Universitat de Lleida

CSIC
Estación Experimental de Aula Dei

Control genético de la floración en cebada: caracterización de los principales loci y relación de patrones de espigado con el rendimiento

Tesis Doctoral

Memoria que para optar al grado de Doctor Ingeniero Agrónomo

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Zaragoza, Abril 2007

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INFORMAN:

Que la tesis doctoral titulada "**Control genético de la floración en cebada: caracterización de los principales loci y relación de patrones de espigado con el rendimiento**" ha sido realizada por el Ingeniero Agrónomo D. ALFONSO CUESTA MARCOS, en el Departamento de Genética y Producción Vegetal, de la Estación Experimental de Aula Dei del Consejo Superior de Investigaciones Científicas bajo su dirección y reúne, a su juicio, las condiciones requeridas para optar al Grado de Doctor Ingeniero Agrónomo

Zaragoza, abril de 2007

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Lleida, abril de 2007

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Agradecimientos

Este trabajo es el resultado del esfuerzo de un gran número de personas. A todas ellas quiero agradecerles su contribución y reconocer el inestimable valor de su trabajo.

Mi agradecimiento en primer lugar es a mis directores de tesis, la Dra. Ana María Casas Cendoya y al Dr. Ernesto Igartua Arregui, por su excelente trabajo, por su disponibilidad en todo momento, por su apoyo y por haberme dado esta gran oportunidad.

Al Profesor Jose Manuel Lasa y a la Dra. Pilar Gracia por sus consejos.

Al personal del Departamento de Genetica y Producción Vegetal de la Estación Experimental de Aula Dei, en particular a Blanca Abad, Tere Acín, Pili Asín, Pili Becerrica, María Jesús Espiau, Clarisa Gracia, Vanesa Martínez, Blanca Medina, Gloria Rodríguez y Marina de la Vega por toda su ayuda en la realización de los experimentos y por el buen ambiente en el trabajo.

A Samia Yahiaoui, por todos los momentos que hemos compartido.

Al Profesor Ignacio Romagosa por aceptar la tutoria de esta tesis.

Al Profesor Patrick M. Hayes por orientarme en los momentos de dudas, al Dr. Peter Szücs, Ann Corey, Tania Filichkina, Jari von Zitzewitz y la Dra. Kelley Richardson, por su ayuda y simpatía.

Al Dr. Francisco Ciudad, a Primitiva Codesal y Maria Cruz Rey por su ayuda con los ensayos de invernadero.

Al Dr. José Luis Molina y a la Dra. Marian Moralejo por suministrar parte de los datos utilizados en este trabajo.

Al Dr. Fred van Eeuwijk y al Dr. Marcos Malosetti por su ayuda en los análisis estadísticos.

A todos los que hicieron que mi estancia en Zaragoza fuera agradable.

A la Dra. Maria Luisa Ruiz, que me enseño el camino de la ciencia.

A la Dra. Lorna Dawson, que me dio la primera oportunidad de dedicarme a la ciencia y a quien nunca estare suficientemente agradecido.

A mi familia. A mis padres, a Teresa, a Jorge y a Eda.

Esta tesis se realizó gracias a una beca de Formación del Personal Investigador del Ministerio de Educación y Ciencia y a la financiación de los proyectos del Plan Nacional de I+D+i, AGF98-0251-C03, AGL2001-2289 y AGL2004-05311.

A mis padres

Resum

La data d'espigolat és un caràcter de gran importància per ser un dels principals factors que determinen l'adaptació i el rendiment dels cereals. Aquest caràcter és especialment important en regions on la disponibilitat d'aigua és un factor limitant, com son les regions de clima mediterrani del Nord d'Espanya. Aquest treball es proposa conèixer el control genètic del caràcter en condicions espanyoles i la seva relació amb el rendiment.

En primer lloc, es van avaluar un conjunt de 120 línies d'haploides duplicats d'ordi, procedents del creuament Beka x Mogador, en una sèrie d'assajos de camp localitzats al Nord d'Espanya i també en condicions controlades de temperatura i fotoperíode. D'aquesta manera es van determinar les principals regions genòmiques que controlen la data d'espigolat, les posicions dels QTL, els seus efectes i les seves interaccions.

El segon pas va ser la validació dels QTL de data d'espigolat identificats en la població Beka x Mogador i en la literatura. Per a això es va avaluar un conjunt de germoplasma d'àmplia base genètica, constituït per 17 petites poblacions interconnectades d'haploides duplicats, els quals parentals eren varietats utilitzades en el programa espanyol de millora d'ordi.

Els resultats dels dos primers estudis van posar de manifest que un conjunt de QTL explica una gran part de la variància fenotípica del caràcter data d'espigolat. A més a més, han confirmat una sèrie de marcadors senzills per a diagnòstic dels QTL, apropiats per al seu ús en millora assistida per marcadors. Aquests QTL són els gens de reposada al fotoperíode *Ppd-H1* i *Ppd-H2*, el gen de precocitat intrínseca Eam6 i els gens de vernalització *Vrn-H1* i *Vrn-H2*.

La interacció epistàtica dels gens *Vrn-H1* i *Vrn-H2* determina el grau de sensibilitat a la vernalització de l'ordi. Aquest model epistàtic es va confirmar en la població Beka x Mogador, en les 17 petites poblacions i també recuperant genotips sensibles a la vernalització en la descendència de creuaments entre varietats insensibles. També es va veure que els diferents graus de sensibilitat a la vernalització podrien ser deguts a diferències en la longitud del primer intró del gen *Vrn-H1*.

També es van identificar les principals regions que contenen QTL de rendiment en la població Beka x Mogador, i es va estudiar l'efecte que els QTL de data d'espigolat tenen sobre el rendiment i sobre la interacció genotip x ambient del rendiment. Es va trobar que existeix una relació canviant entre la data d'espigolat i el rendiment en els diferents assajos però, en general, els espigolats primerencs o tardans no solen afavorir alts rendiments.

Resumen

La fecha de espigado es un carácter de gran importancia por ser uno de los principales factores que determinan la adaptación y el rendimiento de los cereales. Este carácter es especialmente importante en regiones donde la disponibilidad de agua es un factor limitante, como las regiones de clima mediterráneo del Norte de España. Este trabajo se propone conocer el control genético del carácter en condiciones españolas y su relación con el rendimiento.

En primer lugar, se evaluaron un conjunto de 120 líneas de haploides duplicados de cebada, procedentes del cruzamiento Beka x Mogador, en una serie de ensayos de campo en el Norte de España y también en condiciones controladas de temperatura y fotoperiodo. De esta manera se determinaron cuales eran las principales regiones genómicas que controlan la fecha de espigado, las posiciones de los QTL, sus efectos y sus interacciones.

El segundo paso fue la validación de los QTL de fecha de espigado identificados en la población Beka x Mogador y en la literatura. Para ello se evaluó un conjunto de germoplasma de amplia base genética, constituido por 17 pequeñas poblaciones interconectadas de haploides duplicados, cuyos parentales eran variedades utilizadas en el programa español de mejora de cebada.

Los resultados de los dos primeros estudios pusieron de manifiesto que un conjunto de QTL explica una gran parte de la varianza fenotípica del carácter fecha de espigado. Además, han confirmado una serie de marcadores sencillos para diagnóstico de los QTL, apropiados para su uso en mejora asistida por marcadores. Estos QTL son los genes de respuesta al fotoperiodo *Ppd-H1* y *Ppd-H2*, los genes de precocidad intrínseca *Eam6* y *Eam7* y los genes de vernalización *Vrn-H1* y *Vrn-H2*.

La interacción epistática de los genes *Vrn-H1* y *Vrn-H2* determina el grado de sensibilidad a la vernalización en la cebada. Este modelo epistático se confirmó en la población Beka x Mogador, en las 17 pequeñas poblaciones y también recuperando genotipos sensibles a la vernalización en la descendencia de cruzamientos entre variedades insensibles. También se vio que los diferentes grados de sensibilidad a la vernalización podrían ser debidos a diferencias en la longitud del primer intrón del gen *Vrn-H1*.

También se identificaron las principales regiones que contienen QTL de rendimiento en la población Beka x Mogador, y se estudió el efecto que los QTL de fecha de espigado tienen sobre el rendimiento y sobre la interacción genotipo x ambiente del rendimiento. Se encontró que existe una relación cambiante entre la fecha de espigado y el rendimiento en los diferentes ensayos pero, en general, los espigados intermedios favorecen los rendimientos altos.

Summary

Heading date is a trait of major importance, as one of the main determining factors of adaptation and grain yield in cereals. This is especially important in regions with limited water availability, as it is the case for the Mediterranean climate areas of Northern Spain.

In a first step, a set of 120 doubled haploid lines derived from the spring x winter cross between the cultivars Beka and Mogador was evaluated in field trials in Northern Spain, and under controlled temperature and photoperiod conditions in order to determine which are the main genomic regions controlling heading date, the QTL positions, their effects and interactions. The aim of this study is to know the genetic control of flowering under Spanish conditions and its relationship with the grain yield.

In a second step, we validated the heading date QTL identified in the Beka x Mogador population, and other ones reported in the literature, in a germplasm pool of wide genetic base, consisting of a set of 17 small doubled haploid interconnected populations whose parents were cultivars used in the Spanish Barley Breeding Programme.

The results of the two studies revealed a set of QTL that accounts for an important percentage of the phenotypic variation for heading date. Besides, a set of friendly-to-use markers were confirmed as diagnostic markers of these QTL and suitable for being used in marker asisted selection. These QTL are the photoperiod response genes *Ppd-H1* and *Ppd-H2*, the early maturity genes *Eam6* and *Eam7* and the two vernalization genes *Vrn-H1* and *Vrn-H2*.

The epistatic interaction of alleles at the *Vrn-H1* and *Vrn-H2* loci determines the degree of vernalization sensitivity in barley. The epistatic model of these two genes was confirmed in the Beka x Mogador population and also in the set of 17 small populations. A further step was achieved through the recovery of vernalization sensitive genotypes in the offspring of crosses between vernalization insensitive genotypes. We also showed that intron length variation in *Vrn-H1* may account for a continuum of vernalization sensitivity.

We tried to identify the main regions containing QTL for grain yield in the Beka x Mogador population, and explored the effect of heading date QTL on yield and also on the genotype-by-environment interaction of yield. We found a changing relationship between heading date and yield in the different trials but, as a general conclusion, intermediate heading dates usually favour higher yields.

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Capítulo 1

Introducción General

Capítulo 1: Introducción General

1.1 Consideraciones iniciales

La cebada es una de las principales opciones en la agricultura de secano en España, y tiene un papel preponderante en la zona del valle del Ebro. Por ello, en 1982 se comenzó a desarrollar un programa de mejora de cebada en la Estación Experimental de Aula Dei (CSIC) en Zaragoza. Actualmente, este programa forma parte de un programa nacional público de mejora de cebada. Con objeto de superar las limitaciones de los programas clásicos de mejora, se planteó la conveniencia de valorar la incorporación de selección asistida por marcadores, con especial énfasis en la fecha de espigado, al ser ésta uno de los principales determinantes de la adaptación del cultivo al ambiente, especialmente en condiciones semi-áridas y de condiciones climáticas variables, como las de la mayor parte de los climas del interior de España. Para conseguirlo, es necesario que se cumplan dos condiciones: desvelar los factores genéticos que controlan la determinación de la fecha de espigado y conocer la influencia de la fecha de espigado sobre el rendimiento.

En cuanto a la primera de estas condiciones, es necesario identificar los principales loci que controlan este carácter, asi como los marcadores moleculares ligados a los mismos. En primer lugar se buscó la identificación de los principales QTL de fecha de espigado en la población de mapeo Beka x Mogador evaluada en localidades representativas de las condiciones de cultivo de los secanos del norte de España. Posteriormente se procedió a la validación de esos QTL y otros publicados en la literatura, en una serie de pequeñas poblaciones interconectadas de cebada de amplia base genética.

En cuanto a la segunda condición, nos hemos centrado en la relación del rendimiento con el espigado en una población con una segregación extraordinaria en cuanto a fechas de floración. Aunque la serie de ensayos utilizada es corta para llegar a conclusiones inequívocas, es suficiente para observar los patrones de relación más generales.

1.2 La cebada

1.2.1 Origen y domesticación

La cebada (*Hordeum vulgare* ssp. *vulgare*), fue una de las primeras plantas domesticadas. Se supone que su domesticación tuvo lugar hace unos 10.000 años en la zona del Creciente Fértil (Zohary y Hopf, 1993, Lev-Yadun et al., 2000), lo que actualmente corresponde con Israel, Líbano, Siria, Irak y Turquía, a partir de la especie silvestre *Hordeum vulgare* ssp. *spontaneum* (von Bothmer et al., 2003). La zona donde *H. spontaneum* muestra una mayor variabilidad genética se encuentra en Israel y Jordania (Badr et al., 2000), por lo que se supone que ese fue el lugar donde comenzó el cultivo y que desde ahí se difundió hacia el Mediterráneo, Norte de África y Golfo Pérsico. Existen también lugares de gran diversidad genética (también propuestos como centros de origen o diversificación) en Etiopía (Bekele, 1983; Orabi et al., 2007), en el Tíbet (Xu, 1982), en Marruecos (Molina–Cano et al., 2002) y en Asia central (Morrell y Clegg, 2007). La expansión de la cebada por zonas ecogeográficas muy diversas (Knüpffer et al., 2003) se ha realizado gracias a una flexibilidad adaptativa notable de la especie. Los investigadores y mejoradores de este cultivo intentan encontrar y controlar las causas de esta capacidad adaptativa.

1.2.2 Citogenética y taxonomía

La cebada es una planta diploide (2x=2n=14). El tamaño de su genoma haploide es aproximadamente 5000 Mb (Arumuganathan y Earle, 1991), mucho mayor que el de las especies modelo de dicotiledóneas (*Arabidopsis thaliana,* 110 Mb) y monocotiledóneas (*Oryza sativa,* 430 Mb), por lo que es difícil que se llegue a una secuenciación completa del mismo a corto plazo. Sin embargo, se sabe que el tamaño de su genoma no se debe a la presencia de un mayor número de genes que en estas especies, sino a la existencia de grandes regiones de ADN repetitivo, en particular de amplificación de retrotransposones (Bennetzen et al., 1998; Ramakrishna et al., 2002).

La taxonomía de la cebada según von Bothmer et al., 1991, es la siguiente:

Clase: *Angiospermae*

Subclase: *Monocotyledoneae*

Orden: *Glumiflorae*

Familia: *Poaceae*

Género: *Hordeum*

Especie: *vulgare*

Subespecies: *Hordeum vulgare* L. subsp. *spontaneum* Koch

Hordeum vulgare L. subsp. *vulgare*

1.2.3 Importancia del cultivo de la cebada

La cebada es una planta herbácea anual. Es el cuarto cereal en cuanto a superficie cultivada en el mundo, después del maíz, el trigo y el arroz. En cuanto a España, es el principal cultivo tanto en superficie con 3,18 millones de hectáreas, como en producción con 10,6 millones de toneladas (FAOSTAT, datos de 2004). Esto supone el 47,5% del total de superficie dedicada a los cereales y el 43% de la producción.

Las principales razones de la gran extensión del cultivo son una gran adaptabilidad ecológica y el gran número de usos a los que se destina, que incluyen la alimentación humana, bien directamente, bien a través de la producción de malta; la alimentación animal (como pienso y forraje) y usos no alimentarios, como la producción de bioetanol.

1.2.4 Mejora de la cebada

La mejora de la cebada, en su mayor parte, es un proceso empírico. En las primeras generaciones segregantes, los mejoradores suelen centrarse en caracteres de alta heredabilidad, como la altura, forma de espiga y fenología, mientras que caracteres como la calidad o el rendimiento se dejan para las etapas finales (Voltas et al., 2002).

La identificación de los mejores genotipos en los ciclos finales de selección suele hacerse en base a ensayos realizados en múltiples localidades. Esta tarea no es fácil debido a que frecuentemente se produce una interacción genotipo por ambiente (GxE), que es la expresión genotípica diferencial en función de los distintos ambientes. De esta forma, los rendimientos medios obtenidos en una serie de ambientes no debería considerarse como un criterio principal de selección, a no ser que no exista GxE (Voltas et al., 2002).

La interacción GxE juega un papel importante en los programas de mejora (Fox et al., 1997). Su tipo y magnitud condiciona los objetivos de la mejora (adaptación amplia o especifica) y también la elección de las localidades de selección. La identificación de genotipos adaptados a la zona objetivo, y por tanto con tendencia a mostrar menor interacción GxE, es un factor clave para conseguir variedades con rendimiento mayor.

En este sentido, se han publicado diferentes estudios que proponen diversas estrategias de selección. Por un lado, los llevados a cabo en ICARDA (Internacional Center for Agricultural Research in the Dry Areas) son partidarios de la búsqueda de adaptación específica para que se produzca un uso óptimo de los recursos, particularmente en ambientes marginales (Ceccarelli, 1989, 1994; Ceccarelli y Grando, 1996). Ceccarelli (1994) demostró los beneficios de la selección en condiciones similares al ambiente objetivo, proponiendo que los genotipos de cebada mejoradas para zonas de escasas precipitaciones deberían seleccionarse bajo esas mismas condiciones. Por otro lado, estudios llevados a cabo en el CIMMYT (Centro Internacional de Mejora del Maiz y del Trigo), mostraron que genotipos de trigo seleccionados en ambientes favorables, presentaban un rendimiento superior y mejor adaptación a gran escala que los genotipos desarrollados localmente.

La interacción GxE aparece habitualmente de modo significativo en los estudios sobre rendimiento en cereales de invierno. Los factores climáticos que determinan el rendimiento de la cebada en mayor medida son la temperatura y las precipitaciones. Ambos también juegan un papel principal en la aparición de la interacción GxE y, por tanto, en la adaptación (Voltas et al., 2002).

La temperatura tiene una influencia directa sobre las diferentes fases del desarrollo del cultivo, modulando la transición de unas a otras y la duración de las mismas, mediante fenómenos directamente relacionados con ella, como son la precocidad intrínseca, el requerimiento de vernalizacion o la resistencia al frío y otros, como la duración del fotoperiodo. Una consecuencia directa de este control ambiental es la variación en la fecha de espigado cuando el cultivo está expuesto a diferentes ambientes y, por tanto, la aparición de interacción GxE para la fecha de espigado, con sus implicaciones en mejora.

De este modo, la variación en la fecha de espigado es una de las principales causas de la interacción GxE del rendimiento, de forma que las variedades precoces normalmente se comportan mejor en zonas de bajo rendimiento, debido a la mayor

disponibilidad de agua al final del ciclo (van Oosterom et al., 1993; Jackson et al., 1994).

Otros factores también responsables de la interacción GxE del rendimiento en la cebada son las características del suelo (Tewari, 1975), la reacción a diferentes enfermedades (Wright y Gaunt, 1992), el número de carreras (Talamucci, 1975), el uso de cultivares autóctonos respecto a materiales mejorados (Ceccarelli y Grando, 1991), variedades antiguas respecto a modernas (Muñoz et al., 1998) o la estructura genética de las variedades, donde poblaciones compuestas por cruzamientos de varias variedades tienen menor interacción que las líneas puras (Soliman y Allard, 1991).

1.3 Relación de la fecha de espigado con la adaptación y el rendimiento

El rendimiento es el resultado del conjunto de procesos de crecimiento y desarrollo que tienen lugar a lo largo del ciclo de cultivo, con un gran número de factores genéticos que lo condicionan directa o indirectamente. Entre estos, los principales son aquellos que determinan la adaptación al medio de cultivo y los que tienen propiamente efecto sobre el potencial productivo (Slafer, 2003).

En aquellas zonas donde los recursos hídricos son escasos, el factor principal de adaptación de los cultivos es el ajuste fenológico. En el caso de la cebada, la adecuación de la fecha de floración a las épocas del ciclo de cultivo en las que los recursos hídricos y ambientales son más favorables, determina el rendimiento final (van Oosterom y Acevedo, 1992). El papel de la fecha de espigado sobre la adaptación ha sido ampliamente estudiado en los cereales. Hoogendoorn (1985) puso de manifiesto la relación entre la fecha de emergencia de la espiga y la distribución geográfica de las variedades de *Triticum aestivum.* Kato et al. (1998), estudiando *T. dicoccoides* en Israel, demostraron que los genotipos de floración temprana están adaptados a zonas más cálidas y secas, mientras que las tardías lo están a zonas más frías y húmedas. Goldringer et al. (2006) obtuvieron resultados similares con *T. aestivum* en Francia. Young y Elliott (1994), pusieron de manifiesto la importancia de la fecha de espigado en la elección de zonas de cultivo y las fechas de siembra en las condiciones del Oeste de Australia. Por otro lado, es manifiesta la dificultad que supone para los mejoradores la producción de variedades con buena adaptabilidad a zonas con variaciones climáticas anuales, como es el caso de los países de Europa Central (Worland et al., 1998).

En las condiciones de clima mediterráneo en España, la parte final del ciclo de cultivo es seca y cálida. En consecuencia, es importante evitar floraciones tardías, que ocurrirían en épocas en las que el estrés hídrico produce una considerable disminución del rendimiento. Sin embargo, una excesiva precocidad en estas condiciones, impide al cultivo aprovechar al máximo las lluvias de principios de primavera y puede exponerlo a heladas tardías en un momento crucial del crecimiento. De este modo, existe un margen restringido de fechas de espigado en el que el cultivo puede expresar al máximo su potencial de rendimiento.

1.4 Factores que determinan la fecha de floración

Una explicación previa sobre la definición de los caracteres *floración* y *espigado*. Aunque la floración es un hito fisiológico clave del desarrollo de una planta, la determinación del momento exacto de su ocurrencia por métodos visuales es problemática en algunas especies. En una especie autógama como la cebada, la antesis sería el evento clave que definiría la fecha de floración. Sin embargo, ésta ocurre normalmente cuando la espiga aún está encerrada en la vaina, por lo que no se puede estimar de modo práctico en ensayos con múltiples genotipos. Así, se suele tomar el momento del espigado (aparición visual de la espiga o de parte de ella fuera de la vaina) como medida subrogada de la floración. Ambos eventos ocurren muy próximos en el tiempo, y la correlación entre ellos es casi total. En este trabajo se ha estudiado la fecha de espigado.

Los primeros modelos propuestos, para explicar las variaciones en la fecha de floración de la cebada, eran simples modelos mendelianos. Nilan (1964) cita varios estudios que proponían segregaciones 3:1 (tanto a favor de floraciones tempranas como tardías), y otros que proponían modelos basados en dos genes, siendo la floración tardía dominante sobre la temprana. Además, se sabía que este carácter estaba muy influenciado por varios factores ambientales. Ya desde la década de los años 30 del siglo XIX, se sabía que la exposición a bajas temperaturas podía inducir la floración en las plantas (estudios citados en Salisbury, 1963). Por otro lado, Garner y Allard (1920) descubrieron que la floración en muchas plantas estaba condicionada por el fotoperiodo.

Estudios posteriores pusieron en evidencia que la variación en la fecha de floración, entre diferentes genotipos y entre individuos de poblaciones segregantes, era debida a factores como la fecha de siembra, la localización de los experimentos o los

genotipos elegidos como parentales de las poblaciones segregantes. Esto llevó a Bell (1939) a proponer que la época de floración en la cebada suponía una relación compleja entre fisiología, genotipo y ambiente.

Johnson y Taylor (1958) propusieron que la fecha de floración es un carácter cuantitativo determinado por la interacción del genotipo y el ambiente, y en el que el fotoperiodo y la vernalización tenían una influencia directa sobre las tasas de desarrollo.

Posteriormente, el uso de técnicas moleculares de mapeo y análisis de aneuploides, permitió identificar numerosos genes, o regiones cromosómicas, asociados con el control de la época de floración en los cereales de invierno, al menos 20 en trigo y 16 en cebada (Laurie et al., 1995; Worland, 1996; Hayes et al., 1997). Muchos de esos genes, en numerosas ocasiones, interactúan entre sí y con el medio ambiente. El estudio de la floración, suele ir acompañado del estudio de las diferentes fases de desarrollo (estados fenológicos), intentando explicar el efecto del fotoperiodo y la temperatura sobre la duración de las diferentes fases fenológicas y, por tanto, su relación con el adelanto o retraso de la floración (García del Moral et al., 2002), así como el efecto de la interacción genotipo por ambiente y su variación en los diferentes estados fenológicos. Existen diversas clasificaciones de los diferentes estados fenológicos, bien por la apariencia externa de la planta (Large, 1954; Haun, 1973), o bien describiendo los cambios morfológicos que se producen en el meristemo apical (Kirby y Appleyard, 1986). Según García del Moral et al. (2002), el desarrollo de la planta puede dividirse en tres estados o fases fenológicas: vegetativa (desde germinación hasta inicio de la floración), reproductiva (hasta antesis o espigado) y fase de llenado del grano (hasta madurez del grano).

En resumen, se puede afirmar que los principales componentes que influyen en el desarrollo son el fotoperiodo y la temperatura, tanto la temperatura *per se* como la baja temperatura asociada al requerimiento de vernalización. Mientras que el fotoperiodo y la vernalización afectan solamente a la tasa de desarrollo en determinadas fases fenológicas, la temperatura *per se* afecta a todas las fases y no se ha descrito ningún cultivar cuya tasa de desarrollo sea insensible a este factor (García del Moral et al., 2002).

A continuación describiremos los efectos de estas condiciones ambientales sobre el desarrollo y la floración de los cereales, especialmente en la cebada, y el control genético de las respuestas genotípicas a las mismas.

1.4.1 Fotoperiodo

La respuesta de las plantas a los cambios en el número de horas de luz es uno de los principales factores determinantes de la duración de las diferentes fases de desarrollo y, por tanto, de adaptación al ambiente (Evans, 1993).

La respuesta al fotoperiodo es uno de los mecanismos que tienen las plantas para impedir o acelerar la floración cuando las condiciones del medio no son las óptimas. Los cereales de grano pequeño son, en general, plantas de día largo, es decir, florecen cuando el número de horas de luz de los días es creciente (Thomas y Vince-Prue, 1997). En el caso de la cebada, esta generalización está limitada por el hecho de que existe un umbral inferior de fotoperiodo, por debajo del cual la floración no ocurre de ninguna manera (8-10 horas en función de los genotipos), y un umbral superior por encima del cual un incremento en el fotoperiodo no supone un cambio en la tasa de desarrollo (13-18 horas en función de los genotipos, según Roberts et al., 1988). Entre ambos límites, la respuesta de los genotipos de cebada al fotoperiodo creciente permite su clasificación en diferentes grupos, tal como propusieron Boyd et al. (2003):

a) genotipos que no responden, o que lo hacen mínimamente, al incremento en el fotoperiodo. Gallagher et al. (1991) se refieren a estos genotipos como insensibles al fotoperiodo, *Photoperiod insensitive* o PI, en contraposición con aquellos que sí responden (*Photoperiod sensitive* o PS);

b) genotipos cuyo grado de sensibilidad al fotoperiodo varía a lo largo del desarrollo. Estos genotipos sensibles al fotoperiodo son, sin embargo, insensibles durante el periodo de tiempo que sigue inmediatamente a la germinación, denominado periodo preinductivo o periodo básico vegetativo (BVP, Major, 1980), y durante otro periodo que precede a la antesis, denominado postinductivo. El intervalo entre ambos periodos de insensibilidad se denomina periodo inductivo o periodo de sensibilidad al fotoperiodo (PSP, Major, 1980) y en él se produce una relación lineal entre el incremento en el fotoperiodo y el adelanto en el espigado, siendo la tasa de crecimiento variable en función de los genotipos y la temperatura (Ellis et al., 1988).

Por otro lado, también existen diferencias entre los distintos genotipos en función de su sensibilidad a fotoperiodos de corta duración. Los genotipos sensibles a

fotoperiodos de corta duración producen más hojas y permanecen más tiempo en su fase vegetativa, en condiciones de fotoperiodo corto. Sin embargo, pasan a su fase reproductiva y aceleran su floración rápidamente en condiciones de fotoperiodo largo (Mahfoozi et al., 2001).

La respuesta de las plantas a la diferente duración de las fases de luz y oscuridad se debe a la presencia de pigmentos fotorreversibles denominados fitocromos. La forma inactiva del fitocromo, *Pr*, al absorber luz roja, se transforma en otra forma, *Pfr*, fisiológicamente activa pero inestable (Smith y Whitelam, 1990), capaz de absorber luz infrarroja. Este proceso se revierte en condiciones de oscuridad. La inducción de la floración en la cebada está promovida por la absorción de luz infrarroja (Jabben y Deitzer, 1979), con lo que el predominio de la forma *Pfr* sobre la *Pr*, que se da en condiciones de día largo, activa el proceso de inducción de la floración y en el caso contrario lo inactiva. El mecanismo de inducción parece ser debido a la acumulación de estímulos de floración en las hojas, que se translocan a los ápices de los tallos, desencadenando el proceso reproductivo. Hay evidencias del papel de las giberelinas en estos estímulos (Jackson y Thomas, 1997).

1.4.1.1 Control genético de la respuesta al fotoperiodo

Los estudios llevados a cabo por Laurie et al. (1994, 1995) pusieron de manifiesto la presencia de dos genes mayores relacionados con la respuesta al fotoperiodo en la cebada, utilizando marcadores RFLP, en un cruzamiento de una variedad de invierno por una de primavera (Igri x Triumph). Estos genes han sido denominados *Ppd-H1* y *Ppd-H2,* siguiendo la nomenclatura utilizada para el trigo (Law et al., 1993).

Ppd-H1 está situado en el brazo corto del cromosoma 2H a aproximadamente 1 cM del marcador MWG858 y confiere sensibilidad al fotoperiodo largo, de tal manera que el alelo sensible provoca el adelanto de la floración a medida que el fotoperiodo crece, mientras que la variación no es significativa en condiciones de fotoperiodo corto. El gen *Ppd-H2*, situado en el brazo largo del cromosoma 1H, tiene un efecto significativo en la variación de la floración solamente en condiciones de fotoperiodo corto. No existe evidencia de interacciones epistáticas entre ambos genes (Laurie et al., 1995).

Mediante clonaje posicional, se identificó el gen *Ppd-H1* (*pseudo-response regulator*), que está bajo control del ritmo circadiano (Turner et al., 2005). Se dispone

de un marcador STS diagnóstico del gen (un SNP al digerir con *BstUI*). Recientemente, se ha identificado *HvFT3* como candidato para el gen *Ppd-H2* (Faure et al., 2007).

Otros genes que causan diferencias en la fecha de floración en condiciones de fotoperiodo corto son *eam7* (sinónimo ea_7 o e_c) en el brazo corto del cromosoma 6H (Stracke y Börner, 1998), *eam8* (sin. ea_k, *ea-a*, o *mat-a*), en el brazo largo del cromosoma 1H (Franckowiak, 1997), *eam10* (sin. ea_{sp}) en el brazo largo del cromosoma 3H (Börner et al., 2002), y *eam9* (sin. ea_c) en el brazo largo del cromosoma 4H (Franckowiak, 1997; Lundqvist et al., 1997).

1.4.2 Vernalización

El requerimiento de vernalización es un carácter determinante en la adaptación al medio ambiente de algunas plantas, en la medida en que activa el proceso de cambio de la fase vegetativa a la reproductiva. Es una respuesta específica a la temperatura.

En la cebada, así como en otros miembros de la familia *Triticeae*, se ha clasificado tradicionalmente a los genotipos según su hábito de crecimiento en genotipos de invierno y genotipos de primavera. En general, la denominación de "genotipos de invierno" se ha considerado sinónimo de genotipos que requieren vernalización. Actualmente se consideran como genotipos de invierno aquellos que necesitan una señal externa para inducir el paso a la fase reproductiva, pudiendo ser ésta una acumulación de horas de frío (Limin y Fowler, 2002) y/o un fotoperiodo de suficiente duración (Karsai et al., 1999).

Algunos genotipos de cebada precisan de este periodo de exposición a bajas temperaturas para inducir la floración. La respuesta genotípica es variable, desde aquellos en los que la acumulación de horas de frío adelanta la floración en mayor o menor medida, hasta aquellos en los que la vernalización es un requisito indispensable (Roberts et al., 1988). Existen, por otra parte, genotipos puros de primavera en los que el requerimiento de vernalización es nulo.

Salisbury (1963) propuso que la exposición a bajas temperaturas provocaba en la planta la síntesis de una sustancia, a la que denominó *vernalina*, que hacía a las plantas sensibles a los efectos del fotoperiodo. Hoy en día, sin embargo, aún no se ha demostrado la existencia fisiológica de la misma.

Se propusieron varios modelos con intervención de interacción epistática para explicar el mecanismo de respuesta a la vernalización en *Triticum monococcum*

(Dubcovsky et al., 1998; Tranquilli y Dubcovsky, 2000). Yan et al. (2004) propusieron un modelo para el trigo diploide según el cual, en aquellos genotipos que presentan un requerimiento de vernalización, existe un represor de la floración que deja de actuar cuando se ha acumulado un número suficiente de horas de frío, permitiendo el tránsito de la fase vegetativa a la reproductiva.

A diferencia de lo que ocurre con los efectos de la temperatura *per se*, la vernalización no afecta directamente a la tasa de iniciación de las hojas (o lo hace ligeramente), sino al número final de hojas. Por esta razón, a menudo se utiliza el número de hojas como un indicador de la sensibilidad a la vernalización, siempre que se hayan satisfecho los otros requisitos necesarios (Kirby et al., 1985).

Los rangos de temperatura entre los que existe efecto de vernalización en la cebada varían, según los autores, desde -5 hasta 16ºC, con un efecto máximo entre 0 y 8ºC (Roberts et al., 1988), o desde 3 hasta 12ºC, con un efecto óptimo en 7ºC (Trione y Metzger, 1970).

A diferencia de lo que ocurre con el fotoperiodo, aquí no se ha propuesto una translocación de estímulos desde las hojas hasta los ápices de los tallos, sino que las células en fase de división mitótica en el meristemo apical son las únicas capaces de percibir la influencia de la vernalización (Burn et al., 1994).

También es de gran relevancia el requerimiento de vernalización en los procesos de adaptación en la cebada y otros miembros de la familia *Triticeae,* por estar asociado a la resistencia al frío (Skinner et al., 2006).

1.4.3 Control genético de la respuesta a vernalización

La base genética de las necesidades de vernalización ha sido tradicionalmente propuesta como un modelo en el que intervienen 3 loci: Sh_1/sh_1, Sh_2/sh_2 y Sh_3/sh_3, situados en los cromosomas 4HL, 5HL, y 1HL respectivamente (Takahashi y Yasuda, 1971). Diferentes combinaciones alélicas en estos loci son las responsables de la variación en el requerimiento de vernalización. Tanto Sh_2 como Sh_3 son epistáticos sobre el alelo de invierno dominante Sh_1, y el alelo recesivo de primavera sh_1 es epistático sobre sh_2 y sh_3, de manera que solamente aquellos genotipos que presentan una combinación alélica del tipo $Sh_1Sh_1sh_2sh_2sh_3sh_3$ presentan un hábito de crecimiento invernal.

Por otro lado, los diferentes grados de respuesta a la vernalización parecen reflejar la existencia de una serie alélica en el locus *Sh₂* (Takahashi y Yasuda, 1971), donde varios alelos dominantes condicionan variaciones en la respuesta a las bajas temperaturas en aquellos genotipos que no son puramente de invierno. Tanto el alelo recesivo *sh₁* como el dominante *Sh₃*, ambos alelos de primavera, son epistáticos sobre el alelo dominante *Sh₂* y son sólo funcionales en presencia de uno u otro de los alelos dominantes de *Sh₂*, lo cual contribuye a mayores posibilidades de variación fenotípica (Boyd et al., 2003).

La clonación de los genes candidatos *Vrn1A^m* y *Vrn2A^m,* que regulan el requerimiento de vernalización en trigo diploide (Yan et al., 2003, Yan et al., 2004) ha servido para un mejor entendimiento del proceso en los cereales. También ha servido para la clonación de los genes correspondientes en la cebada (*Vrn-H2,* sinónimo de *Sh₁* y *Vrn-H1,* sinónimo de *Sh₂*), basándose en la ortología entre trigo y cebada (von Zitzewitz et al., 2005).

Las variantes alélicas en el locus *Sh₃* (ahora también denominado *Vrn-H3*) habían sido descritas solamente en cebadas de latitudes extremadamente altas o bajas (Takahashi y Yasuda, 1971), con lo que en las variedades comúnmente utilizadas en Europa solamente se esperaba variación en los loci *Sh₁* y *Sh₂.* Recientemente, se ha identificado el gen *Vrn-H3*, que corresponde a un homólogo del gen *FT* de Arabidopsis, *HvFT1* (Yan et al., 2006; Faure et al., 2007). Este gen se ha localizado en el brazo corto del cromosoma 7H.

Según el modelo molecular que explica la interacción epistática entre *Vrn2* y *Vrn1* en la familia *Triticeae,* propuesto por Yan et al. (2004), *Vrn2* codifica un represor dominante de la floración (gen candidato ZCCT1) que inhibe la expresión del gen de floración *Vrn1* (gen candidato *TmAP1*). La vernalización regula la expresión de *Vrn2* en la medida en que la disminuye, permitiendo la expresión de *vrn1* en los genotipos de invierno, mientras que no existe ningún requisito de vernalización en el caso de que no se produzca el represor (genotipo *vrn2*), independientemente del alelo presente en *Vrn1*. De la misma manera, no necesitan vernalización aquellos genotipos que producen el represor (alelo dominante *Vrn2*) pero carecen de un lugar de unión del represor (alelo dominante *Vrn1*).

Los genes candidatos ortólogos en cebada son, según von Zitzewitz et al. (2005), el cluster de genes *ZCCT-H* (correspondientes a *ZCCT1*) y *HvBM5* (correspondiente a

TmAP1). Ambos son factores de transcripción, HvBM5/*Vrn-H1* de tipo "MADS box" y HvZCCT/*Vrn-H2* contiene un "zinc-finger" y un dominio CCT.

La variación alélica en *Vrn-H1* parece que se debe a diferencias en el primer intrón del gen (Fu et al., 2005; von Zitzewitz et al., 2005), aunque también se han propuesto variaciones debidas a diferencias en el promotor (Yan et al., 2003; Beales et al., 2005). Respecto a la variación alélica en *Vrn-H2*, Karsai et al. (2005) comprobaron que se debía a presencia/ausencia de los genes *ZCCT-H* mientras que Dubcovsky et al. (2005) propusieron que es el gen *ZCCT-Ha* el principal responsable. Evidencias posteriores parecen indicar que el responsable de la variación alélica en *Vrn-H2* podría ser *ZCCT-Hb* (Trevaskis et al., 2006, Szücs et al., 2007).

1.4.4 Interacción entre la vernalización y el fotoperiodo

La vernalización y la respuesta al fotoperiodo no son fenómenos independientes. De esta manera, la exposición a fotoperiodos de corta duración en las primeras etapas de crecimiento, en algunos genotipos que responden al incremento del fotoperiodo, puede tener un efecto similar a la exposición a bajas temperaturas. Evans (1987) propuso el nombre de "vernalización de día corto" para este fenómeno.

Tanto los fenómenos de vernalización por bajas temperaturas, como la vernalización de día corto, parece que tienen efecto en la reducción de la duración de la fase pre-inductiva (aquella que sigue a la nascencia, en la que la planta es insensible al incremento del fotoperiodo) y en la reducción del número de hojas del tallo principal (Roberts et al., 1988).

También se ha constatado que, en muchos casos, la inducción de la floración que provoca el incremento del fotoperiodo está condicionada por la etapa de bajas temperaturas inmediatamente anterior (Bernier et al., 1981).

Una exposición moderada a bajas temperaturas tiene además el efecto de incrementar la resistencia al frío, necesaria en aquellas zonas donde se producen temperaturas por debajo de 0ºC. La máxima tolerancia a las bajas temperaturas se consigue en la fase vegetativa del desarrollo (Fowler et al., 2001). Los genotipos de invierno son más resistentes al frío; sin embargo, este proceso de inducción de la resistencia se produce de la misma manera en los genotipos de primavera. Se ha comprobado, en un gran número de genotipos de invierno de cebada, que los fenotipos de respuesta al fotoperiodo, a la vernalización y la resistencia a las bajas temperaturas se

encuentran en todas las combinaciones posibles (Karsai et al., 2001). El hecho de que estos tres fenómenos estén interrelacionados es más probable que sea atribuible a efectos de ligamiento que a pleiotropía (Francia et al., 2004). La identificación de los genes *CBF* en la proximidad de *Vrn-H1* apoya esta observación (Skinner et al., 2006).

1.4.5 Temperatura

De forma general, y como característica común a todos los procesos biológicos, el desarrollo de la cebada y, por tanto, la fecha de floración, se ve adelantado según una relación lineal con el aumento en la temperatura, hasta un valor óptimo por encima del cual no se produce un adelanto mayor, o incluso se retrasa (Roberts et al., 1988). Esta relación se cumple en todos los genotipos y todas las fases de desarrollo de la cebada, si bien el grado de sensibilidad a la temperatura varía en función de los genotipos.

Tradicionalmente, se ha empleado el concepto de integral térmica (grados-día acumulados), basado en la observación de que, en muchas circunstancias, se cumple esta relación lineal:

$$\frac{1}{f} = a + bT$$

Donde f es el tiempo entre siembra y floración (días), y $1/f$ es la tasa de desarrollo. T es la temperatura media diurna (ºC), a es la temperatura base por debajo de la cual no se produce avance en el desarrollo y $1/b$ es la integral térmica, siendo estos dos valores a y b constantes específicas de cada genotipo. Solamente si se cumple esta ecuación se puede afirmar que la floración ocurre cuando la integral térmica necesaria, diferente para cada genotipo, ha sido acumulada. Según Slafer y Rawson (1994), esta ecuación puede aplicarse independientemente a cada fase de desarrollo, y se puede obtener para cada genotipo, para un determinado fotoperiodo y bajo unas determinadas condiciones de vernalización. La temperatura, además de influir en la tasa de desarrollo y, por tanto, en la duración de las diferentes fases, también afecta a la tasa de iniciación de hojas y espiguillas y al filocrono o tasa de aparición de las hojas (Klepper et al., 1982).

Sin embargo, cuando se producen cambios en la duración del fotoperiodo, como ocurre en condiciones de campo, la tasa de desarrollo varía en función de la fenología, con lo que el concepto de integral térmica no es totalmente aplicable (Roberts et al., 1988). Para solventar esta dificultad, Ellis et al. (1988) introdujeron el concepto de integral fototérmica:

$$\frac{1}{f} = a + bT + cP$$

Donde P es el fotoperiodo en horas día^{-1}, T es la temperatura media diurna (ºC) y a, b y c son constantes especificas de cada genotipo. Los límites de esta relación se encuentran entre una temperatura base mínima y una óptima, y entre unos valores de fotoperiodo mínimos (que impiden la floración) y máximos, cuya superación no supone un cambio en la respuesta.

1.4.6 Precocidad intrínseca

Cuando la influencia de la vernalización y el fotoperiodo sobre la fecha de floración es eliminada experimentalmente, satisfaciendo las necesidades de los diferentes genotipos, se observa que aún existe variación en las mismas. Los genes implicados en esta variación, cuyos efectos no son directamente atribuibles a la vernalización o fotoperiodo, se han denominado en general *earliness per se* o genes de precocidad intrínseca (*eps*). Laurie et al. (1995) identificaron 8 loci *eps*: *eps2S* en el brazo corto del cromosoma 2H, próximo al centrómero; *eps3L* en el brazo largo del cromosoma 3H; *eps4L* en el brazo largo del cromosoma 4H; *eps5L* en el brazo largo del cromosoma 5H; *eps6L.1* y *eps6L.2*, ambos en el brazo largo del cromosoma 6H; *eps7S* en el brazo corto del cromosoma 7H y *eps7L* en el brazo largo del cromosoma 7H. Además, se han identificado otros loci causantes de precocidad denominados *Early maturity* QTL o *Eam* (Franckowiak et al., 2003; Franckowiak 2004), algunos de los cuales son sinónimos de los *eps*, aunque otros lo son de los genes de respuesta al fotoperiodo, por ejemplo *Eam1* es sinónimo de *Ppd-H1*.

Las causas de la variación en la fecha de floración por causas distintas al fotoperiodo o la vernalización han sido objeto de diferentes estudios. Roberts et al. (1988) propusieron la relación de esta variación con la duración del periodo pre-inductivo que sigue a la nascencia, en el cual se produce crecimiento y aumento del número de hojas pero no se produce la inducción de la floración. Sin embargo, la duración de este periodo varía muy poco entre genotipos, y su correlación con el número de hojas del tallo principal es baja, con lo que las diferencias entre genotipos serían debidas a diferencias en la tasa de desarrollo (Flood y Halloran, 1984), que estaría asociada a diferencias genéticas en la respuesta a la temperatura, lo cual fue confirmado por Slafer y Rawson (1995). Además, estos autores habían propuesto que

existe una variación en la precocidad, independientemente de la precocidad atribuida a variaciones en el fotoperiodo o la vernalización, y que estaba correlacionada con el número de hojas del tallo principal (Slafer y Rawson, 1994). La duración del efecto provocado por este carácter no era constante ni en días ni en integral térmica, sino que las diferencias entre genotipos eran menores a bajas temperaturas, aumentando hasta los 19ºC sin cambiar el orden, pudiendo éste revertirse a temperaturas superiores. Estos autores concluyeron que las interacciones genotipo por temperatura actuaban en las diferentes fases de desarrollo fenológico afectando a la tasa de desarrollo y, por tanto, a la fecha de floración.

Hay y Ellis (1998), a modo de consenso, propusieron que la variación en el carácter de precocidad intrínseca era debida a una combinación del número de hojas iniciadas en el tallo principal (directamente relacionado con la duración del periodo pre-inductivo propuesto por Roberts et al., 1988) y diferencias en la tasa de desarrollo, la cual era un carácter intrínseco de los genotipos controlado por genes reguladores de la tasa de desarrollo, que a su vez estaban influenciados por la temperatura.

Otros autores parecen haber encontrado evidencias de que la regulación de los genes implicados en la precocidad intrínseca puede ser también diferente en condiciones de fotoperiodo corto o largo (Kato et al., 2002), con lo que el control genético de la respuesta a todos los factores ambientales que influyen en la fecha de floración aparecerían así interrelacionados.

1.4.7 Otros efectos

Existe otra serie de factores, de menor efecto en comparación con los descritos, que afectan también a la fecha de espigado. Entre ellos se pueden citar el estrés hídrico en el suelo (Aspinall, 1961), los niveles de nutrientes (Halse y Weir, 1970), la fotosíntesis y la disponibilidad de asimilados (Dale y Wilson, 1979), y la radiación global (Thompson y Mathews, 1981).

1.5 Métodos de detección de QTL

Tradicionalmente, los estudios de búsqueda de QTL para caracteres de todo tipo en plantas se han llevado a cabo en poblaciones de familias emparentadas, obtenidas mediante el cruzamiento de dos parentales. En cereales, estos dos parentales han sido

casi siempre dos líneas puras. Este enfoque tiene la ventaja de la existencia de sólo dos alelos por locus polimórfico, por lo que su estudio es sencillo. Los dos métodos más ampliamente utilizados por su poder de detección de QTL han sido: mapeo por intervalos (*interval mapping* o IM, por sus siglas en inglés, Lander y Botstein, 1989) y mapeo por intervalos compuesto (*composite interval mapping* o CIM, por sus siglas en inglés, Zeng, 1994), presentando este último algunas ventajas con respecto al IM (Zeng, 1994; Cornforth y Long, 2003).

Sin embargo, el empleo de una población procedente de un cruzamiento simple presenta una serie de desventajas. En la mayoria de los casos, los parentales para este tipo de estudios se seleccionan teniendo en cuenta la capacidad de producir polimorfismo en el cruzamiento, por lo que normalmente se han elegido parentales bastante alejados entre sí. En muchos casos, esta filosofía ha llevado a la realización de cruzamientos con escasa relevancia desde el punto de vista de la mejora (Swanston et al., 2006), y no representativos del germoplasma con el que realmente se trabaja en los programas de mejora (Varshney et al., 2005). Por otro lado, los resultados de un estudio en un cruzamiento simple no siempre pueden ser extrapolados a otra población donde los polimorfismos existentes pueden ser distintos. Por último, el estudio simultáneo de solamente 2 alelos por locus no es representativo del germoplasma de la especie (Flint-Garcia et al., 2003).

Debido a estas limitaciones de los estudios en poblaciones procedentes de cruzamientos simples, se han propuesto otros métodos que acerquen más la detección de QTL a los materiales donde sea más relevante, y también a poblaciones de más amplia base genética. Recientemente, se han empezado a considerar métodos alternativos de construcción de mapas y búsqueda de QTL, más allá de las poblaciones de mapeo basadas en dos parentales. Estos métodos están basados en el principio conocido como *linkage dissequilibrium mapping* (Elsner et al, 1995; Risch, 2000), mediante los que se determinan asociaciones de QTL a marcadores en individuos sin una estructura genética particular, como colecciones de variedades vegetales. Este enfoque ha producido resultados positivos en cultivos: en cebada, Pakniyat et al. (1997) encontraron asociaciones de bandas AFLP con tolerancia a la salinidad; Bahrman et al. (1999) pudieron trazar el origen de genes de resistencia al virus del mosaico leve de la cebada (BaMMV) en variedades francesas. En arroz, Virk et al. (1996) lograron encontrar marcadores relacionados con caracteres cuantitativos, como altura de planta, en una colección de entradas no relacionadas.

Otros enfoques se dirigen a la creación y análisis de poblaciones complejas (Crepieux et al., 2004), incluso de los materiales propios de los programas de mejora (Crepieux et al., 2005). Una alternativa para ampliar la base genética para búsqueda de QTL es el empleo de pequeñas poblaciones de familias con padres en común. En este sentido, Muranty (1996) ya sugirió el uso de progenies de varios parentales para alcanzar una mayor probabilidad de obtener más de un alelo en cada QTL de interés, y también como método para tener una estimación mejor de la varianza debida a cada QTL. En concreto y con este fin, Rae et al. (2006) propusieron el estudio de un conjunto de pequeñas poblaciones de líneas de haploides duplicados de cebada procedentes de cruzamientos entre variedades élites actuales del Reino Unido.

Una ventaja de cualquiera de los nuevos enfoques mencionados es que la detección de QTL tiene lugar en la propia población objetivo (Breseghello y Sorrells, 2006).

El planteamiento que se propone en el presente proyecto para la validación de QTL de espigado detectados en la población Beka x Mogador o conocidos por la literatura, está a medio camino entre el mapeo de QTL en poblaciones procedentes de un cruzamiento y el *linkage disequilibrium mapping*, al ocuparse de pequeñas poblaciones de varios cruzamientos de parentales no necesariamente relacionados genealógicamente.

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Capítulo 2

Objetivos

Capítulo 2: Objetivos

La finalidad de este trabajo es la profundización en el conocimiento del control genético de la fecha de floración en la cebada, al ser éste un factor determinante del rendimiento en las zonas con recursos hídricos escasos. El objetivo general es la identificación de los principales QTL implicados, así como sus interacciones entre sí y con el medio ambiente, y la identificación y desarrollo de marcadores moleculares asociados a los mismos con el fin de ser utilizados en los programas de mejora.

Dentro de este objetivo general, los objetivos específicos que se plantean son los siguientes:

- Identificación de loci de control de espigado, así como marcadores moleculares ligados, en una población de mapeo de amplia diversidad para el carácter (Capítulo 3).
- Validación de estos QTL en un conjunto de 17 poblaciones de líneas haploides duplicadas de amplia base genética, representativas del germoplasma del programa público español de mejora. Estimación de la validez de los marcadores encontrados en el objetivo anterior, y análisis de su diversidad genética. Se trata de evaluar, de esta forma, el comportamiento en diferentes fondos genéticos y su potencial uso en programas de selección asistida por marcadores (Capítulo 4).
- Validación del modelo epistático de los genes *Vrn-H1* y *Vrn-H2,* que determinan la respuesta a la vernalización, al ser ésta una de las principales condicionantes de la fecha de floración (Capítulo 5).
- Identificación de QTL determinantes del rendimiento en una población de mapeo, incluyendo el análisis de la relación de la fecha de espigado con el rendimiento y el estudio de la influencia de los principales genes determinantes de la fecha de espigado sobre el rendimiento (Capítulo 6).

Capítulo 3

Heading date QTL in a spring x winter barley cross evaluated in Mediterranean environments

Capítulo 3: Heading date QTL in a spring x winter barley cross evaluated in Mediterranean environments

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3.1 Abstract

Heading date is a key trait for the adaptation of barley to Mediterranean environments. We studied the genetic control of flowering time under Northern Spanish (Mediterranean) conditions using a new population derived from the spring/winter cross Beka/Mogador. A set of 120 doubled haploid lines was evaluated in the field, and under controlled temperature and photoperiod conditions. Genotyping was carried out with 215 markers (RFLP, STS, RAPD, AFLP, SSR), including markers for vernalization candidate genes, HvBM5 (*Vrn-H1*), HvZCCT (*Vrn-H2*), and HvT SNP22 (*Ppd-H1*). Just four major QTL, and the interactions between them, accounted for most of the variation in both field (71% to 92%) and greenhouse trials (55% to 86%). These were coincident with the location of the major genes for response to vernalization and short photoperiod (*Ppd-H2* on chromosome 1H). A major QTL, near the centromere of chromosome 2H was the most important one under autumn sowing conditions. Although it is detected under all conditions, its action seems not independent from environmental cues. An epistatic interaction involving the two vernalization genes was detected when the plants were grown without vernalization and under long photoperiod. The simultaneous presence of the winter Mogador allele at the two loci produced a marked delay in heading date, beyond a mere additive effect. This interaction, combined with the effect of the gene responsive to short photoperiod, *Ppd-H2*, was found responsible of the phenomenon known as short-day vernalization, present in some of the individuals of the population.

Keywords: *Barley, flowering time, photoperiod, QTL, vernalization*

3.2 Introduction

Barley is often grown under semi-arid conditions. Adjustment of crop phenology to the resources available is the main factor for the determination of grain yield in water limited environments (Ludlow and Muchow 1988). Thus, breeding programs for semi-arid conditions must include the attainment of an appropriate heading date among their objectives.

Barley growing areas in Spain present mostly a Mediterranean climate characterized by mild to cold winters, with temperatures rising rapidly during spring, hot dry summers, and limited rainfall, usually concentrated in mid-autumn and spring. Producers prefer autumn over winter sowings to benefit from a longer growing period, and to make the most of both rain maxima. The decision on the type of cultivar to grow under these conditions is not straightforward. The choice ranges from mid- to late-spring cultivars, with some degree of freezing tolerance, to strict winter cultivars with a strong vernalization requirement, depending on the frequency of occurrence of harsh winters (which follows geographic clines). To make informed decisions on the type of cultivars suited to each specific situation, breeders need to have detailed knowledge on the genetic factors affecting barley development in Mediterranean environments.

It has long been known that vernalization requirement (Takahashi and Yasuda 1971), photoperiod response (Roberts et al. 1988) and earliness *per se* genes (Gallagher et al. 1991) are the main factors controlling heading date in barley. Laurie *et al*. (1995) located several major QTL governing these traits on a barley genetic map. Previous QTL studies were made using neutral markers. Currently, there exist allele-specific markers for some candidate genes controlling these processes (Turner et al. 2005; von Zitzewitz et al. 2005).

After the results of previous studies (Casas et al. 1998; Igartua et al. 1999), the cross between cultivars Beka and Mogador was selected to carry out this study. The parents showed polymorphism at several of the main regions controlling vernalization and photoperiod response.

The aims of this work were: i) to determine the most decisive genetic factors for heading date under Northern Spanish (Mediterranean) conditions, and ii) to assess the effectiveness of closely linked markers and candidate genes, to explain genetic variation in a mapping population grown under Northern Spanish environmental conditions. This

is the first step of a larger study on diversity of genes and QTLs controlling heading time in barley under Mediterranean conditions, and their effect on adaptation and yield.

3.3 Material and methods

Plant material

A population of doubled-haploid (DH) lines of the spring x winter cross between the French two-row cultivars Beka (Bethge XIII x Kneifel) and Mogador (Alpha x Sonja) was used. This population was derived via anther culture from the F_1 of the cross in the framework of the Spanish Barley Breeding Programme. Overall it showed acceptable yield and good agronomic characteristics for the region.

From an original set of 228 lines, a smaller subset of 120 was selected, according to the results of 6 small-plot field experiments carried out prior to this study. Plants were clustered in 5 groups, according to similarities of their heading dates to the behaviour of cultivars with different phenological response. The subset of 120 lines was chosen from the 5 groups, taking into account their proportion in the original set, but imposing a minimum of 15 plants from each group.

Phenotyping

The study was mainly focused on autumn sowings, the most frequent in the area, but other experiments were also performed under contrasting field conditions (winter and spring sowings), and under greenhouse controlled conditions (four different combinations of temperature and photoperiod), to provide assessment of heading date under contrasting field conditions of photoperiod and temperature.

Three autumn-sown trials were carried out at three locations in Northern Spain: Zaragoza, Valladolid and Huesca (latitudes around 41.5ºN) during three seasons (2001-2003). A randomized complete block design with three replicates was used in each location. These trials were coded as AUZA01, AUVA02 and AUHU03, respectively. Three additional trials were carried out at Valladolid, in winter sowing in 2001 (WIVA01), and spring sowing in 1999 and 2001 (SPVA99 and SPVA01). These trials were unreplicated.

For all field trials, plots consisted of six or eight rows, 6 m long, and between 1.2 and 1.5 m wide, depending on locations. Crop husbandry followed local practices at

each location. Days to heading were calculated as the number of days between the 1st of January and the day when approximately 2 cm of awns were visible in 50% of stems. Environmental conditions of each trial are detailed in Table 1. Day length calculation includes periods of twilight (Slafer and Whitechurch 2001).

Table 1. Environmental conditions in the field, growth chambers and greenhouses for every trial. Average daily temperatures were calculated according to nearby meteorological stations. For growth chambers and greenhouses, thermohygrographes were used to have a continuous record of temperatures.

Trial/treatment	SD	CDD	TT	HL-S(1)	HL-G	HL-T(1)	HL-H(1)
SPVA01	28/03/01	100	976	13.2	-	-	15.0
SPVA99	17/03/99	82	954	12.7	-	-	14.8
WIVA01	25/02/01	180	825	11.8	-	-	14.5
AUZA01	21/11/00	434	1323	10.4	-	-	12.9
AUVA02	19/11/01	933	939	10.5	-	-	13.9
AUHU03	04/11/02	693	1240	11.0	-	-	13.2
NV_SP	-	-	1680	-	-	10.2	14.6
NV_LP	-	-	1542	-	-	17.0	17.0
V_SP	-	392	1616	-	9.0	10.8	14.6
V_LP	-	392	1416	-	14.0	17.0	17.0

(1) Natural day length includes civil twilight
SD: Sowing date
CDD: Cooling degree-days (from sowing to average heading date of the trial)
TT: Thermal time (ºC) (from sowing to average heading date of the trial)
HL-S: Hours of light (in sowing date)
HL-G: Hours of light (during stay in growth chamber)
HL-T: Hours of light (when plants were transferred to greenhouse)
HL-H: Hours of light (in the average heading date of the trial)

The population was also tested under controlled conditions in an experiment that combined presence or absence of vernalization, with long and short photoperiod. Treatments were named V_LP (vernalization followed by long photoperiod), V_SP (vernalization followed by short photoperiod), NV_LP (no vernalization, long photoperiod), and NV_SP (no vernalization, short photoperiod), and were applied as follows: For the V_LP and V_SP treatments, four plants per genotype were vernalized for 8 weeks in a growth chamber at 4ºC during light time and 9ºC in the dark, with 14 h light (V_LP) or 9 h light (V_SP). When the vernalization period was completed, two plants per genotype were transferred to glasshouses with day length set to 17 h (V_LP) or approximately 10 h (natural day length, labelled as short photoperiod, V_SP), and temperature set to 20/10ºC (day/night). Two weeks before the end of the vernalization period, another four seeds per cultivar were sown in pots, directly within the long and short photoperiod glasshouses (NV_LP and NV_SP treatments, respectively). By the

end of the vernalization period, both vernalized and unvernalized plants reached approximately the same developmental stage. One plant was tested for each genotype-treatment combination. Final number of leaves on the main stem was recorded for each plant. Cooling degree-days (CDD) were calculated as the sum of daily differences between the average temperature and 12ºC, when average temperature was below 12ºC. A maximum of 9ºC was considered for days with average temperature lower than 3ºC, since there is no increase of the vernalization effect below this temperature (Trione and Metzger 1970).

Vernalization requirement (Ver_LP) was estimated for each line as the difference in number of main stem leaves between the NV_LP and the V_LP treatments. Photoperiod sensitivity (Pho_V) was calculated as the difference in the number of leaves between the V_SP and the V_LP treatments.

Genotyping

Genomic DNA was extracted from young leaf tissue of greenhouse-grown plants as described by Casas et al. (1998). Genotyping was carried out with 215 markers: 10 RFLP, 5 STS (2 from candidate genes), 15 RAPD, 112 AFLP and 73 SSR (15 ESTs and 58 genomic-derived markers). The RFLP loci were named using standard North American Barley Genome Project (NABGP) nomenclature. RFLP probes converted to STS primers were also utilized (Blake et al. 1996; Künzel et al. 2000). Lowercase letters were employed to differentiate them from the RFLP loci. The RAPD loci were mapped using OPERON primer sets. The AFLP markers (EcoRI/MseI) were analyzed following the instructions supplied with the Invitrogen AFLP kit. The AFLP loci were named according to Qi and Lindhout (1997) and Waugh et al. (1997). The SSR loci were identified according to their nomenclature in the literature (Liu et al. 1996; Dávila et al. 1999; Pillen et al. 2000; Ramsay et al. 2000; Moralejo et al. 2004).

Vernalization candidate genes, HvBM5 (*Vrn-H1*) and HvZCCT (*Vrn-H2*) were evaluated as reported by von Zitzewitz et al. (2005). Differences in size at the first intron of HvBM5A were tested: the spring allele (Beka) was analyzed with primers HvBM5.55F and HvBM5.56R; the winter allele (Mogador) was assayed with primers HvBM5.88F (5'-gaatggccgctactgcttag-3') and HvBM5.85R (5'-tctcataggttctagacaaagcatag-3'), or primers HvBM5.66F (5'-ctttagctgttcgacggagg-3') and HvBM5.67R (5'-ctacgccgagcacagaaagc-3'), all within the large size intron.

Linkage map construction was performed using JoinMap 3.0 (van Ooijen and Voorrips 2001). In each chromosome, markers which caused a normalised difference in goodness-of-fit chi-square higher than 5 units after their addition were excluded from the map. Map distances are given in centiMorgan (Kosambi function).

QTL analysis

QTL analysis was performed using the composite interval mapping (CIM) procedure (Zeng 1994) implemented in Windows QTL Cartographer 2.5 (Wang et al. 2005). Up to 21 cofactors for CIM were chosen using a stepwise regression procedure with a significance threshold of 0.05. Walk speed was set to 2 cM, and the scan window to 10 cM beyond the markers flanking the interval tested. Experiment-wise significance (α=0.05) likelihood ratio test (LR) thresholds for QTL identification were determined with 1000 permutations, and expressed as LOD (LOD = 0.217 LR). Epistatic interactions between QTL were evaluated with the Multiple Interval Mapping (MIM, Kao et al. 1999) tool implemented in Windows QTL Cartographer using Bayesian Information Criteria (BIC-M0).

Heading date values used for QTL analysis were calculated using adjusted line means (weighted least square means) for each experiment. The proportion of the total variance explained by the QTL was calculated as the coefficient of determination of the multilocus model for each experiment using MIM. Analyses of variance and regression analyses with markers linked to the QTL were performed using the GLM procedure of SAS v9 (SAS Institute Inc., Cary, NC, USA).

Test for QTL x Environment interactions were performed using NQTL (Windows version of MQTL, Tinker and Mather 1995) at an experiment-wide significance level of 0.05 and 43 background markers.

3.4 Results

Linkage map

A linkage map with 215 markers was constructed. Map density for QTL analysis was reduced to a minimum of 1.5 cM between markers, by removing co-segregant markers (similarity higher than 0.95) and those with poor goodness of fit. The final map for QTL analysis had 126 markers distributed over 7 linkage groups (Fig. 1), at a LOD

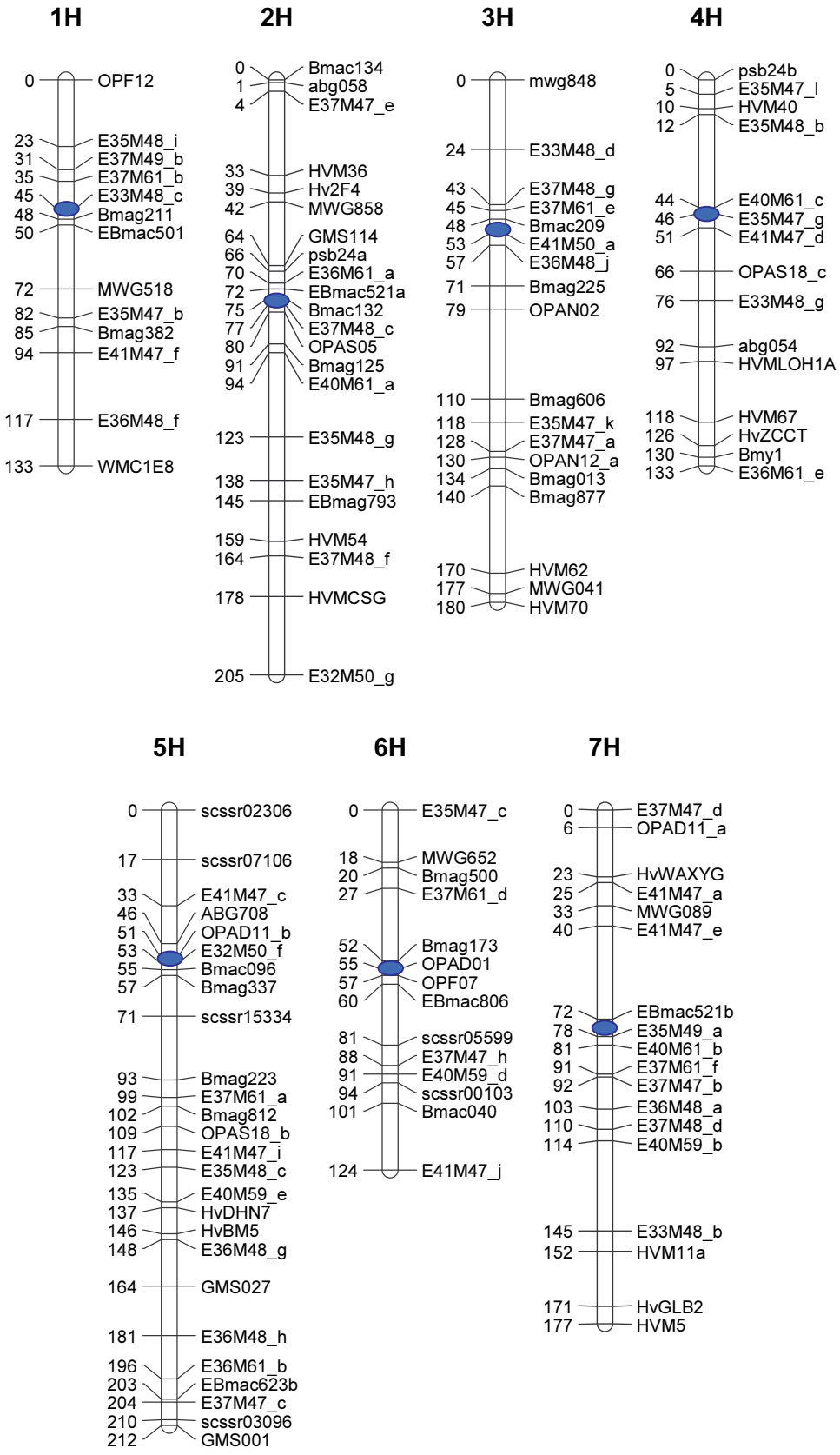


Figure 1. Linkage map of Beka x Mogador doubled haploid population (n = 120). Only 126 spaced markers used for QTL analysis, out of the initial number of 215 are represented (see text). Distances are in Kosambi cM.

score of 5.0. All linkage groups were assigned to barley chromosomes. The linkage map covered 1,163 cM, with an average distance of 9.2 cM per marker. Markers were distributed across the entire genome, except on the distal part of the short arm of chromosome 1H, where no polymorphic markers were found. Segregation distortion was significant (P<0.05) in favour of Beka alleles in regions of chromosomes 1H (E35M48_i - Bmag382), 3H (E36M48_j - OPAN02), 4H (E35M47_l) and 6H (E37M61_d - Bmag173), and in favour of Mogador alleles in chromosomes 2H (E35M47_h - E37M48_f), 5H (scssr07106 - Bmac096 and HVDHN7 - HvBM5) and 7H (E37M47_d - E41M47_a).

Heading time

Frequency distributions of days to heading for field trials and number of leaves for greenhouse treatments showed a quantitative response and transgressive segregation (Fig. 2).

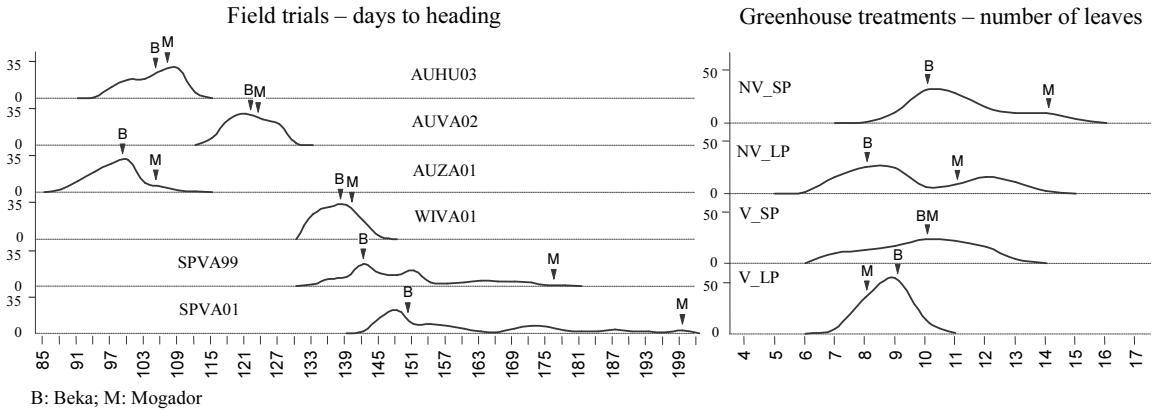


Figure 2. Frequency distribution of days to heading (field) and number of leaves (greenhouse) of the Beka x Mogador DH population experiments. The scale in the vertical axis represents relative frequencies of doubled haploid lines, 0 to 35 % in the field trials and 0 to 50% in the greenhouse trials.

Environmental conditions were very diverse (Table 1). Lack of enough CDD quite possibly led to insufficient vernalization (trials SPVA01 and SPVA99). These treatments, along with greenhouse treatments NV_SP and NV_LP resulted in large heading date differences between the parents, and a wide and flat distribution of DH heading dates (Fig. 2).

All correlation coefficients between field and greenhouse experiments were significant (Table 2). The highest correlation coefficients were obtained between

spring–sown field trials and the greenhouse treatment NV_LP. A high correlation coefficient was also obtained between autumn-sown trials and the V_SP treatment. These correlations are consistent with expectations, based on CDD and hours of daylight at heading time measured at the field trials (Table 1).

Table 2. Pearson correlation coefficients between days to heading in field experiments and number of leaves in greenhouse treatments.

	SPVA01	SPVA99	WIVA01	AUZA01	AUVA02	AUHU03
NV_SP	0.37***	0.40***	0.56***	0.67***	0.54***	0.68***
NV_LP	0.89***	0.85***	0.42***	0.20*	0.21*	0.22*
V_SP	0.20*	0.28**	0.62***	0.70***	0.69***	0.71***
V_LP	0.51***	0.55***	0.65***	0.57***	0.57***	0.57***

Correlation coefficient significant at *P < 0.05, **P < 0.01, ***P < 0.001

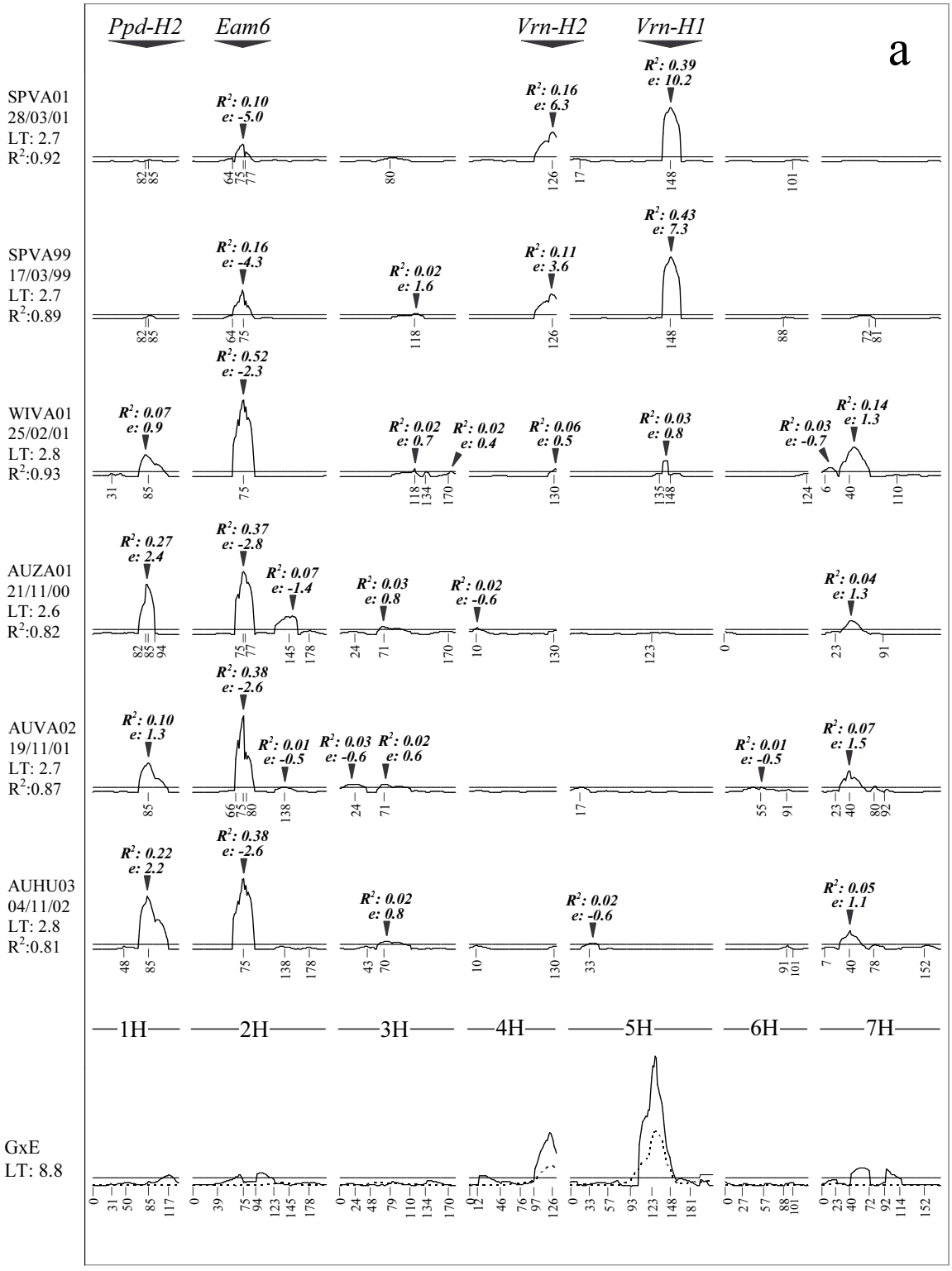
Quantitative trait loci, main effects

Several QTL for heading date were found in the field and greenhouse experiments (Fig. 3).

The amount of phenotypic variation explained jointly by the QTL ranged from 81% to 93% for the field trials, and from 59% to 86% for the greenhouse trials (Fig. 3). Just four major QTL (Fig. 3), and the interactions between them, accounted for most of the variation in both field (71% to 92%) and greenhouse trials (55% to 86%).

An analysis of variance, including the nearest markers to the QTL peaks as sources of variation, and their interactions, is shown in Table 3.

In autumn-sown experiments, a QTL near the centromere of chromosome 2H (bin 8) was the most significant. It was located in the interval E36M61_a – OPAS05, with the peak at Bmac132, in the vicinity of gene *Eam6*. In all cases, the Beka allele conferred later heading. Another large effect QTL was found in the long arm of chromosome 1H (bin 12 -13), in the interval MWG518 – E35M47_b, with the peak at Bmag382, in the vicinity of gene *Ppd-H2*. The Mogador allele conferred late heading. Under greenhouse conditions, it had strong effects under short photoperiod, and a small effect at the V_LP treatment. The third most relevant heading date QTL at all field trials was on chromosome 7H (interval MWG089 – EBmac521b), with Mogador as the later allele.


Ppd-H2
Eam6
Vrn-H2
Vrn-H1
a
SPVA01
28/03/01
LT: 2.7
R2:0.92
SPVA99
17/03/99
LT: 2.7
R2:0.89
WIVA01
25/02/01
LT: 2.8
R2:0.93
AUZA01
21/11/00
LT: 2.6
R2:0.82
AUVA02
19/11/01
LT: 2.7
R2:0.87
AUHU03
04/11/02
LT: 2.8
R2:0.81
1H
2H
3H
4H
5H
6H
7H
GxE
LT: 8.8

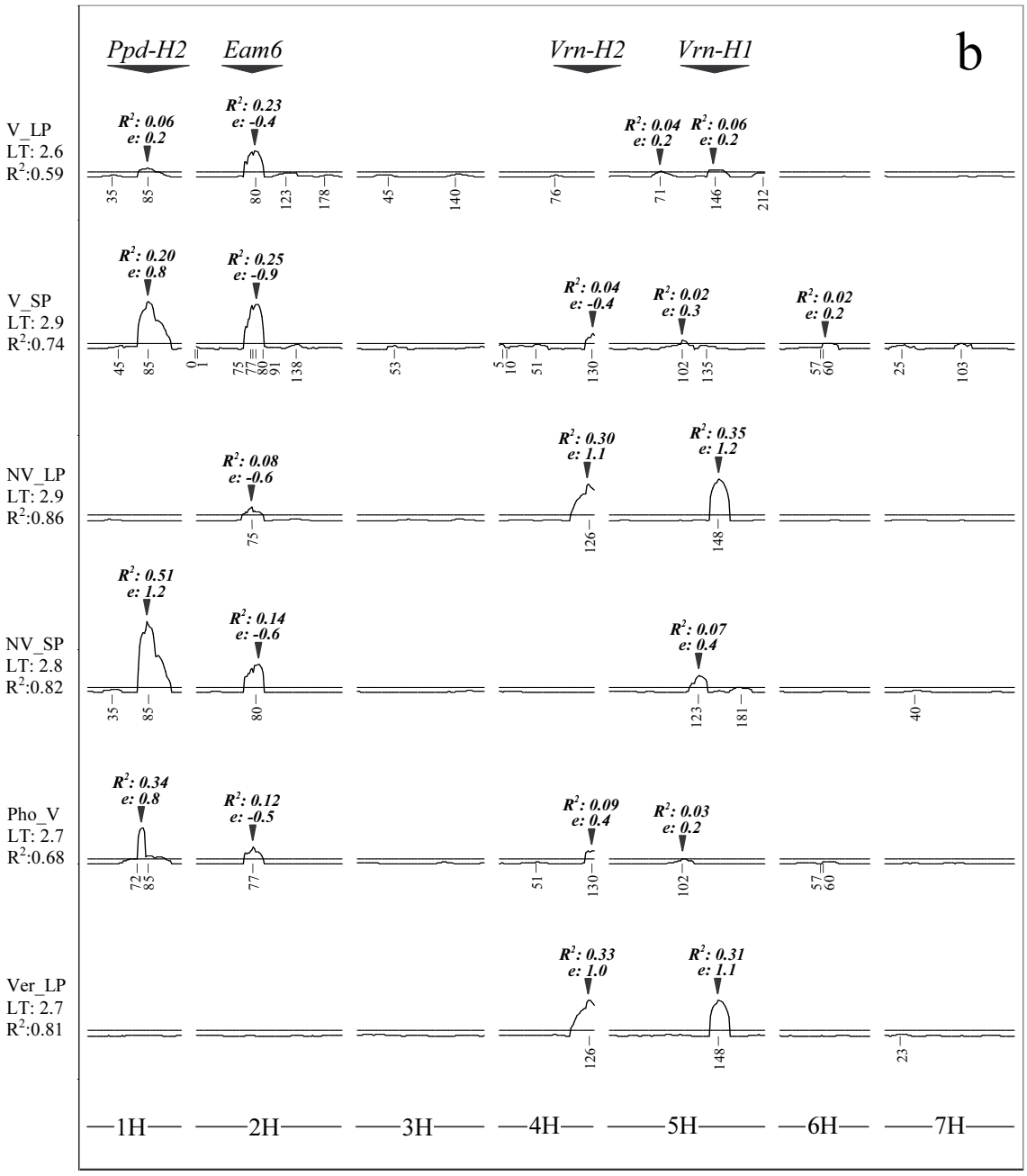


Figure 3. LOD scans of Composite Interval Mapping (CIM) for a) field trials (days to heading) and b) greenhouse treatments and effects (number of leaves). Variable codes are described in the Material and Methods section. On the Y axis, field experiments (3a) are arranged according to sowing date. Also indicated are the code of the experiment, the LOD thresholds (LT) for individual QTL detection based on a experiment-wise error of α=0.05, calculated with 1000 permutations (dotted line), and the R^2 of the multilocus model that includes all significant markers and interactions. For every QTL detected, its individual R^2 and additive effect are indicated. Positive effects indicate later heading of plants carrying the Mogador allele. Chromosomes 1H through 7H are displayed on the X axis, from left to right, showing the positions of the markers included as cofactors in the CIM analysis.
The values of the Genotype x Environment Interaction LOD scans cannot be compared with the ones in the individual trait analysis, since the calculation method is different. Scans for Simple Interval Mapping (SIM – dotted line) and Simplified Composite Interval Mapping (sCIM – full line) are represented (Tinker and Mather, 1995). The LOD threshold in this case corresponds only to the sCIM based on 1000 permutations.

Table 3. Mean squares for the markers significant in the analyses of variance of field and greenhouse experiments. The sources of variation included in the regression models for each experiment are allele-specific or closely linked markers of the QTL detected in the Composite Interval Mapping.

Chr.	Marker	DF	SPVA01	SPVA99	WIVA01	AUZA01	AUVA02	AUHU03	NV_SP	NV_LP	V_SP	V_LP	Pho_V	Ver_LP
1H	Bmag382 (***Ppd-H2***)	1	-	-	110 ***	562 ***	217 ***	615 ***	153.2 ***	-	62.2 ***	3.8 ***	43.6 ***	-
2H	Bmac132 (***Eam6***)	1	1461 ***	1559 ***	585 ***	921 ***	831 ***	727 ***	32.0 ***	30.9 ***	98.4 ***	26.9 ***	25.2 ***	-
2H	E35M47_h	1	-	-	-	103 ***	48 ***	-	-	-	-	-	-	-
3H	E33M48_d	1	-	-	-	-	44 ***	-	-	-	-	-	-	-
3H	Bmag225	1	-	-	-	51 ***	39 ***	34 **	-	-	-	-	-	-
3H	E35M47_k	1	-	144 *	15 **	-	-	-	-	-	-	-	-	-
3H	HVM62	1	-	-	8 *	-	-	-	-	-	-	-	-	-
4H	HVM40	1	-	-	-	17 *	-	-	-	-	-	-	-	-
4H	HvZCCT (***Vrn-H2***)	1	3066 ***	949 ***	17 **	-	-	-	-	99.0 ***	10.7 ***	-	21.6 ***	94.0 ***
5H	E41M47_c	1	-	-	-	-	-	21 *		-	-		-	-
5H	scssr15334	1	-	-	-	-	-	-	-	-	-	6.9 **	-	-
5H	Bmag812	1	-	-	-	-	-	-	-	-	16.3 ***	-	6.5 **	-
5H	E35M48_c	1	-	-	-	-	-	-	9.8 ***	-	-	-	-	-
5H	HvBM5 (***Vrn-H1***)	1	12282 ***	5666 ***	83 ***	-	-	-	-	169.4 ***	-	1.8 **	-	113.7 ***
6H	EBmac806	1	-	-	-	-	11 *	-	-	-	9.2 ***	-	-	-
7H	OPAD11_a	1	-	-	39 ***	-	-	-	-	-	-	-	-	-
7H	E41M47_e	1	-	-	113 ***	81 ***	118 ***	70 ***	-	-	-	-	-	-
	Bmag382*Bmac132	2	-	-	-	-	-	(a)14 *	-	-	-	(a)1.8 **	-	-
	Bmag382*HvZCCT	2	-	-	-	-	-	-	-	-	-	-	(a)3.0 *	-
	Bmag382*HvBM5	2	-	-	-	-	-	-	2.3 *	-	-	(a)1.6 *	-	-
	Bmac132*HvZCCT	2	-	-	-	-	-	-	-	-	6.8 **	-	(a)3.3 *	-
	Bmac132*HvBM5	2	345 **	142 *	-	-	-	-	-	-	-	2.0 **	-	-
	Bmag225*EBmac806	2	-	-	-	-	13 *	-	-	-	-	-	-	-
	E35M47_k*E41M47_e	2	-	-	13 **	-	-	-	-	-	-	-	-	-
	HvZCCT*HvBM5	2	2741 ***	854 ***	-	-	-	-	-	50.3 ***	-	-	-	37.8 ***
	Error df		113	113	109	113	111	113	116	115	110	109	109	115
	Error MS		43	22	2	4	2	4	0.6	0.9	0.7	0.2	0.7	0.9

F value significant at *P < 0.05, **P < 0.01, ***P < 0.001

(a) Non significant interactions according with the BIC criteria using Multiple Interval Mapping

Some interactions were detected only with the MIM procedure: Bmac132 x HvZCCT in SPVA99 y SPVA01, Bmag382 x HvBM5, HVM62 x OPAD11_a and Bmag382 x Bmac132 in WIVA01

Other QTLs, although with minor effect compared to the previous ones, were also detected in autumn sowings (Fig. 3), on chromosomes 2H (at two field trials), 3H (one at three field trials, another one only at AUVA02), 4H, 5H, and 6H (all in just one field trial).

Regarding late sowings, the most important QTL coincided with the positions of markers HvZCCT and HvBM5 in chromosomes 4H and 5H, respectively (Table 3). These positions co-locate with the vernalization genes *Vrn-H2* and *Vrn-H1*, respectively, whose allelic variations determine the requirements of vernalization in temperate grasses (Yan et al. 2003, 2004; von Zitzewitz et al. 2005). In autumn-sown field trials, no effect of these genes was detected (Fig. 3a). As the sowing date advanced, the effect of these markers grew progressively, being smaller at the winter sowing (WIVA01), and larger at the spring-sown trials. Other lesser effect QTL were found in chromosome 3H (peak at 116 cM), and only in the winter-sown trial in chromosomes 3H (peak at 170 cM) and 7H (peak at 12 cM).

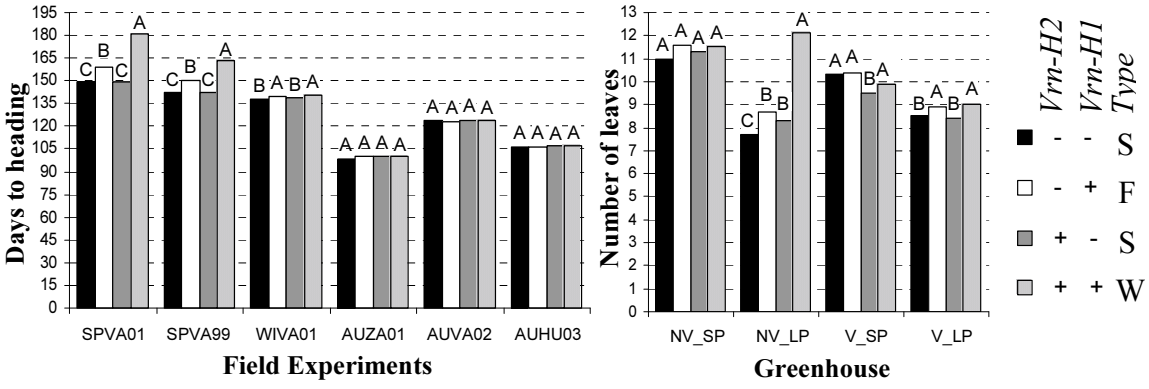
Under controlled conditions, the effects of the QTL at Bmag382, Bmac132, HvZCCT and HvBM5 were very evident. Three other minor QTL were detected in chromosome 5H, at different treatments, and one in chromosome 6H, on the same location as for the AUVA02 field trial. Regarding the Ver_LP effect, the only loci affecting this trait were HvZCCT and HvBM5 and their interaction (Fig. 3b, Table 3). Allelic differences at markers Bmag382, Bmac132, HvZCCT, and (to a lesser extent) Bmag812 were associated to the Pho_V effect.

Interactions between QTL

Some significant interactions between QTLs were found (Fig. 4 and Table 3). The main interaction was found between *Vrn-H1* and *Vrn-H2* in spring-sown field trials, in the NV_LP treatment, and in the VER effect (interaction HvZCCT*HvBM5 in Table 3). The simultaneous presence of the winter Mogador allele at the two loci produced a marked delay in heading date, beyond a mere additive effect. There was also a significant QTL x Environment effect for these two regions (Fig. 3a).

This interaction is further described in Fig. 4, where results have been split into the four classes defined by the combinations of HvZCCT and HvBM5. The allelic composition at the two main vernalization genes was used by von Zitzewitz et al. (2005) to define growth type classes: ‘spring’ when the plants lack the winter allele in *Vrn-H1* (HvBM5), ‘facultative’ if *vrn-H1* winter allele is present but not its repressor *vrn-H2*

(HvZCCT), and 'winter' with both *vrn-H1* and *Vrn-H2* winter alleles functional. We will follow these denominations from now on, bearing in mind that 'spring' actually comprises two classes *(Vrn-H1/Vrn-H2* and *Vrn-H1/vrn-H2*). No differences among classes were found at the autumn-sown trials. Differences were small, though significant, at the winter-sown trial, and rather large at spring-sown ones. In these, 'spring' classes headed at the same time, and significantly earlier than the 'winter' class, 'facultative' being in an intermediate position. In the greenhouse treatments, there were no differences under the less inductive conditions (NV_SP). The two vernalization treatments produced a pattern rather similar to the winter-sown field trial, and at the NV_LP the four classes behaved similar to the spring-sown trials.



- Spring allele (Beka); + Winter allele (Mogador)
S: Spring; F: Facultative; W: Winter

Figure 4. Means of days to heading from January 1st, and number of leaves of the 4 classes of genotypes determined by the allelic composition at *Vrn-H1* and *Vrn-H2*. Due to the strong effect of photoperiod response QTL, means are adjusted for other QTL detected in each experiment. Letters indicate means separation within each experiment. We used the Bonferroni multiple comparison adjustment for the *P*-values and confidence limits for the differences of LS-means with alpha=0.05.

To detect possible repressors, other than *Vrn-H2*, making the facultative class later than the spring one in the spring-sown trials, we performed analyses of variance for each spring-sown trial, within each of the vernalization classes (*spring, facultative*, and *winter*). We used the multilocus model that included the nearest markers to the significant peaks detected in the CIM at each experiment, and their significant interactions (summarized in Table 3a). Then, the rest of the 126 markers were sequentially added and removed to this model, one by one, (data not shown). In this manner, five markers with a consistent significant effect on heading date only for the facultative class lines in the two spring-sown experiments were detected. Three of them

were just above the significance threshold using a False Discovery Rate approach at the chromosome level. The other two presented a much larger effect, and peaked at markers just beside heading time QTL found for the autumn-sown trials (and within the 2-LOD confidence interval). These were OPAN02 in bin 8 of chromosome 3H and MWG089 in bin 3 of chromosome 7H (Table 4). In all cases the Beka allele caused early heading (Table 4) and no interaction was found between them.

Table 4. Joint interaction of Vrn-H1 and Vrn-H2 with other loci. Average days to heading from 1st of January to heading date of two spring sowing experiments. Lines are classified according to their genotype for the vernalization genes Vrn-H1 and Vrn-H2.

			AN02 (3H)					MWG089 (7H)				
Class	*Vrn-H2*	*Vrn-H1*	No	**B**	No	**M**	Pr>t	No	**B**	No	**M**	Pr>t
Spring	B	B	19	145.5	7	145.6	0.9835	11	144.8	15	146.1	0.2923
Facultative	B	M	17	153.0	15	156.7	0.0017	12	152.6	20	156.0	0.0066
Spring	M	B	17	145.7	6	147.0	0.3874	13	145.6	10	146.5	0.5219
Winter	M	M	19	171.5	20	172.7	0.2683	14	170.9	25	172.8	0.0827

B: Beka; M: Mogador; No.: number of lines

Also under spring sowing conditions, we found an interaction between Bmac132 (from now on, *Eam6*) and *Vrn-H1* (Table 3). In both experiments, lines carrying the Beka allele in *Eam6* headed significantly later when the winter allele of *Vrn-H1* was present. Under controlled conditions, *Eam6* also presented significant interactions with vernalization genes, but more so with *Vrn-H2* (HvZCCT), evident at the V_SP treatment. Other significant interactions found were of lesser importance.

We also observed some results in the controlled conditions experiment which agreed with a phenomenon previously described as *short-day vernalization* by Roberts et al. (1988), in which exposure of winter genotypes to short-photoperiod conditions could substitute the effect of vernalization. This is apparent in Fig. 5, as the plants carrying Mogador alleles at the two vernalization loci, and the Beka allele at Bmag382 (from now on, *Ppd-H2,* for simplicity), produced a significantly lower number of leaves until heading at the NV_SP treatment than at the NV_LP one (both marked with an asterisk in Fig. 5).

Actually, the average number of leaves for lines with Beka alleles at *Ppd-H2* was rather similar across all classes of lines (*spring, facultative* and *winter*), at the NV_SP treatment, and the same could be said for the Mogador allele, though it was 2-3 leaves more than Beka overall (Fig. 5).

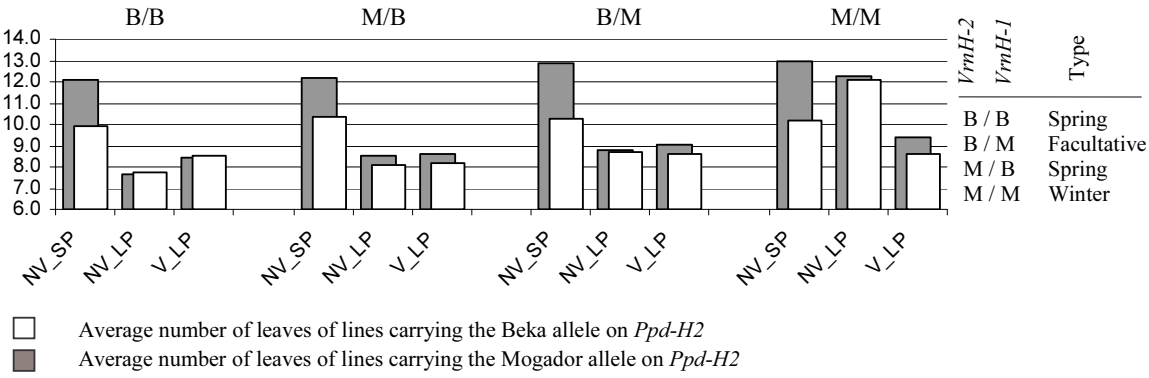


Figure 5. Effect of *Vrn-H1/Vrn-H2* interaction on short-day vernalization. Average number of leaves of the lines divided according to their genotype for the vernalization genes *Vrn-H1* and *Vrn-H2* and the short photoperiod response gene *Ppd-H2.* B: Beka allele; M: Mogador allele.

There was a significantly higher number of leaves for the *winter* class (Mogador at both vernalization loci) at the NV_LP treatment. This increase was evident for both *Ppd-H2* alleles (NV_LP bars at the M/M class in Fig. 5). But the effect it produced for the Beka allele at *Ppd-H2*, when compared with the number of leaves shown by the same lines at the NV_SP treatment, was an apparent shortening of the cycle of unvernalized winter lines, due to the short photoperiod. For other marker combinations, NV_SP was always the treatment where more leaves were produced.

A small quantitative interaction was found between *Ppd-H2* and *Vrn-H1* in non-vernalized plants (Table 3) and we also found that the effect of *Ppd-H2* was larger in the non-vernalized treatment (Fig. 3b). But, no significant three-way interaction among markers for the three loci (*Vrn-H1, Vrn-H2* and *Ppd-H2)* was found.

3.5 Discussion

Distances and marker positions estimated for Beka x Mogador were consistent with other published barley genetic maps (Ramsay et al. 2000; Marquez-Cedillo et al. 2001; Francia et al. 2004).

Major QTL

Genetic control of flowering time in barley has been thoroughly studied and it has long been established that vernalization requirement (Takahashi and Yasuda 1971) and

photoperiod sensitivity (Laurie et al. 1994, 1995) are the most important factors controlling this trait.

Four major QTL, whose positions agree with previously reported genes, were found in this study, at the following locations:

i) Marker Bmac132, on the centromeric region of chromosome 2H (bin 8, bin classification after Kleinhofs and Han 2002), coincident with the earliness *per se* locus *eps2S* (Laurie et al. 1995), whose effect is evident under spring and autumn sowing conditions. It is also coincident with the early maturity locus *Eam6*, which confers early heading under both long- and short-day conditions (Franckowiak and Konishi 2002; Horsley et al. 2006). This was the most important locus for heading time under autumn sowing conditions in our study, and had also a large effect in spring sowings.

The centromeric region of chromosome 2H has been consistently identified in studies searching for heading date QTL, but with large differences in allelic effects. It was first described by Laurie et al. (1994), who reported a large effect of this QTL on heading date (around four days), though the method of analysis available at that time prevented a good estimation of its effect independent of the linked locus *Ppd-H1*.

For *spring x spring* populations, evaluated in spring sowings, the difference between alleles found in a rather large series of studies was always between 1 and 3 days (Qi et al 1998; Marquez-Cedillo et al. 2001; See et al. 2002; Mesfin et al. 2003; Canci et al. 2004; Dahleen et al. 2004; Horsley et al. 2006), whereas for studies involving autumn sowings, its effect was always above 4 days, either in *spring x spring* (Tohno-oka et al. 2000; Boyd et al. 2003; Moralejo et al. 2004) or in *spring x winter* crosses (Laurie et al. 1994; Read et al. 2003). In this last case (population Sloop/Halcyon), the difference between alleles was 6 days, similar to the one found in our study, also a *spring x winter* cross. *Eam6* was identified as the main locus responsible of heading date under Spanish conditions in the population Beka x Logan (Moralejo et al. 2004), and had a large effect in Australian conditions (also a Mediterranean climate), under both short and long photoperiods (Boyd et al. 2003).

The only exceptions to this trend found in the literature were two populations involving a cross with the same *Hordeum spontaneum* accession, evaluated in spring sowings. In these two cases (Pillen et al. 2003, 2004), differences between alleles at this QTL were 9.6 and 12.6 days, with the early allele contributed by the *H. spontaneum* parent, which could be different from the alleles found in barley cultivars.

Eam6 (or *eps2s*) has been defined as an *early maturity* gene, but there is no consensus about its dependence on environmental conditions: it has been described either as photoperiod-insensitive (Boyd et al. 2003) or as photoperiod sensitive (Franckowiak and Konishi 2002; Horsley et al. 2006). Our data support the effect of this locus under a wide variety of conditions, but with the size of its effect modulated by environmental cues that could be photoperiod (an effect on photoperiod sensitivity was reported in Fig. 3) or temperature (we also observed interactions of this locus with the markers for the two vernalization genes, Table 3). It is interesting to note that the effects reported for this locus in the literature, for experiments carried out at lower latitudes and/or early sowings, have always been large; whereas experiments at higher latitudes and/or late sowings always detected a QTL of lesser effect. Consistently with this hypothesis, an AB-QTL study by Talamé et al. (2004) found increasing effects of heading date in a spring x *H. spontaneum* cross (Barke/HOR11508), with large effects (8-9 days) in autumn sowings in north Africa, and lower effect (3.2 days) in a late sowing (February, Italy).

Ppd-H1, also in the short arm of chromosome 2H, has been reported as the main gene affecting heading time in barley under photoperiods of 13 hours or longer (Laurie et al. 1994, Turner et al. 2005). We tested the functional polymorphism in SNP22 of *Ppd-H1* (Turner et al. 2005), and found no polymorphism; therefore, it is likely that both parents of the population carried the same, non-responsive allele. Thus, lack of polymorphism for *Ppd-H1* in this population prevents its study but, on the other hand, allows a clearer resolution of the effect and interactions of *Eam6*, which could have been partially masked by its linkage to *Ppd-H1*, as may have happened in populations segregating for both major QTL on chromosome 2H, such as Steptoe/Morex (Hayes et al. 1993) and Igri/Triumph (Laurie et al. 1994, 1995).

ii) Long arm of chromosome 1H (bin 12-13), marker Bmag382, coinciding with the position of the photoperiod response gene *Ppd-H2*, which causes differences in heading date under short day conditions in field and greenhouse (Laurie et al. 1995; Francia et al. 2004). Its effect was also most influential under Australian field conditions, where cultivars are also exposed to short photoperiods during most of the growing season (Boyd et al. 2003). In our experiments, lines carrying the Mogador allele were always delayed at field autumn sowings, V_SP, and NV_SP treatments, in which most of the vegetative phase elapses with photoperiods around 10 – 11 hours.

iii) Two other main QTLs were found under long photoperiod and no vernalization conditions, in the field and the greenhouse, coinciding with the positions of *Vrn-H1* (marker HvBM5, bin 11 in chromosome 5H) and *Vrn-H2* (marker HvZCCT, bin 13 in chromosome 4H). Allelic variations and interactions of both loci determine the requirements of vernalization in temperate grasses (Yan et al. 2003, 2004; von Zitzewitz et al. 2005).

A significant segregation distortion was found in the *Ppd-H2* and *Vrn-H1* regions. We tested the markers at the distorted regions for a remnant of 108 DH lines of this cross, not used in this study, and found the same distortion which was, therefore, not due to sampling.

Minor QTL

The positions for most of the minor QTL were also coincident with previously reported ones. Only one minor QTL was found in both field and greenhouse conditions, coincident with the position of *eps6L* in bin 7 of chromosome 6H (Laurie et al. 1995). Though it had a more general effect, the largest effect was apparent in the greenhouse under short photoperiods, as in the current study (Table 3). There is also a report of e*ps6L* effect, but with vernalized plants under long photoperiods (Boyd et al. 2003).

Other minor QTLs were found only in the field experiments. The confidence interval of the one detected around 138 cM on chromosome 2H, overlaps with two QTL found in spring barley (Hayes et al. 1993; Pillen et al. 2003). The confidence interval of the QTL found in chromosome 7H spans over bins 3-5 and overlaps with the QTL for heading date *eps7s* (Laurie et al. 1995), which had effect only in spring sowings. Marquez-Cedillo et al. (2001) found a similar effect in the same region (bins 3-4). Pan et al. (1994) also reported a QTL in this region (bins 4-5), in winter sowing conditions. As shown below, loci in this region were associated with differences in heading time also in spring conditions, but only in facultative plants.

Three of the QTLs found in chromosome 3H also agree with other previously reported loci: Hayes et al. (1993) found a QTL in bin 8 in autumn sowings, as our QTL at Bmag225; Mesfin at al. (2003) found another one in bin 12-13 in spring sowing, similar to our QTL with peak at E35M47_k (Table 3); and Gallagher et al. (1991) found a third one in bin 15-16 coincident with the position of our QTL with peak at HvM62, and locus *eam10* (Börner et al. 2001; synonymous of *easp* in Gallagher and Franckowiak 1997, and *eps3L* in Laurie et al. 1995), with effect in short day

photoperiods. It is also coincident with other QTLs for heading date reported in field autumn sowing and 8 hour-photoperiod greenhouse vernalized and non vernalized plants (Pan et al. 1994).

On chromosome 5H, the QTL in bin 6 (peak at scssr15334) agrees with the position of *eps5L* (Laurie et al. 1995), found both under greenhouse and field conditions, under photoperiods longer than 13 hours. The QTL found in bins 8-9 (peak at Bmag812) was in the same position as one found in an autumn-sown field experiment by Pan et al. (1994). The QTL in bin 10 has not been previously reported, but its detection in unvernalized plants grown under short photoperiod, and its proximity, suggests that it could be *Vrn-H1*.

The paramount role of vernalization genes in the Beka x Mogador population

It seems that the autumn sowing conditions provided periods of low temperature long enough to satisfy vernalization requirements in winter genotypes. This caused a narrower range of variation for heading time in the population, compared to the range of variation observed under spring-sown conditions (Fig. 2), in which vernalization requirements for many lines were apparently not met. Thus, it is just coherent that the effect of *Vrn-H1* or *Vrn-H2* was not detected under normal (autumn) sowing conditions, and was mostly evident in spring-sown trials, or in unvernalized treatments in the greenhouse.

The functioning of the two vernalization genes is a clear example of epistasis (Laurie et al. 1995; Yan et al. 2004; Karsai et al. 2005; Koti et al. 2006; Trevaskis et al. 2006). In the spring-sown experiments and at the NV_LP treatment (under long photoperiod and almost absence of vernalization) *Vrn-H1* is repressed by *Vrn-H2* and, therefore, 'winter' lines were remarkably delayed with respect to spring and facultative lines (Fig. 4). This delay of winter genotypes under non-inductive conditions was confirmed when analyzing the number of leaves in the NV_LP greenhouse treatment (Fig. 4). Our results also confirmed another aspect of the hypothesis put forward by Trevaskis et al. (2006), i.e., that r*epression of Vrn-H1 by Vrn-H2 is ineffective when vernalization requirements are fulfilled, and also under short day conditions irrespective of the vernalization.* This was apparent at the autumn-sown trials, and at the short photoperiod treatments (V_SP, NV_SP) in which all vernalization classes reached heading almost at the same time (Fig. 4). Only when there was concurrent lack of

vernalization and long photoperiod, the effect of repression of *Vrn-H2* on *Vrn-H1* was detecTable.

However, we have found that the effect of *Vrn-H2* is not restricted to long day conditions only. There was a QTL at the *Vrn-H2* region in the V_SP treatment (Fig. 3b). We saw that plants with the winter allele in *Vrn-H2* headed earlier, irrespective of the *Vrn-H1* allele, when exposed to vernalization and short photoperiods (treatment V_SP in Fig. 4).

Interactions between photoperiod and vernalization genes, although significant (Table 3), do not seem very important for the determination of heading time under our field conditions. One of these interactions could be responsible for the phenomenon referred to in the literature as *short-day vernalization,* as mentioned in the *Results* section. Laurie et al. (1995), and Igartua et al. (1999) suggested a role of *Ppd-H2* in combination with the two main vernalization genes to explain this phenomenon. Laurie et al. (1995) observed that genotypes with a strong vernalization response under long photoperiod conditions, and carrying a particular allele on *Ppd-H2*, headed at the same time as genotypes that lacked vernalization response when both types were grown under short photoperiods (10 hours). This fact suggests that short day could substitute for cold treatment to hasten heading of these winter genotypes. A similar situation was described by Roberts et al. (1988) for the cultivar Arabi Abiad, which showed an apparent shortening of time to heading in unvernalized plants, when grown under short photoperiod, which was equivalent to about half of the effect due to vernalization. Actually, what we found is that the additive effect of the *Ppd-H2* (Beka allele always earlier than Mogador, under short photoperiod, Fig. 5) with the *Vrn-H1*Vrn-H2* interaction (delay of the M/M class at the NV_LP treatment, Fig. 5) produces an apparent *short-day vernalization* effect, which resembles closely the findings of previous authors, i.e., the fact that *winter* (M/M) lines with the Beka allele at *Ppd-H2,* grown without vernalization, have lower number of leaves under short photoperiod conditions (NV_SP) than under long photoperiod (NV_LP), whereas spring and facultative have more leaves (Fig. 5).

The Beka and Mogador alleles of *Ppd-H2* are possibly the same allele presented by cultivar Triumph and Igri, respectively, in the study by Laurie et al. (1995), according to the RFLP marker MWG518, and the derived STS marker aMWG518, which present the same polymorphism for each pair of cultivars (Beka/Triumph, Mogador/Igri). The behaviour of cultivar Arabi Abiad in Roberts et al. (1988) study was

actually very similar to the behaviour of the most extreme lines in population Beka x Mogador, attending to the number of leaves reported for this cultivar at the treatments most similar to ours.

We searched for other possible interactions with the two main vernalization loci. In spring sowing conditions, *facultative* lines should reach heading at the same time as the spring lines, since they have the winter allele of *Vrn-H1* but lack the repressor *Vrn-H2,* according to the model by Trevaskis et al. (2006). However, facultative lines headed around 10 days later than spring lines in each spring-sown experiment (Fig. 4). Therefore, we propose the hypothesis that there could be other loci interacting with *Vrn-H1*, other than *Vrn*-H2, that could be repressing its action.

Other repressors of *Vrn-H1* have been proposed as *HvVRT2* (Kane et al. 2005), detected on chromosome 7H, in the *facultative* photoperiod-responsive cultivar Dicktoo when exposed to a photoperiod of 8 hours. We did not have comparable conditions in our experiments. Nevertheless, we tested the *HvVRT2* genotype with the same CAPS marker as Szűcs et al. (2006). Both parents showed the same allele, similar to Dicktoo, and thus we could not expect to find a QTL in this region. But we can expect a repressive action of this *HvVRT2* allele (if it is similar to the one from Dicktoo) on *Vrn-H1* in all the population, which could explain, at least partially the intermediate behaviour of *facultative* lines described above.

The results suggest that there could be minor repressors of *Vrn-H1* in two regions: in bin 8 of chromosome 3H, close to marker OPAN02, and in bin 3 of chromosome 7H, close to marker MWG089 (Table 4), which become evident only when *Vrn-H2* is not present. In both cases the Beka allele caused early heading but their effect is still short of accounting for all the delay of the *facultative* lines described above. We cannot tell whether the addition of the effects of these two loci with the effect of the *HvVRT2* repressor is enough to account for the delay.

3.6 Conclusion

Beka x Mogador has been confirmed as an excellent population for vernalization study purposes since it shows polymorphism for both *Vrn-H1* and *Vrn-H2*, and has been genetically characterized with allele specific markers for these genes, which provided an excellent resolution for the detection of the effects of these genes and their interactions, which included a plausible explanation for the phenomenon of *short-day vernalization*.

We have been able to identify the QTLs that explain most of the variation in flowering time of barley in Northern Spanish autumn sowing conditions and we have contrasted and validated these results with field experiments in other sowing times and under controlled conditions of vernalization and photoperiod in the greenhouse. The amount of variation explained by only four allele-specific or closely linked markers to these genes, some of them proposed by the first time in this study, was in all cases over 75%, and we could identify the independent sources of variation making flowering time highly predicTable and almost a qualitative trait. The QTL at the centromere of chromosome 2H deserves further study, as one of the crucial loci controlling heading time under Mediterranean conditions.

Acknowledgements

This work was supported by the Spanish Ministry of Education and Research (Projects AGF98-0251-C03, AGL2001-2289, including a scholarship granted to Mr. Alfonso Cuesta-Marcos, and AGL2004-05311) and by the European Regional Development Fund.

3.7 Supplementary information

Supplementary Table1. Summary of QTL positions (and 2-LOD confidence intervals in brackets), and amount of phenotypic variation explained by them for all the experiments.

	Chromosome							
Trial	**1H**	**2H**	**3H**	**4H**	**5H**	**6H**	**7H**	**R^2(*)**
	---------- Field experiments ----------							
SPVA01	- -	**74** (71-76)	- -	**124** (123-130)	**148** (145-1529)	- -	- -	**0.92**
SPVA99	- -	**74** (72-76)	**116** (79-130)	**124** (122-130)	**148** (146-152)	- -	- -	**0.89**
WIVA01	**82** (77-90)	**75** (73-76)	**116** (99-130) **170** (160-178)	**130** (121-132)	**141** (140-146)	- -	**12** (1-22) **46** (38-53)	**0.93**
AUZA01	**84** (82-86)	**75** (73-79) **149** (145-157)	**67** (58-80)	**10** (4-24)	- -	- -	**40** (34-52)	**0.82**
AUVA02	**86** (81-90)	**75** (74-76) **140** (133-156)	**20** (3-42) **71** (59-80)	- -	- -	**55** (52-77)	**37** (36-40)	**0.87**
AUHU03	**84** (82-87)	**75** (73-76)	**75** (58-80)	- -	**37** (19-45)	- -	**39** (35-65)	**0.81**
	---------- Greenhouse treatments ----------							
NV_SP	**84** (82-87)	**82** (79-87)	- -	- -	**123** (118-132)	- -	- -	**0.82**
NV_LP	-	**75** (72-77)	- -	**122** (119-129)	**148** (145-154)	- -	- -	**0.86**
V_SP	**85** (83-90)	**80** (78-85)	- -	**130** (122-132)	**102** (94-108)	**64** (57-81)	- -	**0.74**
V_LP	**85** (82-99)	**80** (77-84)	- -	- -	**71** (57-88) **141** (134-160)	- -	- -	**0.59**
Pho_V	**78** (73-82)	**77** (73-80)	- -	**130** (126-132)	**102** (86-116)	- -	- -	**0.68**
Ver_LP	- -	- -	- -	**124** (119-130)	**148** (144-155)	- -	- -	**0.81**

(*) R^2 of the multilocus model that includes the QTLs and their significant interactions obtained using Multiple Interval Mapping (MIMapQTL, QTL Cartographer v 2.5)

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Capítulo 4

Joint analysis for heading date QTL in small interconnected barley populations

Capítulo 4: Joint analysis for heading date QTL in small interconnected barley populations

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4.1 Abstract

The purpose of the present work is to validate the effect of the main QTL determining heading date in a set of 281 doubled haploid lines of barley, derived from 17 small interconnected populations, whose parents are cultivars commonly used in the Spanish barley breeding program. We used seventy one molecular markers distributed across the 7 chromosomes, particularly in regions where the presence of flowering time genes or QTL is known. A combined linkage map over the 17 populations was constructed. The lines were evaluated in four field trials: two autumn sowings and two winter sowings, and in one greenhouse trial, under controlled conditions of photoperiod and temperature. We have found that it is possible to carry out QTL detection in a complex germplasm set, representative of the materials used in an active breeding programme. In most cases two alleles per QTL were detected, though polymorphism of flanking markers was notably higher. The results revealed that there is a set of QTL that accounts for an important percentage of the phenotypic variation, amenable for use in marker assisted selection. Also, the role of the photoperiod response genes *Ppd-H1* and *Ppd-H2*, the vernalization response genes *Vrn-H1* and *Vrn-H2*, and the earliness per se locus *Eam6*, of which allele-specific or closely linked markers are available, was confirmed. These results support the use of this kind of approach for the validation of QTL found in biparental studies, or to survey allelic diversity in plant breeding sets of materials.

Keywords: *barley*, *consensus map*, *heading date*, *QTL validation*

4.2 Introduction

Studies on QTL detection in plants have been usually carried out in populations of progenies derived from single crosses. In small grain cereals, these parents are commonly inbred lines. The advantage of this approach is the simplicity of studying only two alleles per polymorphic locus. Although these studies have led to significant advances on genetic control of key traits for many crops, when researchers try to use these QTL in breeding programmes, some drawbacks of the approach become evident. In most cases, parents for QTL studies are selected according to the prospects of finding polymorphism in the cross and, therefore, are chosen among the most extreme ones for the trait of interest. This strategy often led to crosses with little relevance from the plant breeding point of view (Swanston et al. 2006), and/or not adequately representative of germplasm involved in breeding programmes (Varshney et al. 2005). Besides, the results of biparental studies cannot be directly extrapolated to other populations in which polymorphisms may be different (Flint-Garcia et al. 2003), and the resolution obtained in QTL detection is low (Melchinger et al. 2004).

Researchers have sought other approaches to detect QTL over wider arrays of more representative germplasm. Several authors proposed methods of QTL analysis in sets of multiple families, either using some kind of mating design (Muranty 1996; Rebaï and Goffinet 2000; Janninck and Jansen 2001; Jansen et al. 2003), or in multiple families of inbred lines crosses (Xu 1998; Liu and Zeng 2000; Crepieux et al. 2004), such as the ones usually found in breeding programmes. These approaches were put into practice by Crepieux et al. (2005) in wheat, and by Rae et al. (2006) in barley. In this last case, the authors used a set of small populations of doubled haploid lines of barley, derived from crosses between elite varieties currently used in the United Kingdom (*small cross mapping*). The most extreme approach is the search of QTL in sets of individuals with different kinship, even if this kinship is unknown, through association mapping (approach reviewed by Breseghello and Sorrells 2006).

The main advantages of these new approaches are the higher number of alleles explored, and their direct study in target breeding populations (Breseghello and Sorrells 2006). They are also expected to have a deeper impact on marker assisted breeding programmes, detecting marker associations robust enough for successful marker-assisted selection (MAS) beyond its current use for a few genes with outstanding effects.

This study aims at finding useful loci for MAS related to time to flowering in barley. Flowering time is one of the main factors for cultivar adaptation in the dryland Mediterranean agrosystems (van Oosterom and Acevedo 1992). QTL for this trait under Northern Spanish dry land conditions were previously detected in the population Beka x Mogador (Cuesta-Marcos et al. unpublished). This population was extraordinarily polymorphic, and appropriate for flowering time studies.

Before using these QTL for MAS, it is necessary to validate their effect at the germplasm-pool level, and to survey the variability present for each QTL in the breeding pool. For these purposes, we studied a set of small populations of doubled haploid lines, derived from interconnected crosses made with a set of parental lines frequently used in the Spanish National Breeding Programme, and representative of the genetic diversity used by the breeders.

4.3 Materials and Methods

Plant Material

Heading date was evaluated in a set of 281 doubled haploid (DH) lines of barley, derived from the F_1s of 17 small interconnected populations, consisting of biparental crosses among 14 heterogeneous cultivars commonly used in the Spanish barley breeding programme (several parents used more than once). Population size ranged from a minimum of 7 to a maximum of 20 DH lines (Table 1).

Phenotyping

Four field trials were carried out at two provinces in North-Western Spain in 2003 and 2004: Vedado estate in Zuera, province of Zaragoza, and Lupiñén and Alerre, nearby locations of the province of Huesca, all latitudes around 41.5ºN (Table 2). Two of them were sown in autumn in 2002, and harvested in 2003 (coded as AUTVE03 and AUTHU03) and the other two were sown in winter 2004 (coded as WINVE04 and WINHU04). Experimental design followed an alpha lattice with three replicates at each location. Plots consisted of two rows 1.2 m long and 20 cm apart. Days to heading were calculated as the number of days between the 1st of January and the day when approximately 2 cm of awns were visible in 50% of stems. Crop husbandry followed

local practice at each location. Nine DH lines were not sown in 2002 because of lack of seed.

Table 1. Description of the 17 small populations of doubled haploid (DH) lines of barley used in this study. Parent name abbreviations in brackets. Growth type is expressed as follows: S (Spring); F (Facultative); W (Winter).

Population	Parents		Growth type	Row type	DH number	No. markers analyzed	No. polymorphic markers
1	Seira (SEI)	Orria (ORR)	S-F	2-6	20	71	52
2	Seira (SEI)	Alexis (ALE)	S-S	2-2	18	79	29
3	Seira (SEI)	Tipper (TIP)	S-W	2-2	20	71	44
4	Albacete (ALB)	Monlon (MON)	F-F	6-6	7	71	42
5	Albacete (ALB)	Plaisant (PLA)	F-W	6-6	19	71	46
6	Alexis (ALE)	Pané (PAN)	S-F	2-6	20	71	52
7	Angora (ANG)	Clarine (CLA)	W-W	2-2	20	80	29
8	Barberousse (BAR)	Albacete (ALB)	W-F	6-6	10	71	50
9	Barberousse (BAR)	Monlon (MON)	W-F	6-6	12	79	36
10	Barberousse (BAR)	Plaisant (PLA)	W-W	6-6	20	71	22
11	Barberousse (BAR)	Tipper (TIP)	W-W	2-6	8	71	27
12	Beka (BEK)	Monlon (MON)	S-F	2-6	20	71	55
13	Clarine (CLA)	Plaisant (PLA)	W-W	2-6	20	80	39
14	Gaelic (GAE)	Tipper (TIP)	F-W	2-2	8	71	29
15	Nevada (NEV)	Beka (BEK)	S-S	2-2	20	79	31
16	Pané (PAN)	Plaisant (PLA)	F-W	6-6	19	71	46
17	Plaisant (PLA)	Orria (ORR)	W-F	6-6	20	71	38

Table 2. Environmental conditions for every field and greenhouse trial. Average daily temperatures for every field experiment were calculated according to nearby meteorological stations. For growth chambers and greenhouses, thermohygrographes were used to have a continuous record of temperatures.

Trial/treatment	Location	Year	SD	CDD	TT	HL-S(1)	HL-H(1)	RF
AUTHU03	Lupiñén	2002-2003	11-08-2002	687	1291	10.8	14.5	316
AUTVE03	Zuera	2002-2003	11-15-2002	623	1283	10.6	14.5	209
WINHU04	Alerre	2004	01-28-2004	468	932	10.6	15.6	196
WINVE04	Zuera	2004	01-22-2004	427	1055	10.5	15.6	152
NV_LP	greenhouse	2003-2004	-	-	1510	17.0	17.0	-
V_LP	greenhouse	2003-2004	-	392	1147	17.0	17.0	-

(1) Natural day length includes civil twilight

SD: Sowing date

CDD: Cooling degree-days (from sowing to average heading date of the trial)

TT: Thermal time (ºC) (from sowing to average heading date of the trial)

HL-S: Hours of light (in sowing date)

HL-H: Hours of light (in the average heading date of the trial)

RF: Rain fall (mm) from sowing to the average heading date of the trial

Heading date of each line in the field trials was estimated according to the alpha-lattice design, and also according to several spatial analysis models in order to minimize error due to autocorrelation among adjacent plots. These included bidimensional

autoregressive models (AR1 x AR1), either alone or including tiers and columns of the field trial as covariates. The calculation procedure was Restricted Maximum Likelihood (REML), and the Bayesian Information Criterion (BIC, Schwarz 1978) was used to select the best model.

Lines were also tested under controlled conditions in two greenhouse experiments that combined presence or absence of vernalization with long photoperiod. In the vernalization followed by long photoperiod treatment (V_LP), four plants per genotype were vernalized for 8 weeks in a growth chamber at 4ºC during the light time (17 hours), and 7ºC in the dark. When the vernalization period was completed, one plant per DH genotype, and three for the parents, were transferred to a glasshouse with day length set to 17 h, and temperature set to 20/10ºC (day/night).

In the non-vernalized plants and long photoperiod treatment (NV_LP), four seeds per genotype were sown in pots, directly in an adjacent glasshouse with similar conditions of photoperiod and temperature. After 10 days, only one plant per DH genotype and 3 for the parents were retained.

Sowing dates of both treatments were set so that, by the end of the vernalization period, vernalized and unvernalized plants reached approximately the same developmental stage. Plants in the greenhouses were randomly distributed and their positions were rotated weekly.

For each plant and experiment, heading date and number of leaves were recorded. Response to vernalization (VER) was estimated for each line as the difference in the number of main stem leaves between the NV_LP and the V_LP treatments.

No adjustment was made for the number of leaves recorded in the greenhouse experiments since plants were regularly rotated, and hence no spatial effects were expected.

Genotyping

Genomic DNA was extracted from young leaf tissue of greenhouse-grown plants as described by Casas et al. (1998). Eighty markers were used in this study: 44 SSRs, 23 EST derived SSRs, 2 EST InDel, 6 STS and 5 RFLP. Markers were distributed across the 7 chromosomes, particularly on regions where the presence of flowering time genes or QTL are known. Seventy one markers were used for all DH lines and, for those populations that were not segregating for the major vernalization and photoperiod related regions, eight additional markers were placed on chromosome 6H (scssr09398,

Bmac316, Bmag500, scssr02093, Bmag09, Bmac018, scssr05599 y scssr00103). All markers were selected according to the literature and previous studies carried out with the population Beka/Mogador, tested under similar environmental conditions.

Allele specific markers for the two main vernalization response genes *Vrn-H1*, and *Vrn-H2* were used in this study. *Vrn-H1* was characterized with different combinations of primers: HvBM5.42F, HvBM5.43R, HvBM5.55F, HvBM5.56R, HvBM5.85R (von Zitzewitz et al. 2005) and HvBM5.88F, HvBM5.66F, HvBM5.67R, (P. Szücs, pers. comm). These combinations of primers amplify different segments on the first intron of the candidate gene (supplementary Table 1). *VrnH2* was studied analyzing the presence of the candidate genes ZCCT-Ha and ZCCT-Hb with primers HvZCCT 01F/02R (von Zitzewitz et al. 2005). HvT SNP22, which is the allele-specific marker for the long-photoperiod response gene *Ppd-H1* was tested as reported by Turner et al. (2005). HvVRT2 was analyzed as published by Szűcs et al. (2006).

We also used the CAPS marker aMWG518/Nhe I (Primer F 5'-AAAGCTGTC ATACGTCAGC-3' and primer R 5`-CTTGTATCTTTGCTGCACG-3'), derived from the RFLP MWG518 since it is tightly linked to the photoperiod response gene *Ppd-H2* (Laurie et al. 1995).

For the other markers, whose polymorphism is based on amplification product length, alleles of the same size coming from different parents, i.e. identical by state, were also considered as identical by descent.

Consensus map

A consensus map was constructed using the 17 populations. The approximate chromosome location for each marker was previously known. Recombination frequency and LOD score was calculated for each combination of two markers within each chromosome, using Joinmap 3.0 (van Ooijen and Voorrips 2001). The number of individuals considered in each combination of two markers was different, and was set by the number of populations polymorphic for both markers. The consensus map was constructed taking into account the recombination frequency of all possible pairs of markers, and weighting them according to their LOD scores (the higher the number of individuals, the higher the LOD score for a similar recombination frequency), using Joinmap 3.0.

QTL detection

QTL analyses, and calculation of allele effects and marker interactions estimates, were performed using a maximum likelihood method (mixed procedure of SAS v9, SAS Institute Inc., Cary, NC, USA) independently for each field and greenhouse experiment. Analyses were performed for one marker at a time. Each marker parameters where adjusted in the model and, simultaneously, partial regression coefficients where adjusted for other markers (cofactors), in order to reduce the residual variance caused by them. Marker main effects were considered as fixed terms in the model.

Inclusion of other markers (cofactors) for QTL analysis has been reported by other authors such as Jansen (1993), Zeng (1994), or Jansen and Stam (1994). Alleles present in less than 5% of the individuals were excluded from the analysis.

Cofactor selection was carried out independently for each experiment following a forward selection and backward elimination multiple regression procedure (Basten et al. 1995). This method proceeds by performing linear regression of each marker on the studied trait. At each step, the marker with the lowest *P*-value of its F statistic was added to the model. Markers with lowest *P*-value of the partial F statistic were sequentially added to the model until no marker had a *P*-value below the 0.05 threshold. Then, we checked whether all cofactors (markers) included in the model were still significant. Markers with *P*-values above the 0.05 threshold were sequentially removed until all markers left were significant. A likelihood ratio test (LRT) was performed between the maximum likelihood of the model including markers and cofactors and the model including only the cofactors, for each marker. The maximum number of parameters in the model was kept below two times the squared root of the number of individuals (Sakamoto et al. 1986). Cofactors that mapped within a 10 cM window at each side of the marker being tested, were excluded from the model. The significance of the LRT was tested using a χ^2 with a number of degrees of freedom equal to the difference in the number of parameters between the two models (in this case k-1, being k the number of alleles of the marker being tested).

A False Discovery Rate multi-test adjustment (Benjamini and Hochberg, 1995) of the Likelihood Ratio Test *P*-values was performed to declare the significant markers with a genome- wide threshold of 0.05.

Allele effects and interactions

Allele effects of each significant marker were calculated as the adjusted means of days to heading or leaf number, according to the model that included the significant marker and the cofactors for each experiment.

A multilocus model that included the significant markers was tested against the same model plus all possible interactions, added sequentially, and using the BIC to declare significant interactions. In case of significant interactions being present, these were also included in the definitive model for allele effect estimation, and in the LRT in the same way as cofactors.

Classification of detected QTL

We classified the significant markers as primary or secondary, according to the comparison of the BIC between the multilocus model of all significant markers and the multilocus model of all significant markers but the one being tested. The LRT *P*-value of markers with higher number of alleles tended to be more significant, which seems unfair. The use of BIC prevents this flaw to some extent, as it penalizes the number of parameters in the model and, to a much lesser extent, the number of individuals. If the removal of a marker from the multilocus model implies a smaller BIC, it means that the improvement of the LRT caused by that marker was small and/or due to a large number of alleles, i.e number of parameters. Markers presenting this condition were considered as representative of secondary QTL. Conversely, QTL were declared primary when their removal from the multilocus model with all significant markers caused an increment of the BIC value.

4.4 Results

Heading date and leaf number

Ranges of variation for days to heading were similar in the winter-sown and autumn-sown field trials, around 20 days for the DH lines and 6 days for the population means (Fig. 1). In all the experiments, most of the populations showed transgressive segregation.

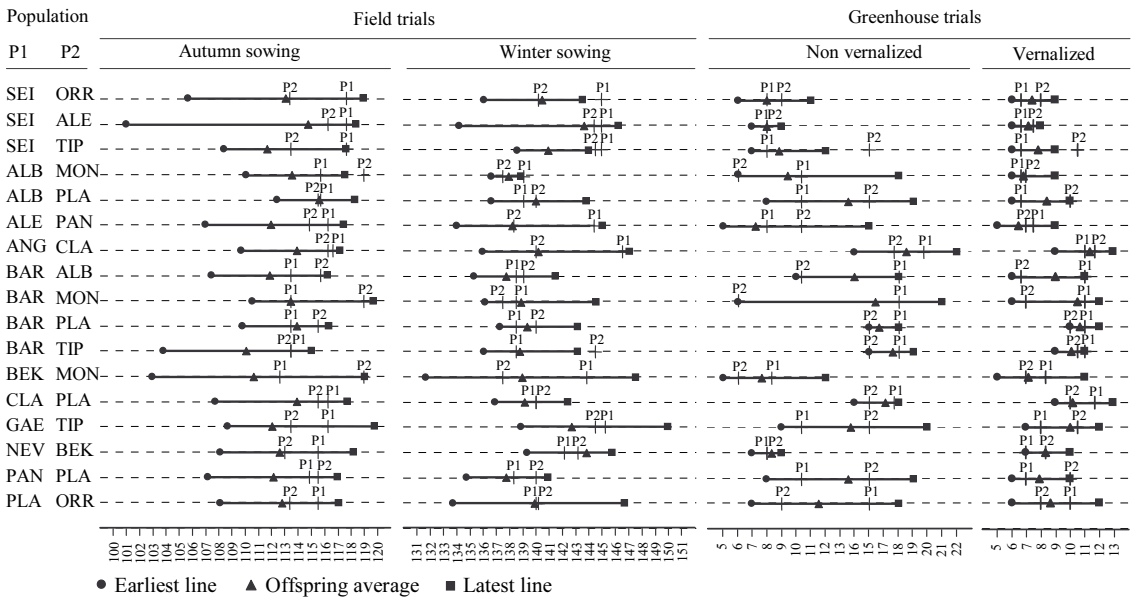


Figure 1. Range of days to heading in the field experiments, and leaf number until heading under greenhouse conditions, for the 17 DH populations evaluated in this study. Days to heading of the DH lines are averaged by sowing season. P1 and P2 are the abbreviations of the parents of each line (see Table 1 for description)

The parents were classified as *winter*, *facultative* or *spring*, according to previous knowledge and cultivar recommendations. Overall, there were minimal differences between parents' heading dates in the autumn-sown trials (averages for the three types of parents differed by less than 1 day). *Spring* parents flowered late in the winter-sown trials, probably as a consequence of the effect of long photoperiod in late sowings. *Spring* cultivars are usually photoperiod insensitive, and thus do not show an advancement of heading date as opposed to most of the sensitive *facultative* and *winter* cultivars. On the other hand, it seems that temperature conditions at winter-sown trials were sufficient to provide enough vernalization for most *winter* cultivars, as most were not delayed in heading date (Fig. 1). The exceptions were cultivars Angora and Tipper, rather late in these trials, suggesting a higher vernalization requirement for them. In the greenhouse, *winter* cultivars were clearly separated from the rest at the NV_LP treatment, where the lack of vernalization made them extremely late. They were also slightly later than *spring* and *facultative* parents at the V_LP treatment. *Spring* parents showed quite homogeneous behaviours across all trials, as expected. *Facultative* parents presented diverse responses, as they comprise a variety of combinations of vernalization requirement and photoperiod sensitivity. Among them, cultivar Monlon was the latest parent at the autumn-sown trials, and the earliest one at the winter-sown ones.

At the V_LP treatment (the most inductive conditions), all populations presented a rather similar response, with a narrow range of variation for all populations. At the NV_LP treatment, the distribution of the populations was influenced by the growth type of the parents, being *winter* x *winter* (ANG/CLA, BAR/PLA, BAR/TIP, CLA/PLA) populations the latest and *spring* x *spring* (NEV/BEK, SEI/ALE) the earliest populations.

Field trials showed a high correlation coefficient between experiments with similar sowing date. The correlation between the two greenhouse trials was also highly significant (Table 3).

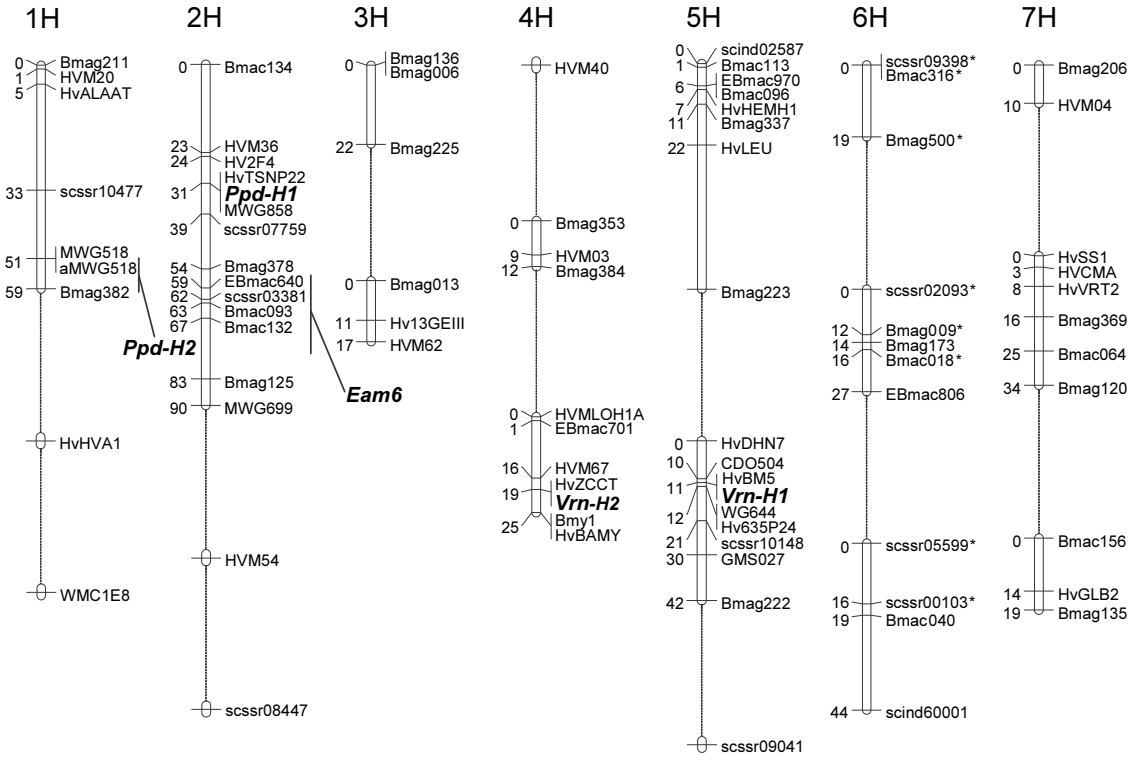
Table 3. Correlation coefficients of heading dates and/or leaf number among the experiments carried out in this study (codes for experiments in the text).

	AUTHU03	WINVE04	WINHU04	NV_LP	V_LP
AUTVE03	0,88***	0,67***	0,65***	0,15*	0.18***
AUTHU03		0,55***	0,57***	0,23***	0.23***
WINVE04			0,88***	0,03	0.19***
WINHU04				-0,03	0.11*
NV_LP					0.86***

*, **, *** Pearson correlation coefficients significant at P < 0.05, P < 0.01, P < 0.001, respectively

Consensus linkage map

A consensus map was constructed with the molecular data of 80 markers analyzed in the 17 DH populations (Fig. 2). Seventy five markers were distributed across 14 linkage groups. Five markers were not grouped since they did not present a significant linkage with, at least, two other markers. Marker distances within each linkage group agree with other published barley maps. Linkage groups and unlinked markers, as well as their relative positions, were assigned to barley chromosomes according to other previously published barley linkage maps (Pillen et al. 2000; Ramsay et al. 2000; Moralejo et al. 2004; Rostoks et al. 2005).



* Markers analyzed only in populations 2, 7, 10, 11, 13, 14 and 15 and excluded from the QTL analysis

Figure 2. Consensus map for the 17 DH populations. Linkage groups are set at a LOD score of 3.0. Distances are in Kosambi cM. Dotted lines bind linkage groups and unlinked markers within each chromosome. Major heading time loci are indicated in bold types, positions according to literature.

Quantitative Trait Loci

Some markers were significantly associated with heading date under all conditions, as Bmac132 and HvBM5, whereas most of the other significant markers had an effect in at least two experiments (Figs. 3 and 4, Table 4). The amount of phenotypic variation explained by the significant QTL and their interactions ranged from 44% to 67% in the field, and was, 93% (NV_LP), 70% (V_LP) and 90% (VER) in the greenhouse. The number of alleles per locus ranged from 2 to 10. Although there were more than two alleles in most of the markers, in most cases the evidence suggested the presence of a biallelic QTL (Table 4). This will be discussed further in the next sections, where QTL will be presented according to their phenotypic effect.

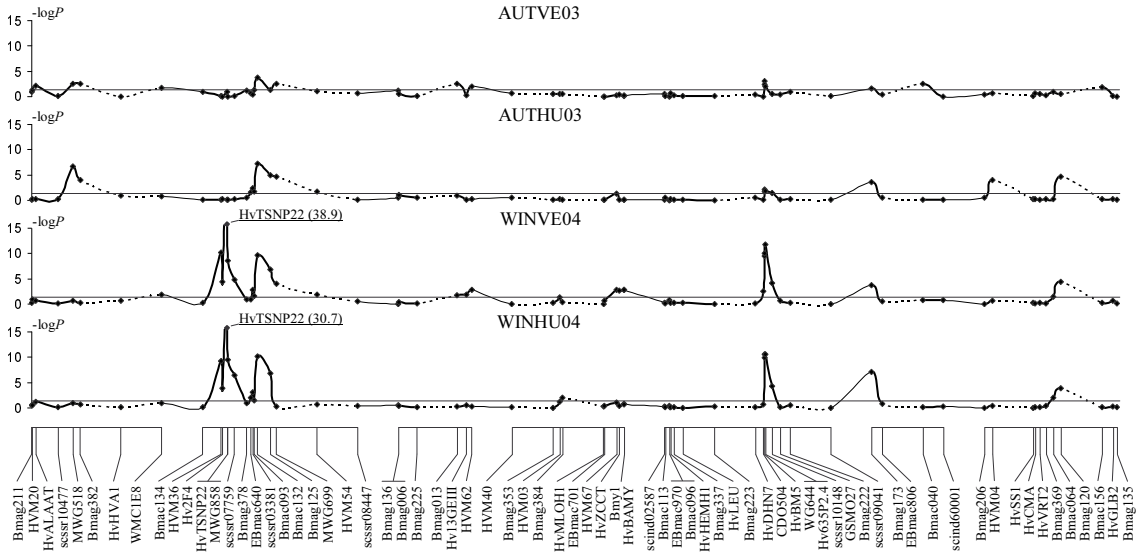


Figure 3. *P* values of the Likelihood Ratio Test (LRT) for all the markers in the field experiments, expressed as –log *P*. Significance threshold for individual QTL detection is based on an experiment-wise error of α=0.05 (-log α=1.3). A False Discovery Rate multi-test adjustment (Benjamini and Hochberg, 1995) of the LRT *P*-values was performed. In brackets are written the values of markers whose value was over 15. Tests were made only at the positions of the markers. Full lines join markers only to facilitate visualization, but they are not indicating test values in the intervals. Dotted lines join linkage groups within the same chromosome.

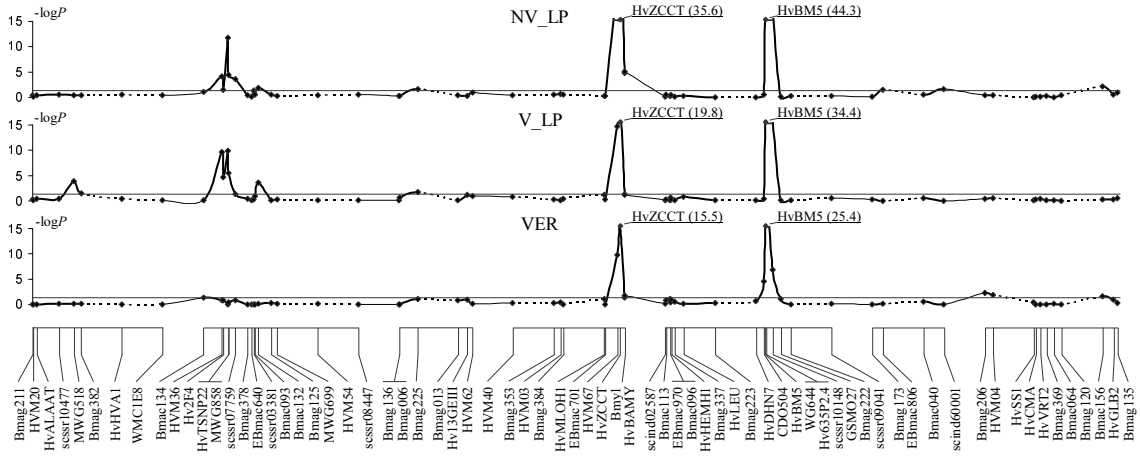


Figure 4. *P* values of the Likelihood Ratio Test (LRT) for all the markers in the greenhouse experiments.

Table 4. Significant markers detected at each experiment. The table shows the number of lines and average days to heading, or leaf number, for the different alleles of the significant markers for each experiment. Means are adjusted for other significant markers in each experiment. Letters indicate means separation. We used a False Discovery Rate multi-test adjustment for the *P*-values and confidence limits for the differences of means with alpha = 0.05. In italics, markers considered as secondary QTL according to the methodology described in the M&M section.

Chr.	Marker		AUTVE03		AUTHU03		WINVE04		WINHU04		NV_LP		V_LP		VER	
	(putative gene)	Allele	No.	Mean	No.	Mean	No.	Mean	No.	Mean	No.	Mean	No.	Mean	No.	Mean
1H	aMWG518 (*Ppd-H2*)	1	99	112.2 b	87	110.0 b	-	- -	-	- -	-	- -	101	6.6 b	-	- -
		2	164	114.5 a	142	113.4 a	-	- -	-	- -	-	- -	168	7.5 a	-	- -
1H	WMC1E8	191	66	112.7 b	-	- -	62	138.5 b	-	- -	-	- -	-	- -	-	- -
		238	197	114.0 a	-	- -	194	139.4 a	-	- -	-	- -	-	- -	-	- -
2H	HvTSNP22 (*Ppd-H1*)	1	-	- -	-	- -	114	142.1 a	115	143.1 a	110	11.0 a	119	7.8 a	-	- -
		2	-	- -	-	- -	142	135.9 b	142	138.1 b	145	9.1 b	152	6.6 b	-	- -
2H	Bmac132 (*Eam6*)	183	27	112.7 b	28	110.4 b	28	138.5 b	28	139.5 b	27	9.8 b	28	6.9 b	-	- -
		189	82	115.5 a	72	114.3 a	80	141.3 a	81	142.3 a	81	10.6 a	85	7.6 a	-	- -
		191	154	111.8 b	129	110.4 b	148	138.2 b	148	139.5 b	147	9.7 b	156	6.6 b	-	- -
2H	MWG699 (*vrs1*)	A	97	114.7 a	89	112.9 a	95	139.8 a	-	- -	-	- -	-	- -	-	- -
		D	41	112.8 b	37	110.2 b	38	137.8 b	-	- -	-	- -	-	- -	-	- -
		K	125	112.6 b	103	119.9 b	124	138.1 b	-	- -	-	- -	-	- -	-	- -
4H	HvZCCT (*Vrn-H2*)	0	-	- -	-	- -	-	- -	95	138.6 b	98	8.1 b	97	6.5 b	89	1.0 b
		1400	-	- -	-	- -	-	- -	162	139.9 a	157	12.0 a	172	7.8 a	163	3.7 a
5H	HvBM5 (*Vrn-H1*)	0	58	111.7 c	51	110.4 b	57	138.0 c	57	138.6 c	56	7.9 c	60	6.2 c	56	1.0 c
		150	25	115.2 ab	28	112.5 ab	27	138.7 bc	28	140.7 b	28	9.5 b	27	7.3 b	25	1.4 bc
		1190	17	112.3 c	18	110.8 b	18	137.3 c	18	138.8 c	19	9.1 b	18	6.4 c	16	2.4 b
		1200	25	112.8 bc	19	111.4 ab	24	139.6 b	24	140.4 b	30	9.9 b	26	6.8 bc	24	2.3 b
		5150	138	114.7 a	113	113.0 a	130	141.2 a	130	142.1 a	122	13.7 a	138	8.5 a	131	4.3 a
6H	Bmag173 (*Eam7*)	124	*134*	*113.2 ab*	118	111.1 bc	135	139.1 a	135	140.4 b	-	- -	-	- -	-	- -
		148	*35*	*111.8 b*	38	109.6 c	39	137.4 b	39	138.6 c	-	- -	-	- -	-	- -
		150	*23*	*113.5 ab*	19	111.5 ab	24	138.8 a	25	140.2 b	-	- -	-	- -	-	- -
		152	*38*	*114.2 a*	37	112.8 a	38	139.8 a	38	141.3 ab	-	- -	-	- -	-	- -
		158	*18*	*115.5 a*	17	113.5 a	20	139.7 a	20	142.3 a	-	- -	-	- -	-	- -
7H	Bmag120	230	-	- -	*58*	*112.6 b*	61	140.0 a	*62*	*141.2 ac*	-	- -	-	- -	-	- -
		232	-	- -	*35*	*110.8 bc*	36	138.9 ab	*36*	*140.1 bd*	-	- -	-	- -	-	- -
		236	-	- -	*43*	*111.9 b*	55	139.8 a	*55*	*141.6 a*	-	- -	-	- -	-	- -
		240	-	- -	*11*	*110.7 bc*	13	137.1 c	*13*	*138.5 d*	-	- -	-	- -	-	- -
		254	-	- -	*15*	*108.7 c*	15	137.4 bc	*15*	*139.8 bcd*	-	- -	-	- -	-	- -
		260	-	- -	*57*	*111.4 b*	65	139.5 a	*65*	*141.2 ab*	-	- -	-	- -	-	- -
		262	-	- -	*10*	*115.7 a*	11	140.2 a	*11*	*141.6 ab*	-	- -	-	- -	-	- -

Vernalization genes and their interaction. Using different primer combinations, five different products, associated with the length of the first intron of *Vrn-H1* were found (supplementary Table 1).

Vrn-H1 showed an important effect in all field trials, with larger effect in the autumn-sown experiments. *Vrn-H2* was significant only in one of the winter-sown experiments. No interaction between both vernalization genes was detected under field conditions. Under greenhouse conditions, however, the vernalization genes presented a strong interaction. The main effects and the interaction among these two loci accounted for most of the genotypic variation: 88% of NV_LP, 77% of V_LP and 91% of the vernalization effect VER.

All possible combinations among *Vrn-H1* and *Vrn-H2* alleles were present, although the number of lines in the different categories was unbalanced (Table 5). Vernalization requirement was maximum in genotypes carrying the repressor allele at *Vrn-H2* (1400 bp) and the 5150 bp allele in *Vrn-H1*, under long photoperiod. This allelic combination is present in the *winter* cultivars (Table 1). When the repressor *Vrn-H2* was present, the response to vernalization decreased as the size of the intron in *Vrn-H1* diminishes (1200, 1190 and 150bp), and was reduced to a minimum in genotypes lacking any amplification product. When *Vrn-H2* was absent, the effect of vernalization was rather similar on all *Vrn-H1* alleles (Table 5).

The 1200 bp allele comes from the Spanish *facultative* cultivars Albacete and Pané (both also have *Vrn-H2*). The 1190 bp allele was present only in the cultivar Orria, selected in Spain from a multicross line of CIMMYT materials. The 0 bp and 150 bp come from *spring* cultivars. Allelic combinations for each of the tested cultivars are shown in Supplementary Table 1.

Table 5. Interaction between markers at the vernalization genes. Number of lines and average leaf number for all allele combinations of the vernalization genes *Vrn-H1* and *Vrn-H2*. Means separation as in Table 4.

HvBM5	HvZCCT	NV_LP		V_LP		VER	
(*Vrn-H1*)	(*Vrn-H2*)	No.	Mean	No.	Mean	No.	Mean
allele size (bp)							
5150	1400	100	18.4 a	115	10.2 a	112	7.2 a
1200	1400	27	12.5 b	24	7.2 bc	22	4.6 b
1190	1400	15	9.8 cd	14	6.9 bcd	14	2.8 c
150	1400	4	11.0 bc	4	8.0 b	2	2.3 cde
0	1400	11	8.4 ef	15	6.6 cd	13	1.2 de
5150	0	22	9.0 de	23	6.8 bcd	19	1.5 d
1200	0	3	7.3 f	2	6.4 bcde	2	0.1 de
1190	0	4	8.4 ef	4	5.9 de	2	2.0 cde
150	0	24	8.0 f	23	6.5 cd	23	0.5 e
0	0	45	7.5 f	45	5.7 e	43	0.8 de

Photoperiod response QTL. Markers aMWG518 and Bmag382 (bin 10-11) showed a significant effect on heading date in both autumn-sown experiments. aMWG518 also showed a significant effect in the V_LP treatment (Table 4, Figs. 3 and 4). The allele that caused delay in heading time is usually present in the winter cultivars (coded as 2, Table 4).

HvT SNP22, on the short arm of chromosome 2H, was the most important marker for heading date variation in winter-sown experiments. This marker showed an important effect also in the greenhouse trials (Table 4, Figs. 3 and 4). *Spring* cultivars usually carried the photoperiod insensitive allele (coded as 1, Table 4).

Earliness per se QTL. The centromeric region of chromosome 2H showed a significant effect in all field and greenhouse trials. Several markers were above the threshold in this region but, in all cases, the peak was located on the SSR marker Bmac132. Lines that presented the 189 bp allele flowered significantly later with respect to the other two alleles (Table 4).

Another remarkable QTL was found in the centromeric region of chromosome 6H, with a peak on the SSR marker Bmag173. This marker was significant in all the field trials. Bmag173 presents 5 alleles in this study. Clearly, the band of 148 bp (from parents Beka and Orria) was associated to earlier heading. The other 4 bands also presented some differences. Although means separations were not clear cut, plants carrying bands of 158 and 152 bp were, on average, between 0.8 to 1.9 days later than those with bands of 124 and 150 bp, suggesting the possible presence of a total of three alleles for the linked QTL.

Other markers (Bmag120, WMC1E8, and MWG699) had a significant effect in at least two experiments. For Bmag120, there were at least two alleles at the linked QTL, represented by bands of 230, 236, 260 and 262 bp (always latest) versus the rest, though a third allele was suggested if this last group was split into band 232 (always intermediate) and bands 240 and 254 (always earliest). Lines carrying the A allele in MWG699, or 238 bp on WMC1E8 (Table 4) flowered later than lines carrying any other allele.

Several other QTL were detected in only one experiment, and they were usually classified as secondary according to the criterion described in the M&M section. The effects of these markers are not reported here.

Besides, when analyzing the effect of those markers on chromosome 6H that were used to characterize only *spring x spring* and *winter x winter* populations, only scssr05599 showed a significant effect, in the WINHU04 and NV_LP experiments and in the VER effect (data not shown).

4.5 Discussion

The aim of the study was to validate the effect of markers detected in biparental populations, so the estimates are reduced to the positions where the markers are located and no interpolation between markers was made. QTL detection may be hampered in this kind of study by the possible presence of homoplasy (bands of similar size but with different sequence). Different studies have revealed its presence and relevance at the intra- or inter-specific level (reviewed by Estoup et al. 2002).

Size homoplasy is a main concern when dealing with phylogenetic relationships among species (Estoup et al. 2002), but for studies involving mapping or gene discovery in biparental crosses, looking at the diversity of accessions within a species (as in this study), or among closely related species, variation at the electromorph level (bands distinguished by electrophoretic mobility) provides sufficient resolution to be both efficient and useful (Chen et al. 2002).

It is essential to get a good consensus linkage map of the markers, in order to obtain accurate results, and especially to determine which cofactors are located in the 10 cM vicinity window. The method used for the consensus map construction, with a maximum of only 20 individuals per population, was different to other methods used for this purpose, usually based on the construction of individual maps for each population (Karakousis et al. 2003; Wenzl et al. 2006). We can conclude that this is an appropriate method, since the outcome agrees well with other barley linkage maps based on single cross populations, and it uses all the information on recombination in the whole set of 281 lines. Another evidence of the accuracy of the map comes from the shape of the LRT score profiles around the QTL. For most cases, the peaks of significance showed a decreasing trend for tightly linked markers at both sides. An exception is the case of marker Hv2F4, in the *Ppd-H1* region (2H), which presented a remarkable reduction in significance between two much more significant flanking markers. The reason for this is not a misplacement of the marker (its position fully agree with other maps), but probably a lack of power in the test at this point caused by the unbalanced number of individuals present in each allele class, as most of the DH lines carried the same allele for marker Hv2F4.

All markers significantly associated to heading date or number of leaves were previously reported as allele-specific or tightly linked to flowering time determining genes. The most significant markers in this study were:

HvT SNP22 - Ppd-H1

Marker HvT SNP22, on the short arm of chromosome 2H, showed the largest effect on heading date in the winter-sown experiments (between 5 and 6 days of heading time difference between the two alleles, Table 4). This is the allele-specific marker for the long photoperiod response gene *Ppd-H1* (Turner et al. 2005). *Ppd-H1* is a pseudo-response regulator, a class of genes involved in circadian clock function and causes an increased expression of *HvFT* with photoperiods over 12 hours (Turner et al. 2005). *HvFT* is the barley orthologue to the key flowering promoters *FT* in Arabidopsis, and *Hd3a* in rice (Turner et al. 2005; Yan et al. 2006). In plants grown under 16 hours photoperiod, under similar photoperiod as for the greenhouse trials of this experiment, peak expression of *HvFT* occurred at the end of the light period in plants carrying the photoperiod response allele *Ppd-H1*, whereas reduced expression of *HvFT* remained constant over the light period for plants carrying the opposite *ppd-H1* allele (Turner et al, 2005). As the greenhouse treatments were carried out under constant long photoperiod, *Ppd-H1* showed a significant effect, also reported in other studies (Laurie et al. 1994, 1995).

aMWG518 - Ppd-H2

The RFLP and the STS-derived marker aMWG518, and its linked SSR marker Bmag382, on the long arm of chromosome 1H, showed a significant effect on autumn-sown field experiments (Table 4). Both are linked to the photoperiod response gene *Ppd-H2*, which causes differences on heading date under short photoperiod conditions (Pan et al. 1994; Laurie et al. 1995; Boyd et al. 2003; Francia et al. 2004). Lines with the allele 2 at aMWG518, or the 105 bp allele at Bmag382, had a later heading date. This QTL is a major determinant of heading date under Northern Spanish autumn sowing conditions, in which most of the vegetative phase elapses with photoperiods around 10-11 hours, decreasing in the first developmental stages (Cuesta-Marcos et. al., unpublished). However, a significant effect of these markers was also detected at the V_LP treatment, where plants were grown in the greenhouse under long photoperiods and we have found no previous report of an effect of *Ppd-H2* under long photoperiod.

Bmac132 - Eam6

The only significant marker in all field and greenhouse experiments was the SSR marker Bmac132, in bin 8 of chromosome 2H (Figs. 3 and 4, Table 4). This marker co-locates in bin 8 with *Eam6* (Franckowiak and Konishi, 2002), which confers early heading under both long and short photoperiod conditions (Horsley et al. 2006). In a similar position, Laurie et al. (1995) identified *eps2,* also with an effect independent of day length. Three Bmac132 alleles were detected in this study. In all experiments, the 189 bp allele showed a marked delay with respect to alleles 183 and 191 bp, which presented similar heading dates. Thus, we clearly found two alleles in the linked QTL in this germplasm pool. The *Eam6* region has also been identified as a major determinant of heading date in the population Beka x Logan (Moralejo et al. 2004; Cuesta-Marcos et al. unpublished) in autumn sowings under Northern Spanish conditions, and also under Australian Mediterranean conditions (Boyd et al. 2003). This study confirms the importance of the *Eam6* gene under Mediterranean conditions, with an overall effect between 3 and 4 days. The peak was consistently present on Bmac132, which has not been widely used thus far, and is not present in the barley genotyping set proposed by Macaulay et al. (2001). Other tightly linked markers, such as Bmac093 and EBmac640, have been used in other studies. Because of the importance of the linked QTL, we propose the use of Bmac132 as a diagnostic marker for *Eam6* in breeding programmes, until this gene is cloned.

Bmag173

The SSR marker Bmag173, in the centromeric region of chromosome 6H, was also significant in all field trials. It is located in the same region of the gene *eam7*, which confers earliness under short-photoperiod conditions (Franckowiak and Gallagher 1997; Stracke and Börner 1998). Although the studies in which this QTL was identified used mutants, in this set of DH lines there is, at least, a consistent effect associated with the allele of 148 bp, which is the earliest in all cases. Canci et al. (2004) described a QTL for heading date, in a similar position, in different mapping populations (Chevron/M69, Stander/MN93 or MS92-299/M81.

MWG699

The STS marker MWG699, on the long arm of chromosome 2H, is tightly linked to the *vrs1* gene, which controls the development and fertility of the lateral spikelets

(Komatsuda et al. 1999). This marker showed a significant effect in one of the winter-sown trials and in both autumn-sown ones. Kjaer et al. (1995) found a similar effect in a 2-row by 6-row population, but they did not have data to prove whether this effect was due to pleiotropy of the *vrs1* gene, or linkage with the QTL now known as *Eam6*. Some populations of this study were 2-row by 6-row crosses, which were the origin of the polymorphism at MWG699. Thus, population structure might influence heading date QTL detection in a set of populations including 2-row by 6-row crosses, as in our study. But, as the model we have used places cofactors at all other significant loci, any carry over effect from an association between MWG699 and *Vrn-H1* (HvBM5), or *Eam6* (Bmac132), has been likely removed, and the effect of MWG699 seems a true one.

The SSR Bmag125 was also significant in all field trials, but with the present QTL analysis method, we could not discern whether it has a direct effect on heading date *per se,* or because of its relative proximity to *Eam6* and *vrs1*.

Vernalization genes and their interaction

The system of the vernalization response genes *VRN2* and *VRN1,* and their epistatic interaction under long photoperiod conditions, is the main factor controlling heading date in temperate grasses (Yan et al. 2003, von Zitzewitz et al. 2005). The epistatic model of the interaction was suggested by Yan et al. (2004), for the *Triticeae* family and was validated with the corresponding barley genes *Vrn-H1* and *Vrn-H2* by von Zitzewitz et al. (2005). A central role for photoperiod in this model has also been proposed (Dubcovsky et al. 2006; Trevaskis et al. 2006). According to this last model, *Vrn-H2* encodes for a dominant flowering repressor that inhibits the expression of *Vrn-H1*, which is a central control point for the transition from vegetative to reproductive growth. Vernalization down-regulates the expression of *Vrn-H2,* allowing *Vrn-H1* expression in the winter cultivars (genotype *vrn-H1*). If the repressor is absent (genotype *vrn-H2*), there would not exist any vernalization requirement. This model has been validated in the populations Dicktoo x Kompolti korai (Karsai et al. 2005), Hardy x Jubilant (Kóti et al. 2006) and also in Dicktoo x Oregon Wolfe Barley Dominant, Dicktoo x Calicuchima-sib and Calicuchima-sib x Oregon Wolfe Barley Dominant (Szűcs et al. 2007). von Zitzewitz et al (2005) identified a 436 bp region in the first intron of *Vrn-H1*, critical for the expression of the vernalization requirement.

Lines with all combinations of several distinct alleles at *Vrn-H1*, combined with presence/absence of the *Vrn-H2* repressor were included in this study, though the number of lines in each class was unbalanced (Table 5).

We identified 5 different alleles in *Vrn-H1,* whose differences were based on different length of deletions in a region within the first intron. The 5150 bp allele, is usually considered as the *winter* allele (von Zitzewitz et al. 2005) and, in this study, presented the strongest vernalization response when the *Vrn-H2* repressor was present. The 1200 bp allele of Albacete and Pané has a large deletion in the first intron, but conserves the critical region (von Zitzewitz et al. 2005). Its effect on vernalization requirement, as long as the *Vrn-H2* repressor was present (1400 bp allele), was intermediate between the typical winter allele (5150), and the alleles carried by spring cultivars (0, 150), as shown in Table 5. These results confirm the findings by Szűcs et al. (2007), who found that the 436 bp critical region is necessary but not sufficient to allow the presence of full vernalization requirement. Therefore, there could be other regulatory regions in *Vrn-H1*. The vernalization effect of lines carrying the 1190 bp allele was similar to (Table 4) or intermediate between the 1200 and 150 bp alleles (Table 5), though data were not conclusive enough to declare whether it corresponds to a functionally distinct allele, or it is the same as the 1200 bp allele. Finally, 0 bp and 150 bp alleles, that correspond with large deletions including the critical region, showed the lowest vernalization requirements (either with or without the *Vrn-H2* repressor). Thus, an allelic series at *Vrn-H1* is suggested, with strength of VER effect related to size of the intron on *Vrn-H1*, when the repressor *Vrn-H2* was present, as in Szűcs et al. (2007).

We have determined that it is possible to carry out QTL detection in a complex germplasm set, representative of the materials used in an active breeding programme. QTL position and allelic effects were consistent with estimates found in the literature. In most cases, apparently the markers presented more diversity than linked QTL. Correspondence between marker and QTL alleles was straightforward when marker allele number was low. Most QTL detected had presumably 2 alleles, with a few exceptions including HvBM5 (*Vrn-H1*). The set of markers used in this study was previously selected to represent regions with heading date QTL. The coverage of the genome achieved, though not complete, was good enough to find QTL that explained a very large proportion of the phenotypic variance. These results support the use of this

kind of approach for the validation of QTL found in biparental studies, or to survey allelic diversity in plant breeding sets of materials.

Acknowledgemments

This work was supported by the Spanish Ministry of Education and Research (Projects AGL2001-2289, including a scholarship granted to Mr. Alfonso Cuesta-Marcos, and AGL2004-05311) and by the European Regional Development Fund. The authors appreciate the critical reading of the manuscript by Prof. Steve Ullrich.

4.6 Supplementary information

Supplementary Table 1. Parental genotypes at loci associated with flowering time in barley

Variety	HvT SNP22	aMWG518	Bmac132	HvBM5[(1)]	HvBM5 55/56	HvBM5 66/67	HvBM5 88/85	HvBM5 42/43	HvZCCT	HvVrT2
Albacete	G=2	C=2	191	1200	1200		650		1400	G=2
Alexis	T=1	A=1	189	0				1900	null	A=1
Angora	T=1	C=2	191	5150	5150	2000	520		1400	G=2
Barberousse	G=2	C=2	191	5150	5150	2000	520		1400	A=1
Beka	T=1	A=1	189	150	150				null	A=1
Clarine	G=2	C=2	191	5150	5150	2000	520		1400	A=1
Gaelic	T=1	C=2	183	150	150				1400	A=1
Monlon	G=2	C=2	191	5150	5150	2000	520		null	G=2
Nevada	T=1	A=1	189	150	150				null	A=1
Orria	T=1	C=2	183	1190	1190				1400	G=2
Pané	G=2	C=2	191	1200	1200		650		1400	G=2
Plaisant	G=2	C=2	191	5150	5150	2000	520		1400	G=2
Seira	T=1	A=1	189	0				1900	null	A=1
Tipper	T=1	A=1	189	5150	5150	2000	520		1400	A=1

[(1)] The genotype at the vernalization gene HvBM5 (Vrn-H1) is the combination of different amplifications covering the first intron of that gene. These are the codes for the HvBM5 alleles reported in the text.

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Capítulo 5

Validation of the VRN-H2/VRN-H1 epistatic model in barley reveals that intron length variation in VRN-H1 may account for a continuum of vernalization sensitivity

Capítulo 5: Validation of the *VRN-H2/VRN-H1* epistatic model in barley reveals that intron length variation in *VRN-H1* may account for a continuum of vernalization sensitivity

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Molecular Genetics and Genomics. Received: 11 August 2006 Accepted: 5 November 2006 Published online: 7 December 2006

5.1 Abstract

The epistatic interaction of alleles at the *VRN-H1* and *VRN-H2* loci determines vernalization sensitivity in barley. To validate the current molecular model for the two-locus epistasis, we crossed homozygous vernalization-insensitive plants harboring a predicted "winter type" allele at either *VRN-H1* (Dicktoo) or *VRN-H2* (Oregon Wolfe Barley Dominant), or at both *VRN-H* (Calicuchima-sib) loci and measured the flowering time of unvernalized F_2 progeny under long-day photoperiod. We assessed whether the spring growth habit of Calicuchima-sib is an exception to the two-locus epistatic model or contains novel "spring" alleles at *VRN-H1* (*HvBM5A*) and/or *VRN-H2* (*ZCCT-H*) by determining allele sequence variants at these loci and their effects relative to growth habit. We found that (a) progeny with predicted "winter type" alleles at both *VRN-H1* and *VRN-H2* alleles exhibited an extremely delayed flowering (i.e. vernalization-sensitive) phenotype in two out of the three F_2 populations, (b) sequence flanking the vernalization critical region of *HvBM5A* intron 1 likely influences degree of vernalization sensitivity, (c) a winter habit is retained when *ZCCT-Ha* has been deleted, and (d) the *ZCCT-H* genes have higher levels of allelic polymorphism than other winterhardiness regulatory genes. Our results validate the model explaining the epistatic interaction of *VRN-H2* and *VRN-H1* under long-day conditions, demonstrate recovery of vernalization-sensitive progeny from crosses of vernalization-insensitive genotypes, show that intron length variation in *VRN-H1* may account for a continuum of vernalization sensitivity, and provide molecular markers that are accurate predictors of "winter vs spring type" alleles at the *VRN-H* loci.

Keywords: *BM5, Cereal, Hordeum vulgare, HvVRT-2, Triticeae, VRN1, VRN-1, VRN2, VRN-2*

5.2 Introduction

The capacity of temperate grasses to survive the winter (i.e. winterhardiness) is associated with an extended exposure to low temperature (i.e. vernalization) in order to properly regulate the transition from vegetative to reproductive growth. Understanding the genetics of vernalization is a prerequisite to improving winterhardiness, and improved winterhardiness will allow for more sustainable and productive cereal crop production.

Based on winterhardiness traits, the growth habits of barley (*Hordeum vulgare* subsp. *vulgare*)—the second most important temperate cereal crop in the world—are classified as winter, facultative, or spring. The term "vernalization requirement" is misleading, as even winter growth habit barley genotypes do not "require" vernalization per se as they eventually will flower under continual warm temperatures and favorable growth conditions. Flowering of winter habit genotypes, while so delayed in the absence of vernalization as to be agronomically unacceptable, is significantly accelerated by vernalization. We utilize the terms "vernalization sensitivity" to indicate the delay in flowering time attributable to a lack of vernalization under long-day conditions and "vernalization response" to indicate the difference in flowering time between unvernalized and vernalized growth conditions.

The genetic basis of vernalization sensitivity in barley was first described as a three-locus epistatic model based on phenotypic data (Takahashi and Yasuda 1971). Using current nomenclature and chromosome designations, the loci are *VRN-H1* (5H), *VRN-H2* (4H) and *VRN-H3* (1H). Winter genotypes have the allelic architecture *Vrn-H2_*/*vrn-H1vrn-H1*/*vrn-H3vrn-H3* and all other allelic configurations lead to a lack of significant vernalization response (i.e. spring or facultative growth habits). There is no allelic variation at *VRN-H3* in most cultivated barley genotypes, reducing the genetic model to a two-locus epistatic model.

HvBM5A, a MADS-box floral meristem identity gene and member of the AP1 transcription factor family (Schmitz et al. 2000), is considered the determinant of the VRN-H1 locus. For nomenclatural simplicity, we use the *VRN-H1* designation to refer to both the locus and its effecter gene. The expression of *VRN-H1* and its wheat orthologs correlates with vernalization response, where increased expression promotes flowering (Danyluk et al. 2003; Dubcovsky et al. 2006; Loukoianov et al. 2005; Murai et al. 2003; Trevaskis et al. 2003, 2006; von Zitzewitz et al. 2005; Yan et al. 2003). In

wheat and barley, allelic variation at *VRN-1* is associated with mutations in the promoter and/or first intron. While promoter mutations in a CArG-like motif were initially reported to account for differences in growth habit of wheat (Yan et al. 2003), this CArG-like motif was found to be invariant in barley genotypes differing in growth habit (von Zitzewitz et al. 2005). Subsequently, *T. monococcum* spring habit genotypes were identified that lack CArG motif mutations like barley (Yan et al. 2004a). Large (□2.8 kb) deletions within the first intron in *VRN-1* were identified as more likely determinants of spring growth habit in both barley and wheat and the proposed key regulatory region has been narrowed down to a highly conserved 0.44 kb "vernalization critical region" (Fu et al. 2005; von Zitzewitz et al. 2005).

A zinc finger-CCT (*CONSTANS*, *CONSTANS*-like, and *TOC*) domain transcription factor (*ZCCT*), encoding a flowering repressor down-regulated by vernalization, is the determinant of the wheat and barley *VRN-2* locus (Yan et al. 2004b). Allelic variation at this locus is ascribed to loss-of-function mutations or complete deletion, leading to recessive inheritance of spring growth habit (Dubcovsky et al. 2005; Karsai et al. 2005; von Zitzewitz et al. 2005; Yan et al. 2004b). There are three tightly linked *ZCCT* genes in barley (*ZCCT-Ha*, *ZCCT-Hb* and *ZCCT-Hc*) and two in diploid wheat (*ZCCT-1* and *ZCCT-2*) at this locus (Dubcovsky et al. 2005; Karsai et al. 2005; Yan et al. 2004b). *ZCCT-1* and *ZCCT-Ha* in wheat and barley, respectively, have been reported to be the most likely candidates for *VRN-2* (Dubcovsky et al. 2005; Yan et al. 2004b).

The two-locus epistatic *VRN* gene interaction has been supported by extensive phenotypic data in wheat and barley populations derived from crosses of vernalization-sensitive (i.e. winter) by vernalization-insensitive (i.e. facultative or spring) growth habits (Dubcovsky et al. 1998, 2005, 2006; Fu et al. 2005; Karsai et al. 2005; Kóti et al. 2006; Laurie et al. 1995; Takahashi and Yasuda 1971; Tranquilli and Dubcovsky 2000; Yan et al. 2003, 2004a, b). Yan et al. (2003, 2004a, b) proposed a molecular model to explain the *VRN-2/VRN-1* epistatic interaction in cereals where *Vrn-2* encodes a dominant repressor—down-regulated by vernalization—that inhibits the expression of *vrn-1* alleles. In this model, no vernalization sensitivity is predicted in genotypes with recessive *vrn-2* alleles (loss-of-function mutations or complete deletions of *ZCCT*), regardless of allelic state at *VRN-1*. Likewise, genotypes with both dominant *Vrn-2* and *Vrn-1* alleles (lacking a repressor binding site in the promoter and/or in the intron 1) are also predicted to show minimal vernalization sensitivity.

There is evidence that the photoperiod and vernalization pathways of flowering are interconnected. High levels of barley and wheat *ZCCT* expression were detected only when photoperiod-responsive plants were grown under long-day photoperiod (Dubcovsky et al. 2006; Trevaskis et al. 2006), providing a molecular basis for explaining quantitative trait locus (QTL) associations between *VRN-H2* and photoperiod (Karsai et al. 2005, 2006; Laurie et al. 1995; Szűcs et al. 2006). *ZCCT* expression is repressed in photoperiod-responsive wheat and barley plants grown under short-day conditions (at both cold and warm temperatures), yet no increase in *VRN-1* expression is observed for several weeks, suggesting the existence of at least one other *VRN-1* repressor (Dubcovsky et al. 2006; Trevaskis et al. 2006). A candidate gene is *VRT-2*, a putative flowering repressor MADS-box gene regulated by both vernalization and day-length that shows higher expression under short-day vs long-day in a photoperiod-sensitive barley (Kane et al. 2005). *HvVRT-2*, on the short arm of chromosome 7H, is associated with photoperiod sensitivity QTL in barley (Szűcs et al. 2006).

Our objective in this study was to genetically validate the *VRN-H2*/*VRN-H1* epistatic model under long-day photoperiod, using genotypic and phenotypic data from three F_2 populations. We examined allelic variation at the dominant *Vrn-H2* and *Vrn-H1* loci to define the role, or roles, of the duplicated *ZCCT-H* genes and the number and structure of regulatory sites in *VRN-H1* and how these relate to the timing of flowering and sensitivity to vernalization.

5.3 Materials and Methods

Plant materials and phenotype evaluation

Dicktoo (Dt) (Hayes et al. 1993), the Oregon Wolfe Barley Dominant genetic stock (hereafter referred to as OWB-D or Od) (Wolfe and Franckowiak 1991), and Calicuchima-sib (Cb) (Hayes et al. 2000) are vernalization-insensitive barley genotypes. Dicktoo has a winter (i.e. recessive) *vrn-H1* allele and a spring (i.e. recessive) *vrn-H2* allele due to deletion of all three *ZCCT-H* genes (Fu et al. 2005; Karsai et al. 2005; von Zitzewitz et al. 2005)—this allele configuration (*vrn-H2vrn-H2*/*vrn-H1vrn-H1*) defines the facultative growth habit. OWB-D has a spring (i.e. dominant) *Vrn-H1* allele due to a large deletion of the intron 1 vernalization critical region (Fu et al. 2005) and a winter (i.e. dominant) *Vrn-H2* allele containing all three *ZCCT-H* genes (see Results). The

spring growth habit Calicuchima-sib, while containing the *ZCCT-H* genes and *VRN-H1* intron 1 vernalization critical region, is deleted for sequence flanking the critical region (see Results).

The three genotypes were crossed to produce three segregating populations (Dicktoo × OWB-D (Dt × Od), Dicktoo × Calicuchima-sib (Dt × Cb), and Calicuchima-sib × OWB-D (Cb × Od)) and test for vernalization sensitivity and determine whether the Calicuchima-sib spring habit is an exception to the two-locus epistatic model or contains novel "spring" alleles at *VRN-H1* and/or *VRN-H2*. F_1 plants derived from each of the three parental crosses were self-fertilized to produce three F_2 populations of 93 plants each. For comparison of flowering time and/or allelic variation at the *VRN-H* loci, three accessions of known winter growth habit were used: Kompolti korai—described by Karsai et al. (2001), Hoody—released in 1994 by the Oregon Agricultural Experiment Station, and the *Hordeum vulgare* subsp. *spontaneum* accession Caesarea 26–24 (also known as OSU11)—described by Karsai et al. (2004).

Vernalization sensitivity was measured based on the method of Takahashi and Yasuda (1971). All plants were grown under greenhouse conditions with supplemental light on a 16 h light/24 h photoperiod and a constant 18 ± 1.5°C day and night temperature, without any vernalization. The number of days from seedling emergence to flowering—Zadock's scale developmental phase 9 and 49, respectively (Tottman and Makepeace 1979)—were scored for each plant. The experiment was terminated 170 days after planting, and plants that had not flowered were assigned a days to flowering value of 170.

Allele sequencing and genotyping

The following primer set was designed to clone and sequence full-length *ZCCT-Ha* and ZCCT-Hb genes: ZCCTH.12F (5′-tcaaatattctagcagtggccttg-3′) to a conserved segment of the 5′ UTRs of published barley partial *ZCCT-H* gene sequences AY485977 and AY485978 and ZCCT2.08R (5′-tggcggtcgtgatggtttactc-3′) to a conserved 3′ UTR region of the *Triticum monococcum* full-length *ZCCT-1* and *ZCCT-2* gene sequences (AY485644). Primers ZCCT.HcF (5′-caccatcgcatgatgcac-3′) and ZCCT.HcR (5′-tcatatggcgaagctggag-3′) were used to amplify a 194 bp long fragment of the *ZCCT-Hc* gene (AY687931). Previously reported primer pairs HvBM5.27F (5′-aggcctattcgtttgcaatgc-3′); HvBM5.06R (5′-atctcgtgcgccttcttgag-3′), and HvBM5.55F (5′-atgcatagaataattggctccagc-3′); HvBM5.56R (5′-cagtaagcactacgatgatgataaac-3′) were

used to clone the promoter region and partial intron 1 of *VRN-H1*, respectively (von Zitzewitz et al. *2005*). For each allele isolated via PCR, cloned amplicons from at least two independent PCRs were sequenced to confirm PCR-based nucleotide substitutions were not present. Sequences of the non-primer portion of each consensus amplicon were deposited with GenBank and accession numbers are given in Table 1.

Table 1 Accession numbers for *ZCCT-H* and *VRN-H1* (*HvBM5A*) sequences

Gene	Allele	Accession	Determined region	Size (bp)
ZCCT-Ha	Calicuchima-sib	DQ492695	Full gene	2,361
ZCCT-Hb	Calicuchima-sib	DQ492696	Full gene	2,353
ZCCT-Ha	OWB-D	DQ492697	Full gene	2,356
ZCCT-Hb	OWB-D	DQ492698	Full gene	2,357
ZCCT-Ha	Kompolti korai	DQ492699	Full gene	2,365
ZCCT-Hb	Kompolti korai	DQ492700	Full gene	2,336
ZCCT-Hb	Caesarea 26-24/OSU11	DQ492701	Full gene	2,357
VRN-H1 (*HvBM5A*)	Calicuchima-sib	DQ492702	Promoter	2,192
VRN-H1 (*HvBM5A*)	OWB-D	DQ492703	Promoter	2,174
VRN-H1 (*HvBM5A*)	Dicktoo	AY785817	Promoter	2,207
VRN-H1 (*HvBM5A*)	Kompolti korai	AY785824	Promoter	2,187
VRN-H1 (*HvBM5A*)	Caesarea 26-24/OSU11	AY785820	Promoter	2,210
VRN-H1 (*HvBM5A*)	Calicuchima-sib	DQ492704	Partial intron 1	1,177
VRN-H1 (*HvBM5A*)	OWB-D	AY750996	Full intron 1	4,364
VRN-H1 (*HvBM5A*)	Dicktoo	AY750994	Full intron 1	10,789
VRN-H1 (*HvBM5A*)	Kompolti korai	AY866487	Partial intron 1	2,625
VRN-H1 (*HvBM5A*)	Caesarea 26-24/OSU11	AY866492	Partial intron 1	2,588

Table 2 shows the gene-specific primers used to assign allele types in individuals of the F_2 segregating populations. The *VRN-H1* locus was genotyped using a co-dominant promoter SSR marker and intron 1-specific dominant markers. The intron 1 primer pairs were designed to be diagnostic for the allele type at the vernalization critical region (see Fig. 5). To develop co-dominant markers for the intron 1 region we combined dominant markers in a single PCR. Triplexing primers HvBM5.68F, HvBM5.87F, and HvBM5.86R distinguished the two parental types and identified heterozygotes for the Cb × Od population. Triplexing primers HvBM5.87F, HvBM5.86R, and HvBM5.89R for Dt × Od, and HvBM5.60F, HvBM5.56R, and HvBM5.89R for Dt × Cb amplified only the shorter parental fragment in the case of heterozygotes. To genotype the *VRN-H2* locus, we used both *ZCCT-H*-specific (dominant) and tightly linked *HvSNF2*-specific (co-dominant) markers (Karsai et al.

2005). The *HvVRT-2* gene was genotyped with a co-dominant CAPS marker (Szűcs et al. *2006*). All genotyping PCRs were replicated at least twice to validate allele scores.

Table 2 Primers used for genotyping *VRN-H1* (*HvBM5A*), *ZCCT-H*, *HvSNF 2*, and *HvVRT-2* in F_2 populations

Gene/region	Primer F (5′→3′)	Primer R (5′→3′)	Amplified allele[a]	F_2 population[b]
VRN-H1 promoter	HvBM5.82 (atatctactccagcctagggtac)	HvBM5.83 (cgcgaatctcccccatattgc)	Cb 173, Dt 189, Od 155	Dt × Od Dt × Cb Cb × Od
VRN-H1 intron 1	HvBM5.87 (gaaaggacgtgtatgttgaggtg)	HvBM5.89 (gtctgagtcggttatatgcagg)	Dt 749	Dt × Od
VRN-H1 intron 1	HvBM5.87 (gaaaggacgtgtatgttgaggtg)	HvBM5.86 (tcccccattctcgtcaaaaagc)	Od 531	Dt × Od Cb × Od
VRN-H1 intron 1	HvBM5.60 (gctttattttcttctctgccgttcc)	HvBM5.89 (gtctgagtcggttatatgcagg)	Dt 305	Dt × Cb
VRN-H1 intron 1	HvBM5.60 (gctttattttcttctctgccgttcc)	HvBM5.56 (cagtaagcactacgatgatgataaac)	Cb 369	Dt × Cb
VRN-H1 intron 1	HvBM5.68 (gtgaggggagctgcaatggtg)	HvBM5.86 (tcccccattctcgtcaaaaagc)	Cb 642	Cb × Od
ZCCT-Ha/b	ZCCTH.06 (cctagttaaaacatatatccatagagc)	ZCCTH.07 (gatcgttgcgttgctaatagtg)	Od and Cb 307/273	Dt × Od Dt × Cb
HvSNF2	HvSNF2.02 (cctggccacaaaaacaatcagc)	HvSNF2.04 (gctgcattatagagaaacaacaacg)	Cb 214, Dt 382, Od 270	Dt × Od Dt × Cb Cb × Od
HvVRT-2	*HvVRT-2*.01 (gagttgcagcagatgg)	*HvVRT-2*.06 (caggtcactaatttgttgcatga)	Cb, Dt and Od 204 (*MspI*)	Dt × Cb Cb × Od

[a] *Cb* Calicuchima-sib, *Dt* Dicktoo, *Od* OWB-D; fragment size (bp) and utilized restriction endonuclease indicated
[b] *Dt* × *Od* Dicktoo × OWB-D; *Dt* × *Cb* Dicktoo × Calicuchima-sib, *Cb* × *Od* Calicuchima-sib × OWB-D

Statistical analyses

Goodness of fit test (χ^2) was used to compare observed and predicted allele segregation patterns at the different loci. Analysis of variance was performed using the general linear model (GLM) procedure of The SAS System for Windows, Release 8.2 (SAS Institute 2001). Sequence analyses were conducted using GeneDoc version 2.6 (Karl and Hugh 1997) and MEGA version 3.1 (Kumar et al. 2004).

5.4 Results

Vernalization-sensitive progeny are recovered from vernalization-insensitive parental crosses

Based on the *VRN-H2*/*VRN-H1* epistatic model, we expected that F_2 progeny with *Vrn-H2_*/*vrn-H1vrn-H1* alleles derived from crosses between vernalization-insensitive (i.e. early flowering) genotypes harboring *Vrn-H2Vrn-H2*/*Vrn-H1Vrn-H1* and *vrn-H2vrn-H2*/*vrn-H1vrn-H1* alleles should exhibit a vernalization-sensitive (i.e. late flowering) phenotype. Unvernalized winter habit controls (Hoody and Kompolti korai) flowered significantly later than facultative (Dicktoo) and spring (Calicuchima-sib and OWB-D) parents under long-day photoperiod (Table 3). There was also variation for flowering time among the parents, and on average, Calicuchima-sib flowered six days later than Dicktoo and 39 days later than OWB-D. The F_1 of each of the three crosses flowered significantly earlier than any of the winter habit controls and flowering times were between those of the respective parents.

Table 3 Days to flowering, growth habit, and *VRN-H* allele combination for control, parental, and F_1 genotypes

Genotype	Days to flowering[a]	Growth habit	*VRN-H2*/*VRN-H1* [b]	Germplasm
Hoody	114.5 ± 6.4	Winter	*V2V2*/*v1v1*	Control
Kompolti korai	110.7 ± 6.1	Winter	*V2V2*/*v1v1*	Control
Dicktoo	65.6 ± 10.3	Facultative	*v2v2*/*v1v1*	Parent
OWB-D	33.2 ± 2.1	Spring	*V2V2*/*V1V1*	Parent
Calicuchima-sib	72.0 ± 2.7	Spring	*V2V2*/*V1V1*	Parent
Dicktoo × OWB-D	53.7 ± 2.1	Spring	*V2v2*/*V1v1*	F_1
Dicktoo × Calicuchima-sib	66.5 ± 3.7	Spring	*V2v2*/*V1v1*	F_1
Calicuchima-sib × OWB-D	39.5 ± 2.6	Spring	*V2V2*/*V1V1*	F_1

Plants were grown under long-day conditions without vernalization
[a] Standard deviation is shown
[b] *V2*: *Vrn-H2*, *v2*: *vrn-H2*, *V1*: *Vrn-H1*, *v1*: *vrn-H1*

Transgressive segregation for flowering time was observed in the Dt × Od and Dt × Cb, but not in the Cb × Od, F_2 populations (Fig. 1). Seventy two Dt × Od plants flowered in 29–64 days, within the range of the vernalization-insensitive OWB-D and Dicktoo parents. Twenty one late flowering transgressive segregants flowered in 105–148 days, values which are as high as or higher than the flowering time of the winter controls, indicating a vernalization-sensitive phenotype. Likewise, in the Dt × Cb F_2 population,

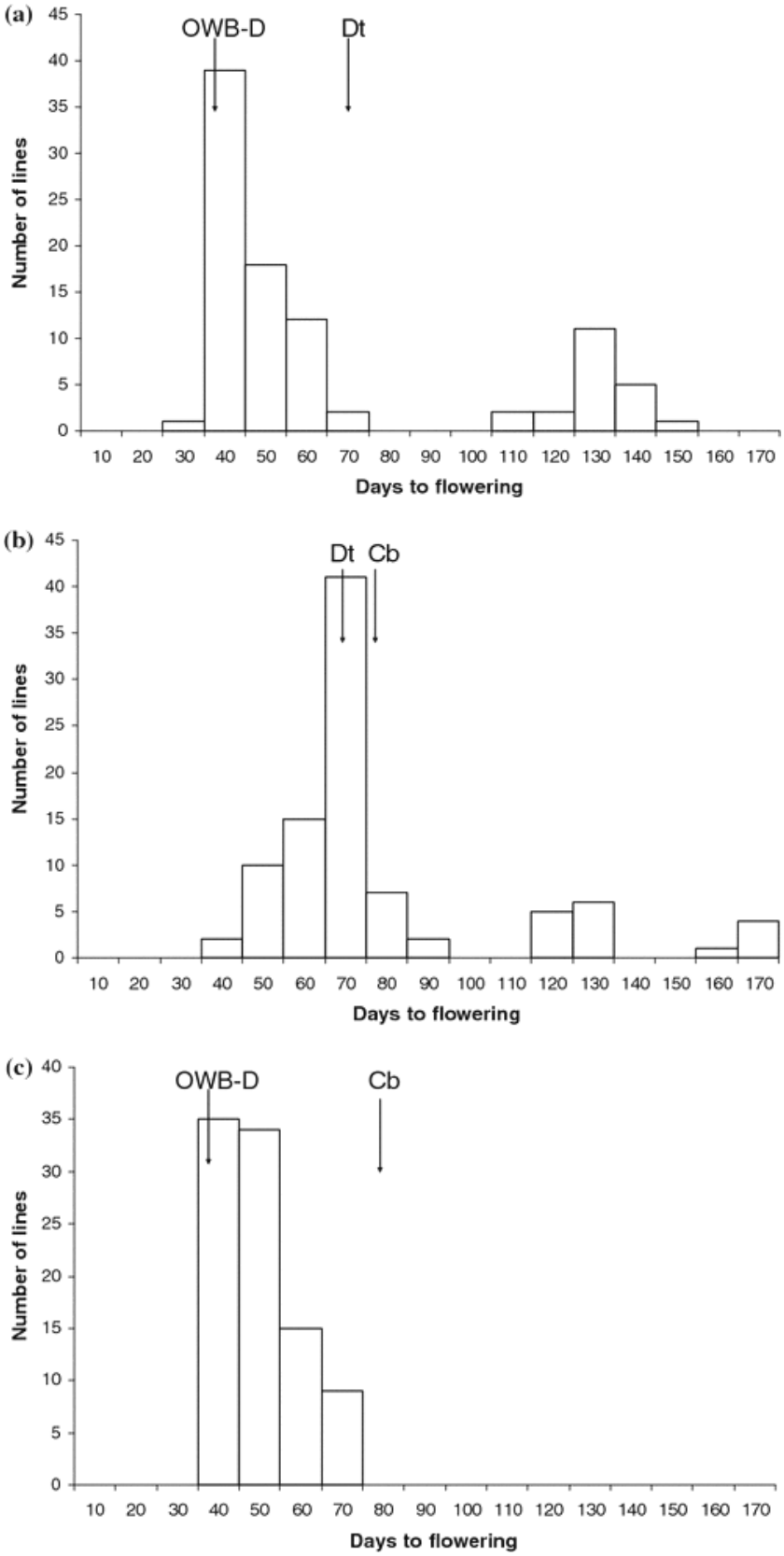

Fig. 1 Frequency distribution of days to flowering in **a** Dicktoo × OWB-D, **b** Dicktoo × Calicuchima-sib, and **c** Calicuchima-sib × OWB-D F_2 populations under long-day photoperiod without vernalization. Plants that had not flowered were assigned a days to flowering value of 170. Days to flowering values of Calicuchima-sib (*Cb*), Dicktoo (*Dt*), and *OWB-D* parents are indicated by *arrows* .

there were early and late transgressive segregants. Seventy seven plants flowered in 36–89 days, most of them as early, or earlier, than the parents. Twelve plants flowered in 113–153 days and four plants died after 158–169 days without flowering. The 93 Cb × Od F_2 plants all flowered after 31–68 days, values within the range of the parents.

Segregation of *VRN-H1*, *VRN-H2*, and *HvVRT2* loci

We determined the allelic architecture at the two *VRN-H* and the *HvVRT-2* loci for the three parental genotypes—sequence accessions are shown in Table 1. All three *ZCCT-H* genes are deleted in Dicktoo and present in Calicuchima-sib and OWB-D. The three parents have different allele sizes at the *ZCCT-H* proximal *HvSNF2* gene (Table 2), which was useful in corroborating the dominant marker data for *VRN-H2*. The three parents differed at *VRN-H1* in the length of a promoter TA-repeat, and the intron 1 vernalization critical region (Fig. 5). We determined □1 kb of *HvVRT-2* genomic sequence: Dicktoo and OWB-D were 100% identical, but differed from Calicuchima-sib in six single nucleotide polymorphisms (SNPs) (data not shown).

Ninety-three F_2 plants from each of the three populations were genotyped for the *VRN-H2* and *VRN-H1* loci using allele-specific primer sets (Table 2). At the *VRN-H2* locus, the co-dominant *HvSNF2* marker confirmed the results obtained with the dominant *ZCCT-H* marker with one exception, indicating a recombination event occurred between these two tightly linked genes. One intragenic recombinant between the *VRN-H1* promoter SSR and the intron 1 vernalization critical region was observed in the Dt × Od population. Due to lack of polymorphism between the Dicktoo and OWB-D *HvVRT-2* alleles, the segregation of alleles at this locus was determined only for the Dt × Cb and Cb × Od populations. There was an excellent fit of observed to expected segregation ratios for alleles at the *VRN-H1*, *VRN-H2*, and *HvVRT2* loci in all tested F_2 populations ($P = 0.08–0.86$ for the χ^2 tests).

***VRN-H* loci are major determinants of flowering time under long-day photoperiod**

Based on the genotype data, *VRN-H1*, *VRN-H2*, and *HvVRT-2* alleles were assigned to each F_2 plant and an analysis of variance was performed using the days to flowering data as an indicator of vernalization sensitivity (Table 4). This analysis revealed that the allele type at *VRN-H1* significantly ($P < 0.0001$) influenced days to flowering in all three populations. The effects of alleles at the *VRN-H2* locus, and the

interaction between the *VRN-H1* and *VRN-H2* loci, were also significant ($P < 0.0001$) for the Dt × Od and Dt × Cb populations. The two *VRN-H* loci, and their interaction, accounted for 97% and 91% of the phenotypic variance in flowering time in the Dt × Od and Dt × Cb populations, respectively. Both Calicuchima-sib and OWB-D have dominant *Vrn-H1* and *Vrn-H2* loci, and as expected, the analysis of variance revealed no significant effect of *Vrn-H2* for the Cb × Od populations. Interestingly, *Vrn-H1* significantly influenced the flowering time—the mean numbers of days to flowering were 40 and 44 for plants harboring a homozygous vs heterozygous OWB-D allele, respectively, and 58 days for those having a homozygous Calicuchima-sib allele. Allelic variation at the *HvVRT-2* locus and its two-way or three-way interactions with the *VRN-H* loci had no significant effect on flowering time under long-day growth conditions (Table 4).

Table 4 Effects of *VRN-H1*, *VRN-H2*, *HvVRT-2*, and their interactions, on days to flowering in F_2 populations

Source of variation[a]	**Degrees of freedom**			**Type III mean square**[b]		
	Dt × Od	**Dt × Cb**	**Cb × Od**	**Dt × Od**	**Dt × Cb**	**Cb × Od**
VRN-H1	2	2	2	23,403****	20,817****	935****
VRN-H2	2	2	2	8,787****	6,235****	21
VRN-H1 × VRN-H2	4	4	4	4,923****	2,702****	68
HvVRT-2	–	2	2	–	85	3
VRN-H1 × HvVRT-2	–	4	4	–	20	22
VRN-H2 × HvVRT-2	–	4	4	–	107	14
VRN-H1 × VRN-H2 × HvVRT-2	–	6	3	–	130	29
Error	84	68	71	49	94	49

Plants were grown under long-day conditions without vernalization; Dt × Od, Dt × Cb, Cb × Od: see Table 2
[a] Due to lack of a polymorphism, *HvVRT-2* was not genotyped in the Dt × Od population
[b] Significance at the 5, 1, 0.1 and 0.01% levels are indicated by *, **, *** and ****, respectively

The observed plant numbers in the nine allele classes at the two *VRN-H* loci showed excellent fit to expected ratios: $P = 0.54$ and $P = 0.60$ for the χ^2 tests of the Dt × Od and Dt × Cb populations, respectively. The mean number of days to flowering in each genotype class was higher for the Dt × Cb population than for the Dt × Od population, and in both populations, there were significant differences in flowering time between certain allele classes (Fig. 2). All plants with winter alleles at both *VRN-H* loci (*Vrn-H2_*/*vrn-H1vrn-H1*) flowered significantly later than plants in any other allele class. Similarly, all late transgressive segregant plants had the winter allele combinations at the two *VRN-H* loci. There were also some significant differences between the seven early flowering allele classes. For example, *Vrn-H2Vrn-H2*/*Vrn-*

H1vrn-H1 plants flowered significantly later than any plant with homozygous dominant *Vrn-H1* alleles in the Dt × Od population, while *vrn-H2vrn-H2*/*Vrn-H1Vrn-H1* plants flowered significantly earlier than any other F_2 class in the Dt × Cb population (Fig. 2).

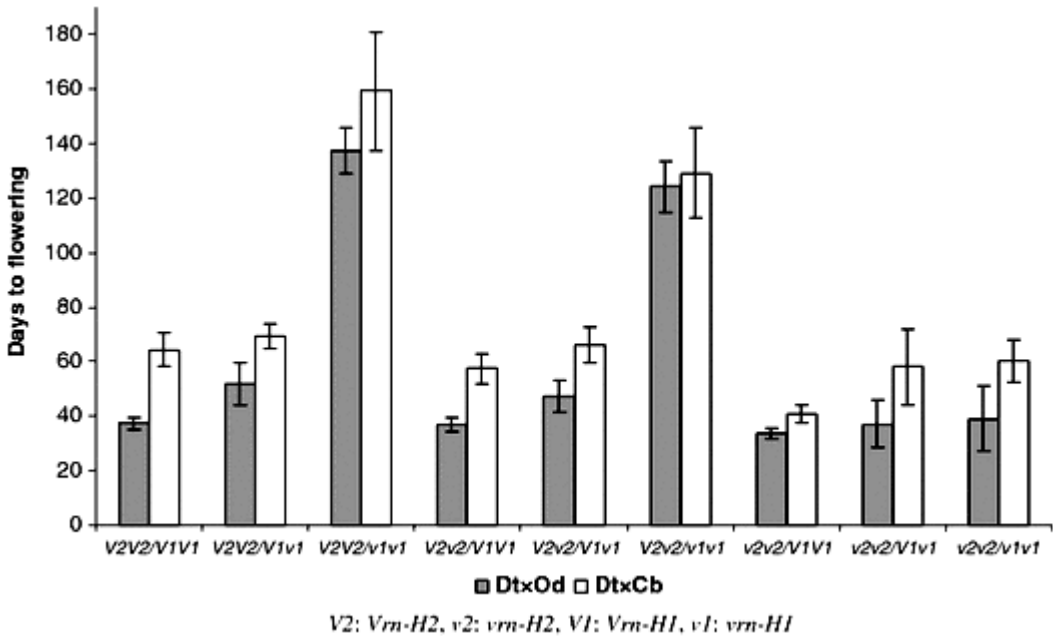

Fig. 2 Mean numbers of days to flowering of the nine different *VRN-H2/VRN-H1* allele classes in the F_2 progeny of the Dicktoo × OWB-D (Dt × Od), and Dicktoo × Calicuchima-sib (Dt × Cb) crosses under long-day photoperiod without vernalization. Plants that had not flowered were assigned a days to flowering value of 170. *Error bars* show standard deviation

Allelic variation at the *VRN-H* loci

Winter habit controls Hoody and Kompolti korai contain all three *ZCCT-H* genes, while Caesarea 26–24 has only *ZCCT-Hb* and *ZCCT-Hc*. Multiple primer pairs specific to various regions of *ZCCT-Ha* failed to amplify a product from Caesarea 26–24, suggesting this gene is absent in this winter habit wild barley accession (data not shown). To investigate the allelic variation at the dominant *Vrn-H2* locus found in different spring and winter habit genotypes, full-length *ZCCT-Ha* and *ZCCT-Hb* gene sequences were determined for Calicuchima-sib, OWB-D, and Kompolti korai, and *ZCCT-Hb* of Caesarea 26–24 (Table 1). Comparison of the three *ZCCT-Ha* alleles showed 35 SNPs and three small (3–6 bp) insertions/deletions (INDELs), while *ZCCT-Hb* alleles displayed even greater allelic diversity with 96 SNPs and eight INDELs (2–18 bp)—the polymorphisms occurred throughout all regions (UTRs, introns and exons) of the genes. All *ZCCT-H* alleles encode full-length polypeptides and the

polymorphisms lead to a number of amino acid substitutions and INDELs (Fig. 3). ZCCT-Ha and ZCCT-Hb are more similar to wheat ZCCT-2 than to ZCCT-1 based on a Neighbor-Joining tree of the full-length polypeptides (Fig. 4); analogous tree topologies were also obtained using the Minimum Evolution and UPGMA clustering methodologies (data not shown).

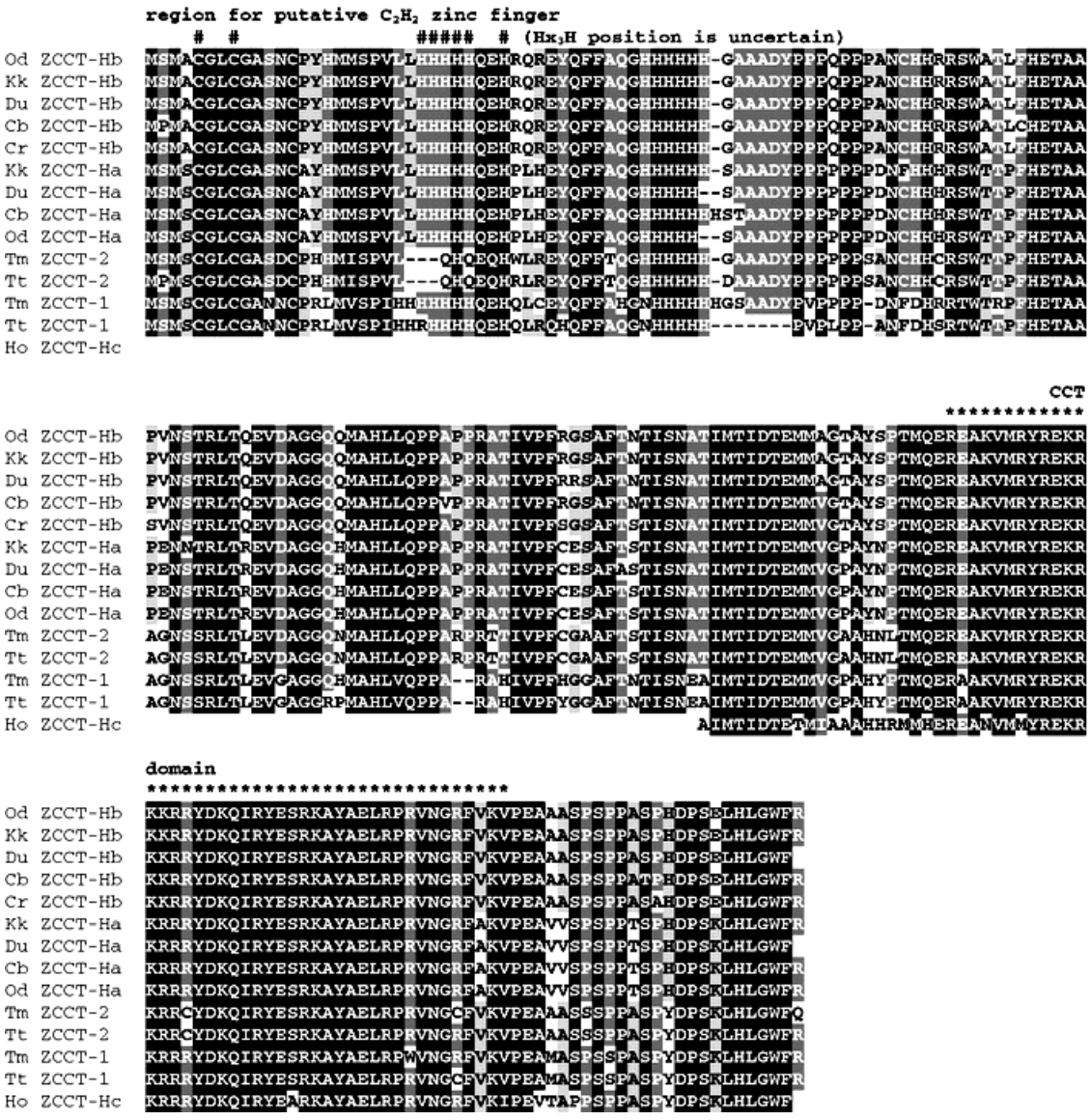

Fig. 3 Sequence alignment of barley and wheat ZCCT family members. Similarity is shown to four levels and regions of putative C_2H_2 zinc finger (#) and CCT domain (*) are indicated. *H. vulgare* sequences shown are from Caesarea 26–24 (Cr; DQ492701), Calicuchima-sib (Cb; DQ492695 and DQ492696), Dairokkaku (Du; AY485977 and AY485978), Hayakiso (Ho; AY687931), Kompolti korai (Kk; DQ492699 and DQ492700), and OWB-D (Od; DQ492697 and DQ492698). Representative wheat sequences shown are from *T. monococcum* (Tm; AY485644) and *T. turgidum* (Tt; AY485979 and AY485980)

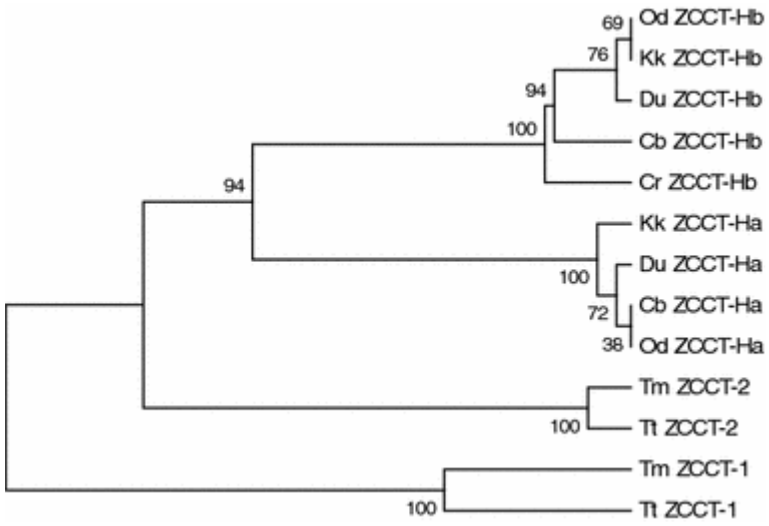

Fig. 4 Neighbor-Joining phylogenetic cluster analyses of barley and wheat ZCCT amino acid sequences. Confidence values on the branches are based on 1,000 bootstraps. See Fig. 3 legend for sequence accessions

We sequenced □2.2 kb of the *VRN-H1* promoter from Calicuchima-sib and OWB-D and aligned these sequences with the eleven previously reported accessions (including Dicktoo, Kompolti korai and Caesarea 26–24) from von Zitzewitz et al. (2005). Calicuchima-sib and OWB-D share the three polymorphisms common to all spring genotypes relative to winter and facultative genotypes, confirming the promoters are of spring habit origin. With the exception of a TA repeat length, the promoter region sequences of Calicuchima-sib and the spring genotypes Harrington and Morex are identical. OWB-D was found to contain a unique SNP (actcatttgg vs cctcatttgg) in the CArG box relative to all other barley genotypes reported to date—this CArG-box was identified as a determinant of spring growth habit in wheat (Yan et al. 2003).

We previously reported that the spring habit OWB-D genotype has a □6.4 kb deletion in the *VRN-H1* intron 1 compared to the intron 1 present in Caesarea 26–24, Dicktoo and Kompolti korai (Fu et al. 2005; von Zitzewitz et al. 2005). Cloning and sequencing a partial *VRN-H1* intron 1 fragment in the spring habit Calicuchima-sib revealed the 436 bp vernalization critical region was intact but the remainder of the 2.8 kb barley-wheat conserved region was deleted—the deletion begins 13 bp downstream from the 3′ end of the vernalization critical region (Fig. 5).

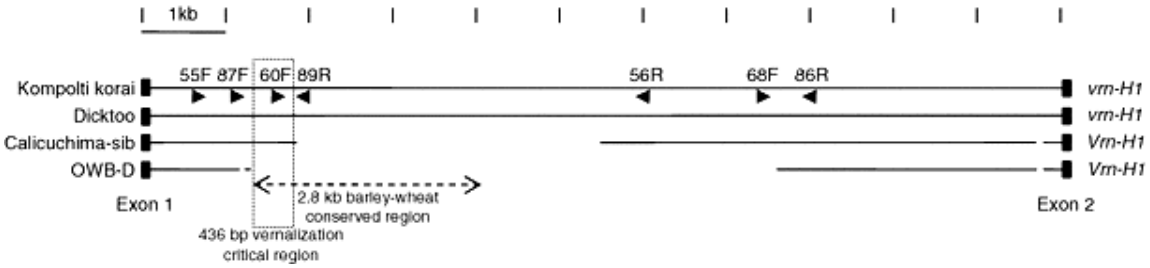

Fig. 5 Schematic representation of the *VRN-H1* intron 1 from four barley genotypes representing dominant and recessive alleles (denoted). Edges of exons 1 and 2 are indicated by *flanking black boxes*. *Gaps* represent deletions relative to the full-length Dicktoo allele (AY750994). The 2.8 kb barley-wheat conserved region (*dashed line*) and the 436 bp vernalization critical region (*dotted box*) are indicated. Positions and orientations of *HvBM5* primers used to characterize the different alleles (Table 2) are indicated by *black triangles*; primer numbers are denoted

5.5 Discussion

We tested and verified the two-locus epistatic model (Takahashi and Yasuda 1971; Yan et al. 2004b) by selecting vernalization-insensitive parents genotyped to harbor a predicted "winter type" allele at either *VRN-H1* or *VRN-H2*, and recovered early and late flowering progeny in the expected ratios in two F_2 populations. We used flowering time under long-day conditions in the absence of vernalization (Takahashi and Yasuda 1971) as a diagnostic indicator of vernalization sensitivity and growth habit. In an F_2 population, it is not possible to have vernalized and unvernalized treatments of the same genotype, since each plant is a unique individual. Proof of vernalization response requires comparison of flowering time in the unvernalized vs vernalized F_3 progeny of *Vrn-H2Vrn-H2*/*vrn-H1vrn-H1* F_2 homozygotes—this experiment is currently being initiated. It is also important to reiterate that these experiments were conducted under long-day photoperiod conditions as recent evidence suggests that the Yan et al. (2004b) molecular model is not applicable under short-day conditions (Dubcovsky et al. 2006; Trevaskis et al. 2006).

Our current results validated a novel dimension of the two-locus epistatic model that was not assessable in F_2 populations derived from crosses of homozygous winter (*Vrn-H2*/*vrn-H1*) by homozygous spring (*vrn-H2*/*Vrn-H1*) growth habit barley genotypes (Dubcovsky et al. 2005; Kóti et al. 2006; Takahashi and Yasuda 1971; Yan et al. 2004b). We show that genotypes inheriting *ZCCT-H* (*Vrn-H2_*) and two repressible *VRN-H1* copies (*vrn-H1vrn-H1*) from vernalization-insensitive backgrounds manifest

significant sensitivity to vernalization. Using *VRN-H*-specific markers our results also confirmed the observation of Reinheimer et al. (2004) where a double haploid population derived from a cross between homozygous vernalization-insensitive parents segregated for growth habit. The use of F_2 segregating progeny in the current study provided an opportunity to assess the degree of dominance at each of the loci and we observed *VRN-H* allele dosage effects. Based on the allele class means, *Vrn-H1Vrn-H1* plants flowered earlier than the *Vrn-H1vrn-H1* plants with the same *VRN-H2* allele configuration, showing incomplete dominance of the *Vrn-H1* allele. This is likely due to expression differences where the *vrn-H1* allele is still susceptible to repression and only the *Vrn-H1* allele is active. The partial dominance of alleles at *VRN-1* has been reported for both barley and wheat (Iwaki et al. 2001; Kóti et al. 2006; Loukoianov et al. 2005). The *Vrn-H2* allele also showed incomplete dominance: *Vrn-H2Vrn-H2* plants flowered later than the *Vrn-H2vrn-H2* plants (given a homozygous recessive or heterozygous allele configuration at *VRN-H1*), suggesting level of available repressor as the basis. Using three F_2 populations we were also able to compare the effects of two different dominant *Vrn-H1* alleles. Flowering time was more delayed for every genotype class for crosses containing a Calicuchima-sib allele relative to an OWB-D allele. This is likely due to the OWB-D allele being a very strong, whereas Calicuchima-sib is a weak, *Vrn-H1* allele.

Under long-day photoperiod conditions, *VRN-H1* played a more significant role in influencing flowering time than *VRN-H2*, corroborating prior reports (Dubcovsky et al. 2005; Kóti et al. 2006) and supporting *VRN-H1*'s function as a central control point for the transition from a vegetative to reproductive meristem. In support of the two locus model, inclusion of the interaction with *VRN-H2* explained nearly all the phenotypic variance in flowering time. While the putative flowering repressor *HvVRT-2* is expressed under both day-lengths in barley (Kane et al. 2005), the *HvVRT-2* locus or its interaction with the *VRN-H* loci had no significant effect on flowering time under long-day conditions.

To better understand the phenotypic variation of vernalization sensitivity, we also explored the allelic variation of the *VRN-H2* and *VRN-H1* genes in the relevant accessions. Comparison of the full-length *ZCCT-Ha* and *ZCCT-Hb* genomic sequences from spring and winter growth habit accessions revealed high polymorphism levels throughout both genes, including at the amino acid level. In contrast, polypeptides encoded by other genes involved in winterhardiness traits (vernalization response—

VRN-H1, *HvVRT-2*; photoperiod perception—phytochromes and cryptochromes; cold tolerance—*CBF*s, *ZFP16*, *ICE2*) show minimal allelic variation (Skinner et al. 2006; Szűcs et al. 2006; von Zitzewitz et al. 2005). The high level of allelic variation at *ZCCT-H* could influence repressor effectiveness and contribute towards variation in flowering time. Each allele encodes a full-length ZCCT-H polypeptide and the putative C_2H_2 zinc finger (Yan et al. 2004b) is invariant while an alanine/valine substitution at the CCT domain (Griffiths et al. 2003) differentiates the ZCCT-Ha vs ZCCT-Hb proteins. Zinc-finger motifs can function as both DNA-binding and protein-protein interaction domains (Takatsuji 1998) and many zinc-finger proteins are involved in regulation of flower development. The CCT domain of CONSTANS, a key gene mediating flowering transition in Arabidopsis, controls nuclear localization (Robson et al. 2001) and binds to the CCAAT binding factor, which can mediate interactions between CONSTANS-like proteins and DNA (Ben-Naim et al. 2006). In ZCCT-1, a mutation of a conserved arginine to a tryptophan is correlated with spring growth habit in multiple *T. monococcum* accessions (Yan et al. 2004b)—all the barley *ZCCT-H* alleles examined in this study encode an arginine at that position.

The highly conserved functional domains of ZCCT-Ha and ZCCT-Hb could indicate related or even redundant functions. There is currently conflicting evidence regarding which gene, or genes, are responsible for repression of *vrn-H1* in barley. We found that while vernalization-responsive wild barley accession Caesarea 26–24 contains *ZCCT-Hb* and *ZCCT-Hc*, it lacks *ZCCT-Ha*, suggesting that *ZCCT-Ha* is not a determinant of the ancestral winter growth habit. The lack of *ZCCT-Ha* is not a general *H. vulgare* subsp. *spontaneum* attribute, winter habit wild barley accession Koch (also known as Erez 8321; OSU6; PBI004-7-0-015) contains all the three *ZCCT-H* genes (data not shown; Dubcovsky et al. 2005). Based on a *vrn-H1vrn-H1* spring genotype containing *ZCCT-Hb* only, Dubcovsky et al. (2005) suggested *ZCCT-Hb* does not encode the *vrn-H1* repressor; although verification of a full-length *ZCCT-Hb* was not reported. Trevaskis et al. (2006) demonstrated that *ZCCT-Hb* expression is down-regulated by vernalization under long-day photoperiod, while *ZCCT-Ha* expression is unaffected. All winter barley accessions analyzed to date have the *ZCCT-Hc* gene based on hybridization or amplification of a small gene fragment (Dubcovsky et al. 2005; Karsai et al. 2005; Yan et al. 2004b); proof of a full-length gene specifying a predicted functional protein has not been obtained to date. Trevaskis et al. (2006) reported *ZCCT-Hc* expression was undetectable in a winter cultivar under both long-day and short-day

growth conditions. Additional experiments will obviously be needed though to determine which of the three barley *ZCCT-H* genes is/are responsible for *vrn-H1* repression under long-day conditions.

Based on sequence comparison of multiple barley accessions, von Zitzewitz et al. (2005) concluded that differences in vernalization response are not due to differences in the polypeptide, but rather in *VRN-H1* regulation and that the major vernalization regulatory site is not the promoter CArG-box, but is located within the first intron. In Fu et al. (2005), we noted that OWB-D has a large deletion in the *VRN-H1* first intron and based on more extensive sequencing in the current study, discovered that OWB-D also has a variant CArG-box—the only barley variety to date to display variation at this motif. OWB-D is one of the earliest flowering spring barley genotypes we have ever studied, and we speculate that this could be attributable to the loss of vernalization regulatory regions in both the *VRN-H1* promoter and first intron. In the *FLC* MADS-box gene—the central vernalization pathway repressor in Arabidopsis (Michaels and Amasino 1999)—mutations in the promoter or first intron each accelerate flowering under long-day conditions, suggesting possible interaction between the two regulatory sites (Sheldon et al. 2002). Similarly, mutations in the regulatory regions located in the *T. monococcum VRN-A^{m} 1* promoter and first intron can both lead to spring growth habit (Dubcovsky et al. 2006; Fu et al. 2005; Yan et al. 2003, 2004a).

A 436 bp segment of the *VRN-H1* first intron has been proposed to be the vernalization critical region based on sequence alignment of multiple barley and wheat genotypes (Fu et al. 2005; von Zitzewitz et al. 2005). Interestingly, we found that spring habit Calicuchima-sib and winter habit Albacete and Pané share an identical 4 kb intron 1 deletion immediately downstream of the intact vernalization critical region. While Spanish cultivars Albacete and Pané have been described as having winter growth habit based on their ability to be fall-sown and survive the mild Mediterranean winters (Lasa et al. 2001), these two genotypes—dominant for *Vrn-H2*—have subsequently been determined to display only a modest vernalization response (A. Casas, personal communication). In a vernalization response survey, Calicuchima-sib was the most vernalization-responsive (16 days) among the spring growth habit barley genotypes, as compared to 0, 4 and 141 days for Morex (spring habit), Dicktoo (facultative habit), and Kompolti korai (winter habit), respectively (I. Karsai, unpublished data).

Vernalization sensitivity in barley is not a presence/absence attribute, but rather a gradated continuum. Takahashi and Yasuda (1971) classified six growth habit classes,

ranked from strongest to weakest spring habit relative to flowering time under 24 h photoperiod during greenhouse growth. They concluded that multiple allelic series at the *Vrn-H1* locus are responsible for the grade of spring habit. We hypothesize the degree of flowering delay in these various classes may represent the degree of repression to which *VRN-H1* is subjected and Albacete, Calicuchima-sib and Pané would represent an intermediate growth habit class of Takahashi and Yasuda (1971). The deletion of the sequence flanking the vernalization critical region might affect the efficiency with which the *VRN-H2* repressor can repress the *vrn-H1* allele critical region, perhaps by deletion of important segments that influence *VRN-H1* locus DNA or chromatin structure. In Arabidopsis, alteration in chromatin structure is a principle means of affecting expression of the MADS-box vernalization repressor *FLC* (Bastow et al. 2004). Expression of another Arabidopsis homeotic MADS box gene, *STK*, is regulated by intron-localized GA-rich sites, where the GA-binding protein BPC1, the *STK* repressor, induced conformational changes when all the GA-rich elements are present—when only the two strongest binding sites are available, no conformational changes were observed (Kooiker et al. 2005). Summarizing natural allelic variation for *VRN-H1* (current study; Fu et al. 2005; von Zitzewitz et al. 2005), deletion of the vernalization critical region results in a strong spring growth habit, deletion of sequence flanking the critical region yields an intermediate spring habit, while presence of both the vernalization critical and flanking region yields a facultative or strong winter habit based on a recessive or dominant *VRN-H2*, respectively. Whether the *VRN-H1* promoter is directly involved in vernalization-based regulation (e.g., as a site of *HvVRT-2* interaction during short days) still remains to be resolved.

In summary, our results (a) validate the molecular model proposed to explain the epistatic interaction of *VRN-H2* and *VRN-H1* under long-day conditions, (b) verify the "winter" vs "spring" *VRN-H2* and *VRN-H1* allele primer sets work as predictors of growth habit class, and (c) demonstrate that hidden vernalization sensitivity can be recovered through appropriate crosses. Analysis of natural allelic variation and its effects relative to growth habit at *VRN-H1* and *VRN-H2* revealed that (d) sequence flanking the vernalization critical region of *VRN-H1* intron 1 likely influences degree of vernalization sensitivity, (e) that the *ZCCT-H* genes have higher levels of allelic polymorphism than other winterhardiness regulatory genes, and (f) a winter habit is retained when *ZCCT-Ha* has been deleted. These results provide grounds for developing and testing hypotheses on the role, or roles, of the duplicated *ZCCT-H* genes and the

number and structure of regulatory regions in *VRN-H1* controlling the timing of flowering and sensitivity to vernalization.

Acknowledgments

We wish to thank Douglas L. Heckart, John L. Helgesson, and Lauren A. Osborn for their technical assistance. This work was supported by the National Science Foundation Plant Genome Research Program (DBI 0110124) and the United States Barley Genome Project.

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Capítulo 6

Relationship between heading date, heading date QTL, and yield in barley grown under Mediterranean conditions.

Capítulo 6: Relationship between heading date, heading date QTL, and yield in barley grown under Mediterranean conditions

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6.1 Abstract

Grain yield is one of the key target traits for cereal breeders. The knowledge on QTL has led to a noticeable advancement in breeding for a variety of traits, but not for yield. Grain yield QTL have not been found consistently across mapping populations and environments, mainly because it is the final outcome of a large number of developmental processes, most of them highly influenced by genotype-by-environment interactions. Under water limiting conditions, phenological adjustment plays a major role on adaptation and yield. The optimum heading date for a given area will optimize final grain yield, as the crop can make the most of moisture available, especially during the critical grain filling stage. In this study we aimed at identifying yield QTL in a series of experiments carried out under Mediterranean conditions of Northern Spain, using the barley doubled haploid population Beka x Mogador, a *spring* by *winter* cross. We found that the relationship between heading date and yield changes depending on the environment. We also studied the effect of heading date QTL on yield and found that some of them have a direct effect, whereas the two main heading date QTL showed a strong QTL-by-environment interaction for yield. Allelic combinations causing early or late heading dates usually did not favour high yield, whereas intermediate heading dates were associated with higher variability and yield potential, being the most interesting from the agronomical point of view. The population was split into three distinct flowering classes, in an attempt to identify yield QTL with less dependence of heading time. Within the intermediate heading date plants, and despite the reduced sample size, we found that the ability to detect yield QTL was higher than for the entire population. Within this class, two heading date QTL presented also a remarkable effect on grain yield.

Keywords: *Barley, yield, heading date, QTL, vernalization*

6.2 Introduction

The final yield of a crop is the product of several growth and developmental processes occurring throughout the life cycle of a crop, with most genes influencing the final outcome directly or indirectly (Slafer, 2003).

Yield QTL have been reported in several barley populations (Thomas, 2003), but their impact in breeding programmes has been negligible so far. Among the causes are the problems of transferability of QTL to different genetic backgrounds, the shrinkage or even disappearance of QTL effects due to the presence of genotype-by-environment (GxE) interaction (both, cases of ascertainment bias), and the small size of the effects of yield QTL (Voltas et al., 2002; Slafer, 2003). GxE interactions are one of the main obstacles for advancement in breeding programmes (Fox et al., 1997). Therefore an accurate identification of the processes determining yield GxE would be of great help for breeders.

Phenological adjustment is one of the key factors for adaptation under water limiting conditions. Actually, adjustment of flowering time to the resources and constraint of the environment determines the final yield for barley (van Oosterom and Acevedo, 1992b).

The main factors influencing phenological responses of barley are photoperiod sensitivity and vernalization requirement. Both have a direct influence on crop adaptation to different geographical areas (Boyd, 1996). Flowering time genes determine the duration of the developmental stages in which the crop cycle is divided and, indirectly, the production of dry matter, the number of structures that contribute to the final yield (tillers, spikes and grains) and also the way in which dry matter is allocated among them (Boyd, 1996). Karsai et al. (1999) showed that *Ppd-H1* and *Vrn-H1*, the two major determinants of flowering time in the Dicktoo x Morex mapping population, had also a significant effect on several agronomic traits, including yield components. This effect is not reduced only to the vegetative phase, but also it has been suggested the effect of photoperiod on the stem elongation phase as a determinant of the number of fertile florets in barley and wheat (Miralles et al., 2000).

On the other hand, photoperiod sensitivity and vernalization requirement do not seem to have a direct intrinsic effect on barley grain yield. Reaching high yield potential is feasible for any combination of genes involved in the control of these processes. This is empirically evident looking at high-yielding cultivars, which can show a wide range

of patterns of phenological development. Kirby and Appleyard (1980) found that photoperiod sensitivity is not associated with differences in yield, studying spring cultivars under different photoperiod conditions in the greenhouse.

Several studies, carried out mainly by ICARDA researchers, focused in the relationships between several agronomic traits (including phenological development) and grain yield in the Mediterranean region. In general, they observed that genetic differences in yield and yield stability are associated with traits as growth habit, vigour, cold tolerance and heading date (Ceccarelli et al., 1991). Other studies showed that heading date must be early enough to allow an appropriate grain filling and avoid drought stress at the end of the cycle, but late enough to avoid cold damage, especially late frosts (van Oosterom and Acevedo, 1992b). Plant ideotypes proposed by these authors are spring types and early heading or winter types and moderately early heading (van Oosterom and Acevedo, 1992a). Their findings cannot be directly applied to the Northern Spanish dryland barley growing areas, however, as the environments are different. Unlike Syrian Mediterranean conditions in which these studies were carried out, in Spain too early cultivars cannot take advantage of a second spring rainfall peak (mainly in May) other than the autumn one.

The aim of the present study was to discover grain yield QTL in a population of barley doubled haploid lines, well adapted agronomically to Northern Spain. Also, we studied the relationship between heading date and yield in this population, and the implication of heading date on the genotype-by-environment interaction of grain yield and its QTL.

6.3 Materials and Methods

Plant Material

The population under study was a set of 120 doubled-haploid (DH) lines of the spring x winter cross between the French two-row cultivars Beka (Bethge XIII x Kneifel) and Mogador (Alpha x Sonja). This population was developed in the framework of the Spanish Barley Breeding Programme. It presents polymorphisms for most of the main loci controlling heading time, acceptable yield, and good agronomic characteristics for the region. The lines were selected from a larger population, as a

representative sample of heading time variation in the cross (procedure described in Chapter 3).

Field Trials

We carried out five autumn sown field trials, at four locations representative of the main barley growing areas of Northern Spain (Table 1). Grain yield was recorded at all five trials, whereas heading dates were recorded only at four of them. The experiments were coded with two letters indicating the province (i.e. HU - Huesca, LE - Lleida, VA - Valladolid, ZA - Zaragoza) and the last two digits of the harvest year (2001, 2002, 2003). Three of the experiments (ZA01, VA02 and HU03) were already described in Chapter 3 (as AUZA01, AUVA02 and AUHU03). Additional trials were carried out in Lleida in 2001, in which heading dates were recorded only in one of the three replicates, and in 2002, for which no heading times were recorded. Days to heading were calculated as the number of days between January 1st and the day when approximately 2 cm of awns were visible in 50% of stems. Experimental design at each trial was an alpha lattice with three replicates. Yield in kilograms per hectare was estimated extrapolating the data of the weight of the grain harvested in the 7.2 m^2 plots.

Table 1. Days to heading and yield of 120 Beka x Mogador DH lines in autumn sowings

Trial	Sowing date	Yield (kg ha^{-1})						Days to heading (from January 1st)					
		Parents		Offspring (DH lines)				Parents		Offspring (DH lines)			
		BEK	MOG	Mean	σ	Min.	Max.	BEK	MOG	Mean	σ	Min.	Max.
LE01	19-12-00	4005	4661	4693	518.5	3515	5862	104.3	104.0	102.4	4.11	95.0	110.0
ZA01	21-11-00	2983	2548	3105	317.8	2153	3811	99.4	104.9	99.1	4.40	89.5	112.5
LE02	01-12-01	5056	5639	5488	613.6	3676	6870	-	-	-	-	-	-
VA02	19-11-01	4060	4651	4829	557.4	3510	6189	122.1	122.6	123.5	3.79	116.1	131.8
HU03	04-11-02	4200	4594	4422	497.9	3195	5748	104.4	106.9	106.2	4.22	97.0	114.2

Genotyping and linkage map

The linkage map of the Beka x Mogador population was constructed with 126 markers and 120 individuals. Genotyping and linkage map construction are described in Chapter 3).

QTL analysis

QTL main effects analysis was performed using the composite interval mapping (CIM) procedure (Zeng, 1994), implemented in Windows QTL Cartographer 2.5 (Wang et al., 2005). We chose up to 21 cofactors for each CIM analysis, using a stepwise regression procedure with a significance threshold of 0.05. Walk speed was set to 2 cM, and the scan window to 10 cM beyond the markers flanking the interval tested. Experiment-wise significance (α=0.05) likelihood ratio test (LR) thresholds for QTL identification were determined with 1000 permutations, and expressed as LOD (LOD = 0.217 LR). Epistatic interactions between QTL were evaluated with the Multiple Interval Mapping (MIM, Kao et al., 1999) tool implemented in Windows QTL Cartographer using Bayesian Information Criterion (BIC-M0).

At each trial, yield for each line was calculated according to the experimental design. Average yield values across trials, used for QTL main effect detection, were obtained averaging line means across the five field trials.

The proportion of the total variance of average grain yield explained by the QTL was calculated as the coefficient of determination of the multilocus model that included the significant QTL and the significant interactions among them, using MIM.

Analyses of variance and regression analyses were performed using the GLM procedure of SAS v9 (SAS Institute Inc., Cary, NC, USA). Genotypes were considered as random factors (representative of the population of possible lines from this cross) and environments were considered as fixed factors (due to a small number of degrees of freedom to consider it as a random factor). The error for the combined analysis of variance of the genotype main effects (including QTL markers) was the Genotype-by-Environment (GXE) term. Tests for QTL-by-environment interactions (QTLxE) were performed using the GxE option of the multitrait mapping method of Jiang and Zeng (1995) that is implemented in Windows QTL Cartographer 2.5. CIM and IM (Interval Mapping, Lander and Botstein, 1989) approaches were used for this analysis. Cofactors were selected manually, and closest markers to the yield and heading date QTL were included. Significance threshold was set through 1000 permutations (α=0.05) only to the IM LOD scores. Environments were treated as fixed factors (Jiang and Zeng, 1995). The joint error for the combined analysis of variance of the GxE and QTLxE interactions was the pooled mean of the errors of the single trials.

6.4 Results

Yield and heading date

The range of variation for grain yield and days to heading of the DH lines was wider than the differences between the parental lines Beka and Mogador, thus showing transgressive segregation at all the experiments (Table 1). Distribution frequencies of heading dates (Chapter 3) and yield (data not shown) of the offspring lines showed a quantitative distribution of the traits. Description of heading date results is reported in Chapter 3. Regarding overall yield level, LE01, LE02 and VA02 showed similar average values and amplitude of the range of variation, whereas ZA01 and LE02 were significantly lower and higher yielding, respectively (Table 1).

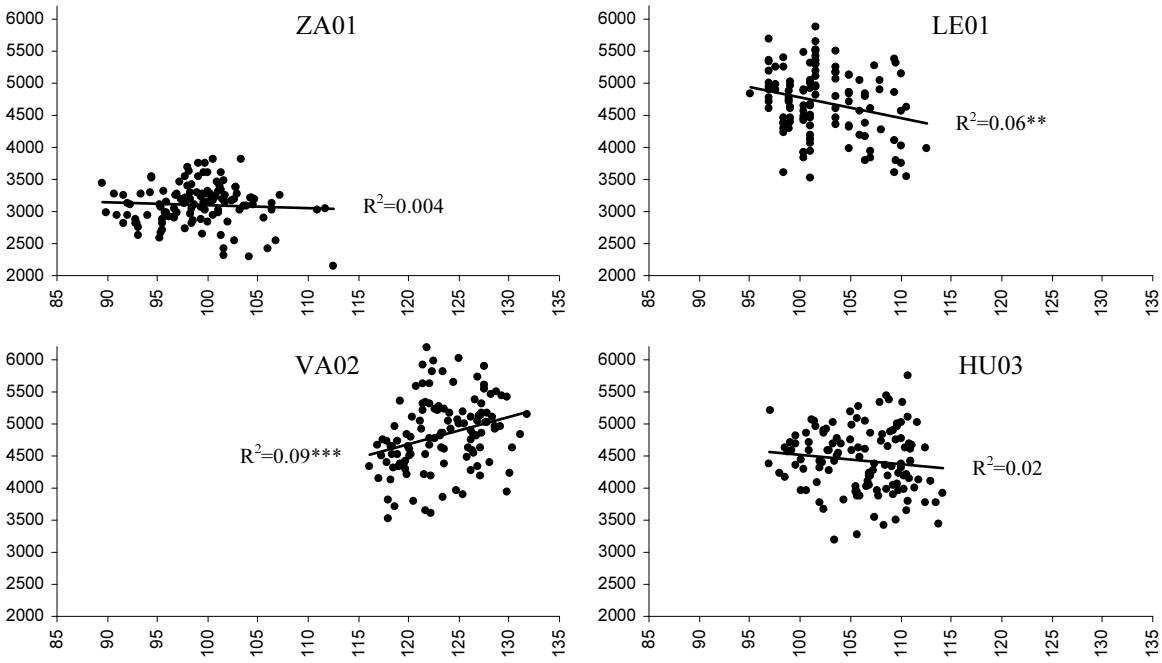


Figure 1. Scatter plots of the heading dates from January 1st (X-axis) and yield in kg ha^{-1} (Y-axis) in four field trials. The 120 DH lines are indicated by dots. Lines show the linear trend between the two variables. R^2 of the linear regressions are also shown. F value of the linear regression significant at *P < 0.05, **P < 0.01, ***P < 0.001.

The relationship between heading date and yield presented different trends at the four experiments in which both variables were recorded (Fig. 1). At LE01, yield decreased with lateness, and the opposite was true at VA02. At the other two trials, though the slope was negative, the relationship was non significant. Overall, the plot of the averages of the 120 lines for yield and heading date presented a slightly quadratic

pattern (Fig. 2), meaning that yield and heading date presented opposite relationships at both ends of the heading date distribution, positive for early lines, and negative for late lines. The highest yielding lines and also the highest variability in the yield occurred at intermediate heading dates (Fig. 2).

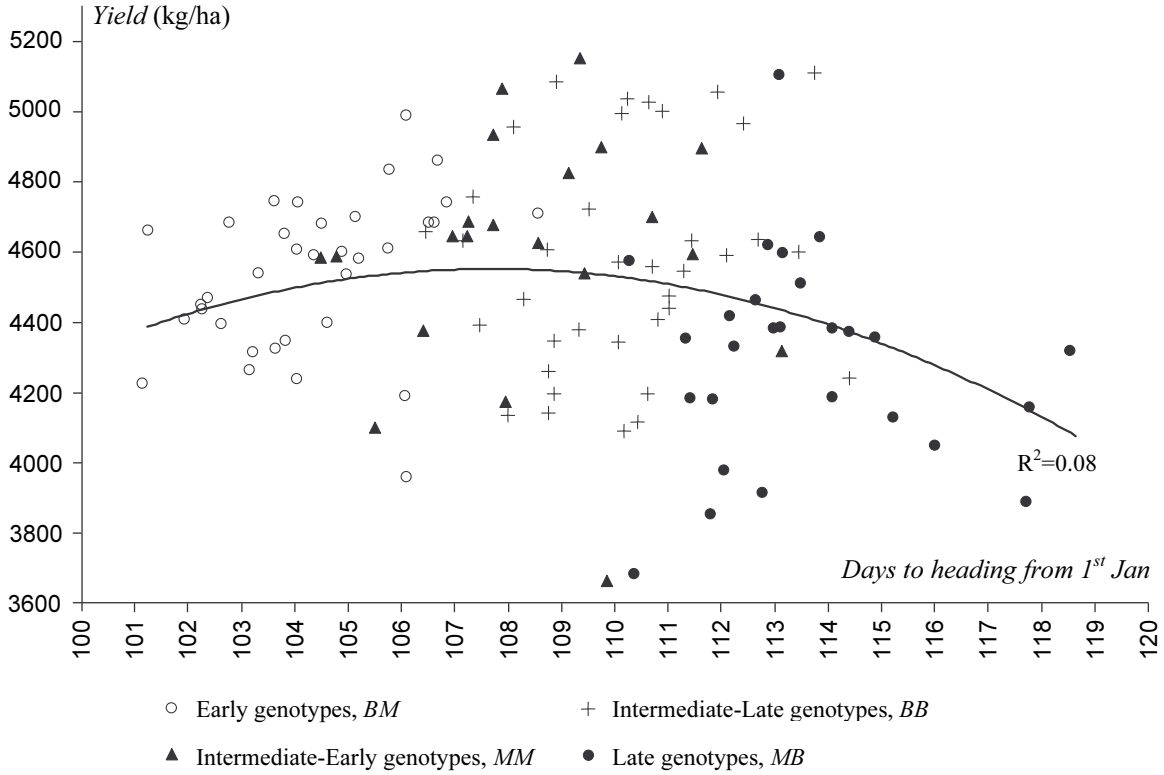
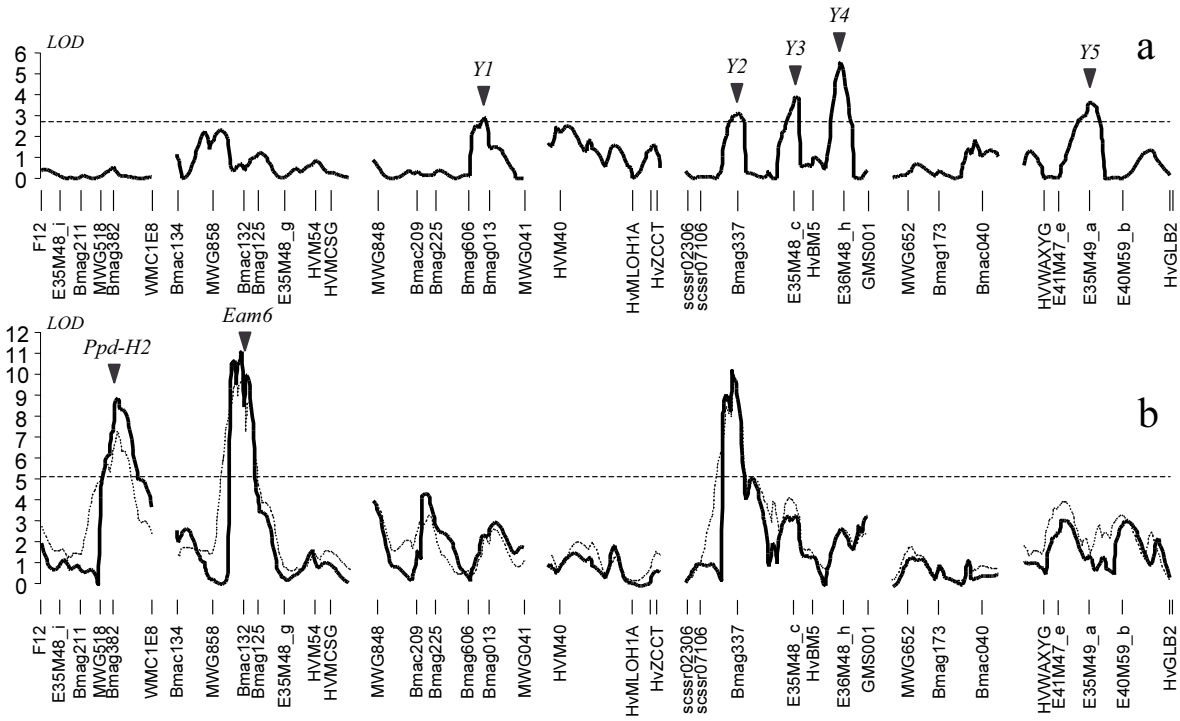


Figure 2. Relationship between heading date and yield for the averages of 120 DH lines of the Beka x Mogador single cross population in four (heading date) or five (yield) environments. R^2 of the quadratic regression is also shown. Symbols stand for haplotypes at the two main heading date loci for this population, represented by markers Bmag382 and Bmac132 (Chapter 3).

QTL main effects and interactions

Five QTL for grain yield were found when analyzing the average yield of the 120 DH lines across the five field trials. Three of them were on the long arm of chromosome 5H (Fig. 2). These QTL, coded as *Y1*-*Y5*, explained 40% of the phenotypic variation for the trait (Table 3). For four of these QTL, higher grain yield was associated with the Mogador allele. The exception was *Y3*, located on bins 9–10 of chromosome 5H, which showed an opposite effect to the other two flanking QTL.

Figure 3. Scans for a) grain yield main effect, CIM analysis with a 2.7 LOD threshold; b) genotype-by-environment interaction, CIM scan (full line) and IM scan (dotted line) analysis for QTL; LOD threshold (5.1) corresponds only to IM.



When testing the interactions among the grain yield QTL detected in the CIM analysis, we found a significant interaction between *Y2* and *Y4,* responsible for an additional 5% of the phenotypic variation (Table 2).

Table 2. Grain Yield QTL detected for average yield of 120 DH lines in 5 environments. LOD scores correspond to the CIM analysis. Multiple Interval Mapping was used to calculate effects and R^2.

QTL Code	Chrom.	Peak position	Closest marker	Bin	2 LOD confidence interval	LOD	Additive effect	R^2
Y1	3H	132.2	Bmag013	13	127.5-160.5	2.90	90.5	0.09
Y2	5H	61.0	Bmag337	5	49.5-70.0	3.12	110.9	0.11
Y3	5H	129.1	E35M48_c	9-10	111.9-134.2	3.90	-84.5	0.06
Y4	5H	181.4	E36M48_h	11-12	170.7-190.8	5.54	95.6	0.10
Y5	7H	77.7	E35M49_a	7	51.1-90.6	3.62	67.9	0.04
Y2 x *Y4*							69.3	0.05

The analysis of genotype-by-environment interaction (GxE) revealed that only one of the grain yield QTL (*Y2*, chromosome 5H) showed a significant QTLxE interaction. Another two regions with a strong QTLxE interaction were detected on the long arm of chromosome 1H, and in the centromeric region of chromosome 2H (Fig. 3).

These regions coincide with the positions of the two major QTL for heading date under autumn sowing conditions detected in this population (markers Bmag382 and Bmac132, in the vicinity of *Ppd-H2* and *Eam6,* respectively, Chapter 3). To further analyze these interactions, we studied the effect of these loci at each trial (Fig. 4). Early alleles (*Beka* for Bmac132, *Mogador* for *Eam6*) were significantly better at LE01, the trial in which earlier lines showed a yield advantage. The opposite was true for VA02, in which late lines presented higher yields.

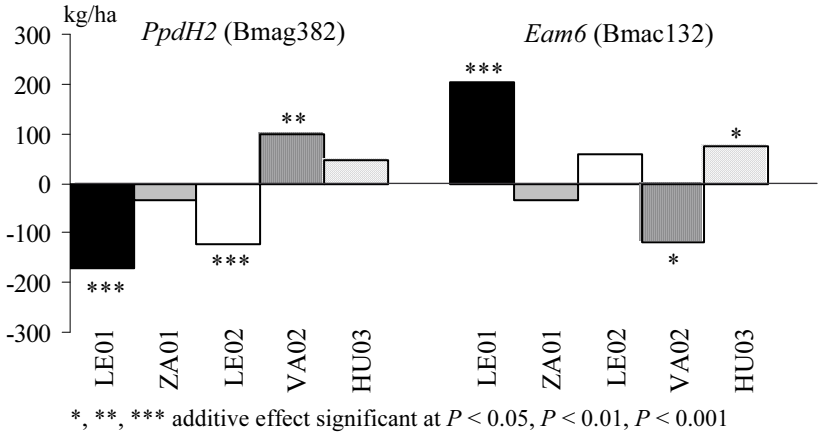


Figure 4. Additive effect of Bmag382 (marker for *PpdH2*) and Bmac132 (marker for *Eam6*) at the five field trials. Positive values indicate higher yield of lines carrying the Mogador allele.

As the two major QTL for heading date in this population (*Ppd-H2* and *Eam6*) also had a primary role on yield GxE, we decided to examine the effect of grain yield QTL in each flowering time class defined by the different allelic combinations at both regions (Table 3). The major effect of the QTL in the regions of *Ppd-H2* and *Eam6* on heading date was used to divide the population into four heading time groups: *early*, *intermediate-early*, *intermediate-late* and *late*, depending on the haplotypes constituted by the alleles present at Bmag382 (*Beka* early and *Mogador* late) and Bmac132 (*Beka* late and *Mogador* early). These four groups presented significantly different heading dates, with the two extreme classes farther apart, and the two intermediate ones more grouped together (Table 3). Regarding yield, both intermediate classes showed the highest values, though only the late lines differed significantly from the rest.

We then run QTL analyses, using CIM within each class (not shown). In this QTL analysis for each heading time class, the two closest classes, *Intermediate-early* and *Intermediate-late,* were considered as a single class (*Intermediate*), to keep sample sizes as large as possible. In this way we could estimate more accurately the effect of these

regions on grain yield independently of its relationship with heading time (Fig. 1, Fig. 2).

Table 3. Average yield and heading date of the three heading time classes defined by the allelic combination at Bmag382 (*Ppd-H2* region) and Bmac132 (*Eam6* region).

	Bmag382 (*Ppd-H2*)	Bmac132 (*Eam6*)	no. lines	Yield (kg ha^{-1})*	Days to heading (from January 1st)
Early	B	M	35	4538 A	103.5 A
Intermediate-early	M	M	37	4603 A	107.2 B
Intermediate-late	B	B	21	4579 A	109.0 C
Late	M	B	27	4295 B	112.3 D

*Means separation LSD; α=0.05; B: Beka allele; M: Mogador allele

Though sample sizes were too small to get reliable thresholds, we used CIM peaks as indicators of presence of further potential QTL. The effect of these additional regions was evaluated by means of ANOVA, including in the model the closest markers to peaks found in the CIM analysis, and markers for yield QTL *Y1* to *Y5* (Table 4, in which the analysis of the entire population is also shown to provide contrast).

Table 4. Analysis of variance of the yield QTL and their significant interactions, for the whole set of lines and also for three subsets of lines according to heading time classes defined by the allelic combinations at Bmag382 and Bmac132. Other QTL detected only in the CIM analysis of the intermediate class are also included (*in italics*). Closest markers to the QTL peaks detected in the CIM analysis are used. Mean squares of yield values, derived from Type III sums of squares, expressed in q ha^{-1}

	Whole set of lines (120)		Early lines (27)		Intermediate lines (58)		Late lines (35)	
Source of variation	DF	MS	DF	MS	DF	MS	DF	MS
GENOTYPE (G)	119	47.8 ***	26	48.8 ***	57	48.3 ***	34	47.4 **
ENVIRONMENT (E)	4	9212.2 ***	4	2686.9 ***	4	4452.6 ***	4	2072.8 ***
Bmag013 (*Y1*)	1	415.0 ***	1	31.9	1	197.1 **	1	41.3
Bmag337 (*Y2*)	1	499.0 ***	1	59.2	1	149.5 **	1	150.8 **
E35M48_c (*Y3*)	1	249.3 ***	1	0.0	1	62.1	1	44.1
E36M48_h (*Y4*)	1	448.6 ***	1	2.0	1	234.6 ***	1	113.2 *
E35M49_a (*Y5*)	1	210.1 **	1	124.2 *	1	59.6	1	82.1 *
Bmag337*E36M48_h	1	225.9 **	1	101.7 *	1	15.6	1	147.0 **
HvM40	-	-	-	-	*1*	*436.1* ***	-	-
HvZCCT	-	-	-	-	*1*	*142.8* **	-	-
E37M61_d	-	-	-	-	*1*	*97.3* *	-	-
G X E	475	20.7 ***	136	21.0 ***	228	20.8 ***	108	20.4 ***
Ens*Bmag337 (*Y2*)	4	216.9 ***	4	23.4 *	4	215.5 ***	4	42.4 ***
Ens*Bmag382 (*Ppd-H2*)	5	123.5 ***	-	-	-	-	-	-
Ens*Bmac132 (*Eam6*)	5	155.6 ***	-	-	-	-	-	-
ERROR	949	8.0	277	8.0	459	8.0	213	8.0

Significant effect at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

We found that the effect of the QTL detected in the whole set of lines was not consistent across heading time classes. Neither the main effect QTL, nor the interaction between *Y2* and *Y4* showed a significant effect at all the three classes (Table 4). According to the results, most of the main effect QTL had an important effect only in the *Intermediate* and *Late* classes, whereas their effect was reduced or non significant for the *Early* class (Table 4, Table 5).

New possible peaks, different from *Y1-Y5*, were evident only at the *Intermediate* class. The closest markers to the QTL peaks found in this class were HvM40, HvZCCT and E37M61_d. HvM40 is located in the short arm of chromosome 4H (bin 2), and its LOD was slightly under the significance threshold in the CIM analysis (Fig. 3a). HvZCCT, in the long arm of chromosome 4H (bin 13) is the allele specific marker for the vernalization gene *Vrn-H2,* and it was not detected in the CIM analysis either as a main effect QTL or in the GxE analysis for grain yield. E37M61_d in bin 4 of chromosome 6H was not detected in the CIM analysis of the whole set of lines either.

Besides of being in the same heading time class, in almost all the cases, yield variations caused by the QTL detected in this study seemed not related to differences in heading date, as their effect on this trait was very low (Table 5).

Table 5. Additive effect of main effect QTL in the different heading time classes.

	Early				Intermediate				Late			
	Number of lines		Additive effect		Number of lines		Additive effect		Number of lines		Additive effect	
Marker	B[(1)]	M[(2)]	Yield[(3)]	Heading[(4)]	B[(1)]	M[(2)]	Yield[(3)]	Heading[(4)]	B[(1)]	M[(2)]	Yield[(3)]	Heading[(4)]
Bmag013 (*Y1*)	20	15	45	0.1	24	34	87**	0.3	17	10	72	-0.2
Bmag337 (*Y2*)	13	22	62	0.1	22	36	79**	0.2	15	12	149**	-0.4
E35M48_c (*Y3*)	13	22	1	0.4	34	24	-53	0.2	9	18	-64	0.4
E36M48_h (*Y4*)	19	16	12	0.3	28	30	104***	0.3	17	10	126*	0.4
E35M49_a (*Y5*)	19	16	87*	-0.1	33	25	50	0.1	17	10	91*	0.4
HVM40	22	13	15	0.1	32	26	-127***	-0.4*	14	13	-16	-0.6
HvZCCT	14	21	35	0.6**	32	26	82**	0.1	12	15	3	0.4
E37M61_d	23	12	-3	-0.2	32	26	-67*	-0.4*	17	10	-0.5	-0.4

(1) Number of lines with the Beka allele
(2) Number of lines with the Mogador allele
(3)Yield additive effect (kg ha^{-1})
(4) Days to heading additive effect
Significant effect at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

6.5 Discussion

Five QTL for grain yield main effect were found in the present study and three additional ones were found for the GxE interaction (Tables 2 and 3, Fig. 2), when considering the entire population. The results also revealed the important role that heading date and several heading date QTL play on yield and its GxE interaction.

The yield main effect QTL were located in the following regions:

- *Y1*, on the long arm of chromosome 3H (bin 13), being Bmag013 the closest marker to the peak. Several studies identified QTL for grain yield in this region (Hayes et al., 1993; Thomas et al., 1995; Bezant et al., 1997; Powell et al., 1997; Yin et al., 1999; Marquez-Cedillo et al., 2001). QTL were also found in this region for ear grain weight and thousand grain weight (Bezant et al., 1997). This is also the region of the semi dwarfing gene *denso* (Laurie et al., 1993), which confers reduced plant height and has been associated with late heading (Barua et al. 1993), low seed weight, high screenings, and high β-glucan content (Powell et al., 1985; Thomas et al., 1995), though it is unlikely that any of the parents carry *denso*.
- We found three QTL on the long arm of chromosome 5H (*Y2, Y3, Y4*), distant enough among them to be considered as independent. On bin 5, we found a QTL near the SSR marker Bmag337 (*Y2*). In this region, Pillen et al. (2003) found a QTL for kernel weight in a cross between a spring cultivar and *Hordeum spontaneum*. Heading date QTL have been reported in the same region, within a 2 LOD confidence interval (Pan et al., 1994; Thomas et al., 1995). Another QTL was in bins 9-10 (*Y3*), and was the only one in which the Beka allele conferred higher yield. A grain yield QTL was also found in a similar position (Barua et al., 1993; Thomas et al., 1995; Powell et al., 1997). The last QTL was in bins 12-14 (*Y4*), co-locating with a grain yield QTL reported by Marquez-Cedillo et al. (2001), a heading date QTL (Pan et al., 1994), and a lodging resistance QTL (Backes et al., 1995).
- Finally, another QTL was located at the centromeric region of chromosome 7H, bin 7 (*Y5*). In the same region, Hayes et al. (1993) found a QTL for grain yield, and Laurie et al. (1995) another one for heading date. QTL for other traits have been reported in this region, and some of them could have an indirect effect on

yield: plant height (de la Pena et al., 1999; Marquez-Cedillo et al., 2001) and also root length (Jefferies et al., 1999).

Besides, a significant interaction between *Y2* and *Y4* was found, explaining an important part of the phenotypic variation. Though both QTL are on chromosome 5H, they are distant enough to be independent. However, the presence of another QTL in repulsion between them (*Y3*) may affect the power of tests in this region.

Several regions also showed an important QTLxE interaction:

- The centromeric region of chromosome 2H (bin 8), coincident with the earliness *per se* locus *eps2S* (Laurie et al., 1995), whose effect is evident under spring and autumn sowing conditions. It is also coincident with the early maturity locus *Eam6*, which confers early heading under both long- and short-day conditions (Franckowiak and Konishi, 2002; Horsley et al., 2006). This was the most important locus for heading time under autumn sowing conditions in this population (Chapter 3), in the population Beka x Logan (Moralejo et al., 2004) and, it is also one of the main determinants of heading date under autumn sowing conditions in a set of small populations whose parents are barley cultivars commonly used in the Spanish Barley Breeding Programme (Chapter 4).
- Marker Bmag382, on the long arm of chromosome 1H (bins 12-13) coinciding with the position of the photoperiod response gene *Ppd-H2*, which causes differences in heading date under short photoperiod conditions (Pan et al., 1994; Laurie et al., 1995; Boyd et al., 2003; Francia et al., 2004). Lines with the Mogador allele have a later heading date (Chapter 3). This QTL is a major determinant of heading date under autumn sowing conditions in Spain (Chapters 3 and 4). It is also the main heading date QTL under Australian field conditions, where cultivars are also exposed to short photoperiods during most of the growing season (Boyd et al., 2003).
- The third region with QTLxE interaction was detected at the *Y2* peak (Table 2; Fig. 2). Thomas et al. (1995) and Pan et al. (1994) also found heading date QTL in this region.

The changing relationship of yield with heading date, and the effect of heading date QTL on yield reveals the complex association of these two variables (Fig. 1, Fig. 2).

The heading date QTL in the regions of *Ppd-H2* and *Eam6* affected yield in different manner, according to the relationship of yield with heading date (Fig. 1), which varied from negative (LE01), to inexistent (ZA01, HU03), to positive (VA02).

Presumably, these environmental differences reflect different conditions of water availability during grain filling. The same allelic combinations at *Ppd-H2* and *Eam6* may produce significant differences in the yield depending on the environment (Fig. 4), unlike heading time, whose variations caused by the allelic combinations at *Ppd-H2* and *Eam6* were similar independently of the environment (Chapter 3).

This population displayed a wide range of heading dates, of about 18 days on average. The scatterplot shown in Fig. 2 reveals that this range is wide enough to encompass the optimum heading date to optimize yield for this population in these environments, with higher yield potential and variability corresponding to intermediate heading dates. Possibly, too early lines or, generalizing, too early cultivars, cannot make the most of spring rains, though they may be a safe bet from the agronomic point of view. Late lines or cultivars, on the other hand, can extend their vegetative phase, thus taking advantage of spring rainfall, and accumulating more biomass to achieve higher yield potential. But their yield can be reduced to a great extent because of the commonly occurring drought stress at the end of the cycle. Under this scenario, usually there is a narrow flowering time window to get optimum yield. The range of heading dates variation of this population is representative of those found at the trials carried out by the Spanish Barley Breeding Programme in the same locations. In these trials, two cultivars are used regularly as early and late checks, to define the acceptable heading date window for the materials in the program. The flowering date of these checks was very similar to the extreme lines of the Beka x Mogador population. Thus, the findings in this study may have a more general relevance for Spanish conditions.

We run further analyses aiming to estimate the effect of the QTL detected for yield, independently of possible confounding effects of heading date, and of heading date QTL. The division of the population based on the haplotypes at *Ppd-H2* (Bmag382) and *Eam6* (Bmac132) clearly produced four heading time classes (Table 2) in which yield QTL could be estimated independently. As higher yield potential was achieved in the intermediate heading dates, and the two intermediate heading time classes showed similar variability and average yield, both were grouped into a single class. Besides, we got a higher number of lines and hence, higher reliability of the results, for the class in which the higher yields and variability are achieved. Yield QTL showed their strongest effect in the intermediate and late heading dates and their effect was reduced to a minimum in the early heading dates (Table 4, Table 5). The effect of the five yield QTL found for the entire population was not equally distributed among

the three heading date classes (Table 4). *Y1* was only evident at the *Intermediate* class, *Y2* and *Y4* at the *Intermediate* and *Late* classes, and *Y5* at the *Early* and *Late* classes. *Y3* was not detected at any of the three subpopulations, which could be caused by either the loss of power inherent to the use of smaller sample sizes, or to the fact that the true differences between lines for this QTL lie only among heading date classes, and not within them.

Besides *Y1*-*Y5*, three other QTL were detected only in the *Intermediate* class. Two of these additional QTL are also heading date QTL that still showed some association with grain yield (HvM40 and HvZCCT, see Chapter 3). HvM40 was detected as a heading time QTL in one of the autumn sowing experiments (ZA01, Chapter 3) and HvZCCT is the allele specific marker for the vernalization gene *Vrn-H2*. These associations were not detected with CIM analysis of the whole set of lines, either as main effect QTL or in the GxE analysis, probably because significance levels and cofactors of the two analyses were different. In the case of HvM40, in the CIM scan (Fig. 3a), there was a peak just below the thresholds for this locus. At the ANOVA shown in Table 4, however, HvM40 presented the largest mean square of all yield QTL.

The winter allele of the *Vrn-H2* gene acts as a repressor of the transition from the vegetative to the reproductive phase regulated by *Vrn-H1* (von Zitzewitz et al., 2005) under long photoperiod conditions (Karsai et al., 2005). The expression of *Vrn-H2* is down-regulated by cold temperatures (Travaskis et al., 2006). It is only possible to speculate at this point as to how the effect of the *Vrn-H2* gene might affect yield, independently of the effect on *Vrn-H1.* The explanation might be in some effect on plant architecture: vernalization genes may affect not only heading time, but also the relative duration of phases of plant development. Thus, different combinations of vernalization alleles might produce different balance of biomass, number of tillers, and spike size, even for the same heading time, as has been indicated by Karsai et al. (2006). These differences in plant architecture could result in differences in grain yield. This effect would be lower in early headings because of lack of time to express the potential for the best allelic combinations, and in late headings because of the strong terminal drought stress. These hypotheses need validation in other materials and experimental confirmation through the study of yield components.

Regarding HvM40, no yield QTL has previously been reported in this region. However, as yield is the outcome of a complex set of factors highly influenced by the

environmental conditions, this region could have an important effect on other traits with a relevant role on yield determination under the Mediterranean conditions of Northern Spain. Besides the effect on heading date detected in this population (Chapter 3), other studies have found heading date QTL in this region (Hayes et al., 1993). It has also been reported the effect of this region on plant height (Zhu et al., 1999; Marquez-Cedillo et al., 2001), which is a trait usually associated with yield under drought conditions (Ceccarelli et al, 1991; Teulat et al., 2001).

Acknowledgements

This work was supported by the Spanish Ministry of Education and Research (Projects AGL2001-2289, including a scholarship granted to Mr. Alfonso Cuesta-Marcos, and AGL2004-05311) and by the European Regional Development Fund.

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Capítulo 7

Discusión General

Capítulo 7: Discusión General

7.1 Identificación de los principales loci determinantes de la fecha de espigado en la cebada

Los diferentes ensayos y análisis realizados en la presente tesis doctoral tienen como base común la identificación de las regiones del genoma de la cebada que determinan la fecha de espigado en las condiciones de secano del norte de España, y su efecto sobre el rendimiento. En una primera etapa se identificaron los principales QTL en una población de mapeo (capítulo 3) y también en la literatura. Posteriormente se procedió a la validación de los mismos, por un lado con un conjunto de pequeñas poblaciones interconectadas de haploides duplicados (capítulo 4) y, específicamente para los genes de vernalización, en una serie de cruzamientos que permitieran un estudio de complementariedad en la descendencia (capítulo 5).). Finalmente se evaluó la influencia de esos QTL de espigado sobre el rendimiento, sobre la detección de QTL para rendimiento, y sobre la interacción genotipo por ambiente del rendimiento (capítulo 6).

Se emplearon diversos sistemas de marcadores en este trabajo (AFLP, RFLP, SSR, STS, RAPD), de los que cabe destacar los marcadores HvBM5, HvZCCT y HvT SNP22, que corresponden a los genes candidato propuestos para *Vrn-H1*, *Vrn-H2* y *Ppd-H1* (Turner et al., 2005; von Zitzewitz et al., 2005). Estos genes fueron identificados durante el desarrollo del trabajo, y fueron incorporados paulatinamente al estudio. Su empleo, tanto en una población de mapeo como en un conjunto de germoplasma de amplia base genética, ha permitido confirmar la correlación de la diversidad alélica a nivel molecular con las respuestas fenotípicas observadas.

Previamente a los análisis para la identificación de QTL, se realizaron dos mapas de ligamiento en la presente tesis doctoral. Tanto el mapa de la población Beka x Mogador (capítulo 3) como el mapa consenso de las 17 poblaciones de DH (capítulo 4) están de acuerdo entre sí y con otros mapas de cebada previamente publicados (Pillen et al., 2000; Ramsay et al., 2000; Moralejo et al., 2004; von Korff et al., 2004; Cuesta-Marcos, 2005; Rostoks et al., 2005, Varshney et al., 2007). El mapa de la población Beka x Mogador tiene una densidad y número de individuos suficientemente elevados como para permitir una correcta identificación de las principales regiones que presentan QTL de espigado y de rendimiento. El mapa consenso, cuya finalidad era la validación,

no presentaba una densidad tan elevada de marcadores, pero el número es suficiente como para ser una herramienta útil en el proceso de validación de QTL. Para la validación específica del modelo de la relación epistática de los genes de vernalización, se siguió la estrategia de recuperar líneas invernales entre la descendencia de una serie de cruzamientos entre líneas de hábito de crecimiento primaveral, que fueron caracterizadas solamente en las dos regiones de los genes de vernalización *Vrn-H1* y *Vrn-H2* y una tercera región *HvVRT2,* cuyo papel en la interacción entre esos dos genes ha sido sugerido en estudios previos (Kane et al., 2005).

Comparando los resultados descritos en la literatura y los obtenidos en el capítulo 4 con los obtenidos mediante el empleo de la población Beka x Mogador (capítulo 3), podemos concluir que en esta última se pudieron detectar la mayoría de los principales loci que controlan la fecha de espigado bajo diferentes condiciones ambientales en campo e invernadero, explicando en cada caso un porcentaje notable de la varianza del carácter (Tabla suplementaria 1, capítulo 3). Así, pudimos establecer cuáles eran los marcadores más cercanos a estos loci, que fueron empleados en el capítulo 4. De hecho, la selección de los parentales de este cruzamiento ya se realizó en base a que presentaban polimorfismo en las principales regiones que, según la literatura, hacían suponer que tuvieran un claro efecto sobre la fecha de espigado en las condiciones de secano del norte de España (Igartua et al., 1999). También fue posible estimar los efectos de los diferentes alelos y sus interacciones. Sin embargo, existen algunas regiones implicadas en la determinación de la fecha de espigado que no son polimórficas en este cruzamiento, especialmente la zona del gen *Ppd-H1*, donde ambos parentales llevan el alelo que no responde a fotoperiodo largo. Como se puso de manifiesto en el capítulo 4, es evidente que el efecto de este locus es importante en el conjunto de materiales empleados en el programa español de mejora.

Para superar estas limitaciones, se realizó un estudio de validación de los principales QTL de fecha de espigado en un conjunto de germoplasma de amplia base genética, compuesto de 17 pequeñas poblaciones de haploides duplicados de cebada en las que intervenían como parentales, normalmente en más de una población, catorce variedades comúnmente utilizadas en el programa español de mejora de la cebada. Un estudio de este tipo sería dificil sin el trabajo previo de localización de marcadores estrechamente ligados a los principales genes que regulan la fecha de floración bajo las mismas condiciones ambientales. Al existir varios parentales, nos encontramos con la presencia de varios alelos por locus, en unas frecuencias muy variables. Dada la

complejidad del análisis, se descartó la posibilidad de realizar mapeo por intervalos, y se optó por realizar los tests de asociación con los caracteres sólo para las posiciones de los marcadores. Por otra parte, es necesario considerar que el hecho de encontrar asociaciones entre marcadores y el carácter fenotípico, en este tipo de estudios basados en el empleo de marcadores moleculares distribuidos por el genoma, hace que sea más propio hablar de regiones de presencia de QTL que de loci propiamente dichos.

De este modo, es posible encontrar asociaciones significativas en regiones en las que existen marcadores, pero no es posible la localización de genes situados entre marcadores e incluso si esa significación es debida a uno o más genes. Esta limitación está atenuada en el caso de los marcadores diagnóstico de los genes candidato, al estar los marcadores situados dentro del propio gen. Este es el caso de *Ppd-H1*, *Vrn-H1* y *Vrn-H2*. Sin embargo, no siempre está claro cuál es la mutación específica que provoca los cambios fenotípicos. Hasta el momento, parece existir acuerdo entre la diversidad molecular y fenotípica en estos genes, lo cual se ha confirmado en este trabajo; sin embargo es imposible predecir si esta situación se mantendrá para alelos no contemplados en los estudios realizados hasta ahora. Por este motivo, en muchos casos es conveniente el empleo de marcadores flanqueantes que permitan seleccionar una región más amplia en las inmediaciones del gen de estudio.

El método de análisis conjunto de QTL en varias poblaciones hace que sea prácticamente imposible la identificación de interacciones en las que intervenga algún loci cuyo efecto principal no haya sido identificado. Además, la estructura genética de los individuos implicados en el estudio hace difícil la validación de interacciones detectadas en poblaciones de mapeo, a no ser que tengan un efecto muy grande, como es el caso de los genes de vernalización.

En una visión general de los dos métodos empleados en los capítulos 3 y 4, se concluye que ambos son complementarios, pues cada uno de ellos suple los puntos débiles del otro. Además, los principales QTL y los efectos de los alelos comunes están plenamente de acuerdo entre ambos estudios. Todo ello nos ha llevado a proponer un conjunto de marcadores moleculares de fácil uso (todos ellos basados en la técnica PCR) que ofrezca a los mejoradores de cebada la posibilidad de controlar el carácter fecha de espigado según sus necesidades (Tabla 3, capítulo 3; Tabla 4, capítulo 4). Su empleo sería posible preferentemente en la elección de los parentales de los cruzamientos, pero también en la selección en las sucesivas generaciones de autofecundación.

Un punto clave de la presente tesis doctoral fue la constatación de la fecha de espigado como uno de los principales causantes de la interacción Genotipo por Ambiente (GxE) del rendimiento (Voltas et al., 2002). En las condiciones en las que se ha realizado el presente estudio, las regiones de *Eam6* y *Ppd-H2* son las principales determinantes de la fecha de espigado en siembras otoñales (capítulos 3 y 4) y a la vez presentan la significación más alta en el análisis GxE del rendimiento (Figura 2, capítulo 6). Aunque se ha descrito que la temperatura y las precipitaciones juegan un papel importante en la aparición del GxE en el rendimiento (Voltas et al., 2002), se han publicado hasta la fecha pocos estudios como el presente, en los que se ponga de manifiesto una clara asociación entre la interacción GxE en este carácter y los patrones de rendimiento.

También se han detectado en el presente estudio una serie de QTL con incidencia directa sobre el rendimiento, aunque con un efecto menor y alguno de ellos, a su vez, con una interacción significativa QTL x ambiente (Figura 2, Tabla 2, capítulo 6). El estudio de los procesos fisiológicos en los que están implicados estos QTL, los componentes del rendimiento sobre los que actúan específicamente, así como una validación de los mismos de manera similar a la realizada para la fecha de espigado, son interesantes propuestas para trabajos futuros a partir de los resultados obtenidos en esta tesis doctoral.

7.2 QTL de fecha de espigado encontrados en este trabajo

En cuanto a los genes de respuesta al fotoperiodo, tanto en la población Beka x Mogador como en el conjunto de pequeñas poblaciones de DHs, la región del gen *Ppd-H2* fue una de los principales determinantes de la fecha de espigado. En ambos casos, aquellas plantas que presentaron en esta región los alelos presentes en los cultivares de invierno fueron las más retrasadas. En el presente estudio se emplearon dos marcadores aparentemente flanqueantes de esta región, el microsatélite Bmag382 y el STS aMWG518, derivado del RFLP MWG518. Esta región resultó significativa en todos los ensayos de siembra otoñal pero, mientras en el caso de la población Beka x Mogador el pico de significación estaba localizado más próximo a Bmag382, en el caso de las pequeñas poblaciones de DHs, el marcador aMWG518 presentó una significación más alta. Esta región es además responsable de una considerable parte de la interacción genotipo por ambiente del rendimiento en la población Beka x Mogador.

Un aspecto interesante de esta región fue el hecho de que fue detectada en los ensayo de invernadero con plantas vernalizadas y fotoperiodo largo constante (Tabla 3, capítulo 3; Tabla 4, capítulo 4). La presencia de un efecto significativo de *Ppd-H2* en condiciones de fotoperiodo largo no ha sido descrita previamente en la literatura. El hecho de que haya sido detectado en dos conjuntos de ensayos totalmente independientes (la población Beka x Mogador y el conjunto de poblaciones de DHs) hace pensar que no se trata de un mero artificio estadístico. Este resultado sugiere la necesidad de replantearse el efecto descrito para este gen (hasta ahora sólo en fotoperiodos cortos), también en condiciones de fotoperiodos largos, quizá mediante su integración en un modelo de interacción con el gen de respuesta al fotoperiodo largo y los genes de vernalización.

Una estrategia correcta para la utilización de este QTL en programas de mejora sería la introgresión de la región entera mediante la selección de los alelos deseados en los dos marcadores flanqueantes del gen propuestos. La reciente identificación del gen *HvFT3* como posible candidato de *Ppd-H2* (Faure et al., 2007), facilitará un estudio más detallado de su efecto en distintas condiciones ambientales y con diferentes fondos genéticos.

Respecto al gen *Ppd-H1*, al no ser polimórfico en la población Beka x Mogador, su inclusión en el ensayo de validación en las poblaciones de DHs se debió a su importancia descrita en otros estudios (Laurie et al. 1994, 1995; Turner et al., 2005). Este gen resultó ser el más determinante para la fecha de espigado en condiciones de siembra invernal. Sin embargo, en las condiciones de siembra otoñal, que son las mayoritarias en los secanos del norte de España, este gen parece no tener un efecto destacable. Una posible explicación vendría dada por el hecho de que la mayor parte del desarrollo vegetativo del cultivo se produce bajo fotoperiodos inferiores a los que este gen necesita para que comience a expresarse su efecto, que son alrededor de 13 horas (Turner et al, 2005). En caso de siembras invernales y primaverales, éste seria uno de los genes principales a tener en cuenta. Además de tener un marcador diagnóstico dentro del propio gen, también existen una serie de marcadores en sus inmediaciones que podrían ser de gran ayuda en una estrategia de selección basada en marcadores flanqueantes.

El hecho de que *Ppd-H1* no fuera polimórfico en Beka x Mogador, permitió sin embargo una mejor caracterización y estimación de efectos asociados a la zona centromérica del cromosoma 2H, próxima a este gen, y en la que según la literatura está

localizado el gen de precocidad intrínseca *Eam6*. No existe un claro consenso en la literatura sobre los factores que podrían afectar a la expresión de este gen, ni sobre el tamaño de su efecto. Esto se puede deber, al menos parcialmente, a que en muchos estudios su efecto puede estar enmascarado por el gran efecto de *Ppd-H1* en su proximidad. En el estudio de Beka x Mogador, se puso de manifiesto el papel preponderante de *Eam6* en la determinación de la fecha de espigado en ensayos de campo sembrados en distintas fechas, y también bajo las diferentes condiciones de los ensayos de invernadero (Figura 3, Tabla 3, capítulo 3). Este efecto fue confirmado en las poblaciones de DHs (Figura 3, Tabla 4, capítulo 4). El efecto en estas poblaciones fue similar al detectado en Beka x Mogador y se demostró su independencia de *Ppd-H1*, en parte porque el desequilibrio de ligamiento que puede impedir la individualización de los efectos causados por QTL que están próximos en poblaciones de cruzamientos simples, se deshace después de unos pocos centimorgan en el análisis conjunto de las pequeñas poblaciones de DHs. Recientemente se ha identificado el gen *HvFT4* en esta región del cromosoma 2H (Faure et al., 2007); a partir del mismo se podrán realizar otros estudios en el futuro.

En el caso de la región de *Eam7*, en el cromosoma 6H, el microsatélite Bmag173 apareció consistentemente en todos los ensayos realizados con las poblaciones de DHs (Tabla 4, capitulo 4). Las plantas portadoras del alelo de 148 pb fueron siempre más tempranas que las que presentaban el alelo de 158 pb. El resto de alelos detectados presentaban comportamientos intermedios. Entre ellos, el alelo de 124 pb, se situaba en una posición intermedia frente a los otros dos. Estos resultados concuerdan con los obtenidos en la población Beka x Mogador. Beka presenta el alelo de 148 pb y Mogador el de 124 pb. La diferencia entre ellos fue significativa sólo en uno de los ensayos de siembra otoñal. También esta región fue significativa en el ensayo de plantas vernalizadas y fotoperiodo corto, el más parecido a los ensayos de campo de siembra otoñal. Comparando ambos alelos en el conjunto de poblaciones de DHs, también se observa que esta diferencia fue significativa sólo en algunos ensayos.

Respecto a los QTL de efecto menor, o detectados sólo en uno de los ensayos, existen algunos comunes entre los trabajos con Beka x Mogador y con las poblaciones de DHs, pero otros son diferentes (Figura 3, capítulo 3; Figura 3, capítulo 4).

Los genes de vernalización juegan un papel preponderante en la determinación de la fecha de floración en los casos en que la exposición a las bajas temperaturas sea inferior a la que necesita la planta. En aquellos genotipos que necesitan vernalización

para que comience su fase reproductiva, esta situación puede producirse cuando el invierno es suave para plantas sembradas en otoño, o cuando las plantas son sembradas demasiado tarde.

El estudio de los genes de vernalización ha sido uno de los aspectos que se han tratado más en profundidad en este trabajo. Su estudio se ha enfocado desde tres perspectivas diferentes. En la población Beka x Mogador, permitió explorar de una forma precisa sus efectos e interacciones, entre sí y con otros genes determinantes de la fecha de floración en una descendencia de un cruzamiento (capítulo 3). Se confirmó también el modelo sobre su interacción epistática (Figura 4, capítulo 3), ya descrito anteriormente (Takahashi y Yasuda 1971; Yan et al., 2004) y completado y ampliado por Dubcovsky et al. (2006) y Trevaskis et al. (2006). Ya se conocía empíricamente que la respuesta a la vernalización no se trata de un fenómeno de mera presencia o ausencia. Takahashi y Yasuda (1971) ya habían propuesto la clasificación de la respuesta a la vernalización en seis tipos diferentes. De esta manera, tanto los resultados de la población Beka x Mogador (capítulo 3), como los obtenidos en estudios realizados con poblaciones de cruzamientos simples entre parentales de primavera e invierno (Laurie et al., 1995, Karsai et al., 2005; Kóti et al., 2006) no permiten un estudio completo del fenómeno.

En la presente tesis doctoral se exponen otras dos aproximaciones a este estudio. Por un lado, se buscó la validación del modelo en la descendencia F_2 de cruzamientos entre variedades no sensibles a la vernalización (capítulo 5), comprobando que aquellos genotipos que heredan una copia de *Vrn-H2* (*Vrn-H2*/_) y dos copias del alelo reprimible de *Vrn-H1* (*vrn-H1/vrn-H1*) presentaban respuesta a la vernalización, aún cuando estos alelos provinieran de genotipos insensibles a la vernalización. También se vieron diferencias en la duración del periodo a espigado según se tratara de individuos homocigotos o heterocigotos para los dos genes implicados (Figura 2, capítulo 5), lo cual no se puede comprobar en los estudios clásicos con poblaciones de mapeo en las que los individuos suelen ser homocigotos. Por otro lado, se analizó una secuencia de nucleótidos dentro del primer intrón del gen *Vrn-H1*, dónde se supone que hay una región clave en la regulación de este gen por parte de *Vrn-H2* (von Zitzewitz et al., 2005). Este estudio puso de manifiesto que entre variedades, hay diferencias en la zona flanqueante a la región propuesta como crítica (Figura 5, capitulo 3), y el tamaño del intrón parece estar relacionado con un diferente grado de sensibilidad a la vernalización. Este fenómeno pudo verificarse también en el experimento de las 17 poblaciones de

DHs. En ellas se constató que líneas con diferentes longitudes del primer intrón de *Vrn-H1* presentaban diferencias en la sensibilidad a la vernalización (Tabla 5, capítulo 4). También se observó que líneas que presentaban la región crítica mostraban diferente sensibilidad a la vernalización, apoyando la hipótesis de que otras regiones del gen podrían estar implicadas en su regulación. Además, gracias a la presencia de varios alelos en *Vrn-H1*, se pudieron comparar los efectos causados por diversas combinaciones alélicas en los dos loci. La interacción epistática entre *Vrn-H1* y *Vrn-H2* explicó prácticamente toda la varianza fenotípica en condiciones de ausencia de vernalización y fotoperiodo largo.

7.3 Influencia de los principales QTL de fecha de espigado sobre el rendimiento

Además de este estudio en profundidad de los genes de vernalización y su influencia sobre la fecha de espigado, también se puso de manifiesto que determinadas combinaciones alélicas podrían también estar asociadas a diferencias significativas en el rendimiento, en determinados rangos de fecha de espigado (Figura 3, Tabla 6, capítulo 6).

Varios QTL fueron detectados en la población Beka x Mogador como causantes de diferencias en rendimiento en las condiciones de secano del norte de España (Tabla 2, Figura 2, capitulo 6). El efecto de estos QTL no es muy grande y además existe una cierta interacción QTL por ambiente. Por otra parte, los principales QTL de fecha de espigado fueron los causantes de que exista una interacción genotipo por ambiente del rendimiento, en las mismas condiciones y aparentemente de mayor importancia que los propios efectos principales (Figura 3, capitulo 6). A pesar de esta fuerte interacción, los resultados del capítulo 6 indican que los mayores rendimientos estuvieron asociados con fechas de espigado intermedias (Figura 2, capítulo 6), por lo que podría existir una ventana óptima de días a espigado que maximizara el rendimiento. La investigación sobre la mejora de la cebada para nuestras condiciones debería abordar como futuro objetivo la acotación de esa ventana (o ventanas, si la interacción genotipo por ambiente tiene un componente geográfico importante).

La consecuencia de todo ello es que una mejora en el rendimiento debería seguir una estrategia que combinara la elección de adecuadas combinaciones alélicas, tanto en los QTL de rendimiento como en los de espigado, aunque parece necesaria también una validación de los QTL de rendimiento en un conjunto de germoplasma de mayor base genética.

7.4 Referencias

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Capítulo 8

Conclusiones

Capítulo 8: Conclusiones

1. Se ha identificado un conjunto de QTL que explican la mayor parte de la variación en la fecha de espigado de la cebada, en siembras otoñales e invernales del Norte de España, así como los factores ambientales a los que responden.
2. Los QTL de fecha de espigado se han validado en un conjunto de germoplasma de amplia base genética, representativo del empleado en el programa español de mejora de la cebada, lo que ha permitido proponer un conjunto de marcadores para la gestión de este carácter en la mejora de la cebada.
3. Se ha demostrado que es posible llevar a cabo una detección de QTL en un conjunto complejo de germoplasma.
4. Se ha validado el modelo epistático de los genes de vernalización, *Vrn-H1* y *Vrn-H2*, mediante la recuperación de genotipos sensibles a la vernalización en la descendencia de cruzamientos entre individuos insensibles. Además, la utilización de marcadores perfectos ha permitido una precisa determinación de sus efectos e interacciones, en tres conjuntos distintos de materiales.
5. Se ha comprobado que la longitud del primer intrón del gen *Vrn-H1* está implicada en el grado de sensibilidad a la vernalización.
6. Las regiones donde se encuentran los genes *Eam6 y Ppd-H2*, son las principales responsables de las diferencias en precocidad en siembras otoñales, las más habituales en España. Así mismo, se ha constatado una ausencia de efecto del gen *Ppd-H1* en esas mismas condiciones. Estos tres genes presentan efectos parcialmente distintos y de distinta magnitud que los detectados en estudios llevados a cabo en latitudes más elevadas.
7. Se ha propuesto un modelo plausible para la explicación del fenómeno de la vernalización en día corto, que combina aditivamente la acción de *Ppd-H2* y la interacción epistática de *Vrn-H1* y *Vrn-H2.*
8. Se han encontrado siete QTL para rendimiento, con efectos individuales pequeños, dos de ellos detectados sólo al estudiar el rendimiento independientemente de la fecha de espigado. Se ha determinado que los principales QTL de espigado son responsables de una parte importante de la interacción genotipo por ambiente en el rendimiento.

9. La relación del rendimiento con el espigado es cambiante según las condiciones de los ensayos. En una población de amplio rango de variación para el espigado, como Beka x Mogador, existe una mayor variabilidad genotípica y un mayor potencial productivo para el rendimiento en las clases de espigado intermedio.

Anexos

Anexos

- Szücs P, JS Skinner, I Karsai, **A Cuesta-Marcos**, KG Haggard, AE Corey, THH Chen, PM Hayes. (2007) Validation of the VRN-H2/VRN-H1 epistatic model in barley reveals that intron length variation in VRN-H1 may account for a continuum of vernalization sensitivity. Molecular Genetics and Genomics 10.1007/s00438-006-0195-8

- Rossi C, **A Cuesta-Marcos**, I Vales, L Gomez-Pando, G Orjeda, R Wise, K Sato, K Hori, F Capettini, H Vivar, X Chen, and P Hayes. (2006) Mapping multiple disease resistance genes using a barley mapping population evaluated in Peru, Mexico, and the USA. Molecular Breeding 18: 355-366

Mol Genet Genomics
DOI 10.1007/s00438-006-0195-8

ORIGINAL PAPER

Validation of the *VRN-H2*/*VRN-H1* epistatic model in barley reveals that intron length variation in *VRN-H1* may account for a continuum of vernalization sensitivity

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Received: 11 August 2006 / Accepted: 5 November 2006

Abstract The epistatic interaction of alleles at the *VRN-H1* and *VRN-H2* loci determines vernalization sensitivity in barley. To validate the current molecular model for the two-locus epistasis, we crossed homozygous vernalization-insensitive plants harboring a predicted "winter type" allele at either *VRN-H1* (Dicktoo) or *VRN-H2* (Oregon Wolfe Barley Dominant), or at both *VRN-H* (Calicuchima-sib) loci and measured the flowering time of unvernalized F_2 progeny under long-day photoperiod. We assessed whether the spring growth habit of Calicuchima-sib is an exception to the two-locus epistatic model or contains novel "spring" alleles at *VRN-H1* (*HvBM5A*) and/or *VRN-H2* (*ZCCT-H*) by determining allele sequence variants at these loci and their effects relative to growth habit. We found that (a) progeny with predicted "winter type" alleles at both *VRN-H1* and *VRN-H2* alleles exhibited an extremely delayed flowering (i.e. vernalization-sensitive) phenotype in two out of the three F_2 populations, (b) sequence flanking the vernalization critical region of *HvBM5A* intron 1 likely influences degree of vernalization sensitivity, (c) a winter habit is retained when *ZCCT-Ha* has been deleted, and (d) the *ZCCT-H* genes have higher levels of allelic polymorphism than other winterhardiness regulatory genes. Our results validate the model explaining the epistatic interaction of *VRN-H2* and *VRN-H1* under long-day conditions, demonstrate recovery of vernalization-sensitive progeny from crosses of vernalization-insensitive genotypes, show that intron length variation in *VRN-H1* may account for a continuum of vernalization sensitivity, and provide molecular markers that are accurate predictors of "winter vs spring type" alleles at the *VRN-H* loci.

Keywords *BM5* · Cereal · *Hordeum vulgare* · *HvVRT-2* · *Triticeae* · *VRN1* · *VRN-1* · *VRN2* · *VRN-2*

Communicated by S. Hohmann.

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Introduction

The capacity of temperate grasses to survive the winter (i.e. winterhardiness) is associated with an extended exposure to low temperature (i.e. vernalization) in order to properly regulate the transition from vegetative to reproductive growth. Understanding the genetics of vernalization is a prerequisite to improving winterhardiness, and improved winterhardiness will allow for more sustainable and productive cereal crop production.

Based on winterhardiness traits, the growth habits of barley (*Hordeum vulgare* subsp. *vulgare*)—the second most important temperate cereal crop in the world—are classified as winter, facultative, or spring. The term "vernalization requirement" is misleading, as even winter growth habit barley genotypes do not "require" vernalization per se as they eventually will flower under continual warm temperatures and favorable growth conditions. Flowering of winter habit genotypes, while so delayed in the absence of vernalization as to be agronomically unacceptable, is significantly accelerated by vernalization. We utilize the terms "vernalization sensitivity" to indicate the delay in flowering time attributable to a lack of vernalization under long-day conditions and "vernalization response" to indicate the difference in flowering time between unvernalized and vernalized growth conditions.

The genetic basis of vernalization sensitivity in barley was first described as a three-locus epistatic model based on phenotypic data (Takahashi and Yasuda 1971). Using current nomenclature and chromosome designations, the loci are *VRN-H1* (5H), *VRN-H2* (4H) and *VRN-H3* (1H). Winter genotypes have the allelic architecture *Vrn-H2_/vrn-H1vrn-H1/vrn-H3vrn-H3* and all other allelic configurations lead to a lack of significant vernalization response (i.e. spring or facultative growth habits). There is no allelic variation at *VRN-H3* in most cultivated barley genotypes, reducing the genetic model to a two-locus epistatic model.

HvBM5A, a MADS-box floral meristem identity gene and member of the AP1 transcription factor family (Schmitz et al. 2000), is considered the determinant of the VRN-H1 locus. For nomenclatural simplicity, we use the *VRN-H1* designation to refer to both the locus and its effecter gene. The expression of *VRN-H1* and its wheat orthologs correlates with vernalization response, where increased expression promotes flowering (Danyluk et al. 2003; Dubcovsky et al. 2006; Loukoianov et al. 2005; Murai et al. 2003; Trevaskis et al. 2003, 2006; von Zitzewitz et al. 2005; Yan et al. 2003). In wheat and barley, allelic variation at *VRN-1* is associated with mutations in the promoter and/or first intron. While promoter mutations in a CArG-like motif were initially reported to account for differences in growth habit of wheat (Yan et al. 2003), this CArG-like motif was found to be invariant in barley genotypes differing in growth habit (von Zitzewitz et al. 2005). Subsequently, *T. monococcum* spring habit genotypes were identified that lack CArG motif mutations like barley (Yan et al. 2004a). Large (~2.8 kb) deletions within the first intron in *VRN-1* were identified as more likely determinants of spring growth habit in both barley and wheat and the proposed key regulatory region has been narrowed down to a highly conserved 0.44 kb "vernalization critical region" (Fu et al. 2005; von Zitzewitz et al. 2005).

A zinc finger-CCT (*CONSTANS*, *CONSTANS*-like, and *TOC*) domain transcription factor (*ZCCT*), encoding a flowering repressor down-regulated by vernalization, is the determinant of the wheat and barley *VRN-2* locus (Yan et al. 2004b). Allelic variation at this locus is ascribed to loss-of-function mutations or complete deletion, leading to recessive inheritance of spring growth habit (Dubcovsky et al. 2005; Karsai et al. 2005; von Zitzewitz et al. 2005; Yan et al. 2004b). There are three tightly linked *ZCCT* genes in barley (*ZCCT-Ha*, *ZCCT-Hb* and *ZCCT-Hc*) and two in diploid wheat (*ZCCT-1* and *ZCCT-2*) at this locus (Dubcovsky et al. 2005; Karsai et al. 2005; Yan et al. 2004b). *ZCCT-1* and *ZCCT-Ha* in wheat and barley, respectively, have been reported to be the most likely candidates for *VRN-2* (Dubcovsky et al. 2005; Yan et al. 2004b).

The two-locus epistatic *VRN* gene interaction has been supported by extensive phenotypic data in wheat and barley populations derived from crosses of vernalization-sensitive (i.e. winter) by vernalization-insensitive (i.e. facultative or spring) growth habits (Dubcovsky et al. 1998, 2005, 2006; Fu et al. 2005; Karsai et al. 2005; Kóti et al. 2006; Laurie et al. 1995; Takahashi and Yasuda 1971; Tranquilli and Dubcovsky 2000; Yan et al. 2003, 2004a, b). Yan et al. (2003, 2004a, b) proposed a molecular model to explain the *VRN-2/VRN-1* epistatic interaction in cereals where *Vrn-2* encodes a dominant repressor—down-regulated by vernalization—that inhibits the expression of *vrn-1* alleles. In this model, no vernalization sensitivity is predicted in genotypes with recessive *vrn-2* alleles (loss-of-function mutations or complete deletions of *ZCCT*), regardless of allelic state at *VRN-1*. Likewise, genotypes with both dominant *Vrn-2* and *Vrn-1* alleles (lacking a repressor binding site in the promoter and/or in the intron 1) are also predicted to show minimal vernalization sensitivity.

There is evidence that the photoperiod and vernalization pathways of flowering are interconnected. High levels of barley and wheat *ZCCT* expression were detected only when photoperiod-responsive plants were grown under long-day photoperiod (Dubcovsky et al. 2006; Trevaskis et al. 2006), providing a molecular basis for explaining quantitative trait locus (QTL) associations between *VRN-H2* and photoperiod (Karsai et al. 2005, 2006; Laurie et al. 1995; Szűcs et al. 2006). *ZCCT* expression is repressed in photoperiod-responsive wheat and barley plants grown under short-day conditions (at both cold and warm temperatures), yet no increase in *VRN-1* expression is observed for

several weeks, suggesting the existence of at least one other *VRN-1* repressor (Dubcovsky et al. 2006; Trevaskis et al. 2006). A candidate gene is *VRT-2*, a putative flowering repressor MADS-box gene regulated by both vernalization and day-length that shows higher expression under short-day vs long-day in a photoperiod-sensitive barley (Kane et al. 2005). *HvVRT-2*, on the short arm of chromosome 7H, is associated with photoperiod sensitivity QTL in barley (Szűcs et al. 2006).

Our objective in this study was to genetically validate the *VRN-H2*/*VRN-H1* epistatic model under long-day photoperiod, using genotypic and phenotypic data from three F_2 populations. We examined allelic variation at the dominant *Vrn-H2* and *Vrn-H1* loci to define the role, or roles, of the duplicated *ZCCT-H* genes and the number and structure of regulatory sites in *VRN-H1* and how these relate to the timing of flowering and sensitivity to vernalization.

Materials and methods

Plant materials and phenotype evaluation

Dicktoo (Dt) (Hayes et al. 1993), the Oregon Wolfe Barley Dominant genetic stock (hereafter referred to as OWB-D or Od) (Wolfe and Franckowiak 1991), and Calicuchima-sib (Cb) (Hayes et al. 2000) are vernalization-insensitive barley genotypes. Dicktoo has a winter (i.e. recessive) *vrn-H1* allele and a spring (i.e. recessive) *vrn-H2* allele due to deletion of all three *ZCCT-H* genes (Fu et al. 2005; Karsai et al. 2005; von Zitzewitz et al. 2005)—this allele configuration (*vrn-H2vrn-H2*/*vrn-H1vrn-H1*) defines the facultative growth habit. OWB-D has a spring (i.e. dominant) *Vrn-H1* allele due to a large deletion of the intron 1 vernalization critical region (Fu et al. 2005) and a winter (i.e. dominant) *Vrn-H2* allele containing all three *ZCCT-H* genes (see Results). The spring growth habit Calicuchima-sib, while containing the *ZCCT-H* genes and *VRN-H1* intron 1 vernalization critical region, is deleted for sequence flanking the critical region (see Results).

The three genotypes were crossed to produce three segregating populations (Dicktoo × OWB-D (Dt × Od), Dicktoo × Calicuchima-sib (Dt × Cb), and Calicuchima-sib × OWB-D (Cb × Od)) and test for vernalization sensitivity and determine whether the Calicuchima-sib spring habit is an exception to the two-locus epistatic model or contains novel "spring" alleles at *VRN-H1* and/or *VRN-H2*. F_1 plants derived from each of the three parental crosses were self-fertilized to produce three F_2 populations of 93 plants each. For comparison of flowering time and/or allelic variation at the *VRN-H* loci, three accessions of known winter growth habit were used: Kompolti korai—described by Karsai et al. (2001), Hoody—released in 1994 by the Oregon Agricultural Experiment Station, and the *Hordeum vulgare* subsp. *spontaneum* accession Caesarea 26–24 (also known as OSU11)—described by Karsai et al. (2004).

Vernalization sensitivity was measured based on the method of Takahashi and Yasuda (1971). All plants were grown under greenhouse conditions with supplemental light on a 16 h light/24 h photoperiod and a constant 18 ± 1.5°C day and night temperature, without any vernalization. The number of days from seedling emergence to flowering—Zadock's scale developmental phase 9 and 49, respectively (Tottman and Makepeace 1979)—were scored for each plant. The experiment was terminated 170 days after planting, and plants that had not flowered were assigned a days to flowering value of 170.

Allele sequencing and genotyping

The following primer set was designed to clone and sequence full-length *ZCCT-Ha* and *ZCCT-Hb* genes: ZCCTH.12F (5′-tcaaatattctagcagtggccttg-3′) to a conserved segment of the 5′ UTRs of published barley partial *ZCCT-H* gene sequences AY485977 and AY485978 and ZCCT2.08R (5′-tggcggtcgtgatggtttactc-3′) to a conserved 3′ UTR region of the *Triticum monococcum* full-length *ZCCT-1* and *ZCCT-2* gene sequences (AY485644). Primers ZCCT.HcF (5′-caccatcgcatgatgcac-3′) and ZCCT.HcR (5′-tcatatggcgaagctggag-3′) were used to amplify a 194-bp long fragment of the *ZCCT-Hc* gene (AY687931). Previously reported primer pairs HvBM5.27F (5′-aggcctattcgtttgcaatgc-3′); HvBM5.06R (5′-atctcgtgcgccttcttgag-3′), and HvBM5.55F (5′-atgcatagaataattggctccagc-3′); HvBM5.56R (5′-cagtaagcactacgatgatgataaac-3′) were used to clone the promoter region and partial intron 1 of *VRN-H1*, respectively (von Zitzewitz et al. 2005). For each allele isolated via PCR, cloned amplicons from at least two independent PCRs were sequenced to confirm PCR-based nucleotide substitutions were not present. Sequences of the non-primer portion of each consensus amplicon were deposited with GenBank and accession numbers are given in Table 1.

Table 2 shows the gene-specific primers used to assign allele types in individuals of the F_2 segregating populations. The *VRN-H1* locus was genotyped using a co-dominant promoter SSR marker and intron 1-specific dominant markers. The intron 1 primer pairs were designed to be diagnostic for the allele type at the vernalization critical region (see Fig. 5). To develop co-dominant markers for the intron 1 region

Table 1 Accession numbers for *ZCCT-H* and *VRN-H1* (*HvBM5A*) sequences

Gene	Allele	Accession	Determined region	Size (bp)
ZCCT-Ha	Calicuchima-sib	DQ492695	Full gene	2,361
ZCCT-Hb	Calicuchima-sib	DQ492696	Full gene	2,353
ZCCT-Ha	OWB-D	DQ492697	Full gene	2,356
ZCCT-Hb	OWB-D	DQ492698	Full gene	2,357
ZCCT-Ha	Kompolti korai	DQ492699	Full gene	2,365
ZCCT-Hb	Kompolti korai	DQ492700	Full gene	2,336
ZCCT-Hb	Caesarea 26-24/OSU11	DQ492701	Full gene	2,357
VRN-H1 (*HvBM5A*)	Calicuchima-sib	DQ492702	Promoter	2,192
VRN-H1 (*HvBM5A*)	OWB-D	DQ492703	Promoter	2,174
VRN-H1 (*HvBM5A*)	Dicktoo	AY785817	Promoter	2,207
VRN-H1 (*HvBM5A*)	Kompolti korai	AY785824	Promoter	2,187
VRN-H1 (*HvBM5A*)	Caesarea 26-24/OSU11	AY785820	Promoter	2,210
VRN-H1 (*HvBM5A*)	Calicuchima-sib	DQ492704	Partial intron 1	1,177
VRN-H1 (*HvBM5A*)	OWB-D	AY750996	Full intron 1	4,364
VRN-H1 (*HvBM5A*)	Dicktoo	AY750994	Full intron 1	10,789
VRN-H1 (*HvBM5A*)	Kompolti korai	AY866487	Partial intron 1	2,625
VRN-H1 (*HvBM5A*)	Caesarea 26-24/OSU11	AY866492	Partial intron 1	2,588

Table 2 Primers used for genotyping *VRN-H1* (*HvBM5A*), *ZCCT-H*, *HvSNF2*, and *HvVRT-2* in F_2 populations

Gene/region	Primer F (5′→3′)	Primer R (5′→3′)	Amplified allele[a]	F_2 population[b]
VRN-H1 promoter	HvBM5.82 (atatctactccagcctagggtac)	HvBM5.83 (cgcgaatctcccccatattgc)	Cb 173, Dt 189, Od 155	Dt × Od, Dt × Cb, Cb × Od
VRN-H1 intron 1	HvBM5.87 (gaaaggacgtgtatgttgaggtg)	HvBM5.89 (gtctgagtcggttatatgcagg)	Dt 749	Dt × Od
VRN-H1 intron 1	HvBM5.87 (gaaaggacgtgtatgttgaggtg)	HvBM5.86 (tcccccattctcgtcaaaaagc)	Od 531	Dt × Od, Cb × Od
VRN-H1 intron 1	HvBM5.60 (gctttattttcttctctgccgttcc)	HvBM5.89 (gtctgagtcggttatatgcagg)	Dt 305	Dt × Cb
VRN-H1 intron 1	HvBM5.60 (gctttattttcttctctgccgttcc)	HvBM5.56 (cagtaagcactacgatgatgataaac)	Cb 369	Dt × Cb
VRN-H1 intron 1	HvBM5.68 (gtgaggggagctgcaatggtg)	HvBM5.86 (tcccccattctcgtcaaaaagc)	Cb 642	Cb × Od
ZCCT-Ha/b	ZCCTH.06 (cctagttaaaacatatatccatagagc)	ZCCTH.07 (gatcgttgcgttgctaatagtg)	Od and Cb 307/273	Dt × Od, Dt × Cb
HvSNF2	HvSNF2.02 (cctggccacaaaaacaatcagc)	HvSNF2.04 (gctgcattatagagaaacaacaacg)	Cb 214, Dt 382, Od 270	Dt × Od, Dt × Cb, Cb × Od
HvVRT-2	HvVRT-2.01 (gagttgcagcagatgg)	HvVRT-2.06 (caggtcactaatttgttgcatga)	Cb, Dt and Od 204 (*MspI*)	Dt × Cb, Cb × Od

[a] *Cb* Calicuchima-sib, *Dt* Dicktoo, *Od* OWB-D; fragment size (bp) and utilized restriction endonuclease indicated

[b] *Dt* × *Od* Dicktoo × OWB-D; *Dt* × *Cb* Dicktoo × Calicuchima-sib, *Cb* × *Od* Calicuchima-sib × OWB-D

we combined dominant markers in a single PCR. Triplexing primers HvBM5.68F, HvBM5.87F, and HvBM5.86R distinguished the two parental types and identified heterozygotes for the Cb × Od population. Triplexing primers HvBM5.87F, HvBM5.86R, and HvBM5.89R for Dt × Od, and HvBM5.60F, HvBM5.56R, and HvBM5.89R for Dt × Cb amplified only the shorter parental fragment in the case of heterozygotes. To genotype the *VRN-H2* locus, we used both *ZCCT-H*-specific (dominant) and tightly linked *HvSNF2*-specific (co-dominant) markers (Karsai et al. 2005). The *HvVRT-2* gene was genotyped with a co-dominant CAPS marker (Szűcs et al. 2006). All genotyping PCRs were replicated at least twice to validate allele scores.

Statistical analyses

Goodness of fit test (χ^2) was used to compare observed and predicted allele segregation patterns at the different loci. Analysis of variance was performed using the general linear model (GLM) procedure of The SAS System for Windows, Release 8.2 (SAS Institute 2001). Sequence analyses were conducted using GeneDoc version 2.6 (Karl and Hugh 1997) and MEGA version 3.1 (Kumar et al. 2004).

Results

Vernalization-sensitive progeny are recovered from vernalization-insensitive parental crosses

Based on the *VRN-H2*/*VRN-H1* epistatic model, we expected that F_2 progeny with *Vrn-H2_*/*vrn-H1vrn-H1* alleles derived from crosses between vernalization-insensitive (i.e. early flowering) genotypes harboring *Vrn-H2Vrn-H2*/*Vrn-H1Vrn-H1* and *vrn-H2vrn-H2*/*vrn-H1vrn-H1* alleles should exhibit a vernalization-sensitive (i.e. late flowering) phenotype. Unvernalized winter habit controls (Hoody and Kompolti korai) flowered significantly later than facultative (Dicktoo) and spring (Calicuchima-sib and OWB-D) parents under long-day photoperiod (Table 3). There was also variation for flowering time among the parents, and on average, Calicuchima-sib flowered six days later than Dicktoo and 39 days later than OWB-D. The F_1 of each of the three crosses flowered significantly earlier than any of the winter habit controls and flowering times were between those of the respective parents.

Transgressive segregation for flowering time was observed in the Dt × Od and Dt × Cb, but not in the Cb × Od, F_2 populations (Fig. 1). Seventy two Dt × Od plants flowered in 29–64 days, within the range of the vernalization-insensitive OWB-D and Dicktoo parents. Twenty one late flowering transgressive segregants flowered in 105–148 days, values which are as high as or higher than the flowering time of the winter controls, indicating a vernalization-sensitive phenotype. Likewise, in the Dt × Cb F_2 population, there were early and late transgressive segregants. Seventy seven plants flowered in 36–89 days, most of them as early, or earlier, than the parents. Twelve plants flowered in 113–153 days and four plants died after 158–169 days without flowering. The 93 Cb × Od F_2 plants all flowered after 31–68 days, values within the range of the parents.

Segregation of *VRN-H1*, *VRN-H2*, and *HvVRT2* loci

We determined the allelic architecture at the two *VRN-H* and the *HvVRT-2* loci for the three parental genotypes—sequence accessions are shown in Table 1. All three *ZCCT-H* genes are deleted in Dicktoo and present in Calicuchima-sib and OWB-D. The three parents have different allele sizes at the *ZCCT-H* proximal *HvSNF2* gene (Table 2), which was useful in corroborating the dominant marker data for *VRN-H2*. The three parents differed at *VRN-H1* in the length of a promoter TA-repeat, and the intron 1 vernalization critical region (Fig. 5). We determined ~1 kb of *HvVRT-2* genomic sequence: Dicktoo and OWB-D were 100% identical, but differed from Calicuchima-sib in six single nucleotide polymorphisms (SNPs) (data not shown).

Ninety-three F_2 plants from each of the three populations were genotyped for the *VRN-H2* and *VRN-H1* loci using allele-specific primer sets (Table 2). At the *VRN-H2* locus, the co-dominant *HvSNF2* marker confirmed the results obtained with the dominant *ZCCT-H* marker with one exception, indicating a recombination event occurred between these two tightly linked genes. One intragenic recombinant between the *VRN-H1* promoter SSR and the intron 1 vernalization critical region was observed in the Dt × Od population. Due to lack of polymorphism between the Dicktoo and OWB-D *HvVRT-2* alleles, the segregation of alleles at this locus was determined only for the Dt × Cb and Cb × Od populations. There was an excellent fit of observed to expected segregation ratios for alleles at the *VRN-H1*, *VRN-H2*, and *HvVRT2* loci in all tested F_2 populations ($P = 0.08$–0.86 for the χ^2 tests).

VRN-H loci are major determinants of flowering time under long-day photoperiod

Based on the genotype data, *VRN-H1*, *VRN-H2*, and *HvVRT-2* alleles were assigned to each F_2 plant and an

Table 3 Days to flowering, growth habit, and *VRN-H* allele combination for control, parental, and F_1 genotypes

Genotype	Days to flowering[a]	Growth habit	*VRN-H2*/*VRN-H1*[b]	Germplasm
Hoody	114.5 ± 6.4	Winter	*V2V2*/*v1v1*	Control
Kompolti korai	110.7 ± 6.1	Winter	*V2V2*/*v1v1*	Control
Dicktoo	65.6 ± 10.3	Facultative	*v2v2*/*v1v1*	Parent
OWB-D	33.2 ± 2.1	Spring	*V2V2*/*V1V1*	Parent
Calicuchima-sib	72.0 ± 2.7	Spring	*V2V2*/*V1V1*	Parent
Dicktoo × OWB-D	53.7 ± 2.1	Spring	*V2v2*/*V1v1*	F_1
Dicktoo × Calicuchima-sib	66.5 ± 3.7	Spring	*V2v2*/*V1v1*	F_1
Calicuchima-sib × OWB-D	39.5 ± 2.6	Spring	*V2V2*/*V1V1*	F_1

Plants were grown under long-day conditions without vernalization

[a] Standard deviation is shown

[b] *V2*: *Vrn-H2*, *v2*: *vrn-H2*, *V1*: *Vrn-H1*, *v1*: *vrn-H1*

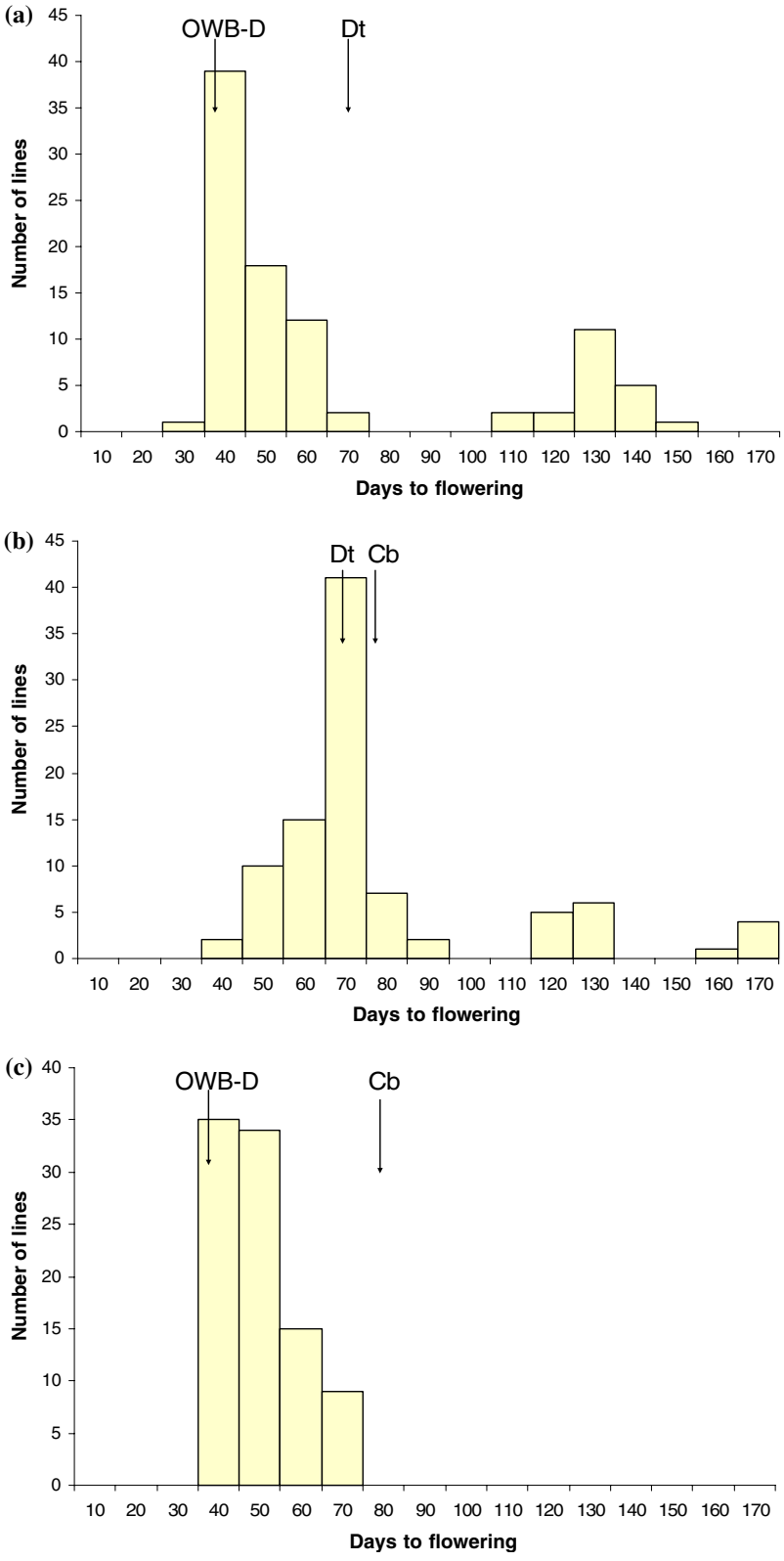

Fig. 1 Frequency distribution of days to flowering in **a** Dicktoo × OWB-D, **b** Dicktoo × Calicuchima-sib, and **c** Calicuchima-sib × OWB-D F_2 populations under long-day photoperiod without vernalization. Plants that had not flowered were assigned a days to flowering value of 170. Days to flowering values of Calicuchima-sib (*Cb*), Dicktoo (*Dt*), and *OWB-D* parents are indicated by *arrows*

analysis of variance was performed using the days to flowering data as an indicator of vernalization sensitivity (Table 4). This analysis revealed that the allele type at *VRN-H1* significantly ($P < 0.0001$) influenced days to flowering in all three populations. The effects of alleles at the *VRN-H2* locus, and the interaction between the *VRN-H1* and *VRN-H2* loci, were also significant ($P < 0.0001$) for the Dt × Od and Dt × Cb

Table 4 Effects of *VRN-H1*, *VRN-H2*, *HvVRT-2*, and their interactions, on days to flowering in F_2 populations

Source of variation[a]	Degrees of freedom			Type III mean square[b]		
	Dt × Od	Dt × Cb	Cb × Od	Dt × Od	Dt × Cb	Cb × Od
VRN-H1	2	2	2	23,403****	20,817****	935****
VRN-H2	2	2	2	8,787****	6,235****	21
VRN-H1 × *VRN-H2*	4	4	4	4,923****	2,702****	68
HvVRT-2	–	2	2	–	85	3
VRN-H1 × *HvVRT-2*	–	4	4	–	20	22
VRN-H2 × *HvVRT-2*	–	4	4	–	107	14
VRN-H1 × *VRN-H2* × *HvVRT-2*	–	6	3	–	130	29
Error	84	68	71	49	94	49

Plants were grown under long-day conditions without vernalization; Dt × Od, Dt × Cb, Cb × Od: see Table 2

[a] Due to lack of a polymorphism, *HvVRT-2* was not genotyped in the Dt × Od population

[b] Significance at the 5, 1, 0.1 and 0.01% levels are indicated by *, **, *** and ****, respectively

populations. The two *VRN-H* loci, and their interaction, accounted for 97% and 91% of the phenotypic variance in flowering time in the Dt × Od and Dt × Cb populations, respectively. Both Calicuchima-sib and OWB-D have dominant *Vrn-H1* and *Vrn-H2* loci, and as expected, the analysis of variance revealed no significant effect of *Vrn-H2* for the Cb × Od populations. Interestingly, *Vrn-H1* significantly influenced the flowering time—the mean numbers of days to flowering were 40 and 44 for plants harboring a homozygous vs heterozygous OWB-D allele, respectively, and 58 days for those having a homozygous Calicuchima-sib allele. Allelic variation at the *HvVRT-2* locus and its two-way or three-way interactions with the *VRN-H* loci had no significant effect on flowering time under long-day growth conditions (Table 4).

The observed plant numbers in the nine allele classes at the two *VRN-H* loci showed excellent fit to expected ratios: $P = 0.54$ and $P = 0.60$ for the χ^2 tests of the Dt × Od and Dt × Cb populations, respectively. The mean number of days to flowering in each genotype class was higher for the Dt × Cb population than for the Dt × Od population, and in both populations, there were significant differences in flowering time between certain allele classes (Fig. 2). All plants with winter alleles at both *VRN-H* loci (*Vrn-H2_/vrn-H1vrn-H1*) flowered significantly later than plants in any other allele class. Similarly, all late transgressive segregant plants had the winter allele combinations at the two *VRN-H* loci. There were also some significant differences between the seven early flowering allele classes. For example, *Vrn-H2Vrn-H2/Vrn-H1vrn-H1* plants flowered significantly later than any plant with homozygous dominant *Vrn-H1* alleles in the Dt × Od population, while *vrn-H2vrn-H2/Vrn-H1Vrn-H1* plants flowered significantly earlier than any other F_2 class in the Dt × Cb population (Fig. 2).

Allelic variation at the *VRN-H* loci

Winter habit controls Hoody and Kompolti korai contain all three *ZCCT-H* genes, while Caesarea 26–24 has

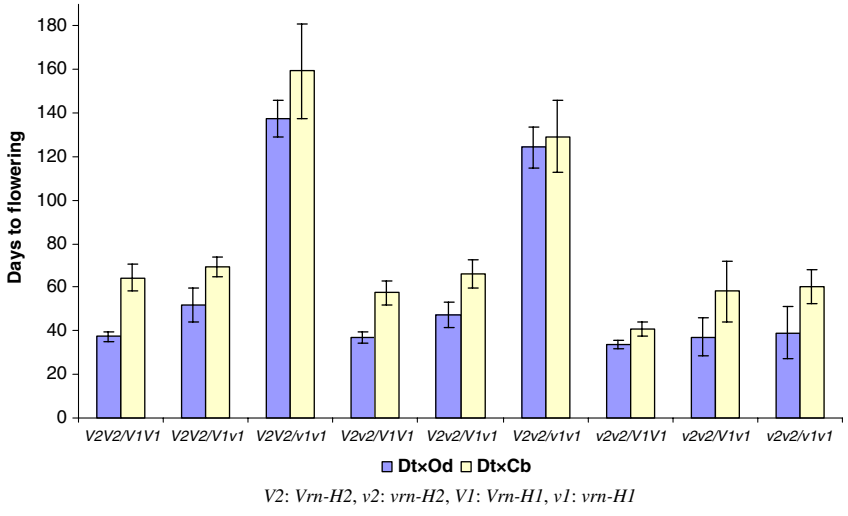

Fig. 2 Mean numbers of days to flowering of the nine different *VRN-H2/VRN-H1* allele classes in the F_2 progeny of the Dicktoo × OWB-D (Dt × Od), and Dicktoo × Calicuchima-sib (Dt × Cb) crosses under long-day photoperiod without vernalization. Plants that had not flowered were assigned a days to flowering value of 170. *Error bars* show standard deviation

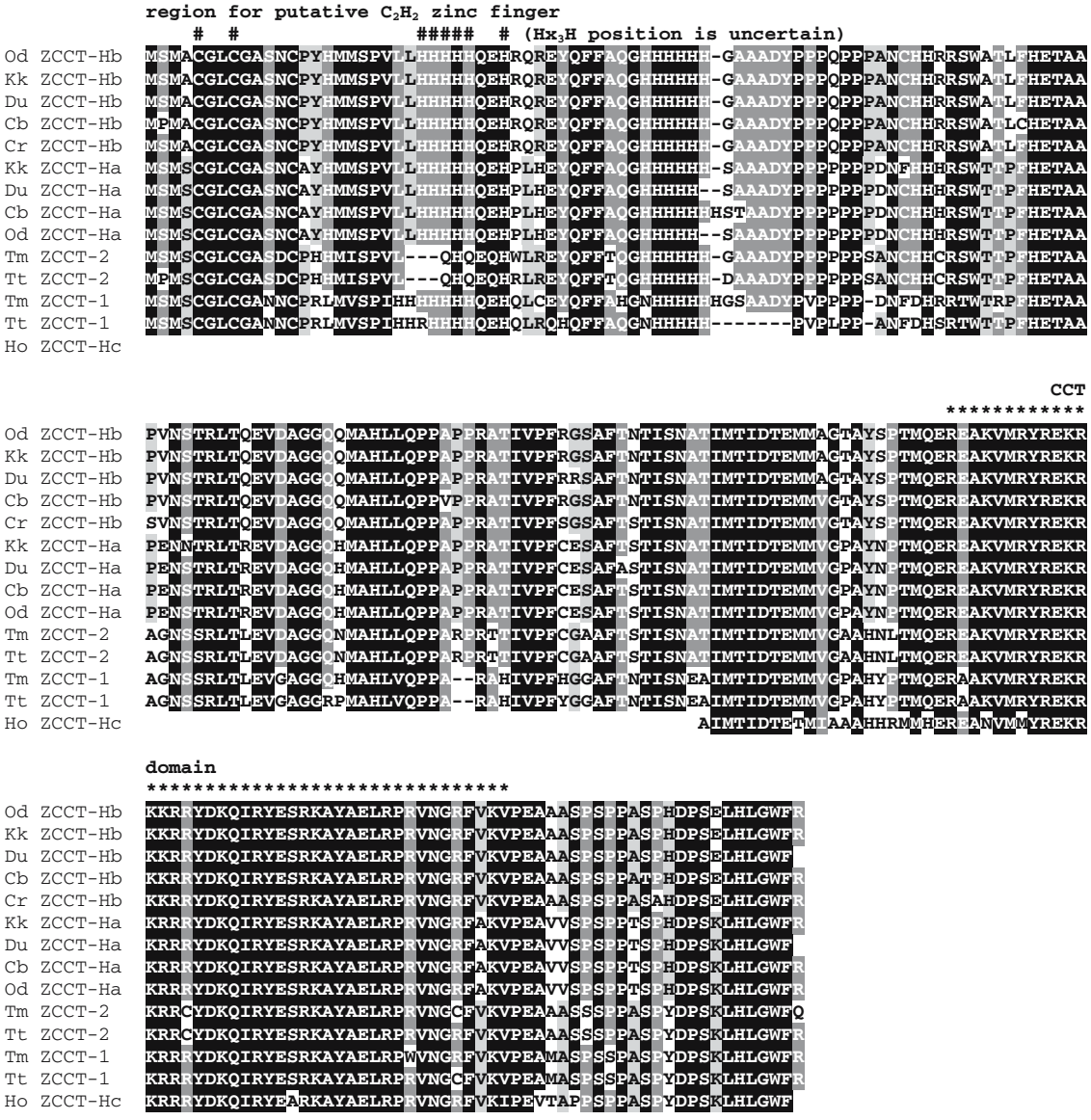

Fig. 3 Sequence alignment of barley and wheat ZCCT family members. Similarity is shown to four levels and regions of putative C_2H_2 zinc finger (#) and CCT domain (*) are indicated. *H. vulgare* sequences shown are from Caesarea 26–24 (Cr; DQ492701), Calicuchima-sib (Cb; DQ492695 and DQ492696), Dairokkaku (Du; AY485977 and AY485978), Hayakiso (Ho; AY687931), Kompolti korai (Kk; DQ492699 and DQ492700), and OWB-D (Od; DQ492697 and DQ492698). Representative wheat sequences shown are from *T. monococcum* (Tm; AY485644) and *T. turgidum* (Tt; AY485979 and AY485980)

only *ZCCT-Hb* and *ZCCT-Hc*. Multiple primer pairs specific to various regions of *ZCCT-Ha* failed to amplify a product from Caesarea 26–24, suggesting this gene is absent in this winter habit wild barley accession (data not shown). To investigate the allelic variation at the dominant *Vrn-H2* locus found in different spring and winter habit genotypes, full-length *ZCCT-Ha* and *ZCCT-Hb* gene sequences were determined for Calicuchima-sib, OWB-D, and Kompolti korai, and *ZCCT-Hb* of Caesarea 26–24 (Table 1). Comparison of the three *ZCCT-Ha* alleles showed 35 SNPs and three small (3–6 bp) insertions/deletions (INDELs), while *ZCCT-Hb* alleles displayed even greater allelic diversity with 96 SNPs and eight INDELs (2–18 bp)—the polymorphisms occurred throughout all regions (UTRs, introns and exons) of the genes. All *ZCCT-H* alleles encode full-length polypeptides and the polymorphisms lead to a number of amino acid substitutions and INDELs (Fig. 3). ZCCT-Ha and ZCCT-Hb are more similar to wheat ZCCT-2 than to ZCCT-1 based on a Neighbor-Joining tree of the full-length polypeptides (Fig. 4); analogous tree topologies were also obtained using the Minimum Evolution and UPGMA clustering methodologies (data not shown).

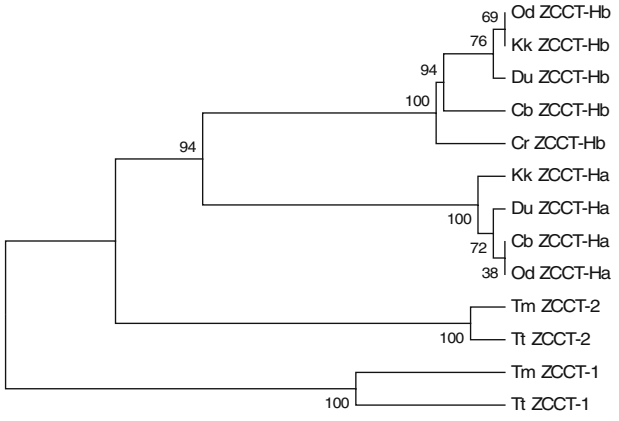

Fig. 4 Neighbor-Joining phylogenetic cluster analyses of barley and wheat ZCCT amino acid sequences. Confidence values on the branches are based on 1,000 bootstraps. See Fig. 3 legend for sequence accessions

We sequenced ~2.2 kb of the *VRN-H1* promoter from Calicuchima-sib and OWB-D and aligned these sequences with the eleven previously reported accessions (including Dicktoo, Kompolti korai and Caesarea 26–24) from von Zitzewitz et al. (2005). Calicuchima-sib and OWB-D share the three polymorphisms common to all spring genotypes relative to winter and facultative genotypes, confirming the promoters are of spring habit origin. With the exception of a TA repeat length, the promoter region sequences of Calicuchima-sib and the spring genotypes Harrington and Morex are identical. OWB-D was found to contain a unique SNP (actcatttgg vs cctcatttgg) in the CArG box relative to all other barley genotypes reported to date—this CArG-box was identified as a determinant of spring growth habit in wheat (Yan et al. 2003).

We previously reported that the spring habit OWB-D genotype has a ~6.4 kb deletion in the *VRN-H1* intron 1 compared to the intron 1 present in Caesarea 26–24, Dicktoo and Kompolti korai (Fu et al. 2005; von Zitzewitz et al. 2005). Cloning and sequencing a partial *VRN-H1* intron 1 fragment in the spring habit Calicuchima-sib revealed the 436 bp vernalization critical region was intact but the remainder of the 2.8 kb barley-wheat conserved region was deleted—the deletion begins 13 bp downstream from the 3′ end of the vernalization critical region (Fig. 5).

Discussion

We tested and verified the two-locus epistatic model (Takahashi and Yasuda 1971; Yan et al. 2004b) by selecting vernalization-insensitive parents genotyped to harbor a predicted "winter type" allele at either *VRN-H1* or *VRN-H2*, and recovered early and late flowering progeny in the expected ratios in two F_2 populations. We used flowering time under long-day conditions in the absence of vernalization (Takahashi and Yasuda 1971) as a diagnostic indicator of vernalization sensitivity and growth habit. In an F_2 population, it is not possible to have vernalized and unvernalized treatments of the same genotype, since each plant is a unique individual. Proof of vernalization response requires comparison of flowering time in the unvernalized vs vernalized F_3 progeny of *Vrn-H2Vrn-H2*/*vrn-H1vrn-H1* F_2 homozygotes—this experiment is currently being initiated. It is also important to reiterate that these experiments were conducted under long-day photoperiod conditions as recent evidence suggests that the Yan et al. (2004b) molecular model is not applicable under short-day conditions (Dubcovsky et al. 2006; Trevaskis et al. 2006).

Our current results validated a novel dimension of the two-locus epistatic model that was not assessable in F_2 populations derived from crosses of homozygous winter (*Vrn-H2*/*vrn-H1*) by homozygous spring (*vrn-H2*/*Vrn-H1*) growth habit barley genotypes (Dubcovsky et al. 2005; Kóti et al. 2006; Takahashi and Yasuda 1971; Yan et al. 2004b). We show that genotypes inheriting *ZCCT-H* (*Vrn-H2_*) and two repressible *VRN-H1* copies (*vrn-H1vrn-H1*) from vernalization-insensitive backgrounds manifest significant sensitivity to

Fig. 5 Schematic representation of the *VRN-H1* intron 1 from four barley genotypes representing dominant and recessive alleles (denoted). Edges of exons 1 and 2 are indicated by *flanking black boxes*. *Gaps* represent deletions relative to the full-length Dicktoo allele (AY750994). The 2.8 kb barley-wheat conserved region (*dashed line*) and the 436 bp vernalization critical region (*dotted box*) are indicated. Positions and orientations of *HvBM5* primers used to characterize the different alleles (Table 2) are indicated by *black triangles*; primer numbers are denoted

vernalization. Using *VRN-H*-specific markers our results also confirmed the observation of Reinheimer et al. (2004) where a double haploid population derived from a cross between homozygous vernalization-insensitive parents segregated for growth habit. The use of F_2 segregating progeny in the current study provided an opportunity to assess the degree of dominance at each of the loci and we observed *VRN-H* allele dosage effects. Based on the allele class means, *Vrn-H1Vrn-H1* plants flowered earlier than the *Vrn-H1vrn-H1* plants with the same *VRN-H2* allele configuration, showing incomplete dominance of the *Vrn-H1* allele. This is likely due to expression differences where the *vrn-H1* allele is still susceptible to repression and only the *Vrn-H1* allele is active. The partial dominance of alleles at *VRN-1* has been reported for both barley and wheat (Iwaki et al. 2001; Kóti et al. 2006; Loukoianov et al. 2005). The *Vrn-H2* allele also showed incomplete dominance: *Vrn-H2Vrn-H2* plants flowered later than the *Vrn-H2vrn-H2* plants (given a homozygous recessive or heterozygous allele configuration at *VRN-H1*), suggesting level of available repressor as the basis. Using three F_2 populations we were also able to compare the effects of two different dominant *Vrn-H1* alleles. Flowering time was more delayed for every genotype class for crosses containing a Calicuchima-sib allele relative to an OWB-D allele. This is likely due to the OWB-D allele being a very strong, whereas Calicuchima-sib is a weak, *Vrn-H1* allele.

Under long-day photoperiod conditions, *VRN-H1* played a more significant role in influencing flowering time than *VRN-H2*, corroborating prior reports (Dubcovsky et al. 2005; Kóti et al. 2006) and supporting *VRN-H1*'s function as a central control point for the transition from a vegetative to reproductive meristem. In support of the two locus model, inclusion of the interaction with *VRN-H2* explained nearly all the phenotypic variance in flowering time. While the putative flowering repressor *HvVRT-2* is expressed under both day-lengths in barley (Kane et al. 2005), the *HvVRT-2* locus or its interaction with the *VRN-H* loci had no significant effect on flowering time under long-day conditions.

To better understand the phenotypic variation of vernalization sensitivity, we also explored the allelic variation of the *VRN-H2* and *VRN-H1* genes in the relevant accessions. Comparison of the full-length *ZCCT-Ha* and *ZCCT-Hb* genomic sequences from spring and winter growth habit accessions revealed high polymorphism levels throughout both genes, including at the amino acid level. In contrast, polypeptides encoded by other genes involved in winterhardiness traits (vernalization response—*VRN-H1*, *HvVRT-2*; photoperiod perception—phytochromes and cryptochromes; cold tolerance—*CBF*s, *ZFP16*, *ICE2*) show minimal allelic variation (Skinner et al. 2006; Szűcs et al. 2006; von Zitzewitz et al. 2005). The high level of allelic variation at *ZCCT-H* could influence repressor effectiveness and contribute towards variation in flowering time. Each allele encodes a full-length ZCCT-H polypeptide and the putative C_2H_2 zinc finger (Yan et al. 2004b) is invariant while an alanine/valine substitution at the CCT domain (Griffiths et al. 2003) differentiates the ZCCT-Ha vs ZCCT-Hb proteins. Zinc-finger motifs can function as both DNA-binding and protein-protein interaction domains (Takatsuji 1998) and many zinc-finger proteins are involved in regulation of flower development. The CCT domain of CONSTANS, a key gene mediating flowering transition in Arabidopsis, controls nuclear localization (Robson et al. 2001) and binds to the CCAAT binding factor, which can mediate interactions between CONSTANS-like proteins and DNA (Ben-Naim et al. 2006). In ZCCT-1, a mutation of a conserved arginine to a tryptophan is correlated with spring growth habit in multiple *T. monococcum* accessions (Yan et al. 2004b)—all the barley *ZCCT-H* alleles examined in this study encode an arginine at that position.

The highly conserved functional domains of ZCCT-Ha and ZCCT-Hb could indicate related or even redundant functions. There is currently conflicting evidence regarding which gene, or genes, are responsible for repression of *vrn-H1* in barley. We found that while vernalization-responsive wild barley accession Caesarea 26–24 contains *ZCCT-Hb* and *ZCCT-Hc*, it lacks *ZCCT-Ha*, suggesting that *ZCCT-Ha* is not a determinant of the ancestral winter growth habit. The lack of *ZCCT-Ha* is not a general *H. vulgare* subsp. *spontaneum* attribute, winter habit wild barley accession Koch (also known as Erez 8321; OSU6; PBI004-7-0-015) contains all the three *ZCCT-H* genes (data not shown; Dubcovsky et al. 2005). Based on a *vrn-H1vrn-H1* spring genotype containing *ZCCT-Hb* only, Dubcovsky et al. (2005) suggested *ZCCT-Hb* does not encode the *vrn-H1* repressor; although verification of a full-length *ZCCT-Hb* was not reported. Trevaskis et al. (2006) demonstrated that *ZCCT-Hb* expression is down-regulated by vernalization under long-day photoperiod, while *ZCCT-Ha* expression is unaffected. All winter barley accessions analyzed to date have the *ZCCT-Hc* gene based on hybridization or amplification of a small gene fragment (Dubcovsky et al. 2005; Karsai et al. 2005; Yan et al. 2004b); proof of a full-length gene specifying a predicted functional protein has not been obtained to date. Trevaskis et al. (2006) reported *ZCCT-Hc* expression was undetectable in a

winter cultivar under both long-day and short-day growth conditions. Additional experiments will obviously be needed though to determine which of the three barley *ZCCT-H* genes is/are responsible for *vrn-H1* repression under long-day conditions.

Based on sequence comparison of multiple barley accessions, von Zitzewitz et al. (2005) concluded that differences in vernalization response are not due to differences in the polypeptide, but rather in *VRN-H1* regulation and that the major vernalization regulatory site is not the promoter CArG-box, but is located within the first intron. In Fu et al. (2005), we noted that OWB-D has a large deletion in the *VRN-H1* first intron and based on more extensive sequencing in the current study, discovered that OWB-D also has a variant CArG-box—the only barley variety to date to display variation at this motif. OWB-D is one of the earliest flowering spring barley genotypes we have ever studied, and we speculate that this could be attributable to the loss of vernalization regulatory regions in both the *VRN-H1* promoter and first intron. In the *FLC* MADS-box gene—the central vernalization pathway repressor in Arabidopsis (Michaels and Amasino 1999)—mutations in the promoter or first intron each accelerate flowering under long-day conditions, suggesting possible interaction between the two regulatory sites (Sheldon et al. 2002). Similarly, mutations in the regulatory regions located in the *T. monococcum VRN-A^{m}1* promoter and first intron can both lead to spring growth habit (Dubcovsky et al. 2006; Fu et al. 2005; Yan et al. 2003, 2004a).

A 436 bp segment of the *VRN-H1* first intron has been proposed to be the vernalization critical region based on sequence alignment of multiple barley and wheat genotypes (Fu et al. 2005; von Zitzewitz et al. 2005). Interestingly, we found that spring habit Calicuchima-sib and winter habit Albacete and Pané share an identical 4 kb intron 1 deletion immediately downstream of the intact vernalization critical region. While Spanish cultivars Albacete and Pané have been described as having winter growth habit based on their ability to be fall-sown and survive the mild Mediterranean winters (Lasa et al. 2001), these two genotypes—dominant for *Vrn-H2*—have subsequently been determined to display only a modest vernalization response (A. Casas, personal communication). In a vernalization response survey, Calicuchima-sib was the most vernalization-responsive (16 days) among the spring growth habit barley genotypes, as compared to 0, 4 and 141 days for Morex (spring habit), Dicktoo (facultative habit), and Kompolti korai (winter habit), respectively (I. Karsai, unpublished data).

Vernalization sensitivity in barley is not a presence/absence attribute, but rather a gradated continuum. Takahashi and Yasuda (1971) classified six growth habit classes, ranked from strongest to weakest spring habit relative to flowering time under 24 h photoperiod during greenhouse growth. They concluded that multiple allelic series at the *Vrn-H1* locus are responsible for the grade of spring habit. We hypothesize the degree of flowering delay in these various classes may represent the degree of repression to which *VRN-H1* is subjected and Albacete, Calicuchima-sib and Pané would represent an intermediate growth habit class of Takahashi and Yasuda (1971). The deletion of the sequence flanking the vernalization critical region might affect the efficiency with which the *VRN-H2* repressor can repress the *vrn-H1* allele critical region, perhaps by deletion of important segments that influence *VRN-H1* locus DNA or chromatin structure. In Arabidopsis, alteration in chromatin structure is a principle means of affecting expression of the MADS-box vernalization repressor *FLC* (Bastow et al. 2004). Expression of another Arabidopsis homeotic MADS box gene, *STK*, is regulated by intron-localized GA-rich sites, where the GA-binding protein BPC1, the *STK* repressor, induced conformational changes when all the GA-rich elements are present—when only the two strongest binding sites are available, no conformational changes were observed (Kooiker et al. 2005). Summarizing natural allelic variation for *VRN-H1* (current study; Fu et al. 2005; von Zitzewitz et al. 2005), deletion of the vernalization critical region results in a strong spring growth habit, deletion of sequence flanking the critical region yields an intermediate spring habit, while presence of both the vernalization critical and flanking region yields a facultative or strong winter habit based on a recessive or dominant *VRN-H2*, respectively. Whether the *VRN-H1* promoter is directly involved in vernalization-based regulation (e.g., as a site of *HvVRT-2* interaction during short days) still remains to be resolved.

In summary, our results (a) validate the molecular model proposed to explain the epistatic interaction of *VRN-H2* and *VRN-H1* under long-day conditions, (b) verify the "winter" vs "spring" *VRN-H2* and *VRN-H1* allele primer sets work as predictors of growth habit class, and (c) demonstrate that hidden vernalization sensitivity can be recovered through appropriate crosses. Analysis of natural allelic variation and its effects relative to growth habit at *VRN-H1* and *VRN-H2* revealed that (d) sequence flanking the vernalization critical region of *VRN-H1* intron 1 likely influences degree of vernalization sensitivity, (e) that the *ZCCT-H* genes have higher levels of allelic polymorphism than other winterhardiness regulatory genes, and (f) a winter habit is retained when *ZCCT-Ha* has

been deleted. These results provide grounds for developing and testing hypotheses on the role, or roles, of the duplicated *ZCCT-H* genes and the number and structure of regulatory regions in *VRN-H1* controlling the timing of flowering and sensitivity to vernalization.

Acknowledgments We wish to thank Douglas L. Heckart, John L. Helgesson, and Lauren A. Osborn for their technical assistance. This work was supported by the National Science Foundation Plant Genome Research Program (DBI 0110124) and the United States Barley Genome Project.

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DOI 10.1007/s11032-006-9043-0

Mapping multiple disease resistance genes using a barley mapping population evaluated in Peru, Mexico, and the USA

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Received: 14 April 2006 / Accepted: 24 August 2006 / Published online: 11 October 2006

Abstract We used a well-characterized barley mapping population (BCD 47 × Baronesse) to determine if barley stripe rust (BSR) resistance quantitative trait loci (QTL) mapped in Mexico and the USA were effective against a reported new race in Peru. Essentially the same resistance QTL were detected using data from each of the three environments, indicating that these resistance alleles are effective against the spectrum of naturally occurring races at these sites. In addition to the mapping population, we evaluated a germplasm array consisting of lines with different numbers of mapped BSR resistance alleles. A higher BSR disease severity on CI10587, which has a single qualitative resistance gene, in Peru versus Mexico suggests there are differences in pathogen virulence between the two locations. Confirmation of a new race in Peru will require characterization using a standard set of differentials, an experiment that is underway. The highest levels of resistance in Peru were observed when the qualitative resistance gene was pyramided with quantitative resistance alleles. We also used the mapping population to locate QTL conferring resistance to barley leaf rust and barley powdery mildew. For mildew, we identified resistance QTL under field conditions in Peru that

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are distinct from the *Mla* resistance that we mapped using specific isolates under controlled conditions. These results demonstrate the long-term utility of a reference mapping population and a well-characterized germplasm array for locating and validating genes conferring quantitative and qualitative resistance to multiple pathogens.

Keywords *Hordeum vulgare* subsp. *vulgare* · *Puccinia striiformis* f. sp. *hordei* · *Puccinia hordei* · *Blumeria graminis* f. sp. *hordei* · Quantitative resistance · Qualitative resistance

Introduction

One of the principal challenges faced by plant breeders is achieving durable disease resistance. Qualitative resistance has been widely used because it shows Mendelian inheritance and can be easily managed in a breeding program. Unfortunately, it is usually not durable and its effective use requires constant monitoring of pathogen virulence and the identification and introgression of new host resistance genes (Vanderplank 1968). There are examples of qualitative (single gene) durability, such as the stem rust resistance in barley conferred by *Rpg1* gene (Ji et al. 1994). However, quantitative resistance is generally thought to have a higher probability of durability than qualitative resistance.

We have mapped genes conferring quantitative and qualitative resistance to barley stripe rust (BSR; incited by *Puccinia striiformis* f. sp. *hordei*) in multiple germplasm combinations (Chen et al. 1994; Hayes et al. 1996; Toojinda et al. 2000; Castro et al. 2003a). We have introgressed these genes singly, and in combination, into susceptible backgrounds and demonstrated that they confer acceptable levels of resistance (Toojinda et al. 1998; Castro et al. 2003a, b; Vales et al. 2005). Similar progress has been made in other crop: pathogen systems, e.g. rice (Hittalmani et al. 2000; Narayanan et al. 2004; Yi et al. 2004) and soybean (Walker et al. 2004).

In 2000, there were reports of a new BSR race in the Andean region (H. Vivar, ICARDA/CIMMYT, personal communication). The same year, we received data from the USDA Barley Stripe Rust Screening Nursery [coordinated by Dr. R. Brown and grown at Huancayo, Peru (HP)] that included some dramatic changes in resistance phenotype for some entries. For example, the BSR severity for CI10587 at HP was 60%, while in repeated tests in the Toluca Valley of Mexico (TVM), CI10587 had shown no disease or very low levels of disease (typically $<1\%$). We have mapped the CI10587 resistance, using phenotype data from TVM, as a single gene on chromosome 7H (Castro et al. 2002).

In this study, we used a well-characterized mapping population to test the effectiveness of BSR resistance quantitative trait locus (QTL) alleles at HP. These QTL were mapped based on disease severity data from TVM and Washington State, USA (WUSA) (Vales et al. 2005). We hypothesized that if we detected the same QTL in Peru that we had mapped using TVM and WUSA data, this would mean that (1) there was no new race, or (2) there is a new race but the same QTL resistance alleles are effective. If the population was uniformly susceptible, this would confirm the presence of a new race and it would demonstrate race-specificity of the QTL. If different resistance QTL were detected in Peru than in Mexico and the USA, this would suggest a shift in virulence, as well as race-specificity of QTL. In addition to the mapping population, we included a set of germplasm of known BSR resistance gene architecture and disease reaction, including CI10587. During the course of the field experiment at HP, the mapping population showed a range of phenotypic responses to natural infection by powdery mildew (caused by *Blumeria graminis* f. sp. *hordei*) and leaf rust (caused by *Puccinia hordei*). We therefore used these data to map QTL conferring resistance to these diseases, and in the case of mildew, we compared the QTL results with those obtained using defined isolates under controlled environment conditions.

Materials and methods

Plant material

This research used two types of germplasm resources: a mapping population and a germplasm

array. The ORO doubled haploid (DH) mapping population (Vales et al. 2005) was derived from the F_1 of the cross of BCD47 and Baronesse. This population consists of 409 lines; 94 were used in the current study. BCD47 is a two-rowed, spring growth habit DH line, developed via marker-assisted selection (MAS) for BSR resistance alleles at QTL on chromosomes 4H and 5H (Castro et al. 2003a). Baronesse is a two-rowed, spring growth habit developed by Nordsaat Saatzucht GmbH and introduced by Westbred, LLC to the Pacific Northwest of the USA, where the variety is widely grown. The germplasm array (Table 1) consisted of 23 varieties and genetic stocks of known BSR resistance gene architecture Castro et al. (2003a, b).

Disease assessments under field conditions

Ninety-four ORO maplines, the two parents, and the germplasm array were evaluated for disease resistance phenotypes at the Universidad Nacional Agraria La Molina research farm at HP in 2005 and 2006 using separate two-replicate Randomized Complete Block Designs. Each plot consisted of two 1-m rows. The HP facility is located at an elevation of 3,320 m, with latitude 11°49′ South and longitude of 75°23′ West. The following diseases occurred in response to natural infection without supplemental inoculation: stripe rust, leaf rust, and powdery mildew. Stripe rust is an endemic disease in this area and susceptible check lines produce the inoculum necessary for infection. All three diseases were scored for disease severity on a plot basis using visual assessment of the percentage of crop canopy infected. Ratings were made when the majority of the test genotypes were at growth stage 55 on the Zadocks scale. For the purposes of comparing resistance QTL number, location, and effect, we used the BSR severity data reported by Vales et al. (2005) for the same 94 lines from the TVM, and Pullman and Mt. Vernon, WUSA.

Disease assessments under controlled conditions

Blumeria graminis f. sp. *hordei* (*Bgh*) isolates 5874 (Torp et al. 1978; Wei et al. 1999; *AvrMla1*, *AvrMla6, AvrMla12*) and A27 (Giese et al. 1981;

Table 1 Barley stripe rust disease severity (%) for 23 barley accessions evaluated at Toluca Valley, Mexico in 2000, and at HP in 2000 and 2004

Accession	Stripe rust resistance alleles	Mexico 2000	Peru 2000	Peru 2004
Harrington	None	77	86	40
Galena	None	73	95	50
Baronesse	2H, 5H, 7H	76	84	70
Calicuchima-sib	**4H**, **5H**, 6H, 7H	20	60	20
Shyri	**1H**, 2H, 3H, 6H	0	0	0
CI10587	**7H**	0	60	20
D1-72	**1H**	15	20	8
D3-6	**7H**	Trace	NA	8
Orca	**4H**, **5H**	17	72	19
BCD47	3H, **4H**, **5H**, 6H	19	43	20
BCD12	**1H**	30	60	25
D3-6/B-23	**7H**	8	30	15
D3-6/B-61	**7H**	4	70	15
OPS 19	**1H**, **7H**	Trace	0	5
OPS 66	**1H**, **7H**	Trace	0.1	10
OPS 78	**1H**, **7H**	Trace	0	10
AJO 44	**4H**, **5H**, **7H**	8	20	25
AJO 59	**4H**, **5H**, **7H**	2	0	8
BU 16	**4H**, **5H**, **7H**	3	10	13
BU 27	**4H**, **5H**, **7H**	Trace	0	8
BU 37	**4H**, **5H**, **7H**	Trace	0.1	0
BU 38	**4H**, **5H**, **7H**	0	0	3
BU 45	**4H**, **5H**, **7H**	0	0.1	5

Stripe rust resistance QTL alleles are numbered according to their chromosomal locations (1–5)

Largest-effect QTL are shown in bold font. 7H denotes a major gene

AvrMla1, *AvrMla7*, *AvrMla10*, *AvrMla13*) were propagated at Iowa State University on *H. vulgare* cv. Manchuria (C.I. 2330) in separate growth chambers at 18°C (16 h light/8 h darkness). The same 94 DH lines that were characterized in Peru were grown in three, 36-cell flats. Groups of three seedlings per DH line were sown per cell in each flat. The Baronesse and BCD47 parents, C.I. 16137 (*Mla1*), C.I. 16151 (*Mla6*), C.I. 16149 (*Mla10*), Sultan5 (*Mla12*), C.I. 16155 (*Mla13*), in addition to the fully susceptible Manchuria (C.I. 2330), were used as checks (Moseman 1972). Seedlings were grown to the second leaf stage with the first leaf unfolded, and inoculation was performed at 16:00 h. US Central Standard Time by tipping the flats at 45° angle and dusting the plants with a high density of fresh conidiospores (84 ± 19 spores/mm^2). This conidial density per unit leaf area routinely results in greater than 50% of epidermal cells that are successfully infected (Bushnell 2002; Collinge et al. 2002). Groups of flats were placed at 18°C (16 h light, 8 h darkness) in separate controlled growth chambers corresponding to the two *Bgh* isolates (5874 and A27). Infection types were scored at 7 and 9 days post-inoculation as described in Wei et al. (1999). The infection types 0, 1, or 2 are considered resistant reactions while the infection types 3 or 4 are considered susceptible.

Genotyping, map construction, and QTL analysis

Markers reported by Vales et al. (2005) that co-segregated or that had ≥10 missing data points were eliminated. Twenty additional markers were added to the ORO data set. The new markers are shown in bold in Fig. 1. Markers k04435, k03512, k08433, k08302, k06257, k04261, k03878, k00688, k04489, k07339, k00088, k02892, k03352, and k07229 are expressed sequence tag (EST)-based marker loci of known location on the barley transcript map (Sato et al. 2004). Markers MWG2180 and ABG54 were developed by the North American Barley Genome Project and were originally scored as Restriction Fragment Length Polymorphisms (RFLPs) (Kleinhofs et al. 1993). They were converted to the Sequence Tagged Site (STS) markers based on the sequences available at the GrainGenes website (http://wheat.pw.usda.gov/GG2/index.shtml). Primers for the kbaal29j18 marker were designed based on unpublished RIB EST sequence of clone rbaal29j18. The EST-derived SSR-markers GBM1071 and GBM1015 were developed by Thiel et al. (2003). The HvSnf2 locus was mapped using the primers and procedures reported by Yan et al. (2002). TC493a locus sequence was obtained from the tentative contig TC112493 sequence in The Institute for Genome Research (TIGR) web page (http://www.tigr.org). The *Mla* locus was mapped using primers based on the sequence for *Mla12* (GenBank Accession AY196347). Primer sequences for unpublished markers are shown in Table 2.

JoinMap® (van Ooijen and Voorrips 2001) was used for linkage map construction, using the Kosambi mapping function (Kosambi 1944). Linkage groups and locus orders were compared with Vales et al. (2005). The two-replicate means for BSR, barley leaf rust, and barley powdery mildew disease severity and BSR infection from HP and the data for *Bgh* isolates 5874 and A27 (Iowa State University) were used for QTL analysis, as were the datasets from TVM and WUSA for the same 94 lines in this study that were used previously by Vales et al. (2005). QTL analyses were performed using the composite interval mapping (CIM) procedure (Zeng 1994) implemented in Windows QTL Cartographer 2.5 (Wang et al. 2005). A forward-selection backward-elimination stepwise regression procedure was used to identify co-factors for CIM for each trait; the LOD threshold values to declare a QTL significant were obtained based on 1,000 permutations, a 10 cM scan window and a type I error of 5%. Tests for epistasis between QTLs were evaluated using the multiple interval mapping (MIM) method of QTL Cartographer. With MIM, the proportion of the phenotypic variance explained for each trait was calculated by fitting a model using all detected QTL and their significant interactions.

Results

Mapping population

The ORO population linkage map (Fig. 1) has 71 markers comprising 11 linkage groups at a LOD

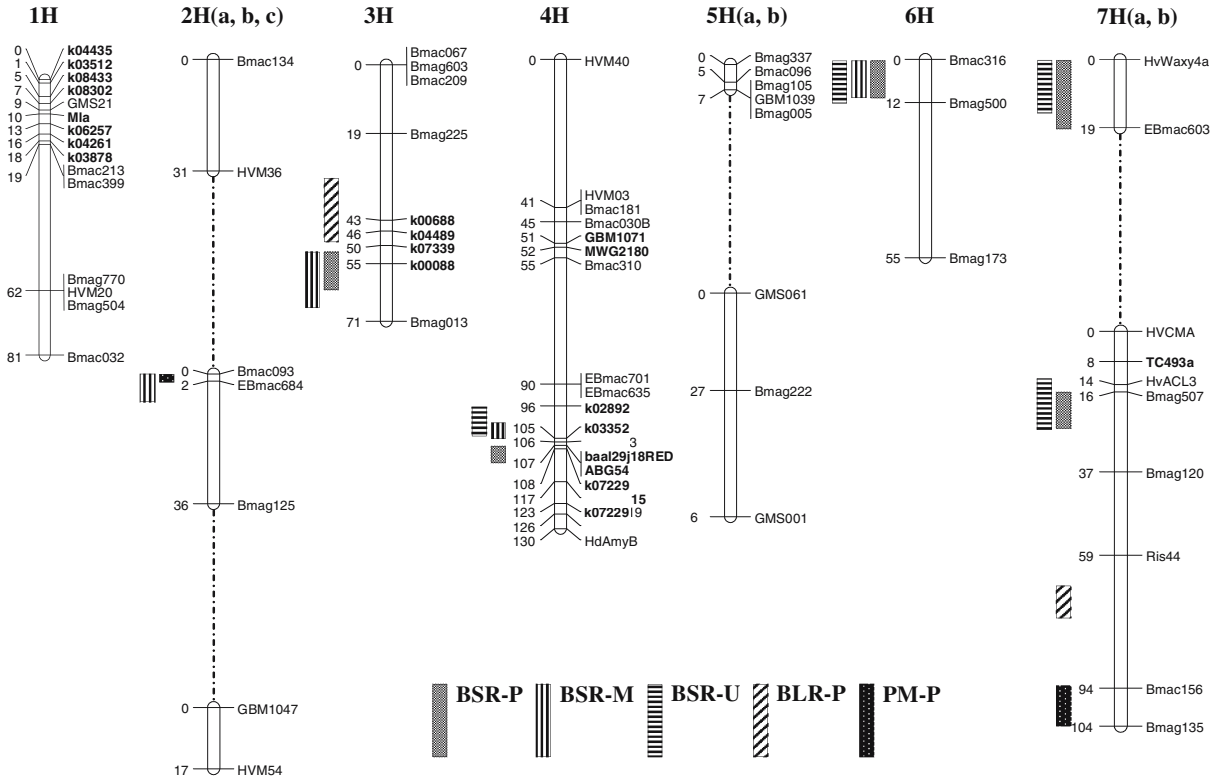

Fig. 1 Linkage map of the ORO (BCD47 × Baronesse) doubled haploid population (n = 94). *Dashed lines* indicate monomorphic regions. Distances are in Kosambi cM. *The bars* to the left of each linkage group indicate 1-LOD intervals for QTL abbreviated as follows: BSR-P, BSR-M, BSR-U (barley stripe rust resistance in Peru, Mexico, and USA, respectively); BLR-P (barley leaf rust resistance in Peru); PM-P (powdery mildew resistance in Peru); and PM-I (powdery mildew infection type in response to inoculation with *Bgh* isolates 5874 and A27 at Iowa State University)

Table 2 Primer sequences used for mapping new marker loci in the ORO (BCD47/Baronesse) population, listed in linkage map order by chromosome (Chr)

Locus	Ch6	Forward primer	Reverse primer
K04435	1H	TTTCCGGGATAAGAGTGTGC	CGACTCGGTTGTTTGCTACA
K03512	1H	CATCCCGACAGAAGCAAAAT	TACCTACCAGTGACCCCTGC
K08433	1H	TACTGGTCTTGGATGCGACA	GCGCCAAATCTGATAGCACT
K08302	1H	GAACGACAAAGATGATGGCA	CATTGATTGACACCACCAGC
K06257	1H	GGCACACATTTACCAAGTGC	CATCCATCAACGTCCTCCTC
K04261	1H	GGGAATTTTCTCCGTTTGGT	AGGTGCTAGACGGTTTCCCT
K03878	1H	AACATGCATGGTGACAAGGA	AAGGGCTTCGTCAATGTTTG
K00688	3H	TAGCCTGGCAGCTTTCTGTT	CTACTTCCCCCGTTTCGAC
K04489	3H	GTGGGCATGAAGAACGCTAC	CGAAGTGGTTCCATGGAGTT
K07339	3H	AGGCACAGGCTCTTTTGCTA	GAGCCTTGCTACTGAATGGG
K00088	3H	ACACGGTCCATGGAAGAAAC	CATAGATGGGCCCTTGAAGA
K02892	4H	AACGTAACAACCGAACGCAT	ATCACGACTGCTCCAATTCC
K03352	4H	CTTTGCATGGCTGATGAAGA	CAATCTGATGGGGAGCACTT
baal29j18	4H	TGTTGATGAATCGCTCTTGC	CACAACAACCACTACGACGG
ABG54	4H	GTGCTTGGCGGTCGACCAGT	GATGTCCAACGGTGGCTTGA
K07229	4H	AGGCACACAAGCAACAACAG	GTTGTGAGCCATCGTGAAGA
TC493a	7H	TTTCGGTTTCTTGGAAATGG	AGCTGCTGGAAGGTGAACTC

threshold grouping value of 4.0. All linkage groups were assigned to barley chromosomes per Vales et al. (2005), with more than one linkage group for chromosomes 2H, 5H, and 7H. The map covers 611.8 cM, corresponding to an average density of 8.7 cM per marker. Segregation distortion ($p < 0.05$) in favor of Baronesse was observed in chromosome 3H (k00688, k04489, k07339, and k00088) and chromosome 4H (Bmac310 and GBM1015); and in favor of BCD47 in chromosome 1H (Bmag770, HVM20, and Bmag504).

The phenotypic frequency distributions for stripe rust, leaf rust, and powdery mildew at HP in each of two years (Fig. 2a–c) reflect quantitative inheritance and for all three diseases even the most resistant lines showed some level of disease. The frequency distribution for leaf rust may indicate the presence of a qualitative gene (or "major" QTL) and one or more minor QTLs. The frequency distributions for reaction type to mildew after inoculation with the two isolates under controlled conditions were discrete and bimodal (Fig. 2d). Infection types for each mapline were nearly identical for both isolates, except for few cases in which one of the isolates gave variable reaction types in a specific mapline. In these cases, the average of the variable reactions always gave an infection type equal to that observed with the other isolate. As a consequence, the classification of resistant (infection types 0–2) and susceptible infection types (3 and 4) was identical for both isolates. These qualitative data showed excellent fit to a 1:1 ratio ($\chi^2 = 0.67$; $p = 0.41$).

In order to properly compare the BSR resistance QTL results from Peru with QTL detected in Mexico and the USA, we re-analyzed all BSR data sets using the new linkage map. In Peru, the LOD threshold for BSR disease severity was 2.5. BSR resistance QTL with the resistance allele tracing to BCD47 were found on chromosomes 3H, 4H, and 6H, and with the resistance allele tracing to Baronesse on chromosome 7H (linkage groups a and b) (Table 3; Fig. 1). The largest effect QTL was on chromosome 4H, and it accounted for 15% of the phenotypic variance, with an additive effect of 10 (percent disease severity). The five QTL explained 70.8% of the

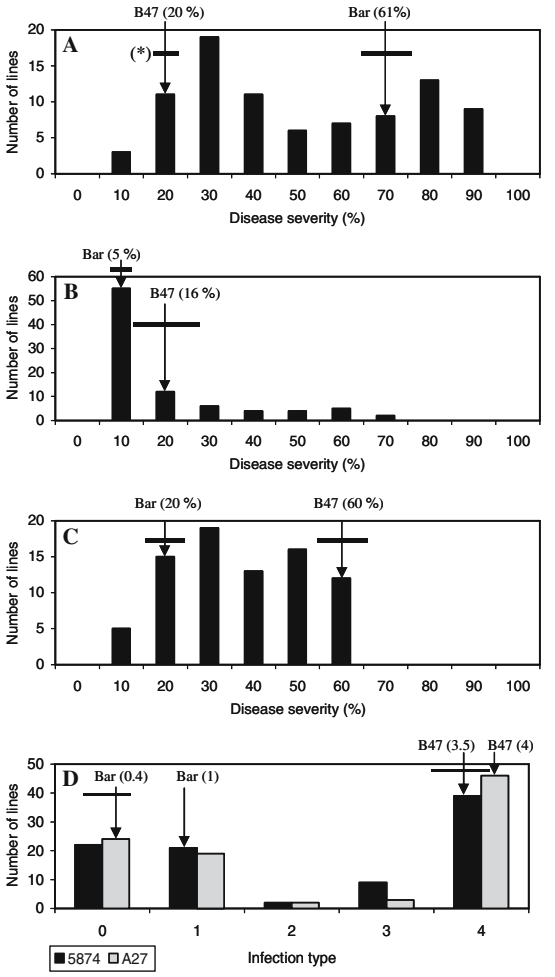

Fig. 2 Phenotypic frequency distributions **a** barley stripe rust, **b** barley leaf rust, and **c** powdery mildew disease severity in BCD47, Baronesse and 94 doubled haploid (*DH*) progeny at Huancayo, Peru in 2004. Panel D shows the phenotypic frequency distribution for mildew infection in response to inoculation with *Bgh* isolates 5874 and A27

phenotypic variance. The reanalysis of the Mexico and USA BSR data with the new linkage map revealed the same total number of QTL as reported by Vales et al. (2005) for a population size of 100. The LOD thresholds were 2.6 and 2.3 for Peru, TVM, and WUSA, respectively.

The BSR resistance QTL on chromosomes 4H and 6H were detected in all three environments. Of the QTL that were significant in Peru, three were also significant in Mexico and four were significant in the USA. Considering all three environments and the population size, more BSR

Table 3 Barley stripe rust disease severity resistance QTL detected in the ORO (BCD47/Baronesse) population at Huancayo, Peru (*HP*) in 2004; the Toluca Valley, Mexico (TVM) in 2001, 2002, and Pullman and Mt. Vernon WUSA in 2002

Location	Chromos	QTL peak position and 1-LOD interval (cM)	LOD[b]	R^2 (%)[c]	Additive effect[d]
TVM	2H(b)[a]	0.0 (0.0–7.5)	5.4	8.3	7.43
TVM	3H	56.7 (51.6–67.0)	7.7	13.4	–9.70
HP		**54.7 (52.0–62.4)**	**5.9**	**9.6**	**–8.03**
TVM	4H	103.8 (99.9–104.4)	11.8	24.6	–13.4
HP		**108.1 (107.3–111.6)**	**8.3**	**15.2**	**–10.37**
WSU		101.8 (95.8–104.2)	4.9	13.0	–3.95
TVM	6H	0.0 (0.0–10.0)	3.0	4.4	–5.78
HP		**2.0 (0.0–10.0)**	**5.0**	**8.3**	**–7.30**
WSU		0.0 (0.0–6.2)	4.0	9.3	–3.41
HP	7H(a)[a]	**18.0 (0.0–19.1)**	**3.6**	**5.7**	**6.07**
WSU		0.0 (0.0–11.9)	4.3	9.9	3.29
HP	7H(b)[a]	**16.2 (14.8–24.9)**	**7.1**	**11.9**	**8.79**
WSU		16.2 (11.6–25.4)	4.9	11.5	3.56
Total (%)[e]	TVM		65.4		
	HP		70.9		
	WSU		52.3		

[a] The letter in parentheses indicates cases where there is more than one linkage group per chromosome (see Fig. 1)

[b] LOD is the log-likelihood at the QTL peak position. The LOD threshold, based on 1,000 permutations and a type I error of 5% was 2.5, 2.6, and 2.3 for Peru, TVM, and WSU, respectively

[c] R^2 is the percentage of phenotypic variation explained by the QTL

[d] Negative and positive values indicate that BCD47 and Baronesse, respectively, contributed the resistance QTL allele

[e] Proportion of the total variance explained by the QTL

resistance QTL were detected in Peru. There were no significant epistatic interactions in any data set.

For barley leaf rust and powdery mildew resistance, the LOD thresholds were 2.6 and 2.5, respectively. Each of the parents contributed a resistance allele at one of two leaf rust resistance QTL (Table 4, Fig. 1). The 7H QTL had the largest effect, accounting for 31% of the phenotypic variance. Considering both QTL and the significant epistatic interaction between them, they accounted for 79% of the phenotypic variance. There were two QTL for powdery mildew resistance (Table 4, Fig. 1); each parent contributed a resistance allele. There was no epistatic interaction between the QTL; the total percentage of phenotypic variance explained was 22%. The results of the segregation based on phenotype data for mildew infection type in response to inoculation with the two isolates and the genotype data based on amplification of the *Mla* sequence were identical and therefore co-segregate. The linkage map position on chromosome 1H (Fig. 1) is indicated as *Mla*.

Germplasm array

In order to look for changes in disease severity that could be diagnostic of a new race, or race shift, we compared data from TVM-2000 (the most recent season when the full germplasm array was grown at this location) with HP-2000 and HP-2004 data. The correlations between TVM-2000 and HP-2000 and HP-2004 data were 0.79 and 0.90, respectively, indicating that most lines showed consistent levels of disease severity. As shown in Table 1, the susceptible checks (Harrington, Galena, and Baronesse) always had the highest disease severity values. The quantitative resistance donor parents showed a range of disease severities: Calicuchima had 20% disease severity in TNV-2000 and HP-2004 but was rated 60% in 2000. Shyri allowed no disease development. CI10587 showed a 60% increase in disease severity in 2000 but only a 20% increase in HP-2004. Lines derived from CI10587 and not known to carry any other resistance genes besides 7H (e.g. D3-6, D3-6/B-23, and D-3-6/B-61) showed smaller increases in disease severity at HP

Table 4 Barley leaf rust (A) and powdery mildew (B) disease severity resistance QTL detected in the ORO (BCD47/Baronesse) population of 94 DH lines at HP in 2004

	QTL peak position and 1-LOD interval (cM)	LOD[b]	R^2 (%)[c]	Additive effect[d]
A				
Chromosome				
3H	42.7 (31.6–49.0)	6.2	21.6	9.48
7H(b)[a]	68.6 (67.4–76.3)	9.4	47.1	–13.96
3H × 7H(b)		5.6	10.4	8.42
Total (%)[e]			79.1	
B				
Linkage group				
2H(b)[a]	0.0 (0.0–2.2)	4.2	12.4	5.36
7H(b)[a]	101.6 (93.5–103.6)	3.6	11.6	–5.11
Total (%)[e]			21.8	

Data for leaf rust are based on the multiple interval mapping procedure of QTL Cartographer, due to the presence of significant QTL × QTL interaction

[a] The letter in parentheses indicates cases where there is more than one linkage group per chromosome (see Fig. 3)

[b] LOD is the log-likelihood at the QTL peak position. The LOD thresholds, based on 1,000 permutations and a type I error of 5% was 2

[c] R^2 is the percentage of phenotypic variation explained by the QTL

[d] Negative and positive values indicate that BCD47 and Baronesse, respectively, contributed the resistance QTL allele

[e] Proportion of the total variance explained by the QTL

in 2004 than in 2000. All lines with multiple resistance alleles tracing to Calicuchima-sib, Shyri, and/or CI10587 (OPS, AJO, and BU) had low and consistent disease severities in all three tests.

Discussion

The coincidence of stripe rust severity QTL detected with the phenotypic data from HP, TVM, and WUSA confirms that the quantitative resistance genes present in the ORO population are effective against the spectrum of virulence encountered in each of the three environments. We had hypothesized that if we detected the same QTL in Peru that we had mapped using TVM and WUSA data, this would mean that (1) there is no new race in Peru, or (2) there is a new race but the same QTL resistance alleles are effective. The changes in disease severity for some genotypes in the germplasm array would lend support to the latter possibility. Additional experiments, including assessment of differentials at HP, are currently underway. Longer term, it would be desirable to develop a more saturated map. More broadly, the effectiveness of these resistance QTL alleles across environments indicates that their introgression into susceptible, but adapted, germplasm may be justifiable. These genes may have a reasonable expectation of durability: they have proven effective over the past 18 years in repeated tests in Mexico and North America.

Strictly defined, quantitative resistance is non-race specific (Vanderplank 1963, 1968) but it not possible to state definitely that the resistance QTL alleles do not show race specificity. The five resistance QTL that were significant with the HP data were detected with either the TVM or WUSA data, but not all five were significant in all environments. Furthermore, the QTL had different magnitudes of effect in the different environments. In some cases, there were ''minor QTL peaks'' (e.g., that did not reach the significance threshold); in others there were no trends whatsoever. Interpretation of QTL trends is very subjective, and higher precision may be achieved in the future by re-analysis of the same data sets using more sophisticated analysis tools, by more rigorous phentoyping, and/or by larger population sizes. In the interest of brevity, we present only significant QTL in this report. For finer analyses, the full phenotype and phenotype data sets are

available from the corresponding author. The difference in number and location of significant QTL could be due to a number of causes. Evidence for some race-specificity, and a degree of race specificity has been reported for some leaf rust resistance QTL (Qi et al. 1998, 1999; Lindhout 2002). It is also true that changes in race specificity can show quantitative rather than qualitative effects (Qi et al. 1998, 1999). Alternatively, differences in environmental effects (e.g., temperature, photoperiod, and nutritional status of the crop) could influence the onset of initial infection. Although purely speculative, it is also possible that qualitative resistance against a specific isolate might appear to be quantitative resistance, either by induced resistance or due to a dilution effect. The most prosaic explanation is that the difference in the number of QTL detected, and the differences in estimates of QTL effect, are biases due to small population size.

The BSR QTL are located in resistance gene-rich regions of the genome (Toojinda et al. 2000; Hayes et al. 2003). There is ample evidence for the existence of resistance gene clusters in plants (Chelkowski et al. 2003; Williams 2003), and for the occurrence of quantitative and qualitative resistance genes within such clusters (Wisser et al. 2005). Of particular interest is the presence of BSR resistance QTL detected in the same regions as the powdery mildew resistance loci: the *Mla* locus on 1H (Toojinda et al. 2000) and the *mlo* locus on 4H (this study).

This linkage map proximity of BSR resistance QTL and qualitative mildew resistance genes, together with mildew resistance QTL mapped with the Peru phenotype data, prompted us to determine if either *Mla* or *mlo* resistance alleles were present in BCD47 and/or Baronesse. BCD47 had not been characterized for its response to specific isolates of mildew. Baronesse is reported to carry *Mla3* resistance (Hovmøller et al. 2000; Dreiseitl 2003), and analysis of its pedigree (Mentor/Minerva// mutant Vada //// Carlsberg/ Union///Opvasky/Salle//Ricardo///// Oriol/6153P40) reveals that the Mentor, Carlsberg, Oriol, and Ricardo parents are reported to carry *Mla12, Mla8, Mla7*, and *Mla3* alleles, respectively (www.scri.sari.ac.uk/cprad). The mildew infection type data cosegregate with the results of the genotyping based on *Mla12*-derived primers. These results clearly confirm the report that Baronesse carries *Mla* resistance, although the exact allele cannot be determined from these data. None of the mildew resistance QTL are coincident with *Mla*. There are no reports of mildew resistance, either qualitative or quantitative, at the position of the 2H(b) QTL. Based on visual alignment of linkage maps, the QTL on chromosome 7H(b) is in the same region as *Mlf* (Schönfeld et al. 1996) and a powdery mildew resistance QTL in *H. vulgare* spp. *spontaneum* (Backes et al. 2003). The two leaf rust resistance QTL alleles, either of which was sufficient to confer resistance, were also found in regions of the barley genome where other resistance genes are reported. The 3H QTL is in the same region as *Rph10* (Feurestein et al. 1990) and the QTL on 7H(b) is located in the same region as the *RphX* gene mapped in Cali-sib and Shyri (Hayes et al. 1996; Toojinda et al. 2000). Also mapping to this region are RphQ9, a QTL with race specificity (Qi et al. 1999; Lindhout 2002), and *Rph3* (Park and Karakousis 2002).

Our rationale for assessing both the mapping population and a germplasm array was that one or more of the genetic stocks could be diagnostic of a new race, or a shift in race frequency. CI10587 showed the most dramatic change in phenotype, with a 60% disease severity rating in HP2000 vs. 0% in TVM2000 (Castro et al. 2003a). However, the disease severity of CI10587 was only 20% in HP2004. This discrepancy merits further study, since the 0–60% difference in disease severity suggests ''defeat'' of a major gene by a new race whereas a 20% increase in disease is more indicative of a resistance gene behaving as a major gene in response to one race and as a QTL to another race, a phenomenon reported in rice with bacterial blight (Li et al. 1999). Alternatively, CI10587 may possess minor genes for resistance to BSR that have heretofore been undetected.

There was a tendency toward increasing levels of resistance when more resistance QTL alleles were pyramided per line, as reported by Castro et al. (2003a), although the differences were not significant. Of particular interest are the lines with 7H qualitative resistance gene from CI10587. This gene did not confer an acceptable level of

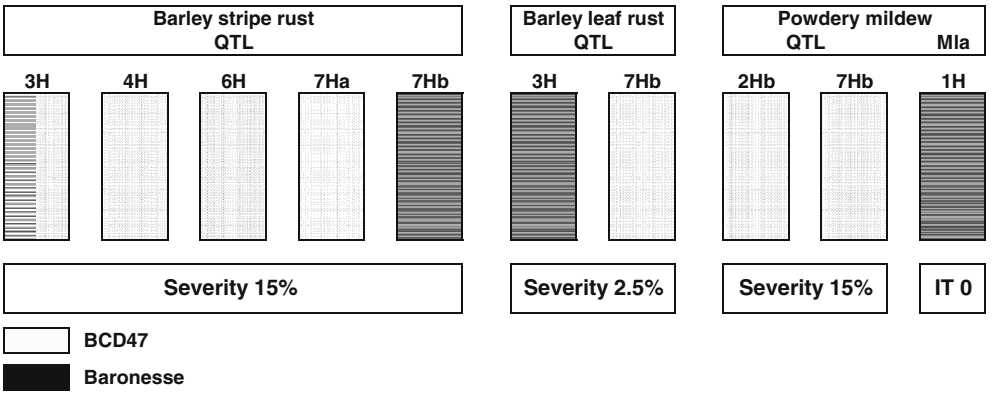

Fig. 3 The graphical genotype of ORO-019 at the *Mla* locus and at barley stripe rust, barley leaf rust and powdery mildew QTL based on field phenotype data from Huancayo, Peru 2004. The split panel for 3H indicates contrasting alleles at the loci flanking the QTL peak

resistance in 2000 and more disease was observed in 2004 than expected based on prior ratings in Mexico. However, when deployed in combination with quantitative resistance alleles at 1H or 4H + 5H lines with this gene had some of the lowest levels of disease severity.

Baronesse, of European origin and moderately susceptible to BSR, contributed resistance alleles effective in the Andean environment. Of two parents, it was also the most resistant to leaf rust and mildew. The presence of positive and negative transgressive segregants is reported in many disease QTL studies and thus the contribution of positive alleles from ''susceptible'' parents is not entirely unexpected (Hayes et al. 2003). The availability of genotype and phenotype information on the ORO population could be useful in introgressing the resistance genes in both Baronesse and BCD47. As shown in Fig. 3, ORO DH line 19 has resistance to all three diseases under field conditions in Peru, and the Baronesse resistance *Mla* allele. This mapline has the expected stripe rust resistance phenotype and allelic configuration at all marker loci bracketing BSR resistance QTL. It is lacking the resistance allele at the small-effect QTL at the 7H(b); this could be remedied by additional crossing. There was a crossover between the markers flanking the 3H QTL, which will assist in future efforts for finer mapping of this locus. This line carries the contrasting target alleles at the two barley leaf rust QTL and the predicted favorable allele at the barley powdery mildew QTL on 7H(b). It is lacking the mildew resistance allele at the 2H(b) QTL; this could be remedied in a subsequent cycle of crossing.

In conclusion, this research was useful in confirming the value of quantitative stripe rust resistance genes that we have mapped and introgressed, via MAS, into North American germplasm. These resistance genes were discovered through collaborative efforts with the ICARDA/CIMMYT program and National Program scientists in the Andean region. Calicuchima sib and Shyri, were identified as BSR resistant and released in the Andean region. It is thus fitting that these genes are returned to the Andean National Programs, with value added via marker information that will allow for their efficient introgression. Optimistically, this will lead to high levels of durable resistance. However, vigilance and continued gene discovery and introgression are essential, because what appears to be a non-race specific QTL today may, in the face of new virulence, become a ''defeated'' major gene, or vice versa.

Acknowledgments We offer special thanks to Ann Corey, Tanya Filichkina, and Kelly Richardson for their technical support in the lab and in the preparation of Peru nurseries.

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