Universitat de Lleida

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Als de casa…

Resum

Les translocacions recíproques (TR) són un dels canvis cromosómics estructurals més comuns en plantes encara que les que succeeixen espontàniament són extremadament inusuals en les varietats d'ordi conreades. De fet, 'Albacete', és l'única varietat coneguda d'ordi conreat que té una TR sense cap efecte aparent en la fitness. Anàlisis preliminars han demostrat que els cromosomes 1H i 3H hi estan involucrats (Lacasa-Benito et al. 2005). Aprofundir en el coneixement d'aquesta TR permetrà saber si està relacionada o no amb la tolerància a la sequera i trobar característiques fenotípiques interessants que es puguin transferir a altres genotips d'ordi per a desenvolupar germoplasma específic adaptat a l'estrès.

En primer lloc, es va desenvolupar una aproximació estadístico-genètica per a la construcció de mapes de lligament en poblacions derivades d'un heterocigot per a una TR. Per aquest fi es va utilitzar una població doble haploide de 230 individus procedents del creuament entre 'Albacete' i 'Barbarrosa' la qual va ser genotipada amb marcadores SSR i DArT®. L'estudi de lligament preliminar va revelar sis grups de lligament principals, un d'ells agrupava els marcadores dels cromosomes 1H i 3H. Aquesta unió va ser deguda a la presència de la TR entre aquests cromosomes en la varietat 'Albacete'. Els resultats d'aquest estudi van revelar que la posició del punt de tall de la translocació està localitzat al voltant de la regió centromérica per a ambdós cromosomes.

En segon lloc, es va combinar tècniques citogenètiques i moleculars per a determinar la grandària dels segments que estan involucrats en l'intercanvi entre ambdós cromosomes. Els resultats van confirmar que els punts de tall estan situats als braços llargs dels cromosomes 1H i 3H resultant en la TR 1HS.1HL-3HL y 3HS.3HL-1HL. El contigut gènic al voltant dels punt de tall va ser estimat aproximadament en 1.100 i 710 models gènics pel 1H i 3H, respectivament.

Per a caracteritzar els efectes de la TR en alguns caràcters d'interès agronòmic es van realitzar assajos en dues localitats durant un període de dos anys utilitzant un gran nombre de línies doble haploides derivades de quatre creuaments amb 'Albacete' com un dels parentals. Els resultats van suggerir que la TR per si sola no és la causant de la tolerància a la sequera present a la varietat 'Albacete'; el pes de mil grans va ser l'únic caràcter significativament superior en les línies doble haploides portadores de la translocació. Per a l'anàlisi de QTL es va construir un mapa de lligament integrat utilitzant una versió modificada de l'algoritme desenvolupat per Jansen (2005). Finalment, es va desenvolupar metodologia específica per a la detecció de QTLs en poblacions interconnectades utilitzant com a exemple dies fins a la floració (caràcter independent de la translocació) i pes de mil grans (afectat per la translocació).

Resumen

Las translocaciones recíprocas (TR) son uno de los cambios cromosómicos estructurales más comunes en plantas aunque las que ocurren espontáneamente son extremadamente inusuales en las cebadas cultivadas. De hecho, 'Albacete' es la única variedad conocida de cebada cultivada que tiene una TR sin ningún efecto aparente en la fitness. Análisis preliminares han demostrado que los cromosomas 1H y 3H están involucrados en la translocación (Lacasa-Benito et al. 2005). Profundizar en el conocimiento de esta TR permitiría saber si está relacionada o no con la tolerancia a la sequía presente en 'Albacete' y encontrar características fenotípicas interesantes que se puedan transferir a otros genotipos de cebada para desarrollar germoplasma específico adaptado al estrés.

En primer lugar, se desarrolló una aproximación estadístico-genética para la construcción de mapas de ligamiento en poblaciones derivadas de un heterocigoto para una TR. Para ello se utilizó una población doble haploide de 230 individuos procedentes del cruzamiento entre 'Albacete' y 'Barbarrosa' genotipada con marcadores SSR y DArT®. El estudio de ligamiento preliminar revelo seis grupos de ligamiento principales, uno de ellos agrupaba los marcadores de los cromosomas 1H y 3H. Está unión fue debida a la presencia de la TR entre estos cromosomas en la variedad 'Albacete'. Los resultados de este estudio revelaron que la posición del punto de corte de la translocación estaba localizada alrededor de la región centromérica para ambos cromosomas.

En segundo lugar, se combinó técnicas citogenéticas y moleculares para determinar el tamaño de los segmentos que están involucrados en el intercambio entre ambos cromosomas. Los resultados confirmaron que los puntos de corte están situados en los brazos largos de los cromosomas 1H y 3H resultando en la TR 1HS.1HL-3HL y 3HS.3HL-1HL. El contenido génico alrededor de los puntos de corte fue estimado aproximadamente en 1.100 y 710 modelos génicos para el 1H y 3H, respectivamente.

Para caracterizar los efectos de la TR en algunos caracteres de interés agronómico se realizaron ensayos en dos localidades durante un periodo de dos años. Para ello se utilizó un gran número de líneas doble haploides derivadas de cuatro cruzamientos con 'Albacete' como uno de los parentales. Los resultados sugirieron que la TR por sí sola no es la causante de la tolerancia a la sequía presente en 'Albacete'; el peso de mil granos fue el único carácter significativamente superior en las líneas doble haploides portadoras de la translocación. Para el análisis de QTL se construyó un mapa de ligamiento integrado utilizando una versión modificada del algoritmo desarrollado por Jansen (2005). Por último, se desarrolló metodología específica para la detección de QTLs en poblaciones interconetadas utilizando como ejemplo días hasta la floración (carácter independiente de la translocación) y peso de mil granos (afectado por la translocación).

Summary

Reciprocal translocations (RT) are one of the most common structural chromosomal rearrangements occurring in plant species. Spontaneous RT are extremely uncommon in cultivated barley. In fact, 'Albacete' is the only extensively cultivated barley variety known to carry a RT without any major reduction in fitness. Previous results have shown that 1H and 3H chromosomes are involved in this translocation which may have a potential interest for plant breeding. A depth understanding of this RT could allowed us to know whether this translocation is involved or not in their drought tolerance and find some interesting phenotypic attributes that could be transfer to other barley genotypes to develop specific germoplasm adapted to stress.

In a first step, a set of 230 doubled haploid lines derived from the cross between 'Albacete' and the non-translocation barley variety 'Barberousse' was used to develop a statistical-genetic approach for the construction of a linkage maps in populations obtained from RT heterozygotes. A preliminary linkage study revealed six main linkage groups, compared to the seven linkage groups on the barley consensus map. One of the linkage groups contained markers of chromosomes 1H and 3H. This union was due to the presence of a RT between chromosomes 1H and 3H in the variety 'Albacete'. The results of this study revealed that the position of the translocation breakpoints was placed around the centromeric regions for both chromosomes.

In a second step, a combination of cytological and molecular genetic approaches was used in order to know how large the segments are that are involved in the interchange between the two chromosomes. The results obtained in this study indicate that the translocation is quite large with breakpoints located on the long arms of chromosomes 1H and 3H resulting in the RT 1HS.1HL-3HL and 3HS.3HL-1HL. The gene content around the translocation breakpoints was estimated at approximately 1,100 and 710 gene models for 1H and 3H, respectively.

A large number of doubled haploid lines derived from four crosses involving 'Albacete' as one of the parents were used to phenotypically characterize the effects of the RT on some interesting agronomic traits. The results suggested that the RT alone is not causing the drought tolerance of 'Albacete'; thousand kernel weight is the only trait which is clearly enhanced in the lines carrying the RT. Further work combining the results from this study with QTL analysis was carried out to phenotypically characterize the effects of the reciprocal translocation and QTL simultaneously. For that, an integrated map was constructed making used of a modified version of an algorithm developed by Jansen (2005). Finally, specific QTL methodology for the simultaneous analysis of these interconnected populations was developed; two traits: days to heading (independent of the RT) and thousand kernel weight (affected by the RT) were used as an example.

Contents

Chapter 1

General introduction

Today, cereals are still the basis of global agriculture by providing more than half the human food consumption. Among the cereals, barley ranks fourth after maize (*Zea mays*), wheat (*Triticum* ssp.) and rice (*Oryza sativa*) with the widest range of production areas in the world (130-140 million tonnes in the recent years; Horsley et al. 2009). In Spain, barley is the cereal with the largest cultivated area (2.8 million ha), 46% of the total area used for growing cereals, with an annual production of 9 million tonnes which corresponds with approximately 51% of the total cereal production (MARM; 2011).

Cultivated barley (*Hordeum vulgare*) is a diploid species (2n = 2x =14), with some tetraploid and hexaploid wild relatives (Martin et al. 1999). It has been derived from the wild progenitor *Hordeum vulgare* ssp *spontaneum*, which continues to grow in the Middle East. Barley is one of the most widely adapted plant species grown in a wide range of environments from near-arctic to semi-desert. Barley varieties have been classified according to their morphology and agronomic adaptation. Morphological, barley varieties can be divided according the number of kernel rows in the head: two-row (only one spikelet at each node is fertile) and six-row (all three spikelets are fertile). Considering agronomic adaptation, varieties can be classified into three types: winter, spring and facultative. Winter barley varieties are tolerant to low temperature, sensitive to daylenght and require vernalization. Spring barley varieties do not require vernalization, and are typically insensitive to short day photoperiod. Facultative barley varieties are tolerant to low temperatures, but do not require vernalization (Zitzewitz et al. 2005).

Despites its large genome (approximately 5,100 Mb; Arumuganathan and Earle 1991; Doležel et al. 1998) barley provides an excellent model for other *Triticeae* species because it is a diploid self-pollinating species with a huge genetic variability. It has been found that the large size of its genome is not due to the presence of a greater number of genes in comparison with other species from the *Triticeae* tribe (family Poaceae), but to the presence of highly repetitive DNA (80% of the genome), mostly retrotransposons (

Bennetzen et al. 1998; Ramakrishna et al. 2002; Schulman et al. 2004). In fact, it has been estimated that barley has around 32,000 genes, which is not very different from rice (41,000 genes, Jung et al. 2008), *Sorghum* (34,500 genes, Paterson et al. 2009) or *Brachypodium* (30,000 genes, The International Brachypodium Initiative 2010). The genetic progress in barley has been focused on improving: yield and stability, resistance against diseases and pests, malting quality and quality as animal feed. Barley is grown for many purposes, but most barley is used as animal feed, human food and the production of malt.

The barley variety 'Albacete'

Conventional plant breeding have been successful in improving yield and yield stability of some important crops under favourable conditions or to conditions which could be made favourable by the use of fertilizers, irrigation, pesticides and fungicides. However, current research is focused on raising yields under low-input conditions, which are more self-sustaining and less harmful to the environment.

In the semiarid areas of Spain crop productivity is not only limited by drought, but also by high temperatures at the end of the growth cycle. The increased use of 'local' germplasm could lead to the selection of varieties adapted to specific regional conditions. So, the use of a barley variety like 'Albacete', with its proven adaptation to semiarid conditions, can be an excellent option.

In Spain 'Albacete' is the most widely grown barley variety during the last decades (up to 1 million ha/year), particularly in semiarid rain-fed areas. This variety was obtained more than 50 years ago by Enrique Sánchez-Monge at the Estación Experimental Aula Dei (Spanish Research Council) in Zaragoza, as an inbred line in a local heterogeneous landrace population from the Albacete province. It is a six-row facultative variety with a relatively long growth cycle and an alternative growth habit, showing lodging at the end of the cycle. Its yield, in optimal environmental conditions, ranks in the average compared to other cultivars. However, its tolerance to cold and drought makes 'Albacete' the best choice in years with low rainfall. For all these properties, it has been grown for decades, particularly in drought-prone areas.

Lasa et al. (1987) investigated different barley populations under drought conditions in Spain using 'Albacete' cultivar as a check. They found significant differences between the populations and between the populations and 'Albacete' for all the traits (related to crop development, growth and yield components). For most of the traits, the local cultivar 'Albacete' gave the best results, with the exception of test weight and lodging. 'Albacete' was the latest in flowering, and had the shortest grain filling period. Later, Arnau et al. (1997) studied the effect of water stress on plant water status and net photosynthetic gas exchange in six barley genotypes differing in productivity and drought tolerance. 'Albacete' showed the highest osmatic adjustment capacity, relative water content and photosynthetic gas exchange. The high osmotic adjustment capacity of 'Albacete' could explain its high yield under very dry conditions.

Intense breeding efforts to cross 'Albacete' with other varieties during the 1970s and 80s failed due to the sterility of the offspring, which gave 'Albacete' a poor reputation as a parent in breeding programmes. Vazquez and Sanchez-Monge (1987) found the cytological cause of this sterility by carrying out meiotic analyse of semi-sterile F_1 hybrids involving 'Albacete'. The presence at metaphase-anaphase I of five bivalents and one quadrivalent, representing 75% of PMC (pollen mother cells) (Figure 1), provided clear evidence of the existence of a reciprocal translocation. This high percentage indicates that the interchanged segments must be of considerable length. They also detected that the coorientation of the quadrivalent in the hybrids of 'Albacete' was mainly of the adjacent type (74%) and the average fertility of the translocation heterozygotes was 54%. However, they did not identify the chromosomes involved. A preliminary study has revealed that chromosomes 1H and 3H are involved in the reciprocal translocation (Lacasa-Benito et al. 2005). 'Albacete' is the only known, widely cultivated barley variety that carries a reciprocal translocation. Until now, it is unknown whether the translocation is responsible for the drought tolerance and whether it has some interesting phenotypic attributes that could be transferred to other barley genotypes to develop specific germplasm adapted to stress.

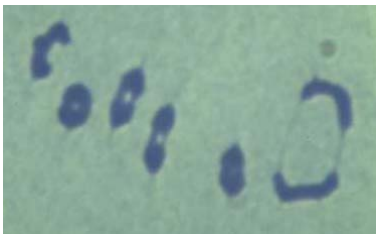

Figure 1. Meiotic metaphase of F_1 hybrid involving 'Albacete' showing 5 II + 1 IV (adjacent coorientation). (provided by L. Cistué)

Chromosome rearrangements

Chromosome rearrangements include deletions, duplications, inversions and translocations have played an important role in plant evolution. Each of these events can be caused by chromosome breakages which are followed by the reunion of broken ends (Figure 2a) or crossing-over between repetitive DNA segments (Figure 2b). Sometimes these breaks will occur within genes generally disrupting gene functions.

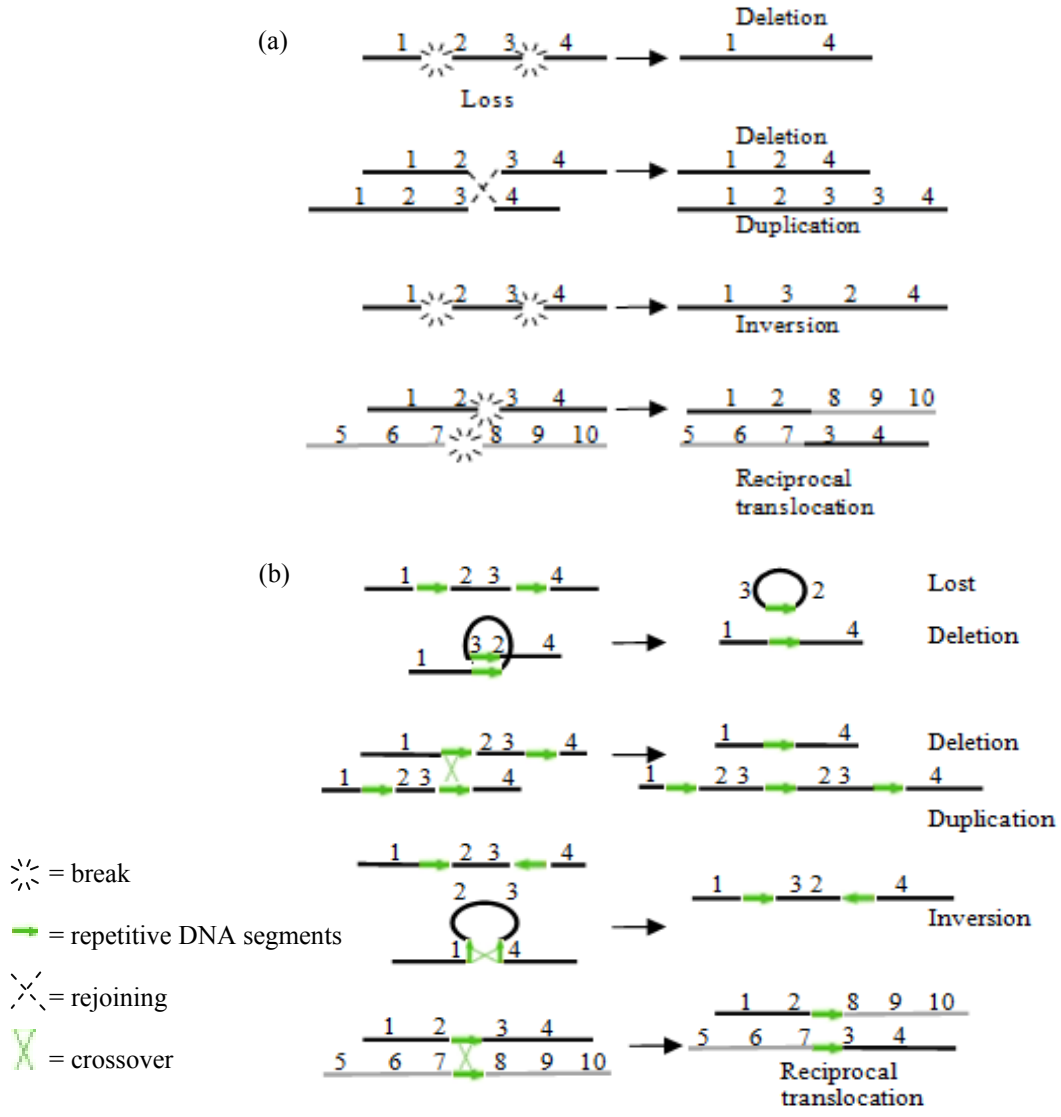

Figure 2. Origins of chromosomal rearrangements: (a) by breakage and rejoining; (b) by crossing-over between repetitive DNA

There are two general types of rearrangements, balanced and imbalanced. Imbalanced rearrangements change the total amount of chromosomal material of the affected chromosomes. The two simple classes of imbalanced rearrangements are deletions and duplications. Some examples in human are the *cri du chat* (del (5p)) or Wolf-Hirschhorn (del (18p); del (18q); del (4p)) syndromes. In contrast, balanced rearrangements change the chromosomal gene order but do not change the total amount of chromosomal material. The two simple classes of balanced rearrangements are inversions and reciprocal translocations.

Reciprocal translocations are produced by an interchange of chromosome segments between two non-homologous chromosomes. Rieger et al. (1968) classified the reciprocal translocations into asymmetrical or symmetrical translocations depending on the way the chromosome segments rejoin after a chromosomal breakage (Figure 3). In an asymmetrical translocation, the segments carrying the centromeres are joined together resulting in an acentric (without centromere) and dicentric (with two centromeres) chromosomes. Standard monocentric chromosomes are formed in symmetrical translocations, which are often viable.

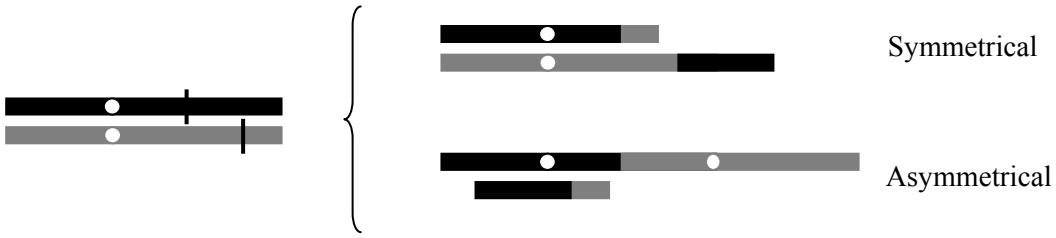

Figure 3. Possible products of a reciprocal translocation interchange

When a symmetrical reciprocal translocation occurs, two new chromosomal arrangements appear, so the individuals can be: (i) homozygotes for the standard arrangement (Figure 4a); (ii) homozygotes for the interchange (Figure 4b) and (iii) heterozygotes for the interchange (Figure 4c). For both homozygotes the meiotic behaviour is regular as they form bivalents. The complex meiosis for the structural heterozygote is explained below.

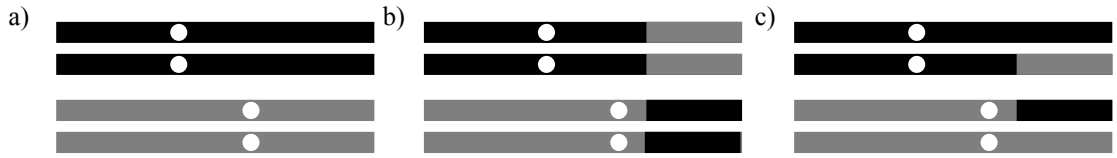

Figure 4. Possible individuals resulting from the reciprocal translocation

Meiosis in heterozygotes having two translocated chromosomes and their normal counterparts causes some important genetic and cytological effects. During meiotic prophase I homologous chromosomes interact with each other through precise synapsis and crossover recombination. In the case of a reciprocal translocation heterozygote, saturated pairing gives rise to a quadrivalent (association of 4 chromosomes) involving the two translocation chromosomes and their non-translocation counterparts instead of two independent bivalents in the normal situation (Burnham 1962; Sybenga 1975). Various configurations can be observed at diakinesis–metaphase I, depending on the number and positions of the crossing overs: zig-zag shapes, rings and open forms of 2–4 chromosomes, as well as univalents. As shown in Figure 5, crossing overs may occur in three types of chromosomal segments: the interchanged segment, the interstitial segment (between translocation points and centromeres) and the non-interchanged segments (chromosome arms distal to the centromeres).

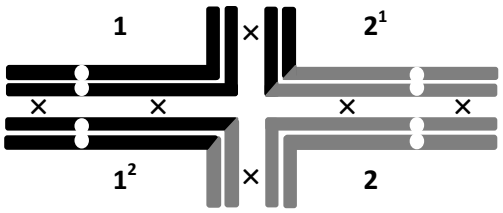

Figure 5. Pairing diagram of translocation heterozygote (1 and 2 are the normal chromosome arrengement, 1^2 and 2^1 are the translocated chromosomes)

If no crossing over takes place in the interstitial segments, the viability of the resulting gametes will depend upon the coorientation of the centromeres at metaphase I. These could be: (i) alternate segregation (corresponding with a zig-zag configuration of the quadrivalent) or (ii) adjacent segregation (corresponding with a ring configuration of the

quadrivalent). The latter type is further divided into two classes: Adjacent-2 and Adjacent-1 segregations, according to the separation of homologous centromeres, and whether they end up into the same Telophase I cell or not, respectively. Adjacent segregation leads to gametes with a translocation and a non-translocation chromosome, containing a duplicated and a deleted segment; this leads to non-viable gametes. Alternate segregation occurs when the chromosomes on opposite sides of the translocation cross segregate into the same pole. In these cases, four balanced gametes are formed: 50% carry the normal chromosome arrangement, and 50% have the translocated chromosomes. Hence, depending on the relative frequency of the alternative orientation, individuals that are heterozygous for a translocation will produce a certain proportion of non-viable gametes. This results in a reduction of fertility; it is called semi-sterility.

If we assume that a crossover occurs in one interstitial segment followed by alternate or adjacent-1 segregations, 50% of the gametes belong to the non-recombinant parental type and the other 50%, recombinant, carry a segment deficiency and duplication and are usually non-viable. The same crossover followed by adjancent-2 will produced non-viable gametes. It is important to note that, in barley, the alternate segregation in the interchange heterozygote is more frequent than the others types of separations, therefore crossing over will further reduce genetic recombination of loci within the interstitial segment because the chromatids carrying the crossover pass to the unbalanced gametes (Burnham, 1964). Therefore the presence of a reciprocal translocation reduces recombination frequencies between the translocation breakpoints (TB) and the centromere. If the TB is close to or in the centromere region, the reduction in recombination that may take place will be due to the interference effects in the flanking TB region.

Lacadena (1989) showed that more complex combinations of crossovers may lead to balanced gametes carrying recombination within the interstitial segment. For instance, if a single crossover occurs in both interstitial segments and in non-interchanged chromosome arms distal to the centromeres, it may happen that with adjacent segregation some gametes results in balanced recombinant chromatids. Following the same reasoning, the alternate segregation could generate unbalanced gametes. Thus, the

possibility of crossing-over in the interstitial region can alter the viability of gametes produced after an adjacent or alternate segregation.

Reciprocal translocations may occur spontaneously or in response to irradiation or treatments with certain chemicals. The first report on a naturally occurring reciprocal translocation was by Belling (1914) in the Florida velvet bean (*Stizolobium deeringianum*), and Muller (1930) was first to report on X-ray-induced translocations in *Drosophila*. In barley, few cases of spontaneous reciprocal translocations have been described in cultivated barley, although they are induced frequently by mutagen treatments. Konishi and Linde-Laursen (1988) conducted an exhaustive study which showed that chromosomal rearrangements such as inversions and translocations rarely occur spontaneously in barley. They investigated 1,240 cultivated barley lines and 120 wild barley lines to detect spontaneous reciprocal translocations. Of the 1,240 cultivated barley lines, four Ethiopian landraces carrying a reciprocal translocation had the same breakpoints at the centromere involving chromosomes 2H and 4H (2HS·4HS and 2HL·4HL), suggesting that the chromosomes involved in the rearrangement had a common origin. Of the 120 wild barley genotypes, three carried translocations between chromosomes 2H and 4H, 3H and 5H and 3H and 6H, respectively.

Translocations have been widely utilized in both applied and fundamental scientific research for chromosome mapping, in particular for assigning linkage groups to chromosomes, development of physical maps in plants (Kim et al. 1993; Sorokin et al. 1994; Marthe and Künzel 1994; Künzel et al. 2000), and to improve our understanding of meiotic chromosome pairing behaviour (Rickards 1983). They have been reported in a number of crop species, such as rye (Benito et al. 1994), soybean (Mahama and Palmer 2003), *Prunus* (Jáuregui et al. 2001), *Lens* (Tadmor et al. 1987), pea (Kosterin et al. 1999), wheat (Naranjo et al. 1987; Badaeva et al. 2007), *Brassica napus* (Osborn et al. 2003) and other crops.

Scope of the thesis

The aim of the thesis is to develop an in depth understanding of the reciprocal translocation present in the widely grown Spanish barley variety 'Albacete'. To achieve this objective different complementary approaches will be followed. First, a statistical-genetic approach will be developed for the construction of a linkage maps in populations obtained from reciprocal translocation heterozygotes using a double haploid population (Chapter 2). Second, the reciprocal translocation present in 'Albacete' will be characterized by a combination of cytological and molecular genetic approaches (Chapter 3). Third, a large number of doubled haploid lines derived from four crosses involving 'Albacete' as one of the parents will be used to phenotypically characterize the effects of the reciprocal translocation on some interesting agronomic traits at two locations in two growing seasons (Chapter 4). Fourth, once we know how to construct a linkage map if one of the parents carries a reciprocal translocation, use will be made of the statistical-genetic approach to construct an integrated genetic linkage map combining five interconnected biparental doubled haploid populations involving four barley varieties ('Albacete', 'Barberousse', 'Plaisant' and 'Orria') (Chapter 5). Fifth, development of specific methodology for the simultaneous analysis of these interconnected populations for QTL analysis in the presence of a reciprocal translocation (Chapter 6).

Chapter 2

Linkage map construction involving a reciprocal translocation

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Abstract

This paper is concerned with a novel statistical-genetic approach for the construction of linkage maps in populations obtained from reciprocal translocation heterozygotes of barley (*Hordeum vulgare* L.). Using standard linkage analysis, translocations usually lead to 'pseudo-linkage': the mixing up of markers from the chromosomes involved in the translocation into a single linkage group. Close to the translocation breakpoints recombination is severely suppressed and, as a consequence, ordering markers in those regions is not feasible. The novel strategy presented in this paper is based on (1) disentangling the "pseudo-linkage" using principal coordinate analysis, (2) separating individuals into translocated types and normal types and (3) separating markers into those close to and those more distant from the translocation breakpoints. The methods make use of a consensus map of the species involved. The final product consists of integrated linkage maps of the distal parts of the chromosomes involved in the translocation.

Keywords Linkage maps · Reciprocal translocation · Translocation breakpoint · Barley · Principal coordinate analysis · Graphical genotypes

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Introduction

At present barley (*Hordeum vulgare* L.) is the fourth most important cereal crop, after maize, wheat and rice. Barley is a diploid (2n = 14) self-pollinating species which can be used as a model plant for other *Triticeae* species providing an excellent system for genome mapping and map-based analyses (Costa et al. 2001). Well-established linkage maps containing morphological, chromosomal, protein and molecular markers are indispensable tools for genetic analysis and for analyzing DNA sequence variation between the crops and its relative accessions and species. In barley, common molecular marker technologies include restriction digest based RFLPs (Restriction Fragment Length Polymorphisms; Graner et al. 1991; Heun et al. 1991; Kleinhofs et al. 1993), and PCR-based markers like AFLPs (Amplified Fragment Length Polymorphism; Waugh et al. 1997), STS (Sequence Tagged Site; Olson et al. 1989), SSRs (Simple Sequence Repeat; Liu et al. 1996; Ramsay et al. 2000; Pillen et al. 2000; Li et al. 2003), and recently, DArT (Diversity Arrays Technology), a technique based on DNA hybridization (Jaccoud et al. 2001; Wenzl et al. 2004). The more recent SNPs (Single Nucleotide Polymorphisms) have become popular thanks to a high density within the genome, easy development from sequence data and high reproducibility. Wenzl et al. (2006) constructed a high-density linkage map which will be used as a reference for future mapping work. The map was based on datasets from multiple populations, comprising 2,935 loci (2,085 DArT, 850 SSR, RFLP and/or STS markers) and spans 1,161 cM. Recently, Varshney et al. (2007) produced a high-density linkage map, using SSR marker data from six mapping populations. A total of 775 microsatellite loci from 688 primer pairs were used to cover 1,068 cM of the barley genome.

Major complications in the construction of genetic maps arose in populations containing reciprocal translocation heterozygotes (Lehmensiek et al. 2005). Reciprocal translocations involve the exchange of two terminal segments between two non-homologous chromosomes. Due to reciprocal translocations recombination between loci around the translocation breakpoints will be suppressed. Consequently, markers in these regions become 'pseudolinked', *i.e.* linkage is detected between markers lying on different chromosomes. Reciprocal translocations are induced spontaneously or artificially by ionizing radiation, mutagens or by transposons and can be confirmed by

aberrant chromosome and banding morphology, by establishing quadrivalent formation in metaphase I complements, and semisterility of pollen in translocation heterozygotes, and by linkage analysis (Jáuregui et al. 2001). Translocations are well-documented in various crops, including rye (Alonso-Blanco et al. 1993; Benito et al. 1994; Catarino et al. 2006), soybean (Mahama and Palmer. 2003), *Prunus* (Jáuregui et al. 2001), *Lens* (Tadmor et al. 1987) and pea (Kosterin et al. 1999). Many bread wheat cultivars contain the 1RS-1BL wheat-rye chromosome translocation, introduced to provide resistance to pests and diseases (Mago et al. 2002; Sharma et al. 2009).

In barley, spontaneous reciprocal translocations were described by Konishi and Linde-Laursen (1988) who investigated 1,240 barley landraces and varieties and 120 wild barley lines, using test-cross and Giemsa chromosome banding analyses. Of the 1,240 landraces and varieties, four carried translocations involving chromosomes 2H and 4H with breakpoints near or at the centromere. Of the 120 wild barley lines, three carried translocations between chromosomes 2H and 4H, 3H and 5H and 3H and 6H, respectively. 'Albacete' is a barley variety that it is known to have a reciprocal chromosomal translocation. Recent results suggest that chromosomes 1H and 3H are involved in this translocation (Lacasa-Benito et al. 2005).

The consensus map of barley (Wenzl et al. 2006) was used as a reference. Preliminary linkage analysis of a doubled haploid population obtained from a cross between 'Albacete' and the non-translocation barley variety 'Barberousse' shows that linkage groups 1H and 3H are indeed involved in the translocation. In order to detect unexpected relationships between markers at those chromosomes we apply a principal coordinate analysis which visualizes marker positions in a tridimensional graph. The next step of our analysis is based on the cytogenetical theory that (1) during meioses two types of viable gametes are produced: a "normal" type (with a balanced set of non-translocation chromosomes) and a "translocated" type (with a balanced set of translocated chromosomes), and (2) that around the translocation breakpoints recombination is suppressed (Burnham, 1962; Sybenga, 1975). The crucial step consists of dividing the doubled haploid population into two subpopulations according to the origin of the alleles at the translocation breakpoints: 'Barberousse' alleles refer to the "normal" type and 'Albacete' alleles refer to the "translocated" type. The position of the

translocation breakpoint is found by a graphical display of recombination frequencies versus positions on the consensus map. At this stage we discuss the problem of extreme segregation distortion around the translocation breakpoints in the two subpopulations, which is solved by removing markers around the translocation breakpoints with severe segregation distortion. Linkage maps for the various chromosome arms are then obtained for each of the subpopulations separately. The final step is integrating the linkage maps of the corresponding arms of linkage groups 1H and 3H of the two subpopulations.

Materials and methods

Plant material

Two six-row parental barley varieties, 'Albacete' and 'Barberousse', were crossed and anther culture lines were produced from the F_1 from which 231 spontaneous doubled haploids were obtained at Estación Experimental de Aula Dei, Zaragoza, Spain. Seed from each doubled haploid line was retained as a reference stock and used to grow the plants for DNA extraction. More than 50 years ago, 'Albacete' was found by Enrique Sánchez-Monge at the Estación Experimental Aula Dei, Spanish Research Council, as an inbred line in a local heterogeneous landrace population from Albacete (province of Spain). It is a six-row barley variety, drought tolerant with a stable grain yield. For these properties, it has been grown for decades on up to 1 million ha/year, especially in drought-prone areas. 'Barberousse' is a six-row winter barley variety released in France in 1977. It was obtained from the cross [259 711/(Hatif de Grignon/Ares)]/Ager. Although it is sensitive to drought, this variety is known for its good productivity and easy adaptation.

DNA isolation

Genomic DNA of the DHs was extracted using two different protocols. For genomic and EST-derived SSR markers, genomic DNA was extracted from 0.1 g of fresh leaf tissue sprayed with liquid nitrogen according to the method of Doyle and Doyle (1990) with some modifications. DNA was diluted in TE buffer pH8 and preserved for a long

period at a temperature of -20ºC. For DArT markers the genomic DNA was extracted from fresh leaf tissue using the Qiagen DNeasy 96 Plant Kit.

Genotyping

Genotypic data were first produced for genomic and EST-derived SSR markers. We refer to the Genomic SSRs markers using the prefix ("Bmac" or "Bmag") followed by four digits and to the ESTs-derived SSR markers using the prefix ("scssr") followed by five digits. Genomic and EST-derived SSR analyses were carried out using the fluorescent fragment detection system on an ABI PRISM® 3100-Avant Genetic Analyzer using Genescan software from Applied Biosystems. For this method, the reverse primer was labeled with a fluorochrome at the 5' end. For all amplifications the PCR programs were used are published in the UK CropNet databases (http://ukcrop.net/db.html).

Subsequently, a genome wide scan was carried out using the Diversity Arrays Technology (DArT, http:\\www.diversityarrays.com). In a single assay, DArT uses microarrays to detect DNA polymorphisms at several hundred genomic loci spread over the entire genome. It is based on hybridising labelled genomic representations of individual DNA samples on a micro-array, which contains a large number of DNA fragments derived from the total genomic DNA of the species under consideration (Wenzl et al. 2004). Polymorphisms were scored as presence (= 1) or absence (= 0) of hybridization to individual array elements. The locus designations of Triticarte Pty. Ltd. were used. DArT markers consisted of prefix ("bPb" or "CloneID") followed by a number corresponding to the particular clone in the genomic representation.

Linkage analysis

For the construction of linkage maps we used the maximum likelihood (ML) algorithm in Jansen et al. (2001) which enables map construction in batch mode and the implementation in JoinMap 4.0 (van Ooijen 2006) which enables the use of many graphical tools. Linkage maps were drawn using MapChart 2.2 (Voorrips 2002).

Statistical techniques

Principal coordinate analysis (Chatfield and Collins 1980), a special form of multidimensional scaling, was used to obtain a 3D representation of the markers on the linkage groups involved in the reciprocal translocation. The similarity between markers was measured by the simple matching coefficient, which for a doubled haploid population is equal to one minus the recombination frequency. Calculations were carried with Genstat version 12 (Payne et al. 2009), using the directives FSIMILARITY and PCO.

Results

Polymorphisms between parents

We applied the following liberal rules for excluding markers: (1) markers with an unknown location on the consensus map (these were later included again when constructing our final maps); (2) markers with an ambiguous chromosome assigment on the consensus map; (3) markers with more than 5% missing data and (4) markers with low quality readings (Q value less than 77). For linkage analysis, 30 polymorphic genomic and EST-derived SSR markers and 309 polymorphic DArT were used. This amounted to an average of 48 markers per chromosome, with an average interlocus distance of 4.4 cM based the consensus linkage map. A few regions had gaps of over 10 cM. Chromosome 4H showed strikingly low levels of polymorphisms, which is in agreement with the findings of Qi et al. (1996), Karakousis et al. (2003), Kleinhofs et al. (1993) and Wenzl et al. (2006).

Preliminary linkage map construction

We obtained six main linkage groups involving 305 markers. Using a recombination frequency threshold of 0.2, 34 markers could not be assigned to one of these linkage groups. Five linkage groups could be related to linkage groups of the consensus map (Wenzl et al. 2006): 2H (48 markers), 4H (20 markers), 5H (48 markers), 6H (49 markers) and 7H (58 markers). The remaining 82 markers had known positions on 1H

and 3H of the consensus map, of which 78 formed one linkage group. Four markers, on the consensus map located at the very end of the long arm of linkage group 1H, were found to be unlinked with the 78 markers of 1H and 3H. One marker showing a consistently high nearest-neighbour stress (Van Ooijen 2006) was also excluded. Figure 1 displays one linkage map with the positions of the remaining 77 markers obtained by the maximum likelihood algorithm. Many different linkage maps were obtained by rerunning the maximum likelihood algorithm. This was a clear indication that it was not possible to obtain a linear order of the markers in an unambiguous way.

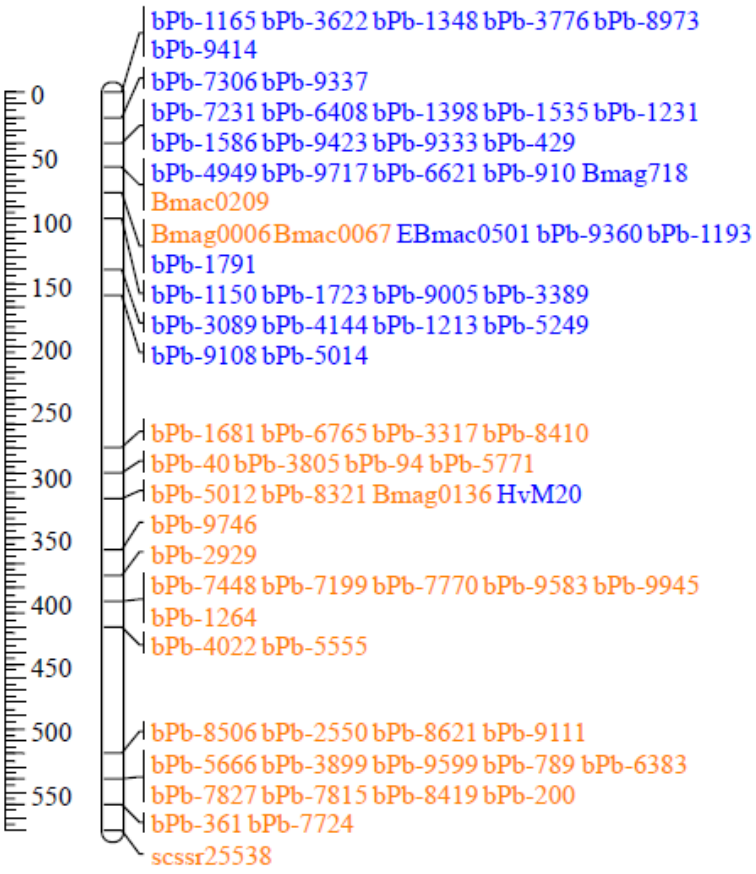

Figure 1. Graphical representation of a linkage map obtained for markers of linkage groups 1H and 3H according to the consensus map of Wenzl et al. (2006); markers of linkage group 1H and 3H are displayed in blue and ochre, respectively.

A spatial representation of the positions of the markers was obtained using principal coordinate analysis. Figure 2 shows a three-dimensional plot in which the points

represent the positions of the markers with regard to the first three principal axes; the percentages variance explained by the first three axes were 27.2, 15.59 and 13.9, respectively. The points have been joined by straight lines according to their position on the consensus map of Wenzl et al. (2004). Markers of linkage group 1H and 3H have been displayed in blue and ochre, respectively. Figure 2 shows that linkage groups 1H and 3H merge at one point (indicated by the circle) and that the short and long chromosome arms divert from this point, and from each other, in three-dimensional space. As a consequence, the translocation breakpoints lie within the circle.

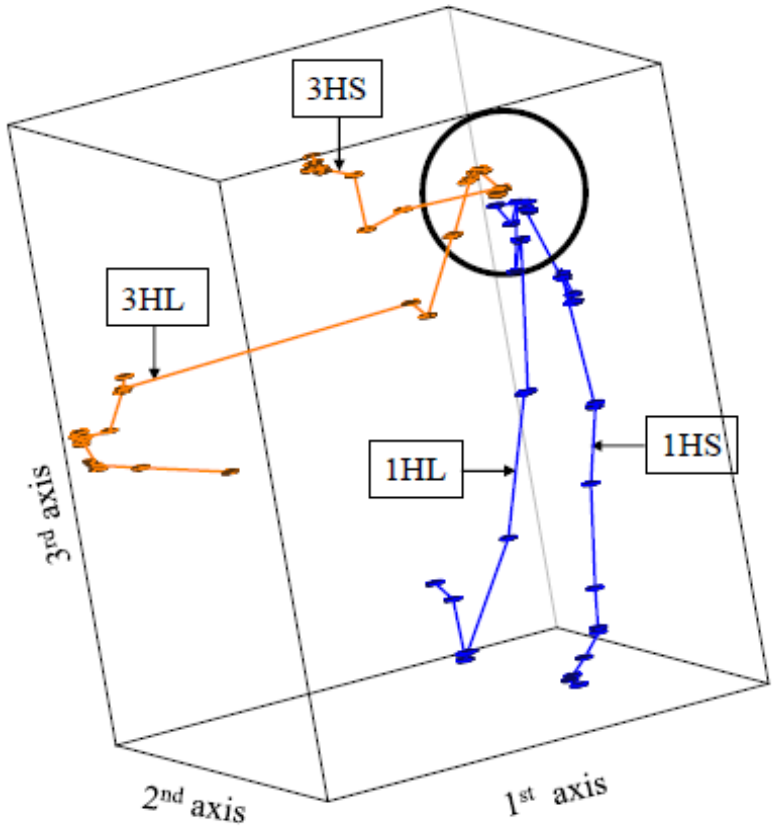

Figure 2. Graphical representation of a principal coordinate analysis using the simple matching coefficient for the markers of linkage groups 1H and 3H according to the consensus map of Wenzl et al. (2006). The percentage variance explained by the first three principal axes are 27.2, 15.5 and 13.9, respectively. The markers have been joined according to their position on the consensus map of Wenzl et al. (2006). The black circle encloses markers of linkage groups 1H and 3H that are close to the translocation breakpoints; they are separated by a small number of recombinations.

Recombination around the translocation breakpoints

To study the recombination behaviour of markers around the translocation breakpoints we focused on estimates of the recombination frequencies between the markers of 1H and 3H, in which the allocation of markers to linkage groups as well as their positions were based on the consensus map. In Figure 3, these recombination frequencies were plotted against the corresponding positions on the consensus map. In Figure 3a, we observed that markers close to the translocation breakpoints have recombination frequencies close to zero. On linkage group 1H the translocation breakpoint was situated at about 60 cM and on linkage group 3H at about 65 cM based on the consensus map. The marker pair with the smallest recombination frequency consisted of HvM20 (1H) and Bmag0136 (3H) with a recombination frequency of 0.017 (4 recombinants out of 231 DHs). Cross-sections of Figure 3a through the positions of markers Bmag0136 and HvM20 are shown in Figure 3b and 3c, respectively.

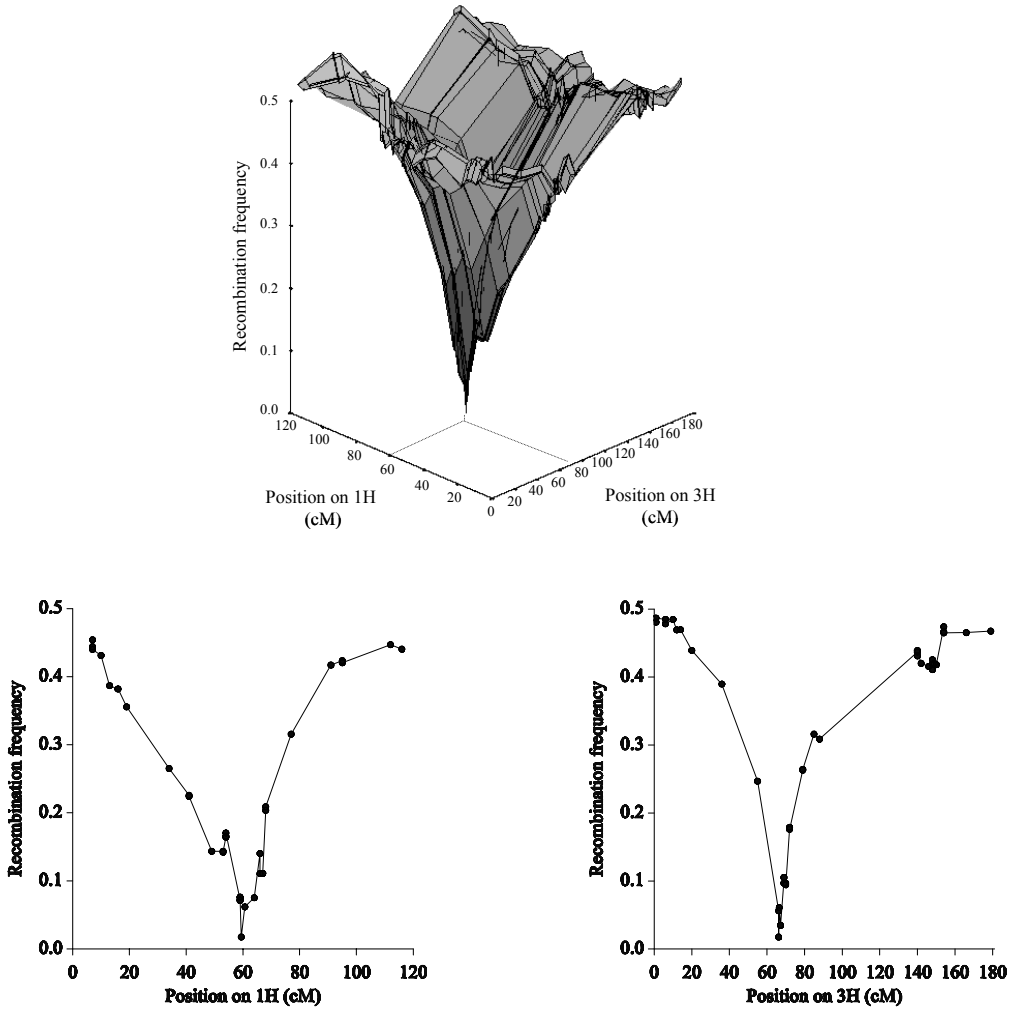

Figure 3. (a) Graphical representation of the recombination frequencies between markers of linkage group 1H and linkage group 3H versus their position on the consensus map of Wenzl et al. (2006). (b) Graphical representation of the recombination frequencies between markers of linkage group 1H with marker Bmag0136 of linkage group 3H versus their position on linkage group 1H of the consensus map of Wenzl et al. (2006). (c) Graphical representation of the recombination frequencies between markers of linkage group 3H with marker HvM20 of linkage group 1H versus their position on linkage group 3H on the consensus map of Wenzl et al. (2006).

Cytogenetical theory suggests that the DH_1 population should be split into two subpopulations: (1) a subpopulation containing individuals in which the alleles close to the translocation breakpoint are identical to 'Albacete', and (2) a subpopulation containing individuals in which the alleles close to the translocation breakpoint are identical to 'Barberousse'. In the first subpopulation the chromosomal arrangement will be that of 'Albacete' (1HS-3HL, 3HS-1HL), in the second subpopulation the chromosomal arrangement will be that of 'Barberousse' (1HS-1HL, 3HS-3HL). The separation of the 231 DH individuals into two subpopulations was based on the data of markers HvM20 and Bmag0136 (Figure 4). First, the markers were divided into linkage groups 1H and 3H and subsequently sorted vertically according to their position within each of the linkage groups based on the consensus map. Secondly, individuals were sorted according to the origin of their allele on marker HvM20; more or less the same effect was obtained if the markers were sorted according to Bmag0136. The split of the individuals into two groups was not perfect; 53 individuals had to be excluded because they had deviating chromosomal combinations within 5 cM from the presumed translocation breakpoints. Some of these deviations concerned so-called 'singletons'; they are likely due to observation errors or caused by gene conversions.

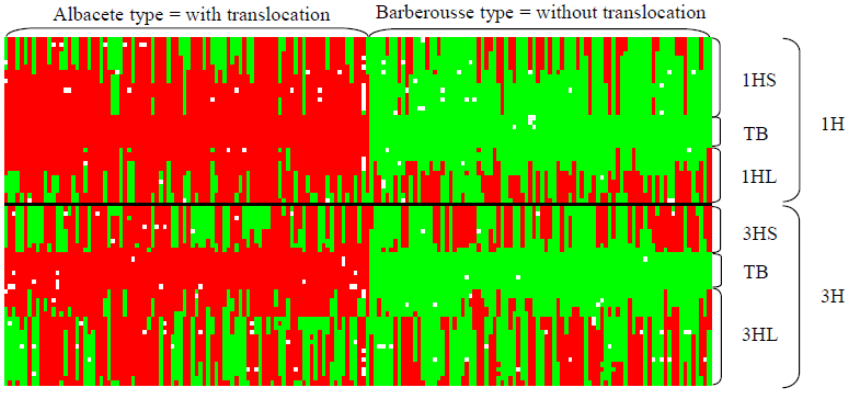

Figure 4. Graphical genotypes of individuals markers on linkage 1H and 3H according to the consensus map of Wenzl et al. (2006). Individuals have been divided into two types with regard to their alleles at the translocation breakpoints: the 'Albacete' type (shown in red), carrying the translocation, and the 'Barberousse' type (shown in green), with 'normal' chromosome arrangement.

In a doubled haploid population markers are expected to segregate according to a 1:1 ratio. However, due to the division into subpopulations, markers near the translocation breakpoints show severe segregation distortion in each of the subpopulations. The distortion decreases as markers are further away from the translocation breakpoints. Severe segregation distortion significantly affects estimation of genetic distances, but will not seriously affect the marker order (Hackett and Broadfoot 2003). Therefore, in order to construct robust linkage maps, markers with severe segregation distortion (in this case markers with segregation ratios below 30% or above 70%) were eliminated. In both subpopulations 37 markers out of 75 were removed, leaving 14 markers for 1H and 24 markers for 3H; these markers were common in both subpopulations.

The two subpopulations were considered separately to construct independent linkage maps for the chromosomes involved in the reciprocal translocation. Subpopulation 1 (with the 'Albacete' allele near or at the translocation breakpoint) contained 92 DHs; Subpopulation 2 ('Barberousse' allele near the translocation breakpoint) contained 86 DHs. The two subpopulations were then subjected to independent linkage analyses. Finally the linkage maps of the two subpopulations were integrated. Results are shown in Figure 5. The order of the markers for the 'Albacete' and the 'Barberousse' population are identical, but differences in distance are present.

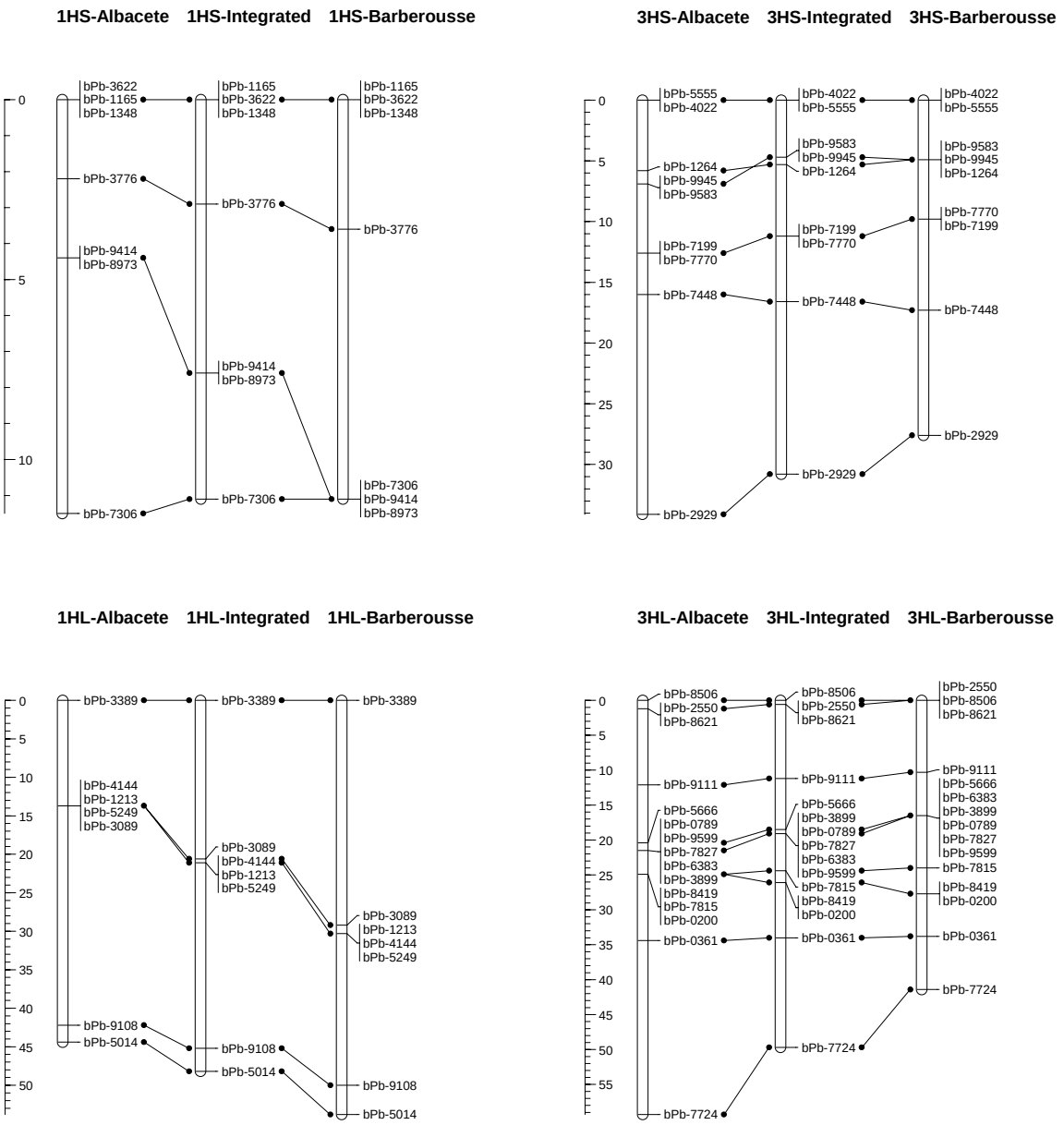

Figure 5. Linkage maps of the short and long arms of linkage groups 1H and 3H obtained for the individuals of type 'Albacete' and for type 'Barberousse' at the translocation breakpoints. The maps of the two types have been connected by their corresponding integrated maps. The maps were obtained for markers with a segregation ratio between 0.3 and 0.7.

Discussion

The methods developed in this paper provide essential tools for disentangling 'pseudo-linkage' between markers in a population derived from a translocation heterozygote. The methods can be implemented using standard software. In this paper a high-quality, consensus map of 'normal' barley (used as a reference) is essential in several steps of

the analysis: (1) interpretation of the three-dimensional principal coordinate plot (Figure 2), (2) determination of the translocation breakpoints on chromosomes 1H and 3H of 'normal' barley (Figure 3), and (3) assignment of markers to the respective chromosome arms (1HS, 1HL, 3HS and 3HL) in 'normal' barley (Figure 4). Without a high-quality, consensus map of 'normal' barley, step (2) will be impossible, and steps (1) and (3) will be more difficult. The chromosome arms designated 1HS, 1HL, 3HS and 3HL become arbitrary chromosome arms.

In a preliminary linkage analysis, six main linkage groups were detected, one of them joining markers from chromosomes 1H and 3H. As indicated by an earlier study this "mixing" of markers of chromosomes 1H and 3H was attributed to the presence of a reciprocal translocation between these chromosomes. In this study the position of the translocation breakpoint could be determined at about 60 cM on chromosome 1H and 65 cM on chromosome 3H (positions refer to the consensus map of 'normal' barley), which correspond with the centromeric regions of these chromosomes. These positions were confirmed in this study when all available markers from 1H and 3H were used to determine in more detail which markers were involved in the translocation breakpoints. In another DH_1 population obtained from a cross of ‘Albacete’ and 'Plaisant' (results not shown), the translocation breakpoints were found at similar positions.

In order to characterize the translocation breakpoint we identified 92 DHs as carrying the translocation and 86 DHs with the standard chromosome arrangement. Fifty three genotypes could not be classified in either of the two subpopulations due to deviating chromosomal combinations which may be caused by scoring errors. In marker studies like these scoring errors should be avoided by using very accurate marker systems. Nevertheless, it is interesting to note that, regardless of the true nature of the 53 non-classified types, the presence of this chromosome rearrangement did not affect the success rate in the double haploid regeneration process. As a result, two different genetic linkage maps were obtained, one with the two interchanged chromosomes together (1HS·3HL + 1HL·3HS, 'Albacete' type) and the other with the two normal chromosomes together (1HS·1HL + 3HS·3HL, 'Barberousse' type).

It is expected that barley varieties carrying chromosomal segments containing the reciprocal translocation between chromosomes 1H and 3H, have an advantage under certain environmental conditions. So, for future breeding purposes, it may be worthwhile to be able to follow individuals carrying interchanged chromosomes. In our case, this is the 'Albacete' haplotype. This requires the development of markers close enough to the translocation breakpoints with allelic combinations that allow differentiation between translocated and non-translocated origin of the chromosomal segments around the translocation breakpoints; in our case marker haplotypes that distinguish between 'Albacete' and all other material used in the breeding program. Away from the translocation breakpoints, a mixing of haplotypes will occur due to recombination. In the end, this would allow the construction of a specific linkage map of the karyotype with a chromosomal rearrangement close to the translocation breakpoints.

A genetic consequence of reciprocal translocation is that linkage relationships in a translocation heterozygote are altered as recombination between loci may be significantly reduced, particularly within the interstitial segment and between genes close to the translocation breakpoint. If the translocation breakpoint is located at or near the centromeric region, the interstitial zone becomes really narrow or even null and, therefore, the recombination that may take place will be due to interference effects near the translocation breakpoint. In our data we have not found a common set of completely linked markers from chromosome 1H and 3H, although a few showed very low levels of recombination. These rare recombinants can be due to scoring errors or, what is more likely, due to the fact that the interstitial zone is practically nonexistent.

The genomic SSR markers linked closely to the translocation breakpoint on chromosome 3H were identified flanking the centromere at 55 cM in the Lina × *Hordeum spontaneum* 'Canada Park' genetic map, between Bmac0067, Bmag0136 and Bmag006 in the short arm and Bmac0209 in long arm covering a genetic distance of 5 cM (Künzel and Waugh 2002). However, this region (with suppressed recombination) covered 36% of the entire chromosome length on the physical map. For chromosome 1H, no articles were found, which relate physical distance to genetic distance using genomic SSR markers along the entire chromosome; only one RFLP-based map

observed this phenomenon (Künzel et al. 2000). The gene density around the translocation breakpoints can be inferred using the synteny observed for barley-rice. Mayer et al. (2009) mentioned that approximately 20% of all genes on barley chromosome 1H are located in centromeric and subcentromeric regions with very low recombination frequencies. A similar gene density can be expected for barley chromosome 3H; Smilde et al. (2001) reported a 30-fold reduction of recombination around the barley 3H centromere.

Livingstone et al. (2000) state that karyotypic rearrangements, such as reciprocal translocations, lead to high variances of inter-marker distances for markers on chromosomes involved in the reciprocal translocation away from the translocation breakpoints, in repeated attempts to construct a linkage map (see also Durrant et al., 2006). Markers close to the translocation breakpoints show small variances of inter-marker distances. However, high variances may also be caused by deficiencies in the data. The methods developed in this paper provide a novel combination of methods available in standard (statistical) software that can be used to identify the true background of 'pseudo-linkage' in a straightforward manner.

In conclusion, the results of this study confirm that there is a reciprocal translocation as the result of an interchange of chromosome arms between 1H and 3H. The markers found to be linked to the translocation breakpoints are HvM20 and EBmac0501 for 1H and Bmag0136, bPb-9746 and a reduced set of genomic SSR markers for 3H, which are located around the centromere. Linkage analysis revealed that alternative arrangements involving the short and long chromosome arms of 1H and 3H may be possible. However, the most frequent arrangement is 1HS·3HL + 3HS·1HL. In addition, regardless of the presence of a reciprocal translocation we have produced a linkage map for the 'Albacete' x 'Barberousse' population that will be used to identify quantitative trait loci (QTLs) for agronomic traits in future work.

Acknowledgements

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Chapter 3

Genetic characterization of a reciprocal translocation present in a widely grown barley variety

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Abstract

Artificially induced translocation stocks have been used to physically map the barley genome; however, natural translocations are extremely uncommon in cultivated genotypes. 'Albacete' is a barley variety widely grown in the last decades in Spain and carrying a reciprocal translocation which obviously does not affect its agronomical fitness. This translocation has been characterized by a combination of cytological and molecular genetic approaches. First, recombination frequencies between markers on chromosomes 1H and 3H were estimated to determine the boundaries of the reciprocal interchange. Secondly, 1H-3H wheat barley telosome addition lines were used to assign selected markers to chromosome arms. Thirdly, fluorescence in situ hybridization (FISH) with rDNA probes (5S and 18S-5.8S-26S) and microsatellite probes [$(ACT)_5$, $(AAG)_5$ and $(CAG)_5$] was used to determine the locations of the translocation breakpoints more precisely. Fourthly, fine-mapping of the regions around the translocation breakpoints was used to increase the marker density for comparative genomics. The results obtained in this study indicate that the translocation is quite large with breakpoints located on the long arms of chromosomes 1H and 3H, between the pericentromeric $(AAG)_5$ bands and above the $(ACT)_5$ interstitial distal bands, resulting

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in the reciprocal translocation 1HS.1HL-3HL and 3HS.3HL-1HL. The gene content around the translocation breakpoints could be inferred from syntenic relationships observed among different species from the grass family Poaceae (rice, *Sorghum* and *Brachypodium*) and was estimated at approximately 1,100 and 710 gene models for 1H and 3H, respectively. Duplicated segments between chromosomes Os01 and Os05 in rice derived from ancestral duplications within the grass family overlap with the translocation breakpoints on chromosomes 1H and 3H in the barley variety 'Albacete'.

Keywords Reciprocal translocation · Barley · Translocation breakpoint · Fluorescence In Situ Hybridization · Comparative genomics

Introduction

Reciprocal translocations, interchanges of chromosome segments between two non-homologous chromosomes, are one of the most common structural chromosomal rearrangements occurring in plant species. Translocations in plants have been widely described by Burnham (1956). Permanent translocation heterozygotes were first observed by Gates (1908) in *Oenothera* species which received generous cytological and genetic studies by Cleland (1922) and Belling and Blakeslee (1926). McClintock (1930) was the first to supply cytological evidence of interchanges in economically important crops such as maize. Translocations have been widely utilized in both applied and fundamental scientific research for chromosome mapping, in particular for assigning linkage groups to chromosomes, development of physical maps in plants (Kim et al. 1993; Künzel et al. 2000; Sorokin et al. 1994; Marthe and Künzel 1994), and to improve our understanding of meiotic chromosome pairing behaviour (Rickards 1983). They have been reported in a number of crop species, such as rye (Benito et al. 1994), soybean (Mahama and Palmer 2003), *Prunus* (Jáuregui et al. 2001), *Lens* (Tadmor et al. 1987), pea (Kosterin et al. 1999), wheat (Naranjo et al. 1987) and *Brassica napus* (Osborn et al. 2003).

Chromosomal interchanges, and translocations in general, can be artificially induced in somatic or meiotic cells by ionizing radiation or mutagens. They may also occur

spontaneously, although there are few cases of spontaneous reciprocal translocations described in cultivated barley. Konishi and Linde-Laursen (1988) investigated 1,240 cultivated barley lines and 120 wild barley lines to detect spontaneous reciprocal translocations, which were identified by both semi-sterility associated to test crosses and subsequent Giemsa banding technique. Of the 1,240 cultivated barley lines, four Ethiopian landraces carrying a reciprocal translocation had the same breakpoints at the centromere involving chromosomes 2H and 4H (2HS·4HS and 2HL·4HL), suggesting that the chromosomes involved in the rearrangement had a common origin. Of the 120 wild barley genotypes, three carried translocations between chromosomes 2H and 4H, 3H and 5H and 3H and 6H, respectively. Xu and Kasha 1991 identified a chromosomal interchange between chromosomes 3H and 4H using N-banding and in situ hybridization techniques in a wild barley cross. The plant heterozygous for the interchange was derived from the backcross of 'Su Pie' with pollen from a triploid interspecific F_1 hibrid of 'Su Pie' × tetraploid *Hordeum bulbosum* accession GBC141.

One of the consequences of a reciprocal translocation is the suppression of genetic recombination in a translocation heterozygote in the interstitial zone (the chromosome segment between the centromere and the breakpoint). The suppression depends on the centromere coorientation frequency at metaphase I (alternate or adjacent segregations) and upon the chiasmata (crossover) frequency expected in both interstitial segments (Hanson and Kramer 1949; Burnham and Hagberg 1956; Kasha and Burnham 1965; Sybenga 1975). Recombination suppression in the interstitial zone affects the linkage relationships in a translocation heterozygote which results in "pseudo-linkage" between the genes of the two chromosomes involved in the reciprocal translocation and subsequent disturbed linkage maps. Another consequence of reciprocal translocations is the occurrence of gametic sterility of which depends on the frequency of the alternate or adjacent orientation in the quadrivalent. In barley, an excess of alternate over adjacent segregation of the chromosomes at meiotic metaphase I would explain the average 25% sterility caused by the interchange (Kakeda and Miyahara 1995).

Plant accessions carrying chromosome rearrangements such as translocations have been identified in the past by its effects on partial pollen and seed sterility (*i.e.* Jáuregui et al. 2001). However, depending on the chromosome breakpoints, a translocation can result

in the disruption or misregulation of normal gene functions. Thus, special interest resides on the characterization of the physical locations of the translocation breakpoints on the genome allowing physical mapping of genes involved in the translocation on the chromosomes. These can be delimited by a combination of cytogenetics and molecular genetics. In barley, chromosome identification can be achieved by using in situ hybridization with ribosomal RNA probes (Brown et al. 1999). In addition, with labelled SSRs it is now possible to cover the physical map with many landmarks distributed along all chromosome arms (Cuadrado and Jouve 2007). Undoubtedly, this rich set of chromosome markers should help to identify the barley breakpoints more precisely than conventional staining techniques.

In the present paper, we aim at determining the positions of the translocation breakpoints in the Spanish six-row barley variety 'Albacete'. Translocations may have dramatic consequences such as modified phenotypes. In fact, 'Albacete' is the only extensively cultivated barley variety known to carry a reciprocal translocation between chromosomes 1H and 3H without any major reduction in fitness. It is adapted to low-yielding West Mediterranean areas and it has been the most widely grown cultivar in the driest Spanish areas for the last decades (over a million ha per year). The translocation was first identified by Cistué (unpublished) upon meiotic analysis of semi-sterile F_1 hybrids involving this variety.

In tracing the possible origin of the reciprocal translocation in 'Albacete' we use syntenic relationships between different families of the grass family Poaceae. For the location and characterization of the translocation breakpoints we used a combination of molecular genetical and cytological techniques. Dense genetic linkage maps of the chromosomes involved in the reciprocal translocation will be used to identify markers in the vicinity of the translocation. Pseudo-linkage arising from suppressed recombination in the interstitial space may result in increased similarities between markers located on the two chromosomes involved in the reciprocal translocation and can be used to fine-map the recombination breakpoints. Further validation and physical characterisation of the translocation breakpoints will be achieved by the use of wheat-barley telosome addition lines and fluorescence in situ hybridization with rDNA probes (5S and 18S-5.8S-26S) and microsatellite probes [$(ACT)_5$, $(AAG)_5$ and $(CAG)_5$] of

'Albacete' and doubled haploid lines derived from crosses between 'Albacete' and cultivars with a standard chromosome arrangement.

Materials and methods

Linkage analysis

Two bi-parental doubled haploid (DH) mapping populations were used. The first mapping population, 'Albacete' × 'Barberousse' (AB), consists of 231 DH lines derived from anther-culture from the cross between the translocation-carrying six-row winter variety 'Albacete' and the six-row winter variety 'Barberousse'. DNA isolation and genotyping data, SSR and DArT®, were performed according to Farré et al. (2011). The second mapping population, ('Albacete' × 'Plaisant') × 'Plaisant' (APP), consists of 94 DH lines derived from the cross of a DH line produced from the 'Albacete' × 'Plaisant' F1, which carried the 'Albacete' reciprocal translocation, backcrossed to 'Plaisant'. DNA was extracted from leaf tissue using kit DNeasy Plant Mini Kit (Quiagen, Valencia, CA, USA). A set of 3072 EST-based high confidence SNP markers were genotyped using GoldenGate BeadArray technology (Illumina) as previously described (Close et al. 2009).

The allocation of markers to linkage groups as well as their genetic map position were based on the published barley consensus map (Wenzl. et al. 2006) for the AB population and Close et al. (2009) for the APP population. The position of the translocation breakpoints was first inferred according to the methods described by Farré et al (2011).

Wheat-barley telosome addition lines

The chromosome arm locations of seven SSR markers on the chromosomes of the barley cultivar 'Betzes' were checked on the 'Chinese Spring'-'Betzes' (CS-B) wheat-barley telosome addition lines for chromosomes 1H and 3H. CS-B telosome addition lines for the chromosome 1H and 3H and chromosome arms 1HS, 3HS and 3HL were used to assign selected markers to chromosome arms. No telosome addition line for

chromosome arm 1HL was available. Genomic DNA from these addition lines were kindly provided by Rafiqul Islam (School of Agriculture, Food and Wine. The University of Adelaide, Australia) and Marion Röder (Leibniz Institute of Plant Genetics and Crop Plant Research. IPK, Germany).

Fluorescence in situ Hybridization

Plant material and root tip and chromosome preparation

Root tips were obtained from seedlings of Hordeum vulgare cv. 'Albacete'. Seeds were germinated on moist filter paper for 24 h at 25ºC and then kept at 4ºC for 72h followed by 25ºC for 24h to synchronize cell division. The seedlings were then transferred to ice-cold water for 24h to accumulate metaphases before tissue fixation in ethanol-glacial acetic acid (3:1) (Cuadrado and Jouve 2007). Chromosome preparations were prepared as described by Schwarzacher et al. (1989). Briefly, root tips were macerated with an enzymatic mixture and then squashed in a drop of 45% acetic acid. After removing the cover slips by quick freezing, the slides were air dried.

DNA probes and in situ hybridization

The three oligodeoxyribonucleotide probes [$(ACT)_5$, $(CAG)_5$ and $(AAG)_5$] were provided by Roche labelled at their 5' and 3' with digoxigenin or biotin. The novel and rapid non-denaturating FISH (ND-FISH) technique developed by Cuadrado and Jouve (2010) was carried out to detect SSR-enriched chromosome regions.

The other probes, pTa71 (a plasmid containing the 18S-5.8S-25S rDNA and the intergenic spacer of Triticum aestivum) and pTa794 (contains a 410-bp BamHI fragment of 5S rDNA isolated from wheat Triticum aestivum) were labelled by nick translation and PCR, respectively (Leitch and Heslop-Harrison 1992; Leitch and Heslop-Harrison 1993; Pedersen and Linde-Laursen 1994; Brown et al. 1999). Chromosome and probe denaturation and the in situ hybridization steps were carried out as described in Cuadrado et al. (2000).

Fluorescence microscopy and imaging

Slides were examined with a Zeiss Axiophot epifluorescence microscope. The separate images from each filter set were captured using a cooled CCD camera (Nikon DS) and processed using Adobe Photoshop, employing only those functions that are applied equally to all pixels in the image.

Results

Similarities between markers on chromosomes 1H and 3H in the AB population

In order to identify DArT and SSR markers in the vicinities of the reciprocal translocation breakpoints, we explored recombination frequencies, between the markers located on chromosomes 1H and 3H. The allocation of markers to linkage groups as well as their genetic map position were based on the published barley consensus map (Wenzl et al. 2006). A total of 82 out of 305 polymorphic markers were mapped on chromosomes 1H and 3H for the AB population. A graphical representation of the recombination frequencies was presented by Farré et al. (2011) which shows that markers on chromosome 1H and 3H were strongly related. This suggests the presence of a reciprocal translocation between these chromosomes. So, for the AB population, the markers with recombination frequencies close to zero were located in the consensus map around 59-64 cM and 66-70 cM for 1H (HvM20 and EBmac0501) and 3H (Bmac209, Bmac067, Bmag0006 and Bmag0136), respectively, which corresponds with the pericentromeric regions of both chromosomes 1H and 3H.

1H and 3H wheat-barley telosome addition lines

Wheat-barley chromosome addition lines were then used to assign the chromosome arm location of the SSR markers found to be closely linked to the translocation breakpoints using their presence/absence as detected in hexaploid wheat *T. aestivum* cv. 'Chinese Spring' (CS) and barley *H. vulgare* cv. 'Betzes' using PCR . For chromosome 3H, 3 out of 4 SSR markers were found to be located on the short arm and the other one on the long arm (positions reported by Künzel and Waugh 2002). Using 1H wheat-barley addition lines the two SSR markers used mapped on different chromosome arms.

HvM20 amplified in both CS and Betzes and, therefore, did not have any diagnostic value.

Fluorescence in situ hybridization analysis

Further progress in localizing the translocation breakpoints can be expected from direct cytological observations of barley chromosomes by FISH using probes that were used successfully as anchored chromosomal markers. The following locations of pTa794 (5SrDNA) were reported: interstitial on chromosome arms 2HL and 3HL, distal on 4HL and proximal on 7HS (Leitch and Heslop-Harrison 1993). In addition to the two NOR-bearing barley chromosomes, 5HS and 6HS, the locations of four minor rDNA loci with pTa71 (18S-5.8S-26S rDNA) were reported on chromosome arms 1HS, 2HS, 4HS and 7HS differentiated by their position and intensity (Pedersen and Linde-Laursen 1994). Thus the use of these two ribosomal probes should allow easy identification of all barley chromosomes including chromosomes 1H and 3H.

First, two-colour FISH was carried out with pTa71 and pTa794 in pollen mother cells of a hybrid between 'Albacete'×'Plaisant'. The expected chromosome pairing occurred during meiotic I prophase for a heterozygous genotype for the chromosomic arrangement, in which five bivalents and one quadrivalent were observed (Fig. 1a). Bivalents for the two satellited chromosomes, 5H and 6H, with the strongest pTa71 signals, and the bivalents formed by chromosomes labelled with both probes, 2H, 4H and 7H, were identified despite the weak signals observed in the Figure 1. Thus, chromosome 1H, with the stronger pTa71 signal between the non-satellited chromosomes, and 3H are the chromosomes involved in the quadrivalent (Fig. 1b,c).

We subsequently analysed mitotic metaphases of 'Albacete' (Fig. 1d-h). Chromosomes 2H, 4H, 5H, 6H and 7H showed the expected and distinctive FISH pattern of ribosomal probes. In addition, one pair of submetacentric chromosomes with a rather strong pTa71 signal on its short chromosome arm and pTa794 signal on its long chromosome arm and another pair of metacentric chromosomes without any ribosomal signals, confirmed the presence of a reciprocal translocation resulting in two chromosomal combinations 1HS-3HL and 3HS-1HL, respectively (Fig. 1f). These two chromosomes

are shown in Fig. 1i. Therefore, the translocation breakpoints are located below the pTa71 signal on 1HS and above the pTa794 signal on 3HL.

More information about the exact position of the translocation breakpoints can be obtained by identifying relocated FISH landmarks on chromosomes 1H and 3H. To this aim three microsatellites probes, $(ACT)_5$, $(CAG)_5$ and $(AAG)_5$ were chosen for their characteristic patterns on these chromosomes (Cuadrado and Jouve 2007). As expected, $(ACT)_5$ resulted in a distinct pattern of FISH signals on chromosomes 2H, 3H, 4H, 5H, and 6H. The characteristic signals on the long arm of chromosome 3H were found on the translocated chromosomes 1HS-3HL (Fig 1g and i). These results suggest that the translocation breakpoints are located below the pTa71 signal on 1HS and above the $(ACT)_5$ signal on 3HL (Fig. 1e). The $(AAG)_5$ microsatellite probe facilitates the identification of all barley chromosomes thanks to a rich pattern of signals of different intensities located in the pericentromeric and interstitial chromosomal regions (Fig. 1h). The characteristic patterns obtained in the translocated chromosomes are similar to the patterns found in other barley varieties with normal 1H and 3H chromosomes and suggest that the translocation breakpoints are located below the pericentromeric AAG signals on the long arms of chromosomes 1HL and 3HL (Fig. 1i-j). Results obtained with the $(CAG)_5$ probe suport the detection and identification of the translocations 1HS.1HL-3HL and 3HS.3HL-1HL. The signals obtained in the centromere of translocated chromosomes 3HS-1HL were stronger than on 1HS-3HL (Fig 1j).

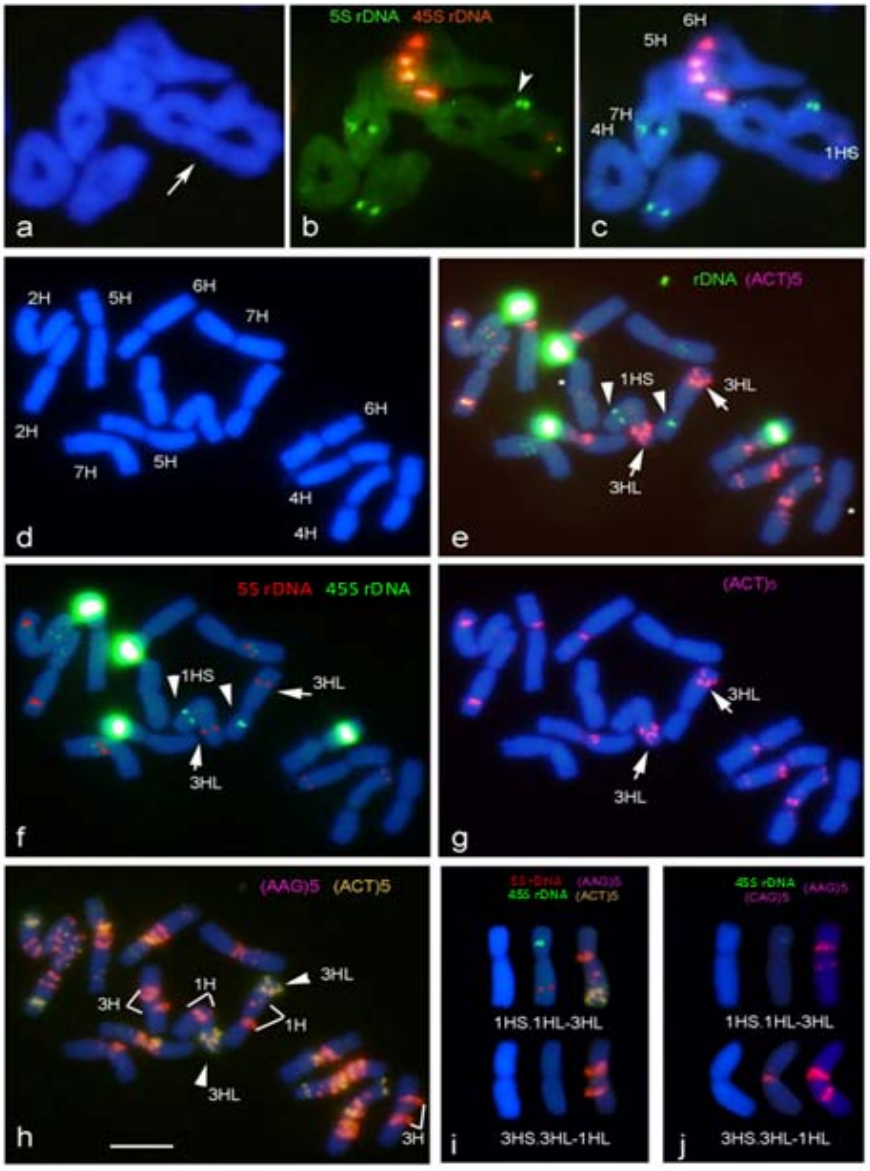

Figure 1. FISH results with rDNA probes (5S and 18S-5.8S-26S) and microsatellite probes ($(ACT)_5$, $(AAG)_5$ and $(CAG)_5$). (a-c) In situ hybridization of rDNA probes pTa71 (red) and pTa794 (green) in pollen mother cells of hybrid between 'Albacete' × 'Plaisant' (Arrows indicate the quadrivalent): (a) DAPI staining for DNA; (b) pTa71 and pTa794 probes; (c) combination of DAPI and pTa71 and pTa794 probes. (d-h) Root-tip metaphase chromosomes from barley (*Hordeum vulgare* cv. 'Albacete') after DAPI staining and in situ hybridization with biotin-labelled probes (detected by red Cy3) or digoxigenin-labelled probes (detected by green FITC): (d) DAPI staining for DNA; (e) rDNA probes and $(ACT)_5$ (arrows indicate the characteristic ACT signals on 3HL and arrowheads the 45S rDNA loci on 1HS); (f) pTa71 and pTa794 (arrows indicate the 5S rDNA loci on 3HL and arrowheads the 45S rDNA loci on 1HS); (g) $(ACT)_5$ (arrows indicate the characteristic ACT signals on 3HL); (h) $(AAG)_5$ and (ACT)5 (arrowsheads indicate the characteristic ACT signals on 3HL. Lines indicated the pericentromeric

AAG signals on chromosomes 1H and 3H); (i-j) translocated chromosomes hybridized with pTa71, pTa794, $(AAG)_5$, $(ACT)_5$ and $(CAG)_5$. Scale bar represent 10 μm.

Fine-mapping of the translocation breakpoint region

In order to increase the marker density around the translocation breakpoints the APP population genotyped with SNP was used. As $(AAG)_5$ and $(ACT)_5$ microsatellite probes have proved to be useful to determine the translocation breakpoints on 1H and 3H chromosomes, they were also chosen for detailed cytogenetic characterization of 8 out of 94 DH lines from the APP population (Figure 2). These DH lines were selected according to the 'Albacete' SNP allele distribution along 1H and 3H aiming to narrow down the position of the translocation breakpoints. The allocation of markers to linkage groups as well as their genetic map position were based on the published barley consensus map (Close et al. 2009). 755 out of 3072 SNP markers were polymorphic for the APP population; of these 212 markers mapped on chromosomes 1Hand 3H. From Figure 2 it can be observed that 8 markers around 50-52.5 cM on 1H (11_20427, 11_20660, 12_11209, 12_31208, 11_31381, 11_20912, 11_21312 and 12_30350) and 30 markers around 43.2-55.6 cM on 3H (12_21533, 11_11002, 11_21101, 11_11086, 11_11501, 11_10137, 11_10328, 11_20970, 12_30039, 12_30130, 12_30318, 12_31008, 12_31372, 11_10365, 12_10155, 12_31502, 11_10008, 11_20102, 11_10224, 11_20333, 11_20428, 11_20439, 11_10456, 11_20796, 11_20856, 11_20890, 11_21062, 11_21147, 11_11124 and 11_11337) were close to translocation breakpoints. This set of SNP markers was used to determine the gene content around the translocation breakpoints with rice, *Brachypodium* and *Sorghum* using Strudel (http://bioinf.scri.ac.uk/strudel/; Bayer et al. 2011). Inferred gene contents for rice, *Brachypodium* and *Sorghum* yielded around 1,100, 600 and 550 for the region on 1H and 710, 410 and 430 gene models for 3H, respectively.

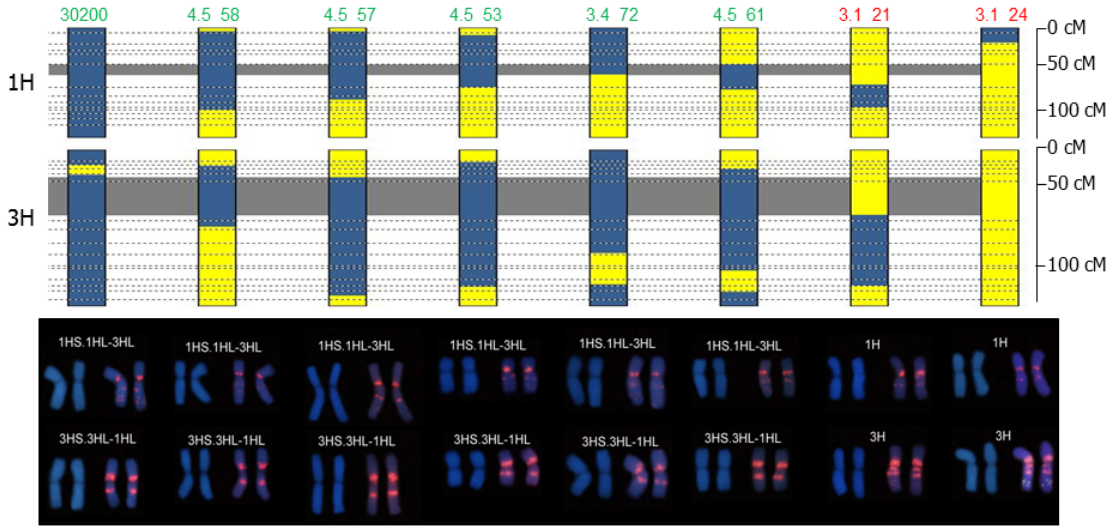

Figure 2. Fine-mapping of the translocation breakpoint region using eight selected lines from the APP mapping population. Graphical genotypes of the eight selected DH lines (top). Blue and yellow colours indicate 'Albacete' and 'Plaisant' parental alleles respectively. Genomic region in the vicinity of the reciprocal translocation breakpoints are indicated with grey colour. Horizontal dashed lines are drawn every 10 cM with the distance proportional to the number of SNPs markers. Line number are shown using green and red colours according to the presence or not of the reciprocal translocation, respectively (line nº30200 was used as a positive control whereas 3.1 24 as a negative). On the bottom, In situ hybridization of $(ACT)_5$ (red or green) and $(AAG)_5$ (red) in root-tip metaphase chromosomes 1H and 3H after DAPI staining and in situ hybridization with biotin-labelled probes (detected by red Cy3) or digoxigenin-labelled probes (detected by green FITC).

Discussion

The combination of cytogenetics and molecular genetics allowed us to determine the translocation breakpoints on chromosomes 1H and 3H of the barley variety 'Albacete' more accurately than with conventional staining techniques. The success of the combination of methods depends on the locations of the translocation breakpoints and on the presence of differential signals on the chromosomes involved (Xu and Kasha, 1991).

In the present study, the positions of the translocation breakpoints have been determined using a number of approaches. First, an analysis involving recombination frequencies

between markers was used to determine markers located near the translocation breakpoints with a high degree of precision (Farré et al. 2011). SNP and microsatellite markers putatively flanking the translocation breakpoints were identified in the AB and APP mapping populations in the pericentromeric regions of chromosomes 1H and 3H. Secondly, 1H and 3H wheat-barley telosome addition lines were used to assign a number of markers closely linked to the translocation breakpoints to their proper chromosome arms and to validate the genetic mapping results. For the genomic SSR markers on 3H chromosome, the positions are in good agreement with the positions reported by Künzel and Waugh (2002). They placed 24 microsatellite loci onto the physical RFLP map of barley chromosome 3H using the map position of the translocation breakpoints as reference (Künzel et al. 2000). Our genomic SSR markers on 3H chromosome were identified to be flanking the centromere (positioned at 55 cM) in the 'Lina'×*Hordeum spontaneum* Canada Park genetic map, where Bmac0067, Bmag0136 and Bmag0006 on the short arm and Bmac0209 on the long arm cover a genetic distance of 5 cM. It is well known that genetic map distances between markers along chromosomes correlate poorly with physical distances, particularly in the large cereal genomes where closely linked markers which genetically map near the centromere represent considerable physical distances (Schwarzacher 2003). As a consequence, most of the chromosomal gene content is trapped in chromosome segments with severely suppressed recombination. According to Künzel and Waugh (2002) the region flanked by our four SSR covers 36% of the entire 3H chromosome length on the physical map. For chromosome 1H, no studies were found relating genetic to physical distance using the genomic SSR we identified close to the translocation breakpoint. Although being pericentromeric we may expect results similar to those reported for 3H. In general, Triticeae centromeres are characterised by suppressed recombination (Schwarzacher 2003; Rostoks et al. 2002). In barley, suppressed recombination rates in proximal segments and high recombination rates in distal regions were demonstrated by Pedersen et al. (1995). Later, Künzel et al. (2000) reported that most recombination was confined in a relatively small chromosomal region, mostly at the arm ends, alternating abruptly with regions of severely suppressed recombination.

Our approach allowed us to identify and genetically delimit the physical regions in which the reciprocal translocation took place. However, as the regions involved in the

translocation are located in the vicinity of the centromeres, given the recombination suppression, they represent fairly large physical regions on both chromosomes 1H and 3H. FISH was used to validate the positions of the translocation breakpoints on the chromosomes involved in the chromosomal interchanges, which was found to be a useful approach to characterize non-recombinant regions.

The proposed positions of the breakpoints on the long arms of chromosomes 1H and 3H can be attributed to the combined use of rDNA and microsatellite probes. Once the translocation between chromosome 1H and 3H was confirmed using the ribosomal probes (pTa71 and pTa794) and $(ACT)_5$, the reasons that led us to concluding that the breakpoints are on the long arms of chromosomes 1H and 3H (resulting in the reciprocal translocation 1HS.1HL-3HL and 3HS.3HL-1HL) are multiple. Firstly, the signals obtained using the $(AAG)_5$ probe (which were similar to banding patterns obtained by C-banding) on the short arms of both chromosomes are the same as those observed in other barley varieties which indicates that the short arms are complete until the centromere and not involved in the reciprocal interchange (Pedersen and Linde-Laursen 1994; Cuadrado and Jouve 2007). However, a discrepancy in the number of bands on 3HL among different barley varieties was found; some barley lines such as 'Plaisant', 'Gaelic' and 'Hispanic' carried two bands compared with just one for varieties as 'Albacete', 'Dobla' and 'Golden Promise' (Á. Cuadrado, *personal communication*). The lacking of a band near the centromere could be a polymorphism in these varieties (unpublished data). Besides that, it is important to note that the signal intensity of the pericentromeric band present on 3HL is stronger than the one present on 1HL, which is characteristic for all barley varieties. Therefore, these results suggest that the translocation breakpoints are located below this band. Secondly, the differences in intensity found in the centromeres of the translocated chromosomes with the $(CAG)_5$ probe are consistent with those obtained for Plaisant (a variety with an standard chromosome arrangement). Signals on chromosome 3H were stronger than on 1H (Cuadrado and Jouve 2007) suggesting that the translocation breakpoints are located below the centromere on both chromosomes. Thirdly, the interchanged chromosomes 1HS.1HL-3HL and 3HS.3HL-1HL are more similar in total length and more submetacentric and metacentric, respectively, compared with published 'normal' barley karyotypes in which centromeres of chromosome 1H, the smallest barley chromosome, and 3H are located at 41 and 44% FL, respectively (arm ratios for 1H and 3H were

estimated at 1.4 and 1.3 milliGeNomes (mGN), respectively, and chromosome arms are the following: 51 mGN (1HS), 72 mGN (1HL), 64 mGN (3HS) and 83 mGN (3HL); Taketa et al. 2003). To summarize, the different patterns of these SSR probes have proven to be of a great value for localizing and validating the translocation breakpoints on 1H and 3H chromosomes in the Spanish barley variety 'Albacete'. The results obtained in this study suggest that the translocation breakpoints are located on the long arms of both chromosomes between the $(AAG)_5$ and $(ACT)_5$ pericentromeric bands for 3HS.3HL-1HL and below the $(AAG)_5$ pericentromeric band for 1HS.1HL-3HL leading to a interstitial zone with suppressed recombination.

For barley and other not yet sequenced species synteny conservation with related Poaceae species sequenced genomes such as rice, *Brachypodium*, maize or *Sorghum* (Mayer et al. 2011) can be explored and exploited for studying genome evolution and identification of candidate genes for traits of interest. So, an interesting point is to use the shared syntenic relationships observed among different species from the grass family Poaceae to determine the gene content around the translocation breakpoints. To explore genome co-linearity of barley with rice, *Brachypodium* and *Sorghum* we employed Strudel (http://bioinf.scri.ac.uk/strudel/), a standalone Java desktop application that allows the simultaneous multi-way comparison of several genomes (Bayer et al. 2011), on a reduced set of SNP markers selected using a cytogenetical and molecular characterization of the APP population.

The gene contents, as inferred from rice Os01/Os10 and Os05 chromosomes, around the translocation breakpoints were estimated as approximately 1,100 and 710 for chromosomes 1H and 3H, respectively. As a proportion of the genes represent retroelement-like components and pseudo-genes, the final number of functional genes in barley must be smaller. Inferred gene contents for *Sorghum* and *Brachypodium* yielded similar figures, around 600 (Sb09/Sb01) and 550 (Bradi02/Bradi03) genes for the region on 1H and 410 (Sb02) and 430 (Bradi03) gene models for 3H, respectively. This gene content is in good agreement with the gene content reported by Mayer et al. (2011) who estimated that the barley genome contains in the order of 32,000 genes. With such a large gene content the use of a candidate gene approach aiming at identifying which

gene(s) may have been altered by the translocation conferring a higher drought tolerance to 'Albacete' is not practical.

Whole-genome duplication analyses have been documented in grass species. Thiel et al. (2009) identified shared duplicated segments that originate from the last common ancestor and that remain in the barley genome. A large degree of synteny existed between barley chromosome Hv1H and rice chromosome Os05 as well as between Hv3H and Os01. The barley chromosome Hv3H corresponded to the ancestral chromosome type of rice chromosome Os01. The structure of chromosome 1H could be explained by combining the ancestral type of two rice chromosomes, where the first (A10) appears to be nested in the second (A05). An interesting observation arising from the comparative mapping of several Poaceae species is the presence of duplicated segments between chromosomes Os01 and Os05 in rice derived from ancestral duplications within the grass family (Guyot and Keller 2004) which are overlapping with the translocation breakpoints on chromosomes 1H and 3H in 'Albacete'. Moreover, there are also large grass ancestral genome duplications affecting rice chromosomes Os03 and Os07 homologous to barley chromosomes 2H and 4H. Smaller ancestral duplications are present in regions of the rice genome homologous to barley chromosomes 3H-5H and 3H-6H, which are also involved in spontaneous reciprocal translocations in barley (Konishi and Linde-Laursen 1988).

In conclusion, the widely grown Spanish barley variety 'Albacete' carries a large pericentric chromosome rearrangement between chromosomes 1H and 3H without any major change in fitness. SNP and microsatellite markers located in the proximity of the reciprocal translocation breakpoints were identified in both AB and APP mapping populations within the pericentromeric regions of both chromosomes. The physical characterization suggested that the translocation breakpoints are located on the long arms of both chromosomes between the $(AAG)_5$ and $(ACT)_5$ pericentromeric bands for 3HS.3HL-1HL and below the $(AAG)_5$ pericentromeric band for 1HS.1HL-3HL. The gene content in this region was estimated at approximately 1,100 and 710 gene models for 1H and 3H respectively. Presence of duplicated segments between chromosomes Os01 and Os05 in rice derived from ancestral duplications within the grass family (Guyot and Keller 2004) overlaps with the translocation breakpoints on 1H and 3H chromosomes in the barley variety 'Albacete'. However, no current evidence is

available of whether similar gene contents on different chromosomes arising from ancestral duplications play a role in spontaneous chromosomal exchanges through non-homologous chromosome pairing.

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Chapter 4

Agronomic effects of a large reciprocal translocation present in a widely grown Spanish barley variety

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Abstract

A large spontaneous reciprocal translocation is present in a widely grown Spanish barley cv. 'Albacete'. It has been hypothesized that high popularity of 'Albacete' with farmers, particularly in semi-arid areas where barley is grown under rainfed conditions, may be due to the presence of this translocation. Agronomic effects of this translocation were studied at two locations and two growing seasons in a set of 245 doubled haploid lines derived from the F_1s of four crosses involving 'Albacete'. The results have shown a significant positive main effect of the translocation on the thousand kernel weight and a significant environment by translocation interaction for the thousand kernel weight, lodging and tiller number. However, the results do not support the hypothesis that this chromosomal structural change alone provides an increased adaptation to low-yielding sites.

Keywords Reciprocal translocation · Barley · Breeding · Adaptation

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Introduction

Spontaneous reciprocal translocations seldomly occur in cultivated barley; only a few cases have been described (Konishi and Linde-Laursen 1988). Translocations usually reduce the agronomic value; 'Albacete' is the only extensively cultivated barley variety carrying a reciprocal translocation without apparent loss of agronomic value. In Spain it has been grown for decades on up to 1 million ha/year. The reciprocal translocation was identified in a meiotic analysis of semi-sterile F_1 hybrids involving 'Albacete' (Vazquez and Sanchez-Monge 1989; see also Farré et al. (2011)). Farré et al. (2012) performed a molecular and cytogenetic characterization of the reciprocal translocation and determined the position of the translocation breakpoints. Drought is the main factor limiting the yield of cereals in environments with high temperatures and limited rain during the grain-filling period (López-Castañeda and Richards 1994). It is unknown whether the reciprocal translocation has a positive effect on drought tolerance and other traits that make it worth to be introduced in the barley germplasm. In the present study, 248 doubled haploid (DH) lines from four crosses involving 'Albacete' as one of the parents will be used to phenotypically characterize the effects of the presence of the reciprocal translocation.

Materials and methods

Plant material

Different agronomic traits were evaluated in 245 DH lines of barley derived from the F_1s between 'Albacete' and 'Barberousse', 'Plaisant' and 'Orria' and a DH line derived from 'Plaisant'×'Orria'. 'Albacete' is a variety with a long cycle and an alternative growth habit. It is drought tolerant with a stable grain yield production. 'Barberousse' is known for its good productivity and easy adaptation; it is sensitive to drought. 'Plaisant' shows good adaptation and high-yield under Spanish conditions. 'Orria' is a Spanish variety of CIMMYT origin, well adapted to fertile, rainfed environments. The DH lines were scored for the presence of the reciprocal translocation using molecular data (Farré et al. 2011). The number of lines carrying/not carrying the translocation were 41/54, 18/20, 40/27, 36/9 for A×B, A×O, A×P and A×(P×O), respectively.

Phenotyping

Four field trials were carried out at two rainfed locations in North-Eastern Spain in 2008/2009 and 2009/2010: Gimenells (41º 37'N, 0º 22'E, 248m) and Foradada (41º 51'N, 1º 0'E, 407m). Experiments contained one or two replicates per DH line augmented by four replicated checks in a rectangular set-up. The traits measured were: days to heading, days to jointing, days to maturity, number of spikes in 50 cm, yield, thousand kernel weight (TKW), early vigour, till number, total height and lodging.

Data analysis

For each population the average broad sense heritability was estimated. For each trait, Best Linear Unbiased Estimates (BLUEs) of DH individuals were estimated by removing spatial effects. The BLUEs were further analysed using the mixed model facilities of Genstat version 13 (Payne et al. 2009), heterogeneous variances within population were corrected.

Results and Discussion

(Table 1) Broad sense heritabilities (H^2) ranged from 0.27 to 0.84. A highly significant main effect of the reciprocal translocation was obtained for TKW; DH lines carrying the reciprocal translocation had a greater TKW than those with a standard chromosome arrangement (34.8 vs 32.9 gr, respectively). No significant main effects were found for the other traits. For TKW, lodging and till number a significant environment by translocation interaction was found. More lodging was recorded at Gimenells and for the RT genotypes. Differences in the response of the RT to till number may be associated to specific meteorological conditions. In conclusion, the results do not support the hypothesis that the reciprocal translocation alone provides an increased adaptation to low-yielding sites; TKW is the only trait which is clearly enhanced by the reciprocal translocation. Future work combining the results from this study with QTL analysis will be carried out to characterize the effects of the reciprocal translocation and QTL simultaneously.

Table 1. (a) Summary of the mixed model analysis performed for all the agronomic traits studied comprising 982 genotypes carrying a reciprocal translocation (RT) or the standard chromosome arrangement (no RT) in four trials (the significant levels are based on the Wald test). (b) Average values for the two groups in 4 trials (carrying or not the reciprocal translocation).

(a)

		Traits associated to development						**Yield and yield components**					
Source of variation	d.f.[1]	**Days to Jointing**		**Days to Heading**		**Days to Maturity**		**Nº spikes 50 cm**		**Yield (T/ha)**		**TKW (g)**	
		Wald-test	p-value	Wald-test	p-value	Wald-test	p-value	Wald-test	p-value	Wald-test	p-value	Wald-test	p-value
Environment (E)	3	14689.0	<0.001	62871.4	<0.001	73360.5	<0.001	195.2	<0.001	559.6	<0.001	2982.0	<0.001
Population (Pop)	3	3.8	0.292	27.0	<0.001	4.1	0.263	19.7	<0.001	11.0	0.016	37.4	<0.001
RT	1	0.2	0.625	0.2	0.667	1.5	0.218	2.7	0.103	0.7	0.402	21.0	<0.001
Pop.RT	3	2.3	0.512	2.3	0.518	1.7	0.641	4.8	0.200	1.2	0.768	2.9	0.411
E.Pop	9	13.3	0.040	24.1	0.005	13.3	0.152	14.7	0.024	6.4	0.694	62.8	<0.001
E.RT	3	3.6	0.165	1.7	0.625	1.4	0.713	5.0	0.082	10.6	0.014	17.6	<0.001
E.Pop.RT	9	17.7	0.008	13.5	0.142	1.6	0.996	5.1	0.534	10.6	0.309	23.9	0.005
H^2		0.73		0.84		0.57		0.27		0.60		0.81	

(b)

Environment	no RT	RT	no RT	RT	no RT	RT	no RT	RT	no RT	RT	no RT	RT
F-2009/2010	149.5	148.1	183.0	183.1	223.0	223.4	44.5	45.6	4.9	5.1	35.6	37.9
F-2008/2009	-	-	166.6	166.8	196.0	196.3	57.7	55.8	5.4	5.6	29.8	32.3
G-2009/2010	126.9	127.1	161.8	161.9	202.0	202.4	56.4	59.5	6.0	6.3	38.3	40.0
G-2008/2009	109.4	109.2	151.3	151.2	184.6	184.8	-	-	4.7	4.4	27.8	28.4
Average s.e.d	0.63		0.35		0.27		1.80		0.13		0.51	

[1] The degrees of freedom for Days to Jointing, number of spikes in 50 cm, Early Growth and Till Number should be equal to two, as data was not recorded in one trial

(Table 1. Continued)

(a)

Source of variation	d.f.[1]	Architecture traits							
		Early Growth		**Till Number**		**Total Height (cm)**		**Lodging**	
		Wald-test	p-value	Wald-test	p-value	Wald-test	p-value	Wald-test	p-value
Environment (E)	3	393.4	<0.001	1357.0	<0.001	176.5	<0.001	231.0	<0.001
Population (Pop)	3	4.1	0.263	13.9	0.005	19.3	<0.001	36.0	<0.001
RT	1	1.3	0.262	1.3	0.263	0.0	0.923	0.1	0.745
Pop.RT	3	1.6	0.662	6.0	0.123	2.0	0.579	1.6	0.654
E.Pop	9	13.0	0.044	20.3	0.003	17.4	0.045	18.3	0.034
E.RT	3	0.5	0.765	12.6	0.002	1.1	0.781	13.7	0.004
E.Pop.RT	9	6.0	0.425	4.7	0.584	7.1	0.624	7.8	0.559
H^2		0.46		0.49		0.41		0.60	

(b)

Environment	no RT	RT	no RT	RT	no RT	RT	no RT	RT
F-2009/2010	3.1	3.1	2.7	2.8	90.1	88.7	4.3	4.3
F-2008/2009	-	-	-	-	102.8	104.8	4.78	3.9
G-2009/2010	2.7	2.7	5.2	5.8	93.8	94.1	4.6	5.2
G-2008/2009	3.8	3.7	7.4	7.0	99.0	97.0	6.8	7.2
Average s.e.d	0.09		0.23		1.82		0.41	

[1] The degrees of freedom for Days to Jointing, number of spikes in 50 cm, Early Growth and Till Number should be equal to two, as data was not recorded in one trial

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Chapter 5

Construction of an integrated genetic linkage map of barley (*Hordeum vulgare* L.) involving a parent carrying a reciprocal translocation

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Abstract

Using DArT® markers, an integrated genetic linkage map has been constructed by combining five interconnected biparental doubled haploid (DH) populations of barley involving four barley varieties. Three populations share 'Albacete' as a parent. 'Albacete' carries a reciprocal translocation between chromosomes 1H and 3H which complicates the construction of genetic linkage maps due to 'pseudo-linkage' between markers on the chromosomes involved in the translocation. Levels of polymorphism varied considerably among populations; the highest level was observed for 'Albacete' × 'Barberousse' (65.68%) and 'Albacete' × 'Plaisant' (60.86%) whereas 'Barberousse' × 'Plaisant' (22.08%) showed the lowest level. Only markers polymorphic in at least two populations were used for constructing the integrated map. As a consequence, construction of an integrated linkage map involves huge numbers of missing data. The algorithm used for constructing the integrated map is based on minimization of the number of recombination events between adjacent markers using hidden inheritance vectors. This algorithm enabled us to develop a robust integrated linkage map involving complex chromosomal rearrangements. The integrated map consists of 310 markers covering 938 cM for all chromosomes except 1H and 3H. The number of markers per chromosome varied between 17 (4H) and 92 (7H).

Keywords Integrated map · DArT · Reciprocal translocation · Barley

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Introduction

High-density genetic linkage maps have been constructed for many crop species, including barley (*Hordeum vulgare* L.). They are increasingly used in plant research to understand genomic organization and evolution, the determination of synteny relationships, gene tagging and map-based cloning. One of the most important objectives for developing high-density genetic maps is to identify precise locations of genes and quantitative trait loci (QTL) associated with traits of interest. This objective can be achieved by constructing consensus maps using information from multiple populations. In principle, the use of multiple populations has many advantages over the use of a single population. In particular, a larger number of markers can be placed on a single map providing greater genome coverage. Marker order and intermarker distances can be estimated more accurately, and possible chromosomal rearrangements and gene duplication can be determined.

Although many consensus maps have been produced in barley (Karakousis et al. 2003; Langridge et al. 1995; Marcel et al. 2007; Qi et al. 1996; Stein et al. 2007; Varshney et al. 2007; Rostoks et al. 2005), the consensus map which provided us the opportunity to compare the order of the DArT markers on our integrate linkage maps with the order of the same markers on a high-density genetic map was constructed by Wenzl et al. (2006). This map was based on segregation data from seven DH populations and three populations of RIL, comprising 2,935 loci (2,085 DArT, 850 SSR, RFLP and/or STS markers) and spans 1,161 cM. Later, this map was improved by incorporating 607 newly developed DArT markers to create a new integrated map of the barley genome containing 3542 markers (Alsop et al. 2010). Other saturated genetic maps were developed using DArT markers in barley for instance the one which was constructed by Hearnden et al. (2007). This was developed from an F_1-derived doubled haploid population generated from a cross between cultivated barley (*Hordeum vulgare*) and the subspecies H. *vulgare* ssp. *spontaneum*. The map comprises 1,000 loci, amplified using 536 SSR (558 loci) and 442 DArT markers.

The construction of high-density genetic linkage map requires powerful methods, which can perform the necessary calculations in a limited amount of time. There are several

programs that can be used to build consensus maps, including JoinMap, Carthagene and PhenoMap (Stam 1993; De Givry et al. 2005; (GeneFlow Inc., Centreville, VA.). The most important point to keep in mind when building a consensus map is that markers are sorted correctly. For that, two aspects have to be considered: the mapping criterion and the mapping algorithm (Van Os et al. 2005). The mapping criteria that have been proposed include the maximum likelihood, the minimum sum of adjacent recombination fractions (SARF), the maximum sum of adjacent LOD scores (SALOD), the minimum number of crossovers and the 'least square locus order'. For the mapping algorithms several approaches have been proposed like branch-and-bound, seriation and simulated annealing. A widely used platform for the construction of genetic linkage maps is provided by JoinMap®. The map integration facilities were developed in a period that segregating markers were scarce and many populations had to be combined to obtain adequate genome coverage. However, the regression algorithm of JoinMap® fails when it is required to integrate linkage maps involving large numbers of markers.

Jansen (2005) introduced an algorithm for constructing genetic linkage maps for full-sib families of outbreeding species, which typically involves the integration of maternal and paternal maps. In outbreeding species, markers may have different segregation types, *i.e.* some markers only segregate only in the mother, and other markers only segregate in the father. This leads to missing information about the inheritance of genomic fragments. The algorithm is based on minimization of the number of recombination events between adjacent markers using hidden inheritance vectors. Inheritance vectors are used to indicate the grandparental origins of alleles.

The aim of the present study was to adapt the algorithm developed by Jansen (2005) to cope with multiple populations derived from crosses between pure lines. In this situation missing data occur due to the fact that markers may segregate in some populations but not in others. This paper presents the results of integrating marker data obtained using DArT technology from five interconnected DH populations of barley into a single consensus map. Three of these DH populations have one parent in common: 'Albacete' which is known to carry a reciprocal chromosomal translocation between chromosomes 1H and 3H. To construct linkage maps in the populations obtained from reciprocal translocation heterozygotes we followed the novel statistical-

genetic approach developed by Farré et al. (2011) which disentangled the 'pseudo-linkage' between the markers from the chromosomes involved in the reciprocal translocation making use of the consensus map obtained by Wenzl et al. (2006).

Materials and methods

Mapping populations

Five connected DH populations were derived from crosses between four parents: 'Albacete', 'Barberousse', 'Plaisant' and 'Orria'. 'Albacete' is a six-row facultative variety selection from a local Spanish landraces with a relatively long cycle and an alternative growth habit. It is drought tolerant showing a relatively stable grain yield production. 'Barberousse' ('Ager' × ['W.259-711' × ('Hatif' × 'Ares')]), a six-row feed winter variety released in France in 1977, is known for its good productivity and easy adaptation although it is more sensitive to drought. 'Plaisant' ('Ager' × 'Nymphe') is a six-row French winter malting cultivar released in 1979. 'Orria' ({[('Api' × 'Kristina') × 'M66.85'] × 'Sigfrido's} × '79W40762') is a Spanish six-row facultative variety obtained from a cross made at CIMMYT; it is well adapted to fertile, rainfed environments.

DH lines were obtained from the F_1s using the anther-culture technique (Cistué et al. 2003). The five DH populations that will be used to construct the integrated linkage map are shown in Figure 1, together with the numbers of DH lines per population and polymorphic DArT markers.

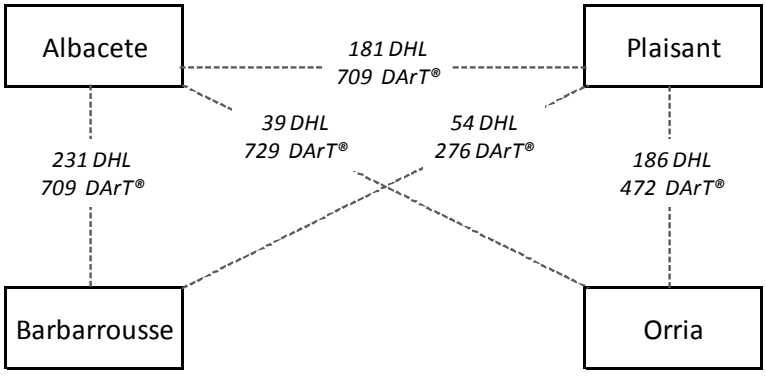


Figure 1. Set-up of the five DH populations with the number of DH lines (top) and the number of DArT markers (bottom).

DNA isolation and genotyping

Genomic DNA of the DH lines was extracted from fresh leaf tissue using the Qiagen DNeasy 96 plant Kit. A genome wide scan was carried out using Diversity Arrays Technology (DArT®) (*www.diversityarrays.com*). This technique is based on DNA hybridization, does not require a priori sequence information and is carried out on a microarray platform which allows high-throughput screening of hundreds of molecular markers simultaneously (Jaccoud et al. 2001, Wenzl et al. 2004). The polymorphisms scored are the presence (1) or absence (0) of hybridizations to individual array elements. The locus designations used by Triticarte Pty. Ltd. were adopted in this paper. DArT marker names consist of the prefix "bPb", followed by numbers corresponding to a particular clone in the genomic representation, where b stands for barley, P for P*st*I (primary restriction enzyme used) and b for B*st*NI (secondary restriction enzyme).

Genetic linkage map construction

The first step in the construction of the consensus map was to assess each DH population separately. For each DH population quality-filtered datasets were prepared by removing markers and DH lines in two different steps: pre-mapping and post-mapping diagnostics. For pre-mapping diagnostics markers were removed according to the following criteria: (1) Q value (estimate of marker quality) less than 77, (2) markers with more than 10% missing data and (3) markers with segregation ratio less than 0.2 or greater than 0.8. DH lines were discarded according to high segregation distortion ratio (above 80%). For post-mapping diagnostics, JoinMap® 4.0 (Van Ooijen, 2006) was used to: (1) determine the number of linkage groups present in each population using a recombination frequency of 0.20, (2) remove markers that do not fit well by the "nearest-neighbour stress" criterion and (3) discard DH lines with large number of recombinations per individual (more than 20).

The novel statistical-genetic approach developed by Farré et al (2011) was followed to construct linkage maps in the populations obtained from reciprocal-translocation heterozygotes making use of the consensus map obtained by Wenzl et al. (2006). Briefly, the five chromosomes showing a standard arrangement were analyzed

separately. Then, for chromosomes 1H and 3H four stages were followed: (i) the recombination frequencies between the markers from 1H and 3H were calculated, which allowed us to select the markers close to the TB; (ii) based on the genotype for those markers the DH lines were separated into two distinct subpopulations: S1 which around the translocation breakpoint the DH lines are identical to 'Albacete', so they are expected to carry the translocation between 1H and 3H chromosomes (1HS·3HL + 3HS·1HL) and S2 which close to the TB the alleles are identical to standard type ('Plaisant', 'Barberousse' or 'Orria'), this indicates that they have a standard chromosomal arrangement (1HS·1HL + 3HS·3HL); (iii) the segregation ratios for each marker were calculated for each of the two subpopulations, markers showing segregation ration less than 0.3 or greater than 0.7 were removed; (iv) the two subpopulations were considered separately to obtain independent linkage maps for the short and long arms of 1H and 3H. The final product consists of integrated linkage maps of the chromosome parts away from the translocation breakpoint.

The markers used for constructing the consensus map were those which segregated in more than 300 DH lines for all chromosomes except for 1H and 3H; in that case, the markers selected were those that mapped in more than 150 DH lines. Therefore, all markers used were segregating in at least two mapping populations.

Integrated linkage maps were obtained by a simplified version of the algorithm described by Jansen (2005) and developed for full-sib families of outbreeding species. The algorithm has also been applied succesfully in F_2 populations with dominant markers (Jansen 2009). The algorithm aims at obtaining the marker order with the smallest number of recombinations between adjacent markers on hidden inheritance vectors. Inheritance vectors represent the grandparental origin of alleles in binary form: 0 = allele of DH line is identical to allele of grandmother (= female parent of cross), 1 = allele of DH line is identical to allele of grandfather (= male parent of cross). Since DH lines contain information from a single meiosis, inheritance vectors for DH lines consist of a single digit. If the parents differ with regard to hybridization with DArT probes (*i.e* polymorphic parents) and hybridization data are present for the DH lines, hybridization data can be converted uniquely into inheritance vectors according to the scheme in Table 1. In other cases information about the transmission of alleles is missing and the

inheritance vectors may assume the value zero or one. Two steps were performed to obtain the integrated maps for each linkage group separately. In the first step, a framework map is constructed for a set of markers which are selected because they cover the whole linkage map using the spatial sampling procedure. In the next step, all non-selected markers will be attached on the previous map close to the nearest selected marker. Linkage maps were drawn using MapChart 2.2 (Voorrips, 2002).

Table 1. Conversion of hybridization data into inheritance vectors

	DH line: Hybridization	DH line: No-hybridization	DH line: Missing data
Grandmother: hybridization Grandfather: no hybridization	0	1	0, 1
Grandmother: no hybridization Grandfather: hybridization	1	0	0, 1

Results

Single populations

Pre-mapping diagnostics

Linkage maps are strongly affected by scoring errors. Therefore, high quality genotypic data is critical. Thus, in order to get robust estimates very stringent criteria were used for data filtering. For each DH population separately, markers and DH lines were excluded according to different criteria. In the case of the markers, although a total of 2,895 DArT® polymorphic markers were found by the Australian company Triticarte Pty for the five DH populations, 545 markers were removed according to: Q value (195 markers), missing data (230 markers) and high segregation distortion ratios (120 markers). The results are shown in Table 2. Population A×O has a remarkably large number of markers with an extreme segregation ratio. This may be due to the small population size (only 39 DH lines). As a result, 2,350 DArT markers can be used for the map construction. In the case of DH lines, a total of 691 DH lines were available for the construction of linkage maps but 7 individuals were discarded due to the high segregation distortion ratios (above 80%) caused by very limited recombination as they

consisted almost entirely of one parental haplotype (5 like 'Albacete' and 2 like 'Plaisant' from A×B and P×O crosses, respectively).

Table 2. Summary of individual mapping data used to construct the consensus map (* = in the case of A×O population markers with more than 5% of missing data were removed; Pop = population).

Pop.	No. polymorphic Markers	Number of markers excluded per criterion			Available polymorphic markers
		Q value	Missing data	Segregation ratio	
A×B	709	16	85	8	600
A×P	709	64	51	2	592
A×O	729	51	34*	108	536
P×O	472	50	40	0	382
B×P	276	14	20	2	240
Total	2,895	195	230	120	2,350

Post-mapping diagnostics

Markers that according to the information supplied by the Australian company Triticarte Pty belonged to more than one chromosome or mapped on different loci within the same chromosome were not removed and location was attempted in the correct chromosome (16, 5, 13, 13 and 8 markers from A×B, A×O, A×P, P×O and B×P crosses, respectively). 38 out of 684 DH lines were discarded because of an extremely large number of recombinations per individual (6, 2, 1, 28 and 1 for A×B, A×P, A×O, P×O and B×P crosses, respectively). As a result, a total of 646 DH lines were used for the linkage maps construction.

Polymorphisms between parents

The first step required in the construction of a linkage map is to check the parental lines for polymorphism. A high level of polymorphism between parents facilitates the construction of high-density consensus map. The level of polymorphism among populations for each chromosome is represented in Figure 2 and SEM I_Table 3. The location of markers to chromosomes and their positions was obtained from the consensus map of barley (Wenzl et al. 2006). This clearly shows the highest level of

polymorphism revealed by DArT markers between parents of the A×B (65.68%) and A×P (60.86%) mapping populations. However, B×P exhibits the lowest level (22.08%), in almost all chromosomes followed by A×O (38.66%). For the parental lines 'Plaisant' and 'Orria' (52.89%), 1H was the highest level (92.23%) while the lowest is observed on 4H (9.62%). In the present study, the percentage of polymorphism of the individual chromosomes for all mapping populations ranged from 13% (4H) to 95.8% (6H).

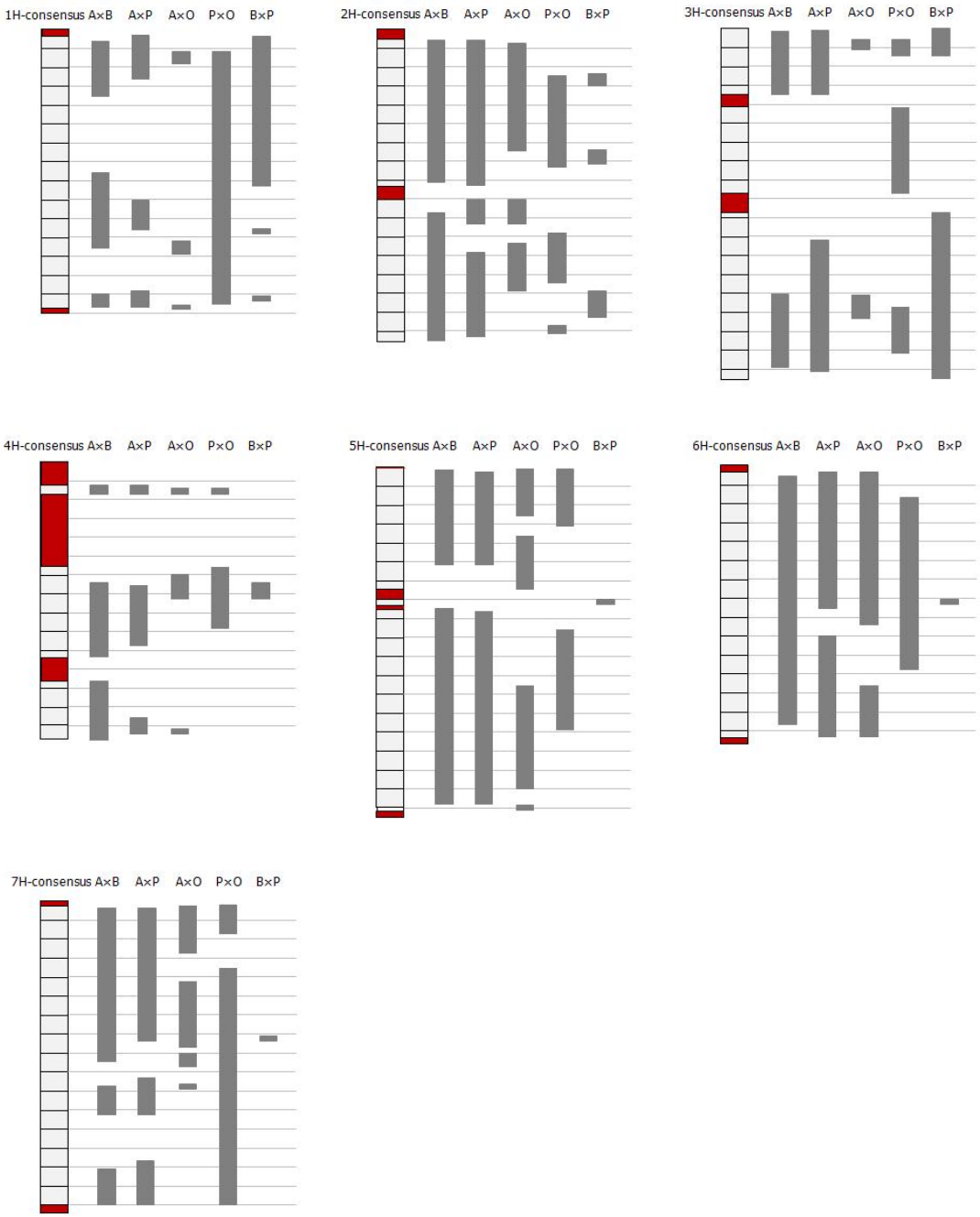


Figure 2. Polymorphism among populations for each chromosome. The allocation of markers as well as their position were estimated based on the consensus map of barley (Wenzl et al.

2006) (horizontal lines are drawn every 10 cM; red colour indicated the regions of the consensus map without coverage).

Individual maps

In all populations and for the recombination value used (r = 0.2), more than seven linkage groups corresponding to each of the barley chromosomes were obtained because in a lot of cases each chromosome was split in more than one linkage group. It is important to note that markers near the centromeric region for chromosomes 1H and 3H based on the consensus map appeared together in the same linkage group for the DH lines derived from the cross between 'Albacete' and another parental (A×B, A×P, and A×O). This confirms the presence of the reciprocal translocation previously studied in the DH population derived from the cross between 'Albacete' and 'Barberousse' (Farré et al. 2012). Table 4 shows the selected markers close to the translocation breakpoint used to divide the DH line for each population into two distinct subpopulations; not all selected markers coincide among populations, this is because some markers were removed according to the criteria described in Farré et al. 2011 or they were not within the 5 cM from the presumed translocation breakpoints.

The mapping populations with the highest number of mapped loci were A×B (521 markers) and A×P (514 markers) while the lowest was observed for B×P (225 markers). The total length of the individual maps ranged from 1,623 cM (A×P) to 683 cM (B×P). For the three populations that share 'Albacete' as a parent, 5H was the individual linkage group with the highest map length (252, 296 and 243 cM from A×B, A×P and A×O crosses, respectively); for P×O and B×P population was 7H (243 cM) and 3H (221 cM), respectively; whereas the smallest linkage group varied among mapping populations. In the case of A×B and A×P, 1H was the linkage group with the smallest map length (149 and 160 cM, respectively). For A×O and P×O it corresponded with 7H (85 cM) and 4H (92 cM), respectively. And for B×P, the map length for the linkage groups 5H and 6H was 0 cM because they had 4 markers which mapped in the same position. The greatest gap in all mapping populations was on chromosome 3H (104, 76, 130, 81 and 60 cM from A×B, A×P, A×O, B×P and P×O crosses, respectively).

It is important to note that some markers that according to the information supplied by the Australian company Triticarte Pty could be mapped in more than one chromosome or multiple loci on the same chromosome were located correctly (9, 5, 6, 2 and 2 markers from A×B, A×P, A×O, B×P and P×O population, respectively). However, some markers mapped on different chromosomes according to the consensus map (bPb-0413, bPb-6063, bPb-5389 and bPb-4963 mapped on 5H instead of 2H; bPb-6109 and bPb-7181 on 7H instead of 3H and 6H, respectively).

Comparison of the individual maps with the consensus map of Wenzl et al (2006)

The individual map distances generated by the algorithm developed by Jansen et al. (2001, 2005 and 2009) are larger than those on the consensus map of barley (Wenzl et al. 2006). For population, the overestimation of distances relative to the consensus map distance was calculated. The A×P and A×B populations had the highest percentage, 40 and 22%, respectively, while the smallest percentage was for A×O (4%) and B×P (-41%).

Table 4. Summary of the selected markers used to divide the DH lines for each mapping population and subpopulation data (S1: 1HS·3HL + 3HS·1HL and S2: 1HS·1HL + 3HS·3HL).

Population	S1		S2		Linkage group			
					1H		3H	
	No. lines	Markers with segregation distortion (<30%, >70%)	No. lines	Markers with segregation distortion (<30%, >70%)	Marker loci	Consensus map position (cM)	Marker loci	Consensus map position (cM)
A×B	88	65	85	69	bPb-9717	58.7	bPb-6329	66.5
					bPb-6621	59.1	bPb-0094	69.3
					bPb-4949	59.2	bPb-5771	69.3
					bPb-0910	59.4	bPb-5012	69.8
					bPb-9957	63.3	bPb-8321	70.4
					bPb-9360	64.4		
A×P	66	50	80	60	bPb-5749	63.3	bPb-0094	69.3
					bPb-9767	63.3	bPb-5771	69.3
					bPb-9957	63.3	bPb-8283	69.6
					bPb-1193	62.2	bPb-5012	69.8
					bPb-3382	62.2	bPb-8321	70.4
					bPb-1791	67.1		
A×O	18	61	15	55	bPb-5749	63.3	bPb-0040	72.2
					bPb-9767	63.3	bPb-3805	72.2
					bPb-9957	63.3	bPb-3317	78.6
					bPb-1193	62.2	bPb-8410	78.6
					bPb-3382	62.2		

Multiple populations

Integrated maps

The construction of the consensus map was carried out for each chromosome separately using the algorithm developed by Jansen et al. (2001, 2005, and 2009). For chromosomes 1H and 3H two independent integrated maps were obtained using the markers from A×B, A×P, A×O and B×P, P×O mapping populations separately.

The integrated maps of seven barley chromosomes are presented by Figure 4 and Table 5. This map has a total length of 938 cM with 310 markers for all chromosomes except 1H and 3H providing an average density of one marker every 3.02 cM. The individual linkage groups ranged from 228 cM (5H) to 148 cM (4H). The numbers of common markers varied greatly between linkage groups: 17 on 4H compared to 92 on 7H. On the integrated map, linkage group 7H had the greatest number of markers (92) with an average marker density of 2.19 cM followed by linkage group 6H (75) with an average marker density of 2.14 cM. Linkage group 4H had the smallest number of markers (17) and the highest marker density (8.70 cM). The highest gap is in linkage group 4H (41 cM). The resulting maps for each population compared with the integrated are shown on SEM II_Figure 3 and Table 5.

Comparison of integrated map with the consensus map of Wenzl et al (2006)

At completion of construction of the map a comparison was made between the order of loci on the consensus map and the integrated map which showed a high level of conservation of DArT marker order with the exception on 4H and for bPb-7335 marker on 3HS for A×O/A×P/A×B integrated map which is located on 3HL on the consensus map. The linkage group distances are larger than those on the consensus map. Linkage group 1H obtained using markers data from P×O and B×P mapping populations had the highest percentage (27%) followed by 7H (22%). The smallest percentage was in 1H obtained using markers data from A×B, A×P and A×O mapping populations (-27%).

Table 5. Summary of individual and integrated mapping data (I = integrated and CN = consensus) (length of disjoint intergroup segments within a linkage group determined based on the consensus map; a= map obtained using marker data from A×O, A×B and A×P populations; b= map obtained using markers data from P×O and B×P mapping populations).

Pop		1H	2H	3H	4H	5H	6H	7H	Total
A×B	No. of segments	3	2	2	3	2	1	3	
	Length segments (cM)	87	238	82	104	240	207	170	1,128
	No. of markers	41	98	55	43	79	101	104	521
	Consensus intergap	62	7	105	58	12	-	45	
	Estimate total	149	245	187	162	252	207	215	1,417
A×O	No. of segments	3	3	2	3	4	2	4	
	Length segments (cM)	25	192	20	92	180	133	59	701
	No. of markers	14	91	16	38	61	59	65	344
	Consensus intergap	115	33	130	104	63	32	26	
	Estimate total	140	225	150	196	243	165	85	1,204
A×P	No. of segments	3	3	2	3	2	2	3	
	Length segments (cM)	62	243	146	101	278	206	235	1,271
	No. of markers	41	95	54	37	82	94	111	514
	Consensus intergap	98	39	76	74	18	14	33	
	Estimate total	160	282	222	175	296	220	268	1,623
P×O	No. of segments	1	3	3	2	3	1	2	
	Length segments (cM)	226	120	135	53	143	126	236	1,039
	No. of markers	73	41	44	20	41	51	108	378
	Consensus intergap	-	60	88	39	100	-	14	
	Estimate total	226	180	223	92	243	126	250	1,340
B×P	No. of segments	3	3	2	1	1	1	2	
	Length segments (cM)	131	33	140	22	0	0	86	412
	No. of markers	61	40	28	16	4	4	72	225
	Consensus intergap	59	104	81	-	-	-	27	
	Estimate total	190	137	221	22	0	0	113	683
Integrated	No. of segments	3[a]	1[b]	2	2[a]	2[b]	3	2	1	1	
	Length segments (cM)	45[a]	193[b]	180	86[a]	114[b]	80	203	161	202	
	No. of markers	40[a]	63[b]	64	51[a]	30[b]	17	62	75	92	
	Consensus intergap	66[a]	-	19	104[a]	41[b]	68	25	-	-	
	Estimate total	111[a]	193[b]	199	190[a]	155[b]	148	228	161	202	
	I/CN (%)	-27[a]	27[b]	19	3[a]	-5[b]	0	17	9	22	

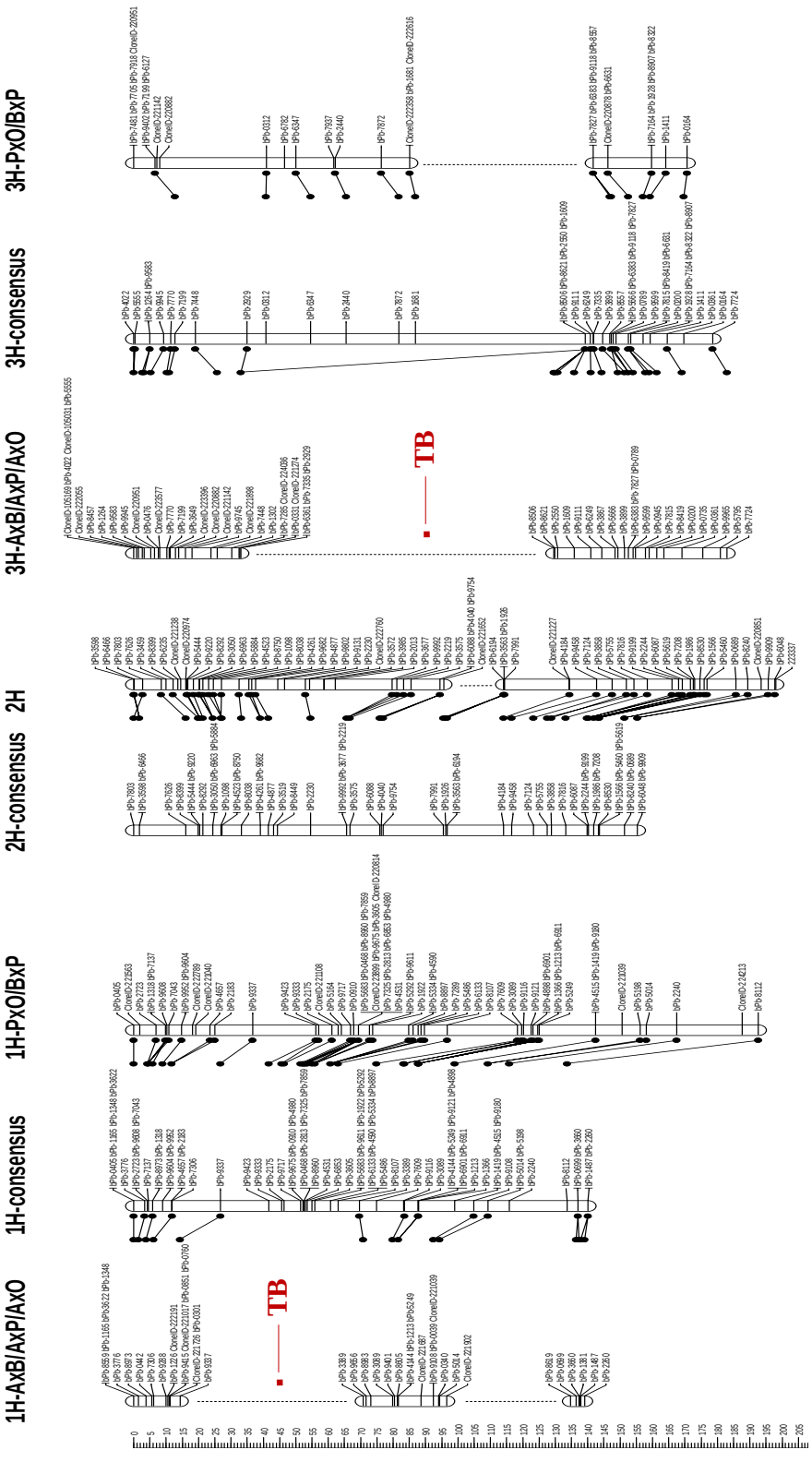


Figure 4. Comparison of the integrated maps with the consensus maps constructed by Wenzl et al. 2006 (only common markers are illustrated). Chromosomes are oriented with the short arm at the top. The genetic distances are expressed in map distances (cM). (TB= translocation breakpoint).

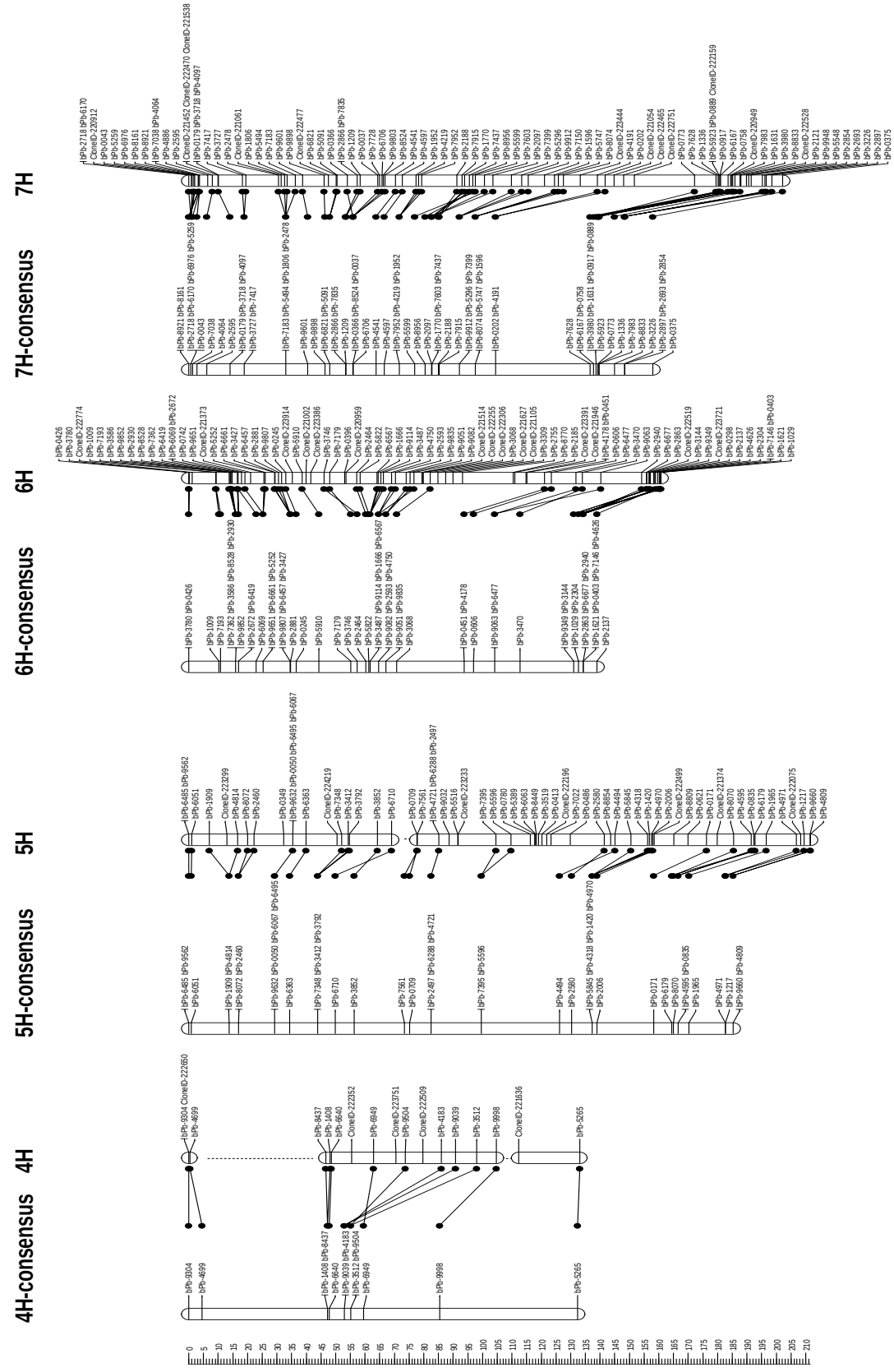


Figure 4. (continued)

Discussion

High density genetic linkage maps provide a greater probability that the entire genome is covered with molecular markers avoiding large gaps in the map without markers. For the construction of high-density genetic linkage maps is of particular importance to have several loci in common between populations. This requires a high level of polymorphism between parents. In the present study, the highest level of polymorphism among populations was observed for A×B and A×P whereas B×P showed the lowest level. This appears to be in good agreement with the results obtained by Casas et al. (1998). They estimated the genetic resemblance among a representative set of 37 barley cultivars, including our four parents, grown in Spain using RFLP markers. A dendrogram constructed from UPGMA cluster analysis split the 37 barley cultivars into five distinct germplasm groups that were consistent with the history of the cultivars: winter European (including 'Barberousse' and 'Plaisant'), spring European, CIMMYT-ICARDA materials (including 'Orria'), the single cultivar 'Dobla' and Spanish local materials (including 'Albacete'). Parental lines 'Plaisant' and 'Barberousse' demonstrated the highest level of similarity in the dendrogram compared with the other parents. This could be explained because they share 'Ager' as parent. The level of polymorphism between populations is determined by the level of genetic divergence among parents.

The integrated map developed in this paper was obtained by assembling marker data from five interconnected DH populations which allowed us to fill a number of gaps present in maps of individual populations. The order of the markers on the integrated map as well as on maps of individual populations are consistent with the order on the consensus map (Wenzl et al. 2006) except for some markers on chromosome 4H and one marker on chromosome 3H. In our study, map distances are slightly larger than those on the consensus map. The discrepancy in map lengths may be due to difference in "data curation" possibly in combination to the use of different mapping criteria and mapping algorithms.

For map integration it is very important to create high-quality datasets free of errors because genotyping errors (which produce unexpected crossovers) and missing data

lead to map expansion, especially in regions with a high marker density. The filtered high quality datasets from the consensus map used in this paper to verify our integrated map were obtained following different steps: (i) JoinMap 3.0 was used to distribute loci into linkage groups and remove redundant lines; (ii) loci order within individual population was determined using RECORD programme which employs a marker-ordering criteria based on minimization of the total number of recombination events and the algorithm applied combine elements of branch-and-bound with local reshuffling (Van Os et al. 2005a) and (iii) lines and loci with an excess of singletons were identified and removed using a purpose-built Perl script. Map expansion could also be reduced by removing markers that do not fit well by "nearest-neighbour stress" and/or had high segregation ratios. However, a more accurate method will be necessary to remove the appropriate DH lines.

Differences in map lengths could also arise due to the different methods used by the software packages to calculate map lengths. Whereas the consensus map was derived using the regression mapping algorithm we focused on a multipoint maximum likelihood (ML) mapping algorithm developed by Jansen et al. (2001, 2005 and 2009). JoinMap® algorithm fails when it is required for integrating linkage maps with large numbers of markers, missing data, typing errors and pairwise recombination frequencies are not present for all marker combinations. Therefore, the algorithm applied in this study is an efficient method for constructing good-quality dense linkage maps. Spatial sampling used to obtain a small set of markers homogenously distributed in the linkage group provided an efficient way to reduce map expansion.

The percentage of genome coverage of our integrated map compared with the consensus map of Wenzl et al. 2006 from the three populations with the highest number of individuals (A×B, A×P and P×O) are 82.0, 10.6, 86.6, 94.5 and 95.5% for 2H, 4H, 5H, 6H and 7H chromosomes respectively, this percentage improved for chromosome 2H up to 7% more when B×P and A×O populations with the smallest number of individuals were included in the analysis.

In conclusion, the consensus map constructed with the useful method develop by Jansen (2005) is consistent compared with the consensus map of barley (Wenzl et al. 2006).

This will be used for QTL mapping for quantitative agronomic characters allowing us to compare QTL between different genetic backgrounds. A high density genetic linkage map allows a greater precision of QTL location than in a sparse map (fewer markers) because the greater the distance between markers, the greater the chance of recombination between the marker and the QTL. These cause that the magnitude of the effect of a QTL to be underestimated (Collard et al. 2005).

Acknowledgments

This study has been supported by the Spanish Ministry of Science and Innovation (Project AGL2008-05541-C02 and the Agrogenomics CONSOLIDER).

SUPPLEMENTARY MATERIAL I - POLYMORPHISMS AMONG PARENTS

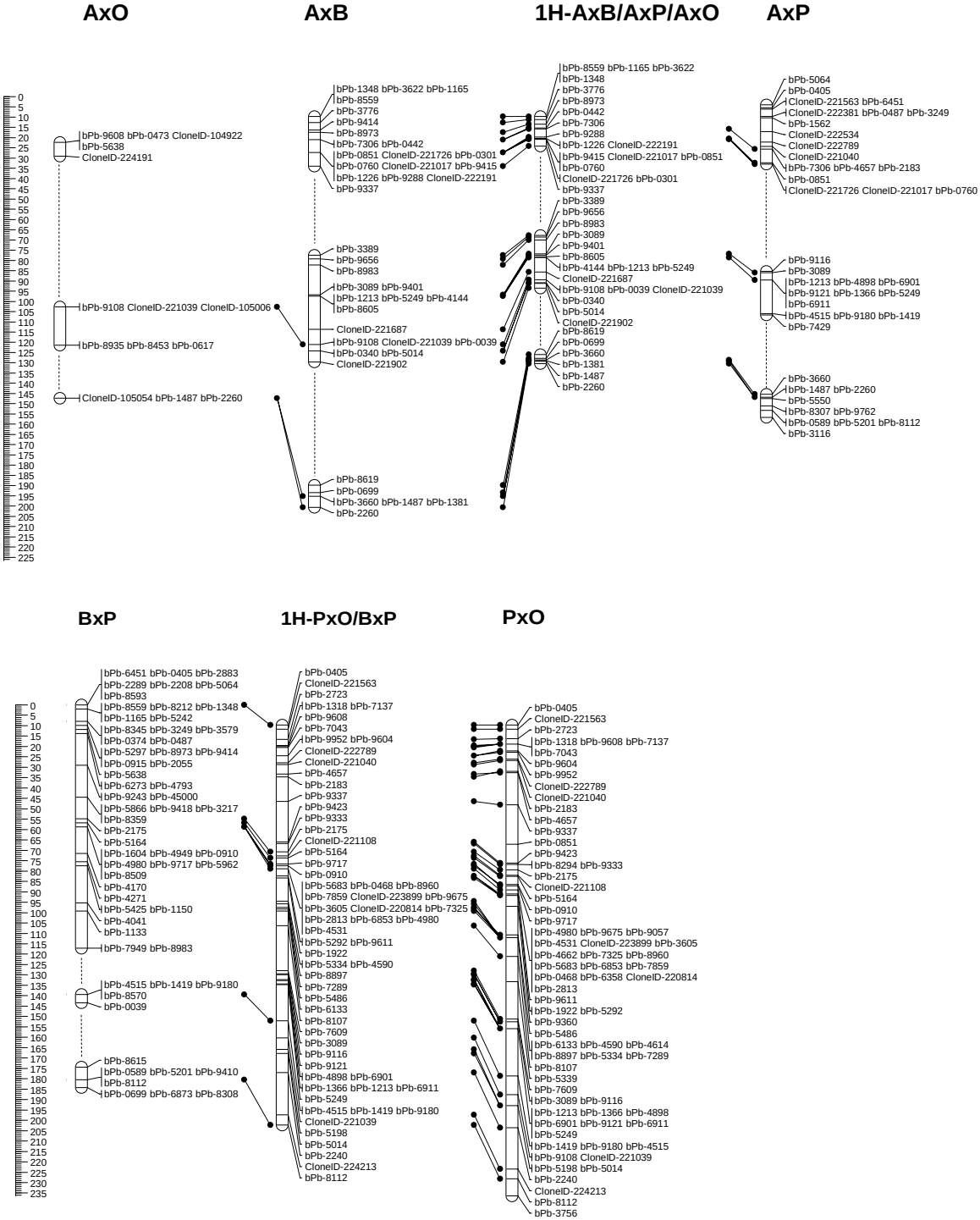
Table 3. Level of polymorphism among populations and chromosomes (values in brackets are expressed as percentages).

Chromosome	Consensus (cM)	A×B (cM)	A×P (cM)	A×O (cM)	P×O (cM)	B×P (cM)
1H	151.90	40.80 (26.86)	39.30 (25.87)	14.60 (9.61)	140.10 (92.23)	82.10 (54.05)
2H	166.70	144.60 (86.74)	132.60 (79.54)	118.40 (71.03)	63.50 (38.09)	42.87 (25.72)
3H	184.59	74.10 (40.14)	88.30 (47.84)	17.40 (9.43)	76.50 (41.44)	63.20 (34.24)
4H	148.58	63.90 (43.01)	46.40 (31.23)	14.90 (10.03)	14.30 (9.62)	8.10 (5.45)
5H	194.15	185.30 (95.44)	168.20 (86.63)	103.0 (53.05)	89.50 (46.1)	0.30 (0.15)
6H	147.08	139.0 (94.51)	117.60 (79.96)	108.80 (73.97)	86.80 (59.02)	2.40 (1.63)
7H	165.42	113.20 (68.43)	112.60 (68.07)	70.80 (42.8)	142.0 (85.84)	56.80 (34.34)
Total (%)		65.68	60.86	38.66	52.89	22.08

SUPPLEMENTARY MATERIAL II- COMPARISON OF THE INTEGRATED MAP WITH THE INDIVIDUAL MAPS

Figure 3. (A-G). Individual maps and their integrated map of the seven barley chromosomes. Chromosomes are oriented with the short arm at the top. Lines are indicated the common markers, which are presented in black and underline.

(A) Chromosome 1H



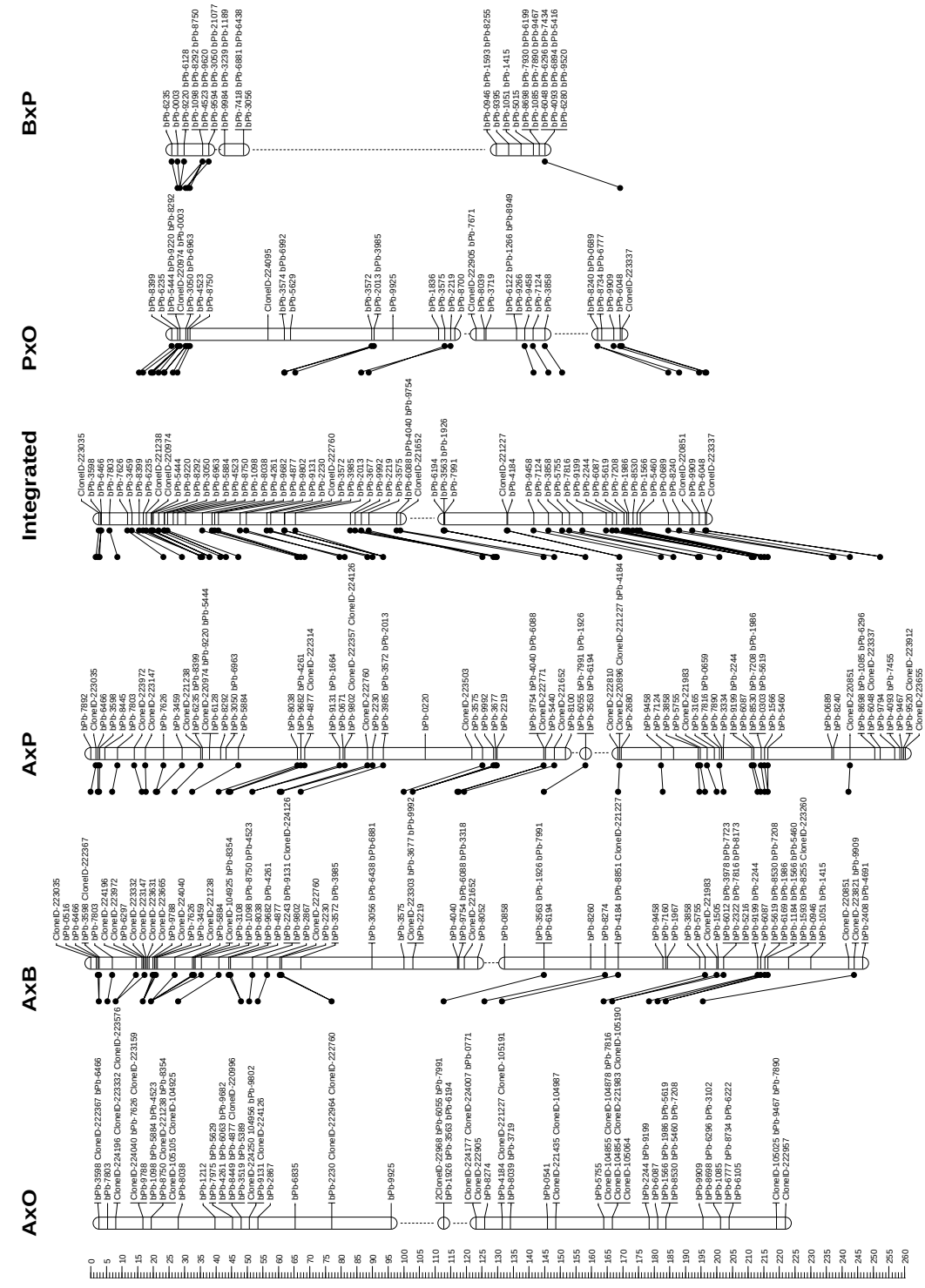


Figure 3. (continued)

(B) Chromosome 2H

Figure 3. (continued)

(C) Chromosome 3H

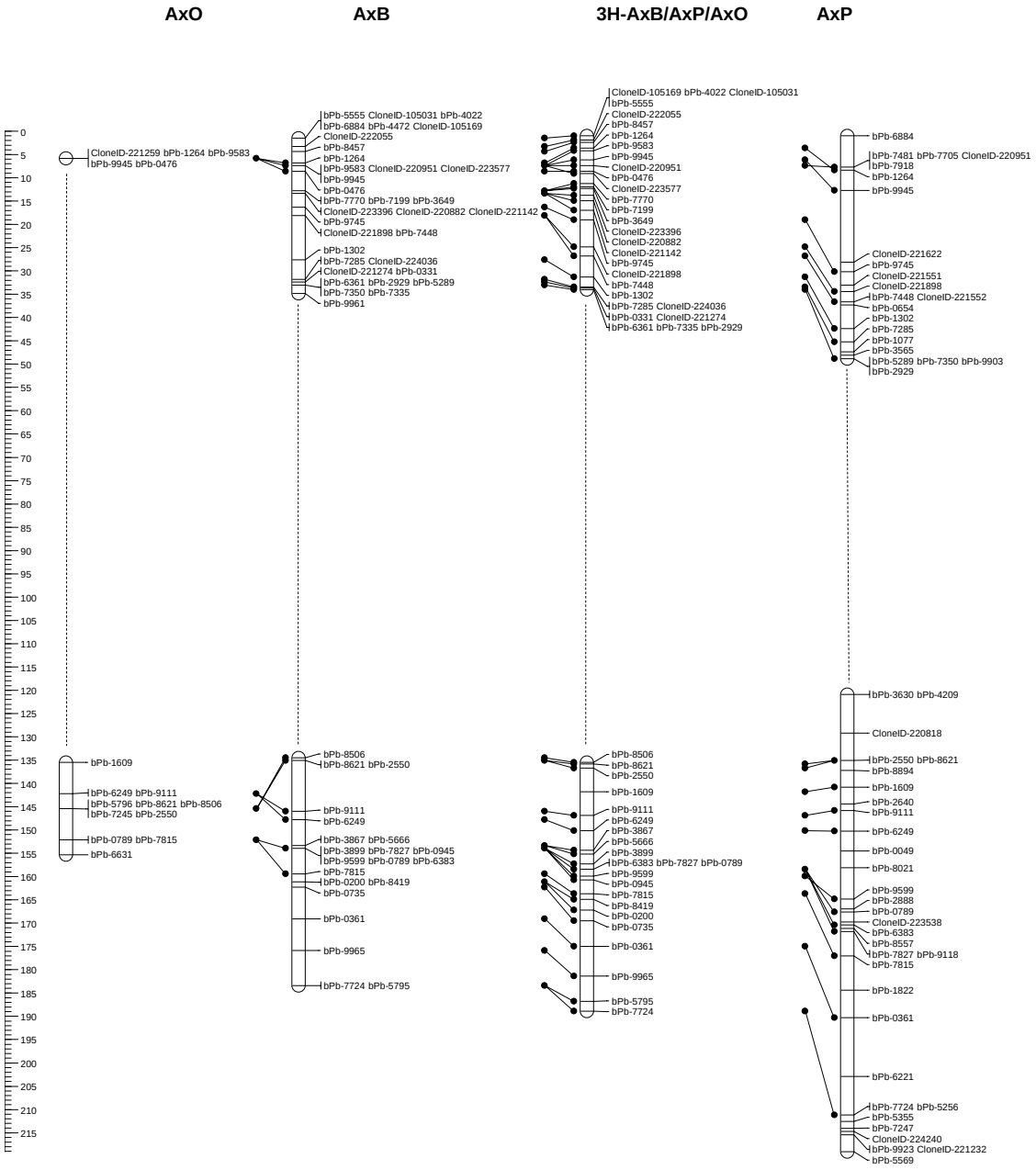
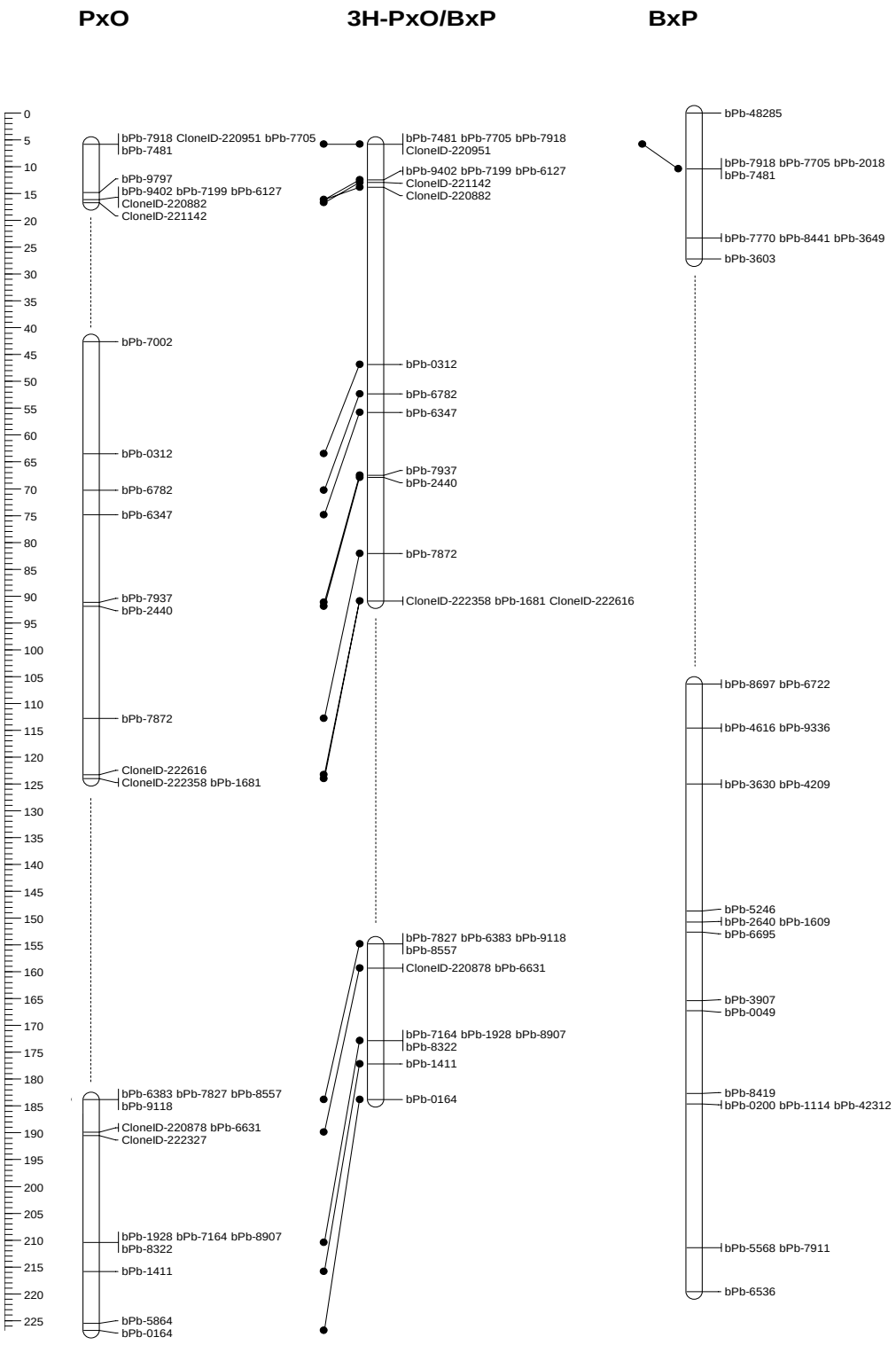


Figure 3. (continued)



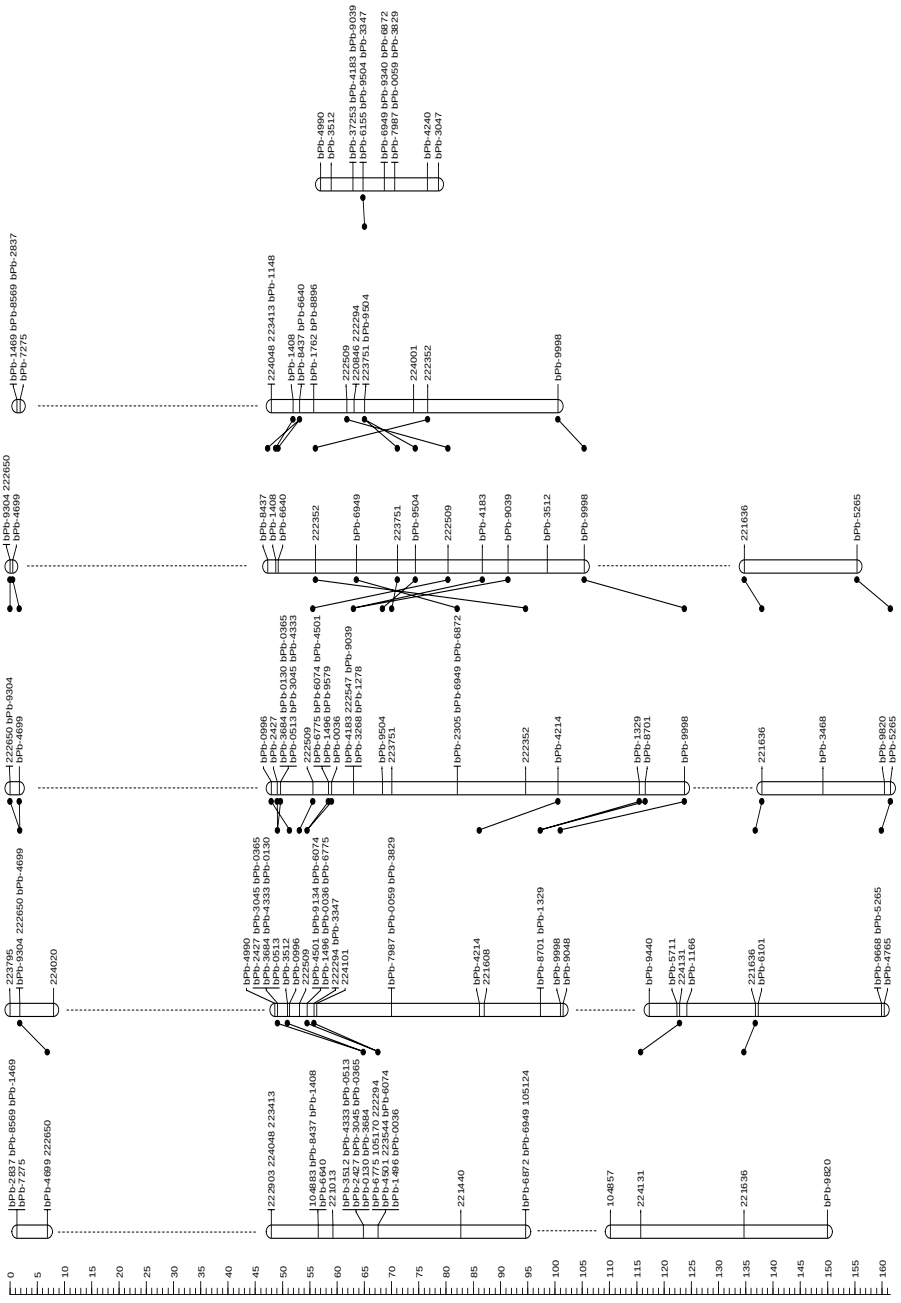


Figure 3. (continued)

(D) Chromosome 4H

Figure 3. (continued)

(E) Chromosome 5H

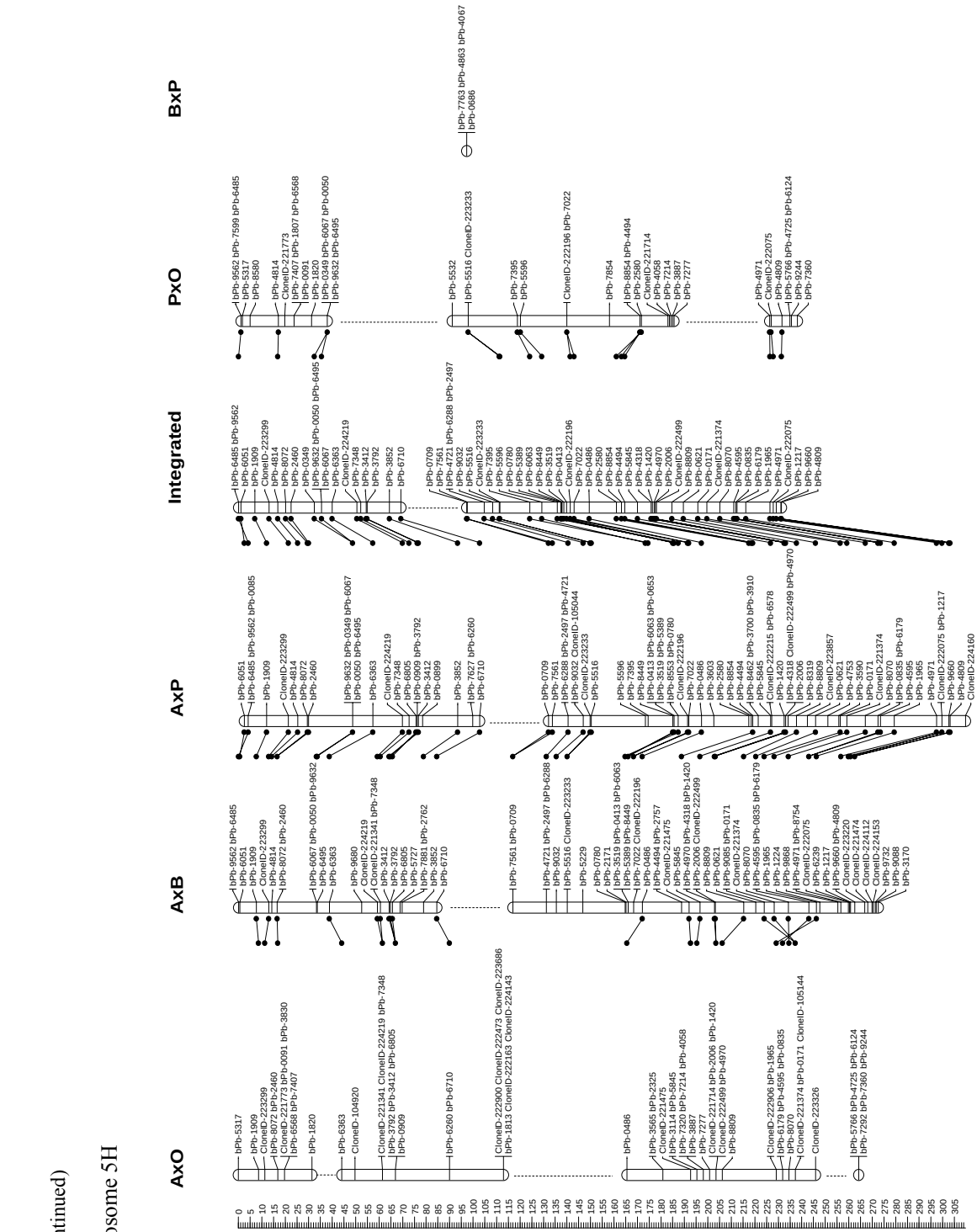
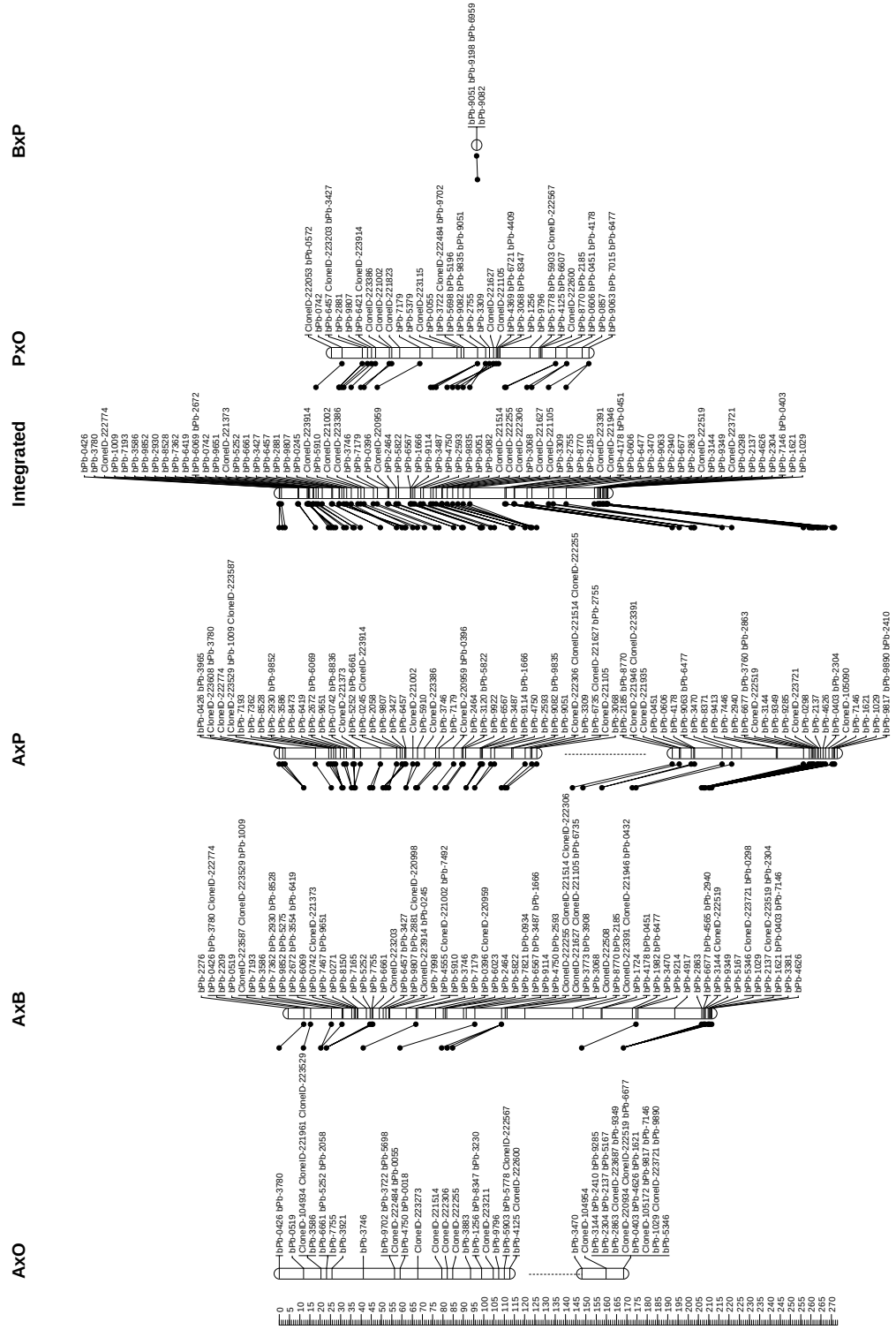


Figure 3. (continued)

(E) Chromosome 6H



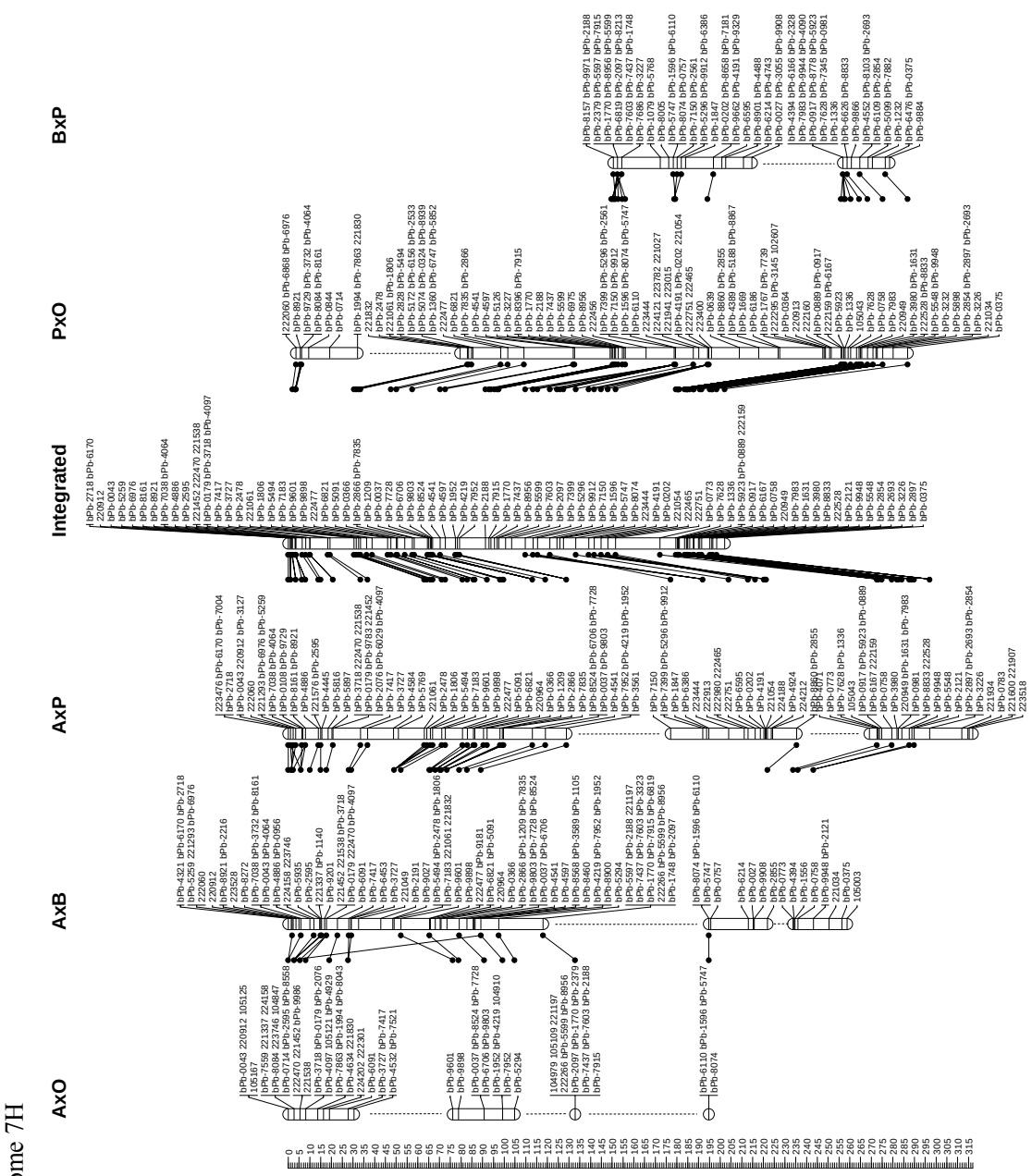


Figure 3. (continued)

(E) Chromosome 7H

Chapter 6

QTL analysis in multiple interconnected barley doubled haploid populations in the presence of a reciprocal translocation

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Abstract

Multiple connected populations address broader diversity than biparental populations which have been widely used for QTL analysis. The use of multiple connected populations allows a more global comparison of the effects of all QTL alleles segregating in the populations simultaneously and allows investigation of interactions between QTL and genetic background. In this study, a new approach was developed for QTL detection in a multi-population and a multi-environment analysis. Five interconnected biparental doubled haploid (DH) populations of barley derived from crosses between pure lines were used. Three of the populations have a common parent which is known to carry a reciprocal chromosomal translocation between chromosomes 1H and 3H. Days do heading (DtH) and thousand kernel weight (TKW) will be used to develop the methods. The QTL found for DtH and TKW are consistent with the results of previous QTL studies. The integral approach used in this study provided a direct comparison of the effects of the four parental alleles. Only for TKW a direct effect of the reciprocal translocation was found; DH lines carrying the reciprocal translocation had a greater TKW than those with a standard chromosome arrangement.

Keywords Reciprocal translocation · Barley · Translocation breakpoint · Connected populations · Quantitative trait locus

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Introduction

In breeding programs most traits of agronomic interest display continuous variation due to the contributions of many genes with small effects. Such genes are usually called quantitative trait loci (QTL). Quantitative traits, also called complex traits in contrast to simple Mendelian traits, are traits where the relation between genotype and phenotype cannot be observed directly. Examples are yield, quality or some forms of disease resistance. QTL mapping has been widely used during the last two decades thanks to the abundance of molecular markers in combination with the development of powerful methods for statistical analysis (Kearsey and Farquhar, 1998; Bernardo 2008, Zhu et al. 2008) implemented in user-friendly software (*i.e.* MapMaker/QTL (Lincoln and Lander 1990), QTLCartographer (Basten et al. 1999), MapQTL®5 (Van Ooijien 2004), Genstat (Payne et al. 2011). QTL mapping tools are useful for dissecting complex traits and for identifying favourable alleles in diverse germplasm, to estimate several genetic parameters that underlie phenotypic variation, including the number of loci, the type and magnitude of their effects, interactions between genes (epistasis), and gene-by-environment interactions (Symonds et al. 2005). Understanding the response of QTLs in different environments or genetic backgrounds can lead to the development of improved crop varieties through marker-assisted selection (MAS) which is the most evident application of QTL analysis together with QTL cloning. But while thousands of marker-trait association studies have been conducted in many plant species for many traits (Holland 2007), only a few QTLs have been cloned (Liu et al. 2002, 2003; Price 2006; Salvi et al. 2005; Ramsay et al. 2011). Until recently mainly biparental populations (derived from single crosses of two inbred lines) were used to detect and map QTLs in plants. Genetic analysis of biparental populations only allows differentiation between alleles for which the parents differ. In general unrelated parents were carefully selected to maximise marker polymorphism and trait divergence, *i.e.*, Oregon Wolfe barley mapping population (Costa et al. 2001), barley crosses between wild and cultivated varieties (Backes et al. 2003), winter and spring varieties (Laurie et. 1995; Hayes et al. 1993; Francia et al. 2004) or resistant and susceptible (Arru et al. 2003). As a result, some QTLs explaining quantitative variability in the population can be missed because only the effect of major genes can be studied in such biparental populations (Edwards et al. 1992). Moreover, results based on biparental populations are based on a small part of

the germplasm, results may not apply to other genetic backgrounds (Parisseaux and Bernardo 2004). So, following this strategy only a small part of the total diversity available to breeders is explored.

As an alternative, multiple connected biparental populations may be developed. These are particularly interesting because: (i) the genetic variability addressed is increased, (ii) they allow a global comparison of the effects of all QTL alleles segregating in the populations simultaneously thereby increasing the power of QTL detection, but also allowing the identification of the parental origins of favourable alleles, and (iii) interactions between QTL and genetic background, which may be an indication of epistatis, may be investigated (Muranty et al. 1997). For all these advantages, studies involving connected populations have been reported in cereals. For example, in barley Cuesta-Marcos et al. (2008a) used 281 doubled haploid lines derived from 17 F_1 involving 14 different parental cultivars, to validate the effect of the main QTL determining heading date. In maize, studies based on multiparental connected populations were carried out to compare multiparental connected designs with biparental populations with regard to MAS and phenotypic selection (Blanc et al. 2006, 2008). Here a diallel cross design consisting of six connected F_2 populations, derived from four parental inbreds, was used. Recently, Liu et al. (2010) used three connected RIL populations derived from the crosses among three parents to find QTLs for the number of spikelets per panicle and 1,000-grain weight in rice. Other more complex populations have been proposed, for example, MAGIC (multiparent advanced generation intercross, Cavanagh et al. 2008), NAM (nested association mapping; Yu et al. 2008; Buckler et al. 2009) and AMPRIL (a multiparent recombinant inbred line population, Huang et al. 2011). These studies require genome-wide a high density of markers and use re-sequencing technology.

The aim of the present paper is to develop an approach for a multi-population and multi-environment QTL analysis in the presence of a reciprocal translocation. To illustrate our approach, we analysed days to heading (DtH) and thousand kernel weight (TKW) using a large number of doubled haploid lines from the F_1 of five crosses produced from different combinations of four parents, one of which was ‘Albacete’. DtH was selected

because is independent of the reciprocal translocation and TKW because it was the only trait showing a significant positive effect of the reciprocal translocation in 'Albacete' (Farré et al 2012b). 'Albacete' is known to carry a reciprocal chromosomal translocation between chromosomes 1H and 3H (Vazquez and Sanchez-Monge, 1987; Lacasa-Benito et al. 2005; Farré et al. 2011; Farré et al. 2012). This complicates the QTL analysis due to 'pseudo-linkage' between markers on chromosomes involved in the interchange. Close to the translocation breakpoints recombination is suppressed (Burnham, 1962; Sybenga, 1975) and, as a consequence, markers in those regions were removed; see also Farré et al. (2011). The final linkage maps consist of the distal parts of the chromosomes involved in the translocation (1HS-1HL and 3HS-3HL). The genotyping was performed using Diversity Arrays Technology (DArT®). The DArT markers were positioned on the barley chromosomes according to the barley consensus map (Wenzl et al. 2006).

Materials and methods

Plant material

Agronomic traits were evaluated on anther-culture derived DH lines of barley. The lines were derived from five F_1s obtained by crossing four parents: 'Albacete', 'Barberousse', 'Orria' and 'Plaisant'. 'Albacete' is a landrace variety with a relatively long cycle and an alternative growth habit. It is drought tolerant showing relatively stable grain yield production under Spanish conditions. 'Albacete' carries a reciprocal translocation between chromosomes 1H and 3H (Farré et al 2011; Farré et al. 2012a). 'Barberousse' is known for its good productivity and easy adaptation, although it is sensitive to drought in Spain. 'Orria' is a Spanish variety of CIMMYT origin that is well adapted to fertile, rainfed environments. 'Plaisant' shows good adaptation and has a high yield under Spanish conditions. The vernalisation and photoperiod characteristics of the parents are given in SEM_Table1. The allele representation within each population for 'Albacete', 'Barberousse', 'Plaisant' and 'Orria' were 336, 166, 138 and 218 descendants, respectively.

Genotyping

Genomic DNA of the DH lines was extracted from fresh leaf tissue using the Qiagen DNeasy 96 plant Kit. A genome wide scan was carried out using Diversity Arrays Technology (DArT®) (www.diversityarrays.com). In a single assay, DArT uses microarrays to detect DNA polymorphisms at several hundred genomic loci spread over the entire genome. It is based on hybridising labelled genomic representations of individual DNA samples on a micro-array, which contains a large number of DNA fragments derived from the total genomic DNA of the species under consideration (Wenzl et al. 2004). Polymorphisms are scored as the presence (= 1) or absence (= 0) of hybridization to individual array elements. The locus designations used by Triticarte Pty. Ltd. were adopted in this paper. DArT marker names consist of the prefix "bPb", followed by numbers corresponding to a particular clone in the genomic representation, where b stands for barley, P for PstI (primary restriction enzyme used) and b for BstNI (secondary restriction enzyme). For each DH population quality-filtered datasets were prepared by removing markers and DH lines according to Farré et al. 2011. Individual linkage maps were constructed for the three largest populations (A×B, A×P and P×O). For A×B and A×P, linkage groups 1H and 3H were split in two as they carry the reciprocal translocation (Farré et al. 2011). For P×O, 1H and 3H formed a single linkage group, respectively. DArT markers were located on the barley chromosomes according to the barley consensus map (Wenzl et al. 2006). The markers used for constructing the consensus map were those which segregated in at least two mapping populations. DArT markers located around the translocation breakpoints were removed according to Farré et al. (2011) resulting in two sub-linkage groups for chromosome 1H and 3H (1HS-1HL and 3HS-3HL).

Experimental design

Four field trials were carried out at two locations (Gimenells (41º 37'N, 0º 22'E, 248m, dry, rainfed); Foradada (F; 41º 51'N, 1º 0'E, 407m, dry, rainfed) in North-Eastern Spain (Catalonia) in two growing seasons (2008/2009 and 2009/2010). Details of the environmental conditions are given in SEM_Table2. Experimental designs followed a latinized row and column alpha design with a single replication for most DH lines

augmented by four replicated checks (parents) that were randomly included in a diagonal fashion along rows and columns. The experiments contained a total of 600 plots arranged in 30 rows and 20 columns, except Foradada-2008/2009 which contained 630 plots arranged in 15 rows and 42 columns. Plots consisted of eight rows, 4 m long and 15 cm apart and were sown at a density of 300 seeds m^2 and an accoding with standard crop practise in terms of plant density, herbicide treatment and fertilizanty rates.

Phenotyping

To illustrate our approach we analysed two traits: days to heading and thousand kernel weight. Days to heading (measured as the number of days between 1st of January until 50% of spikes on a plot had extruding anthers) was selected because it is a major selection criterion in all plant breeding programs of barley as it determines crop adaptation. Besides that, it is a highly heritable trait with significant QTLs and its biological basis has been well characterised in model plants (Arabidopsis; Greenup et al. 2009) with many barley homologues identified (Higgins et al. 2010). None of the major genes known to affect flowering time in barley are located in the region of the reciprocal translocation (Laurie et al. 1995) so days to heading should be independent of the reciprocal translocation. Thousand kernel weight (measured in grams) was selected because it was the only trait showing a significant positive effect of the reciprocal translocation in 'Albacete' (Farré et al 2012b).

Data analysis

The observations of each experiment (different site and year combinations) were analysed by means of a mixed model using Genstat 14 procedures (Payne et al. 2011). Spatial effects were eliminated by incorporating rows and columns in the random part of the mixed model. We allowed the mixed model to have unequal variances for populations, which made it possible to estimate the heritabilities of the traits for all combinations of sites, years and populations separately. Finally, for each trait Best Linear Unbiased Estimates (BLUEs) of the contributions of the genotypes were obtained for further analysis. Genotype × environment interactions were studied by

fitting an AMMI model (Van Eeuwijk 1995; Gauch y sobel) to tables with genotypes as rows and experiments as columns. The BLUEs and the scores of the genotypes on the first two principal axes for each trait were subjected to QTL analysis which were estimated using the method developed by Gabriel and Zamir (1979) and Romagosa et al. (1996).

QTL analysis

QTL analysis has been carried out using Genstat 14 (Payne et al. 2011). For all individuals the probabilities associated with the descent states 0 (maternal allele) and 1 (paternal allele) at marker loci were obtained using the procedure QIBDPROBABILITIES, based on the DArT genetic linkage map (Wenzl et al. 2006). Here, maternal and paternal refer to the parents of the cross (F_1) from which each DH population was derived. The descent probabilities for individual j (= 1, 2 ... N_i) of population i (= 1, 2 ... 5) at locus k were arranged in vectors $\mathbf{p}_{0k}$ and $\mathbf{p}_{1k}$, respectively; k = 1 ... M. Subsequently, the descent probabilities were used to calculate regressors $\mathbf{x}_{ik}$, accounting for allelic differences within populations (irrespective of the parents). Here, $\mathbf{x}_{ik} = \mathbf{p}_{0k} - \mathbf{p}_{1k}$ if the corresponding individual belongs to population i and $\mathbf{x}_{ik} = 0$, elsewhere. For each marker, the regressors $\mathbf{x}_{1k}$, $\mathbf{x}_{2k}$ ... $\mathbf{x}_{5k}$ represent 4 degrees of freedom.

The regressors for the general (additive) effect of the four parents, denoted by $\mathbf{a}_{pk}$ (p = 1, 2, 3, 4), were obtained as follows:

$$\mathbf{a}_{1k} = \mathbf{x}_{1k}+\mathbf{x}_{2k}+\mathbf{x}_{3k},$$
$$\mathbf{a}_{2k} = -\mathbf{x}_{1k} \quad +\mathbf{x}_{4k},$$
$$\mathbf{a}_{3k} = \quad -\mathbf{x}_{2k} \quad -\mathbf{x}_{5k},$$
$$\mathbf{a}_{4k} = \quad -\mathbf{x}_{3k}-\mathbf{x}_{4k}+\mathbf{x}_{5k}.$$

Parent 1 (‘Albacete’) is the mother of populations 1, 2 and 3, parent 2 (‘Barberousse’) is the father of population 1 and the mother of population 4, parent 3 (‘Orria’) is the father of populations 2 and 5, and finally parent 4 (‘Plaisant’) is the father of populations 3 and 4 and the mother of population 5. It should be noticed that $\sum_p \mathbf{a}_{pk} = \mathbf{0}$, *i.e.* the regressors for the general additive effects of the parents represent 3 degrees of freedom. Differences in ‘fit’ between the sets of regressors $\{\mathbf{x}_{1k}, \mathbf{x}_{2k} \ldots \mathbf{x}_{5k}\}$ on the one hand, and

the sets of regressors $\{\mathbf{a}_{1k}, \mathbf{a}_{2k}, \mathbf{a}_{3k}, \mathbf{a}_{4k}\}$ are due to additive x genetic background interaction or specific additive effects, and may be an indication of epistasis (Jannink and Jansen, 2001; Jansen et al. 2009).

Interval mapping was carried out using a mixed model. The mixed model accounted for presence/absence of the reciprocal translocation in the populations that were derived from 'Albacete' and allowed heterogeneous variances between populations. Tests for putative QTL were based on the Wald statistic with the appropriate numbers of degrees of freedom. Initially a forward selection of marker loci was carried out using a liberal threshold of 3.0 for minus the logarithm of the p-value associated with the Wald statistic. Only the most significant marker loci selected in this way were added to the set of cofactors used in the mixed model. This was followed by backward elimination of the marker loci selected in the previous step using a threshold of 3.5 (which conforms to the usual threshold obtained by Li and Ji (2005) for this situation). It should be noticed that in a standard QTL analysis the Wald statistics involves one degree of freedom for the numerator; here the number equals three). In the output, contrasts between parents were presented as differences from parent 1 ('Albacete') and appropriated standard errors were calculated. The effect of the reciprocal translocation was tested by incorporating the effects of the final set of cofactors obtained by backward elimination.

Results

Genetic map

A total of 364 previously mapped markers (Wenzl et al. 2006) were polymorphic across all DH populations with between 75 (B×P) and 274 markers (P×O) polymorphic in individual populations. No markers were segregating in all five populations; 14 markers were polymorphic in four populations although the highest number of polymorphic markers was found across three populations (171 markers). The number of markers per chromosome varied between 30 (1H) and 86 (4H). The map used in this study covered 1,111 cM for all seven chromosomes. Chromosomes 1H and 3H were split in two linkage groups due to the presence of the reciprocal translocation (1HS-1HL and 3HS-3HL; Farré et al. 2011). The DArT markers located around the translocation breakpoints

were removed according to Farré et al. (2011). The greatest gap was on chromosome 4H (64 cM).

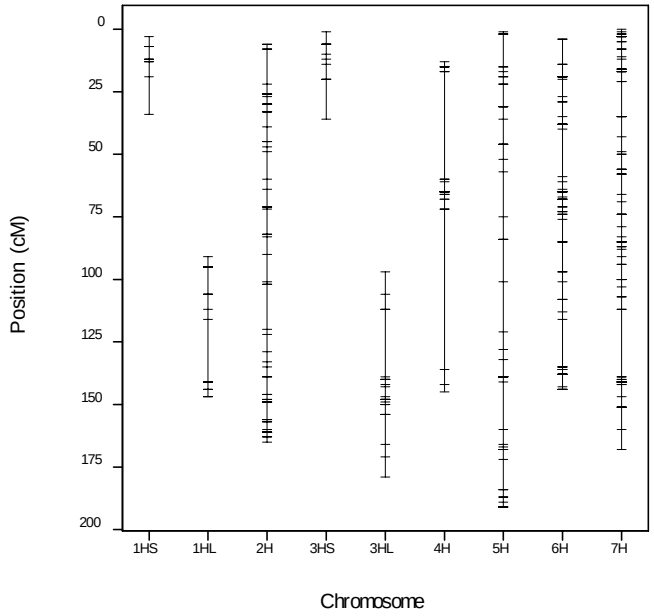

Figure 1. Genetic linkage map. Map distances based on the barley consensus map (Wenzl et al. 2006). Linkage groups 1H and 3H were slipt in two sub-linkage groups: 1HS-1HL and 3HS-3HL.

Agronomic results

For DtH, Barberousse was on average the earliest and Albacete the latest parent, with a difference of three days. For TKW, Albacete had on average the highest parental value and Barberousse the lowest, with a difference of 3.1 g.

Means of the DH lines for DtH values ranged from 109.5 to 124.7 days at G9 and F10, respectively. For TKW, the values ranged from 27.2 gr at G9 to 39.5 g at G10. For both traits, the magnitude of the correlations varied across environments but not the sign which is always positive for all the trials. The highest correlation coefficient (0.76) was observed between G10 and F9 while the lowest (0.29) was between G9 and F10 which could be explained by the extremely different mean values for DtH. However, for TKW the lowest values were observed between G9 and the other environments (0.40 to 0.46). The correlation between traits (DtH and TKW) for the various environments was -0.22 indicating that late flowering genotypes were associated with higher TKW.

Genotype × Environment interaction

The results of a principal component analysis on the TKW and DtH are shown in Figure 1. The first two axes, PCA1 and PCA2, explained 84.33 and 78.31% of the DtH and TKW variation, respectively. For DtH, the most contrasting environments were F10 and G9, the latter having an earlier flowering time. For TKW, the most contrasting environments were G9 and G10, the former having a lower TKW. PCA2 was not used in the next steps of the analysis because accounted for a small proportion of variation.

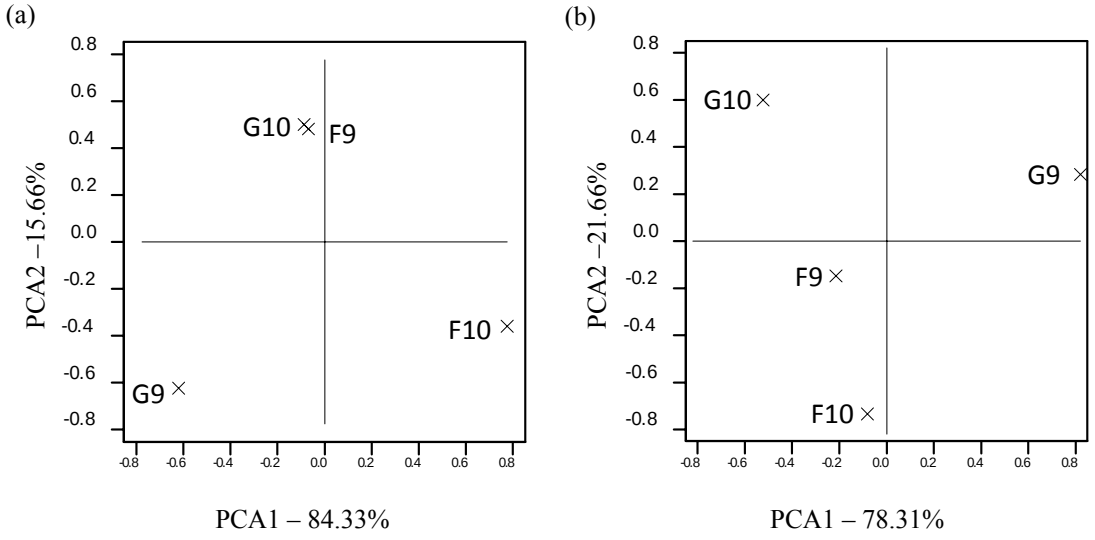

Figure 1. Environmental loadings for DtH (a) and TKW (b) as obtained from a principal component analysis of the genotype x environment table.

QTL analysis in single populations across environments

First a multi-environment QTL mapping was performed for the three largest populations (A×B, A×P and P×O) for DtH and TKW to detect QTL and QTL×E interaction. The results are presented in Table 1 and Figure 2. For DtH, the numbers of QTL detected were 4, 2 and 4 for A×B, A×P and P×O, respectively. None of the QTL were present in all three populations. In A×B, four QTLs were detected on chromosomes 2H, 3H, 5H and 6H with Albacete alleles delaying flowering date. Only one QTL found on chromosome 2H at 47 cM (consensus map of Wenzl et al. 2006) was detected in all four environments (with effects ranging from -0.37 to -1.00 days). For A×P, two QTLs were

detected on chromosome 2H (68cM) and 3H (10 cM) with delayed flowering time for Albacete alleles. Both are stable QTLs as they did not show any difference for additive effects between trials. For P×O, four QTLs were determined on chromosomes 2H(2x), 3H and 5H. When more than one peak was found on a chromosome, the peaks were well spaced; the two peaks on chromosome 2H were located at 49 and 122 cM. The first one presented an effect in opposite directions for F10 and G9 showing QTL×E interaction. The delay of flowering time in F10 is due to the Orria allele and in G9 due to the Plaisant allele. Apart from this QTL all the others did not show any difference between trial for additive effects (stable QTLs). The QTLs on 2H (49 cM) and 5H (127 cM) coincide with the ones detected in A×B population.

For TKW, QTLs were only detected in A×B and P×O. In A×B, only one QTL was detected on 3H chromosome with the Barberousse allele giving an increasing of TKW. In P×O, four QTLs were found: on chromosomes 3H, 5H(2x) and 6H. The QTLs on 3H (97 cM) and 5H (120 cM) showed effects that were opposite to the effects for the other QTLs in which Plaisant alleles gave an increase of TKW.

Table 1. QTLs, their locations and effects for DtH and TKW from the three DH populations (A×B, A×P and P×O) for 4 trials in Gimenells (G) and Foradada (F), 2009 and 2010 (Pop = population; CR = chromosome; se = standard error; additive effect: a positive value means that the second parent allele (B, P or O) increases the trait; the chromosome locations were referred to Wenzl et al. 2006).

Trait	Pop	CR	cM	Marker	-Log10(P)	Additive effect (se)			
						F10	F9	G10	G9
DtH	A×B	2H	47	bPb-4877	9.48	-0.37 (0.141)	-0.88 (0.137)	-0.79 (0.130)	-1.00 (0.175)
		3H	19	bPb-9745	5.46	-0.06 (0.160)	-0.55 (0.155)	-0.77 (0.147)	-0.70 (0.198)
		5H	127	bPb-4494	6.02	-0.31 (0.147)	-0.42 (0.142)	-0.52 (0.135)	-0.08 (0.182)
		6H	64	bPb-5822	4.41	-0.10 (0.142)	-0.62 (0.138)	-0.43 (0.131)	-0.78 (0.177)
	A×P	2H	68	bPb-0220	6.63	-0.64 (0.124)	-0.64 (0.124)	-0.64 (0.124)	-0.64 (0.124)
		3H	10	bPb-9945	5.52	-0.58 (0.124)	-0.58 (0.124)	-0.58 (0.124)	-0.58 (0.124)
	P×O	2H	49	bPb-6992	10.71	0.75 (0.202)	0.29 (0.145)	0.16 (0.159)	-0.81 (0.200)
		2H	122	bPb-1266	4.25	0.62 (0.154)	0.62 (0.154)	0.62 (0.154)	0.62 (0.154)
		3H	66	bPb-2440	4.11	0.58 (0.146)	0.58 (0.146)	0.58 (0.146)	0.58 (0.146)
		5H	127	bPb-4494	6.92	-0.73(0.137)	-0.73(0.137)	-0.73(0.137)	-0.73(0.137)
TKW	A×B	3H	1	bPb-5555	4.31	0.73 (0.340)	1.27 (0.366)	2.01 (0.406)	0.77 (0.375)
	A×P	-	-	-	-	-	-	-	-
	P×O	3H	97	bPb-6722	5.08	1.08 (0.242)	1.08 (0.242)	1.08 (0.242)	1.08 (0.242)
		5H	1	bPb-6485	10.10	-1.67 (0.362)	-1.82 (0.371)	-2.48 (0.355)	-0.96 (0.285)
		5H	120	bPb-7854	8.33	1.44 (0.245)	1.44 (0.245)	1.44 (0.245)	1.44 (0.245)
		6H	68	bPb-5698	7.82	-1.27 (0.224)	-1.27 (0.224)	-1.27 (0.224)	-1.27 (0.224)

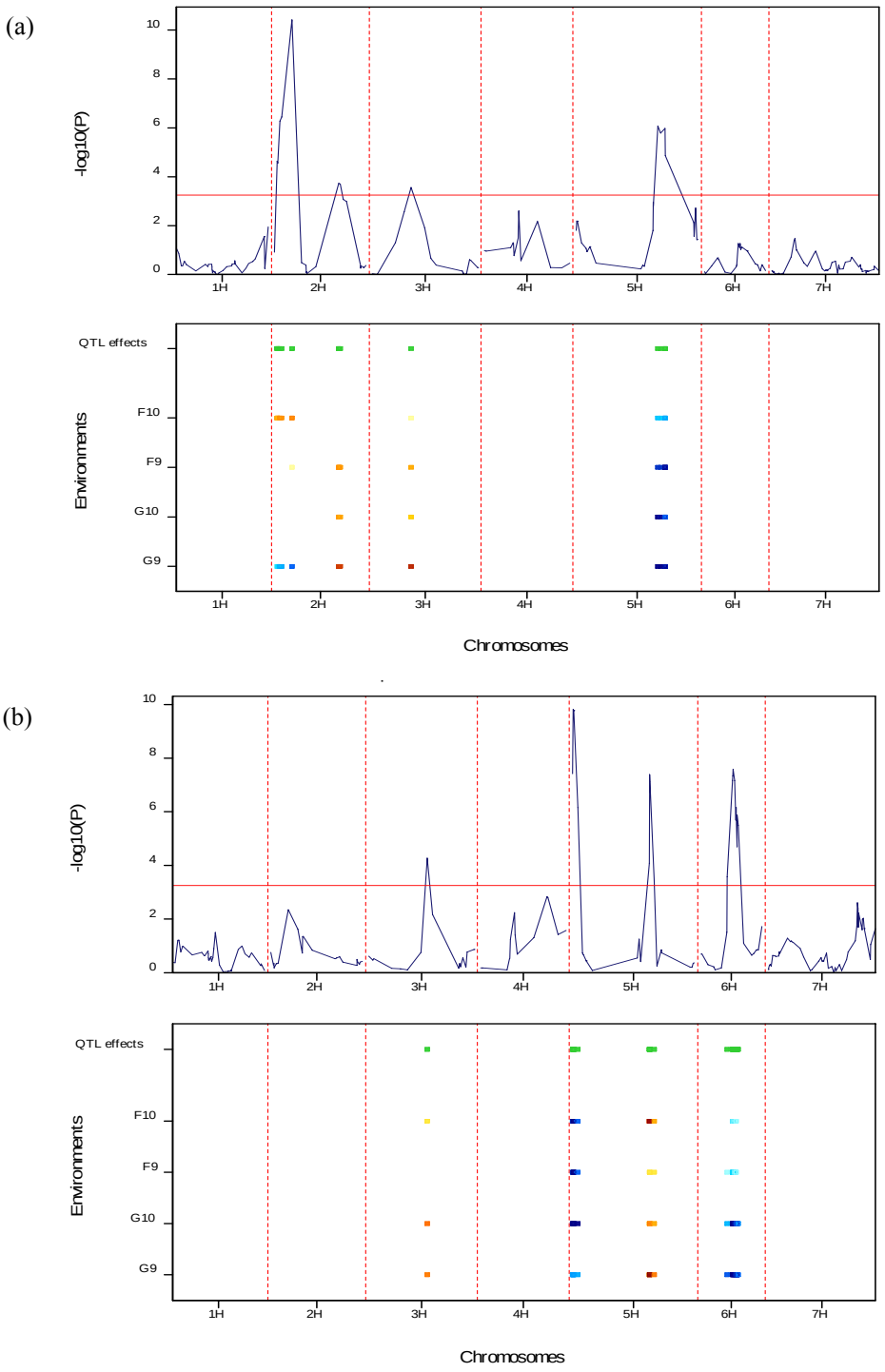

Figure 2. Example of multi-environment QTL analysis using the data from P×O population for 4 trials in Gimenells (G) and Foradada (F), 2009 and 2010 which was analyzed using Genstat 14 ((a) DtH and (b) TKW). Top panel shows the genome-wide profile, the blue line indicates the profile of –log10 (P-value) for composite interval mapping scan where test is for QTL expression in at least one environment. The red horizontal line shows the threshold value for significance. Low panel shows the representation of QTL allele effects in individual environments. Blue and yellow indicate the size and direction of the QTL effect in each environment for the first and the second parent, respectively. The green line at the top shows the significance for the overall test on QTL presence in the upper panel

QTL analysis of separate environments

Table 2 lists the QTLs found in each environment separately. A total of nine different QTLs were detected for DtH on chromosomes 2H(5x), 3H, 5H(2x) and 6H. The QTL with the largest and most consistent effect was located on chromosome 3H at 10 cM. This QTL had a consistent effect across all environments (only the magnitude changed), with the allele of 'Albacete' delaying flowering. A second important QTL with an effect in G9 and G10 was located on chromosome 2H at 72 cM. At this locus, compared with the Albacete allele, the three parental alleles reduced time to heading. Other QTLs for DtH that were specific to individual environments were located on chromosomes 2H, 5H and 6H.

For TKW, a total of five different QTLs were found on chromosomes 2H, 4H, 5H(x2) and 6H. No QTLs were detected in common in all environments. The QTL with the largest effect was located on 5H at 17 cM; compared to Albacete, Barberousse allele increased TKW whereas Plaisant allele decreased it in G10 and F9. The Orria allele had opposite effects on TKW in both environments. No significant QTLs were found in G9 for any marker. Other TKW QTL detected in specific environments were located on chromosomes 2H, 5H and 6H.

Table 2. Estimates of environment specific effects for DtH and TKW QTL across all five DH connected populations for 4 trials separately (F= Foradada; G= Gimenells; CR= chromosome; the chromosome location were referred to Wenzl et al. 2006; positive and negative signs mark superior versus inferior contributions as compared to the respective Albacete allele; se = standard error).

Trait	Env	CR	mm	cM	-log10(P)	Parental additive effects (se)		
						Barberousse	Orria	Plaisant
DtH	F9	2H	bPb8038	39	4.50	-0.64 (0.202)	-1.05 (0.285)	0.02 (0.168)
		2H	bPb9925	64	3.61	-0.20 (0.260)	0.80 (0.286)	-0.56 (0.206)
		3H	bPb9945	10	7.61	-0.68 (0.152)	-0.50 (0.193)	-0.72 (0.139)
		5H	bPb5596	101	4.19	-0.69 (0.237)	-0.82 (0.223)	-0.43 (0.161)
	F10	2H	bPb5755	133	5.52	0.46 (0.145)	1.07 (0.232)	0.32 (0.151)
		3H	bPb9945	10	3.82	-0.31 (0.143)	-0.65 (0.227)	-0.65 (0.147)
	G9	2H	bPb3575	72	6.23	-0.92 (0.220)	-0.40 (0.302)	-0.72 (0.189)
		2H	bPb7208	148	3.62	0.45 (0.201)	-0.77 (0.296)	0.17 (0.178)
		3H	bPb9945	10	6.08	-0.55 (0.205)	-0.70 (0.294)	-0.95 (0.182)
	G10	2H	bPb3575	72	9.75	-0.91 (0.154)	-0.38 (0.229)	-0.63 (0.165)
		3H	bPb9945	10	5.82	-0.58 (0.147)	-0.50 (0.214)	-0.64 (0.149)
		5H	bPb2580	132	4.86	-0.41 (0.171)	-0.93 (0.220)	-0.26 (0.151)
		6H	bPb3230	73	4.17	-0.35 (0.150)	0.08 (0.213)	-0.44 (0.145)
TKW	F9	4H	bPb6640	61	3.62	0.23 (0.338)	1.09 (0.421)	-0.34 (0.341)
		5H	bPb8072	19	6.27	1.56 (0.371)	-1.27 (0.402)	0.27 (0.322)
	F10	2H	bPb6222	156	3.94	-0.93 (0.327)	-1.35 (0.358)	-0.48 (0.296)
		5H	bPb5317	1	5.12	0.56 (0.335)	-1.48 (0.405)	0.53 (0.349)
	G10	4H	bPb3512	68	4.51	1.71 (0.382)	0.96 (0.497)	0.36 (0.394)
		5H	bPb7407	17	8.63	1.62 (0.422)	-1.15 (0.532)	0.82 (0.464)
		6H	bPb5698	68	3.80	0.51 (0.362)	-0.80 (0.479)	0.68 (0.390)

QTL analysis across environments

For the multi-population QTL analysis across environments, the data from the five populations and the seven chromosomes were used together to detect QTLs using the overall mean for each genotype from the BLUEs obtained for each trial and the scores on the first two principal components from the AMMI model, if significant (Table 3).

For DtH, a total of 4 QTLs were detected. Three were found using the mean and the other one was detected using PCA1. In the first case, all three QTLs showed negative additive effects for the three parental alleles (ranged from -0.33 to -0.79 days) indicating an early flowering time compared to Albacete allele. The QTL with the biggest effect was located on 3H at 10 cM. In order to study the QTL×E interaction, PCA1 was used to detect QTLs and one peak was found on chromosome 2H at 148 cM. Compared to Albacete the other three parental alleles had positive effects on DtH (0.07 to 1.03 days).

For TKW, only one QTL was detected for the overall mean on chromosomes 5H at 17 cM. In this case the effect was highly dependent on the parental allele: two increasing TKW alleles (0.47 to 0.96 g) were specific to Plaisant and Barberousse and one decreasing TKW allele (-0.98 g) came from Orria. No QTL was found on chromosome 2H, 4H and 6H with this analysis although they were present in the previous analysis. No QTL×E interaction (PCA1) was found for TKW. No QTLs were found for both traits in the same position.

Table 3. Comparison of QTLs for DtH and TKW using multi-population multi-environment analysis (CR= chromosome; the chromosome location were referred to Wenzl et al. 2006; additive effect: a positive value means that the parent allele increase the trait compared to Albacete; se = standard error).

Trait		**mm**	**CR**	**cM**	**-log10(P)**	**Parental additive effects (se)**		
						Barberousse	**Orria**	**Plaisant**
DtH	Mean	bPb3575	2H	72	6.05	-0.61 (0.147)	-0.33 (0.204)	-0.52 (0.134)
		bPb9945	3H	10	7.26	-0.54 (0.135)	-0.51 (0.193)	-0.65 (0.123)
		bPb5596	5H	101	3.84	-0.52 (0.210)	-0.79 (0.209)	-0.34 (0.133)
	PCA1	bPb7208	2H	148	6.98	0.07 (0.128)	1.03 (0.185)	0.11 (0.110)
TKW	Mean	bPb7407	5H	17	7.10	0.96 (0.303)	-0.98 (0.334)	0.47 (0.282)

Effect of the reciprocal translocation

For the populations in which Albacete was one of the parents (A×B, A×P and A×O), the presence of the reciprocal translocation was included in the QTL analyses to check if the reciprocal translocation had an effect on DtH and TKW. Table 4 shows that there was no evidence of a significant effect of the reciprocal translocation on DtH but for TKW the lines carrying the reciprocal translocation showed higher TKW than the ones without it (0.47 to 2.19 g). A positive additive effect of the reciprocal translocation was found in F9, F10 and G10 for TKW.

Table 4. Estimates of the effects of the reciprocal translocation using the data from A×B, A×O and A×P populations (se = standard error).

Trait		**-log10(P)**	**RT vs no RT (se)**
DtH	F9	0.487	0.27 (0.275)
	F10	0.323	-0.17 (0.245)
	G9	0.148	-0.12 (0.337)
	G10	0.251	0.16 (0.272)
	Mean	0.024	0.02 (0.244)
	PCA1	0.105	-0.00 (0.005)
TKW	F9	5.187	2.16 (0.479)
	F10	5.750	2.19 (0.459)
	G9	-	-
	G10	0.349	0.47 (0.616)
	Mean	3.331	1.39 (0.396)
	PCA1	-	-

So far the general additive effects of parents were estimated. Specific additive effects were estimated to analyse background interaction. No interactions between QTL and genetic background were found.

Discussion

In the Mediterranean climate, characterized by low rainfall, high temperatures and high light intensity leading to high evaporation, it is important to develop cultivars well adapted to drought-prone conditions by improving characters related to yield. The differences observed across environments for DtH and TKW can be attributed to differences in climatic and other environmental conditions, especially differences in rainfall. Usually, Foradada is colder and wetter than Gimenells. G9 was the earliest to flower (12-30 April) whereas F10 the latest (2-14 May). The lowest values for both DtH and TKW were found in G9, whereas the highest values were obtained in F10 and G10 for DtH and TKW, respectively. The main differences between years and locations were due to the distribution of rainfall during the growth cycle. During the grain-filling period (May-June), 2010 had a higher cumulated rainfall compared with 2009 which could explain the higher TKW values obtained in 2010. Besides, the grain-filling period was longer in 2010 than in 2009 (40 and 32 days, respectively). This difference in the days of grain-filling, combined with limited rainfall and higher temperatures could explain the low TKW values obtained for G9 (27 g) compared to the other environments (30, 36 and 39 g for F9, F10 and G10, respectively).

The four parents ('Albacete', 'Barberousse', 'Orria' and 'Plaisant') used in the present study show differences in the vernalization and photoperiod genes (Table 1, supplementary material). All of them are winter varieties with a full or partial vernalization requirement, performing similar when sown in autumn. Under these conditions, 'Albacete' shows slightly later heading dates than the other parents (Igartua et al. 1999). Under winter or spring sowing conditions, 'Barberousse' and 'Plaisant' are clearly delayed. So, under the conditions of the experiments carried out in this study major effects of flowering genes cannot be expected.

It is known that genetic variation for genes controlling flowering time has provided adaptation to different environments maximizing grain yield in cereal varieties (Cockram et al. 2007). The genetic factors determining flowering time in barley have been exhaustively studied and can be divided into: photoperiod-responsive genes (day length), vernalization-responsive genes (response to a low temperature period to induce transition from vegetative to reproductive stage) and 'earliness per se' genes (plant response to a temperature sum over a certain period) (Boyd et al. 2003). In the QTL analysis for each population separately, no QTLs were common among all three populations. A×B and P×O had two QTL in common and A×B and A×P had one. The most significant QTL for DtH was located on 2HS at 47-49 cM. By comparing genetic maps (Wenzl et al. 2006; Cuesta-Marcos et al. 2008a; Close et al. 2009; Aghnoum et al. 2010) we assumed that this QTL corresponded to the location of *PpdH1*, one of the major genes responsible for photoperiodic response expressed under long days (Laurie et al. 1995; Turner et al. 2005; Sameri et al. 2011; Borràs-Gelonch et al. 2012). Yet, in another study in the Mediterranean area, using a large panel of genotypes, Comadran et al. (2008) concluded that under autumn sowing and latitudes under 43ºN it is unlikely that *PpdH1* might be effective. In P×O, *PpdH1* had an opposite effect depending on the environment. In F10 the delay of flowering time is due to 'Orria' allele whereas in G9 is because of 'Plaisant'. Similar findings were reported for the 'SBCC145'×'Beatrix' population (Ponce-Molina et al. 2012). They found an effect of this gene in an autumn sowing with the insensitive allele ('Beatrix') conferring earliness; the effect changed in winter sowing where the sensitive allele ('SBCC145') led to earlier flowering. This QTL was also found in other studies using 'Scarlett'×'ISR42-8' (Wang et al. 2010) and 'Steptoe'×'Morex' (Borràs-Gelonch et al. 2012). An interesting finding was observed

for *PpdH1* in A×B population in which 'Albacete' allele somewhat delayed flowering time. However, both parents carry the dominant, long photoperiod responsive allele, with the same polymorphism in the SNP that is used to differentiate the dominant/recessive alleles (Turner et al. 2005). This result suggests that, there may be more alleles for *PpdH1*. Resequencing the *PpdH1* gene, Jones et al. (2008) identified 83 polymorphisms in a set of 266 barley accessions (194 European landraces and 72 wild barley accesions). Among them 10 Spanish landraces were present. Although they carry the dominant, long photoperiod responsive SNP allele they show other polymorphisms along the PpdH1 gene which could be related with different phenotypic effects.

The other common QTL was detected on 5HL at 127 cM , where is located *Vrn-H1*, a major gene which determines the vernalization requirement (Laurie et al. 1995; Trevaskis et al. 2003; Cuesta-Marcos et al. 2008a,b). In our study, 'Barberousse' and 'Plaisant' are two typical winter varieties with full vernalization requirement as they carry a recessive allele at *vrnH1* and a dominant allele at *VrnH2*. In contrast, 'Albacete' and 'Orria' carry an intermediate (*VRNH1-4*) allele, which has been found to be associated with a reduced vernalization requirement (Casao et al. 2011). In P×O, 'Orria' alleles conferred earliness. Different results were obtained in A×B population in which 'Albacete' alleles delayed flowering time. The flowering difference in A×B is between 0.16 to 1 day whereas in P×O is 1.5 days. For A×B and A×P, the common QTL was found on 3HS at 10 cM, which we predict to be in the region of QHea.S42-3H.a (von Korff et al. 2006). A QTL on chromosome 2H (122 cM detected in P×O) is in the same region that a QTL found in two association mapping studies (Comadran et al. 2011; Wang et al. 2012). Other QTLs such as that found on 3H (66 cM detected in P×O) or 6H (64 cM detected in A×B) could be specific to backgrounds of these populations.

Concerning TKW, no QTL were found in common between populations, although the greatest number of QTL was found in the P×O population. Two other QTL on 5H (120 cM) and 6H (68 cM) are in similar regions to those reported by von Korff et al. (2008) in the 'Tadmor' × 'ER/Apm' population.

Until recently the populations used to detect and map QTLs in plants were biparental populations which have been widely employed by selecting two contrasting parents to guarantee marker polymorphism and trait divergence (e.g. Backes et al. 2003). This approach is not particularly applicable to plant breeding because QTLs can be missed as they do not segregate in the particular cross. Nowadays, the development of multiple connected populations for QTL analysis is increasing because it: (i) improves the power to detect QTL and the accuracy of the estimation of their effects and positions; (ii) increases variability addressed; (iii) permits direct comparison of the epistatic interactions between parental alleles at specific genome regions across genetic backgrounds (Rebaï et al. 1997; Verhoeven et al. 2006; Blanc et al. 2006, 2008; Cuesta-Marcos et al. 2008a; Liu et al. 2010). For that reason, it is necessary to develop new QTL mapping methodologies for populations derived from more than two parents due to the fact that most current statistical methods developed for QTL mapping are based on bi-parental populations (Hackett 2002).

In this study, a new approach was developed for QTL detection in multi-population analysis in the presence of a reciprocal translocation. This approach was applied using DtH and TKW providing us with the opportunity to directly compare general additive effects among four parental alleles. In this paper we mainly looked at the contrast of Albacete with the other parents involved in creating the populations. The number and location of QTLs found in each environment separately could be influenced by the heterogeneity between all the environments. Indeed, seven and three QTLs were identified in one particular environment for DtH and TKW, respectively (Table 2), whereas only one QTLs was found in all environments for DtH, none for TKW. For instance, the QTL of chromosome 3H at 10 cM was involved in the variation of DtH in all environments. In the multi-environment analysis, the some QTLs found were at locations where QTLs had been already identified in one or several environments separately.

With this approach, three major QTLs, whose positions agree with previously studies, were found at the following locations: on chromosome arm 2HS at 64-72 cM in the region of *Eam6*, identified as the main heading date QTL in Mediterranean conditions

(Cuesta-Marcos et al. 2008a,b; von Korff et al. 2008; Comadran et al. 2011; Wang et al. 2012); on chromosome 3HS at 10 cM, which we predict to be in the region of QHea.S42-3H.a (von Korff et al. 2006) and finally on chromosome 5HL at 101 cM, in the region of a minor QTL found in 'Beka' × 'Mogador' (Cuesta-Marcos et al. 2008b). For TKW, a QTL on the short arm of chromosome 5H could be related to that on detected in 'TX9425'×'Franklin' (Wang et al. 2010b). QTLs only found in the present study could be specific to one environment or, when detected across environments, specific to the genetic backgrounds employed (*i.e.* for DtH, the QTL found on chromosome 2HL using PCA1 values). None QTL were found for both traits at the same position.

The results obtained when we included the presence of the reciprocal translocation in the QTL analyses are in agreement with the results obtained by Farré et al. (2012b) in which the reciprocal translocation affected only TKW. Other studies in 'Blenheim'×'E224/3' detected QTL in the region of the translocation (Thomas et al. 1995; Powell et al. 1997). DH lines carrying the reciprocal translocation had a greater TKW than those with a standard chromosome arrangement. Identifying the regions that improve traits related to yield (*i.e.* TKW) allows the breeders to use marker-assisted selection for the introgression of favourable alleles into their germplasm (in our case the 'Albacete' haplotype). All genes present in the interstitial zone of a translocation heterozygote are tightly linked due to the suppression of genetic recombination, and as a consequence they can be transferred as a block to other backgrounds. In breeding programs, the use of hybrids derived from one parent carrying a reciprocal translocation in breeding programs could lead to problems in seed production as a consequence of semi-sterility. Although semi-sterility disappears once the individuals become homozygous for the translocation.

Acknowledgments

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SUPPLEMENTARY MATERIAL

Table 1. Summary of the vernalization and photoperiod gene structure of the parents used in the production of the doubled haploid populations.

	Growth type	HvFT3 (PpdH2;1H)	SNP22 (PpdH1;2H)	HvZCCT (VrnH2;4H)	HvBM5 (VrnH1;5H)	HvFT1 (VrnH3;7H)
'Albacete'	Facultative	dominant	dominant	dominant	intermediate	recessive
'Barberousse'	Winter	recessive	dominant	dominant	recessive	recessive
'Orria'	Facultative	recessive	recessive	dominant	intermediate	recessive
'Plaisant'	Winter	recessive	dominant	dominant	recessive	recessive

Table 2. Means of temperature (T) and cumulated rainfall (R) during the months of the growth cycle of the four trials: F9= Foradada 2008/2009, F10= Foradada 2009/2010, G9= Gimenells 2008/2009 and G10= Gimenells 2009/2010 (http://xarxes.meteocat.com).

Trial		Nov	Dec	Jan	Feb	Mar	Apr	May	June	July	Total
F9	R (mm)	33.8	30.6	50.6	34.6	52.2	115.2	9.4	29.5	28.7	384.6
	T (ºC)	5.4	3.1	3.5	4.9	8.1	10.2	17.0	21.6	24.1	10.9
F10	R (mm)	10.0	67.8	61.6	32.6	64.1	14.6	54.0	102.1	4.9	411.7
	T (ºC)	8.3	3.7	2.8	3.9	6.6	11.3	13.7	18.8	24.4	10.4
G9	R (mm)	23.6	25.6	21.6	13.6	31.0	90.4	8.6	16.4	31.2	262.0
	T (ºC)	6.8	4.4	4.4	6.8	9.8	12.1	18.9	22.9	24.6	12.3
G10	R (mm)	5.5	45.5	65.8	26.7	38.4	19.1	27.3	54.3	0.2	282.8
	T (ºC)	10.4	5.5	5.1	5.7	8.8	13.2	15.8	20.3	25.5	12.3

Chapter 7

General discussion

Alba Farré

Breeding programs in Mediterranean environments are focused on the development of varieties with high yield and yield stability across environments. However, crop productivity is limited by drought, one of the most important abiotic stress factors in the Mediterranean basin (Araus et al. 2003). A Spanish barley variety, 'Albacete', which is drought tolerant and shows adaptation to low-yielding sites was the basis of the present thesis. 'Albacete' was selected because it is an extensively cultivated variety in the poorest Spanish areas. It is known to carry a reciprocal translocation between chromosomes 1H and 3H without apparent loss of agronomic value. One of the main objectives of the present thesis was to investigate whether the reciprocal translocation has a positive effect on drought tolerance and other traits that make it worth to introduce genes in the vicinity of the translocation breakpoints into the barley germplasm.

Balanced chromosomal rearrangements such as reciprocal translocations or inversions seem to be the most common rearrangements in plants (see interchanges in individual species in the summary by Burnham, 1956, pp.459-522) which not always produce phenotypic effects either in the heterozygous or homozygous state, other than reducing heterozygote fertility as a result of duplications and deficiencies. Their maintenance largely depends on their effects upon two properties: (i) the survival of individuals and (ii) their fertility (Rees 1961). Wallace (1959) postulates that at the margin of a species distribution, a translocation heterozygote with one of the better marginal genotypes may have a relative advantage that compensates for the expected semi-sterility. Furthermore, the semi-sterility disappears once the individuals become homozygous for the translocation, *i.e.* once a pure line is produced. Deleterious effects may arise when a breakpoint occurs within genes, generally disrupting gene functions; this is called a 'position effect'. The best established example of a position effect in plants is in

Oenothera and involves the factors P^s (broad red and narrow green stripes on the sepals) and P^r (red or rubricalyx); the translocation of a segment carrying the factor for broad red calyx stripes (P^s) to a new position resulted in variegated patches of red (P^r). When the locus was transferred back to the normal chromosome by crossing-over, the normal phenotype was restored. (Catcheside, 1947). Other examples were found in Drosophila (Ephrussi and Sutton, 1944; Lewis, 1950; Muller, 1954) and maize (Brink, 1932, 1954; Roberts, 1942; Jones, 1944; McClintock; 1951, 1953).

The emergence of new species is often accompanied by structural chromosome changes (Stebbins, 1971; Levin, 2002) which have played an important role in plant evolution showing adaptive features of many natural populations. For instance, some species of the genus *Clarkia* are notorious for a high frequency of translocation heterozygotes (*C. williamsonii* (Wedberg et al. 1968); *C. amoena* (Snow, 1963); *C. unguiculata* (Mooring, 1958); *C. exilis* (Vasek, 1960); *C. elegans* (Lewis, 1951); *C. dudleyana* (Snow, 1960)). The frequency of translocation heterozygotes in wild populations of *Clarkia williamsonii* was found to be strongly correlated with habitat; heterozygotes are abundant in populations in foothill woodlands and infrequent in populations at higher elevations in the yellow pine forest. The constancy of the distributions of heterozygotes strongly suggests that they have an adaptive role in populations (Wedberg et al. 1968). This adaptive role has not only been studied in plants but also in other species like the yeast *Saccharomyces cerevisiae*. Colson et al. (2004) investigated the effect of a reciprocal translocation involving chromosomes VI, VII and XVI on the whole organism's evolutionary fitness. They found that this naturally occurring rearrangement had the potential to provide a selective advantage in certain environmental conditions. These results support the idea of chromosomal evolution involving fixation of major rearrangements by natural selection. In barley, Konishi and Linde-Laursen (1988) identified four Ethiopian cultivated landraces carrying a reciprocal translocation with the translocation breakpoints at the centromere region involving chromosomes 2H and 4H, suggesting a common origin.

An interesting example is given by the T5B:7B-1 translocation which is frequent in hexaploid wheat. It was first identified in French bread wheat (T. *aestivum* 'Cappelle-

Desprez'; Riley et al. 1967) and was found later in many other varieties of T. *aestivum* (Baier et al. 1974; Bourgeois et al. 1978; Seal, 1982; Vega and Lacadena 1983; Lange et al. 1987; Schlegel and Schelgel 1989; Friebe and Gill 1994; Hohmann et al. 1996; Schlegel 1996). Its broad distribution can result from selective advantages of rearranged genotypes in diverse environmental conditions which may have significant breeding potential (Miura et al. 1992; Law and Worland 1997). For instance, Law and Worland (1997) indicate that the 5BS-7BS translocation was responsible for a substantial part of the adult plant yellow rust resistance of these varieties although it cannot be solely responsible for the resistance. These authors suggest that the gene(s) involved in resistance to yellow rust are closely linked to the translocation breakpoints adding an important attribute to the translocation favouring its selection. They also found some indication for a yield advantage associated with the 5BL arm. Yasumuro et al. (1998) suggest that most commercial bread wheat cultivars inherited translocations from their landrace ancestors; the maintenance of these translocations in landraces and their transmission to commercial varieties may reflect the significant breeding potential of translocated chromosomes. A reciprocal chromosomal transposition in *Brassica napus* between interstitial homeologous regions on linkage groups N7 and N16 gave significantly higher seed yields (Osborn et al. 2003).

In the present study, the agronomic effects of the presence of the reciprocal translocation on some interesting agronomic traits (associated to development, yield and yield components and architecture traits) were tested using a mixed model procedure dividing a set of 248 doubled haploid lines in two groups (carrying or not the reciprocal translocation) (Chapter 4). A highly significant main effect of the translocation was obtained for TKW; no significant main effects could be detected for other agronomic traits. It has been hypothesized that the high level of farmer's acceptance of 'Albacete' particularly for semi-arid rainfed areas may be due to the presence of this translocation. However, the adaptation to low-yielding sites cannot be attributed to this chromosomal structural change alone.

Plant accessions carrying chromosome rearrangements such as translocations have been identified by the resulting partial pollen and seed sterility and by the apparent linkage of

genes whose normal loci are on separate chromosomes (*i.e.* Jáuregui et al. 2001). Special interest resides in the identification of chromosomes involved in the interchanges and determination of the translocation breakpoints as it will provide new information on the origin of chromosomal rearrangements. It will also allow physical mapping of genes involved in the translocation on the chromosomes. These can be achieved by a combination of cytogenetics and molecular genetics (Chapter 3). The translocation breakpoints on 1H and 3H chromosomes in the barley variety 'Albacete' overlaps with duplicated segments between chromosomes Os01 and Os05 in rice derived from ancestral duplications within the grass family (Guyot and Keller 2004; Thiel et al. 2009). Among other causes the original interchange may have originated from a crossing-over between duplicated segments in non-homologous chromosomes (Burnham, 1956). Identification of structural changes is crucial in utilizing genetic pools of wild and cultivated varieties in breeding programs (Badaeva et al. 2007). Due to the suppression of genetic recombination, the genes in the interstitial zone of a translocation heterozygote are linked and can be transferred as a block to other backgrounds. So, chromosome segments carrying the reciprocal translocation are worth to be transferred in the barley germplasm. The time needed to incorporate favourable alleles into the germplasm can be reduced due to the development of specific molecular markers.

The incorporation of molecular technologies in plant breeding programs allows linkage analysis of agronomically important traits. For that purpose, linkage map construction using data from molecular markers is an indispensable tool. Major complications arose in populations based on reciprocal translocation heterozygotes as recombination between loci around the translocation breakpoints is suppressed (Burnham, 1962; Sybenga, 1975). Consequently, markers in these regions become 'pseudo-linked'. In order to disentangling this 'pseudo-linkage' a novel statistical-genetic approach was developed making use of a high-quality consensus map of 'normal' barley (Wenzl et al. 2006) (Chapter 2). An alternative study investigated the effects of karyotypic differences on the construction of linkage maps (Livingstone et al. 2000). They used Mapmaker/EXP to create several genetic maps to examine intermarker distances using two simulated datasets for F_2 progeny which differed by a reciprocal translocation or a reciprocal translocation and an inversion. They found alternative maps corresponding to different chromosomal rearrangements around the translocation breakpoints. Using this

study as a reference, Durrant et al. (2006) introduced QuadMap, a software application, to analyze linkage maps affected by translocations. Quadmap constructs and compares multiple maps using Mapmaker/EXP. It generates multiple maps, permutates the marker order, and analyzes the data from the linkage groups involved in the translocation simultaneously using a large number of iterations. It gives the frequencies, average intermarker centimorgan distance and centimorgan distance variance of each marker pair. Large intermarker distance variance was proposed as an indication of the presence of a breakpoint. However, high variances may also be caused by deficiencies in the data. The methods developed in the present study can be implemented using standard statistical software.

The first steps for the introgression of favourable alleles into germplasm are the construction of a high-quality linkage maps (Chapter 5) follow by QTL analysis (Chapter 6) which allows the identification of the precise location of chromosome regions associated with traits of interest. This goal can be achieved by constructing consensus maps using information from multiple populations which provides greater genome coverage avoiding large gaps in the map without markers and the marker order and intermarker distances can be estimated more accurately. This requires having common markers among populations which depends on the high level of polymorphism between parents. In the present study, the highest level of polymorphism among populations was observed for A×B and A×P whereas B×P showed the lowest level. This appears to be in good agreement with the results obtained by Casas et al. (1998). They demonstrated that parental lines ‘Plaisant’ and ‘Barberousse’ showed the highest level of genetic similarity compared with the other parents. This could be explained because they share ‘Ager’ as parent. So, to enhance the chance to detect more polymorphic markers the parents should be divergent. But this can result in populations that are not representative of germplasm used in breeding programs. This also have a direct consequence on the QTL analysis because if marker alleles are non-segregating in B×P population also QTL will have a higher chance of being non-segregating.

High-quality genetic linkage maps, including marker order within linkage groups, are of great importance for robust QTL mapping. For that, high-quality datasets free of errors

are essential as genotyping errors and missing data led to map expansion, especially in regions with a high marker density. In our study discrepancies in map lengths, compared with the reference consensus map (Wenzl et al. 2006), may be due to difference in "data curation" possibly in combination to the use of different mapping criteria and mapping algorithms (Van Os et al. 2005). The algorithm applied in this study which was adapted from the algorithm developed by Jansen (2005), is an efficient method for constructing good-quality dense, integrated linkage maps. This algorithm enabled us to develop a robust integrated linkage map involving complex chromosomal rearrangements. Dense linkage maps are used to reduce the confidence interval for a QTL and the distance between the marker and the QTL so avoiding recombinations between QTL and the nearest marker.

New QTL mapping methodologies have to be developed to cope with multi-population, multi-environment analysis. Until now, most QTL studies were performed using biparental populations (F_2 or $F_\times$ derived families, backcross, recombinant inbred lines, near-isogenic lines and double haploids) which were obtained by crossing two inbred parents with contrasting difference in phenotypic traits of interest (Semagn et al. 2010). Consequently, a small part of the total variability for breeders is explored. So, the development of multiple connected populations has become useful to: (i) increase the variability addressed; (ii) allow global comparison of the effects of all QTL alleles segregating in the different populations increasing the power to detect QTL, the accuracy of the estimation of their effects and position and identifying the parental origin(s) of favorable alleles at each QTL; and (iii) addressed interactions between QTL and the genetic backgrounds (Muranty et al. 1997). As an alternative, other more complex populations have been proposed to exploit the advantages of both linkage analysis and association mapping such as the MAGIC populations (multiparent advanced generation intercross) (Cavanagh et al. 2008), the nested association mapping populations (NAM) design based on a huge set of recombinant inbred lines derived from a large number of founder genotypes (Yu et al. 2008; Buckler et al. 2009) and AMPRIL populations (a multiparent recombinant inbred line population) (Huang et al. 2011). For instance, NAM population captured approximately 136,000 crossover events, corresponding, on average, to three crossover events per gene (McMullen et al. 2009). This NAM was derived from the crosses between 25 diverse inbred maize lines

and a reference line (B73) comprising 5000 RILs, 200 RILS per family. This allows genetic factors to be mapped to very specific regions of the genome. These studies genome-wide require a high density of markers and use re-sequencing technology.

The results obtained using the new methodology implemented in this study for a multi-population, multi-environment QTL analysis was found useful to directly compare additive effects among four parental alleles (Albacete, Barberousse, Orria and Plaisant) (Chapter 6). The reciprocal translocation affected thousand kernel weight. DH lines carrying the reciprocal translocation had a greater TKW than those with a standard chromosome arrangement. These results are in concordance with the results found by Farré et al. 2012 (Chapter 3).

The progress made with new marker technologies (such as DArT and SNP markers) allow high throughput and low cost genotyping which provides the opportunity to develop high-quality linkage maps. Currently, the new challenge in plant science is the plant phenotyping which is considered to be the new bottleneck. The 'phenotyping bottleneck' can be addressed by combining novel technologies such as robotics, noninvasive imaging and analysis using powerful computing. This brings us to the age of 'phenomics'.

The present study was focused on the characterization of a reciprocal translocation present in the widely grown Spanish barley variety 'Albacete'. The combination of different complementary approaches (cytogenetic, linkage and QTL mapping) led us to develop an in-depth understanding of the reciprocal translocation and its consequences.

Chapter 8

General conclusions

1. The reciprocal translocation present in ‘Albacete’ involves chromosomes 1H and 3H. The markers found to be linked to the translocation breakpoints are HvM20 and EBmac0501 for 1H and Bmag0136, bPb-9746 and a reduced set of genomic SSR markers for 3H, which are located around the centromere.
2. The novel statistical-genetic approach developed for the construction of linkage maps in populations obtained from reciprocal translocation heterozygotes of barley (*Hordeum vulgare* L.) can be used to identify the true background of 'pseudo-linkage' in a straightforward manner.
3. The physical characterization suggested that the translocation breakpoints are located on the long arms of both chromosomes between the $(AAG)_5$ and $(ACT)_5$ pericentromeric bands for 3HS.3HL-1HL and below the $(AAG)_5$ pericentromeric band for 1HS.1HL-3HL.
4. Duplicated segments between chromosomes Os01 and Os05 in rice derived from ancestral duplications within the grass family overlap with the translocation breakpoints on chromosomes 1H and 3H in the barley variety ‘Albacete’.
5. A highly significant main effect of the translocation was obtained for TKW; DH lines carrying the reciprocal translocation had a greater TKW than those with a standard chromosome arrangement. However, the results do not support the hypothesis that this chromosomal structural change alone provides an increased adaptation to low-yielding sites.
6. The algorithm applied in this study, adapted from Jansen et al. (2001, 2005 and 2009), is an efficient method for constructing good-quality dense linkage maps. The integrated map constructed is consistent compared with the consensus map of barley (Wenzl et al. 2006).
7. The integral approach for a multi-population and multi-environment QTL analysis in the presence of a reciprocal translocation provided a direct comparison of the effects of the four parental alleles.

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