Barley adaptation to stress prone environments

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Barley Adaptation to Stress Prone Environments

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Summary

Multi environment trials conducted over mapping population are often used to test genotypes in a set of environments that represent the target environmental range. The first part of this work is the evaluation of the 'Nure' x 'Tremois' double-haploid mapping population, together with an association panel comprising 185 barley varieties representative of the barley germplasm cultivated in the Mediterranean basin. Plant material was tested across eighteen site by year field trials combination, in six countries across the Mediterranean basin. Trials were growth at sites contrasting for natural rainfall (high vs low on the base of past meteorological data) or at the same site with one being rainfed and the other with supplementary irrigation. Trials conducted for two years in each one of the sites and this allowed tocollect a huge data series comprising agronomical traits defining grain yield and yield components, phenological and environmental data, subsequently used to identify genomic regions involved in barley adaptation. The 118 doubled haploid lines of the mapping population were genotyped with Diversity Array Technology® (DaRT) marker assay and subsequently a total of 15 CAPS and SSCP marker for candidate genes involved in phenology regulation and abiotic stress response were added to the linkage map based on DaRT markers. Data collected were firstly used to perform QTLs analysis with composite interval mapping for any environment/ trait combination, results showed eight QTLs for grain yield, days to heading and grain yield components. . The two mostly frequents QTLs for grain yield and days to heading were located on barley chromosome 1H (3 trials), 2H (8 trials) and 5H (5 trials) overlapping respectively HvFT3 gene, the earliness per se locus (eam6/Eps-2) and the vernalization gene Vrn_H1. A further QTL multi-environment analysis was performed and revealed that across the 18 field trials QTL for eam6/Eps-2 (2H) and Vrn-H1 (5H) were commons for days to heading and grain yield. We use all the environmental information collected to check QTLs sensitivities to co-environmental co-variables. Most of significant associations collected were related to temperature and temperature-based variables troughtout the growing cycle. Eam6/Eps-2 showed non-crossover QTL.E interaction, while for Vrn-H1 crossover interactions were revealed. The 185 barley accession were genotyped with 1536 SNPs and data collected for this population for cold resistance in two field trials in Spain an Italy, the first trial was characterized by an exceptional winter, while the second was previously know has frost-prone environment. Results from genome wide association analysis showed 13 positive associations with specific genomic regions. Interestingly several of these QTL were coincident with the position of previously mapped loci for cold tolerance, on chromosomes 2HL, 4HL and 5HL.

Resumen

Los ensayos en localidades múltiplas de poblaciones de mapeo se utilizan frecuentemente para testar genotipos en un conjunto de ambientes representativos de la condiciones climáticas donde se quieren introducir dichos genotipos. La primera parte de esto trabajo ha sido la evaluación de la población de mapeo 'Nure x Tremois' constituida de 118 de doble haploides de cebada, junto con panel de asociación que comprende 185 variedades de cebada representativas del germoplasma cultivado en la cuenca Mediterránea. El material vegetal ha sido ensayado en una combinación de dieciocho campos por año dislocados en seis países de la cuenca mediterránea.

Los ensayos se han llevado a cabo en campos con diferente disponibilidad de agua, clasificados en base a los datos relativos a las frecuencia y cantidad de las precipitaciones o en el mismo sitio con un campo en secano y otro regado. Los ensayos se llevaron a cabo por dos años en cada localidad y esto permitió la recogida de un gran volumen de datos que comprenden caracteres agronómicos relacionados con rendimiento y componentes del rendimiento, datos fenológicos y ambientales. Dichos datos se utilizaron después para la identificación de regiones genomicas involucradas en la adaptación de la cebada al ambiente. Los 118 dobles haploides de la población 'Nure x Tremois' se genotiparon con marcadores DaRT (Diversity Array Technology), después un set de 15 marcadores CAPS Y SCCP para genes candidatos involucrados en la regulación de las fases fenológicas fueron añadidos al mapa de ligamento construido con los marcadores DaRT. Los datos fueron utilizados para hacer una análisis de QTL con procedimiento 'Composite Interval Mapping' para cada combinación ambiente/ carácter. Se encontraron varios QTLs por rendimiento y fecha de espigado y componentes del rendimiento. Los QTL mas frecuentes encontrados por rendimiento y fecha de floración y componentes del rendimiento están localizados en los cromosomas 1H (3 campos), 2H (8 campos) y 5H(5 campos) coincidentes respectivamente con HvFT3 locus, eam6/Eps-2 (earliness per se) locus y con el locus de vernalización Vrn-H1. Una ulterior análisis de QTL hecha con el método "Multi Environment Trial" ha revelado que los QTL localizados en el locus eam6/Eps-2 (cromosoma 2H) y Vrn-H1 (cromosoma 5H) son comunes por rendimiento y fecha de floración en los 18 campos de ensayo. Por esto utilizamos todos lo datos ambientales coleccionadas durante todo el ciclo del cultivo para investigar la sensibilidad de dichos QTL a las co-variables ambientales. La mayoría de las asociaciones encontradas están relacionadas con temperaturas y variables relacionadas con estas. Eam6/Eps-2 muestra una interacción de tipo cuantitativo con dichas variables mientras Vrn-H1 muestra una interacción de tipo cualitativo con dichas variables. Las 185 variedades ensayadas fueron genotipadas con 185 SNPs y fenotipadas por resistencia a frío en dos ensayos uno en España y otro en Italia. El primer ensayo fue caracterizado por un invierno excepcionalmente frío, mientras el de Italia ha sido utilizado en pasado por testar resistencia a frío debido a los inviernos rígidos que suelen registrarse en dicha localidad. Los datos fueron utilizados para llevar a cabo la analisis GWAS "Genome Wide Association Analysis". Los resultados permitieron identificar 13 regiones genomicas

involucradas en la resistencia a frio. Entre ellas tres regiones coinciden con loci ya mapeados y conocidos por ser involucrados en la respuesta a frio en los cromosomas 2HL, 4HL y 5HL.

Resum

Els assajos en localitats múltiplas de poblacions de mapeo s'utilitzen freqüentment per a testar genotips en un conjunt d'ambients representatius de la condicions climàtiques on es volen introduir aquests genotips. La primera part d'això treball ha estat l'avaluació de la població de mapeo 'Nure x Tremois' constituïda de 118 de doble haploides d'ordi, juntament amb panell d'associació que comprèn 185 varietats d'ordi representatives del germoplasma conreat en la conca Mediterrània. El material vegetal ha estat assajat en una combinació de divuit camps per any deslloregats en sis països de la conca mediterrània. Els assajos s'han portat a terme en camps amb diferent disponibilitat d'aigua, classificats sobre la base de les dades relatives a les freqüència i quantitat de les precipitacions o en el mateix lloc amb un camp en secà i altre regat. Els assajos es van portar a terme per dos anys en cada localitat i això va permetre la recollida d'un gran volum de dades que comprenen caràcters agronómicos relacionats amb rendiment i components del rendiment, dades fenológicos i ambientals. Aquestes dades es van utilitzar després per a la identificació de regions genomicas involucrades en l'adaptació de l'ordi a l'ambient. Els 118 dobles haploides de la població 'Nure x Tremois' es genotiparon amb marcadors DaRT (Diversity Array Technology), després un set de 15 marcadors CAPS I SCCP per a gens candidats involucrats en la regulació de les fases fenológicas van ser afegits al mapa de lligament construït amb els marcadors DaRT. Les dades van ser utilitzats per a fer una anàlisi de QTL amb procediment 'Composite Interval Mapping' para cada combinació ambienti/ caràcter. Es van trobar diversos QTLs per rendiment i data d'espigolat i components del rendiment. Els QTL mes freqüents trobats per rendiment i data de floració i components del rendiment estan localitzats en els cromosomes 1H (3 camps), 2H (8 camps) i 5H (5 camps) coincidents respectivament amb HvFT3 locus, eam6/Eps-2 (earliness per se) locus i amb el locus de vernalización Vrn-H1. Una ulterior anàlisi de QTL feta amb el mètode "Multi Environment Trial" ha revelat que els QTL localitzats en el locus eam6/Eps-2 (cromosoma 2H) i Vrn-H1 (cromosoma 5H) són comunes per rendiment i data de floració en els 18 camps d'assaig. Per això utilitzem tots el dades ambientals col·leccionades durant tot el cicle del cultiu per a investigar la sensibilitat de dites QTL a les co-variables ambientals. La majoria de les associacions oposades estan relacionades amb temperatures i variables relacionades amb aquestes. Eam6/Eps-2 mostra una interacció de tipus quantitatiu amb aquestes variables mentre Vrn-H1 mostra una interacció de tipus qualitatiu amb aquestes variables. Les 185 varietats assajades van ser genotipadas amb 185 SNPs i fenotipadas per resistència a fred en dos assajos uneixo a Espanya i altre a Itàlia. El primer assaig va ser caracteritzat per un hivern excepcionalment fred, mentre el d'Itàlia ha estat utilitzat en passat per testar resistència a fred a causa de els hiverns rígids que solen registrar-se en aquesta localitat. Les dades van ser utilitzats per a portar a terme la analisis GWAS "Genome Wide Association Analysis" . Els resultats van permetre identificar 13 regions genomicas involucrades en la resistència a frio. Entre

elles tres regions coincideixen amb loci ja mapeados i coneguts per ser involucrats en la resposta a frio en los cromosomes 2HL, 4HL i 5HL.

Index of figures

Index of tables

Chapter 1
Introduction

1.1 Barley (Hordeum vulgare L.) a brief overview.

1.1.1 Origin and Domestication

Figure 1.1. barley growing areas (green) and region of origin (red). (http://frontiers-of-anthropology.blogspot.it)

Barley (Hordeum vulgare L.) was one of the first plants domesticated together with Einkorn and Emmer wheat, lentils, peas and chickpeas, bitter vetches and flax. Barley domestication occurred in the Fertile Crescent Region about 10.000 years ago (Zohary and Hopf 1993; Lev-Yadun et al. 2000) from the wild ancestor Hordeum vulgare L. spp. spontaneum C. Koch (von Bothmer et al. 2003). This ancestor of barley still grows in the Fertile Crescent Region in countries such as Israel, Jordan, Iran, Iraq and Turkey (Harlan and Zohary 1968). Domestication can be defined as the transitional phase from preferential reaping of wild plants to purposely performed cultivation (Fishbeck 2002). During the process of domestication, barley has gradually accumulated traits that facilitated agricultural production, the first trait domesticated being the increased seed size followed by non-shattering rachis (Purugganan and Fuller 2009). Two variant genes control whether or not the ear shatters: genes Btr1 and Btr2 tightly linked on chromosome 3H (Takahashi and Hayashi, 1964). A recessive mutation in either gene locus leads to the domesticated condition (non-shattering). The early form of cultivated barley was certainly a two-rowed type with hulled kernels and grown in the Near East about 8000 B.C.

Zohary and Hopf (1993) reported that the four major types of cultivated barley, namely, hulled versus naked kernels and two-rowed versus six-rowed barleys, appeared in the Fertile Crescent region during the early phases of cultivation in the Old Word.
Hulled and Naked barley have different use: hulled barley is mainly used for animal feeding and brewing malts, while the naked one can be used as human food. Naked barley is largely diffused in East Asian countries (Bothmer et al., 2003). Vavilov (1926), due to its low frequency in Western Europe, considered southern Asia to be a centre of origin for naked barley. However, subsequent studies reported that was grown in Anatolia (Turkey) and in Northern Europe in ancient years (Helbaek 1969). The hulled vs. naked caryopsis character is a key trait to follow the origin and domestication process of barley (Harlan 1995; Salamini et al. 2002). Experimental and archaeological evidences suggest that naked barley appeared after domestication of hulled barley, in Southern Iran about by 6500 B.C. (Zohary and Hopf 2000; Taketa et al. 2004). Hulled barley has caryopses with the husk cemented to the grain, while naked barley grows with easily separable husks. This is under the control of a single recessive gene â nudâ on chromosome 7H, recently cloned by Taketa et al. (2008). The gene underlying the recessive mutation is a defective allele of an ethylene response factor (ERF) family transcription factor. A deletion in the sequence of 17 Kb was found only in naked barley whereas the deletion was found in none of hulled barley cultivars used in the study. The transcription factor protein might activate production of special lipids in the testa, thus produced lipids are transported through the pericarp layers and are secreted out of the pericarp epidermids. In naked barley, the lack of the lipid layer probably blocks adhesion, thereby rendering free-threshing caryopses.
Diffusion of six-rowed barleys is probably the result of selective pressure due to the major and stable production, for yield of six-rowed barleys can be up to three times higher than two-rowed barley (Fujimara et al. 2006). The first archaeological evidence of six-rowed barley was found in Ali Kosh (Iran) where, with remains of two-rowed barley a rchaeologists found sporadic presence of six-rowed barley these remains were dated as 9000 B.P. (Helbaek 1969). Wild barley is two-rowed, and this suggests that the two-rowed spike is the ancestral form, which was changed to a six-rowed spike in cultivated barley by mutation during domestication. The lateral spikelets in two-rowed wild and

cultivated barley are sterile, while are fertile in the six-rowed cultivated barley. The Six-rowed spike 1 (vrs1) recessive gene is observed in all six-rowed cultivars and is located on chromosome 2HL. Wild barleys and two-rowed cultivated barleys have dominant alleles for Vrs1. The Vrs1 gene has been recently cloned by Komatsuda et al. (2007). Vr s1 encodes a transcription factor that includes a homeodomain with a closely linked leucine zipper motif. Its expression is localized in the lateral-spikelet primordia of immature spikes, suggesting that the Vrs1 protein suppresses development of the lateral rows. Loss of function results in into fully developed fertile spikelets in the six-rowed phenotype. Other loci responsible in quantitative variation in the size and fertility of the lateral spikelets have also been reported, particularly in progenies of two- by six-rowed crosses (Lundqvist and Lundqvist 1989). This variation seems to be regulated by the INT-C, ortholog of maize domestication gene THEOSINTE BRANCHED 1 (TB1) and located in chromosome 4H (Ramsay et al. 2011). From these four types of ancestral barley (hulled versus naked kernels and two-rowed versus six-rowed spike), thanks to mutations and hybridization with both cultivated barley and accompanying weedy form of Hordeum spontaneum, a multitude of new barley genotypes differing in morphological and physiological traits were created and then subjected to natural selection. With the expansion of agriculture and the subsequent diffusion out of the Fertile Crescent Region, in addition to natural selection, barley was subjected to human selection and consequently the restriction of population size and the mass selection contributes to introduce genetic drift in the different growing areas. Archaeological evidence showed that Barley geographical expansion, together with Einkorn and Emmer wheat and often weedy forms of wild barley, started from Fertile Crescent Region to Aegean region and subsequently to the eastern part of the Mediterranean basin, to reach countries of the Caucasian and Trans-Caucasian regions. Further expansion to east from the Fertile Crescent then proceeded to the highlands of Indian subcontinent and Tibet (Zohary and Hopf, 1993; Lisitsina et al. 1984; Costantini 1984). Cultivated barleys domesticated in the west and east regions of the Fertile Crescent carry different mutations associated to brittle rachis. Western lines carry brittle-1 whereas Eastern lines carry Brittle-2 suggesting independent domestication events. For this reason, Central Asia have been proposed as secondary center of origin or domestication of barley (Saisho and

Purugganan 2007) this due to the presence of a broad genetic variability and for clues of presence of wild ancestors. Recently Morrell and Clegg (2012) reported that barley was firstly domesticated in the Fertile Crescent Area and then between 1500 and 3000 km farther east in Central Asia. Cultivated forms of barley are also found in across Egypt and the Ethiopian/Eritrean regions, were continuous cultivation practices developed a secondary center of genetic diversity (Lakev et al. 1997). Diffusion in the western part of Mediterranean basin happened later than in its eastern part, although Morocco has been proposed by Molina-Cano et al. (1999) as secondary center of domestication, independently from the Fertile Crescent Region sources. In 1980 Molina-Cano and Conde discovered in Morocco Hordeum spontaneum C.Koch the wild ancestor of cultivated barley and 25 additional populations, identified later as Hordeum spontaneum (F. Kh. Bakhtheyev, personal communication; J. R. Harlan, personal communication).

Various studies based on agromorphological traits, RFLPs (Restriction Fragment Length Polymorphism), chloroplast DNA SSR (Simple Sequence Repeat) (Molina-Cano 1987, 1999, 2005) and a more recent based on 1536 SNPs (Single Nucleotide Polimorfism) to characterize 107 accession of wild and cultivated barley from Western Mediterranean, Fertile Crescent, Ethiopia and Tibet; showed clear genetic differences between Moroccan H. spontaneum both wild and cultivated barley with other non-western Mediterranean origins. These results favored the hypothesis of polyphyletic origin of cultivated barley, with additional centers of origin in Western Mediterranean and Ethiopia, apart from the widely accepted Near Eastern centre (Igartua et al. 2012). Despite the location of secondary domestication centers the most important thing arising from all these studies, for researcher and breeders, is the adaptability of barley to an incredibly broad spectrum of environments, starting from Mediterranean and semi-arid ones. The understanding of genetic and physiologic mechanisms that regulate adaptability of barley across environments is therefore one of the most important aims of geneticists and breeders to maximize the barley yield in stress prone environments.

1.1.2 Barley diffusion and economic importance

Amongst the major cereal crops, following the much larger cultivation area and overall crop yield of wheat, rice and maize, barley occupied the fourth position (FAOstat 2008). This is principally due to the great adaptability to very different environmental conditions, comprising extreme latitudes and altitudes (Ullrich 2002). The total world production in 2008 was 136 millions of tons (FAOstat www.faostat.fao.org). Europe is in the leading position with 62% of the worldwide production, followed by Asia (15%) and North America (14%), while the fraction of barley production in other regions is less than 5%. Average yield range from 0.78 t/ha to 2.8 t/ha, with a global average of 2,3 t/ha and with the higher average yield in Europe (Kim and Dale 2004). In general great fluctuation in yield is observed, data from 2006 (www.faostat.fao.org) showed that barley production ranged from an average yield of 5.9 t/ha in Western Europe (which show a nearly ideal climate for barley with relatively high inputs of fertilizer and pesticides) to 1.04 t/ha observed in Bolivia and Peru, where barley is growth in high altitude. This due to a large number of factors such as adaptation to climate, soil, biotic and abiotic factors including the level of farmer inputs of cultivar, fertilizers, pesticides, and irrigation. Data from FAO (2010) show that the most important barley production region in Europe is Germany (10. 4 million tons) followed by France (10.1 million tons), and Ukraine (8.5 million tons). Spain was the second European country in 2007 for grain production (11.9 million tons), and the fifth in 2008 with 12 million tons with a production similar to France, Germany, and Ukraine. Considering Spain, during year 2008 barley was the main crop with 20% of the entire cultivated crop area, which corresponds to 3.5 million hectares (notably, 51. 6% of the cereal growing area in Spain (MARM 2009)). In recent years, barley cultivated area and production in Spain has decreased to 2.5 million hectares with an average grain yield for the last five years of 3.1 t/ha, taking total production to 7.8 million tons, after Ukraine (MARM 2012). Barley principal use in Spain is for animal feed or forage. Barley was firstly used for human nutrition during the rise of Old Word agriculture, but with the development of bakery, the preference for wheat flour soon reduced the use of barley for human consumption, mainly for the lack of gluten among the storage proteins of barley kernels (Fischbeck 2002). Furthermore barley has been used for since many centuries as a food source related to soup and porridge dishes in Central and Southwest Asia, Africa and Ethiopia (von Bothmer et al 2003), while in Tibet

is still the main source of cereal-based starch food. Barley was also used, since the early phases of ancient agriculture, for the production of alcoholic drinks and became the major source of raw material for brewing in Europe during medieval times. Nowadays 18 million tons are used yearly in the brewing industry (Fischbeck 2002). In recent years barley also have been used for bio-ethanol production. About 3.4% of barley in the world, 3:7 Tg (Teragrams), is lost as waste. As reported by Kim and Dale (2004) if wasted barley could be fully utilized to produce bio-ethanol, then 1:5 GL (Gigaliters) of bio-ethanol could be produced globally, replacing 1:1 GL of gasoline if ethanol is used as fuel.

1.1.3 Barley: a model plant for study stress response mechanisms in Triticeae

Barley (Hordeum vulgare L.) is cultivated in a wide geographic area, from Northern European countries just below the polar circle, to semi deserted areas and, in general, in less favorable geographic areas where other cereals survive with difficulty. The genetic base of abiotic stress response is very difficult to study due to the intrinsic complexity of various types of stresses such as cold, heat, drought and salt stress that often occur simultaneously or in different combinations. Different response of genotypes depends on the genetic make-up of the line, by the phenological phase in which the stress occurs, and by the length and severity of the stress episodes. Often multiple abiotic stresses greatly affects crop yield in many growing areas, with most common ones low temperature and drought stress, but also water-logging in East Asian regions. In less favorable areas barley, with low energy input shows higher yield than wheat or other cereals (Stanca 1989). The good adaptability of barley to large climate differences between different growing areas prove that in the barley gene pool there are numerous genes for adaptability and resistance. Among cereals, barley can be considered as a good genetic model to study plant response to adverse conditions (Tondelli et al. 2006). Its inbreeding behavior and diploidy make the genetic studies simpler to perform than in other Triticeae family members such as polyploid wheat; or also respect open-pollinated crops like maize; both major cereal crops in economic importance and global production. Barley is prepared to cope with more numerous abiotic stress than maize that is not resistant to cold. Barley is also more resistant to drought and cold stress than rice, a

model plant for genomic studies in monocots. By the way it is demonstrated that is the most salt tolerant crop (Rawson et al. 1988; Forster et al. 1990; Munns et al. 200; Walia et al. 2007). Barley shows broad adaptability, the availability of wide range of genetic stocks and the extended colinearity â conservation of gene content and order - with other Triticeae members are additional advantages as a model (Hayes et al. 2003).

1.1.4 Taxonomy

Barley taxonomy proposed by Integrated Taxonomic Information System (ITIS http://www.itis.gov/) is the following:

Kingdom: Plantae´

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Subkingdom: Tracheobionta´

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Superdivision: Spermatophyta

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Division: Magnoliophyta

Class: Liliopsida´

Subclass: Commelinidae´

´

Order: Cyperales´

´

Family: Poaceae´

´

Genus: Hordeum´

´

Species: vulgare

Subspecies: Hordeum vulgare L. subsp. Spontaneum C.´ Koch

Hordeum vulgare L. subsp. Vulgare

The Hordeum subspecies Hordeum vulgare L. subsp. Vulgare Hordeum vulgare L. subsp. Spontaneum C.´ Koch refer to both cultivated and wild forms of barley. The Hordeum genus contains 14 species more: Hordeum arizonicum, Hordeum bogdanii, Hordeum brachyantherum, Hordeum brevisubulatum, Hordeum bulbosum, Hordeum comosum, Hordeum depressum, Hordeum intercedens, Hordeum jubatum, Hordeum marinum, Hordeum murinum, Hordeum parodii, Hordeum pusillum, Hordeum secalinum. Although the genus Hordeum contains 16 species and 26 taxa in total here we reported only the most important species and sub-species of cultivated barley (GRAMENE, www.gramene.org).

1.1.5 Citogenetics

Barley is autogamous, annual and true diploid (2n=2x=14) with large chromosomes (6-8 ´µm). Both cultivated and wild forms of barley have seven pairs of distinct chromosomes, originally designated using the Arabic numbers 1-7 (Nilan 1974; Ramage 1985). A new nomenclature of barley chromosomes based on morphological, biochemical and molecular studies was adopted, and the barley chromosomes were named according to their homeologous relationships with other members of the Triticeae tribe (Linde-Laursen 1997). Chromosomes 1, 2, 3, 4, 5, 6 and 7 were thus renamed respectively as 7H, 2H, 3H, 4H, 1H, 6H and 5H. The haploid genome size of barley is 5.100 Mb with 32.000 estimated genes (Arumuganathan and Earle 1991; Mayer et al. 2011), and at present day 22500 complete gene sequences have been identified using new generation sequencing technology (Matsumoto et al. 2011). Barley genome size (~5.100 Mb) is very large if compared to other model species such as Arabidopsis thaliana (~110 Mb), Oryza sativa (~430 Mb), Brachypodium distachyon (~270 Mb) and Sorghum bicolor (~730 Mb), this due to the presence of large repetitive DNA regions. Despite large genome, the number of estimated barley genes is similar to other Triticeae memb

ers, and quite similar to other model plants such as Oryza sativa (41.000; Jung et al. 2008), Brachypodium distachyon (30.000; The international Brachypodium Initiative 2010), Sorghum bicolour (34.500; Patterson et al. 2009). Extensive presence of higly repetitive DNA is delaying barley genome assembly. However, despite the difficulties expected, the complete barley genome sequence â or at least the physical order of barley gene space - will be available in a near future(International Barley Sequencing Consortium (IBSC); http://barleygenome.org; Shulte et al. 2009)

1.2. Mechanisms of the molecular response to abiotic stress

Acclimation can be defined as the plant response to environmental stress conditions at cellular level. It involves a large number of molecular and physiological processes, controlled by the interaction between several regulatory mechanisms (Bagnaresi et al. 2004). Many genes involved in stress response are not constitutively expressed, this means that a plant, in order to acquire the maximum degree of stress tolerance, needs a period of acclimation (e.g. hardening in the case of cold tolerance). After acclimation plants show more tolerance to the negative effects of stress. Response to stress is mediated by different sets of genes with redundant and additive effects that may interact. There are various metabolic pathways that may concur or cross to respond to stress induced by complex climatic and pedological conditions and their interactions.

Figure 1.2. General overview of various events that lead to the activation of effector genes of abiotic stress response (modified from Mastrangelo et al. 2005).

1.2.1 Perception of environmental variation and signal transduction.

The mechanisms that regulate the plant perception of environmental signals are not well known. However, although the nature of primary sensors is still unknown they may be located on the cell wall like other receptors do. Alterations of characteristic membrane fluidity and alteration of cito-skeleton have been observed during exposition to low temperature, in this way, both may actuate as sensors of environmental changes (Orvar et al, 2000; Sangwan et al. 2001; Wang y Nick, 2001). The chloroplasts also seem to be involved in perception of external stimuli. The absorbed light is transformed in chemical energy (ATP y NADPH) that must be continuously compensated with metabolic process, which uses ATP and NADPH. Otherwise this energy is dispersed as heat. Cold and drought stress do not affect plant irradiation but can affect the plantâ s ability to use the energy absorbed by inhibiting various metabolic pathways and producing an excess of reducing power, such as NADPH, in the photosynthetic apparatus. This excess of reducing power may act as a redox (reduction-oxidation) signal activating physiological, morphological and molecular adaptations (Gray et al., 1997; Huner et al., 1996). Abiotic stress promotes a cascade of events of different chemical nature from cellular wall to the nucleus, where transcription of stress response-specific genes is induced. Between sensing and stress response, essential is the phase of signal transduction, in which is important the protein phosphorilation activity of protein kinases. Protein kinases play thus a very important role in the response to abiotic stress, specially the MAPK (Mitogen Activated Protein Kinase) class. In Arabidopsis many MAPK genes have been identified as involved in abiotic stress response, and phosforylated proteins that regulate MAPK activity (Ligterink y Hirt, 2001). The increase of Ca+ ion concentration in the citosol promotes the transduction of stress signals mediated by other proteins such as calmodulin, calcium dependent protein kinases (CDPK) and calcium regulated phosfatases. In fact, in Arabidopsis, an increase of Ca+ ion concentration is usually observed when plants are exposed to salinity, drought or to a fast decrease of temperature (Sandres et al., 1999; Knight 2000, Plieth et al., 1999). Genes that encode for proteins of the CDPK family (induced by salt and drought stress) have been identified by Urao et al. (2004). Abscissic acid (ABA) also plays a role in abiotic stress response (Palva and Heino 1998), by promoting mechanisms that avoid plant dehydration such as decreasing plant transpiration through stomatal closure. In

Arabidopsis, a stronger response to drought stress is induced by ABA over-expression. Iuchi et al. (2001) reported reduced stomatal conductivity, reduced loss of water from leaves and in general a better resistance to water deficit in plants over-expressing the 9-cis-epoxycarotenoid dioxygenase (NCED) a key enzyme in ABA biosynthesis. AtNCED3 induces increase of endogenous ABA levels that promote expression of drought and soil salinty responsive genes. Stomatal closure reduces CO2 availability and consequently a reduced use of NADP, produced by photosynthesis. This entails an important increase of cellular O2- that is fastly transformed to hydrogen peroxide (H2O2). An increase of H2O2 has been observed as consequence of drought and salt stress and temperature changes (Mittler 2002). The production of reactive oxygen species (ROS) with high cito-toxic effect and alteration of macromolecules, such as polyunsaturated fatty acids in membrane lipids, proteins and DNA have been observed (Mittler 1992). Low levels of ROS generated during the first steps of damage may act as stress signal transductors to activate mechanisms of enzymatic protection for both ROS molecules and the stress. High ROS concentration induces cell programmed death (Vac ca et al. 2004; Mittler 2002).

1.2.2. Role of transcription factors

Transcription factors of a number of classes were identified as key nodes, in the middle between sensing/signal transduction and activation of protecting proteins, essential to acquire tolerance and adaptation. Transcription in eukaryotes is a very complex mechanism, where transcription is mediated by many proteins called transcription factors (Lewin 1999). Transcription factors (TFs) are sequence-specific DNA-binding proteins that bind to specific DNA sequences and act as a switch of molecular events by controlling the flow (or transcription) of genetic information from specific genes (Riechmann et al. 2000). Using different combinations of TFs, which acts as control module, the cells can control gene expression with high precision and in a very specific way (Zawel and Reinberg, 1993; Harrison and Sauer, 1994). Most important TFs are inducible, because they fine-regulate transcription and allow to modulate gene expression in response to changing plant needs. TFs are syntetized or activated only in response to certain stimuli, during a specific developmental phase or in specific tissues,

thus promoting the transcription of effector genes (such as stress response genes). TFs can be syntetized only in certain types of cells, or may be activated by modification such as phosphorylation or proteolysis or by specific binding proteins and through dimers formation (Lewin 1999).

1.2.3. Abscissic acid and transcription factors

Abiotic stress response is a very complex and finely regulated mechanism that comprises different and apparently independent pathways. The plant hormone abscisic acid (ABA) mediates a variety of physiological processes, including the response to drought and salt stress. ABA is produced under water deficit conditions, which causes stomata closure and tolerance to drought and salt stress (reviewed by Bray, 1997; Busk and Pages, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000). Analysis of the promoter sequences from first genes cloned induced under drought and cold stress allowed to identify different regulation pathways, that could be divided in ABA-dependent and ABA-independent (Shinozaky et al. 2000). Many genes involved in abiotic stress response are activated by exogenous treatments with abscissic acid (ABA), suggesting that ABA plays an important role in stress response. Abiotic stress response is a very complex and finely regulated mechanism that comprises different and apparently independent pathways. Analysis of the promoter sequences from first genes cloned induced under drought and cold stress allowed to identify different regulation pathways, that could be divided in ABA-dependent and ABA-independent (Shinozaky et al. 2000). In general the major part response to drought is ABA-dependent, while response to cold is basically ABA-independent. However, effects of both stresses are inter-related cold temperatures as well drought stress may reduce availability of liquid water thus generating a dehydration response, as showed figure 1.3. Genes involved in stress response function not only in stress tolerance but also in the regulation of gene expression and signal transduction of the response (Bray, 1997; Hasegawa et al. 2000). The ABA dependent pathway promotes the expression of those genes that carry in their promoter the ABRE â ABA responsive element -5â -PyACGTGGC-3â sequence. ABA mediated activation of genes is also induced by others cis-elements also known as Coupling

Elements (CE). Shen and HO (1995) reported that the barley ABA responsive gene HvA 22 carry on its promoter an ABRE3 (GCCACGTACA) and a CE1 (TGCCACCGG). Thes e two sequences are required for high-leve1 ABA induction, and replacement of either of these sequences abolishes ABA responsiveness. These cis-elements carry the binding site for other TFs that may confer an ABA dependent tissue-specific expression based on the state of plant development. Another pathway requires the sinthesys of TFs, such as the MYC/MYB to enhance the expression of genes induced by drought and high soil salinity (Urao et al. 2003). The drought responsive gene Rd22 (figure 1.3) carry on its promoter two bindings sites MYC and MYB, that acts as cis-elements in the expression of Rd22 under drought stress. Transegenic arabidopsis overexpressing both AtMYC2 and AtMYB2 transcription factors, which interact specifically with MYC and MYB recognition sites, showed ABA hypersensitivity and increased ABA-induced expression of Rd22. These results indicate that both AtMYC2 and AtMYB2 proteins play important roles as transcription factors in ABA-regulated gene expression under drought and salt stress (Abe et al. 2002). Studies based on ABA deficient mutants or ABA insensitive mutants, firstly demonstrated that several genes are activated under cold and drought in an ABA-independent way. However, their expression can also be induced by treatment with exogenous ABA (Ingram and Bartels, 1996; Thomashow, 2000). ABA- independent genes are known to carry the DRE (Dehydratation Responsive Elements; Shinozaky and Yamaguchy-Shinozaky 1996) and LTRE (Low Temperature Responsive Elements) or â C-repeatâ (CRT; Baker et al. 1994; Medina et al. 1999) elements in their promoter. Subsequent studies allowed to identify TFs that directly interact with the DRE sequences in response to water deficit called â DRE-binding elementsâ (DREB) and those who legate the CRT motif during exposition to low temperatures called â C-Repeat Binding Factorsâ or CBF (Gilmour et al. 1998). Both factors recognize the same cis-element and seem to be very similar in their sequences but are characterized by different expression patterns; in this way nomenclature was changed in DREB1 (induced by low temperatures, CBFs) and DREB2 induced by water deficit (Liu et al. 1998). Constitutive over-expression of CBF1/ DREB1 and CBF3/DREB1a in transgenic Arabidopsis, demonstrated that both induced the expression of many genes that contain DRE/CRT elements in absence of low

temperatures. Transformed plants were more tolerant to both drought and frost stress. This confirmed the role of DRE/CRT regulatory element in genes involved in drought and cold stress response (Jaglo-Ottensen et al. 1998; Kasuga et al. 1999). â DREB/ CBF likeâ elements have been identified in many species like barley, wheat and tomato, suggesting a high level of conservation of stress response pathways also in evolutionary divergent species (Xue et al. 2002; Choi et al. 2002; Jaglo et al. 2001; Skinner et al. 2005).

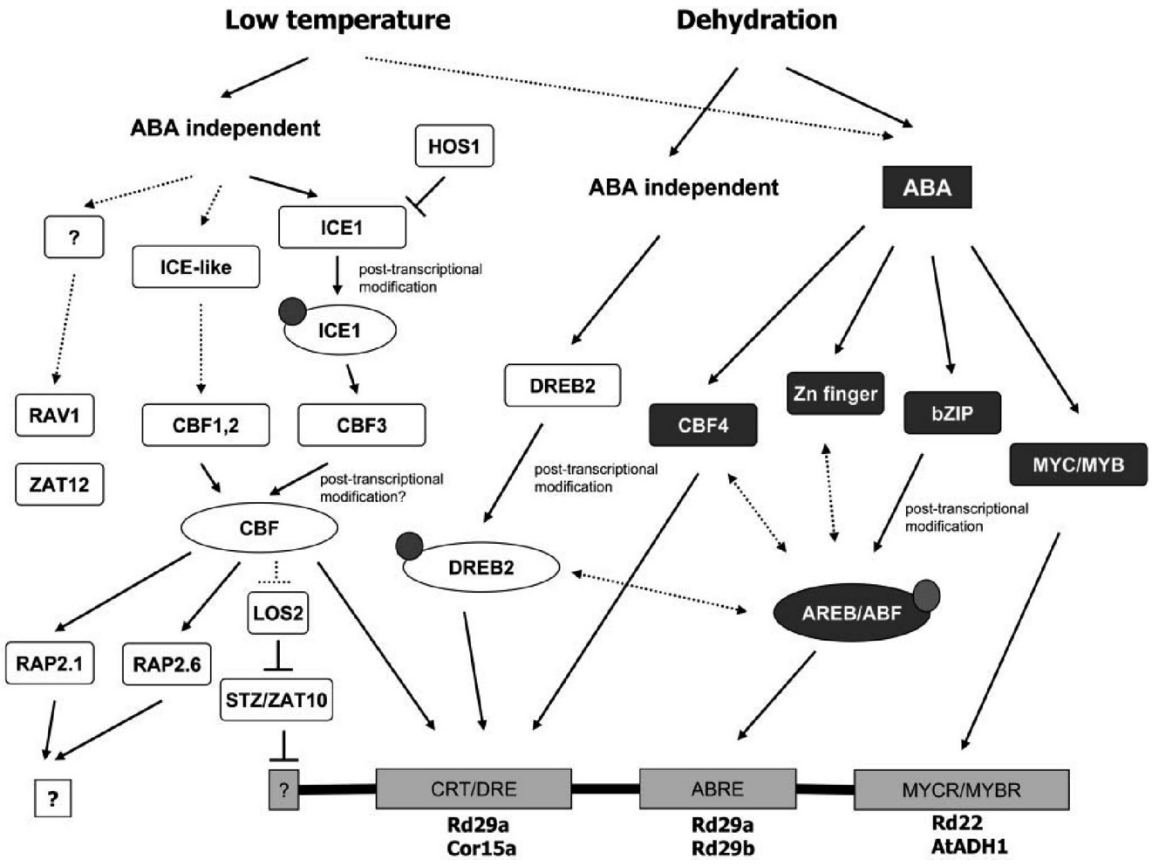

Figure 1.3. Schematic representation of cold and drought stress pathways and the different binding sites of transcription factors (Zhang et al. 2004).

1.2.4. Changes in gene expression and modifications of the cellular metabolism in response to stress

â Stress specificâ genes (such as Rd29a, Cor15a or Rd22 from the previous scheme)

can be considered the final target of signal transduction mechanisms leading to cell response to stress. The expression of this set of genes establishes the plants degree of stress tolerance. Some of these genes are activated only during the occurrence of a specific stress, while others are expressed in response to various types of stress. Because of the molecular response triggered by the stress, under stress conditions a crowd of enzymes are regulated in different way respect normal condition, to allow plants adaptation to unfavorable conditions. This change in expression patterns of a cascade of enzymes often results in profound changes at the basic metabolic level and structural make-up of the cell. These metabolic changes depend on the type and intensity of the stress occurred. In the case of low temperature a higher synthesis of ribosomal proteins, and chloroplast proteins is observed. This suggests that the plants, in order to respond to cold stress, need a radical re-organization of the protein synthesis complex (Baldi et al. 2001). Drought stress induces protein denaturation, thus enzymes that repair damaged cellular proteins and structures are needed to mitigate consequent negative effects, thus converting the damaged residues of proteins in residues suitable for metabolic needs. Enzymes like L-isoaspertil methiltranferase are able to repair cellular protein damage by increasing conversion of damaged L-isoaspartil residues in viable residues for methabolic needs. (Mudgett and Clark 1996). Cellular walls are the first cellular structures damaged when plants are exposed to low temperature. Therefore we can observe an increased crio-stability in response to cold temperatures exposure mainly due to changes in the phospholipidic composition of the plasmatic membrane (Steponkus and Webb, 1992). Some Arabidopis studies have confirmed the importance of poly-unsaturated fatty acid in cold resistance. Plants with a deficit in the production of this class of compounds cannot survive to prolonged exposition to low temperatures. Furthermore mutants that produces poly-unsaturated fatty acids only in the chloroplast, showed altered development of chloroplast under cold stress; this suggest that poly-unsaturated fatty acids may play a role in cold stress tolerance (Hugly and Sommerville, 1992). Plastids are also damaged by abiotic stress, this due to oxidation caused by reactive oxygen species such as O2 and H2O2, which causes alterations of reducing power. In order to mitigate these effects plants synthesized proteins that preserve the structure of plastids (Monte et al. 1999). Enzymes that reduce

the levels of ROS were also observed during exposition to stress. Plant species or particular accessions within a species with higher drought tolerance to stress produce higher levels of these enzymes. Plants under stress produce a class of antioxidant molecules called â scavengersâ (ascorbate, reduced glutathione, tocofenol, tirodisine and carotenoids (Millar, 2002). Furthermore under stress condition accumulation of various low molecular weights compounds has been observed in cytoplasm: amminoacids, sugars and poly-oils, called compatible solutes. These compounds usually are accumulated in high concentrations, without damage cells, to reduce the hydric potential of cells. Accumulation of high quantity of these compounds protects the original protein configuration. In wheat, a strong correlation between genes involved in proline accumulation and drought stress response has been found (Mastrangelo et al. 2000). Furthermore the accumulation of compatible solutes such as betaines, proline sugars, alcohols, in cells during stress seems to be one of most promising approach to obtain more resistant genotypes for drought and salt stress; but unfortunately not for cold stress. Dehydrins are another class of proteins involved in cold and drought stress response. Dehydrins are highly hydrophilic and belong to the LEA (Late Embryogenesis Abundant) protein family. LEA proteins are expressed during exposition to stress and cold acclimation and have an osmo-protective function. Accumulation of dehydrins near the cell wall has been shown during exposition to low temperatures suggesting a possible crio-protective role (Danyluk et al. 1998). â Antifreezing proteinsâ (AFP) bind crystal-ice during formation, modifying the crystal structure and thus blocking aggregation and consequently the growth. Some authors highlighted that proteins with similar properties have been found in some artic fishes (Urrutia et al. 1992; Griffith et al. 1997). AFPs have been found in lymph of more than 20 species monocots and dicots (Griffith et al. 1997). Effects of AFP during cold acclimation have been demonstrated in barley, rice and rape but not in cold sensitive varieties of maize and tobacco (Yeh et al. 2000; Tomczak et al. 2002; Antikainen and Marilyn 2006).

1.3. Breeding for abiotic stress tolerance and environmental adaptation

Plant responses to abiotic stresses such as drought, cold (chilling and freezing) and soil salinity are major factors limiting crop genetic yield potential, thus regulating their

geographical distribution and has critical implications for agriculture. Genetic adaptation implies the shaping of gene pools in response to environmental challenges due to climate and soil (Cattivelli et al. 2002). Plants, in order to adapt to environmental changes such as temperatures, rainfall, and nutrient availability have developed the ability to modify their metabolism. A very complex network in which many pathways are involved in monitoring environmental changes, signaling and transduce environmental variation allow to activate the adequate cell response to facethese environmental constraints. The capacity of barley to survive in hostile and stress prone environments depends on these sophisticated mechanisms. The genetic variability plays a central role in adaptation to environmental stresses and in supporting the spread of various barley genotypes to extreme climate condition (Cattivelli et al 1994). Understanding how genotypes interact with the photo-thermal environmental cues driving crop adaptation is a difficult task. The main reasons being (1) the unpredictability, in terms of timing, intensity and duration of abiotic stresses, (2) the highly polygenic nature of the traits controlled by many genes with small additive effects and (3) the strong genotype-by-environment interaction (Romagosa et al. 1996; Voltas et al. 1999; Rizza et al. 2011). At present days several loci responsible of barley adaptation and stress response have been identified and classified as loci controlling heading date, growth habit, frost resistance, drought tolerance and salt tolerance (Cattivelli et al. 2003). These genetic lo ci under the control of traits that allow plants to tailor their life cycle to the surrounding environment have been object of selection by breeders to improve crops grain yield.

1.3.1. Loci controlling plant adaptation

An adaptive trait is an aspect of the developmental pattern of the organism, which enables or enhances the probability of that organism surviving and reproducing (Dobzhansky 1956). Some adaptive traits are key traits for local adaptation very specific to certain environments such as boron tolerance Jefferies et al. 1999) or salt tolerance Mano and Takeda 1997). Among the most important traits controlling barley adaptation to different growing seasons in different climatic regions are heading date and growth habit. These two traits are strongly correlated. The first of them, heading date, is considered a crucial aspect of plants and crops adaptation as its genetic regulation ensures that flowering occurs in the optimal conditions for pollination and seed

development (Karsai et al. 2008). Heading date shows a strong interaction with the environment and is the final result of a number of interacting characters including vernalization requirement, photoperiod sensitivity and earliness per se (Karsai el al. 1997). Vernalization response is the induction of flowering by exposure of plants to extended periods of low temperature. Vernalization occur during winter when plants are exposed to temperatures between 0â and 10â promote inflorescence initiation, whic h is the first step of transition to vegetative to reproductive phase (Takahashy and Yasuda 1971; Flood and Halloran 1984). Usually crops with winter habit requires several weeks of low temperature exposition before advancing to reproductive phase. Vernalization response is strongly affected by photoperiod (Takahashi and Yasuda 1972). Photoperiod sensitivity refers to the ability of the plant to synchronize flowering according to short days (winter in European latitudes) or long days (summer in European latitudes). Genes that controls photoperiod affected the timing of terminal spikelet production and stem elongation and these effects interacts with sowing date (Snape et al. 2001). Earliness per se explains differences in heading date when vernalization and photoperiod requirements are fully satisfied (Appendino and Slafer 2003). Mechanisms, which control earliness per se, are not very well understood and furthermore the loci under the control of such traits have been so far elusive to genetic dissection by classical genetic mapping procedures. Vernalization, photoperiod and earl iness per se also shows other effects on factors that controls plant growth and development. In the early steps of domestication two major types of barley arose associated to their respective growth habit: winter and spring barleys. Growth habit is controlled by the same genetic factors that regulate vernalization requirement and photoperiod sensitivity (Cattivelli et al. 2002). In addition, winter barleys are also carrying genetic factors for adaptation to the cold temperatures occurring during winter when the crop is grown. After barley domestication in the Middle Crescent, spring growth habit genotypes expanded to higher latitudes and to mountainous regions to avoid the damage caused by very cold winters, and into regions where the air temperature is too high to induce vernalization (Kole et al. 2006). By having a crop already established when the good weather conditions arrive, in general, winter barleys yield more than spring types. However, spring barley is more prevalent than winter habit

barley in cold-to-temperate growing areas, in part due to the superior malting quality of those cultivars. Vernalization sensitivity typical from winter barley is the necessary induction of flowering by exposition of plants to low temperatures. As reviewed by Michaels and Amasino (2000) the range of effective temperatures at which cold promotes flowering ranges from 1´° to 7´° C, and in some cases vernalization temperature can be as low as -6´° C. Barley varieties are classified as winter, spring and facultative on the base of the genetic make-up of their vernalization requirement and photoperiod sensivity. Winter barley highly sensitive to vernalization but may vary in ph otoperiod sensivity and is considered as winterhardy (tolerant to cold temperatures); spring barley does not respond to vernalization requirement, may be insensible to long day photoperiod and shows no or very low tolerance to low temperatures. Facultative varieties are vernalization insensitive and may be short photoperiod sensitive, so that they do not flower in the short day conditions experienced in winter. Winter varieties are autumn sowing and the vernalization requirement avoids spike development during the winter providing protection to vegetative tissues against cold and frost and minimizing future negative effects on yield potential. Vernalization requirement is related to cold tolerance, and when the vernalization requirement is fully satisfied the transcription of genes related with cold resistance decrease (Stockinger et al. 2007). Spring varieties in general are low tolerant to cold exposition. Early spring frost that may occur, in certain latitudes, during reproductive phase and may damage the anthers, grain development and can produce spike abortion (Reinheimer et al. 2004; Chen et al. 2009). The use of facultative varieties may thus represent an optimal strategy to postpone the vegetative to reproductive phase transition until the danger of frost occurrence is reduced (von zitz ewitz 2011).

1.3.2. Vernalization genes

Growth habit in barley and wheat is under the control of three vernalization genes known as: VRN1, VRN2 and VRN3 located respectively on chromosome 5H, 4H and 7H. The first model to explain genetic control of growth habit was proposed by Takahashi and Yasuda (1971), where the vernalization genes were originally called Sh (VRN2), Sh 2 (VRN1) and Sh3 (VRN3). VRN-H1 is HvBM5A (Danyluk et al. 2003; Trevaskis et al.

2003; Yan et al 2003), a transcription factor that belongs to â MADS boxâ family, which promotes the transition of the apex from vegetative to reproductive phase. In barley the natural variation for vernalization sensitivity is associated with a deletion in the first intron of HvBM5A (Fu et al. 2005; von Zitzewitz et al. 2005). Basal expression of HvBM 5A is high in plants that carry the spring allele, while winter allele expression results repressed until plants are exposed to low temperature and short day photoperiod to fulfill the vernalization requirement. The candidate region for VRN-H2, on the long arm chromosome 4H, has a cluster of three ZCCT-H genes (ZCCT-Ha, ZCCT-Hb and ZCCT-Hc) (von Ziztewitz et al. 2005). Actually, still not clear which one of them is the functional gene for VRN-H2 (Dubcovsky et al. 2005; Trevaskis et al. 2006), while the allelic variation is due to the presence/absence of the whole ZCCT-H cluster. The spring allele arises from a complete deletion of the three genes, which has been found in most varieties characterized at present day (Yan et al. 2004; Dubcovsky et al. 2005; Karsai et al. 2005; von Ziztewitz 2005). Cockram et al. (2007) in a subsequent study, made with 429 spring, winter and facultative barley cutivars from 13 EU countries, selected to represent cultivated EU germplasm grown over the last 60 years, demonstrated that presence of just two allelic states at the ZCCT-H locus. This suggests that only spring-associated variants in which all three ZCCT-H genes are deleted have been widely utilized in European spring barley germplasm. Although a minority of spring European cultivars still carry ZCCT-H locus, further experimental evidences demonstrated that the presence of winter allele at ZCCT-H in combination with Vrn_H1 spring allele, and vice versa, results in identical flowering time in a â Morexâ x H. vulgare ssp. spontaneum F2 population (Dubcovsky et al. 2005). The third vernalization gene, VRN-H3, is located on the short arm of chromosome 7H and has as candidate the HvFT1 gene (Yan et al. 2006) an homolog of Arabidopsis thaliana FLOWERING LOCUS T (Turck et al. 2008). Vrn-H3 plays a key role as a mobile floral induction signal that initiates the floral transition and in integrating flowering signals because is regulated antagonistically by the vernalization and photoperiod pathways (Jeong et al. 2009; Faure et al. 2007). The allelic variation of this locus is related to two functional polymorphisms, one in the promoter and one in the first intron, where the dominant allele is the spring allele (Yan et al. 2006; Casas et al. 2011). VRN-H3 expression is induced by exposition to low

temperatures and mediates the long-day flowering response. It has been proposed as connection between the vernalization and photoperiod response during flowering (Yan et al. 2006; Trevaskis et al. 2007; Hemming et al. 2008). VRN-H3 activity is mediated by the CONSTANS gene family that in Arabidopsis and barley has an important role regulating flowering (Griffiths et al. 2003; Turner et al. 2005).

1.3.3 Epistatic interaction of vernalization genes

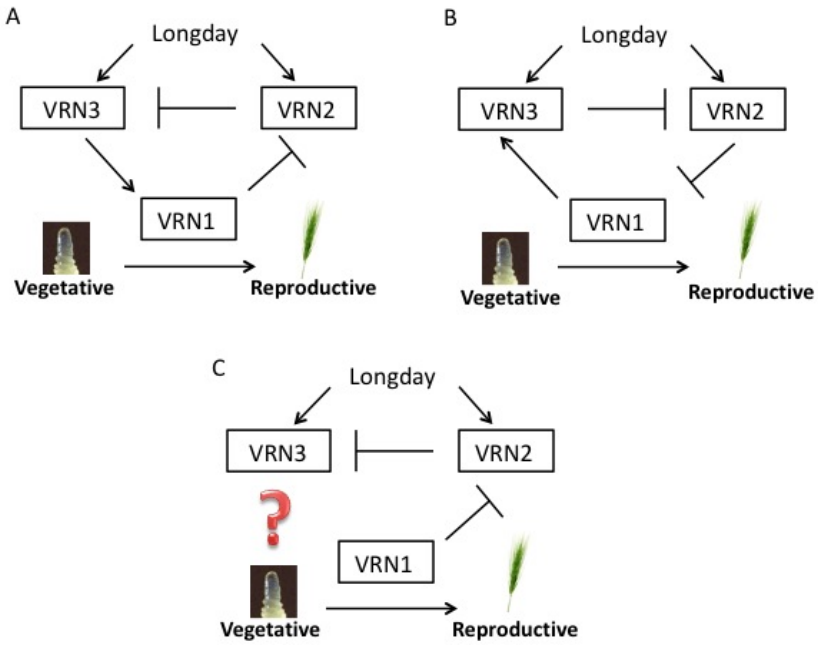

Figure 1.4. Epistatic model for the vernalization genes

The winter growth habit in barley requires the combination of VRN-H1 recessive allele together with the active allele of VRN-H2 and the winter allele of VRN-H3, which seems to show a central role in the integration of vernalization and photoperiod pathways (Casas et al. 2011). Two models have been proposed to explain the epistatic interaction between these three loci, thus the question arising from current knowledge is how vernalization (i.e. a prolonged stimulus by non-freezing low temperatures) regulates flowering time in barley. In both models VRN-H1 expression promotes flowering and is regulated by low temperatures; while long day photoperiod promotes the expression of VRN3 and VRN2. The first hypothesis is that VRN-H2, under long day photoperiod, act

as repressor of VRN-H3 and in this way VRN-H3 expression enhances VRN-H1 which in turn acts as a repressor of VRN-H2 (Trevaskis et al. 2007; Distfield et al. 2009). In the second model VRN-H1 promotes the expression of VRN-H3 that repressVRN-H2; VRN-H2 in this case act as repressor of VRN-H1 (Shimada et al. 2009). There is an alternative model where VRN-H 3, induced by long day photoperiod, plays a more central role promoting flowering. This hypothesis is also supported by the fact that Ppd_ H1 has a strong influence on flowering time in barley lines carry the deletion at the VRN-H2 locus. The induction of VRN-H3 requires the expression of VRN-H1 and is expressed only after plants have been vernalized. VRN-H1 also down-regulates VRN-H2 that act as repressor of flowering, allowing the log day photoperiod induction of VRN-H3 (Trevaskis et al. 2007; Hemmings et al. 2008).The molecular basis of dominant allele still unclear, but Yan et al. (2006) reported that in the â Sloopâ x â Halcyonâ DHs mapping population, segregate for two HvFT1 sequences, suggested to correspond to two alleles of VRN_H3. A Further hypothesis argued by Hamming et al. (2008), is that sequence changes in the region proximal to the promoter and transcribed sequences may provide the molecular basis for the dominant allele of VRN_H3. However, with VRN-H3 being mostly fixed in elite germplasm pools, the vernalization pathway can be reduced to a two genes epistatic interaction involving VRN-H1 and VRN-H2 (Karsai 2005).

1.3.4 Photoperiod response genes

Photoperiod together with vernalization controls the transition to reproductive phase. The most important genes controlling photoperiod response are Ppd-H1 and Ppd-H2 located respectively on chromosome short arm of chromosome 2H and in the long arm of chromosome 1H. Ppd-H1 encodes for a "pseudo response regulator" gene (HvPRR7), member of a gene family that plays a number of critical roles in the function of the plant circadian clock (Laurie et al. (2005)) and is responsible of long day photoperiod response. Differences in long day sensitivity are associated to a SNP that produce an amino acid change in the CTT domain (Turner et al. 2005) . This amino acidic change results in a functional polymorphism, that allow to discriminate between alleles that are responsive to long day and insensitive to long day .The dominant allele (long day

responsive) hasten flowering time as day-length increase. In long growing season such as Western Europe and much of North America this allows a prolonged growing period in longer springs, and consequently higher yield. The dominant allele of Ppd-H1 accelerates flowering and may induce VRN-H3 (Hemming et al. 2008). Another member of Flowering Locus T family, HvFT3, collocates with the genetic position of Ppd-H2, but has not been validated yet as the most likely candidate. Allelic variation at Ppd-H2 has been attributed to deletions. The functional allele that is present in sprig varieties and confers sensitivity to short day conditions (Faure et al. 2007; Kikuchi et al. 2009). Expression of HvFT3 have been detected principally in short day conditions. However, Kikuchi et al. (2009), also detected expression of HvFT3 under long day conditions suggesting that this gene may also play a role under these conditions.

1.3.5 Loci controlling â earliness per seâ

Variation in flowering time is also affected by additional genes (the so-called Earliness per se loci (Eps) loci), whose effects are not specifically dependent on photoperiod nor vernalization and that usually responsible for the fine tuning of flowering time for specific local adaptation (Bullrich et al., 2002). Several eps loci have been identified in barley: es p2S on chromosome 2H (bin 6), eps3L on 3H (bin 13), eps4L on 4HL, eps5L on 5H (bin 6) eps6L.1, esp6L.2 on 6H (bin 7,13), eps7S and eps7L on 7H (bin 3,12) (Laurie et al. 1995). Earliness per se genes regulates flowering time independently of the previous environmental signals. Thus, they can be defined as the difference in flowering time between varieties when the vernalization and photoperiod requirements are satisfied (Hoogendorm 1985; Masle et al. 1989; Penrose et al. 1991; Worland et al.1994; Slafer & Rawson 1994; Laurie et al. 1995; Slafer 1996). This is the result of the integration of differences in the duration of several developmental phases, including the transition from the vegetative to the reproductive apices, early and late spike development, and stem elongation (Slafer 1996, Lewis et al., 2008, Borras et al., 2009). No candidate genes for the eam/eps genes have been identified so far in barley.

1.4. Loci controlling abiotic stress tolerance

1.4.1. Frost resistance

Various QTL for frost resistance for winter hardiness (crown fructan content, photoperiod sensitivity and low temperature tolerance) have been mapped on chromosome 5H, in the predicted position of VRN-H1 (Karsai et al. 1997) in the Dicktoo x Morex mapping population (Hayes et al. 1993). The coincidence of low temperature tolerance QTL (quantitative trait locus) with VRN-H1 has been an interesting focus of research due to the parallelism of VRN-H1 expression with either cold tolerance or flowering time. Low temperature tolerance is induced by cold acclimation, which occurs during the induction of vernalization response (low temperature) as well as photoperiod sensitivity (short day). Furthermore cold temperature tolerance is gradually lost once plants switch from the vegetative to the reproductive phase (Galiba et al. 2009). The principal determinants of low temperatures tolerance in barley are Frost-Resistance loci (Fr-H1 and Fr-H2); both located on the long arm of chromosome 5H and approximately 30 cM apart from each other in the Nure x Tremois mapping population (Francia et al. 2004; Skinner et al. 2005; Galiba et al. 2009). Fr-H1 co segregates with VRN-H1 candidate gene HvBM5A. For instance, the cleaved amplified polymorphic sequence (CAPS) marker, targeting HvBM5A was reported as the best marker available nowadays for marker assisted selection for cold resistance in a panel of highly frost tolerant Turkish barleys (Akar et al 2009). Fr-H2 collocates with a cluster of CBF genes (C-Repeat Binding Factors), which as mentioned in section 1.2.3 are a family transcription factors involved in low temperature tolerance (Vˆ¡gˆ”jfalvi et al. 2003; Skinner et al. 2006; Tondelli et al. 2006; Francia et al. 2007). In wheat and barley CBF expression is induced by cold and drought stress (Choi et al. 2002; Vˆ¡gˆ”jfalvi et al. 2003). It has been debated in the past, especially in wheat, and nowadays it is still not clear if the coincident positions of the QTL for frost resistance (Fr-H1) and vernalization requirement (VRN-H1) are due to true pleiotropy of the MADS-box gene, or to tight linkage. Recent results from Dhillon et al. (2010) indicate that is due to pleiotropy rather than the effect of separately closely linked locus by using two diploid wheat mutants, ma intained vegetative phase (mvp), carrying a deletion encompassing VRN-1. Homozygou s mvp/mvp never flowers while mutants carrying at least one copy of Mvp/- exhibit normal flowering and high transcript levels of VRN-1 under long day, but reduced

freezing tolerance that could be associated with reduced transcripts levels of CBFs and COR genes. The mvp/mvp genotypes showed higher levels of CBFs and COR transcrip ts and consequently an improved freezing tolerance. In this way differences in freezing tolerances, previously due to the separated FR-1 locus, can be explained only on the base of allelic variation at the VRN-1 locus. Results are in agreement with a previous work from Limin and Flower (2006), who reported that in spring wheat genotypes the repression of VRN-1 is associated with increased freezing tolerance. However, although the maximum cold tolerance is coincident with vernalization saturation (Limin and Fowler 2007), there is evidence that low temperature tolerance is independent from vernalization, facultative cultivars that are not vernalization sensitive and can achieve a high degree of cold resistance (Limin et al. 2007). The Vrn-H1-mediated genetic control of flowering time may have a role in down regulating the expression of CBF genes at Fr-H2, as suggested by Stockinger et al. (2007), and by postponing the exposition of the reproductive tissues to frost that are more sensitive to frost damage than vegetative tissues. Both vernalization and photoperiod genes play an important role in cold tolerance, the allelic combination of these two loci controls the beginning of reproductive phase has an important effect on the degree of frost resistance (Turner et al. 2005; Trevaskis et al. 2003; Yan et al. 2003, 2004). More than 13 genes have been identified in the CBFs cluster on chromosome 5H (Tondelli et al. 2011). These genes code for CBF transcription factors which bind highly conserved regions in promoters of genes involved in drought and cold stress response (Stockinger et al. 1997; Liu et al. 1998; Skinner et al. 2006; Tondelli et al. 2006; Francia et al. 2007). Furthermore expression of CBFs is also mediated by photoperiod, being higher under short days (Stockinger et al. 2007).

1.4.2 Loci controlling drought

In Mediterranean regions barley undergoes drought stress during grain filling phase, reducing the number of tillers, spikes, grains per plant and individual grain weight (Samarah et al. 200). As barley cultivation area covers rain-fed environments prone to yield losses due to drought, drought stress tolerance as been and is going to be one of major goals of plant breeding (Cattivelli et al. 2008). The limited progress achieved with

direct selection for grain yield depends by low heritability, polygenic control, epistasis, significant genotype by environment interaction (G x E), and quantitative trait loci by environment (QTL x E) interaction (Piepho et al. 2000). Many morphological and physiological traits are found to be linked with drought "resistance", that could be thus dissected into several components: tillering, root development, plant early vigor, leaf water potential, relative water content and water use efficiency, stomata number and size, membrane stability, osmotic adjustment, desiccation tolerance, leaf rolling, waxiness (presence and absence of wax), leaf and canopy temperature, accumulation of metabolites or hormones, small plant size, reduced leaf area, early maturity and prolonged stomatal closure (Fisher and Wood 1979; Karamanos and Papatheohari 1999). Several QTL associated with drought tolerance have been reported in the literature for some of those traits: water use efficiency on chromosome 4H (Handley et al. 1994), relation between relative water content and growth parameters on chromosome 7H (Teulat et al. 1997), and several regions for relative water content have been identified with the most important QTL detected on chromosome 7H, that was also collinear with a QTL for the same character on rice chromosome 8 and wheat chromosome 7A (Teulat et al. 1998). ABA mediated many components of plant drought stress response; the major QTL for drought-induced ABA production in wheat is located on chromosome 5A closest to the frost resistance locus Fr-2 (Quarrie et al. 1994). Tondelli et al. (2006) mapped several candidate genes for drought tolerance in a consensus map drawn from three mapping populations (â Nure x Tremoisâ , â Proctor x Nudinkaâ , and â Steptoe x Morexâ); they found that four and two QTLs previously mapped were associated to regulatory CGs and effectors genes respectively on chromosomes 2H, 5H, 7H, and 5H and 6H. They also reported that on chromosome 5H are located the major part TFs and regulators of cold and drought induced genes.

1.4.3. Loci controlling salt stress

A saline soil is defined as having a high concentration of soluble salts, high enough to affect plant growth (www.plantstress.com). Salt stress effects are visible at whole plant

levels and severely affects yield. As approximately the 10 % of wordâ s arable land surface consists of saline soils (Kovda and Szabolcs, 1979), plant salt tolerance is a major topic of applied research. High salt concentration inhibits plant growth through both osmotic stress and ionic toxicity and also increases the concentration of ROS, which produce oxidative stress in plants cells (Munns 2005; Munns and Tester 2008). Among cereals, barley is the most tolerant to salt stress, Walia et al. (2007), on the basis of phenotypic data, clearly classify barley as more salt-tolerant than wheat, and as salt-tolerant member of the Triticeae tribe. Higher level of tolerance in barley may depen d from its rapid growth and fast phenological development that lead to early maturity (Munns et al. 2006). Kuel and Bright in 1982 found salt tolerant barley mutants that over-accumulate proline, that belong to the class of compatible compound involved in maintenance of osmotic potential of cells under various abiotic stress. Salt tolerance is physiologically complex and shows the characteristics of multigenic trait and requires changes in many biochemical pathways and in all the major processes like photosynthesis, protein synthesis, energy and lipid metabolism (Parida and Das 2005). High salt concentration in soil affect roots efficiency in water extraction whereas high co ncentration of salt within plants has toxic effects. Plant response to salinity should be divided into an osmotic phase that inhibits growth of young leaves and an ionic phase where senescence of mature leaves is accelerated. As reviewed by Munns and Tester (2008) salt tolerance mechanisms should be divided into three categories: (i) tolerance to osmotic stress, that immediately reduce cell expansion in root tips and young leaves and causes stomatal closure; (ii) Na+ exclusion from leaf blades and roots, to avoid accumulation at toxic concentration; (iii) tissue tolerance to accumulated Na+ and in some case Cl-, this requires compartmentalization of these ions at both cellular and intracellular levels to avoid toxic concentration. NaCl is the most soluble salt present in soil in this way is expected that plant have been evolved mechanisms to control its accumulation and in favor of other nutrients commonly presents in minor concentration such as K+ NO3-. Salt tolerance at the germination and seedling stages affects the initial plant stand and has been used in the past to screen plants for salt tolerance. QTL for salt tolerance at germination stage have been reported on chromosome 4H, 5H and 6H in the Steptoe x Morex mapping population; and for salt tolerance at germination

stage on chromosomes 1H, 4H, 5H and 6H and at seedling stage on chromosomes 1H, 2H, 5H and 6H (Mano and Takeda 1997). Due to the scarce information about QTL controlling salt stress in literature, physiological traits have been used to screening salinity tolerant genotypes: plant yield and growth under stress conditions (Munns et al. 2002), Na+ and K+ concentration in tissues (Chen et al. 2005), and K+/Na+ discrimination in ion transport (Chen et al. 2007). In recent years using genetic approac hes many genes, associated with salt tolerance, have been identified. These genes have been divided into three groups: (i) genes enhancing osmotic protection and scavenging of ROS, like Osmoregulatory Threalose Synthetase (OTS) (Garg et al. 2002) ; (ii) gene involved in Na+ and K+ transport such as SOS involved in Na+/H+ antiport sistems (Apse et al. 1999; Shi et al. 2000); and transcription factors that funcioning in signal trasduction pathways such as CBFs (Zu et al. 2001; Morran et al. 2011). Recently published study by Wu et al. (2011) demonstrated by association analysis that HvCBF4 in Tibetan annual wild barley is associated enhanced salt tolerance.

1.5. Genotype x environment interaction and QTL x environment interaction

Genotype x environment interaction (GE) can be defined as the nonâ parallelism between phenotypic responses to key environmental factors and genotypes (Malosetti et al. 2004). This lack of correlation limits breeding efforts to obtain crops with adaptation to a broad range of environments. Thus, one of the final goals in plant breeding is to assess the suitability of plant material across a range of agro-ecological conditions. QTL x environment interaction (QTL.E) refers to the differential effect of a quantitative trait locus across environments, which may be favorable in one or more environments and irrelevant or unfavorable in others. Plant adaptation, was firstly described by Allard (1960), as a complex chain of physico-chemical reaction and interactions, initiated by genes and then controlled or modified by other genes and by the environment that lead to the final phenotype. Plant evaluation is generally made in a diverse set of locations that may results in changes in varietal rank. The term environment refers to the agro-ecological conditions where plants are grown and can be

defined as a set of soil, climatic conditions, biotic and abiotic factors. Usually those factors are not known and the term environment involves broad descriptions of the trial sites such as trial location, years, management practices or combinations of these factors (Romagosa and Fox 1993; Romagosa et al 2012). Crop breeders look for "high yield stability", i.e. for cultivars that have the potential to grow over a relatively wide range of environments with stable performance in terms of yield and with low response to soil and climate variation. Breeders, in order to facilitate the selection of best performing genotypes use multi-environment trials (MET) that represent the target environmental range. MET data from a mapping population is typically summarized in the form of a Genotype x environment (G.E) table of means (Romagosa et al 2009) and analyzed for QTL analysis. QTL mapping has the potential to dissect complex traits into their individual genetic determinants so that their genetic effects can be explored across environments. The magnitude of the effect variation in different environments can be expressed as the amount of G.E explained by any individual QTL. We have to distinguish two types of G.E interaction: (1) a quantitative or non-crossover interaction; in this case genotypes with superior means are recommendable for all tested environments, and (2) a qualitative or crossover interaction that implies changes in the genotypes rankings across environments. The absence of G.E crossover interaction means that the genotypic means collected across environments in a MET are adequate indicators of genotypes performance.

Various statistical methods have been proposed to evaluate QTL.E interactions in plant breeding, among them: (i) Finlay-Wilkinson regression, (ii) linear-bilinear models like AMMI and GGE, and (iii) regression models (factorial regression model incorporating explicit genotypic information and factorial regression model incorporating explicit environmental and genotypic information).

1.5.1. Finlay-Wilkinson regression model

The Finlay-Wilkinson approach, also called joint regression analysis, is a simple regression method widely used in plant breeding for characterizing GE. In the model

yield adaptability is defined as the slope of regression of yield for an individual cultivar on the mean yield, over all cultivars, across environments. Genotypes performance is explained in terms of: (i) main effects for genotypes and environments; (ii) the product of environmental main effects multiplied by the regression coefficients of genotypes. GE term obtained with the analysis of variance is partitioned between heterogeneity and of regressions and deviations from regressions. In absence of explicit environmental information the average phenotypic performance of all genotypes in an environment may be used as good estimate of agronomical value of that environment. The model is summarized in the equation:

$$yij = vi + bi \hat{} \ ej + error$$

where vi are variety means, ej are environment effects (with â ej =0) and bi are the sensitivity parameters (with mean(bi)=1). A breeder is going to look for varieties with large genetic means (yield potential) and small sensitivities to make sure you have stable crop yields over the range of environments the varieties were tested. As mentioned above Finley-Wilkinson regression model has been widely used, many examples are available in literature. Kraakman et al. (2004) investigated associations between markers and complex quantitative traits such as mean yield, adaptability (Finlay-Wilkinson slope), and stability (deviations from regression). Results for regression of those traits on individual marker data disclosed marker-trait associations for mean yield and yield stability; demonstrating that association mapping approaches can be a viable alternative to classical QTL approaches especially for complex traits with costly measurements. As reviewed by Cattivelli et al. (2008) Finlay Wilkison has been used to describe yield performance of a given genotype under stress and non-stress conditions or in comparison with the average yield or the yield of a superior genotype. Lacaze et al. (2008) used the Finlay-Wilkinson regression model to check phenotypic plasticity (variation in phenotypic traits produced by a genotype in different environments). Results showed that Finlay-Wilkinson slope is able to discriminate between different types of QTL affecting plasticity.

1.5.2. Factorial regression model incorporating explicit environmental and/or genotypic information

More powerful predictive GE study models are based on factorial regression models introducing agro-ecological variables and/or genetic information that could be used as in dependent explanatory variables to modeling GE interaction. Environmental co-variables may have effects on plant growth, development, biomass and grain yield. Continuous monitoring of environments are currently more frequents and this allows collecting huge series of environmental data. In this context the central question is the choice of co-variables to describe GE (Copper and Hammer 1996). Furthermore the order of co-variables used must reflect the sequence of growth stages and the eco-physiological understanding of genotypes and environments under study should complement statistical considerations; and may drive the collection of potentially useful sets of environmental co-variables (Voltas et al. 1999). This model allows predicting different genotypes sensitivities to environmental changes. The model is:

yij = xiˇ + Ej + xi (¸»Zj) + Eij

where the differential QTL expression to environments is iˇ , can be regressed on any environmental co-variable, z, to relate the differential QTL expression directly to key environmental co-variable responsible for GxE. This is done by substituting the QTLxE term, xiˇ j,with a linear regression xi (¸»Zj) and residual term. ¸»is a constant that determines the extent to which a unit change in z, the environmental co-variable, influence the effect of a QTL allele substitution. This allows, once a physiological base is found, to model the phenotypic behavior in form of QTL-dependent response as curves to environmental characterization (Hammer et al. 2006; Malosetti et al. 2006; van Eeuwijk et al. 2007).

1.5.3 Factorial regression models incorporating explicit genotypic information

Genetic co-variables in form of molecular markers may also be included in regression models to define partitioning of G and GE terms. The most used genetic co-variables

are molecular markers such as DNA polymorphisms for anonymous and/or for functional genes, such as DarT (Diversity Array Technology; www.triticarte.com.au) markers and recently SNPs (Single Nucleotide Polymorphisms). Using multiple markers across the whole genome factorial regression allow to detect, locate and estimate QTLs main effects and QTL.E interactions. Markers adjacent to QTLs allows to estimated the effect of allelic substitutions; and to partitioning of GE into a term for different effects across environments and a residual GE interaction (Romagosa et al. 20 09). QTLs for plant adaptations, which have been reported in several mapping populations, usually shows different effects in different in different environments. When a QTL underlying GE is coincident with the position of a QTL with genotypic main effect, within a given populations, this can be used for breeding programs and in MAS (Marker Assisted Selection). Factorial regression models allows to perform full genome scan by fitting in on a grid genomic position on markers and between markers; furthermore virtual markers can be easily generated from flanking marker information (Lynch and Walsh 1998). Factorial regression models can be potentially used for each set of genotypes for which genetic predictors can be constructed. Multiple QTLs models can be constructs using CIM (Composite Interval Mapping) and incorporating cofactors o markers that correct the QTL position in the genome. CIM combines interval mapping with linear regression and includes additional molecular markers in the statistical model, in addiction to adjacent pair of linked markers this allow a major precision of detection of QTLs (Jansen 1993; Jansen and Stam 1994).

1.5.4 Factorial regression model incorporating explicit environmental and genotypic information

QTL responsible for adaptation shows different effects in different environments. MET is used by breeders to explain GE interaction in terms of differential sensitivity of QTLs or genes to environmental co-variables. In presence of QTL.E interaction and when environmental co-variables derived from geographical and weather information are available, QTLs effects across environments can be tested for the dependence on a particular environment co-variables (Crossa et al. 1999; Malosetti et al. 2004; Vargas et

al. 2006). Factorial Regression model can be used to determine the degree of which each of these factors influence GE interaction or QTL.E interaction (van Eeuwijk et al. 1996). A model proposed by Romagosa et al. (2009) allows predicting different genotypes sensitivities to environmental changes relating differential QTL expression directly to key environmental variables responsible for GE.

1.6. Use of bi-parental cross mapping populations vs. large germplasm collections in QTL mapping.

Mapping a gene to a certain location on a chromosome, requires a linkage map of the whole genome made using a segregating mapping population - which can be a backcross / advanced backcross population, doubled haploids, an F2 or more advanced recombinant inbreed lines. The identification of genetic linkage between the genetic markers and the location of genes governing the traits of interest can be used to identify the genomic location of individual QTL. Nowadays there are various types of molecular markers that can be used to build a linkage map: RFLP (restriction fragment length polymorphisms), RAPD (random amplified polymorphisms DNAs), AFLP (amplified fragment length polymorphisms), SSRs (simple sequence repeat or microsatellites), STS (sequence tagged sites), DArTs (Wenzl et al. 2004) and SNPs (single nucleotide polymorphisms). Amongst the different marker technologies available, some have fallen in disuse in favor of new high-throughput reliable genotyping platforms. DArTs enable whole-genome profiling of crops without the need of sequence information; is based on microarray hybridizations that detect the presence versus absence of individual fragments in genomic representations as described by Jaccoud et al. (2001). In species like barley characterized by a large genome with at least 80% of higly repeated DNA, expressed sequence tags (ESTs) and sequenced PCR amplicons, provide an easy way to find single nucleotide polymorphisms (SNPs) in protein encoding transcribed genes that may allow to identify gene of interest (Close et al. 2009). High resolution SNP based map offer the possibility to improve efficiency in identifying genes related with important traits such as gene controlling complex traits. In last years a

many consensus map have been draw combining different bi-parental population and various types of markers AFLP, DaRT, SSR, STS and SNP (Wenzl et al. 2006; Marcel et al. 2007; Stein et al. 2007; Varshney et al. 2007; Potokina et al. 2008). Unfortunately, despite marker intersection between these maps is significant, the accuracy of the map merging process and the resolution of synteny between barley and other genomes was limited due to missing data, non-uniform data quality and anonymity of many markers. S NP high-throughput genotyping platforms are the platform of choice in most of present projects and are rapidly substituting all the other marker platforms due to their robustness, transferability and comparability of the results across projects and most important as they are sequence-based markers we can benefit from synteny (conservation of gene content and order among the Triticeae) thus enabling sequence similarity searches to find orthologs in other model plants. SNP can be considered ideal markers to quantitative trait locus (QTL) discovery, assessment of genetic diversity, association analysis, and marker-assisted selection (Hayden et al. 2008). In the near future, SNP genotyping platforms are going to be substituted by genotyping by sequencing technologies (Kilian et al 2005). Large scale SNP discovery has lead to establish the level of DNA sequence variation and the effects that evolutionary events have had on that variation (Choi et al. 2007; Hyten et al. 2006; Schmid et al. 2003; Tenaillon et al. 2002).

Bi-parental cross mapping population have been widely used during the last 20 years to dissect the genetic basis of many quantitative traits in barley and discover the genomic location of major loci with strong G.E interaction for traits such as yield (Romagosa et al. 1996; Teulat et al 2001; Voltas et al. 2001), winter-hardiness (Hayes et al 1994; Francia et al. 2004; Reinheimer et al 2004; Skinner et al. 2006), frost resistance (Francia et al 2007; Stockinger et al. 2007), flowering time (von Ziztewitz et al. 2005; Sz^{-}–cs et al 2007; Cockram et al. 2007; Cuesta-Marcos et al. 2008) or photoperiod response (laurie et al. 1995; Turner et al. 2005) amongst others. Bi-parental mapping populations allow the detection of QTL position, with a confidence interval, and the estimation of QTL effects and their interaction without a high marker density (Piepho 2000). Despite all progress and the successful identification of many important gene controlling quantitative trait locus, these population only capture a portion of genetic diversity of the species that

depend by the genetic diversity between parents chose to build the population; and that may be not representative of the diversity present in germplasm pools. Most of variation is driven by few genes with big pleiotropic effects, which may mask smaller and stronger effects arising from interaction between genes. Limitations in the use of bi-parental populations arise from the reduced sample size of population (which is enough to detect major loci with mendelian segregation but that may run into statistical power limitations for smaller effects and QTL.QTL interactions), narrow genetic base (only two parental lines used) and the consequent limited scope of inference (usually the two parental lines of choice are not representative of the breeding genepools), and broad confidence intervals for QTL position and effects with their consequent negative effects in further application of QTL linked markers in marker assisted selection (Darvasi et al. 1993; Hyne et al. 1995; Crepiuxet et al. 2005; Vales et al. 2005). SNPs were defined as the most abundant molecular markers identified in genome studies (Brookes et al. 1999). The emergence of high throughput SNP marker genotyping platforms containing many thousands of markers allows fine-mapping QTL governing a trait of interest with genome wide association analysis (GWAS) instead of classical biparental mapping. It has been shown that with enough markers and individuals, we can exploit the accumulated recombination events within the cultivated genepool to map traits to gene resolution (Cockram et al. 2010; Ramsay et al. 2011). GWAS can be considered a complementary or alternative approach to bi-parental mapping. GWAS approaches involve the use of the diversity present in germplasm collections. This allows to exploit all the recombination events that occurred during the evolutionary history of each genotype present in the population sample (Zhu et al., 2008). Different cultivated barley types (such as 2-rowed springs, 6-rowed winters, etc...) can be defined as variety collections of homozygous â eliteâ inbreed cultivars, characterised by: high autogamy, long history of recombination and conserved linkage disequilibrium; and therefore they may be considered optimal genepools for association genetics studies targeting traits still segregating within the breeding pools. QTL detection by means of GWAS is based on existence of linkage disequilibrium (LD) between QTL and markers (von Zitzewitz 2011). As reviewed by Flint-Garcia et al. (2003) factors responsible of LD are recombination, mutation, admixture, and different degree of relatedness among

individuals, genetic drift and selection. The results of all the processes may create a population structure that can lead to false positives and false negative discovery (Pritchard et al. 2000); although various methods have been developed to accurately model population structure (Kang et al. 2010; Zhang et al. 2010). Recently published studies on elite cultivars were successful to map genetic variants associated with simple and quantitative traits (Cockram et al. 2010; Comadran et al. 2011; Ramsay et al. 2011).

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Chapter 2
Scope of Thesis

2 Scope of the thesis

The aim of this work was perform a wide-eyed genetic study of barley adaptability in stress prone environments such as Mediterranean basin. Thus we followed both a biparental and an association mapping approach. These used a segregating, doubled haploid, population derived from the cross between two elite barley cultivars â Nureâ and â Tremoisâ (Francia et al. 2004), together with an association mapping panel of 185 genotypes comprising the past and present of cultivated barley genetic diversity in the Mediterranean basin. The collection used represents a geographically diverse range of spring, winter and facultative cultivated barley forms (better described in Comadran et al. 2011). To better understand the barley adaptability to stress prone environments we thus performed:

(i) Development of a new DArT-based molecular linkage map that harbours the genomic position of genes involved in the regulation of barley flowering time and abiotic stress response.

(ii) Evaluation of the â Nure x Tremoisâ mapping population in a wide range of drought prone environment trials (Mediterranean environmental conditions) to identify QTL with major and stable effects for grain yield and other morpho-physiological and phenological traits important for yield potential and yield stability. Detected loci would provide new knowledge and molecular tools improving yield potential and broad adaptation of barley to Mediterranean conditions.

(iii) Evaluation of QTL sensitivities to a series of environmental co-variables in the â Nure x Tremoisâ mapping population for some major flowering time and grain yield QTL. An study of the relationship between QTL effects and environmental co-variables as logical consequence of previous chapter. Grain yield in cereals is greatly influenced by GE interaction, and the basis of GE involves the modelling of quantitative trait loci

expression in relation to their dependence on environmental factors.

(iv) Assessment of a GWAS approach for a quantitative complex trait such as cold resistance in the 185 barleys association panel. Recently a great interest has been focused on genome wide association analysis (GWAS) as new methodology to study traits in higher plants, especially in crops. Despite the advantages respect bi-parental population mapping nowadays only few studies have been published in barley for complex traits such as yield and yield related traits such as abiotic stress tolerance.

(v) Identification of barley varieties within our association panel, with superior cold tolerance and advance our understanding of the genetics of winter-hardiness in autumn sowing conditions. SNP markers closely linked to positive associations could be useful to develop new molecular marker tools for MAS.

Chapter 3

QTLs for yield adaptation in the â Nureâ x â Tremoisâ DArT map in multi-environmental barley trials across the Mediterranean Basin.

3.1 Introduction

Barley (Hordeum vulgare L.) is the predominant crop of the driest Mediterranean areas (< 300 mm annual rainfall), where it often represents the main source of livelihood. In these environments yield and quality of barley, durum wheat and other crops are heavily affected by drought, whose recurrence is even likely to increase in the future, in terms of both frequency and severity (Bolle et al. 2003). Improving the exploitation of water in agriculture involves mainly training farmers on the use of soil moisture conservative cultural practices and breeding for varieties with higher productivity under water limiting conditions. Even if breeding activities have led to some yield increase in drought-prone environments for barley and other cereals, a gap is still present between yields in optimal and stressed environments. Breeding strategies in such environments should consider the nature, timing and intensity of the stress events can vary significantly across regions and years, thus plants designed to cope with a specific type of drought

events may under-perform when the stress conditions are different or absent (Cattivelli et al. 2008). Because of this strong genotype by environment (GxE) interaction, selection for yield potential in high yielding conditions has frequently led to some breeding progress under moderate drought conditions (Araus et al. 2002; 2008). This implies that traits maximizing productivity normally expressed in the absence of stress, can still sustain a significant yield improvement under mild to moderate drought (Slafer et al. 2005; Tambussi et al. 2005). In a typical Mediterranean environment of South Italy, Rizza et al. (2004) observed a highly significant yield response of 89 barley genotypes, that included cultivars released during 40 years in several EU countries, as a function of a water-stress index (WSI, based on soil water balance calculation). The strong yield increase in response to water availability shown by modern cultivars compared to old varieties let the authors conclude that selection based on the absolute performance of the genotypes across environments is more successful than selecting for the minimum yield decrease under stress with respect to favorable conditions (Rizza et al. 2004). In small grain cereals, genetic gain in yield potential has been associated to changes in physiological traits related to time to flowering and plant height, biomass production and partitioning, and yield components such as number of fertile ears per plant and grain number and size (Araus et al. 2008). In particular, the synchronization of crop cycle with the most favorable environmental conditions is fundamental for maximizing yield potential and adaptiveness through the best use of resources (e.g. water and radiant energy) and the avoidance of stress events during growth and grain filling (Slafer et al. 2005; Reynolds et al. 2009). For example, it is well known that a good level of earliness is an effective breeding strategy for enhancing yield in Mediterranean environments where wheat and barley are commonly exposed to terminal drought stress, even if extreme earliness could lead to yield penalty in fertile conditions (Cuesta-Marcos et al. 2008a). In cereals, phenological adjustments are mainly driven by a few well-known photoperiod (Ppd) and vernalization (Vrn) responsive genes, as well as early maturity or earliness per se loci (Eam/Eps) that affect life-cycle timing independently from these stimuli (Cockram et al. 2007; Distelfeld et al. 2009; Faricelli et al. 2010; Higgins et al. 2010). Changes in physiological traits associated with main yield components have been revealed from retrospective studies in wheat. For example, it has been observed

that selection for high yield under Mediterranean drought-prone conditions mainly resulted in increasing the number of grains per unit land area rather than mean grain weight (Acreche et al. 2008). This could be related to changes in growth partitioning over the phase of stem elongation, immediately before anthesis (Araus et al 2008). Although differences in the determination of grains per spike in barley compared to wheat exist, also in barley the amount of assimilates partitioned towards the spike during this phase seems to have a major role for the establishment of fertile florets and grains per unit area (Arisnabarreta and Miralles 2008). The advancements in crop physiology and genomics allow nowadays a multidisciplinary approach for the study of cereal adaptation to water-limiting conditions (as reviewed by Tuberosa and Salvi 2006; Cattivelli et al. 2008; Reynolds et al. 2009). In particular, multi-environment trials (METs) conducted over populations of genetically related individuals (i.e. mapping populations), or wide germplasm collections can help in understanding the genetic basis of grain yield, as well as the morpho-physiological and phenological traits determining yield potential and stability in dry and wet conditions, while dissecting the genetic basis of the genotype by environment interaction (GE). Agronomic evaluation of experimental populations under Mediterranean environments resulted in the identification of genomic regions underlying QTL with major and stable effects (Teulat et al. 2001; Baum et al. 2003; Talamˆ' et al. 2004; Comadran et al. 2011; Cuesta-Marcos et al. 2008a; von Korff et al. 2008). Such loci provide the breeders with new knowledge and molecular tools for improving small-grain cereals in terms of yield potential and broad adaptation to the environment. For QTL mapping, as an alternative to functionally neutral molecular markers, in plants with large and not yet sequenced genomes such as barley (5,000 Mb) , the generation of linkage maps based on candidate genes (function maps) can shorten the way towards the identification of the genetic determinants of QTLs (Tondelli et al. 2006; Stein et al. 2007). As an example, the HvABI5 gene encoding for a bZIP transcription factor has been mapped in a genomic region associated to the tolerance to multiple abiotic stresses (Tondelli et al. 2006; Pecchioni et al. 2011), and its functional role has been recently reinforced by the report of Kobayashi et al. (2008), in which the authors found a positive role of a wheat HvABI5 ortholog (namely Wabi5) in response to low temperature, drought and exogenous ABA treatment. In recent years, the EU INCO-

MED funded project MABDE (â Mapping Adaptation of Barley to Droughted Environmentsâ) has brought significant advancements in understanding the processes underlying barley adaptation to Mediterranean environments and the consequences of barley breeding carried out in the last century, through the accumulation of exhaustive agronomic, physiologic and molecular marker datasets (Pswarayi et al. 2008a, 2008b; Comadran et al. 2008, 2009, 2011; Borrˆ s-Gelonch et al. 2010; Francia et al. 2011). Data collected on a barley segregating population deriving from the cross between two barley elite cultivars representative of the Mediterranean winter and Central European spring barley germplasm-pools, namely â Nureâ and â Tremoisâ (Francia et al. 2004), have been already used to study the ecophysiological performance of the population and to describe the relationships among a series of characters defining grain yield as a function of the length of the different barley developmental phases (Francia et al. 2011). In the present study, we aim to further exploit the â Nureâ x â Tremoisâ biparental population through 1) increasing the marker density of its molecular linkage map (Francia et al. 2004), and 2) identifying QTL responsible for the adaptation of barley crop to a wide range of Mediterranean environments in terms of grain yield, yield components and phenology.

3.2 Materials and methods

3.2.1 Plant material

The â Nureâ x â Tremoisâ (NT) population is composed of 118 Doubled Haploid (DH) lines derived by anther culture from the F1 of the â Nureâ x â Tremoisâ cross (Francia et al. 2004). â Nureâ - [(â Fior 40â x â Alphaâ 2) x â Barakaâ] - is a winter, two-rowed variety, adapted to South European environments, showing high yield potential and yield stability in irrigated as well as in moderately stressed conditions (400 mm rainfall; Rizza et al. 2004). â Tremoisâ - [(â Dramâ x â Aramirâ) x â Berarâ] - is a spring, high yielding two-rowed malting cultivar, adapted to fertile environments. Pure stock seed of

the â Nureâ x â Tremoisâ DH lines (NTs) was multiplied at ICARDA in the harvest year 2003 to allow for multi-environment trials in the subsequent harvest seasons 2004 and 2005. Genomic DNAs extracted from the same population were used for molecular marker analyses in the present study.

3.2.2 Genotyping

A â Nureâ x â Tremoisâ low resolution linkage map was previously described by Francia et al. (2004). Here, Diversity Array Technology´fi (DArT) marker assays were performed by Triticarte Pty Ltd (Australia) to enhance map coverage. The NTs were genotyped with an identical set of DArT markers from a PstI/BstNI genomic representation (’bPb’ markers) described by Wenzl et al. (2004). Candidate genes known to be involved in regulation of barley phenology and abiotic stress response have been previously located on the NT map (von Zitzewitz et al. 2005; Tondelli et al. 2006; Francia et al., 2007). Following the approach reported by Tondelli et al. (2006), 15 further candidates were mapped in the present work. Nucleotide sequences of the genes were downloaded from public databases and specific PCR primer pairs were designed by using the software Primer3 (Rozen and Skaletsky, 2000) (Table 3.1). Amplification and fragment sequencing were performed as previously described (Tondelli et al. 2006). Sequence assembly as implemented in the software package Sequencher (Gene Codes Corp., Ann Arbor MI) assisted in the identification of Single Nucleotide Polymorphisms (SNPs) and Insertion/DELetions (INDELs). Based on the polymorphism type, new CAPS (Cleaved Amplified Polymorphic Sequence) markers were developed for mapping 13 candidate genes (Table 3.1). Five ¸…l of PCR product were incubated for 1.5 h with 1U of restriction enzyme, 1X reaction buffer and 0.1 mg/ml of bovine serum albumin, and then separated on a standard 2% agarose gel. The remaining TC-MYB1 and HvBPBF candidate genes were genotyped by SSCP (Single Strand Conformation Polymorphism) in acrilamyde gels as described by Tondelli et al. (2006). Protocols described above were also adopted for SNP detection and mapping of three â scsnpâ markers (scsnp02737, scsnp00177, scsnp15296; Rostoks et al., 2005),

after amplification with primers downloaded from the Germinate database (http://germinate.scri.ac.uk/barley_snpdb/index.html). In addition, fluorescently labelled primer pairs for PCR amplification of nine â scssrâ and one â scindâ markers (Rostoks et al., 2005; Varshney et al., 2007) were obtained from the Germinate database. Reactions were performed in a 20 ¸…l final volume containing: 40 ng of â Nureâ and â Tremoisâ genomic DNA as template, 1X PCR buffer, 1.5 mM of MgCl2, 5% DMSO, 0.25 mM of each dNTP, 0.4 ¸…M of each primer, and 1U of Taq DNA Polymerase (Promega). Reactions were incubated for 2 min at 94´℃, followed by 40 cycles of 94´℃ for 30 s, 55´° C for 30 s, 72´℃ for 1 min, and a final extension of 72´℃ for 7 min. Fragment length polymorphisms were separated on a ABI PRISM 3130 Genetic Analyzer.

Table 3.1. Candidate genes added to the NT Italian Barley Mapping Population. BIN is assigned according to Aghnoum et al. (2010).

Marker Name	Accession Number	Chr_ BIN a	Forward Primer Sequence (5’-	Reverse Primer Sequence (5’-	Marke r Type b	Referen ce

HvZFP16-1	BQ761311, BI777789	1H_03.2	ttcttcttccatttggccatc	aaagtaattacattgggccac	CAPS, MseI	Skinner et al. 2006
Hv347D22_HvFT3	DQ411319	1H_11.3	ccaaggtaggtccaatgttca	ctcctgtatggggatctgaca	CAPS, AlwNI	Faure et al. 2007
HvHDAC2-1	EU348775	1H_14.3	gaagaggagaggagaggagagg	gcatagcatagcataccaccaa	CAPS, MboII	Demetriou et al. 2009
Hv673I14_Ppd-H1	AY943294	2H_04.2	aagaaagagaaggagggtgtcc	ccttttcagagctgcgtctact	CAPS, PvuI	Turner et al. 2005
HvFT4	DQ411320	2H_06.2	gcataattgcaccaaacttctg	tgatcctcaaatacgttggaag	CAPS, MboII	Faure et al. 2007
HvBM3	AJ249143 and unpubl.	2H_06.2	acacggtttttgtattccatcc	tttttaccacgccttccatc	CAPS, DdeI	Schmitz et al. 2000
HvBM8	AJ249146 and unpubl.	2H_07.2	tcagattcagtagcccacct	cttgttctcctcctgcagtg	CAPS, NaeI	Schmitz et al. 2000
TC-MYB1	TC174935	3H_03.1	gatcagggtcttcagtgtggtc	gtattccctgtctgctcgtctc	SSCP	-
wca11a2	N. Christov, unpubl.	3H_06.1	gctgtcggggaagaagagt	ctagcacaacgggattattga	CAPS, TspEI	-
HvHDAC2-2	EU348776	3H_12.2	gggaagatgacggagaatcac	gcaacagctcaaactccttttt	CAPS, PagI	Demetriou et al. 2009

HvBPBF	AJ000991	5H_05.1	cggtggtgttgttggattag	gaaaatgaccgagcgaaatac	SSCP	Mena et al. 2002
HvCRY2	AF348460	6H_06.1	ggacatgagcttggtcgtc	ccaagttcttacgtattcac	CAPS, AluI	Perrotta et al. 2001
HvSAD	AJ312297	6H_07.1	agcaatcccactacggtcat	ggcaagaacacagcaacaag	CAPS, RsaI	La Moneda et al. 2003
TC-MYB2	TC178195	7H_01.1	ccacaacaacctcctcatatcg	ctcagtcgcatcagaagttagc	CAPS, RsaI	-
HVP1	AB032839	7H_12.1	gctcaacatcctcatcaagctc	ccctctgctaccactactacagc	CAPS, HhaI	Fukuda et al. 2004

3.2.3 Phenotyping

In the frame of MABDE project, 18 multi-environment field trials were conducted in six countries around the Mediterranean basin: Algeria (DZA), Italy (ITA), Jordan (JOR), Spain (ESP), Syria (SYR) and Turkey (TUR), for two harvest seasons (2004 and 2005; Table 3.2). In each country, trials were grown at sites contrasting for natural rainfall (high vs. low; based on past meteorological data, not shown), or at the same site with one trial being rainfed and the other supplied with supplementary irrigation (I) (Table 3.2, see also Francia et al. 2011). Suffix W (wet) and D (dry) were used to name the contrasting water availabilities. Field experimental designs consisted of a replicated trial with two replicates for â Nureâ , â Tremoisâ and the 118 NTs, augmented by four check entries repeated 15 times in a systematic diagonal fashion to adjust for spatial

variation. The first check (cv â Harmalâ) was common to all sites and the other three checks were a landrace, a local old and a local new cultivar relevant for each country. At each site, trials were sown in a rectangular grid of 15 rows and 20 columns, with 6 m 2 plots, and were grown according to local practice for sowing rate and other inputs. The following traits defining grain yield, yield components and plant phenology were recorded on a plot basis for each trial: grain yield (Yld) in t ha-1, number of spikes per square meter (Ssm), number of grains per spike (Gps), 1,000 grain weight (Tgw) in grams, harvest index (Hi), early growth vigour (Ev) as a visual score from 0 = poor vigour to 5 = good vigour, frost resistance (Fr) as a visual score from 0 = no damage to 9 = all plants killed, plant height (Ht) in cm from soil to the bottom of the spike, days from sowing to heading (Hd), days from sowing to physiological maturity (Md), spike length (Sl) in cm, peduncle length (Pl) in cm from the last node to the bottom of the spike, peduncle extrusion (Pe) in cm from the ligule of the flag leaf to the bottom of the spike, reaction to powdery mildew (Pm) as a visual score from 0 = free to 3 = severe attack. For each trait, the number of analysed field trials depends on the availability of suitable data (Table 3.2).

4W 07 0

Table 3.2. Characters available for each trial site, with sites ordered for growing grain yield. Field mean values are presented; â -â indicates missing or discarded data, irrigated fields are indicated with (I). a DZ A, Algeria; ESP, Spain; ITA, Italy; JOR, Jordan; SYR, Syria; TUR, Turkey.

#	Code a	Water input (mm)	Yield and yield components		Phenology and plant development												
			Yld	Tgw	Hi	Sl	Ssm	Gps	´	Hd	Md	Ht	Ev	Fr	Pl	Pe	Pm
			(t ha-1)	(g)	(%)	(cm)	(m-2)	´	´	´ (d)	´ (d)	(cm)	(0-5)	(0-5)	(cm)	(cm)	(0-9)
1	JOR_	194	0.	27.	-	-	-	15	´	-	-	-	-	-	-	-	-

2	ESP_5D	167	0.48	-	-	-	-	-	´	178	-	-	-	3.2	-	-	-
3	JOR_5D	140	0.51	33.3	13.0	5.4	-	-	´	108	127	37.7	-	-	-	-	-
4	JOR_5W	217	0.81	34.7	-	8.0	-	-	´	96	128	63.1	-	-	-	-	-
5	JOR_4D	151	1.32	-	25.9	-	-	-	´	-	-	-	-	-	-	-	-
6	SYR_4D	204	1.36	40.4	41.8	7.8	-	-	´	-	-	47.3	2.4	-	13.3	-	-
7	SYR_5D	143	2.35	37.2	33.1	8.3	-	-	´	-	-	53.3	-	-	-	-	-
8	ITA_4D	258	3.19	31.8	-	-	476	17	´	113	145	78.9	4.7	-	-	-	3.9
9	TUR_4D	232	3.28	41.9	-	7.8	493	17	´	203	-	67.5	-	-	23.5	-	-
10	DZA_5W	130	3.50	-	33.3	-	-	-	´	96	-	53.4	-	-	-	-	-
11	ITA_4W (I)	327	3.77	34.2	-	-	497	18	´	119	154	-	4.1	-	-	-	4.9
12	ITA_5D	268	3.81	38.2	-	-	467	-	´	136	-	79.2	-	-	-	-	-
13	TUR_5	174	3.86	34.0	31.4	6.7	627	-	´	71	-	67.0	2.7	-	-	-	-
14	SYR_4W	290	4.13	48.4	-	8.9	-	-	´	125	-	66.7	2.7	-	-	-	-
15	TUR_4W(I)	282	4.45	41.8	-	9.2	606	20	´	-	-	74.9	3.2	-	-	3	-
16	ITA_5F	292	4.56	-	-	-	-	-	´	177	-	81.0	-	1.8	-	-	1.9

17	ITA_5W (I)	362	4.86	47.7	-	-	-	-	´	135	-	-	4.2	-	-	-	-
18	SYR_5W	290	5.43	43.9	43.8	8.3	-	-	´	122	-	91.2	2.3	-	-	-	-

3.2.4 Map Construction, statistical and QTL analyses

Genotyping information was recorded for each marker and segregation data entered into a population file (available from GrainGenes at http://wheat.pw.usda.gov/GG2/index.shtml) that also included previously published marker data. Software JoinMap 4 (Van Ooijen 2006) was used for grouping markers (LOD score = 4.0) and subsequent determination of marker order (minimum LOD score = 1.0, recombination threshold = 0.4, ripple value = 1, jump threshold = 5). The Kosambi mapping function was applied for converting recombination units into genetic distances through the regression mapping algorithm. In order to avoid a contradictory placement of loci that occurred occasionally, individual maps were recalculated by setting individual loci at â fixed orderâ . The complete set of marker segregation data and map will be made public through the Graingenes database (http://wheat.pw.usda.gov). The collected phenotypic data were analysed in GenStat version 11 (Payne et al. 2006) by a mixed model with entries and repeated checks as fixed effects and rows, columns and entries as random effects, in order to generate Best Linear Unbiased Estimates (BLUEs) for each NT line. Genotypic BLUEs were used to calculate broad sense heritability (h2) and for all the subsequent analyses. Main Genotypic and Environmental effects, GE interaction and correlations for any pair of characters of the same dataset have been already described in Francia et al. (2011). In the present paper we focused on the QTL analyses in the IBMP, by using the software MapQTL 5 (Van Ooijen 2004). For any trait/environment combination, LOD threshold values defining the genome-wide significance ($P < 0.05$) of a putative QTL was obtained by permutation tests (1,000 replications). Simple interval mapping

analysis was performed at a 1 cM interval and the marker closest to each LOD peak was selected as cofactor in a composite interval mapping (CIM) analysis. As a measure for yield adaptability (Ya) and yield stability (Ys), Finlay-Wilkinson regression coefficient, bi, and mean squared deviation from regressions, si2, were calculated for each trial as described by Kraakman et al. (2004). Both statistics were based on the regressions of yields for individual genotypes in a trial on an environmental index, here represented by the trial average yield. For QTL analyses, values of si2 were log-transformed.

3.3 Results

3.3.1 A new DArT-based linkage map of the â Nureâ x â Tremoisâ Mapping Population

A total of 396 DArT, 18 STS-SNP and 10 SSR loci (424) were added to the â Nureâ x â Tremoisâ molecular linkage map already available (Figure 3.1). The NxT map is now composed of 542 markers (with 394 non co-segregating loci), spanning a total length of 1,114 cM, with an average resolution of one marker every 2.8 cM (Fig. 3.2; http://wheat.pw.usda.gov). Individual linkage group length ranges from 117.7 cM (1H) to 203.3 cM (5H), and alignment with the barley consensus map built by Wenzl et al. (2006) showed a high level of conservation of DArT locus order (data not shown). The same NxT genotypic dataset has also been recently used for the construction of a high resolution consensus map (Aghnoum et al. 2010). However, large gaps are still present on the â NxTâ linkage map, six of them being larger than 20 cM. In particular, only 15 markers have been placed on chromosome 4H. Segregation distortion was observed in several genomic regions, especially on chromosomes 1H (long arm) and 6H, even if this is expected not to affect QTL analyses, as described by Xu (2008). Thirty-four candidate genes mainly encoding barley transcription factors have been mapped at present on the â NxTâ linkage function map (in bold italic in Fig. 3.2). For the purpose of the work, mapping genes with a well characterized or a putative role in the regulation of barley flowering is noteworthy. On chromosome 2H the â Nureâ and â Tremoisâ sequences

of the genomic region spanning HvPRR, the genetic determinant of PPD-H1 photoperiod responsive locus (Turner et al. 2005), did not reveal any polymorphism. Both parents carry the same recessive, late-flowering ppd-H1 allele. In order to place the gene on our IBMP map the first useful polymorphism was identified 7.5 Kb upstream the HvPRR start codon, based on the sequence of the â Morexâ BAC clone Hv673I14 (containing HvPRR; Turner et al. 2005). No sequence polymorphisms have also been detected among parents within the coding region of HvFT1, the candidate gene for VRN-H3 vernalization responsive locus on barley chromosome 7H (Yan et al. 2006). Both â Nureâ and â Tremoisâ carry the recessive vrn-H3 allele, and due to the absence of polymorphisms it was not possible to map the locus. A CAPS marker has been developed for mapping the â Morexâ BAC clone Hv347D22, harbouring HvFT3, the candidate gene for Ppd-H2 photoperiod responsive locus on chromosome 1H (Faure et al. 2007) (Fig. 3.2). Finally, a third gene belonging to the FT gene family (HvFT4; Faure et al. 2007), together with two MADS-Box genes (HvBM3 and HvBM8; Schmitz et al. 2000) have been positioned at the pericentromeric region of chromosome 2H (Fig. 3.2; Table 3.1).

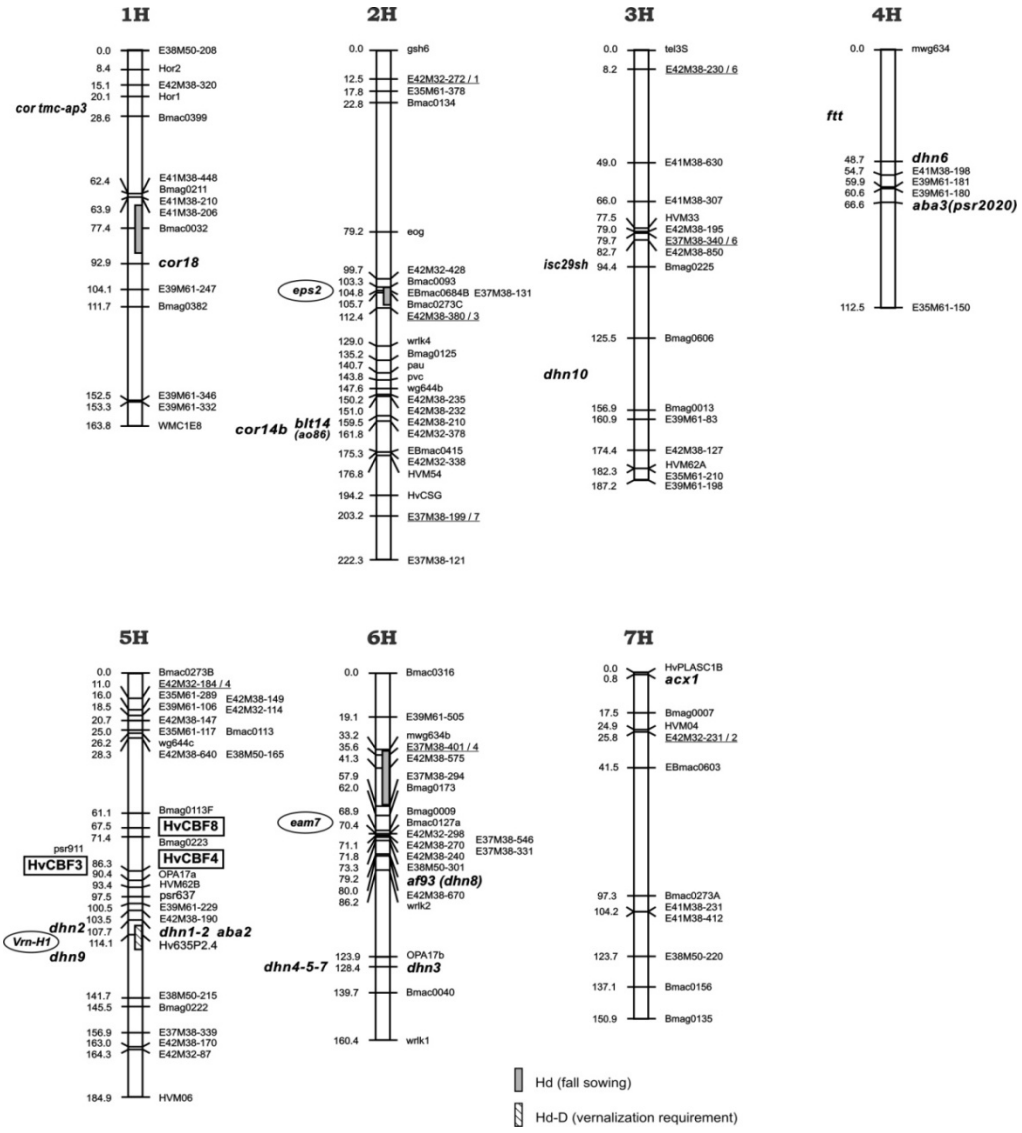

Figure 3.1. Low resolution linkage map of the â Nureâ x â Tremoisâ barley population. Distances are in Kosambi cM and linkage groups are oriented with short arms at the top. Cosegregating markers are placed to the right and inferred positions of markers mapped in other populations are shown to the left of each cartoon. COR genes are in bold italic, whereas CBF transcription factor genes are in bold type highlighted by boxes. AFLP markers are named according to Qi and Lindhout (1997); in addition, AFLP loci that are in common with both the â Proctorâ x â Nudinkaâ and the â L94â x â Vadaâ map are underlined and have the numerical suffix number assigned by Becker et al. (1995). Gray boxes inside chromosomes 1H, 2H and 6H represent heading date QTLs. Hatched box inside chromosome 5H represents a vernalization requirement QTL. Ellipses indicate the inferred positions of major genes affecting flowering time (Vrn-H1, eam7 and eps2).

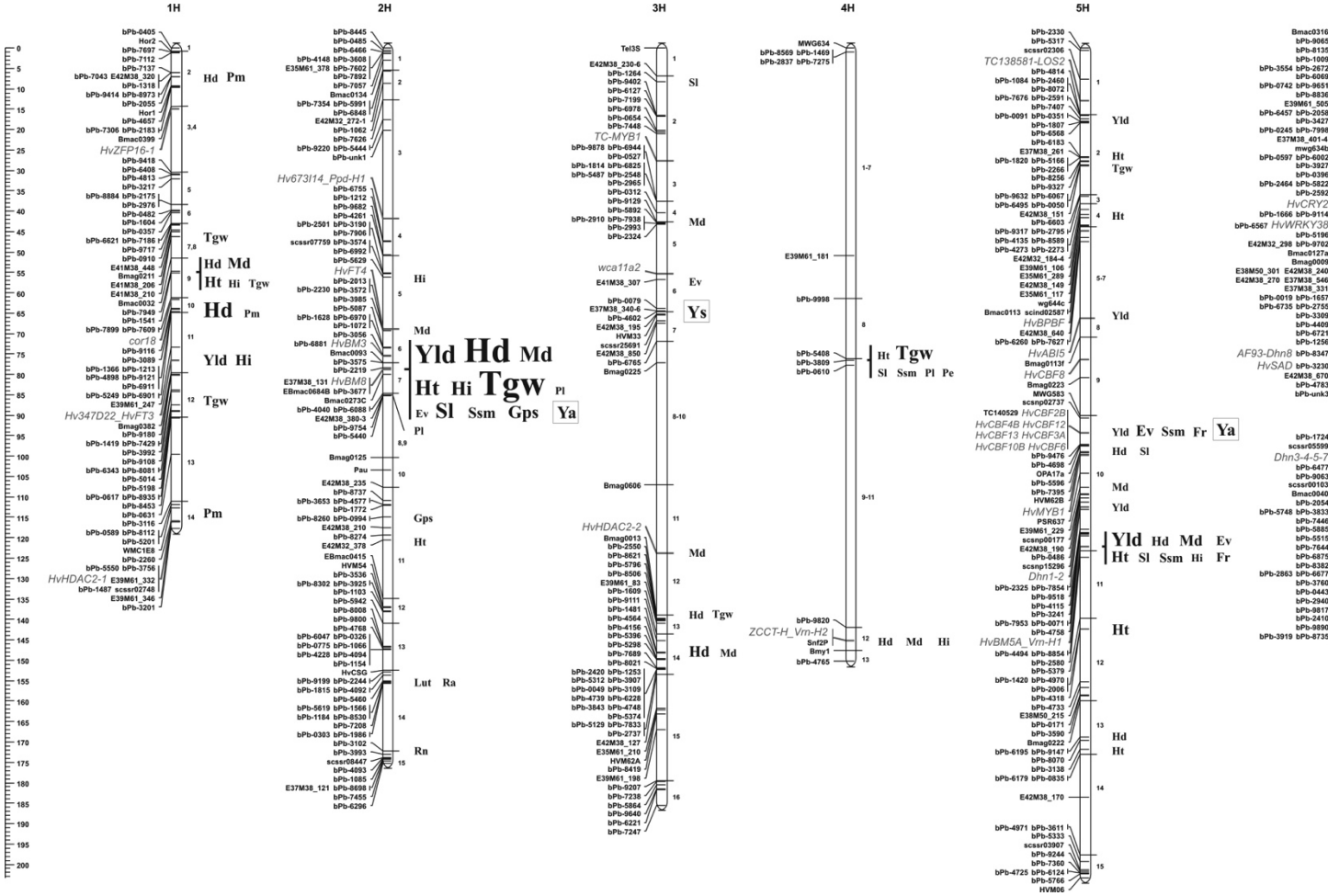

Figure 3.2. The IBMP genetic linkage map. Chromosomes are oriented with short arms on the top and distances on the left ruler are in Kosambi cM. Chromosome BIN assignment derives from Aghnoum et al. (2010). Candidate genes are in grey, bold italic; QT loci nomenclature follows abbreviations reported in the Materials and methods section and in Table S4. With the exception of yield adaptability (Ya) and stability (Ys), the font size for QTL names is proportional to the number of occurrences of the QTL across the 18 environments (see the legend in the bottom-right side).

3.3.2 NT yield evaluation across the Mediterranean basin

In the different Mediterranean environments, average field yield ranged between 0.07 t ha-1 (#1-JOR_4W) and 5.43 t ha-1 (#18-SYR_5W) (Table 3.3). In seven trials this value was below 2.5 t ha-1, reported in literature as the so-called crossover point, at which

cultivars with high yield potential could produce less than cultivars with lower yield potential, but better adapted to stress (Ceccarelli and Grando 1991; Blum 2005; von Korff et al 2008). With the exception of #6- SYR_4D, #10-DZA_5W and #17-ITA_5W, the â Nureâ parent always outperformed the â Tremoisâ parent in the different field trials. Transgressive segregation has been observed in all 18 trials, and in both directions, towards low and high yields (data not shown). Broad sense heritability (h2) calculated for grain yield ranged from 35% (#10-DZA_5W) to 99% in the highly drought-stressed site #1-JOR_4W, where completely crop failure was observed for some entries, and very low yields for a few others (Table 3.3). A more detailed description of the environments, including meteorological data collected over the two-years of experiments, and their impact on the NT performance has already been reported elsewhere (Francia et al. 2011).

Table 3.3. Summary statistics for Yld and Hd in the NT population

#	Cod	Gr	´	D

		Min	Mean	Max	SD	Nure	Tremois	h2´	´	Min	Mean	Max	SD	Nure	Tremois	h2´
1	JOR_4W	0.00	0.07	0.31	0.08	0.03	0.01	0.99		-	-	-	-	-	-	-
2	ESP_5D	0.16	0.48	0.78	0.12	0.62	0.47	0.48		172	178	185	3.2	175	179	0.89
3	JOR_5D	0.00	0.51	1.24	0.26	0.88	0.47	0.62		100	108	116	5.0	105	115	0.79
4	JOR_5W	0.19	0.81	1.76	0.29	1.32	1.00	*		90	96	101	2.5	96	98	0.52
5	JOR_4D	0.43	1.32	2.11	0.39	1.69	1.34	0.47		-	-	-	-	-	-	-
6	SYR_4D	0.19	1.36	2.49	0.37	1.20	1.80	0.42		-	-	-	-	-	-	-
7	SYR_5D	0.23	2.35	4.05	0.84	2.90	1.71	0.73	´	-	-	-	-	-	-	-
8	ITA_4D	2.34	3.19	4.16	0.36	3.34	3.11	0.53		105	113	122	3.3	113	114	0.96
9	TUR_4D	1.07	3.28	5.57	1.01	5.18	3.05	0.58		192	203	212	5.4	191	209	0.77
10	DZA_5W	1.06	3.50	5.84	1.12	2.51	2.53	0.35		92	96	98	1.1	97	95	0.42

11	ITA_4W	2.72	3.77	4.73	0.43	4.69	3.92	0.88		112	119	126	2.7	118	120	0.92
12	ITA_5D	2.45	3.81	5.22	0.50	4.31	3.60	0.62		130	136	141	2.5	133	138	0.92
13	TUR_5	1.79	3.86	5.78	0.80	3.77	3.48	0.39		64	71	82	4.4	72	72	0.95
14	SYR_4W	2.74	4.13	5.30	0.49	5.14	4.17	0.99		117	125	135	3.8	124	125	0.95
15	TUR_4W	1.51	4.45	6.99	1.11	6.18	3.75	0.73		-	-	-	-	-	-	-
16	ITA_5F	3.03	4.56	5.65	0.54	5.23	3.56	0.64		170	177	185	1.9	172	184	0.90
17	ITA_5W	3.01	4.86	5.98	0.47	4.17	5.05	0.39		131	135	139	3.2	135	137	0.81
18	SYR_5W	4.07	5.43	6.59	0.53	6.18	5.37	0.52	´	115	122	128	4.1	121	123	´ 0.97

3.3.3 QTLs for barley adaptation to Mediterranean conditions

Composite Interval Mapping (CIM) analyses for yield revealed eight QTLs on four barley chromosomes (Table 3.4 and Fig. 3.2). Among them, five loci were uniquely identified in single field trials, while three were consistently mapped from multiple environment data. The most frequently detected QTL maps on chromosome 2H, BIN_07.2 (common to 8 trials), followed by a QTL on chromosome 5H, BIN_10.5 (5 trials), and a region of chromosome 1H, BIN_11.3 (3 trials). The HvBM8 MADS-Box gene was the peak marker of the most significant QTL on chromosome 2H (Fig. 3.2), with the â Nureâ allele showing a positive effect on grain yield in both low and high yielding environments (Table 3.4). The QTL was responsible for 13.7 to 45.8% of the observed phenotypic variance for yield and, most importantly, the QTL peak coincided with the eam6/Eps-2

locus, conferring early maturity per se, i.e. under both long and short day conditions, and theoretically independently from vernalization (Laurie et al. 1995; Horsley et al. 2006). A number of highly significant QTLs for days to heading (up to LOD 37.0 and 72. 6% of explained phenotypic variance) were detected at the same genomic region in 12 out of 13 sites where the trait was recorded (Table 3.3). On average, NT lines carrying the â Nureâ allele at HvBM8 flowered 5.7 days before DH progenies carrying the â Tremoisâ allele. Moreover, a plant height decrease was associated with the â Nureâ allele at HvBM8, in 6 environments (Table 3.4). The second most frequently detected QTL for grain yield mapped on the long arm of chromosome 5H and co-segregated with Vrn-H1 (Fig. 3.2). It was observed for the QTL an inversion in the additive effect between two field groups, suggesting the existence of a significant QTL by environment interaction. In three autumn sown trials (#2-ESP_5D, #9-TUR_4D and #15-TUR_4W) a positive contribution on grain yield was reported for the recessive (vernalization responsive) â Nureâ allele, opposite to the results from the two late winter sowing sites: #10-DZA_5W and #13-TUR_5. The same HvBM5A gene represented the peak marker for the QTL in #8-ITA_4D and #13-TUR_5 environments (Table 3.3). Finally, a significant yield increase was always associated with the â Tremoisâ allele at the Hv347D22_HvFT3 marker on chromosome 1H. This QTL was detected in two low-yielding environments, #5-JOR_4D and #7-SYR_5D, as well as in a high-yielding trial # 12-ITA_5D, and explained 8.4 - 11.9% of the observed phenotypic variance.

Five environment-specific QTLs for grain yield were detected on the NT map (Table 3.4), all in high yielding sites (above 2.5 t/ha). Four of them were all located in different regions of chromosome 5H, bPb-0351 (BIN 2.2) in the short arm, HvABI5 (BIN 8.2), HvCBF-Fr-H2 (BIN 9.1), bPb-2325 (BIN 10.4) in the long arm, with a positive contribution from the â Nureâ parent. On the contrary, a positive effect on Yld from the â Tremoisâ parent was observed at the bPb-6735 locus on chromosome 6H, BIN_06.2 in #11-ITA_4W. Not coinciding with known phenology-related genes, they could interestingly represent yield per se loci, specific for single environments. In fact, as a general trend, we were not able to identify QTLs common for the group of low- , neither for the high-yielding environments.

A cluster of yield component QTLs, not associated to phenology/developmental loci was

also observed at a single locus of chromosome 4H, BIN 9.1 (Fig. 3.1). We observed, in five environments, a positive contribution on thousand grain weight from the â Nureâ allele at the bPb-3809 DArT marker, and this QTL did not co-map with other grain yield determinants. Interestingly, the same QTL was responsible for plant height, peduncle length and peduncle extrusion, which were considered by literature as adaptive traits under drought conditions.

NuTr-5H.3	09.1	FrH-2 (95.0)

Table 3.4. Grain yield and heading date QTL detected in the Italian Barley Mapping Population. Field trial number is according to Table 3.1, and chromosome BINs follow Aghnoum et al. 2010. Data of the last four columns, separated by commas, refer to and are ordered based on the â Field trialâ column. For the additive effect, positive values indicate that â Nureâ allele increases the trait value.

QTL	Field trial (#) a	Chr_BIN b	Peak marker (cM)	Peak position (cM) c	LOD c	R2% c	Additive c, d
Grain Yield							
QYld.NuTr-1H.1	5, 7, 12	1H_11.3	Hv347D22_HvFT3 (76.6)	74.2, 76.2, 79.6	3.5, 4.5, 3.4	11.9, 8.8, 8.4	-0.14, -0.27, -0.16
QYld.NuTr-2H	1, 3, 5, 7, 11, 12, 16, 18	2H_07.2	HvBM8 (78.7)	78.7, 78.3, 78.7, 78.7, 78.7, 78.7, 78.3, 78.7	3.3, 9.8, 5.1, 17.3, 8.1, 11.2, 4.6, 5.3	13.7, 35.5, 17.1, 45.8, 27.9, 32.6, 13.9, 21.0	0.03, 0.16, 0.17, 0.59, 0.23, 0.30, 0.20, 0.24
QYld.NuTr-5H.1	16	5H_02.2	bPb-0351 (17.9)	17.9	3.9	11.7	0.18
QYld.NuTr-5H.2	8	5H_08.2	HvABI5 (66.3)	62.4	4.0	18.2	0.15
QYld.	15	5H_	HvCBF_	95	5.2	13.4	0.45

	14, 16,			7, 78.7, 78.7, 78.	0, 20.8, 37.0, 22.	35.8,	2.69,
QYld.NuTr-5H.4	17, 18	5H_10.4	bPb-2325 (113.1)	117.5	6.5	19.4	0.23
QYld.NuTr-5H.5	2, 9, 10, 13, 15	5H_10.5	HvBM5A_VrnH1 (122.2)	119.6, 122.2, 122.2, 119.6, 122.2	5.8, 7.2, 6.3, 3.0, 5.9	22.9, 27.5, 24.5, 12.4, 15.4	0.06, 0.52, -0.56, -0.29, 0.48
QYld.NuTr-6H	11	6H_06.2	bPb-6735 (52.5)	52.5	2.9	9.0	-0.17
Heading Date							
QHd.NuTr-1H.1	8	1H_02.1	bPb-9414 (7.0)	7.0	5.8	5.7	0.82
QHd.NuTr-1H.2	2, 16	1H_09.2	bPb-7949 (54.6)	54.6, 54.6	5.2, 7.8	9.0, 7.2	0.98, 1.13
QHd.NuTr-1H.3	8, 11, 12, 14, 17, 18	1H_11.1	bPb-5249 (64.7)	64.7, 64.7, 64.7, 64.7, 64.7, 64.7	10.1, 5.1, 6.2, 5.4, 4.0, 5.2	11.0, 6.8, 5.7, 8.8, 6.4, 5.9	1.15, 0.72, 0.62, 1.13, 0.48, 0.78
QHd.NuTr-2H	2, 3, 4, 8, 9, 11, 12, 13,	2H_07.2	HvBM8 (78.7)	78.7, 78.7, 78.7, 78.7, 78.7, 78.7, 78.7, 78.	21.1, 11.7, 11.3, 26.9, 8.4, 25.1, 33.3, 22.	53.7, 37.6, 39.6, 45.0, 22.8, 54.8, 61.6,	-2.36, -3.10, -1.62, -2.26, -2.66, -2.05, -2.03, -

						49.5, 72.6, 57.0, 66.7	-2.70, -3.60, -1.44, -2.63
QHd.NuTr-3H.1	16	3H_13.1	Bmag0013 (138.9)	133.7	5.2	5.3	-0.80
QHd.NuTr-3H.2	8, 11, 12	3H_14.2	bPb-1481 (148.0)	148.0, 148.0, 147.2	6.3, 6.2, 4.5	6.3, 8.5, 4.2	-0.84, -0.80, -0.51
QHd.NuTr-4H	13	4H_12.3	ZCCT-H_VrnH2 (145.2)	145.2	6.4	7.1	1.18
QHd.NuTr-5H.1	9	5H_10.2	bPb-5596 (98.9)	98.9	7.9	21.2	-2.53
QHd.NuTr-5H.2	8, 13	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2, 121.6	7.3, 18.2	7.4, 26.8	0.93, 2.27
QHd.NuTr-5H.3	14	5H_13.3	bPb-6195 (168.7)	168.7	3.4	5.3	0.86
QHd.NuTr-6H	12	6H_06.1	HvCRY2 (46.5)	46.5	3.1	2.7	-0.56

Since we observed a main effect on both grain yield and heading date of a relatively small number of major loci, we decided to further investigate the behavior in terms of the two main adaptive traits, of the eight haplotype classes in which the NxT population

can be partitioned. Haplotype classes were based on the allelic state at the peak markers of the most represented QTLs: HvBM8 on chromosome 2H and HvBM5A on chromosome 5H for Yld, and HvBM8 and Pb-5249 on chromosome 1H for Hd (Fig. 3.3). A mixed model was thus fitted on all the available grain yield and days to heading data, considering both haplotypes and environments as fixed effects, while entries within haplotype random (Lacaze et al. 2009). A highly significant effect ($P < 0.0001$) of the QTL peak marker haplotypes on grain yield was observed, with a predominant role of HvBM8_eam6 /Eps-2 in determining higher grain yield in contrasting Mediterranean environments. Genotypes carrying the â Nureâ allele at this locus outperform the other two classes, independently from the alternate allelic state at HvBM5A_VRN-H1 and bPb-5249. As suggested by the co-mapping of a major QTL for earliness in 2H, these effects should be in large part related to differences in the number of days from sowing to heading. In fact, a highly significant effect of the haplotype classes was observed also on heading date, over 13 different environments ($P < 0.0001$). The four earliest flowering haplotypes also showed the highest grain yield, even if they resulted statistically more similar between each other with respect to what we have found considering Hd (Fig. 3.3)
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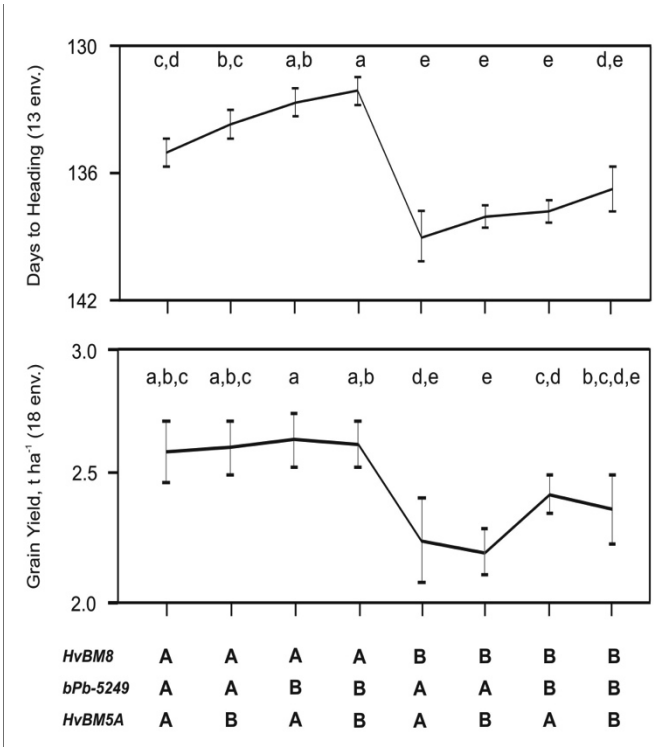

Figure 3.3. Average grain yield and heading date of the 118 NT-DH lines, divided into eight haplotypes. Haplotype classes were defined by the allelic state at the peak markers most frequently detected by QTL analyses for Hd (HvBM8 and bPb-5249) and Yld (HvBM8 and HvBM5A). Haplotypes are formed based on least square means differences after Tukey test (¸–=0.05). â Nureâ and â Tremoisâ alleles are indicated as â Aâ and â Bâ , respectively.

The single trait-single environment QTL analysis reported in this study suggests a strong relationship between loci responsible for yield components traits and the major developmental genes described above. In fact, the increase in grain yield associated to the â Nureâ allele at HvBM8 is related to a higher thousand grain weight in twelve locations out of fourteen, and to an increased number of spikes per square meter in three environments out of six, confirming, in a significant number of environments, the pleiotropic nature of the 2H QTL (Fig. 3.2 and Table 3.5). Notably, in the highest yielding environment #18-SYR_5W, the locus explained 71.5% of the recorded variation for Tgw. Harvest index was significantly controlled by the same â Nureâ allele in five

sites out of seven, with LOD scores reaching up to 19.9. Interestingly, an opposite negative effect was observed for the traits spike length (6 trials) and number of grains per spike (4 trials)

On chromosome 1H, Hv347D22_HvFT3 was the peak marker of a Hi QTL detected at three locations, with a positive contribution of the â Tremoisâ allele, as already observed for grain yield (Table 3.4). On the contrary, â Nureâ allele at three distinct regions of chromosome 1H (from 46 cM to 87 cM) was revealed by CIM analysis as associated to higher thousand grain weight, with a maximum additive effect of 1.49 (g). overlapping QTLs for grain yield and other yield components were also observed at the peak marker HvBM5_VRN-H1 (Fig. 3.2). In this QTL region, the â Nureâ allele increased the number of spikes per square meter in the TUR_4 dry and wet experiments (trials #9-TUR_4D and #15-TUR_4W), while having a negative effect on spike length (#4-JOR_5W and #15-TUR_4W) and harvest index (TUR_5). A further QTL for grain yield was mapped on chromosome 5H, at the HvCBF_FR-H2 frost resistance locus (Francia et al. 2004); it accounts for 13.4% of the phenotypic variance observed in #15-TUR_4W environment, with the â Nureâ allele increasing grain yield of 0.9 t ha-1. Co-location of QTL for frost resistance, early vigour, heading date, spike length and number of spikes per square meter has been detected at the same genomic region (Fig. 3.2 and Table 3.5).

A genotype by environment table of means has been used to estimate yield adaptability (Ya) and yield stability (Ys) parameters for the parental genotypes â Nureâ and â Tremoisâ and for the NT barley lines. QTL analyses then revealed that HvBM8 (eam6/ Eps-2) on 2H and HvCBF_FR-H2 on 5H were responsible for 10.2% and 11.7% of the phenotypic variation for yield adaptation, respectively (Fig. 3.2 and Table 3.5). About yield stability, only a rather low significant (LOD 2.7) effect has been detected for the bPb-4602 DArT marker on chromosome 3H, in a genomic region where no other QTLs were mapped (Fig. 3.2 and Table 3.5).

5D 07.2 2

Table 3.5. QTL detected in the NxT Mapping Population

a Chromosome BINs follow Aghnoum et al. 2010

b Positive values indicate that the â Nureâ allele increases the trait value

QTL	field trial	Chr_ BINa	Peak Marker (cM)	Peak position (cM)	LOD	R2%	Additiveb
Grain Yield	´	´	´	´	´	´	´
QYld.NuTr-1H.1	#5-JOR_4D	1H_11.3	Hv347D22_HvFT3 (76.6)	74.2	3.5	11.9	-0.14
	#7-SYR_5D	1H_11.3	Hv347D22_HvFT3 (76.6)	76.2	4.5	8.8	-0.27
	#12-ITA_5D	1H_11.3	Hv347D22_HvFT3 (76.6)	79.6	3.4	8.4	-0.16
QYld.NuTr-2H	#1-JOR_4W	2H_07.2	HvBM8 (78.7)	78.7	3.3	13.7	0.03
	#3-JOR_5D	2H_07.2	HvBM8 (78.7)	78.3	9.8	35.5	0.16
	#5-JOR_4D	2H_07.2	HvBM8 (78.7)	78.7	5.1	17.1	0.17
	#7-SYR_5D	2H_07.2	HvBM8 (78.7)	78.7	17.3	45.8	0.59
	#11-ITA_4W	2H_07.2	HvBM8 (78.7)	78.7	8.1	27.9	0.23
	#12-ITA_	2H_	HvBM8 (78.7)	78.7	11.	32.6	0.30

	#16-ITA_5F	2H_07.2	HvBM8 (78.7)	78.3	4.6	13.9	0.20
	#18-SYR_5W	2H_07.2	HvBM8 (78.7)	78.7	5.3	21.0	0.24
QYld.NuTr-5H.1	#16-ITA_5F	5H_02.2	bPb-0351 (17.9)	17.9	3.9	11.7	0.18
QYld.NuTr-5H.2	#8-ITA_4D	5H_08.2	HvABI5 (66.3)	62.4	4.0	18.2	0.15
QYld.NuTr-5H.3	#15-TUR_4W	5H_09.1	HvCBF_Fr-H2 (95.0)	95.0	5.2	13.4	0.45
QYld.NuTr-5H.4	#16-ITA_5F	5H_10.4	bPb-2325 (113.1)	112.5	6.5	19.4	0.23
QYld.NuTr-5H.5	#2-ESP_5D	5H_10.5	HvBM5A_VrnH1 (122.2)	119.6	5.8	22.9	0.06
	#9-TUR_4D	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	7.2	27.5	0.52
	#10-ALG_5W	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	6.3	24.5	-0.56
	#13-TUR_5	5H_10.5	HvBM5A_VrnH1 (122.2)	119.6	3.0	12.4	-0.29
	#15-TUR_4W	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	5.9	15.4	0.48
QYld.NuTr-6H	#11-ITA_4W	6H_06.2	bPb-6735 (52.5)	52.5	2.9	9.0	-0.17
Days to Heading	´	´	´	´	´	´	´

QHd.NuTr-1H.1	#3-ITA_4D	1H_02.1	bPb-9414 (7.0)	7.0	5.8	5.7	0.82
QHd.NuTr-1H.2	#2-ESP_5D	1H_09.2	bPb-7949 (54.6)	54.6	5.2	9.1	0.98
	#16-ITA_5F	1H_09.2	bPb-7949 (54.6)	54.6	7.8	7.2	1.13
QHd.NuTr-1H.3	#8-ITA_4D	1H_11.1	bPb-5249 (64.7)	64.7	10.1	11.0	1.15
	#11-ITA_4W	1H_11.1	bPb-5249 (64.7)	64.7	5.1	6.8	0.72
	#12-ITA_5D	1H_11.1	bPb-5249 (64.7)	64.7	6.2	5.7	0.62
	#14-SYR_4W	1H_11.1	bPb-5249 (64.7)	64.7	5.4	8.8	1.13
	#17-ITA_5W	1H_11.1	bPb-5249 (64.7)	64.7	4.0	6.4	0.48
	#18-SYR_5W	1H_11.1	bPb-5249 (64.7)	64.7	5.2	5.9	0.78
QHd.NuTr-2H	#2-ESP_5D	2H_07.2	HvBM8 (78.7)	78.7	21.1	53.7	-2.36
	#3-JOR_5D	2H_07.2	HvBM8 (78.7)	78.7	11.7	37.6	-3.10
	#4-JOR_5W	2H_07.2	HvBM8 (78.7)	78.7	11.3	39.6	-1.62
	#8-ITA_4D	2H_07.2	HvBM8 (78.7)	78.7	26.9	45.0	-2.26
	#9-TUR_4D	2H_07.2	HvBM8 (78.7)	78.7	8.4	22.8	-2.66
	#11-ITA_	2H_	HvBM8 (78.7)	78.7	25.	54.8	-2.05

	#12-ITA_5D	2H_07.2	HvBM8 (78.7)	78.7	33.3	61.6	-2.03
	#13-TUR_5	2H_07.2	HvBM8 (78.7)	78.7	22.0	35.8	-2.69
	#14-SYR_4W	2H_07.2	HvBM8 (78.7)	78.7	20.8	49.5	-2.70
	#16-ITA_5F	2H_07.2	HvBM8 (78.7)	78.7	37.0	72.6	-3.60
	#17-ITA_5W	2H_07.2	HvBM8 (78.7)	78.7	22.8	57.0	-1.44
	#18-SYR_5W	2H_07.2	HvBM8 (78.7)	78.7	30.7	66.7	-2.63
QHd.NuTr-3H.1	#16-ITA_5F	3H_13.1	Bmag0013 (138.9)	133.7	5.2	5.3	-0.80
QHd.NuTr-3H.2	#8-ITA_4D	3H_14.2	bPb-1481 (148.0)	148	6.3	6.3	-0.84
	#11-ITA_4W	3H_14.2	bPb-1481 (148.0)	148	6.2	8.5	-0.80
	#12-ITA_5D	3H_14.2	bPb-1481 (148.0)	147.2	4.5	4.2	-0.51
QHd.NuTr-4H	#13-TUR_5	4H_12.3	ZCCT-H_VrnH2 (145.2)	145.2	6.4	7.1	1.18
QHd.NuTr-5H.1	#9-TUR_4D	5H_10.2	bPb-5596 (98.9)	98.9	7.9	21.2	-2.53
QHd.NuTr-5H.2	#8-ITA_4D	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	7.3	7.4	0.93
	#13-TUR_5	5H_10.5	HvBM5A_VrnH1 (122.2)	121.6	18.2	26.8	2.27

QHd.NuTr-5H.3	#14-SYR_4W	5H_13.3	bPb-6195 (168.7)	168.7	3.4	5.3	0.86
QHd.NuTr-6H	#12-ITA_5D	6H_06.1	HvCRY2 (46.5)	46.5	3.1	2.7	-0.56
Plant Height	´	´	´	´	´	´	´
QHt.NuTr-1H.1	#8-ITA_4D	1H_09.1	bPb-1541 (55.1)	58.1	2.9	12.7	-1.89
QHt.NuTr-1H.2	#10-ALG_5W	1H_10.2	bPb-7609 (61.2)	61.2	3.2	7.0	1.32
	#14-SYR_4W	1H_10.2	bPb-7609 (61.2)	61.2	2.9	12.2	2.02
QHt.NuTr-2H.1	#6-SYR_4D	2H_07.2	HvBM8 (78.7)	78.7	4.2	15.0	-1.62
	#9-TUR_4D	2H_07.2	HvBM8 (78.7)	78.7	4.1	16.7	-3.16
	#13-TUR_5	2H_07.2	HvBM8 (78.7)	78.7	12.6	43.2	-4.50
	#15-TUR_4W	2H_07.2	HvBM8 (78.7)	78.3	5.9	23.2	-3.91
	#17-ITA_5F	2H_07.2	HvBM8 (78.7)	78.7	16.5	52.2	-3.66
	#18-SYR_5W	2H_07.2	HvBM8 (78.7)	78.7	4.4	12.6	-1.46
QHt.NuTr-2H.2	#10-ALG_	2H_11.3	E42M32_378 (120.6)	119.3	5.1	11.8	-1.63

3H	TUR_5	06.1	(56.7)				
QHt.NuTr-4H	#18-SYR_5W	4H_09.1	bPb-3809 (76.5)	76.2	4.1	11.5	1.39
QHt.NuTr-5H.1	#6-SYR_4D	5H_02.3	bPb-6183 (26.7)	26.7	3.4	12.0	-1.42
QHt.NuTr-5H.2	#7-SYR_5D	5H_05.1	E35M61_117 (43.3)	43.3	3.8	13.7	-1.46
QHt.NuTr-5H.3	#8-ITA_4D	5H_11.2	bPb-2580 (124.7)	128.7	6.6	30.9	-2.91
	#10-ALG_5W	5H_11.2	bPb-2580 (124.7)	128.7	10.4	29.6	-2.63
	#18-SYR_5W	5H_11.2	bPb-2580 (124.7)	129.7	9.4	35.3	-2.43
QHt.NuTr-5H.4	#1-JOR_4D	5H_12.1	bPb-4318 (142.4)	151.4	4.6	20.3	-2.30
	#4-JOR_5W	5H_12.1	bPb-4318 (142.4)	145.4	3.2	14.7	-1.48
	#7-SYR_5D	5H_12.1	bPb-4318 (142.4)	142.4	3.5	12.5	-1.39
QHt.NuTr-5H.5	#3-JOR_5D	5H_13.3	bPb-3138 (171.8)	171.8	3.9	16.1	-1.48
Early growth Vigour	´	´	´	´	´	´	
QEv.NuTr-2H	#18-SYR_5W	2H_07.2	HvBM8 (78.7)	78.7	6.6	18.5	-0.16
QEv.NuTr-	#13-	3H_	E41M38_307	56.7	3.0	10.2	-0.11

2H.2	5D	07.2					
QEv.NuTr-5H.1	#14-SYR_4W	5H_09.1	HvCBF_Fr-H2 (95.0)	95	4.4	17.8	0.20
	#15-TUR_4W	5H_09.1	HvCBF_Fr-H2 (95.0)	95	5.1	13.4	0.31
	#18-SYR_5W	5H_09.1	HvCBF_Fr-H2 (95.0)	95	5.8	15.9	0.15
QEv.NuTr-5H.2	#13-TUR_5	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	4.8	16.6	-0.14
	#15-TUR_4W	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	5.5	14.7	0.32
QEv.NuTr-6H	#6-SYR_4D	6H_06.1	HvWRKY38 (46.7)	46.7	3.5	14.6	-0.18
	#18-SYR_5W	6H_06.1	HvWRKY38 (46.7)	46.7	3.7	9.7	-0.16
Days to Maturity	´	´	´	´	´	´	´
QMd.NuTr-1H	#4-JOR_5W	1H_09.1	bPb-1541 (55.1)	56.1	4.1	9.5	0.60
	#8-ITA_4D	1H_09.1	bPb-1541 (55.1)	61.1	11.6	15.8	1.15
	#11-ITA_4W	1H_09.1	bPb-1541 (55.1)	55.1	9.5	15.6	1.02
QMd.NuTr-2H.1	#11-ITA_4W	2H_06.2	HvFT4 (68.9)	68.9	4.1	6.0	0.94
QMd.NuTr-	#3-JOR_	2H_	HvBM8 (78.7)	78.7	5.3	18.3	-2.12

	#4-JOR_5W	2H_07.2	HvBM8 (78.7)	78.7	13.5	37.2	-1.19
	#5-JOR_4D	2H_07.2	HvBM8 (78.7)	78.3	4.1	16.7	-0.79
	#8-ITA_4D	2H_07.2	HvBM8 (78.7)	78.7	18.0	32.5	-1.61
	#11-ITA_4W	2H_07.2	HvBM8 (78.7)	78.7	11.1	20.7	-1.74
	#13-TUR_5	2H_07.2	HvBM8 (78.7)	78.7	13.3	27.1	-1.73
QMd.NuTr-3H.1	#3-JOR_5D	3H_05.1	bPb-2324 (43.1)	48.1	3.5	11.6	1.66
QMd.NuTr-3H.2	#11-ITA_4W	3H_14.2	bPb-1481 (148.0)	148	6.3	8.8	-0.82
	#8-ITA_4D	3H_14.2	bPb-1481 (148.0)	148	3.3	4.9	-0.52
QMd.NuTr-4H	#13-TUR_5	4H_12.3	ZCCT-H_VrnH2 (145.2)	142.9	7.6	15.7	1.29
QMd.NuTr-5H.1	#4-JOR_5W	5H_10.3	E39M61_229 (107.6)	107.6	5.4	12.4	0.69
QMd.NuTr-5H.2	#13-TUR_5	5H_10.5	HvBM5A_VrnH1 (122.2)	120.6	10.3	19.8	1.47
QMd.NuTr-5H.3	#8-ITA_4D	5H_11.2	bPb-2580 (124.7)	133.7	5.6	9.8	0.91
	#11-ITA_4W	5H_11.2	bPb-2580 (124.7)	129.7	6.2	10.6	0.81
QMd.NuTr-6H	#8-ITA_4D	6H_06.1	HvCRY2 (46.5)	46.5	9.5	14.0	-1.42
	#11-ITA_4W	6H_06.1	HvCRY2 (46.5)	46.5	10.4	17.6	-1.30

Spikes per spike length	´	´	´	´	´	´	´
QSt.NuTr-2H	#6-SYR_4D	2H_07.2	HvBM8 (78.7)	78.7	4.1	16.8	-0.47
	#9-TUR_4D	2H_07.2	HvBM8 (78.7)	78.3	7.1	23.0	-0.70
	#13-TUR_5	2H_07.2	HvBM8 (78.7)	78.3	11.2	35.3	-0.56
	#14-SYR_4W	2H_07.2	HvBM8 (78.7)	78.7	2.8	11.9	-0.31
	#15-TUR_4W	2H_07.2	HvBM8 (78.7)	78.3	14.7	42.3	-1.09
	#18-SYR_5W	2H_07.2	HvBM8 (78.7)	78.7	7.2	27.6	-0.44
QSl.NuTr-3H	#3-JOR_5D	3H_01.1	bPb-1264 (8.2)	6.9	3.6	14.9	0.52
QSl.NuTr-4H	#13-TUR_5	4H_09.1	bPb-3809 (76.5)	76.5	4.2	11.3	0.32
QSl.NuTr-5H.1	#9-TUR_4D	5H_10.2	bPb-7395 (99.1)	99.1	3.8	11.4	-0.48
QSl.NuTr-5H.2	#4-JOR_5W	5H_10.5	HvBM5A_VrnH1 (122.2)	123.1	2.9	12.3	-0.23
	#15-TUR_4W	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	6.6	16.0	-0.67
Number of	´	´	´	´	´		

QSsm.NuTr-2H	#8-ITA_4D	2H_07.2	HvBM8 (78.7)	78.7	3.1	10.6	26.61
	#9-TUR_4D	2H_07.2	HvBM8 (78.7)	78.7	3.0	8.4	44.23
	#15-TUR_4W	2H_07.2	HvBM8 (78.7)	78.7	3.1	8.7	46.28
QSsm.NuTr-4H	#8-ITA_4D	4H_09.1	bPb-3809 (76.5)	77.5	5.5	20.0	-36.03
QSsm.NuTr-5H.1	#9-TUR_4D	5H_09.1	HvCBF_Fr-H2 (95.0)	95	3.1	8.5	48.60
	#15-TUR_4W	5H_09.1	HvCBF_Fr-H2 (95.0)	95	3.9	11.0	56.85
QSsm.NuTr-5H.2	#9-TUR_4D	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	3.9	11.0	54.54
	#15-TUR_4W	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	2.9	8.2	48.32
Number of grains per spike	´	´	´	´	´	´	
QGps.NuTr-2H.1	#8-ITA_4D	2H_07.2	HvBM8 (78.7)	78.7	12.5	42.8	-1.19
	#9-TUR_4D	2H_07.2	HvBM8 (78.7)	78.7	5.0	20.2	-1.62
	#11-ITA_4W	2H_07.2	HvBM8 (78.7)	78.3	10.9	37.2	-1.36

	#15-TUR_4W	2H_07.2	HvBM8 (78.7)	78.3	4.9	19.7	-1.55
QGps.NuTr-2H.2	#11-ITA_4W	2H_11.2	bPb-0994 (114.9)	114.9	5.0	14.8	0.84
Harvest Index	´	´	´	´	´	´	´
QHi.NuTr-1H.1	#6-SYR_4D	1H_09.1	bPb-1541 (55.1)	58.1	3.8	14.7	-2.36
QHi.NuTr-1H.2	#7-SYR_5D	1H_11.3	Hv347D22_HvFT3 (76.6)	76.6	12.5	21.5	-4.63
	#13-TUR_5	1H_11.3	Hv347D22_HvFT3 (76.6)	79.6	4.8	14.8	-1.93
	#18-SYR_5W	1H_11.3	Hv347D22_HvFT3 (76.6)	79.6	5.8	17.9	-1.53
QHi.NuTr-2H.1	#13-TUR_5	2H_05.2	bPb-5629 (56.2)	56.2	3.2	9.7	1.46
QHi.NuTr-2H.2	#3-JOR_5D	2H_07.2	HvBM8 (78.7)	78.3	7.3	28.7	2.55
	#5-JOR_4D	2H_07.2	HvBM8 (78.7)	78.7	9.7	30.5	3.64
	#6-SYR_4D	2H_07.2	HvBM8 (78.7)	78.7	5.9	20.7	2.79
	#7-SYR_5D	2H_07.2	HvBM8 (78.7)	78.7	19.9	40.3	6.08
	#18-SYR_5W	2H_07.2	HvBM8 (78.7)	78.7	4.9	16.6	1.38
QHi.NuTr-4H	#10-ALG_	4H_12.3	ZCCT-H_VrnH2 (145.2)	143.9	4.3	18.9	3.83

	5D	07.2			7		
QHi.NuTr-5H	#13-TUR_5	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	3.3	10.1	-1.50
Thousand Grain Weight	´	´	´	´	´	´	
QTgw.NuTr-1H.1	#9-TUR_4D	1H_08.1	E41M38_206 (46.2)	46.2	7.1	21.1	1.32
	#14-SYR_4W	1H_08.1	E41M38_206 (46.2)	46.2	4.1	6.6	0.86
QTgw.NuTr-1H.2	#13-TUR_5	1H_09.1	bPb-1541 (55.1)	57.1	8.8	25.7	1.49
QTgw.NuTr-1H.3	#4-JOR_5W	1H_12.2	bPb-6343 (87.5)	87.5	5.1	14.9	1.09
	#15-TUR_4W	1H_12.2	bPb-6343 (87.5)	84.2	5.1	11.6	1.18
QTgw.NuTr-2H	#4-JOR_5W	2H_07.2	HvBM8 (78.7)	78.7	6.1	18.3	1.14
	#6-SYR_4D	2H_07.2	HvBM8 (78.7)	78.7	9.9	32.4	1.71
	#7-SYR_5D	2H_07.2	HvBM8 (78.7)	78.7	20.8	60.5	2.50
	#8-ITA_4D	2H_07.2	HvBM8 (78.7)	78.7	9.2	30.3	1.59
	#9-TUR_4D	2H_07.2	HvBM8 (78.7)	78.7	7.0	21.0	1.29
	#11-ITA_4W	2H_07.2	HvBM8 (78.7)	78.7	9.0	31.0	2.01
	#12-ITA_	2H_	HvBM8 (78.7)	78.3	10.	38.7	2.36

7H	4D	11.2	110.6)				
	#13-TUR_5	2H_07.2	HvBM8 (78.7)	78.7	8.3	22.5	1.40
	#14-SYR_4W	2H_07.2	HvBM8 (78.7)	78.7	21.6	53.6	2.47
	#15-TUR_4W	2H_07.2	HvBM8 (78.7)	78.7	11.9	32.1	1.82
	#17-ITA_5W	2H_07.2	HvBM8 (78.7)	78.7	21.2	57.4	3.37
	#18-SYR_5W	2H_07.2	HvBM8 (78.7)	78.7	28.1	71.5	3.24
QTgw.NuTr-3H	#13-TUR_5	3H_13.1	Bmag0013 (138.9)	138.9	3.0	7.2	0.78
QTgw.NuTr-4H	#4-JOR_5W	4H_09.1	bPb-3809 (76.5)	77.5	3.1	8.9	0.78
	#8-ITA_4D	4H_09.1	bPb-3809 (76.5)	77.5	3.3	9.6	0.88
	#14-SYR_4W	4H_09.1	bPb-3809 (76.5)	77.5	5.1	8.1	0.95
	#15-TUR_4W	4H_09.1	bPb-3809 (76.5)	77.5	5.9	13.7	1.17
	#17-ITA_5W	4H_09.1	bPb-3809 (76.5)	77.5	3.4	5.9	1.07
QTgw.NuTr-5H	#6-SYR_4D	5H_02.4	bPb-6495 (28.7)	28.7	3.0	8.3	0.85
QTgw.NuTr-	#9-TUR_	7H_	bPb-0889 (	109.7	4.0	11.6	0.96

	#11-ITA_4W	7H_11.2	bPb-0889 (110.6)	110.6	2.9	8.6	1.04
Peduncle length	´	´	´	´	´	´	´
QPl.NuTr-2H.1	#15-TUR_4W	2H_07.2	HvBM8 (78.7)	78.3	5.9	23.1	-3.29
QPl.NuTr-2H.2	#6-SYR_4D	2H_08.1	bPb-5440 (85.2)	93.2	5.7	28.8	1.27
QPl.NuTr-4H	#9-TUR_4D	4H_09.1	bPb-3809 (76.5)	76.5	3.0	12.5	0.96
Peduncle extrusion	´	´	´	´	´	´	
QPe.NuTr-4H	#15-TUR_4W	4H_09.1	bPb-3809 (76.5)	77.5	2.8	11.8	0.80
Frost resistance	´	´	´	´	´	´	´
QFr.NuTr-5H.1	#2-ESP_5D	5H_09.1	HvCBF_Fr-H2 (95.0)	95.0	10.4	20.4	-0.56
	#16-ITA_5F	5H_09.1	HvCBF_Fr-H2 (95.0)	95.0	3.5	6.5	-0.19
QFr.NuTr-5H.2	#2-ESP_5D	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	9.6	18.5	-0.53
	#16-ITA_5F	5H_10.5	HvBM5A_VrnH1 (122.2)	122.2	13.9	33.1	-0.42
Reaction to powdery mildew	´	´	´	´	´	´	

QPm.NuTr-1H.1	#11-ITA_4W	1H_02.1	bPb-8973 (6.9)	8.9	8.0	20.3	0.43
	#16-ITA_5F	1H_02.1	bPb-8973 (6.9)	7.9	9.0	29.5	1.08
QPm.NuTr-1H.2	#11-ITA_4W	1H_11.1	bPb-5249 (64.7)	64.5	5.2	12.5	-0.33
QPm.NuTr-1H.3	#11-ITA_4W	1H_14.2	bPb-0589 (111.4)	111.4	3.1	7.0	-0.25
QPm.NuTr-1H.4	#8-ITA_4D	1H_14.3	bPb-2260 (115.3)	115.3	3.3	11.5	-0.34
QPm.NuTr-5H	#8-ITA_4D	5H_12.1	bPb-5379 (139.8)	139.8	4.4	15.7	-0.39
QPm.NuTr-6H	#11-ITA_4W	6H_06.1	bPb-5196 (47.0)	47.0	6.0	14.6	-0.51
Length of Grain Filling Period	´	´	´	´	´	´	
QGfp.NuTr-1H	#8-ITA_4D	1H_02.1	bPb-8973 (6.9)	7	3.1	11.3	-0.54
QGfp.NuTr-2H	#8-ITA_4D	2H_07.2	HvBM8 (78.7)	78.7	4.3	15.8	0.64
	#11-ITA_4W	2H_07.2	HvBM8 (78.7)	78.7	8.3	27.1	0.94
	#13-TUR_5	2H_07.2	HvBM8 (78.7)	78.7	8.9	25.1	0.97
QGfp.NuTr-5H	#13-TUR_5	5H_10.5	HvBM5A_VrnH1 (122.2)	120.6	7.6	20.0	-0.85
QGfp.NuTr-6H	#11-ITA_4W	6H_07.2	bPb-4783 (57.4)	57.4	3.9	11.5	-0.74
Yield Stability	´	´	´	´	´	´	´

QYs.NuTr-3H	Across 18 sites	3H_07.1	bPb-4602 (64.6)	64.6	2.7	10.6	0.07
Yield Adaptability	´	´	´	´	´	´	´
QYa.NuTr-2H	Across 18 sites	2H_07.2	Bmac0273C (79.9)	80.9	2.8	10.2	0.03
QYa.NuTr-5H	Across 18 sites	5H_09.1	HvCBF_Fr-H2 (95.0)	95	3.2	11.7	0.03

3.3 Discussion

The first objective of this study was to increase the marker density of the â Nureâ x â Tremoisâ linkage map. The DArT marker platform allowed us to build a genetically denser NT map, made publicly available (http://wheat.pw.usda.gov/GG2/index.shtml), that was also used to integrate powdery mildew resistance QTLs into a high density consensus map (Aghnoum et al. 2010). Even if the completion of the barley genome sequence is expected in the near future, the development of gene-targeted markers (GTM; Andersen and Lˆ…bberstedt 2003) starting from the great deal of available ESTs is still worthy for a candidate gene approach aiming at identifying the genetic basis of complex traits. The dense barley transcript maps published by Rostoks et al. (2005), Varshney et al. (2007), Stein et al. (2007) and Sato et al. (2009), as well as the recently available gene-based OPA map (Szucs et al. 2009) could represent a starting point for the identification of associations between QTLs for agronomic traits and GTMs with polymorphisms designed on genes with known or putative function. Considering the importance of phenology in barley adaptation to drought-prone areas, in this study we followed the same approach by targeted mapping of transcription factors that may be involved in the regulation of flowering time, plant development and adaptation to the environment (Table 3.1). The genetic determinants of barley response to environmental stimuli (such as increasing day length and temperature changes) have in fact been widely recognized as major components of barley adaptation under water-limiting

conditions (Cuesta-Marcos et al. 2008a; Wang et al. 2010). The interaction between the vernalization and photoperiod response pathways results in a gradient of phenological adjustments that can be used for fine-tuning the regulation of heading date. One of the main interests for the development and characterization of the IBMP population was to study in one of the few "winter x spring" habit bi-parental cross the genetic relationships between Vrn/Fr, Ppd and Eam/Eps genes. In a previous report on the same experimental dataset, Francia et al. (2011) interpreted the grain yield variation observed in the IBMP population in terms of mean differences between genotypes (G), environments (E), and genotypeˆ environment (GE) interaction. Moreover, by studying grain yield and its components as a function of length of the different developmental phases from sowing to maturity, it was confirmed that in environments characterized by terminal drought events the best performing (adapted) NT genotypes were those capable of rapidly reaching the most critical stage for grain yield determination in barley, i.e. the period prior anthesis (Francia et al. 2011). In the present work a classical quantitative genetic study has been pursued to search, with a map-referenced and allele-weighting methodology, the strong relationship between Yld and Hd QTLs. Moreover, to study map-referenced association of grain yield QTLs with a significant set of other developmental, morpho-agronomic and yield component traits collected from one of the largest multi-environment field trial surveys made for a Triticeae species across the Mediterranean basin (Comadran et al. 2008).

As a key finding, a large effect on both grain production and flowering time was associated with the early flowering allele from the winter parent â Nureâ at the eam6/ Eps-2 locus, in coincidence with the HvBM8 GTM (Fig. 3.2 and Table 3.4). The same allele significantly increased grain yield in four poorly yielding, high stressed environments where the average yield was <2.5 t ha-1, as well as in four >2.5 t ha-1 mo re fertile and water supplied sites. Most importantly in fact, no yield penalty due to the early heading was observed in the highest yielding environments (>4.5 t ha-1; Table 3.4) . Even if the importance of earliness for escaping terminal drought and heat stress that frequently occur under Mediterranean-like conditions is well recognized (e.g. Araus et al. 2008), the stable effect of Eam6 we have observed across very different agro-meteorological conditions reveals its more general role in wide adaptation. This is

further confirmed by the detection of a QTL for yield adaptability at the same genomic locus (Fig. 3.2). The importance of this chromosomal region, most likely due to the same locus, has also been highlighted in other studies on bi-parental populations evaluated in METs (Cuesta-Marcos et al. 2008a; von Korff et al. 2008), as well as in a genome-wide association study performed on a panel of diverse cultivated barley lines (Comadran et al. 2011). In developed agricultures, breeders' work led to a relative optimization of crop flowering time in Triticeae, and from this experience it was proposed to focus more the near future research on optimizing pre-heading phases (i.e. from sowing to the onset of stem elongation, and from then to heading), as on managing biomass and ensuring effective remobilization of assimilates to grain (Borrˆ s-Gelonch et al. 2010; Fleury et al. 2010). The available data on the NT population did not allow the dissection of the stem elongation phase, that some authors described as of special relevance for grain yield determination (Araus et al. 2008). However, earliness per se loci were shown to represent an untapped source of variation for targeted breeding, when precise markers available, and can play a crucial role in determining early heading/early ripening to maximize yield potential both when vernalization and photoperiod requirement are satisfied and in the insensitive genotypes. Noteworthy, Eps genes can influence other grain yield components as shown in this work, and as also shown in diploid wheat, where the cloning of Eps-Am1, a gene affecting the timing of the transition between the vegetative and reproductive stages, the duration of spike development and the number of spikelets and grains per spike is underway (Faricelli et al. 2010). In our study, earliness due to Eam6 was associated to a shorter spike and to a smaller number of grains per spike, but on the contrary to a higher thousand grain weight, higher harvest index, and a higher number of spikes per square meter (Table 3. 5). The results seem to point out that â lower value" alleles associated with earliness (i.e. shorter spike, low number of grains per spike) can be largely counterbalanced by " higher value" alleles for tgw and grains per square meter in determining yield adaptation of the NT population. Further experiments are underway for a better description of eam6 (J. Comadran, unpublished); however, the lack of recombination we observed in the centromeric region of chromosome 2H could make it difficult to test the single gene or multi-genic nature of the locus, and to verify how the HvBM8 MADS-box gene could be

a potential candidate to explain the locus. In this work we also found a clear involvement of another region, from 64.7 to 79.6 cM, on the long arm of chromosome 1H to determine both grain yield and heading date(Fig. 3.2 and Table 3.4); however, Yld and Hd QTL are not exactly overlapping at this locus. Based on these observations, we can hypothesize that Ppd-H2, a major determinant of heading date in Mediterranean environments (Iguarta et al. 1999; von Korff et al. 2008; Cuesta-Marcos et al. 2008a, 2008b), or a second relevant gene are segregating in the NT population.

As already observed by Slafer (2008), MAS may be pertinent for manipulating phenology when breeding for adaptation to Mediterranean rain-fed environments. Based on the most significant detected loci we tried to define the best Hd loci haplotype to get yield adaptation to such contrasting environments. Our results showed that the â Nureâ allele at HvBM8 (eam6/Eps-2) was sufficient to determine a higher grain yield in contrasting Mediterranean environments, predominantly on Ppd-H2 (bPb-5249) and Vrn-H1 alleles (Fig. 3.3). In addition, a general pattern due to the positive contribution of the early (Tremois) allele at bPb-5249 can be noticed, at least in absolute terms. Comadran et al. (2011), in a genetically wide association mapping panel representative of the Mediterranean gene-pool, found Eam6 as being the main driver of flowering time in the same environments. The NT population has been widely exploited to study barley tolerance to low temperatures, due to the segregation of both Frost resistance-H1 (Fr-H1), coincident with Vrn-H1, and Frost resistance-H2 (Fr-H2), coincident with a cluster of CBF genes (Francia et al. 2004, 2007; Knox et al. 2010). Interestingly, HvCBF_FR-H2 coincides with the most important yield adaptability (Ya) QTL we found across the 18 field trials. Even if the involvement of CBF/DREB transcription factor in barley response to drought stress has been reported (Skinner et al. 2005) and cannot be excluded here, we hypothesize FR-H loci could have had a prominent role in the best establishment of plant juvenile phase after autumn sowing, as suggested by the co-mapping of other QTLs for frost resistance, early vigour and number of spikes per square meter. The only yield stability (Ys) QTL was mapped at the BIN 7_1 on chromosome 3H, at a genomic position where plasticity QTL has been previously detected in barley by Lacaze et al. (2009), by using a similar stability parameter based on Finlay and Wilkinsonâ s regression. Plasticity was intended by the authors as the

variation in phenotypic traits produced by a genotype in different environments (Lacaze et al. 2009). Alleles at loci that affect this phenotypic variation should therefore be considered as determinants of plasticity. However, unlike Lacaze et al. (2009), in the present paper no other individual trait QTL were mapped in its coincidence. Among the genetic models proposed by Via et al. (1995) for explaining phenotypic plasticity, our data could support a gene regulation model where loci affecting only plasticity and involving regulatory, environment-sensitive genes, mediate the expression of constitutive genes that actually determine the trait. Several abiotic stress responsive genes and their regulators are present on the NT map (Fig. 3.2; Tondelli et al. 2006). However, only the â Nureâ allele of the HvABI5 bZIP transcription factor significantly increased barley yield in environment #8-ITA_4D (Table 3.4, LOD: 4.0, R2%: 18.2) HvABI5 maps to chromosome 5H-BIN8, a genomic region where multiple loci for abiotic stress tolerance co-locate (Pecchioni et al. 2011). As observed by Araus et al. (2008), selection under drought conditions can result in plants with high dehydration tolerance, but lower yield potential, and for this reason the development of drought-resistant cultivars has benefited more from genes that control constitutive traits than from drought-responsive genes. However, in the future the introduction in high yielding genotypes of traits able to improve drought tolerance per se without detrimental effects on yield potential is crucial for future progress in cereal rainfed cropping around the Mediterranean basin (Cattivelli et al. 2008). In conclusion, the present work represents one of the most detailed QT characterizations of a barley segregating population grown under different water regimes, for several agronomic traits. Despite the heterogeneity of the environmental conditions, we have identified genomic regions consistently associated to yield. The results here shown complement by a linkage mapping approach the detailed analyses on genotype x environment and QTL x environment (meteo variable) interactions carried out on the same multi-environment dataset (Francia et al. 2011; A. Visioni et al. unpublished). Moreover, they provide a valuable source of knowledge and tools for explaining the genetic bases of barley yield adaptation across the Mediterranean basin. Further advancements could be reached exploiting complementary approaches of linkage and association mapping through a joint analysis of data coming from different barley populations evaluated in the same environments,

namely a second bi-parental population (â Henniâ x â Meltanâ , Borras et al. 2010) and a Diverse Barley Germplasm panel (Comadran et al. 2008, 2009, 2011).

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Chapter 4

Effect of temperature on eam6/Eps2 and Vrn-H1 loci by environment interaction in multi-environment barley trials across the Mediterranean Basin.

4.1 Introduction

Under rainfed Mediterranean environments growth and grain yield are influenced by the ability of crops to escape unfavorable conditions, by avoiding stress events or by avoiding the coincidence of the most sensitive growing phases with occurrence of stress. Barley is more sensitive to drought stress just before and during spike emergence, during anthesis and at the initial stages of grain development (Snape et al. 2001). The severity of drought stress from the beginning of anthesis to maturity could be prejudicial for grain development and consequently for yield determination. Drought stress reduces the net leaf photosynthetic rate, leaf transpiration rate and leaf water potential (Samarah, 2005). For this reasons one of the strategies that have been proposed to maximize grain yield (GY) of cereals, is tailoring the life cycle of the plants to the agro-environments in which they are grown (Cockram et al, 2007). In fall-sown cereals like barley, the number of days occurring from sowing to heading (days to heading - DtH) is the final result of a number of interacting characters that include vernalization requirement, photoperiod sensitivity, and earliness per se (Karsai et al., 1997). These three components also have pleiotropic effects on other factors that control plant growth and development. Vernalization has major effects on the rate of primordia production,

whilst photoperiod affects the timing of terminal spikelet production and stem elongation, and these effects are influenced by sowing date (Snape et al. 2001). Earliness per se explains differences in heading when vernalization and photoperiod requirements are fully satisfied (Appendino and Slafer 2003). For these reasons DtH is considered one of the most important adaptative characteristics of plants, and genetic regulation of this physiological process acts to ensure that flowering occurs at seasonal optima for pollination and seed development (Karsai et al. 2008). DtH also determines the duration of other crop development phases and, indirectly, the production of dry matter, the numbers of structures that contribute to final yield (tillers, spikes and grains) and also the way in which dry matter is portioned (Slafer et al. 1994, Boyd et al. 1996, Borras et al. 2009). Several Quantitative Trait Loci (QTLs) and underlying genes regulating this important trait have been detected and cloned in barley. Among them the two major vernalization genes Vrn-H1 and Vrn-H2 on chromosome 5H and 4H, respectively - control transition from vegetative to reproductive phase and growth habit in most of the cultivated germplasm (Francia et al. 2004; von Ziztewitz et al. 2005; Sz¯–cs et al. 2007; Cockram et al. 2007). Ppd-H1 and Ppd-H2 on chromosomes 2H and 1H are main actors of photoperiod response, respectively in long day and short day conditions (Laurie et al. 1995; Turner et al. 2005). Variation in flowering time is also influenced by the additional loci whose effects were not specifically dependent on photoperiod or vernalization, and thus called Earliness per se (Eps) genes. Their number is higher respect to the previous master switches, and in barley at least eight have been identified as esp2S on chromosome 2H (bin 6), eps3L on 3H (bin 13), eps4L on 4HL, eps5L on 5H (bin 6) eps6L.1, esp6L.2 on 6H (bin 7,13), eps7S and eps7L on 7H (bin 3, 12) (Laurie et al. 1995). Under water or nutrient deficit the loci regulating DtH often become grain yield determinants because the duration of crop cycle affect the timing and intensity of the stress experienced by plants (Reynolds and Tuberosa 2008; Francia et al. 2011). During pre-anthesis, the success of floret set defines the potential grain number (Gonzalez et al. 2003, Slafer et al. 2007), while final grain weight relies on post-anthesis conditions favoring grain filling (Ugarte et al. 2007). Grain yield in cereals is influenced by genotype (G), environment (E) and genotype by environment (GE) interaction. GE can be defined as the variation in relative performance of genotypes in

different environments (Cooper and Bith 1996) and is important in plant breeding because it complicates testing and selection of superior genotypes, thereby reducing genetic progress (Romagosa 2009). Generally there are two types of GE interaction called quantitative and qualitative interaction (De Kroon and van der Laan, 1981). Quantitative interaction is a change of magnitude of differences among genotypes in different test environments without any rank changes. Change in rank orders, or " crossover" interaction, is the qualitative interaction, and is the most important in plant breeding because it prevents prediction of genotype performance in different locations (genotype by location interaction), during different years (genotype by year interaction), or both (Baker 1988). In the last two decades many QTLs for DtH and GY have been identified, but only a small part of these had direct impact on breeding programs, most likely because of GE interaction, more important for these traits than for other ones. Several studies have been published on GE interaction for yield, but there is no much empirical data on GE studies that introduce external environmental, physiological and / or genetic information as factors underlying GE in the form of co-variables useful to describe GE patterns (see, e.g. Romagosa et al. 2009). The initial study of the genetic bases of GE in barley, simply mapped QTLs responsible of yield GE variation to chromosomes 2H, 3H, 5H and 6H (Romagosa et al. 1999). A second approach focused at the modeling of QTL expression in its dependence by environmental (E) factors (Malosetti et al. 2004). For a better understanding of the genetic architecture of quantitative traits as observed across environments, genotype by environment (QTLE) interaction should in fact be investigated with statistical models that use explicit information on genotypes and environments (van Eeuwijk et al. 2005; Boer et al. 2007). Malosetti et al. (2004) newly defined GE interaction as non-parallelism between phenotypic responses to key environmental factors and genotypes. QTLs effects across environments can be tested for dependence on particular environment covariables (Crossa et al. 1999, Malosetti et al. 2004, Vargas et al 2006). If climatic data are available (e.g. precipitations, temperature and solar radiation), Factorial Regression models can be used to determine the degree to which each of these factors influence GE interaction or QTLE interaction (van Eeuwijk et al., 1996). Bi-parental populations and wide germplasm collections have been intensively used by breeders to dissect

complex traits such as grain yield (GY) and flowering time (DtH), and to understand crop adaptability to stress-prone environments through the combination of multi-environment trials and QTLs analysis or genome wide association studies (Psawarayi et al 2008; Comadran et al. 2008, 2009). Among barley bi-parental populations, the â Nureâ x â Tremoisâ (NT) population shows high level of diversity between parental lines due to no ancestors in common, different end uses (malting and feeding), and growth habit. This allowed to map loci affecting the various segregating traits such as winter hardiness, flowering time, vernalization requirement, biotic and abiotic stress tolerance and malting quality (Francia et al. 2004; von Zitzewitz et al 2005; Laidˆ‡ et al. 2009; Aghnoum et al. 2010). All entries were characterized with molecular markers associated to four major loci that regulate the phenological adjustment in barley: Vrn-H1, Vrn-H2, Ppd-H2 and eam6/Eps2. Francia et al. (2011) found that allelic variation at three of them (Vrn-H1, Ppd-H2 and eam6/Eps2) explained together 42% of G and 26% of GE sum of squares for grain yieldGY. Further studies with the population, aimed at identify QTLs responsible for the adaptation of barley in a wide range of Mediterranean environments in terms of phenology, grain yield and its components were then performed (Tondelli et al. submitted; see Chapter 3). Results revealed that major QTLs controlling DtH and GY in various environment overlap the Vrn-H1 and eam6/Eps2 loci, while for Ppd-H2 only "pleiotropic" QTLs affecting GY were detected. During the last years, relations between flowering time and yield have been deeply investigated in barley (Cuesta-Marcos 2009 et al. 2009; Borrˆ¡s et al. 2009). We focused in this study on relationships between QTL (G) effects and environmental (E) co-variables for QTLs commonly determining flowering time and grain yield (eam6/Eps2 and Vrn-H1). Accordingly, it was our aim to assess how a series of environmental co-variables collected in a wide range of drought-prone environments across the Mediterranean Basin may influence yield and DtH QTL effects. This work may be considered as logical consequence of previous works published on NT mapping population.

4.2 Materials and Methods

4.2.1 Plant material and field trials

One hundred and eighteen doubled-haploid (DH) lines derived by anther culture from

the F1 cross of â Nureâ x â Tremoisâ , NT, (Francia et al., 2004) were used in the present study. The winter parent â Nureâ [(Fior 40 _ x Alpha2) _ x Baraka] is a modern, high yielding two-rowed feed-barley, with a wide range of adaptability and cold tolerance. This cultivar was released by the Istituto Sperimentale per la Cerealicoltura, Fiorenzuola dâ Arda, Italy. The spring parent â â Tremoisâ [(Dram x_ Aramir) x_ Berar] is a modern, high yielding French two-rowed malting variety, adapted to fertile environments and susceptible to low temperatures. A low resolution linkage map of this population was previously described by Francia et al. (2004). All the 118 entries were genotyped with Diversity Array Technology´fi (DArT) marker assay by Triticarte Pty Ltd (Australia), as already described in Chapter 3 (Tondelli et al. submitted). In addition, candidate genes known to be involved in regulation of barley phenology and abiotic stress response have been mapped in the same NxT linkage map (Francia et al., 2011, Tondelli et al. submitted). In the frame of the EU ICO-MED funded project MABDE (â Mapping Adaptation of Barley to Drought Environmentsâ , ICA3-CT22002-10026), all entries were grown in replicated yield trials in harvest years 2004 and 2005 at 18 sites in Algeria (DZA), Italy (ITA), Jordan (JOR), Spain (ESP), Syria (SYR), and Turkey (TUR) as showed in Figure 1.

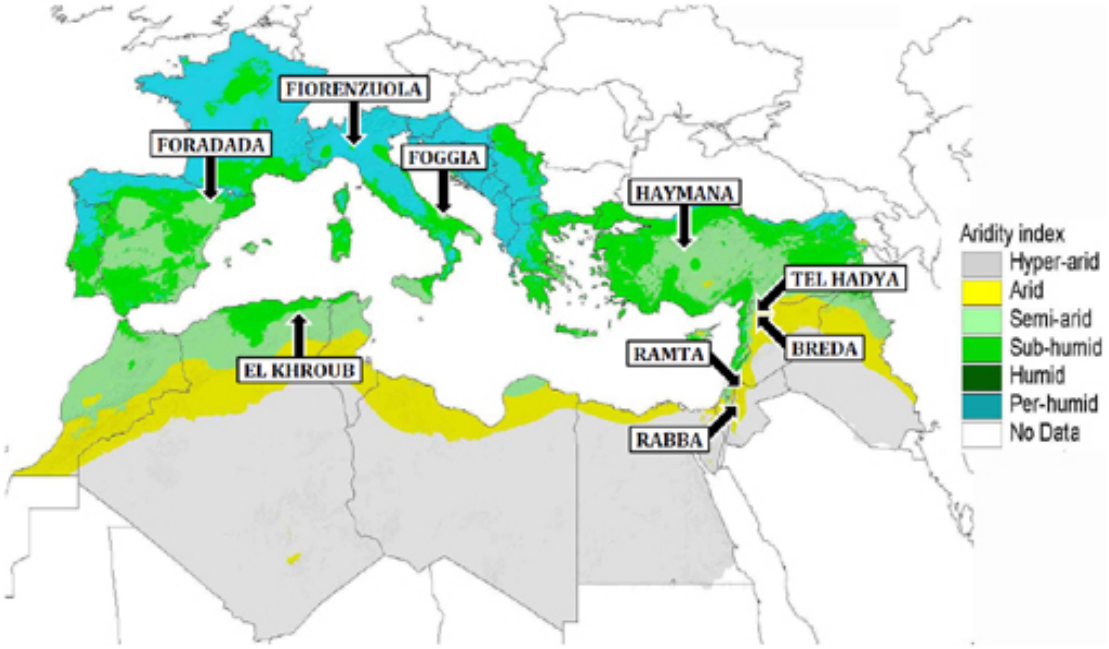

Figure 4.1. Location of field trials across the Mediterranean basin

In each country trial sites have been selected according to past meteorological data to explore a wide range of Mediterranean environments, from very low to moderately high rainfall regions, indicated as Wet and Dry. In some case two trials were sown at the same site with one being rainfed and the other supplied with supplementary irrigation (Table 4.1). At each site of our multi environment trial (MET), a 15 by 20 rectangular grid with 6 m22 plots was applied. Agronomic practices, including sowing rate, weed control and fertility management, were conducted in accordance with local practices. The experimental design consisted of two replicates for the 120 entries (118 DHs plus â Nureâ and â Tremoisâ) augmented by four checks repeated 15 times and included in a diagonal fashion within the rectangular grid. The first check was â Harmalâ which was grown in all sites, while the other checks varied across the sites, with one being a landrace and two being an old and a modern cultivar, that were specific to the region in which the trial was being grown. The two replications as the repeated checks served to estimate the experimental error and to correct for any spatial patterns.

Table 4.1. NT barley doubled haploid population in six countries in of Mediterranean Basin for harvest in years 2004 and 2005. Geographic referentiation is given together with a few relevant informations about sowing date, meteo data, DtH and average GY.

Code	Location	Country	Latitude	Longitude	Altitude	Site/Watering[1]	Sowing date	Average Temperature[2] (°C)		Water input[3]	Days t
								min	Max	(mm)	Headir
DZA_5W	El Khroub	Algeria	36°15'N	06°42'E	596	Wet/Rainfed	19/02/05	10,2	24,6	130	95,6
ITA_4D	Foggia	Italy	41°28'N	15°33'E	57	Dry/Rainfed	13/01/04	6,6	16,8	258	113,2
ITA_4W	Foggia	Italy	41°28'N	15°33'E	57	Wet/Irrigated	08/01/04	6,6	17,5	327	118,9
ITA_5D	Foggia	Italy	41°28'N	15°33'E	57	Dry/Rainfed	16/12/04	8,1	19,5	268	136,4
ITA_5W	Foggia	Italy	41°28'N	15°33'E	57	Wet/Irrigated	16/12/04	8,1	19,4	362	134,8
ITA_5F	Fiorenzuola	Italy	44°56'N	9°54'E	80	Wet/Rainfed	17/11/04	5,3	18	292	176,8
JOR_4D	Ramta	Jordan	32°32'N	36°02'E	561	Dry/Rainfed	16/12/03	6,8	20,6	151	
JOR_4W	Rabba	Jordan	31°16'N	35°44'E	616	Wet/Rainfed	23/12/04	5,6	18	194	
JOR_5D	Ramta	Jordan	32°32'N	36°02'E	561	Dry/Rainfed	28/12/04	7,1	20,3	140	108,4
JOR_5W	Rabba	Jordan	31°16'N	35°44'E	616	Wet/Rainfed	28/12/04	5,9	17,8	217	96,3
ESP_5D	Foradada	Spain	41°39'N	01°23'E	318	Dry/Rainfed	11/11/04	3,8	19,2	167	177,9
SYR_4D	Breda	Syria	35°56'N	37°10'E	300	Dry/Rainfed	07/12/03	6,7	21,3	204	
SYR_4W	Breda	Syria	35°56'N	37°10'E	300	Wet/Rainfed	11/12/03	5,5	20	290	124,6
SYR_5D	Breda	Syria	35°56'N	37°10'E	300	Dry/Rainfed	02/01/05	8,2	23,3	143	
SYR_5W	Tel Hadya	Syria	36°01'N	36°56'E	362	Wet/Rainfed	13/12/04	3,8	17,8	192	121,7
TUR_4D	Haymana	Turkey	39°26'N	32°30'E	1214	Dry/Rainfed	03/11/03	8,3	19,8	232	203,1
TUR_4W	Haymana	Turkey	39°26'N	32°30'E	1214	Wet/Irrigated	03/11/03	8,3	19,8	282	
TUR_5	Haymana	Turkey	39°26'N	32°30'E	1214	Dry/Rainfed	21/03/04	9,4	21,9	174	

1 Sites are classified according to previous meteorological data Wet (suffix â Wâ) and Dry (suffix â Dâ); in some case the Wet site was created artificially by supplementary irrigation supplied during the growing season.

2 Average minimum and maximum temperatures (´”C) registered from sowing to harvest.

3 Total rainfall plus irrigation (mm) from sowing to harvest

4.2.2 Data collection and analysis

Phenotypic data were collected for days to heading (DtH, d; from sowing date) and grain yield (GY, t ha-1), whereas environmental co-variables were recorded during the growing cycle. Three main growth phases were considered: vegetative, sink determination and grain filling. Since DtH depends only barely on environmental and physiological factors influencing of the vegetative and sink determination phases, only environmental data from these two growth phases were considered for the. The environmental co-variables, collected during all the cycle of the crops for data analysis were : average daily maximum temperature (Tmax), average daily minimum temperature (Tmin), difference between average daily maximum and minimum temperature (Tdif), number of days with temperature under 0´” C (dT0), number of days with temperature above 30´” C (dTa30), Growing Degree Days [calculated by subtracting Tbase (10´” C) to the average of the daily maximum and minimum

temperatures, GDD], Rainfall (indicated in mm, Rf), total water input (mm, WT), Solar radiation (W/m2, SR), Potential evapotranspiration (mm/day ET), Photothermal Quotient (radiation per unit area per day, PQ) defined as solar radiation to average daily temperature ratio and Water Deficit (WD), defined as WT to ET ratio (Table 4.2). Average means for each environmental co-variable was then calculated for each one of the three considered growth phases. Data analysis was conducted using Genstat13th edition software (Payne et al., 2008). Firstly, Best Linear Unbiased Estimators (BLUEs) were generated for available characters in each trial by mixed model analysis, spatially adjusting for rows and columns effects. QTL multi environment analysis was performed by Genstat 13, using Composite Interval Mapping (CIM) with data proceeding from each trial. We used data for all 18 sites for grain yield. However, Days to Heading was recorded only in 12 trials and analyzed accordingly. Additive effects of QTL, from each trial, were regressed with all the environmental co-variables, collected for each growth period, to determine QTL sensitivities to all meteorological variables. Genotypic BLUEs were organized in a two-way genotype (entry) x environment (trial) table of the means to evaluate G and E main effects, GE and their partition to individual QTL. We used a reduced interaction linear model for partitioning phenotypic variability, where a factorial regression model was fitted with genotypic co-variables, i.e. marker alleles at QTL peaks in common for DtH and GY. The genetic markers used were HvBM8 for eam6/ Eps2 and HvBM5A for Vrn-H1. Semipartial R2 of GE interaction was calculated from the sum of squares of GE, this representing the percentage of GE that can be associated to differences in QTLs sensitivities to each specific environmental co-variable. P-value of the mixed model was calculated from the mean square. We studied the relationships between environmental main effects and allele variation at QTL marker peaks with the explicit physiological and meteorological characterization of all the environments.

Table 4.2. Average mean of meteo-variables collected for each field trial: average daily maximum temperature (Tmax), average daily minimum temperature (Tmin), difference between average daily maximum and minimum temperature (Tdif), number of days with temperature under 0´” C (dT0), number of days with temperature above 30´” C (dTa30), Growing Degree Days [calculated by subtracting Tbase (10´” C) to the average of the daily maximum and minimum temperatures, GDD], Rainfall (indicated in mm, Rf), total water input (mm, WT), Solar radiation (W/m2, SR), Potential evapotranspiration (mm/day ET), Photothermal Quotient (radiation per unit area per day, PQ) defined as solar radiation to average daily temperature ratio and Water Deficit (WD), defined as WT to ET ratio

		Meteo-variables											
	Field trial	Tmx	Tmin	Tdif	dT0	dTa30	GDD	Rf	WT	Sr	ET	PQ	WD
Vegetative phase	DZA_5W	17.8	6.0	11.9	6.0	2.0	861.0	110.2	110.2	15289.4	233.4	1187.6	47.2
	ESP_5D	10.7	-2.1	12.8	104.0	0.0	533.2	96.3	96.3	18581.0	184.8	1115.7	52.1
	ITA_4D	12.6	3.2	9.4	20.0	0.0	648.0	71.4	71.4	10150.0	195.3	1005.5	36.6
	ITA_4W	13.3	3.6	9.7	20.0	0.0	744.3	87.8	127.8	10993.3	209.8	1095.7	60.9
	ITA_5D	12.4	3.1	9.3	14.0	0.0	761.8	203.0	203.0	14553.8	209.0	1344.5	97.1
	ITA_5F	9.8	-1.6	11.4	101.0	0.0	488.2	179.6	179.6	16075.4	124.5	912.4	144.3
	ITA_5W	12.3	3.0	9.3	14.0	0.0	745.3	201.8	246.8	14289.3	207.9	1328.5	118.7
	JOR_4D	15.2	5.0	10.2	0.0	1.0	891.3	145.3	145.3	10365.6	184.5	1017.2	78.8
	JOR_4W	13.9	4.0	10.0	4.0	0.0	667.3	188.1	188.1	6107.8	82.4	681.8	228.3
	JOR_5D	15.1	5.0	10.1	0.0	0.0	773.9	128.1	128.1	9243.3	131.8	889.3	97.2
	JOR_5W	14.0	4.8	9.2	4.0	0.0	671.0	215.5	215.5	5529.3	78.1	525.1	275.9
	SYR_4D	13.1	4.8	8.3	10.0	0.0	893.3	177.0	177.0	9887.1	161.5	1011.3	109.6
	SYR_4W	12.8	3.3	9.5	18.0	0.0	785.4	250.9	250.9	10515.5	151.6	1105.6	165.5
	SYR_5D	15.2	3.8	11.3	16.0	0.0	923.5	128.8	128.8	15059.0	251.2	1459.4	51.3
	SYR_5W	11.3	1.7	9.6	26.0	0.0	600.4	144.3	144.3	9591.4	129.0	940.1	111.9
	TUR_4D	10.4	1.4	8.9	85.0	0.0	920.7	193.8	193.8	19902.7	201.8	1244.9	96.0
	TUR_4W	10.3	1.4	8.9	85.0	0.0	906.2	193.8	193.8	19790.4	200.0	1237.2	96.9
	TUR_5	11.0	1.9	9.2	82.0	0.0	925.5	236.5	236.5	21311.6	227.6	1697.5	103.9
Sink determination phase	DZA_5W	26.2	10.2	16.0	0.0	4.0	382.2	7.1	7.1	6487.3	115.1	364.6	6.2
	ESP_5D	23.3	5.2	18.2	1.0	1.0	311.7	2.2	2.2	5688.7	85.2	394.4	2.6
	ITA_4D	17.2	7.6	9.7	0.0	0.0	255.4	51.2	51.2	3307.5	51.2	263.9	100.0
	ITA_4W	19.1	8.5	10.6	0.0	0.0	284.7	53.4	53.4	4074.6	63.7	293.9	83.8
	ITA_5D	20.3	8.8	11.5	0.0	0.0	305.8	24.4	24.4	5139.7	96.3	369.8	25.3
	ITA_5F	21.1	8.0	13.1	0.0	0.0	306.4	45.4	45.4	4594.4	70.8	307.5	64.1
	ITA_5W	20.2	8.8	11.4	0.0	0.0	304.0	25.6	25.6	5122.5	89.7	370.4	28.5
	JOR_4D	21.7	7.4	14.3	0.0	2.0	305.4	1.0	1.0	4837.4	101.8	354.5	1.0
	JOR_4W	18.1	5.2	12.8	1.0	0.0	244.8	4.4	4.4	3624.9	48.1	340.8	9.1
	JOR_5D	20.2	6.2	14.1	0.0	0.0	277.0	2.7	2.7	4697.4	73.8	363.9	3.7
	JOR_5W	18.1	5.0	13.1	0.0	0.0	249.9	0.0	0.0	3715.1	49.6	319.2	0.0
	SYR_4D	23.7	5.7	18.1	1.0	1.0	309.9	0.0	0.0	4710.6	74.1	338.1	0.0
	SYR_4W	23.7	5.2	18.5	2.0	2.0	304.3	0.0	0.0	4866.9	85.1	364.3	0.0
	SYR_5D	23.8	9.4	14.4	0.0	0.0	348.5	8.8	8.8	5294.0	100.8	323.9	8.7
	SYR_5W	19.7	2.7	17.0	6.0	0.0	236.0	17.4	17.4	4701.4	65.6	452.8	26.5
	TUR_4D	22.0	10.2	11.8	0.0	0.0	345.8	32.8	32.8	3839.1	59.1	230.7	55.5
	TUR_4W	21.7	10.2	11.5	0.0	0.0	340.1	32.8	32.8	3718.8	57.3	226.9	57.2
	TUR_5	26.8	12.4	14.5	0.0	3.0	422.1	3.0	3.0	4241.9	71.5	210.8	4.2
Grain filling phase	DZA_5W	29.9	14.3	15.6	0.0	5.0	309.6	0.0	0.0	4423.6	83.6	1756.0	0.0
	ESP_5D	23.6	8.4	15.2	0.0	0.0	222.1	58.8	58.8	3700.2	54.1	1744.6	108.7
	ITA_4D	20.7	9.0	11.8	0.0	0.0	207.8	17.4	17.4	3182.1	58.9	1484.4	29.5
	ITA_4W	20.2	7.7	12.5	0.0	0.0	197.8	12.2	12.2	3417.9	73.7	1630.9	16.6
	ITA_5D	25.7	12.5	13.2	0.0	1.0	267.5	1.4	1.4	2859.7	83.2	1866.4	1.7
	ITA_5F	23.0	9.4	13.7	0.0	0.0	235.3	31.2	31.2	3527.7	57.2	1429.1	54.5
	ITA_5W	25.6	12.6	13.0	0.0	1.0	267.8	1.4	1.4	2856.1	86.3	1850.6	1.6
	JOR_4D	24.8	8.1	16.7	0.0	2.0	230.9	2.8	2.8	3850.5	79.4	1611.4	3.5
	JOR_4W	21.9	7.5	14.5	0.0	0.0	205.7	0.0	0.0	3157.5	46.6	1242.9	0.0
	JOR_5D	25.7	10.0	15.6	0.0	2.0	249.9	0.0	0.0	3696.6	72.1	1464.0	0.0
	JOR_5W	21.4	8.0	13.4	1.0	1.0	206.0	0.0	0.0	3049.9	46.3	1067.2	0.0
	SYR_4D	27.2	9.5	17.6	0.0	4.0	256.9	24.6	24.6	3200.2	64.4	1526.3	38.2
	SYR_4W	23.4	8.0	15.4	0.0	2.0	219.8	27.9	27.9	3055.6	59.7	1665.8	46.7
	SYR_5D	30.8	11.4	19.4	0.0	9.0	295.0	0.0	0.0	4093.8	95.0	1981.1	0.0
	SYR_5W	22.5	7.0	15.5	0.0	1.0	206.1	17.3	17.3	3295.1	53.7	1629.0	32.2
	TUR_4D	27.8	13.4	14.4	0.0	2.0	299.8	2.6	2.6	2981.5	50.4	1615.1	5.2
	TUR_4W	27.5	13.2	14.3	0.0	1.0	294.6	2.6	52.6	2978.0	49.8	1605.8	105.6
	TUR_5	27.8	13.8	14.0	0.0	3.0	298.1	23.1	23.1	2838.0	46.7	2040.8	49.5

4.3 Results

Performing CIM genome searches using QTL peak markers as cofactors, we found five QTLs for DtH and seven for GY when analyzing the average days to heading and grain yield of the 118 DH lines (Figure 4.2). This work should be considered a logical consequence of the previous work reported in Chapter 3, due to the importance of dynamic relationship between DtH and GY. Major QTLs for flowering time were located on the centromeric region of chromosomes 2H (peak marker HvBM8 at ~ cM 79) and on chromosome 5HL (peak marker HvBM5 at ~ cM 122), overlapping with the eam6/Eps2 â

earliness per seâ locus and with the vernalization gene Vrn-H1, respectively. QTLs for GY were detected on chromosomes 1H, 2H, 4H, 5H and 6H (Figure 4.2).

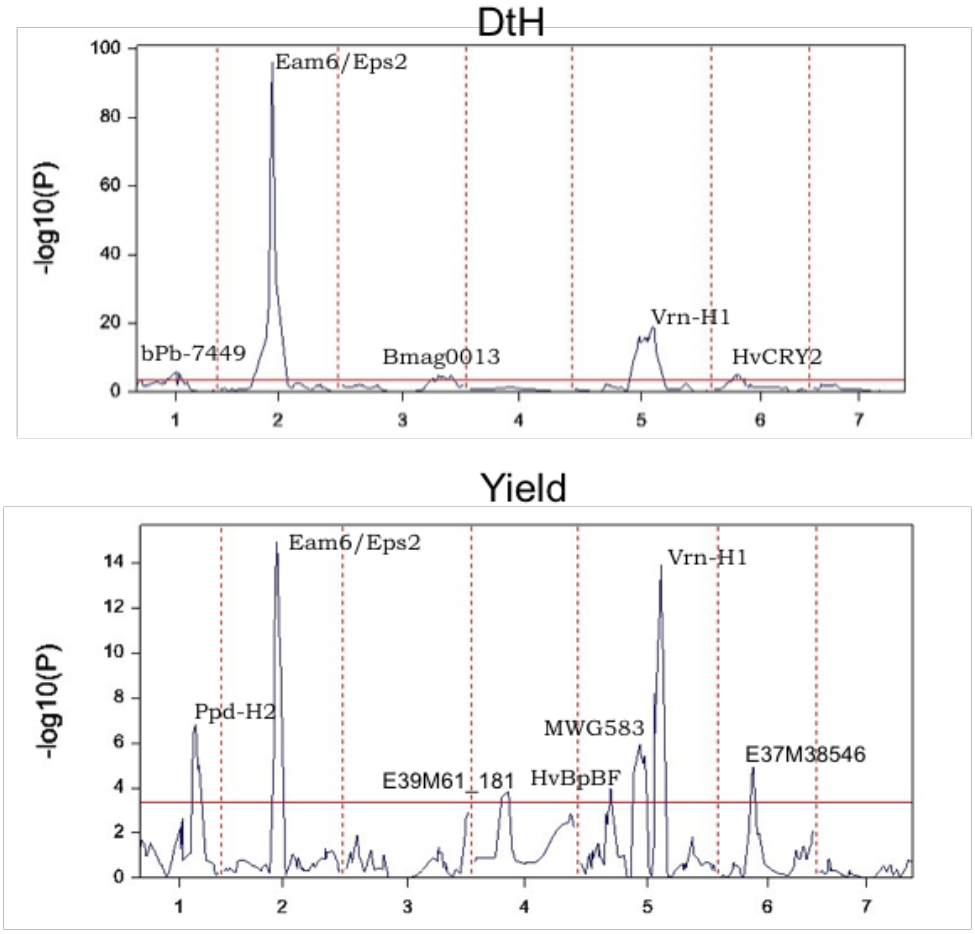

Figure 4.2. Results of QTL scans with composite interval mapping for GGE for the NT mapping population grown at 18 sites in Mediterranean basin for two years (2004/2005).

As for DtH, QTLs with greater significance were located on chromosome 2H (eam6/ Eps2) and on the chromosome 5H (Vrn-H1). Therefore we focused our attention on modeling DtH and GY QTL sensitivity to environmental variables to these two major loci. The allelic effects for the two common QTLs for DtH and GY in each environment are shown in Table 4.3.

Table 4.3. eam6/Eps2 and Vrn-H1 gene effects for the â Tremoisâ allele for days to heading and grain yield.

Environment	Days to heading (Days)		Yield (Kg/ha)	
	eam6/Eps2	*Vrn-H1*	*eam6/Eps2*	*Vrn-H1*
DZA_5W	0.21	-0.22	-140.00	620,00*
ESP_5D	2,39*	0.21	40.00	-50,00*
ITA_4D	2,42*	-1,05*	-50.00	0.00
ITA_4W	2,20*	-0.68	-170,00*	-40.00
ITA_5D	2,16*	-0.35	-320,00*	-10,00
ITA_5F	3,61*	0.71	-120,00*	-140,00
ITA_5W	1,49*	-0.22	-170.00	0.00
JOR_4D			-170,00*	-10,00
JOR_4W			-40,00*	0.00
JOR_5D	3,19*	-1.53	-170,00*	50.00
JOR_5W	1,61*	-0.6	0.00	-50.00
SYR_4D			-180.00	30.00
SYR_4W	2,77*	-0.47	-120.00	10.00
SYR_5D			-550,00*	20.00
SYR_5W	2,71*	-0.57	-290,00*	-20,00
TUR_4D	2,97*	1.81	-480.00	-380,00*
TUR_4W			-320.00	-490,00*
TUR_5			-20.00	220,00*

In the reduced linear model we used the allelic variation at eam6/Eps2 and Vrn-H1 loci (Table 4.3) as external factors to dissect G and GE for both DtH and GY. Results obtained from GGE partitioning based on a simple two gene model showed that for DtH the model explained 69% of the G main effects. Eam6/Eps2 was significant in all field trials and explained alone two thirds of main effect for flowering time, while Vrn-H1 was not significant. Semipartial R2 for GE interaction for the two genes model is 31.4% and both loci explained almost the same percentage of GE sum of square 45.5 % and 46. 6% for Vrn-H1 and eam6/Eps2, respectively. (Table 4.4). Results of GGE partitioning based on two genes model interaction, also showed that the main effect explain more than 40% of the genotype differences for GY. The eam6/Eps2 locus explained again

most (93.5%) of the main effects for the two gene model and this highlighted the importance of allelic variation at this gene in rainfed Mediterranean conditions (Table 4. 4). Together, the two loci explained 24.8% of GE. Partitioning between the two is different respect to DtH, in fact the eam6/Eps2 explained only 24.9% of GE sum of squares for grain yield, while Vrn-H1 the vast majority (72.0%). As for DtH, no epistatic interactions were detected.

Table 4.4. Partitioning of the G+GE variation for Days to Heading and Grain Yield for the â Nureâ x â Tremoisâ barley doubled haploid population grown in a series of Mediterranean environments, based on a two gene model (HvBM8 and HvBM5A_Vrn-H1).

	Days to Heading						Grain Yield					
Source of variation	degrees of freedom	Sum of Squares ‡	Semi-partial R^2	Mean Squares	F-test	p-value	degrees of freedom	Sum of Squares ‡	Semi-partial R^2	Mean Squares	F-test	p-value
Genotype	119	10148.5	59.8	85.3	16.36	<0.0001	119	136.9	16.0	1.15	3.24	<0.0001
Two-gene model	3	7005.2	69.0	2335.1	86.17	<0.0001	3	58.7	42.9	19.58	29.04	<0.0001
Vrn-H1	*1*	*12.9*	*0.2*	*12.9*	*0.47*	*0.4922*	*1*	*3.1*	*5.3*	*3.12*	4.63	*0.0334*
eam6/Eps2	*1*	*6992.2*	*99.8*	*6992.2*	*258.04*	<0.0001	*1*	*54.9*	*93.5*	*54.92*	81.45	<0.0001
Vrn-H1.Eam6	*1*	*0.1*	*0.0*	*0.1*	*0.01*	*0.9424*	*1*	*0.7*	*1.2*	*0.69*	1.03	*0.3132*
Residual G	116	3143.3	31.0	*27.1*	*5.20*	<0.0001	116	78.2	57.1	*0.67*	1.90	*0.0003*
Genotype.Environment	1309	6822.7	40.2	5.21			2023	718.6	84.0	0.36		
Env.Two-gene model	33	2138.9	31.4	64.8	17.66	<0.0001	51	178.3	24.8	3.50	12.76	<0.0001
Env.Vrn-H1	*11*	*972.4*	*45.5*	*88.4*	*24.08*	<0.0001	*17*	*128.4*	*72.0*	*7.55*	27.57	<0.0001
Env.eam6/Eps2	*11*	*997.7*	*46.6*	*90.7*	*24.71*	<0.0001	*17*	*44.5*	*24.9*	*2.62*	9.55	<0.0001
Env.Vrn-H1.eam6/Eps2	*11*	*168.8*	*7.9*	*15.3*	*4.18*	<0.0001	*17*	*5.4*	*3.1*	*0.32*	1.17	*0.2830*
Residual GE	1276	4683.8	68.6	*3.7*			1972	540.3	75.2	*0.27*		

QTL on chromosome 2H (eam6/Eps2) showed greater effects on DtH and was always associated with delay in flowering time due to the â Tremoisâ allele (Table 4.3). Additivity associated to eam6/Eps-2 locus and GE interactions associated to Vrn-H1 loc us are in full agreement to the putative earliness per se and vernalization nature of the two loci. No epistatic effects between the two genes were found for both DtH and GY. Significant regression models for the partitioning of GGE effects for both loci are showed in Table 4.4.

Table 4.5. Significant simple linear first and second degree factorial regression models for the partitioning of GGE effects for eam6/Eps2 and Vrn-H1 determined for the â Nureâ x â Tremoisâ barley population grown in a series of Mediterranean environments, using a collection of environmental variables at three growth stage.

			Days to Heading			Grain Yield		
Locus	*Growth Stage*	*Meteovar*	*Model*	R^2	*p-value*	*Model*	R^2	*p-value*
eam6/Eps2	*Vegetative stage*	Tmax	Lineal	48.7	0.011			
		ET	Cuadratic	77.5	0.001			
	Sink determination	Tmax	Cuadratic	52.0	0.018			
		Sr	Cuadratic	58.9	0.018			
	Grain filling	Tmax				Lineal	30.6	0.018
Vrn-H1	*Vegetative stage*	Tmax	Cuadratic	67.6	0.006	Lineal	47.8	0.001
		Tmin	Cuadratic	41.6	0.017			
		dT01	Lineal	58.8	0.003			
		Sr	Lineal	63.4	0.001			
		GDD	Cuadratic	76.0	0.001			
	Sink determination	Tmax				Cuadratic	47.4	0.008
		dTa30				Cuadratic	57.9	0.001
		Sr				Cuadratic	46.6	0.009
		ET				Cuadratic	43.0	0.014
	Grain filling	Sr				Cuadratic	46.2	0.009

For the same dataset of meteorological variables, a preliminary study by Romagosa et al. (2008) reported that Principal Component Analysis highlighted a high correlation between environmental co-variables across growth phases. In our experiment, the factors affecting DtH were different temperature-based variables such as Tmax, Tmin, dT0 and dTa30 and GDD, together with SR and ET. (Table 4.5). In particular, Tmax was the variable more often detected as significant for any of the two loci at the different growth phases. QTL effects for the eam6/Eps2 locus showed a delay in DtH associated with the allele contributed by â Tremoisâ that ranged from 0.21 to 3.6 days. The lowest effect was detected in DZA_5W, in which the trial was sown in early spring (Table 4.1). Effects of eam6/Eps2 on DtH in the majority of field trials were between two and three days. Effects for Vrn_H1 associated to the â Tremoisâ allele were negative in nine field trials and ranged from -0.22 and -1.05 days. Positive effects were found in ESP_5D (0. 21 d), ITA_5F (0.71 d) and TUR_4D (1.81 days). Figure 4.3 summarizes the relationships between the environmental variables and the effect of the â Tremoisâ allele on DtH when it is harbored at eam6/Eps-2 and Vrn-H1 loci.

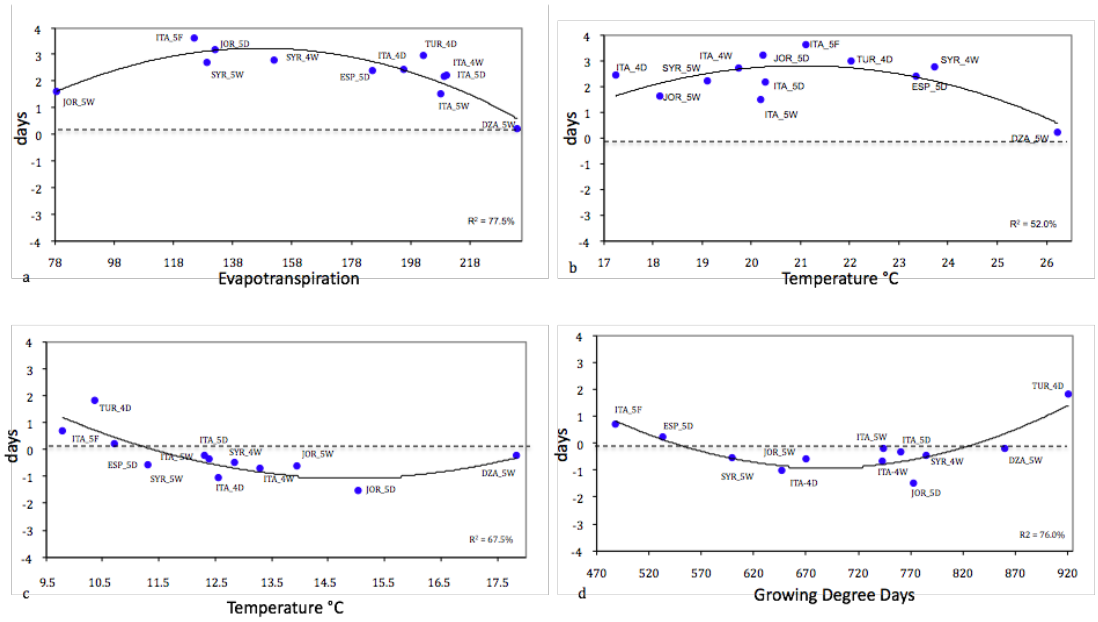

Figure 4.3. Factorial regression between DtH QTL effects (measured in days) for the â Tremoisâ allele and a set of environmental co-variables: (a) ET during vegetative phase for eam6/Eps2. (b) Tmax during sink determination phase for eam6/Eps2. (c) Tmax during vegetative phase for Vrn-H1. (d) GDD during vegetative phase for Vrn-H1. Graphs are fitted with the effects of â Tremoisâ allele.

According to a second degree polynomial model, the most important environmental factors affecting DtH considering the eam6/Eps2 locus, were ET during vegetative phase (R2 = 77.5 %) and Tmax during sink determination phase (R2 = 52 %) (Figure 4. 3A and 4.3B). As expected, significant associations for Vrn-H1 were only detected at the vegetative growth phase, with a R2 of 67.6 % for maximum temperature and 76.0 % for GDD (Figure 4.3C and 4.3D). For eam6/Eps2, the effects on days to heading showed a non-crossover QTLE interaction. In fact, variation in DtH across environments is quantitative and the â Tremoisâ spring allele always caused a delay in DtH. On the other hand, Vrn-H1 effects showed a crossover QTL.E interaction, and in this case variation in flowering time was qualitative. Figure 4.3C shows that, as temperature increase above 10´”C, the effects of the â Tremoisâ allele at Vrn-H1 is to reduces flowering time, whereas in field where maximum temperature recorded during vegetative phase was under 10´℃ (ITA_5F and TUR_4D) the same allele delayed flowering time (Figure 4.3C). The same response was found for GDD (Figure 4.3D), in fields with lower GDD during the vegetative phase (ITA_5F and TUR_4D) the spring

allele from the parent â Tremoisâ delayed DtH. QTL for GY on chromosome 2H (eam6/ Eps2) showed negative effects due the â Tremoisâ allele in 16 field trials. Negative effects ranged from -20 to -550 kg ha-1. A low positive effect of 40 kg ha-1 was found in ESP_5D, while no effects were detected in JOR_5D (Table 4.3). Vrn-H1 showed negative effects for GY in nine field trials, and the range was from -10.0 to -490.0 kg ha-1. Five fields showed positive effects between 10.0 and 620.0 kg ha-1, while in three fields no effects were recorded (ITA_4D, ITA_5W and JOR_4W). Interestingly, the highest positive effects were recorded in the two fields in which sowing was performed late, DZA_5W and TUR_5 (Table 4.1). Regression analysis for GY QTL effects with environmental co-variables is shown in Table 4.4. The most relevant environmental factors associated to differential sensitivity sensibility of the two genes for GY were again the temperature-related -variables (Tmax, Tmin, dT0, dTa30 and GDD) and SR across all growth stages. In this case the most important environmental factors were Tmax during grain filling for eam6/Eps2 (R2 = 30.6 %) and Tmax measured during the vegetative phase for Vrn-H1 with a R2 = 47.83 % (Figure 4.4). As for DtH the eam6/ Eps2 locus showed a non-crossover interaction; Figure 4.4A shows how, as temperatures increase above 24 ´℃, the â Tremoisâ allele is associated to a progressive reduction of GY. Lower negative effects on GY, despite the high temperatures recorded, were observed in TUR_5 and DZA_5W, once again attributable to their late sowing. Figure 4.4B shows a crossover interaction for Vrn-H1, in those fields (ITA_5F, TUR_4D and TUR_4W) where Tmax during vegetative phase was under 10 ´℃, and in which the â Tremoisâ allele was associated to a reduction of GY. On the other hand, the â Nureâ allele took advantage in these fields and increased GY yield probably as a consequence of its effect in accelerating flowering time.

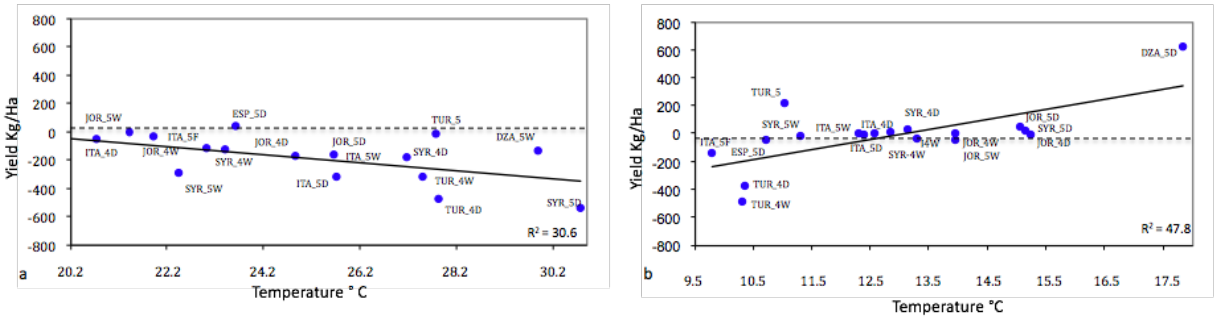

Figure 4.4. Factorial regression between grain yield QTL effects (Kg ha-1) for the â Tremoisâ allele and a set of environmental co-variables: (a) Maximum temperature during grain filling phase for eam6/Eps2. (b) Maximum temperature during vegetative phase for Vrn-H1.

As expected, R2 values obtained from regression of environmental co-variables with GY effects were lower than for DtH, this could be explained by the higher complexity of this trait respect to days to heading. Results also showed a very similar response of both QTLs to environmental co- variables. These two loci explained together the 25 % of GE interaction for GY and 31% for DtH and this may underlay the dynamic relationship existing between these two characters. Furthermore eam6/Eps2 seemed to be the locus controlling the major part of genetic variability for DtH and GY. For DtH, both loci showed the same magnitude of GE interaction while Vrn-H1 explained the major part of GE interaction for GY (Table 4.4). The eam6/Eps2 locus showed non-crossover interaction for both DtH and GY, in this locus the allele from the winter parent â Nureâ seems to be favorable and increase GY by hastening flowering time in all field trials except DZA_5W probably due to late sowing. The sensitivity of Vrn-H1 locus to Tmax for both DtH and GY is interesting because, the winter allele from â Nureâ in fields with temperature under 10´℃ (ITA_5F and TUR_4D) is associated with early flowering (3.6 and 2.9 days) and the same field trials showed a positive high effects on GY yield (120 kg ha-1 and 380 kg ha-1 respectively) due to the same allele. While in fields where sowing was performed late, such as DZA_5W and TUR_5D, the spring allele from â Tremoisâ showed a positive effects on GY (660 kg ha-1 and 220 kg ha-1, respectively) but not significant effect on flowering time in DZA_5W was recorded.

4.4 Discussion

To maximize grain yield potential, breeders must optimize crop flowering time, basically through a life cycle tailored to the target environment. As we underlined in the premises, the three key genetic factors that control cereal adaptation, and consequently flowering time and yield, are photoperiod response, vernalization requirement and earliness per se (Eps). Eps is defined as the difference in flowering time between varieties when vernalization and photoperiod requirements are satisfied (Hoogendorm 1985; Masle et

al. 1989; Penrose et al. 1991; Worland et al.1994; Slafer & Rawson 1994; Laurie et al. 1995; Slafer 1996). However, these differences are the result of the integration of differences in the duration of several developmental phases, including the transition from the vegetative to the reproductive apices, early and late spike development, and stem elongation (Slafer 1996, Lewis et al., 2008, Borras et al., 2009). Variation in earliness per se loci is also found both within and between spring and winter varieties, and may represent an untapped source of variation for targeted breeding, thus allowing a â fine tuningâ of flowering time within these types (Cockram et al., 2007). Particularly under Mediterranean conditions, heading date is closely related with crop GY. Applying the multi-environmental QTL analysis we found two major loci determining DtH and GY that overlap the eam6/Eps2 earliness per se locus on the long arm of chromosome 2H and the Vrn-H1 vernalization locus on the long arm of chromosome 5H. Eam6/Eps2 has been shown to regulate heading date in other barley mapping populations growth under Mediterranean conditions (Moralejo et al., 2004, Cuesta-Marcos et al., 2008a,2008b, von Korff et al., 2008, Francia et al., 2011), while in the same NT mapping population we described its effects on other developmental processes like plant early growth vigor and basic yield components (see Chapter 3). Results obtained from G+GE partitioning based on simple two gene model, using the allele variation at eam6/Eps2 and Vrn-H1, showed that model explain the 69 % of G variation for DtH and 42.9 % for GY (Table 4. 4). Surprisingly, the 98 % for DtH and 93.5 % for GY of genotypic variation are explained by eam6/Eps2. This mean that allelic variation at Vrn-H1 is not related with DtH and GY, in this multienvironment study and in this population, probably due to variability of conditions across field trials where in some case vernalization requirement was not necessary or not fully satisfied. A physiological explanation may be that Vrn-H1 is responsible only of the transition of apex from vegetative to reproductive phase (Trevaskis et al. 2003; Yan et al. 2003; Preston and Kellogg 2008), while earliness per se loci are expected to affects both vegetative and early reproductive phase. A definitive clarification of these hypotheses could come from a biparental study where eam6/eps2 is not segregating. Furthermore differences in DtH due to earliness per se genes have been attributed, as rewieved by Lewis et al. (2008), to integration of differences in the duration of several developmental phases such as (i) the transition from the vegetative

to the reproductive apices, (ii) early and late spike development, and (iii) stem elongation. Semipartial R2 for DtH is 31.4 % and both loci explained almost the same percentage of GE sum of square (45.5 % and 46.6 %); this may depend by the fact that different loci that control flowering time that interact with environmental cues are part of the same complex network. Partitioning for GY was different, semipartial R2 of GE was 24.8 % and was controlled, as expected and in the vast majority, by Vrn-H1 (72.0 %)(Table 4.3).Our results confirm the role of Vrn-H1 in DtH and in determining yield and its GE interaction in Mediterranean environment as reported in literature (Karsai et al. 1999; Snape et al. 2001). The introduction of co-variables (environmental and physiological) in modern G.E studies allow to develop statistical model that offer better possibilities for implementation of QTL selection in breeding programs (Spiertz et al. 2007; Romagosa et al. 2008). Once a physiological base is found for each significant environmental co-variable, this may allow a better understanding of how major genes interact with different environments, and of which co-variables are the most influent across a range of environments. Factorial regression between QTL effects and environmental co-variables for days to heading (Figure 4.3) showed a non cross-over QTL.E interaction for eam6/Eps2 and a cross-over interaction for Vrn-H1. In case of a non-crossover QTLE interaction variation of QTL effects, across environments, is quantitative and in the same direction such as the case of eam6/Eps2. Effects of â Tremoisâ spring alleles on this locus are always positive and associated with late flowering in each field. While a cross-over QTLE interaction shows qualitative variation of QTL effect across environments, this mean that QTL effects may change not only in magnitude but also in direction, from an environment to another as observed for Vrn-H1. For HvBM8, the marker peak at eam6/Eps2, factorial regression showed that variation at this locus is sensible to temperature, SR and ET(Table 4.5). The most interesting variables driving QTL effects across environments were Tmax during sink determination phase and ET during vegetative phase (Figure 4.3a and 4.3b). Bullrich et al. (2002) found that in diploid wheat the earliness per se gene Eps-Am1 showed significant interaction with temperature that result in significant differences in flowering time between accession carry different allelic variants at the Eps-Am1 locus. Differences in the number of spikelets per spike, an important component of GY, between Eps-Am1 alleles were

correlated with the differences in heading time (Lewis et al. 2008). Variation in DtH due to eam6/Eps2 locus, across environments ranged from 0.2 to 3.6 days, similar to what various authors already reported in the Triticeae, where differences in heading date for earliness per se genes were just of few days (Flood and Halloran 1983; Scarth and Law 1983; Hoogendoorn 1985; Miura and Worland 1994; Laurie et al. 1995; Worland 1996; Kato et al. 1999). The lowest effect was found in DZA_5W, where the trial was sown in late winter (Table 4.1). The second degree nature of the response curve to the two environmental variables, particularly for ET (Table 4.5) may be unexpected. ET is used to describe the sum of soil evaporation and plant transpiration from crop to atmosphere, and is related with water stress and temperature. In literature we do not found references of a direct relationship between DtH and ET, furthermore Angus & Moncur (1977) reported that mild stress can speed-up flowering in wheat. They proposed that increased leaf temperature, which is known to accompany water stress, has an effect in hastening development similar to that of an increase in ambient temperature and that plants adapts to stress by modifying the normal sequence of development, perhaps so that fewer cell division are required before anthesis. On the other hand, severe stress delays flowering and this is due to cessation of development of the shoot apex and possibly cessation of all cell division. In Figure 4.3A, a significant difference in DtH between field with mild ET and fields where ET was high is evident. The effects of ET on eam6/Eps2 are higher in field were ET range from 118 and 158 mm/day, while in fields where ET was higher (from 178 to 218 mmm/day) a reduced hastening of DtH was observed. Therefore a high ET seems to depress the effect of eam6/Eps2. This means that with high ET â Nureâ accelerate flowering time but not as under mild ET. These results are in part in accordance with those reported by Angus and Moncur because we only observed a reduction of eam6/Eps2 effects, this probably due to the growth stage; severe drought stress are unlikely during vegetative phase in Mediterranean environments. Tmax and ET showed similar effects on eam6/Eps2 locus this may be in part due to the fact that Tmax is one of biggest forces driving ET together with wind. Solar radiation also showed significant effect on eam6/Eps2 locus, this may due to dependence of Tmax from this variable. The allele of eam6/Eps2 carried by the Southern Mediterranean winter barley â Nureâ promotes earlier flowering. Temperature,

aside from its effects on vernalization, has the most obvious effect on duration of a crop, in that crops generally flower earlier at high temperature (Slafer and Rawson 1995). Earliness per se genes regulate flowering theoretically independently of the two major environmental signals, and are usually responsible for the fine tuning of flowering time, e.g., in wheat (Bullrich et al., 2002). Different growing sub-phases are differentially sensitive to temperature among genotypes and this may be related with the genetic component of earliness per se. As reviewed by Cockram et al. (2007), once Eps loci are resolved in a backgrounds in which effects of additional flowering time genes have been removed, it should be possible to demonstrate their no â trulyâ independence from environmental signals. Francia et al. (2004; 2011) showed that the eam6/Eps2 locus is greatly affecting flowering time in the NT mapping population. Lewis et al. (2008) showed that eam6/Eps2 influence vegetative phase, early and late spike development and stem elongation, and that its effects are modulated by temperature. GY components are determined during vegetative and early reproductive phase (Slafer and Whitechurch, 2001) and earliness per se seems to play an important role driven by temperature in these phases. We found in NT population several QTL for GY yield components overlapping eam6/Eps2 locus (Chapter 3; Table 3.5) such as plant height, spike lenght, spike number for square meter and number of grains for spike that may be influenced by temperatures. The QTL effect of Vrn-H1 for days to heading showed cross-over interaction with environment, in particular with Tmax and GDD co-variables during vegetative phase. Graphs of Figure 4.3c and Figure 4.3d show the effects of the â Tremoisâ allele on DtH. Figure 4.3c shows the results of factorial regression between Vrn-H1 QTL effects and Tmax during vegetative phase according to a second degree polynomial. The spring allele â Tremoisâ seemed to accelerate heading when maximum temperature increased. In fact the â Nureâ (winter) allele, sensitive to vernalization, was expected to drive this sensitivity. At the coolest sites, with average maximum temperature during vegetative stage below 11´”C, vernalization requirement was realistically fulfilled and the presence of â Nureâ allele thus translated into a faster heading. The second degree nature of the sensibility was driven by short-day vernalization (Slafer and Rawson, 1995). In DZA_5W where sowing took place in late winter, short-day photoperiod replaced vernalization requirements and, so we

hypothesize that the presence of the â Nureâ allele did not delay heading as much as we would expect under relatively higher temperatures during the vegetative stage. Figure 4.3d shows results of factorial regression between QTL effects and GDD for Vrn-H1, GDD being strictly related to temperature. In this case the â Tremoisâ allele seems to accelerate heading in most of the field trials, with mild effects on hastening days to heading (Table 4. 3). Vernalization saturation represses the activity of dominant allele Vrn-H2, allowing the expression of the recessive alleles at Vrn-H3 and Vrn-H1. Vrn-H3 enhances the activity of Vrn-H1 under a long photoperiod, resulting in earlier flowering (Karsai et al., 2008). The â Tremoisâ allele of Vrn-H1 delayed flowering time in three field trials (ITA_5F, ESP_5D and TUR-4D) in coincidence with lowest Tmax, where GDD calculated for vegetative phase were the smallest (ITA_5F, ESP_5D) and the highest (TUR_4D) (Fig. 4.3c and 4.3d). The apparent contradiction between the same effect driven by low average Tmax and high GDD for TUR_4D may be explained by the early fall sowing date in this site, that translated into a high value of GDD accumulated from sowing. In these three trials the lowest temperatures and the highest number of days with temperature under 0´° C (dT0) were recorded and this should explain the Tmax effect on the Vrn-H1 winter allele from â Nureâ to accelerate flowering time. Increased expression of Vrn-H1 in leaves and apices of vernalized plants is correlated with reduction of flowering time and it is likely that Vrn-H1 acts to promote flowering in vernalized plants (Trevaskis et al., 2006). Barley is sensitive to drought stress from pre-anthesis stage to grain filling. High temperatures usually cause a drop in duration of grain filling, resulting in a smaller grain size (Sofield et al., 1977; Chowdhury and Wardlaw 1978; Wardlaw et al., 1989). It is well known that short periods of high temperature and drought are quite common in Mediterranean environments during the grain filling period of cereal growth (Aspinall 1965; Nix 1975; Macnicol et al., 1993; Stone and Nicolas 1994; Savin and Nicolas 1996). Figure 4.4a shows the sensitivity of eam6/Eps2 QTL for grain yield to increasing Tmax during grain filling. This GE effect was undoubtedly mediated by heading date (Figure 4.3) and could not be described independently from this phenological trait. Figure 4.4a shows that when temperature increased above 22´”C, the effect on the â Tremoisâ allele at eam6/Eps2 took to a progressive reduction of grain yield. Negative effects on yield ranged from 120 kg ha-1

to 548 kg ha-1, when Tmax reached 30´”C. Under high temperature both plant growth and development are affected by temperature (Porter and Moot 1998). In wheat a general reduction in yield per ear of 3-4% for each 1´℃ rise in temperature above a mean of 15´℃ has been observed (Wardlaw et al., 1989). In two late-sowing field trials (DZA_5D and TUR_5), despite high temperatures (28´”C and 30´”C), sensitivity of eam6/ Eps2 QTL was negligible. On the other hand, the â Nureâ eam6/Eps2 allele seemed to answer to growing Tmax by increasing yield. This could be due by per se early flowering that allowed escaping the reduction of grain size mediated by temperature. Since Lewis et al. (2008), suggested that variability in earliness per se loci could be exploited by breeding to increase yield potential in different environments, here we estimated which size could be the yield gain given by a Southern European allele of eam6/Eps2 when Tmax during grain filling ranges from above 20.2´° to above 30.2´℃. Generally crops with winter grow habit require vernalization, that promotes transition from vegetative phase to flowering. Crops need several weeks of low temperature before advancing to the reproductive stage. In this work we showed how the â Tremoisâ spring allele at Vrn-H1 locus was affecting negatively yield in interaction with temperature only when Tmax in vegetative phase was under 11´”C (Figure 4.4b). In three out of four environments where Tmax was below this threshold (ITA_5F, ESP_5D and TUR_4D) the population also showed late flowering compared with the other field trials (Figure 4.3c and Table 4. 3). In TUR_4W the behavior could have been the same, however DtH data were not available for the field (Table 4.3). Respect to eam6/Eps2, the effect of temperature on the QTL is significant in the vegetative vs. the grain filling phase; the effect on the â Tremoisâ allele is approximately specular, i.e. the Tmax increase takes to a yield increase rather than to a decrease, and lastly, only in the case of Vrn-H1 there is a crossover effect. In the colder locations as ITA_5F, ESP_5D, TUR_4D the Vrn-H1 â Nureâ allele could have increased yield by accelerating flowering time due to its response to vernalization. In the same way, in the DZA_5W field a later sowing with respect to other field trials (Table 4.1) could have taken the â Nureâ allele to lower yields due to the not fully satisfied vernalization, associated to later heading. Wang et al. (2010) reported that the Vrn-H1 locus on chromosome 5H maps in a region that harbors QTLs with significant effect on Thousand kernel weight and grain yield. In the same

position of chromosome 5H, QTLs for cold, drought and salt stress tolerance were found (Francia et al., 2004, Weidner et al., 2006, Tondelli et al., 2006). Recently, Dhillon et al. (2010) reported that the frost tolerance QTLs previously mapped in this region of chromosome 5H are likely a pleiotropic effects of Vrn-H1 rather than the effect of separated closely linked locus (Frost Resistance-H1). This could suggest another reason for the effect of Tmax over Vrn-H1 for grain yield. The â Nureâ winter allele could have contributed to higher frost tolerance in colder places during vegetative phase (Table 4.1) respect to the â Tremoisâ spring allele, and led to the yield decrease effect in ITA_5F, ESP_5D and TUR_4D (Fig. 4.4). This work highlights the effect of meteorological co-variables on two Eps and Vrn loci important for growth cycle regulation in barley, and contributing both to DtH and GY in multilocation field trials across the Mediterranean. Among the meteorological variables analyzed, only ET, Tmax (during both vegetative and grain filling period) and GDD showed a significant QTLE interaction in the NT segregating population. While it could be obvious that temperature, and temperature-related variables such as ET and GDD would have influenced QTL effects on DtH and yield, it is not known why other important meteorological variables such as rainfall or total water input did not give origin to significant interactions. If this could be due to the genetic materials used, i.e. a single bi-parental population, should be clarified by means of association mapping panels of unrelated germplasm. The relationships between QTLs and meteorological co-variables were both lineal and cuadratic, and in some cases a cross-over was evident with a QTL allele effect both decreasing and increasing the trait in dependence from the meteorological co-variable considered. The weighting of such QTL sensitivity is relevant for barley breeding for the Mediterranean Basin, and such a study is an example of what could be identified on a wider scale with genome-wide association scans (GWAS) in barley, or in other crops. The responses to low and high meteorological values, together with the identification of specific environmental thresholds in case of cross-overs, can in fact help breeders to generate predictive values for the introgression of specific alleles into cultivars, to tailor new cultivar design for expected average values of meteorological variables.

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Chapter 5

Genome wide association analysis for frost tolerance

5.1 Introduction

The genetic components underlying the response of plants to the photo-thermal environmental cues driving seasonal and local adaptation are key traits limiting cropsâ geographical distribution. Therefore, as they have critical implications for agriculture, such components have been important focus of applied research. The winter hardiness

of cereal crops refers to the ability of plants to withstand the freezing temperatures that occur during the winter season (frost tolerance), and is also associated with other traits that regulate flowering in temperate climates, namely vernalization requirement and photoperiod sensitivity. The genetic control of low temperature tolerance is complex and is the final manifestation of several component traits. Frost tolerance depends on the intrinsic capacity of the plants for fast acclimatization to cold, the capacity of vegetative tissues to survive freeze-induced desiccation and the ability of the plant to recover from the stress (for a review, see Pecchioni et al. 2012). The process of hardening - by which an individual plant becomes tolerant to the effects of freezing (Flower et al. 1999; Hayes et al. 1993; Giorni et al. 1999) is a relatively slow, adaptive response during autumn, when the temperature, day length and light intensity decrease gradually, and comprises a series of biochemical changes that enable tissues to enhance their resistance. Moreover, strong genotype x environment interactions involving the response of plants to photoperiod and the duration and intensity of cold events may complicate the interpretation of the tolerance response. In the Triticeae, major efforts to dissect the genetic basis of flowering time have been based on the use of bi-parental mapping populations of random recombinant lines in wheat and barley, and led to the discovery of major and minor loci responsible for the traits. Major quantitative trait loci (QTL) â especially those with strong genotype x environment interaction â emerged as logical targets for gene cloning and the responsible candidate genes have been identified for lociVrn-1 and Vrn-2 (yan et al. 2003; Yan et al 2004; von Zitzewitz et al. 2005), Ppd-1 (T urner et al. 2005), Ppd-2 (Faure et al. 2008) andVrn-3 (Yan et al. 2006). However, in the case of freezing tolerance, the literature in barley is scarce with most of the original QTL studies based on small populations with some major developmental genes also segregating. The â Nureâ (winter) x â Tremoisâ (spring) barley population (Francia et al. 2004) is at present the only example where both Frost Resistance-1, Fr-1, and Frost Resistance-2, Fr-2 are segregating in the Triticeae (Galiba et al. 2009). Fr-1 and Fr-2 ar e located approximately 30 cM apart on the long arm of chromosome 5H and co-segregate with Vrn-1 and a cluster of at least 13 C-repeat binding factor (CBF) genes respectively (Francia et al. 2007). Other loci with minor effects on freezing tolerance at the vegetative stage have been mapped on 1HL, 4HS and 4HL in the â Dicktooâ (

facultative) x â Morexâ (spring) barley mapping population (Skinner et al. 2006), whereas a locus for frost induced sterility at the reproductive stage has been mapped distally on 2HL in the â Haruna Nijoâ (facultative) x â Galleonâ (spring) barley mapping population (Reiheimer et al. 2004). Progenies derived from all these crosses segregate for Vrn-1 and Vrn-2 (major genes governing the vernalization requirement in cultivated barley genepool (Cockram et al. 2007); which may obscure and complicate interpretation of the results. Despite being very successful for the identification of the key genetic switches underlying winter hardiness of the barley crop, which has been an important focus of applied research, it can be argued that the mapping populations utilized capture only a portion of the genetic diversity of the species and thus may not be representative of the diversity present in the breeding germplasm pools. Also, the usually large pleiotropic and epistatic effects involving major genes segregating within the mapping populations limit our capacity to detect other loci with smaller effects and significant interactions amongst themselves. There is currently great interest in Genome Wide Association Studies (GWAS) where a germplasm collection of individual lines, instead of segregants from pair crosses, are used to fine-map traits of interest. Most of the research on GWAS has been applied in human and animal genetics where directed crosses and large progenies are not possible. In humans, GWAS performed with thousands of SNP markers has led to the identification of hundreds of genetic variants associated with complex human diseases and traits, a large proportion of which correspond to previously unknown loci. GWAS utilises recombination events accumulated over many life cycles in natural and breeding populations to map traits with unprecedented resolution. The restriction is that highly saturated genetic maps are often needed to ensure that Linkage Disequilibrium (LD) persists for more than the average distance between markers. Such mapping has only been possible over the past decade due to the development of highly multiplex marker platforms, which provide robust and informative low cost genotyping. The emergence of high throughput SNP marker genotyping platforms with many thousands of markers (Close et al. 2009) and those related to next generation sequencing technologies17 will accelerate the implementation of GWAS approaches in crop plants, where the interest is considerable. Barley is a diploid autogamous crop plant where linkage disequilibrium (LD) is predicted

to be extensive(Caldwell et al. 2006; Comadran et al. 2009;Comadran et al. 2011a);. Therefore medium-resolution GWAS amongst cultivated germplasm can potentially be used to capture significant genetic effects segregating in the cultivated gene-pool (Rostocks et al. 2006)and successful examples considering simple and quantitative traits have recently been published (Cockram et al. 2010; Ramsay et al. 2011). Both these studies demonstrated that there is enough accumulated recombination within the cultivated gene-pool to map to gene resolution, identify and validate the candidate genes responsible for the traits. For instance, Cockram et al. (2010) used a collection of 500 elite UK barley lines genotyped with 1536 SNP markers to identify the causal polymorphism for ANT-2, a major switch governing anthocyanin production in barley. Ramsay et al. (2011) used a genetically broader germplasm collection consisting of 192 American / European elite cultivars genotyped with 4608 SNP markers as starting point to identify the candidate gene for INT-C, one of the genes controlling barley spike morphology. The candidate gene was then validated using a collection of well-characterized mutant stocks (Ramsay et al 2011; Lundqvist et al. 1988). Only three association mapping approaches for studying frost tolerance have been published to date in the Triticeae: two in barley and one in rye, the most frost tolerant species. The allelic variation of four barley CBF genes in a panel of 216 accessions was studied to identify two nucleotide variants of HvCBF14 and one nucleotide variant of HvBM5A (barl ey Vrn-1 gene candidate) as being significantly associated with frost tolerance (Fricano et al. 2009). Von Zitzewitz et al. (2011) performed a genome-wide association mapping study of winter hardiness traits in a different germplasm collection and found all the significant associations were on chromosome 5H. In the Fr-2 region, two SNPs were significantly associated with the trait, one representing the HvCBF9 gene, and the other located in an EST encoding for a heat shock transcription factor (HSF). At the Fr-1 locus, a specific HvBM5A intron 1 amplicon showed the most significant association, whereas a third significant SNP, about 8 cM proximal to HvBM5A, targeted a Glu-tRNA aminotransferace subunit C, a gene with no obvious relationship with frost tolerance. Finally, Li et al. (2011) studied eleven candidates involved in frost response (including several ScCBFs, ScICE2, and ScVRN1) in a panel of 201 rye lines (Ly ey al. 2011a; Li et al. 2011b). Two SNPs in ScCBF15 and one in ScCBF12, all leading to amino acid

changes, were related to frost tolerance. Although no association was found for ScVRN 1, this gene showed significant gene ˆ gene interaction for frost tolerance. Comadran et al. (2011b) utilized a panel of barley accessions representative of the Mediterranean basin and NW Europe to study barley adaptation to drought environments by analyzing genotypic, phenotypic and environmental data from 28 site x year combinations collected during harvest years 2004 and 2005. They found strong genotype x environment interactions complicate the interpretation of the data as different associations may be detected in different environments and proposed joint analysis of all the data as a way to identify and prioritize main QTL effects that were robust over a broad range of environmental conditions(Comadran et al. 2011b). In this way, two to seven QTL for characters such as yield components, heading date, harvest index and plant height were identified, many linked to known major developmental loci. Interestingly, one of the 28 trials located in Foradada (Spain) experienced extremely cold winter conditions with severe and long freezing events where minimum temperatures ranging from -5´℃ to -10´℃ were recorded for several consecutive weeks . In order to get a more reliable evaluation of cold damage, the same dataset was assessed for winter survival in the growing season 2007/08 in Fiorenzuola dâ Arda (Italy) . The data from these trials presents a valuable opportunity to identify important genetic regions associated with cold tolerance by GWAS. Therefore the objective of the current study was to identify barley varieties with superior cold tolerance and advance our understanding of the genetics of low temperature tolerance in barley.

5.2 Materials and methods

5.2.1 Plant material and genotyping

The germplasm consisted of 192 genotyped barley accessions that represented a survey of the breeding history of the Mediterranean basin as well as NW Europe and has been described in full by Comadran et al (2009). DNA was extracted from leaf tissue of two-week-old single plants using the DNeasy Plant DNA miniprep kit (Qiagen, Hilden, Germany) and 185 of the 192 accessions genotyped with Barley Oligo Pooled

Array 1 (BOPA1, consisting of 1536 SNPs) using the Illumina GoldenGate platform as described by Close et al. (2009). Genotypes and SNP markers with more than 10 % of missing data and minimum allele frequency (MAF) <10% were removed from the dataset and omitted from further analyses. QMVREPLACE procedure, implemented in Genstat v.14 (VSN International), which replaces missing marker scores with one of the scores of the most similar genotype(s), was used to infer missing genotypic data of the remaining 1,307 SNP subset. Using the default values for QMVREPLACE, we had 184 accessions with genotypes for 1,307 SNPs and only 51 missing marker scores remaining and these were used for further analyses.

5.2.2 Phenotyping

The whole set of accessions described by Comadran et al. (2009) was sown in November 2004 in Foradada (Spain, 41´”39â N, 01´”29â E) following an augmented cyclical design with an incomplete block size of 60. Each incomplete block was planted in 5 rows of 12 columns and included 4 checks, each replicated three times with one located in each column in a diagonal fashion at fixed intervals. We used four incomplete blocks so sow one of full replicate of all the 192 entries that were phenotyped and used a fifth incomplete block with a random selection of 48 of the 192 entries to provide partial replication. The checks (a local landrace, a local old variety and a local modern variety and an improved variety â Rihaneâ were used to detect and correct for any spatial variation across rows and column and the partial replication provided an estimate of the trial error. Plots were 6 m2 and were grown according to local management practice in terms of sowing rate, weed and disease control, and fertilizer inputs. Winter survival was evaluated at the end of March 2005 by visual estimation on a 0-5 scale as described in figure 5.1.a. (Akar et al 2009). Minimum and Maximum temperatures were recorded during the growing season (Figure 5.1.b.). In order to get a more reliable evaluation of cold damage, the same dataset was assessed for winter survival in the growing season 2007/08 in Fiorenzuola dâ Arda (Italy, 44´°55â N, 9´°53â E). The 192 entries were planted in two 1-m long rows, with each genotype replicated twice. Minimum and Maximum temperatures were recorded during the growing season (

Figure 5.1.a.) and cold damages were evaluated following a 0-5 visual scale as described in figure 5.1.b.

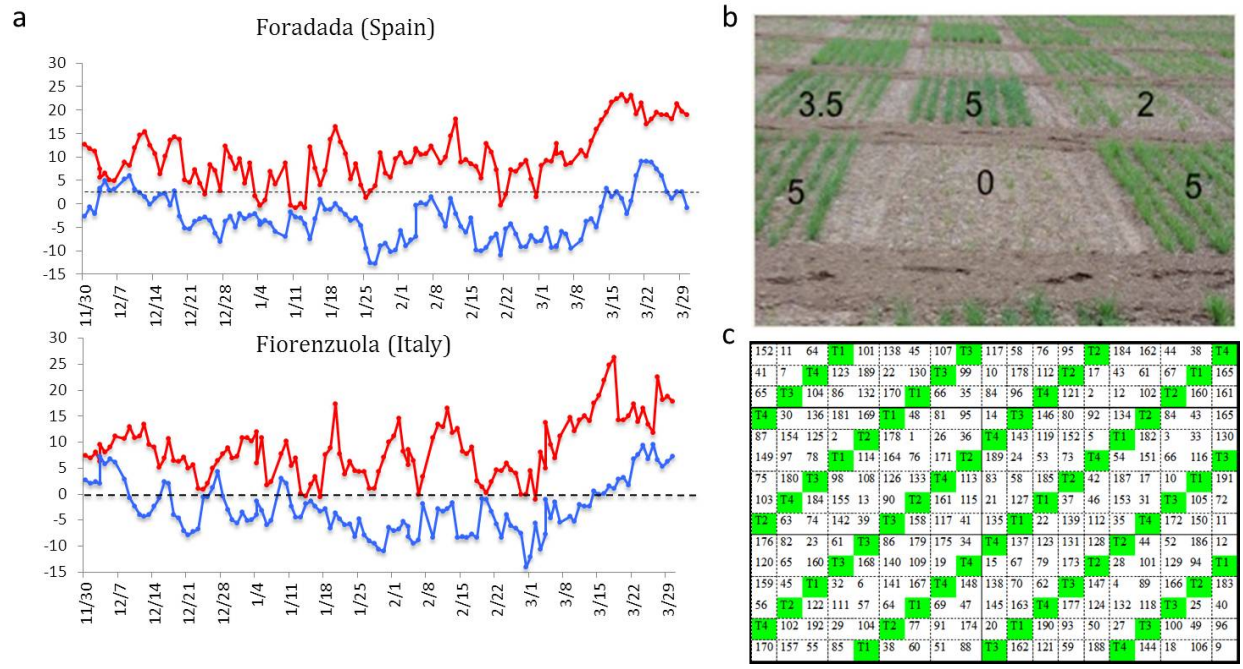

Figure 5.1. Frost episodes in Foradada (Spain, winter 2004/05) and Fiorenzuola (Italy, winter 2007/08). a. Daily maximum and minimum temperatures from sowing until the end of March when cold tolerance was scored. The recorded average mean of minimum temperature were -1.1â℃ and -2.68â℃ for Foggia and Foradada trials respectively, while the absolute lowest temperature -8.6â℃ and -12.7â℃ for Fiorenzuola and Foradada trials respectively. An alternation of freezing and thaw periods were observed. b. Differentia l frost damage in winter barley plots (Foradada, Spain). Cold injury was visually estimated on a 0-5 scale: 0. all plants were killed; 1. whole plants yellowed and 50% of plant mortality was observed; 2. whole plants yellowed and 20 % of plant mortality was observed; 3. fully yellowed basal leaves; 4. half yellowed basal leaves; 5. no damage was observed. C. experimental design in Foradada (Spain).

5.2.3 Statistical Analysis and GWA mapping

DARwin v.5.0 (Perrier, X. and Jacquemoud-Collet, J.P. (2006), http://darwin.cirad.fr/) was used to construct a Neighbour joining tree of the 184 barley cultivars from simple matching distances of 1,307 SNPs with MAF > 10%. Linkage Disequilibrium and haplotype analyses of the positive SNPs in relation to germplasm clusters were performed with HAPLOVIEW v.4.2 (http://www.broadinstitute.org/haploview/haploview). Best linear unbiased predictions (BLUPs) of the cold resistance for each accession were calculated using Restricted Maximum Likelihood (REML) directive in Genstat v.14 (VSN International). In the model, checks were used as a fixed effect, and columns, rows and test entries were used as random effects From the variance components obtained by REML, repeatability (H2) was estimated as H2 = [˘ 2g / (˘ 2g + ˘ 2e)], where ˘ 2e, is the residual component derived from the replicated entries and ˘ 2g is the genotypic component derived by subtracting ˘ 2e from the phenotypic component. The BLUPs for each accession were classified according to their geographic origin, growth habit and ear morphology, the main drivers of genetic divergence of barley germplasm and tested for differences by Analysis of Variance using Genstat v.14 (VSN International). GWAS were carried out by using a mixed linear regression model which accounts for multiple levels of genetic relatedness due to historical population substructure and kinship (Yu et al. 2006). To correct for population substructure, we used either the Eigenstrat relationship model with PCA scores as random terms (Price et al. 2006) or a kinship matrix (Yu et al. 2006) in the association mapping routines implemented in Genstat v.14 (VSN International). TASSEL v. 3.0 (Bradpury et al. 2007) was used to estimate the kinship matrix (K) from a stratified subset of 631 random markers with unique map positions so that we did not over-estimate sub-population divergence. A threshold of (-log10 p â¥ 3) was set for identifying significant SNP associations. SNPs that were either monomorphic or had a minor allele frequency of <10% were excluded, leaving 1307 polymorphic markers for GWAS. The Turkish low temperature tolerant facultative line â Tokakâ and the elite winter two-rowed cultivar â Introâ were used as reference genotypes.

5.3 Results

5.3.1. Environmental conditions and natural variation for cold tolerance in the GWA

population.

The exceptional environmental conditions experienced in Foradada (Spain) in the 2004/05 winter season enabled us to collect valuable information on winter survival under natural field Autumn-sown conditions in the GWA population. Minimum and maximum temperatures recorded in Foradada trial during the growing seasons show the severity of the frost episodes experienced by the GWA population with long and extreme freezing periods, where minimum temperature values below 0â℃ were recorded for several consecutive weeks (Figure 5.1). A less severe winter was experienced by the same genotypes when grown in Fiorenzuola (Italy) in 2007/08 season with multiple events of temperature going over 0â℃ and then dropping to -5â℃ (Figure 5.1). Repeatability (H2) of cold tolerance measures in both trials was high (H2 = 0.84 and 0.81 for Foradada and Fiorenzuola respectively).

Substantial genotypic variation in cold tolerance was observed ($P<0.001$). As was expected, analysis of variance of cold tolerance revealed highly significant main effects of germplasm type (landraces, old cultivars and elite cultivars), geographic origin, ear type, and seasonal growth habit but no significant interactions were detected (Table 5.1). We believe the significance of germplasm type is an indirect result of most of the elite cultivars in our GWA panel being spring types. Winter lines are generally cold tolerant but great fluctuation of cold tolerance values can be observed amongst the spring lines, consistent with little selection pressure amongst them for cold tolerance. Interestingly, the most cold-tolerant lines were detected amongst Turkish facultative Types (Akar et al. 2009) (Table 5.2), suggesting a cold tolerance mechanism in these accessions may be independent of the allelic states at the vernalisation loci of these lines.

Table 5.1. ANOVA for the phenotypic data in relation to the main drivers of germplasm genetic divergence in barley: geographic origin of the germplasm, growth habit (spring/ winter / facultative) and ear morphology (two-rowed / six-rowed types) and germplasm type (landraces / old cultivars / modern

cultivars.

ANOVA fixed term	n.d.f.	Foradada (Spain)	Fioren zuola (Italy)		
		Wald statistic	F pr	Wald statistic	F pr
Germplasm type	2	30.83	<0. 001	3.38	0.188
Growth habit	2	76.45	<0. 001	36.88	<0.001
Region of origin	4	43.56	<0. 001	42.17	<0.001
Ear type	1	7.85	0.006	2.15	0.144

Table 5.2a. Summary statistics for Foradada (Spain). Genotypic means and standard error for cold resistance for the fixed terms in the model.

Region of origin	Grow th Habit	Germplas m type	Ear type				
Class	Mean s	Class	Mean s	Class	Mean s	Class	Mean s
Turkey	4,83	winter	4,37	Landrace	4.22	6 Rows	4.25
North EU Winter	4,34	Spring	3,44	Old cv.	3.80	2 Rows	3.59
South West Med.	4,22	Facultativ e	3,13	Modern cv.	3.52		
Syrian and Jordan	4.16						

North EU Spring	2,91						
St. Error	0,073		0,085		0,078		0,074

Table 5.2b. Summary statistics for Fiorenzuola (Italy). Genotypic means and standard error for cold resistance for the fixed terms in the model.

Region of origin	Growth Habit	Germplasm type	Ear type				
Class	Means	Class	Means	Class	Means	Class	Means
Turkey	3.955	winter	3.534	Landrace	3.593	6 Rows	3.346
North EU Winter	3.834	Spring	3.360	Old cv.	3.504	2 Rows	3.577
South West Med.	3.469	Facultative	3.490	Modern cv.	3.288		
Syrian and Jordan	3.066						
North EU Spring	2,983						
St. Error	0, 2024		0. 1368		0, 1208		0, 1572

5.3.2 GWA mapping

For the Foradada dataset, 14 and 6 SNPs exceeded the significance threshold for EIGENSTRAT and kinship mixed models respectively (Table 5.3). Several of these QTL

map in the same regions as loci previously reported to be involved in cold tolerance. Significant marker 11_20320, on the long arm of chromosome 5H (108 cM) is a SNP in HvCBF6 located within a physically linked cluster of at least 13 CBF family members, also known as DRE binding protein 1 (DREB1) which correspond to the cold tolerance â Fr-H2â locus 9. The QTL on the long arm of chromosome 2H at 128 cM is in the same region as the â FLT-2Lâ flowering locus (Chen et al. 2009). Interestingly, the â FLT-2Lâ locus has been reported to be closely linked to a QTL controlling frost induced sterility at the reproductive stage (Reiheimer et a. 2004; Chen et al. 2009b), thus SNPs mapping in this region could provide a means of detecting further recombinants in the region. SNP 11_11019 located on chromosome 4H at 123 cM was coincident with results of another GWA study of cold tolerance in barley (von Ziztewitz 2011), in which SNP 12_30824 had the most significant association with low temperature tolerance and targets the same barley unigene as 11_11019 (von Ziztewitz 2011), The unigene has been mapped ~4 cM from the barley vernalization gene â VRN-H2â . Both SNPs are located in HvBmy1 a beta-amylase protein. In Arabidopsis, one specific beta-amylase has been shown to have a key role for the cold-temperature dependent increase in soluble sugars and the associated protection of the photosynthetic electron transport chain and proteins in the chloroplast stroma during freezing stress (Kaplan et al. 2004). A similar role could be suggested for the HvBmy1 underlying our association hit. The same three SNP associations were also significant at Fiorenzuola, where a total of 9 and 4 significant SNP associations were detected (Table 5.3). Apart from the three above loci, all our significant associations are in regions which have not previously been reported as being associated with cold tolerance. The most significant hit for the Fiorenzuola trial (for both structure models) was located in the pericentromeric region of chromosome 2H. The lack of a significant association around the â Fr-H1â locus on chromosome 5H at 132 cM was unexpected. GWAS from the Foradada site without population structure correction (Figure 5.2.1) identified a significant association within 5 cM of a gene candidate for â Fr-H1â , the vernalization gene â VRN-H1â on chromosome 5H at 137 cM whose role on low temperature tolerance has already been reported in the literature (Dhillon et al. 2010). Two other highly significant associations were found for SNPs 11_21428 and 11_20409 on chromosome 3H at 136.66 cM.

Based on synteny and colinearity with rice sequence data, these two SNP are located 11 and 14 genes away from INDUCER of CBF EXPRESSION 2 (ICE2), a known regulatory gene of the cold tolerance pathway in Arabidopsis. Interestingly, an association mapping study of cold tolerance in rye using gene candidate approach identified a rye ICE2 homologue SNP as the most significant hit in field trials (Ly et al. 2011a). The barley homologue of ICE2 was mapped in the same position in a previous study(Skinner et al. . The region is not significantly important in the Fiorenzuola site uncorrected GWAS (Figure 5.2.2), suggesting a Genotype x Environment interaction related to the severity of the stress.

Table 5.3. Summary of significant (-log10 p â¥ 3) marker trait associations identified by genome-wide association scans where a. EIGENSTRAT analysis. b. Kinship analysis. Spring (â Tokakâ) and winter (â Introâ) are low temperature tolerant reference cultivars genotypes with maximum cold tolerance scores in our trial conditions.* S, Foradada (Spain); I, Fiorenzuola (Italy). ** reported allele effects are relative to the most frequent allele

	Trial*	Peak marker name	Chromosome	GWA statistics	SNP diversity	Reference cultivars

		20531					136)				
				-LOG 10(P)	Effect	s.e.	Alleles	MAF*	â Tokkâ	â Introâ	
a		´	´	´	´	´	´	´	´	´	´
	S	11_ 21067	1H	1.5	3.36	-0. 20	0.06	G/A	A (0. 293)	G/G	G/G
	S	11_ 10216	2H	26.5	3.4	-0. 21	0.06	A/C	C (0. 467)	A/A	A/A
	S	11_ 10498	2H	49.1	3.08	-0. 36	0.11	G/A	A (0. 207)	G/G	G/G
	S	11_ 20366	2H	128.3	4.23	0.31	0.08	A/G	G (0. 402)	A/A	G/G
	S	11_ 10565	3H	19.1	3.13	-0. 27	0.08	A/G	G (0. 247)	A/A	A/A
	S	11_ 20168	3H	114	3.77	0.44	0.12	G/A	A (0. 103)	G/G	G/G
	S	11_ 11019	4H	123.3	3.93	-0. 35	0.09	A/G	G (0. 125)	A/A	A/A
	S	11_ 11048	5H	29.9	3.12	-0. 28	0.08	T/A	A (0. 147)	T/T	A/A
	S	11_ 20320	5H	108.2	5.65	-0. 31	0.07	A/C	C (0. 310)	A/A	A/A
	S	11_ 10817	6H	45.4	3.29	-0. 24	0.07	C/A	A (0. 473)	A/A	C/C
	S	11_ 10013	6H	45.4	4.29	-0. 40	0.10	A/G	G (0. 212)	A/A	A/A
	S	11_	6H	97.4	3.90	0.34	0.09	G/A	A (0.	G/G	A/A

	S	11_11111	6H	128.5	4.88	0.35	0.08	A/G	G (0.332)	A/A	G/G
	S	11_20168	U	U	3.77	0.44	0.12	G/A	A (0.103)	G/G	G/G
	I	11_21126	1H	73.9	3.25	-0.20	0.06	G/C	C(0.152)	G/G	C/C
	I	11_10919	2H	39.1	4.29	-0.21	0.05	G/A	A(0.462)	G/G	G/G
	I	11_11522	2H	53.5	4.32	-0.27	0.07	A/G	G(0.185)	A/A	A/A
	I	11_21388	2H	55	7.51	-0.40	0.07	A/C	C(0.141)	A/A	A/A
	I	11_20366	2H	128.3	3.29	0.22	0.06	A/G	G(0.402)	A/A	G/G
	I	11_21130	4H	116.9	3.02	-0.24	0.07	C/A	A(0.158)	C/C	C/C
	I	11_20320	5H	108.2	6.10	-0.26	0.05	A/C	C(0.310)	A/A	A/A
	I	11_21168	5H	109.6	3.69	-0.21	0.06	G/A	A(0.440)	G/G	G/G
	I	11_21271	6H	105.6	3.48	0.25	0.07	C/A	A(0.321)	A/A	C/C
b			´	´				´	´	´	´
	S	11_21192	1H	88.2	3.31	-0.27	0.08	A/T	T (0.277)	A/A	A/A
	S	11_21187	2H	29.2	3.08	-0.33	0.10	G/A	A (0.141)	G/G	G/G
	S	11_10565	3H	19.1	3.65	-0.32	0.09	A/G	G (0.247)	A/A	A/A

	S	11_ 11019	4H	123.3	3.68	-0. 38	0.10	A/G	G (0. 125)	A/A	A/A
	S	11_ 20320	5H	108.2	4.48	-0. 30	0.07	A/C	C (0. 310)	A/A	A/A
	S	11_ 10013	6H	45.4	3.40	-0. 39	0.11	A/G	G (0. 212)	A/A	A/A
	I	11_ 10919	2H	39.1	3.33	-0. 19	0.06	G/A	A(0.462)	G/G	G/G
	I	11_ 11522	2H	53.5	3.37	-0. 26	0.07	A/G	G(0.185)	A/A	A/A
	I	11_ 21388	2H	55	6.26	-0. 40	0.08	A/C	C(0.141)	A/A	A/A
	I	11_ 20320	5H	108.2	4.69	-0. 25	0.06	A/C	C(0.310)	A/A	A/A

Figure 5.2.1. Manhattan plots for frost tolerance in barley in the Foradada location (Spain) where the frost episode was long and severe. (a) â Uncorrectedâ naive analysis. (b) EIGENSTRAT and (c) Kinship analysis. The -log10 (p-values) from a genome-wide scan are plotted against the position on each of the 7 barley chromosomes. The horizontal line indicates the genome-wide significance threshold (-log10 p â¥ 3). Two of the three top â uncorrectedâ hits fail to reach the significance threshold in the kinship analysis despite being closely linked to FLT-2L-linked â frost sensitivity in reproductive tissuesâ locus (), ICE2 regulatory gene () and the Fr-H1 locus (), known to be involved with cold tolerance. () Top hit in the â uncorrectedâ analysis also significant in the kinship analysis. (O) Robust hits previously reported in the literature 35.

Figure 5.2.2. Manhattan plots for winter survival in barley in the Fiorenzuola location (Italy) where accessions experienced less severe cold conditions. (a) â Uncorrectedâ naive analysis. (b) EIGENSTRAT and (c) Kinship analysis. The -log10 (p-values) from a genome-wide scan are plotted against the position on each of the 7 barley chromosomes. The horizontal line indicates the genome-wide significance threshold (-log10 p â¥ 3). Similar to Foradada data, â uncorrectedâ hits closely linked to FLT-2L-linked â frost sensitivity in reproductive tissuesâ locus (), and the Fr-H1 locus () fail to reach the significance threshold in the kinship analysis despite being, known to be involved with cold tolerance. () Top hit in the â uncorrectedâ analysis also significant in the kinship analysis. (O)

Robust hits previously reported in the literature 35 also detected in the Foradada trial.

5.3.2. QTL frequencies and population structure.

Classic LD parameters (Dâ and r2) as implemented by HAPLOVIEW v.4.2 (http://www.broadinstitute.org/haploview/haploview) were used to test whether the positive SNPs were in strong linkage disequilibrium (LD) with each other. The presence of very strong LD would raise concerns about a high rate of false positives present in our results whilst complete absence of LD between the positive SNPs would provide evidence of complete independence between the SNPs. We did not observe signs of strong inter-QTL LD resulting from population sub-structure and admixture within GWA panels even for closely linked SNPs such as SNPs 11_10817 and 11_10013 both mapping on chromosome 6H at 45 cM. With an average r2 value of 0.07, most observed r2 values were <0.3 with only four (between SNPs 11_11019, 11_10013, 11_21187 and 11_10498) just greater than 0.3, all of them having significant positive associations in the kinship model. Such r2 values are not strong enough to suggest a high false positive rate but we believe they add some evidence of co-selection, a reasonable situation when measuring traits of high agronomic / economic importance subjected to a long history of breeding and selection. In order to understand which genotypes possess favourable cold-tolerance alleles, it is important to check QTL diversity and distribution in the different genetic clusters of our GWA panel. We evaluated the genetic relationships among the accessions by generating a neighbour-joining population tree based on simple matching of allelic distances as implemented in DARwin v.5.0 which produced clear separated branches corresponding to each of our germplasm origin groupings (Northern European springs, Turkish, Syrian and Jordan, Northern European winters and South-West Mediterranean accessions). This analysis supported the same groupings as the Bayesian cluster analysis implemented in the program STRUCTURE (Pritchard et al. 2000) and Principal Coordinates results described in previous publications (Comadran et al. 2009;Comadran et al. 2011b) (Figure 5.3). Subsequent QTL haplotype analysis for the eight significant SNPs detected with the kinship analysis in the Foradada and / or the Fiorenzuola field trials highlighted a large number of allelic combinations within the â Northern European springsâ germplasm, with most of the

QTL fixed or nearly fixed in both winter clusters (â Northern European wintersâ and â South-West Mediterraneanâ lines). A high degree of allelic fixation was also observed within the cold tolerant â Turkishâ and â Syrian and Jordanâ clusters (Figure 5.3).

Figure 5.3. QTL haplotype analysis across germplasm clusters. (1) Left figure: Neighbour joining tree of the selected 184 barley cultivars constructed from simple matching distance of 1307 SNP markers. Lines are coloured according to population structure clusters described in Comadran et al. 2009: a. Northern European springs; b. Turkish; c. Syrian and Jordan; d. South-West Mediterranean accessions; e. Norther n European winters. (2) Right figure: QTL and QTL haplotype frequencies within population structure clusters for the eight significant SNPs detected with the kinship analysis in the Foradada and / or the Fiorenzuola field trials (Table 1). Reported allele frequencies correspond to the SNP allele increasing winter survival (blue). The alternative allele is shown in red.

5.4. Discussion and main conclusions

The cold tolerance component of winter-hardiness is a key trait limiting the geographical distribution of the crop and the transfer of quality traits from spring to winter crop types. Despite the world economical and agronomic importance of barley, reports of cold tolerance studies are few and limited to a few bi-parental QTL mapping studies (Pecchio ni et al. 2011). Those studies usually involve genetically broad winter (or facultative) x spring growth habit type crosses which also segregate for some major developmental lo ci. Such crosses enabled the detection of the two main cold tolerance QTL in the Tritice ae, Frost Resistance-1 (FR-1) and Frost Resistance-2 (FR-2). A cluster of CBF genes co-segregates with the wheat (Fr-A2) and the barley (Fr-H2) orthologous loci (Francia et al. 2007). The CBF gene family has been shown to have a critical role in stress response in Arabidopsis and encodes a small family of transcription factors that have been described to regulate cold acclimation response, controlling the level of COR (cold-regulated) expression, which in turn promotes tolerance to freezing. In barley, it has been shown variation in both expression levels and copy number of CBF genes is

associated with low temperature tolerance differences amongst cultivars (Knox et al. 2010). Fr-H1 is also a major switch of plant expression. Recent evidence suggests that the genetic determinant underlying Fr-H1 is the same of that of VRN-H1, a major locus governing barley vernalization requirement. Dhillon et al. (2010) demonstrated that allelic variation at the wheat VRN-1 locus is sufficient to trigger the regulatory cascade that down-regulates the cold acclimatization pathway. Progress in understanding the genetic basis of barley tolerance to low temperatures is often hindered by the unpredictability of the number, length and intensity of the frost episodes in field conditions and the difficulty in reproducing field conditions under a controlled environment conditions. Moreover, the trait is usually measured as percentage of plants surviving freezing conditions, which may dilute the effects of other components of cold tolerance such as cold acclimatization or recovery after the stress. Genome wide association data has arisen as a powerful tool to dissect quantitative traits which promises higher resolution mapping by identifying SNPs tightly linked to the trait of interest for both academic and commercial sectors (Rafalsky et al. 2002; Waugh et al. 2009). A recent GWAS study using 148 advanced breeding barley lines identified Fr-H1 and Fr-H2 QTL. Low temperature toleranceQTL at both loci explained 25 % of the phenotypic variation which suggested undetected genetic variation elsewhere 35. Von Zitzewitz et al. (2011) utilized advanced breeding material where linkage disequilibrium (LD) was extensive - 5 cM and 15 cM with r2 LD values higher than 0.6 around Fr-H2 an d Fr-H1 locations, respectively. Strong LD values across long genetic distances such as those reported in the study usually arise from inbreeding and recent selection (usual within breeding programs) and are an indication of strong population stratification. Both aspects affect alleleâ s random segregation within the genome and constitute real handicaps for association mapping studies. The approach from von Zitzewitz et al. (2011), sampling advanced breeding material may bring those issues to the extreme. The germplasm set used in this study (landraces and old cultivars from distinct geographical origins) samples a longer history of recombination events which will dilute the effects on LD related to inbreeding. However, by sampling spring, facultative and winter genotypes we are inevitably introducing sources of population structure and the need to statistically deal with them. Current genome map coverage and knowledge

about the role of Fr-H1 and Inducer of CBF expression 2, ICE2, in cold tolerance 26 sug gest that the strong associations in the â uncorrectedâ approach tightly linked to both lo ci are more than plausible false negatives introduced by population structure correction in the analysis (Figure 5.2). In a similar way, the FLT-2L related hits - with extensive literature on the involvement of this genomic region in frost sensitivity in reproductive tissues (Reinheimer et al. 2004; Chen et al. 2009b;Chen et al. 2009c)- in both Foradada and Fiorenzuola EIGENSTRAT analyses do not reach the statistical threshold using the kinship approach. Correction for population structure in GWAS performed in highly stratified populations can result in important associations being undetected. Whilst it is necessary to avoid an inflated rate of false positives arising from the genetic stratification of the germplasm, we have found that association hits of interest can match the population structure of the germplasm. In this example, 2 of the 3 top hits (tightly linked to major known genetic determinants of cold tolerance) identified in the â uncorrectedâ approach fail to reach the significance threshold in both EIGENSTRAT and kinship analyses. This study identified 6 and 3 positive associations, considering the most restrictive kinship analysis, for Foradada and Fiorenzuola trials respectively. Fr-H2 was consistently detected in both trials. It was the most significant hit under the severe cold conditions experienced in Foradada and although it was also significant in the mild winter conditions experienced in Fiorenzuola it is interesting the most significant hit, in the pericentromeric region of 2H (55 cM), had not been reported in the past. This region of the genome is not linked to the more distal Ppd-H1 (SNP on the gene on chromosome 2H 26.6 (cM) or the EPS-H2 locus (centromeric on chromosome 2H 63 cM). The extra regions detected are interesting findings that are worth pursuing in the near future. Exploration of the allelic frequencies of the significantly associated SNPs revealed most QTL are genetically fixed or nearly fixed within the winter barley germplasm (Figure 5.3). Positive alleles for cold tolerance are also fixed within the Syrian and Jordan landraces and the Turkish facultative lines both known to be winter hardy. In contrast, most of the QTL are freely segregating within the spring germplasm. Two conclusions follow these observations: First, the majority of spring x winter crosses are going to sample a portion of the variation on cold tolerance mostly dependant on the genetic make-up of the spring line. And second, work within un-adapted spring

germplasm pools is a viable alternative which will avoid complications linked to the pleiotropic effects of major developmental genes segregating in spring x winter crosses, and it will minimize the statistical issues relative to the inherent population structure of the germplasm. In any case, use of the cold adapted winter gene pool to capture additional alleles should not be discarded. The information we generated is also valuable to chose parental lines to use in crosses (in the form of bi-parental or MAGIC populations) to complement the GWAS data and take some of the hits further. However, as the genetic determinants underlying major loci Fr-H1 and Fr-H2 have already been identified population numbers weâ ll need to be increased in order to capture effects that can be masked by the pleiotropic effects of the major loci Fr-H1 and Fr-H2 and to detect stronger effects arising from epistasis that have naturally been accumulated in cultivated germplasm but broken in the development of the mapping population. An attractive alternative which does not involve the development of new plant material to be tested involves the ultra-saturation of the genome by emerging new generation sequencing technologies (methods reviewed in Davey et al. 2011) such as genotyping by sequencing (GbS)(Elshire et al. 2011). These technologies promise a deeper coverage of polymorphic sequence information and therefore improve the mapping resolution to the extent of being able to provide shortlists of gene candidates to be functionally tested. At the same time, they will provide an unbiased assessment of the diversity at every single gene which will result in a fast implementation of the results arising from that data in the form of specific haplotype and allele information tightly linked to the regions of interest to be used as new molecular tools in pre-breeding marker assisted selection (MAS) programs aiming to have the right alleles in acceptable genetic backgrounds to be use in commercial breeding programs.

5.5. References

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Chapter 6
General discussion

6.1 General discussion

The complex nature of quantitative traits studied by QTL (Quantitative Trait Locus) mapping, requires adequate analytical tools to overcome limiting factors such as QTL detection power and QTL effects on quantitative trait variation. Dissection of the genetic basis of complex traits has been greatly enhanced by the introduction of the genomics platforms that allow identifying genes and QTLs governing genetic variation at higher numbers of relevant loci. However, often quantitative trait variation shows strong genotype x environment interaction. Despite progresses made in last years, most of the mechanisms that control wide environmental adaptation and abiotic stress response are poorly understood (Roy et al. 2011). The aim of this thesis work was performing a wide-eyed genetic study of barley adaptability in stress prone environments, such as those around the Mediterranean basin, using both a bi-parental and an association mapping

approach. Both approaches have extensively used to map genetic determinants of abiotic stress response in crops. We used a segregating, doubled haploid, population derived from the cross between two elite barley cultivars â Nureâ and â Tremoisâ (Francia et al. 2004), together with an association mapping panel of 185 genotypes comprising the past and present of cultivated barley genetic diversity in the Mediterranean basin and described more in details by Comadran et al. (2011). The â Nureâ x â Tremoisâ double haploid mapping population shows high level of diversity of parents due to no ancestors in common, and has been widely exploited to study various quantitative traits such as winter hardiness, flowering time and malting quality (Francia et al. 2004; von Zitzewitz et al 2005; Laidˆ‡ et al. 2009). After improving the marker density of NT linkage map with several new markers added; the population has been used to map QTL for yield and yield adaptation in Mediterranean environments. We also checked QTL sensitivities to environmental co-variables of two major loci controlling both grain yield and days to heading on chromosomes 2H (eam6/Eps-2) and 5H (Vrn-H1). In turn, the association mapping panel represents a geographically diverse range of spring, winter and facultative cultivated barleys. We used this panel to perform genome wide association analysis (GWAS) for frost tolerance using data collected in Spain during the season 2004/2005 and in Italy during season 2007/2008. As reviewed by Maccaferri et al (2009) association mapping provides an additional tool to identify genes and/or QTLs for target traits (Gupta et al. 2005; Burke et al. 2007; Veyrieras et al. 2007). Despite this nowadays most of published works are limited to resistance to biotic stress and qualitative traits (Roy et al. 2010; Cockram et al. 2010; Ramsay et al. 2011). Furthermore only three GWAS approaches have been published for study frost tolerance two in barley and one in rye, which are considered the most tolerant species (von Zitezewitz et al. 2011; Li et al. 2011a; Li et al. 2011b). Our association panel was successfully used to map QTLs for major components of grain yield linked to major developmental loci (Comadran et al. 2011). Plant materials were tested in 18 multi-environment field trials (MET) conducted in six countries around the Mediterranean basin: Algeria (DZA), Italy (ITA), Jordan (JOR), Spain (ESP), Syria (SYR) and Turkey (TUR), for two harvest seasons (2004 and 2005). In each country, trials were grown at sites contrasting for natural rainfall (high vs. low; based on past

meteorological data, not shown), or at the same site with one trial being rainfed and the other supplied with supplementary irrigation. The environmental co-variables, collected during all the cycle of the crops for data analysis were : average daily maximum temperature (Tmax), average daily minimum temperature (Tmin), difference between average daily maximum and minimum temperature (Tdif), number of days with temperature under 0´" C (dT0), number of days with temperature above 30´" C (dTa30), Growing Degree Days [calculated by subtracting Tbase (10´" C) to the average of the daily maximum and minimum temperatures, GDD], Rainfall (indicated in mm, Rf), total water input (mm, WT), Solar radiation (W/m2, SR), Potential evapotranspiration (mm/ day ET), Photothermal Quotient (radiation per unit area per day, PQ) defined as solar radiation to average daily temperature ratio and Water Deficit (WD), defined as WT to ET ratio. Average means for each environmental co-variable was then calculated for each one of the three considered growth phases (vegetative phase, sink determination phase and grain filling phase). Winter survival was evaluated in Foradada (Spain) after an exceptional at the end of March 2005 by visual estimation on a 0-5 scale as described by Akar et al. 2009. In order to get a more reliable evaluation of cold damage, the same dataset was assessed for winter survival in the growing season 2007/08 in Fiorenzuola dâ Arda (Italy) previously known as frost-prone environment.
The two genetic approaches allowed to:

improve the 'Nuure'x'Tremois' biparental mapping population;
identify QTLs for yield and yield adaptation in the biparental population
identify and measure QTL sensitivity to meteo (environmental) variables
identify additional loci responsible of frost tolerance by GWAS in the association panel

6.2 Development of new DaRT- based linkage map

Using the DArT´fi marker platform we made genetically denser the â Nureâ x â Tremoisâ linkage map with a total of 396 DaRT, 18 STS-SNP and 10 SSR markers were integrated into a medium-high density map. Fourteen SNP derived markers were added to the map as 13 Cleaved Amplified Polymorphic Sequence (CAPS) and 1 Single Strand Conformational Polymorphism (SSCP). The total length of the map is now of 1114 cM, with an average resolution of one marker every 2.8 cM. Individual linkage group length ranges from 117.7 cM (1H) to 203.3 cM (5H), and alignment with the barley consensus map built by Wenzl et al. (2006) showed a high level of conservation of DaRT locus order. Gaps on the map, larger than 20 cM, were found in six regions. Segregation distortion was also found on chromosome 1HL and on chromosome 6H, but as described by Xu et al. (2008) this is not expected to affect QTL detection. High density consensus maps could represent a starting point to identify association between QTL for agronomics trait and gene targeted markers (GTM). GTM introgressed (Andreson and Lˆ…bberstedt, 2003) are useful for candidate gene approach to dissect complex traits. Mapped GTM usually can preferentially be transcription factors, which may be involved in barley flowering time regulation, development and adaptation to the environment. GTM are developed starting from ESTs (Expressed Sequence Tags) and are an optimal tool for candidate gene (CGs) approach. The CG strategy can then be deployed starting from GTMs, and it has been recently used to identify genetic determinants of quantitative trait loci (Thornsberry et al. 2001; Palaisa et al. 2003). TFs are preferred as CGs and considered better than effectors genes due to their key role in modulating cascades of signal transduction, such as in response to abiotic stress, where a single TF can regulate a pathway that can lead to an enhanced tolerance (Tondelli et al. 2006).

6.2.1 Genome scans for yield adaptation in Mediterranean environments

Multi-environment trials conducted over mapping populations represent a valuable tool for the identification of the genetic basis of barley grain yield potential and yield stress

adaptation. The evaluation of the â Nureâ x â Tremoisâ population in eighteen sitebyy ear field trial combinations across the Mediterranean basin, allowed the identification of the genomic regions responsible for barley adaptation in terms of phenology, grain yield and yield component traits. We performed composite interval mapping genome scan, using putative QTLs as cofactors for any environment / trait combination. The most frequently detected yield (GY) and days to heading (DtH) QTL overlapped with the early maturity eam6/Eps2 locus (chromosome 2H), showing a positive effect from the early winter parent â Nureâ in eight field trials, and explaining up to 45.8% of the observed phenotypic variance. Earliness was generally associated to a shorter spike and a smaller number of grains per spike, but to a higher thousand grain weight and harvest index. Moreover, no yield penalty due to the early heading was observed in the highest (i.e. >4.5 t ha^{-1}) yielding environments. The importance of the genomic region has also been highlighted in previous studies on bi-parental populations evaluated in METs (Cuesta-Marcos et al. 2008a; von Korff et al. 2008); and in our association panel assayed in 28 site by year combination (Comadran et al. 2011). The â Nureâ allele at E am6/Eps-2 was sufficient to determine an higher grain yield in contrasting Mediterranean environments probably by hastening flowering time; thus representing an interesting source of variation in early heading /early ripening to maximize yield potential in Mediterranean environments once vernalization and photoperiod requirement are fully satisfied. The Vrn-H1 locus on chromosome 5H associated to GY and DtH in five and three field trials respectively. QTL for yield components such as spike length, number of spikes for square meters and length of grain filling phase were found for both eam6/Eps-2 and Vrn-H1 loci thus, highlighting again the importance of these two loci in Mediterranean environments. Other significant QTL at the eam6/Eps-2 locus were found for numbers of grain for spike, thousand grain weight, days to maturity and peduncle length confirming again the importance of fine tuning in flowering time and consequently to maximize yield potential. Overlapping of yield and heading date QTL suggested the observed effects are mainly related to differences in the number of days from sowing to heading, as it was further confirmed by partitioning the population in haplotype classes based on the most important loci detected. Environment-specific QTLs for grain yield, and cluster of yield component QTLs not related to phenology/

developmental genes (e.g. chromosome 4H, BIN 9) were observed as well. The NT population is the only bi-parental population segregating for both Frost resistance-H1 (F r-H1), coincident with Vrn-H1, and Frost resistance-H2 (Fr-H2) loci, the second coincident with a cluster of CBF genes (Francia et al. 2004, 2007; Knox et al. 2010). Interestingly, HvCBF_FR-H2 coincides with the most important yield adaptability QTL we found across the 18 field trials. This could be due to the fact that CBFs locus is involved in both drought and cold stress response (Skinner et al. 2005; Francia et al. 2007). The co-mapping of QTLs for frost resistance, early vigour and number of spikes per square meter can then lead to hypothesize that FR-H loci could have had a prominent role in the best establishment of plant juvenile phase. The only one yield stability QTL was mapped at the BIN 7_1 on chromosome 3H, at a genomic position where a plasticity QTL has been previously detected in barley by Lacaze et al. (2009). Despite the heterogeneity of the environmental conditions, we have identified genomic regions consistently associated to yield. The results here shown complement and reinforce the detailed analyses on genotype x environment and QTL x environment interactions carried out on the same multi-environmental trial dataset (Francia et al. 2011).

6.3 QTLs sensitivity to environmental co-variables

In stress-prone environments, such as Mediterranean basin, optimizing flowering time is crucial maximize grain yield. QTL that drives plant adaptation, usually show different effects across environments. In presence of QTLE interaction, when environmental co-variables are available, QTL effects across environments can be tested for their sensitivity to a particular co-variable (Crossa et al. 1999; Malosetti et al. 2004; Vargas et al 2006). Regression of QTL effects has been used to check sensitivity to environmental co-variables, in the â Nureâ x â Tremoisâ double haploid mapping population tested in 18 field trial across the Mediterranean basin. We used two QTLs on chromosomes 2H and 5H, located in the same position for both grain yield (GY) and days to heading (DtH) . These loci correspond respectively to the well-known development genes eam6/Eps2, the earliness per se or early maturity locus and a vernalization gene, Vrn-H1. Both loci

are involved in control of flowering time in barley and consequently in grain yield. Relationship between flowering time and grain yield in Mediterranean environments have been highlighted by Cuesta-Marcos et al. (2008a; 2008b). Results obtained from G+GE partitioning based on simple two gene model, using the allele variation at eam6/ Eps2 and Vrn-H1, showed that model explain the 69 % of G variation for DtH and 42.9 % for GY (Table 4.4). Surprisingly, the 98 % for DtH and 93.5 % for GY of genotypic variation are explained by eam6/Eps2. This mean that allelic variation at Vrn-H1 is apparently not related with DtH and GY, in this multi-environment study and in this population, probably due to variability of conditions across field trials where in some case vernalization requirement was not necessary or not fully satisfied. Another explanation, from a physiological point of view, could be that Vrn-H1 is responsible only of the transition of apex from vegetative to reproductive phase (Trevaskis et al. 2003; Yan et al. 2003; Preston and Kellogg 2008), while earliness per se loci are expected to affect both vegetative and early reproductive phase. A definitive clarification of these hypotheses could come from a biparental study where eam6/eps2 is not segregatingwhi le Vrn-H genes are. For the same dataset of meteorological variables, a preliminary study by Romagosa et al. (2008) reported that Principal Component Analysis highlighted a high correlation between environmental co-variables across growth phases. Once a physiological basis is found for each significant environmental co-variable, this may allow a better understanding of how major genes interact with different environments, and of which co-variables are the most influent across a range of environments. Results of regression between QTL effects and environmental co-varibles collected in all field trials during the whole life cycle of plant showed that maxim um temperature was the variable often detected both loci at the different growth stage. Variation in DtH associated to eam6/Eps2 locus across environments ranged from 0.2 to 3.6 days, and is in full agreement with previous studies on effects of earliness per se in triticeae (Flood and Halloran 1983; Scarth and Law 1983; Hoogendoorn 1985; Miura and Worland 1994; Laurie et al. 1995; Worland 1996; Kato et al. 1999). The most significant environmental co-variables related with DtH for eam6-Eps-2 locus were maximum temperature (Tmax) during sink determination phase and evapotranspiration (ET) during vegetative phase. Both variables showed non-crossover QTLE interaction. In

this case variation of QTL effects across environments, is quantitative and in the same direction. Effects of â Tremoisâ spring alleles on this locus are always positive and associated with late flowering in each field. Bullrich et al. (2002) found that in diploid wheat the earliness per se gene Eps-Am1 showed significant interaction with temperature that result in significant differences in flowering time between accessions carrying different allelic variants at the Eps-Am1 locus. ET is used to describe the sum of soil evaporation and plant transpiration from crop to atmosphere, and is related with water stress and temperature. Relationship between ET and DtH in literature was never reported, furthermore as reported by Angus and Moncur (1979) mild stress speed-up flowering time in wheat, while severe stress delay DtH. We observed that in fields with relatively mild ET the effects on flowering were higher than in fields with higher ET. This mean that the allele from the parent â Nureâ always hasten flowering time but in field with mild ET its effect higher than in fields were ET recorded was high. We only observed a partial reduction of eam6/Eps-2 locus effects with high ET, this probably due to the growth phase; severe drought stress is unlikely in during vegetative phase in Mediterranean environments. On the other hand Vrn-H1 shows a crossover interaction, in this case the spring allele from â Tremoisâ seemed to accelerate heading date in mayor part of field trials, when subjected to high temperatures during vegetative phase. In case of a crossover interaction, variation associated to a locus is qualitative; this means that QTL effects changes in magnitude and in direction across fields. In three trials, characterized by lowest temperatures and for the highest number of days with temperature under 0´℃, the recessive winter allele vrn-h1 from â Nureâ seems to be favorable by hastening flowering time. As reported by Trevaskis et al. (2006) increased expression of Vrn-H1 in vernalized plants is correlated with reduction of flowering time and that is it likely that Vrn-H1 acts as promoter of flowering. The most significant environmental co-variables, detected at both loci for GY were again Tmax. Regression for GY showed a quantitative QTL.E interaction for eam6/Eps2. In this case the allele from â Nureâ seems to increase GY due to early flowering mediated by temperatures. Benefits arising from good levels of earliness per se in Mediterranean environments, where crops are exposed to terminal drought stress have been reported also by Cuesta-Marcos et al. (2008a). As for DtH a crossover interaction was found for Vrn-H1. The vrn-

h1 allele from â Nureâ seemed to increase GY in trials with lowest temperature during vegetative phase, due his effects on vernalization under short day. On the other hand, in two field where sowing was performed late, and temperature during the vegetative step were higher, we found significant positive effect on GY associated to the Vrn-H1 all ele from â Tremoisâ that is a spring modern high yield cultivar. Ellis and Russell (1984) reported that spring growth habit genotypes develop faster in spring sowing, this allow to escape to unfavorable conditions that frequents occurs, at this latitudes, in late phases of growing cycle. Results highlighted the usefulness of study QTLs sensitivity to environmental factors to better understand the genetic architecture of complex trait and to predict genotypes performances. The relationships between QTLs and meteorological co-variables were both lineal and quadratic, and in some cases a cross-over was evident with a QTL allele effect both decreasing and increasing the trait in dependence from the meteorological co-variable considered.

6.4 Genome wide association analysis for cold tolerance

The genetic control of cold tolerance is complex and results from the manifestation of many components traits. In barley the major efforts to dissect the basis of cold tolerance have been based on classical bi-parental crosses-mapping populations. These populations captures only a portion of genetic diversity and that may not be representative of the diversity present in breeding pools thus limiting advances in understanding of genetic control of complex traits. Increasing interest in Genome Wide Association Scans (GWAS) due to published studies, done with elite germplasm, that were successful to identify genetic variants associated to simple and quantitative traits induce us to attempt GWAS for cold tolerance. To identify barley varieties with superior cold tolerance and to advance our understanding of the genetics of low temperature tolerance, we used 185 barley accessions, sampling the cultivated diversity across the Mediterranean basin, genotyped with 1536 SNPs. Analysis of the phenotypic data for cold resistance collected during an extraordinary cold season in Spain during year 2005 and in another field trial in Italy during year 2007, previously known as frost-prone

environment. Genome scan was performed using a mixed linear regression model which account for multiple levels of genetic relatedness due to historical population substructure and kinship. To correct for population substructure, we used either the EIGENSTRAT relationship model with PCA (Principal Component Analysis) scores as random terms (Yu et al. 2006) or a kinship matrix (Price et al. 2006) in the association mapping routines implemented in Genstat v.14 (VSN International).

Some of the detected associations involved SNPs tightly linked to known major genes and loci determining cold resistance in barley such as the vernalization gene Vrn-H2 loc ated on chromosome 4H previously reported in other GWAS study (von Ziztevitz 2011), and the frost resistance quantitative trait locus â Fr-H2â located in the long arm of chromosome 5H (Francia et al. 2004) that overlap the CBFs locus a family of transcription factors involved in abiotic stress response such as cold, drought and salinity (Skinner at al. 2005; Francia et al. 2007; Wu et al. 2011). Another QTL detected corresponded to a QTL on the long arm of chromosome 2H affecting t frost tolerance at reproductive stage (Reinheimer et al. 2004). Further studies demonstrated that the low temperature tolerance locus was genetically separable from the close â FTL-2Lâ flower ing time locus (Chen et al. 2009). Surprisingly, no association was found for the frost resistance locus â Fr-H1â probably due to the strong population structure effect that could have generated â false negativesâ . Checking genome scan results without correction for population structure we found strong associations tightly linked to â Fr-H1â locus (chromosome 5H) and on the long arm of chromosome 3H for ICE2 the inducer of CBFs expression (Li et al. 2011). In highly stratified populations a correction for population structure is necessary to avoid false positive discovery arising from the genetic stratification of populations. This may produce an overcorrection in associations that match the population structure.

Other positive associations identified on chromosomes 1H, 2H, 3H, 4H and 6H showed that the genetic basis underlying cold tolerance in autumn sown winter growing conditions is genetically richer than what thought before. Turkish facultative entries showed the higher cold resistance scores, while â Syrian and Jordanâ spring entries surprisingly showed a higher degree of cold tolerance respect to all others spring cultivars comparable with â North Mediterranean Winterâ and â South West

Mediterraneanâ lines. Checking the allele frequencies of the QTL SNPs is evident that most QTL are genetically fixed / nearly fixed within the winter germplasm and though they are freely segregating in the spring germplasm. Working on un-adapted spring and facultative germplasm may be an interesting alternative for barley breeding, which could also lead by a "mapping-on-the-go" approach to identify new genomic regions involved in cold resistance.

6.5 Final Remarks

Considering the importance of phenology in barley adaptation to drought-prone areas, in this study we successfully developed a new molecular linkage map with improved resolution was drawn for the â Nure x Tremoisâ mapping population. The map now comprises a total of 542 molecular markers. Considering the importance of phenology in barley adaptation to drought-prone areas, in this study we successfully added to the previous ones 14 transcription factors that may be involved in the regulation of flowering time, plant development and adaptation to the environment. By performing composite interval mapping we then detected yield (GY) and days to heading (DtH) QTLs overlapped with the early maturity eam6/Eps2 locus (chromosome 2H), showing a positive effect from the early winter parent â Nureâ in eight field trials, and explaining up to 45.8% of the observed phenotypic variance. The Vrn-H1 locus on chromosome 5H associated to GY and DtH in five field trials with positive effect of the parent â Nureâ allele. Overlapping of yield and heading date QTL on loci on chromosomes 2H and 5H, together with several QTL for yield components seems to confirm, especially for eam6/ Eps-2, results published in other MET studies in both bi-parental population and in GWAS (Cuesta-Marcos 2008a; von Korff et al. 2008; Comadran et al. 2011), highlighting the importance of research focused in pre-heading phases, in managing biomass and in ensuring effective remobilization of assimilates to grain (Borras-Gelonch et al. 2010; Fleury et al. 2010). Furthermore an yield adaptability QTL was found across the 18 field trials on chromosome 5H overlapping with the CBF-Fr-H2 locus. A yield stability QTL was mapped on chromosome 3H, at a genomic position where a plasticity QTL has been previously detected in barley by Lacaze et al. (2009). Further QTLs for

yield were detected on chromosome chromosome 1H for yield in three field trails also with positive effect of the parent â Nureâ allele, that explaining 8.4 - 11.9% of the observed phenotypic variance, one on chromosome 6H with positive effect on yield from the parent â Tremoisâ . A QTL associated with several yield components (Plant Height, Spike Length, Number of Spike for Square Meters, TKW, Peduncle Length and Peduncle Extrusion) not related to phenology/developmental genes was observed in addition on chromosome 4H.

Performing QTL multi-environment analysis using composite interval mapping and putative QTLs as cofactors we found two commons QTL for days to heading and grain yield. QTL effect for days to heading ranged from 0.2 to 3.36 and from 40 kg/Ha to 620 Kg/Ha. Both loci explained together 31% of GE for days to heading (45.4 % and 46.7% for eam6/Eps-2 and Vrn-H1 respectively) and 25% of GE for grain yield (24.9% for eam 6/Eps-2 and 72% for Vrn-H1). We found significant sensibilities for these two QTLs related with temperature and temperature based variables throughout the growing cycle. The most important meteo-variables that drove QTL effects in our METs were temperature and temperature-related variables such as ET and GDD and SR for both DtH and GY. Non-cross over interaction were detected for eam6/Eps-2 locus, where the allele from the winter parent â Nureâ always shows positive effects on GY by hastening flowering time. Cross-over interactions were revealed for Vrn-H1 for DtH and GY. The vrn-h1 allele from the winter parent â Nureâ accelerate flowering in fields with temperature of vegetative phase were under 10´℃ degrees, due his effects on vernalization under short day; these effect results in increased GY. In the other hand the allele from the spring parent â Tremoisâ seemed to accelerate heading date in mayor part of field trials, when subjected to high temperatures during vegetative phase. Whereas in two field where sowing was performed late, and temperature during the vegetative step were higher, we found significant positive effect on GY associated to the Vrn-H1 allele from â Tremoisâ that is a spring modern high yield cultivar. The responses to low and high meteorological values, together with the identification of specific environmental thresholds in case of cross-overs, can in fact help breeders to generate predictive values for the introgression of specific alleles into cultivars, to tailor new cultivar design for expected average values of meteorological variables. Results of

genome wide association analysis for cold tolerance, performed in our association mapping panel comprising landraces old and modern cultivars we found, correcting population structure with EIGENSTRAT and Kinship, thirteen significant associations with specific genomic regions. Of particular interest are positions of several QTLs coincident with previously known locations of loci involved in cold tolerance such as the vernalization gene â Vrn-H2â , the frost resistance locus â Fr-H2â and â FTL-2Lâ flowering locus. We also found new significant associations never reported before on chromosomes 1H, 2H, 3H, 4H and 6h showed that the genetic of cold tolerance is genetically richer than a priori through. QTLs are genetically fixed o nearly fixed within the winter barley germplasm, but positives alleles for cold tolerance are also fixed within Syrian and Jordan landraces and Turkish spring lines both known to be winter hardy. The aim of this work was study barley adaptation to Mediterranean environments where yield and quality of barley and other crops are heavily affected by abiotic stress such as frost and drought. Identification of QTL responsible for the yield adaptation, yield components, phenology and stress tolerance of barley in a wide range of Mediterranean environmentsrepresent an important challenge to maximize crop production; in a scenario where frequency and severity of this limiting factors are expected to increase in the future. We successful used two approaches, a bi-parental mapping population and an association mapping panel to describe, from a genetic point of view, the effect of genetic component (G) that controls adaptation to drought and cold tolerance and the effect the GE interaction and how is this modulated by environmental cues. The new NT high density linkage map and QTL detected for yield adaptation, phenology and stress tolerance may be used in Marker Assisted Selection of new varieties of barley for these environments. In our opinion studies like this will give the bases to improve the connection between genetic, physiology and agronomy. Evaluation of QTL effects on large scale, such as in present work, may be used to develop agronomic models to predict production together with agronomic and environmental variables.

6.6 Conclusions

1 We draw a new high density linkage map comprising 542 DaRT markers and 14 Gene Targeted Marker (GTM) encoding transcription factors that may be involved in the

regulation of flowering time, plant development and adaptation to the environment. GTM are useful for candidate gene approach to dissect complex traits.

2 The evaluation of the NT mapping population in MET across the Mediterranean basin and the subsequent QTL analysis has led to map several QTL with major and stable effects for grain yield and other morpho physiological and phenological traits important for yield potential and yield stability. In particular several QTL detected for yield, yield components and heading date overlaps the eam6/Eps-2 (Chromosome 2H) and Vrn-H1 (Chromosome 5H) underlying the importance of these two loci in Mediterranean environments.

3 Using a regression model incorporating explicit environmental information and genotypic information, we studied sensitivities of QTL located on eam6/Eps-2 and Vrn-H1 loci to environmental co-variables. Results showed as QTL effects are driven by environmental co-variables such as temperature and evapotranspiration and growing degree days.

4 Performing GWAS for complex traits we successfully map previously know QTL involved in frost tolerance; in an association panel comprising 185 barley varieties representative of genetic variability of cultivated barley in Mediterranean basin.

5 GWAS also allows identifying barley varieties with superior cold tolerance within our association mapping panel. Several new QTL for cold tolerance were mapped and this represent an advance in knowledge of mechanisms that controls frost resistance that could be useful to develop new molecular markers for MAS.

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