The Expression Patterns of Three *VRN* Genes in Common Wheat (*Triticum aestivum* L.) in Response to Vernalization

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Common wheat is a widely planted cereal in China, and vernalization is a crucial phase in wheat development. Although three major genes (*VRN1, VRN2* and *VRN3*) are associated with the vernalization response, little is known about their expression profiles during wheat growth. In this study, we observed the spike differentiation process in spring wheat variety XC2 and winter wheat variety J841 and used qRT-PCR to analyze the expression patterns of three *VRN* genes in the leaves of these wheat varieties during development under vernalization versus non-vernalization treatment under long-day conditions. We also analyzed the expression patterns of *VRN1* and *VRN3* in the apical meristem. In both spring and winter wheat, the spikes remained at the single ridge state and did not differentiate under vernalization treatment. Spike differentiation completed one week earlier in XC2 spring wheat after vernalization treatment compared with non-vernalization conditions. Vernalization treatment significantly upregulated *VRN1* and *VRN3* expression in leaves under long-day conditions, whereas *VRN2* expression was sharply reduced. The expression of *VRN3* was low in shoot apical meristems, while *VRN1* was highly expressed in shoot apical meristems when floral primordia began to differentiate and develop, suggesting that *VRN1* functions independently in leaves and shoot apical meristems.

Keywords: apical meristems, common wheat, expression, leaf, spike differentiation, vernalization

Introduction

The transition from the vegetative phase to the reproductive phase is crucial for the life cycle of a plant (Michaels and Amasino 2000). This transition occurs when internal and external conditions are appropriate for successful plant reproduction. Wheat (*Triticum aestivum* L.) is the most widely planted cereal, and its adaptability to diverse growing conditions is associated with the strong variability of its genetic background (Shewry 2009; Cockram et al. 2007). The genetic mechanism controlling wheat heading time is primarily classified into three components: vernalization requirement, photoperiod sensitivity and narrow-sense earliness (Kato and Yamagata 1988). Vernalization requirement refers to the requirement for a prolonged period of cold treatment to accelerate plants

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flowering, which is essential for protecting vulnerable floral meristems from frost damage on cold days. Three major genes related to wheat vernalization response have been identified, namely, *VRN1, VRN2* and *VRN3* (Yan et al. 2003, 2004, 2006). Recently, another gene, *VRN4*, was precisely mapped on the D genome, and its response to vernalization was analyzed (Yoshida et al. 2010). *VRN1*, which promotes flowering, encodes a MADS-box transcription factor and is highly similar to the meristem identity gene *AP1* in Arabidopsis (Yan et al. 2003). *AP1* is a key temperature-responsive gene that regulates the initiation of the vegetative shoot transition to reproductive apices (Ferrándiz et al. 2000; Trevaskis et al. 2003). Deletion of *VRN1* causes a loss of flowering ability in Einkorn wheat (*Triticum monococcum*) (Shitsukawa et al. 2007). However, a recent study suggests that *VRN1* downregulates the flowering repressor gene *VRN2* in leaves but is not essential for flowering (Chen and Dubcovsky 2012). Common wheat, an allohexaploid, contains three homoeologous copies of *VRN1*, which are located on the long arms of chromosomes 5A, 5B and 5D, respectively (Yan et al. 2003). Allelic variations in the three homoeologous genes (*Vrn-A1*, *Vrn-B1* and *Vrn-D1*) are the main factors that determine vernalization requirements (Fu et al. 2005; Loukoianov et al. 2005; Zhang et al. 2008). Recently, Zhang et al. (2012) found that a single nucleotide variation in the promoter region of *Vrn-D1* is associated with the vernalization response in common wheat. The function of *VRN1* is thought to differ in temperate cereal depending on the organ (Trevaskis 2010).

VRN2 is a flowering repressor gene, which includes two linked *ZCCT* genes, *ZCCT1* and *ZCCT2*, encoding proteins with a zinc finger motif and a CCT domain (Yan et al. 2004). Simultaneous deletions and mutations in these two *ZCCT* genes are associated with the spring growth habit in wheat (Distelfeld et al. 2009b). Tetraploid wheat (*T. turgidum*, AABB) contains two *ZCCT* genes (*ZCCT1, ZCCT2*), which are present on each copy of the A and B genomes, respectively. Plants exhibit spring characteristics only when both of these *ZCCT* genes are nonfunctional. In hexaploid wheat, only when all three *VRN1* loci are homozygous recessive can both dominant and recessive *Vrn-2* function in regulating plant growth habit. As *vrn-2* is the only gene that suppresses reproductive growth, the expression of this gene is activated only under long-day conditions, and it is inhibited under short-day conditions and cold-temperatures (Trevaskis et al. 2006).

VRN3, a flowering promoter gene, encodes a Raf kinase inhibitor-like protein that is highly similar to Arabidopsis protein FLOWERING LOCUS T (FT) (Yan et al. 2006). FT is highly conserved as a universal signal for flowering among plant species and travels from the leaves to the apical meristem through the phloem (Corbesier et al. 2007; Tamaki et al. 2007; Zeevaart 2008). The insertion of a repetitive element in the regulatory region of *Vrn-B3* in wheat variety Chinese Hope produces a dominant allele and early flowering. Transgenic winter wheat overexpressing the *Vrn-B3* allele flowers earlier than the control without vernalization treatment (Yan et al. 2006), suggesting that the dominant *VRN3* allele can overcome the repression of *VRN2* and bypass the vernalization requirement, while downregulation of *FT1* expression by RNA interference results in late flowering in wheat (Lv et al. 2014).

Great progress has been made in elucidating the interactions between *VRN1*, *VRN2* and *VRN3* (Distelfeld et al. 2009a). However, the mechanisms underlying these complicated interactions are still not completely understood. Chen and Dubcovsky (2012) developed a genetic network model based on Trevaskis' work. This model describes how *VRN2* is highly expressed in leaves before vernalization and prevents the induction of *VRN3* transcription under long-day conditions (Li et al. 2011). The expression of *VRN1* is promoted, and *VRN2* transcription is downregulated, at the end of vernalization, resulting in upregulation of *FT* transcription under long-day conditions. FT protein is produced in leaves and transported to the apical meristem, which it interacts with FDL2 (Li and Dubcovsky 2008) and upregulates *VRN1* transcription to levels required for the transition of the shoot apex from the vegetative phase to the reproductive phase (Chen and Dubcovsky 2012). Chen and Dubcovsky (2012) also proposed that vernalization can repress the expression of *VRN2* and *VRN1*, thereby playing an important role in maintaining low levels of *VRN2* transcription after vernalization. This model also suggests that the interaction between *VRN3* and *VRN1* may differ depending on the plant organ (leaf or shoot apex).

Previous studies on *VRN* genes were mainly conducted in *Triticum monococcum* or tetraploid wheat (Greenup et al. 2009; Shimada et al. 2009; Higgins et al. 2010; Chen and Dubcovsky 2012). Hexaploid wheat is widely planted in China, and high wheat yield is essential for social and economic security. Developmental characteristics of wheat are one of the main factors that limit the possibility of procuring excellent germplasm. Therefore, identifying genotypes and expression patterns of *VRN* genes would be beneficial for altering the developmental characteristics of wheat at the molecular level. In this study, we examined the expression patterns of three *VRN* genes in leaves and spikes differentiation of common wheat under long-day conditions and different vernalization treatments, and we also analyzed *VRN1* and *VRN3* transcript levels in apical meristems during plant development. The results of this study increase our understanding of the relationship between *VRN* gene expression patterns and both floral induction and plant development.

Materials and Methods

Plant materials and experimental treatments

Two varieties of wheat (*Triticum aestivum* L.) were selected to represent different types of vernalization-responsive growth habits: Xinchun No. 2 (hereafter referred to as XC2), a spring variety, and Jing841 (hereafter referred to as J841), a winter variety. The genotypes of these varieties regarding three *VRN* genes and *Ppd* gene are shown in Table 1 (related article unpublished). Plump seeds were selected and germinated in laboratory dishes. When seedlings grew to 2–3 cm tall, they were transplanted to pots containing 3:2:1 of sphagnum: turf: vermiculite. Twenty-five seedlings were uniformly planted in each pot, and eight pots were planted per variety. The plants were grown in a phytotron under a 16 h light/8 h dark photoperiod and 22 °C day/20 °C night temperatures under 200 µmol m^{-2} s^{-1} light intensity at pot level. Three weeks after germination, when plants reached the three-leaf stage, pots containing each variety were divided into two groups.

Table 1. Genotypes of XC2 and Jing841 wheat

	XC2	Jing841
Phenotype	Spring	Winter
VRN1	*Vrn1(Vrn-A1vrn-B1vrn-D1)*	*vrn1(vrn-A1vrn-B1vrn-D1)*
VRN2	*Vrn2*	*Vrn2*
VRN3	*vrn-B3*	*vrn-B3*
Ppd	*Ppd-D1a Ppd-B1b Ppd-A1b*	*Ppd-D1aPpd-B1b Ppd-A1b*

One group was maintained in the phytotron for non-vernalization (control) treatment, while the other group was transferred to a cold cabinet at 3–4 °C and grown under the same photoperiod for 5 weeks. Eight-week-old vernalized plants were then transferred to the same phytotron (16 h light/8 h dark photoperiod and 22 °C day/20 °C night temperatures) until flowering or plant aging. Flowering time is defined as the date at which the inflorescence overtops the flag leaf; aging time is defined as the period during which the rate of new leaf growth is lower than the death rate of mature leaves.

 Shoot apices and leaves were sampled every week for each treatment. Sample times were set as 0 (three-week-old seedlings, just prior to treatment), 1, 2, 3, 4, 5 weeks (during treatment) and so on, until plant flowering or aging (post-treatment). Samples were collected in the middle of the light period to avoid the influence of circadian rhythms as much as possible. Each sample consisted of five plants, thereby reducing individual variance. All samples were immediately frozen in liquid nitrogen and stored at –80 °C until use.

RNA extraction and cDNA synthesis

Total RNA was extracted from leaves and apical meristems using Trizol Reagent (Invitrogen, USA) following the manufacturer's instructions. RNA concentration was measured using a spectrophotometer (NanoDrop ND-1000, USA). Then, cDNA was synthesized using a PrimeScript RT cDNA synthesis kit with gDNA eraser (TaKaRa, China) according to the manufacturer's instructions.

Real-time quantitative PCR

Real-time quantitative RT-PCR was performed using an Eppendorf Realplex4 Mastercycler Epgradient S (Eppendorf, Germany). Reactions were carried out with a SYBR Green quantitative RT-PCR kit (TaKaRa, China) according to the manufacturer's instructions and repeated three times. Cycling conditions were 2 min at 95 °C, 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A melting curve protocol was performed for each primer to detect its specificity. The relative expression of each target gene relative to *β-ACTIN* (endogenous control gene) was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Primers used for real-time quantitative RT-PCR of *VRN1*, *VRN2*, *VRN3* and *ACTIN* are listed in Table 2.

Table 2. Primers used for gene expression analysis

Prime names	Primer sequences (5'-3')	Annealing temperature (%)	References
VRN1-F	GGCGAAGGTTGAGACAATACA	60	Winfield et al. 2009
VRN1-R	TCTCCTCCTGCAGTGACCTCT		
VRN2-F	TTCCGGTAATTTATAGCACAAGC	60	Winfield et al. 2009
VRN2-R	GGCTCCAATCGATCAATCAC		
V3-ABD-F	GCGGCAATGAGATGAGGA	60	Yan et al. 2006; Bonnin et al. 2008
V3-ABD-R	CTGTCACAAGCCAGTGGAGATA		
WAC-F	TTTGAAGAGTCGGTGAAGGG	56	Winfield et al. 2009
WAC-R	TTTCATACAGCAGGCAAGCA		

Spike differentiation

For each sampling time, the differentiation of spikes of each sample was observed. Shoot apices were isolated under a dissecting microscope and digitally photographed using a Leica DMRB microscope digital camera.

Results

Spike differentiation

Spike differentiation was observed at each sampling time for the two varieties under different treatments (Fig. 1A–D). In only five weeks, XC2 spikes went from the single ridge stage to finishing differentiation under long-day and non-vernalization conditions (Fig. 1A). XC2 spikes were in the early elongation stage when seedlings were three weeks old (0 week, Fig. 1A, a); one week after the first sampling, the spikes were in the double ridge stage (Fig. 1A, b). A distinct spike with stigmas on enlarging spikelets and early glumes was evident on the fifth week (Fig. 1A, f). Under vernalization treatment, the spikes were in the elongation stage and did not differentiate during the five-week vernalization treatment (Fig. 1B, b–f). When plants were transferred to room temperature (the phytotron), the spike primordia differentiated quickly and reached the same stage as that shown in Fig. 1A–f after four weeks, indicating that the spring wheat variety does not require vernalization treatment for flowering.

The winter variety, J841 did not flower until the end of the experiment under non-vernalization treatment, and the spikes were maintained at the single ridge stage (Fig. 1C, a–l). During five weeks of cold treatment, the spikes did not differentiate and remained in the single ridge stage (Fig. 1D, b–f). However, when plants were transferred into the phytotron, the spikelets differentiated quickly, producing stigmas and glumes after five weeks (Fig. 1D, g–k). Therefore, the winter wheat variety is more sensitive to cold temperatures than the spring wheat variety.
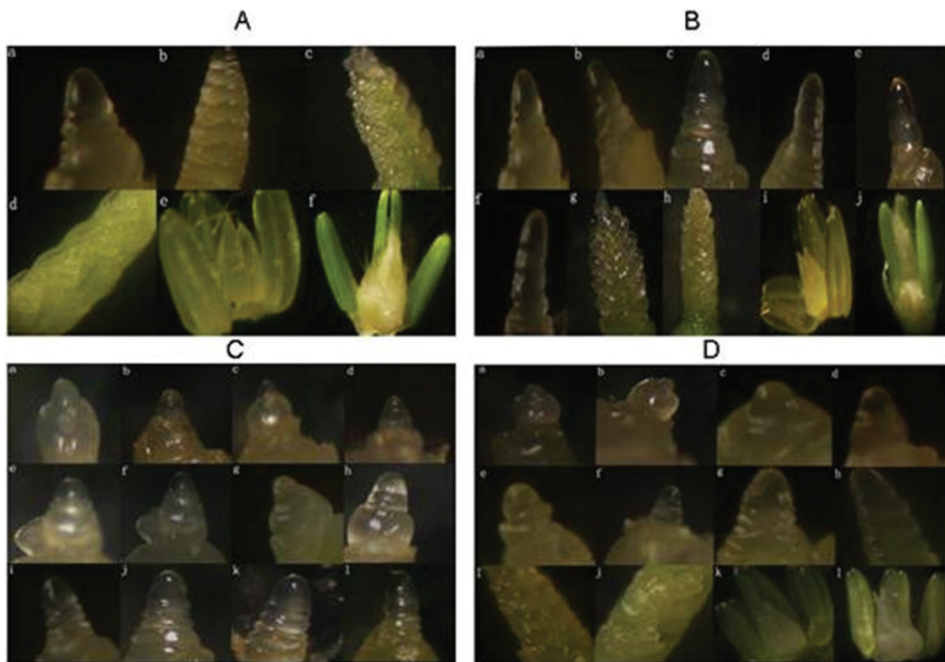
Figure 1. Spike differentiation process in spring wheat cultivar XC2 and winter wheat cultivar J841. A and C indicate spike differentiation processes for XC2 and J841 under non-vernalization treatment; B and D indicate spike differentiation processes for XC2 and J841 under vernalization treatment. Note: a, state of the shoot apices of 3-week-old seedlings (0 week); b–f, every sampling time from one week (1 w) to five weeks (5 w) during vernalization treatment; g–l, sampling time for each week (6–10 w) after vernalization treatment

VRN gene expression patterns in leaves

We analyzed the expression patterns of three *VRN* genes in the two lines (XC2, J841) under long-day conditions after vernalization and non-vernalization treatments (Fig. 2). *VRN1* transcript levels were higher in XC2 than that in J841 over the course of the experiment under both vernalization and non-vernalization treatments. Under non-vernalization treatment, *VRN1* expression levels in XC2 increased gradually and reached the highest levels at 5 w. *VRN1* expression levels in J841 were negligible relative to those in XC2 (Fig. 2B). During the vernalization process, *VRN1* expression levels increased gradually in XC2 (Fig. 2A). Interestingly, *VRN1* expression in both varieties reached their highest levels during the last period of vernalization, while it decreased when the plants were transferred to the phytotron (16 h light/8 h dark photoperiod and 22 °C day/20 °C night temperatures). *VRN1* expression increased gradually during plant development but did not reach its highest levels until flowering.

Under each treatment, *VRN2* expression in both varieties decreased over the course of the experiment. The expression of *VRN2* in XC2 was negligible as compared to that in
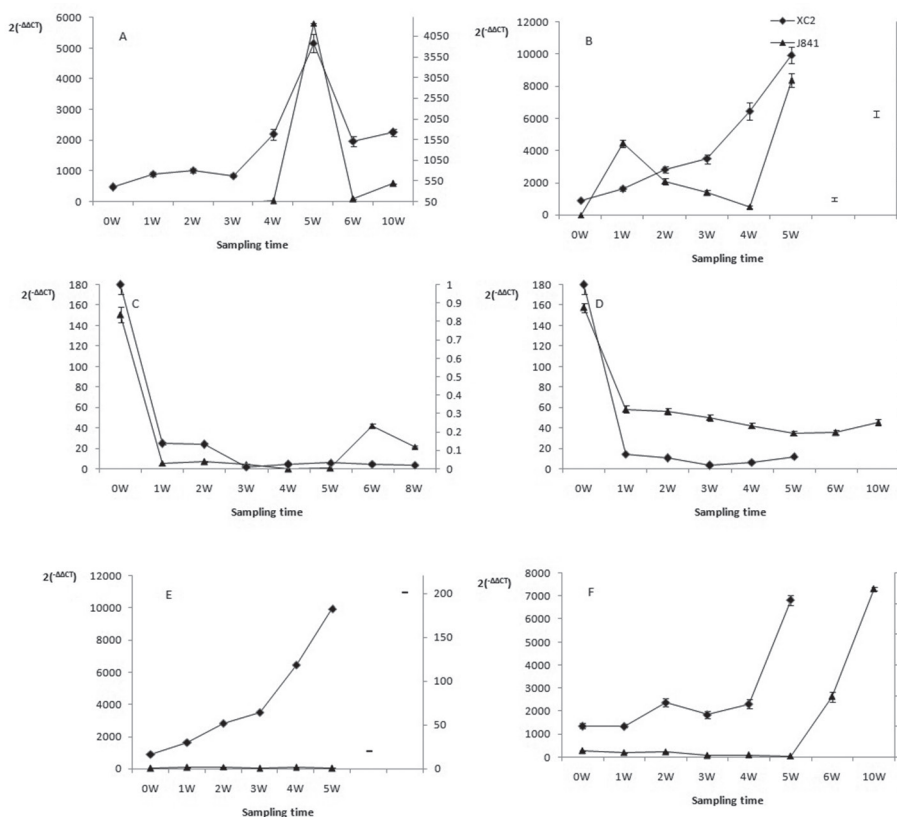
Figure 2. Relative expression levels of *VRN1* (A, B), *VRN2* (C, D) and *VRN3* (E, F) detected by qRT-PCR in spring wheat cultivar XC2 and winter wheat cultivar J841at 5 weeks of vernalization treatment (A, C, E) or non-vernalization (B, D, F) treatment under long-day conditions. Sample times were set as 0 (three-week-old seedlings, immediately before treatment), 1, 2, 3, 4 and 5 weeks (during treatment) and 6 and 8 weeks (post-treatment), except for unvernalized XC2, which flowered at 5 w under these conditions. The data represent the means of three replicates and the error bars indicate the SD

J841 under both vernalization and non-vernalization conditions (Fig. 2C, D). The transcription of *VRN2* in J841 was maintained at high levels during the experiment, although transcription was reduced sharply during the earlier stage of plant development under non-vernalization treatment (Fig. 2D). Cold temperatures powerfully repressed *VRN2* expression in J841. Notably, there was a transitory increase in *VRN2* expression when the vernalized plants were moved to the phytotron (Fig. 2C).

In general, *VRN3* expression was higher in XC2 than in J841 (Fig. 2E, F). Under non-vernalization treatment, *VRN3* expression was quite low in J841; however, in XC2, *VRN3* expression increased gradually (Fig. 2F). Under vernalization treatment, *VRN3* expression was low for both genotypes (Fig. 2E). Nevertheless, when the plants were moved to the phytotron, *VRN3* transcription increased rapidly.

Comprehensive analysis of *VRN* gene expression patterns revealed that *VRN3* transcript levels lagged behind those of *VRN1* in XC2 and J841 during vernalization (Fig. 2A and E), while the transcription of *VRN2* exhibited an opposite trend to that of *VRN1* and *VRN3,* with lower expression in response to vernalization (Fig. 2C). *VRN2* expression was low in XC2, even without vernalization treatment (Fig. 2F), which might have resulted from the higher transcript levels of *VRN1*. Under non-vernalization treatment, *VRN1* and *VRN3* exhibited low levels of expression in J841 during plant development, whereas *VRN2* expression was maintained at a certain level, although it decreased during the early stage of growth (Fig. 2B, D, F).

VRN1 and VRN3 expression patterns in apical meristems

We analyzed the expression patterns of *VRN1* and *VRN3* in apical meristems of XC2 and J841 during both vernalization and non-vernalization treatments using qRT-PCR (Fig. 3A–D). As the flowering time differed between genotypes, the sampling numbers also differed.

For unvernalized XC2 plants, the expression of *VRN1* in apical meristems increased gradually during the experiment, reaching the highest level at 5 w (Fig. 3, A1–5 w); at this stage, stigmas appeared in the spikelets. For vernalized XC2, the expression of *VRN1* in the apical meristem was lower at 0–5 w, while it increased sharply when the vernalized plants were returned to the phytotron one week later (Fig. 3A, 6–8 w). At this stage, the apical meristems exhibited enlargement of the terminal spikelets. For vernalized winter wheat J841, the expression of *VRN1* in the apical meristems gradually increased, reaching
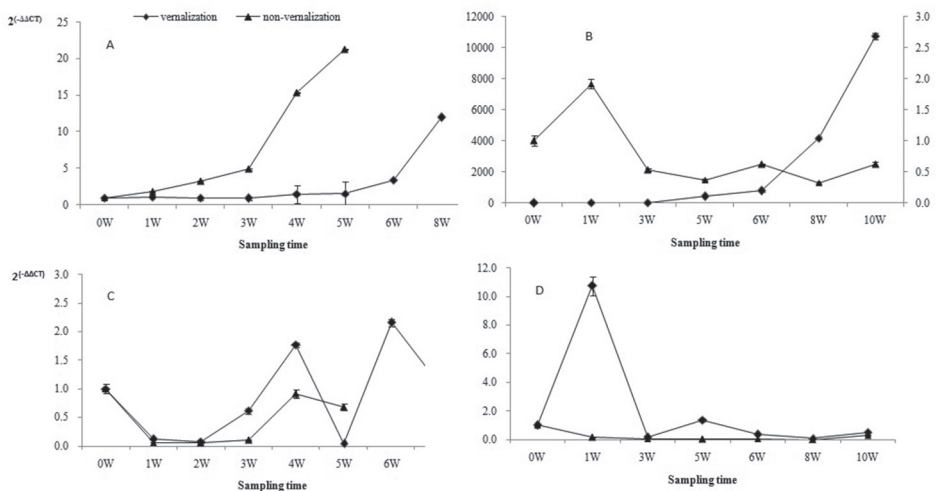

Figure 3. Relative expression levels of *VRN1* (A, B) and *VRN3* (C, D) in shoot apical meristems assayed by qRT-PCR in spring wheat cultivar XC2 (A, C) and winter wheat cultivar J841 (B, D) after vernalization and no vernalization treatment. The data represent the means of three replicates and the error bars indicate the SD

its highest level at 10 w. For unvernalized winter wheat J841, the expression of *VRN1* in the apical meristems was quite low, approaching zero (Fig. 3B).

Compared with the expression of *VRN1* in the apical meristems, the expression of *VRN3* in the apical meristems was quite low in XC2 under both vernalization and non-vernalization treatment (Fig. 3C), and it was also quite low in non-vernalized J841 plants (Fig. 3D). The expression of *VRN3* in apical meristems in vernalized J841 plants increased sharply during week 1 but decreased quickly (to nearly zero) during week 3–10 (Fig. 3D, 1 w–10 w), indicating that the transcription of *VRN3* in apical meristems was quite low in both spring and winter wheat.

Discussion

Vernalization is one of the most critical phases of wheat development. This highly complex physiological and biochemical process is controlled by multiple *VRN* genes. Typically, spring wheat does not require a cold period to heading while in winter varieties it is a crucial requirement for flowering (Casao et al. 2011). This may be associated with the composition of *VRN1* on the A, B, D genome. The *VRN1* composition of XC2 is *Vrn-A1vrn-B1vrn-D1*, and the presence of the *Vrn-A1* allele is sufficient for the plant to exhibit a spring habit (Yuan et al. 2008). Preston and Kellogg (2008) suggested that *VRN1* genes play distinct roles in flowering competence, and the upregulation of *VRN1* in cereal leaves may make plants competent to flower during vernalization treatment. According to this viewpoint, the induction of *VRN1* in leaves by cold treatment leads to flowering competence. When plants are moved to warm temperatures, the *VRN1* transcription center is transferred to the shoot apical meristem and induces inflorescence production, resulting in a drop in *VRN1* levels in leaves post-vernalization. However, the expression of *VRN1* rebounds quickly and is maintained at a certain level in leaves, which may prevent *VRN2* upregulated under warm temperature and long-day conditions (Chen and Dubcovsky 2012). This model is consistent with the current result. When the expression of *VRN1* dropped to low levels immediately post-vernalization, the expression of *VRN2* increased quickly, reaching a high level, followed by a decrease as the transcription of *VRN1* in leaves again increased.

To investigate whether *VRN1* plays different roles in shoot apices versus leaves, we examined *VRN1* transcript levels in leaves and apical meristems. *VRN1* transcript levels were higher in leaves than in shoot apices when the shoot meristems were maintained in the vegetative state under vernalization treatment. The transcription of *VRN1* in shoot apical meristems was activated only when the plants were transferred to warm temperatures after the expression of *VRN1* reached peak levels in leaves, and the spikelets were undergoing a rapid transitional developmental stage. These expression profiles are similar to those reported in oat (*Avena sativa*) and wheat (*Triticum monococcum*) (Preston and Kellogg 2008). In this study, when XC2 vernalized plants were transferred to the phytotron, *VRN1* transcript levels in shoot apices at 6 w (equivalently to 1 w for spike differentiation) and 8 w were higher than that in unvernalized plants at 1 w and 3 w, this

may explain why spike differentiation in XC2 from the three-leaf stage to heading under a five-week cold treatment completed one week earlier compared to unvernalized XC2.

The expression of *VRN3* in leaves was lower than that of *VRN1* (Fig. 2A, E), suggesting that *VRN3* is regulated by *VRN1* downstream in leaves, which is in accordance with the view of Shimada (Shimada et al. 2009). The upregulation of *VRN3* in both varieties of leaves appeared to be synchronized with inflorescence initiation in the shoot apical meristem, indicating that the main function of *VRN3* is to produce a florigen that takes part in floral primordium differentiation and development.

By examining the expression patterns of *VRN* genes during the development of common wheat, we deduced the relationship between three *VRN* genes. In the autumn, the transcription of *VRN2* prevents flower initiation in winter wheat. In the winter, *VRN1* is induced in leaves by cold temperatures, and the gradual increase of *VRN1* transcripts level inhibits *VRN2* expression. During the winter, *VRN1* is not activated in the shoot apical meristems, so the meristems are still present at vegetative stage zero (Gardner 1985). In the spring, the transcriptional activation of *VRN1* in leaves helps maintain low levels of *VRN2*. Thus, a large amount of FT protein is produced under long-day conditions and warm temperatures in leaves (Corbesier et al. 2007; Distelfeld et al. 2009a). FT protein is then transported to the apices where it interacts with FDL2 protein, inducing the transcription of *VRN1* (Li and Dubcovsky 2008). The high *VRN1* transcript levels in shoot meristems promote the transition from vegetative growth to reproductive development.

Acknowledgements

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