

Association of barley photoperiod and vernalization genes with QTLs for flowering time and agronomic traits in a BC₂DH population and a set of wild barley introgression lines

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Abstract The control of flowering time has important impacts on crop yield. The variation in response to day length (photoperiod) and low temperature (vernalization) has been selected in barley to provide adaptation to different environments and farming practices. As a further step towards unraveling the genetic mechanisms underlying flowering time control in barley, we investigated the allelic variation of ten known or putative photoperiod and vernalization pathway genes between two genotypes, the spring barley elite cultivar ‘Scarlett’ (*Hordeum vulgare* ssp. *vulgare*) and the wild barley accession ‘ISR42-8’

(*Hordeum vulgare* ssp. *spontaneum*). The genes studied are *Ppd-H1*, *VRN-H1*, *VRN-H2*, *VRN-H3*, *HvCO1*, *HvCO2*, *HvGI*, *HvFT2*, *HvFT3* and *HvFT4*. ‘Scarlett’ and ‘ISR42-8’ are the parents of the BC₂DH advanced backcross population S42 and a set of wild barley introgression lines (S42ILs). The latter are derived from S42 after backcrossing and marker-assisted selection. The genotypes and phenotypes in S42 and S42ILs were utilized to determine the genetic map location of the candidate genes and to test if these genes may exert quantitative trait locus (QTL) effects on flowering time, yield and yield-related traits in the two populations studied. By sequencing the characteristic regions of the genes and genotyping with diagnostic markers, the contrasting allelic constitutions of four known flowering regulation genes were identified as *ppd-H1*, *Vrn-H1*, *vrn-H2* and *vrn-H3* in ‘Scarlett’ and as *Ppd-H1*, *vrn-H1*, *Vrn-H2* and a novel allele of *VRN-H3* in ‘ISR42-8’. All candidate genes could be placed on a barley simple sequence repeat (SSR) map. Seven candidate genes (*Ppd-H1*, *VRN-H2*, *VRN-H3*, *HvGI*, *HvFT2*, *HvFT3* and *HvFT4*) were associated with flowering time QTLs in population S42. Four exotic alleles (*Ppd-H1*, *Vrn-H2*, *vrn-H3* and *HvCO1*) possibly exhibited significant effects on flowering time in S42ILs. In both populations, the QTL showing the strongest effect corresponded to *Ppd-H1*. Here, the exotic allele was associated with a reduction of number of days until flowering by 8.0 and 12.7%, respectively. Our data suggest that *Ppd-H1*, *Vrn-H2* and *Vrn-H3* may also exert pleiotropic effects on yield and yield-related traits.

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Introduction

The appropriate timing of flowering is a critical adaptive trait for the propagation and survival of a plant species. To

ensure that flowers occur at an optimum time for pollination, seed development and dispersal, plants have evolved sophisticated mechanisms capable of responding to environmental cues such as day length (photoperiod) and exposure to low temperature (vernalization). In *Arabidopsis*, photoperiod, vernalization, gibberellic acid and autonomous pathways have been defined as the genetic basis of flowering time regulation (Hayama and Coupland 2004; Baurle and Dean 2006). In temperate cereals, such as barley and wheat, the principal genes from vernalization and photoperiod regulatory pathways are also active in controlling flowering time (Cockram et al. 2007; Distelfeld et al. 2009).

The photoperiod pathway is well conserved between *Arabidopsis*, a long-day (LD) dicot plant and rice, a short-day (SD) monocot plant, with the gene *CONSTANS* (*CO*) playing a central role (Putterill et al. 1995; Hayama et al. 2003; Trevaskis et al. 2007; Greenup et al. 2009). In *Arabidopsis*, *CO* activates the transcription of the *FLOWERING LOCUS T* (*FT*) gene and promotes flowering under long days. *GIGANTEA* (*GI*) is likely to act upstream of *CO* in the pathway (Suarez-Lopez et al. 2001). In rice, *OsGI*, *Hd1* and *Hd3a* are determined as orthologs of *Arabidopsis* *GI*, *CO* and *FT*, respectively (Yano et al. 2000; Hayama et al. 2002; Kojima et al. 2002). It was shown that *Hd1* represses *Hd3a* expression under long days, but promotes *Hd3a* expression under short days, resulting in flowering. In both species, increased *FT* expression is crucial to the induction of flowering. These results suggest that components of the photoperiod regulatory network are conserved among different plant species, but that their regulation can be modified to generate different phenotypic responses.

Barley is an LD plant, like *Arabidopsis*, but is phylogenetically closer to rice. Several photoperiod pathway homologs have been identified in barley, such as *HvCO1* to *HvCO9* (Griffiths et al. 2003), *HvGI* (Dunford et al. 2005) and *HvFT1* to *HvFT5* (Faure et al. 2007). Among the nine *CO*-like genes isolated from barley, *HvCO1* and *HvCO2* were reported to be the most *CO*-like genes. *HvFT1*, *HvFT2*, *HvFT3* and *HvFT4* were shown to be highly homologous to *OsFTL2* (the *Hd3a* QTL), *OsFTL1*, *OsFTL10* and *OsFTL12*, respectively, while no rice equivalent was found for *HvFT5* (Faure et al. 2007). At least three *FT*-like genes in rice are known to be expressed and capable of promoting flowering (Izawa et al. 2002). Although these photoperiod pathway genes have conserved roles and generate substantial natural variation of flowering in *Arabidopsis* and rice, so far, it has only been reported that variation in *HvFT1* (*VRN-H3*) could provide sources of adaptive variation in flowering behavior in barley (Yan et al. 2006). *Ppd-H1*, the major determinant of LD response in barley (Laurie et al. 1995), does not correspond to either of the barley *CO*-like, *GI*-like, or *FT*-like genes. Positional

cloning identified it as a pseudo-response regulator, a class of genes involved in circadian clock function (Turner et al. 2005).

Many temperate cereals can be broadly divided into winter and spring types based on their growth habit. Winter varieties require several weeks of low temperature to flower (vernalization), whereas spring varieties lack the need of this stimulus. In barley and wheat, variation in the requirement for vernalization is determined by three genes, *VRN1*, *VRN2* and *VRN3* (Sasani et al. 2009; Distelfeld et al. 2009). The *VRN1* gene encodes an APETALA1 and *FRUITFULL*-like MADS-box transcription factor required for the initiation of reproductive development at the shoot apex (Trevaskis et al. 2003; Yan et al. 2003; Preston and Kellogg 2008). The *VRN2* gene is a dominant repressor of flowering and encodes a protein with a zinc finger motif and a CCT (*CO*, *CO*-like and *TOC1*) domain (Yan et al. 2004). The *VRN3* gene is a homolog of the *Arabidopsis* *FT* gene (Yan et al. 2006; Faure et al. 2007). The *VRN3* gene exhibits an elevated expression level if its dominant allele is present, resulting in an accelerated flowering and a bypass of the vernalization requirement (Yan et al. 2006). In vernalization-requiring cereals, *VRN1* is expressed at low levels until its expression is raised upon vernalization, with the level of expression being correlated with the length of cold treatment (Yan et al. 2003; Trevaskis et al. 2006). Vernalization promotes an active chromatin state at *VRN1* (Oliver et al. 2009). The expression of *VRN1* is independent of day length and remains high after vernalization (Sasani et al. 2009). *VRN1* down-regulates *VRN2*, and allows long-day induction of *VRN3* to accelerate subsequent stages of floral development (Trevaskis et al. 2006; Hemming et al. 2008; Sasani et al. 2009). Mutations in the promoter or deletions within the first intron of *VRN1* are associated with high levels of *VRN1* expression and reduced vernalization requirement (Yan et al. 2003; Fu et al. 2005). Allelic variation at *VRN2* and *VRN3* can also influence vernalization requirement. Loss-of-function mutations at *VRN2* allow expression of *FT1* without prior vernalization, causing rapid flowering under long days. However, this requires an active *Ppd-H1* gene, which promotes long-day induction of *HvFT1* (Yan et al. 2004; Karsai et al. 2005; Turner et al. 2005; Hemming et al. 2008).

In a previous study, ten QTLs for flowering time were detected in the BC₂DH population S42, which is derived from the cross between a German elite barley cultivar and a wild barley accession from Israel (von Korff et al. 2006). A set of 39 wild barley introgression lines was subsequently developed from S42 and utilized for QTL verification (Schmalenbach et al. 2008, 2009). As a first step towards unraveling the genetic mechanisms underlying flowering time variation in both populations, we investigated, in the

Table 1 Primer details used for PCR amplification and sequencing of candidate genes

| Target genes | Primer names | Sequences (5'–3') ^a | GenBank accession ^b | Annealing temperature (°C) | PCR fragment size (bp) |
|---------------|--------------|--------------------------------|--------------------------------|----------------------------|------------------------|
| <i>Ppd-H1</i> | PP04 | GTGCAAAGCATAATATCAGTGTCC | AY970701, AY943294 | 61 | 1,012 |
| | PP05 | GGCCAAAGACACAAGAATCAG | | | |
| <i>VRN-H1</i> | Intr1/H/F1 | GCTCCAGCTGATGAAACTCC | AY750996 | 64 | 477 |
| | Intr1/H/R1 | CTTCATGGTTTTGCAAGCTCC | | | |
| | Intr1/H/F3 | TTCATCATGGATCGCCAGTA | AY750994 | 60 | 383 |
| | Intr1/H/R3 | AAAGCTCCTGCCAACTACGA | | | |
| <i>VRN-H2</i> | ZCCT.06 | CCTAGTTAAAACATATATCCATAGAGC | AY485977 | 50 | 306 |
| | ZCCT.07 | GATCGTTGCGTTGCTAATAGTG | | | |
| <i>VRN-H3</i> | VRN3-654-F | CCATTCACCACCTCCTCAGT | DQ898515 | 64 | 770 |
| | VRN3-1423-R | CGCTAGGACTTGAGCATCT | | | |
| <i>HvCO1</i> | CO19 | TCGCTCCATACACAAAAATCTC | AF490467, AF490468 | 59 | 883 |
| | CO23 | AGCATCGATTTCGCTTGAAATAC | | | |
| <i>HvCO2</i> | HvCO2-164-F | TTTTGGAGAAGGAAGCTGGA | AF490470 | 60 | 651 |
| | HvCO2-814-R | TCCATAATTGCTCCCTTGC | | | |
| | HvCO2-774-F | CCCATTTCCGCGTTAGAATA | AF490470 | 60 | 823 |
| | HvCO2-1596-R | GCACTGGCATCTGAAGTGAA | | | |
| <i>HvGI</i> | HvGI-5433-F | CCTTTGCAAGAGTGCAACAA | AY740524 | 64 | 753 |
| | HvGI-6185-R | TGCCAGAGCAATGAGACAAC | | | |
| <i>HvFT2</i> | HvFT2-4319-F | GGGTGCTTGAGATTGTCCAT | DQ297407 | 64 | 534 |
| | HvFT2-4852-R | TCGTAGACGCATCTTTGTCTG | | | |
| <i>HvFT3</i> | HvFT3-1186-F | TTTTGCCCATCCTTAACACC | DQ411319 | 60 | 662 |
| | HvFT3-1847-R | CTGATCCACCTTCCCTTTGA | | | |
| <i>HvFT4</i> | HvFT4-165-F | CGTTGAGATTGGTGGTGATG | DQ411320 | 64 | 554 |
| | HvFT4-718-R | GTACGGGGATGTTGTACGG | | | |

^a PCR primers for amplification of *VRN-H1* and *VRN-H2* were taken from Fu et al. (2005) and Szücs et al. (2006), respectively

^b GenBank accession number used to select the primer sequences

present study, the allelic variation and the precise genetic map location of ten known or putative photoperiod and vernalization pathway genes and, in addition, examined the coincidence between these candidate genes and QTLs for flowering time and yield-related traits. Our emphasis was on assessing whether photoperiod pathway homologs in barley, such as *HvCO1*, *HvCO2*, *HvFT2*, *HvFT3*, *HvFT4* and *HvGI* are positional candidates for flowering time QTLs detected in the BC₂DH population and the introgression lines.

Materials and methods

Plant materials

The doubled haploid (DH) population S42 and a set of derived introgression lines (S42ILs) were utilized for genetic mapping and QTL analyses. The development of the advanced backcross population S42 with 301 BC₂DH lines generated from a primary cross between ‘Scarlett’ and

‘ISR42-8’ is described in detail in von Korff et al. (2004). The approach for the development of a set of 39 selected BC₃S₄ wild barley introgression lines (S42ILs), each containing a single marker-defined chromosomal introgression from accession ‘ISR42-8’ in the uniform genetic background of cultivar ‘Scarlett’, is outlined by Schmalenbach et al. (2008). ‘ISR42-8’ is a wild barley accession (*Hordeum vulgare* ssp. *spontaneum*, hereafter abbreviated with *Hsp*) from Israel and ‘Scarlett’ is a German spring barley cultivar (*Hordeum vulgare* ssp. *vulgare*, hereafter abbreviated with *Hv*). Both genotypes were used for allele sequencing to detect single nucleotide polymorphisms (SNPs) between the candidate flowering time genes.

Sequencing of candidate genes and identification of allelic variation

Publicly available genomic sequence information of the candidate genes in GenBank (<http://www.ncbi.nlm.nih.gov/>) was utilized to design primers for amplification of 500–1,100-bp fragments from genomic DNA of ‘Scarlett’

and ‘ISR42-8’. The PCR products were sequenced using BigDye sequencing technology at the Automatic DNA Isolation and Sequencing (ADIS) facility of the Max-Planck-Institute of Plant Breeding Research. Amplicons from at least two independent PCR reactions were sequenced from both ends with forward and reverse primers to eliminate PCR-based artifacts. In case of discrepancies, the ambiguous sequence was compared with other reads from the same parent and a conserved base rather than a SNP was accepted. Sequence analyses, such as assembly of forward and reverse reads and identification of polymorphisms, were done with the software package Geneious, Biomatters Ltd., Auckland, New Zealand.

Primer details for PCR amplification and sequencing are listed in Table 1.

Genotyping of candidate genes

Sequence polymorphisms between the parents ‘Scarlett’ and ‘ISR42-8’ were transformed to either indel (insertion/deletion) markers, CAPS (cleaved amplified polymorphisms) markers or SNP markers (see Table 2) in order to genotype 301 BC₂DH lines from population S42 and 39 S42ILs. Indels were genotyped after PCR amplification on a Li-Cor DNA Sequencer 4200, LI-COR, Bad Homburg, Germany, as stated in von Korff et al. (2004). CAPS were

Table 2 Primers and methods used for genotyping candidate genes

| Target genes | Primer names | Sequences (5′–3′) ^a | T (°C) ^b | PCR size ^c | Genotyping method ^d | Scarlett ^e | ISR42-8 ^e |
|---------------|---------------|--------------------------------|---------------------|-----------------------|--|-----------------------|----------------------|
| <i>Ppd-H1</i> | PH1-3113-F | CCATGCTGCCAACTATGGTA | 53 | 209 | Indel at SNP20 | Insertion | 9-bp deletion |
| | PH1-3321-R | TCCCAAAGTTCCTCTCTTTTCTC | | | | | |
| <i>VRN-H1</i> | Intr1/H/F1 | GCTCCAGCTGATGAAACTCC | 64 | 477 | PCR fragment presence (+) or absence (–) | + | – |
| | Intr1/H/R1 | CTTCATGGTTTTGCAAGCTCC | | | | | |
| | Intr1/H/F3 | TTCATCATGGATCGCCAGTA | 60 | 383 | | | |
| | Intr1/H/R3 | AAAGCTCCTGCCAACTACGA | | | | | |
| <i>VRN-H2</i> | ZCCT.06 | CCTAGTTAAAACATATATCCATAGAGC | 50 | 306 | PCR fragment presence (+) or absence (–) | – | + |
| | ZCCT.07 | GATCGTTGCGTTGCTAATAGTG | | | | | |
| <i>VRN-H3</i> | VRN3-1185-F | CCATTTTTCTGTGCTCTCTGG | 64 | 206 | Indel at position 326-bp of intron 1 | Insertion | 4-bp deletion |
| | VRN3-1390-R | CCTGCAGGCAGTATAAAGCA | | | | | |
| <i>HvCO1</i> | HvCO1-3111-F | TCATGCAAACAGGGAAGAAG | 60 | 188 | Pyrosequencing | CAT <u>A</u> TTA | CAC <u>A</u> TTA |
| | HvCO1-3298-R | Biotin-GCTGGACTGGACCGTATTGT | | | | | |
| | HvCO1-AS-3191 | GCAATATCAATATGATCA | | | | | |
| <i>HvCO2</i> | HvCO2-394-F | GCACTATGTACCGCTGTGA | 65 | 191 | Pyrosequencing | GC <u>A</u> GAGG | GCG <u>G</u> AGG |
| | HvCO2-584-R | Biotin-CTGAGGAGCCAAGAGTCCAC | | | | | |
| | HvCO2-AS-493 | CCAGCTGCCTCTGGCTTT | | | | | |
| <i>HvGI</i> | HvGI-5566-F | GGCATCCTGGAAGCTCTTT | 65 | 201 | Pyrosequencing | GTC <u>G</u> CAG | GTT <u>G</u> CAG |
| | HvGI-5766-R | Biotin-GGATGATGCCCTGGTAGAAA | | | | | |
| | HvGI-AS-5623 | TATAGTTCAAATGAGATA | | | | | |
| <i>HvFT2</i> | HvFT2-4319-F | GGGTGCTTGAGATTGTCCAT | 64 | 534 | CAPS (<i>Nde</i> I) | 534 bp | 328 + 206 bp |
| | HvFT2-4852-R | TCGTAGACGCATCTTTGTCTG | | | | | |
| <i>HvFT3</i> | HvFT3-1186-F | TTTTGCCCATCCTTAACACC | 60 | 662 | CAPS (<i>Swa</i> I) | 662 bp | 417 + 245 bp |
| | HvFT3-1847-R | CTGATCCACCTTCCCTTTGA | | | | | |
| <i>HvFT4</i> | HvFT4-165-F | CGTTGAGATTGGTGGTGATG | 64 | 554 | CAPS (<i>Bst</i> NI) | 554 bp | 421 + 133 bp |
| | HvFT4-718-R | GTACGGGGATGTTTGTACGG | | | | | |

^a PCR primers for genotyping *VRN-H1* and *VRN-H2* were taken from Fu et al. (2005) and Szücs et al. (2006), respectively

^b Annealing temperature for PCR

^c PCR fragment size in bp

^d Genotyping methods are explained under “Materials and methods”. The restriction enzyme used to differentiate the two alleles for CAPS markers is indicated in brackets

^e Resulting genotyping alleles for Scarlett and ISR42-8

Table 3 List of agronomic traits evaluated in von Korff et al. (2006); Schmalenbach et al. (2009) and this study

| Abbr. | Trait | Method of measurement | S42 | S42ILs |
|-------|-------------------------------------|---|---|--|
| | | | Environments tested by von Korff et al. (2006), (i.e. seasons 2003 and 2004) ^a | Environments tested by Schmalenbach et al. (2009), (i.e. season 2007) and this study (i.e. season 2008) ^a |
| EAR | Ears per square meter | Number of ears counted from a row of 50 cm or 100 cm | D03, D04, G03, G04, M03 | D07, H07, M08 |
| GEA | Grains per ear | Number of grains per ear calculated from a row of 50 cm or 20 average ears | | D07, G07, H07 |
| HEA | Days until heading (flowering time) | Number of days from sowing until emergence of 50% of ears on main tillers | D03, D04, G03, G04, I03, I04, M03, M04 | D07, D08, G07, H08, M08 |
| HEI | Plant height | Average plant height measured from soil surface to tip of spike (including awns) 2 weeks after flowering | D03, D04, G03, G04, I03, I04, M03, M04 | D07, D08, G07, H07, M08 |
| HI | Harvest index | Ratio of generative to vegetative biomass, calculated from a row of 50 cm at maturity | D03, D04 | |
| LAH | Lodging at harvest | Visual rating of the severity of lodging at harvest (one represents no lodging and nine represents total lodging of plot) | D03, D04, G03, G04, I03, I04, M03, M04 | D07, D08, G07, H07, M08 |
| TGW | Thousand grain weight | Average weight of 1,000 kernels calculated from two samples of 250 kernels | D03, D04, G03, I03, I04, M04 | D07, D08, G07, G08, H07, M08 |
| YLD | Grain yield | Weight of barley grain harvested per plot and dried for 1-2 days | D03, D04, G03, G04, I03, I04, M03, M04 | D07, D08, G07, G08, H07, M08 |

^a Combination of location [Dikopshof (D), Gudow (G), Herzogenaurach (H), Irlbach (I), Morgenrot (M)] and year [2003 (03), 2004 (04), 2007 (07), 2008 (08)]

PCR amplified as stated in von Korff et al. (2004), cleaved with the appropriate restriction enzyme, stated in Table 2, and electrophoretically separated on 1.0% agarose gels. The pyrosequencing assay for SNP genotyping was carried out on a PyroMark ID system, Biotage AB, Uppsala, Sweden. The experimental procedure for pyrosequencing assays, including PCR optimization of the pyrosequencing template, sequencing primers, annealing plate preparation, immobilization of PCR products to streptavidin beads and the preparation of single stranded pyrosequencing template DNA were essentially done as described by Royo et al. (2007). The pyrosequencing reaction was performed according to the manufacturer's instructions. Pyrograms were generated and the genotypes were assigned to samples at the end of the run by the pyrosequencing software.

Phenotypic evaluation of traits

Phenotypic investigation and QTL mapping for flowering time and yield-related traits were carried out in population S42 under field conditions at four different locations in Germany during the seasons 2003 and 2004 and published

by von Korff et al. (2006). For S42ILs, field tests for seven agronomic traits including flowering time were conducted in three environments in 2007 and published by Schmalenbach et al. (2009). In the present study, we re-investigated the S42ILs in 2008 under the same conditions as in 2007. The test locations in the 2008 growing season were the experimental field station Dikopshof (D08, University of Bonn, West Germany) and the breeders' experimental field stations in Gudow (G08, Nordsaat Saatzucht, North Germany), Herzogenaurach (H08, Saatzucht Josef Breun, Southeast Germany) and Morgenrot (M08, Saatzucht Josef Breun, East Germany). The field tests were designed in three randomized complete blocks (replications of S42ILs) per environment. In addition, the recurrent parent 'Scarlett' was tested as a control in four replications per block. Net plot sizes (4.5–6.0 m²), seed density (300–390 kernels/m²) and nitrogen fertilization (30–80 kg N/ha) taking into account the N_{min} content of the soil and field management were in accordance with the local practice. The grain was harvested with a plot harvester at total maturity (EC 92). The agronomic traits evaluated in this study are presented in Table 3. For all experiments mentioned above, seed

sowing was carried out in early spring (March) when temperatures reached 5–8°C.

Data processing and statistical analyses

The statistical analyses were performed with SAS Enterprise Guide 4.1 (SAS Institute 2006). The candidate genes were placed on a linkage map from the BC₂DH population S42 that consisted of 98 SSR markers by looking for linkage disequilibrium. The basic assumption for each marker pair tested was linkage equilibrium. The independent assortment of the alleles for each marker pair was tested with a Chi-square test. The candidate gene was placed in the map interval between the two markers which exhibited the highest Chi-square values, indicating a strong deviation from linkage equilibrium due to genetic linkage. The map location was verified with introgression lines and compared to the literature.

The detection of QTLs associated with the tested candidate genes in population S42 was carried out using the general linear model (GLM) procedure as described by von Korff et al. (2006). The three-factorial mixed model included the marker as a fixed factor and the line, environment and marker × environment interaction as random factors. Marker main effects and marker × environment interactions were accepted as QTLs, if the *P* value calculated by the Type III sums of squares was less than 0.01 (Pillen et al. 2003). Linked significant markers, with a distance of less than or equal to 20 cM which showed the same direction of effect, were treated as a single QTL.

For detection of QTLs in S42ILs, a two-factorial mixed model ANOVA was carried out with the GLM procedure as described in Schmalenbach et al. (2009). Here, the GLM model included the line as a fixed factor and the environment and line × environment interaction as random factors. When the analysis revealed significant differences between lines or line × environment interactions, a Dunnett multiple comparison of least squares means differences between the ILs and the recurrent parent ‘Scarlett’ as the control was conducted (Dunnett 1955). The presence of a QTL due to an *Hsp* introgression was accepted, if the trait value of a particular IL was significantly (*P* < 0.05) different from ‘Scarlett’ either across all environments (line main effect) or in a particular environment (line × environment interaction effect).

Results

Allelic polymorphisms of candidate genes

The sequence comparison of the two parents of population S42 and the S42IL set revealed polymorphism for all

candidate genes investigated. The allelic constitutions of ‘Scarlett’ and ‘ISR42-8’ are presented in Table 2 and in the following.

Ppd-H1

The 880-bp genomic region spanning from the 7th to the 8th exon of *Ppd-H1* was sequenced from ‘ISR42-8’ and ‘Scarlett’. In total, eight polymorphisms including seven SNPs and one indel were revealed (Table 4). Among them, three SNPs (SNP 19, 22 and 23) resulted in amino acid changes. In addition, ‘ISR42-8’ had a 9-bp deletion at SNP 20, causing a deletion of three amino acids. According to the genotype at SNP 22, which Turner et al. (2005) identified as the most likely causal basis of the *ppd-H1* mutation, ‘ISR42-8’ contains a typical dominant early-flowering *Ppd-H1* allele, whereas ‘Scarlett’ harbors a recessive late-flowering *ppd-H1* allele (Table 4).

VRN-H1

It has been reported that large deletions within the first intron of the dominant *Vrn-H1* allele are associated with spring growth habit in barley. Specific PCR primers, designed by Fu et al. (2005), were used to test for the presence or absence of the large deletion in intron 1 of *VRN-H1*. Primer pair Intr1/H/F1 and Intr1/H/R1 amplified a 477-bp fragment only in ‘Scarlett’, while primers Intr1/H/F3 and Intr1/H/R3 produced a 383-bp band only in ‘ISR42-8’, confirming the presence of a deletion in the ‘Scarlett’ *Vrn-H1* allele and the absence of the deletion in the ‘ISR42-8’ *vrn-H1* allele. This finding has been further approved by testing additional primer combinations stated by Hemming et al. (2009). As shown in Table 5, primer combinations B + S, F + S, G + X, T(ic) + U, U(ic) + V and A + S produced PCR fragments of the expected size in ISR42-8. The primer combinations A + T and B + T did not reveal PCR fragments in ISR42-8, presumably, because the expected PCR fragments were too large (>3.5 kb). On the contrary, from the primer combinations revealing expected PCR fragments in ISR42-8 only T(ic) + U revealed a PCR fragment in ‘Scarlett’. This fragment corresponded to the expected size. It can thus be inferred that there are no indications for a deletion within intron 1 of the ISR42-8 sequence. The ‘Scarlett’ sequence for intron 1 of the *VrnH1* gene indicates a deletion at least between primers X and T, reducing the size of intron 1 by at least 5 kb.

VRN-H2

Previous reports showed that deletions of the complete *VRN-H2* gene result in recessive alleles for spring growth

Table 4 Barley haplotype scoring from seven SNPs and one indel at the *Ppd-H1* locus

| Cultivar/accession | Position of polymorphism ^a | | | | | | | | Allele |
|--------------------|---------------------------------------|-----------|--------|--------|-----------|-----------|--------|--------|---------------|
| | SNP 17 | Pos. 2939 | SNP 19 | SNP 20 | Pos. 3239 | Pos. 3317 | SNP 22 | SNP 23 | |
| ‘Scarlett’ | T | C | A | G | T | C | T | A | <i>ppd-H1</i> |
| ‘Triumph’ | T | C | A | G | T | C | T | A | <i>ppd-H1</i> |
| ‘ISR42-8’ | G | T | G | – | C | T | G | G | <i>Ppd-H1</i> |
| ‘Igri’ | G | C | G | A | T | C | G | G | <i>Ppd-H1</i> |

^a The SNP numbers and the haplotypes of ‘Triumph’ and ‘Igri’ are taken from Turner et al. (2005). Positions “2939”, “3239” and “3317” refer to the genomic sequence of ‘Igri’ (GenBank accession AY970701). SNP19, SNP22 and SNP23 produced an Ala-to-Thr, a Gly-to-Trp and an Ala-to-Thr change from ‘ISR42-8’ to ‘Scarlett’, respectively. In addition, ‘ISR42-8’ had a 9-bp deletion, indicated by “–”, at SNP20 and thus caused a three-amino-acid (Ala-Ala-Ala) deletion in the predicted protein

Table 5 PCR results used to determine the presence of deletions in intron 1 of the *VRN-H1* gene in ‘Scarlett’ and ‘ISR42-8’

| Forward and Reverse Primers (5'–3') | Pos. | Expected product size (bp) | Observed product size (bp) | | |
|-------------------------------------|----------------------------|----------------------------|----------------------------|---------|-------|
| | | | Scarlett | ISR42-8 | |
| B | GCTCCAGCTGATGAACTCC | 3046 | 2,421 | – | 2,421 |
| S | AAAGCTCCTGCCAACTACGA | 5467 | | | |
| A | TTCATCATGGATCGCCAGTA | 5084 | 3,572 | – | * |
| T | CTTCATGGTTTTGCAAGCTCC | 8656 | | | |
| F | AGGAACTCTGTGATGGGTCTATG | 4437 | 1,030 | – | 1,030 |
| S | AAAGCTCCTGCCAACTACGA | 5467 | | | |
| G | GTTCTCCACCGAGTCATGGT | 2306 | 988 | – | 988 |
| X | CGCTGGACGAGAATTATTGA | 3294 | | | |
| T (ic) | GGAGCTTGCAAAACCATGAAG | 8656 | 1,368 | 1,368 | 1,368 |
| U | TTCGTCCTACCTTCGTCGGTTGTGCC | 10024 | | | |
| U (ic) | GGCACAACCGACGAAGGTAGGACGAA | 10024 | 2,054 | – | 2,054 |
| V | CTCTCCGTCCTCAGCCAC | 12078 | | | |
| A | TTCATCATGGATCGCCAGTA | 5084 | 383 | – | 383 |
| S | AAAGCTCCTGCCAACTACGA | 5467 | | | |
| B | GCTCCAGCTGATGAACTCC | 3046 | 5,610 | 477 | * |
| T | CTTCATGGTTTTGCAAGCTCC | 8656 | | | |

The primer sequences and PCR protocols are taken from Hemming et al. (2009)

“ic” indicates a primer sequence which is inverse complementary compared to the original primer. The position (Pos.) is given based on cultivar ‘Strider’ (AY750993) in Hemming et al. (2009). Asterisk a fragment which is presumably too large for PCR amplification (>3 kb)

habit in barley (Yan et al. 2004; Dubcovsky et al. 2005). With the ZCCT gene specific primers ZCCT.06 and ZCCT.07, taken from Szücs et al. (2006), a 306-bp fragment was amplified from ‘ISR42-8’; however, no PCR product was amplified from ‘Scarlett’. This indicates that ‘ISR42-8’ carries the dominant winter type *Vrn-H2* allele and ‘Scarlett’ carries the recessive spring type *vrn-H2* allele.

VRN-H3

In barley, mutations in the first intron of the *VRN-H3* gene, which is synonymous with *HvFT1*, differentiate plants in

regard to spring and winter growth type (Yan et al. 2006). Two SNPs at positions 270 and 384 are reported to be completely associated with the dominant *Vrn-H3* allele (A and G), promoting flowering and the recessive *vrn-H3* allele (T and C), delaying flowering (Yan et al. 2006). We thus sequenced the corresponding 770-bp genomic fragment from ‘ISR42-8’ and ‘Scarlett’, which contained the end of the promoter, exon 1 and intron 1 of *VRN-H3*. Three SNPs and one indel were found in intron 1 between the ‘ISR42-8’ and ‘Scarlett’ alleles (Table 6). ‘Scarlett’ carries a typical recessive intron 1 haplotype (*vrn-H3*). However, the intron 1 genotype of ‘ISR42-8’ was different from those reported previously, indicating a novel haplotype

Table 6 Barley haplotype scoring from four SNPs and one indel at the *VRN-H3* locus

| Cultivar/accession | Position of SNP in intron 1 ^a | | | | | Allele |
|--------------------|--|----|-----|-----|-----|---------------|
| | 63 | 80 | 270 | 326 | 384 | |
| ‘Scarlett’ | T | C | T | in | C | <i>vrn-H3</i> |
| ‘Igr1’ | T | C | T | in | C | <i>vrn-H3</i> |
| ‘ISR42-8’ | C | T | T | del | G | ? |
| ‘BGS213’ | C | C | A | in | G | <i>Vrn-H3</i> |

^a Letters “in” and “del” indicate a 4-bp indel (GCTC). Numbers on the top indicate the base pairs from the SNP to the start of the first intron (based on the *vrn-H3* allele in ‘Igr1’). The abbreviations “A”, “C”, “T”, “G”, “in” and “del” indicate the polymorphic nucleotide, insertion or deletion, respectively. Information for the genotypes of ‘BGS213’ and ‘Igr1’ is taken from Yan et al. (2006)

(Table 6). At this time, we cannot judge whether ‘ISR42-8’ carries a dominant *Vrn-H3* allele or a recessive *vrn-H3* allele.

HvCO1

We sequenced 883 bp of *HvCO1* from ‘ISR42-8’ and ‘Scarlett’, containing the end of intron 1, exon 2 and a portion of the 3’ UTR. Comparison of the alleles revealed a single SNP in intron 1 at position 1,816 bp of GenBank accession AF490467. Here, ‘Scarlett’ and ‘ISR42-8’ possessed a T and a C, respectively.

HvCO2

We sequenced 1,065 bp of *HvCO2* from ‘ISR42-8’ and ‘Scarlett’, encompassing exon 1 (partial) and intron 1 (partial) and found a single SNP in exon 1 at position 513 bp. Here, ‘Scarlett’ and ‘ISR42-8’ possessed an A and a G, respectively. The SNP was silent at the amino acid level.

HvGI

We sequenced 753 bp of the *HvGI* exon 10 fragment from ‘ISR42-8’ and ‘Scarlett’ and detected one SNP at position 5,643 bp. Here, ‘Scarlett’ and ‘ISR42-8’ possessed a C and a T, respectively. The SNP was silent at the amino acid level. In addition, no differences between ‘ISR42-8’ and ‘Scarlett’ were found in a fragment spanning about 600 bp of exon 13, intron 13, exon 14 and the 3’ UTR of *HvGI* (data not shown).

HvFT2

We sequenced 534 bp of the 3’ UTR region of the *HvFT2* sequence. Three SNPs, C/T, G/A and T/C were detected

between ‘Scarlett’ and ‘ISR42-8’ at positions 4,415, 4,647 and 4,753 bp, respectively. In addition ‘ISR42-8’ revealed a 3-bp deletion at position 4,464–4,466 bp as compared to ‘Scarlett’.

HvFT3

We sequenced 662 bp of the *HvFT3* gene spanning intron 3 and exon 4 from ‘ISR42-8’ and ‘Scarlett’. One SNP in intron 3 was revealed at position 1,603 bp, where ‘Scarlett’ and ‘ISR42-8’ possessed a C and an A, respectively.

HvFT4

We sequenced 554-bp of the *HvFT4* gene extending from the end of exon 1 through the end of intron 3. One SNP in intron 1 was revealed at position 298 bp, where ‘Scarlett’ and ‘ISR42-8’ possessed an A and a C, respectively.

Genetic mapping of ten candidate genes

After transforming the identified polymorphisms into marker assays (see Table 2) the genotypes for 301 BC₂DH lines of the advanced backcross population S42 were determined. Applying a Chi-square test for linkage equilibrium, all ten candidate genes were integrated into the original SSR map of von Korff et al. (2004, see Fig. 1 and Table 7), placing the new genes into the interval between those two SSRs, which revealed the highest Chi-square values. Following the order of the chromosomes, *HvFT3* was mapped to the long arm of chromosome 1H, between markers GBMS12 and HVABAIP. *Ppd-H1* and *HvFT4* were placed on the short arm of chromosome 2H, in the intervals GBM1035–GBM1052 and GBM1052–MGB391, respectively. *HvGI* and *HvFT2* were tightly linked to each other and mapped to the short arm of chromosome 3H, flanked by markers MGB410 and Bmag603. *Vrn-H2* was assigned to the long arm of chromosome 4H, between markers HVM67 and HDAM-YB. *Vrn-H1* was mapped to the long arm of chromosome 5H, adjacent to marker GMS61. *HvCO2* was located close to the centromere of chromosome 6H, between markers EBmac624 and Bmag613. *Vrn-H3* and *HvCO1* were mapped to the short arm of chromosome 7H, in the intervals Bmag7-EBmac603 and HVA22S and Bmag11, respectively. The genetic location of all but one candidate gene has been confirmed after genotyping the set of S42IL introgression lines, which carry single marker-defined chromosomal segments from accession ‘ISR42-8’ (Table 8). The only exception was *VRN-H1*. In this chromosomal region, none of the 39 S42ILs possessed an exotic introgression.

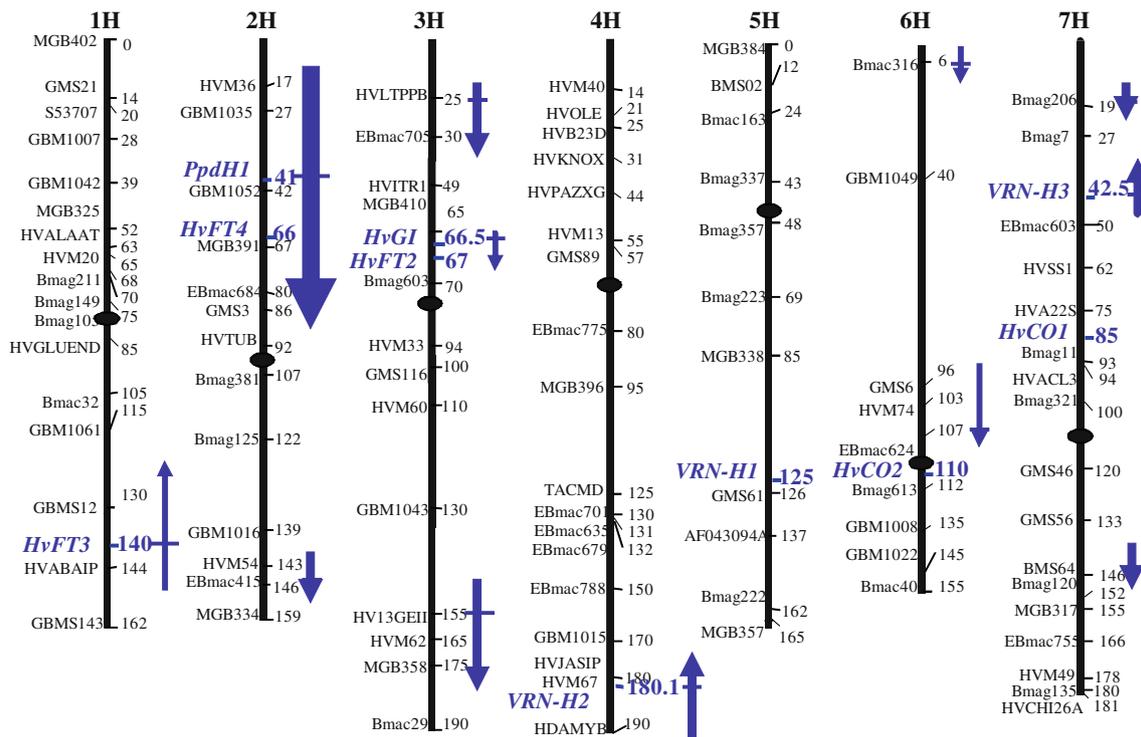


Fig. 1 Location of candidate genes and QTLs for flowering time regulation in the SSR map S42. The candidate genes are highlighted in bold. Their genetic position in cM is based on von Korff et al. (2004). QTLs are indicated by solid arrows right to the chromosome. The horizontal dashes in the arrows indicate the marker with the

highest F value. The upward and downward orientation of the arrow head indicates an increasing and decreasing effect of the Hsp allele, respectively. The width of the arrows indicates the strength of the Hsp effect. QTL effects from non-candidate genes are taken from von Korff et al. (2006)

Association of candidate genes with QTLs for flowering time and further agronomic traits in the BC₂DH population S42

In order to test whether the ten candidate genes potentially exert effects on regulation of flowering time (heading date) as well as yield and yield-related traits, the 301 BC₂DH genotypes of each candidate gene were subjected to a three-factorial mixed model analysis using the phenotype data from von Korff et al. (2006). Altogether 40 significant QTL effects of Hsp alleles from nine candidate genes could be detected. Seven candidate genes (*Ppd-H1*, *VRN-H2*, *VRN-H3*, *HvGI*, *HvFT2*, *HvFT3* and *HvFT4*) were associated with QTLs ($P < 0.01$) exerting effects on flowering time in population S42 (Table 7; Fig. 1). In case of *Ppd-H1*, *VRN-H2*, *VRN-H3*, *HvGI* and *HvFT3*, the candidate gene revealed the strongest effect on flowering time, based on F values in a QTL interval, supporting the hypothesis that the candidate genes might be causative for the measured effects on flowering time (see Fig. 1). The strongest effect on flowering time regulation was observed at the *Ppd-H1* locus. The *Ppd-H1* gene explained 22.7% of the genetic variation and the exotic allele reduced the average number of days until flowering by 8.0% or 5.8 days (Table 7; Fig. 1).

As presented in Table 7, nine candidate genes also revealed significant associations with yield or yield-related traits in population S42. The *Ppd-H1* gene on chromosome 2H was associated with QTL effects on heading (HEA), height (HEI), lodging at harvest (LAH) and thousand-grain weight (TGW). The Hsp allele revealed a decreased value in HEA, HEI and LAH and an enhanced performance in TGW. The *VRN-H1* gene on chromosome 5H corresponded to a region that harbored QTLs with significant effects on TGW and yield (YLD). The exotic allele was associated with an improved performance in TGW, but a reduced value in YLD. The *VRN-H2* locus on chromosome 4H coincided with a region showing QTL effects on HEA, ears per square meter (EAR), HEI, harvest index (HI), LAH, TGW and YLD. The exotic allele was associated with a reduced performance in HEI, LAH and TGW, but an enhanced value in HEA, EAR, HI and YLD. The *VRN-H3* locus on chromosome 7H coincided with a region showing QTL effects on HEA, HI and YLD. The exotic allele was associated with an increased value in HEA, but a reduced performance in HI and YLD. No effects were associated with *HvCO1* on chromosome 7H. *HvCO2* on chromosome 6H was in a region that contained QTLs for EAR and TGW. The Hsp allele was associated with a reduced value in EAR, but an enhanced performance in TGW. The

Table 7 List of ten candidate genes associated with significant effects on flowering time (HEA) and potential pleiotropic effects on agronomic traits in the advanced backcross population S42

| Candidate gene ^a | Trait ^b | Effect ^c | <i>F</i> value ^d | <i>R</i> ² (%) ^e | [<i>Hv</i>] ^f | [<i>Hsp</i>] ^g | RP [<i>Hsp</i>] ^h |
|------------------------------|--------------------|---------------------|-----------------------------|--|----------------------------|-----------------------------|--------------------------------|
| <i>Ppd-H1</i> (2H, 41.1 cM) | HEA | M + I | 28.9 | 22.7 | 72.7 | 66.9 | -8.0 |
| | HEI | M + I | 10.2 | 3.5 | 80.9 | 71.0 | -12.3 |
| | LAH | I | 4.0 | 0.1 | 2.7 | 2.2 | -19.7 |
| | TGW | I | 4.9 | 1.1 | 42.5 | 43.6 | 2.6 |
| <i>VRN-H1</i> (5H, 125.1 cM) | HEA | n.s. | | | | | |
| | TGW | M | 8.2 | 3.0 | 42.5 | 44.4 | 4.6 |
| | YLD | M | 11.9 | 3.8 | 59.3 | 50.9 | -14.2 |
| <i>VRN-H2</i> (4H, 180.1 cM) | HEA | M | 18.7 | 5.9 | 72.1 | 73.5 | 2.0 |
| | EAR | M + I | 23.5 | 22.5 | 745.4 | 841.3 | 12.9 |
| | HEI | M + I | 23.2 | 8.6 | 82.4 | 75.5 | -8.4 |
| | HI | M | 29.4 | 11.9 | 0.583 | 0.629 | 7.9 |
| | LAH | M | 9.2 | 2.9 | 2.9 | 2.0 | -33.0 |
| | TGW | M | 31.8 | 12.0 | 43.1 | 41.3 | -4.1 |
| | YLD | I | 8.3 | 1.1 | 58.0 | 61.2 | 5.5 |
| <i>VRN-H3</i> (7H, 42.5 cM) | HEA | I | 5.1 | 0.3 | 72.4 | 74.3 | 2.6 |
| | HI | I | 13.6 | 0.7 | 0.597 | 0.564 | -5.5 |
| | YLD | M | 7.5 | 2.5 | 59.2 | 51.8 | -12.5 |
| <i>HvCO1</i> (7H, 85.0 cM) | HEA | n.s. | | | | | |
| <i>HvCO2</i> (6H, 110.0 cM) | HEA | n.s. | | | | | |
| | EAR | I | 4.6 | 2.4 | 787.4 | 722.6 | -8.2 |
| | TGW | M | 13.4 | 5.4 | 42.3 | 43.6 | 3.2 |
| <i>HvGI</i> (3H, 66.5 cM) | HEA | I | 7.2 | 0.4 | 72.5 | 71.8 | -1.0 |
| | EAR | M | 22.4 | 9.5 | 782.3 | 665.2 | -15.0 |
| | HEI | M + I | 22.7 | 7.5 | 79.5 | 91.2 | 14.7 |
| | HI | I | 11.5 | 0.7 | 0.603 | 0.517 | -14.2 |
| | LAH | M + I | 10.4 | 3.4 | 2.5 | 4.4 | 74.2 |
| | YLD | M + I | 59.1 | 30.4 | 60.5 | 41.8 | -30.9 |
| <i>HvFT2</i> (3H, 67.0 cM) | HEA | I | 6.0 | 0.4 | 72.6 | 71.2 | -1.8 |
| | EAR | M | 20.6 | 9.5 | 780.4 | 649.7 | -16.7 |
| | HEI | M + I | 21.0 | 6.6 | 79.7 | 92.1 | 15.6 |
| | HI | I | 8.8 | 0.5 | 0.601 | 0.513 | -14.6 |
| | LAH | M + I | 15.7 | 4.9 | 2.5 | 5.1 | 101.5 |
| | YLD | M + I | 53.8 | 27.2 | 60.1 | 39.7 | -33.9 |
| <i>HvFT3</i> (1H, 140.0 cM) | HEA | M + I | 11.6 | 5.0 | 72.2 | 73.8 | 2.1 |
| | EAR | I | 5.2 | 2.7 | 760.9 | 827.8 | 8.8 |
| | HEI | I | 3.3 | 0.2 | 81.0 | 76.9 | -5.1 |
| | HI | M | 21.7 | 8.1 | 0.589 | 0.633 | 7.5 |
| | TGW | I | 6.7 | 1.6 | 42.5 | 42.7 | 0.5 |
| | YLD | I | 51.7 | 7.0 | 60.1 | 55.1 | -8.3 |
| <i>HvFT4</i> (2H, 66.0 cM) | HEA | I | 19.5 | 1.2 | 72.6 | 71.5 | -1.6 |
| | EAR | I | 4.3 | 2.1 | 759.7 | 834.1 | 9.8 |
| | HEI | M | 8.8 | 2.8 | 81.3 | 76.2 | -6.3 |
| | HI | M | 11.0 | 4.5 | 0.589 | 0.626 | 6.2 |

^a The map position (based on von Korff et al. 2004) is indicated in parentheses

^b Abbreviation of traits, see Table 3

^c Significant marker main effect (M) or marker × environment interaction effect (I) in 3-factorial ANOVA. *n.s.* not significant at $P = 0.01$

^d *F* value of the target candidate gene in the 3-factorial ANOVA

^e R_M^2 and $R_{(M \times E)}^2$: Proportion of the genetic variance, which is explained by the marker main effect (if effect contains 'M') or the M × E interaction effect (if effect = 'I'), respectively, as calculated by von Korff et al. (2006)

^f Least squares means of trait value across all tested environments for BC₂DH lines carrying the elite genotype (*Hv*) at the target candidate gene locus

^g Least squares means of trait value across all tested environments for BC₂DH lines carrying the exotic genotype (*Hsp*) at the target candidate gene locus

^h Relative performance (in %) of the *Hsp* genotype = $100 \times ([Hsp] - [Hv])/[Hv]$

Table 8 List of twelve S42ILs carrying introgressions with candidate genes from ‘ISR42-8’ which reveal significant effects on flowering time (HEA) and further agronomic traits

| Candidate gene | Introgression line ^a | Trait ^b | Effect ^c | [IL] ^d | Diff. ^e | RP [IL] ^f | |
|------------------------------|---|----------------------------|---------------------|-----------------------|--------------------|----------------------|-----|
| <i>HvFT3</i> | S42IL-138 (1H, 140 cM & 7H, 166-181 cM) | HEA | n.s. | | | | |
| | | LAH | L | 2.7 | -1.1 | -30.1 | |
| <i>Ppd-H1</i> | S42IL-107 (2H, 17-42 cM) | HEA* | L + I | 56.1 | -8.1 | -12.7 | |
| | | EAR | I | 1365.4 ^{D07} | 397.4 | 41.1 | |
| | | GEA | L + I | 16.8 | -6.1 | -26.8 | |
| | | HEI* | L + I | 67.7 | -12 | -15.1 | |
| | | TGW* | L + I | 46.1 | 2.0 | 4.6 | |
| | | YLD | L + I | 54.0 | -5.1 | -8.6 | |
| <i>Ppd-H1</i> & <i>HvFT4</i> | S42IL-108 (2H, 17-92 cM) | HEA* | L + I | 57.1 | -7.2 | -11.2 | |
| | | EAR* | L + I | 1193.4 | 253.8 | 27.0 | |
| | | GEA | L + I | 19.7 | -3.3 | -14.2 | |
| | | HEI* | L | 75.4 | -4.2 | -5.3 | |
| | | TGW* | I | 38.1 ^{H07} | 4.4 | 13.1 | |
| | | YLD | L + I | 54.1 | -5.0 | -8.5 | |
| <i>HvFT4</i> | S42IL-109 (2H, 66-92 cM) | HEA | n.s. | | | | |
| | | EAR* | L + I | 1191.5 | 251.9 | 26.8 | |
| | | GEA | L + I | 18.4 | -4.5 | -19.5 | |
| | | HEI* | L + I | 72.1 | -7.6 | -9.5 | |
| | | LAH | L | 2.7 | -1.1 | -30.1 | |
| <i>HvGI</i> & <i>HvFT2</i> | S42IL-111 (3H, 65-70 cM) | HEA | n.s. | | | | |
| | | GEA | L + I | 19.1 | -3.8 | -16.6 | |
| | | YLD* | L | 55.5 | -3.6 | -6.1 | |
| <i>VRN-H2</i> | S42IL-124 (4H, 170-190 cM) | HEA* | L + I | 66.5 | 2.2 | 3.4 | |
| | | GEA | I | 18.3 ^{D07} | -3.9 | -17.6 | |
| | | HEI* | L | 76.1 | -3.5 | -4.4 | |
| | | TGW* | L + I | 41.1 | -2.9 | -6.6 | |
| <i>HvCO2</i> | S42IL-128 (6H, 40-112 cM) | HEA | n.s. | | | | |
| | | TGW | L | 42.0 | -2.0 | -4.6 | |
| | | YLD | L | 55.7 | -3.4 | -5.8 | |
| | S42IL-129 (6H, 96-112 cM) | HEA | n.s. | | | | |
| | | S42IL-130 (6H, 110-155 cM) | HEA | n.s. | | | |
| | | | TGW* | L + I | 46.6 | 2.6 | 5.9 |
| YLD | L + I | 55.3 | -3.8 | -6.4 | | | |
| <i>VRN-H3</i> | S42IL-133 (7H, 42.5-50 cM) | HEA* | L | 66.1 | 1.9 | 2.9 | |
| | | GEA | I | 18.3 ^{D07} | -3.9 | -17.6 | |
| | | YLD* | L + I | 53.7 | -5.4 | -9.1 | |
| <i>HvCO1</i> | S42IL-134 (7H, 62-85 cM) | HEA | L + I | 60.4 | -3.9 | -6.0 | |
| | | GEA | L + I | 20.7 | -2.3 | -9.8 | |
| | | HEI | L + I | 87.0 | 7.3 | 9.2 | |
| | | YLD | L + I | 54.0 | -5.1 | -8.6 | |
| | | S42IL-135 (7H, 75-155 cM) | HEA | n.s. | | | |

^a The map extent of *Hsp* introgressions, based on von Korff et al. (2004) and Schmalenbach et al. (2008) is indicated in parentheses

^b Abbreviation of traits, see Table 3. Asterisk the effect was also detected in S42, see Table 7

^c Significant line main effect (L) or line × environment interaction effect (I) in 2-factorial ANOVA. *n.s.* not significant at $P = 0.05$

^d Least squares means of the IL calculated either across all environments, if a line main effect (L) or both a line main effect and an interaction effect (L + I) were detected, or from a particular environment, if only a line × environment interaction effect (I) was identified

^e Deviation of IL performance from ‘Scarlett’: LSMEANS[IL] – LSMEANS[Scarlett]

^f Relative performance (in %) of the *Hsp* carrying IL = $100 \times (\text{LSMEANS[IL]} - \text{LSMEANS[Scarlett]}) / \text{LSMEANS[Scarlett]}$

closely linked genes *HvGI* and *HvFT2* on chromosome 3H both were associated with significant effects on HEA, EAR, HEI, HI, LAH and YLD. The *Hsp* alleles resulted in a reduced performance for HEA, EAR, HI and YLD, while an increased performance in HEI and LAH. The *HvFT3* gene on chromosome 1H was in a region that contained QTLs for HEA, EAR, HEI, HI, TGW and YLD. The exotic allele was associated with a reduced value in HEI and YLD, but an increased performance in HEA, EAR, HI and TGW. Finally, the *HvFT4* gene on chromosome 2H coincided with a region that harbored QTLs for HEA, EAR, HEI and HI. The exotic allele was associated with an improved value in EAR and HI, but a reduced performance in HEA and HEI.

Verifying associations of candidate genes with QTL effects using S42ILs

In order to verify possible effects of alleles from the exotic barley accession ‘ISR42-8’ on flowering time and yield as well as yield-related traits, the performance of the set of 39 S42ILs was phenotyped through two seasons in up to six environments (see Table 3). The presence of exotic candidate genes in *Hsp* introgressions was confirmed by genotyping with the appropriate assay (see Table 2). The S42ILs with chromosomal introgressions of candidate genes and their effects on flowering time as well as further agronomic traits are shown in Table 8. Altogether 34 significant effects among ten *Hsp* introgressions which contain exotic candidate genes could be detected. Out of these, 16 effects in total (47%), and 4 HEA effects in particular (57%), confirmed QTLs already detected in the BC₂DH population S42 (Table 7). As an example, the phenotypic differences between the control ‘Scarlett’ and eight ILs, each containing a selected candidate gene, are shown in Fig. 2 for the trait HEA. In the following, the effects detected in S42ILs are presented according to the chromosomal order of the introgressions.

Compared with the control ‘Scarlett’, the S42IL-138, containing the exotic *HvFT3* allele, showed a significant reduction in lodging (LAH). Two ILs, S42IL-107 and -108, carrying the exotic *Ppd-H1* allele, revealed significantly increased performance in EAR and TGW and reduced values in HEA, grains per ear (GEA), HEI and YLD. The latter IL possessed, in addition, the exotic *HvFT4* allele. Consistent with the results obtained in population S42, the S42IL-107 and -108, revealed the strongest effects on HEA. Compared with the recurrent parent ‘Scarlett’, the number of days until flowering was reduced by 8.1 (12.7%) and 7.2 (11.2%) days, respectively. S42IL-109, also harboring the exotic *HvFT4* allele, exhibited a significant reduction in GEA, HEI and LAH but an enhanced performance in EAR. S42IL-111, containing both the exotic

HvGI and *HvFT2* alleles, displayed a significant decrease in both GEA and YLD. S42IL-124, harboring the exotic *VRN-H2* allele, showed a significant reduction in GEA, HEI and TGW but an increase in HEA. Two lines, S42IL-128 and -130, carrying the exotic *HvCO2* allele revealed significantly reduced performances in YLD but showed contrasting effects on TGW. A third line, S42IL-129, which also contains the exotic *HvCO2* allele, lacked significant effects. S42IL-133, containing the exotic *VRN-H3* allele, displayed a significant reduction in both GEA and YLD but an increase in HEA. S42IL-134, harboring the exotic *HvCO1* allele, exhibited a significant reduction in HEA, GEA and YLD but an increase in HEI. S42IL-135, which also harbored the exotic *HvCO1* allele, did not show any effect.

Discussion

The control of flowering has important impacts on grain yield in crop species. Variation in responses to environmental cues such as day length and vernalization has been selected in many temperate cereals to provide adaptation to different environments and farming practices. In our study, the parents of the advanced backcross population S42 and its derivative, the collection of S42IL introgression lines, are an elite spring barley and, based on our genotyping data, an exotic winter barley. Both are of contrasting geographic origin revealing substantial differences in flowering time behavior. In order to dissect the genetic and molecular mechanisms which control flowering time variation in

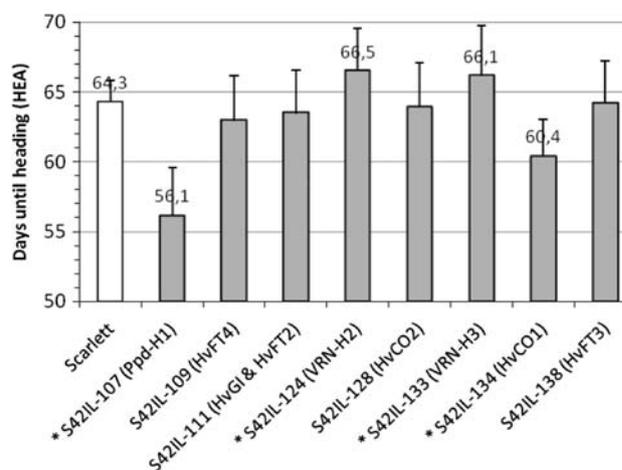


Fig. 2 Least squares means of number of days until heading (HEA) of ILs containing candidate genes compared to the recurrent parent ‘Scarlett’. The name of the candidate gene is placed in brackets behind the name of the IL which contains the respective exotic allele. ILs which significantly ($P < 0.05$) deviate from ‘Scarlett’ are indicated with an asterisks (*) and their least squares mean is given on top of the respective column (for details: see Table 8)

barley, we investigated in these two data sets the association of known and putative photoperiod and vernalization genes with QTLs for flowering time and other yield-related agronomic traits.

By means of linkage equilibrium mapping, we could locate ten candidate genes to individual marker intervals on the seven barley chromosomes (see Fig. 1; Table 7). We had to choose this strategy for mapping with the advanced backcross population since a regular linkage analysis with common software programs usually resulted in artificial linkage. This is most likely due to the over-representation of the elite *Hv* alleles in the BC₂DH population S42, where in general at each locus 87.5% of all individuals comprised the *Hv* genotype. Nevertheless, the placement of the candidate genes based on Chi-square values proved to be successful since for each candidate gene markers which strongly deviated from the assumption of linkage equilibrium could be restricted to a single chromosomal region. In addition, the chromosomal placement of the candidate genes could be validated with S42IL introgression lines (Table 8) and in the literature (Laurie et al. 1995; Yan et al. 2003, 2004, 2006; Griffiths et al. 2003; Dunford et al. 2005; Faure et al. 2007).

Subsequently, all candidate genes were tested for effects on flowering time, yield and yield-related traits in the advanced backcross population S42 and the collection of S42ILs. The latter collection contained *Hsp* introgressions from the donor accession, which was used to generate the S42 population. The first candidate gene, we investigated, was *Ppd-H1*. It is known to be a major determinant of LD response in barley (Turner et al. 2005). By sequencing the functional CCT domain of the *Ppd-H1* locus, it was concluded that ‘ISR42-8’ contains a typical early-flowering *Ppd-H1* allele, whereas ‘Scarlett’ harbors a late-flowering *ppd-H1* allele. This finding has been confirmed in our QTLs analysis. In both populations, S42 and the S42ILs, the strongest *Hsp* effect, associated with a reduction of days until flowering, was mapped to the top of chromosome 2H, corresponding to the *Ppd-H1* locus. The number of days until flowering was reduced by 8.0% in S42 and 12.7% in S42ILs. Our findings are supported by other authors who found in association studies that the *Ppd-H1* locus was the major component to explain variation in flowering time among wild barley accessions and domesticated barley landraces (Jones et al. 2008) and spring barley accessions (Stracke et al. 2009). As described by Laurie et al. (1994), the *Ppd-H1* locus also exerts pleiotropic effects on plant height and yield components, eventually as a direct result of the effect on flowering time. The *Hsp* allele was also associated with a significantly reduced plant height and yield in S42ILs. These results are in agreement with the fact that ‘Scarlett’ is a spring cultivar with high yield performance. In long growing seasons, such

as in Western Europe, the late flowering of the *ppd-H1* allele allows spring cultivars to extend the period of vegetative growth and to increase the source capacity in order to support productivity. In contrast, ‘ISR42-8’ is a wild barley accession from Israel. The strong responses of the exotic *Ppd-H1* allele to LD have probably evolved as an adaptation strategy to escape from drought prone environments, such as hot and dry summers. This assumption is also supported by Lister et al. (2009) who found that LD-responsive and non-responsive alleles at the *Ppd-H1* locus are clearly divided latitudinally where the majority of non-responsive *ppd-H1* alleles are present in the north of Europe.

In the European-cultivated germplasm, most variation in vernalization requirement is accounted for by alleles at the *VRN-H1* and *VRN-H2* loci, as the majority of European varieties are thought to be fixed for winter alleles at the *VRN-H3* locus (Yasuda et al. 1993; Cockram et al. 2007). Spring alleles at *VRN-H3* confer extremely early flowering and are found mostly in exotic barley genotypes (Takahashi and Yasuda 1971). By genotyping with diagnostic PCR markers, we concluded that the allelic constitutions in ‘Scarlett’ and ‘ISR42-8’ are *Vrn-H1/vrn-H2* and *vrn-H1/Vrn-H2*, respectively. At the *VRN-H3* locus, the sequence polymorphism in the putative regulatory intron 1 region suggested that ‘Scarlett’ carries a typical winter recessive *vrn-H3* allele while ‘ISR42-8’ carries a novel, previously not reported, haplotype (Table 6). The spring growth habit of ‘Scarlett’ can thus be extended to the allelic combination *Vrn-H1*, *vrn-H2* and *vrn-H3*. Our QTL analysis revealed that *VRN-H2* and *VRN-H3* are associated with flowering time QTLs, whereas *VRN-H1* did not exert any significant effect in the S42 population. Unfortunately, we could not validate the latter finding in the S42IL population since no introgression line for *Vrn-H1* was available at this time. The *Hsp* alleles at the two QTLs corresponding to *VRN-H2* and *VRN-H3* exhibited late flowering effects which have also been confirmed in S42ILs. The coincidence of *VRN-H2* and *VRN-H3* with late-flowering time QTLs might suggest that wild barley tends to require a period of low temperature as an impulse for flowering (Laurie 1997), or vernalization genes may also play a role in photoperiod responses (Szücs et al. 2006, 2007). In case of *VRN-H3*, the late flowering phenotype indicates, that the *Hsp* allele might also represent a winter recessive *vrn-H3* allele. It should be pointed out that all three vernalization genes possibly exert pleiotropic effects on yield and yield-related traits. The *Hsp* alleles were associated with a yield reduction at *VRN-H1* and *VRN-H3* but with a yield increase at *VRN-H2*, respectively. Similar findings for *VRN-H2* are reported by Karsai et al. (2006) who reported pleiotropic effects of the *VRN-H2* locus on traits like heading date, tiller number, thousand-kernel weight and yield under

different vernalization and photoperiod treatments. Judged from our experiments, the *Hsp* allele at *VRN-H2* may be promising for barley breeding since it was associated with an increase in yield, number of ears and harvest index.

For the photoperiod pathway homologs analyzed in this study, the detected polymorphisms between ‘Scarlett’ and ‘ISR42-8’ were located either in non-coding regions (such as *HvCO1*, *HvFT2*, *HvFT3* and *HvFT4*) or were typically silent at the amino acid level (such as *HvCO2* and *HvGI*). Although no direct polymorphisms at the peptide level could be found between ‘Scarlett’ and ‘ISR42-8’, four candidate genes (*HvFT2*, *HvFT3*, *HvFT4* and *HvGI*) were associated with QTLs, exhibiting small but significant effects on flowering time in S42. Kikuchi et al. (2009) also found that overexpression of *HvFT1*, *HvFT2* and *HvFT3* in transgenic rice resulted in early flowering phenotypes, which suggests that these genes can act as promoters of floral transition. In our barley study, it remains open if the observed effects are caused by the genes itself or by linked genes which are not yet identified. For instance, *HvFT3* is considered to be a good candidate for the *Ppd-H2* gene on chromosome 1H, a major QTL affecting flowering time under SD (Faure et al. 2007; Kikuchi et al. 2009). We found a late flowering effect of the *Hsp* allele in S42, however, no effect was found in the corresponding S42IL-138 which contained *HvFT3*. Likewise, the flowering time QTL effects in S42 for *HvFT4* on chromosome 2H and for *HvGI* and *HvFT2* on chromosome 3H could not be verified in the corresponding S42IL introgression lines, which contained the respective candidate genes. We thus conclude, that it is more likely, that the original QTL effects may dependent on background loci or environmental effects since the S42 population and the S42ILs differ in their genetic background and were tested in different environments. As a further line of evidence, we recently started to isolate the *Hsp* alleles of *VRN-H2*, *VRN-H3* and *HvFT3* and used them for transformation of the ‘Scarlett’ wild type to ultimately prove whether the QTL effects are caused by these candidate genes or not.

HvCO1 and *HvCO2* are the most *CO*-like genes, potentially playing important roles in the photoperiod pathway of barley. For example, Turner et al. (2005) demonstrated that reduced photoperiod responsiveness of the *ppd-H1* mutant can be explained by altered circadian expression of the downstream genes *HvCO1* and *HvCO2*. In our study, both *HvCO1* and *HvCO2* did not reveal significant effects on flowering time in S42. Although, the introgression line S42IL-134, which carry the exotic allele at *HvCO1*, exhibited an early flowering phenotype, we assume that this effect is not caused by *HvCO1* itself, since the sister line S42IL-135 did not confirm this effect. In case of *HvCO2*, the S42 results were validated by S42IL data since all three introgression lines containing the *Hsp* allele

exhibited no effect on flowering time regulation. In conclusion, the barley photoperiod pathway homologs from two different geographic origins probably encoded conserved polypeptides and revealed either non-significant or significant but relatively small effects on flowering time, likely due to their fundamental biological functions. On the other hand, it is interesting to note that most of the photoperiod pathway homologs were in regions shown to harbor QTLs for yield or yield-associated traits in both populations. However, simple coincidence between map position and a QTL effect is not a sufficient proof of candidacy, especially when more than one candidate gene is present at the same locus. To investigate further, we are analyzing the roles of barley photoperiod pathway homologs using transgenic approaches (see above).

In future, we also plan to study epistatic interactions of candidate genes by studying flowering time phenotypes and gene expression patterns after crossing single ILs and selecting the resulting double IL combinations. For verified QTLs like QHea.S42IL-7H.c (Schmalenbach et al. 2009), exhibiting strong effects on flowering time which cannot be explained by a known candidate gene, we plan to launch a map-based cloning approach based on the appropriate introgression line to ultimately clone the causative gene.

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