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ORIGINAL PAPER

Deciphering the genetics of flowering time by an association study on candidate genes in bread wheat (*Triticum aestivum* L.)

Michel Rousset · Isabelle Bonnin · Carine Remoué · Matthieu Falque · Bénédicte Rhoné · Jean-Baptiste Veyrieras · Delphine Madur · Alain Murigneux · François Balfourier · Jacques Le Gouis · Sylvain Santoni · Isabelle Goldringer

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Abstract Earliness is very important for the adaptation of wheat to environmental conditions and the achievement of high grain yield. A detailed knowledge of key genetic components of the life cycle would enable an easier control by the breeders. The objective of the study was to investigate the effect of candidate genes on flowering time. Using a collection of hexaploid wheat composed of 235 lines from diverse geographical origins, we conducted an association study for six candidate genes for flowering time and its components (vernalization sensitivity and earliness per se). The effect on the variation of earliness components of polymorphisms within the copies of each gene was tested in ANOVA models accounting for the underlying genetic structure. The collection was structured in five groups that minimized the residual covariance. Vernalization requirement and lateness tend to increase according to the mean latitude of each group. Heading date for an autumnal sowing was mainly determined by the earliness per se. Except for the *Constans* (*CO*) gene orthologous of the barley *HvCO3*, all gene polymorphisms had a significant impact on earliness components. The three traits used to quantify vernalization requirement were primarily associated with polymorphisms at *Vrn-1* and then at *Vrn-3* and *Luminidependens* (*LD*) genes. We found a good correspondence between spring/winter types and genotypes at the three homeologous copies of *Vrn-1*. Earliness per se was mainly explained by polymorphisms at *Vrn-3* and to a lesser extent at *Vrn-1*, *Hd-1* and *Gigantea* (*GI*) genes. Vernalization requirement and earliness as a function of geographical origin, as well as the possible role of the breeding practices in the geographical distribution of the alleles and the hypothetical adaptive value of the candidate genes, are discussed.

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M. Rousset (✉) · I. Bonnin · C. Remoué · M. Falque · B. Rhoné · J.-B. Veyrieras · D. Madur · I. Goldringer
UMR de Génétique Végétale, INRA/CNRS/AgroParisTech/ Univ. Paris XI, Ferme du Moulon, 91190 Gif sur Yvette, France
e-mail: mrousset@moulon.inra.fr

I. Bonnin
Fondation pour la Recherche sur la Biodiversité (FRB)
195 rue Saint Jacques, 75005 Paris, France

F. Balfourier · J. Le Gouis
UMR Génétique, Diversité et Ecophysiologie des Céréales, INRA/UBP, 234 Avenue du Brézet,
63100 Clermont-Ferrand, France

S. Santoni
UMR Diversité et Génomes des Plantes Cultivées,
INRA Domaine de Melgueil, 34130 Mauguio, France

A. Murigneux
Biogemma 8 Rue des Frères Lumière,
63028 Clermont Ferrand Cedex 2, France

A. Murigneux
Limagrain Europe ZAC Les Portes de Riom BP 173,
63720 Chappes, France

Introduction

Major genes influencing plant development play an important role in plant adaptation to environmental conditions and as a consequence in grain yield and quality. To maximize yield expression in a given environment, wheat cultivars must have an appropriate flowering time and life cycle duration, which 'fine-tunes' the life cycle to the

target environment (Hanocq et al. 2002). In such a way, earliness can be considered as an adaptive trait (Worland 1996). Its control by breeders has allowed for the gradual extension of wheat cultivation. A detailed knowledge of key genetic components of the life cycle would enable an easier control by the breeder (Snape et al. 2001).

The initiation of flowering in bread wheat is determined both by external signals—cold temperatures (vernalization requirement), long days (photoperiod sensitivity)—and internal signals such as intrinsic earliness (earliness per se) (Sun 1985; Snape et al. 2001). Previous mapping and segregation analyses have shown that vernalization requirement and photoperiod sensitivity are mainly regulated by major genes (see Law and Worland 1997 for a review), whereas earliness per se is more polygenic. Recent genomic studies have confirmed these results. In wheat, vernalization requirement is controlled by four major genes designated by *Vrn-1*, *Vrn-2*, *Vrn-3* and *Vrn-4* (Yoshida et al. 2010). For example, three homeologous *Vrn-1* genes, the *Vrn-A1*, *Vrn-B1* and *Vrn-D1*, respectively, located on the long arms of chromosomes 5A, 5B and 5D were found to control the winter (vernalization required)/spring (vernalization not required) growth habit (Yan et al. 2003). The expression of *Vrn-1* in winter genotypes is controlled by repressors interacting with regulatory sequences. The presumed target sites are the promoter region of the copy on genome A (*Vrn-A1*) and the first intron of the copy on genome B (*Vrn-B1*) and D (*Vrn-D1*) (Yan et al. 2004; Fu et al. 2005). Mutations altering repressor recognition sites at one of these sites lead to the expression of the gene in unvernalized genotypes (Loukoianov et al. 2005; Pidal et al. 2009). This mechanism explains the epistatic interaction between the three copies and the dominance found for the spring allele in the literature. Recent quantitative trait locus (QTL) meta-analyses (Hanocq et al. 2007; Griffiths et al. 2009) revealed that the genetic control of earliness and its components involves also other genomic regions of chromosomes 4 and 7 with lesser effects. On chromosome 7, an orthologous gene of *Arabidopsis thaliana*, *FLOWERING TIME LOCUS T* (*FT*), was recently isolated. Formerly known as *Vrn-5* or *Vrn-B4* (McIntosh et al. 2003), it is now referred to as *Vrn-3*, (Yan et al. 2006). The dominant allele at the *Vrn-B3* locus (the genome B copy of *Vrn-3*), found in the variety Hope, is associated with the insertion of a transposable element in the *Vrn-B3* promoter. *Vrn-3* promotes the transcription of *Vrn-1* and accelerates flowering (Li and Dubcovsky 2008), and mutations in the A and D copies were found to be mostly associated with flowering time without vernalizing treatment (Bonnin et al. 2007).

Identifying genes underlying agronomic traits is a major topic for plant breeders and crop genetic improvement. Until recently, QTL mapping was the single available method, requiring controlled crossing and maintenance of a large number of progeny to develop a robust linkage map, before associating phenotypes with specific identifiable regions of the genome (e.g., Gervais et al. 2003; Hanocq et al. 2007; Buckler et al. 2009). Conversely, new approaches such as linkage disequilibrium mapping rely on surveys of genetic polymorphism data from a collection of mostly unrelated samples (inbred lines, accessions, individuals or populations) to test for statistical associations between these genetic markers and particular phenotypes. This approach is based on the hypothesis that significantly associated markers are in linkage disequilibrium with a causal polymorphism of the phenotype or even reveal in fact the causal mutation itself. Unlike QTL mapping, linkage disequilibrium mapping approaches do not require the time-consuming and expensive generation of specific populations, but involve the control of cryptic population structure, which can lead to false positives. Using candidate genes, this method has successfully been applied in several model or crop species (e.g., Thornsberry et al. 2001; Nachman et al. 2003; Ehrenreich et al. 2007; Ravel et al. 2007).

In this paper, using different vernalization treatments, we conducted an association study in hexaploid wheat for six candidate genes for flowering time and the variation for vernalization sensitivity and earliness per se. A collection of 235 lines representing diverse geographical origins and status (landraces, old or recent cultivars) was used. For clarity and brevity, day length sensitivity was not included in this study, but it will be analyzed independently using appropriate phenotypic assessment in a forthcoming study.

Three genes out of six have already been described in bread wheat: *Vrn-1* (Yan et al. 2004; Sherman et al. 2004; Fu et al. 2005; Rhoné et al. 2008; Zhang et al. 2008; Pidal et al. 2009), *Vrn-3* (Yan et al. 2006; Bonnin et al. 2007), and *TaHd-1* (Nemoto et al. 2003). Transformation of rice with *TaHd-1* (=*CO2* in the Griffiths nomenclature (Griffiths et al. 2003)) showed that this gene is a functional homolog of the *Hd1* gene (=*CO*) in rice, which is involved in the photoperiod sensitivity in this species. Unlike *Vrn-1* and *FT* genes, no association study with earliness variation in wheat has been conducted for this gene before. Given that *CO2* gene is part of a large gene family in *Arabidopsis*, rice and barley (Griffiths et al. 2003), we also studied another orthologous gene fragment, different from *TaHd-1* and called *CO* in this paper. The two other gene fragments studied were orthologous sequences of *Arabidopsis thaliana* genes, known in this species to be involved either in the autonomous pathway, i.e., an endogenous pathway functioning independently of environmental signals and related to the developmental state of the plant, such as *LUMINI-DEPENDENS*: *LD* (Lee et al. 1994), or in the photoperiod pathway such as *GIGANTEA*: *GI* (Dunford

et al. 2005). The analysis of the epistatic interactions between all these genes and their effect on the earliness component variation is worthwhile. This study contributes to highlight the mechanisms involved in the genetic control of different quantitative traits describing the flowering time in hexaploid wheat. The study of a highly diversified core collection of 235 accessions increased the allele richness maximizing allelic combinations of genes of flowering pathways.

Materials and methods

Plant material

A sample of 235 bread wheat accessions was chosen within the INRA bread wheat core collection of 372 accessions (372CC) (Balfourier et al. 2007). This 372CC was previously selected from 4,000 accessions by maximizing both the number of alleles at SSR loci and the number of geographical origins. For the present study, 235 accessions were sub-sampled from this 372CC, using passport data and field evaluation data on the core, to cover an expected wide range of variation for earliness and growth habit. This sub-sample of 235 accessions, which captures more than 88% of the alleles observed at 38 SSR loci among the 4,000 accessions, includes landraces and cultivars from 56 different countries (Table supplementary S1).

Phenotyping

Phenotypic traits related to earliness (Table 1) were assessed in field experiments at Le Moulon (48.4°N, 2.1°E) in a 3-year experiment (2004, 2005 and 2006), using the same protocol as described by Goldringer et al. (2006) and Bonnin et al. (2007). In the autumn nursery experiment, the 235 genotypes were sown at the end of October in 2004 and beginning of November in 2005 and 2006, in a two-replicate complete block design, where they received natural full vernalization during winter. Each genotype was sown in two 1.20-m long single-row plots with 20 seeds per plot. Average heading dates were derived for each accession and denoted as HdField. In the 2004, 2005 and 2006 spring nursery experiments, young plantlets of the 235 genotypes were transplanted in the field in mid-April (photoperiod is $\sim$14 h and is increasing) after a vernalization treatment of 4 or 8 weeks at 4°C. The 235 genotypes were also sown between the 4th and the 11th of April (non-vernalized treatment) and observations continued until mid-August. The last headings for the non-vernalized treatment were observed at the beginning of August at a date corresponding to a maximum of 1,934, 1,984 and 2,089°d, respectively, since seedling emergence in 2004, 2005 and 2006, respectively. We attributed an arbitrary value of 2,000°d (for years 2004 and 2005) or 2100°days (for 2006) to genotypes that did not head by the time the experiment was terminated.

Heading dates observed in the field after no, partial or full vernalization treatments were denoted as HdNV, HdV4 and HdV8, respectively. For these nursery treatments, heading dates were scored on five to six individuals per accession averaged over the values obtained. Heading dates were transformed in thermal times (Celsius degrees days) starting from the emergence.

In this paper, we used the HdV8 parameter to estimate earliness per se, the remaining earliness when requirements in vernalizing temperatures and long days are satisfied (Goldringer et al. 2006; Bonnin et al. 2007). The terms "narrow-sense earliness" or "intrinsic earliness" can be considered as synonymous of "earliness per se". According to Worland et al. (1994), earliness per se genes are those that act independently from environmental stimuli, and for Slafer and Rawson (1995) earliness per se is the minimum time betwen sowing and maturity independently from sensibility to vernalization and photoperiod. We will use "earliness per se" all along this paper.

Partial vernalization sensitivity (PVS) was determined as the quantitative response to cold temperature exposure. It was calculated as the difference between the mean heading time in partially vernalized treatment and the mean heading time in fully vernalized treatment: PVS = HdV4-HdV8 (Goldringer et al. 2006). We also characterized the wheat accessions according to their HdNV mean value. As shown by Rhoné et al. (2008) on experimental populations of wheat, HdNV averages exhibited a bimodal distribution. For the present data, the value of 1,450°d was considered as a critical threshold to split the whole 235 core collection into two subpopulations. The accessions heading for the

Table 1 Core collection phenotyping for vernalization and earliness parameters

Trait/character	Treatment	Variable ($\Sigma\theta$°d)
Vernalization requirement	Spring planting (long days) without vernalization	HdNV
Vernalization requirement	Spring planting (long days) with a partial 4-week vernalization at 4°C	HdV4
Earliness per se	Spring planting (long days) with a full 8-week vernalization at 4°C	HdV8
Earliness in the field	Autumn planting in the field	HdField
Partial vernalization sensitivity		PVS = HdV4–HdV8

unvernalized April planting with less than 1,450°d were classified in the spring subpopulation and the accessions heading for the unvernalized April planting with more than 1,450°d or not heading at all were classified in the winter subpopulation. We stopped observations of heading while some genotypes (very winter types) were still in a vegetative stage. We attributed an arbitrary value of 2,000°d (for years 2004 and 2005) or 2,100°d (for 2006) to genotypes not having headed at the end of the experiment.

Molecular tools and genotyping

DNA isolation

All the seeds used for DNA extraction were obtained from self-pollinated ears coming from the Clermont-Ferrand Genetic Resources Center (http://www.clermont.inra.fr/umr1095). For SSR markers study, fresh leaves of five to six plants per accession were pooled and bulk genomic DNA was extracted using a cetyltrimethylammonium bromide (CTAB) protocol as previously described (Tixier et al. 1998). For SNP study, total DNA from one plant per accession was extracted from 200 mg of young leaves following a protocol derived from the DNeasy 96 Plant kit of Qiagen (QIAGEN, Basel, Switzerland).

SNP discovery

Vrn-1 and Vrn-3 genes For genes *Vrn-1* and *Vrn-3*, previously described polymorphisms were studied, consisting for *Vrn-1* in: (1) duplication, insertion and deletion in the promoter of *Vrn-1* genome A (denoted as $Vrn\text{-}A1_{pr}$ in the following) in Yan et al. (2004), (2) a substitution in the 7th exon of *Vrn-1* genome A ($Vrn\text{-}A1_{ex7}$) in Sherman et al. (2004), (3) a 4-kb deletion in the first intron of *Vrn-1* genome B ($Vrn\text{-}B1_{int1}$) and (4) a 4-kb deletion in the first intron of *Vrn-1* genome D ($Vrn\text{-}D1_{int1}$) in Fu et al. (2005) (see Table supplementary S2 for the Vrn-1 allele nomenclature). For *Vrn*-3, studied polymorphic sites were: (1) a substitution in the first intron of genome A (*Vrn-A3-Y*), (2) a variable dinucleotide microsatellite in the second intron of genome A ($Vrn\text{-}A3\text{-}(TG)_{8or9}$), (3) a substitution in the third exon of genome A (*Vrn-A3-K*) and (4) an insertion-deletion in a poly G in the third exon of genome D ($Vrn\text{-}D3\text{-}(G)_{3or4}$) as described in Bonnin et al. (2007).

Lumini-dependens (LD), Gigantea (GI) and Constans (CO) genes Both Gigantea (*GI*) and Luminidependens (*LD*) genes have been significantly associated with flowering time in an association study conducted on *Arabidopsis thaliana* for 51 genes of the flowering time pathway (Ehrenreich et al. 2009). Luminidependens (*LD*), Gigantea (*GI*) and Constans (*CO*) wheat gene fragments were isolated from sequence homology analyses between rice genomic sequences and wheat ESTs. Gene-specific primers were designed in conserved regions of putative exons using Oligo 6 software (Medprobe, Oslo, Norway). To discard primers giving no band or a smear, amplifications were performed on one hexaploid AABBDD accession (*Triticum aestivum* var. Récital) and three diploid accessions: *T. urartu* (AA genome), *T. speltoides* (BB genome, selfed progeny) and *T. tauschii* (DD genome). Each genomic copy from hexaploid wheat was then isolated by cloning amplification products into pGEM-T Vector system I (Promega, Madison, Wisconsin, USA) and sequenced (subcontracted by Genome Express, Meylan, France). All sequences were analyzed using the Staden software package (Staden et al. 2000). Design of genome-specific PCR primers was achieved by comparing diploid and hexaploid sequences. Amplification of the four genotypes with these primers was used to check genome specificity. Furthermore, PCR on nullitetrasomic, ditelosomic and deletion lines of the 'Chinese Spring' variety (Sourdille et al. 2004) allowed assigning amplification product to one chromosome, one chromosome arm and one deletion bin, respectively. More details on the PCR conditions for each primer couple can be found in Rhoné et al. (2010). Amplification products were separated on a 1% agarose gel and visualized under UV light following ethidium bromide staining.

For LD genes of genomes A and B, a BLASTN search (National Centre for Biotechnology Information: http://www.ncbi.nlm.nih.gov/BLAST/) was used to reveal the location of the five SNPs within the gene sequence on a set of 40 lines and then to compare it to the rice LD gene.

TaHd-1 gene We studied the wheat *CO2* gene published in Nemoto et al. (2003) and provided as three genomic sequences from the cultivar Chinese Spring: *TaHd-1-1*, *TaHd-1-2* and *TaHd-1-3* for genomes A, B and D respectively. Sequence alignment was used to define genome-specific primers (Table supplementary S3) and genome specificity of amplified products was checked as above.

For each gene fragment, polymorphism was then screened on a sub-sample of 40 out of 235 accessions using primers and PCR conditions from the literature for *Vrn-1* and *Vrn-3* genes and from Table supplementary S3 for the other genes. Except for *Vrn-1*, all PCR products were purified and sequenced with the Big Dye Sequencing kit according to the manufacturer's specifications (Applied Biosystems, Courtaboeuf, France), then loaded onto ABI3700 96 capillary sequencers. Mutations detected in only one line (singleton) were checked by re-sequencing an independent amplification product.

SNP genotyping For the *Vrn-1* polymorphic sites, PCR conditions and PCR product digestion protocols were the

same as defined by the authors. Unlike Yan et al. (2004), polymorphism at *Vrn-A1*$_{pr}$ was revealed by migration on acrylamide gel as in Rhoné et al. (2008).

For the *TaHd-1* and *Vrn-3* polymorphic sites, PCR products of the 195 (235-40) remaining lines were upstream or downstream sequenced only, using ABI3700 96 capillary sequencers.

For the fragments of the three candidate genes, *LD*, *GI* and *CO*, initial genome-specific PCR amplifications of the genes were carried out as described before. PCR products were then diluted 1/50 and 2 µl was used for subsequent SNP genotyping by competitive allele-specific PCR. Reactions were carried out in 25 µl of total volume, with 3 mM $MgCl_2$, 0.6 mM of each dNTP, 1U Taq polymerase (Qiagen), Taq polymerase buffer (Qiagen), and the three following primers:

1. 0.5 µM of a (short) forward primer designed from the sequence immediately upstream to the SNP, with the base at its 3′ extremity corresponding to the SNP position (TTGTCATCTTGTGTACGAATTC, CAGA AGAGGCTAGGCGAAAC and CAAGCACTCTTG GTCGATCTC 5′ to 3′ for LD, GI and *CO*, respectively),
2. 0.25 µM of a (long) similar forward primer, but with the base at the 3′ extremity corresponding to the alternate allele of the SNP and with a 5′ tail added, so that PCR products originating from this long forward primer are 19 bp longer than those originating from the short forward primer (CACGACGTTGTAAAAC GACTTGTCATCTTGTGTACGAATTT, CACGAC GTTGTAAAACGACCAGAAGAGGCTAGGCGAA AT and CACGACGTTGTAAAACGACCAAGCAC TCTTGGTCGATCTT 5′ to 3′ for *LD*, *GI* and *CO*, respectively), and
3. 0.5 µM of a reverse primer designed to get an amplified product with either the short or the long forward primer (CTGGAAGAACACAAATAATGA CG, AGCCTCGATAACCCCCATT and AATTCTA TGGACTTGATCTCTAT 5′ to 3′ for *LD*, *GI* and *CO*, respectively). The competition between both forward primers results in a much higher priming efficiency for the primer with a correct match at the 3′ base, so that depending on the allele at the SNP position, either the short or the long forward primer is preferentially involved in the product. Thermal cycling conditions were: 94° 5 min, 35 cycles: (94° 30 s, 65° or 60° 30 s, 72° 30 s), final extension 72° 5 min. The products were electrophoresed on 4% MetaPhor™ (TEBU, Le Perray, France) agarose gel with 1 mg/ml ethidium bromide during 4 h at 120 V, so that the 19-bp size differences between the two SNP alleles were easy to read. Genotyping results on these three genes were completed and checked by using a variant of competitive allele-specific PCR: here, a 5′ tail containing a restriction site was added to the short forward primer, so that both forward primers had the same size. Competition bias due to primer size differences was then reduced. The long forward primer and the reverse primer were the same as above. Competitive allele-specific PCRs were then carried out as described above, except for the reaction volume, which was 22 µl. These products were then entirely digested with three units of restriction enzyme in a total volume of 27 µl at 37°C overnight, then electrophoresed as described above. For *LD* and *GI*, the restriction enzyme used was EcoRV and the 5′ tail for short forward primers was (5′ to 3′) CACGACGTTGTAAGATATC. For *CO*, the restriction enzyme used was EcoRI and the 5′ tail for short forward primers was (5′ to 3′) CACGACGTTGT AAGAATTC.

Data analysis

The core collection structure: Data from 38 genomic SSR loci (Roussel et al. 2004, 2005) and 44 SSR loci derived from EST (Balfourier et al. 2007) were simultaneously used to detect the underlying genetic structure of the sample of 235 accessions. Because of their low frequency and to simplify analyses, heterozygous data (1–3% of the data) were considered as missing data. We used an expectation maximization (EM)-algorithm procedure based on recent theoretical development of discrete principal component analysis (DPCA) from the frequentist framework devised by Tang et al. (2005) to extract from marker data set population components on which individuals are probabilistically assigned (Veyrieras et al. 2006; Stracke et al. 2009). The results derived from this procedure were compared to those obtained with the Structure program V2.2 (Pritchard et al. 2000) using the admixture model. Both approaches are equivalent and rely on the same assumption: the genome of individuals is a mixture of genes originating from *K* unknown 'ancestral' populations that may have undergone introgression events. Under this model, the EM-algorithm based on DPCA and the Structure algorithm estimate the proportion of membership (genome ancestry) of each individual in each of the *K* ancestral populations. Because microsatellites derived from EST are less polymorphic than genomic SSR (Balfourier et al. 2006), which could lead to different genetic structures, inferences were carried out on the complete data set and on the two types of markers separately.

The admixture analysis using the EM-algorithm was run using a maximum number of 1,500 iterations and a convergence tolerance of 10^{-8}. To choose the 'optimal'

number of subpopulations, K (minimal number of population components which capture most of the linkage disequilibrium due to population structure), we applied the procedure for $K = 1$–10 with ten independent runs. Among those runs, we selected the one leading to the minimum residual linkage disequilibrium, i.e., linkage disequilibrium between markers not explained by the corresponding admixture model, while also considering the dispersion of values. The optimal number of group K was then inferred by comparison of the corresponding residual linkage disequilibrium with the one estimated after 1,000 bootstrap permutations (Veyrieras et al. 2006; Stracke et al. 2009).

As recommended by the authors for the Structure algorithm, the length of the burn-in period and the number of iterations were fixed at 10,000 and 100,000, respectively. This number of iterations permitted to obtain less variable likelihood estimates across different runs. Ten independent runs were performed at each K value, from $K = 1$ to $K = 10$, with K referring to the number of groups to be inferred. The choice of the appropriate K value was conducted as recommended by the authors: the K value at which a maximum log likelihood of data was reached was retained.

Analyses of variance, correlations: All variables assessed from the field experiments (HdField, HdNV, HdV4, HdV8, PVS) were available for the 3 years of experimentation except for some missing data corresponding to accidental loss of plants in part of the trial. A simple ANOVA with a fixed year and a random genotype effect was performed to assess heritabilities over years and to derive least-square-means (LSM using the general linear model, GLM, procedure of the SAS software package, SAS Institute 2000) for the 235 accessions. Phenotypic correlations were computed between the LSM of HdField, HdNV, HdV4, HdV8 and PVS.

Association genetics: Association between the nucleotide polymorphisms (SNP for short) and phenotypic traits was tested with the GLM procedure of the SAS software package, considering the proportions of admixture to the K ancestor groups of each individual accession as covariates in the model. The contribution of the structure (groups) to explain the variations of the different traits was first estimated through the determination coefficient (R^2) of a model without any other genetic effects.

Then we used the following procedure to identify the model(s) that best explain each trait variation using the SNPs of the six genes:

(i) the effects of SNPs were tested for each gene at a time using the following linear model to explain the trait value, Y, of genotype j grown during year i:

$$Y_{ij} = \mu + yr_i + \sum_{k=1}^{K-1} a_k g_{(j)}^k + \sum_{s=1}^{S} P_{(j)}^s + R_{ij}$$

with yr the fixed year effect, $g_{(j)}^k$ the proportion of the genotype j genome attributed to group k, a_k the effect of group k, $P_{(j)}^s$ the effect of the genotype carried by j at SNP s, and R the residual. For each gene, pairwise interactions between SNPs were also tested when the sample sizes of all genotypic classes defined were large enough (≥5 individuals). Otherwise, di-locus haplotypes of the genotypic classes with sufficient sample size were defined. Note that the sample size and thus the statistical power of the variance analysis could be reduced when haplotypes were considered:

(ii) for each gene, the SNPs and their interactions (or di-locus haplotypes) that yielded the highest level of explanation of the data variation (R^2) using the largest sample size and the lowest number of parameters were identified as the "gene model" for each trait;

(iii) finally, to best describe each trait variation, all SNPs and interactions (or di-locus haplotypes) from the six "gene models" were included in a single "global model" considering pairwise interactions between genes. For each character, we also present results from simplified "global model" retaining the significant effects only. Note that by applying this method, different "global models" can give the same level of explanation of a trait variation.

To control for multiple testing, we have applied the conservative Bonferroni correction based on the fact that all SNPs within each of the six genes were tested together in the first step (gene model) and considered as potentially not independant, thus the alpha risk was set to 0.05/6 ~ 0.008 in all ANOVA (gene model and global model).

Linkage disequilibrium between loci

Linkage disequilibrium between all SNPs was estimated as the correlation coefficient between standardized individual allele frequencies. In addition to these "gross" values, linkage disequilibria were also corrected to take into account the specific linkage disequilibrium that might be due to the identified structure of the data, by standardizing allele frequencies with their different values conditional to the groups. First, for each locus, we estimated the allelic frequencies in each of the K identified groups. Then, individual expected frequencies were computed based on the proportion of admixture to the groups. Finally, the true individual allelic frequencies (0 or 1) were corrected by their expected values. Linkage disequilibrium was estimated as the correlation between corrected frequencies at two loci. Significance of the correlation coefficients was assessed using bootstraping of alleles.

Table 2 Number, adjusted mean, minimum, maximum, standard deviation of the growth habit traits estimated on the 235 accessions, the spring types (lsmNV < 1,450°d) and the winter types (lsmNV > 1,450°d)

Variable	Type	*N*	Mean	Minimum	Maximum	SD
lsmHdNV	All	235	1425	745	2060	494
	Spring	120	965***	745	1339	145
	Winter	115	1904	1409	2060	164
lsmHdV4	All	234	1066	652	2116	291
	Spring	119	864***	652	1157	105
	Winter	115	1276	829.4	2116	280
lsmHdV8	All	235	959	703.2	1492	122
	Spring	120	890***	703.2	1142	90
	Winter	115	1031	794.8	1492	107
lsmPVS	All	233	89	−301	980	215
	Spring	118	−51***	−301	237	60
	Winter	115	233	−130	980	223
lsmHdField	All	235	1184	934	1444	103
	Spring	120	1138***	934	1444	92
	Winter	115	1232	1012	1413	93

*** Lsmeans difference between spring and winter types is significant at a 0.0001 level

Results

The genetic structure of the collection

The EM-algorithm approach identified five ancestral groups (*K*) that minimized the residual covariance of the dataset when the 82 SSR markers were included. The analysis with the Struture software led to the same number of groups. When using the sub-samples of markers (38 neutral/44 EST), we found rather similar genetic structures with both methods. The genetic structure inferred with the 82 SSR markers was the following (see also Figure supplementary S1): all Nepalian (NPL) accessions were gathered in a first group (group 1), together with accessions from Afghanistan, Tajikistan and some from India and Pakistan and other Asian countries; the major part of accessions from northwest European countries (AUT, CHE, DEU, DNK, FRA, GBR, POL, SWE) formed a second group of accessions (group 2), while a third group (group 3) possibly included accessions related to the worldwide CIMMYT breeding program based on common genetic resources and scattered in very diverse regions like Mexico (MEX), Australia (AUS), Israel (ISR) South America (ARG, COL, URY), Africa (EGY, KEN, TUN, ZAF) and even North America (CAN, USA). Most of Japanese and Chinese accessions form a fourth group (group 4) with some Mediterranean accessions (ESP, ITA). A majority of European, east European, southeast European accessions (BEL, CSK, DEU, FIN, FRA, HUN, ROM, RUS, UKR) together with Australian, Canadian and North American accessions were clustered in a fifth group (group 5) including some accessions from the Middle East (LBN, TUR). Consistently, these five groups could also be discriminated in the projection on the plan of the two first axes of a PCA based on the 82 markers (see Figure supplementary S3).

Earliness phenotyping of the core collection

Using the critical threshold of 1,450°d for HdNV, we partitioned the core collection into winter/spring groups and about half of the accessions of the whole core collection were included in each of the spring and the winter groups (Table 2). For earliness per se measures (HdV8), HdField and variables related to vernalization requirement (HdNV, HdV4), the spring genotypes headed significantly earlier on average (Table 2). They also showed a lower partial vernalization sensitivity. Conversely, the later winter group exhibited a larger variance for HdV4 and partial vernalization sensitivity (Table 2).

It is noticeable that the vernalization requirements were not equal in the five groups identified from neutral markers. Indeed, we observed a decrease in the mean heading date under no vernalization treatment as measured by HdNV (Figure supplementary S1) with cluster2 > cluster5 > cluster1 > cluster4 > cluster3. This ranking corresponded to a decreasing trend in the proportion of "winter" *Vrn-1* haplotypes with the relative proportions of winter versus spring of 53/14, 32/27, 9/19, 12/26 and 9/34 for clusters 2, 5, 1, 4 and 3, respectively.

Overall, correlations between adjusted genotypic means of the different heading date measures were all highly

Table 3 Correlations between phenotypical traits according to the growth habit group

	HdV4			HdV8			PVS			HdField		
	S	W	All	S	W	All	S	W	All	S	W	All
HdNV	0.645****	0.519****	0.782****	0.536****	0.453****	0.678****	0.145 ns	0.444****	0.708****	0.416****	0.469****	0.554****
HdV4				0.800****	0.687****	0.789****	0.205*	0.909****	0.919****	0.814****	0.544****	0.662****
HdV8							−0.358****	0.357****	0.505****	0.903****	0.881****	0.908****
PVS										−0.204*	0.241**	0.382****
HdField												

S spring (HdNV < 1450°d), *W* winter (HdNV > 1450°d), *All* for the whole 235 core collection
ns not significant
* <0.05
** <0.01
*** <0.001
**** <0.0001

significant (Table 3). HdField and HdV8 appeared to be highly positively correlated whatever the growth habit group considered (S, W or all) (Figure supplementary S2) suggesting that heading date scored in the field with an autumn sowing included a large contribution of earliness per se. Conversely, lower but significant correlations were observed between this HdV8 variable and partial vernalization sensitivity in the winter group and overall, suggesting that earliness per se and vernalization requirement were not independent in our experiment. Yet negative correlations were observed between the same traits within the spring group, which were due to a very low variation for partial vernalization sensitivity in this group (Table 3). Other variables related to vernalization requirement (HdNV, HdV4) were highly correlated to each other whatever the level studied and with partial vernalization sensitivity at the winter group level and overall.

Allelic variation of the candidate genes

The number of genotypes found in the collection for each polymorphism, as well as a brief description of the polymorphisms with the different allele nomenclatures, is presented in Tables 4, 5 and 6. The polymorphisms of the collection for the *Vrn-A3* and *Vrn-D3* copies of the *Vrn-3* gene have already been described in Bonnin et al. (2007). For the *Vrn-1* promoter on genome A, the three alleles described in Yan et al. (2004) (alleles 1, 2 and 5) and the two additional new alleles found by Rhoné et al. (2008) (alleles 3 and 4) were present within the 235 core collection, the "winter" $Vrn\text{-}A1_{pr}$ "allele 2" being the most frequent. The three other studied loci, $Vrn\text{-}A1_{ex7}$, from Sherman et al. (2004) and $Vrn\text{-}B1_{int1}$ and $Vrn\text{-}D1_{int1}$ from Fu et al. (2005), were also polymorphic in the collection, with two alleles each and the lowest level of diversity observed for $Vrn\text{-}D1_{int1}$.

We did not succeed in obtaining genome-specific amplification for the D copy of *LD*. Out of 2,000 sequenced bases for *LD* genomes A and B on the 40 lines, five SNPs were found overall. The BLASTN search revealed that the five SNPs were located inside a wheat intron and between two exons that are orthologous to the 11th and 12th exon of the rice *LD* gene. They were designated as *LD-A-Y1* and *LD-A-Y3* on the genome A (C/T polymorphisms) and were separated by 340 bases. Unfortunately, given that they were encountered in less than 5 individuals out of 235 (2%), these SNPs were not further used for association study. Around 600 bases downstream on genome B, the SNP *LD-B-Y*, was observed followed by *LD-B-M* (300 bases from *LD-B-Y*) and *LD-B-R* (147 bases from *LD-B-M*). Given that *LD-B-Y* and *LD-B-R* were in total linkage disequilibrium (Table 7), allelic variations for both sites distinguished the same lines (31 out of 235 for *LD-B-Y*). Considering the three SNPs together, four haplotypes were observed overall.

Table 4 Number and type of polymorphisms detected for each of the gene in the 235 wheat core collection

Gene	Sequenced bases on the sub-sample of 40 wheats	Physical mapping (chromosome, arm, bin)	Polymorphism name (SNPs are designed by EUB code)	Polymorphism localization (5′–3′)	Observed genotypes (see nomenclature in Tables 5 and 6) and number of accessions in the 235 core collection in parenthesis	Diversity of Nei(He)
Vrn-1 genome A	–	5A L (Yan et al. 2004)	*Vrn-A1*$_{pr}$	Promoteur	11 (31), 22 (163), 25 (3), 33 (13), 44 (4), 55 (19)	0.475
		Sherman et al. (2004)	*Vrn-A1*$_{ex7}$	Exon 7 (+12 Kbases/*Vrn-1A*$_{pr}$)	11 (141), 12 (5), 22 (89)	0.478
Vrn-1 genome B	–	5B L (Fu et al. 2005)	*Vrn-B1*$_{int1}$	Intron 1 (+1 Kbases/*Vrn-1A*$_{pr}$)	11 (170), 22 (44)	0.328
Vrn-1 genome D	-	5D L (Fu et al. 2005)	*Vrn-D1*$_{int1}$	Intron 1 (+1 Kbases/*Vrn-1A*$_{pr}$)	11 (201), 12 (3), 22 (30	0.234
Vrn-3 genome A	791	7A S (Bonnin et al. 2007)	*Vrn-A3Y*	Intron 1	CC (212), CT (2), TT (18)	0.151
			Vrn-A3 K	Exon 3	GG (21), TT (211)	0.165
			Vrn-A3(TG)$_{8or9}$	Intron 2	88 (77), 89 (3), 99 (152)	0.450
Vrn-3 genome B	1064	7B S (Bonnin et al. 2007)	–	–	–	–
Vrn-3 genome D	828	7D S (Bonnin et al. 2007)	*Vrn-D3(G)*$_{3or4}$	Exon 3 (+30 bases/*Vrn-A3 K*)	33 (178), 44 (53)	0.355
GI genome A	1300	3A S 4	*GI-A-R*	Intron 2	AA (212), GG (6)	0.054
			GI-A-S	Exon 5 (+690 bases/*GI A R*)	CC (213), GG (6)	0.054
GI genome B	1300	3B S 9	*GI-B-Y*	Exon 5 (+90 bases/*GI A S*)	CC (154), CT (2), TT (79)	0.451
GI genome D	1300	3D S 3	–	–	–	–
LD genome A	2000	3A L 5	*LD-A-Y1*	Intron	CC (2), TT (209)	0.019
			LD-A-Y3	Intron (+340 bases/*LD- A Y1*)	CC	0.035
LD genome B	2000	3B L 7	*LD-B-Y*	Intron (+600 bases/*LD- A Y3*)	CC (31), TT (204)	0.230
			LD-B-M	Intron (+300 bases/*LD- B-Y*)	AA (144), AC (2), CC (84)	0.468
			LD-B-R	Intron (+147 bases/*LD- B-M*)	AA (30), GG (189)	0.238
TaHd-1-1 genome A	1800	6A L (Nemoto et al. 2003)	*Hd-1A-K*	Intron	GG (12), GT (1), TT (211)	0.106
TaHd-1-2 genome B	1900	6B L (Nemoto et al. 2003)	*Hd-1B-(G)*$_{0or1}$	Exon 1 (−1160 bases/*Hd-1 A K*)	11 (217), 00 (10)	0.085
TaHd-1-3 genome D	1800	6D L (Nemoto et al. 2003)	–	–	–	–
CO genome A	300	5A L 12	–	–	–	–
CO genome B	300	5B L 6	*CO-B-Y*	Intron	CC (51), TT (179)	0.347
CO genome D	300	5D L 1	–	–	–	–

Table 5 Polymorphisms detected for the VRN-1 gene in the 235 wheat core collection, synonymous allele nomenclatures

Physical mapping (chromosome, arm, bin)	Gene, locus	Allele nomenclature		Polymorphism, GenBank #
		Yan et al. (2004), Sherman et al. (2004), Fu et al. (2005)	Rhoné et al. (2008)	
5A L (Yan et al. 2004)	*Vrn-A1*$_{pr}$	*Vrn-A1a*	*Vrn-A1pr-1* = *Vrn*$_{(1)}$ = 11	Insertion and duplication in the promoter region (GenBank AY616458), (GenBank AY616459)
		Vrn-A1b	*Vrn -A1pr-5* = *Vrn*$_{(5)}$ = 55	Deletion of 20-bp and SNP, in the 5′UTR regions. (GenBank AY616461)
		vrn-A1	*vrn-A1pr-2* = *vrn*$_{(2)}$ = 22	"Wild" winter allele. (GenBank AY616455)
		Not described	*vrn-A1pr-3* = *vrn*$_{(3)}$ = 33	"High fragment" (GenBank EU127902) and "low fragment" (GenBank EU127903)
		Not described	*vrn-A1pr-4* = *vrn*$_{(4)}$ = 44	(GenBank: EU127901)
Sherman et al. (2004)	*Vrn-A1*$_{ex7}$	*vrn-A1*	*vrn-A1*$_{ex7}$ -1 = *vrn* $_{(1)}$ = 11	"Wild" winter allele
		Vrn-A1	*Vrn-A1*$_{ex7}$ -2 = *Vrn* $_{(2)}$ = 22	"Mutated" spring allele: two one bp indels in introns and one substitution in exon 7 val⇨ala
5A L (Fu et al. 2005)	*Vrn-A1*$_{int1}$	*vrn-A1*	No polymorphism observed	"Wild" winter allele non-deleted
		Vrn-A1		"Mutated" spring allele deleted
5B L (Fu et al. 2005)	*Vrn-B1*$_{int1}$	*vrn-B1*	*vrn-B1-1* = *vrn* $_{(1)}$ = 11	"Wild" winter allele non-deleted (GenBank AY747604)
		Vrn-B1	*Vrn-B1-2* = *Vrn* $_{(2)}$ = 22	"Mutated" spring allele deleted deleted (GenBank AY747603)
5D L (Fu et al. 2005)	*Vrn-D1*$_{int1}$	*vrn-D1*	*vrn-D1-1* = *vrn* $_{(1)}$ = 11	"Wild" winter allele non-deleted deleted (GenBank AY747606)
		Vrn-D1	*Vrn-D1-2* = *Vrn* $_{(2)}$ = 22	"Mutated" spring allele deleted deleted (GenBank AY747597)

Table 6 Polymorphisms detected for the *VRN-3*, *GI*, *LD*, *TaHd* and *CO* genes in the 235 wheat core collection, synonymous allele nomenclatures

Physical mapping (chromosome, arm, bin)	Gene, locus	Allele nomenclature	Polymorphism
7A S (Bonnin et al. 2007)	*Vrn-A3* $_{int1}$ = *Vrn-A3 Y*	*Vrn-A3 Y a* = CC = 22	A substitution C⟺T in the first intron of genome A
		Vrn-A3 Y b = TT = 44	
	Vrn-A3 $_{ex3}$ = *Vrn-A3 K*	*Vrn-A3 Ka* = TT = 44	A substitution T⟺G in the third exon of genome A (*Vrn-A3-K*)
		Vrn-A3 Kb = GG = 33	
	Vrn-A3 $_{int2b}$ = *Vrn-A3* $(TG)_{8or9}$	*Vrn-A3* $(TG)_9$ = *Vrn-A3a* = 99	A variable dinucleotide microsatellite in the second intron of genome A (*Vrn-A3-*$(TG)_{8or9}$)
		Vrn-A3 $(TG)_8$ = *Vrn-A3b* = 88	
7B S (Bonnin et al. 2007)	No polymorphism observed		
7D S (Bonnin et al. 2007)	*Vrn-D3* $_{ex3}$ = *Vrn-D3* $(G)_{3or4}$	*Vrn-D3* $(G)_3$ = *Vrn-D3a* = 33	An insertion-deletion in a poly G in the third exon of genome D (*Vrn-D3-*$(G)_{3or4}$)
		Vrn-D3 $(G)_4$ = *Vrn-D3b* = 44	
3A S 4	*GI-A-R*	*GI-A-Ra* = AA = 11	A substitution A⟺G in the second intron of genome A
		GI-A-Rb = GG = 33	
	GI-A-S	*GI-A-Sa* = CC = 22	A substitution C⟺G in the fifth exon of genome A
		GI-A-Sb = GG = 33	
3B S 9	*GI-B-Y*	*GI-B-Ya* = CC = 22	A substitution C⟺T in the fifth exon of genome B
		GI-B–Yb = TT = 44	
3D S 3	No polymorphism observed	–	
3A L 5	*LD-A-Y1*	*LD-A-Y1a* = TT = 44	A substitution T⟺C in intron of genome A
		LD-A-Y1b = CC = 22	
3B L 7	*LD- B-Y* = *LD- B* $_{int\ 11a}$ = *LD-DgB_SNP1*	*LD- B-Ya* = TT = 44	A substitution T⟺C in the eleventh intron of genome B
		LD- B–Yb = CC = 22	
	LD- B-M = *LD- B* $_{int\ 11b}$ = *LD-*DgB_{i11}*_SNP2*	*LD- B-Ma* = AA = 11	A substitution A⟺C in the eleventh intron of genome B
		LD- B-Mb = CC = 22	
	LD-B-R	*LD-B–Ra* = GG = 33	A substitution G⟺A in intron of genome B
		LD-B-Rb = AA = 11	
6A L (Nemoto et al. 2003)	*TaHd-A1-K*	*TaHd-A1-Ka* = TT = 44	A substitution T⟺G in intron of genome A
		TaHd-A1-Kb = GG = 33	
6B L (Nemoto et al. 2003)	*TaHd-B1-*$(G)_{0or1}$	*Hd-B1a* = $(G)_0$ = 00	One insertion-deletion of one G in the first exon of genome B
		Hd-B1b = $(G)_1$ = 11	
5A L 12	–	–	
5B L 6	*CO-B-Y*	*CO-B-Ya* = TT = 44	A substitution T⟺C in intron of genome B
		CO-B–Yb = CC = 22	
5D L 1	–	–	

For *GI*, out of 1,300 sequenced bases on a subset of 40 lines for the three genomes, three SNPs were found overall: two for genome A (*GI-A-R* and *GI-A-S*) that were rare (frequency less than 5%) and one for genome B (*GI-B-Y*). A BLASTN search revealed that *GI-A-S* and *GI-B-Y* were localized inside a wheat exon, which is orthologous to the fifth exon of the *GI* genes from rice and from barley (Dunford et al. 2005). Considering either genome A or B, these sites were around 90 bases apart. *GI-A-R* was located 690 bases upstream from *GI-A-S* in the second intron. In contrast with *GI-B-Y*, few allelic variations were observed for both *GI-A-R* and *GI-A-S*, which discriminated the same six lines.

Only 300 bases were sequenced for the *CO* gene fragment on the three wheat genomes. One SNP *CO-B-Y* was observed on genome B only. According to the BLASTN search, it was localized inside the intron of the gene, between two exons that are orthologous to the two exons of the barley gene, *HvCO-3* (Griffiths et al. 2003).

As orthologous *CO* genes in barley, rice and Arabidopsis, the *TaHd-1* gene showed two exons and one intron (Griffiths et al. 2003; Nemoto et al. 2003). Although no polymorphism was observed for genome D, genomes A and B showed, respectively, 1 SNP in the intron called *Hd-A1-K* and one insertion-deletion of one G in the first exon denoted *Hd-B1-*$(G)_{0or1}$. Both sites were only slightly polymorphic (Nei diversity index of 0.106 and 0.085, respectively).

Overall, out of the six genes and the 19 polymorphic sites studied, five sites from four genes showed the highest level of variability (He index around 0.40): *Vrn-A3-*$(TG)_{8or9}$, *GI-B-Y*, *LD-B-M* and the two loci from *Vrn-A1* (for *Vrn-*$A1_{pr}$ this value was probably increased by the multi-allelic state of this locus).

Linkage disequilibrium between loci

Overall, coefficients estimated from data corrected for the genetic structure of the population were very similar to the coefficients estimated from raw data (Table 7). Thus, in our study, ignoring the population genetic structure has no impact on the correlation coefficient estimation. Total linkage disequilibrium was found between the two polymorphisms of the same gene, *LD-B-Y* and *LD-B-R*. Significant linkage disequilibrium was also found between *Vrn-A1* polymorphisms. Except in those cases, no high linkage disequilibrium was found between polymorphisms located within the same gene, whereas significant and high linkage disequilibrium ($R > 0.1$) was found between polymorphisms of genes located on different chromosomes (for example, between *Vrn-*$D1_{int1}$ and *Vrn-A3-Y* or between *Vrn-*$D1_{int1}$ and *GI-B-Y*).

Table 7 LD estimated for all SNPs

	Vrn-$A1_{pr}$	*Vrn-*$A1_{ex7}$	*Vrn-*$B1_{int1}$	*Vrn-*$D1_{int1}$	*Vrn-A3-*$(TG)_{8or9}$	*Vrn-A3-Y*	*Vrn-A3-K*	*Vrn-D3-*$(G)_{3or4}$	*GI-A-S*	*GI-B-Y*	*LD-B-R*	*LD-B-M*	*LD-B-Y*	*CO-B-Y*	*Hd-A1-K*	*Hd-B1-*$(G)_{0or1}$
Vrn-$A1_{pr}$		***0.164***	***0.042***	0.009	0.000	0.000	0.000	0.000	0.003	0.007	*0.018*	0.004	*0.022*	0.007	0.001	0.008
Vrn-$A1_{ex7}$	***0.166***		*0.036*	*0.042*	0.017	0.018	0.001	0.008	*0.046*	*0.046*	*0.032*	0.000	*0.038*	0.001	0.028	0.025
Vrn-$B1_{int1}$	**0.041**	*0.034*		0.008	*0.035*	0.005	0.007	0.013	0.008	0.000	*0.054*	0.007	**0.058**	0.001	0.000	0.002
Vrn-$D1_{int1}$	0.009	*0.043*	0.009		*0.041*	***0.280***	0.012	***0.091***	0.004	***0.171***	0.001	0.019	0.001	0.012	*0.050*	0.000
Vrn-A3-$(TG)_{8or9}$	0.000	0.023	0.024	*0.042*		*0.036*	*0.040*	**0.057**	0.013	***0.075***	0.001	0.001	0.001	***0.070***	0.001	0.015
Vrn-A3-Y	0.001	0.019	0.004	***0.278***	*0.037*		0.007	0.001	0.002	*0.038*	0.010	**0.056**	0.011	**0.052**	***0.148***	0.004
Vrn-A3-K	0.000	0.001	0.005	0.011	*0.040*	0.006		***0.277***	0.003	***0.210***	0.008	***0.093***	0.007	0.003	0.006	0.005
Vrn-D3-$(G)_{3or4}$	0.000	0.012	0.016	***0.088***	***0.068***	0.001	***0.280***		0.010	***0.109***	0.002	0.024	0.000	0.009	0.001	0.001
GI-A-S	0.003	*0.050*	0.012	0.003	0.018	0.002	0.002	0.015		0.004	0.000	0.016	0.000	***0.108***	0.002	***0.133***
GI-B-Y	0.007	**0.057**	0.000	***0.170***	***0.093***	*0.038*	***0.197***	***0.117***	0.005		0.001	**0.056**	0.002	*0.042*	0.001	0.004
LD-B-R	*0.019*	*0.036*	*0.042*	0.000	0.004	0.009	0.007	0.000	0.001	0.004		**0.060**	***1.000***	0.011	0.001	0.001
LD-B-M	0.004	0.000	0.004	0.018	0.002	**0.058**	***0.100***	0.029	0.013	**0.057**	**0.055**		**0.061**	*0.033*	0.001	0.016
LD-B-Y	*0.023*	*0.041*	*0.048*	0.001	0.002	0.010	0.007	0.000	0.001	0.004	***1.000***	**0.056**		0.008	0.001	0.001
CO-B-Y	0.007	0.002	0.001	0.012	***0.076***	**0.053**	0.003	0.011	***0.110***	*0.048*	0.009	*0.033*	0.006		0.000	*0.040*
Hd-A1-K	0.001	0.025	0.000	*0.046*	0.000	***0.143***	0.004	0.001	0.002	0.002	0.000	0.001	0.000	0.001		0.003
Hd-B1-$(G)_{0or1}$	0.008	0.024	0.001	0.000	0.015	0.004	0.007	0.001	***0.124***	0.005	0.000	0.021	0.001	*0.040*	0.004	

Correlation coefficients were computed both on raw data (*upper triangle*) and on data corrected for the genetic structure of the population (*lower triangle*). Level of significance obtained by permutation

Italicized values signify P value <0.01

Bold values signify P value <0.001

Bolditalics values signify P value <0.0001

Table 8 Haplotypes reconstructed from the four polymorphic *Vrn-1* loci and association with the growth habit in the 235 core collection

Haplotypes	*Vrn-A1*$_{pr}$	*Vrn-A1*$_{ex7}$	*Vrn-B1*$_{itn1}$	*Vrn-D1*$_{itn1}$	Growth habit phenotype in the core collection	
	(5 alleles)	(2 alleles)	(2 alleles)	(2 alleles)	Expected	Observed
h1	*Vrn* $_{(1)}$	*Vrn* $_{(2)}$	*Vrn* $_{(2)}$	*vrn* $_{(1)}$	S	S(12)
h1bis	*Vrn* $_{(1)}$	*Vrn* $_{(2)}$	*Vrn* $_{(2)}$	*Vrn* $_{(2)}$	S	S(2)
h2	*Vrn* $_{(1)}$	*Vrn* $_{(2)}$	*vrn* $_{(1)}$	*vrn* $_{(1)}$	S	S(10)
h3	*vrn* $_{(2)}$	*vrn* $_{(1)}$	*vrn* $_{(1)}$	*vrn* $_{(1)}$	W	W(84) + S(9)
h4	*vrn* $_{(2)}$	*vrn* $_{(1)}$	*vrn* $_{(1)}$	*Vrn* $_{(2)}$	S	S(6) + W(2)
h5	*vrn* $_{(2)}$	*vrn* $_{(1)}$	*Vrn* $_{(2)}$	*vrn* $_{(1)}$	S	S(15)
h6	*vrn* $_{(2)}$	*Vrn* $_{(2)}$	*vrn* $_{(1)}$	*vrn* $_{(1)}$	W	W(4) + S(6)
h7	*vrn* $_{(2)}$	*Vrn* $_{(2)}$	*Vrn* $_{(2)}$	*vrn* $_{(1)}$	S	S(2)
h7bis	*vrn* $_{(2)}$	*Vrn* $_{(2)}$	*vrn* $_{(1)}$	*Vrn* $_{(2)}$	S	S(14) + W(1)
h7ter	*vrn* $_{(2)}$	*Vrn* $_{(2)}$	*Vrn* $_{(2)}$	*Vrn* $_{(2)}$	S	S(1)
h8	*Vrn* $_{(5)}$	*Vrn* $_{(2)}$	*vrn* $_{(1)}$	*vrn* $_{(1)}$	S	S(12) + W(1)
h10	*Vrn* $_{(5)}$	*Vrn* $_{(2)}$	*Vrn* $_{(2)}$	*vrn* $_{(1)}$	S	S(5)
h11	*vrn* $_{(3)}$	*vrn* $_{(1)}$	*vrn* $_{(1)}$	*vrn* $_{(1)}$	W	W(11)
h12	*vrn* $_{(3)}$	*vrn* $_{(1)}$	*Vrn* $_{(2)}$	*vrn* $_{(1)}$	S	S(1)
h14	*vrn* $_{(4)}$	*Vrn* $_{(2)}$	*vrn* $_{(1)}$	*vrn* $_{(1)}$	W	W(4)

The column "expected" indicates the growth habit associated with the corresponding *Vrn-A1*$_{pr}$, *Vrn-B1*$_{int1}$ *Vrn-D1*$_{int1}$ haplotype, considering the supposed effect of each allele individually or combined to the others. *h1* to *h10*: haplotypes with an allele described in Yan et al. (2004) at *Vrn-A1*$_{pr}$. *h11* to *h14*: haplotype with an allele described in Rhoné et al. (2008) at *Vrn-A1*$_{pr}$

vrn allele associated with winter growth habit, *Vrn* allele associated with spring growth habit (according to Yan et al. 2004; Sherman et al. 2004; Fu et al. 2005 and the present study). $_{(1)}$, $_{(2)}$, etc. refer to the allele code number attributed in the article

S spring growth habit, *W* winter growth habit

Diversity at the *Vrn-1* genes according to the genetic structure

The "winter" *Vrn-A1*$_{pr}$ "allele 2" was at a very high frequency in the core collection, occurring in more than half of the cases in all the groups and in particular group 1 was almost monomorphic for this allele (25/28). The "winter" allele *Vrn-A1*$_{pr}$ "allele 3" was observed only in 13 accessions originating from various regions around the globe but none belonging to group 1. The "winter" allele *Vrn-A1*$_{pr}$ "allele 4" was observed in only four accessions originating from northern Europe: 'Birgitta' from Sweden, 'Lesczynska Wczesna' from Poland (both in group 2), 'Rouge de Marchissy' from Switzerland and 'Prince Leoplold' from Belgium (both in group 5). The spring *Vrn-A1*$_{pr}$ "allele 1" was at a low frequency in the core collection, mostly found in group 3 (South American + African) with 13/43 accessions issued from 'breeders programs' and in group 5 (Southeast European + North American) with 14/59 accessions mostly issued from northern regions. The "spring" *Vrn-A1*$_{pr}$ "allele 5" was at a low frequency in the core collection, very rare in group 1 and present at a low frequency in the other groups. Yet, this was the only spring *Vrn-A1*$_{pr}$ allele in group 2. The rarer *Vrn-D1*$_{int1}$ "allele 2" was the most frequent in groups 1 and 4, but absent or seldomly found in groups 5 and 2, i.e., in Europe and North America.

For the *Vrn-1* gene, we found that it was important to study the multi-locus haplotypes based on the four polymorphisms analyzed for this gene (Table 8) to take into account their epistatic relations. Our study revealed much less haplotypes (15) than the 40 possible allelic combinations (10 on 5A × 2 on 5B × 2 on 5D). Twelve out of the 15 haplotypes were common to Rhoné et al. (2008). The presumed "native" *h3* haplotype was observed in all five groups with the highest frequency in the northwest European group 2 (44/62) and in the southeast European + North American group 5 (23/49). The *h8* and *h10* haplotypes (that share *Vrn-A1*$_{pr}$ "allele 5" and *Vrn-A1*$_{ex7}$ "allele 2") were present at a low frequency in all five groups.

Associations between SNPs, haplotypes and phenotypes

Predicting the growth habit type from the Vrn-1 genotype

For the *Vrn-1* promoter, the *Vrn-A1*$_{pr}$-1 and *Vrn-A1*$_{pr}$-5 alleles were associated with spring growth habit, the *Vrn-A1*$_{pr}$-2 allele, ancestral form of the gene, as well as *Vrn-A1*$_{pr}$-3 and *Vrn-A1*$_{pr}$-4, the two additional new alleles found by Rhoné et al. (2008), were associated with winter growth habit (Table 8). For the other regions, the ancestral forms of *Vrn-B1*$_{int1}$ and *Vrn-D1*$_{int1}$ with no

deletion were associated with a winter phenotype and the mutated forms with a large deletion were associated with a spring phenotype. Based on this information, and knowing that a good genetic model for these three polymorphisms is all spring alleles are dominant at a given locus, as well as on the two other loci (epistasis), we compared the expected phenotype based on the allelic variation at the three *Vrn-1* loci simultaneously and the observed phenotype based on mean HdNV. We found a rather good correspondence between expected and observed phenotypes for 12 out of 15 haplotypes corresponding to 183 genotypes out of the 202 completely phenotyped and genotyped. There were five haplotypes for which some of the observed phenotypes did not correspond with the expected ones (19 genotypes out of 202). Among them 4 "expected spring" genotypes (sharing the h4, h7bis and h8 haplotypes) exhibited a winter phenotype, while 15 "expected winter" genotypes (haplotype h3 and h6) exhibited a spring phenotype. Most of these 15 conflicting genotypes can be tentatively explained by allelic variation at other loci implicated in growth habit (see discussion below).

Association analysis considering all the earliness traits as quantitative characters

As already shown by Bonnin et al. (2007), the large broad sense heritability h^2 found for HdNV, HdV4, HdV8 and HdField (Table 9), and to some extent for PVS, underlined the major contribution of genetic variation to the phenotypic variability of these traits. Considering the variance analysis without gene effect, the R^2 values indicated that the genetic structure of the collection explained a larger part of the variation for heading date in the field ($R^2 = 0.36$) and earliness per se ($R^2 = 0.41$) than for vernalization requirement. Up to 50% of HdField variation was explained by both the genetic structure and the effect of the year of experimentation (Table 9).

The effect of each gene on the variation of earliness components was tested in different analyses of variance (ANOVA) models (Table supplementary S4). Epistatic interactions between two polymorphisms from the same genomic copy and between polymorphisms from different copies of the same gene were tested in the different "gene models", while interactions polymorphisms from different

Table 9 Association tests for each earliness trait combining the different genes

	HdNV	HdV4	PVS	HdV8	HdField
Heritability (h^2)	0.97	0.92	0.75	0.91	0.96
R^2 (*structure*)	0.25	0.26	0.10	0.41	0.36
R^2 (*yr + structure*)	0.28	0.26	0.15	0.41	0.50
R^2 (*full model*)	0.75	0.48	0.37	0.54	0.59
haplo-$Vrn\text{-}A1_{pr}Vrn\text{-}B1_{int1}$	<0.0001 (11.80%)	<0.0001 (6.04%)	<0.0001 (7.52%)		
haplo-$Vrn\text{-}A1_{pr}Vrn\text{-}D1_{int1}$					0.0005 (1.59%)
$Vrn\text{-}A1_{ex7}$	<0.0001 (2.11%)	0.0006 (1.78%)	0.0004 (2.97%)		
$Vrn\text{-}B1_{int1}$				0.0023 (1.25%)	
$Vrn\text{-}D1_{int1}$	<0.0001 (1.41%)	0.0002 (1.62%)			
$Vrn\text{-}A1_{ex7}$*$Vrn\text{-}D1_{int1}$	0.0002 (0.84%)				
$Vrn\text{-}A3_{int2a} = Vrn\text{-}A3\ K$					0.0055 (0.60%)
$Vrn\text{-}A3_{int2b} = Vrn\text{-}A3\ (TG)_{8or9}$	0.0001 (0.73%)	0.0080 (0.82%)		0.0002 (1.85%)	<0.0001 (1.61%)
$Vrn\text{-}D3_{ex3} = Vrn\text{-}D3\ (G)_{3or4}$				<0.0001 (3.29%)	<0.0003 (1.02%)
GI- B Y				0.0035 (1.15%)	
LD- B-Y = LD- B int 11a = LD-DgB_SNP1	0.0038 (0.40%)				
$LD\text{-}B\text{-}M = LD\text{-}B_{int\ 11b} = LD\text{-}DgB_{i11}\_SNP2$			0.0004 (2.40%)		
Hd-1 A K				<0.0001 (2.42%)	<0.0001 (2.22%)
$Hd\text{-}1\ B\ (G)_{0or1}$					0.0036 (0.66%)

Full models include all the nucleotide polymorphisms which were significantly associated with the variation of the character in the analysis performed per gene (see full models showing the highest value of R^2 in the table supplementary S4). In the analyses below, we took into account linkage disequilibrium between genes, testing the effect of their epistatic interactions

h^2 is the broad sense heritability in the experiment; R^2 (*structure*). R^2 (*yr + structure*) and R^2 (*full model*) are the determination coefficients issued from the ANOVA model restricted to the group effect only, to the group and year effects and from the full model including the group and year effects and the nucleotide polymorphism effect, respectively; *: only SNP with *P* values lower than 0.008 were conserved. Earliness traits: HdNV, HdV4 and HdV8 heading dates after 0, 4 and 8 weeks of vernalization, respectively

PVS partial vernalization sensitivity, *HdField* heading date in the field experiment with an autumn sowing

genes were tested in the "global model". For the *Vrn-1* gene, whereas the *Vrn-A1*$_{pr}$ polymorphism was associated with HdNV variation only, the three other loci had a significant effect on four characters out of five, with only the locus *Vrn-D1* being associated with the HdField variation. For all traits, introducing the effect of the haplotypes of the *Vrn-A1*$_{pr}$ locus and either the *Vrn-1B* locus or the *Vrn-D1* locus allowed to increase the values of R^2. Conversely, for *Vrn-3*, *GI*, *LD* and *Hd-1* genes, taking into account interaction or haplotype effects did not increase the R^2 values more than 1%. Except for *Vrn-3K*, each nucleotide polymorphism from *Vrn-3* gene had a significant effect on at least one character, with the polymorphisms *Vrn-A3-*$(TG)_{8or9}$ and *Vrn-D3-*$(G)_{3or4}$ affecting four and two characters, respectively. Conversely, *Vrn-A3-Y* was specifically associated with HdField. In comparison to the basic ANOVA model (without any SNP effects), taking into account SNP from *GI* and *LD* genes allowed to increase the R^2 values for HdNV, HdV4 and HdV8, the SNP *LD-B-Y* being also largely associated with PVS variation. Polymorphisms for *Hd-1* gene were mostly related to HdField, the *Hd-B1-*$(G)_{0or1}$ locus alone or within the haplotype *Hd-A1K-Hd-B1-*$(G)_{0or1}$ exhibited some effect on HdV8 and a highly significant effect on HdField. The allelic variation for the SNP *CO-B-Y* was not associated with any earliness component variation (results not shown).

When combining the significant nucleotide polymorphisms from different genes and their pairwise interactions in the "global model" (Table 9), we found that 9% (HdField) to 47% (HdNV) of the total phenotypic variation was explained by the gene effects. The three vernalization requirement traits were mostly associated to polymorphisms from *Vrn-1* but also from *Vrn-3* and *LD* genes, with PVS showing the lowest number of significant polymorphism effects. HdField and earliness per se were both explained by polymorphisms from *Vrn-1*, *Vrn-3* and *Hd-1* genes, while *GI* gene also contributed to earliness per se variation.

Differences between genotypes or haplotypes at the different loci significantly associated with the trait variation appeared quite noteworthy (Table supplementary S 5). The most extreme haplotypes for *Vrn-A1*$_{pr}$*Vrn-B1*$_{int1}$ (22 22 and 33 11) for HdNV (resp. HdV4) differed on average for 656°C days (resp. 178°C days), that is around 33 days (resp. 9 days) within the experimental conditions (Table supplementary S 5). The genotypes of the other significant polymorphisms differed by a maximum of 413°C days for HdNV in the case of *Vrn-D1*, which is around 21 days. For HdV8 and HdField, the largest difference (91°C days, 5 days and 80°C days, 4 days) was found between the two *Hd-1-A K* genotypes. Thus for all traits, the association mapping study allowed to detect QTLs with moderate to strong effects.

Discussion

Using a structured population of 235 accessions of hexaploid wheat, we conducted for six candidate genes (*Vrn-1*, *Vrn-3*, *TaHd-1*, *LD*, *GI*, *CO*) an association study on plant growth habit and earliness. While we did not consider here the day length sensitivity, the traits studied characterized sensitivity to vernalization and earliness per se to get a better understanding of the genetic basis of the heading date of the wheat plant in the field with an autumn planting.

Several studies have looked for the genetic determinism of growth habit and earliness characters in the *Graminaea* family mostly using QTL or meta-QTL approaches (Sourdille et al. 2000; Börner et al. 2002; Hanocq et al. 2002, 2004, 2007; Quarrie et al. 2006; Snape et al. 2007; Griffiths et al. 2009). More recently, the association mapping approach was proposed as an alternative means of identifying QTL or genes on wheat (Beales et al. 2005) and other species (Ivandic et al. 2002; Skøt et al. 2005; Neumann et al. 2011; Stracke et al. 2009). In a recent study, Eagles et al. (2009, 2010), estimated the effects of *Vrn-A1*, *Vrn-B1*, *Vrn-D1* and *Ppd-D1* genes on days to heading on a set of 1,085 homozygous cultivars and breeding lines issued from southern Australian wheat-breeding programs observed in a large multilocation network situated in a range of latitudes of −34.41°S to −36.67°S over 24 years. The bi-allelism polymorphism for the four genes accounted for ∼45% of the genotypic variance for days to heading with a strong epistatic effect of the *Vrn-A1*, *Vrn-B1*, *Vrn-D1* and *Ppd-D1* genes, but most of these lines had a spring growth habit (only 9 genotypes among the 1085 exhibiting a photoperiod-sensitive *ppd-D1* allele combined with the three *vrn-A1*, *vrn-B1*, *vrn-D1* winter alleles). As compared to these studies, our work involved a population already described in Bonnin et al. (2007) with more variation, especially for growth habit parameters and earliness traits, and consequently with a higher allelic richness (diversity). In addition, our approach analyzed the earliness and growth habit traits more deeply, considering flowering time through qualitative and quantitative aspects of vernalization requirements, as well as earliness with different growing conditions. To get more insights into the genetic bases of flowering time and reach a better understanding of the way they interplay to determine the different components and the final trait, we have addressed the following issues: (1) the structure of the core collection and its relation to earliness components, (2) the correlations observed between the different traits, and finally (3) the genetic basis of earliness in the field with an autumn planting.

The core collection structure and its relation to flowering time variability

In this study, we detected five ancestral subpopulations by using the EM-algorithm procedure developed by Veyrieras et al. (2006), and obtained similar results with the STRUCTURE program (Pritchard et al. 2000). Our results were consistent with those obtained by Balfourier et al. (2007) analyzing a larger sample of accessions (744 accessions, including the 235 accessions studied) with exactly the same set of SSR markers. They were also consistent with results obtained by Horvath et al. (2009) analyzing the structure of the whole core collection (i.e., a sample of 372 accessions including the present sub-sample) with 578 DArT markers. In these studies, five groups were detected, i.e., group 1 with all Nepalian and Central Asian accessions, group 2 with accessions from northwest European countries, group 3 including accessions related to the worldwide CIMMYT breeding program, group 4 with most of Japanese and Chinese accessions with some Mediterranean accessions and group 5 gathering a majority of European, east European, southeast European accessions together with Australian, Canadian and North American accessions and a few accessions from the Middle East. As discussed in Balfourier et al. (2007), the two European groups may be the result of both the historical process of the spread of initial wheat germplasm from the Fertile Crescent through Europe, and a biological process of adaptation to specific environmental conditions. The South American and African group of accessions together with some accessions of the Middle East might reflect recent breeding practices developed by CIMMYT and ICARDA like the use of Japanese progenitors (Akakomugi, Saitama 27) involved in the famous Strampelli's breeding program or 'Norin 10'/'Brevor' progenitors (Worland et al. 1994, 1998). Finally, the proximity between Asian and Mediterranean groups may be explained by recent introductions of Italian bread wheats to improve Chinese bread wheat cultivars.

We found that vernalization requirement and lateness tend to increase according to the mean latitude of each group: group 3 (South American + African) was the earliest with the lowest vernalization requirement followed by groups 4 (Mediterranean) and 1 (Central Asia), while group 5 (southeast European + North American) was later and group 2 (northwest European) was the latest with the largest vernalization requirement. The vernalization requirements and earliness per se are key traits for the adaptation of the wheat crop to environmental conditions, in particular climate × sowing date interaction. Alleles controlling wheat flowering times are good markers to follow the world colonization by wheats (Pozzi and Salamini 2007), since there is a correlative increase of the "winter" genotype frequencies from the lowest latitudes to the highest. The wheat genus was domesticated in the Fertile Crescent in a time frame comprised between 8,000 and 12,000 years from present (BP) and winter habit is prevalent in wild relatives of wheat while spring habit is the derived grown form (Beales et al. 2005).

Phenotypic correlation between earliness traits

Because the phenotypic variability for the growth habit components differed according to the group of accessions that was considered, i.e., "Spring" (S), "winter" (W) or "all" (A), it is interesting to consider the correlation structure separately for each group.

Since the correlation between HdV4 and HdV8 was high ($r = 0.80$) for the S group, we assumed that for these accessions, vernalization requirements were fully satisfied for both the 4- and 8-week treatments. Then the variation left was probably mainly due to earliness per se variability and, maybe, to some extent to sensitivity to photoperiod. For the W group, a lower correlation, ($r = 0.69$) was observed between HdV4 and HdV8. This was probably due to a lack of vernalization treatment resulting in a relatively lower effect of the earliness per se on the HdV4 trait.

HdV8 was a good estimate of earliness per se. An 8-week cold treatment at 4–5°C applied for HdV8 was enough to fully satisfy vernalization requirements of most of the accessions with, maybe, the two exceptions 'Lammas' and 'Non-plus-extra'. These two accessions exhibited a deviation from the regression line between HdV8 and HdField due to a delay in their heading with 8 weeks of vernalization treatment (Figure supplementary S2). We can hypothesize that they have high vernalization requirements, which were not satisfied with the 8-week cold treatment.

Masle et al. (1989) and Worland (1996) reported that heading date in the field with an autumn sowing reflected photoperiod sensitivity and earliness per se together in north European conditions. In our experiment, the high correlations observed between HdV8 and HdField, whatever the group of accessions considered (spring, winter or all), indicated that heading date for an autumnal sowing was rather strongly determined by the earliness per se. The difference was probably due to the much larger range of variation within this core collection than in the previous studies.

Distribution of the dominant *Vrn-1* and recessive *vrn-1* alleles in the groups

Because of the different frequencies of *Vrn-1* alleles observed in different parts of the world, Zhang et al. (2008) suggested that their allele combinations have an adaptive value. In our study, considering all possible allele

combinations, we detected only a small sub-sample of haplotypes (15/40). The missing haplotypes are rather those of expected low frequency based on allele frequencies at individual loci and those that could arise from very improbable recombinational events. Yet, some alleles or haplotypes were present at different frequencies according to the group. This indicated that either for historical reasons or due to divergent selective pressures in some groups, some haplotypic combinations correlate slightly with the genetic structure. For instance, the rare spring *Vrn-D1*$_{int1}$ "allele 2" is frequent in Chinese and Japanese spring wheats that were very rarely used in Europe, and thus it is specific to the Asian accessions. This is consistent with results cited by Zhang et al. (2008) reporting on the high frequency of this allele in commercial cultivars from Asian countries, i.e., Japan, Central Asia, Republic of the former Soviet Union, China and countries with a Mediterranean climate. In our study, the only non-Asian accessions exhibiting this allele were observed in group 3: 'Mexique 9', 'Estanzuela Dorado', 'BT 2296', 'Menflo' and 'G66257'. This was probably due to an introgression from Asian progenitors through crosses made by breeders particularly in the CIMMYT or ICARDA regional programs.

The strong dominant "spring" *Vrn-A1*$_{pr}$ "allele 1" was at a low frequency in this core collection, detected mainly in group 5 (14 accessions). On the contrary, Zhang et al. (2008) observed that the *Vrn-A1*-1 allele was frequent in improved cultivars from Europe and Siberia probably as a response to breeding practices. Wheat breeders in high northern latitudes have incorporated the vernalization-insensitive gene *Vrn-A1*$_{pr}$ "allele 1" into spring wheat by selecting for early flowering/maturity genotypes that complete their life cycles within the short growing season (Stelmakh 1993). Vernalization non-responsiveness appears as an interesting phenotype for short growing season regions like western Canada (Jeddel 1994) where the climatic conditions during winter (frost and snow cover) are inappropriate for winter wheat growing, which is in agreement with observation of this type of wheats in groups 3 and 5. On the contrary, most accessions of group 2 came from medium latitude European countries, mostly France and neighboring countries where true spring growth habit types cannot be grown in winter because of cold winter conditions and where spring sowing is not a widely used practice. In addition, during the selection of the accessions to build our core collection, possibly, the spring growth habit types were under-represented.

The genetic basis of growth habit and earliness

A large proportion of the genetic variation for the three traits linked to vernalization requirement, HdNV, HdV4 and PVS, was explained by the genetic variation at the *Vrn-1* gene on the three genomes. For example, introducing a combination of *Vrn-1* gene effects in the ANOVA model of HdNV led to increase R^2 by 47%. In addition to principal effects of *Vrn-A1*$_{pr}$, *Vrn-B1*$_{int1}$ and *Vrn-D1*$_{int1}$, interactions between *Vrn-A1*$_{pr}$ and *Vrn-B1*$_{int1}$, and between *Vrn-A1*$_{pr}$ and *Vrn-D1*$_{int1}$ were also observed. We found a rather good correspondence between expected and observed phenotypes for the *Vrn-1* haplotypes (Table 8). For the 15 expected winter genotypes and observed spring (haplotype h3 and h6), we tested the possible role of the *Vrn-1A*$_{int1}$ polymorphism. We genotyped them for the *Vrn-A1* intron 1 deletion identified in the Afghanistan wheat landrace IL369 (Fu et al. 2005). This complementary study revealed no polymorphism for 227 out of the 235 accessions (8 missing data) of our core collection, all carrying the non-deleted allele. However, *Vrn-A1*$_{ex7}$, *Vrn-A3*$(TG)_{8or9}$, *LD-B-Y* gene (SNP1) or any other dominant "spring" allele could explain the difference. Among the spring genotypes originally classified as winter phenotypes, there are two genotypes in h4 and one in h7bis, which in fact could be classified as semi-winter or "intermediate" growth habit types. Their vernalization requirement was perfectly satisfied with the 4-week vernalization treatment with HdV4 values close to those of the spring genotypes. This behavior is easily explained by their *Vrn-D1* allele which is the weakest "spring" allele. The last exception concerned the accession 'P. de Brollon', which has the h8 haplotype with an "expected spring" growth habit due to the *Vrn-A1b* or *Vrn-A1*$_{pr}$*-5* allele but exhibiting a winter HdNV value. A more detailed analysis of its growth habit revealed a "semi-winter" or intermediate phenotype with 1089dd and 1078dd for their lsmHd4V and lsmHd8V, respectively. A vernalization treatment of 4 weeks was sufficient to satisfy the vernalization requirement. This observation is not surprising if we consider that the *Vrn-A1*$_{pr}$-5 allele is not as strong as the *VRN-A1a* allele (J. Dubcovsky 2011, personal communication).

Finally, other gene variations with minor effects could be involved in the residual HdNV variation not explained by the *Vrn-1* genes (Rhoné et al. 2008). Up to four genes have been identified, *Vrn-1*, *Vrn-2*, *Vrn-3*, *Vrn-4*, in the bread wheat vernalization pathway (Chen et al. 2010; Yoshida et al. 2010). In the present study, we considered the allelic composition only at the *Vrn-1* and *Vrn-3* genes. The effect of *Vrn-2* may not be direct (e.g., mediated by *Vrn-1*) but is down-regulated by vernalization (Yan et al. 2004). Deletions of this gene in diploid and tetraploid wheat have been shown to determine spring growth habit. In hexaploid wheat, a triple recessive is required to see a spring effect (J. Dubcovsky 2011, personal communication), which would indicate that it is unlikely that *Vrn-2* plays a role in the vernalization response during winter. According to Bonnin et al. (2007), the *Vrn-3* gene, mapped on the short arm of chromosomes of group 7, could explain part of the variation for growth habit and vernalization

requirements. We found a significant association between polymorphism *Vrn-A3*$(TG)_{8or9}$ and in *Vrn-D3*$(G)_{3or4}$ and HdNV and HdV4. However, the spring "Hope" allele (*Vrn-B3*) described in Yan et al. (2006) is probably very rare or even totally absent from our core collection because these authors did not find this allele in a collection of 19 tetraploid spring wheats, 29 hexaploid winter wheats and 77 hexaploid spring wheats. They concluded that this mutation had not yet been used extensively in commercial varieties.

A number of other genes may also be involved in the control of vernalization requirements, such as the hypothetical gene co-locating the meta-QTL detected on the 2B chromosome by Hanocq et al. (2007) where none of our candidate genes were located. Except for *CO*, all gene polymorphisms had a significant impact on earliness components. There was a possible complementary role of genes with a minor effect, especially *LD-B-Y* gene (SNP1). In bread wheat, *LD* and *GI* genes were mapped on group 3 chromosomes and QTLs for flowering time traits were mapped on 3A and 3B chromosomes, but not on 3D which could co-localize with *LD* gene (Griffiths et al. 2009; Sourdille et al. 2004; Habash et al. 2007; Baga et al. 2009; Wang et al. 2009). To our knowledge, none of the *GI* regions (3AL-5, 3BL-7) have been shown to be linked to flowering time QTLs in wheat.

While all the genes tested were "good" candidates to be involved in the control of flowering time traits, significant association between a polymorphism and trait variation is not proof that these polymorphisms are really the genetic cause of the variation and we cannot rule out that the "causal" polymorphisms are in fact only in linkage disequilibrium with them. According to Horvath et al. (2009), the extent of the linkage disequilibrium in the same collection on chromosome 3B is quite low: less than 1% of marker pairs are significantly associated ($\alpha < 0.001$) in the 0–5 cM range. A reason may be that the core collection was built to maximize genetic diversity. As a comparison term, in rice, according to Huang et al. (2010) the linkage disequilibrium decay rate was measured as the chromosomal distance at which the average pairwise correlation coefficient (r^2) dropped to half its maximum value. Genome-wide linkage disequilibrium decay rates of indica and japonica were estimated at $\sim$123 and $\sim$167 kb, where the r^2 drops to 0.25 and 0.28, respectively. To further investigate the risk of detecting association due to linkage disequilibrium, we tested 46 of the neutral SSR markers (due to numerous alleles with few individuals, many data were declared as missing and thus the loci discarded) among the 82 studied, for their association to earliness traits. Only 2 of the 46, i.e., 4.3%, were significantly associated with earliness traits: cfe273 on chromosome 6A and gpw7433 on chromosome 6D were both associated with Hdfield. Using highly recombined populations such as the so-called MAGIC (multi-parents advanced generation intercrossed) might offer a complementary approach for candidate gene validation. By limiting linkage disequilibrium, such populations present a greater mapping precision and increase the allelic combinations that can be studied within a unified germplasm platform (Mackay and Powell 2007; Cavanagh et al. 2008).

Conclusion

Vernalization requirement is a major trait to consider for characterizing the growth habit of bread wheat. Allelic variation at the *Vrn-1* locus strongly influences vernalization requirement and a large part of the phenotypic variation for growth habit was associated with variation in the promoter and exon 7 regions of *Vrn-A1* and in the intron 1 of *Vrn-B1* and *Vrn-D1*. At a significant but lesser level, we demonstrated a clear effect of the *Vrn-A3* and *Luminidependens* genes on vernalization requirement traits. We also showed that it was possible to predict in most cases the vernalization requirement of a genotype from its allelic composition at the three homeologous copies of *Vrn-1*. All the combinations between *Vrn-1* alleles were not observed, i.e., 15 among the 40 possible haplotypes. Moreover, we found a significant linkage disequilibrium between the polymorphisms *Vrn-A1*$_{pr}$ and *Vrn-A1*$_{ex7}$, *Vrn-A1*$_{pr}$ and *Vrn-B1*$_{int1}$, and to a lesser extent between *Vrn-A1*$_{ex7}$ and *Vrn-B1*$_{int1}$, *Vrn-A1*$_{ex7}$ and *Vrn-D1*$_{int1}$. Consequently, it was not optimal to assess the exact individual role of each allelic type on the earliness phenotypic traits.

Heading date for an autumnal sowing appeared strongly influenced by earliness per se in this collection. This observation at the phenotypic level was clearly confirmed at the gene level as allelic variation at the three loci *Vrn-A3* $_{int2b}$ = *Vrn-A3* $(TG)_{8or9}$, *Vrn-D3* $_{ex3}$ = *Vrn-D3* $(G)_{3or4}$ and *Hd-1 A K* exhibited a similar significant effect on both earliness per se and heading date in the field with an autumn sowing. A complementary explanation of the genetic variation of the Hdfield trait is expected through the study of the photoperiodic sensitivity trait (day length sensitivity) and the genotyping of the well-known *Ppd-1* candidate genes.

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