Identification of a large deletion in the first intron of the \textit{Vrn-D1} locus, associated with loss of vernalization requirement in wild wheat progenitor \textit{Aegilops tauschii} Coss.

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Vernalization promotes flowering in winter wheat cultivars, whereas spring wheat cultivars are able to transition from vegetative to reproductive phase without vernalization. The wheat vernalization requirement is mainly controlled by the major locus \textit{Vrn-1}, an APETALA1/FRUITFULL MADS-box gene homolog. To study natural variation of the vernalization requirement in a wild progenitor of common wheat, we sequenced the \textit{Vrn-Dt1} locus in four accessions of \textit{Aegilops tauschii} Coss. Some structural mutations were found in the promoter and first intron regions of \textit{Vrn-Dt1}, and haplotype analysis was conducted to examine the distribution of each identified mutation within 211 accessions of \textit{Ae. tauschii} germplasm. Out of the total, nine accessions, which were originally collected in Afghanistan and Pakistan, contained deletions of a 5.4-kb sequence in the critical region of the \textit{Vrn-D1} first intron. The 5.4-kb deletion mutation appeared independently of the dominant allele of the common wheat \textit{Vrn-D1} locus. The large deletion was absolutely associated with a lack of vernalization requirement for flowering under long-day conditions, but had no influence on heading date under field growth conditions. The levels of \textit{Vrn-1} and \textit{WFT} transcript increased in the \textit{Ae. tauschii} accessions having the large deletion. Identification of natural mutant accessions with a loss of vernalization requirement indicates the agricultural significance of \textit{Ae. tauschii} as a genetic resource for wheat breeding.

Key words: flowering time, intron, natural variation, vernalization, wheat

INTRODUCTION

Timing of flowering is a reproductive trait that has significant impact on fitness in higher plants. Regulation of flowering time is not only ecologically but also agronomically important. Many temperate plants with winter growth habit need to be exposed to low temperature for certain periods to transition from vegetative to reproductive growth phase. Exposure to low temperature can accelerate flowering, and this phenomenon is called vernalization. In cultivated grasses, winter-type varieties require vernalization for heading and flowering, and loss of the vernalization requirement makes it possible to sow seeds in spring. Spring-type varieties are able to transition from vegetative to reproductive growth phase without vernalization.

In common wheat (\textit{Triticum aestivum} L.) and barley (\textit{Hordeum vulgare} L.), flowering time is a complex trait under the control of three genetic characteristics, vernalization requirement, photoperiodic sensitivity, and narrow-sense earliness (Kato and Yamagata, 1988). The vernalization requirement is generally controlled by a major locus, \textit{Vrn-1}, in wheat and barley (Flood and Halloran, 1986; Worland et al., 1987; Dubcovsky et al., 1998). Previous genetic studies revealed that other two loci, \textit{Vrn-2} and \textit{Vrn-3}, also determine the vernalization requirement (Takahashi and Yasuda, 1971; Law and Worland, 1997; McIntosh et al., 1998). Common wheat is an allohexaploid species that originated from natural hybridization between tetraploid wheat (\textit{T. turgidum} L.) and \textit{Aegilops tauschii} Coss. (Kihara, 1944; McFadden and Sears, 1944), and therefore the hexaploid genome contains three homoeologous \textit{Vrn-1} loci, \textit{Vrn-A1}, \textit{Vrn-B1} and \textit{Vrn-D1}, on the long arms of homoeologous group 5 chromosomes (McIntosh et al., 1998). The wheat and barley...
Vrn-1 loci encode an APETALA1/FRUITFULL (AP1/FUL)-like MADS-domain transcription factor (Yan et al., 2003; Trevaskis et al., 2003; Murai et al., 2003). 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 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., 2004; Fu et al., 2005; von Zitzewitz et al., 2005; Dubcovsky et al., 2006). At the Vrn-1 loci, double-stranded break repair through nonhomologous end-joining, resulting in the structural mutations, creates novel alleles selected to confer adaptation to annual cropping systems in barley and wheat (Cockram et al., 2007a).

Many mutation events inducing large deletions in the first intron of Vrn-1 have been found in the spring-type cultivars of wheat and barley (Fu et al., 2005; von Zitzewitz et al., 2005; Cockram et al., 2007b). However, little information has been reported on wild wheat relatives. Recently, spring growth habit accessions were identified from wild wheat species such as *T. araraticum* Jakubz., *T. urartu* Thum., *T. boeoticum* Boiss. and *Ae. tauschii* (Golovnina et al., 2010). Some of the identified spring-type accessions contained a dominant allele with deletion mutations of the Vrn-A1 promoter in *T. boeoticum* and *T. araraticum*, whereas the spring-type accessions from *T. urartu* and *Ae. tauschii* carried a recessive allele of Vrn-1, and the causal gene for spring growth habit remains unknown.

Wheat and barley were originally winter growth habit, and Vrn-1 expression is cold-inducible in leaves and shoot apices of the winter-type cultivars (Danyluk et al., 2003; Yan et al., 2003; Trevaskis et al., 2003; Dubcovsky et al., 2006). The increased Vrn-1 transcript level during vernalization is associated with the phase transition from vegetative to reproductive growth (Trevaskis et al., 2003). The Vrn-1 expression pattern is altered under low temperature conditions in the spring-type varieties (von Zitzewitz et al., 2005; Trevaskis et al., 2006; Ishibashi et al., 2007), and large deletions in the first intron affect accumulation of Vrn-H1 transcripts but not cold induction in barley (Hemming et al., 2009). The critical region within the Vrn-H1 first intron might be required to maintain low levels of Vrn-H1 expression in barley. Under long-day conditions, Vrn-1 up-regulates expression of the downstream Vrn-3 gene, an *Arabidopsis* FLOWERING LOCUS T (FT) homolog (Yan et al., 2006), together with a CONSTANS homolog (Hemming et al., 2008; Shimada et al., 2009). FT is well known as an integrator of the vernalization and photoperiod pathways for flowering (Searle and Coupland, 2006), and the FT protein activates floral meristem-identity genes together with the FD bZIP transcription factor at the shoot apex (Abe et al., 2005; Wigge et al., 2005).

*Ae. tauschii*, a wild relative of wheat, is the D-genome progenitor of hexaploid wheat (Eig, 1929; Van Slageren, 1994). The birthplace of common wheat is considered to lie within the area comprising Transcaucasia and the southern coastal region of the Caspian Sea (Tsunewaki, 1966; Nishikawa et al., 1980; Dvorak et al., 1998). In the southern coastal region of the Caspian Sea, *Ae. tauschii* grows as a weed in fields of cultivated tetraploid wheat (Matsuoka et al., 2008a). *Ae. tauschii* has a wide natural species range in central Eurasia, spreading from northern Syria and Turkey to western China. The species’ putative primary region of origin is the Transcaucasus, where diploid species of the genus *Aegilops* radiated 2.5–4.5 million years ago (Lubbers et al., 1991; Van Slageren, 1994; Dvorak et al., 1998; Huang et al., 2002). Therefore, the eastern accessions were dispersed from the western habitats (Matsuoka et al., 2008b). Population structure and intraspecific diversification of *Ae. tauschii* have been well studied, and recent Bayesian population structure analysis showed that phylogenetic lineages one (L1) and two (L2) were respectively divided into six and three sublineages in *Ae. tauschii* (Mizuno et al., 2010a). Only four out of the six L1 sublineages had diverged from those of western habitats in the Transcaucasian and northern Iran regions to eastern habitats such as Pakistan and Afghanistan, whereas the *Ae. tauschii* populations involved in the origin of common wheat have been narrowed down to L2, probably sublineage 2–3 (Mizuno et al., 2010a, 2010b). *Ae. tauschii* is genetically and morphologically diverse (Dudnikov and Goncharov, 1993; Dvorak et al., 1998; Matsuoka et al., 2007, 2008b, 2009; Takumi et al., 2009). Latitudinal and longitudinal clines were found for natural flowering time variation. The early-flowering accessions were spread mainly in the eastern habitats, implying that the early-flowering phenotype contributed to eastward dispersal and adaptation to these habitats (Matsuoka et al., 2008b). This recent progress implies that *Ae. tauschii* is suitable and useful for molecular genetic analyses of genes associated with environmental adaptation.

Vernalization-insensitive accessions were formerly found in the *Ae. tauschii* germplasm collected from Afghanistan and Pakistan (Tanaka and Yamashita, 1957; Tsunewaki, 1966; Golovnina et al., 2010). Nevertheless, there is little information about the genetic relationship between the spring growth habit and Vrn-D1, and about the geographic and phylogenetic distribution of the causal genes in the *Ae. tauschii* population. In addition, most spring-type landraces of common wheat in South China and Southwest Japan carry a dominant allele of the Vrn-D1 locus (Gotoh, 1979; Iwaki et al., 2000). However, it is not clear whether the causal mutation in the vernalization-insensitive accession of *Ae. tauschii* corresponds with that in the dominant allele of Vrn-D1 in common wheat. In the present study, we reported the genomic
sequences of the Vrn-D1 locus in several *Ae. tauschii* accessions, and examined the association of the structural variations at Vrn-D1 with vernalization requirement. Together with these results, we compared mutation events spontaneously occurring during the west-to-east disperal in wild populations and accumulating in landraces after crop domestication.

**MATERIALS AND METHODS**

**Plant materials and evaluation of vernalization requirement** In total, 211 accessions of *Ae. tauschii* were used in this study (Table 1). Their passport data including geographical coordinates and chloroplast DNA haplogroups have been provided in previous reports (Matsuoka et al., 2007, 2008b). This sample set consisted of 205 accessions representing the entire natural habitat range and six accessions (AT47, AT55, AT60, AT76, AT80, and PI508264) representing adventive populations in the Shaanxi and Henan provinces of China (Matsuoka et al., 2007). When geographical coordinates of sampling sites were not available, we estimated latitude and longitude by means of Kashmir 3D software (http://www.kashmir3d.com/) on scanned paper maps (scales 1:4,000,000-1:1,000,000) based on locality information. For four accessions (CGN10731, CGN10732, CGN17333, and CGN10734), geographical coordinates were not estimated due to lack of locality information. In the original collection, most of the accessions were not obtained by population sampling. For each accession, we used seeds propagated from a single plant by selfing.

For estimation of vernalization requirement, sprouted seeds were sown in soil-filled containers, and then grown in a growth cabinet at 20°C with a 16-h photoperiod. Five plants per accession were grown without vernalization, and the number of days to heading from sowing was scored for each plant.

**Direct sequencing and data analyses of the Vrn-D1 locus** Total DNA was extracted from leaves of four *Ae. tauschii* accessions, KU-20-3, KU-20-6, KU-20-9, and KU-20-10. For direct sequencing of the Vrn-D1 locus, gene-specific primer sets were designed based on reference to the Vrn-D1 sequence in the DDBJ/EMBL/GenBank database (accession no. AT747605). Nucleotide sequences were determined by an automated fluorescent Dye Deoxy terminator cycle sequencing system using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The four genomic sequences of the Vrn-D1 locus were deposited into the DDBJ database under these accession numbers: AB630961 to AB630964.

For molecular population genetic analysis of the Vrn-D1 locus, total DNA was extracted from leaves of 23 *Ae. tauschii* accessions (Table 1), and two regions within Vrn-D1 were amplified. One genomic region included a ca. 1-kb 5'-upstream sequence, the 1st exon and the 5'-end of the 1st intron. For amplification of the promoter and exon 1 region, a primer set, 5'-TATGGATGTCTGGTGTTACCA-3' and 5'-GCAGAAAATCGAATCGAGG-3', was designed, and the annealing temperature and PCR cycle conditions were 62°C/35 cycles. Another region amplified contained a genomic region from the 3'-end of the 2nd intron to the 8th exon, and three primer sets, 5'-TAACTCAGCTTATTTGTACC-3' and TACTTGTGCTGAACCTCTCTTG, 5'-AAACATATCAATCCAGGAGG-3' and 5'-CGGTGCAACTTTGTTACTCTCTA-3', and 5'-TTCTCTCTCCTCATGAT-3' and 5'-AAGATCCTGTATTATTTATT-3', were designed for amplification of the exon 3 to 8 region. Their annealing temperature and PCR cycle conditions were respectively 62, 55 and 53°C, each for 35 cycles. The PCR products from the two regions of Vrn-D1 were directly sequenced as mentioned above. Multiple sequence alignments were carried out using the CLUSTAL W computer program (Thompson et al., 1994), and the alignments were revised manually. Population genetic analyses were conducted with the DnaSP program (Rozas et al., 2003) so that within-species diversity could be estimated. Values of Tajima’s D statistic (Tajima, 1989) and Fu and Li’s D statistic (Fu and Li, 1993) were also estimated with DnaSP.

**Haplotype analysis of the *Ae. tauschii* accessions** For genotyping of three structural mutations at the Vrn-D1 locus, total DNA was extracted from the 210 *Ae. tauschii* accessions except KU-20-3, and the following four primer sets were designed: 5'-TAGGACTTGGGCGAGTATCCTTCC-3' and 5'-GGTTTTCTTTAGCATTTTTCCT-3' for amplifying a 8-bp insertion/deletion (indel) mutation in the promoter region, 5'-TGATCTGCTCATTCAAAATCTCGTCG-3' and 5'-AGATTAGGAGCCGAGACACAGG-3' for amplifying sequences lacking a large fragment in the first intron, 5'-GGTTGCTGCTGCTCAATACC-3' and 5'-AAAATGCAGAAACACAGGC-3' for amplifying sequences with no deletion of the large fragment in the 1st intron, and 5'-GCAAGAGCGACACGACGG-3' and 5'-GTGATCCCAATGTGAGCCAC-3' for amplifying a 109-bp duplication in the first intron. The upper primer is located within the 109-bp duplication, and the PCR products in the duplication-containing accessions show two amplified bands. The third primer set for detecting the non-deletion allele in the 1st intron was that of Fu et al. (2005). The annealing temperature and PCR cycle conditions were 60°C/35 cycles. The PCR products were separated by electrophoresis through a 1.5 or 2.0% agarose gel and stained with ethidium bromide.

**Gene expression analysis** For gene expression analysis, 14-day-old seedlings grown under standard conditions (23°C) were transferred to 4°C and kept for various time periods with a 12 h photoperiod at a light intensity
<table>
<thead>
<tr>
<th>Country</th>
<th>Accessions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Armenia</td>
<td>IG126273, IG126280, IG126293, IG126333, IG126991, IG127015, IG48747, IG48748, IG48758, KU-2809, KU-2810, KU-2811, KU-2814, KU-2816, KU-2821, KU-2822, KU-2823, KU-2824, CGN10734.</td>
</tr>
<tr>
<td>Azerbaijan</td>
<td>IG47182, IG47186, IG47192, IG47193, IG47194, IG47196, IG47199, IG47202, IG47203, IG47204, KU-2801, KU-2804, KU-2806, CGN10731, CGN10732.</td>
</tr>
<tr>
<td>China</td>
<td>AT47, AT55, AT76, AT80, PI499262, PI508262, PI508264.</td>
</tr>
<tr>
<td>Dagestan</td>
<td>IG120863, IG120866, IG48274, KU-20-1.</td>
</tr>
<tr>
<td>Kazakhstan</td>
<td>AE1090.</td>
</tr>
<tr>
<td>Kyrgyzstan</td>
<td>IG131606.</td>
</tr>
<tr>
<td>Syria</td>
<td>IG46823, IG47259.</td>
</tr>
<tr>
<td>Tajikistan</td>
<td>AE1038, IG4554, IG4555, IG48564.</td>
</tr>
<tr>
<td>Turkmenistan</td>
<td>IG120735, IG12837, IG12848, IG48508, IG48518, CGN10733.</td>
</tr>
<tr>
<td>Uzbekistan</td>
<td>IG120736, IG12839, IG48539, IG48565, IG48567, KU-20-3.</td>
</tr>
</tbody>
</table>

Underlined accessions were used for molecular population genetic analysis of the Vrn-D1 locus.

KU: Plant Germ-Plasm Institute, Faculty of Agriculture, Kyoto University, Japan.
P: National Small Grains Research Facility, USDA-ARS, USA.
IG: International Center for Agricultural Research in the Dry Areas (ICARDA).
CGN: Centre for Genetic Resources, The Netherlands.
AE: Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK).
AT: Kenji Kato, Okayama University.
*large deletion in the 1st intron (n = 9), *110-bp duplication (n = 7), *8-bp insertion (n = 43).
*ssp. strangulata (n = 12), **var. meyeri (n = 5).
of 110–120 μm photons m⁻² s⁻¹ provided by cool white fluorescent lamps. Total RNA was extracted from the seedlings, and quantitative RT-PCR was performed using LightCycler 480 System II (Roche, Basel, Switzerland) and gene-specific primer sets for Vrn-D1 and WFT. The WFT primer set was 5’-TCAGCAAATTATCCAGTG-GCT-3’ and 5’-GTGGATCCATATGCATGC-3’ (Shimada et al., 2009). The wheat actin gene (Act) was used as an internal control. The rate of amplification was monitored using THUNDERBIRD SYBR qPCR mix (TOYOBO, Japan) according to the manufacturer’s protocol. Results were obtained as 2⁻ΔCt, where Ct is the difference in number of PCR cycles required to reach the log phase of amplification for the target gene relative to Act; values were expressed relative to the transcript levels in the first samples of each time course experiment.

RESULTS

Genomic structure of the Vrn-D1 locus in Ae. tauschii  A genomic sequence of the Vrn-D1 locus of Ae. tauschii has already been deposited as a BAC clone sequence (AT_BAC22J2) in the DDBJ/EMBL/GenBank database. Based on the reported Vrn-D1 sequence, four genomic sequences for Vrn-D1 were newly determined in Ae. tauschii. The Vrn-D1 locus of Ae. tauschii contained 8 exons and 7 introns (Fig. 1A). In the ca. 13-kb Vrn-D1 sequence of KU-20-3, the first intron was 8,650 bp in length. Multiple alignments of the five Vrn-D1 sequences showed many mutation sites including indels and single nucleotide polymorphisms (SNPs) (Fig. 1B). All the Vrn-D1 sequences were postulated to encode a functional API/FUL-like MADS-domain protein. The polymorphic patterns of the five Vrn-D1 sequences implied that the two sequences from KU-20-3 and KU-20-6 were distinct from the other three sequences. Most of the identified indel mutations and SNPs were localized in the intron regions, and only four synonymous substitutions were found in the exon 1 to 7 regions (Fig. 1B). Among the identified indel mutations, two large structural mutations, a 5,437-bp deletion in KU-20-6 and a 109-bp tandem duplication in KU-20-10, were observed in the 1st
intron. The 5,437-bp deletion fragment fully covered the critical region for loss of vernalization requirement identified in the spring-type cultivars of wheat and barley (Fu et al., 2005; von Zitzewitz et al., 2005; Cockram et al., 2007b). The positions of the large deletions in the Vrn-D1 allele of common wheat did not correspond to the deletion site of KU-20-10.

**Haplotype analysis of the structural mutations in the 210 Ae. tauschii population** To examine distribution of the structural mutations at the Vrn-D1 locus, three mutations, including an 8-bp indel in the promoter
Structural mutations of \( Vrn-D1 \) in wild wheat

region, the large deletion in the 1st intron and the 109-bp duplication in the 1st intron, were selected (Fig. 2A), and haplotype analysis based on PCR amplification was performed using the 210 \( Ae. tauschii \) accessions except KU-20-3.

A primer set was designed to amplify the flanking region of the large deletion mutation in KU-20-3. A single fragment was successfully amplified for most \( Ae. tauschii \) accessions, indicating no large deletion in the critical region (Fig. 2B). An amplified fragment for the 1st intron region was absent in only nine accessions, KU-2627, KU-20-6, and KU-2008 from Pakistan. To confirm whether the nine accessions possessed an identical deletion mutation, a primer set was designed at the flanking regions of the large deletion. All nine accessions showed single fragments of the same size (Fig. 2C). One Turkmenistan accession (IG126489) had a novel deletion mutation, a 41-bp deletion that occurred within the 1st intron region flanking the 4-bp repeat (TGGT) (Fig. 2B).

Amplified fragments for the 1.2-kb 5' upstream region showed a monomorphic pattern in the 210 accessions (Fig. 2D). An 8-bp deletion (AAAAATTT) in the promoter region was originally found in KU-20-3 and KU-2006 and KU-2008 from Pakistan. To confirm whether the nine accessions possessed an identical deletion mutation, a primer set was designed at the flanking regions of the large deletion. All nine accessions showed single fragments of the same size (Fig. 2C). One Turkmenistan accession (IG126489) had a novel deletion mutation, a 41-bp deletion that occurred within the 1st intron region flanking the 4-bp repeat (TGGT) (Fig. 2B).

Vernalization requirement of accessions with the large deletion To examine the vernalization requirement in the nine \( Ae. tauschii \) accessions with the large deletion, heading and flowering times were assessed under long-day conditions without vernalization treatment. All accessions transitioned from vegetative to reproductive phase despite the lack of vernalization treatment (Table 2), indicating that the large deletion in the 1st intron of \( Vrn-D1 \) results in loss of vernalization requirement in the wild progenitor as well as in common wheat. IG126489, which contains a 41-bp deletion mutation outside the critical region of the 1st intron, required vernalization for heading.

The \( Ae. tauschii \) accessions in Afghanistan and Pakistan generally show an early flowering phenotype.

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### Table 2. Flowering feature of the \( Ae. tauschii \) accessions with the large deletion (5,437-bp) in the \( Vrn-D1 \) 1st intron

<table>
<thead>
<tr>
<th>Accession#</th>
<th>( Vrn-D1 ) genotype</th>
<th>Field*</th>
<th>long day (24-h light)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Heading time</td>
<td>Flowering time</td>
</tr>
<tr>
<td>KU-2010</td>
<td>large deletion</td>
<td>156</td>
<td>159</td>
</tr>
<tr>
<td>KU-2012</td>
<td>large deletion</td>
<td>156</td>
<td>160</td>
</tr>
<tr>
<td>IG108561</td>
<td>large deletion</td>
<td>156</td>
<td>160</td>
</tr>
<tr>
<td>KU-2006</td>
<td>large deletion</td>
<td>152</td>
<td>167</td>
</tr>
<tr>
<td>KU-2008</td>
<td>large deletion</td>
<td>153</td>
<td>157</td>
</tr>
<tr>
<td>KU-20-6</td>
<td>large deletion</td>
<td>153</td>
<td>159</td>
</tr>
<tr>
<td>KU-2627</td>
<td>large deletion</td>
<td>162</td>
<td>172</td>
</tr>
<tr>
<td>CGN10767</td>
<td>large deletion</td>
<td>153</td>
<td>158</td>
</tr>
<tr>
<td>CGN10769</td>
<td>large deletion</td>
<td>159</td>
<td>160</td>
</tr>
<tr>
<td>IG126489</td>
<td>small deletion</td>
<td>153</td>
<td>159</td>
</tr>
<tr>
<td>IG46663</td>
<td>no deletion</td>
<td>151</td>
<td>157</td>
</tr>
<tr>
<td>KU-20-9</td>
<td>no deletion</td>
<td>163</td>
<td>171</td>
</tr>
<tr>
<td>KU-20-10</td>
<td>no deletion</td>
<td>165</td>
<td>171</td>
</tr>
</tbody>
</table>

* Heading/flowering days after sowing in the field of Kobe University at the 2003–2004 season.
** Heading/flowering days after sowing under the long-day condition without vernalization.
- Not heading and flowering until 130 days after sowing.

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### Table 3. Relationship between presence/absence of the large deletion (5,437-bp) in the \( Vrn-D1 \) 1st intron and heading time in the field

<table>
<thead>
<tr>
<th>Collection country</th>
<th>No. accessions</th>
<th>( Vrn-D1 ) genotype</th>
<th>Heading time*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afghanistan</td>
<td>3</td>
<td>large deletion</td>
<td>158.0 ± 3.46</td>
</tr>
<tr>
<td>Afghanistan</td>
<td>34</td>
<td>no deletion</td>
<td>160.0 ± 7.42</td>
</tr>
<tr>
<td>Pakistan</td>
<td>6</td>
<td>large deletion</td>
<td>154.3 ± 2.66</td>
</tr>
<tr>
<td>Pakistan</td>
<td>7</td>
<td>no deletion</td>
<td>154.4 ± 1.77</td>
</tr>
<tr>
<td>Afghanistan + Pakistan</td>
<td>9</td>
<td>large deletion</td>
<td>155.6 ± 3.28</td>
</tr>
<tr>
<td>Afghanistan + Pakistan</td>
<td>41</td>
<td>no deletion</td>
<td>158.9 ± 7.09</td>
</tr>
<tr>
<td>Others</td>
<td>160</td>
<td>no deletion</td>
<td>164.6 ± 7.95</td>
</tr>
</tbody>
</table>

* Heading days after sowing in the field of Kobe University at the 2003–2004 season.
(Matsuoka et al., 2008b). To confirm the effect of the large deletion in the 1st intron on early flowering, heading days after sowing were compared between accessions in Afghanistan and Pakistan. The accessions with the large deletion exhibited similar heading time to the non-deletion accessions in both Afghanistan and Pakistan (Table 3). The large deletion in the 1st intron of Vrn-D1 showed no significant effect on flowering time under common field conditions when the seeds were sown in autumn.

**DNA polymorphism at the Vrn-D1 locus** Nucleotide sequences of two genomic fragments at the Vrn-D1 locus were determined in 23 accessions of *Ae. tauschii*. One

Table 4. Summary of nucleotide variation in two regions of the Vrn-D1 locus in the *Ae. tauschii* population

<table>
<thead>
<tr>
<th></th>
<th>promoter + exon 1</th>
<th>exon 3 to exon 8</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sites</td>
<td>1,099</td>
<td>1,611</td>
<td>2,747</td>
</tr>
<tr>
<td>Number of accessions</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Number of variable sites</td>
<td>23</td>
<td>32</td>
<td>53</td>
</tr>
<tr>
<td>Total number of mutation</td>
<td>23</td>
<td>32</td>
<td>53</td>
</tr>
<tr>
<td>Number of haplotypes</td>
<td>11</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Haplotype diversity</td>
<td>0.810 ± 0.080</td>
<td>0.938 ± 0.039</td>
<td>0.957 ± 0.034</td>
</tr>
<tr>
<td>Nucleotide diversity (Pi)</td>
<td>0.00478 ± 0.00093</td>
<td>0.00701 ± 0.00104</td>
<td>0.00599 ± 0.00103</td>
</tr>
<tr>
<td>Tajima's D</td>
<td>−0.67280 (P &gt; 0.10)</td>
<td>1.13184 (P &gt; 0.10)</td>
<td>0.43054 (P &gt; 0.10)</td>
</tr>
<tr>
<td>Fu and Li's D</td>
<td>−1.62089 (P &gt; 0.10)</td>
<td>0.17899 (P &gt; 0.10)</td>
<td>−0.46824 (P &gt; 0.10)</td>
</tr>
</tbody>
</table>

Fig. 3. Effects of the large deletion in the Vrn-D1 first intron on gene expression patterns. (A, B) Comparison of Vrn-D1 transcript accumulation between accessions with and without the large deletion. Quantitative RT-PCR was conducted using total RNA from seedling leaves after treatment with low temperature for 1 and 7 days. The Act gene was used as an internal control. Each transcript level was represented as a mean value and standard deviation relative to that of the KU-2069 level. Student’s *t*-test was used to test for statistical significance (*P < 0.05, **P < 0.01). (C) Vrn-D1 transcript accumulation under normal growth temperature in accessions with and without the large deletion. (D, E) Comparison of WFT transcript accumulation in accessions with and without the large deletion.
fragment included about 1 kb 5’ upstream from the start codon and the 1st exon, and another contained the genomic region from the third to eighth exon.

In total, only 26 polymorphisms were found in a fragment containing the non-coding region of the promoter to exon 1 of Vrn-D1. Of these, 11 nucleotide substitutions and two length variants were singletons. Two indel mutations such as an 8-bp insertion and a 16-bp deletion in KU-2058 were excluded from later analyses, and nucleotide substitution from AA to TG in KU-2111 was treated as one mutation event. The nucleotide diversity (π) of the promoter to exon 1 region of Vrn-D1 was 0.0048 (Table 4). Tajima’s D (1989) and Fu and Li’s D (1993) tests were conducted to examine evolutionary neutrality at the Vrn-D1 locus. The D test statistics were negative in the promoter to exon 1 region, although the results were not significant.

Thirty-six polymorphisms were detected in the exon 3 to 8 fragment of Vrn-D1. In these variants, six nucleotide substitutions and two length variants were singletons. From the following analyses, four gaps such as a 4-bp deletion were excluded. π of the exon 3 to 8 region of Vrn-D1 was 0.0070 (Table 4). Tajima’s D and Fu and Li’s D statistics were positive in the exon 3 to 8 region. None of the neutrality tests gave a significant result.

Effect of the large deletion mutation on Vrn-D1 and WFT expression To study the effect of the 5,437-bp deletion in the 1st intron on Vrn-D1 expression, we compared the expression profile of Vrn-D1. This quantitative comparison showed significant differences in levels of Vrn-D1 transcript between KU-2069, a non-deletion accession, and IG108561, an accession with the deletion, under low temperature conditions (Fig. 3, A and B). Four weeks after germination, Vrn-D1 transcript was more abundant in IG108561 even under normal temperature conditions (23°C) (Fig. 3C). WFT transcript was also more abundant in IG108561 than in KU-2069 after a 28-day low temperature treatment, although no significant difference in WFT transcript levels was observed 21 days after treatment (Fig. 3, D and E).

DISCUSSION

Vernalization requirement is thought to be an important trait for environmental adaptation in some higher plants in temperate regions, such as wheat (Golovnina et al., 2010). Ae. tauschii is widely distributed in central Eurasia (Van Slageren, 1994), and carries abundant natural variation in various traits including flowering time and spike morphology (Matsuoka et al., 2008b, 2009; Takumi et al., 2009). Here, we showed some of its genetic variation in vernalization requirement. In several Triticum species, some structural mutations found at the Vrn-1 loci including the promoter region are associated with spring habit (Yan et al., 2004; Fu et al., 2005; Golovnina et al., 2010). Based on these observations, the critical region for spring habit was determined to be in the first intron of Vrn-1 (Fu et al., 2005; von Zitzewitz et al., 2005). No structural differences were found in the promoter region of Vrn-D1 among the Ae. tauschii accessions, which corresponded with the previous report (Golovnina et al., 2010). In total, nine Ae. tauschii accessions shared the same large deletion, 5,437 bp in length, in the critical region of the first intron of Vrn-1 (Figs. 1 and 2), implying that the deletion event was derived from a single mutation. In addition, the mutation site did not correspond to any large deletion reported in the Vrn-D1 dominant alleles of common wheat. Therefore, the large deletion identified from the Ae. tauschii population has not contributed to spring habit in common wheat varieties. Our discovery of the large deletion indicates that artificial improvement after domestication of wheat and barley has at least partly taken advantage of similar mutation events occurring in natural habitats.

Other winter growth habit loci containing Vrn-2 and Vrn-3 have been reported in barley and common wheat (Takahashi and Yasuda, 1971; Law and Worland, 1997). Recent study using a lot of barley varieties showed that three major vernalization genes, Vrn-1, Vrn-2 and Vrn-3, partially explained the variations in vernalization requirement and other known genes contributed in the remainder of the variations (Saisho et al., 2011). Presence of spring-type Ae. tauschii accessions has been previously reported (Tanaka and Yamashita, 1957; Tsunewaki, 1966; Golovnina et al., 2010). Here, we showed that all nine accessions with the large deletion in the Vrn-D1 first intron lost their vernalization requirement for flowering under long-day conditions (Table 2), indicating that the large deletion results in the loss of vernalization requirement. Golovnina et al. (2010) identified some spring-type accessions without any mutation in Vrn-D1. We confirmed that at least six Ae. tauschii accessions exhibited loss of vernalization requirement in spite of no structural mutation in Vrn-D1 (our unpublished results). Therefore, the large deletion in the Vrn-D1 first intron explains some of the natural variation in vernalization requirement in Ae. tauschii. The molecular basis of the Vrn-D1-independent spring-habit phenotype should be elucidated in future studies.

Vrn-D1 transcript increased in the Ae. tauschii accessions carrying the large deletion (Fig. 3). Alteration of Vrn-1 transcript levels was previously reported in spring-type varieties of einkorn wheat and barley (Dubcovsky et al., 2006; Hemming et al., 2009). As Hemming et al. (2009) mentioned, the critical region within the Vrn-1 first intron might be required to maintain low levels of Vrn-1 expression. According to Hemming et al. (2009), the critical region contains a number of short AT-rich
The scaffold/matrix attachment regions are found within the first intron of plant genes, and associated with transcriptional down-regulation (Rudd et al., 2004). The putative scaffold/matrix attachment region of the Vrn-D1 large deletion is also observed for the Vrn-I repression in Ae. tauschii. The increase in the Vrn-D1 transcript level was related to abundant accumulation of WFT transcript (Fig. 3). Similar results have been reported for common wheat (Yan et al., 2006; Shimada et al., 2009). These observations indicated that the enhanced expression of Vrn-1 in leaves might rapidly induce abundant expression of wheat FT orthologs following vernalization-independent phase transitioning from vegetative to reproductive growth. On the other hand, flowering time was not significantly different between accessions with and without the deletion in a common garden experiment (Table 3), implying that low temperature in winter efficiently negates the effect of the loss of vernalization requirement via the large deletion in the Vrn-D1 first intron. This field observation is agreed with previous reports (Kato and Yamashita, 1991; Fujita et al., 1995).

The Ae. tauschii population consists of three major genealogical lineages, L1, L2 and HG17 (Matsuoka et al., 2008b; Mizuno et al., 2010a). L2 and HG17 lineages consisted of 75 accessions, whereas L1 included 135 accessions. Based on nucleotide polymorphisms, the Vrn-D1 sequences were generally divided into two types; one contained KU-20-3 and KU-20-6, and the other contained KU-20-9 and KU-20-10 (Fig. 1). There were two major lineages, L1 and L2, in Ae. tauschii according to a population structure analysis (Mizuno et al., 2010a), and the two types of Vrn-D1 sequences identified corresponded to the diversification of the two lineages. Therefore, most of the nucleotide polymorphisms in Vrn-D1 reflect the nuclear genome diversification between L1 and L2 in Ae. tauschii. On the other hand, the three structural mutations at the Vrn-D1 locus, the large deletion and 109-bp duplication in the first intron, and the 8-bp insertion in the promoter, occurred after the L1 and L2 diversification of Ae. tauschii (Fig. 4). Based on chloroplast DNA variation, the 210 accessions were classified into 17 haplogroups (Matsuoka et al., 2008b). The chloroplast DNA haplogroups were also previously determined based on data from biallelic single nucleotide polymorphism and minisatellite sites that were found in the flanking regions of chloroplast microsatellite loci (Matsuoka et al., 2007, 2008b). On the basis of the network topology, four

![Fig. 4. Haplotype analysis based on structural differences at the Vrn-D1 locus and on chloroplast DNA variation.](image-url)

(A) The Ae. tauschii accessions were genealogically divided into two large lineages, L1 and L2, and others belonging to chloroplast haplogroup 17 (HG17) (Mizuno et al., 2010a). (A) Distribution of the 8-bp insertion and the 5,437-bp deletion in the cp-haplogroup network. The cp-haplogroup network for 210 Ae. tauschii accessions was previously constructed based on nine biallelic polymorphisms of the wheat cpDNA sequence (Matsuoka et al., 2008b, 2009). (B) Relationship among the genealogical lineage, the 8-bp insertion and the 109-bp duplication. The number of accessions with each haplotype is indicated.
intraspecific lineages were defined as follows: the HG7 lineage (HG7 and its derivatives HG2, HG4, HG5, HG6, HG8, HG10, HG11, HG12, HG13 and HG14), the HG9 lineage (HG9 and its derivatives HG1, HG3 and HG18), the HG16 lineage (HG16 and its derivative HG15), and the HG17 lineage (HG17) (Matsuoka et al., 2008b, 2009). The major haplgroup lineage information of the 205 accessions should be referred to the previous reports (Matsuoka et al., 2008b, 2009). Most of the haplogroups belonged to either L1 or L2, and the largest haplgroup (HG7) contained both L1 and L2 accessions. The nine accessions with the large deletion in the 1st intron belonged to HG8, HG13 or HG16 in L1, whereas the 43 accessions with the 8-bp insertion were broadly distributed within HG3, HG4, HG5, HG7 and HG9 in L2 (Fig. 4A). All seven accessions with the 109-bp duplication possessed the 8-bp insertion and were distributed in Syria, Iran and Armenia (Fig. 4B). The accessions with the large deletion belonged to L1, and had a limited distribution in Afghanistan and Pakistan. The limited distribution of the large deletion formed a contrast to the wide distribution of the 8-bp insertion in the Vrn-D1 promoter within the L2 accessions, suggesting that the large deletion is a comparatively recent mutation that occurred during the west-to-east dispersal of Ae. tauschii. Significantly, Ae. tauschii accessions in the eastern habitats showed an early-flowering phenotype (Matsuoka et al., 2008b). It was assumed that the early-flowering phenotype had played an important role in Ae. tauschii adaptation to the eastern regions of its native range (Matsuoka et al., 2008b). As mentioned above, however, the large deletion in the Vrn-D1 first intron did not directly contribute to the early flowering in the Afghanistan and Pakistan accessions. The nucleotide variation pattern in Vrn-D1 was evolutionarily neutral in the Ae. tauschii population (Table 4), indicating no evidence for presence of any selection pressure at Vrn-D1, indicating no evidence for presence of any selection pressure at Vrn-D1 (Table 4). Relationship between the large deletion in the Vrn-D1 first intron and the west-to-east dispersal of Ae. tauschii should be clarified in further studies.

The 109-bp duplication in the Vrn-D1 first intron was also identified in only limited accesses of the Ae. tauschii population (Fig. 4). The seven accessions with the 109-bp duplication were originally collected from Syria, Iran and Armenia. Importantly, the 109-bp duplication was found at the same site of Vrn-D1 in the common wheat cultivar ‘Chinese Spring’ (Fu et al., 2005). The correspondence suggests that the Ae. tauschii accesses with the 109-bp duplication in the Vrn-D1 first intron might be a D-genome donor of common wheat. Subspecies strangulata and variety meyeri probably contributed to the D-genome of hexaploid wheat (Nishikawa et al., 1980). Subspecies strangulata and variety meyeri are closely related in L2 (Mizuno et al., 2010a), and never show any abnormal phenotype in triploid hybrids crossed into tetraploid wheat (Matsuoka et al., 2007; Mizuno et al., 2010b). These observations suggest that variety meyeri and its closely related accesses of Ae. tauschii could be a D-genome progenitor of common wheat. In fact, hexaploid synthetic wheat lines can be obtained from crosses between variety meyeri and tetraploid wheat (Kajimura et al., 2011). Distribution of the 109-bp duplication should be elucidated in the D-genome of common wheat to better understand the origin and speciation of hexaploid wheat.

Identification of natural mutant accessions with a loss of vernalization requirement indicates agricultural significance of Ae. tauschii as a genetic resource for wheat breeding. The large-deletion-containing Vrn-D1 allele identified here might function in the hexaploid genetic background similarly to the dominant Vrn-D1 allele of common wheat. The effect of the novel Vrn-D1 allele with the large deletion on flowering characteristics should be clarified in the hexaploid wheat background.

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