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Study of genetic bases of traits involved in resistance to biotic and abiotic stresses in durum wheat (*T. turgidum L.* subsp. *durum (Desf.) Husn.*)

Scientific-disciplinary Area
AGR/07

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INTRODUCTION

Durum wheat (*T. turgidum* subsp. *durum*) is a tetraploid species (2n=28, genomes AABB), a very important crop for the human diet, particularly in the Mediterranean basin where about 75% of the world's durum grain is produced. With about 21.0 million hectares under cultivation (about 8% of the total wheat cultivated area), durum wheat ranks eighth among all cereals. Except for the small amount used in manufacturing couscous and local bread in some Mediterranean countries, its only significant finished product is represented by alimentary pasta.

Grain yield is estimated around 31.9 million tons for 2009/10, up 2.3 million tons from 2008/09 and up 5.7 million tons from 2007/08 (USDA-FAS) with wide variation due to drought stress, diseases and pests. The major exporting countries of the European Union (EU), Canada, and the United States combined, account for 51 percent of total durum production, followed by Syria, Algeria, Morocco, Russia, Turkey, Mexico and India.

The EU's 2009/10 durum crop is estimated to be 8.0 million tons. Europe's durum crop is concentrated along the drier Mediterranean Basin area with Italy, France, Spain, and Greece being the largest durum producing countries. Italy is the main market for the European Community durum wheat. With a growth area larger than 1.5 million hectares, and a production of about 4t million tons, Italy is one of the most important durum wheat producing countries in Europe. Southern and insular regions represent seventy-five percent of durum wheat growing areas in Italy, even if they only account for about 66% of the total Italian production with an average yield 52 and 26% lower than in the Northern and central areas, respectively. The cause of such differences is mainly due to the different pedology and climatic conditions. In fact, durum wheat is primarily grown under rainfed conditions, and grain yield is strongly limited by the frequent and irregular occurrence of drought combined with heat stress at the late phases of the

growth cycle (Araus et al. 2002; Condon et al., 2004). Furthermore, fungal pathogens and other diseases also concur to reduce the field performance with losses reaching 30-50% in grain yield. For these reasons, the improvement of plant capacity to cope with water stress and the accumulation of disease resistance genes into the same genotype are the main objects of the breeding programs for durum wheat in order to reduce the gap existing between potential and actual yield.

As compared to hexaploid wheat, durum wheat underwent a more limited selection until 1960, when more intense breeding programs based on innovative germplasm introgressions and multi-environment testing for wide adaptation were applied also to durum wheat. Accordingly, the genetic gains obtained after 1970 in grain yield (GY) of durum wheat are comparable to those obtained for hexaploid wheat. These gains have mainly been attributed to a balanced improvement in fertility because of higher allocation of assimilates to the growing tillers and ears concomitant with a general increase in total biomass production, with the harvest index remaining practically unchanged (Slafer and Andrade 1993; Slafer et al. 1996; Pfeiffer et al. 2000;De Vita et al. 2007; Slafer and Araus 2007). As suggested by Pfeiffer et al. (2000), GY components have reached a near-optimal balance in modern elite durum wheat cultivars. While the improvement of GY under optimal growing conditions has prevailingly been attributed to increased spike fertility, under Mediterranean- like conditions the importance of traits at the basis of growth plasticity, such as early vigor and a finely tuned heading date that allows the plant to escape from terminal drought, has been universally recognized (Richards 2000; Spielmeyer et al. 2007).

High-yielding cultivars endowed with drought tolerance and disease resistance, high grain yield, in addition to high commercial and technological value, are therefore highly desirable. Due to the complex genetic basis and the high genotype – environment interaction of quantitative traits as the yield capacity and the tolerance to abiotic and some biotic

stresses, the success obtained with traditional breeding approaches have been somehow limited. Therefore, the integration of the traditional breeding methods with modern technologies based on molecular markers is expected to open new opportunities for selection of high yielding durum wheat cultivars in environments characterized by biotic and abiotic constraints. In particular, plant physiology has provided new insights and developed new tools to understand the complex network of drought-related traits, and molecular genetics allows to map disease resistance genes and QTLs affecting the expression of drought tolerance-related traits. This kind of studies makes possible to dissect the genetic mechanisms of analysed traits and the identification of linked molecular markers that can be used to transfer useful alleles into elite cultivars in order to reduce the gap between yield potential and actual yield.

A wide research program is in course at the CRA-Cereal Research Center of Foggia (CRA-CER), aimed to the genetic analysis of traits of agronomic relevance for durum wheat, and funded by the national project "AGROGEN - Laboratorio di GENomica per caratteri di importanza AGROnomica in frumento duro: identificazione di geni utili, analisi funzionale e selezione assistita con marcatori molecolari per lo sviluppo della filiera sementiera nazionale". A number of segregating populations, together with the corresponding genetic maps have been developed in the frame of this project, by starting from crosses between durum wheat varieties contrasting for the traits of interest. The work described in this thesis was carried out by using some of these genetic materials, and was focused on studying genetic basis of yield-related traits, and resistance to biotic and abiotic stresses for the improvement of field performance of durum wheat in the Mediterranean environment.

CHAPTER 1

Development of the linkage map Ofanto x Cappelli: a tool for the genetic dissection of traits of agronomic value in durum wheat

Genetic map. Genetic linkage maps are a fundamental tool for several purposes, such as evolutionary genomics, understanding the biological basis of complex traits, dissection of genetic determinants underlying the expression of agronomically important traits.

The construction of a genetic map requires a segregating plant population and molecular markers. Genetic linkage maps indicate the position and relative genetic distances between markers along chromosomes. Genes or markers that are close together or tightly-linked will be transmitted together from parent to progeny more frequently than genes or markers that are located further apart.

There are three major types of genetic markers: (1) morphological markers which themselves are phenotypic traits or characters; (2) biochemical markers, which include allelic variants of enzymes called isozymes; and (3) DNA (or molecular) markers, which reveal sites of variation in DNA.

Wheat genetic maps were first comprised of restriction fragment length polymorphisms (RFLPs) and later on PCR-based markers were adopted, including random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) (Gale et al. 1995; Messmer et al. 1999; Peng et al. 2000; Paillard et al. 2003; Blanco et al. 1998). In contrast to hexaploid wheat (AABBDD), for which several linkage maps have been developed (Gill et al. 1991; Lagudah et al. 1991; Dubcovsky et al. 1996; Röder et al. 1998; Somers et al. 2004; Sourdille et al. 2004), relatively little attention has been given to developing genetic linkage maps for durum wheat. The first full genetic linkage map for this species, based on RFLP markers, was presented by Blanco et al. (1998), and subsequently integrated with SSRs from hexaploid wheat (Röder et al. 1998; Korzun et al. 1999). Later on, intra and inter-specific linkage maps based on RFLPs, SSRs and AFLPs were developed (Peng et al. 2000; Nachit et al. 2001; Maccaferri et al. 2008).

SSRs, also known as microsatellites, in particular have become the markers of choice for cereal genetic analysis and mapping (Röder et al. 1998; Song et al. 2005; Gupta et al. 2002). To date, over 2,000 SSR markers on 21 hexaploid wheat chromosomes have been published (Ganal and Röder 2007). Microsatellite markers generally exhibit a higher level of polymorphism that is critical for detecting differences among related crop cultivars, and they can even discriminate among closely related wheat breeding lines (Plaschke et al. 1995). In addition to their high polymorphism, microsatellites are stable and usually inherited in a co-dominant Mendelian manner. The abundance of information derived from such markers, together with the ease by which they can be identified, make them ideal markers for the construction of genetic linkage maps and useful in marker-assisted selection experiments. Nevertheless, more recently new molecular markers types have been developed in order to satisfy the need of high-throughput assays, able to analyse a great number of markers and genotypes with a reduced cost. Diversity Array Technology (DArT-www.diversityarrays.com) is an array-based platform for high-throughput analysis of DNA polymorphism and molecular markers for genetic mapping (Jaccoud et al. 2001). The genotyping technology involves the use of methylation sensitive restriction enzymes to digest genomic DNA, thereby reducing genome complexity and enriching for low copy sequences for marker development. DNA samples enriched for low copy DNA sequences from parents and individuals of mapping populations are hybridized to a microarray panel of clones representing low copy sequences from the same plant species. Restriction site polymorphisms between individuals from mapping populations are detected through differences in hybridization signal, with clones on the microarray panel scored as dominant markers and providing for allele attribution to one or the other parental genotype. DArT and other DNA markers have been used to produce genetic maps for a range of crop species including rice (Jaccoud et al. 2001), barley (Wenzl et al. 2004;

Hearnden et al. 2007) sorghum (Mace et al. 2008) and wheat (Semagn et al. 2006). Furthermore, genomic representations from diverse accessions also have been developed for durum wheat (Mantovani et al. 2008; Peleg et al. 2008).

Linkage maps have been widely utilised for identifying chromosomal regions that contain genes controlling simple traits (controlled by a single factor) and quantitative traits by using QTL analysis.

Once the genomic determinants of the target trait have been identified, close molecular markers can be used to transfer useful alleles to elite cultivars by marker-assisted selection (MAS). MAS based on using the allelic status of a marker as a substitute for or to assist in phenotypic selection, in a more efficient, effective, reliable and cost-effective way compared to the conventional plant breeding methodology.

QTL mapping. Many agriculturally important traits such as yield, quality and some forms of disease resistance are controlled by many genes and are known as quantitative traits (also 'polygenic,' 'multifactorial' or 'complex' traits). The process of constructing linkage maps and conducting QTL analysis, to identify genomic regions associated with traits, is known as QTL mapping, and it is based on the integration of genotypic and phenotypic data obtained for a segregating population.

After the construction of the linkage map and phenotyping we can use three methods for detecting QTLs: single-marker analysis, simple interval mapping (SIM) and composite interval mapping (CIM). Single-marker analysis (also 'single-point analysis') is the simplest method for detecting QTLs associated with single markers. The statistical methods used for single-marker analysis include *t*-tests, analysis of variance (ANOVA) and linear regression. Linear regression is most commonly used because the coefficient of determination (R^2) from the marker explains the phenotypic

variation arising from the QTL linked to the marker. This method does not require a complete linkage map and can be performed with basic statistical software programs. However, the major disadvantage with this method is that the further a QTL is from a marker, the less likely it will be detected, due to recombination events that may occur between the marker and the QTL.

The simple interval mapping (SIM) method makes use of linkage maps and analyses intervals between adjacent pairs of linked markers along chromosomes simultaneously, instead of analyzing single markers (Lander and Botstein, 1989). The use of linked markers for analysis compensates for recombination between the markers and the QTL, and is considered statistically more powerful compared to single-point analysis (Lander and Botstein, 1989; Liu, 1998). More recently, composite interval mapping (CIM) has become popular for mapping QTLs. This method combines interval mapping with linear regression and includes additional genetic markers in the statistical model in addition to an adjacent pair of linked markers for interval mapping. The main advantage of CIM is that it is more precise and effective at mapping QTLs compared to single-point analysis and interval mapping, especially when linked QTLs are involved.

Interval mapping methods produce a profile of the likely sites for a QTL between adjacent linked markers. In other words, QTLs are located with respect to a linkage map. The results of the test statistic for SIM and CIM are typically presented using a logarithmic of odds (LOD) score.

Plant resistance to abiotic stresses. While natural selection has favoured mechanisms for adaptation and survival, breeding activity has directed selection towards increasing the economic yield of cultivated species. Wheat production is adversely affected by drought in 50% of the area under production in the developing and 70% in the developed countries (Trethowan and Pfeiffer 2000). The Mediterranean region of Europe is

particularly sensitive to drought and potentially very vulnerable to future climate changes, due to frequent occurrence of drought combined with heat stress (Araus et al. 2002, 2003a,b; Condon et al. 2004). In the Mediterranean basin, durum wheat is cultivated across a number of macro environments that differ widely in the quantity of rainfall as well as in their thermo-pluviometrical patterns during the crop cycle (Leemans and Cramer 1991; Loss and Siddique 1994; Dunkeloh and Jacobeit 2003).

Water deficit, often accompanied by high temperature, is the main environmental factor limiting wheat productivity in many parts of the world (Boyer 1982; Araus et al. 2008). Drought is by far the most important environmental stress in agriculture and many efforts have been made to improve crop productivity under water-limiting conditions. Drought is a multidimensional stress affecting plants at various levels of their organization. Drought tolerance is defined as the ability of a plant to live, grow, and reproduce satisfactorily with limited water supply or under periodic conditions of water deficit (Turner 1979). Nevertheless, crop plants should not only have the ability to survive under drought but also the ability to produce a harvestable yield, therefore, it is necessary to set up breeding strategies according to the level of stress of different drought-prone environments. In particular, a more general ‘‘xerophytic’’ breeding strategy can be applied in extremely harsh environments to improve plant survival through the limitation of evapotranspiration. Nevertheless, in a typical Mediterranean environment, years with ample water availability during the main cereal growing season alternate with years in which terminal drought occurs as well as years with early drought during vegetative growth and flowering. In mild to moderate drought conditions characterized by a wheat/barley grain yield between 2 and 5 mg $ha^{-}1$, selection for high yield potential has frequently led to some yield improvements under drought conditions (Araus et al. 2002). In these cases the breeders have selected plants characterized by high yield potential and high yield stability, with the

latter being attributed to a minimal G x E interaction. 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). An example is the success of wheat and rice varieties bred at CIMMYT and IRRI where selection under stress-free environments identified genotypes with high yield in a wide range of conditions including regions with a low yield potential (Trethowan et al. 2002). Drought tolerance is a quantitative trait, with complex phenotype and genetic control (McWilliam 1989). Understanding the genetic basis of drought tolerance in crop plants is a prerequisite for developing superior genotypes through conventional breeding. Given the complexity of the genetic control of drought tolerance (multigenic, low-heritability, epistasis, significant genotype-by-environment interaction and quantitative trait loci-by-environment interaction), traditional breeding methods have not fully contributed to cultivar improvement for dry environments and a new integration between direct phenotypic selection and selection based on genotype is needed.

An additional problem in investigating the genomics of drought tolerance in species such as wheat is the size and complexity of the genome. Breeding for drought tolerance is further complicated by the fact that several types of abiotic stress can challenge crop plants simultaneously. At the molecular scale, pathways and gene networks between abiotic stresses overlap; about 40% of drought or high salinity inducible genes are also induced by cold stress in rice (Shinozaki and Yamaguchi-Shinozaki 2007). Crop species of the *Poaceae* display a remarkable level of genetic similarity despite their evolutionary divergence 65 million years ago. Molecular markers have been used to develop comparative chromosome maps for several members of the *Gramineae* and represent a valuable tool to explore germplasm through segregation and association mapping to identify useful alleles in both cultivated varieties and wild relatives.

Traits that increase water use, water use efficiency or harvest index are likely to enhance grain yield and maintain grain size. A large number of studies have reported QTL for yield and yield components in wheat in different environments including water-limiting stress environments; QTL for grain yield, heading date, plant height, grain weight, grain number have been reported (Huang et al. 2006; Marza et al. 2006; Snape et al. 2007; Maccaferri et al. 2008; Quarrie et al. 2006). Maccaferri et al. (2008) detected in Kofa x Svevo durum wheat mapping population, tested in Mediterranean environments, two major QTLs for grain yield on chromosomes 2B and 3B.

Classically, plant resistance to drought has been divided into escape, avoidance and tolerance strategies (Levitt 1972; Turner 1986). Nevertheless, these strategies are not mutually exclusive and, in practice, plants may combine a range of response types (Ludlow 1989). Plants that escape drought exhibit a high degree of developmental plasticity, being able to complete their life cycle before physiological water deficits occur. Escape strategies rely on successful reproduction before the onset of severe stress. This is important in arid regions, where native annuals may combine short life cycles with high rates of growth and gas exchange, using maximum available resources while moisture in the soil lasts (Mooney et al. 1987; Maroco et al. 2000). A short life cycle is particularly advantageous in environments with terminal drought stress or where physical or chemical barriers inhibit root growth (Turner 1986; Blum 1988; Bidinger and Witcombe 1989).

Plants can also endure drought conditions by avoiding tissue dehydration, while maintaining tissue water potential as high as possible. Dehydration avoidance is common to both annuals and perennials and is associated with a variety of adaptative traits. These involve minimizing water loss and maximizing water uptake. Water loss is minimized by closing stomata or by a dense trichome layer increasing reflectance (Larcher 2000) or by decreasing canopy leaf area through reduced growth and shedding of older

leaves. Closing the stomata helps to decrease the loss of water and maintain turgor under conditions of low soil water potential.

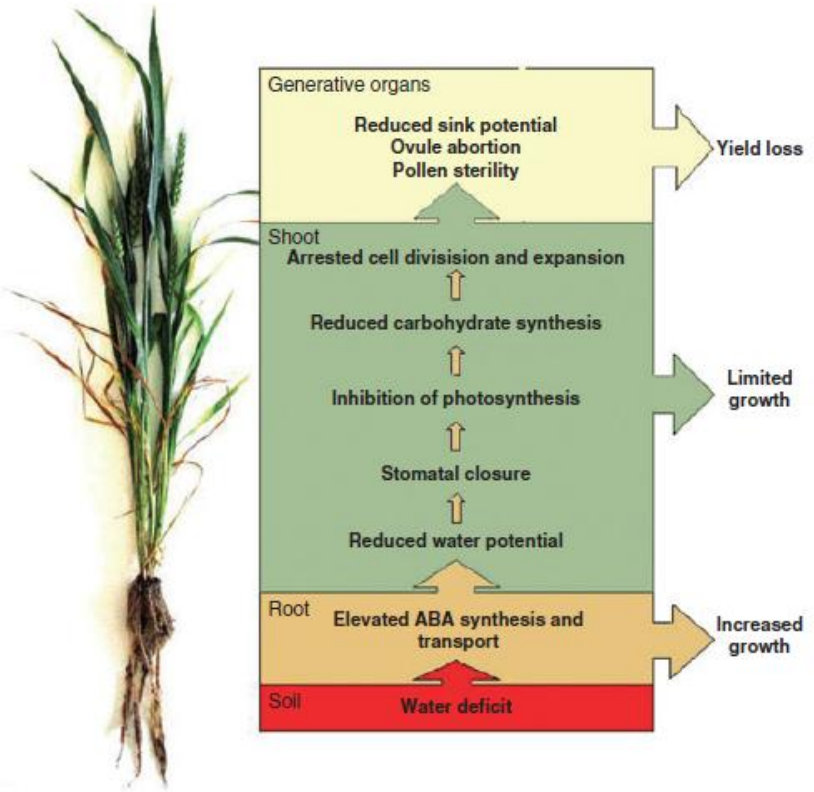


Figure 1: Drought-induced abscisic acid (ABA)-dependent plant responses (Barnabàs et al. 2008).

The two mechanisms will conflict when high temperature and drought occur simultaneously, which is frequently the case in a Mediterranean climate.

Water uptake is also maximized by adjusting the allocation pattern, namely increasing investment in the roots (Jackson et al. 2000).

Finally, tolerance to low tissue water potential may involve osmotic adjustment (Morgan 1984), more rigid cell walls, or smaller cells (Wilson et al. 1980). Partial plant dormancy to survive the dry season is another tolerance strategy; dormancy is evident by the repression of genes encoding photosynthetic proteins. Drought tolerance may also be associated with the

efficient scavenging of reactive oxygen species (ROS) formed as a consequence of disturbed metabolism (Sairam and Saxena 2000). Plants respond to drought stress at the molecular, cellular and physiological levels (fig. 1).

The response depends on the species and genotype (Rampino et al. 2006), the length and severity of water loss (Araus et al. 2002; Bartels and Souer 2004), the age and stage of development (Zhu et al. 2005), the organ and cell type (Verdoy et al. 2004; Cominelli et al. 2005; Zhou et al. 2007) and the sub-cellular compartment (Battaglia et al. 2007).

Fundamental research has provided significant gains in the understanding of the physiological and molecular responses of plants to water deficits, but there is still a large gap between yields in optimal and stress conditions. Minimizing the 'yield gap' and increasing yield stability under different stress conditions are of strategic importance in guaranteeing food for the future.

At the whole plant and crop levels, the plant response to drought is complex because it reflects the integration of stress effects and responses at all underlying levels of organization over space and time; the effect of stress is usually perceived as a decrease in photosynthesis and growth. The rate of CO_2 assimilation in the leaves is reduced at moderate water deficits. Several lines of evidence indicate that a decrease in photosynthesis due to water deficits can be attributed to both stomatal and non-stomatal limitations (Graan and Boyer 1990; Ort at al. 1994; Shangguan et al. 1999). Stomatal closure is the first line of defense against desiccation, since it is much quicker than e.g. changes in roots growth, leaf area, chloroplast ultrastructure and pigment proteins. In many cases the most drought tolerant species control stomatal function to allow some carbon fixation at stress, thus improving WUE or open stomata rapidly when water deficit is relieved. Water deficit reduces transpiration rate, stomatal conductance, net CO_2 uptake and growth of crop plants. During the period of water deficit, water

potential and relative water content decrease with an associated decrease of Rubisco activity and chlorophyll content. Chlorophyll content is positively correlated with photosynthetic rate. Under drought stress in wheat the decreased photosynthesis can be associated with the perturbations of the biochemical processes of photosystem II (Lu and Zhang 1998, 1999; Inoue et al. 2004; Tambussi et al. 2005). Changes of biochemical reaction of PSII can be sensitively characterized by the chlorophyll fluorescence that is strongly correlated, in rainfed conditions, with cholorophyll content (Yang et al. 2007). Under drought stress conditions, genotypes with a stay-green phenotype (high chlorophyll content) retain their leaves in an active photosynthetic state.

Water use efficiency is an important determinant of plant adaptation under limited water availability, often represented by carbon isotope ratio, $\delta^{13}C$ (Farquhar and Richards 1984). $\delta^{13}C$ reflects a time-integrated measure of quantitative changes in the relationships between stomatal conductance and photosynthetic capacity (Condon and Hall 1997). Carbon isotopic discrimination, through its negative relationship with transpiration efficiency, has been used in selection of higher wheat yields in breeding for rainfed environments (Rebetzke et al. 2008).

Few studies, in wheat, reported QTLs associated with specific components of drought response (Yang et al. 2007; Peleg et al. 2009; Zhang et al. 2009). Peleg et al. (2009) in particular, carried out a deep genetic dissection of 11 traits linked to drought stress detecting 110 QTLs, 20 out of which were partially or exclusively expressed under the dry treatement. Actually, various tools are available to integrate progress in physiology with genetics and molecular breeding. For yield and drought-related traits, which are controlled by multiple loci, the availability of new crosses between parents contrasting for the traits of interest is needed to identify new useful alleles associated to the observed phenotypic variation in the segregating

population. At the same time, screening methods applicable to a large number of samples in field conditions is desirable.

Determining how plants sense water deficits at molecular level is complex because signals may be different for different processes. Signaling can either occur locally or at long distance.

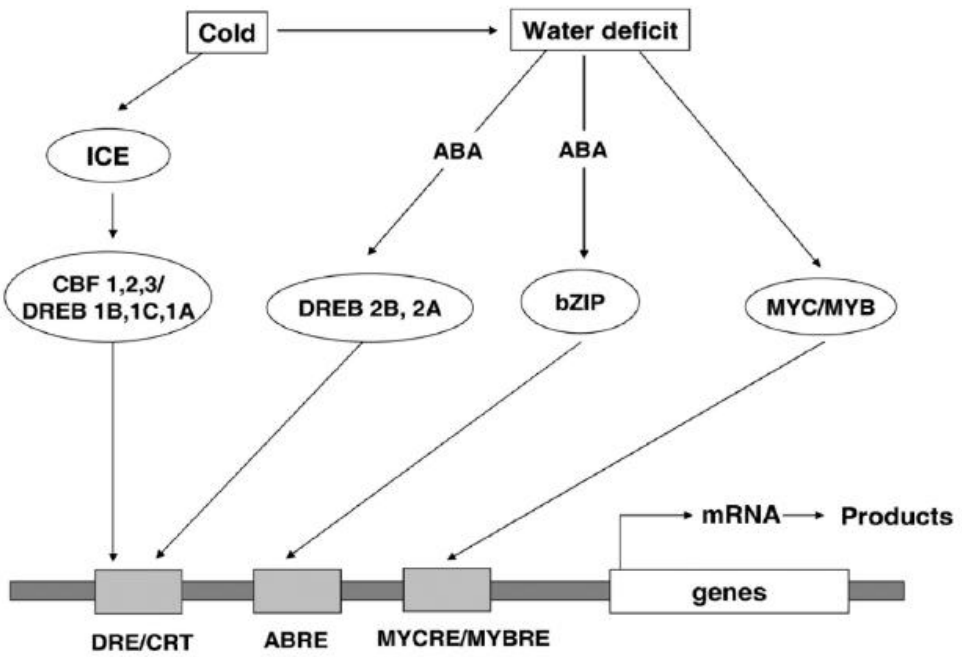


Figure 2: Involvement of various signal transduction elements in the adaptation response to cold and drought (Beck et al. 2007).

The first step in switching on a molecular response in response to an environmental signal is its perception by specific receptors. Plant responses to drought may rely on several mechanisms that sense water status, turgor, bound water, hormones (e.g. ABA), alteration in cell membranes. Upon activation, these receptors initiate (or suppress) a cascade response to transmit the information through a signal-transducing pathway (fig. 2).

A transmembrane histidine kinase receptor (ATHK1) and associated proteins forming a potential 'osmosensor' have already been implicated in the

perception of water deficit in *Arabidopsis* (Urao et al. 1999; Urao, Shinozaki and Yamaguchi-Shinozaki 2001).

The stimulation of the 'osmosensor', and/or other drought-sensing mechanisms, may trigger signal transduction cascades involving protein phosphorylation/ dephosphorylation mediated by kinases or phosphatases up-regulated by water stress (Bray 2002; Kaur and Gupta 2005; Mishra et al. 2006). Changes in the cytoplasmic calcium concentration are likely to mediate the integration of different signalling pathways (Bray 2002; Kaur and Gupta 2005). Several Ca^{2+}-dependent protein kinases have already been implicated in water stress-related signalling (Bray 2002; Kaur and Gupta 2005; Klimecka and Muszynska 2007). The active signal receptor activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphat to yield the second messengers inositol 1,4,5-trisphosphat ($InsP_3$) and diacylglycerol (Mahajan and Tuteja 2006). $InsP_3$ releases calcium from internal stores and the Ca^{2+}-sensor (Calcineurin B-like protein, CBL) activates downstream protein kinases and phosphatases. Drought-activated kinase cascades finally result in the phosphorylation and activation of transcription factors regulating gene expression. Drought-inducible genes display characteristic promotor *cis*-acting-elements, the dehydration-responsive elements (DREs) which at least partially resemble those of the cold-induced genes (Bray 1997). Activation of the abscisic acid responsive elements (ABREs) by several transcription factors such as the DRE-binding factors and bZIP-proteins leads to the expression of drought stress tolerance effectors such as dehydrins or enzymes catalyzing low molecular weight osmolytes.

Traits associated to yield improvement in durum wheat. Development of high yielding varieties with good end-use quality is a major focus in wheat breeding programs. Grain yield and agronomic traits related to yield such as plant height, maturity and thousand-grain weight, kernel shape, and quality traits such as protein content, composition and strength are complex quantitative traits controlled by multiple genes, and exhibit high genotype-environment interaction.

Thanks to the breeding activity carried out during the past century in Italy, leading cultivars have been selected to perform well under intensive crop management and characterized by an increased yield potential (De Vita et al. 2007; Martos et al. 2005). During the 1970s, after the introgression of the *Rht* genes from Norin 10 (*Triticum aestivum L.*) a series of CYMMIT short straw recombinant lines were introduced in the Italian Breeding programs (Bozzini et al. 1998). This strategy led to the release of new durum wheat cultivars with a high yield potential and high pasta making quality (De Vita et al. 2007). Comparisons of cultivars raised in different periods can provide interesting knowledge on the evolutionary trend in morpho-physiological, agronomical and qualitative characteristics of the wheats grown in a given region and allow to directly estimate the breeding progress.

Studies from several countries have provided a direct comparison of yield, dry matter accumulation and partitioning among old and modern bread wheat cultivars grown simultaneously in the same field trial, and recently some similar trials also have been performed for durum wheat, indicating a genetic gain for durum wheat from 10 to 18 kg ha^{-1} yr^{-1} similar to the value reported in many cases for bread wheat (Calderini et al. 1999). A similar genetic gain for potential GY (25 kg ha^{-1} $year^{-1}$) was found by De Vita et al. (2010) by comparing 64 durum wheat genotypes, a result also in agreement with a previous experiment conducted using 14 Italian durum wheat cultivars (20 kg ha^{-1} $year^{-1}$, De Vita et al. 2007). The superior

performance in terms of grain yield of the modern wheat cultivars has been attributed largely to changes in harvest index, often associated with few key genes affecting plant height, mainly *Rht* (Slafer et al. 1994), with small or negligible increases in total biomass production and in number of grains per unit of land (Austin et al. 1989; Siddique et al. 1989; Slafer and Andrade 1993; Brancourt-Hulmel et al. 2003). A large variation for heading time was observed during the last century, by starting from landraces at the beginning of the breeding history. In particular, a progressive reduction of vegetative phase was first observed due to breeding improvement until Appulo was released, followed by a lengthening of heading time with the introduction of Creso and Valnova. After that, a new reduction was registered in the vegetative phase. Notably, although Appulo, Creso and Valnova were released in the same years (1973–1975) they belong to two different breeding phases, since the CIMMYT short straw recombinant lines were used as progenitor starting from Creso and Valnova onwards. The earliness in heading time was accompanied by a rise in the grain filling period since that the length of the total life cycle was only marginally reduced. Plant height decreased dramatically passing from old landreaces to modern varieties. Interestingly, a remarkable reduction of height was already attained with Capeiti 8 and Appulo, before the introduction of the dwarfing genes from bread wheat. With the introduction of these genes, modern varieties characterized by height below 80 cm were obtained. Among yield components, kernels m^{-2} increased throughout the century, and the same trand was observed for the number of kernels $spike^{-1}$, while the thowsand kernel weight did not exhibit a clear trend due to breeding improvements (De Vita et al. 2007).

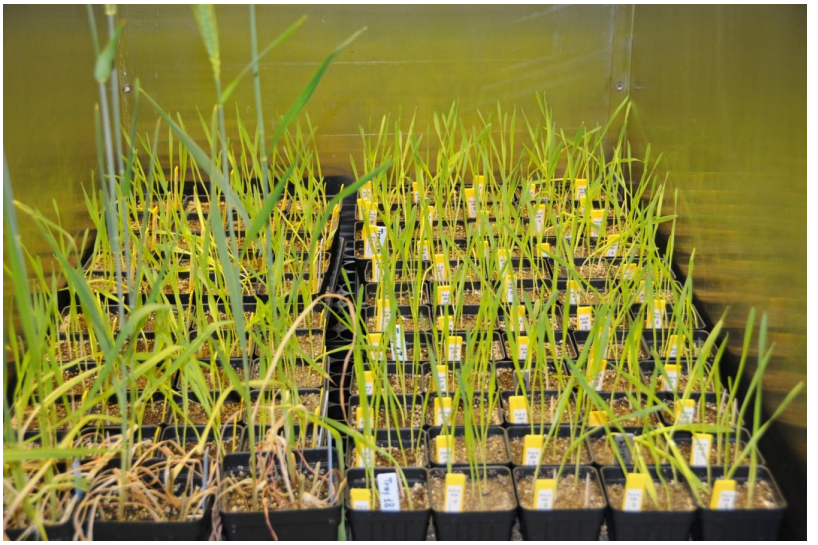
Modern wheat cultivars, even if characterized by better yield performance, frequently present lower grain protein concentration than their predecessors (Austin et al. 1980; Calderini et al. 1995) suggesting a not-desired reduction in grain quality because of successful breeding for higher yields. In fact, the

increase of C availability to the grains, is correlated to a decrease in protein content and other minor components, by dilution effects (Martre et al. 2003). In durum wheat protein content is an essential trait for quality, although this trait alone does not necessarily determine pasta cooking quality (Dexter and Matsuo, 1980; Autran and Galtiero, 1989), which is also strongly affected by the allelic composition for endosperm storage proteins as gliadins and glutenins influencing strenght and elasticity of the dough for pasta processing. In fact, the reduced grain protein concentration registered at the endo of a century of genetic improvement of durum wheat as a consequence of improved yield, not necessarily has negatively affected pasta cooking-quality (Motzo et al. 2004), suggesting that the genetic improvement could have positively modified grain protein composition concomitantly with the yield related traits. Furthermore, quality is strongly influenced by environment and genotype x environment interactions (Garrido-Lestache et al. 2005), a fact that hamper the evaluation of the quality genetic progress. Climatic conditions, genotype effect, nitrogen fertilizer rate, time of nitrogen application, residual soil nitrogen and available moisture during grain filling are the most important factors influencing protein accumulation in grains (Rharrabti et al. 2001, 2003).

Morphological features of seeds also have been investigated for ther relationship with grain yield. While contrasting results have been reported about the effect of selection for kernel weight and size on grain yield (Alexander et al. 1984; Baril 1992), more clear seems to be the importance of kernel weight and size because of their relationships with milling quality (Wiersma et al. 2001; Marshall et al. 1984; Berman et al. 1996). Geometrical models indicated that changes in kernel shape and size could result in increases in flour yield of up to 5% (Marshall et al. 1984). Furthermore, larger kernels could have a favorable effect on seedling vigor and consequently promote yield increase (Botwright et al. 2002; Chastain et al. 1995).

With the advent of molecular markers, several researchers have used QTL analysis to study the genetic control of yield components, including kernel weight and kernel morphology (Campbell et al. 1999; Prasad et al. 1999; Varshney et al. 2000; Zanetti et al. 2001; Groos et al. 2003; McCartney et al. 2005; Sun et al. 2009), but the genetic basis for most of these traits is not well elucidated and QTL analysis for kernel traits of wheat, except for thousand kernel weight (TKW), have not been extensively studied. QTL for yield and yield –related traits most frequently account for between 2 and 10% of the total phenotypic variation, in fact QTL with R^2 values >15% have seldom been described (Quarrie et al. 2005; Dilbirligi et al. 2006). Therefore, further investigation is needed in order dissect genetic determinants of these traits that can be successfully used in genetic improvement of durum wheat in the near future.

MATERIALS AND METHODS



Plant material and DNA extraction. A population of 161 F8-F9 recombinant inbred lines (RILs) was developed at the CRA-CER cereal research center in Foggia (Italy) by single-seed decent from a cross between 2 durum wheat (*Triticum turgidum* ssp. *durum*) cultivars: Ofanto and Cappelli. After the last selfing, every line was bulk-harvested to provide seed for field experiments and DNA extraction.

Cultivar Ofanto originated from a cross between Appulo and Valnova, released in Italy in 1990. The cultivar Cappelli derived from a selection of exotic landraces "Tunisia", released in Italy in 1915.

High-quality genomic DNA from parents and RILs was extracted from young leaves using CTAB method (Hoisington et al. 1994).

The DNA concentration was adjusted to 20 ng/ul and DNA samples were stored at -20°C to be used for the analysis of microsatellites, EST-derived and DArT markers.

SSR and EST-derived assay. A total of 512 genomic SSRs and 315 EST-derived markers (eSTSs and eSSRs) (table 1) were used to screen DNA from parents and RILs belonging to the mapping population. Molecular markers were amplified from 80 ng of genomic DNA in 15 µl reactions containing 1X PCR Buffer Promega (10 mM Tris-HCL – pH 8.3, 50 mM KCL, 1.5 mM $MgCl_2$), 0.4 µM reverse primer labelled with a fluorochrome (HEX, FAM, NED o TET), 0.4 µM unlabelled forward primer, 0.2 mM each dNTP, 5% DMSO and 1U GoTaq DNA Polimerase (Promega). PCR amplifications were performed in a thermocycler Applied Biosystem 2720 using the following conditions according to Röder et al. (1998): 3 min at 94°C, followed by 45 cycles of 1 min 94°C, 1 min annealing (between 50 and 65°C, depending on the optimal annealing temperature of the primers), 2 min 72°C, and a final extension of 10 min at 72°C. The ampilifications products were analysed by means of capillary electrophoresis (ABI3130),

multiplexing different fluorescent dyes. Electropherograms were analysed with GeneMapper version 4.0.

Genomic DNA of parental lines and individuals of mapping populations were sent to Triticarte Pty Ltd. for DArT marker genotyping.

	class	Number	References
gSSR	GWM	266	(Röder et al. 1998)
	GPW	4	(Sourdille et al. 2001)
	WMC	87	(Gupta et al. 2002)
	BARC	132	(Song et al. 2005)
	CFD	14	(www.graingenes.com)
	CFA	9	(Sourdille et al. 2003)
EST-derived markers	DuPw	14	(Eujayl et al. 2002)
	CWEM	12	(Peng et al. 2005)
	SWES	41	(Li et al. 2008)
	CINAU	11	(Zhuang et al. 2008)
	KSUM	9	(Yu et al. 2004)
	F	71	(Gadaleta et al. 2009a)
	EST-SSR	24	(Gadaleta et al. 2009b)
	TC on genes	3	
	BCD	1	(Anderson et al. 1992)
	MAG	129	(Xue et al. 2008)

Table 1: Molecular markers used to screen parental lines.

DArT assay. DArT markers were generated by Triticarte Pty. Ltd. (Canberra, Australia; http://www.triticarte.com.au), which is a whole-genome profiling service laboratory, as described by Wenzl et al. (2004) and Akbari et al. (2006). Briefly, a genomic representation of a mixture of 13 cultivars was produced after *Pst*I–*Taq*I digestion, spotted on microarray slides. Genomic representations of parents and RILs were generated with the same complexity reduction method used to prepare the library spotted on the array. DNA from the parents was first screened for polymorphism and the individual RILs were genotyped and scored for the presence (1) or absence

(0) of hybridization based on fluorescence signal intensities. The locus designations used by Triticarte Pty. Ltd. were adopted in this paper. DArT markers consisted of the prefix "wPt", followed by numbers corresponding to a particular clone in the genomic representation, where w stands for wheat, P for *Pst*I (primary restriction enzyme used) and t for *Taq*I (secondary restriction enzyme).

Segregation analysis and map construction. The significance of deviation of observed allelic frequencies of the marker loci from the expected ratio (1:1) were tested by chi-square. Linkage groups were established using a minimum LOD score of 4.0 using the software JoinMap 4 (Van Ooijen and Voorrips 2004). The Kosambi function was used to calculate genetic distances in CentiMorgan units from recombinant fraction (Kosambi 1944). Linkage groups were assigned to the chromosomes by comparison with the other published durum genetic maps (Korzun et al. 1999; Elouafi et al. 2001; Nachit et al. 2001; Elouafi and Nachit 2004; Blanco et al. 1998; 2004) and the bread wheat SSR consensus map developed by Somers et al. (2004).

Mapping of pirrolyne-5-carboxilate synthase (P5CS) gene. A couple of primers (Fw: CTCTGTGCGAGGAATGAAGAAA; Rev: AATCACGGAACTAGATAGCGACATAG) was designed within the 5' region of the sequence corresponding to the wheat TC297220. Amplification reation was carried out with the following conditions: 3 min at 94°C followed by 35 cycle of 1 min 94°C, 30 sec annealing at 56°C, 30 sec at 72°C and a final extension of 10 min at 72°C. The resulting 185 bp fragment was analysed by means of capillary electrophoresis (ABI3130), and electropherograms were analysed with GeneMapper version 4.0. The amplification was carried out on DNA from lines belonging to two segregating populations: Ofanto x Cappelli and Creso x Pedroso, whose genetic map was previously developed (Marone et al. 2009).

Statistical analysis of phenotypic data. The STATISTICA version 9.0 statistical package was used for statistical analyses. All phenotypic variables were tested for normal distribution. A factorial model was employed for the analysis of variance (ANOVA), with RILs and blocks as random effects.

Phenotypic measurements. Phenological parameters for Ofanto x Cappelli population were recorded during the seasons for each plot. The field trials were conducted, for parent and for RILs, at two locations across two major durum wheat-growing areas of Italy (Foggia –FG- and Sassari –Sas-). The trials were carried out during four consecutive growing seasons, 2006-2007, 2007-2008, 2008-2009 and 2009-2010 according to a randomised block design with 3 replicates sown in a single row 1 m long with 25 cm. All recommended agronomic practices were followed according to local standards.

Heading date (HD), considered as days from first April, was measured as the time the first spike of 50% of the plants in a plot were fully exposed. The measure was done for years 2006-2007, 2007-2008 and 2008-2009. Plant height (PH) was measured at maturity from the soil surface to the end of the spike, and was recorded in trials carried out in 2006-2007 and 2008-2009.

Seeds harvested at Foggia in 2006-2007 and 2008-2009 were used for evaluating morphological features of seeds. About that, at the "Stazione consorziale sperimentale di granicoltura per la Sicilia" prof. Venora measured single kernel dimension, shape and weight (about 100 grains per plot) using the image analysis software Zeiss KS-400 V3.0 (Carl Zeiss Vision GmbH, Hallbergmoos, Germany, 2001).

This software can be customized, by users to develop specific applications by editing appropriate image analysis algorithms in ''Macros'', able to automate the analysis. The macro utilized in this work were edited by Venora et al. (2009).

Images were acquired by a Zeiss AxioCam MRc5 digital camera (Carl Zeiss Vision GmbH, Hallbergmoos, Germany, 2005) with 5 Megapixel resolution, 36-bit RGB colour depth, a dynamic range of 1:1300 for optimal capture of various colour intensities, integration times of 1 ms up to 60 s, a system to minimize background noise connected with a 50 mm objective lens (Canon Inc. Tokyo, Japan, 2000). Successively the images were stored with Zeiss AxioVision AC Rel. 4.5 software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany, 2005).

The acquiring hardware (camera + lens) was mounted on a specially constructed light table to capture trans-illuminated kernel images. The light table was constructed using a sub-stage lamp (Koninklijke Philips Electronics. N.V., 75W Philips Softone, Eindhoven, Netherlands, 2007) covered with a satinized double Plexiglas layer, the lower layer to diffuse the light and the upper layer simply as a tray for samples. The intensity of a new lamp was maintained constant using a voltage transformer Ministatic TS400n (IREM SpA, Turin, Italy, 2000) to minimize disturbances such as voltage variation spikes and high frequency interferences affecting the external power supply.

In the present work, using the data from Venora experiments, area (SA), volume (SV), maximum (MaxD) and minimum (MinD) diameter of seeds and thousand kernel weight (TKW), were evaluated.

At the Dipartimento di Scienze Agronomiche e Genetica Vegetale Agraria of University of Sassari prof. F. Giunta measured, in growing seasons 2007-2008 and 2008-2009, water stress related physiological traits (chlorophyll content, stomatal resistance and carbone isotope discrimination).

For leaf chlorophyll content analyses, flag leaves were taken from five plants per plot at tillering stage (ChlCtill) and heading stage (ChlChea).

Leaf greenness was assessed with a hand-held meter (SPAD 502, Minolta, Spectrum Technologies Inc., Plainfield, IL), which generates a measure

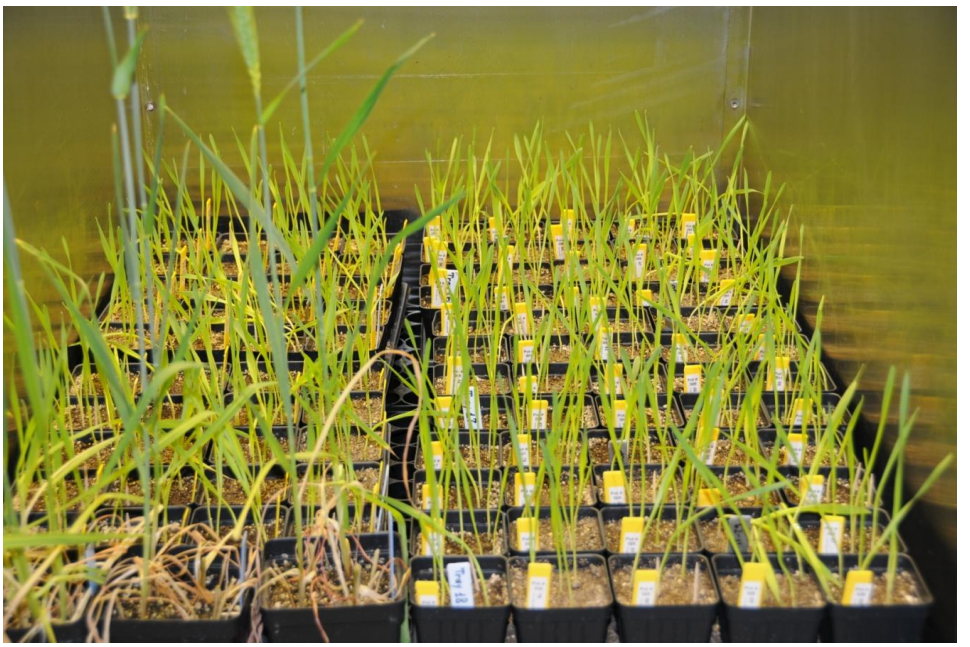
predictive of chlorophyll concentration (Yadava 1986; Marquard and Tipton 1987).

An indirect, but rapid measure of stomatal opening was obtained using an air flow porometer (Thermoline Scientific Equipment, Wetherill Park, Australia), which times the passage of a fixed volume of air through the leaf under pressure. This time is proportional to the leaf resistance to air flow (LR, arbitrary units) (Rebetzke et al. 2000), while $\log_{10}$ of LR (logLR) has a close negative linear relationship to the diffusive conductance of the leaf (Fischer et al. 1998). Five to eight leaves per plot were sampled within 60 s in this way.

Samples were evaluated on different occasions during the pre-anthesis period (14/2, 15/2, 26/2, 03/3 and 13/3 in 2007/08 trial and 30/03, 07/4, 15/4 and 12/5 in 2008/09 trial). For year 2008 the reading was only for 1 plant, while for year 2009 the readings was for 5 plants for inbreed line.

Carbon isotope discrimination (Δ) of vegetative tissues was measured according to the methodology reported by Condon et al. (1987) on samples of 10 plants per plot harvested on 12/03/08 and 9/04/09. At peak tillering (DC30 – Zadoks et al. 1974), leaf laminae were collected from all plants and dried at 70°C for three days. Dried samples were ground to pass a 0.5mm sieve and the ^{13}C:^{12}C composition determined by ratio mass spectrometry using a Micromass Isochrom mass spectrometer. Carbon isotope discrimination (Δ) was calculated following Farquhar and Richards (1984) assuming the ^{13}C:^{12}C composition of CO_2 in air equals -8‰. Accuracy of the measurements on the mass spectrometer was ± 0.1‰.

RESULTS AND DISCUSSION



Development on the Ofanto x Cappelli linkage map. Dense genetic maps are a very useful tool in the identification of molecular markers closely linked to genes or QTLs of interest, isolation of genes via map based cloning, comparative mapping, and genome organization studies (Varshney et al. 2007). The Ofanto x Cappelli genetic map was developed with the aim of studying a number of morpho-phenologycal and agronomic traits for which the two parents were contrasting. Cappelli is an old (year of release 1915), low-yielding durum wheat variety selected by Nazareno Strampelli from a late-maturing pure line selected from the North African landrace Jean Rhetifah (Scarascia Mugnozza 2005), while Ofanto (Appulo x Adamello) is a high-yielding modern cultivar (released in 1990). Therefore, the parents of the segregating population represent the starting and end points of nearly a century of breeding of durum wheat in Italy and are contrasting for a number of traits including yield and yield related traits, earliness, plant height, harvest index, stomatal conductance and morphological features of seeds, as reported by studies comparing a number of durum wheat cultivars released in different steps of the breeding activity (De Vita et al. 2007; 2010; Giunta et al. 2007; 2008; Royo et al. 2007).

The relatively population size used for construction of the genetic map presented in this work (161 RILs) is higher as compared with other studies (62–120; Blanco et al. 1998; 2004; Röder et al. 1998; Nachit et al. 2001; Elouafi and Nachit 2004; Quarrie et al. 2005; Akbari et al. 2006), and it is highly advantageous to improve the resolution of QTL mapping for the agronomic traits taken into consideration.

Among the 512 gSSR, 315 EST-derived, and 1000 DArT markers used (tab. 2) to screen the parental lines for polymorphism, 166 gSSR , 39 EST-derived and 464 DArT showed a different allelic composition (an example is shown in fig. 3). Furthemore, 1 TRAP marker (Liu et al. 2005) was tested in this population, producing 4 polymorphic loci. Polymorphism frequencies of 27.5 and 46.4% were observed for PCR-based and DArT markers

respectively, in line with values previously found in other works for crosses between elite cultivars (Peleg et al. 2008; Semagn et al. 2006).

	class	Number	References	Polymorphic markers
gSSRs	GWM	266	(Röder et al. 1998)	100
	GPW	4	(Sourdille et al. 2001)	3
	WMC	87	(Gupta et al. 2002)	31
	BARC	132	(Song et al. 2005)	30
	CFD	14	(www.graingenes.com)	0
	CFA	9	(Sourdille et al. 2003)	2
EST-derived markers	DuPw	14	(Eujayl et al. 2002)	5
	CWEM	12	(Peng et al. 2005)	1
	SWES	41	(Li et al. 2008)	1
	CINAU	11	(Zhuang et al. 2008)	0
	KSUM	9	(Yu et al. 2004)	0
	F	71	(Gadaleta et al. 2009a)	7
	EST-SSR	24	(Gadaleta et al. 2009b)	8
	TC on genes	3		3
	BCD	1	(Anderson et al. 1992)	1
	MAG	129	(Xue et al. 2008)	13

Table 2: Molecular markers, references and polymorphisms on Ofanto x Cappelli

Among the polymorphic markers, 157 gSSR, 35 EST-derived and 464 DArT produced specifically the expected single-locus fragment, while 9 gSSR (BARC187, BARC361, WMC382, WMC597, WMC826, XGWM131, XGWM328, XGWM526, XGWM959), 4 EST-derived (DuPw43, BCD348, 1 TC on genes, MGBE895), and 1 TRAP (W01T03) marker produced from one to three additional polymorphic fragments, leading a total of 178 gSSR, 47 EST-derived, 4 TRAP and 464 DArT loci. The final map was constituted by 619 loci (151 gSSR, 26 EST-derived, 437 DArT, 1 morphological and 4 TRAP), distributed over 30 linkage groups, after removal of 75 markers presenting a too high number of missing data, or remained unlinked following the segregation analysis.

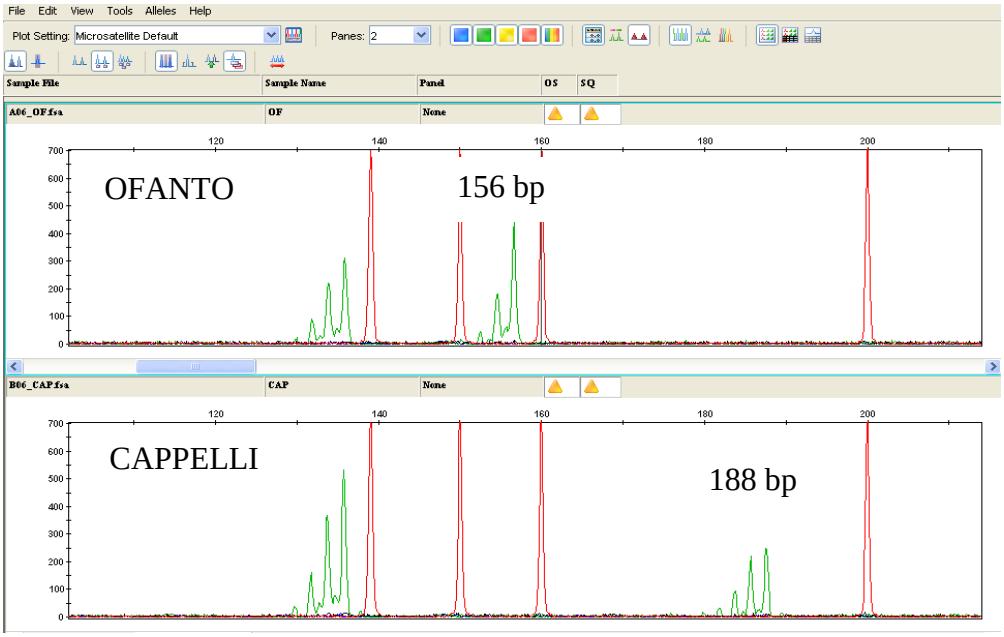


Figure 3: Polymorphism between Ofanto and Cappelli

The linkage groups identified in this work were assigned to all chromosomes of the durum wheat genome, except for chromosome 1A, for which no linkage group with more that 2 markers could be identified, probably due to absence of polymorphism and/or recombination between parents for this chromosome. The parental genotypes of our cross cannot be considered as highly divergent in evolutionary terms and this could explain the lack of polymorphism for 1A.

The final map (fig. 13) spanned 1651.3 cM. with the chromosomes of the tetraploid wheat genome varying in the number of markers, map length and marker density (tab. 3). First of all, a clear difference was found between A and B genomes in terms of both length and number of markers, with the B genome more represented as found in many other published maps of wheat (Mantovani et al. 2008; Peleg et al. 2008). Map length of genomes A and B was 688.5 and 962.8 cM, respectively, with 247 (40%) markers on the A genome and 371 (60%) markers on the B genome. The difference observed in the number of markers regarded the total number of markers, as well as

the different classes of molecular markers employed in this work. The average chromosome length was 117.9 cM; in particular, 1B was the longest chromosome (183.9 cM) while the shortest was 5B (52.9 cM). The total number of mapped loci per chromosome ranged 17 (chr. 3A and chr. 5B) to 81 (chr. 6B), with an average of 47.4 loci per chromosome. The density of markers ranged 1.65 cM/marker on chromosome 7B to 6.48 cM/marker on chromosome 3A, with an average density of 2.67 cM/marker.

Chromosome	SSR	TRAP	DArT	EST-derived	Markers	Lenght (cM)	cM/marker
1A	2	-	-	-	2	1.9	1.9
2A	11	-	35	4	50	109.3	2.18
3A	5	1	10	1	17	110.3	6.48
4A	14	-	56	2	72	106.8	1.48
5A	12	-	11	4	27	160.6	5.94
6A	10	-	27	-	37	99.7	2.69
7A	10	-	30	2	42	99.9	2.37
genome A	64	1	169	13	247	688.5	2.78
1 B	17	-	28	3	48	183.9	3.83
2 B	21	1	45	2	69	182.7	2.64
3 B	13	-	45	-	58	170.1	2.93
4 B	9	-	8	1	18	83.7	4.65
5 B	4	1	11	1	17	52.9	3.11
6 B	12	-	68	1	81	156.9	1.93
7 B	11	1	63	5	80	132.6	1.65
genome B	87	3	268	13	371	962.8	2.59
Total	151	4	437	26	618	1651.3	2.67

Table 3: Distribution and density of markers across the durum wheat chromosomes.

A different distribution was observed for the different classes of molecular markers utilised to construct the map. In particular, the number of loci per chromosome ranged 8 (4B) to 68 (6B) for DArT markers, and 6 (5B) to 24 (2B) in the case of PCR-based markers. The higher number of loci per chromosome found for DArT with respect to the PCR-based markers, actually was due to clustering events observed for the Ofanto x Cappelli linkage map, according to data available for previously developed maps in

both bread and durum wheat. Clustering phenomena are very common in wheat linkege maps, even if different chromosomes can be involved in the different maps (Mantovani et al. 2008; Peleg et al. 2008). Clusters of DArT markers were found on most of the chromosomes of the A and B genomes, but particularly on chromosomes 1B, 2A, 2B, 3B, 4A, 6A, 6B, 7B. In particular, 43 DArT markers were grouped in just 6.3 cM on the chromosome 6B. DArT clustering was observed mainly on the telomeric regions, but DArT clusters spanning the whole chromosome were identified on chromosomes 2B, 3B and 7B. Map distance between adjacent markers varied from 0.1 to 43.6 cM; the largest distance between adjacent markers, observed on chromosome 5A between 349142 (DArT marker) and XGWM865 (gSSR), was 43.6 cM.

Several differences in terms of map length, number and density of markers also were observed among homeologous groups (tab. 4).

Group 4 and 2 showed the shortest (190.5 cM) and longest (292 cM) map length, respectively. The number of mapped markers was the highest in group 7 (122 loci) whereas homoeologous group 5 (44 loci) was characterized by the lowest number of markers.

Homeologous group	Markers	Lenght (cM)	cM/marker
1	50	185.8	3.71
2	119	292	2.45
3	75	280.4	3.73
4	90	190.5	2.11
5	44	213.5	4.85
6	118	256.6	2.17
7	122	232.5	1.9

Table 4: Distribution and marker density across the homeologous groups.

Segregation analysis. Segregation analysis data indicated that 109 markers (15.7%) out of the total number of molecular markers used for developing the Ofanto x Cappelli map were significantly ($P<0.01$) distorted from an expected 1:1 ratio. This value is lower than values found in previous mapping studies on bread (Paillard et al. 2003; Semagn et al. 2006; Sing et al. 2007; Xue et al. 2008) and durum wheat (Blanco et al. 1998; Nachit et al. 2001; Mantovani et al. 2008; Peleg et al. 2008). All homoeologous groups showed segregation distortion, except group 1 and 6. DArT markers showed a higher proportion of distorted segregation (71 markers) than PCR-based markers (38) in the present map. Distorted regions were observed in particular on chromosomes: 2B, 3B, 4A, 4B, 5B, 7A and 7B.

Out of these, the chromosome 7B showed the highest number of distorted loci (40), all characterized by the predominance of the Ofanto allele. The presence of loci with segregation distorsion in the Ofanto x Cappelli, as well as in other wheat genetic maps can be explained by a variety of mechanisms including meiotic drive and preferential abortion of gametes, effect of usual gametophyte factors, non-random fertilization, and viability selection at post-syngamic stages.

Ofanto x Cappelli: a genetic tool for mapping drought-related genes. Twenty-six EST-derived molecular markers previously developed have been localized on the linkage map Ofanto x Cappelli (Table 3). The majority of these sequences correspond to genes coding for transcriptional factors and other proteins involved in pathways of signal transduction. A microsatellite marker (F118 – Gadaleta et al. 2009a), in particular, putatively corresponding to a *RAS-related* protein belonging to the GTPase family and involved in secretion pathways, has been positioned on the chromosome 5A. Furthermore, the map has been implemented with a molecular marker developed in this work for a drought-related gene. The accumulation at cellular level of osmotically active compounds as amino acids has been

largely recognized as one of the main mechanisms to tollerate abiotic stress constraints characterized by an osmotic component, such as drought, low temperature and salt (Jimenez-Bremont et al. 2006). Proline in particular is one of the most studied osmolyte in plants. Pirrolyne-5-carboxilate synthase (P5CS) is the key enzyme for biosynthesis of proline and its expression is up-regulated in plant cells subject to abiotic stresses (Hu et al. 1992). A couple of primer was designed within the 5' region of the sequence corresponding to the wheat TC297220, producing the amplification of a 185 bp fragment that revealed a presence/absence polymorphism between the parents of the mapping population. The P5CS gene was therefore mapped on the long arm of the chromosome 3B, within the perycentromeric region. The position of the gene has been confirmed by mapping the same polymorfism also in the segregating population Creso x Pedroso, already available at the CRA-CER (Fig. 4).

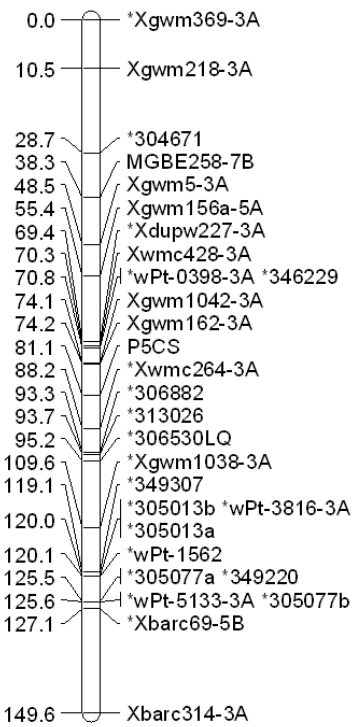


Figure 4: Creso x Pedroso 3A chromosome with P5CS gene mapping.

Mapping of a locus responsible for wax accumulation on leaves. Ofanto and Cappelli are contrasting for many morphological traits. One of these is the accumulation of wax on leaves and spike: Ofanto is characterized by an extreme viridescent colour due to the absence of glaucosity, which is present in the cultivar Cappelli. Glaucosity is described as a white, waxy substance that coats green parts of the plant. The traits was scored in the field-grown segregating population as presence/absence of the wax for two years giving stable results, and the locus controlling the viridescence, corresponding to a single dominant gene, was mapped on the telomeric region of short arm of chromosome 2B (Fig. 13), confirming previous results obtained by Simmonds et al. (2008) for common wheat. These authors, in particular, showed a significant association between the viridescent gene and a delay in senescence, resulting in an extension of the grain filling period. Furthermore, a stable QTL for grain yield co-localized with the *Vir* gene in the segregating population utilized for this study. The yield and stay-green advantages associated with viridescence emphasize the importance of introducing this trait in the breeding programs for durum wheat. As non glaucous variants are common in wild emmer, but rare in cultivated bread and durum wheat, the cultivar Ofanto represents a valuable tool for transferring this trait into new elite varieties without association with less useful alleles of wild genotypes.

QTL analysis for traits of agronomic importance for the genetic improvement of durum wheat

Morpho-phenological traits. RILs and parents of the population were evaluated in field trials for two morfo-phenological traits (plant height and heading date), which are strictly correlated with yield performance of durum wheat especially in Mediterranean environments (De Vita et al. 2007). Results of statistical analysis are reported in Table 5.

Trait	Year	Parent			RIL			
		Ofanto	Cappelli	LSD	Average	SD	Min	Max
Heading date (HD)	2007	23	31	3.355	29.434	3	22.5	35
Heading date (HD)	2008	21	30	2.84	25.246	2.976	20.3	31.3
Heading date (HD)	2009	28	35	2.103	30.913	2.467	27	37.333
Plant height, cm (PH)	2007	80	152	22.152	136.109	20.826	67	157.5
Plant height, cm (PH)	2009	85	154	11.184	133.565	17.313	73.33	158.33

Table 5: Phenotyping performance for phenological traits. Heading date (considered as days from first April) and Plant height (expressed in cm) recorded in Foggia trials.

The ANOVA analysis showed significant differences between parents and among RILs ($P<0.001$) for both traits. Data from two years of trial were available for plant height, with Cappelli showing allways much higher values than Ofanto. A bimodal distribution (fig. 5) was observed in the segregating population, even if the population was unbalanced with a majority of tall plants, indicating a simple genetic basis for this trait.

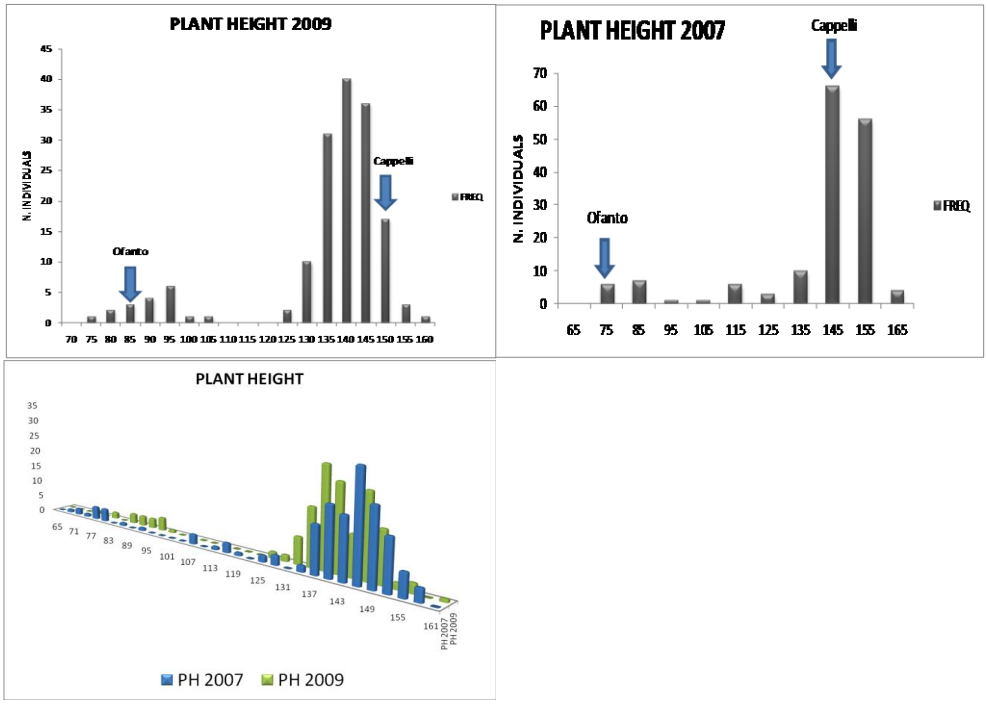


Figure 5: Phenotypic distribution (single e multiple) for plant height 2007, 2009; multiple distribution for every year.

The QTL analysis indicated that PH was controlled by a single major QTL located on short arm of chromosome 4B, according with data previously published (McCartney et al. 2005; Somers et al. 2004), and explaining more than 90% of observed variability. Specific analysis with marker for *Rht* genes indicated that the variation for height in the Ofanto x Cappelli segregating population was due to the *Rht-B1* gene. The Ofanto allele reduced plant height up to 32.96 cm. The LOD score peak for this trait was 40.79 for year 2007 and 31.21 for year 2009. Reduced plant height in wheat, via the introduction of dwarfing genes (*Rht-B1b* and *Rht-D1b*), has been associated with increased yield potential because of greater harvest index and lodging resistance (Araus et al. 2008).

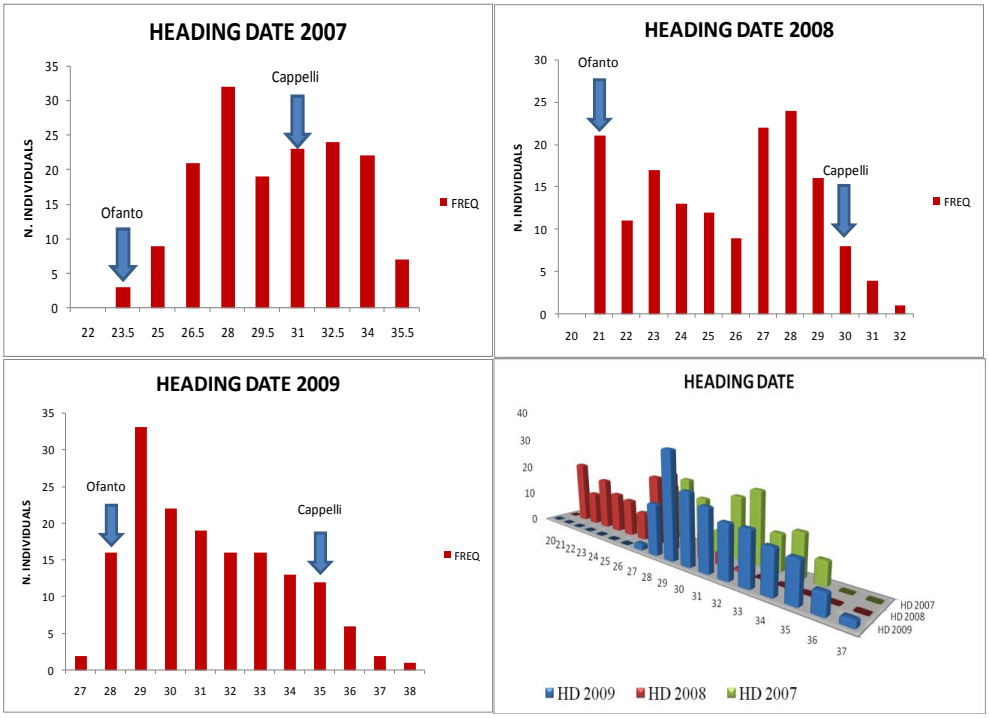


Figure 6: Phenotypic distribution for heading date 2007, 2008, 2009 (red single distribution); multiple distribution for every year (colored multiple distribution).

The data for heading date were available for three years of field trials. Ofanto was allways more precocious with respect to Cappelli, with differences ranging 7 to 9 days (Table 5).

The frequency distributions for heading date (fig. 6) also, indicated the presence of a major gene influencing the trait, but with a more complex genetic basis compared to plant height.

QTL	Peak marker	Chr*	Trait	LOD	R2 (%)	Add. Eff. **
1	374299	2B	HD 2009	25.86	44.5	-1.684
			HD 2008	34.2	61.9	-2.347
			HD 2007	13.72	32.7	-1.711
2	wpt-6047	3B	HD 2008	4.29	5.2	0.663
3	Xgwm595	5A	HD 2009	4.4	5.7	-0.588
4	345499	6A	HD 2009	5.74	7.3	-0.691
			*HD 2008**	*2.84*	*8.1*	*-0.855*
5	BARC193	4B	PH 2007	40.79	90.8	-32.96
			PH 2009	31.21	84.9	-24.57

*Chr: chromosome

** referred to Ofanto allele

Table 6: Results of QTL analysis for phenological traits. Traits with * indicate that the LOD value is under threshold value.

In fact, four QTLs for heading date were identified on chromosomes 2B, 3B, 5A and 6A (table 6).

A major QTL, explaining 32.7-61.9% of the total variability for this trait, was identified on chromosome 2B for all three years.

Considering the major homeologous gene series controlling the photoperiod response in tetraploid and hexaploid wheats (*Ppd-A1* on chr. 2A, *Ppd-B1* on chr. 2B and *Ppd-D1* on chr. 2D), the major QTL for HD detected in our population, on the basis of common molecular markers, could correspond to the *Ppd-B1* gene (Mohler et al. 2004; Hanocq et al. 2004; Kuchel et al. 2006). QTLs on chromosomes 3B, 5A and 6A showed only a marginal effect, with lower LOD values, and being evident in one year. Only the QTL on chromosome 6A was found in both 2008 and 2009 trials, even if the LOD value was just below the significant treshold in 2008. In particular, the QTL located on the chromosome 3B and linked to wpt-6047 DArT marker was detected only for year 2008 with a LOD value of 4.29 and a R^2 of 5.2%. The QTLs on 5A and 6A, linked to markers Xgwm595 and 345499, respectively, showed R^2 values of 5.7 and 7,3% for 5A and 6A, respectively.

Plant phenology (earliness) manipulation is a key breeding strategy for Mediterranean environments (Araus et al. 2008), where water deficit and high temperature during flowering and grain-filling period (i.e. terminal drought) are very common (Loss and Siddique 1994). Earliness (drought

escape) improves fitness in environments with a short or an unpredictable growing season (Mediterranean-like), whereas late flowering promotes accumulation of resources in a favourable environment. Productivity was associated with earliness in drought-affected wild emmer germplasm collection; however, accessions with mid-early (not the earliest) flowering time exhibited the highest productivity levels (Peleg et al. 2007), indicating that a good balance between escape from terminal drought stress and length of growing cycle to accumulate photosynthates into seeds is at the basis of good yield levels in Mediterranean environments. The minor QTLs identified in this work, even with a lower effect on the trait compared to the *Ppd-B1* gene, could be helpful in genetic improvement of durum wheat to further regulate earliness in this species.

Mapping physiological traits related to response to water stress. Besides pheno-morphological traits, physiological aspects of the plant response to environmental conditions are fundamental in determining grain yield performance in field. Many drought-related physiological traits have been studied and measured in controlled conditions, but fast, stable and easy measures are needed when analysing a high number of samples as a segregating population in field trial conditions. In order to study physiological aspects related to photosynthetic activity in the Ofanto x Cappelli mapping population, measures predicting chlorophyll content in leaves, stomatal resistance and carbon isotopic discrimination have been carried out in cooperation with the University of Sassari. Phenotypic performance for these traits of RIL and parents in two years are given in table 7.

Trait	Year	Parent			RIL			
		Ofanto	Cappelli	LSD	Average	SD	Min	Max
Stomatal resistence (logRS)	2008	1.469	1.648	0.125	1.655	0.093	1.474	2.079
Stomatal resistence, March 30 reading (logRS March 30)	2009	1.456	1.543	0.247	1.477	0.066	1.313	1.665
Stomatal resistence, April 7 reading (logRS April 7)	2009	1.449	1.647	0.161	1.469	0.066	1.333	1.669
Stomatal resistence, April 15 reading (logRS April 15)	2009	1.371	1.862	0.396	1.557	0.147	1.27	1.917
Stomatal resistence, May 12 reading (logRS May 12)	2009	1.641	1.862	0.303	1.783	0.129	1.533	2.268
Chlorophyll content, tillering stage (ChlCtill)	2008	52.168	46.125	3.578	49.111	2.798	42.1	56.575
Chlorophyll content, tillering stage (ChlCtill)	2009	53.065	44.515	3.62	48.303	2.331	42.99	54.33
Chlorophyll content, tillering stage Foggia (ChlCtillFG)	2009	619.5	561.6	73.72	550.147	35.359	457.3	662.3
Chlorophyll content, heading stage (ChlChea)	2008	57.468	45.218	3.03	49.361	2.802	39.787	56.825
Chlorophyll content, heading stage (ChlChea)	2009	58.82	44.42	5.457	50.823	3.45	42.05	60.25
Isotopic carbon discrimination (DC)	2008	23.079	22.247	0.587	22.589	0.229	22.107	23.189
Isotopic carbon discrimination (DC)	2009	22.935	22.482	0.586	22.886	0.217	22.314	23.374

Table 7: Phenotyping performance for physiological traits; chlorophyll content at tillering stage Sassari (ChlCtill), chlorophyll content at heading stage Sassari (ChlChea), chlorophyll content at tillering stage Foggia (ChlCtillFG), stomatal resistance (logRS) and carbon isotopic discrimination (DC).

Stomatal resistance was measured at different dates in two years at Sassari. Cappelli had larger values than Ofanto for stomatal resistance for all measures carried out, and differences were statistically significant between parents as well as among RILs ($P<0.001$). Frequency distributions very near to a continuous one were observed in all cases (Fig. 7), suggesting a quantitative control for this trait.

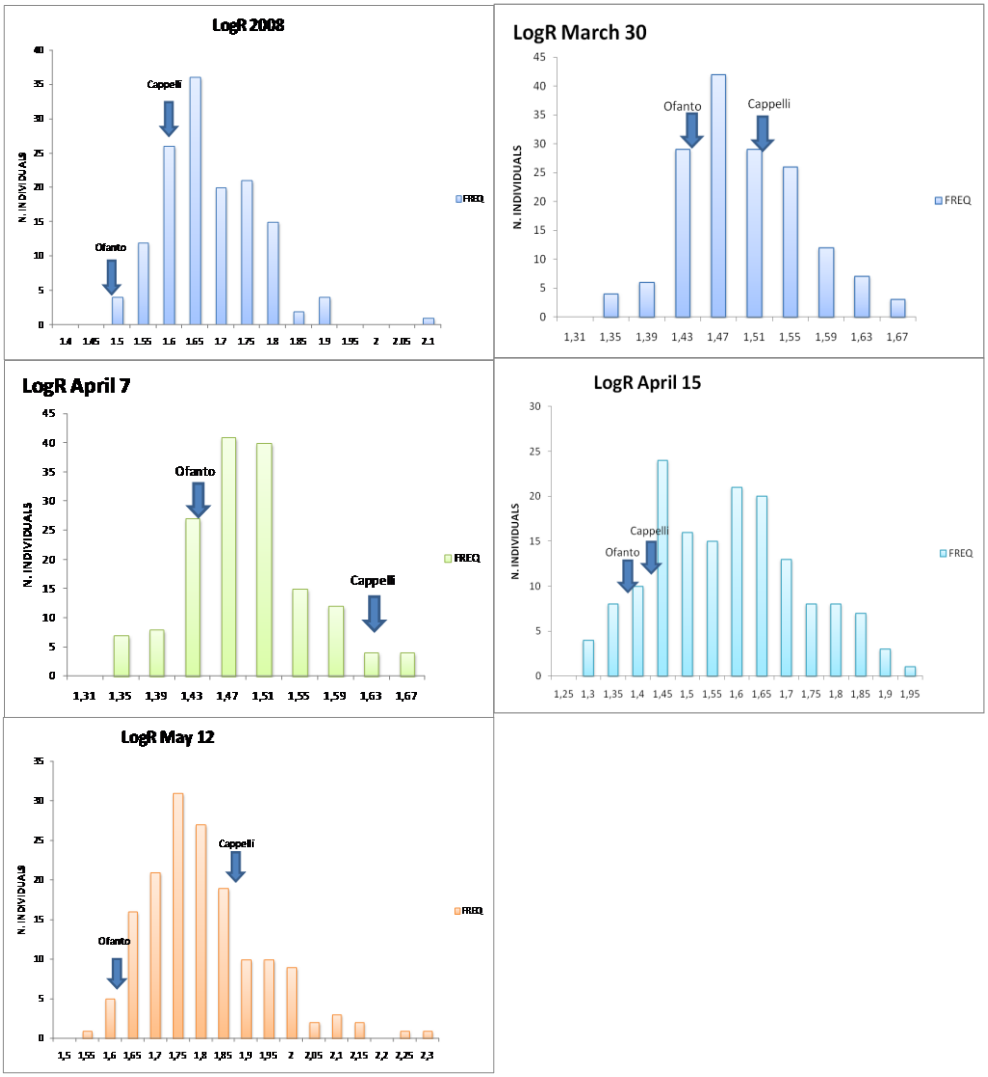


Figure 7: Phenotypic distribution for logRS. Year 2008: LogR2008; year 2009: March 30, April 7, April 15, May 12.

QTL	Peak marker/interval	Chr*	Trait	LOD	R2 (%)	Add. Eff. **
1	349352	2A	logRS May 12 2009	3.73	9.5	0.04
			*logRS 2008**	*2.09*	*4.6*	*-0.02*
2	wpt1159 - Xgwm285	3B	logRS 2008	5.81	14.9	-0.0365
		3B	logRS May 12 2009	3.46	9.9	-0.021
		3B	logRS April 7 2009	4.19	12.1	-0.047
3	Xgwm1081	4A	logRS 2008	3.53	8.6	-0.027
4	Xgwm63	7A	log RS 2008	5.22	13.3	-0.034
5	Xgwm900	7A	DC 2008	3.9	12.2	0.081

*Chr: chromosome

** referred to Ofanto allele

Table 8: Results of QTL analysis for stomatal resistance and carbon isotopic discrimination; Stomatal resistance (logRS) and carbon isotopic discrimination (DC). Traits with * indicate that the LOD value is under threshold value.

Four QTLs were detected for stomatal resistance (table 8). The most important was detected on chromosome 3B. This QTL explained from 9.9% to 14.9% of the total variability for this trait and also was stable, controlling expression of the trait for both years 2008 and 2009 (reading May 12 2009 and April 7 2009). The LOD value ranged 3.46 to 5.81.

Interestingly, this QTL was in the same region in which one of the minor QTLs for heading date was identified in this work. Four more QTLs were identified providing a minor contribute to the control of the stomatal resistance in field conditions only for one year. The first one, located on chromosome 2A was involved in the control of stomatal resistance only for year 2009 with a LOD value of 3.73 and explaining 9.5% of observed variability. Two QTLs, on chromosomes 4A and 7A, controlled, only for year 2008, stomatal resistance; about 13% of the observed variability for stomatal resistance was controlled by the QTL on chromosome 7A, while less than 10% was explained by the minor QTL identified on chromosome 4A.

Data available in literature suggest that group 3 in wheat (Bobo et al. 1992) was involved in control of stomatal resistance, but further information about precise mapping of genetic determinants for this trait in wheat is still not available, therefore the QTLs identified in this work, can be considered an important source for the improvement of durum wheat performance in

Mediterranean environments. The QTL located on the chromosome 3B in particular, seems to have a major importance and stability with respect to the other ones, and different microsatellite markers have been positioned within the interval of the QTL or in the vicinity, that could represent a useful tool in programs of selection assisted by molecular markers (Rizza et al. 2004).

Chlorophyll content was estimated by SPAD measures in filed trials carried out at Sassari (2008 and 2009) at stages of tillering and heading, and at Foggia (2009) only at the stage of tillering. In all cases, higher values were registered for Ofanto, with differences statistically significant between parents as well as among RILs ($P<0.001$). Also in this case a normal frequency distribution was observed for the trait (Fig. 8 and 9). Consistently with this result, a total of four QTLs were detected for chlorophyll content on chromosomes 3A, 3B, 4A and 6B (Table 9).

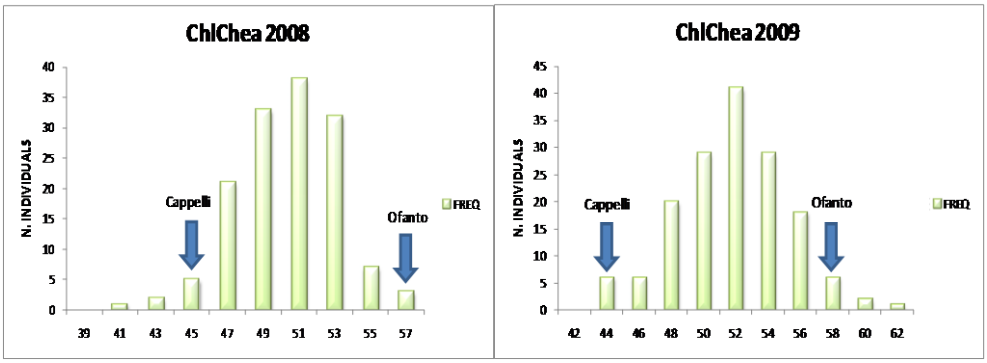


Figure 8: Phenotypic distribution for chlorophyll content. Chlorophyll content at tillering stage Sassari (ChlCtill), chlorophyll content at tillering stage Foggia (ChlCtillFG) and chlorophyll content at heading stage Sassari (ChlChea).

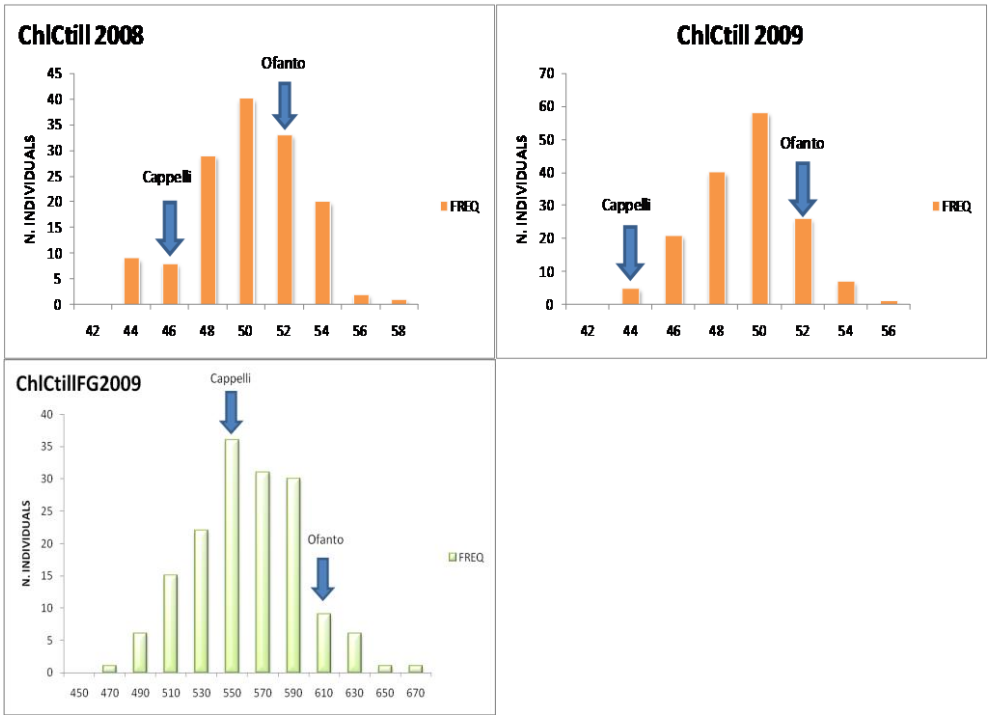


Figure 8: Continued.

QTL	Peak marker/interval	Chr*	Trait	LOD	R2 (%)	Add. Eff. **
1	348145	3A	ChlChea2008	4.03	12.7	-1.018
2	Xgwm247	3B	ChlCtill2009	3.72	8.9	0.702
3	wpt-9342	4A	ChlChea2009	3.17	9.4	1.096
4	wpt1287 - wpt1730	6B	ChlCtill2008	3.52	11	0.948
		6B	ChlCtill2009	5.87	16.2	1
		6B	ChlCtillFG2009*	2.48	9	10.65

*Chr: chromosome
** referred to Ofanto allele

Table 9: Results of QTL analysis for chlorophyll content at tillering stage (ChlCtill), chlorophyll content at heading stage (ChlChea). Chlorophyll content at tillering stage recorded in Foggia (ChlCtillFG). Traits with * indicate that the LOD value is under threshold value.

Among them, the QTL located on the chromosome 6B was particularly interesting as it was involved in control of the trait, in both years (2008 and 2009) and locations (Sassari and Foggia) of field trials, at the tillering stage even if at Foggia the LOD value was just below the significant treshold. This QTL explained 9-16.2% of the total variability observed for this trait, and the positive contribute was given by the allele of Ofanto. Minor QTL were identified on chromosome 3A, 3B and 4A, acting only in one field trial. The QTL detected on chromosome 3B explained 8.9% of the observed variability for chlorophyll content recorded during tillering stage in Sassari for year 2009. Very similar values characterized the QTL on the chromosome 4A; in both cases the Ofanto allele contribute positively in increasing chlorophyll content. A positive contribution was provided by the Cappelli allele for the QTL located on chromosome 3A, which was involved in controlling chlorophyll content recorded at Sassari during heading stage only for year 2008, and explained 12.8% of the observed variability. The majority of traits associated with plant photosynthetic physiology are quantitative and controlled by multiple genes and environmental variation (Fracheboud et al. 2002; Cao et al. 2004; Juenger et al. 2005).

Zhang et al. (2009) and Peleg et al. (2009) mapped genetic determinants for chlorophyll content on chromosomes 4A e 6B but it is not possible, based on common molecular markers, to establish if these chromosomal regions correspond to the ones identified in this work on the map Ofanto x Cappelli.

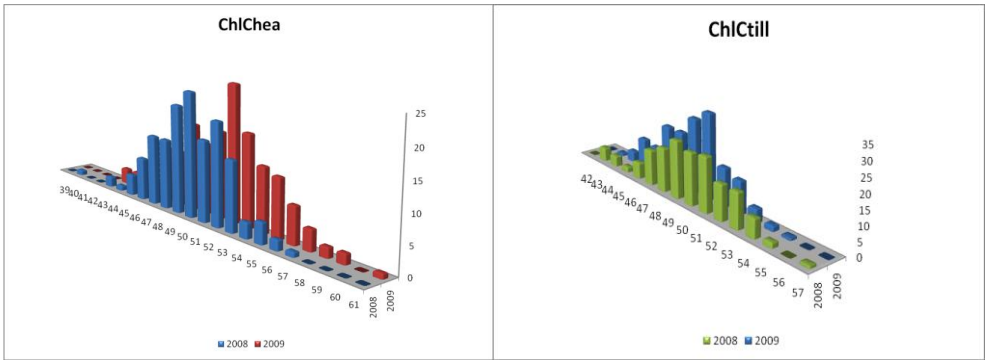


Figure 9: Phenotypic multiple distribution for chlorophyll content. Chlorophyll content at tillering stage (ChlCtill) and chlorophyll content at heading stage (ChlChea).

Under drought stress conditions, genotypes with a stay-green phenotype (i.e. delayed senescence) retain their leaves in an active photosynthetic state during the grain filling period. This enhances the stress tolerance by increasing the assimilate supply for grain filling and, therefore, could result in higher plant productivity. Therefore, understanding the genetic mechanism of chlorophyll content would be very important for yield and drought-tolerance improvement in wheat, and the identification of many QTLs controlling the accumulation of chlorophyll in leaves represents a genetic resource of great importance for the further improvement of wheat from this point of view.

Isotopic carbon discrimination, finally, was determined on leaves at vegetative stage in field trials carried out at Sassari in 2008 and 2009. Also in this case a normal frequency distribution was observed for the trait (Fig. 10). Unfortunately, significant differences were found for parents and RILs only in 2008 (DC 2009 $P>0.05$), and only a QTL located on chromosome 7A and explaining 12.2% of the observed variability was identified, with a positive contribute from the Ofanto allele.

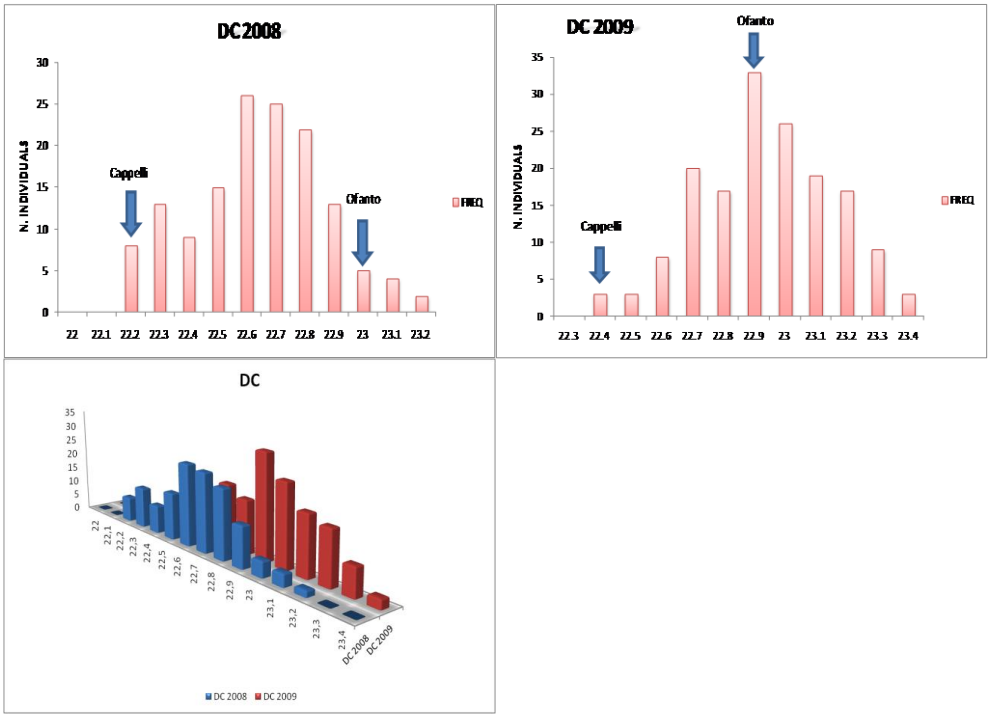


Figure 10: Phenotypic distribution (single and multiple) for carbon isotopic discrimination.

In wheat, genotypic increases in isotopic carbon discrimination were associated with increases in aerial biomass and yield in favorable environments (Condon et al. 1987; 2004). High isotopic carbon discrimination has been associated with higher leaf conductance, increased water use and growth (Fischer et al. 1998; Condon et al. 2004). Hence positive relationships for isotopic carbon discrimination and yield have been obtained in favorable irrigated environments where water supply was not a major constraint to yield (Fischer et al. 1998), or in Mediterranean environments where soil water up to anthesis was plentiful (Araus et al. 2003b).

Rebetzke et al. (2008) detected for isotopic carbon discrimination many loci controlling this trait confirming its polygenic inheritance in wheat (Peleg et

al. 2009). Rebetzke et al. (2008) mapped a QTL for isotopic carbon discrimination on chromosome 7A in DH populations, from Cranbrook/Halberd, Sunco/Tasman, CD87/Katepwa, but in a different position with respect to the Ofanto x Cappelli map.

Mapping of morphological features of seeds. Few QTL studies have been conducted in wheat for morphological features of seeds (Breseghello and Sorrels 2007; Breseghello et al. 2005; Campbell et al. 1999; Sun et al. 2009; Tsilo et al. 2010). The traits for kernel morphology are difficult to manipulate by conventional breeding due to pronounced effect of environment. Morphological features of seeds of RIL and parents were evaluated on grain samples harvested in field trials carried out at Foggia in two years (Table 10).

Trait	Year	Parent			RIL			
		Ofanto	Cappelli	LSD	Average	SD	Min	Max
Thousand kernel weight (g), TKW	2007	46.744	48.095	5.453	43.602	7.012	21.43	56.285
	2009	45.4	60.578	4.314	51.566	3.019	43.251	60.326
Seed area (mm^2), SA	2007	29.04	30.4	1.72	27.368	2.639	18.099	32.182
	2009	18.162	22.19	0.977	19.592	0.802	17.477	22.324
Seed volume (mm^3), SV	2007	43.486	46.145	3.773	40.234	5.89	23.376	51.464
	2009	42.738	57.252	3.715	48.219	2.883	39.736	57.378
Diameter max (mm), DMax	2007	9.171	9.585	0.288	8.983	0.398	6.987	9.763
	2009	7.55	8.145	0.189	7.72	0.209	7.221	8.338
Diameter min (mm), DMin	2007	4.213	4.266	0.152	4.058	0.249	3.242	4.473
	2009	3.246	3.624	0.104	3.421	0.082	3.125	3.631

Table 10: Phenotypic performance for morphological features of seed.

Significant differences were observed among RILs for all traits for both years ($P<0.001$), while the parents showed to be statistically different for all traits in 2009, but only for Dmax in 2007. Continuous distributions were common for all traits (fig. 11), indicating a polygenic inheritance. Multiple distributions are also showed to compare results from two years for each trait (fig. 12) and the large differences in means, observed in the two years, for area, Dmax, Dmin and volume, indicate a strong genotype-environment interaction for these traits.

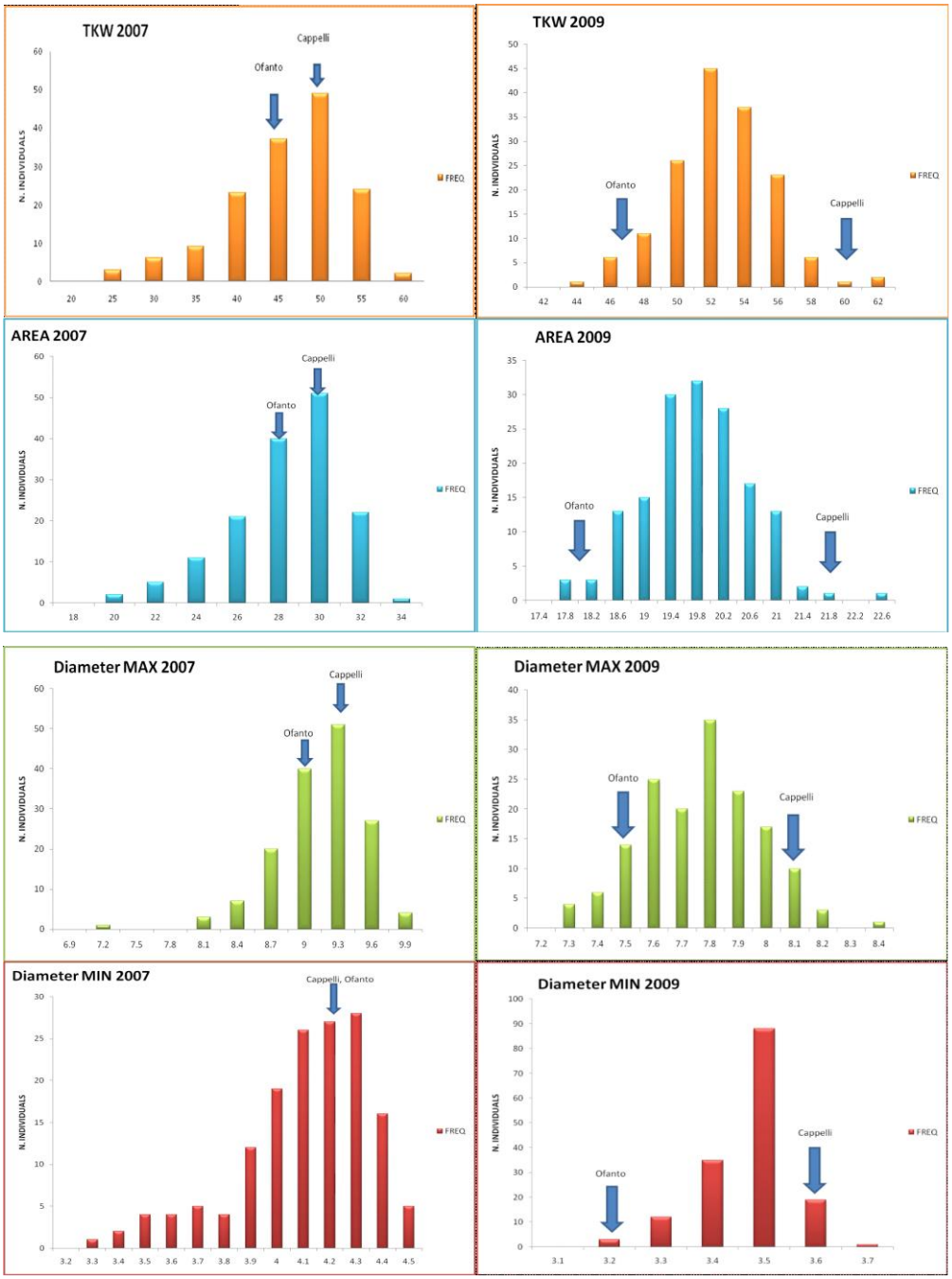
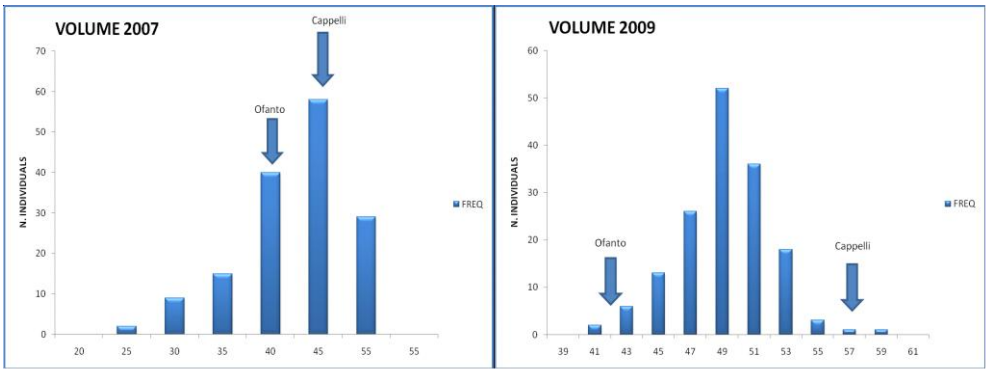


Figure 11: Phenotypic distribution for: TKW, SA, DMax, DMin, SV for years 2007 and 2009



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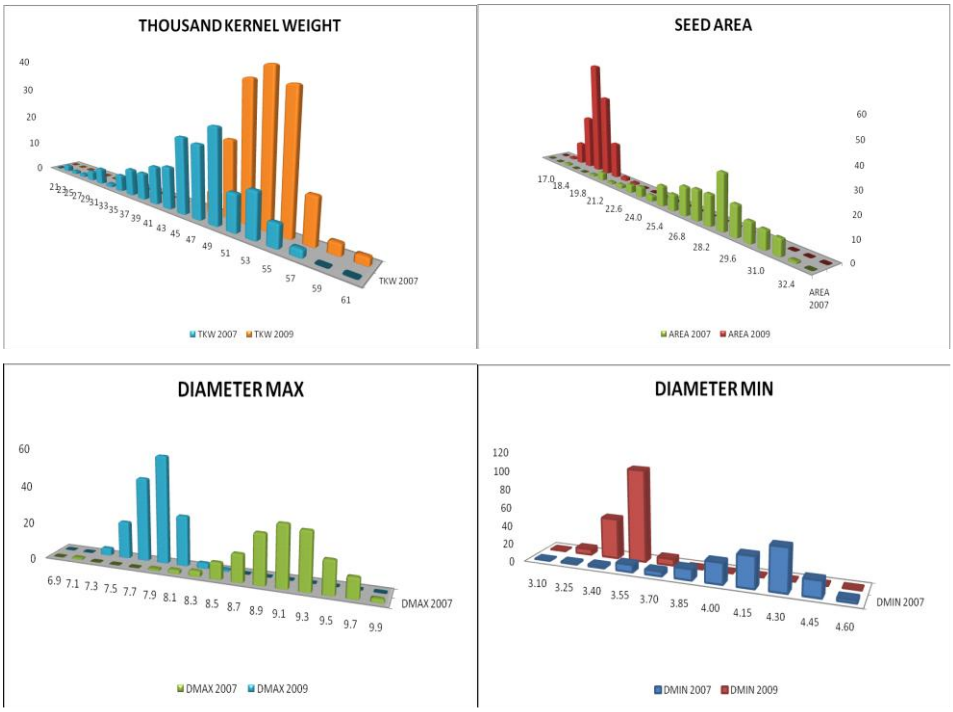


Figure 12: Phenotypic multiple distribution: TKW, SA, DMax, DMin and SV for years 2007 and 2009

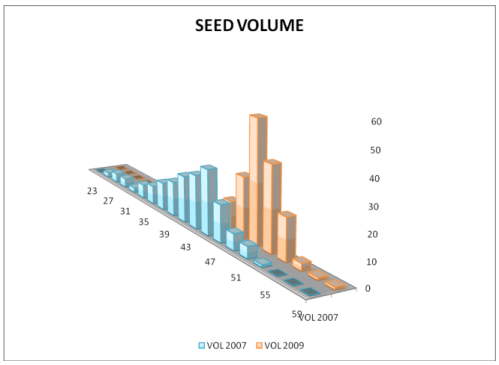


Figure 12: Continued

QTL	Peak marker/interval	Chr*	Trait	LOD	R2 (%)	Add. Eff. **
1	wpt-6142	1B	DMax 2009	4.72	7.7	0.059
2	WMC 382a	2A	SV 2009	3.16	8.9	-0.884
3	WMC477	2B	SA 2007	3.56	7.8	0.73
			DMin 2007	4.91	11.2	0.082
			DMin 2009	3.33	8.8	0.023
			SV 2007	4.06	9.7	1.796
4	Xgwm682	2B	DMax 2009	4.27	6.9	-0.056
5	374339 - wpt1482	4A	SA 2009	4.15	9.2	-0.244
			DMax 2007	3.14	9.2	-0.127
			DMax 2009	8.1	14.4	-0.083
6	380739 - 378423	4B	TKW 2007	5.62	20.1	-4.366
			*TKW 2009**	*2.17*	*12.2*	*-1.279*
			SV 2009	3.53	13.4	-1.49
			SA 2007	3.67	8.9	-0.965
			*SA 2009**	*2.25*	*7.1*	*-0.305*
			DMin 2007	6.15	15.8	-0.119
			DMin 2009	4.68	14.2	-0.036
			SV 2007	5.43	14.6	-2.7
7	WMC705 - XMAG3794	5A	SA 2007	3.28	6.2	-0.721
			SA 2009	5.3	11.9	-0.285
			DMax 2007	4.26	11.1	-0.134
			DMax 2009	5.43	9.2	-0.067
8	wpt1437 - 381689	6B	TKW 2007	3.96	9.8	2.18
			SA 2007	3.82	8.2	0.748
			DMin 2007	4.62	10.3	0.078
			SV 2007	4	9.7	1.83
9	BARC134	6B	DMax 2009	4.3	6.9	-0.057

*Chr: chromosome
** referred to Ofanto allele

Table 11: Results of QTL analysis for morphological features of seed: SA – Seed Area (mm^2); SV – Seed Volume (mm^3); MinD and MaxD – Minimum and Maximum Diameter (mm); TKW – thousand Kernel Weight (g). Traits with * indicate that the LOD value is under threshold value.

Many studies in the past have demonstrated that all seven chromosome groups are involved in the genetic control of yield and yield-related traits in wheat

The QTL analysis produced 9 QTLs distributed on 7 chromosomes, 1B, 2A, 2B, 4A, 4B, 5A and 6B (Table 11). The LOD score ranged from 3.14 for DMax 2007 (chr. 4A) to 8.1 for DMax 2009 (chr. 4A). The most important QTL was located on chromosome 4B between DArT markers 380739 and 378423. This QTL explained from 8.9 to 20.1% of the total variation in TKW, SA, DMin and SV and for DMin and SV was detected in both 2007 and 2009. Actually, this QTL also explained TKW and SA for both years of trials, even if in the case of TKW 2009 and SA 2009 the LOD value was below the significance thresold (but >2). This QTL colocalizes with the major QTL for plant height identified in this work (Fig. 12), indicating the importance of the effect of *Rht-B1* gene, not only on grain yield per se (Araus et al. 2008), but also on yield components and kernel features in particular. In wheat genes *Rht* have been generically involved with differences in kernel size (Borner et al. 1993). These results also were confirmed at level of QTL analysis.

QTLs for TKW were previously reported on chromosomes 1B, 3B, 4A, 4B, 5A, 6B, and 7A (Elaoufi and Nachit 2004; McCartney et al. 2005; Huang et al. 2006; Patil et al. 2008). Previously Patil et al. (2008) and McCartney et al. (2005) mapped TKW in the same interval of chromosome 4B in two different mapping populations. A second QTL for TKW was identified in this work on chromosome 6B between markers wpt-1437 and 381689. This QTL was involved, but only for year 2007, in controlling TKW, SA, DMin and SV explaining 9.8-10.3% of the total variability for this traits. Elouafi et al. (2004), Quarrie et al. (2005) mapped, previously, TKW on 6B, but the chromosomal region was different respect to the Ofanto x Cappelli mapping population. No QTLs were detected previously on chromosome 6B for SA, DMin and SV. The QTL on 2B linked to marker WMC477 controlled

expression of SA and SV for year 2007 and DMin both 2007 and 2009; this QTL explained from 7.8 to 11.2% of the total variability.

Previously Breseghello (2007) and Campbell (1999) mapped seed area on chromosome 2B, but it is not possible to understand if the region is the same found in this work based on common molecular markers. SV was also controlled by a QTL on chromosome 2A. On 2B another QTL in a distinct region, with a LOD value 4.27 and 6.9% of variability, was involved in controlling DMax for year 2009. The QTL identified on chromosome 4A between 374339-wpt1482 marker and the QTL on chromosome 5A within the interval WMC705-XMAG3794 showed a role in controlling SA and DMax. In particular, the QTL on chromosome 5A controlled Dmax and SA in both years 2007 and 2009, while the QTL on chromosome 4A controlled Dmax for both years, but SA only for 2009. QTLs on chromosome 1B (peak marker wpt-6142) and 6B (peak marker BARC134) controlled a minor component of DMax (7.7% and 6.9%, respectively).

Breseghello et al. (2007) mapped kernel width on chromosome 1B but in a different region with respect to the map Ofanto x Cappelli.

The work carried out in this thesis, based on a very accurate evaluation of the different components of the seed morphology, allowed to clarify as the final results of the morphology of seeds is the results of a complex regulation acting at different levels:

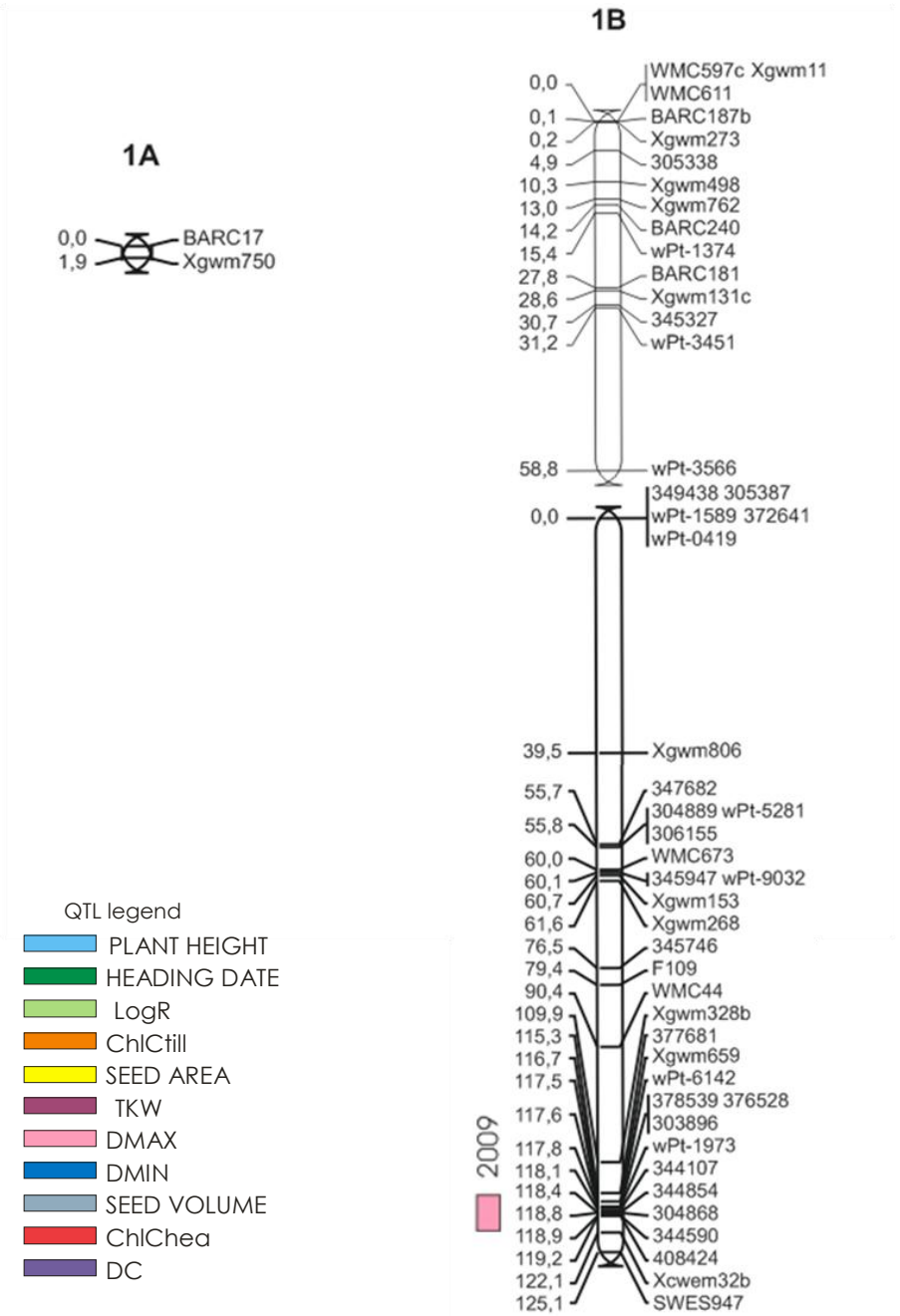
1. each feature of the seed morphology can be controlled by different genomic regions: for example 5 different QTLs were found only for Dmax, 3 for Dmin and so on;

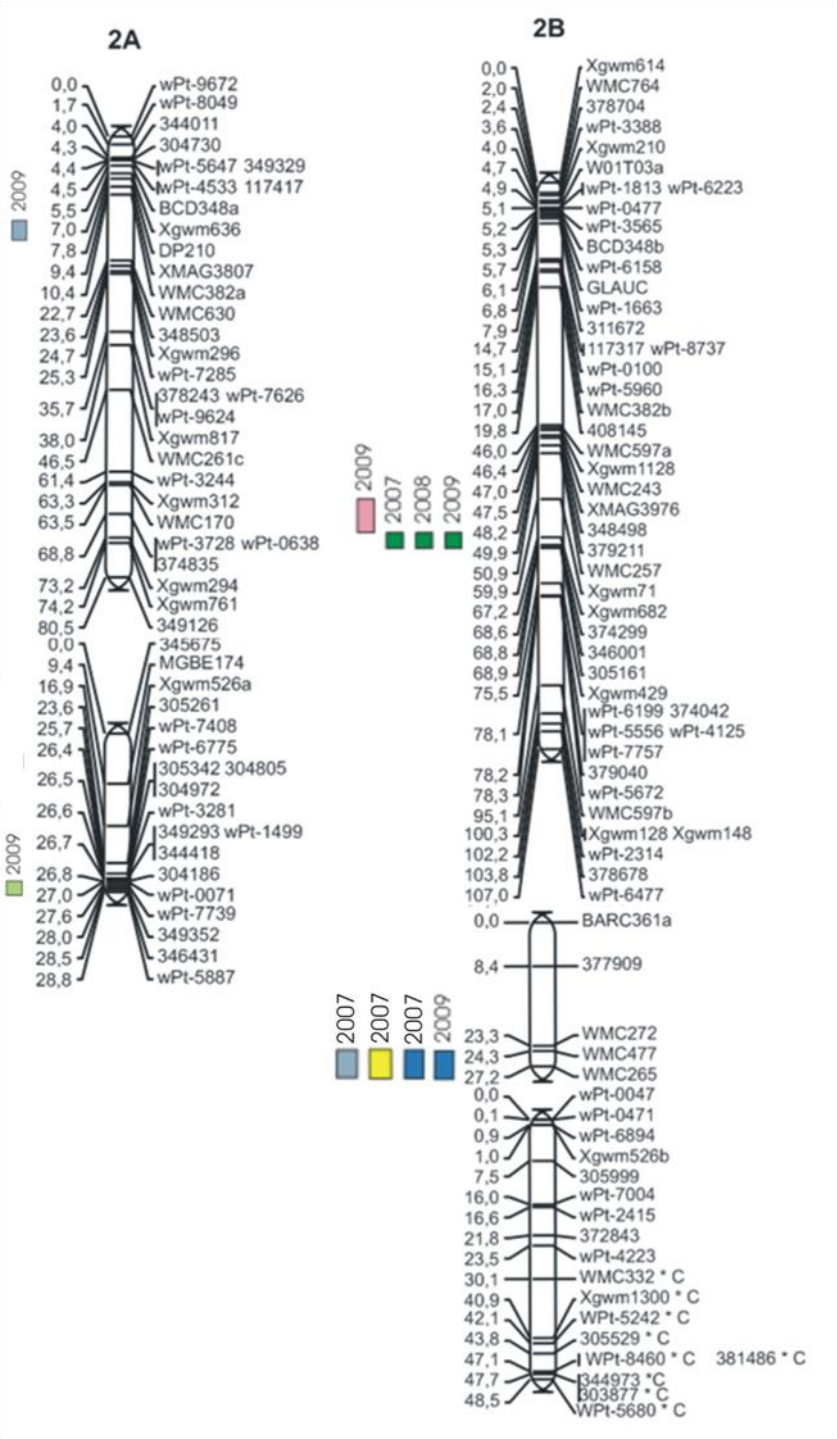
2. some of these QTLs can be specific for one feature (for example, QTLs on chromosomes 1B and 6B are specific for Dmax), or explain part of the observed variability for different features (the QTL on chromosome 4B controls for example TKW, SA, SV and Dmin, but not Dmax).

All these components act together for the fine regulation of the seed morphology, which is strictly connected to grain yield and quality. The

knowledge of the position of these QTLs and the linked molecular markers are of great importance for the transfer of useful alleles at multiple loci to improve the characteristics of seeds.

Figure 13: Linkage genetic map, with QTLs, of tetraploid wheat constructed from RIL population derived from a cross between durum wheat cv Ofanto and cv Cappelli. Markers are show on the right with map distances in cM on the left. Short arms are at the top. Markers that showed distorted segregation in favor of Ofanto and Cappelli were marked on the right close to marker with "*" and O or C, respectively. QTLs are show on the left side by colored squares with above the year for significative trait.





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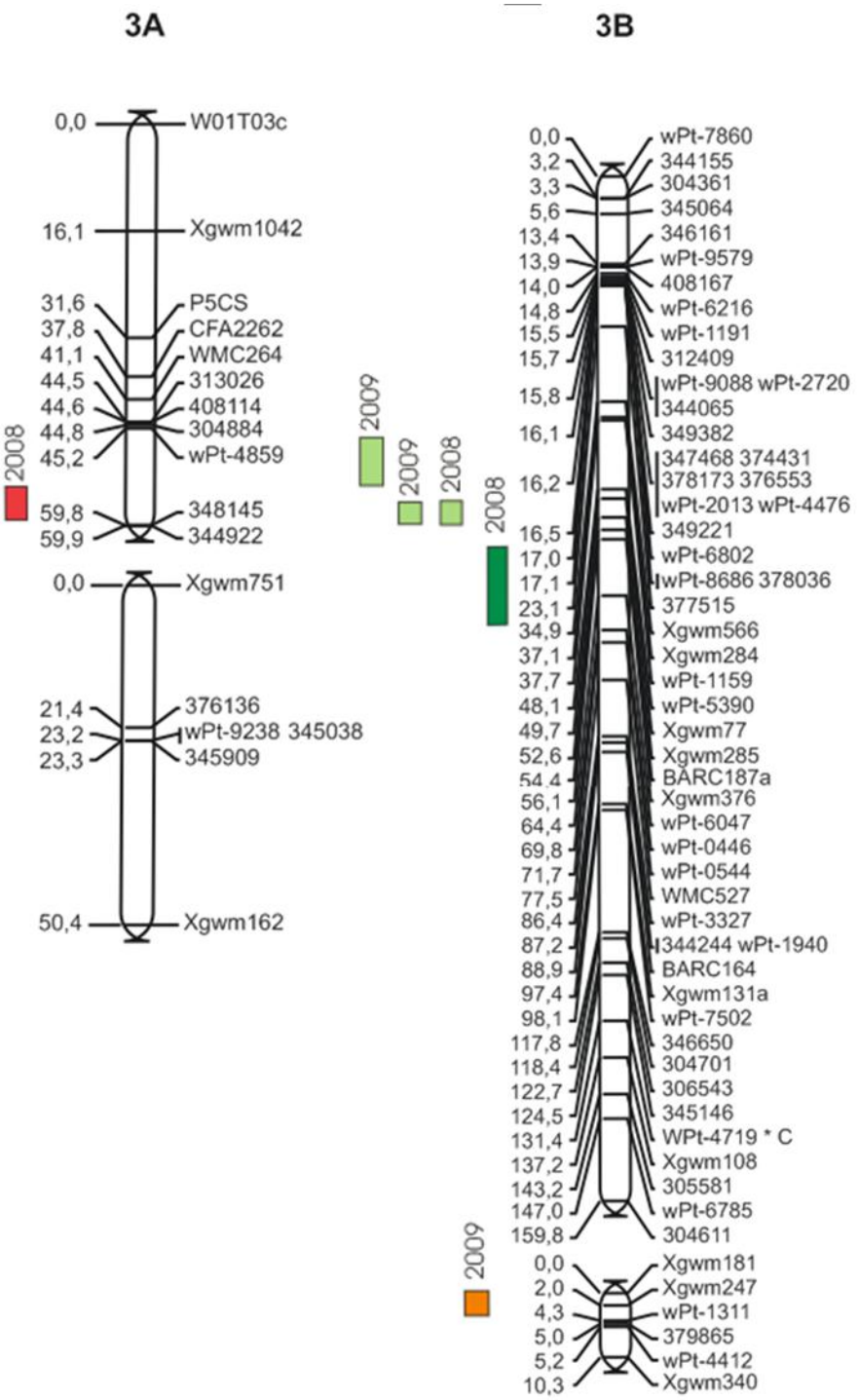

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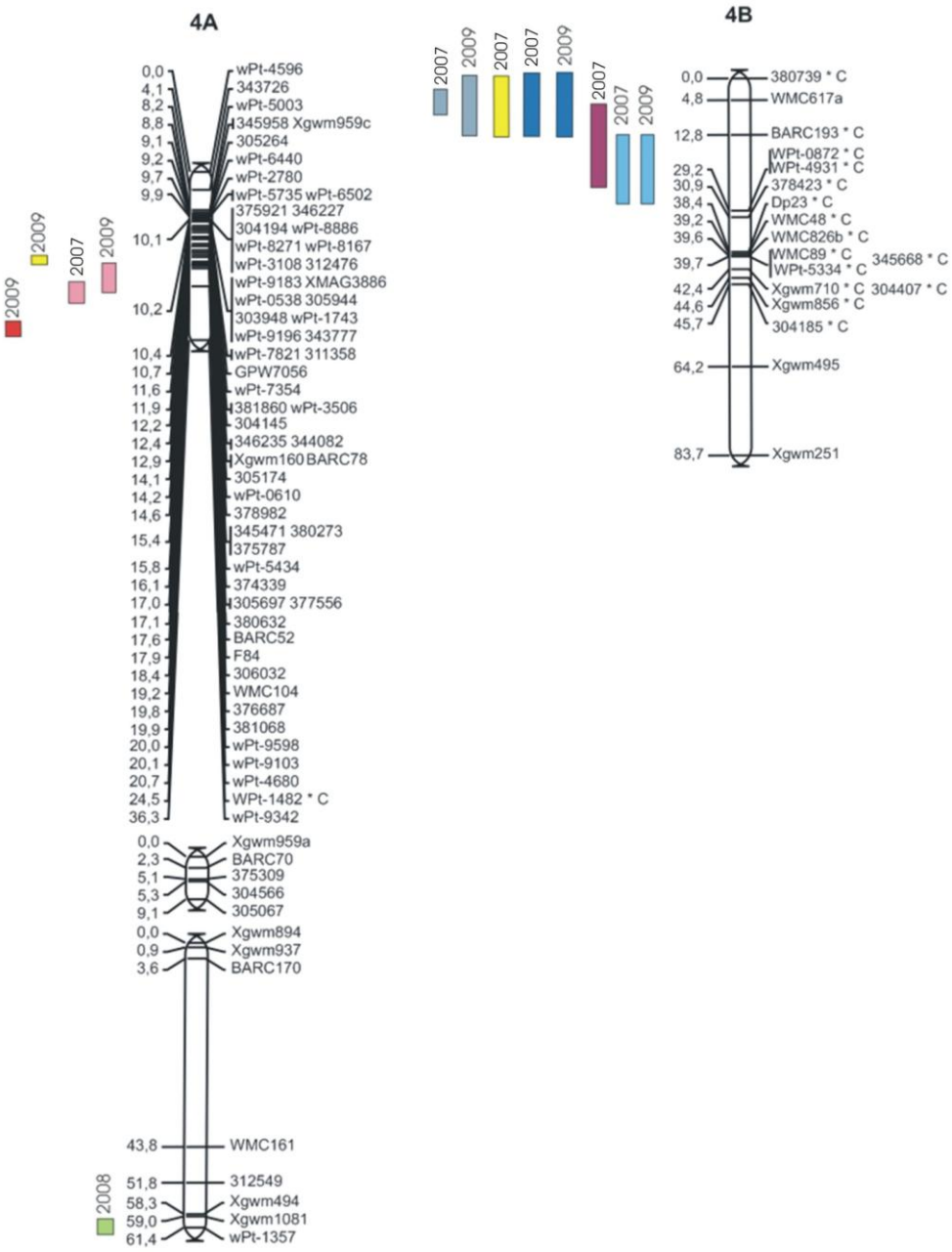

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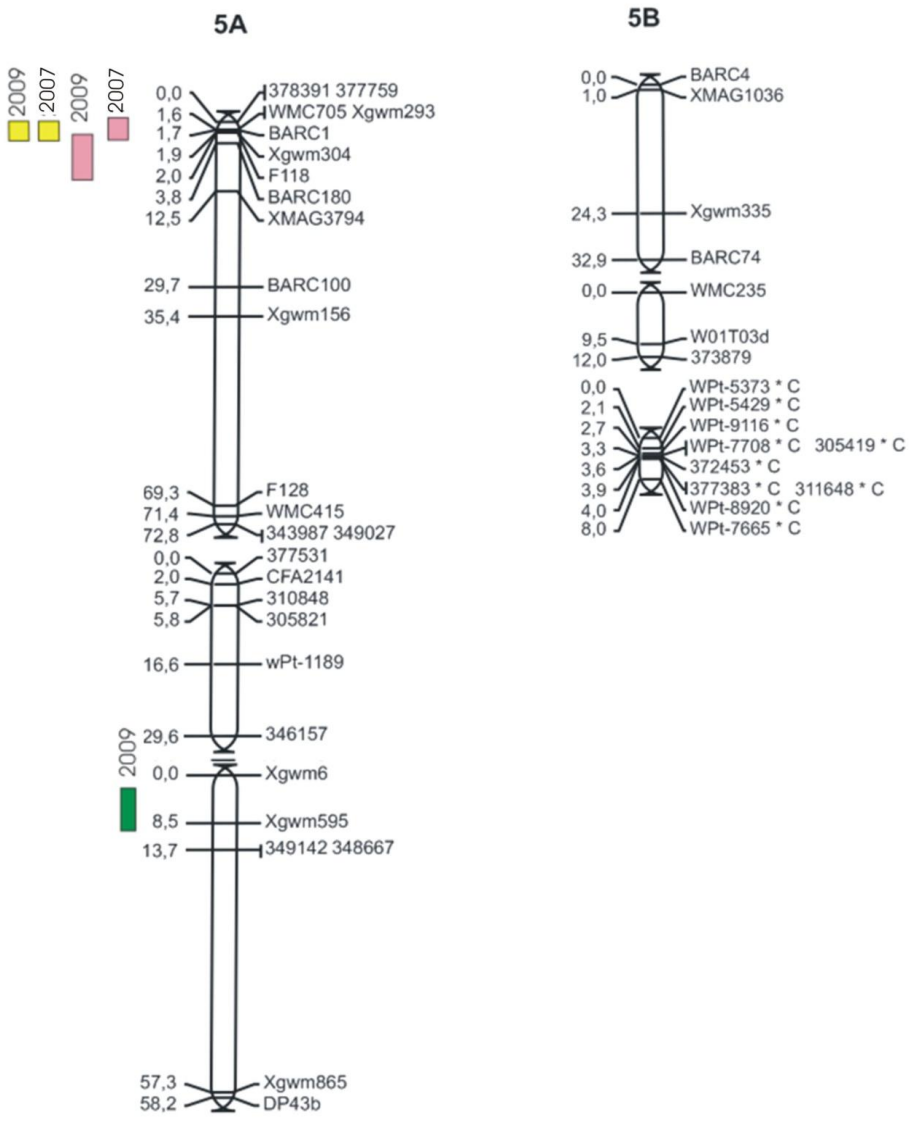

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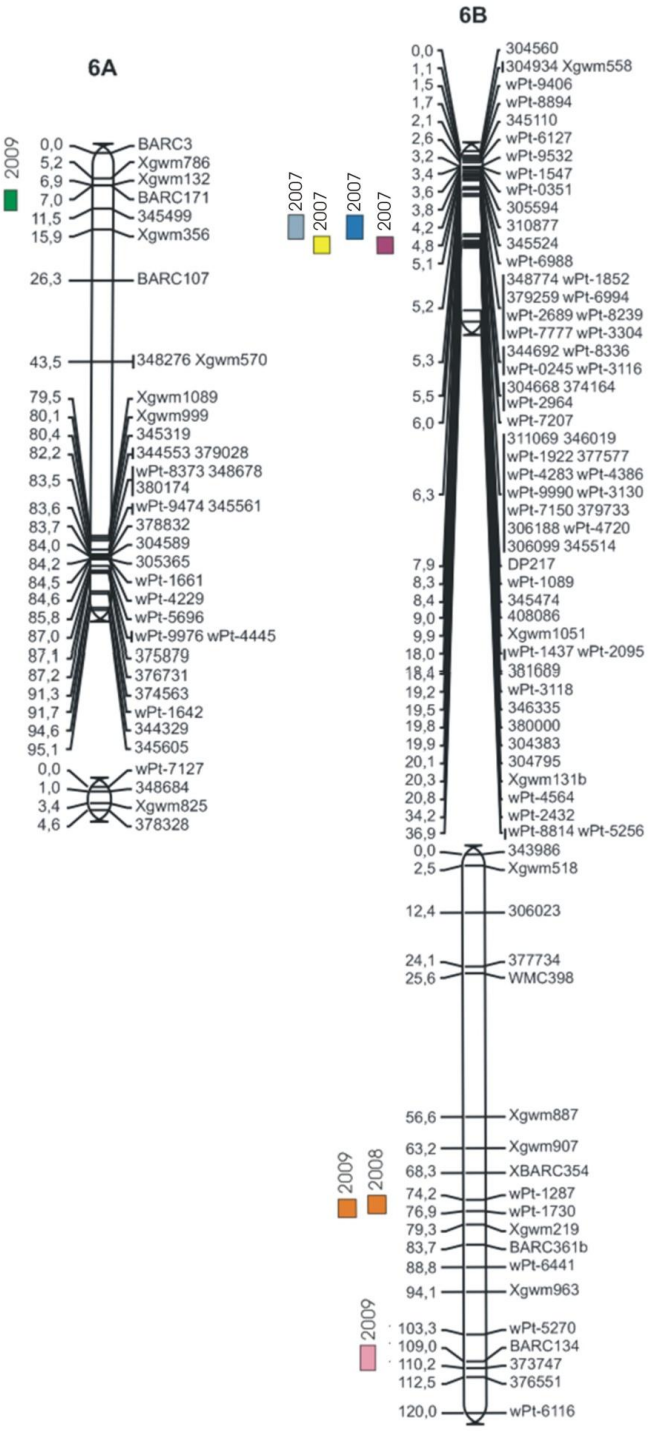


Figure 12: continued

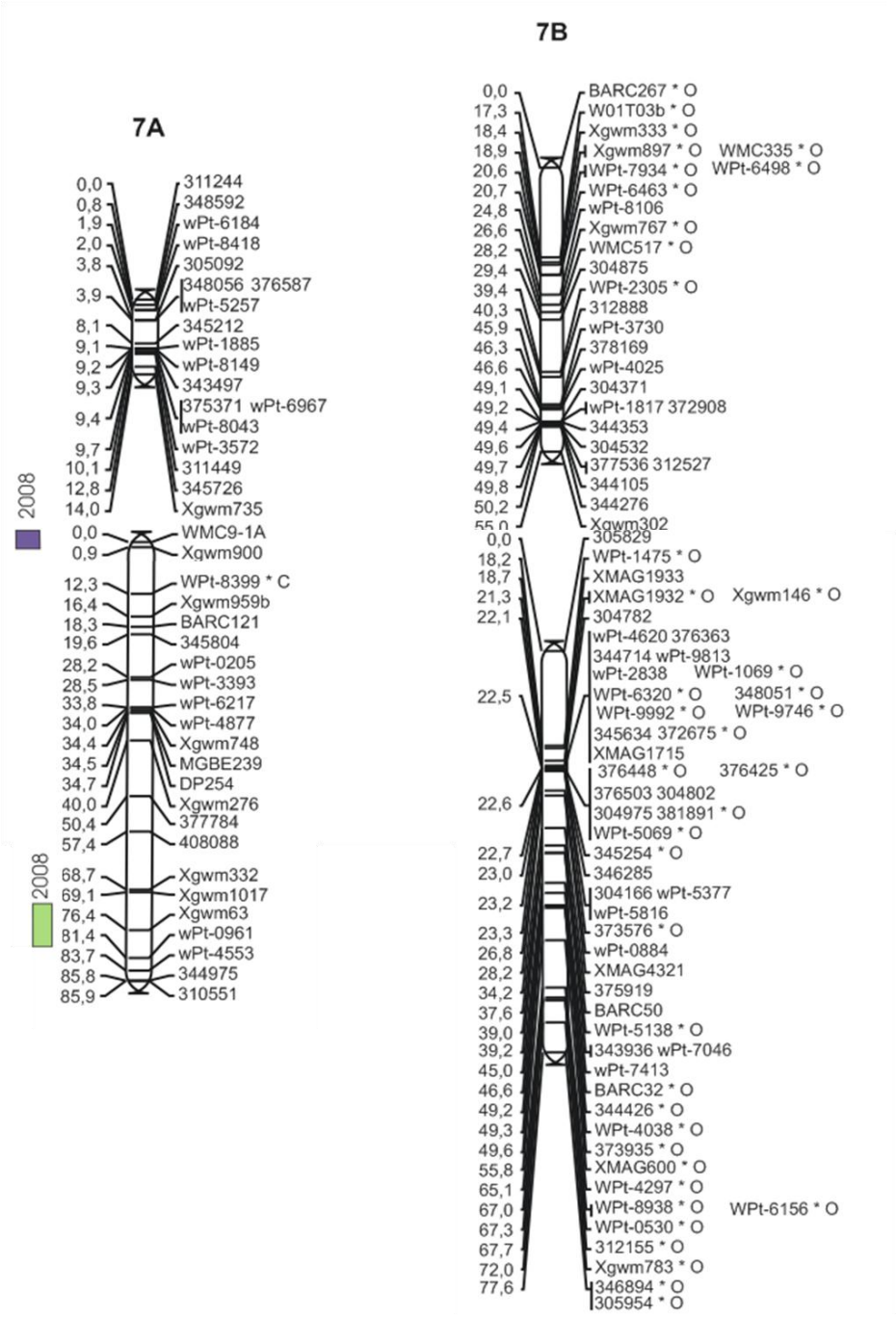


Figure 12: continued

CHAPTER 2

Stem Rust

Resistance Gene

Mapping

Plant resistance to biotic stresses. Common and durum wheat are vulnerable to attack by many different pathogens and pests and some of these pathogens and pests have a broad range of occurrence whereas others may be very localised; some are extremely damaging to crop production whereas others cause relatively little damage despite widespread occurrence. Plant pathogens can be broadly divided into those that kill the host and feed on the contents (necrotrophs) and those that require a living host to complete their life cycle (biotrophs). Microbial necrotrophy is often accompanied by production of toxins.

The comprehensive information on pathogen virulence and variation, and the epidemiological information on pathogen movements, provide a basis for the development of an early warning system to farmers growing potentially susceptible cultivars. Flor (1942) was first to realise that incompatibility between a host and a pathogen involved corresponding genes in each organism. More in detail, plant–pathogen interactions, particularly those involving biotrophic parasites, are governed by specific interactions between pathogen *avr* (avirulence) gene loci and alleles of the corresponding plant disease resistance (*R*) locus. When corresponding *R* and *avr* genes are present in both host and pathogen, the result is disease resistance. If either is inactive or absent, disease results.

Plants are constantly exposed to pathogens. To be pathogenic, most microbes must access the plant interior, either by penetrating the leaf or root surface directly or by entering through wounds or natural openings such as stomata, pores in the underside of the leaf used for gas exchange. Once the plant interior has been breached, microbes are faced with another obstacle: the plant cell wall, a rigid, cellulose-based support surrounding every cell. Penetration of the cell wall exposes the host plasma membrane to the microbe, where they encounter extracellular surface receptors that recognize pathogen-associated molecular patterns (PAMPs). Perception of a microorganism at the cell surface can initiate PAMP-triggered immunity

(PTI), which usually halts infection before the microbe gains a hold in the plant.

PAMP-triggered immunity may be the plant's first active response to microbial perception. PTI is initiated upon recognition of conserved microbial features by plant cell-surface receptors (receptor like kinase-RLKs belong to resistance proteins), and its induction is associated with MAP kinase signaling, transcriptional induction of pathogen-responsive genes, production of reactive oxygen species, and deposition of callose to reinforce the cell wall at sites of infection, all of which contribute to prevention of microbial growth (Nurnberger et al. 2004). Basal defences inhibit pathogen spread after successful infection and onset of disease. Pathogenic microbes have evolved the means to suppress PTI by interfering with recognition at the plasma membrane or by secreting effector proteins into the plant cell cytosol that presumably alter resistance signaling or manifestation of resistance responses. Once pathogens acquired the capacity to suppress primary defenses, plants developed a more specialized mechanism to detect microbes, referred as effector-triggered immunity (ETI). Effector-triggered immunity involves the direct or indirect recognition of the very microbial proteins used to subvert PTI by plant resistance (R) proteins. Activation of R protein-mediated resistance also suppresses microbial growth, but not before the invader has had an opportunity for limited proliferation (Chisholm et al. 2006).

Most of the disease resistance genes (R genes) in plants cloned to date encode nucleotide-binding site leucine-rich repeat (NBS-LRR) and the extracellular LRR (eLRR) resistance proteins. About eLRRs three subclasses have been classified according to their domain structure: RLPs, RLKs, and PGIPs. NBS-LRR are characterized by nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains as well as variable amino- and carboxy-terminal domains. The NBS-LRR class is the most abundant, and members

can possess amino-terminal coiled-coil (CC) or Toll-interleukin-1 receptor (TIR) domains. Plant NBS-LRR proteins are similar in sequence to members of the mammalian nucleotide-binding oligomerization domain (NOD)-LRR protein family (also called 'CARD, transcription enhancer, R (purine)-binding, pyrin, lots of leucine repeats' (CATERPILLER) proteins), which function in inflammatory and immune responses (Inohara et al. 2005).

There are approximately 150 NBS-LRRencoding genes in Arabidopsis thaliana, over 400 in Oryza sativa (Meyers et al. 2003; Functional and Comparative Genomics of Disease Resistance Gene Homologs [http://niblrrs.ucdavis.edu]; Monosi et al. 2004), and probably considerably more in larger plant genomes that have yet to be fully sequenced.

The most important diseases causing heavy losses in durum wheat grain yield and quality are caused by fungi, viruses, bacteria, nematodes, insects and mites that intercept the photosynthates produced by plants. Leaf, stem and yellow rust in particular, are fungal diseases widespread that heavely affect plant productivity also in Mediterranean environments. Research on these diseases has focused on host plant resistance, and several sources of resistance have been identified and utilized in the durum wheat breeding programs. Nevertheless, the continuous evolution of pathogens leads to the diffusion of new virulent rust isolates that overcome single-gene based resistances. A valuable approach to growth resistant varieties consists in identifying durable resistances or in pyramiding a number of simple resistances into the same genotype by means of selection assisted by molecular markers. In both cases the identification/mapping of new resistance determinants and of closely linked molecular markers is needed.

Stem rust. Stem rust (fig. 19), also called black rust, caused by *Puccinia graminis* Pers.:Pers. f. sp. *Tritici* Eriks. & E. Henn., is a major, devastating disease of common or bread wheat and durum wheat and belongs to one of several *formae speciales* in *P. graminis*.

The occurrence of rust diseases in cultivated cereals has significantly influenced the development of human civilisation. Urediospores of *Puccinia graminis* taken from excavations in Israel were dated at about 1300 BC (Kislev 1982); biblical accounts, at about 1870 BC, indicate that rust epidemics forced the family of patriarch Jacob to seek refuge in Egypt; the scourge of rust is recorded in the early Greek and Roman literatures where, in about 500 BC, ceremonial details indicate liturgies to appease Robigus, the Corn God, in an attempt to prevent crop failure (McIntosh RA 1995).

Although the biology was not understood, an observed relationship between rusted cereals and the proximity of diseased barberry was appreciated from early times. In 1660, a law enacted in Rouen, France, required the removal of barberries from the vicinity of grain production fields. Measures that followed to control barberry in other countries such as England and the USA, culminated in the expensive and often controversial barberry eradication program in the USA in the early years of the last century (Roelfs 1982; Christensen 1984)

The first detailed reports of wheat stem rust were given independently by Italian scientists Fontana and Tozzetti in 1767 and the causal organism was named as *Puccinia graminis* in 1797 by Persoon. However, it was not until the 19th century that a distinction among the rust diseases was made. This was noted by de Candolle, who described the leaf rust pathogen as *Uredo rubigo-vera* and thereby distinguished it from Persoon's *Puccinia graminis.* De Bary in the 1860s provided proof of heteroecism of *P. graminis* on cereals and barberry. Eriksson in Sweden defined *formae speciales* to describe "special forms" (f. sp.) of the wheat stem rust and stripe rust pathogens that showed specialisation on different host species (Stakman and Piemeisel 1917). These races varied in their ability to infect different wheat varieties which later were found to carry distinct resistance genes.

Kingdom	Fungi
Phylum	Basidiomycota
Class	Pucciniomycetes
Subclass	Incertae sedis
Order	Pucciniales (Uredinales)
Family	Pucciniaceae
Genus	Puccinia
Species	P. graminis

Thus, by the beginning of the 20th century, it was generally accepted that the different rusts, also described as 'mildew' or 'blast' in some publications, were caused by distinctive fungal species with contrasting host ranges. The massive effort directed at cereal rusts and their control since the 1880s, both in terms of basic science and in practical efforts to curtail losses, led Large to observe that "the greatest single undertaking in the history of plant pathology was to be the attack on rust in cereals". In the mid of 20th century, yield losses reached 20 - 30% in eastern and central Europe (Zadoks 1963) and many other countries, including Australia, China and India (Rees 1972; Joshi and Palmer 1973; Roelfs 1977; Leonard and Szabo 2005). In the United States, from 1920 to 1960, stem rust epidemics in Minnesota, North Dakota and South Dakota caused average yield losses over 20% and up to 50% in 1935 (Leonard 2001b). Stem rust resistance genes were successfully deployed in commercial cultivars worldwide from the middle 1950s, effectively controlling the disease.

The fungus, *Basidiomycetes* of the order *Uredinales*, is heteroecious, alternating between a telial host in *Poaceae* and an aecial host in *Berberidaceae*, and macrocyclic, with five spore states that are distinct in morphology and function:

Telium (n + n): teliospores (n + n) after karyogamy (2n)

Basidium (2n): basidiospores (n)

Pycnium (Spermagonium): pycniospore (Spermatia) (n)

Aecium (n + n): aeciospores (n+n)

Uredium (n + n): uredospores (n + n)

Some of this spore stages infect one host while the others must infect and parasitize a different, alternate host (Agrios GN 2005).

Some macrocyclic rusts complete their life cycles on a single host and are called autoecious. Others, such as stem rust of cereals, require two different or alternate hosts for completion of their full life cycle and are called heteroecious.

Wheat is the primary host where the pathogen spends most time, and barberry is the secondary host. The fungus can reproduce itself only in living host plants. Crop species as primary hosts include bread wheat, durum wheat, barley, and triticale. There are a large number of species in *Berberis* and *Mahonia* that are susceptible to *P. graminis* (Roelfs 1985), but the common barberry, *B. vulgaris*, is considered to be the most important alternate host. Since common barberry has been largely removed after the 1920s, barberry bushes have not been a major source of disease inoculum (Kolmer et al. 2007), even if in recent years, barberry plants have reappeared in a few original eradication sites in south-eastern Minnesota (Peterson et al. 2005).

The symptoms appear as elliptical blisters or pustules, known as uredia, that develop parallel with the long axis of the stem, leaf or leaf sheat. Blisters

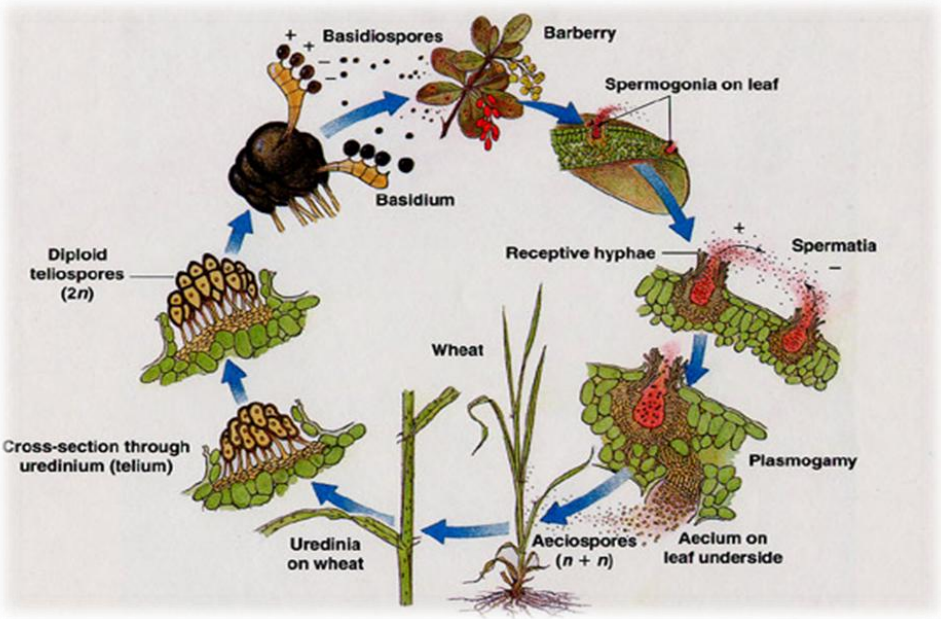


Figure 14: *Puccinia graminis* cycle

may also appear on the neck and glumes of the wheat spike. The epidermis covering the pustules is later ruptured irregularly and pushed back, revealing red-colored uredospores. Later in the season, as the plant approaches maturity, the pustules turn black as the fungus produces teliospores.

The disease cycle (fig. 14) starts with the exposure of the new wheat crop to stem rust inoculum. The source of the inoculum is different under different environments. In regions with warm climates, the volunteer plants carry the spores over summer and urediniospores from the infected volunteer wheat are the source of initial inoculum for wheat planted in the next fall to start a disease cycle (Leonard and Szabo 2005). In the regions with a cold winter the fungus overwinters as teliospores on infected wheat debris. Teliospores germinate in the spring and produce a basidium on which form four basidiospores. The basidiospores are ejected into the air and are carried by air currents for a few hundred meters. Basidiospores landing on young barberry leaves (fig. 15) germinate and penetrate the epidermal cells. After that, the mycelium grows mostly intercellularly. Within 3 or 4 days the mycelium develops into a spermagonium, which ruptures the epidermis, and its opening emerges on the surface of the plant tissue. Receptive hyphae from the spermagonium extend beyond the opening, and spermatia, embedded in a sticky liquid are exuded through the opening. Visiting insects become smeared with spermatia and carry them to other spermagonia. Spermatia may also be spread by rain-water or dew

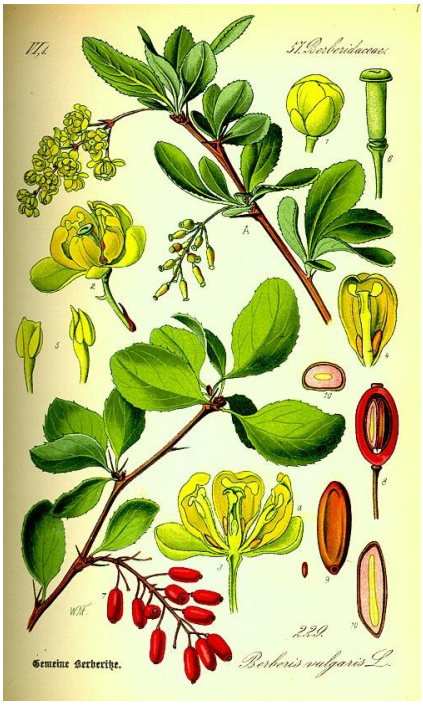


Figure 15: ***Berberis vulgaris***

running off the plant surface. When pycniospores (spermatia) are paired with receptive hyphae, cross-fertilization occurs successfully and dikaryotic mycelium forms. Then a cup-shaped dikaryotic aecium forms to release aeciospores. Normally aeciospores stalked in chains produced on barberry are transported by wind to start a new disease cycle in susceptible wheat cultivars (Roelfs 1985; Leonard and Szabo 2005).

In wheat, rust infection mainly occurs on stems and leaf sheaths. Within two weeks after inoculation, a brick-red structure, called a rust pustule also known as a uredium containing urediospores, appears at the point of inoculation. In heteroecious rusts, urediospores can reproduce themselves and re-infect wheat multiple times, which can lead to severe damage on wheat production. The uredospores are easily blown away by air currents, sometimes for many kilometers, even hundreds of kilometers , from the point of their origin. In a later developmental stage, another type of spore called teliospore, which is a black overwintering spore, is produced in telia to conclude the disease cycle of stem rust in wheat and to start a new life cycle in barberry. Teliospores do not germinate immediately and do not infect wheat. Teliospores also serve as the stage in which fusion of the two nuclei takes place and, after meiosis in the basidium, results in the production of new combinations of genetic characters of the fungus through genetic recombination.

The development of stem rust in wheat is favored by warm temperature (18-30°C) with high relative humidity. The symptoms of the disease are visualized as erumpent uredia pustules mainly on the stems and leaf sheaths about 7 to 10 days after infection (Leonard 2001a).

Rust-infected wheat plants show increased water loss because they transpire more and because more water evaporates through the ruptured epidermis. In addition, the fungus itself removes much of the nutrients and water that would normally be used by the plant. The respiration of infected plants increases rapidly during the development of the uredia, but subsequently

respiration drops to slightly below normal. Photosynthesis of diseased plants is reduced considerably due to the destruction of much of the photosynthetic area and to the interference of fungal secretions with the photosynthetic activity of the remaining green areas on the plant. The fungus also seems to interfere with normal root development and uptake of nutrients by the roots. All these effects reduce the normal number and size of seeds on the plant. The fungus also induced earlier maturity of the plant, resulting in decreased time available for the seed to fill. Heavy rust infections before or at the flowering stage of the plant are extremely damaging and may cause total yield loss, whereas if heavy infections occur later, the damage to yield is much smaller (Agrios GN 2005).

Several hundred races of the stem rust fungus are known to date, and new ones appear every year.

Pathogen race UG99 and resistance genes. Leonard and Szabo (2005) listed major losses to stem rust that occurred in the twentieth century, primarily before 1960, in Europe, China, Australia, and North America. In the United States, from 1920 to 1960, stem rust epidemics in Minnesota, North Dakota and South Dakota caused average yield losses over 20% and up to 50% in 1935 (Leonard 2001b). Stem rust resistance genes were successfully deployed in commercial cultivars worldwide from the middle 1950s, effectively controlling the disease.

By the 1990s, the incidence of stem rust had dramatically fallen, due in large part to the deployment of stem rust resistance (*Sr*) genes in cultivars (Singh et al. 2006). At present about 50 different stem resistance genes are catalogued and multiple alleles are known for three gene loci (tab. 12). Several of these genes were incorporated into wheat from alien wheat relatives. All designated genes, except *Sr2*, are race specific and are expressed in both seedling and adult plants.

Combination of *Sr2* with other unknown slow rusting resistance genes possibly originating from Thatcher and Chris, commonly known as the ‘‘*Sr2-Complex*’’, provided the foundation for durable resistance to stem rust in germplasm from the University of Minnesota in the United States, Sydney University in Australia, and the spring wheat germplasm developed by Dr. N. E. Borlaug (McIntosh 1988; Rajaram et al. 1988). Unfortunately, not much is known about the other genes in the *Sr2* complex and their interactions. The importance of stem rust declined worldwide with the deployment of various other alien resistance genes such as *Sr24, Sr26, Sr31* and more recently *Sr38*. One of the most important *Sr* genes was *Sr31*,

	Stem rust resistance (*Sr*) genes	
Origin of *Sr* genes	Ineffective	Effective
Triticum aestivum	*5, 6, 7a, 7b, 8a, 8b, 9a, 9b, 9f, 10, 15, 16, 18, 19, 20, 23, 30, 41, 42, Wld-1*	*28*[1], *29*[2], *Tmp*
Triticum turgidum	*9d, 9e, 9g, 11, 12, 17*	*2*[2], *13*[1,2], *14*[1]
Triticum monococcum	*21*	*22, 35*
Triticum timopheevi		*36*[1], *37*
Triticum speltoides		*32, 39*
Triticum tauschii		*33*[2], *45*
Triticum comosum	*34*	
Triticum ventricosum	*38*	
Triticum araraticum		*40*
Thinopyrum elongatum		*24*[1], *25, 26, 43*
Thinopyrum intermedium		*44*
Secale cereale	*31*	*27*[1], *1A.1R*

[1]Virulence for the gene is known to occur in other races.
[2]Level of resistance conferred in the field usually not enough.

Table 12: *Sr* genes and origin

which was deployed worldwide in many cultivars (Singh et al. 2006). In 1999, virulence to *Sr31* was detected in nurseries in Uganda (Pretorius et al. 2000) and the race was designated as Ug99. *Sr31*, a strong resistance gene derived from *Secale cereale* introduced to bread wheat through a 1B/1RS translocation, has been deployed in winter and spring wheat varieties in China, Europe, India, and USA (Zeller 1973; Zeller and Hsam 1983; McIntosh et al. 1995; Das et al. 2006).

Based on the North American stem rust nomenclature (tab. 13) system (Roelfs and Martens 1988), Wanyera et al. (2006) designated this virulence

type as TTKS. With the recent identification of new variants of TTKS, the original race Ug99 was redesignated as TTKSK after a fifth set of differentials was added to further expand the characterization (Jin et al. 2008b).

The most striking feature of race Ug99 is that it not only carries virulence to gene *Sr31* but also this unique virulence is present together with virulence to most of the genes of wheat origin, and virulence to gene *Sr38* (chromosome

		Infection types produced on near-isogenic Sr lines			
	Host set 1	*Sr5*	*Sr21*	*Sr9e*	*Sr7b*
	Host set 2	*Sr11*	*Sr6*	*Sr8a*	*Sr9g*
	Host set 3	*Sr36*	*Sr9b*	*Sr30*	*Sr17*
	Host set 4	*Sr9a*	*Sr9d*	*Sr10*	*SrTmp*
Pgt code[a]	Host set 5	*Sr24*	*Sr31*	*Sr38*	*SrMcN*
B		L	L	L	L
C		L	L	L	H
D		L	L	H	L
F		L	L	H	H
G		L	H	L	L
H		L	H	L	H
J		L	H	H	L
K		L	H	H	H
L		H	L	L	L
M		H	L	L	H
N		H	L	H	L
P		H	L	H	H
Q		H	H	L	L
R		H	H	L	H
S		H	H	H	L
T		H	H	H	H

[a]Adopted from Roelfs and Martens (1988); Jin et al. (2007)
L= low/resistant infection type (0 to 2+); H= high/susceptible infection type (3- to 4+)

Table 13: North American stem rust nomenclature (Jin et al. 2007)

segment from *T. ventricosum* carries stem rust resistance gene *Sr38* together with genes *Yr17* and *Lr37*) introduced into wheat from *Triticum ventricosum* that is present in several European and Australian cultivars and a small portion of new CIMMYT germplasm (Jin et al. 2007). Preliminary studies of inheritance of seedling resistance about *Sr7a* to Ug99 indicated that Ug99 resistance in Chris (cv including *Sr7a*) is controlled by two complementary recessive genes (Jin 2007), and the same seedling resistance is present in AC Barrie (a Canadian spring wheat cultivar), Thatcher, and Bonza 65 (a CIMMYT-derived cultivar). Singh and McIntosh (1987) indicated the

possibility that the adult plant resistance to *Sr7a*-avirulent Australian races may involve interaction of the moderately effective gene *Sr7a* and other unknown adult plant resistance genes. Seedling tests indicated that Ug99 is virulent on the *Sr7a*-tester line (Jin et al. 2007) although Chris did show seedling resistance. Singh and McIntosh (1987) indicated that resistance conferred by *Sr7a* is difficult to evaluate both in seedlings and adult plants when the gene is present alone. Therefore, at this stage we cannot determine the role *Sr7a* may have played in resistance of ‘‘Chris’’ observed in Kenya. Even though seedling tests indicate that *Sr23*, another gene whose expression is difficult to evaluate in seedlings and adult plants when present alone, may be ineffective against Ug99, adequate resistance in ‘‘Selkirk’’ may involve interactions of moderately effective genes *Sr2* and *Sr23* (linked to leaf rust resistance gene *Lr16*) and perhaps additional unknown adult plant resistance genes.

Race TTKSK is causing alarm for three reasons. First, this race has broad virulence to currently deployed *Sr* genes (Jin et al. 2007; Singh et al. 2006). Jin and Singh (2006) found that among North American cultivars, only 16% of hard red spring wheats, 48% of hard red winter wheats, and 28% of soft winter wheats had resistance to race TTKSK. Second, race TTKSK has continued to evolve. In 2006–2007, variants of TTKSK, designated TTKST and TTTSK (i.e., with added virulence to *Sr24* and *Sr36*, respectively) were detected in Kenya (Jin et al. 2008b; 2009). The third concern was the rapid movement of TTKSK from Africa. A predicted path for dispersal of TTKSK from eastern Africa to the Arabian Peninsula and ultimately to the Indian subcontinent, was proposed by Singh et al. (2006). Race TTKSK has closely followed this path, appearing in Yemen in 2006 (Jin et al. 2008a) and Iran in 2007 (Nazari et al. 2009). More recently, Ug99 has spread to major wheat production areas of the Middle East, such as Iran, Afghanistan, India, Pakistan, Turkmenistan, Uzbekistan and Kazakhstan (http://www.fao.org/newsroom/en/news/2008/1000805/). The estimated

annual losses due to race Ug99 reached to approximately USD $3 billions in Africa, the Middle East and South Asia (http://www.seedquest.com/News/releases/2007/january/18117.htm).

Urediniospores are produced in huge number during the crop season and they subsequently are transmitted onto the same or new host plants by wind dispersion. Typically, most spores will be deposited close to the source (Roelfs et al. 1984), however long-distance dispersal is well documented, with three principal modes of dispersal known to occur.

The first mode of dispersal is single event, extremely long-distance (typically cross-continent) dispersal (fig. 16) that results in pathogen colonization of new regions; dispersion of this type is rare under natural conditions. Several examples of long distance dispersal have been described by Brown and Hovmøller (2002).

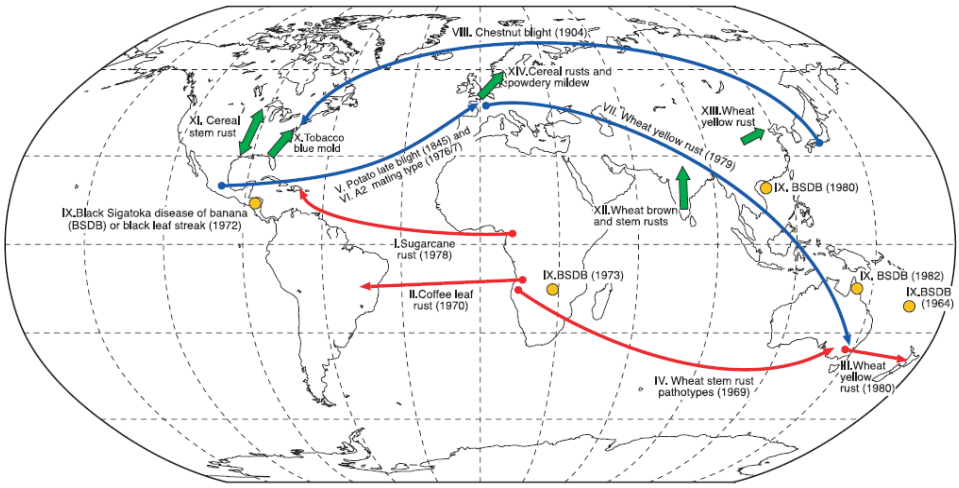


Figure 16: Selected dispersal events of fungal pathogens. Red and blue arrows indicate invasions of new territories (first year recorded in brackets). Red arrows indicate dispersal that probably occurred by direct movement of airborne spores. Blue arrows indicate pathogens that were probably transported to the new territory in infected plant material or by people and spread thereafter as airborne spores. Green arrows indicate periodic migrations of airborne spores in extinction-recolonization cycles. [Background world map © C. Lukinbeal, Southern Connecticut State University, New Haven, Connecticut] (Brown and Hovmøller 2002).

The second major mode of dispersal for pathogens like rusts is step-wise range expansion (fig. 17). This typically occurs over shorter distances, within country or region, and has a much higher probability than the first described dispersal mode. This probably represents the most common or normal mode of dispersal for rust pathogens.

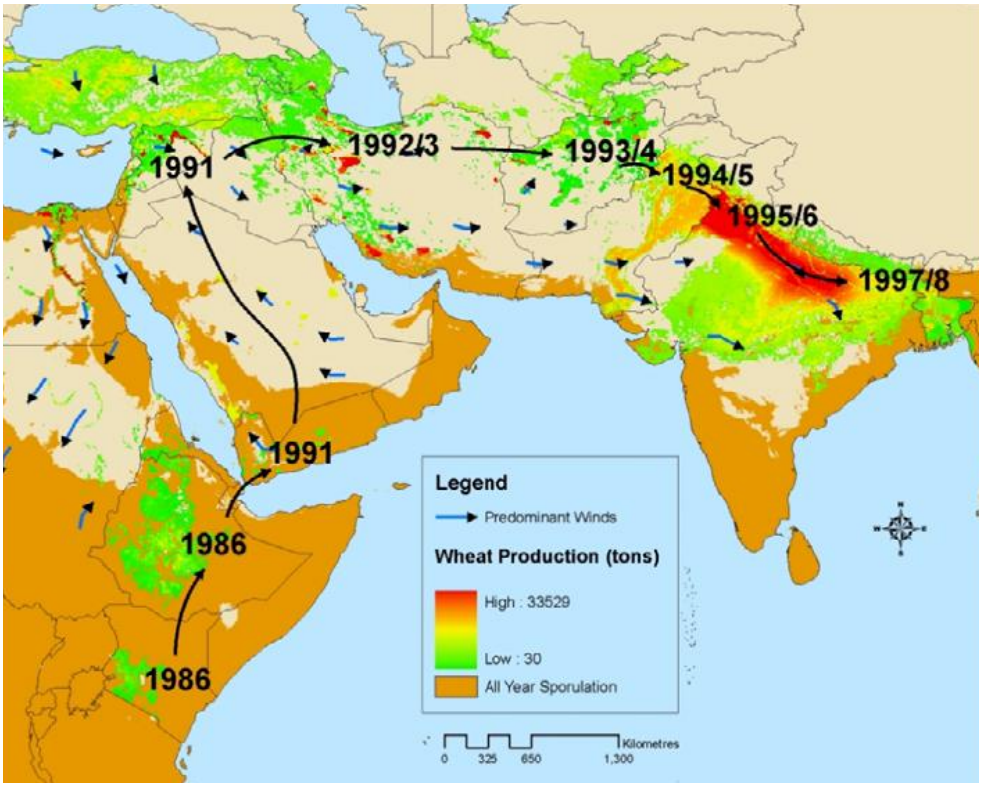


Figure 17: Example of step-wise (year-by-year) movement of a *Yr9*-virulent race of the yellow rust pathogen that evolved in East Africa and migrated to Asia (Singh et al. 2006).

The third mode of dispersal, extinction and re-colonization, could perhaps be considered a sub-mechanism of step-wise range expansion. This mechanism occurs in areas that have unsuitable conditions for year-round survival. Typically, these are temperate areas with inhospitable winter conditions or seasonal absence of host plants.

A good examples of this mechanism is the '*Puccinia pathways*' of North America–a concept that arose from the pioneering work of Stakman and Harrar (1957) in which rust pathogens over-winter in the southern USA or

Mexico and re-colonize wheat areas further north following the prevailing south–north winds as the wheat season progresses (fig. 18).

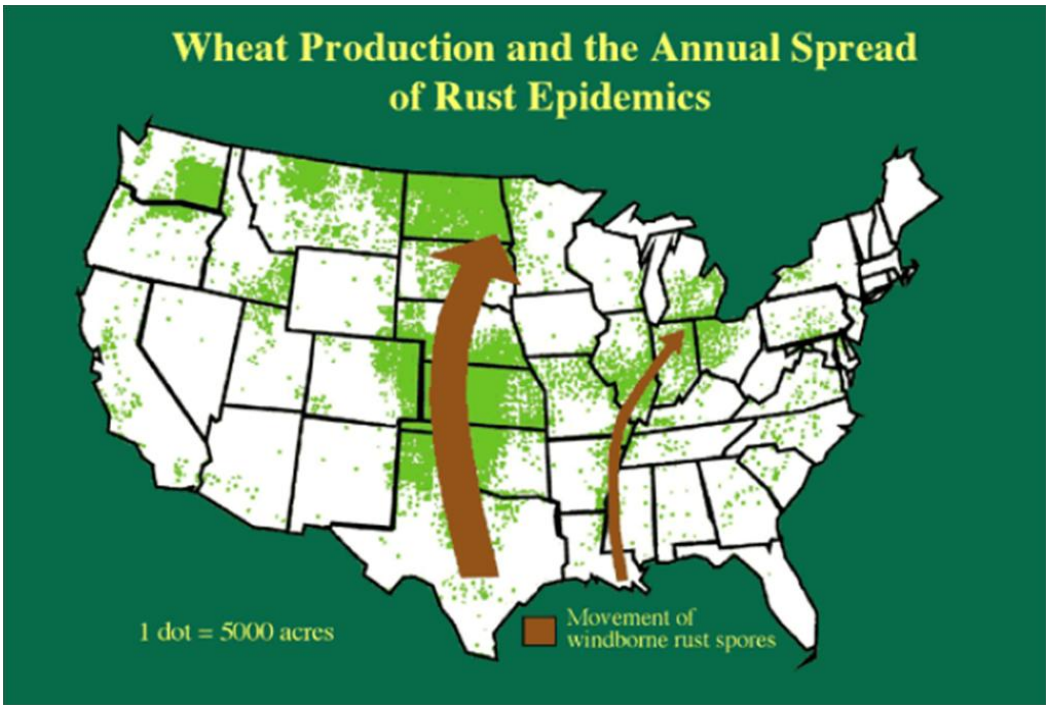
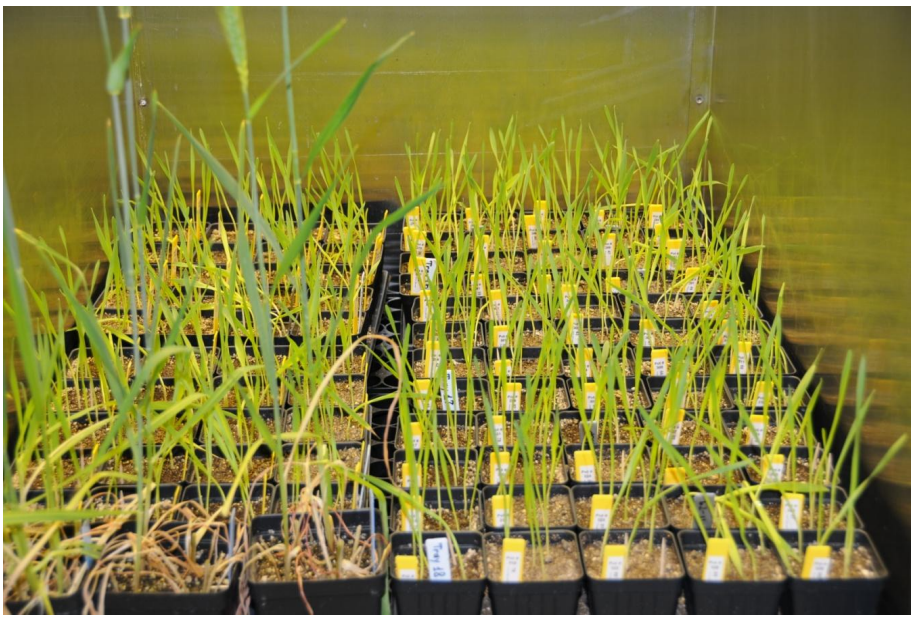


Figure 18: Rust re-colonization movements (Leonard KJ and Szabo LJ 2005)

Figure 19: in order i)barberry leaf with rust, ii) wheat kernels, iii) uredinia on wheat leaf, iv) stem rust on wheat

MATERIALS AND METHODS



Plant material. A collection of 240 genotypes mostly belonging to different subspecies of tetraploid wheat, as described in Table 14, were evaluated, at the Department of plant pathology of the University of Minnesota (USA) with prof. Brian Steffenson, for resistance to Ug99.

A population of 178 F8-F9 recombinant inbred lines (RILs) was developed at the CRA-CER cereal research center in Foggia (Italy) by single-seed decent from a cross between 2 durum wheat cultivars: Cirillo and Neodur.

Wheat genotypes gen. *Triticum*	**N° genotypes**
T. turgidum ssp. *turgidum* var. *durum*	128
T. turgidum ssp. *turgidum* var. *turanicum*	20
T. turgidum ssp. *turgidum* var. *polonicum*	20
T. turgidum ssp. turgidum var. *turgidum*	19
T. turgidum ssp. *carthlicum*	12
T. turgidum ssp. *dicoccum*	20
T. turgidum ssp. *dicoccoides*	17
T. aestivum	3
T. monococcum spp. Monococcum	1
Total	**240**

Table 14: Composition of the wheat collection evaluated in this study

Cultivar Cirillo originated from a cross between (Jucci x Polesine) x (Creso x Montanari), released in Italy in 1992. The cultivar Neodur derived from a cross between (184/7 x Valdur) x Edmore, released in France in 1987. The corresponding genetic map also was available.

Infection protocol for stem rust (*Puccinia graminis*). BL3 (bio-level 3) facility at the Department of plant pathology of the University of Minnesota (USA), capable of containing micro-organisms like bacteria and fungi, was

employed to carry out infection experiments with the stem rust TTKSK (Ug99) isolate.

Seeds were sown in pots containing 50:50 (Soil: metro Mix).

1. Inoculation was performed at the 1 leaf stage by using fresh rust spores (14 mg/capsule), previously controlled by a germination test on agar.
2. After inoculation, plants were placed in a mist chamber for 30 minutes with humidifiers running continuously, with the aim of creating a very fine layer of water micro-droplets, covering all surfaces of the plants, and then kept wet overnight for 16-18 hours at 20-22°C in the dark.
3. After this period, plants were allowed to dry in the light, removed from the mist chamber and transferred to greenhouse (MCC = 22°C – 25°C). Reading of infection was performed 12 days later.

TTKSK (Ug99) testing and phenotypic measurements. Seedlings belonging to the Cirillo x Neodur segregating population were rated for their infection type (IT) using a 0–4 scale (Stakman et al. 1962) (fig. 20); ITs 0, ;, 1, 2, or any combination of these indicated resistance, while ITs 3 or 4 indicated susceptibility. Observed segregation ratios (resistant vs. susceptible) were evaluated using χ^2 to test for the probability of their goodness of fit to various theoretical ratios. The same reading was for 240 genotypes.

In each test, 5 seedling plants were evaluated; the experiment was repeated twice, and only differential hosts that produced similar infection types in the two experiments were considered for the data analysis. When infection type 0 (immune reaction) occurred, the test was repeated to exclude the possibility of disease escape.

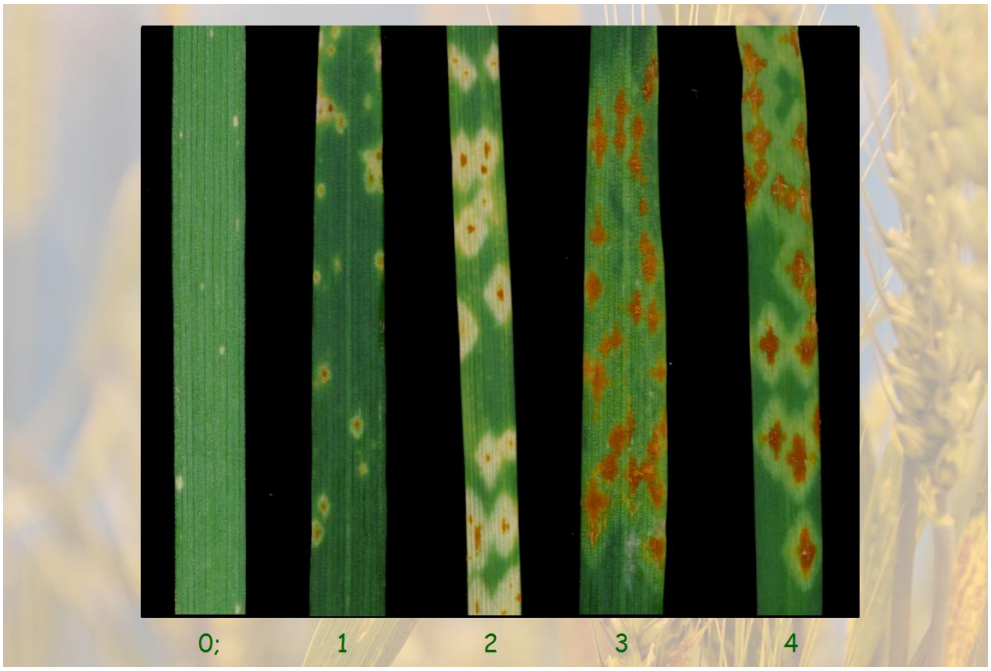
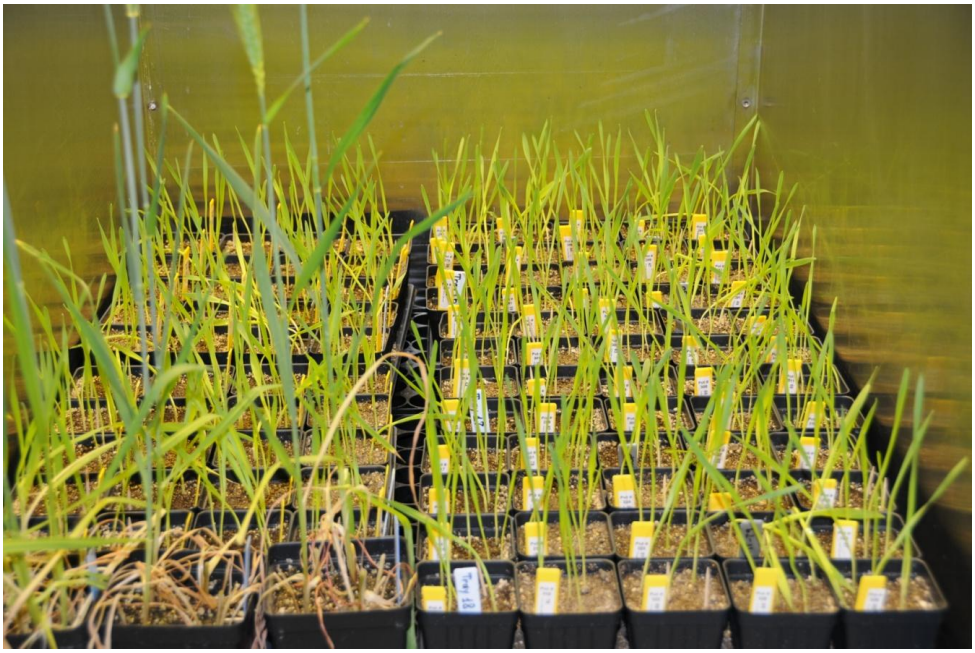


Figure 20: ITs using the system of Stakman et al. (1962)

RESULTS and DISCUSSION



Evaluation of biodiversity for resistance to Ug99 in the tetraploid wheat collection. A great variability was observed for resistance to Ug99 in the tetraploid wheat collection examined (table 15). In particular, if genotypes belonging to single subspecies were taken into consideration, the largest variability was observed for the group of the durum wheat commercial varieties (Fig. 21).

Therefore, even if wild genotypes represent a valuable source of new resistance genes for stem rust Ug99, it is still possibile to identify useful resistences within the genepool of cultivated elite cultivars of durum wheat.

Species Agrogen collection	resistant lines	susceptible lines
T. turgidum var. *durum*	38	90
T. turgidum var. *turanicum*	1	19
T. turgidum var. *polonicum*	3	17
T. turgidum var. *turgidum*	2	17
T. turgidum ssp. *carthlicum*	3	9
T. turgidum ssp. *dicoccum*	3	17
T. turgidum ssp. *dicoccoides*	0	16
T. aestivum spp. *Spelta*	0	1
T. monococcum spp. *monococcum*	1	0
T. aestivum	0	2

Table 15: Agrogen collection with resistant and susceptible lines.

Looking at results within the group of durum wheat commercial varieties, unfortunately, no differences were observed between the parents of the mapping population described in the first part of the thesis Ofanto and Cappelli, being both susceptible. This result actually made impossibile to utilize the Ofanto x Cappelli genetic map to identify the map position of a gene for resistance to Ug99. On the contrary, a clear difference was observed for two durum wheat cultivars, Cirillo (resistant) and Neodur (susceptible), which were parents of a segregating population previously developed,

together with the genetic map, at the CRA-CER. For this reason, the work continued by mapping the resistance gene of Cirillo.

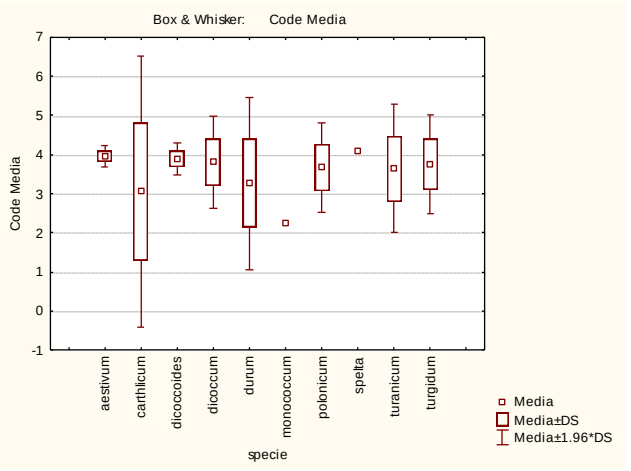


Figure 21: Representation of the variability observed for resistance to Ug99 within each subspecies taken into consideration.

TTKSK resistance gene mapping. Cirillo was characterized by an infection type (IT) ;1 while an IT 3/4 was observed for Neodur. As the evaluation of IT was carried out on the whole segregating population, each RIL could easily been classified as resistant or susceptible, showing IT completely similar to that of one parent or the other one. For this reason, the resistance to Ug99 of Cirillo was mapped as a single, mendelian gene. The ratio of phenotypic scores against race TTKSK (Ug99) agreed with single gene segregation in the population (χ^2 = 0.68, P<0.05). The segregation data were integrated into the first version of the Cirillo x Neodur linkage map, spanning 1013 cM and comprising 259 molecular markers (SSR and DArT) spread on 28 linkage group. The linkage analysis lead to the localization of the resistance gene in a linkage group assigned to the long arm of the chromosome 6A (fig. 22), 3 cM proximal to DArT marker wPt-8124, and 5.2 cM proximal to SSR marker Xdupw167, producing a 222 bp fragment in

Cirillo and a 240 bp fragment in Neodur, consisting in a clear polymorphism, very simple to score in progenies deriving from MAS programs.

Simons et al. (2010) mapped a gene of resistance to Ug99 on the distal region of the long arm of chromosome 6A, according to the previously reported location of *Sr13* (McIntosh 1972). *Sr13*, which was assigned to the long arm of chromosome 6A by cytogenetic methods (McIntosh 1972), was derived from *T. turgidum* var. *dicoccum* cv. Khapli C.I. 4013, and was later transferred to the common wheat line, Khapstein (McIntosh et al. 1995). *Sr13* is an important source of resistance to TTKSK (Ug99) race, but it is important to note that some virulent races are reported to *Sr13* in some countries (Huerta- Espino 1992; McIntosh et al. 1995). It has already been exploited in some Australian wheat cultivars as a source of resistance, but cultivars carrying this gene, in general, have low 1000 grain weight (McIntosh et al. 1995).

The durum wheat cultivar Cirillo showed an infection type ;1 for TTKSK when evaluated in this work, while the line carrying the gene *Sr13* identified by Simons et al. (2010) was characterized by an infection type 2/2+ against the Ug99 isolate. These results suggest that two different but very close loci could be positioned in the two works, or that two different alleles, with different properties in terms of resistance, were identified. Furthermore, Liu et al. (2010) mapped the *Sr26* gene in the same genomic region of chromosome 6AL identified by Simons et al. (2010) and in this work. The resistant parent of the segregating population utilized by Liu et al. (2010) showed an IT 1 like Cirillo, suggesting that Cirillo could have a common resistance determinant.

Sr26 is among the few major genes effective against the TTKS lineage that includes races TTKSK (Ug99 first race), TTKST and TTTKS (Sing et al. 2006; Jin et al. 2007).

Due to its high resistance and effectiveness against TTKSK, this gene in Cirillo x Neodur population, would be an excellent source of resistance alone or for pyramiding with other resistance genes to provide durable resistance against stem rust. The use of molecular markers will assist breeders in the efforts of combining multiple resistance genes during the breeding process. New and more closely linked markers will be identified on chromosome 6A by using the Cirillo x Neodur genetic map, in order to have more PCR-based markers to be utilized in MAS programs.

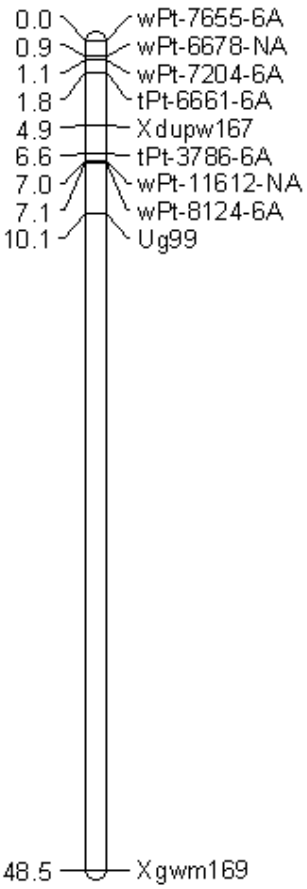


Figure 22: Chromosome 6A from CirilloxNeodur mapping population and mapping TTKSK (Ug99) resistance gene.

CONCLUSIONS

In this work, a detailed genetic dissection was carried out for different traits of agronomic value by using different durum wheat genetic materials. In particular, a gene for resistance to Ug99 stem rust was identified and located on the chromosome 6A on a segregating population already available at the CRA-Cereal Research Center of Foggia (Cirillo x Neodur). QTLs involved in the control of physiological traits related to water stress response and genomic regions controlling features of seed morphology were localized on a new genetic map developed in the frame of this thesis and based on the segregating population Ofanto x Cappelli, a modern and an old durum wheat variety respectively, which are contrasting for a number of morphological and physiological traits.

The intense breeding activities carried out during the last century for durum wheat led to the release of new varieties characterized by high yield potential. Although the improvement of yield potential remains one of the main targets of genetic improvement, great results in terms of plant productivity and quality can be obtained by filling the gap between potential and actual yield, and this means to improve, without penalizing quality, durum wheat for resistance to the most common environmental constraints: diseases and abiotic stresses.

With regard to the resistance to plant pathogens, simple and single-based resistance genes are relatively more easy to identify and transfer to elite cultivars by MAS programs, therefore, a huge amount of work has been carried out in order to study genetic determinants of resistance. Nevertheless, the co-evolution of the plant-pathogen system, a very good example of which is represented by the new strains of stem rust, requires a continuous search for new alleles and genes effective in resistance against pathogens. In fact, a good resistance level only can be reached by pyramiding more different resistance genes into the same genotype (Raj and Sanghamitra 2010). The identification of a new allele for resistance to Ug99 in the durum

wheat cultivar Cirillo can provide a new genetic tool useful for contrasting the diffusion of the new virulent strains of stem rust.

Much more difficult is the genetic improvement of durum wheat for complex and quantitative traits. In this work, a number of QTLs, some of which not previously identified for our knowledge, have been mapped throughout the genome, effective in controlling morpho-phenological and physiological traits of the plant related to the response to water stress, and morphological features of seeds related to grain yield capacity. Different QTLs for each trait were positioned on the Ofanto x Cappelli genetic map, each of them explaining up to 15-17% of the variability observed for the trait. Even if the breeding programs based on selection assisted by molecular markers have not been as successful for quantitative traits as for traits with a simple genetic bases, some examples have been reported in literature, in which grain yield capacity in water shortage conditions has been significantly improved in cereals by following 4-5 QTLs for a key trait, each explaining around 10% of the variability of the trait (Borrelli et al. 2009; Mastrangelo et al. 2011). For these reasons, works in which QTLs with a limited effect on the trait of interest are characterized, are useful for the genetic improvement of durum wheat, and segregating populations deriving from crosses between varieties contrasting for these traits are wellcome to expand the estate of QTLs and closely linked molecular markers to start MAS programs. At this pourpose, the Ofanto x Cappelli linkage map represents a very useful tool that has been used in this work to map QTLs for a number of traits, but that can be utilized in the near future to explore the genetic basis of many other traits of interest.

Finally, the use of the segregating populations utilized in this work allowed the identification of genes and QTLs directly in commercial and high-yielding (except for Cappelli) cultivars of durum wheat, that can be used to transfer desired alleles into more recent elite varieties minimizing the risck of introducing alleles with negative effects together with the alleles of

interest, a problem to take into account when wild genotypes are utilized as donors.

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