New germplasm for barley hybrid breeding in Spain: yield and phenological adaptation

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A Casilda y Javier

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Abstract

Plant breeders must continuously deliver high-yielding and stable varieties adapted to increasingly unfavourable and variable environmental conditions. Nowadays, hybrid varieties are increasingly cultivated for their ability to cope with dynamic environments through their improved stability and potential. In particular, hybrid barley cultivation is gaining ground in recent years. However, in the case of Spain, a major consumer of feed barley, the potential of hybrid barley has not been exploited. This is because the current cultivars have not been optimised for local adaptation, which may include reduced vernalization requirement, early flowering and maturity. In the light of these circumstances, this thesis seeks to explore, understand, and facilitate the exploitation of Spanish germplasm and its adaptive traits for the improvement of hybrid barley under Southern European conditions. The work carried out aimed at: (1) creating a hitchhiker's guide for barley breeders including the allelic diversity available at major flowering time genes; (2) quantifying the potential agronomic problem that could arise in hybrid barleys combining alternative mutations at the *non-brittle rachis* (*btr*) genes; (3) exploring the inheritance and effect on the plant cycle of major barley flowering time genes in heterozygosis, and their dynamics in relation to insufficient vernalization; and (4) finding promising Spanish germplasm contributing to the development of high-yielding hybrid varieties adapted to Southern Europe. To accomplish these objectives, the following studies were carried out.

Firstly, I conducted a literature review that summarized the allelic series, effects, interactions between genes and with the environment, for the major flowering time genes that drive phenological adaptation of barley.

Secondly, I evaluated rachis fragility in hybrid crosses with different compositions at the *btr* genes, through a mechanical test, and under natural conditions. This experiment revealed higher brittleness in hybrids bearing alternative mutations. Moreover, we identified an increase in the number of disarticulated rachis nodes with time post-maturation.

Thirdly, we assessed the phenology and gene expression of major genes in a set of hybrid barleys and their parents, subjected to three vernalization treatments: complete, moderate, and low. We observed a gradation in responses to vernalization, mostly additive, concentrated in the phase until the initiation of stem elongation, and proportional to the allele constitution and dosage present in *VRN-H1*. These responses were further modulated by the presence of *PPD-H2*. The duration of the late reproductive phase presented more dominance towards earliness, and was affected by the rich variety of alleles at *VRN-H3*.

Lastly, a set of locally adapted breeding lines were evaluated for their potential to widen the germplasm available for hybrid barley development. A subset of lines was introduced into three-way hybrid combinations and tested in a field trial network of up to 4 locations and 2 years. The hybrid performance of the rest of the lines was estimated based on genomic prediction models. No three-way hybrid exceeded the best check, but we succeeded in identifying high GCA parental lines, and widely adapted, for the development of promising two-way hybrids.

The work reported in this thesis shows the wide variety of allelic effects that provide enormous plasticity in barley flowering behaviour, and are available to breeders for fine-tuning barley phenology. Moreover, it confirms an actual risk of grain loss in hybrid cultivars with alternative brittle mutations. Furthermore, our results conclude that hybrid combinations provide further opportunities for fine-tuning total and phasal growth duration, beyond what is currently feasible in inbred cultivars. Lastly, this thesis shows a successful strategy of exploiting local germplasm for the development of hybrids adapted to new target areas.

Resumen

Los mejoradores deben desarrollar continuamente variedades estables y de alto rendimiento, adaptadas a condiciones ambientales cada vez más desfavorables y cambiantes. Actualmente, las variedades híbridas se cultivan de manera creciente dada su capacidad para prosperar en ambientes dinámicos, resultado de una mayor estabilidad y potencial. Particularmente, el cultivo de cebada híbrida ha ganado terreno en los últimos años. Sin embargo, en el caso de España, uno de los principales consumidores de cebada forrajera, el potencial de la cebada híbrida no se ha llegado a explotar. Esto es debido a que los cultivares actuales no han sido optimizados para la adaptación local, lo que incluiría la reducción del requerimiento de vernalización y una floración y maduración tempranas. Ante estas circunstancias, esta tesis pretende explorar, comprender y facilitar la explotación del germoplasma español y sus caracteres adaptativos, para la mejora de la cebada híbrida en las condiciones del sur de Europa. Los trabajos realizados tienen como objetivo: (1) crear un catálogo para los mejoradores de cebada que recoja la diversidad alélica disponible en los genes mayores que controlan el tiempo a floración; (2) cuantificar el potencial problema agronómico que podría surgir en cebadas híbridas que combinen mutaciones alternativas en los genes *non-brittle rachis* (*btr*); (3) explorar la herencia y el efecto en el ciclo de la planta de los principales genes que controlan el tiempo a floración de la cebada en heterocigosis, y su respuesta a la vernalización insuficiente; e (4) identificar germoplasma español con potencial para el desarrollo de variedades híbridas de alto rendimiento adaptadas al sur de Europa. Para alcanzar estos objetivos, llevamos a cabo los siguientes estudios.

En primer lugar, realicé una revisión bibliográfica para recopilar las series alélicas, los efectos, las interacciones entre genes y con el ambiente, de los principales genes de floración que impulsaron la adaptación fenológica de la cebada.

En segundo lugar, evalué la fragilidad del raquis en híbridos con diferentes composiciones en los genes *btr*, mediante un test mecánico, y en condiciones naturales. Este experimento reveló una mayor fragilidad en los híbridos con mutaciones alternativas. Además, identificamos un incremento en el número de nudos desarticulados conforme aumentó el tiempo post-maduración.

En tercer lugar, evaluamos la fenología y la expresión génica de genes mayores en un conjunto de cebadas híbridas y sus parentales, sometidos a tres tratamientos de vernalización: completa, moderada y baja. Observamos una gradación en las respuestas a la vernalización, mayormente aditiva, concentrada en la fase hasta el inicio de la elongación del tallo, y proporcional a la constitución y dosis del alelo presente en *VRN-H1*. Estas respuestas fueron además moduladas

por la presencia de *PPD-H2*. La duración de la fase reproductiva tardía presentó una mayor dominancia hacia la precocidad, y se vio afectada por la rica variedad de alelos en *VRN-H3*.

Por último, se evaluó un conjunto de líneas de mejora localmente adaptadas con el fin de ampliar el germoplasma disponible para el desarrollo de cebadas híbridas. Un subconjunto de líneas se introdujo en híbridos tres vías y se evaluó en una red de ensayos de campo, en hasta 4 localidades y durante 2 años. El rendimiento híbrido del resto de las líneas se estimó a partir de modelos de predicción genómica. Ningún híbrido tres vías superó al mejor testigo, pero logramos identificar líneas parentales con alta ACG y amplia adaptación, útiles para el desarrollo de híbridos de dos vías con potencial.

El trabajo presentado en esta tesis muestra una amplia gama de efectos alélicos, los cuales proporcionan enorme plasticidad en el tiempo a floración de la cebada, y que están disponibles para los mejoradores con el fin de optimizar la fenología del cultivo. Además, confirma un riesgo real de pérdida de grano en los híbridos con mutaciones alternativas en los genes *non-brittle rachis*. Por otro lado, nuestros resultados concluyen que las combinaciones híbridas ofrecen más oportunidades para afinar la duración del crecimiento total y fásico, más allá de lo que es actualmente factible en las líneas puras. Por último, esta tesis muestra una estrategia exitosa de explotación del germoplasma local para el desarrollo de híbridos adaptados a nuevas zonas objetivo.

Resum

Els milloradors han de desenvolupar contínuament varietats estables i d'alt rendiment, adaptades a condicions ambientals cada cop més desfavorables i canviants. Actualment, les varietats híbrides es cultiven de manera creixent atesa la seva capacitat per prosperar en ambients dinàmics, resultat de més estabilitat i potencial. Particularment, el cultiu d'ordi híbrid ha guanyat terreny en els darrers anys. Tot i això, en el cas d'Espanya, un dels principals consumidors d'ordi farratger, el potencial de l'ordi híbrid no s'ha arribat a explotar. Això és degut a que els cultivars actuals no han estat optimitzats per a l'adaptació local, cosa que inclouria la reducció del requeriment de vernalització i una floració i maduració primerenques. Davant d'aquestes circumstàncies, aquesta tesi pretén explorar, comprendre i facilitar l'explotació del germoplasma espanyol i els seus caràcters adaptatius, per millorar l'ordi híbrid en les condicions del sud d'Europa. Els treballs realitzats tenen com a objectiu: (1) crear un catàleg per als milloradors d'ordi que reculli la diversitat al·lèlica disponible dels gens majors que controlen el temps a floració; (2) quantificar el potencial problema agronòmic que podria sorgir en ordi híbrid que combini mutacions alternatives als gens *non-brittle rachis* (*btr*); (3) explorar l'herència i l'efecte en el cicle de la planta dels principals gens que controlen el temps de floració de l'ordi en heterocigosi, i la resposta a la vernalització insuficient; i (4) identificar germoplasma espanyol amb potencial per al desenvolupament de varietats híbrides d'alt rendiment adaptades al sud d'Europa. Per assolir aquests objectius, duem a terme els estudis següents.

En primer lloc, vaig fer una revisió bibliogràfica per recopilar les sèries al·lèliques, els efectes, les interaccions entre gens i amb l'ambient, dels principals gens de floració que van impulsar l'adaptació fenològica de l'ordi.

En segon lloc, vaig avaluar la fragilitat del raquis en híbrids amb diferents composicions als gens btr, mitjançant un test mecànic, i en condicions naturals. Aquest experiment va revelar una fragilitat més gran en els híbrids amb mutacions alternatives. A més, identifiquem un increment en el nombre de nusos desarticulats conforme va augmentar el temps post-maduració.

En tercer lloc, avaluem la fenologia i l'expressió gènica de gens majors en un conjunt d'ordi híbrid i els seus parentals, sotmesos a tres tractaments de vernalització: completa, moderada i baixa. Observem una gradació en les respostes a la vernalització, majoritàriament additiva, concentrada a la fase fins a l'inici de l'elongació de la tija, i proporcional a la constitució i dosi de l'al·lel present a *VRN-H1*. Aquestes respostes van ser modulades per la presència de *PPD-H2*. La durada de la

fase reproductiva tardana va presentar una major dominància cap a la precocitat, i es va veure afectada per la rica varietat d'al·lels a *VRN-H3*.

Finalment, es va avaluar un conjunt de línies de millora localment adaptades per tal d'ampliar el germoplasma disponible per al desenvolupament d'ordi híbrid. Un subconjunt de línies es va introduir en híbrids tres vies i es va avaluar en una xarxa d'assajos de camp, fins a 4 localitats i durant 2 anys. El rendiment híbrid de la resta de línies es va estimar a partir de models de predicció genòmica. Cap híbrid tres vies va superar al millor testimoni, però vam aconseguir identificar línies parentals amb alta ACG i àmplia adaptació, útils per al desenvolupament d'híbrids de dues vies amb potencial.

El treball presentat en aquesta tesi mostra una àmplia gamma d'efectes al·lèlics, els quals proporcionen una enorme plasticitat en el temps a floració de l'ordi, i que estan disponibles per als milloradors per tal d'optimitzar la fenologia del cultiu. A més, confirma un risc real de pèrdua de gra als híbrids amb mutacions alternatives als gens *non-brittle rachis*. D'altra banda, els nostres resultats conclouen que les combinacions híbrides ofereixen més oportunitats per afinar la durada del creixement total i fàsic, més enllà del que és actualment factible a les línies pures. Finalment, aquesta tesi mostra una estratègia d'explotació d'èxit del germoplasma local per al desenvolupament d'híbrids adaptats a noves zones objectiu.

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Chapter I. General Introduction

1. Chapter I. General Introduction

1.1. Barley (*Hordeum vulgare* L.)

1.1.1. Importance of the crop

Barley (*Hordeum vulgare* L.) as one of the oldest crops, played a key role in the development of agriculture, civilizations and cultures (Zohary et al. 2012).

Currently, the main use of this cereal is animal feed, which represents 60–75% of the total production, essentially as grain, but also as fodder (Langridge 2018). Barley composition makes it a particularly suitable ingredient for feed manufacture, since it is highly energetic, and stands out over other cereals due to its superior content in protein and fibre (Ullrich 2014).

Feed is followed in importance by barley used for malting, food, and industrial purposes, such as paper, textile or biofuel production (Tricase et al. 2018).

In addition, this crop has been used as an experimental model for temperate climate cereals of the *Triticeae* tribe (wheat, rye, triticale) (Kumlehn and Stein 2014). Barley has featured prominently in genetic research. The origin and domestication of crops, phylogeny and systematics have been widely studied through it, and its grain has served as a physiological and anatomical model for other species (Langridge 2018).

Finally, apart from its versatile utilization, much of barley's success lies in its adaptation to a wide variety of environments. Barley is able to develop at higher altitudes and latitudes compared to other grasses and has good tolerance to drought, cold and salinity. While other temperate climate cereals drastically reduce their performance in the face of climatic or fertilization limitations, barley is profitable even in semi-arid areas. Therefore, it is a relevant crop in the Mediterranean region, central and northern Europe, the Middle East, northern Africa and the Andean region of South America (Newton et al. 2011).

1.1.2. World and national production

Barley is grown on more than 51 million hectares worldwide, thereby representing the fourth most widely grown cereal crop after wheat, maize, and rice, with 159 million tonnes harvested in 2019. About 60% of the global production is obtained in Europe, while Asia and America produce around 16 and 14%, respectively (Figure 1.1). The average world yield is 3.1 $t \cdot ha^{-1}$, varying between

8 t·ha^{-1} in optimal conditions without water and nutrient limitation, and 1–2 t·ha^{-1} in African countries bordering the Sahara Desert (FAOSTAT 2019).

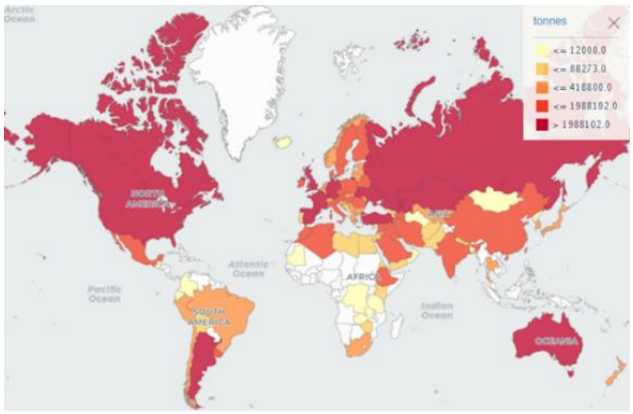

Figure 1.1. World barley production in 2019 (FAOSTAT 2019).

Spain is the eighth world barley producer, after Russia and near France, Germany, Canada, Ukraine, Australia, and UK. Within the Spanish territory, barley is the rainfed crop presenting the greatest production and acreage (MAPA 2021a). The Spanish barley cultivation area in 2020 exceeded 2.7 million hectares, yielding 7.4 million tonnes harvested mainly in the two central plateaus and Aragón (Figures 1.2, 1.3).

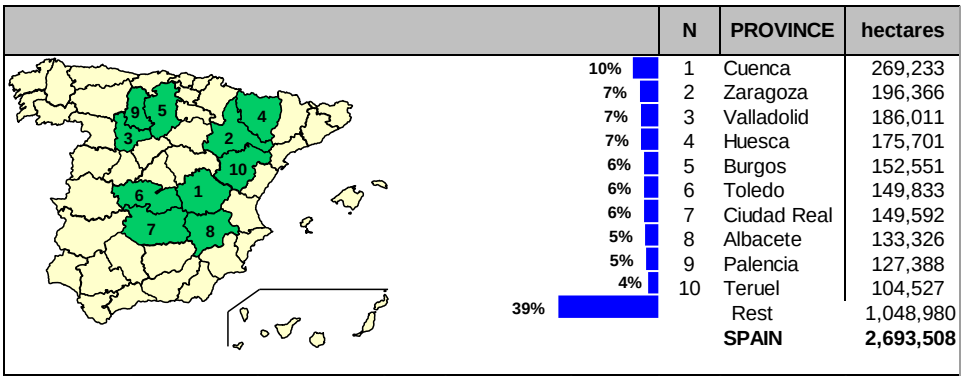

Figure 1.2. Provincial analysis of Spanish barley grain acreage in 2020 (MAPA 2021a).

Approximately 92% of the Spanish barley acreage is devoted to grain for animal feed, which represents around 3.5 million tonnes, which is one-third of the total national cereal production (Martínez-Moreno et al. 2017). Spain is Europe's leading pig producer (FAOSTAT 2019), which contributes to the crop's relevance. However, the national barley production does not meet the feed manufacturing industry internal needs, forcing Spain to address the shortfall through imports, mainly from the EU (MAPA 2021b).

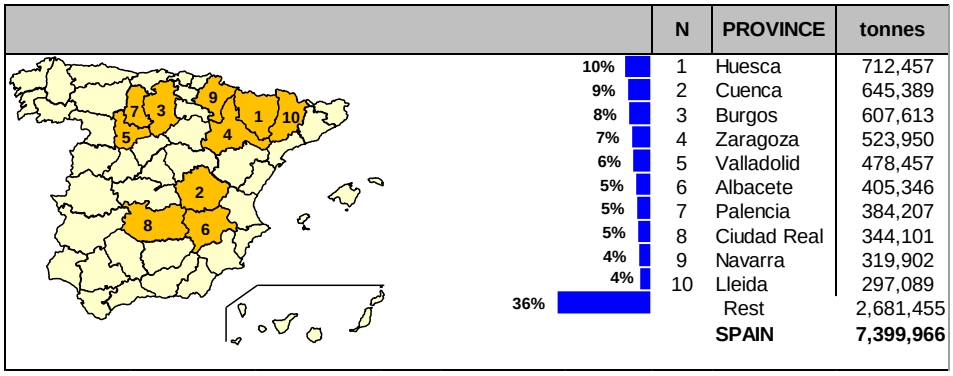

Figure 1.3. Provincial analysis of Spanish barley grain production in 2020 (MAPA 2021a).

Thus, Spain would benefit from an increase of barley production. To this effect, the most reasonable option is the use of higher yielding varieties, as arable land is limited and priority is given to crops yielding greater economic returns.

1.1.3. Origin and domestication

Barley is part of the *Triticeae* tribe, a globally spread and economically important group of plants, within the *Poaceae* family. This tribe is characterized by spike-shaped inflorescences, a base chromosome number x = 7 and large genomes. In addition to barley, the *Triticeae* tribe includes major small grain and temperate climate cereals such as wheat (*Triticum* spp.), rye (*Secale cereale* L.), and triticale (*Triticosecale* Wittm.) (Al-Saghir 2016). Cultivated and wild barleys are classified in the genus *Hordeum*, which split apart from wheat species around 13 million years ago (von Bothmer and Komatsuda 2011).

The origin of barley (*Hordeum vulgare* L. ssp. *vulgare*) is a topic that has stirred up an ongoing debate in the scientific community (Azhaguvel and Komatsuda 2007). It is widely accepted that barley originated in the Fertile Crescent, domesticated from its wild progenitor *H. vulgare* ssp. *spontaneum* (C.Koch) Thell. about 10,000 years ago (Badr et al. 2000). The multiple crosses combinations between cultivated barley and the wild ancestral form do not show any incompatibility barrier, so it is used as a source of gene transfer for crop improvement.

During the barley domestication process, humans selected those plants capable of producing an ever-increasing amount of harvestable grain. Wild barley has a brittle rachis that allows seed dispersal, while the tough rachis of cultivated barley prevents spontaneous disarticulation of mature spikelets, representing an adaptive change that ensures an efficient harvest (Pankin and von Korff 2017). Probably, the loss of the grain dispersal natural mode was the most important single event in the barley domestication process (Pourkheirandish et al. 2015).

Nevertheless, it was precisely the study of rachis fragility, as well as that of other diagnostic traits of the barley domestication syndrome, which boosted the current polyphyletic theory of barley origin (Morrell and Clegg 2007). This hypothesis postulates that the fixation of domestication traits in cultivated populations was a slow process and that barley origin was not restricted to a specific geographic centre (Pankin and von Korff 2017).

Unlike wheat and other crops, wild barley has been found widely dispersed from the Middle East to Central Asia and the Tibetan Plateau, a fact that does not agree with a single origin centre. Recent work suggests that the ancestry of barley is 'mosaic', resulting from genetic interaction between multiple wild or proto-domesticated lineages (Poets et al. 2015; Pankin et al. 2018). Although probably the strongest argument for at least a diphyletic origin of barley comes from sequence divergence at the *non-brittle rachis* loci, suggesting two independent origins of a key domestication trait (Pourkheirandish et al. 2015). Collectively, these data suggest a diphyletic, but admixed origin of domesticated barley inside the Fertile Crescent (Haas et al. 2019).

1.1.4. Botanical description

Barley is an annual, self-pollinating grass. It has two types of root systems: seminal roots develop from germination to tillering and adventitious roots grow from the crown from tillering phase onwards. Its stem reaches between 60 and 120 cm in height, it is a cylindrical reed consisting of alternating hollow internodes and solid nodes, which bear the leaves. Barley has several tillers arising from basal leaves axils, which have the same structure as the main stem. Barley leaves consist of a smooth blade and a tubular sheath that surrounds the stem, which are joined by the ligule and have two membranous extensions, called auricles, which distinguish them from other species. These are glabrous, embrace the stem and may be pigmented with anthocyanins. A leaf emerges from each node alternately and in the opposite position to the previous one. The inflorescence of barley is referred to as the ear, head or spike. It consists of units called spikelets attached to the rachis (i.e., extension of the stem that supports the spike). There are three spikelets at each node, named triplets, alternating on opposite sides of the spike. Each spikelet is made up of two glumes, which are empty bracts, and one floret that includes the lemma, the palea, and the enclosed, female and male, reproductive components. Depending on the variety, each lemma extends as an awn or, more rarely, a hood (Briggs 1978). In terms of barley spike morphology, two-rowed and six-rowed types can be distinguished. Two-rowed barleys have reduced and sterile lateral spikelets, while in six-rowed barleys the three spikelets of each node are fertile (Komatsuda et al. 2007). The growing preference for one type or another is mainly due to historical reasons. Given Europe's brewing and distilling tradition, two-row barley, valued in the industry for its

plump, uniform grains, predominates in this area. In North Africa, the Iberian Peninsula, and eastern Asia, where malting is less common, six-row barley prevails. Both types are grown in regions where barley is used as feed and food. Currently, in both North America and the Iberian Peninsula, there has been a major shift towards the cultivation of two-row barley, either due to the introduction of European cultivars or due to changing preferences of malting companies (Martínez-Moreno et al. 2017; Hernandez et al. 2020). Finally, barley's fruit is a caryopsis, with the palea and lemma attached, except in the case of naked barley (Briggs 1978).

1.1.5. Development and adaptation

Barley is a facultative long-day plant, flowering earlier under increasing day-lengths. Attending to the growth habit, barley cultivars are classified as winter or spring, although existing variation is more complex, as we will see later on. Winter cultivars are sown in autumn and are exposed to freezing temperatures in the winter. To avoid exposure of the reproductive structures to frost, the transition from the vegetative to the reproductive stage in winter barleys must be delayed until frost probability recedes. This is achieved through the mechanism of vernalization, the requirement of a plant to go through a low temperature period to induce reproductive stage. Thus, the exposure of frost-sensitive floral organs to freezing winter temperatures is prevented, and flowering occurs under warmer conditions, in spring. Spring cultivars, on the other hand, are sown in late winter or spring (depending on regions), in areas with harsh winters, and usually show no vernalization requirement. If conditions permit, winter varieties are preferred over spring varieties because of their yield advantage due to the longer growing season. However, in areas that experience low temperatures during a longer winter season, the cultivation of spring varieties is essential (Verstegen et al. 2014).

Barley growth and development is a complex process, composed of several overlapping stages (Figure 1.4), which can be divided into pre- and post-anthesis phases. Pre-anthesis development is classified into three major phases based on morphological changes of the shoot apical meristem: the vegetative phase (leaf initiation), the early reproductive phase (spikelet initiation), and the late reproductive phase (spike growth and floral development) (Slafer and Rawson 1994; González et al. 2002). The length of these stages and the balance between them impact yield components (Figure 1.4). The duration of the vegetative and early reproductive phases determines the final number of spikelets, while the late reproductive phase determines the number of fertile florets, thus the number of grains and potential yield (Alqudah and Schnurbusch 2014; Digel et al. 2015). Several studies in wheat and barley have shown that the length of the pre-anthesis phenological

phases is genetically controlled, and that these developmental periods show different sensitivity to environmental stimuli (Slafer and Rawson 1994; Miralles and Richards 2000; González et al. 2002; Gol et al. 2017; Ochagavía et al. 2018). The ability of barley to alter its developmental program in response to environmental stimuli is mainly regulated by genes belonging to the vernalization and photoperiod pathways (Campoli and von Korff 2014). A detailed description of the major flowering time regulators, and their allelic diversity available for barley breeding is reported in the third chapter of this thesis. After anthesis, the grain-filling phase starts, which determines the weight of the grain. Hence, each of the developmental phases has a particular role in barley growth, development, and yield (Alqudah and Schnurbusch 2017).

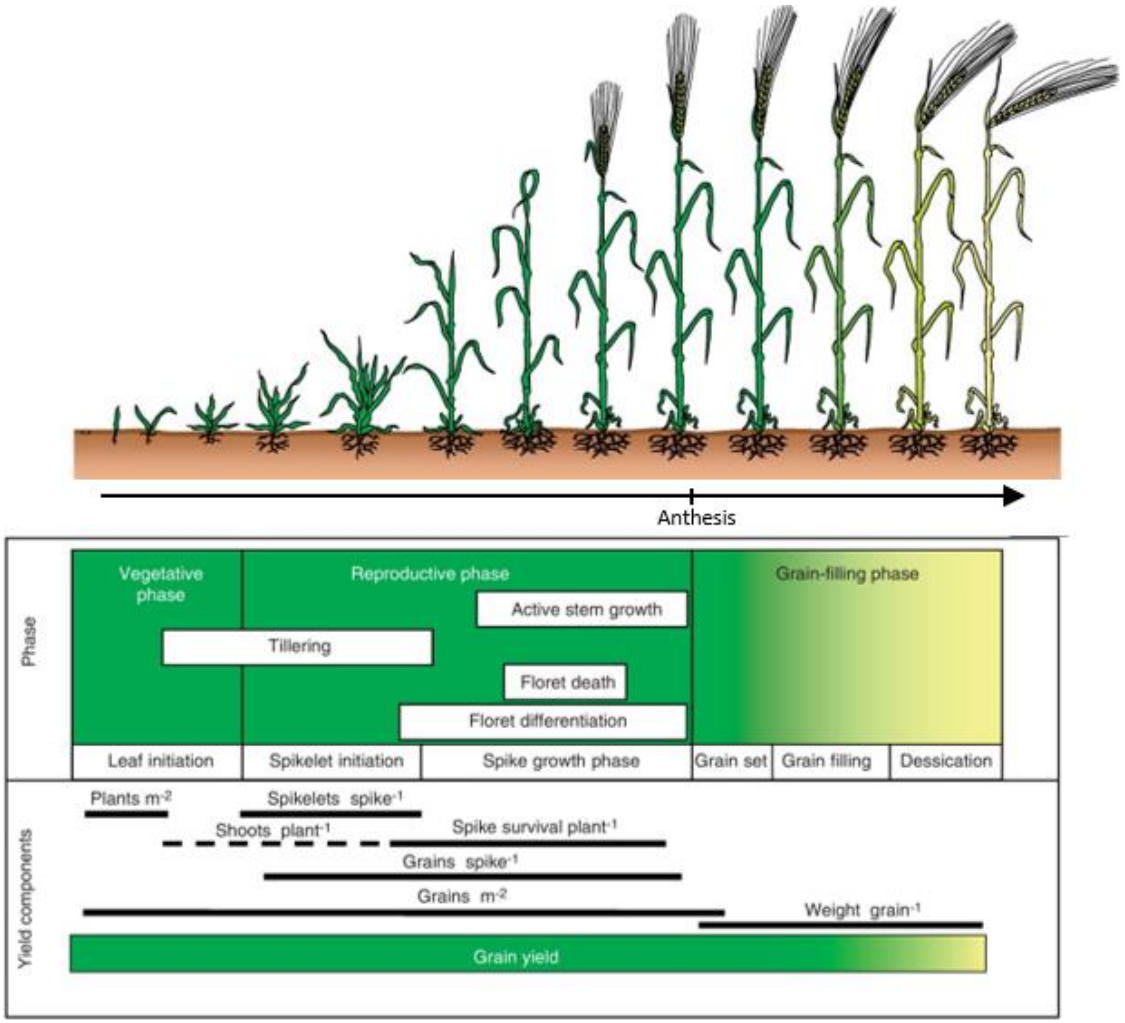

Figure 1.4. Barley developmental cycle in relation to the establishment of components of grain yield. The upper part shows the crop morphology during development. The middle part shows the extension of the different developmental phases pre- and post-anthesis. The bottom diagram yield formation duration crop cycle. Adapted from Sreenivasulu and Schnurbusch (2012).

Barley wide adaptation has been achieved through natural selection of pre-existing diversity as the crop colonised new niches with new climatic requirements. The strict vernalization requirement of wild barley was modulated to extend barley cultivation to areas where spring sowing is necessary to avoid winter frost damage or where winters are warmer (von Bothmer et al. 2003; Cockram et al. 2011). The selection for frost tolerance has also been key for extending barley growing areas, a trait controlled by a number of genes in the CBF (*C-repeat binding factor*) cluster (Guerra et al. 2021). Lastly, spring barleys were selected for long photoperiod insensitivity allowing vegetative growth to be extended in long days, favouring its expansion towards higher latitudes (von Bothmer and Komatsuda 2011). These adaptations, and surely others yet to be discovered, have occurred largely through allele selection on the major genes that regulate barley development, which is described in the third chapter of this thesis.

1.1.6. Barley genome

Barley is a diploid species with a low number (2n = 2x = 14) of relatively large size chromosomes (Graner et al. 2011). Its genome is composed of approximately 5 billion base pairs, which is double the size of the corn genome (*Zea mays* L.) and twelve times that of rice (*Oryza sativa* L.), of which 80% is made up of repetitive DNA. Consequently, it was not until 2012 that the first draft of the barley genome sequence (Mayer et al. 2012) was published. Five years later, a high-quality reference assembly genome was released (Mascher et al. 2017). This version provided a highly contiguous and ordered sequence, unravelling previously inaccessible pericentromeric regions. After that, other two versions have become available (Monat et al. 2019; Mascher et al. 2021), which have considerably improved the quality of the assemblies and gene annotation. Very recently, the barley pangenome was published (Jayakodi et al. 2020), expanding the genetic variation accessible to genetic studies and breeding. Moreover, high-throughput genotyping arrays have been developed for barley, whose information is routinely used by plant breeders (Bayer et al. 2017).

The integration of sequencing, expression and phenotyping data is key to understanding biological processes and identifying functional elements. Several resources have been developed to accelerate the search and interpretation of the large data sets resulting from this integration (Riaz et al. 2021). These tools enable available knowledge to be quickly summarized and new hypotheses to be formulated (Beier et al. 2018). Among the most important genomic resources of barley are the following: Ensembl Plants, BARLEX, GrainGenes, BARTv1.0, EoRNA, and Barleymap. The web portal Ensembl Plants contains genomic data for various plant species and has been used to explore differences and similarities between barley and its related species

(http://plants.ensembl.org, Bolser et al. 2017). BARLEX is a barley genome explorer that collects all available barley sequences (http://barlex.barleysequence.org, Colmsee et al. 2015). GrainGenes is a genetic database primarily containing data on barley and wheat, which provides access to the pan-genome resources (https://wheat.pw.usda.gov/GG3/, Blake et al. 2019). BARTv1.0 the barley reference transcript (https://ics.hutton.ac.uk/barleyrtd/index.html, Rapazote-Flores et al. 2019). EoRNA is a barley expression database that includes transcript abundance from different cultivars and tissues (https://ics.hutton.ac.uk/eorna/index.html, Milne et al. 2021). Finally, Barleymap was designed to search the position of barley genetic markers and sequences, on the different versions of the barley reference genome (http://floresta.eead.csic.es/barleymap/, Cantalapiedra et al. 2015). The most recent effort being made in this direction is the construction of a barley pan-transcriptome. This resource will allow precise quantification and comparison of allelic variant expression across barley cultivars, and holds promise to become an essential tool in barley breeding programs (https://www.barleyhub.org/pan-bart/).

1.1.7. Barley breeding challenges

The current breeding objectives depend on the crop end use. In the case of malting barley, the most important aspect is malting quality, while in feed barley, the starch content, raw fibre, and protein is prioritized. However, the key barley breeding trait is still grain yield. The main current challenge for breeders is the development of new cultivars that improve productivity under harsher or less predictable environments (Voss-Fels et al. 2019; Reynolds et al. 2021). To achieve this, it is essential to increase both yield potential and yield stability, breeding crops for stress tolerance (Tollenaar and Lee 2002; Ceccarelli et al. 2004).

Within abiotic stresses, heat and drought are the two most limiting factors for the global crop production (Fahad et al. 2017), and with increasing frequency in the Mediterranean region (Rosenzweig et al. 2001; Hoerling et al. 2012). Although heat and drought have a negative impact at any stage of the cycle, their most detrimental effect on yield occurs when the stress coincides with reproductive development. Therefore, one of the most successful mechanisms of crop adaptation has been stress escape (Kooyers 2015). Breeders have mimicked this strategy by modifying the flowering date. Thus, by selecting earlier cultivars, plants reproduce, and yield is formed before terminal stress occurs at the end of the cycle (Shavrukov et al. 2017), which is frequent in the Mediterranean climate (Cammarano et al. 2019). Besides, research has improved stress tolerance by using both conventional and molecular breeding approaches. Conventional breeding has benefited from the high degree of genetic variability for stress tolerance that the barley germplasm harbours (Forster et al. 2000; Stanca et al. 2003). This variability is now accessible

thanks to large collective efforts to characterize the barley germplasm, including core collection development (Muñoz-Amatriaín et al. 2014; Milner et al. 2018). The current challenge is to create a redundancy-free atlas of available barley biodiversity worldwide. To this end, a network connecting gene banks across the world has recently been set up. Its aim is to design a systematic and globally applicable approach to the management of genetic resources that maximises their value and use (https://www.agent-project.eu/).Together with heat and drought, one of the greatest threats to crop production in this century is the proliferation and dispersion of pests and diseases affected by climate change (Rosenzweig et al. 2001). Fungal diseases are responsible for the greatest losses in barley world production (Oerke and Dehne 2004). These diseases are contended by cultivation of resistant varieties combined with appropriate agronomic practices. In many cases, resistant cultivars are the only option for disease control, due to the widespread withdrawal of crop protection chemicals. However, pathogen's high variability together with the genetic homogeneity of cultivars and, in some cases, a narrow genetic base of the crop, favours a rapid overcoming of resistances. Indeed, Barley yellow dwarf virus and Ramularia are a standout problem for barley nowadays (Walters et al. 2008; Choudhury et al. 2017; Stam et al. 2019). Therefore, maintaining resistance to the ever-evolving spectrum of pests and diseases is one of the most impactful barley breeding objectives.

1.1.8. Omics-aided breeding and future perspectives

Traditional plant breeding is based on phenotypic selection. Although very effective, traditional breeding is time and resource consuming. Over the last few decades, several omics technologies including genomics, transcriptomics, phenomics, proteomics, and metabolomics have emerged. The use of these omics-based approaches helps to increase genetic gain and reduce the breeding cycle (Kaur et al. 2021). Moreover, the integration of different omics technologies has led to improved understanding of genetic architecture, molecular network, and physiological basis of complex traits (Yang et al. 2021).

The remarkable rise in throughput and accuracy of genome sequencing technologies has opened up new avenues and tools that increase the efficiency and precision of conventional barley breeding. Following the reduction in sequencing costs and the proliferation of thousands of markers, genetic analysis, such as QTL mapping and genome wide association studies (GWAS), have led to the identification of genomic regions and novel genes associated with main agronomic traits. In addition, marker-assisted backcrossing and marker-assisted recurrent selection provide really powerful tools to accurately transfer an allele of interest to an elite background or pyramid

QTLs to integrate several complementary traits in a cultivar (Langridge and Fleury 2011). However, despite the large number of reported QTLs associated with stress tolerance (Kebede et al. 2019), their use in breeding is limited, since their effect is not conserved across genetic backgrounds.

The availability of a large number of molecular markers allows the adoption of a strategy that does not require mapping genes or QTLs for crop improvement, namely genomic selection. Genomic selection uses the marker information distributed across the whole genome to predict the performance of selection candidates for economically important complex traits (Meuwissen et al. 2001). To do so, first a prediction model is developed based on genotypic and phenotypic data of a training population. Then, the model is used to derive the genetic value of unphenotyped individuals through their genomic profile only. The implementation of genomic selection is now routine in both public and private breeding programmes because it allows increasing genetic gain by addressing several components of the breeder's equation simultaneously: reducing the breeding cycle by replacing phenotyping by genotyping, increasing accuracy by integrating information from relatives and multiple environments, and increasing selection intensity by assessing more candidates and eliminating those predicted to perform worst (Gholami et al. 2021). Its full realization will come from the hand of the acceleration of the breeding process though the doubled haploid and the 'speed breeding' techniques (Voss-Fels et al. 2019). The latter uses controlled environmental conditions and extended photoperiods to achieve up to five generations per year, in the case of winter types (Cha et al. 2021), and six, for spring types (Watson et al. 2018), and can be used to speed up the development of inbred lines following a cross. The integration of genomic selection and speed breeding can accelerate the genetic gains needed for rapid improvement of complex traits in crop plants (Krishnappa et al. 2021). Besides, in the present century, gene editing is expected to be the most powerful biotechnological tool allowing specific changes to be made through directed mutagenesis, precise gene editing, multigenic transformation, and favourable alleles stacking (Zhang et al. 2018). However, these techniques must gain general and legal approval before they impact plant breeding.

Despite the above-mentioned advances, there are still certain limitations that slow down crop improvement progress. One of the main bottlenecks hindering crop breeding and functional genomics studies is phenotyping (Yang et al. 2020). Despite the immense progress in phenomics, facilitated by the development of robotics, advanced imaging, spectroscopy, data processing and automation, and its integration with machine learning/deep learning models, there is still room for improvement, particularly in root phenotyping, which represents the frontier of field phenotyping

(Araus et al. 2021). Alongside roots, knowledge gaps in hormone crosstalk, recombination rate, maintenance respiration, and source–sink balance prevent promising opportunities to accelerate genetic gain from being exploited (Reynolds et al. 2021). To conclude, the current challenges facing crop breeding are: 1) processing and making the most efficient use of the vast amount of environmental, sequencing, and phenotyping platforms data generated (Zhao et al. 2021; Gholami et al. 2021), and 2) creating science-based regulation policies that allow exploiting the potential of new technologies available for breeding and guaranteeing global food security (Anders et al. 2021).

1.2. Hybrid barley

A hybrid variety results from the cross of two or more different (inbred or not) genotypes. Hybrid varieties cultivation has represented a real step forward for several cross-pollinated species such as corn, sunflower, sorghum, beet and rye (Coors and Pandey 1999), and a revolution in their breeding methods. The main objective of hybrid breeding is the exploitation of heterosis (Whitford et al. 2013), a phenomenon whereby the performance of hybrid offspring is higher than the average of their parents (Falconer and Mackay 1996; Bernardo 2002). Additional advantages of hybrid varieties are: greater yield stability, especially in marginal environments (Hallauer et al. 1988); the ease of stacking major dominant genes; and a higher return of investment for seed companies due to the intrinsic variety protection by inbreeding depression (Edwards 2001). Hybrid breeding for autogamous cereals, instead, has been less successful due to the lower heterosis, the difficulties to implement a cost-effective system for hybrid seed production, the lack of knowledge about high yielding heterotic patterns, and the lower selection gain for hybrid compared to line breeding (Edwards 2001; Oettler et al. 2005; Lu and Xu 2010). However, despite these drawbacks, in recent decades, there have been important attempts, both in the public and in the private sphere, to develop hybrid-breeding programs in autogamous cereals (Yuan 2017; Gupta et al. 2019; Miedaner and Laidig 2019). These efforts have recently been stimulated by the demand for an increase in agricultural productivity per unit area despite the increasing abiotic stress problems caused by climate change, and the need for breeders to ensure an investment return against the increased farm-saved seed use and the lack of political solutions in this regard (Edwards 2001; Rajaram 2001). To achieve satisfactory hybrid breeding, a suitable heterosis level for economically important traits and a cost-effective hybridization mechanism preventing female parent self-pollination, although allowing cross-pollination between the female and male parent are required. In addition, the setup of an efficient system for the identification of superior hybrid combinations is critical (Mühleisen et al. 2013; Xu et al. 2014).

The availability of a suitable male-sterility system for hybrid seed production was true for barley only recently. From 1940 to 1980, many male sterility genes were reported, but their use in hybrid development was met with little success (Wiebe 1960; Ramage 1965, 1983). In 1979, Ahokas described a cytoplasmic male sterility (CMS) system in an Israeli strain of wild barley with a reliable single dominant fertility restorer gene (Rizzolatti et al. 2017). However, it was not until 1994 that Paul Bury, a breeder at Syngenta Seeds, transferred the CMS system to European breeding lines and began the systematic hybrid breeding in barley.

Heterosis (hybrid vigour) boosts the productivity and resilience of crops above the levels of both parents, yet there is no universal explanation for this phenomenon. Although the dispersion of favourable alleles between parents, which show directional dominance, is one of the most widespread underlying mechanisms (Mackay et al. 2021). In hybrid barley, heterosis varies between crosses. The mid-parent heterosis of barley grain yield averaged 11.3%, with a range from 0.7 to 19.9%, while better-parent heterosis was slightly lower with an average of 9.2%. Commercial heterosis (i.e., hybrid superiority with respect to the most productive line in the market) was 7.6%, underlying the relevance of hybrid barley breeding (Mühleisen et al. 2013). In addition to a sufficiently high heterosis amount, recurrent and systematic hybrid breeding requires an efficient system to identify parents of superior hybrid combinations. Traditionally, hybrid performance has been predicted based on the mid-parent value or the general combining ability (GCA) effects of the parental lines (Bernardo 2002; Hallauer et al. 2010; Zhang et al. 2015c). However, the predictability based on both strategies is low (Mühleisen et al. 2013), and in the case of the latter, it requires that both parental components have been previously evaluated in a hybrid background. This is not necessary in the case of genomic prediction, which is currently the method of choice for hybrid prediction of complex traits such as grain yield (Huang et al. 2016). Applying genomic prediction, moderate to high prediction abilities were obtained (Liu et al. 2016; Philipp et al. 2016; Zhao et al. 2021; Zhang et al. 2022), demonstrating the time and resource savings of this approach in hybrids.

Hybrid barley cultivation is gaining ground in Europe in recent years. It is increasingly important in the UK, France, and Germany (Longin et al. 2012), where it covers up to 30% of the winter six-row feed barley acreage (Philipp et al. 2016; SolACE 2017). This interest is motivated by hybrid cultivars producing a yield bonus that pays off the increase in seed cost, above a certain production level. The price of hybrid seed is 50% higher than that of conventional seed. Even though the sowing density is reduced by 25%, hybrid seed represents an additional investment of 90 €·ha^{-1}. However, this extra cost for farmers is offset by a reduction of 15 €·ha^{-1} in fungicide costs and a

78 €·ha^{-1} profit from the surplus straw production. Under these circumstances, the higher productivity of hybrid barley compared to inbred lines (+0.6 t·ha^{-1}) translates into a net benefit for farmers of about 86 €·ha^{-1} (Syngenta 2020). According to Syngenta AG®, the agronomic benefits with respect to the inbred lines consist in a rapid crop establishment, avoiding diseases and competition with weeds; a vigorous root system, resulting in improved water and nutrient absorption; a greater tillering capacity; and a better tiller maintenance under stress conditions (Syngenta 2017). Moreover, hybrid combinations release genetic variation contained in the pericentromeric region, otherwise trapped in extensive linkage blocks that are refractory to recombination (Mascher et al. 2017).

1.2.1. Hybrid barley in Spain

Despite barley relevance in Spain, its breeding by the private sector has been almost non-existent until recently. The reason is the low profit obtained from the seed sales, given that only 35% of the seed sown is certified, a figure that contrasts with the 60% of our European neighbours (Fuentes 2020). However, after the success achieved in other European countries and the economic return it implies for breeders, in 2014 Syngenta AG presented its Hyvido® line in Spain dedicated to hybrid crop breeding, marketing for the first time in the country the Jallon hybrid barley variety. The Hyvido® barleys are winter six-rowed type and are intended for high yielding (average yield potentials above 5 t·ha^{-1}) drylands of inland Northern Spain (Figure 1.5, Syngenta 2016), where the yield advantage of hybrids offsets the increased cost of their seeds.

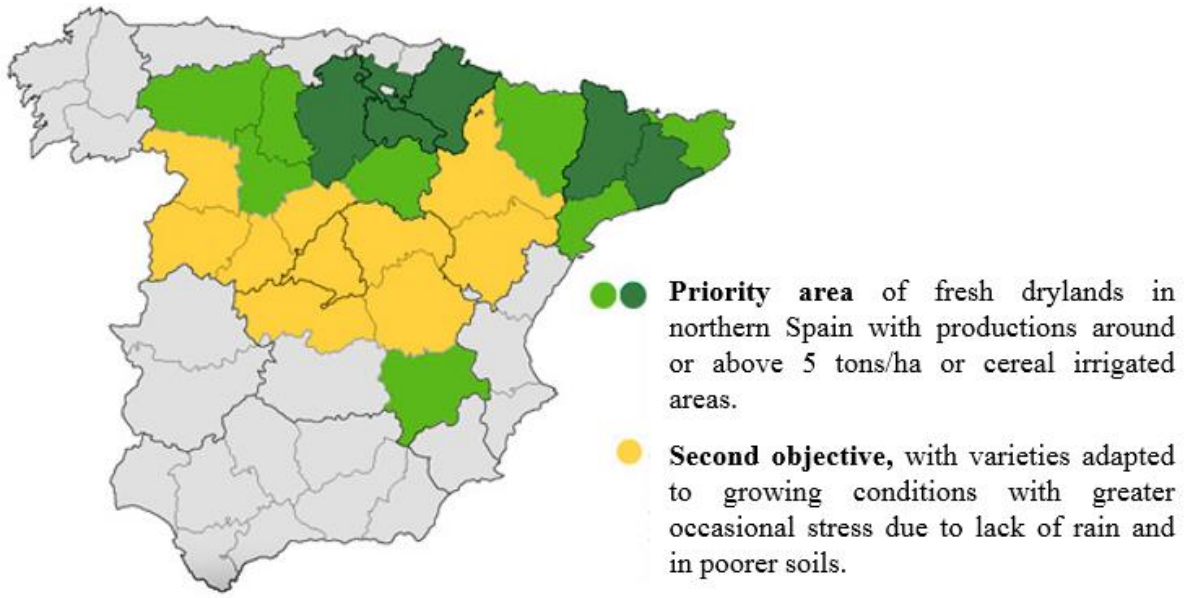

Figure 1.5. Hyvido potential in Spain (Syngenta 2016).

1.2.2. Potential problems in hybrids

In hybrid varieties it is important to evaluate the possible deleterious phenotypes resulting from heterozygous genes that are fixed in the conventional varieties. This is the case of the loss of the natural grain dispersal system, one of the most relevant events occurred during barley domestication. A mutation in either one of two linked genes, *Btr1* and *Btr2*, turns the fragile rachis (brittle) of the wild form into the tough rachis phenotype (non-brittle) (Pourkheirandish et al. 2015). All cultivated barleys carry either one or the other mutation, promoting grain retention. The mutation *btr1* is widely spread in Europe and Central Asia, while the mutation *btr2* is distributed around Oriental Asia and North Africa (Pourkheirandish et al. 2015). In the Iberian Peninsula, both converge. This situation was irrelevant when the target cultivars of breeding programs were inbred lines, but is highly relevant now, with the advent of hybrid barley. In this context, the cross of parents with alternative mutations in the *btr* genes would lead to an F_1 hybrid with trend to show a fragile rachis and, thus, that might present grain retention problems.

1.3. Why was this thesis proposed?

This thesis project is part of the current interest in hybrid barley cultivation as an efficient way to increase both yield potential and yield stability, without resorting to additional inputs. In Spain, the potential of hybrid barley has not been fully realized, due to the lack of hybrid varieties adapted to the warmer and highly variable Mediterranean growing conditions, where the occurrence of occasional stresses due to lack of rainfall or heat waves are more frequent than in North-western Europe (Cammarano et al. 2019). Under these circumstances, the opportunity arises to resort to the genetic diversity and adaptation contained in locally adapted breeding lines (e.g., reduced vernalization requirement, early flowering, and early maturity) for the development of high-yielding hybrid varieties adapted to Southern Europe. In this context, this thesis is the result of a collaborative project with the company Syngenta AG, which is interested in obtaining hybrids adapted to the conditions of the Iberian region.

1.3.1. Genetic diversity to achieve local adaptation

Barley landraces are valuable breeding resources in the Mediterranean region (Ceccarelli et al. 1998; Comadran et al. 2009), given their long history of selection under stress conditions. The Spanish Barley Core Collection is a representative set of barley genotypes formerly cultivated in Spain, selected on the basis of agro-ecological growing zones (Igartua et al. 1998). These accessions contain unique alleles compared to barley genotypes used in modern barley breeding in Europe, particularly in the six-row barley pool (Yahiaoui et al. 2008). Making use of this gene pool and foreign germplasm, the Spanish National Research Council (CSIC) developed, through the

National Barley Breeding Programme, advanced material adapted to the Spanish and Mediterranean growing regions (Gracia et al. 2012). These advanced breeding lines can be used as potential donors of local adaptation traits, which are essential for the development of hybrid cultivars suitable for Southern Europe.

The study of Spanish local genetic diversity has revealed the importance of phenological processes and the genes that control them for barley adaptation (Casas et al. 2011; Casao et al. 2011c; Contreras-Moreira et al. 2019). Moreover, tight coordination of plant cycle to environmental conditions to match resource availability with the most sensitive growth stages has a major effect on yield (Tondelli et al. 2014; Flohr et al. 2018; Cammarano et al. 2021). Understanding the adaptive mechanisms and the genes that control phenology allows optimising the duration of the different developmental phases, avoiding abiotic stress at the most critical stages, and maximising yield in the target environments (Gouache et al. 2017). In this context, fine-tuning the phenology of hybrids is key to the development of varieties adapted to Southern Europe. For this, we must first understand flowering time gene action in heterozygosis.

1.3.2. Heterotic patterns

Hybrid breeding depends on the discovery, establishment, and management of high-yielding heterotic patterns. A heterotic pattern is formed by two heterotic groups whose cross results in superior hybrid performance. In turn, a heterotic group is defined as a pool of genotypes that display similar combining ability and heterotic response when crossed with genotypes from other genetically distinct germplasm group (Melchinger and Gumber 1998). Grouping germplasm into divergent heterotic groups has been associated with maximizing heterosis and hybrid performance, and with a lower ratio of specific combining ability (SCA) to GCA variance. Thus, before the advent of genomic prediction, superior hybrids could be identified and selected mainly based on their prediction from GCA effects (Schulthess et al. 2017).

The clearest example of the establishment of a successful heterotic pattern is that used for temperate maize breeding (Melchinger and Gumber 1998). Hybrid maize breeders maximised heterosis through the development of heterotic groups by reciprocal recurrent selection. This method aims, in addition to improving populations *per se*, to increase the heterotic response between two populations. In this way, the two complementary populations or heterotic groups coevolved and diverged (Duvick and Smith 2004). As a result, the crosses 'Reid' x 'Lancaster' in the US corn belt, and 'European Flint' x 'Corn Belt Dent' in Europe brought about unprecedented yield improvements.

The significant yield increase in maize hybrids encouraged hybrid breeding in other cross-pollinated crops, including sunflower, sugar beet, and rye (Coors and Pandey 1999). In the case of self-pollinated crops, the traditional breeding methods themselves, and the intensive exchange of germplasm between breeders complicate the establishment of genetically distinct heterotic groups. Despite this, major efforts have been made to establish heterotic patterns for the main cereals that feed the world (Longin et al. 2013; Zhao et al. 2015; Boeven et al. 2016; Beukert et al. 2017; Gupta et al. 2019; Adhikari et al. 2020). It is clear that well-developed heterotic patterns of mature breeding programs are artificial buildings created by breeders, and improved by the breeding hybrid process. In the case of wheat or rice, crops for which hybrid breeding is relatively recent, the use of genomic prediction to define potential heterotic patterns is a promising approach (Zhao et al. 2015; Beukert et al. 2017).

Hybrid barley breeding has been successful in North western Europe, where heterotic groups have been developed (Li et al. 2017; Sommer et al. 2020), whose cross gives rise to high-yielding hybrids. However, the vast majority of barley germplasm has not been tested in a hybrid format, including the Spanish germplasm. Therefore, a broad exploration for heterotic patterns across the global barley germplasm should be carried out.

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Chapter II. Objectives

2. Chapter II. Objectives

The general objective of the thesis is to explore, understand, and facilitate the exploitation of Spanish germplasm and its adaptive traits for the improvement of hybrid barley under Southern Europe conditions.

The specific objectives are:

1. To create a hitchhiker's guide for barley breeders and geneticists including the allelic diversity available at major flowering time genes.

2. To quantify the potential agronomic problem that could arise in single and three-way hybrids derived from crosses with different compositions at the *non-brittle rachis* genes.

3. To explore the inheritance and effect on the plant cycle of major barley flowering time genes in heterozygosis, and their dynamics in relation to insufficient vernalization.

4. To find promising Spanish advanced breeding lines contributing to the development of high-yielding hybrid varieties adapted to Southern Europe, by combining multi-location agronomic testing of a training subset, and genomic prediction for the whole set.

Chapter III. Major flowering time genes of barley: allelic diversity, effects, and comparison with wheat

3. Chapter III. Major flowering time genes of barley: allelic diversity, effects, and comparison with wheat

3.1. Introduction

Phenological adjustment is critical for maximizing yields during crop adaptation. Synchronizing the plant cycle to the prevailing environmental conditions was key to enable the expansion of crops to agricultural environments far distant from those found in their progenitors' domestication centres (Evans 1996; Knüpffer et al. 2003; Cockram et al. 2007b; Zohary et al. 2012). Currently, plant breeders are challenged to develop new cultivars allowing a profitable production under increasingly unfavourable and shifting environmental conditions, due to climate change (Verstegen et al. 2014). Under these circumstances, the timing of the developmental milestones, with flowering first and foremost, is essential to achieve adaptation to increasingly prevalent temperature and water deficit stresses (Rosenzweig et al. 2001; Kazan and Lyons 2016). Fine-tuning crop phenology will be critical to reduce the impacts of these limiting factors on yield, minimizing the exposure of the most sensitive growth stages to climate extremes (Craufurd and Wheeler 2009).

Barley (*Hordeum vulgare* L.) represents a relevant model for agroecological adaptation since it has been cultivated in all temperate regions from the Arctic Circle to the tropics (Ullrich 2011). Besides, it belongs to the *Triticeae* tribe, an economically and socially important group of species providing a significant share of food and feed (Al-Saghir 2016).

Flowering time is a complex trait, tightly controlled by genetic networks that integrate environmental cues. In barley, the transition to the reproductive stage is mainly controlled by genes affected by two main seasonal cues (Laurie 2009): day length (photoperiod) and extended periods of low temperature (vernalization) (Figure 3.1). The allelic richness at these genes is the basis for barley wide adaptation (Campoli and von Korff 2014). A thorough understanding of the genetic and environmental control of flowering time, and better knowledge and utilization of the genetic diversity, will enable breeders to develop cultivars adapted to specific areas and climates, by deploying appropriate phenology gene combinations (Wilczek et al. 2010; Nazim Ud Dowla et al. 2018).

Depending on the vernalization requirement, barley cultivars are roughly classified as having winter or spring growth habit, although this scale is oversimplified, as we will see later on. "Winter" varieties are usually sown in autumn and need vernalization for timely flowering. This adaptive feature delays apex transition, preventing the exposure of frost-sensitive floral organs to freezing winter temperatures, ensuring flowering occurs only under warm conditions, in spring. Spring types are sown in spring, in regions with too harsh winters, and show null or reduced vernalization

requirement. Almost all wild barleys are winter type, so one of the prerequisites for barley production expansion to spring sowing areas was the development of lines lacking vernalization requirements (Pourkheirandish and Komatsuda 2007). The geographical distribution of winter and spring varieties is mainly mediated by winter harshness, although the need to avoid unfavourable conditions for grain filling at the end of the season is also determinant in the Mediterranean region (Yahiaoui et al. 2008; Verstegen et al. 2014). In addition to temperature, flowering time also depends on photoperiod (Laurie 1997). In wild barleys, photoperiods over 12 hours trigger a rapid switch to reproductive growth, a phenomenon called photoperiod sensitivity. This behaviour was also typical of the first domesticated barleys, and slowed down their spread to areas with winter temperatures too low for barley to survive. In these areas, spring sowing was the only option, and photoperiod sensitivity reduced vegetative growth to a minimum over spring and summer, insufficient to attain acceptable agronomic performance. Therefore, photoperiod insensitivity enabled the expansion of barley cultivation into higher latitudes (Komatsuda 2014).

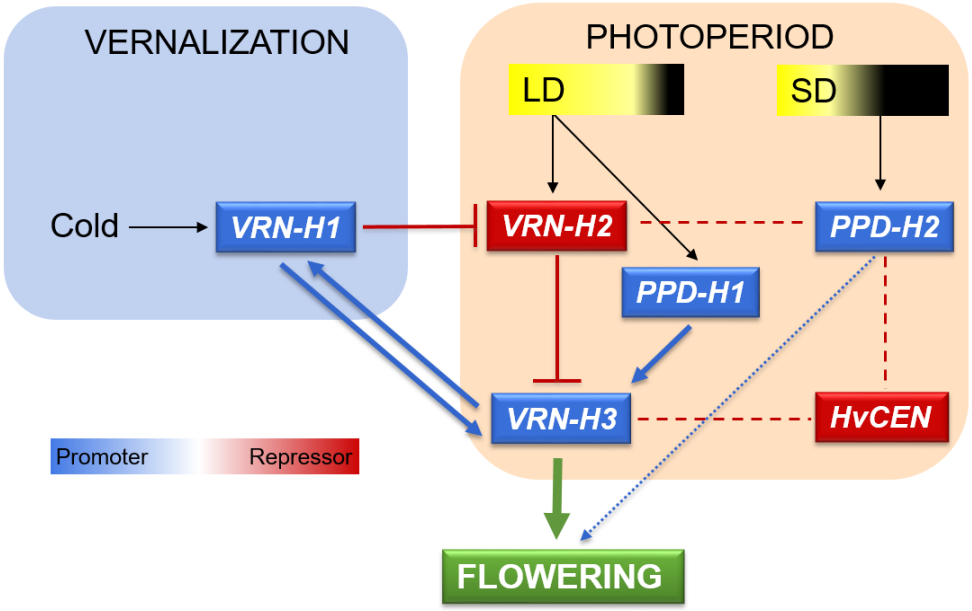

Figure 3.1. Flowering time control in barley: main genes, environmental cues and regulatory pathways. Reproductive transition in barley is regulated by genetic networks that respond to extended periods of low temperature (vernalization, blue frame) and day length (photoperiod, orange frame). Genes depicted in blue promote flowering, whereas genes depicted in red act as repressors. Blue and green arrows indicate induction. Red lines with blunt ends indicate repression. Antagonistic relationships between genes reported in the literature are represented as dashed red lines. *PPD-H2* connection with flowering is represented as a dashed blue line because it induces spikelet initiation but not floral development (Mulki et al. 2018). LD: long days, SD: short days.

The purpose of this review is to describe the catalogue of alleles found in QTL studies by barley geneticists, which likely correspond to the genetic diversity at major flowering time genes. We will summarize the diversity found associated with *VRN-H1* (*HvBM5A*), *VRN-H2* (*HvZCCTa-c*), *VRN-H3* (*HvFT1*), *PPD-H1* (*HvPRR37*), *PPD-H2* (*HvFT3*), and *eam6/eps2* (*HvCEN*), as the main drivers of phenological adaptation of barley during its long history of expansion starting in the Neolithic. We will also cover briefly some genes that have become relevant in modern barley breeding, with large effects on phenology, namely, *denso*, *eam8* (*EARLY FLOWERING3* or *HvELF3*) and *eam5* (*HvPHYTOCHROME C* or *HvPHYC*). In addition, we will outline parallelisms, differences of the main flowering time genes, and allelic variation between the most important *Triticeae* cultivated species, barley and wheat (*Triticum* species).

Two disclaimers are needed. First, heading date has been commonly used as a surrogate for flowering time in barley, although this equivalence is not fully correct (Alqudah and Schnurbusch 2017). Different authors have used slightly different methods to record the moment of "flowering". The most common has been the recording of awn tipping (Z49) and heading (Z55), according to the Zadoks growth scale (Zadoks et al. 1974). For the sake of simplicity, and to facilitate communication, "heading" and "flowering", are used as synonyms in this article. The slight differences of timing of occurrence between those physiological stages do not affect the purpose of this review. Second, in QTL studies it is almost impossible to be certain about the actual gene underlying each effect detected. However, authors make informed guesses which, in most major flowering time genes cases, are eventually confirmed with functional proofs. We have summarized QTL studies following the authors' judgement regarding underlying genes. When QTL detection preceded the declaration of candidate genes in the region, we have used later literature or our own judgement to declare possible underlying major genes.

3.2. Vernalization response

The genetic control of vernalization in winter barley is based on three genes: *VRN-H1* (Yan et al. 2003; Trevaskis et al. 2003), *VRN-H2* (Yan et al. 2004b), and *VRN-H3* (Yan et al. 2006), which take part in a feedback regulatory loop through epistatic interactions (Distelfeld et al. 2009a) (Figure 3.1). According to the currently accepted model, the high levels of *VRN-H2* during the long days of fall repress flowering by preventing the expression of *VRN-H3*, which limits the up-regulation of *VRN-H1*. The up-regulation of *VRN-H1* during winter results in the down-regulation of *VRN-H2*, the release of *VRN-H3* from its repression and, under long days, the *VRN-H3* up-regulation of *VRN-H1* transcripts beyond the threshold required to initiate

flowering. Loss of *VRN-H2* results in earlier expression of *VRN-H3* under long-day conditions, and promotion of flowering without vernalization (Trevaskis et al. 2006; Distelfeld et al. 2009a).

3.2.1. VRN-H1

VRN-H1 is the central regulator of vernalization-induced flowering in barley (Trevaskis et al. 2007; Distelfeld et al. 2009a). In winter cultivars (with an active *VRN-H2* allele), the expression of this gene is induced by vernalization and accelerates flowering by the promotion of inflorescence initiation at the shoot apex (Trevaskis et al. 2003). *VRN-H1* encodes an *AP1-like* MADS-box transcription factor and is located on chromosome 5HL. In winter cultivars, a prolonged cold period induces *VRN-H1* transcription, eventually leading to phase transition from vegetative to reproductive growth (Yan et al. 2003; Danyluk et al. 2003; Trevaskis et al. 2003). Activation of *VRN-H1* is quantitative, with longer cold treatments inducing higher levels of expression (Yan et al. 2003; Danyluk et al. 2003; Trevaskis et al. 2003; von Zitzewitz et al. 2005; Sasani et al. 2009), which results in earlier transition to the reproductive phase (Sasani et al. 2009). The vernalization-induced transcription of *VRN-H1* is mediated by epigenetic regulation involving changes in chromatin state, through particular modifications in the pattern of histone methylation, whose maintenance provides a memory of cold exposure in winter barley plants (Oliver et al. 2009). Deng et al. (2015) identified binding targets of the VRN1 protein and demonstrated that it regulates flowering repressors *OS2* and *VRN-2*, and flowering promoter *VRN-3*. VRN1 also binds to the promoters of *CBF* (*C-repeat Binding Factor*) genes that play critical roles in low-temperature induction of freezing tolerance and to *VRS1*, which regulates spike architecture. Thus, in addition to controlling flowering, VRN1 directly targets genes in pathways that control other key traits such as frost tolerance.

The previous paragraph describes the classic hypothesis, which still holds, but there is evidence of the presence of a wide allelic diversity at this gene, with more nuanced phenotypic effects. The wild-type *vrn-H1* allele, found in winter barleys, is induced by cold exposure and development, and is characterized by an intact first intron. Other reported alleles differ in the first intron structure, containing deletions or insertions, which affect the length of the cold period needed to reach full de-methylation of the gene (Fu et al. 2005; von Zitzewitz et al. 2005; Cockram et al. 2007a; Hemming et al. 2009). While this is the main regulatory mechanism of this gene, there may be more. Recently, the presence of additional intron regulatory elements in *VRN-H1,* differentiating winter, spring, and wild barleys, has been advocated (Wiegmann et al. 2019). Hemming et al. (2009) characterized at least eleven different alleles based on the size of the first intron (11 kb in the wild-

type *vrn-H1*) (Table S3.1). Alleles characterized by insertions or large deletions within *VRN-H1* intron 1, that disrupt putative cis-regulatory regions presumably required for repression of *VRN-H1*, are associated with increased *VRN-H1* transcript levels, and with earlier flowering without vernalization. In contrast, alleles lacking small segments of the intron, have been associated with moderate basal transcript levels and a weaker flowering stimulation (Szűcs et al. 2007; Hemming et al. 2009; Casao et al. 2011a; Oliver et al. 2013). Therefore, the various *VRN-H1* alleles display a continuum gradation in the strength of flowering promotion (Takahashi and Yasuda 1971; Szűcs et al. 2007). Regarding the gene action of the *VRN-H1* allelic series, the accepted model states that the winter allele is recessive, while the rest are dominant (Takahashi and Yasuda 1971; Haas et al. 2020), although additive effects in F_1 crosses have been observed for non-strict spring alleles (Chapter V).

The vernalization requirement determines the cultivar adaptation range in barley. Mutations in *VRN-H1* and the loss of strong cold requirements allowed the expansion of cultivated barley to areas where spring types are more suitable (von Bothmer et al. 2003; Cockram et al. 2011), although this explanation can be extended to encompass the role of less strict winter types, adapted to fall sowings in areas with warm winters. In fact, several studies have reported ample allelic variation at *VRN-H1* and its relation with geographical distribution, in accordance with this hypothesis (Cockram et al. 2007b, a; Saisho et al. 2011; C.H. Zhang et al. 2015; Dondup et al. 2016; Contreras-Moreira et al. 2019). Besides vernalization response, the *VRN-H1* region has also been associated with winter survival in the field and frost tolerance (Francia et al. 2004; Cuesta-Marcos et al. 2015), with deep implications on the geographical distribution of barley cultivars. In autumn-sown trials subjected to frost stress, the winter *vrn-H1* frost-resistance allele provided a yield advantage (Tondelli et al. 2014). Recently, Rizza et al. (2016) established that the structure of *VRN-H1* intron 1 was strongly correlated not only with vernalization response but also with frost tolerance. In general terms, the higher the vernalization requirement, the higher the frost tolerance levels. However, this is not always true. Some alleles inducing similar vernalization response were associated with different levels of frost tolerance. The alleles *VRN-H1-1*, *VRN-H1-2*, *VRN-H1-3*, and *VRN-H1-4* all showed similarly low frost tolerance levels. The alleles *VRN-H1-6* (medium-high vernalization requirement, Casao et al. 2011b), and *vrn-H1 (5200)* (high vernalization requirement) showed medium-high levels of frost tolerance, whereas allele *vrn-H1 (5300)* was associated with a higher level of frost tolerance. In principle, *vrn-H1 (5200)* and *vrn-H1 (5300)*, which are differentiated by partial amplifications of the first intron, are considered functionally similar variants of the wild type winter allele, both displaying a high vernalization requirement. However, they present sequence differences; *vrn-H1 (5200)* has a small deletion (118 bp) of a region

including a MITE (miniature inverted-repeat transposable element), which could affect epigenetic regulation (von Zitzewitz et al. 2005). In fact, these apparent discrepancies between vernalization and frost tolerance may be a result of lack of experiments run at the sensitivity needed to discriminate all the effects on both traits. Interestingly, from a breeding point of view, Casao et al. (2011a) demonstrated that it is possible to manipulate vernalization requirement with only minor effects on frost tolerance, by taking advantage of the known interaction between *VRN-H1/Fr-H1* and *Fr-H2* (Galiba et al. 2009; Dhillon et al. 2010). This finding opens the path to breed new cultivars that are better suited to a range of winter harshness, especially in a climate-change scenario, by combining reduced vernalization requirement alleles and the frost resistant *Fr-H2* allele from strict winter lines.

An interesting hypothesis argues that vernalization, despite its well-proven adaptive role, could carry an agronomic burden when sowing dates are uncertain. Under these circumstances, frost-tolerant facultative cultivars could be advantageous (Muñoz-Amatriaín et al. 2020).

To summarize the results of flowering time QTL in the *VRN-H1* region (Table 3.1), we followed the terminology of Hemming et al. (2009) for the allelic series (Table S3.1). This region has been strongly associated with vernalization response in controlled conditions experiments in which, in the absence of cold, the winter *vrn-H1* allele consistently delayed flowering (Laurie et al. 1995; Cuesta-Marcos et al. 2008b; Karsai et al. 2008). There is evidence of gradually decreasing vernalization responses of alleles *VRN-H1-6* (Casao et al. 2011a) and *VRN-H1-4* (Casao et al. 2011a, b). The late-flowering effect of the winter *vrn-H1* allele was also found in field trials, apparently when the conditions prevent the completion of the vernalization requirement (e.g. spring sowings) (Laurie et al. 1995; Francia et al. 2004; Cuesta-Marcos et al. 2008b; Tondelli et al. 2014), although the actual measurement of the vernalization potential in field trials is rare. Some studies were sensitive enough to reveal phenotypic differences between *VRN-H1* alleles with more similar vernalization requirements (Cuesta-Marcos et al. 2008a; Rollins et al. 2013; Afsharyan et al. 2020). Genome wide association studies (GWAS) carried out on large germplasm collections also detected important associations between *VRN-H1* and flowering time, of the same kind as for biparental populations (Table 3.1).

Table 3.1. *VRN-H1* polymorphisms and effects on flowering. Surveys in which associations between flowering time and the *VRN-H1* locus region were detected are reported. It includes linkage mapping studies performed in biparental populations segregating for *VRN-H1*, and genome wide association analyses.

		***VRN-H1* allele**[b]			
Population	**Environment/Conditions**[a]	**Parent 1**	**Parent 2**	***VRN-H2* segregating**[c]	**Additive effect**[d]
Biparental populations					
Igri x Triumph[1]	Controlled conditions	*vrn-H1*	***VRN-H1-3***	yes	
Igri x Triumph[1]	Field, spring sowing	*vrn-H1*	***VRN-H1-3***	yes	1.10 days
Dicktoo x Morex[2]	Controlled conditions, uv	*vrn-H1*	***VRN-H1-1***	no	9.00-24.00 days
Mogador x Beka[3]	Controlled conditions	*vrn-H1*	***VRN-H1-1***	yes	0.20-1.20 leaves
Mogador x Beka[3]	Field, spring sowing	*vrn-H1*	***VRN-H1-1***	yes	7.30-10.20 days
Mogador x Beka[3]	Field, winter sowing	*vrn-H1*	***VRN-H1-1***	yes	0.80 days
Nure x Tremois[4]	Field, spring sowing	*vrn-H1*	***VRN-H1-7***	yes	2.30 days
Nure x Tremois[4]	Field, winter sowing	*vrn-H1*	***VRN-H1-7***	yes	0.90 days
Arta x Keel[5]	Field, winter sowing	*VRN-H1-6*	***VRN-H1-4***	yes	1.10-6.50 days
Arta x Keel[5]	Field, autumn sowing	*VRN-H1-6*	***VRN-H1-4***	yes	0.30-1.00 days
Plaisant x Orria[6]	Field, winter sowing	*vrn-H1*	***VRN-H1-4***	no	3.70 days
Plaisant x Orria[6]	Field, autumn sowing	*vrn-H1*	***VRN-H1-4***	no	0.80-1.20 days
Plaisant x (Candela x 915006)[7]	Controlled conditions, uv	*vrn-H1*	***VRN-H1-4***	yes	11.60 days
GWAS					
HEB-25[8]	Field, spring sowing	wild	***VRN-H1-3***	yes	3.80 days
HEB-25[9]	Field, winter sowing	wild	***VRN-H1-3***	yes	3.00 days
HEB-25[10]	Field, autumn sowing	wild	***VRN-H1-3***	yes	2.70 days
HEB-YIELD[11]	Field, spring sowing	wild	***VRN-H1-3***	yes	ns
HEB-YIELD[11]	Field, winter sowing	wild	***VRN-H1-3***	yes	2.50 days
HEB-YIELD[11]	Field, autumn sowing	wild	***VRN-H1-3***	yes	2.20 days
Phenology diversity panel[12, 13]	Field, autumn sowing			yes	6.30 days
MAGIC[14]	Field, spring sowing	*VRN-H1-6*	***VRN-H1-3***	no	2.70 days

[a]Environmental conditions (uv: unvernalized), [b]*VRN-H1* alleles, [c]*VRN-H2* segregation state in the population, and [d]*VRN-H1* additive effect were collected from the original sources (ns: non-significant effect). [b]Alleles contributing to earliness are highlighted in bold.

[1]Laurie et al. (1995), [2]Karsai et al. (2008), [3]Cuesta-Marcos et al. (2008b), [4]Tondelli et al. (2014), [5]Rollins et al. (2013), [6]Mansour et al. (2014), [7]Malosetti et al. (2011), [8]Maurer et al. (2015), [9]Saade et al. (2016), [10]Merchuk-Ovnat et al. (2018), [11]Wiegmann et al. (2019), [12]He et al. (2019), [13]Hill et al. (2019), [14]Afsharyan et al. (2020).

The adaptive role of *VRN-H1* is confirmed by its influence on yield and yield-related traits (Wang et al. 2010; Rollins et al. 2013; Mansour et al. 2014; Tondelli et al. 2014). The study of Rollins et al. (2013) showed that in short-season environments, faster development associated with low vernalization requirement alleles was beneficial for yield. These results are in agreement with those from Mansour et al. (2014) and Tondelli et al. (2014), who found an important QTL by environment interaction at *VRN-H1* for grain yield. In the population Nure (*vrn-H1*) x Tremois (*VRN-H1-7*), a positive contribution on grain yield was reported for the winter allele of Nure in autumn-sown trials, whereas opposite results were found in the late sowing sites (Tondelli et al. 2014). In the case of the population Orria (*VRN-H1-4*) x Plaisant (*vrn-H1*), the winter *vrn-H1* allele from Plaisant reduced grain yield significantly at the three trials which experienced higher temperatures (Mansour et al. 2014). On the contrary, no effect of *VRN-H1* on grain yield was found in a study carried out under similar Mediterranean conditions with the spring x winter population Beka x Mogador (Cuesta-Marcos et al. 2009). In this last case, all trials were sown in autumn and vernalization requirements were probably fulfilled. From the latter studies, it seems clear that the winter *vrn-H1* allele is detrimental for yield at warm sites prone to terminal stress (probably by not meeting the vernalization requirements on time).

Most recently, Voss-Fels et al. (2018) reported that natural allelic variation at *VRN-H1* modulates root growth angle and root length. Compared to the wild-type allele, spring alleles in barley were associated with reduced root elongation and maximum root length between anthesis and maturity. Therefore, the authors suggested a role for this gene in the adaptation of barley to drought. Multi-parental population studies are also a relevant source of evidence for the pleiotropic effects of *VRN-H1* on multiple agronomic traits (Maurer et al. 2016; Saade et al. 2016; Nice et al. 2017; Sharma et al. 2018; Wiegmann et al. 2019). Abdel-Ghani et al. (2019) identified the *VRN-H1* region as hotspot controlling shoot and root architecture under osmotic stress in a spring barley collection. These findings are in agreement with Rollins et al. (2013) and Voss-Fels et al. (2018), who reported *VRN-H1* as an important region under drought conditions, with pleiotropic effects on root architecture, biomass and yield. When the nested association mapping (NAM) population HEB-25 (Halle Exotic Barley) was evaluated with salt stress in field conditions, wild alleles at the *VRN-H1* locus increased height, reduced harvest index, grains per ear and yield under stress and control treatments (Saade et al. 2016). The yield reduction effect of the wild *vrn-H1* alleles was associated with a decreased number of ears but larger grains, supported by Sharma et al. (2018) findings.

The *VRN-H1* region is involved in epistatic interactions affecting heading time and other agronomic traits. A combination of the winter *vrn-H1* allele and the insensitive *ppd-H1* allele resulted in the latest flowering genotypes in a population segregating for both genes (Karsai et al. 2008). Besides, the most significant epistatic interaction under a high temperature conditions experiment (foil tunnel) was among regions that corresponded to *VRN-H3* and *VRN-H1* (Afsharyan et al. 2020). Several studies have found a significant interaction between *VRN-H1* and *HvCEN*, with effects on heading time and yield (Laurie et al. 1995; Cuesta-Marcos et al. 2008b; Mansour et al. 2014; Boudiar et al. 2016), reviewed below in the '*HvCEN*' section. Although probably the most important interaction in which *VRN-H1* is involved is that with the repressor *VRN-H2*, reviewed in the next section, devoted to that gene.

In wheat, *VRN-1* presents homoeologous copies in chromosomes 5A, 5B and 5D. Polymorphisms at this gene are richer in wheat than in barley. Besides deletions in the first intron (Fu et al. 2005), like in barley, many mutations have been described in other regulatory regions and coding sequence, all associated with increased expression of the gene and accelerated flowering in the absence of vernalization (Yan et al. 2003, 2004a; Chu et al. 2011; Li et al. 2013; Muterko et al. 2015; X. Zhang et al. 2015; Kippes et al. 2018). These mutations give rise to spring dominant alleles, with the *VRN-A1* allele showing the strongest effect on flowering time (lack of vernalization requirement), and *VRN-B1* and *VRN-D1* alleles showing a weaker effect (reduced vernalization requirement) (Trevaskis et al. 2003). Moreover, copy number variation has also been described for *VRN-1* in subgenome A, influencing vernalization requirement duration and flowering time of wheat (Díaz et al. 2012; Li et al. 2013; Würschum et al. 2015; Dixon et al. 2019). Besides, the translocation of the region from chromosome 5A that contains the *VRN-1* gene to the chromosome 5DS gave rise to the gene *VRN-D4*, which also reduces vernalization requirement (Kippes et al. 2015).

Summarizing, *VRN-H1* is the major flowering promoter in the vernalization pathway. It is induced by cold exposure and development. There is a large number of *VRN-H1* alleles, which are defined by the length of the first intron, and present a whole gradation of responses to vernalization, from strict winter to spring growth habits. *VRN-H1* effect on flowering time is mainly detected when vernalization requirements are not fully satisfied or are met too late. *VRN-H1* has a wide influence on barley agronomics, through extensive pleiotropic effects (frost tolerance, root architecture, yield…), revealing an adaptive role beyond flowering. The direction and magnitude of *VRN-H1* effects on grain yield vary depending on the environment, particularly on a delicate balance between *VRN-H1* allele, probability of frost occurrence, and vernalizing potential.

3.2.2. VRN-H2

VRN-H2 is the central flowering repressor of the vernalization mechanism. When active, it delays flowering until plants have satisfied their cold needs, when *VRN-H1* represses it (Laurie et al. 1995; Yan et al. 2004b). This epistatic system is clearly a major factor controlling the time to flowering in winter barley (Yan et al. 2003; von Zitzewitz et al. 2005). It has been validated in genetic studies with biparental populations (Karsai et al. 2005; Kóti et al. 2006; Szűcs et al. 2007) and is supported by the results observed in a number of QTL studies (Cuesta-Marcos et al. 2008a, b; Malosetti et al. 2011; Maurer et al. 2015). Recently, ChIP-seq analyses have confirmed the direct regulation of *VRN-H2* by *VRN-H1* (Deng et al. 2015).

VRN-H2 encodes a cluster of three *ZCCT-H* genes, which contain a zinc finger and a *CONSTANS*-like domain, and are located on chromosome 4HL. Functional diversity at *VRN-H2* is the result of the presence or absence of the whole *ZCCT-H* gene cluster (Karsai et al. 2005) (Table S3.1). Winter barleys carry the functional dominant allele (Distelfeld et al. 2009a). The null recessive allele of *VRN-H2* largely bypasses the requirement for vernalization and causes early flowering, regardless of the allelic state at *VRN-H1*. The facultative growth habit is the result of the deletion of the *VRN-H2* locus and the presence of a winter *vrn-H1* allele. These cultivars show winter hardiness but lack an obligate vernalization requirement (Dubcovsky et al. 2005; Karsai et al. 2005; von Zitzewitz et al. 2005). Recent results suggest that facultative barleys, with very high frost tolerance, may contain full or partial deletions of some of the *HvZCCT* genes (Muñoz-Amatriaín et al. 2020).

Its high expression is only achieved in long days (Yan et al. 2004b; Karsai et al. 2005; Trevaskis et al. 2006). However, it has been recently reported that it is also expressed, at lower levels, at day-lengths below 12 h (Monteagudo et al. 2019b), or under conditions in which plants are deceived to sense that they are in long days (Turner et al. 2013). Therefore, this gene is not under the direct control of the light sensing mechanism, but is instead under the control of clock-regulated downstream components (Turner et al. 2013; Mulki and von Korff 2016).

Actually, the regulation of *VRN-H2* is not fully unravelled. Besides its repression by *VRN-H1*, recent shreds of evidence indicate that high expression of *VRN-H2* necessitates of long days and induction by *HvCO1/CO2*, the barley orthologues of the *Arabidopsis CONSTANS* (*CO*) gene, and *PPD-H1*. The VRN2 protein is instrumental in the repression of *VRN-H3* and, hence, of flowering in winter barley, before vernalization (Mulki and von Korff 2016). In addition, Casao et al. (2011b) suggested that *VRN-H2* could also down-regulate *PPD-H2* expression under long days. The

antagonism between the expression of these two genes is clear, but the direction of the repression is not.

There is ample evidence on the presence of flowering time QTL in the region of *VRN-H2*, in a variety of barley biparental populations and association panels (Table 3.2). In general, no effect was detected in fully vernalized experiments, whereas QTL were detected when vernalization was not complete, under long days, and not under short days. This agrees with the dynamics of its expression explained above. *VRN-H2* presents a broad range of additive effects on flowering time, detected in spring-sown trials. It depends on the presence of at least a winter *VRN-H1* allele in the population, which causes wide segregation of vernalization requirements, and on the sowing date and location, which determines the degree of vernalization fulfilment. Karsai et al. (2006) found that the effect of *VRN-H2* on flowering time became significant when the photoperiod was 12h or longer, which agrees with the day-length threshold leading to a marked rise in *VRN-H2* expression that Monteagudo et al. (2019b) determined, and was suggested as the deadline to fulfil the vernalization requirement in winter barley. However, some studies have detected flowering QTL on the *VRN-H2* region under conditions apparently non-inductive for this gene, like vernalized plants (Karsai et al. 2005, 2006, 2008), possibly due to an incomplete vernalization treatment (6 weeks) (Table 3.2). Also, a subtle but consistent effect in short days has been reported (Laurie et al. 1995; Karsai et al. 2005, 2006; Cuesta-Marcos et al. 2008b; Rollins et al. 2013) (Table 3.2).

In addition, *VRN-H2* exerts pleiotropic effects on several developmental and agronomic traits. As expected, when vernalization cannot be completed timely, the presence of *VRN-H2* is deleterious for grain yield and yield components (Rollins et al. 2013). However, positive effects of the presence allele have also been reported. Lines with this allele showed more reproductive tillers, greater thousand grain weight (TGW) and grain yield, when fully vernalized (Karsai et al. 2006). This interesting finding should be confirmed in field trials with appropriate plant materials. Some evidence of field effects of *VRN-H2* on spring barleys is provided by Wang et al. (2010). Unique introgressions carrying *VRN-H2* showed delayed flowering (Table 3.2), reduced height, lodging severity and TGW, but an enhanced value in ears per square meter, harvest index and yield.

Table 3.2. *VRN-H2* polymorphisms and effects on flowering. Surveys where associations between flowering time and the *VRN-H2* locus region have been detected are reported. It includes linkage mapping studies performed in biparental populations, as well as genome wide association analyses.

Population	Environment/ Conditions[a]	Vernalization[b]	Photoperiod[c]	***VRN-H2* allele[d]**		***VRN-H1* segregating[e]**	Additive effect[f]
				Parent 1	**Parent 2**		
---------- Biparental populations ----------							
Igri x Triumph[1]	Controlled conditions	6w - 0w	16h	*VRN-H2*	***vrn-H2***	yes	
Igri x Triumph[1]	Field, spring sowing		LD	*VRN-H2*	***vrn-H2***	yes	1.00 days
Igri x Triumph[1]	Field, autumn sowing		SD	*VRN-H2*	***vrn-H2***	yes	0.90 days
Kompolti Korai x Dicktoo[2]	Controlled conditions	null	8h	*VRN-H2*	***vrn-H2***	no	4.50 days
Kompolti Korai x Dicktoo[2]	Controlled conditions	null	16h	*VRN-H2*	***vrn-H2***	no	12.20 days
Kompolti Korai x Dicktoo[2]	Controlled conditions	incomplete (6w)	16h	*VRN-H2*	***vrn-H2***	no	3.30 days
Kompolti Korai x Dicktoo[2]	Field, spring sowing		LD	*VRN-H2*	***vrn-H2***	no	1.70 days
Kompolti Korai x Dicktoo[3]	Controlled conditions	incomplete (6w)	10h	*VRN-H2*	***vrn-H2***	no	3.00 days
Kompolti Korai x Dicktoo[3]	Controlled conditions	incomplete (6w)	12h	*VRN-H2*	***vrn-H2***	no	13.50 days
Kompolti Korai x Dicktoo[3]	Controlled conditions	incomplete (6w)	14h	*VRN-H2*	***vrn-H2***	no	12.40 days
Kompolti Korai x Dicktoo[3]	Controlled conditions	incomplete (6w)	16h	*VRN-H2*	***vrn-H2***	no	15.80 days
Kompolti Korai x Dicktoo[3]	Controlled conditions	incomplete (6w)	18h	*VRN-H2*	***vrn-H2***	no	17.40 days
Kompolti Korai x Dicktoo[4]	Controlled conditions	incomplete (6w)	24h, constant T[a]	*VRN-H2*	***vrn-H2***	no	12.00 days
Kompolti Korai x Dicktoo[4]	Controlled conditions	incomplete (6w)	16h, constant T[a]	*VRN-H2*	***vrn-H2***	no	12.00 days
Kompolti Korai x Dicktoo[4]	Controlled conditions	incomplete (6w)	16h, thermocycle	*VRN-H2*	***vrn-H2***	no	7.00 days
Mogador x Beka[5]	Controlled conditions	complete (8w)	10h	***VRN-H2***	*vrn-H2*	yes	0.40 leaves
Mogador x Beka[5]	Controlled conditions	null	17h	*VRN-H2*	***vrn-H2***	yes	1.10 leaves
Mogador x Beka[5]	Field, spring sowing		LD	*VRN-H2*	***vrn-H2***	yes	3.6-6.3 days
Mogador x Beka[5]	Field, winter sowing		SD	*VRN-H2*	***vrn-H2***	yes	0.50 days

[a]Environmental conditions, [b]vernalization treatment (w: weeks), [c]photoperiod length (LD: long days, SD: short days), [d]*VRN-H2* alleles, [e]*VRN-H1* segregation state in the population, and [f]*VRN-H2* additive effect were collected from the original sources. [d]Alleles contributing to earliness are highlighted in bold.
[1]Laurie et al. (1995), [2]Karsai et al. (2005), [3]Karsai et al. (2006), [4]Karsai et al. (2008), [5]Cuesta-Marcos et al. (2008b).

Table 3.2. (continued)

Population	Environment/ Conditions[a]	Vernalization[b]	Photoperiod[c]	*VRN-H2* allele[d]		*VRN-H1* segregating[e]	Additive effect[f]
				Parent 1	Parent 2		
Biparental populations							
17 interconnected populations[6]	Controlled conditions	null	17h	*VRN-H2*	***vrn-H2***	yes	2.00 leaves
17 interconnected populations[6]	Controlled conditions	complete (8w)	17h	*VRN-H2*	***vrn-H2***	yes	0.70 leaves
17 interconnected populations[6]	Field, winter sowing		LD	*VRN-H2*	***vrn-H2***	yes	0.70 days
ISR42-8 x Scarlett[7]	Field, spring sowing		LD	*VRN-H2*	***vrn-H2***	yes	0.70 days
Nure x Tremois[8]	Field, spring sowing		LD	*VRN-H2*	***vrn-H2***	yes	1.20 days
KNG x Azumamugi[9]	Field, spring sowing		LD	*VRN-H2*	***vrn-H2***	yes	7.10 days
Arta x Keel[10]	Field, autumn sowing		SD	*VRN-H2*	***vrn-H2***	yes	0.50 days
Arta x Keel[10]	Field, winter sowing		LD	*VRN-H2*	***vrn-H2***	yes	3.70 days
Plaisant x (Candela x 915006)[11]	Controlled conditions	null	LD	*VRN-H2*	***vrn-H2***	yes	2.40 days
GWAS							
HEB-25[12]	Field, spring sowing		LD	*VRN-H2*	***vrn-H2***	yes	2.20 days
HEB-25[13]	Field, winter sowing		LD	*VRN-H2*	***vrn-H2***	yes	1.50 days
HEB-25[14]	Field, spring sowing		LD	*VRN-H2*	***vrn-H2***	yes	1.20 days

[a]Environmental conditions, [b]vernalization treatment (w: weeks), [c]photoperiod length (LD: long days, SD: short days), [d]*VRN-H2* alleles, [e]*VRN-H1* segregation state in the population, and [f]*VRN-H2* additive effect were collected from the original sources. [d]Alleles contributing to earliness are highlighted in bold.
[6]Cuesta-Marcos et al. (2008a), [7]Wang et al. (2010), [8]Tondelli et al. (2014), [9]Sameri et al. (2011), [10]Rollins et al. (2013), [11]Malosetti et al. (2011), [12]Maurer et al. (2015), [13]Saade et al. (2016), [14]Herzig et al. (2018)

There is a particularly rich stream of experimental evidence for the pleiotropic effects of *VRN-H2* on multiple traits coming from the study of NAM populations. Besides lengthening of the stem elongation phase, shortening of the ripening phase, and the corresponding delay in flowering time (Table 3.2), wild barley alleles at *VRN-H2* (presence) were associated with reductions in plant height (Maurer et al. 2016; Nice et al. 2017; Herzig et al. 2018), particularly under high ambient temperature and salt stress (Saade et al. 2016).

In wheat, the *VRN-2* locus encodes two tandemly repeated *ZCCT* genes (Yan et al. 2004b). Deletions or recessive *vrn-2* loss-of-function alleles result in spring growth habit in both diploid and tetraploid wheat (Yan et al. 2004a; Distelfeld et al. 2009b). However, the combination of mutations in all three *VRN-2* homeologues, that would give rise to spring growth habit in hexaploid wheat, has not been observed in nature (Kippes et al. 2016). Apparently, there is no natural variation for this gene in the A and D subgenomes. Natural variation in gene copy number has been revealed for the *VRN-B2* locus, which also shows a stronger effect on vernalization requirement than other homeologues (*VRN-B2* > *VRN-D2*) (Distelfeld et al. 2009b; Kippes et al. 2016). *VRN-2* variation in wheat does not have the same clear-cut effect on growth habit as it has in barley, probably due to the complexity of polyploidy gene effect compensations. Variation at this locus could be used to expand allelic diversity for heading time and to broaden the adaptation of polyploid wheat (Kippes et al. 2016).

In summary, the epistatic interaction between *VRN-H2* and *VRN-H1* is the main factor controlling vernalization response in barley. *VRN-H2* repressing effect depends on the length of low temperature exposure and photoperiod regime. Its effect on flowering is mostly visible in spring-sown trials or in insufficiently vernalized plants followed by long photoperiods. Additionally, *VRN-H2* exerts pleiotropic effects on agronomic traits such as height or grain yield components. This was proven in winter barleys under incomplete vernalization and deserves further investigation in spring barleys.

3.2.3. VRN-H3

VRN-H3 (*HvFT1*), on 7HS, is the barley orthologue of the *Arabidopsis FLOWERING LOCUS T* gene (Yan et al. 2006; Faure et al. 2007; Kikuchi et al. 2009), the main integrator of the photoperiod and vernalization signals leading to the transition from vegetative to reproductive state of the apical meristem. Its expression requires induction by long days, and increased transcript levels correlate with earlier flowering times (Turner et al. 2005; Yan et al. 2006). Mulki and Von Korff (2016) hypothesized that once the vernalization requirements are satisfied, *PPD-H1* and *HvCO1/CO2* up-regulate *VRN-H3*, inducing flowering under long-day conditions. On the other hand, the

photoperiod insensitive *ppd-H1* allele, typical of spring types, has been associated with lower transcript levels of *VRN-H3* and delayed flowering under long days compared with the sensitive *PPD-H1* allele (Turner et al. 2005; Hemming et al. 2008).

FT encodes a mobile protein (florigen) produced in the leaves, then transported to the apices, where it triggers flowering (Corbesier et al. 2007; Li and Dubcovsky 2008). The induction of flowering is the result of complex interactions occurring in the shoot apical meristem (SAM). At the SAM, the FT protein interacts with the *bZIP* transcription factor FD to activate expression of the floral meristem identity genes *AP1* in *Arabidopsis* (Abe et al. 2005; Wigge et al. 2005), and *VRN-1* in wheat (Li and Dubcovsky 2008). Later, the same authors demonstrated that FT, other FT-like proteins and different FD-like proteins could interact with multiple wheat and barley 14-3-3 proteins (Li et al. 2015).

The regulation of *VRN-H3* expression is affected by some known transcription factors, which can result in the occurrence of QTL interactions in studies with mapping populations. In *A. thaliana*, Tiwari et al.(2010) described that the flowering time regulator CO binds to the promoter of *FT*, via a unique cis-element. Although this tight relationship has not been described in barley, there is evidence of an enhanced *VRN-H3* expression caused by *HvCO2* (Mulki and von Korff 2016). Also, Deng et al. (2015) showed that the VERNALIZATION 1 protein binds to the promoter of *VRN-H3* in barley, up-regulating its expression.

Ample allelic variation at *VRN-H3* has been described, arising from sequence polymorphisms in the promoter and first intron (Yan et al. 2006; Hemming et al. 2008; Casas et al. 2011), and from copy number variation (Nitcher et al. 2013; Loscos et al. 2014). However, a clear, unique nomenclature for *VRN-H3* alleles gathering all these polymorphisms has not been developed. Therefore, we propose a new *VRN-H3* allele designation that defines alleles based on their promoter and intron haplotypes, and specifies the number of copies of *HvFT1*, the gene underlying *VRN-H3* (Table S3.1). We aim at introducing a unifying allele nomenclature to ease the knowledge transfer between breeders and plant scientists, and to be routinely used in future studies.

Several reports in different biparental populations have detected flowering time QTL on the *VRN-H3* region of chromosome 7H, representing all types of polymorphism at *VRN-H3* (Table 3.3). Studies involving large germplasm collections also detected an important association between *VRN-H3* and flowering time (Pasam et al. 2012; Alqudah et al. 2014; Sharma et al. 2020, and other references in Table 3.3). The *VRN-H3* region also presented the most significant association with flowering time in multi-parent advanced generation inter-cross (MAGIC) population studies (Sannemann et al., 2015; Afsharyan et al., 2020).

Table 3.3. Polymorphisms at *VRN-H3* and effects on flowering. Surveys where associations between heading time and the *VRN-H3* locus region were detected are reported. It includes linkage mapping studies performed in biparental populations segregating for *VRN-H3*, and genome wide association analyses.

	Differential polymorphism[a]			*VRN-H3* allele[b]		Additive	Interaction (days)[d]	
Population	**P**	**I**	**CNV**	**Parent 1**	**Parent 2**	**effect (days)[c]**	***VRN-H1***	***vrn-H1***
Biparental populations								
H. spontaneum x BGS213[1]	Late *vs* Early	TC *vs* AG	1 *vs* 4*	*vrn-H3d(1)*	***VRN-H3a(T)***	33.00		
Igri x BGS213[1]	Late *vs* Early	TC *vs* AG	1 *vs* 4*	*vrn-H3d(1)*	***VRN-H3a(T)***	35.50		
IMC x BGS213[2]			1 *vs* 4*	*vrn-H3a(1)*	***VRN-H3a(T)***	41.50		
H. spontaneum x Morex[2]	Late *vs* Early	TC *vs* AG		*vrn-H3d(1)*	*vrn-H3a(1)*	ns		
Hayakiso 2 x IMC[2]		TC *vs* AG		*vrn-H3c(1)*	*vrn-H3a(1)*	ns		
H. spontaneum x E878[2]	Late *vs* Early			*vrn-H3d(1)*	***vrn-H3c(1)***		4.8	19.5
H. spontaneum x U672[2]	Late *vs* Early			*vrn-H3d(1)*	***vrn-H3c(1)***		30.0	8.5
Hayakiso 2 x *H. spontaneum*[2]	Early *vs* Late			*vrn-H3c(1)*	***vrn-H3d(1)***	7.00		
SBCC016 x Esterel[3]		AG *vs* TC		*vrn-H3b(1)*	***vrn-H3d(1)***	3.50		
Beatrix x SBCC145[4]	Late *vs* Early			*vrn-H3d(1)*	***vrn-H3c(1)***	2.40		
Mogador x Beka[5, 6]			1 *vs* 2	*vrn-H3d(1)*	***vrn-H3d(2)***	1.10		
SBCC154 x Beatrix[6]		AG *vs* TC	4 *vs* 1	*vrn-H3b(4)*	***vrn-H3d(1)***	1.30		
Henni x Meltan[6, 7]	Late *vs* Early			*vrn-H3d(3)*	***vrn-H3c(3)***	1.50		
Beka x Logan[8]	Late *vs* Early		2 *vs* 1	*vrn-H3d(2)*	***vrn-H3c(1)***	1.30		
Steptoe x Morex[9]	Late *vs* Early	TC *vs* AG		*vrn-H3d(1)*	***vrn-H3a(1)***	0.40		
GWAS								
140 winter landraces (SBCC)[3]	Late *vs* Early	AG *vs* TC		AG intron	**TC intron**	3.50		
HEB-25[10]	Late *vs* Early			*vrn-H3d(1)*	***vrn-H3c(1)***	2.10		
MAGIC [11,12]	Late *vs* Early	AG *vs* TC	?	AG intron	**TC intron**	5.80/-0.20		
AB-NAM[13]	?	?	?	wild	**Rasmusson**	0.70		
Phenology diversity panel[14, 15]	?	AG *vs* TC	?	AG intron	**TC intron**	0.10		

[a]Type/s of polymorphism differencing the parents (P: promoter, I: intron and CNV: copy number variation). Contrasting haplotypes for each differential polymorphism are shown. *For CNV, the asterisk indicates the unique feature of having a single copy of the promoter and several copies of the transcribed region. [b]*VRN-H3* alleles arise from the combination of polymorphisms at the P and I, and from CNV, as reported in Table S3.1. Alleles contributing to earliness are highlighted in bold. [c]*VRN-H3* additive effects were collected from the original sources (ns: non-significant effect). The populations cited were phenotyped under field conditions except for those from references 1 and 2, which were phenotyped under LD conditions and nonvernalizing temperatures. [d]The effect of the interaction with *VRN-H1* alleles is presented (*VRN-H1*: spring allele, *vrn-H1*: winter allele).

[1]Yan et al. (2006), [2]Nitcher et al. (2013), [3]Casas et al. (2011), [4]Ponce-Molina et al (2012), [5]Cuesta-Marcos et al. (2008b), [6]Loscos et al. (2014), [7]Borràs-Gelonch et al. (2010), [8]Casas et al. (2021), [9]Borràs-Gelonch et al. (2012), [10]Maurer et al. (2015), [11]Sanneman et al. (2015), [12]Afsharyan et al. (2020), [13]Nice et al. (2017), [14]He et al. (2019), [15]Hill et al. (2019).

Multiple copies of *VRN-H3* have only been detected in spring and facultative genotypes lacking *VRN-H2* (Loscos et al. 2014). If the VRN2 protein interacts directly with the mechanism of promotion of *VRN-H3* (Li et al. 2011), it could be hypothesized that *VRN-H3* CNV has not been found in winter cultivars because the VRN2 protein produced would not be able to repress several copies of *VRN-H3*. Nitcher et al. (2013) showed that the presence of multiple copies of certain spring barley *VRN-H3* allele was associated with earlier up-regulation of *VRN-H3*, earlier flowering, and an overriding effect of the vernalization mechanism, later confirmed by Cuesta-Marcos et al. (2015). This overriding effect of vernalization came only from the *VRN-H3* allele present in the barley genetic stock BGS213 (derived from the Finnish cultivar Tammi), and not from other CNV alleles. This allele, hereafter named *VRN-H3a(T)* (T from Tammi) had the unique feature of having a single copy of the promoter and several copies of the transcribed region (Nitcher et al. 2013). *VRN-H3a(T)* is dominant over the rest of *VRN-H3* alleles described (Yan et al. 2006). It was reportedly found only in spring cultivars originating from regions of extremely high latitude or high altitude, where it seems to be particularly beneficial (Takahashi and Yasuda 1971). Loscos et al. (2014) found no clear relation between CNV, gene expression and flowering time for other alleles present in spring/facultative barleys.

Regarding sequence variation, Yan et al. (2006) described two promoter haplotypes characterized by seven linked SNPs and two InDels (insertion/deletion) in the first 550 bp upstream of the start codon (InDel 1-InDel 2: insertion-deletion *vs* deletion-insertion), and two first intron haplotypes characterized by two linked polymorphisms (AG *vs* TC). They reported a strong phenotypic effect associated only with the first intron polymorphism. While the AG allele was initially featured as conferring earliness, this was later corrected when more data from mapping populations were available (Casas et al. 2011), and now the TC allele is currently acknowledged as the "early" allele. Another source of confusion could stem from the strand that the *VRN-H3* intron SNP markers from the Illumina 9K and 50K chips (12_30894 and 12_30895) are called. These markers are targeting the bottom strand, where the early TC allele would be read as AG. Casas et al. (2011) analysed natural variation for promoter and intron 1 haplotypes in a landrace collection of predominantly winter barleys (SBCC). In this latter survey, four main *VRN-H3* haplotypes (*vrn-H3a-d*, Table S3.1) were associated with flowering time differences, which geographical distribution strongly correlated with latitude. The intron TC haplotype showed significantly earlier flowering (6-8 days) than the AG haplotype. The prevalence of the early allele (TC) in southern Spanish barley landraces suggests an adaptation role for the *VRN-H3* gene. The presence of the TC allele may be convenient for plants growing in mid-spring in Mediterranean climates, to escape from rapidly rising temperatures and the risk of terminal drought and heat stress. Conversely,

barley landraces from Northern Europe carry predominantly the AG haplotype (Aslan et al. 2015), suggesting that the geographical distribution of *VRN-H3* allelic diversity plays a role in adaptation. In addition, Casas et al. (2011) found that polymorphisms at the *VRN-H3* promoter also contributed to the gene effect on flowering time. The deletion in InDel 1 (early promoter hereafter) was associated with earlier heading (2-3 days) than the insertion (late promoter hereafter) in autumn sowings. The landraces carrying the combination of the early promoter with the TC intron were associated with the earliest flowering (Casas et al. 2011; Ponce-Molina et al. 2012). This class, *vrn-H3c*, actually represents a distinct allele with more polymorphisms in the promoter compared to other classes, as later confirmed by Nitcher et al. (2013). Likewise, in the population Beka (*vrn-H3d(2)*) x Logan (*vrn-H3c(1)*), the Logan *VRN-H3* allele was associated with earlier flowering (Casas et al. 2021). Borràs-Gelonch et al. (2012) detected a QTL for flowering time in the population Steptoe (*vrn-H3d(1)*) x Morex (*vrn-H3a(1)*) close to the *VRN-H3* region. This QTL was only significant in some environments, indicating the expected balancing effect of the two polymorphisms, intron and promoter.

Under spring-sown field conditions, a strong epistatic interaction was found between the regions corresponding to *VRN-H3* and *PPD-H1*, with a strong flowering delay caused by the combination of the insensitive *ppd-H1* allele and the late *vrn-H3a* allele (Afsharyan et al. 2020). Ponce-Molina et al. (2012) also found this interaction in an autumn sowing. As in the study of Afsharyan et al. (2020), the allelic effect at *VRN-H3* was maximized in the presence of the insensitive allele *ppd-H1*. However, under autumn sowing conditions, the photoperiod insensitive allele accelerated flowering time. Finally, there is a recent report by Bi et al. (2019), suggesting that *HvCEN* genetically interacts with *VRN-H3* to modulate floral development.

In addition to flowering time, pleiotropic effects of *VRN-H3* have been reported on duration of developmental phases, plant height, low temperature tolerance, yield and yield-related traits (Wang et al. 2010; Chutimanitsakun et al. 2013; Mansour et al. 2014; Maurer et al. 2016; Nice et al. 2017; Sharma et al. 2018). These effects probably stem from the relationship between earliness and yield. A rich source of experimental evidence of the pleiotropic effects of *VRN-H3* comes from the study of barley populations derived from a spring elite cultivar x wild accession(s) cross (Wang et al. 2010; Maurer et al. 2015; Nice et al. 2017). Wild alleles at the *VRN-H3* region have been associated with delayed development, including shooting, stem elongation, heading and maturity phases (Maurer et al. 2016), increased height (Maurer et al. 2016; Nice et al. 2017), and reduced performance in harvest index and yield (Wang et al. 2010; Sharma et al. 2018).

The richness of polymorphisms and flowering time effects found at *VRN-H3* may provide breeders with additional genetic variability to fine-tune plant development to local environmental conditions.

As in barley, *VRN-3* plays a central role in the integration of signals from vernalization and photoperiod pathways in wheat, with a similar mechanism. This gene presents homeologue copies in subgenomes A, B and D. Sequence variation has been reported for *VRN-A3* (non-synonymous substitution in exon 3) and *VRN-D3* (InDel in exon 3), both having small effects on flowering time (Bonnin et al. 2008). A natural insertion of a retrotransposon element in the promoter region of *VRN-B3* is associated with a stronger early flowering effect under long-day photoperiods (Nitcher et al. 2014), and induced mutations at *VRN-A3* and *VRN-B3* also affect flowering time (Lv et al. 2014). This richness of genetic variation makes this gene one of the main breeding targets to adjust wheat heading time to changing environments.

Summarizing, this key flowering promoter integrates the vernalization and photoperiod pathways. Its expression requires long days and fulfilment of vernalization requirements in winter barleys. Recent studies have revealed ample allelic variation, likely indicating different regulation mechanisms, associated with phenotypic effects. Further additional variation for barley flowering is provided by epistatic interactions with *PPD-H1* and *VRN-H1*. The allelic richness at this locus and its central role in the flowering pathways suggest that it plays a key role in adaptation and agronomic fitness, and offers a large catalogue of options for plant breeders.

3.3. Photoperiod response

Barley is a long day plant, with genetic sensitivities to both long and short photoperiod (Laurie et al. 1995). Two main genes, *PPD-H1* and *PPD-H2*, have been proposed as the main drivers of these responses.

3.3.1. PPD-H1

The *PPD-H1* locus has been identified as the major determinant of long photoperiod response in barley (Turner et al. 2005). Both wild barley and landraces from south-west Asia, southern Europe, and the Mediterranean basin carry a dominant allele, which induces an early occurrence of flowering under increasing day length in spring. Spring landraces from central and northern Europe carry a recessive photoperiod-insensitive *ppd-H1* allele, which confers delayed flowering and maturity under long days (Turner et al. 2005; Hemming et al. 2008; Jones et al. 2008). The emergence of the nonresponsive *ppd-H1* allele, in combination with other mutations at different genes, clearly favoured the expansion of barley production to higher latitudes (von Bothmer and

Komatsuda 2011), by extending the period of vegetative growth of spring-sown plants, thus allowing higher accumulation of biomass, potentially supporting higher yields.

The *PPD-H1* locus encodes a *PSEUDO-RESPONSE REGULATOR* (*HvPRR37*) gene, orthologous to the *Arabidopsis* gene *PRR7*, and maps to the short arm of chromosome 2H. *HvPRR37* is part of the plant circadian clock and its activity causes an increased expression of *VRN-H3*, the main promoter of flowering, when photoperiods rise above 12 h (Turner et al. 2005; Campoli et al. 2012b). On the one hand, *PPD-H1* acts in parallel to *HvCO1* (Campoli et al. 2012a; Shaw et al. 2020). After vernalization, *PPD-H1* and *HvCO1/CO2* up-regulate *VRN-H3*, inducing flowering under long-day conditions (Mulki and von Korff 2016). On the other hand, mutations at evening complex genes *HvELF3* and *HvLUX1*, and *HvPHYC* modulate the expression of *PPD-H1*. Mutations in any of these genes result in a day-neutral up-regulation of *VRN-H3* and early flowering (Zakhrabekova et al. 2012; Faure et al. 2012; Nishida et al. 2013a; Campoli et al. 2013; Pankin et al. 2014). Turner et al. (2005) identified a single nucleotide polymorphism (G/T) at the *PPD-H1* locus (Table S3.1), leading to a change of amino acid in the CCT-domain, as potentially responsible for long photoperiod insensitivity, which has been confirmed recently (Sharma et al. 2020).

Polymorphisms at this gene abound, and its phylogeny has been well studied (Russell et al. 2016; Sharma et al. 2020). However, the phenotypic effects rarely indicate the presence of more than the two functionally distinct alleles described above, the sensitive (*PPD-H1*) and the insensitive (*ppd-H1*) ones. Some studies hint at the presence of alleles that are functionally different from those two (Hemshrot et al. 2019; Bustos-Korts et al. 2019). On the one hand, several private alleles were found in Asian barleys conferring both positive and negative effects, which are not due to the same causative variant for European barley flowering time variation (Hemshrot et al. 2019). On the other hand, from the eight *PPD-H1* haplotypes described by Bustos-Korts et al. (2019) in a global barley panel, haplotype g, classified as photoperiod-sensitive, accelerated flowering both under short and long-day conditions, indicating a response different from that typical of a photoperiod-responsive allele.

Several association-based studies involving wide germplasm collections have identified *PPD-H1* as a major player responsible for flowering time variation (Jones et al., 2008; Russell et al., 2016; He et al., 2019 and references in Table 3.4). Moreover, several of these studies showed a clear latitude-dependent geographical distribution of the two main *PPD-H1* alleles, with the nonresponsive (or, better, less responsive) form predominant in the North (Jones et al. 2008; Lister et al. 2009; Russell et al. 2016; Bustos-Korts et al. 2019). It is well established that *PPD-H1* shows stronger effects on

heading date under long photoperiod conditions (e.g., winter or spring sowings), with the sensitive allele conferring earliness (Laurie et al. 1994, 1995; Boyd et al. 2003; Cuesta-Marcos et al. 2008a; Maurer et al. 2015; Boudiar et al. 2016; Mikołajczak et al. 2016). However, a crossover interaction between *PPD-H1* and the environment has been reported independently for several barley populations (Table 3.4), namely Dicktoo × Morex (Pan et al. 1994), Steptoe × Morex (Borràs-Gelonch et al. 2012), SBCC145 x Beatrix (Ponce-Molina et al. 2012), and Plaisant x Orria (Mansour et al. 2014). In these studies, a significant QTL by environment interaction for heading date was detected in the region of *PPD-H1*, with the sign and magnitude of the *PPD-H1* effect varying depending on the environment. The insensitive *ppd-H1* allele conferred earliness in autumn sowings in the Mediterranean region, in which most of the growing season occurred under short days. On the contrary, in winter or spring sowings, or autumn sowings with a larger proportion of the growing season under long days, the sensitive *PPD-H1* allele conferred earliness. The delaying effect of the sensitive *PPD-H1* allele in early flowering trials is small, but it is credible, given its consistency across four different populations. Field-based GWAS studies confirm this interaction (Bustos-Korts et al., 2019). Likewise, Wiegmann et al. (2019) found a latitude and photoperiod-dependent *PPD-H1* effect. The wild (sensitive) allele of *PPD-H1* accelerated flowering time only in locations exceeding 12h photoperiod during the shooting phase, and the effect was higher with increasing latitude. In addition, the interaction was evident when comparing results of the HEB-25 population from spring-sown German trial, in which the sensitive *PPD-H1* allele reduced time to heading by 9.5 days (Maurer et al. 2015) with autumn-sown Israel (Merchuk-Ovnat et al. 2018) and Dubai trials (Saade et al. 2016), where the sensitive *PPD-H1* allele increased heading time by 6.7 and 2 days, respectively.

Figure 3.2 summarizes the effects of QTL at the *PPD-H1* region found in biparental populations. A change in the direction of the *PPD-H1* effect occurs at approximately 112 Julian days. This could be valid for a certain range of temperatures and latitudes. All the studies summarized in the graph come from latitudes between 40 and 50°N because Julian dates were available only for those. The crossover point may vary for trials at lower or higher latitudes, and different temperatures and pace of thermal time accumulation. Therefore, it is not surprising that this *PPD-H1* x environment interaction was also observed in Scotland for two trials sown in autumn and spring, flowering in May-June (Bustos-Korts et al. 2019). For photoperiod sensitive genotypes (*PPD-H1* allele) to benefit from the accelerating effect of long days, the rhythm of accumulation of growing degree-days has to be such that the occurrence of the inducing photoperiod coincides with the leaf initiation phase, and this depends not only on the latitude but also on the local climate. Studying

the causes of the *PPD-H1* x environment interaction could shed further light on the mechanism of barley response to photoperiod.

PPD-H1 is a central gene in the photoperiod developmental pathway, and is rich in interactions with genes upstream and downstream. The interaction between *PPD-H1* and *VRN-H3* (or, at least, QTL with those underlying genes as candidates) has strong experimental backing, as explained in the *VRN-H3* section. von Korff et al. (2010) found an interaction, between *PPD-H1* and *HvCO2*. The *PPD-H1* sensitive allele accelerated flowering only in presence of an exotic allele at *HvCO2*, while it did not show an effect in combination with the most common allele at this locus. In the latter work, an interaction between *PPD-H1* and *VRN-H2* was also found. The wild (sensitive) *PPD-H1* allele only promoted flowering in a genetic background lacking *VRN-H2*. Mulki and von Korff (2016) found again a link between these two genes, whose nature depended on whether it takes place before or after vernalization (reviewed in '*VRN-H2*' section). Ejaz and von Korff (2017) demonstrated that under high ambient temperature, flowering time is controlled by interactions between *PPD-H1* and *VRN-H1*. Only in the background of a spring *VRN-H1* allele or after up-regulation of *vrn-H1* by vernalization, the wild-type *PPD-H1* allele is capable of accelerating early reproductive development under high ambient temperatures.

In addition to heading time, pleiotropic effects of *PPD-H1* have been reported on many relevant agronomic and morphological traits, like plant height, leaf size, root growth or yield components (Laurie et al., 1994; Karsai et al., 1999; von Korff et al., 2006; Bauer et al., 2009; Wang et al., 2010; Mansour et al., 2014; Maurer et al., 2016; Digel et al., 2016; Alqudah et al., 2018; Abdel-Ghani et al., 2019; Wiegmann et al., 2019).

PPD-H1 seems to act in a location-specific manner on yield-related traits, mostly (but not only) in connection with earliness. At those locations where earliness is beneficial (e.g., early plants can escape higher temperatures and terminal drought at the end of the growing season), the responsive/sensitive allele of *PPD-H1* has been associated with an increase in yield. The yield effect may be explained through pleiotropic effects of the responsive *PPD-H1* allele, which shortens the overall growing season, increases the period of grain filling and increases grain size. On the other hand, at those locations where lateness is preferable to achieve higher yields, the nonresponsive *ppd-H1* allele has been associated with increases in yield-related traits (Wiegmann et al. 2019). However, the current long growing season characteristic of Northern Europe might increasingly change towards Mediterranean conditions as a consequence of climate change, and the ecological advantages of *ppd-H1* could thus disappear in some regions (Herzig et al. 2018).

Table 3.4. Interaction of *PPD-H1* effect and environment on flowering time. Surveys where associations between flowering time and the *PPD-H1* locus region were detected are reported. It includes linkage mapping studies performed in biparental populations segregating for *PPD-H1*, and genome wide association analyses.

Population	Environment (sowing/photoperiod)[a]	Location[b]	Latitude[c]	*PPD-H1* allele[d]		Additive effect[e]	Sowing date	Heading date	DTH[f]	ZD[g]
				Parent 1	Parent 2					
---------- Biparental populations ----------										
Dicktoo x Morex[1]	Phytotron, 8h	Martonvasar (HU)	47° 18' N	*PPD-H1*	*ppd-H1*	ns			105.0	
Dicktoo x Morex[1]	Phytotron, 16h	Martonvasar (HU)	47° 18' N	***PPD-H1***	*ppd-H1*	7.80			45.0	
Dicktoo x Morex[1]	Greenhouse	Oregon (US)	44° 24' N	***PPD-H1***	*ppd-H1*	16.10			52.5	
Igri x Triumph[2]	Field, autumn	Norwich (UK)	52° 38' N	***PPD-H1***	*ppd-H1*	6.00				Z55
Igri x Triumph[2]	Field, spring	Norwich (UK)	52° 38' N	***PPD-H1***	*ppd-H1*	10.80				Z55
Igri x Triumph[2]	Greenhouse, 10h	Norwich (UK)	52° 38' N	*PPD-H1*	*ppd-H1*	ns				Z55
Igri x Triumph[2]	Greenhouse, 18h	Norwich (UK)	52° 38' N	***PPD-H1***	*ppd-H1*	10.00				Z55
Dicktoo x Morex[3]	Phytotron, 16h, 18°C	Martonvasar (HU)	47° 18' N	***PPD-H1***	*ppd-H1*	12.00			44.0	
Dicktoo x Morex[3]	Phytotron, 16h, 18/16°C	Martonvasar (HU)	47° 18' N	***PPD-H1***	*ppd-H1*	13.00			74.0	
Dicktoo x Morex[3]	Phytotron, 24h, 18°C	Martonvasar (HU)	47° 18' N	***PPD-H1***	*ppd-H1*	9.00			42.0	
17 interconected pop.[4]	Field, autumn	Lupinén (ES)	42° 10' N	*PPD-H1*	*ppd-H1*	ns	Nov-08	Apr-19	110.0	Z49
17 interconected pop.[4]	Field, autumn	Zuera (ES)	42° 09' N	*PPD-H1*	*ppd-H1*	ns	Nov-15	Apr-19	110.0	Z49
17 interconected pop.[4]	Field, winter	Alerre (ES)	41° 00' N	***PPD-H1***	*ppd-H1*	2.50	Jan-28	May-19	140.0	Z49
17 interconected pop.[4]	Field, winter	Zuera (ES)	42° 09' N	***PPD-H1***	*ppd-H1*	3.10	Jan-22	May-19	140.0	Z49
Azumamugi x KNG[5]	Phytotron, 12h	Tsukuba (JP)	36° 01' N	***PPD-H1***	*ppd-H1*	19.11			189.0	Z49
Azumamugi x KNG[5]	Field, autumn	Tsukuba (JP)	36° 01' N	*PPD-H1*	*ppd-H1*	ns			185.0	Z58
Azumamugi x KNG[5]	Field, spring	Tsukuba (JP)	36° 01' N	*PPD-H1*	*ppd-H1*	ns			54.0	Z58
SBCC145 x Beatrix[6]	Field, autumn	Zaragoza (ES)	41° 43' N	*PPD-H1*	***ppd-H1***	-1.09	Oct-29	Apr-11	102.0	Z49
SBCC145 x Beatrix[6]	Field, winter	Zaragoza (ES)	41° 43' N	***PPD-H1***	*ppd-H1*	3.32	Feb-08	May-14	135.2	Z49
Steptoe x Morex[7]	Field, autumn	Lleida (ES)	41° 37' N	*PPD-H1*	***ppd-H1***	-0.82	Nov-21	Apr-25		Z55
Steptoe x Morex[7]	Field, autumn	Gimenells (ES)	41° 38' N	*PPD-H1*	***ppd-H1***	-0.60	Nov-30	Apr-20		Z55
Steptoe x Morex[7]	Field, autumn, ext. PD	Lleida (ES)	41° 37' N	***PPD-H1***	*ppd-H1*	0.59	Nov-21	Apr-19		Z55
Steptoe x Morex[7]	Field, winter	Gimenells (ES)	41° 38' N	***PPD-H1***	*ppd-H1*	2.43	Feb-26	May-09		Z55
Steptoe x Morex[7]	Greenhouse, spring	Lleida (ES)	41° 37' N	***PPD-H1***	*ppd-H1*	3.66	Mar-23	May-20		Z55
Plaisant x Orria[8]	Field, autumn	Gimenells (ES)	41° 39' N	*PPD-H1*	***ppd-H1***	-0.30	Dec-01	Apr-16	107.7	Z49
Plaisant x Orria[8]	Field, autumn	Bell-lloc (ES)	41° 37' N	*PPD-H1*	***ppd-H1***	-0.40	Nov-02	Apr-21	112.9	Z49

[a]Environmental conditions (ext. PD: extended photoperiod), [b]location, [c]latitude, [d]*PPD-H1* alleles and [e]additive effects in days were collected from the original sources. [d]Alleles contributing to earliness are highlighted in bold. [e]Additive effect on heading time in days (substitution of one sensitive *PPD-H1* allele by one insensitive *ppd-H1* allele). Negative sign indicates that *ppd-H1* promotes flowering, positive sign indicates that *ppd-H1* delays flowering (ns: non-significant effect). [f]Days to heading from sowing (underlined) or from January 1st; [g]Zadoks stage, developmental phase measured as flowering time in each experiment.

[1]Pan et al. (1994), [2]Laurie et al. (1995), [3]Karsai et al. (2008), [4]Cuesta-Marcos et al. (2008a), [5]Sameri et al. (2011), [6]Ponce-Molina et al. (2012), [7]Borràs-Gelonch et al. (2012).

Table 3.4. (continued)

Population	Environment (sowing/photoperiod)[a]	Location[b]	Latitude[c]	*PPD-H1* allele[d]		Additive effect[e]	Sowing date	Heading date	DTH[f]	ZD[g]
				Parent 1	Parent 2					
Biparental populations										
Plaisant x Orria[8]	Field, autumn	Sádaba (ES)	42° 17' N	***PPD-H1***	*ppd-H1*	1.30	Nov-22	May-01	122.3	Z49
Plaisant x Orria[8]	Field, autumn	Sádaba (ES)	42° 17' N	***PPD-H1***	*ppd-H1*	0.50	Nov-26	Apr-25	116.5	Z49
Plaisant x Orria[8]	Field, winter	Fiorenzuola(IT)	44° 56' N	***PPD-H1***	*ppd-H1*	2.70	Mar-01	May-23	144.9	Z49
SBCC073 x Orria[9]	Field, autumn	Zuera (ES)	42° 09' N	***PPD-H1***	*ppd-H1*	1.50	Nov-11	Mar-02	111.0	Z49
Cam x Maresi[10]	Field, spring	Cerekwica (PL)	52° 31' N	***PPD-H1***	*ppd-H1*	2.79	Apr-10	May-31	51.4	Z51
Cam x Lubuski[10]	Field, spring	Cerekwica (PL)	52° 31' N	***PPD-H1***	*ppd-H1*	2.42	Apr-10	May-30	50.7	Z51
Harmal x Georgie[10]	Field, spring	Cerekwica (PL)	52° 31' N	***PPD-H1***	*ppd-H1*	1.68	Apr-09	May-26	47.9	Z51
GWAS										
HEB-25[11]	Field, autumn	Rehovot (IL)	31° 54' N	*PPD-H1*	***ppd-H1***	-3.40	Dec-03	Mar-16	102.5	Z49
HEB-25[12]	Field, autumn	Dubai (AE)	25° 05' N	*PPD-H1*	***ppd-H1***	-1.00	Dec-08	Feb-26	82.6	Z55
HEB-25[13]	Field, spring	Halle (DE)	51° 29' N	***PPD-H1***	*ppd-H1*	4.75	Mar/Ap		68.1	Z49
HEB-25[14]	Field, spring	Dundee (UK)	56° 28' N	***PPD-H1***	*ppd-H1*	3.00	Apr		78.4	Z49
HEB-25[14]	Field, spring	Halle (DE)	51° 29' N	***PPD-H1***	*ppd-H1*	3.90	Mar		69.4	Z49
WHEALBI subset[15]	Field, autumn	Martonvasar (HU)	47° 17' N	*PPD-H1*	***ppd-H1***	-3.20	Oct-20	May-03	195.1	Z55
WHEALBI subset[15]	Field, autumn	Fiorenzuola (IT)	44° 53' N	*PPD-H1*	***ppd-H1***	-1.90	Oct-27	May-02	187.2	Z55
WHEALBI subset[15]	Field, autumn	Dundee (UK)	56° 30' N	*PPD-H1*	***ppd-H1***	-1.70	Oct-29	Jun-07	222.3	Z55
WHEALBI subset[15]	Field, winter	Martonvasar (HU)	47° 17' N	***PPD-H1***	*ppd-H1*	1.30	Mar-11	May-22	72.7	Z55
WHEALBI subset[15]	Field, winter	Dundee (UK)	56° 30' N	***PPD-H1***	*ppd-H1*	2.50	Mar-03	May-25	83.1	Z55
HEB-YIELD[16]	Field, autumn, 11h	Dubai (AE)	25° 05' N	*PPD-H1*	*ppd-H1*	ns	Dec		89.4	Z49
HEB-YIELD[16]	Field, autumn, 10.5h	Adelaide (AU)	35° 19' S	*PPD-H1*	*ppd-H1*	ns	May/Jun		124.5	Z49
HEB-YIELD[16]	Field, spring, 16h	Dundee (UK)	56° 28' N	***PPD-H1***	*ppd-H1*	3.80	Mar/Ap		84.6	Z49
HEB-YIELD[16]	Field, spring, 15h	Halle (DE)	51° 29' N	***PPD-H1***	*ppd-H1*	4.40	Mar		66.1	Z49
HEB-YIELD[16]	Field, winter, 12h	Al-Karak (JO)	31° 16' N	***PPD-H1***	*ppd-H1*	3.40	Dec/Jan		108.9	Z49
AB-NAM[17]	Field, spring	Minnesota (US)	47° 46' N	***PPD-H1***	*ppd-H1*	3.00	May-08	Jun-28	51.2	Z55
BRIDG6[18]	Field, spring	Minnesota (US)	47° 46' N	***PPD-H1***	*ppd-H1*	4.50	May-04	Jun-24	50.8	Z58
MAGIC[19]	Field, spring	Bonn (DE)	50° 36' N	***PPD-H1***	*ppd-H1*	0.36	Apr-07	Jun-14	68.5	Z49

[a]Environmental conditions (ext. PD: extended photoperiod), [b]location, [c]latitude, [d]*PPD-H1* alleles and [e]additive effects in days were collected from the original sources. [d]Alleles contributing to earliness are highlighted in bold. [e]Additive effect on heading time in days (substitution of one sensitive *PPD-H1* allele by one insensitive *ppd-H1* allele). Negative sign indicates that *ppd-H1* promotes flowering, positive sign indicates that *ppd-H1* delays flowering (ns: non-significant effect). [f]Days to heading from sowing (underlined) or from January 1st; [g]Zadoks stage, developmental phase measured as flowering time in each experiment.

[8]Mansour et al. (2014), [9]Boudiar et al. (2016), [10]Mikołajczak et al. (2016), [11]Merchuk-Ovnat et al. (2018), [12]Saade et al. (2016), [13]Maurer et al. (2015), [14]Herzig et al. (2018), [15]Bustos-Korts et al. (2019), [16]Wiegmann et al. (2019), [17]Nice et al. (2017), [18]Hemshrot et al. (2019), [19]Afsharyan et al. (2020).

In this regard, *PPD-H1* not only perceives day length but also seems to interact with temperature to regulate plant development in barley (Borràs-Gelonch et al. 2012; Hemming et al. 2012; Ford et al. 2016; Ejaz and von Korff 2017; Herzig et al. 2018). Ejaz and von Korff (2017) found that the sensitive allele of *PPD-H1* accelerated floral development and maintained the seed number under high ambient temperatures, whereas the insensitive *ppd-H1* allele delayed floral development and reduced the number of florets and seeds per spike. In addition, Gol et al. (2021) recently showed that variation at *PPD-H1* interacts with drought to control flowering time and yield. Lines with a photoperiod responsive *PPD-H1* allele showed higher trait stability in response to drought. Considering the upcoming environmental conditions, the sensitive *PPD-H1* allele may gain more importance in spring barleys in latitudes North of the Mediterranean region, although possible negative effects on tillering should be compensated.

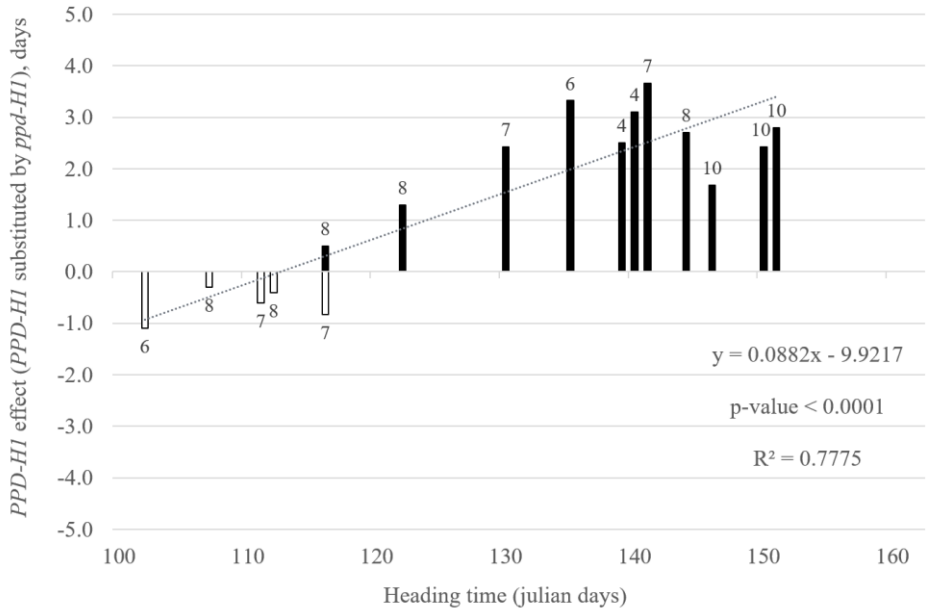

Figure 3.2. Interaction of the effect of *PPD-H1* with environment. Additive effect of *PPD-H1* detected in several barley mapping populations plotted according to average field heading date. The additive effect of *PPD-H1* is calculated as the average effect in flowering time when one sensitive *PPD-H1* allele is substituted by one insensitive *ppd-H1* allele. White bars indicate earliness conferred by the insensitive *ppd-H1* allele. Black bars indicate earliness conferred by the sensitive *PPD-H1* allele. Numbers above or below bars indicate the study from which the data was obtained. The correspondence between numbers and references is located in the footnote of Table 3.4. The regression line (dotted line), the linear equation, the coefficient of determination (R^2), and the significance of the regression analysis are shown.

The wheat homologues of *PPD-H1* are *PPD-A1*, *PPD-B1* and *PPD-D1* located on chromosomes 2A, 2B and 2D, respectively (Laurie 1997; Beales et al. 2007). Wild-type alleles ('b' suffix, e.g., *PPD-A1b*; Mcintosh et al. 2003) are associated with day-length-sensitivity, whereas mutations in *PPD-1*

genes ('a' suffix) result in photoperiod insensitivity. Apparently, there are differences between wheat and barley photoperiod responses. In wheat, genotypes carrying the photoperiod-insensitive allele flower rapidly regardless of whether they are exposed to short or long-day conditions, whereas in barley, the consensus names as "insensitive alleles" those that delay flowering under long days (Turner et al. 2005). Photoperiod-sensitive alleles in wheat and barley substantially delay heading under short days. It is worth noting that both barley and wheat will accelerate flowering to some extent under long days, even in genotypes with the alleles of *PPD-1* that confer strong day-length insensitivity (Hyles et al. 2020). As in barley, wheat *PPD-1* interacts with temperature to accelerate flowering (Hemming et al. 2012).

The molecular mechanisms underlying photoperiod sensitivity may differ between the two species. Allelic diversity in *PPD-1* results from deletions or a transposon insertion in the promoter, and from copy-number variation (*PPD-B1*) (Wilhelm et al. 2009; Bentley et al. 2011; Díaz et al. 2012; Bentley et al. 2013; Nishida et al. 2013b; X. Zhang et al. 2015; Würschum et al. 2019). The homeologue in D subgenome is the major factor affecting flowering time in hexaploid wheat germplasm (Kiss et al. 2014; Langer et al. 2014; Würschum et al. 2018). Moreover, there is a dosage effect, lines combining photoperiod-insensitive alleles on two or three genomes had enhanced earliness (Shaw et al. 2012; Ochagavía et al. 2017).

In wheat, higher expression of some *PPD-1* alleles confers earliness (Shaw et al. 2012; Kiss et al. 2017), something not seen in barley. This is consistent with the type of polymorphisms found, in regulatory regions or CNV for wheat, and in the coding region (CCT domain) of barley. Plant breeders using genome editing may use the knowledge of these different mechanisms underlying photoperiod response in barley and wheat in the future.

In brief, *PPD-H1* is the major gene responsible for photoperiod response in barley. Two main functional alleles have been reported, although recent evidence suggests that there might be more. Finding out the effects of new *PPD-H1* alleles should be prioritized in barley research. The marked latitudinal distribution of the two main alleles and their effects on relevant agronomic traits supports its strong adaptive role. *PPD-H1* effect on flowering time shows a crossover interaction with the environment. The sensitive allele of *PPD-H1* confers earliness under long days. However, a delay of flowering by the responsive allele under short days has been consistently reported. *PPD-H1* interacts with temperature and drought to regulate plant development and acts in a location-specific manner on yield-related traits. New conditions arising from climate change may call for redefining the agronomic fitness of *PPD-H1* alleles for each region.

3.3.2. PPD-H2

The *PPD-H2* locus was first identified as a modifier of flowering time, manifested in response to short days (Laurie et al. 1995). *HvFT3*, another *FT-like* member of the PEBP family, is the candidate gene underlying this locus and was mapped to chromosome 1H (Faure et al. 2007; Kikuchi et al. 2009). Attending to its phenotypic effect, only two allelic variants are known: a dominant one, with a functional copy of the gene, and a recessive allele, with most of the gene missing and non-functional (Kikuchi et al. 2009) (Table S3.1). The dominant, functional allele is prevalent in spring barley and winter barley landraces and cultivars from southern Europe (<44°N). Its effect is more complex than initially thought and promotes flowering in short-day conditions, or even long-day conditions when vernalization requirements have not been fully satisfied (Casao et al. 2011a, c). The non-functional recessive allele is mainly found in central and northern European winter barley (Kikuchi et al. 2009). This uneven distribution across geographic and germplasm divides points at a relevant adaptation role for *PPD-H2*, confirmed by environmental association studies which identified *PPD-H2* as a divergent selection signature between groups of barley landraces (Contreras-Moreira et al. 2019; Lei et al. 2019).

The non-functional *ppd-H2* allele originated pre-domestication (Cockram et al. 2011). Within winter barleys, the mutated *ppd-H2* allele was favoured in northern latitudes, characterized by longer seasons where sufficient vernalization is ensured, and early transition to reproductive growth would expose the plants at a high risk of winterkill. The null, late-flowering allele helps autumn-sown cultivars maintain the vegetative growth phase longer (Pan et al. 1994), perhaps through maintaining the expression of genes that confer tolerance to low temperature (Fowler et al. 2001). However, the dominant ancestral *PPD-H2* allele was conserved in southern latitudes characterized by higher temperatures, where it might help to induce flowering when the vernalization requirement has not been satisfied in full. In spring cultivars, *PPD-H2* can facilitate flowering and ensure timely completion of such a short vital cycle, particularly in combination with "late" alleles at other loci (for instance, *HvCEN*), providing an adequate balance of duration of phenological phases to optimize yield. Therefore, *PPD-H2* likely plays an important adaptive role in spring barleys, and also in winter barleys, where it seems to act as a compensatory mechanism to accelerate flowering and ensure it occurs at the optimal time (Casao et al. 2011c).

During the last years, the view of *PPD-H2* as the "short-photoperiod" gene has given way to a more complex regulation and function. *PPD-H2* is actually expressed both under short and long days, although its expression is more pronounced under short-day conditions (Faure et al. 2007; Kikuchi et al. 2009; Casao et al. 2011a). Kikuchi et al. (2009) reported that overexpression of *PPD-*

H2 resulted in early heading. However, its effect on heading time was weaker than that of *VRN-H3*, suggesting an indirect role of *PPD-H2* in the promotion of floral transition (Kikuchi et al. 2009). Recently, the findings of Mulki et al. (2018) supported this role: overexpression of *PPD-H2* accelerated the initiation of spikelet primordia and the early reproductive development, independently of photoperiod length. However, overexpression of *PPD-H2* did not accelerate floral development, and inflorescences aborted under short days, suggesting that *PPD-H2* controls spikelet initiation but not floral development, which necessitates of additional factors.

Regarding the regulation of *PPD-H2* expression, it has been hypothesized that it is repressed by *VRN-H2* (Casao et al. 2011a). In winter genotypes, with an active *VRN-H2* gene, its transcripts must be absent or clearly receding (either because lack of induction under short days, or repression by expression of *VRN-H1*) for *PPD-H2* to be expressed (Casao et al. 2011a), and this happens only after some cold exposure, and increasingly with plant age (Monteagudo et al. 2019b). In this last study, it was demonstrated that *PPD-H2* expression in a winter genotype is not induced merely by short days. In spring genotypes, most of them involving the deletion of *VRN-H2*, *PPD-H2* is expressed without restriction, even under long days, although to a lesser extent than under short days. Casao et al. (2011a) demonstrated that the up-regulation of the *PPD-H2* transcript correlated with increased levels of *VRN-H1* and *VRN-H3* expression. In contrast, Mulki et al. (2018) reported that overexpression of *PPD-H2* was associated with a strong up-regulation of *VRN-H1*, but not *VRN-H3* in the leaf under both photoperiods. Additionally, *PPD-H2* upregulated the expression of barley row-type genes *VRS4*, *VRS1*, and *INT*-C, which suggested that *FT*-like genes may control spike architecture in addition to modulating developmental timing (Mulki et al. 2018).

PPD-H2 was identified originally as a major heading time QTL in winter × spring barley crosses under short photoperiod conditions (Laurie et al., 1995 and references in Table 3.5). The dominant *PPD-H2* allele was associated with earliness under early-sown field and short-day glasshouse experiments. Several association-based studies involving large germplasm collections have also identified *PPD-H2* as a major flowering time QTL in a worldwide survey of barley germplasm (Alqudah et al., 2014 and references in Table 3.5). The largest effects on growth occurred until the stage of awn tipping, although, in the *PPD-H1* group, it was still visible until anther extrusion. Under controlled conditions, an apparent substitution of the vernalization requirement by exposure to short-photoperiod conditions in winter genotypes was observed (Laurie et al. 1995; Cuesta-Marcos et al. 2008b), phenomenon that was previously named "short-day vernalization" (Roberts et al. 1988). Mulki et al. (2018) concluded that *PPD-H2* does not only counteract the repressive effect of the vernalization pathway, but also induces early reproductive development of

winter barley under short-day conditions. *PPD-H2* seems to play a dual role in the induction of flowering by promoting spikelet initiation under short days and by reducing the requirement for vernalization under long days, as *PPD-H2* seemed to cause a down-regulation of *VRN-H2* in the absence of vernalization. Therefore, *PPD-H2* constitutes an adaptive mechanism to mild winters (at least milder than central-European ones).

For this reason, the *PPD-H2* effect is most influential in Mediterranean latitudes, where autumn-sown cultivars experience short photoperiods during most of the growing season. In early-sowings, it has been identified as one of the two largest effect QTL affecting flowering time, together with *HvCEN* (Boyd et al. 2003; Cuesta-Marcos et al. 2008a, b; Malosetti et al. 2011) (Table 3.5). However, a lower magnitude but significant effect of *PPD-H2* was also detected in vernalized plants grown under long photoperiods (Cuesta-Marcos et al. 2008a, b). This quantitative QTLxE interaction at the *PPD-H2* region is clearly exemplified in the study by Borràs-Gelonch et al. (2012). The effect of *PPD-H2* was gradual, larger in autumn sowings than in winter sowings, while it was absent in spring sowings. In addition, this survey reported that the effect of *PPD-H2* was higher on the leaf and spikelet initiation phase than in the stem elongation phase, which is in agreement with Mulki et al. (2018) findings. Karsai et al. (2008) also identified the *PPD-H2* locus as a significant determinant of flowering time under long photoperiods, but it presented an interaction with temperature, as its effect was only seen when synchronous photo and thermo cycles were applied, and not under constant temperature. *PPD-H2* effect also depended on the allelic configurations at *PPD-H1* and *VRN-H1*, with largest effect on winter-type haplotypes, particularly with the insensitive *ppd-H1* allele. The effect of this gene has been observed outside the temperate regions. A flowering time QTL with a strong effect under short photoperiod was observed in subtropical latitudes (Sameri et al. 2011).

The adaptive role of *PPD-H2* is confirmed by its influence on key agronomic traits, as mentioned recurrently in the literature. Cuesta-Marcos et al. (2009) reported that *PPD-H2* affected grain yield indirectly, through flowering date, under Mediterranean conditions. As expected, its effect was dependent on the environment. The dominant allele was significantly superior in environments where earliness conferred a yield advantage (e.g. terminal stress), whereas the opposite was true for the recessive allele. Mansour et al. (2018) confirmed the beneficial effect of dominant *PPD-H2* on yield of winter types evaluated in Egyptian conditions, by hastening development under short days. Two populations developed from the cross of a Spanish landrace and the elite cultivar Cierzo shared a QTL hotspot on the *PPD-H2* region (Monteagudo et al. 2019a). The QTL contributed to variation in flowering time, TGW, soil coverage, and hectolitre weight. In both populations,

flowering was accelerated by the dominant *PPD-H2* allele, which also increased TGW. In the same region, better soil coverage was contributed by the landrace SBCC042 but coincident with a lower hectolitre weight. On the negative side, a dominant *PPD-H2* seems to reduce frost tolerance in winter and facultative genotypes (Cuesta-Marcos et al. 2015; Rizza et al. 2016). In the HEB-25 population, Sharma et al. (2018) found pleiotropic effects of the *PPD-H2* region (though co-location was not fully certain) on grain area, grain length, and grain roundness.

Table 3.5. Polymorphisms at *PPD-H2* and effects on flowering time in barley mapping populations. Surveys where associations between flowering time and the *PPD-H2* locus region were detected are reported. It includes linkage mapping studies performed in biparental populations segregating for *PPD-H2*, and genome wide association analyses.

Population	Environment / Conditions[a]	*PPD-H2* allele[b]		Additive effect (days)[c]
		Parent 1	Parent 2	
---------- Biparental populations ----------				
Igri x Triumph[1]	Field, autumn sowing	*ppd-H2*	***PPD-H2***	3.40
Igri x Triumph[1]	Field, spring sowing	*ppd-H2*	***PPD-H2***	0.70
Mogador x Beka[2]	Field, autumn sowing	*ppd-H2*	***PPD-H2***	2.00
Mogador x Beka[2]	Field, winter sowing	*ppd-H2*	***PPD-H2***	0.90
Mogador x Beka[2]	Field, spring sowing	*ppd-H2*	***PPD-H2***	ns
17 interconnected populations[3]	Field, autumn sowing	*ppd-H2*	***PPD-H2***	1.40
17 interconnected populations[3]	Field, winter sowing	*ppd-H2*	***PPD-H2***	ns
Steptoe x Morex[4]	Field, autumn sowing	*ppd-H2*	***PPD-H2***	2.10
Steptoe x Morex[4]	Field, winter sowing	*ppd-H2*	***PPD-H2***	0.60
Steptoe x Morex[4]	Field, spring sowing	*ppd-H2*	***PPD-H2***	ns
Plaisant x (Candela x 915006)[5]	Field, autumn sowing	*ppd-H2*	***PPD-H2***	1.60
Azumamugi x KNG[6]	Field, autumn sowing	*ppd-H2*	***PPD-H2***	3.30
Cierzo x SBCC073[7]	Field, autumn sowing	*ppd-H2*	***PPD-H2***	0.60
Cierzo x SBCC042[7]	Field, autumn sowing	*ppd-H2*	***PPD-H2***	1.30
---------- GWAS ----------				
HEB-25[8]	Field, autumn sowing	wild	***PPD-H2***	1.00
Spring world collection[9]	Field, spring sowing	*ppd-H2*	***PPD-H2***	2.50
WHEALBI subset[10]	Field, autumn sowing	*ppd-H2*	***PPD-H2***	1.00
WHEALBI subset[10]	Field, winter sowing	*ppd-H2*	***PPD-H2***	0.70

[a]Environmental conditions, [b]*PPD-H2* alleles, and [c]additive effect were collected from the original sources (ns: non-significant effect). [b]Alleles contributing to earliness are highlighted in bold.
[1]Laurie et al. (1995), [2]Cuesta-Marcos et al. (2008b), [3]Cuesta-Marcos et al. (2008a), [4]Borràs-Gèlonch et al. (2012), [5]Malosetti et al. (2011), [6]Sameri et al. (2011), [7]Monteagudo et al. (2019a), [8]Saade et al. (2016), [9]Pasam et al. (2012), [10]Bustos-Korts et al. (2019).

Orthologues of *PPD-H2* have been identified in the A, B and D genomes of hexaploid and tetraploid wheat (Halliwell et al. 2016). As in barley, these genes are upregulated under short photoperiods (Halliwell et al. 2016; Zikhali et al. 2017). The whole deletion of *TaFT3-B1* gene was associated with late flowering, paralleling the results of genotypes carrying the recessive *ppd-H2* in barley. Wheat *TaFT3-B1* gene, however, presents more allelic variation, with CNV and a non-

synonymous substitution (also associated with late flowering), besides the presence/absence alleles similar to barley (Zikhali et al. 2017).

In summary, *PPD-H2* induces early reproductive development under non-inductive conditions. Its prevailing effect is found under short photoperiod conditions, e.g., autumn sowings. However, its effect has also been detected under long days, when vernalization is incomplete, e.g., winter or spring sowings. The functional allele (*PPD-H2*) predominates in spring and southern winter barley, whereas the non-functional (*ppd-H2*) does it in central and northern winter barley. This distribution hints at an adaptive role in spring and winter barleys, where it could promote spikelet initiation, and ensuring that flowering occurs at the optimal time. Expression of *PPD-H2* in winter barleys has a complex regulation. It has an antagonistic relationship with *VRN-H2*, but it also needs some time of cold exposure. This gene presents large pleiotropic effects on agronomic traits beyond flowering time. Its presence/absence should be a question to be addressed in any breeding program aiming at winter genotypes for temperate regions.

3.4. Earliness *per se* genes

Besides vernalization and photoperiod response genes, the rest of QTL detected affecting flowering time were classically grouped under the generic term "earliness *per se*" or "*eps*" (Laurie et al., 1995). Over the last years, several of these genes have been cloned. Some of them actually have major effects on phenology, and the most important one affecting adaptation is *HvCEN*.

3.4.1 HvCEN

The *eam6* or *eps2* locus, located in the centromeric region of chromosome 2H (Laurie et al. 1995), has been identified as an orthologue of the *Antirrhinum CENTRORADIALIS* gene, designated *HvCEN* (Comadran et al. 2012). This gene is orthologous to *Arabidopsis TFL1*, a member of the *FT*-like gene family, but in contrast to *FT*, encodes a flowering repressor.

A world-wide survey of *HvCEN* genetic variation across wild and cultivated barley detected 14 SNPs that defined 13 haplotypes, 3 prevalent (HI, HII and HIII), shared between wild and domesticated barleys, and several minor ones. Phylogenetic analyses indicate that HIII was selected from wild barley and became fixed in European spring barley cultivars, whereas HII predominates in wild barleys from the eastern Mediterranean and in cultivated winter barleys. Therefore, *HvCEN* has been identified as a relevant contributor to the expansion of barley cultivation into diverse habitats, and as a signature of divergent selection between spring and winter cultivars (Comadran et al. 2012).

A single SNP in the last exon of *HvCEN* encodes a Pro135Ala amino-acid change that differentiates barleys with the two main growth habits. Haplotypes HI and HIII harbour the mutation encoding Ala135, and have been associated with later flowering than haplotype HII-Pro135 (Comadran et al. 2012) (Table S3.1). HI and HIII differ in non-coding regions, two SNPs in intron 2 and one in the 3'UTR. Each allele would be beneficial under different environmental conditions, as follows: in Mediterranean rain-fed conditions (hot and dry summers), the winter Pro135-encoding allele would accelerate development, providing a mechanism to escape terminal drought. On the contrary, in long cool seasons, the spring Ala135-encoding allele would confer an advantage because it would delay flowering, lengthening grain filling under well-watered conditions (Comadran et al. 2012). Exome sequencing of geographically diverse barley landraces and wild relatives indicated that the Pro135Ala mutation in *HvCEN* was the most associated with latitude of all tested flowering- associated gene SNPs (Russell et al. 2016), supporting the hypothesis that *HvCEN* natural variation played an important role in environmental adaptation of cultivated barley.

It is not clear, whether the phenotypic effects of this gene stem only from modifications in the proteins or if gene expression levels are also involved. Comadran et al. (2012) pointed out that protein sequence changes were sufficient to justify the flowering phenotypes, but they also found a constitutive higher expression of HIII (late) than HII (early) alleles, which could also affect the phenotype. Bi et al. (2019) also found expression differences of *HvCEN* haplotypes. They found differential tissue expression of *HvCEN* HI (Bowman) and HIII (Bonus), which are identical for the proposed diagnostic polymorphism (Ala135). These haplotypes also present differences in regulatory regions, which could underlie the distinct expression patterns. Recently, the barley pan-genome has revealed an inversion tightly linked to the *HvCEN* region, which was possibly selected during the barley geographical range expansion and seems exclusive of the HIII carriers. Further research is required to determine whether this inversion has direct functional consequences, for instance, by modulating *HvCEN* expression (Jayakodi et al. 2020).

Flowering time QTL at the *HvCEN* region were first detected by Laurie et al. (1995). They found a QTL with the largest effect on flowering time, both in autumn and spring sowings, identified as *eps2*. The lines with the spring allele (Triumph, HIII) were consistently associated with later flowering than the lines with the winter allele (Igri, HII), with an effect between 2.5 days in the autumn sowing to 3.2 days in the spring one (Table 3.6). Since then, a good number of linkage studies in barley populations, tested on a wide variety of environments, have detected flowering time QTL on the *HvCEN* region (Boyd et al. 2003; Pillen et al. 2003, 2004; Sameri and Komatsuda

2004; Horsley et al. 2006; von Korff et al. 2006, 2008; Castro et al. 2008 and references in Table 3.6), and the same has been reported in GWAS studies (Pasam et al., 2012; Muñoz-Amatriaín et al., 2014; Alqudah et al., 2014; Maurer et al., 2016; Russell et al., 2016 and references in Table 3.6).

Two field experiments carried out with winter x spring populations, with polymorphisms similar to Igri x Triumph, detected QTL at the *HvCEN* region as the most important for flowering time variation in Southern Europe, for sowing dates ranging from autumn to spring (Cuesta-Marcos et al. 2008b; Tondelli et al. 2014). Certainly, the most conspicuous effect of *HvCEN* on flowering time has been identified in autumn sowings in Mediterranean latitudes/climates (Moralejo et al. 2004; Cuesta-Marcos et al. 2008a, b; Ponce-Molina et al. 2012), including Australian environments, where it was identified as the major contributor to heading date variation for several mapping populations (Boyd et al. 2003). All these studies detected effects due to the polymorphism between HII and HIII. Fewer studies focused on polymorphisms involving HI. A phenotypic difference attributable to haplotypes I and III was found in crosses involving North-American germplasm (Marquez-Cedillo et al. 2001; Moralejo et al. 2004; Borràs-Gelonch et al. 2012; Casas et al. 2021). In all of them, HI conferred earliness over HIII (Table 3.6). The difference in flowering time between HI and HIII found in these studies indicates that the amino-acid change is not solely responsible for phenotypic variation. Considering the differential tissue expression of HI and HIII found by Bi et al. (2019), a polymorphism in the 3' region of *HvCEN* could be relevant for regulation of gene expression with potential phenotypic effects.

The question of which is the developmental phase most affected by this gene is not settled yet in the literature. Boyd et al. (2003) hypothesized that this locus was associated with variation in the timing of floral initiation (i.e., duration of the vegetative phase). However, other studies have reported a *HvCEN* effect mainly in the length of the stem elongation phase (Borràs-Gelonch et al. 2012; Castro et al. 2017). In GWAS studies, it was reported that all developmental phases were shortened, except ripening phase, when the spring Barke (HIII) elite alleles were substituted with the exotic alleles at the *HvCEN* region (Maurer et al., 2015). Moreover, Herzig et al. (2018) suggested that one of the wild alleles tested in their study offered a considerable lengthening of the ripening phase in Northern European environments.

HvCEN seems to be in a central position of flowering pathways, due to its involvement in many interactions with other known flowering time genes. Laurie et al. (1995) detected a small but significant interaction between *HvCEN* and *PPD-H1,* and *HvCEN* and *VRN-H3* in a spring-sown trial. Besides, they found a contrasting effect of the *HvCEN* x *VRN-H1* interaction between autumn and spring sowings. Later, other studies hinted at the presence of an interaction between

HvCEN and *VRN-H1* (Table 3.6). Cuesta-Marcos et al. (2008b) only found this interaction in spring sowings. Lines carrying the HIII allele headed significantly later only when the winter allele of *VRN-H1* was present. Mansour et al. (2014) also identified an interaction between these genes in the mapping population Plaisant (HII, *vrn-H1*) x Orria (HI, *VRN-H1-4*), as shown in Figure 3.3, although it was not published in that article. HI allele conferred earliness compared to HII, only in the presence of a winter allele at *VRN-H1*, with differences increasing in parallel to the average Julian date of flowering of the field trials (solid line, Figure 3.3). The winter *vrn-H1* allele delayed heading time in all trials, but especially in the March-sown trial that experienced high temperatures. However, the delay in flowering time associated with that allele was reduced in the presence of the HI at *HvCEN*, only in late flowering trials. These results could indicate that the effect of *HvCEN* is influenced by day-length, or some other environmental feature correlated with it.

It is possible that other genes take part in this interaction but, given the population size, no definitive conclusions could be drawn. In the population SBCC073 (HII, *VRN-H1-4*) x Orria (HI, *VRN-H1-4*) studied by Boudiar et al. (2016), the Orria allele (HI) at *HvCEN* was associated with late flowering time, which agrees with the late effect of the HI in the presence of the *VRN-H1-4* allele found in Orria x Plaisant. In both studies (Mansour et al. 2014; Boudiar et al. 2016), there were significant grain yield QTL interactions involving the *HvCEN* region, indicating its potential interest for plant breeding.

HvCEN also presented significant interactions with *VRN-H2*, in plants that had been vernalized and then grown under short photoperiod (Cuesta-Marcos et al. 2008b). Recently, the study of Bi et al. (2019) indicated that *HvCEN* interacts with *PPD-H2* to control spikelet initiation and with *VRN-H3* to repress floral development. Given the known relationship between *VRN-H2* and *PPD-H2*, these two findings could be connected. Finally, Casas et al. (2021) found several epistatic interactions involving *HvCEN*, *HvELF3*, *HvFD-like* and *VRN-H3*. In the triple interaction *HvELF3* x *HvCEN* x *VRN-H3*, *VRN-H3* had a large effect, particularly when all three alleles came from Logan, producing marked earliness. In the *HvELF3* x *HvCEN* x *HvFD-like* case, the effect of *HvFD-like* was overridden by the presence of Beka alleles at the other two QTL, resulting in late flowering.

Apparently, external cues affect the size of the effect of flowering time QTL coincident with *HvCEN*. van Eeuwijk et al. (2010) indicated that lower minimum temperatures during heading were associated with larger QTL effects in the population Steptoe x Morex, evaluated across North-America and Scotland locations.

Table 3.6. Polymorphisms at *HvCEN* and effects on flowering time in barley mapping populations. Surveys where associations between flowering time and the *HvCEN* locus region were detected are reported. It includes linkage mapping studies performed in biparental populations segregating for *HvCEN*, and genome wide association analyses.

Population	Environment / Conditions[a]	***HvCEN* haplotype[b]**		Additive effect (days)[c]	Interaction ***VRN-H1*[d]**
		Parent 1	**Parent 2**		
---------- Biparental populations ----------					
Triumph x Igri[1]	Field, autumn sowing	III	**II**	2.50	
Triumph x Igri[1]	Field, spring sowing	III	**II**	3.20	
Harrington x Morex[2]	Field, autumn sowing	III	**I**		
Beka x Logan[3]	Field, autumn sowing	III	**I**	2.20	
Beka x Logan[3]	Field, spring sowing	III	**I**	2.50	
KNG x Azumamugi[4]	Field, autumn sowing	III	**II**	2.40	
KNG x Azumamugi[4]	Field, spring sowing	III	**II**	1.70	
Beka x Mogador[5]	Field, autumn sowing	III	**II**	2.70	*vrn-H1*
Beka x Mogador[5]	Field, winter sowing	III	**II**	2.30	*vrn-H1*
Beka x Mogador[5]	Field, spring sowing	III	**II**	4.70	*vrn-H1*
17 interconnected pop.[6]	Field, autumn sowing	III	**I, II**	1.80	
17 interconnected pop.[6]	Field, winter sowing	III	**I, II**	1.40	
Beatrix x SBCC145[7]	Field, autumn sowing	III	**VI**	3.10	
Beatrix x SBCC145[7]	Field, winter sowing	III	**VI**	2.90	
Steptoe x Morex[8]	Field, autumn sowing	III	**I**	2.50	
Steptoe x Morex[8]	Field, winter sowing	III	**I**	2.50	
Tremois x Nure[9]	Field, autumn sowing	III	**II**	2.50	
Tremois x Nure[9]	Field, winter sowing	III	**II**	2.30	
Tremois x Nure[9]	Field, spring sowing	III	**II**	2.70	

[a]Environmental conditions, [b]*HvCEN* alleles, and [c]additive effect were collected from the original sources. [b]Alleles contributing to earliness are highlighted in bold. [d]The effect of the interaction with *VRN-H1* alleles is presented (*VRN-H1-4*: reduced vernalization requirement allele, *vrn-H1*: winter allele).
[1]Laurie et al. (1995), [2]Márquez-Cedillo et al. (2001), [3]Casas et al. (2021), [4]Sameri and Komatsuda (2004), [5]Cuesta-Marcos et al. (2008b), [6]Cuesta-Marcos et al. (2008a), [7]Ponce-Molina et al (2012), [8]Borràs-Gelonch et al. (2012), [9]Tondelli et al. (2014).

Table 3.6. (continued)

Population	Environment / Conditions[a]	*HvCEN* haplotype[b]		Additive effect (days)[c]	Interaction *VRN-H1*[d]
		Parent 1	Parent 2		
---------- Biparental populations ----------					
Baronesse x Full Pint[10]	Field, autumn sowing	III	**II**	1.80	
Baronesse x Full Pint[10]	Field, winter sowing	III	**II**	2.00	
Orria x SBCC073[11]	Field, autumn sowing	I	**II**	1.30	*VRN-H1-4*
---------- GWAS ----------					
HEB-25[12]	Field, autumn sowing	III	**wild**	3.00	
HEB-25[13]	Field, autumn sowing	III	**wild**	3.80	
HEB-25[14]	Field, spring sowing	III	**wild**	1.50	
HEB-25[15]	Field, spring sowing	III	**wild**	1.20	
AB-NAM[16]	Field, spring sowing	wild	**Rasmusson**	0.50	
WHEALBI subset[17]	Field, autumn sowing	late (III, I)	**early (II)**	5.00	
WHEALBI subset[17]	Field, winter sowing	late (III, I)	**early (II)**	2.50	
MABDE[18]	Field	III, I	**II**	3.70	
Uruguay panel[19]	Field, winter sowing	III	**II**	2.40	
Phenology diversity panel[20, 21]	Field, autumn sowing	III	**II**	1.70	

[a]Environmental conditions, [b]*HvCEN* alleles, and [c]additive effect were collected from the original sources. [b]Alleles contributing to earliness are highlighted in bold. [d]The effect of the interaction with *VRN-H1* alleles is presented (*VRN-H1-4*: reduced vernalization requirement allele, *vrn-H1*: winter allele).
[10]Castro et al. (2017), [11]Boudiar et al. (2016), [12]Saade et al. (2016), [13]Merchuk-Ovnat et al. (2018), [14]Maurer et al. (2015), [15]Herzig et al. (2018), [16]Nice et al. (2017), [17]Bustos-Korts et al. (2019), [18]Comadran et al. (2011), [19]Locatelli et al. (2013), [20]He et al. (2019), [21]Hill et al. (2019)

Several pleiotropic effects have been detected for flowering time QTLs on the *HvCEN* region (Pillen et al. 2003, 2004; Moralejo et al. 2004; Horsley et al. 2006; Castro et al. 2008; Cuesta-Marcos et al. 2009; Borràs-Gelonch et al. 2012; Rollins et al. 2013; Tondelli et al. 2014; Mansour et al. 2014; Boudiar et al. 2016; Obsa et al. 2017). In most cases, they are a consequence of the large influence of earliness on other traits. The *HvCEN* region has been described as a hotspot for grain yield-related traits in several populations. A study with three interconnected genetic populations developed from the cross of Australian elite barley genotypes, confronting HII and HIV (deduced from authors' data), detected a yield QTL on *HvCEN* region (Obsa et al. 2017), even though both haplotypes are Pro135. Haplotype II parents contributed the high yield allele, with no relation to maturity. Yield and yield-components (mostly, TGW) QTLs have been commonly found in populations tested in Mediterranean environments, confronting HII and HIII (Nure x Tremois, Tondelli et al., 2014; Beka x Mogador, Cuesta-Marcos et al., 2009). In another population, confronting HIII and HI (Beka x Logan), the *HvCEN* region harboured a QTL by environment (QTLxE) for TGW (Moralejo et al. 2004), with an increasingly larger favourable effect of the early allele (HI) on TGW the later the flowering date of the trial. Other studies have reported grain yield QTL on the *HvCEN* region presenting a crossover interaction between environments (Francia et al. 2011; Mansour et al. 2014; Boudiar et al. 2016). In populations confronting HI and HII (Orria x Plaisant, Mansour et al., 2014; SBCC073 x Orria, Boudiar et al., 2016), HII reduced yield in a late flowering spring-sown trial, while increasing it in an early flowering autumn-sown trial. All these relationships seemed a consequence of significant correlations between earliness and yield. This relationship was usually negative, although QTL x environment qualitative interactions were evident in some cases, depending on the sign of the earliness-yield relationship.

There is a large body of experimental evidence for the pleiotropic effects of *HvCEN* on multiple traits from the study of the HEB-25 NAM population (Maurer et al., 2016; Saade et al., 2016; Merchuk-Ovnat et al., 2018; Herzig et al., 2018; Pham et al., 2019). Briefly, their findings confirm the effects of *HvCEN* alleles studied in biparental populations, although it is not clear in some cases (Merchuk-Ovnat et al. 2018) if the effects detected are caused by *HvCEN* or by some closely linked gene. It is also worth mentioning that *HvCEN* was reported as the gene underlying yield-related traits QTL when this population was tested under field stress conditions. Both under drought (Merchuk-Ovnat et al. 2018; Pham et al. 2019) and salinity (Saade et al. 2016), *HvCEN* wild alleles offered better agronomic performance, under control and stress conditions, compared to the allele contributed by the cultivated parent (Barke, carrying HIII).*HvCEN* is yet another example of the benefits of genetic diversity in adaptation. The stable effect observed across very different agrometeorological conditions revealed its more general role in wide adaptation, and this

is further confirmed by the detection of a QTL for yield adaptability at the same genomic locus (Tondelli et al. 2014).

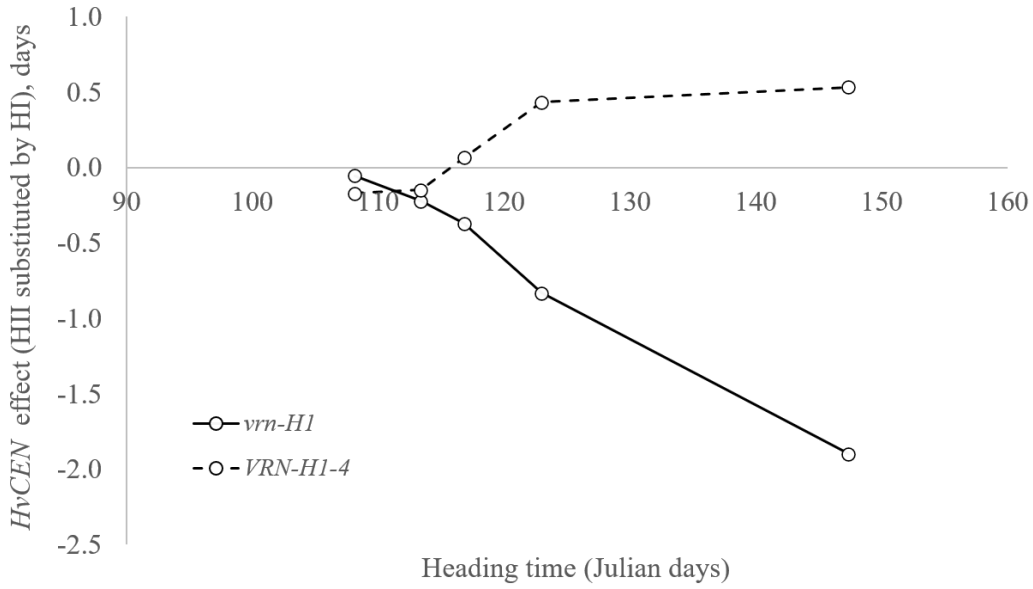

Figure 3.3. Interaction effect between *HvCEN* and *VRN-H1* on heading time in the Orria x Plaisant population, estimated in 5 field trials. The x-axis represents the average Julian days to flowering per trial. The additive effect of *HvCEN* represented in the y-axis is calculated as the average effect in flowering time when one HII allele is substituted by one HI allele. The solid line represents the *HvCEN* effect across heading times in the presence of the winter *vrn-H1* allele. The dashed line represents the *HvCEN* effect across heading times in the presence of the reduced vernalization requirement *VRN-H1-4* allele. The difference in flowering time between homozygous genotypes (HI-HII) would be double of the additive effect shown. Data reanalysed from Mansour et al. (2014).

This gene has not been highlighted in wheat as underlying any QTL of agronomic relevance. A search of genomic databases using *HvCEN* as a template finds three hits, corresponding by sequence and position to two orthologues on chromosomes 2B and 2D, and a third orthologue with no position assigned, annotated as "Terminal flower 1". There were very similar sequences in all six whole genome sequenced wheat varieties for two of these genes. In genome D, there was a predicted amino acid change differentiating them.

In summary, *HvCEN* contributes to the differentiation between spring and winter cultivars. Three main haplotypes have been described (I, II and III). The early haplotype II predominates in winter barleys, whereas the haplotype III does in spring-types. These two haplotypes differ at a single amino acid (Pro135Ala), and show a clear latitudinal distribution, suggesting an adaptive role. Differences in flowering time between haplotypes I and III, however, may be regulatory. Although

haplotype II has been classically acknowledged as the "early" allele, plants carrying haplotype I in some genetic backgrounds, have been associated with even earlier flowering. The *HvCEN* region is frequently identified as a hotspot of QTL, QTL x environment, and QTL x QTL effects on flowering, and on yield-related traits, suggesting a central position for *HvCEN* in the flowering pathways. These interactions should be investigated further for their potential application in breeding.

3.4. Other genes affecting flowering time used in modern barley breeding

Besides vernalization and photoperiod pathways, circadian clock-related *earliness per se* genes have had a relevant role in the barley breeding history to expand the agroecological range of the crop even further (Faure et al. 2012). *HvCO1* and *HvCO2* are LD-flowering promoters modulated by circadian clock and day-length (Griffiths et al. 2003; Campoli et al. 2012a; Mulki and von Korff 2016). In wheat, CO2 competes with VRN2 to bind the NF-Y proteins, in a mechanism to integrate environmental cues through regulation of *VRN-H3* (Li et al. 2011). *HvPHYC* is a phytochrome receptor, functioning as a red and far-red light sensor, key to entrain the circadian clock and perceive the photoperiod (Franklin and Quail 2010). *HvPHYC* is involved in a complex gene interaction network (Pankin et al. 2014; He et al. 2019). Interestingly, an early *PHYC-e* allele was selected in barley cultivars from Japan, where it may provide a selective advantage (Pankin et al. 2014). Several population analysis studies support the role of *HvPHYC* in flowering time variation (Mikołajczak et al. 2016; Ibrahim et al. 2018; Hu et al. 2019; Hill et al. 2019; Sato et al. 2020), as well as its effect in other agronomic traits, including yield (Tesso Obsa et al. 2016; Gong et al. 2016; Hill et al. 2019). In the same pathway, mutations at *HvELF3* (*Mat-a* or *eam8*) within recessive *ppd-H1* stocks, were identified as a major earliness factor facilitating adaptation of barley to very short seasons, at high latitudes (Zakhrabekova et al. 2012; Faure et al. 2012).

Recently, increasing attention is being paid to the Gibberellic acid (GA)-dependant pathway, as an important regulator of key development stages in short-day conditions (Pham et al. 2020). Genes within this pathway enabled the adaptation to modern agriculture, thereby have been widely used in barley improvement, as is the case of the dwarfing or semi-dwarfing genes *Slender 1* (*SLN1*) (Chandler et al. 2002), *breviaristatum-e* (*ari-e*) (Liu et al. 2014), and *semi-dwarf 1* (*sdw1/denso*) (Mickelson and Rasmusson 1994; Hellewell et al. 2000). The semi-dwarfing varieties have better lodging resistance, higher harvest index, and more efficient utilization of the environment (Milach and Federizzi 2001). The *sdw1/denso* gene, however, has been associated with deleterious effects such as late heading and maturity, decreased TGW, and decreased grain weight (Thomas et al. 1991, 1995; Mickelson and Rasmusson 1994; Powell et al. 1997; Hellewell et al. 2000; Jia et al. 2011). The

combination of both semi-dwarfing genes, *sdw1* and *ari-e*, in lines of a recently developed MAGIC population suggest a compensation of flowering time between the two genes, further shortening plant height, and maintaining or slightly increasing grain yield (Dang et al. 2020).

3.5. Concluding remarks

This review summarizes the allelic variation, effects, interactions between genes and with the environment, for the six major flowering time players that have driven barley adaptation to diverse growing environments (Figure 3.4). Considering the wide catalogue of alleles and effects described above, it seems clear that the flowering time genetic variation available to date is enough to tune-up phenology to different formats. However, more extreme ideotypes will be needed with the upcoming environmental conditions brought about by climate change. One potential source of flowering time variation could stem from CNV. Until recently, CNV in flowering time genes in barley had been described in *VRN-H2* (Dubcovsky et al. 2005) and *VRN-H3* (Nitcher et al. 2013; Loscos et al. 2014), whereas in wheat, it was detected in *VRN-A1*, *VRN-B2*, *PPD-B1*, and *TaFT3-B1* (Díaz et al. 2012; Würschum et al. 2015, 2018, 2019; Kippes et al. 2016; Zikhali et al. 2017). However, the recent publication of the barley pan-genome (Jayakodi et al. 2020) allows searching for new variation accessible to scientists and breeders. We identified several mutations responsible for amino acid changes within *ZCCT-Ha*, *ZCCT-Hb*, or *PPD-H1* genes, which could be further explored to assess possible phenotypic differences. Another source of plasticity in flowering behaviour could derive from refining the phases that comprise the plant cycle. The duration of the vegetative and early reproductive phases determines the final number of spikelets, while the late reproductive phase (LRP) determines the number of fertile florets, thus the number of grains and potential yield (Alqudah and Schnurbusch 2014; Digel et al. 2015). The length of the preanthesis phenological phases is genetically controlled, and these developmental periods show different sensitivity to environmental stimulus (Slafer and Rawson 1994; Miralles and Richards 2000; González et al. 2002; Gol et al. 2017; Ochagavía et al. 2018). *VRN-H1*, *VRN-H2*, and *PPD-H2* have been associated mainly with the length of the vegetative and early reproductive phases. *VRN-H3*, *PPD-H1*, and *HvCEN*, however, seem to affect the length of the LRP. Breeders to fine-tune varietal vernalization needs to the target environments can use the gradual vernalization responses provided by the allelic series at *VRN-H1*. The choice of *VRN-H1* allele for a specific environment should balance the vernalizing potential of the environment and the risk of frost. The winter *vrn-H1* allele can be paired with *PPD-H2* as a safeguard to promote flowering in case of incomplete vernalization, but it must also be combined with genes enhancing frost tolerance, because the rapid development linked to the functional *PPD-H2* allele could enhance frost susceptibility in

facultative and winter barleys. Allele *VRN-H1-6* combines high frost tolerance and medium vernalization requirement; therefore, it could be useful for climates where the risk of frost is high but also the possibility of vernalization incompletion. To control floret survival, breeders can play with the *PPD-H1* allelic variation. The sensitive *PPD-H1* allele may gain more importance in central Europe, considering the increases in climate extremes and water and heat stresses derived from climate change. This allele is more stable in the presence of heat and drought. Regarding *VRN-H3*, the *vrn-H3c* allele showed a short LRP independently of the vernalization treatment, therefore it could be useful to escape extreme drought and heat at the end of the season. In contrast, the allele *vrn-H3d* was associated with a consistently long LRP across complete and incomplete vernalization treatments (Chapter V), which could result in increased number of grains and yield under optimum conditions. Finally, *HvCEN* haplotypes offer options to modify the length of the ripening phase. A thorough understanding of the control of each stage might allow fine-tuning phenology for an optimum proportion of phases and allocation of resources, thereby, yield. Similarities and differences of the mechanisms acting in wheat and barley reveal possible new avenues for exploring further ways of fine-tuning phenology of cereal crops.

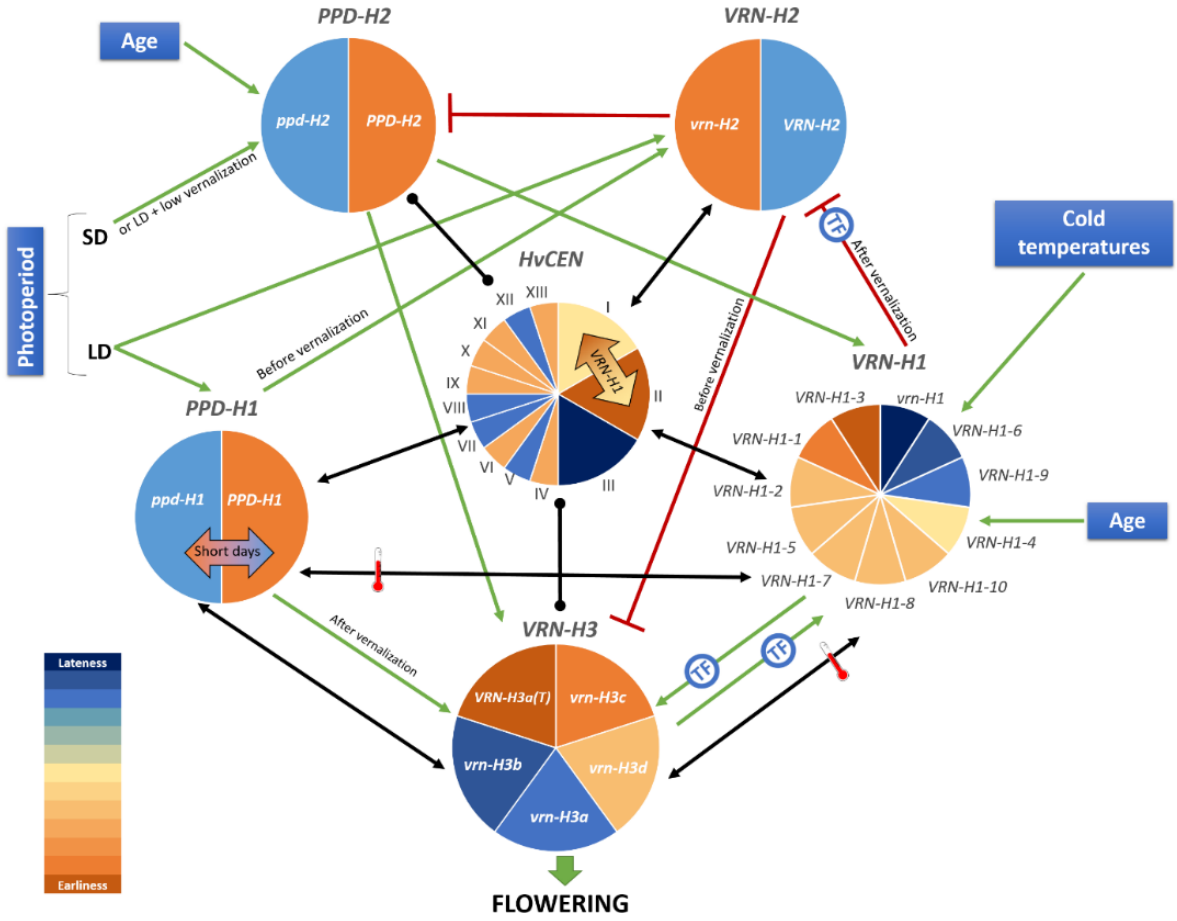

Figure 3.4. Allelic series, effects, interactions between genes and with the environment for six major flowering time genes of barley. Each gene is represented by a circle, sectors represent the alleles that have been reported with phenotypic effect for each gene. The scale of colours indicates the degree of promotion or repression for each allele, between brown (early) and blue (late). Blue boxes indicate external or internal cues. Green lines with arrows and red lines with blunt ends, respectively indicate positive and negative regulatory actions. Black lines indicate epistatic interactions detected in different types of studies: round, gene x gene interaction; arrowed, QTL x QTL interaction. The TF badge (transcription factor) indicates evidence for protein-DNA interaction. The thermometer icon indicates that the QTL x QTL interaction was observed under high temperature. The arrow within the *PPD-H1* circle indicates earliness conferred by the insensitive *ppd-H1* allele under short days, and the opposite under long days. The arrow within the *HvCEN* circle indicates a crossover interaction of the effect of haplotypes I and II dependent on the *VRN-H1* allele. LD: long days, SD: short days.

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3.7. Supplementary material

Table S3.1. Polymorphisms at the major flowering time genes of barley. Locus and gene nomenclature, type and position of polymorphisms, allelic variants, allele nomenclature and effects on flowering time are shown.

Locus	Gene	Polymorphism			Variant			Allele/ Haplotype	Effect	Reference
		Type[a]	Gene position	Description	Promoter	Intron	Coding sequence			
						Intron 1 insertion /deletion size				
VRN-H1	*HvBM5A*	SV (Insertion/ Deletion)	intron	deletions or insertions of the intron 1 of *HvBM5A*		5154 bp deletion		*VRN-H1-1*		Hemming et al. (2009)
						6414 bp deletion		*VRN-H1-2*		
						8898 bp deletion		*VRN-H1-3*		
						4079 bp deletion		*VRN-H1-4*		
						3984 bp deletion		*VRN-H1-5*		
						489 bp deletion		*VRN-H1-6*		
						692 bp insertion		*VRN-H1-7*		
						6604 bp deletion		*VRN-H1-8*		
						2416 bp deletion		*VRN-H1-9*		
						5785 bp deletion		*VRN-H1-10*		
						-		*vrn-H1*		
							Presence/absence of *HvZCCT* genes			
VRN-H2	*HvZCCTa-c*	SV (PAV)	coding	presence/ absence of the *HvZCCT* genes			presence	*VRN-H2*		Karsai et al. (2005)
							absence	*vrn-H2*		
					Promoter inDel 1-inDel 2 haplotype	Intron 1 SNPs haplotype	*HvFT1* CNV			
VRN-H3	*HvFT1*	SNP and SV (inDel and CNV)	promoter, intron, coding	several linked SNPs and 2 inDels in the promoter, two linked SNPs at the first intron and CNV	d-i[b], Early	AG	one promoter and multiple copies of the transcribed region	*VRN-H3a(T)*		Yan et al. (2006), Casas et al. (2011), Nitcher et al. (2013), Loscos et al. (2014)
					d-i, Early	AG	number of *HvFT1* copies	*vrn-H3a(n[c])*		
					i-d, Late	AG	number of *HvFT1* copies	*vrn-H3b(n)*		
					d-i, Early	TC	number of *HvFT1* copies	*vrn-H3c(n)*		
					i-d, Late	TC	number of *HvFT1* copies	*vrn-H3d(n)*		
							SNP in exon 8 (G>W)			
PPD-H1	*HvPRR37*	SNP	coding	missense mutation in exon 8			G, Gly	*PPD-H1*		Turner et al. (2005)
							T, Trp	*ppd-H1*		
							Presence/absence of *HvFT3*			
PPD-H2	*HvFT3*	SV (PAV)	coding	presence/absence of *HvFT3*			presence	*PPD-H2*		Kikuchi et al. (2009)
							absence	*ppd-H2*		
						Intron 2 SNP 63 Intron 2 SNP 93	SNP in exon 4 (P135A)			
eam6/eps2	*HvCEN*	SNP	intron, coding	two SNPs in intron 2 and missense mutation in exon 4		C A	G, Ala135	I		Comadran et al. (2012)
						T G	C, Pro135	II		
						T G	G, Ala135	III		
						T G	C, Pro135	IV, VI, IX, X, XI, XIII		
						variable variable	G, Ala135	V, VII, VIII, XII		

[a]SV, structural variation; PAV, presence–absence variantion; CVN, copy number variation; SNP, single nucleotide polymorphism. [b]d, deletion; i, insertion. [c]n, number of copies of the *HvFT1* gene

Chapter IV. Rachis brittleness in a hybrid-parent barley (Hordeum vulgare) breeding germplasm with different combinations at the non-brittle rachis genes

4. Chapter IV. Rachis brittleness in a hybrid-parent barley (*Hordeum vulgare*) breeding germplasm with different combinations at the *non-brittle rachis* genes

4.1. Introduction

Increased agricultural production needed to meet food demand can only be achieved by "sustainable intensification" (Tilman et al. 2011) of existing croplands, by adopting yield-increasing technologies. In this context, hybrid barley is attracting growing interest as a way to increase productivity per unit area, due to its greater yield potential and yield stability compared to conventional varieties, especially under stress conditions (Longin et al. 2012; Mühleisen et al. 2013, 2014a).

Hybrid barley is increasingly important in Europe, with a significant market share in Germany, France and the United Kingdom, where it covers between 10 and 25% of the acreage devoted to winter six-row feed barley (Longin et al. 2012). The increased productivity of hybrids is the result of the heterosis due to the presence of a number of genes in heterozygosis (Semel et al. 2006). Hybrid yield gain over inbred parental lines has been estimated at about 10% (Longin et al. 2012; Mühleisen et al. 2013). However, it is important to evaluate the possible deleterious phenotypes resulting from heterozygous genes that are fixed in the conventional varieties. This is the case of the loss of the natural grain dispersal system (Pourkheirandish et al. 2015), one of the most relevant events occurred during barley domestication.

Wild barley (*Hordeum vulgare* ssp. *spontaneum*) has a fragile rachis facilitating seed dissemination, whereas the tough rachis of cultivated barley (*Hordeum vulgare* ssp. *vulgare*) prevents spontaneous disarticulation of mature spikelets, ensuring an efficient harvest (Pankin and von Korff 2017). Rachis brittleness is controlled by two dominant, closely linked and complementary genes, located on chromosome 3H, *Btr1* and *Btr2*, involved in the thinning and collapse of the cell walls under the rachis node (Ubisch 1915). In addition to *Btr1* and *Btr2* genes, secondary QTLs for brittle rachis have been detected on chromosomes 5H and 7H (Komatsuda et al. 2004). Independent recessive mutations in any of the *Btr* genes, *Non-brittle rachis 1* (*btr1*) or *Non-brittle rachis 2* (*btr2*), turn the fragile rachis (brittle) into the tough rachis phenotype (non-brittle). All cultivated barleys present a non-brittle genotype, carrying a mutation in one of these two genes (Pourkheirandish et al. 2015). There is a clear pattern in the geographical distribution of *btr* mutations among cultivated barleys. Barley grown in Europe essentially carries the *btr1* mutation, while *btr2* is more frequent in other world regions (Pourkheirandish et al. 2015). Recently, a new non-brittle causal mutation (*btr1b*) has been described in some landraces of Serbia and Greece (Civáň and Brown 2017). Hence,

the cross of parents with alternative mutations in the *Non-brittle rachis* genes (*btr1btr1Btr2Btr2* by *Btr1Btr1btr2btr2*) would lead to a F_1 hybrid (*Btr1btr1Btr2btr2*) which shows a fragile rachis and, thus, might present grain retention problems (Figure 4.1).

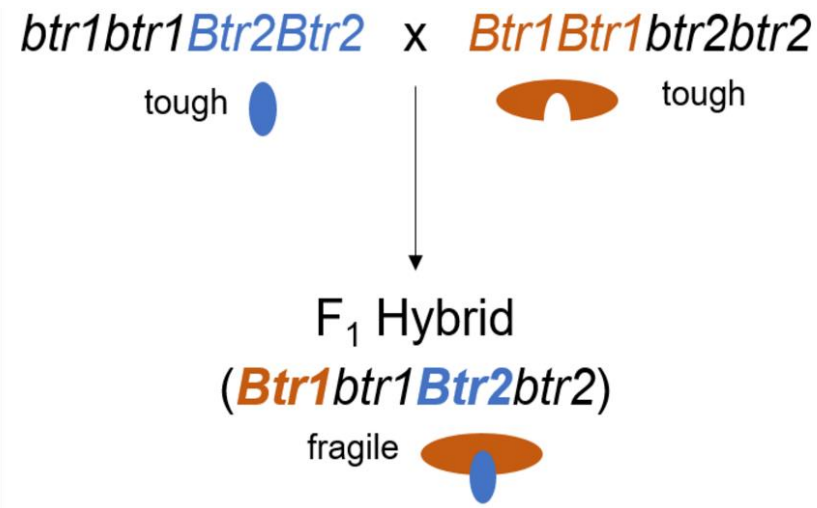

Figure 4.1. Diagram of the *non-brittle rachis* genotype and phenotype of a potential barley cross and resultant hybrid. In the figure, the *non-brittle rachis* genotype of a possible barley cross between inbred lines bearing alternative mutations and its resultant hybrid are represented. The *Btr1* and *Btr2* genes are hypothesized to act as receptor and ligand (Pourkheirandish et al. 2015). Gene products are depicted following graphical representation of Haberer and Mayer (2015).

The aim of this study was to quantify the potential agronomic problem that could arise in F_1 crosses from a real breeding program, testing crosses with different compositions at the *Non-brittle rachis* genes, and to develop a repeatable phenotyping method that could be used routinely in barley breeding programs aiming at hybrid cultivars.

4.2. Material and methods

4.2.1. Plant material

Twenty-three barley (*Hordeum vulgare* L.) F_1 crosses and their twenty-five parents, previously generated in the framework of the Spanish National Public Barley Breeding Program (Gracia et al. 2012), were chosen to represent the three possible combinations at the *Non-brittle rachis* genes. Six crosses *btr1*x*btr1* (short for *btr1btr1Btr2Btr2* x *btr1btr1Btr2Btr2*), six crosses *btr2*x*btr2* (short for *Btr1Btr1btr2btr2* x *Btr1Btr1btr2btr2*), eleven crosses *btr1*x*btr2* (short for *btr1btr1Btr2Btr2* x *Btr1Btr1btr2btr2* or its reciprocal), together with eighteen *btr1* (short for *btr1btr1Btr2Btr2*) and seven *btr2* (short for *Btr1Btr1btr2btr2*) parental lines were selected (Table 4.1).

Table 4.1. Selected F_1 crosses for rachis brittleness assessment, *Non-brittle rachis* genes genotype and presence in experiments.

Cross	Female genotype	Male genotype	Hybrid genotype	Block 1 GR[a]	Block 2 GR[b]	Field[c]
CNE-106 x Esterel	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+		
CNE-126 x Esterel	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+		
02V017-Z10 x 93Z074-Z1	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+		
02V017-Z10 x Lavinia	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+	+	+
04Z001-Z107 x 93Z074-Z1	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+		
02V017-Z10 x 97V115-Z7	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+	+	
CNE-73 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	+		
CNE-75 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	+		
CNE-89 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	-	+	
CNE-123 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	+	+	+
CNE-145 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	+		
CNE-81 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	+		
CNE-6 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-37 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	+
CNE-49 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-58 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-79 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	-	+	
CNE-98 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-106 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-110 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-138 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	-	+	
CNE-135 x Plaisant	*Btr1Btr1btr2btr2*	*btr1btr1Btr2Btr2*	*Btr1btr1Btr2btr2*	-		
CNE-145 x Plaisant	*Btr1Btr1btr2btr2*	*btr1btr1Btr2Btr2*	*Btr1btr1Btr2btr2*	+		

[a]First replicate of the greenhouse experiment, [b]second replicate from the greenhouse experiment, and [c]field nursery experiment. + Presence of that cross and respective parents in a certain experiment, - plant failure.

4.2.2. Experimental setup

Two experiments were conducted at the facilities of the EEAD-CSIC located in Zaragoza (41°43'N, 00°49'W), one in a greenhouse and another one in the field. The greenhouse experiment had two replicates, placed in separate cabinets. In the first replicate (block 1) all genotypes (23 F_1 crosses and 25 parents) were evaluated; in the second replicate (block 2) only the genotypes for which remaining F_1 seed was available were assessed. Also, a sample composed of 3 hybrids (one with each combination of the *Non-brittle rachis* genes) and their respective parents were grown under field conditions (Table 4.1).

For the controlled conditions experiment, seven to ten seeds of each genotype were sown in paper-pot trays (block 1 on 24th Nov 2017, block 2 on 30th Jan 2018) and vernalized for 52 days in a cold chamber (4 – 8 ºC, 16-h light/8-h dark photoperiod). After the cold treatment (with the plants at the three-leaf stage), seven to ten plants of each genotype were transplanted to a 60x20x15 cm pot and transferred to a heated sunlit glasshouse (23°C day/18°C night). The transplant mix composition was 2 x 70 L bales of black peat, 1 bag of vermiculite type 3 (100 L), 2.5 bags of sand (6 kg/bag) and 250 g of slow-release fertilizer Plantacote® 14-9-15 (SQM Vitas, Cádiz, Spain). In addition, plants were fertilized during jointing stage with 3 g/L of Fertipron 20-20-20 (Probelte, S.A., Murcia, Spain). The first block of the greenhouse experiment suffered a powdery mildew (*Blumeria graminis* f. sp. *hordei*) attack, which was controlled with fungicide Bayfidan® (Bayer Hispania, S.L., Barcelona, Spain). The second block was sprayed with fungicides Bayfidan® and Aviator® Xpro (Bayer Hispania, S.L., Barcelona, Spain) as a preventive measure. To avoid spatial effects, the positions of the pots were shuffled every week. Irrigation was applied daily. At maturation stage, the greenhouse temperature was risen to 33 ºC and irrigation was stopped.

Regarding the natural conditions experiment, between 24 and 35 seeds of each genotype were sown in paper-pot trays in 1st Dec 2017. Once emerged (18th Dec 2017), seedlings were transplanted to a field nursery.

4.2.3. Genotyping

Leaf tissue from individual plants of the parental lines and F_1 crosses was sampled, frozen in liquid nitrogen and homogenized (Mixer Mill model MM301, 140 Retsch). Genomic DNA was extracted using the NucleoSpin® Plant II protocol (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Delaware, USA). Finally, samples were diluted to a final concentration of 50 ng/µl using TE buffer.

Genotypes were checked using specific KASP™ markers (Table S4.1) for the *Non-brittle rachis* genes developed in-house (via LGC Genomics Ltd, Hertfordshire, UK), which detect the canonical mutations of *btr1* (1 nucleotide deletion), and *btr2* (11 nucleotides deletion). The assay mix preparation and PCR protocols were conducted according to LGC Genomics protocols in an ABI7500 real-time PCR system (Applied Biosystems, Foster City, CA).

4.2.4. Phenotyping

For the greenhouse experiment, rachis brittleness was assessed through mechanical processing of spikes in an adapted threshing machine equipped with cooking grade silicone toothed rotor blades. Spikes were threshed for five seconds at 900 rpm. The threshed material was collected in a removable plastic tray. Rachis fragility, in percentage, was calculated as previously reported by Komatsuda et al. (2004), i.e., the percentage of rachis nodes disarticulated over the total number of rachis nodes in a spike, measured in five F_1 plants per genotype, using two spikes per plant, at two different times (2 and 4 weeks after ripening, determined as stage Z91 (Zadoks et al. 1974)) (Video S1).

In the field nursery, all the spikes from three to ten plants of each genotype were bagged with breathable and translucent bags (Fito Agrícola S.L., Castellón, Spain). Spontaneous spikelet disarticulation was measured, at three different times (two, three, and four weeks after Z91), through the counting of the number of disarticulated rachis nodes per number of spikes inside the bag.

In addition, the disarticulation scars from a representative sample of brittle and non-brittle spikes were evaluated with the aid of a Nikon SMZ 745 T stereomicroscope connected to a Nikon DS-Fi camera.

4.2.5. Statistical analysis

In order to satisfy the assumptions required for later analyses (i.e. normality distribution of residuals and homoscedasticity of variances), the variable percentage of brittleness was transformed using an arcsin $\sqrt{x}$ function, suitable for percentage data (Sokal and Rohlf 1969). All statistical procedures were performed with the transformed data. However, actual percentages are presented in tables and figures, as their interpretation is more intuitive. Differences in rachis brittleness between genotypes, sampling times (two and four weeks after Z91) and blocks (block 1 and block 2) were evaluated using the analysis of variance (ANOVA) "type III" procedure for unbalanced designs in JMP (SAS Institute, Cary, North Carolina, United States). The ANOVA model included genotype, sampling time, block and genotype by time interaction. Genotype, time

and block were all considered fixed factors. The ten spikes sampled per genotype (five plants, two spikes from each) were considered replicates. The contrasts defined were: brittle *vs.* non-brittle types[1], hybrids *vs.* parents within non-brittle type[2], *btr1 vs. btr2* alleles[3], hybrids *btr1*x*btr1 vs. btr2*x*btr2*[4], and finally, parents *btr1 vs.* parents *btr2*[5]. The interactions of all these contrasts with time were also tested. Means were compared using least significant difference (LSD) test ($P<0.05$).

4.3. Results

4.3.1. Rachis brittleness differences between brittle and non-brittle types

The analysis of the phenotypic data showed significantly higher rachis brittleness in crosses bearing alternative mutations in the *Non-brittle rachis* genes (*Btr1btr1Btr2btr2*) compared to hybrids and inbred parents carrying one of the deletions conferring the non-brittle phenotype (*btr1btr1Btr2Btr2* or *Btr1Btr1btr2btr2*) in the mechanic test (Table 4.2, contrast "brittle *vs.* non-brittle").

Considering the two sampling times (two and four weeks after maturation), two blocks, parents and hybrids, the overall percentage of rachis nodes disarticulated shown by brittle types was 55% *vs.* 17% of non-brittle types[1] (Figure 4.2A, Table S4.2). Moreover, there was a visual difference in the disarticulation scar morphology between types (Figure S4.1). For the brittle types, 80% of the rachis nodes disarticulated easily into individual triplets, leaving a smooth surface. On the contrary, non-brittle rachises remained almost intact after mechanical processing, even if most grains became separated from the floral axis. Whenever these broke, breaks were mostly harsh (65%), leaving a jagged surface.

Differences between blocks were detected, probably due to the fungal infection mentioned above. Rachis brittleness was distinctly higher in block 2 than in block 1[6]. This was probably influenced by the length and overall volume of the spikes. Plants in block 1 produced spikes of smaller size than plants in block 2. In addition, there were visible differences in grain filling between the blocks. It was optimum in the second block, whereas spikes in the first block presented many shrivelled grains. The spike size difference is clear from the comparison of the average number of triplets per spike between blocks (Table 4.3), i.e., spikes in block 1 showed, on average, 20% less internodes than in block 2.

Despite dissimilarities in the range of values, the division between brittle and non-brittle types was clear in both data sets. Brittle types presented significantly higher rachis fragility values than non-brittle types[1] (Table 4.4). It is clear that the block had an effect on rachis brittleness[6], but the trends

were consistent, as indicated by a positive correlation (r = 0.61, in both sampling times) between rachis fragility scores shown by common genotypes assessed in both blocks.

Table 4.2. Effects of genotype, block, time, genotype by time interaction, and contrasts on rachis brittleness.

Source of Variation	df[a]	SS[b]	MS[c]	F[d]	p-value[e]	
Genotype	44	18.540	0.42	29.7	2.44E-158	***
brittle *vs.* non-brittle[1]	1	10.950	11.00	771.1	9.29E-131	***
within brittle	9	0.375	0.04	2.9	1.90E-03	**
within non-brittle	34	4.544	0.13	9.4	9.81E-42	***
Hybrids (*btr*) *vs.* Parents[2]	1	0.217	0.22	15.3	9.79E-05	***
within Hybrids (*btr*)	11	0.720	0.07	4.6	6.72E-07	***
within Parents	22	3.611	0.16	11.6	5.44E-37	***
btr1 vs. btr2[3]	1	0.000	0.00	0.0	9.73E-01	
within *btr1*	22	2.138	0.10	6.8	1.07E-19	***
within *btr2*	11	2.426	0.22	15.5	1.30E-28	***
Hybrids *btr1 vs.* Hybrids *btr2*[4]	1	0.022	0.02	1.5	2.13E-01	
within Hybrids *btr1*	5	0.217	0.04	3.1	9.54E-03	**
within Hybrids *btr2*	5	0.534	0.11	7.5	5.76E-07	***
Parents *btr1 vs.* Parents *btr2*[5]	1	0.101	0.10	7.1	7.76E-03	**
within Parents *btr1*	16	1.844	0.12	8.1	9.76E-19	***
within Parents *btr2*	5	1.769	0.35	24.9	6.48E-24	***
Repetition	9	0.015	0.00	0.1	9.99E-01	
Block[6]	1	6.308	6.31	444.2	7.77E-84	***
Time[7]	1	2.336	2.34	164.5	2.50E-35	***
Genotype * Time	44	2.353	0.05	3.8	1.02E-14	***
brittle *vs.* non-brittle * Time[8]	1	0.510	0.51	35.9	2.76E-09	***
within brittle * Time	9	0.386	0.04	3.02	1.42E-03	**
within non-brittle * Time	34	1.916	0.06	4.0	4.67E-13	***
Hybrids (*btr*) *vs.* Parents * Time[9]	1	0.142	0.14	10.0	1.62E-03	**
within Hybrids (*btr*) * Time	11	0.196	0.02	1.2	2.46E-01	
within Parents * Time	22	1.051	0.05	3.4	2.76E-07	***
btr1 vs. btr2 * Time	1	0.003	0.00	0.2	6.46E-01	
within *btr1* * Time	22	1.146	0.05	3.7	2.65E-08	***
within *btr2* * Time	11	0.649	0.06	4.1	4.85E-06	***
Hybrids *btr1 vs.* Hybrids *btr2* * Time	1	0.002	0.00	0.1	7.08E-01	
within Hybrids *btr*1 * Time	5	0.153	0.03	2.2	5.66E-02	
within Hybrids *btr2* * Time	5	0.042	0.01	0.6	7.03E-01	
Parents *btr1 vs.* Parents *btr2* * Time	1	0.001	0.00	0.1	7.91E-01	
within Parents *btr1* * Time	16	0.557	0.04	2.4	1.17E-03	**
within Parents *btr2* * Time	5	0.456	0.09	6.4	6.69E-06	***
Residuals	1169	16.600	0.01			

[a]Degrees of freedom, [b]sum of squares, [c]mean squares, [d]F-statistic, [e]*P<0.05 **P<0.01 ***P<0.001. Superscript numbers designate each contrast and will be used throughout the text to facilitate tracking.

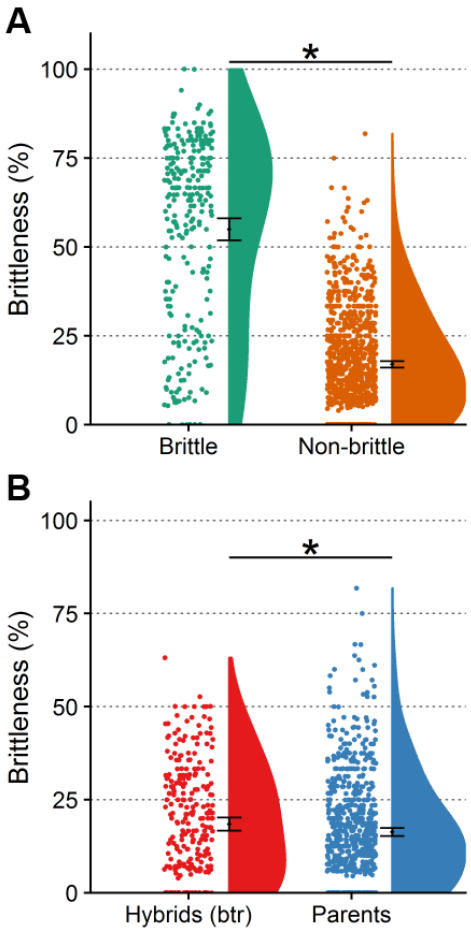

Figure 4.2. Contrasts on rachis fragility between (A) brittle and non-brittle types[1], and (B) between hybrids (*btr*) and parents[2]. "Brittle" includes all hybrids *btr1*x*btr2*; "non-brittle" comprises hybrids *btr1*x*btr1*, hybrids *btr2*x*btr2*, parents *btr1* and parents *btr2*. "Hybrids (*btr*)" includes hybrids *btr1*x*btr1* and hybrids *btr2*x*btr2*; "parents" comprises parents *btr1* and parents *btr2*. Raw measurement data points (left), probability density distribution (right), and mean ± 95 % confidence interval of rachis fragility in percentage are represented for each genotypic class within each contrast. Means are averaged for the two sampling times. Asterisks indicate significantly different group means at P<0.05 according to the contrast performed for the overall ANOVA with transformed data.

Table 4.3. Number of rachis internodes per spike for each block and *btr* genotype class.

Number of rachis internodes				
	Block 1		**Block 2**	
***btr* genotype**	**N[a]**	**Mean ± CI[b]**	**N[a]**	**Mean ± CI[b]**
Parents *btr1*	304	14.28 ± 0.39	240	18.09 ± 0.36
Parents *btr2*	100	13.13 ± 0.67	60	16.88 ± 0.47
Hybrids *btr1*x*btr1*	105	15.84 ± 0.59	40	20.08 ± 0.93
Hybrids *btr2*x*btr2*	94	14.40 ± 0.52	40	19.63 ± 1.00
Hybrids *btr1*x*btr2*	104	15.91 ± 0.62	180	18.08 ± 0.39
TOTAL	707	14.60 ± 0.25	560	18.21 ± 0.23

[a]Number of spikes assessed within each genotypic class and block, [b]mean of the number of rachis internodes ± 95 % confidence interval.

4.3.2. Rachis brittleness differences within non-brittle types

Significant differences in rachis fragility were found within non-brittle genotypes, in the controlled conditions experiment. Non-brittle hybrids (*btr1*x*btr1* and *btr2*x*btr2*) presented a significantly higher percentage of rachis brittleness (two sampling times averaged) than parents (inbred lines *btr1* and *btr2*)[2] (Figure 4.2B).

Table 4.4. Rachis brittleness for levels of brittle-type and block factors.

Rachis brittleness (%)						
	Block 1			**Block 2**		
Brittle-type	**N[a]**	**Mean ± CI[b]**	**Groups[c]**	**N[a]**	**Mean ± CI[b]**	**Groups[c]**
Brittle	104	28.15 ± 4.27		180	70.45 ± 1.98	
Non-brittle	603	12.62 ± 1.07		380	23.86 ± 1.41	
TOTAL[1]	707	14.91 ± 2.14	b	560	38.83 ± 1.18	a

[a]Number of spikes assessed within each factor level, [b]rachis fragility mean (in percentage) ± 95 % confidence interval, [c]means in the same row followed by the same letter were not different at P<0.05.

No gene-specific effect on rachis brittleness was detected when comparing genotypes carrying the *btr1* mutation with genotypes bearing the *btr2* mutation[3], regardless of whether they were hybrids or parents. Furthermore, no significant differences in rachis fragility were found between hybrids *btr1*x*btr1* and *btr2*x*btr2*[4]. However, the effect of the *btr* gene on the percentage of rachis nodes disarticulated was significant when contrasting parents *btr1 vs. btr2*[5] (Table 4.5), though the size of the effect (1.24%) was probably too low to bear agronomic relevance.

Table 4.5. Rachis brittleness means (in percentage) ± 95% confidence intervals, averaged for two sampling times, for the genotypic contrasts considered.

Rachis brittleness (%) **Contrast**	**N[a]**	**Mean ± CI[b]**	**Groups[c]**
btr1 allele *vs. btr2* allele[3]			
btr1	689	16.96 ± 1.03	a
btr2	294	17.00 ± 1.91	a
Hybrids (*btr1*) *vs.* Hybrids (*btr2*)[4]			
*btr1*x*btr1*	145	18.08 ± 2.28	a
*btr2*x*btr2*	134	18.89 ± 2.82	a
Parents *btr1 vs.* Parents *btr2*[5]			
Parents *btr1*	544	16.65 ± 1.16	a
Parents *btr2*	160	15.41 ± 2.59	b
Time[7]			
2w[d]	647	20.82 ± 1.77	b
4w[e]	620	30.35 ± 1.91	a

[a]Number of spikes assessed within each level, [b] rachis brittleness means (in percentage) ± 95% confidence intervals, [c]means followed by the same letter in this column were not significantly different at P<0.05 in the analysis of variance. [d]Two weeks post-maturation, [e]four weeks post-maturation.

Regarding within groups variation, genotypes homozygous for *btr2* (both hybrids and parents) showed higher variability in brittleness than genotypes homozygous for *btr1* (F-test within *btr2 vs.* within *btr1* = 2.27, P=0.049).

4.3.3. Effect of time post-maturation on rachis fragility

We tested the influence of time after maturation on rachis brittleness. When considering the overall means for the whole set of genotypes, the percentage of rachis nodes disarticulated four weeks after maturation was significantly higher than after two weeks[7] (Table 4.5).

Furthermore, we found a significant interaction between brittle-type and time[8]. Both types increased their fragility with time. This notwithstanding, the increase in the percentage of disarticulated nodes over time for the brittle types doubled that of non-brittle types (Figure 4.3A). The contrast of the interaction of hybrids (non-brittle only) against parents by time was significant[9]. While hybrids (*btr*) and parents presented similar rachis fragility at the two weeks sampling, rachis brittleness at the four weeks sampling increased 15% for the non-brittle hybrids and only 5% for the parents (Figure 4.3B).

Finally, we tested the interaction of *Non-brittle rachis* genes as a whole (*btr1 vs. btr2*) with time, and of hybrids (*btr*) and parents, independently (Table 4.2). We found no significant interaction with time for any of these contrasts.

4.3.4. Spontaneous disarticulation under natural conditions

Spontaneous spikelet disarticulation was assessed in the field nursery for three hybrids (*btr1*x*btr2*, *btr1*x*btr1* and *btr2*x*btr2*) and their parents (Table S4.3). The weather during spike maturation was stormy and windy; therefore, the conditions were favourable for spike breakage.

We found significant differences in spontaneous disarticulation for brittle and non-brittle types (Table 4.6). Spikes of non-brittle plants were all intact, regardless of the time passed after maturation. On the contrary, we found broken spikes for some of the brittle type plants bagged starting from 3 weeks after Z91 (Figure 4.4). The fragments found were both big pieces and individual triplets. Because breakage occurs in the rachis, a single breakage results in total loss of the rest of the spike above that point. Moreover, spontaneous disarticulation rose with time in the brittle types.

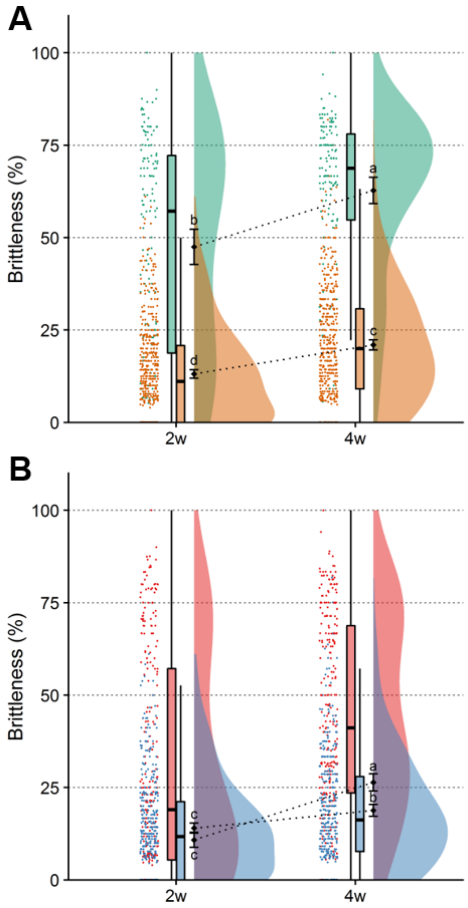

Figure 4.3. Genotype by time interaction. (A) Brittle-type by time interaction[8], in which "brittle" includes all hybrids *btr1*x*btr2*; "non-brittle" comprises hybrids *btr1*x*btr1*, hybrids *btr2*x*btr2*, parents *btr1* and parents *btr2;* and (B) hybrids (*btr*) *versus* parents by time interaction[9], in which "hybrids (*btr*)" includes hybrids *btr1*x*btr1* and hybrids *btr2*x*btr2*; "parents" comprises parents *btr1* and parents *btr2*. In both panels, raw measurement data points (left), boxplots with medians and interquartile range (centre), mean ± 95% confidence interval of rachis fragility in percentage and probability density distribution (right), are represented for each genotypic class, 2 and 4 weeks after maturation. Points with different letter are significantly different at P<0.05 according to means separation by LSD.

Table 4.6. Effects of genotype, time, genotype by time interaction, and contrasts on spontaneous disarticulation (the ratio of number of rachis nodes disarticulated to number of spikes inside the bag) in the field nursery.

Source of Variation	df[a]	SS[b]	MS[c]	F[d]	p-value[e]	
Genotype	7	0.824	0.118	6.78	3.02E-06	***
Brittle *vs.* non-brittle	1	0.819	0.819	47.14	1.57E-09	***
Time	2	0.110	0.055	3.16	4.79E-02	*
Repetition	8	0.104	0.013	0.75	6.49E-01	
Genotype*Time	14	0.879	0.063	3.61	1.40E-04	***
Residuals	76	1.320	0.017			

[a]Degrees of freedom, [b]sum of squares, [c]mean squares, [d]F-statistic. [e]*, **, *** factors significant at P<0.05, P<0.01, and P<0.001, respectively.

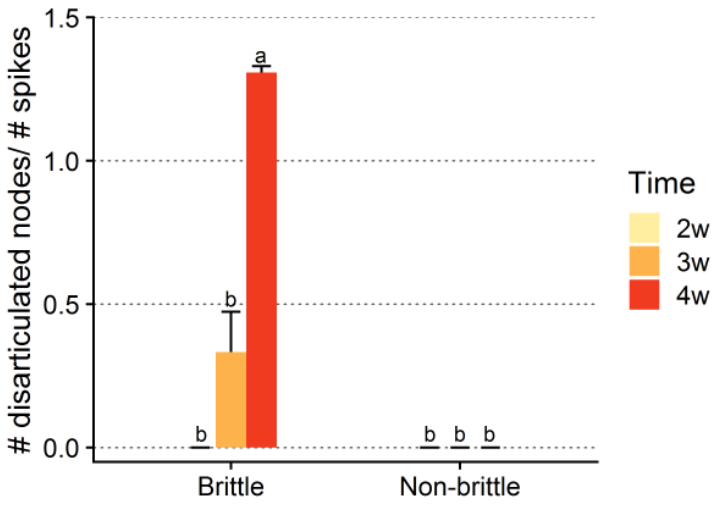

Figure 4.4. Spontaneous disarticulation on brittle and non-brittle types assessed 2, 3 and 4 weeks after ripening in field conditions. Spontaneous disarticulation was measured as the number of disarticulated nodes per number of spikes inside a plant bagged. "Brittle" includes all hybrids *btr1*x*btr2*; "non-brittle" comprises hybrids *btr1*x*btr1*, hybrids *btr2*x*btr2*, parents *btr1* and parents *btr2*. Error bars represent 95 % confidence interval. Bars with different letter are significantly different at P<0.05 according to the contrast performed for the overall ANOVA with transformed data.

4.4. Discussion

Rachis brittleness has been thoroughly studied for the understanding of barley origin and domestication process (Zohary 1999; Pourkheirandish et al. 2015). However, limited attention has been paid to the consequences this trait could have on current agriculture because it was not identified as a major problem until the recent development of hybrid cultivars.

We assessed rachis fragility in F_1 hybrids and parents from a breeding program, with different compositions at the *Non-brittle rachis* genes, both under controlled and field conditions. Moreover, we developed a fully standardized protocol for rachis brittleness assessment that could replace other operator-dependent methods (Komatsuda and Mano 2002; Nalam et al. 2006; Watanabe et al. 2006). A previously published method also made use of an electrical thresher (Jiang et al. 2014), but the blade modification and the optimization of time of operation of our study allow the replication of the method with complete reliability. To the best of our knowledge, this is one of few studies based on the possible impact of this trait on plant breeding.

4.4.1. Brittle rachis could limit the range of potential crosses for the development of barley hybrids

Rachis fragility was significantly higher in crosses bearing alternative mutations compared to genotypes carrying one of the deletions conferring the non-brittle phenotype, both in the mechanic

test and under natural conditions. These results agree with those obtained by Komatsuda et al. (2004), when evaluating rachis brittleness in F_1 plants from testcrosses between lines from a biparental population and two testers (one *btr1*, and the other *btr2*), as well as with those reported by Pourkheirandish et al. (2015) in test hybrids of cultivars from a world core collection. The latter study reported an average rachis fragility of 54.99% in brittle hybrids contrasting with 7.92% in non-brittle hybrids. Our analysis shows similar overall means for the brittle types (54.96%) and slightly higher values for the non-brittle types (16.97%), being this difference probably due to the higher aggressiveness of our phenotyping approach.

Furthermore, the morphology of the disarticulation scars between brittle (smooth) and non-brittle types (jagged), coincided with that already reported by Pourkheirandish et al. (2015) between a brittle wild barley accession (OUH602) and its non-brittle mutant (M96-1), as well as with difference between wild (*H. vulgare* ssp. *spontaneum*) and domesticated (*H. vulgare* ssp. *vulgare*) barley archaeological remnants (Zohary et al. 2012).

Finally, not only could we observe higher rachis breakage in the brittle types through mechanical test, but also spontaneous rachis disarticulation in a *Btr1btr1Btr2btr2* genotype when grown under natural conditions in the field nursery, but only four weeks after maturity (Z91). Senthil and Komatsuda (2005) detected no differences in rachis brittleness between greenhouse and field conditions, suggesting rainfall and temperature have no significant effects on rachis fragility. We only analysed one brittle hybrid under field conditions and, therefore, we cannot calculate a correlation with the experiment under controlled conditions.

Therefore, rachis fragility in hybrids derived from crosses of lines bearing alternative mutations in the *Non-brittle rachis* genes could jeopardize the efficient harvest of this type of hybrids and its acceptance in the market. This fact could reduce the choice of possible crosses for hybrid barley breeding.

4.4.2. The exploitation of certain potential heterotic patterns could be hampered by rachis brittleness

The success of hybrid barley breeding requires defining good heterotic patterns. Barley genetic diversity has not yet been explored from the point of view of finding heterotic patterns (Longin et al. 2012). However, considering that hybrid vigour is the result of the cross of genetically distinct germplasm groups (Melchinger and Gumber 1998), and that barley genetic differentiation has a geographic basis (Morrell et al. 2003; Muñoz-Amatriaín et al. 2014; Pasam et al. 2014; Poets et al. 2015; Russell et al. 2016), promising heterotic patterns between geographically isolated populations could arise (Melchinger and Gumber 1998).

There is an overlap between the geographical distribution of the *Non-brittle rachis* genes mutations and the geographical differentiation of barley. For instance, several authors have reported the genetic divergence of western and eastern barleys (Morrell and Clegg 2007; Saisho and Purugganan 2007; Morrell et al. 2014; Poets et al. 2015; Milner et al. 2018), and their intercross may give rise to promising genetic combinations. However, 'Occidental' barley lines mostly bear the *btr1* mutation, while the 'Oriental' lines mainly carry the *btr2* mutation (Komatsuda et al. 2004; Saisho and Purugganan 2007; Pourkheirandish et al. 2015; Pankin and von Korff 2017). Therefore, if a promising combination between predominantly *btr1* and *btr2* carrying pools was found, its exploitation could be prevented by the risk of grain loss when crossing barley lines with alternative mutations in the *Non-brittle rachis* genes.

4.4.3. Differences in rachis fragility within non-brittle rachis types could indicate a more complex genetic control of the rachis brittleness trait

The degree of rachis toughness in the non-brittle group can be variable (Åberg and Wiebe 1948). We also found rachis fragility variation within non-brittle types. On the one hand, non-brittle hybrids (*btr1*x*btr1* and *btr2*x*btr2*) showed higher percentage of rachis disarticulation than inbred parents. Although brittle rachis is well explained by a two complementary gene model, the existence of further genetic factors involved in the control of this trait cannot be ruled out (Smith 1951).

Several mechanisms involved in grain dispersal in the *Poaceae* probably coexist in barley. The brittle rachis character is specific to species within the *Triticeae* tribe, species that produce a spike-shape inflorescence (Chen et al. 1998; Nalam et al. 2006; Li and Gill 2006; Avni et al. 2017; Pourkheirandish et al. 2018). Intermediate seed dispersal mechanisms have also been described, as the "weak rachis", characterized for one or two rachis breaks, resulting in the loss of a spike segment (Kaufmann and Shebeski 1954). Brittle rachis, weak rachis and grain shattering (breakage of grains above the glumes within the rachilla (Sakuma et al. 2011)) have all been reported as dominant in barley (Kandemir et al. 2004). Schiemann (1921), concluded that, in addition to the brittleness factors B and R (now the genes *Btr1* and *Btr2*) of wild barley, at least another brittleness factor acting in the same direction existed. Kandemir et al. (2000) mapped a major QTL for weak rachis, *Hst-3*, on the short arm of chromosome 3H. Nonetheless, it was the QTL analyses performed by Komatsuda and Mano (2002) and Komatsuda et al. (2004) which represented a major step forward in the study of the genetic control of rachis brittleness. According to this latter analysis, non-brittle rachis of oriental lines would be controlled by the major gene *btr2* on chromosome 3H and two additional QTLs on chromosomes 5HL and 7H. An unlinked inhibitor

gene, designated D, was suggested for the QTL on chromosome 7H, preventing rachis fragility in its *dd* condition. Later, the dense spike 1 (*dsp1*) gene (Taketa et al. 2011) was identified as the candidate gene behind this QTL. It reduces spike internode length (increasing spike density), and is correlated with a lower degree of rachis fragility compared with normal (or lax) spikes (Takahashi and Yamamoto 1949). Lastly, Kandemir et al. (2004) also supported the complex inheritance of the rachis brittleness trait, concluding that there must be at least five genes involved. Besides *btr1* and *btr2* genes, and the D locus reported by Komatsuda and Mano (2002), two additional dominant factors affected brittleness, with the alleles for higher brittleness occurring in the *btr2* gene pool.

All these reports indicate that rachis brittleness is controlled by several genes interacting with each other to control the trait. Two of them are major genes, *btr1* and *btr2*. The hypothesis of additional dominant genetic factors described above agrees with the difference we found between non-brittle hybrids and inbred parents.

We found a broader dispersion range of brittleness values in the *btr2*x*btr2* hybrids (0–63%) in contrast to the *btr1*x*btr1* hybrids (0–50%). Pourkheirandish et al. (2015) reported similar results when assessing rachis fragility in F_1 plants derived from the cross of 274 cultivars from a world core collection and two testers (one *btr1* and one *btr2*). Their results also showed higher dispersion in the *btr2*x*btr2* hybrids (5 – 37%) compared to the *btr1*x*btr1* genotypes (2 – 17%). We used the test of homogeneity of variances of Bartlett to assess the heteroscedasticity between the *btr1* and *btr2* pools, both in Pourkheirandish et al.'s and in our own data. In both cases, variation within *btr2* non-brittle hybrids was significantly larger than within *btr1*x*btr1* hybrids. Likewise, in our results, rachis brittleness variation was significantly higher within all *btr2* genotypes (hybrids plus parents) compared to *btr1* genotypes. Again, this finding supports the existence of further genetic factors related to the control of rachis fragility with higher prevalence in the *btr2* pool.

4.4.4. Rachis brittleness changes over time and the response is higher in hybrid genotypes

Rachis fragility increased with time post-maturation. Nonetheless, the effect of time was higher on brittle types than on non-brittle ones, both under controlled conditions and in the field nursery test. This dissimilarity over time was presumably not identified before because previous surveys did not consider time after maturation as a factor (Komatsuda and Mano 2002; Komatsuda et al. 2004). However, this increase in rachis brittleness with time is in agreement with the increase of smooth scars observed by Snir and Weiss (2014) for several wild barleys, due to the gradual collapse of the thin cell walls around the 'constriction groove', detected in the brittle-types rachis nodes (Pourkheirandish et al. 2015).

Furthermore, we also found a significant effect of time on rachis brittleness in non-brittle hybrids compared to inbred parents (7.6% less brittleness for inbreds 4 weeks after maturation), once again, indicating possible additional dominant genes involved in the control of the trait. This effect could be linked to the specific *btr2* parents used in this study. In fact, Kandemir et al. (2004) suggested that dominant alleles at additional loci affecting brittleness (besides *btr* genes) might confer rachis fragility in hybrids and not in inbred lines.

We do not know whether this effect could lead to spike loss in production fields and, therefore, potential agronomic losses for hybrid barley, and is something that deserves further investigation. However, we observed no spike breakage in non-brittle hybrids in the field evaluation and, therefore, we cannot support a non-brittle hybrid disadvantage with field data.

4.5. Conclusions and further prospects

Rachis brittleness in hybrids from parents carrying alternative mutations in the *Non-brittle rachis* genes was significantly higher in relation to the rest of genotypes, confirming an actual risk of seed loss in hybrid cultivars with this particular gene combination. Therefore, the search of heterotic patterns for hybrid barley will have to take into account the *btr* genotype of the components of each heterotic group. This situation reduces the choice of possible crosses for hybrid barley breeding, and should be amended through pre-breeding approaches. Moreover, the higher percentage of rachis nodes disarticulated in non-brittle hybrids (*btr1*x*btr1* or *btr2*x*btr2*) compared to parents, indicates the existence of further dominant genetic factors involved in the control of the rachis brittleness trait, whose effect increases with time. This effect, however, was small, and we do not know if these differences in non-brittle genotypes will result in yield penalties in the field. The possible agronomic consequences should be assessed accordingly. The phenotyping method here described will facilitate screening for differences in rachis brittleness in cereals.

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4.7. Supplementary material

Table S4.1. Competitive allele-specific forward primers and common reverse primer designed based on *btr1* and *btr2* indels sequence.

Gene	Primer_AlleleFAM	Primer_AlleleHEX	Common Primer
Non-brittle rachis 1	GACTATGAAACC GGAGAGG	GCTGACTATGAAA CCGGAGAGC	TGACCCACGTCG AGCACGCAT
Non-brittle rachis 2	GTTCCAGGCCGT GCTGGG	GGTTCCAGGCCGT GCTGGT	CCCTGGACCTGG AGCCCGAT

Table S4.2. Means and 95% confidence intervals for rachis brittleness percentage, for each genotype and sampling time assessed in each block of the controlled conditions greenhouse experiment.

Genotype	Block	Time[a]	N[b]	Rachis brittleness (%) ± CI[c]
02V017-Z10	1	2w	10	8.91 ± 07.56
		4w	10	32.52 ± 21.19
02V017-Z10 x 93Z074-Z1	1	2w	2	0.00 ± 00.00
		4w	3	8.10 ± 18.22
02V017-Z10 x 97V115-Z7	1	2w	10	16.32 ± 08.09
		4w	10	24.16 ± 07.13
02V017-Z10 x Lavinia	1	2w	10	5.64 ± 06.19
		4w	10	31.01 ± 10.56
04Z001-Z107	1	2w	10	4.00 ± 05.01
		4w	10	3.17 ± 03.75
04Z001-Z107 x 93Z074-Z1	1	2w	10	4.27 ± 05.22
		4w	10	23.31 ± 08.28
93Z074-Z1	1	2w	10	11.90 ± 05.12
		4w	10	24.22 ± 05.67
97V115-Z7	1	2w	10	6.59 ± 04.52
		4w	6	12.99 ± 09.48
CIERZO	1	2w	10	9.51 ± 07.25
		4w	10	30.79 ± 13.06
CNE-106	1	2w	10	10.81 ± 08.52
		4w	10	18.72 ± 05.47
CNE-106 x CIERZO	1	2w	6	8.29 ± 09.64
		4w	4	17.83 ± 39.47
CNE-106 x ESTEREL	1	2w	10	1.50 ± 02.28
		4w	10	21.99 ± 06.85
CNE-110	1	2w	10	11.06 ± 04.15
		4w	8	27.02 ± 10.74
CNE-110 x CIERZO	1	2w	5	34.40 ± 18.16
		4w	4	46.27 ± 33.55
CNE-123	1	2w	10	1.77 ± 02.70
		4w	10	7.72 ± 06.82
CNE-123 x CIERZO	1	2w	10	6.26 ± 05.08
		4w	10	14.50 ± 08.31
CNE-126	1	2w	10	12.82 ± 06.26
		4w	10	9.27 ± 08.22

[a]2w, two weeks sampling; 4w, four weeks sampling; [b]number of spikes assessed for each combination of genotype, sampling time and block; [c]– missing data due to disease.

Table S4.2. (continued)

Genotype	Block	Time[a]	N[b]	Rachis brittleness (%) ± CI[c]
CNE-126 x ESTEREL	1	2w	10	8.23 ± 06.20
		4w	10	19.46 ± 09.35
CNE-135	1	2w	0	-
		4w	0	-
CNE-135 x PLAISANT	1	2w	0	-
		4w	0	-
CNE-138	1	2w	0	-
		4w	0	-
CNE-138 x CIERZO	1	2w	0	-
		4w	0	-
CNE-145	1	2w	10	8.90 ± 03.97
		4w	10	36.14 ± 11.42
CNE-145 x CIERZO	1	2w	10	8.66 ± 07.54
		4w	4	22.50 ± 28.52
CNE-145 x PLAISANT	1	2w	10	15.70 ± 08.97
		4w	10	46.69 ± 19.68
CNE-37	1	2w	10	0.71 ± 01.62
		4w	10	7.56 ± 03.37
CNE-37 x CIERZO	1	2w	0	-
		4w	0	-
CNE-49	1	2w	10	0.00 ± 00.00
		4w	10	7.66 ± 04.07
CNE-49 x CIERZO	1	2w	4	12.83 ± 26.68
		4w	1	-
CNE-58	1	2w	10	10.45 ± 04.15
		4w	10	12.40 ± 07.24
CNE-58 x CIERZO	1	2w	10	15.23 ± 08.22
		4w	10	55.51 ± 10.28
CNE-6	1	2w	10	5.78 ± 06.40
		4w	7	5.86 ± 07.88
CNE-6 x CIERZO	1	2w	10	13.54 ± 07.99
		4w	10	29.33 ± 12.83
CNE-73	1	2w	10	0.00 ± 00.00
		4w	10	9.46 ± 03.02
CNE-73 x CIERZO	1	2w	10	7.59 ± 07.65
		4w	10	24.03 ± 09.04
CNE-75	1	2w	0	-
		4w	0	-
CNE-75 x CIERZO	1	2w	10	0.00 ± 00.00
		4w	10	12.63 ± 04.94
CNE-79	1	2w	10	1.83 ± 02.78
		4w	9	29.66 ± 07.12
CNE-79 x CIERZO	1	2w	0	-
		4w	0	-
CNE-81	1	2w	10	0.00 ± 00.00
		4w	10	6.86 ± 05.45
CNE-81 x CIERZO	1	2w	10	16.81 ± 11.76
		4w	10	29.77 ± 07.56

[a]2w, two weeks sampling; 4w, four weeks sampling; [b]number of spikes assessed for each combination of genotype, sampling time and block; [c]– missing data due to disease.

Table S4.2. (continued)

Genotype	Block	Time[a]	N[b]	Rachis brittleness (%) ± CI[c]
CNE-89	1	2w	0	-
		4w	0	-
CNE-89 x CIERZO	1	2w	0	-
		4w	0	-
CNE-98	1	2w	10	8.21 ± 06.50
		4w	5	23.34 ± 19.87
CNE-98 x CIERZO	1	2w	10	15.50 ± 08.13
		4w	10	46.06 ± 09.71
ESTEREL	1	2w	10	10.95 ± 05.53
		4w	10	9.58 ± 04.34
Lavinia	1	2w	10	7.66 ± 05.32
		4w	10	18.38 ± 04.23
PLAISANT	1	2w	10	14.12 ± 05.71
		4w	9	23.76 ± 16.33
02V017-Z10	2	2w	10	23.05 ± 05.19
		4w	10	23.54 ± 05.43
02V017-Z10 x 97V115-Z7	2	2w	10	22.12 ± 06.37
		4w	10	34.85 ± 07.50
02V017-Z10 x Lavinia	2	2w	10	15.78 ± 04.41
		4w	10	31.15 ± 06.80
97V115-Z7	2	2w	10	25.28 ± 08.93
		4w	10	3.70 ± 03.36
CIERZO	2	2w	10	46.35 ± 05.24
		4w	10	40.04 ± 06.25
CNE-106	2	2w	10	26.74 ± 14.03
		4w	10	39.69 ± 12.05
CNE-106 x CIERZO	2	2w	10	76.37 ± 07.21
		4w	10	79.00 ± 03.09
CNE-110	2	2w	10	30.95 ± 09.10
		4w	10	23.98 ± 04.49
CNE-110 x CIERZO	2	2w	10	69.38 ± 08.64
		4w	10	73.24 ± 05.52
CNE-123	2	2w	10	17.97 ± 04.83
		4w	10	12.51 ± 04.52
CNE-123 x CIERZO	2	2w	10	8.50 ± 04.07
		4w	10	38.40 ± 06.79
CNE-138	2	2w	10	20.49 ± 05.64
		4w	10	14.64 ± 07.45
CNE-138 x CIERZO	2	2w	10	68.44 ± 08.00
		4w	10	69.32 ± 10.33
CNE-37	2	2w	10	25.18 ± 09.00
		4w	10	27.76 ± 06.15
CNE-37 x CIERZO	2	2w	10	60.82 ± 14.09
		4w	10	69.89 ± 05.02
CNE-49	2	2w	10	15.10 ± 05.46
		4w	10	17.74 ± 05.78
CNE-49 x CIERZO	2	2w	10	65.21 ± 08.90
		4w	10	75.44 ± 05.92

[a]2w, two weeks sampling; 4w, four weeks sampling; [b]number of spikes assessed for each combination of genotype, sampling time and block; [c]– missing data due to disease.

Table S4.2. (continued)

Genotype	Block	Time[a]	N[b]	Rachis brittleness (%) ± CI[c]
CNE-58	2	2w	10	15.42 ± 03.93
		4w	10	21.65 ± 04.09
CNE-58 x CIERZO	2	2w	10	73.48 ± 05.68
		4w	10	76.19 ± 04.41
CNE-6	2	2w	10	17.98 ± 07.37
		4w	10	14.99 ± 04.59
CNE-6 x CIERZO	2	2w	10	69.89 ± 08.37
		4w	10	68.92 ± 04.50
CNE-79	2	2w	10	16.71 ± 02.26
		4w	10	5.40 ± 04.57
CNE-79 x CIERZO	2	2w	10	43.04 ± 15.64
		4w	10	76.59 ± 05.97
CNE-89	2	2w	10	13.88 ± 06.16
		4w	10	4.67 ± 03.69
CNE-89 x CIERZO	2	2w	10	32.14 ± 09.75
		4w	10	44.83 ± 05.95
CNE-98	2	2w	10	28.90 ± 09.16
		4w	10	34.90 ± 06.90
CNE-98 x CIERZO	2	2w	10	74.56 ± 07.05
		4w	10	78.24 ± 05.66
Lavinia	2	2w	10	34.98 ± 05.68
		4w	10	34.73 ± 04.93

[a]2w, two weeks sampling; 4w, four weeks sampling; [b]number of spikes assessed for each combination of genotype, sampling time and block; [c]– missing data due to disease.

Table S4.3. Means and 95% confidence intervals for the spontaneous disarticulation of spikes, for each genotype and sampling time assessed in the field nursery test.

Genotype	Time[a]	N[b]	Spontaneous disarticulation ± CI
02V017-Z10	2w	5	0.00 ± 0.00
	3w	5	0.00 ± 0.00
	4w	5	0.00 ± 0.00
02V017-Z10 x Lavinia	2w	4	0.00 ± 0.00
	3w	4	0.00 ± 0.00
	4w	4	0.00 ± 0.00
CIERZO	2w	5	0.00 ± 0.00
	3w	5	0.00 ± 0.00
	4w	5	0.00 ± 0.00
CNE-123	2w	5	0.00 ± 0.00
	3w	5	0.00 ± 0.00
	4w	5	0.00 ± 0.00
CNE-123 x CIERZO	2w	4	0.00 ± 0.00
	3w	4	0.00 ± 0.00
	4w	4	0.00 ± 0.00
CNE-37	2w	4	0.00 ± 0.00
	3w	4	0.00 ± 0.00
	4w	4	0.00 ± 0.00

[a]2w, two weeks sampling; 3w, three weeks sampling; 4w, four weeks sampling; [b]number of plants assessed.

Table S4.3. (continued)

Genotype	Time[a]	N[b]	Spontaneous disarticulation ± CI
CNE-37 x CIERZO	2w	9	0.00 ± 0.00
	3w	4	0.17 ± 0.31
	4w	5	0.62 ± 0.71
Lavinia	2w	3	0.00 ± 0.00
	3w	3	0.00 ± 0.00
	4w	3	0.00 ± 0.00

[a]2w, two weeks sampling; 3w, three weeks sampling; 4w, four weeks sampling; [b]number of plants assessed.

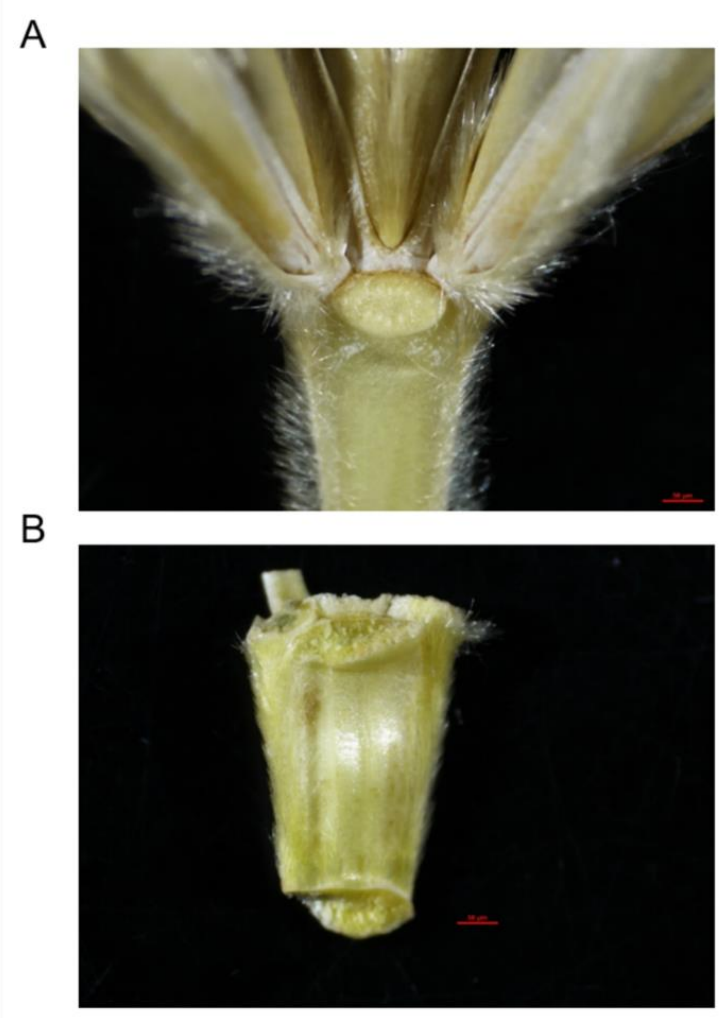

Figure S4.1. Disarticulation scars at one rachis node after the threshing procedure. (A) Smooth disarticulation scar from a brittle type spike (x20, scale bar: 50 μm). (B) Rough disarticulation scar from a non-brittle type spike (x20, scale bar: 50 μm).

Video S4.1. Rachis brittleness phenotyping. Assessment through mechanical processing of spikes in an adapted threshing machine equipped with cooking grade silicone toothed rotor blades. Video S3.1 can be found in the following link: https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2Fpbr.12776&file=pbr12776-sup-0003-VideS1.mp4

Chapter V. Hybrids provide more options for fine-tuning flowering time responses of winter barley

5. Chapter V. Hybrids provide more options for fine-tuning flowering time responses of winter barley

5.1. Introduction

Higher and more stable crop yields are the main targets for cereal breeders. This goal is increasingly challenging in temperate regions, where major crops face growing threats from the impact of climate change, particularly from drought and heat events at critical developmental milestones during the crop cycle (Olesen et al. 2010; Porter et al. 2014; Trnka et al. 2014). Tight coordination of plant cycle to environmental conditions to match resource availability with the most sensitive growth stages is crucial for crop adaptation (Craufurd and Wheeler 2009), and has a major effect on yield (Bolaños and Edmeades 1993; Evans 1996; González et al. 1999; Cockram et al. 2007b; Tondelli et al. 2014; Flohr et al. 2018; Wiegmann et al. 2019). In this context, the current variety formats for cultivation should be re-assessed, as they may no longer be the highest yielding ones. Further research on crop plasticity is necessary to adapt cereal crops to the range of future climatic conditions (Fatima et al. 2020). Winters in the temperate zone are projected to be warmer, so the vernalization requirement of current winter cultivars may be excessive, i.e., may not be met on time, due to a lower vernalizing potential of the environment (Saadi et al. 2015; Yang et al. 2019). Future ideotypes will have to combine specific vernalization and photoperiod responses fine-tuned to the projected climatic conditions prevalent for each region (Stratonovitch and Semenov 2015; Tao et al. 2017; Gouache et al. 2017). Allelic variation at the *VRN-H1* gene already induces a gradation of vernalization needs to the barley plants, which have had large impact on barley adaptation to regional climates (Casao et al. 2011b; Contreras-Moreira et al. 2019). Breeders must aim at deploying appropriate phenology gene combinations to optimize the crop foundation phase (vegetative and early reproductive), and construction phase (late reproductive) growth periods, as well as avoiding abiotic stresses at critical developmental stages, thus optimizing yield potential in target environments (Gouache et al. 2017).

Nowadays, there is growing interest in breeding hybrid cereal varieties, including barley. Hybrids have shown greater yield potential than inbred lines, due to exploitation of heterosis, greater yield stability under fluctuating environmental conditions, and the ease of pyramiding strategic combinations of dominant major genes (Longin et al. 2012; Mühleisen et al. 2013, 2014a). Therefore, optimizing phenology in hybrid cultivars is a strategy to improve yields under current and future climate conditions. However, there is lack of knowledge about flowering time gene action in a hybrid context.

Flowering time in barley is tightly regulated by genetic networks that respond predominately to day-length (photoperiod) and prolonged exposure to cold temperature (vernalization). Barley is a facultative long-day plant, flowering earlier under increasing day-lengths, and characterized by two major growth types: winter and spring. Winter barleys need vernalization for timely flowering (Campoli and von Korff 2014).

Vernalization genetic control is based on the epistatic system composed of flowering inducer *VRN-H1* (Yan et al. 2003; Trevaskis et al. 2003), and repressor *VRN-H2* (Yan et al. 2004b). *VRN-H1* corresponds to gene *HvBM5A*, orthologue of MADS box *AP1* from Arabidopsis (Trevaskis et al. 2007), whereas *VRN-H2* has no clear correspondence in Arabidopsis. Winter barleys carry the functional dominant *VRN-H2* allele, accompanied by a cold-sensitive *VRN-H1* allele. Activation of *VRN-H1* is quantitative, with longer cold treatments inducing higher levels of expression (von Zitzewitz et al. 2005; Sasani et al. 2009), which results in earlier transition to the reproductive phase (Sasani et al. 2009). It presents a large number of alleles, which are defined by the length of the first intron (11 kb in the wild-type *vrn-H1*), and present a gradation of responses to vernalization (Takahashi and Yasuda 1971; Szűcs et al. 2007), roughly proportional to the first intron length (Szűcs et al. 2007; Hemming et al. 2009; Casao et al. 2011a; Oliver et al. 2013; Guerra et al. 2021). Regarding the gene action of the *VRN-H1* allelic series, the accepted model states that the winter allele is recessive, while the rest are dominant (Takahashi and Yasuda 1971; Haas et al. 2020). *VRN-H3* (*HvFT1*) is the key flowering inducer that integrates the photoperiod and vernalization pathways (Yan et al. 2006; Faure et al. 2007; Kikuchi et al. 2009), whose expression is induced under long-day conditions and promotes flowering (Turner et al. 2005; Hemming et al. 2008), and is an orthologue of *FT1* in Arabidopsis (Yan et al. 2006). Ample allelic variation at *VRN-H3* has been described, arising from sequence polymorphisms in the promoter and first intron (Yan et al. 2006; Hemming et al. 2008; Casas et al. 2011, 2021), and copy number variation (Nitcher et al. 2013; Loscos et al. 2014), as summarized in Fernández-Calleja et al. (2021), but there is no information on its gene action. According to the currently accepted model, during autumn, when temperate cereals germinate, *VRN-H2* represses *VRN-H3* expression. During winter, vernalization induces *VRN-H1* expression, resulting in *VRN-H2* repression in leaves and, consequently, activation of *VRN-H3* transcription in spring, which promotes the transition from the vegetative to the reproductive stage (Trevaskis et al. 2006; Distelfeld et al. 2009a). At the whole plant level, this transition is visible as the appearance of the first node at the main stem, and the beginning of stem elongation (jointing stage). Besides the *VRN* genes, *HvODDSOC2* also plays a repressor role in the vernalization pathway (Greenup et al. 2010). This gene is the monocot orthologue of *Arabidopsis thaliana FLOWERING LOCUS C* (*FLC*, Ruelens et al., 2013). It is

downregulated by prolonged cold exposure, was identified as a binding target of the VRN1 protein in barley, together with *VRN-H2* and *VRN-H3* genes (Deng et al. 2015), and plays a repressor role in absence of full vernalization (Monteagudo et al. 2019b). Genes *PPD-H1* and *PPD-H2* control photoperiod sensitivity. *PPD-H1* (*HvPRR37*, orthologue of *PRR7* in Arabidopsis) is the major determinant of long photoperiod response in barley (Turner et al. 2005). Its activity causes an increased expression of *VRN-H3* after vernalization fulfilment, promoting flowering under long-day conditions (Turner et al. 2005; Campoli et al. 2012b; Mulki and von Korff 2016). *PPD-H2* (*HvFT3*), which belongs to the FT gene family, induces early reproductive development in short-day conditions, or even long-day conditions when vernalization requirements have not been fully satisfied (Laurie et al. 1995; Faure et al. 2007; Casao et al. 2011a, c; Mulki et al. 2018). Phylogeographic and genetic studies suggest an adaptive role for this gene in winter barleys (Kikuchi et al. 2009; Casao et al. 2011c).

Barley breeding for the near future requires understanding the genetic mechanisms of adaptation, including vernalization responses, in a hybrid context. This work is intended to explore the inheritance and effect of several major flowering genes in heterozygosis, and their dynamics in relation to insufficient vernalization. For this purpose, an experiment with different vernalization treatments was designed aiming to evaluate the phenology and gene expression of key genes in the plant cycle duration, in a set of hybrid barleys and their parents. Here we show that hybrid combinations extend the available catalogue of genetic responses to vernalization, opening new possibilities for optimizing phenology to specific areas using hybrids.

5.2. Material and methods

5.2.1. Plant material

Eleven barley (*Hordeum vulgare* L.) genotypes were used in this study: two female parents (Female A and Female B), 3 pollinators (Male 1, Male 2, and Male 3), and 6 hybrids derived from their crosses (Hybrid A1, Hybrid A2, Hybrid A3, Hybrid B1, Hybrid B2, and Hybrid B3). The female parents are cytoplasmic male sterile (CMS) inbred lines used in the development of 6-row winter barley hybrids for Europe by Syngenta AG. The pollinators are advanced inbred lines developed in the framework of the Spanish Barley Breeding Program (Gracia et al. 2012), well adapted to the Mediterranean conditions, and without fertility restorer genes. The resultant offspring are male-sterile hybrids (female hybrids, F_1F), an intermediate step in the production of a three-way hybrid, after further crossing with a fertility restorer genotype. F_1F seed was produced by Syngenta AG in isolation plots by open pollination of the CMS female line by the adjacent pollinator, planted in alternating strips. For maintenance of the CMS female parent, both its male-sterile and male-fertile

version (maintainer line) were planted in alternating strips. Plot sizes and amounts of seed used in these operations were not disclosed by the company.

The genotypes studied present different *VRN-H1* alleles, which are defined by the length of the first intron of the gene, and have different vernalization requirements, ranking from low to high cold needs. These alleles are *VRN-H1-4*, which presents a vernalization requirement of around 2 weeks, *VRN-H1-6* requires approximately 30 days, and *vrn-H1* requires not less than 7 weeks (Casao et al. 2011a). These alleles represent the main allelic diversity that is spread across Western European six-row winter barleys. The strict winter allele (*vrn-H1*) prevails in North-western European barleys, whereas alleles *VRN-H1-4* and *VRN-H1-6* correspond to the two largest germplasm groups found in Spanish barley landraces (Casao et al. 2011a). The geographical distribution of these two alleles coincides with the harshness of winters in the Iberian region (Yahiaoui et al. 2008; Casao et al. 2011a; Contreras-Moreira et al. 2019). *VRN-H1-4* predominates in Southern and coastal Spanish landraces, while *VRN-H1-6* is frequently found in the continental inlands of Spain. Besides, the genotypes studied also present different alleles at other major genes involved in the control of vernalization responses and day-length sensitivity (Table 5.1).

Table 5.1. Genotypes for the genes associated with responses to vernalization and photoperiod in the parent lines under study.

Parent lines	**Vernalization, photoperiod, and *earliness per se* genes**				
	***VRN-H1*[a]**	***VRN-H2*[b]**	***VRN-H3*[c]**	***PPD-H1*[d]**	***PPD-H2*[e]**
Female A	*vrn-H1*	*VRN-H2*	*vrn-H3d(1)*	*PPD-H1*	*ppd-H2*
Female B	*VRN-H1-6*	*VRN-H2*	*vrn-H3a(1)*	*PPD-H1*	*ppd-H2*
Male 1	*VRN-H1-4*	*VRN-H2*	*vrn-H3d(1)*	*PPD-H1*	*ppd-H2*
Male 2	*VRN-H1-6*	*VRN-H2*	*vrn-H3c(1)*	*PPD-H1*	*ppd-H2*
Male 3	*vrn-H1*	*VRN-H2*	*vrn-H3a(1)*	*PPD-H1*	*PPD-H2*

[a]Alleles based on the size of intron 1, following Hemming et al. (2009). [b]Presence (dominant)/absence (recessive) of *HvZCCT*, following Karsai et al. (2005). [c]Alleles based on two indels in the first 550 bp upstream of the start codon, two SNPs in intron 1, and CNV, coded as in Fernández-Calleja et al. (2021). *vrn-H3a*(1) = promoter deletion-insertion, intron 1 AG, CNV 1 copy; *vrn-H3c*(1) = deletion-insertion, TC, 1 copy; *vrn-H3d*(1) = insertion-deletion, 1 TC, 1 copy. [d]Alleles based on SNP22 of Turner et al. (2005), dominant = G, recessive = T. [e]Presence (dominant)/absence (recessive), as in Faure et al. (2007).

5.2.2. Plant growth conditions, phenotyping, and sampling

A study under controlled conditions was designed to assess differences in development and gene expression in key genes controlling the duration of the plant cycle, after three vernalization treatments: complete (8 weeks of cold period, V8), moderate (4 weeks, V4), and low (2 weeks, V2).

Genotypes were exposed to treatments of 14 (low), 28 (intermediate), and 58 days (full vernalization) at 6±2°C, under a short-day regime (8 h light/16 h dark). After vernalization, seedlings were transferred to a growth chamber with conditions set to long photoperiod (16 h

light/8 h night), 220 μmol m^{-2} s^{-1} light intensity, and 20ºC day/16ºC night temperatures. The duration of the vernalization treatments was set according to previous experiments. Fourteen days are enough for genotypes carrying the *VRN-H1-4* allele, 58 days are sufficient for cultivars with strict winter growth habit, and 28 days is an intermediate condition.

The study was carried out in two stages, growing plants independently for gene expression and phenotyping, using the same growth chamber. For phenotyping, plants were grown in trays of 12 cells (650 cc per cell) from sowing until flowering. The number of days to the appearance of the first node at the base of the main stem, or stage 31 on the Zadoks scale (Z31), and the number of days to awn tipping or Z49 (Zadoks et al. 1974) were recorded. Four plants were assessed for each genotype and treatment. After discarding dead plants and outliers, data from the best three plants per treatment were kept. The phenotyping experiment lasted 130 days.

The gene expression experiment was carried out in two batches due to growth chamber capacity, split according to the females, due to space limitations. One subset comprised the hybrids derived from Female A and respective parents (Batch A), and the other subset included the hybrids coming from Female B and respective parents (Batch B). Therefore, the three male parents were present in the two batches. Plants were grown in trays of 35 cells (200 cc per cell), from sowing until sampling. In the second stage (phenotyping), an experiment with undisturbed plants from all eleven genotypes were simultaneously assessed for developmental traits.

The last expanded leaf of four independent plants was sampled 17 and 35 days after the entry in the growth chamber (i.e., after the end of the respective vernalization treatment), 14 h into the light period (2 h before the end of the day). Samples were frozen in liquid nitrogen, homogenized (Mixer Mill model MM400, Retsch) and conserved at − 80 °C until RNA isolation. In principle, three plants were analysed. If they were clearly dissimilar, the fourth plant was also analysed, and the best three were kept for further analysis.

5.2.3. Gene expression analysis

RNA extraction was carried out using Total RNA Mini Kit for Plants (IBI Scientific) following manufacturer instructions. Total RNA (1 μg) was employed for cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen) and oligo (dT) 20 primer (Invitrogen). Real-time PCR quantification (ABI 7500, Applied Biosystems) was performed for samples from each time point and vernalization treatment. Four plants (biological replicates) per sampling time, treatment and genotype were sampled. Three biological replicates and two technical replicates were tested per sample and pair of primers (*VRN-H1*, *VRN-H2*, *VRN-H3*, *PPD-H1*, *HvODDSOC2*, and *PPD-*

H2). When outliers were detected, the fourth plant was also analysed, and the three replicates which showed the best agreement were kept. Primer sequences and conditions are specified in Table S5.1. Gene expression levels were normalized to *Actin* expression, considering primer efficiencies. The average of the two technical replications of ΔCt (Ct *Actin* – Ct target gene) was used as the experimental unit for statistical analyses, to protect against small pipetting errors.

5.2.4. Statistical analysis

Statistical analyses were carried out using R software (Team 2013). Differences in Z31, Z49, and the lag between the latter stages (Lag Z31-Z49) between genotypes and treatments were evaluated using the analysis of variance (ANOVA) procedure in R. The ANOVA model included genotype, vernalization treatment, and genotype by treatment interaction, all taken as fixed factors. The three plants sampled per genotype were considered biological replicates. The genotype factor was broken down into the most informative contrasts: hybrids *vs.* parents, females *vs.* males, and finally, hybrids from Female A *vs.* hybrids from Female B. Multiple comparisons were obtained by Fisher's protected Least Significant Differences (LSD) with the R package 'emmeans' (Lenth et al. 2018).

Using the same statistical procedure, differences in vernalization sensitivity for Z31, Z49 and lag Z31-Z49 between genotypes and treatments (V8-V4, V4-V2, and V8-V2) were tested. Vernalization sensitivity comparing V8 and V4 treatments was calculated by subtracting the value for an individual observation in the 8-week vernalization treatment from the mean value of that genotype in the 4-week vernalization treatment, for each suitable variable. The same procedure was followed for each variable considering the V4-V2 or V8-V2 treatments.

For gene expression results, Batch A and Batch B were analysed separately. The ANOVA model included genotype, treatment, sampling time, and factorial interactions. The analyses of variance were performed considering all factors (genotype, sampling time, and treatment) as fixed. The three biological replications were considered replicates. The contrasts defined were: hybrids *vs.* parents, hybrids *vs.* females, hybrids *vs.* males, and females *vs.* males. Means were compared using the least significant difference (LSD) test ($P < 0.05$).

A correlation network analysis was carried out with the R package 'qgraph' (Epskamp et al. 2012). We performed a multiple factorial analysis (Pagès 2002) using R packages 'FactoMineR' (Lê et al. 2008) and 'factoextra' (Kassambara and Mundt 2017). This method summarizes and displays a complex data table in which individuals are described by several sets of variables (quantitative and /or qualitative) structured into groups. It requires balancing the influences of each set of variables. Therefore, the variables are weighted during the analysis. Variables in the same group are

normalized using the same weighting value, which can vary from one group to another. In our case, we summarized the observations described by a set of variables structured into four groups (*Treatment*, *Genotype*, *Development,* and *Gene expression*). *Genotype* and *treatment* are groups based on categorical variables specifying the genotype identity of each individual and the vernalization treatment to which they were subjected. *Development* and *gene expression* quantitative variables were considered as active groups and their contribution was considered to define the distance between individuals. Each variable within a group was equally weighted, so the influence of each set of variables in the analysis was balanced.

Except for seed production, experimental work, phenotyping, gene expression, and statistical analysis were performed at EEAD-CSIC.

5.3. Results

Insufficient vernalization markedly extended the growth cycle of plants. At the complete vernalization treatment (V8), the duration of the two phases considered (time until Z31 and lag Z31-Z49) was rather similar. With increasingly insufficient vernalization, time to awn tipping (Z49) raised progressively. Most of this lengthening occurred in the period until first node appearance (Z31), although additional delays were observed in the late reproductive phase (lag Z31-Z49), particularly for the female genotypes (Figures 5.1, 5.2).

5.3.1. Vernalization response of parent and hybrid genotypes

Differences in the length of developmental phases between genotypes were also detected (Tables S5.2, S5.6). Male parents (Mediterranean adapted) were earlier than female parents. In general, hybrids showed an intermediate phenotype between both parents for days to reach the jointing stage (Z31), and days to awn tipping in all vernalization treatments. However, the length of the late reproductive phase of the hybrids was closer to that of the male parents (Figures 5.1, 5.2, Table S5.3).

Parents and hybrids presented different vernalization responses (Figure 5.2). This differential behaviour was mostly explained by changes in the duration of the phase until jointing, which was associated with the *VRN-H1* allele present (negative correlation indicated in Figure S5.1), and their dosage in the case of the hybrids. Increasing vernalization treatments minimized these differences between genotypes, especially in those triads (female, hybrid, male) where different vernalization alleles were crossed (Figure 5.2).

With complete vernalization (V8), all genotypes reached the Z31 stage in a similar range, although some differences were still evident. Male 1 and Male 2, carrying low (*VRN-H1-4*) and medium

(*VRN-H1-6*) vernalization requirement alleles, were the earliest until Z31. Winter parents (*vrn-H1*), Male 3 and Female A, and Female B (*VRN-H1-6*), showed intrinsic lateness, reaching Z31 stage ten days later than the other male parents did. All six hybrids reached first node appearance at approximately the same time, independently of their *VRN-H1* allele, and significantly earlier than their female parents (Figure 5.2).

When vernalization was moderate (V4), the differences in days to first node appearance between genotypes were accentuated (Figure 5.2). Male 1 (*VRN-H1-4*) maintained a short Z31 phase, only 8 days longer compared to V8. Male 2 (*VRN-H1-6*) and Male 3 (*vrn-H1*) were more affected by the reduction of the cold treatment, although they only delayed their Z31 date around 15 days compared to the V8 treatment. The female parents, in contrast, experienced a remarkable delay in days to first node appearance, particularly large for the winter Female A (40 days, Figure S5.2). Interestingly, both Male 3 and Female A carry the strict winter *vrn-H1* allele, but they differed almost 30 days at Z31 for the V4 treatment (Figure 5.2). This finding indicates that early alleles of Male 3 at genes other than *VRN-H1* are having a shortening effect on the Z31 phase. This gene could be *PPD-H2*, as will be discussed later. For hybrids, in general we observed intermediate phenotypes between the behaviours of their parents. Hybrids A1 and B1, carrying one copy of the *VRN-H1-4* allele, were the least affected by the reduction in the cold treatment, reaching Z31 earlier than any other hybrid. Hybrids from Male 2 and Male 3 showed a longer delay in Z31 with reduced vernalization (Figure 5.2).

When vernalization was low (V2), the Z31 phase was prolonged more than 15 days for most genotypes (compared to V4). The exception were the genotypes carrying the *VRN-H1-4* allele, which reached Z31 considerably earlier than the rest of the genotypes (Figure 5.2), and had a low vernalization sensitivity (Figure S5.2). Winter genotypes suffered the largest changes in time until Z31 when comparing V4 and V2, particularly Male 3 and its crosses. The differences between the females were even more pronounced in this treatment (V2), Female A reached Z31 thirty days later than Female B (Figure 5.2). This agrees with the alleles that these genotypes carry at *VRN-H1*. Female A carries the winter allele (*vrn-H1*), characterized by a higher sensitivity to vernalization than the *VRN-H1-6* allele of female B. This dissimilarity in Z31 dates between females translated into differences in Z31 duration also between A and B hybrids (Figure 5.2). For instance, Hybrid A2 (*vrn-H1*/*VRN-H1-6*) delayed the jointing stage 16 days more than Hybrid B2 (*VRN-H1-6*/*VRN-H1-6*) when reducing the vernalization treatment from 4 weeks to 2 weeks (Figure S5.2), indicating a dosage effect of winter *VRN-H1* alleles. Particularly interesting in this treatment is the change in ranking observed in the slower developing parents. Male 3, which bears the active allele

at *PPD-H2*, reached Z31 earlier than both female parents under moderate (V4) to complete (V8) vernalization. By contrast, at V2, Male 3 (*vrn-H1*) delayed significantly its early development, reaching the Z31 stage later than Female B (*VRN-H1-6*) and almost at the same time as Female A (*vrn-H1*) (Figure 5.2). The striking reduction in time to jointing of Male 3 and Hybrid B3 with moderate vernalization, but not in the low vernalization treatment, agrees well with the hypothesis that *PPD-H2* needs some cold to come into play (Monteagudo et al. 2019b).

In summary, the Z31 phase was clearly the most sensitive period to the cold treatment. Vernalization sensitivity differed between genotypes (Tables S5.2, S5.7), and seemed related to the *VRN-H1* allele present and proportional to the *VRN-H1* allele dosage. Genotypes carrying the low vernalization requirement allele *VRN-H1-4*, reached the jointing stage earlier than the rest of the genotypes regardless of the vernalization treatment. In contrast, genotypes carrying winter *vrn-H1* alleles increased steeply the time to reach Z31 stage in the low vernalization treatment. Genotypes with a *VRN-H1-6* allele delayed jointing stage under insufficient vernalization, but not as much as strict winter types. We recoded *VRN-H1* alleles as a categorical variable with integer numbers roughly proportional to their associated vernalization response (1-2-3, for *VRN-H1-4*, *VRNH1-6*, and *vrn-H1*, respectively). We calculated a synthetic vernalization score by adding the values for the two alleles carried by each genotype. A regression of vernalization sensitivity (in this case, the difference V8-V2 for Z31) on the genotypic score produced a very good fit (Figure 5.3), supporting the dosage effect of *VRN-H1*.

The differences observed for the jointing phase were maintained until awn tipping, but modulated by the effect of other genes on the late reproductive phase, likely *VRN-H3* and *PPD-H2* (Figure S5.1). Male 1 and its crosses showed a constant and rather long lag Z31-Z49 phase across cold treatments. Conversely, Male 2 and its hybrids stood out for a consistent short late reproductive phase across treatments, probably because they carry the fast *vrn-H3c(1)* allele at *VRN-H3*. This additional precocity allowed them to be the fastest genotypes in reaching Z49 in V8, among parents and hybrids respectively, even earlier than *VRN-H1-4* carriers. Male 3 and its crosses also showed a particularly short lag Z31-Z49 phase, but only at V8. Interestingly, female parents differed in their late reproductive phase duration patterns. Female A showed a constant duration of the late reproductive phase across treatments, whereas Female B showed a lag Z31-Z49 phase increasingly long with decreasing vernalization treatments (Figure 5.2). This observation was not translated into the hybrids, as they more closely resembled their male parents in the duration of the late reproductive phase.

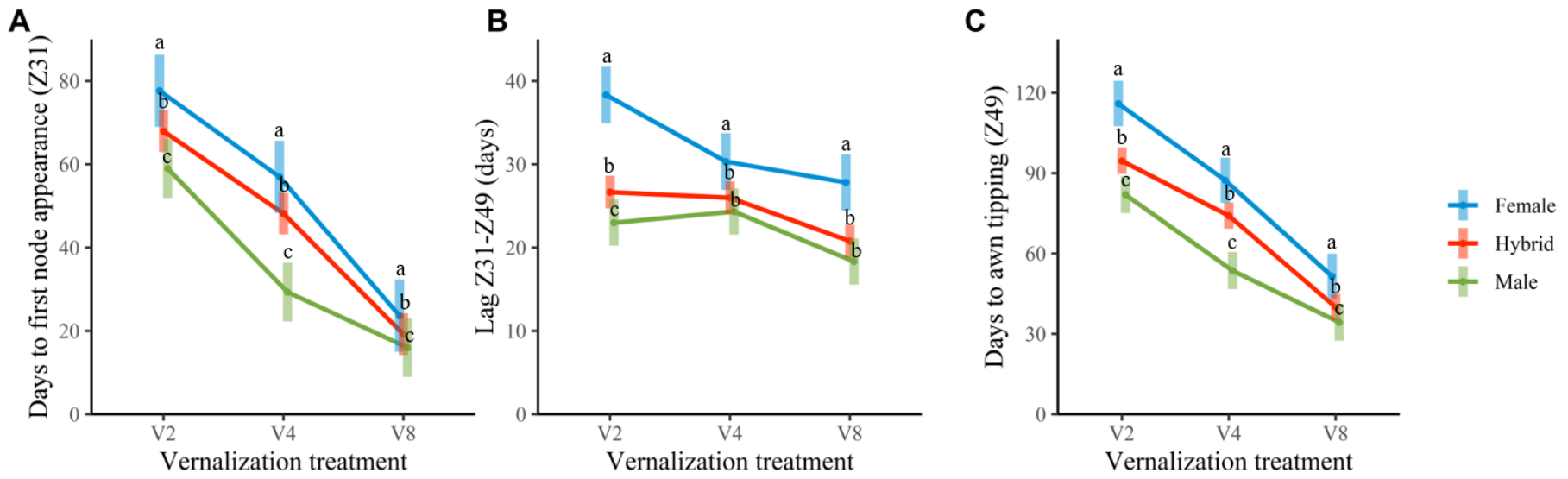

Figure 5.1. From left to right, average duration of time until Z31, lag Z31-Z49 and total time until Z49 for the three groups of genotypes, male parents, female parents and hybrids. Error bars are 95% confidence intervals. For each developmental variable and treatment, group means with a different letter are significantly different at $P < 0.05$ according to the contrasts performed for the overall ANOVA.

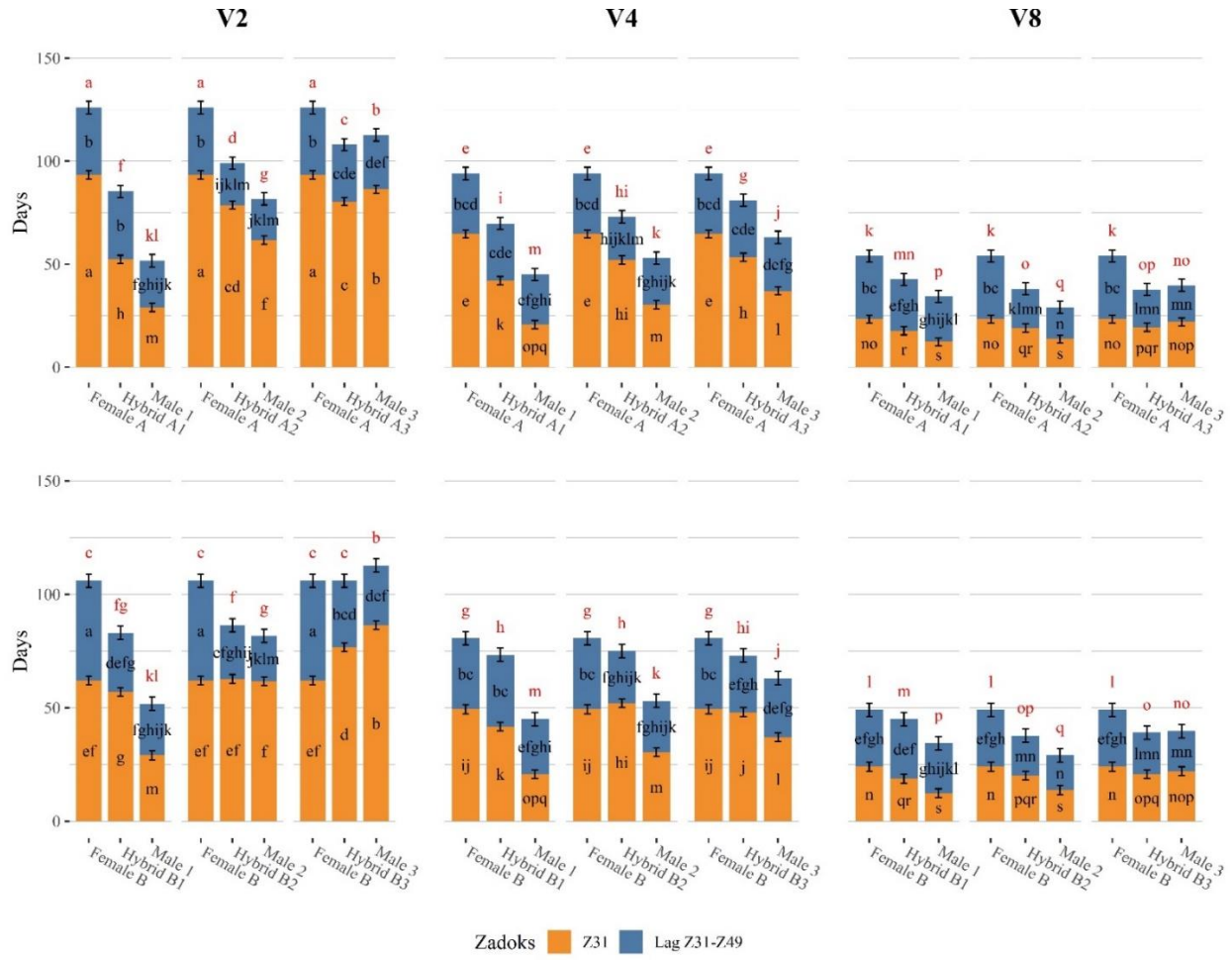

Figure 5.2. Developmental differences of triads of genotypes (Female, Hybrid, Male) grown under 16 h light, in response to different vernalization treatments (V2: 2 weeks of vernalization, V4: 4 weeks of vernalization, V8: 8 weeks of vernalization; 4-8 °C, 8 h light). Days to first node appearance (Z31) are represented as the height of the orange bars, days to awn tipping (Z49) are represented as the total height of the bars, and the lag period between Z31 and Z49 (LagZ31-Z49) is represented as the segment in blue. Genotypes from Batch A are shown in the upper part of the graph, whereas genotypes from Batch B are shown in the bottom part of the graph. Each of the three columns of facets represents a vernalization treatment, low on the left, moderate in the middle, and complete on the right. Each subplot within each facet contains one triad of genotypes composed of one female parent on the left, the male parent on the right, and the hybrid in the middle. Error bars are 95% confidence intervals for Z31 and LagZ31-Z49. For each developmental variable, bars with a different letter are significantly different at $P < 0.05$. Letters in red represent the means comparison for Z49.

Focusing on inheritance, hybrids derived from Male 1 and Male 2 showed an intermediate phenotype for Z31 and Z49, between the early male parents and the late female parents, in all vernalization treatments. Nevertheless, the lag Z31-Z49 phase of Male 2 hybrids was as short as that of their male parent (Figure 5.2), indicating dominance of the Male 2 early allele controlling this phase. Hybrids A3 and B3, in contrast to the other hybrids, did not show a consistent intermediate phenotype between their parents. In the low and complete vernalization treatments, hybrids A3 and B3 headed Z49 as early as the early parent, due to a dominant short late reproductive phase similar to that of Male 3 (Figure 5.2).

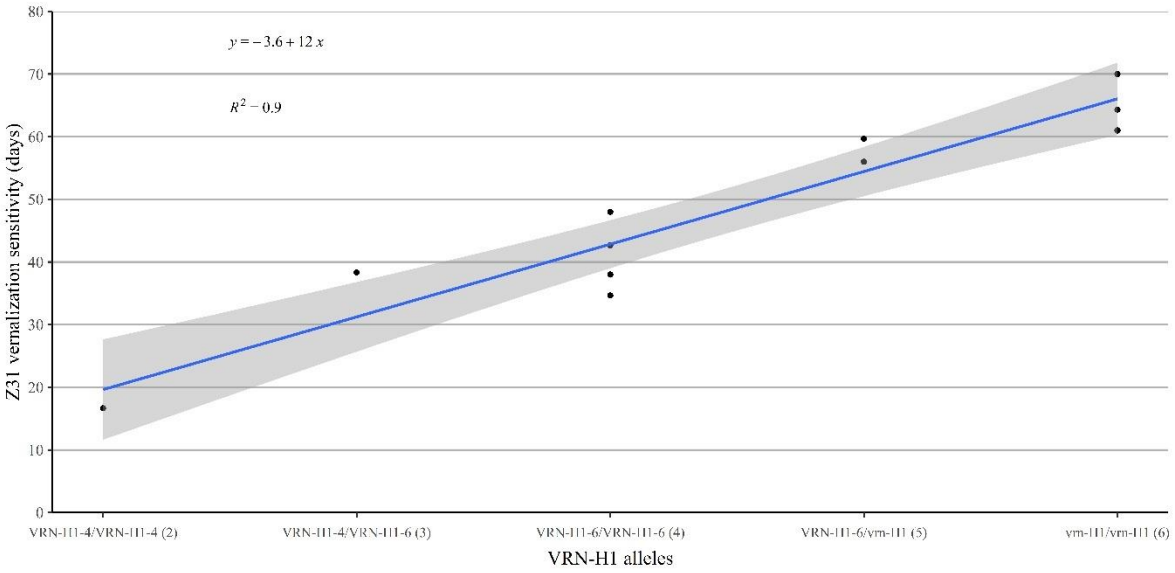

Figure 5.3. Regression of vernalization sensitivity (duration until Z31 at V2 minus duration until Z31 in V8) on allelic constitution at *VRN-H1*, coded as 1 (*VRN-H1-4*), 2 (*VRN-H1-6*), or 3 (*vrn-H1*), proportional to the vernalization requirement induced by each allele. The number within brackets indicates the vernalization requirement score of the genotype, according to their *VRN-H1* alleles.

5.3.2. Gene expression

Differences among genotypes were detected for the expression of all genes tested (Tables S5.4, S5.5, S5.8, S5.9).

In all genotypes, *VRN-H1* expression increased gradually with increasing duration of vernalization (Figures 5.4, S5.3), although differences between *VRN-H1* alleles were evident. Male 1 and its hybrids, carrying *VRN-H1-4*, were the only genotypes showing upregulated *VRN-H1* expression after just two weeks of vernalization. After vernalization for 4 weeks, *VRN-H1* expression also reached high levels in Male 2 and Hybrid B2, both carrying the medium vernalization requirement allele *VRN-H1-6*. The rest of the parents and hybrids required 8 weeks of cold to show high *VRN-*

H1 transcript levels (Figure 5.4A, D). However, not all differences in *VRN-H1* expression levels were due to allelic differences. For instance, both Female B and Male 2 carry the *VRN-H1-6* allele, but present different *VRN-H1* expression at V4 (Figure 5.4A, D). Hybrid B2 had the same (higher) *VRN-H1* expression as Male 2, indicating higher repression of *VRN-H1* in Female B, which is lost in the hybrid. In general, *VRN-H1* expression levels paralleled plant development patterns across genotypes and treatments.

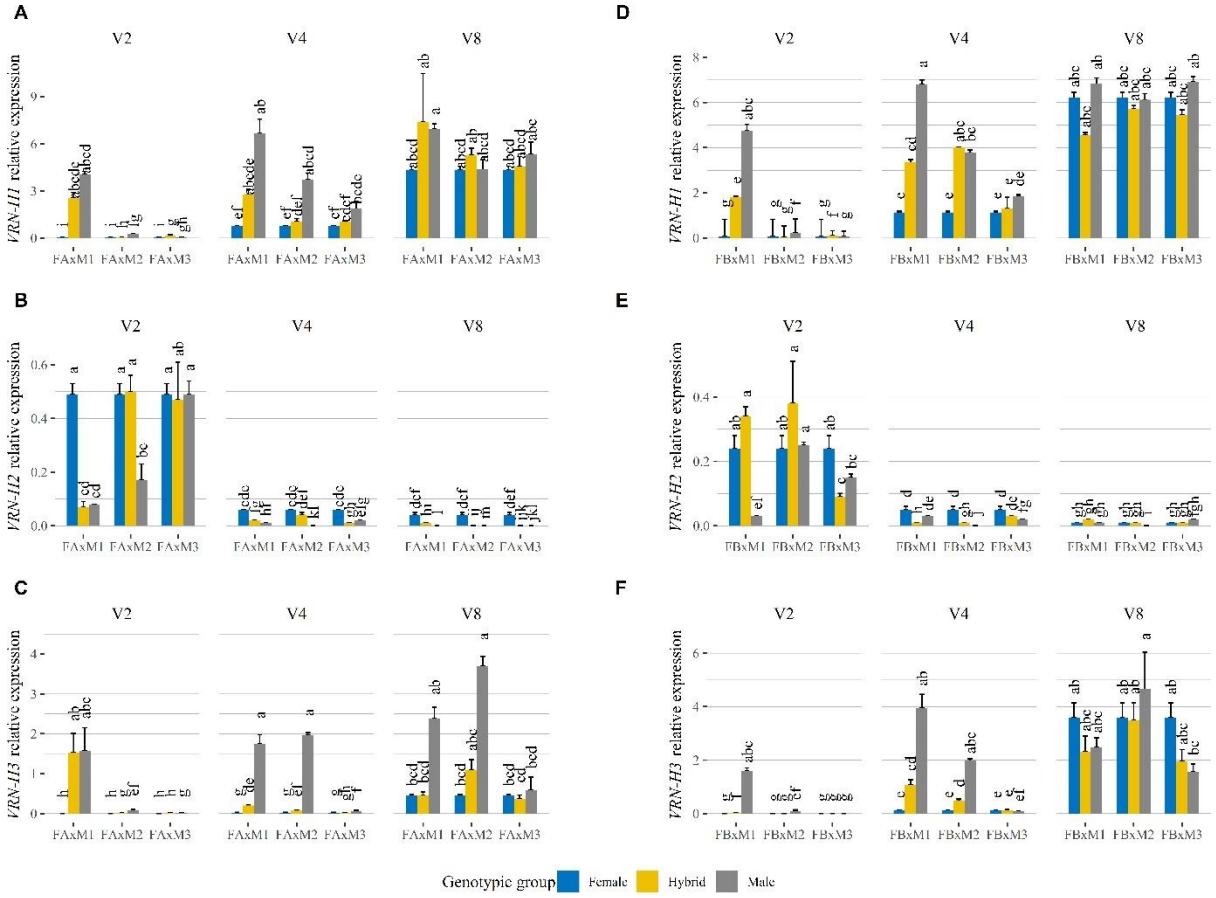

Figure 5.4. Relative expression levels, at 35 d of growth in each treatment, of *VRN-H1* (A, D), *VRN-H2* (B, E) and *VRN-H3* (C, F) assayed by qRT-PCR in triads of barley genotypes (Female, Hybrid, Male) grown under 16 h light, in response to different vernalization treatments (V2: 2 weeks of vernalization, V4: 4 weeks of vernalization, V8: 8 weeks of vernalization; 4-8 °C, 8 h light). Plots A, B, and C correspond to Female A crosses (Batch A). Plots D, E, and F correspond to Female B crosses (Batch B). Each plot is divided into three facets, each of them containing gene expression assayed for one vernalization treatment, and the three triads of genotypes composed of one female parent in blue, the male parent in grey, and the hybrid in yellow. The triads are represented as abbreviations of the crosses between the parents, e.g., FAxM1: Female A x Male 1. The results shown are normalized to the level of the housekeeping gene *Actin* for each genotype and treatment. Mean of 3 biological replicates. Error bars represent the SEM. For each gene and batch, bars with a different letter are significantly different at $P < 0.05$, according to ANOVA that included genotypes and all treatments.

When vernalization was complete, all parents and hybrids showed a high *VRN-H1* expression, probably caused by saturating vernalization requirements. With incomplete vernalization, however, *VRN-H1* expression in hybrids was, in general, intermediate between their parents (Figure 5.4A, D). Additive gene action caused by a dosage effect of *VRN-H1* was visible at gene expression level, supporting the hypothesis that the duration of the phase until jointing is related to the effect of this gene. This was apparent when comparing Hybrid A2 (*vrn-H1*/*VRN-H1-6*), which did not peak until V8 (Figure 5.4A), with Hybrid B2 (*VRN-H1-6*/*VRN-H1-6*), in which *VRN-H1* expression peaked already with 4 weeks of vernalization (Figure 5.4D).

All lines carried the active *VRN-H2* allele, but differences in its expression were observed (Figure 5.4B, E). As expected, *VRN-H2* expression decreased with increasing duration of the vernalization treatment, and inversely correlated to the expression of *VRN-H1*. A similar trend was observed for *HvODDSOC2* (Figure S5.4B, E). Nevertheless, there were some differences in *VRN-H2* expression among genotypes carrying the same winter *VRN-H1* allele. Indeed, at V4 and V8 treatments, the repression of *VRN-H2* in Male 3 and Hybrid A3 was higher than in the Female A (Figure 4B), despite all being winter types. This result agrees with an antagonistic relationship between *VRN-H2* and *PPD-H2* (present only in Male 3 and its hybrids).

VRN-H3 expression increased with increasing duration of vernalization (Figure 5.4C, F), and followed closely that of *VRN-H1*. There were no apparent differences in expression between *VRN-H3* alleles. Again, we could observe differences in expression among the two winter parents (Female A and Male 3), with Male 3 showing the highest *VRN-H3* expression in all vernalization treatments.

PPD-H1 expression was consistently high across vernalization treatments and genotypes. All genotypes assessed in the experiment carry the photoperiod sensitive *PPD-H1* allele (Figure S5.4D).

Expression of *PPD-H2* was detected in all genotypes that carried the gene, i.e., Male 3, Hybrid A3, and Hybrid B3 (Figures S5.4, S5.5). *PPD-H2* expression was detected after 4 and 8 weeks of cold, but not in the 2-week vernalization treatment, confirming that a cold period is needed to induce its expression in winter genotypes. Expression in Male 3 and Hybrid B3 in the V4 and V8 treatments was similar, indicating dominance of the active *PPD-H2* allele (Figure S5.5F).

5.3.3. Associations between developmental phases and flowering time genes expression

We performed a multiple factorial analysis (MFA) to examine patterns of relationships between developmental phases and gene expression averaged over the two sampling dates (Figure S5.6). The expression of flowering inducers *VRN-H1*, *PPD-H1* (ns), *VRN-H3*, and *PPD-H2* showed a negative correlation with the length of developmental phases (Figures 5.5, S5.1), i.e., higher expression of gene inducers was related to earliness. On the contrary, the expression of flowering repressors *VRN-H2* and *HvODDSOC2* showed a positive correlation with the length of developmental phases and negative correlations with the flowering inducers, positioned in the opposite semicircle (Figure 5.5). The first dimension, accounting for almost 60% of the variance, summarized the time to reach the jointing stage and awn tipping, and the expression of the regulators involved in the control of the length of these periods, i.e., vernalization genes *VRN-H1* and *VRN-H2*, and *HvODDSOC2* and *VRN-H3*. The closeness of Z49 and Z31 vectors is explained by a correlation coefficient of 0.98 between the two variables. The duration of the lag Z31-Z49 had large loadings on the two dimensions, and was relatively independent of the duration until Z31. The second dimension (12% of the variance) was related to the duration of the lag Z31-Z49, the expression of *PPD-H2* and, to a lesser extent, *VRN-H2* and *HvODDSOC2*, reflecting the negative correlation between *PPD-H2* expression and the duration of the lag Z31-Z49. The variable *PPD-H1* was located close to the origin, indicating a poor representation on the factor map.

When plotting the individuals in the MFA, we could observe that the first axis mainly opposed the genotypes in the V8 and V2 treatments (Figure S5.7). The genotypes Female A and Hybrid A3, carrying winter alleles at *VRN-H1*, showed the highest positive coordinates in the x-axis, which were positively correlated with a longer Z31 phase and a lower expression of *VRN-H1*. In contrast, genotypes characterized by a lower vernalization sensitivity, Male 1 and Male 2, showed the lowest negative coordinates, indicating shorter periods until the first node appearance and higher expression of *VRN-H1*. The second axis was essentially associated with genotypes Hybrid A2, Hybrid B3, Male 2, and Male 3, characterized by a short lag Z31-Z49 phase and low values of *VRN-H2* expression. On the opposite side of this axis, genotypes Female B and Hybrid A1 showed a consistently long late reproductive phase.

We detected variation in the length of the late reproductive phase, and its response to vernalization, which seemed related to the *VRN-H3* allele. We observed that those genotypes carrying the *vrn-H3c(1)* allele presented a short late reproductive phase independently of vernalization. Male 2 and its hybrids carry this allele and are represented by horizontal ellipses located in the positive side of

the y-axis of the individuals' MFA for genotypes (Figure S5.8). In contrast, those genotypes carrying one or two copies of the *vrn-H3d(1)* allele (Female A, Male 1, and derived hybrids) showed a constant and long duration of the late reproductive phase across treatments, and their ellipses are located in the negative coordinates of the y-axis (Figure S5.8). Besides, Male 1 and Hybrid A1 were represented by small ellipses, pointing out a reduced variance in their responses across vernalization treatments. In contrast, Hybrid B3 showed a more vertical distribution, indicating higher variance for the second axis, related to lag Z31-Z49 phase, *PPD-H2,* and *VRN-H2* expression (Figure S5.8).

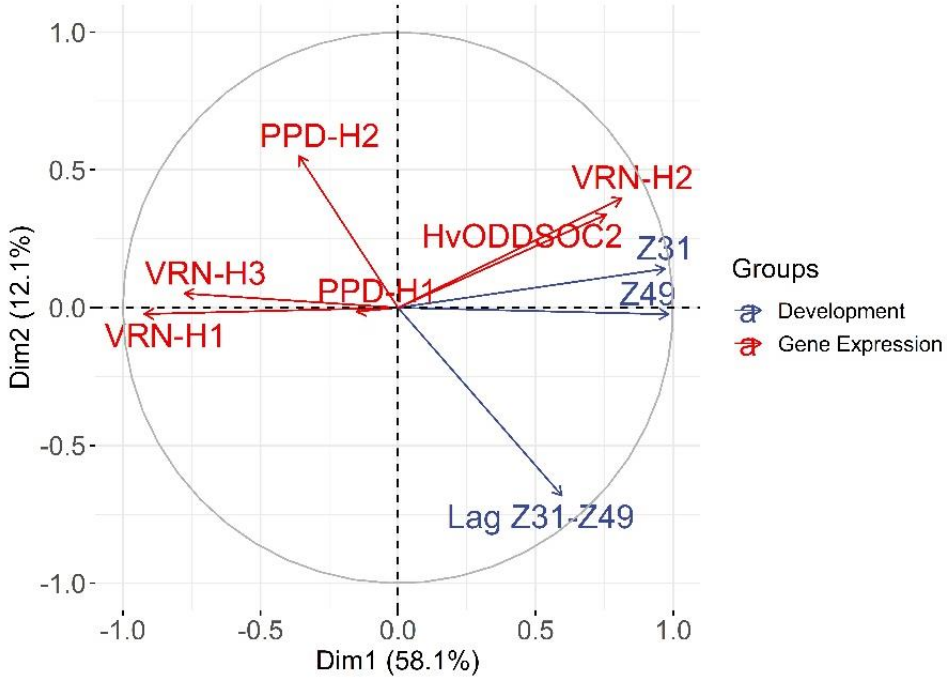

Figure 5.5. Multiple factorial analysis (MFA), variable correlation circle. The plot shows the correlation of the quantitative variables with the MFA axes. Variables related to developmental phases are depicted in blue and variables related to gene expression are depicted in red. The expression of flowering promoters *VRN-H1*, *PPD-H1* (ns), *VRN-H3*, and *PPD-H2* shows negative correlation with the length of developmental phases. On the contrary, the expression of flowering repressors *VRN-H2* and *HvODDSOC2* shows a positive correlation with the length of developmental phases and negative correlations with the promoters, positioned in the opposite semicircle. The first dimension represents mainly the time to reach the jointing stage and the genes that influence it, whereas the second dimension is more related to the late reproductive phase.

5.4. Discussion

Climate change is challenging current agricultural practices, posing questions about the best combinations of genotype x environment x management options for the near future (Cooper et al. 2021). Sheehan and Bentley (2021) recently pointed out the need for greater flexibility in varietal flowering time to sustain UK wheat productivity, a view that can be easily extended to barley, and

to other geographical areas. Other adaptation strategies that extend the catalogue of possibilities include shifts in the sowing date. Recent studies suggest shifting towards earlier sowings to offset climate change impacts and increasing cereal yields in the future scenario (Zheng et al. 2012; Hunt et al. 2019). Phenological adjustment of barley hybrids requires acquiring detailed knowledge of the functioning of major flowering time genes in heterozygosis. Our experiment supposes a first step in this direction. We exposed a set of hybrids and their parents to a range of vernalization conditions, which has provided some new insights for phenology management in hybrid barley breeding.

5.4.1. Flowering time in hybrids is intermediate between parents

The Mediterranean-adapted lines used as pollinators were earlier than the central European elite lines used as female parents, whereas the hybrids were intermediate. This was true for both Z31 (jointing) and Z49 (awn tipping) stages, across all vernalization treatments, indicating the presence of additive inheritance. Additivity was not complete, however, as hybrids reached the Z31 stage significantly later than the average of parental lines (1.33 days, p-value<0.001). In contrast, hybrids headed significantly earlier than the parental average (1.21 days, p-value<0.01), due to a shorter late reproductive phase of hybrids, compared to the parental average (2.55 days, p-value<0.001). The addition of the two phases resulted in slight heterosis towards earliness for Z49, which is a common finding in cereals, as reported for wheat (Borghi et al. 1988; Barbosa-Neto et al. 1996; Ahmed et al. 2000; Corbellini et al. 2002; Dreisigacker et al. 2005; Longin et al. 2013; Zhao et al. 2014; Al-Ashkar et al. 2020), triticale (Oettler et al. 2001), and barley (Zali and Allard 1976; Oury et al. 2000; Bernhard et al. 2017). However, we revealed a distinct gene action at each developmental stage, with prevalence of additivity in the foundation phase (up to jointing), and a trend towards dominance for earliness in the construction phase. This was not unexpected, as the different genetic control of the length of the preanthesis phenological phases in winter cereals is well supported by strong experimental evidence (Slafer and Rawson 1994; Miralles and Richards 2000; González et al. 2002; Gol et al. 2017; Ochagavía et al. 2018).

5.4.2. Vernalization mostly affects the foundation growth period, which is controlled by allelic variation at VRN-H1/VRN-H2 genes

The vernalization treatments reduced the duration of the time until Z31 (72% on average, comparing V8 with V2), and lag Z31-Z49 (23% on average). Therefore, the sensitivity to vernalization mostly affected the vegetative and early reproductive phases, largely in agreement with the literature (Flood and Halloran 1984; Griffiths et al. 1985; Roberts et al. 1988; Slafer and Rawson 1994; Whitechurch et al. 2007), although strong effects of vernalization on the duration

of the construction phase have also been reported (González et al. 2002). We observed this last effect only for the females.

Despite testing very different genotypes, the agreement between phenological development and gene expression supported our assumption that the range of responses to vernalization can largely be traced to the effect of the alleles present in *VRN-H1*. The multifactorial analysis indicated that the length until the reproductive transition (Z31) was associated with the pattern of expression of *VRN-H1* and *VRN-H2* genes, whose epistatic interaction controls the response to vernalization (von Zitzewitz et al. 2005). A novel finding of this study is that the length of the cold treatment needed to induce the expression of *VRN-H1*, as well as the degree of promotion towards flowering, depended on the allele constitution and dosage at *VRN-H1*. The dynamics of expression of *VRN-H1* alleles in the parents responded to the expectations of the gradual vernalization requirements induced by the three alleles. Thus, parents carrying the *VRN-H1-4* allele showed higher *VRN-H1* expression and accelerated development after just 2 weeks of cold; *VRN-H1-6* parents needed at least 4 weeks to reach the same point; whereas those carrying the *vrn-H1* allele required 8 weeks. The comparisons between homozygotes (parents and hybrids) indicated gradually decreasing vernalization requirements induced by alleles *vrn-H1*, *VRN-H1-6* and *VRN-H1-4*, which confirms the gradation in the strength of flowering promotion displayed by the allelic series at *VRN-H1* (Takahashi and Yasuda 1971; Szűcs et al. 2007; Casao et al. 2011a).

5.4.3. Additive inheritance of VRN-H1 winter alleles

The use of parental lines with different *VRN-H1* alleles provided the opportunity to assess the gene action at this locus. When *VRN-H1* alleles were confronted in the crosses, we observed intermediate Z31 and Z49 phenotypes (and *VRN-H1* expression) between the early and late allele indicating additivity of the effect of winter *VRN-H1* alleles. In triads where there was no variation for *VRN-H1*, the phenotypic differences between genotypes were small, regardless of the vernalization treatment, and most likely due to other genes.

The prevalent view among geneticists indicates dominance of the spring growth habit over the winter type (Takahashi and Yasuda 1971; Fu et al. 2005; Dubcovsky et al. 2005). This view is supported by a dominant inheritance of the gene *VRN-H1* at the expression level in spring x winter crosses (Haas et al. 2020). Our results challenge this view. In fact, a review of the literature finds other results in agreement with ours. Some studies found hybrids with intermediate flowering date between parents carrying spring *VRN-H1* and winter *vrn-H1* alleles. While complete dominance may occur in particular environmental conditions, experiments covering a wider and more realistic range of conditions revealed that additivity is the rule more than the exception in

the vernalization process (Kóti et al. 2006; Szűcs et al. 2007). The additivity in the inheritance of winter *VRN-H1* alleles has agronomic implications. It expands the range of barley flowering time and vernalization responses available using hybrid combinations, and can be used by breeders to fine-tune varietal vernalization needs to the target environments.

A reduced vernalization requirement, matching winter harshness level, may cause timely flowering and enhance yield. In fact, earliness conferred by *VRN-H1-4* was associated with increased grain yield in warm sites prone to occurrence of terminal water stress (Mansour et al. 2014). Therefore, it seems a good choice to deploy in barley breeding for future scenarios in which current vernalization potential will be reduced, either in homozygosis or in hybrid combinations with other alleles.

5.4.4. The construction growth period shows a dominant inheritance controlled by FT-family genes

VRN-H1, *VRN-H2*, and *PPD-H2* effects have been associated mainly with the length of the vegetative and early reproductive phases (Gol et al. 2017; Mulki et al. 2018). *VRN-H3* and *PPD-H1*, however, seem to affect the length of the late reproductive phase (Alqudah et al. 2014). In this experiment, *PPD-H2* expression apparently affected the duration of the jointing phase, but mostly the late reproductive phase, as also noticed by Casas et al., (2011).

We observed that the presence of *PPD-H2* modulated the responses of the *VRN-H1* alleles. In the two genotype triads involving a functional *PPD-H2* allele (including Male 3), we detected differences in the duration of development until the initiation of the jointing phase, which did not match the expectations based solely on their *VRN-H1* alleles. Male 3 and its hybrids showed a steeper reduction in development time, in response to moderate and complete vernalization, than the female parents (both carrying a non-functional *ppd-H2* allele). However, this effect was absent in the low vernalization treatment: Female B (*VRN-H1-6*) was earlier at Z49 than Male 3 (*vrn-H1*) at V2, but this order was reversed at V4. Concurrent with this crossover of cycle duration, we detected *PPD-H2* expression, in the male or in the hybrid, only after 4 and 8 weeks of cold, but not in the 2-week vernalization treatment. This suggests that *PPD-H2* responds not only to photoperiod, but also to vernalization, and helps to accelerate development only after some vernalization has occurred (between 2 and 4 weeks in this case). This result agrees with Monteagudo et al., (2019) who found that *PPD-H2* expression required some developmental trigger (either a cold period or advanced plant age) in winter barleys. Moreover, the earliness effect of *PPD-H2* was highly conspicuous in the shortening of the late reproductive phase of the hybrid and male when fully vernalized. As a result, hybrids carrying *PPD-H2* flowered at least as early as

the earlier parent when vernalization was fully satisfied, indicating dominance of the *PPD-H2* functional allele.

PPD-H2 is predominant in spring barleys, where it boosts development ensuring timely completion of the cycle. However, its agronomic merit in winter barley is not clear. Previous studies indicate that this gene acts as a safeguard mechanism in winter barleys, promoting spikelet initiation under short days, and reducing vernalization requirement under long days (Casao et al. 2011c; Mulki et al. 2018). Also, *PPD-H2* seems to have an adaptive role, confirmed by its influence on key agronomic traits (Cuesta-Marcos et al. 2009; Mansour et al. 2018; Sharma et al. 2018; Monteagudo et al. 2019a). We have shown that one single functional *PPD-H2* allele in a winter barley hybrid does accelerate flowering under insufficient vernalization (provided a minimum vernalization threshold is supplied). Therefore, our data support the role of *PPD-H2* as a source of earliness, to promote timely growth in warm winters with incomplete vernalization, which can be included in the formulation of hybrids for areas with mild winters.

VRN-H3 allelic variation also contributed to differences in the length of the late reproductive phase. The *vrn-H3c(1)* allele was associated with a short late reproductive phase independently of vernalization, showing partial dominance in the hybrids. This allele combines an early promoter with an early intron haplotype, and has been associated with the earliest flowering in both, a landrace collection of predominantly winter barleys (SBCC) (Casas et al. 2011), and a cross of two spring cultivars (Casas et al. 2021). In contrast, the *vrn-H3d(1)* allele, characterized by a late promoter and early intron haplotype, seems to confer a long and constant duration of the reproductive phase, independently of vernalization. The late reproductive phase determines the potential number of grains; therefore, this allele could be a stable resource for breeders aiming at high-yielding varieties.

5.4.5. Suggestions and perspectives

This work shows the wide range of vernalization responses and flowering times that barley, and in particular hybrids, can display. From this information, the breeder can choose the earliness combination that best suits the conditions of each target environment. Here are some suggestions for allelic combinations that might work well, depending on the environment.

In climates where winters are long and cold, similar to our V8 treatment, and where we could expect that the vernalization needs will be completely satisfied, any of the tested hybrids would reach heading in a suitable date. However, to maximize yield, the breeder should choose a hybrid that matches the length of the LRP with the resource availability. In this sense, in environments

prone to terminal stress, hybrids with a short LRP would have a better chance of escaping stress, which could be achieved with the *vrn-H3c(1)* allele or the *PPD-H2* allele. In contrast, in those environments where the end of the cycle benefits from optimal conditions, hybrids with a long LRP could enhance yield. In this scenario, the *vrn-H3d(1)* or *vrn-H3a(1)* allele could provide the effect we are looking for.

In those climates where winters are becoming warmer, comparable to our V4 treatment, where the cold needs might be compromised, the hybrid options that would flower on time are reduced. Hybrids carrying a dominant *PPD-H2* allele would be a suitable option when at least 4 weeks of cold were ensured. However, if the vernalization period decreased under 4 weeks, the delay in flowering time could negatively affect yield. A similar situation could be expected in hybrids with the *VRN-H1-6/vrn-H1* allelic combination. These could stand a certain decrease in the cold period duration, but not less than 4 weeks.

Under those circumstances, the safest option to ensure prompt flowering in the climate change scenario would be to use a hybrid with at least one *VRN-H1-4* allele or two *VRN-H1-6* alleles. Using these hybrid combinations should guarantee to maintain a suitable flowering even when the cold period is reduced under 4 weeks (V2 treatment).

To conclude, although based on a small set of genotypes, we have demonstrated that hybrids can show a more nuanced response to insufficient vernalization than inbred lines. We also show that these phenotypic responses agree with the expression levels of main developmental genes. New options are proposed to manage time to flowering based on specific alleles and, particularly, the duration of developmental phases that build yield potential in hybrid barley. Our results highlight that hybrid combinations extend the available catalogue of genetic responses to vernalization, which would be useful for adaptation to environmental conditions representing expected climate change trends.

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5.6. Supplementary material

Table S5.1. Primer sequences for gene expression assay.

Gene	Primer sequence (5'-3')	Reference
VRN-H1[a]	Forward: TGAAGCTCAGAAATGGATTCG Reverse: TATGAGCGCTACTCTTATGC	Trevaskis et al. (2006)
VRN-H2[a]	Forward: GAGCCACCATCGTGCCATTC Reverse: GCCGCTTCTTCCTCTTCTC	Trevaskis et al. (2006)
VRN-H3[a]	Forward: ATCTCCACTGGTTGGTGACAGA Reverse: TTGTAGAGCTCGGCAAAGTCC	Yan et al. (2006)
PPD-H1[a]	Forward: CAAATCAAAGAGCGGCGATC Reverse: TCTGACTTGGGATGGTTCACA	Hemming et al. (2008)
HvODDSOC2[a]	Forward: CAATGCTGATGACTCAGATGCT Reverse: CGCTATTTCGTTGCGCCAAT	Greenup et al. (2010)
PPD-H2[b]	Forward: GGTTGTGGCTCATGTTATGC Reverse: CTACTCCCCTTGAGAACTTTC	F: Kikuchi et al. (2009) R: Faure et al. (2007)
Actin[a]	Forward: GCCGTGCTTTCCCTCTATG Reverse: GCTTCTCCTTGATGTCCCTTA	Trevaskis et al. (2006)

[a]For these genes, each reaction contained 5 µl of PowerUp SYBR Green Master Mix (Applied Biosystems), 0.5 µM of each primer and 250 ng of cDNA in a volume of 10 µl. Reactions were run with the following conditions: 2 min at 50°C, 2 min at 95°C, 44 cycles of 15 s at 95°C and 1 min at 60°C, followed by a melting curve program (60-95°C) implying temperature increases of 1°C each minute.
[b]For this gene, each reaction contained 5 µl of PowerUp SYBR Green Master Mix (Applied Biosystems), 0.5 µM of each primer and 250 ng of cDNA in a volume of 10 µl. Reactions were run with the following conditions: 2 min at 50°C, 2 min at 95°C, 44 cycles of 15 s at 95°C, 15 s at 60°C and 45 s at 72°C, and a melting curve program (60-95°C) of 1°C of temperature increment for each minute.

Table S5.2. Effects of genotype, repetition, treatment, genotype by treatment interaction, and contrasts on developmental variables. The values under each variable's heading correspond to mean squares.

Source of variation	Df[a]	Z31	Z49	Lag Z31-Z49	Sensitivity Z31	Sensitivity Z49	Sensitivity Lag Z31-Z49
Genotype	10	976.0***	1373.2***	183.2***	375.2***	440.2***	48.4***
F_1 *vs.* Parents	1	241.0***	64.0***	56.6**	7.2	0.2	5.0
within Parents	4	2008.0***	3246.7***	314.7***	686.5***	764.8***	81.5***
Females *vs.* Males	1	3499.2***	8636.0***	1140.8***	217.7***	510.0***	61.2**
within Females	1	1058.0***	734.7***	29.4*	768.2***	168.7***	216.7***
within Males	2	1737.4***	1808.1***	44.3**	880.2***	1190.2***	24.0
within Hybrids	5	297.3***	136.2***	103.3***	199.7***	268.6***	30.6**
F_1 (Fem A) *vs.* F_1 (Fem B)	1	50.1***	42.7**	0.3	83.9***	103.4***	1.0
within F_1 (Fem A)	2	518.5***	211.8***	160.6***	330.1***	297.2***	27.6*
within F_1 (Fem B)	2	199.7***	107.4***	97.4***	127.2***	322.7***	48.4**
Repetition	2	1.0	11.0	10.9	5.0	3.7	10.0
Treatment	2	19126.4***	24628.5***	370.7***	133.9***	661.8***	200.4***
Genotype*Treatment	20	248.2***	267.5***	33.1***	363.9***	284.4***	53.7***
Residuals	64	2.8	4.9	6.6	2.8	4.6	7.5

[a]Degrees of freedom, P < 0.1 * P < 0.05 ** P <0.01 *** P <0.001.

Table S5.3. Means and 95% confidence intervals of developmental phases for each genotypic set by treatment combination. For each developmental variable and treatment, group means with a different letter are significantly different at $P < 0.05$ according to the contrasts performed for the overall ANOVA.

Genotypic set	Treatment	Z31±CI[a]	Z49±CI	LagZ31-Z49±CI
Female	V2	77.7 ± 8.7 [a]	116.0 ± 8.5 [a]	38.3 ± 3.4 [a]
Hybrid	V2	67.9 ± 5.1 [b]	94.6 ± 4.9 [b]	26.7 ± 2.0 [b]
Male	V2	59.0 ± 7.1 [c]	82.0 ± 6.9 [c]	23.0 ± 2.8 [c]
Female	V4	57.0 ± 8.7 [a]	87.3 ± 8.5 [a]	30.3 ± 3.4 [a]
Hybrid	V4	48.2 ± 5.0 [b]	74.2 ± 4.9 [b]	26.0 ± 2.0 [b]
Male	V4	29.3 ± 7.2 [c]	53.7 ± 7.0 [c]	24.3 ± 2.8 [b]
Female	V8	23.7 ± 8.7 [a]	51.5 ± 8.5 [a]	27.8 ± 3.4 [a]
Hybrid	V8	19.2 ± 5.1 [b]	40.0 ± 4.9 [b]	20.8 ± 2.0 [b]
Male	V8	16.0 ± 7.1 [c]	34.3 ± 7.0 [c]	18.3 ± 2.8 [b]

[a]CI, 95% confidence interval.

Table S5.4. Effects of genotype, repetition, treatment, sampling time, factorial interactions, and contrasts on gene expression for Batch A. The values under each variable's heading correspond to mean squares.

Source of variation	**Df**[a]	**dCt *VRN-H1***	**dCt *VRN-H2***	**dCt *VRN-H3***	**dCt *PPD-H1***	**dCt *PPD-H2***	**dCt *HvODDSOC2***
Genotype	6	86.6***	48.6***	122.6***	4.9***	3.4	74.3***
F_1 *vs.* Parents	1	2.9	31.6***	5.7*	4.2	2.6	105.0***
F_1 *vs.* Females	1	105.0***	29.8***	170.5***	0.0	0.9	1.4
F_1 *vs.* Males	1	7.4	91.8***	83.5***	6.3*	3.4	177.6***
within Parents	3	122.6***	83.4***	215.8***	7.7***	0.8	97.7***
Females *vs.* Males	1	148.1***	149.6***	381.1***	2.9	0.8	112.2***
within Males	2	109.9***	50.3***	133.2***	10.1***		90.6***
within Hybrids	2	74.4***	4.9**	41.1***	1.0		23.9*
Repetition	2	2.0	2.3	1.4	0.2	5.7	4.4
Treatment	2	866.0***	506.4***	271.1***	6.0**	5.7	586.3***
Genotype*Treatment	12	48.8***	18.0***	22.3***	1.3	23.7	12.3*
Sampling Time (ST)	1	142.8***	44.4***	162.1***	8.8**	0.8	249.7***
Genotype*Sampling Time	6	8.9**	2.9**	12.1***	1.2	24.7	7.3
Treatment*Sampling Time	2	112.9***	12.9***	5.0*	0.6	8.5	117.1***
Genotype*Treatment*ST	12	9.1***	2.1*	5.2***	1.1	30.0	10.9*
Residuals	82	2.4	0.9	1.4	1.1	341.1	5.4

[a]Degrees of freedom, P < 0.1 * P < 0.05 ** P <0.01 *** P <0.001.

Table S5.5. Effects of genotype, repetition, treatment, sampling time, factorial interactions, and contrasts on gene expression for Batch B. The values under each variable's heading correspond to mean squares.

Source of variation	Df[a]	dCt *VRN-H1*	dCt *VRN-H2*	dCt *VRN-H3*	dCt *PPD-H1*	dCt *PPD-H2*	dCt *HvODDSOC2*
Genotype	6	46.9***	12.8***	87.8***	5.3***	156.2***	117.2***
F_1 *vs.* Parents	1	0.0	10.1***	41.9***	0.8*	23.9	303.1***
F_1 *vs.* Females	1	52.7***	0.1	23.6***	3.5***	161.9**	7.2
F_1 *vs.* Males	1	10.1***	16.7***	107.3***	0.1	18.2	418.2***
within Parents	3	68.6***	22.1***	150.3***	6.8***	288.6***	114.3***
Females *vs.* Males	1	90.3***	9.8***	148.3***	2.9***	288.5***	138.9***
within Males	2	57.8***	28.2***	151.3***	8.8***	0.0	102.1***
within Hybrids	2	37.7***	0.3	17.0***	5.3***	0.0	28.5***
Repetition	2	0.0	0.3	0.8	0.0	19.6	17.6**
Treatment	2	479.5***	204.6***	717.1***	1.9***	155.3***	461.6***
Genotype*Treatment	12	22.9***	10.7***	25.5***	2.0***	58.6**	18.8***
SamplingTime (ST)	1	51.5***	0.6	337.8***	6.3***	28.7	124.7***
Genotype*SamplingTime	6	4.7***	3.0***	3.5**	2.6***	23.4	15.0***
Treatment*SamplingTime	2	20.8***	25.3***	6.2**	0.0	18.9	8.1
Genotype*Treatment*ST	12	3.0***	3.2***	3.0***	1.3***	30.5	7.9**
Residuals	82	0.3	0.2	0.9	0.2	14.7	2.8

[a]Df, degrees of freedom; P < 0.1 * P < 0.05 ** P <0.01 *** P <0.001.

Table S5.6. Means and standard deviation of developmental variables for each genotype by treatment combination.

Genotype	Treatment	Z31±SD[a]	Z49±SD	LagZ31-Z49±SD
Female A	V2	93.33±0.58	126.00±2.00	32.67±2.08
Female B	V2	62.00±2.65	106.00±1.00	44.00±1.73
Hybrid A1	V2	52.33±0.58	85.33±2.08	33.00±1.73
Hybrid A2	V2	78.67±3.79	99.00±3.61	20.33±1.53
Hybrid A3	V2	80.33±1.53	108.00±1.00	27.67±0.58
Hybrid B1	V2	57.00±1.00	83.00±2.65	26.00±1.73
Hybrid B2	V2	62.67±0.58	86.33±0.58	23.67±1.15
Hybrid B3	V2	76.67±0.58	106.00±5.00	29.33±5.51
Male 1	V2	29.00±1.00	51.67±2.08	22.67±1.53
Male 2	V2	61.67±0.58	81.67±2.52	20.00±2.00
Male 3	V2	86.33±0.58	112.67±0.58	26.33±0.58
Female A	V4	64.67±4.04	94.00±0.00	29.33±4.04
Female B	V4	49.33±1.15	80.67±3.79	31.33±4.93
Hybrid A1	V4	42.00±3.00	69.67±3.06	27.67±3.51
Hybrid A2	V4	52.00±1.00	73.00±1.00	21.00±1.73
Hybrid A3	V4	53.33±0.58	81.00±1.00	27.67±0.58
Hybrid B1	V4	41.67±0.58	73.33±0.58	31.67±1.15
Hybrid B2	V4	52.00±1.00	75.00±2.00	23.00±1.73
Hybrid B3	V4	48.00±3.46	73.00±0.00	25.00±3.46
Male 1	V4	20.67±1.53	45.00±1.00	24.33±0.58
Male 2	V4	30.33±3.21	53.00±0.00	22.67±3.21
Male 3	V4	37.00±1.73	63.00±0.00	26.00±1.73
Female A	V8	23.33±1.53	54.00±1.00	30.67±0.58
Female B	V8	24.00±1.00	49.00±1.73	25.00±2.65
Hybrid A1	V8	17.67±0.58	42.67±4.16	25.00±3.61
Hybrid A2	V8	19.00±0.00	38.00±3.46	19.00±3.46
Hybrid A3	V8	19.33±0.58	37.67±4.04	18.33±4.51
Hybrid B1	V8	18.67±0.58	45.00±2.00	26.33±2.52
Hybrid B2	V8	20.00±1.00	37.67±2.52	17.67±3.21
Hybrid B3	V8	20.67±1.15	39.00±2.00	18.33±2.31
Male 1	V8	12.33±0.58	34.33±0.58	22.00±1.00
Male 2	V8	13.67±0.58	29.00±0.00	15.33±0.58
Male 3	V8	22.00±0.00	39.67±2.08	17.67±2.08

[a]SD, standard deviation.

Table S5.7. Means and standard deviation of vernalization sensitivity of developmental phases for each genotype by treatment combination.

Genotype	Treatment	Sensitivity Z31±SD[a]	Sensitivity Z49±SD	Sensitivity Lag Z31-Z49±SD
Female A	V4-V2	-28.67±4.04	-32.00±0.00	-3.33±4.04
Female B	V4-V2	-12.67±1.15	-25.33±3.79	-12.67±4.93
Hybrid A1	V4-V2	-10.33±3.00	-15.67±3.06	-5.33±3.51
Hybrid A2	V4-V2	-26.67±1.00	-26.00±1.00	0.67±1.73
Hybrid A3	V4-V2	-27.00±0.58	-27.00±1.00	0.00±0.58
Hybrid B1	V4-V2	-15.33±0.58	-9.67±0.58	5.67±1.15
Hybrid B2	V4-V2	-10.67±1.00	-11.33±2.00	-0.67±1.73
Hybrid B3	V4-V2	-28.67±3.46	-33.00±0.00	-4.33±3.46
Male 1	V4-V2	-8.33±1.53	-6.67±1.00	1.67±0.58
Male 2	V4-V2	-31.33±3.21	-28.67±0.00	2.67±3.21
Male 3	V4-V2	-49.33±1.73	-49.67±0.00	-0.33±1.73
Female A	V8-V4	-41.33±1.53	-40.00±1.00	1.33±0.58
Female B	V8-V4	-25.33±1.00	-31.67±1.73	-6.33±2.65
Hybrid A1	V8-V4	-24.33±0.58	-27.00±4.16	-2.67±3.61
Hybrid A2	V8-V4	-33.00±0.00	-35.00±3.46	-2.00±3.46
Hybrid A3	V8-V4	-34.00±0.58	-43.33±4.04	-9.33±4.51
Hybrid B1	V8-V4	-23.00±0.58	-28.33±2.00	-5.33±2.52
Hybrid B2	V8-V4	-32.00±1.00	-37.33±2.52	-5.33±3.21
Hybrid B3	V8-V4	-27.33±1.15	-34.00±2.00	-6.67±2.31
Male 1	V8-V4	-8.33±0.58	-10.67±0.58	-2.33±1.00
Male 2	V8-V4	-16.67±0.58	-24.00±0.00	-7.33±0.58
Male 3	V8-V4	-15.00±0.00	-23.33±2.08	-8.33±2.08

[a]SD, standard deviation.

Table S5.8. Means and standard error of relative gene expression for each genotype by treatment combination of Batch A.

Genotype	Treatment	Sampling Time	*VRN-H1*±SE[a]	*VRN-H2*±SE	*VRN-H3*±SE	*PPD-H1*±SE	*PPD-H2*±SE	*HvODDSOC2*±SE
Female A	V2	17	0.000±0.000	0.4705±0.1154	0.0000±0.0000	0.125±0.007	0.0000±0.0000	0.4699±0.0898
Hybrid A1	V2	17	1.025±0.089	0.1861±0.1116	0.0152±0.0053	0.430±0.114		0.1174±0.0284
Hybrid A2	V2	17	0.000±0.000	0.5337±0.0680	0.0108±0.0073	0.279±0.133		0.2285±0.0177
Hybrid A3	V2	17	0.046±0.041	0.4237±0.1268	0.0142±0.0067	0.230±0.063	0.0000±0.0000	0.7640±0.1806
Male 1	V2	17	3.160±0.347	0.0772±0.0069	0.2816±0.0068	0.318±0.146		0.0161±0.0006
Male 2	V2	17	0.000±0.000	0.2735±0.0324	0.0070±0.0027	0.284±0.024		0.0703±0.0098
Male 3	V2	17	0.008±0.004	0.4323±0.0890	0.0003±0.0001	0.185±0.020	0.0000±0.0000	0.5344±0.0835
Female A	V4	17	0.682±0.199	0.1137±0.0338	0.0006±0.0003	0.165±0.050	0.0000±0.0000	0.2236±0.0295
Hybrid A1	V4	17	1.872±0.351	0.0437±0.0077	0.0362±0.0023	0.289±0.039		0.1929±0.0957
Hybrid A2	V4	17	1.283±0.170	0.2280±0.0907	0.0836±0.0190	0.352±0.041		0.1495±0.0334
Hybrid A3	V4	17	0.982±0.101	0.0524±0.0033	0.0048±0.0025	0.157±0.069	0.0019±0.0018	0.7823±0.0505
Male 1	V4	17	5.751±0.279	0.0115±0.0014	0.7049±0.0408	0.434±0.021		0.0011±0.0001
Male 2	V4	17	2.250±0.107	0.0588±0.0075	0.2588±0.0334	0.336±0.091		0.0224±0.0015
Male 3	V4	17	1.174±0.239	0.1051±0.0184	0.0217±0.0057	0.300±0.114	0.0000±0.0000	0.0877±0.0024
Female A	V8	17	2.954±0.037	0.0325±0.0076	0.0785±0.0025	0.206±0.013	0.0000±0.0000	0.0322±0.0026
Hybrid A1	V8	17	4.039±0.455	0.0136±0.0049	0.1765±0.0707	0.139±0.050		0.0160±0.0023
Hybrid A2	V8	17	5.281±0.033	0.0042±0.0013	0.0852±0.0021	0.277±0.008		0.0282±0.0055
Hybrid A3	V8	17	5.825±1.134	0.0081±0.0022	0.1222±0.0079	0.183±0.109	0.0000±0.0000	0.0233±0.0042
Male 1	V8	17	7.312±0.505	0.0044±0.0004	0.4832±0.1755	0.321±0.054		0.0030±0.0004
Male 2	V8	17	3.576±0.697	0.0000±0.0000	0.9338±0.1077	0.323±0.058		0.0007±0.0001
Male 3	V8	17	4.228±0.235	0.0020±0.0003	0.1132±0.0230	0.066±0.016	0.0120±0.0120	0.0592±0.0087
Female A	V2	35	0.023±0.018	0.4869±0.0402	0.0011±0.0001	0.152±0.008	0.0000±0.0000	0.4979±0.0297
Hybrid A1	V2	35	2.569±0.335	0.0684±0.0169	1.5313±0.4782	0.234±0.110		0.1043±0.0545
Hybrid A2	V2	35	0.059±0.025	0.4978±0.0605	0.0116±0.0034	0.203±0.057		0.4203±0.1559
Hybrid A3	V2	35	0.154±0.048	0.4706±0.1408	0.0113±0.0062	0.191±0.078	0.0000±0.0000	0.6826±0.1664
Male 1	V2	35	4.054±0.197	0.0822±0.0044	1.5653±0.5805	0.254±0.023		0.0296±0.0104
Male 2	V2	35	0.265±0.020	0.1733±0.0650	0.0837±0.0191	0.159±0.021		0.0634±0.0059
Male 3	V2	35	0.063±0.005	0.4886±0.0453	0.0113±0.0020	0.138±0.008	0.0000±0.0000	0.5175±0.1820
Female A	V4	35	0.748±0.040	0.0558±0.0033	0.0234±0.0121	0.162±0.015	0.0000±0.0000	0.2822±0.0447
Hybrid A1	V4	35	2.750±0.359	0.0185±0.0044	0.1956±0.0098	0.180±0.006		0.0155±0.0148
Hybrid A2	V4	35	1.060±0.174	0.0444±0.0110	0.0659±0.0110	0.171±0.003		0.0759±0.0354
Hybrid A3	V4	35	1.016±0.066	0.0085±0.0007	0.0051±0.0029	0.145±0.043	0.0000±0.0000	0.0600±0.0081
Male 1	V4	35	6.687±0.882	0.0050±0.0008	1.7546±0.2178	0.348±0.024		0.0615±0.0530
Male 2	V4	35	3.727±0.415	0.0010±0.0004	1.9667±0.0638	0.392±0.007		0.0029±0.0021
Male 3	V4	35	1.856±0.451	0.0207±0.0039	0.0567±0.0218	0.157±0.061	0.0000±0.0000	0.1054±0.0287
Female A	V8	35	4.354±0.221	0.0370±0.0120	0.4580±0.0059	0.134±0.042	0.0000±0.0000	0.0015±0.0015
Hybrid A1	V8	35	7.402±3.080	0.0062±0.0020	0.4535±0.0902	0.070±0.041		0.0020±0.0007
Hybrid A2	V8	35	5.282±0.426	0.0035±0.0015	1.0869±0.2596	0.062±0.002		0.0162±0.0124
Hybrid A3	V8	35	4.552±0.635	0.0030±0.0012	0.3687±0.0925	0.144±0.053	0.0000±0.0000	0.0029±0.0022
Male 1	V8	35	6.928±0.346	0.0008±0.0001	2.3866±0.2753	0.228±0.010		0.0010±0.0006
Male 2	V8	35	4.361±0.619	0.0000±0.0000	3.6885±0.2538	0.172±0.038		0.0000±0.0000
Male 3	V8	35	5.337±0.758	0.0026±0.0011	0.5876±0.3238	0.092±0.020	0.0000±0.0000	0.0000±0.0000

[a]SE, standard error of the mean.

Table S5.9. Means and standard error of relative gene expression for each genotype by treatment combination of Batch B.

Genotype	Treatment	Sampling Time	*VRN-H1*±SE[a]	*VRN-H2*±SE	*VRN-H3*±SE	*PPD-H1*±SE	*PPD-H2*±SE	*HvODDSOC2*±SE
Female B	V2	17	0.003±0.001	0.1060±0.0063	0.0005±0.0003	0.084±0.001	0.0000±0.0000	0.0531±0.0136
Hybrid B1	V2	17	1.761±0.068	0.0807±0.0054	0.0050±0.0005	0.717±0.023		0.1247±0.0035
Hybrid B2	V2	17	0.005±0.002	0.1588±0.0086	0.0001±0.0000	0.130±0.001		0.1445±0.0263
Hybrid B3	V2	17	0.029±0.011	0.1448±0.0012	0.0005±0.0001	0.179±0.029	0.0000±0.0000	0.1111±0.0370
Male 1	V2	17	3.621±0.564	0.0340±0.0117	0.2950±0.0421	0.656±0.161		0.0051±0.0004
Male 2	V2	17	0.006±0.001	0.2690±0.0369	0.0043±0.0030	0.247±0.004		0.0102±0.0019
Male 3	V2	17	0.030±0.004	0.2114±0.0044	0.0002±0.0001	0.175±0.007	0.0000±0.0000	0.0796±0.0173
Female B	V4	17	0.267±0.061	0.0851±0.0061	0.0013±0.0006	0.156±0.018	0.0000±0.0000	0.1662±0.0339
Hybrid B1	V4	17	1.212±0.084	0.0235±0.0057	0.0077±0.0028	0.271±0.013		0.0386±0.0125
Hybrid B2	V4	17	0.640±0.018	0.1168±0.0068	0.0179±0.0018	0.294±0.009		0.3471±0.0094
Hybrid B3	V4	17	1.630±0.387	0.0769±0.0075	0.0240±0.0141	0.508±0.087	0.0014±0.0007	0.0222±0.0081
Male 1	V4	17	5.901±0.251	0.0172±0.0027	0.6981±0.0068	0.517±0.103		0.0008±0.0001
Male 2	V4	17	1.945±0.101	0.0208±0.0049	0.2501±0.0264	0.188±0.019		0.0037±0.0010
Male 3	V4	17	1.169±0.073	0.1108±0.0026	0.0218±0.0020	0.320±0.011	0.0218±0.0217	0.0927±0.0098
Female B	V8	17	3.376±0.795	0.0028±0.0015	0.2173±0.0820	0.159±0.055	0.0000±0.0000	0.0053±0.0032
Hybrid B1	V8	17	10.682±1.064	0.0156±0.0011	0.4715±0.0034	0.235±0.020		0.0074±0.0027
Hybrid B2	V8	17	7.879±1.405	0.0043±0.0012	0.5999±0.1565	0.239±0.020		0.0114±0.0012
Hybrid B3	V8	17	4.025±0.637	0.0053±0.0014	0.3453±0.1011	0.169±0.046	0.0040±0.0020	0.0007±0.0004
Male 1	V8	17	7.228±1.199	0.0129±0.0007	0.4734±0.0777	0.407±0.122		0.0010±0.0002
Male 2	V8	17	11.234±0.087	0.0005±0.0000	0.9459±0.0553	0.572±0.108		0.0013±0.0008
Male 3	V8	17	4.173±0.512	0.0139±0.0008	0.2158±0.0286	0.144±0.013	0.0088±0.0036	0.0010±0.0005
Female B	V2	35	0.068±0.022	0.2448±0.0354	0.0023±0.0009	0.213±0.044	0.0000±0.0000	0.0338±0.0050
Hybrid B1	V2	35	1.816±0.040	0.3362±0.0255	0.0313±0.0087	0.697±0.105		0.0579±0.0097
Hybrid B2	V2	35	0.043±0.013	0.3849±0.1273	0.0010±0.0003	0.146±0.030		0.0827±0.0475
Hybrid B3	V2	35	0.122±0.011	0.0894±0.0082	0.0023±0.0008	0.068±0.006	0.0000±0.0000	0.0800±0.0167
Male 1	V2	35	4.736±0.996	0.0278±0.0035	1.6047±0.1054	0.343±0.042		0.0031±0.0004
Male 2	V2	35	0.231±0.097	0.2535±0.0116	0.0851±0.0462	0.159±0.026		0.0027±0.0006
Male 3	V2	35	0.065±0.010	0.1498±0.0125	0.0018±0.0012	0.057±0.008	0.0000±0.0000	0.0242±0.0036
Female B	V4	35	1.135±0.026	0.0499±0.0055	0.1120±0.0017	0.284±0.007	0.0000±0.0000	0.0200±0.0016
Hybrid B1	V4	35	3.339±0.324	0.0101±0.0011	1.0726±0.2006	0.260±0.047		0.0010±0.0005
Hybrid B2	V4	35	4.001±0.161	0.0143±0.0029	0.4654±0.0687	0.292±0.015		0.1028±0.0150
Hybrid B3	V4	35	1.317±0.370	0.0336±0.0008	0.1076±0.0465	0.127±0.010	0.0000±0.0000	0.0188±0.0042
Male 1	V4	35	6.800±0.786	0.0346±0.0020	3.9462±0.5083	0.209±0.017		0.0001±0.0001
Male 2	V4	35	3.766±0.261	0.0006±0.0001	1.9697±0.0824	0.402±0.027		0.0008±0.0001
Male 3	V4	35	1.859±0.079	0.0193±0.0044	0.0801±0.0035	0.106±0.008	0.0034±0.0017	0.0369±0.0006
Female B	V8	35	6.196±1.271	0.0142±0.0027	3.5810±0.5596	0.187±0.043	0.0000±0.0000	0.0007±0.0007
Hybrid B1	V8	35	4.574±0.333	0.0157±0.0021	2.3064±0.5789	0.195±0.035		0.0012±0.0001
Hybrid B2	V8	35	5.705±0.602	0.0123±0.0030	3.4826±0.6685	0.270±0.015		0.0038±0.0006
Hybrid B3	V8	35	5.458±0.830	0.0109±0.0012	1.9484±0.4403	0.167±0.018	0.0761±0.0629	0.0138±0.0118
Male 1	V8	35	6.825±1.254	0.0139±0.0015	2.4662±0.3629	0.215±0.037		0.0001±0.0001
Male 2	V8	35	6.117±1.252	0.0014±0.0001	4.6541±1.3786	0.130±0.017		0.0004±0.0004
Male 3	V8	35	6.908±0.914	0.0165±0.0024	1.5454±0.3053	0.129±0.005	0.0008±0.0008	0.0045±0.0012

[a]SE, standard error of the mean.

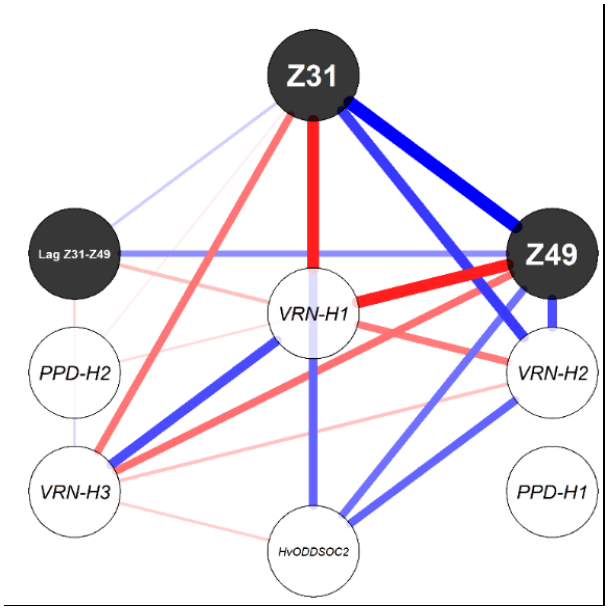

Figure S5.1. Correlation network of developmental phases and flowering time regulators gene expression (averaged across two sampling times per genotype by treatment combination, 33 data points). Red and blue lines represent negative and positive correlations, respectively. Line width is proportional to the strength of the correlation. Only significant (P<0.05) correlations are shown. Developmental phases depicted in black: days to first node appearance (Z31), days to awn tipping (Z49), and late reproductive phase (Lag Z31-Z49). Flowering time genes expression depicted in white: *VRN-H1*, *VRN-H2*, *PPD-H1*, *HvODDSOC2*, *VRN-H3*, and *PPD-H2*.

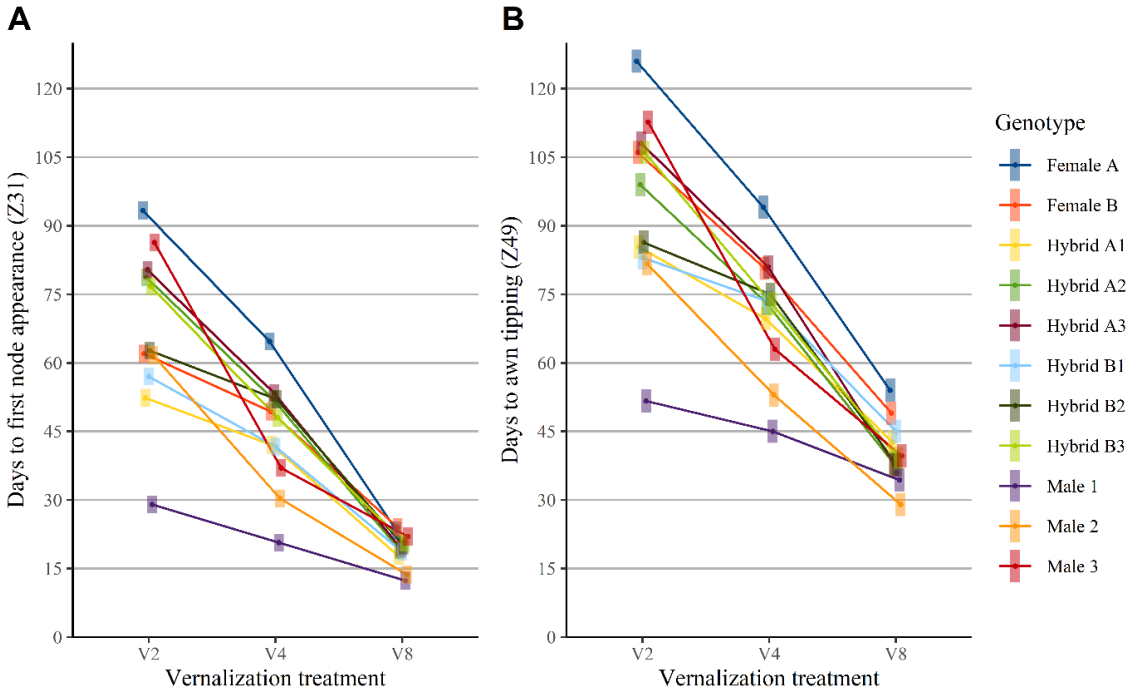

Figure S5.2. Vernalization sensitivity of days to first node appearance (A) and days to awn tipping (B). Dots represent means for each genotype and vernalization treatment (V2: 2 weeks of vernalization, V4: 4 weeks of vernalization, V8: 8 weeks of vernalization). Each colour line connects the treatment means from one genotype. Error bars are 95% confidence intervals.

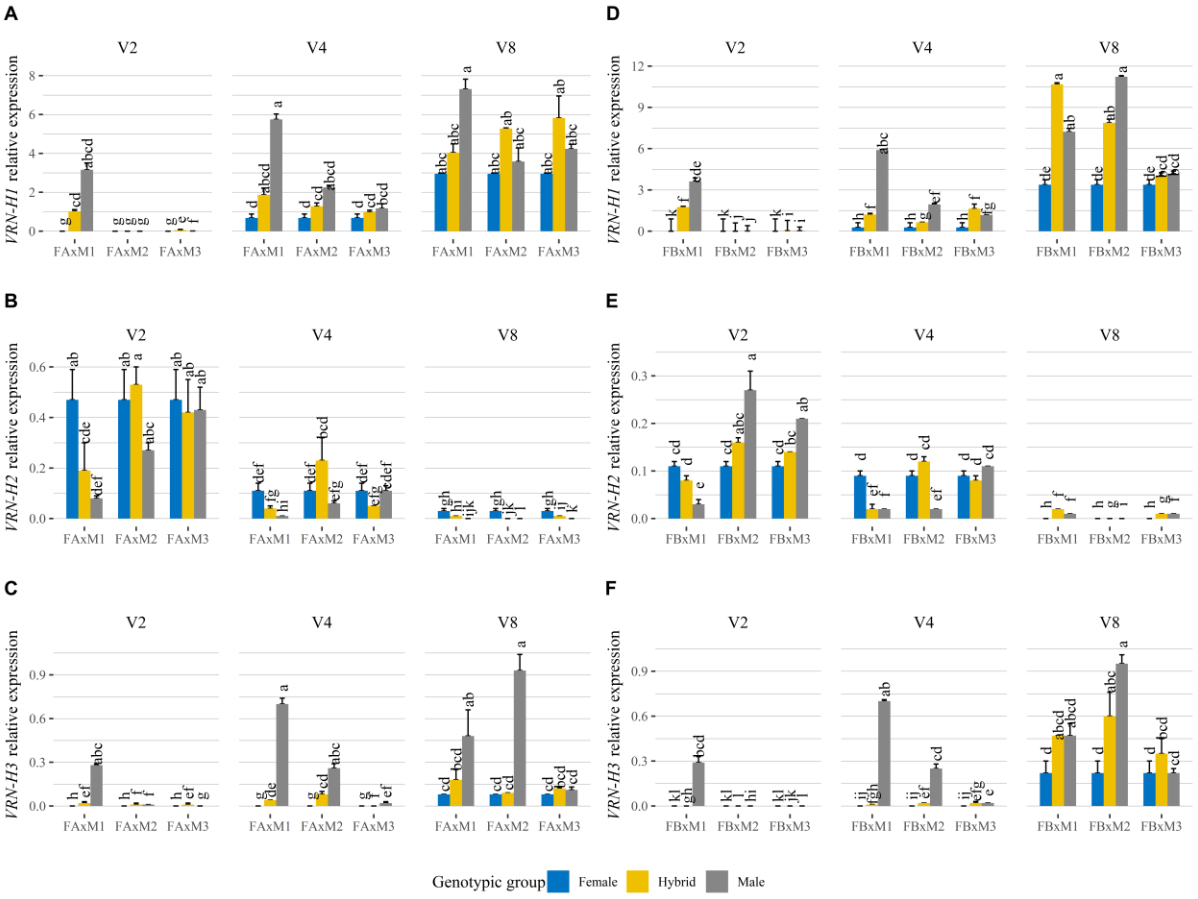

Figure S5.3. Relative expression levels of *VRN-H1* (A, D), *VRN-H2* (B, E) and *VRN-H3* (C, F) assayed by qRT-PCR in triads of barley genotypes (Female, Hybrid, Male) grown under 16 h light, in response to different vernalization treatments (V2: 2 weeks of vernalization, V4: 4 weeks of vernalization, V8: 8 weeks of vernalization; 4-8 °C, 8 h light). Plots A, B and C correspond to female A crosses (Batch A). Plots D, E and F correspond to female B crosses (Batch B). Each plot is divided in three facets, each of them containing gene expression assayed for one vernalization treatment, and the three triads of genotypes composed of one female parent in blue, the male parent in grey, and the hybrid in yellow. The triads are represented as abbreviations of the crosses between the parents, e.g., FAxM1: Female A x Male 1. The results shown are normalized to the level of the housekeeping gene *Actin* for each genotype and treatment. Samples were taken from plants after 17 d of growth under each treatment. Mean of 3 biological replicates. Error bars represent the SEM. For each gene and batch, bars with a different letter are significantly different at $P < 0.05$, according to ANOVA that included genotypes and all treatments.

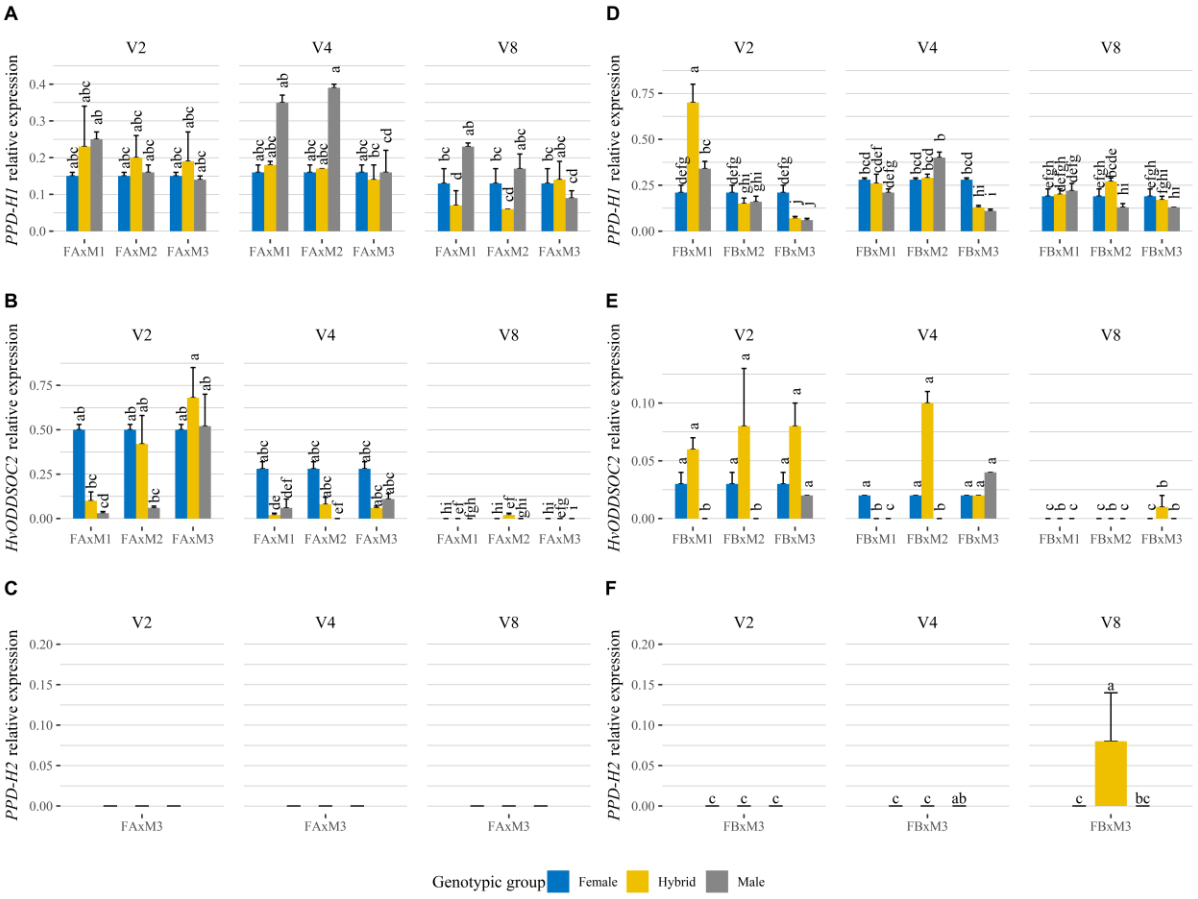

Figure S5.4. Relative expression levels of *PPD-H1* (A, D), *HvODDSOC2* (B, E) and *PPD-H2* (C, F) assayed by qRT-PCR in triads of barley genotypes (Female, Hybrid, Male) grown under 16 h light, in response to different vernalization treatments (V2: 2 weeks of vernalization, V4: 4 weeks of vernalization, V8: 8 weeks of vernalization; 4-8 °C, 8 h light). Plots A, B and C correspond to female A crosses (Batch A). Plots D, E and F correspond to female B crosses (Batch B). Each plot is divided in three facets, each of them containing gene expression assayed for one vernalization treatment, and the three triads of genotypes composed of one female parent in blue, the male parent in grey, and the hybrid in yellow. The triads are represented as abbreviations of the crosses between the parents, e.g., FBxM1: Female B x Male 1. The results shown are normalized to the level of the housekeeping gene *Actin* for each genotype and treatment. Samples were taken from plants after 35 d of growth under each treatment. Mean of 3 biological replicates. Error bars represent the SEM. For each gene and batch, bars with a different letter are significantly different at $P < 0.05$, according to ANOVA that included genotypes and all treatments.

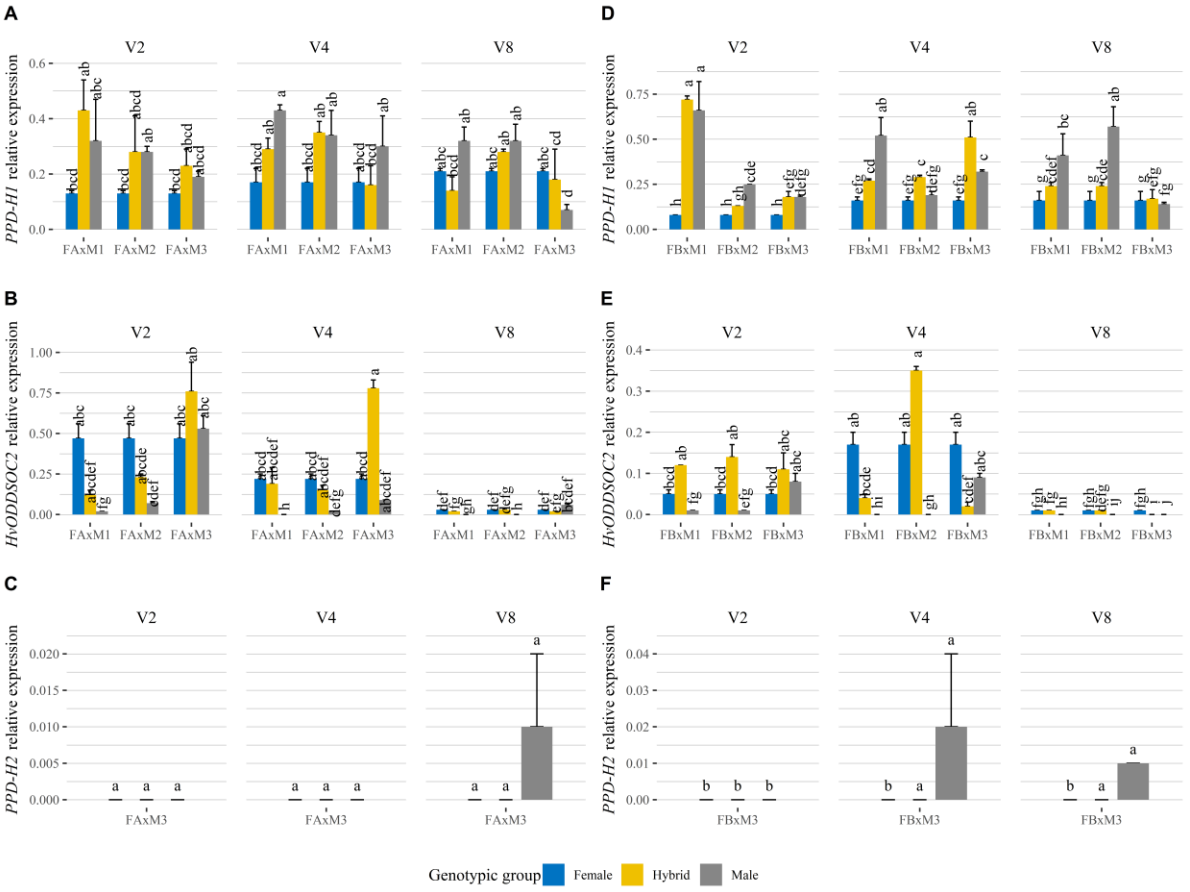

Figure S5.5. Relative expression levels of *PPD-H1* (A, D), *HvODDSOC2* (B, E) and *PPD-H2* (C, F) assayed by qRT-PCR in triads of barley genotypes (Female, Hybrid, Male) grown under 16 h light, in response to different vernalization treatments (V2: 2 weeks of vernalization, V4: 4 weeks of vernalization, V8: 8 weeks of vernalization; 4-8 °C, 8 h light). Plots A, B and C correspond to female A crosses (Batch A). Plots D, E and F correspond to female B crosses (Batch B). Each plot is divided in three facets, each of them containing gene expression assayed for one vernalization treatment, and the three triads of genotypes composed of one female parent in blue, the male parent in grey, and the hybrid in yellow. The triads are represented as abbreviations of the crosses between the parents, e.g., FBxM1: Female B x Male 1. The results shown are normalized to the level of the housekeeping gene *Actin* for each genotype and treatment. Samples were taken from plants after 17 d of growth under each treatment. Mean of 3 biological replicates. Error bars represent the SEM. For each gene and batch, bars with a different letter are significantly different at $P < 0.05$, according to ANOVA that included genotypes and all treatments.

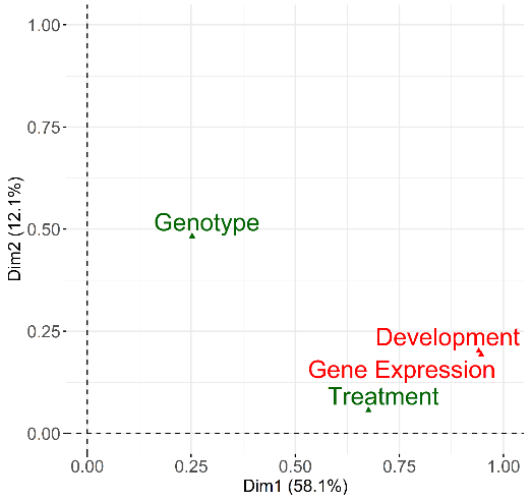

Figure S5.6. Multiple factorial analysis (MFA), plot of variable group correlation with axes. The plot represents the correlation between groups of variables and axes. *Development* and *Gene Expression* (red) are active groups based on quantitative variables used to define the dimensions of the MFA. *Genotype* and *treatment* (green) are supplementary groups based on categorical variables specifying the genotype identity of each individual and the vernalization treatment to which they were subjected. Triangles represent the correlation of groups with the axes.

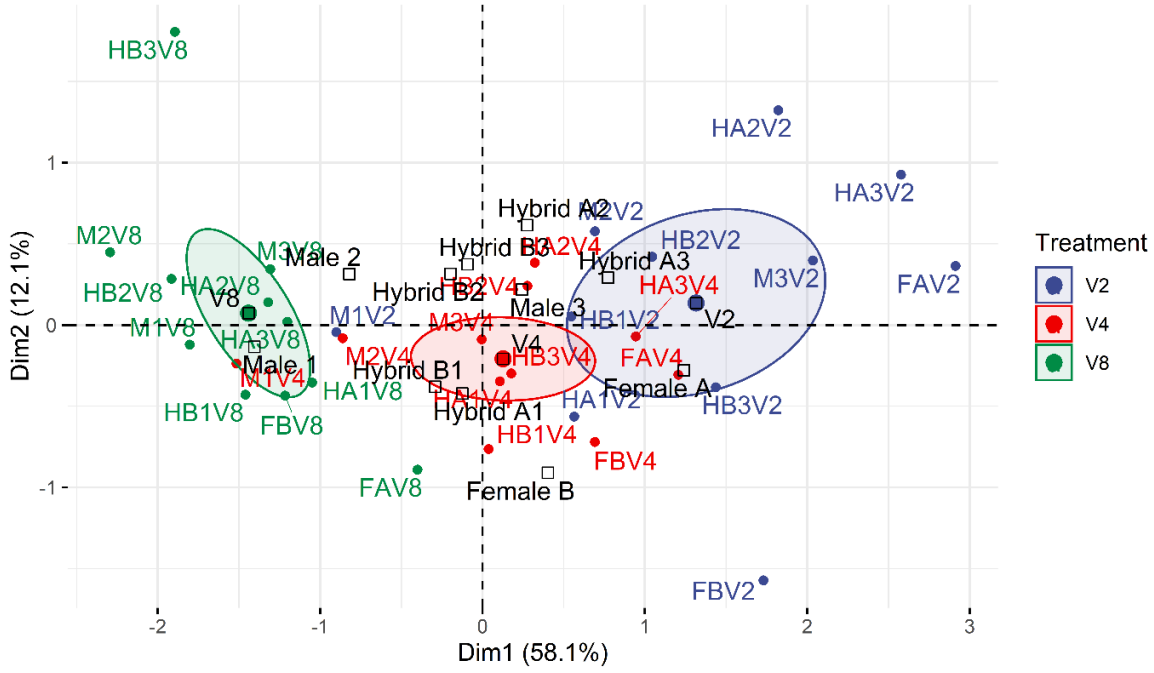

Figure S5.7. Multiple factorial analysis (MFA). Individuals grouped by vernalization treatment. This plot exhibits the position of individuals in the MFA by the vernalization treatment. Dots represent individuals (e.g., FAV2: genotype Female A in 2-week vernalization treatment). Squares represent group mean points for categorial variables. Confidence interval ellipses around each vernalization treatment were added. V2: 2 weeks of cold treatment; V4: 4 weeks of cold treatment; V8: 8 weeks of cold treatment.

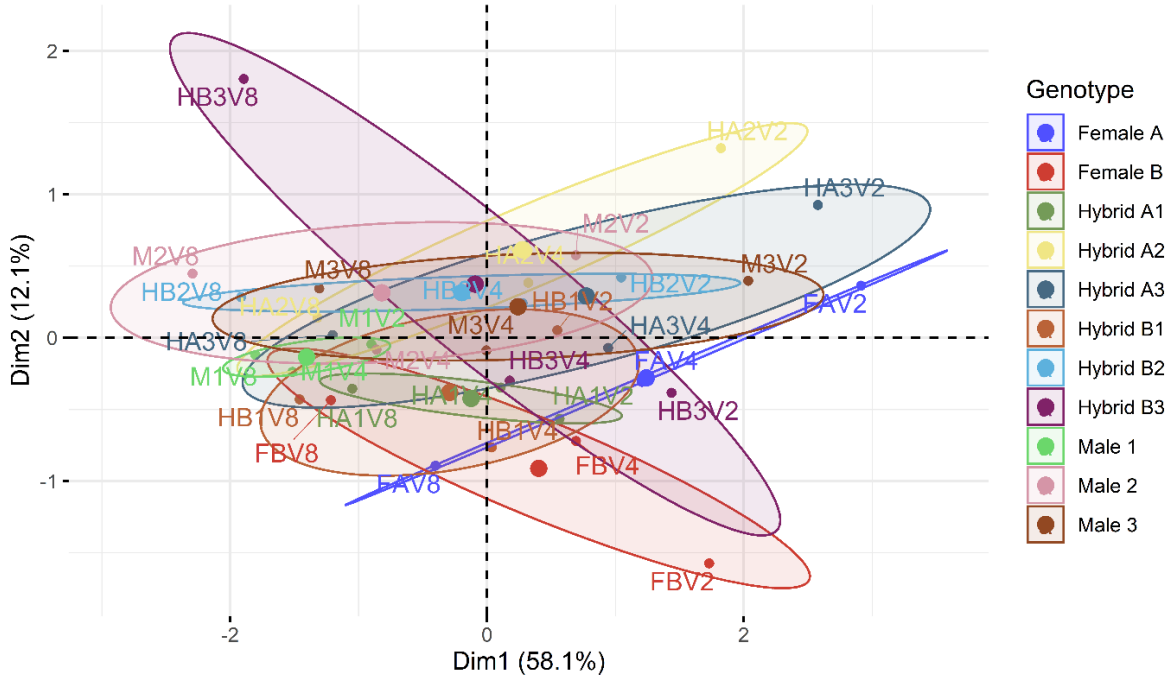

Figure S5.8. Multiple factorial analysis (MFA). Individuals grouped by genotype. This plot exhibits the position of individuals in the MFA by the genotype variation. Dots represent individuals (e.g., HB3V8: genotype Hybrid B3 in 8-week vernalization treatment). Confidence interval ellipses around each genotype were added.

Chapter VI. Identification of adapted breeding lines to improve barley hybrids for Spain

6. Chapter VI. Identification of adapted breeding lines to improve barley hybrids for Spain

6.1. Introduction

Expanding the genetic base of elite breeding programs is key to the continued success of breeding (Cobb et al. 2019; Sommer et al. 2020). This is even more urgent when the current cultivars are exposed to changing and increasingly unfavourable conditions due to climate change, something already visible in the Mediterranean basin (Cammarano et al. 2019).

Hybrid barley cultivation is gaining ground in Europe in recent years. It is increasingly important in the UK, France, and Germany, where it covers up to 30% of the winter six-row feed barley acreage. This interest is motivated by hybrid cultivars producing a yield bonus that pays off the increase in seed cost, above a certain production level. For this reason, hybrid barley could be successfully grown in the most productive areas of the Iberian Peninsula, mostly located in the northern half of Spain. However, hybrid barley breeding has not focused on these areas historically and, therefore, current cultivars have not been optimized for local adaptation, which may include reduced vernalization requirement or even facultative growth habit, and early flowering and maturity. Under these circumstances, the opportunity arises to resort to the genetic diversity and adaptation contained in locally adapted breeding lines, for the development of high-yielding hybrid varieties adapted to Southern Europe.

Hybrid breeding depends on the discovery and management of heterotic patterns showing good general combining ability. This was accomplished historically for temperate maize and is under development in rice, wheat (Gupta et al. 2019), and barley (Sommer et al. 2020). Barley heterotic groups have been developed for Central and Atlantic Europe (Li et al. 2017), but there is no information about the performance of winter barley from Southern Europe. The expansion of the crop further south may benefit from using new germplasm sources, better adapted to Mediterranean climates.

A major challenge of hybrid breeding is the evaluation of inbred lines in numerous cross combinations, which is a time, labour, and cost-prohibitive process. For this reason, predicting hybrid performance is a major issue for hybrid breeding programs (Schrag et al. 2009), which is currently supported through genomic prediction strategies.

One particular issue of hybrid barley is the possible loss of one of the critical domestication traits of the crop. During barley domestication, the natural grain dispersal system was lost. A mutation in either one of two linked genes, *btr1* and *btr2*, turns the fragile rachis (brittle) of the wild form

into the tough rachis phenotype (non-brittle). All cultivated barleys carry just one mutated gene, which is enough to produce full grain retention (accompanied by the effects of several modifier genes). The mutation *btr1* is widely spread in Europe and Central Asia, while the mutation *btr2* is distributed around Oriental Asia and North Africa (Pourkheirandish et al. 2015). In the Iberian Peninsula, both mutations converge. This situation was irrelevant when the target cultivars of breeding programs were inbred lines, but is highly relevant now, with the advent of hybrid barley. In this context, the cross of parents with alternative mutations in the *Btr* genes could lead to an F_1 hybrid with rachis fragility issues and, thus, that might present grain retention problems and reduced yield.

This study presents the result of testing advanced breeding lines developed in the public Spanish National Barley Breeding Program (Gracia et al. 2012), which are winter and facultative six-rowed lines with good local adaptation. They were crossed with lines coming from the European breeding program of Syngenta AG, all of them carrying six-rowed winter genetics. Hybrids combine adapted Spanish lines and Syngenta AG genetics carrying the cytoplasmic male sterility (CMS) system and male fertility restoration genes (*Rfm1*). Since the adapted Spanish material is neither male-sterile (non-CMS) nor does it have the ability to restore fertility (*rfm1*), the only possibility to produce enough seeds for multi-location yield trials in a short time, and have a rapid evaluation of the genetic potential of lines, is the three-way hybrid approach.

The objective was to find promising lines that contribute to the development of high-yielding hybrid varieties adapted to the Iberian region, by combining multi-location agronomic testing of a training subset, and genomic prediction for the entire set.

6.2. Material and methods

6.2.1. Plant materials

Plant material used in this study comprised a set of six-rowed winter barley three-way hybrids developed in the framework of the European barley breeding program of Syngenta, AG. The three-way hybrids were produced using a CMS system as:

$$hybrid = (female\ x\ Spanish\ pollinator)\ x\ male\ restorer$$

where $female$ belongs to the CMS elite lines' pool, $Spanish\ pollinator$ is an advanced inbred line without restorer genes, and $male\ restorer$ belongs to a pool of elite genotypes carrying restorer genes guaranteeing fertility of hybrid plants.

Initially, a set of 140 Spanish advanced inbred lines, well adapted to Mediterranean growing conditions, resultant of the Spanish National breeding program (Gracia et al. 2012), were considered as potential parents of hybrids. Based on historic grain yield *per se* performance estimates and flowering time nick, twenty-four lines were selected to produce three-way hybrids. The remaining 116 unpromoted lines were then subjected to genomic prediction.

One single elite female (α) was pollinated with 24 Spanish lines (1-24), resulting in 24 sterile female hybrids (F_1F). These 24 F_1F were crossed by 3 male restorers (A-C) in a full factorial mating design, generating the 72 three-way hybrids (1A-24C). Hybrids were evaluated together with six commercial checks, including both inbred lines and hybrids (Table S6.1).

Hybrid seed was produced by Syngenta AG. F_1F seed was produced in isolation plots by open pollination of the CMS line by the adjacent pollinator, planted in alternating strips. Three-way hybrid seed was produced with a simple blend of 95% F_1F and 5% male restorer seed sowed. The harvested seed contained sufficient hybrid seed (>85%) to comply with regulations for hybrid commercialization. Hybrid seed needed for each year of trials (8 plots: 4 locations x 2 replicates) was around 0.7-0.8 kg. Other details on seed production plots were not disclosed by the company.

6.2.2. Field experiments

Three-way hybrids were evaluated in a multi-environment field trial network. The data was structured into 3 different trials divided by the male restorer line (A, B, C). Each trial included 30 entries: 24 three-way hybrids derived from one of the male restorers, and the 6 common checks. Trials A and B were evaluated in 8 environments, defined as the combination of 4 locations and 2 years. Trial C was evaluated in 2 environments, 1 location in 2 years (Table 6.1). The experimental design at each trial was an alpha lattice with two replicates, each arranged in six incomplete blocks of size five, including one check per incomplete block. Plot sizes ranged between 10.6 and 12.0 m^2, plot width between 1.2 and 1.33 m, plot length between 8 and 9 m, and the number of rows between 6 and 8. Sowing density varied from 180 to 200 seeds·m^{-2} for hybrids, and 260 to 350 seeds·m^{-2} for inbred lines, depending on local growth practice and optimized Syngenta AG growing protocols for hybrids. Crop management followed local practices at each location.

Plots were scored for developmental traits, yield and components, and grain quality traits (Table 6.2). For details on the phenotyping, see Table S6.2.

Table 6.1. Description of field trials.

Year	Location	Environment	Trials	Planting date	Harvest date	Latitude	Longitude	masl[a]	Season rainfall [mm]
2019	Berzosa de Bureba (Burgos)	Burgos_19	A, B	12-Dec	19-Jul	42° 38' N	3° 15' W	681	390
	Grisolles (Tarn et Garonne)	Grisolles_19	A, B	13-Nov	26-Jun	43° 53' N	1° 14' E	102	270
	Sádaba (Zaragoza)	Sadaba_19	A, B, C	26-Dec	01-Jul	42° 15' N	1° 15' W	454	180
	Zamadueñas (Valladolid)	Valladolid_19	A, B	19-Nov	19-Jun	41° 42' N	4° 41' W	695	160
2020	Berzosa de Bureba (Burgos)	Burgos_20	A, B	27-Nov	-	42° 37' N	3° 16' W	681	520
	Grisolles (Tarn et Garonne)	Grisolles_20	A, B	06-Dec	16-Jun	43° 47' N	1° 16' E	102	300
	Sádaba (Zaragoza)	Sadaba_20	A, B, C	07-Jan	06-Jun	42° 15' N	1° 15' W	454	265
	Zamadueñas (Valladolid)	Valladolid_20	A, B	29-Oct	22-Jun	41° 42' N	4° 41' W	695	375

[a]Meters above sea level.

Table 6.2. List of scored traits, abbreviations, units, and trait group to which they belong.

Trait group	Abbreviation	Trait	Unit
Developmental	**DAW**	Deficiencies after winter	[1-9 score]
	PV	Plant vigour	[1-5 score]
	GH	Growth habit	[1-9 score]
	DTH	Days to heading	[days after January 1st]
	HT	Plant height	[cm]
	GFP	Grain filling period	[days]
Yield and components	**LODG**	Lodging	[1-9 score]
	GY	Grain yield	[t·ha^{-1}]
	BIO	Biomass	[g·m^{-2}]
	GPE	Grains per ear	[grain number·ear^{-1}]
	GM2	Number of grains	[grain number·m^{-2}]
	TM2	Number of tillers	[tiller number·m^{-2}]
	EM2	Number of ears	[ear number·m^{-2}]
	HI	Harvest index	[%]
	BTRS	Brittleness score	[0-2 score]
	NECK	Necking	[%]
	FERT	Fertility	[%]
	MOI	Moisture	[%]
Grain quality	**TGW**	Thousand-grain weight	[g]
	TW	Specific weight	[kg·hL^{-1}]
	PROT	Protein content	[%]
	GRA2_5	Plumpness 2.5	[%]
	GRA2_8	Plumpness 2.8	[%]
	Area	Grain area	[mm]
	Width	Grain width	[mm]
	Length	Grain length	[mm]

6.2.3. Phenotypic data analyses

We performed a one-stage analysis of the phenotypic data by fitting the following linear mixed model:

$$y_{klmno} = \mu + g_k + e_l + (ge)_{kl} + t_{ml} + b_{onml} + \varepsilon_{klmno}, \quad (1)$$

where y_{klmno} is the phenotypic observation for the kth genotype in the oth incomplete block within the nth replicate of the mth trial in the lth environment, μ is the intercept term, g_k is the genotypic effect of the kth genotype, e_l is the effect of the lth environment, $(ge)_{kl}$ reflects the genotype and environment interaction effect, t_{ml} is the effect of the mth trial nested in the lth environment, b_{onml} is the effect of the oth incomplete block nested in the nth replicate of the mth trial in the lth environment, and ε_{klmno} is the residual term. The replicate effect nested in trial within environment was captured by the block effect, as reflected in a lower Bayesian

Information Criterion (BIC) of the reduced model, and thus was omitted from all analyses. We inspected the data set for outliers using the re-scaled median absolute deviation of standardized residuals combined with a Bonferroni-Holm test (p-value < 0.05) to correct for multiple testing (Bernal-Vasquez et al. 2016). The outliers were removed from the dataset. Finally, best linear unbiased estimations (BLUEs) for the genotypes were estimated by fitting model (1) on the outlier corrected data, assuming genotypes as fixed, and the rest of the effects as random. Multiple comparisons were obtained by Fisher's protected Least Significant Differences (LSD). Planned comparisons between groups of genotypes were tested using the appropriate contrasts. For prediction of best linear unbiased predictions (BLUPs) and estimation of variance components, the genotype effect was also set as random.

Group-specific variance components were estimated using dummy variables for checks and hybrids (Piepho et al. 2006), fitting the following model:

$$y_{dkjlmno} = a_d + c_k + h_j + (ce)_{kl} + (he)_{jl} + e_l + t_{ml} + b_{onml} + \varepsilon_{djklnmo}, \quad (2)$$

where $y_{dkjlmno}$ is the phenotypic observation for the kth check or jth hybrid of the dth group in the oth incomplete block within the nth replicate of the mth trial in the lth environment, a_d is a group effect for checks and hybrids, c_k is the effect of the kth check, h_j is the effect of the jth hybrid, $(ce)_{kl}$ is the interaction effect between the kth check and the lth environment, $(he)_{jl}$ is the interaction effect between the jth hybrid and the lth environment, and $\varepsilon_{dkjlnmo}$ the residual effect. The effects e_l, t_{ml}, and b_{onml} follow the same notation as above. The group effect was modelled as fixed, whereas other effects were considered random.

We further partitioned the variance due to hybrids into general (GCA) and specific combining ability (SCA) effects. Three-way hybrids were decomposed as single crosses between a female hybrid (elite female x Spanish pollinator) and a male restorer parent. Thus, the genotypic value of three-way hybrids was estimated as the sum of the GCA effect of the female hybrid, the GCA of the male restorer, and the SCA effect of their cross. We implemented the following model, which extends model (2):

$$y_{dkijlmno} = a_d + c_k + g'_i + g''_j + s_{ij} + (ce)_{kl} + (g'e)_{il} + (g''e)_{jl} + (se)_{ijl} + e_l + t_{ml} + b_{onml} + \varepsilon_{dkijlnmo}, \quad (3)$$

where $y_{dkijlmno}$ is the phenotypic observation for the kth check or cross between female hybrid i and male restorer j, of the dth group in the oth incomplete block within the nth replicate of the mth trial in the lth environment, g'_i is the GCA effect of the ith female hybrid, g''_j is the GCA effect of the jth male restorer parent, s_{ij} is the SCA effect of the cross between female hybrid i and male restorer j, $(g'e)_{il}$ and $(g''e)_{jl}$ are the GCA-by-environment interaction effects of female hybrids and male restorer parents, $(se)_{ijl}$ is the SCA-by-environment interaction effect, and $\varepsilon_{dkijlnmo}$ the residual effect. $a_d, c_k, (ce)_{kl}, e_l, t_{ml,}$ and b_{onml} follow the same notation as above.

The group effect was modelled as fixed, all other effects were considered as random. Variance components were estimated using restricted maximum likelihood (REML). Significances of the variance components were tested through model comparison based on a likelihood ratio test (Stram and Lee 1994). All linear mixed models were implemented using ASReml-R (Butler et al. 2017). Broad-sense heritability was calculated separately for checks and hybrids using group-specific variance components following Piepho & Möhring (Piepho and Möhring 2007):

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{ge}^2}{E} + \frac{\sigma_\varepsilon^2}{E * R}},$$

where σ_g^2 refers to the variance of genotypes in each group, σ_{ge}^2 to the interaction variance of group genotypes and environment, σ_ε^2 to the error variance, and E and R to the number of environments and replicates, respectively, where the group genotypes were tested.

In addition, all traits were subjected to principal component analysis (PCA) to obtain a general overview of the structure of variation among hybrids (inbred line checks were not included). A second PCA was performed standardizing all variables by male restorer parent, to focus only on the variation due to the female side (F_1Fs). The latter analyses were conducted using R packages 'FactoMineR' (Lê et al. 2008) and 'factoextra' (Kassambara and Mundt 2017).

An additive main effect and multiplicative interaction (AMMI) analysis was carried out to describe the genotype-by-environment interaction (GxE) for grain yield. A regression analysis of additional phenotypic and environmental (climatic) variables on the principal component scores of the AMMI axes was performed to study the relationship of these variables with the GxE of grain yield. We inspected the stability of the genotypes using the weighted average of absolute scores (WAAS) index proposed by Olivoto et al. (Olivoto et al. 2019). The WAAS index of a certain genotype is calculated as the average of the loadings of the genotype on the significant axes derived from the

GxE decomposition, and weighted by the amount of variance explained by each axis. Thus, genotypes with a low WAAS index, deviate little from the average performance across environments and are considered more stable. The latter analyses were performed using the R package 'metan' (Olivoto and Lúcio 2020).

6.2.4. Genomic data

The parental lines of the yield-tested three-way hybrids were fingerprinted using a 22k SNP array based on an Illumina Infinium assay (Illumina Inc., San Diego, CA). Hence, marker information could be deduced for the 72 three-way hybrids evaluated in the field. Moreover, SNP profiles were also available for other 116 Spanish pollinators that were unpromoted during the three-way hybrid development. Hybrid profiles were deduced for 348 untested three-way hybrids derived from the cross of 116 F_1Fs (α female x 116 pollinators) and the 3 male restorers (A-C). Genotyping was carried out at Syngenta AG's laboratory facilities in Saint-Sauveur (Toulouse).

Markers were excluded if missing values were >10%. For the remaining 15,349 SNP markers, missing marker data were imputed using the linkage disequilibrium-based k-nearest neighbour genotype imputation method, LD KNNi (Money et al. 2015). After imputation, monomorphic markers were removed, and 11,389 polymorphic SNPs remained.

6.2.5. Genomic prediction

We used the data of the 72 field-tested three-way hybrids to train a genome-wide prediction model. Then, this model was applied to predict the performance of 348 untested three-way hybrids, which were derived from 116 unpromoted Spanish pollinators. We applied two statistical approaches:

The first approach was to apply a genomic best linear unbiased prediction (GBLUP) model including additive and dominance effects. The GBLUP model is defined as:

$$y = 1_n\mu + g_a + g_d + \varepsilon, \quad (4)$$

where y is a vector of BLUEs across environments, 1_n is a vector of ones and n is the number of genotypes, μ refers to the overall mean across environments, g_a and g_d represent the additive and dominance effects, and ε is a vector of residuals. We assume that μ is a fixed parameter, $\varepsilon \sim N[0, I\sigma_\varepsilon^2]$, $g_a \sim N[0, K_a\sigma_a^2]$, and $g_d \sim N[0, K_d\sigma_d^2]$, where the matrices K_a and K_d are the relationship matrices corresponding to additive and dominance effects, calculated according to VanRaden (VanRaden 2008) and Álvarez-Castro & Carlborg (Álvarez-Castro and Carlborg 2007).

The SNP profile of the three-way hybrids was calculated as the mean of the SNP status between the Spanish pollinator and the male restorer, as the female parent was shared between all the hybrids. The GBLUP model was fitted using the R package BGLR (Pérez and de los Campos 2014) that solves mixed models as a special case of reproducing kernel Hilbert space regression in a Bayesian framework, using a Gibbs sampler. We used 10000 iterations of which 1000 iterations were treated as the burn-in phase.

Because hybrid profiles were deduced from parents' profiles, a strong male restorer effect was noticed (Figure S6.1). Thus, we extended model (4) including the male restorer parent as fixed effect.

The second approach is an extension of the ridge regression best linear unbiased prediction (RRBLUP) model, which allows for general and subpopulation-specific additive marker effects (GSA-RRBLUP: general and subpopulation-specific additive RRBLUP)(Li et al. 2017). The GSA-RRBLUP model for the three-way hybrids is:

$$y = 1_n\mu + Z_A a_0 + \frac{1}{2}Z_{AM}a_{MS} + \frac{1}{4}Z_{AP}a_{PS} + Z_D d + \varepsilon, \quad (5)$$

where y, 1_n, μ, and ε are defined as outlined above for the GBLUP model. Z_A and Z_D are design matrices of the hybrids for the additive and dominance effects of the markers. a_0 and d represent the general additive and dominance effects of the markers, where $a_0 \sim N[0, I\sigma^2_{a_0}]$ and $d \sim N[0, I\sigma^2_d]$. Z_{AM} and Z_{AP} are design matrices of the male restorer and Spanish pollinator parents of the three-way hybrids for the additive effects of the markers, and $Z_A = \frac{1}{2}Z_{AM} + \frac{1}{4}Z_{AP}$. The elements of Z_{AM} and Z_{AP} are −1, 0, and 1. a_{MS} and a_{FS} represent the subpopulation-specific additive marker effects of the restorer and pollinator, where $a_{MS} \sim N[0, I\sigma^2_{a_{MS}}]$ and $a_{PS} \sim N[0, I\sigma^2_{a_{PS}}]$. Because marker effects are available only for the pollinator population, marker effects for the female population cannot be considered in the prediction, and the coefficient for Z_{AP} is $\frac{1}{4}$. The GSA-RRBLUP model was fitted using the 'BRR' argument of the BGLR package (Pérez and de los Campos 2014).

We also extended model (5) including the male restorer parent as a fixed effect for improved accuracy.

We evaluated the accuracy of genome-wide prediction models using the 72 field-tested hybrid data set. We applied 100 runs of 5-fold cross-validation. A random sample of 80% of the hybrids (58) was used as the training set to predict the performance of the remaining 20% of hybrids (14, test set). Random sampling was repeated 100 times for each validation scenario. Prediction ability was estimated as Pearson's correlation coefficient between the predicted genotypic values and the observed phenotypic values.

Syngenta AG contributed seed production, genotyping, and phenotyping of Grisolles' trials. The Instituto Tecnológico Agrario de Castilla y León (ITACyL), managed and collaborated in the phenotyping of the trials carried out in Burgos and Valladolid. CSIC managed and phenotyped the rest of the trials, collaborated in the trials of Burgos and Valladolid, and performed the genomic and phenotypic data analysis.

6.3. Results

6.3.1. Multi-environmental field trial network reflects the heterogeneity of the Iberian region

Season 2019 in Southern Europe was characterized by a dry winter, warm spring, and heatwaves during June (Figure S6.2), accelerating grain filling period and penalizing TGW, especially at Valladolid (Table 6.3). Season 2020 was marked by an extremely wet autumn, which delayed sowings (Table 6.1), and a warm winter. Precipitations in 2020 were, in general, more abundant and more evenly distributed than the year before. The end of the season was again characterized by high temperatures at Valladolid (Figure S6.2), which reduced spike fertility and resulted in a lower TGW (Tables 6.3, S6.3).

Table 6.3. Main traits' averages within and across environments.

Environment	GY[a] [t·ha^{-1}]	DTH[b] [Julian days]	HT[c] [cm]	TW[d] [kg·hL^{-1}]	TGW[e] [g]	GFP[f] [days]
Burgos_19	7.833	135	98	70	42	43
Grisolles_19	9.105	112	119	72	45	-
Sadaba_19	4.614	125	75	71	42	42
Valladolid_19	5.421	113	81	67	35	36
Burgos_20	-	123	-	-	-	-
Grisolles_20	6.483	111	116	64	37	-
Sadaba_20	7.007	122	101	67	38	35
Valladolid_20	6.417	105	121	67	34	-
Grand mean	6.587	121	97	68	39	39

[a]Grain yield, [b]days to heading, [c]plant height, [d]specific weight; [e]thousand-grain weight, [f]grain filling period.

In terms of the productive potential of the environments, in 2019 we observed a clear distinction between locations, associated with water availability. Burgos and Grisolles stood out for their high productivity, reaching 8 t·ha^{-1} on average. On the other hand, Sádaba and Valladolid, locations where plants experienced a prolonged water deficit, showed low productivity, around 5 t·ha^{-1}. Season 2020, in contrast, was characterized by more homogeneous conditions between locations, and the yield of the three locations harvested (Burgos_20 was not harvested) was similar, between 6.5 and 7 t·ha^{-1} (Table 6.3). The same behaviour was observed for yield components as biomass, number of grains, or tillers (Table S6.3). Regarding flowering time, Valladolid and Grisolles locations showed earlier heading than Burgos and Sádaba (Table 6.3). This is explained by a faster thermal time accumulation and earlier sowings in Valladolid and Grisolles (Table 6.1).

6.3.2. General combining ability was more relevant than specific combining ability in hybrid barleys

We inspected the population structure of the parental lines of the study by applying a principal component analysis. The first two principal components explained 16% of the molecular variation (Figure 6.1).

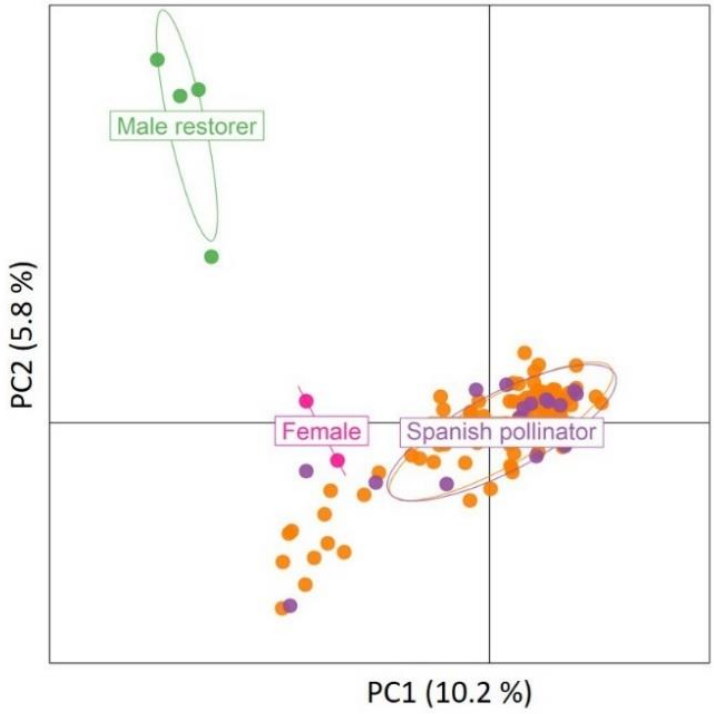

Figure 6.1. Marker-based principal component analysis of six-rowed winter barley germplasm involved in the development of hybrids for the Iberian region. Male restorer and female pools represent the heterotic groups on which European hybrid barley breeding is based. Spanish pollinator pool includes 140 advanced inbred lines with local adaptation to Mediterranean climates. The 24 Spanish pollinators used in the development of three-way hybrids tested in the field are highlighted in purple, the rest of the Spanish lines (orange) were subjected to genomic prediction.

The elite male restorer lines formed a narrow genetic cluster, which was clearly separated from the female elite lines. The Spanish lines were more scattered but still clearly separated by the first and second axes from the male restorer pool. Since the Spanish lines were more genetically similar to the elite female pool, they were used on the female side of the cross for the development of three-way hybrids. Moreover, the 24 Spanish pollinators tested in the field in a hybrid format were widely dispersed within the Spanish pool, illustrating the potential of Mediterranean-adapted barley germplasm to broaden the elite breeding pools.

The 72 three-way hybrids produced were examined for relevant developmental, agronomic, and grain quality traits across up to 8 environments (Table S6.2). Environment (σ^2_{ENV}) explained a large proportion of the phenotypic variation, accounting for more than 50% of the main agronomic traits (Table 6.4).

Table 6.4. Estimates of variance components (σ^2_{ENV} environment, σ^2_G genotypic, σ^2_{GxE} genotype-by-environment interaction, σ^2_ε error) and heritability for the main traits. Genotypic and GxE interaction variances are divided into hybrids and checks. Genotypic variance of hybrids is split into general combining ability of male restorers (GCA_M) and females (GCA_F), and specific combining ability (SCA). The same applies to the interaction effects.

Source	GY[a] [t·ha^{-1}]	DTH[b] [Julian	HT[c] [cm]	TW[d] [kg·hL^{-1}]	TGW[e] [g]	GFP[f] [days]
σ^2_{ENV}	2.15***	93.4***	343***	8.12***	15.4***	13.7***
---------- Hybrids ----------						
σ^2_G	0.0212***	0.782***	0.669*	0.097	0.433**	0.012
$\sigma^2_{GCA\_M}$	0.0144	1.012**	2.567*	0.674***	2.857***	0.271
$\sigma^2_{GCA\_F}$	0.0097	0.204**	0.072	0.038	0.310**	0.000
σ^2_{SCA}	0.0054	0.090*	0.492	0.000	0.010	0.000
σ^2_{GxE}	0.0254*	0.254*	0.340	0.000	0.000	0.001
$\sigma^2_{GCA\_MxE}$	0.0307	0.442***	0.571	0.090	0.000	0.466*
$\sigma^2_{GCA\_FxE}$	0.0332***	0.255**	0.395	0.000	0.129	0.130
σ^2_{SCAxE}	0.0000	0.015	0.110	0.195	0.489	0.000
h^2	0.58	0.86	0.71	0.78	0.88	0.47
---------- Checks ----------						
σ^2_G	0.0892*	15.1***	40.6***	2.26***	5.49***	6.50***
σ^2_{GxE}	0.2088***	2.90***	7.36***	2.27***	3.21***	2.78***
σ^2_ε	0.1749	1.65	12.3	1.11	2.38	2.43
h^2	0.68	0.97	0.95	0.82	0.87	0.91

[a]Grain yield, [b]days to heading, [c]plant height, [d]specific weight; [e]thousand-grain weight, [f]grain filling period. * p<0.05, ** p<0.01, *** p<0.001

Genotypic variation (σ^2_G) was significant for most traits (except for GM2, BIO, HI, FERT, PV, and DAW), although most of it was due to differences among checks (Tables 6.4, S6.4). Despite

the high degree of relatedness between hybrids, which shared 25 to 50% of the pedigree, the genotypic variation within hybrids was still significant for several relevant traits, including GY, DTH, HT, and TGW. The genotype-by-environment interaction variance (σ^2_{GxE}) was significant and, in the case of GY, even higher than the genotypic variance. Variance components were more pronounced for general (σ^2_{GCA}) than for specific combining ability effects (σ^2_{SCA}). Moreover, the GCA variance of the male restorers ($\sigma^2_{GCA\_M}$) was, in general, larger than the GCA variance of the females ($\sigma^2_{GCA\_F}$), as expected by the lower genetic variation within the female pool (25% common). Overall, moderate to high heritabilities were obtained, ranging from 0.47 for grain filling period in the hybrids to 0.98 for grain length in the checks.

6.3.2.1. Male restorer parents explained most phenotypic differences. Further variation was contributed by Spanish pollinators

On average, hybrids yielded significantly less than the checks (Figure 6.2). Overall, checks yielded 2.4% more than hybrids, this is 0.17 t·ha^{-1} (Table S6.5). However, the variation between hybrids was not negligible (Table 6.4), and 25% of them outperformed the checks mean (Table S6.6).

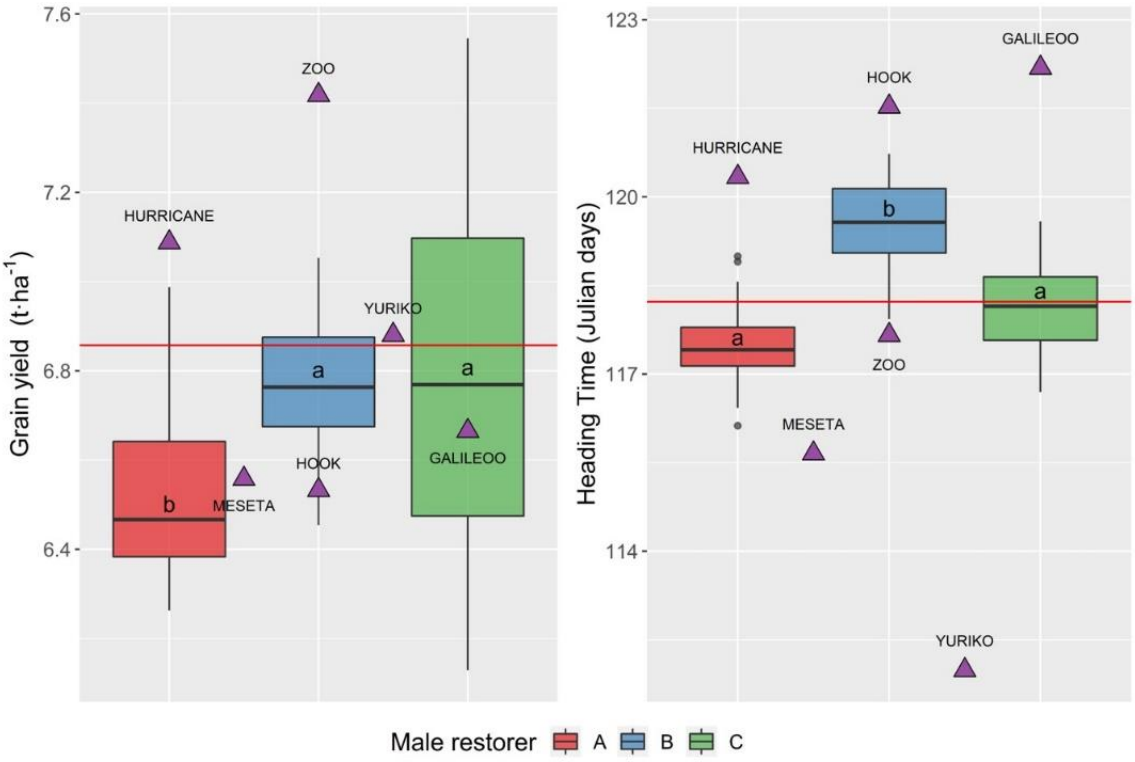

Figure 6.2. Grain yield (left) and heading time (right) across environments. Boxplots represent the distribution of hybrids divided by male restorer parent: hybrids from Male restorer A (red), hybrids from Male restorer B (blue), and hybrids from Male restorer C (green). The middle line in the boxplot represents the mean. The red line represents the checks average. Purple triangles depict the mean across environments of each check. Groups of hybrids with different letters are significantly different at $p<0.05$.

Yield differences between hybrids were due to both the male restorer and female parents (Table 6.4). Hybrids from Male restorer B and C outyielded hybrids from Male restorer A by 3.7% (0.254 t·ha^{-1}, Figure 6.2). Indeed, the proportion of hybrids that surpassed the checks average differed between male restorer parents: 5 % for Male restorer A, 30% for Male restorer B, and 40% for Male restorer C (Table S6.6).

We also detected differences in grain yield due to the female hybrids. The range of yields displayed by the hybrids grouped by F_1Fs was wide, around 0.7 t·ha^{-1} (Table 6.5). Hybrids derived from F_1Fs 2, 19, and 18 outyielded the checks average (Table S6.5). These three lines produced high-yielding hybrids with all the three tested male restorers (Table S6.6). Moreover, the combination of the top female (F_1F 2) and male restorer (Male restorer C) parents outperformed the best check *ZOO* by 0.13 t·ha^{-1} (Table S6.6), although this difference was not significant.

In this data set, the general combining ability of females and male restorers was more relevant than specific combining ability, indicating low interaction between the male restorer parent and the F_1F. Male restorers B and C were identified as high-yielding male restorer parents, contributing a 0.06 and 0.04 t·ha^{-1} advantage, respectively, to their crosses. Male restorer A, in contrast, stood out by a poor GCA. Hybrids derived from this male restorer showed a yield reduction of 0.1 t·ha^{-1} compared to the population mean (Figure S6.3A). The top-yielding female parents, lines 2, 18, and 19 increased grain yield between 0.11 and 0.13 t·ha^{-1} in the crosses they were involved in. On the opposite side, three lines (12, 14, and 15) were highlighted as inferior female parents, reducing grain yield in their crosses by 0.1 t·ha^{-1} (Figure S6.3B).

The range of heading times shown by the hybrids was narrow, with a difference of 5 days between the earliest and latest flowering hybrid (Table S6.5). On average, checks and hybrids reached heading time similarly (Table S6.5), although differences due to the male restorer parent were evident in hybrids (Table 6.4). Male restorer A and C hybrids were significantly earlier than Male restorer B hybrids. Most hybrids from Male restorer A, and some of Male restorer C, flowered earlier than the earliest hybrid check *ZOO*. However, when comparing the heading time of hybrids to *YURIKO*, an inbred line check well adapted to Mediterranean conditions, *YURIKO* was around 6 days earlier than any hybrid. The 25% of the pedigree contributed by the Spanish pollinators also explained differences in heading time of hybrids (Table S6.5). Nevertheless, due to this low percentage of variation, the range of hybrid averages grouped per F_1F was narrow; around 2 days difference (Table 6.5).

Table 6.5. Grain yield (GY) and heading time (DTH) hybrid means grouped per F_1F, averaged across male restorer parents and environments. F_1Fs followed by a different letter are significantly different at $p<0.05$.

F_1F	GY [t·ha^{-1}]		F_1F	DTH [Julian days]	
2	7.073	a	11	117.2	a
19	7.004	ab	7	117.4	ab
18	7.003	ab	22	117.9	abc
9	6.826	abc	2	117.9	abc
24	6.808	abc	4	118.0	abcd
11	6.790	abc	13	118.1	abcd
17	6.789	abc	5	118.1	abcd
5	6.737	abcd	17	118.1	abcd
13	6.718	abcd	3	118.2	abcd
4	6.693	bcde	23	118.2	abcde
16	6.683	bcde	8	118.2	abcde
21	6.678	bcde	9	118.2	abcde
23	6.676	bcde	12	118.3	abcde
6	6.660	bcde	20	118.3	abcde
22	6.660	cde	16	118.3	abcde
3	6.649	cde	24	118.5	bcdef
7	6.646	cde	19	118.7	cdef
20	6.636	cde	10	118.7	cdef
8	6.587	cde	14	118.8	cdef
10	6.524	cde	15	118.9	cdef
1	6.497	cde	6	118.9	cdef
12	6.486	cde	21	119.1	def
15	6.416	de	18	119.3	ef
14	6.357	e	1	119.6	f

6.3.2.2. Spanish pollinators presented different specific combining ability patterns

We observed a variety of specific combining ability patterns within the tested hybrids (Figure 6.3). Although GCA was dominant in the set, we observed different reactions of Spanish pollinators depending on the male restorer parent with whom they were crossed. For instance, lines 2 and 7 showed an intermediate performance with Male restorers A and B, while when crossed with Male restorer C, their reaction was contrasting. Other lines, both high- (19) and low-yielding (15), showed no specific affinity with any male restorer parent. Particularly good combinations with each of the male restorer parents were identified, i.e., 18A, 2C, and 7B (Figure S6.4). These differences in SCA between hybrids suggest the presence of different heterotic patterns within the Spanish panel.

6.3.3. Different combinations of traits gave rise to high-yielding hybrids

A principal component analysis of all measured phenotypic variables offers a better insight into the structure of variation among hybrids and the relationships between traits (Figure 6.4). PC1 and

PC2 explained together 41 % of the total variation (25.2 % and 16.1%, respectively). The first dimension was mostly influenced by yield components and opposed grain weight against grain number. The distribution of genotypes on this axis showed a clear distinction of hybrids based on their male restorer parent (A, B *vs.* C), where Male restorer C hybrids showed bigger and heavier grains, although fewer in number, compared to Male restorer A and B hybrids. Phenology explained most of the variation on the second axis, although grain volume had also a relevant contribution. PC2 distinguished between the late flowering hybrids with plump grains derived from Male restorer B (blue ellipse) and the early hybrids with thin grains derived from Male restorer A (red ellipse). Although more scattered, Male restorer C hybrids were also located in the lower part of the graph (green ellipse), indicating their earliness compared to the Male restorer B hybrids.

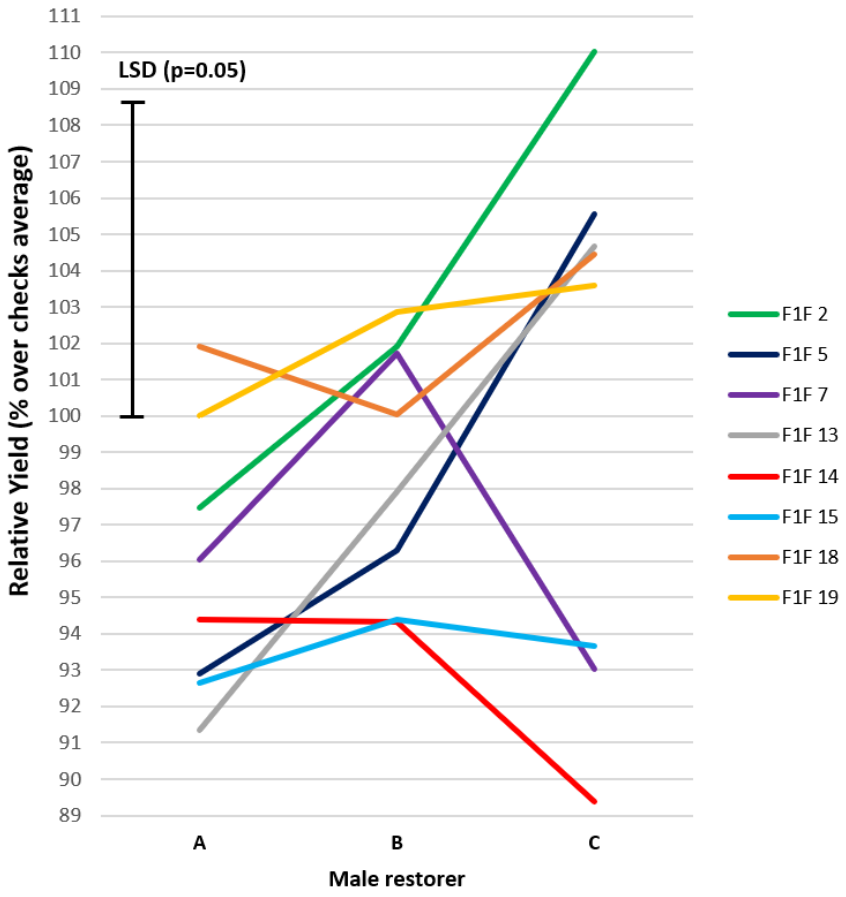

Figure 6.3. Interaction female hybrid (coloured lines) x male restorer parent. Relative yield was calculated as the hybrid yield percentage over the checks average across environments. Particular F_1Fs were chosen to show different specific combining ability patterns. Least significant difference (LSD) at P=0.05 is represented as a black line.

Interestingly, different combinations of traits resulted in several formats producing high-yielding hybrids. On the one hand, those that maximized the weight of the grains at the expense of spike

number. On the other hand, those hybrids that showed a high tillering ability, compensating a lower TGW. Furthermore, different heading dates resulted in high yields. This is consistent with the independence observed in this dataset between flowering time and most yield components, evidenced by the 90-degree angle between the latter traits (Figure 6.4).

From Figure 6.4, most of the variation seems related to the male restorer parent. However, when standardizing all traits by male restorer parent, we unravelled further variation due to the female pool (Figure S6.5). Moreover, the relationship between certain traits changed. Once the effect of the male restorer parent was removed, earliness was beneficial to yield through increased grain size and volume. Yield building in the top-yielding Spanish pollinators came from different combinations of traits. F_1F 2 stood out for its grain size and volume, while F_1F 18 invested more in grain number (Figure S6.5). Spanish pollinators showed a continuous gradient of responses including more balanced trait combinations, expanding the useful variability for hybrids.

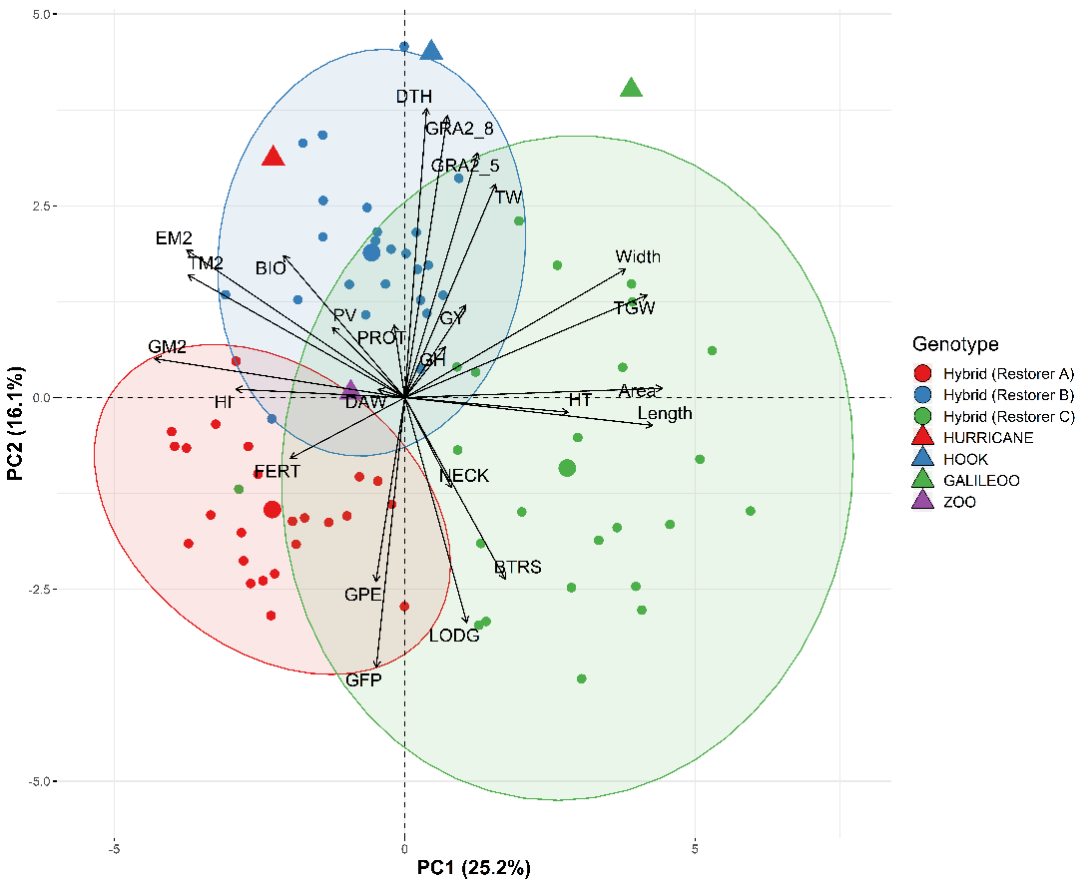

Figure 6.4. Biplot of the first two axes of a principal component analysis carried out with all measured phenotypic variables averaged across environments. Arrows show the contribution of each trait to the PCA axes. Dots represent test hybrids, whereas triangles depict hybrid checks. Check and test hybrids sharing male restorer parent are coloured similarly. Ellipses enclose hybrids derived from the same male restorer parent. Big dots indicate the centroid of the ellipse, representing the mean of each group of test hybrids.

6.3.4. Genotype-by-environment interaction played an important role in hybrid performance in the Iberian region

Once we identified the best genotypes across environments, we focused on the interaction with the environment. We performed an AMMI analysis to decompose the genotype-by-environment interaction for grain yield. Only the first two components were significant, accounting for 61.6 % of the variation (Table S6.7). The first axis explained most of the variation (42.5 %) and was mainly caused by the differences between Valladolid_20 and Sadaba_19 environments (Figure 6.5). The distribution of the environments along the PC1 was related to precipitation, as evidenced by the high correlations between the loadings of the environments on the first AMMI axis and the total season rainfall (r=-0.79), the precipitation recorded in December (r=-0.78), or particularly, that recorded in April (r=-0.87) (Figure S6.6B-D). The first axis clearly separated the test hybrids according to their male restorer parent (Figure 6.5). This was because the aforementioned disadvantage of the Male restorer A hybrids compared to the Male restorer B hybrids was markedly large at Sadaba_19, and almost non-existent at Valladolid_20. The reason behind this sharp distinction seems related to the advantage of certain phenological formats depending on the water availability of the environment, as indicated the correlation between heading time and the AMMI PC1 for yield (Figure S6.6A). The early phenotype of the Male restorer A hybrids allowed them to leverage the abundant rainfall recorded in Valladolid_20 before flowering, increasing the number of grains per ear compared to the late Male restorer B hybrids (Table S6.8). In Sadaba_19, the earliness of Male restorer A hybrids penalized them, as they flowered before the rains came and after an extremely dry winter, resulting in a reduction of the number of ears and the final grain yield (Table S6.8).

PC2 (19.1 %) was related to the productive potential of the environment (r=-0.82, Figure S6.7). This axis separated the low-yielding environments prone to terminal stress from the cooler high-yielding environments (Figure 6.5). PC2 did not split the test hybrids as clearly as PC1, although contrasting responses related to the F_1F parent were observed. The most extreme reactions were shown by F_1Fs 7 and 9, whose hybrids, independently of the male restorer parent, were benefited in low- and high-yielding environments, respectively (Table S6.9). Interestingly, hybrids derived from the top-yielding F_1Fs were located towards the positive side of the axis, indicating adaptation to low potential environments. For checks, the division along PC2 was obvious (Figure 6.5). *HOOK* and *GALILEOO*, the later checks, were favoured in highly productive environments. The inbred checks, *MESETA* and *YURIKO*, both well adapted to the Mediterranean conditions, and the early hybrid checks, *ZOO* and *HURRICANE*, were favoured in the lower yielding environments.

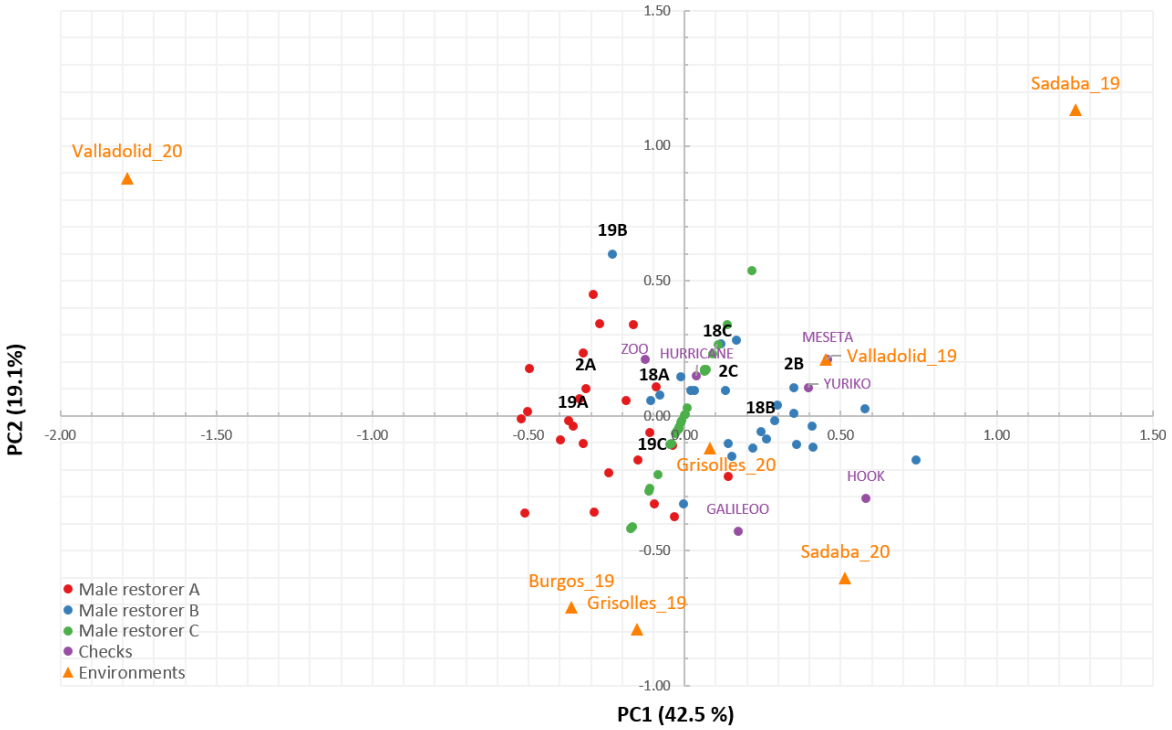

Figure 6.5. Plot of the first two principal components of the AMMI analysis of grain yield for 78 barley genotypes, evaluated in 7 environments. Coloured dots represent genotypes: checks (purple) and test hybrids divided by male restorer parent (Male restorer A in red, Male restorer B in blue, and Male restorer in green). Environments represented as orange triangles. Hybrids derived from top-yielding F_1Fs highlighted in boldface.

Finally, we compared the stability and performance of genotypes by inspecting simultaneously the WAAS index and average grain yield (Figure S6.8). Although based on few genotypes, hybrids (WAAS=0.19) were more stable than inbred lines (WAAS=0.34), at equal yields. Within hybrids, three-way hybrids (WAAS=0.18), although inferior in yield, were less responsive to the environment than two-way hybrid checks (WAAS=0.24). Stability in three-way hybrids varied according to the male restorer parent. Male restorer A hybrids showed low stability and a responsive nature to the environment. Male restorer B and Male restorer C hybrids were more stable and also more productive. Within the top-yielding F_1Fs, lines 19 and 2 produced environment-responsive hybrids (Figure S6.8, quadrant II), able to translate favourable conditions into an improvement in performance. In contrast, hybrids derived from F_1F 18 were broadly adapted and performed well regardless of the environment (Figure S6.8, quadrant IV).

6.3.4.1. Full exploitation of hybrid vigour in this set required long cycles

In the two seasons in which this set of hybrids was evaluated, the correlation between yield and earliness was low and environmentally dependent (Table S6.10). In those environments where

sowing was early and plants were subjected to terminal stress, earliness was beneficial for yield (e.g., Valladolid_20). On the other hand, in environments where water availability was guaranteed throughout and, particularly, at the end of the season, genotypes with longer cycles resulted in higher yields (e.g., Grisolles_19). In addition to the environment, the relationship between flowering time and grain yield varied depending on the male restorer parent. For Male restorer B and C hybrids, earliness was, in general, beneficial for yield. In contrast, within the already early Male restorer A hybrids, earliness was not an advantage (Figure S6.9).

As already mentioned, the performance of the test hybrids was poor compared to the checks, reaching a similar level only in two environments. The test hybrids yielded relatively better the longer the cycle (Figure 6.6). Therefore, full exploitation of hybrid vigour in this set of genotypes requires long cycles. However, earlier hybrids were less sensitive to the length of the period from sowing to heading (Table S6.11), indicating that hybrids with shorter cycles should be addressed for Spanish conditions.

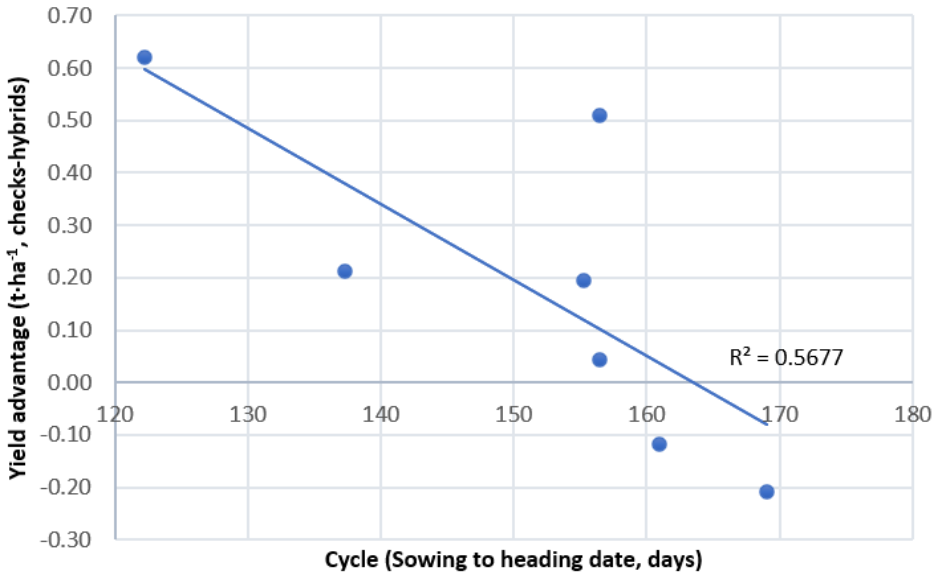

Figure 6.6. Grain yield advantage of checks over test hybrids vs. the length of the cycle. Grain yield advantage calculated as the difference between the averages of checks grain yield and test hybrids grain yield. Cycle was calculated as the days elapsed between sowing and heading date, averaged across trials for each environment. The regression line and coefficient of determination (R^2) are presented. Each dot represents one environment.

6.3.4.2. Spike disarticulation caused environment-dependent yield loss in three-way hybrids derived from btr2 pollinators

We found differences in grain yield between three-way hybrids depending on the genotype of their Spanish pollinator at the *non-brittle rachis* genes. Hybrids derived from *btr2* pollinators showed a 3% lower yield than those from *btr1* pollinators. Furthermore, the decrease in yield of the brittle

hybrids (*btr1*x*btr2*) was higher in environments where harvest was late, or where extreme heat was experienced at the end of the season, indirectly evidenced by the moisture content at harvest (Table 6.6).

Table 6.6. Effect of non-brittle rachis genotype on hybrids grain yield within and across environments. Grain yield ($t \cdot ha^{-1}$) of the three-way hybrids according to the genotype of the Spanish pollinator, grain yield difference (%) between genotypic classes, harvest date, and moisture at harvest for each environment are presented.

Environment	Grain yield crosses (*btr1*x*btr1*) x *btr1*	Grain yield crosses (*btr1*x*btr2*) x *btr1*	Difference (%) between hybrid classes		Harvest date	Moisture (%)
Grisolles_20	6.479	6.422	0.9	ns	16-Jun	14.5
Grisolles_19	9.193	9.102	1.0	ns	26-Jun	14.1
Sadaba_20	6.932	6.853	1.1	ns	06-Jun	11.6
Valladolid_20	6.595	6.439	2.4	ns	22-Jun	10.1
Burgos_19	7.991	7.713	3.5	*	19-Jul	11.2
Valladolid_19	5.567	5.349	3.9	*	19-Jun	9.2
Sadaba_19	4.677	4.445	5.0	**	01-Jul	7.6
Across	6.820	6.639	2.7	**		

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns non-significant

We complemented the yield evaluation with a visual scoring of rachis disarticulation in plot samples. Brittleness score showed a positive and strong correlation with the *non-brittle rachis* genotype of the Spanish pollinator ($n=72$, $r=0.78$, $p<0.001$), supporting the risk of spike disarticulation in hybrids derived from *btr1*x*btr2* crosses.

6.3.5. Genomic prediction identified promising untested pollinators for hybrid development

None of the 72 test three-way hybrids reliably (>105%) outperformed the best check *ZOO*. Therefore, we aimed at assessing whether there would be any other untested three-way hybrid, derived from the remaining 116 unpromoted Spanish pollinators, with potential for the Iberian Region. For this purpose, we used the phenotypic data of the 72 three-way hybrids evaluated in the field (training set) to train prediction models, and forecast the performance of the 348 untested three-way hybrids (test set).

The prediction ability mostly varied depending on the trait (Figure 6.7). We obtained high prediction abilities for heading time (0.77), moderate for plant height (0.65), and grain yield (0.45). Differences between models were minor, although the best-performing model varied depending on the trait modelled. For grain yield, accounting for general, and male restorer and pollinator-specific additive marker effects (GSA_RRBLUP model), increased the prediction ability 5%

compared to the GBLUP model, which relies on the relationship between individuals. The addition of the male restorer parent as fixed effect to the model improved the prediction ability in all cases. The inclusion of this term in the model was necessary because, since the SNP profiles of the hybrids were derived from the parental profiles, the population structure due to the male restorer parent was strong, as evidenced by the clusters in the additive relationship matrix (Figure S6.1). For heading time and plant height, the GBLUP+Male restorer model, which involves additive and dominance relationship matrices, resulted in the highest prediction abilities.

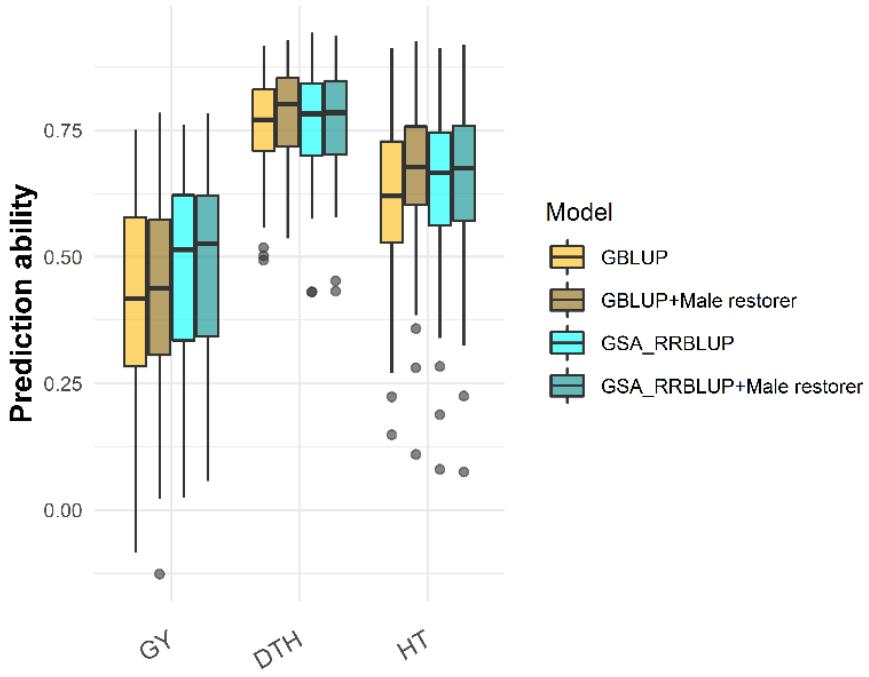

Figure 6.7. Prediction abilities for grain yield (GY), days to heading (DTH), and plant height (HT) according to different models. Prediction abilities result from 100 runs of five-fold cross-validation using the same training and test set across models. The middle line in the boxplot represents the mean.

After training the models, we used those most accurate (highest predictive ability) to predict the performance of untested hybrids (Figure 6.8). In general, the test set (unphenotyped hybrids) presented a narrow distribution, within the range of the training set (phenotyped hybrids), because of the shrinkage of the predictions towards the mean. For heading time and height, the mean of the test set was similar to that of the training set. However, for grain yield, the mean of predicted untested hybrids was lower than the field-assessed hybrids. This result could be due to the preselection for high yield *per se* of the pollinator lines made in the training set. The differences between male restorers were clear and consistent with the results observed in the field. Untested hybrids did not outperform the best hybrid tested in the field (Table S6.12). However, some untested Spanish pollinators were predicted to produce high-yielding hybrids across male restorers

(Table S6.13). Particularly, lines 26 and 55 were predicted to be in the top 10% of both the most productive and earliest lines, holding promise for future hybrid development.

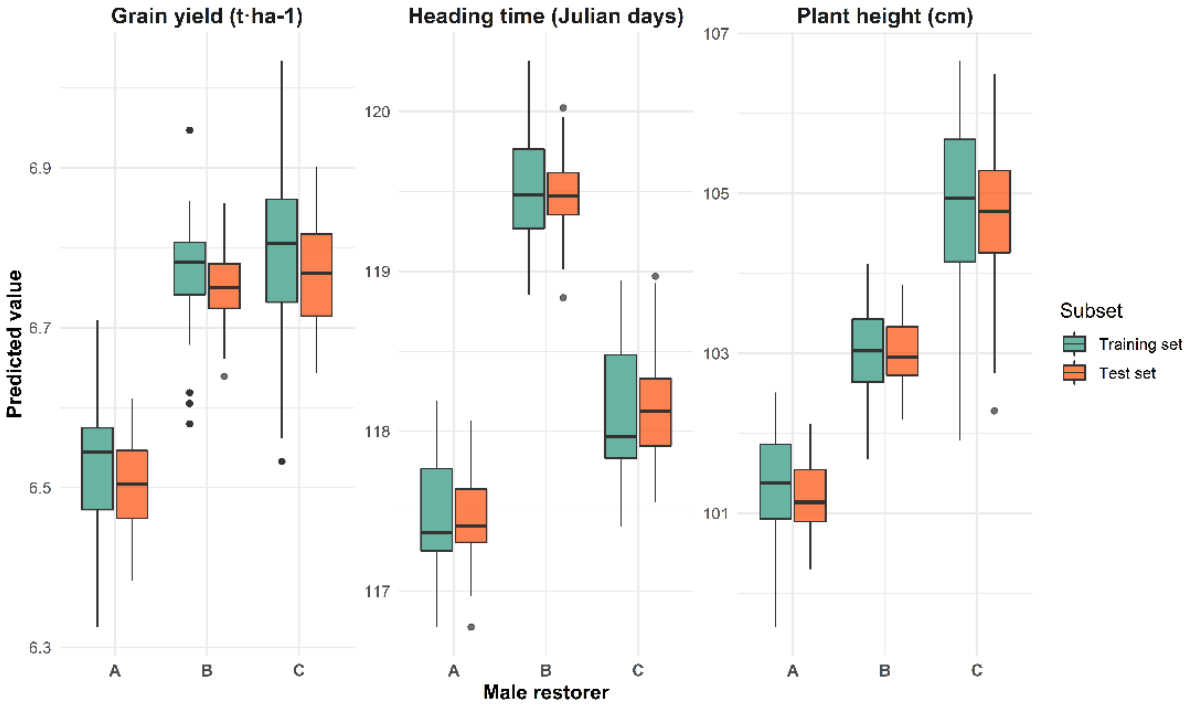

Figure 6.8. Distribution of the observed versus the predicted phenotypes for grain yield, days to heading, and plant height split by male restorer parent. Predicted values of the 72 field-assessed hybrids are depicted in green (training set), predicted values of the 348 untested hybrids (test set) are represented in orange. For each trait, the model with the highest prediction ability was used. The middle line in the boxplot represents the mean.

6.4. Discussion

6.4.1. Spanish pollinators hold promise for two-way hybrid development

Genotypic variation within three-way hybrids was low. This was due to the limited genetic variability at play, since three-way hybrids only differed in 25 to 50% of the pedigree. The three-way hybrid strategy was applied because it allowed evaluating the potential of the Spanish germplasm in a hybrid context, prior to their embedding in a CMS cytoplasm, which is a time- and cost-intensive process (Trini et al. 2021).

Despite this, phenotypic variation for the main agronomic traits was found. Most of this variation was driven by the male restorer parent, particularly for traits controlled by major genes as days to heading or plant height. However, for complex traits as grain yield, the parental contribution was more balanced, and Spanish pollinators harboured further variability unexplained by the male restorer parents.

General combining ability was predominant over specific combining ability. This occurs when two genetically distinct germplasm pools are crossed (Melchinger and Gumber 1998), as is the case for the male restorer and female elite pools on which the three-way hybrids of our study are based. The low SCA to GCA ratio found is in line with other surveys based on the same hybrid barley breeding program (Philipp et al. 2016; Li et al. 2017; Sommer et al. 2020). The general combining ability of the female pool was buffered in the three-way hybrid format because we only played with 25% of the genome contributed by the Spanish germplasm. Notwithstanding that, we found three Spanish pollinators that stood out for their high GCA, and gave rise to top-yielding three-way hybrids, regardless of the male restorer parent to whom they were crossed. The male restorer parents, however, showed a moderate GCA. The hybrids tested did not consistently outperform the best check. Therefore, no three-way hybrid was promoted for registration. Yet, the identification of top-yielding Spanish pollinators is a step forward in barley hybrid breeding for the Iberian region. After CMS conversion, these lines can be used as female parents of two-way hybrids, where their GCA will be doubled. In addition, if crossed with a more suitable restorer, we can expect highly productive two-way hybrids, presumably adapted to the conditions of the Iberian region.

6.4.2. Spanish germplasm contributes new genetic diversity to the hybrid breeding program

Our study proved that the Spanish germplasm tested was diverse, and contributed new and distinct variation to that provided by the elite germplasm. Other surveys have also highlighted the potential of genetic resources to boost the diversity of elite breeding pools (Longin and Reif 2014; Allier et al. 2020; Sommer et al. 2020; Sharma et al. 2021). Spanish pollinators presented different ways to build high yields. On the one hand, lines 2 and 19 maximized grain filling. Line 18, on the other hand, allocated more resources to building structures: more tillers, more ears, and therefore, more grains. However, it was not able to realize its full yield potential, as shown by the small size and volume of its grains. The poor grain filling of line 18 indicates an inferior adaptation. In fact, this line is one of the latest in the panel, which makes it more prone to experience terminal stress conditions. Therefore, lines 2 and 19 seem safer options for future hybrid development.

In general, the germplasm accessible to hybrid barley breeding is limited due to the potential yield losses that can occur in crosses that involve alternative *non-brittle rachis* mutations (*btr1* x *btr2*). Our results proved that rachis brittleness is indeed linked to a reduction in performance in three-way barley hybrids, which may present up to 50% of fragile ears. More importantly, in those two-way hybrids derived from the cross of a *btr2* Spanish female and a *btr1* elite male restorer, up to 100%

of ears would be brittle. Widening the germplasm accessible for hybrid barley breeding requires management of *non-brittle rachis* genes through pre-breeding.

The different specific combining ability patterns in the Spanish material also evidence its diversity. Moreover, it could indicate a bad fit of the Spanish germplasm in the current heterotic groups. To find out whether these lines actually belong to the existent female parent pool or should constitute a new pool, further evaluation of testcross performance with a larger number of testers should be conducted (Melchinger and Gumber 1998). Thus, there is still room for improvement and future development of hybrids with these materials.

6.4.3. Widely adapted hybrids seem a feasible option for the heterogeneous Iberian region

The field trial network comprised of four locations, no more than 3° of latitude apart. However, they showed very contrasting agro-climatic conditions. This high heterogeneity of the Iberian region was reflected in the large GxE component for yield. High stability can be used as a proxy for the selection of broadly adapted genotypes (Bassi and Sanchez-Garcia 2017). In our study, although based on few data, the stability analysis illustrated that the WAAS index was substantially higher for hybrids compared to inbred lines. This indicates higher yield stability of hybrids and is in line with previous studies that thoroughly investigated yield stability in barley (Mühleisen et al. 2014b, a). Like Mühleisen et al. (2014a), we observed higher yield stability of three-way hybrids compared with two-way hybrids. This agrees well with the higher buffering ability attributed to three-way hybrids, which is the result of a higher genetic heterogeneity (Becker and Léon 1988). Adapted hybrids, therefore, seem a feasible option for an area as diverse as the Iberian region, including well-designed three-way hybrids.

6.4.4. Best hybrids result from the combination of specific heterotic patterns and the appropriate earliness

Test hybrids showed an overall poor performance compared to checks. This is the result of a combination of two factors: on the one hand, the absence of high-yielding heterotic responses when crossing F_1Fs and male restorer parents in the three-way hybrids. On the other hand, the inability of the test hybrids to fully exploit their potential in short cycles, as already reported in other studies involving hybrids (Capristo et al. 2007; Akinnuoye-Adelabu and Modi 2017). Test hybrids were more advantageous the longer the period from sowing to heading. However, as supported by several studies, the climatic conditions to come, and particularly those of the Mediterranean basin, push crops into shorter cycles (Dettori et al. 2017; Funes et al. 2021). Therefore, earlier hybrids should be sought for the Iberian region. This shortening of the cycle

could be compensated for in the final yield through earlier sowings (Baum et al. 2020; Kim and Lee 2020). In this context, it is important to define an optimal sowing window, which is not too early as to expose the ear to the first frost (Zheng et al. 2012), but early enough to exploit the full tillering potential of the hybrids (Adhikari et al. 2020). Regarding flowering time, the variation provided by the Spanish pollinators was narrow but, still, earliness was associated with better grain filling in the tested environments. This association came as no surprise, since modern cultivars adapted to the Mediterranean basin have been selected for early flowering, and long grain filling periods, resulting in greater harvest index and final yield (Royo et al. 2021). The Spanish lines succeeded in bringing the test hybrids' flowering time closer to the ranges of the most productive checks, such as *ZOO* or *HURRICANE*. However, when combined with late male restorer parents, the earliness conferred by 25% of the Spanish germplasm was not enough, and they were behind well adapted checks such as *YURIKO*. In order to obtain hybrids with a cycle similar to that of Mediterranean-adapted checks, earlier Spanish material should be evaluated. To do so, male parents must also carry genes leading to stronger earliness, in this way synchronizing flowering with the early females. Therefore, earliness should be built on both sides of the cross to expand the range of potentially positive flowering times for the Iberian region. *ZOO* was the best-performing check, even superior than *YURIKO*, an excellent cultivar adapted to Mediterranean conditions. What is particular about this check is its balance. *ZOO* presents a good heterotic pattern, as suggests its perfect trade-off between tilling ability and grain weight. Moreover, it presents an early to intermediate flowering time, which allows it to maintain yield even at environments prone to terminal stress. Therefore, this genotype is a clear example of how a strong heterotic pattern combined with a suitable cycle result in top grain yields.

6.4.5. Genomic prediction of hybrid performance drives selection of Spanish parents

Resource constraints limit the number of inbred lines assessable in hybrid combinations. Therefore, we predicted the performance of a set of Spanish lines that were not tested in hybrid combinations in the field.

Considering the small size of our training set, the prediction accuracies obtained were acceptable and comparable to other studies with a similar number of hybrids (Zhao et al. 2013). More accurate estimates were obtained in studies that predicted hybrid performance using a larger training set and/or a higher number of tested environments (Liu et al. 2016; Philipp et al. 2016; Zhao et al. 2021; Zhang et al. 2022). The differences in accuracy between prediction models were small. Although, the architecture of the trait defined the best performing model. In conclusion, GBLUP quickly and accurately predicted the performance of hybrids for any trait, whereas GSA-RRBLUP

increased the prediction ability of complex traits as grain yield. The generally good performance of GBLUP is supported by a large body of experimental evidence (de los Campos et al. 2013; Montesinos-López et al. 2018). The suitability of the RRBLUP for traits controlled by several loci with minor effects is consistent with the model assumptions, i.e., all markers have equal variances with small effects (Krishnappa et al. 2021). However, in hybrid breeding, the parental lines come from genetically diverse populations to maximize the exploitation of heterosis. In the case of hybrid barley, these are the male restorer and female pools, which have been subjected to different selection processes. During this time, markers and QTL will have recombined, losing their association or even changing in sign, and QTL allele frequencies may have drifted into different directions. Under these circumstances, it may be necessary to model marker effects as population-specific (Technow et al. 2012). This is exactly what the GSA-RRBLUP model allows. The implementation of the GSA-RRBLUP model has been associated with an improved genome-wide hybrid prediction for scenarios of genetically diverse parental populations (Philipp et al. 2016; Li et al. 2017), as is the case of the elite male restorer pool and Spanish pollinators of the three-way hybrids tested.

We succeeded in implementing genomic prediction for the selection of superior Spanish parental lines. We identified a number of promising untested lines, based on the genomic prediction of their hybrid performance. These lines, together with the best ones identified in the field network, could be used for the development of superior two-way hybrids combined with the appropriate male restorer line.

6.5. Conclusions

In this study, we explored the potential of locally adapted Spanish material in hybrid development. The expression of Spanish diversity and potential heterosis was limited due to the low contribution of the Spanish germplasm in the three-way hybrid approach, which hampered the obtention of superior hybrids. Even so, we identified high-yielding and widely adapted parental lines for the development of promising two-way hybrids, both tested in the field, and untested, the latter facilitated by genomic prediction. Earliness seems to be a key trait for yield realization in environments prone to terminal stress, as were some of those tested in the Iberian region. Thus, the success of hybrid breeding in Southern Europe requires further investigation of the underlying heterotic patterns in the Spanish germplasm and expanding the range of flowering times by manipulating both sides of the cross. Moreover, management of *non-brittle rachis* genes through pre-breeding is needed to widen the germplasm accessible for hybrid barley breeding.

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6.7. Supplementary material

Table S6.1. List of plant materials evaluated. For each hybrid, parents, row number, and *non-brittle rachis* genotype of their components is presented.

Genotype	Elite female	Spanish pollinator	Male restorer	Type	Rows	*btr* female	*btr* pollinator	*btr* male
MESETA				Inbred line (check)	2	*btr1*		
YURIKO				Inbred line (check)	6	*btr2*		
HURRICANE	β		A	2-way hybrid (check)	6	*btr1*		*btr1*
HOOK	γ		B	2-way hybrid (check)	6	*btr1*		*btr1*
GALILEOO	γ		C	2-way hybrid (check)	6	*btr1*		*btr1*
ZOO	α		D	2-way hybrid (check)	6	*btr1*		*btr1*
1A	α	1	A	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
2A	α	2	A	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
3A	α	3	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
4A	α	4	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
5A	α	5	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
6A	α	6	A	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
7A	α	7	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
8A	α	8	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
9A	α	9	A	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
10A	α	10	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
11A	α	11	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
12A	α	12	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
13A	α	13	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
14A	α	14	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
15A	α	15	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
16A	α	16	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
17A	α	17	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
18A	α	18	A	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
19A	α	19	A	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
20A	α	20	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
21A	α	21	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
22A	α	22	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
23A	α	23	A	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
24A	α	24	A	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
1B	α	1	B	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
2B	α	2	B	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
3B	α	3	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
4B	α	4	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
5B	α	5	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
6B	α	6	B	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
7B	α	7	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
8B	α	8	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
9B	α	9	B	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
10B	α	10	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
11B	α	11	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
12B	α	12	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
13B	α	13	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
14B	α	14	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
15B	α	15	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
16B	α	16	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*

Table S6.1. (continued)

Genotype	Elite female	Spanish pollinator	Male restorer	Type	Rows	*btr* female	*btr* pollinator	*btr* male
17B	α	17	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
18B	α	18	B	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
19B	α	19	B	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
20B	α	20	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
21B	α	21	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
22B	α	22	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
23B	α	23	B	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
24B	α	24	B	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
1C	α	1	C	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
2C	α	2	C	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
3C	α	3	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
4C	α	4	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
5C	α	5	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
6C	α	6	C	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
7C	α	7	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
8C	α	8	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
9C	α	9	C	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
10C	α	10	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
11C	α	11	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
12C	α	12	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
13C	α	13	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
14C	α	14	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
15C	α	15	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
16C	α	16	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
17C	α	17	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
18C	α	18	C	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
19C	α	19	C	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
20C	α	20	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
21C	α	21	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
22C	α	22	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*
23C	α	23	C	3-way hybrid (test)	6	*btr1*	*btr1*	*btr1*
24C	α	24	C	3-way hybrid (test)	6	*btr1*	*btr2*	*btr1*

Table S6.2. List of scored traits.

Abbreviation	Trait group	Trait	Unit	Method of measurement	Environments tested[a]							
					B19	G19	S19	V19	B20	G20	S20	V20
DAW	Developmental	Deficiencies after winter	[1-9 score]	Visual scoring of the validity of the plot after winter. 1 (100% plot present) to 9 (0% plot present).	2	2	2	2	2	2	2	0
PV		Plant vigour	[1-5 score]	Visual scoring of early vigour. 1 (high vigour) to 5 (low vigour). Recorded at Z25-Z29 (Zadoks et al., 1974).	2	0	2	2	2	0	2	2
GH		Growth habit	[1-9 score]	Visual scoring of growth habit. 1 (erect) to 9 (prostrate). Recorded at Z25-Z29.	2	0	2	2	2	0	2	2
DTH		Days to heading	[days after January 1st]	Number of days from January 1st until half of the ear is visible above the flag leaf (Z55) for 50 % of all ears in a plot.	2	1	2	2	2	1	2	1
HT		Plant height	[cm]	Distance from ground level to tip of the erected ear, excluding awns. Recorded at Z80-Z95. Average of 3 plants/plot.	2	1	2	1	0	1	2	2
GFP		Grain filling period	[days]	Grain filling period duration, calculated as the number of days elapsed between heading and ripening*.	2	0	2	2	2	0	2	2
LODG	Yield and components	Lodging	[1-9 score]	Visual scoring for extent of lodging. 1 (all plants standing) to 9 (plants totally flat). Recorded at >Z65.	2	2	0	0	2	2	2	2
GY		Grain yield	[t·ha^{-1}]	Weight of grain combine-harvested per plot, converted to tonnes per hectare and adjusted to 15% moisture by taking the harvested plot area and harvest moisture into account.	2	2	2	2	0	2	2	2

[a]B19, Burgos_19; G19, Grisolles_19; S_19, Sadaba_19; V19, Valladolid_19; B20, Burgos_20; G20, Grisolles_20; S_20, Sadaba_20; V20, Valladolid_20. 0, Trait not measured; 1, trait measured only in 1 replication; 2, trait measured in 2 replications.

*Ripening date was estimated as the date when NDVI=0.2, which has been associated with physiological maturity. NDVI was measured in four dates (time lapse ≈ 7days) from the beginning (≈0.80) until the end of the senescence period (≈0.15) through a Greenseeker device. A regression of NDVI on days after heading was fitted to find the ripening date.

Table S6.2. (continued)

Abbreviation	Trait group	Trait	Unit	Method of measurement	Environments tested[a]							
					B19	G19	S19	V19	B20	G20	S20	V20
BIO	Yield and components	Biomass	[g·m^{-2}]	Weight of above-ground dry mass of a 0.1 m^2 plot sample† at maturity (Z90), extrapolated to 1 m^2.	2	0	2	2	2	0	2	2
GPE		Grains per ear	[grain number·ear^{-1}]	Number of grains divided by the number of ears counted on the 0.1 m^2 plot sample.	2	0	2	2	2	0	2	2
GM2		Number of grains	[grain number·m^{-2}]	Number of grains counted on the 0.1 m^2 plot sample, extrapolated to 1 m^2.	2	0	2	2	2	0	2	2
TM2		Number of tillers	[tiller number·m^{-2}]	Number of stems counted on the 0.1 m^2 plot sample, extrapolated to 1 m^2.	2	0	2	2	2	0	2	2
EM2		Number of ears	[ear number·m^{-2}]	Number of ears counted on the 0.1 m^2 plot sample, extrapolated to 1 m^2.	2	0	2	2	2	0	2	2
HI		Harvest index	[%]	Ratio of grain dry weight divided by the above-ground dry mass per plot sample (0.1 m^2). Expressed as percentage.	2	0	2	2	2	0	2	2
BTRS		Brittleness score	[0-2 score]	Visual scoring of rachis disarticulation. 0=no breaks, 2=high frequency of breaks. Recorded on the 0.1 m^2 plot sample.	2	0	2	2	2	0	2	2
NECK		Necking	[%]	Percentage of tillers showing creasing of the straw just below the ear. Assessed on the 0.1 m^2 plot sample.	2	0	2	2	2	0	2	2
FERT		Fertility	[%]	Ratio of grain dry weight divided by the dry weight of the whole ear. Expressed as percentage. Assessed on the 0.1 m^2 plot sample.	2	0	2	2	2	0	2	2

[a]B19, Burgos_19; G19, Grisolles_19; S_19, Sadaba_19; V19, Valladolid_19; B20, Burgos_20; G20, Grisolles_20; S_20, Sadaba_20; V20, Valladolid_20. 0, Trait not measured; 1, trait measured only in 1 replication; 2, trait measured in 2 replications.

†Plot samples of 0.1 m2 were manually harvested at maturity (Z90). A template that delimited 25 cm of each of two contiguous rows was placed in the 2 central rows of the plot, and the area covered by the template was reap at ground level. The harvested sample was introduced in paper bags for subsequent moisture extraction in dryers. Samples were dried until constant weight for at least 24 hours at 80 °C.

Table S6.2. (continued)

Abbreviation	Trait group	Trait	Unit	Method of measurement	Environments tested[a]							
					B19	G19	S19	V19	B20	G20	S20	V20
MOI	Yield and components	Moisture	[%]	Moisture content at harvest, as recorded with a Dickey–John analyser model GAC-II.	2	2	2	2	0	2	2	2
TGW	Grain quality	Thousand grain weight	[g]	Weight of 1000 grains randomly selected from 2 kg seed sample.	1	1	1	1	0	1	1	1
TW		Specific weight	[kg·hL^{-1}]	Hectolitre weight measured with a Dickey–John analyser model GAC-II on seed samples previously cleaned.	1	1	1	1	0	1	1	1
PROT		Protein content	[%]	Percentage of grain protein content, estimated as 6.25*grain nitrogen content, measured by NIR.	1	1	1	1	0	1	1	1
GRA2_5		Plumpness 2.5	[%]	Percentage of grains over 2.5 mm sieve. Seed samples previously cleaned over 1.8 mm.	1	1	1	1	0	1	1	1
GRA2_8		Plumpness 2.8	[%]	Percentage of grains over 2.8 mm sieve. Seed samples previously cleaned over 1.8 mm.	1	1	1	1	0	1	1	1
Area		Grain area	[mm]	Average grain area, measured with Marvin Digital Seed Analyzer (GTA Sensorik GmbH).	1	1	1	1	0	1	1	1
Width		Grain width	[mm]	Average grain width, measured with Marvin Analyzer.	1	1	1	1	0	1	1	1
Length		Grain length	[mm]	Average grain length, measured with Marvin Analyzer.	1	1	1	1	0	1	1	1

[a]B19, Burgos_19; G19, Grisolles_19; S_19, Sadaba_19; V19, Valladolid_19; B20, Burgos_20; G20, Grisolles_20; S_20, Sadaba_20; V20, Valladolid_20. 0, Trait not measured; 1, trait measured only in 1 replication; 2, trait measured in 2 replications.

Table S6.3. Additional traits averages within and across environments (trait abbreviations as in Table 6.2).

Environment	GM2 [grains·m^{-2}]	BIO [g·m^{-2}]	GPE [grains·ear^{-1}]	HI [%]	TM2 [tillers·m^{-2}]	EM2 [ears·m^{-2}]	BTRS [0-2 score]	FERT [%]	PROT [%]	Area [mm]
Burgos_19	22407	1617	36	52.8	721	635	0.89	87.9	9.6	20.1
Grisolles_19	-	-	-	-	-	-	-	-	8.3	20.5
Sadaba_19	15500	1177	44	56.3	451	366	0.87	85.1	12.9	20.1
Valladolid_19	17477	1115	36	48.9	565	504	0.59	85.3	10.5	19.0
Burgos_20	19750	1412	43	51.5	516	470	0.26	87.6	-	-
Grisolles_20	-	-	-	-	-	-	-	-	14.5	22.5
Sadaba_20	20951	1524	42	50.6	552	506	0.19	83.4	12.4	22.4
Valladolid_20	15910	1588	33	31.4	583	500	0.23	74.3	12.4	20.1
Grand mean	18603	1397	39	49.3	556	488	0.51	84.0	11.6	20.8

Table S6.3. (continued)

Environment	Width [mm]	Length [mm]	GRA2_5 [%]	GRA2_8 [%]	LODG [1-9 score]	GH [1-9 score]	PV [1-5 score]	DAW [1-9 score]	NECK [%]
Burgos_19	3.4	8.8	78.6	32.6	2	5	3	1	11.8
Grisolles_19	3.6	8.3	94.9	70.4	1	-	-	1	-
Sadaba_19	3.5	8.6	78.7	54.4	-	4	2	2	19.0
Valladolid_19	3.2	8.7	44.7	13.2	-	6	4	1	10.9
Burgos_20	-	-	-	-	2	5	2	1	8.8
Grisolles_20	3.5	9.4	74.1	39.7	7	-	-	3	-
Sadaba_20	3.5	9.4	63.6	26.7	5	5	2	2	8.4
Valladolid_20	3.4	8.7	58.4	29.2	7	5	1	-	14.4
Grand mean	3.5	8.9	70.5	38.3	4	5	2	2	12.4

Table S6.4. Estimates of variance components (σ^2_{ENV} environment, σ^2_G genotypic, σ^2_{GxE} genotype-by-environment interaction, σ^2_ε error) and heritability for additional traits. Genotypic and GxE interaction variances are divided into hybrids and checks.

	GM2	**BIO**	**GPE**	**HI**	**TM2**	**EM2**	**BTRS**	**FERT**	**PROT**	**Area**
Source	[grains·m^{-2}]	[g·m^{-2}]	[grains·ear^{-1}]	[%]	[tillers·m^{-2}]	[ears·m^{-2}]	[0-2 score]	[%]	[%]	[mm]
σ^2_{ENV}	7527988***	44329***	20.8***	75.5***	7794***	7275***	0.097**	22.3***	4.56***	1.56***
	---------- Hybrids ----------									
σ^2_G	1.58	0.007	0.636	0.000	0.001	0.001	0.060***	0.000	0.000	0.106***
σ^2_{GxE}	206971	0.004	4.34**	3.90**	0.006	0.006	0.065***	10.2***	0.042	0.000
h^2	0.000	0.000	0.220	0.000	0.000	0.000	0.666	0.000	0.000	0.859
	---------- Checks ----------									
σ^2_G	1168687*	2565	58.0***	0.000	29020***	28518***	0.000	3.16**	0.177*	1.27***
σ^2_{GxE}	9.23	0.007	1.24	0.000	1501	850	0.037	0.000	0.359***	0.077*
σ^2_ε	15645363	72222	22.8	19.7	13245	11253	0.289	30.5	0.316	0.244
h^2	0.511	0.332	0.970	0.000	0.962	0.969	0.000	0.592	0.706	0.978

* p<0.05, ** p<0.01, *** p<0.001

Table S6.4. (continued)

	Width	**Length**	**GRA2_5**	**GRA2_8**	**LODG**	**GH**	**PV**	**DAW**	**NECK**
Source	[mm]	[mm]	[%]	[%]	[1-9 score]	[1-9 score]	[1-5 score]	[1-9 score]	[%]
σ^2_{ENV}	0.016***	0.150***	272***	354***	7.00***	0.383***	0.502***	0.585***	15.0***
	---------- Hybrids ----------								
σ^2_G	0.000*	0.028***	2.49	3.54**	0.102	0.003	0.000	0.000	1.41
σ^2_{GxE}	0.000	0.000	0.000	0.000	0.162	0.000	0.052*	0.001	4.68
h^2	0.476	0.938	0.484	0.610	0.429	0.055	0.000	0.000	0.264
	---------- Checks ----------								
σ^2_G	0.004***	0.217***	2.01	48.1**	0.113	0.368*	0.037	0.000	2.43
σ^2_{GxE}	0.004***	0.013***	79.6***	82.5***	1.05***	0.606***	0.245***	0.000	4.73
σ^2_ε	0.004	0.026	37.2	31.6	1.58	0.695	0.399	0.224	45.7
h^2	0.820	0.983	0.125	0.774	0.301	0.730	0.368	0.000	0.382

* p<0.05, ** p<0.01, *** p<0.001

Table S6.5. Means and 95% confidence interval (CI) across environments for the several planned contrasts on genotypes: checks *vs.* hybrids, hybrids grouped per male restorer, and hybrids grouped per F_1F.

	GY [t·ha^{-1}]		DTH [Julian days]		HT [cm]		TW [kg·hL^{-1}]		TGW [g]		PROT [%]		GM2 [grains·m^{-2}]		BIO [g·m^{-2}]		GPE [grains·ear^{-1}]		HI [%]	
Checks *vs.* Hybrids	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
Checks	6.857	0.364	118.2	4.1	98.4	6.7	69.0	1.7	39.9	2.6	12.0	0.5	18157	1474	1381	78	34.7	8.0	49.4	0.9
Hybrids	6.691	0.064	118.4	0.3	103.1	0.5	68.1	0.2	39.5	0.4	11.4	0.1	18312	417	1406	22	39.9	0.5	48.0	0.5
Male restorers																				
A	6.522	0.073	117.5	0.3	101.3	0.5	67.2	0.2	37.9	0.4	11.2	0.1	19492	471	1391	27	40.7	0.9	49.1	0.8
B	6.763	0.073	119.5	0.3	103.1	0.5	68.8	0.2	39.3	0.4	11.5	0.1	18618	471	1438	27	38.9	0.9	48.2	0.8
C	6.789	0.147	118.1	0.3	104.9	0.9	68.3	0.4	41.3	0.7	11.5	0.3	16826	780	1388	53	40.3	1.1	46.6	0.9
F_1F																				
1	6.497	0.580	119.6	1.6	102.7	4.1	68.3	2.4	39.5	4.6	11.8	1.3	18356	6273	1417	140	39.9	6.0	48.0	4.5
2	7.073	1.085	117.9	1.9	102.7	2.8	68.6	3.5	41.3	6.8	10.8	1.5	19032	4704	1449	298	40.8	2.8	49.9	5.5
3	6.649	0.554	118.2	4.4	102.9	8.7	67.8	1.6	40.1	6.7	11.2	1.0	17823	1446	1369	196	41.4	3.4	49.0	1.7
4	6.693	0.240	118.0	3.5	102.4	4.3	68.4	0.8	40.8	5.2	11.6	1.4	16897	5830	1334	154	37.4	6.4	46.7	6.4
5	6.737	1.116	118.1	2.5	102.6	1.3	68.5	1.3	39.3	4.6	11.1	1.2	17731	6182	1341	331	40.9	10.9	47.5	4.4
6	6.660	0.815	118.9	2.7	104.8	7.5	68.2	1.7	40.0	3.8	11.3	1.0	17588	3455	1381	350	40.8	3.8	47.4	7.6
7	6.646	0.751	117.4	3.6	103.0	9.5	68.6	2.6	39.9	5.3	11.4	0.8	16861	3678	1351	126	39.6	7.5	46.8	7.8
8	6.587	0.491	118.2	3.0	102.4	4.2	68.4	3.6	39.7	4.2	11.7	0.7	18542	3894	1427	251	38.0	4.9	47.2	1.6
9	6.826	0.818	118.2	1.1	104.1	4.3	68.2	3.8	38.9	4.1	11.4	0.1	17637	7218	1339	347	40.7	2.8	48.9	3.3
10	6.524	0.200	118.7	3.7	103.0	7.8	67.9	2.3	38.4	1.6	11.9	1.0	18717	4375	1436	124	41.2	6.5	49.0	6.1
11	6.790	0.413	117.2	1.9	103.1	5.6	67.9	2.5	39.0	3.2	11.2	0.7	17999	6639	1409	234	41.2	3.7	46.9	13.2
12	6.486	0.503	118.3	4.0	103.6	8.5	67.9	1.6	38.4	2.3	11.2	0.2	17486	3719	1346	21	39.3	6.9	47.0	5.7
13	6.718	1.135	118.1	3.9	102.9	2.9	67.9	2.2	38.9	2.4	11.4	1.4	18094	3830	1403	80	40.6	2.3	48.3	2.6
14	6.357	0.491	118.8	3.9	103.5	2.8	68.0	1.5	39.5	7.2	11.2	1.1	18494	5770	1388	14	40.3	11.6	47.2	7.6
15	6.416	0.148	118.9	1.4	102.4	3.5	67.5	2.4	37.7	3.5	11.5	0.5	19005	5790	1402	222	39.3	0.8	47.8	4.5
16	6.683	0.610	118.3	1.9	101.5	7.6	68.0	3.4	40.0	6.0	11.5	0.5	19836	1195	1495	305	41.5	1.2	49.3	1.5
17	6.789	0.689	118.1	3.1	105.4	6.4	67.9	1.4	39.5	3.1	11.7	1.9	18114	2674	1375	142	40.0	2.2	49.3	2.1
18	7.003	0.377	119.3	3.1	104.1	7.0	67.9	2.8	37.8	1.8	12.1	1.8	19956	6267	1522	478	39.9	6.4	48.4	1.2
19	7.004	0.323	118.7	4.2	103.6	5.8	68.5	1.9	39.8	3.6	11.4	0.6	19366	3922	1488	263	39.1	8.3	47.1	2.0
20	6.636	0.450	118.3	3.2	103.3	1.6	67.9	1.7	39.4	3.9	11.8	1.5	18475	2562	1423	28	40.1	7.2	48.2	1.4
21	6.678	0.129	119.1	1.3	104.1	7.9	67.5	2.7	41.0	8.3	11.3	0.3	17679	6952	1386	301	38.4	2.7	47.8	4.7
22	6.660	0.498	117.9	2.8	103.4	4.9	67.3	1.8	39.7	2.2	11.3	0.5	17740	3646	1406	130	37.4	7.1	46.2	6.8
23	6.676	0.582	118.2	1.4	101.6	7.5	69.2	4.5	40.0	5.7	11.4	0.7	19456	2873	1471	126	40.5	7.1	49.4	1.9
24	6.808	0.748	118.5	2.3	100.6	5.2	67.6	0.9	39.3	7.1	11.4	1.2	18600	3831	1377	201	40.3	2.9	48.0	7.0

Table S6.5. (continued)

	LODG [1-9 score]		TM2 [tillers·m^{-2}]		EM2 [ears·m^{-2}]		BTRS [0-2 score]		GH [1-9 score]		PV [1-5 score]		DAW [1-9 score]		NECK [%]		FERT [%]	
Checks *vs.* Hybrids	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
Checks	3.5	0.6	620	181	565	179	0.03	0.03	4.6	0.7	2.5	0.3	1.6	0.1	9.2	2.4	85.0	2.2
Hybrids	4.5	0.3	538	10	468	10	0.69	0.09	5.0	0.1	2.4	0.1	1.6	0.1	12.9	0.8	83.3	0.6
Male restorers																		
A	4.5	0.2	556	12	485	12	0.65	0.13	4.9	0.1	2.4	0.1	1.6	0.0	12.9	1.0	84.7	1.1
B	3.6	0.2	558	12	485	12	0.57	0.13	5.1	0.1	2.5	0.1	1.6	0.0	13.2	1.0	83.0	1.1
C	5.3	0.6	498	14	435	17	0.87	0.18	5.0	0.2	2.3	0.1	1.6	0.1	12.6	2.0	82.4	1.1
F_1F																		
1	3.3	1.2	522	98	469	82	0.15	0.29	5.0	0.4	2.4	0.6	1.6	0.2	10.1	4.9	82.0	9.5
2	4.5	2.8	524	102	472	84	0.20	0.25	4.8	1.1	2.0	0.9	1.5	0.3	9.9	3.5	83.9	8.6
3	5.3	4.1	501	44	436	52	0.84	1.48	5.3	0.9	2.4	0.6	1.5	0.5	13.0	3.6	83.1	3.3
4	4.1	2.3	520	80	457	59	0.73	0.62	4.9	0.6	2.4	0.8	1.7	0.3	12.2	7.4	81.3	4.4
5	4.3	1.5	541	207	455	203	0.73	0.32	5.3	0.8	2.4	0.4	1.5	0.1	15.2	6.3	81.8	6.9
6	4.5	1.4	505	103	435	104	0.40	0.68	5.2	1.3	2.4	0.5	1.5	0.1	13.5	8.3	83.5	4.6
7	4.3	3.7	531	98	446	108	0.95	0.25	4.6	0.8	2.4	0.3	1.7	0.6	15.5	6.6	82.7	10.9
8	4.4	2.5	566	129	494	114	0.87	0.46	4.9	0.7	2.6	0.6	1.6	0.3	11.9	2.4	83.8	9.0
9	4.0	0.5	523	200	456	207	0.39	0.26	5.0	0.3	2.4	0.6	1.6	0.3	13.7	7.8	84.1	4.8
10	5.3	3.9	523	159	469	131	0.79	0.54	5.1	1.1	2.4	0.4	1.7	0.3	10.8	4.8	83.1	3.3
11	4.5	1.5	529	129	445	157	0.98	0.13	4.7	0.7	2.5	0.3	1.5	0.6	17.2	15.4	84.7	5.1
12	4.8	4.4	543	67	455	109	0.84	0.97	5.1	0.8	2.4	0.3	1.7	0.4	16.3	11.3	82.2	5.5
13	4.5	2.7	527	98	453	61	1.09	0.35	5.4	0.1	2.5	0.3	1.5	0.5	13.7	7.0	84.0	3.8
14	4.3	1.9	539	105	462	92	0.84	0.56	4.9	0.7	2.5	0.1	1.5	0.4	15.4	3.4	83.8	16.9
15	5.4	3.9	557	157	488	132	0.67	0.23	5.1	0.9	2.4	1.2	1.7	0.5	12.5	2.8	83.4	5.9
16	4.2	1.2	554	26	488	47	0.95	1.47	4.7	0.4	2.3	0.8	1.5	0.4	11.6	11.1	85.0	4.7
17	4.3	1.3	526	76	462	17	0.98	0.31	4.5	0.5	2.4	0.3	1.6	0.2	12.0	9.9	84.5	0.9
18	5.7	5.0	575	82	512	87	0.09	0.38	4.8	0.5	2.3	1.7	1.6	0.5	11.1	3.8	84.2	2.2
19	3.8	1.4	569	100	508	112	0.37	0.34	5.1	0.5	2.2	0.6	2.1	1.8	10.9	3.5	81.7	3.4
20	4.7	4.2	539	23	468	81	0.65	0.85	5.0	0.6	2.3	0.1	1.7	0.4	12.8	14.0	84.3	4.8
21	4.9	4.2	548	126	473	130	0.87	0.44	5.2	0.1	2.5	0.5	1.6	0.3	14.3	10.4	83.3	4.1
22	5.1	3.8	559	118	483	36	0.92	0.73	5.2	0.8	2.5	0.5	1.6	0.2	12.8	15.9	82.8	5.0
23	4.4	1.0	555	142	486	116	0.45	0.74	4.7	1.0	2.5	0.7	1.7	0.3	12.1	5.1	83.9	2.6
24	3.3	1.9	526	58	465	44	0.92	1.29	5.1	0.3	2.5	0.4	1.7	0.3	11.4	2.4	82.8	8.3

Table S6.5. (continued)

	Area [mm]		Width [mm]		Length [mm]		GFP [days]		GRA2_5 [%]		GRA2_8 [%]	
Checks *vs.* Hybrids	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
Checks	20.8	1.2	3.5	0.1	8.8	0.5	39.7	2.8	73.3	4.2	39.8	8.2
Hybrids	21.0	0.2	3.4	0.0	9.0	0.1	38.9	0.2	69.4	0.9	37.0	0.9
Male restorers												
A	20.4	0.1	3.4	0.0	8.8	0.1	39.5	0.2	68.0	1.3	35.7	1.4
B	20.7	0.1	3.4	0.0	8.9	0.1	38.1	0.2	71.5	1.3	39.6	1.4
C	21.8	0.3	3.5	0.0	9.3	0.1	39.1	0.3	68.7	2.2	35.6	1.7
F_1F												
1	20.5	1.1	3.4	0.1	8.8	0.5	38.1	1.3	69.3	9.0	35.3	3.5
2	21.2	2.1	3.5	0.1	9.0	0.7	38.7	1.9	74.0	8.1	41.0	5.7
3	21.4	2.2	3.5	0.1	9.1	0.8	38.6	3.0	68.8	4.2	34.5	4.7
4	21.4	2.4	3.5	0.1	9.2	1.1	39.0	3.1	71.1	0.6	38.4	1.2
5	20.9	2.1	3.4	0.1	9.0	0.9	39.1	0.8	70.9	2.0	36.9	5.3
6	20.9	2.3	3.4	0.1	9.0	0.9	39.0	0.5	72.2	8.8	38.6	8.8
7	21.3	2.4	3.5	0.2	9.1	0.8	39.0	3.1	72.1	10.8	39.1	4.5
8	20.6	1.3	3.4	0.1	8.9	0.7	39.3	2.9	67.2	11.6	35.6	12.3
9	20.6	1.3	3.4	0.1	8.8	0.5	38.9	2.0	69.3	9.8	37.7	9.1
10	20.6	0.8	3.4	0.1	8.9	0.3	39.1	3.0	67.9	9.2	35.6	8.3
11	20.6	1.8	3.4	0.1	8.8	0.6	39.1	2.2	69.8	7.2	37.1	6.4
12	20.9	2.1	3.4	0.1	9.0	0.7	38.9	3.6	67.4	9.9	35.2	10.1
13	21.2	2.4	3.4	0.1	9.1	1.0	39.0	2.2	68.6	10.3	36.2	9.2
14	21.2	2.1	3.4	0.1	9.1	0.8	38.4	3.8	69.4	7.1	36.3	3.0
15	20.5	1.1	3.4	0.1	8.9	0.4	38.7	0.6	65.3	12.3	33.2	11.3
16	21.0	1.9	3.4	0.2	9.0	0.5	39.1	2.1	70.2	9.8	38.7	9.1
17	20.9	2.1	3.4	0.1	9.0	0.9	39.0	2.5	68.6	9.4	36.9	8.4
18	20.4	0.5	3.4	0.1	8.8	0.1	38.2	2.4	63.7	9.9	32.9	13.6
19	20.9	1.7	3.4	0.1	9.0	0.7	38.8	3.3	71.7	8.4	40.1	16.0
20	21.1	2.1	3.4	0.0	9.1	1.0	39.2	3.6	67.8	13.0	37.2	8.0
21	21.6	2.8	3.5	0.1	9.3	1.3	39.1	1.1	70.5	11.0	39.2	11.3
22	21.3	2.3	3.5	0.0	9.2	1.1	39.8	1.7	69.9	10.5	38.6	11.2
23	20.9	1.4	3.5	0.2	8.9	0.2	38.8	0.6	72.1	17.3	38.8	14.6
24	21.2	3.3	3.4	0.2	9.1	1.0	38.5	0.5	67.3	12.6	34.3	12.9

Table S6.6. Best linear unbiased estimates and 95% confidence interval (CI) across environments for the 78 genotypes tested in the field. Least significant difference (LSD) is presented for each trait, in the bottom row. Green cells indicate genotypes whose yield was higher than the checks average. Red bold numbers indicate genotypes whose yield was not significantly different from ZOO.

Genotype	GY [t·ha^{-1}]		DTH [Julian days]		HT [cm]		TW [kg/hL^{-1}]		TGW [g]		GFP [days]	
	Mean	CI	Mean	CI	Mean	CI	Mean	CI	Mean	CI	Mean	CI
GALILEOO	6.665	2.288	122.2	13.7	105.6	27.9	67.3	4.6	42.8	6.3	36.7	7.8
HOOK	6.533	2.285	121.5	13.7	101.7	27.9	67.7	4.6	40.5	6.3	37.6	7.8
HURRICANE	**7.088**	2.289	120.3	13.7	101.0	27.9	68.0	4.6	39.3	6.3	38.0	7.8
1C	6.339	2.534	119.6	14.1	101.7	29.1	68.9	5.7	41.5	8.0	37.5	8.5
2C	**7.545**	2.538	117.4	14.1	101.4	29.1	69.0	5.7	44.4	8.0	38.8	8.5
3C	6.751	2.537	116.7	14.1	106.9	29.1	67.6	5.7	43.1	8.0	39.9	8.5
4C	6.638	2.537	118.3	14.1	104.4	29.1	68.5	5.7	43.2	8.0	38.4	8.5
5C	**7.239**	2.533	118.2	14.1	103.1	29.1	68.6	5.7	41.4	8.0	39.2	8.5
6C	**6.926**	2.533	119.0	14.1	108.0	29.1	67.9	5.7	41.5	8.0	39.1	8.5
7C	6.380	2.538	117.0	14.1	106.4	29.1	69.8	5.7	42.3	8.0	39.3	8.5
8C	6.696	2.537	118.0	14.1	104.3	29.1	68.4	5.7	41.4	8.0	39.1	8.5
9C	**7.096**	2.537	118.1	14.1	105.9	29.1	68.4	5.7	40.5	8.0	38.7	8.5
10C	6.433	2.538	118.4	14.1	105.7	29.1	68.6	5.7	38.4	8.0	39.6	8.5
11C	**6.977**	2.537	117.4	14.1	105.2	29.1	68.1	5.7	40.2	8.0	39.4	8.5
12C	6.702	2.537	117.5	14.1	107.2	29.1	67.8	5.7	39.2	8.0	39.5	8.5
13C	**7.177**	2.537	117.0	14.1	102.8	29.1	67.3	5.7	39.7	8.0	39.7	8.5
14C	6.129	2.537	119.2	14.1	104.7	29.1	68.4	5.7	42.8	8.0	37.9	8.5
15C	6.422	2.536	119.0	14.1	103.0	29.1	67.8	5.7	38.9	8.0	38.7	8.5
16C	6.800	2.538	118.3	14.1	103.6	29.1	68.5	5.7	42.5	8.0	39.4	8.5
17C	**7.109**	2.533	117.6	14.1	108.0	29.1	67.6	5.7	40.9	8.0	39.2	8.5
18C	**7.161**	2.536	118.6	14.1	107.3	29.1	68.4	5.7	38.0	8.0	38.5	8.5
19C	**7.102**	2.534	117.8	14.1	106.3	29.1	68.8	5.7	40.5	8.0	39.2	8.5
20C	6.787	2.536	118.1	14.1	104.0	29.1	67.6	5.7	40.4	8.0	39.6	8.5
21C	6.624	2.533	118.6	14.1	107.5	29.1	67.7	5.7	44.4	8.0	39.6	8.5
22C	6.450	2.536	118.7	14.1	105.2	29.1	66.9	5.7	40.1	8.0	40.3	8.5
23C	6.483	2.533	117.8	14.1	104.2	29.1	70.9	5.7	42.5	8.0	38.7	8.5
24C	**6.969**	2.533	118.8	14.1	99.7	29.1	67.7	5.7	42.6	8.0	38.3	8.5
1B	6.765	2.317	120.2	13.7	104.6	28.1	68.7	4.7	39.3	6.5	38.2	8.0
2B	**6.989**	2.317	118.8	13.7	103.4	28.1	69.8	4.7	40.4	6.5	37.8	8.0
3B	6.802	2.317	120.2	13.7	100.9	28.1	68.6	4.7	37.8	6.5	37.4	8.0
4B	6.804	2.318	119.3	13.7	101.4	28.1	68.8	4.7	39.9	6.5	38.3	8.0
5B	6.602	2.317	119.1	13.7	102.7	28.1	68.9	4.7	38.8	6.5	38.7	8.0
6B	6.762	2.322	119.9	13.7	104.5	28.1	68.9	4.7	39.9	6.5	38.8	8.0
7B	**6.975**	2.317	118.9	13.7	103.8	28.1	68.2	4.7	39.1	6.5	37.7	8.0
8B	6.706	2.317	119.5	13.7	101.7	28.1	69.8	4.7	39.8	6.5	38.3	8.0
9B	**6.923**	2.317	118.7	13.7	103.9	28.1	69.7	4.7	39.2	6.5	38.2	8.0
10B	6.559	2.317	120.3	13.7	103.7	28.1	68.2	4.7	39.0	6.5	37.7	8.0
11B	6.732	2.317	117.9	13.7	103.2	28.1	68.8	4.7	39.2	6.5	38.2	8.0
12B	6.454	2.317	120.2	13.7	103.0	28.1	68.6	4.7	38.6	6.5	37.2	8.0
13B	6.714	2.380	119.9	13.9	104.1	28.4	69.0	5.0	39.2	7.0	38.0	8.2
14B	6.469	2.317	120.1	13.7	103.2	28.1	68.2	4.7	38.1	6.5	37.2	8.0
15B	6.472	2.317	119.4	13.7	100.8	28.1	68.3	4.7	38.1	6.5	38.4	8.0
16B	**6.848**	2.317	119.1	13.7	103.0	28.1	69.1	4.7	39.6	6.5	38.2	8.0
17B	6.639	2.317	119.6	13.7	105.4	28.1	68.6	4.7	39.0	6.5	37.9	8.0
18B	**6.859**	2.317	120.7	13.7	102.0	28.1	68.7	4.7	38.4	6.5	37.2	8.0
19B	**7.053**	2.317	120.6	13.7	102.5	28.1	69.1	4.7	40.8	6.5	37.3	8.0
20B	6.687	2.317	119.7	13.7	103.3	28.1	68.6	4.7	40.3	6.5	37.6	8.0
21B	6.728	2.317	119.7	13.7	103.6	28.1	68.5	4.7	41.0	6.5	39.0	8.0
22B	**6.849**	2.317	118.4	13.7	103.6	28.1	68.1	4.7	40.3	6.5	39.0	8.0
23B	**6.937**	2.317	118.9	13.7	102.3	28.1	69.4	4.7	39.5	6.5	39.0	8.0
24B	**6.994**	2.317	119.2	13.7	103.0	28.1	67.9	4.7	38.0	6.5	38.4	8.0
1A	6.387	2.317	118.9	13.7	101.7	28.1	67.2	4.7	37.8	6.5	38.5	8.0
2A	6.685	2.317	117.6	13.7	103.3	28.1	67.1	4.7	39.1	6.5	39.4	8.0
3A	6.393	2.317	117.7	13.7	100.8	28.1	67.4	4.7	39.4	6.5	38.5	8.0
4A	6.635	2.317	116.5	13.7	101.4	28.1	68.1	4.7	39.3	6.5	40.5	8.0
5A	6.371	2.317	117.1	13.7	102.1	28.1	67.9	4.7	37.8	6.5	39.3	8.0
6A	6.294	2.317	117.8	13.7	102.0	28.1	67.6	4.7	38.5	6.5	39.2	8.0
7A	6.585	2.317	116.1	13.7	98.9	28.1	67.9	4.7	38.3	6.5	40.1	8.0
8A	6.359	2.318	117.2	13.7	101.1	28.1	67.0	4.7	38.0	6.5	40.6	8.0
9A	6.459	2.317	117.9	13.7	102.4	28.1	66.7	4.7	37.2	6.5	39.8	8.0
10A	6.582	2.317	117.4	13.7	99.6	28.1	66.9	4.7	37.7	6.5	39.9	8.0
11A	6.660	2.318	116.4	13.7	100.7	28.1	66.8	4.7	37.6	6.5	39.9	8.0
12A	6.301	2.318	117.2	13.7	100.4	28.1	67.3	4.7	37.4	6.5	39.8	8.0
13A	6.263	2.317	117.3	13.7	101.8	28.1	67.6	4.7	37.8	6.5	39.4	8.0
14A	6.473	2.317	117.0	13.7	102.5	28.1	67.3	4.7	37.5	6.5	40.1	8.0
15A	6.353	2.317	118.3	13.7	103.4	28.1	66.5	4.7	36.2	6.5	38.9	8.0
16A	6.401	2.317	117.6	13.7	98.0	28.1	66.5	4.7	37.7	6.5	39.8	8.0
17A	6.620	2.317	117.2	13.7	102.8	28.1	67.6	4.7	38.6	6.5	39.9	8.0
18A	**6.987**	2.318	118.6	13.7	103.1	28.1	66.6	4.7	37.0	6.5	39.0	8.0
19A	**6.857**	2.317	117.6	13.7	102.1	28.1	67.7	4.7	38.1	6.5	39.8	8.0
20A	6.435	2.317	117.2	13.7	102.7	28.1	67.4	4.7	37.6	6.5	40.5	8.0
21A	6.683	2.317	119.0	13.7	101.2	28.1	66.3	4.7	37.7	6.5	38.7	8.0
22A	6.682	2.317	116.6	13.7	101.3	28.1	66.9	4.7	38.7	6.5	40.2	8.0
23A	6.608	2.317	117.9	13.7	98.3	28.1	67.4	4.7	38.0	6.5	38.5	8.0
24A	6.460	2.317	117.4	13.7	99.1	28.1	67.2	4.7	37.3	6.5	38.7	8.0
MESETA	6.558	2.288	115.7	13.7	87.3	27.9	71.5	4.6	42.1	6.3	42.6	7.8
YURIKO	**6.880**	2.289	112.0	13.7	94.9	27.9	70.4	4.6	36.0	6.3	42.9	7.8
ZOO	**7.418**	2.288	117.7	13.7	100.0	27.9	69.2	4.6	38.8	6.3	40.6	7.8
LSD	0.596		1.9		4.5		1.9		2.7		2.4	

Table S6.6. (continued)

Genotype	GM2 [grains·m^{-2}]		BIO [g·m^{-2}]		GPE [grains·ear^{-1}]		HI [%]		TM2 [tillers·m^{-2}]		EM2 [ears·m^{-2}]		BTRS [0-2 score]	
	Mean	CI	Mean	CI	Mean	CI	Mean	CI	Mean	CI	Mean	CI	Mean	CI
GALILEOO	16446	5452	1386	401	37.7	8.8	48.1	15.0	492	164	443	155	0.04	0.78
HOOK	17852	5291	1439	391	36.8	8.7	48.8	14.9	551	159	493	152	0.02	0.76
HURRICANE	19809	5453	1487	401	38.5	8.8	49.5	15.0	571	164	520	155	0.00	0.78
1C	15612	9567	1366	672	37.1	13.7	45.9	18.0	498	277	444	257	0.02	1.40
2C	17622	9598	1435	674	41.2	13.8	47.4	18.0	486	277	444	257	0.27	1.40
3C	17473	9591	1460	674	42.8	13.7	48.2	18.0	495	277	422	257	1.52	1.40
4C	14193	9583	1263	674	34.7	13.7	43.8	18.0	494	277	432	257	1.02	1.40
5C	15251	9558	1187	672	44.6	13.7	47.9	18.0	445	277	361	257	0.77	1.40
6C	16873	9551	1431	671	39.3	13.7	43.9	18.0	495	277	439	257	0.53	1.40
7C	15520	9589	1293	674	41.6	13.7	46.7	18.0	491	277	397	257	1.02	1.40
8C	16868	9591	1404	674	39.9	13.7	47.5	18.0	508	277	446	257	1.02	1.40
9C	14501	9583	1179	674	41.7	13.7	47.4	18.0	437	277	373	257	0.52	1.40
10C	16846	9600	1381	675	43.8	13.8	47.7	18.0	450	277	409	257	1.02	1.40
11C	14932	9591	1369	674	42.3	13.7	40.7	18.0	485	277	375	257	1.02	1.40
12C	16208	9583	1340	674	41.7	13.7	45.1	18.0	513	277	404	257	1.27	1.40
13C	16313	9581	1371	674	39.7	13.7	47.1	18.0	482	277	425	257	1.02	1.40
14C	16863	9590	1394	674	40.9	13.7	47.2	18.0	497	277	420	257	1.02	1.40
15C	16382	9583	1307	673	39.3	13.7	46.3	18.0	484	277	427	257	0.77	1.40
16C	20391	9599	1621	675	41.2	13.8	49.2	18.0	550	277	510	257	1.52	1.40
17C	17008	9557	1346	671	39.1	13.7	50.0	18.0	499	277	459	257	1.01	1.40
18C	22124	9582	1735	674	42.6	13.7	49.0	18.0	590	277	528	257	0.26	1.40
19C	19146	9569	1578	672	42.7	13.7	47.3	18.0	526	277	458	257	0.52	1.40
20C	17918	9573	1432	673	36.7	13.7	47.9	18.0	540	277	495	257	0.27	1.40
21C	14552	9554	1247	671	37.2	13.7	45.6	18.0	500	277	414	257	1.02	1.40
22C	16158	9582	1364	674	34.4	13.7	43.1	18.0	505	277	467	257	1.26	1.40
23C	18253	9557	1417	671	42.7	13.7	48.7	18.0	489	277	433	257	0.76	1.40
24C	16822	9551	1396	671	39.0	13.7	44.8	18.0	501	277	445	257	1.52	1.40
1B	18875	6560	1407	473	41.6	9.9	48.8	15.6	500	194	457	182	0.17	0.91
2B	21184	6559	1576	473	41.7	9.9	50.9	15.6	568	194	510	182	0.09	0.91
3B	17502	6557	1330	473	41.4	9.9	49.3	15.6	486	194	425	182	0.58	0.91
4B	18093	6563	1370	474	37.5	9.9	47.5	15.6	556	194	478	182	0.58	0.91
5B	17716	6563	1424	474	36.1	9.9	45.6	15.6	582	194	504	182	0.58	0.91
6B	19191	6558	1490	473	40.9	9.9	49.4	15.6	550	194	474	182	0.09	0.91
7B	16615	6562	1380	474	36.1	9.9	43.8	15.6	570	194	482	182	0.84	0.91
8B	19974	6565	1538	474	35.9	9.9	46.5	15.6	608	194	538	182	0.66	0.91
9B	18173	6561	1406	473	40.8	9.9	49.3	15.6	536	194	455	182	0.34	0.91
10B	18962	6560	1479	473	38.5	9.9	47.4	15.6	569	194	494	182	0.75	0.91
11B	19833	6563	1517	474	39.5	9.9	49.5	15.6	586	194	499	182	1.00	0.91
12B	17116	6559	1342	473	36.3	9.9	46.3	15.6	566	194	476	182	0.75	0.91
13B	18980	8156	1435	577	40.6	11.9	49.1	16.7	549	238	471	222	1.25	1.17
14B	17465	6562	1387	474	35.4	9.9	44.1	15.6	581	194	491	182	0.59	0.91
15B	19793	6562	1485	473	38.9	9.9	49.8	15.6	596	194	523	182	0.67	0.91
16B	19583	6560	1489	473	41.2	9.9	50.0	15.6	546	194	479	182	0.34	0.91
17B	19159	6560	1440	473	40.9	9.9	48.3	15.6	559	194	470	182	0.84	0.91
18B	17187	6562	1361	474	37.5	9.9	48.1	15.6	537	194	471	182	0.00	0.91
19B	17908	6564	1371	474	36.1	9.9	47.8	15.6	573	194	521	182	0.34	0.91
20B	17843	6568	1411	474	41.6	9.9	48.8	15.6	529	194	432	182	0.92	0.91
21B	18534	6559	1450	473	38.7	9.9	49.1	15.6	543	194	489	182	0.67	0.91
22B	18005	6560	1464	473	37.6	9.9	48.0	15.6	574	194	495	182	0.76	0.91
23B	19558	6564	1518	474	37.3	9.9	49.4	15.6	590	194	520	182	0.17	0.91
24B	19574	6560	1446	473	40.4	9.9	50.0	15.6	548	194	480	182	0.59	0.91
1A	20582	6560	1477	473	41.1	9.9	49.3	15.6	567	194	507	182	0.25	0.91
2A	18290	6558	1337	473	39.5	9.9	51.4	15.6	518	194	463	182	0.25	0.91
3A	18495	6565	1317	473	40.1	9.9	49.5	15.6	521	194	460	182	0.42	0.91
4A	18405	6563	1370	473	39.8	9.9	48.8	15.6	510	194	463	182	0.58	0.91
5A	20228	6718	1411	484	42.1	10.0	49.1	15.7	596	198	500	186	0.83	0.91
6A	16701	6559	1222	473	42.3	9.9	48.8	15.6	469	194	390	182	0.59	0.91
7A	18450	6562	1381	474	41.1	9.9	50.0	15.6	532	194	458	182	1.00	0.91
8A	18783	6562	1340	474	38.1	9.9	47.7	15.6	581	194	498	182	0.91	0.91
9A	20237	6560	1433	473	39.5	9.9	50.0	15.6	597	194	540	182	0.33	0.91
10A	20343	6557	1447	473	41.3	9.9	51.8	15.6	550	194	504	182	0.59	0.91
11A	19231	6563	1341	474	41.9	9.9	50.3	15.6	517	194	460	182	0.92	0.91
12A	19133	6563	1356	474	40.0	9.9	49.6	15.6	549	194	484	182	0.50	0.91
13A	18987	6559	1404	473	41.6	9.9	48.7	15.6	550	194	463	182	1.00	0.91
14A	21154	6559	1383	473	44.6	9.9	50.3	15.6	540	194	474	182	0.92	0.91
15A	20840	6719	1412	473	39.5	10.0	47.4	15.7	592	194	515	182	0.58	0.91
16A	19534	6559	1375	473	42.1	9.9	48.9	15.6	565	194	476	182	1.00	0.91
17A	18176	6566	1337	474	39.9	9.9	49.4	15.6	522	194	458	182	1.08	0.91
18A	20558	6560	1469	473	39.6	9.9	48.2	15.6	598	194	536	182	0.00	0.91
19A	21043	6557	1514	473	38.5	9.9	46.2	15.6	607	194	545	182	0.25	0.91
20A	19665	6562	1427	473	41.9	9.9	47.9	15.6	547	194	478	182	0.75	0.91
21A	19949	6560	1462	473	39.3	9.9	48.7	15.6	601	194	515	182	0.91	0.91
22A	19057	6561	1390	473	40.1	9.9	47.5	15.6	596	194	486	182	0.75	0.91
23A	20559	6556	1479	473	41.6	9.9	50.2	15.6	587	194	506	182	0.42	0.91
24A	19403	6558	1288	473	41.3	9.9	49.2	15.6	528	194	469	182	0.67	0.91
MESETA	16633	5487	1300	401	19.4	8.9	50.0	15.0	962	164	906	155	0.00	0.78
YURIKO	19159	5454	1302	401	35.6	8.8	50.4	15.0	615	164	542	155	0.04	0.78
ZOO	19042	5454	1373	401	40.0	8.8	49.4	15.0	530	164	482	155	0.08	0.78
LSD	4327		295		5.9		5.6		123		113		0.66	

Table S6.6. (continued)

	FERT [%]		PROT [%]		Area [mm]		Width [mm]		Length [mm]		GRA2_5 [%]		GRA2_8 [%]	
Genotype	Mean	CI	Mean	CI	Mean	CI	Mean	CI	Mean	CI	Mean	CI	Mean	CI
GALILEOO	84.2	10.3	12.0	3.3	22.47	1.94	3.60	0.21	9.38	0.61	74.73	26.07	44.44	29.51
HOOK	84.1	10.1	12.1	3.3	21.42	1.93	3.55	0.20	9.02	0.61	73.56	26.00	49.24	29.44
HURRICANE	86.4	10.3	11.8	3.3	20.64	1.94	3.48	0.21	8.71	0.61	72.64	26.07	40.60	29.51
1C	77.6	16.7	12.5	4.2	20.99	2.38	3.47	0.27	9.08	0.77	72.02	32.12	35.00	34.82
2C	80.0	16.7	10.1	4.2	22.16	2.39	3.55	0.27	9.33	0.77	76.70	32.12	42.71	34.84
3C	84.0	16.7	11.0	4.2	22.46	2.39	3.53	0.27	9.49	0.77	69.48	32.12	35.56	34.84
4C	80.5	16.7	12.1	4.2	22.49	2.38	3.52	0.27	9.66	0.77	71.23	32.12	37.94	34.82
5C	81.4	16.6	10.6	4.2	21.91	2.39	3.48	0.27	9.46	0.77	71.82	32.12	34.76	34.84
6C	81.4	16.6	11.5	4.2	21.95	2.38	3.50	0.27	9.44	0.77	71.46	32.12	36.47	34.82
7C	84.5	16.7	11.4	4.2	22.41	2.38	3.55	0.27	9.48	0.77	76.56	32.12	41.01	34.82
8C	85.2	16.7	12.0	4.2	21.16	2.39	3.45	0.27	9.19	0.77	63.03	32.12	30.80	34.84
9C	82.2	16.7	11.5	4.2	21.23	2.38	3.47	0.27	9.05	0.77	67.31	32.12	34.79	34.82
10C	81.6	16.7	12.3	4.2	20.74	2.39	3.45	0.27	8.97	0.77	63.78	32.12	32.10	34.84
11C	82.3	16.7	11.5	4.2	21.46	2.38	3.47	0.27	9.13	0.77	68.85	32.12	35.78	34.82
12C	80.5	16.7	11.1	4.2	21.78	2.39	3.46	0.27	9.32	0.77	63.57	32.12	31.91	34.84
13C	82.5	16.7	12.0	4.2	22.22	2.39	3.48	0.27	9.57	0.77	64.13	32.12	32.46	34.84
14C	90.2	16.7	10.7	4.2	22.13	2.38	3.49	0.27	9.43	0.77	72.65	32.12	37.70	34.82
15C	80.7	16.7	11.6	4.2	20.99	2.39	3.41	0.27	9.09	0.77	60.10	32.12	29.56	34.84
16C	83.1	16.7	11.3	4.2	21.90	2.38	3.52	0.27	9.27	0.77	70.22	32.12	37.76	34.82
17C	84.8	16.6	12.5	4.2	21.88	2.38	3.47	0.27	9.41	0.77	64.25	32.12	33.19	34.82
18C	84.0	16.7	12.9	4.2	20.49	2.38	3.40	0.27	8.86	0.77	59.98	32.12	27.66	34.82
19C	82.0	16.7	11.2	4.2	21.71	2.38	3.46	0.27	9.33	0.77	70.98	32.12	36.40	34.82
20C	82.2	16.7	12.5	4.2	22.03	2.38	3.44	0.27	9.53	0.77	62.34	32.12	34.00	34.82
21C	81.6	16.6	11.2	4.2	22.66	2.38	3.49	0.27	9.83	0.77	69.71	32.12	37.30	34.82
22C	80.9	16.7	11.5	4.2	22.30	2.39	3.47	0.27	9.66	0.77	65.38	32.12	34.03	34.84
23C	84.5	16.7	11.0	4.2	21.51	2.39	3.57	0.27	8.91	0.77	79.08	32.12	44.73	34.84
24C	79.1	16.6	11.2	4.2	22.69	2.38	3.54	0.27	9.54	0.77	73.15	32.12	40.18	34.82
1B	83.5	11.6	11.6	3.3	20.19	2.02	3.42	0.22	8.73	0.64	70.58	26.75	36.82	30.19
2B	85.2	11.6	11.3	3.3	20.68	2.02	3.45	0.22	8.86	0.64	74.95	26.75	41.82	30.20
3B	81.5	11.6	11.7	3.3	20.85	2.02	3.46	0.22	8.91	0.64	66.85	26.75	32.36	30.19
4B	80.1	11.6	11.6	3.3	20.82	2.02	3.45	0.22	8.96	0.64	70.82	26.75	38.91	30.19
5B	79.2	11.6	11.6	3.3	20.51	2.02	3.41	0.22	8.89	0.64	70.32	26.75	39.05	30.19
6B	84.2	11.6	11.6	3.3	20.69	2.02	3.44	0.22	8.91	0.64	76.06	26.75	42.73	30.20
7B	77.7	11.6	11.8	3.3	20.67	2.02	3.40	0.22	9.02	0.64	67.83	26.75	37.43	30.19
8B	79.7	11.6	11.5	3.3	20.43	2.02	3.45	0.22	8.76	0.64	72.26	26.75	40.71	30.20
9B	86.0	11.6	11.4	3.3	20.55	2.02	3.48	0.22	8.73	0.64	73.89	26.75	41.82	30.20
10B	83.7	11.6	12.0	3.3	20.85	2.02	3.44	0.22	9.02	0.64	70.93	26.75	38.74	30.19
11B	85.9	11.6	11.1	3.3	20.28	2.02	3.44	0.22	8.72	0.64	73.09	26.75	40.07	30.19
12B	81.4	11.6	11.3	3.3	20.73	2.02	3.43	0.22	8.93	0.64	71.51	26.75	39.75	30.19
13B	83.9	14.2	10.9	3.5	21.11	2.13	3.45	0.23	9.09	0.68	72.37	28.39	39.89	31.57
14B	76.7	11.6	11.6	3.3	21.06	2.02	3.44	0.22	9.08	0.64	67.18	26.75	35.48	30.19
15B	84.8	11.6	11.5	3.3	20.32	2.02	3.39	0.22	8.79	0.64	69.97	26.75	38.31	30.19
16B	86.8	11.6	11.6	3.3	20.64	2.02	3.40	0.22	8.96	0.64	74.21	26.75	42.74	30.20
17B	84.6	11.6	11.6	3.3	20.60	2.02	3.45	0.22	8.82	0.64	70.79	26.75	39.80	30.20
18B	83.3	11.6	11.8	3.3	20.60	2.02	3.44	0.22	8.83	0.64	67.87	26.75	38.58	30.19
19B	83.0	11.6	11.6	3.3	20.66	2.02	3.44	0.22	8.85	0.64	75.42	26.75	47.56	30.19
20B	86.1	11.6	11.4	3.3	20.65	2.02	3.44	0.22	8.90	0.64	72.78	26.75	40.41	30.19
21B	83.5	11.6	11.5	3.3	21.64	2.02	3.51	0.22	9.20	0.64	75.28	26.75	44.40	30.19
22B	82.7	11.6	11.3	3.3	21.07	2.02	3.47	0.22	9.08	0.64	73.73	26.75	43.04	30.19
23B	82.7	11.6	11.6	3.3	20.77	2.02	3.47	0.22	8.90	0.64	71.94	26.75	38.67	30.20
24B	85.6	11.6	12.0	3.3	20.39	2.02	3.37	0.22	8.95	0.64	64.22	26.75	32.24	30.19
1A	84.8	11.6	11.4	3.3	20.23	2.02	3.41	0.22	8.70	0.64	65.18	26.75	34.03	30.19
2A	86.6	11.6	11.0	3.3	20.73	2.02	3.45	0.22	8.84	0.64	70.40	26.75	38.38	30.19
3A	83.7	11.6	11.0	3.3	20.95	2.05	3.44	0.22	8.97	0.65	69.98	26.75	35.69	30.20
4A	83.4	11.6	11.0	3.3	20.78	2.02	3.45	0.22	8.86	0.64	71.21	26.75	38.40	30.19
5A	84.7	11.8	11.0	3.3	20.40	2.02	3.43	0.22	8.74	0.64	70.55	26.75	36.93	30.20
6A	85.0	11.6	10.8	3.3	20.14	2.02	3.39	0.22	8.73	0.64	69.09	26.75	36.75	30.20
7A	85.9	11.6	11.2	3.3	20.75	2.02	3.46	0.22	8.83	0.64	71.99	26.75	38.87	30.19
8A	86.5	11.6	11.5	3.3	20.16	2.02	3.41	0.22	8.70	0.64	66.39	26.75	35.30	30.19
9A	84.2	11.6	11.4	3.3	20.16	2.02	3.38	0.22	8.73	0.64	66.83	26.75	36.44	30.20
10A	84.1	11.6	11.5	3.3	20.27	2.02	3.39	0.22	8.78	0.64	68.94	26.75	35.84	30.19
11A	85.9	11.6	11.0	3.3	20.14	2.05	3.41	0.22	8.68	0.65	67.55	26.75	35.52	30.19
12A	84.7	11.6	11.2	3.3	20.13	2.02	3.38	0.22	8.77	0.64	67.11	26.75	33.99	30.20
13A	85.6	11.6	11.2	3.3	20.26	2.02	3.40	0.22	8.77	0.64	69.28	26.75	36.32	30.20
14A	84.5	11.6	11.2	3.3	20.42	2.02	3.41	0.22	8.78	0.64	68.49	26.75	35.85	30.20
15A	84.8	11.8	11.3	3.3	20.11	2.02	3.36	0.22	8.78	0.64	65.78	26.75	31.81	30.19
16A	85.1	11.6	11.5	3.3	20.46	2.02	3.39	0.22	8.87	0.64	66.32	26.75	35.62	30.19
17A	84.1	11.6	11.0	3.3	20.31	2.02	3.42	0.22	8.70	0.64	70.84	26.75	37.75	30.20
18A	85.1	11.6	11.5	3.3	20.24	2.02	3.37	0.22	8.78	0.64	63.25	26.75	32.39	30.19
19A	80.2	11.6	11.4	3.3	20.46	2.02	3.40	0.22	8.82	0.64	68.80	26.75	36.40	30.19
20A	84.7	11.6	11.5	3.3	20.48	2.02	3.43	0.22	8.80	0.64	68.21	26.75	37.27	30.19
21A	84.8	11.6	11.4	3.3	20.44	2.02	3.42	0.22	8.79	0.64	66.54	26.75	35.97	30.20
22A	84.9	11.6	11.1	3.3	20.45	2.02	3.43	0.22	8.79	0.64	70.63	26.75	38.65	30.19
23A	84.6	11.6	11.5	3.3	20.42	2.02	3.41	0.22	8.77	0.64	65.18	26.75	33.01	30.19
24A	83.7	11.6	11.2	3.3	20.44	2.02	3.39	0.22	8.82	0.64	64.49	26.75	30.42	30.19
MESETA	83.9	10.4	12.8	3.3	20.36	1.94	3.58	0.21	8.30	0.61	80.09	26.07	43.20	29.51
YURIKO	88.5	10.3	11.9	3.3	19.00	1.94	3.45	0.21	8.12	0.61	69.24	26.07	27.78	29.51
ZOO	82.8	10.3	11.2	3.3	20.78	1.94	3.44	0.21	8.98	0.61	69.50	26.07	33.61	29.51
LSD	7.5		1.2		0.73		0.10		0.25		10.36		10.06	

Table S6.6. (continued)

	LODG [1-9 score]		GH [1-9 score]		PV [1-5 score]		DAW [1-9 score]		NECK [%]	
Genotype	Mean	CI	Mean	CI	Mean	CI	Mean	CI	Mean	CI
GALILEOO	4.0	4.5	5.1	1.3	2.4	1.4	1.6	1.2	10.4	8.9
HOOK	3.4	4.5	4.8	1.3	2.4	1.3	1.6	1.2	10.2	8.6
HURRICANE	4.1	4.5	4.6	1.3	2.7	1.4	1.6	1.2	8.9	8.9
1C	2.9	6.3	5.2	2.2	2.3	2.0	1.7	1.5	10.4	16.6
2C	5.3	6.3	4.3	2.2	1.6	2.0	1.5	1.5	8.3	16.6
3C	7.0	6.3	5.5	2.2	2.2	2.0	1.3	1.5	14.5	16.6
4C	4.3	6.3	4.6	2.2	2.5	2.0	1.8	1.5	13.1	16.6
5C	3.6	6.3	5.5	2.2	2.3	2.0	1.5	1.5	17.9	16.6
6C	5.1	6.3	5.7	2.2	2.4	2.0	1.6	1.5	10.6	16.6
7C	5.9	6.3	4.7	2.2	2.2	2.0	2.0	1.5	18.3	16.6
8C	5.5	6.3	4.8	2.2	2.4	2.0	1.5	1.5	11.4	16.6
9C	4.1	6.3	5.0	2.2	2.2	2.0	1.6	1.5	15.3	16.6
10C	7.0	6.3	4.8	2.2	2.5	2.0	1.7	1.5	9.3	16.6
11C	5.0	6.3	4.5	2.2	2.4	2.0	1.3	1.5	24.0	16.6
12C	6.5	6.3	4.8	2.2	2.5	2.0	1.8	1.5	21.0	16.6
13C	5.7	6.3	5.5	2.2	2.4	2.0	1.3	1.5	10.9	16.6
14C	5.0	6.3	4.6	2.2	2.4	2.0	1.3	1.5	16.4	16.6
15C	6.8	6.3	5.5	2.2	1.8	2.0	1.7	1.5	11.2	16.6
16C	4.6	6.3	4.6	2.2	2.0	2.0	1.3	1.5	6.8	16.6
17C	4.8	6.3	4.3	2.2	2.3	2.0	1.4	1.5	7.8	16.6
18C	8.0	6.3	4.5	2.2	1.6	2.0	1.8	1.5	10.0	16.6
19C	3.9	6.3	5.2	2.2	2.0	2.0	3.0	1.5	12.5	16.6
20C	6.4	6.3	5.3	2.2	2.4	2.0	1.6	1.5	7.3	16.6
21C	6.5	6.3	5.3	2.2	2.4	2.0	1.5	1.5	18.1	16.6
22C	6.9	6.3	5.5	2.2	2.5	2.0	1.6	1.5	5.9	16.6
23C	4.8	6.3	4.4	2.2	2.3	2.0	1.8	1.5	11.5	16.6
24C	2.5	6.3	5.2	2.2	2.7	2.0	1.8	1.5	10.3	16.6
1B	3.3	4.7	4.9	1.5	2.7	1.5	1.7	1.3	8.0	10.8
2B	3.2	4.7	5.1	1.5	2.4	1.5	1.7	1.3	10.8	10.8
3B	3.6	4.7	5.5	1.5	2.6	1.5	1.6	1.3	13.1	10.8
4B	3.1	4.7	5.0	1.5	2.6	1.5	1.8	1.3	14.6	10.8
5B	4.7	4.7	5.5	1.5	2.3	1.5	1.5	1.3	12.9	10.8
6B	4.0	4.7	5.0	1.5	2.6	1.5	1.5	1.3	12.8	10.8
7B	3.0	4.7	4.9	1.5	2.5	1.5	1.5	1.3	15.2	10.8
8B	4.2	4.7	5.2	1.5	2.7	1.5	1.5	1.3	11.4	10.8
9B	3.7	4.7	5.1	1.5	2.6	1.5	1.8	1.3	15.6	10.8
10B	3.8	4.7	5.6	1.5	2.4	1.5	1.6	1.3	13.0	10.8
11B	3.9	4.7	5.0	1.5	2.6	1.5	1.5	1.3	15.7	10.8
12B	2.9	4.7	5.4	1.5	2.5	1.5	1.6	1.3	15.9	10.8
13B	3.7	5.3	5.4	1.9	2.4	1.7	1.7	1.3	13.6	13.9
14B	3.5	4.7	5.0	1.5	2.5	1.5	1.6	1.3	15.9	10.8
15B	3.7	4.7	5.0	1.5	2.7	1.5	1.6	1.3	13.1	10.8
16B	3.7	4.7	4.8	1.5	2.6	1.5	1.6	1.3	12.4	10.8
17B	3.7	4.7	4.4	1.5	2.5	1.5	1.6	1.3	15.8	10.8
18B	4.3	4.7	4.9	1.5	2.4	1.5	1.4	1.3	12.9	10.8
19B	3.3	4.7	5.3	1.5	2.1	1.5	1.6	1.3	9.9	10.8
20B	3.0	4.7	5.0	1.5	2.3	1.5	1.5	1.3	18.5	10.8
21B	3.1	4.7	5.3	1.5	2.7	1.5	1.7	1.3	9.8	10.8
22B	4.0	4.7	5.2	1.5	2.6	1.5	1.5	1.3	13.9	10.8
23B	4.0	4.7	5.1	1.5	2.5	1.5	1.5	1.3	10.4	10.8
24B	3.5	4.7	5.0	1.5	2.4	1.5	1.8	1.3	12.0	10.8
1A	3.8	4.6	4.9	1.5	2.2	1.5	1.6	1.3	11.9	10.8
2A	5.0	4.6	4.9	1.5	2.0	1.5	1.4	1.3	10.7	10.8
3A	5.3	4.6	4.9	1.5	2.3	1.5	1.6	1.3	11.5	10.8
4A	4.9	4.6	5.0	1.5	2.0	1.5	1.6	1.3	8.9	10.8
5A	4.6	4.6	4.9	1.5	2.6	1.5	1.6	1.3	14.7	11.1
6A	4.3	4.6	4.7	1.5	2.2	1.5	1.5	1.3	17.2	10.8
7A	4.0	4.6	4.3	1.5	2.4	1.5	1.7	1.3	13.1	10.8
8A	3.5	4.6	4.7	1.5	2.8	1.5	1.7	1.3	13.1	10.8
9A	4.1	4.6	4.9	1.5	2.6	1.5	1.6	1.3	10.0	10.8
10A	5.1	4.6	5.0	1.5	2.2	1.5	1.8	1.3	10.0	10.8
11A	4.8	4.6	4.6	1.5	2.5	1.5	1.7	1.3	11.8	10.8
12A	4.9	4.6	5.0	1.5	2.3	1.5	1.8	1.3	11.9	10.8
13A	4.1	4.7	5.4	1.5	2.7	1.5	1.5	1.3	16.6	10.8
14A	4.3	4.6	5.1	1.5	2.5	1.5	1.5	1.3	13.8	10.8
15A	5.8	4.6	4.7	1.5	2.6	1.5	1.9	1.3	13.3	10.8
16A	4.2	4.6	4.9	1.5	2.4	1.5	1.6	1.3	15.6	10.8
17A	4.3	4.6	4.7	1.5	2.5	1.5	1.6	1.3	12.4	10.8
18A	4.8	4.6	4.9	1.5	2.9	1.5	1.5	1.3	10.5	10.8
19A	4.4	4.6	4.9	1.5	2.5	1.5	1.9	1.3	10.4	10.8
20A	4.7	4.6	4.8	1.5	2.3	1.5	1.8	1.3	12.5	10.8
21A	5.0	4.6	5.2	1.5	2.3	1.5	1.5	1.3	15.0	10.8
22A	4.5	4.6	4.8	1.5	2.3	1.5	1.7	1.3	18.6	10.8
23A	4.3	4.6	4.5	1.5	2.8	1.5	1.7	1.3	14.3	10.8
24A	4.0	4.6	5.1	1.5	2.4	1.5	1.6	1.3	11.9	10.8
MESETA	2.9	4.5	4.9	1.3	2.6	1.4	1.7	1.2	5.2	8.9
YURIKO	3.9	4.5	3.2	1.3	2.9	1.4	1.5	1.2	11.9	8.9
ZOO	2.7	4.5	4.8	1.3	2.0	1.4	1.6	1.2	8.8	8.9
LSD	2.1		1.0		0.8		0.5		7.8	

Table S6.7. Additive main effect and multiplicative interaction (AMMI), within the analysis of variance of grain yield ($t \cdot ha^{-1}$) of barley genotypes in 7 environments.

Source	Df[a]	Sum Sq[b]	Mean Sq[c]	F value[d]	P-value	GxE explained (%)	Cumulative (%)
Environment	6	1793.2	298.874	1382.57	0.000		
Repetition (Environment)	7	10.3	1.473	6.81	0.000		
Block (Repetition*Environment)	70	33.5	0.478	2.21	0.000		
Genotype	77	113.2	1.470	6.80	0.000		
Genotype*Environment (GxE)	339	160.6	0.474	2.19	0.000		
PC1	83	58.1	0.701	3.24	0.000	42.5	42.5
PC2	81	26.1	0.323	1.49	0.007	19.1	61.6
PC3	79	22.3	0.282	1.30	0.054	16.3	77.8
PC4	77	13.0	0.169	0.78	0.910	9.5	87.4
PC5	75	10.5	0.140	0.65	0.989	7.6	95.0
PC6	73	6.8	0.094	0.43	1.000	5.0	100.0
PC7	71	0.0	0.000	0.00	1.000	0.0	100.0
Residuals	457	98.8	0.216				
Total	1495	2346.5	1.570				

[a]Degrees of freedom, [b]sum of squares, [c]mean squares, [d]F-statistic.

Table S6.8. Comparison of yield components between Sadaba_19 and Valladolid_20 environments for checks, and hybrids split by male restorer parent.

	EM2 [ears·m^{-2}]		TGW [g]		GPE [grains·ear^{-1}]		GY [t·ha^{-1}]	
	Sadaba_19	Valladolid_20	Sadaba_19	Valladolid_20	Sadaba_19	Valladolid_20	Sadaba_19	Valladolid_20
Male restorer A	332	437	39.6	33.2	46.2	36.6	4.106	6.417
Male restorer B	394	533	41.4	35.0	44.4	30.2	4.747	6.557
Male restorer C	319		44.5		44.5		4.684	
Checks	436	560	43.1	34.8	37.5	29.2	5.021	6.277

Table S6.9. Scores of principal components of AMMI (Additive Main effect and Multiplicative Interaction) analysis for grain yield (GY), and weighted average of absolute scores (WAAS) index for genotypes and environments.

Type	Code	GY ($t \cdot ha^{-1}$)	PC1	PC2	PC3	PC4	PC5	PC6	PC7	WAAS index
Genotype	10A	6.63	-0.51	-0.36	0.03	0.10	-0.03	0.15	0.00	0.47
Genotype	10B	6.43	-0.11	0.06	-0.16	-0.02	0.19	0.19	0.00	0.09
Genotype	10C	6.36	-0.02	-0.05	-0.15	0.04	0.00	-0.08	0.00	0.03
Genotype	11A	6.71	-0.50	0.02	0.00	0.09	0.07	-0.07	0.00	0.35
Genotype	11B	6.59	0.15	-0.15	-0.27	0.04	-0.10	-0.08	0.00	0.15
Genotype	11C	7.21	-0.12	-0.28	-0.13	-0.02	0.04	0.00	0.00	0.17
Genotype	12A	6.41	-0.10	-0.33	0.06	-0.06	-0.04	0.01	0.00	0.17
Genotype	12B	6.35	0.74	-0.16	0.35	0.38	0.26	0.28	0.00	0.56
Genotype	12C	6.55	0.00	0.01	-0.04	0.02	0.00	-0.03	0.00	0.00
Genotype	13A	6.40	-0.15	-0.16	0.19	-0.16	-0.24	-0.05	0.00	0.16
Genotype	13B	6.61	0.17	0.28	-0.08	0.08	0.19	-0.06	0.00	0.20
Genotype	13C	7.37	-0.11	-0.27	-0.07	-0.04	0.04	0.03	0.00	0.16
Genotype	14A	6.56	-0.34	0.06	-0.35	0.38	0.06	0.00	0.00	0.25
Genotype	14B	6.31	0.22	-0.12	0.00	0.32	0.08	-0.03	0.00	0.19
Genotype	14C	6.31	-0.09	-0.22	-0.32	0.07	0.02	-0.14	0.00	0.13
Genotype	15A	6.44	-0.29	-0.36	0.16	-0.24	0.05	0.02	0.00	0.31
Genotype	15B	6.46	0.14	-0.10	0.25	-0.06	0.12	-0.16	0.00	0.13
Genotype	15C	6.53	-0.01	-0.02	-0.07	0.02	0.00	-0.04	0.00	0.01
Genotype	16A	6.45	-0.27	0.34	-0.36	0.10	0.03	-0.09	0.00	0.29
Genotype	16B	6.74	0.41	-0.04	-0.18	-0.06	-0.05	0.07	0.00	0.29
Genotype	16C	6.84	0.01	0.03	0.06	-0.02	0.00	0.03	0.00	0.01
Genotype	17A	6.76	-0.16	0.34	-0.13	-0.13	0.21	-0.03	0.00	0.22
Genotype	17B	6.52	0.29	-0.02	0.14	-0.12	0.14	-0.06	0.00	0.21
Genotype	17C	7.51	-0.17	-0.42	-0.17	-0.03	0.06	0.00	0.00	0.25
Genotype	18A	7.05	-0.09	0.11	0.40	-0.21	-0.14	-0.15	0.00	0.10
Genotype	18B	6.71	0.24	-0.06	-0.10	0.10	-0.07	-0.16	0.00	0.19
Genotype	18C	7.17	0.09	0.23	0.34	-0.07	-0.02	0.15	0.00	0.13
Genotype	19A	6.89	-0.36	-0.04	-0.25	-0.25	-0.15	0.10	0.00	0.26
Genotype	19B	6.98	-0.23	0.60	-0.18	0.01	-0.13	0.03	0.00	0.34
Genotype	19C	7.22	-0.03	-0.06	0.08	-0.05	0.01	0.07	0.00	0.04
Genotype	1A	6.52	-0.52	-0.01	0.16	0.03	-0.11	-0.17	0.00	0.36
Genotype	1B	6.73	0.00	-0.33	0.63	-0.19	0.33	-0.28	0.00	0.10
Genotype	1C	6.22	0.11	0.26	0.10	0.02	-0.04	0.00	0.00	0.15
Genotype	20A	6.50	-0.04	-0.11	0.18	-0.10	0.18	0.28	0.00	0.06
Genotype	20B	6.55	0.35	0.01	-0.30	0.06	0.10	-0.16	0.00	0.25
Genotype	20C	6.80	-0.02	-0.05	-0.03	0.00	0.01	0.00	0.00	0.03
Genotype	21A	6.83	-0.40	-0.09	-0.07	0.11	0.06	-0.11	0.00	0.30
Genotype	21B	6.70	0.13	0.09	-0.29	-0.39	0.36	0.07	0.00	0.12
Genotype	21C	6.73	0.07	0.17	0.16	-0.02	-0.02	0.06	0.00	0.10
Genotype	22A	6.74	-0.19	0.06	0.21	0.32	-0.08	0.01	0.00	0.15
Genotype	22B	6.85	0.02	0.09	0.25	-0.07	0.21	-0.20	0.00	0.04
Genotype	22C	6.19	0.14	0.34	0.17	0.02	-0.05	0.01	0.00	0.20
Genotype	23A	6.67	-0.29	0.45	0.14	0.11	0.00	0.10	0.00	0.34
Genotype	23B	6.91	0.30	0.04	-0.23	0.65	0.10	0.18	0.00	0.22
Genotype	23C	6.24	0.07	0.17	0.03	0.03	-0.03	-0.03	0.00	0.10
Genotype	24A	6.50	-0.32	-0.10	-0.10	-0.23	-0.42	0.30	0.00	0.26
Genotype	24B	6.89	-0.01	0.14	-0.14	-0.35	-0.18	0.22	0.00	0.05
Genotype	24C	6.88	0.00	0.00	0.05	-0.02	0.00	0.03	0.00	0.00
Genotype	2A	6.81	-0.32	0.10	-0.11	-0.29	0.16	-0.58	0.00	0.25
Genotype	2B	6.93	0.35	0.10	-0.22	0.17	0.18	-0.23	0.00	0.27
Genotype	2C	7.76	0.06	0.17	0.45	-0.13	-0.01	0.24	0.00	0.10

Table S6.9. (continued)

Type	Code	GY (t·ha^{-1})	PC1	PC2	PC3	PC4	PC5	PC6	PC7	WAAS index
Genotype	3A	6.46	0.14	-0.22	-0.02	0.23	-0.23	0.21	0.00	0.17
Genotype	3B	6.80	0.03	0.09	-0.13	0.08	0.27	0.13	0.00	0.05
Genotype	3C	6.78	0.06	0.16	0.17	-0.03	-0.02	0.06	0.00	0.10
Genotype	4A	6.68	-0.11	-0.06	0.29	-0.04	-0.28	-0.04	0.00	0.10
Genotype	4B	6.69	0.12	0.27	-0.13	0.04	-0.11	0.01	0.00	0.16
Genotype	4C	6.56	-0.04	-0.10	-0.14	0.03	0.01	-0.06	0.00	0.06
Genotype	5A	6.46	-0.24	-0.21	0.40	0.30	0.13	0.04	0.00	0.23
Genotype	5B	6.44	0.41	-0.12	0.11	-0.12	-0.31	0.08	0.00	0.32
Genotype	5C	7.33	-0.05	-0.10	0.07	-0.05	0.02	0.07	0.00	0.06
Genotype	6A	6.39	-0.37	-0.02	0.06	0.13	0.06	0.15	0.00	0.26
Genotype	6B	6.72	0.26	-0.09	0.05	-0.27	0.31	0.06	0.00	0.21
Genotype	6C	6.89	-0.01	-0.02	0.03	-0.02	0.00	0.02	0.00	0.02
Genotype	7A	6.68	-0.50	0.17	0.09	0.12	0.11	0.12	0.00	0.40
Genotype	7B	6.91	-0.08	0.08	-0.04	0.02	-0.03	-0.11	0.00	0.08
Genotype	7C	6.24	0.22	0.54	0.37	-0.01	-0.07	0.09	0.00	0.31
Genotype	8A	6.52	-0.33	0.23	-0.05	0.28	0.17	0.00	0.00	0.30
Genotype	8B	6.56	0.58	0.03	-0.26	-0.15	0.19	0.12	0.00	0.41
Genotype	8C	6.91	-0.02	-0.04	0.01	-0.02	0.01	0.02	0.00	0.03
Genotype	9A	6.57	-0.03	-0.37	-0.04	0.13	-0.01	-0.12	0.00	0.14
Genotype	9B	6.78	0.36	-0.11	0.21	-0.01	-0.12	-0.15	0.00	0.28
Genotype	9C	7.15	-0.17	-0.41	-0.26	0.00	0.05	-0.06	0.00	0.24
Genotype	*GALILEOO*	6.71	0.17	-0.43	-0.16	-0.14	-0.20	0.18	0.00	0.25
Genotype	*HOOK*	6.55	0.58	-0.31	-0.16	0.00	-0.15	0.05	0.00	0.50
Genotype	*HURRICANE*	7.01	0.04	0.15	0.01	0.16	-0.31	-0.28	0.00	0.07
Genotype	*MESETA*	6.56	0.46	0.21	-0.23	-0.43	0.09	-0.24	0.00	0.38
Genotype	*YURIKO*	6.81	0.40	0.10	0.01	0.22	-0.75	-0.34	0.00	0.31
Genotype	*ZOO*	7.39	-0.13	0.21	-0.36	-0.34	-0.16	0.37	0.00	0.15
Environment	Burgos_19	7.87	-0.36	-0.71	0.89	-0.09	-0.73	-0.64	0.00	0.47
Environment	Grisolles_19	9.18	-0.15	-0.79	0.56	-0.47	0.77	0.66	0.00	0.35
Environment	Grisolles_20	6.51	0.08	-0.12	-0.15	1.18	-0.44	0.63	0.00	0.09
Environment	Sadaba_19	4.55	1.25	1.13	0.46	-0.48	-0.28	0.19	0.00	1.22
Environment	Sadaba_20	6.92	0.51	-0.60	-1.37	-0.52	-0.25	-0.12	0.00	0.54
Environment	Valladolid_19	5.46	0.45	0.21	-0.06	0.62	0.91	-0.74	0.00	0.38
Environment	Valladolid_20	6.50	-1.79	0.88	-0.32	-0.22	0.02	0.01	0.00	1.51

Table S6.10. Linear correlation coefficients between heading time and grain yield. Significant coefficients in bold type.

	Across environments	Burgos_19	Grisolles_19	Sádaba_19	Valladolid_19	Grisolles_20	Sádaba_20	Valladolid_20
Hybrids (Restorer A)	0.04	-0.10	0.09	-0.12	-0.07	-0.22	-0.06	-0.32
Hybrids (Restorer B)	-0.26	-0.18	0.17	-0.33	0.07	-0.10	-0.16	-0.10
Hybrids (Restorer C)	-0.39			**-0.47**			-0.27	
All genotypes	0.03	0.00	**0.46**	0.03	0.23	-0.17	-0.01	-0.17

Table S6.11. Grain yield advantage of checks over test hybrids according to the length of the cycle. Grain yield advantage was calculated as the difference between the grain yield of the checks and the grain yield of the test hybrids. Cycle was calculated as the difference between sowing and heading date. Pearson correlation coefficient between grain yield advantage and cycle length is presented.

Yield advantage ($t \cdot ha^{-1}$)	Sadaba_19	Burgos_19	Grisolles_19	Valladolid_19	Sadaba_20	Grisolles_20	Valladolid_20	r (Yield advantage, cycle)
Checks-Restorer A hybrids	0.91	0.29	-0.04	0.20	0.91	0.21	-0.14	-0.62
Checks-Restorer B hybrids	0.27	0.10	-0.20	-0.10	0.52	0.21	-0.28	-0.87
Checks-Restorer C hybrids	0.34				0.43			
Checks-Hybrids	0.51	0.20	-0.12	0.04	0.62	0.21	-0.21	-0.75
Cycle (days)	156	155	161	156	122	137	169	

Table S6.12. Predicted values for days to heading (DTH, days), height (HT, cm), and grain yield (GY, $t \cdot ha^{-1}$) of field-assessed (training set, 72) and untested (test set, 348) hybrids. For each trait, the model with the highest prediction ability was used.

Hybrid	Female	Pollinator	Male restorer	DTH	HT	GY	Subset
1C	α	1	C	118.94	102.88	6.640	Training set
2C	α	2	C	117.81	102.63	7.034	Training set
3C	α	3	C	117.40	106.02	6.739	Training set
4C	α	4	C	118.21	104.27	6.805	Training set
5C	α	5	C	117.97	104.01	6.870	Training set
6C	α	6	C	118.94	106.64	6.748	Training set
7C	α	7	C	117.48	105.76	6.775	Training set
8C	α	8	C	117.94	104.68	6.790	Training set
9C	α	9	C	118.11	105.56	6.878	Training set
10C	α	10	C	118.22	105.65	6.712	Training set
11C	α	11	C	117.90	105.23	6.827	Training set
12C	α	12	C	117.75	106.30	6.684	Training set
13C	α	13	C	117.55	103.89	6.880	Training set
14C	α	14	C	118.75	104.83	6.533	Training set
15C	α	15	C	118.71	104.06	6.561	Training set
16C	α	16	C	117.96	104.25	6.834	Training set
17C	α	17	C	117.83	105.55	6.837	Training set
18C	α	18	C	117.96	106.52	6.895	Training set
19C	α	19	C	117.95	105.58	6.882	Training set
20C	α	20	C	117.82	104.34	6.858	Training set
21C	α	21	C	118.55	106.66	6.772	Training set
22C	α	22	C	118.45	105.06	6.807	Training set
23C	α	23	C	118.14	104.17	6.697	Training set
24C	α	24	C	118.56	101.91	6.831	Training set
1B	α	1	B	119.80	104.07	6.701	Training set
2B	α	2	B	119.18	103.37	6.947	Training set
3B	α	3	B	120.05	101.68	6.749	Training set
4B	α	4	B	119.31	102.67	6.796	Training set
5B	α	5	B	119.27	102.85	6.782	Training set
6B	α	6	B	119.75	104.12	6.740	Training set
7B	α	7	B	119.23	102.99	6.783	Training set
8B	α	8	B	119.42	102.29	6.765	Training set
9B	α	9	B	119.27	103.55	6.816	Training set
10B	α	10	B	120.07	103.26	6.678	Training set
11B	α	11	B	118.85	103.02	6.756	Training set
12B	α	12	B	119.66	102.71	6.619	Training set
13B	α	13	B	119.59	103.86	6.810	Training set
14B	α	14	B	119.87	102.91	6.580	Training set
15B	α	15	B	119.47	102.18	6.605	Training set
16B	α	16	B	119.29	103.40	6.784	Training set
17B	α	17	B	119.53	103.94	6.742	Training set
18B	α	18	B	119.85	102.22	6.832	Training set
19B	α	19	B	120.32	102.55	6.858	Training set
20B	α	20	B	119.49	103.50	6.774	Training set
21B	α	21	B	119.62	103.05	6.794	Training set
22B	α	22	B	118.98	103.39	6.806	Training set
23B	α	23	B	119.15	102.38	6.793	Training set
24B	α	24	B	119.33	103.40	6.812	Training set
3A	α	3	A	117.87	100.94	6.388	Training set
2A	α	2	A	117.30	102.49	6.710	Training set
1A	α	1	A	118.19	101.46	6.465	Training set
4A	α	4	A	116.92	101.31	6.557	Training set
5A	α	5	A	117.18	101.81	6.550	Training set
6A	α	6	A	117.75	101.85	6.442	Training set
7A	α	7	A	116.77	100.37	6.539	Training set
8A	α	8	A	117.22	101.20	6.531	Training set
9A	α	9	A	117.50	101.88	6.571	Training set
10A	α	10	A	117.56	100.36	6.475	Training set
11A	α	11	A	116.78	101.03	6.532	Training set
12A	α	12	A	117.34	100.89	6.396	Training set

Table S6.12. (continued)

Hybrid	Female	Pollinator	Male restorer	DTH	HT	GY	Subset
17A	α	17	A	117.39	102.09	6.576	Training set
16A	α	16	A	117.68	99.62	6.325	Training set
15A	α	15	A	118.09	101.85	6.326	Training set
14A	α	14	A	117.25	101.78	6.551	Training set
13A	α	13	A	117.24	101.98	6.511	Training set
22A	α	22	A	117.32	100.94	6.607	Training set
21A	α	21	A	118.12	101.01	6.639	Training set
20A	α	20	A	117.29	102.04	6.549	Training set
19A	α	19	A	117.78	101.82	6.588	Training set
18A	α	18	A	117.86	102.51	6.588	Training set
23A	α	23	A	117.76	99.57	6.485	Training set
24A	α	24	A	117.32	100.30	6.574	Training set
25A	α	25	A	117.23	101.25	6.534	Test set
26A	α	26	A	117.26	101.63	6.612	Test set
27A	α	27	A	117.30	101.79	6.584	Test set
28A	α	28	A	117.18	100.64	6.537	Test set
29A	α	29	A	117.57	102.12	6.569	Test set
30A	α	30	A	117.79	100.40	6.464	Test set
31A	α	31	A	117.67	102.06	6.524	Test set
32A	α	32	A	117.57	100.92	6.447	Test set
33A	α	33	A	117.75	101.34	6.457	Test set
34A	α	34	A	117.36	101.15	6.461	Test set
35A	α	35	A	117.50	101.06	6.519	Test set
36A	α	36	A	117.23	100.86	6.494	Test set
37A	α	37	A	117.19	100.43	6.519	Test set
38A	α	38	A	118.07	101.48	6.428	Test set
39A	α	39	A	117.36	101.73	6.483	Test set
40A	α	40	A	117.81	101.04	6.465	Test set
41A	α	41	A	117.59	100.80	6.383	Test set
42A	α	42	A	117.41	101.22	6.548	Test set
43A	α	43	A	117.63	100.44	6.442	Test set
44A	α	44	A	117.74	100.30	6.430	Test set
45A	α	45	A	117.86	100.99	6.480	Test set
46A	α	46	A	117.55	101.83	6.560	Test set
47A	α	47	A	117.80	100.30	6.462	Test set
48A	α	48	A	117.44	100.74	6.478	Test set
49A	α	49	A	117.29	101.50	6.470	Test set
50A	α	50	A	117.31	101.29	6.482	Test set
51A	α	51	A	117.48	101.05	6.454	Test set
52A	α	52	A	117.35	100.86	6.563	Test set
53A	α	53	A	117.19	101.26	6.592	Test set
54A	α	54	A	117.22	101.67	6.579	Test set
55A	α	55	A	117.21	101.84	6.601	Test set
56A	α	56	A	117.37	101.48	6.487	Test set
57A	α	57	A	117.15	101.09	6.555	Test set
58A	α	58	A	117.39	101.62	6.586	Test set
59A	α	59	A	117.28	100.60	6.457	Test set
60A	α	60	A	117.43	101.69	6.588	Test set
61A	α	61	A	117.75	101.54	6.491	Test set
62A	α	62	A	117.32	101.60	6.572	Test set
63A	α	63	A	117.30	101.64	6.585	Test set
64A	α	64	A	117.33	101.67	6.559	Test set
65A	α	65	A	117.49	101.27	6.551	Test set
66A	α	66	A	117.28	101.39	6.540	Test set
67A	α	67	A	117.37	101.81	6.567	Test set
68A	α	68	A	117.34	101.18	6.557	Test set
69A	α	69	A	117.27	101.74	6.529	Test set
70A	α	70	A	117.73	100.82	6.436	Test set
71A	α	71	A	117.56	102.10	6.511	Test set
72A	α	72	A	117.35	100.58	6.482	Test set
73A	α	73	A	117.14	101.13	6.567	Test set
74A	α	74	A	117.33	101.07	6.451	Test set
75A	α	75	A	117.37	101.01	6.496	Test set

Table S6.12. (continued)

Hybrid	Female	Pollinator	Male restorer	DTH	HT	GY	Subset
76A	α	76	A	117.18	100.96	6.510	Test set
77A	α	77	A	117.24	101.11	6.453	Test set
78A	α	78	A	116.97	100.75	6.534	Test set
79A	α	79	A	117.49	101.43	6.468	Test set
80A	α	80	A	117.36	100.92	6.418	Test set
81A	α	81	A	117.59	102.00	6.507	Test set
82A	α	82	A	117.39	102.09	6.576	Test set
83A	α	83	A	117.39	102.09	6.576	Test set
84A	α	84	A	117.37	101.83	6.544	Test set
85A	α	85	A	117.29	101.62	6.529	Test set
86A	α	86	A	117.78	101.79	6.448	Test set
87A	α	87	A	117.66	101.72	6.473	Test set
88A	α	88	A	117.58	101.54	6.463	Test set
89A	α	89	A	117.56	101.64	6.462	Test set
90A	α	90	A	117.70	101.09	6.428	Test set
91A	α	91	A	117.36	100.90	6.541	Test set
92A	α	92	A	117.39	100.84	6.561	Test set
93A	α	93	A	117.51	100.87	6.523	Test set
94A	α	94	A	117.32	101.31	6.455	Test set
95A	α	95	A	117.53	100.83	6.526	Test set
96A	α	96	A	117.30	101.16	6.509	Test set
97A	α	97	A	117.23	101.42	6.522	Test set
98A	α	98	A	117.83	101.42	6.549	Test set
99A	α	99	A	117.63	101.26	6.551	Test set
100A	α	100	A	117.33	100.99	6.512	Test set
101A	α	101	A	117.54	100.99	6.563	Test set
102A	α	102	A	117.44	100.70	6.446	Test set
103A	α	103	A	117.32	101.43	6.522	Test set
104A	α	104	A	117.21	101.20	6.529	Test set
105A	α	105	A	117.22	101.14	6.509	Test set
106A	α	106	A	117.11	100.82	6.491	Test set
107A	α	107	A	117.26	100.86	6.509	Test set
108A	α	108	A	117.74	101.18	6.522	Test set
109A	α	109	A	117.91	100.84	6.436	Test set
110A	α	110	A	117.35	100.73	6.483	Test set
111A	α	111	A	117.58	101.56	6.458	Test set
112A	α	112	A	117.90	100.72	6.454	Test set
113A	α	113	A	117.53	101.11	6.485	Test set
114A	α	114	A	116.77	100.89	6.529	Test set
115A	α	115	A	117.68	101.03	6.484	Test set
116A	α	116	A	117.50	101.03	6.495	Test set
117A	α	117	A	117.31	101.99	6.565	Test set
118A	α	118	A	117.82	101.09	6.494	Test set
119A	α	119	A	117.43	101.12	6.533	Test set
120A	α	120	A	117.39	101.20	6.592	Test set
121A	α	121	A	117.73	101.92	6.517	Test set
122A	α	122	A	117.71	101.61	6.429	Test set
123A	α	123	A	117.44	101.04	6.545	Test set
124A	α	124	A	117.29	101.16	6.473	Test set
125A	α	125	A	117.52	100.90	6.391	Test set
126A	α	126	A	117.28	101.49	6.565	Test set
127A	α	127	A	117.33	101.07	6.496	Test set
128A	α	128	A	117.29	100.55	6.513	Test set
129A	α	129	A	117.67	100.98	6.392	Test set
130A	α	130	A	117.40	100.66	6.448	Test set
131A	α	131	A	117.41	100.93	6.409	Test set
132A	α	132	A	117.65	101.03	6.482	Test set
133A	α	133	A	117.79	100.62	6.459	Test set
134A	α	134	A	117.83	100.36	6.426	Test set
135A	α	135	A	117.67	100.75	6.478	Test set
136A	α	136	A	117.88	101.22	6.433	Test set
137A	α	137	A	117.68	101.08	6.501	Test set
138A	α	138	A	117.40	101.53	6.573	Test set

Table S6.12. (continued)

Hybrid	Female	Pollinator	Male restorer	DTH	HT	GY	Subset
139A	α	139	A	117.76	101.04	6.447	Test set
140A	α	140	A	117.48	101.95	6.499	Test set
25B	α	25	B	119.26	102.73	6.796	Test set
26B	α	26	B	119.37	102.75	6.856	Test set
27B	α	27	B	119.30	103.61	6.841	Test set
28B	α	28	B	119.65	102.17	6.776	Test set
29B	α	29	B	119.13	102.53	6.800	Test set
30B	α	30	B	119.33	103.81	6.765	Test set
31B	α	31	B	119.11	103.39	6.761	Test set
32B	α	32	B	119.28	103.46	6.688	Test set
33B	α	33	B	119.19	102.85	6.727	Test set
34B	α	34	B	119.63	103.66	6.698	Test set
35B	α	35	B	119.43	102.71	6.780	Test set
36B	α	36	B	119.48	102.66	6.719	Test set
37B	α	37	B	119.38	102.56	6.746	Test set
38B	α	38	B	119.93	103.24	6.719	Test set
39B	α	39	B	119.50	102.94	6.724	Test set
40B	α	40	B	119.27	102.80	6.744	Test set
41B	α	41	B	119.38	102.97	6.668	Test set
42B	α	42	B	119.64	103.15	6.777	Test set
43B	α	43	B	119.41	103.63	6.748	Test set
44B	α	44	B	119.26	103.31	6.728	Test set
45B	α	45	B	119.32	102.98	6.724	Test set
46B	α	46	B	119.61	102.97	6.781	Test set
47B	α	47	B	119.47	103.53	6.750	Test set
48B	α	48	B	119.53	103.15	6.734	Test set
49B	α	49	B	119.66	103.48	6.716	Test set
50B	α	50	B	119.65	103.24	6.727	Test set
51B	α	51	B	120.02	102.79	6.724	Test set
52B	α	52	B	119.48	103.22	6.808	Test set
53B	α	53	B	119.17	103.72	6.849	Test set
54B	α	54	B	119.39	102.63	6.812	Test set
55B	α	55	B	119.36	102.96	6.834	Test set
56B	α	56	B	119.33	102.70	6.758	Test set
57B	α	57	B	119.44	102.94	6.794	Test set
58B	α	58	B	119.47	103.63	6.827	Test set
59B	α	59	B	119.33	102.75	6.724	Test set
60B	α	60	B	119.52	103.55	6.824	Test set
61B	α	61	B	119.01	103.33	6.775	Test set
62B	α	62	B	119.31	102.45	6.800	Test set
63B	α	63	B	119.47	102.93	6.818	Test set
64B	α	64	B	119.43	102.55	6.793	Test set
65B	α	65	B	119.65	102.43	6.797	Test set
66B	α	66	B	119.30	102.61	6.781	Test set
67B	α	67	B	119.50	102.84	6.800	Test set
68B	α	68	B	119.41	103.13	6.783	Test set
69B	α	69	B	119.45	102.96	6.764	Test set
70B	α	70	B	119.36	103.80	6.744	Test set
71B	α	71	B	118.84	103.55	6.749	Test set
72B	α	72	B	119.45	102.32	6.740	Test set
73B	α	73	B	119.36	102.78	6.804	Test set
74B	α	74	B	119.25	103.03	6.750	Test set
75B	α	75	B	119.56	102.76	6.726	Test set
76B	α	76	B	119.37	102.65	6.731	Test set
77B	α	77	B	119.44	102.55	6.675	Test set
78B	α	78	B	119.17	102.90	6.756	Test set
79B	α	79	B	119.89	102.60	6.688	Test set
80B	α	80	B	119.65	102.73	6.639	Test set
81B	α	81	B	119.52	103.73	6.771	Test set
82B	α	82	B	119.59	103.86	6.810	Test set
83B	α	83	B	119.59	103.86	6.810	Test set
84B	α	84	B	119.54	103.52	6.778	Test set
85B	α	85	B	119.36	103.43	6.769	Test set

Table S6.12. (continued)

Hybrid	Female	Pollinator	Male restorer	DTH	HT	GY	Subset
86B	α	86	B	119.68	103.11	6.715	Test set
87B	α	87	B	119.62	102.88	6.729	Test set
88B	α	88	B	119.67	103.19	6.721	Test set
89B	α	89	B	119.59	103.18	6.721	Test set
90B	α	90	B	119.18	103.50	6.719	Test set
91B	α	91	B	119.63	102.62	6.771	Test set
92B	α	92	B	119.71	102.49	6.786	Test set
93B	α	93	B	119.60	102.45	6.750	Test set
94B	α	94	B	119.39	102.55	6.684	Test set
95B	α	95	B	119.73	102.49	6.756	Test set
96B	α	96	B	119.56	102.41	6.733	Test set
97B	α	97	B	119.44	103.52	6.759	Test set
98B	α	98	B	119.97	102.90	6.795	Test set
99B	α	99	B	119.89	103.72	6.777	Test set
100B	α	100	B	119.66	102.81	6.735	Test set
101B	α	101	B	119.77	102.40	6.791	Test set
102B	α	102	B	119.45	102.75	6.696	Test set
103B	α	103	B	119.40	102.86	6.757	Test set
104B	α	104	B	119.50	103.20	6.763	Test set
105B	α	105	B	119.44	102.84	6.740	Test set
106B	α	106	B	119.50	103.43	6.727	Test set
107B	α	107	B	119.41	103.00	6.738	Test set
108B	α	108	B	119.62	102.81	6.777	Test set
109B	α	109	B	119.48	103.53	6.712	Test set
110B	α	110	B	119.73	102.59	6.704	Test set
111B	α	111	B	119.87	103.00	6.726	Test set
112B	α	112	B	119.61	102.75	6.737	Test set
113B	α	113	B	119.74	103.19	6.716	Test set
114B	α	114	B	119.07	103.07	6.770	Test set
115B	α	115	B	119.19	103.28	6.736	Test set
116B	α	116	B	119.21	103.36	6.747	Test set
117B	α	117	B	119.45	103.71	6.797	Test set
118B	α	118	B	119.31	102.91	6.772	Test set
119B	α	119	B	119.11	102.86	6.778	Test set
120B	α	120	B	119.72	102.48	6.814	Test set
121B	α	121	B	119.82	102.77	6.770	Test set
122B	α	122	B	119.79	103.44	6.680	Test set
123B	α	123	B	119.59	102.68	6.772	Test set
124B	α	124	B	119.56	102.91	6.714	Test set
125B	α	125	B	119.36	103.60	6.680	Test set
126B	α	126	B	119.47	102.86	6.791	Test set
127B	α	127	B	119.69	102.58	6.711	Test set
128B	α	128	B	119.59	103.09	6.744	Test set
129B	α	129	B	119.50	102.80	6.661	Test set
130B	α	130	B	119.39	102.70	6.689	Test set
131B	α	131	B	119.43	103.45	6.676	Test set
132B	α	132	B	119.33	103.28	6.749	Test set
133B	α	133	B	119.24	103.20	6.749	Test set
134B	α	134	B	119.35	103.49	6.724	Test set
135B	α	135	B	119.51	103.22	6.759	Test set
136B	α	136	B	119.18	103.00	6.732	Test set
137B	α	137	B	119.45	102.68	6.774	Test set
138B	α	138	B	119.55	102.70	6.802	Test set
139B	α	139	B	119.48	103.09	6.735	Test set
140B	α	140	B	119.70	102.84	6.764	Test set
25C	α	25	C	118.04	104.26	6.801	Test set
26C	α	26	C	117.66	104.46	6.901	Test set
27C	α	27	C	118.54	104.03	6.883	Test set
28C	α	28	C	117.83	105.37	6.786	Test set
29C	α	29	C	118.12	105.20	6.837	Test set
30C	α	30	C	118.86	102.28	6.709	Test set
31C	α	31	C	118.80	106.46	6.759	Test set
32C	α	32	C	118.25	104.59	6.676	Test set

Table S6.12. (continued)

Hybrid	Female	Pollinator	Male restorer	DTH	HT	GY	Subset
33C	α	33	C	118.44	105.33	6.678	Test set
34C	α	34	C	118.26	105.05	6.720	Test set
35C	α	35	C	117.94	104.75	6.767	Test set
36C	α	36	C	118.17	106.12	6.768	Test set
37C	α	37	C	117.91	105.95	6.782	Test set
38C	α	38	C	118.83	106.42	6.662	Test set
39C	α	39	C	118.29	105.61	6.782	Test set
40C	α	40	C	118.30	104.10	6.715	Test set
41C	α	41	C	118.51	104.42	6.644	Test set
42C	α	42	C	117.94	105.66	6.807	Test set
43C	α	43	C	118.74	104.53	6.667	Test set
44C	α	44	C	118.26	103.96	6.645	Test set
45C	α	45	C	118.48	106.39	6.687	Test set
46C	α	46	C	117.96	105.59	6.840	Test set
47C	α	47	C	118.22	104.08	6.675	Test set
48C	α	48	C	117.78	103.96	6.759	Test set
49C	α	49	C	118.38	103.71	6.773	Test set
50C	α	50	C	118.06	104.71	6.776	Test set
51C	α	51	C	117.91	105.58	6.746	Test set
52C	α	52	C	118.46	103.39	6.817	Test set
53C	α	53	C	118.46	102.76	6.865	Test set
54C	α	54	C	117.73	104.37	6.864	Test set
55C	α	55	C	117.67	104.27	6.884	Test set
56C	α	56	C	118.35	104.59	6.750	Test set
57C	α	57	C	118.24	104.33	6.805	Test set
58C	α	58	C	118.16	103.63	6.865	Test set
59C	α	59	C	118.54	104.23	6.686	Test set
60C	α	60	C	118.04	103.80	6.872	Test set
61C	α	61	C	118.80	103.56	6.731	Test set
62C	α	62	C	117.84	104.69	6.844	Test set
63C	α	63	C	117.78	104.87	6.868	Test set
64C	α	64	C	117.81	104.90	6.847	Test set
65C	α	65	C	117.71	104.68	6.825	Test set
66C	α	66	C	117.97	104.21	6.798	Test set
67C	α	67	C	118.02	104.70	6.836	Test set
68C	α	68	C	117.87	104.27	6.828	Test set
69C	α	69	C	117.69	104.48	6.816	Test set
70C	α	70	C	118.93	102.81	6.702	Test set
71C	α	71	C	118.97	103.86	6.757	Test set
72C	α	72	C	118.48	105.02	6.724	Test set
73C	α	73	C	117.72	104.84	6.828	Test set
74C	α	74	C	118.21	105.12	6.708	Test set
75C	α	75	C	118.02	105.42	6.768	Test set
76C	α	76	C	117.96	105.46	6.803	Test set
77C	α	77	C	118.00	106.25	6.736	Test set
78C	α	78	C	118.03	105.37	6.823	Test set
79C	α	79	C	118.00	106.49	6.741	Test set
80C	α	80	C	117.79	106.34	6.712	Test set
81C	α	81	C	117.77	103.88	6.808	Test set
82C	α	82	C	117.55	103.89	6.880	Test set
83C	α	83	C	117.55	103.89	6.880	Test set
84C	α	84	C	117.99	104.52	6.816	Test set
85C	α	85	C	118.05	104.76	6.831	Test set
86C	α	86	C	118.50	105.91	6.676	Test set
87C	α	87	C	118.42	105.94	6.695	Test set
88C	α	88	C	118.53	106.41	6.700	Test set
89C	α	89	C	118.51	106.27	6.695	Test set
90C	α	90	C	118.53	103.90	6.662	Test set
91C	α	91	C	117.90	105.30	6.835	Test set
92C	α	92	C	117.74	105.88	6.858	Test set
93C	α	93	C	117.79	105.45	6.810	Test set
94C	α	94	C	118.24	105.11	6.736	Test set
95C	α	95	C	118.08	104.90	6.761	Test set

Table S6.12. (continued)

Hybrid	Female	Pollinator	Male restorer	DTH	HT	GY	Subset
96C	α	96	C	117.94	105.13	6.785	Test set
97C	α	97	C	117.77	104.71	6.820	Test set
98C	α	98	C	118.12	105.50	6.783	Test set
99C	α	99	C	118.16	104.60	6.799	Test set
100C	α	100	C	117.87	105.09	6.788	Test set
101C	α	101	C	117.76	105.77	6.818	Test set
102C	α	102	C	118.38	104.53	6.694	Test set
103C	α	103	C	118.19	104.81	6.808	Test set
104C	α	104	C	117.86	104.80	6.822	Test set
105C	α	105	C	117.87	104.90	6.791	Test set
106C	α	106	C	118.03	104.62	6.773	Test set
107C	α	107	C	118.24	104.82	6.776	Test set
108C	α	108	C	118.20	104.78	6.761	Test set
109C	α	109	C	118.59	103.72	6.682	Test set
110C	α	110	C	118.09	105.61	6.758	Test set
111C	α	111	C	118.05	105.28	6.765	Test set
112C	α	112	C	118.41	105.14	6.699	Test set
113C	α	113	C	118.04	104.81	6.744	Test set
114C	α	114	C	117.81	105.12	6.771	Test set
115C	α	115	C	118.30	104.06	6.733	Test set
116C	α	116	C	118.25	103.71	6.737	Test set
117C	α	117	C	117.71	104.09	6.865	Test set
118C	α	118	C	118.24	104.25	6.729	Test set
119C	α	119	C	118.26	104.44	6.776	Test set
120C	α	120	C	117.74	105.38	6.884	Test set
121C	α	121	C	118.10	104.91	6.796	Test set
122C	α	122	C	118.07	104.65	6.738	Test set
123C	α	123	C	118.11	104.75	6.802	Test set
124C	α	124	C	118.03	104.26	6.744	Test set
125C	α	125	C	118.59	103.66	6.664	Test set
126C	α	126	C	117.84	104.32	6.841	Test set
127C	α	127	C	118.22	105.13	6.741	Test set
128C	α	128	C	118.30	104.82	6.769	Test set
129C	α	129	C	118.46	104.97	6.674	Test set
130C	α	130	C	118.46	105.23	6.705	Test set
131C	α	131	C	118.41	103.81	6.668	Test set
132C	α	132	C	118.29	104.88	6.720	Test set
133C	α	133	C	118.28	104.26	6.683	Test set
134C	α	134	C	118.32	104.23	6.649	Test set
135C	α	135	C	118.23	105.10	6.683	Test set
136C	α	136	C	118.31	104.41	6.660	Test set
137C	α	137	C	118.04	104.84	6.732	Test set
138C	α	138	C	117.79	105.60	6.874	Test set
139C	α	139	C	118.28	104.39	6.731	Test set
140C	α	140	C	118.14	104.99	6.764	Test set

Table S6.13. Predicted values for grain yield (GY, t·ha^{-1}), days to heading (DTH, days), and height (HT, cm) of field-assessed (training set, 24) and untested (test set, 116) F_1F female hybrids. The predicted value of each F_1F is averaged across the three male parents (A, B, and C). The 10% of the most productive lines are highlighted in green. In bold, those lines that are in the top 10% in both grain yield and flowering time. Earliness is considered as a positive trait.

F_1F	GY	DTH	HT	Subset
2	**6.897**	**118.10**	**102.83**	**Training Set**
26	**6.790**	**118.10**	**102.95**	**Test Set**
19	6.776	118.68	103.32	Training Set
55	**6.773**	**118.08**	**103.03**	**Test Set**
18	6.772	118.56	103.75	Training Set
27	6.769	118.38	103.14	Test Set
53	6.769	118.27	102.58	Test Set
120	6.764	118.28	103.02	Test Set
60	6.761	118.33	103.02	Test Set
58	6.759	118.34	102.96	Test Set
63	6.757	118.19	103.15	Test Set
83	6.756	118.18	103.28	Test Set
82	6.755	118.18	103.28	Test Set
9	6.755	118.29	103.66	Training Set
54	6.752	118.11	102.89	Test Set
138	6.750	118.25	103.28	Test Set
117	6.742	118.16	103.26	Test Set
22	6.740	118.25	103.13	Training Set
24	6.739	118.40	101.87	Training Set
62	6.739	118.16	102.91	Test Set
29	6.735	118.28	103.29	Test Set
21	6.735	118.76	103.57	Training Set
92	6.735	118.28	103.07	Test Set
67	6.734	118.30	103.12	Test Set
5	6.734	118.14	102.89	Training Set
13	6.734	118.13	103.24	Training Set
64	6.733	118.19	103.04	Test Set
73	6.733	118.07	102.92	Test Set
126	6.732	118.20	102.89	Test Set
52	6.730	118.43	102.49	Test Set
20	6.727	118.20	103.30	Training Set
46	6.727	118.38	103.47	Test Set
65	6.724	118.28	102.79	Test Set
101	6.724	118.36	103.05	Test Set
68	6.723	118.20	102.86	Test Set
4	6.719	118.15	102.75	Training Set
17	6.718	118.25	103.86	Training Set
57	6.718	118.28	102.79	Test Set
91	6.716	118.30	102.94	Test Set
84	6.713	118.30	103.29	Test Set
42	6.711	118.33	103.34	Test Set
25	6.710	118.18	102.74	Test Set
85	6.710	118.23	103.27	Test Set
99	6.709	118.56	103.20	Test Set
98	6.709	118.64	103.28	Test Set
123	6.707	118.38	102.82	Test Set
66	6.706	118.19	102.74	Test Set

Table S6.13. (continued)

F_1F	GY	DTH	HT	Subset
11	6.705	117.85	103.09	Training Set
104	6.705	118.19	103.07	Test Set
78	6.704	118.06	103.01	Test Set
69	6.703	118.14	103.06	Test Set
97	6.700	118.14	103.21	Test Set
28	6.700	118.22	102.73	Test Set
7	6.699	117.83	103.04	Training Set
103	6.696	118.30	103.04	Test Set
8	6.695	118.20	102.72	Training Set
81	6.695	118.29	103.20	Test Set
119	6.695	118.27	102.81	Test Set
121	6.695	118.55	103.20	Test Set
93	6.695	118.30	102.92	Test Set
114	6.690	117.88	103.03	Test Set
35	6.689	118.29	102.84	Test Set
108	6.687	118.52	102.93	Test Set
37	6.682	118.16	102.98	Test Set
31	6.681	118.53	103.97	Test Set
76	6.681	118.17	103.02	Test Set
95	6.681	118.45	102.74	Test Set
105	6.680	118.18	102.96	Test Set
100	6.678	118.29	102.96	Test Set
96	6.676	118.27	102.90	Test Set
140	6.676	118.44	103.26	Test Set
128	6.675	118.39	102.82	Test Set
107	6.674	118.30	102.89	Test Set
71	6.672	118.45	103.17	Test Set
137	6.669	118.39	102.87	Test Set
61	6.666	118.52	102.81	Test Set
118	6.665	118.46	102.75	Test Set
56	6.665	118.35	102.92	Test Set
106	6.664	118.22	102.96	Test Set
75	6.663	118.32	103.06	Test Set
39	6.663	118.38	103.43	Test Set
50	6.662	118.34	103.08	Test Set
36	6.660	118.29	103.21	Test Set
116	6.660	118.32	102.70	Test Set
23	6.658	118.35	102.04	Training Set
48	6.657	118.25	102.62	Test Set
49	6.653	118.44	102.90	Test Set
115	6.651	118.39	102.79	Test Set
132	6.650	118.42	103.06	Test Set
111	6.650	118.50	103.28	Test Set
127	6.649	118.41	102.93	Test Set
72	6.649	118.42	102.64	Test Set
113	6.648	118.44	103.03	Test Set
110	6.648	118.39	102.98	Test Set
16	6.648	118.31	102.42	Training Set
30	6.646	118.66	102.16	Test Set
124	6.644	118.29	102.78	Test Set
6	6.643	118.82	104.21	Training Set

Table S6.13. (continued)

F_1F	GY	DTH	HT	Subset
51	6.641	118.47	103.14	Test Set
40	6.641	118.46	102.65	Test Set
135	6.640	118.47	103.02	Test Set
139	6.638	118.51	102.84	Test Set
74	6.636	118.26	103.07	Test Set
79	6.632	118.46	103.51	Test Set
87	6.632	118.57	103.51	Test Set
45	6.630	118.55	103.45	Test Set
133	6.630	118.44	102.69	Test Set
112	6.630	118.64	102.87	Test Set
47	6.629	118.50	102.64	Test Set
88	6.628	118.59	103.71	Test Set
70	6.627	118.67	102.47	Test Set
34	6.626	118.42	103.29	Test Set
89	6.626	118.55	103.70	Test Set
3	6.625	118.44	102.88	Training Set
94	6.625	118.32	102.99	Test Set
59	6.622	118.38	102.53	Test Set
10	6.622	118.62	103.09	Training Set
77	6.621	118.23	103.30	Test Set
33	6.621	118.46	103.18	Test Set
43	6.619	118.59	102.87	Test Set
122	6.616	118.52	103.23	Test Set
130	6.614	118.42	102.86	Test Set
86	6.613	118.65	103.60	Test Set
102	6.612	118.42	102.66	Test Set
109	6.610	118.66	102.70	Test Set
136	6.608	118.46	102.88	Test Set
32	6.604	118.37	102.99	Test Set
90	6.603	118.47	102.83	Test Set
38	6.603	118.94	103.71	Test Set
1	6.602	118.98	102.80	Training Set
44	6.601	118.42	102.53	Test Set
134	6.600	118.50	102.69	Test Set
80	6.590	118.27	103.33	Test Set
131	6.584	118.42	102.73	Test Set
125	6.578	118.49	102.72	Test Set
129	6.575	118.54	102.92	Test Set
12	6.566	118.25	103.30	Training Set
41	6.565	118.49	102.73	Test Set
14	6.554	118.62	103.17	Training Set
15	6.497	118.75	102.69	Training Set

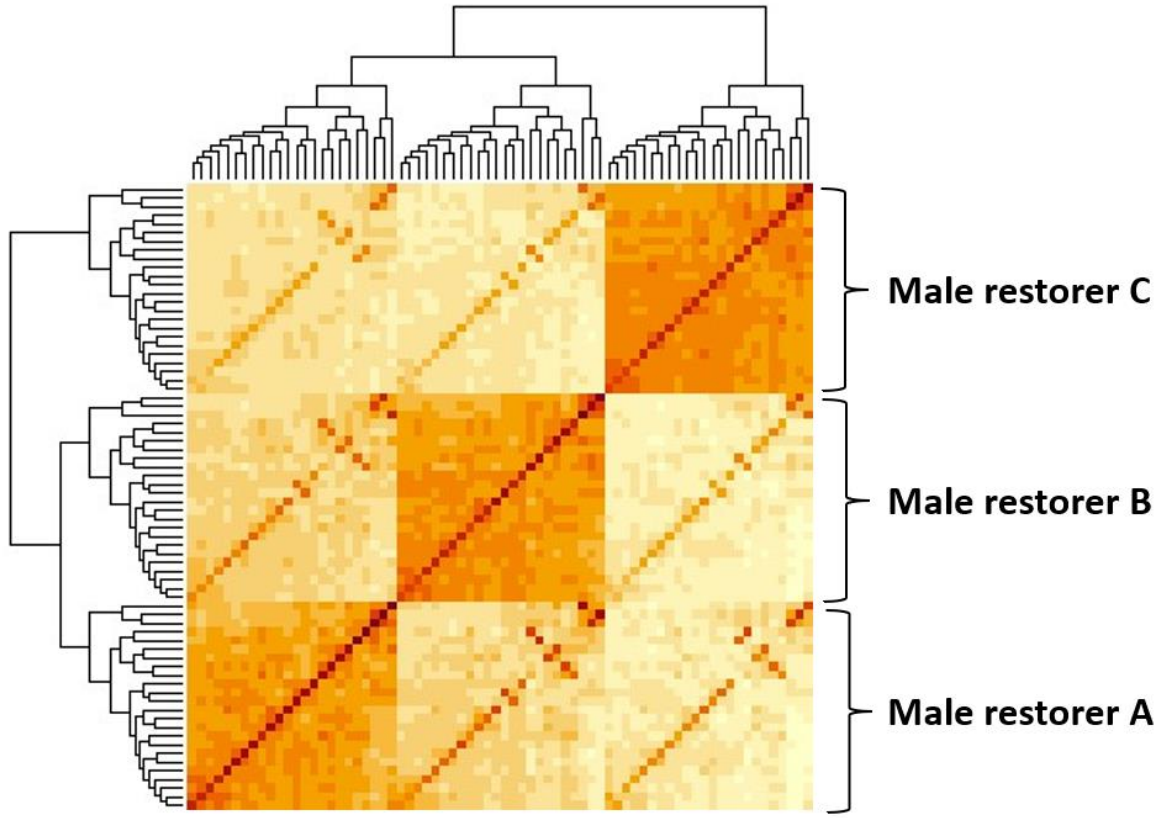

Figure S6.1. Additive relationship matrix of 72 three-way hybrids, calculated according to VanRaden (2008, "first method"). Blocks of rows for hybrids derived from the same male restorer are indicated.

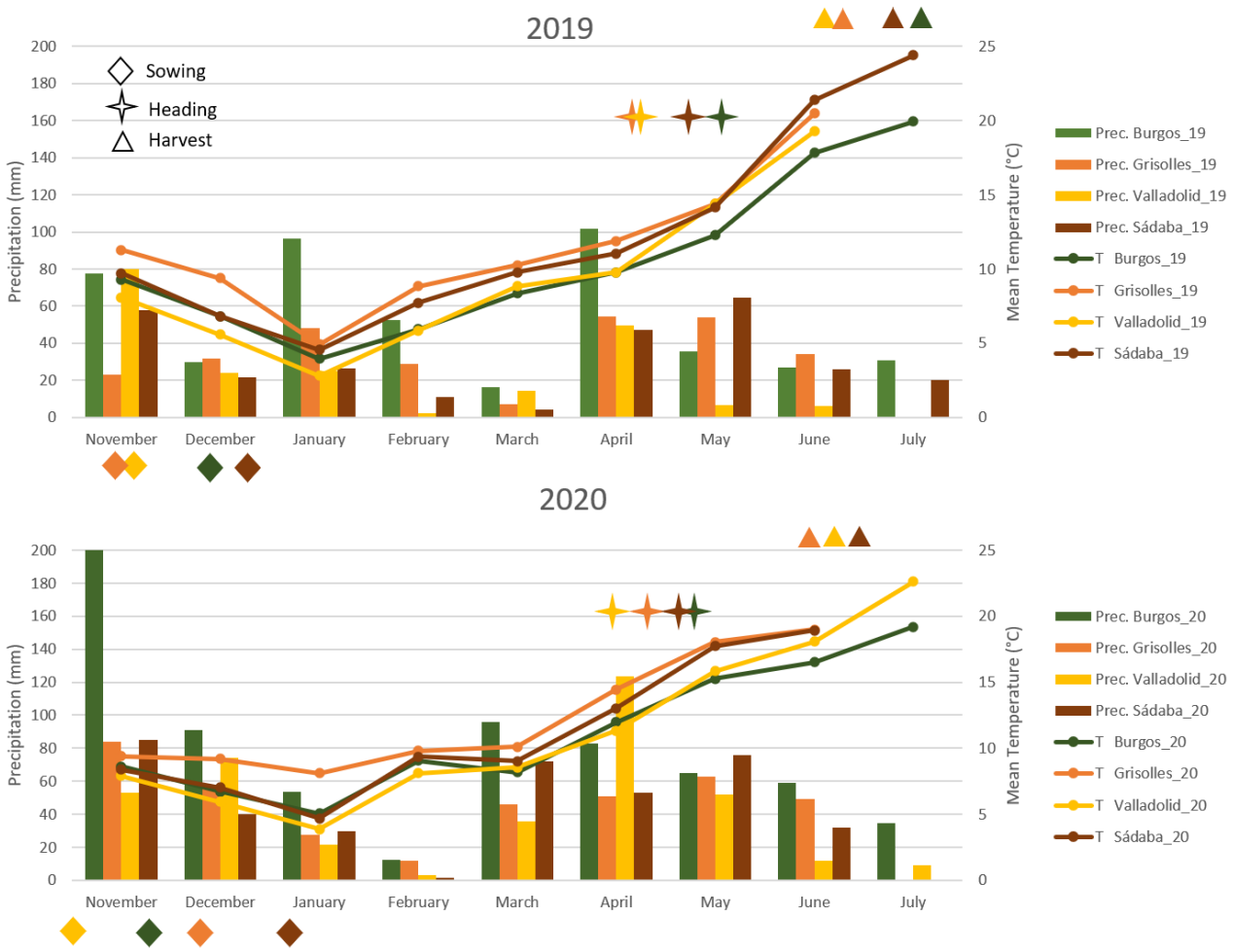

Figure S6.2. Climate data summary of seasons 2019 (upper plot) and 2020 (bottom plot) for each field trial location. Local monthly mean temperature, and cumulative rainfall are colour coded and represented by lines and bars, respectively. Sowing, heading, and harvest date are depicted as diamonds, stars, and triangles, respectively.

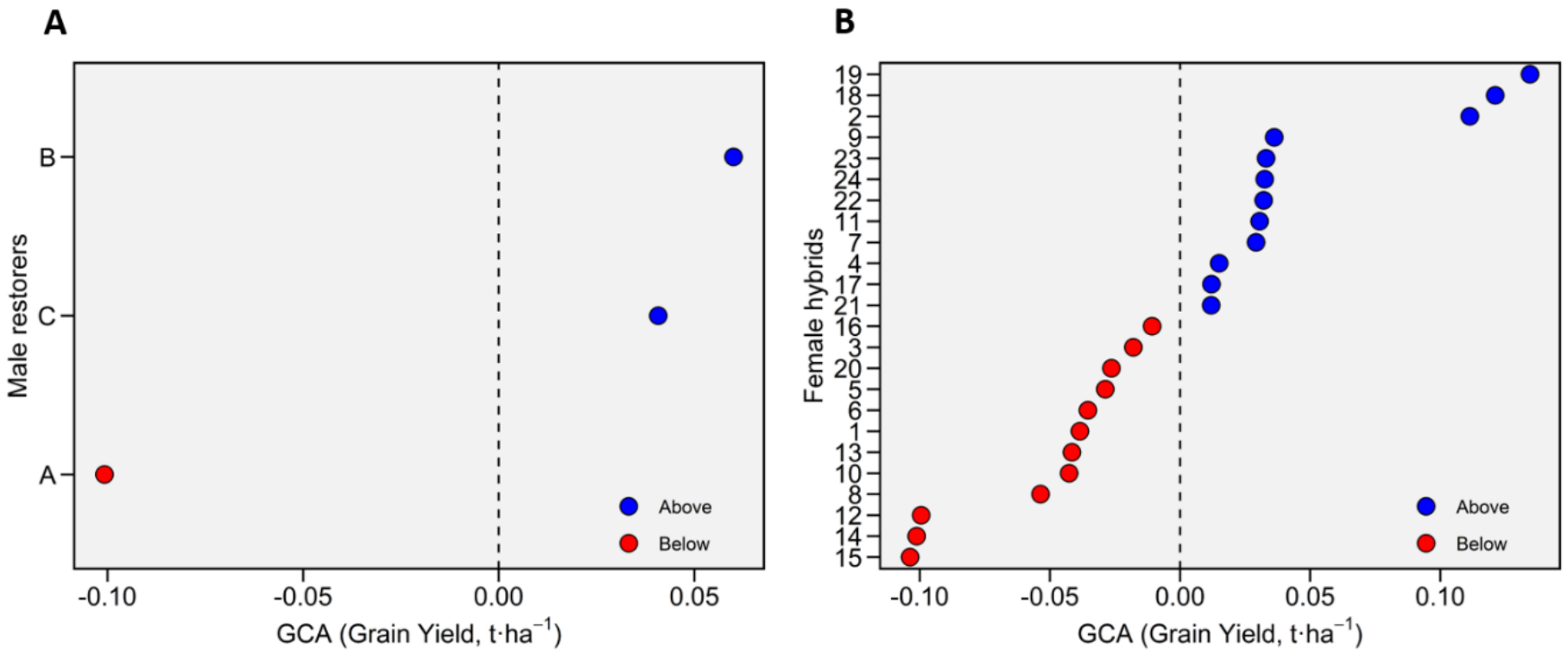

Figure S6.3. General combining ability (GCA) of male restorer (A) and female (B) parents for grain yield (t·ha^{-1}). The GCA of the female side corresponds to the female male-sterile hybrid (F_1F=elite female x Spanish pollinator). Parents that contributed a yield advantage over the population mean are presented in blue, whereas those that contributed a yield disadvantage over the population mean are shown in red.

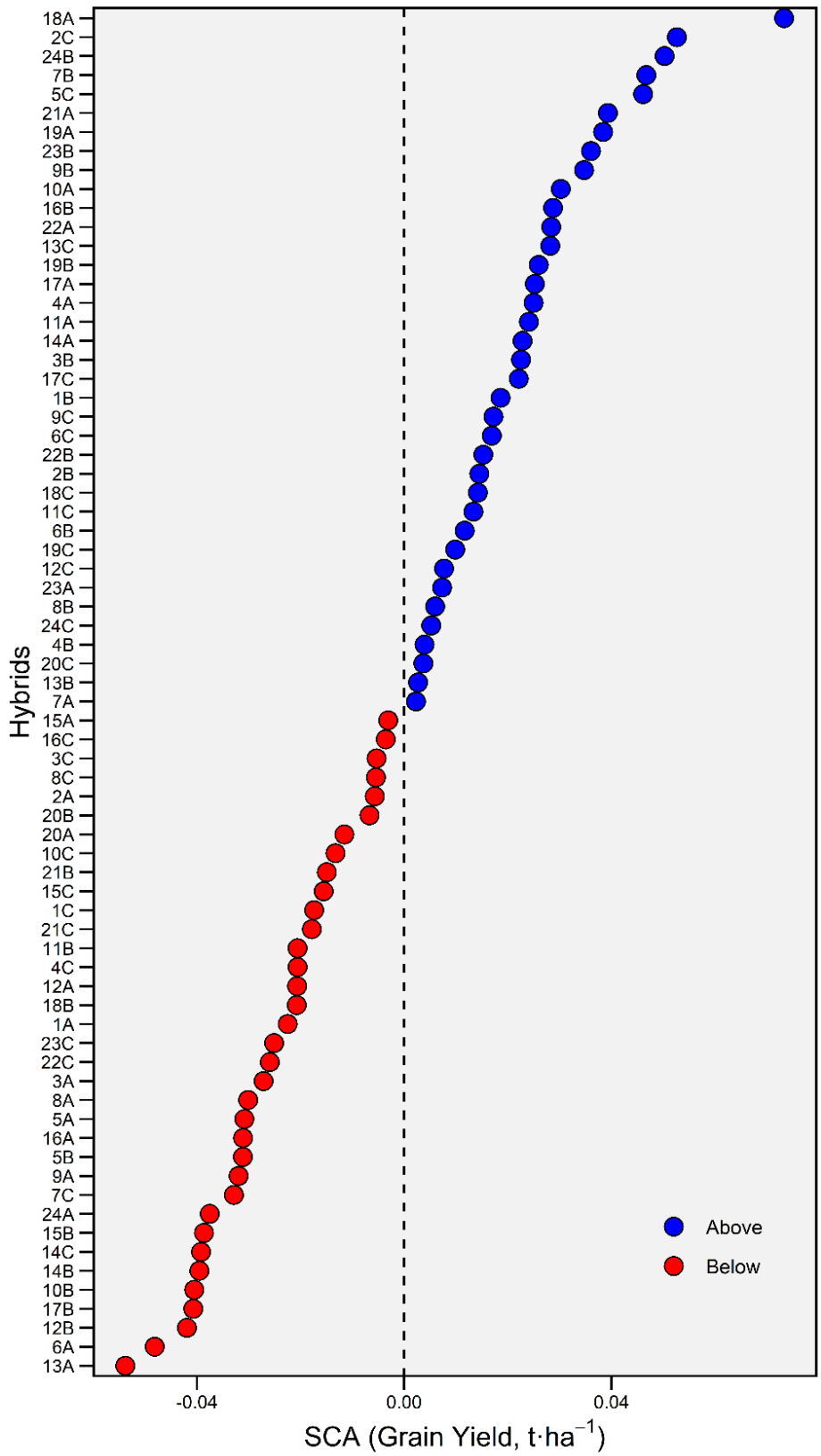

Figure S6.4. Specific combining ability (SCA) of hybrid crosses for grain yield (t·ha^{-1}). SCA is calculated for the 72 hybrids derived from the cross of 24 F_1F and 3 male restorer parents. Positive SCAs in blue, negative SCAs in red.

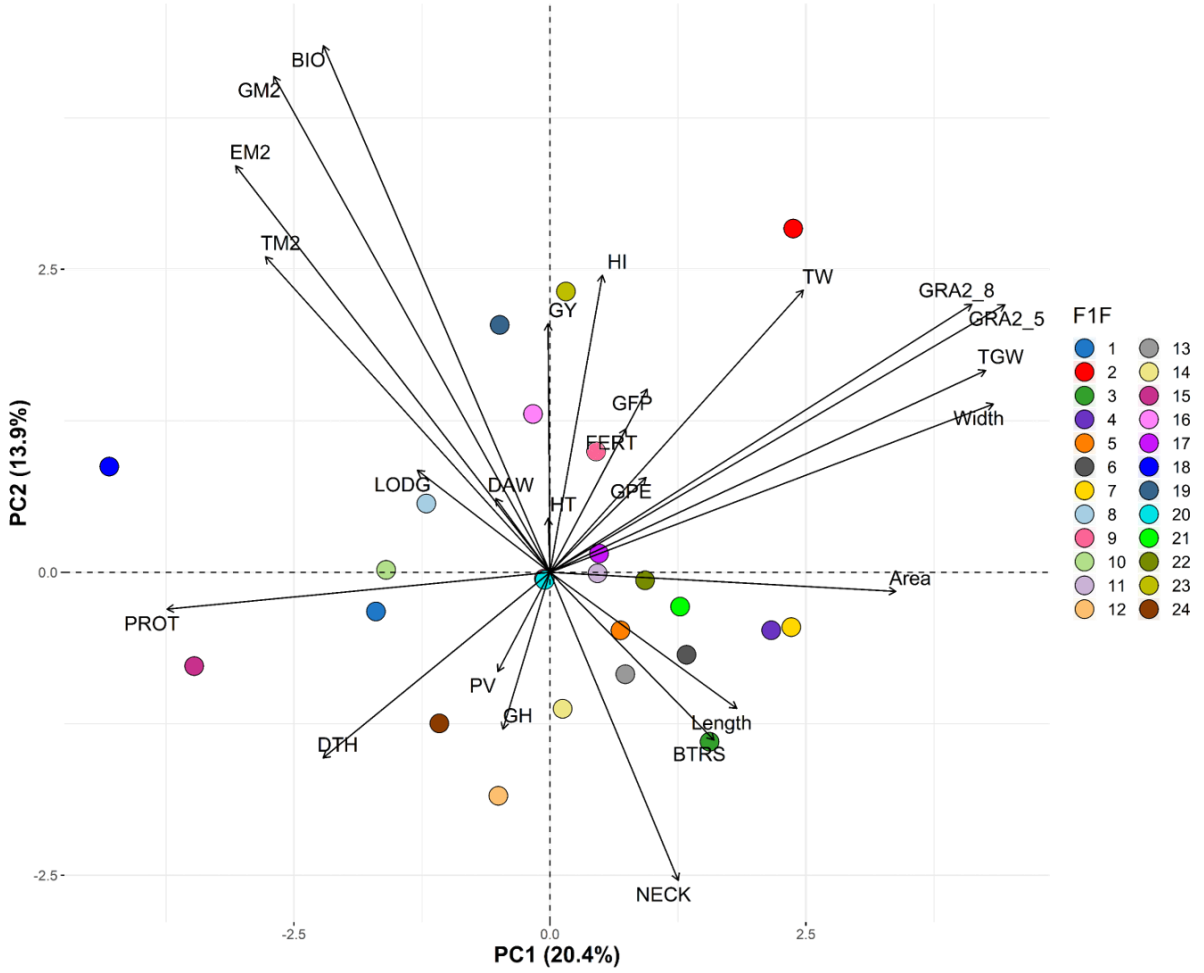

Figure S6.5. Biplot of the first two axes of a principal component analysis carried out with all measured phenotypic variables, averaged across environments, and standardized by male restorer parent. Trait abbreviations as in Table 6.2. Arrows show the contribution of each trait to the PCA axes. Dots represent the mean of test hybrids grouped per F_1F parent (averaged across male restorer parents). Checks were not included in the analysis.

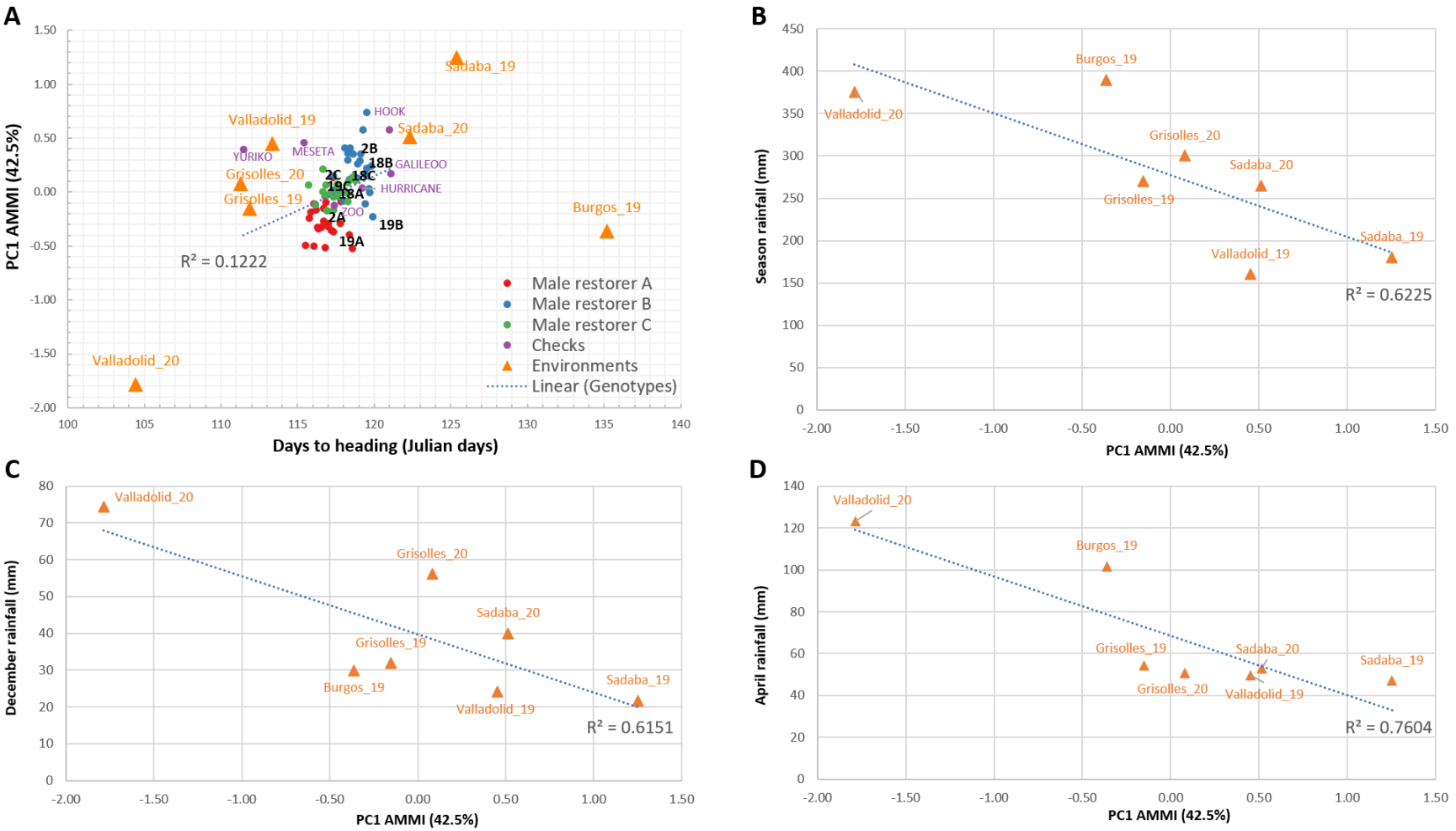

Figure S6.6. Regression analysis of phenotypic and environmental variables on the scores of the first principal component (PC1) of the AMMI analysis for grain yield. (A) Regression of AMMI PC1 scores of genotypes on days to heading. Genotypes (dots) and environments (triangles) are presented. Checks are depicted in purple. Test hybrids are coloured by male restorer parent. Hybrids derived from top-yielding F_1F are highlighted in bold face. (B) Regression of total season rainfall on AMMI PC1 scores of environments. (C) Regression of rainfall recorded in December on AMMI PC1 scores of environments. (D) Regression of rainfall recorded in April on AMMI PC1 scores of environments. Regression lines and coefficients of determination (R^2) are shown.

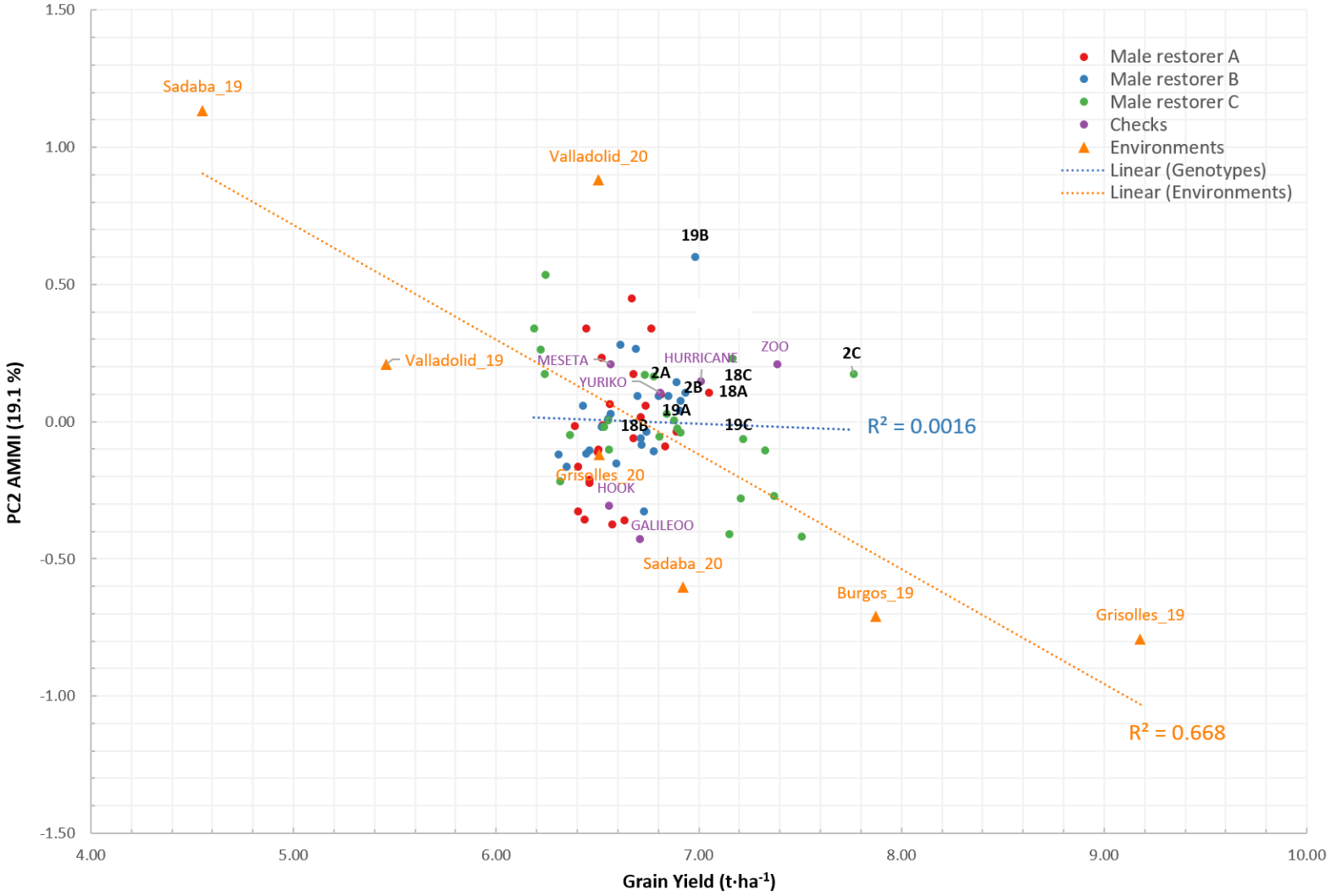

Figure S6.7. Regression analysis of phenotypic variables on the scores of the second principal component (PC2) of the AMMI analysis for grain yield. Regression of AMMI PC2 scores on grain yield. Genotypes (dots) and environments (triangles) are presented. Checks are depicted in purple. Test hybrids are coloured by male restorer parent. Hybrids derived from top-yielding F_1F are highlighted in bold face. Regression lines and coefficients of determination (R^2) for environments (orange line) and genotypes (blue line) are shown.

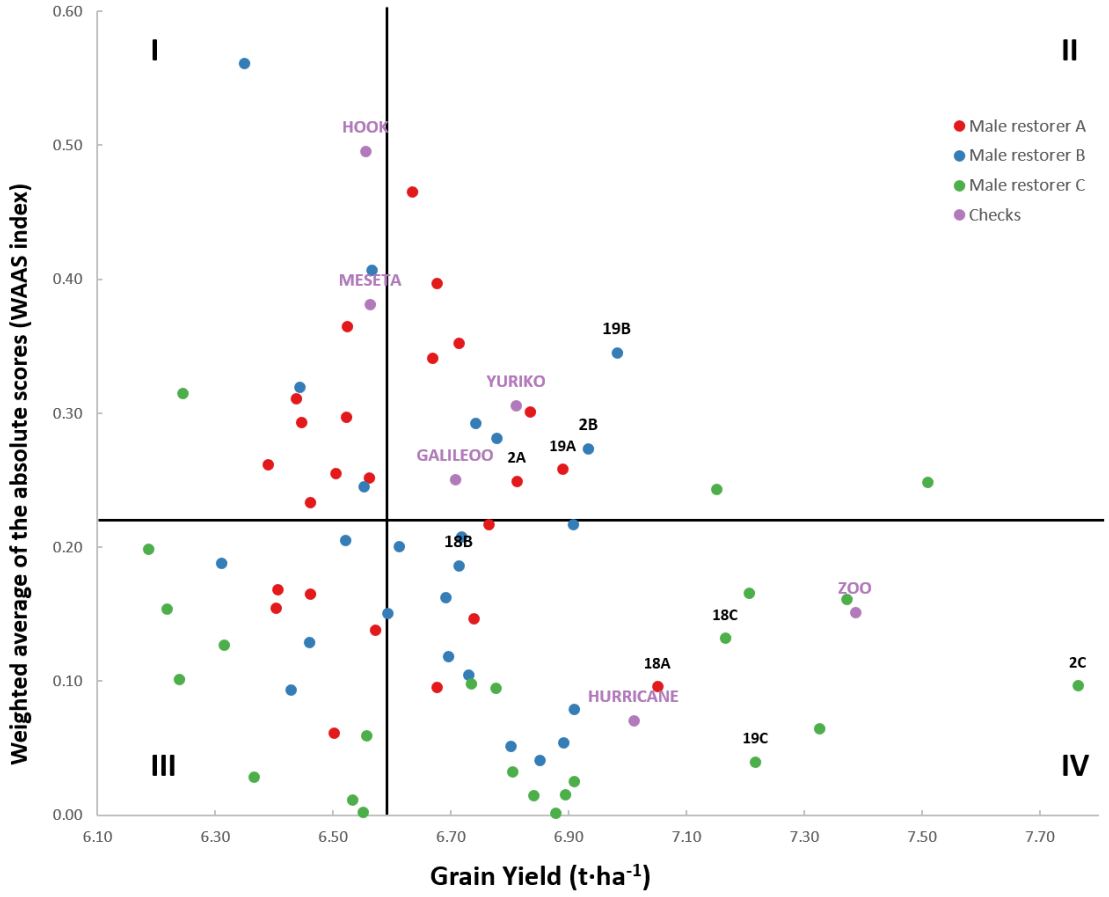

Figure S6.8. Stability *vs.* performance analysis. Weighted average of the absolute scores (WAAS index) is presented on the vertical axis, and average grain yield on the horizontal axis. Genotypes are depicted as dots: checks in purple, and test hybrids are coloured by male restorer parent. Hybrids derived from top-yielding F_1F are tagged and highlighted in bold face. Population means for WAAS index and grain yield are presented as black lines. The cross of these lines defines four quadrants that characterize genotypes based on the joint interpretation of stability and performance.

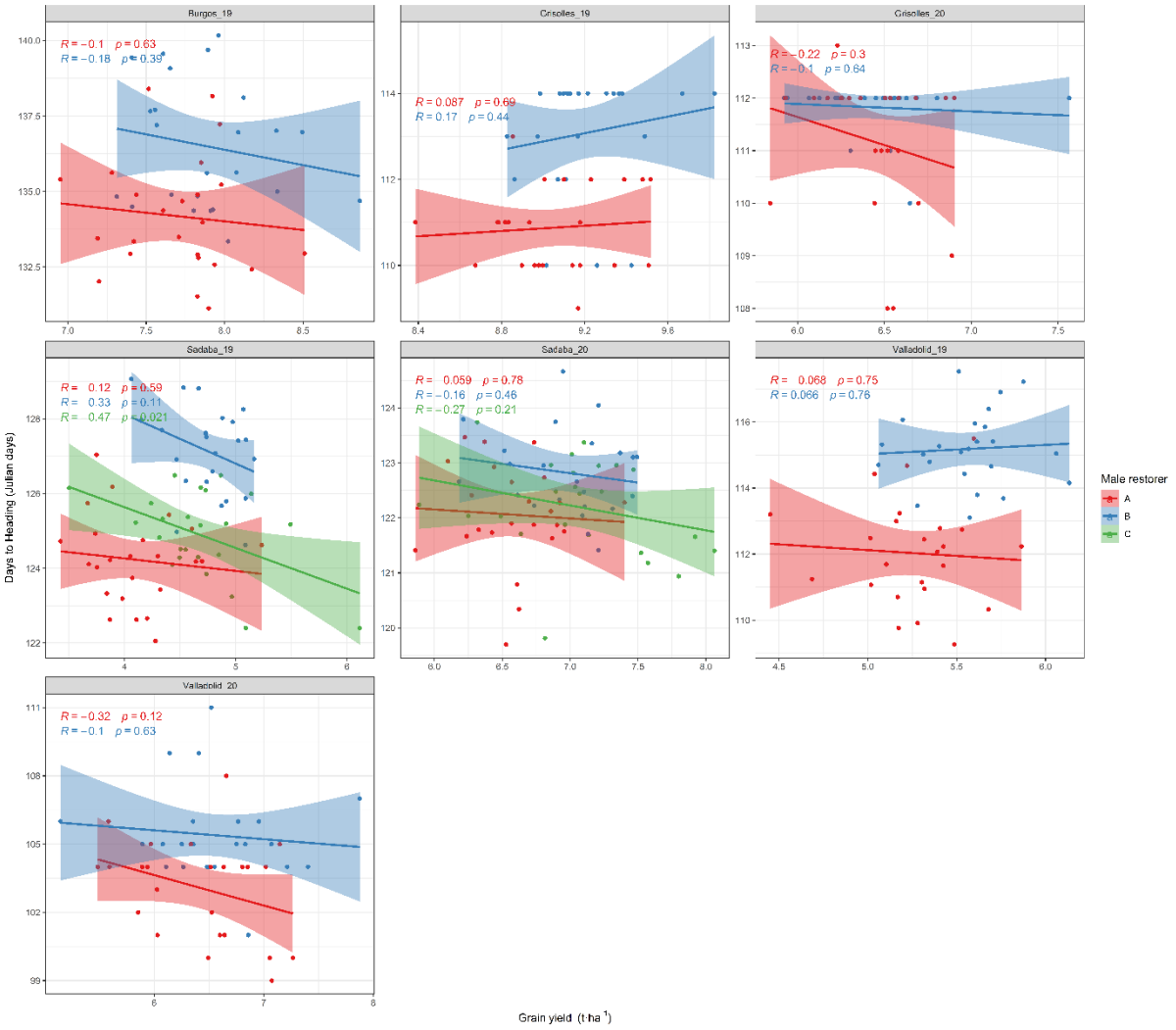

Figure S6.9. Correlation between heading time and grain yield for hybrids split by environment and male restorer parent. Regression lines, Pearson correlation coefficients (R), and p-values for the correlation (p) are shown for each male restorer parent x environment combination.

Chapter VII. General Discussion

7. Chapter VII. General Discussion

Through the development of this thesis, we have explored, understood, and facilitated the exploitation of Spanish germplasm and its adaptive traits for their use in hybrid barley breeding. However, the resultant hybrids did not outyield the best hybrid check and, therefore, were not promoted as candidate cultivars. The main reasons behind this are: (1) the absence of an established heterotic group(s) for the Spanish germplasm, (2) the lack of identification of high-yielding heterotic patterns involving Spanish material, (3) a phenological cycle too long for Southern European conditions in the hybrids developed to date, and (4) the reduced expression of Spanish diversity and potential heterosis due to the low contribution of the Spanish germplasm in the three-way hybrid approach. The rest of the discussion is aimed at guiding breeders to find solutions to these issues.

7.1. Identification of high-yielding heterotic patterns in the global barley diversity

Both the absence of defined heterotic pools, and the lack of patterns with a strong heterotic response affect not only to the Spanish materials but to all barley germplasm. Indeed, the characterization and utilization of barley global diversity for hybrid development are restricted to a couple of examples (Sommer et al. 2020). The heterotic pattern on which current commercial barley hybrids are based relies on the narrow North western European germplasm. As in maize (Melchinger and Gumber 1998), the two barley heterotic pools were separated based on characteristics for efficient seed production. Thus, the female pool carries the CMS system, whereas the male pool carries the fertility restoration genes (Li et al. 2017). The combination of some of the techniques available today in the breeder's toolbox (Varshney et al. 2021), such as marker-assisted backcrossing, doubled haploids, and speed breeding, allow for quick and precise transfer of the male-sterility/fertility-restoration system, and the brittle locus of choice, to any barley cultivar. In this sense, any six-row barley variety, irrespective of its origin, could be potentially used for barley hybrid breeding.

The study of barley germplasm worldwide reflects a vast diversity, both in landraces and breeding cultivars (Muñoz-Amatriaín et al. 2014; Poets et al. 2015; Russell et al. 2016; Pankin et al. 2018; Milner et al. 2018; Hill et al. 2019; Bustos-Korts et al. 2019). However, this diversity is untapped in a hybrid context. We propose exploring global barley diversity to enhance the current European heterotic pools, and to look for unexploited high-yielding heterotic patterns. We will limit the diversity to be explored to the six-row barley pool to ensure efficient seed production. The latter could be hampered by the intrinsic cleistogamy of two-row barley.

Heterosis can be considered a function of heterozygosity and consequently genetic diversity between heterotic groups increases the level of heterosis (Falconer and Mackay 1996). Therefore, Melchinger and Gumber (1998) suggested that the first step to identify heterotic pools was grouping diverse germplasm based on genetic similarity. Genetically distinct groups were identified in barley a long time ago (Graner et al. 2003). More recently, the falling of sequencing costs, followed by the proliferation of genome-wide molecular markers, allowed geneticists to analyse the structure and diversity of large global barley germplasm collections stored in genebanks (Milner et al. 2018). These analyses have shown that patterns of genetic differentiation reflect geographic origins and major germplasm divisions created by agricultural practices (Russell et al. 2016). Besides, modern plant breeding has further diversified barley germplasm (Fischbeck 2003).

Although diversity contained in landraces and wild barleys is wide, we will focus on that of breeding material, which has already been selected for traits essential for intensive cultivation. Within the six-row barley breeding material, several distinct groups have been reported, mainly divided by geographical origin (Milner et al. 2018; Bustos-Korts et al. 2019). These include materials from Europe, North Africa, Ethiopia, Asia (from China, Japan, Korea, and the Tibetan plateau), the Middle East, and the Northern Mediterranean Basin. Groups of six-rowed barleys from the USA, Canada, and South America should also be considered. Although their materials mostly derive from Mediterranean, European, and Asian germplasm, introduced after the 15th century (Knüpffer et al. 2003), their genetics have been reshaped recently by breeding. In addition to geographic factors, annual growth habit is a major determinant of genetic divergence (Comadran et al. 2012). On this basis, any of these dissimilar groups, besides the European ones, already being exploited, could be explored for the benefit of hybrid barley breeding.

Since heterotic groups based on adapted European germplasm are already established, we could use the most genetically similar materials to reinforce the existing pools with novel diversity, and the most distinctive groups to search for new heterotic patterns. First, based on the molecular characterization of the global six-row barley germplasm, it would be possible to estimate their genetic distances to the two established groups. Lines must be assessed for flowering time and *non-brittle rachis* (*btr*) genotype. Having compatible combinations of genes governing these two traits is essential for hybrid development. On the one hand, synchronization of flowering time between the components of a hybrid is crucial for cost-efficient seed production (Zhao et al. 2015). On the other hand, the cross of alternative *btr* mutations leads to ear fragility and potential yield loss (chapter IV). This is a real concern, as global barley germplasm carries different mutations at the *non-brittle rachis* genes.

Fingerprinting of germplasm candidates will also allow the genomic prediction of their GCA or the hybrid performance (Zhao et al. 2015; Beukert et al. 2017). Lines with genetic similarity to either of the two established groups, and a predicted high GCA with the respective alternative group would be identified as promising candidates to enhance the recognised pools. At this stage, the candidate lines must be converted to females or males before test-crosses are made. Moreover, marker-assisted selection based on optimum flowering time, high frost tolerance, and *non-brittle rachis* genotype will also be performed at this point. Then, the selected lines must be crossed with tester lines from the complementary heterotic group to assess their combining ability. Finally, genotypes with a high field-observed GCA could become part of the corresponding heterotic group. In addition to enhancing the current heterotic groups, the analysis of genetic distances followed by GCA tests could also result in the identification of new heterotic patterns. This occurs when a genetically divergent group of lines shows superior hybrid performance with one or both initial pools (Boeven et al. 2016). In this way, different heterotic patterns could be established for different target zones, as it was done for temperate maize in Europe and USA (Duvick and Smith 2004).

Once a heterotic pattern is established, it must be enhanced through breeding. Since barley hybrid breeding is still in its infancy, the shaping of its heterotic groups is still far behind that of cross-pollinated crops. Following the lessons from maize, the use of long-term reciprocal recurrent selection will enable barley pools to coevolve into increasingly divergent populations, maximizing GCA and resultant heterosis (Duvick and Smith 2004). In fact, recurrent selection has already been successfully implemented in barley breeding in the past (Delogu et al. 1988), with the use of genetic male sterility for efficient crossing in a self-pollinated crop (Ramage 1983; Falk 2010).

Following the proposed strategy, promising unexplored heterotic patterns could be found. This may happen, for instance, between the European and Asian barleys, the two most divergent germplasm groups (Pasam et al. 2012; Muñoz-Amatriaín et al. 2014; Russell et al. 2016; Milner et al. 2018). Particular attention should be given to the *non-brittle rachis* genotype of this cross. European barley germplasm mainly carries the *btr1* mutation, while Asian barleys mostly bear the *btr2* mutation (Komatsuda et al. 2004; Pourkheirandish et al. 2015). Thus, brittle hybrids must be expected from their cross. To avoid this potential problem, Asian parents could be converted to *btr1* genotype through marker-assisted backcrossing.

7.2. Optimization of phenological cycle in hybrid barleys

Plant material entering a hybrid breeding program is limited by phenology. Hybrid seed production among the parental lines requires synchronization of flowering time, which is optimized if the

female line flowers 3–4 days earlier than the male line (Zhao et al. 2015). In the European hybrid barley breeding program of Syngenta, the phenology of female and male lines is already optimized for their flowering time nick. However, when trying to introduce Spanish germplasm into the program, early candidate materials were discarded because their flowering times were too far away from those of the two established pools. Under these circumstances, the Spanish diversity that entered the breeding program was restricted from the outset, especially those allelic combinations in the genes controlling flowering time that were more suitable for Mediterranean conditions. As a consequence, the shift towards earliness of the hybrids developed was not enough for Southern Europe, and showed a narrow range of flowering times.

In order to expand the diversity available for hybrid barley, and the range of phenological responses, breeding must act on both sides of the cross. Earliness in the current established pools could be achieved through introgression of early flowering time alleles by marker-assisted backcrossing. Then, their effect on phenological cycle and yield should be evaluated in the field. Firstly, on the parental lines to ensure crossability, and later, on the resultant hybrids.

So far, hybrid barley breeding has focused on six-row winter germplasm, to produce cultivars for feed. There are, however, six-row barley groups which are not strict winter types, like semi-winter (as described by Knüpffer et al. 2003), and spring, which are widely spread over Northern Africa, Asia, and America. Warming winters are causing a shift of sowing dates from spring or winter towards autumn, at least in Southern, Western, and Central Europe (Fisk et al. 2013). Including semi-winter (as done in this thesis) or spring parents in hybrid formulations would widen the genetic diversity amenable for hybrid breeding, and could produce phenological types with good adaptation to environments with all kinds of winters. Actually, a potential heterotic pattern has been suggested between spring and winter cultivars (Koekemoer et al. 2011). For these two types to cross, both must be sown in autumn so that flowering occurs evenly in spring. However, we must ensure that varieties sown in autumn, of any growth habit, still carry enough frost resistance to withstand winter frosts. One way to ensure good levels of frost tolerance in hybrids is the use of facultative varieties, some of which have been associated with high levels of frost tolerance (Muñoz-Amatriaín et al. 2020). Hybrids made of winter by facultative types should be explored, in the same manner that winter by semi-winter crosses have been explored in this thesis. A winter by facultative cross would have *VRN-H2* in heterozygosis, which is a conformation whose phenotypic effect has not been sufficiently put to test.

In this thesis we have explored a number of genes that could be used to achieve earliness quickly and easily. For instance, the *VRN-H1-4* allele seems to be a great candidate. This allele confers

earliness even under insufficient vernalization. This situation is becoming more likely in the Mediterranean region, as winters are getting warmer due to climate change. On the other side of the cross, another of the multiple alleles described for *VRN-H1* (Guerra et al. 2021), characterized by a continuous range of responses to vernalization (Hemming et al. 2009), could be used. As we have seen, the inheritance of winter *VRN-H1* alleles is additive, while that of spring alleles is dominant (Takahashi and Yasuda 1971). Thus, crosses of winter alleles will result in an intermediate phenotype, while crosses involving a spring allele will result in the earliest phenotype. *VRN-H1* largely controls the duration of the vegetative phase (Trevaskis et al. 2003), and it is during this period that hybrids build much of their yield advantage by exploiting their tillering potential. Therefore, maintaining certain duration of the vegetative phase by using winter *VRN-H1* alleles seems beneficial for maximising yield in hybrids. We have observed, in field trials not presented in this thesis, that hybrids carrying one *VRN-H1-4* allele flower earlier than hybris with any other combinations. This would confirm that an early fulfilment of vernalization requirement accelerates the whole growth cycle, although this observation should be confirmed with ad hoc plant materials, and should examine whether it entails any yield penalty.

PPD-H2 is another potential candidate gene to induce early flowering. This gene played a key adaptive role both in spring and winter barleys in the Mediterranean region (Casao et al. 2011c). Furthermore, we confirmed its function as a safeguard mechanism in case of incomplete vernalization and its dominant inheritance (Kikuchi et al. 2009). However, its effect on hybrid varieties needs to be explored under field conditions. During the progress of this project, we were unable to do so because the Spanish materials with the functional *PPD-H2* allele were discarded, as they were too early to favour correct synchronization with the European parents. Nevertheless, other allelic combinations conferring similar earliness could be used to ensure crossability with a *PPD-H2* carrier. This gene has been associated with low frost tolerance (Rizza et al. 2016), so it must be paired with frost resistance genes or avoided in northern latitudes.

Another gene that provides a wide catalogue of options is *VRN-H3*. This gene comes into play after vernalization fulfilment (Mulki and von Korff 2016), towards the late reproductive phase (LRP), when the potential yield is realized (Sreenivasulu and Schnurbusch 2012). In the Mediterranean Basin, terminal abiotic stress is one of the major limitations of yield (Cammarano et al. 2019), so it is relevant to accelerate the finalization of the cycle. Spanish barley landraces showed a clear geographical distribution pattern of *VRN-H3* alleles, suggesting a key role of this gene in adaptation and agronomic fitness (Casas et al. 2011). Furthermore, we showed that *VRN-H3* alleles were associated with different LRP duration and different dynamics in response to

vernalisation. For the development of Southern European adapted parents, we propose the *vrn-H3c* allele. This allele showed a short and partially dominant LRP independent of vernalization. Therefore, it could be useful to escape the rapid rise in temperatures and the risk of terminal drought and heat stress at the end of the Mediterranean season.

HvCEN haplotypes have been associated with differences in the length of the ripening phase (Maurer et al. 2015; Herzig et al. 2018). This gene is a hotspot of interactions with other flowering time genes and environmental cues (Mansour et al. 2014; Bi et al. 2019; Casas et al. 2021). We propose investigating the effect of the haplotype I, particularly in combination with the winter *vrn-H1* allele, to seek for earliness in parental lines and resultant hybrids.

Besides the genotype (G), both the environment (E) and the management (M), and particularly the complex GxExM interactions determine crop performance (Cooper et al. 2021). In this sense, we suggest a practical application to achieve synchronized flowering times between parents through the management of sowing dates and *PPD-H1* alleles. As we have seen, *PPD-H1*, the major determinant of long photoperiod response in barley, shows a crossover interaction with the environment. The sensitive *PPD-H1* allele accelerates flowering time under long days, whereas it delays it under short days. To achieve synchronized earliness in parental lines, a late male parent with a sensitive *PPD-H1* could be crossed with an early female parent (*VRN-H1-4*, *vrn-H3c* or *PPD-H2* carrier) but with an insensitive *ppd-H1* allele. To synchronize flowering times and facilitate the nick, sowing must be delayed until mid-November in Mediterranean latitudes. In that way, the allele conferring earliness would be the sensitive *PPD-H1* and not the insensitive allele (Chapter III).

In addition to the main drivers of barley adaptation, other genes modifying phenology enabled its agro-ecological range to be extended even further (Faure et al. 2012). Within these, *HvPHYC* or *HvELF3* could be explored as donors of earliness for the established heterotic pools. However, the effect conferred by these two genes might be too drastic for Southern Europe. Indeed, the strong effect of the early *PHYC-e* allele was associated with a negative impact on yield when tested in the Mediterranean conditions of Australia (Hill et al. 2019).

In this section, we have shown options to optimize flowering time for Southern Europe in the short term, but these can be useful for norther latitudes in the middle term, due to the effect of climate change.

7.3. Pitfalls and lessons from Iberian hybrid barley

The expression of the Spanish diversity shown by the three-way hybrids both in phenological and agronomic traits was limited. This was mainly due to the low contribution (25%) of the Spanish germplasm in the three-way hybrid approach. The three-way hybrid strategy was applied because it allows rapidly obtaining sufficient hybrid seed for multi-environment evaluations, and thus testing the potential of new germplasm in a hybrid format in a time- and cost- efficient way. By applying this strategy, it avoids the time-consuming process of transferring the male-sterilising cytoplasm or the fertility restoration genes into untested germplasm (Trini et al. 2021). Moreover, three-way hybrids combine heterozygosity and heterogeneity, thus they have been associated with improved yield stability in a number of surveys (Patanothai and Atkins 1974; Becker and Léon 1988; Smithson and Lenné 1996; Mühleisen et al. 2014a), including ours. Nevertheless, the three-way hybrid approach limited the expression of Spanish diversity and potential heterosis, since only 25% of the hybrid was contributed by the Spanish parent. Three-way hybrids ((AxB)xC)) could show higher or lower yield than single crosses (AxC/BxC) (Wricke and Weber 1986). This depends on the similarity between its components. If A and B are similar, we can expect higher percentage of heterozygous loci and potential heterosis in the three-way hybrid. On the contrary, if A and B are dissimilar, we can expect that one of the single crosses (AxC or BxC) performs better than the three-way hybrid. In this sense, we could hypothesize that the female parent and Spanish pollinator of the three-way hybrids tested are not so genetically similar. Therefore, we could be losing potential heterosis compared to the best single cross. Moreover, in self-pollinated species, such as barley, the importance of epistasis, in the form of complexes of co-adapted genes, is high. In the case of hybrids, the additive-by-additive epistasis component is reduced from two- to three-way hybrids following a linear trend (Wricke and Weber 1986). Therefore, by using the three-way hybrid approach we could also have lost some of these favourable interactions between loci.

Despite this, we have proved that the three-way hybrid approach is a successful strategy for mining local germplasm to develop hybrids adapted to new target areas. Through it, high-yielding parents were identified that can be used in the production of two-way hybrids, where their general combining ability effect will be doubled.

The ease of commercially producing two-way *vs.* three-way hybrids is why, in the long term, breeders are more interested in developing single crosses. Thus, in parallel to the evaluation of the complete set of Spanish materials as three-way hybrids, a subset of Spanish lines, selected on the basis of *per se* performance and flowering time, was used to produce single crosses. The two-way hybrids were evaluated in the same multi-environment trial network in Southern Europe, for one

year, and although their analysis is not part of this thesis, it is relevant to comment on their outcome. The Spanish candidate lines were used as female parents because they were more genetically similar to the female pool than to the restorers. The time and resources needed to transfer the CMS system into the Spanish lines was intensive, but the results were satisfactory. We were able to confirm the good general combining ability of the top-yielding Spanish pollinators that had been identified in the three-way hybrid trials. What is more, the single cross derived from the Spanish line 2 reliably outyielded the best check (though by a non-significant margin), and showed wide adaptation to Southern European conditions.

The analysis of the two-way hybrids confirmed our hypotheses. It allowed us to verify that the phenological and agronomic variation in the two-way hybrids was much wider than in the three-way hybrids. The distribution of flowering dates in the single crosses was broader than in the three-way hybrids, and earliness conferred by the females was partially transferred to the hybrids. In addition, several two-way hybrids showed greater yield performance (10%) and stability than their best parent, justifying the growing interest in the hybrid strategy. As demonstrated by a number of studies, hybrid varieties show both yield increase and stability across multiple environments, especially under low yielding conditions due to abiotic stresses (Oettler et al. 2005; Okada and Whitford 2019). Enhanced yield stability of hybrids compared to inbred lines facilitates coping with increasing abiotic stress expected from the predicted climate change (Mühleisen et al. 2014a). Growing hybrids enables exploiting heterogeneity without losing uniformity. In this way, we can gain stability and conserve diversity for the future.

As shown in part of this thesis, the implementation of genomic prediction in hybrid breeding programmes is a promising approach to increase genetic gains (Krishnappa et al. 2021). It allows: on the one hand, advancing only those parents with a high predicted GCA, which results in a better allocation of field-testing resources (Albrecht et al. 2014); on the other hand, predicting the hybrid performance of all possible crosses, widening the scope of diversity (Kadam et al. 2021).

To conclude, this thesis provides a number of useful lessons for hybrid breeding programmes seeking to expand into new agro-ecological niches. In particular, the information obtained in this thesis may be useful for hybrid barley breeding in the rest of Europe, where the growing conditions may resemble Mediterranean ones with the effect of climate change.

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Chapter VIII. Conclusions

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1. The allelic diversity in the major genes controlling flowering time in barley is enormous. In addition, the effect of interactions with other genes and the environment further broadens the range of phenotypes. The available genetic variation is therefore enough to fine-tune flowering time for current and future conditions. Particular allelic combinations will allow the duration of the different phenological phases to be adjusted according to the availability of resources, maximising yields in the target regions.

2. Hybrids showed intermediate earliness. The foundation phase was the most sensitive to vernalization treatment. It tended towards intermediate inheritance in the hybrids, due to the additive action of *VRN-H1* winter alleles. In contrast, the constitutional phase showed dominance towards earliness, conferred by *PPD-H2* and *VRN-H3* genes. Thus, hybrid combinations extend the available catalogue of genetic responses to vernalization, providing new opportunities for fine-tuning barley phenology.

3. The relationship between earliness and yield in the Southern European conditions was important, especially in environments prone to terminal stress. Earliness in this data set of hybrids showed no heterosis. Therefore, full exploitation of hybrid potential requires expanding the range of flowering times by looking for earliness in both sides of the cross.

4. The combination of alternative mutations at the *non-brittle rachis* genes in hybrids leads to yield losses in some environments. Management of *non-brittle rachis* genes through pre-breeding is needed to widen the germplasm accessible for hybrid barley breeding.

5. The three-way approach proved to be a successful strategy for mining local germplasm to develop hybrids adapted to new target areas in a time- and cost- efficient way. However, the reduced expression of Spanish diversity and potential heterosis prevented the obtention of superior hybrids. Even then, the combination of phenotypic field evaluation and genomic prediction allowed identifying high-yielding and widely adapted parental lines for the development of promising two-way hybrids.

6. This work proves that advanced breeding germplasm adapted to Spanish conditions is a valuable source of genetic diversity for adaptive traits to be used in hybrid barley breeding.