

Vernalization requirement duration in winter wheat is controlled by *TaVRN-A1* at the protein level

Genqiao Li[†], Ming Yu[†], Tilin Fang[†], Shuanghe Cao, Brett F. Carver and Liuling Yan*

Department of Plant and Soil Sciences, Oklahoma State University, Stillwater, OK 74078, USA

Received 21 July 2013; revised 16 August 2013; accepted 6 September 2013; published online 14 October 2013.

*For correspondence (e-mail liuling.yan@okstate.edu).

[†]These authors contributed equally to this work.

SUMMARY

Winter wheat requires a period of low temperatures to accelerate flowering (vernalization). This requirement could make winter wheat more vulnerable to elevated global temperature via insufficient vernalization. All known vernalization genes are cloned according to qualitative variation in vernalization requirement between spring and winter wheat, but the genes controlling quantitative variation for more or less vernalization requirement among winter wheat cultivars remain unknown. We report here that the gene for the vernalization requirement duration in winter wheat was cloned using a BC₁F_{2:3} population that segregated in a 3:1 ratio of early-flowering plants and late-flowering plants after vernalization for 3 weeks. The positional cloning of the gene for vernalization requirement duration demonstrated that this trait is controlled by *TaVRN-A1* at the protein level. The Ala¹⁸⁰ in *vrn-A1a*, encoded by the dominant allele for 3-week vernalization, was mutated to Val¹⁸⁰ in *vrn-A1b*, encoded by the recessive allele for 6-week vernalization. Further studies with *in vitro* protein pull-down assays and immunoprecipitation analyses indicated that the mutated Val¹⁸⁰ in *vrn-A1b* protein decreased the ability to bind with *TaHOX1* (the first homeobox protein in *Triticum aestivum*). The direct binding of *TaVRN-A1* and *TaHOX1* proteins was confirmed in the nucleus of living plant cells by bimolecular fluorescence complementation (BiFC) analyses. The *TaHOX1* gene was found to be upregulated by low temperatures, and to have a significant genetic effect on heading date, suggesting that *TaHOX1* functions in the flowering pathway in winter wheat.

Keywords: vernalization requirement duration, VRN-1, homeobox protein, winter wheat, protein interaction, *Triticum aestivum*.

INTRODUCTION

Triticum aestivum (wheat, $2n = 6x = 42$, AABBDD) is cultivated across more land area than any other grain crop. Qualitatively, wheat cultivars are classified as two general types: winter wheat, with a variable low-temperature requirement for a proper flowering time (vernalization) and thus successful grain reproduction, and spring wheat, without this requirement (Chouard, 1960; Pugsley, 1971; Amasino, 2004). Quantitatively, winter wheat cultivars are classified as three types according to the low temperature duration required to reach the vernalization saturation point or to achieve the maximum vernalization effect: a weak winter type that is stimulated to flower by brief exposure to low temperature (for <2 weeks); a semi-winter type that requires 2–4 weeks of cold exposure for flowering; and a strong winter type that requires more than 4 weeks of cold exposure for timely flowering (Crofts, 1989).

Vernalization usually occurs at temperatures of <8°C (Chouard, 1960; Pugsley, 1971; Crofts, 1989; Amasino,

2004). Recent studies have shown that the average global surface air temperature rose by 0.5°C in the 20th century, and is projected to continue its increase by roughly 3°C or 5°C by the end of the 21st century (Kerr, 2007; Semenov and Halford, 2009). As various simulation models have shown, winter wheat is more vulnerable to increasing temperatures during winter seasons because of its low temperature requirement to ensure proper flowering time and successful seed reproduction (Miglietta *et al.*, 1995; Humphreys *et al.*, 2006). A shortened duration at low temperature, caused by global warming, could result in a failed or insufficient vernalization in winter wheat. The cloning of genes for quantitative vernalization requirement durations in winter wheat cultivars would provide new knowledge and molecular tools to breed novel cultivars to adapt to changing climates.

Three vernalization genes have been cloned from wheat by using a positional cloning approach, *VRN1* (Yan *et al.*,

2003), *VRN2* (Yan *et al.*, 2004a) and *VRN3* (Yan *et al.*, 2006), and each of them was cloned in the context of growth habit as a discrete trait of winter and spring types in diploid wheat and *Hordeum vulgare* (barley). *VRN1* (=AP1) is a central promoter for spring cultivars to flower without vernalization (Danyluk *et al.*, 2003; Murai *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003), and dominant *Vrn-A1* alleles originated from mutations in the promoter or the first intron of the wild recessive *vrn-A1* in hexaploid wheat (Yan *et al.*, 2004b; Fu *et al.*, 2005). A recent study reported that the increased copy number of *TaVRN-A1* resulted in an increased requirement for vernalization in winter wheat (Díaz *et al.*, 2012). *VRN2* (=ZCCT1) is a flowering repressor, and a recessive *vrn2* allele was caused by a point mutation at the conserved CCT domain or complete deletion of the wild dominant *Vrn2* in diploid wheat (Yan *et al.*, 2004a). *VRN3* (=FT1) is another flowering promoter, and the early-flowering plants carry *Vrn3* alleles originating from mutations in the promoter or the first intron in wheat and barley (Yan *et al.*, 2006). The genetic and molecular mechanisms to account for the qualitative difference in the three genes between the two divergent types of wheat are not expected to explain the quantitative variation in vernalization requirement duration among winter wheat cultivars, because all winter wheat cultivars are supposed to have the same winter allele for each of the three cloned vernalization genes: recessive *vrn1*, recessive *vrn3* and dominant *Vrn2* alleles (Pugsley, 1971; Tranquilli and Dubcovsky, 2000). No exception was reported to invalidate this genetic model, when numerous cultivars/germplasm from different wheat species varying in ploidy level were screened using molecular markers for each of the three genes (Yan *et al.*, 2003, 2004a,b; Fu *et al.*, 2005; Bonnin *et al.*, 2008; Zhang *et al.*, 2008; Santra *et al.*, 2009; Chen *et al.*, 2010).

The molecular mechanism underlying quantitative vernalization requirement in a winter type of Arabidopsis is not yet known, but could be explained by three models. The first model is that vernalization results in a quantitative reduction in *FLC* mRNA levels, which negatively correlates with flowering time (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). The second model is that the transcript level of *FLC* is upregulated or downregulated by multiple genes, including *VIN3* and *FRI* in the vernalization pathway, as well as *GI*, *CO* and *FT* in the photoperiod pathway (Reeves and Coupland, 2001; Levy *et al.*, 2002; Corbesier *et al.*, 2007; Heo and Sung, 2011). The signals from these pathways are integrated at *FLC* to induce *AP1* for flowering. The last model is that quantitative vernalization requirement is modulated by the accumulation of the Polycomb-based epigenetic silencing complexes and histone modifications at the *FLC* gene (Angel *et al.*, 2011), and the quantitative modulation of Polycomb silencing is associated with natural variation in the sequence of *FLC* (Coustham *et al.*, 2012). Previous studies have demonstrated that vernalization has

evolved different mechanisms between winter wheat and Arabidopsis (Yan *et al.*, 2003). The gene(s) responsible for various vernalization requirement durations in winter wheat should be cloned from a population generated using two winter wheat cultivars with different vernalization saturation points.

In previous studies, we developed a population of recombinant inbred lines (RILs), generated from a cross between two winter wheat cultivars: 'Jagger' and '2174'. When different sets of the same population were tested in field, the RIL population was segregated in stem elongation, heading date and physiological maturity (Chen *et al.*, 2009). In this previous study, a polymerase chain reaction (PCR) marker developed for allelic variation in exon 4 of *TaVRN-A1* was found to have genetic association with a major quantitative trait locus (QTL) for the phenotypes, but it was not known whether *TaVRN-A1* caused the QTL in winter wheat. If *TaVRN-A1* was indeed responsible for this QTL, it could have different mechanisms in regulating the developmental process in winter wheat (as opposed to spring wheat). Otherwise, another gene at the *TaVRN-A1* locus should be responsible for the QTL regulating developmental process in winter wheat. In the present study, we tested this RIL population under thermal- and photo-controlled glasshouse conditions, and found a major QTL for vernalization requirement duration. We then generated a large backcross population and cloned the first gene for vernalization requirement duration in winter wheat.

RESULTS

The discovery of a major gene for vernalization requirement duration

We tested the two parental lines, Jagger and 2174, for variation in vernalization requirement duration to reach the vernalization saturation point or gain the maximum vernalization effect on heading under long-day conditions (16-h day/8-h night). Jagger required 3 weeks at 4°C to reach the maximum vernalization effect on heading, whereas 2174 required 6 weeks under the same conditions (Figure 1a). When vernalized for 3 weeks, Jagger flowered 101 days after planting but 2174 flowered 136 days after planting. When vernalized for 6 weeks, Jagger flowered 106 days after planting but 2174 flowered 115 days after planting. A major QTL for vernalization requirement duration that segregated in the mapping population of Jagger × 2174 RILs was mapped to the long arm of chromosome 5A in a genomic region encompassing the *vrn-A1* locus (Figure 1b), and this QTL was designated *QVrd.osu-5A*. The *QVrd.osu-5A* locus explained 63.4% (logarithm of the odds, LOD = 18.8) of the total phenotypic variation in the population vernalized for 3 weeks, 43.2% (LOD = 11.3) in the population vernalized for 6 weeks and 20.9% (LOD = 4.3) in the control population with no vernalization.

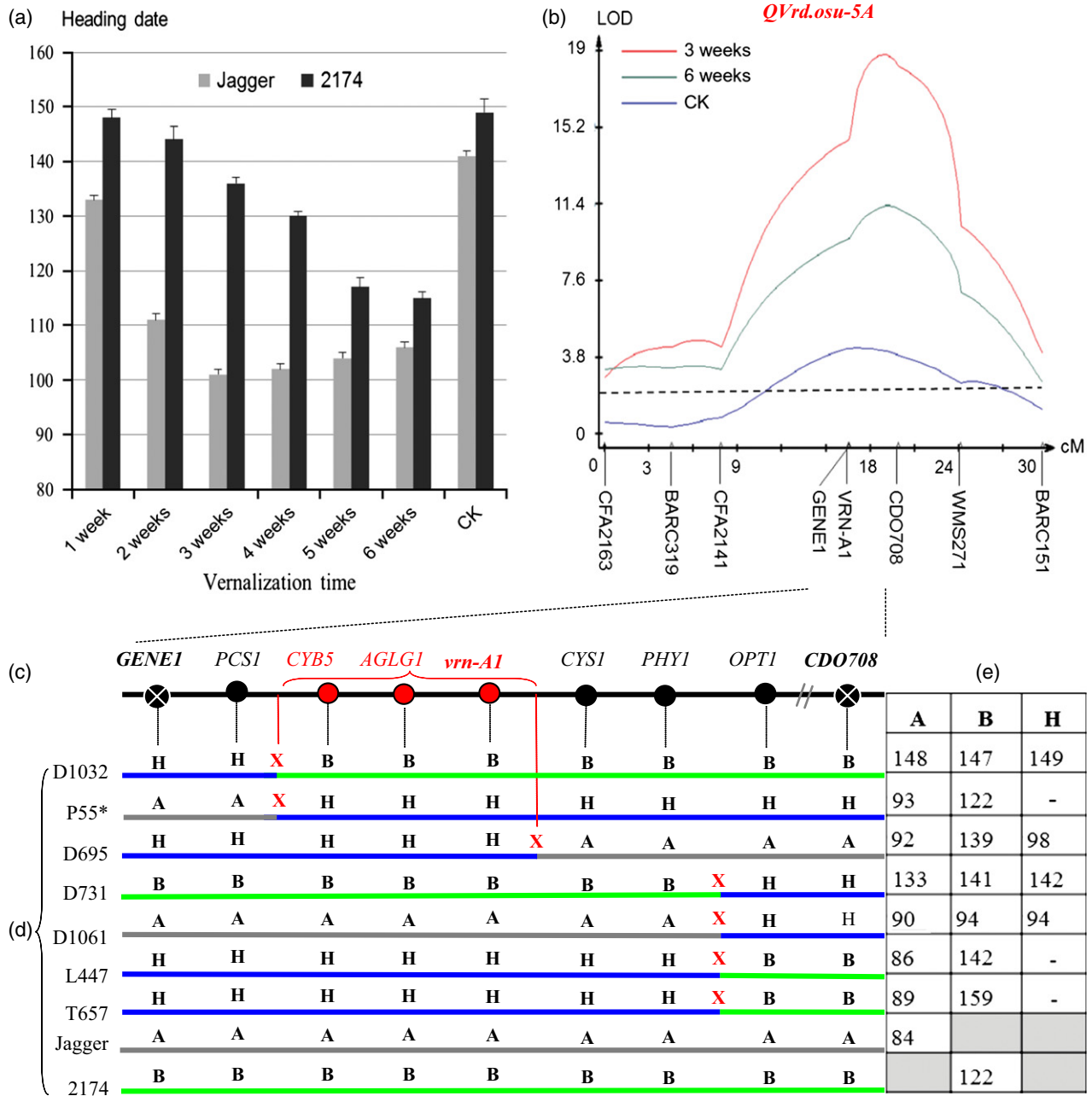


Figure 1. Genetic and physical maps of the gene for *QVrd.osu-5A*.

(a) Differential effects of vernalization requirement duration on heading date between ‘Jagger’ and ‘2174’. The plants were vernalized for a varying number of weeks (1–6 weeks), and non-vernalized plants were used as the control (CK). Heading date was the date of emergence of the first spike of each plant. The values represent mean heading date ($n = 8$), and error bars indicate standard errors.

(b) Mapping of the *QVrd.osu-5A* locus. Two sets of the Jagger \times 2174 RIL populations were vernalized for 3 and 6 weeks, and one set of the same population was not vernalized as a control (CK). The horizontal dotted line represents a threshold value of 2.5 LOD, logarithm of the odds.

(c) The physical location of the *QVrd.osu-5A* locus.

(d) The critical crossovers in the *QVrd.osu-5A* locus. ‘A’ represents the Jagger allele, ‘B’ represents the 2174 allele and ‘H’ represents heterozygosity at the given marker locus; ‘X’ indicates the recombinant site.

(e) Days to heading of each critical recombinant plant. The detailed statistical analyses on the phenotype of the recombinant plants are provided in Table S1.

A recombinant inbred line, RIL23, carrying the Jagger *vrn-A1* allele, was backcrossed with the parental line 2174 to generate a BC₁F₂ population, in which the *QVrd.osu-5A* locus was heterozygous but each of *PPD-D1* and *VRN-D3*

were fixed at the homozygous allele. The latter two genes showed significant effects on heading date in the Jagger \times 2174 RIL population when tested in the field (Chen et al., 2009). The resulting BC₁F₂ population was used to

test genetic effects of the *QVrd.osu-5A* locus on vernalization requirement in winter wheat.

Ninety plants of the RIL23 × 2174 BC₁F₂ population were tested for heading date when the population was vernalized for 3 weeks. On average, 24 plants homozygous for the Jagger *vrn-A1* allele headed at 110 days after planting, 20 plants homozygous for the 2174 *vrn-A1* allele headed at 138 days and 46 plants heterozygous at *vrn-A1* headed at 118 days. In order to avoid confusion of gene/allele in the terminology (*Vrn-A1a*, *Vrn-A1b*, *Vrn-A1c*, *Vrn-A1d* and *Vrn-A1e* were designed for the dominant or spring alleles; Yan *et al.*, 2004b), the Jagger *vrn-A1* allele is hereafter designated as *vrn-A1a* and the 2174 *vrn-A1* allele is designated as *vrn-A1b*, and *TaVRN-A1* rather than *Tavrn-A1* is used for both *vrn-A1a* and *vrn-A1b*.

The 70 plants either homozygous or heterozygous for the Jagger *vrn-A1a* allele for early heading showed a significant difference from the 20 plants homozygous for the 2174 *vrn-A1b* allele for late heading ($P < 0.001$). The observed segregation ratio between the earlier-heading and later-heading groups was not significantly different from a 3:1 ratio ($\chi^2 = 0.37$, d.f. = 1, $P = 0.54$), and fits with a one-gene model. We genotyped a total of 6410 BC₁F₃ plants derived from BC₁F₂ plants heterozygous at *vrn-A1* using PCR markers for *GENE1* and *CDO708* that flanked the gene responsible for *QVrd.osu-5A* (Figure 1c), and the progeny of the resulting seven critical recombinant plants that exhibited a crossover between *PCS1* and *OPT1* (Figure 1d) were tested for heading date after vernalization for 3 weeks. The results clearly showed that each of the progeny population was segregated in a 3:1 ratio of plants that headed early and plants that headed later, indicating the dominance of the *vrn-A1a* allele for early heading over the *vrn-A1b* allele for late heading (Figure 1e; see Table S1).

The positional cloning of *QVrd.osu-5A*

We determined the locations of crossovers using PCR markers for nine genes at the *QVrd.osu-5A* locus (see Figures S1–S9). Subsequently, the gene responsible for *QVrd.osu-5A* was positioned within a genomic region flanked by *PCS1* and *CYS1*, and included three candidate genes: *CYB5*, *AGLG1* and *vrn-A1*. Gene expression results indicated that the vernalization requirement duration was not associated with any of the candidate genes at the transcriptional level (see Figure S10), suggesting that this trait was controlled by a candidate gene at the protein level.

No difference was observed in the *CYB5* protein encoded by the two alleles, eliminating the possibility that *CYB5* is responsible for *QVrd.osu-5A*. One amino acid substitution occurred in *AGLG1* but the substituted amino acids are similar, serine in Jagger and alanine in 2174, and *AGLG1* may be involved in spike differentiation but not in flowering time (Yan *et al.*, 2003). In contrast, two amino acid substitutions occurred in *vrn-A1*, and the substituted

amino acids are very different. In *vrn-A1*, one single nucleotide polymorphism (SNP) occurred in exon 4, producing a leucine (Leu) at position 117 in *vrn-A1a*, encoded by the Jagger allele, but a phenylalanine (Phe) at the same position in *vrn-A1b*, encoded by the 2174 allele (Figure 2a). The second SNP occurred in exon 7, producing an alanine (Ala) at position 180 in *vrn-A1a*, but a valine (Val) at the same position in *vrn-A1b* (Figure 2a). The *vrn-A1* is a MADS-box protein (Mandel *et al.*, 1992), and the Leu¹¹⁷/Phe¹¹⁷ substitution occurred within the conserved K-box at positions 89–174, whereas the Ala¹⁸⁰/Val¹⁸⁰ substitution occurred in the divergent C terminal. In addition, *VRN1* functions in the vernalization pathway in wheat (Danyluk *et al.*, 2003; Murai *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003); therefore, we further tested and validated the hypothesis that allelic variation in the *vrn-A1* protein is responsible for *QVrd.osu-5A*.

A differential interaction of *vrn-A1a* and *vrn-A1b* with *TaHOX1*

To determine whether the protein properties of *vrn-A1a* or *vrn-A1b* have been altered by the presence of the two mutations, we first expressed *vrn-A1a* and *vrn-A1b* proteins of full length using the pSUMO vector. However, after the pSUMO portion containing 6xHIS-Tag was removed, *vrn-A1b* was almost insoluble, whereas *vrn-A1a* was soluble. The decrease in *vrn-A1b* protein solubility could result in altered function. To investigate if *vrn-A1a* or *vrn-A1b* has different interactions with any protein partners, we used *vrn-A1a* as bait to screen a yeast-two hybrid (Y2H) library generated from Jagger.

Positive clones identified from the Y2H library included *TaSOC1* (*T. aestivum* wheat suppressor of overexpression of constans 1) and *TaVRT2* (*T. aestivum* wheat vegetative to reproductive transition 2), both of which have physical interactions with other MADS proteins because of the presence of a K-box in these proteins (Mandel *et al.*, 1992; Yang *et al.*, 2004; Kane *et al.*, 2005). Strong interactions between *TaVRN-A1* and *TaSOC1* and between *TaVRN-A1* and *TaVRT2* were detected in our pull-down assays and immunoprecipitation analyses (Figure 3), but no significant difference was found in the interaction of *vrn-A1a* and *vrn-A1b* with *TaSOC1* or *TaVRT2*, suggesting that the interaction of *TaVRN-A1* with *TaSOC1* or *TaVRT2* was not directly involved in controlling vernalization requirement duration in winter wheat.

We discovered, however, that a protein *TaHOX1* not only interacted with *TaVRN-A1* but also interacted differently with *vrn-A1a* and *vrn-A1b*. Three independent cDNA clones identified from the Y2H library were predicted to encode 127 amino acids [100–226 aa (end)] of the *TaHOX1* protein (Figure 2g). In pull-down assays, both *vrn-A1a* and *vrn-A1b* proteins interacted with *TaHOX1*, but the *vrn-A1b* protein showed a visible decrease in its interaction ability with

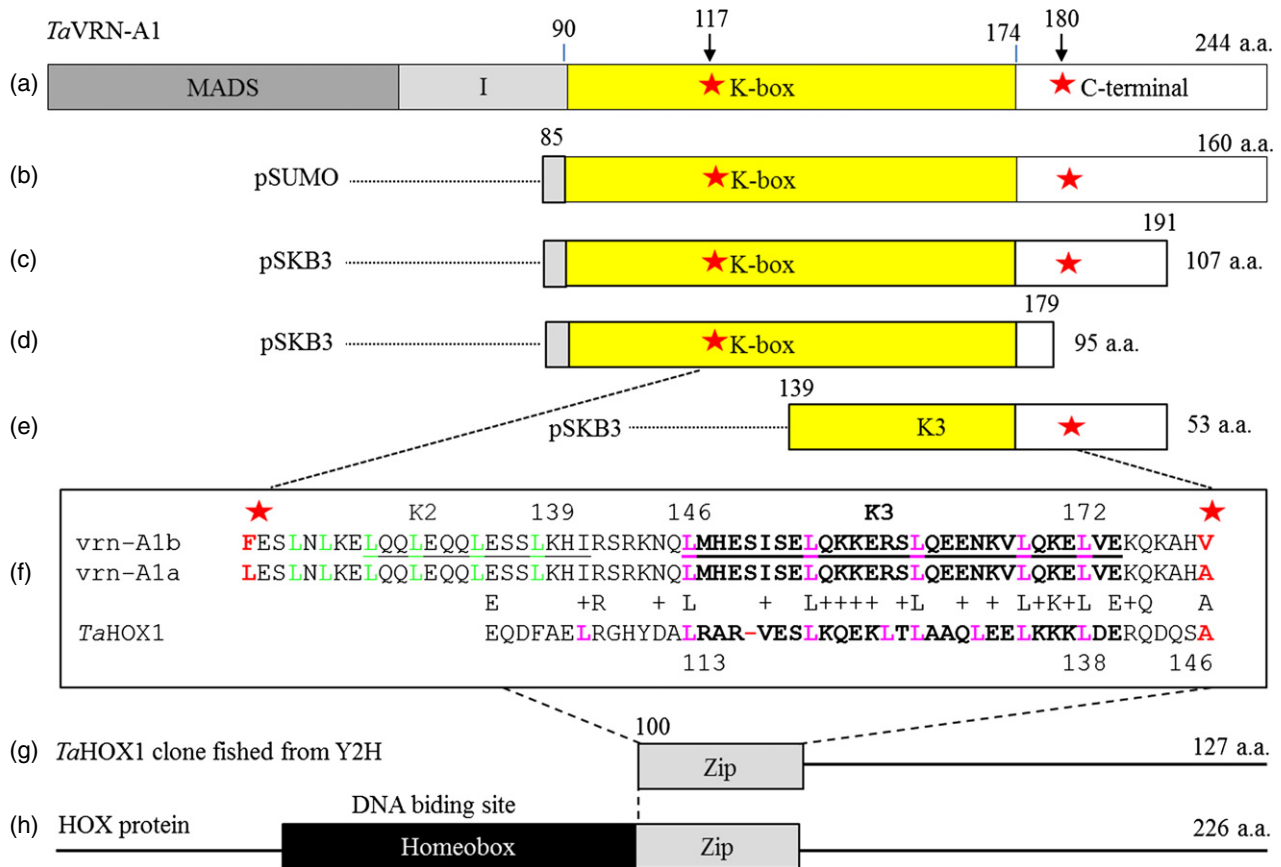


Figure 2. Interacting site of *TaVRN-A1* and *TaHOX1* proteins.

(a) The locations of the conserved domains in *TaVRN-A1* and two altered sites in the amino acid sequence are indicated with red stars.

(b–e) Four *TaVRN-A1* fragments covering either one or two mutations were expressed to test differential interactions with *TaHOX1*.

(f) Sequence comparison between the *TaVRN-A1* and *TaHOX1* proteins. Leu¹¹⁷/Phe¹¹⁷ or Ala¹⁸⁰/Val¹⁸⁰ residues are highlighted in red, and the conserved leucine residues are highlighted in pink.

(g) The cDNA of *TaHOX1* fished out from the Y2H library was expressed to test protein interactions with *TaVRN-A1*.

(h) Positions of conserved HD and Zip domains in *TaHOX1* proteins in plants.

TaHOX1 (Figure 4a). To determine whether the Leu¹¹⁷/Phe¹¹⁷ or Ala¹⁸⁰/Val¹⁸⁰ substitution in the *TaVRN-A1* proteins caused the differential interaction with *TaHOX1*, three additional protein fragments for each of *vrn-A1a* and *vrn-A1b* were expressed. These fragments included: (i) the Leu¹¹⁷/Phe¹¹⁷ and Ala¹⁸⁰/Val¹⁸⁰ substitutions together (Figure 2b, c) (ii) the Leu¹¹⁷/Phe¹¹⁷ substitution (Figure 2d) alone; and (iii) the Ala¹⁸⁰/Val¹⁸⁰ substitution alone (Figure 2e). Comparative *in vitro* interaction studies indicated that *vrn-A1a* and *vrn-A1b* protein fragments with both substitutions differed in their ability to interact with *TaHOX1* (Figure 4b), as did the *vrn-A1a* and *vrn-A1b* protein fragments including only the Ala¹⁸⁰/Val¹⁸⁰ substitution (Figure 4c). However, the *vrn-A1a* and *vrn-A1b* protein fragments including only the Leu¹¹⁷/Phe¹¹⁷ substitution interacted similarly with *TaHOX1* (Figure 4d). The decreased interactive ability of *vrn-A1b* with *TaHOX1* in pull-down assays was confirmed by protein immunoprecipitation analyses (Figure 4e,f). We concluded

the Ala¹⁸⁰/Val¹⁸⁰ substitution accounted for the differential interactions of *vrn-A1a* and *vrn-A1b* with *TaHOX1*.

The *TaHOX1* protein found in this study has the same structure of the homeodomain (HD) and the leucine zipper domain (Zip) (Figure 2h) as HOX proteins reported in animals (Ariel *et al.*, 2007), and *TaHOX1* and *TaVRN-A1* shared five leucine residues present in the Zip domain (Figure 2f). It is likely that the physical interaction between *TaHOX1* and *TaVRN-A1* is attributable to the presence of the zipper in the two proteins; however, the Ala¹⁸⁰ in *vrn-A1b* represented the same amino acid residue as *TaHOX1*, but Val¹⁸⁰ caused a mismatch between *vrn-A1b* and *TaHOX1* (Figure 2f). This mismatch explained the decreased ability of *vrn-A1b* to interact with *TaHOX1*, indicating the Ala¹⁸⁰/Val¹⁸⁰ substitution outside the K-box in *TaVRN-A1* was involved in its physical interaction with *TaHOX1*.

We confirmed that *TaVRN-A1* and *TaHOX1* proteins exhibited direct binding in living cells by using a transient expression system in tobacco leaves (Lu *et al.*, 2010). The

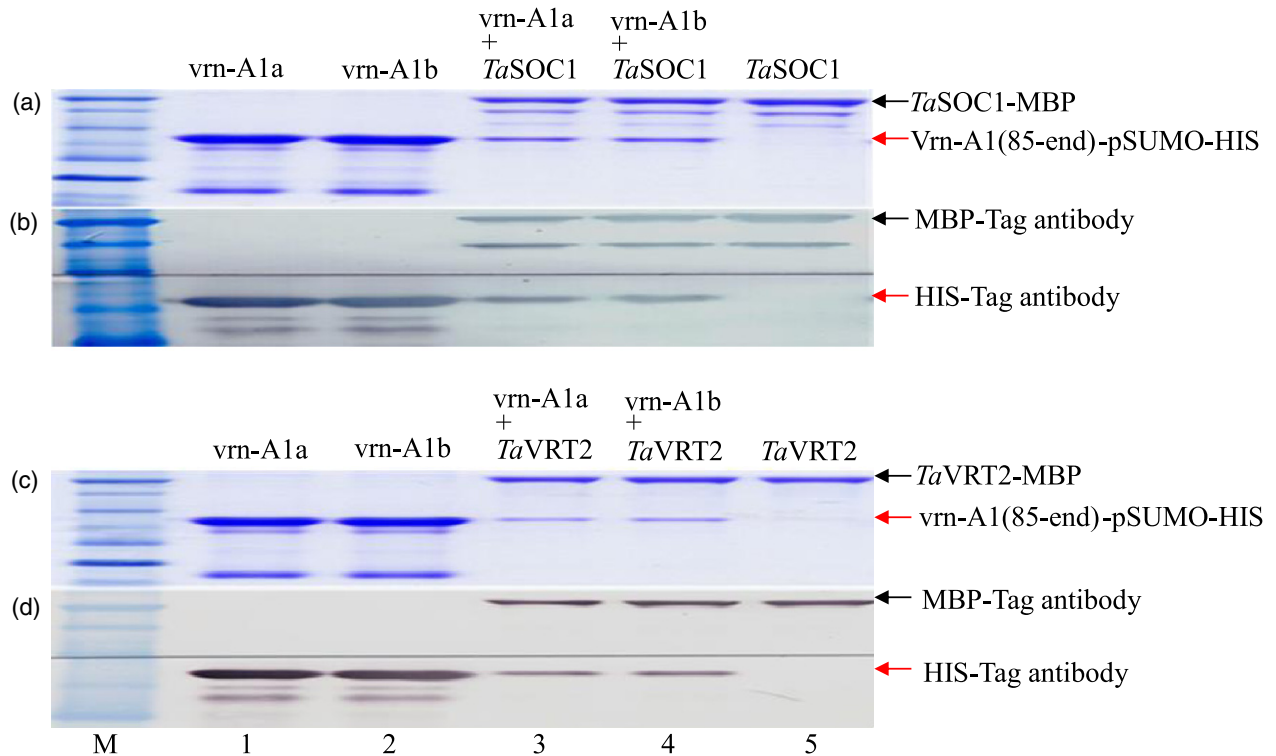


Figure 3. *In vitro* interactions of *TaVRN-A1* with *TaSOC1* and *TaVRT2* proteins.

(a, c) Pull-down assays of *TaVRN-A1* with *TaSOC1* and *TaVRT2* interactions.

(b, d) Protein immunoprecipitation analyses of *TaVRN-A1* with *TaSOC1* and *TaVRT2*. *TaVRN-A1*(85-end) with *TaSOC1* (a and b) and *TaVRT2* (c and d). *TaVRN-A1* was detected by anti-HIS antibody, and *TaSOC1* or *TaVRT2* was detected by anti-MBP antibody: lane 1, purified Jagger *vrn-A1a*-HIS-tag; lane 2, purified 2174 *vrn-A1b*-HIS-tag; lane 3, interaction of *TaVRN-A1a*-HIS-tag with *TaSOC1*-MBP-tag or *TaVRT2*-MBP-tag; lane 4, interaction of *TaVRN-A1b*-HIS-tag with *TaSOC1*-MBP-tag or *TaVRT2*-MBP-tag; lane 5, purified *TaSOC1*-MBP-tag or *TaVRT2*-MBP-tag; M, protein marker. Arrowheads represent the expressed or interacting proteins. Six independent reactions for each interaction were performed, and one representative reaction is shown.

full-length *TaVRN-A1* was expressed by pEG101-YFP vector, and enriched yellow fluorescent signals were detected predominantly in the nucleus (Figure 5b). The full-length *TaHOX1* was not expressed by the pEG101-YFP vector or by a series of bimolecular fluorescence complementation (BiFC) vectors (Waadt *et al.*, 2008); therefore, we expressed a protein fragment, *TaHOX1*(1–180), to localize the protein in living cells. When *TaHOX1*-YFP(1–180) alone was expressed, enriched yellow fluorescent signals were detected only in the nucleus (Figure 5b). Then, we cloned *TaVRN-A1* into pEG201-YN vector and *TaHOX1*(1–180) into pEG202-YC vector and analyzed *in vivo* protein interactions by BiFC. When *TaVRN-A1*-YN and *TaHOX1*(1–180)-YC were simultaneously expressed in the same cell, yellow fluorescence was observed in the nucleus with a confocal microscope (Figure 5c,e). We observed that when *TaVRN-A1*-YN and *TaHOX1*(100-end)-YC were simultaneously expressed in the same cell, *TaVRN-A1* and *TaHOX1* had a very strong interaction (Figure 5f). Furthermore, *TaHOX1*(100-end) showed more interacting fluorescence with *vrn-A1a* than *vrn-A1b* in multiple images on a large scale. These results were consistent with the results for the differential *in*

in vitro interactions of *TaHOX1*(100-end) with *vrn-A1a* and *vrn-A1b*.

The upregulation of *TaHOX1* by low temperature and its genetic effects on heading date

We isolated the complete *TaHOX1* gene from Jagger (JQ915061) and 2174 (JQ915062) for functional characterization. Using RT-PCR, we found that the *TaHOX1* transcripts in leaves were greatly upregulated, and remained at a high level during vernalization (Figure 6a). This result indicated that *TaHOX1* had a similar expression pattern as the recessive *vrn1* allele characterized in previous studies (Danyluk *et al.*, 2003; Murai *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003). *TaHOX1* and *TaVRN-A1* were concomitantly upregulated by low temperature, indicating that they functioned in the flowering pathway.

We developed a PCR marker for one SNP (see Figure S11A), and mapped *TaHOX1* to chromosome 6B using the Jagger × 2174 RIL population (see Figure S11B). A significant difference in heading date was observed between the Jagger *TaHOX1a* and the 2174 *TaHOX1b* alleles (120 versus 109 days, respectively) in the RIL population vernalized

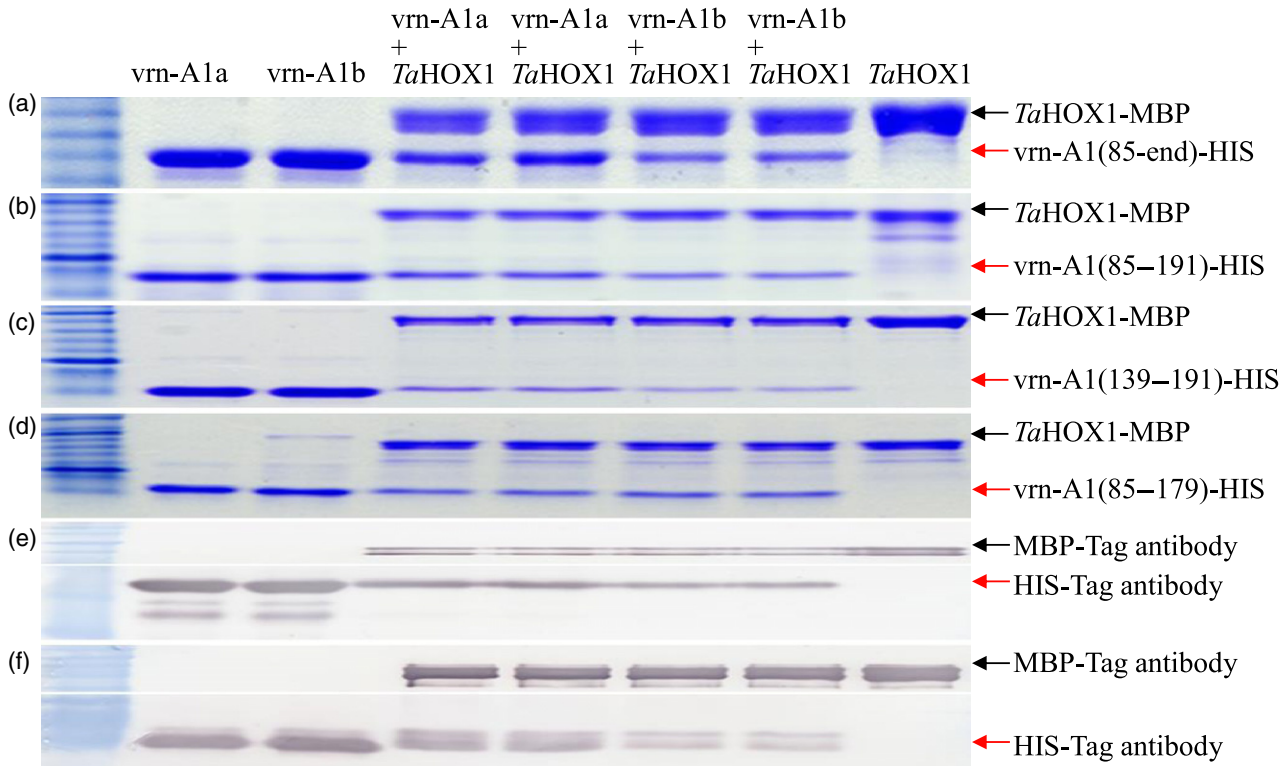


Figure 4. *In vitro* interaction between *TaVRN-A1* and *TaHOX1* proteins.

(a–d) Pull-down assays of *TaHOX1*-MBP and *TaVRN-A1* interactions. *In vitro* interaction between *TaHOX1*(100–end) and *TaVRN-A1*(85–end) (a), *TaVRN-A1*(85–191) (b), *TaVRN-A1*(139–191) (c), and *TaVRN-A1*(85–179) (d).

(e, f) Protein immunoprecipitation analyses of *TaHOX1* and *TaVRN-A1*. *TaHOX1*-MBP was detected by anti-MBP antibody (the upper panel, e and f). *TaVRN-A1*(85–end) and *TaVRN-A1*-HIS(139–191) were detected by anti-HIS antibody (the lower panel, e and f). Lane 1, purified Jagger *vrn-A1a*-HIS-tag; lane 2, purified 2174 *vrn-A1b*-HIS-tag; lanes 3 and 4, interaction of *TaVRN-A1a*-HIS-tag and *TaHOX1*-MBP-tag; lanes 5 and 6, interaction of *TaVRN-A1b*-HIS-tag and *TaHOX1*-MBP-tag; lane 7, purified *TaHOX1*-MBP-tag; M, protein marker. Arrowheads represent expressed or interacting proteins. Six independent replicates were performed for each interaction, and two replicates are shown to indicate consistency among replicates.

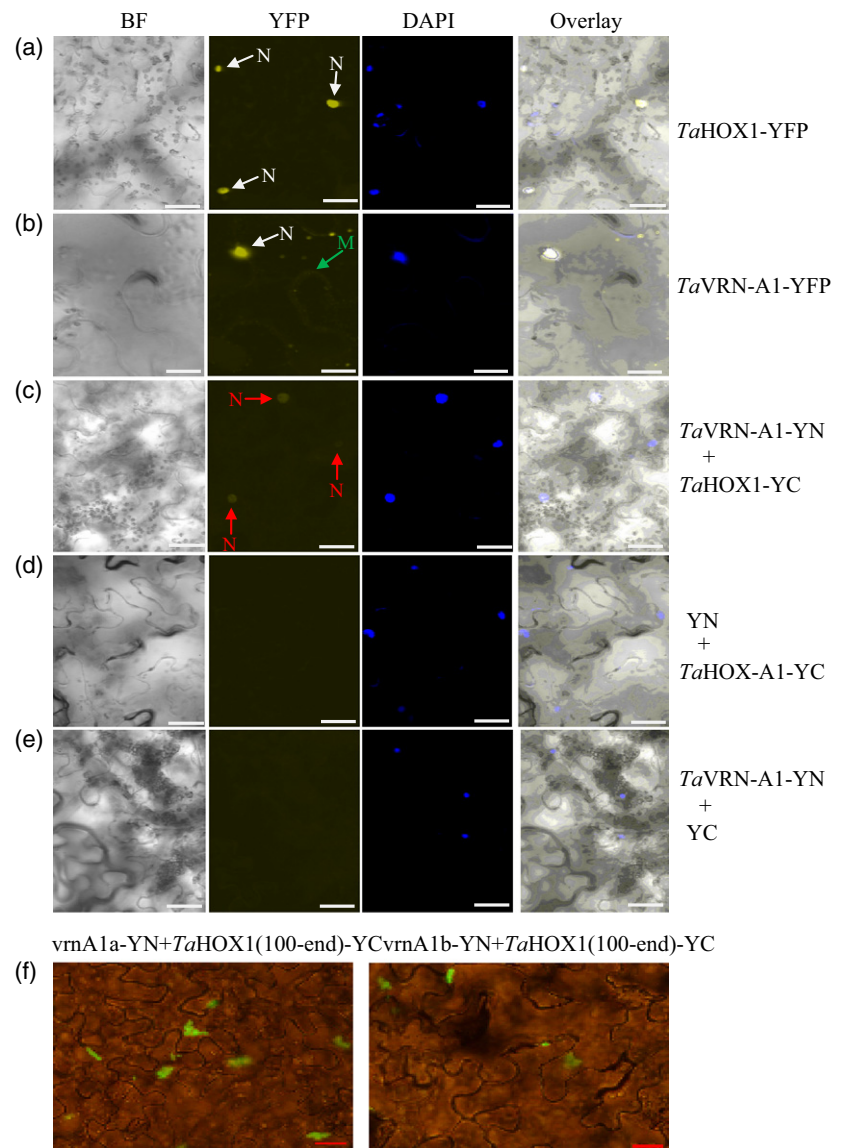
for 3 weeks ($P < 0.05$), but not in the RIL population vernalized for 6 weeks or the control population (Figure 6b). The SNP in exon 1 resulted in a 'leucine' residue at position 99 in *TaHOX1b* and a 'proline' residue at the same position in *TaHOX1a* (Figure S12A). This point mutation in *TaHOX1* did not affect its interaction with *vrn-A1* (Figure S12B). In contrast, the higher *TaHOX1* transcriptional level was associated with earlier heading, suggesting that the regulation of heading date by *TaHOX1* was at the transcriptional level.

DISCUSSION

All of the vernalization genes that were previously cloned are related to segregation between spring wheat and winter wheat, which can differ by as many as several months in phenotype (Danyluk *et al.*, 2003; Murai *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003, 2004a, 2006). The phenotypic segregation in flowering time among winter wheat cultivars usually spans a few days when the plants are tested in the field (Chen *et al.*, 2009, 2010). The present study allowed us to observe more than 1 month

for flowering time segregated between the two winter wheat cultivars when a 3-week vernalization was performed under thermal- and photoperiodic-controlled conditions. This approach was used to separate the effect of low temperature in accelerating the developmental transition from the effect of low temperature in delaying the growth rate in a semi-winter cultivar that required only 3 weeks at low temperatures for full vernalization. If the semi-winter wheat cultivar was vernalized for 6 weeks, as has been performed in numerous studies, the over-vernalized plant to the prolonged low temperatures after 3 weeks would not be accelerated flowering, but would instead result in delayed flowering. Winter wheat, for which any requirement for vernalization has been fully satisfied, will have similar responses to ambient temperature as spring wheat (Snape *et al.*, 2001). If the fully vernalized plants continue to stay at low temperatures, however, the developmental process of these over-vernalized plants will be delayed because of a longer phyllochron and a slower growth rate (McMaster, 2005). The phenotyping approach used in this study has facilitated

Figure 5. Subcellular localization and *in vivo* interaction of *TaVRN-A1* and *TaHOX1* proteins. (a) The subcellular location of *TaHOX1*(1–180)-YFP protein expressed by pEG101 in the nucleus (N) of living cells in *Nicotiana tabacum* (tobacco) leaves. (b) The subcellular location of *TaVRN-A1*(full-length)-YFP protein expressed by pEG101 predominantly in the nucleus (N) as well as partly on the cytoplasmic membrane (M). (c) Bimolecular fluorescence complementation (BiFC) analysis of *TaVRN-A1* and *TaHOX1* (1–180) proteins. The yellow fluorescent proteins (YFPs) resulted from the *in vivo* interaction between *TaVRN-A1*-YN and *TaHOX1*(1–180)-YC in the nucleus are arrowed 'N'. (d–e) No YFP was observed in a negative control of the co-transformation of pEG202-YC with *TaVRN-A1*-pEG201-YN (d) or pEG201-YN with *TaHOX1*-pEG202-YC (e). The YFPs resulting from the *in vivo* interaction between *TaVRN-A1*-YN and *TaHOX1*(1–180)-YC in the nucleus are arrowed by 'N'. (d, e) No YFP was observed in a negative control of the co-transformation of pEG202-YC with *TaVRN-A1*-pEG201-YN (d) or pEG201-YN with *TaHOX1*-pEG202-YC (e). YN, YFP fragment at the N-terminal end expressed from pEG201-YN vector; YC, YFP fragment at the C-terminal end expressed from pEG202-YC vector. Images were visualized under a confocal microscope. (f) BiFC analysis of *TaVRN-A1* and *TaHOX1* (100–end) proteins. The green fluorescent proteins resulted from the *in vivo* interaction between *TaVRN-A1*-YN and *TaHOX1*(100–end)-YC. YN, YFP fragment at the N-terminal end expressed from pEG201-YN vector; YC, YFP fragment at the C-terminal end expressed from pEG202-YC vector. Images were taken under a fluorescent microscope. *TaHOX1*(100–end) was also tested for interaction with the vectors as negative controls, and no interaction signal was observed (data not shown).



the positional cloning of the major gene for vernalization requirement duration in winter wheat.

In a previous study, we found a C/T polymorphism in exon 4 that is responsible for the amino acid change at Leu¹¹⁷/Phe¹¹⁷ in *TaVRN-A1* between the Jagger allele and the 2174 allele, and we developed a PCR marker for this polymorphism that showed an association with developmental variation in the Jagger × 2174 RIL population tested in the field (Chen *et al.*, 2009). It was recently reported that *TaVRN-A1* was duplicated in winter wheat cv. 'Hereward' (JF965397) but not in 'Claire' (JF965395) (Díaz *et al.*, 2012). In the population of Claire × Hereward, plants homozygous for the Claire allele flowered earlier and plants homozygous for the Hereward allele flowered later, whereas heterozygotes had an intermediate flowering time; therefore, the authors concluded that the increased copy number of *TaVRN-A1* in Hereward resulted in an increased requirement for

vernalization (and thus late flowering). We found that at the polymorphic sites of both exon 4 and exon 7, Jagger has the same allele as Claire and 2174 has the same allele as Hereward. We also observed the duplication event of *vrn-A1b* in 2174 for more vernalization, but not in Jagger for less vernalization (Figure S13). Thus, on the surface, it seems that the two studies have consistent results and that the phenomenon that Díaz *et al.* described in their paper, that plants with an increased copy number of *TaVRN-A1* have an increased requirement for vernalization, was correct. However, we affirm that a greater vernalization requirement in 2174 was not because 2174 has one more *vrn-A1* copy than Jagger, but because Jagger produces a different *vrn-A1* protein than 2174. Our results from several independent progeny populations clearly and consistently demonstrated the Jagger *vrn-A1a* allele for early flowering was dominant over the 2174 *vrn-A1b* allele for late flowering, with pheno-

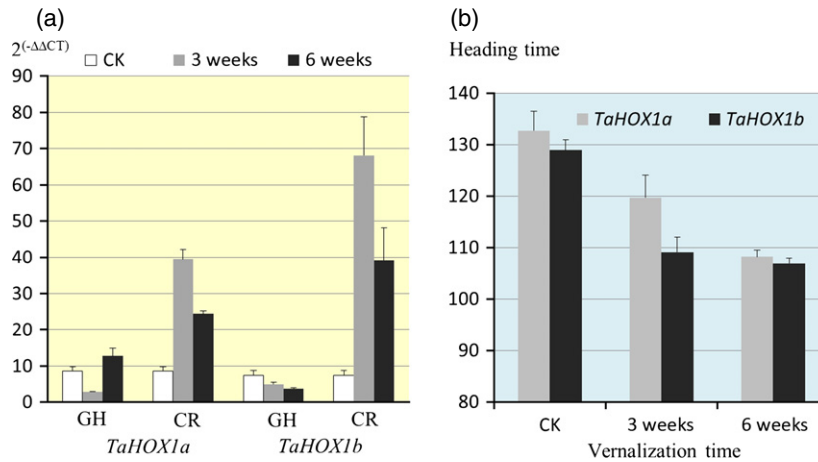


Figure 6. Expression profiles of *TaHOX1* and its genetic effect on flowering time.

(a) Expression pattern of *TaHOX1* in the glasshouse (GH) and in the cold room (CR). Transcript levels of *TaHOX1a* (the Jagger allele) and *TaHOX1b* (the 2174 allele) in leaves of vernalized plants for 3 and 6 weeks, as well as non-vernalized plants (CK) are shown using the values calculated by the $2^{(-\Delta\Delta CT)}$ method, where CT is the cycle threshold, and *actin* was used as an endogenous control. The primers for *TaHOX1* and *actin* are provided in Table S2. The values represent mean expression levels ($n = 15-20$), and the error bars indicate standard errors.

(b) Genetic effect of *TaHOX1* on heading date. The heading date was from each line of the population ($n = 96$) that was vernalized for 3 or 6 weeks, or used for the control in the glasshouse (CK). The significant effects of the *TaHOX1* gene on heading date were determined using one-way analysis of variance (ANOVA). Error bars indicate standard errors.

typic segregation of 3:1 (Figure 1e; Table S1). The two studies have come to different conclusions for several reasons. First, in the Diaz *et al.* study, vernalization was performed under short-day conditions (7°C, 8 h of light), and then the vernalized plants were grown under long-day conditions (18 h of light) in a glasshouse (no temperature condition was provided). In our study, however, vernalization was performed at 4°C with 16 h of light, and then the vernalized plants were moved to 25°C and 16 h of light. The same long day was set throughout the experiment in our study to avoid any disruption of photoperiod, which is well known to have significant effects on flowering in winter wheat (Dubcovsky *et al.*, 2006; Wang *et al.*, 2009). Second, in the Diaz *et al.* study a small mapping population of 96 F₂ lines was used to test the association between the *vrn-A1* copy number and flowering time. By contrast, we generated a backcross population and screened 6500 plants to find several lines that had crossovers at the *vrn-A1* locus. The flowering time in the progeny of these recombinant lines was segregated by a single gene *vrn-A1a*, without any disruption by other genetic factors for this phenotype. Third, *vrn-A1* is the promoter of flowering, as shown in wheat and barley (Danyluk *et al.*, 2003; Murai *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003), and in Arabidopsis (Ng and Yanofsky, 2001). The dosage effect of two copies from such a flowering promoter should not result in a phenotype of a later flowering time, as suggested in the previous study (Díaz *et al.*, 2012). In this study, we used the positional cloning strategy to prove that *vrn-A1a* in Jagger was dominant for early flowering, regardless of the function of the duplicated *vrn-A1b* in 2174. Therefore, we concluded that less vernalization requirement by Jagger resulted from its *vrn-*

A1a protein form, or that more vernalization requirement in 2174 was not due to the presence of its two copies of *vrn-A1b*.

Sequence comparison analyses showed that the A genome of the diploid donor (*Triticum urartu*, GenBank accession DQ291016) and the tetraploid donor (*Triticum turgidum*, GenBank accession AY747598) had exactly the same cDNA sequence of *vrn-A1a* as Jagger, suggesting that the *vrn-A1a* allele in Jagger is the wild type. The two amino acids, Phe¹¹⁷ and Val¹⁸⁰, in *vrn-A1b* in cultivar 2174 were mutated during the domestication and adaptation of hexaploid wheat. Previous studies revealed that the recessive *vrn-A1* allele for winter growth habit is the wild type, and the dominant *Vrn-A1* allele for spring growth habit was mutated via insertions or deletions in the promoter or the first intron (Yan *et al.*, 2004b; Fu *et al.*, 2005). This study revealed that winter wheat cultivars Jagger and 2174 carried the same recessive *vrn-A1* allele but encoded different *vrn-A1* proteins that underlie various vernalization requirement durations. Altogether, these studies demonstrate that a single gene may evolve differential mechanisms to enable adaptation to multiple environmental conditions. Through one mechanism evolution occurred at the transcriptional level by mutations in the promoter or first-intron region of *vrn-A1*, producing *Vrn-A1* that confers spring growth habit without vernalization requirement (Yan *et al.*, 2004b; Fu *et al.*, 2005). Through a second mechanism, the wild-type *vrn-A1a* allele for less vernalization requirement in semi-winter wheat cultivars like Jagger evolved at the protein level through mutations in the coding region, producing *vrn-A1b* for greater vernalization requirement in strong winter wheat cultivars like 2174. The proposed mechanism underlying the quantitative vernali-

zation requirement in winter wheat differs from the mechanisms that explain the various vernalization requirement durations in winter types of *Arabidopsis* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999; Reeves and Coupland, 2001; Levy *et al.*, 2002; Corbesier *et al.*, 2007; Angel *et al.*, 2011; Heo and Sung, 2011; Coustham *et al.*, 2012).

TaHOX1 is an orthologue of rice homeobox *HOX24* (NP_001047582.1) and *Arabidopsis thaliana* homeobox 7 (*AtHB-7*) (NP_182191.1). *AtHB7* was characterized as a member of HD-Zip class I involved in responses to water and light stresses, and overexpression of *AtHB7* resulted in the early flowering of transgenic *Arabidopsis* plants (Olsson *et al.*, 2004), providing supporting evidence for the role of the *HOX* genes in the regulation of flowering time in plants. *TaHOX1* is one of the *HOX* proteins known to function as an on-off switch in controlling development, including specialization of regional identities along the anterior/posterior axis in a wide range of phyla in animals (Manak and Scott, 1994), whereas *vrn-A1* (=AP1) is one of the MADS-box proteins known to act as floral switches for the transition from vegetative to reproductive development in plants (Ng and Yanofsky, 2001). This study presented one example in which MADS and *HOX* proteins involving homeosis have a direct binding in higher plants.

In winter wheat, two models were established to explain the relationship between the three vernalization genes that have been cloned so far: the first model supposes that *VRN3* positively regulates *VRN1*, which then negatively downregulates *VRN2* (Yan *et al.*, 2006); the second model hypothesizes that *VRN1* activates its downstream gene *VRN3*, which then downregulates *VRN2* (Shimada *et al.*, 2009). In this study, we focused on the positional cloning of the gene for vernalization requirement duration in winter wheat. In the cloning population, flowering time segregated according to a single gene, *vrn-A1*, and no genetic effect was observed from *VRN2* or *VRN3*. When *vrn-A1* acts as a protein in either of the two models, it should appear as a *TaVRN1-TaHOX1* protein complex because of a direct binding between them. *TaHOX1* protein and *TaVRN-A1* protein should also have direct interaction in spring wheat cultivars, although the role of the protein complex in regulating flowering time in spring wheat needs to be investigated.

Previous studies suggested that the gene for vernalization requirement duration was postulated as a sensor to code for a temperature-sensitive protein (Roberts, 1990; Amasino, 2004; Sung and Amasino, 2004). The findings from this study indicated that the *vrn-A1* protein could function as such a sensor, to measure cold duration. Although the accumulation of *vrn-A1a* was accelerated by a 3-week vernalization duration, its activity reached a threshold for flowering. Compared with *vrn-A1a*, *vrn-A1b* needs a longer time at the low temperature to reach the same activity. The point mutation at Val¹⁸⁰ of *vrn-A1a* is critical in retaining *vrn-A1* activity. The *TaHOX1* protein

was suggested to function in the flowering pathway, based on its direct interaction with *TaVRN-A1* and genetic association with flowering time; however, the role of *TaHOX1* in regulating flowering time needs to be confirmed in transgenic plants, and further experiments are also needed to test if the *TaVRN-A1-TaHOX1* protein complex is sensed by low temperature in winter wheat. The information on the role of *TaVRN-A1* in controlling vernalization requirement duration is particularly useful for directing breeding cultivars for adaptation to global warming in future climate scenarios for winter wheat, which represents approximately 75% of the wheat grown worldwide.

EXPERIMENTAL PROCEDURES

Plant materials and vernalization experiments

Jagger and 2174 were initially grown in a glasshouse at 20–25°C under long days (LD, 16-h day/8-h night). At the fifth-leaf stage, the parental plants were moved into a cold room with 4°C and the LD photoperiod. The LD photoperiod was applied throughout this study to avoid any disruption from photoperiod effects on vernalization (Wang *et al.*, 2009). The two parental lines were vernalized for varying numbers of weeks. The vernalized plants were returned to the glasshouse, whereas non-vernalized plants that continuously remained in the glasshouse were used as controls. The heading date for eight plants for each treatment was scored.

Three populations from the same Jagger × 2174 RILs were initially grown in the same glasshouse as used for the parental lines. At the fifth-leaf stage, two of these populations were vernalized for 3 and 6 weeks, respectively. The vernalized populations were returned to the glasshouse for comparison with the third population as a non-vernalized control. The heading date was scored for three plants of each line in these three populations.

Development of PCR markers for the genes flanking *QVrd.osu-5A*

A total of nine genes in the *QVrd.osu-5A* locus were converted to orthologous genes in chromosome 5A of hexaploid wheat tested in this study. PCR markers for each of nine genes at the *QVrd.osu-5A* locus were developed and used to determine the locations of critical crossovers in the recombinant plants (Figures S1–S9).

Allelic variation and expression of candidate genes for *QVrd.osu-5A*

Each of the two alleles for each of three candidate genes was sequenced. Two SNPs were found in *CYB5* between the Jagger allele and the 2174 allele, 10 SNPs were found in *AGLG1* between the Jagger allele and the 2174 allele, and 29 SNPs were found in *vrn-A1* between the Jagger allele and the 2174 allele. Transcript levels for *vrn-A1*, *AGLG1* and *CYB5* were determined from the same cDNA sample collected from five plants for the vernalized and non-vernalized treatments, and *actin* was used as an endogenous control (see Figure S10). The specific primers for expression of these genes are listed in Table S2.

Identification of proteins interacting with *vrn-A1*

The 'Matchmaker™' Library Construction & Screening System (Clontech, <http://www.clontech.com>) was used to construct a Y2H 'prey' library for Jagger. RNA was extracted from pooled samples

of young leaves and apices from vernalized plants for 1, 2 and 3 weeks, and the *vrn-A1a* was used as 'bait' to screen the 'prey' library. The constructing and screening procedures were described in a previous study in which 2174 was used as a host plant (Cao and Yan, 2013).

Protein pull-down assay

The complete *vrn-A1a* and *vrn-A1b* cDNA sequences were cloned into pSUMO vectors with an N-terminal 6XHIS-tag (Deng *et al.*, 2008), and *vrn-A1* fragments (85-end) were also cloned into the pSUMO vector. Three shorter fragments for each of *vrn-A1a* and *vrn-A1b*, encoding different mutations, were cloned into pSKB3 with an N-terminal 6XHIS-tag (Figure 2c–e). The *TaSOC1*, *TaVRT2* and *TaHOX1* cDNA obtained from the Y2H library were cloned into the pMAL-c2X vector with a MBP-tag (New England BioLabs, <http://www.neb.com>). The primers used in cloning are listed in Table S3. The protein constructs were expressed in *Escherichia coli* (BL21 DE3). The expressed proteins from pSUMO and pSKB3 were dialyzed and purified using Ni-NTA column (QIAGEN, <http://www.qiagen.com>). The proteins fused with MBP-tag were dialyzed and purified with amylose column (New England BioLabs).

A protein fused with the MBP-tag (500 µg ml⁻¹, 50 µl) was incubated with amylose resin (40 µl) for 1 h at 4°C in the column buffer, and the MBP-tag (500 µg ml⁻¹, 50 µl) alone was used as a negative control. A protein fused with the 6XHIS-tag (500 µg ml⁻¹, 100 µl) was added to the amylose resins and incubated for 4 h at 4°C. The interacting proteins in the resin were then washed with the column buffer between six and eight times, depending on when the protein tagged with the 6XHIS-tag was no longer observed in the negative control. The protein that bound to the resin was eluted by boiling the samples in 100 µl of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer for 5 min. The eluted protein samples (80 µl) were analyzed by 10% SDS-PAGE stained with Coomassie Brilliant blue.

Western blot was performed using the protocol previously described by Earley *et al.* (2006). Immunodetection was performed using primary antibodies (1:1000 dilution): anti-Maltose Binding Protein (MBP) (M1321; Sigma-Aldrich, <http://www.sigmaaldrich.com>) to detect HOX1-MBP, and anti-6XHIS (SAB4600048; Sigma-Aldrich) to detect *TaVRN-A1*-HIS, *TaSOC1*-HIS or *TaVRT2*-HIS. After washing, anti-Mouse IgG-Alkaline Phosphatase antibody (A3562; Sigma-Aldrich) was used as the secondary antibody. The membranes were washed three times before color was developed with Alkaline Phosphatase Conjugate Substrate Kit (170-6432; Bio-Rad, <http://www.bio-rad.com>).

Subcellular localization and *in vivo* interaction of *TaVRN-A1* and *TaHOX1* proteins

The complete *TaVRN-A1* cDNA was cloned into pDONR207 with the BP cloning kit (Invitrogen, now Life Technologies, <http://www.lifetechnologies.com>), and then transferred to pEarleygate 101 (pEG101) using the LR cloning kit for subcellular localization of *TaVRN-A1*. *TaVRN-A1* in pDONR207 was fused to the N-terminal 174 amino acid portion (1–174 aa) of YFP in the pEarleyGate201-YN vector (pEG201-YN) to test *in vivo* interaction with *TaHOX1* [1–180 aa, 100–226 aa (end)] fused to the C-terminal amino acid portion (175–239 aa) of YFP in the pEarleyGate202-YC vector (pEG202-YC). Empty vectors were also used as negative controls for interaction with *TaVRN-A1* or *TaHOX1* proteins. *Agrobacterium tumefaciens* strains (GV3101) carrying the BiFC constructs were used together with the p19 strain for infiltration of *Nicotiana benthamiana* leaves (at 5 weeks old). Leaf discs were cut for BiFC for imaging 3 days after infiltration. Images were visualized under a confocal microscope (TCS SP2; Leica, <http://www.leica-microsystems.com>).

The images (Figure 5a–e) were also taken with a bright filter (BF) to indicate the background of the leaves infiltrated with *A. tumefaciens* carrying constructs, or with an ultraviolet filter to indicate the position of the nucleus stained with 4',6-diamidino-2-phenylindole (DAPI). The overlay images align the locations of YFP with the DAPI-stained nucleus. Images (Figure 5f) were taken under a fluorescent microscope (BX51; Olympus, <http://www.olympus-global.com>) with GFP filter to indicate the presence of fluorescent proteins. The scale bars in all images are 50 µm.

ACKNOWLEDGEMENTS

We thank J. Deng (Oklahoma State University) for his assistance with the development of the *in vitro* protein interaction systems, Y Cui (Agriculture and Agri-Food Canada) for providing pEarleygate201-YN and pEarleygate202-YC vectors and F. Zhang (Oklahoma State University) for his assistance with the development of the *in vivo* protein interaction systems in tobacco. We thank Y. Chen for technical assistance in generating backcross populations. This project was supported by the National Research Initiative Competitive Grants CAP project 2011-68002-30029 from the US Department of Agriculture (USDA) National Institute of Food and Agriculture and from the Oklahoma Center of Advanced Science and Technology (OCAS, PS07-032). This research project was partially funded by the Oklahoma Agricultural Experiment Station and the Oklahoma Wheat Research Foundation.

SUPPORTING INFORMATION

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

Figure S1. PCR marker for *GENE1*.

Figure S2. PCR marker for *PCS1*.

Figure S3. PCR marker for *CYB5*.

Figure S4. PCR marker for *AGLG1*.

Figure S5. PCR marker for *vrn-A1a*.

Figure S6. PCR marker for *CYS1*.

Figure S7. PCR marker for *PHY1*.

Figure S8. PCR marker for *OPT1*.

Figure S9. PCR marker for *CDO708*.

Figure S10. Comparison of gene expression levels of three candidate genes for *QVrd.osu-5A* in Jagger versus 2174.

Figure S11. Mapping of *TaHOX1*.

Figure S12. Location of a point mutation in *TaHOX1*.

Figure S13. Copy number of *TaVRN-A1*.

Table S1. Progeny test of critical recombinant plants for heading date.

Table S2. List of primers used for gene expression.

Table S3. List of primers used for protein expression.

REFERENCES

- Amasino, R. (2004) Vernalization, competence, and the epigenetic memory of winter. *Plant Cell*, **16**, 2553–2559.
- Angel, A., Song, J., Dean, C. and Howard, M. (2011) A polycomb-based switch underlying quantitative epigenetic memory. *Nature*, **476**, 105–108.
- Ariel, F.D., Manavella, P.A., Dezar, C.A. and Chan, R.L. (2007) The true story of the HD-Zip family. *Trends Plant Sci.*, **12**, 419–426.
- Bonnin, I., Rousset, M., Madur, D., Sourdil, P., Dupuits, C., Brunel, D. and Goldringer, T. (2008) FT genome A and D polymorphisms are associated with the variation of earliness components in hexaploid wheat. *Theor. Appl. Genet.*, **116**, 383–394.
- Cao, S. and Yan, L. (2013) Construction of a high-quality Yeast two-hybrid (Y2H) library and its application in identification of interacting proteins with key vernalization regulator *TaVRN-A1* in wheat. *BMC Res. Note*, **6**, 81–86.

- Chen, Y., Carver, B.F., Wang, S., Zhang, F. and Yan, L. (2009) Genetic loci associated with stem elongation and winter dormancy release in wheat. *Theor. Appl. Genet.*, **118**, 881–889.
- Chen, Y., Carver, B.F., Wang, S., Cao, S. and Yan, L. (2010) Genetic regulation of developmental phases in winter wheat. *Mol. Breed.*, **118**, 1339–1349.
- Chouard, P. (1960) Vernalization and its relation to dormancy. *Annu. Rev. Plant Physiol.*, **11**, 191–238.
- Corbesier, L., Vincent, C., Jang, S. *et al.* (2007) FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. *Science*, **316**, 1030–1033.
- Coustham, V., Li, P., Strange, A., Lister, C., Song, J. and Dean, C. (2012) Quantitative modulation of Polycomb silencing underlies natural variation in vernalization. *Science*, **337**, 584–587.
- Crofts, H.J. (1989) On defining a winter wheat. *Euphytica*, **44**, 225–234.
- Danyluk, J., Kane, N.A., Breton, G., Limin, A.E., Fowler, D.B. and Sarhan, F. (2003) *TaVRT-1*, a putative transcription factor associated with vegetative to reproductive transition in cereals. *Plant Physiol.*, **132**, 1849–1860.
- Deng, J.P., Lewis, P.A., Greggio, E., Sluch, E., Beilina, A. and Cookson, M.R. (2008) Structure of the ROC domain from the Parkinson's disease-associated leucine-rich repeat kinase 2 reveals a dimeric GTPase. *Proc. Natl Acad. Sci. USA*, **105**, 1499–1504.
- Díaz, A., Zikhali, M., Turner, A.S., Isaac, P. and Laurie, D.A. (2012) Copy number variation affecting the photoperiod-B1 and vernalization-A1 genes is associated with altered flowering time in wheat (*Triticum aestivum*). *PLoS One*, **7**, e33234. doi:10.1371/journal.pone.0033234.
- Dubcovsky, J., Loukoianov, A., Fu, D., Valarik, M., Sanchez, A. and Yan, L. (2006) Effect of photoperiod on the regulation of wheat vernalization genes *VRN1* and *VRN2*. *Plant Mol. Biol.*, **60**, 469–480.
- Earley, K.W., Haag, J.R., Pontes, O., Oppen, K., Juehne, T., Song, K. and Pikaard, C.S. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.*, **45**, 616–629.
- Fu, D., Szucs, P., Yan, L., Helguera, M., Skinner, J.S., Hayes, P. and Dubcovsky, J. (2005) Large deletions in the first intron of the *VRN1* vernalization gene are associated with spring growth habit in barley and polyploid wheat. *Mol. Genet. Genomics*, **273**, 54–65.
- Heo, J.B. and Sung, S. (2011) Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. *Science*, **331**, 76–79.
- Humphreys, M.W., Yadav, R.S., Cairns, A.J., Turner, L.B., Humphreys, J. and Skot, L. (2006) A changing climate for grassland research. *New Phytol.*, **169**, 9–26.
- Kane, N.A., Danyluk, J., Tardif, G., Ouellet, F., Laliberte, J.F., Limin, A.E., Fowler, D.B. and Sarhan, F. (2005) *TaVRT-2*, a member of the StMADS-11 clade of flowering repressors, is regulated by vernalization and photoperiod in wheat. *Plant Physiol.*, **138**, 2354–2363.
- Kerr, R.A. (2007) Climate change: global warming is changing the world. *Science*, **316**, 188–190.
- Levy, Y.Y., Mesnage, S., Mylne, J.S., Gendall, A.R. and Dean, C. (2002) Multiple roles of Arabidopsis *VRN1* in vernalization and flowering time control. *Science*, **297**, 243–246.
- Lu, Q., Tang, X., Tian, G. *et al.* (2010) Arabidopsis homolog of the yeast TREX-2 mRNA export complex: components and anchoring nucleoporin. *Plant J.*, **60**, 259–270.
- Manak, J.R. and Scott, M.P. (1994) A class act: conservation of homeodomain protein functions. *Dev. Suppl.* 61–71.
- Mandel, M.A., Gustafsonbrown, C., Savidge, B. and Yanofsky, M.F. (1992) Molecular characterization of the Arabidopsis floral homeotic gene *Apeta1*. *Nature*, **360**, 273–277.
- McMaster, G.S. (2005) Phytomers, phyllochrons, phenology and temperate cereal development. *J. Agric. Sci.*, **143**, 137–150.
- Michaels, S.D. and Amasino, R.M. (1999) FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell*, **11**, 949–956.
- Miglietta, F., Tanasescu, M. and Marica, A. (1995) The expected effects of climate change on wheat development. *Global Change Biol.*, **1**, 407–415.
- Murai, K., Miyamae, M., Kato, H., Takumi, S. and Ogihara, Y. (2003) *WAP1*, a wheat *APETALA1* homolog, plays a central role in the phase transition from vegetative to reproductive growth. *Plant Cell Physiol.*, **44**, 1255–1265.
- Ng, M. and Yanofsky, M.F. (2001) Function and evolution of the plant MADS-box gene family. *Nature Rev. Genet.*, **2**, 186–195.
- Olsson, A.S., Engström, P. and Söderman, E. (2004) The homeobox genes *ATHB12* and *ATHB7* encode potential regulators of growth in response to water deficit in Arabidopsis. *Plant Mol. Biol.*, **55**, 663–677.
- Pugsley, A.T. (1971) A genetic analysis of the spring-winter habit of growth in wheat. *Aust. J. Agric. Res.*, **22**, 21–31.
- Reeves, P.H. and Coupland, G. (2001) Analysis of flowering time control in Arabidopsis by comparison of double and triple mutants. *Plant Physiol.*, **126**, 1085–1091.
- Roberts, D.W.A. (1990) Identification of loci on chromosome 5A of wheat involved in control of cold hardiness, vernalization, leaf length, rosette growth habit, and height of hardened plants. *Genome*, **33**, 247–259.
- Santra, D.K., Santra, M., Allan, R.E., Campbell, K.G. and Kidwell, K.K. (2009) Genetic and molecular characterization of vernalization genes *Vrn-A1*, *Vrn-B1*, and *Vrn-D1* in spring wheat germplasm from the Pacific Northwest region of the USA. *Plant Breed.*, **128**, 576–584.
- Semenov, M. A. and Halford, N.G. (2009) Identifying target traits and molecular mechanisms for wheat breeding under a changing climate. *J. Exp. Bot.*, **60**, 2791–2804.
- Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J. and Dennis, E.S. (1999) The *FLF* MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. *Plant Cell*, **11**, 445–458.
- Shimada, S., Ogawa, T., Kitagawa, S. *et al.* (2009) A genetic network of flowering-time genes in wheat leaves, in which an *APETALA1/FRUIT-FULL*-like gene, *VRN1*, is upstream of FLOWERING LOCUS T. *Plant J.*, **58**, 668–681.
- Snape, J.W., Sarma, R., Quarrie, S.A., Fish, L., Galiba, G. and Sutka, J. (2001) Mapping genes for flowering time and frost tolerance in cereals using precise genetic stocks. *Euphytica*, **120**, 309–315.
- Sung, S.B. and Amasino, R.M. (2004) Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. *Nature*, **427**, 159–164.
- Tranquilli, G.E. and Dubcovsky, J. (2000) Epistatic interactions between vernalization genes *Vrn-Am 1* and *Vrn-Am 2* in diploid wheat. *J. Hered.*, **91**, 304–306.
- Trevaskis, B., Bagnall, D.J., Ellis, M.H., Peacock, W.J. and Dennis, E.S. (2003) MADS box genes control vernalization-induced flowering in cereals. *Proc. Natl Acad. Sci. USA*, **100**, 13099–13104.
- Waadt, R., Schmidt, L.K., Lohse, M., Hashimoto, K., Bock, R. and Kudla, J. (2008) Multicolor bimolecular fluorescence complementation reveals simultaneous formation of alternative CBL/CIPK complexes in planta. *Plant J.*, **56**, 505–516.
- Wang, S., Carver, B. and Yan, L. (2009) Genetic loci in the photoperiod pathway interactively modulate reproductive development of winter wheat. *Theor. Appl. Genet.*, **118**, 1339–1349.
- Yan, L., Loukoianov, A., Tranquilli, G., Helguera, M., Fahima, T. and Dubcovsky, J. (2003) Positional cloning of wheat vernalization gene *VRN1*. *Proc. Natl Acad. Sci. USA*, **100**, 6263–6268.
- Yan, L., Loukoianov, A., Tranquilli, G. *et al.* (2004a) The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. *Science*, **303**, 1640–1644.
- Yan, L., Helguera, M., Kato, K., Fukuyama, S., Sherman, J. and Dubcovsky, J. (2004b) Allelic variation at the *VRN1* promoter region in polyploid wheat. *Theor. Appl. Genet.*, **109**, 1677–1686.
- Yan, L., Fu, D., Li, C., Blechl, A., Tranquilli, G., Bonafede, M., Sanchez, A., Valarik, M., Yasuda, S. and Dubcovsky, J. (2006) The wheat and barley vernalization gene *VRN3* is an orthologue of *FT*. *Proc. Natl Acad. Sci. USA*, **103**, 19581–19586.
- Yang, Y., Fanning, L. and Jack, T. (2004) The K domain mediates heterodimerization of the Arabidopsis floral organ identity proteins, *APETALA3* and *PISTILLATA*. *Plant J.*, **33**, 47–59.
- Zhang, X.K., Xia, X.C., Xiao, Y.G., Dubcovsky, J. and He, Z.H. (2008) Allelic variation at the vernalization genes *Vrn-A1*, *Vrn-B1*, *Vrn-D1* and *Vrn-B3* in Chinese common wheat cultivars and their association with growth habit. *Crop Sci.*, **48**, 458–470.