

MADS box genes control vernalization-induced flowering in cereals

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By comparing expression levels of MADS box transcription factor genes between near-isogenic winter and spring lines of bread wheat, *Triticum aestivum*, we have identified *WAP1* as the probable candidate for the *Vrn-1* gene, the major locus controlling the vernalization flowering response in wheat. *WAP1* is strongly expressed in spring wheats and moderately expressed in semispring wheats, but is not expressed in winter wheat plants that have not been exposed to vernalization treatment. Vernalization promotes flowering in winter wheats and strongly induces expression of *WAP1*. *WAP1* is located on chromosome 5 in wheat and, by synteny with other cereal genomes, is likely to be collocated with *Vrn-1*. These results in hexaploid bread wheat cultivars extend the conclusion made by Yan *et al.* [Yan, L., Loukoianov, A., Tranquilli, G., Helguera, M., Fahima, T. & Dubcovsky, J. (2003) *Proc. Natl. Acad. Sci. USA* 100, 6263–6268] in the diploid wheat progenitor *Triticum monococcum* that *WAP1* (*TmAPI*) corresponds to the *Vrn-1* gene. The barley homologue of *WAP1*, *BM5*, shows a similar pattern of expression to *WAP1* and *TmAPI*. *BM5* is not expressed in winter barleys that have not been vernalized, but as with *WAP1*, expression of *BM5* is strongly induced by vernalization treatment. In spring barleys, the level of *BM5* expression is determined by interactions between the *Vrn-H1* locus and a second locus for spring habit, *Vrn-H2*. There is now evidence that *AP1*-like genes determine the time of flowering in a range of cereal and grass species.

Many plants from temperate regions are induced to flower by an extended exposure to low temperature: vernalization. In winter cereal crops, such as wheat and barley, plant breeders have selected for variation in vernalization responsiveness to produce cultivars suited to plantings in different climatic zones. Winter cultivars are sown in autumn, vernalized by the low temperatures of winter, and subsequently flower and develop grain in spring. Spring cultivars do not require vernalization and usually are planted in the late winter period (reviewed in refs. 1–3).

The genetics of the vernalization response have been studied in a number of cereals. In hexaploid bread wheat, where the effects of recessive traits are masked by the redundancy resulting from the three genomes, the dominant *Vrn-1* gene has been found to be the major determinant of vernalization responsiveness. *Vrn-1* is located on chromosome 5 in each of the A, B, and D genomes of wheat. Winter wheats carry only winter *Vrn-1* alleles (*vrn-A1/vrn-B1/vrn-D1*), and without vernalization are late flowering. Spring alleles of *Vrn-1* are dominant and reduce the requirement for vernalization. Spring alleles of the *Vrn-1* gene on the A genome, *Vrn-A1*, have the strongest effect on flowering time, and plants with the *Vrn-A1* spring allele do not require any vernalization (4). The effects of spring alleles of the *Vrn-1* genes from the B and D genomes are weaker. Plants that carry *Vrn-B1* or *Vrn-D1* spring alleles (in the absence of a *Vrn-A1* spring allele) flower earlier than winter wheats but still show some acceleration in flowering time when vernalized. Such plants are classed as semispring wheats.

The *Vrn-1* locus has been mapped to the long arm of chromosome 5 (5–8). Orthologous genes have been identified in *Triticum monococcum* (*Vrn-A^{m1}*) (9) and in barley (*Vrn-H1*)

(10). In these diploid cereals recessive alleles of a second gene, *Vrn-2* (*vrn-2* for the recessive allele), can also confer the spring habit phenotype. The *Vrn-H2* locus of barley maps to the long arm of chromosome 4(H) (10, 11) and the *Vrn-A^{m2}* locus maps to a syntenic region on the long arm of chromosome 5 in *T. monococcum* (9). *vrn-A^{m2}* is epistatic to *Vrn-A^{m1}*, suggesting that these genes operate in a single genetic pathway controlling the vernalization response (12). Similar genetic interactions have been observed between *Vrn-H1* and *vrn-H2* in barley (11).

Recently, the *Vrn-A^{m1}* locus of *T. monococcum* was mapped to a small region of chromosome 5 that includes two MADS box genes (13). One of these MADS box genes, *TmAPI*, has an expression pattern consistent with a role in determining the vernalization response. In winter lines of *T. monococcum*, *TmAPI* expression is low in plants that have not been vernalized, but increases after vernalization. In spring cultivars that have *Vrn-A^{m1}* spring alleles and flower early without vernalization, *TmAPI* expression is high. *TmAPI* is a strong candidate for the *Vrn-1* gene (13). Sequencing of the promoter region of the *TmAPI* gene from *Vrn-A^{m1}* spring cultivars revealed small deletions of a region that includes a putative MADS box transcription factor binding site. Yan *et al.* (13) suggested that the removal of this binding site renders the promoter of *TmAPI* immune to the action of a proposed second MADS box protein that acts as a repressor to ensure that *TmAPI* is expressed only after vernalization treatment. Spring alleles of the *Vrn-A^{m1}* locus would be caused by constitutively derepressed *TmAPI* expression.

In this paper, we have compared the expression of MADS box transcription factor genes in near-isogenic winter and spring cultivars of hexaploid bread wheat, *Triticum aestivum*. We have identified 10 wheat MADS box genes that are expressed in vegetative tissues before the floral transition, including two vernalization-responsive MADS box genes. One of these is the hexaploid wheat orthologue of *TmAPI*, *WAP1*, and we suggest that this gene corresponds to the *Vrn-1* locus of hexaploid wheat. We also suggest that the barley orthologue of *WAP1*, *BM5*, corresponds to the *Vrn-H1* gene of barley.

Materials and Methods

Plant Growth. Seeds for the various wheat and barley cultivars were obtained from the Australian Winter Cereal Collection (www.dpi.qld.gov.au/extra/asp/auspgrs). Plants were grown in pots of soil in glasshouse conditions under a 16-h light/8-h dark photoperiod. Average temperature was 19°C. Nonvernalized seedlings were grown for 14 days. Vernalized seedlings were imbibed overnight in water, then planted in pots and kept at 4°C for 60 days under long-day conditions. At the end of this treatment, vernalized seedlings were equivalent in size to 7-day-old nonvernalized seedlings and were placed under glasshouse conditions for 7 days before harvesting. Barley cultivars of known genotype are described in the Barley Germplasm Data-

Abbreviations: TDA, Triple Dirk A; TDC, Triple Dirk C.

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base (www.shigen.nig.ac.jp/barley/Barley.html). Summaries of information pertaining to the wheat and barley cultivars used in this study are included in Tables 2 and 3, which are published as supporting information on the PNAS web site, www.pnas.org.

Genomic DNA Extraction and Southern Blots. Genomic DNA was extracted by using cetyltrimethylammonium bromide and purified on a cesium chloride gradient (14). DNA digestion, electrophoresis, blotting, and hybridization were performed by using standard methods (14). The 3' untranslated region of *WAP1* was used for a gene specific probe.

RNA Extraction and Gel Blot Analysis. Total RNA was extracted by using the RNA Extraction protocol of Chang *et al.* (15). Twenty micrograms of total RNA was separated in formaldehyde gels and then blotted to Hybond-N membrane as described in Ausubel *et al.* (14). RNA blots were hybridized with riboprobes and washed by using the protocol of Dolferus *et al.* (16). A *WAP1/BM5* specific riboprobe was amplified from the 3' end of the *WAP1* cDNA clone (BE431095) by using the sequences CTGAAACATATCAGATCCAGG and TATACACATCGCTGCAGCTTGC. Riboprobes were synthesized by using the Promega T7 *in vitro* transcription kit following the protocol provided. Blots were exposed to autoradiograph film (Fuji) for varying lengths of time, dependent on signal intensity.

RT-PCR Analysis. A oligo(T) primer (T₁₈[G/C/A]) was used to prime first-strand cDNA synthesis from 5 µg of total RNA by using the SuperScript reverse transcriptase enzyme (Invitrogen) according to the manufacturer's instructions. For PCR, primers were designed against the untranslated regions of cDNA sequences of wheat MADS box genes (Table 4, which is published as supporting information on the PNAS web site). Negative control reactions that lacked template or reverse transcriptase enzyme and a positive control reaction using genomic DNA as template were run simultaneously for each primer and template combination. Amplification of a fragment from the wheat *Ubiquitin* gene (X56601) was used to compare loading for each sample. Reactions were run for 30 cycles of 20 s at 94°C, 20 s at 60°C, and 30 s at 72°C, by using *Taq* polymerase (Fisher Biotechnology) in the polymerase buffer supplied by the manufacturer with 2.5 mM magnesium chloride. Fragments were visualized by agarose gel electrophoresis. For the *BM5* gene, the same reaction conditions were used with the primer pair TGAAGCTCAGAAATGGATTCG and TATGAGCGCTACTCTTATGC, which does not amplify a product from genomic DNA under these conditions but amplifies the predicted fragment size from first-strand cDNA. The resulting fragments were hybridized at high stringency with a *BM5*-specific DNA probe to demonstrate specificity of the PCR.

Results

An RT-PCR assay was used to compare the expression levels of 25 different MADS box genes (Fig. 8, which is published as supporting information on the PNAS web site) between two near-isogenic wheat lines that differ for spring or winter habit (4). The Triple Dirk C line (TDC) is a winter wheat (*vrn-A1/vrn-B1/vrn-D1*) that flowers late when not vernalized, whereas the Triple Dirk A line (TDA) has *Vrn-A1* and *Vrn-B1* spring alleles (*Vrn-A1/Vrn-B1/vrn-D1*) and flowers early without vernalization (17). Ten MADS box genes were expressed in vegetative (2-week-old) nonvernalized wheat plants before the floral transition. Differences in gene expression levels were observed between the spring and winter genotypes for two of these genes (Table 1). Strong expression of the *WAP1* gene (18) was detected in the spring cultivar TDA, but no expression of *WAP1* was detected in the winter line TDC (Fig. 1). The second MADS box

Table 1. Summary of RT-PCR results

Sequence	EST	Winter	Spring
<i>WAP1</i>	AB007504*		++
<i>TaMADS12</i>	AB007505*		
<i>TaMADS51</i>	AB007506*		
<i>TaMX1</i>	BJ316096		
<i>TaMX2</i>	BE423660		
<i>TaMX3</i>	BJ208073		
<i>TaMX4</i>	BJ233536	++	++
<i>TaMX5</i>	BM137344		
<i>TaMX6</i>	BF483385		
<i>TaMX8</i>	BE445262		
<i>TaMX9</i>	BE499520	++	++
<i>TaMX10</i>	BF484557	++	++
<i>TaMX11</i> [†]	BJ245749		
<i>TaMX12</i>	BJ245437		
<i>TaMX13</i>	BJ224514		
<i>TaMX14</i>	BJ314788	++	++
<i>TaMX15</i>	BJ247111		
<i>TaMX16</i>	BQ806253	++	++
<i>TaMX17</i>	BF483481	++	++
<i>TaMX18</i>	BJ245890		
<i>TaMX19</i>	BJ233463		
<i>TaMX20</i>	AL823256	++	++
<i>TaMX21</i>	BJ258612	++	++
<i>TaMX22</i>	BE445505		
<i>TaMX23</i>	BJ258117	++	+

The source ESTs for the MADS box sequences and whether the sequence was expressed in winter or spring plants. ++, strong amplification; +, weak amplification.

*Previously characterized gene.

[†]Amplification was unsuccessful with *TaMX11* primer set.

sequence, *TaMX23*, was more strongly expressed in TDC (winter) than in TDA (spring) (Fig. 1).

The near-isogenic Triple Dirk cultivar series generated by Pugsley (4, 17) includes winter, semispring, and spring lines, all of known genotype for the *Vrn-1* locus on each of the three genomes. RNA gel blot analysis was used to examine the expression of *WAP1* across the Triple Dirk series. In seedlings that were not vernalized, the level of *WAP1* expression was found to be related to the effect of the *Vrn-1* genotype on heading date (Fig. 2). High *WAP1* expression was observed in the spring Triple Dirk lines TD, TDA, and TDD, which carry the strong *Vrn-A1* spring allele and flower early. Moderate expression was detected in the semispring lines TDB and TDE, which carry either of the *Vrn-B1* or *Vrn-D1* spring alleles. No expression of *WAP1* was detected in the winter line TDC, which flowers late.

Analysis of *WAP1* gene expression was extended to other wheat cultivars. Spring, semispring, and winter lines of the cultivar Festival have been defined on the basis of heading dates. The exact *Vrn-1* genotypes of these lines are unknown, although, on the basis of the winter habit phenotype, the winter Festival line must lack spring *Vrn-1* alleles and therefore is likely to have the genotype *vrn-A1/vrn-B1/vrn-D1*. The pattern of *WAP1* expression in the different Festival lines parallels that observed in the Triple Dirk series (Fig. 2). High expression of *WAP1* was found in the spring Festival line, which flowers early without vernalization, moderate expression of *WAP1* was detected in semispring Festival and no *WAP1* expression was detected in winter Festival (Fig. 2). Furthermore, no expression of *WAP1* was detected in the winter wheat Cheyenne (Fig. 3) or in three lines of the winter wheat Black Hull (Fig. 3).

Expression of *WAP1* was examined in spring, semispring, and winter wheats that had been vernalized for 60 days. *WAP1* expression was strongly induced in winter lines by vernalization

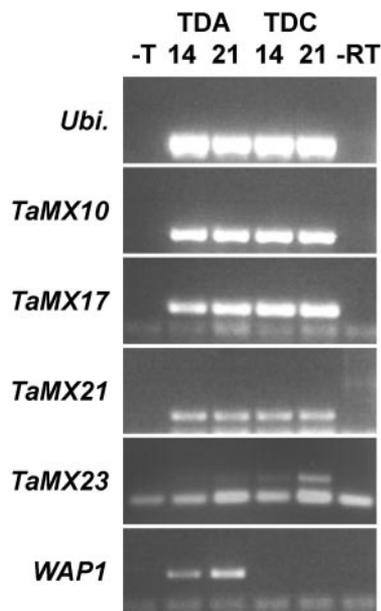


Fig. 1. RT-PCR analysis of wheat MADS box gene expression in 2-week-old plants. Shown are RT-PCR results for the positive control gene *Ubiquitin* and four of the MADS box genes expressed in wheat plants before floral transition, *TaMX10*, *TaMX17*, *TaMX21*, *TaMX23*, and *WAP1*. Control samples were water as template (–T) or without addition reverse transcriptase (–RT). Test samples included first-strand cDNA from 14- or 21-day old TDA (a strong spring wheat with the genotype *Vrn-A1/Vrn-B1/vrn-d1*) and 14- or 21-day-old TDC (a vernalization-responsive winter wheat, genotype *vrn-a1/vrn-b1/vrn-d1*). None of the plants were vernalized. The products were all ≈150 bp and match the sizes predicted on the basis of sequence.

treatment (Fig. 3). Likewise, expression of *WAP1* was induced in semispring lines and some induction was observed in the vernalized spring lines. After vernalization, the heading dates of the winter, semispring, and spring wheats examined were all similar (≈35 days after vernalization). Thus, the induction of *WAP1* expression in winter and semispring wheats correlates with the promotion of flowering.

The second MADS box gene *TaMX23*, which was found by RT-PCR to be also differentially expressed between TDA and TDC, was repressed by vernalization in the TDC and Cheyenne winter wheats (Fig. 3). Whereas the expression pattern of *WAP1* matches that which would be expected for a dominant promoter of flowering and is consistent across a number of wheat cultivars, the effect of vernalization on *TaMX23* expression was found to vary depending on which cultivar was examined. Furthermore, the rice homologue of *TaMX23* is located on chromosome 1 (marker S10304) in a region not known to be syntenic with any vernalization response genes in winter cereals. For these reasons, the role of *TaMX23* in the vernalization response of cereals was not characterized further in this study.

The role of *WAP1*-like genes in controlling the vernalization response of cereals was further examined in the important (diploid) cereal crop barley. The barley MADS box gene *BM5* shares a high degree of predicted amino acid similarity (95%) with *WAP1* (19). Furthermore, during plant development, *BM5* has a gene expression pattern similar to *WAP1* and is likely to be the barley orthologue of *WAP1* (18, 19). No expression of *BM5* was detected in six nonvernalized winter barley cultivars. Vernalization treatment strongly induced *BM5* expression in all six lines (Fig. 4, lanes 7–12 and 19–24). Thus, *BM5* shows a response to vernalization similar to *WAP1* and *TmAPI*.

Spring barley lines of known genotype for the *Vrn-H1*, *Vrn-H2*, and *Vrn-H3* genes were used to determine whether expression of

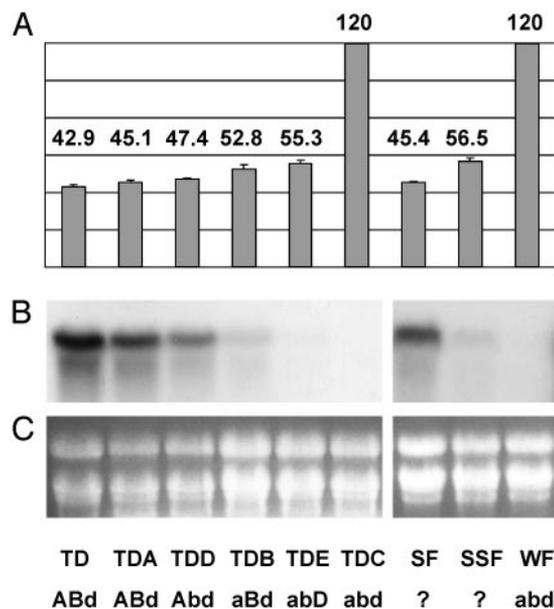


Fig. 2. Expression of *WAP1* compared with head emergence time for plants from the Triple Dirk and Festival cultivar series. (A) Days until head emergence for nonvernalized plants from each of the following lines: Triple Dirk (TD), Triple Dirk A (TDA), Triple Dirk D (TDD), Triple Dirk B (TDB), Triple Dirk E (TDE), Triple Dirk C* (TDC), Spring Festival (SF), Semispring Festival (SSF), and Winter Festival* (WF). The winter lines (*) did not show head emergence within the 120-day time period assayed. (B) Expression of *WAP1* in 2-week-old, nonvernalized plants from each line. *WAP1* expression was assayed by high stringency hybridization of RNA gel blots with a *WAP1*-specific riboprobe. Ethidium bromide staining of ribosomal RNA is shown in C as a loading comparison. The genotype of each line is included underneath each line with uppercase letters indicating dominant spring *Vrn-1* alleles. Lowercase letters represent recessive winter *vrn-1* alleles.

BM5 correlates with the spring habit phenotype of barley. All five genotypic combinations for spring habit that occur naturally in spring barleys were assayed for *BM5* expression (10). Expression of *BM5* was detected in lines that carry spring habit alleles at both the *Vrn-H1* and *Vrn-H2* loci (*Vrn-H1*/–, *vrn-H2/vrn-H2*) (Fig. 5), but not in spring barleys that carry spring habit alleles at only one of *Vrn-H1* or *Vrn-H2*. By using the more sensitive RT-PCR assay of *BM5* gene expression, we were able to detect expression of *BM5* in all spring barleys examined, including those lines that carry spring habit alleles at either of the *Vrn-H1* or *Vrn-H2* genes (Fig. 6). We found one winter barley line (Bollo) that also showed a low level of *BM5* activity detectable by RT-PCR analysis. We are not able to explain why this winter line, in contrast to all of the other winter lines we have examined, has some expression of *BM5*.

In six spring barley lines of unknown genotype, Northern analyses showed two (presumably containing spring alleles at both *Vrn-H1* and *Vrn-H2*) to have high expression levels of the *BM5* gene, equivalent to vernalized winter lines, and one with a moderate level of expression of the gene (Fig. 4, lanes 1, 4, and 15). In the other three lines, the Northern analyses did not detect any expression of *BM5* (Fig. 4, lanes 3, 13, and 17), but RT-PCR showed a low level of activity (Fig. 6). After vernalization treatment, *BM5* transcript levels in all six lines were equivalent to those of vernalized winter lines (Fig. 4, lanes 4, 6, 8, 14, 16, and 18).

Our results suggest that *Vrn-H1* and *Vrn-H2* interact to regulate *BM5* expression. A spring genotype at either the *Vrn-H1* or *Vrn-H2* locus leads to a low level of *BM5* expression detectable by RT-PCR. This low level of *BM5* expression is sufficient to cause an early flowering phenotype. The combination of spring

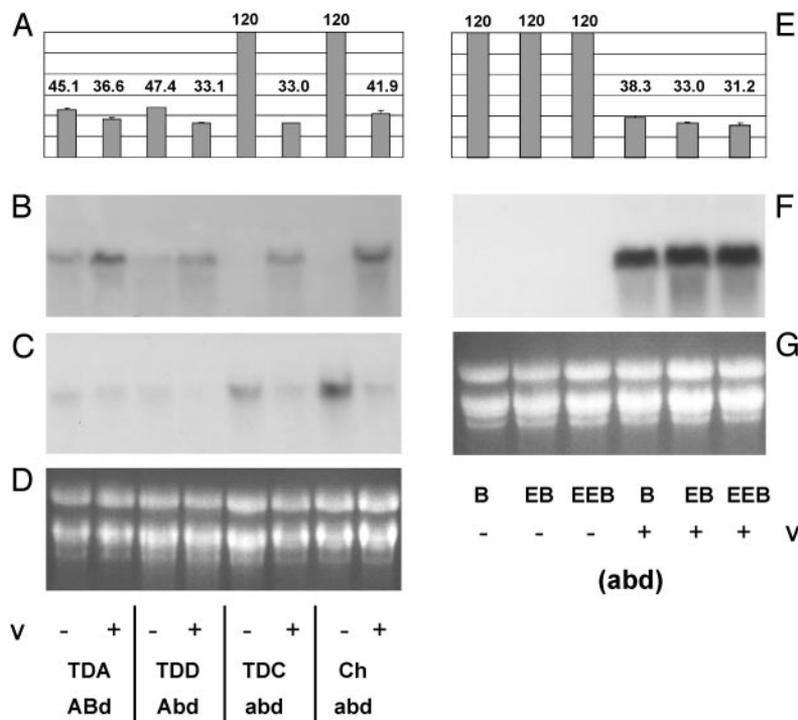


Fig. 3. Expression of *WAP1* compared with head emergence dates for vernalized and nonvernalized spring and winter wheat cultivars. (A–D) Data from nonvernalized and vernalized plants from the lines TDA, TDD, Triple Dirk C (TDC)*, and Cheyenne* (CH, a winter wheat). (E–G) Data from nonvernalized plants from three lines of the winter wheat Blackhull [Blackhull (B)*, Early Blackhull (EB)*, and Extra Early Blackhull (EEB)*], and then vernalized plants from each of the same three lines. Days until heading are shown for each combination of line and treatment in A and E. This does not include the 60-day vernalization period for the vernalized plants. Without vernalization, winter lines (*) showed no head emergence within the 120-day time period assayed. RNA gel blot analysis of *WAP1* expression in 2-week-old plants from each combination of line and treatment is shown in B and F. (C) Expression of *TaMX23* in 2-week-old vernalized or nonvernalized plants from the Triple Dirk series and winter wheat Cheyenne, analyzed by hybridization of RNA blots with a *TaMX23* specific riboprobe. Ethidium bromide staining of ribosomal RNA is shown in D and G as a loading comparison. The genotype of each line is included (a capital letter indicates dominant spring *Vrn-1* allele from the respective genome), as is vernalization status by + for vernalized and – for nonvernalized.

alleles at both loci (*Vrn-H1*/–, *vrn-H2*/*vrn-H2*) has a synergistic effect on *BM5* gene expression, leading to greatly increased *BM5* transcript levels that are then detectable by both Northern analysis and by RT-PCR.

Discussion

Our results show that the *WAP1* gene is likely to correspond to the *Vrn-1* locus of bread wheat. The *WAP1* genes are located on chromosomes 5A, 5B, and 5D in hexaploid wheats (Fig. 9, which is published as supporting information on the PNAS web site), the same chromosomes as *Vrn-A1*, *Vrn-B1*, and *Vrn-D1*, and by synteny with rice and *T. monococcum*, are most probably collocated with the *Vrn-1* loci. In nonvernalized plants, *WAP1* is strongly expressed in spring wheats, moderately expressed in semispring wheats, and is not expressed in winter wheats. Furthermore, the nearest rice homologue to *WAP1*, *RAP1b* (*OsMADS14*), causes an extreme early-flowering phenotype when overexpressed in rice (20), suggesting that *WAP1* expression promotes flowering in this cereal. These data are consistent with the results of Yan *et al.* (13), which led them to suggest that the *TmAPI* gene encodes the *Vrn-A^{m1}* gene of *T. monococcum*.

The Triple Dirk set of near-isogenic *Vrn-1* genotypes in bread wheat enabled us to examine the levels of *WAP1* expression associated with the *Vrn-1* alleles of each of the three genomes of wheat. The A genome locus, *Vrn-A1*, generates the strongest spring phenotype, and plants carrying the *Vrn-A1* allele show the strongest expression of *WAP1* in the absence of vernalization. Spring alleles of *Vrn-B1* and *Vrn-D1*, on the B and D genomes, respectively, produce semispring phenotypes and low levels of *WAP1* gene expression. Comparison of *WAP1* expression be-

tween spring and semispring lines shows that there is not a linear correlation between the level of *WAP1* activity and flowering times; TDA has strong expression of *WAP1* and a flowering time (heading date) of 45 days; the two semispring lines, TDB and TDE, have much lower *WAP1* expression levels but a flowering time of ≈54 days. It seems that even a low level of *WAP1* expression can elicit early flowering. Increases in *WAP1* expression above the effective threshold level seem to have a limited effect on flowering time. This is shown in semispring wheats, where vernalization causes a large increase in *WAP1* transcript level but has only a minor effect on heading date.

BM5, in barley, shows a similar expression pattern to *TmAPI* and *WAP1*. *BM5* is generally not expressed in nonvernalized winter barleys, but it is induced by vernalization. As is the case for *TmAPI*, *BM5* is expressed in spring barleys that have not been exposed to vernalization. Barley lines carrying dominant spring *Vrn-H1* alleles have a low level of *BM5* expression. Likewise, barleys homozygous for the recessive *vrn-H2* spring allele have a low level of *BM5* expression. Cultivars with the spring genotype at both the *Vrn-H1* and *Vrn-H2* loci have a higher level of *BM5* transcript, similar to that found in vernalized plants.

Small deletions occur in the promoter of the *TmAPI* genes from spring lines of *T. monococcum* that carry the dominant *Vrn-A^{m1}* for spring habit (13). Spring wheats and *Vrn-H1* spring barley lines may also carry alterations in the promoter of *WAP1* and *BM5*. Yan *et al.* (13) suggested that the small deletions found in the promoter of *TmAPI* from spring *T. monococcum* accessions render the promoter of *TmAPI* immune to the action of the *Vrn-A^{m2}* repressor. This seems not to be the case in barley. The level of *BM5* expression is much higher in plants with spring

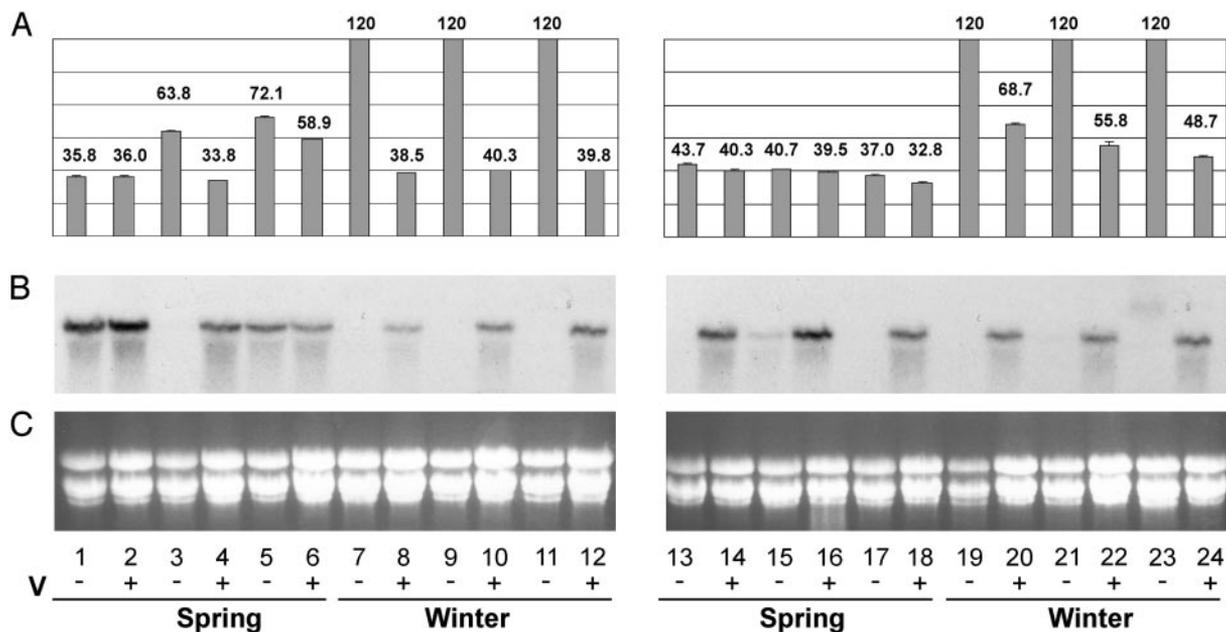


Fig. 4. Expression of *BM5* in vernalized and nonvernalized seedlings of 12 barley cultivars. Days until heading for nonvernalized or vernalized plants are shown in A. Lane 1, Morgenrot; lane 2, vernalized Morgenrot (+V); lane 3, Randolph; lane 4, Randolph +V; lane 5, Malta; lane 6, Malta +V; lane 7, Sonja*; lane 8, Sonja +V; lane 9, Hudson*; lane 10, Hudson +V; lane 11, Mirra*; lane 12, Mirra +V; lane 13, Perga; lane 14, Perga +V; lane 15, Dunja; lane 16, Dunja +V; lane 17, Will; lane 18, Will +V; lane 19, Hydra*; lane 20, Hydra +V; lane 21, Bollo*; lane 22, Bollo +V; lane 23, Igri*; lane 24, Igri +V. This does not include the 60-day vernalization period for the vernalized plants. Winter lines (*) did not show head emergence within the 120-day time period assayed when not vernalized. (B) Expression of *BM5* determined by RNA gel blot analysis for 2-week-old plants from the same combinations of lines and treatments. Ethidium bromide staining of ribosomal RNA is shown in C as a loading comparison. Vernalization status (–, nonvernalized; +, vernalized) and spring habit phenotype are shown beneath each cultivar/treatment combination.

genotype at both the *Vrn-H1* and *Vrn-H2* loci than in those that carry the *Vrn-H1* (or *vrn-H2*) spring genotype alone.

We suggest that *BM5* expression may be controlled by both an activator and a repressor, which interact to determine the response to vernalization. In winter barleys that have not been vernalized, the *BM5* promoter activity is low and is repressed by the *Vrn-H2* protein. We propose that vernalization treatment removes repression by *Vrn-H2* and further enhances the activity of the *BM5* promoter through the induction of a transcription factor that promotes *BM5* expression. A MADS box gene similar to *SOC1* or *AGL24*, which are vernalization-

responsive and promote flowering in *Arabidopsis* (21, 22), could be the activator of the *BM5* promoter in vernalized barley plants. The combined effect of these two regulatory systems would maintain a low level of *BM5* expression in unvernallized plants, and ensure strong induction of *BM5* by vernalization (Fig. 7). In *Vrn-H1* spring barleys, the *BM5* promoter would be active, but the action of the *Vrn-H2* repressor would repress this activity and the result would be a low level of *BM5* transcription. In a *vrn-H2* spring barley, the *Vrn-H2* repressor would not be active, but the activity of the *BM5* promoter would remain low in the absence of vernalization, resulting in a low level of *BM5* expression. The presence of spring alleles at both loci (*Vrn-H1*/–, *vrn-H2*/*vrn-H2*)

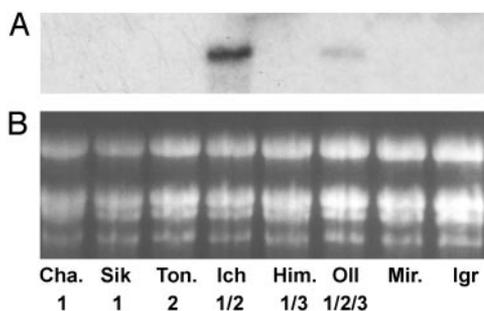


Fig. 5. Expression of *BM5* in seedlings of barleys of known spring habit genotype. Expression of *BM5* in nonvernalized barley cultivars was determined by hybridizing RNA gel blots with a *BM5*-specific riboprobe. Cha., Chame 11 (*Vrn-H1* spring); Sik, Sikangense Type 15 (*Vrn-H1* spring); Ton., Tongyeong Covered (*vrn-H2* spring); Ich, Icheon Naked (*Vrn-H1*/*vrn-H2* spring); Him., Himalayense Type 5 (*Vrn-H1*/*Vrn-H3* spring); Oll, Olli (*Vrn-H1*/*vrn-H2*/*Vrn-H3* spring); Mir., Mirra (winter); Igr, Igri (winter). Numbers below each lane indicate locus or loci where spring alleles are present: 1, *Vrn-H1*; 2, *vrn-H2*; or 3, *Vrn-H3*. Ethidium bromide staining of ribosomal RNA is shown in B as a loading comparison.

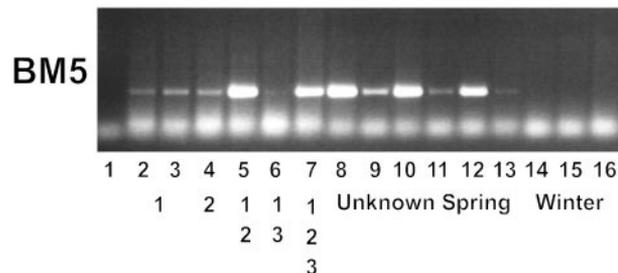


Fig. 6. RT-PCR assay of *BM5* expression in spring and winter barley lines. RT-PCR was performed with 12 spring and 3 winter barley cultivars, and the resulting fragments were separated by gel electrophoresis. Lane 1, No template; lane 2, Chame 11; lane 3, Sikangense Type 15; lane 4, Tongyeong Covered; lane 5, Icheon Naked; lane 6, Himalayense Type 5; lane 7, Olli; lane 8, Morgenrot; lane 9, Randolph; lane 10, Malta; lane 11, Perga; lane 12, Dunja; lane 13, Will; lane 14, Hudson; lane 15, Mirra; lane 16, Hydra. For spring barleys of known genotype, the presence of spring alleles is shown beneath each sample as 1 (*Vrn-H1*), 2 (*vrn-H2*), or 3 (*Vrn-H3*).

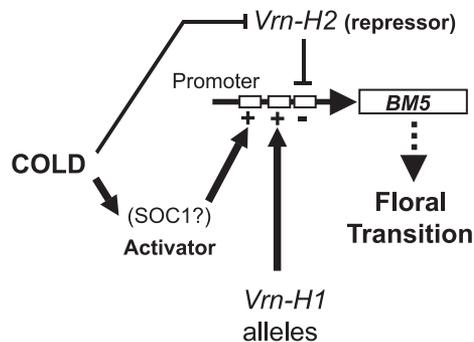


Fig. 7. A model for the regulation of *BM5* expression by vernalization and spring habit genotypes. Vernalization controls two genetic systems that regulate *BM5* transcription through the *BM5* promoter. One represses *BM5* expression in plants that have not been vernalized. Extended cold treatment counteracts this repression and activates a second regulatory mechanism that activates *BM5* transcription. Known flowering time genes such as the barley equivalent of *SOC1* are likely to be involved in this activation pathway. Recessive spring habit alleles of the *Vrn-H2* locus abolish the repression pathway and allow some expression of *BM5*. Dominant alleles of *Vrn-H1* activate expression regardless of vernalization status, but do not completely counteract repression, leading to some *BM5* activity. Combining both spring habit genotypes results in *BM5* expression levels that approach those in vernalized plants, through simultaneous activation and de-repression of the *BM5* promoter.

would result in greatly enhanced expression of *BM5*, relative to barleys with *Vrn-H1* or *vrn-H2* genotype alone. In the *Vrn-H1/vrn-H2* spring lines, the promoter of *BM5* would be both active and derepressed. It has been suggested that, in *T. monococcum*, the *Vrn-2* repressor is a MADS box gene (13).

Experiments in *Lolium temulentum* offer further evidence for the involvement of *API*-like genes in the floral transition in all grasses. Long days promote flowering in *L. temulentum*, and an *API*-like MADS box gene, *LtMADS1*, closely related in sequence to *BM5* and *WAP*, is one of the first genes to be induced after exposure to long day conditions (23). This raises the possibility that *WAP*-like genes could also promote flowering in response to photoperiod in wheat and barley.

In cereals, two MADS box proteins with opposite effects appear to be involved in the regulation of the vernalization response. This pathway resembles the vernalization response pathway of *Arabidopsis*, where *SOC1*, a MADS box gene that promotes flowering, is repressed by *FLC*, also a MADS box gene, in plants that have not been vernalized (21, 24, 25). No *FLC*-like genes have been identified in cereals, making it unlikely that *Vrn-2* is closely related to *FLC*. There is no evidence that *API*-like genes mediate the vernalization response in *Arabidopsis*, but overexpression of *API* does result in early flowering (26). The vernalization response may have evolved separately in the ancestors of the cereals (monocots) and the *Brassicaceae* (dicots) through the recruitment of different MADS box transcription factor genes into a cold regulated switch that promotes flowering in vernalized plants.

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