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Molecular characterization of the duplicated meristem identity genes \textit{HvAP1a} and \textit{HvAP1b} in barley

Liuling Yan, Jarislav von Zitzewitz, Jeffrey S. Skinner, Patrick M. Hayes, and Jorge Dubcovsky

Abstract: The vernalization gene \textit{VRN-1} has been identified as a MADS-box transcription factor orthologous to the meristem identity gene \textit{APETALA1} (\textit{AP1}). A single copy of this gene was found in diploid wheat, but 2 copies were reported in barley. In this study, we present a detailed characterization of these 2 copies to understand their respective roles in the vernalization response. We identified 2 groups of barley bacterial artificial chromosomes (BACs), each containing 1 \textit{AP1} copy designated hereafter as \textit{HvAP1a} and \textit{HvAP1b}. A physical map of the \textit{VRN-H1} region showed that the \textit{HvAP1a} BACs were part of the \textit{VRN-H1} region but that the \textit{HvAP1b} BACs were not. Numerous structural changes were observed between the barley and wheat \textit{VRN-1} physical maps. In a population segregating for \textit{VRN-H1}, the \textit{HvAP1a} gene cosegregated with growth habit, suggesting that \textit{HvAP1a} is the barley vernalization gene \textit{VRN-H1}. The other copy, \textit{HvAP1b}, was mapped on the centromeric region of chromosome 1H, the chromosome where vernalization gene \textit{VRN-H3} was previously mapped. We developed a mapping population segregating for \textit{VRN-H3} and showed that 2 molecular makers flanking \textit{HvAP1b} locus were not linked to growth habit. The \textit{HvAP1b} copy has a complete deletion of the first 2 exons, suggesting that it is a truncated pseudogene and not a candidate for \textit{VRN-H3}. In summary, this study contributed a detailed physical map of the barley \textit{VRN-H1} region, showed several structural differences with the orthologous wheat region, and clarified the identity of the barley \textit{VRN-H1} gene.

Key words: barley, vernalization, \textit{Vrn-1}, physical map.

Résumé : Le gène de vernalisation \textit{VRN-1} a été identifié comme étant un facteur de transcription MADS-box orthologue au gène d’identité méristématique \textit{APETALA1} (\textit{AP1}). Une seule copie de ce gène a été trouvée chez le blé diploïde, mais 2 copies ont été rapportées chez l’orge. Dans ce travail, les auteurs présentent une caractérisation détaillée de ces 2 copies afin de comprendre leurs rôles respectifs dans la vernalisation. Deux groupes de clones BAC, chacun avec une copie de gène \textit{AP1}, appelés \textit{HvAP1a} et \textit{HvAP1b}, ont été identifiés. Une carte physique de la région \textit{VRN-H1} a révélé que les BAC du gène \textit{HvAP1a} en formaient partie, mais pas ceux de \textit{HvAP1b}. De nombreux changements structuraux ont été observés entre les cartes physiques \textit{VRN-1} chez le blé et l’orge. Dans une population en ségrégation pour \textit{VRN-H1}, le gène \textit{HvAP1a} coségréait avec le type de croissance, ce qui suggère que \textit{HvAP1a} serait le gène de vernalisation chez l’orge. L’autre copie, \textit{HvAP1b}, a été localisé dans la région centromérique du chromosome 1H, le chromosome sur lequel avait été localisé le gène \textit{VRN-H3}. Les auteurs ont développé une population de cartographie en ségrégation pour \textit{VRN-H3} et ont montré que 2 marqueurs bordant \textit{HvAP1b} n’étaient pas liés au type de croissance. La copie \textit{HvAP1b} a une délétion complète des 2 premiers exons, ce qui suggère qu’il s’agirait d’un pseudogène tronqué et non pas d’un candidat pour \textit{VRN-H3}. En résumé, ce travail a contribué une carte physique détaillée de la région \textit{VRN-H1}, montré plusieurs différences de structure par rapport à l’orthologue du blé et a clarifié l’identité du gène \textit{VRN-H1} chez l’orge.


[Traduit par la Rédaction]
**Introduction**

Both barley and wheat are divided into winter and spring varieties according to their requirements for vernalization, which is the acceleration of the transition from the vegetative to the reproductive state by a long exposure to low temperatures. The 2 main genes controlling the vernalization response in these 2 species, **VRN-1** and **VRN-2**, map on colinear chromosome locations and show similar dominance and epistatic interactions suggesting that they are orthologous genes (Dubcovsky et al. 1998; Laurie et al. 1995; Takahashi and Yasuda 1971; Tranquilli and Dubcovsky 2000). Allelic variation in a 3rd vernalization gene designated **VRN-H3** has been observed only in barley (Takahashi and Yasuda 1971).

The **VRN-H3** gene was mapped on the long arm of chromosome 1H by its linkage with the **Blp** (black lemma and pericarp) locus (recombination fraction =0.36) (Takahashi and Yasuda 1971). Like **VRN-H1, VRN-H3** is dominant for spring-growth habit and has similar epistatic interactions with **VRN-H2**. The dominant **Vrn-H3** allele determines a spring-growth habit phenotype regardless of the allelic variation at **VRN-H2**, whereas the recessive **vrn-h2** determines a spring-growth habit phenotype regardless of the allelic variation at **VRN-H3** (Takahashi and Yasuda 1971). Spring barley accessions having only the dominant **Vrn-H3** gene have never been found, and those carrying the **Vrn-h1 Vrn-H3** genotype occur most frequently in North Pakistan, North India, Tibet, Ethiopia, and Northern Europe (Yasuda et al. 1986). The dominant **Vrn-H3** allele is most commonly observed in barley accessions from regions of extremely high latitude or high altitude (Takahashi and Yasuda 1971).

**VRN-H3** is the only 1 of the 3 barley vernalization genes that has not yet been cloned. **VRN-A** was recently cloned from diploid wheat *Triticum monococcum* L., and was shown to be a zinc finger–CCT domain transcription factor (**ZCCT**) downregulated by vernalization (Yan et al. 2004b). Analysis of a large collection of spring barley varieties carrying the recessive **vrn-h2** allele showed that they all have a deletion of the **ZCCT** gene. Linkage analysis confirmed that **VRN-A** and **VRN-H2** were orthologous (Dubcovsky et al. 2005). The **VRN-A** gene was also first cloned from *T. monococcum* and was shown to be a MADS-box transcription factor orthologous to the Arabidopsis meristem identity gene **APETALA1 (AP1)** (Yan et al. 2003). The dominant **Vrn-1** alleles in diploid and pentaploid wheat were associated with mutations in the promoter or intron 1 regions of the wheat **VRN-1** gene (Fu et al. 2005; Yan et al. 2004a; Yan et al. 2003).

However, the relationship between the wheat and barley **VRN-1** gene is not clear, because 2 copies of the **HvAP1** gene exist in barley. A probe for the last exons (excluding the MADS- and K-boxes) of the barley **BM5** cDNA clone corresponding to the **VRN-A** gene (Yan et al. 2003) revealed 2 bands in Southern blots of barley genomic DNA digested with different restriction enzymes (Schmitz et al. 2000). These 2 **HvAP1** copies were not mapped in previous studies because of lack of DNA polymorphism (Schmitz et al. 2000). Recent studies have shown that the transcripts of **BM5** are regulated by vernalization in winter barley but not in spring barley (Danyluk et al. 2003; Trevaskis et al. 2003).

However, these studies did not consider the **HvAP1** duplication and, therefore, it is not possible to determine from which of the 2 **HvAP1** copies these transcripts originated.

In this present study, we provide a detailed genetic and molecular characterization of these 2 copies of **HvAP1** and identify the 1 corresponding to the barley **VRN-H1** gene. We also test the hypothesis that the 2nd copy of **HvAP1** corresponds to the **VRN-H3** vernalization gene. This hypothesis was based on the following indirect evidence: (i) both the **HvAP1** duplication and the **VRN-H3** gene have been reported in barley but not in wheat, (ii) a duplicated **HvAP1** gene was expected to be dominant for a spring-growth habit as reported for **VRN-H3**, and (iii) a duplicated **HvAP1** gene would provide a simple explanation for the identical epistatic interactions of **VRN-H1** and **VRN-H3** with **VRN-2**. To test this hypothesis we determined the chromosome location of the 2nd **HvAP1** gene and mapped 2 markers flanking this gene in a population segregating for **VRN-H3**.

**Materials and methods**

**Plant materials**

Three barley mapping populations were used in this study. The 1st one was an **F2** population (91 plants) produced from the cross between *Hordeum vulgare* L. spring ‘Morex’ and winter *H. spontaneum* C. Koch (OSU6, PBI004-7-0-015). This population was kindly provided by Dr A. Kleinhofs and was used to map the **HvAP1** gene linked to **VRN-H1**. ‘Morex’ is the source of the 1st barley bacterial artificial chromosome (BAC) library (Yu et al. 2000), which was used in this study for the construction of the **VRN-H1** region physical map. ‘Morex’ has a dominant **Vrn-H1** allele and recessive **vrn-2** and **vrn-H3** alleles, whereas *H. spontaneum* has recessive **vrn-h1** and **vrn-H3** alleles and a dominant **Vrn-2** allele (Dubcovsky et al. 2005). The **F2** plants were grown in the greenhouse without vernalization. Under these conditions, plants carrying the allele combinations for winter-growth habit flowered on average 37 days later than plants with the alleles for spring-growth habit (Dubcovsky et al. 2005).

The doubled haploid mapping from the cross ‘Dicktoo’ × ‘Morex’ (Hayes et al. 1997) was used to map the 2nd **HvAP1** copy, because this copy showed no polymorphism between the parents of the 1st mapping population. In addition, a complete genetic map was available for the ‘Dicktoo’ × ‘Morex’ doubled haploid population facilitating the location of the 2nd **HvAP1** copy on the linkage map.

The 3rd mapping population was an **F2** population from the cross between the spring genetic stock BG213 and the same winter accession of *H. spontaneum* as the one used in the 1st mapping population. BG213 is of genetic stock for **VRN-H3** and has a dominant **Vrn-H3** allele for spring-growth habit combined with recessive **vrn-h1** and dominant **Vrn-H2** alleles for winter-growth habit (Takahashi and Yasuda 1971). Since the 3 alleles for *H. spontaneum* are for winter-growth habit (**vrn-H1, Vrn-H2, vrn-H3**), this population was expected to segregate only for **VRN-H3**.

**Methods**

Molecular markers previously mapped in the **VRN-1** region in wheat (WG644, PCS, CYB5, AGLG1, and **AP1**) were
used to screen the ‘Morex’ BAC library (Yan et al. 2003). Hereafter, we will use the prefix Tm and Hv to designate the *T. monococcum* and barley loci, respectively. Bacterial artificial chromosome DNA extraction, fingerprinting, and assembly into physical contigs were performed as described before (Dubcovsky et al. 2001; Yan et al. 2003). Southern blots from BAC HindIII fingerprints were hybridized with the different markers to determine their location within the contigs. Bacterial artificial chromosome ends were subcloned using inverse PCR and plasmid rescue; and these BAC ends were used as hybridization probes to confirm BAC contigs as described before (Yan et al. 2003).

Primers designed based on the sequences of several genes from the *VRN-I* region in *T. monococcum* (Yan et al. 2003) were used to amplify the orthologous genes from barley. The primer sequences and the ‘Morex’ barley BAC clones that were used as a DNA template are summarized in Table 1.

The *TmAP1* primers Ex4F1 and Ex7R1 were used to isolate and sequence the regions between exons 4 and 7 from the 2 different BAC groups including *HvAP1* genes. These copies were designated *HvAP1a* and *HvAP1b*. A primer-walking approach was used to sequence the rest of the *HvAP1* genes from the BAC clones. Primers *HvAP1*-Ex1-F and *HvAP1*-Ex1-R were used to amplify part of the 5′ UTR and the compete exon 1; whereas primers *HvAP1*-Ex2-F and *HvAP1*-Ex2-R were used to amplify the complete exon 2 and part of the 2nd intron.

To map the *HvAP1b* gene we sequenced the parental alleles from ‘Dicktoo’ and ‘Morex’ and developed an *Alu* I-based cleavable amplified polymorphic sequence (CAPS) marker using primers *HvAP1B*-F and *HvAP1B*-R. The *HvAP1b* locus was not polymorphic in the BG213 × *H. spontaneum* populations so we mapped instead the *HvAP1b* flanking loci *Tri* and *bcd454.

All PCR programs included a denaturation cycle at 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 30 s; and a final extension cycle at 72 °C for 7 min. PCR products were purified and cloned into the pCR 4-TOPO® TA vectors (Invitrogen Corporation, Carlsbad, California, USA) following manufacturer’s instructions. Ligation reactions were transformed into Electro MAX TMDH10B competent cells ( Gibco BRL) and inserts were sequenced. *HvAP1a* and *HvAP1b* sequence differences were used to investigate their transcription profile by analyzing the expressed sequence tags (ESTs) clones present in GenBank EST databases.

A barley probe prepared with a genomic DNA fragment including the region between exons 4 and 7 was used to screen a collection of 85 barley accessions to evaluate the distribution of the *HvAP1* duplication. This same collection was used before to evaluate the variation at the *VRN-H2* locus (Dubcovsky et al. 2005).

| Results |

**Physical map of the *VRN-H1* region**

A total of 8 BAC clones were obtained from the ‘Morex’ BARC library, 7 using the *TmAP1*-Exons 4–7 probe (Fig. 1B) and the last 1 (BAC 31B22) using the *HvAP1*-Exon 2 probe. Southern blots from HindIII digested BAC fingerprints hybridized with the *TmAP1*-Exons 4–7 probe revealed that these 8 BACs were organized in 2 nonoverlapping groups (Fig. 2A and 2B). The 1st group, including the *HvAP1a* copy, consisted of 6 BACs (31B22, 597D19, 317M7, 631P8, 460O15, and 472K16), whereas the 2nd group including the *HvAP1b* copy showed only 2 BACs (143P9 and 315F20). Hybridization of the Southern blots from the HindIII fingerprints using 143P9 and 315F20 BAC ends probes prepared by inverse PCR and plasmid rescue showed no connection between the 2 *HvAP1* contigs suggesting that the 2 *HvAP1* genes were not adjacent.

To test the relative position of the *HvAP1a* and *HvAP1b* BAC contigs within the barley VRN-H1 region we compared the wheat *VRN-A1* physical contig with the 2 groups of barley physical contigs (Fig. 1). The probe for *T. monococcum* Phytochrome-C gene (PHY-C, AAM34402.1) hybridized with 5 of the BACs including the *HvAP1a* gene indicating that this *HvAP1* copy was the one located in the *VRN-H1* region. The *HvAP1b* BAC fingerprints showed no connection with any of the BACs of the *VRN-H1* region and did not hybridize with any of the probes generated from this region, suggesting that they were located outside this region.

**Comparison between the barley and *T. monococcum* VRN-A1 physical maps**

The PHY-C probe hybridized with 2 HindIII fragments, 1 present in 5 of the *HvAP1a* BACs, and the other present in only 3 of these BACs. The presence of 2 *HvPHY-C* copies in barley was confirmed by hybridization of EcoRI-digested BACs with the PHY-C probe. These 2 copies of the *HvPHY-C* gene were tentatively designated *HvPHY-C1* and *HvPHY-C2* (Fig. 1). Screening of the *T. monococcum* BAC library with the *TmPHY-C* probe yielded only 2 BACs, both with the same gene. Hybridization of genomic DNAs from ‘Morex’ barley and *T. monococcum* digested with restriction enzymes *HindIII*, *SacI* showed a single copy of *PHY-C* in *T. monococcum* and 2 in ‘Morex’ suggesting that *PHY-C* is duplicated in barley but not in *T. monococcum*.

An additional difference between diploid wheat and barley was found for the *Cysteine Proteinase* gene (CYS, AY244510) gene. Although the *Cysteine Proteinase* gene was present between *API* and *PHY-C* in wheat and rice (Yan et al. 2003), the probe for this gene did not hybridize with the barley BACs including *HvAP1* and *HvPHY-C* or with any of the other BACs of the *HvAP1* physical contigs (Fig. 1). This result suggests the presence of a deletion including the *HvCYS* gene in the barley *HvAP1a* region compared with the other species.

On the distal side of *HvAP1a*, the *TmAGLG1* MADS-box gene (AY188333) hybridized with 7 ‘Morex’ barley BAC clones (398F16, 420B3, 221P8, 606J6, 731K10, 673M15, and 749N15). These BACs formed a single contig, indicating that *TmAGLG1* is a single copy gene in barley as it is in diploid wheat. Sequencing of a 1096-bp fragment covering the *TmAGLG1* gene region between exons 4 and 8 from BAC 606J6 (GenBank accession No. AY916506) revealed 85% identity to *TmAGLG1*, suggesting they are orthologous genes. Bacterial-artificial-chromosome ends from BACs of the *HvAGLG1* contig showed no hybridization with the flanking *HvAP1a* and *HvCYS* contigs, suggesting the presence of large repetitive regions flanking this gene as was also observed in *T. monococcum* (Yan et al. 2003).
The probe for the Cytochrome B5 gene (TmCYB5, AY188332) detected a single copy gene in diploid wheat and also in barley. The 7 'Morex' BAC clones containing HvCYB5 (249E2, 312K18, 637F7, 422A20, 115N22, 715N9, and 604I5) formed a single contig connected to previously sequenced BAC 635P2 (Dubcovsky et al. 2001).

In T. monococcum and Sorghum bicolor there are 2 adjacent copies of the Phytochelatin synthetase gene designated PCS1 and PCS2 (AY188332 and AY188330, (Yan et al. 2003)) with the same relative position to CYB5. However, in wheat, the TmPCS1 gene is no longer functional and in rice it was eliminated from this region (Yan et al. 2003). In barley, we detected 38 BACs by hybridization with a probe from the TmPCS2 gene. The PCS genes found in 2 of the contigs located within the HvAP1a region (Fig. 1) were designated HvPCSa (BAC 432G22) and HvPCSb (GenBank AY887090 from BAC 115N22). The complete sequence of HvPCSa (GenBank AY887089) revealed that this is likely a pseudogene with premature stop codons in exons 4 and 6 and a mutated splicing site at the beginning of exon 5 (AY887089). A 643-bp segment from the HvPCSb gene showed 92% identity with the corresponding HvPCSa region and suggested that this duplication was relatively recent. Both HvPCSa and HvPCSb sequences showed higher iden-

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**Table 1.** Primer sequences for HvAP1 genes and genes in the HvAP1a region in barley.

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Sequences (5′-3′)</th>
<th>Target genes</th>
<th>BAC clones*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TmAGLG1-F1</td>
<td>AAGACCTTGAGGCAAATAAG</td>
<td>HvAGLG1</td>
<td>606J6</td>
</tr>
<tr>
<td>TmAGLG1-R1</td>
<td>GTTTTGGATAGTATACGCC</td>
<td>HvAGLG1</td>
<td>606J6</td>
</tr>
<tr>
<td>TmPCS1-F1</td>
<td>ATGCTGCTCTCCTCTCCAG</td>
<td>HvPCS1</td>
<td>115N22</td>
</tr>
<tr>
<td>TmPCS1-R1</td>
<td>GCAGGTTACATTCCATGACA</td>
<td>HvPCS1</td>
<td>115N22</td>
</tr>
<tr>
<td>TmAP1-Ex4Fl</td>
<td>TCTCATGGGAGAGGATCTT</td>
<td>HvPCS1-a-b</td>
<td>631P8 &amp; 315I20</td>
</tr>
<tr>
<td>TmAP1-Ex7R1</td>
<td>GGAGAGCCTCCTCCTGAGAA</td>
<td>HvPCS1-a-b</td>
<td>631P8 &amp; 315I20</td>
</tr>
<tr>
<td>HvAP1-Ex1-F</td>
<td>CAACCCCTGAAGCCGATGG</td>
<td>HvPCS1</td>
<td>631P8</td>
</tr>
<tr>
<td>HvAP1-Ex1-R</td>
<td>CATGACTGCGGAGGAGAAGCTC</td>
<td>HvPCS1</td>
<td>631P8</td>
</tr>
<tr>
<td>HvAP1-Ex2-F</td>
<td>TGGACAAAATTCTTGGAAAGGC</td>
<td>HvPCS1</td>
<td>631P8</td>
</tr>
<tr>
<td>HvAP1-Ex2-R</td>
<td>ACCATGCCTAAGCATTCATC</td>
<td>HvPCS1</td>
<td>631P8</td>
</tr>
<tr>
<td>HvAP1B-F</td>
<td>TCGGAAATGAAACAAAGAGACTT</td>
<td>HvAP1b</td>
<td>315I20 &amp; ‘Dicktoo’ DNA</td>
</tr>
<tr>
<td>HvAP1B-R</td>
<td>GAAACTTGAACAAACCCAGAACC</td>
<td>HvAP1b</td>
<td>315I20 &amp; ‘Dicktoo’ DNA</td>
</tr>
</tbody>
</table>

*The ‘Morex’ barley BAC or the barley variety used as a DNA source for the amplification is listed in the last column.
tity with the \( TmPCS2 \) gene (97% and 96%) than with the \( TmPCS1 \) pseudogene (86% and 86%). A similar result was obtained when the 2 barley copies were compared with the \( ShbPCS2 \) (84%–85%) or \( ShbPCS1 \) gene (82%–81%). Therefore, it was not possible to establish a clear correspondence between the 2 barley \( PCS \) copies and the wheat and sorghum genes (Fig. 1).

None of the markers flanking \( HvAP1a \) hybridized with the \( HvAP1b \) BAC clones, suggesting the \( HvAP1 \) duplication event involved a single gene rather than a genome segment.

**Genetic mapping of the \( HvAP1 \) genes**

We first investigated whether the \( HvAP1 \) duplication was a common feature among cultivated barleys. For this purpose, we studied a collection of 23 winter and 61 spring barley varieties from a wide geographical distribution, previously used to characterize the \( VRN-H2 \) allelic variation (Dubcovsky et al. 2005). Hybridization of Southern blots from \( HindIII \)-digested DNAs from these 84 varieties with the \( TmAP1 \)-Exons 4–7 probe showed the presence of 2 fragments in all accessions suggesting that this duplication preceded the domestication of barley. The duplication was also found in the single accession of \( H. spontaneum \) included in this study.

To better characterize this duplication, we genetically mapped the 2 \( HvAP1 \) genes. The \( HvAP1a \) gene was mapped completely linked to \( Xwg644 \) (Fig. 1) in the \( F_2 \) population from the cross between ‘Morex’ and \( H. spontaneum \) using a polymorphic deletion located within the 1st intron of the \( HvAP1a \) gene (Fu et al. 2005). The ‘Morex’ \( HvAP1a \) and \( Xwg644 \) alleles cosegregated with the spring-growth habit in this population, confirming that the \( HvAP1a \) copy was the one orthologous to the wheat \( TmAP1 \) gene previously identified as \( VRN-1 \) (Yan et al. 2003).

The \( HvAP1b \) locus was mapped in the ‘Dicktoo’ × ‘Morex’ population using CAPS primers \( HvAP1B-F \) and \( HvAP1B-R \) (see Materials and methods) and it cosegregated with RFLP marker \( ABG452 \) in the centromeric region of chromosome 1H (BIN 7). This was an interesting result, because the \( VRN-H3 \) locus was reported to be on chromosome 1H, 36 recombination units from the \( BLP \) locus (Takahashi and Yasuda 1971). To test whether \( HvAP1b \) was a good candidate for the \( VRN-H3 \) gene we tried to map it in the BG213 (dominant \( Vrn-H3 \) × \( H. spontaneum \) (recessive \( vrn-H3 \)) mapping population. Even though the \( HvAP1b \) locus was not polymorphic in this population, we were able to map loci \( TRI \) and \( BCD454 \), which are known to flank \( ABG452 \) within a 10 cM interval in other mapping populations (Dubcovsky et al. 1995). Neither \( TRI \) nor \( BCD454 \) were linked with the segregation for growth habit in the \( BG213 \) × \( H. spontaneum \) population. Therefore, we inferred that the \( HvAP1b \) and \( ABG452 \) loci flanked by these 2 markers would also be unlinked with the segregation for growth habit. This result suggests that the \( VRN-H3 \) gene is not in the centromeric region of chromosome 1H, the region where \( HvAP1b \) was mapped in the ‘Dicktoo’ × ‘Morex’ population.

The 314 \( F_2 \) plants from the \( BG213 \) × \( H. spontaneum \) mapping population showed a clear 3:1 ratio for growth habit (232 spring: 82 winter; \( \chi^2 P = 0.65 \)) confirming the segregation for a single dominant gene for spring-growth habit. To rule out the possibility of an incorrect cross with a barley line carrying the \( Vrn-H1 \) dominant allele instead of the \( Vrn-H3 \) allele (BG213 genetic stock), we developed a marker for \( HvAP1a \) (= \( Vrn-H1 \)). This marker showed no linkage with the segregation for growth habit in the \( BG213 \) × \( H. spontaneum \) mapping population, confirming that the dominant gene for spring-growth habit segregating in this mapping population was not \( Vrn-H1 \).

**Sequencing of the \( HvAP1 \) genes**

\( HvAP1a \): A 12 360-bp region from ‘Morex’ BAC 631P8 (AY750995) was subcloned and sequenced. This region included the complete \( HvAP1a \) gene, 2139-bp from the 5’ end and 656-bp from the 3’ end regions. \( HvAP1a \) showed the same gene structure as the wheat \( VRN-1 \) gene (AY188331) including identical exon sizes, similar intron sizes, and 3 identical split codons at the end of exons 1, 3, and 7 (Fig. 3, see Fu et al. 2005 for better comparison of intron 1). The shorter intron 1 observed in ‘Morex’ \( HvAP1a \) (Fig. 3) relative to diploid wheat was originated by a 5.2-kb deletion (Fu et al. 2005). Independent deletions including a conserved region of intron 1 have been described before in other spring wheat and barley varieties and were always associated with the presence of dominant \( Vrn-I \) alleles (Fu et al. 2005). The similar gene structure (Fig. 3), together with a high level of sequence identity in the coding (96%) and noncoding regions (88%), suggests that \( HvAP1a \) is the barley orthologue of wheat \( VRN-1 \). This result is consistent with a previous phylogenetic analysis of MADS-box genes, which showed that BM5 was the barley gene closest to wheat \( Vrn-I \) (Yan et al. 2003).

The \( HvAP1a \) sequence from the barley variety ‘Morex’ has a 99.7% identity with the cDNA BM5 from barley variety ‘Atlas’ (HUV249144, Schmitz et al. 2000), suggesting that the BM5 cDNA corresponds to the \( HvAP1a \) locus.

\( HvAP1b \): The \( HvAP1b \) sequence was determined from ‘Morex’ BAC 315I20 (GenBankAY887088). The last 6 exons of \( HvAP1b \) had the same structure as \( HvAP1a \) (Fig. 3). Good similarity was observed between the introns of the 2 barely copies (93% identical) suggesting that this was a recent duplication.

However, the first 2 exons of the ‘Morex’ \( HvAP1b \) gene were deleted. Comparison of the \( HvAP1a \) and \( HvAP1b \) se-
Fig. 3. Comparative structure of AP1 genes in diploid wheat (TmAP1) and barley (HvAP1a and HvAP1b). The length of the exons (boxes) is identical in the 3 genes and intron lengths are similar. Dark gray boxes indicate the location of the MADS-box domain, whereas light gray boxes indicate the location of the K-box domain. Arrowheads indicate the presence of conserved split codons at the end of exons 1, 3, and 7. The HvAP1b gene shows a 2.2-kb deletion between AAG direct repeats including exon 2 and a large deletion including exon 1 and most of the 1st intron. Vertical dotted lines indicate conserved regions of intron 1 (200-bp) and intron 2 (400-bp) between HvAP1a and HvAP1b (94%–96% identical). Sequence in front of the 3rd exon of HvAP1b shows the presence of in-frame stop codons (indicated by *).

Fig. 4. Screening blots of ‘Morex’ and H. spontaneum DNAs digested with (1–2) AseI, (3–4) BamHI, (5–6) EcoRI, (7–8) EcoRV, (9–10) HindIII, (11–12) SacI, and (13–14) XbaI. For each enzyme, the 1st sample is ‘Morex’ and the 2nd sample is H. spontaneum. The Southern blot was hybridized with probe HvAP1a exon 2. Note the presence of a single fragment for most of the enzymes.

sequences revealed a 2.2-kb deletion 400-bp upstream from the start of exon 3 (Fig. 3). This deletion includes exon 2 and the last 73-bp of intron 1 and is flanked on the 5’ side by a 200-bp region of exon 1 that shows good identity between HvAP1a and HvAP1b (95%). The 2.2-kb deletion is flanked by AAG direct repeats, which suggest that the deletion could have been originated by slippage replication.

The 1859-bp upstream from the 200-bp conserved region of intron 1 showed no significant similarity between HvAP1a and HvAP1b sequences, suggesting the presence of a 2nd deletion in HvAP1b (Fig. 3). Two probes developed from HvAP1a exon 1 and 2 failed to hybridize with the Southern blots from the HvAP1b BAC fingerprints indicating that HvAP1b exons 1 and 2 were not present in the 2 BAC clones carrying the 2nd AP1 copy. Strong hybridization signals were detected in the HvAP1a BAC clones used as a positive control (Fig. 2C).

To test whether the HvAP1b first 2 exons were present outside these 2 BACs, we developed a probe including the 77-bp of exon 2 and 212-bp of the flanking introns from HvAP1a. Screening of the ‘Morex’ BAC library with this probe returned only the 6 BAC clones previously assigned to HvAP1a, suggesting that the HvAP1b 2nd exon was not present in the BAC library. The same probe from exon 2 was used to screen Southern blots of ‘Morex’ genomic DNA digested with 11 different restriction enzymes. A single fragment (HvAP1a) hybridized with this probe in 10 of the 11 restriction enzymes tested (Fig. 4) indicating that only 1 copy of the HvAP1a second exon is present in the ‘Morex’ genomic DNA. We concluded that the ‘Morex’ HvAP1b is a truncated gene missing the complete MADS-box and part of the K-box (Fig. 3).

Analysis of the sequence upstream from the start of the exon 3 revealed the presence of 3 stop codons before the 1st possible in-frame start codon (ATG), suggesting that a shorter version of the protein is not translated (Fig. 3). However, it is not possible to rule out the presence of a different initial exon somewhere upstream from the 3rd exon.

We performed a similar hybridization analysis of the HvAP1b locus in the spring line BG213, which is the genetic stock for the dominant Vrn-H3 allele (Franckowiak and Konishi 1997). BG213 showed 2 fragments when hybridized with the TmAP1-Exons 4–7 probe but only a single fragment when hybridized with the probe from exon 2 and the flanking introns. These results indicated that HvAP1b was also truncated in BG213 and, together with the previous genetic analysis, confirmed that HvAP1b was not a good candidate gene for Vrn-H3.

Discussion

Duplication of HvAP1 gene

The HvAP1 gene (=BM5) was 1st reported to be duplicated by Schmitz et al. (2000) and this observation was extended to a large collection of cultivated barleys in this present study. We have previously detected a partial duplication of the wheat TaAP1 gene in the A genome of several hexaploid wheat accessions. This duplication included the promoter region, the 1st exon, and part of the 1st intron (Fu et al. 2005; Yan et al. 2004a). Hybridization of wheat genomic DNAs from lines differing in this duplication using the TmAP1-Exons 4–7 probe confirmed that the duplication did not extend to the last exons.

The 2 copies of the HvAP1 genes in barley were more similar to each other than to any of the AP1 copies present

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in the different wheat genomes indicating that the barley duplication occurred after the wheat–barley divergence 11–15 million years ago (Ramakrishna et al. 2002). However, the duplication is not that recent, because it is present in all the analyzed cultivated barley varieties and in the single H. spontaneum included in this study.

We showed here that the HvAP1a copy is the one that corresponds to the VRN-H1 gene by its inclusion in the VRN-H1 physical map and its linkage with growth habit in the ‘Morex’ × H. spontaneum population. This agrees with reports showing that the BM5 transcripts are upregulated by vernalization in winter barley varieties (Trevaskis et al. 2003). We also showed that the 2nd HvAP1b copy is a truncated gene lacking exons 1 and 2, which include the critical MADS-box domain and part of the K-box (Fig. 3). In the frame, stop codons were found upstream from the 3rd exon before any ATG codon, suggesting that the HvAP1b copy is not transcribed.

Indirect evidence that HvAP1b is not transcribed was obtained from the analysis of the available barley ESTs for AP1. HvAP1a and HvAP1b differ in 2 bases at the 5th exon, facilitating the assignment of ESTs to each of these 2 genes. A BLASTN search of the barley EST database with the AP1 5th exon showed that the 16 significant barley ESTs form ‘Morex’ and all the ESTs from other barley varieties belong to the HvAP1a copy. No ESTs with 2 characteristic single nucleotide polymorphisms from HvAP1b were found, suggesting that no HvAP1b transcripts are present in the extensive barley EST collection. Lack of transcription of the HvAP1b copy was also confirmed in different tissues collected at different growth stages (von Zitzewitz et al. 2005). Taken together, these data suggest that HvAP1b is an inactive pseudogene.

This truncated HvAP1 gene was present not only in the lines with the recessive vrn-H3 allele but also in the BG213 line carrying the dominant Vrn-H3 allele, indicating that HvAP1b was not a good candidate gene for VRN-H3. In addition, the linkage analysis between the molecular markers for the 1H centromeric region encompassing the HvAP1b locus and the segregation for growth habit in the BC213 × H. spontaneum population confirmed that VRN-H3 was not linked to the chromosome 1H centromeric region.

In spite of the negative result in relation to our original VRN-H3 hypothesis, this study provided the 1st detailed physical map of the VRN-H1 region, resolved the map positions of the duplicated HvAP1 gene in barley, identified which of the 2 copies is VRN-H1, developed a useful mapping population for the VRN-H3 gene, and showed that VRN-H3 is not linked to the centromeric region of barely chromosome 1H.

Comparative analysis of the VRN-I genomic regions in wheat and barley

An additional contribution of this study is the detailed analysis of the microcolinearity of the VRN-I genomic region between wheat and barley. We showed before that the TmAP1 gene was located within a region of very low gene density (>70-kb per gene). The TmAP1 gene was the only gene detected within a 134-kb BAC clone, whereas the TmAGLG1 gene was the only gene in a 112-kb BAC clone (Yan et al. 2003). A similar situation was observed in barley. The HvAGLG1 gene was located within an 8-BAC contig that was not connected with the BACs including the 2 flanking genes. These observations indicate that the VRN-I locus is located in low-density gene regions in both species. However, the barley HvAP1a contig showed a relatively higher abundance of genes than the wheat orthologous region since 3 of the HvAP1a BACs also included 2 copies of the PHY-C gene.

The closer proximity of the PHY-C and HvAP1 genes in barley relative to the orthologous genes in T. monococcum seems to be in part the result of a deletion that included the CYS gene. The change is more likely a deletion in ‘Morex’ than an insertion in T. monococcum, because the CYS gene is also located between AP1 and PHY-C in rice (Yan et al. 2003). The rearrangement of this region also involved the duplication of the PHY-C gene in barley relative to the single copy observed in T. monococcum.

Additional rearrangements differentiate barley and diploid wheat in the HvPCS gene region. The distance between 2 TmPCS genes in T. monococcum is approximately 15 kb, whereas the same genes are separated by at least 60 kb in barley. In addition, the cloned segments from both barley genes are more similar to the TmPCS2 gene than to the TmPCS1 pseudogene when the intron 2 region is compared. Therefore, it is possible that additional structural changes differentiate the PCS genes from wheat and barely.

Other confirmed gene rearrangements included the inversion of HvGENE2 relative to its flanking genes when compared with T. monococcum and rice, and the HvWG644 gene duplication in barley, which was not observed in wheat (Ramakrishna et al. 2002). In summary, the barley rearrangements included 6 to 7 (depending on the inclusion of the PCS putative changes) of the 11 genes analyzed within this region. In most of these cases, the T. monococcum structure seemed more similar to rice, suggesting that the barley VRN-H1 genomic region might be more unstable than the orthologous region in T. monococcum. A similar result was observed in the comparison of the VRN-2 region between these 2 species. Sequencing of the barley ‘Morex’ genomic region showed deletions of the HvZCCT, the P450, and the HvSEC14 genes relative to the orthologous T. monococcum sequence including only 9 genes (Yan et al. 2004b). Analysis of additional genomic regions will be necessary to confirm the frequent changes observed in the previous 2 regions in barley. It will be interesting to investigate whether these frequent structural changes are related to the intensive selection pressure of modern barley breeding.

The frequent changes observed at the microcolinearity level between T. monococcum and barley contrasts with the broad colinearity observed between the maps from these 2 organisms. One major translocation and 3 small inversions were the major differences detected between the barley and T. monococcum RFLP maps (Dubcovsky et al. 1996). The results from this present study indicate that some caution should be used when using the gene order in 1 of these 2 species to predict the gene order in the other.

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