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Characterization of three VERNALIZATION INSENSITIVE3-like (VIL) homologs in wild wheat, Aegilops tauschii Coss.

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Control of flowering time is an adaptive trait of plants for different growth habitats. A vernalization requirement is a major genetic component determining wheat flowering time. Arabidopsis VERNALIZATION INSENSITIVE3 (VIN3) and VIN3-like 1 (VIL1) play critical roles in the vernalization pathway of flowering, and three wheat VIL homologs are upregulated by vernalization in einkorn wheat. To study the relationship between vernalization and wheat VIL homologs in Aegilops tauschii, the D-genome progenitor of common wheat, we isolated three cDNAs orthologous to the einkorn wheat VIL genes. The three Aegilops tauschii VIL genes showed many single nucleotide polymorphisms including non-synonymous substitutions relative to the einkorn orthologs. In addition, high rates of non-synonymous and synonymous substitutions were revealed by intraspecific variation analysis of the AetVIL sequences, suggesting adaptive evolution at the AetVIL loci. Quantitative RT-PCR analysis was conducted to examine the time course of expression of the VIL genes during vernalization. Of the three AetVIL genes, AetVIL2 was upregulated after one week of low-temperature treatment, and its expression pattern was distinct for winter and spring habit accessions. These observations strongly suggest that AetVIL2 is associated with the vernalization-responsive pathway in A. tauschii.

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Timing of flowering is a significant trait influencing fitness in higher plants. The transition from vegetative to reproductive growth is a critical developmental switch and a key adaptive trait in both crop and wild cereal species (Cockram et al. 2007). Many temperate plants with a winter growth habit need to be exposed to low temperature for certain periods to transition from vegetative to reproductive growth phases (Amasino 2004). Vernalization is a phenomenon for acceleration of flowering by prolonged exposure to low temperature (Trevaskis et al. 2007). In cultivated grasses, winter-type varieties require vernalization for heading and flowering, whereas spring-type varieties are able to transition from the vegetative to reproductive growth phase without vernalization.

In wheat and barley, flowering time is a complex trait controlled by three genetic characteristics, vernalization requirement, photoperiodic sensitivity and narrow-sense earliness (Kato and Yamagata 1988), and the vernalization requirement for flowering is generally controlled by a major locus, Vrn-1 (Flood and Halloran 1986; Worland et al. 1987; Dubcovsky et al. 1998). Two other loci, Vrn-2 and Vrn-3, are also associated with determination of the vernalization requirement (Takahashi and Yasuda 1971; Law and Worland 1997; McIntosh et al. 1998). The wheat and barley Vrn-1 loci encode an APETALA1/FRUITFULL-like MADS-domain transcription factor (Murai et al. 2003; Trevaskis et al. 2003; Yan et al. 2003). Vrn-2 encodes ZCCT duplicated proteins with CONSTANS (CO), CO-like and TOC1 (CCT) domains (Yan et al. 2004a), and Vrn-3 is an Arabidopsis FLOWERING LOCUS T (FT) homolog (Yan et al. 2006). Wheat genome contains at least two FT homologs, Vrn-3 and WFT, and transcript accumulation levels of these homologs were upregulated during vernalization treatment (Yan et al. 2006; Shimada et al. 2009). WFT functions as a flowering accelerator (Shimada et al. 2009). Dominant alleles of the Vrn-1 loci contribute to the vernalization-insensitive spring habit, and structural mutations at the Vrn-1 loci, such as insertion/deletion (indel) events at the promoter region and large deletions in the first intron, generate the dominant alleles in the spring-type cultivars of barley and of einkorn, durum and common wheat (Yan et al. 2004b; Fu et al. 2005; von Ziteczewiz et al. 2005; Dubcovsky et al. 2006). Spring growth habit accessions have also been found in wild wheat species including Triticum araraticum Jakubz., Triticum urartu Thum., Triticum boeoticum Boiss. and Aegilops tauschii Coss. (Tsunewaki 1966; Golovnina et al. 2010; Takumi et al. 2011). Of the spring-type accessions in wild wheat species, some of T. boeoticum, T. araraticum and Ae. tauschii contain a dominant allele in Vrn-1, whereas other spring-type accessions from T. urartu and Ae. tauschii carry a winter-type allele of Vrn-1; the causal gene for spring growth
habit remains unknown (Golovnina et al. 2010; Takumi et al. 2011). Recent study using many barley varieties showed that three major vernalization genes, Vrn-1, Vrn-2, and Vrn-3, partially explain the variation in vernalization requirement and that other known genes contribute to the remainder of the variation (Saisho et al. 2011).

Activation of barley Vrn-1 after vernalization is associated with histone modification of the Vrn-1 locus (Oliver et al. 2009). In Arabidopsis, FLOWERING LOCUS C (FLC), acting as a flowering repressor, plays a central role in the vernalization pathway, and vernalization promotes flowering through silencing FLC (Michaels and Amasino 1999). The epigenetic silencing of FLC expression is accompanied by chromatin modification (Bastow et al. 2004), which is initiated by a protein complex involving a VERNALIZATION INSENSITIVE3 (VIN3) plant homeodomain (PHD)-containing protein (Sung and Amasino 2004). Another PHD finger protein, VIN3-like 1 (VIL1), is also associated with the epigenetically silenced state of FLC chromatin (Sung et al. 2006). The Arabidopsis FLC floral repressor is a MADS-box gene similar to cereal Vrn-1. Therefore, the relationship between transcriptional activation of Vrn-1 during vernalization and cereal VIL genes is worth studying (Hemmings and Trevaskis 2011). Three VIL homologs, TmVIL1, TmVIL2 and TmVIL3, have been identified in the einkorn wheat (Triticum monococcum L.) genome, and their exon/intron structure was distinct between TmVIL3 and others (Fu et al. 2007). TmVIL1, TmVIL2 and TmVIL3 are respectively assigned to the centromeric regions of chromosome 5A, 6A and 1A, and the TmVIL expression is upregulated by vernalization with a peak after 4 to 6 weeks of cold treatment (Fu et al. 2007).

Ae. tauschii, a wild relative of wheat, is the D-genome progenitor of hexaploid wheat (Van Slageren 1994). Ae. tauschii has a wide natural species range in central Eurasia, spreading from northern Syria and Turkey to western China, and latitudinal and longitudinal clines have been found for natural flowering time variation (Matsuoka et al. 2008). Most early-flowering accessions were distributed in the eastern habitats such as Transcaucasia-Middle East (Matsuoka et al. 2008; Mizuno et al. 2010). Vernalization-insensitive accessions were found in the Ae. tauschii germplasm collected from Afghanistan and Pakistan (Tsunewaki 1966; Golovnina et al. 2010; Takumi et al. 2011). Some of the Ae. tauschii accessions exhibited loss of vernalization requirement in spite of having no structural mutation in Vrn-D1 (Golovnina et al. 2010; Takumi et al. 2011). The accessions having natural mutations in vernalization requirement are useful resources to elucidate the molecular mechanism behind regulation of vernalization-dependent flowering time. Therefore, we attempted to isolate cDNA clones of Ae. tauschii homologs of the einkorn wheat VIL genes. We also compared the expression pattern of Ae. tauschii VIL homologs between accessions sensitive and insensitive to vernalization.

MATERIAL AND METHODS

Plant materials

Seven Ae. tauschii accessions, KU-2039 (Afghanistan), KU-2050 (Afghanistan), KU-2069 (Iran), KU-2810 (Armenia), IG108561 (Pakistan), PI476874 (Afghanistan) and IG47182 (Azerbaijan) were used in this study (the country of collection is given in parentheses). IG108561 lacks the vernalization requirement due to a large deletion in the Vrn-D1 1st intron (Takumi et al. 2011). KU-2039 and KU-2050 are spring-type accessions despite having no structural mutation in Vrn-D1. KU-2069 requires a 40-day vernalization treatment, and KU-2810 also requires vernalization for stable heading (unpubl.). PI476874 and IG47182 were used as parental varieties for a linkage map. These two winter-type accessions showed distinct flowering times; PI476874 and IG47182 were early and late heading time accessions, respectively (Matsuoka et al. 2008). Previous population structure analysis indicated that PI476874 belonged to a genealogically diverged lineage (L1) from the IG47182’s one (L2) (Mizuno et al. 2010). Habitats of the Ae. tauschii accessions in L1 were covered from the eastern to western regions in central Eurasia, whereas the L2 accessions distributed in the Transcaucasus area (Mizuno et al. 2010). Here, we selected PI476874 and IG47182 for the mapping parental varieties as representatives of L1 and L2, respectively. KU-2069 is also included in L2 and other accessions are in L1.

cDNA cloning and sequencing

VIL cDNA clones were isolated by reverse transcription-polymerase chain reaction (RT-PCR) with a gene-specific primer pair designed based on einkorn wheat VIL sequences (Fu et al. 2007). Total RNA from seedlings of KU-2069 was used as a template in RT-PCR. VIL homologs were isolated using the following primer pairs: 5′-ATGGAGTCGACCGGAGGA-3′ and 5′-AGTGTTCGCTATCCGGATT-3′ for a TmVIL1 homolog, 5′-GCCATGGATCCTCCATAC-3′ and 5′-GGCGTATAATGCCAGAGAT-3′ for a TmVIL2 homolog, and 5′-AGACATGTCCCAAATCTACTCC-3′ and 5′-GAGTTAGGGAGACTCTTGAAATTG-3′ for a TmVIL3 homolog. Amplified cDNAs of VIL were cloned into the pGEM-T vector
(Promega, Madison, WI), and nucleotide sequences were determined by an automated fluorescent Dye-Deoxy terminator cycle sequencing system using an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, Foster City, CA). Nucleotide sequences of the isolated cDNA clones and their predicted amino acid sequences were analyzed by GENETYX-MAC version 12.00 software (Whitehead Inst. for Biomedical Research, Cambridge, MA). The cDNA sequences in KU-2069, named respectively AetVIL1 (2112-bp), AetVIL2 (2257-bp) and AetVIL3 (1866-bp) based on their homology to the einkorn wheat VIL genes, were deposited in the DDBJ database under the accession numbers AB691581 (AetVIL1), AB691582 (AetVIL2), and AB691583 (AetVIL3). The accession numbers of additional cDNA sequences of these AetVIL genes are AB691584-AB691591 in the DDBJ database.

Mapping

For a mapping population, 104 F2 individuals from a single F1 plant between two Ae. tauschii accessions, PI476874 and IG47182, were used. For simple sequence repeat (SSR) genotyping, 40 cycles of PCR were performed using 2× Quick Taq HS DyeMix (TOYOBO, Osaka, Japan) and the following conditions: 30 s at 94°C, 30 s for annealing, and 30 s at 68°C. The last step was incubation for 1 min at 68°C. Information on SSR markers and their annealing temperature was provided from the National BioResource Project (NBRP) KOMUGI web site (<http://wheat.pw.usda.gov/GG2/maps.shtml>) and GrainGene web site (<http://wheat.pw.usda.gov/GG2/maps.shtml>) and Genomic DNA was isolated from the F2 individuals using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA). Nucleotide sequences of the isolated cDNA sequences of these AetVIL genes were AB691584-AB691591 in the DDBJ database.

Expression analysis

Seeds were germinated for 48 h at 23°C in darkness. Four or five synchronously germinated seeds were transferred to pots containing soil and incubated at 23°C under short-day conditions (12 h light/12 h darkness). Seedlings 14 days old were treated with 4°C under long-day conditions (16 h light/8 h darkness) for four weeks. Total RNA was extracted from leaves using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). First-strand cDNA was synthesized from DNase I-treated mRNA samples with oligo-dT primers using the high fidelity ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). The transcript accumulation of each gene was detected by quantitative RT-PCR using a LightCycler 480 System II (Roche, Basel, Switzerland) with the following gene-specific primer sets: 5′-TTCTTC TTCCTCTCATGAT-3′ and 5′-AAGATCGTGATTAT TTATT-3′ for Vrn-1 (Takumi et al. 2011), 5′-TCAG CAAATTATCAACGTGCT-3′ and 5′-GTTGATGG CATATATGCATGCGG-3′ for WFT (Shimada et al. 2009), 5′-GGTCTCTTCTAGATGGCCGCGT-3′ and 5′-ACCTAAGCTCAGAGTGCCAAACCTCAGC-3′ for AetVIL1, 5′-GTTATTTTGTACTGCTGAAA TC-3′ and 5′-ATTAAGCCTCTCTAATCCAAGCTG-3′ for AetVIL2, 5′-TCGTAAGCAGGTTATCTGTTG-3′ and 5′-GGATGATAAACAGGAGACG-3′ for AetVIL3, and 5′-GCCGTGCATCCTCGATTTG-3′ and 5′-GCTTCTCTGTAGTTCTCAGT-3′ for Actin. The Actin gene was used as an internal control. The annealing temperature conditions were respectively 60, 53 and 53°C for AetVIL1, AetVIL2 and AetVIL3. The rate of

TCGTCAAGTC-3′, and one lower primer, 5′-GCAGC ACAGCAATAGTACC-3′, were used. The annealing temperature and PCR cycle conditions for AetVIL1 were 59°C and 35 cycles. Using the two upper primers, a 12-bp indel mutation found in AetVIL1 was genotyped. The PCR products were separated by electrophoresis through a 1.5% agarose gel and stained with ethidium bromide. The primer sets for mapping of AetVIL2 was 5′-GGGTCTGGAAATTCAGT-3′ and 5′-ATAGGCTCTCTCATCAAAGCTG-3′, and the set for AetVIL3 was 5′-TCGAACAGGTATCTGCTTG GG-3′ and 5′-GGATGATAAACAGGAGACG-3′. The annealing temperature and PCR cycle conditions for AetVIL2 and AetVIL3 were 53°C and 35 cycles. After amplification, PCR products were digested with the restriction enzyme Hinfl, and the digested fragments were separated by electrophoresis through a 2.0% agarose gel and stained with ethidium bromide. The targeted Hinfl site was observed in AetVIL2 of PI476874 and AetVIL3 of IG47182.
amplification was monitored using THUNDERBIRD SYBR qPCR mix (Toyobo) according to the manufacturer’s protocol. The relative expression level was calculated as $2^{-\Delta Ct}$, where $\Delta Ct$ is the difference in number of PCR cycles required to reach the log phase of amplification of the target gene relative to Actin; representative values were expressed relative to the transcript levels in KU-2069 samples obtained at 0 h.

RESULTS AND DISCUSSION

Cloning of three VIL homologs

Three cDNA sequences highly homologous to TmVIL1, TmVIL2 and TmVIL3 were isolated from leaves of an Ae. tauschii accession KU-2069 by RT-PCR with locus-specific primer sets. Each of the isolated cDNA clones, AetVIL1, AetVIL2 and AetVIL3, contained a complete...

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**Fig. 1A–C.** Three VIL cDNA sequences isolated from seedling leaves of *Ae. tauschii* KU-2069. (A) Phylogenetic tree based on amino acid sequences, showing the relationship of the three *Ae. tauschii* VIL proteins to other plant VIN3/VIL proteins. The phylogenetic tree was constructed by the neighbor-joining method based on Nei’s genetic distances which are shown above branches. *At*, *Arabidopsis thaliana*; *Os*, *Oryza sativa*; *Tm*, *T. monococcum*; *Aet*, *Ae. tauschii*. (B) Multiple alignment of amino acid residues of the PHD finger domain in plant VIN3/VIL proteins. (C) Sequence homology among the three AetVIL genes based on percentages of their cDNA sequences (above the diagonal) and amino acid sequences (below the diagonal).
open reading frame (ORF), and the ORFs putatively encoded PHD finger proteins of 697, 750 and 615 amino acid residues, respectively. A phylogenetic tree of the PHD finger proteins homologous to *Arabidopsis* VIN3 was constructed by the neighbor–joining method based on Nei’s genetic distance (Fig. 1A). Previously, five *VIL* genes were identified in *Arabidopsis* and four in rice (Sung et al. 2006; Fu et al. 2007). The three VIL amino acid sequences of *Ae. tauschii* showed the highest level of identity with their einkorn wheat counterparts. A multiple alignment of these PHD finger proteins indicated that homology was especially high in the PHD regions (Fig. 1B). The three *VIL* genes of *Ae. tauschii* were clearly distinct from each other at both the nucleotide and amino acid sequence levels (Fig. 1C). 

*AetVIL1*, *AetVIL2* and *AetVIL3* were classified to the *VIL1*, *VIL2* and *VIL3* groups, respectively, of rice and einkorn wheat in the phylogenetic tree (Fig. 1A). These observations implied that *AetVIL1*, *AetVIL2* and *AetVIL3* can be considered orthologs of *TmVIL1*, *TmVIL2* and *TmVIL3*.

In the three *VIL* ORFs, many single nucleotide polymorphisms (SNPs) were found between einkorn wheat and *Ae. tauschii* (Fig. 2). *VIL3* was the most conserved of the three *VIL* cDNAs. Out of the 34, 45 and 14 SNPs respectively found within the *VIL1*, *VIL2* and *VIL3* ORFs, 13 (38.2%), 13 (28.9%) and 5 (35.7%) non-synonymous substitutions were included. The VIL proteins of einkorn wheat contain three conserved domains: a PDH finger domain, a fibronectin type III (FNIII) domain and a VIN3 interacting domain (VID) (Sung et al. 2006; Fu et al. 2007). In *AetVIL1*, four non-synonymous substitutions were found in the PHD finger region compared with *TmVIL1*, and in *AetVIL2*, six were found compared with *TmVIL2*. Two to four non-synonymous substitutions between einkorn wheat and *Ae. tauschii* were observed in the FNIII region of all *VIL* genes. Two non-synonymous substitutions occurred in the VID region of *VIL1*. These non-synonymous substitutions in the conserved domains might affect target DNA binding and protein–protein interaction, and the encoded proteins might be functionally differentiated in spite of their high homology between einkorn wheat and *Ae. tauschii* at the nucleotide sequence level. The functional differences between these wheat *VIL* genes should be the subject of future studies.

To study intraspecific variation of the *VIL* nucleotide sequences, the *AetVIL1*, *AetVIL2* and *AetVIL3* cDNA sequences were respectively determined in four, two and two additional accessions of *Ae. tauschii*. The four additional *AetVIL1* cDNA sequences shared five SNPs, and a 12-bp deletion within the ORF referred to the KU-2069 *AtVIL1* sequence (Fig. 3A). Four of the five SNPs were non-synonymous substitutions compared with *AtVIL1* of KU-2069, and two were located in the VID

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**Fig. 2.** Sites of the three *VIL* cDNAs that are polymorphic between *T. monococcum* and *Ae. tauschii* KU-2069. Vertical lines represent SNPs and filled circles represent non-synonymous substitutions. Open boxes indicate conserved domains in the *T. monococcum* VIL proteins. PHD, plant homeodomain; FNIII, fibronectin type III domain; VID, VIN3 interacting domain.
region of \textit{VIL1}. The 12-bp deletion was found outside the conserved regions of \textit{VIL1}. In \textit{AetVIL2}, four SNPs were observed in PI476874, all synonymous, and twelve in IG47182, of which seven were non-synonymous (Fig. 3B). Fewer SNPs were detected in \textit{AetVIL3} than in the other \textit{AetVIL} genes, and two of the three SNPs were non-synonymous in IG47182 (Fig. 3C).

The ratio of non-synonymous and synonymous substitution ($K_a/K_s$) is a useful parameter to assess gene evolution, and the likely occurrence of adaptive evolution for a given gene is indicated by a $K_a/K_s$ value greater than 1 (Li 1993). Comparing KU-2069 with the other four \textit{Ae. tauschii} accessions, the $K_a/K_s$ values of \textit{AetVIL1} were 2 to 4. Between KU-2069 and IG47182, the $K_a/K_s$ value of \textit{AetVIL2} was 1.4 and \textit{AetVIL3} was 2. These high $K_a/K_s$ values suggest that adaptive evolution has occurred in the \textit{AetVIL} genes during intraspecific diversification of \textit{Ae. tauschii}. Based on common garden and greenhouse experiments, longitudinal and latitudinal clines for flowering time in \textit{Ae. tauschii} were found to be significant (Matsukura et al. 2008). The degree of association of the high $K_a/K_s$ values in the \textit{AetVIL} genes with flowering time variation should be elucidated using many accessions of \textit{Ae. tauschii}.

\textbf{Chromosome assignment of the three VIL homologs}

A linkage map of \textit{Ae. tauschii} was constructed using wheat SSR markers (data not shown). In total, 345 SSR primer sets were tested for detection of polymorphisms.

\textbf{Fig. 3.} Intraspecific variation of the three \textit{VIL} ORF sequences in \textit{Ae. tauschii}. A 12-bp deletion in \textit{AetVIL1} is indicated by triangles. Vertical lines represent SNPs and filled circles represent non-synonymous substitutions. Open boxes indicate conserved domains in the \textit{VIL} proteins of KU-2069. Arrows and filled triangles represent primer positions and \textit{Hinfl} sites for mapping of \textit{AetVILs}, respectively. PHD; plant homeodomain; FNIII, fibronectin type III domain; VID, VIN3 interacting domain.
between PI476874 and IG47182, and 121 sets (35.1%) showed polymorphism. The genetic map was constructed by the segregation of 93 SSR loci and three genes using the 104 F2 individuals, and 10 linkage groups were generated. Each 2D, 4D and 7D chromosome was separated into two linkage groups. The total map length was 1,370.8 cM with an average spacing of 14.3 cM between markers. Positions of centromeres were assumed according to the wheat linkage maps reported by Somers et al. (2004) and Koyama et al. (2010).

Based on the indel mutation and SNPs of the AetVIL genes between PI4768874 and IG47182, gene-specific primer sets were designed for genotyping. Using the constructed linkage map, AetVIL1, AetVIL2 and AetVIL3 were assigned to the centromeric regions of chromosomes 5D, 6D and 1D, respectively (Fig. 4). These map locations corresponded to those of TmVIL1, TmVIL2 and TmVIL3 (Fu et al. 2007), indicating chromosomal syney was conserved in the VIL gene regions between the A and D genomes. Fu et al. (2007) observed that the chromosomal location of TmVIL1 is close to the vernalization gene Vrn-D5. It was reported that the Ae. tauschii accessions without any structural mutation in Vrn-1 exhibited a loss of vernalization requirement (Golovnina et al. 2010; Takumi et al. 2011). The Ae. tauschii mutants affected in vernalization requirement are also attractive for studying the relationship between the AetVIL genes and vernalization requirement.

Expression patterns of the three VIL homologs

The VIL genes of einkorn wheat are transcriptionally upregulated under low temperature conditions (Fu et al. 2007). TmVIL2 expression gradually increases during a six-week cold treatment under the short-day condition, and the transcript levels of TmVIL1 and TmVIL3 reach a maximum level after four weeks in leaves. The significant upregulation of the three VIL genes were also observed in the four-week vernalized einkorn wheat plants under the long-day condition (Fu et al. 2007). To examine cold-responsive gene expression of VIL genes in Ae. tauschii, the specificity of the primer sets for the VIL homologs was first confirmed by PCR using plasmid DNA templates of AetVIL1, AetVIL2 and AetVIL3. No visible band was amplified in the non-identical VIL plasmids (Fig. 5A), supporting specificity of the primers.

Two-week-old seedlings of two Ae. tauschii accessions, KU-2069 and IG108561, were treated with low temperature under the long-day condition. The AetVIL1 transcript level slightly increased within 1 day of cold treatment in IG108561, whereas no cold-responsive expression of AetVIL1 was observed in KU-2069 (Fig. 5B). AetVIL2 was upregulated after one week of cold treatment in IG108561, but no change in expression was found in KU-2069 (Fig. 5C). No obvious change in AetVIL3 expression was detected in either KU-2069 or IG108561 (Fig. 5D). The cold-responsive expression patterns of VIL genes in Ae. tauschii differed from those in einkorn wheat. KU-2069 showed no obvious cold responsiveness in VIL expression at least under the long-day condition. Gene expression studies of VIL genes in einkorn wheat showed that the VIL transcript levels were generally higher under short-day condition than those under the long-day condition (Fu et al. 2007). Therefore, comparative expression analyses under the short-day condition should be required to clarify the photoperiodic response of the cold-inducible expression of AetVILs.

Expression patterns of two vernalization requirement-related genes, Vrn-1 and WFT, were compared between KU-2069 and IG108561. The transcript level of Vrn-1 increased after two weeks of cold exposure in IG108561,
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Three VIL homologs in wild wheat

Fig. 5A–D. Expression profiles of the three VIL genes in Ae. tauschii during vernalization treatment. (A) Validation of PCR primer specificity for each VIL-containing plasmid template. (B, C, D) Time-course analysis of VIL gene expression. Transcript levels of AetVIL1 (A), AetVIL2 (B) and AetVIL3 (C) were estimated by quantitative RT-PCR. Total RNA was extracted from seedling leaves after treatment with low temperature for the indicated number of days. Actin was used as an internal control. Each transcript level is represented as the mean value relative to that of KU-2069 at 0 days. Error bars indicate standard deviation.

whereas no response in Vrn-1 expression was found in KU-2069 (Fig. 6A). WFT, a wheat homolog of Arabidopsis FT (SHIMADA et al. 2009), was also upregulated after three weeks of vernalization in IG108561, but no change in WFT mRNA levels was observed in KU-2069 (Fig. 6B).

As HEMMING and TREVASKIS (2011) mentioned, it is unlikely that a VIN3-like gene is responsible for cold-induced activation of Vrn-1, as wheat VIN3-like genes are not downregulated by cold to allow induction of Vrn-1, nor are wheat VIN3-like genes strongly induced by cold (Fu et al. 2007). The VIL gene expression analyses in Ae. tauschii showed that out of three VIL genes, only AetVIL2 was transcriptionally upregulated, and only in IG108561, not in KU-2069 (Fig. 5). KU-2069 requires a 40-day vernalization for flowering, whereas IG108561 is a spring growth habit accession due to a large deletion in the first intron of Vrn-1 (TAKUMI et al. 2011; unpubl.). The upregulation of AetVIL2 was prior to Vrn-1 activation in IG108561 (Fig. 5C, 6A). In Arabidopsis, VIN3 and VILI act as negative regulators of the FLC flowering repressor gene, and their expression is activated in response to vernalization (SUNG and AMASINO 2004; SUNG et al. 2006). The relationship between AetVIL2 expression and vernalization remains unclear, whereas a major wheat vernalization requirement gene, Vrn-1, seemed to be activated followed by AetVIL2 upregulation in leaves (Fig. 5C, 6A). The timing of WFT activation occurred afterward the AetVIL2 upregulation (Fig. 6B). WFT is assumed to act downstream of Vrn-1 (SHIMADA et al. 2009). Therefore, AetVIL2 seems to function upstream of Vrn-1 in the vernalization-responsive pathway.

In Ae. tauschii, vernalization-insensitive mutant accessions have been found (GOLOVNINA et al. 2010; TAKUMI et al. 2011). The expression pattern of AetVIL2 was examined during vernalization in two additional accessions of Ae. tauschii. The two spring-type accessions, KU-2039 and KU-2050, showed higher levels of the AetVIL2 transcript accumulation than KU-2069 (Fig. 5C), and Vrn-1 upregulation was observed within 4 weeks of vernalization in spring-type accessions. Thus, the AetVIL2 expression pattern was distinct for winter and spring habit accessions, strongly suggesting an association of AetVIL2 with the vernalization-responsive
pathway. Elucidating the molecular mechanism behind the \textit{AtVIL2} vernalization response should clarify the role of \textit{AetVIL2} in the vernalization-responsive pathway of wheat.

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