**WAP1, a Wheat APETALA1 Homolog, Plays a Central Role in the Phase Transition from Vegetative to Reproductive Growth**

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Heading time in bread wheat (*Triticum aestivum* L.) is determined by three characters: vernalization requirement, photoperiodic sensitivity and narrow-sense earliness, which are involved in the phase transition from vegetative to reproductive growth. We identified and characterized the APETALA1 (API)-like MADS box gene in wheat (*WAP1*) as an activator of phase transition. Its expression starts just before the phase transition and is maintained during the reproductive phase. Inhibition of *WAP1* expression in the transgenic plants by co-suppression affected neither vernalization requirement nor photoperiodic sensitivity, but resulted in delayed narrow-sense earliness, indicating that *WAP1* accelerates autonomous phase transition. Analyses of the *WAP1* expression in the near-isogenic lines (NILs) for spring habit genes (*Vrn*) revealed that *WAP1* transcripts were induced by vernalization strongly in the NILs with *Vrn* dominant alleles and weakly with the recessive alleles. Furthermore, *WAP1* expression was up-regulated by a long photoperiod in both NILs with and those without a photoperiod-insensitive gene (*Ppd*). These results suggest that *WAP1* is a key gene in the regulatory pathway controlling the phase transition from vegetative to reproductive growth in wheat.

**Keywords:** APETALA1 — Heading time — MADS box gene — Phase transition — *Triticum aestivum* L. — Wheat.

**Introduction**

Heading time associated with the timing of phase transition from vegetative to reproductive growth is an important character for cereal crops because of its influence on the adaptability to various environmental conditions. For example, the wide adaptability of bread wheat (*Triticum aestivum* L., 2n = 6x = 42, genome constitution AABBDD) results from its varietal variation in the heading time. The genetic control of heading time in wheat is determined by three component characters, i.e., vernalization requirement, photoperiodic sensitivity and narrow-sense earliness (earliness per se) (Yasuda and Shinozoyama 1965, Hoogendoorn 1985, Kato and Yamagata 1988). Vernalization requirement means the sensitivity of the plant to cold temperature for accelerating spike primordium formation, and vernalization-insensitive (spring habit) genes, *Vrn-A1, Vrn-B1* and *Vrn-D1*, were genetically identified on chromosomes 5A, 5B and 5D, respectively (for review see Flood and Halloran 1986, Worland et al. 1987, gene designation after McIntosh et al. 1998, Iwaki et al. 2002). The photoperiodic (long-day) response is determined by dominant genes, *Ppd1, Ppd2* and *Ppd3*, controlling insensitivity to a long-day photoperiod, located on chromosomes 2D, 2B and 2A, respectively (Welsh et al. 1973, Law et al. 1978). On the other hand, narrow-sense earliness or earliness per se is the earliness of fully vernalized plants grown under long-day conditions, and is controlled by polygenes with minor effects (Kato and Wada 1999). Although a lot of genetic studies have been done, little is known about the molecular mechanism of the phase transition from vegetative to reproductive growth in bread wheat.

Over the past decade, extensive studies in Arabidopsis have revealed the genetic and molecular mechanisms of the phase transition from vegetative growth to flowering (for review see Araki 2001, Mouradov et al. 2002). According to the current understanding in Arabidopsis, there are four major floral promotion pathways. The photoperiod and vernalization pathways integrate environmental signals into the phase transition, whereas the autonomous and gibberellin (GA) pathways act independently of external stimuli. The photoperiod path-
way promotes the phase transition in response to a long-day photoperiod, and CONSTANS (CO) and FLOWERING LOCUS T (FT) genes together with the circadian clock system mainly act in this pathway (reviewed by Yanovsky and Kay 2003). The vernalization pathway mediates the promotion of phase transition induced by low temperatures. Vernalization results in the stable reduction of the levels of floral repressor FLOWERING LOCUS C (FLC) by the epigenetic regulation. Genes involved in the autonomous pathway such as FCA also negatively regulate FLC expression, indicating that the vernalization and autonomous pathways are connected in the FLC gene. These two pathways commonly promote phase transition by reducing the level of FLC expression, which then promote the expression of SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1). The growth regulator GA promotes flowering by up-regulating the transcription level of a floral meristem identity gene LEAFY (LFY). FT, SOC1 and LFY in turn activate floral meristem identity gene APETALA1 (AP1) which promotes the formation of a floral meristem (reviewed by Sung et al. 2003). Among these genes involved in the phase transition from vegetative to reproductive growth in Arabidopsis, FLC, SOC1 and AP1 are members of the MADS box gene family encoding a large family of transcription factors (for review see Riechmann and Meyerowitz 1997). Although MADS box gene family was originally identified as the key gene in determining floral organ identity, it is now known to regulate many developmental processes such as fruit and root development (reviewed by Ng and Yanofsky 2001). Other than FLC, SOC1 and AP1, several MADS box genes were also identified to regulate flowering time in Arabidopsis; SHORT VEGETATIVE PHASE (SVP) (Hartmann et al. 2000), CAULIFLOWER (CAL), FRUITFULL (FUL) (Ferrandiz et al. 2000), and FLOWERING LOCUS M (FLM) (Scortecci et al. 2001). FLM is also referred to as MADS AFFECTING FLOWERING1 (MAF1), a member of five FLC paralogs which are involved in the vernalization response (Ratcliffe et al. 2003). These facts indicate that the MADS box gene family plays an important role in many steps of the phase transition from vegetative growth to flowering.

Previously, we reported that wheat MADS box genes constitute a multigene family which are dispersed throughout the
APETALAI homolog controlling wheat flowering

WAP1  MGRKVLKRIENKRIKQRTFSSRSLKLXHESLYLCDAEVLGLIFSTSKKLYFSTE
ToVRT1 MGRKVLKRIENKRIKQRTFSSRSLKLXHESLYLCDAEVLGLIFSTSKKLYFSTE
VRN1  MGRKVLKRIENKRIKQRTFSSRSLKLXHESLYLCDAEVLGLIFSTSKKLYFSTE
BMS  MGRKVLKRIENKRIKQRTFSSRSLKLXHESLYLCDAEVLGLIFSTSKKLYFSTE
L-MADS1 MGRKVLKRIENKRIKQRTFSSRSLKLXHESLYLCDAEVLGLIFSTSKKLYFSTE
RAP1B MGRKVLKRIENKRIKQRTFSSRSLKLXHESLYLCDAEVLGLIFSTSKKLYFSTE

WAP1  SCMDKILEERYSEYAEKVLSSYSEQWHEYNKRKLXKXQIKQKQHMEDLES
ToVRT1 SCMDKILEERYSEYAEKVLSSYSEQWHEYNKRKLXKXQIKQKQHMEDLES
VRN1  SCMDKILEERYSEYAEKVLSSYSEQWHEYNKRKLXKXQIKQKQHMEDLES
BMS  SCMDKILEERYSEYAEKVLSSYSEQWHEYNKRKLXKXQIKQKQHMEDLES
L-MADS1 SCMDKILEERYSEYAEKVLSSYSEQWHEYNKRKLXKXQIKQKQHMEDLES
RAP1B SCMDKILEERYSEYAEKVLSSYSEQWHEYNKRKLXKXQIKQKQHMEDLES

WAP1  NLKELQLEQQLSXLHRSNQKLMLHESLYELEQKQSLQVEELVQELVKQKA
ToVRT1 NLKELQLEQQLSXLHRSNQKLMLHESLYELEQKQSLQVEELVQELVKQKA
VRN1  NLKELQLEQQLSXLHRSNQKLMLHESLYELEQKQSLQVEELVQELVKQKA
BMS  NLKELQLEQQLSXLHRSNQKLMLHESLYELEQKQSLQVEELVQELVKQKA
L-MADS1 NLKELQLEQQLSXLHRSNQKLMLHESLYELEQKQSLQVEELVQELVKQKA
RAP1B NLKELQLEQQLSXLHRSNQKLMLHESLYELEQKQSLQVEELVQELVKQKA

WAP1  QAQQODQTQTOQSSSSSFMRRDAPPAAATSJHAGAAPAAGAVQPAAPRTGLPLW
ToVRT1 QAQQODQTQTOQSSSSSFMRRDAPPAAATSJHAGAAPAAGAVQPAAPRTGLPLW
VRN1  QAQQODQTQTOQSSSSSFMRRDAPPAAATSJHAGAAPAAGAVQPAAPRTGLPLW
BMS  QAQQODQTQTOQSSSSSFMRRDAPPAAATSJHAGAAPAAGAVQPAAPRTGLPLW
L-MADS1 QAQQODQTQTOQSSSSSFMRRDAPPAAATSJHAGAAPAAGAVQPAAPRTGLPLW
RAP1B QAQQODQTQTOQSSSSSFMRRDAPPAAATSJHAGAAPAAGAVQPAAPRTGLPLW

WAP1  MYSHING
ToVRT1 MYSHING
VRN1  MYSHING
BMS  MYSHING
L-MADS1 MYSHING
RAP1B MYSHING

Fig. 2  Comparison of amino acid sequence within the monocot subclade of AP1-like genes including WAP1. Sequences were taken from DDBJ database and aligned by the computer program CLUSTALS W. Amino acid residues identical and similar in all sequences are indicated by asterisks and dots, respectively. Dashes indicate gaps inserted to maximize alignment.

genome, and are preferentially expressed in young spikes (Murai et al. 1997). To investigate the function of wheat MADS box genes, we have cloned class A, B, and C MADS box genes; WAP1 (wheat AP1, formerly TaMADS#11), WAP3 (wheat AP3, formerly TaMADS#51) (Murai et al. 1998, Murai et al. 2002) and WAG (wheat AGAMOUS) (Meguro et al. 2003). To obtain an insight into the molecular mechanisms controlling the phase transition in wheat, we performed a transgenic study coupled with the expression analysis of WAP1. Recently, the expression of ToVRT-1, an AP1-like MADS box gene possibly identical to WAP1, was reported to be associated with the formation of spike primordia (Danyluk et al. 2003). However, transgenic analyses to investigate the function of this gene have not been reported. In this study, we produced co-suppression transgenic wheat lines in which the expression of endogenous WAP1 was reduced by the transgene even when the plants were vernalized. Inhibition of the WAP1 expression affected neither vernalization requirement nor photoperiodic sensitivity, but resulted in delayed narrow-sense earliness, indicating that WAP1 is involved in accelerating the autonomous phase transition. Together with the results that the level of WAP1 expression was increased just before the phase transition and was positively associated with vernalization and long photoperiod, our transgenic study demonstrated that WAP1 is a key gene in the regulatory pathway controlling the phase transition from vegetative to reproductive growth in wheat.

**Results**

**Phylogenetic study of WAP1, an AP1 homolog in wheat**

WAP1 (DDBJ accession no. AB007504) was isolated by screening of a cDNA library from young spikes of common wheat cv. Norin 26 with the degenerate PCR products of genomic DNA corresponding to the MADS box region as probes (Murai et al. 1998). The deduced amino acid sequence of WAP1 reveals a protein of 244 amino acid residues highly homologous to AP1. To examine the relationship between WAP1 gene and other members of the AP1-like gene family, a phylogenetic tree was reconstructed by using the amino acid...
sequences (Fig. 1). Arabidopsis MADS box genes controlling flowering time, FLC (Michaels and Amasino 1999, Sheldon et al. 1999), SVP (Hartmann et al. 2000), SOC1 (Borner et al. 2000, Lee et al. 2000, Samach et al. 2000), and FLM/MAF1 (Scortecci et al. 2001) were used as outgroup. The reconstructed tree indicated that the AP1-like gene family was clearly separated into dicot and monocot clades. The monocot clade was then divided into two subclades. WAP1 gene belongs to one subclade together with TaVRT-1 of wheat (Danyluk et al. 2003), VRN1 (unrelated to the gene with similar name in Arabidopsis) of T. monococcum (Yan et al. 2003), BM5 of barley (Schmitz et al. 2000), LmA d1 of Lolium temulentum (Gocal et al. 2001), and RAP1B of rice (Kyozuka et al. 2000). In addition to a serine-rich motif and LPPWMLSHL/IN sequence in the C-terminal region (Gocal et al. 2001), high sequence similarity among these genes throughout the coding region suggests that they are all orthologous genes (Fig. 2). WAP1 is 98.8% identical to TaVRT-1 and has only three amino acid changes in the C region. WAP1 was isolated from spring wheat cv. Norin 26 (Murai et al. 1998, Murai et al. 2002), whereas TaVRT-1 from winter wheat cv. Fredrick (Danyluk et al. 2003), suggesting that they should represent the same gene and the difference of sequence is due to varietal polymorphism.

Wheat genome contains three homoeologous WAP1 genes

To assign the copy number of the WAP1 gene in the wheat genome, we conducted Southern blot analysis using total DNAs digested by BamHI, EcoRI, EcoRV or HindIII (Fig. 3). Using WAP1 gene-specific probes derived from the 3′-region for hybridization with the DNA gel blot, three bands were detected in the blots of all enzymes (Fig. 3A), suggesting that there are three copies of the WAP1 gene in the wheat genome. Southern blot analysis of BamHI-digested DNAs from wheat cv. Chinese Spring (CS) nulli-tetrasomics was also performed to determine the chromosomal location of WAP1 genes. Nulli-tetrasomics are defined as a series of lines missing a pair of chromosomes that are replaced by an extra pair of their homoeologous chromosomes (Sears 1966). Chromosome locations of the genes should be thus determined by a lack of hybridizing band(s) for nullisomics, and an increased intensity of that for...
tetrasomics, against the pure line. Fig. 3B shows that in the CS line of nulli-5A tetra-5B the third hybridizing band was missing and the intensity of the second band was increased. On the other hand, the second band was missing and the first band increased the intensity in the nulli-5B tetra-5D line. Furthermore, the first band replaced the second band in the nulli-5D tetra-5B line. These results clearly demonstrate that the first band corresponds to the WAP1 gene located on chromosome 5D, the second on chromosome 5B, and the third on chromosome 5A. Consequently, in the wheat genome there are three homoeologous WAP1 genes located on the homoeologous group 5 chromosomes. We have cloned three cDNAs corresponding to three homoeologous genes of WAP1, which are more than 97% identical with each other in amino acid sequence (unpublished data).

Expression pattern of WAP1 in non-transformed ‘Akadaruma’ wheat

A transgenic study was performed using wheat cv. Akadaruma to identify the function of WAP1 in the phase transition from vegetative to reproductive growth. ‘Akadaruma’ wheat shows a winter habit. The progeny tests of F2 generation between ‘Akadaruma’ andtester lines with spring habit genes, Vrn-A1, Vrn-B1 and Vrn-D1, revealed that ‘Akadaruma’ has none of dominant alleles of the Vrn genes (data not shown). Before analysis of the transformants, we examined the expression pattern of WAP1 in the non-transformed ‘Akadaruma’ wheat. To examine the effects of developmental stage on the level of WAP1 mRNA, we performed RT-PCR analyses using the non-vernalized plants at the 1-, 3-, 5-, 7-, and 9-leaf stages under a 24-h light condition (Fig. 4A). These plants had formed 11 leaves in long-day conditions. Since WAP1 is expressed in the vegetative organs as well as inflorescence (spikes) (Murai et al. 1998), total RNA was extracted from leaves and used for RT-PCR. The RT-PCR analyses were performed with the primer set which detects the transcripts of all three homoeologous genes of WAP1. The transcripts of WAP1 were not observed in early stages and were increased significantly around the 5- to 7-leaf stage, when the growth phase changed from vegetative into reproductive, namely double-ridge stage. This is consistent with the report by Danyluk et al. (2003) that TaVRT-1 expression was associated with the timing of the double-ridge formation. The effect of vernalization on the WAP1 expression was also determined (Fig. 4B). Sprouted seeds were vernalized for 0-42 d at intervals of 7 d, and then transferred to a growth chamber at 20°C. In the plants at the 3-leaf stage, in which mRNA of WAP1 is not detected under the non-vernalized condition, the WAP1 mRNA was clearly detected after 21 d of cold treatment. A high level of WAP1 expression was maintained under the growth chamber at 20°C, indicating that WAP1 is not just a cold stress-induced gene. These results suggest that the expression of WAP1 is associated with the phase transition from vegetative to reproductive growth.

Analysis of transformed ‘Akadaruma’ wheat revealed the WAP1 function as an activator in phase transition

The WAP1 cDNA driven by rice actin1 gene (Act1) promoter was introduced through particle bombardment, and 12 lines were identified as the WAP1 transformants. Line #25 was selected for further study because of its high fertility and single locus integration of the WAP1 transgene. Among the progeny of #25 T0 plant (primary transformant), we selected two T1 lines, #25-2(2) and #25-5(2), homozygous to the WAP1 transgene by the progeny test of T2 generation. The integration of the WAP1 transgene was assessed by the PCR assay with a WAP1-specific primer set, by which transgene and endogenous WAP1 gene were distinguishable because the PCR products were different in size due to insertion/deletion of intron region (Fig. 5). The T1 lines of two T1 plants homozygous to the WAP1 transgene (T-WAP1+) line were used to examine the heading characters in a growth chamber. A non-transformed ‘Akadaruma’ wheat and the T1 lines of two T1 plants, which have not taken over the WAP1 transgene, #25-2(13) and #25-5(7), that is null segregant (T-WAP1- line), were used as the controls.

Table 1 shows the relationship between D2h (days to heading from the second leaf unfolding) and the duration of vernalization treatment for two each transgenic lines with WAP1 transgene (T-WAP1+) and without the transgene (T-WAP1-) together with ‘Akadaruma’ control. D2h reduced to a minimum value with increase of the length of the vernalization treatment. Vernalization requirement is represented by the minimum duration of vernalization which reduces D2h to the minimum value, and narrow-sense earliness is estimated by the average of D2h values after full vernalization. T-WAP1+ lines
sense earliness (Table 1, 2). This observation demonstrates that
* Indication of the treatment corresponding to vernalization requirement.
RT-PCR using the plants at the 3-leaf stage after 42 d of
periodic sensitivity is observed in the T-W AP1+ lines (Table 2).
tion to a short-day condition. No significant change in photope-
shown). On the other hand, photoperiod sensitivity was exam-
number of the T-W AP1+ lines tended to be increased, but not
showed the same level of vernalization requirement as T-
activate the autonomous phase transition from vegeta-
tive to reproductive growth in wheat.

**Relationships between WAP1 and Vrn or Ppd genes in phase transition**

We carried out expression studies using near-isogenic lines (NILs) for Vrn genes in wheat cv. Triple Dirk (TD) (Pugsley 1971, Pugsley 1972) to examine the up-regulation of WAP1 by vernalization more precisely. The effect of vernalization on the heading time of the Vrn-NILs of TD is shown in Fig. 6A. The NILs with Vrn-A1, Vrn-B1 and Vrn-D1 showed early-heading without vernalization, indicating that these Vrn genes activate the phase transition. Vernalization for 35 d advanced the heading time in NIL with all recessive alleles (Fig. 6A), in which the expression of WAP1 was induced by the cold treatment (Fig. 6B). This clearly indicates that the expression of WAP1 under the vernalization condition is associated with early heading. Contrary to the expression pattern in the NIL with vrn alleles, the expression of WAP1 was slightly detected in the non-vernalized plants of NILs with Vrn

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Days to heading (mean ± SE) from the second leaf unfolding (D2h) with a 24-h day length regime (20°C) after different lengths of vernalization treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of vernalization (days)</td>
<td>T-WAP1+ #25-2(2)</td>
</tr>
<tr>
<td>0</td>
<td>52.3±0.3 b</td>
</tr>
<tr>
<td>7</td>
<td>64.0±1.5 a</td>
</tr>
<tr>
<td>14</td>
<td>38.3±0.7 c</td>
</tr>
<tr>
<td>21</td>
<td>29.7±1.3 d</td>
</tr>
<tr>
<td>28</td>
<td>22.0±0.6 e*</td>
</tr>
<tr>
<td>35</td>
<td>22.3±0.3 e</td>
</tr>
<tr>
<td>42</td>
<td>22.0±0.6 e</td>
</tr>
<tr>
<td>49</td>
<td>21.7±0.3 e</td>
</tr>
<tr>
<td>Average</td>
<td>22.0 a</td>
</tr>
</tbody>
</table>

a,b Duncan’s new multiple-range test (5% level).
* Indication of the treatment corresponding to vernalization requirement.
† Mean D2h value in the treatments corresponding to full vernalization.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Days to heading from the second leaf unfolding (D2h) with a 24-h or 12-h day length regime (20°C) after 49 d of vernalization treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photoperiodic condition</td>
<td>T-WAP1+ #25-2(2)</td>
</tr>
<tr>
<td>24-h (A)</td>
<td>21.3</td>
</tr>
<tr>
<td>12-h (B)</td>
<td>25.7</td>
</tr>
<tr>
<td>(B)/(A)</td>
<td>1.13</td>
</tr>
</tbody>
</table>

a Data from one plant.
b Photoperiodic sensitivity.
were vernalized for 0, 7, 14 and 35 d. The NILs with different pathways in promoting the phase transition.

The NILs with \( WAP1 \) locus (–) were vernalized for 0 and 35 d. (B) RT-PCR analysis of the \( WAP1 \) gene with and without the TaVRT-1 gene acts to reduce the delay in heading under short-day conditions (Fig. 7A). Both NILs with \( Vrn-A1 \) dominant allele and with all recessive alleles of the three locus (–) were vernalized for 0 and 35 d. (B) RT-PCR analysis of the \( WAP1 \) gene is used as a control. The NILs with \( Vrn-A1 \) dominant allele and with all recessive alleles (–) were vernalized for 0, 7, 14 and 35 d.

The level of expression was greatly increased by a short cold treatment (Fig. 6B). Danyluk et al. (2003) did not observe the up-regulation of TaVRT-1 by vernalization in wheat lines with \( Vrn \) genes, because they used plant materials given cold treatment for 3 d.

To examine the effect of photoperiod on the expression of \( WAP1 \) and the relationship between \( WAP1 \) and \( Ppd \) genes, we analyzed the expression of \( WAP1 \) using NILs for the \( Ppd \) gene. Wheat is a quantitative long-day plant, and short-day conditions delay the heading time. \( Ppd \) gene acts to reduce the delay of heading under short-day conditions (Fig. 7A). Both NILs with and without the \( Ppd \) gene exhibited the expression of \( WAP1 \) under long-day conditions, but not under short-day conditions (Fig. 7B), indicating that \( WAP1 \) and \( Ppd \) genes act on different pathways in promoting the phase transition.

**Discussion**

We characterized the wheat \( AP1 \) homolog \( WAP1 \), which was previously cloned from a cDNA library of wheat young spikes (Murai et al. 1998, Murai et al. 2002). Phylogenetic study clearly indicated that the \( AP1 \)-like MADS box genes were classified into the dicot and monocot clades (Fig. 1). Furthermore, the monocot clade can be classified into two sub-clades. The first group includes \( WAP1 \) together with \( TaVRT-1 \) of bread wheat (Danyluk et al. 2003), \( VRN1 \) of diploid wheat \( T. monococcum \) (Yan et al. 2003) and \( LiMADS1 \) of \( L. temulentum \) (Gocal et al. 2001). Expression analyses suggested that both \( TaVRT-1 \) and \( LiMADS1 \) are associated with the phase transition from vegetative to reproductive growth. In addition, \( VRN1 \) in diploid wheat was recently identified as a vernalization-related gene by positional cloning (Yan et al. 2003). Since the sequence homology of the MADS box genes is highly related to functional homology (Purugganan et al. 1995, Theissen et al. 1996), these findings suggest that the subclade with \( WAP1 \) could be consistent with monocot MADS box genes associated with the phase transition. In Arabidopsis, \( AP1 \) acts to specify the identity of the floral meristem and to determine sepal and petal development (Mandel et al. 1992). In a previous study, we reported that \( AGAMOUS \) (\( AG \)) group of MADS box genes in monocots were also divided into two groups, which are likely to be correspond to the two \( AG \) functions; specification of stamina and carpel identities, and determination of floral meristem (Meguro et al. 2003). Therefore, it can be hypothesized that \( AP1 \) activity is also shared or separated by two classes of MADS box genes in monocots, for example \( LiMADS1 \) and \( LiMADS2 \) in \( L. temulentum \). Gocal et al. (2001) revealed that \( LiMADS2 \) has the ability to partially complement mutant phenotype when introduced into Arabidopsis \( ap1 \) mutant, whereas \( LiMADS1 \) has not, supporting the above hypothesis. More precise studies on the functional difference between BM5 and BM8 in barley, and \( RAP1B \) and \( OsMADS15 \) (possibly the same as \( RAP1A \)) in rice, as well as \( LiMADS1 \) and \( LiMADS2 \), are necessary to test this hypothesis.

Bread wheat is a hexaploid species with genome constitution AABBDD, which originated from three diploid relative species: A genome from \( T. urartu \), B genome from \( Ae. speltoides \) or other species classified into Sitopsis section, and D genome from \( Ae. tauschii \) (Feldman 2001). Allopolyploidy leads to the generation of duplicated homoeologous genes, which is opposed to paralogous genes. Consequently, the hexaploid wheat genome contains triplicated homoeologous genes derived from the ancestral diploid species. In this study, we demonstrated that \( WAP1 \) has three homoeologous genes located on group 5 homoeologous chromosomes, 5A, 5B and 5D (Fig. 3). We also previously identified three homoeologous genes of the wheat \( AG \)-like MADS box gene \( WAG \) in the wheat homoeologous group 1 chromosomes, 1A, 1B and 1D (Meguro et al. 2003). There are three possibilities for the evolutionary fates of homoeologous genes in polyploidy: functional diversification, gene silencing, and retention of original and similar function (Wendel 2000). Among them, the functional diversification of homoeologous genes is believed to be a reason for the evolutionary success of polyploid species. Did the functional diversification occur among \( WAP1 \) homoeologous genes as well as \( WAG \) in the hexaploid wheat genome? Studies on the genomic constructions, especially in the promoter
APETALA1 homolog controlling wheat flowering

The present study revealed that WAP1 is expressed just before the phase transition from vegetative to reproductive growth (Fig. 4A), and is up-regulated by vernalization and long photoperiod (Fig. 4B, 6B, 7B). Together with these findings, our transgenic study indicated that the WAP1 gene products act as an activator in the phase transition and that the timing when WAP1 expression is induced determines the earliness of heading time. Recently, Yan et al. (2003) reported that VRN1, an orthologous gene of Vrn-A1 on 5A chromosome of bread wheat, is an AP1-like gene located on 5A* chromosome of diploid wheat T. monococcum. WAP1 is 98% identical to VRN1 and has only five amino acid changes (Fig. 2). Furthermore, our nulli-tetrasomic analysis indicated that WAP1 has three homoeologous genes located on group 5 homoeologous chromosomes in bread wheat (Fig. 3). These findings strongly suggested that WAP1 is ortholog of VRN1, and consequently three homoeologous genes of WAP1 located on 5A, 5B and 5D correspond to Vrn-A1, Vrn-B1, and Vrn-D1 genes, respectively. The level of WAP1 expression was increased by vernalization in the NIL with vrn recessive alleles. How is WAP1 (= Vrn genes) up-regulated by vernalization? In diploid wheat, Dubcovsky et al. (1998) identified a second gene affecting vernalization response and named it Vrn-A*2 (renamed VRN2 now). Contrary to the VRN1 dominant allele for spring growth habit, VRN2 allele for winter growth habit is dominant (Tranquilli and Dubcovsky 2000). The effect of VRN1 on heading time was significant only when the dominant VRN2 allele was present, that is VRN2 is epistatic to VRN1. Although the VRN2 gene has not been cloned, Yan et al. (2003) proposed a model of the vernalization pathway in diploid wheat according to the knowledge of the epistatic interactions between VRN1 and VRN2 and the available results. In their model, VRN2 encodes a repressor of VRN1 expression, which binds to the promoter region of the VRN1 gene. As the vernalization process reduces the abundance of the VRN2 gene product, VRN1 transcription gradually increased, leading to the competence to flower. The 20-, 34-, or 48-bp deletion within the promoter region was present in spring habit accessions in diploid wheat, whereas none of the winter accessions showed deletions in this region (Yan et al. 2003), supporting this model. Does the VRN2 gene also act in bread wheat? It is interesting that the VRN2 locus has not been identified in bread wheat. It is possible that in hexaploid wheat there are three homoeologous orthologs of VRN2 which have redundant functions each other, resulting in unlikely event of finding mutant phenotype. Assuming that the expression of WAP1 is repressed by VRN2 orthologs strongly in vrn recessive allele and weakly in Vrn dominant allele in bread wheat, we can explain why WAP1 is up-regulated by vernalization in the spring habit NIL as well as in the winter habit NIL (Fig. 6B). Promoter analysis of the WAP1 homoeologous genes and cloning of the VRN2 orthologs are necessary to understand
vernalization requirement in bread wheat. Based on the results of this study, we present a model of regulation of the phase transition from vegetative to reproductive growth in bread wheat (Fig. 8). In this model, WAP1 (≡ Vrn genes) plays a pivotal role in the control of phase transition with the vernalization and photoperiodic pathways merging to activate WAP1. The present study revealed that there is no difference in the WAP1 expression pattern in the NILs between Ppd dominant and recessive alleles (Fig. 7B), indicating that the Ppd gene is involved in a pathway different from the WAP1-related pathway in the phase transition. Investigation of the regulation of WAP1 expression by cold and long photoperiod may lead to an understanding of the phase transition in bread wheat.

Materials and Methods

Plant materials and growth conditions

A bread wheat (Triticum aestivum L.) cv. Norin 26 (N26) was used for cDNA cloning and Southern/northern blot analysis in the present as well as the previous study (Murai et al. 1998). Nulli-tetrasomics of a common wheat cv. Chinese Spring (CS), which are defined as a series of lines missing a pair of chromosomes that are replaced by an extra pair of their homoeologous chromosomes (Sears 1966) were also used for Southern blot analysis. N26 and CS are spring habit cultivars and are known to have "winter" genes, and are vernalization insensitive. These NILs of TD were developed by particle bombardment with the plasmids containing cDNA of WAP1 gene and the bar gene as a selectable marker under the control of the rice actin1 gene (Ubi-1) promoter according to the method of Takumi and Shimada (1996). The integration of the WAP1 transgene was assessed by PCR with a primer set WAP1-553L (5’-AAAGATCAGACTCGCCCTCAA-3’) and WAP1-982R (5’-TAGAGACGGGTATCATGGGAA-3’). As a control, a fragment from the wheat ubiquitin gene (Ubi-1) was amplified using the primers Ubi-1L (5’-GCATGCAGATATTTGTGAA-3’) and Ubi-1R (5’-GGGACTTTGCGCCAC-3’). The PCR conditions were as follows: initial denaturation at 94°C for 3 min; 27–33 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min; final extension at 72°C for 3 min. The PCR products in the exponential range of amplification were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed.

Transgenic study

Twenty-eight transgenic lines of bread wheat (T. aestivum L.) cv. Akadaruma were produced by particle bombardment with the plasmids containing cDNA of WAP1 gene and the bar gene as a selectable marker under the control of the rice actin1 gene (Act1) promoter according to the method of Takumi and Shimada (1996). The integration of the WAP1 transgene was assessed by PCR with a primer set WAP1-553L (5’-AAAGATCAGACTCGCCCTCAA-3’) and WAP1-1003R (5’-TTCACATAAACAACATCCCA-3’). Transgene and endogenous WAP1 gene were distinguishable because the PCR products were different in size due to insertion/deletion of the intron region. Twelve lines were selected as the WAP1 transformants and line #25 was used for the following experiments because of its high fertility and single integration of the WAP1 transgene. Selfed progeny (T₁ and T₂ plants) of T₁ plants (primary transformants) were examined for the transmission of the WAP1 transgene. The T₂ lines of two T₁ plants homozygous to the WAP1 transgene (#25-2-2) and #25-5-2: T-WAP1+ lines) were used for characterization of the heading traits. Non-transformed ‘Akadaruma’ wheat and the T₁ lines of two T₁ plants, which have not taken over the WAP1 transgene, that is null segregant (#25-2-13) and #25-5-7: T-WAP1- lines), were used as the controls. Seedlings of the 3-leaf stage after 42 d of cold treatment were used for expression analysis.
Evaluation of heading characters using growth chamber

Vernalization requirement and narrow-sense earliness of transgenic lines were evaluated based on the method developed by Kato and Yamagata (1988). Sprouted seeds were sown in small soil-filled containers and then vernalized at 4°C for various periods ranging from 0 to 49 d at intervals of 7 d. The second leaf did not unfold during the vernalization treatment for 0–49 d. After the vernalization treatment, they were grown in a growth chamber under a 24-h day length regime at 20°C. Three plants per line were allotted to each treatment, and the number of days to heading from the second leaf unfolding (D2h) was scored for each plant. The differences between D2hs at various vernalization conditions were statistically evaluated by using Duncan’s new multiple range tests. Vernalization requirement was estimated by the minimum duration of the vernalization treatment, which reduces D2h to a constant value. Narrow-sense earliness was also statistically evaluated based on the differences in the constant value of the D2h.

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