Mapping-by-Sequencing Identifies HvPHYTOCHROME C as a Candidate Gene for the early maturity 5 Locus Modulating the Circadian Clock and Photoperiodic Flowering in Barley

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ABSTRACT Phytochromes play an important role in light signaling and photoperiodic control of flowering time in plants. Here we propose that the red/far-red light photoreceptor HvPHYTOCHROME C (HvPHYC), carrying a mutation in a conserved region of the GAF domain, is a candidate underlying the early maturity 5 locus in barley (Hordeum vulgare L.). We fine mapped the gene using a mapping-by-sequencing approach applied on the whole-exome capture data from bulked early flowering segregants derived from a backcross of the Bowman (eam5) introgression line. We demonstrate that eam5 disrupts circadian expression of clock genes. Moreover, it interacts with the major photoperiod response gene Ppd-H1 to accelerate flowering under noninductive short days. Our results suggest that HvPHYC participates in transmission of light signals to the circadian clock and thus modulates light-dependent processes such as photoperiodic regulation of flowering.

Many plants use seasonal cues, such as photoperiod or vernalization, to coincide the timing of reproductive development with optimal climate conditions. Cultivated barley (Hordeum vulgare L. subsp. vulgare), like most temperate cereal crops, is a long day (LD) plant with two growth types, winter and spring. Winter types accelerate flowering after a prolonged period of cold (vernalization), whereas spring barley does not respond to vernalization. The growth habit is determined by the interaction of two genes, Vrn-H2, a strong inhibitor of flowering under long day conditions and Vrn-H1 (also known as HvVRN1). Vrn-H1 is upregulated during vernalization and represses Vrn-H2 (Yan et al. 2003, 2004). A deletion of the Vrn-H2 locus and deletions in a regulatory region of Vrn-H1 cause a spring growth habit (Hemming et al. 2009; Rollins et al. 2013). In spring or vernalized winter barley, LDs strongly promote flowering, whereas short days (SDs) delay reproductive development. Flowering under LDs is controlled by the major photoperiod response gene Ppd-H1 (Turner et al. 2005). Ppd-H1 is a homolog of the PSEUDO-RESPONSE REGULATOR (PRR) genes implicated in the circadian clock of the model species Arabidopsis thaliana (L.) Heynh. (hereafter Arabidopsis). A natural mutation in the conserved CCT domain of Ppd-H1 causes a reduced response to LDs and was selected in cultivation areas with long growing seasons (Turner et al. 2005; Von Korff et al. 2006, 2010; Jones et al. 2008; Wang et al. 2010). Ppd-H1, Vrn-H1, and Vrn-H2 converge on the floral inducer HvFT1 (Vrn-H3), a homolog of Arabidopsis florigen FLOWERING LOcus T (FT). In barley, expression levels of HvFT1 in the leaf correlate with flowering time. Vrn-H2 represses HvFT1 to counteract induction of HvFT1 by
Ppd-H1 under LDs before vernalization (Hemming et al. 2008). After vernalization, Ppd-H1 becomes dominant and controls HvFT1 expression and flowering time under LDs (Turner et al. 2005; Campoli et al. 2012a).

In addition to the vernalization and photoperiod response genes, reproductive development is controlled by the early maturity (eam; also referred to as earliness per se) loci. The environmental effect on flowering phenotypes controlled by variation at these genes is reduced or completely removed. Two barley eam genes, HvELF3 and HvLUX1, have recently been identified as homologs of the Arabidopsis circadian clock regulators EARLY FLOWERING 3 (ELF3), and LUX/ARRHYTHMO (LUX), respectively (Faure et al. 2012; Zakhrebekova et al. 2012; Campoli et al. 2013). Mutations in these genes were linked to reduced photoperiod response and early flowering under both LD and noninductive SD conditions.

The circadian clock is an autonomous oscillator that produces endogenous biological rhythms with a period of ~24 hr. Conceptually, a circadian system can be divided into three parts: the central oscillator and input and output pathways. In Arabidopsis, the circadian system comprises at least three interlocking feedback loops. The core oscillator is composed of three negative feedback loops: (a) the inhibition of evening complex (EC) genes ELF3, EARLY FLOWERING 4 (ELF4), and LUX by the rise of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) late at night; (b) the inhibition of PRR genes by the EC early at night; and (c) the inhibition of LHY/CCA1 by TIMING OF CAB EXPRESSION 1 (TOC1) in the morning (Kolmos et al. 2009; Huang et al. 2012; Pokhilko et al. 2012). Furthermore, the evening-expressed GIGANTEA (GI) protein was proposed as a negative regulator of the EC, which in turn inhibits TOC1 expression (Herreréro et al. 2012; Pokhilko et al. 2012).

Light provides the circadian clock with diurnal entrainment signals. In plants, light is perceived and transduced by multiple photoreceptors including phytochromes, cryptochromes, and phototropins (Davis 2002). The phytochromes (PHY), which are apoproteins covalently bound to the chromophore, primarily detect and interpret the levels and ratio of red and far-red light in the environment. In darkness, phytochrome is synthesized as the physiologically inactive red-absorbing form, Pr. Upon illumination with red light, Pr is converted to the active far-red absorbing form, Pfr, which can be transformed back to Pr by far-red light. In the dark, Pfr-active phytochrome reverts to the inactive Pr form in a process referred to as “dark reversion” (Butler and Lane 1965). These interconvertible forms provide the plant with a cellular switch that can interpret information on spectral quantity and quality into a suitable response.

The diversity of phytochromes is organized in three major clades of PHYA, PHYB, and PHYC, with the former two present in all studied seed plant taxa (Mathews 2010). Plant phytochromes have been intensively studied, since they contribute to various developmental processes in plants such as flowering, shade avoidance, dormancy, germination, and stomatal development (Franklin and Quail 2010). Mutations in phytochrome genes affect flowering time in Arabidopsis, sorghum, and rice (Childs et al. 1997; Takano et al. 2005; Balasubramanian et al. 2006; Saidou et al. 2009; Osugi et al. 2011; Hu et al. 2013). However, very little is known about the diversity, function, and signaling pathways of barley phytochromes. Szücs et al. (2006) mapped the barley orthologs of PHYA (HvPHYA) and -B (HvPHYB) both to the short arm of chromosome 4H, and PHYC (HvPHYC) to the long arm of chromosome 5H at the same location as the vernalization gene Vrn-H1. According to Hanumappa et al. (1999), a chemically mutagenized barley genotype BMDR-1 contains a light-labile phyB. They demonstrated that it was responsible for the photoperiod insensitivity of this genotype and additionally implicated phyA in regulation of flowering via a distinct but interrelated pathway. A recent study in barley has suggested that variation at HvPHYC, an amino-acid substitution in the GAF domain, affected flowering time only under LDs and independently of the circadian clock (Nishida et al. 2013).

We identified the same candidate mutation in HvPHYC, which underlies the early maturity 5 (eam5) locus, using whole-exome capture and a mapping-by-sequencing approach (Schneeberger et al. 2009; Mascher et al. 2013a) applied on a backcross population between the spring barley Bowman and the introgression line Bowman(eam5) (Druka et al. 2011). We add important information to the recent findings of Nishida et al. (2013) by demonstrating that HvPHYC-eam5 genetically interacts with the major photoperiod response gene Ppd-H1 to accelerate flowering under LDs and in particular under SDs. In contrast to findings by Nishida et al. (2013), expression analyses showed that HvPHYC-eam5 disrupts the circadian clock and acts in the same pathway as the evening complex genes HvELF3 and HvLUX1. Diversity analysis indicated the presence of two major HvPHYC haplotypes separated by a synonymous (s) SNP and reduced nucleotide diversity at this locus. Interestingly, the HvPHYC-eam5 allele was selected in barley cultivars from Japan despite the strong effect of this mutation on the barley clock. This invites further research into comparing physiological effects and the overall significance of the circadian clock on plant adaptation.

Materials and Methods

Plant material and growth conditions

Flowering time (days to awn emergence on the main spike) of the spring cultivar Bowman and three Bowman-derived introgression lines Bowman(Ppd-H1), Bowman(eam5), and Bowman(Ppd-H1 + eam5) (kindly provided by R. Waugh, James Hutton Institute and by David Laurie, John Innes Centre) was recorded for 10–15 plants per genotype. To score flowering, plants were grown in soil in a glasshouse under SDs (10 hr light, 20°:14 hr dark, 18°) and LDs (16 hr light, 20°:8 hr dark, 18°). To investigate expression levels of HvFT1, replicate leaves were sampled 2 hr before light off
14 days and 28 days after sowing (DAS) under LDs and SDs, respectively. In addition, expression changes during development were analyzed in replicate samples of Bowman and Bowman(eam5) harvested once per week (2 hr before light off) for 3 weeks under LDs and 9 weeks under SDs. Diurnal and circadian expression of core clock and flowering time genes was tested under SDs (8 hr light, 20°:16 hr dark, 18°) and free-running conditions in Bowman and Bowman(eam5) grown in a controlled environment growth chamber (CEGC). After 14 days under SDs, leaf material was harvested every 2 hr for a total of 24 hr from the start of the light period (time point T0). Night samples (T10–T22) were collected in the dark. After SDs, plants were released into continuous light (light-light, LL) and constant temperature (20°) and sampled every 2 hr or 4 hr for 48 hr starting after 8 hr of continuous light (T8). Two biological replicates, comprising the second youngest leaves of three independent plants, were analyzed. In addition, expression of HvCCA1 was analyzed in the barley cultivars Azumamugi (F380S), Hayachinemugi (F380S), and its parental lines Kaikei 84 (F380S) and Yukiwarimugi (without F380S) under constant conditions after entrainment under SDs.

Meristem development was scored in Bowman and Bowman(eam5) grown in soil in a CEGC. The main stem of three plants per genotype was dissected starting 16 days after germination every 2–3 days under LDs and every 3–4 days under SDs until flowering. The experiment was stopped after 70 days under SDs when the shoot apical meristem (SAMs) of Bowman(eam5) had flowered, and those of Bowman did not further develop. Meristem development was scored following the Waddington scale (Waddington 1983).

To investigate natural diversity of the candidate gene, a set of 110 wild and cultivated barley genotypes were selected from germplasm collections at the Max Planck Institute of Plant Breeding Research (MPIPZ) in Cologne (Badr et al. 2000), the barley 1K collection (Hübner et al. 2009), the barley Germplasm Center at Okayama University (http://www.shigen.nig.ac.jp/barley), and the University of Curkurova, Turkey (Hakan Özkan) (Supporting Information, Table S3).

**Gene expression analysis**

Gene expression was analyzed in leaf samples harvested from Bowman, Bowman(Ppd-H1), Bowman(eam5), and Bowman(Ppd-H1 + eam5) grown under LD and SD conditions, from a developmental series of leaf samples from Bowman and Bowman(eam5) and from diurnal and circadian sampling of Bowman and Bowman(eam5) leaves. In addition, expression of HvCCA1 was analyzed in circadian samples of the barley cultivars Azumamugi, Hayachinemugi, Kaikei 84, and Yukiwarimugi. Total RNA extraction, cDNA synthesis, and qRT-PCRs using gene-specific primers were performed as explained in Campoli et al. (2012a,b; 2013) and Habte et al. (2014). Additional primers are listed in Table S4.

**Identification of a candidate gene using mapping by sequencing and segregation analysis**

To determine the position of eam5 on genetic and physical maps, we found closest flanking RFLP markers with known nucleotide sequences using the GrainGenes CMap browser (BinMap 2005; http://wheat.pw.usda.gov/cgi-bin/cmap). Illumina’s Barley Oligo Pool Array (BOPA) markers flanking the Bowman(eam5) introgression were as determined by Druka et al. (2011). The RFLP and BOPA markers were anchored to the Morex genomic contigs (IBSC 2012) using Blastn. Genetic and physical locations of the Morex contigs were extracted from Mascher et al. (2013a) and IBSC (2012), respectively. To refine the candidate region carrying the causative mutation, we used a mapping-by-sequencing approach applied on Bowman, Bowman(eam5), and a pool of 204 BC1F2 lines enriched for early-flowering genotypes. The BC1F2 lines flowering together or close to Bowman (eam5) were selected from 846 BC1F2 lines sown in March 2012 in the field at MPIPZ and scored for heading date together with the parental lines. Genomic DNA was extracted from leaves using the BioSprint 96 kit (Qiagen) according to the manufacturer’s recommendations. DNA samples were quantified using Quant-iT PicoGreen assay (Invitrogen) with the Synergy 4 microplate reader (Biotek). DNA from 204 BC1F2 lines was pooled in equal amounts and along with Bowman, Bowman(eam5) enriched for a 61.6 megabase pair coding sequence target using in-solution whole-exome capture (Roche NimbleGen, Madison, WI; Mascher et al. 2013b).

Illumina sequencing of the enriched libraries generated 93 M, 80 M, and 211 M reads for the Bowman, Bowman(eam5), and the pool of BC1F2 lines, respectively. Reads were trimmed and aligned to the barley reference sequence (IBSC 2012) using BWA 0.59 with default parameters (Li and Durbin 2009). Only those reads that uniquely mapped to the reference sequence were retained. Samtools 0.1.19 was used to generate consensus pileup information (Li et al. 2009). SNPs distinguishing the two parental samples were extracted using VarScan 2.3.5 (Koboldt et al. 2009). SNPs supported by at least 30 reads with the nonreference allele frequency >95% in either Bowman or Bowman(eam5) were considered in downstream analyses. In the BC1F2 pool data, we estimated the frequencies of the Bowman(eam5) SNP alleles. Next, we estimated the median allele frequency for all SNPs within individual reference contigs that were anchored to the barley physical map (IBSC 2013) and calculated a locally weighted scatterplot smoothing (LOWESS) regression. We used SHOREmap 2.1 (http://www.shoremapped.org) to calculate a mapping interval based on the median allele frequencies and the corresponding coefficients of variation (Schneeberger et al. 2009; Galvão et al. 2012).

Barley genes within the candidate mapping interval were extracted using the map published by Mascher et al. (2013a) and characterized by the gene ontology (GO) terms using Blast2GO 2.5 pipeline (Conesa et al. 2005). Genes related to
flowering and circadian clock were selected as candidate genes. To narrow the list of candidate genes down, we designed allele-specific codominant markers (sequence characterized amplified region, SCAR and cleaved amplified polymorphic sequence, CAPS) distinguishing candidate gene alleles from Bowman and Bowman (eam5) and analyzed their segregation with the flowering phenotype using BC1 F2:3 lines. To extract allele-specific polymorphisms in the candidate genes, Bowman and Bowman (eam5) reads were assembled de novo into contigs using ABySS 1.3.7 assembler (single end, k = 25; Simpson et al. 2009). The contigs homologous to the candidate genes were identified using Blastn and aligned to extract SNPs to design allele-specific PCR markers. Allele-specific markers for the barley VIP4-like genes, which were absent from the exome-enrichment assay, were designed based on the Sanger sequencing data of PCR fragments (see primers in Table S4).

To create the BC1 F2:3 population, one seed of each of the early BC1 F2:3 plants was sown in the greenhouse under 10-hr short days. Flowering time was scored and leaf material was harvested for DNA extraction and genotyping. PCR reactions [1 × Colorless GoTaq Buffer, 0.2 μM dNTPs, 0.5 μM primers, 1 unit GoTaq polymerase (Promega, Mannheim, Germany), 50 ng DNA] were incubated in the PTC-200 DNA Engine thermocycler (Bio-Rad, Hercules, CA) and visualized using agarose gel electrophoresis. The primer sequences and incubation regimes were as in Table S4. Restriction of the CAPS PCR fragments was performed using endonucleases (New England Biolabs, Frankfurt am Main, Germany) following the manufacturer's recommendations.

Natural diversity and population-genetic analyses

Barley genomic DNA was extracted using the DNAeasy 96 Plant kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). A 2045-bp fragment covering exon 1 of HvPHYC was amplified from a set of 113 wild and cultivated barley genotypes using the primer pairs Ex1seq_1f + Ex1seq_1r and Ex1seq_2f + Ex1seq_2r (Table S4). PCR reactions [1 × Q5 buffer, 0.2 μM dNTPs, 0.5 μM primers, 1 unit Q5 High-Fidelity DNA Polymerase (New England Biolabs), 50 ng DNA] were incubated in a PTC-200 DNA Engine thermocycler (Bio-Rad). PCR fragments were purified using 1.8× Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany) following the manufacturer's recommendations and Sanger sequenced. Three additional HvPHYC sequences were extracted from National Center for Biotechnology Information (NCBI) GenBank (DQ201145, DQ201146, and DQ238106). Haplotype analysis was performed as described in Campoli et al. (2013). Nucleotide diversity π was calculated for the coding region using DnaSAM v. 20100621 (Eckert et al. 2010).

Motif conservation analysis and protein modeling

Positions of the GAF and PAS domains at HvPHYC were determined using InterProScan (Quevillon et al. 2005). A set of 4419 protein sequences of the PHY homologs from plant and bacterial species were extracted using Blastp search in the NCBI “nr” database with a 70-bp conserved fragment of the HvPHY GAF domain as a query (cut-off e-value 1e-7). The PHY homologs were aligned using MAFFT v6.851b (“auto” model selection) and PRANK v.130820 (default parameters, +F) (Loytynoja and Goldman 2005; Katoh and Toh 2008). Amino-acid polymorphisms at HvPHYC were discovered by translating exon 1 fragments comprising nonsynonymous (ns) SNPs using ExPASy Translate tool (http://web.expasy.org/translate). The 8- to 15-aa subalignments around these polymorphic sites were submitted to the WebLogo generator (Crooks et al. 2004). Visually misaligned regions flanking the polymorphic amino-acid residues outside the GAF domain were iteratively realigned in a smaller subset of the PHY sequences. In addition, the functional effect of the amino-acid substitutions was predicted using PROVEAN (cut-off score −2.5) (Choi et al. 2012).

Structural modeling was carried out with the I-TASSER approach (Zhang 2008). Models were generated for the chromophore-bearing region of HvPHYC for both the Bowman and Bowman(eam5) alleles. Structural alignment of the models was performed with the University of California San Francisco Chimera package (Pettersen et al. 2004).

Statistical analysis

Significant differences in flowering time, meristem development, and gene expression between Bowman, Bowman(eam5), and Bowman(eam5 + Ppd-H1) were calculated using a paired t-test (P < 0.05). For diurnal and circadian gene expression, statistical significance was calculated using a general linear model in the SAS software 9.1.3 (SAS Institute 2009) with the factors genotype, time point, biological replicate, and first-order interaction effects. Significant differences (P < 0.05) between least-squares means of the genotype-by-time interactions were calculated using a Tukey–Kramer adjustment for multiple comparisons.

Results

Bowman(eam5) is early flowering under SD and LD conditions

We analyzed the effects of eam5 on photoperiod-dependent flowering in barley and its genetic interaction with the major barley photoperiod response gene Ppd-H1. Bowman, Bowman (eam5), Bowman(Ppd-H1), and Bowman(Ppd-H1 + eam5) plants were scored for flowering time under LD and SD conditions in controlled greenhouse settings (Figure 1A). Under LDs, Bowman(Ppd-H1) and Bowman(Ppd-H1 + eam5) flowered first, both at 28 DAS. Bowman(eam5) flowered at 42 DAS, followed by Bowman at 45 DAS. Under SDs, Bowman (Ppd-H1 + eam5) flowered 57 DAS, followed by Bowman (eam5) with 65 DAS, while Bowman and Bowman(Ppd-H1) flowered on average 88 and 91 DAS, respectively. Thus eam5 accelerated flowering time under both LD and SD conditions.
It is notable that, under SDs, Ppd-H1 accelerated flowering time in the background of eam5. We determined the effect of eam5 on the development of the SAM by scoring its morphological changes in Bowman and Bowman(eam5) plants starting 2 weeks after germination until heading (Figure S1). Under both LD and SD conditions, the SAM of Bowman(eam5) developed significantly faster than that of Bowman. Under SDs, differences in SAM development between the genotypes became manifest only after Waddington stage 4: the SAM of Bowman(eam5) developed until flowering, while the SAM of Bowman remained at this stage until 70 DAS. Under LDs, the SAM started to develop significantly faster in Bowman(eam5) than in Bowman after Waddington stage 3. These differences in SAM development suggested that eam5 primarily affects inflorescence development and stem elongation.

To test whether differences in the expression of HvFT1 correlated with the observed flowering-time phenotypes, we measured its expression in the different Bowman introgression lines under LDs and SDs, respectively (Figure 1, C and D). After 2 weeks under LDs, HvFT1 showed a low but detectable expression in Bowman and Bowman(eam5), which was significantly lower than HvFT1 expression in Bowman(Ppd-H1) and Bowman(Ppd-H1 + eam5). After 4 weeks under SDs, HvFT1 expression was detected only in Bowman(Ppd-H1 + eam5) and Bowman(eam5). Therefore, we suggest that the eam5 mutation accelerated flowering time under both LDs and SDs through an upregulation of HvFT1.

Identification of barley PHYC as a candidate gene underlying eam5

The eam5 locus was described as a mutation of unknown origin isolated from an ICARDA/CIMMYT selection CMB85-533 (Hguerilla*2/Gobernadora) and mapped onto chromosome 5H (Jain 1961; Franckowiak 2002). Like many other barley QTL, eam5 has been introgressed into the spring barley cultivar Bowman (Druka et al. 2011). The resultant BC6 introgression line Bowman(eam5) (also referred to as BW285) was genotyped using the SNP-based array (Illumina's BOPA) with 3072 SNPs (Close et al. 2009). This revealed a single
introgression on chromosome 5H of Bowman(eam5) flanked by the BOPA markers 2_0533 (9.3 cM as morex_contig_64122) and 1_0336 (149.8 cM as morex_contig_2550061). eam5 was mapped between RFLP markers MWG522 (80.3 cM as morex_contig_2549712) and MWGS83 (89.9 cM as morex_contig_1583223) (Figure 2A). To delineate the candidate gene underlying the eam5 mutation, we backcrossed the introgression line Bowman(eam5) with Bowman and scored heading date in the field. Of the 846 phenotyped BC1F2 lines, 204 genotypes were selected as flowering at the same time as Bowman(eam5), which flowered on average 4 days earlier than Bowman and the remaining population.

To refine the eam5 interval, we used a mapping-by-sequencing approach applied on the parental lines Bowman and Bowman(eam5) and the pool of BC1F2 lines enriched for early flowering genotypes. We reduced the complexity of the barley genome using whole-exome capture (Mascher et al. 2013b), which targeted sequencing on the gene space. On
average, 92% of the Illumina reads aligned against the targeted regions and were used to discover SNPs between the samples and the reference sequence. We identified 3884 SNPs that were specific for either Bowman or Bowman(eam5). Of those, 2929 SNPs resided in 640 contigs located on the barley physical map (IBSC 2012). To estimate Bowman (eam5) allele frequency at each of these marker sites within the pool of BC1F2, we used SHOREmap (Schneeberger et al. 2009). Using the median-allele frequency of SNPs within each contig and their physical location, it identified a probabilistic QTL mapping interval of 8 Mb located on the chromosome arm 5HL and comprising 210 genes according to the most current POPSEQ barley map (Figure 2B; Mascher et al. 2013a). The maximum SNP allele frequencies of Bowman(eam5) approached 75% due to the presence of heterozygous genotypes in the pool of BC1F2 lines selected from the field experiment. The presence of heterozygotes in the pools was verified by phenotyping and genotyping in BC1F2:3 lines derived from the pooled plants as explained below.

The GO analysis identified five candidate genes related to flowering time or circadian clock within this 8-Mb interval. Four of them, HvPHYC (MLOC_824), VRN-H1 (AK360697), HvVIP4.1 (MLOC_17672), and HvVIP4.2 (MLOC_17943), which are, respectively, homologs of Arabidopsis genes PHYC (AT5G35840), APETALA 1 (AT1G691120), and VERNALIZATION INDEPENDENCE 4 (AT5G61150), mapped to the same location on the chromosome arm 5HL. The fifth gene HvCK2α (MLOC_55943), a homolog of Arabidopsis CASEIN KINASE 2 ALPHA (AT2G23070), resided 2 Mb downstream of this cluster of four genes. Another flowering-related gene HvPIF-like (AK362162), a homolog of Arabidopsis PHYTOCHROME-INTERACTING FACTOR genes, located 9 Mb upstream of the unresolved cluster of the four candidate genes was also selected for the fine mapping.

Segregation analysis of the candidate genes was conducted in BC1F2:3 lines, derived from the early flowering BC1F2 plants. Analysis of flowering time in 180 BC1F2:3 under SD conditions in the glasshouse revealed that the phenotype followed a trimodal distribution. A total of 108 lines were scored as early, which flowered within 10 days after Bowman (eam5), whereas 17 lines flowered significantly later frequently showing abnormalities in the spike development. The rest of the 55 lines did not flower until the end of the experiment. Therefore, we suggested that eam5 was a semidominant locus with the heterozygote exhibiting an intermediate phenotype.

We reconstructed Bowman and Bowman(eam5) alleles of the candidate genes from the de novo assembly of the exome reads. Allele-specific polymorphisms were tagged with codominant SCAR and CAPS markers used for screening of the BC1F2:3 lines. The segregation analysis revealed that both Vrn-H1 and HvPHYC were tightly linked to the early flowering phenotype; all plants carrying Bowman(eam5) alleles at these genes flowered, plants with Bowman alleles did not flower until the end of the experiment, and heterozygotes exhibited an intermediate phenotype. No recombinants were detected between Vrn-H1 and HvPHYC (Figure 2C). Vrn-H1 and HvPHYC reside only five genes models apart based on the better resolved Brachypodium map. Using allele-specific markers reported by Hemming et al. (2009), we discovered that Bowman carries the HvVRN1-1 spring allele with a 5154-bp deletion in the first intron, whereas Bowman(eam5) contains the HvVRN1 winter allele without an intron deletion. This winter allele is known to strongly delay flowering in the absence of vernalization (Hemming et al. 2009).

HvPHYC carried a nonsynonymous mutation (T/C) in Bowman(eam5). This caused the missense substitution that leads to a change of the hydrophobic phenylalanine to the hydrophilic serine (mutation F380S) within a previously uncharacterized extremely conserved motif in the GAF domain of phytochromes (Figure 3). At this position, phenylalanine is exclusively present in 4267 phytochrome homologs from 2799 species of Plantae and Bacteria kingdoms present in GenBank. The conservation analysis using the PROVEAN tool predicted that the F380S mutation could be functional (observed score –7.673; cut-off score –2.5). Taken together, our analyses strongly suggested that the F380S substitution could be critical for the HvPHYC function and thus we propose HvPHYC as the candidate gene underlying eam5.

Diversity analysis of HvPHYC and its linkage with VRN-H1 alleles

To explore natural diversity of barley HvPHYC alleles, we sequenced a 2045-bp fragment of the first exon comprising conserved domains from 52 wild (H. vulgare L. subsp. spontaneum (K. Koch) Thell.), 56 cultivar and landrace, and 3 H. agriocriton A. E. Åberg genotypes. In addition, three HvPHYC sequences from cultivars were extracted from the NCBI GenBank. We identified 15 haplotypes, of which 6 haplotypes were specific for cultivated accessions, 6 haplotypes for wild, and 3 haplotypes were common to both groups (Figure 4; Table S1). The nucleotide diversity of HvPHYC haplotypes, which were defined by 13 ns and 8 s SNPs within the coding region, was low (π = 0.46 × 10⁻³). Haplotypes 1 and 2 were most frequent; 84 of 114 genotypes carried these two haplotypes.

We attempted to predict a functional effect of the observed nonsynonymous SNPs based on the protein conservation patterns. The motif conservation analysis revealed two extremely conserved amino-acid substitutions, which were additionally identified by PROVEAN as deleterious (Table S2). One of the mutations, F380S, which was found in Bowman(eam5), also appeared in 10 Japanese cultivars (haplotype 4; Table S3), whereas another mutation, L364D, was found in addition to F380S in Japanese cultivar Azumamugi (haplotype 7).

Based on the result of the segregation analysis, we assumed that the Bowman(eam5) HvPHYC allele and wild-type HvVRN1 are tightly linked. To verify this fact, we screened 10 other “haplotype 4” genotypes with markers specific for the wild-type HvVRN1 allele. Without exception, the Bowman(eam5) HvPHYC allele was linked to the winter type HvVRN1 allele (Table S3).
Previous studies have shown that the circadian clock mediates light signaling to downstream components of the photoperiod-response pathway in Arabidopsis and barley (Onai and Ishiura 2005; Faure et al. 2012; Campoli et al. 2013). To investigate whether the eam5 mutation affected the barley circadian clock, we studied diurnal (under SDs) and circadian LL expression of barley clock homologs, HvCCA1, Ppd-H1 (HvPpR37), HvPpRR73, HvPpRR59, HvPpRR95, HvGI, HvELF3, HvELF4, and HvLUX1 in Bowman(eam5) (Figure 5A, Figure S2).

Under SDs, Bowman(eam5) showed significantly reduced expression of HvPpRR73, HvELF3, HvELF4, and HvPHYC compared to Bowman at the peak time of expression. The SD expression of all other tested clock genes was not significantly different between genotypes. Under LL conditions, the expression patterns of HvCCA1, Ppd-H1 (HvPpR37), HvPpRR73, HvGI, and HvPpRR1 were significantly different between Bowman and Bowman(eam5). Under these conditions, in Bowman(eam5), circadian amplitude of HvCCA1 expression was strongly reduced during the subjective days, while Ppd-H1 was significantly upregulated in Bowman(eam5) compared to Bowman at most time points. In contrast to Ppd-H1, expression of its homolog HvPpR73 was significantly lower in Bowman(eam5) than in Bowman during the subjective day. Expression of the evening expressed genes HvGI and HvPpRR1 was significantly higher in Bowman(eam5) than in Bowman during subjective nights. Finally, HvPHYC expression was significantly lower in Bowman(eam5) during the subjective day. Circadian expression of HvLUX1, HvPpRR59, HvPpRR95, HvELF3, and HvELF4 was not strongly affected by the eam5 mutation. In Bowman(eam5), peak expression of HvCCR2, encoding the barley ortholog of the GRP77/CCR2), characterized as a slave (nonself-sustaining) oscillator (Schöning and Staiger 2005), was reduced under SD and LL compared to Bowman (Figure 5B). Taken together, these results indicate that eam5 alters the expression of barley homologs of Arabidopsis clock genes, in particular the expression of HvCCA1 and Ppd-H1.

In Arabidopsis, expression of genes implicated in regulation of photoperiod-dependent flowering is under circadian control. We therefore tested whether eam5 changed the diurnal and circadian expression of the photoperiod response genes HvCO1, HvFT1, and Vrn-H1 (Figure 5B). Under SDs, HvCO1 was significantly higher expressed in Bowman(eam5) than in Bowman in SDs and LL (Figure 5B). HvFT1
expression was not detected under SDs in any of the two genotypes at 14 DAS, whereas Bowman (eam5) exhibited a strong upregulation of HvFT1 during the subjective day in LL compared to Bowman. The spring Vrn-H1 allele was expressed and showed circadian oscillations in Bowman, but the winter HvVRN1 allele in Bowman (eam5) was not expressed at 14 DAS under SDs (Figure 5B). This corroborates our suggestion that HvVRN1 was not involved in the early flowering of Bowman (eam5). In addition, we have previously shown that variation between the winter and spring HvVRN1 alleles did not affect the circadian clock (Faure et al. 2012). Taken together, flowering time data and the expression analysis excluded HvVRN1 as a gene underlying eam5.

To corroborate the link between eam5/HvPHYC and the circadian clock, we examined different lines carrying the HvPHYC F380S allele (haplotypes 4 and 7) for variation in the circadian expression of the marker gene HvCCA1 (Figure S3). The expression of HvCCA1 in Hayachinemugi and Kaikei 84 (both with F380S, haplotype 4) was markedly dampened during the subjective day compared to Yukiwarimugi (without F380S). Therefore, the effect of F380S in Japanese lines was similar to the HvCCA1 expression differences between Bowman and Bowman (eam5). Expression of HvCCA1 in Azumamugi (with F380S and L364D, haplotype 7) was also significantly lower than in Yukiwarimugi, but higher than in Kaikei 84 and Hayachinemugi. Based on these data, we conclude that the F380S mutation in HvPHYC correlated with variation in circadian expression of HvCCA1 also in Japanese cultivars. However, quantitative variation in HvCCA1 expression between different HvPHYC F380S lines suggested the presence of modifying genes.

Discussion

**HvPHYC is a candidate gene underlying eam5**

In this study, we describe the barley locus eam5, which accelerated flowering under LDs and in addition, led to flowering under noninductive SDs. To fine map the eam5 mutation, we used mapping-by-sequencing of bulked early flowering BC1F2 lines, followed by candidate-gene mapping in BC1F2. Fine mapping-by-deep sequencing has been successfully applied in model species to map and identify
Figure 5 Expression patterns of circadian clock and clock output genes. Expression of (A) circadian clock genes HvCCA1, Ppd-H1 (HvPRR37), HvGI, HvPRR1, HvPRR73, HvELF3, HvELF4, and HvPHYC and (B) the clock output gene HvCCR2 and the flowering genes HvCO1, HvVRN1, and HvFT1 in Bowman (black lines) and Bowman(eam5) plants (red lines) under SD and constant conditions. White, black, and gray bars indicate days, nights, and
induced mutations underlying a specific phenotype in a single step (James et al. 2013). We demonstrate that this method is also effective in fine mapping a mutation in the large genome of a crop plant. We found that the use of an introgression line with prior mapping information and exome enrichment greatly reduced the complexity of the task (Druka et al. 2011; IBSC 2012; Mascher et al. 2013b). Mascher et al. (2013b) have demonstrated that fine mapping of barley genes has become feasible due to the recent release of the barley gene space reference sequence and advances in the physical and genetic mapping (IBSC 2012; Mascher et al. 2013a). As a proof of concept, using a simulated in silico bulk-segregant analysis, they showed that a qualitative row-type gene Vrs1 could be fine mapped to a relatively small interval containing 128 genes (Mascher et al. 2013b). We demonstrate that fine mapping through exome capture and deep sequencing of a BC1F2 pool was successful, even though the phenotype was quantitative, subtle, and obscured by the segregation of another tightly linked flowering gene. The identification of a mutation in the extremely conserved motif of the PHY GAF domain strongly suggested that HvPHYC is the gene underlying the eam5 locus. Protein modeling revealed that the amino-acid change occurred at a prominent position in the GAF domain at the end of a helix coming to the chromophore pocket, potentially affecting conformational flexibility of the protein (Figure S4). A mutation in this region causes a period lengthening, whereas overexpression of phytochromes control the period of the circadian clock; deficiency of phytochromes causes a period shortening, whereas overexpression of phytochromes results in a period shortening (Somers et al. 1998; Hu et al. 2013). Additionally, Hu et al. (2013) demonstrated that phyABDE and phyABCDE Arabidopsis mutants retained robust clock rhythms and thus argued that phytochromes are not required for clock maintenance. By contrast, we showed that a mutation in HvPHYC was linked to the disruption of circadian oscillations of barley clock genes. Our results offer new perspectives on the role of PHYC in controlling the circadian clock and downstream light signaling pathways. As has been shown for Bowman(eam8) and Bowman (eam10) (Faure et al. 2012; Campoli et al. 2013), early flowering of Bowman(eam5) was associated with an upregulation of HvFT1 under both LD and SD conditions (Figure 2). Consequently, eam5 caused an induction of the LD photoperiod pathway under noninductive SD conditions. Interestingly, a recent study in wheat demonstrated that the loss of a functional Phyc resulted in a drastic downregulation of PPD1 and TaFT1 and late flowering under LDs and SDs (Chen et al. 2014). This is in line with our findings, which suggest that Phyc is important in light perception or signaling to downstream components of the photoperiod pathway.

HvPHYC modulates the circadian clock

The F380S mutation did not correspond to any known loss- or gain-of-function phytochrome allele in Arabidopsis (Nagatani 2010). However, the mutation was identical to the HvPHYC-e allele recently described in the Japanese winter barley Hayakiso 2 (Nishida et al. 2013). The authors of that study found that this allele accelerated flowering time under 16-hr and 20-hr LDs, but not under 12-hr SDs. However, we observed the strongest effect of eam5 under 8-hr SDs, where flowering time of Bowman(eam5) and Bowman differed by >60 days. It is noteworthy that the loss-of-function PHYC mutants in Arabidopsis and rice also showed acceleration of flowering in noninductive photoperiods (Monte et al. 2003; Takano et al. 2005; Balasubramanian et al. 2006).

Osugi et al. (2011) have shown that light signals mediated by the PHYB/PHYC heterodimer in rice can induce expression of Ghd7, a floral repressor of Hd3, which is the rice ortholog of FT, and thus delay flowering. They also argued that phytochromes do not control the gating of Ghd7 and therefore do not affect flowering through entrainment of the circadian clock. Likewise, the complete loss of three phytochromes in rice did not influence the expression of circadian-clock genes, while it affected the expression of Hd3 (Izawa et al. 2002). Similarly, Nishida et al. (2013) reported that, in barley, HvPHYC F380S (HvPhyC-e) caused an upregulation of HvFT1 under LDs, but did not change the expression of clock genes under the diurnal LD regime. Hence they suggested that HvPHYC F380S does not affect the circadian clock. Based on the expression patterns of clock genes measured under circadian LL conditions, we conclude that HvPHYC F380S markedly disturbed the circadian expression of clock genes in Bowman(eam5) and the Japanese cultivars carrying the F380S mutation. For example, HvCCA1 did not oscillate under LL conditions. Changes in clock gene expression, especially the absence of HvCCA1 oscillations in Bowman (eam5), are reminiscent of expression changes observed in the eam8 and eam10 mutants carrying mutations in HvELF3 and HvLUX1, respectively (Faure et al. 2012; Campoli et al. 2013; Figure S5).

It is known that Arabidopsis phytochromes control the period of the circadian clock; deficiency of phytochromes causes a period lengthening, whereas overexpression of phytochromes results in a period shortening (Somers et al. 1998; Hu et al. 2013). Additionally, Hu et al. (2013) demonstrated that phyABDE and phyABCDE Arabidopsis mutants retained robust clock rhythms and thus argued that phytochromes are not required for clock maintenance. By contrast, we showed that a mutation in HvPHYC was linked to the disruption of circadian oscillations of barley clock genes. Our results offer new perspectives on the role of PHYC in controlling the circadian clock and downstream light signaling pathways.

As has been shown for Bowman(eam8) and Bowman (eam10) (Faure et al. 2012; Campoli et al. 2013), early flowering of Bowman(eam5) was associated with an upregulation of HvFT1 under both LD and SD conditions (Figure 2). Consequently, eam5 caused an induction of the LD photoperiod pathway under noninductive SD conditions. Interestingly, a recent study in wheat demonstrated that the loss of a functional Phyc resulted in a drastic downregulation of PPD1 and TaFT1 and late flowering under LDs and SDs (Chen et al. 2014). This is in line with our findings, which suggest that Phyc is important in light perception or signaling to downstream components of the photoperiod pathway.

In Arabidopsis, ELF3 together with ELF4 and LUX form the so-called evening complex (EC) and repress transcription...
of PRRs (Dixon et al. 2011; Herrero et al. 2012). The transcriptional targets of the EC genes seem conserved in barley. HvELF3 and HvLUX1 act as repressors of the barley PRY gene Ppd-H1, which in turn affects HvFT1 expression and flowering time (Faure et al. 2012). Indeed, we observed genetic interactions between ema5 and Ppd-H1 as demonstrated for ema8 and ema10 (Figure 1). Additionally, upregulation of Ppd-H1 in Bowman(eama5) suggested that ema5, like ema8 and ema10, controlled the expression of this gene (Figure S5). In this context, it is interesting to note that variation at ema5 had similar effects on SAM development as variation at Ppd-H1. Both affected primarily inflorescence development (Figure S1, Campoli et al. 2012b).

Similarities in the effects of ema5, ema8, and ema10 on the expression of circadian clock genes and HvFT1 (Figure S5) suggest that all three genes may act in the same pathway. This is in line with the findings that phytochromes modulate the function of ELF3 in Arabidopsis. There it is noted that PHYB interacts with the ELF3 protein and represses its activity (Liu et al. 2001; Kolmos et al. 2011). We thus speculate that HvPHYC might modulate the repressive activity of the evening complex genes. However, further mechanistic evidence is required to solidify the hypothesis that HvPHYC F380S affects accumulation and activity of the HvELF3 protein.

To investigate genetic diversity at HvPHYC, we resequenced in addition to Bowman and Bowman(eama5) a diverse set of 109 HvPHYC genotypes and added three HvPHYC alleles from the GenBank. The prevalence of two major haplotypes and the nucleotide diversity index, which was 2 to 16 times lower than reported for other barley genes (Russell et al. 2004; Morrell et al. 2005, 2013; Kilian et al. 2006; Xia et al. 2012), indicated that PhyC was conserved and presumably under selective constraints. It is tempting to speculate that reduced nucleotide diversity at HvPHYC could be an indirect effect of its tight linkage to VRN-H1, which mediates an adaptive trait such as vernalization response (Beales et al. 2005; Hemming et al. 2009). It is intriguing that the mutant PhyC380 allele was detected in 11 genotypes with a common geographic origin in Japan, suggesting that this mutant allele was targeted by breeders.

Molecular taxonomists have widely used phytochromes in phylogenetic studies (Mathews et al. 1995). This led to the accumulation of a large number of PHY sequences from very diverse plant and bacteria species. We used these data to infer a functional effect of the observed amino-acid substitutions based on the extent of their conservation. Remarkably, except for the two amino-acid substitutions, L364D and F380S (haplotypes 4 and 7), which we linked to the functionally distinct HvPHYC alleles, other substitutions were within variable motifs and thus presumably nonfunctional.

In summary, we successfully applied a mapping-by-sequencing approach to pinpoint a mutation in HvPHYC as a candidate underlying the ema5 locus in barley. We demonstrated that this mutation disrupts the circadian clock and results in an acceleration of flowering under LDs and noninductive SDs. Such interaction of phytochromes and the circadian clock has not been reported before and opens new perspectives on the role of PHYC in controlling the circadian clock and downstream light signaling pathways. We demonstrate that HvPHYC is characterized by low levels of genetic diversity in wild and cultivated barley germplasm. Interestingly, HvPHYC F380S was selected in cultivars from Japan, and may thus provide a selective advantage in these environments.

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Mapping-by-Sequencing Identifies *HvPHYTOCHROME C* as a Candidate Gene for the *early maturity 5* Locus Modulating the Circadian Clock and Photoperiodic Flowering in Barley

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**Figure S1** Meristem development in wild type Bowman and Bowman(eam10) mutant plants. Apex development assessed by the Waddington scale (WS) under (A) long- (LD; 16 h) and (B) short- (SD; 8 h) day conditions in Bowman (black lines) and Bowman(eam5) (red lines). Double ridge formation (WS2) and the initiation of internode elongation (WS3) are indicated by full and dashed lines, respectively. Values are means of three plants plus/minus standard deviation. Significant differences in meristem development are indicated by asterisks (*P < 0.05).
Figure S2  Expression patterns of circadian clock genes. Expression of HvPRR59, HvPRR95 and HvLUX1 in Bowman (black lines) and Bowman (eam5) plants (red lines) under short-day and continuous-light conditions. White, black and grey bars indicate days, nights and subjective nights, respectively. Expression values represent averages of two biological and two technical replicates relative to HvActin plus/minus standard deviation. Significant differences in gene expression are indicated by asterisks (*P < 0.05).
Figure S3  Expression levels of HvCCA1 in WT and mutant HvPHYC F380S lines. Expression of HvCCA1 in Azumamugi (orange), Hayachinemugi (green) and its parental lines Kaikai 84 (purple) and Yukiwarimugi (blue). Full and dashed lines indicate genotypes with a wild type or a mutated HvPHYC allele, respectively. Plants were grown under short days for two weeks, and then released into continuous light. Samples were taken every 4 hours starting from the beginning of the first subjective night (T8). White and grey bars indicate days and subjective nights, respectively. Expression values are averages of two biological and two technical replicates relative to HvActin plus/minus standard deviation.
Figure S4  Structural model from I-TASSER of barley HvPHYC. In red is the wild type model and this is overlaid in blue with the eamS-derived variant (HvPHYC F380S). The amino-acid change is at the end of an alpha helix and outside of the chromophore binding pocket.
Figure S5  Expression profiles of the clock genes in eam5 and previously described clock mutants. Expression of HvCCA1 and Ppd-H1 (HvPRR37) in Bowman (black lines), Bowman(eam5) (red lines), Bowman(eam10) (blue lines; Campoli et al., 2013) and Bowman (eam8) (green lines; Faure et al., 2012). Plants were grown under short days for two weeks, and then released in continuous light. Samples were taken every 2 hours starting from the beginning of the first subjective night (T8). White and grey bars indicate days and subjective nights, respectively. Values represent average of two biological and two technical replicates of expression values relative to HvActin plus/minus standard deviation.
Table S1  Fifteen polymorphic haplotypes of *HvPHYC*.

| Haplotypes | 99 | 2006 | 2009 | 2010 | 2015 | 2018 | 2019 | 2021 | 2022 | 2023 | 2024 | 2025 |
|------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
|            | Position (bp) relative to the start codon of the reference Morex *HvPHYC* gene (DQ238106) |          |          |          |          |          |          |          |          |          |          |          |
| 1          | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 70 |
| 2          | - | - | - | - | - | - | - | - | - | - | - | - | - | T | 14 |
| 3          | - | - | - | - | - | - | A | - | - | - | - | - | - | T | 4 |
| 4          | - | - | - | - | - | - | - | - | - | - | - | - | - | C | 11 |
| 5          | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | 2 |
| 6          | - | - | - | - | - | - | - | - | - | - | - | - | G | - | - | 2 |
| 7          | - | - | - | - | - | - | C | - | C | - | - | - | - | - | - | 1 |
| 8          | - | - | - | - | - | - | - | - | - | - | - | - | G | - | - | 1 |
| 9          | - | - | A | C | - | - | - | - | - | A | - | - | - | - | - | 1 |
| 10         | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| 11         | - | - | - | - | T | - | T | A | - | - | T | - | - | - | A | 1 |
| 12         | - | - | - | - | - | - | - | - | - | - | - | - | T | A | - | 1 |
| 13         | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 3 |
| 14         | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| 15         | - | - | - | - | - | - | T | - | - | - | - | T | - | - | - | 1 |

\[ n = 114 \]

Non-synonymous single nucleotide polymorphisms (nsSNP) are highlighted in red. Position of the nsSNP found in *HvPHYC* from Bowman(eam5) is underlined.
Table S2  Non-synonymous SNPs in the exon 1 of HvPHYC and conservation of the corresponding amino-acid (a.a.) residues.

| SNP ID | A.a. substitution | # of sequences in the logo | Motif logo |
|--------|-------------------|-----------------------------|------------|
| 149    | S50Y              | 72                          |            |
| 175    | S59T              | 231                         |            |
| 209-210| Y70F              | 249                         |            |
| 415    | V139I             | 966                         |            |
| 920    | S307L             | 4298                        |            |
| 1027   | I343V             | 4331                        |            |
| 1091   | L364D             | 4340                        |            |
| 1139   | F380S             | 4353                        |            |
| 1403   | K466R             | 4107                        |            |
| 1921   | V641I             | 1610                        |            |
| 1945   | G649S             | 1610                        |            |

1 - SNP positions given from the first nucleotide of the HvPHYC start codon;
2 - Deleterious a.a. substitutions as predicted by PROVEAN (cut-off score <2.5) are highlighted in red;
3 - Substituted a.a. residues marked within the motifs with asterisks; letters in black, green and blue refer to hydrophobic, neutral and hydrophilic a.a. residues, respectively.
Table S3  Accessions used for the *HvPHYC* re-sequencing and haplotype analysis.

| *Hordeum* species | Genotype* | Status     | Growth habit | Origin      | *HvPHYC* haplotype |
|-------------------|-----------|------------|--------------|-------------|--------------------|
| *vulgare* subsp. *vulgare* | Arta      | cultivar   | winter       | Syria       | 1                  |
|                   | Asahi 5   | cultivar   | n.d.**       | Japan       | 4                  |
|                   | Azumamugi | cultivar   | n.d.         | Japan       | 7                  |
|                   | B.E. 22 (ASA) | cultivar | n.d.         | Pakistan    | 2                  |
|                   | B1K-70-01 | cultivar   | spring       | Israel      | 1                  |
|                   | B1K-70-02 | cultivar   | spring       | Israel      | 1                  |
|                   | Bowman    | cultivar   | n.d.         | USA         | 14                 |
|                   | Bowman(eam5) | introgression line | spring | n.d. | 4 |
|                   | Dicktoo   | cultivar   | n.d.         | USA         | 15                 |
|                   | Erectoides 16 | cultivar | n.d.         | Sweden      | 1                  |
|                   | G-391 F   | cultivar   | spring       | Italy       | 2                  |
|                   | G-413 I   | cultivar   | n.d.         | Czech Republic | 1 |
|                   | Ghara 1 (1609) | cultivar | n.d.         | Nepal       | 3                  |
|                   | Hamidiye 85 | cultivar | spring       | Turkey      | 1                  |
|                   | Haruna Nijo | cultivar | spring       | Japan       | 4                  |
|                   | Hayachinemugi | cultivar | n.d.         | Japan       | 4                  |
|                   | Hayakiso 2 | cultivar   | winter       | Japan       | 4                  |
|                   | Hayakiso 3 | cultivar   | n.d.         | Japan       | 1                  |
|                   | Indian dwarf | cultivar | n.d.         | n.d.        | 1                  |
|                   | Indo Omugi | cultivar   | spring       | Taiwan      | 1                  |
|                   | Ishuku Shirazu | cultivar | spring       | Japan       | 4                  |
|                   | Kagoshima Gold | cultivar | n.d.         | Japan       | 1                  |
|                   | Kaitai 84  | cultivar   | n.d.         | Japan       | 4                  |
|                   | Kanto Nijo 3 | cultivar | n.d.         | Japan       | 4                  |
|                   | Kawasaiogoku | cultivar | spring       | Japan       | 4                  |
|                   | Keel      | cultivar   | spring       | Australia    | 1                  |
|                   | Kinai 5   | cultivar   | n.d.         | Japan       | 1                  |
|                   | Kindoku   | cultivar   | n.d.         | Sweden      | 1                  |
|                   | Kompolti  | cultivar   | n.d.         | Hungary     | 2                  |
|                   | L871      | cultivar   | spring       | Egypt       | 1                  |
|                   | Mari      | cultivar   | n.d.         | Sweden      | 1                  |
|                   | Marthe    | cultivar   | spring       | Germany     | 1                  |
|                   | Mota 4 (1-24-13) | cultivar | n.d.         | Ethiopia    | 1                  |
|                   | Mota 6 (1-24-15) | cultivar | n.d.         | Ethiopia    | 1                  |
|                   | Omugi 15  | cultivar   | n.d.         | Japan       | 4                  |
| Variety                          | Type   | Origin     | notes         | Count |
|---------------------------------|--------|------------|---------------|-------|
| Rum                             | cultivar | n.d.        | Jordand       | 1     |
| **Saikai Kawa 24**              | cultivar | n.d.        | Japan         | 4     |
| Shiga Hayakiso 1                | cultivar | n.d.        | Japan         | 1     |
| Sladoran                        | cultivar | winter      | Turkey        | 2     |
| Tadmor                          | cultivar | winter      | Syria         | 1     |
| Tainan 2                        | cultivar | n.d.        | Taiwan        | 1     |
| Turkey 759                      | cultivar | n.d.        | Turkey        | 1     |
| Yarmouk                         | cultivar | n.d.        | Lebanon       | 2     |
| Yercil 147                      | cultivar | winter      | Turkey        | 2     |
| Zairai 1                        | cultivar | n.d.        | Taiwan        | 1     |
| Zairai 2                        | cultivar | n.d.        | Taiwan        | 1     |
| B1K-55-01                       | landrace | n.d.        | Israel        | 2     |
| B1K-55-02                       | landrace | n.d.        | Israel        | 1     |
| B1K-55-06                       | landrace | spring      | Israel        | 2     |
| G-1573 A                        | landrace | n.d.        | Syria         | 1     |
| G-398 H                         | landrace | n.d.        | Ethiopia       | 2     |
| G-400 H                         | landrace | n.d.        | Egypt          | 1     |
| G-404 H                         | landrace | spring      | Tibet          | 3     |
| G-423 H                         | landrace | n.d.        | Ethiopia       | 1     |
| G-434 H                         | landrace | n.d.        | Ethiopia       | 1     |
| G-439 H                         | landrace | spring      | Yemen          | 1     |
| G-440 E                         | landrace | n.d.        | n.d.           | 1     |
| LR 1887                         | landrace | n.d.        | n.d.           | 1     |
| **vulgare subsp. spontaneum**   |        |             |               |       |
| B1K-02-18                       | wild    | n.d.        | Israel        | 9     |
| B1K-03-07                       | wild    | n.d.        | Israel        | 1     |
| B1K-05-13                       | wild    | n.d.        | Israel        | 5     |
| B1K-08-13                       | wild    | n.d.        | Israel        | 5     |
| B1K-08-18                       | wild    | n.d.        | Israel        | 10    |
| B1K-13-01                       | wild    | n.d.        | Israel        | 2     |
| B1K-17-13                       | wild    | n.d.        | Israel        | 1     |
| B1K-21-02                       | wild    | n.d.        | Israel        | 1     |
| B1K-22-06                       | wild    | n.d.        | Israel        | 1     |
| B1K-22-10                       | wild    | n.d.        | Israel        | 1     |
| B1K-33-13                       | wild    | n.d.        | Israel        | 1     |
| HID-1                           | wild    | winter      | Iraq           | 1     |
| HID-10                          | wild    | n.d.        | Iraq           | 1     |
| HID-101                         | wild    | winter      | Syria          | 1     |
| HID-104                         | wild    | n.d.        | Syria          | 13    |
| HID-107                         | wild    | winter      | Jordan         | 2     |
| HID-109                         | wild    | winter      | Syria          | 12    |
| HID-114                         | wild    | n.d.        | Lebanon        | 1     |
| Genotype   | Type  | Season | Country   | Year |
|------------|-------|--------|-----------|------|
| HID-122    | wild  | n.d.   | Jordan    | 11   |
| HID-136    | wild  | winter | Iran      | 1    |
| HID-137    | wild  | n.d.   | Turkey    | 13   |
| HID-140    | wild  | winter | Iraq      | 13   |
| HID-145    | wild  | n.d.   | Israel    | 2    |
| HID-2      | wild  | n.d.   | Iraq      | 1    |
| HID-24     | wild  | n.d.   | Iran      | 3    |
| HID-257    | wild  | n.d.   | Iran      | 1    |
| HID-301    | wild  | n.d.   | Iran      | 1    |
| HID-309    | wild  | n.d.   | Iran      | 2    |
| HID-330-1  | wild  | n.d.   | n.d.      | 1    |
| HID-366    | wild  | n.d.   | Iran      | 1    |
| HID-377-1  | wild  | n.d.   | Israel    | 1    |
| HID-377-2  | wild  | n.d.   | Israel    | 1    |
| HID-44     | wild  | n.d.   | Iran      | 1    |
| HID-46     | wild  | n.d.   | Iran      | 1    |
| HID-54     | wild  | n.d.   | Turkey    | 2    |
| HID-55     | wild  | n.d.   | Turkey    | 1    |
| HID-56     | wild  | n.d.   | Turkey    | 1    |
| HID-70     | wild  | n.d.   | Turkey    | 1    |
| HID-85     | wild  | n.d.   | Turkey    | 1    |
| HID-96     | wild  | n.d.   | Jordan    | 1    |
| HID-99     | wild  | winter | Syria     | 1    |
| HP-02-3    | wild  | n.d.   | Turkey    | 1    |
| HP-03-2    | wild  | n.d.   | Turkey    | 1    |
| HP-10-4    | wild  | n.d.   | Turkey    | 1    |
| HP-10-5    | wild  | n.d.   | Turkey    | 1    |
| HP-11-1    | wild  | n.d.   | Turkey    | 1    |
| HP-13-2    | wild  | n.d.   | Turkey    | 8    |
| HP-15-3    | wild  | winter | Turkey    | 6    |
| HP-15-5    | wild  | winter | Turkey    | 6    |
| HP-24-1    | wild  | winter | Turkey    | 1    |
| HP-26-1    | wild  | winter | Turkey    | 1    |
| HP-27-2    | wild  | winter | Turkey    | 1    |
| B1K-52-01  | wild  | n.d.   | Israel    | 1    |
| HID-383-1  | wild  | winter | China     | 3    |
| HID-383-3  | wild  | n.d.   | China     | 1    |

* - genotypes carrying the wild-type HvVRN1 allele are highlighted in bold; allelic composition at HvVRN-H1 in other genotypes is unknown; ** - n.d., no data.
Table S4  SCAR and CAPS markers for genotyping, sequencing and real-time experiments: PCR primers and amplification regimes.

| Primer                     | Sequence (5’-3’)                  | Fragment size (bp)* | Ta** | Cycle *** | Restriction enzyme | Digested fragment size* |
|----------------------------|-----------------------------------|---------------------|------|-----------|--------------------|-------------------------|
| Allele-specific SCAR and CAPS markers |                                  |                     |      |           |                    |                         |
| PIFcaps_f       | GAGCAGTACGCGCACTTC                | 301                 | 58   | A         | Hgal               | 30+270/30+80+190         |
| PIFcaps_r       | CTTTGTGTTGCGATGTCGC              |                     |      |           |                    |                         |
| PHYCcaps_f      | GGTCTAATGCAGAAGCATGT              | 880                 | 62   | A         | BstI               | 650+230/650+160+70       |
| PHYCcaps_r      | CTCTTGCGTGACGCTGTC                |                     |      |           |                    |                         |
| CK2Acaps_f      | GTTTGTCTGCAGCATGGTGG             | 410                 | 60   | B         | Acu                | 300+110/410              |
| CK2Acaps_r      | ATGGTGACAGAAACATTCCAC             |                     |      |           |                    |                         |
| VIP4.1f         | TGCTGGGATGTTATCCAT                | 200/350             | 57   | B         | none               |                         |
| VIP4.1r         | GTGAATTGTAACAGCTCGC               |                     |      |           |                    |                         |
| VIP4.2f         | CATGGGTGTTGGAATAATTG              | 180/200             | 57   | B         | none               |                         |
| VIP4.2r         | ACCAAATGTATTACAGATCT              |                     |      |           |                    |                         |
| VRN-H1f         | AATACGACTCAGATAGGGAACATTCAACACC   | 320/360             | 50   | C         | none               |                         |
| VRN-H1r         | TTCTGCAATAAAGAGTACGGC             |                     |      |           |                    |                         |
| VIP4 sequencing  |                                   |                     |      |           |                    |                         |
| seqVIP4.1f      | ATGCAACTCTGATTGGCG                | 450                 | 61   | A         | none               |                         |
| seqVIP4.1r      | CTTAATCTTTCTTGTATGG               |                     |      |           |                    |                         |
| seqVIP4.2f      | ATGCACTTAACACATTCC                | 900                 | 63   | A         | none               |                         |
| seqVIP4.2r      | ACTCACTGATAGGTTG                  |                     |      |           |                    |                         |
| HvPHYC exon 1 sequencing |                                   |                     |      |           |                    |                         |
| Ex1seq_1f       | CCCGCTTCTTCTCACAAGA               | 1100                | 62   | A         | none               |                         |
| Ex1seq_1r       | GAGCCACAGAGGCTGATAGG              |                     |      |           |                    |                         |
| Ex1seq_2f       | ACTACCGGAACAGACATTCC              | 1200                | 62   | A         | none               |                         |
| Ex1seq_2r       | ACAGAATACACCCTACAGAG              |                     |      |           |                    |                         |
| qRT-PCR primers |                                   |                     |      |           |                    |                         |
| ELF3_DL3060F3   | TGCTGTCAAGGTGGTGGC                | 242                 | 60   | D         | none               |                         |
| ELF3_DL4483R3   | CCTGTTCTCTGCGTGGT                 |                     |      |           |                    |                         |
| ELF4_280F       | AAGAACAMGATGAGTGC                | 140                 | 60   | D         | none               |                         |
| ELF4_419R       | CAGGAGAGGCTGAGTCA                |                     |      |           |                    |                         |
| PHYC_0986F      | ACTACCGGAAACATGACAT              | 142                 | 60   | D         | none               |                         |
| PHYC_1127R      | GAGCCACAGAGGCTGATAGG              |                     |      |           |                    |                         |

* Expected allele size is given in the following format: Bowman / Bowman(em5).

** (A) – 98°C for 2 m; 4 touchdown cycles of 98°C – 30 s, (Ta+4) – 30 s (-1°C/cycle), 72°C – 1 m; 31 cycles of 98°C – 30 s, Ta – 30 s, 72°C – 1 m; final extension 72°C – 10 m; (B) – 94°C for 2 m; 4 touchdown cycles of 94°C – 30 s, (Ta+4) – 30 s (-1°C/cycle), 72°C – 30 s; 31 cycles of 94°C – 30 s, Ta – 30 s, 72°C – 30 s; final extension 72°C – 10 m; (C) – 94°C for 2 m; 9 touchdown cycles of 94°C – 30 s, (Ta+9) – 30 s (-1°C/cycle), 72°C – 30 s; 30 cycles of 94°C – 30 s, Ta – 30 s, 72°C – 30 s; (D) – 95°C for 5 m; 45 cycles of 95°C – 10 s, Ta – 10 s, 72°C – 10 s, 82°C – 10 s.