For all lines except PI531718 and Chinese Spring, expression of *CBF12* at Z22 2w was significantly downregulated compared to Z22 24 h (Appendix 32). All wheatgrass, wheat and perennial wheat lines follow a similar expression pattern (Figure 3.2 A). Expression of *CBF12* in meristem tissue followed a similar expression pattern to leaf tissue, with *CBF12* being the most highly upregulated at Z22 6 h and Z22 24 h (Figure 3.2 B). At stage Z22 2 w, *CBF12* expression decreased and was significant for most cultivars compared to Z22 24 h (Appendix 33). *CBF12* had a similar expression level to Z22 for the rest of the first sexual cycle (Figure 3.2 B). The expression pattern of *CBF12* in meristem tissue was similar for all lines (Figure 3.2 B). In both leaf and meristem tissue Norstar and Chinese Spring had the highest *CBF12* expression early on during vernalization, while PI206624 had relatively low expression, especially in meristem tissue (Figure 3.2 A, B).

In leaf tissue, *CBF14* was the most upregulated early on during vernalization, at stages Z22 6 h and Z22 24 h (Figure 3.3 A). *CBF14* expression began to decrease at Z22 2 w and was significantly less than Z22 24 h for most cultivars (Figure 3.3 A, Appendix 34). At post-vernalization stage Z31, *CBF14* became downregulated and stayed that way for the rest of the first sexual cycle (Figure 3.3 A). *CBF14* expression in leaf tissue had a

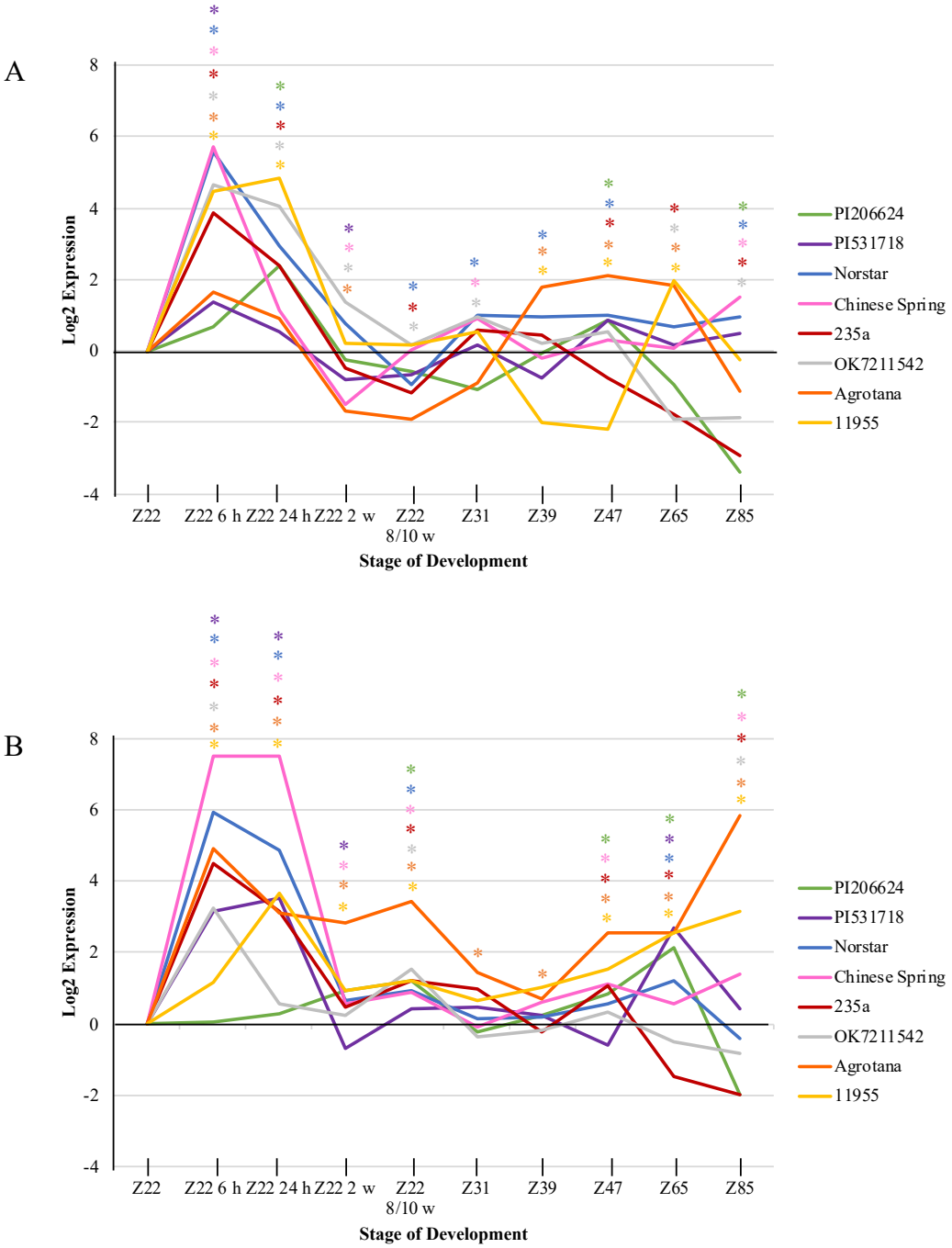


Figure 3.2 Log2 gene expression trends of *CBF12* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

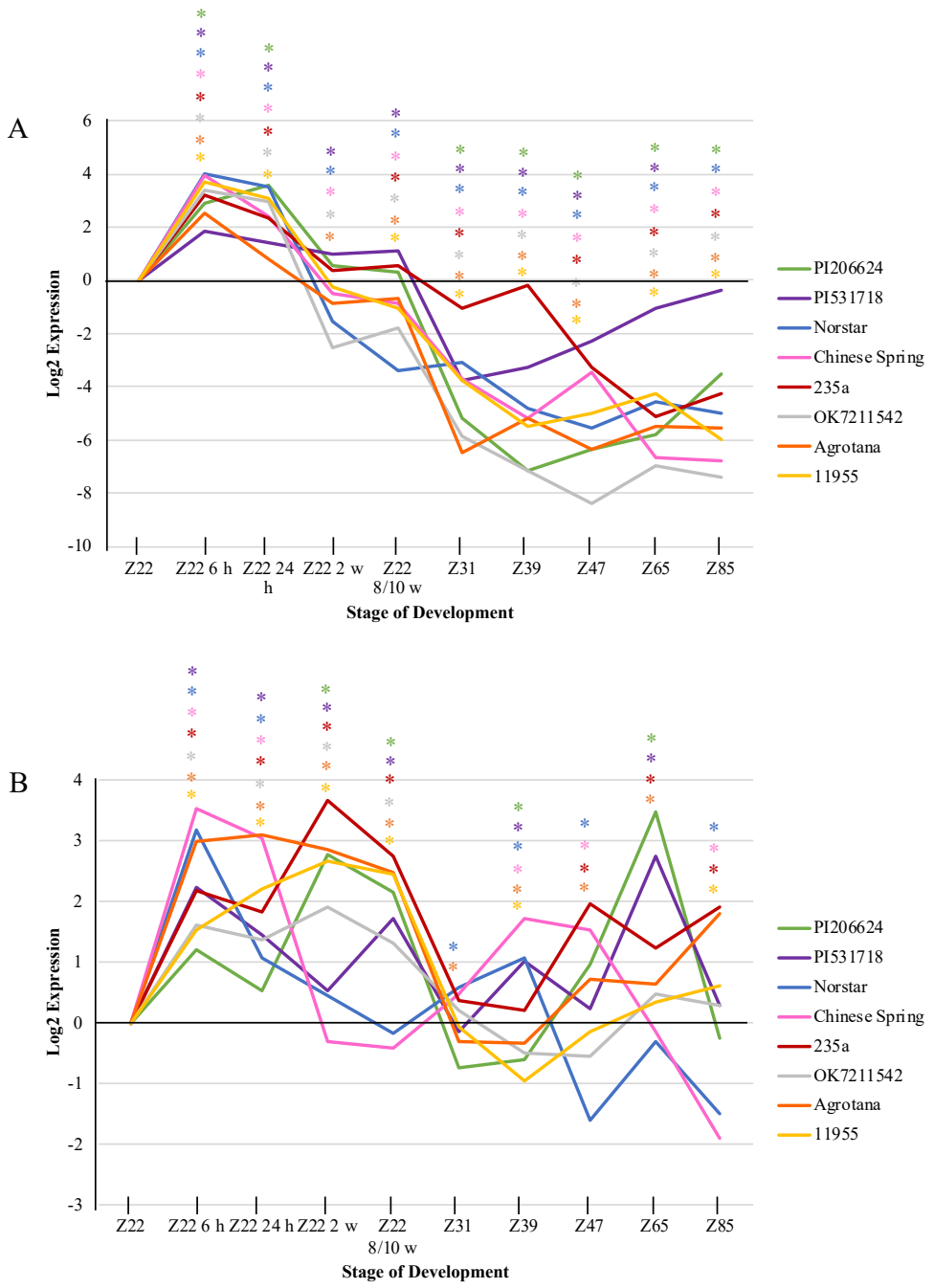


Figure 3.3 Log2 gene expression trends of *CBF14* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

similar trend for all lines (Figure 3.3 A). Expression of *CBF14* in meristem tissue was significantly upregulated in most lines from Z22 6 h – Z22 8/10 w (Figure 3.3 B). Post vernalization, at Z31, *CBF14* expression decreased, and was significantly downregulated compared to Z22 8/10 w (Figure 3.3 B, Appendix 35). For most wheatgrass and perennial wheat lines, *CBF14* remained slightly upregulated until the end of the first sexual cycle, although standard error bars are quite large (Figure 3.3 B, Appendix 30 B).

COR genes are upregulated by *CBF* genes under cool temperatures, and lead to cold tolerance in cereals and perennial grasses (Zhao and Bughrara 2008, Galiba et al. 2009). Cold tolerance genes are expressed in both leaf and meristem tissue, although most studies only assess their expression in leaf tissue (Dhillon et al. 2010). *COR14* in leaf tissue starts to become upregulated at Z22 6 h and expression increased to Z22 24 h and then remained highly upregulated throughout the rest of the vernalization period (Figure 3.4 A). At Z31 the *COR14* expression became significantly downregulated compared to 8/10 w and remained downregulated until the end of the first sexual cycle at Z85 (Appendix 36, Figure 3.4 A). In PI206624 *COR14* at Z31 and Z85 was not detected by REST. Expression of *COR14* in the leaf tissue has a similar pattern in all lines (Figure 3.4 A). The expression pattern of *COR14* in meristem tissue was similar to its expression in leaf tissue, although *COR14* became more downregulated in leaf tissue post vernalization. *COR14* in meristem tissue wass highly upregulated during the vernalization period, followed by a sharp decrease in expression after vernalization at Z31 that was significant compared to Z22 8/10 w (Figure 3.4 B, Appendix 37). Expression remained lower than Z22 until the end of the cycle for all lines (Figure 3.4 B). All lines experienced similar

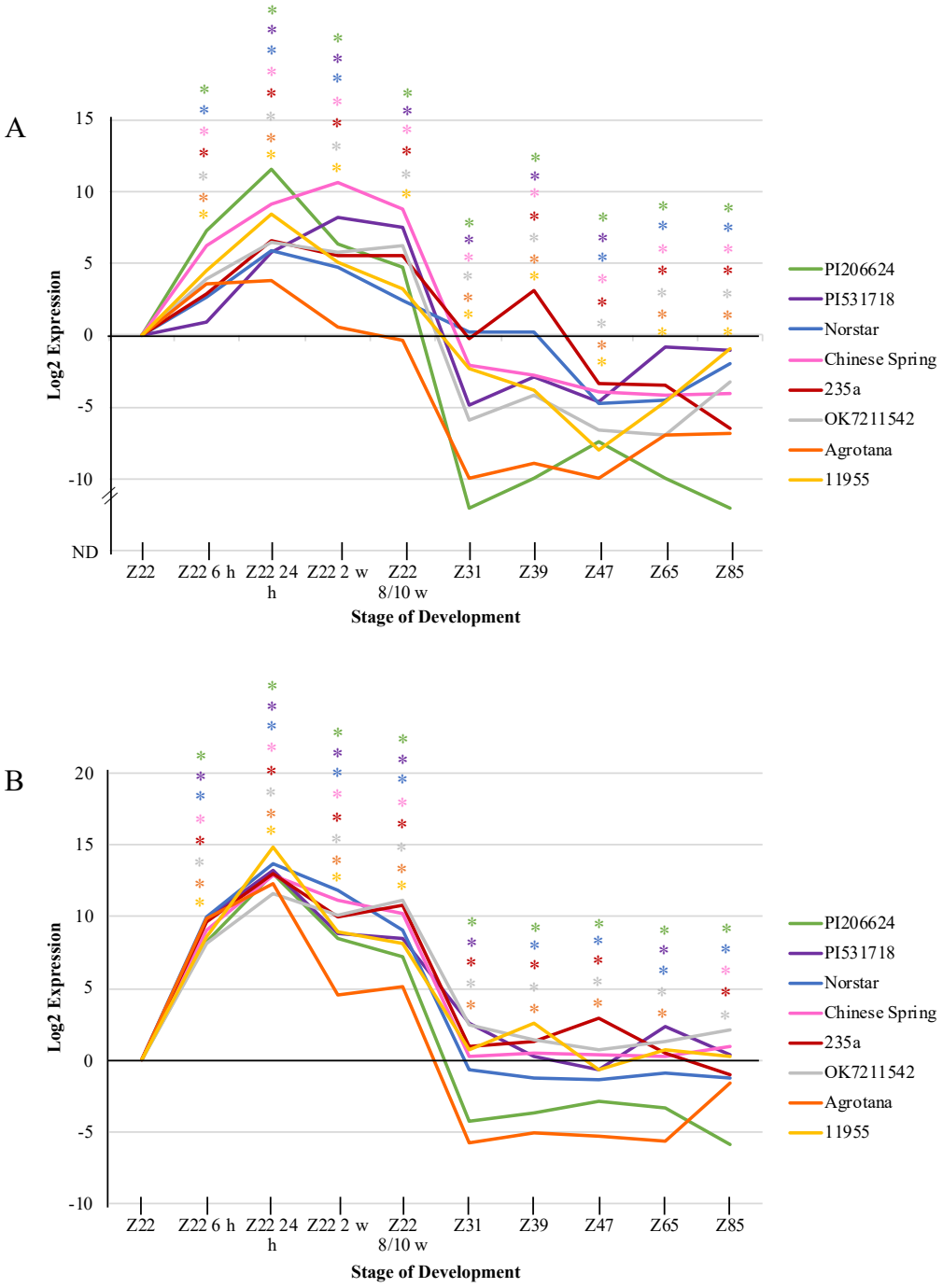


Figure 3.4 Log2 gene expression trends of *COR14* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

trends of *COR14* expression in meristem tissue (Figure 3.4 B). In both leaf and meristem tissue Agrotana had slightly lower expression than the rest of the lines (Figure 3.4 A, B).

3.3.4 Cold tolerance gene expression of plants exposed to varying photoperiods and temperatures

Wheatgrass PI206624, winter wheat Norstar, and perennial wheat 235a were grown under LD conditions until stage Z85, at which point the plants were separated into LDs, SDs and CSDs in order to simulate 3 different fall growing conditions. The plants were sampled for 8 weeks under these conditions, and at the eighth week it was assumed that they began their second lifecycle. Expression relative to Z22, which was represented as zero (log2 scale), was used to analyze qPCR results of cold tolerance genes *CBF12*, *CBF14* and *COR14*. Either Z22 or Z85 could have been used for comparison as 235a plants grew few new tillers and had older tissue sampled that was closer to Z85, while PI206624 grew many young tillers that were around Z22. Z22 was chosen as PI206624, the known perennial, had tissue at that stage of development and it allowed for comparison to gene expression during the first vegetative and sexual cycle. The log2 of the relative expression value was graphed for different stages of plant development. Graphs of leaf and meristem tissue were made to show cold tolerance gene expression. Significant results have a p-value less than 0.05 compared to stage Z22 in the first cycle, which appears as a value of zero in the graphs. Standard error bar graphs for this data set were included in Appendices 38 – 40.

In winter cereals and perennial grasses, *CBF* genes are quickly upregulated in leaf and meristem tissue after cold exposure and *COR* genes also become upregulated, leading to increased cold tolerance (Xiong and Fei 2006, Campoli et al. 2009, Galiba et al. 2009). *CBF12* in leaf tissue was upregulated during Z22 6 h and Z22 24 h, and expression then decreased at Z22 2 w and remained at a low expression level until Z85 (Figure 3.5 A). After Z85, when the plants were grown under different conditions, *CBF12* in 235a for LDs and SDs was more downregulated than in CSDs (Figure 3.5 A). *CBF12* in PI206624 had similar expression in LDs and CSDs, that was less downregulated than *CBF12* in SDs (Figure 3.5 A). Wheatgrass, wheat and perennial wheat leaf tissue followed a similar expression pattern, while meristem tissue had a slightly different pattern in PI206624 (Figure 3.5 B). In meristem tissue of Norstar and 235a, *CBF12* was the most highly upregulated at Z22 6 h and Z22 24 h (Figure 3.5 B). At Z22 2 w, *CBF12* expression decreased and it remained at around the same level until Z85 (Figure 3.5 B). During the vernalization period *CBF12* in PI206624 meristem tissue was only upregulated at Z22 8/10 w (Figure 3.5 B). After Z85, 235a plants grown under CSDs had a higher expression of *CBF12* than plants grown under LDs or SDs, while PI206624 plants had a higher expression level when grown under CSDs and LDs, which were expressed similarly, than when grown under SDs. Both 235a and PI206624 plants grown under SDs had a similar downregulation of *CBF12* in meristem tissue (Figure 3.5 B). In both leaf and meristem tissue when the reference is Z85 instead of Z22, a similar expression pattern of *CBF12* can be seen (Appendix 41).

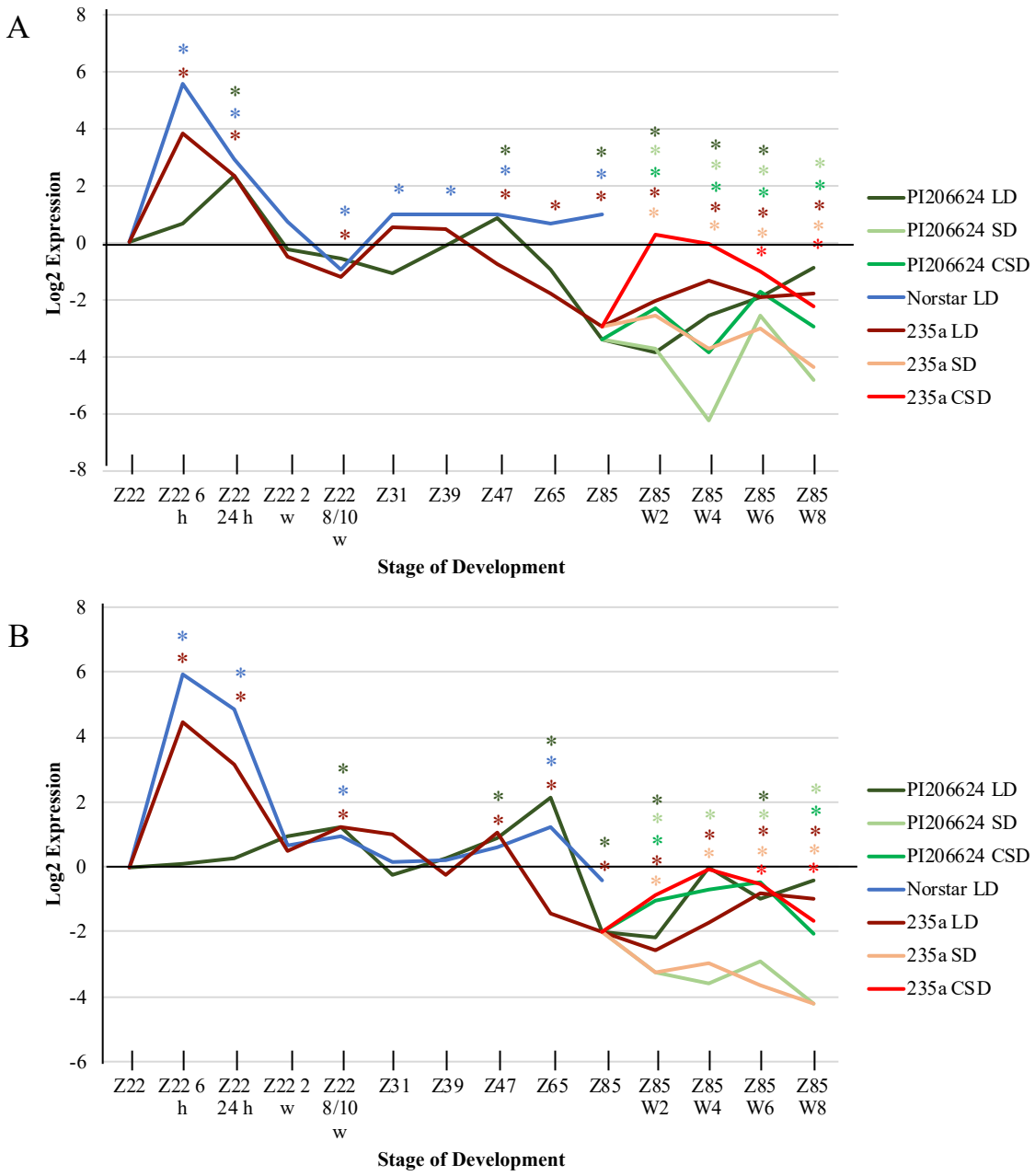


Figure 3.5 Log2 gene expression trends of *CBF12* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

In Norstar, PI206624 and 235a *CBF14* in leaf tissue was highly upregulated 6 h and 24 h after vernalization, with expression decreasing by Z22 2 w (Figure 3.6 A). At Z31, *CBF14* became downregulated until Z85 W8 (Figure 3.6 A). In the PSCR stage, *CBF14* became more downregulated in LDs and SDs than in CSDs in 235a (Figure 3.6 A). In PI206624 plants *CBF14* was more downregulated in SDs than in CSDs and LDs, although *CBF14* was downregulated under all conditions. Both PI206624 and 235a had a similar downregulation pattern for *CBF14* in SDs, although PI206624 was more downregulated than 235a (Figure 3.6 A). *CBF14* expression in the meristem was upregulated throughout the entire vernalization period in 235a, earlier in vernalization for Norstar, and later in vernalization for PI206624 (Figure 3.6 B). At Z31 *CBF14* expression decreased and remained around the same expression level until Z85 (Figure 3.6 B). After Z85 of PSCR, expression of *CBF14* in 235a and PI206624 decreased, and was more downregulated under SDs than under LDs and CSDs (Figure 3.6 B). A similar trend of *CBF14* expression in leaf and meristem tissue was shown when Z85 was used as the reference instead of Z22 (Appendix 42).

COR genes are upregulated after exposure to cool temperatures in leaf and meristem tissue, leading to cold tolerance (Zhao and Bughrara 2008, Galiba et al. 2009, Dhillon et al. 2010). At Z22 6 h and Z22 24 h, *COR14* in leaf tissue of all lines began to be upregulated and remained upregulated throughout the vernalization period (Figure 3.7 A). Post vernalization, at Z31, expression of *COR14* decreased and remained low or downregulated for Norstar and 235a leaves and was much more downregulated for

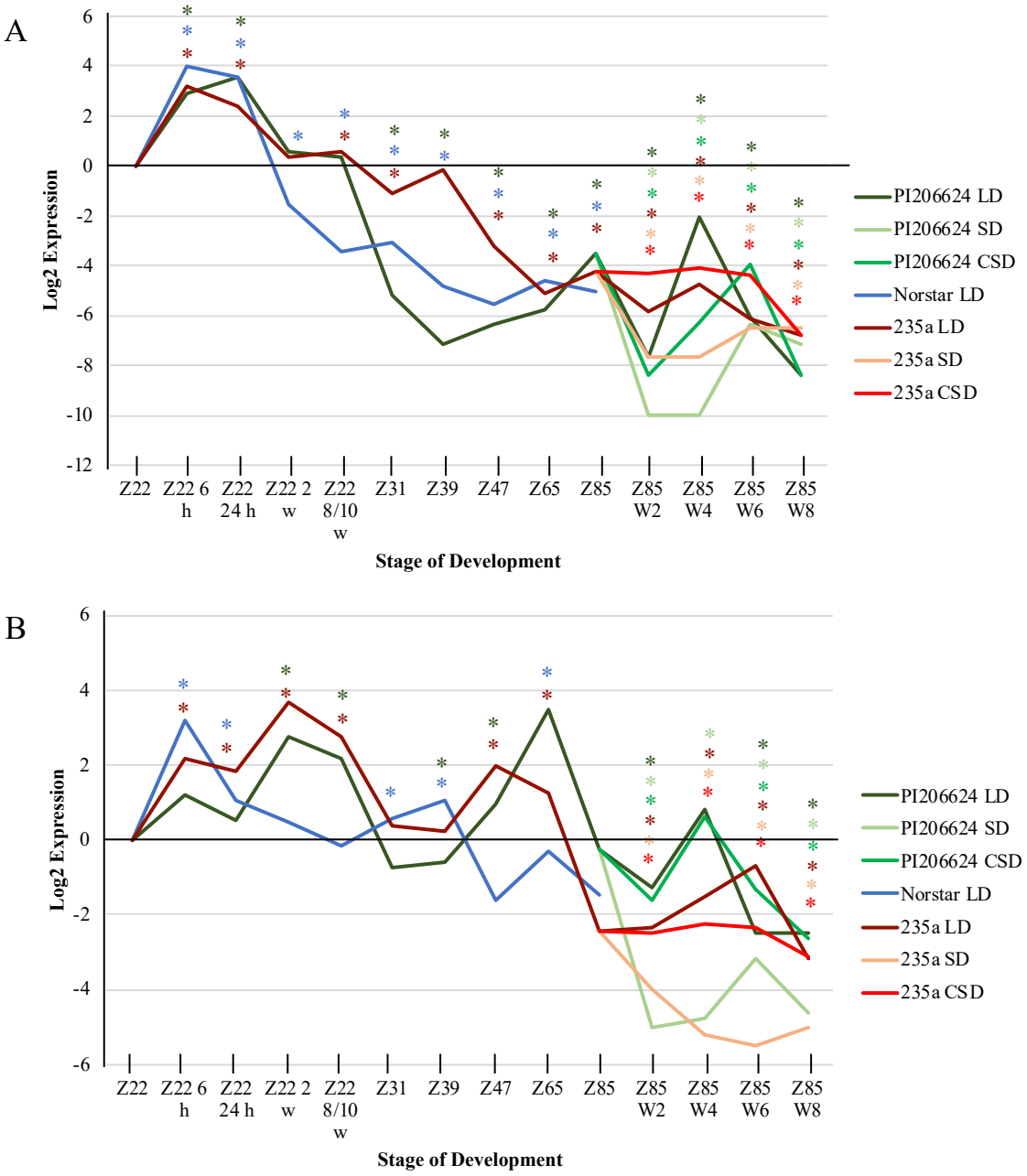


Figure 3.6 Log2 gene expression trends of *CBF14* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

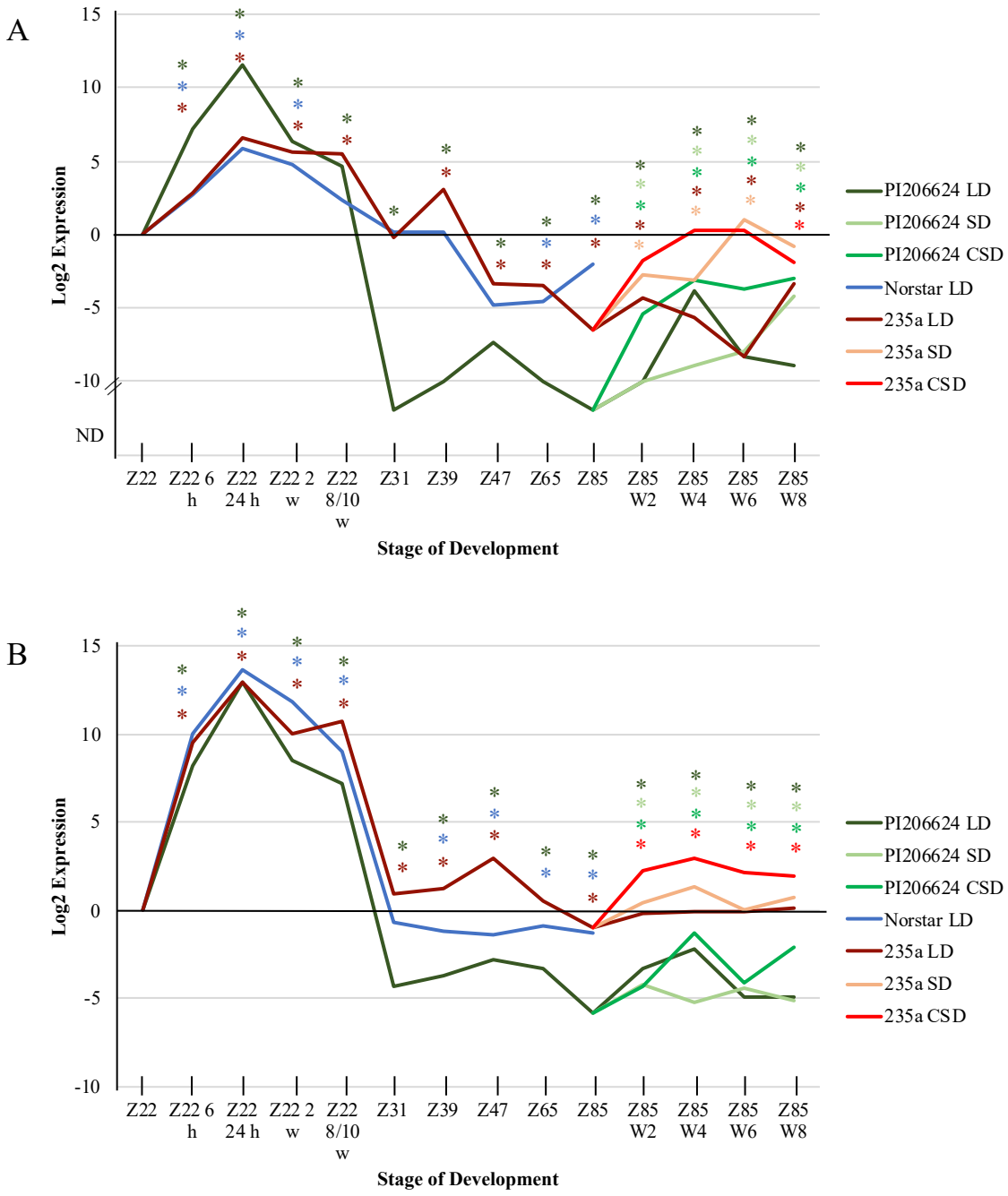


Figure 3.7 Log2 gene expression trends of *COR14* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. * indicates significant gene expression compared to stage Z22, $p<0.05$. Significance is shown in the order of plant lines displayed on the legend.

PI206624 leaves until Z85 (Figure 3.7 A). After Z85 of PSCR in 235a, *COR14* expression under CSDs became more upregulated, to around the same expression level as the Z22 comparison stage and was significantly upregulated under CSDs compared to stage Z85 (Figure 3.7 A, Appendix 43). *COR14* expression remained slightly downregulated under SDs and more downregulated under LDs when compared Z22 (Figure 3.7 A). In PI206624 leaves *COR14* was more downregulated in LDs and SDs than in CSDs (Figure 3.7 A). In meristem tissue of all lines, *COR14* became upregulated at Z22 6 h and remained upregulated throughout vernalization. *COR14* expression decreased at Z31 and remained below or similar to Z22 in 235a and Norstar meristem and was downregulated in PI206624 meristem until Z85 (Figure 3.7 B). After Z85 of PSCR, *COR14* became upregulated in CSDs for 235a tissue and was significantly upregulated when compared to Z85, while under LDs and SDs meristem expression levels were similar to Z22 (Figure 3.7 B, Appendix 43). *COR14* in PI206624 meristem was downregulated under LDs, SDs and CSDs and had similar expression (Figure 3.7 B). Overall, compared to *COR14* expression in 235a, PI206624 *COR14* expression was more downregulated after stage Z85 of PSCR (Figure 3.7 B). Comparing all stages of development to Z85 rather than Z22 resulted in a similar expression pattern of *COR14* in leaf and meristem tissue (Appendix 43).

3.4 Discussion

3.4.1 Gene sequencing

Comparison of coding sequences of *COR14*, *CBF14* and *CBF12* in wheatgrass accession PI206624 (*Th. ponticum*) and PI531718 (*Th. elongatum*) to wheat, barley and rye sequences, indicate that these genes are highly conserved. *CBF14* and *CBF12* in wheat have been shown to have a conserved *AP2* domain, which is found in members of the *AP2/EREBP* family of transcription factors (Campoli et al. 2009). The conserved *AP2* domain has a role in plant development and stress response, like cold tolerance (Campoli et al. 2009). Wheatgrass sequences of both *CBF* genes contained this domain. Both *CBF* sequences were highly conserved when aligned with the wheat sequence, and only had a few SNPs, that did not cause any major changes to the *AP2* domain or the rest of the coding sequence (Appendix 26 – 28). Cladograms indicated that the cold tolerance genes in wheatgrass were closely related to other cereal crops. *COR14* cladograms show that the wheatgrass sequences are closely related to wheat, while for both *CBF* genes the cladograms indicate that the *Th. ponticum CBF* sequences are more closely related to wheat, while the *Th. elongatum* sequences were more closely related to rye. A major difference in sequence between the annual and perennial could reveal how annual and perennial cereals cold acclimate and develop cold tolerance differently. The high conservation of *CBF12*, *CBF14* and *COR14* sequences between wheatgrass and wheat suggest that their function is likely to be similar in the two species. For example, wheat and barley have slight sequence differences in *CBF12*, *CBF14* and *COR14*; however, the function of *CBF* and *COR* genes is known to be same in both wheat and barley (Rapacz et

al. 2008, Galiba et al. 2009, Novák et al. 2015, Erath et al. 2017). This further suggests that the function of these cold tolerance genes is likely similar in wheat and wheatgrass.

3.4.2 Freezing test and LT_{50}

When freezing tolerance was compared using Norstar, Chinese Spring, wheatgrass (PI206624 and PI531718) and perennial wheat lines (235a, OK7211542, Agrotana and 11955), only Norstar showed considerable cold tolerance. Results indicate that Norstar had the greatest freezing tolerance, with an LT_{50} value of -24.1 °C, while all other lines had similar LT_{50} values that ranged from -14.8 °C to -15.6 °C. Similar results were shown by Fowler et al. (1999) for Norstar, the most cold hardy winter wheats, which had an LT_{50} value of -24.1 °C. According to Limin and Fowler (2006), the winter allele, (*vrn-A1*), allowed winter wheat plants to tolerate a temperature 11 °C colder than spring wheat, as was confirmed by Norstar LT_{50} values in these results. Chinese Spring in this experiment had an LT_{50} of -14.9 °C, a lower value than that reported in the literature (-11.3 °C) (Limin and Fowler 1988). The slight differences in LT_{50} values might have been due to different methods in freezing (Limin and Fowler 1988). Studies conducted on timothy and perennial ryegrass revealed more similar LT_{50} to those of PI206624 and PI531718 found in this experiment (Höglind et al. 2010, Goslee et al. 2017). Goslee et al. (2017) had similar results in perennial ryegrass with LT_{50}'s ranging from -12.9 °C to -20.8 °C for varying cultivars. These results are comparable to the results observed in this experiment. It has been suggested that wheatgrass plants are very cold hardy and can survive temperatures cooler than some winter wheats (Limin and Fowler 1988). For example, a line of *Th. ponticum* native to North America (Orbit) had an LT_{50} value of -23.3 °C, while

two lines of intermediate wheatgrass native to North America (Chief and Clarke) had LT_{50} values of -21.8 °C and -21.5 °C (Limin and Fowler 1988, Chen et al. 1998). Wheatgrasses in this experiment had much higher LT_{50} values with PI206624 having an LT_{50} of -14.8 °C, and PI531718 having an LT_{50} of -15.6 °C. These differences could be due to the geographical origins of the accessions of *Th. ponticum* and *Th. elongatum* used in our study. Both PI206624 and PI531718 are from warmer countries, Turkey and Tunisia respectively, and are from areas of these countries that generally experience Mediterranean climates (GRIN 2007a, b, Di Castri and Mooney 2012, Iyigun et al. 2013). Findings of several studies have shown that northern-adapted perennial grass populations were able to tolerate colder temperatures and sustain a higher freezing tolerance than southern-adapted populations (Larsen 1994, Junttila 1996, Dalmannsdottir et al. 2016). This could explain why Mediterranean adapted accessions PI206624 and PI531718 did not have LT_{50}'s as low as Norstar's. None of the perennial wheat lines flowered without vernalization, suggesting that the control of flowering in these lines is more similar to winter wheat. However, under these experimental conditions, perennial wheat lines have similar LT_{50} values to spring wheat and wheatgrass. The pedigree of 235a is Madsen//Chinese Spring/PI531718, which suggests that 235a had similar LT_{50} values to the spring wheat and PI531718 parent (Hayes et al. 2012). The pedigrees of OK7211542, Agrotana and 11955 are wheat/*Th. ponticum*, indicating that these lines have more similar LT_{50} values to PI206624 (Hayes et al. 2012). The LT_{50} values of the perennial wheat lines are far from Norstar values, suggesting that these perennial wheat lines likely are not able to survive cold temperatures as well as Norstar.

3.4.3 Wheat, wheatgrass and perennial wheat have comparable cold tolerance gene expression during the first vegetative and sexual cycle

Cold tolerance gene expression of *CBF12*, *CBF14* and *COR14* was compared in the different plant lines during their first vegetative and sexual cycle. Similar expression patterns were observed in all lines, including spring types, suggesting that wheat, wheatgrass and perennial wheat have comparable cold tolerance gene expression over the first vegetative and sexual cycle (Figure 3.8). Although spring wheat does not require vernalization, some cultivars of spring cereals can still respond to vernalization (Kamran et al. 2014). Chinese Spring has a dominant *Vrn1-D1* allele, so although it does not require vernalization to flower, its flowering time is still partially sensitive to vernalization (Zhang et al. 2008, Kamran et al. 2014). Cold tolerance genes in all lines followed the expected pattern observed in annual cereals and perennial grasses, with *CBF12* and *CBF14* being rapidly upregulated within a few hours of vernalization, followed by an increase in *COR14* expression for the rest of the vernalization period (Fowler 2008, Rapacz et al. 2008, Zhao and Bughrara 2008, Campoli et al. 2009, Dhillon et al. 2010). *VRN1* and cold tolerance genes have also been shown to interact, in that as *VRN1* is upregulated, cold tolerance genes become downregulated (Limin and Fowler 2006, Dhillon et al. 2010). Consistent with this mechanism occurring in cereals, *VRN1* becomes rapidly upregulated just after vernalization and *COR14* becomes downregulated at the same time. Since *VRN1* is responsible for the vegetative to reproductive transition and cereals are unable to induce cold tolerance genes when in the reproductive phase, this indicates that the plants have lost their cold acclimation ability (Dhillon et al. 2010). The similar expression pattern of cold tolerance genes in wheat, wheatgrass and perennial

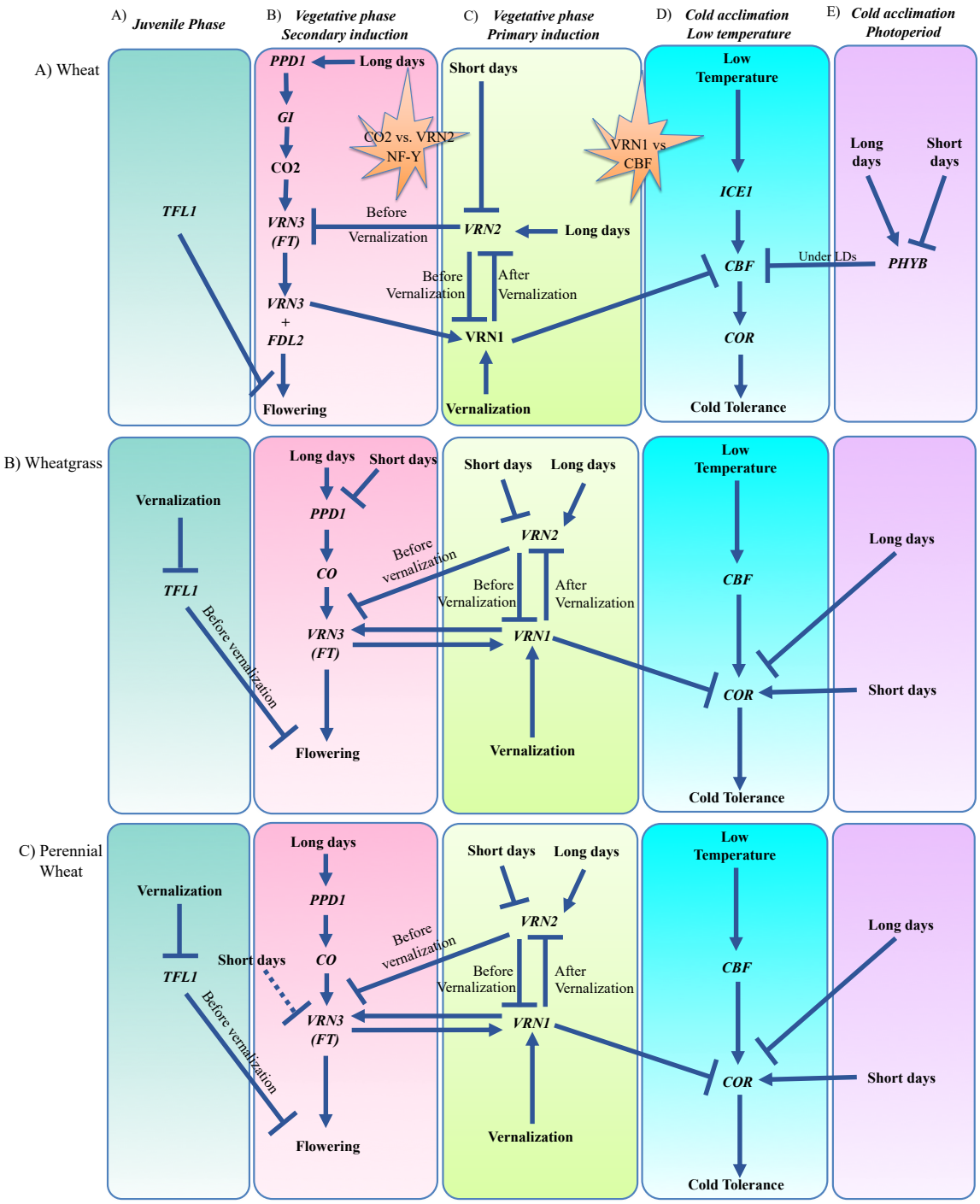


Figure 3.8 Updated cold tolerance pathway in (A) cereal, (B) wheatgrass and (C) perennial wheat.

wheat throughout the first life cycle suggests that all the species employ a similar mechanism for cold acclimation.

3.4.4 Expression of cold tolerance genes in plants exposed to varying photoperiods and temperatures

COR14 generally was more downregulated in PI206624 than in 235a, although the expression pattern was similar. Under CSD conditions *COR14* was not as upregulated relative to Z22 as anticipated and as a short photoperiod and cooler temperatures have been shown to upregulate cold tolerance genes, this is somewhat unexpected (Limin and Fowler 2006, Malyshev et al. 2014). Since perennials do not have a juvenile phase in the second vegetative cycle, they could potentially not need the same level of upregulation of cold tolerance genes to signal the plant as they would need during their first vegetative cycle (Bäurle and Dean 2006, Friedman and Rubin 2015). Higher expression of *COR* and *CBF* genes has been shown to be correlated with better low temperature tolerance and winter survival in winter wheat and barley (Todorovska et al. 2014, Vítámvás et al. 2019). Because both PI206624 and 235a had relatively high LT_{50} values, indicating that they had much lower survival than winter wheat Norstar, this may correspond with low *COR14* expression under CSDs. Also, floral promoters *VRN1* and *VRN3* were upregulated at the same time indicating that PI206624 and 235a were likely at least partially in the reproductive phase (Figures 2.8, 2.9) (Limin and Fowler 2006). The upregulation of *VRN1* in annual cereals and perennial grasses has also been shown to cause cold tolerance genes to become downregulated and causes the plant to lose its cold tolerance (Limin and Fowler 2006, Dhillon et al. 2010, Ergon et al. 2016, Kovi et al. 2016) (Figure 3.8).

Expression of cold tolerance genes in PI206624 and 235a follow a similar expression pattern that indicates that the plants were at least partially in the reproductive phase during CSDs. The low expression of *COR14* in SDs is supported by the literature, as SDs alone do not induce cold tolerance as efficiently as SDs combined with cooler temperatures (Limin and Fowler 2006, Dalmannsdottir et al. 2016, Dalmannsdottir et al. 2017). LDs are not efficient at inducing cold tolerance and *COR14* remained downregulated during LD exposure (Dhillon et al. 2010). Since *VRN3* remained upregulated during LD exposure, it is likely that 235a and PI206624 are still in the reproductive phase when cold tolerance cannot be induced (Danyluk et al. 2003, Yan et al. 2006, Dhillon et al. 2010).

Expression results of cold tolerance genes *CBF12*, *CBF14* in PI206624 and 235a plants exposed to different photoperiods and temperatures (LDs, SDs, and CSDs) display similar expression patterns. However, plants were first sampled 2 weeks into vernalization and not within a few hours. As *CBF* genes have been shown to only be rapidly upregulated within a few hours of cold exposure and then not expressed after a few days, the possibly more relevant expression pattern of *CBF12* and *CBF14* was not fully be determined (Campoli et al. 2009).

Either Z22 or Z85 could have been chosen as a reference point as 235a had older tissue sampled that was closer to Z85, while PI206624 had new tillers and young tissue that was around Z22. Since perennial wheatgrass PI206624 had tissue at the Z22 stage of development and gene expression could be better compared between the first vegetative and sexual cycle to PSCR, Z22 was chosen as the reference. Similar gene expression

trends for *CBF12, CBF14* and *COR14* genes in both PI206624 and 235a were shown if either Z22 or Z85 was used as the reference suggesting that regardless of the reference point, expression trends are the same.

3.4.5 Summary

Freezing tests determined that winter wheat Norstar had the greatest freezing tolerance, while spring wheat, wheatgrass and perennial wheat lines had similar freezing tolerance and LT_{50} values suggesting that that these lines likely are not able to survive cold temperatures as well as Norstar. Cold tolerance gene expression of *CBF12*, *CBF14* and *COR14* was compared in the different plant lines during their first vegetative and sexual cycle. Similar expression patterns were observed in all lines, suggesting that wheat, wheatgrass and perennial wheat have comparable cold tolerance gene expression over the first cycle (Figure 3.8). Gene expression results from PI206624 and 235a plants during PSCR using Z85 as a reference resulted in similar cold tolerance gene expression in both PI206624 and 235a, suggesting that regardless if Z22 or Z85 was used as a reference, expression trends are the same. Expression results of the cold tolerance gene *COR14* in plants PI206624 and 235a exposed to different photoperiods and temperatures display similar expression patterns although the gene was more downregulated in PI206624 than in 235a. *COR14* was downregulated or had low expression compared to Z22 in PI206624 and 235a when exposed to SDs and LDs, as cold tolerance genes are most efficiently upregulated when exposed to a combination of cold temperatures and short days (Limin and Fowler 2006, Dalmannsdottir et al. 2016, Dalmannsdottir et al. 2017). *COR14* was not as upregulated in plants exposed to CSDs as expected. *VRN1* and

VRN3 were upregulated at the same time under CSDs, suggesting that PI206624 and 235a were partially in the reproductive phase.

CHAPTER FOUR: SUMMARY OF CONCLUSIONS

4.1 Conclusions

Understanding the life cycle, persistence and cold acclimation ability of perennial wheat would be an important advancement in agriculture. Perennial wheat lines currently do not seem to revert back to the vegetative state in the fall, have poor winter survival rates, and generally die after the first year of growth, suggesting that they are weak perennials (Hayes et al. 2018). Not much is known about flowering and cold tolerance in perennial grasses and most information about flowering and cold tolerance is based on annual cereal crops, and although thought to be conserved, some traits may differ suggesting that this information may not be sufficient for understanding the perennial life cycle (Seppänen et al. 2010, Sandve et al. 2011, Jokela et al. 2014, Wingler 2014). Understanding flowering and cold tolerance in perennials could allow researchers and breeders to improve or develop new varieties of perennial cereals.

The goals of this project were to establish flowering time and cold tolerance gene expression in the first vegetative and sexual cycle of perennial wheat lines, and to extend gene expression data into the second vegetative and sexual cycle after exposing plants to varying photoperiods and temperatures at the end of their first cycle. Flowering time and cold tolerance gene expression of *VRN1*, *VRN2*, *VRN3*, *PPD1*, *TFL1*, *CBF12*, *CBF14* and *COR14* was compared in the different plant lines through the first vegetative and flowering cycle. Similar expression patterns were observed in all lines, suggesting that wheat, wheatgrass and perennial wheat have comparable flowering time and cold

tolerance gene expression over the first cycle. The function of *VRN2* as a floral repressor was previously unknown in perennial grasses and results suggest that *VRN2* likely has a similar function to cereals in wheatgrass and perennial wheat during the first life cycle. In 235a, expression pattern of genes especially *VRN1* and *VRN3,* was closest to that in wheatgrass lines, suggesting that it may be better at transitioning to the vegetative phase after flowering than other perennial wheat lines.

Expression results of cold tolerance genes *CBF12*, *CBF14* in plants PI206624 and 235a exposed to different photoperiods and temperatures display similar expression patterns with expression following exposure to LDs, SDs and CSDs being comparable. *CBF* genes have been shown to be rapidly upregulated within a few hours of cold exposure and then downregulated several days later (Campoli et al. 2009). Because I assessed gene expression 2 weeks after vernalization, the gene expression patterns may not be indictive of the immediate response to cold treatment. A future study that included *CBF* gene expression at 6 and 24 hours during vernalization would help provide more information into how *CBF* genes are expressed after the first sexual cycle.

Expression of flowering time and cold tolerance genes seem to suggest PI206624 and 235a grown under LDs are likely in the reproductive phase. Floral promoters *VRN1* and *VRN3* were upregulated, while floral repressor *VRN2* was downregulated, and *TFL1* had an expression level similar to the Z22 comparison stage. In the fall, when exposed to short photoperiods, perennial plants begin to transition to the vegetative phase from the reproductive phase in order to prepare for winter (Moore et al. 1991, Wang et al. 2009, Friedman and Rubin 2015). PI206624 and 235a are likely at least partially in the

vegetative phase as floral promoters *VRN1* and *VRN3* are downregulated, likely due to the SD conditions (Yan et al. 2003, Yan et al. 2006, Seppänen et al. 2010, Jokela et al. 2014). *VRN2* and *TFL1*, floral repressors, are also downregulated though, suggesting that the plants are not fully in the vegetative phase (Jensen et al. 2001, Yan et al. 2004). PI206624 and 235a grown in CSD conditions seem to be at least partially in the reproductive phase, as *VRN1* and *VRN3* are upregulated, while *VRN2* and *TFL1* are downregulated (Jensen et al. 2001, Yan et al. 2003, Yan et al. 2006, Seppänen et al. 2010, Jokela et al. 2014). Cold tolerance gene *COR14* was also downregulated. As short photoperiods and cool temperatures have been shown to cause floral promoters to be downregulated and cold tolerance genes to be upregulated, this is somewhat unexpected (Danyluk et al. 2003, Yan et al. 2003, Yan et al. 2006, Dhillon et al. 2010, Seppänen et al. 2010, Jokela et al. 2014). Plants may have remained partially in the reproductive phase because the light intensity of the plants in the vernalization room exposed to cold days was higher (575 μmol/m^2/s) than the plants exposed to SDs (340 μmol/m^2/s). A barley variety, Dicktoo, was shown to have a significant flowering delay when exposed to lower light intensities between 210 and 290 μmol/m^2/s, while it flowered much quicker when exposed to higher light intensities (Karsai et al. 2008). This could suggest that reproductive growth was caused by the higher light intensity in the CSD treatment.

While expression of cold tolerance genes *CBF12, CBF14* and *COR14* was relatively similar between lines, expression results of flowering time genes *VRN1*, *VRN2*, *VRN3*, *PPD1* and *TFL1* in plants PI206624 and 235a exposed to different photoperiods and temperatures displayed some similarities, as well as some important differences. *VRN2*, *PPD1* and *TFL1* have relatively similar expression between PI206624 and 235a

under LDs, SDs and CSDs, while some differences are observed in *VRN1* and *VRN3* expression levels. 235a had higher relative expression levels of these *VRN1* and *VRN3* than PI206624 when exposed to SDs and CSDs As perennial plants have been shown to become vegetative in the fall to prevent floral organs from freezing and to cold acclimate to survive the winter a result, expression of *VRN1* and *VRN3* is expected to be downregulated since *VRN1* is responsible for the vegetative to reproductive transition, and *VRN3* induces flowering (Yan et al. 2003, Distelfeld et al. 2009a, Seppänen et al. 2010, Andrés and Coupland 2012, Malyshev et al. 2014). Although 235a had a decrease in expression when exposed to a short photoperiod, its expression of *VRN1* and *VRN3* was much higher than in PI206624, suggesting that it may not be as efficient at transitioning to the vegetative phase as PI206624. 235a seemed to perform better than other perennial wheat lines, as flowering time gene expression was the most similar to wheatgrass. However, results still suggest that 235a may not be as efficient at responding to photoperiod and temperature cues and therefore likely is not able to transition to the vegetative phase as completely as perennial PI206624 indicating that it may be a weak perennial. Other results also suggest that the perennial wheat lines are weak perennials. Perennial wheat lines all had LT_{50} values around -15°C. Although results indicated that the perennial wheat lines were winter type, they could not survive as low of a temperature as winter wheat Norstar at -24.1°C, providing evidence that they might not have a high winter survival rate.

Results from these experiments suggest that perennial wheat lines are not as efficient at responding to photoperiod and temperature cues and as a result, do not likely transition to the vegetative phase as effectively as wheatgrass plants. Results also suggest

that perennial wheat lines have similar freezing tolerance to wheatgrass, but do not have as great of freezing tolerance as Norstar. This provides further evidence that current perennial wheat lines likely are not able to survive the winter as well as Norstar and could contribute to them being weak perennials.

Future work could involve more closely studying cold tolerance genes within a few hours of vernalization and crossing wheatgrass with wheat that has higher freezing tolerance levels to create perennial wheat lines with better freezing tolerance and winter survival. More flowering time and cold tolerance genes could also be examined. *PEP1*, a floral repressor discovered in perennial *A. alpina* that is orthologous to *Arabidopsis FLC*, allows some SAMs to remain vegetative as it prevents flowering before and after vernalization (Wang et al. 2009). Although no genes similar to *PEP1* have been discovered in cereal crops, approaching floral repression from a polycarpic point of view in perennial grasses and perennial wheat lines may to help to gain more knowledge of how flowering is repressed in perennials.

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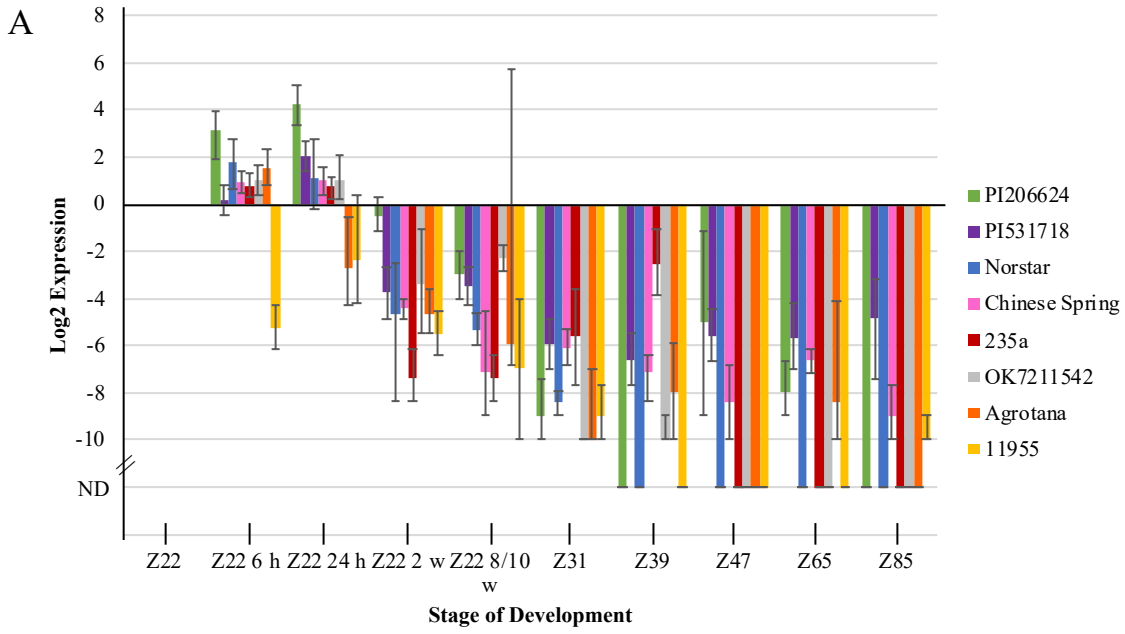
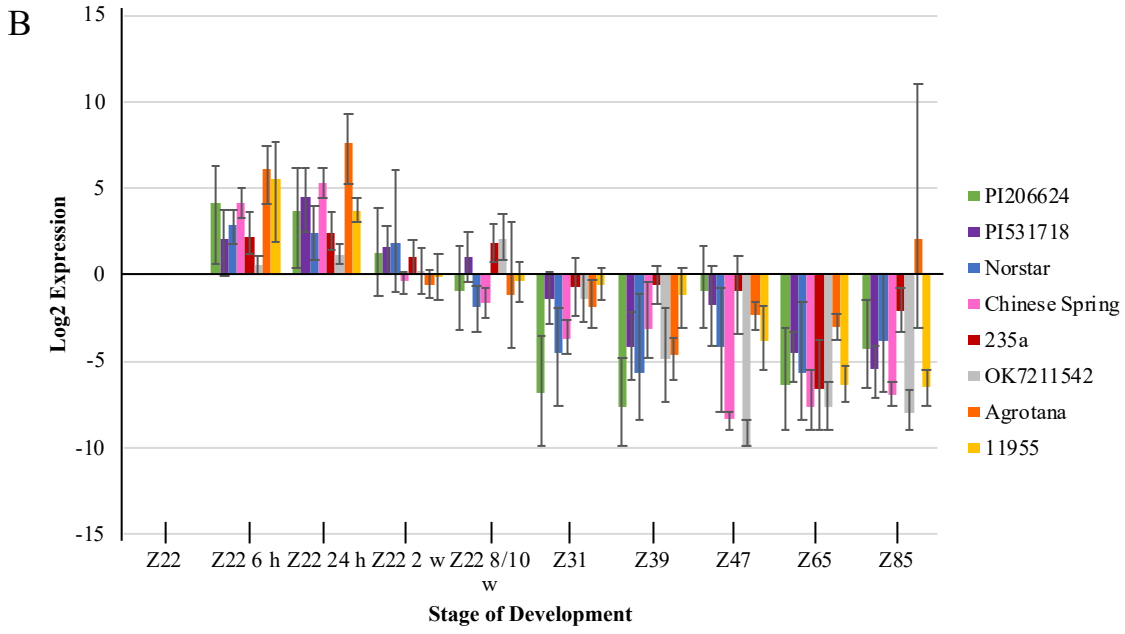
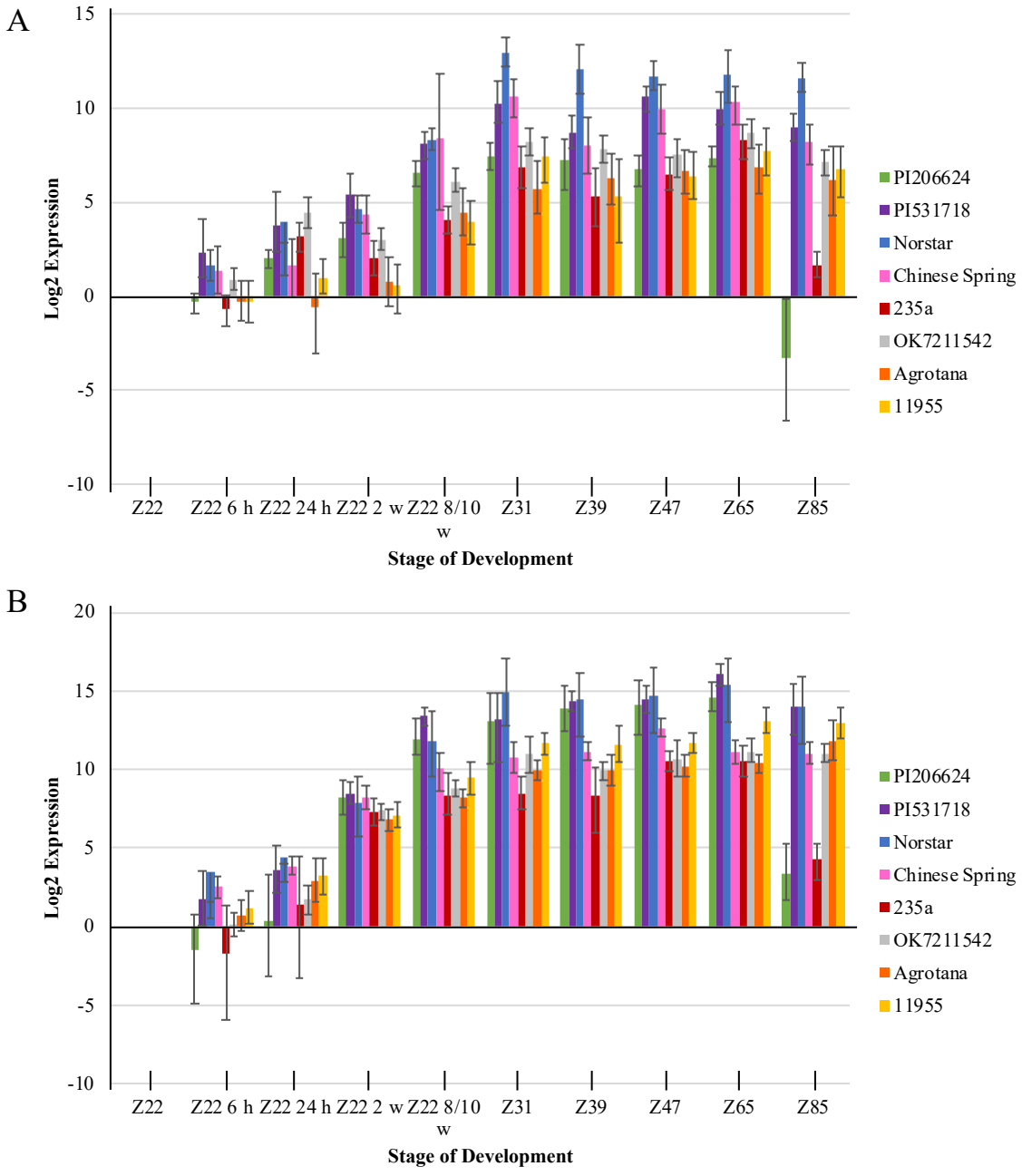
Appendix 1. Log2 gene expression of *VRN2* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Error bars indicate standard error.

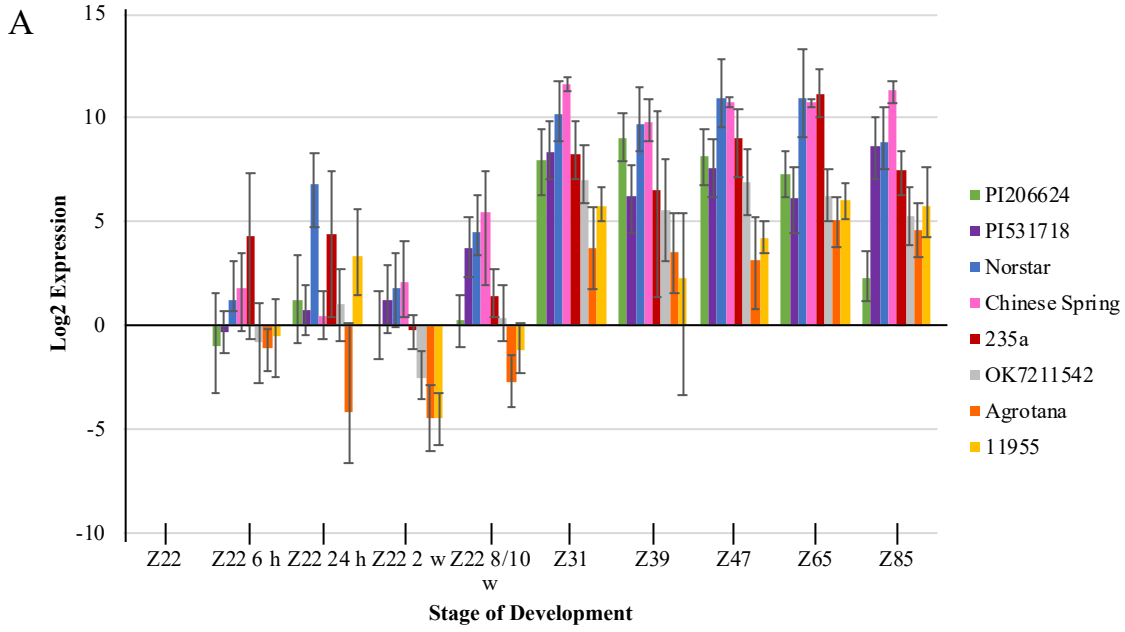


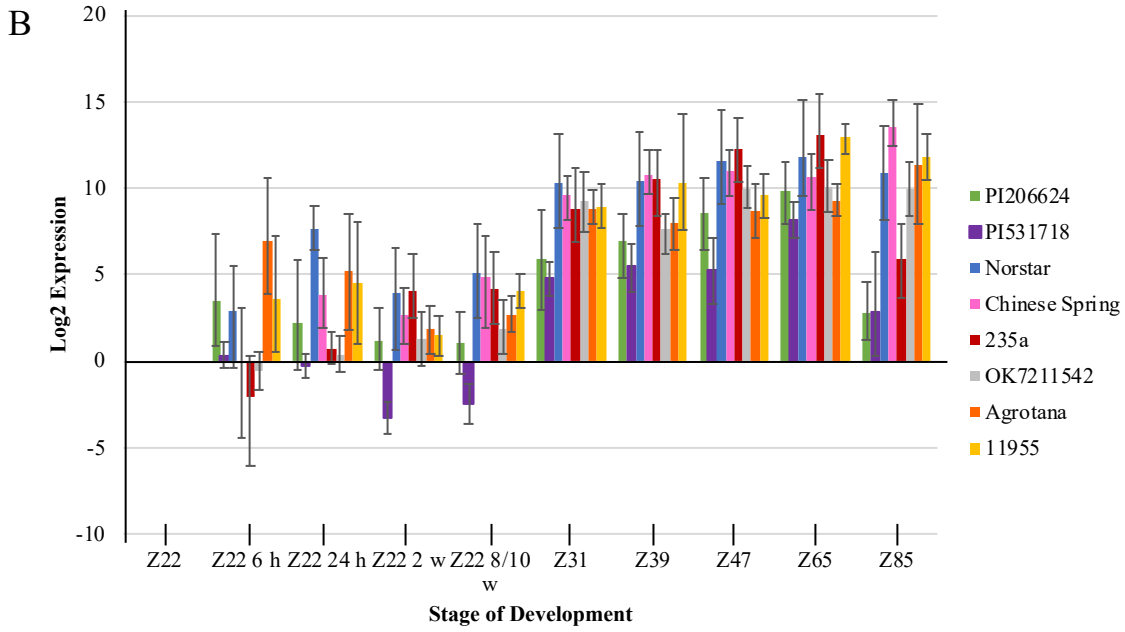


Appendix 2. Log2 gene expression of *VRN1* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Error bars indicate standard error.

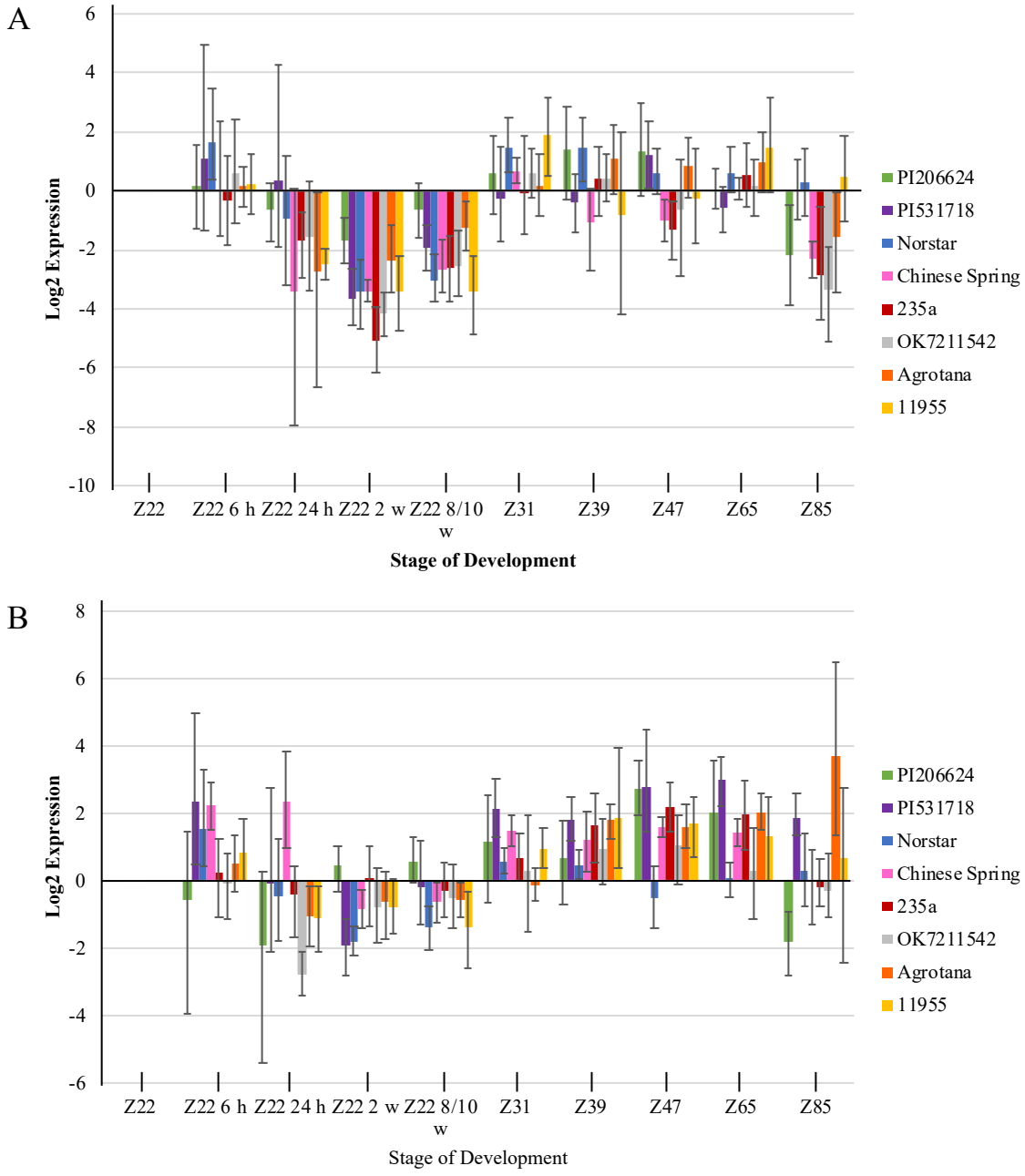


Appendix 3. Log2 gene expression of *VRN3* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Error bars indicate standard error.

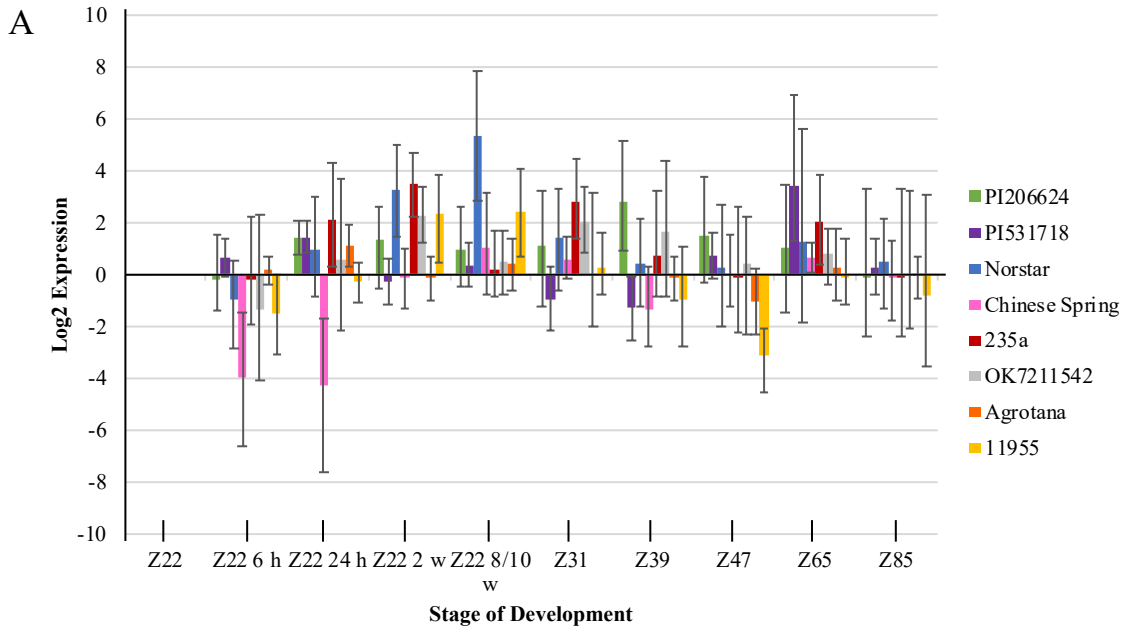


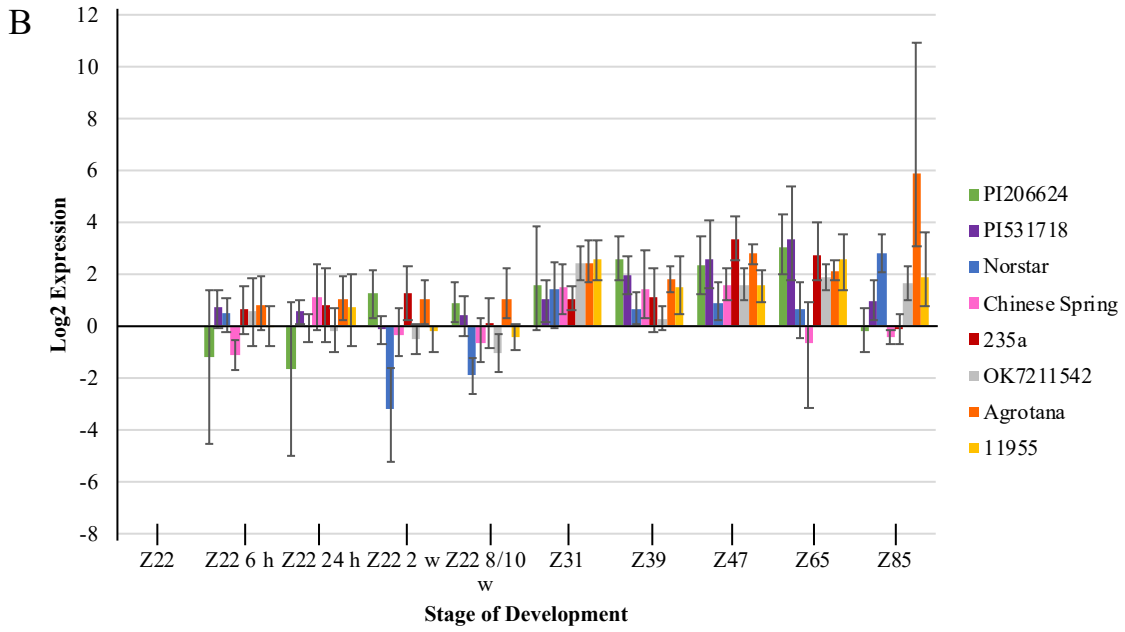


Appendix 4. Log2 gene expression of *PPD1* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Error bars indicate standard error.



Appendix 5. Log2 gene expression of *TFL1* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Error bars indicate standard error.





Appendix 6. Significance ($p<0.05$) of *VRN2* expression between stages of development in leaf tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) - 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	0.0010	0.0125	0.9382	0.10
	24 h	2 w	0.0792	0.0125	<0.0001	8.25
	2 w	8/10 w	-0.0827	0.0125	<0.0001	-7.94
	8/10 w	Z31	0.2106	0.0100	<0.0001	23.44
	Z31	Z39	-0.1512	0.0046	<0.0001	-14.03
	Z39	Z47	0.2158	0.0063	<0.0001	24.09
	Z47	Z65	-0.0188	0.0271	0.9447	-1.86
PI206624	Z65	Z85	-0.6419	0.0372	0.0859	-47.37
	6 h	24 h	0.0412	0.0127	0.0014	4.21
	24 h	2 w	0.1028	0.0127	<0.0001	10.83
	2 w	8/10 w	-0.0200	0.0127	0.1167	-1.98
	8/10 w	Z31	0.0636	0.0127	<0.0001	6.57
	Z31	Z39	-0.0185	0.0127	0.1456	-1.83
	Z39	Z47	0.0104	0.0127	0.4143	1.04
	Z47	Z65	-0.0388	0.0127	0.0026	-3.80
PI531718	Z65	Z85	0.0611	0.0127	<0.0001	6.30
	6 h	24 h	-0.0380	0.0114	0.0011	-3.73
	24 h	2 w	0.1154	0.0287	<0.0001	12.23
	2 w	8/10 w	-0.0338	0.0271	0.2146	-3.32
	8/10 w	Z31	0.1572	0.0078	<0.0001	17.02
	Z31	Z39	-0.1694	0.0064	<0.0001	-15.58
	Z39	Z47	0.0000	-	-	0.00
	Z47	Z65	0.0000	-	-	0.00
Norstar	Z65	Z85	0.0000	-	-	0.00
	6 h	24 h	-0.0009	0.0151	0.9506	-0.09
	24 h	2 w	0.1511	0.0148	<.0001	16.31
	2 w	8/10 w	-0.1257	0.0319	0.0001	-11.81
	8/10 w	Z31	-0.0236	0.0314	0.4536	-2.33
	Z31	Z39	-0.0192	0.0118	0.1060	-1.90
	Z39	Z47	-0.0215	0.0183	0.2407	-2.13
Chinese	Z47	Z65	0.1118	0.0182	<.0001	11.83
Spring	Z65	Z85	-0.1714	0.0137	<.0001	-15.75
	6 h	24 h	-0.0385	0.0110	0.0006	-3.77
	24 h	2 w	0.1995	0.0110	<.0001	22.08
	2 w	8/10 w	0.0328	0.0110	0.0033	3.33
	8/10 w	Z31	0.0143	0.0415	0.7315	1.44
	Z31	Z39	0.1027	0.0410	0.0133	10.82
	Z39	Z47	-0.2093	0.0064	<.0001	-18.88
	Z47	Z65	-0.0500	0.0098	<.0001	-4.88
235a	Z65	Z85	0.1136	0.0094	<.0001	12.03
	6 h	24 h	-0.0406	0.0106	0.0002	-3.98
	24 h	2 w	0.0824	0.0225	0.0003	8.59
	2 w	8/10 w	0.0419	0.0254	0.1005	4.28
	8/10 w	Z31	0.3328	0.0132	<.0001	39.49
	Z31	Z39	0.0267	0.0123	0.0320	2.71
	Z39	Z47	-0.0556	0.0124	<.0001	-5.41
	Z47	Z65	-0.0301	0.0188	0.1116	-2.96
OK7211542	Z65	Z85	-0.0410	0.0256	0.1106	-4.02
	6 h	24 h	-0.1450	0.0400	0.0004	-13.50
	24 h	2 w	0.0904	0.0392	0.0226	9.46
	2 w	8/10 w	-0.0247	0.0123	0.0453	-2.44
	8/10 w	Z31	0.1363	0.0281	<.0001	14.60
	Z31	Z39	0.0193	0.0322	0.5502	1.94
	Z39	Z47	-0.0718	0.0312	0.0226	-6.93
	Z47	Z65	0.0263	0.0336	0.4349	2.66
Agrotana	Z65	Z85	-0.0477	0.0223	0.0338	-4.65
	6 h	24 h	0.1082	0.0270	<0.0001	11.43
	24 h	2 w	0.0151	0.0290	0.7172	1.52
	2 w	8/10 w	-0.0613	0.0446	0.1712	-5.95
	8/10 w	Z31	0.1262	0.0442	0.9855	13.45
	Z31	Z39	-0.0469	0.0116	0.0006	-4.58
	Z39	Z47	-0.3714	0.0186	0.0475	-31.02
	Z47	Z65	0.0274	0.0199	0.1712	2.78
11955	Z65	Z85	0.0028	0.0139	0.8414	0.28

Appendix 7. Significance ($p<0.05$) of *VRN2* expression between stages of development in meristem tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) - 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	-0.0269	0.0152	0.0783	-2.66
	24 h	2 w	0.0359	0.0152	0.0194	3.66
	2 w	8/10 w	-0.0627	0.0152	<0.0001	-6.08
	8/10 w	Z31	0.2023	0.0108	<0.0001	22.42
	Z31	Z39	0.0139	0.0089	0.1193	1.40
	Z39	Z47	0.1566	0.0089	<0.0001	16.95
	Z47	Z65	-0.1212	0.0112	<0.0001	-11.41
PI206624	Z65	Z85	0.1277	0.0123	<0.0001	13.62
	6 h	24 h	0.0498	0.0127	0.0001	5.11
	24 h	2 w	0.0276	0.0127	0.0308	2.80
	2 w	8/10 w	-0.0269	0.0127	0.0353	-2.65
	8/10 w	Z31	0.1580	0.0127	<0.0001	17.12
	Z31	Z39	-0.0265	0.0127	0.9997	-2.62
	Z39	Z47	0.0799	0.0127	<0.0001	8.31
	Z47	Z65	-0.1042	0.0127	<0.0001	-9.90
PI531718	Z65	Z85	0.0000	0.0127	1.0000	0.00
	6 h	24 h	-0.0165	0.0105	0.1197	-1.63
	24 h	2 w	-0.0036	0.0233	0.8762	-0.36
	2 w	8/10 w	-0.1017	0.0267	0.0002	-9.67
	8/10 w	Z31	0.0936	0.0315	0.0034	9.81
	Z31	Z39	-0.0290	0.0434	0.5053	-2.85
	Z39	Z47	0.0393	0.0483	0.4170	4.01
	Z47	Z65	-0.0516	0.0484	0.2873	-5.03
Norstar	Z65	Z85	0.0350	0.0449	0.4366	3.57
	6 h	24 h	0.0441	0.0132	0.0010	4.51
	24 h	2 w	0.1193	0.0091	<.0001	12.67
	2 w	8/10 w	-0.0502	0.0222	0.0248	-4.90
	8/10 w	Z31	0.0965	0.0208	<.0001	10.13
	Z31	Z39	0.0143	0.0128	0.2653	1.44
	Z39	Z47	-0.1500	0.0128	<.0001	-13.93
Chinese Spring	Z47	Z65	0.0000	-	-	0.00
	Z65	Z85	0.0000	-	-	0.00
	6 h	24 h	-0.0147	0.0078	0.0617	-1.46
	24 h	2 w	0.0944	0.0078	<.0001	9.90
	2 w	8/10 w	0.0169	0.0078	0.0326	1.70
	8/10 w	Z31	0.0994	0.0066	<.0001	10.45
	Z31	Z39	0.0223	0.0079	0.0052	2.25
	Z39	Z47	-0.0592	0.0103	<.0001	-5.75
	Z47	Z65	-0.1397	0.0076	<.0001	-13.04
235a	Z65	Z85	0.0272	0.0096	0.0053	2.76
	6 h	24 h	0.0275	0.0075	0.0004	2.79
	24 h	2 w	0.1120	0.0090	<.0001	11.85
	2 w	8/10 w	0.0691	0.0123	<.0001	7.16
	8/10 w	Z31	0.1589	0.0114	<.0001	17.22
	Z31	Z39	-0.0613	0.0278	0.0290	-5.94
	Z39	Z47	-0.1211	0.0271	<.0001	-11.41
	Z47	Z65	0.0000	-	-	0.00
OK7211542	Z65	Z85	0.0000	-	-	0.00
	6 h	24 h	0.0622	0.0402	0.1238	6.42
	24 h	2 w	0.2214	0.0346	<.0001	24.78
	2 w	8/10 w	-0.0878	0.0109	<.0001	-8.40
	8/10 w	Z31	-0.0246	0.0102	0.0170	-2.43
	Z31	Z39	-0.0584	0.0067	<.0001	-5.67
	Z39	Z47	0.0292	0.0086	0.0009	2.96
	Z47	Z65	0.0098	0.0111	0.3790	0.98
Agrotana	Z65	Z85	0.0205	0.0091	0.0253	2.07
	6 h	24 h	-0.1612	0.0460	0.0006	-14.89
	24 h	2 w	0.0566	0.0328	0.0861	5.82
	2 w	8/10 w	-0.0243	0.0113	0.0331	-2.40
	8/10 w	Z31	0.0372	0.0078	<0.0001	3.79
	Z31	Z39	-0.0512	0.0051	<0.0001	-4.99
	Z39	Z47	0.0064	0.0356	0.8570	0.64
	Z47	Z65	-0.1360	0.0353	0.0002	-12.72
11955	Z65	Z85	0.0000	-	1.0000	0.00

Appendix 8. Significance ($p<0.05$) of *VRN1* expression between stages of development in leaf tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) - 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	0.0489	0.0119	0.0003	5.01
	24 h	2 w	-0.1030	0.0119	<0.0001	-9.79
	2 w	8/10 w	0.1231	0.0119	<0.0001	13.10
	8/10 w	Z31	-0.0149	0.0124	0.2315	-1.47
	Z31	Z39	-0.0262	0.0101	0.0105	-2.59
	Z39	Z47	-0.8893	0.0339	0.0096	-58.91
	Z47	Z65	0.1477	0.0410	0.0004	15.92
PI206624	Z65	Z85	-0.0669	0.0291	0.0228	-6.47
	6 h	24 h	0.0388	0.0117	0.0011	3.96
	24 h	2 w	0.0013	0.0117	0.9125	0.13
	2 w	8/10 w	0.0616	0.0117	<0.0001	6.35
	8/10 w	Z31	-0.9111	0.0099	<0.0001	-59.79
	Z31	Z39	-0.0572	0.0122	<0.0001	-5.56
	Z39	Z47	0.0073	0.0132	0.5805	0.73
	Z47	Z65	-0.0295	0.0145	0.0431	-2.91
PI531718	Z65	Z85	0.0083	0.0137	0.5436	0.84
	6 h	24 h	0.0708	0.0200	0.0005	7.34
	24 h	2 w	-0.0666	0.0206	0.0015	-6.44
	2 w	8/10 w	0.1025	0.0104	<0.0001	10.79
	8/10 w	Z31	-0.1135	0.0133	<0.0001	-10.73
	Z31	Z39	-0.0194	0.0191	0.3104	-1.92
	Z39	Z47	0.0056	0.0218	0.7967	0.57
	Z47	Z65	0.0173	0.0285	0.5438	1.75
Norstar	Z65	Z85	-0.0549	0.0254	0.0320	-5.34
	6 h	24 h	0.0081	0.0171	0.6374	0.81
	24 h	2 w	-0.0783	0.0157	<.0001	-7.53
	2 w	8/10 w	0.1073	0.0321	0.0010	11.33
	8/10 w	Z31	-0.0594	0.0341	0.0830	-5.77
	Z31	Z39	-0.0647	0.0172	0.0002	-6.27
	Z39	Z47	0.0755	0.0172	<.0001	7.84
Chinese Spring	Z47	Z65	0.0650	0.0172	0.0002	6.72
	Z65	Z85	-0.1461	0.0172	<.0001	-13.59
	6 h	24 h	0.0993	0.0122	<.0001	10.44
	24 h	2 w	-0.0148	0.0122	0.2292	-1.47
	2 w	8/10 w	0.1284	0.0122	<.0001	13.70
	8/10 w	Z31	-0.0217	0.0289	0.4527	-2.15
	Z31	Z39	-0.0633	0.0413	0.1279	-6.13
	Z39	Z47	0.1398	0.0363	0.0002	15.00
	Z47	Z65	0.0167	0.0280	0.5528	1.68
235a	Z65	Z85	-0.0904	0.0211	<.0001	-8.64
	6 h	24 h	0.0916	0.0069	<.0001	9.60
	24 h	2 w	-0.0168	0.0069	0.0159	-1.66
	2 w	8/10 w	0.1329	0.0069	<.0001	14.21
	8/10 w	Z31	-0.0820	0.0087	<.0001	-7.87
	Z31	Z39	-0.0390	0.0075	<.0001	-3.82
	Z39	Z47	0.0389	0.0118	0.0012	3.96
	Z47	Z65	0.0631	0.0118	<.0001	6.51
OK7211542	Z65	Z85	-0.0943	0.0187	<.0001	-9.00
	6 h	24 h	0.0214	0.0146	0.1455	2.16
	24 h	2 w	-0.0714	0.0107	<.0001	-6.89
	2 w	8/10 w	0.1487	0.0062	<.0001	16.03
	8/10 w	Z31	-0.0472	0.0168	0.0054	-4.61
	Z31	Z39	-0.0358	0.0190	0.0614	-3.51
	Z39	Z47	0.0611	0.0104	<.0001	6.30
	Z47	Z65	-0.0641	0.0403	0.1137	-6.21
Agrotana	Z65	Z85	-0.0054	0.0463	0.9075	-0.54
	6 h	24 h	0.0430	0.0225	0.0577	4.40
	24 h	2 w	-0.0813	0.0218	0.0003	-7.80
	2 w	8/10 w	0.1100	0.0109	<0.0001	11.63
	8/10 w	Z31	-0.0873	0.0094	<0.0001	-8.36
	Z31	Z39	-0.0275	0.0160	0.0882	-2.71
	Z39	Z47	0.0471	0.0273	0.0873	4.82
	Z47	Z65	0.0199	0.0229	0.3865	2.00
11955	Z65	Z85	-0.0750	0.0108	<0.0001	-7.22

Appendix 9. Significance ($p<0.05$) of *VRN1* expression between stages of development in meristem tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	0.0512	0.0197	0.0103	5.2512
	24 h	2 w	-0.1307	0.0197	<0.0001	-12.2519
	2 w	8/10 w	0.1313	0.0197	<0.0001	14.0310
	8/10 w	Z31	0.0118	0.0475	0.8045	1.1840
	Z31	Z39	0.0740	0.0495	0.1366	7.6807
	Z39	Z47	-0.0262	0.0224	0.2446	-2.5821
	Z47	Z65	0.0556	0.0204	0.0070	5.7196
PI206624	Z65	Z85	-0.0450	0.0221	0.0439	-4.3974
	6 h	24 h	0.0531	0.0108	<0.0001	5.4556
	24 h	2 w	0.0399	0.0108	0.0003	4.0696
	2 w	8/10 w	0.1580	0.0108	<0.0001	17.1166
	8/10 w	Z31	0.1122	0.0481	0.0211	11.8737
	Z31	Z39	0.0914	0.0499	0.0686	9.5674
	Z39	Z47	0.0247	0.0162	0.1410	2.5038
	Z47	Z65	0.0160	0.0121	0.1876	1.6108
PI531718	Z65	Z85	-0.0420	0.0200	0.0377	-4.1101
	6 h	24 h	0.0844	0.0076	<0.0001	8.8108
	24 h	2 w	-0.0335	0.0224	0.1374	-3.2926
	2 w	8/10 w	0.1500	0.0231	<0.0001	16.1834
	8/10 w	Z31	-0.0976	0.0142	<0.0001	-9.3016
	Z31	Z39	0.0136	0.0124	0.2774	1.3652
	Z39	Z47	0.0019	0.0096	0.8415	0.1927
	Z47	Z65	0.0168	0.0099	0.0895	1.6983
Norstar	Z65	Z85	-0.0706	0.0142	<0.0001	-6.8119
	6 h	24 h	0.0426	0.0075	<.0001	4.3500
	24 h	2 w	-0.0889	0.0098	<.0001	-8.5063
	2 w	8/10 w	0.0542	0.0101	<.0001	5.5696
	8/10 w	Z31	-0.0008	0.0159	0.9612	-0.0770
	Z31	Z39	0.0086	0.0205	0.6765	0.8621
	Z39	Z47	0.0634	0.0205	0.0024	6.5464
Chinese Spring	Z47	Z65	-0.0826	0.0205	<.0001	-7.9299
	Z65	Z85	-0.0241	0.0205	0.2424	-2.3812
	6 h	24 h	0.0850	0.0122	<.0001	8.8695
	24 h	2 w	-0.1125	0.0122	<.0001	-10.6403
	2 w	8/10 w	0.0386	0.0122	0.0019	3.9365
	8/10 w	Z31	0.0287	0.0289	0.3221	2.9085
	Z31	Z39	0.0274	0.0413	0.5079	2.7799
	Z39	Z47	0.0210	0.0363	0.5634	2.1263
	Z47	Z65	0.0212	0.0280	0.4493	2.1467
235a	Z65	Z85	-0.0478	0.0211	0.0249	-4.6666
	6 h	24 h	0.0578	0.0106	<.0001	5.9471
	24 h	2 w	-0.1187	0.0106	<.0001	-11.1926
	2 w	8/10 w	0.0784	0.0106	<.0001	8.1512
	8/10 w	Z31	-0.0359	0.0193	0.0643	-3.5234
	Z31	Z39	0.0073	0.0203	0.7188	0.7342
	Z39	Z47	0.0423	0.0120	0.0005	4.3218
	Z47	Z65	-0.0740	0.0223	0.0011	-7.1300
OK7211542	Z65	Z85	0.0117	0.0229	0.6104	1.1748
	6 h	24 h	0.0764	0.0224	0.0008	7.9437
	24 h	2 w	-0.2012	0.0225	<.0001	-18.2251
	2 w	8/10 w	-0.0122	0.0344	0.7241	-1.2106
	8/10 w	Z31	-0.1180	0.0361	0.0013	-11.1304
	Z31	Z39	0.0468	0.0230	0.0439	4.7923
	Z39	Z47	-0.0554	0.0208	0.0084	-5.3893
	Z47	Z65	0.0253	0.0121	0.0382	2.5633
Agrotana	Z65	Z85	-0.1000	0.0676	0.1406	-9.5163
	6 h	24 h	-0.0079	0.0225	0.7274	-0.7829
	24 h	2 w	-0.2517	0.0218	<0.0001	-22.2522
	2 w	8/10 w	0.0775	0.0109	<0.0001	8.0593
	8/10 w	Z31	-0.0479	0.0094	<0.0001	-4.6752
	Z31	Z39	-0.0424	0.0160	0.0088	-4.1542
	Z39	Z47	0.1166	0.0273	<0.0001	12.3670
	Z47	Z65	-0.0250	0.0229	0.3768	-2.4690
11955	Z65	Z85	-0.0020	0.0108	0.8560	-0.1958

Appendix 10. Significance ($p<0.05$) of *VRN3* expression between stages of development in leaf tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	0.0415	0.0119	0.0006	4.24
	24 h	2 w	0.1043	0.0119	<0.0001	10.99
	2 w	8/10 w	0.0108	0.0119	0.3642	1.09
	8/10 w	Z31	-0.2307	0.0086	<0.0001	-20.60
	Z31	Z39	0.0170	0.0028	<0.0001	1.71
	Z39	Z47	-0.1079	0.0257	<0.0001	-10.23
	Z47	Z65	0.1010	0.0353	0.0048	10.63
PI206624	Z65	Z85	-0.4504	0.0312	0.1501	-36.26
	6 h	24 h	0.0180	0.0110	0.1035	1.81
	24 h	2 w	0.0279	0.0101	0.0066	2.83
	2 w	8/10 w	0.0508	0.0077	<0.0001	5.21
	8/10 w	Z31	-0.1648	0.0155	<0.0001	-15.19
	Z31	Z39	-0.0732	0.0209	0.0006	-7.06
	Z39	Z47	-0.0103	0.0209	0.6223	-1.02
	Z47	Z65	-0.0527	0.0209	0.0126	-5.13
PI531718	Z65	Z85	0.1227	0.0209	<0.0001	13.05
	6 h	24 h	0.1463	0.0232	<0.0001	15.75
	24 h	2 w	-0.0270	0.0128	0.0362	-2.66
	2 w	8/10 w	0.0876	0.0113	<0.0001	9.15
	8/10 w	Z31	-0.1789	0.0068	<0.0001	-16.38
	Z31	Z39	-0.0118	0.0075	0.1197	-1.17
	Z39	Z47	0.0788	0.0135	<0.0001	8.20
	Z47	Z65	0.0236	0.0278	0.3975	2.39
Norstar	Z65	Z85	-0.1611	0.0260	<0.0001	-14.88
	6 h	24 h	-0.0488	0.0283	0.0863	-4.76
	24 h	2 w	-0.0368	0.0182	0.0452	-3.61
	2 w	8/10 w	0.1021	0.0281	0.0004	10.75
	8/10 w	Z31	-0.2479	0.0263	<.0001	-21.96
	Z31	Z39	-0.0566	0.0183	0.0023	-5.50
	Z39	Z47	0.0634	0.0138	<.0001	6.54
Chinese	Z47	Z65	0.0751	0.0198	0.0002	7.79
Spring	Z65	Z85	-0.0819	0.0271	0.0029	-7.86
	6 h	24 h	-0.0188	0.0458	0.6825	-1.86
	24 h	2 w	-0.0548	0.0322	0.0904	-5.33
	2 w	8/10 w	0.0825	0.0187	<.0001	8.60
	8/10 w	Z31	-0.1514	0.0192	<.0001	-14.05
	Z31	Z39	-0.0484	0.0573	0.3993	-4.73
	Z39	Z47	0.1564	0.0617	0.0122	16.93
	Z47	Z65	0.0332	0.0431	0.4423	3.37
235a	Z65	Z85	-0.1570	0.0396	0.0001	-14.53
	6 h	24 h	0.0383	0.0204	0.0626	3.90
	24 h	2 w	0.0752	0.0204	0.0003	7.81
	2 w	8/10 w	0.0922	0.0204	<.0001	9.66
	8/10 w	Z31	-0.2386	0.0164	<.0001	-21.23
	Z31	Z39	-0.0749	0.0250	0.0032	-7.21
	Z39	Z47	0.1008	0.0260	0.0002	10.61
	Z47	Z65	-0.0286	0.0109	0.0093	-2.82
OK7211542	Z65	Z85	-0.0521	0.0182	0.0047	-5.08
	6 h	24 h	-0.0564	0.0419	0.1799	-5.48
	24 h	2 w	0.1102	0.0416	0.0089	11.65
	2 w	8/10 w	0.0674	0.0087	<.0001	6.97
	8/10 w	Z31	-0.2196	0.0192	<.0001	-19.72
	Z31	Z39	-0.0649	0.0259	0.0134	-6.28
	Z39	Z47	0.0291	0.0260	0.2650	2.95
	Z47	Z65	-0.0003	0.0356	0.9924	-0.03
Agrotana	Z65	Z85	0.0036	0.0324	0.9125	0.36
	6 h	24 h	0.0952	0.0282	0.0009	9.99
	24 h	2 w	0.0744	0.0298	0.0136	7.72
	2 w	8/10 w	0.0941	0.0853	<0.0001	9.87
	8/10 w	Z31	-0.2025	0.0140	<0.0001	-18.33
	Z31	Z39	-0.0770	0.0209	0.0003	-7.41
	Z39	Z47	0.0757	0.0222	0.0008	7.86
	Z47	Z65	0.0367	0.0149	0.0149	3.74
11955	Z65	Z85	-0.0405	0.0224	0.0724	-3.97

Appendix 11. Significance ($p<0.05$) of *VRN3* expression between stages of development in meristem tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	-0.0544	0.0128	<0.0001	-5.29
	24 h	2 w	-0.0345	0.0128	0.0075	-3.40
	2 w	8/10 w	0.0008	0.0128	0.9480	0.08
	8/10 w	Z31	-0.0944	0.0290	0.0014	-9.00
	Z31	Z39	0.0677	0.0325	0.0384	7.01
	Z39	Z47	0.0276	0.0192	0.1536	2.80
	Z47	Z65	0.0745	0.0133	<0.0001	7.73
PI206624	Z65	Z85	-0.0554	0.0129	<0.0001	-5.39
	6 h	24 h	-0.0422	0.0110	0.0002	-4.13
	24 h	2 w	0.0365	0.0101	0.0004	3.72
	2 w	8/10 w	0.0143	0.0077	0.0665	1.44
	8/10 w	Z31	-0.1211	0.0155	<0.0001	-11.41
	Z31	Z39	0.0787	0.0209	0.0002	8.18
	Z39	Z47	0.0143	0.0209	0.4930	1.44
	Z47	Z65	0.0610	0.0209	0.0040	6.29
PI531718	Z65	Z85	-0.1501	0.0209	<0.0001	-13.94
	6 h	24 h	0.1353	0.0232	<0.0001	14.49
	24 h	2 w	0.0350	0.0128	0.0068	3.56
	2 w	8/10 w	0.0704	0.0113	<0.0001	7.29
	8/10 w	Z31	-0.0188	0.0068	<0.0001	-1.86
	Z31	Z39	0.0142	0.0075	0.0622	1.43
	Z39	Z47	0.0407	0.0135	0.0030	4.15
	Z47	Z65	0.0048	0.0278	0.8627	0.48
Norstar	Z65	Z85	-0.0715	0.0260	0.0066	-6.90
	6 h	24 h	0.1149	0.0283	<.0001	12.18
	24 h	2 w	0.0195	0.0182	0.2864	1.97
	2 w	8/10 w	0.0697	0.0281	0.0143	7.22
	8/10 w	Z31	-0.1559	0.0263	<.0001	-14.44
	Z31	Z39	0.0433	0.0183	0.0193	4.42
	Z39	Z47	0.0270	0.0138	0.0525	2.74
Chinese Spring	Z47	Z65	-0.0552	0.0198	0.0059	-5.37
	Z65	Z85	0.1061	0.0271	0.0001	11.19
	6 h	24 h	0.0680	0.0319	0.0347	7.03
	24 h	2 w	-0.0091	0.0112	0.4176	-0.91
	2 w	8/10 w	0.0727	0.0327	0.0275	7.54
	8/10 w	Z31	-0.1253	0.0134	<.0001	-11.78
	Z31	Z39	0.0831	0.0070	<.0001	8.66
	Z39	Z47	0.0021	0.0204	0.9195	0.21
	Z47	Z65	0.0505	0.0357	0.1595	5.18
235a	Z65	Z85	-0.0482	0.0319	0.1334	-4.70
	6 h	24 h	0.0338	0.0135	0.0133	3.43
	24 h	2 w	0.0203	0.0135	0.1340	2.05
	2 w	8/10 w	0.0263	0.0135	0.0525	2.67
	8/10 w	Z31	-0.2029	0.0130	<.0001	-18.36
	Z31	Z39	-0.0167	0.0102	0.1033	-1.65
	Z39	Z47	0.0987	0.0072	<.0001	10.38
	Z47	Z65	-0.0742	0.0241	0.0024	-7.15
OK7211542	Z65	Z85	0.0111	0.0257	0.6665	1.12
	6 h	24 h	-0.0446	0.0126	0.0005	-4.36
	24 h	2 w	-0.0416	0.0145	0.0047	-4.08
	2 w	8/10 w	-0.0253	0.0338	0.4551	-2.50
	8/10 w	Z31	-0.2344	0.0330	<.0001	-20.90
	Z31	Z39	0.0088	0.0225	0.6952	0.89
	Z39	Z47	-0.0287	0.0252	0.2550	-2.83
	Z47	Z65	0.0359	0.0145	0.0146	3.65
Agrotana	Z65	Z85	-0.0747	0.0472	0.1152	-7.20
	6 h	24 h	-0.2872	0.0282	0.3104	-24.96
	24 h	2 w	-0.0735	0.0298	0.0148	-7.08
	2 w	8/10 w	0.0636	0.0185	0.0008	6.57
	8/10 w	Z31	-0.1336	0.0140	<0.0001	-12.51
	Z31	Z39	0.0117	0.0209	0.5776	1.17
	Z39	Z47	0.0738	0.0222	0.0110	7.65
	Z47	Z65	0.0572	0.0149	0.0002	5.89
11955	Z65	Z85	-0.0412	0.0224	0.0678	-4.04

Appendix 12. Significance (p<0.05) of *PPD1* expression between stages of development in leaf tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	-0.0753	0.0111	<0.0001	-7.25
	24 h	2 w	0.0212	0.0111	0.0056	2.14
	2 w	8/10 w	0.0482	0.0111	<0.0001	4.94
	8/10 w	Z31	-0.0300	0.0099	0.0029	-2.96
	Z31	Z39	0.0128	0.0093	0.1715	1.29
	Z39	Z47	-0.0934	0.0246	0.0002	-8.91
	Z47	Z65	0.0981	0.0386	0.0121	10.31
PI206624	Z65	Z85	-0.0361	0.0362	0.3982	-3.54
	6 h	24 h	-0.0502	0.0112	<0.0001	-4.89
	24 h	2 w	0.1375	0.0112	<0.0001	14.74
	2 w	8/10 w	0.0315	0.0112	0.0057	3.20
	8/10 w	Z31	-0.0733	0.0213	0.0007	-7.07
	Z31	Z39	-0.0053	0.0239	0.8256	-0.52
	Z39	Z47	-0.0012	0.0179	0.9488	-0.11
	Z47	Z65	-0.0734	0.0146	<0.0001	-7.07
PI531718	Z65	Z85	0.0670	0.0106	<0.0001	6.93
	6 h	24 h	-0.1090	0.0206	<0.0001	-10.33
	24 h	2 w	0.0544	0.0224	0.0163	5.59
	2 w	8/10 w	0.0064	0.0121	0.5986	0.64
	8/10 w	Z31	-0.1208	0.0050	<0.0001	-11.38
	Z31	Z39	0.0077	0.0154	0.6176	0.77
	Z39	Z47	-0.0157	0.0177	0.3753	-1.56
	Z47	Z65	0.0170	0.0179	0.3453	1.71
Norstar	Z65	Z85	-0.0635	0.0164	0.0002	-6.16
	6 h	24 h	-0.1147	0.0280	<.0001	-10.84
	24 h	2 w	0.0032	0.0264	0.9044	0.32
	2 w	8/10 w	0.0047	0.0123	0.7031	0.47
	8/10 w	Z31	-0.1139	0.0175	<.0001	-10.77
	Z31	Z39	-0.0433	0.0180	0.0172	-4.24
	Z39	Z47	0.0174	0.0180	0.3350	1.76
Chinese	Z47	Z65	0.1035	0.0180	<.0001	10.90
Spring	Z65	Z85	-0.1785	0.0180	<.0001	-16.35
	6 h	24 h	-0.0702	0.0133	<.0001	-6.78
	24 h	2 w	0.0115	0.0133	0.3874	1.16
	2 w	8/10 w	0.1059	0.0133	<.0001	11.17
	8/10 w	Z31	-0.0055	0.0250	0.8272	-0.54
	Z31	Z39	0.0170	0.0250	0.4968	1.72
	Z39	Z47	0.0038	0.0156	0.8095	0.38
	Z47	Z65	0.0153	0.0249	0.5403	1.54
235a	Z65	Z85	-0.0981	0.0224	<.0001	-9.35
	6 h	24 h	-0.0992	0.0104	<.0001	-9.45
	24 h	2 w	-0.0002	0.0093	0.9797	-0.02
	2 w	8/10 w	0.0467	0.0079	<.0001	4.78
	8/10 w	Z31	-0.0880	0.0134	<.0001	-8.42
	Z31	Z39	-0.0176	0.0176	0.3177	-1.75
	Z39	Z47	0.0017	0.0176	0.9253	0.17
	Z47	Z65	0.0273	0.0176	0.1224	2.77
OK7211542	Z65	Z85	-0.1220	0.0176	<.0001	-11.49
	6 h	24 h	-0.0746	0.0238	0.0020	-7.19
	24 h	2 w	0.0205	0.0234	0.3832	2.07
	2 w	8/10 w	0.0521	0.0075	<.0001	5.35
	8/10 w	Z31	-0.0456	0.0116	0.0001	-4.46
	Z31	Z39	-0.0206	0.0147	0.1647	-2.04
	Z39	Z47	0.0297	0.0102	0.0041	3.01
	Z47	Z65	-0.0543	0.0298	0.0697	-5.29
Agrotana	Z65	Z85	-0.0577	0.0339	0.0905	-5.60
	6 h	24 h	-0.1530	0.0276	0.0002	-14.19
	24 h	2 w	0.0126	0.0280	0.6540	1.26
	2 w	8/10 w	-0.0111	0.0104	0.2894	-1.10
	8/10 w	Z31	-0.1247	0.0081	<0.0001	-11.72
	Z31	Z39	-0.3814	0.0098	0.0001	-31.71
	Z39	Z47	0.0185	0.0237	0.4352	1.87
	Z47	Z65	0.0275	0.0232	0.2375	2.79
11955	Z65	Z85	-0.0572	0.0182	0.0020	-5.56

Appendix 13. Significance ($p<0.05$) of *PPD1* expression between stages of development in meristem tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	-0.0753	0.0165	<0.0001	-7.26
	24 h	2 w	0.0651	0.0165	0.0001	6.73
	2 w	8/10 w	0.0110	0.0165	0.5062	1.11
	8/10 w	Z31	0.0299	0.0480	0.5342	3.04
	Z31	Z39	0.0228	0.0507	0.6541	2.30
	Z39	Z47	0.0481	0.0228	0.0365	4.93
	Z47	Z65	0.0106	0.0126	0.3984	1.07
PI206624	Z65	Z85	-0.5668	0.0148	0.0002	-43.27
	6 h	24 h	-0.1162	0.0112	<0.0001	-10.97
	24 h	2 w	0.0694	0.0112	<0.0001	7.18
	2 w	8/10 w	0.0506	0.0112	<0.0001	5.18
	8/10 w	Z31	0.0317	0.0213	0.1397	3.22
	Z31	Z39	0.0501	0.0239	0.0371	5.14
	Z39	Z47	0.0600	0.0179	0.0010	6.18
	Z47	Z65	-0.0395	0.0146	0.0077	-3.87
PI531718	Z65	Z85	-0.0071	0.0106	0.5067	-0.70
	6 h	24 h	-0.0794	0.0089	<0.0001	-7.63
	24 h	2 w	0.0773	0.0163	<0.0001	8.04
	2 w	8/10 w	0.0451	0.0164	0.0066	4.62
	8/10 w	Z31	-0.0608	0.0046	<0.0001	-5.90
	Z31	Z39	0.0004	0.0025	0.8646	0.04
	Z39	Z47	-0.0447	0.0122	0.0003	-4.38
	Z47	Z65	0.0173	0.0140	0.2201	1.74
Norstar	Z65	Z85	-0.0168	0.0206	0.4147	-1.67
	6 h	24 h	-0.0001	0.0280	0.9976	-0.01
	24 h	2 w	0.0591	0.0264	0.0263	6.09
	2 w	8/10 w	0.0014	0.0123	0.9098	0.14
	8/10 w	Z31	-0.0482	0.0175	0.0064	-4.71
	Z31	Z39	-0.0123	0.0180	0.4937	-1.23
	Z39	Z47	0.0267	0.0180	0.1398	2.70
Chinese	Z47	Z65	-0.0403	0.0180	0.0266	-3.95
Spring	Z65	Z85	-0.0341	0.0130	0.0097	-3.35
	6 h	24 h	-0.0408	0.0133	0.0025	-4.00
	24 h	2 w	0.0073	0.0133	0.5851	0.73
	2 w	8/10 w	-0.0216	0.0133	0.1067	-2.13
	8/10 w	Z31	-0.0094	0.0250	0.7069	-0.94
	Z31	Z39	0.0502	0.0250	0.0461	5.15
	Z39	Z47	-0.0367	0.0156	0.0202	-3.60
	Z47	Z65	0.0092	0.0249	0.7116	0.93
235a	Z65	Z85	-0.0764	0.0224	0.0008	-7.35
	6 h	24 h	-0.0893	0.0104	<.0001	-8.54
	24 h	2 w	-0.0321	0.0093	0.0007	-3.16
	2 w	8/10 w	0.0141	0.0079	0.0758	1.42
	8/10 w	Z31	0.0210	0.0134	0.1194	2.12
	Z31	Z39	0.0596	0.0176	0.0009	6.14
	Z39	Z47	0.0066	0.0176	0.7060	0.67
	Z47	Z65	-0.0864	0.0176	<.0001	-8.28
OK7211542	Z65	Z85	-0.0054	0.0176	0.7598	-0.54
	6 h	24 h	-0.0633	0.0110	<.0001	-6.13
	24 h	2 w	0.0354	0.0120	0.0036	3.60
	2 w	8/10 w	-0.0495	0.0269	0.0673	-4.83
	8/10 w	Z31	-0.0513	0.0264	0.0534	-5.00
	Z31	Z39	0.0928	0.0152	<.0001	9.72
	Z39	Z47	-0.0542	0.0179	0.0028	-5.28
	Z47	Z65	0.0266	0.0160	0.0974	2.70
Agrotana	Z65	Z85	-0.0789	0.0431	0.0694	-7.58
	6 h	24 h	-0.1498	0.0276	<0.0001	-13.91
	24 h	2 w	-0.7978	0.0280	0.0050	-54.97
	2 w	8/10 w	-0.0310	0.0104	0.0033	-3.05
	8/10 w	Z31	-0.0430	0.0081	<0.0001	-4.21
	Z31	Z39	-0.0023	0.0098	1.0000	-0.23
	Z39	Z47	0.0820	0.0237	0.0007	8.55
	Z47	Z65	-0.0744	0.0232	0.0016	-7.17
11955	Z65	Z85	-0.0160	0.0182	0.3806	-1.59

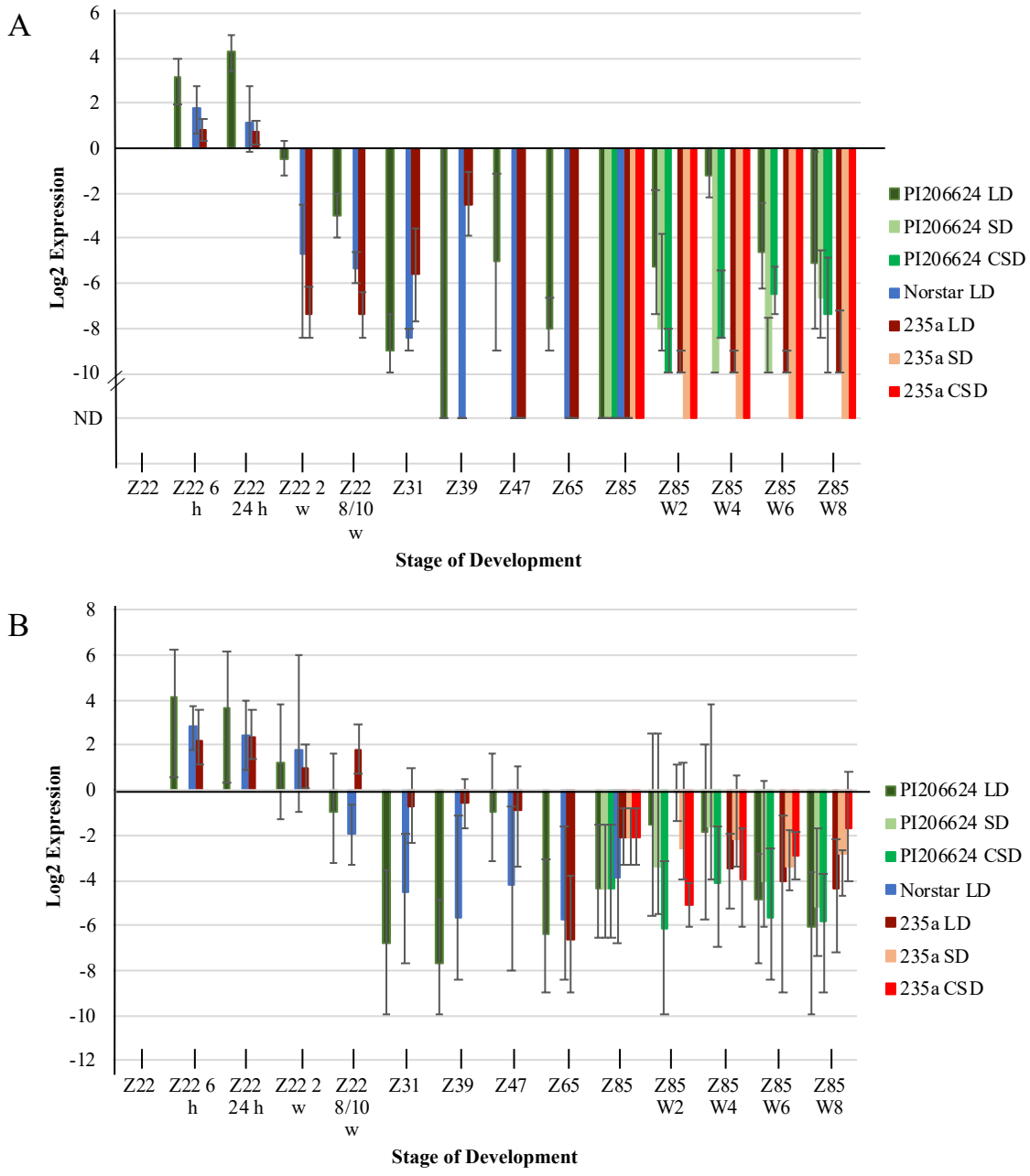
Appendix 14. Significance ($p<0.05$) of *TFL1* expression between stages of development in leaf tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	0.0169	0.0146	0.2481	1.70
	24 h	2 w	0.0325	0.0082	0.0001	3.30
	2 w	8/10 w	-0.0033	0.0075	0.6631	-0.33
	8/10 w	Z31	0.0094	0.0249	0.7057	0.95
	Z31	Z39	0.0311	0.0251	0.2179	3.15
	Z39	Z47	-0.0947	0.0152	<0.0001	-9.03
	Z47	Z65	0.0875	0.0265	0.0012	9.14
PI206624	Z65	Z85	0.0160	0.0341	0.6389	1.61
	6 h	24 h	0.0098	0.0079	0.2198	0.98
	24 h	2 w	0.0701	0.0079	<0.0001	7.26
	2 w	8/10 w	-0.0072	0.0079	0.3692	-0.71
	8/10 w	Z31	0.0244	0.0221	0.2707	2.47
	Z31	Z39	-0.0093	0.0239	0.6979	-0.92
	Z39	Z47	0.0096	0.0160	0.5519	0.96
	Z47	Z65	0.0688	0.0269	0.0114	7.12
PI531718	Z65	Z85	-0.0542	0.0256	0.0359	-5.27
	6 h	24 h	0.0561	0.0118	<0.0001	5.77
	24 h	2 w	0.0044	0.0238	0.8535	0.44
	2 w	8/10 w	0.0545	0.0262	0.0393	5.60
	8/10 w	Z31	0.1364	0.0201	<0.0001	14.61
	Z31	Z39	-0.0206	0.0201	0.3077	-2.04
	Z39	Z47	0.0101	0.0201	0.6171	1.01
	Z47	Z65	0.0360	0.0201	0.0754	3.67
Norstar	Z65	Z85	-0.0565	0.0201	0.0057	-5.49
	6 h	24 h	-0.0104	0.0170	0.5414	-1.03
	24 h	2 w	0.0329	0.0170	0.0542	3.35
	2 w	8/10 w	0.0116	0.0170	0.4965	1.16
	8/10 w	Z31	0.0197	0.0124	0.1146	1.99
	Z31	Z39	-0.0311	0.0105	0.0035	-3.06
	Z39	Z47	0.0431	0.0148	0.0042	4.40
Chinese Spring	Z47	Z65	0.0512	0.0113	<.0001	5.26
	Z65	Z85	-0.0691	0.0222	0.0022	-6.67
	6 h	24 h	0.0444	0.0136	0.0013	4.54
	24 h	2 w	-0.1631	0.0136	<.0001	-15.05
	2 w	8/10 w	-0.1107	0.0136	<.0001	-10.48
	8/10 w	Z31	-0.0070	0.0233	0.7655	-0.69
	Z31	Z39	-0.0845	0.0282	0.0032	-8.10
	Z39	Z47	0.0496	0.0319	0.1223	5.08
	Z47	Z65	0.0305	0.0414	0.4616	3.10
235a	Z65	Z85	-0.0899	0.0350	0.0112	-8.60
	6 h	24 h	0.0326	0.0140	0.0207	3.31
	24 h	2 w	-0.0716	0.0140	<.0001	-6.91
	2 w	8/10 w	-0.0804	0.0140	<.0001	-7.73
	8/10 w	Z31	-0.0484	0.0178	0.0072	-4.73
	Z31	Z39	-0.0297	0.0365	0.4160	-2.93
	Z39	Z47	-0.0086	0.0415	0.8370	-0.85
	Z47	Z65	0.0162	0.0275	0.5569	1.63
OK7211542	Z65	Z85	-0.0372	0.0432	0.3903	-3.65
	6 h	24 h	0.0852	0.0277	0.0024	8.89
	24 h	2 w	0.1279	0.0302	<.0001	13.64
	2 w	8/10 w	0.0457	0.0144	0.0018	4.68
	8/10 w	Z31	0.0096	0.0295	0.7443	0.97
	Z31	Z39	-0.0683	0.0299	0.0237	-6.60
	Z39	Z47	0.0089	0.0095	0.3537	0.89
	Z47	Z65	-0.0212	0.0290	0.4652	-2.10
Agrotana	Z65	Z85	0.0103	0.0295	0.7284	1.03
	6 h	24 h	0.0492	0.0293	0.0947	5.04
	24 h	2 w	-0.1569	0.0268	<0.0001	-14.52
	2 w	8/10 w	-0.0105	0.0152	0.4932	-1.04
	8/10 w	Z31	0.1522	0.0176	<0.0001	16.44
	Z31	Z39	0.0254	0.0499	0.6118	2.57
	Z39	Z47	-0.0995	0.0529	0.0618	-9.47
	Z47	Z65	0.0906	0.0287	0.0019	9.48
11955	Z65	Z85	-0.0589	0.0354	0.0985	-5.72

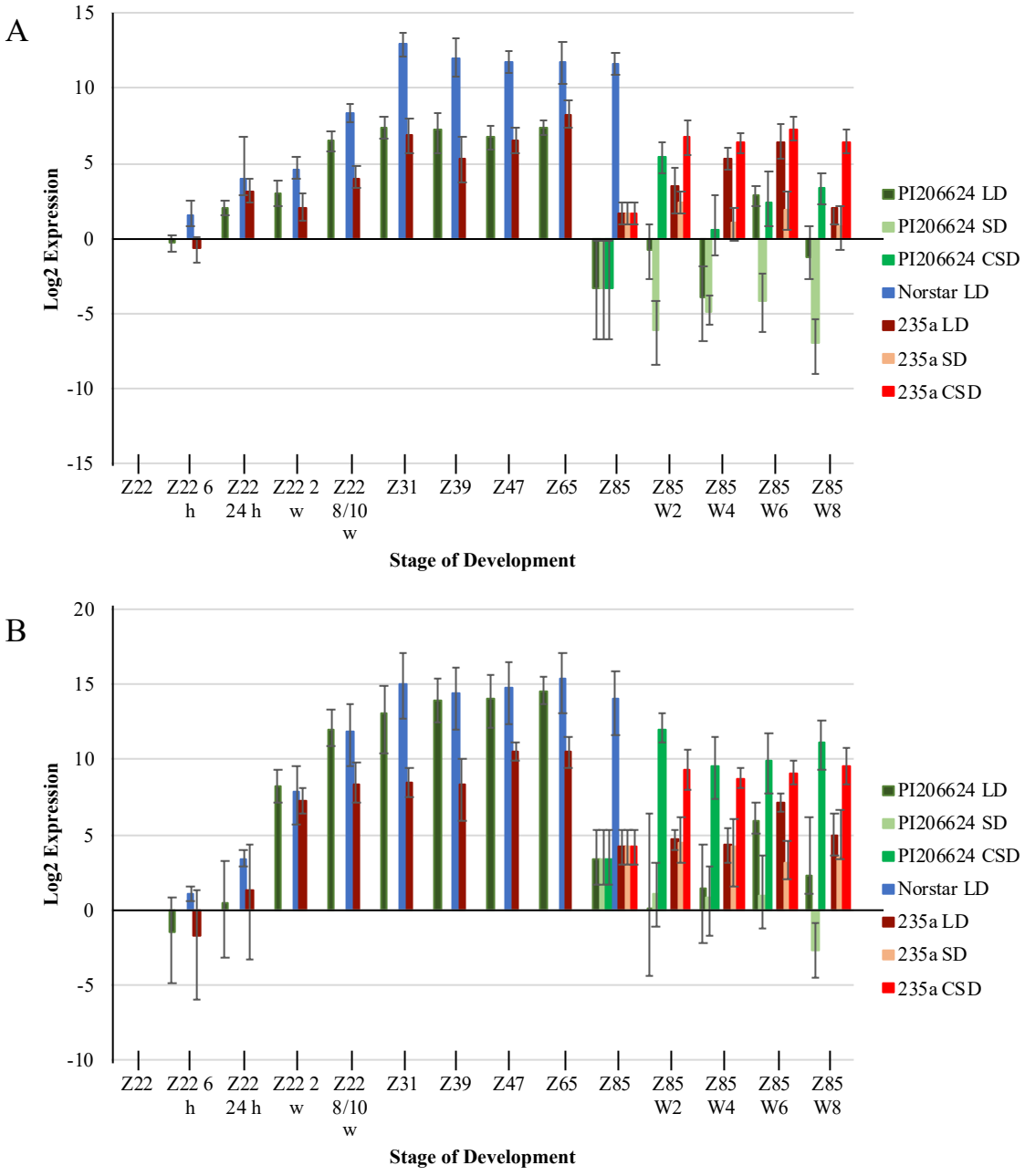
Appendix 15. Significance ($p<0.05$) of *TFL1* expression between stages of development in meristem tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	-0.0481	0.0146	0.0012	-4.70
	24 h	2 w	0.0686	0.0082	<0.0001	7.10
	2 w	8/10 w	-0.0076	0.0075	0.3159	-0.75
	8/10 w	Z31	0.0249	0.0249	0.3190	2.52
	Z31	Z39	0.0694	0.0251	0.0063	7.19
	Z39	Z47	-0.0310	0.0152	0.0426	-3.06
	Z47	Z65	0.0547	0.0265	0.0406	5.62
PI206624	Z65	Z85	-0.0710	0.0341	0.0387	-6.85
	6 h	24 h	-0.0356	0.0079	<0.0001	-3.49
	24 h	2 w	0.0742	0.0079	<0.0001	7.70
	2 w	8/10 w	0.0066	0.0079	0.4094	0.66
	8/10 w	Z31	0.0765	0.0221	0.0007	7.95
	Z31	Z39	0.0776	0.0239	0.0014	8.07
	Z39	Z47	0.0372	0.0160	0.0215	3.79
	Z47	Z65	-0.0112	0.0269	0.6779	-1.11
PI531718	Z65	Z85	-0.4528	0.0256	0.0789	-36.42
	6 h	24 h	-0.0333	0.0118	0.0054	-3.27
	24 h	2 w	0.1688	0.0238	<0.0001	18.39
	2 w	8/10 w	0.0600	0.0262	0.0235	6.18
	8/10 w	Z31	-0.0914	0.0201	<0.0001	-8.73
	Z31	Z39	-0.0195	0.2014	0.3354	-1.93
	Z39	Z47	0.0010	0.0201	0.9601	0.10
	Z47	Z65	-0.0131	0.0201	0.5161	-1.30
Norstar	Z65	Z85	0.0487	0.0201	0.0168	4.99
	6 h	24 h	0.0723	0.0130	<.0001	7.49
	24 h	2 w	0.1268	0.0130	<.0001	13.52
	2 w	8/10 w	-0.0148	0.0130	0.2564	-1.47
	8/10 w	Z31	-0.0418	0.0180	0.0218	-4.09
	Z31	Z39	-0.0026	0.0238	0.9122	-0.26
	Z39	Z47	0.0148	0.0184	0.4218	1.50
Chinese	Z47	Z65	-0.0970	0.0229	<.0001	-9.25
Spring	Z65	Z85	-0.0119	0.0239	0.6195	-1.18
	6 h	24 h	-0.0104	0.0136	0.4457	-1.03
	24 h	2 w	0.0305	0.0136	0.0261	3.10
	2 w	8/10 w	-0.0761	0.0136	<.0001	-7.33
	8/10 w	Z31	-0.0163	0.0233	0.4865	-1.61
	Z31	Z39	0.0336	0.0282	0.2360	3.41
	Z39	Z47	0.0242	0.0319	0.4493	2.45
	Z47	Z65	-0.0020	0.0414	0.9617	-0.20
235a	Z65	Z85	-0.0865	0.0350	0.0146	-8.29
	6 h	24 h	-0.0258	0.0158	0.1053	-2.55
	24 h	2 w	0.0632	0.0158	0.0001	6.52
	2 w	8/10 w	-0.0137	0.0158	0.3882	-1.36
	8/10 w	Z31	-0.2343	0.0154	<.0001	-20.89
	Z31	Z39	-0.0433	0.0161	0.0080	-4.24
	Z39	Z47	0.0750	0.0117	<.0001	7.79
	Z47	Z65	-0.0878	0.0193	<.0001	-8.40
OK7211542	Z65	Z85	0.0072	0.0233	0.7593	0.72
	6 h	24 h	0.0065	0.0146	0.6542	0.66
	24 h	2 w	0.0563	0.0165	0.0008	5.79
	2 w	8/10 w	-0.0851	0.0357	0.0184	-8.16
	8/10 w	Z31	-0.1332	0.0339	0.0001	-12.47
	Z31	Z39	0.0255	0.0104	0.0153	2.58
	Z39	Z47	-0.0305	0.0148	0.0400	-3.01
	Z47	Z65	-0.0088	0.0156	0.5739	-0.88
Agrotana	Z65	Z85	-0.0319	0.0455	0.4841	-3.14
	6 h	24 h	-0.8401	0.0293	0.0046	-56.83
	24 h	2 w	-0.0248	0.0268	0.3576	-2.44
	2 w	8/10 w	-0.0308	0.0152	0.0446	-3.03
	8/10 w	Z31	-0.1031	0.0176	<0.0001	-9.80
	Z31	Z39	-0.0977	0.0499	0.0519	-9.31
	Z39	Z47	0.1372	0.0529	0.0104	14.71
	Z47	Z65	-0.0460	0.0287	0.1108	-4.49
11955	Z65	Z85	-0.0311	0.0354	0.3812	-3.06

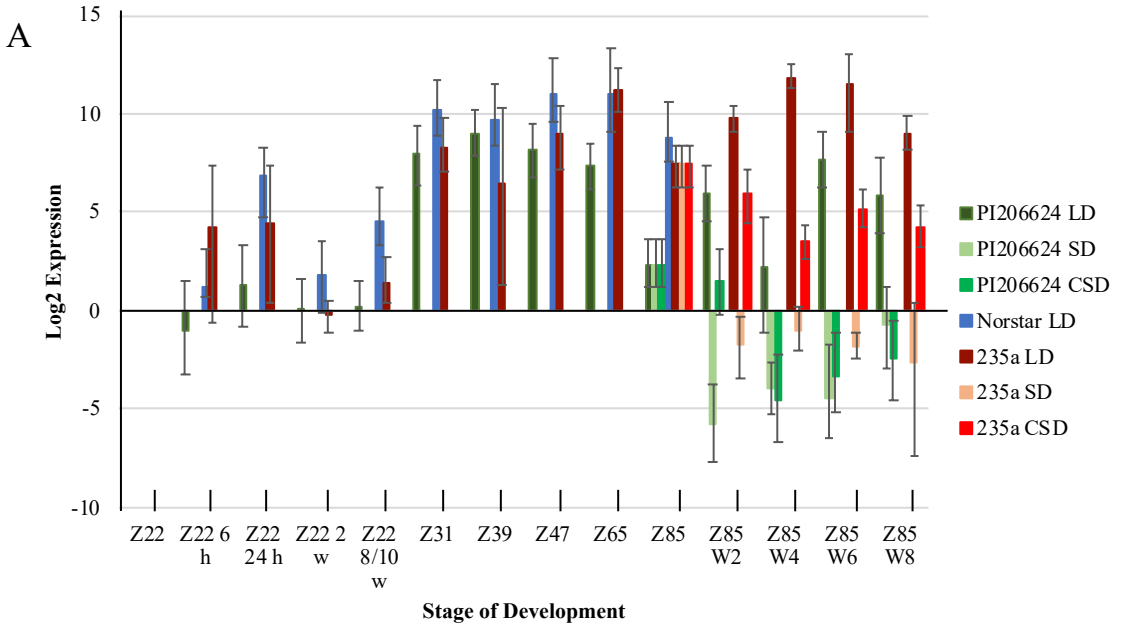
Appendix 16. Log2 gene expression of *VRN2* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. Error bars indicate standard error.

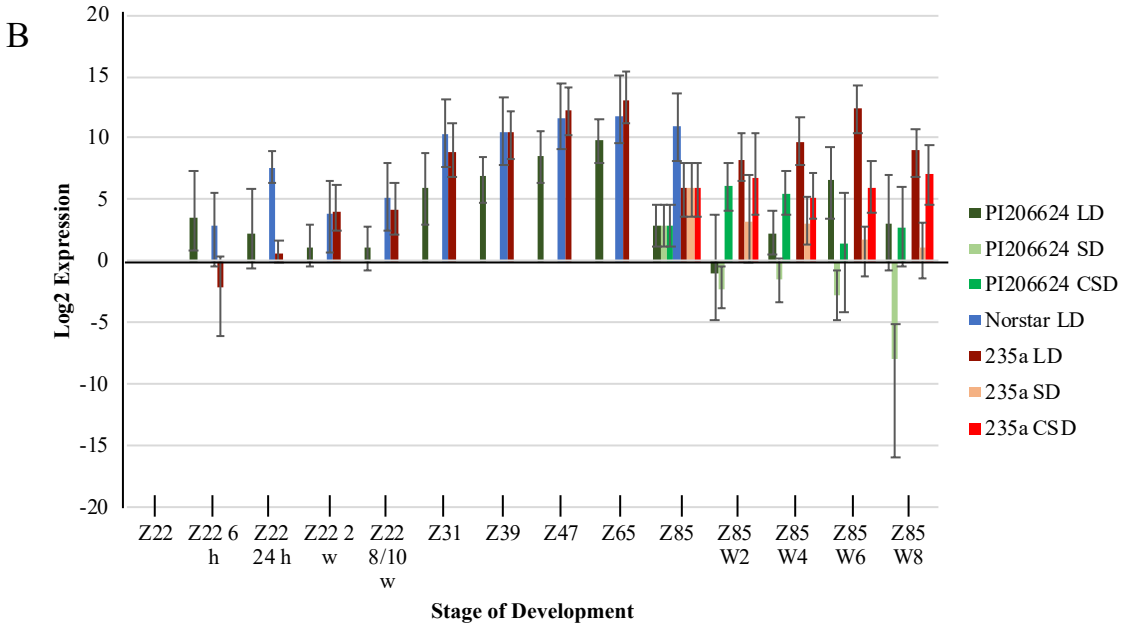


Appendix 17. Log2 gene expression of *VRN1* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. Error bars indicate standard error.

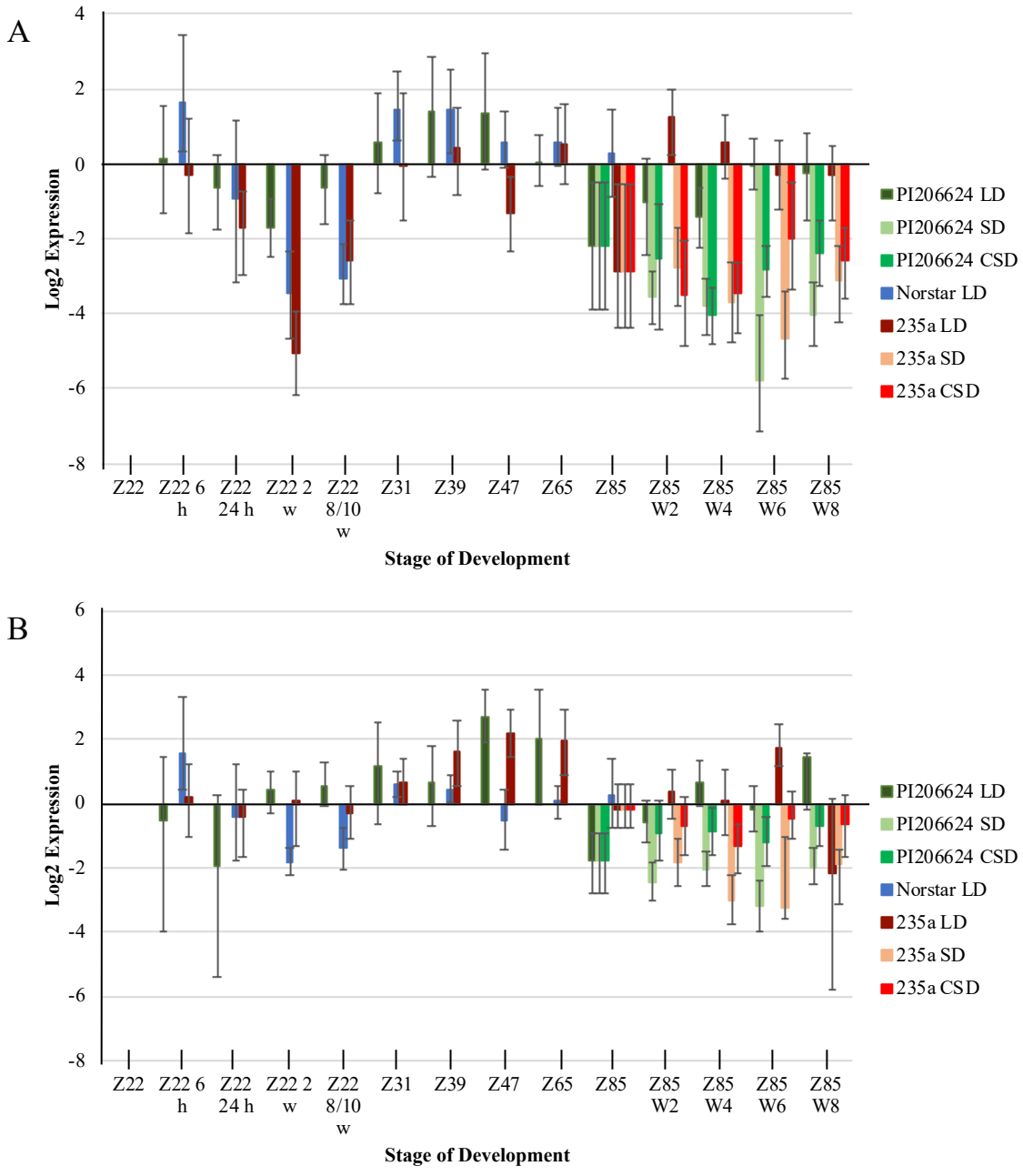


Appendix 18. Log2 gene expression of *VRN3* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. Error bars indicate standard error.

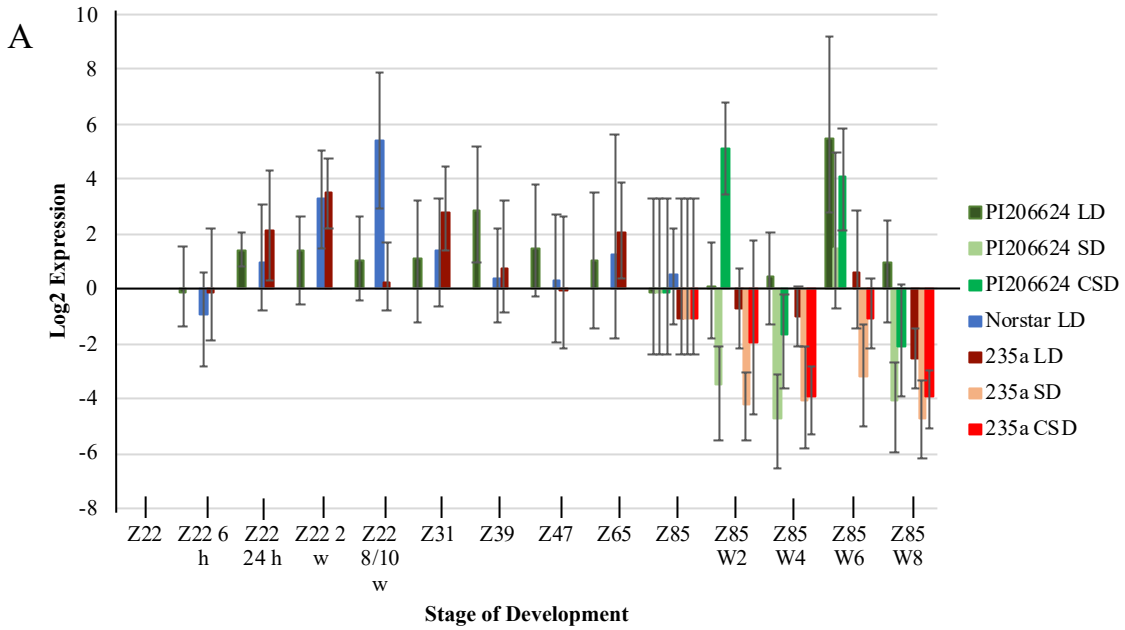


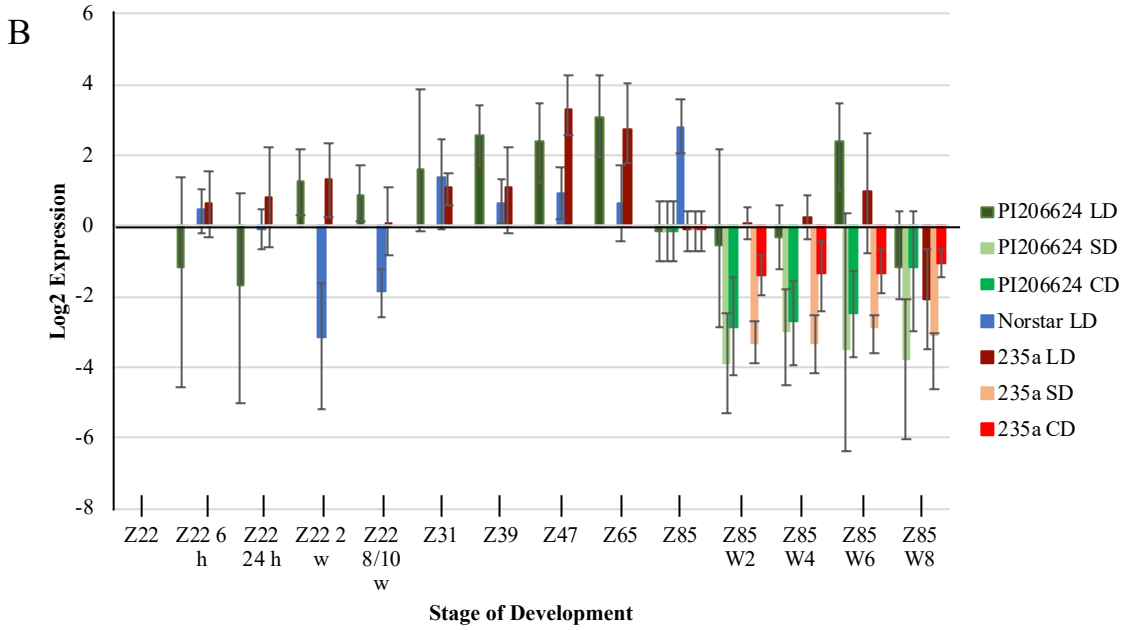


Appendix 19. Log2 gene expression of *PPD1* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. Error bars indicate standard error.



Appendix 20. Log2 gene expression of *TFL1* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. Error bars indicate standard error.





Appendix 21. Significant ($p<0.05$) relative expression and up or downregulation of *VRN2* between stages of development in leaf (A) and meristem (B) tissue of wheatgrass and perennial wheat lines exposed to LDs, SDs or CSDs compared to stage Z85. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling.

A

Line	Stage of Development	Expression	Standard Error	P Value	Regulation
	Z85 W2	0.364	0.074 - 2.715	0.099	
	Z85 W4	5.198	3.144 - 8.347	0.000	UP
	Z85 W6	0.568	0.166 - 1.661	0.145	
PI206624 LD	Z85 W8	0.415	0.059 - 8.660	0.266	
	Z85 W2	0.045	0.004 - 0.157	0.000	DOWN
	Z85 W4	0.01	0.002 - 0.030	0.000	DOWN
	Z85 W6	0.006	0.001 - 0.025	0.000	DOWN
PI206624 SD	Z85 W8	0.122	0.032 - 0.236	0.000	DOWN
	Z85 W2	0.011	0.004 - 0.040	0.000	DOWN
	Z85 W4	0.038	0.001 - 0.220	0.000	DOWN
	Z85 W6	0.131	0.064 - 0.209	0.000	DOWN
PI206624 CSD	Z85 W8	0.075	0.006 - 0.410	0.000	DOWN
	Z85 W2	144.495	77.344 - 245.848	0.000	UP
	Z85 W4	117.384	53.124 - 259.145	0.000	UP
	Z85 W6	127.511	77.958 - 205.920	0.000	UP
235a LD	Z85 W8	152.095	42.168 - 774.538	0.000	UP
	Z85 W2	72.827	44.511 - 129.309	0.000	UP
	Z85 W4	17.238	10.231 - 29.259	0.000	UP
	Z85 W6	41.602	26.310 - 60.732	0.000	UP
235a SD	Z85 W8	55.302	30.987 - 107.032	0.000	UP
	Z85 W2	0.601	0.446 - 0.800	0.001	DOWN
	Z85 W4	27.782	1.287 - 242.045	0.001	UP
	Z85 W6	19.606	4.824 - 57.924	0.000	UP
235a CSD	Z85 W8	40.62	14.348 - 143.451	0.000	UP

B

Line	Stage of Development	Expression	Standard Error	P Value	Regulation
	Z85 W2	0.364	0.074 - 2.715	0.099	
	Z85 W4	5.198	3.144 - 8.347	0.000	UP
	Z85 W6	0.568	0.166 - 1.661	0.145	
PI206624 LD	Z85 W8	0.415	0.059 - 8.660	0.266	
	Z85 W2	0.045	0.004 - 0.157	0.000	DOWN
	Z85 W4	0.01	0.002 - 0.030	0.000	DOWN
	Z85 W6	0.006	0.001 - 0.025	0.000	DOWN
PI206624 SD	Z85 W8	0.122	0.032 - 0.236	0.000	DOWN
	Z85 W2	0.011	0.004 - 0.040	0.000	DOWN
	Z85 W4	0.038	0.001 - 0.220	0.000	DOWN
	Z85 W6	0.131	0.064 - 0.209	0.000	DOWN
PI206624 CSD	Z85 W8	0.075	0.006 - 0.410	0.000	DOWN
	Z85 W2	144.495	77.344 - 245.848	0.000	UP
	Z85 W4	117.384	53.124 - 259.145	0.000	UP
	Z85 W6	127.511	77.958 - 205.920	0.000	UP
235a LD	Z85 W8	152.095	42.168 - 774.538	0.000	UP
	Z85 W2	72.827	44.511 - 129.309	0.000	UP
	Z85 W4	17.238	10.231 - 29.259	0.000	UP
	Z85 W6	41.602	26.310 - 60.732	0.000	UP
235a SD	Z85 W8	55.302	30.987 - 107.032	0.000	UP
	Z85 W2	0.601	0.446 - 0.800	0.001	DOWN
	Z85 W4	27.782	1.287 - 242.045	0.001	UP
	Z85 W6	19.606	4.824 - 57.924	0.000	UP
235a CSD	Z85 W8	40.62	14.348 - 143.451	0.000	UP

Appendix 22. Significant ($p<0.05$) relative expression and up or downregulation of *VRN1* between stages of development in leaf (A) and meristem (B) tissue of wheatgrass and perennial wheat lines exposed to LDs, SDs or CSDs compared to stage Z85. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling.

A

Line	Stage of Development	Expression	Standard Error	P Value	Regulation
	Z85 W2	3.365	0.736 - 24.274	0.054	
	Z85 W4	0.708	0.120 - 6.786	0.581	
	Z85 W6	22.073	3.908 - 128.189	0.000	UP
PI206624 LD	Z85 W8	2.997	0.394 - 11.470	0.069	
	Z85 W2	0.143	0.016 - 1.653	0.020	DOWN
	Z85 W4	0.329	0.034 - 2.689	0.116	
	Z85 W6	0.531	0.072 - 5.511	0.382	
PI206624 SD	Z85 W8	0.081	0.013 - 1.245	0.005	DOWN
	Z85 W2	398.227	39.843 - 4,124.938	0.000	UP
	Z85 W4	14.157	1.523 - 116.020	0.006	UP
	Z85 W6	48.818	3.932 - 308.407	0.000	UP
PI206624 CSD	Z85 W8	97.785	12.456 - 962.701	0.000	UP
	Z85 W2	3.339	1.103 - 7.157	0.003	UP
	Z85 W4	11.851	8.436 - 16.249	0.000	UP
	Z85 W6	28.328	15.521 - 61.834	0.000	UP
235a LD	Z85 W8	1.358	0.672 - 2.464	0.166	
	Z85 W2	2.682	1.740 - 4.392	0.000	UP
	Z85 W4	0.971	0.334 - 2.143	0.923	
	Z85 W6	1.924	0.578 - 5.391	0.092	
235a SD	Z85 W8	0.693	0.167 - 2.490	0.388	
	Z85 W2	33.376	15.782 - 69.551	0.000	UP
	Z85 W4	25.486	19.372 - 33.230	0.000	UP
	Z85 W6	48.6	33.389 - 66.387	0.000	UP
235a CSD	Z85 W8	27.466	20.948 - 36.798	0.000	UP

B

Line	Stage of Development	Expression	Standard Error	P Value	Regulation
	Z85 W2	0.164	0.018 - 6.664	0.073	
	Z85 W4	0.357	0.068 - 2.021	0.095	
	Z85 W6	3.832	1.662 - 8.880	0.001	UP
PI206624 LD	Z85 W8	1.199	0.342 - 4.867	0.689	
	Z85 W2	0.2	0.044 - 1.123	0.013	DOWN
	Z85 W4	0.163	0.041 - 0.858	0.004	DOWN
	Z85 W6	0.177	0.028 - 1.724	0.020	DOWN
PI206624 SD	Z85 W8	0.015	0.003 - 0.077	0.000	DOWN
	Z85 W2	386.388	102.281 - 1,135.039	0.000	UP
	Z85 W4	73.197	15.482 - 372.114	0.000	UP
	Z85 W6	93.175	23.287 - 480.590	0.000	UP
PI206624 CSD	Z85 W8	220.101	64.273 - 849.918	0.000	UP
	Z85 W2	1.467	0.783 - 3.098	0.105	
	Z85 W4	1.072	0.409 - 2.886	0.752	
	Z85 W6	7.537	4.179 - 16.651	0.000	UP
235a LD	Z85 W8	1.612	0.647 - 3.956	0.137	
	Z85 W2	1.611	0.455 - 5.095	0.326	
	Z85 W4	1.287	0.108 - 6.323	0.699	
	Z85 W6	0.479	0.168 - 1.247	0.040	DOWN
235a SD	Z85 W8	0.64	0.162 - 2.573	0.260	
	Z85 W2	126.253	29.539 - 352.928	0.000	UP
	Z85 W4	75.609	37.204 - 203.748	0.000	UP
	Z85 W6	115.91	47.798 - 315.222	0.000	UP
235a CSD	Z85 W8	156.494	53.501 - 387.468	0.000	UP

Appendix 23. Significant ($p<0.05$) relative expression and up or downregulation of *VRN3* between stages of development in leaf (A) and meristem (B) tissue of wheatgrass and perennial wheat lines exposed to LDs, SDs or CSDs compared to stage Z85. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling.

A

Line	Stage of Development	Expression	Standard Error	P Value	Significance
	Z85 W2	8.041	3.382 - 16.252	0.000	UP
	Z85 W4	1.028	0.126 - 4.331	0.951	
	Z85 W6	22.211	9.065 - 56.243	0.000	UP
PI206624 LD	Z85 W8	8.133	2.318 - 25.428	0.000	UP
	Z85 W2	0.004	0.001 - 0.012	0.000	DOWN
	Z85 W4	0.013	0.006 - 0.030	0.000	DOWN
	Z85 W6	0.01	0.002 - 0.056	0.000	DOWN
PI206624 SD	Z85 W8	0.122	0.027 - 0.442	0.001	DOWN
	Z85 W2	0.58	0.152 - 1.839	0.159	
	Z85 W4	0.008	0.002 - 0.034	0.000	DOWN
	Z85 W6	0.02	0.006 - 0.071	0.000	DOWN
PI206624 CSD	Z85 W8	0.039	0.007 - 0.146	0.000	DOWN
	Z85 W2	4.306	2.364 - 7.436	0.000	UP
	Z85 W4	16.394	9.558 - 29.459	0.000	UP
	Z85 W6	13.632	2.665 - 44.749	0.000	UP
235a LD	Z85 W8	2.698	1.406 - 5.113	0.001	UP
	Z85 W2	0.001	0.000 - 0.002	0.000	DOWN
	Z85 W4	0.001	0.000 - 0.003	0.000	DOWN
	Z85 W6	0.001	0.000 - 0.001	0.000	DOWN
235a SD	Z85 W8	0	0.000 - 0.004	0.000	DOWN
	Z85 W2	0.346	0.105 - 1.054	0.005	DOWN
	Z85 W4	0.065	0.031 - 0.131	0.000	DOWN
	Z85 W6	0.198	0.083 - 0.368	0.000	DOWN
235a CSD	Z85 W8	0.101	0.046 - 0.195	0.000	DOWN

B

Line	Stage of Development	Expression	Standard Error	P Value	Significance
	Z85 W2	0.117	0.019 - 2.526	0.010	DOWN
	Z85 W4	0.7	0.405 - 1.153	0.071	
	Z85 W6	8.98	1.652 - 30.667	0.001	UP
PI206624 LD	Z85 W8	1.497	0.165 - 12.370	0.548	
	Z85 W2	0.029	0.019 - 0.045	0.000	DOWN
	Z85 W4	0.053	0.019 - 0.160	0.000	DOWN
	Z85 W6	0.02	0.007 - 0.042	0.000	DOWN
PI206624 SD	Z85 W8	0.001	0.000 - 0.005	0.000	DOWN
	Z85 W2	9.239	3.867 - 24.444	0.000	UP
	Z85 W4	6.116	3.178 - 12.347	0.000	UP
	Z85 W6	0.384	0.013 - 2.996	0.230	
PI206624 CSD	Z85 W8	0.947	0.138 - 11.161	0.935	
	Z85 W2	4.36	1.571 - 15.353	0.003	UP
	Z85 W4	11.219	4.336 - 33.757	0.000	UP
	Z85 W6	71.393	21.527 - 207.737	0.000	UP
235a LD	Z85 W8	7.843	2.355 - 19.974	0.000	UP
	Z85 W2	0.12	0.011 - 2.646	0.019	DOWN
	Z85 W4	0.106	0.032 - 0.474	0.001	DOWN
	Z85 W6	0.036	0.007 - 0.161	0.000	DOWN
235a SD	Z85 W8	0.021	0.004 - 0.069	0.000	DOWN
	Z85 W2	1.95	0.237 - 34.100	0.425	
	Z85 W4	0.57	0.150 - 2.290	0.265	
	Z85 W6	1.068	0.238 - 5.288	0.906	
235a CSD	Z85 W8	2.628	0.418 - 14.510	0.097	

Appendix 24. Significant ($p<0.05$) relative expression and up or downregulation of *PPD1* between stages of development in leaf (A) and meristem (B) tissue of wheatgrass and perennial wheat lines exposed to LDs, SDs or CSDs compared to stage Z85. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling.

A

Line	Stage of Development	Expression	Standard Error	P Value	Significance
	Z85 W2	2.788	0.958 - 9.953	0.020	UP
	Z85 W4	2.291	0.884 - 6.489	0.014	UP
	Z85 W6	5.882	2.353 - 16.682	0.000	UP
PI206624 LD	Z85 W8	4.505	1.886 - 14.834	0.001	UP
	Z85 W2	0.38	0.118 - 1.233	0.014	DOWN
	Z85 W4	0.325	0.098 - 1.011	0.007	DOWN
	Z85 W6	0.083	0.016 - 0.275	0.000	DOWN
PI206624 SD	Z85 W8	0.283	0.098 - 0.994	0.006	DOWN
	Z85 W2	0.781	0.228 - 3.871	0.598	
	Z85 W4	0.271	0.084 - 0.853	0.002	DOWN
	Z85 W6	0.64	0.186 - 1.933	0.210	
PI206624 CSD	Z85 W8	0.877	0.266 - 2.734	0.719	
	Z85 W2	11.911	3.455 - 31.232	0.000	UP
	Z85 W4	8.23	2.426 - 21.287	0.000	UP
	Z85 W6	4.909	1.509 - 13.667	0.000	UP
235a LD	Z85 W8	4.744	1.392 - 13.159	0.001	UP
	Z85 W2	0.72	0.233 - 2.168	0.361	
	Z85 W4	0.357	0.093 - 0.942	0.007	DOWN
	Z85 W6	0.242	0.068 - 0.742	0.000	DOWN
235a SD	Z85 W8	0.545	0.139 - 1.517	0.123	
	Z85 W2	0.633	0.166 - 2.483	0.327	
	Z85 W4	0.668	0.162 - 2.030	0.298	
	Z85 W6	1.836	0.347 - 7.086	0.193	
235a CSD	Z85 W8	1.238	0.294 - 3.743	0.596	

B

Line	Stage of Development	Expression	Standard Error	P Value	Significance
	Z85 W2	3.028	1.703 - 5.690	0.000	UP
	Z85 W4	7.062	3.952 - 13.215	0.000	UP
	Z85 W6	3.666	1.994 - 6.732	0.000	UP
PI206624 LD	Z85 W8	6.261	3.208 - 12.610	0.000	UP
	Z85 W2	0.632	0.364 - 1.136	0.035	DOWN
	Z85 W4	0.834	0.489 - 1.454	0.370	
	Z85 W6	0.385	0.189 - 0.722	0.003	DOWN
PI206624 SD	Z85 W8	0.886	0.492 - 1.546	0.582	
	Z85 W2	1.842	0.845 - 3.877	0.031	UP
	Z85 W4	1.881	0.963 - 3.695	0.012	UP
	Z85 W6	1.503	0.752 - 2.967	0.092	
PI206624 CSD	Z85 W8	2.153	1.180 - 3.994	0.002	UP
	Z85 W2	1.415	0.984 - 2.124	0.017	UP
	Z85 W4	1.231	0.710 - 1.945	0.250	
	Z85 W6	3.196	2.382 - 4.201	0.000	UP
235a LD	Z85 W8	0.306	0.041 - 1.337	0.054	
	Z85 W2	0.285	0.178 - 0.455	0.000	DOWN
	Z85 W4	0.142	0.089 - 0.227	0.000	DOWN
	Z85 W6	0.137	0.083 - 0.227	0.000	DOWN
235a SD	Z85 W8	0.274	0.162 - 0.482	0.000	DOWN
	Z85 W2	0.756	0.417 - 1.350	0.190	
	Z85 W4	0.523	0.299 - 0.887	0.003	DOWN
	Z85 W6	0.761	0.496 - 1.245	0.122	
235a CSD	Z85 W8	0.793	0.390 - 1.609	0.325	

Appendix 25. Significant ($p<0.05$) relative expression and up or downregulation of *TFL1* between stages of development in leaf (A) and meristem (B) tissue of wheatgrass and perennial wheat lines exposed to LDs, SDs or CSDs compared to stage Z85. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling.

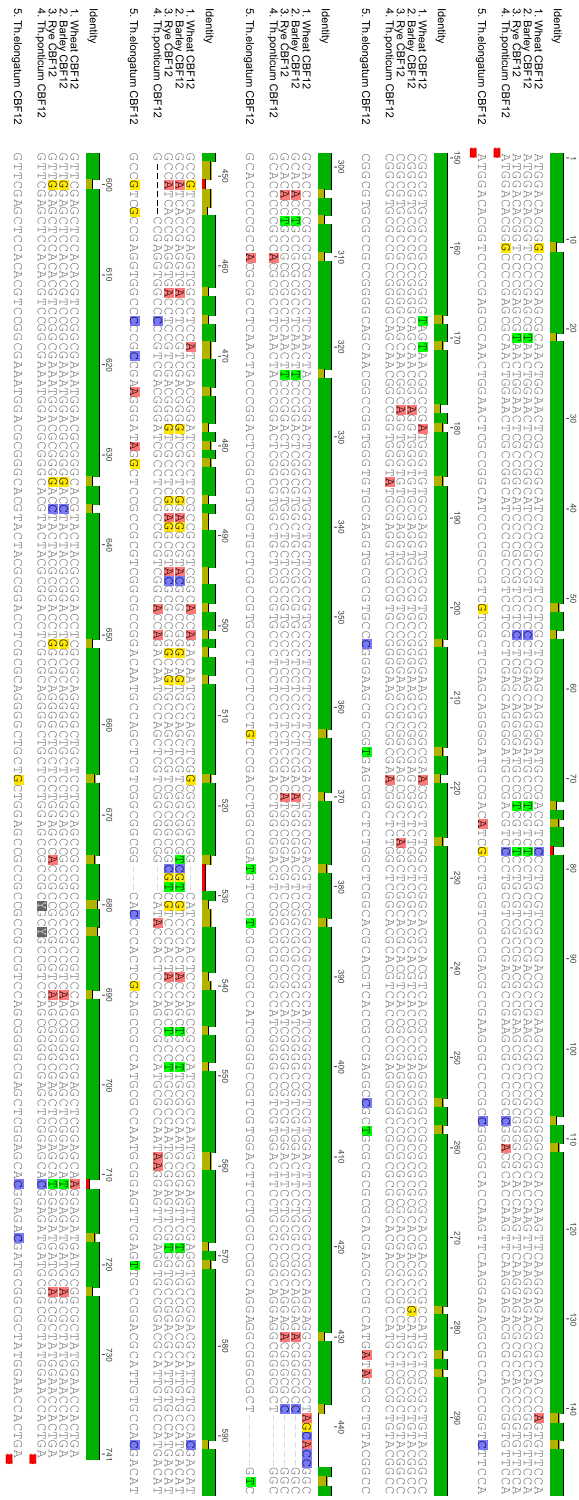
A

Line	Stage of Development	Expression	Standard Error	P Value	Significance
	Z85 W2	1.108	0.244 - 5.363	0.822	
	Z85 W4	1.472	0.325 - 6.852	0.420	
	Z85 W6	26.623	3.458 - 194.626	0.000	UP
PI206624 LD	Z85 W8	2.028	0.359 - 8.315	0.152	
	Z85 W2	0.097	0.013 - 0.501	0.000	DOWN
	Z85 W4	0.042	0.007 - 0.251	0.000	DOWN
	Z85 W6	2.999	0.401 - 25.431	0.171	
PI206624 SD	Z85 W8	0.064	0.010 - 0.379	0.000	DOWN
	Z85 W2	38.081	6.577 - 258.641	0.000	UP
	Z85 W4	0.339	0.049 - 1.906	0.074	
	Z85 W6	18.245	2.625 - 105.393	0.000	UP
PI206624 CSD	Z85 W8	0.261	0.055 - 2.592	0.069	
	Z85 W2	1.337	0.316 - 4.827	0.529	
	Z85 W4	0.98	0.266 - 3.349	0.953	
	Z85 W6	4.199	0.516 - 38.310	0.038	UP
235a LD	Z85 W8	0.323	0.084 - 1.074	0.011	DOWN
	Z85 W2	0.069	0.016 - 0.292	0.000	DOWN
	Z85 W4	0.1	0.013 - 0.825	0.006	DOWN
	Z85 W6	0.207	0.026 - 1.302	0.018	DOWN
235a SD	Z85 W8	0.047	0.011 - 0.227	0.000	DOWN
	Z85 W2	0.55	0.083 - 6.651	0.389	
	Z85 W4	0.14	0.042 - 0.379	0.000	DOWN
	Z85 W6	1.033	0.372 - 3.184	0.936	
235a CSD	Z85 W8	0.139	0.046 - 0.371	0.000	DOWN

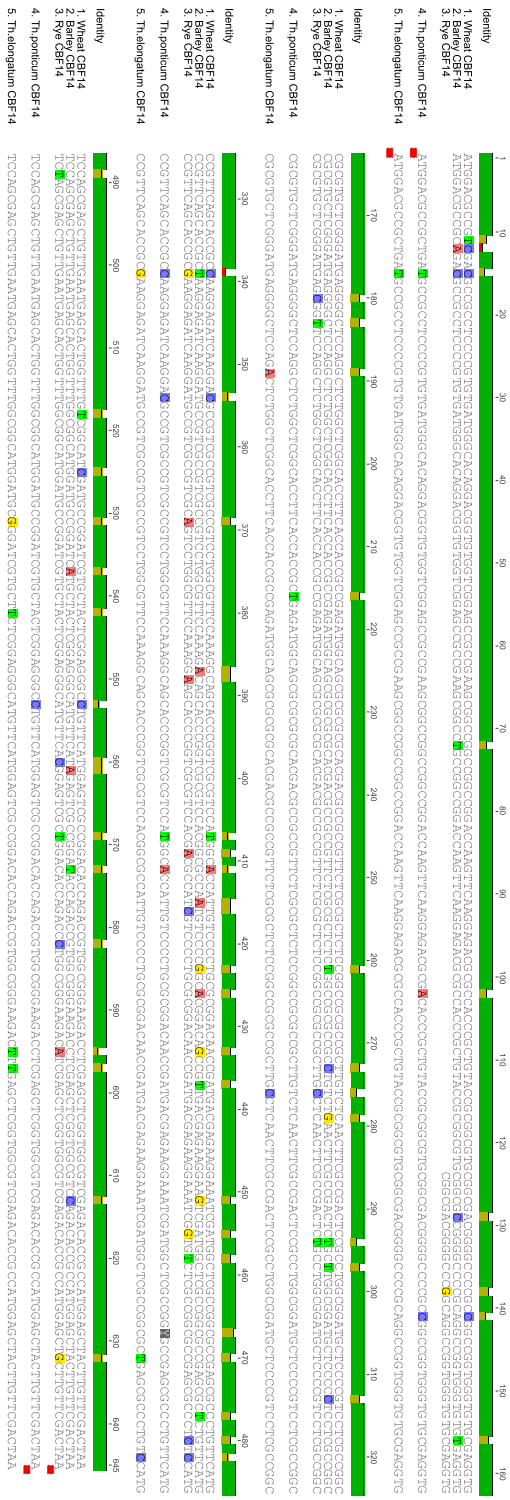
B

Line	Stage of Development	Expression	Standard Error	P Value	Significance
	Z85 W2	0.811	0.210 - 4.443	0.645	
	Z85 W4	0.932	0.576 - 1.493	0.644	
	Z85 W6	4.697	2.195 - 8.010	0.000	UP
PI206624 LD	Z85 W8	0.737	0.335 - 1.410	0.201	
	Z85 W2	0.077	0.035 - 0.233	0.000	DOWN
	Z85 W4	0.141	0.057 - 0.289	0.000	DOWN
	Z85 W6	0.1	0.016 - 1.472	0.009	DOWN
PI206624 SD	Z85 W8	0.083	0.019 - 0.298	0.000	DOWN
	Z85 W2	0.153	0.069 - 0.440	0.000	DOWN
	Z85 W4	0.172	0.078 - 0.395	0.000	DOWN
	Z85 W6	0.204	0.098 - 0.476	0.000	DOWN
PI206624 CSD	Z85 W8	0.498	0.158 - 1.473	0.049	DOWN
	Z85 W2	1.332	0.969 - 1.776	0.015	UP
	Z85 W4	1.303	0.782 - 1.926	0.081	
	Z85 W6	2.586	0.523 - 9.412	0.045	UP
235a LD	Z85 W8	0.194	0.037 - 0.678	0.000	DOWN
	Z85 W2	0.057	0.029 - 0.119	0.000	DOWN
	Z85 W4	0.057	0.025 - 0.120	0.000	DOWN
	Z85 W6	0.087	0.012 - 0.315	0.000	DOWN
235a SD	Z85 W8	0.07	0.044 - 0.120	0.000	DOWN
	Z85 W2	0.285	0.173 - 0.492	0.000	DOWN
	Z85 W4	0.299	0.137 - 0.591	0.000	DOWN
	Z85 W6	0.337	0.212 - 0.627	0.000	DOWN
235a CSD	Z85 W8	0.391	0.283 - 0.594	0.000	DOWN

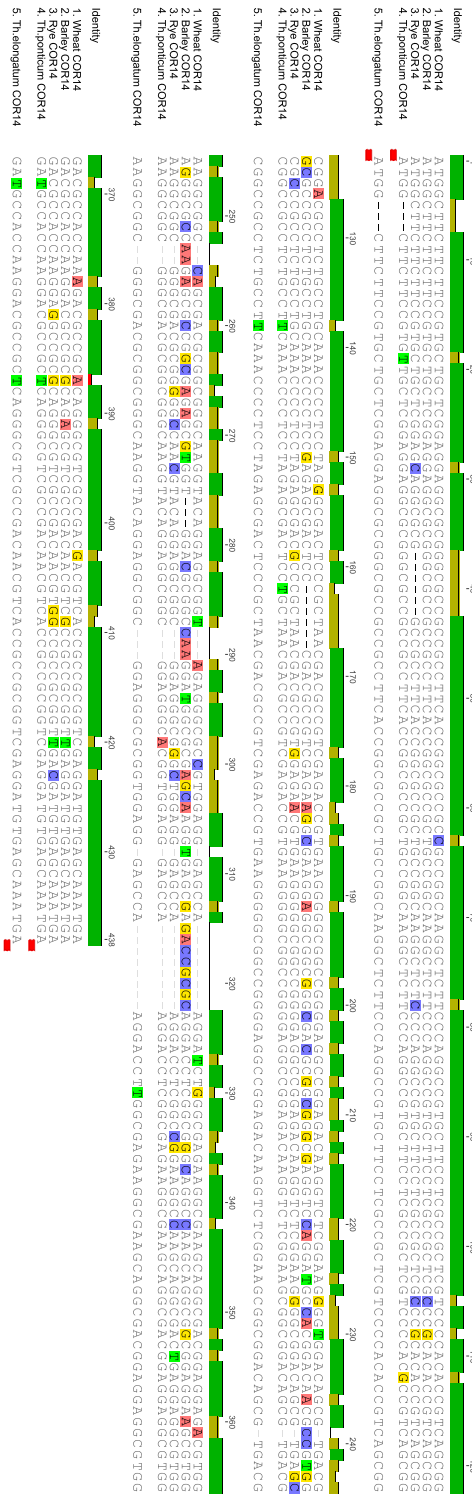
Appendix 26. *CBF12 Thinopyrum ponticum* and *elongatum* sequences compared to wheat, barley and rye.



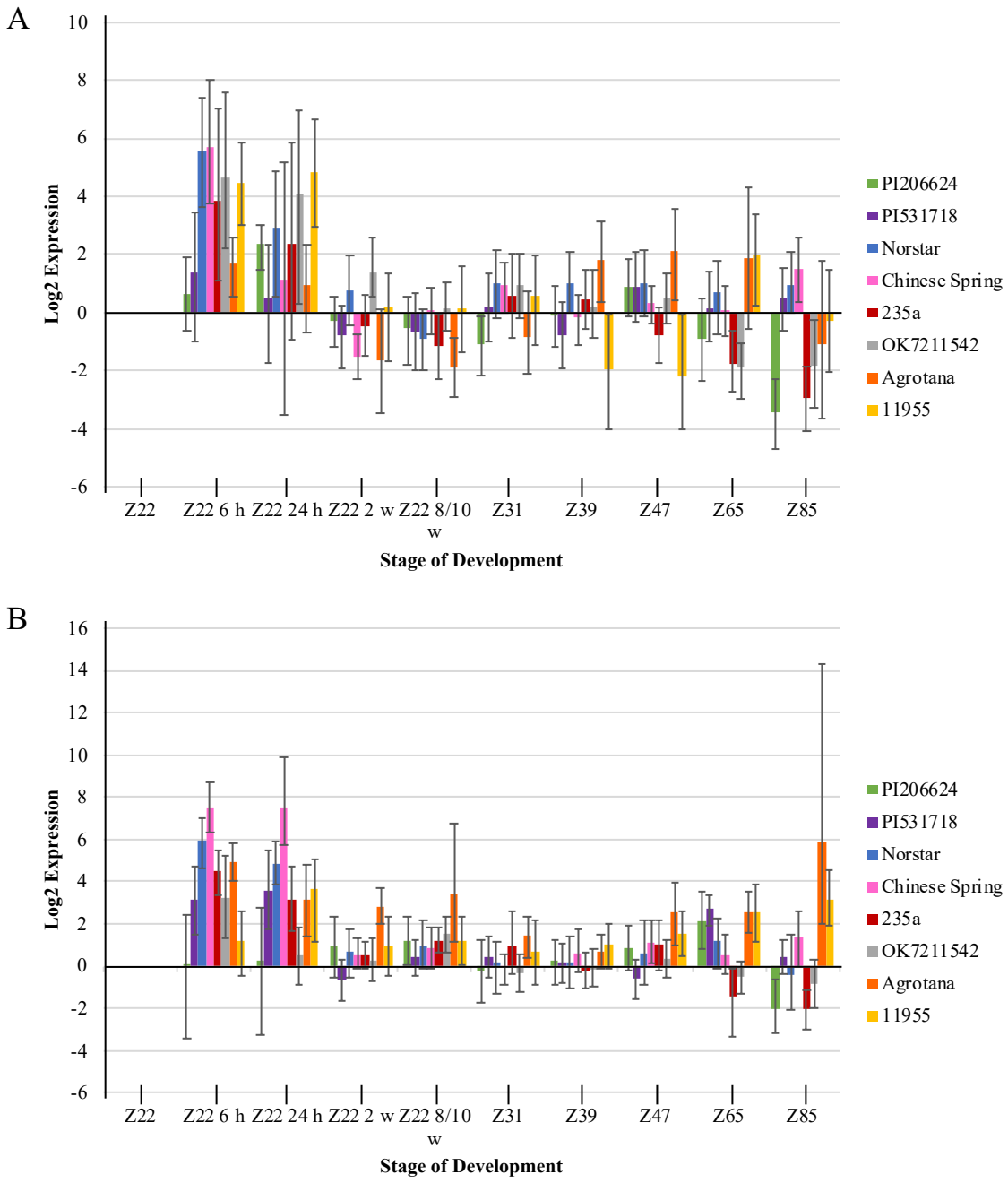
Appendix 27. *CBF14 Thinopyrum ponticum* and *elongatum* sequences compared to wheat, barley and rye.



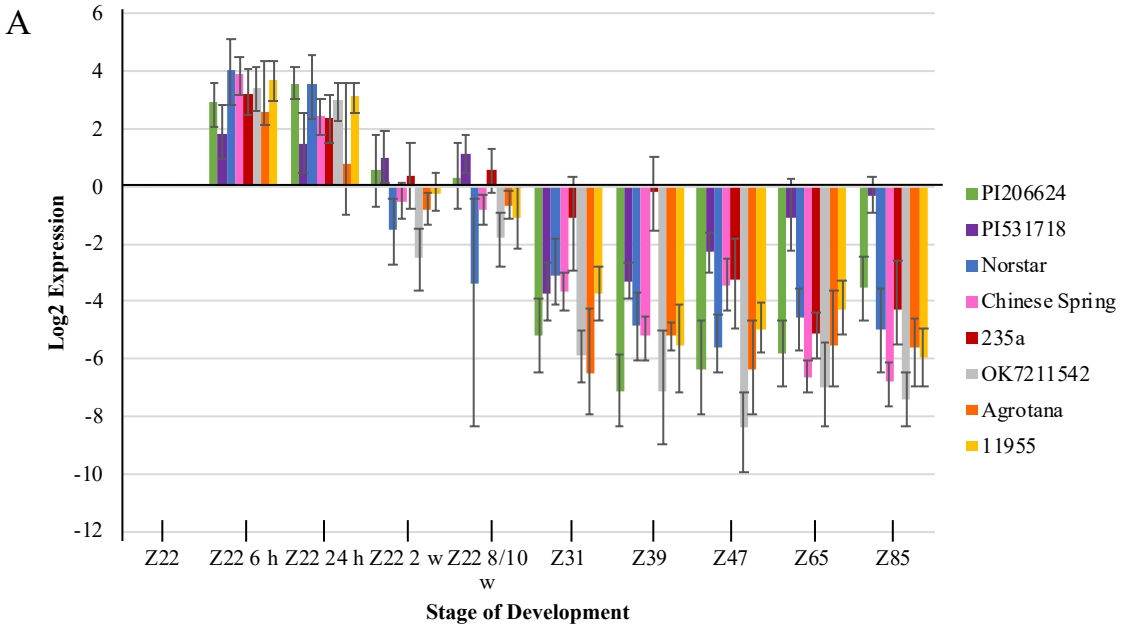
Appendix 28. *COR14 Thinopyrum ponticum* and *elongatum* sequences compared to wheat, barley and rye.

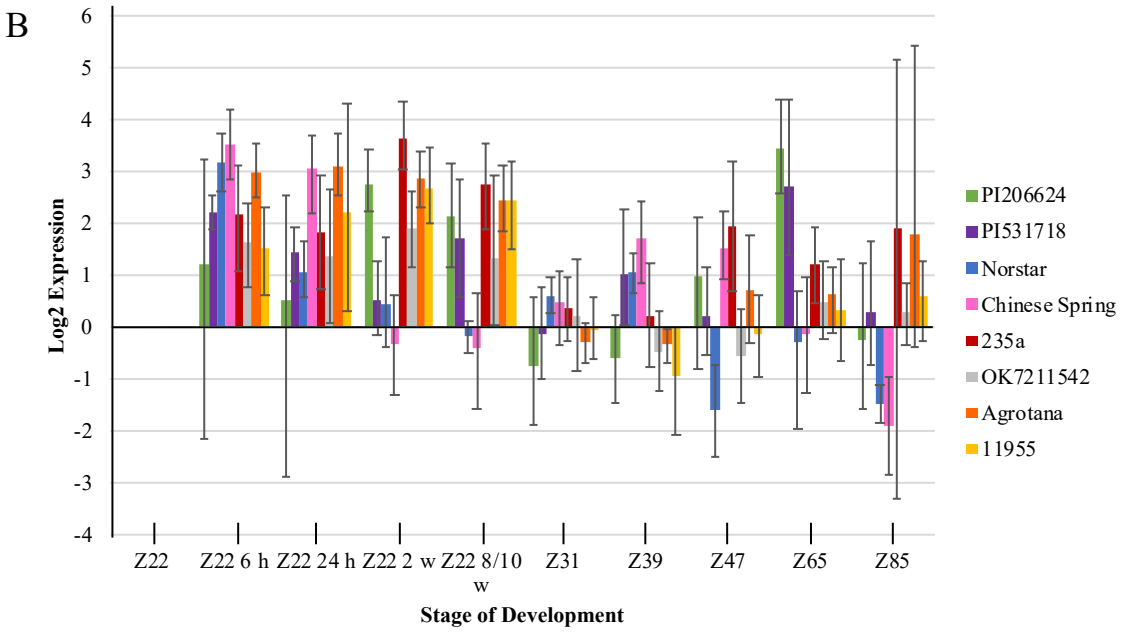


Appendix 29. Log2 gene expression of *CBF12* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Error bars indicate standard error.

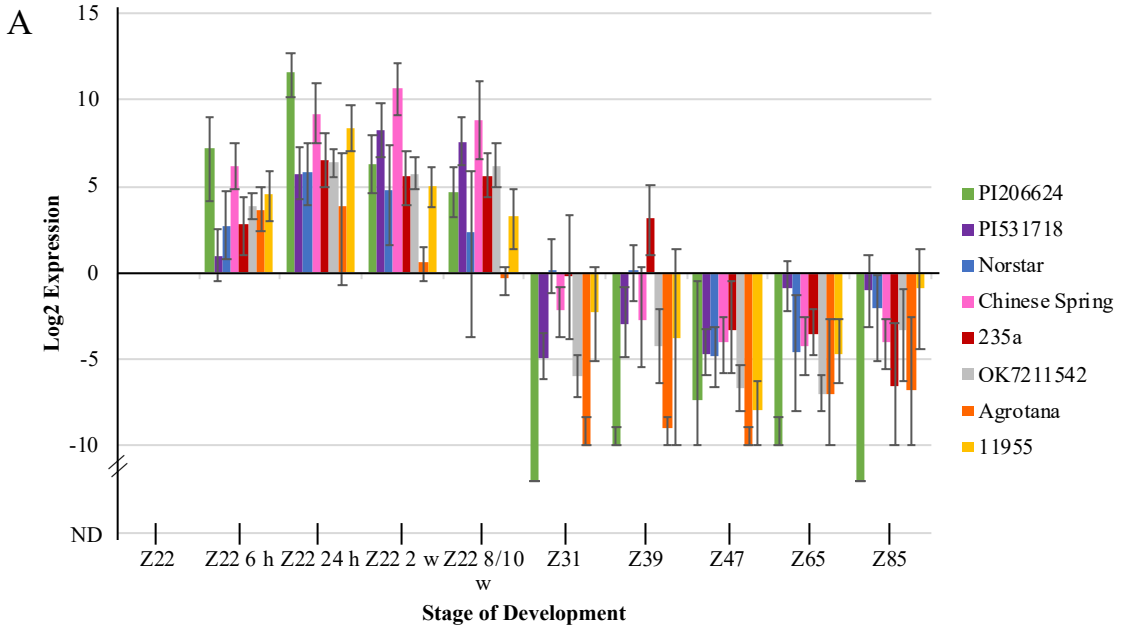


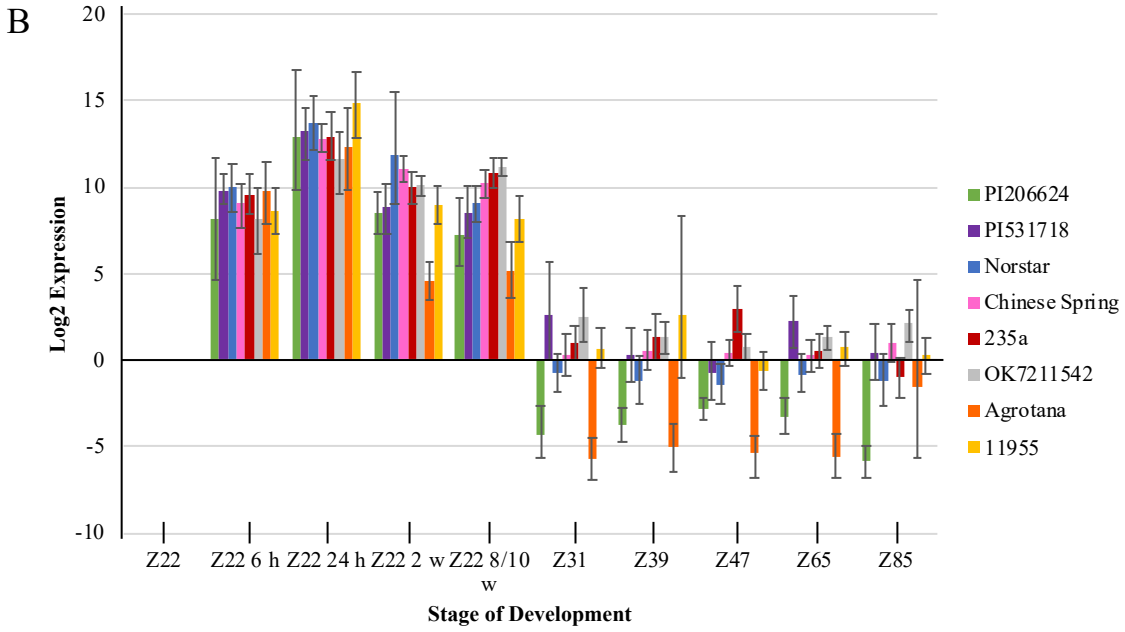
Appendix 30. Log2 gene expression of *CBF14* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Error bars indicate standard error.





Appendix 31. Log2 gene expression of *COR14* over various Zadoks stages of development for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Error bars indicate standard error.





Appendix 32. Significance ($p<0.05$) of *CBF12* expression between stages of development in leaf tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	0.0182	0.0083	0.0292	1.84
	24 h	2 w	-0.0256	0.0083	0.0024	-2.52
	2 w	8/10 w	-0.0021	0.0083	0.7965	-0.21
	8/10 w	Z31	0.0299	0.0128	0.0209	3.04
	Z31	Z39	0.0150	0.0160	0.3503	1.51
	Z39	Z47	-0.0378	0.0301	0.2104	-3.71
	Z47	Z65	0.0568	0.0401	0.1587	5.84
PI206624	Z65	Z85	-0.0061	0.0326	0.8515	-0.61
	6 h	24 h	0.0437	0.0128	0.0008	4.47
	24 h	2 w	0.0171	0.0128	0.1843	1.72
	2 w	8/10 w	-0.0193	0.0128	0.1333	-1.91
	8/10 w	Z31	-0.0342	0.0225	0.1302	-3.36
	Z31	Z39	-0.0277	0.0218	0.2052	-2.73
	Z39	Z47	-0.0005	0.0089	0.9559	-0.05
	Z47	Z65	-0.0250	0.0100	0.0134	-2.47
PI531718	Z65	Z85	0.0432	0.0109	0.0001	4.42
	6 h	24 h	-0.0971	0.0272	0.0005	-9.26
	24 h	2 w	-0.0843	0.0242	0.0006	-8.08
	2 w	8/10 w	-0.0534	0.0121	<0.0001	-5.20
	8/10 w	Z31	-0.0222	0.0103	0.0330	-2.19
	Z31	Z39	0.0040	0.0142	0.7797	0.40
	Z39	Z47	0.0141	0.0142	0.3243	1.41
	Z47	Z65	0.0033	0.0142	0.8178	0.33
Norstar	Z65	Z85	-0.0317	0.0142	0.0269	-3.12
	6 h	24 h	0.0135	0.0137	0.3254	1.36
	24 h	2 w	-0.0382	0.0264	0.1494	-3.75
	2 w	8/10 w	0.0245	0.0134	0.0688	2.48
	8/10 w	Z31	-0.0156	0.0158	0.3242	-1.55
	Z31	Z39	-0.0132	0.0115	0.2545	-1.31
	Z39	Z47	0.0239	0.0098	0.0163	2.42
Chinese	Z47	Z65	0.0364	0.0119	0.0025	3.71
Spring	Z65	Z85	-0.0213	0.0163	0.1939	-2.10
	6 h	24 h	-0.0574	0.0239	0.0176	-5.58
	24 h	2 w	-0.0418	0.0230	0.0709	-4.09
	2 w	8/10 w	0.0095	0.0108	0.3791	0.96
	8/10 w	Z31	0.0219	0.0112	0.0527	2.21
	Z31	Z39	-0.0001	0.0130	0.9931	-0.01
	Z39	Z47	0.0212	0.0177	0.2314	2.15
	Z47	Z65	-0.0779	0.0215	0.0004	-7.49
235a	Z65	Z85	-0.0355	0.0164	0.0316	-3.49
	6 h	24 h	-0.0346	0.0228	0.1314	-3.40
	24 h	2 w	-0.0839	0.0209	<.0001	-8.05
	2 w	8/10 w	-0.0446	0.0122	0.0003	-4.36
	8/10 w	Z31	-0.0118	0.0132	0.3745	-1.17
	Z31	Z39	-0.0368	0.0147	0.0130	-3.62
	Z39	Z47	0.0472	0.0147	0.0016	4.83
	Z47	Z65	-0.0784	0.0147	<.0001	-7.54
OK7211542	Z65	Z85	-0.0076	0.0147	0.6036	-0.76
	6 h	24 h	0.0019	0.0172	0.9125	0.19
	24 h	2 w	0.0516	0.0172	0.0032	5.29
	2 w	8/10 w	0.0097	0.0172	0.5739	0.98
	8/10 w	Z31	-0.0300	0.0172	0.0834	-2.96
	Z31	Z39	0.0313	0.0172	0.0712	3.18
	Z39	Z47	0.0453	0.0172	0.0094	4.63
	Z47	Z65	-0.0661	0.0172	0.0002	-6.39
Agrotana	Z65	Z85	-0.0616	0.0172	0.0005	-5.97
	6 h	24 h	0.0098	0.0157	0.5325	0.98
	24 h	2 w	0.0328	0.0157	0.0376	3.34
	2 w	8/10 w	-0.0100	0.0157	0.5225	-1.00
	8/10 w	Z31	0.0246	0.0157	0.1190	2.49
	Z31	Z39	-0.0273	0.0157	0.0836	-2.69
	Z39	Z47	-0.0055	0.0157	0.7274	-0.55
	Z47	Z65	0.0915	0.0157	<0.0001	9.58
11955	Z65	Z85	-0.0848	0.0157	<0.0001	-8.13

Appendix 33. Significance ($p<0.05$) of *CBF12* expression between stages of development in meristem tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	-0.0097	0.0185	0.6010	-0.97
	24 h	2 w	0.0192	0.0185	0.3011	1.94
	2 w	8/10 w	0.0137	0.0185	0.4602	1.38
	8/10 w	Z31	0.0875	0.0357	0.0154	9.14
	Z31	Z39	0.0487	0.0345	0.1595	4.99
	Z39	Z47	-0.0057	0.0154	0.7124	-0.57
	Z47	Z65	0.0682	0.0140	<0.0001	7.06
PI206624	Z65	Z85	-0.0383	0.0170	0.0258	-3.76
	6 h	24 h	-0.0065	0.0128	0.6122	-0.65
	24 h	2 w	0.0037	0.0128	0.7757	0.37
	2 w	8/10 w	0.0249	0.0128	0.0536	2.52
	8/10 w	Z31	0.0930	0.0225	<0.0001	9.75
	Z31	Z39	0.0406	0.0218	0.0642	4.14
	Z39	Z47	-0.0078	0.0089	0.3810	-0.78
	Z47	Z65	0.0643	0.0100	<0.0001	6.64
PI531718	Z65	Z85	-0.0414	0.0109	0.0002	-4.05
	6 h	24 h	-0.0438	0.0129	0.0008	-4.28
	24 h	2 w	0.0583	0.0166	0.0006	6.00
	2 w	8/10 w	0.0328	0.0172	0.0576	3.34
	8/10 w	Z31	0.0321	0.0137	0.0204	3.26
	Z31	Z39	0.0052	0.0175	0.7667	0.52
	Z39	Z47	0.0055	0.0175	0.7553	0.55
	Z47	Z65	0.0145	0.0175	0.4090	1.46
Norstar	Z65	Z85	-0.0650	0.0175	0.0003	-6.29
	6 h	24 h	-0.0043	0.0265	0.8715	-0.43
	24 h	2 w	0.0547	0.0264	0.0394	5.63
	2 w	8/10 w	0.0043	0.0134	0.7490	0.43
	8/10 w	Z31	0.0511	0.0158	0.0014	5.25
	Z31	Z39	0.0174	0.0115	0.1333	1.75
	Z39	Z47	0.0253	0.0098	0.0110	2.56
	Z47	Z65	-0.0435	0.0119	0.0003	-4.26
Chinese Spring	Z65	Z85	0.0078	0.0163	0.6349	0.78
	6 h	24 h	-0.0540	0.0239	0.0253	-5.26
	24 h	2 w	-0.0004	0.0230	0.9872	-0.04
	2 w	8/10 w	0.0152	0.0108	0.1625	1.53
	8/10 w	Z31	0.0331	0.0112	0.0036	3.37
	Z31	Z39	-0.0190	0.0130	0.1467	-1.88
	Z39	Z47	-0.0130	0.0177	0.4620	-1.29
	Z47	Z65	-0.0663	0.0215	0.0024	-6.41
235a	Z65	Z85	-0.0567	0.0164	0.0007	-5.51
	6 h	24 h	-0.0730	0.0228	0.0017	-7.04
	24 h	2 w	-0.0672	0.0209	0.0016	-6.50
	2 w	8/10 w	0.0535	0.0122	<.0001	5.49
	8/10 w	Z31	0.1175	0.0132	<.0001	12.47
	Z31	Z39	0.0484	0.0147	0.0012	4.96
	Z39	Z47	-0.0736	0.0147	<.0001	-7.10
	Z47	Z65	-0.0955	0.0147	<.0001	-9.11
OK7211542	Z65	Z85	0.0022	0.0147	0.8785	0.22
	6 h	24 h	-0.0658	0.0135	<.0001	-6.37
	24 h	2 w	0.0360	0.0135	0.0083	3.67
	2 w	8/10 w	-0.0372	0.0135	0.0065	-3.65
	8/10 w	Z31	0.0159	0.0135	0.2395	1.60
	Z31	Z39	0.0101	0.0135	0.4533	1.02
	Z39	Z47	0.0088	0.0135	0.5154	0.88
	Z47	Z65	0.0129	0.0135	0.3402	1.30
Agrotana	Z65	Z85	-0.0139	0.0135	0.3029	-1.38
	6 h	24 h	0.0050	0.0145	0.7279	0.51
	24 h	2 w	0.0202	0.0145	0.1635	2.04
	2 w	8/10 w	-0.0044	0.0145	0.7592	-0.44
	8/10 w	Z31	0.0416	0.0145	0.0046	4.24
	Z31	Z39	-0.0173	0.0145	0.2337	-1.71
	Z39	Z47	0.0913	0.0145	<0.0001	9.55
	Z47	Z65	-0.0259	0.0145	0.0752	-2.56
11955	Z65	Z85	0.0213	0.0145	0.1421	2.16

Appendix 34. Significance ($p<0.05$) of *CBF14* expression between stages of development in leaf tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	-0.0255	0.0155	0.1031	-2.51
	24 h	2 w	-0.0435	0.0114	0.0002	-4.25
	2 w	8/10 w	-0.0013	0.0167	0.9366	-0.13
	8/10 w	Z31	0.2219	0.0239	<0.0001	24.84
	Z31	Z39	-0.0769	0.0259	0.0034	-7.40
	Z39	Z47	-0.0438	0.0259	0.0926	-4.28
	Z47	Z65	0.1348	0.0259	<0.0001	14.43
PI206624	Z65	Z85	0.0320	0.0259	0.2179	3.25
	6 h	24 h	-0.0490	0.0117	<0.0001	-4.78
	24 h	2 w	-0.0526	0.0117	<0.0001	-5.13
	2 w	8/10 w	-0.0287	0.0117	0.0152	-2.83
	8/10 w	Z31	0.1672	0.0221	<0.0001	18.20
	Z31	Z39	0.0145	0.0226	0.5228	1.46
	Z39	Z47	-0.0231	0.0130	0.0780	-2.28
	Z47	Z65	0.0363	0.0175	0.0393	3.70
PI531718	Z65	Z85	0.0696	0.0188	0.0003	7.21
	6 h	24 h	-0.0502	0.0201	0.0134	-4.90
	24 h	2 w	0.0672	0.0137	<0.0001	6.95
	2 w	8/10 w	-0.0669	0.0403	0.0991	-6.47
	8/10 w	Z31	0.0343	0.0414	0.4088	3.49
	Z31	Z39	-0.0506	0.0140	0.0004	-4.94
	Z39	Z47	-0.0084	0.0140	0.5503	-0.84
	Z47	Z65	0.0454	0.0140	0.0015	4.64
Norstar	Z65	Z85	-0.0587	0.0140	<0.0001	-5.70
	6 h	24 h	-0.0769	0.0115	<.0001	-7.40
	24 h	2 w	-0.0098	0.0115	0.3976	-0.97
	2 w	8/10 w	-0.0333	0.0115	0.0043	-3.28
	8/10 w	Z31	0.1047	0.0134	<.0001	11.04
	Z31	Z39	-0.0282	0.0118	0.0176	-2.78
	Z39	Z47	0.0666	0.0083	<.0001	6.89
Chinese Spring	Z47	Z65	-0.0521	0.0107	<.0001	-5.08
	Z65	Z85	-0.0720	0.0165	<.0001	-6.94
	6 h	24 h	-0.0838	0.0142	<.0001	-8.03
	24 h	2 w	-0.0510	0.0142	0.0004	-4.97
	2 w	8/10 w	0.0565	0.0142	<.0001	5.81
	8/10 w	Z31	0.1724	0.0361	<.0001	18.82
	Z31	Z39	0.0333	0.0358	0.3528	3.39
	Z39	Z47	-0.0487	0.0199	0.0153	-4.75
	Z47	Z65	-0.1255	0.0280	<.0001	-11.79
235a	Z65	Z85	0.0307	0.0280	0.2750	3.12
	6 h	24 h	-0.0608	0.0159	0.0002	-5.90
	24 h	2 w	-0.0184	0.0159	0.2482	-1.82
	2 w	8/10 w	0.0272	0.0159	0.0886	2.75
	8/10 w	Z31	0.1854	0.0135	<.0001	20.37
	Z31	Z39	-0.0592	0.0175	0.0009	-5.75
	Z39	Z47	-0.0119	0.0170	0.4841	-1.18
	Z47	Z65	0.0583	0.0131	<.0001	6.00
OK7211542	Z65	Z85	-0.0291	0.0124	0.0197	-2.87
	6 h	24 h	-0.0459	0.0114	<.0001	-4.49
	24 h	2 w	-0.0710	0.0083	<.0001	-6.86
	2 w	8/10 w	0.0384	0.0044	<.0001	3.91
	8/10 w	Z31	0.2476	0.0190	<.0001	28.09
	Z31	Z39	-0.0071	0.0204	0.7305	-0.70
	Z39	Z47	-0.0022	0.0149	0.8842	-0.22
	Z47	Z65	-0.0373	0.0284	0.1918	-3.66
Agrotana	Z65	Z85	0.0173	0.0307	0.5733	1.75
	6 h	24 h	-0.0321	0.0043	<0.0001	-3.16
	24 h	2 w	-0.1017	0.0159	<0.0001	-9.67
	2 w	8/10 w	-0.0546	0.0178	0.0025	-5.32
	8/10 w	Z31	0.1662	0.0098	<0.0001	18.08
	Z31	Z39	-0.0083	0.0218	0.7032	-0.83
	Z39	Z47	0.0207	0.0224	0.3568	2.09
	Z47	Z65	-0.0073	0.0080	0.3683	-0.72
11955	Z65	Z85	-0.9167	0.0075	<0.0001	-60.02

Appendix 35. Significance ($p<0.05$) of *CBF14* expression between stages of development in meristem tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	-0.0651	0.0155	<0.0001	-6.30
	24 h	2 w	0.0150	0.0114	0.1886	1.51
	2 w	8/10 w	-0.1721	0.0167	0.3032	-15.81
	8/10 w	Z31	0.1590	0.0239	<0.0001	17.23
	Z31	Z39	0.0455	0.0259	0.0808	4.65
	Z39	Z47	0.0279	0.0259	0.2833	2.82
	Z47	Z65	0.1310	0.0259	<0.0001	14.00
PI206624	Z65	Z85	-0.0301	0.0259	0.2466	-2.96
	6 h	24 h	-0.0741	0.0117	<0.0001	-7.14
	24 h	2 w	-0.0026	0.0117	0.8251	-0.26
	2 w	8/10 w	0.0351	0.0117	0.0031	3.57
	8/10 w	Z31	0.1870	0.0221	<0.0001	20.56
	Z31	Z39	0.1001	0.0226	<0.0001	10.53
	Z39	Z47	-0.0077	0.0130	0.5531	-0.77
	Z47	Z65	0.0513	0.0175	0.0039	5.26
PI531718	Z65	Z85	-0.0591	0.0188	0.0020	-5.74
	6 h	24 h	-0.1168	0.0143	<0.0001	-11.02
	24 h	2 w	0.0056	0.0125	0.6573	0.56
	2 w	8/10 w	0.0096	0.0066	0.1453	0.97
	8/10 w	Z31	-0.0151	0.0100	0.1334	-1.50
	Z31	Z39	0.0240	0.0128	0.0637	2.42
	Z39	Z47	-0.1051	0.0128	<0.0001	-9.98
	Z47	Z65	0.0419	0.0128	0.0013	4.28
Norstar	Z65	Z85	-0.0656	0.0128	<0.0001	-6.35
	6 h	24 h	-0.0317	0.0115	0.0065	-3.12
	24 h	2 w	0.0394	0.0115	0.0008	4.02
	2 w	8/10 w	-0.0108	0.0115	0.3509	-1.07
	8/10 w	Z31	-0.0040	0.0134	0.7661	-0.40
	Z31	Z39	0.0414	0.0118	0.0006	4.23
	Z39	Z47	0.0057	0.0083	0.4972	0.57
Chinese	Z47	Z65	-0.0906	0.0107	<.0001	-8.66
Spring	Z65	Z85	-0.0748	0.0165	<.0001	-7.20
	6 h	24 h	-0.0452	0.0142	0.0017	-4.42
	24 h	2 w	-0.1050	0.0142	<.0001	-9.97
	2 w	8/10 w	-0.0573	0.0142	<.0001	-5.57
	8/10 w	Z31	0.1336	0.0361	0.0003	14.29
	Z31	Z39	0.0192	0.0358	0.5921	1.94
	Z39	Z47	0.0019	0.0199	0.9221	0.19
	Z47	Z65	-0.0084	0.0280	0.7653	-0.83
235a	Z65	Z85	-0.0318	0.0280	0.2581	-3.13
	6 h	24 h	-0.0089	0.0159	0.5762	-0.88
	24 h	2 w	-0.0668	0.0159	<.0001	-6.46
	2 w	8/10 w	-0.0162	0.0159	0.3078	-1.61
	8/10 w	Z31	0.1140	0.0135	<.0001	12.08
	Z31	Z39	0.0225	0.0175	0.2019	2.27
	Z39	Z47	0.0028	0.0170	0.8671	0.28
	Z47	Z65	-0.0389	0.0131	0.0034	-3.81
OK7211542	Z65	Z85	0.0067	0.0124	0.5890	0.67
	6 h	24 h	-0.0016	0.0048	0.7468	-0.16
	24 h	2 w	-0.0967	0.0033	<.0001	-9.22
	2 w	8/10 w	-0.0989	0.0354	0.0058	-9.42
	8/10 w	Z31	0.0618	0.0368	0.0952	6.37
	Z31	Z39	0.0441	0.0145	0.0027	4.51
	Z39	Z47	-0.0209	0.0115	0.0710	-2.07
	Z47	Z65	0.0162	0.0128	0.2062	1.64
Agrotana	Z65	Z85	-0.1245	0.0479	0.0102	-11.71
	6 h	24 h	-0.0718	0.0239	0.0031	-6.93
	24 h	2 w	-0.0225	0.0235	<0.0001	-2.23
	2 w	8/10 w	-0.0315	0.0124	0.0120	-3.10
	8/10 w	Z31	0.1548	0.0122	<0.0001	16.74
	Z31	Z39	-0.0726	0.0311	0.0207	-7.00
	Z39	Z47	0.1429	0.0349	<0.0001	15.36
	Z47	Z65	-0.0628	0.0171	0.0003	-6.08
11955	Z65	Z85	0.0171	0.0171	0.3177	1.72

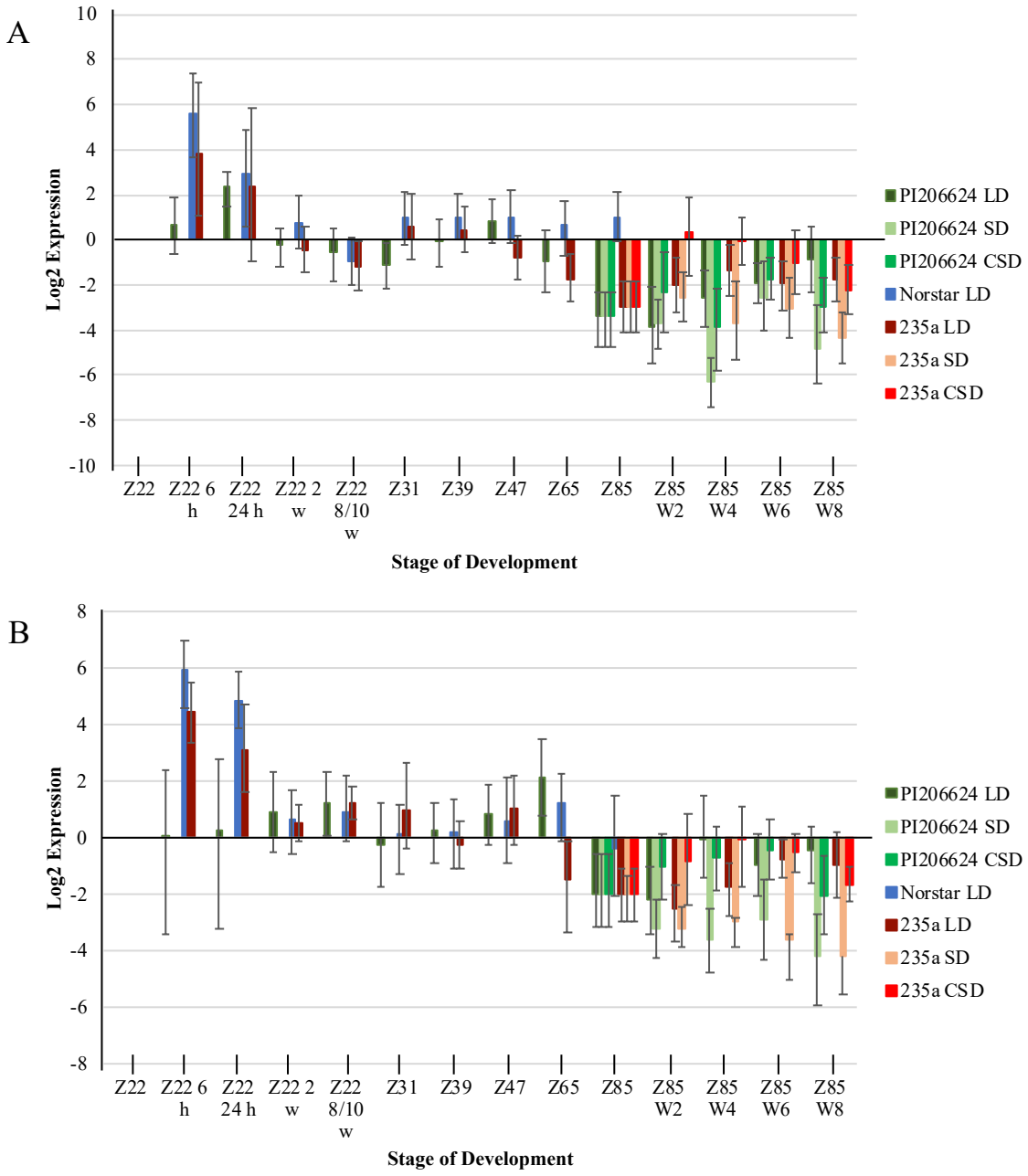
Appendix 36. Significance ($p<0.05$) of *COR14* expression between stages of development in leaf tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	0.1070	0.0243	<0.0001	11.29
	24 h	2 w	-0.1790	0.0243	<0.0001	-16.39
	2 w	8/10 w	-0.0632	0.0243	0.0101	-6.12
	8/10 w	Z31	0.5665	0.0277	<0.0001	76.21
	Z31	Z39	0.0395	0.0229	0.0833	4.03
	Z39	Z47	0.0167	0.0184	0.3653	1.69
	Z47	Z65	0.0260	0.0201	0.1969	2.64
PI206624	Z65	Z85	0.0040	0.0162	0.8079	0.40
	6 h	24 h	0.1364	0.0146	<0.0001	14.61
	24 h	2 w	-0.1399	0.0146	<0.0001	-13.06
	2 w	8/10 w	-0.0623	0.0146	<0.0001	-6.04
	8/10 w	Z31	0.3880	0.0117	<0.0001	47.40
	Z31	Z39	0.0548	0.0191	0.0047	5.63
	Z39	Z47	-0.0925	0.0202	<0.0001	-8.84
	Z47	Z65	0.0984	0.0123	<0.0001	10.34
PI531718	Z65	Z85	0.0301	0.0203	0.1411	3.05
	6 h	24 h	0.1034	0.0210	<0.0001	10.89
	24 h	2 w	-0.0569	0.0287	0.0490	-5.53
	2 w	8/10 w	-0.1072	0.0636	0.0937	-10.17
	8/10 w	Z31	0.1523	0.0622	0.0154	16.45
	Z31	Z39	0.0057	0.0262	0.8288	0.57
	Z39	Z47	-0.1609	0.0262	<0.0001	-14.86
	Z47	Z65	0.0477	0.0262	0.0702	4.88
Norstar	Z65	Z85	0.0142	0.0262	0.5893	1.43
	6 h	24 h	0.1272	0.0164	<.0001	13.56
	24 h	2 w	-0.0936	0.0169	<.0001	-8.94
	2 w	8/10 w	-0.1182	0.0278	<.0001	-11.15
	8/10 w	Z31	0.4193	0.0292	<.0001	52.09
	Z31	Z39	0.0003	0.0171	0.9843	0.03
	Z39	Z47	-0.0263	0.0171	0.1268	-2.59
Chinese Spring	Z47	Z65	0.0384	0.0171	0.0262	3.91
	Z65	Z85	-0.0600	0.0171	0.0006	-5.83
	6 h	24 h	0.1433	0.0154	<.0001	15.41
	24 h	2 w	-0.0012	0.0154	0.9362	-0.12
	2 w	8/10 w	0.0567	0.0154	0.0003	5.84
	8/10 w	Z31	0.3296	0.0485	<.0001	39.04
	Z31	Z39	0.1342	0.0489	0.0067	14.36
	Z39	Z47	-0.1950	0.0222	<.0001	-17.72
	Z47	Z65	-0.0611	0.0267	0.0234	-5.93
235a	Z65	Z85	0.2253	0.0275	<.0001	25.27
	6 h	24 h	0.0840	0.0118	<.0001	8.76
	24 h	2 w	-0.0680	0.0118	<.0001	-6.58
	2 w	8/10 w	0.0226	0.0118	0.0569	2.29
	8/10 w	Z31	0.5638	0.0135	<.0001	75.73
	Z31	Z39	0.0505	0.0286	0.0788	5.18
	Z39	Z47	-0.0477	0.0284	0.0950	-4.66
	Z47	Z65	-0.0082	0.0104	0.4335	-0.81
OK7211542	Z65	Z85	0.1215	0.0213	<.0001	12.92
	6 h	24 h	0.0424	0.0315	0.1799	4.33
	24 h	2 w	-0.1356	0.0361	0.0002	-12.68
	2 w	8/10 w	-0.0212	0.0209	0.3138	-2.09
	8/10 w	Z31	0.4587	0.0210	<.0001	58.20
	Z31	Z39	-0.0234	0.0225	0.3000	-2.31
	Z39	Z47	0.0211	0.0135	0.1189	2.14
	Z47	Z65	0.0389	0.0265	0.1440	3.96
Agrotana	Z65	Z85	0.0414	0.0563	0.4634	4.23
	6 h	24 h	0.1560	0.0188	<0.0001	16.88
	24 h	2 w	-0.0663	0.0210	0.0019	-6.42
	2 w	8/10 w	-0.1278	0.0241	<0.0001	-12.00
	8/10 w	Z31	0.3279	0.0358	<0.0001	38.81
	Z31	Z39	0.0028	0.0424	0.9480	0.28
	Z39	Z47	-0.1626	0.0424	0.0002	-15.01
	Z47	Z65	0.0931	0.0424	0.0298	9.75
11955	Z65	Z85	0.1190	0.0424	0.0057	12.64

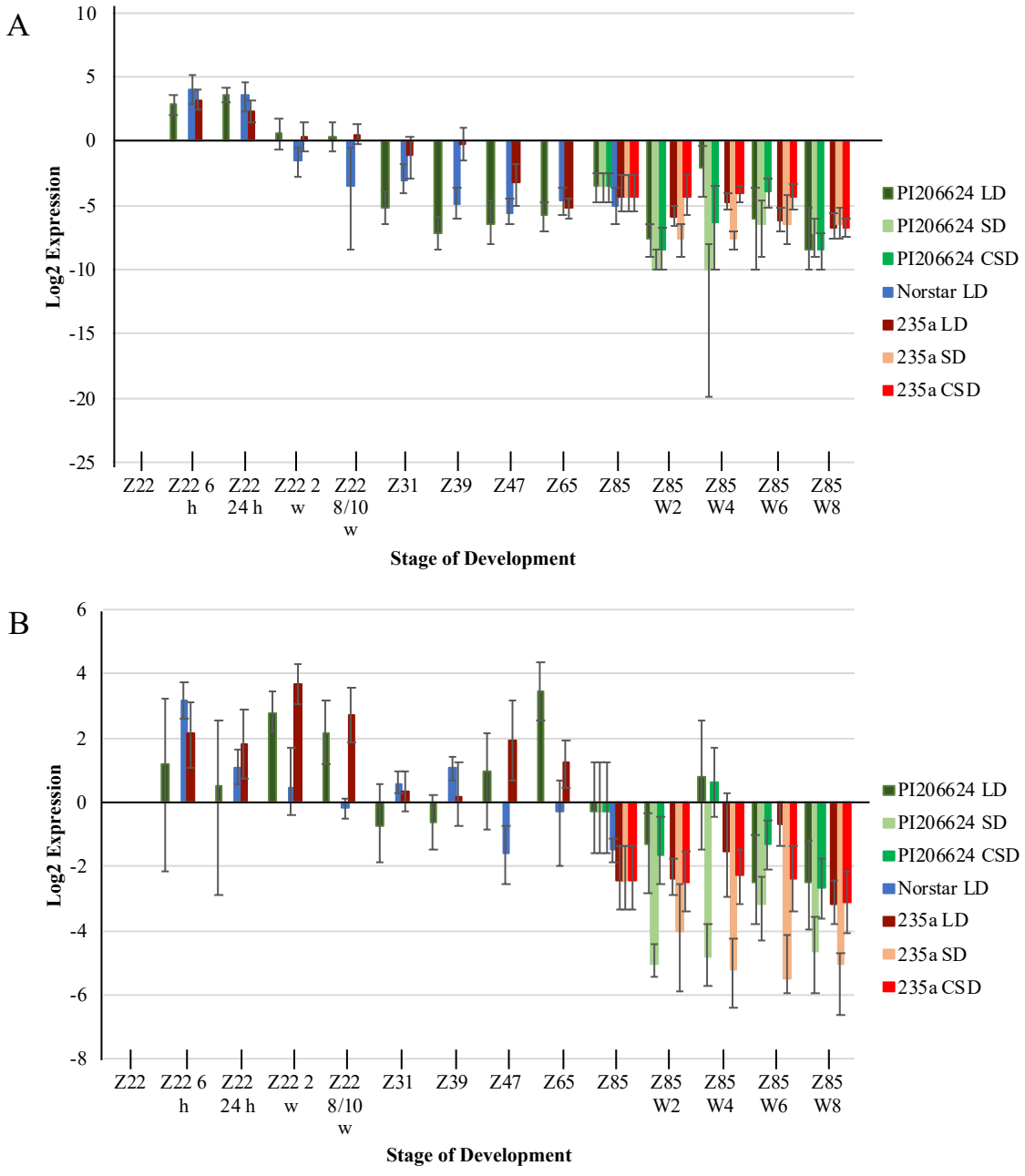
Appendix 37. Significance ($p<0.05$) of *COR14* expression between stages of development in meristem tissue of wheatgrass, wheat, and perennial wheat lines. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Estimates demonstrate differences in natural logarithms and represent unitless ratios that can be expressed as a percent. Percent difference was calculated using the formula: 100 x (*e*^(Estimate) – 1) in order to determine the percent difference in gene expression between two stages of development.

Cultivar	Stage of Development		Estimate	Standard Error	P value	Difference (%)
	6 h	24 h	0.1322	0.0243	<0.0001	14.13
	24 h	2 w	-0.0787	0.0243	0.0014	-7.57
	2 w	8/10 w	-0.0424	0.0243	0.0827	-4.15
	8/10 w	Z31	0.3918	0.0277	<0.0001	47.96
	Z31	Z39	0.0442	0.0227	0.0532	4.52
	Z39	Z47	0.0028	0.0184	0.8782	0.28
	Z47	Z65	0.0128	0.0201	0.5253	1.29
PI206624	Z65	Z85	-0.0150	0.0162	0.3564	-1.49
	6 h	24 h	0.0809	0.0146	<0.0001	8.42
	24 h	2 w	-0.0826	0.0146	<0.0001	-7.93
	2 w	8/10 w	-0.0225	0.0146	0.1258	-2.22
	8/10 w	Z31	0.2709	0.0117	<0.0001	31.11
	Z31	Z39	-0.0122	0.0191	0.5233	-1.21
	Z39	Z47	-0.0120	0.0202	0.5556	-1.19
	Z47	Z65	0.0465	0.0123	0.0002	4.76
PI531718	Z65	Z85	-0.0126	0.0203	0.2012	-1.25
	6 h	24 h	0.1209	0.0130	<0.0001	12.85
	24 h	2 w	-0.0864	0.0253	0.0008	-8.27
	2 w	8/10 w	-0.0911	0.0251	0.0004	-8.71
	8/10 w	Z31	0.3672	0.0098	<0.0001	44.37
	Z31	Z39	-0.0107	0.0119	0.3704	-1.06
	Z39	Z47	-0.0129	0.0119	0.2788	-1.29
	Z47	Z65	0.0124	0.0119	0.3006	1.24
Norstar	Z65	Z85	-0.0311	0.0119	0.0100	-3.06
	6 h	24 h	0.1480	0.0164	<.0001	15.95
	24 h	2 w	-0.0088	0.0169	0.6042	-0.88
	2 w	8/10 w	-0.0464	0.0278	0.0974	-4.53
	8/10 w	Z31	0.3772	0.0292	<.0001	45.82
	Z31	Z39	0.0048	0.0171	0.7786	0.48
	Z39	Z47	0.0046	0.0171	0.7871	0.46
	Z47	Z65	-0.0294	0.0171	0.0875	-2.90
Chinese Spring	Z65	Z85	0.0040	0.0171	0.8140	0.40
	6 h	24 h	0.1203	0.0154	<.0001	12.78
	24 h	2 w	0.0028	0.0154	0.8588	0.28
	2 w	8/10 w	0.0253	0.0154	0.1033	2.56
	8/10 w	Z31	0.4302	0.0485	<.0001	53.76
	Z31	Z39	0.0319	0.0489	0.5144	3.25
	Z39	Z47	-0.0015	0.0222	0.9479	-0.15
	Z47	Z65	-0.0643	0.0267	0.0170	-6.23
235a	Z65	Z85	-0.0084	0.0275	0.7612	-0.83
	6 h	24 h	0.1405	0.0148	<.0001	15.08
	24 h	2 w	-0.0370	0.0148	0.0136	-3.63
	2 w	8/10 w	0.0667	0.0148	<.0001	6.90
	8/10 w	Z31	0.4337	0.0153	<.0001	54.30
	Z31	Z39	0.0061	0.0175	0.7280	0.61
	Z39	Z47	-0.0176	0.0173	0.3120	-1.74
	Z47	Z65	-0.0475	0.0145	0.0013	-4.64
OK7211542	Z65	Z85	0.0379	0.0110	0.0007	3.86
	6 h	24 h	0.0824	0.0279	0.0036	8.59
	24 h	2 w	-0.1474	0.0268	<.0001	-13.71
	2 w	8/10 w	-0.0697	0.0305	0.0237	-6.73
	8/10 w	Z31	0.4040	0.0300	<.0001	49.78
	Z31	Z39	0.0656	0.0211	0.0022	6.78
	Z39	Z47	-0.0663	0.0244	0.0073	-6.42
	Z47	Z65	0.0056	0.0221	0.7992	0.56
Agrotana	Z65	Z85	-0.0149	0.0278	0.5921	-1.48
	6 h	24 h	0.1340	0.0528	0.0121	14.34
	24 h	2 w	-0.1691	0.0521	0.0014	-15.56
	2 w	8/10 w	-0.0662	0.0226	0.0038	-6.41
	8/10 w	Z31	0.3630	0.0293	<0.0001	43.76
	Z31	Z39	0.0398	0.0271	0.1437	4.06
	Z39	Z47	-0.0256	0.0271	0.3469	-2.52
	Z47	Z65	-0.0199	0.0271	0.4642	-1.97
11955	Z65	Z85	-0.0124	0.0271	0.6487	-1.23

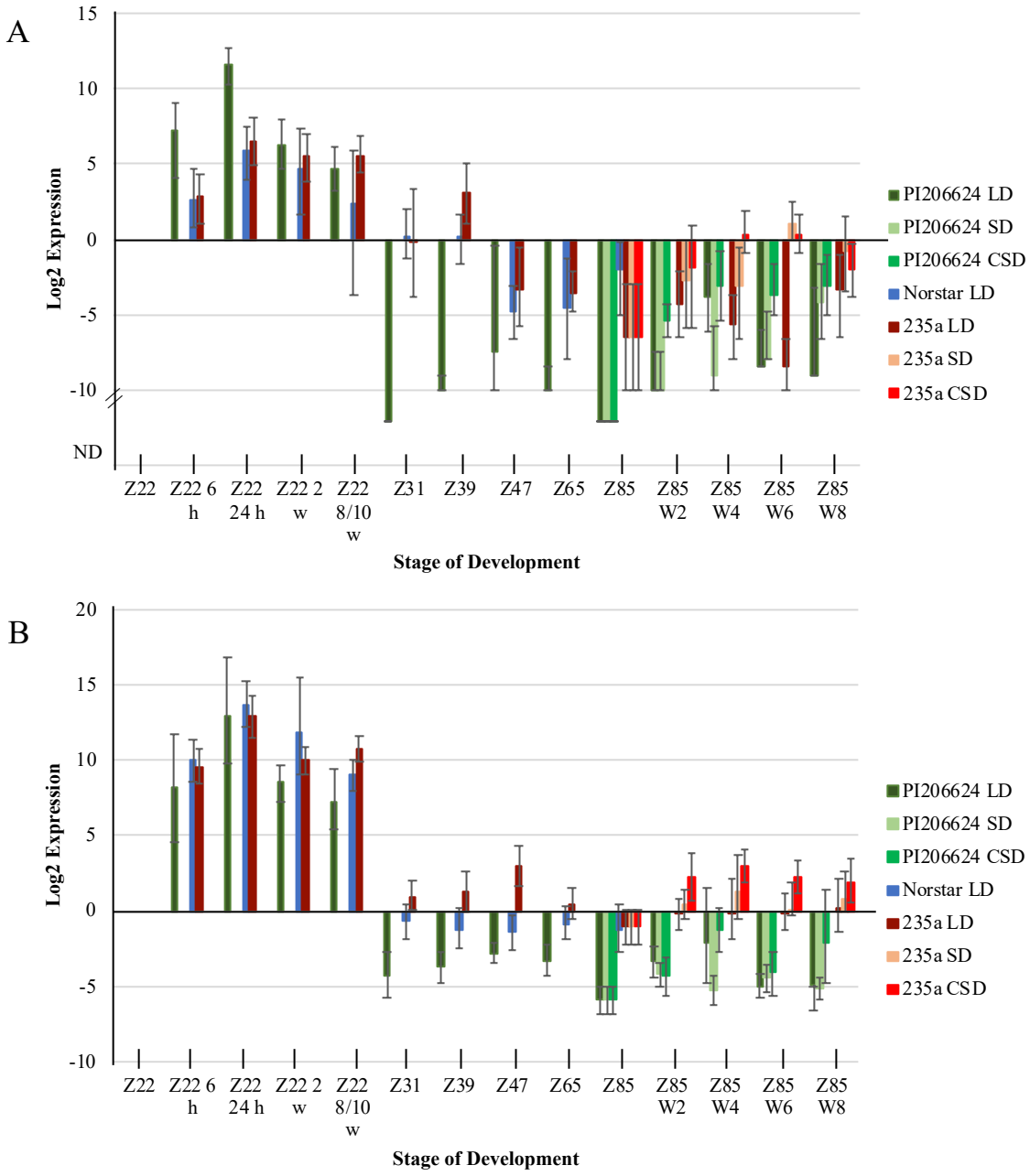
Appendix 38. Log2 gene expression of *CBF12* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. Error bars indicate standard error.



Appendix 39. Log2 gene expression of *CBF14* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. Error bars indicate standard error.



Appendix 40. Log2 gene expression of *COR14* over various Zadoks stages of development in the first sexual cycle and beginning of the second sexual cycle for leaf (A) and meristem (B) tissue of wheatgrass, wheat, and perennial wheat lines exposed to different photoperiods and temperatures compared to stage Z22. Z22 6 h, 24 h, 2 w, 8/10 w represents a vernalization time of 6 h, 24 h, 2 weeks and 8/10 weeks respectively. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling. Error bars indicate standard error.



Appendix 41. Significant ($p<0.05$) relative expression and up or downregulation of *CBF12* between stages of development in leaf (A) and meristem (B) tissue of wheatgrass and perennial wheat lines exposed to LDs, SDs or CSDs compared to stage Z85. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling.

A

Line	Stage of Development	Expression	Standard Error	P Value	Regulation
	Z85 W2	0.834	0.302 - 2.642	0.606	
	Z85 W4	1.995	0.959 - 4.784	0.030	UP
	Z85 W6	2.964	1.403 - 5.676	0.001	UP
PI206624 LD	Z85 W8	5.713	2.406 - 13.732	0.000	UP
	Z85 W2	0.813	0.356 - 1.938	0.457	
	Z85 W4	0.142	0.060 - 0.307	0.000	DOWN
	Z85 W6	1.8	0.580 - 6.549	0.158	
PI206624 SD	Z85 W8	0.374	0.117 - 1.608	0.026	DOWN
	Z85 W2	2.135	0.635 - 8.737	0.121	
	Z85 W4	0.73	0.206 - 2.617	0.478	
	Z85 W6	3.215	1.358 - 7.088	0.001	UP
PI206624 CSD	Z85 W8	1.376	0.598 - 3.461	0.309	
	Z85 W2	1.885	0.869 - 4.160	0.038	UP
	Z85 W4	3.004	1.700 - 5.942	0.001	UP
	Z85 W6	2.15	1.225 - 4.055	0.004	UP
235a LD	Z85 W8	2.428	1.428 - 4.103	0.000	UP
	Z85 W2	1.347	0.667 - 2.941	0.279	
	Z85 W4	0.571	0.198 - 2.441	0.196	
	Z85 W6	0.981	0.349 - 2.831	0.953	
235a SD	Z85 W8	0.34	0.154 - 0.772	0.002	DOWN
	Z85 W2	9.55	2.575 - 26.431	0.001	UP
	Z85 W4	7.448	4.088 - 14.038	0.000	UP
	Z85 W6	3.824	1.568 - 9.550	0.001	UP
235a CSD	Z85 W8	1.634	0.856 - 3.247	0.042	UP

B

Line	Stage of Development	Expression	Standard Error	P Value	Regulation
	Z85 W2	0.973	0.454 - 2.237	0.923	
	Z85 W4	3.855	1.577 - 8.713	0.000	UP
	Z85 W6	2.084	0.981 - 4.349	0.005	UP
PI206624 LD	Z85 W8	2.935	1.469 - 5.482	0.000	UP
	Z85 W2	0.412	0.194 - 0.973	0.004	DOWN
	Z85 W4	0.326	0.133 - 0.779	0.002	DOWN
	Z85 W6	0.521	0.177 - 1.470	0.098	
PI206624 SD	Z85 W8	0.216	0.067 - 0.652	0.001	DOWN
	Z85 W2	1.925	0.868 - 4.854	0.041	UP
	Z85 W4	2.392	0.954 - 5.552	0.008	UP
	Z85 W6	2.865	1.266 - 6.872	0.001	UP
PI206624 CSD	Z85 W8	0.962	0.343 - 2.729	0.914	
	Z85 W2	0.719	0.360 - 1.585	0.209	
	Z85 W4	1.222	0.654 - 2.445	0.465	
	Z85 W6	2.418	1.293 - 4.297	0.001	UP
235a LD	Z85 W8	2.157	0.938 - 4.578	0.006	UP
	Z85 W2	0.386	0.189 - 0.833	0.002	DOWN
	Z85 W4	0.487	0.201 - 1.188	0.020	DOWN
	Z85 W6	0.29	0.119 - 0.623	0.004	DOWN
235a SD	Z85 W8	0.18	0.084 - 0.421	0.001	DOWN
	Z85 W2	2.579	0.653 - 11.137	0.075	
	Z85 W4	4.553	1.130 - 15.003	0.002	UP
	Z85 W6	3.167	1.642 - 7.351	0.001	UP
235a CSD	Z85 W8	1.334	0.666 - 2.843	0.245	

Appendix 42. Significant ($p<0.05$) relative expression and up or downregulation of *CBF14* between stages of development in leaf (A) and meristem (B) tissue of wheatgrass and perennial wheat lines exposed to LDs, SDs or CSDs compared to stage Z85. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling.

A

Line	Stage of Development	Expression	Standard Error	P Value	Regulation
	Z85 W2	0.074	0.033 - 0.192	0.000	DOWN
	Z85 W4	2.892	0.917 - 7.653	0.009	UP
	Z85 W6	0.219	0.030 - 1.251	0.015	DOWN
PI206624 LD	Z85 W8	0.052	0.011 - 0.468	0.000	DOWN
	Z85 W2	0.015	0.009 - 0.030	0.000	DOWN
	Z85 W4	0.017	0.006 - 0.041	0.000	DOWN
	Z85 W6	0.14	0.028 - 0.427	0.000	DOWN
PI206624 SD	Z85 W8	0.079	0.029 - 0.196	0.000	DOWN
	Z85 W2	0.038	0.016 - 0.098	0.000	DOWN
	Z85 W4	0.15	0.008 - 0.974	0.004	DOWN
	Z85 W6	0.743	0.388 - 1.485	0.237	
PI206624 CSD	Z85 W8	0.032	0.015 - 0.070	0.000	DOWN
	Z85 W2	0.311	0.120 - 0.733	0.000	DOWN
	Z85 W4	0.7	0.235 - 1.436	0.215	
	Z85 W6	0.272	0.105 - 0.638	0.000	DOWN
235a LD	Z85 W8	0.177	0.054 - 0.547	0.000	DOWN
	Z85 W2	0.074	0.020 - 0.258	0.000	DOWN
	Z85 W4	0.071	0.022 - 0.193	0.000	DOWN
	Z85 W6	0.19	0.029 - 1.666	0.007	DOWN
235a SD	Z85 W8	0.175	0.035 - 0.812	0.003	DOWN
	Z85 W2	0.966	0.251 - 3.567	0.946	
	Z85 W4	1.133	0.357 - 2.667	0.681	
	Z85 W6	0.949	0.345 - 2.574	0.881	
235a CSD	Z85 W8	0.18	0.060 - 0.397	0.000	DOWN

B

Line	Stage of Development	Expression	Standard Error	P Value	Regulation
	Z85 W2	0.563	0.280 - 1.584	0.119	
	Z85 W4	2.101	0.689 - 7.612	0.101	
	Z85 W6	0.264	0.072 - 0.859	0.004	DOWN
PI206624 LD	Z85 W8	0.228	0.055 - 0.812	0.006	DOWN
	Z85 W2	0.037	0.013 - 0.092	0.000	DOWN
	Z85 W4	0.043	0.013 - 0.120	0.000	DOWN
	Z85 W6	0.131	0.048 - 0.380	0.000	DOWN
PI206624 SD	Z85 W8	0.047	0.016 - 0.143	0.000	DOWN
	Z85 W2	0.384	0.107 - 0.944	0.022	DOWN
	Z85 W4	1.837	0.578 - 5.894	0.126	
	Z85 W6	0.479	0.149 - 1.149	0.041	DOWN
PI206624 CSD	Z85 W8	0.191	0.050 - 0.589	0.000	DOWN
	Z85 W2	1.121	0.578 - 1.938	0.534	
	Z85 W4	1.965	0.735 - 7.392	0.079	
	Z85 W6	3.543	1.654 - 7.653	0.000	UP
235a LD	Z85 W8	0.617	0.321 - 1.122	0.051	
	Z85 W2	0.304	0.088 - 1.222	0.018	DOWN
	Z85 W4	0.116	0.045 - 0.330	0.000	DOWN
	Z85 W6	0.096	0.031 - 0.308	0.000	DOWN
235a SD	Z85 W8	0.134	0.053 - 0.343	0.000	DOWN
	Z85 W2	1.008	0.383 - 2.368	0.982	
	Z85 W4	1.19	0.524 - 2.746	0.539	
	Z85 W6	1.084	0.412 - 3.037	0.791	
235a CSD	Z85 W8	0.609	0.241 - 1.660	0.121	

Appendix 43. Significant ($p<0.05$) relative expression and up or downregulation of *COR14* between stages of development in leaf (A) and meristem (B) tissue of wheatgrass and perennial wheat lines exposed to LDs, SDs or CSDs compared to stage Z85. Z85 W2, W4, W6, W8 represents 2, 4, 6 and 8 weeks of sampling.

A

Line	Stage of Development	Expression	Standard Error	P Value	Regulation
	Z85 W2	2.372	0.453 - 15.991	0.196	
	Z85 W4	119.498	31.186 - 543.416	0.000	UP
	Z85 W6	7.802	1.676 - 43.329	0.004	UP
PI206624 LD	Z85 W8	7.559	0.649 - 197.412	0.047	UP
	Z85 W2	5.911	1.115 - 40.969	0.024	UP
	Z85 W4	9.397	0.999 - 87.477	0.006	UP
	Z85 W6	18.072	2.150 - 206.472	0.005	UP
PI206624 SD	Z85 W8	233.016	37.014 - 1,968.858	0.000	UP
	Z85 W2	99.641	16.548 - 387.579	0.000	UP
	Z85 W4	480.249	96.657 - 3,338.013	0.000	UP
	Z85 W6	315.559	37.191 - 1,723.978	0.000	UP
PI206624 CSD	Z85 W8	504.808	98.115 - 3,200.876	0.000	UP
	Z85 W2	4.853	0.200 - 82.983	0.077	
	Z85 W4	1.756	0.090 - 24.717	0.493	
	Z85 W6	0.265	0.012 - 2.655	0.129	
235a LD	Z85 W8	10.646	0.981 - 193.051	0.028	UP
	Z85 W2	35.623	3.227 - 843.672	0.005	UP
	Z85 W4	29.429	2.198 - 1,153.441	0.014	UP
	Z85 W6	1,052.54	37.385 - 10,103.943	0.000	UP
235a SD	Z85 W8	208.655	14.200 - 3,535.647	0.000	UP
	Z85 W2	26.338	2.609 - 544.877	0.007	UP
	Z85 W4	116.627	8.223 - 721.960	0.000	UP
	Z85 W6	116.581	7.404 - 745.752	0.000	UP
235a CSD	Z85 W8	24.105	1.663 - 213.416	0.002	UP

B

Line	Stage of Development	Expression	Standard Error	P Value	Regulation
	Z85 W2	4.331	2.299 - 8.314	0.000	UP
	Z85 W4	8.807	1.894 - 67.450	0.000	UP
	Z85 W6	1.839	1.028 - 3.239	0.010	UP
PI206624 LD	Z85 W8	1.091	0.607 - 1.969	0.659	
	Z85 W2	3.228	1.635 - 6.153	0.000	UP
	Z85 W4	1.579	0.702 - 3.196	0.083	
	Z85 W6	2.808	1.399 - 5.910	0.002	UP
PI206624 SD	Z85 W8	1.712	0.896 - 3.090	0.022	UP
	Z85 W2	2.91	1.127 - 8.301	0.005	UP
	Z85 W4	23.993	7.878 - 74.795	0.000	UP
	Z85 W6	3.455	1.269 - 9.558	0.003	UP
PI206624 CSD	Z85 W8	14.05	2.530 - 151.079	0.000	UP
	Z85 W2	1.964	0.965 - 4.129	0.017	UP
	Z85 W4	2.093	0.682 - 8.999	0.091	
	Z85 W6	2.012	0.878 - 4.954	0.032	UP
235a LD	Z85 W8	2.438	0.829 - 9.282	0.020	UP
	Z85 W2	4.417	1.998 - 8.432	0.000	UP
	Z85 W4	9.746	2.179 - 82.873	0.000	UP
	Z85 W6	3.047	1.305 - 6.217	0.001	UP
235a SD	Z85 W8	5.738	2.530 - 13.937	0.000	UP
	Z85 W2	18.83	4.635 - 78.016	0.000	UP
	Z85 W4	34.59	13.692 - 97.649	0.000	UP
	Z85 W6	18.846	7.321 - 50.830	0.000	UP
235a CSD	Z85 W8	14.12	3.963 - 63.609	0.000	UP