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Molecular characterization of the duplicated meristem identity genes *HvAP1a* and *HvAP1b* in barley

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Abstract: The vernalization gene *VRN-1* has been identified as a MADS-box transcription factor orthologous to the meristem identity gene *APETALA1* (*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 *AP1* copy designated hereafter as *HvAP1a* and *HvAP1b*. A physical map of the *VRN-H1* region showed that the *HvAP1a* BACs were part of the *VRN-H1* region but that the *HvAP1b* BACs were not. Numerous structural changes were observed between the barley and wheat *VRN-1* physical maps. In a population segregating for *VRN-H1,* the *HvAP1a* gene cosegregated with growth habit, suggesting that *HvAP1a* is the barley vernalization gene *VRN-H1*. The other copy, *HvAP1b,* was mapped on the centromeric region of chromosome 1H, the chromosome where vernalization gene *VRN-H3* was previously mapped. We developed a mapping population segregating for *VRN-H3* and showed that 2 molecular makers flanking *HvAP1b* locus were not linked to growth habit. The *HvAP1b* copy has a complete deletion of the first 2 exons, suggesting that it is a truncated pseudogene and not a candidate for *VRN-H3*. In summary, this study contributed a detailed physical map of the barley *VRN-H1* region, showed several structural differences with the orthologous wheat region, and clarified the identity of the barley *VRN-H1* gene.

Key words: barley, vernalization, *Vrn-1*, physical map.

Résumé : Le gène de vernalisation *VRN*-1 a été identifié comme étant un facteur de transcription MADS-box orthologue au gène d'identité méristématique *APETALA1* (*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 *AP1*, appelés *HvAP1a* et *HvAP1b*, ont été identifiés. Une carte physique de la région VRN-H1 a révélé que les BAC du gène *HvAP1a* en faisaient partie, mais pas ceux de *HvAP1b*. De nombreux changements structuraux ont été observés entre les cartes physiques VRN-1 chez le blé et l'orge. Dans une population en ségrégation pour *VRN-H1*, le gène *HvAP1* coségrégait avec le type de croissance, ce qui suggère que *HvAP1a* serait le gène de vernalisation chez l'orge. L'autre copie, *HvAP1b*, a été localisé dans la région centromérique du chromosome 1H, le chromosome sur lequel avait été localisé le gène *VRN-H3*. Les auteurs ont montré que 2 marqueurs bordant *HvAP1b* n'étaient pas liés au type de croissance. La copie *HvAP1b* a une délétion complète des 2 premiers exons, ce qui suggère qu'il s'agirait d'un peudogène tronqué et non pas d'un candidat pour *VRN-H3*. En résumé, ce travail a contribué une carte physique détaillée de la région *VRN-H1*, montré plusieurs différences de structure par rapport à l'orthologue du blé et a clarifié l'identité du gène *VRN-H1* chez l'orge.

Mots clés : orge, vernalisation, *Vrn-1*, carte physique.

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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^m2* 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. 2004*b*). 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^m2* and *VRN-H2* were orthologous (Dubcovsky et al. 2005). The *VRN-A^m1* 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 polyploid 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. 2004*a*; Yan et al. 2003).

However, the relationship between the wheat and barley *VRN-1* genes 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^m1* 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 F_2 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 F_2 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 F_2 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-H1Vrn-H2vrn-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 *Hin*dIII 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-1* 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* population 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' BAC 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 *Hin*dIII 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 315I20). Hybridization of the Southern blots from the *Hin*dIII fingerprints using 143P9 and 315I20 BAC end 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-A^m1* 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 *Hin*dIII 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 *Eco*RI-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 *Hha*I, *Hin*dIII, and *Sac*I 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 (*CYS,* AY244510) gene was present between *AP1* 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, 731I10, 673M15, and 749N15). These BACs formed a single contig, indicating that *HvAGLG1* is a single copy gene in barley as it is in diploid wheat. Sequencing of a 1096-bp fragment covering the *HvAGLG1* 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 *HvCYB5* contigs, suggesting the presence of large repetitive regions flanking this gene as was also observed in *T. monococcum* (Yan et al. 2003).

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

Primer names	Sequences (5′-3′)	Target genes	BAC clones[a]
TmAGLG1-F1	AAGACCTTGAGGCAAATAAG	*HvAGLG1*	606J6
TmAGLG1-R1	GTTTTTGAGATAGATAATCGC	*HvAGLG1*	606J6
TmPCS1-F1	ATGCTGCTTCCTCCTTCCAG	*HvPCS1*	115N22
TmPCS1-R1	GCAGGTTACATTCCATGACA	*HvPCS1*	115N22
TmAP1-Ex4F1	TCTCATGGGAGAGGATCTT	*HvAP1a-b*	631P8 & 315I20
TmAP1-Ex7R1	GGGAGCATCCCTCATCATGAA	*HvAP1a-b*	631P8 & 315I20
HvAP1-Ex1-F	CAACCACCTGACAGCCATGG	*HvAP1a*	631P8
HvAP1-Ex1-R	CATGACTCGGTGGAGAACTC	*HvAP1a*	631P8
HvAP1-Ex2-F	TGGACAAAATTCTTGAACGG	*HvAP1a*	631P8
HvAP1-Ex2-R	ACCATGCCTAAGCATTCATC	*HvAP1a*	631P8
HvAP1B-F	TCGGAAATGAACAAAAGAGACTT	*HvAP1b*	315I20 & 'Dicktoo' DNA
HvAP1B-R	GAAAACTTGAACAACACCAGAACC	*HvAP1b*	315I20 & 'Dicktoo' DNA

[a]The 'Morex' barley BAC or the barley variety used as a DNA source for the amplification is listed in the last column.

Fig. 1. Comparative physical maps of the *VRN-1* region between *T. monococcum* and barley. Names of gene markers are as reported by Yan et al. (2003). *CYS* indicates the *Cysteine proteinase* gene detected in diploid wheat but not in barley. (A) Physical map of the *VRN-1* region on chromosome 5A^m of *T. monococcum* (Yan et al. 2003). (B) Physical map of the colinear *VRN-1* region in chromosome 5H of barley. Order of bacterial artificial chromosomes (BAC) clones from left to right is: 75D16, 666E12, 55G5, 635P2, 94K12, 491K18, 161J1, 423G22, 249E2, 312K18, 637F7, 422A20, 115N22, 715N9, 604I5, 398F16, 420B3, 221P8, 606J6, 731I10, 673M15, 749N15, 31B22, 597D19, 317M7, 631P8, 460O15, and 472K16. Bacterial artificial chromosomes in red were completely or partially sequenced. *Hvgene4* is the same as *wg644*. (C) Genetic location on *HvAP1a* on barley chromosome 5H. (D) BAC clones 143P9 and 315I20 including the *HvAP1b* gene. (E) Genetic location of *HvAP1b* on barley chromosome 1H.
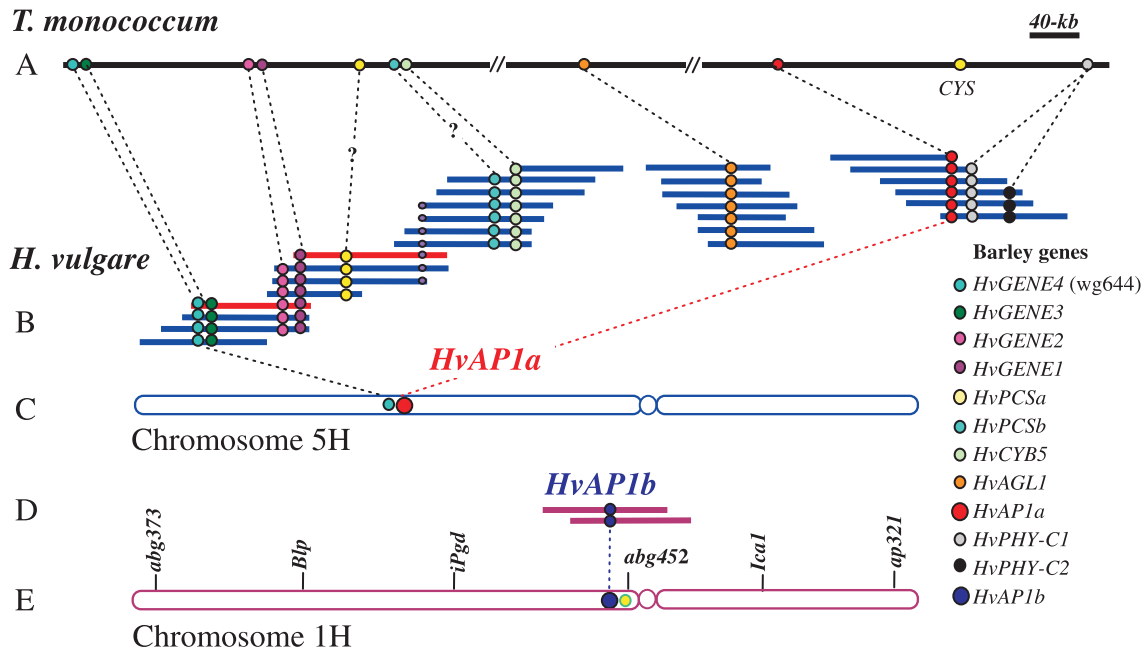

The probe for the *Cytochrome B5* gene (*TmCYB5*, AY188332) detected a single copy gene in diploid wheat and also in barley. The 7 'Morex' BAC clones containing *HvCYB5* (249E2, 312K18, 637F7, 422A20, 115N22, 715N9, and 604I5) formed a single contig connected to previously sequenced BAC 635P2 (Dubcovsky 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 bar-

ley, 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-

Fig. 2. Identification of duplicated *HvAP1* genes in barley. (A) *Hin*dIII fingerprints of 2 groups of *HvAP1* BAC clones. 1–3. *HvAP1a* BAC clones 31B22, 597D19, 472K16; 4–5. *HvAP1b* BAC clones 143P9 and 315I20. (B–C) Hybridization patterns of the same BACs with the (B) TmAP1 Exons 4–7 probe. (C) HvAP1a Exon 2 probe. Note the absence of hybridization of the last probe with the *HvAP1b* BAC clones.
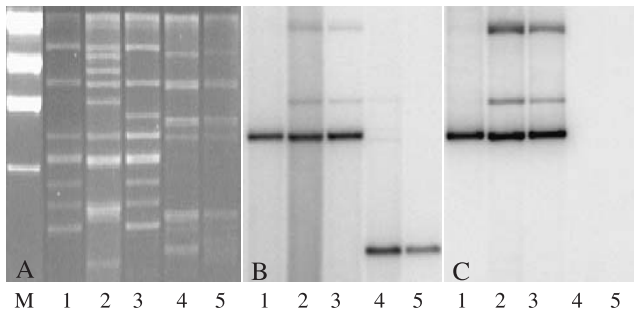
tity with the *TmPCS2* gene (97% and 96%) than with the *TmPCS1* pseudogene (86% and 86%). A similar result was obtained when the 2 barley copies were compared with the *SbPCS2* (84%–85%) or *SbPCS1* gene (82%–81%). Therefore, it was not possible to establish a clear correspondence between the 2 barley *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 *Hin*dIII-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; χ^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-1* 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 phylogenic analysis of MADS-box genes, which showed that BM5 was the barley gene closest to wheat *VRN-1* (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' (HVU249144, Schmitz et al. 2000), suggesting that the BM5 cDNA corresponds to the *HvAP1a* locus.

HvAP1b: The *HvAP1b* sequence was determined from 'Morex' BAC 315I20 (GenBank AY887088). 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 doted 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 *).
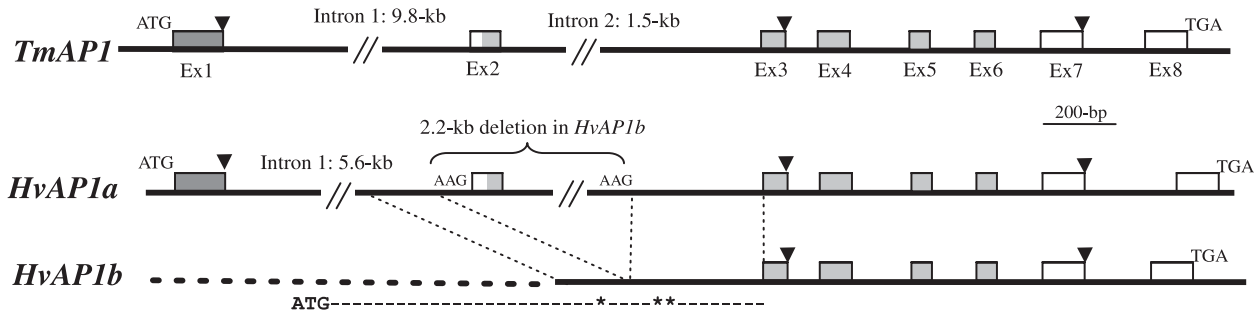
quences revealed a 2.2-kb deletion 400-bp upstream from the start of exon 3 (Fig. 3). This deletion includes exon 2 and the last 73-bp of intron 1 and is flanked on the 5′ side by a 200-bp region of intron 1 that shows good identity between *HvAP1a* and *HvAP1b* (95%). The 2.2-kb deletion is flanked 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

Fig. 4. Screening blots of 'Morex' and *H. spontaneum* DNAs digested with (1–2) *Ase*I, (3–4) *Bam*HI, (5–6) *Eco*RI, (7–8) *Eco*RV, (9–10) *Hin*dIII, (11–12) *Sac*I, and (13–14) *Xba*I. 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.
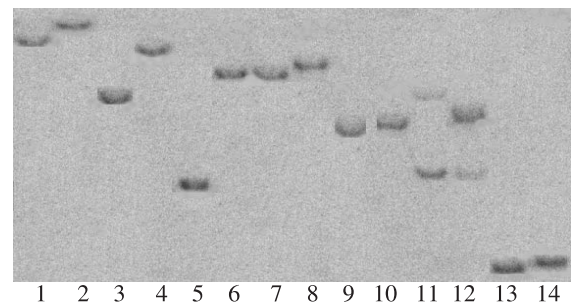
with the TmAP1-Exons 4–7 probe but only a single fragment when hybridized with the probe from exon 2 and the flanking introns. These results indicated that *HvAP1b* was also truncated in BG213 and, together with the previous genetic analysis, confirmed that *HvAP1b* was not a good candidate gene for *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. 2004*a*). 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

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 the 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-1* genomic regions in wheat and barley

An additional contribution of this study is the detailed analysis of the microcolinearity of the *VRN-1* 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-1* 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. 2004*b*). 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 organism. 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.

Acknowledgements

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