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Overexpression of *TaVRN1* in *Arabidopsis* Promotes Early Flowering and Alters Development

Hélène Adam, François Ouellet, Ndjido A. Kane, Zahra Agharbaoui, Geneviève Major, Yoko Tominaga and Fathey Sarhan *

Université du Québec à Montréal, Département des Sciences biologiques, Case Postale 8888, Succursale Centre-ville, Montréal, Québec, Canada H3C 3P8

***TaVRN1*, a member of the *APETALA1* (*AP1*) subfamily of MADS-box transcription factors, is a key gene that controls transition from vegetative to reproductive phase in wheat. The accumulation of *TaVRN1* transcripts in winter wheat probably requires the down-regulation of TaVRT2, a MADS-box factor that binds and represses the *TaVRN1* promoter, and of the flowering repressor *TaVRN2*. However, the molecular mechanisms by which TaVRN1 functions as an activator of phase transition is unknown. To address this, a combination of gene expression and functional studies was used. RNA in situ hybridization studies showed that *TaVRN1* transcripts accumulate in all meristems and primordia associated with flower development. An interaction screen in yeast revealed that TaVRN1 interacts with several proteins involved in different processes of plant development such as transcription factors, kinases and a cyclophilin. *Arabidopsis* plants overexpressing *TaVRN1* flower early and show various levels of modified plant architecture. The ectopic expression causes an overexpression of the *AP1* and *MAX4* genes, which are associated with flowering and auxin regulation, respectively. The induction of gene expression probably results from the binding of TaVRN1 to CArG motifs present on the *AP1* and *MAX4* promoters. In contrast, *Arabidopsis* plants that overexpress *TaVRT2*, which encodes a putative flowering repressor, show an opposite late flowering phenotype. Together, the data provide molecular evidence that TaVRN1 may have pleiotropic effects in various processes such as control of axillary bud growth, transition to flowering and development of floral organs.**

Keywords: *Arabidopsis* — Development — Flowering — MADS-box — Vernalization — Wheat.

Abbreviations: bHLH, basic helix–loop–helix; EMSA, electrophoretic mobility shift assay; GUS, β-glucuronidase; RLK, receptor-like kinase; RRM, RNA recognition motif; RT–PCR, reverse transcription–PCR; SAM, shoot apical meristem.

Introduction

Flowering is a complex biological process that is regulated by both environmental and developmental factors. Extensive studies in *Arabidopsis* have revealed genetic and molecular mechanisms of the transition from vegetative growth to flowering (Araki 2001, Mouradov et al. 2002). In *Arabidopsis*, there are four major floral promotion pathways in which several genes are involved: (i) *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) act in the photoperiod pathway; (ii) the vernalization and autonomous pathways promote floral transition via the down-regulation of *FLOWERING LOCUS C* (*FLC*) and up-regulation of *SUPPRESSOR OF OVEREXPRESSION OF CO* (*SOC1*); (iii) the growth regulator gibberellic acid promotes flowering by up-regulating the transcription level of the floral meristem identity gene *LEAFY* (*LFY*); and (iv) *FT*, *SOC1* and *LFY* activate the floral meristem identity gene *APETALA1* (*AP1*) which promotes the formation of a floral meristem (Blazquez 2000). Among these floral transition genes, *FLC*, *SOC1* and *AP1* are members of the MADS-box gene family (Riechmann and Meyerowitz 1997). Examples of other MADS-box genes that are also recognized as flowering time genes include *SHORT VEGETATIVE PHASE* (*SVP*) and *AGAMOUS-like 24* (*AGL24*), while *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*) are associated with the development of floral meristems.

Varieties of hexaploid wheat, *Triticum aestivum* ($2n = 6x = 42$ chromosomes), are classified as winter and spring growth habit types, based on their vernalization requirement. Winter wheat requires vernalization, a period of cold exposure, to induce flowering. In contrast, spring varieties are basically vernalization insensitive and become competent to flower in the early stage of vegetative development. Both habit types show a long-day

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*Corresponding author: E-mail, sarhan.fathey@uqam.ca; Fax, +1-514-987-4647.

photoperiod requirement to achieve floral development. *VRN1* (also called *TaVRT1*), *VRN2* and *TaVRT2* are three genes that determine spring/winter type in hexaploid bread wheat (Danyluk et al. 2003, Kane et al. 2005, Yan et al. 2004). To avoid confusion with the *Arabidopsis VRN1* gene, which is different from the wheat *VRN1* gene, we suggest renaming the latter '*TaVRN1*'.

The identification of the wheat *TaVRN1* gene was independently reported by ourselves and other groups (Danyluk et al. 2003, Murai et al. 2003, Yan et al. 2003). This gene encodes a MADS-box protein belonging to the *AP1* subfamily. *AP1*-like genes have been identified in diverse dicotyledonous, monocotyledonous and other basal angiosperms. The *Arabidopsis AP1* gene is the best characterized member of this subfamily. This gene, first characterized as a class A gene, encodes a protein that is necessary for the correct development of sepals and petals, and for the determination of the identity of flower meristems (Irish and Sussex 1990, Mandel and Yanofsky 1995). AP1 is involved in the determination of inflorescence architecture together with the closely related FUL and CAL proteins. AP1, FUL and CAL act redundantly in the control of meristem identity; therefore, only the *ap1 ful cal* triple mutant plants exhibit a dramatic non-flowering phenotype where flower meristems are converted into leafy shoots (Ferrandiz et al. 2000). Very little is known about these genes in monocotyledonous plants, which include the economically important cereals. Cereals are different from *Arabidopsis* since the first meristem that branches from the inflorescence meristem has a distinct identity: it is not a floral meristem but a spikelet meristem (McSteen et al. 2000). In rice, four MADS-box genes (*OsMADS20*, *OsMADS14*, *OsMADS15* and *OsMADS18*) show high sequence homology with *AP1*. Ectopic expression of *OsMADS14* in rice induces the development of calli into shoot- or embryo-like structures, on which abnormal flowers subsequently develop (Jeon et al. 2000). In contrast, overexpression of *OsMADS18* in *Arabidopsis* causes a phenotype that closely resembles the *ap1* mutant (Fornara et al. 2004). In monocotyledonous species such as barley (*Hordeum vulgare*) and wheat, *AP1*-like genes such as *TaVRN1* are constitutively expressed in spring varieties. However, they are not expressed in winter varieties unless plants are vernalized. Spring growth habit is dominant to winter habit: a spring allele at any of the homoeologous *VRN-A1*, *B1* or *D1* loci in hexaploid wheat will promote flowering in the absence of vernalization. These observations support a role for this protein in floral transition (Trevaskis et al. 2003, Yan et al. 2003). We recently reported that TaVRT2 is likely to regulate the transition from the vegetative to reproductive phase negatively by binding to the promoter region of *TaVRN1*, thereby repressing its expression (Kane et al. 2007). However, the mechanism by which *TaVRN1* itself promotes flowering at the molecular level is still unclear. To address this, we used a combined approach of gene expression and functional studies. The data obtained demonstrate that TaVRN1 accelerates flowering and modifies the plant's architecture in *Arabidopsis*, and that these effects may be mediated by the overexpression of the key positive regulator *AP1* and the hormone regulation gene *MAX4*. In addition, we show that TaVRT2 delays flowering in *Arabidopsis*, probably through the up-regulation of the *FLC* repressor.

Results and discussion

RNA in situ localization of TaVRN1 in wheat

The development of the wheat shoot apical meristem (SAM) into a mature flower proceeds through many different stages, leading to the appearance of various organs (Fig. 1A). These developmental stages are identical in spring and winter wheat, provided winter wheat is vernalized and plants are exposed to a long-day photoperiod. To determine the spatial and temporal expression of *TaVRN1* during floral development, RNA in situ hybridization analyses were performed on spring wheat tissues. The accumulation of transcripts was determined in longitudinal sections of the SAM at different stages of spikelet and flower development. The results show that *TaVRN1* transcripts are detected in cells of the SAM and young leaves during the vegetative phase and floral transition (Fig. 1B–D). During the development of the spikelet, *TaVRN1* transcripts are detected in cells of the apical and spikelet meristems (Fig. 1E). Later, *TaVRN1* transcripts are localized in cells of the spikelet but not in the rachis (Fig. 1F). At the floret stage, *TaVRN1* transcripts are present in cells of the glume primordia and flower primordia (Fig. 1G). During flower development, *TaVRN1* transcripts are present in cells of the primordia of all floral organs (Fig. 1H). When the flower organs are fully developed, *TaVRN1* transcripts are detected in cells of lemma and palea, but are not detectable in stamens and carpels (Fig. 1I). The expression localization data suggest that, in addition to regulating flowering time, *TaVRN1* might play roles in the development of spikelet and floral organs.

TaVRN1 is a wheat MADS-box transcription factor belonging to the *AP1/SQUA* clade of MADS-box genes (Danyluk et al. 2003). Grass genes that belong to the *AP1* clade appear to fall into three distinct lineages (Schmitz et al. 2000, Munster et al. 2002). *TaVRN1* is related to a lineage which includes *Zmm4* and *Zmm15* from maize, *OsMADS14* from rice and *BM5* from barley (Fischer et al. 1995, Moon et al. 1999, Schmitz et al. 2000, Munster et al. 2002). As observed for *TaVRN1*, *BM5* transcripts are also detected in vegetative tissues (nodes, leaves)

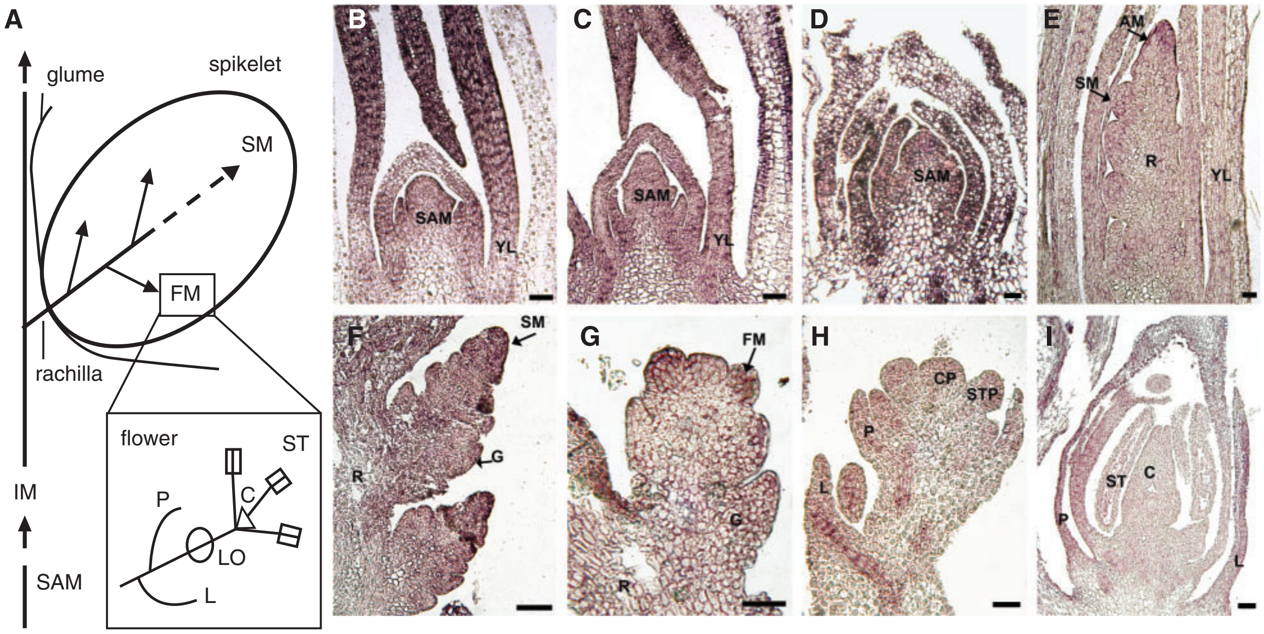

Fig. 1 RNA in situ localization of *TaVRN1* transcripts at different stages of wheat inflorescence development. (A) Diagram illustrating the development of the various inflorescence organs. The shoot apical meristem (SAM) first differentiates into an inflorescence meristem (IM) then into spikelet meristems (SM) on lateral branches. The floret meristems (FM) form on these lateral branches. (B) SAM during the vegetative phase. (C,D) SAM during the floral transition. (E) Apical meristem with spikelet meristem. (F) Spikelet stage. (G) Floret stage. (H) Flower with organ primordia. (I) Flower with all developed organs. AM, apical meristem; C, carpel; CP carpel primordium; G, glume; L, lemma; LO, lodicules; P, palea; R, rachilla; ST, stamen; STP, stamen primodium; YL, young leaf. Bars = 100 μm.

(Schmitz et al. 2000). The barley gene *BM5* is expressed at a high level in all organ primordia and vascular tissues of the floret throughout inflorescence development. Expression of *TaVRN1* in the later phases of floral development becomes restricted to perianth organs, including the glumes, lemma and palea. This pattern was also shown for the maize (Mena et al. 1995) and rice (Kyozuka et al. 2000) *AP1*-like genes. Based on the available data, it is difficult to establish a consensus pattern of expression of *AP1*-like genes due to the fact that the in situ hybridization studies were performed at different stages of inflorescence development for the different grass species.

TaVRN1 expression patterns suggest that the encoded protein could have a function similar to that of SQUA and AP1 in dicots. As for *AP1*/*SQUA*-related genes, *TaVRN1* transcripts are detected in the apical dome of the shoot apex in vegetative plants, and in the floral meristem and perianth organs. We have found that *TaVRN1* is expressed within the provascular strands in the central core of the meristem and the lemmas. Provascular expression has also been observed for *SaMADSB* (Menzel et al. 1996). This expression in the vascular strands may indicate a function in the regulation of the transport of flowering-specific substances, or in development of the vascular bundles. The expression of *TaVRN1* in the various organs and cell types indicates that this gene may have several functions during plant development, including floral transition, spikelet development, floral meristem identity and floral organ identity.

Identification of proteins interacting with TaVRN1

The identification of protein partners is an important tool to gain insight into the cellular function of proteins. Heterodimerization greatly increases the number of possible DNA-binding complexes. The identity of the interactors bound to transcription factors may determine their DNA-binding characteristics, for example if they act as activators or repressors of transcription. Heterodimerization therefore allows for the fine tuning of the regulation of gene expression. MADS-box proteins are known to form homo- and heterodimers as well as ternary or higher order complexes. To identify proteins that interact with TaVRN1, a yeast two-hybrid screen was performed. The TaVRN1 protein lacking the MADS domain was used as a bait against a cDNA library constructed from RNA populations prepared from wheat tissues collected at different stages of development (vegetative phase and various stages of spike formation). The screening resulted in the detection of several putative interactors, identified by growth on selective media and by X-Gal assay (Supplementary Fig. S1). Specific interactions between TaVRN1 and each putative interactor were confirmed by

Table 1 TaVRN1 interactors from wheat identified by yeast two-hybrid screening

TaVRN1 interactor	Description	Accession No.	Interaction strength[a]
TabHLH	Putative bHLH transcription factor	EF040600	+
TaWRKY	WRKY transcription factor	EF040599	+
TaRLK	Putative RLK	BJ215404	++
	Serine/threonine protein kinase	EF040601	++
TaRNAb	RNA-binding protein	CK170456	++
TaCyclo	Cyclophilin	AAP80861	++
TaZIM	Protein with ZIM motif	EF040602	+
TaRZF	Putative ring zinc finger	BJ283295	+
TaWD40	WD-40 protein	CK206161	+
TaDEAD	DEAD box protein	CK161729	++

[a] Interaction strength was assessed by comparing the growth of the transformants and control strains on medium lacking tryptophan, leucine and uracil, and by β-galactosidase assays (see Supplementary Fig. S1).
+, weak interaction; ++, moderately strong interaction.

re-transformation of yeast cells, and cDNAs encoding the positive interactors were sequenced. Homology search allowed us to identify 10 genuine interactors of TaVRN1 (Table 1).

Reverse transcription–PCR (RT–PCR) experiments were performed to determine if expression of the genes encoding the interactors is associated with developmental stages of spring wheat (Fig. 2). Only genes for which it was possible to design specific primers were studied. *TabHLH* transcripts are detected in spikelets and weakly in leaves (Fig. 2). Members of the bHLH (basic helix–loop–helix) family are involved in controlling cell proliferation and in the development of specific cell lineages (Heim et al. 2003). The mechanism by which they regulate transcription often involves the formation of homo- or heterodimeric configurations that can bind specifically to the G-box DNA sequence motif CACGTG (Toledo-Ortiz et al. 2003). The *Arabidopsis thaliana* genome contains 133 genes with a bHLH motif (Heim et al. 2003), and interactions between MADS-box proteins and cofactors such as bHLH proteins, HMG (high mobility group) proteins or bZIP factors have been suggested to regulate the activity and specificity of the MADS proteins (Davies et al. 1996, Messenguy and Dubois 2003).

TaWRKY transcripts accumulate from the floral transition stage to flower maturity, and transcripts are more abundant in spikelets compared with leaves and embryos (Fig. 2). In *Arabidopsis*, genes belonging to the WRKY family are involved in salicylic acid signaling and in response to pathogens (Eulgem et al. 2000). Very little is known about the physiological processes and developmental programs that require the functions of WRKY proteins. *WRKY* genes are often detected preferentially in leaves, and the different family members share similar expression patterns (Schmid et al. 2005). Even though the majority of WRKY genes have been described as being involved in the pathogen response, some of these genes could also have a function during plant development. Overexpression of *AtWRKY6*, *AtWRKY18*, *AtWRKY53* or *AtWRKY70* results in small stunted transgenic plants, and nearly all lines show altered leaf morphologies and changes in flowering time (Chen and Chen 2002, Robatzek and Somssich 2002, Li et al. 2004).

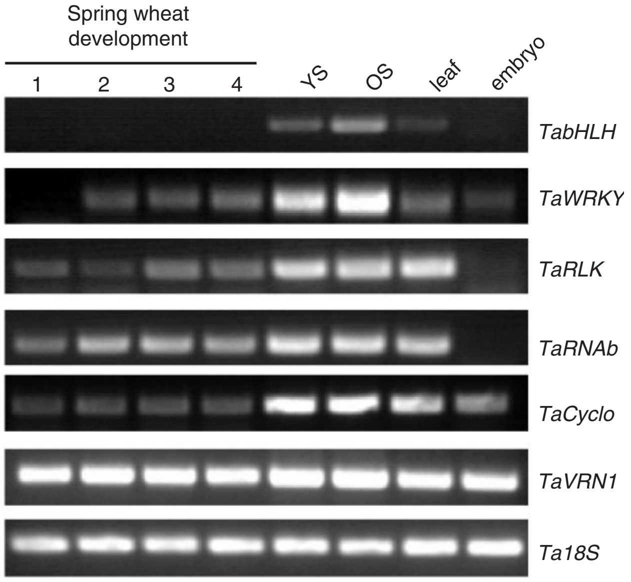

Fig. 2 Expression levels of wheat genes encoding the TaVRN1 interactors identified by the yeast interaction screen. Spring wheat plants were grown and tissues were collected throughout the life cycle. Stage 1, vegetative development; stage 2, floral transition; stage 3, development of spikelet; stage 4, flower maturity; YS, young spikelet; OS, old spikelet. RNA was extracted and RT–PCR analysis was performed using gene-specific primers (see Table 1 for description of genes). The 18S rRNA (*Ta18S*) was used as a control.

TaRLK is expressed at all stages of leaf and flower development (Fig. 2). In addition to this member of the RLK (receptor-like kinase) family, a serine-threonine protein kinase also interacts with TaVRN1. These two proteins often work together to transduce intercellular signals, an essential event in plant development. RLKs perceive extracellular signals and transduce them to intracellular downstream factors by a protein phosphorylation cascade and, as such, they play important roles in intercellular communication (Becraft et al. 1996, Takasaki et al. 2000). It is known that the activity of MADS-box transcription factors can be regulated post-translationally (Ornatsky et al. 1999). Previous studies have shown that the *Arabidopsis* MADS-box protein AGL24 interacts with the kinase domain of meristematic receptor-like kinase (MRLK; Fujita et al. 2003). This raises the possibility that TaVRN1 could be post-translationally modified by TaRLK or by a serine-threonine protein kinase in addition to being transcriptionally regulated. There is also a possibility that the formation and/or subcellular localization of the complexes might be regulated, in part, by phosphorylation by a protein kinase.

TaRNAb is expressed at all stages of leaf and flower development (Fig. 2). RNA-binding proteins play key roles in post-transcriptional regulation, participating in mRNA splicing, transport, localization, stability and translocation (Keene 2001). RNA-binding proteins are a group of evolutionarily conserved proteins that have distinct RNA-binding motifs, such as RRMs (RNA recognition motifs), KH motifs, zinc fingers, zinc knuckles, RGG-boxes and DEAD-boxes (Makeyev and Liebhaber 2000, Lorkovic and Barta 2002). Among these, the RRMs and KH motifs are most frequently found in *Arabidopsis* proteins, but few proteins have been functionally characterized. The only two plant-specific RRM-containing proteins of known function, FCA and FPA, are proteins that promote flowering (Macknight et al. 1997, Schomburg et al. 2001). A function has been established for only two KH-domain RNA-binding proteins, HUA ENHANCER 4 and FLK, with FLK promoting flowering (Lim et al. 2004). The abundance of *Arabidopsis* genes encoding RNA-binding proteins, and the recent identification of developmental and hormone response mutations in *Arabidopsis* genes that encode RNA-binding proteins, suggest that these will emerge as important players in plant morphogenesis and cellular regulation. Recently, it has been shown that *LEAFY HEAD2* encodes a putative RNA-binding protein that could be involved in the regulation of rice shoot development through *KNOX* (a homeobox gene) and hormone-related genes (Xiong et al. 2006). Also recently, the *Arabidopsis* FCA RNA-binding protein, which is homologous to the barley ABA-P1 protein, was found to be an ABA receptor involved in RNA metabolism and in the control of flowering time in the autonomous pathway (Razem et al. 2006).

TaCyclo is expressed at all stages of leaf and flower development, and in embryos (Fig. 2). Cyclophilins are ubiquitous proteins (Galat 1999, Romano et al. 2004) present in all subcellular compartments and are involved in a wide variety of processes including receptor complex stabilization (Leverson and Ness 1998), receptor signaling (Brazin et al. 2002, Yurchenko et al. 2002), RNA processing (Krzywicka et al. 2001) and spliceosome assembly (Bourquin et al. 1997, Mortillaro and Berezney 1998, Horowitz et al. 2002). Some plant cyclophilins are induced by a variety of biotic and abiotic stresses, suggesting that they could play a role in environmental responses (Chou and Gasser 1997, Kurek et al. 1999). *Arabidopsis* plants lacking *AtCYP40* show a defect in the transition from the juvenile to adult stages of vegetative development (Berardini et al. 2001). This suggests that *TaVRN1* may need to interact with a cyclophilin to promote the transition between the various stages of floral development.

Other TaVRN1-interacting partners, for which RT–PCR could not be performed because we could not design specific primers, include TaZIM, TaRZF, TaWD40 and TaDEAD. The ZIM motif is found in a variety of plant transcription factors that contain GATA domains as well as other motifs. The most conserved ZIM motif, TIFF/YXG, may be involved in the binding of DNA. Previous studies on proteins similar to RNA-binding proteins, WD40 proteins and ring zinc finger proteins (RZFs) have shown that these proteins may play important roles in the post-transcriptional regulation of transcription factors. WD-40 proteins contain 4–16 repeated short motifs of ~40 amino acids, often ending with a Trp–Asp (W–D) dipeptide. They form a large family found in all eukaryotes and are implicated in a variety of functions such as signal transduction and transcription regulation. The underlying common function of WD-repeat proteins is the coordination of the assembly of multiprotein complexes, where the repeating units serve as a rigid scaffold for protein interactions. DExH/D-box proteins are a large family of ATPases that have been proposed to mediate RNA structural rearrangements in a variety of cellular processes, including nuclear pre-mRNA splicing, ribosome assembly, protein synthesis, nuclear transport and RNA degradation.

Although the above discussion is speculative in nature, the information regarding the TaVRN1-interacting partners is of value for future work that aims to understand the cellular functions of TaVRN1 during plant development.

TaVRN1 accelerates flowering in Arabidopsis

To investigate further the function and regulation of *TaVRN1*, the gene was constitutively expressed in *Arabidopsis*. A total of 33 independent *35S:TaVRN1*

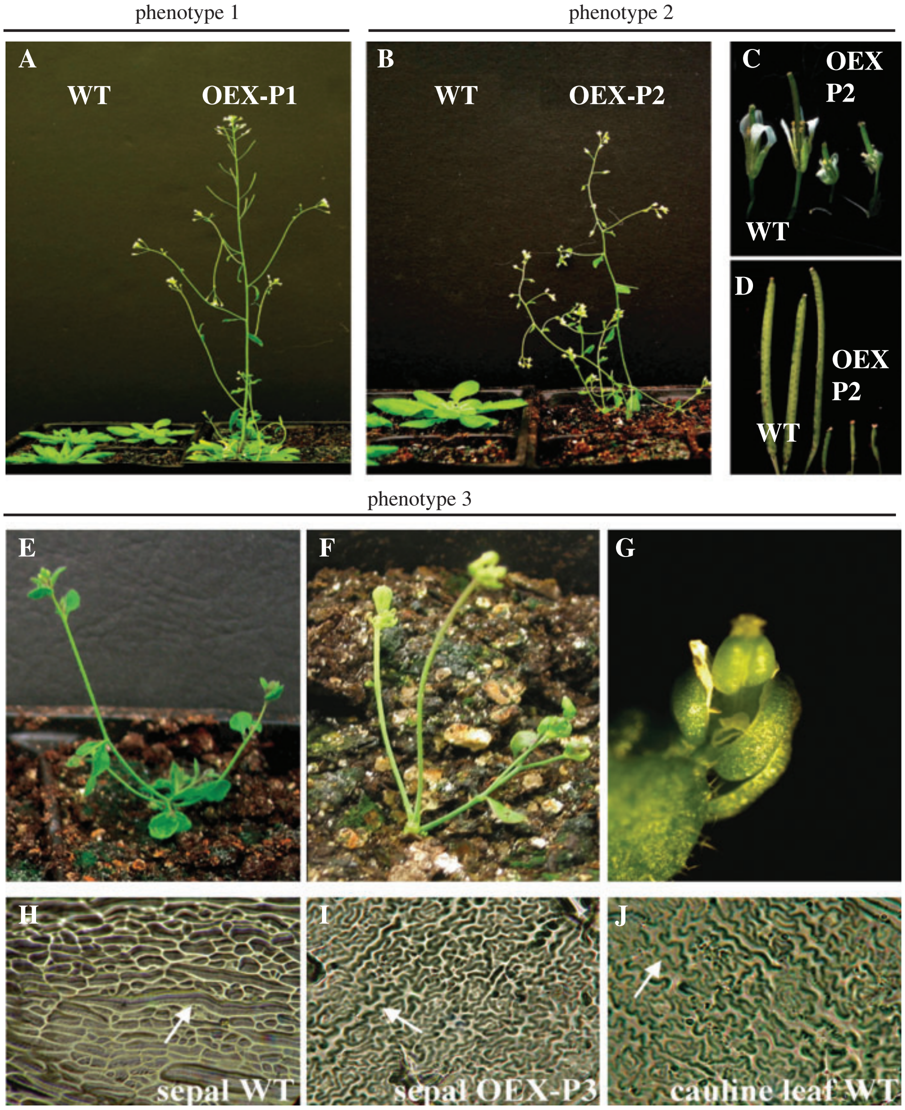

Fig. 3 Phenotypic effect of *TaVRN1* overexpression in *Arabidopsis*. Transformed plants show early flowering and varying levels of defects in architecture. This allowed us to classify them into three phenotypic groups. (A) Phenotype1 plants (OEX-P1) flower early compared with wild-type plants (WT), and are relatively normal in appearance. (B) Phenotype 2 plants (OEX-P2) flower early and display a reduced apical dominance and enhanced shoot branching, and rosette leaves are smaller. All organs of the OEX-P2 flowers are smaller compared with the wild type (C), as are the siliques (D). (E, F) Phenotype 3 plants (OEX-P3) flower early, display a loss of apical dominance and are smaller. (G) The flowers of OEX-P3 plants have shorter stamens and the carpels are abnormal. (H, I) Epidermal cells of flower sepal in WT and OEX-P3 plants. (J) Epidermal cells of a cauline leaf in the WT. The arrows show cells that display a typical epidermal cell morphology.

transformants were selected at the T_1 generation, of which 22 (66%) showed an early flowering phenotype compared with wild-type plants or *Pro35S:GUS*-expressing lines. In addition, some of these 22 lines showed other modifications of their development, which allowed us to classify the transgenic plants into three categories. Two plants per phenotype were brought to the T_2 generation and the selfed plants showed the same phenotypes. Phenotype 1 plants (30% of the transgenic plants) show an early flowering phenotype without modification of plant architecture and development (Fig. 3A). Phenotype 2 plants (21% of the transgenic plants) show an early flowering phenotype and reduced apical dominance (Fig. 3B). Flowers and siliques of these plants are shorter compared with wild-type plants (Fig. 3C, D). Phenotype 3 plants (15% of the transgenic plants) show an early flowering phenotype and a strong modification of their architecture (Fig. 3E, F). Plants are small and show reduced apical dominance and small rosette leaves. In addition, flower sepals show homeotic modifications: epidermal cells are similar to those of cauline leaves from wild-type plants (Fig. 3G–J). We hypothesized that the severity of the phenotype could result from varying numbers of T-DNA insertions in the different plants, but Southern analysis showed that this is not the case (data not shown). RT–PCR analysis revealed that *TaVRN1* transcripts accumulate in rosette leaves and floral meristems, and that the severity of the phenotype appears to be slightly associated with the transcript levels (Fig. 6, *TaVRN1*).

Since several members of the MADS-box transcription factors family are involved in flowering, it could be argued that the early flowering phenotype observed in the *TaVRN1*-expressing plants may not be specific to the transgene. However, we hereafter show that an opposite phenotype is oberved when another wheat MADS-box gene is overexpressed in *Arabidopsis*.

TaVRT2 delays flowering in Arabidopsis

We recently reported that the TaVRT2 MADS-box factor binds and represses *TaVRN1* promoter activity (Kane et al. 2007). Based on the expression patterns and molecular data, we suggested that TaVRT2 could be a negative regulator of flowering that would delay floral transition by the repression of *TaVRN1* transcription. *Arabidopsis* plants that constitutively express *TaVRT2* were generated and, contrary to the phenotype observed for *TaVRN1*-overexpressing lines, showed a delayed flowering time phenotype under long-day photoperiod. From these, 19 lines were randomly selected, brought to the T_2 generation, and the flowering time phenotype was again assessed. Finally, four lines were selected based on the strength of their phenotype and brought to the T_3 generation (Fig. 4A). The L1 and L5 lines flower 7–10 d later than the control line, while lines L17 and L23 flower 15 d later than the control lines. In addition to their flowering phenotype, lines L17 and L23 have shorter siliques compared with control plants (Fig. 4A). Lines L17 and L23 have slightly higher *TaVRT2* transcript levels compared with lines L1 and L5 (Fig. 4B), suggesting that the extent of flowering delay is associated with the level of *TaVRT2* transcript accumulation. Among the MADS-box family members, the closest TaVRT2 homologs are *SVP* (*Short Vegetative Phase*) in *Arabidopsis* (Hartmann et al. 2000) and *INCO* (*Incomposita*) in *Antirrhinum* (Masiero et al. 2004). Interestingly, overexpression of *SVP* or *INCO* in *Arabidopsis* and *Antirrhinum* also results in a delay in flowering time. Floral morphogenesis is affected in these plants, with flowers showing leaf-like features such as branched trichomes on sepals, petals and carpels, and sepaloid petals (Masiero et al. 2004). No such effects were seen in the *TaVRT2*-overexpressing plants. Together, our data show that *TaVRN1* and *TaVRT2* act in an opposite manner in the control of flowering when ectopically expressed in *Arabidopsis*, and suggest that a similar

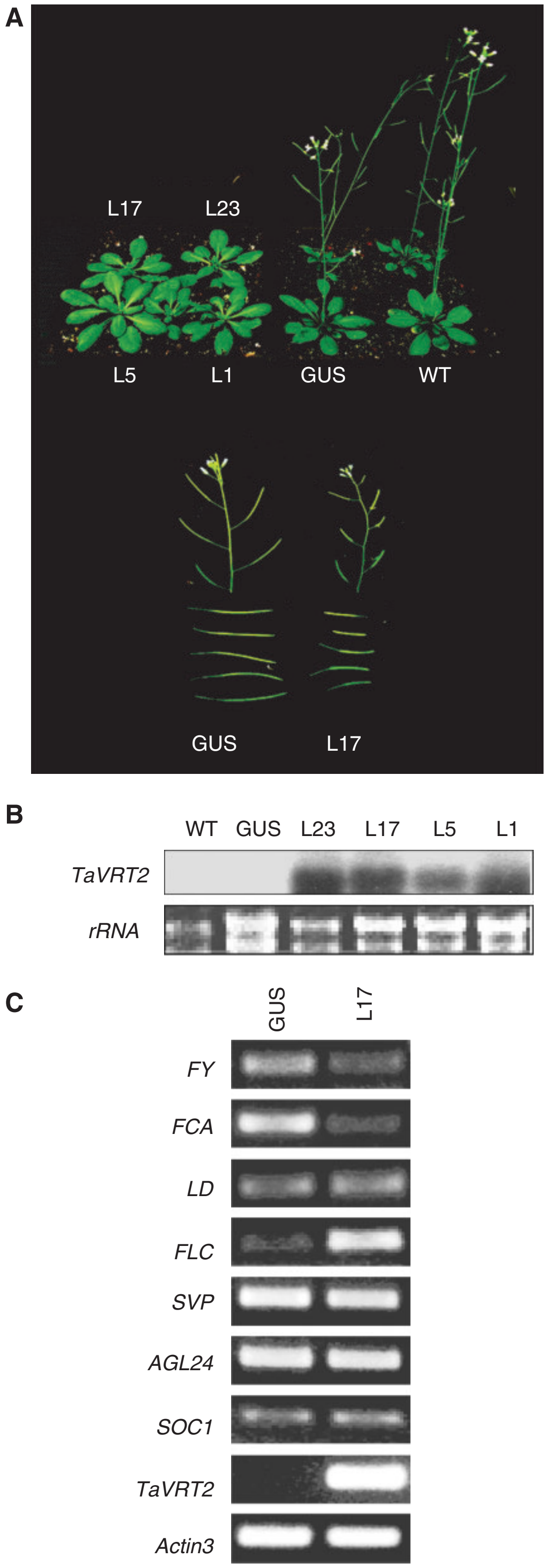

Fig. 4 *TaVRT2* overexpression in *Arabidopsis*. (A) Phenotypic effect of *TaVRT2* overexpression. Wild-type (WT) and transgenic plants expressing β-glucuronidase (GUS) or *TaVRT2* (L1–L23)

mechanism could possibly occur in wheat. This, however, remains to be demonstrated.

The delay in flowering may result from a modulation of the expression of downstream genes or from the titration of proteins that positively regulate floral transition. To determine the impact of *TaVRT2* overexpression in *Arabidopsis*, we have measured the accumulation level of several flowering-associated genes. The RT–PCR results show that the expression of FCA and FY is lower while that of FLC is higher in the transgenic plants (Fig. 4C). This is consistent with flowering regulation by the autonomous pathway. In wild-type plants, genes of the autonomous pathway such as *FCA* and *FY* induce flowering by reducing the level of the repressor *FLC* at both the mRNA and protein levels. This suggests that the delay in flowering observed in the transgenic *TaVRT2*-overexpressing plants is due to the accumulation of FLC via the autonomous pathway. Similarly, it was reported that overexpression of the flowering repressors *SVP* (Hartmann et al. 2000, Gregis et al. 2006) and *FLC* (Searle et al. 2006) in *Arabidopsis* modifies the expression of downstream genes, providing a molecular mechanism for their repressing effect on floral induction. FLC represses the expression of the floral integrator *SUPPRESSOR OF OVEREXPRESSION OF CONTSTANS* (*SOC1*). Intriguingly, *SOC1* expression is not affected in the *TaVRT2*-overexpressing plants even though FLC is down-regulated. This suggests that the delay in flowering in these plants results from the repression by FLC of other, as yet unidentified, target genes.

Flowering time can be measured by the number of rosette leaves at the time of bolting. Plants that flower early will show fewer rosette leaves, whereas a higher number of leaves is expected when flowering is delayed. The results clearly show that TaVRN1-expressing plants flower earlier while TaVRT2-expressing plants flower later than wild-type plants or GUS (β-glucuronidase)-expressing plants used as negative control (Fig. 5). It could be argued that the results obtained in our functional studies in *Arabidopsis* do not necessarily reflect the function of the TaVRN1 and TaVRT2 proteins in wheat. However, the fact that these two MADS-box genes lead to opposite phenotypes when ectopically expressed in *Arabidopsis* supports a specific effect of these genes on flowering.

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were grown for 34 d under long-day conditions at 20°C. Lower panel: comparison of silique size between the GUS and L17 lines. (B) Northern blot analysis of control and *TaVRT2*-overexpressing lines. (C) Effect of *TaVRT2* overexpression on the accumulation of flowering-associated transcripts. Total RNA was extracted from leaves of 15-day-old L17 transgenic *Arabidopsis* plants and transcript levels were measured by RT–PCR. *Actin3* was used as a control. Each experiment was repeated three times using RNA prepared from two biological samples. Similar results were obtained for the three other lines.

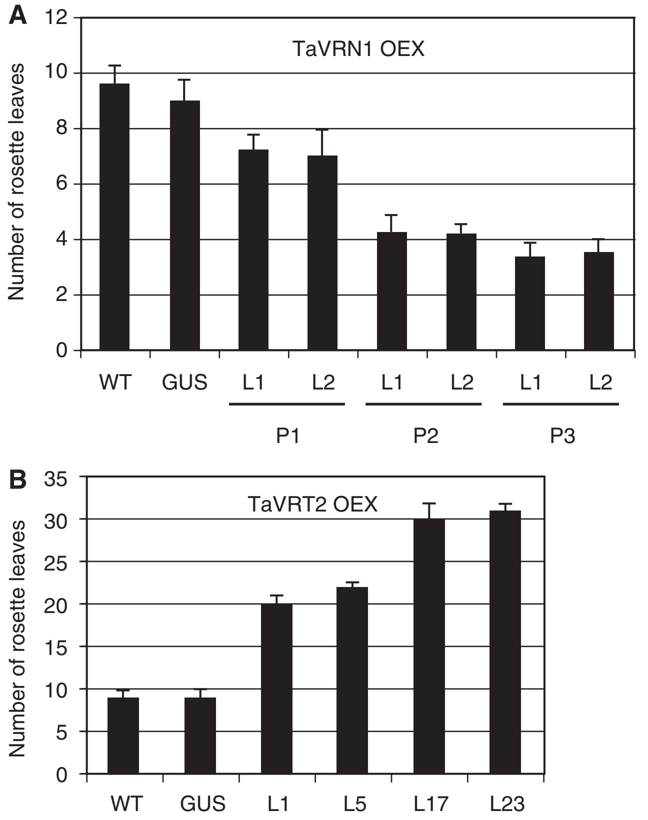

Fig. 5 Number of rosette leaves at the time of bolting. (A) Wild-type (WT) and transgenic plants expressing β-glucuronidase (GUS) or *TaVRN1* (L1–L6) were grown under long-day conditions at 20°C and leaves were counted when the first bolt became visible. L1 and L2 are lines of phenotype 1, L3 and L4 are lines of phenotype 2, L5 and L6 are lines of phenotype 3. The number of leaves is expressed as means ± SEM ($n = 12$ plants). (B) Wild-type (WT) and transgenic plants expressing β-glucuronidase (GUS) or *TaVRT2* (L1–L23) were grown under the same conditions as described in (A). The number of leaves is expressed as means ± SEM ($n \geq 15$ plants). Statistical significance was evaluated using Student's *t*-test relative to the WT plants.

Overexpression of TaVRN1 in Arabidopsis affects AP1 and MAX4 expression

Since the overexpression of *TaVRN1* affects flowering and apical dominance, it was of interest to determine if this overexpression affects transcript levels of genes that are known to be associated with these two physiological processes. In rosette leaves, the presence of TaVRN1 leads to the overexpression of *AP1*, a gene encoding a positive regulator of floral identity (Fig. 6A). All the other flowering-associated genes tested (*FUL*, *FT*, *AGL24*, *SVP* and *SOC1*) do not show differences in their accumulation pattern compared with wild-type plants. Expression levels of flowering-associated genes were also measured in floral meristems (Fig. 6B). Again, there is an increased accumulation of *AP1* transcripts in the *TaVRN1*-overexpressing

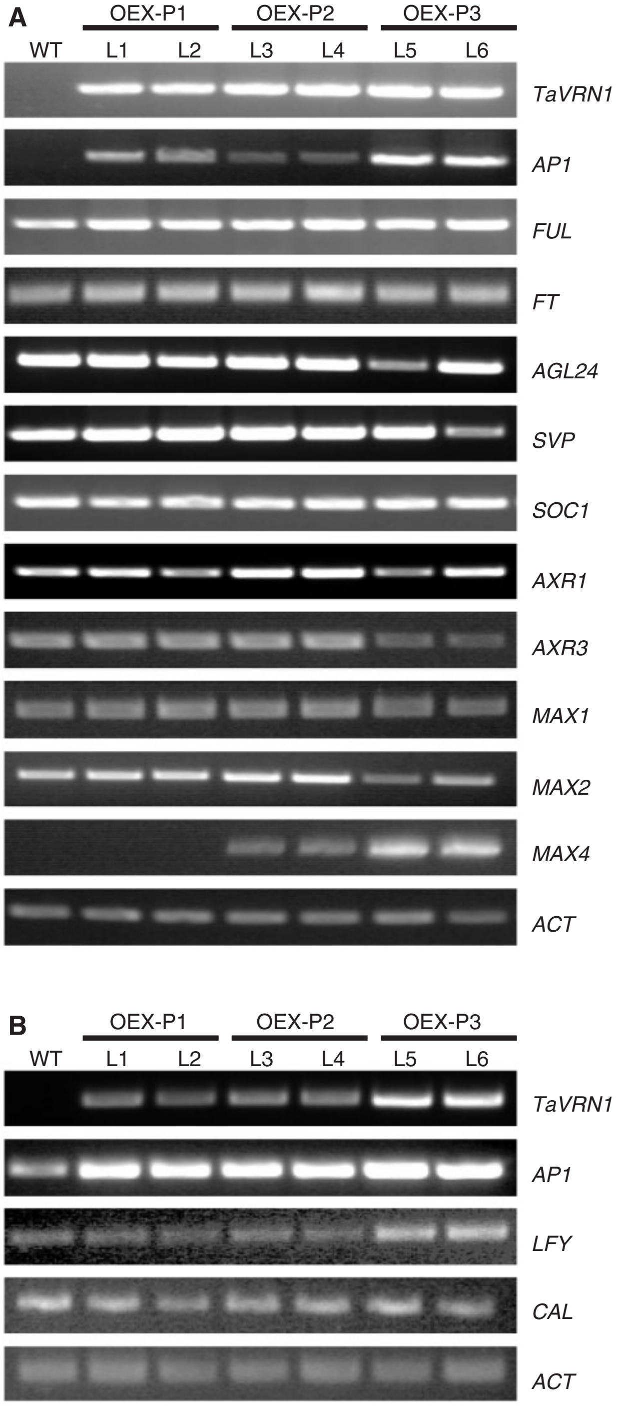

Fig. 6 Expression levels of selected *Arabidopsis* genes in the *TaVRN1*-overexpressing plants. Plants were grown under long-day conditions at 24°C. RNA was extracted from rosette leaves (A) or floral meristems (B) of wild-type and overexpressing plants representative of the three phenotypes, and analyzed by RT–PCR using gene-specific probes. Target genes were selected based on their reported involvement in the determination of floral identity or in auxin signaling. Analyses were performed on two independent lines per phenotype, and all PCRs were performed at least three times. The lower signal seen for *AGL24* in L5 and for *SVP* in L6 was not reproducible. *CAL* and *MAX3* transcripts were not detected in rosette leaves. *FUL* and *FT* were not detected in floral meristems. *Actin3* was used as a control.

plants compared with wild-type plants. There is a slightly higher level of *LFY*, but no differences was observed for the expression of *CAL*.

Apical dominance is associated with auxin regulation, therefore we tested if *TaVRN1* overexpression affects the expression of six *AXR* and *MAX* genes encoding proteins associated with auxin response. The results show that only *MAX4* is up-regulated by TaVRN1 in transgenic plants that exhibit phenotypes 2 and 3, the two categories that show defects in architecture (Fig. 6A). This indicates that these defects in the transgenic plants could result from the abnormal presence of *MAX4*.

For RT–PCR analyses, we initially collected tissues from wild-type and *TaVRN1*-overexpressing plants at the same biological, not physiological, age. However, since *TaVRN1*-overexpressing plants show an accelerated development compared with wild-type plants, one could argue that the difference in *AP1* and *MAX4* gene expression that we observed is the result of a difference in developmental stage between the two plants. RT–PCR analyses were thus performed on tissues collected at three stages of development for wild-type plants and compared with the expression in leaves of transgenic line L3, which shows phenotype 2 (Fig. 7). No differences were observed between the three stages in the wild type, except that *LFY* is expressed, as expected, after floral transition. These results indicate that the modification in expression of the flowering-associated *AP1* gene and of the auxin-associated *MAX4* gene is not a consequence of the modulation of plant development but is truly dependent on the ectopic expression of the wheat *TaVRN1* gene.

TaVRN1 transcripts are only localized in active growing tissues in both vegetative and reproductive organs, indicating that *TaVRN1* gene expression may be required for cell division and growth. Indeed, like the *TaVRN1* gene, several *AP1* subfamily genes are also expressed in different areas of the plant. For example, *MdMADS2* (Sung et al. 1999), *PFG* (Immink et al. 1999), *NtMADS11* (Jang et al. 2002) and *POTM1* (Kang et al. 2003) are all expressed in vegetative and floral organs. Overexpression of *TaVRN1* in *Arabidopsis* results in plants exhibiting reduced apical dominance. In potato, transgenic plants with reduced levels of the *AP1* gene *POTM1* exhibited decreased apical dominance accompanied by a compact growth habit and a reduction of leaf size (Rosin et al. 2003). In the axillary buds of these plants, cytokinin levels are higher than in the wild-type plants, suggesting that *POTM1* mediates the control of axillary bud development by regulating cell growth in vegetative meristems.

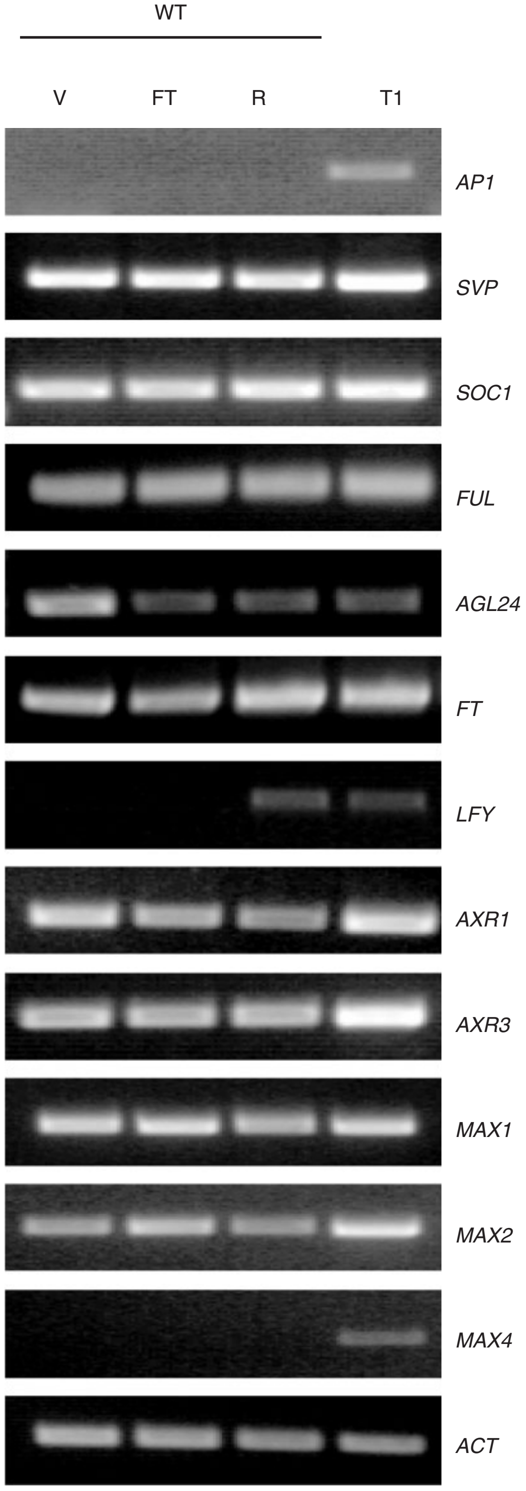

Fig. 7 Expression levels of selected *Arabidopsis* genes in wild-type and *TaVRN1*-overexpressing plants of phenotype 2. Plants were grown under long-day conditions at 24°C. RNA was extracted from rosette leaves at different stages of development (V, vegetative; FT, floral transition; R reproductive phase) and RT–PCR analyses were performed using gene-specific probes. *Actin3* was used as a control.

An attractive hypothesis is that overexpression of *TaVRN1* may alter the ratio of auxins and cytokinins to activate growth of lateral meristems. However, this remains to be determined.

The SAM is responsible for primary shoot growth, whereas lateral branching is initiated by the development of axillary meristems. Shoot branches are usually formed in two developmental steps: the initiation of axillary shoot meristems, then the outgrowth of axillary buds (Sussex and Kerk 2001). After axillary meristem initiation, the SAM maintains its role as the primary site of growth by maintaining a high auxin to cytokinin ratio in the axillary bud meristem, thereby inhibiting the growth of axillary meristems, a process called apical dominance (Leyser 2003). Molecular genetic analyses of *AXR1* and *AXR2* have revealed that the two genes are important players in the inhibitory effect of auxin on axillary bud growth (Leyser 2003). Recently, a novel pathway controlling the growth of axillary buds and shoot branching, called the MAX pathway, was identified in *Arabidopsis* (McSteen and Leyser 2005). Mutations in the four genes, *MAX1*, *MAX2*, *MAX3* and *MAX4*, result in an enhanced shoot branching phenotype (Booker et al. 2005). The observation that *35S:TaVRN1* plants show apparent modifications in apical dominance raised the possibility that TaVRN1 has an influence on the expression of *AXR* and *MAX* genes. Our expression studies showed that *MAX4* is overexpressed in plants that display a loss of apical dominance (enhanced shoot branching); therefore, plants that express *MAX4* ectopically show the same phenotype as plants that do not express *MAX4*. Intriguingly, it was reported that overexpression of *MAX4* in *Arabidopsis* does not lead to major phenotypic effects and that overexpression of other components of the MAX pathway may be needed to reduce bud outgrowth (Bainbridge et al. 2005). The level of *MAX1* and *MAX2* is not affected by the overexpression of *TaVRN1*, and we were not able to detect *MAX3*. It is therefore likely that TaVRN1 affects the expression of another, as yet unidentified gene involved in the regulation of shoot branching. The fact that *MAX4* knock-out and overexpressing plants do not exhibit different phenotypes could possibly be explained by the hormonal dose–response relationship, whereby the hyperstimulation of a hormone signal transduction pathway leads to antagonistic physiological effects. This phenomenon has been observed in studies describing auxin responses. For example, the *axr3-1* and *axr3-3* mutants were originally isolated as auxin-resistant mutants, but further characterization showed that the mutations actually increase the sensitivity to auxins (Leyser et al. 1996). The *axr3* mutant plants show enhanced apical dominance, a phenotype opposite to that of the *MAX4* mutant and *TaVRN1*-overexpressing plants.

AP1CArG1 -1350 ATTTATTTTT**CTAAAATTAG**TATAATTGTTG
TAAATAAAAA**GATTTTAATC**ATATTAACAAC

MAX4CArG1 -121 ATCGTCATTTACGCGTAT**CTAAAAAATG**AATAT
TAGCAGTAAATGCGCATA**GATTTTTTAC**TTATA

MAX4CArG2 -659 GTATAGAATACACC**CATATATATG**TATAT
CATATCTTATGTGG**GTATATATAC**ATATA

MAX4CArG3 -1360 CAAC**CTTTAAATAG**ATGAATC
GTTG**GAAATTTATC**TACTTAG

MAX4CArG4 -1534 ATGTCAGTATTT**CTATATATTG**ACGGTTATC
TACAGTCATAAA**GATATATAAC**TGCCAATAG

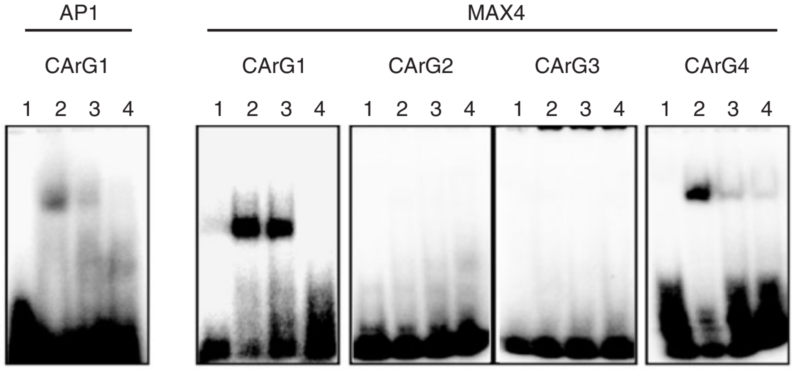

Fig. 8 Binding of TaVRN1 to CArG motifs of *Arabidopsis* promoters. (A) Double-stranded oligomers corresponding to the CArG motifs found in the *AP1* and *MAX4* promoters were synthesized and used as probes in electrophoretic mobility shift assays. (B) Bacterial extracts containing the recombinant TaVRN1 protein were incubated without (lane 1) or with ^{32}P-labeled oligomers (lanes 2–4). To assess specificity, two concentrations of unlabeled oligomers were used as competitors (lanes 3 and 4). The complexes were resolved by PAGE.

The up-regulation of *AP1* and *MAX4* in *35S:TaVRN1* plants suggested that the TaVRN1 transcription factor has the ability to interact physically with the *AP1* and *MAX4* promoters to trigger the accumulation of the corresponding transcripts. The *AP1* promoter contains one CArG motif, the *cis*-element bound by MADS-box transcription factors, in the proximal 1,700 bp upstream of the predicted transcription start site. Electrophoretic mobility shift assays (EMSAs) revealed that TaVRN1 binds the CArG motif, and that the binding is competed out by an excess of unlabeled DNA probe (Fig. 8). The *MAX4* promoter contains four CArG motifs in the proximal 1,700 bp upstream of the predicted transcription start site. EMSA analysis showed that TaVRN1 binds the CArG1 and CArG4 motifs, but not CArG2 and CArG3. These results show that the ectopically expressed TaVRN1 protein acts as a transcriptional activator of *AP1* and *MAX4* expression in *Arabidopsis* and suggests that it could induce the expression of wheat genes that possess CArG motifs in their promoters. There is a CArG motif in the *TaVRN1* promoter, which would suggest autoregulation in wheat. However, data obtained recently showed that the TaVRN1 protein does not appear to bind the *TaVRN1* promoter (Kane et al. 2007). The TaVRN1 target genes in wheat remain to be determined.

Our results provide evidence to support that (i) the early-flowering phenotype resulting from ectopic expression of the wheat *TaVRN1* MADS-box gene in *Arabidopsis* could be due to the interaction between the TaVRN1 protein and the *AP1* promoter; (ii) the modification in architecture could result from the overaccumulation of *MAX4* transcript and corresponding protein; and (iii) the late-flowering phenotype resulting from ectopic expression of the wheat *TaVRT2* MADS-box gene in *Arabidopsis* probably results from the up-regulation of the expression of the *FLC* repressor. The data reported here therefore suggest that ectopic expression of *TaVRN1* and *TaVRT2* could be used to modulate flowering time in various species.

Materials and methods

In situ RNA hybridization

Hexaploid spring wheat (*T. aestivum* L.) cv. Bounty was grown in environmentally controlled growth chambers under a long-day (16 h) photoperiod at 20°C and a light intensity of 150 μmol m^{-2} s^{-1} (Danyluk et al. 2003). Tissues were collected at different stages of development, as indicated in the figure legends, and used for in situ RNA hybridization. A specific probe corresponding to *VRN1* transcripts was PCR-amplified using primers containing the

T7 RNA polymerase initiation site (for primers, see Supplementary Table S1). This PCR fragment was used as template for the synthesis of an antisense digoxigenin-labeled riboprobe using the UTP-DIG (Roche) and T7 MAXIscript (Ambion) kits. The amplification product was tested by Southern blotting to confirm the specificity of the probe (data not shown). In situ RNA hybridization experiments were performed as described (Adam et al. 2007) except that tissue sections were de-paraffinized with xylene. Images were captured on a Leica Laborlux-S microscope using a Nikon Coolpix 4500 digital camera.

Yeast interaction screen

An interaction screen was performed in yeast using the Proquest two-hybrid system with GATEWAY technology (Invitrogen). To generate the bait construct, a truncated *TaVRN1* coding region lacking the MADS domain was cloned in pENTR4 (for primers, see Supplementary Table S1) then transfered to pDEST32 (GAL4-binding domain fusion; Invitrogen) by LR recombination, and selected clones were sequenced to verify the integrity of the construct. The bait did not auto-activate the system, indicating that this construct could be used for the screening. The interaction library was generated from a mix of four mRNA populations purified from aerial parts of winter wheat (cv Norstar): 1 cm crown sections after 30 d of vernalization, 1 cm vernalized crown sections that were exposed to flower-inducing conditions for 11 d, different developmental stages of spike formation (5–50 mm) before emergence from the flag leaf (dissection required) and different developmental stages of spike and seed formation after emergence from the flag leaf (visible) (Library 5, Houde et al. 2006). The corresponding cDNAs were cloned in pCMVSPORT6 to generate the original libraries, which were then transferred to pDEST22 [GAL4 activation domain (AD) fusion; Invitrogen] by LR recombination. The screening of 3×10^6 independent clones was carried out in the yeast strain MaV203, which contains three GAL4-inducible reporter genes (*HIS3*, *URA3* and *LACZ*). The transformants were plated on medium lacking tryptophan, leucine and histidine, and containing 25 mM 3-aminotriazole. Following incubation at 30°C, transformants containing putative interactors were selected by their growth on the selective medium. The plasmids carrying the GAL4-AD fusions were isolated from the yeast transformants and retransformed in *Escherichia coli*. Interactors were re-tested by two-hybrid assays, sequenced, and identified by homology-based search.

For RT–PCR, total RNA was extracted from wheat tissues collected at various stages of development and from different tissues using the RNAeasy kit (QIAGEN), and was reverse transcribed using the SuperScript$^{\text{TM}}$ II Reverse Transcriptase kit (Invitrogen). Expression of the genes encoding the interactors was detected by RT–PCR, and expression of the ribosomal subunit *Ta18S* was used as the internal control. The specific primers used for these genes are indicated in Supplementary Table S1. PCR amplification products were analyzed by electrophoresis on 1% agarose/ethidium bromide gels.

Overexpression of TaVRN1 and TaVRT2 in Arabidopsis

Pro_{35S}:*TaVRN1* and Pro_{35S}:*TaVRT2* constructs were generated in pGreen vector (for primers, see Supplementary Table S1), then electroporated in *Agrobacterium tumefaciens* for transformation of *A. thaliana* ecotype Columbia by floral dipping (Clough and Bent 1998). Transformants were selected on medium containing MS salts and vitamins supplemented with 50 mg l^{-1} kanamycin, and resistant T_1 seedlings were transferred to potting mixture and grown to maturity under long-day conditions (16 h photoperiod) at 24/20°C (day/night). Wild-type Columbia and plants transformed with pBIN19 (*Pro35S:GUS*) were used as controls. Transgenic lines showing a phenotype different from the wild-type plants were selected to produce homozygous lines. Seedlings were grown for 2 weeks in MS agar, transferred to short-day conditions at 20°C for 7 d and then transferred to long-day conditions until flowering. The epidermal cells of organs were observed by agarose imprints of surfaces. Tissues were collected, dissected and laid on a microscope slide dipped into a 3% agarose solution. Slides were chilled for 10 min at 4°C. After removing the tissues, images of the imprints were captured on a Leica Laborlux-S microscope using a Nikon Coolpix 4500 digital camera.

The flowering time of *TaVRN1*-overexpressing and *TaVRT2*-overexpressing plants was measured as the number of rosette leaves produced on the main shoot at the time of bolting. Seedlings were grown for 2 weeks in MS agar and transferred to long-day conditions at 20°C. Leaves were counted as soon as the first bolt became visible. A minimum of 12 plants from each line was used and the experiment was repeated twice. Statistical significance was determined following Student's *t*-test.

The expression of several genes associated with flowering and auxin signaling was detected by RT–PCR as described above and in the figure legends, and expression of the *Actin3* gene was used as the internal control. The plants used for these experiments were collected in the morning at least 3 h after the beginning of the light cycle.

Gel shift assays

The full-length *TaVRN1* coding region was PCR-amplified (for primers, see Supplementary Table S1) and cloned in pTrcHisB, then the histidine-tagged fusions proteins were expressed in *E. coli* by isopropyl-β-D-galactopyranoside (IPTG) induction following the manufacturer's recommendations (Invitrogen). The recombinant TaVRN1 proteins were released by sonication in a buffer containing 50 mM potassium phosphate pH 7.8, 100 mM NaCl, 50 mM KCl, 10 mM imidazole, 10% glycerol and lysozyme. A bacterial strain carrying the empty vector was treated similarly to provide a negative control for the gel shift assays.

Sense and complementary oligonucleotides (Fig. 8A) corresponding to the three CArG motifs found in the *AP1* promoter region (AT1G69120.1) and the CArG motif in the *MAX4* promoter (AT4G32810) were annealed and radiolabeled with [γ-^{32}P]ATP (PerkinElmer) to generate double-stranded probes. DNA binding reactions were performed in a total volume of 20 µl of buffer (10 mM Tris–HCl pH 7.5, 4% glycerol, 20 mM KCl, 20 mM dithiothreitol) containing 1 µM poly(dI·dC), 0.2% (v/v) Triton X-100, 2 ng ($\sim 5 \times 10^4$ c.p.m.) of probe, and 5 µl of the total bacterial protein extracts. The binding specificity was assessed by competition with 50 and 100 ng (*AP1* probe) or with 100 and 300 ng (*MAX4* probe) of unlabeled double-stranded oligonucleotides. Binding reaction mixtures were incubated for 15 min at room temperature and then resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel, prepared in 0.5× TBE, at 100 V for 90 min. The gels were dried, exposed to K screens and the signal was detected with a Personal Molecular Imager FX System (Bio-Rad).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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