TaVRT-2, a Member of the *St*MADS-11 Clade of Flowering Repressors, Is Regulated by Vernalization and Photoperiod in Wheat[1][w]

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The initiation of the reproductive phase in winter cereals is delayed during winter until favorable growth conditions resume in the spring. This delay is modulated by low temperature through the process of vernalization. The molecular and genetic bases of the interaction between environmental factors and the floral transition in these species are still unknown. However, the recent identification of the wheat (*Triticum aestivum* L.) *TaVRT-1* gene provides an opportunity to decipher the molecular basis of the flowering-time regulation in cereals. Here, we describe the characterization of another gene, named *TaVRT-2*, possibly involved in the flowering pathway in wheat. Molecular and phylogenetic analyses indicate that the gene encodes a member of the MADS-box transcription factor family that belongs to a clade responsible for flowering repression in several species. Expression profiling of *TaVRT-2* in near-isogenic lines and different genotypes with natural variation in their response to vernalization and photoperiod showed a strong relationship with floral transition. Its expression is up-regulated in the winter genotypes during the vegetative phase and in photoperiod-sensitive genotypes during short days, and is repressed by vernalization to a level that allows the transition to the reproductive phase. Protein-protein interaction studies revealed that *Ta*VRT-2 interacts with proteins encoded by two important vernalization genes (*TaVRT-1/VRN-1* and *VRN-2*) in wheat. These results support the hypothesis that *Ta*VRT-2 is a putative repressor of the floral transition in wheat.

Flowering is one of the most crucial developmental programs that plants use to ensure survival and reproductive success. The timing of this process is under the control of several interdependent pathways (for review, see Mouradov et al., 2002; Simpson and Dean, 2002; Henderson et al., 2003). The photoperiod and vernalization pathways respond to environmental signals, whereas the autonomous and GA-dependent pathways integrate the endogenous developmental state of the plant. In the model system Arabidopsis (*Arabidopsis thaliana*), the effect of low temperature (LT) on flowering time was found to be mediated in part by FLOWERING LOCUS C (*At*FLC), a MADS-box protein that delays floral transition (Michaels and Amasino, 1999). Recent evidence indicates that *At*FLC functions in part by directly repressing the expression of the Arabidopsis gene *SUPPRESSOR OF CONSTANS 1* (*AtSOC1*; for review, see Boss et al., 2004), which encodes a MADS-box protein that accelerates flowering. Interestingly, the *AtSOC1* gene was also shown to be the target of CONSTANS (*At*CO; Hepworth et al., 2002). The convergence of *At*CO and *At*FLC at the promoter of *AtSOC1* could be one mechanism by which photoperiodic and cold signaling pathways, respectively, are integrated to ensure an appropriate seasonal control of flowering time (Hepworth et al., 2002). Different regions of the *AtSOC1* promoter have been found to mediate activation by *At*CO under long-day photoperiod and repression by *At*FLC, which itself is negatively regulated by vernalization (Hepworth et al., 2002).

In winter cereals, flowering is delayed during winter until the favorable growth conditions of spring. This delay allows plants to fulfill their vernalization requirement, an important trait in winter growth habit cereal species. The recent cloning and characterization of *Vrn-A^{m}1* from *Triticum monococcum* (Yan et al., 2003) and *TaVRT-1* from *Triticum aestivum* (Danyluk et al., 2003) provides an opportunity to unravel the molecular basis of the floral inductive pathway in cereals. Expression profiling and genetic analysis of *VRN-1/TaVRT-1* showed that transcript accumulation is associated with the vernalization response, the transition from vegetative to reproductive phase, the expression of cold-regulated (*COR*) genes, and the degree of freezing tolerance (FT). The gene encodes a MADS-box

[1] This work was supported by a Natural Sciences and Engineering Research Council of Canada discovery grant (to F.S.), and by Genome Canada, Genome Québec, and Genome Prairie grants (to F.S. and D.B.F.).

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[w] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.061762.

protein that belongs to the AP1/SQUA-like clade of transcriptional regulators whose members have been implicated in functions such as determining inflorescence meristem identity and flower development. It is becoming apparent that variations detected in different *VRN-1* alleles of temperate cereals are important for specifying vernalization requirement (Yan et al., 2003, 2004a; Beales et al., 2005; Fu et al., 2005). However, the exact molecular mechanisms involved in the interaction between environmental factors and allelic variants at the *Vrn-1* locus remain unknown.

Several other components of the floral pathway identified in Arabidopsis seem to have functional equivalents in cereals. The genes underlying the rice (*Oryza sativa*) flowering-time quantitative trait loci (QTLs) *Hd1*, *Hd3a*, and *Hd6* are closely related to the Arabidopsis genes *AtCO*, *FLOWERING LOCUS T* (*AtFT*), and *α-subunit of protein kinase CK2*, respectively, and their products play key roles in the photoperiodic flowering pathway (Yano et al., 2000; Takahashi et al., 2001; Izawa et al., 2002). In addition, an *SOC1*-like gene from rice, *OsSOC1*, can complement the Arabidopsis *soc-1* mutant and promote flowering (Tadege et al., 2003), whereas overexpression of *OsGI*, a rice ortholog of the Arabidopsis photoperiod pathway gene *GIGANTEA* (*AtGI*), was shown to regulate the expression of the downstream genes *OsCO* and *OsFT* (Hayama et al., 2003).

There are, however, differences between the components of the flowering pathway in cereals and Arabidopsis. *FLC*-like genes have so far been identified only in the Brassicaceae (Becker and Theissen, 2003), and the recent identification of the floral repressor *Vrn-A^{m}2* gene in *T. monococcum* showed that it encodes a substantially diverged member of a zinc-finger family of transcription factors present only in temperate cereal species (Yan et al., 2004b). These observations raise the question whether similar interactions of transcriptional activator/repressor proteins regulate flowering time in cereals as has been shown in Arabidopsis. To understand these interactions and identify the genes involved in the floral inductive pathways in cereals, we pursued molecular analysis of other MADS-box genes found in the Functional Genomics of Abiotic Stress (FGAS; a Genome Canada project) wheat expressed sequence tag (EST) database. Expression profiling, bioinformatics analysis, and phylogenetic and genetic studies revealed that a MADS-box gene, which we have named *TaVRT-2*, encodes a potential repressor of the floral inductive pathway in hexaploid wheat. The putative function of this gene in the control of the transition from vegetative to reproductive phase in wheat is discussed.

RESULTS

Identification and Molecular Characterization of *TaVRT-2*

To identify components of the regulatory network involved in the vernalization pathway in hexaploid wheat, we selected MADS-box genes by homology-based search of the FGAS wheat EST collection. Expression profiling of seven MADS-box genes revealed that three of them, including the previously published *TaVRT-1* gene (Danyluk et al., 2003), were differentially regulated by LT (data not shown). One of the other two genes showed an inverse pattern of expression relative to that of *TaVRT-1* in that the level of expression is high during the early stage of LT exposure but starts to decline toward the vegetative/reproductive transition point. This decrease is concomitant with the increase in expression observed for *TaVRT-1*. This suggests possible interactions between the two genes and could indicate that their products act in an opposite manner in the flowering inductive pathway. This gene was therefore named *TaVRT-2* (*Triticum aestivum* vegetative to reproductive transition gene-2).

The *TaVRT-2* gene encodes a predicted protein of 226 amino acids (Supplemental Table I). A search in public databases for conserved domains in the *Ta*VRT-2 protein revealed an MIKC structure (M, MADS domain; I, intervening region; K, K box; C, C-terminal domain), a conserved bipartite nuclear targeting sequence in the MADS domain, and several putative phosphorylation sites (Fig. 1). Sequence comparison with several MADS-box proteins from monocots and dicots revealed that *Ta*VRT-2 is homologous to MADS-box proteins from the *St*MADS11-like clade (Carmona et al., 1998; Becker and Theissen, 2003). *Ta*VRT-2 shows 96% amino acid sequence identity with its reconstructed ortholog from barley (*Hordeum vulgare*; named *Hv*VRT-2), 77% with two proteins from maize (*Zea mays*; *Zm*M19 and *Zm*M26), 73% with a protein from rice (*Os*MADS22), and 51% with the SHORT VEGETATIVE PHASE protein from Arabidopsis (*At*SVP), the closest predicted homolog from dicot species (Supplemental Table II).

A phylogenetic analysis was performed using several members of the AP1/SQUA-, FLC-, *St*MADS11-, and AG-like clades, the latter being used as an outgroup. The results of this analysis classified *Ta*VRT-2 into the *St*MADS11-like regulators clade (Fig. 2). Transcription factors of this clade have been associated with the repression of transition of the shoot apex from the vegetative to the reproductive phase (Carmona et al., 1998; Becker and Theissen, 2003).

Mapping of the *TaVRT-2* Gene

To determine the location of *TaVRT-2* in relation to *TaVRT-1*, we performed a DNA gel-blot analysis on ditelocentric chromosome lines of hexaploid wheat (cv Chinese Spring). The results show that *TaVRT-2* is located on the short arms of chromosomes 7A and 7B, as indicated by the missing bands in lines 7AL and 7BL (Fig. 3A). The ditelocentric 7DL line was unavailable, but, since no bands were missing in the 7DS line, it was likely that the D genome copy was on the short arm of chromosome 7D. The presence of a copy of *TaVRT-2* on the 7D chromosomes was confirmed by

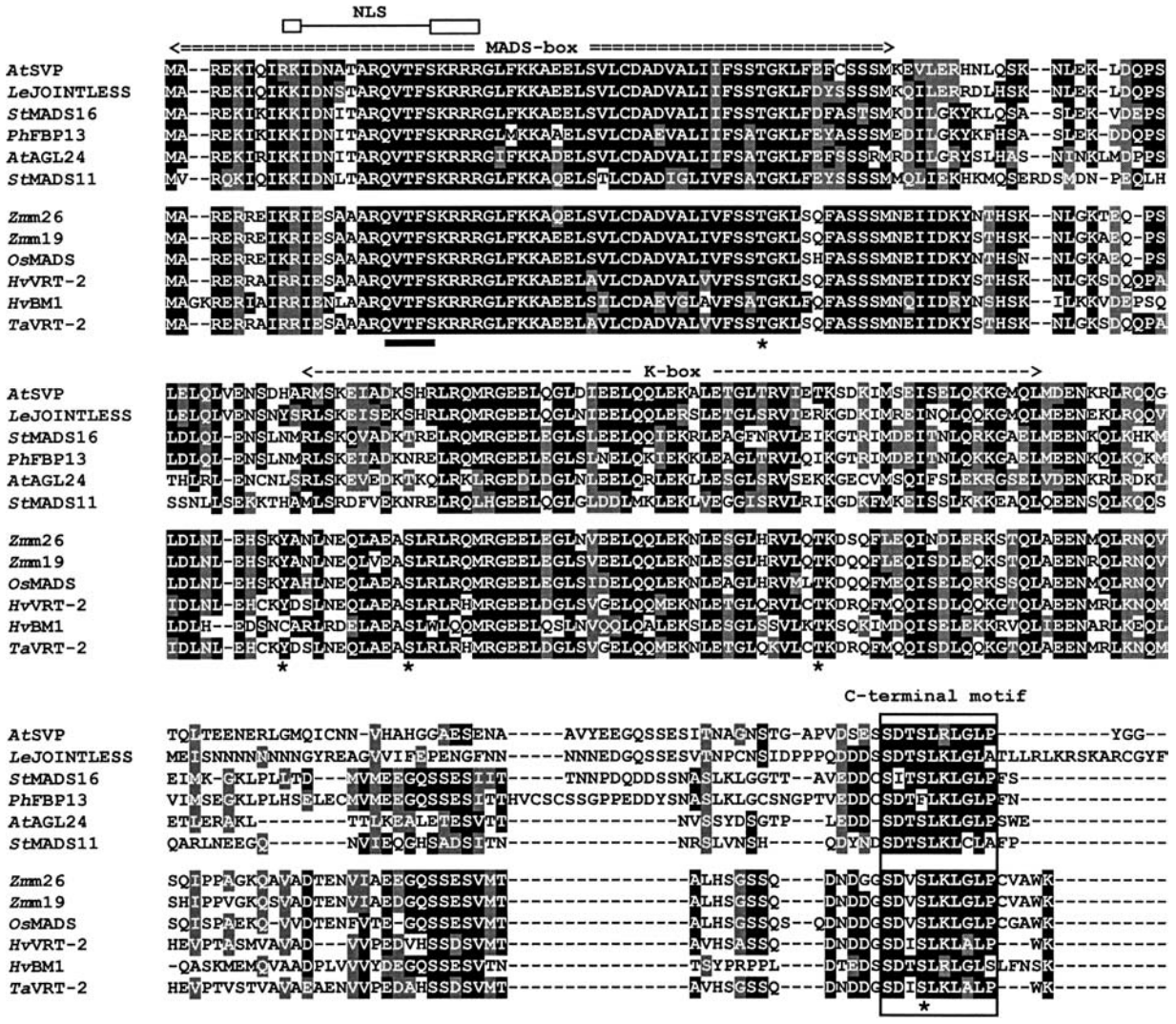

Figure 1. Alignment of *Ta*VRT-2 with related MADS-box proteins from other species. The deduced amino acid sequence of proteins from *T. aestivum* (*Ta*VRT-2), rice (*Os*MADS), maize (*Zm*m19, *Zm*m26), barley (*Hv*VRT-2, *Hv*BM1), *Solanum tuberosum* (*St*MADS16, *St*MADS11), *Petunia x hybrida* (*Ph*FBP13), Arabidopsis (*At*AGL24, *At*SVP), and *Lycopersicon esculentum* (*Le*JOINTLESS) were aligned using ClustalW (gap opening = 10.00; gap extension = 0.10). Accession numbers are given in Supplemental Table II. The double and single dash bars indicate the location of the MADS and K boxes, respectively. The arrow indicates the *Ta*VRT-2 nuclear localization signal (NLS) identified by PSORT (Nakai and Horton, 1999). The bar indicates the potential phosphorylation site (QVTFS) for calmodulin-dependent protein kinases (Carmona et al., 1998). The asterisks represent other predicted phosphorylation sites using ScanProsite (Gattiker et al., 2002). A conserved C-terminal motif found in these proteins is boxed. Black shading indicates identical residues in at least four proteins, gray boxes are similar residues, and dashes indicate gaps introduced to optimize the alignment.

the data obtained when the group-7 nullisomic-tetrasomic lines were examined (Fig. 3B). Together, these results show that *TaVRT-2* is located on the short arms of the group-7 chromosomes in each of the three genomes of hexaploid wheat.

Expression of *TaVRT-2* in Parental and Near-Isogenic Lines of Wheat Differing at the *Vrn-A1*/*vrn-A1* Locus

To determine if *TaVRT-2* expression is associated with the vernalization response in cereals, northern-blot analyses were performed using two wheat genotypes having different responses to vernalization and different degrees of FT. We used the *WCS120* and *TaVRT-1* genes as markers to determine the relationship between the expression of *TaVRT-2*, vernalization, and developmental stages in cereals. Previous studies had identified *WCS120* as a cold-specific gene likely involved in FT (Limin et al., 1997) and *TaVRT-1* as a marker of the transition from the vegetative to the reproductive phase (Danyluk et al., 2003). The results show that the *TaVRT-2* transcript level remains low and stable in spring wheat Manitou during LT exposure (Fig. 4A). Together with the low level of *TaVRT-2*

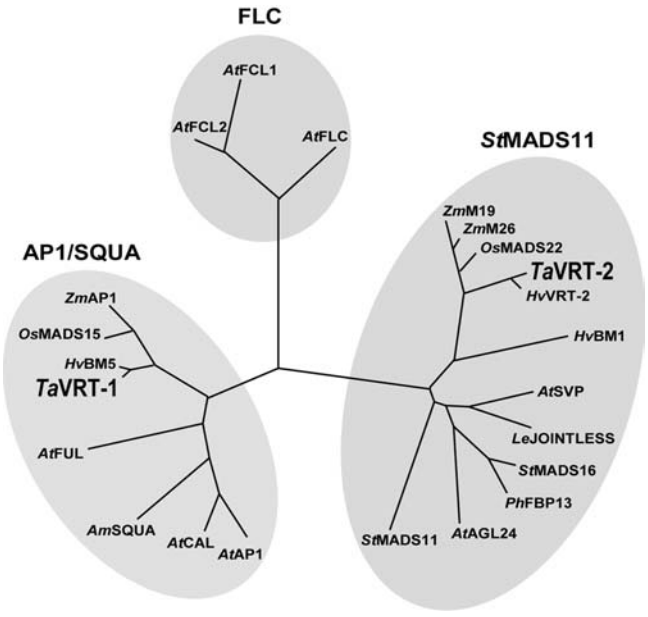

Figure 2. Unrooted phylogenetic tree illustrating the relationship between *Ta*VRT-2 and other major MADS-box regulatory proteins in monocots and dicots. The full-length sequence of *Ta*VRT-2 and several sequences from the AP1/SQUA, FLC, *St*MADS11, and AG clades were aligned using ClustalX version 1.83 as described in ''Materials and Methods.'' Only three clades are represented. Branch lengths are proportional to the number of amino acid substitutions.

expression, the spring genotype shows constitutive expression of *TaVRT-1* and lower levels of *WCS120* transcript. By contrast, the *TaVRT-2* transcript level in winter wheat Norstar is higher than in spring Manitou early in the vegetative phase, and this expression level remains for 35 d of LT exposure (Fig. 4B). This period is associated with higher *WCS120* transcript levels and increased FT compared to spring Manitou (Danyluk et al., 2003). Toward the floral transition point, *TaVRT-2* is down-regulated to a very low level, whereas *TaVRT-1* starts to accumulate (Fig. 4B). After this transition, *WCS120* transcript levels decrease and FT is gradually lost.

Since the *TaVRT-2* regulation appears to be associated with the vernalization saturation point, we also investigated its accumulation profile in the previously described Manitou/Norstar reciprocal near-isogenic lines (NILs) for the *Vrn-A1* locus (Limin and Fowler, 2002). In these two sets of NILs, the recessive winter habit allele (*vrn-A1*) in Norstar was replaced with the dominant spring allele (*Vrn-A1*) from Manitou (to produce ''spring Norstar NIL''), and the spring allele in Manitou was replaced with the winter allele from Norstar (to produce ''winter Manitou NIL''). In the spring habit plants (spring Manitou and spring Norstar NIL), the *TaVRT-2* gene shows similar low levels of constitutive expression (Fig. 4, A, C, and E). By contrast, the *TaVRT-2* transcript level is higher in winter habit plants (winter Norstar and winter Manitou NIL) until 35 d of LT exposure, after which it decreases to the low level observed in the spring habit plants (Fig. 4, B, D, and F). A clear inverse relationship is observed in the expression patterns of *TaVRT-1* and *TaVRT-2* after 35 to 49 d of LT exposure (vernalization). This expression pattern corresponds in time to the period of vernalization saturation, after which the plant has achieved competence to flower. Analyses of several spring and winter genotypes confirmed that the accumulation of *TaVRT-2* is higher in winter cultivars, which require a vernalization period (data not shown). Overall, these results suggest that a higher level of *TaVRT-2* expression is required to maintain winter wheat cultivars in the vegetative growth phase and that there is a possible interrelationship between *TaVRT-1* and *TaVRT-2*, where one may be repressing the expression of the other (discussed below).

To study the expression of the two *TaVRT* genes in the reproductive tissues, we used wheat inflorescences at different development stages. The results show that *TaVRT-2* is weakly or not expressed, whereas *TaVRT-1* is highly expressed at all stages (Fig. 5). The *TaVRT-2*

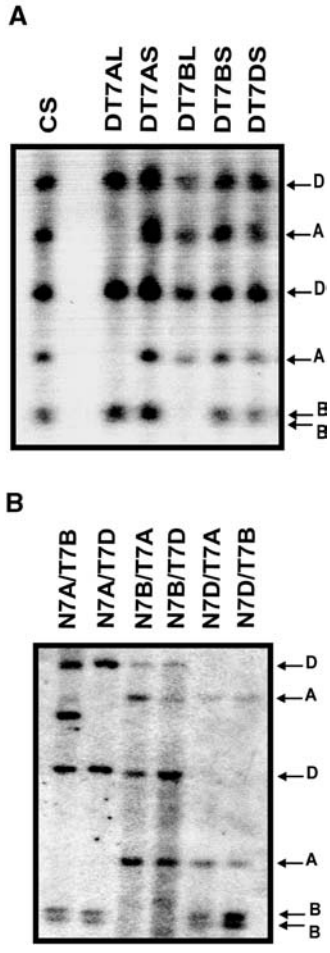

Figure 3. Mapping of the *TaVRT-2* homologous genes on wheat group-7 chromosomes. DNA-blot analysis of *Dra*I-digested genomic DNA from ditelocentric (A) and nullisomic-tetrasomic lines (B) of hexaploid wheat (cv Chinese Spring) hybridized with a *TaVRT-2*-specific probe. The arrows indicate which of the A, B, or D genome contributes to the bands seen on the blots.

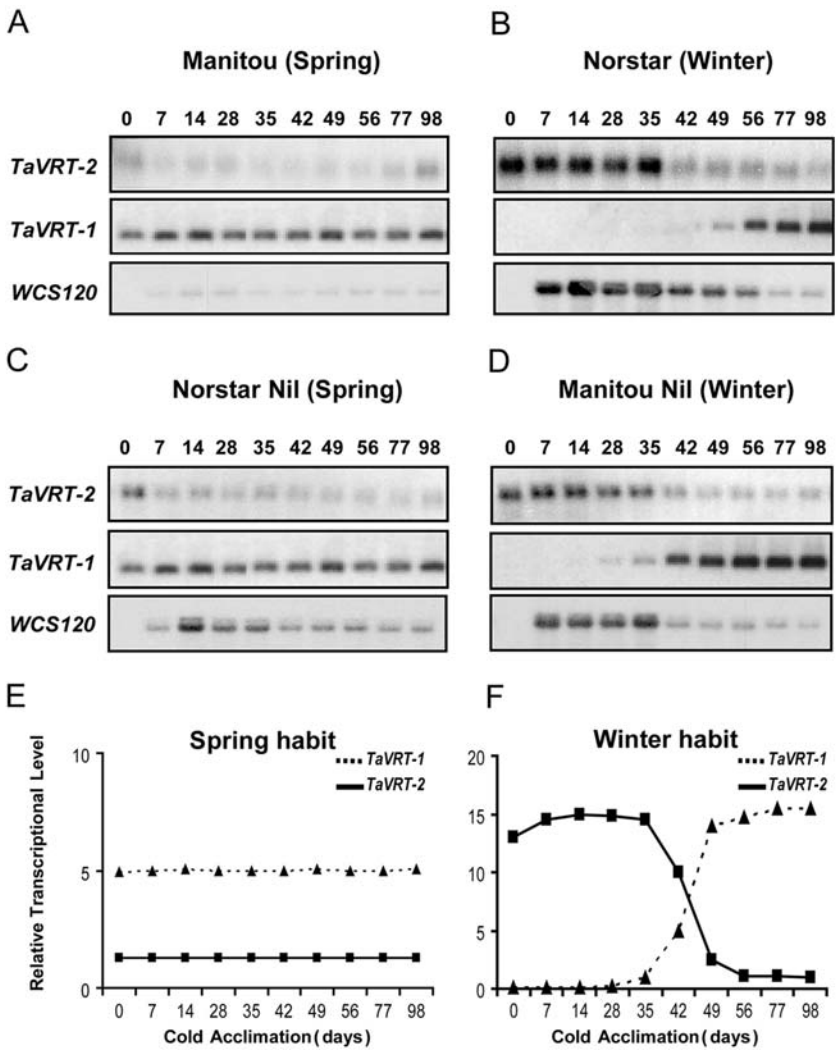

Figure 4. Expression of *TaVRT* and *COR* genes during LT exposure in parental and near-isogenic lines of wheat. Parental lines spring Manitou (A) and winter Norstar (B) and NILs spring Norstar (C) and winter Manitou (D) were exposed for the indicated period (in days) at LT, then RNA was extracted for northern-blot analyses. Blots were first hybridized with a *TaVRT-2*-specific probe, then with a specific *TaVRT-1* probe, and finally with a *WCS120* probe. Panels A to D are scanned x-ray films. The same blots were also exposed to a phosphor screen that was scanned in a Molecular Imager FX (Bio-Rad) for densitometric measurement. Values are expressed as relative arbitrary units of the *TaVRT* genes' accumulation in spring (E) and winter (F) parental genotypes.

expression pattern is in agreement with that of most of the other members of the *StMADS11* clade, which is restricted to vegetative tissues (Becker and Theissen, 2003). Further northern-blot analysis demonstrated that *TaVRT-2* transcripts accumulate preferentially in the aerial part of wheat plants compared to roots (data not shown).

Influence of Photoperiod on *TaVRT-1* and *TaVRT-2* Expression in Cereals

Norstar winter wheat and photoperiod-sensitive spring Dicktoo barley plants were grown under short- or long-day conditions at 4°C for 98 d to determine the effect of photoperiod on *TaVRT-1* and *TaVRT-2* expression in association with flowering competency and FT. Norstar winter wheat shows a higher accumulation of *TaVRT-2* transcripts under long day and to a lesser extent under short day at 14 and 35 d of LT exposure (Fig. 6A). At these time points, Norstar plants are still in the vegetative phase and development has not reached the double-ridge stage, the morphological indicator of flowering competence (Danyluk et al., 2003). In photoperiod-sensitive spring Dicktoo barley, *HvVRT-2* (the *TaVRT-2* ortholog) is expressed at higher levels under short days at 14, 35, and 56 d of LT exposure and is down-regulated after 77 d (Fig. 6B). Interestingly, the double-ridge formation and reproductive phase initiation occurs at 70 d of LT under short-day conditions (Fowler et al., 2001; Danyluk et al., 2003). On the other hand, *HvVRT-2* is expressed at low levels during long-day LT treatments (Fig. 6B), where Dicktoo barley has already entered the reproductive stage. These data also provide further evidence of the inverse relationship between *TaVRT-1* and *TaVRT-2* expression and flowering capacity in wheat and barley (Fig. 6, A and B), and suggest that *TaVRT-2* up-regulation is associated with the maintenance of plants in the vegetative phase.

Protein-Protein Interactions

Proteins involved in the same biological process frequently form complexes, and members of the

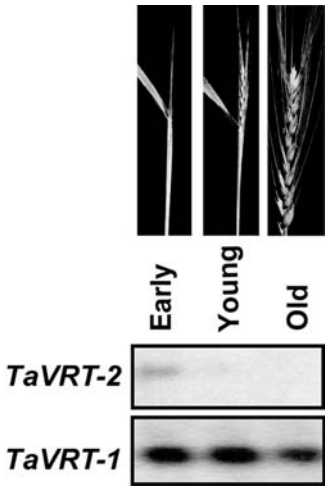

Figure 5. Expression of *TaVRT-2* at different developmental stages of wheat. RNA gel-blot analyses were performed with specific *TaVRT-2* and *TaVRT-1* probes using RNA extracted from wheat inflorescences (Early = very young spike dissected out of the stem; Young = boot stage of development during emergence; Old = fully emerged spike and florets).

MADS-box family are known to form specific homodimers and heterodimers (Davies et al., 1996; Favaro et al., 2003; Immink et al., 2003). To determine if *Ta*VRT-2 can interact with itself or with other proteins involved in the flowering pathway, we used the yeast two-hybrid system. Fusion constructs were made with the GAL4 binding domain (BD) and GAL4 activation domain (AD) using various MADS proteins (Fig. 7). The results show that *Ta*VRT-2 can homodimerize as well as heterodimerize with *Ta*VRT-1 (AP1/SQUA clade), *Ta*57H08 (*St*MADS11 clade), and *Ta*73C21 (TM3/SOC1 clade; Table I; Supplemental Table III). Additional assays showed that *Ta*VRT-1 can also homodimerize and heterodimerize with five other wheat MADS-box proteins with a similar apparent affinity, whereas the *Ta*VRT-1/*Ta*VRT-2 pair shows a higher apparent affinity (Table I; Supplemental Table III).

The *VRN-2* gene, which encodes a zinc-finger protein that acts as a flowering repressor, was recently identified in diploid wheat (Yan et al., 2004b). The significant epistatic interactions observed between *VRN1* and *VRN2* indicated that these two genes act in the same pathway. The *VRN-2* cDNA was thus cloned from hexaploid wheat (A. Diallo, N.A. Kane, and F. Sarhan, unpublished data) and used in the yeast two-hybrid studies to determine if it has any affinity with the two *Ta*VRT proteins. The results show that VRN-2 has a higher apparent affinity with *Ta*VRT-2 than with *Ta*VRT-1 (Table I). The interaction of the *Ta*VRT proteins with another wheat zinc-finger protein, *Ta*HD1, was also tested, and the results indicate that the proteins interact with each other but with a lesser apparent affinity (Table I). Together, these data indicate that *Ta*VRT-2 interacts with several transcription factors involved in flowering control and vernalization in wheat and suggest that the transition to the reproductive phase could be mediated by complex protein-protein interactions.

DISCUSSION

We have characterized a wheat MADS-box gene, *TaVRT-2*, that has an expression pattern opposite to that of the previously identified *TaVRT-1* gene, a key gene that regulates the vegetative to reproductive phase transition in hexaploid winter wheat (Danyluk et al., 2003). While *TaVRT-1* is located in the phenotypically defined *Vrn-A1* region on chromosome 5A, *TaVRT-2* was mapped to the short arm of the group-7 chromosomes. In Triticeae, the latter chromosomes have not been found to carry major genes affecting flowering time and have therefore often been overlooked in this regard. A survey of the literature, however, reveals that this chromosome group is in fact highly involved in the continuous variation of flowering time found in nature. QTLs associated with ear emergence, vernalization, heading date, and photoperiod sensitivity have been located on the group-7 chromosomes in wheat and barley (Bezant et al., 1996; Sourdille et al., 2000; Boyko et al., 2002; Baum et al., 2003; Shindo et al., 2003). The mapping of *TaVRT-2* and *HvVRT-2* to group-7 chromosomes suggests that they

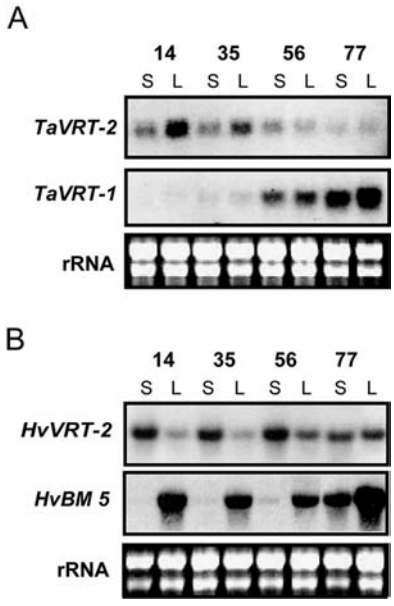

Figure 6. Expression of *TaVRT* genes in Norstar wheat and their orthologs in Dicktoo barley in response to LT and photoperiodic regimes. Norstar winter wheat (A) and photoperiod-sensitive spring barley plants (B) were grown at 4°C under short-day (S) or long-day (L) photoperiod for the indicated number of days, and RNA-blot analyses were performed as described in Figure 4. Under these growth conditions, flowering competence based on final leaf number is reached by day 49 under both S and L photoperiod conditions in Norstar (Mahfoozi et al., 2001). In long-day-grown Dicktoo barley, the morphological indicator of flowering competence, double-ridge formation, is present at the start of treatment (0 d LT), whereas in short-day-grown barley it appears after 70 d of LT exposure (Danyluk et al., 2003).

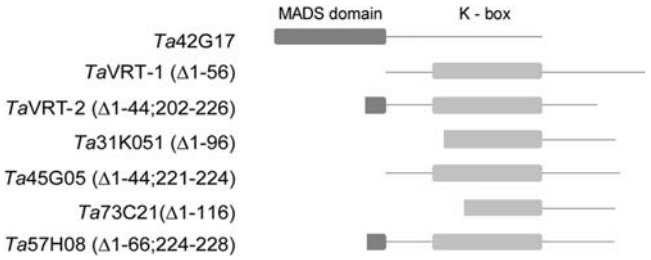

Figure 7. Schematic representation of the constructs used in the protein-protein interaction studies in yeast. Except for *Ta*42G05, the MADS domain was deleted to avoid toxicity to the yeast strain used.

may be involved in these QTLs. However, direct experimental evidence is needed for verification of such an association.

Phylogenetic analysis has positioned *Ta*VRT-2 in the *St*MADS11-like clade. Members of this clade are expressed strictly in vegetative organs and seem to have an ancestral function in the maintenance of vegetative phase or in the timing of the transition from vegetative to reproductive phase (Becker and Theissen, 2003). In Arabidopsis, the two *StMADS11*-like genes *SVP* and *AGL24* act in an opposite manner in the floral transition, despite their close phylogenetic relationship. SVP is a negative regulator of flowering because its inactivation causes early flowering (Hartmann et al., 2000), whereas AGL24 acts as a promoter of flowering because loss-of-function plants show a late flowering phenotype (Yu et al., 2002). In Antirrhinum, the *StMADS11*-like gene *INCO* is also capable of repressing flowering when overexpressed (Masiero et al., 2004). Therefore, the higher levels of *TaVRT-2* transcripts found to be associated with the vegetative growth phase in winter habit wheat or short-day-photoperiod-sensitive barley are in line with functions such as maintaining vegetative growth or repressing the transition to the reproductive phase.

The inverse expression pattern between *TaVRT-2* and *TaVRT-1* suggests that *Ta*VRT-2 could suppress *TaVRT-1* transcription or that, inversely, *Ta*VRT-1 could suppress *TaVRT-2*. Since *TaVRT-2* belongs to the clade of genes encoding suppressors of flowering and because *TaVRT-1* appears to shortcut the vernalization process (Fu et al., 2005), it seems logical that *Ta*VRT-2 may be suppressing *TaVRT-1* transcription in winter habit genotypes by interaction with the recessive winter allele. This allele is believed to be the ancestral type (Yan et al., 2003), and it is possible that the spring allele may have arisen from mutational changes affecting its susceptibility to the possible repressor effect of *Ta*VRT-2. Alternatively, we cannot rule out the possibility that, once induced, *TaVRT-1* could either directly or indirectly repress *TaVRT-2* expression seen in the winter wheat genotypes. This suppression is associated with vernalization saturation and competence to flower (Limin and Fowler, 2002), which is coincidental with *TaVRT-1* up-regulation (Danyluk et al., 2003). In the spring habit genotypes, *TaVRT-1* is constitutively expressed at a high level and *TaVRT-2* is down-regulated. However, the decrease in *TaVRT-2* may also be caused by other factors. Induction of genes from the AP1/SQUA clade (such as *TaVRT-1*) is believed to be one of the furthest downstream events in the flowering pathway in plants (Mouradov et al., 2002; Becker and Theissen, 2003). Genes from this clade were shown to be important in determining inflorescence meristem identity and flower development. Once these proteins are induced, the plant is committed to reproductive growth and it is likely that integration of most developmental and environmental signals has taken place. Therefore, factors involved in regulation of the vegetative/reproductive transition, such as members of the *St*MADS11 clade, should become redundant and likely to be down-regulated by their upstream regulators. Further identification of genes involved in wheat floral transition will be necessary to establish the full extent of these interactions.

Although the evidence seems to suggest that most of the members of the *St*MADS11-like clade function in repressing the transition to the reproductive phase, their exact roles in the flowering pathway remain unclear. Recently, double mutant analyses in Arabidopsis have shown that *AtSVP* is positioned in the same pathway as another MADS-box gene, *FLOWERING LOCUS M* (Scortecci et al., 2003). These genes were shown to interact with the photoperiod pathway downstream of *AtGI* and *AtCO* (Scortecci et al., 2003). If simi-

Table I. *Protein-protein interactions between candidates that could be associated with the floral induction pathway*

The interactions between several wheat MADS-box and other flowering proteins were investigated using the yeast two-hybrid assay. β-Galactosidase-specific activity was determined using *o*-nitrophenyl-β-d-galactopyranoside as a substrate. Data are an average of two experiments performed in triplicate. Autoactivation control results are presented in Supplemental Table V.

GAL4 BD and AD Fusions	β-Galactosidase Activity
	nmol min^{-1} mg^{-1} protein
BD-VRN-2 + AD-*Ta*VRT-2	85 ± 5
BD-*Ta*VRT-2 + AD-*Ta*VRT-2	73 ± 6
BD-*Ta*VRT-1 + AD-*Ta*VRT-2	73 ± 4
BD-*Ta*31K05 + AD-*Ta*VRT-2	Not tested
BD-*Ta*57H08 + AD-*Ta*VRT-2	110 ± 9
BD-*Ta*42G17 + AD-*Ta*VRT-2	29 ± 3
BD-*Ta*45G05 + AD-*Ta*VRT-2	25 ± 3
BD-*Ta*73C21 + AD-*Ta*VRT-2	47 ± 1
BD-*Ta*VRT-1 + AD-*Ta*VRT-1	21.3 ± 0.7
BD-*Ta*VRT-2 + AD-*Ta*VRT-1	61 ± 4
BD-*Ta*31K05 + AD-*Ta*VRT-1	23.2 ± 0.4
BD-*Ta*57H08 + AD-*Ta*VRT-1	22.7 ± 0.3
BD-*Ta*42G17 + AD-*Ta*VRT-1	17.3 ± 0.3
BD-*Ta*45G05 + AD-*Ta*VRT-1	18.3 ± 0.2
BD-*Ta*73C21 + AD-*Ta*VRT-1	24.8 ± 0.7
BD-VRN-2 + AD-*Ta*VRT-1	49.6 ± 3.7
BD-VRN-2 + AD-VRN-2	12.4 ± 0.3
BD-*Ta*VRT-2 + AD-VRN-2	98 ± 3
BD-*Ta*VRT-1 + AD-VRN-2	50 ± 4
BD-*Ta*VRT-2 + AD-*Ta*HD1	33 ± 3
BD-*Ta*VRT-1 + AD-*Ta*HD1	20 ± 4

lar regulatory networks exist in cereals, this could explain the higher expression of *TaVRT-2*/*HvVRT-2* long-day-grown winter wheat and short-day-grown photoperiod-sensitive barley, where a high level of repression would be required to maintain plants in the vegetative stage. Thus, in dicot and monocot species, genes such as *AtSVP* and *TaVRT*-2 may have a similar function in the repression of flowering by integrating photoperiodic signals. Arabidopsis has at least one additional photoperiodic pathway (*At*CO that converges on the promoter of the *AtSOC1* gene) that may offer plants the observed variation in photoperiod sensitivity necessary for environmental adaptation. The significance of this interplay in determining flowering time (adaptation) should not be underestimated in wheat because of the importance in maintaining up-regulation of the LT tolerance genes, a requirement of which is the extension of the vegetative state (Mahfoozi et al., 2001; Danyluk et al., 2003). Although the evidence suggests that some members of the *St*MADS11 clade may function in a parallel photoperiodic pathway that plays a role in timing the transition to the reproductive phase, it is not yet known how they exert their molecular action.

A possible mode of action can be proposed for *Ta*VRT-2 that is based on common properties of MADS-box proteins and from information gained from this and previous studies. Available wheat genomic sequence data indicate the presence of a CArG-box (a MADS-box binding motif) in the promoter of *Vrn-1* (*TaVRT1*) in all winter wheat genotypes tested (Yan et al., 2003, 2004a; Beales et al., 2005; Fu et al., 2005; N.A. Kane, F. Ouellet, and F. Sarhan, unpublished data). Analysis of promoter and gene sequences of different alleles of *Vrn-1* revealed that spring accessions contain either (1) a deletion or insertion in the vicinity of this CArG-box, suggesting that these variations could interfere with the regulation mediated by this motif; or (2) a deletion of a conserved 440-bp sequence in the first intron, which suggests that the regulation of *VRN-1* expression is complex and may involve multiple regulatory elements. We speculate that the *Vrn-1/TaVRT-1* CArG-box could be bound by a homodimer of *Ta*VRT-2, thereby contributing to repressing its expression. VRN-2, a dominant repressor of flowering identified in *T. monococcum* (Yan et al., 2004b), is a zinc-finger protein that is unlikely to directly bind the CArG-box but that interacts strongly with *Ta*VRT-2. Therefore, VRN-2 may exert its repressor effect by first binding unidentified elements in the first intron and/or by being recruited by *Ta*VRT-2. Once bound to the CArG-box, the complex composed of these two factors and possibly others would repress the expression of the *VRN-1/TaVRT-1* gene and result in vegetative growth. After vernalization of winter wheat genotypes, the expression of *VRN-2* is repressed and levels of *TaVRT-2* transcripts decrease. This would lead to a reduction of a functional repressive complex, allowing expression of *VRN-1/TaVRT-1* and the switch to the reproductive phase. In spring varieties of wheat, mutations in the promoter or intron of *Vrn-1* alleles could preclude the binding of the repressors and other transcription factors, and this in turn would result in a constitutive expression of *VRN-1/TaVRT-1* and flowering competence.

The significance of *Ta*VRT-2 interaction with *Ta*VRT-1, a member of the AP1/SQUA clade, is an intriguing question that has arisen from this study. Specific homodimerization and heterodimerization have been reported for a few MADS-box proteins from various plant species (Davies et al., 1996; Moon et al., 1999; Pelaz et al., 2001; Immink et al., 2003). These studies have demonstrated that protein-protein interactions and formation of complexes are at the basis of MADS-box transcription factor function (Messenguy and Dubois, 2003). Other members of the *St*MADS11 clade have also been found to interact with proteins of the AP1/SQUA clade in petunia (Immink et al., 2003), rice (Fornara et al., 2004), Antirrhinum, and Arabidopsis (Masiero et al., 2004). Therefore, the interaction between members of these two clades may represent an evolutionarily conserved property that is important for their function. Recently, de Folter et al. (2005) have proposed that *At*AP1 (*Ta*VRT-1) could serve as a hub between the flower induction pathway interacting proteins, such as *At*SVP, *At*SOC1, and *At*AGL24, and the floral organ identity proteins. It could be speculated that *Ta*VRT-2 homodimerization during the early stages of LT exposure in winter wheat, when other high affinity MADS proteins are unavailable for interaction, could lead to a complex that can repress flowering. With the induction of *Ta*VRT-1, there could be a preference for heterodimerization and, consequently, a change of function. In support of this, genetic and transgenic studies in Antirrhinum and Arabidopsis have lead to the proposal that the INCO homodimer acts as a repressor of flowering, whereas the INCO/SQUA heterodimer acts as an activator (Masiero et al., 2004). However, this might not represent a general property for this clade of proteins since *At*SVP does not homodimerize (Masiero et al., 2004). Such differences may be based on nonidentical conservation of homodimerization capacity of MADS-box proteins in plants following the appearance and evolution of heterodimerization capacity in angiosperms (Kaufmann et al., 2005). This suggests that there will be differences among the interactions of transcriptional activator/repressor MADS-box proteins that regulate flowering time in plants. Overall, the expression data and identification of the physical association of *Ta*VRT-2 with *Ta*VRT-1 and VRN-2 further our knowledge of the regulation of flowering transition in temperate cereals.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Two spring cultivars of hexaploid wheat (*Triticum aestivum*; cv Glenlea and Manitou) and four winter cultivars (cv Absolvent, Fredrick, Monopole, and

Norstar) were used in the initial comparative gene expression studies. For detailed gene expression analyses, the non-hardy spring habit wheat cv Manitou, the very cold-hardy winter habit wheat cv Norstar, and two reciprocal near-isogenic wheat lines that differ in vernalization requirement were used. The reciprocal NILs were produced using the cv Manitou (dominant *Vrn-A1* allele) and cv Norstar (recessive *vrn-A1* allele) as described previously (Limin and Fowler, 2002). After four backcrosses, heterozygous plants were selected and selfed. Homozygous lines (*vrn-A1/vrn-A1* and *Vrn-A1/Vrn-A1*) with theoretically 96.9% of the recurrent parent genome were recovered. This procedure produced a spring growth habit Norstar with the *Vrn-A1* allele of Manitou and a winter habit Manitou with the *vrn-A1* allele of Norstar. Ditelocentric (DT) chromosome lines and the group-7 nullisomic-tetrasomic series (NT) of Chinese Spring wheat were used to locate and confirm the presence of *Ta*VRT-2 homoeologs on chromosome arms of each of the three genomes.

The experimental design for these studies was a 4 (genotypes) $\times$ 11 (acclimation periods) factorial in a two replicate randomized complete block design. All NILs and parental material were evaluated for 11 LT exposure periods (0, 7, 14, 21, 28, 35, 42, 49, 56, 77, and 98 d). Growth conditions for plants were as described previously (Limin and Fowler, 2002; Danyluk et al., 2003). For photoperiod studies, Norstar winter wheat and Dicktoo barley (*Hordeum vulgare*) were grown for 13 d at 20°C under either long-day (20 h) or short-day (8 h) photoperiod, transferred at 4°C under identical photoperiods, and then sampled at regular intervals for RNA extraction.

Identification and Molecular Characterization of *TaVRT-2*

A cDNA library was prepared from an mRNA mixture from cold-acclimated and nonacclimated crown and leaf tissues of cv Norstar. For cDNA synthesis, the SuperScript plasmid system with Gateway Technology and cloning kit (Invitrogen, Carlsbad, CA) was used, except that the precipitation steps without yeast carrier tRNA were replaced by the QIAquick PCR purification procedure (QIAGEN, Valencia, CA). The cDNAs were directionally cloned into the pCMV.SPORT6 vector with the *Sal*I adaptor (GTCGACCCACGC-GTCCG) on the 5′ end and the *Not*I primer-adaptor (GCGGCCGCCC(T_{15})) on the 3′ end.

All the MADS-box genes present in the FGAS wheat EST database were completely sequenced using the CEQ 2000 DNA analysis system (Beckman Instruments, Fullerton, CA) and analyzed by northern blotting (Danyluk et al., 2003) to determine their expression patterns. One of these genes was found to be cold regulated, and its expression pattern was associated with the vernalization response. This gene, named *TaVRT-2*, was selected for detailed molecular characterization.

For Southern analysis, genomic DNA was extracted by the cetyl-trimethyl-ammonium-bromide method from several wheat cytogenetic lines in the Chinese Spring background. In the ditelocentric lines, the long or short arms of a specific chromosome pair are missing. For example, DT7AL represents a line where only the long arms of the 7A pair are present (therefore, the short arms are missing). In the nullisomic-tetrasomic lines, a pair of chromosomes is missing, but the loss is compensated by the homoeologous chromosome pair from another genome. For example, the N7A/T7B line is missing the chromosome pair 7A, and there are two pairs of 7B. Genomic DNA (5 μg) was digested with *Dra*I and separated overnight using a FIGE mapper (Bio-Rad, Hercules, CA). A gene-specific *TaVRT-2* probe lacking the MADS domain was amplified with *Pfx*-polymerase (Invitrogen) using the primers 5′-ATTCAAA-GAACCTGGGGAAATCTG-3′ and 5′-TCCAAGGTAACGCTAGTTTCAGG-GATA-3′. Blotting and probe hybridizations were performed as described previously (Danyluk et al., 2003). All filters were washed at high stringency (0.1$\times$ SSC, 0.1% SDS) and exposed to Molecular Imager FX screens (Bio-Rad) and to x-ray films (Kodak BioMax-MS; Rochester, NY).

Phylogenetic Analysis

The amino acid sequences of *Ta*VRT-2 and several other MADS-box sequences from dicots and monocots were aligned using ClustalX version 1.83 (Thompson et al., 1997) with the following parameters: gap opening penalty of 10.00, gap extension penalty of 0.20, and substitution scoring matrix Gonnet. A distance matrix was computed from the alignment using the Protdist program under the model JTT (Jones et al., 1992). Using several members of the AP1/SQUA-, FLC-, *St*MADS11-, and AG-like clades (Supplemental Table II), a tree was computed with the Neighbor program using the neighbor-joining method (Saitou and Nei, 1987) and then adjusted manually. The same analysis was repeated under 1,000 bootstrap replicates to assess the reliability of branches. The extended-majority rule consensus tree was created by the Consense program. All the programs used to generate the trees were from the PHYLIP package version 3.6 (Felsenstein, 1989).

Yeast Two-Hybrid Analysis

Protein interaction assays were performed with the GAL4 yeast two-hybrid system (CLONTECH, Palo Alto, CA). The *Saccharomyces cerevisiae* strains used were AH109 (*MAT*α, *trp1*, *leu2*), which contains the *ADE2*, *HIS3*, *lacZ*, and *MEL1* reporter genes, and Y187 (*MAT*α, *trp1*, *leu2*), which contains the *lacZ* and *Mel1* reporters. All the reporter genes in the two strains are under the control of their own GAL4-responsive promoter. The vectors used for the protein-protein interaction assays were pGADT7 (activation domain fusion; AD) and pGBKT7 (binding domain fusion; BD). The cDNAs encoding the proteins tested were PCR amplified (Supplemental Tables III and IV) and then fused in frame with the GAL4 AD- or BD-encoding vector using the Gap repair technique. The recombinant pGBKT7 and pGADT7constructs were introduced in Y187 and AH109, respectively. Following conjugation between haploids cells (Kaiser et al., 1994), diploid cells were spread in triplicate on selective medium (SD/Gal/Raffinose without adenine, Leu, Trp, and uracil) and supplemented with 10 mg/L 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside. β-Galactosidase-specific activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (Sigma, St. Louis) as a substrate (Rose et al., 1990).

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number DQ022679.

ACKNOWLEDGMENTS

We are grateful to Dr. P. Gulick (Concordia University) for critical reading and suggestions on the manuscript. We also thank G. Schellhorn (University of Saskatchewan), G. Cormack and A.O. Diallo (Université du Québec à Montréal) for technical help. Dr. C. Chauve and M. Pelletier (Université du Québec à Montréal) are acknowledged for their help with the phylogenetic analysis.

Received February 21, 2005; revised April 22, 2005; accepted April 29, 2005; published July 15, 2005.

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