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ORIGINAL PAPER

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Positional relationships between photoperiod response QTL and photoreceptor and vernalization genes in barley

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Abstract Winterhardiness has three primary components: photoperiod (day length) sensitivity, vernalization response, and low temperature tolerance. Photoperiod and vernalization regulate the vegetative to reproductive phase transition, and photoperiod regulates expression of key vernalization genes. Using two barley mapping populations, we mapped six individual photoperiod response QTL and determined their positional relationship to the phytochrome and cryptochrome photoreceptor gene families and the vernalization regulatory genes *HvBM5A*, *ZCCT-H*, and *HvVRT-2*. Of the six photoreceptors mapped in the current study (*HvPhyA* and *HvPhyB* to 4HS, *HvPhyC* to 5HL, *HvCry1a* and *HvCry2* to 6HS, and *HvCry1b* to 2HL), only *HvPhyC* coincided with a photoperiod response QTL. We recently mapped the candidate genes for the 5HL *VRN-H1* (*HvBM5A*) and 4HL *VRN-H2* (*ZCCT-H*) loci, and in this study, we mapped *HvVRT-2*, the barley *TaVRT-2* ortholog (a wheat flowering repressor regulated by vernalization and photoperiod) to 7HS. Each of these three vernalization genes is located in chromosome regions determining small photoperiod response QTL effects. *HvBM5A* and *HvPhyC* are closely linked on 5HL and therefore are currently both positional candidates for the same photoperiod effect. The coincidence of photoperiod-responsive vernalization genes with photoperiod QTL suggests vernalization genes should also be considered candidates for photoperiod effects.

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Introduction

Winterhardiness in temperate cereals consists of three primary components—low temperature (LT) tolerance capacity, photoperiod (PPD) (or day length) sensitivity, and vernalization (VRN) response. In cereals, the vegetative growth phase is associated with maximum LT tolerance and the transition to a reproductive state is associated with a loss in LT tolerance capacity (Fowler et al. 2001; Limin and Fowler 2002). In responsive genotypes the VRN and PPD regulatory inputs prevent premature meristem transition to a reproductive state until winter conditions have passed, and thus minimize the risk of freezing injury or death.

Barley (*Hordeum vulgare* subsp. *vulgare*) is a long-day (LD) plant requiring a critical minimum day length to flower (Vince-Prue 1975). The barley germplasm can be broadly divided into winter, facultative, and spring growth habits. In general, winter varieties are PPD sensitive while spring varieties are insensitive to short day PPD. The "facultative" habit may or may not be sensitive to PPD. In PPD-sensitive genotypes grown under non-vernalizing short-day (SD) regimes, production of an inflorescence is delayed and plants keep producing leaves from the vegetatively fated meristem until a genetically predetermined maximum leaf number is attained, at which point the meristem converts to an inflorescence fate (Mahfoozi et al. 2001). In contrast, LD growth conditions accelerate the conversion to an inflorescence meristem and reduce the total leaf number formed. Major loci affecting PPD response have been mapped in barley, with the *Ppd-H1* locus (2HS) controlling flowering under LD but having no effect under

SD, while *Ppd-H2* (1HL) controls flowering only under SD (Laurie et al. 1995). Recently, Turner et al. (2005) have reported cloning of the candidate gene for the LD-responsive *Ppd-H1* locus.

Plant growth and development, including photoperiod-dependent flowering, is regulated by the products of the red/far-red light phytochrome and the blue/UV-A light cryptochrome photoreceptor gene families (Cashmore et al. 1999; Lin 2000; Quail 2002). Both gene classes consist of a small family of related chromoproteins and in the Arabidopsis (*Arabidopsis thaliana* L.) genome five phytochromes (*PhyA* through *PhyE*) and two cryptochromes (*Cry1* and *Cry2*) are encoded. All five *Arabidopsis* phytochromes are involved in regulation of flowering time (Lin 2000; Monte et al. 2003) and mutations in *PhyA* and *Cry2* genes have marked effects on the photoperiodic control of *Arabidopsis* flowering (Mockler et al. 2003; Sullivan and Deng 2003). Hanumappa et al. (1999) demonstrated a chemically mutagenized PPD-insensitive barley line, which flowered early, contained a light-labile PhyB and suggested that in barley both PhyA and PhyB are involved in the normal regulation of flowering. These results suggest that photoreceptor genes are involved in PPD control in other plant systems (Mouradov et al. 2002) and are possible candidates for PPD effects in barley.

Using RFLP analysis on barley-wheat substitution lines, Biyashev et al. (1997) mapped five phytochrome loci to barley chromosomes 5H, 4H, and 1H (loci *phy1*, *phy2*, *phy3*, respectively), and a *phy4* locus as duplicate loci on chromosomes 2H and 5H. These *phy* loci were arbitrarily designated and it is unknown which phytochrome gene form each encodes and how their precise map locations correspond to PPD response loci. Also, only three Phy genes (*PhyA*, *PhyB,* and *PhyC*) have been identified in monocots (Mathews and Sharrock 1996), suggesting some of the *phy* loci could be pseudogenes. Little information is available on cryptochrome genes in barley, but based on the conserved Cry1 and Cry2 flavin-binding domain sequences in *Arabidopsis* and cloning of partial gene fragments, Perrotta et al. (2001) demonstrated that barley has at least three cryptochrome genes designated *Cry1a*, *Cry1b*, and *Cry2*; their genetic map location is unknown.

In temperate cereals, the floral meristem identity gene *HvBM5A* and its orthologs, members of the AP1 transcription factor family, appear to function as a central control point through which the environmental PPD and VRN signals regulate the transition from a vegetative to an inflorescence meristem by directly affecting *HvBM5A* expression. The expression of *HvBM5A* and its wheat orthologs correlates with both genotypic VRN requirement and PPD sensitivity (Danyluk et al. 2003; Murai et al. 2003; Trevaskis et al. 2003; von Zitzewitz et al. 2005; Yan et al. 2003). The barley *ZCCT-H* gene is orthologous to the diploid wheat *Triticum monococcum* *VRN-A*^{m}*2* locus *ZCCT1* gene (Dubcovsky et al. 2005; Yan et al. 2004). *ZCCT1* is down regulated by vernalization, and is implicated as a repressor of the wheat *HvBM5A* ortholog *TmAP1* (Yan et al. 2004). The effect of PPD on *ZCCT-H* expression is unknown, although in a facultative × winter barley mapping population, the *VRN-H2* locus was the sole vernalization response determinant and its effect was modulated by photoperiod after vernalization requirement fulfillment (Karsai et al. 2005). HvVRT-2, the barley ortholog to wheat TaVRT-2, a putative flowering repressor MADS box protein regulated by VRN and PPD, shows higher expression under SD (vs. LD) in PPD-sensitive barley (Kane et al. 2005).

To investigate the possible role(s) of the photoreceptor and photoperiod-responsive vernalization genes in barley photoperiod response, our objectives were to determine (1) the genetic map location of the barley photoreceptor genes and the unmapped vernalization gene *HvVRT-2*, (2) the degree of the allelic variation in the coding region sequences, and (3) whether barley photoreceptor and/or vernalization genes were positional candidates of photoperiod response QTL.

Material and methods

Plant material

Hordeum vulgare subsp. *vulgare* varieties Dicktoo, Morex, and Kompolti korai were used for allele sequencing and genetic mapping. The Hungarian cultivar Kompolti korai is a frost tolerant winter genotype with a strong VRN requirement, but is less sensitive to SD PPD than Dicktoo (Karsai et al. 2005). The US cultivar Dicktoo is a frost tolerant facultative winter genotype that lacks a strong VRN response but is SD PPD sensitive. The US cultivar Morex is a spring genotype with poor frost tolerance, no vernalization response, and is insensitive to SD photoperiods (Hayes et al. 1997). Two doubled haploid (DH) mapping populations were utilized for linkage mapping and QTL analyses—the 92 DH line Dicktoo × Morex (D×M) population (Hayes et al. 1997) and the 95 DH line Dicktoo × Kompolti korai (D×K) population (Karsai et al. 2005).

Isolation of barley photoreceptor and HvVRT-2 alleles

Publicly available full-length rice (*Oryza sativa* L.) and partial barley phytochrome and cryptochrome gene sequences present in GenBank (http://www.ncbi.nlm.nih.gov/) were used for EST collection database analysis to identify barley clones harboring the respective genes. Representative cDNA clones (Table 1) were obtained and sequenced. For the six photoreceptor genes, the determined EST sequence information was used to amplify, clone, and sequence intron-containing alleles from genomic DNA of Dicktoo, Morex, and Kompolti korai. For 5′ truncated EST clones, raw GenBank sequence data of barley and wheat EST alleles containing 5′ untranslated region (UTR) sequence information

was directly utilized to design primers to obtain the missing 5′ region of the respective genes. Dicktoo and Morex ESTs harboring the *HvVRT-2* barley ortholog to *TaVRT-2* (DQ022679) (Kane et al. 2005) were identified and directly sequenced. The Morex BAC clone 631P8, which contains the *VRN-H1* candidate gene *HvBM5A* (von Zitzewitz et al. 2005) and two *PhyC*-hybridizing fragments (Yan et al. 2005) was sequenced and annotated in the laboratory of Dr. Olin D. Anderson (USDA-ARS, Albany, CA). Dicktoo alleles to the two *HvPhyC* sequence remnants present in BAC 631P8 were amplified, cloned, and sequenced from genomic DNA. For each gene and allele isolated via PCR, cloned amplicons of at least two independent PCR reactions were sequenced to confirm PCR-based nucleotide substitutions had not occurred. Sequence data from this article has been deposited with GenBank and accession numbers are given in Table 1.

Linkage mapping and photoperiod response QTL analysis

The photoreceptor genes and *HvVRT-2* were mapped by allele polymorphisms in the D×K and/or D×M linkage mapping populations. When no polymorphisms were detected in either population, the locus was assigned to chromosome arms using barley-wheat disomic substitution lines (Islam et al. 1981). Mapping strategies and primer sequences relative to each gene and population are provided in Table 2. JoinMap 3.0 and the Kosambi mapping function were used for linkage map construction (Van Ooijen and Voorrips 2001). PPD response data were calculated as in Karsai et al. (2005) as the flowering time difference (FT8-16 and FT8-24) for plants grown under vernalized SD (8 h light/24 h) versus vernalized LD (16 h or 24 h light/24 h). The flowering time of the D×K population lines and parents were characterized in a phytotron (Martonvásár, Hungary) as described in Karsai et al. (2005). Flowering time data for D×M population and the parents are available at GrainGenes (http://www.wheat.pw.usda.gov/ggpages/DxM/). The QTL analyses were performed with QTL Cartographer Version 2.5 (Wang et al. 2005) using the standard Composite Interval Mapping (CIM) model. A forward-selection backward-elimination stepwise regression procedure was used to identify control markers for CIM. The analyses were performed with a 10 cM window size and a 2 cM walk speed. The threshold LOD value was set by 1,000 permutations and a Type I error of 5%.

Table 1 Accessions of determined barley photoreceptor and *HvVRT-2* allele sequences

Gene	Allele[a]	Accession	Determined region	Size (bp)
HvPhyA	Dicktoo	DQ201139	Full gene	6,652
HvPhyA	Morex	DQ201140	Full gene	6,652
HvPhyA	Kompolti korai	DQ201141	Full gene	6,659
HvPhyA	Morex	DQ201158	EST HVSMEc0008C13f	2,485
HvPhyB	Dicktoo	DQ201142	Partial gene	2,271
HvPhyB	Morex	DQ201143	Partial gene	2,271
HvPhyB	Kompolti korai	DQ201144	Partial gene	2,271
HvPhyB	H602 (*spontaneum*)	DQ201159	EST Bah21c13	2,633
HvPhyC	Dicktoo	DQ201145	Full gene	4,914
HvPhyC	Morex	DQ238106	Full gene	4,938
HvPhyC	Kompolti korai	DQ201146	Full gene	4,914
HvPhyC	Dicktoo	DQ201160	EST UCRHV18_03dh02	370
HvPhyC	Morex	DQ201161	EST HVSMEg0011G23f	1,116
HvPhyCΨ1a	Dicktoo	DQ201147	Full pseudo gene	1,112
HvPhyCΨ1b	Dicktoo	DQ201148	Full pseudo gene	1,147
HvCry1a	Dicktoo	DQ201149	Full gene	3,808
HvCry1a	Morex	DQ201150	Full gene	3,808
HvCry1a	Kompolti korai	DQ201151	Full gene	3,808
HvCry1a	Morex	DQ201162	EST HVSMEg0015F23f	1,415
HvCry1a	CI16155	DQ201163	EST HV_CEa0001F24f	1,400
HvCry1b	Dicktoo	DQ201152	Full gene	4,753
HvCry1b	Morex	DQ201153	Full gene	4,753
HvCry1b	Kompolti korai	DQ201154	Full gene	4,753
HvCry1b	Morex	DQ201164	EST HVSMEm0022D07f	1,150
HvCry1b	CI16151	DQ201165	EST HV_CEb0010E01f	1,736
HvCry2	Dicktoo	DQ201155	Partial gene	2,662
HvCry2	Morex	DQ201156	Partial gene	2,735
HvCry2	Kompolti korai	DQ201157	Partial gene	2,686
HvVRT-2	Dicktoo	DQ201166	cDNA	1,047
HvVRT-2	Dicktoo	DQ201167	EST UCRHV18_07ce10	970
HvVRT-2	Morex	DQ201168	EST HVSMEn0013H24f	1,402
HvBM5A, HvPhyCΨ1a, HvPhyCΨ1b, HvKCO1	Morex	DQ249273	BAC 631P8	101,158

[a] *Hordeum vulgare* subsp. *vulgare* genotypes or subsp. *spontaneum* genotype (indicated)

Table 2 Populations, primers and methods used for gene mapping

Gene	Mapping population[a]	Primer F (5′ → 3′)	Primer R (5′ → 3′)	Mapping method[b]
HvPhyA	D×K	HvPhyA.17 (cacccctgctagaaatatagag)	HvPhyA.16 (gtcactagattcttcaaactcagc)	CAPS (*SfaNI*)
HvPhyA[c]	D×K	HvPhyA.15 (gggtaaaggaaggttgtgtgg)	HvPhyA.08 (ttgcccaagtacatatcacagc)	InDel
HvPhyB	D×K	HvPhyB.02 (agggagatggttaggttgatcg)	HvPhyB.20 (atgagaaacaaacccataaagcatc)	CAPS (*BccI*)
HvPhyC	D×K	HvPhyC.19 (ggctatgacagggtgatgg)	HvPhyC.08 (gctcgtgtgatggcaaacc)	CAPS (*BsphI*)
HvPhyC	D×M	HvPhyC.05 (atgagcggcacggtacagta)	HvPhyC.06 (gctaagctcctcctcaacca)	InDel
HvPhyCΨ1b	D×M	HvPhyCΨ1.03 (ggcagcagcagttcaaggaa)	HvPhyCΨ1.04 (cacaaggagtcgcagatatgg)	InDel
HvCry1a	D×K	HvCry1a.19 (gaacaccaccatcccacctc)	HvCry1a.18 (acacgtacgctggcaccac)	CAPS (*HpyCH4V*)
HvCry1b	Betzes	HvCry1b.11 (gtgttggtggaattggaacttg)	HvCry1b.05 (ttctgattgcacaaaaccgtcc)	+/−
HvCry2	D×K	HvCry2.07 (ctgtgatcaaaaagtgcccactg)	HvCry2.12 (ctcaccagacatcttgcagtg)	InDel
HvCry2	D×M	HvCry2.07 (ctgtgatcaaaaagtgcccactg)	HvCry2.10 (caccatatcgaccaacaagaatg)	InDel
HvBM5A	D×K	HvBM5.82 (atatctactccagcctagggtac)	HvBM5.83 (cgcgaatctcccccatattgc)	InDel
HvBM5A	D×M	HvBM5.35 (gaaaacttgaacaacaccagaacc)	HvBM5.43 (ttctgcataagagtagcgctcat)	InDel
ZCCT-Ha/b	D×K	ZCCT.06 (cctagttaaaacatatatccatagagc)	ZCCT.07 (gatcgttgcgttgctaatagtg)	+/−
HvVRT-2	D×M	HvVRT-2.01 (gagttgcagcagatgg)	HvVRT-2.06 (caggtcactaatttgttgcatga)	CAPS (*MspI*)

[a]Barley mapping population used to determine map or chromosome location: Dicktoo × Kompolti korai (D×K), Dicktoo × Morex (D×M), and barley-wheat substitution lines (Betzes)
[b]Mapping method used to determine map or chromosome location: cleaved amplified polymorphic sequences (CAPS) (restriction endonuclease indicated), sequence insertion/deletion event (InDel), PCR fragment presence/absence (+/−)
[c]*HvPhyA* was mapped in D×K population based on both 5′ and 3′ UTR polymorphisms

Results

Allelic variation of barley phytochrome genes

We determined 6.6 kb of genomic sequence for the full-length barley *HvPhyA* gene (Table 1), which contains three introns in the coding region and one each in the 5′ and the 3′ UTRs. The Dicktoo and Morex *HvPhyA* alleles are 100% identical, while Kompolti korai harbors 35 SNPs and a 7 bp insertion relative to the other two cultivars. Along with the 7 bp insertion, the majority of the Kompolti korai SNPs are located in the introns and UTRs. Of the seven coding region SNPs, only three lead to amino acid substitutions in the predicted polypeptides. We sequenced a 5′-truncated *HvPhyB* EST clone that began within the first exon and isolated 2.3 kb of allelic genomic sequence corresponding to portions of exons 1 and exon 2 and the complete intervening intron (Table 1). The Dicktoo and Morex *HvPhyB* alleles are 100% identical, while Kompolti korai only differed by a single SNP in intron 1. We determined the genomic sequence (4.9 kb) for the full-length *HvPhyC* gene alleles from the three genotypes (Table 1). Seven SNPs are present between Dicktoo, Morex, and Kompolti korai, and additionally, Morex contains a 24 bp (8 aa) insertion relative to Dicktoo and Kompolti korai in exon 4. Besides the 8 aa Morex insertion, two Dicktoo SNPs lead to amino acid substitutions relative to the other two alleles.

In Yan et al. (2005), we noted the presence of two *HvPhyC*-hybridizing bands in Morex BAC clones harboring *HvBM5A*, suggesting the possible presence of duplicated *HvPhyC* genes. We sequenced one of these BACs (Table 1), and found that the 101.1 kb Morex BAC clone 631P8 (DQ249273) contains a single truncated *HvPhyC* pseudogene divided into two segments (designated *HvPhyCΨ1a* and *HvPhyCΨ1b*) via a 17 kb insertion. *HvPhyCΨ1a* lies 10 kb downstream of *HvBM5A* (AY750995) and relative to *HvPhyC*, contains a partial intron 2 (279 bp) and partial exon 3 (180 bp) fragment. *HvPhyCΨ1b* sits 17 kb downstream of *HvPhyCΨ1a* and contains the remainder of exon 3 (114 bp) and a portion of intron 3 (129 bp). Using the non-*PhyC*-based sequence flanking the Morex *HvPhyCΨ1a* and *HvPhyCΨ1b* segments, we amplified the corresponding Dicktoo genomic regions (1.1 kb each) (Table 1). We confirmed Dicktoo harbors the same two pseudogene fragments and relative to Morex, SNPs are present both within and flanking the pseudogene fragments, as well as a 68 bp insertion in the *HvPhyCΨ1b* flanking segment. Besides *HvBM5A* and the *HvPhyCΨ1* pseudogene, BAC clone 631P8 also contains the outward-rectifying potassium channel gene *HvKCO1* (AY770627) and multiple repetitive DNA elements.

Allelic variation of barley cryptochrome genes

Amplification and analysis of the full-length 3.8 kb *HvCry1a* sequence revealed the Dicktoo and Morex alleles are 100% identical and only one 3′ UTR-localized SNP in Kompolti korai (vs. Dicktoo and Morex). The full-length 4.7 kb *HvCry1b* alleles are 100% identical among the three cultivars. The 2.7 kb sequence of *HvCry2* encompasses the 3′ portion of the gene from exon 4 (partial) through the 3′ UTR, with the intervening introns. The Dicktoo and Morex alleles are identical except for a repeat length in intron 5, while relative to these two cultivars, the Kompolti korai allele also differs in the intron 5 repeat length, has a MITE (ID No. TREP30) in intron 5, and 11 SNPs that are mainly localized to the introns; only a single Kompolti korai *HvCry2* SNP results in an amino acid substitution.

Allelic variation of the barley HvVRT-2 gene

A full-length *HvVRT-2* Morex EST and an incompletely 5′ processed Dicktoo EST were identified; the remainder of the Dicktoo cDNA 5′ end was obtained via RT-PCR (Table 1). Comparison of the alleles revealed only a single SNP in the fifth exon that was silent at the amino acid level, indicating Dicktoo and Morex encode identical HvVRT-2 polypeptides.

Map locations of barley photoreceptor genes and HvVRT-2

In the Dicktoo × Morex (D×M) mapping population, we could only map the *HvPhyC* and *HvCry2* photoreceptors, as well as *HvVRT-2*, due to a lack of polymorphisms between the remaining Dicktoo and Morex photoreceptor alleles (Table 2). In the Dicktoo × Kompolti korai (D×K) population, we mapped all the functional barley phytochrome and cryptochrome genes except for *HvCry1b* (Table 2). Based on these two populations, *HvPhyA* and *HvPhyB* are present on 4HS, *HvPhyC* is on 5HL, and *HvCry1a* and *HvCry2* are on 6HS (Fig. 1); *HvPhyC* and *HvCry2* were mapped in both populations. In the D×K population, *HvBM5A* and *HvPhyC* co-segregate and we could not establish the gene order on chromosome 5HL. In contrast, a single recombination event separates the two genes in the D×M population. In the D×M population, *HvBM5A*, *HvPhyCΨ1b*, and Morex BAC clone 635P2 co-segregate, also verifying that the *HvPhyC* gene and *HvPhyCΨ1* pseudogene are distinct loci. Examination of these three markers in the extended D×M population (an additional 144 DH lines) did not reveal any other recombination events separating these three loci (not shown). *HvVRT-2* was mapped to chromosome 7HS in the D×M population (Fig. 1). While the Dicktoo, Morex and Kompolti korai alleles were not polymorphic, *HvCry1b* was assigned to chromosome 2HL via the barley–wheat substitution lines (Table 2).

Positional relationships between PPD response QTL and photoreceptor and VRN genes

Using both a facultative × winter (D×K) and a facultative × spring (D×M) barley mapping population, we mapped six individual PPD response QTL (Table 3). In the D×K population, QTL analyses revealed PPD effects on chromosome 3HL (LOD 3.6–4.2) and 4HL (LOD 3.1–3.5), with Dicktoo contributing the higher PPD response allele (Table 3). In the D×M population, we found QTL effects on chromosomes 1HL (LOD 7.8–11.6), 2HS (LOD 4.6–8.6), and 7HS (LOD 3.9–5.8), with Dicktoo again contributing the higher PPD response allele (Table 3). The D×M population FT8-24 data set also showed a significant QTL effect on chromosome 5HL, with Morex contributing the higher PPD response allele (Table 3). Of the six photoreceptors (*HvPhyA-C*, *HvCry1a/b*, *HvCry2*), only *HvPhyC* coincided with a photoperiod response QTL position (5HL). In contrast, the map positions of all three VRN regulatory genes (*HvBM5A*, *ZCCT-H*, and *HvVRT-2*) are located in chromosome regions determining small photoperiod response QTL effects (5HL, 4HL, and 7HS, respectively) (Table 3).

Discussion

While multiple gene classes can be considered candidates for PPD response QTL, in the current study, our primary goal was to map and determine the location of the barley photoreceptor genes relative to PPD response QTL and therefore, which of the photoreceptor genes should be considered or excluded as candidates for PPD QTL effects. The recent cloning of the *Ppd-H1* effecter gene, a member of the CCT-domain pseudo-response regulator (PRR) family, demonstrates that additional gene family classes also need to be investigated relative to PPD QTL effects (Turner et al. 2005). The sequences of the Dicktoo and Morex photoreceptor alleles were highly monomorphic and we mapped only two photoreceptors in the D×M population. While the alleles from Kompolti korai, the other parent of the D×K population and of a different geographic origin than Dicktoo (Hungarian vs. North American), were in general more diverged compared to the North American alleles, the detected polymorphisms were mainly located in non-coding regions and the few coding region polymorphisms were typically silent at the polypeptide level. We mapped five photoreceptor genes in the D×K population. In sum, the photoreceptor genes from two different geographic origins and/or PPD sensitivity encoded highly conserved polypeptides, likely due to their fundamental biological function. Comparison of the hexaploid wheat A, B, and D genome *PhyC* genes also found that the homoeologous *TaPhyC* gene sequences are highly conserved (Devos et al. 2005).

Previously, Biyashev et al. (1997) determined that five undefined barley loci hybridizing to an oat *PhyA* cDNA were present individually on chromosomes 1H, 2H, 4H, and with two on 5H. Here we have shown functional *HvPhyA* and *HvPhyB* genes are each located on chromosome 4HS and confirmed that a functional *HvPhyC* gene is located on chromosome 5HL. These results are in agreement with the rice *PhyA* and *PhyB* location in chromosome 3 (Takano et al. 2005)—barley chromosome 4H and rice chromosome 3 are homoeologous—and the wheat *PhyC* location on chromosome 5A (Beales et al. 2005; Yan et al. 2003). The other phytochrome loci reported by Biyashev et al. (1997) on chromosomes 1HL, 2H, and 5HS likely represent hybridization to pseudogenes, and in combination with the recent cloning of the *Ppd-H1* gene (Turner et al. 2005), the location of these three loci do

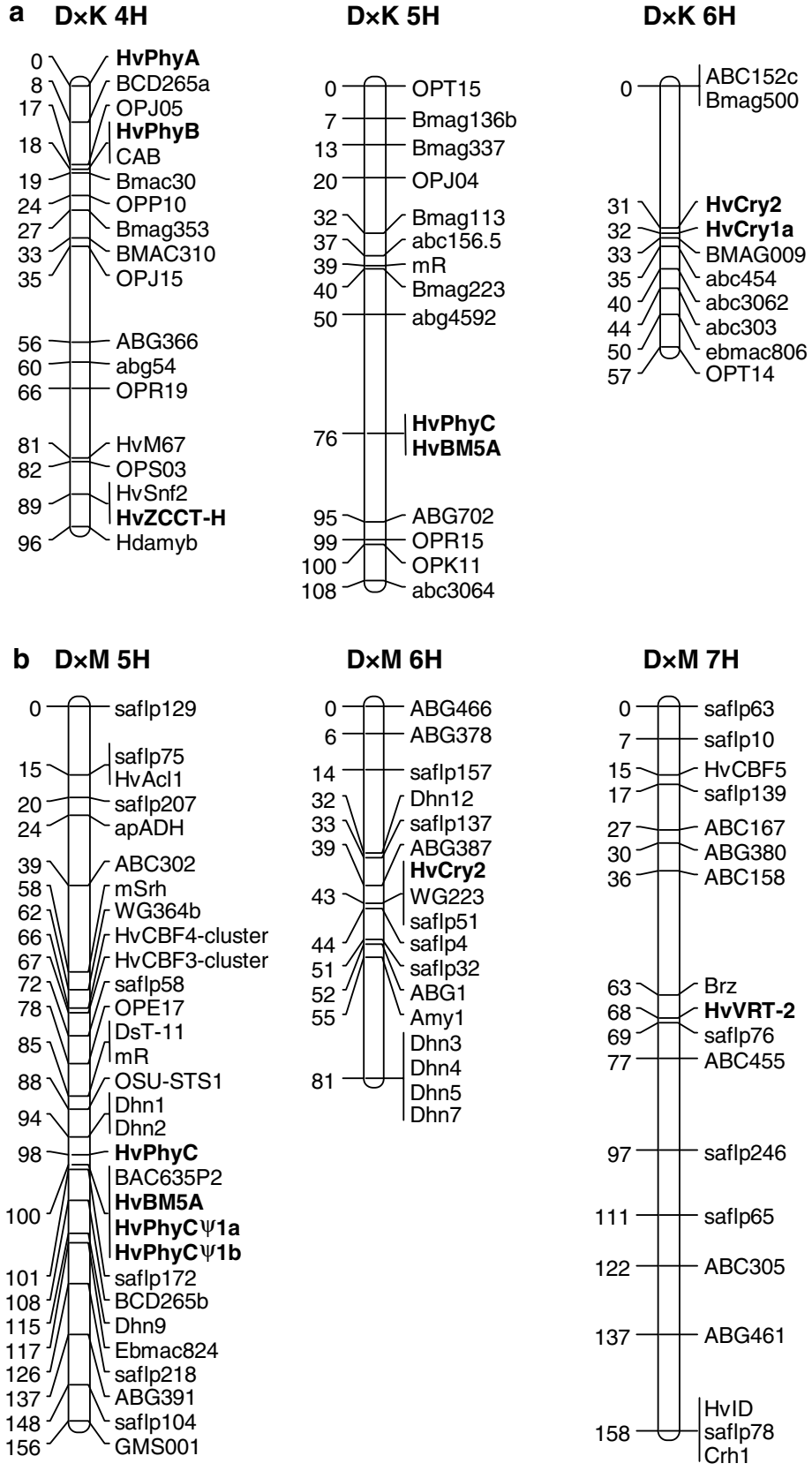

Fig. 1 Linkage maps of **a** chromosomes 4H, 5H, and 6H of the Dicktoo × Kompolti korai; and **b** chromosomes 5H, 6H, and 7H of the Dicktoo × Morex barley mapping populations. Photoreceptor and vernalization genes are in **bold face**

Table 3 PPD response QTL peak summary

Population[a]	Trait[b]	Chromosome	BIN[c]	QTL Position (cM)	2-LOD Interval (cM)	LOD	Marker [d]	Additive Effect	R^2
D×K	FT8-16	3H-L	12	55	47–67	4.2	*OPO20*	16.3	0.15
D×K	FT8-16	4H-L	12	93	82–95	3.1	***ZCCT-H***	14.0	0.11
D×K	FT8-24	3H-L	12	57	46–68	3.6	*OPO20*	15.1	0.13
D×K	FT8-24	4H-L	12	89	82–93	3.5	***ZCCT-H***	14.4	0.13
D×M	FT8-16	1H-L	11	86	84–94	11.6	*Saflp164*	8.8	0.38
D×M	FT8-16	2H-S	3	12	3–23	4.6	*ABG8*	4.7	0.11
D×M	FT8-16	7H-S	7	81	71–89	5.8	***HvVRT-2***	5.9	0.17
D×M	FT8-24	1H-L	12	107	86–118	7.8	*BCD265c*	7.7	0.14
D×M	FT8-24	2H-S	4	25	17–29	8.6	*PSB993*	9.6	0.14
D×M	FT8-24	5H-L	11	98	95–101	7.8	***HvPhyC/HvBM5A***	-11.9	0.12
D×M	FT8-24	7H-S	7	75	71–84	3.9	***HvVRT-2***	7.1	0.07

[a]Mapping populations used to determine PPD response QTL effects are Dicktoo × Kompolti korai (D×K) and Dicktoo × Morex (D×M)
[b]PPD response data was calculated as the difference of the flowering time under vernalized short day (8 h light/24 h) and vernalized long day (16 h or 24 h light/24 h) grown plants (FT8-16 and FT8-24)
[c]QTL peaks assigned to BINs using the BIN map concept of Kleinhofs and Graner (2001)
[d]Nearest marker or relevant candidate gene (bold) to the determined PPD response QTL

not correspond to reported PPD QTL effects. Analysis of all barley EST sequence information in GenBank demonstrated that the multiples ESTs to each of the three phytochromes appear to represent transcripts from a single gene each. Thus, the presence of more than one functional copy of *PhyA*, *PhyB*, or *PhyC* was not indicated by the current EST data. Verification whether these additional phytochrome-hybridizing loci are detecting psuedogenes or functional genes will, however, require isolation and sequencing of the corresponding genomic regions. While we did not attempt to systematically verify all three presumptive phytochrome pseudogene loci as such, sequencing of *HvBM5A*-harboring Morex BAC clone 631P8 confirmed the presence of a phytochrome C pseudogene on chromosome 5HL. As the functional *HvPhyC* gene (mapped with a 3′ gene segment absent from the pseudogene) and the *HvPhyCΨ1* pseudogene map to distinct 5HL locations, neither of the *HvPhy-C1* and *HvPhy-C2* bands reported in Yan et al. (2005) appear to correspond to a functional *HvPhyC* gene, and rather represent the two 17 kb-spaced *HvPhyCΨ1* pseudogene segments *HvPhyCΨ1a*, and *HvPhyCΨ1b*. Since Dicktoo has the same two *HvPhyCΨ1* pseudogene segments as Morex, this verifies Dicktoo does not have a second functional gene at this alternate locus and indicates that the 5HL PPD response difference between Dicktoo versus Morex likely does not rely on these *HvPhyC* pseudogene sequences.

Perrota et al. (2001) verified the presence of three cryptochrome genes in barley, via amplification of small gene fragments to a highly conserved region. In this study, we report for the first time the full genomic sequence of the *HvCry1a* and *HvCry1b* genes, as well as the map or chromosome arm locations for all three barley *Cry* genes. *HvCry1a* and *HvCry2* map as adjacent loci on 6HS, while *HvCry1b* was assigned to chromosome 2HL via the barley–wheat substitution lines due to a lack of polymorphisms. The presence of the two closely related *HvCry1* genes on different chromosomes verifies they did not arise from a localized duplication event.

QTL analyses revealed that genes other than photoreceptors are determinants of the phenotypic variation for the PPD response traits in the D×K and D×M populations. In the D×K population, two loci with small PPD QTL effects were present on chromosomes 3HL and 4HL. The 4HL locus coincides with the *VRN-H2* locus, the major flowering time determinant of the population (Karsai et al. 2005). In the D×M population, the major PPD response QTL are on chromosome 1HL at the *Ppd-H2* locus and on chromosome 2HS at the *Ppd-H1* locus. These loci were previously reported in the Igri (winter) × Triumph (spring) population as the major genetic variation source of barley PPD response (Laurie et al. 1995). The detection of six PPD QTL validated the use of the different winterhardiness population genotype combinations. The observation that the same genetic loci are the major PPD determinates in both winter × spring and facultative × spring populations supports the facultative genotype as a subclass of the winter genotype (Skinner et al. 2005; von Zitzewitz et al. 2005). In addition to these two *Ppd-H* loci, we also detected significant QTL effects on chromosome 5HL at the *VRN-H1* locus and on chromosome 7HS. Flowering time QTL have also been reported at the *VRN-H1* locus (Hayes et al. 1997) and on chromosome 7HS (summarized in Kane et al. 2005).

Relative to the six PPD response QTL, only the *HvPhyC* photoreceptor linkage map position is coincident with one of the barley PPD QTL, lying under the D×M FT8-24 PPD response QTL peak, and can be considered a candidate gene for the effect. However, simple coincidence between map position and a QTL effect is not proof of candidacy, especially when more than one valid candidate gene is present at the same locus (Beales et al. 2005). *HvBM5A* and *HvPhyC* map to the same locus and can both be considered candidates. Due to the small size of the D×M and D×K populations, only one recombinant line between *HvBM5A* and *HvPhyC* was present in the D×M population (and none in the D×K population), and hence we could not

statistically analyze the PPD response of the recombinant lines. We recently determined *HvPhyC* transcription level was essentially constant under both SD and LD regimens and across multiple genotypes of all three barley growth habits, including Dicktoo and Morex (Stockinger et al. submitted), indicating if *HvPhyC* is influencing PPD response, it is not at the transcriptional level. However, it is notable that there are several differences between the predicted Dicktoo, Morex and Kompolti korai HvPhyC polypeptides that could account for differences in functional properties, with the eight amino-acid duplication-based insertion at the C-terminal region of Morex HvPhyC one possible source. The C-terminal region of phytochromes is thought to be responsible for signal transfer and interacts with different protein cofactors (Huq and Quail 2002). Mutant analyses in *Arabidopsis*, another LD plant, indicates that *PhyC* has an inhibitory role in flowering induction under SD conditions and by contrast, a promotive effect under LD conditions—the opposite effects of *AtPhyC* on floral initiation in SD versus LD conditions may reflect a unique property of *AtPhyC* compared to the other phytochromes (Monte et al. 2003). The 5HL PPD QTL effect (attributable to the Morex parent) is observed in the D×M population, which contrasts for the alternate HvPhyC C-terminal allele, but not the D×K population, which does not. However, this *HvPhyC* insertion presence is not limited to or consistent within genotypes displaying the spring growth habit based on a germplasm screen (data not shown). Likewise though, Dicktoo and Morex contrast for the *HvBM5A* allele while Dicktoo and Kompolti korai do not (von Zitzewitz et al. 2005). Thus, which of these two genes is the better candidate is still in question.

Expression of the *VRN-H1* candidate gene *HvBM5A* is repressed under pre-vernalized SD conditions, but promoted under LD in Dicktoo (Danyluk et al. 2003). It is constitutively expressed under both PPD conditions in Morex (von Zitzewitz et al. 2005), validating it as a candidate for the PPD QTL. The *VRN-H2* locus is the major D×K population flowering time determinant (Karsai et al. 2005) and detailed PPD experiments gave strong evidence that PPD also has a significant effect on the activity of this locus, where a significant difference is observed in the two *VRN-H2* allele classes (presence/absence of the *ZCCT-H* genes) to PPD regime changes (Karsai et al. submitted). Kane et al. (2005) determined that the wheat VRN regulatory gene *TaVRT-2* is located on the short arms of the A, B, and D group 7 chromosomes and that Dicktoo *HvVRT-2* (the *TaVRT-2* ortholog) is regulated by day length. We determined the genetic map location of *HvVRT-2* and based on the 2-LOD confidence interval it is directly adjacent to the 7HS PPD QTL. Besides *HvVRT-2*, the inferred map position of the *CONSTANS*-like gene *HvCO1* (Griffiths et al. 2003) is also near the 7HS PPD QTL and thus both genes are currently positional candidates for the QTL. None of the other barley *CONSTANS*-like gene family members (Griffiths et al. 2003) are considered positional candidates for PPD response QTL reported in this study. Taken together, these results indicate some of the PPD QTL could be pleiotropic effects of the PPD-responsive *VRN* genes. Pleiotropy among the winter-hardiness components is also seen with LT tolerance QTL that are coincident with *VRN–H1* and *Ppd-H2* loci in the D×M population (Skinner et al. 2006).

In summary, the combination of EST database screens, allele sequencing, and the barley-wheat substitution lines let us assign chromosome positions for the six functional barley photoreceptor genes, two phytochrome pseudogene fragments, and the *HvVRT-2* gene. The photoreceptor genes, which have a fundamental role in plant light perception and photoperiod response (Mouradov et al. 2002), show limited allelic variation in cultivated barley according to our allele comparison from representative genotypes. Based on the results of the winter × spring (Igri × Triumph; Laurie et al. 1995), facultative × spring (Dicktoo × Morex), and facultative × winter (Dicktoo × Kompolti korai) mapping populations, we concluded that five of the six barley photoreceptors are not positional candidates for the different PPD response QTL determined to date. While these genes are likely not candidates for marker assisted selection (MAS) in a PPD-response breeding program, the key amino acid substitutions could make them tractable targets for other MAS programs and genetic engineering. The 5HL map position of both *HvPhyC* and *HvBM5A* coincides with a PPD response QTL in the D×M population and both genes are currently candidates for this effect based on protein structure differences (*HvPhyC*) or gene expression differences (*HvBM5A*). We also confirmed the positional candidacy of *HvVRT-2* and *Hv*CO1 for the 7HS PPD response QTL and *ZCCT-H* for the PPD response 4HL QTL. The common occurrence of distinct PPD-responsive VRN regulatory genes at PPD QTL positions indicates that some PPD QTL effects may be pleiotropic effects of the PPD-responsive VRN genes. However, three other PPD QTL, including the major *Ppd-H1* and *Ppd-H2* loci (Laurie et al. 1995), are not coincident with VRN candidate genes. Therefore, determination of candidate genes for these other PPD QTLs will require the investigation of additional gene classes.

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