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Molecular markers validation to develop a Marker Assisted Selection programme in durum wheat (*T. turgidum* L. subsp. *durum* (Desf.) Husn.)

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If you aren't pushing limits,

you aren't going far enough

and you still don't know who you are.

Paulo Coelho

To (Virginia) [2]*,*

as well as Myself and my dear Grandmother.

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CONTENTS

ABSTRACT

Molecular markers validation to develop a Marker Assisted Selection programme in durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf) Husn.)

Recognizing the enormous potential of DNA markers in plant breeding, many agricultural research centers and plant breeding institutes have adopted the capacity for marker development and marker-assisted selection (MAS). DNA markers have enormous potential to improve the efficiency and precision of conventional plant breeding via marker-assisted selection. Progress has been made in mapping and tagging many agriculturally important genes with molecular markers which forms the foundation for MAS in crop plants.

Molecular markers have several advantages over the traditional phenotypic markers that were previously available to plant breeders. They offer great scope for improving the efficiency of conventional plant breeding by carrying out selection not directly on the trait of interest but on molecular markers linked to that trait. This, of course, would require a molecular marker to be tightly linked to the trait of interest. Besides, these markers are not environmentally regulated and are, therefore, unaffected by the conditions in which the plants are grown and are detectable in all stages of plant growth.

The use of marker-assisted selection for improving complex traits is one of the challenges facing wheat breeders. Wheat is one of the major food crops utilised worldwide. Modern cultivars of bread wheat (*Triticum* L. subsp. *aestivum*) and durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf) Husn.) are the result of extensive selection by breeders to meet the agronomic and quality requirements of the diverse environments under which they are cultivated and the wide range of products for which they are utilised.

In the Italian context, pasta is the worldwide appreciated end product made from durum wheat and improvement of end use quality in durum wheat is very essential. In the efforts on durum wheat improvement, we focused on developing new Italian varieties with improved diseases resistance and grain quality traits that affects the final product. Here, we present a practical validation of the use of MAS for durum wheat breeding, producing gene-pyramided lines via assembling markers linked to interesting agronomic traits as quality traits (lipoxygenase activity. protein content, yellow pigment content) and main disease host and non-host resistances (leaf rust, powdery mildew and

soil borne cereal mosaic virus and systemic acquired resistance) from multiple donor lines into a genetic background of Italian durum cultivars.

Keywords: Plant breeding, durum wheat, marker-assisted selection, gene pyramiding, TILLING, NPR1.

RIASSUNTO

Validazione di marcatori molecolari per lo sviluppo di un programma di *Marker Assisted Selection* in frumento duro (*Triticum turgidum* L. subsp. *durum* (Desf) Husn.)

Riconoscendo il potenziale di un programma di miglioramento genetico (*breeding*) basato sull'applicazione dell'analisi del DNA (marcatori molecolari), molti centri e istituti di ricerca si sono impegnati e si impegnano nell'attività di ricerca volta all'ottenimento di strumenti innovativi da applicare nell'attività di miglioramento genetico. E' iniziato così un approccio di ricerca che prevede l'applicazione dell'analisi del DNA (marcatori molecolari) per la selezione, con lo scopo di ottenere materiali innovativi attraverso programmi di selezione assistita da marcatori (MAS, *Marker Assisted Selection*). I marcatori molecolari hanno un'enorme potenzialità nel migliorare l'efficienza e la precisione del miglioramento genetico convenzionale attraverso la MAS. Sono stati compiuti progressi nella mappatura e identificazione di molti geni di interesse agronomico per i quali i marcatori molecolari identificati come associati, costituiscono il requisito fondamentale per sviluppare un programma di *breeding* assistito.

I marcatori molecolari costituiscono lo strumento ideale per un'identificazione varietale attendibile e costante nel tempo grazie ai numerosi vantaggi. L'utilità dei marcatori molecolari nel miglioramento genetico è basata essenzialmente sulla presenza di associazione fra marcatore e geni di interesse; tali associazioni permettono di seguire la segregazione del gene in questione saggiando la presenza del marcatore. Inoltre, sono indipendenti da fattori ambientali e dalle diverse fasi fenologiche della pianta.

La possibilità di applicare la MAS per migliorare caratteri bio-agronomici è una delle sfide che devono affrontare i *breeders* in una specie come il frumento. Il frumento è la coltura più estesamente coltivata nel mondo e di elevatissimo interesse alimentare. Varietà moderne di frumento tenero (*Triticum* L. subsp. *aestivum*) e frumento duro (*Triticum turgidum* L. subsp. *durum* (Desf) Husn.) rappresentano il risultato di una ampia attività di *breeding* svolta per soddisfare le caratteristiche agronomiche e qualitative riscontrate negli ambienti interessati dalla coltivazione e l'ampia gamma di prodotti per i quali esse vengono utilizzate.

Il frumento duro è una specie di elevatissimo interesse per l'Italia, soprattutto considerando i vertici mondiali per la produzione e il consumo di pasta che occupa il

nostro Paese, diventando quindi essenziale la capacità di migliorare la qualità del prodotto finale.

Nell'intento di avviare un programma di *breeding* per i principali caratteri agronomici del frumento duro, ci siamo concentrati sulla possibilità di sviluppare nuove varietà italiane con una migliore resistenza a malattie e migliori caratteristiche qualitative della granella che incidano sulle proprietà del prodotto finale. In questo studio presentiamo una valida applicazione dell'uso della MAS per il miglioramento genetico del frumento duro, attraverso la validazione e l'introgressione, partendo da linee donatrici multiple, di marcatori associati a caratteri di interesse agronomico come la qualità (attività lipossigenasica, contenuto proteico e accumulo di micronutrienti, contenuto di carotenoidi) e le principali resistenze a malattie ospite e non ospite specifiche (ruggine bruna, oidio, virus del mosaico dei cereali, resistenza sistemica acquisita), in un background genetico di cultivar italiane di frumento duro, secondo uno schema di *gene-pyramiding*.

Keywords: Miglioramento genetico, frumento duro, selezione assistita da marcatori, *gene-pyramiding*, TILLING, NPR1.

1. INTRODUCTION

Wheat is the major food crop in the world, grown in most countries except in the hot, humid tropical regions. Approximately 684 millions tons was the world production of wheat in the years 2008-2009 (http://www.fas.usda.gov/wap/current/toc.asp). Most of this production, around 90%, is represented by common or bread wheat *Triticum aestivum* L. subsp. *aestivum* ($2n = 6x = 42$, genome hexaploid AABBDD in which each subgenome has 7 chromosomes). Due to a wide range of intrinsic quality it is not surprising that a myriad of flour products are produced and consumed throughout the world.

Durum wheat, *Triticum turgidum* L. subsp. *durum* (Desf) Husn. ($2n = 4x = 28$, genome tetraploid AABB), accounts for about 10% of the total wheat production. Altough durum wheat as a class might be considered a minor crop relative to common wheat, the diets of millions of people in the Middle East and North Africa are based on durum wheat. In Western Europe and North America durum wheat is consumed primarily in the form of pasta products, while in the Near Est and North Africa it is consumed in various products as couscous and burghul (Matsuo, 1994). With a production, approximately, of 3.2 million tons per year (http://www.pasta-unafpa.org/ingstatistics5.htm), Italy is the first world producer of pasta from durum wheat and an intense breeding activity has been conducted over the last century to support the long tradition of pasta making (De Vita et al., 2007).

Losses due to diseases, pests, and environmental constraints each year strongly limit durum wheat production and quality, with respect to the potential 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 multienvironment testing for wide adaptation were applied also to this species. Accordingly, the genetic gain obtained after 1970 in grain yield (GY) of durum wheat is comparable to that 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).

The demands for increasing global crop production have prompted the development of new approaches relying on molecular marker technologies to investigate and improve the plant genome. It is expected that the use of molecular techniques will speed the development of improved varieties and enable creation of novel germplasm that cannot be obtained by classical approaches. However, isolation of important genes in wheat is a major challenge and a prerequisite for the exploitation of such molecular techniques (Kubalàkovà et al., 2005). The merits of molecular markers make them valuable tools in a range of research areas in wheat as the assessment of the genetic variation among plant individuals, accessions, populations and species, the determination of evolutionary relationships and genetic distances and the construction of genetic and physical maps to localize genes or genomic regions responsible for the expression of a trait of interest (Gupta et al., 1999). The general characteristics of the main generations of molecular markers and their applications in wheat have been extensively reviewed (Alexandrova et al., 1999; Gupta et al., 1999, 2001; Joshi et al., 1999; Langridge et al., 2001; Korzun and Ebmeyer, 2003; Feuillet and Keller, 2004; Mohler and Schwarz, 2004; Rakoczy-Trojanowska and Bolibok, 2004; Röder et al., 2004; Bonnett et al., 2005; Kuchel et al., 2005; Varshney et al., 2005a; Khlestkina and Salina, 2006; William et al., 2007).

Plant breeding, in its conventional form, is based on phenotypic selection of plants with traits of interest, with the final goal of assembling desirable combinations of genes in new varieties. These practices have been very effective in improving crop productivity during the past decades. However, conventional methods often encounter difficulties related principally to genotype x environment (G x E) interactions that can reduce the effectiveness of phenotypic selection and complicate the identification of superior genotypes. In addition, several phenotyping procedures are often expensive, time consuming or sometimes unreliable for particular traits (i.e. for some traits related to abiotic stress tolerance or disease resistance) (Ribaut and Hoisington, 1998; Gupta and Varshney, 2000; Francia et al., 2005; Collard and Mackill, 2008; Torres, 2010). As a consequence, the average length of a breeding program from hybridisation and selection of favourable genetic combinations to testing in the field and introduction into the market can vary from 10 to 15 years.

Marker-assisted selection (MAS) involves the use of genetic markers to follow regions of the genome that encode specific characteristics of a plant. For example, a marker genetically linked to a disease resistance locus can be used to predict the presence of the resistant or the susceptible allele. Breeding strategies including marker-assisted backcrossing, forward breeding, MAS involving doubled haploid technology and F_2 enrichment have been successfully utilized for this purpose (Varshney et al., 2005, 2006; Gale, 2005; Kuchel et al., 2005, 2007; Collard et al., 2005; Collard and Mackill, 2008; Gupta et al., 2010). However, for improvement of complex polygenic traits, newer technologies based on high throughput genotyping associated with new marker systems (e.g. DArT and SNP), and new selection strategies such as AB-QTL (advanced backcross quantitative trait locus), mapping-as-you-go, marker-assisted recurrent selection and genome-wide selection will have to be tried in future (Varshney et al., 2005; Heffner et al., 2009; Jannink et al., 2010; Gupta et al., 2010; Varshney and Dubey, 2009).

As marker-trait associations are now known for a number of simple traits, MAS has been found useful to improve several important economic traits (for biotic and abiotic stress resistance, and quality). In contrast to hexaploid wheat, little attention has been given to developing MAS schedules for durum wheat. The following section will present a general overview of the application of marker-trait associations in wheat breeding, and discusses the potential of considering the identified markers as tool to enhance the impact of MAS in the near future and specially as novel and successful application in Italian breeding programmes of durum wheat.

1.1 Molecular marker technologies

Table 1 shows the key features of common molecular marker technologies (Edwards and McCouch, 2007; Barr, 2009). The earlier types of molecular markers include anonymous or neutral markers based on hybridization, such as restriction fragment length polymorphisms (RFLPs) (Botstein et al., 1980; Siedler et al., 1994; Paull et al., 1998), later followed by markers based on the polymerase chain reaction (PCR; Mullis et al., 1986), a faster and less expensive technology. PCR-based DNA markers include random amplified polymorphic DNAs (RAPDs) (Williams et al., 1990; Welsh and McClelland, 1990; Devos and Gale, 1992; Khan et al., 2005a), amplified fragment length polymorphisms (AFLPs) (Vos et al., 1995; Law et al., 1998), cleaved amplified polymorphic sequences (CAPS; Konieczny and Ausubel, 1993), and sequence

characterized amplified regions (SCARs; Paran and Michelmore, 1993), which have been extensively used in different applications.

The main drawbacks of using RFLPs are the high cost and the low throughput of genotyping. RAPDs and AFLPs have also been widely used in genetic diversity studies and gene mapping. Both technologies are particularly useful when there is a need to assay loci across the entire genome. Nevertheless, their dominant nature, the lack of reproducibility of RAPDs compared with AFLPs and the lack of specificity in both cases, are limiting factors for their application in accurate MAS breeding approaches. However, random techniques such as RAPDs and AFLPs are highly useful for finding new markers linked to desirable alleles. Once such markers are identified, the corresponding bands can be sequenced and used to develop more specific and reliable markers such as CAPS or SCARs that simplify the screening of large progenies (Edwards and McCouch, 2007).

A significant development in PCR marker technology is evident when the DNA sequence is available and it is possible to design primers to amplify across a highly variable locus. These highly variable features include tandem repeats such as microsatellites or SSRs (Hearne et al., 1992; Plaschke et al., 1995; Donini et al., 1998; Stachel et al., 2000; Röder et al., 2002; Chebotar et al., 2003; Alamerew et al., 2004; Landjeva et al., 2006a, b; Orford et al., 2006), and dispersed complex repeats such as transposable elements (Queen et al., 2004). Microsatellites are relatively simple and cheap to use and have been employed for a multitude of genetic projects due to the highly reproducible and reliable identification of alleles. In recent years, the availability of whole genome sequences of a few selected crops and the sequence information generated by expressed sequence tags (ESTs) has also led to the development of a new generation of gene-targeted markers (also called candidate gene markers) and functional markers (Andersen and Lübberstedt, 2003; Bagge et al., 2007).

Gene-targeted markers and functional markers are often based on the discovery of single nucleotide polymorphisms (SNPs) between alleles. SNPs provide the most abundant source of sequence variants encountered in most genomes (Cho et al., 1999; Picoult-Newberg et al., 1999), and are often the only option for finding markers that are very close to or within a gene of interest. Their development costs are similar to those of SSRs, but there is a myriad of SNP assay technologies which constitute some of the most highly automated, efficient and relatively inexpensive genotyping methods (Edwards and Mogg, 2001; Somers et al., 2003; Henikoff and Comai, 2003; Kwok and Chen, 2003).

Importantly, new array based screening methods, such as DArT (Diversity Arrays Technology) appear to offer still cheaper assays due to their very high multiplexing capability. Diversity array technology is a modification of the AFLP procedure using a microarray platform (Jaccoud et al., 2001) that greatly increases throughput. In DArT, DNA fragments from one sample are arrayed and used to detect polymorphisms for the fragments in other samples by differential hybridization (Wenzl et al., 2004).

Each method analyses different aspects of DNA sequence variation and different regions of the genome. For example, RFLPs were detected using cDNA clones, namely coding sequence, but frequently detected variation that lay in regions flanking the genes. SSR markers have generally been developed on non-coding regions although recently the attention has moved to three-base repeats and the use of ESTs as the source of SSR markers. Other markers such as RAPD and AFLP markers appear to frequently target repetitive regions of the genome. The stability of the sequence difference may also be an issue in some cases. SSRs are seen as being too unstable for some applications since the mutation rate may in some cases be high. The decision about the most appropriate marker system to use will vary greatly depending on the species, the objective of the marker work and resources available (Edwards and McCouch, 2007).

1.2 Breeding with molecular markers

The wide range of markers currently available has dramatically increased our knowledge of the genetic diversity within many plant species, and has greatly facilitated mapping of genomic regions that contribute to trait variation.

Using the marker maps, putative genes affecting traits of interest have been detected by testing for statistical associations between marker variants and traits (Paterson et al., 1991). Following their identification, useful genes or quantitative trait loci (QTLs) can be introgressed into desirable genetic backgrounds via MAS, using markers physically located close to or even within genes of interest. The potential of MAS as a tool for crop improvement has been extensively explored (Tanksley et al., 1989; Ribaut et al., 2002; Servin et al., 2004). MAS offers promise for:

1. early screening of genotypes in the seedling stage, important for traits that are expressed late in the life cycle of the organism;
2. screening for rare recombinants between closely linked genes;
3. effective screening for traits that are extremely difficult, expensive or time consuming to score phenotypically;

a. indirect selection of desirable plants avoiding environmental, pleiotrophic or epistatic effects;

b. discriminating between homo- and heterozygous individuals in a single generation without the need for progeny testing;

c. monitoring single or multiple trait/QTL introgression in backcrossing programs (known as gene pyramiding).

A successful application of molecular markers to assist breeding procedures rely on several factors: (i) a genetic map with molecular markers linked to the major gene(s) or QTLs of agronomic interest; (ii) a tight association between the markers and the major gene(s) or the QTLs; (iii) adequate recombinations between the markers associated to the trait(s) of interest and the rest of the genome and (iv) the possibility of analyzing a large number of individuals in a time and cost effective manner. The success of MAS also depends on the localization of the marker with respect to the target gene. In a first case, the molecular marker can be located directly within the gene of interest. This kind of relationship is clearly the most favourable and in most cases requires the availability of the target gene cloned. In a second case, the marker is genetically associated to the trait of interest. In this case lower is the genetic distance between the marker and the gene and more reliable is the application of the marker in MAS because only in few cases the selected marker allele will be separated from the desired trait by a recombination event. In a third case, the target gene(s) can be represented by one or more QTLs. In this case genomic regions to be selected are often chromosome segments; it is therefore preferable either to have two polymorphic markers flanking the target QTL, and/or one or more markers within the QTL genomic region (Mohan et al., 1997; Gupta et al., 1999; Francia et al., 2005; Collard and Mackill, 2008; Torres, 2010).

Moreover several factors need to be considered when choosing traits, for which MAS is appropriate and desirable. MAS is particularly preferred for traits, which have low heritability (effect of environment), are recessive in nature, involve difficult and cost-prohibitive phenotyping, and require desired pyramiding of genes as in case of disease resistance (Gupta et al., 2009).

1.3 Next-generation sequencing technologies and their applications for breeding

Abstractly speaking, next generation sequencing (NGS) technologies enable the quick, inexpensive and comprehensive analysis of complex nucleic acid populations. In other words, they produce DNA sequence reads, and a lot of them. The production,

assembly and analysis of these sequence reads require different experimental approaches from sequencing library generation to new bioinformatics tools for post-sequencing procedures (Metzker, 2010; Brautigam and Gowik, 2010). There are currently four commercially available NGS technologies: 454 Life Sciences (acquired by Roche), Solexa (acquired by Illumina), ABI SOLID (acquired from Agencourt Biosciences), and Helicos Biosciences. Although all have their specific features, generally, they can be grouped into two classes based on the lengths of the sequence reads produced. Solexa, ABI SOLID, and Helicos all produce very short reads (about 30-100 bp) in very large quantities, while the 454 platform can produce a more moderate amount of sequence, but with longer read lengths (about 400-500 bp). Several of the platforms have already gone through multiple rounds of upgraded specifications, and improvements are likely to continue (Rounsley et al., 2009). NGS applications include resequencing reference genomes (Wheeler et al., 2008), *de novo* sequencing of small bacterial genomes (McCutcheon and Moran, 2007), assessing microbial diversity (Sogin et al., 2006), and gene expression, small RNA, and methylation analyses (Lister et al., 2008).

Currently, Roche/454, Solexa and AB SOLiD are the technologies predominantly used in crop genetics and breeding applications. NGS and high-throughput marker genotyping technologies are considered to have greater impact on plant genetics research and breeding programmes. The development of large-scale genomic resources, including transcript and sequence data, molecular markers and genetic and physical maps, is significant, in addition to other potential applications. Transcriptome and genome sequencing (both resequencing and *de novo*) using NGS technology is increasing for crop plants (Varshney et al., 2009). Although the initial aim of NGS technologies was resequencing, they are currently being used to explore *de novo* genome sequencing in several crop species, including wheat. The challenge of *de novo* sequencing with larger genomes is that assembly becomes difficult as repeat content increases, and many larger genomes, particularly those of crop plants, have significant repetitive content (Rounsley et al., 2009). It is possible now to mine large scale SNPs in major as well as under-resourced crop species and to undertake molecular breeding (Varshney et al., 2009). NGS technologies have been applied for identification of SNPs in several crops including maize (Barbazuk et al., 2007) and soybean (Hyten et al., 2008) as well as under resourced crops like chickpea (May et al., 2008).

Apart from developing new molecular markers, NGS technologies can be and are being used for other applications such as *de novo* sequencing, association mapping,

alien introgression, transcriptome expression and polymorphism, population genetics, evolutionary biology and genome-wide assembly in several crop species (Varshney and Dubey, 2009; Varshney et al., 2009).

The availability of large numbers of genetic markers developed through NGS technologies is facilitating trait mapping and making marker-assisted breeding more feasible. Metagenomics approaches and the sequencing of pooled amplicons generated for a large number of candidate genes across large populations offer possibilities to better understand population biology and to study genome-wide association genetics. Therefore on one hand, genome-wide sequence data should greatly facilitate our understanding of complex phenomena, such as heterosis and epigenetics, which have implications for crop genetics and breeding; on the other hand, these genomics data will also enable breeders to visualize which fragment of a chromosome is derived from which parent in the progeny line, thereby identifying clear crossover events occurring in every progeny line and placing markers on genetic and physical maps without ambiguity. Eventually, this will help in introducing specific chromosome regions from one cultivar to another.

NGS technologies will be particularly useful for developing and confirming introgression lines for a trait of interest. In addition to facilitating genomics-assisted breeding, NGS can also accelerate the development of transformation technologies for crops because it will become easier to modify genes with the increasing availability of genomic data. Although large-scale NGS data analysis remains a challenge at present, significant progress is being made in improving existing tools and in developing new approaches for this task (Varshney et al., 2009).

1.3.1 Wheat genome project

While rice and maize improvement is profiting already from information derived from their genome sequences, wheat has been lagging behind for the past decade. The wheat genome has always been viewed as impossible to sequence because of its large amount of repetitive sequences (>80%) and its size of 17 Gb (common wheat genome; 13Gb, durum wheat genome; Bennett and Leitch, 1995), which is five times larger than the human genome. The largest wheat chromosome (3B) alone is more than twice the size of the entire 370-Mb rice genome (Itoh et al., 2007), whereas the entire maize genome (2.6 Gb) is about the size of three wheat chromosomes. Further complicating the challenge, common wheat is a relatively recent hexaploid containing three homoeologous A, B, and D genomes of related progenitor species, meiotic

recombination is not distributed homogeneously along the chromosomes, and intervarietal polymorphism is very low (Paux et al., 2008).

Nevertheless, it became a target for genome sequencing thanks to the International Wheat Genome Sequencing Consortium (IWGSC, www.wheatgenome.org). To overcome the difficulties related to the size and complexity of the bread wheat genome, the IWGSC decided to develop a strategy based on (1) the isolation of individual chromosomes by laser-flow-cytometry and the construction of BAC libraries for each of the 21 wheat chromosomes, (2) the construction of physical maps anchored to genetic maps using these BAC libraries and (3) the sequencing of each chromosome. Scientific leadership from several countries is working to develop a physical map of each wheat chromosome. Physical maps are essential for high-quality sequence assembly regardless of the sequencing strategy used, such as bacterial artificial chromosome (BAC)–by–BAC or whole-genome shotgun strategies, and they will remain pivotal for *de novo* sequencing even with the advent of short-read technologies (Warren et al., 2006). In 2008, Paux et al. published the physical map of the chromosome 3B (1 Gb, i.e. 2.5 times the rice genome), led by C. Feuillet (INRA, France) and established the proof of concept for this chromosome-based approach. Italy is involved into sequencing of wheat genome developing a physical map of chromosome 5A, project led by M. Stanca (Agricultural Research Council), to develop a first draft of the 5A sequence and focusing on the functional analysis of 5A located genes involved in the determination of quality aspects, biotic and abiotic stress tolerance.

Genome sequencing is the foundation for understanding the molecular basis of phenotypic variation, accelerating breeding, and improving the exploitation of genetic diversity to develop new crop varieties with increased yield and improved resistance to biotic and abiotic stresses.

1.4 Agronomic traits pyramiding for breeding

After the discovery of interesting traits in specific donor lines, desirable genes or QTLs can be combined through crossing of these genotypes into a common genetic background and followed by MAS. Pyramiding is the process of combining several genes together into a single genotype, based on the idea of efficiently accumulating beneficial genes or QTLs using MAS (Ashikari and Matsuoka, 2006; Ye and Kevin, 2008; Joshi and Nayak, 2010; Francis et al., 2011).

Pyramiding may be possible through conventional breeding but it is usually not easy to identify the plants containing more than one gene. Using conventional

phenotypic selection, individual plants must be evaluated for all traits tested. Therefore, it may be very difficult to assess plants from certain population types (e.g. F_2) or for traits with destructive bioassays. DNA markers can greatly facilitate selection because DNA marker assays are non-destructive and markers for multiple specific genes can be tested using a single DNA sample without phenotyping (Collard and Mackill, 2008).

Recent exploitation of DNA markers of desirable trait genes facilitates construction of high-degree, gene-pyramided lines via assembling markers from multiple donor lines. In such a program, a plant that has all the target markers in a heterozygous state must be produced first (Ishii and Yonezawa, 2007). A number of markers that are known to be associated with QTL/genes for some major economic traits are being deployed for MAS in wheat breeding programs (Gupta et al., 2010).

DNA markers, once validated via appropriately designed experiments (Li et al., 2001; Zhou et al., 2003; Glover et al., 2004; Landi et al., 2005), could be effectively used for accumulating into single genotypes useful genes that have been detected separately in different plant lines (Liu et al., 2000; Singh et al., 2001; Datta et al., 2002; Castro et al., 2003; Jiang et al., 2004).

Multigene pyramided lines thus produced will be of high practical use as parents for new inbred as well as hybrid market cultivars. With a wide variety of gene-pyramided lines becoming available, it will become possible to breed superior market cultivars solely by marker-based selection without phenotypic test, just by assembling markers from a number of gene-pyramided stock lines, as planned by Bonnett et al. (2005) for wheat breeding.

Procedures in a marker-based gene accumulation program proceed in two steps. First, all target markers in the donor lines are assembled into the genome of a single plant in a heterozygous state, and second, a plant that has all the markers in a homozygous state is selected from among the progeny of the heterozygous plant produced in the first step (Fig. 1). Different parameters determine the efficiency of the two steps; the schedule (pattern and order) of crossing between donor lines is important in the first step, and the scheme of selection, in the second step (Ishii et Yonezawa, 2007).

The most widespread application for pyramiding has been for combining multiple disease resistance genes (i.e. combining qualitative resistance genes together into a single genotype). The motive for this has been the development of ‘durable’ or stable disease resistance since pathogens frequently overcome single gene host resistance over time due to the emergence of new plant pathogen races. Some evidence suggests that

the combination of multiple genes (effective against specific races of a pathogen) can provide durable resistance (Kloppers & Pretorius, 1997; Shanti et al., 2001; Singh et al., 2001). The ability of a pathogen to overcome two or more effective genes by mutation is considered much lower compared with the 'conquering' of resistance controlled by a single gene. In the past, it has been difficult to pyramid multiple resistance genes because they generally show the same phenotype, necessitating a progeny test to determine which plants possess more than one gene. With linked DNA markers, the number of resistance genes in any plant can be easily determined. The incorporation of quantitative resistance controlled by QTLs offers another promising strategy to develop durable disease resistance. Castro et al. (2003) referred to quantitative resistance as an insurance policy in case of the breakdown of qualitative resistance. Examples of successful use of marker-assisted gene pyramiding in wheat are showed in Table 2 (Gupta et al., 2009).

1.5 Target traits for MAS in durum wheat

MAS has shown to be effective for relatively simple traits that are controlled by a small number of genes, as resistance to disease controlled by major disease resistance (R) genes. Most of the traits of agronomic importance, such as yield, some classes of quantitative disease resistance, abiotic stress tolerance and quality traits, are indeed complex and regulated by several genes. Difficulties in manipulating these traits are derived from their genetic complexity, principally the number of genes involved, the interactions between genes (epistasis) and environment-dependent expression of genes.

In the present study, in order to develop an assisted-breeding program in durum wheat, we focused on quality traits including lipoxygenase activity, protein content and yellow pigment content, and on main disease resistances distinguishing between the host resistance (leaf rust, powdery mildew and soil borne cereal mosaic virus) mediated by the products of plant resistance (R) genes which establish pathogen race- or cultivar-specific resistance, and the broad-spectrum nature of non-host resistance (systemic acquired resistance) which closely parallels that exhibited by the innate immune system of animals.

Quality traits

1.5.1 Yellow pigment content

One of the primary quality traits targeted by durum wheat breeding programs is the bright yellow colour of semolina and pasta products as this trait becomes

increasingly important in global markets (Dexter and Marchylo, 2000). The degree of yellowness is influenced by several factors, including the presence of carotenoid pigments (Hentschel et al., 2002; Panfili et al., 2004), semolina extraction rate (Matsuo and Dexter, 1980), processing conditions (Borrelli et al., 1999), and oxidative degradation by lipoxygenases (Manna et al., 1998; Borrelli et al., 1999). Yellow pigment content (YPC) is a trait affected by environment (Miskelly, 1984) and in durum, it is largely controlled by additive gene effects and is highly heritable (Johnston et al., 1983; Elouafi et al., 2001; Clarke et al., 2006).

QTLs for endosperm colour have been mapped to at least seven chromosomes. Major genes exist on the group 2 chromosomes (Joppa and Williams, 1988) with minor effect QTLs being reported on chromosomes 4A and 5A (Hessler et al., 2002) and on 3BS (Mares and Campbell, 2001). However, chromosomes of the group 7 appear to contain genes most critical to yellow colour.

In durum wheat, major QTLs for YP content were found on chromosomes 7A and 7B (Elouafi et al., 2001; Pozniak et al., 2007). QTLs for YP content were also detected on homoeologous group 1 chromosomes (Ma et al., 1999), chromosomes 4A and 5A (Hessler et al., 2002), 1B and 6A (Zhang et al., 2008), and 2A, 4B and 6B (Pozniak et al., 2007), indicating multigenic control of YP content in wheat grain in addition to the major genes on homoeologous group 7 chromosomes.

Carotenoids are the main components of flour yellow pigment (Miskelly, 1984), with lutein being the most abundant type, followed by zeaxanthin and β-cryptoxanthin, which contribute to both pasta quality and nutritional value (Hentschel et al., 2002; Adom et al., 2003; Panfili et al., 2004). The biosynthetic pathway of lutein, zeaxanthin and β-cryptoxanthin involves more than ten enzymatic steps (Hirschberg, 2001), among which the step catalyzed by phytoene synthase (Psy), dimerizing two geranylgeranyl pyrophosphate molecules, is assumed to be ratelimiting in the process (Lindgren et al., 2003). In the grass family, duplicated PSY genes were identified (Gallagher et al., 2004) and designated as *Psy1* and *Psy2*, respectively. In maize (*Zea mays* L.), Palaisa et al. (2003) and Gallagher et al. (2004) demonstrated that *Psy1*, but not *Psy2*, exhibited a strong association with YP content of endosperm. Pozniak et al. (2007) localized the durum wheat *Psy1* and *Psy2* genes to homoeologous group 7 and 5 chromosomes, respectively, and demonstrated that *Psy1*, rather than *Psy2*, was associated with grain YP content. A similar conclusion was made by Zhang and Dubcovsky (2008). These reports led to a conclusion that the genes responsible for the QTLs detected on chromosomes 7A and 7B were orthologues of *Psy1*.

Psy1 gene on chromosome 7A was cloned and designated *Psy-A1*, and two allelic variants, *Psy-A1a* and *Psy-A1b*, were detected in Chinese winter wheat cultivars (He et al. 2008); subsequently, Zhang et al. (2009) demonstrated a significant influence of *Psy-A1* on flour YP content. Moreover, He et al. (2009) identified allelic variants at the *Psy-B1* locus, developing functional markers for the different alleles, and determining the associations of the allelic variants with grain YP content, in common wheat cultivars and advanced lines.

1.5.2 Lipoxygenase activity

Lipoxygenases (LOX, linoleate:oxygen oxidoreductase, EC 1.13.11.12) are non-haem iron-containing dioxygenases presents in both plants and animal kingdom. These enzymes catalyze the region and stereo-specific insertion of molecular oxygen into polyunsatured lipids containing a *cis*,*cis*-1,4-pentadiene system such as linoleic, linolenic, and arachidonic acids to yield conjugated hydroperoxide products (Porta et al., 2002). During substrate peroxidation radical forms are produced and they might led to the degradation of many molecules as well as carotenoid pigments, the main components of flour yellow pigment that is an important criterion for assessment of the commercial and nutritional quality of the durum wheat end-products (Borrelli et al., 2006; Leenhardt et al., 2006a).

Since LOX enzymes have been identified as important elements affecting the technological properties and the nutritional values of the cereal and products, many studies have been dedicated to the analysis of this enzyme family in rice (Wang et al., 2008), barley (Holtman et al., 1996), wheat (Leenhardt et al., 2006a; McDonald et al., 1979; Hsieh and McDonald, 1984; Pastore et al., 2000; Borrelli et al., 2006) and maize (Cho et al., 2007). Various plant LOX isoforms were found within a single tissue, each with distinct stereo-specificity and substrate preferences, kinetic parameters, pH profile and subcellular localization suggesting common and/or specific functions for each isoform (Saravitz and Siedow, 1996). Multiple isoforms of seed expressed LOXs have been identified in cereal species. In barley, three LOX cDNAs (*LoxA*, *LoxB* and *LoxC*) have been isolated in germinating grains.

The wheat genes encoding the lipoxygenases, named *Lpx* have been assigned to wheat chromosomes 4 (*Lpx-1 and Lpx-3*) and 5 (*Lpx-2*) (Hart and Langstom, 1977; Li et al., 1999). In particular, the *Lpx-1* and *Lpx-3 loci* map to colinear regions on chromosomes from homoeologous group 4 (Garbus et al., 2009). On the basis of sequence similarity the wheat *Lpx-1* has been associated to barley *LoxA* gene (Hessler et

al., 2002), while *Lpx-2* and *Lpx-3* loci correspond to the barley *LoxC* and *LoxB,* respectively. Carrera et al. (2007) found in the durum wheat cv. Kofa two different genes corresponding to barley *LoxA* (*Lpx-B1.1* and *Lpx-B1.2*) derived from a duplication of the *Lpx-B1* locus and identified a molecular marker able to highlight a deletion at the *Lpx-B1.1* locus (mapped on 4BS chromosome of durum wheat) associated to a reduction in lipoxygenase (LPX) activity in durum wheat varieties.

Recently, Verlotta et al. (2010) analysed the role of *Lpx-1* in the determination of LOX activity in mature durum wheat grains, performing an in-depth characterization of five genes and alleles at the *Lpx-B1* locus present in a germplasm collection. The full-length sequences of the *Lpx-B1* genes/alleles were isolated and characterized. On the basis of sequence polymorphisms and map positions, a new gene designated *Lpx-B1.3* was identified in addition to the already known *Lpx-B1.1* and *Lpx-B1.2* genes, and three different alleles at the *Lpx-B1.1* locus were distinguished. According to the distribution of the *Lpx-B1* genes/alleles in the germplasm collection, three distinct groups were identified that correspond to three different haplotypes and are characterized by different *Lpx-B1* expression profiles and LOX activity in mature grains.

1.5.3 Grain protein content

Grain protein content (GPC) of wheat is important for improved nutritional value and is also one of the major factors affecting breadmaking and pasta quality (Dick and Youngs, 1988; Finney et al., 1987). In spite of its importance, progress in breeding for high GPC has been slow and difficult. The first limitation is that genetic variation for protein content is small compared with variation due to differences in growing environments. The second limitation is that there is a strong negative correlation between GPC and grain yield; cultivars with high GPC tend to be low yielders.

As a result of a well documented negative correlation between grain yield (GY) and GPC (Blanco et al., 2002; Feil, 1992; Gonzalez-Hernandez et al., 2004; Groos et al., 2003; Kibite and Evans, 1984; Levy and Feldman, 1987; Mesfin et al., 2000; Simmonds, 1995), selection for increased GY has probably countered gains in GPC during the past decades. Studies comparing wheat cultivars of different release periods have shown that modern cultivars have reduced GPC compared to older cultivars (Austin et al., 1980; Slafer et al., 1990; Fufa et al., 2005). The improvement of GPC in modern wheat cultivars without associated penalties on grain yield will require higher N use efficiency (NUE) by increasing either N uptake or remobilization. In fact there are exceptional genotypes that combine excellent yield potential and high GPC, probably

by a more efficient relocation of nitrogen from senescing tissues to grain, or by a more efficient uptake of nitrate and ammonia from the soil (Blackman and Payne, 1987).

An additional constraint to GPC improvement is the limited range of genetic variation controlling protein quantity in modern wheat cultivars (Blanco and De Giovanni, 1995). Gene introgressions from wild relatives into cultivated genotypes have expanded the genetic diversity for this trait providing new alternatives to increase GPC. Wild emmer wheat, *Triticum turgidum* ssp. *dicoccoides* (DIC hereafter), is a valuable source of genetic variation in GPC, since some accessions exhibit much higher GPC than most of the commercial wheat cultivars (Avivi, 1978). A good example of the contribution of DIC to the improvement of GPC in commercial wheat varieties is the *Gpc-B1* gene which consistently contributed to increase GPC (on average 14 g kg^{-1}) in both tetraploid and hexaploid wheat (Mesfin et al., 1999; Chee et al., 2001) as well as across diverse environments (Joppa et al., 1997; Olmos et al., 2003). Joppa and Cantrell (1990) developed substitution lines of the DIC chromosomes in the cultivar 'Langdon' (LDN) and showed that a locus for high GPC was present on chromosome 6BS. Using isogenic recombinant lines and a large number of replications, Olmos et al. (2003) mapped this QTL as a single Mendelian locus within a 2.7 cM region. A more precise map was later produced by Distelfeld et al. (2004) using wheat–rice micro-colinearity, which narrowed the *Gpc-B1* region to a 0.3 cM interval.

During field experiments aimed to map the *Gpc-B1* gene, differences in senescence among the tetraploid RILs segregating for the *Gpc-B1* locus were observed. This was an important observation because senescence, the programmed degradation of cell constituents, makes nutrients available for remobilization to developing seeds (Mae, 2004; Waters et al., 2009) and therefore can have a significant impact on GPC. Previous work by Kade et al. (2005) showed increased levels of soluble proteins and amino acids in flag leaves at anthesis and increased efficiency in N remobilization in lines carrying the DIC *Gpc-B1* allele. These results suggested that the effect of this locus on GPC could be a pleiotropic effect of the observed differences in senescence. The positional cloning of *Gpc-B1* revealed that this gene code for a NAC (domain present in NAM, ATAF and CUC genes) transcription factor designated NAM1 that is closely related to a group of three related *Arabidopsis* proteins including the No Apical Meristem (NAM) protein (Uauy et al., 2006b; Brevis et al., 2010). Wild tetraploid wheat has a functional *Gpc-B1* allele, whereas commercial tetraploid and hexaploid wheat cultivars analyzed by Uauy et al. (2006b) showed a non-functional copy of the gene as a result of a frame-shift mutation or a deletion at this locus. The DIC allele accelerated senescence and

increased protein, zinc and iron concentration in the grain compared to the non-functional allele (Uauy et al., 2006a, b; Brevis and Dubcovsky, 2010). These effects make the DIC *Gpc-B1* introgression an interesting source to improve the nutritional value and the quality properties of the wheat grain, increasing grain mineral concentrations for biofortification of food (Waters et al., 2009).

Comparisons between near isogenic lines (NILs) with different *Gpc-B1* alleles in tetraploid and hexaploid wheat have been recently used to show that the functional *Gpc-B1* allele is associated with increases in both protein concentration and total protein yield. The increased N accumulation in the grain was paralleled by a decrease of the residual N in the straw, suggesting a more efficient N remobilization (Brevis and Dubcovsky, 2010; Waters et al., 2009). The same set of NILs was used by Brevis et al. (2010) to investigate the effect of the functional *Gpc-B1* allele on the major milling, bread-baking and pasta-making quality traits used for quality characterization of common and durum wheat. This set of NILs included varieties with contrasting levels of GPC and high-molecular weight glutenin subunit composition and therefore, were particularly valuable to test the effect of the *Gpc-B1* alleles on quality in different genetic backgrounds. The presence of the *Gpc-B1* introgression was associated with a consistent increase on GPC across genotypes and environments, and with a positive effect on several bread-baking and pasta-making quality parameters. The full pasta analyses included in their study showed that the *Gpc-B1* introgression was also associated with additional benefits in spaghetti firmness and cooking loss, two critical traits for pasta quality.

The accelerated maturity associated with the functional *Gpc-B1* allele can shorten the grain filling period and result in potential grain yield penalties in certain genotype-environment combinations. Some preliminary studies have analyzed the effect of the functional *Gpc-B1* allele on yield components using single or two-row plots (Blanco et al., 2002; Chee et al., 2001; Joppa et al., 1997). However, the interpretation of these results could be affected by the fact that the stripe rust (*Puccinia striiformis* Westend. f. sp. *tritici* Erikson) resistance gene *Yr36* is tightly linked to the functional *Gpc-B1* allele (Uauy et al., 2005; Fu et al. 2009; Brevis and Dubcovsky, 2010).

Host disease resistance

1.5.4 Powdery mildew resistance

Powdery mildew, caused by *Blumeria graminis* (DC) Speer f. sp. *tritici* Em. Marchal (syn. *Erysiphe graminis* f. sp. *tritici*), is one of the most destructive foliar

diseases in temperate climates and usually leads to 5 to 34% yield losses (Conner et al., 2003). The use of resistant cultivars has proven to be an effective and environmentally safe strategy for controlling wheat pathogens and eliminating the use of fungicides. However, since several wheat resistance genes tend to become ineffective within a short period due to frequent changes in the pathogen population, it is necessary to search for new sources of resistance and to use available genes in combinations that will provide effective and more durable resistance.

Up to now, more than 55 powdery mildew resistant alleles designated at 39 loci (*Pm1-39*) on wheat chromosomes (Huang and Röder, 2004; Miranda et al., 2007; Lillemo et al., 2008; He et al., 2009) have been described. Some of the genes were transferred from wild relatives of wheat, such as *T. turgidum* var. *dicoccoides* and var. *dicoccum*, *T. timopheevii*, *T. monococcum*, *Ae. squarrosa*, *Ae. speltoides*, *Ae. longissima*, *Ae. ovata*, or from more distant species, like *Secale cereale* and *Dasypyrum villosum* (see review by Huang and Röder, 2004).

The wild emmer wheat progenitor of tetraploid and hexaploid wheats shows particular promise as a donor of useful genetic variation for several traits, including disease resistances, drought tolerance, yield components, protein quality and quantity (Feldman and Millet, 1993). A number of accessions of emmer wheat was found to carry resistance to several pathogens, including powdery mildew, stripe rust, leaf rust and stem rust (Dinoor et al., 1991). The powdery mildew resistance genes *Pm16*, *Pm26*, *Pm30* and *Pm31*, located on chromosomes 4A, 2B, 5B and 6A, respectively, were transferred from var. *dicoccoides* to cultivated wheats (Reader and Miller, 1991; Rong et al., 2000; Liu et al., 2002; Xie et al., 2003).

Though several tens of wheat powdery mildew *R* genes have been genetically studied, only *Pm3b* was molecularly cloned (Yahiaoui et al., 2004). Besides R genes, some genes of other types possibly associated with powdery mildew resistance reactions were also isolated in wheat. However, the molecular studies on wheat powdery mildew resistance are limited and scattered. Niu and He (2009) provided an overall review of the related studies.

A novel powdery mildew resistance gene designated *Pm36* (Blanco et al., 2008), was introgressed from *Triticum turgidum* var. *dicoccoides* into durum wheat. In order to investigate the inheritance of the powdery mildew resistance derived from var. *dicoccoides*, a segregating population was developed by crossing a resistant backcross inbred line (5BIL-42) with the recurrent susceptible parent cv. Latino of durum wheat. The segregation pattern supported the hypothesis that the resistance was controlled by a

single, dominant gene mapped on chromosome arm 5BL, and an EST–SSR marker (BJ261635) tightly linked to *Pm36* was identified. Molecular markers tightly linked to genes of interest can be used in breeding programs to facilitate selection and as starting point for the map-based cloning of such genes. Marker-assisted selection (MAS) would be particularly effective to develop stable resistance to powdery mildew in wheat, where simultaneous or even sequential screening of plants with several pathogen isolates is difficult or impractical.

1.5.5 Leaf rust resistance

Leaf rust (*Puccinia triticina* Eriks.) is one of the most damaging foliar pathogens of wheat. Leaf rust infections may cause up to 50% yield losses, mainly associated with a reduction in biomass, harvest index, and kernels per square meter (Herrera-Foessel et al., 2006). Sources of genetic resistance are valuable to increase the sustainability of cereal production, from both economic and environmental standpoints (Reynolds and Borlaug, 2006).

To date, more than 50 leaf rust resistance genes that originate from *Triticum* species have been characterized (http://genes.pp.ksu.edu/Main/docs.htm?docid=9915; McIntosh et al., 2004). Most of these genes belong to the race-specific gene class where the incompatible interaction is controlled by a relatively simple gene-for-gene recognition pattern (hypersensitive resistance). As a consequence, single *R*-genes are easily overcome by rapidly changing *Puccinia triticina* populations with the spread of new virulent pathotypes (Kolmer et al., 2007). Obtaining cultivars with durable resistance is a major target for wheat geneticists, pathologists and breeders. For this purpose, two approaches have been suggested: (1) pyramiding more than two *Lr* genes, mainly through marker-assisted selection (Chelkowski and Stepien, 2001), and (2) pursuing the genetic characterisation and mapping of durable resistance.

Many leaf rust resistance genes have been mapped in wheat during the past decade by means of linkage mapping using molecular markers and recombinant inbred populations (http://www.cdl.umn.edu/res_gene/wlr.html). Only few were fine-mapped to more specific genetic locations. *Lr21* (Huang et al., 2003), *Lr10* (Feuillet et al., 2003) and *Lr1* (Cloutier et al., 2007) are three leaf rust resistance genes recently cloned in bread wheat, encoding typical resistance proteins containing coiled coil (CC), nucleotide-binding-site (NBS), and leucine-rich-repeat (LRR) motifs. Despite the great advances in the characterization of leaf rust resistance loci in bread wheat, limited progress has been achieved in the identification of the durum wheat corresponding

genes. Some evidences suggest that leaf rust resistance in durum wheat is based on different genetic determinants compared to the *Lr* genes known in bread wheat (Zhang and Knott, 1990; 1993; Martìnez et al., 2007).

Some strategies have been adopted to extend the effectiveness of the resistance, as growing cultivar mixtures or pyramiding different *Lr* genes into the same genotype (Kolmer and Liu, 2001; McDonald and Linde, 2002). A more desirable alternative is based on the utilization of sources of resistance that are intrinsically more likely to last longer.

The Italian durum wheat cultivar Creso, released in 1974, was obtained by crossing a CIMMYT's advanced line with a semi-dwarf Cappelli mutant (Cp B14). Due to its positive characteristics for yield potential, gluten quality and leaf rust resistance, Creso has been largely used in breeding programs throughout the Mediterranean Basin (Scarascia Mugnozza, 2005; De Vita et al., 2007). Resistance to leaf rust in Creso under field conditions has remained effective since 1975 in cultivation environments characterised by recurrent leaf rust epidemics (Pasquini and Casulli, 1993; Martinez et al., 2007), thus fulfilling the basic requirement for being considered as a durable resistance, according to the definition provided by Johnson (1984). There are evidences that this durability is based on a combination of hypersensitive and non-hypersensitive resistance (Martìnez and Rubiales, 2002).

Marone et al. (2009) identified a major QTL, coincident with QTL *QLr.ubo-7B.2* detected in a durum wheat background derivative of Creso (cv. Colosseo) by Maccaferri et al. (2008), on 7BL chromosome in durum wheat, underling the possibility to use the Italian durum wheat cultivar Creso like donor parent of resistance to *P. triticina*. They named this allele *Lr14c* in order to distinguish it from *Lr14a* and *Lr14b* alleles previously identified (Herrera-Foessel et al., 2005; Oelke and Kolmer, 2005; Ordoñez and Kolmer, 2007a, b).

1.5.6 Soil borne cereal mosaic virus (SBCMV) resistance

Soil-borne cereal mosaic virus (SBCMV), a Furovirus transmitted by *Polymyxa graminis* Led., is responsible for an important disease of wheat (Koenig and Huth, 2000) and it is widespread in the main wheat growing areas of the world. Although most of the durum wheat cultivars grown in Italy and in the Mediterranean region are characterized by a disease response ranging from susceptible to medium-resistant, valuable sources of resistance have been identified in the cultivated durum germplasm (Rubies et al, 2006; Ratti et al, 2006). SBCMV is transmitted by the plasmodiophorid

Polymyxa graminis, a eukaryotic soil-borne microorganism that has been detected down to a soil depth of 60 cm and colonises roots of *Gramineae* plants (Rao and Brakke, 1969). Following transmission by *P. graminis*, SBCMV is translocated into the upper parts of susceptible plants causing stunting and mosaic symptoms on leaves that are most prominent in early spring.

Chemical control except soil fumigation, which is unacceptable for economical and ecological reasons, is ineffective against *P. graminis*. Furthermore, as virus-containing resting spores of *P. graminis* are distributed by wind, water and machinery and can survive in the soil for decades (Brakke and Langenberg, 1988), crop rotation is not an effective option for disease control either. Therefore, the only possibility of controlling this disease on infested fields is growing resistant cultivars.

Resistant cultivars have been known in France for many years, but until now no comprehensive information on the genetic control of resistance in these cultivars have been available. A locus for resistance to SBCMV, designated as *Sbm1*, has been mapped to the long arm of chromosome 5D in the UK wheat cv. Cadenza (Bass et al., 2006). However, it appears that Cadenza shares no common ancestry, regarding donors of resistance, with SBCMV-resistant cultivars commonly grown in France (e.g., Tremie, Claire and Moulin) (Bayles and Napier, 2002). This source of genetic resistance is not readily available to durum wheat breeders, due to its location on the D genome. Maccaferri et al. (2008) reported in durum wheat a major QTL (*QSbm.ubo-2BS*) located on the distal end of chromosome 2BS, coincident with that of the recently reported *Sbm2* locus detected in the hexaploid wheat background (cv. Cadenza) by Bayles et al. (2007).

In most species, bulked segregant analysis (BSA, proposed by Michelmore et al. 1991) is the classical way to find genetic markers of disease resistance genes, by screening two DNA pools of phenotypically distinct plants for markers with skewed allele frequencies. Through a BSA, Russo et al. (unpublished) have selected ten susceptible and ten resistant lines, from a RIL population obtained crossing two durum wheat cultivars, Neodur (highly resistant) and Cirillo (highly susceptible). Phenotypic data for the SBCMV resistance obtained by visual scoring of genotypes grown in SBCMV-infested fields and DAS-ELISA were used for composing resistant and susceptible DNA bulks according to Michelmore et al. (1991). Russo (personal communication 2010) found that the SBCMV resistance in the durum wheat variety Neodur is conferred by a major gene located on the telomeric region of the short arm

of chromosome 2B, coincident with the major QTL detected by Maccaferri et al. (2008).

The development of molecular markers which can be employed for efficient marker assisted selection (MAS) in breeding for SBCMV resistance is of special importance in wheat breeding as field based phenotypic selection is expensive, laborious and time consuming.

Non-host disease resistance

1.5.7 Systemic acquired resistance

Plants have evolved sophisticated defence mechanisms to respond to microbial pathogens. Systemic acquired resistance (SAR) is a defence mechanism that is characterized by the systemic activation of a broad spectrum of host defence responses in uninfected parts of the plant as a result of the localized induction of defence responses upon pathogen recognition (Zhang and Klessig, 1997). SAR was first described by Ross (1961) in tobacco leaves infected with tobacco mosaic virus (TMV). He demonstrated that the spread TMV infections were reduced in plants that were previously infected.

SAR can provide a long lasting resistance against diverse organisms such as fungi, bacteria and viruses. It is associated with induced defence reactions, including biochemical and cytological changes, and depends on the production of a signal that is translocated systemically. Recognition of a pathogen can trigger a localized resistance reaction, known as hypersensitive response (HR), which is often characterized by a rapid cell death that contributes to block the infection of biotrophic microbes (Hammond-Kosack and Jones, 1996). Although plants do not possess immunoglobulins, the general phenomenon of SAR is comparable to the acquired immune system in animals and human. In addition SAR provides a broad spectrum resistance that is effective not only against the primary infecting agent, but also against a wide array of pathogens (Sticker et al., 1997).

SAR depends on the signal molecule salicylic acid (SA; Gaffney et al., 1993; Cao et al., 1994; Glazebrook et al., 1996; Shah et al., 1997). SA and its functional analogs 2,6- dichloroisonicotinic acid (INA) and benzo (1,2,3) thiadiazole-7- carbothioic acid S-methyl ester (BTH) can induce SAR in absence of pathogen infection (Kogel et al., 1994; Görlach et al., 1996; Rairdan et al., 2001; Rairdan and Delaney, 2002; Schweizer et al., 1997; Morris et al., 1998). Transgenic plants expressing a bacterial salicylate hydroxylase (NahG), which converts SA to the biologically inactive catechol,

accumulate very little SA after pathogen infection, fail to express *pathogenesis related* genes (PR), and are impaired in SAR (Delaney, 1994; Gaffney et al., 1993). Loss of function of phenylalanine ammonia lyase (PAL), which is required for the SA synthesis, leads to reduction of SAR (Pallas et al., 1996).

Accumulation of SA in plant tissues results in the induction of *PR* genes expression, in local and systemic tissues. These proteins were first described in the 1970s by Van Loon and Van Kammen, who observed accumulation of various novel proteins after infection of tobacco with TMV (Van Loon and Van Strien, 1999; Van Loon and Van Kammen A., 1970; Ryals et al., 1996). PR proteins include glucanases, chitinases, and peroxidases. Some of these proteins may have their individual role against fungal or bacterial pathogens via hydrolytic action on their cell walls.

The activation of SAR is mediated by the Non-expresser of *Pathogenesis-Related* gene 1 (*NPR1*) protein (Shah et al., 1997; Datta and Muthukrishnan, 1999; Dong, 2004). *NPR1* (Cao et al., 1994) also known as *NIM1* (non-inducible immunity; Delaney et al., 1995) and *SAI1* (salicylic acid–insensitive; Shah et al., 1997) is essential for transduction of the SA signal to activate *PR* genes and to induce SAR (Shah et al., 1997; Cao et al., 1998; Dong, 2001; Mètraux, 2001).

NPR1 gene was identified in *Arabidopsis* through a genetic screen for SAR compromised mutants (Cao et al., 1994; Glazebrook et al., 1996; Shah et al., 1997; Delaney et al., 1995). *Arabidopsis npr1* mutants fail to respond to various SAR inducing agents and, thus, exhibit enhanced susceptibility to pathogens (Cao et al., 1997; Chern et al., 2001). *NPR1* homologs were found in other plants like tobacco, rice, barley, soybean, apple, banana and cacao (Liu et al., 2002; Chern et al., 2005b; Kogel and Langen, 2005; Sandhu et al., 2009; Malnoy et al., 2007; Zhao et al., 2009, Shi et al., 2010). Overexpression of *Arabidopsis NPR1* (*AtNPR1*) in *Arabidopsis*, rice, tomato, wheat and apple enhanced pathogens resistance by elevation of PR genes expression (Cao et al., 1998; Lin et al., 2004; Fitzgerald et al., 2004; Chern et al., 2001, 2005b; Friedrich et al., 2001; Makandar et al., 2006; Malnoy et al., 2007). In rice and tobacco, silencing of *NPR1* via RNA interference (RNAi) results in the higher susceptibility to pathogens and herbivores (Rayapuram and Baldwin, 2007; Yuan et al., 2007).

NPR1 encodes a protein with a bipartite nuclear localization sequence and two potential protein–protein interaction domains: an ankyrin repeat domain and a BTB/POZ (Broad complex, Tramtrack and Bric-a-brac/Pox Virus and Zink finger motif; Cao et al., 1997) (Fig. 2). Activity of *NPR1* depends on the cellular redox state. Increasing SA concentration after pathogen infection leads to change of redox state of

the cell (Chen et al., 1993; Noctor et al., 2002; Vanacker et al., 2000). After the induction of defence responses, plant cells attain a more reducing environment thanks to the accumulation of antioxidants like SA; in a reducing state *NPR1* is converted from an oligomeric form to a monomeric form through the reduction of intermolecular disulfide bonds. The monomeric *NPR1* then moves into the nucleus to activate SAR-associated gene expression. Mutations of Cys^{82} and Cys^{216} result in constitutive expression of monomeric nuclear *NPR1* and *PR1* expression even in the absence of SAR inducer (Mou et al., 2003; Tada et al., 2008).

The presence of two protein-protein interaction domains in *NPR1* suggests that it regulates SAR related gene expression through interaction with other proteins, including transcription factors that are present in the nucleus (Mou et al., 2003). *NPR1* interacts with several members of the TGA subclass of basic domain/leucine zipper transcription factors (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). These TGA factors are able to bind to the SA-responsive elements present in *PR* genes promoters (Lebel et al., 1998). In *Arabidopsis,* NPR1 interacts with three TGA transcription factors (TGA2, TGA5 and TGA6) and a triple-mutation in all of them (*tag2*, *tga5*, *tga6*) is essential to suppress *NPR1* regulation of *PR* gene expression (Zhang et al., 2003). *In vitro* gel mobility shift assay showed that the DNA binding activity of TGA2 is enhanced by *NPR1* (Després et al., 2000).

Because of its key central role as mediator of SAR initiation and progression, *NPR1* represents an ideal target for engineering broad-spectrum pathogen resistance in crop plants. The study of the rice *NPR1* homolog (*OsNH1*) revealed that although rice and *Arabidopsis* share conserved defence pathways (Chern et al., 2001), the regulation of these pathways and the links to other pathways may be quite divergent (Chern et al., 2005a). The genetic and physiological bases of SAR in wheat have not been explored yet.

Following an internship from November 6th 2009 to June 1st 2010 undertook in the laboratory of Prof. Jorge Dubcovsky (Department of Plant Sciences, University of California, Davis), we isolated the full-length *NH1* cDNA of durum wheat cultivar Langdon (hereafter called *TdNPR1*) to start exploring the protein interaction network of NPR1 in wheat. We also applied a TILLING reverse genetic approach to functionally characterize NPR1 in wheat, identifying novel allelic variants as markers in the gene proposed as global regulator of broad spectrum disease resistance.

1.6 Specific issues in MAS for wheat breeding

There are also factors that are limiting the use of MAS for wheat breeding. Two primary limitations to MAS are (i) the biparental mapping populations used in most QTL studies do not readily translate to breeding applications and (ii) statistical methods used to identify target loci and implement MAS have been inadequate for improving polygenic traits controlled by many loci of small effect (Heffner et al., 2009).

The most common method of QTL detection is the use of a biparental mapping population. While these studies are important to the understanding of genetic architecture, building mapping populations distinct from breeding populations often strains the resources of a breeding program. Available resources limit the size of mapping populations and, consequently, the accuracy of QTL position and effect estimates (Dekkers and Hospital, 2002; Schön et al., 2004). Also, allelic diversity and genetic background effects that are present in a breeding program will not be captured with a single biparental population. Therefore, multiple mapping populations are needed, QTL positions require validation, and QTL effects must be reestimated by breeders in their specific germplasm. The validation in locally adapted germplasm is important because poor estimates of the numerous small-effect QTLs will lead to gains from MAS that are inferior to traditional phenotypic selection (Bernardo, 2008). Therefore, the resources required for QTL detection coupled with validation and effect reestimation limit the effectiveness of biparental population derived QTLs for MAS in plant breeding populations (reviewed by Holland, 2004). Linkage disequilibrium (LD)–based mapping represents an alternative approach that can be used for dissecting complex traits in breeding populations for which extensive phenotypic data across locations and years are available (Jannink et al., 2001; Rafalski, 2002). This strategy avoids the need to develop special mapping populations that impose an additional burden on breeding programs.

Other factors were identified by the breeders as the major reasons for limited application of MAS for wheat improvement:

- Trait-marker relationships: markers are not always available for the major traits or alleles of interest to wheat breeders. Breeders identified as major limitations the lack of reliable markers for abiotic stress tolerance, such as drought tolerance and quantitative disease resistance and for specific end-uses, such as noodle quality. These traits were also highlighted as traits that were particularly difficult to screen using conventional approaches.

- Cost of MAS: cost of marker assays remains an issue although the recent shift to SNP and DArT-based platforms will help address this limitation (William et al., 2007). However, the cost of using MAS compared with conventional phenotypic selection may vary considerably, although only a relatively small number of studies have addressed this topic. Landmark papers by Dreher et al. (2003) and Morris et al. (2003) showed that the cost–benefit ratio of MAS will depend on several factors, such as the inheritance of the trait, the method of phenotypic evaluation, the cost of field and glasshouse trials and labour costs. It is also worth noting that large initial capital investments are required for the purchase of equipment, and regular expenses will be incurred for maintenance. Intellectual property rights, for example, licensing costs due to patents, may also affect the cost of MAS (Jorasch, 2004; Brennan et al., 2005). One approach to this problem is to contract the marker work out to larger laboratories that can benefit from economies of scale and high-throughput equipment (Collard and Mackill, 2008) .

- Genome structure: the complexity of the wheat genome is another issue, where a detailed understanding of the genetics of the target traits can be critical for effective deployment of MAS in a breeding program (Powell and Langridge, 2004). Further, the QTL/genes for many key traits for wheat improvement are present in alien segments, so that devising strategies to enhance introgression and recombination involving these alien segments are of particular importance (Able et al., 2007; Feuillet et al., 2008). Approaches are needed that would allow the management of large linkage blocks and are able to deal with traits that may be linked in repulsion.

- Number of loci for MAS: dealing with large numbers of loci is also an issue for several programs that seek to expand the use of molecular markers. As shown in Fig. 3 , more than 60 loci are being tracked with markers in wheat breeding programmes. This is greatly increasing the complexity of breeding and driving the search for new breeding strategies, where multiple traits can be introgressed (Gupta et al., 2010).

The large size, complex arrangement of repetitive sequences and the poliploid nature of the wheat genome (Appels et al., 2003), makes the development of genomic resources and application to breeding programs a challenging task. Nevertheless, the global economic importance of wheat and the need to develop higher yielding wheat varieties has seen the development of latest genomic tools and technologies to understand the genetic control of a range of morphological characteristics, grain quality and tolerances to biotic and abiotic stresses for adaptation. Traits are either quantitatively or qualitatively inherited and traditional strategies have heavily relied on selecting the desired phenotype for the target environment throughout the breeding

process. The development of genetic maps and subsequent QTL and linkage disequilibrium (LD) mapping is a prerequisite to identify DNA markers linked to genes controlling qualitative and quantitative traits prior to implementation in marker-assisted selection (Francki et al., 2010).

1.7 Marker-assisted wheat breeding

Several examples of successful use of MAS are now available in wheat, and more examples will become available in future (Dubcovsky, 2004; Bonnett et al., 2005; Kuchel et al., 2005, 2007, 2008). One should, however, recognize that currently MAS is largely practiced for simple traits that are difficult to score, and not for the complex polygenic quantitative traits like yield, for which MAS involving marker-assisted recurrent selection (MARS) and genome-wide selection (GS) seem to be more appropriate (Bernardo and Yu, 2007; Heffner et al., 2009).

A large number of relatively simple traits have been targeted for wheat improvement through MAS. These include (1) disease/pest resistance, including resistance against various rusts, *Fusarium* head blight, barley yellow dwarf virus (BYDV), nematodes and Hessian fly/Russian wheat aphid; and (2) quality traits including grain protein content, grain hardness, tolerance to pre-harvest sprouting, grain colour, bread making quality, grain texture and gluten strength. Wheat breeding programs targeting improvement in these traits have been in progress in several countries including Australia, USA, Canada and at CIMMYT (Mexico) (for details see Table 3; Gupta et al., 2010). There are other traits like tolerance to drought, heat, salinity, water logging, and metal toxicity (e.g., boron, aluminium and arsenic), which will be the future targets for wheat improvement using MAS.

Table 4 shows a main index of marker/trait associations collected in the USA program MASwheat (http://maswheat.ucdavis.edu). This project contribute in useful materials and information relating to the use of MAS for improvement of complex traits in wheat. Is a project recently funded by the United States Department of Agriculture (USDA) under the Coordinated Agricultural Project (CAP) program entitled ‘‘Applied Wheat Genomics’’. Coordinated by Prof. J. Dubcovsky at the University of California (Davis), it builds on a project entitled ‘‘Bringing Genomics to the Wheat Fields’’ involving wheat breeding programs across the US (Sorrells, 2007).

Several strategies for effective use of MAS have been suggested and tested for wheat breeding. These strategies include crossing two parental genotypes, either to combine desirable attributes of both the parents into one genotype, or to transfer

desirable allele(s) from a donor to an otherwise elite wheat cultivar used as recipient. The strategy for further use of the F_1 derived from such a cross may differ and one of the following alternative strategies may be used: (1) repeated backcrossing of the F_1's to reconstitute the recipient genome without losing the desirable gene; (2) forward breeding MAS involving a top cross or a three-way cross, where superior genes from both parents are combined, and background selection is only rarely practiced; (3) development of doubled haploids (DH) or inbreeding to increase homozygosity, or (4) F_2 enrichment or recurrent selection to increase the relative frequency of the desirable allele(s). These different approaches have been tested experimentally (Bonnett et al., 2005) and discussed by Gupta et al. (2010).

A wide research program is in course at the CRA - Cereal Research Centre of Foggia (CRA-CER), aimed to the genetic analysis of traits of agronomic relevance for durum wheat, and funded by Ministero dell'Università e della Ricerca (MiUR) of Italy special grant AGROGEN "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" (http://www.agrogen.it/default.asp). A number of segregating populations, together with the corresponding genetic maps have been developed by starting from crosses between durum wheat varieties contrasting for the traits of interest. A number of useful alleles have been positioned on genetic maps, and closely linked molecular markers have been identified for traits. Based on these molecular markers together with other ones already developed and for which information was available in literature, a molecular breeding program was initiated.

1.8 Future strategies for marker-assisted wheat breeding

The specific issues using MAS for wheat breeding (mentioned above) are being addressed through a number of strategies. In this connection modelling of QTL effects for MAS may prove useful (Cooper et al., 2007). Similarly, improved breeding strategies like AB-QTL, mapping-as-you-go, F_2 enrichment, marker-assisted recurrent selection (MARS) and genomic selection (GS) have not been tried yet to exploit the full potential of MAS in wheat. There are also several new approaches to identify loci controlling complex traits and addressing some of the difficulties associated with low levels of polymorphism, such as the use of new, high-throughput marker systems and novel populations (Gupta et al., 2010).

- Multiparent advanced generation intercross (MAGIC): in the past, most QTL studies involved the use of biparental mapping populations, thus putting a restriction on the genetic diversity that is sampled in each mapping population. Multi-parental mapping populations may therefore be used in future, so that genetic and phenotypic diversity involving breeding material from around the world may be exploited. Multiparent advanced generation intercross (MAGIC) approach in particular is being employed in UK and Australia to develop multi-parent recombinant inbred lines (RILs) (Cavanagh et al., 2008; Cavanagh and Morell, 2008). These populations will prove to be useful in QTL analysis but will only be suitable for some traits. A limitation with such populations is that they are likely to show extensive segregation for developmental traits, such as maturity and plant height thus greatly limiting their use in the analysis of complex traits such as components of yield or drought tolerance (Gupta et al., 2010).

- High throughput marker technology (SNP and DArT; perfect markers): considerable progress in achieving high throughput in marker technology has been made during the last decade. Array-based high-throughput low-cost marker systems such as single nucleotide polymorphisms (SNPs) and diversity array technology (DArT) have become the markers of choice for whole genome profiling, and therefore, for background screening. DArT markers, developed by Triticarte (Australia), provide extensive genome coverage, ultra-high-throughput and low cost. Such complete genotypic information would allow the breeding program to select those individuals that not only have the QTLs of interest but also contain the maximal amount of recurrent parent genome. Thus, DArT markers can be used effectively for introgression of one or more transgenes into a new variety. A single DArT genotyping array with around 100 markers would provide a low-cost method to determine how similar a particular backcross individual is to the desired recurrent parent (Gupta et al., 2009). Moreover, the rapidly expanding use of Next-Generation Sequencing technologies offers the ability to rapidly identify SNP markers, which may, therefore, dominate marker-assisted wheat breeding during the next few years allowing for drastically quicker and cheaper variant discovery, and leading towards a far more comprehensive view of the genome (Schuster, 2008; Ganal et al., 2009). The possibility to develop and analyze a huge number of SNPs in crop species opens new perspectives for the use of these molecular markers to accelerate selection of improved genotypes. New platforms are now available for the rapid genotypization of individuals with SNPs. The Infinium assay by Illumina (http://www.illumina.com/technology/beadarray_technology.ilmn) can provide the analysis with several hundred thousands SNPs in a single assay. The KBiosciences

Competitive Allele Specific PCR SNP genotyping system (KASPar) is a novel homogeneous fluorescent genotyping system (http://www.kbioscience.co.uk) providing very fast and cheap analyses.

- AB-QTL analysis and mapping-As-You-Go (MAYG): AB-QTL analysis and Mapping-As-You-Go (MAYG) are two novel marker-assisted approaches for crop breeding involving simultaneously QTL detection and MAS, and requiring no validation (Tanksley and Nelson, 1996; Podlich et al., 2004). The advanced backcross quantitative trait locus (AB-QTL) strategy was introduced by Tanksley and Nelson (1996) in order to combine the mapping of favourable exotic QTL alleles and the transfer of these alleles into elite breeding lines. In order to achieve this goal, the authors utilized wild species as the donor parents for improvement of quantitative agronomic traits and collected the phenotypic and genotypic data in advanced backcross generations. AB-QTL has recently been used in wheat (Kunert et al., 2007; Naz et al., 2008) and may allow gene pyramiding through inter-mating best AB-lines with each other and combining a series of favourable exotic QTL alleles in a single line using MAS (Kunert et al., 2007).

MAYG is a mapping-MAS strategy that explicitly recognizes that alleles of QTL for complex traits can have different values as the current breeding material changes with time (Podlich et al., 2004). This method results in substantial increases in MAS efficiency compared with standard approaches based on the evaluation of the QTL effects only at the beginning of the breeding program, particularly when epistasis and/or genotype-environment interactions play a significant role. MAYG approach is currently being evaluated by several wheat breeding programmes.

- Marker-assisted recurrent selection (MARS) and genomic selection (GS): one limitation of marker-assisted backcross breeding (MABB) is that introgression of only one or few alleles controlling a trait is undertaken in a breeding program. One would, however, like to simultaneously select for a number of QTLs controlling either a solitary trait or a number of traits. Two selection strategies to deal with this problem in future wheat breeding include marker-assisted recurrent selection approach (Xie and Xu, 1998; Charmet et al., 1999, 2001) and genomic selection (GS), which are briefly discussed below.

(i) Marker-assisted recurrent selection (MARS): two related approaches have been proposed and used to increase the frequency of favourable QTL alleles at multiple loci: (i) F_2 enrichment followed by inbreeding (Howes et al., 1998; Bonnett et al., 2005; Wang et al., 2007) and (ii) marker-assisted recurrent selection (MARS; Edwards and

Johnson, 1994; Hospital et al., 1997; Koebner, 2003; Johnson, 2004; Bernardo and Charcosset, 2006). In both approaches the base generation is usually an F_2 population from the cross between two inbreds, although backcrosses, three-way crosses, or double crosses may also be used. The objective is to develop a recombinant inbred with superior per se performance for self-pollinated crops or with superior testcross performance for hybrid crops. Whereas F_2 enrichment usually involves only one generation of marker-based selection, MARS involves several cycles of marker-based selection (Bernardo, 2008). The approach has also been effectively used by Monsanto for improvement of several traits in corn, soybean and sunflower (Eathington et al., 2007) and will certainly be used in future wheat breeding programs.

(ii) Genomic selection and GSBV-based MAS for quantitative traits: a quantitative trait is generally controlled by a few major genes and many minor QTLs/genes, and only major QTLs/genes are generally used for MAS, so that the benefit from MAS is limited by the proportion of the genotypic/phenotypic variance explained by these marker-associated major QTLs. However, it would be desirable to utilize all the QTLs affecting the trait for MAS. Genomic selection (GS) is a form of MAS, where marker effects across the entire genome (explaining entire phenotypic variation) are simultaneously estimated and used to calculate genomic estimated breeding values (GEBVs; Meuwissen et al., 2001; Heffner et al., 2009). Selection is then based on this breeding value rather than on a subset of significant markers, that are generally used in MAS; moreover, the introduction of genomic selection (GS) can leads to more rapid and lower cost gains from breeding (Jannink et al., 2010).

A key component for the future success of wheat breeding will lie in the strategies and innovations that come through the application of molecular technologies. For these advances to be realized, breeders will need to be directly involved in defining targets and identifying key germplasm for analysis. The molecular groups should act in a support capacity and should challenge the breeders by questioning their methods and breeding strategies. It seems probable that innovations in breeding strategies will be a key driver to the future role of MAS and new breeding methods are already seeing that were not feasible prior to marker application. Wheat does present some special challenges through the complexities of working with two (durum wheat) or three (common wheat) genomes and the major role played by alien germplasm and chromosome segments in improvement. However, these challenges also represent major opportunities for achieving significant genetic gains (Gupta et al., 2010).

2. AIMS OF THE WORK

The specific objective of the present study was to develop an assisted-breeding program in durum wheat for quality traits and disease resistance.

To develop the marker assisted breeding program three subsequently steps were carried out:

i) the collection of existing as well as the development of new molecular markers for traits of interest. This step also include a work dedicated to the identification of new alleles at the gene *TdNPR1*, a sequence controlling a broad spectrum (non-host) defence response, by means of a TILLING approach (work carried out at the UCDavis c/o Prof. J. Dubcovsky);

ii) markers validation to check their portability in Italian cultivars;

iii) planning and development of a Marker Assisted Selection schedule. When useful alleles originally present in wild accessions where taken into considerations, lines derived from a pre-breeding activity were used as donor in the present breeding program in order to reduce the risk of transferring alien segments associated with undesirable agronomic characteristics together with useful alleles.

3. MATERIALS AND METHODS

3.1 Genetic materials and growth conditions

A number of donor lines carrying the desirable genes (Creso for leaf rust resistance; 5BIL-42 for powdery mildew resistance; UC1113 for high protein content; Pedroso and Primadur for yellow pigment content; Neodur for soil borne cereal mosaic virus resistance) (Tab. 5) and the recipient line PR22D89 were used in the MAS breeding programme. The plants were grown in open field, in greenhouse and in the growth chamber of the experimental station of the CRA-CER of Foggia following standard cultural practices. The pyramiding of the genes *Gpc-B1*, *Pm36*, *Lr14c*, *Psy-A1* and QTL *QSbm.ubo-2BS* into the elite cultivar was realized in conformity with the scheme in Figure 4.

During the 2007 the F_1 populations UC1113 x PR22D89 and 5BIL-42 x PR22D89 were grown in the growth chamber. In the same year another F_1 population was developed crossing UC1113 with 5BIL-42.

During the 2008 the F_1 UC1113 x 5BIL-42, F_2 UC1113 x PR22D89 and F_2 5BIL-42 x PR22D89 populations were grown in the greenhouse. In the same year another F_1 population was developed crossing Creso with PR22D89.

During the 2009 the F_2 UC1113 x 5BIL-42, F_3 UC1113 x PR22D89 and F_3 5BIL-42 x PR22D89 populations were grown in the open field. The F_2 UC1113 x 5BIL-42 population was grown with plants 10 cm apart per row and 50 cm between rows; the parental lines UC1113, 5BIL-42 and PR22D89, F_1 and F_3 plants were evaluated for GPC and powdery mildew resistance using a randomized complete block. Each progeny was planted with 3 g of seeds in a single row 2 m long with 50 cm between rows. In the same year the F_1 population Creso x PR22D89 was crossed with UC1113 and 5BIL-42; moreover, F_3 plants 5BIL-42 x PR22D89 homozygous for *Pm36* gene were crossed with F_3 plants UC1113 x PR22D89 homozygous for *Gpc-B1* allele.

During 2010 the F_4 families 5BIL-42 x PR22D89 homozygous for *Pm36* gene, F_4 families UC1113 x PR22D89 homozygous for *Gpc-B1* allele, F_3 plants UC1113 x 5BIL-42 homozygous for both genes, F_1 (5BIL-42 x PR22D89) x (UC1113 x PR22D89), F_1 (Creso x PR22D89) x UC1113 and F_1 (Creso x PR22D89) x 5BIL-42 populations were grown in the open field. In the same year F_4 plants UC1113 x PR22D89 homozygous for *Gpc-B1* allel and F_3 plants UC1113 x 5BIL-42 homozygous for *Gpc-B1* allele and *Pm36* gene were crossed with the durum wheat cultivars Creso, Pedroso, Primadur and Neodur; the following F_1 populations were grown in the growth chamber.

Actually the F_5 5BIL-42 x PR22D89, F_5 UC1113 x PR22D89, F_4 UC1113 x 5BIL-42, F_2 (5BIL-42 x PR22D89) x (UC1113 x PR22D89), F_2 (Creso x PR22D89) x UC1113, F_2 (Creso x PR22D89) x 5BIL-42, F_2 (UC1113 x PR22D89) x Creso, F_2 (UC1113 x PR22D89) x Pedroso, F_2 (UC1113 x PR22D89) x Primadur, F_2 (UC1113 x PR22D89) x Neodur, F_2 (UC1113 x 5BIL-42) x Creso, F_2 (UC1113 x 5BIL-42) x Pedroso, F_2 (UC1113 x 5BIL-42) x Primadur, F_2 (UC1113 x 5BIL-42) x Neodur populations are growing in the open field of the experimental station in Foggia.

3.2 DNA extraction and molecular markers analysis

The DNA extraction was performed using an high-throughput platform to achieve a rapid genotyping of lines from different crosses developed in this research. An automated protocol to get genomic DNA from each genotype using a robotic platform (Beckman Coulter, Biomek® 3000 Laboratory Automation Workstation) was developed in this work. Genomic DNA was extracted from about 50 mg of leaves in

racks of 96 x 1.2 ml collection microtubes using the Wizard Magnetic 96 DNA Plant System (Promega, WI, USA). Ball bearings (3.0 mm) were used to crush frozen samples by shaking 30 s at a frequency of 25 rpm in a MM300 Mixer Mill (Retsch, Germany) and DNA was extracted as recommended by the manufacturer.

PCR amplification was performed using the Applied Biosystems 2720 Thermal Cyclers (PCR primer information and PCR cycling conditions are described in the Tab. 6). The amplification products were analyzed by means of 2% agarose gel or capillary electrophoresis multiplexing different fluorescent dyes. In that case the fluorescently-labelled amplification PCR products were diluted 1:300 in water. 1.5 µl of this dilution was added to 8.5 µl of formamide and Gene Scan 500 ROX size standard (Applied Biosystems, Foster City, CA) mixture (respectively, 8.45 µl and 0.05 µl), placed in a 96 well microplate, denatured for 2 min at 94 °C, immediately cooled to 4 °C. Samples were run in a capillary electrophoresis instrument (3130xl Genetic Analyzer, Applied Biosystems Inc. Foster City, CA USA). Fragment size data were analyzed using GeneMapper version 4.0 software (Applied Biosystems Inc. Foster City, CA USA) to determine marker size.

3.3 Molecular markers available at the CRA - Cereal Research Centre of Foggia (CRA-CER): *molecular markers associated with leaf rust resistance, SBCMV resistance and yellow pigment content*

A number of segregating populations, together with the corresponding genetic maps have been developed by starting from crosses between durum wheat varieties contrasting for the traits of interest. A number of useful alleles have been positioned on genetic maps, and closely linked molecular markers have been identified for leaf rust, soil borne cereal mosaic virus resistance (SBCMV) and yellow pigment content.

Sequences and features of primers utilized in this work are summarized in Table 6. The amplification reactions were performed following the protocol described by Röder et al. (1998), slightly modified. The PCR mixture (15 µl) contained 50-100 ng of genomic DNA, 1X Taq Buffer (10 mM Tris – HCl - pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) (GoTaq Buffer, Promega), 0.2 mM of each dNTP, 0.4 µM labelled reverse primer (both FAM), 0.4 µM unlabelled forward primer, 5% DMSO and 1 U Taq Polymerase (GoTaq Polymerase, Promega). PCR amplification conditions were as follows: 3 min at 94 °C; 45 cycles of 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C, followed by final extension of 10 min at 72 °C.

3.4 Validation of other molecular markers

3.4.1 Molecular marker associated with low lipoxygenase activity

Primers InDel *LOXA-4BSL2* and *LOXA-4BSR* published by Carrera et al. (2007) and Hessler et al. (2002) were used to evaluate the distribution of the *Lpx-B1.1* deletion on an Italian durum wheat germplasm collection (Tab. 7).

PCR amplification conditions were as follow: 3 min at 94 °C; 5 touchdown cycles (-1 °C each) of 30 s at 94 °C, 30 s at 62–58 °C and 45 s at 72 °C. After that, 35 cycles of 45 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C, followed by final extension of 10 min at 72 °C. The PCR products were separated in 2% agarose gel with 1X TBE and visualized by GelRed™.

3.4.1.1 Field trial

Seventy-one durum wheat genotypes (Tab. 7), including old (released before 1971), intermediate (released between 1971 and 1990) and modern varieties (released between 1991 and 2005) were grown during the 2004-2006 growing season at Foggia, Italy. The genotypes were arranged in a randomized complete block with three replications. Each experimental unit consisted of a 10.2 m^2 plot. The grains were ground into whole meal with an experimental mill (Tecator Cyclotec 1093) and used for lipoxygenase activity measurements. For more complex biochemical analysis (kinetics and pH profile), four selected cultivars (Trinakria, Primadur, Creso, Cosmodur), bred at Foggia during the 2005-2006 growing season, were used.

3.4.1.2 Pasta processing

Experimental semolina samples were produced from 10 kg of durum wheat grains conditioned overnight to 16.5% moisture content and processed in a laboratory mill (MLU202, Bühler, Uzwill, Switzerland), fitted with six breaking and six sizing passages. Semolina was mixed with water at room temperature to reach a total dough moisture content of 33-34%. Dough was processed into spaghetti with a diameter of 1.7 mm using a 2 kg capacity laboratory press (Namad, Rome, Italy). The mixing time was 10 min (with constant mixing speed), while the extrusion was performed with a pressure of 9.1-12.1 MPa and vacuum of 0.09 MPa. A low temperature drying procedure (50° C for 18 h) was applied using a pilot drying plant (Giussani, Fara D'Adda, Bergamo, Italy).

3.4.1.3 LOX enzymatic assay

In order to compare LOX activity across the cultivars, wholemeal flour (5 g) was homogenized with 10 ml of 0.1 M sodium phosphate buffer (pH=7.0) in an ice-water bath for 1 hr and stirred for 1 min at 15 min intervals. Homogenates were immediately centrifuged at 35000 x g for 15 min at 4 °C. The supernatant was centrifuged in the same conditions. Protein content of extracts was evaluated by the method of Lowry et al. (1951) and used for enzymatic assay. For the assay, the reaction mixture (2 ml) contained 0.05 M sodium phosphate buffer (pH=6.6) and 0.1 mg of protein of enzyme extract. The reaction started by adding 1 mM of linoleic acid substrate solution. One unit of enzymatic activity (linoleate hydroperoxidation activity) corresponded to the production of 1μmol of conjugated diene per min at 25 °C normalised for dry content.

3.4.1.4 Yellow pigment content

The evaluation of yellow pigment content (YPC) was made according to AACC Approved Method 14-50 (AACC, 2000). Samples were extracted and analysed in triplicate. Pigment concentration was calculated using the extinction coefficient of lutein (Rodriguez- Amaya and Kimura, 2004). The reported YPC data are the means of three replications. Moisture (%) was determined by oven drying for 3 h at 130° C on 5 g of sample in triplicate (Approved Method 44-19, AACC, 2000).

3.4.2 Molecular marker associated with grain protein content

The InDel marker *Xuhw89-F/R* is located at 0.1-cM from the *Gpc-B1* gene; it reveals a 4-bp deletion linked to the gene and is absent in most common and durum wheat lines.

Prof. Dubcovsky (University of California, Davis) kindly provided the breeding durum line UC1113 (UC selection from CIMMYT cross CD52600 [Kifs//RSS/BD1419/3/Mexis-CP/4/Wahas/5/Yav79]), carrying *Yr36–Gpc-B1* genes. The amplification reactions were performed following the protocol available on website http://maswheat.ucdavis.edu/.

3.4.2.1 Determination of protein content and single kernel weight

The protein content (N*5.7) was determined by Kjedhal analysis in duplicate by the AACC approved method 46-13 (American Association of Cereal Chemists, 2000). The values are referred as GPC (Grain Protein Content) throughout the text.

The weight of kernels was determined with manual count of 100 seeds after removing by handpicking broken kernels and foreign materials. The values are referred as SKW (Single Kernel Weight) throughout the text.

3.4.3 Molecular marker associated with powdery mildew resistance

Primers *mgbe684-F/R* were designed on EST-SSR (Simple Sequence Repeat derived from Expression Sequence Tag) BJ261635 by Blanco et al. (2008). This marker identify the powdery mildew resistance gene *Pm36* in the backcross inbred line 5BIL-42, make available by Prof. Blanco (University of Bari, Italy). The amplification reactions were performed following the protocol of Blanco et al. (2006), lightly modified.

3.4.3.1 Evaluation of powdery mildew resistance

Resistance screening was performed on adult plants in greenhouse (2008) and in field (2009) conditions following natural infections. The intensity of symptoms was recorded between the heading and flowering stages following the rating scale described by Saari and Prescott (1975). The level of infection was expressed as the percentage of plant surface infected (0 = 0-9%; 1 = 10-19%; 2 = 20-29%; …; 9 = 90-100%). Individual plants were considered resistant if no symptoms or less than 20% infection was observed.

3.4.4 Molecular markers associated with yellow pigment content

SSR molecular markers were validated to better resolve the correlation between genotype and phenotype. Blanco (personal communication 2010) identified two flanking markers to phytoene synthase gene *Psy-A1*, *Xgwm1061* and *Xgwm344*, on the distal region of chromosome arm 7AL. Another QTL also were mapped by Blanco (personal communication 2010) on the upper region of same chromosome 7AL, associated with the linked SSR marker *Xgwm282*. These three markers were mapped using a segregant population of 121 progenies derived by crossing the durum wheat cultivars Latino and Primadur characterized by low and high values of yellow pigment content, respectively.

3.5 Statistical analysis

Data from this study were reported as mean ± standard deviation (SD). Results were subjected to analysis of variance (ANOVA) using STATISTICA software

(StatSoft version 7.1 StatSoft, Inc., Tulsa, Oklahoma, USA). Means were identified as being significantly different on the basis of Fisher's protected least significant differences (LSD) at a probability level of 5%.

3.6 Identification of new alleles at the *NPR1* gene of durum wheat: a sequence controlling the non-host resistant response to disease (work carried out at UCDavis c/o Prof. J. Dubcovsky)

Full-length *TdNPR1* cDNA cloning

3.6.1 Plant material and RNA extraction

Total RNA was isolated from young leaves of durum wheat cultivar Langdon using Spectrum™ Plant Total RNA Kit. The cDNA was amplified by reverse transcription polymerase chain reaction (SuperScript II Reverse Transcripatase, Invitrogen) using the total RNA as template.

3.6.2 PCR-based cloning

Primer pairs were designed based on the sequences of the barley *NPR1*-homolog (*HvNPR1*; GenBank: AM050559.1) and of two wheat ESTs (GenBank: CJ906714 and CJ906122) obtained by similarity searches with BLASTN (http://www.ncbi.nlm.nih.gov/BLAST).

cDNA was denatured at 94°C for 5 min, followed by 37 cycles of 30 s at 94°C, 30 s at the specific primer annealing temperature and variable time at 72°C (depend on the size: 1 min for a 1-kb product), with final extension at 72°C for 10 min by using the Applied Biosystems 2720 Thermal Cyclers. The PCR products were separated in 1% agarose gel with 1X TAE and the residual PCR product was used as a template for sequencing. PCR samples (2 µL/sample) were cleaned using ExoSAP-IT according to manufacturer's instructions, and subsequently sequenced using BigDye Terminator sequencing kit and an ABI 3730 DNA sequencer.

We used the cDNA of Langdon to get a part of the coding sequence through the primers 201F_5' CACCTGCTCGACTACCTGTACAGC and 69R_5' AGCTATCAATATGGCAAGAATGG. Because of the high CG content of the first part of the gene (73%), we failed to clone the complete coding sequence directly from cDNA. To solve this problem, we applied a two step approach where the first part of the gene (from start codon, nucleotides 1-485) and the second part (nucleotides 365-1770, falling in 3'UTR) were cloned from a BAC clone and from cDNA, respectively (Fig. 5).

3.6.3 BAC library screening and BAC DNA isolation

A pair of gene-specific primers (56F_5' CGGCATGCTACTTGTAACAG; 69R_5' AGCTATCAATATGGCAAGAATGG) were used for screening the BAC library of tetraploid durum wheat cultivar Langdon. Eight BAC clones were identified and one of these was chosen for sequencing to extend the sequence of the gene. For isolation of BAC DNA free of bacterial genomic DNA the QIAGEN® Large-Construct Kit was used. We used the genomic DNA extracted from the BAC to get the first part of the coding sequence (485-bp from ATG) using primers 197F_5' CACCATGGAGGCCCCGAGCAGCC and 164R_5' CGACCTGGAAGGTGGATG.

3.6.4 Isolation of TdNPR1 cDNA and cloning of the full-length protein

The two PCR products (one fragment from BAC clone and the other one from Langdon cDNA) were each cloned into pENTR™ Directional TOPO® plasmid vector (Invitrogen) and transformed into competent *E. coli* DH5α. As recommended in the manual for the directional cloning, we included the 4 base pair sequence CACC at the 5′ end of the forward primers developing blunt-end PCR products that were amplified using Phusion™ High-Fidelity DNA Polymerase reaction (Finnzymes).

Plasmid DNA purifications were carried out using the QIAprep Miniprep kit (QIAGEN). Clones with correct *TdNPR1* sequences were identified by PCR using vector-based primers M13 F/R (F_5' GTAAAACGACGGCCAG; R_5' CAGGAAACAGCTATGAC), followed by sequencing.

The vectors identified with both correct fragments were digested with restriction enzyme *Not*I. Then, the plasmid with the Langdon cDNA insert was dephosphorylated using calf intestinal alkaline phosphatase (Promega). In-frame insert derived from BAC clone was then ligated inside the plasmid dephosphorylated getting the full-length *TdNPR1* gene (Fig. 6a, b).

The Gateway® LR Clonase II™ reaction (Invitrogen) was used to clone the *TdNPR1* sequence in yeast cloning vectors pGADT7 and pGBKT7 (Clontech) to generate prey and bait constructs, respectively, for future yeast two-hybrid screens. Prior to *E. coli* transformation unrecombined pENTR were digested using the *Asi*SI restriction enzyme (NEB). The bacteria might contain both un-recombined and recombined plasmids, impossible to select because both vectors (pENTR and pGBKT7) include kanamycin resistance gene for selection in *E. coli*. In order to be sure to select for the bait construct recombined, we linearized pENTR vector and transformed bacteria selecting for the antibiotic resistance present only in the pGBKT7 vector.

The ligations were transformed into competent *E. coli* DH5α and clones with correct *TdNPR1* sequences were identified by PCR of insert using vector-based primers T7-F/pGADT7-R (F_5' TAATACGACTCACTATAGGGCG; R_5' AGATGGTGCACGATGCACAG) and pGBKT7-R (R_5' TTTTCGTTTTAAAACCTAAGAGTC) followed by plasmids purification.

3.6.5 Sequence alignment and phylogenetic analysis

The full-length cDNA sequence was translated using the ExPASy Translate Tool (http://www.expasy.ch/tools/dna.html) and conserved domains were identified through searches in PROSITE (http://www.expasy.ch/prosite/). The BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST) was used to search for protein sequence homolog to barley NPR1. Multiple sequence alignment was done by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Phylogenetic analysis and construction of a neighbour-joining tree were performed by using the MEGA 4.0 software (http://www.megasoftware.net/) using the bootstrap method with 1000 bootstrap iterations (Felsenstein, 1985).

Characterization of *TdNPR1* mutants through TILLING approach

3.6.6 Genetic material

gDNA of diploid wheat species *Triticum urartu* (genome AA), *Aegilops spletoides* (genome BB), *Aegilops taushii* (genome DD) were used to develop genome-specific primers. A tetraploid TILLING population (1368 M_2 plants) of durum wheat cultivar Kronos EMS-mutagenized available in the laboratory of Prof. Dubcovsky (Uauy et al., 2009) was used to produce NPR1 mutants. The tetraploid TILLING population is currently being expanded to 1536 lines.

3.6.7 Primer design

We designed primers to amplify a region including the ankyrin repeat domain, a necessary domain for NPR1 function in diploid *T. urartu* (donor of the A genome) and *Ae. speltoides* (closest species to the B genome of tetraploid wheat). Based on the *T. urartu* sequence we designed a pair of primers (176F_5' TCCTTGATTTCCTTGATAAT; 11R_ AGGTACAGTAACTTCCCACGAAGA) to amplify the region of the exon 2 surrounding the domain of interest from the A genome. Using the *Ae. spletoides* sequence we designed a new forward primer (175F_5'

AGTACTAACCCATGTTATGC) complementary to intron sequence flanking the exon target to amplify the B genome sequence.

Using nulli-tetrasomic lines of chromosome 3 (N3AT3B, N3BT3D and N3DT3A), *Triticum urartu* and *Aegilops spletoides* genomic DNA, we validated the genome specificity of the *NPR1* primers.

3.6.8 Screening technique and two-step strategy

A two-step screening approach was used to screen for mutations using polyacrylamide vertical gels and ethidium bromide staining (Uauy et al., 2009). The first PCR screen of the complete set of DNA pools was carried out in a 25-µl reaction volume using 50–100 ng of pooled DNA, 1 U of Taq polymerase and the following cycling conditions: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, at 50°C for 30 s and extension at 72°C for 1 min. A denaturing and re-annealing step was included at the end of the PCR reaction (99°C for 10 min, 70 cycles of 70°C for 20 s decreasing 0.3°C per cycle) to allow the formation of heteroduplexes if a mutation was present in the pool.

After PCR amplification, 12 µl of sample (~500 ng) were digested with celery juice extract (CJE) which was obtained using the protocol described by Till et al. (2006). The digestion was performed making a mix of: 12 µl PCR product, 0.12 µl CJE, 1.7 µl 10X digestion buffer (Till et al., 2006) and 3.18 µl dH2O for a final volume of 17 µl. The digestion was carried out at 45°C for 30 min and stopped immediately by adding 5 µl of 0.225 M EDTA per sample and mixing thoroughly. Two µl of bromophenol blue loading dye were added and the complete volume (24 µl) was loaded on the gel. Samples were visualized on a 3% polyacrylamide gel in 0.5X TBE running buffer with ethidium bromide; gel images were analyzed manually on PowerPoint and pools including a mutant individual were identified by the presence of cleaved CJE products whose combined size was similar to the original PCR product.

The second screen was performed using individuals DNAs only from the DNA pools that showed cleaved products in the first screen. PCR amplifications were as described before with the exception that the heteroduplex formation step was postponed. First, 6 µl from each PCR sample were pooled in four pairs (a+b, c+d, a+c and b+d) following the diagram described by Uauy et al. (2009). The heteroduplex formation step was then performed on the mixed pairs and the samples followed the same detection protocol as described above. The pooling step is necessary to detect homozygous mutations in the M_2 plants since combining two samples allows

heteroduplex formation and detection which would otherwise go undetected in a single homozygous sample. In addition to the identification of the individual from the pool that carries the mutation, this step also provides an independent validation of the mutations and a better estimation of its location within the target region (Uauy et al., 2009).

3.6.9 Sequence analysis

To characterize the individual mutations, the residual PCR products from the selected individuals were used as a template for sequencing. To predict the effect of EMS mutations in the amplicons we used CODDLE program (Choose codons to Optimize the Detection of Deleterious Lesions) (http://www.proweb.org/coddle/); whereas, to identify the EMS mutations we used Pregap4 and Gap4 software.

3.6.10 DNA exctraction

For genotyping, genomic DNA was isolated from young leaves of M_3 mutants, selected from A genome, with cetyltrimethylammonium bromide (CTAB)-based method (Doyle and Doyle, 1987) slightly modified to be more rapid and economical with small samples of plant tissue.

4. RESULTS

4.1 Validation of molecular marker linked to *Lpx-B1.1* deletion across an Italian durum wheat germplasm collection

4.1.1 Genetic variability of YPC and LPX activity in durum wheat germplasm

The YPC and the LPX activity of the 71 durum wheat genotypes cultivated under the same environmental conditions are presented in Table 6. The range in YPC within the whole set of genotypes tested in this study was 3.68-9.43 µg/g of dry weight (dw) with about 2.5-fold differences. The genotypes with the highest YPC (9.43 µg/g dw and 9.33 µg/g dw in Zenit and Brindur, respectively) belonged to the group of modern genotypes, while two old genotypes, Polesine (3.68 µg/g dw) and Timilia (3.75 µg/g dw), showed the lowest YPC values. Overall, a wide range in YPC was observed among the modern genotypes (3.78-9.43 µg/g dw) released between 1991 and 2005. A general trend toward an increase of YPC could be noticed when the mean values of the three groups of cultivars (Tab. 7; Fig. 7) were compared. Mean YPC rised from 5.4 µg/g dw in the old varieties to 6.0 µg/g dw in the cultivars released between 1971 and 1990 and

to 6.6 µg/g dw in the group of modern genotypes (released between 1991 and 2005). Significant differences in LPX activity were found among the evaluated durum wheat genotypes ($p < 0.001$). The mean LPX activity was 1.32 EU/g dw with values ranged from 0.02 (cv. Lesina) to 7.91 EU/g dw (cv. Matarese) showing, also for this trait, a considerable variability. When the durum wheat genotypes were grouped according to their year of release, the results indicated that old varieties had a significantly higher LPX activity (3.65 EU/g dw) than intermediate (0.87 EU/g dw) and modern genotypes (0.84 EU/g dw), an effect of a probably unconscious selection. In contrast to Manna et al. (1998) that observed a strong negative correlation (r = -0.95***) between LPX activity at pH 10.2 and semolina yellow index, in our study (De Simone et al., 2010) YPC was not associated with LPX activity (r = -0.12ns) supporting the possibility to screen for modern genotypes with higher carotenoid concentration and low level of LPX activity (Tab. 7; Fig. 7). These results provide strong support for the durum wheat chain to preserve the carotenoid content during milling and pasta processing in order to improve the pigment level of final products.

4.1.2 Distribution of the Lpx-B1.1 deletion across durum wheat germplasm

Carrera et al. (2007) reported that differences in LPX activity are generated by a deletion at the *Lpx-B1* locus. This polymorphism was therefore assessed on germplasm collection employed in the present work to extend the knowledge on the relation between *Lpx-B1.1* deletion and LPX activity. Our findings (De Simone et al., 2010) confirmed the *Lpx-B1.1* deletion as valid target for durum wheat breeding (Tab. 7). A highly significant decrease in LPX activity was observed in genotypes carrying the *Lpx-B1.1* deletion with a clear correlation between high/low LPX activity and presence/absence of the band for the samples evaluated. Many genotypes with LPX activity mean value lower than 0.5 EU/g dw showed the *Lpx-B1.1* deletion, while most of the genotypes with LPX activity value higher than 0.5 EU/g dw showed the duplication at the *Lpx-B1* locus. A few exceptions were also detected across the large set of germplasm examined: in the cultivars Latino, Bronte and Karel the low LPX activity was associated with the *Lpx-B1.1* duplication, while the cultivars Solex, Messapia, Produra, Cannizzara and Saadi showed a high LPX activity although the *Lpx-B1.1* fragment was not detected. Some exceptions were also reported in the previous work carried out by Carrera et al. (2007).

Notably, among the fragments amplified with the primers LOXA-4BSL2/R, an additional polymorphism was detected. While the majority of the genotypes with high

LPX activity showed a sequence fully matching the GenBank accession DQ474240 corresponding to *Lpx-B1.1* locus (Carrera et al., 2007), the LOXA-4BSL2/R amplicon sequence of six genotypes (Trinakria, Matarese, Timilia, Capeiti 8, Nefer and Kiperounda) was different from previously published lipoxygenase (*LoxA*) cDNA from barley (van Mechelen et al., 1995). The *Lpx-B1.1* sequences from the indicated genotypes differed by a large deletion in the intron region (and consequently show a smaller size in respect to control UC1113, see Fig. 8) and are identical to the sequence "Jennah Khetifa" (J4.2 *Lpx* genomic sequence) reported by Hessler et al. (2002), where only the first half of the Stowaway element and the last 11-bp inverted repeat was present. The polymorphism seen among durum wheat genotypes evaluated at this site indicates potential instability caused by the MITE insertion in the Stowaway elements (Hessler et al., 2002).

4.1.3 Effect of processing on pasta colour in selected genotypes contrasting for YPC and LPX activity

Semolina samples obtained from four durum wheat genotypes selected for contrasting value of endogenous YPC and LPX activity were used to evaluate the role of LPX enzymes on pigment loss during pasta processing. Creso and Cosmodur with extremely low levels of endogenous LPX activity in the wholemeal (0.20 and 0.22 EU/g dw, respectively), showed undetectable activities in semolina and in the final product. On the contrary, Trinakria and Primadur, showing a high LPX activity on wholemeal (7.85 and 4.52 EU/g dw, respectively), were subjected to a reduction of LPX activity after milling (62.5 and 52.2%, respectively) and pasta processing (Fig. 9A). YPC also showed a marked reduction, moving from wholemeal to semolina (Fig. 9B). On average, the reduction in carotenoid content was about 12%. Primadur and Cosmodur showed the highest and the lowest YPC loss (20% and 4.3%, respectively).

A low-temperature drying procedure (50 °C for 18 h) was applied after pasta extrusion to evaluate the effect of a long drying period on pasta YPC. A strong reduction was observed for pasta samples produced with semolina of Trinakria and Primadur. Pasta YPC was 22.2 and 22.9% less than semolina for Trinakria and Primadur, respectively. Samples from Creso and Cosmodur showed a much reduced carotenoid loss (8 and 4.9%, respectively).

Therefore the *Lpx-B1.1* deletion seems to be a valid tool for durum breeding programs aimed to improve pasta colour as is responsible for the observed differences

in LOX activity that influence YPC changes occurring during pasta processing besides the differences in the degradation of pigments during grain development.

4.2 Identification of new alleles at the *NPR1* gene of durum wheat: a sequence controlling the non-host resistant response to disease (work carried out at UCDavis c/o Prof. J. Dubcovsky)

Full-length *TdNPR1* cDNA cloning

4.2.1 Isolation of the full-length TdNPR1 cDNA

Analysis of nullisomic-tetrasomic DNAs suggests that the wheat NH1 homolog is located on chromosome 3 (Fig. 10a, b). In rice NH1 is found on chromosome 1, which is orthologous of chromosome 3 in wheat (Sorrells et al., 2003). The syntheny between *TdNPR1* and the *OsNH1* confirms the orthology between the sequence target of our cloning effort and the rice NH1.

The full-length *TdNPR1* cDNA sequence was reconstituted by cloning the first part from DNA of BAC clone (from start codon, nucleotides 1-485) and the second part from cDNA of Langdon cultivar (nucleotides 365-1770, falling in 3'UTR) (Fig. 5). The ORF of 1734-bp encoded a protein of 578 amino acids. Both fragments obtained and fused (DNA from BAC clone and cDNA from) are more similar to *T. urartu* than to *Ae. speltoides* confirming that they are both from the A genome and not chimeric of homeologous copies (Fig. 11).

Comparison of the cDNA sequence and the genomic sequence revealed that the wheat *TdNPR1* gene has four exons and three introns. The same gene structure was observed in *HvNPR1* and *OsNH1* with the identical position of the introns in all orthologous forms, indicating the *NPR1* is structurally conserved (Fig. 2).

4.2.2 Phylogenetic analysis

Sixtheen different *NPR1* homologs genes from different plant species were retrieved through BLASTP searches (http://www.ncbi.nlm.nih.gov/BLAST and http://www.brachybase.org/blast/). Both duplicated genes *NH1* and *NH2* of rice were used as search queries (the two proteins shared limited identity, 44%). TdNPR1 was 79% identical to OsNH1 and 43% identical to OsNH2. Plant *NPR1* genes could be grouped into two clusters, showing the relationship between *NH1* and *NH2* in dicots and monocots species (Fig. 12). The rice *NH1*, durum wheat, barley and *Brachipodium* hortologues grouped together in a cluster that included the *Arabidopsis NPR1* gene.

These results suggest that *NH1* and *NH2* genes are a result of a duplication that preceded the separation between monocots and dicots.

Characterization of *TdNPR1* mutants by TILLING

4.2.3 Mutations detected in T. urartu and Ae. speltoides genomes

The two pairs of specific primers, designed in this work, amplified fragments of 730-bp and 774-bp in TdNPR-A1 and TdNPR-B1 copies, respectively (Fig. 10a, b). The fragment of 730-bp derived completely from the exon 2; the other one contained the first 37-bp of the intron 1 and after the rest of the sequence of exon 2. In the tetraploid library, we detected 7 and 9 mutants for A and B genomes, respectively. Six of the 7 mutants selected from the A genome, were missense or non-silent mutations and 1 was silent; 5 mutations were in heterozygous and 2 in homozygous state. For the 9 mutants identified in the B genome, 7 were missense or non-silent and 2 were silent mutations; seven were in homozygous state (Tab. 8). Sequencing confirmed that all mutations were G to A or C to T transition as expected from alkylation by EMS.

The sequence of TdNPR1 protein and the homologs from barley (577 aa; accession no. CAJ19095.1), rice (583 aa; accession no. AAP92751.1), maize (480 aa; accession no. NP_001152107.1), *Brachypodium* (578 aa; accession no. Bradi2g05870.1) and *Arabidopsis* (584 aa; accession no. NP_176610) were aligned using ClustalW2 (Fig. 13a) to identify the conserved regions of the protein. BTB/POZ and ankyrin repeat domain are indicated above the sequences.

Putting focus on the coding sequence from exon 2 surrounding the domain of interest, we reported also in figure 13b an enlargement of alignment, to show 13 mutations selected in A (*T. urartu*) and B (*Ae. speltoides*) genomes, in a perfectly conserved position among the analyzed sequences. In order to obtain an approximate quantitative measure for the degree of conservation of these 13 changes, we projected them onto the BLOSUM62 matrix (Henikoff and Henikoff, 1992) (Tab. 8). This matrix is derived from counting the frequency of amino acid pairs at a given position in sequence alignments by focusing on evolutionarily conserved sequence blocks (Henikoff et al., 2000). Residues that are conserved completely on sequences alignment of protein are expected to be important for function, and even a conservative substitution at one of these residues may affect protein function. Strongly conserved positions are expected to be unable to tolerate most substitutions, whereas weakly conserved positions are expected to tolerate more substitutions. Amino acid substitutions with BLOSUM62 scores ≥ 0 are classified as conservative substitutions

(Cargill et al., 1999) these substitutions are predicted as tolerated. Substitutions with negative scores are classified as non-conservative changes (Cargill et al., 1999), and these changes are observed less frequently than expected by chance; these substitutions are predicted as deleterious (Ng and Henikoff, 2001).

The 15 M_3 progeny, corresponding to heterozygous mutants selected from A genome, were genotyped. Homozygous individuals were backcrossed to wild type Kronos to reduce the EMS-induced mutations in non target loci. For the A genome we selected mutant T4-2383 which had the lowest BLOSUM62 score (-2) and was present in a relatively conserved position among the grasses (all G) but that was different in *Arabidopsis* (C, G to C change BLOSUM62 = -3). As back up mutations we selected 577 and 813. These mutations showed positive BLOSUM62 values (1) and therefore were not predicted to have strong effects on the protein structure and function. For the B genome copy of *NPR1* we selected mutant T4-308 because of its low BLOSUM62 score (-2) (Tab. 8) in a perfectly conserved position among the analyzed sequences (Fig. 13b). We have selected the homozygous mutants 577, 813, 2383 (A genome) and 308, 2368 (B genome).

The selected mutants will be backcrossed twice to the non-mutagenized Kronos lines to reduce the load of background mutations, before testing the effect of the mutations.

4.3 Marker-based procedures to develop a gene-pyramiding schedule in durum wheat

The whole procedure followed to establish the marker assisted breeding programme is summarized in figure 4.

4.3.1 Effects of the chromosome region including the Gpc-B1 locus on kernel weight and grain protein content

The amplification profile of the molecular marker *Xuhw89*, was assessed in four genotypes (UC1113, 5BIL-42, Creso, PR22D89); UC1113, the breeding durum line carrying the *Yr36–Gpc-B1* gene was used as donor line. In particular, a peak of 120-bp was amplified in UC1113, while a peak of 124-bp was found in 5BIL-42, Creso and PR22D89. On the light of this polymorphism, UC1113 was crossed to the three genotypes in order to transfer the *Gpc-B1* gene by following the linked allele at the *Xuhw89* locus. The marker showed a codominant inheritance, therefore allowing selection of homozygous plants for the functional allele during the selfing generations.

In fact, F_1 plants were characterized by the presence of both peaks (120 and 124-bp; Fig. 14).

Segregation ratios in F_2 plants of the cross UC1113 x PR22D89 (26 homozygous for *Gpc-B1* allele, 56 heterozygous, 22 homozygous for non functional allele and 15 missing data) and F_2 plants of the cross UC1113 x 5BIL-42 (24 homozygous for *Gpc-B1* allele, 50 heterozygous, 29 homozygous for non functional allele) fit a 1:2:1 genotypic ratio thus confirming that the grain protein content in UC1113 was controlled by a single dominant gene (Tab. 9). Hereafter, the lines carrying the *Gpc-B1* introgression will be referred to as *Gpc-B1* lines.

As the DIC *Gpc-B1* allele was shown to decrease grain size in UC1113 making the grain-filling period shorter (Uauy et al., 2006a), during 2008 SKW was evaluated in both parents and F_2 lines grown in the greenhouse to test if the differences in the length of the grain-filling period were associated with differences in grain size. The single kernel weight was determined on 50 plants UC1113, 50 plants PR22D89 and in our population F_2, determining also the protein content (Tab. 10). UC1113 confirmed lower kernel weight than PR22D89 (without DIC segment), characterized by high kernel weight. The average weight for UC1113 plants was 0.03 mg; while the average GPC was 15.8%. PR22D89 showed an average weight of 0.05 mg and an average GPC of 14.7%. The average weight observed in F_2 plants was 0.04 mg; instead, the average GPC observed was 16.5%. Based on these results, F_2 plants UC1113 x PR22D89 *Gpc-B1* that showed higher GPC and SKW values with respect to both parents (Fig. 15a) were chosen and crossed to plants F_2 5BIL-42 x PR22D89 (homozygous for *Pm36* gene) which were resistant to powdery mildew, in order to obtain plants with both functional *Gpc-B1* allele and *Pm36* gene.

During the 2009, following self-pollination of UC1113 x PR22D89 F_2 plants, 101 families F_3 were obtained. To ensure the actual genotypic state of F_3 families, to evaluate the effect of the DIC *Gpc-B1* allele on single kernel weight and GPC, and to observe its variation, 15 families homozygous for *Gpc-B1* allele, 20 families heterozygous and 15 families homozygous for non functional allele were randomly chosen. The profiles obtained sustained the validity of InDel marker *Xuhw89*. SKW and GPC were evaluated in both parents, in F_1 plants and F_3 families chosen (Tab. 11). UC1113 confirmed again lower kernel weight than PR22D89; the average single kernel weight for UC1113 plants was 0.042 mg; while the average GPC was 16.2%. PR22D89 showed an average weight of 0.055 mg and an average GPC of 14.3%. The average

weight observed in F_1 plants was 0.049 mg; instead, the average GPC observed was 16.1%. F_3 plants showed an average weight of 0.048 mg and an average GPC of 16.4% .

GPC was higher in the lines carrying the DIC *Gpc-B1* introgression relative to the controls (Tab. 12). Within the F_2 population UC1113 x 5BIL-42 the *Gpc-B1* lines exhibited an increase in GPC of 11.1% in the homozygous and 6.5% in the heterozygous; within the F_2 population UC1113 x PR22D89 the *Gpc-B1* lines were with higher GPC of 8.7% in the homozygous and 5.4% in the heterozygous and, ultimately, within the F_3 population UC1113 x PR22D89 the *Gpc-B1* lines exhibited an increase in GPC of 10.1% in the homozygous and 4.4% in the heterozygous. Moreover, among 262 plants F_3 UC1113 x PR22D89, 76 lines (including homozygous and heterozygous for *Gpc-B1* allele) showed an higher GPC and SKW than both parents (Fig. 15b).

The presence of the DIC *Gpc-B1* segment is also associated with significant differences in maturity dates as reflected by earlier dates of flag leaf senescence in the *Gpc-B1* lines (Uauy et al., 2006a, b; Brevis and Dubcovsky, 2010). Since heavy infection of leaf rust occurred in the field (2009), it was impossible to score differences in earlier flag leaf senescence relatively to *Gpc-B1* lines and the lines homozygous for non functional allele of F_3 population UC1113 x PR22D89.

Moreover, during 2010 the homozygous state for *Gpc-B1* allele and *Pm36* gene in 200 plants F_2 UC1113 x 5BIL-42 was confirmed using both markers *Xuhw89* and *mgbe684*. Some plants were chosen and crossed with the durum wheat cultivars Creso, Pedroso, Primadur and Neodur. The homozygous state for *Gpc-B1* allele in 162 plants F_4 UC1113 x PR22D89 was also confirmed; we have randomly chosen 54 plants.

4.3.2 Validation of molecular marker linked to powdery mildew resistance

5BIL-42, the powdery mildew resistant parental line, was used as donor line. To check the polymorphism revealed by molecular marker *mgbe684*, the founder genotypes used in the breeding programme (UC1113, 5BIL-42, Creso, PR22D89) were used. Also in this case a polymorphism was found between 5BIL-42 and the other genotypes, allowing to set up of a program of crosses for transferring the resistance to powdery mildew. In particular, two peaks of 220+227-bp were amplified in 5BIL-42, while a peak of 121-bp was found in UC1113, Creso and PR22D89. On the light of this polymorphism, 5BIL-42 was crossed to the three genotypes in order to transfer the *Pm36* gene by following the linked allele at the *mgbe684* locus. Also this marker showed a codominant inheritance, therefore allowing selection of resistant homozygous

plants during the selfing generations. In fact, F_1 plants were characterized by the presence of all three peaks (120, 121 and 124-bp; Fig. 16).

The segregation patterns in F_2 populations 5BIL-42 x PR22D89 grown in the greenhouse (2008) and UC1113 x 5BIL-42 grown in field (2009) confirmed that resistance was controlled by a single dominant gene (Tab. 13-14). Segregation analysis in F_2 plants of the cross 5BIL-42 x PR22D89 (80 resistant, 137 segregating, 65 susceptible and 4 missing data) and F_2 plants of the cross UC1113 x 5BIL-42 (22 resistant, 40 segregating, 15 susceptible and 26 missing data) fit a 1:2:1 genotypic ratio.

Temperature and moisture conditions both in greenhouse and field were favourable for initiation and development of a natural infection of powdery mildew, therefore it was possible to asses the resistance level of plants. The segregation pattern of F_2 population 5BIL-42 x PR22D89 tested in the greenhouse and F_2 population UC1113 x 5BIL-42 tested in the field, confirmed again that resistance was controlled by a single dominant gene (Tab. 13-14). Segregation in F_2 plants of the cross 5BIL-42 x PR22D89 (204 resistant and 82 susceptible) and F_2 plants of the cross UC1113 x 5BIL-42 (69 resistant and 34 susceptible) fit a 3:1 phenotypic ratio.

During the 2009, following self-pollination in field of 5BIL-42 x PR22D89 F_2 plants, 235 families F_3 were obtained. To ensure the actual homozygous state of *Pm36* gene, 35 F_3 families were chosen that showed a correspondence of resistance across the phenotypic and genotypic screening; the profiles obtained sustained the validity of EST-SSR marker *mgbe684*.

4.3.3 Stacking for grain protein content and powdery mildew resistance

During 2008, the evaluation of SKW and GPC allowed to identify some F_2 plants UC1113 x PR22D89 *Gpc-B1* that showed the higher values of GPC than both parents (Fig. 15a). In the following generation these plants were crossed with plants F_3 5BIL-42 x PR22D89 *Pm36*.

During 2010, the alleles heterozygous state of 231 plants F_1 (UC1113 x PR22D89) x (5BIL-42 x PR22D89) were analyzed using both markers *Xuhw89* and *mgbe684*; 35 heterozygous plants were randomly chosen to select, in the next months, the homozygous plants for *Gpc-B1* and *Pm36* genes, at the same time.

4.3.4 Stacking for leaf rust resistance

Creso, the leaf rust resistant parental line, was used as donor line. To check the polymorphism revealed by molecular markers *SWES619* and *Xgwm146*, the founder

genotypes used in the breeding programme (UC1113, 5BIL-42, Creso, PR22D89) were used.

Marone et al. (2009) indicated a major QTL able to confers nearly complete and durable resistance to leaf rust; it was located within the 6 cM interval comprised between markers *Xgwm344a* and *Mag4362*, with *Mag4362* as peak marker. Both markers were not able to reveal an useful polymorphism in the nucleotide sequence of the genotypes used. *Xgwm344a* showed an unclear profile and *Mag4362* was monomorphic between the donor line Creso and the recipient lines UC1113 and 5BIL-42, while was polymorphic with the cultivar PR22D89. To overcome this limit, other two flanking markers to leaf rust resistance gene *Lr14c* were chosen: *SWES619* and *Xgwm146* (Fig. 17a, b). The EST-SSR marker *SWES619* (spaced from the *Lr14c* gene 7-cM down) showed a dominant inheritance; while, the SSR marker *Xgwm146* (spaced from the gene 14-cM up) showed a codominant inheritance, useful to allow selection of resistant homozygous during the selfing generations.

During 2010, the allele state of 95 plants F_1 (Creso x PR22D89) x UC1113, and 38 plants F_1 (Creso x PR22D89) x 5BIL-42 was analyzed using, respectively, markers *Xuhw89* and *mgbe684,* and including markers *Xgwm146* and *SWES619*. We have selected 24 and 16 heterozygous plants, respectively.

The allele state of 120 plants F_1 (UC1113 x PR22D89) x Creso, and 54 plants F_1 (UC1113 x 5BIL-42) x Creso was also analyzed; 75 and 33 plants were selected, respectively. The electropherograms shown in Fig. 17b highlight the possibility to screen and select, within F_1 (UC1113 x PR22D89) x Creso, the genotypes heterozygous for *Lr14c* gene but homozygous for the allele of PR22D89 (167-bp; 38 plants) or UC1113 (157-bp; 37 plants).

4.3.5 Stacking for yellow pigment content

Pedroso and Primadur two cultivars with high grain yellow pigment content (YPC) were used as donor lines. To check the polymorphism revealed by molecular markers *Xgwm786b* and *Xgwm1061*, *Xgwm344* and *Xgwm282* the founder genotypes used in the breeding programme (UC1113, 5BIL-42, Creso, PR22D89) were used. Not all these markers were polymorphic if considering all genotypes involved in the pyramiding (Fig. 18a, b, c, d).

During 2010 the allele state of 81 plants F_1 (UC1113 x PR22D89) x Pedroso, and 32 plants F_1 (UC1113 x 5BIL-42) x Pedroso was analyzed using the marker *Xgwm786b*; 51 and 22 plants heterozygous were selected, respectively.

The allele state of 85 plants F_1 (UC1113 x PR22D89) x Primadur, and 29 plants F_1 (UC1113 x 5BIL-42) x Primadur was also analyzed using the markers *Xgwm1061*, *Xgwm344* and *Xgwm282*; 56 and 7 plants heterozygous were selected, respectively.

The electropherograms shown in Fig. 18a highlight the possibility to screen and select, within F_1 population (UC1113 x PR22D89) x Pedroso, the genotypes heterozygous for YPC QTL but homozygous for the allele of PR22D89 (139-bp) or UC1113 (151-bp); Fig. 18b and 18c highlight the possibility to screen and select, within F_1 population (UC1113 x PR22D89) x Primadur, the genotypes heterozygous for *Psy-A1* gene but homozygous for the allele of PR22D89 (165.7-bp, 14 plants; 124-bp, 17 plants).

Phenotyping data for the evaluation of the yellow pigment content showed that PR22D89 has an optimal semolina and pasta colour (De Vita, personal communication 2010), UC1113 has an intermediate pasta colour (Carrera et al., 2007), Creso and 5BIL-42 has a low semolina yellow colour (De Simone et al., 2010; De Vita, personal communication 2010).

4.3.6 Stacking for soil borne cereal mosaic virus (SBCMV) resistance

Neodur, the SBCMV resistant parental line, was used as donor line. To check the polymorphism revealed by molecular markers *bcd348* and *Xgwm1128*, the founder genotypes used in the breeding programme (UC1113, 5BIL-42, Creso, PR22D89) were used (Fig. 19a, b).

Russo et al. (unpublished) identified a major QTL for resistance to SBCMV in the durum cultivar Neodur on the short arm of chromosome 2B. The highest LOD values were registered for two DArT markers, while two SSR markers were found flanking the QTL in distal and proximal position. The marker *bcd348*, mapping at 13 cM from the peak marker in distal position, is associated to the resistance phenotype in the segregating population Cirillo x Neodur, utilized for the QTL analysis. The second marker, *Xgwm1128*, positioned at 26 cM from the peak marker in proximal position, is not associated to the resistance phenotype in the segregating population Cirillo x Neodur, but revealed to be effective in predicting resistance/susceptibility in a panel of 25 durum wheat varieties for which information on level of resistance to the virus was available.

The two markers were taken into consideration together in this work to transfer the Neodur resistance to PR22D89, even if different cycles of phenotypic evaluation

will be necessary besides molecular analysis due to the high probability of a double recombination event between the two markers and the resistance gene.

5. DISCUSSION

5.1 Distribution of the Lpx-B1.1 deletion across durum wheat germplasm

In the present work YPC, LPX activity and the polymorphism at the *Lpx-B1.1* locus were assessed on a durum wheat germplasm collection. The preliminary screening of 71 durum wheat genotypes showed a great genetic variability for YPC and LPX activity. The range of variation detected for YPC is similar to that described in other studies (Digesù et al., 2009; Konopka et al., 2005; Hidalgo et al., 2006; Leenhardt et al., 2006b) suggesting that genetic variability still exists in modern durum wheat germplasm to further increase YPC content. The general trends toward an increased YPC and a decreased LPX activity that can be observed in the cultivar released during the XX century (Fig. 7) confirms that "yellow colour" has became a sign of quality and that breeders have focused particular attention on high YPC/low LPX activity during selection of new genotypes.

The differences in pasta colour associated with *Lpx-B1* locus were most likely due to differences in the degradation of the pigments during pasta processing rather than differences in the degradation of pigments during grain development. Indeed pasta products made from raw material with a high LPX activity developed an undesirable loss of colour during processing (Fig. 8) (Borrelli et al., 2003; Trono et al., 1999). The amount of pigment losses during pasta processing can be correlated to the LPX activity of the corresponding semolina.

A part few exceptions, the molecular marker validated was able to highlight the deletion at the *Lpx-B1.1* locus, confirming a clear correlation between high/low LPX activity and presence/absence of the band for the samples evaluated. The selection of genotypes carrying the *LpxB1.1* deletion can therefore be considered an essential component to ensure a yellow colour in the final durum wheat products. These results were published in the manuscript: De Simone V., Menzo V., De Leonardis A.M., Ficco D.B.M., Trono D., Cattivelli L., De Vita P., 2010. Different mechanisms control lipoxygenase activity in durum wheat kernels. *Journal of Cereal Science* 52: 121-128.

5.2 Characterization of TdNPR1

The key regulator of SA-mediated resistance, *NPR1* or *NH1*, is functionally conserved in plant species across the plant kingdon (Durrant and Dong, 2004). Homolog of *AtNPR1* gene have now been isolated from several plants species (Kogel and Langen, 2005; Liu et al., 2002; Zhu et al., 2003; Chern et al., 2005b; Meur et al., 2006; Malnoy et al., 2007; Endah et al., 2008), but functional analysis has been carried out primarily only in the model plants *Arabidopsis* and rice. The description of NH1 in rice provided the closest reference for other grasses, including wheat.

In this study we report the cloning of the full-length *NPR1*-like gene in durum wheat, from cv. Langdon. The deduced amino acid sequence of *TdNPR1* had 93% sequence identity with *HvNPR1* (Kogel and Langen, 2005) and 79% sequence identity with *OsNH1* (Quanhong et al., 2003). These levels of divergence are close to the average distances observed between proteins from those species suggesting and average rate of divergence. As *NPR1* of barley, rice, maize, *Brachipodium* and *Arabidopsis*, *NPR1* in wheat contained a predicted BTB/POZ domain (amino acids 54-131) and an ankyrin repeats domain (amino acids 290-365) (Fig. 13c). The sequence showed a higher identity with barley (Fig. 13a) both for BTB/POZ domain (90%) and ankyrin repeat domain (98%) which is slightly more conserved than the entire protein.

Amino acid crucial for the NPR1 function as defined by genetic mutants, such as *npr1-1* (H), *npr1-2* (C) (Cao et al., 1997) and *nim1-4* (R) (Ryals et al., 1997) were also conserved in *TdNPR1*. Other conserved cysteins such as Cys^{82}, Cys^{150}, Cys^{155}, Cys^{160} and Cys^{216} that have been shown in previous studies to be involved in oligomer-monomer transition (Mou et al., 2003; Tada et al., 2008) were also conserved in the predicted wheat protein. Cys^{82} present in BTB/POZ domain is required for SA-mediated activation of *PR1* (Rochon et al., 2006); Cys^{306} is a crucial amino acid within the ankyrin repeats to mediate the interactions with the TGA transcription factors. Mutations in this amino acid abolishes the NPR1-TGA complex formation, PR gene expression, and SAR (Cao et al., 1997; Ryals et al., 1997; Zhang et al., 1999; Després et al., 2000, 2003) (Fig. 13a). Both motifs mediate the interactions of NPR1 protein with other proteins (Sandhu et al., 2009).

NPR1 proteins belong to a multigene family. Gene duplication may have resulted in either functional diversification or functional redundancy of NPR1. In the *Arabidopsis* genome there are five paralogs of *NPR1* (*NPR2*, *NPR3*, *NPR4*, *BOP1* and *BOP2*; Zhang et al., 2006); five *NPR1*-like genes were also found in rice (*NH1*, *NH2*, *NH3*, *NH4*, *NH5*) and phylogenetic analysis showed that *OsNH1* is the closest member

of the rice family to *NPR1* (Chern et al., 2005b). Moreover, using HvNPR1 protein to BLASTP search (http://www.brachybase.org/blast/) in the *Brachypodium* genome database (http://www.brachypodium.org/) were retrieved five NPR1-like genes (Bradi2g05870.1, Bradi2g51030.1, Bradi1g12870.1, Bradi2g60710.1, Bradi4g43150.1) with Bradi2g05870.1 that appeared as the highest hit and Bradi2g51030.1 in second, most likely the putative *Brachypodium* orthologs of *Arabidopsis* NPR1 and NPR2, respectively.

5.2.1 TdNPR1 mutants

Many traits that are important for wheat production and quality would benefit from the ability to modify and understand gene function that in wheat is still not fully developed due to several limitations. The large size of the wheat genome and its high content of repetitive DNA are important obstacles for the complete genome sequencing of wheat. In addition, wheat is a polyploidy species with most genes represented by two (in tetraploid) or three (in hexaploid) homoeologous copies that share approximately 93–96% sequence identity (Uauy et al., 2009). Gene duplication limits the use of forward genetics phenotypic screens as the effect of single-gene knockouts are frequently masked by the functional redundancy of homoeologous genes present in the other wheat genomes (Lawrence and Pikaard, 2003).

Despite these barriers, a broad range of genomic resources have been developed for wheat (EST, BAC library) and have facilitated the positional cloning of several agronomically important genes, but the functional validation of the candidate genes has relied mainly in transgenic approaches that are laborious, low throughput and require regulatory oversight. The ability to determine the function of these and other genes will ultimately depend on the establishment of robust, flexible and high-throughput reverse genetic tools.

Reverse genetic approaches use sequence information to identify candidate genes and then study the phenotype of the mutant alleles to determine gene function. TILLING (targeting induced local lesions in genomes) is a flexible reverse genetics approach that generates a lasting resource that can be utilized to screen multiple targets. With TILLING, a library of DNA samples from thousands of individuals can be screened in a high-throughput manner for induced or naturally occurring single-nucleotide polymorphisms (SNPs) (Colbert, 2001; McCallum et al., 2000), to rapidly generate and identify many novel alleles, some of which could have a phenotypic effect

and represent a rich resource of genetic diversity at the target loci for potential modulation of characteristics.

Recently, a powerful reverse genetics approach was implemented in wheat through the combination of ethyl methane sulphonate (EMS)-mediated mutagenesis and TILLING technology (Slade et al., 2005). Briefly, a TILLING screen starts with PCR amplification of a target region from pooled DNA of mutagenized plants. This PCR product is heated and reannealed to allow heteroduplexes to form between mutated and wild-type DNA. Heteroduplexes are identified through cleavage of mismatched sites by the *Cel*I endonuclease (Colbert, 2001; Oleykowski et al., 1998). Cleavage products can be visualized by size separation from the full-length PCR product on a polyacrylamide gel to identify mutant individuals. The individuals composing the positive pools are sequenced to determine which individual carries the mutation and to reveal the exact nature of the mutation. Using this technology, large populations can be screened rapidly to obtain an allelic series that contains numerous point mutations in any targeted gene. Gene function is assigned based on phenotypic evaluation of the mutant individuals.

Alleles generated by TILLING can be readily used in traditional breeding programs since the technology is non-transgenic and the mutations are stably inherited. These advantages are reflected by the successful implementation of TILLING in several plant species such as *Arabidopsis* (McCallum et al., 2000), maize (Till et al., 2004b), wheat (Slade et al., 2005; Dong et al., 2009), barley (Caldwell et al., 2004), rice (Till et al., 2007, Wu et al., 2005), pea (Dalmais et al., 2008), potato (Muth et al., 2008), *Lotus japonicus* (Perry et al., 2003; 2009), soybean (Cooper et al., 2008) and *Medicago truncatula* (Le Signor et al., 2009).

The ability to understand gene function will become increasingly important as more sequence information is generated in wheat. Thus, there is a need for a diverse set of publicly available reverse genetic resources in wheat to assist with the functional validation of candidate genes.

We decided to screen for *TdNPR1* mutation in our tetraploid wheat TILLING population because it is then easier to cobine the mutations in two genomes than in three. As reported by Uauy et al. (2009), each mutant library is characterized by TILLING multiple genes, revealing high mutation densities in both the hexaploid (~1/38 kb) and tetraploid (~1/51 kb) populations for 50% GC targets. These mutation frequencies predict that screening 1536 lines for an effective target region of 1.3 kb with 50% GC content will result in ~52 hexaploid and ~39 tetraploid mutant alleles. In our analysis, by screening 1368 lines for a 730- and 774-bp target regions we would expect

to recover approximately 20 mutant alleles (50% GC content); whereas our screening yielded 13 mutations with an average GC content of 42% in the regions targeted.

The effects on the coding sequence of the identified EMS mutations were predicted with CODDLE program. In the tetraploid screening the predicted effects were 6 truncation changes (nonsense or alternative splicing sites) for a 1300-bp target region. Since we did not identify any of these in our targeted fragments it might be worth to till another region of the protein, screening for a ~1500-bp target region including the junction sites intron/exon in the remaining part of the sequence, beyond exon 2.

We identified 13 mutations within the coding region of exon 2, our targeted region of the genome including the ankyrin repeat domain, that can potentially alter the capability to mount a SAR response. We analyzed the distribution of amino acids that were replaced by EMS mutagenesis in our collection of alleles changes in functionally impaired mutant lines and we asked the question whether replacements in some amino acids were more likely to result in a non-functional protein than others. To obtain an approximate quantitative measure for the degree of conservation of these 13 possible changes, we projected them onto the BLOSUM62 matrix. Among the 13 mutations only 5 EMS inducible amino acid replacements have a negative score (i.e. are infrequently observed in evolution) and hence are more likely than conservative exchanges to have a detrimental effect, suggesting an high probability of substantial modifications in the protein structure and/or function. Replacements in functionally defective EMS alleles coul be represented by mutant we have selected (577, 813, 2383 for the A genome copy and 308, 2368 for the B genome copy of NPR1), located in a perfectly conserved position among the analyzed sequences surrounding Cys^{306}; mutation 2368 adjacent to Cys^{216} (S to F change BLOSUM62= -2) could impair the crucial role in which this C is involved, about the oligomer-monomer transition.

If both the A and B genome selected mutations result in non-functional alleles, then plants homozygous for both A and B mutations should be impaired in their ability to induce *PR* gene expression and mount a SAR response, after treatment with SA or INA or challenge with pathogens. If this response is confirmed, we will also analyze the single A genome or B genome mutants to determine the effect of dosage of this critical gene.

Since there are several paralogs of NPR1 we cannot rule out a potential functional redundancy of paralogs, which may complicate the detection of a phenotype in the TdNPR1 TILLING mutants. RNAi lines with suppressed *NH1* accumulation were more susceptible to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) compared with the wild-type,

indicating that the functionality of *OsNH1* is required for the basal resistance to *Xoo*. The role of NH2 and NH3 in *Xoo* resistance was ruled out also by a lack of increased resistance when these where over-expressed in a susceptible background (Yuan et al., 2007).

The clear-cut phenotypes of NPR1 deficient rice and *Arabidopsis* lines suggest that TdNPR1 mutants may also show a phenotype that will start elucidating the role of these important proteins in wheat.

5.3 Gene Pyramiding using molecular markers

Developing elite breeding lines and varieties often requires plant breeders to combine desirable traits from multiple parental lines. The process of gene pyramiding can be accelerated by using molecular markers to identify and keep plants that contain the desired alleles combinations. Furthermore, reducing the number of generations to be evaluated is another key-point to accelerate the breeding process and to decrease costs.

In the present work we develop strategies for marker-assisted gene pyramiding of six desired traits in durum wheat (Fig. 4). A first aim was to select breeding lines having both beneficial genes *Gpc-B1* and *Pm36* in the genetic background of the elite durum cultivar PR22D89, characterized by a high gluten quality and good yield. Considering that both donor lines, UC1113 and 5BIL-42, are the product of a extended pre-breeding activity, there should not be a negative effect on the genetic background of the elite cultivar PR22D89. Moreover, the future development of the breeding programme genotypes will further increment the proportion of the genome of PR22D89 through several backcrosses already scheduled.

The *Pm36* gene from 5BIL-42 line confers a good protection against powdery mildew in wheat. We have succeeded to combined powdery mildew resistance and high-GPC into durum wheat lines and selected homozygous genotypes. In order to increase our stacking schedule, we have used these homozygous lines to carry out new cross with other donor cultivars (Creso, Pedroso, Primadur, Neodur) and we have confirmed the heterozygous state for the respective desirable genes (leaf rust resistance, yellow pigment content and soil borne cereal mosaic virus response).

The *Lr14c* locus from Creso cultivar confers nearly complete and durable resistance to leaf rust. Durum wheat Creso is still grown in marginal areas of Italy, with good grain yield performance and high grain quality, therefore it represents an optimal donor cultivar to transfer leaf rust resistance to more recent and productive genotypes

by MAS without the risk of introducing undesired traits together with the resistance gene(s).

A relatively wide ranges of variation within genotypes was found in this work for YPC without a direct correlation between the alleles of markers used and phenotype. This result can be justified with the multigenic control of YPC for which several QTLs were identified, at least three out of which with major effect on chromosomes 6A, 7A and 7B. For this reason is necessary to validate others markers associated with others major QTLs, to verify if combining several markers could be possible to define better the correlation between genotype and phenotype.

The markers used to confer resistance to SBCMV should be useful for reliable and errorless identification of resistant genotypes in the laboratory and their direct use in molecular breeding strategies should allow to enhance virus resistance, evaluating the effective introgression of the major QTL *QSbm.ubo-2BS* for SBCMV-response in experimental conditions under SBCMV infection.

Our results showed that *Gpc-B1* lines of the cv. PR22D89 and 5BIL-42 line, exhibited a good increase in GPC. Moreover, the *Gpc-B1* lines of the cv. PR22D89 showed also a no negative effects on grain weight, suggest that the potential negative impact of the *Gpc-B1* DIC allele on grain size could be limitated. The deployment of the *Gpc-B1* allele from *T. turgidum* ssp. *dicoccoides* into wheat breeding programs has the potential of improving GPC in a wide range of germplasm due to the absence of the functional allele in most of the modern tetraploid and hexaploid commercial cultivars (Uauy et al., 2006b). Although the favorable changes in GPC and beneficial effects on a number of bread and pasta-making traits described recently (Brevis and Dubcovsky, 2010; Brevis et al., 2010), the negative effect of the *Gpc-B1* allele on grain weight should be expected since, in spite of the reductions in grain weight, a significant increase in total grain protein (grain yield x protein concentration) is showed due to better N remobilization from leaves to the grain (Brevis and Dubcovsky, 2010; Kade et al., 2005; Uauy et al., 2006a; Waters et al., 2009). Therefore, on the basis of gene x genotype and gene x environment interactions, only developing near-isogenic lines (NILs, >99% identical to the recurrent parent PR22D89) will be possible to investigate better the effect of DIC *Gpc-B1* introgression on grain weight and yield penalties.

6. CONCLUSION

Marker-based gene pyramiding is a "popular" approach to develop improved breeding lines carrying specific traits on interest, usually with simple genetic bases. In this work, we designed and developed an efficient marker-based gene pyramiding strategy for durum wheat using available (and newly identify) molecular markers. Markers associated to quality traits as lipoxygenase activity, protein content and yellow pigment content, and to main disease resistances as leaf rust, powdery mildew and soil borne cereal mosaic virus.

A molecular breeding program in durum wheat was set up at CRA-CER of Foggia and it will be continued in the future by incorporating new agronomically important traits. In this way the marker-assisted breeding schedule will become a routine approach to produce new valuable durum wheat varieties that will be transferred to farmers.

During an internship undertook in the laboratory of Prof. Dubcovsky, we identified mutations within the coding region of *NPR1* gene as markers that leads to constitutive expression of defence genes in plants. These studies suggest that manipulated expression of *NPR1* or its orthologues can create broad spectrum resistance in crop plants, and therefore, could be a suitable strategy in improving crop plants for disease resistance and utilized as a molecular markers for induced resistance.

7. APPENDIX: TABLES and FIGURES

Table 1 - Comparison of major marker systems.

Marker system	PCR-based	Uses restriction enzymes	DNA amount	Loci per assay	Approx. time per assay	Specialized equipment	Comments
RFLP	no	yes	5.0 g	1 to few	5 days	Radioactive isotope	Co-dominant, reliable, often low-level polymorphism.
RAPD	yes	no	0.2 g	many	5 hours	Agarose gels	Dominant, unreliable in some situations.
AFLP	yes	no	0.2 g	many	1 day	Polyacrylamide gels/capillary	Mostly dominant, reliable, low level of polymorphism but high multiplexing capacity.
SSR	yes	no	0.2 g	1 to about 20	5 hours	Polyacrylamide gels/capillary	Co-dominant, reliable, large number of alleles.
CAPS	yes	yes	0.2 g	single	5 hours	Agarose gels	Co-dominant, reliable, low development cost.
SCAR	yes	no	0.2 g	single	5 hours	Agarose gels	Co-dominant or dominant, low level of polymorphism, low development cost.
SNP	yes	no	0.05 g	1 to thousands	1 to 5 hours	Variable	Co-dominant or dominant, very rapid result depending on technology platform. High development cost.
DArT	yes	yes	5 ng	many	1.5 to 2 days	Microarray	Dominant, ideal for fingerprinting as many loci generated from single sample.

Figure 1 - Procedural flow of marker-based gene pyramiding from k donor lines (Ishii and Yonezawa, 2007).

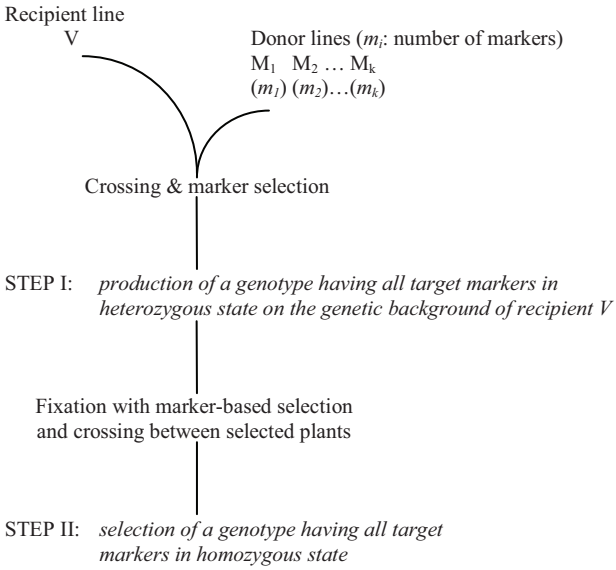


Table 2 - Examples of successful use of marker-assisted gene pyramiding in wheat (Gupta et al., 2009).

Target trait(s)	Target loci	Marker type	Effect of selection	Reference
Powdery mildew resistance	3 gene combinations	RFLP	Higher resistance in pyramided lines	Liu et al., 2000
Powdery mildew resistance	4 genes	–	Increased resistance	Wang et al., 2001
Leaf rust resistance	2 genes	STS	Successful pyramiding in F_3 lines	Singh et al., 2004
Powdery mildew resistance	3 genes	–	–	Gao et al., 2005
FHB resistance	3 QTL	SSR	Maximum gain from phenotypic selection following marker-based selection	Miedaner et al., 2006
Cereal cyst nematode resistance	2 genes (*CreX*, *CreY*)	SCAR	Higher resistance in the pyramided line	Barloy et al., 2007
FHB resistance and DON content	3 QTL	SSR	Increased gains for major QTL only	Wilde et al., 2007
PHST and GPC	One QTL for each trait	CAPS, SSR	Increased GPC or high level of PHS tolerance in BC_3F_1 plants	Gupta et al., 2008b
FHB resistance	Multiple QTL	SSR	Successful pyramiding of QTL	Shi et al., 2008
FHB resistance	3 QTL	SSR	Enhanced mean FHB resistance	Wilde et al., 2008
FHB resistance	3 QTL	SSR	Marker selection led to a slightly higher selection gain on an annual basis	Miedaner et al., 2009

Figure 2 - The comparison of the genomic structure of durum wheat, barley (accession no. AM050559.1), rice (accession no. DQ450948) and *Arabidopsis* (accession no. U87794) *NPR1*-like genes. The length of the exons of genes are indicated below them. A BTB (Broad complex, Tramtrack and Bric-a-brac) and an ankyrin repeat domain (ANK_REP_REGION) are indicated in exon 1 and exon 2, respectively.

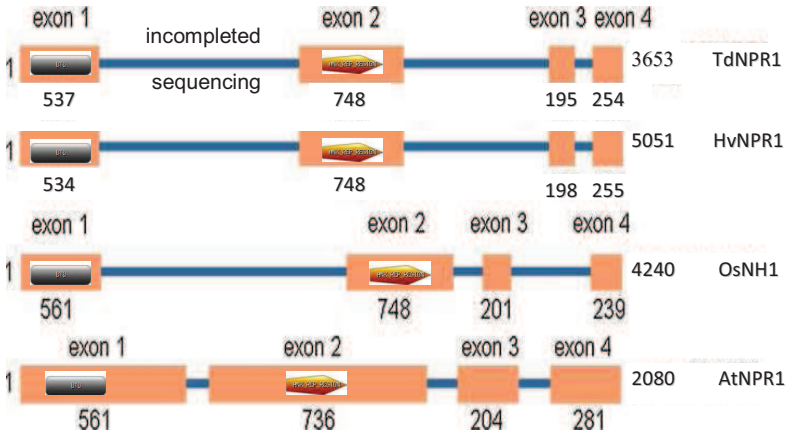


Figure 3 - Loci currently being tracked with molecular markers in wheat breeding programmes (based on information from the Wheat CAP project the Australian Winter Cereals Molecular Marker Programme and the Projects undertaken in India). **Glu** - high molecular weight glutenin loci, **Lr** - leaf rust resistance, **Yr** - yellow rust resistance, **Sr** - stem rust resistance, **Cre** - cereal cyst nematode resistance, **Rht** - dwarfing (reduced height), **Fhb** - Fusarium head scab resistance, **Cr** - crown rot resistance, **PHS** - preharvest spouting tolerance, **AlmT** - aluminium tolerance, **Yls** - yellow leaf spot resistance, **Pin** - puroindoline (grain hardness), **Gpc** - grain protein content, **Yfc** - yellow flour colour, **Rlnn** - root lesion nematode resistance, **Bo** - boron tolerance, **Pch** - *Pseudocerosporella herpotrichoides* resistance, **Bdv** - barley yellow dwarf, **Stb4** - *Septoria tritici* blotch, **Bx7** - high molecular weight glutenin subunit-gene, **BGGP** - Beta-1-3-galactosyl-o-glycosyl-glycoprotein, **GBS** - granule bound starch synthase loci, **LMA** - late maturity a-amylase, **PPO** = polyphenol oxidase, **Gw.ccsu-1A.1 & Gw.ccsu-1A.3** - QTL for grain weight, **QPhs.ccsu-3A.1** - QTL for pre-harvest sprouting, **Qfhs.ndsu-3AS** - QTL for resistance to Fusarium head blight, **Qss.msub-3BL** - QTL for resistance to wheat stem sawfly. The genes/QTL placed in the bins are shown with fraction lengths in parentheses; the genes/QTL, which could not be placed in bins are shown with no fraction lengths (Gupta et al., 2010).

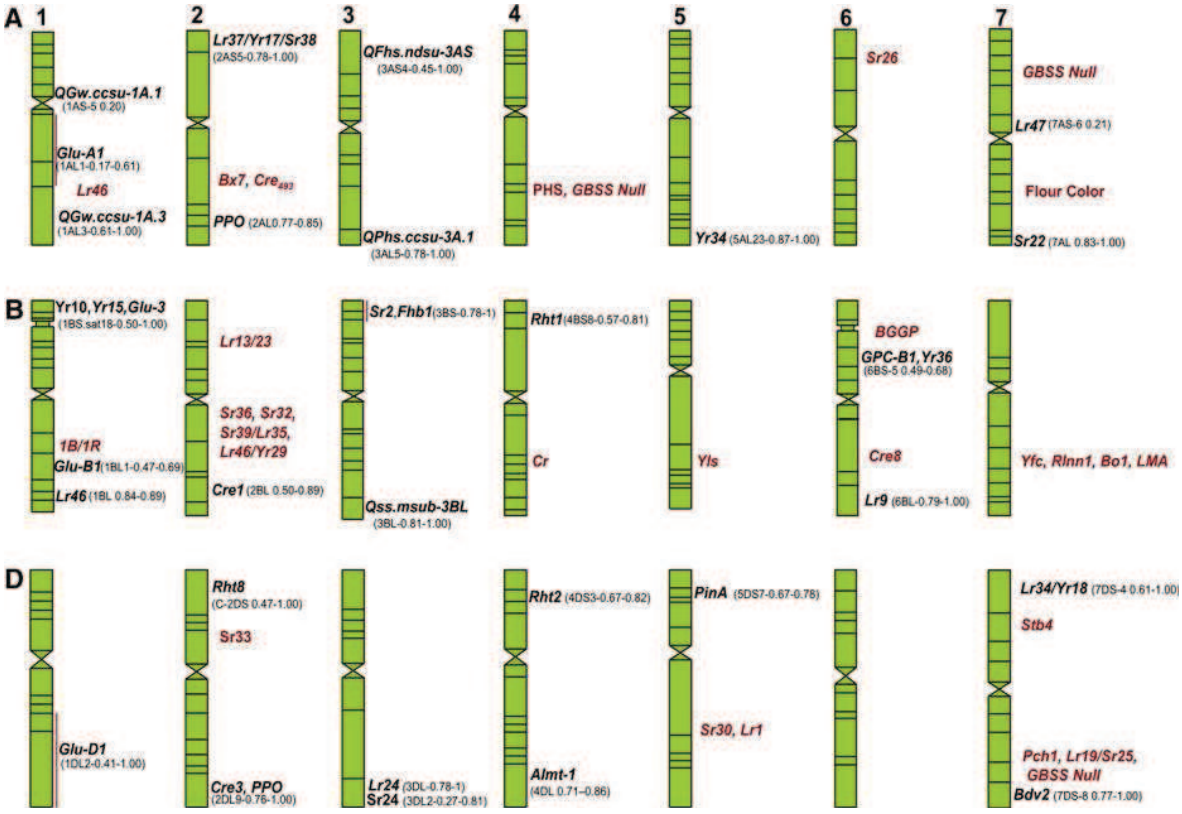


Table 3 – Targeted traits of wheat for marker assisted selection in different countries (Gupta et al., 2010).

***** http://www.csiro.au/science/psmg.html

Country	**Program**	**Targeted traits**				
		Disease resistance	**Insect**	**Virus**	**Quality traits**	**Others**
Australia	National Wheat Molecular Marker Program (NWMMP)	Cereal cyst nematode, Karnal bunt, rusts and yellow spot (tan spot)	Russian wheat aphid and Hessian fly	Barley yellow dwarf virus	Pre-harvest sprouting, grain hardness, flour color, GBSS Null	Phenology (*Rht1* and *Rht2*), boron tolerance, *salinity tolerance, *drought tolerance
USA	MASwheat	Stripe rust, leaf rust, stem rust,, *Fusarium* head blight, *Septoria* blotch, tan spot, eyespot, powdery mildew, glume blotch	Hessian fly, wheat swafly, orange wheat blossom midge, russian wheat aphid	Wheat streak mosaic virus, barley yellow dwarf virus	Gluten strength, grain protein content, grain texture, color, starch quality, nutritional value	Aluminium tolerance, drought tolerance, dwarfing genes, grain shattering, growth habit, herbicide resistance
UK	-	*Fusarium* head blight (FHB), *Septoria* tritici blotch (STB) and yellow rust	Orange blossom midge	Soil-borne mosaic virus (SBMV), barley yellow dwarf virus	Grain texture	-
CIMMYT	-	Stem rust, crown root, *Fusarium* head blight, resistance to cereal cyst nematode, flour color/*P. neglectus*	Russian wheat aphid and Hessian fly	Barley yellow dwarf virus	Swelling volume, grain hardness, dough strength, high protein content	Boron tolerance
Canada	-	Leaf rust, stem rust, common bunt, loose smut	Orange blossom midge	-	Grain protein content	Low cadmium uptake
India	DBT funded project	Leaf rust, stripe rust, stem rust	-	-	Pre-harvest sprouting, grain hardness, grain protein content, grain weight, dough strength	-

Table 4 - Molecular markers main index for Marker Assisted Selection in wheat based on information from the Wheat CAP project (**http://maswheat.ucdavis.edu/**).

* Gene/QTL not include in MASWheat website.

Trait	Gene/ QTL	Marker type	Designation	Chromosomal location	Reference
Fungi					
Leaf rust resistance	**Lr14c*	EST-STS SSR	*Mag4362* *Xgwm344a* *SWES619* *Xgwm146*	7BL	Marone et al., 2009
	Lr19	AFLP STS RFLP	Gb BF145935 *Xmwg2062* *Xpsr547* *Xwg420*	7AL 7DL	Friebe et al., 1994 Prins et al., 2001 Zhang et al., 2005 Liu et al., 2010
	Lr21	RFLP	KSUD14	1DS	Huang and Gill, 2001
	Lr29 - Lr25	SCAR	Lr29F24/R24 Lr25F20/R19	7DS (*Lr29*) 4A (*Lr25*)	Procunier et al., 1995
	Lr39 (*Lr41*)	SSR	*Xgdm35*	2DS	Pestsova et al., 2000
	Lr47	CAPS	PS10L2/R	7AS	Helguera et al., 2000
	Lr50	SSR	*Xgwm382* *Xgdm87*	2BL	Brown-Guedira et al., 2003
	Lr51 (*LrF7*)	CAPS	S30-13L/ AGA7-759R	1BL	Helguera et al., 2005
Stripe rust resistance	*Yr5*	STS	Yr5STS-7/8 Yr5STS-9/10	2BL	Chen et al., 2003
	Yr15	RFLP SSR	*XTri* *Xcdo1173* *Xbarc8* *Xgwm273*	1BS	Peng et al., 2000 http://maswheat.ucdavis.edu/
	Yr36	InDel	*Xuhw89*	6BS	Distelfeld et al., 2006
Stem rust resistance	*Sr2*	SSR	*Xgwm533* *Xgwm389*	3BS	McNeil et al., 2008
	Sr13	SSR EST-marker	*Xbarc104b* *Xbarc104c* *Xwmc580* *Xdupw167* *XCK207347* *XCD926040* *XBE403950*	6AL	Simons et al., 2010
	Sr22	SSR	*Xbarc121* *Xcfa2019* *Xcfa2123* *Xwmc633*	7AL	Olson et al., 2010 Yu et al., 2010
	Sr24	SSR AFLP	*Xbarc71* *Sr24#12* *Sr24#50*	3DL	Mago et al., 2005
	Sr25	STS	*BF145935*	7DL	Friebe et al., 1994 Zhang et al., 2005 Liu et al., 2010
	Sr26	AFLP EST-marker	*Sr26#43* *BE518379*	6A	Liu et al., 2010

	Sr35	SSR InDel	*Xcfa2193* *Xwmc559* *Xcfa2170* *Xcfa2076* *Xwmc169* *Xgwm480* *XBE423242* *XBF485004* *XAK335187* *XBE405552*	3AL	Zhang et al., 2010
	Sr36	SSR	*Xgwm319* *Xwmc477* *Xstm773-2*	2BS	Tsilo et al., 2008
	Sr39	AFLP EST-marker	*Sr39#22r* *Sr39#50s* *BE500705*	2B	Mago et al., 2009
	SrCad	PCR-marker	FSD/RSA	6DS	Laroche et al., 2000 Hiebert et al., 2011
	SrWeb	SSR	*Xgwm47*	2BL	Hiebert et al., 2010
Powdery mildew resistance	*Pm34*	SSR	*Xbarc177* *Xbarc144* *Xgwm272*	5DL	Miranda et al., 2006
	Pm35	SSR	*Xcfd26*	5DL	Miranda et al., 2007
	**Pm36*	EST-SSR	BJ261635	5BL	Blanco et al., 2008
Fusarium head blight resistance (FHB)	**Fhb1* *(Qfhs.ndsu-3BS)*	PCR-marker SSR	*UMN10* *Xgmw533* *Xgmw493*	3BS	Anderson et al., 2001 Liu et al., 2008 Buerstmayr et al., 2009
	**Qfhs.ifa-5A*	SSR	*Xbarc117* *Xbarc186* *Xbarc100* *Xbarc40*	5A	Buerstmayr et al., 2003a ; 2009
	Qfhs.ndsu-3AS	SSR RFLP	*Xgwm2* *Xmwg14* *Xbcd828*	3AS	Otto et al., 2002
Eyespot resistance	*Pch1*	endopeptidase marker RFLP	Ep-D1b *Xpsr121*	7DL	McMillian et al., 1986 Chapman et al., 2008
Septoria tritici blotch resistance	*Stb2*	SSR	*Xgwm389* *Xgwm533* *Xgwm493*	3BS	Adhikari et al., 2004
	Stb4	SSR	*Xgwm111*	7D	Adhikari et al., 2004
	Stb5	SSR gene for red coleoptile	*Xgwm44* *Rc*	7DS	Arraiano et al., 2001
	Stb7	SSR	*Xwmc313*	4AL	McCartney et al., 2003
	Stb8	SSR	*Xgmw146* *Xgwm577*	7BL	Adhikari et al., 2003
Ptr ToxA and Sn ToxA insesitivity	*Tsn1*	SSR	*Xfcp1* *Xfcp2* *Xfcp393* *Xfcp394*	5BL	Faris et al., 1996 Haen et al., 2004
Viruses					
Wheat streak mosaic virus (WSMV) resistance	*Wsm1*	STS	J15 G43	4DS	Talbert et al., 1996 http://maswheat.ucdavis.edu/
Soil-borne cereal mosaic virus (SBCMV) resistance	*Sbm1*	SSR	*Xgwm469*	5DL	Perovic et al., 2009

	**QSbm.ubo-2BS*	SSR	*Xgwm1128* *Xbarc35*	2BS	Russo, personal communication, 2010
Insects					
Hessian fly resistance	*Hdicoccum*	SSR	*Xgwm136* *Xbarc263*	1AS	Liu et al., 2005
	H9 *H10* *H11*	SCAR SSR	$SOPO05_{909}$ *Xcfa2153* *Xbarc263*	1AS	Kong et al., 2005 Liu et al., 2005
	H13	SSR	*Xcfd132* *Xgdm36* *Xcfd42* *Xgdm141*	6DS	Liu et al., 2005
	H25	SSR	*Xgwm610* *Xgwm397*	4A	http://maswheat.ucdavis.edu/
	H26 *H32*	STS	*Xrwgs10* *Xrwgs11* *Xrwgs12*	3D	Yu et al., 2009 ; 2010
	H31	STS	*Xupw4148*	5BS	Williams et al., 2003
Russian wheat aphid (RWA) resistance	*Dn2*	SSR SCAR	*Xgwm437* *Xgwm111* *B10880* *N1400*		Myburg et al., 1998 Liu et al., 2000 Miller et al., 2001
	Dn4	SSR	*Xgwm106* *Xgwm337*	1DS	Liu et al., 2002
Wheat stem sawfly (WSS) resistance	*Qss.msub-3BL*	SSR	*Xgwm340* *Xgwm247* *Xgwm547*	3BL	Cook et al., 2004
Greenbug resistance	*Gb3*	SSR	*Xwmc634*	7DL	Weng et al., 2005
Quality					
High grain protein content	*Gpc-B1*	InDel	*Xuhw89*	6BS	Distelfeld et al., 2006
Grain Texture	*Pina*	CAPS	PinaD1-F/PinaD1-R	5DS	Tranquilli et al., 1999
	Pinb	CAPS	PinbD1-F/PinbD1-R	5DS	Tranquilli et al., 1999
Gluten Strength-High Molecular Weight (HMW)	**Glu-D1* *Dx2, Dx5*	PCR-marker	P1/P2	1DL	Ahmad, 2000
	**Glu-D1* *Dy10, Dy12*	PCR-marker	P3/P4	1DL	Ahmad, 2000
	**Glu-B1* *Bx7*	PCR-marker	P5/P6	1BL	Ahmad, 2000
	**Glu-B1* *By8*	PCR-marker	ZSBy8F5/R5	1BL	Lei et al., 2006
Gluten Strength-Low Molecular Weight (LMW)	**Gli-B1 γ-42* *(Glu-B3 LMW1)*	PCR-marker	PCR-primers	1BS	D'Ovidio , 1993 D'Ovidio and Porceddu, 1996
	**Gli-B1 γ-45* *(Glu-B3 LMW2)*	PCR-marker	PCR-primers	1BS	D'Ovidio , 1993 D'Ovidio and Porceddu, 1996
Pre-harvest sprouting tolerance (PHST)	**QPhs.ccsu-3A.1*	SSR	*Xgwm155* *Xwmc153*	3AL	Kulwal et al., 2005 Kumar et al., 2010
Semolina color	QTL	STS	*FC7*	7A	Parker and Langridge, 2000
	QYP-7B	SSR	*Xgwm* 344	7BL	Elouafi et al., 2001
	Lpx-B1.1	InDel	LOXA-4BS/L2R	4BS	Carrera et al., 2007

	**Psy-A1*	STS SSR	*YP7A* *Xwmc809*	7AL	He et al., 2008
	**Psy-B1b*	STS	*YP7B-1*	7BL	He et al., 2009
	*QTL	SSR	*Xgwm786b*	6AL	Mastrangelo, personal communication, 2009
	**Psy-A1*	SSR	*Xgwm1061* *Xgwm344*	7AL	Blanco, personal communication, 2010
	*QTL	SSR	*Xgwm282*	7AL	Blanco, personal communication, 2010
Reduced grain cadmium concentration	*Cdu1*	CAPS	*usw47*	5BL	Wiebe et al., 2010 http://maswheat.ucdavis.edu/
Abiotic stress					
Vernalization requirement	*Vrn-B3*	CAPS	Vrn-B3 F/R	7BS	http://maswheat.ucdavis.edu/
Aluminum tolerance	*Alt_{BH} (Alt2)*	RFLP SSR	*Xpsr914* *Xgdm125*	4DL	Riede and Anderson, 1996

Table 5 - List of the molecular markers employed in the Marker Assisted Selection schedule developed in the present study. For each marker the target gene linked, trait introgressed, durum wheat genotype used as donor variety, chromosome position, polymorphism type and references are reported.

Marker	Target gene	Trait	Genotype	Chromosome	Polymorphism type	Primers source
SWES619 Xgwm146	*Lr14c*	Leaf rust resistance	Creso	7BL	EST-SSR; SSR	Marone et al., 2009
Xgwm1128 Xbarc35	*QSbm.ubo-2BS*	Soil borne cereal mosaic virus (SBCMV)	Neodur	2BS	SSR	Russo, personal communication, 2010
Xgwm786b	QTL	Yellow pigment content	Pedroso	6AL	SSR	Mastrangelo, personal communication, 2009
LOXA-4BS/L2R	*Lpx-B1.1*	Lipoxygenase activity	UC1113	4BS	InDel	Carrera et al., 2007
Xuhw89	*Gpc-B1*	Protein, zinc and iron content; stripe rust resistance	UC1113	6BS	InDel	http://maswheat.ucdavis.edu/
mgbe684	*Pm36*	Powdery mildew resistance	5BIL-42	5BL	EST-SSR	Blanco et al., 2008
Xgwm1061 Xgwm344	*Psy-A1*	Yellow pigment content	Primadur	7AL	SSR	Blanco, personal communication, 2010
Xgwm282	QTL	Yellow pigment content	Primadur	7AL	SSR	Blanco, personal communication, 2010

Figure 4 - Marker-based gene-pyramiding scheme applied in durum wheat.

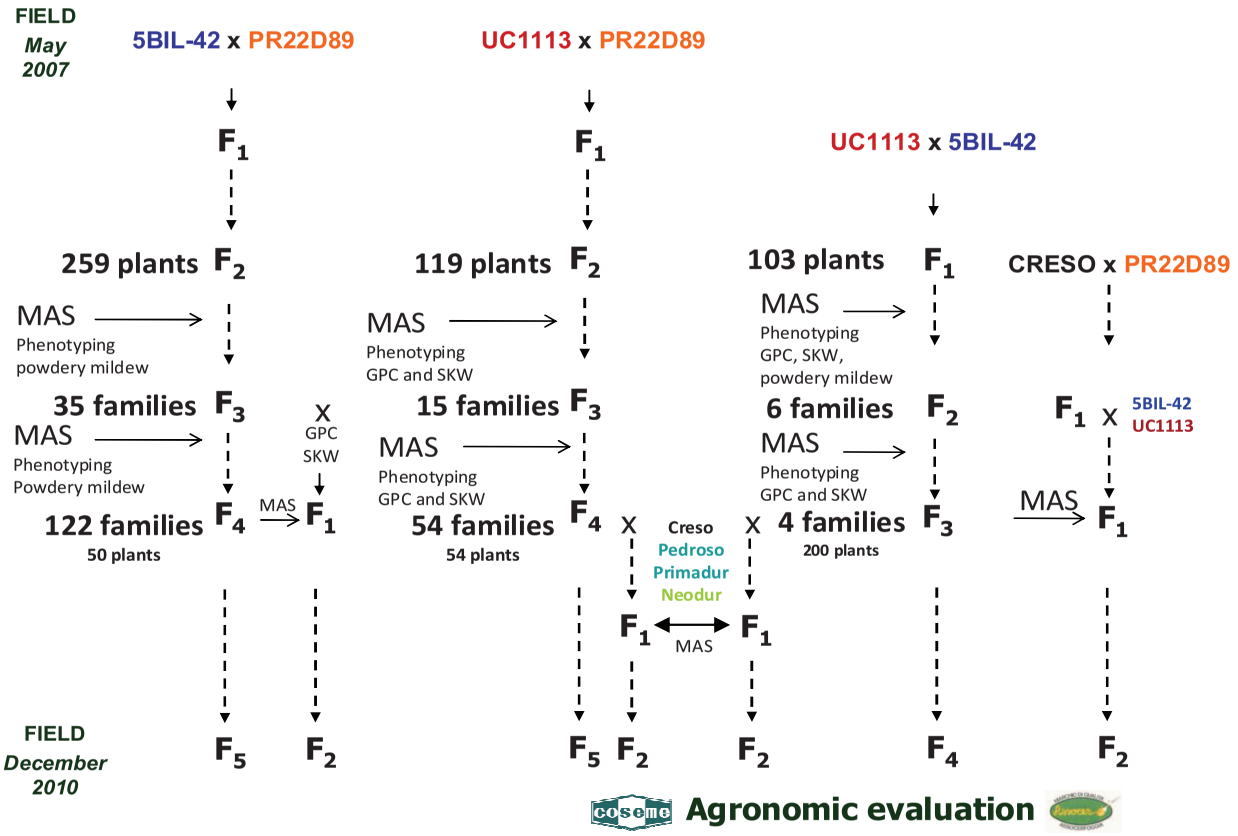


Table 6 - PCR primer information and PCR cycling conditions for the amplification of markers used for Marker Assisted Selection schedule in durum wheat.

Marker	PCR product (bp)	Forward and reverse PCR primer sequence 5'-3'	Marker type	PCR components	PCR cycling
LOXA-4BS/L2R	~1000	F: TCTCATCAAGAGGTACCTACTTA R: ATGATGGTCTGGATCTG	Dominant	1units/*Taq* 1X PCR buffer 0.1µM/primer 0.2mM/dNTPs 75-100ng/DNA	1 X 94 °C 3'; touchdown (-1°C each) 5 X 94 °C 30'', 62-58 °C 30'', 72 °C 45''; 35 X 94 °C 45'', 55 °C 45'', 72 °C 45''; 1 X 72 °C 10'; 1 X 4 °C hold
Xuhw89	120	F: TCTCCAAGAGGGGAGAGACA R*: TTCCTCTACCCATGAATCTAGCA (*labelled with FAM fluorescent dye)	Co-dominant	2.5units/*Taq* 1X PCR buffer 0.2µM/primer 0.2mM/dNTPs 50-100ng/DNA	1 X 94 °C 5'; 37 X 94 °C 30'', 59 °C 30'', 72 °C 45''; 1 X 72 °C 5'; 1 X 4 °C hold
mgbe684	220+227	F: TAGCCTGGTACCATTCTGCC R*: TGTAATGGAGGTGCAGCTTG (*labelled with HEX fluorescent dye)	Co-dominant	1units/*Taq* 1X PCR buffer 0.2µM/primer 0.2mM/dNTPs 50-100ng/DNA	1 X 94 °C 3'; 35 X 94 °C 1', 55 °C 1', 72 °C 2'; 1 X 72 °C 10'; 1 X 4 °C hold
SWES619	298	F: AACGGCTTCCAAACCTTA R*: ACCACGCACCTTTCTTCT (*labelled with FAM fluorescent dye)	Dominant	1units/*Taq* 1X PCR buffer 0.4µM/primer 0.2mM/dNTPs 5% DMSO 50-100ng/DNA	1 X 94 °C 3'; 45 X 94 °C 1', 60 °C 1', 72 °C 2'; 1 X 72 °C 10'; 1 X 4 °C hold
Xgwm146	159+174	F: CCAAAAAAACTGCCTGCATG R*: CTCTGGCATTGCTCCTTGG (*labelled with FAM fluorescent dye)	Co-dominant	1units/*Taq* 1X PCR buffer 0.4µM/primer 0.2mM/dNTPs 5%DMSO 50-100ng/DNA	1 X 94 °C 3'; 45 X 94 °C 1', 60 °C 1', 72 °C 2'; 1 X 72 °C 10'; 1 X 4 °C hold
Xgwm786b	141	F: GGCGACCGGAGTCTGAC R*: GATCCGCCGTCAGAGAG (*labelled with TET fluorescent dye)	Co-dominant	1units/*Taq* 1X PCR buffer 0.4µM/primer 0.2mM/dNTPs 5%DMSO 50-100ng/DNA	1 X 94 °C 3'; 45 X 94 °C 1', 60 °C 1', 72 °C 2'; 1 X 72 °C 10'; 1 X 4 °C hold
Xgwm1061	161	F: TCTCTCCAGCAAGACCCTGT R*: CGGTGATGTGTCTGTATGCC (*labelled with FAM fluorescent dye)	Co-dominant	1units/*Taq* 1X PCR buffer 0.4µM/primer 0.2mM/dNTPs 5%DMSO 50-100ng/DNA	1 X 94 °C 3'; 45 X 94 °C 1', 60 °C 1', 72 °C 2'; 1 X 72 °C 10'; 1 X 4 °C hold
Xgwm344	122	F: CAAGGAAATAGGCGGTAACT R*: ATTTGAGTCTGAAGTTTGCA (*labelled with HEX fluorescent dye)	Co-dominant	1units/*Taq* 1X PCR buffer 0.4µM/primer 0.2mM/dNTPs 5%DMSO 50-100ng/DNA	1 X 94 °C 3'; 45 X 94 °C 1', 55 °C 1', 72 °C 2'; 1 X 72 °C 10'; 1 X 4 °C hold
Xgwm282	225	F: TTGGCCGTGTAAGGCAG R*: TCTCATTCACACACAACACTAGC (*labelled with TET fluorescent dye)	Co-dominant	1units/*Taq* 1X PCR buffer 0.4µM/primer 0.2mM/dNTPs 5%DMSO 50-100ng/DNA	1 X 94 °C 3'; 45 X 94 °C 1', 55 °C 1', 72 °C 2'; 1 X 72 °C 10'; 1 X 4 °C hold

Xgwm1128	158	F: ACAAATTACCGCAACTCTAA R*: AGAACCATTTGGGAGCTTTG (*labelled with HEX fluorescent dye)	Co-dominant	1units/Taq 1X PCR buffer 0.4µM/primer 0.2mM/dNTPs 5%DMSO 50-100ng/DNA	1 X 94 °C 3'; 45 X 94 °C 1', 55 °C 1', 72 °C 2'; 1 X 72 °C 10'; 1 X 4 °C hold
bcd348	330+334	F: TTCACCGCCAAACACAGAGC R*: CCCCTACCAAAGACTCCAAACG (*labelled with HEX fluorescent dye)	Co-dominant	1units/Taq 1X PCR buffer 0.4µM/primer 0.2mM/dNTPs 5%DMSO 50-100ng/DNA	1 X 94 °C 3'; 45 X 94 °C 1', 55 °C 1', 72 °C 2'; 1 X 72 °C 10'; 1 X 4 °C hold

Table 7 - YPC, LPX activity and distribution of the *Lpx-B1.1* locus deletion in a durum wheat germplasm collection. The cultivars are grouped on the bases of their year of release to allow an evaluation of the breeding evolution. Measurement were carried out as described in *Experimental*. In bold type are represent the four genotype selected for LPX analysis (De Simone et al., 2010).
Wt: wild type *LpxB1.1*; **del**: presence of a deletion at the *LpxB1.1* locus.

Group	Genotype	YPC (*μg/g dw*)	LPX (*UE/g dw*)	*Lpx-B1.1*
Old (*before 1971*)	Matarese	4.03	7.91	wt
	Trinakria	**6.45**	**7.85**	**wt**
	Kiperounda	6.02	4.08	wt
	Polesine	3.68	3.24	wt
	Cannizzara	4.53	2.91	del
	Grifoni	6.15	2.86	wt
	Timilia	3.75	2.74	wt
	Taganrog	5.35	2.70	wt
	Cappelli	4.90	2.59	wt
	Aziziah	6.32	2.34	wt
	Capeiti 8	6.85	2.30	wt
	Russello	6.79	2.27	wt
	Mean	***5.40***	***3.65***	
Intermediate (*1971-1990*)	**Primadur**	**8.03**	**4.52**	**wt**
	Tresor	6.72	2.69	wt
	Neodur	7.47	1.71	wt
	Produra	5.28	1.27	del
	Magrebi 72	6.29	1.22	wt
	Duilio	5.72	0.79	wt
	Sansone	4.50	0.52	wt
	Valgerardo	5.21	0.26	del
	Creso	**6.48**	**0.20**	**del**
	Grazia	6.68	0.18	del
	Karel	5.04	0.18	wt
	Ofanto	6.41	0.12	del
	Valforte	5.10	0.08	del
	Simeto	7.31	0.07	del
	Latino	5.03	0.06	wt
	Valnova	4.79	0.05	del
	Mean	***6.00***	***0.87***	
Modern (*1991-2005*)	Saadi	6.51	3.66	del
	Brindur	9.33	3.13	wt
	Messapia	4.27	2.81	del
	Italo	6.76	2.31	wt
	Rusticano	6.50	2.26	wt
	Claudio	5.37	2.19	wt
	Nefer	6.83	2.00	wt
	Giotto	7.36	1.60	wt
	Vitromax	6.36	1.32	wt
	Marco	6.38	1.31	wt
	Vetrodur	6.14	1.28	wt
	Tiziana	5.97	1.27	wt
	Solex	5.68	1.16	del

Gianni	4.88	1.16	wt
L83	5.60	0.94	wt
Torrebianca	6.63	0.91	wt
Dupri	8.20	0.89	wt
Svevo	8.61	0.82	wt
Parsifal	5.19	0.67	wt
Iride	8.24	0.64	wt
Arcobaleno	7.57	0.58	wt
Verde	8.67	0.58	wt
Gargano	5.78	0.45	del
Adamello	5.26	0.24	del
Cosmodur	**8.53**	**0.22**	**del**
CTA 503	6.59	0.14	del
Cirillo	6.66	0.13	del
Colorado	8.83	0.13	del
Zenit	9.43	0.12	del
Bronte	5.11	0.12	wt
Fortore	6.69	0.11	del
San Carlo	6.91	0.11	del
Colosseo	5.30	0.11	del
Quadrato	6.64	0.10	del
Carpio	4.99	0.09	del
Platani	6.92	0.08	del
Ciccio	6.90	0.08	del
Varano	5.26	0.07	del
Vesuvio	6.33	0.07	del
CTA 432	4.22	0.06	del
Giusto	3.78	0.06	del
PR22D89	8.97	0.03	del
Lesina	5.69	0.02	del
Mean	***6.55***	***0.84***	
$LSD_{0.05}$			

Figure 5 – The full-length *TdNPR1* cDNA sequence reconstituted by cloning the first part from DNA of BAC clone (from start codon, nucleotides 1-485) and the second part from cDNA of Langdon cultivar (nucleotides 365-1770, falling in 3'UTR). The part of both sequences in *yellow* indicate a region of 150-bp, in common between the two PCR products that include the restriction site of *Not*I.

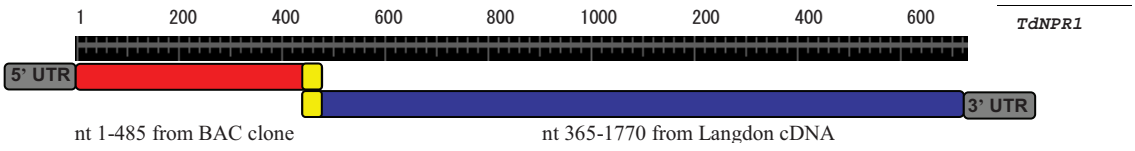


Figure 6 - A two step approach for generating the full-length *TdNPR1* gene. **a)** In the first step, the two PCR products (one fragment from BAC clone and the other one from Langdon cDNA) are inserted into an entry vector pENTR/D-TOPO which uses a TOPO-based method to generate entry clones. The vectors identified with both correct fragments were digested with restriction enzyme *Not*I. Then, the plasmid with the Langdon cDNA insert was dephosphorylated using calf intestinal alkaline phosphatase (CALF). **b)** In the second step, in-frame insert derived from BAC clone was then ligated inside the plasmid dephosphorylated getting the full-length *TdNPR1* gene. The part of both sequences in *yellow* indicate a region of 150-bp, in common between the two PCR products that include the restriction site of *Not*I.

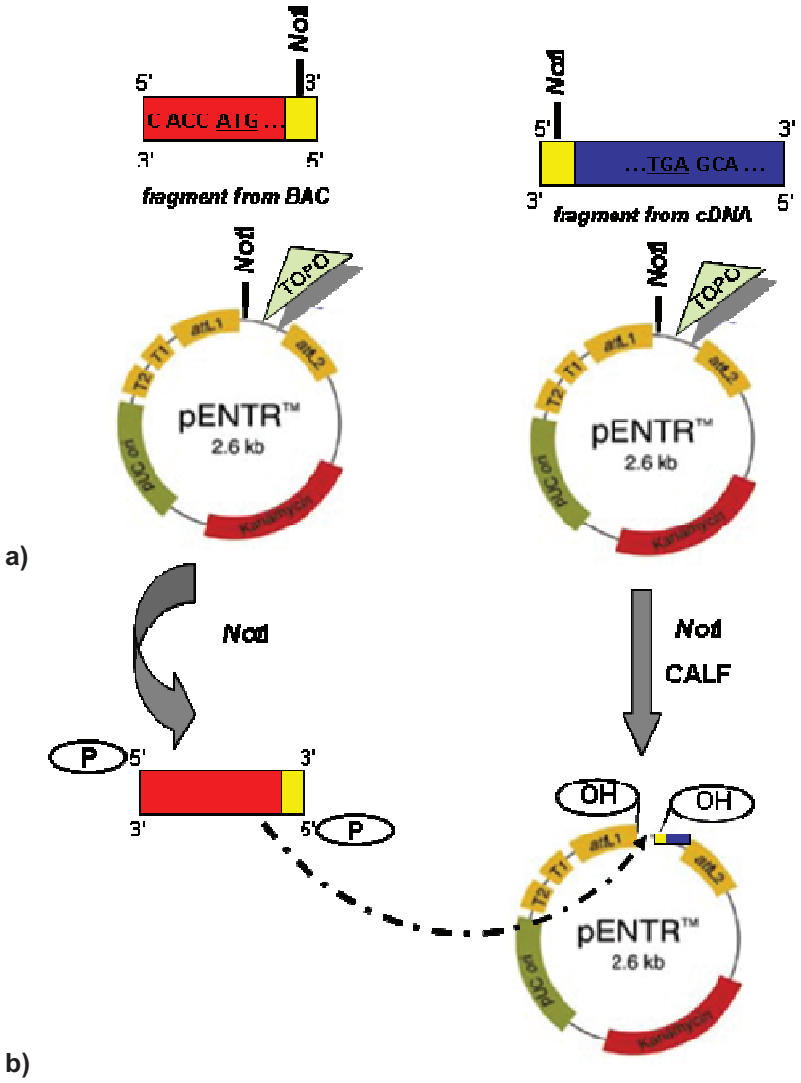


Figure 7 - Trend of YPC **(A)** and LPX activity **(B)** in old and modern wheat genotypes released in Italy during the XX century. Data from Table 7. Bars represent ±SE (De Simone et al., 2010).

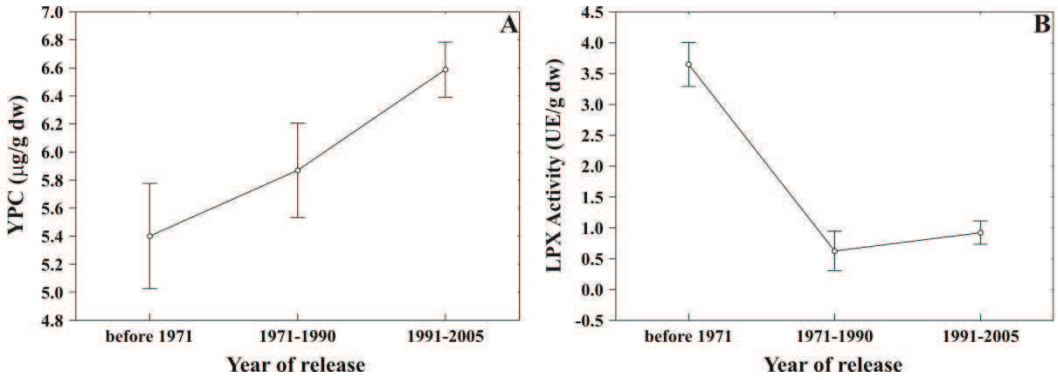


Figure 8 - Polymorphism detection at the *Lpx-B1.1* locus performed with LOXA-4BSL2/R primers. Four genotypes representing all variability detected in the germplasm collection are reported: UC1113 (control), Creso and Cosmodur with *Lpx-B1.1* locus deletion, Primadur with *Lpx-B1.1* identical sequence and Trinakria with J4.2 identical sequence (De Simone et al., 2010).

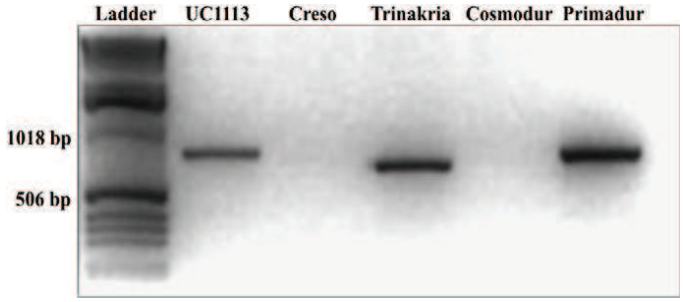


Figure 9 - LPX activity **(A)** and YPC **(B)** of wholemeal, semolina and pasta products obtained from four durum wheat genotypes with contrasting YPC and LPX activity. Measurements were carried out as described in Section 3. Bars represent ±SD (De Simone et al., 2010).

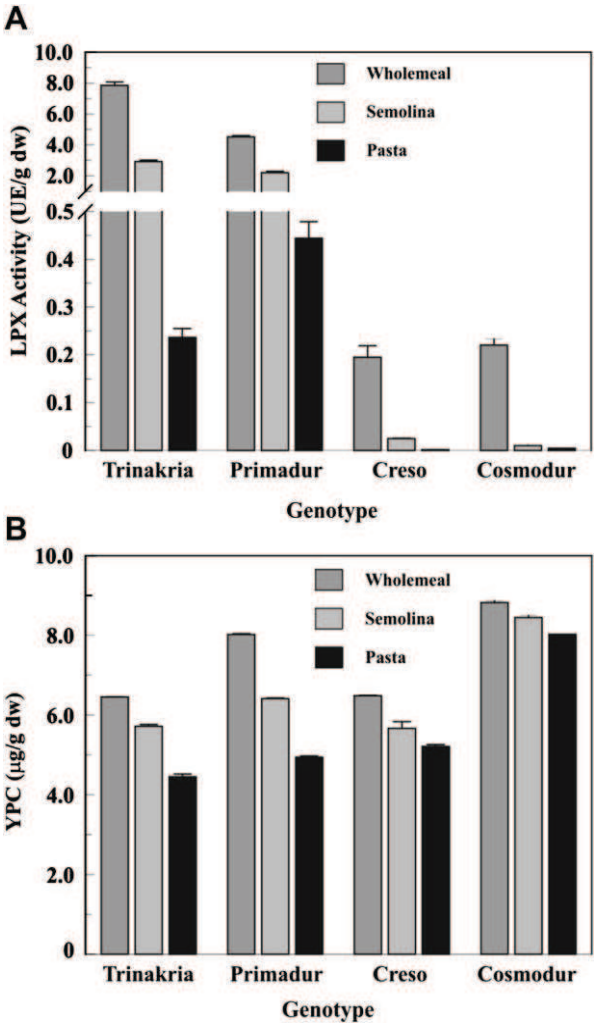


Figure 10 - Electrophoretic profiling in 1% agarose gel of amplified products of *NPR1*-like gene obtained using primers **(a)** A genome-specific 176F/11R and **(b)** B genome-specific 175F/11R. M, molecular weight marker; A, *T. urartu* genomic DNA; B, *Ae. speltoides* genomic DNA; D, *Ae. taushii* genomic DNA; N3A, N3B and N3D tetraploid nulli-tetrasomic lines N3AT3D, N3BT3D and N3DT3B.

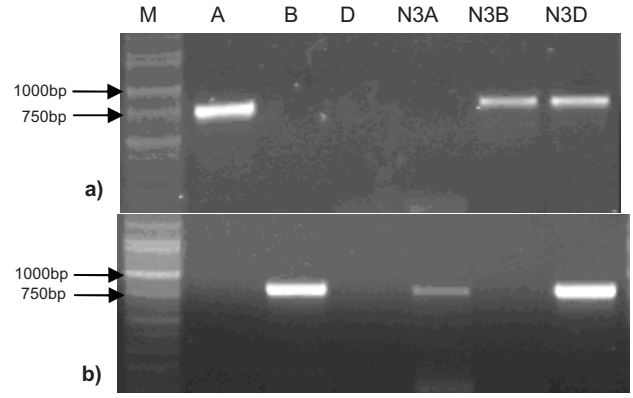


Figure 11 - Electrophoretic profiling in 1% agarose gel of amplified products of *TdNPR1* gene obtained using genome-specific primers for the A genome (176F/11R) and the B genome (175F/11R) to amplify the fragments of coding sequence from Langdon cDNA (lanes 1-3) and A genome BAC clone (lanes 2-4). The amplified fragment of 730-bp in *TdNPR1-A1* copy is showed.

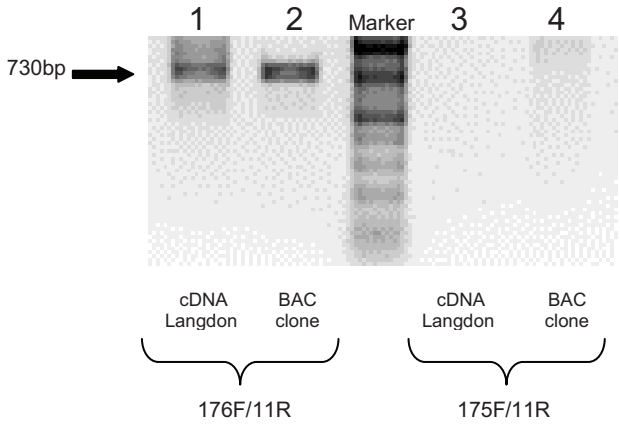


Figure 12 - Phylogenetic three of the known NPR1 and NPR2-like proteins from different monocots and dicots. GeneBank accession numbers are given for each sequence following species name (Hv: *Hordeum vulgare*, GenBank CAJ19095.1; Td: *Triticum durum*; Bradi: *Brachypodium distachyon*, GenBank Bradi2g05870.1, Bradi2g51030.1, Bradi1g12870.1, Bradi2g60710.1, Bradi4g43150.1; Os: *Oryza sativa*, GenBank AAP92751.1, ABE11616.1; Zm: *Zea mays*, GenBank NP_001152107.1; Ma: *Musa acuminate*, GenBank ABL63913.1; Pt: *Populus trichocarpa,* GenBank XP_002308281.1, XP_002322351.1; Nt: *Nicotiana tabacum*, GenBank AAM62410.1, AAT57641.1; At: *Arabidopsis thaliana,* GenBank NP_176610, AAT57641.1, NP_199324.2, NP_193701.2; Le: *Lycopersum esculentum*, AAT57638.1, AAT57639.1). Rice NPR1 and NPR2-like are indicated by a *rectangle*; *Arabidospis* NPR1 is *underlined*; durum wheat NPR1-like is *circled*. The *numbers* on the branches indicate bootstrap values.

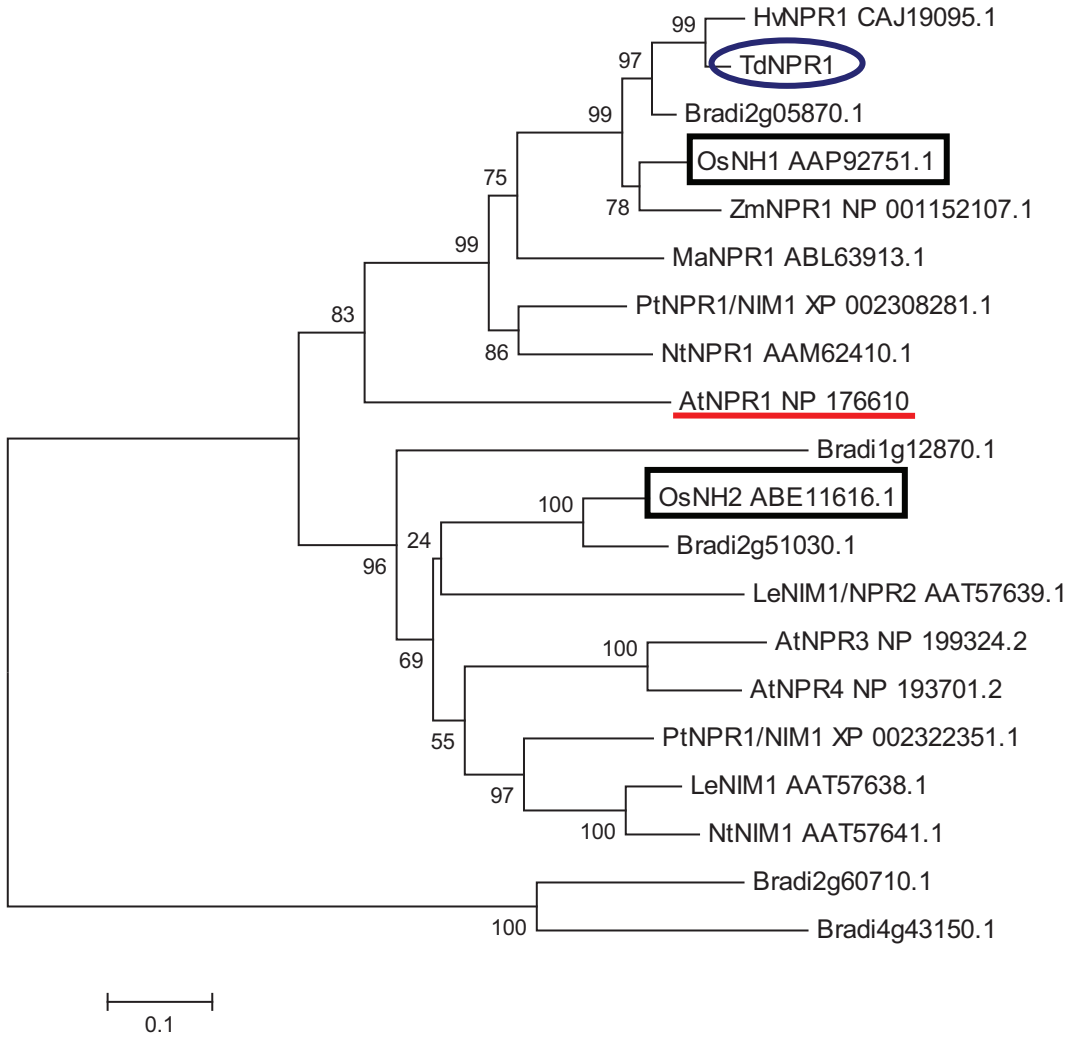
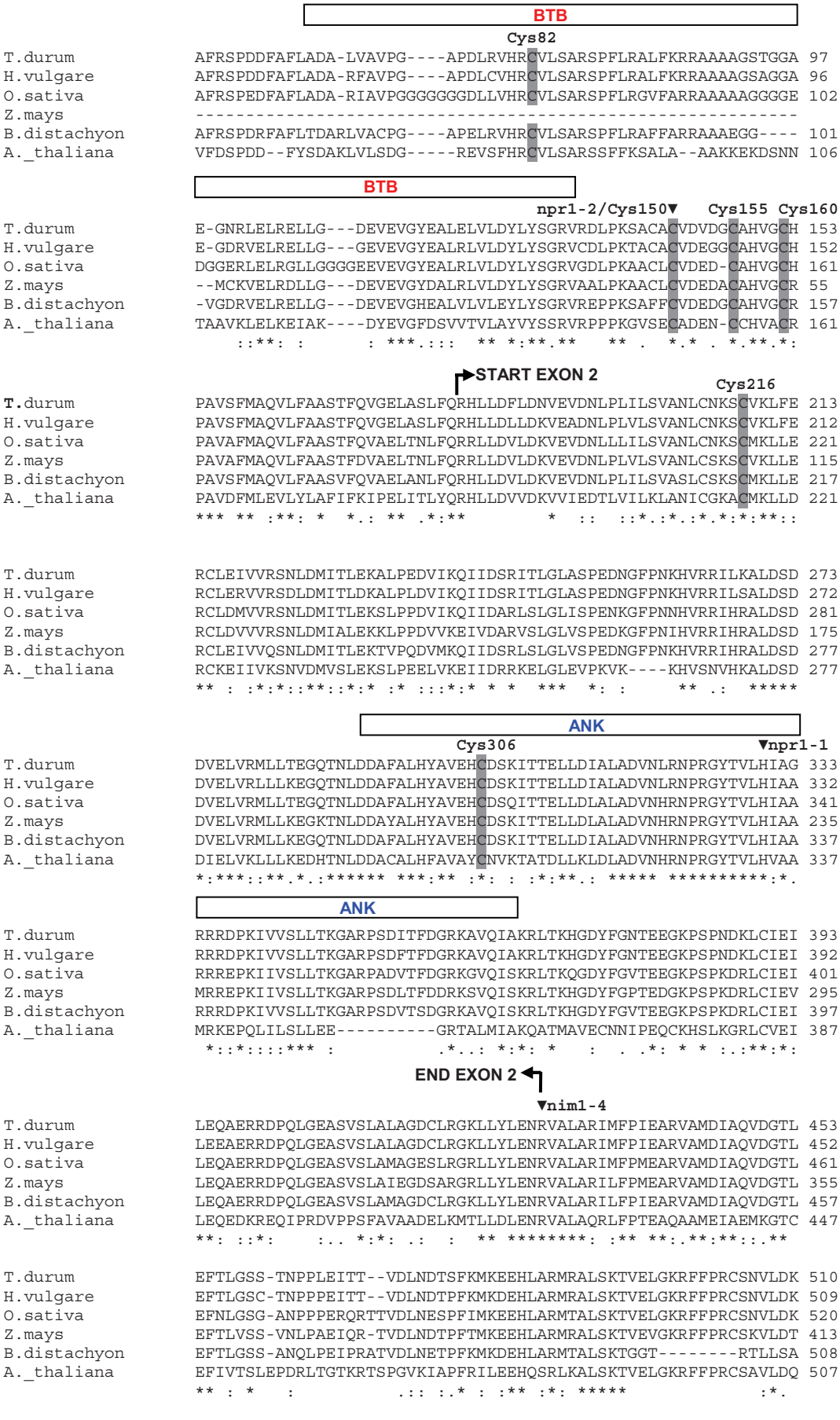


Table 8 - Summary of selected *TdNPR1* mutations. In the nucleotide change column, the position is relative to the first nucleotide of the exon II target (containing ANK domain) since we do not have the complete genomic sequence for *TdNPR1* gene. In the amino acid change column, the position is relative to the first amino acid on start methionine based on the predicted amino acid sequence of TdNPR1.

Genome	M_3 line	Nucleotide Change	Amino Acid Change	Blosum62	Genotype
A	T4-708	G397A	D312N	1	Hetero
A	T4-577	G412A	D317N	1	Hetero
A	T4-813	G289A	E276K	1	Hetero
A	T4-941	G646A	E395K	1	Homo
A	T4-803	G659A	R399K	2	Hetero
A	T4-2383	G605A	G381E	-2	Homo
A	T4-826	G288A	V275V	silent	Hetero
B	T4-101	G506A	G345D	-1	Homo
B	T4-454 *no seeds*	C565T	L365F	0	Homo
B	T4-359 *no seeds*	G37A	D189N	1	Hetero
B	T4-308	C279T	S269F	-2	Homo
B	T4-2368	C83T	S204F	-2	Homo
B	T4-2231	G388A	E306K	1	Homo
B	T4-2711	G437A	G322D	-1	Homo
B	T4-2138	G300A	R276R	silent	Homo
B	T4-2254	G465A	K331K	silent	Homo

Figure 13 - ClustalW2-produced alignment of durum wheat, barley (accession no. CAJ19095.1), rice (accession no. AAP92751.1), maize (accession no. NP_001152107.1), *Brachypodium* (accession no. Bradi2g05870.1) and *Arabidopsis* (accession no. NP_176610) NPR1-like proteins. **a)** The protein domains are indicated *above* the sequence. The amino acids changed in *npr1-1* (H), *npr1-2* (C) and *nim1-4* (R) mutants are marked with *filled triangles*. Cys82, Cys150, Cys155, Cys160, Cys216 and Cys306 are shaded in grey. Two arrows indicate the coding sequence from exon 2, reported also in figure **b)** as enlargement of alignment, to show the mutations selected in A (*T. urartu*) and B (*Ae. speltoides*) genomes in a perfectly conserved position among the analyzed sequences, shaded in yellow and red respectively. **c)** Domain structure of durum wheat, barley and rice NPR1-like proteins. A BTB and an ankyrin repeat domain (ANK_REP_REGION) are indicated.

```
T.durum            ----------MEAP-SSHVTASFSDCDD-SVSMGD-----AAPDADVEALRRLSDNLAA 42
H.vulgare          ----------MEAP-SSHVTTSFSDCD--SVSMED-----AAPDADVEALRRLSDNLAA 41
O.sativa           ----------MEPP-TSHVTNAFSDSDSASVEEGG-----ADADADVEALRRLSDNLAA 43
Z.mays             ------------------------------------------------------------
B.distachyon       ----------MEAPLTSHVTTAFSDCDSAPMEMEDDAAAAAADAADVEALRRLSDNLAA 49
A._thaliana        MDTTIDGFADSYEISSTSFVATDNTDSSIVYLAAEQ-----VLTGPDVSALQLLSNSFES 55
```


BTB
Cys82
T.durum AFRSPDDFAFLADA-LVAVPG----APDLRVHRCVLSARSPFLRALFKRRAAAAGSTGGA 97
H.vulgare AFRSPDDFAFLADA-RFAVPG----APDLCVHRCVLSARSPFLRALFKRRAAAAGSAGGA 96
O.sativa AFRSPEDFAFLADA-RIAVPGGGGGGGDLLVHRCVLSARSPFLRGVFARRAAAAAGGGGE 102
Z.mays ----------
B.distachyon AFRSPDRFAFLTDARLVACPG----APELRVHRCVLSARSPFLRAFFARRAAAEGG---- 101
A._thaliana VFDSPDD--FYSDAKLVLSDG-----REVSFHRCVLSARSSFFKSALA--AAKKEKDSNN 106
BTB
npr1-2/Cys150▼ Cys155 Cys160
T.durum E-GNRLELRELLG---DEVEVGYEALELVLDYLYSGRVRDLPKSACACVDVDGCAHVGCH 153
H.vulgare E-GDRVELRELLG---GEVEVGYEALRLVLDYLYSGRVCDLPKTACACVDEGGCAHVGCH 152
O.sativa DGGERLELRGLLGGGGEEVEVGYEALRLVLDYLYSGRVGDLPKAACLCVDED-CAHVGCH 161
Z.mays --MCKVELRDLLG---DEVEVGYDALRLVLDYLYSGRVAALPKAACLCVDEDACAHVGCR 55
B.distachyon -VGDRVELRELLG---DEVEVGHEALVLVLEYLYSGRVREPPKSAFFCVDEDGCAHVGCR 157
A._thaliana TAAVKLELKEIAK----DYEVGFDSVVTVLAYVYSSRVRPPPKGVSECADEN-CCHVACR 161
START EXON 2
Cys216
T.durum PAVSFMAQVLFAASTFQVGELASLFQRHLLDFLDNVEVDNLPLILSVANLCNKSCVKLFE 213
H.vulgare PAVSFMAQVLFAASTFQVGELASLFQRHLLDLLDKVEADNLPLVLSVANLCNKSCVKLFE 212
O.sativa PAVAFMAQVLFAASTFQVAELTNLFQRRLLDVLDKVEVDNLLLILSVANLCNKSCMKLLE 221
Z.mays PAVAFMAQVLFAASTFDVAELTNLFQRRLLDVLDKVEVDNLPLVLSVANLCSKSCVKLLE 115
B.distachyon PAVSFMAQVLFAASVFQVAELANLFQRHLLDVLDKVEVDNLPLILSVASLCSKSCMKLLE 217
A._thaliana PAVDFMLEVLYLAFIFKIPELITLYQRHLLDVVDKVVIEDTLVILKLANICGKACMKLLD 221
T.durum RCLEIVVRSNLDMITLEKALPEDVIKQIIDSRITLGLASPEDNGFPNKHVRRILKALDSD 273
H.vulgare RCLERVVRSDLDMITLDKALPLDVIKQIIDSRITLGLASPEDNGFPNKHVRRILSALDSD 272
O.sativa RCLDMVVRSNLDMITLEKSLPPDVIKQIIDARLSLGLISPENKGFPNNHVRRIHRALDSD 281
Z.mays RCLDVVVRSNLDMIALEKKLPPDVVKEIVDARVSLGLVSPEDKGFPNIHVRRIHRALDSD 175
B.distachyon RCLEIVVQSNLDMITLEKTVPQDVMKQIIDSRLSLGLVSPEDNGFPNKHVRRIHRALDSD 277
A._thaliana RCKEIIVKSNVDMVSLEKSLPEELVKEIIDRRKELGLEVPKVK----KHVSNVHKALDSD 277
ANK
Cys306
▼npr1-1
T.durum DVELVRMLLTEGQTNLDDAFALHYAVEHCDSKITTELLDIALADVNLRNPRGYTVLHIAG 333
H.vulgare DVELVRLLLKEGQTNLDDAFALHYAVEHCDSKITTELLDIALADVNLRNPRGYTVLHIAA 332
O.sativa DVELVRMLLTEGQTNLDDAFALHYAVEHCDSQITTELLDLALADVNHRNPRGYTVLHIAA 341
Z.mays DVELVRMLLKEGKTNLDDAYALHYAVEHCDSKITTELLDLALADVNHRNPRGYTVLHIAA 235
B.distachyon DVELVRMLLKEGQTNLDDAFALHYAVEHCDSKITTELLDIALADVNHRNPRGYTVLHIAA 337
A._thaliana DIELVKLLLKEDHTNLDDACALHFAVAYCNVKTATDLLKLDLADVNHRNPRGYTVLHVAA 337
ANK
T.durum RRRDPKIVVSLLTKGARPSDITFDGRKAVQIAKRLTKHGDYFGNTEEGKPSPNDKLCIEI 393
H.vulgare RRRDPKIVVSLLTKGARPSDFTFDGRKAVQIAKRLTKHGDYFGNTEEGKPSPNDKLCIEI 392
O.sativa RRREPKIIVSLLTKGARPADVTFDGRKGVQISKRLTKQGDYFGVTEEGKPSPKDRLCIEI 401
Z.mays MRREPKIIVSLLTKGARPSDLTFDDRKSVQISKRLTKHGDYFGPTEDGKPSPKDRLCIEV 295
B.distachyon RRRDPKIVVSLLTKGARPSDVTSDGRKAVQISKRLTKHGDYFGVTEEGKPSPKDRLCIEI 397
A._thaliana MRKEPQLILSLLEE----------GRTALMIAKQATMAVECNNIPEQCKHSLKGRLCVEI 387
END EXON 2
▼nim1-4
T.durum LEQAERRDPQLGEASVSLALAGDCLRGKLLYLENRVALARIMFPIEARVAMDIAQVDGTL 453
H.vulgare LEEAERRDPQLGEASVSLALAGDCLRGKLLYLENRVALARIMFPIEARVAMDIAQVDGTL 452
O.sativa LEQAERRDPQLGEASVSLAMAGESLRGRLLYLENRVALARIMFPMEARVAMDIAQVDGTL 461
Z.mays LEQAERRDPQLGEASVSLAIEGDSARGRLLYLENRVALARILFPMEARVAMDIAQVDGTL 355
B.distachyon LEQAERRDPQLGEASVSLAMAGDCLRGKLLYLENRVALARILFPIEARVAMDIAQVDGTL 457
A._thaliana LEQEDKREQIPRDVPPSFAVAADELKMTLLDLENRVALAQRLFPTEAQAAMEIAEMKGTC 447
T.durum EFTLGSS-TNPPLEITT--VDLNDTSFKMKEEHLARMRALSKTVELGKRFFPRCSNVLDK 510
H.vulgare EFTLGSC-TNPPPEITT--VDLNDTPFKMKDEHLARMRALSKTVELGKRFFPRCSNVLDK 509
O.sativa EFNLGSG-ANPPPERQRTTVDLNESPFIMKEEHLARMTALSKTVELGKRFFPRCSNVLDK 520
Z.mays EFTLVSS-VNLPAEIQR-TVDLNDTPFTMKEEHLARMRALSKTVEVGKRFFPRCSKVLDT 413
B.distachyon EFTLGSS-ANQLPEIPRATVDLNETPFKMKDEHLARMTALSKTGGT--------RTLLSA 508
A._thaliana EFIVTSLEPDRLTGTKRTSPGVKIAPFRILEEHQSRLKALSKTVELGKRFFPRCSAVLDQ 507

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T.durum           IMD-DEPELASLGRDASSER----RRRFHDLQDALLKAFSEDKEEFNKTTTLSSSSSSTS 565
H.vulgare         IMD-DEPELASLGRDASSER----KRRFHDLHDTLLKAFSEDKEEFARSATLSASSSSTP 564
O.sativa          IMD-DETDPVSLGRDTSAEK----RKRFHDLQDVLQKAFHEDKEENDRSG-LSSSSSS-- 572
Z.mays            IMD-DEAEMASLGRDTSAEK----KRRFHDLQDLVQKAFSEDKEENDRSA-ARSPSSSTR 467
B.distachyon      LFK-SAGHDHGNGYRVGFPR----TGYIHREEEEIPPARRASEGIQRGQGGVCPVGPFVF 563
A._thaliana       IMNCEDLTQLACGEDDTAEKRLQKKQRYMEIQETLKKAFSEDNLELGNSSLTDSTSSTSK 567
                  ::. .     . *      :           .: :  *   .:          . ..

T.durum           TVARNLTGRPRR---- 577
H.vulgare         TVARNLTGRPRR---- 576
O.sativa          --TSIGAIRPRR---- 582
Z.mays            TTTSIGAVRPRR---- 479
B.distachyon      LIIGDVDRRSPPEM-- 577
A._thaliana       STGGKRSNRKLSHRRR 583
                         *
```

a)

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                                                  START EXON 2
T.urartu          PAVSFMAQVLFAASTFQVGELASLFQRHLLDFLDNVEVDNLPLILSVANLCNKSCVKLFE 213
Ae.speltoides     -------------------------RASP---FPVEVDNLPLILSVANLCNKSCVKLFE 210
H.vulgare         PAVSFMAQVLFAASTFQVGELASLFQRHLLDLLDKVEADNLPLVLSVANLCNKSCVKLFE 212
O.sativa          PAVAFMAQVLFAASTFQVAELTNLFQRRLLDVLDKVEVDNLLLILSVANLCNKSCMKLLE 221
Z.mays            PAVAFMAQVLFAASTFDVAELTNLFQRRLLDVLDKVEVDNLPLVLSVANLCSKSCVKLLE 115
B.distachyon      PAVSFMAQVLFAASVFQVAELANLFQRHLLDVLDKVEVDNLPLILSVASLCSKSCMKLLE 217
A._thaliana       PAVDFMLEVLYLAFIFKIPELITLYQRHLLDVVDKVVIEDTLVILKLANICGKACMKLLD 221
                  *** ** :**: *  *.: ** .*:**        *  ::  ::*.:*.:*.*:*:**::

T.urartu          RCLEIVVRSNLDMITLEKALPEDVIKQIIDSRITLGLASPEDNGFPNKHVRRILKALDSD 273
Ae.speltoides     RCMEMVVRSNLDMITLEKALPQDVIKQITDLRITLGLASPEDNGFPNKHVRRILRALDSD 270
H.vulgare         RCLERVVRSDLDMITLDKALPLDVIKQIIDSRITLGLASPEDNGFPNKHVRRILSALDSD 272
O.sativa          RCLDMVVRSNLDMITLEKSLPPDVIKQIIDARLSLGLISPENKGFPNNHVRRIHRALDSD 281
Z.mays            RCLDVVVRSNLDMIALEKKLPPDVVKEIVDARVSLGLVSPEDKGFPNIHVRRIHRALDSD 175
B.distachyon      RCLEIVVQSNLDMITLEKTVPQDVMKQIIDSRLSLGLVSPEDNGFPNKHVRRIHRALDSD 277
A._thaliana       RCKEIIVKSNVDMVSLEKSLPEELVKEIIDRRKELGLEVPKVK----KHVSNVHKALDSD 277
                  ** : :*:*::**::*:* :* :::*:* * *  ***  *: :     ** .:  *****

                                  ANK
                                                                  ▼npr1-1
T.urartu          DVELVRMLLTEGQTNLDDAFALHYAVEHCDSKITTELLDIALADVNLRNPRGYTVLHIAG 333
Ae.speltoides     DVELVRMLLTEGQTNLDDAFALHYAVEHCDSKITTELLDIALADVNLRNPRGYTVLHIAA 330
H.vulgare         DVELVRLLLKEGQTNLDDAFALHYAVEHCDSKITTELLDIALADVNLRNPRGYTVLHIAA 332
O.sativa          DVELVRMLLTEGQTNLDDAFALHYAVEHCDSQITTELLDLALADVNHRNPRGYTVLHIAA 341
Z.mays            DVELVRMLLKEGKTNLDDAYALHYAVEHCDSKITTELLDLALADVNHRNPRGYTVLHIAA 235
B.distachyon      DVELVRMLLKEGQTNLDDAFALHYAVEHCDSKITTELLDIALADVNHRNPRGYTVLHIAA 337
A._thaliana       DIELVKLLLKEDHTNLDDACALHFAVAYCNVKTATDLLKLDLADVNHRNPRGYTVLHVAA 337
                  *:***::**.*.:****** ***:** :*: : :*:**.: ***** **********:*.

                  ANK
T.urartu          RRRDPKIVVSLLTKGARPSDITFDGRKAVQIAKRLTKHGDYFGNTEEGKPSPNDKLCIEI 393
Ae.speltoides     KRRDPKIVVSLLTKGARPSDFTFDGRKAVQISKRLTKHGDYFGNTEEGKPSPNDKLCIEI 390
H.vulgare         RRRDPKIVVSLLTKGARPSDFTFDGRKAVQIAKRLTKHGDYFGNTEEGKPSPNDKLCIEI 392
O.sativa          RRREPKIIVSLLTKGARPADVTFDGRKGVQISKRLTKQGDYFGVTEEGKPSPKDRLCIEI 401
Z.mays            MRREPKIIVSLLTKGARPSDLTFDDRKSVQISKRLTKHGDYFGPTEDGKPSPKDRLCIEV 295
B.distachyon      RRRDPKIVVSLLTKGARPSDVTSDGRKAVQISKRLTKHGDYFGVTEEGKPSPKDRLCIEI 397
A._thaliana       MRKEPQLILSLLEE----------GRTALMIAKQATMAVECNNIPEQCKHSLKGRLCVEI 387
                   *::*::::*** :           .*..: *:*: *   :  . .*: * * :.:**:*:

                                  END EXON 2
                                                  ▼nim1-4
T.urartu          LEQAERRDPQLGEASVSLALAGDCLRGKLLYLENRVALARIMFPIEARVAMDIAQVDGTL 453
Ae.speltoides     LEQAERRDPQLGEASLSLALAGDCLRGKLLYLENR------------------------- 425
H.vulgare         LEEAERRDPQLGEASVSLALAGDCLRGKLLYLENRVALARIMFPIEARVAMDIAQVDGTL 452
O.sativa          LEQAERRDPQLGEASVSLAMAGESLRGRLLYLENRVALARIMFPMEARVAMDIAQVDGTL 461
Z.mays            LEQAERRDPQLGEASVSLAIEGDSARGRLLYLENRVALARILFPMEARVAMDIAQVDGTL 355
B.distachyon      LEQAERRDPQLGEASVSLAMAGDCLRGKLLYLENRVALARILFPIEARVAMDIAQVDGTL 457
A._thaliana       LEQEDKREQIPRDVPPSFAVAADELKMTLLDLENRVALAQRLFPTEAQAAMEIAEMKGTC 447
                  **: ::*:    :.. *:*: .:  :  ** ********: :** **:.**:**::.**
```
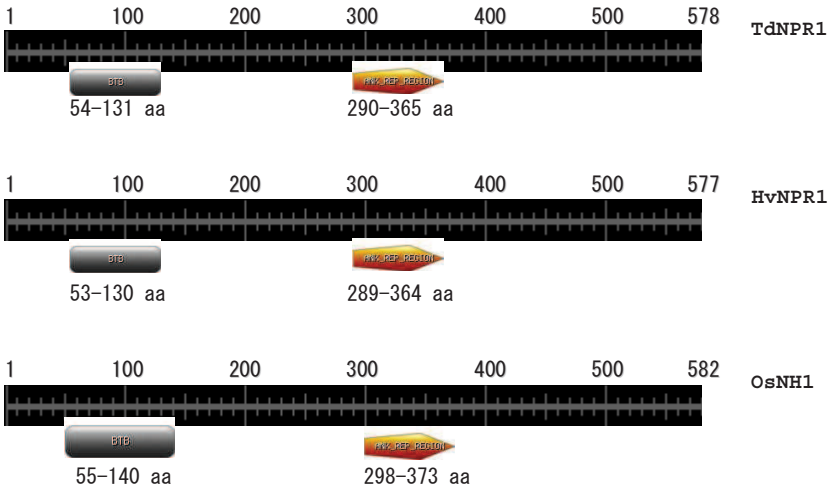

b



c)

Figure 14 - Electropherograms of *Xuhw89* InDel marker amplified on (a) the durum breeding line UC1113 with the functional *Gpc-B1* allele; on (b), (c), (d) the tetraploid wheat genotypes 5BIL-42, Creso and PR22D89 with a non-functional copy and on (e) one of the heterozygous F_1 populations.
The PCR products were separated by an automated DNA sequencer (ABI-PRISM 3100).

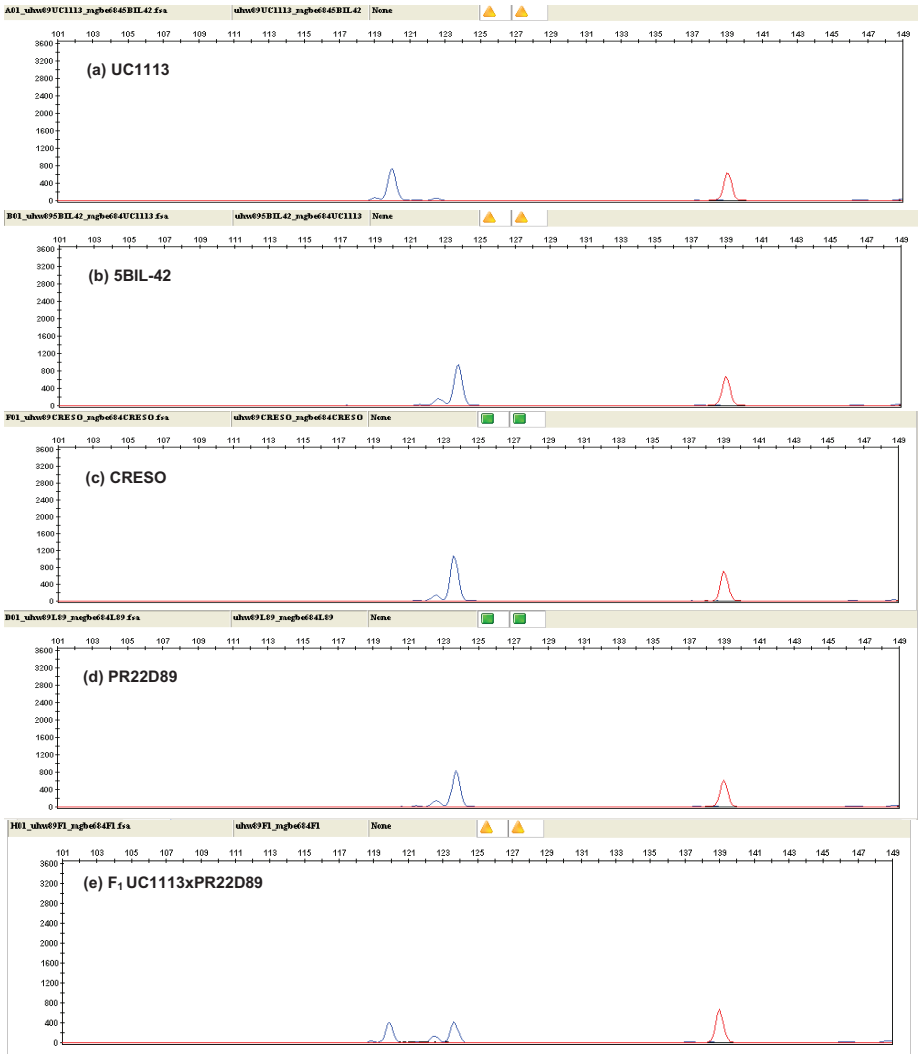


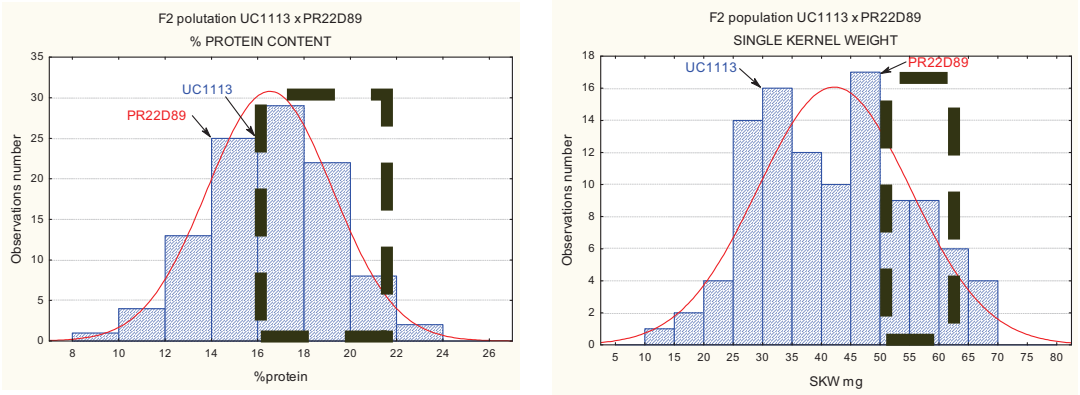
Table 9 - Segregation analysis of distribution of the functional *Gpc-B1* allele in F_2 populations of the crosses UC1113 x PR22D89 and UC1113 x 5BIL-42 through *Xuhw89* marker.

Segregating population	Number of plants	Number of F_2 plants				Expected ratio	χ^2 value	Probability
		HOMOZYGOUS for Gpc-B1 allel	*HETEROZYGOUS*	*HOMOZYGOUS for non-functional allel*	*Missing Data*			
UC1113 x PR22D89	119	26	56	22	15	1:2:1	0.92	*0.50>P>0.30*
UC1113 x 5BIL-42	103	24	50	29		1:2:1	0.57	*0.90>P>0.70*

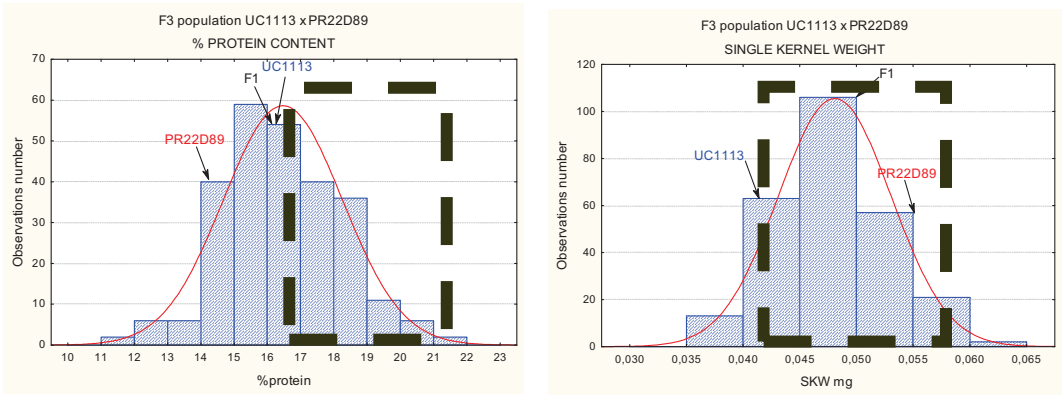
Table 10 - Mean values, included min and max levels in *brackets*, of single kernel weight (SKW) and grain protein content (GPC) observed between the durum donor line UC1113 with the functional *Gpc-B1* allele, the durum recipient line PR22D89 and the F_2 population UC1113 x PR22D89, grown in the 2008 in greenhouse.

Genotype	Number of plants	SKW	GPC
UC1113	50	0.03 mg (0.02 – 0.04 *mg)*	15.8% *(14.1 – 18.4%)*
PR22D89	50	0.05 mg *(0.03 – 0.07 mg)*	14.7% *(12.7 – 17.2%)*
F_2 UC1113xPR22D89	119	0.04 mg *(0.01 – 0.07 mg)*	16.5% *(9.7 – 23.4%)*

Figure 15 - Histograms to Gaussian distribution observed on the GPC (left) and SKW (right) **(a)** in F_2 population UC1113 x PR22D89. *Boxed* frequency classes represent 16 genotypes (3 homozygous and 13 heterozygous) with higher GPC and SKW than both parents; **(b)** in F_3 population UC1113 x PR22D89. *Boxed* frequency classes represent 76 genotypes with higher GPC and SKW than both parents.



a)



b)

Table 11 - Mean values, included min and max levels in *brackets*, of single kernel weight (SKW) and grain protein content (GPC) observed between the durum donor line UC1113 with the functional *Gpc-B1* allele, the durum recipient line PR22D89, the F_1 and F_3 populations UC1113 x PR22D89, grown in the 2009 in field.

Genotype	Number of plants	SKW	GPC
UC1113	50	0.04 mg (*0.03 – 0.05 mg)*	16.2% *(14.7 – 19.2%)*
PR22D89	50	0.055 mg *(0.05 – 0.06 mg)*	14.3% *(11.9 – 15.8%)*
F_1 UC1113xPR22D89	41	0.05 mg *(0.04 – 0.07 mg)*	16.1% *(13.7 – 18.3%)*
F_3 UC1113xPR22D89	262	0.05 mg *(0.04 – 0.06 mg)*	16.4% *(11.5 – 21.5%)*

Table 12 - Mean values of single kernel weight (SKW) and grain protein content (GPC) observed between the genotype homozygous for *Gpc-B1* allel, heterozygous and homozygous for non-functional allel in F_2 populations UC1113 x PR22D89 and UC1113 x 5BIL-42, and F_3 population UC1113 x PR22D89.
* Δ, mean change between *Gpc-B1* lines and control.

F_2 UC1113 x 5BIL-42 103 plants	**Genotype**		
	HOMOZYGOUS for Gpc-B1 allel	*HETEROZYGOUS*	*HOMOZYGOUS for non-functional allel*
SKW (mg)	0.04	0.05	0.05
GPC (%)	16.3	15.5	14.5
*** Δ (%)**	**+11.1**	**+6.5**	
$LSD_{0.05}$			

F_2 UC1113 x PR22D89 119 plants	**Genotype**		
	HOMOZYGOUS for Gpc-B1 allel	*HETEROZYGOUS*	*HOMOZYGOUS for non-functional allel*
SKW (mg)	0.04	0.04	0.05
GPC (%)	17	16.4	15.6
*** Δ (%)**	**+8.7**	**+5.4**	
$LSD_{0.05}$			

F_3 UC1113 x PR22D89 101 families	**Genotype**		
	HOMOZYGOUS for Gpc-B1 allel 15 families	*HETEROZYGOUS* 20 families	*HOMOZYGOUS for non-functional allel* 15 families
SKW (mg)	0.045	0.05	0.05
GPC (%)	17.3	16.3	15.6
*** Δ (%)**	**+10.1**	**+4.4**	
$LSD_{0.05}$			

Figure 16 - Electropherograms of *mgbe684* EST-SSR marker amplified on (a) the powdery mildew resistant parental line 5BIL-42; on (b), (c), (d) the susceptible tetraploid wheat genotypes UC1113, Creso and PR22D89 and on (e) one of the heterozygous F_1 populations.
The PCR products were separated by an automated DNA sequencer (ABI-PRISM 3100).

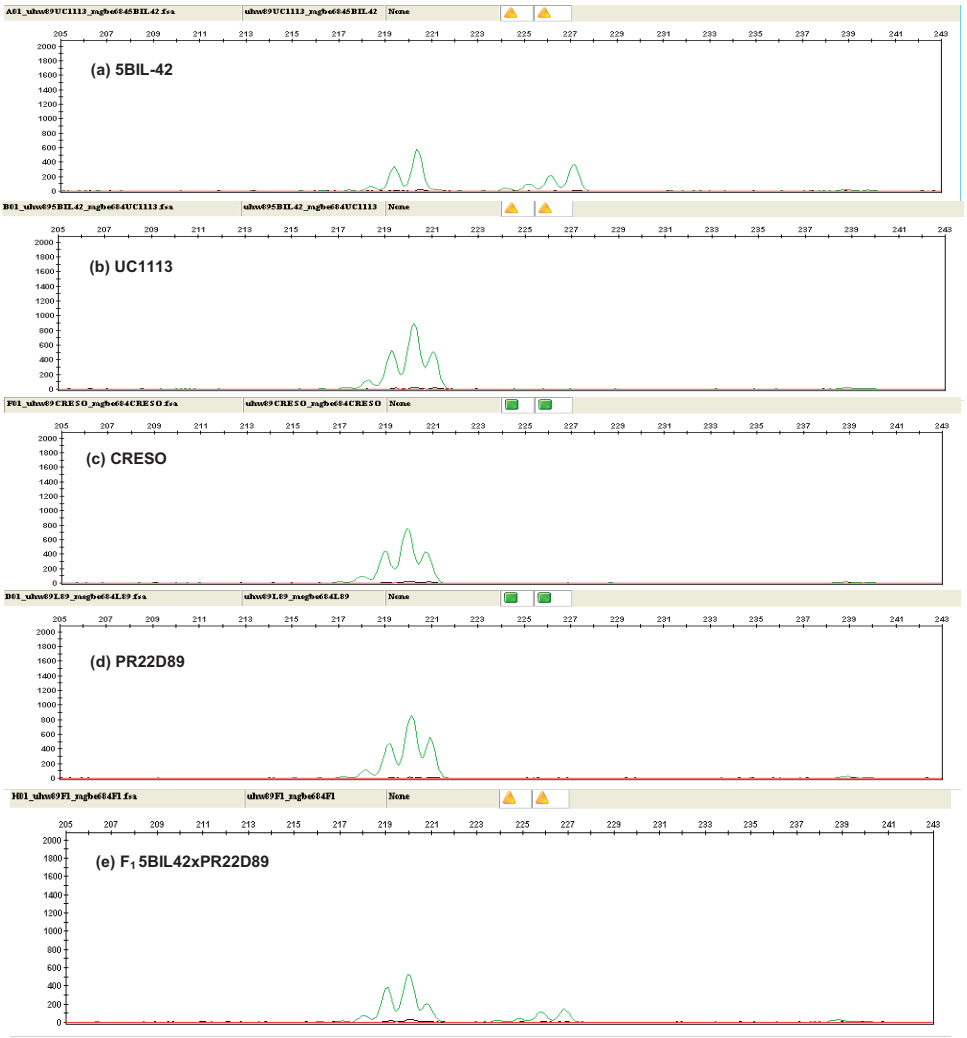


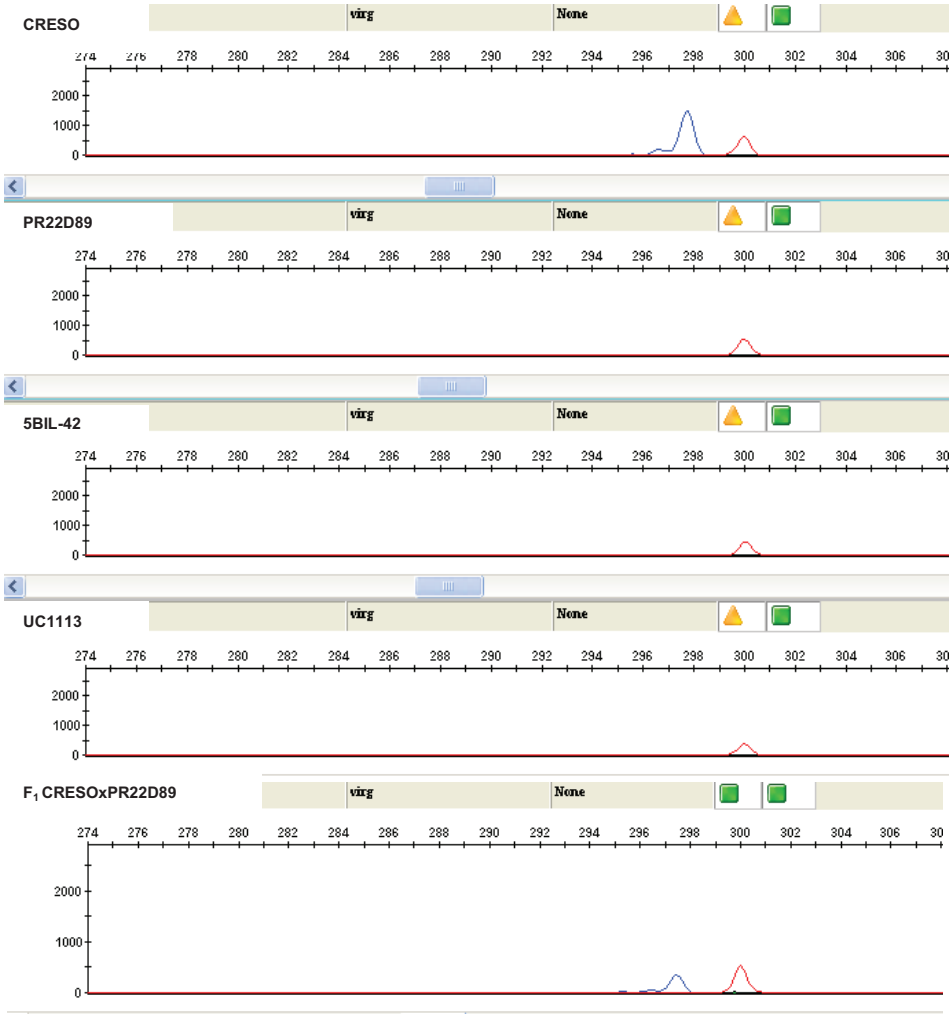
Table 13 - Segregation analysis of the powdery mildew resistance in F_2 population of the cross 5BIL-42 x PR22D89 grown in the 2008 in greenhouse.

F_2 5BIL-42 x PR22D89 286 genotypes				**Expected ratio**	**χ^2 value**	**Probability**
RESISTANTS	*SEGREGATING*	*SUSCEPTIBLES*	*Missing Data*			
80	137	65	4	1:2:1	1.8	*0.50>P>0.30*
F_2 5BIL-42 x PR22D89 286 phenotypes				**Expected ratio**	**χ^2 value**	**Probability**
RESISTANTS		*SUSCEPTIBLES*				
204		82		3:1	2.06	*0.20>P>0.10*

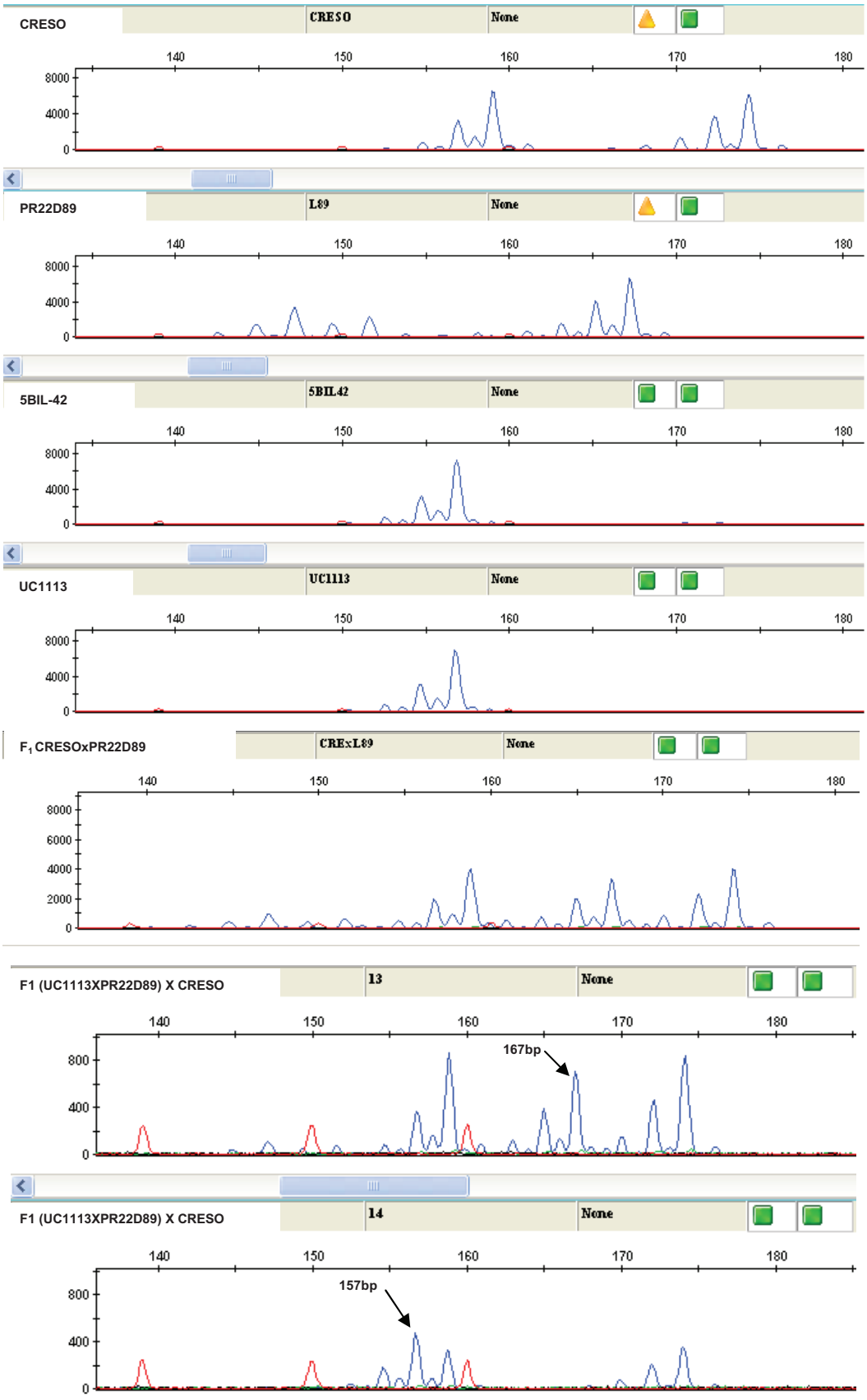
Table 14 - Segregation analysis of the powdery mildew resistance in F_2 population of the cross UC1113 x 5BIL-42 grown in the 2009 in field.

F_2 UC1113 x 5BIL-42 103 genotypes				**Expected ratio**	**χ^2 value**	**Probability**
RESISTANTS	*SEGREGATING*	*SUSCEPTIBLES*	*Missing Data*			
22	40	15	26	1:2:1	1.4	*0.50>P>0.30*
F_2 UC1113 x 5BIL-42 103 phenotypes				**Expected ratio**	**χ^2 value**	**Probability**
RESISTANTS		*SUSCEPTIBLES*				
69		34		3:1	3.05	*0.10>P>0.05*

Figure 17 - Electropherograms of **(a)** *SWES619* EST-SSR marker and **(b)** *Xgwm146* SSR marker amplified on the leaf rust resistant parental line Creso, on the susceptible tetraploid wheat genotypes PR22D89, 5BIL-42 and UC1113 and one of the heterozygous F_1 populations. *Black arrows* shows the genotypes heterozygous for *Lr14c* gene but homozygous for the allel of PR22D89 (167bp) and UC1113 (157bp), observed within F_1 (UC1113xPR22D89) x Creso population.
The PCR products were separated by an automated DNA sequencer (ABI-PRISM 3100).

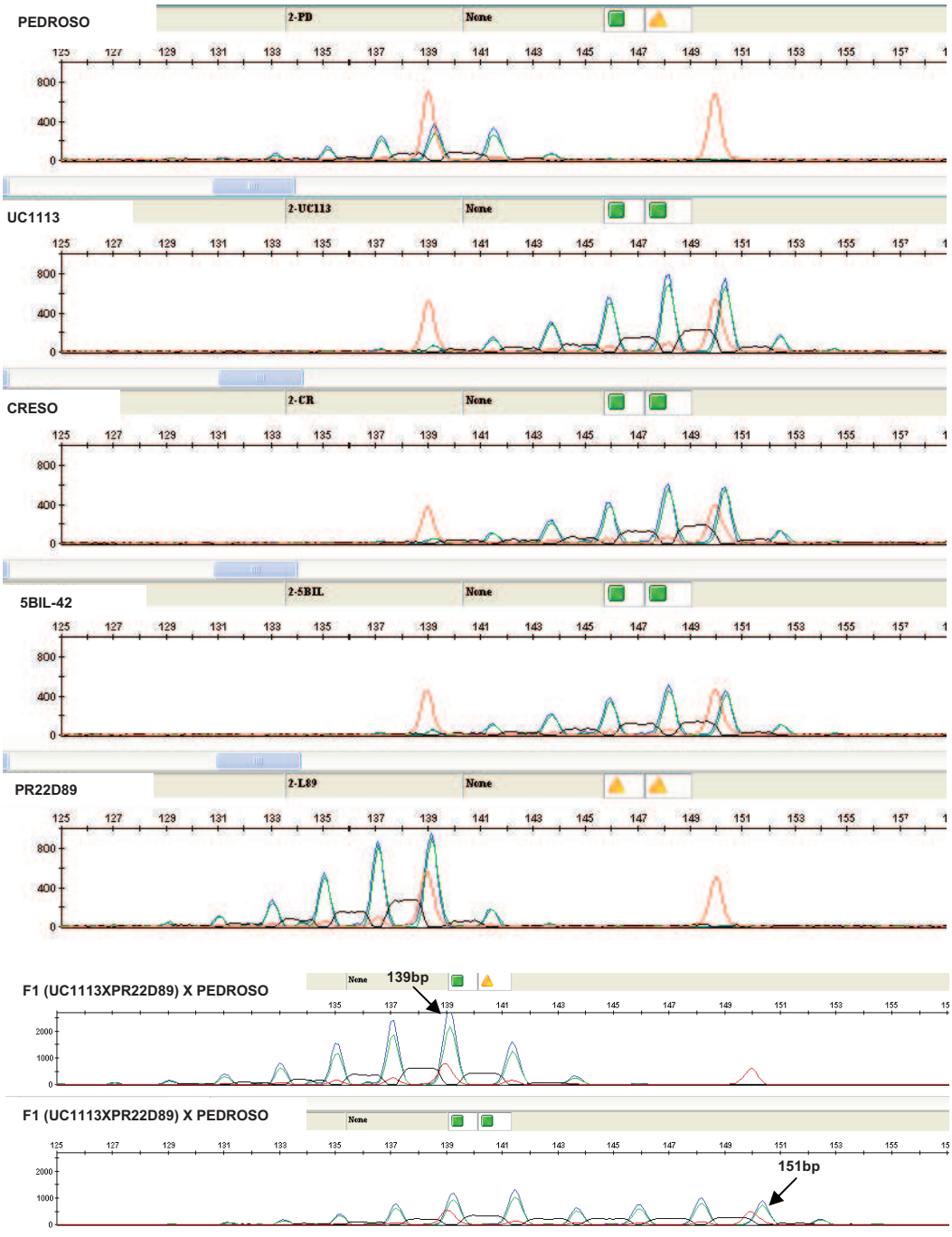


a)

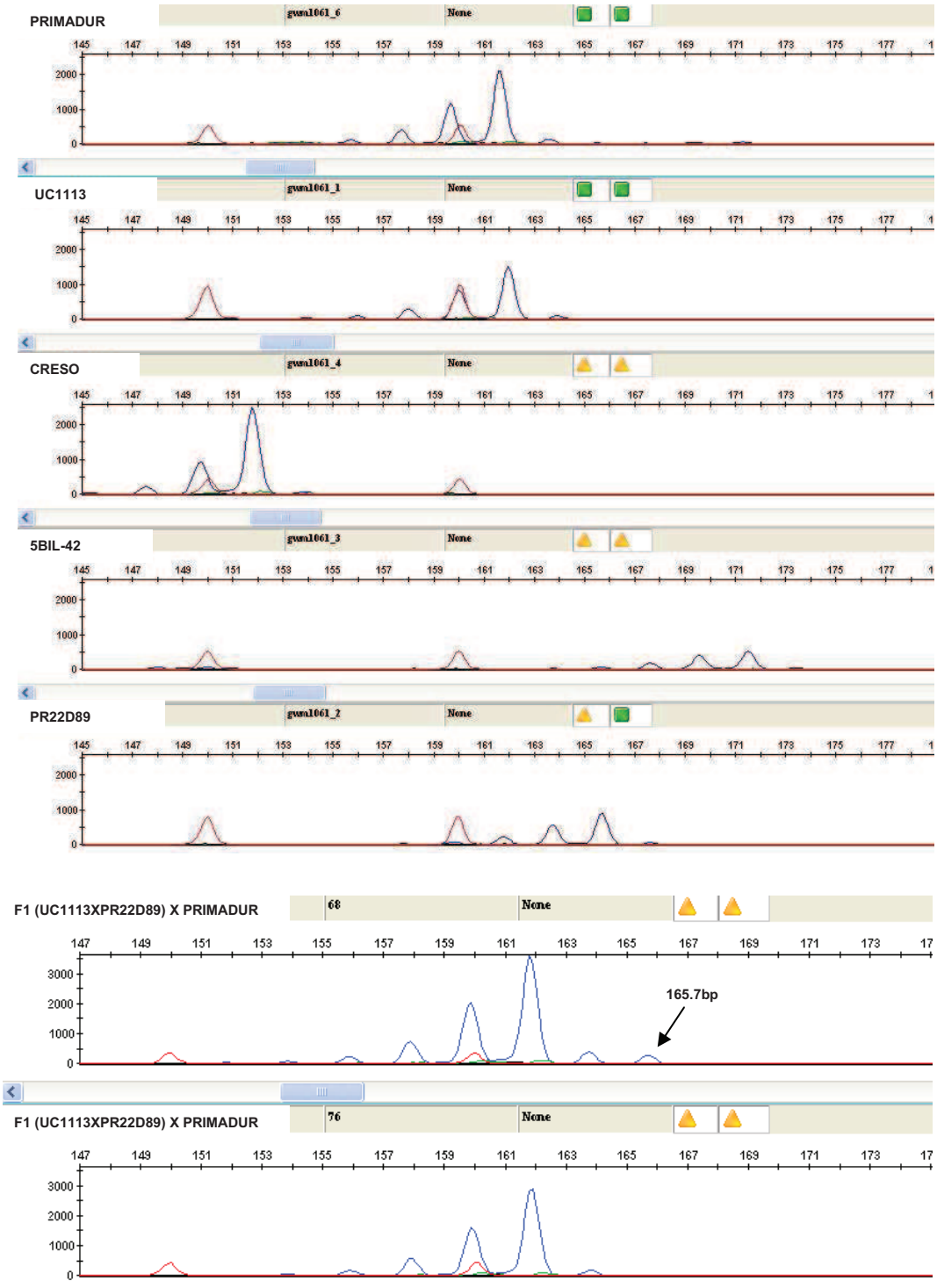

CRESO
CRESO
None
140
150
160
170
180
8000
4000
0
PR22D89
L89
None
5BIL-42
5BIL42
None
UC1113
UC1113
None
F1 CRESOxPR22D89
CRExL89
None
6000
2000
F1 (UC1113XPR22D89) X CRESO
13
None
167bp
800
400
14
157bp

b)

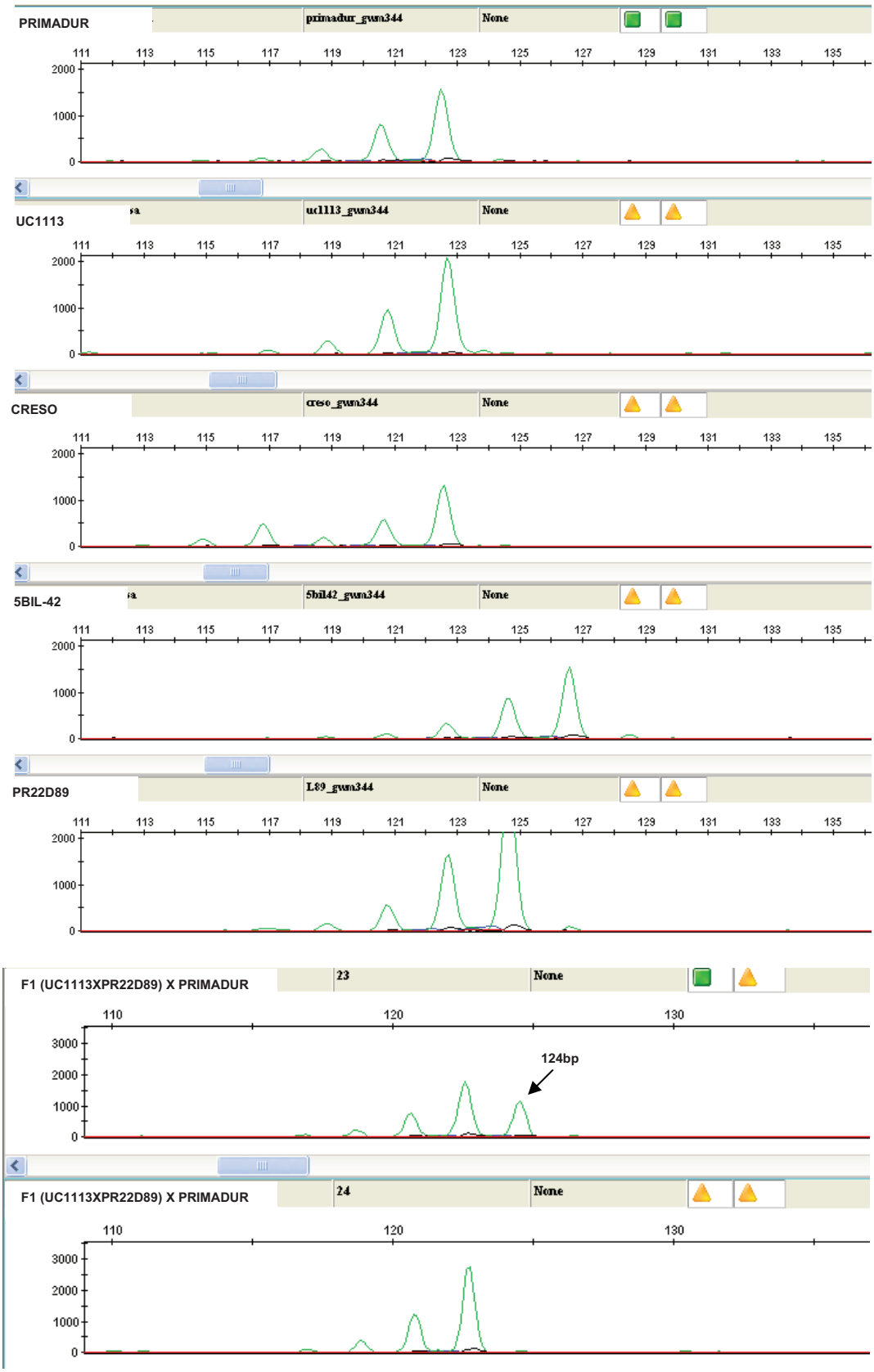
Figure 18 - Electropherograms of **(a)** *Xgwm786b* and **(b)** *Xgwm1061,* **(c)** *Xgwm344* and **(d)** *Xgwm282* SSR markers amplified, respectively, on the durum wheat cultivars Pedroso and Primadur with an high grain yellow pigment content (YPC) and on the tetraploid wheat genotypes UC1113, Creso, 5BIL-42 and PR22D89, and the heterozygous F_1 populations. *Black arrows* shows **(a)** the genotypes heterozygous for YPC QTL but homozygous for the allel of PR22D89 (139bp) and UC1113 (151bp), observed within F_1 (UC1113xPR22D89) x Pedroso population; **(b)** the genotypes heterozygous for *Psy-A1* gene but homozygous for the allel of PR22D89 (165.7bp), **(c)** (124bp).
The PCR products were separated by an automated DNA sequencer (ABI-PRISM 3130).



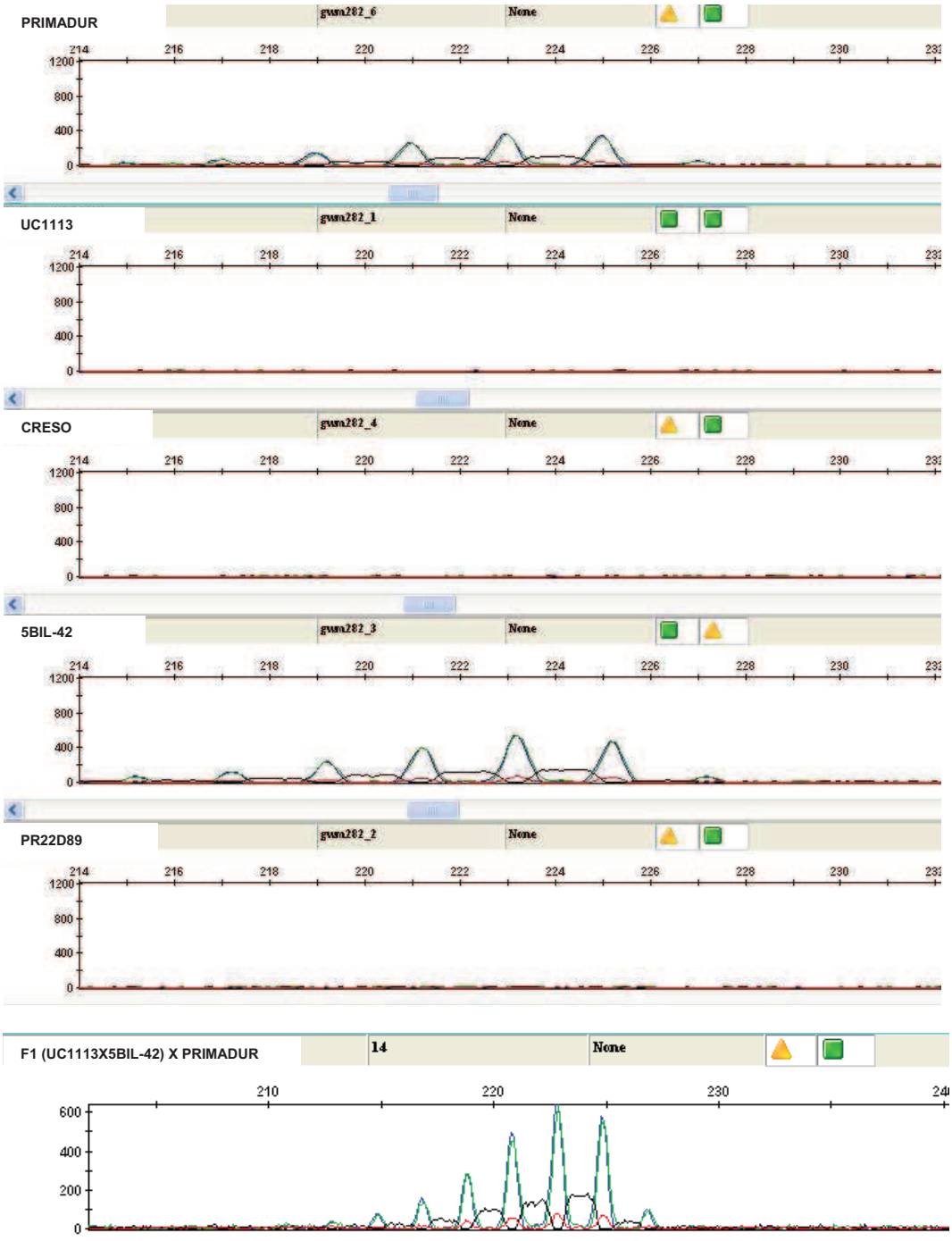
a)


PRIMADUR
gwm1061_6
None
UC1113
gwm1061_1
None
CRESO
gwm1061_4
None
5BIL-42
gwm1061_3
None
PR22D89
gwm1061_2
None
F1 (UC1113XPR22D89) X PRIMADUR
68
None
165.7bp
F1 (UC1113XPR22D89) X PRIMADUR
76
None

b)

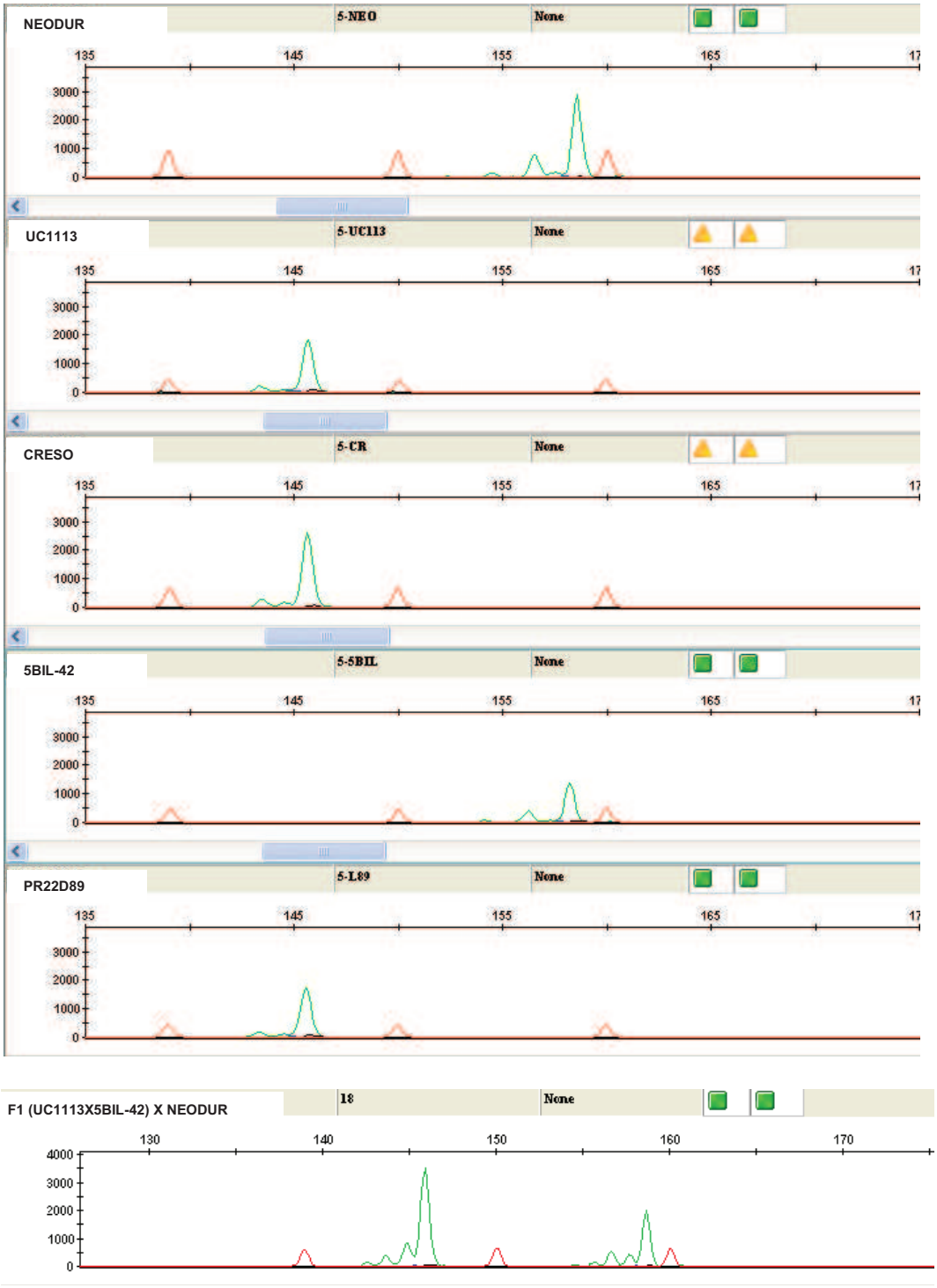

PRIMADUR
primadur_gwm344
None
UC1113
uc1113_gwm344
None
CRESO
creso_gwm344
None
5BIL-42
5bil42_gwm344
None
PR22D89
L89_gwm344
None
F1 (UC1113XPR22D89) X PRIMADUR
23
None
124bp
F1 (UC1113XPR22D89) X PRIMADUR
24
None

c)

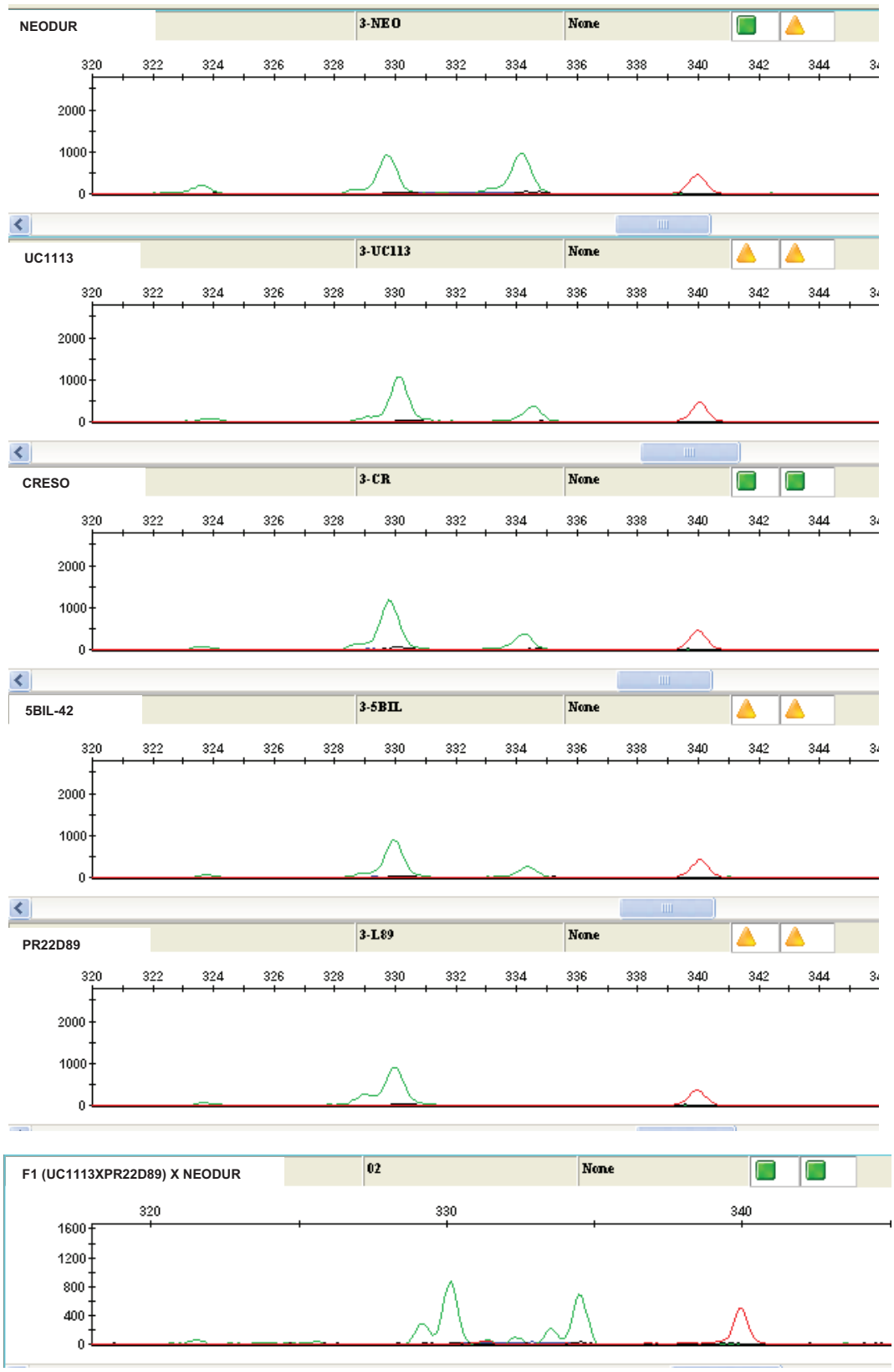

PRIMADUR
gwm282_6
None
UC1113
gwm282_1
None
CRESO
gwm282_4
None
5BIL-42
gwm282_3
None
PR22D89
gwm282_2
None
F1 (UC1113X5BIL-42) X PRIMADUR
14
None

d)

Figure 19 - Electropherograms of **(a)** *Xgwm1128* and **(b)** *bcd348* SSR marker amplified on the leaf rust resistant parental line Neodur, on the susceptible tetraploid wheat genotypes UC1113, Creso, 5BIL-42 and PR22D89, and one of the heterozygous F_1 population. The PCR products were separated by an automated DNA sequencer (ABI-PRISM 3100).



a)


NEODUR
3-NEO
None
UC1113
3-UC113
None
CRESO
3-CR
None
5BIL-42
3-5BIL
None
PR22D89
3-L89
None
F1 (UC1113XPR22D89) X NEODUR
02
None

b)

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