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ORIGINAL ARTICLE

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Vernalization-induced flowering in wheat is mediated by a lectin-like gene *VER2*

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Abstract A vernalization-related gene *VER2* was isolated from winter wheat (*Triticum aestivum* L.) using a differential screening approach. The deduced VER2 is a lectin-like protein of 300 amino acids, which contains the presence of a jacalin-like GWG domain. RNA in situ hybridization results demonstrated that *VER2* gene expression is restricted to the marginal meristems of immature leaves in vernalized wheat seedlings. No hybridization signal was detected in the epidermal tissue and vascular bundles. However, "devernalization" resulted in the silencing of *VER2* gene activity. The gene expression pattern of *VER2* induced by jasmonate was similar to that induced by vernalization. Antisense inhibition of *VER2* in transgenic wheat showed that heading and maturation time were delayed up to 6 weeks compared with non-transformed wheat and the pBI121empty-vector-transformed wheat. Tissue degeneration at the top of the spike was also noticed in the antisense inhibited transgenic wheat. These results suggest that *VER2* plays an important role in vernalization signaling and spike development in winter wheat.

Keywords Flowering · Lectin-like gene · Transgenic wheat · *Triticum* · *VER2* · Vernalization

Abbreviations A-VER2TW: antisense *VER2* transgenic wheat plant · DIG: digoxigenin · GUS: β-glucuronidase · JA: jasmonate · ORF: open reading frame · RACE: rapid amplification cDNA ends · S-VER2TW: sense *VER2* transgenic wheat plant · UTR: untranslated region · *VER*: vernalization-related gene

Introduction

The flowering of higher plants is a complex biological process and is regulated by both environmental and developmental factors. Winter annual and biennial plants, such as winter wheat, require vernalization, a period of cold treatment (0–10 °C) for induction of flowering (Curtis and Chang 1930; Gregory and Purvis 1938). The shoot apex is believed to be the site of perception of the cold temperature (Curtis and Chang 1930). During the 2–8 weeks of vernalization, several metabolic changes are observed before morphological changes in the shoot apex appear. These metabolic changes appear to follow a strictly programmed and multi-step sequence. The altered morphological patterns as well as the dramatic physiological changes induced by vernalization can be reversed by "devernalization", i.e. treatment at a temperature of 33 ± 2 °C for 5 days. On the other hand, the cold-conditioned physiological status of plant tissues can be maintained through the mitotic divisions, but diminishes once meiosis or the reproductive process is initiated (Yong et al. 1999). The molecular mechanisms underlying vernalization-induced winter annual and biennial plant flowering are still largely unknown.

Arabidopsis thaliana has been reported to have at least four loci that are related to vernalization, i.e. *VRN1*, *VRN2*, *VRN3* and *VRN4*. Thus far, the *VRN2* and *VRN1* genes have been cloned (Gendall et al. 2001; Levy et al. 2002). Levy et al. (2002) reported that *VRN1* encodes a protein that binds DNA in vitro in a

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non-sequence-specific manner and functions in stable repression of the major target of the vernalization pathway, the floral repressor *FLC*. Overexpression of *VRN1* reveals a vernalization-independent function for VRN1 that is mediated predominantly through the floral pathway integrator FT, and demonstrates that VRN1 requires vernalization-specific factors to target FLC. Other studies suggested that VRN1 gene may function upstream of the gibberellic acid (GA_3) signaling pathway (Chandler et al. 1996). In addition, many other flowering-related *Arabidopsis* genes, identified using the map-based cloning method, such as *FCA* (Macknight et al. 1997), *FLC* (*FLOWERING LOCUS C*; T-DNA tagged by Sheldon et al. 1999; map-based cloning by Michaels and Amasino 1999), *FRI* (*FRIGIDA*; Johanson et al. 2000) and *FKF1* (*FLAVIN-BINDING, KELCH REPEAT, F BOX*; Nelson et al. 2000), have also been suggested to play a role in vernalization. The transition from the vegetative to the flowering state may also be under the control of biological clock-regulated genes (Nelson et al. 2000), and genetic studies also suggested that *FLC* is probably a key gene in regulating vernalization-induced flowering (Sheldon et al. 2000).

Genetic analysis has revealed several *VRN* loci involved in the control of flowering in winter wheat (*Triticum aestivum* L.; Law et al. 1993). These *VRN* loci have been mapped to chromosomes 5A, 5B, 5D and 7D (Law et al. 1993), but, heretofore, the cloning of a VRN gene from wheat has not been reported. We have identified a large number of vernalization-induced genes from winter wheat using RNA differential screening (Yong et al. 1999). Among these vernalization-related cDNAs (*VERC*; Chong et al. 1994a, 1994b, 1995; Zhao et al. 1999), a fragment of a cDNA, *VERC203* in antisense orientation inhibited cold-induced flowering (Chong et al. 1998). To further investigate the function of the *VER203* gene family in controlling vernalization-induced flowering, we isolated a homologue of this gene, named *VER2* (AF420243), using both RACE (rapid amplification of cDNA ends) and wheat cDNA library screening. *VER2* was localized in specific regions in the shoot apex through RNA in situ hybridization, and the function of this gene in vernalization-induced flowering was probed using antisense RNA in transgenic wheat.

Materials and methods

Plant materials

Seeds of winter wheat (*Triticum aestivum* L. cv. Jingdong No.1) were surface-sterilized in 2% NaOCl for 20 min. After extensive washing in water overnight, the seeds were germinated on moist filter paper under the following incubation conditions: (i) in the dark at 25 °C for 5 days (non-vernalization, V0), (ii) at 4 °C for 14 days (V14), (iii) at 4 °C for 21 days (V21), (iv) at 4 °C for 28 days (V28) and (v) at 4 °C for 21 days and subsequently at 35 °C for 5 days (DV).

The germinated seeds were incubated in a solution of jasmonic acid (4 μmol l^{-1}) for 2 min and grown at 25 °C for 12 h (JA).

Molecular cloning of the *VER2* gene

For reverse transcription–polymerase chain reaction (RT–PCR), total RNA was isolated from the plumules with Trizol reagent according to the manufacturer's protocol (Gibco BRL). The mRNA was purified using the poly(A) Tract mRNA Isolation System (Promega). The 5′ primer of PCR was the sequence from *VERC203* (5′ TAG AAT TCC CAC ACC CCG CCA CTC ACC 3′) and the 3′ primer was oligo d(T). The PCR product recovered from low-melting-point agarose (Sigma) was subcloned into pGEM-4Z using a T-vector method (Boehringer Mannheim).

A cDNA library was constructed into the λTriplEX2 vector with the SMART cDNA Library Construction Kit (Clontech). The cDNA library was screened with the α-[^{32}P]dCTP labeled cDNA probe of the *VER2* gene fragment.

DNA sequencing and analysis

The *VER2* cDNA was sequenced by the di-deoxy chain-termination method, using an autofluorescence sequencer (Model 377; ABI, Foster City, Calif., USA). Structural and homological analyses of the sequence were carried out.

Extraction of genomic DNA and Southern blot analysis

Genomic DNA was extracted from tissue as described by Dellaporta et al. (1983) with minor modifications. DNA (10 μg) was digested with appropriate restriction enzymes and fractionated on a 1.0% agarose gel. After blotting the DNA onto a positively charged nylon membrane (Boehringer Mannheim), the filters were pre-hybridized and hybridized as previously described (Sambrook et al. 1989; Ge et al. 2000). The blot was washed with 2×SSC (0.3 mol l^{-1} NaCl, 0.03 mol l^{-1} sodium citrate, pH 7.0) plus 0.5% sodium dodecyl sulphate (SDS) at room temperature for 5 min followed by 2×SSC plus 0.1% SDS at room temperature for 15 min and another washing in 0.1×SSC plus 0.1% SDS at 65 °C for 20 min. The autoradiograph of DNA was obtained by exposing X-ray film to the blot at −80 °C.

Extraction of RNA and Northern blot analysis

Total RNA was isolated using the Qiagen RNeasy plant Mini Kit and measured by a UV detector (Genequant; Pharmacia Biotech). Electrophoresis of the RNA transferred and cross-linked onto a nylon membrane (Hybrid N+) was performed as described by Sambrook et al. (1989) and Ge et al. (2000). About 15 μg of total RNA was loaded on each lane. The probe labeled with [^{32}P]dCTP was synthesized for hybridization. After hybridization for 20 h at 68 °C, the membrane was washed with 2×SSC plus 0.1% SDS at 68 °C for 20 min and with 1×SSC plus 0.1% SDS at 37 °C for 30 min. The X-ray film was exposed to the membrane at −70 °C for 3–7 days.

RNA in situ hybridization

Plumules excised from seedlings were fixed in a solution of 50% (v/v) ethanol containing 3.7% (v/v) formaldehyde and 5% (v/v) acetic acid at room temperature for 16 h. The fixed tissues were dehydrated in a graded ethanol series, cleared with xylene and embedded in Paraplast (Sigma). Sections (10 μm) were placed on poly-lysine-coated slides (Sigma). After dewaxing and rehydrating, the sections were digested for 30 min at 37 °C in a solution containing 100 mmol l^{-1} Tris–HCl (pH 7.5), 50 mmol l^{-1} EDTA, and 5 μg ml^{-1} proteinase K; acetylated by incubating in 0.25% acetic anhydride in 100 mmol l^{-1} triethanolamine–HCl (pH 8.0), for 5 min; dehydrated in a graded ethanol series to 100% and dried at room temperature.

Hybridization in situ was carried out as previously described (Xu et al. 2002). The antisense and sense RNAs of *VER2* were labeled with digoxigenin (DIG) through in vitro transcription of linearized pVER2 (DIG Northern Starter Kit; Roche, Mannheim, Germany) and hydrolyzed to a mean length of approx. 100 bases in carbonate buffer at 60 °C. Hybridization was carried out at 42 °C overnight with a solution containing 0.2 ng μl^{-1} probe, 50% formamide, 300 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris–HCl (pH 7.5), 1 mmol l^{-1} EDTA, 5% dextran sulfate, 1% blocking reagent (Roche), 150 μg ml^{-1} tRNA, 500 μg ml^{-1} poly(A). Labeled probe was detected through alkaline phosphatase coupled to anti-DIG antibody Fab fragments and 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP; Roche). When the color reaction was complete, the section was dehydrated in an ethanol series, cleared in xylene and mounted in Canada gum. Sections were observed and photographed by bright-field microscopy.

Construction of antisense and sense expression vectors

The expression plasmids of both the sense (p2-Sense) and the antisense (p2-Anti) *VER2* were constructed in pBI121. The cDNA, with *Bam*HI and blunt end, was inserted into the *Bam*HI–*Sma*I sites of pBI121 to create the p2-Sense expression vector. The same insert was ligated in the vector with *Bam*HI-blunt (derived from *Xba*I) end to produce the p2-Anti expression plasmid. The GUS (β-glucuronidase) gene was a marker. The *VER2* fusion gene was driven by the cauliflower mosaic virus (CaMV) 35S promoter.

Gene transformation in wheat

Gene transformation in wheat was carried out by the pollen-tube-pathway method, which transfers the plasmids via the pollen tube into the ova at the moment of fertilization (Chong et al. 1998). In the experiment, a stigma was excised within 30 min to 3 h after pollination and a droplet of the plasmid DNA solution was pipeted onto the end of the style. Potential transformants were marked with indelible ink on the glume. The seeds were harvested at maturity, and planted into soil after a short after-ripening period.

Histochemical assay of GUS

The whole or sectioned tissues were stained with 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Gluc) according to Jefferson (1987).

Results

Isolation and molecular characterization of VER2

The 3′ end of the *VER2* cDNA, about 1.2 kb in length, was obtained by using the RACE approach. To isolate the full-length transcripts of members of the *VER* (vernalization-related) gene homologue, a cDNA library was first constructed using mRNA extracted from the plumule tissue of the vernalized wheat seedlings and screened with a probe of the cDNA fragment. Out of 1 million lambda phage clones, three putative *VER* cDNA clones were selected. DNA sequence analysis of one of these cDNA clones revealed a 1.25-kb long cDNA insert (Fig. 1a) containing a 903-bp-long open reading frame (ORF) with 61% nucleotide identity to the *VER203* cDNA. This cDNA was then named *VER2* (*vernalization-related gene 2*). The 5′-upstream untranslated region (UTR) was 84-bp long, whereas the 3′-downstream UTR was 263-bp long. The specific tailing sequences, such as AATAA, appeared at the 3′ UTR of the cDNA sequence (Fig. 1a). The ORF was predicted to encode a 300-amino acid-long polypeptide of a molecular mass of 32,427 and a pI of 6.77.

Comparative alignment of the amino acid sequence of the *VER2* gene with sequences deposited in protein databases (GenBank and SwissPro) suggested that *VER2* is closely related to a lectin-like protein, agglutinin (AF319617) and β-galactosidase aggregation factor precursor (AF232008), as shown in Fig. 1b and Table 1. Interestingly, a novel motif GWG was repeated four times in the VER2 protein (Fig. 1b). The domain of GPWGGNGG, a member of the GWG motif, is involved in a conservative amino acid sequence that is nearly identical to the salt- and drought-inducible *SalT* gene in rice (Hirano et al. 2000). Alignment of the C-terminal end (187–300 amino acids) of the VER2 protein with those protein sequences deposited in the databases revealed a similarity to jacalins, a subfamily of lectin-like proteins. The conservative GWG motif appeared in lectin-like proteins.

Spatial and temporal expression of the *VER2* gene in wheat

RNA hybridization in situ revealed that *VER2* mRNA accumulated in immature leaves around the shoot apex after 2–4 weeks of cold treatment, whereas no in situ hybridization signal was detected in tissues of the untreated group (Fig. 2a, c, d; Fig. 3). In situ hybridization signals were positively correlated with the length of vernalization. Under the same hybridization conditions except for using the sense probe, no signals were detected (Fig. 2b), which indicates that the experimental system of in situ hybridization worked well. In contrast, the *VER2* mRNA was not found in the cold-treated coleoptile or in any tissues of "devernalized" plants (Fig. 2a–e). When seedlings of winter wheat were treated with JA, the intensity and distribution of in situ hybridization signals of the *VER2* gene in the apex were similar to those in the vernalized wheat tissues (Fig. 2f). Curiously, the in situ hybridization signals of the *VER2* gene were not detected in the apical meristem of vernalized wheat. This was in clear contrast to the signals detected in immature leaves around the apex. Transverse sections (Fig. 3) showed clearly that hybridization signals were gradually weaker from the tip to the base in the same immature leaf. Similar trends in signal strength from stronger to weaker appeared in different leaves from the outside to the inner circle. In contract, no signal appeared in a coleoptile around immature leaves or in the epidermal tissue or vascular bundles of immature leaves (Fig. 3). The *VER2* transcripts accumulated specifically in the marginal meristem of young leaves.

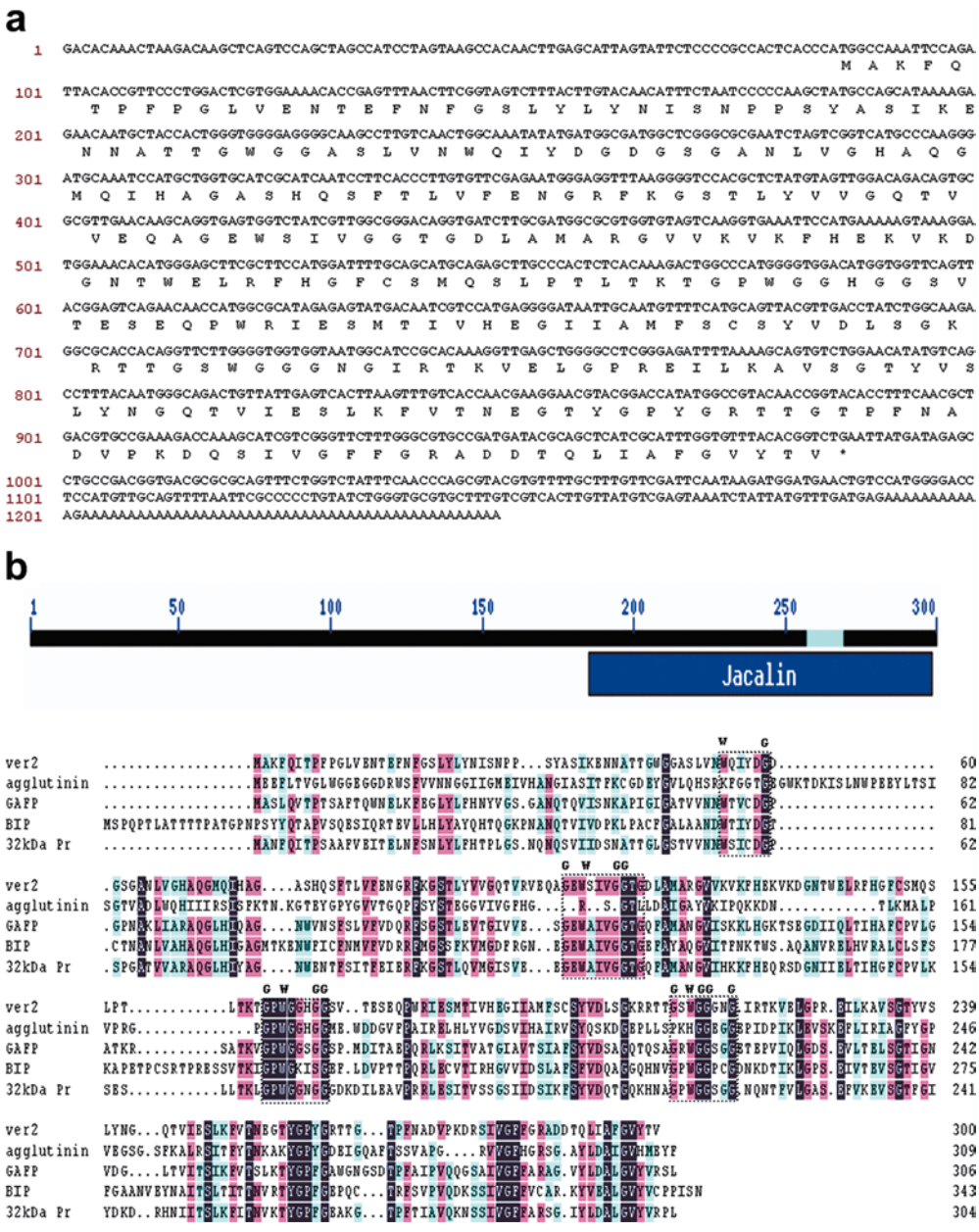

Fig. 1a b DNA coding and amino acid sequence of *VER2*, and amino acid alignments of VER2 and other proteins containing the jacalin-like domain. **a** cDNA sequence and deduced amino acid sequence of *VER2*. The *asterisk* indicates the translation stop codon (985 TGA). The sites of poly(A)-tail addition (AATAA) locate in 1071–1075. **b** The amino acid sequence of VER2 (accession number AB012103) aligned with other proteins containing the jacalin-like domain is shown. *Agglutinin*, agglutinin from *Castanea crenata* (AF319617); *GAFP*, *β*-glucosidase aggregating factor precursor from *Zea mays* (AF232008); *BIP*, benzothiadiazole-induced protein from *Triticum aestivum* (U32427); *32 kDa Pr*, 32-kDa jasmonate-induced protein from *Hordeum vulgare* (AF021256). Shading highlights the homology level: *black* = 100%, *pink* > 75%, *blue* > 50%

A possible regulatory function for VER2 in vernalization-induced flowering

In order to investigate the possibility of a regulatory function for the *VER2* gene in monocot plant flowering, a genetic transformation was carried out in wheat. To construct an expression vector of the target gene, a cDNA fragment of 1.2 kb at the 3′ end of the *VER2* gene was inserted between downstream of the CaMV 35S promoter sequence and upstream of the GUS gene in the pBI121 vector, and placed in both sense and antisense orientations. These recombinant constructs, including the control pBI121 empty vector, were then transformed into winter wheat using a pollen-tube-pathway method (Chong et al. 1998). The number of seeds carrying the sense *VER2* cDNA (named p2-Sense), antisense *VER2* cDNA (named p2-Anti) and the control DNA (pBI121) were 398, 359 and 133, respectively. A total of 887 seeds was obtained from 1,091 florets inoculated with the recombinant DNA carrying either sense or antisense *VER2* gene fragments, and the ratio of transformed seeds to florets was 79%–83% (Table 2). Histochemical staining of GUS activity identified 68 transgenic wheat plants obtained from the germinated wheat seeds, suggesting that an average of about 8.5% of the harvested seeds were successfully transformed (Table 2, Fig. 4d). Transformation of wheat plants with positive GUS signals was further confirmed by PCR, Southern blot and Northern blot analysis. The PCR amplified 1.4-kb and 0.6-kb fragments, respectively, that were specific to p2-sense and p2-antisense constructs (Fig. 4a). Southern blot analysis with a GUS gene probe

Table 1 Homology between VER2 from wheat (*Triticum aestivum*) and related proteins

Accession number	Gene coding product	Identities (%)	Positives (%)
AF420243	VER2	-	-
AF319617	Agglutinin protein	27	42
AF232008	β-Glucosidase aggregating factor precursor	35	50
G7489661	BIP (WCI-1, benzothiadiazole-induced protein in *T. aestivum*)	34	47
AF021256	32-kDa protein, JA-induced	43	56

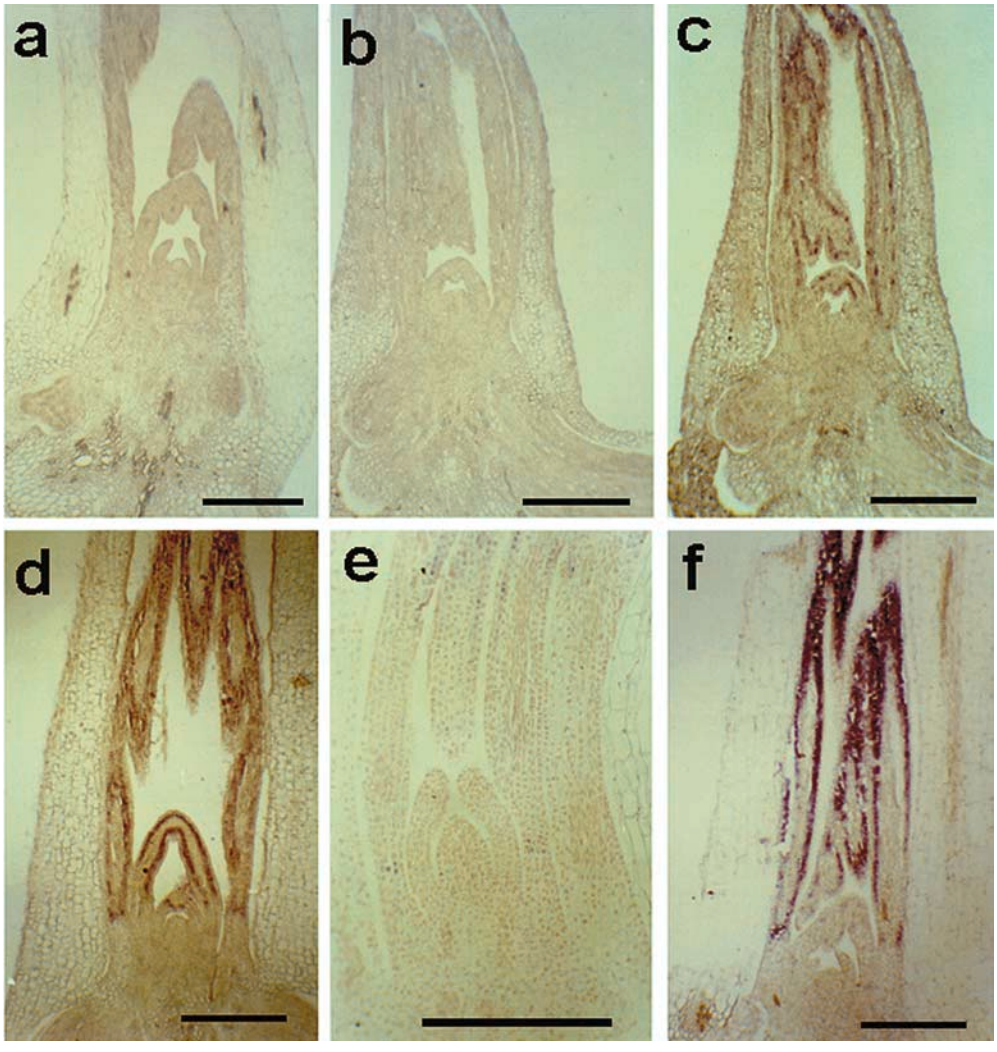

Fig. 2a–f RNA in situ hybridization analysis to show temporal pattern of the *VER2* gene expression during vernalization in wheat (*Triticum aestivum*). **a** Non-vernalized apex; **b** vernalization treatment for 14 days, followed by hybridization with the sense probe as a control; **c** vernalization treatment for 14 days; **d** vernalization treatment for 28 days; **e** devernalization treatment after vernalization for 28 days; **f** treatment with JA. Bars = 500 μm

also showed that there were 3.0-kb and 1.9-kb GUS gene-containing nuclear DNA fragments in sense (S-VER2TW) and antisense (A-VER2TW) configurations, respectively (Fig. 4b). Northern blot data showed reduction patterns of mRNA expression only in the representative lines of antisense transgenic plants compared with non-transformed control plants and the sense transgenic plants (Fig. 4c).

Having obtained these transgenic wheat lines, we examined the number of days required for heading following vernalization in both S-VER2TW and A-VER2TW lines compared with the control. The heading time for A-VER2TW lines was 149 ± 8 days (mean ± SD). This was nearly 6 weeks behind the heading time of the control pBI121 transgenic wheat, the sense transgenic lines and the non-transformed controls (Table 3, Fig. 5a). The S-VER2TW lines showed a normal flowering phenotype for the T2 generation although some S-VER2TW transgenic plants showed a delay in flowering in the T1 generation (data not shown). Degeneration also was observed at the top of spikes in the A-VER2TW group (Fig. 5d). The degeneration of some A-VER2TW lines became more apparent even if vernalized, with the degeneration phenotype expanding

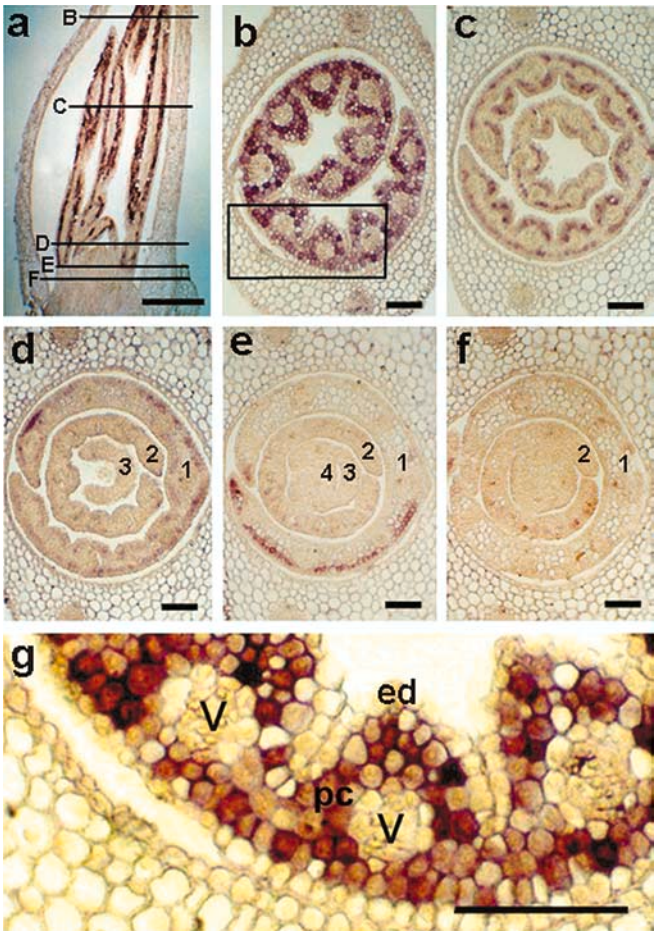

Fig. 3a–g RNA in situ hybridization analysis to show the spatial pattern of *VER2* gene expression in different positions from the top to the base of a wheat leaf (vernalized for 21 days). **a** longitudinal section; **b–f** transverse sections at positions *B–F* shown in **a**, respectively; **g** Enlargement of the area marked in **b**. Numbers *1–4* indicate developmental stages of leaves. *ed* Epidermal tissue, *pc* parenchyma cells, *v* vascular bundles. Bars = 500 μm (**a**), 100 μm (**b–g**)

from the first and second spikelet to the middle of the wheat spike, or in some instances, even to the whole spike (Fig. 5c). The delayed-flowering phenotype, as well as the abnormal spike phenomenon following vernalization, was maintained throughout three generations in these transgenic monocots. Examination of T4 antisense transgenic wheat showed that the defective vernalization response of these plants was similar to that of T1 plants (Fig. 6). Three weeks of cold treatment failed to induce flowering of the antisense *VER2* transgenic lines. Slight differences in heading percentage between the control (non-transformed), the sense transgenic lines and the antisense transgenic plants existed under both non-vernalization and vernalization treatments for 7 days, but they did not reach a statistically significant level. Thus, VER2 may have a role in promoting heading and spike development in wheat.

Discussion

Morphological changes of the stem apex occur during transition from vegetative growth to reproductive development. The vernalization speed (D) of the stem apex is related to the duration (t) and temperature (T) of vernalization. The relationship can be expressed as a function of $dD/dt = \int[T(t)]$ (status of vernalization was determined by diameter of stem apex; Wurr et al. 1993). In fact, serial changes in gene expression and metabolic response to temperature appear in plant cells before morphological changes take place. In anticipation, a series of genetic and metabolic events may occur in response to temperature change, and a multifactor-control model can therefore be proposed (Fig. 7). There are three stages during the development of the vernalization response based on morphological changes to heading (i.e. inducing period of vernalization, accelerating period of vernalization and steadying period), which are involved in five programmed metabolic characteristics (Li and Tan 1987; Li et al. 1987). The VER2 gene may have several functions and may play a direct or indirect role in accelerating vernalization, as suggested by its expression pattern (Fig. 2) and its effect on flowering (Fig. 5).

In our experiments, flowering of the antisense inhibited transgenic wheat was delayed up to 6 weeks compared with the positive controls (vernalized pBI121 control wheat, and the sense transgenic plants). The first and second spikelet from either the top or the base of the spike degenerates in winter wheat if vernalization is insufficient, and degeneration can be reduced by sufficient vernalization (Liang et al. 2001). The antisense *VER2* transgenic plants showed abnormal development of the compound spike. These phenotypes suggest that VER2, or a signal generated from the VER2 protein, could also function in spike development.

Comparison of the deduced C-terminal region of VER2 indicated that the protein has a jacalin domain and may also belong to a jacalin subfamily (data not shown). Jacalin, isolated from the seed of the jackfruit,

Table 2 Statistics for gene transformation in wheat

Plasmid	Number of transformed florets	Harvested seeds		Germinated seeds		GUS-positive plants	
		No.	%	No.	%	No.	%
pBI121	40	33	83	30	92	5	12
p2-Sense	506	398	79	310	78	29	5.7
p2-Anti	359	359	82	320	89	34	7.8

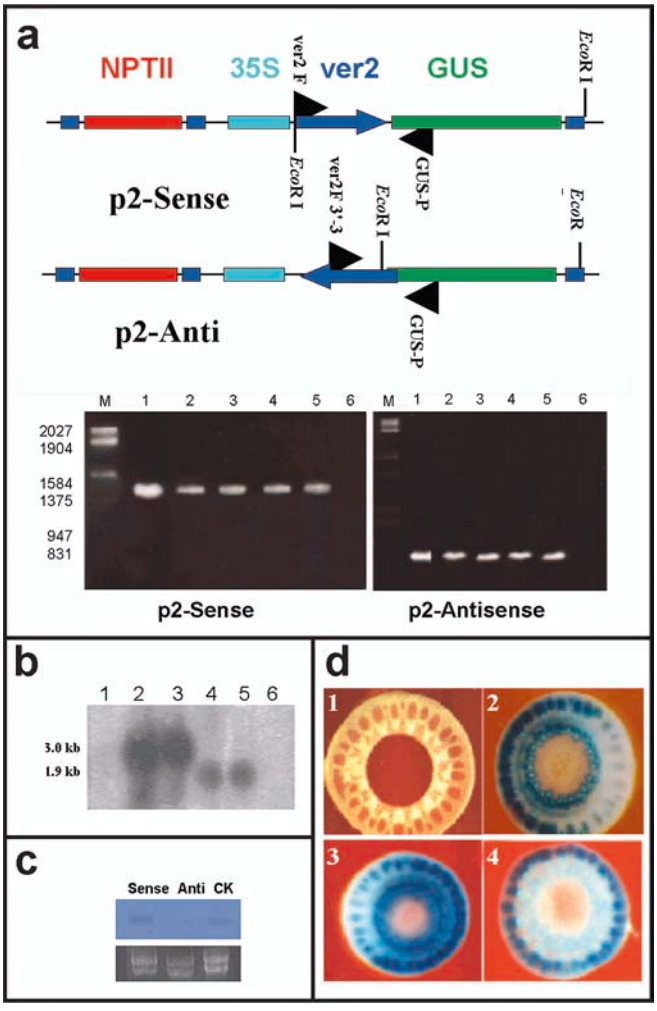

Fig. 4a–d Identification of a transgenic plant by PCR, Southern blot, Northern blot and GUS histochemical analyses. **a** *Top*: a model of the sense and the antisense construct to show locus of the insert and the PCR primers in the pBI121 vector. *Bottom*: electrophoreses maps of PCR products. *Lane M*, molecular maker; *lane 1*, positive control with plasmid DNA as template; *lanes 2–5*, transgenic lines (p2-Sense: p2-S-47, p2-S-53, p2-S-105 and p2-S-216; p2-Antisense: p2-A-79, p2-A-108, p2-A-275 and p2-A-293); *lane 6*, negative control, template DNA from an non-transformed plant. Primers used in PCR analysis were the following: GUS-P, GCG TCG ACC AAC GCT GAT CAA TTC CACA; *VER2* F primer, TAG AAT TCC ACA CCC AGC CAC TCA CCC; *VER2* F 3′-3 primer: CGATAGACCACTCACCTGC TTGTTC. **b** Southern blot analysis of transgenic wheat. *Lane 1*, MW marker; *lanes 2, 3*, *Eco*RI digestion of DNA from the sense transgenic plant lines (p2-S-105, p2-S-216); *lanes 4, 5*, *Eco*RI digestion of DNA from antisense transgenic plant lines (p2-A-79, p2-A-108); *lane 6*, normal plant control. **c** Northern blot analysis of transgenic wheat. *CK*, non-transformed plant; *Anti*, antisense transgenic plant; *Sense*, sense transgenic plant. **d** GUS histochemical assay of the representative transgenic wheat lines. *1*, non-transformed; *2*, pBI121 transgenic plant; *3*, sense transgenic plant and; *4*, anti-sense transgenic plant

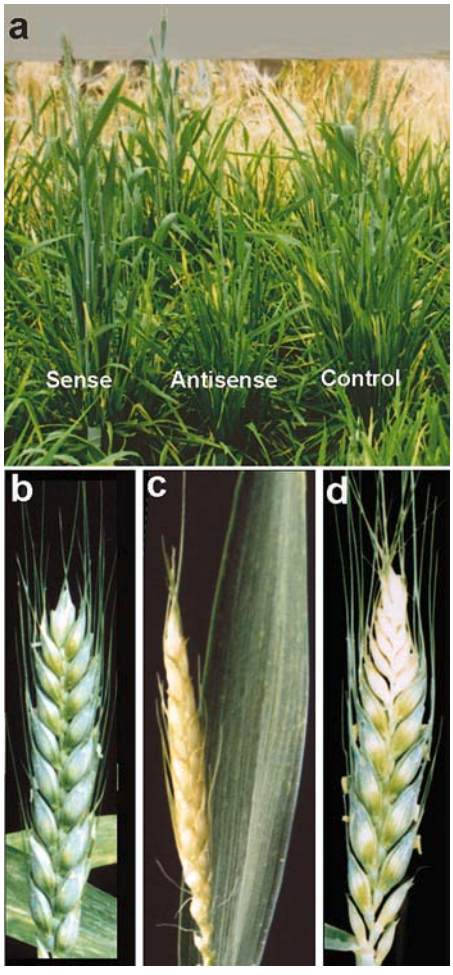

Fig. 5a–d Phenotype of transgenic wheat. **a** Growth and development status of the control (pBI121 transformed), the antisense and the sense T4 transgenic plants (120 days from germination). **b** Wheat spike of the control. **c,d** Degenerate spikes of the representative antisense transgenic lines

is one member of several families of plant lectins that are specific for binding to galactose, although its function in plants is still unknown (Young et al. 1989; Kabir and Daar 1994). The structure of jacalin is similar to that of lectins that stimulate distinct T- and B-cell functions (Pecanha and Dos Reis 1989). Some JA-inducible proteins, the salt-stress induced protein from rice and an animal prostatic spermine-binding protein also belong to this lectin-superfamily (Geshi and Brand 1998; Zhang et al. 2000). Discovery that *VER2* encodes a lectin-like protein may constitute an important clue to its biochemical function in plant cells.

The florigen (Chailakhyan 1936) and nutrient division hypotheses have been proposed for many years to address the mechanisms underlying the developmental and environmental control of flowering. The shoot apex has long been considered the sensor for vernalization (Curtis and Chang 1930); however, our in situ hybridization data appear to contradict the prevailing concept (Fig. 2). It seems that it is the immature leaves that perceive the vernalization signal. The supporting data come from the following: first, the expression of *VER2* was induced by vernalization (Fig. 2); second, the *VER2* gene product is required for cold-induced

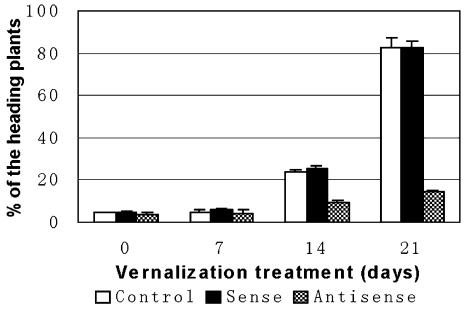

Fig. 6 Response of the T4 transgenic plant heading phenotype to vernalization. Data are means and SD (more than 60 plants were used in each statistical group)

Table 3 Phenotypic analysis of vernalized transgenic wheat plants. Non-transformed plants were used as a positive control

Lines	Response to GUS activity and PCR	Average heading time (days)[a]	Degeneration of spikelets
Antisense	+	149 ± 8	Yes
Sense	+	105 ± 5	No
pBI121	+	103 ± 7	No
Control	–	108 ± 6	No

[a]Time for the appearance of a discernible spike protruding above the leaf sheath; mean ± SD

flowering (Figs. 5, 6; Table 3); and third, *VER2* expression was limited to young leaves surrounding the apical meristem throughout the vernalization period (Fig. 2).

The molecular basis for vernalization has been investigated through characterization of a set of genes in *Arabidopsis*. *FRIGIDA* (*FRI*), *FLOWERING LOCUS* (*FLC*) and *VRN* genes (such as *VRN*2, *VRN1*) are key members in a network of genes that play a signaling role in vernalization. FRI increases the mRNA level of FLC, a MADS-box protein that functions as a floral repressor (Sheldon et al. 1999; Johanson et al. 2000). After cold treatment, the expression of FLC is reduced and remains at low levels throughout the growth cycle, but returns to high levels in the next generation. Transgenic plants constitutively expressing FLC exhibit late flowering after cold treatment (Michaels and Amasino 1999, 2000; Sheldon et al. 1999). The *VRN2* gene mediates vernalization and encodes a nuclear-localized zinc-finger protein with similarity to Polycomb group (PcG) proteins. *VRN2* function stably maintains FLC repression after a cold treatment, serving as a mechanism for cellular memory of vernalization (Gendall et al. 2001). Cold-induced posttranslational modifications of VRN1 and changes in cellular location may change VRN1 activity. Alternatively, vernalization-induced accessory proteins may recruit VRN1 into a complex, possibly including VRN2, which can target FLC (Levy et al. 2002). Demethylation is speculated to repress expression of the FLC gene. Gibberellin (GA) action and repression of *FLC* also promote *LFY* expression (Sheldon et al. 2000). Thus, investigation of the role of *VER2* in VRN2, VRN1, FRI, FLC and LFY signaling pathways during vernalization-induced flowering should provide new insights into the vernalization-signaling network in flowering of winter wheat. For example, an approach of overexpression of *VER2* in different genetic mutants may reveal the relationship between *VER2* and other genes which function in a genetic pathway in floral development.

Vernalization decreases DNA methylation, and the methylation status of plant genomes is known to affect vernalization-induced flowering in higher plants (Burn et al. 1993; Brock and Davidson 1994; Finnegan et al. 1996, 1998; Ronemus et al. 1996). Jasmonate (JA), a recently found plant growth regulator (Creelman and Mullet 1997), can induce both flowering and expression of an *O*-methyltransferase gene in barley (Lee et al. 1997). Because expression of *VER2* was induced by treatment with JA (Fig. 2), it is possible that *VER2* may play a role downstream of JA signaling to regulate flowering in a methylation-dependent manner. Demethylation is speculated to reduce expression of FLC (Michaels and Amasino 2000), indicating one site of regulation. Moreover, our unpublished data suggest that *VER2* is also GA-inducible. Because a GA synthesis-related gene has been known to induce flowering (Burn et al. 1993; Brock and Davidson 1994) and to respond to vernalization, it will be interesting to determine the relationship between GA and *VER2* gene function.

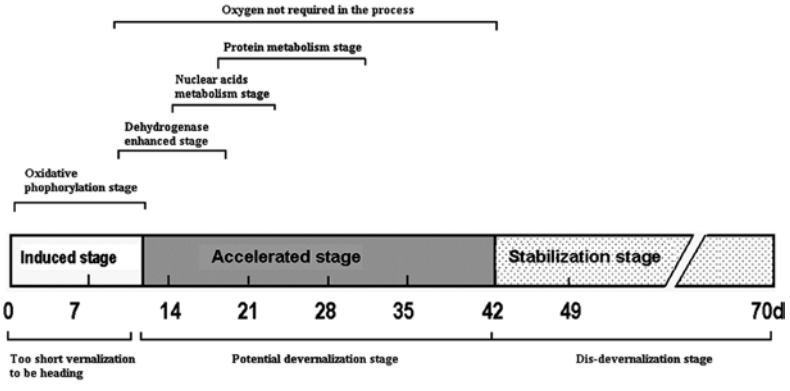

Fig. 7 Metabolic progress during vernalization of wheat

Our data suggest that vernalization of wheat is like the photoperiodism-regulated flowering response, in which the leaf perceives the photoperiodic signal and transduces the signal to the shoot apex. The shoot apex responds and undergoes morphological changes (Curtis and Chang 1930; Gregory and Purvis 1936, 1938). However, it is also possible that vernalization signaling could be more complex in wheat flowering. There may exist a signal exchange between the shoot apex and young leaves to regulate flowering. The shoot apex could still be the primary cold-temperature sensor: it perceives the cold signal and transduces that signal to the young leaf, which then expresses *VER2*. In turn, the young leaf sends VER2 signals to the shoot apex, which undergoes morphological changes. Thus, VER2 becomes the intermediate signaling component in this apex versus young leaf communication model. Nevertheless, the 70-year-old hypothesis by Curtis and Chang (1930) regarding the location of the primary site that perceives vernalization signal may have to be re-examined as the molecular evidence accumulates.

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