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Molecular and phenotypic characterization of the alternative seasonal growth habit and flowering time in barley (Hordeum vulgare ssp. vulgare L.)

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Abstract:

Barley can be classified into three major agronomic types, based on its seasonal growth habit (SGH): spring, winter and alternative. Winter varieties require exposure to vernalization to promote subsequent flowering and are autumn-sown. Spring varieties proceed to flowering in the absence of vernalization and are sown in the spring. The 'alternative' (also known as 'facultative') SGH is only loosely defined and can be sown in autumn or spring. Here, we investigate the molecular genetic basis of alternative barley. Analysis of the major barley vernalization (VRN-H1, VRN-H2) and photoperiod (PPD-H1, PPD-H2) response genes in a collection of 386 varieties found alternative SGH to be characterized by specific allelic combinations. Spring varieties possessed spring loci at one or both of the vernalization response loci, combined with long-day nonresponsive ppd-H1 alleles and wild-type alleles at the short-day photoperiod response locus, PPD-H2. Winter varieties possessed winter alleles at both vernalization loci, in combination with the mutant ppd-H2 allele conferring delayed flowering under short-day photoperiods. In contrast, all alternative varieties investigated possessed a single spring allele (either at VRN-H1 or at VRN-H2) combined with mutant ppd- H2 alleles. This allelic combination is found only in alternative types and is diagnostic for alternative SGH in the collection studied. Analysis of flowering time under controlled environment found alternative varieties flowered later than spring control lines, with the difference most pronounced under short-day photoperiods. This work provides genetic characterization of the alternative SGH phenotype, allowing precise manipulation of SGH and flowering time within breeding programmes, and provides the molecular tools for classification of all three SGH categories within national variety registration processes.

Keywords: Flowering time, Vernalization Requirement,Triticeae,Diagnostic genetic markers, Crop adaptation, Marker assisted selection

Introduction

Barley (Hordeum vulgare ssp. vulgare L.) was one of the first cultivated grains and is currently the fourth most important cereal in the world, in terms of both quantity produced and area of cultivation. Flowering time is a critical component of barley adaptation to agricultural environment, as alignment of crop development with favourable environmental conditions helps maximize grain yield (Cockram et al. 2007a). Wild barley (H. vulgare ssp. spontaneum C. Koch) possesses a winter seasonal growth habit (SGH), requiring exposure to prolonged periods of low temperature (vernalization, typically 6–8 weeks at 4 °C) for competence to flower during inductive conditions the following summer. In the wild, this response allows vegetative growth to be established prior to the spring and prevents the development of sensitive floral organs over winter months, thus avoiding low-temperature damage. Cultivated barley varieties which retain this ancestral response are classified as possessing a 'winter' SGH. Four major genetic loci are known to control a large proportion of flowering time variation in barley. These are the vernalization response loci VERNALIZATION-H1 (VRN-H1) and VRN-H2, and the photoperiod response loci PHOTOPERIOD-H1 (PPD-H1) and PPD-H2 (reviewed by Bentley et al. 2013). VRN-H1 is encoded by the MADS-box transcription factor HvBM5A which shows homology to the Arabidopsis meristem identity gene APETALA 1 (AP1), although functionally it is more similar to the Arabidopsis gene, FRUITFUL (FUL). In barley, deletions or insertions within VRN-H1 intron I that disrupt putative cisregulatory regions are thought to confer dominant spring alleles, which result in VRN-H1 expression and subsequent floral transition in the absence of vernalization (Cockram et al. 2007b, c, 2011; Sz}ucs et al. 2007; Hemming et al. 2009; Trevaskis 2010). At the VRN-H2 locus, deletion of three closely linked genes (ZCCT-Ha, Hb, Hc) is thought to be responsible for the creation of recessive vernalization insensitive vrn-H2 alleles (Yan et al. 2004; Karsai et al. 2005; Distelfeld et al. 2009). Cereal ZCCT genes encode a zinc-binding domain and a CCT domain and have evolved as part of a chromosomal duplication event that predates the divergence of cereal species (Cockram et al. 2010a). Based on analysis of CCT motifs, they belong to a family of plant flowering time genes belonging to the CONSTANS-like Group I clade (Cockram et al. 2012a), which also contains the rice flowering time gene Ghd7. Truncation/deletion of Ghd7 removes floral delay under long-day (LD) photoperiods and is one of the key variations which have allowed rice to grow in temperate LD agricultural environments (Xue et al. 2008). Similarly, VRN-H2 is thought to repress flowering under LDs, as it shows little or no expression in SD photoperiods (Trevaskis et al. 2006; Turner et al. 2013). Current flowering time models hypothesize that VRN-H2 represses the floral promoter FLOWERING LOCUS T 1 (FT1), with VRN-H1 repressing VRN-H2 expression in vernalization sensitive accessions under LDs (Trevaskis 2010). VRN-H1 is gradually upregulated during vernalization, repressing VRN-H2 and allowing expression of FT1 under LDs. To confer a winter SGH, barley varieties must carry wild-type winter alleles at both VRN-H1 and VRN-H2, while spring varieties must possess vernalization insensitive spring alleles at one or both loci (Laurie et al. 1995). The PPD-H1 locus controls flowering in response to LDs. It is encoded by gene

belonging to the PSEUDO RESPONSE REGULATOR (PRR) gene family (Turner et al. 2005), which in Arabidopsis acts close to the central oscillator. In barley, the photoperiod insensitive ppd-H1 allele is thought to be due to a nonsynonymous G/T mutation within the CCT domain (Turner et al. 2005). Plants carrying the ppd-H1 allele (T nucleotide) are delayed in flowering under LD, allowing them to take advantage of the long growing seasons prevalent in north-west Europe. The PPD-H2 locus modulates flowering under short-day (SD) photoperiods and is thought to be encoded by the FT-like gene, HvFT3 (Faure et al. 2007). Complete or partial deletion of HvFT3 is associated with recessive ppd-H2 alleles that result in delayed flowering under SDs.

During barley domestication, early farmers selected types that lack a vernalization requirement and so are able to progress to flowering in the absence of vernalization (Jones et al. 2011). These possess a 'spring' SGH, germinate and flower in the same year. This selection has led to two largely distinct breeding pools, with associated partitioning of genome-wide genetic diversity (Cockram et al. 2008, 2012b). However, a third classification exists: the 'alternative' SGH. Alternative varieties are only loosely described, but can be used agronomically either as spring or as winter types. At the molecular level, classification of alternative types has received little attention and has been variously described. For example, von Zitzewitz et al. (2005) described an alternative (also termed 'facultative') variety as containing a winter vrn-H1 allele combined with a spring vrn-H2 allele. The resulting plant would likely retain the frost tolerance loci linked to VRN-H1 on the long arm of chromosome 5H, while displaying a spring (vernalization non-responsive) phenotype conferred by vrn-H2 alleles. Similarly, others describe alternative varieties as carrying ''only a modest vernalization response'' (Szucs et al. 2007), delaying flowering by only a few days, which can again be predicted by known allele combinations at VRN-H1 and VRN-H2 (Karsai et al. 2005). During the process of awarding Plant Breeders' Rights, new barley submissions are classified as belonging to one of the three SGH classes according to internationally recognized Distinct Uniform and Stable (DUS) protocols, as outlined by the Union for the Protection of Varieties (UPOV) (Jones et al. 2013). Within the DUS system, alternative types are defined as lines that flower later than spring lines in the absence of vernalizing temperatures, and are based on field assessment of flowering time. For DUS purposes, the scoring of this characteristic currently involves planting out significant areas of submitted winter and alternative varieties in spring time in order to confirm their seasonal growth habit (SGH). Winter varieties do not flower at all, and since the material does not produce heads or grains, the trial is not used to score any other characters. A molecular test for the vernalization characteristic in barley would serve to demonstrate to the international community the viability and advantages of using a molecular approach to assess functional traits of relevance to DUS. It is also clearly attractive as a direct replacement for the existing field trials on a cost– benefit basis. Furthermore, molecular characterization of the alternative SGH will allow breeders to track and combine relevant flowering time alleles for the development of new alternative varieties. This study uses a collection of 386 barley varieties to identify the flowering time haplotypes diagnostic for alternative SGH, as well as

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for spring and winter types, and shows that alternative varieties flower later than spring types under both LD and SD photoperiods.

Methods

Barley germplasm and growth conditions

Six alternative UK barley varieties were sourced from germplasm stored at NIAB. An additional 380 spring and winter UK varieties were sourced, as previously described (Cockram et al. 2010b) (Supplementary Table 1). Ten varieties were selected for investigation of flowering time under controlled environment conditions (where known, application for protection [AFP] numbers is shown in brackets): alternative varieties, Gaelic (2/1117), Novetta, SW Alison (2/ 1788), 52B4 (2/1797) and BR5593C3 (2/2159); winter varieties, Igri (2/129), Tempo (2/1170) and Express (2/ 894); spring varieties, Felicie (2/1091, early spring control), Indola (2/1874, late spring control) and Rebecca (2/1871). Seeds were germinated on moist filter paper for 24 h at 4 "C, and the resulting seedlings grown in one-litre pots filled with M2 compost. Five replicates were grown per variety, with 10 replicates grown for the early (Felicie) and late (Indola) spring control varieties. Plants were arranged in a randomized bock design and grown for 140 days under controlled environment conditions, with day/night temperatures of 20/18 °C. Long-day and short-day experiments received 16 and 12 h of light, respectively. Plant developmental stages were recorded according to Zadok's growth stage (GS). Flowering time was recorded as date of awn emergence (GS 47). Apical meristems of all plants that had not flowered by the end of each SD and LD experiment were dissected using a stereomicroscope and imaged using a mounted camera.

DNA extraction, genotyping and sequencing

Genomic DNA was extracted from leaf material using the DNeasy Kit (Qiagen). DNA quality was assessed using a Nanodrop 200 spectrophotometer (Thermo Scientific) and diluted to a final concentration of 10 ng/ll. VRN-H1 haplotypes were determined using the diagnostic multiplex PCR/agarose gel multiplex assay described by Cockram et al. (2009), as well as the single nucleotide polymorphisms (SNPs)/simple sequence repeats (SSRs)/insertion–deletions (InDels) described by Cockram et al. (2007b) (Fig. 1). VRN-H1 intron I from the variety Eminant was investigated using primer pairs that tile across the region, as described by Cockram et al. (2007b). VRN-H1 sequencing was performed by direct sequencing of PCR products using BigDye Kit v3.5 (Applied Biosystems) in 10 ll sequencing reactions using the primers and methods described by Cockram et al. (2007b) on a 3730 DNA Analyser (Applied Biosystems). Sequence outputs were processed using sequence analysis (Applied Biosystems), analysed using the Vector NTI Advance package v10.1.1 (Invitrogen) and manipulated using GENEDOC v2.6 (http://www.nrbsc.org/gfx/genedoc/). DNA and protein alignments were performed using CLUSTALW (Thompson et al. 1994) with additional manual

adjustment. Genomic sequences were submitted to GenBank under accessions KT247893 to KT247898 (Supplementary Table 2). Presence/absence of the three ZCCT-H candidate genes underlying the VRNH2 locus was genotyped using the PCR/agarose gel assay described by Karsai et al. (2005). The nonsynonymous T3081/G SNP in the CCT domain of PPD-H1 (Turner et al. 2005) was genotyped by direct sequencing of PCR products amplified using primers PPD9F (50-AACACCAATAACGGGAGCAC-30) and PPR9R (50 CCGGCATGTTCTATGGTAGG-30). The presence/absence of the HvFT3 candidate gene at the PPD-H2 locus was genotyped using primers described by Faure et al. (2007) that amplify an 874-bp product when HvFT3 is present, with the inclusion of an additional internal positive control using primers BM5A1intF (50-TCCCAAGAAAACTTGAACAACACCAG-30) and BM5A2intR (50 GGTATTAGGTTACATCATTCGACCA-30), which additionally assays for the 42-bp insertion–deletion (InDel) in VRN-H1 intron 1 (resulting in amplicons of 616 and 574 bp, respectively) (Fig. 2).

Results

Genotyping major flowering time loci in spring, winter and alternative barleys

We previously showed that SGH for spring and winter barley can be predicted by their multi-locus haplotypes at VRN-H1 and VRN-H2 (Cockram et al. 2007b, 2009). Here, we hypothesize that differences in haplotypes at the vernalization loci VRN-H1 and VRNH2 and the photoperiod response loci PPD-H1 and PPD-H2 largely determine the differentiation in flowering time of alternative varieties from winter and spring forms. Genotyping VRN-H1 and VRN-H2 in the 380 UK winter and spring varieties (Supplementary Table 1) found all winter accessions to possess winter alleles at both loci, as predicted by the epistatic interaction between the two loci (Table 1). The majority of winter varieties (95 %) were found to possess the winter haplotype 1A allele. The remaining 11 varieties possessed the 5C haplotype which contains a 0.5 kb deletion within the Lolaog LTR in intron 1 (as well as numerous other SNPs) and has previously been associated with a weak vernalization requirement (Cockram et al. 2007b; Hemming et al. 2009). Winter lines varied for alleles at PPD-H1, with the majority (52 %) possessing the wild-type LD photoperiod-responsive Ppd-H1 allele, with a G nucleotide at position +3081 (Table 1). The remaining 48 % possessed a T nucleotide at position 3081, thought to confer the non-responsive ppd-H1 allele (Turner et al. 2005). Finally, all but three winter varieties (Adonis, Gaulois and Tempo) show a deletion of the PPD-H2 candidate gene, HvFT3. Spring Vrn-H1 alleles and LD photoperiod non-responsive ppd-H1 alleles were found in all of the 170 spring varieties investigated. However, while the majority of spring varieties carried spring vrn-H2 alleles, 18 % were found to possess winter alleles. All but one spring variety (Rebecca) were predicted to possess wild-type Ppd-H2 alleles. Thus, with the exception of the four lines highlighted above, spring and winter varieties were found to be partitioned for the presence of Ppd- H2 and ppd-H2 alleles, respectively. Using the two multiplex assays for VRN-H1 and VRN-H2, five of the six

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alternative varieties were predicted to be phenotypically spring. In addition, all six alternative lines carried a deletion of HvFT3, indicating that they carry the ppd-H1 allele (found in 99 % of winter varieties screened here) previously shown to delay flowering under SDs (Laurie et al. 1995; Faure et al. 2007). Although alternative varieties showed specific allelic distributions at VRN-H1, VRN-H2 and PPD-H2, genotypic evaluation of PPD-H1 showed LD-responsive and non-responsive alleles to vary between alternative lines, with the wild-type Ppd-H1 allele found in two of the six alternative lines investigated (Novetta and 52B4).

Flowering time under SD and LD photoperiods

Following the identification of specific VRN-H1/VRNH2/ PPD-H2 multi-locus haplotypes characteristic for spring/winter/alternative SGH, ten varieties with varying multi-locus haplotypes were selected for phenotypic evaluation of flowering time under LD and SD photoperiods (Fig. 3). These included control winter and spring varieties with varying allelic combinations at the four major flowering time loci. The three winter control lines consisted of Igri, which carries the standard multi-locus configuration at the four major flowering time loci genotyped [vrn-H1 (haplotype 1A), Vrn-H2, Ppd-H1, ppd-H2], Tempo (differing to Igri by the presence of a dominant Ppd- H2 allele) and Express (differing to Igri by the presence of the 'weak' haplotype 5C winter allele at VRN-H1). Additionally, three spring control varieties were evaluated, including two varieties used as controls within DUS phenotypic evaluation of flowering time: Felicie, the early flowering DUS spring control, which possesses the standard spring multilocus haplotype (Vrn-H1, vrn-H2, ppd-H1, Ppd-H2), and Indola, the late flowering DUS control, which differs to Felicie by the presence of a dominant late flowering Vrn-H2 allele. In addition, the spring variety Rebecca was included, which differs from the early flowering control Felicie (and all other spring varieties investigated) by the presence of a late flowering ppd-H2 allele. Grown alongside the control varieties were the alternative varieties Gaelic (2/1117), Novetta, SW Alison (2/1788), 52B4 (2/1797), BR5593C3 (2/2159), all of which possessed one spring VRN allele in combination with a recessive ppd-H2 allele that delays flowering under SDs. Under LD photoperiods in the absence of vernalization, two of the three winter varieties remained vegetative by experiment end, with Tremois (GS 32) more advanced relative to Igri (GS 31), although meristem development for both was at the double ridge stage (Fig. 3b). The winter variety Express flowered at 87 ± 4.2 days, significantly later than the late spring control variety, indicating that it possesses a weak spring SGH. All other varieties flowered, with the early (Felicie) and late (Indola) spring control lines flowering at 67 ± 0.8 and 75 ± 4.0 days, respectively. The five alternative varieties showed a range of mean flowering times, ranging from 52 ± 0.4 (52B4) to 95 ± 4.0 (BR5593C3). Alternative lines with the LD responsive Ppd-H1 allele (Novetta, 52B4) flowered significantly earlier than the early spring control, while those with the non-responsive allele flowered along with the late spring control, or significantly after (BR5593C3). Rebecca, the only spring variety found to possess a mutated ppd-H2 allele, flowered after the late DUS spring control (77 ± 4 days), although this

difference was not significant. Under SDs, development was delayed for all varieties, in comparison with LDs (Fig. 3a). The winter varieties Igri and Tremois were developmentally most delayed, as determined by GS and meristem dissection. In contrast, the early control Felicie (flowered at 104 ± 11.8 days) and late spring control Indola (GS 45) controls were the most advanced, followed by the anomalous spring variety Rebecca (GS 43). The five alternative varieties were considerably later in their development than the spring lines, ranging between GS 37 (SW Alison, Gaelic) and GS 31 (Novetta, BR5593C3). This difference was also seen in apical development, with alternative lines at GS 31, 33 and 37 all showing inflorescence development.

VRN-H1

To further investigate VRN-H1 genetic variation in alternative varieties, seven polymorphic features (six SNPs and one SSR), previously used by Cockram et al. (2007b) to assign VRN-H1 haplotypes, were genotyped (Table 2). The SNPs, located in the promoter region (SNP1, SNP2 and SNP3) and 30 end of the gene (SNP4, SNP5 and SNP6), were identified by direct sequencing, returning a total of 33 kb of sequence (Supplementary Figures. 2-3). Nineteen polymorphic features were identified in the 1.1 kb sequenced region of the VRN-H1 promoter, while eleven polymorphisms were found in the 1.7 kb region spanning VRNH1 exon 3 to the 3'UTR. Analysis of the polymorphisms showed all lines belonged to one of the three previously identified VRN-H1 haplotypes, according to the designations described by Cockram et al. (2007b). Alternative varieties Gaelic and SW Alison were found to possess the previously characterized spring VRN-H1 haplotype 5A, with sequencing across the intron I breakpoint confirming that the major deletion detected is identical to the 5.2 kb deletion found in the spring haplotype 5A variety, 'Golf' (Supplementary Figure 4). Alternative varieties Angela and 52B4 have genotype configurations identical to that of the previously identified haplotype 5C allele found in 'Express', thought to confer weak vernalization requirement. Sequencing the intron I breakpoint in these alternative SGH varieties showed the location of the 0.5 kb deletion to be exactly the same as that previously identified in 'Express' (Supplementary Figure 5). The remaining three alternative varieties (Eminant, BR5593C3 and Novetta) displayed the winter 1A haplotype. However, as BR5593C3 and Novetta were shown to carry spring Vrn-H2 alleles, their lack of vernalization requirement is explained by their VRN-H1/VRN-H2 multi-locus haplotype (Table 2). In contrast, the VRN-H1/VRN-H2 multi-locus haplotype of Eminant (1A ? Z) predicted a winter SGH. Although the VRN-H1 multiplex deployed in this study did not detect an intron 1 deletion, it is possible that a previously un-described InDel is present in these varieties. Accordingly, a series of 13 PCR amplicons that tile across the ~11 kb winter vrn-H1 allele (Cockram et al. 2007b) were used to further investigate intron I configuration in Eminant. PCR amplification was achieved for all but one primer pair: HvBM5a-intron 1-F2/HvBM5a intron 1-R2. This amplicon spans the vernalization critical region (Fu et al. 2005) and suggests the presence of a disruption likely to result in spring SGH. Thus,

while all six of the alternative varieties investigated were predicted to possess a spring SGH, they possess just one spring allele, either at VRN-H1 or at VRN-H2.

Discussion

VRN-H1 haplotypes

The development of spring varieties during the domestication of barley has been due to the selection of mutated alleles at VRN-H1 and/or VRN-H2. In both instances, these novel alleles are thought to have arisen via insertion–deletion (Cockram et al. 2007b, c, 2011; Fu et al. 2005; von Zitzewitz et al. 2005; Szucs et al. 2007; Hemming et al. 2009). In the case of VRNH1, these rearrangements are thought to disrupt putative cis-regulatory regions within intron I. By overlaying regions of intron I sequence conservation between barley and the related cereal species, wheat (Triticum aestivum L.) with the positions of the major intron I deletions in barley thought to confer spring alleles, putative vernalization critical regions have been identified and refined (Supplementary Figure 1). The first half of intron I (~5 kb) shows high levels of conservation between these species (Fu et al. 2005). Initially, a~2.8 kb at the 5' end of intron I was identified as possessing particularly high sequence conservations, with the first 436 bp of this region suggested as the region most likely to harbour a 'Triticeae-shared vernalization critical motif' (Fu et al. 2005; von Zitzewitz et al. 2005; Sz}ucs et al. 2007). Subsequently, expression analyses have highlighted an adjacent 0.5 kb region associated with high VRN-H1 expression in non-vernalized plants (Hemming et al. 2009). The Eminant VRN-H1 intron I rearrangement identified in this study prevents amplification of a region that spans both the 'vernalization critical' motif and the region associated with high VRN-H1 expression. Given that we failed to amplify across this region in Eminant despite numerous primer pairs being attempted, it may be more likely to represent a large DNA insertion. To date, two instances of transposable element (TE) insertion into Triticeae VRN1 intron I associated with spring alleles are known: a 0.7 kb insertion 170 bp downstream from the start of intron I in barley [Haplotype 2 (Cockram et al. 2007b), also known as HvVRN1-7 (Hemming et al. 2009)], and a 0.8 kb TE, inserted ~1 kb downstream of the start of intron I in Vrn-D1s alleles from the hexaploid wheat species, T. spelta and T. compactum (Muterko et al. 2014). Interestingly, the barley allele carrying the TE insertion has been shown to correlate with the highest level of VRN-H1 expression under unvernalized LD conditions of all alleles studied to date (Hemming et al. 2009; Oliver et al. 2013), indicating that TE insertion may be more effective than deletion in the formation of strong spring Vrn-H1 alleles. Thus, while the precise nature of the intron I rearrangement present in Eminant was not determined, it is very likely to result in a spring Vrn-H1 allele. Controlled environment phenotyping showed that Express, predicted to be a weak winter variety from its VRN-H1/VRN-H2 haplotype (5C ? Z), was under stem extension in SDs, and although significantly later than the late spring control, flowered under LDs. It has been previously noted that the 5C VRN-H1 allele may confer a weak vernalization response (Cockram et al. 2007b; Hemming et al. 2009). While the vernalization response of Express was not formally investigated here, these results indicate the 5C allele result in plants that are able to flower in the absence of vernalization.

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Multi-locus haplotypes for SGH and effect on flowering time

Analysis of the genetic variation at VRN-H1, VRN-H2, PPD-H1 and PPD-H2 allowed the identification of diagnostic multi-locus haplotypes for all three SGH classes. As expected, all winter varieties carried winter alleles at both vernalization loci (vrn-H1/Vrn-H2). In addition, 99 % of winter accessions were predicted to carry recessive ppd-H2 alleles that delay flowering under SDs. This vrn-H1/Vrn-H2/ppd-H2 allelic combination presumably helps ensure vegetative growth under winter SD conditions, even after vernalization requirement has been met, thus helping avoid cold damage to reproductive tissues. The three exceptions all had spring varieties in their pedigree, either as one of the parents (Gaulois, Adonis) or grandparents (Tempo). However, allelic state at PPD-H1 was more varied, with an overall ratio of 121:70 LD-responsive versus non-responsive alleles observed. However, when assessed over time, allelic ratios have changed from ~2:1 (in years ≤1992, 1993–1996 and 1997–2000) to 1:1 (2001–2004) over time (Supplementary Table 3), indicating possible changes in breeder selection for LD photoperiod response over the timescale studied.

The predominance of spring alleles at both VRN-H1 and VRN-H2 in the UK spring varieties investigated is in agreement with previous reports on European germplasm (Cockram et al. 2007b), as is the occurrence of a small minority of spring varieties with allelic combination Vrn-H1/Vrn-H2 (spring/winter). Here, we define a wider multi-locus haplotype for spring barley, in which spring alleles at VRN-H1 are combined with photoperiod insensitive ppd-H1 and Ppd-H2 alleles. This allelic combination would prolong the growth season of spring barley in north-west European conditions, allowing maximum benefit to be gained from the extended growing seasons prevalent in this area. Just one exception to this spring multilocus haplotype was identified (Rebecca), which carried a ppd-H2 allele conferring delayed flowering under SDs. Growth room experiments confirmed that Rebecca showed delayed flowering under SDs in comparison with both the early and late spring controls, but was more advanced than all of the alternative varieties investigated, as predicted by its VRN-H1/VRN-H1 (spring/winter) haplotype. The majority of varieties carry recessive spring vrn-H2 alleles (ZCCT genes deleted) in combination with wild-type PPD-H2 alleles (HvFT3 present). HvFT3 is normally expressed in SDs only (Faure et al. 2007; Turner et al. 2005). However, in lines lacking VRNH2, HvFT3 is also expressed in LDs (Casao et al. 2011), so it has been suggested that the day-length specificity of HvFT3 might be mediated by VRN-H2 (Fjellheim et al. 2014). Therefore, the predominance of Vrn-H2 alleles in spring varieties may be due to selection of accelerated flowering in LDs due to upregulation of HvFT3.

A diagnostic multi-locus haplotype was also identified for the alternative SGH, characterized by a spring allele at either VRN-H1 or VRN-H2, in combination with mutant ppd-H2 alleles. This multi-locus haplotype is predicted to be genetically spring, with delayed flowering under SDs. SD-controlled environment phenotyping confirmed this, showing that while none of the alternative varieties flowered, all were in stem extension stages by the end of the experiment, and that their developmental stage was intermediate to the winter and late spring controls. Under autumn-sown field

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conditions, the presence of just a single spring vernalization allele combined with ppd-H2 is assumed to delay reproductive development sufficiently until the onset of LDs in the spring. Autumn planting also means that alternative varieties must be cold hardy. This definition of the alternative SGH agrees with (and expands on) previous genetic descriptions of facultative types, described as having a winter vrn-H1 allele combined with a spring vrn-H2 allele (Karsai et al. 2005; von Zitzewitz et al. 2005). It should be noted that previous investigation of the facultative variety 'Dicktoo' crossed with the winter line 'Komptai Korai' found a flowering time QTL at VRN-H2 (due the spring vrn-H2 allele carried by the facultative parent), while no QTL was identified at PPD-H2 (Karsai et al. 2005). However, this is due to both parents carrying SD insensitive alleles at PPDH2 and agrees with the findings concerning allelic variation at PPD-H2 in winter and alternative/facultative varieties identified here. This limitation raises the importance of exhaustively defining multi-locus flowering time locus haplotypes in the winter and spring germplasm, as well as in alternative/facultative types. Here, we not only identify multi-locus haplotypes for alternative varieties, but also multi-locus haplotypes for large numbers of spring and winter varieties. Indeed, it is this sample size that allows us to highlight the novelty of the observed haplotypes in the alternative lines, even though the alternative type is uncommon (here we include all alternative varieties released in the UK over a 25-year period). This rarity explains why the alternative type has not previously been well defined genetically. Similarly, such lack of genetic understanding may also, at least partially, explain why they are not often released as varieties. The baseline data provided here will help informed investigation of the effect of currently rare haplotype classes on agronomic traits.

Two major cold hardiness loci have been identified, both on the long arm of chromosome 5H. FROST RESISTANCE-H1 (Fr-H1) is linked to VRN-H1 (von Zitzewitz et al. 2011; Fricano et al. 2009), while Fr-H2 is thought to be encoded by one of the clusters of Crepeat binding factor (CBF) transcription factors that cosegregate with the locus (Francia et al. 2007; Knox et al. 2010). VRN-H1 expression is known to be rapidly and transiently activated after exposure to short periods of cold temperature and is thought to be involved in cold acclimatization pathway leading to frost tolerance (Oliver et al. 2013). VRN-H1 upregulation in response to both short and long exposure to vernalizing temperatures is thought to be modulated by loss of histone H3 lysine 4 trimethylation (H3K4me3) and increased H3 acetylation (H3Ac) at VRN-H1 chromatin (Oliver et al. 2009, 2013). Interestingly, changes in histone methylation and VRN-H1 upregulation after vernalization are true for both winter (VRN-H1 haplotype 1A) and spring (5A) varieties (Oliver et al. 2009). Indeed, the two regions with the strongest increase in H3Ac (exon I, and at the 50 end of intron I between the VRN-H1 1B and 4A 50 deletion breakpoints) are present in all of the VRN-H1 alleles present in alternative varieties. While all three SGH classes can now be classified using VRN and PPD multi-locus haplotypes, more work is needed to determine the frost tolerance loci and alleles deployed.

Conclusions

The ability to track alleles at multiple flowering time loci allows precise manipulation of flowering time and SGH within breeding programmes. Understanding the multi-locus haplotypes underlying alternative SGH allows precise and rapid development of new varieties with the specific flowering time characteristics. Similarly, the wide range of alternative flowering times under LDs in comparison with spring varieties will likely inform future studies aimed at determining the genetic basis behind this. Such knowledge will inform future genetic studies on flowering time, yield and yield components, and help the production of barley for cultivation adapted to a range of agricultural environments. It is likely to be of particular relevance under future climate scenarios predicted by global climate change.

TABLE LEGENDS

Table 1. Allelic ratios for four *VRN-H1, VRN-H2, PPD-H1* and *PPD-H2* (*HvFT3*) in winter, spring and alternative barley varieties. Wild-type alleles are underlined.SGH (seasonal growth habit).

Table 2. Extended haplotypes for the six alternative varieties, along with the winter varieties Igri and Express (weak vernalization requirement). W (winter), A (alternative), SGH (seasonal growth habit), Mpx (multiplex), del (deletion), +Z/-Z (presence/absence of the three *ZCCT* genes). [a] According to Cockram *et al.* (2007b). [b] SNP 22 (G/T, Turner *et al.* 2005). [c] 1 or 0 (presence or absence of *HvFT3*). [d] PCR analysis of intron I finds an unknown rearrangement affecting the vernalization critical region, as delimited by Fu *et al.* (2005), predicted to result in a spring allele. Alternative *VRN-H1* intron 1 major deletion nomenclature (Hemming *et al.* 2009): 0 kb (*HvVRN1*), 0.5 kb (*HvVRN1-6*) and 5.2 kb (*HvVRN1-1*) deletions.

FIGURE LEGENDS

Figure 1. Diagram of the genomic winter *vrn-H1* allele from the cultivar 'Strider' (AY750993), indicating the positions of primer used for the *VRN-H1* multiplex assay (primer pairs 1, 2 and 3, Cockram *et al* 2009), the (CGCT) SSR in the 5'UTR, SNPs 1 to 3 (promoter), SNP4 and SNP5 (intron 7) and SNP6 (3'UTR). Solid black boxes denote exons. The position of the Lolaog soloLTR within intron I is indicated by the hatched box. The 5.2 kb intron I deletion characteristic of haplotype 5A is indicated by the dashed line box.

Figure 2. The multiplex PCR/agarose-gel genetic marker for *HvFT3*. The presence/absence of the 873 bp amplicon assays for *HvFT3*, while the 616/574 bp amplicon acts as a positive control, assaying for the 42bp InDel within *VRN-H1* intron 1. L = 1 kb DNA ladder.

Figure 3. Barley flowering time and development under varying photoperiod. (a) Short-day photoperiod (12 hours)**.** (b) Long-day photoperiod (16 hours). Black, white and grey bars denote winter, spring and alternative varieties, respectively. Standard deviations (+1) for days to flowering are shown. Varieties which did not flower by the end of the experiment are recorded as having a flowering time of 140 days, and their Zadock's growth stage and am example of a main tiller meristem are shown. Ig (Igri), Te (Tremois), Ex (Express), Fe (Felicie), Re (Rebecca), SWA (SW Alison), Ga (Gaelic), No (Novetta). (c) Multi-locus haplotypes at the vernalization loci *VRN-H1* and *VRN-H2* and photoperiod loci *PPD-H2* (responsive to short-days) and *PPD-H1* (responsive to long days). 1A and 5C haplotypes at *VRN-H1* denote winter alleles, all others denote spring. +Z and –Z at *VRN-H2* denote winter and spring alleles, respectively. +F and –F at *PPD-H2* denote wild-type and mutated (delayed flowering under short days) alleles at the *PPD-H2* candidate gene, *HvFT3*. (d) Predicted 30 seasonal growth habit, based on multilocus haplotype. W (winter), S (spring), A (alternative), SA (spring-like alternative).

Table 1

SGH	Number of varieties	*Vrn-H1/vrn-H1* allele frequency	*Vrn-H2/vrn-H2* allele frequency	*Ppd-H1/ppd-H1* allele frequency	*HvFT3* presence / absence
Winter	210	0/210	210/0	121/58 (U=26)	3/207
Spring	170	170/0	31/139	0/134 (U=36)	169/1
Alternative	6	2/4	3/3	2/4	0/8

Table 2

Variety	SGH	VRN-H1 Mpx	SNP1	SNP2	SNP3	SSR	Intron 1 del. (kb)	SNP4	SNP5	SNP6	VRN-H2	VRN-H1/-H2 haplotype[a]	Predicted SGH	PPD-H1[b]	PPD-H2[c]
Igri	W	1A	T	A	C	4	0	T	G	C	+Z	1A+Z	winter	G	0
Express	W	5C	T	G	C	5	0.5	C	A	G	+Z	5C+Z	weak winter	U	U
Eminant	A	1A[d]	T	A	C	4	0	T	G	C	+Z	1A[d]+Z	spring	T	0
BR5593C3	A	1A	T	A	C	4	0	T	G	C	-Z	1A-Z	spring	T	0
Novetta	A	1A	T	A	C	4	0	T	G	C	-Z	1A-Z	spring	G	0
52B4	A	5C	T	G	C	5	0.5	C	A	G	-Z	5C-Z	spring	G	0
Gaelic	A	S	T	G	C	5	5.2	C	A	G	+Z	5A+Z	spring	T	0
SW Alison	A	S	T	G	C	5	5.2	C	A	G	+Z	5A+Z	spring	T	0

Figure 1
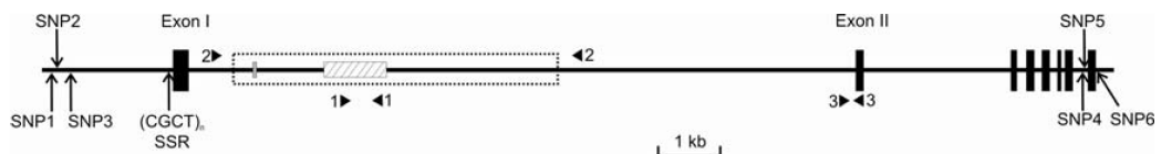
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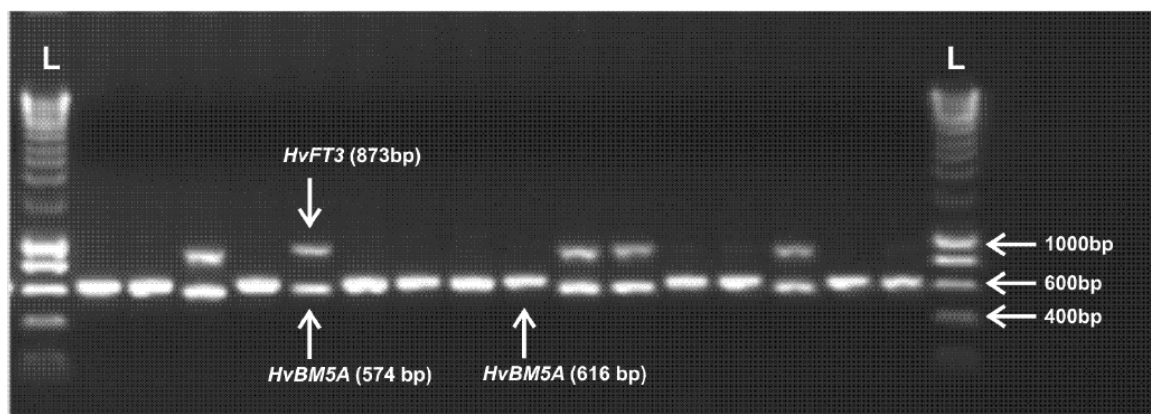
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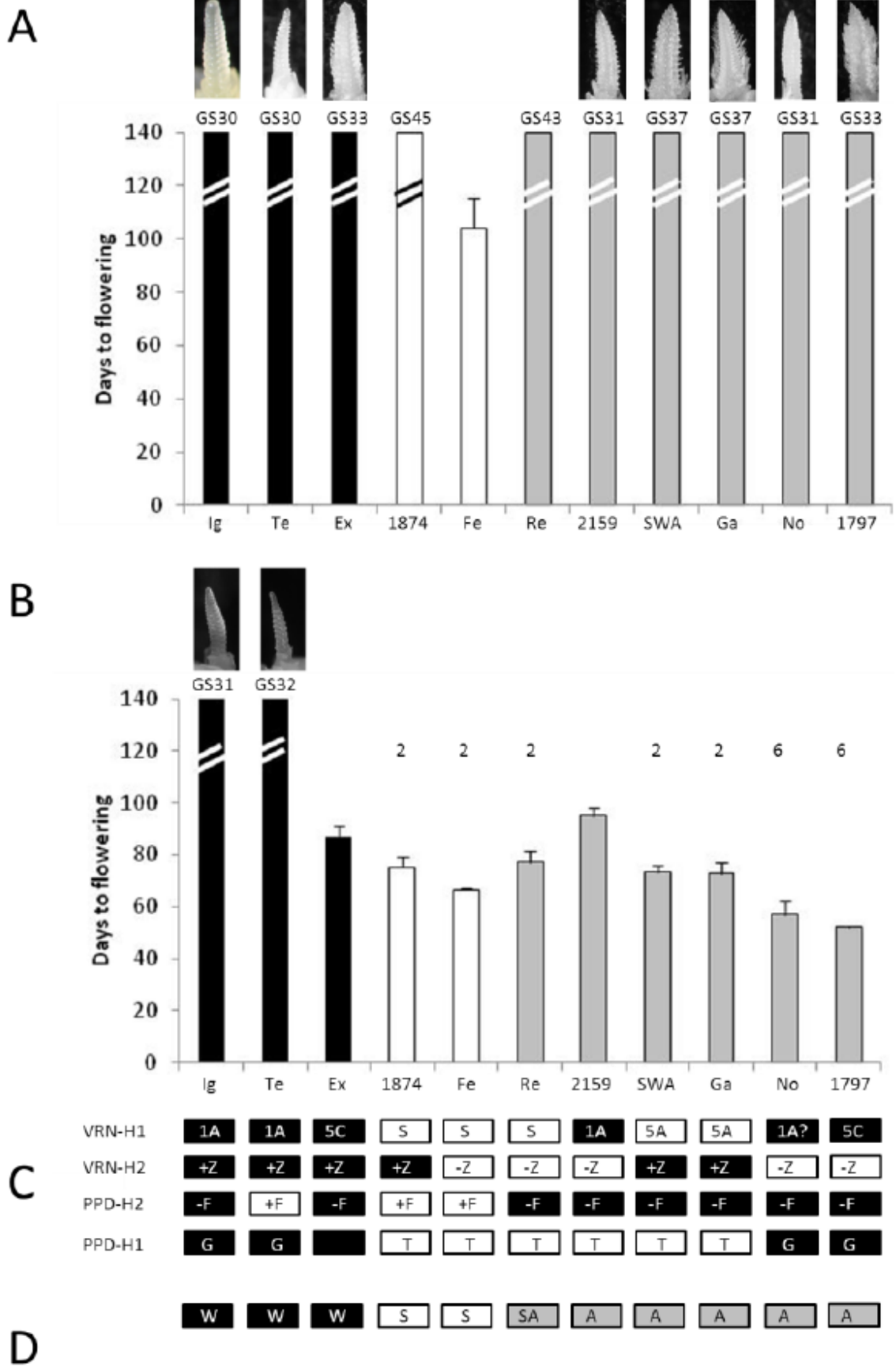
Author contributions

J.C., C.N. and D.O.S. devised research,

J.C., E.S., R.H. undertook experimental work, J.C. analysed data

and wrote the manuscript, all authors reviewed the manuscript.

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