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ORIGINAL PAPER

Development of COS-SNP and HRM markers for high-throughput and reliable haplotype-based detection of *Lr14a* in durum wheat (*Triticum durum* Desf.)

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Abstract Leaf rust (*Puccinia triticina* Eriks. & Henn.) is a major disease affecting durum wheat production. The *Lr14a*-resistant gene present in the durum wheat cv. Creso and its derivative cv. Colosseo is one of the best characterized leaf-rust resistance sources deployed in durum wheat breeding. *Lr14a* has been mapped close to the simple sequence repeat markers *gwm146*, *gwm344* and *wmc10* in the distal portion of the chromosome arm 7BL, a gene-dense region. The objectives of this study were: (1) to enrich the *Lr14a* region with single nucleotide polymorphisms (SNPs) and high-resolution melting (HRM)-based markers developed from conserved ortholog set (COS) genes and from sequenced Diversity Array Technology (DArT®) markers; (2) to further investigate the gene content and colinearity of this region with the *Brachypodium* and rice genomes. Ten new COS-SNP and five HRM markers were mapped within an 8.0 cM interval spanning *Lr14a*. Two HRM markers pinpointed the locus in an interval of <1.0 cM and eight COS-SNPs were mapped 2.1–4.1 cM distal to *Lr14a*. Each marker was tested for its capacity to predict the state of *Lr14a* alleles (in particular, *Lr14-Creso* associated to resistance) in a panel of durum wheat elite germplasm including 164 accessions. Two of the most informative markers were converted into KASPar® markers. Single assay markers *ubw14* and *wPt-4038-HRM* designed for agarose gel electrophoresis/KASPar® assays and high-resolution melting analysis, respectively, as well as the double-marker combinations *ubw14/ubw18*, *ubw14/ubw35* and *wPt-4038-HRM–ubw35* will be useful for germplasm haplotyping and for molecular-assisted breeding.

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Introduction

Durum wheat (*Triticum turgidum* ssp. *durum* Desf.) is an important cereal crop adapted, among others, to the Mediterranean region where it is the main cultivated cereal and produces staple foods for tens of millions of people. It is also grown in the Northern Prairies of USA and Canada, Southwestern USA, Mexico, Central Asian regions, Central India and Australia. Throughout these regions, crop

production and grain quality are often affected by rust infections (Huerta-Espino et al. 2011). Leaf rust, caused by *Puccinia triticina* Eriks. is a major foliar disease of wheat (*Triticum* species), endemic in its growing areas worldwide, with distinct races or virulence phenotypes found on durum, and common wheat (*Triticum aestivum* L.), as reported by Mantovani et al. (2010) and by Ordoñez and Kolmer (2007). In durum wheat leaf rust was reported to cause up to 60 % yield losses (Singh et al. 2004; Herrera-Foessel et al. 2006). The pioneering cereal rust researchers Elvin C. Stackman and Norman Borlaug afterward, stated that "*rust never sleeps*" emphasizing the compelling threat that rusts represent for crops worldwide and for human food production (Vietmeyer 2011).

Breeding of new leaf-rust-resistant durum varieties is the most economical and environmentally friendly strategy to control this disease. For many developing countries, or for cultivation in low-returning environments, it could be the only option for control. Molecular markers tagging the known leaf-rust resistance (*Lr*) genes would provide diagnostic tools useful to characterize the parental lines, therefore allowing the breeders to identify and stack multiple effective genes into new varieties. Second, it has the potential to dramatically increase the efficiency of the breeding process by allowing selection in the early phases for multiple useful alleles via marker-assisted selection (MAS; Varshney and Tuberosa 2007; Gupta et al. 2010).

Although 70 *Lr* genes have been identified (McIntosh et al. 2011), to date, very few are known to be present in the elite durum wheat germplasm. Detailed genetic analyses of resistance factors present in *T. durum* have been undertaken only recently (Herrera-Foessel et al. 2007a, b, 2008a, b, c; Maccaferri et al. 2008, 2010; Marone et al. 2009). Survey studies based on tests of seedlings with different rust isolates and molecular genotyping have shown the presence of *Lr1*, *Lr3*, *Lr10*, *Lr14a*, *Lr16*, *Lr17a*, *Lr19*, *Lr23*, *Lr25*, *Lr33*, *Lr61* and *Lr64* in the elite durum wheat germplasm (Singh et al. 1992, 2004; Aguilar-Rincón et al. 2001; Herrera-Foessel et al. 2007a, b, 2008b; Huerta-Espino and Roelfs 1989, 1992; Martinez et al. 2007; Singh et al. 2010; Shynbolat and Aralbek 2010; Goyeau et al. 2010; Kassem et al. 2011; Karim Ammar and James Kolmer, personal communication). Some of the reported genes are not native to durum wheat, having being transferred from common wheat, such as *Lr3*, *Lr16*, *Lr17a* (Dyck and Kerber 1971, 1977; Haggag and Dyck 1973) or from wheat relatives (as for *Lr19*, *Lr25*, *Lr47* and *Lr64*, Driscoll and Anderson 1967; Browder 1972; Dubcovsky et al. 1998; James Kolmer, unpublished data). Furthermore, only a few of these genes remain effective over years and across regions (Huerta-Espino et al. 2011).

Out of the 11 genes mentioned above, only three, i.e., *Lr14a*, *Lr23* and *Lr33* were directly identified in the cultivated tetraploid germplasm in the early 1970s and 1980s and then frequently detected in the durum wheat elite germplasm by gene postulation (McIntosh and Dick 1975; Dyck 1994; Autrique et al. 1995). Herrera-Foessel et al. (2008b) recently identified the new *Lr61* gene directly in the elite germplasm, from the durum wheat cultivar (cv.) Guayacan-INIA. *Lr19* and *Lr47* have been transferred to durum wheat from wild wheat donors (*Thinopyrum ponticum* and *Triticum speltoides*, respectively) through chromosome (chr.) engineering techniques, followed by marker-assisted backcross. Both resistance alleles are not yet widely deployed in the durum elite germplasm (Ceoloni et al. 1996; Helguera et al. 2000). Other loci, such as *Lr10* and *Lr23*, have lost their effectiveness upon the rise and spread of correspondingly new virulent races (Singh et al. 2004; Huerta-Espino et al. 2011).

Lr14a, native to the tetraploid wheat germplasm, has been transferred to the modern bread wheat germplasm from emmer wheat (*Triticum dicoccum* L. accession Yaroslav; McFadden 1930).

The relevance of *Lr14a* in the elite durum wheat germplasm as useful source of leaf-rust resistance has been reported in 2008 and 2009 by three independent studies characterizing and mapping by means of molecular markers the leaf-rust resistance factors carried by the Chilean cvs. Llareta-INIA (Herrera-Foessel et al. 2008a) and the Italian cultivars Creso and Colosseo (Maccaferri et al. 2008; Marone et al. 2009). The resistant alleles *Lr14a-Ll* and *Lr14a-Creso* were mapped in the identical 7BL distal chromosome regions with a common set of simple sequence repeat (SSR) markers. Later, Maccaferri et al. (2010) provided the evidence that Llareta-INIA and Creso share an identical by descent long-range SSR haplotype in the chromosome region surrounding *Lr14a*. The Italian cv. Creso, obtained by ENEA-Casaccia in Rome from Cappelli CpB144 mutant and a semi-dwarf CIMMYT durum line (Creso's pedigree: CpB144 9//Yt54-N10-B/Cp263Tc3) was one of the Italian genotypes most widely exploited by durum breeding programs in Italy and other Mediterranean countries, including the ICARDA breeding program. Creso has been successfully used since its release in 1975 as an effective source of leaf-rust resistance under field conditions in a wide range of Mediterranean environments (Martinez and Rubiales 2002; Amaro et al. 2007; De Vita et al. 2007). The cv. Colosseo (Mexa Mutant/Creso), directly related to Creso, but with improved agronomic value, was also exploited as donor of leaf-rust resistance (Maccaferri et al. 2007; De Vita et al. 2010).

The presence of *Lr14a* in the durum germplasm should date back to the earlier attempt to transfer dwarfing genes from bread wheat. It was very rare in the CIMMYT germplasm distributed until 2002. After the appearance of the race BBG/BN in Mexico in 2001 and the resulting

breakdown of *Lr-Altar*, the gene that individually protected 85 % of the CIMMYT germplasm (Herrera-Foessel et al. 2005), an extensive effort was conducted to diversify the genetic basis of the resistance by systematically crossing the CIMMYT elite germplasm to non-CIMMYT-derived, leaf-rust-resistant parental lines, primarily from Italy, Spain, France and ICARDA. However, subsequent surveys using SSR markers have indicated that *Lr14a* was present at high frequencies in the resistant genotypes from the latter germplasm groups (Maccaferri et al. 2010; Karim Ammar, personal communication). This resulted in a sharp increase of its frequency in the new CIMMYT germplasm, at one point approaching 90 %.

The genetic vulnerability of the durum germplasm globally, due to the overdependence on *Lr14a*, needs to be urgently considered and addressed by breeding programs worldwide, especially in light of a possible generalized breakdown of the resistance on durum wheat, recently documented in France (Goyeau et al. 2010). In other southern European countries or in North Africa, while susceptible reactions have been occasionally observed on the *Lr14a*-protected cultivars such as Creso (Italy, Andrea Massi, personal communication) or lines such as Sooty/Rascon//Llareta INIA (Tunisia, Mohamed S. Gharbi, personal communication), this gene appears to be still effective as *Lr14a* virulence appears to be limited in distribution and impact. To date, *Lr14a* continues to provide protection against the Mexican (Maccaferri et al. 2010; Huerta-Espino et al. 2011), the Latin-American, the Central African, Middle-Eastern and Southern Asian races (Karim Ammar, personal communication).

In summary, high-throughput markers that can reliably diagnose the presence of *Lr14a* would be very valuable, first to avoid cross combinations resulting in populations segregating exclusively for *Lr14a*, second to be able to pyramid it with other marked genes (*Lr19*, *Lr47*, *Lr37*, *Lr68* and others) and finally, to avoid the deployment of cultivars protected exclusively by *Lr14a* in target areas where it is likely to breakdown in the near future (i.e., Southern Europe, North Africa).

By means of quantitative trait locus (QTL) analysis starting from a bi-parental recombinant inbred line (RIL) population, Maccaferri et al. (2008) identified *QLr-ubo.7B.2* (=*Lr14a*) as the major genetic factor controlling leaf-rust resistance in Creso. *Lr14a* has been mapped in the most distal region of the long arm of chr. 7B (deletion bin 7BL10-0.78-1.00) in a 7 cM interval (LOD minus 2 confidence interval) flanked by the SSR markers *barc340* and *gwm146* on the proximal side and by *gwm344* and by DArT markers *wPt4038*, *wPt1085* and *wPt4259* on the distal side (Maccaferri et al. 2008), a chromosomal location that coincides with the reported location of *Lr14a* in cv. Llareta-INIA by Herrera-Foessel et al. (2008a). The presence of this major locus for leaf-rust resistance in cv. Creso was further confirmed by QTL analysis in the cross Creso × Pedroso (Marone et al. 2009).

Currently, MAS to track *Lr14a* in breeding programs is mostly based on the SSR markers *gwm146* and *gwm344* (Susanne Dreisigacker, Diane Mather, personal communications), with some drawbacks caused by a relatively high recombination rate between *gwm146* and the resistance locus, due to an estimated distance of 4.0–4.5 cM (Maccaferri et al. 2008; Marone et al. 2009). In addition, high frequency of null alleles, hence dominance, is frequent in the durum germplasm at *gwm344*, reducing MAS effectiveness. Therefore, the availability of co-dominant markers more tightly linked to *Lr14a* and more amenable for cost-effective MAS than the currently available ones would certainly be useful.

For more than a decade, linkage mapping in wheat has relied on SSR markers Somers et al. 2004, and later on microarray-based genomic probes such as the Diversity Arrays Technology® (DArT) markers (Akbari et al. 2006; Mantovani et al. 2008). Regrettably, these marker classes are either low throughput (as for SSRs) or not available in the density required for high-resolution mapping (both SSR and DArT markers), due to the lack of an assembled whole-genome sequence. In wheat, targeted enrichment of molecular markers in regions harboring the genes of interest is now mainly carried out by exploiting the syntenic relationships with the sequenced grass species *Brachypodium distachyon* L. and rice, *Oryza sativa* L. (Feuillet and Keller 2002; Sorrells et al. 2003; Bossolini et al. 2007). Conserved ortholog set (COS) markers from expressed sequences (EST) can be identified in high number in the target regions (Quraishi et al. 2009), thus compensating for their lower polymorphism as compared to SSR and DArT markers. The selected ESTs are then re-sequenced in the wheat lines of interest to search for homeologous sequence variants (HSVs) and varietal SNPs.

Upon SNP identification, various systems have been devised for SNP profiling, such as the development of allele-specific oligonucleotides for agarose gel electrophoresis (Wallace et al. 1979) and, more recently, fluorescence-based systems such as the KASPar® (KBioscience Competitive Allele-Specific polymerase chain reaction) system and the fluorescent high resolution DNA melting (HRM) analysis, among the others (Sobrino et al. 2005). The KASPar assay (Orrù et al. 2009; Allen et al. 2011) is a cost-effective genotyping assay providing a highly specific fluorescent assay with an improved signal-to-background fluorescence ratio. The HRM analysis is a post-PCR analysis method based on dsDNA-binding dyes and high-resolution analysis of the PCR melting (dissociation) curves (Dong et al. 2009). This assay allows for the discrimination of a single SNP included in short PCR amplicons.

In this work, we describe the enrichment of the region surrounding the *Lr14a* locus with newly identified COS-SNP and HRM markers more suitable for cost-effective MAS and more accurate haplotyping for a better diagnosis of the presence of *Lr14a* in durum wheat.

Materials and methods

Plant materials

A total of 176 durum RILs ($F_{7:8}$) obtained from the cross of the Italian cv. Colosseo (leaf-rust-resistant parent; hereafter referred to as C) and the North American cv. Lloyd (susceptible parent; hereafter referred to as L) were previously produced through single seed descent (Maccaferri et al. 2008) and herein used for linkage mapping analysis.

A panel of 164 durum cvs. and advanced breeding lines bred in Mediterranean countries, South-western USA and Mexico (Maccaferri et al. 2010) have been used for evaluating the accuracy of the PCR-based markers mapped near the *Lr14a* locus in predicting leaf-rust resistance. The complete list of the panel accessions is available as supplementary material in Table S1. *T. aestivum* cv. Chinese Spring (CS) and its nulli-tetrasomic-derived lines (CS N7BT7D and CS N7AT7D) were kindly provided by Beat Keller (University of Zurich, Schweiz) while *Triticum urartu* (AA genome) accession MG29422-1558 was kindly provided by Antonio Blanco (University of Bari, Italy).

A ditelosomic line of wheat *T. aestivum* L. cv. Chinese Spring carrying the 7AL chromosome arm as a telocentric chromosome ($2n = 40 + 2t7AL$), and a double ditelosomic line of Chinese Spring carrying both arms of chromosome 7B as telosomes ($2n = 40 + 2t7BS + 2t7BL$) were used to flow sort the 7AL and 7BL chromosome arms (Dolezel et al. 2007). The seeds were kindly provided by Dr. Bikram Gill (Kansas State University, Manhattan, USA).

Adult-plant resistance screening

The C × L RILs were evaluated in 2006 for leaf-rust response in a field trial conducted in Argelato (44°39′N 11°20′E, Italy). RILs were evaluated in 2.5 m long rows with three replicates arranged in a randomized complete block design (RCBD, for details see Maccaferri et al. 2008). Plots were artificially inoculated with a mixture of 16 Italian *P. triticina* isolates collected from durum wheat, whose avirulence/virulence formula has been reported in Mantovani et al. (2010). All the isolates were avirulent on *Lr14a* as assessed on the North American Thatcher differential set (Mantovani et al. 2010). Three visual scores of percentage of infected leaf area were carried out during the disease development cycle and the area under the disease progress curve (AUDPC) value was then calculated for each RIL.

The accessions of the durum panel were evaluated for leaf-rust response in five trials. Two trials were conducted in Argelato, Italy, in 2006 and 2007 by inoculating the mixture of the 16 isolates used for the C × L RIL evaluation. Three additional trials were conducted in El Batan (19°31′N 98°50′E, Mexico) in 2006 and Ciudad Obregon (27°33′N 109°09′E, Mexico) in 2007 and 2008, by inoculating the two Mexican races BBG/BN and BBG/BP, both avirulent on *Lr14a*; methodologies and results are reported in Maccaferri et al. (2010). Similarly to the C × L RILs, AUDPC values were obtained for each field trial and reported as relative disease severity indexes (rAUDPC) by setting '100' as the reference value of the susceptible cv. Kofa. This allowed for a more meaningful comparison of results across trials inoculated by the same races.

Seedling resistance screening

Single-uredinial leaf rust isolates were used for *Lr14-Creso* screening at the seedling stage. The C × L RILs were inoculated under controlled greenhouse conditions using the PSB_1–3 isolate provided by Produttori Sementi Bologna (PSB, Bologna, Italy). Infection types (ITs) were recorded following both the 0–4 scale described by Long and Kolmer (1989) as well as the 0–9 scale of McNeal et al. (1971); methodologies and results are reported in Maccaferri et al. (2008).

For the durum wheat accession panel, previously known to have other major genes in addition to *Lr14a* as well as uncharacterized minor genes, up to 25 isolates collected from both tetraploid and hexaploid wheat cvs in Italy, Europe and from the worldwide isolate collection held at the USDA-ARS Cereal Disease Laboratory, St. Paul MN, USA, were used.

Scoring was recorded using the 0–4 scale (Long and Kolmer 1989). The IT responses from four isolates selected for their clear avirulence against *Lr14a* were used for further analysis.

Phenotypic data analysis

The field AUDPC responses of all the C × L RILs were converted into Colosseo-like (resistant) or Lloyd-like (susceptible) scores on the basis of the 95th percentile confidence intervals of the distributions observed in the two groups of RILs with the complete parental haplotypes (C and L, respectively) at the *Lr14a* chromosomal region. For the seven RILs with phenotypic values falling outside these confidence intervals, missing (undetermined) scores were imposed. Similarly, the IT scores of the RILs assessed

with the Italian leaf rust isolate PSB_1–3 were mendelized based on the responses of the two parental lines.

The leaf-rust responses of the 164 durum panel accessions were determined as IT scores obtained at the seedling stage and as the rAUDPC value in five artificially inoculated field trials.

The rAUDPC values of the accessions were converted to binomial resistant (R) or susceptible (S) responses based on the following criteria: (1) for each trial, the rAUDPC threshold for declaring an accession as resistant was set based on the maximum values observed for Creso and the group of derivatives known to carry *Lr14-Creso* (Arcangelo, Bicre, Colosseo, Italo, Plinio and Radioso), (2) the accessions with rAUDPC values higher than 50 % up to 100 % were considered as susceptible, (3) the accessions with intermediate rAUDPC values (either medium resistant and medium susceptible) were considered as missing (undetermined) scores for further analyses.

Since the repeatability of the field responses observed over years in Italy and in Mexico was high (Maccaferri et al. 2010), the results from the two Italian trials and the three Mexican trials were merged in two single R/S score phenotypic series for the Italian and Mexican environments, respectively, based on the consensus of the disease response observed over years.

Based on the results of the joint phenotypic and molecular analysis reported in Maccaferri et al. (2010) from the isolate survey, four of these isolates (namely, Eth6.1-1, LR#Td1649, MX14.3 and PSB_1–3) were selected for their discriminating features of the *Lr14-Creso* allele. The IT of the accessions evaluated with the four selected isolates at the seedling stage was scored as resistant (R) for values between 0 and 2, while those with IT equal to 3 and 4 were considered as susceptible (S).

Synteny analysis

The orthologous genes to the *Lr14-Creso* interval in wheat were predicted from the two model species *O. sativa* L. and *B. distachyon* L. through a reciprocal BLASTN analysis. The sequences of the sequence tagged sites (STS) markers identified as *mag1811* and *mag1932* were searched against the 22,000 unique full-length EST sequences of the *T. aestivum* Gene Index Project (TaGI) database release 12.0 (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=wheat). The full-length cDNA sequences were then BLASTN searched against the *B. distachyon* database (Brachybase, The International Brachypodium Initiative 2010; http://www.brachypodium.org/) and against the Rice Annotation Project (RAP) database (Ouyang et al. 2007; http://rice.plantbiology.msu.edu/index.shtml). The best hit with expected values smaller than e^{-15} was considered as the putative ortholog. We considered the orthologous relationships to be confirmed only if the identity of the reciprocal alignments of the retrieved genes was above 85 % and the expected values were still below e^{-15}. Once the colinearity was confirmed, the regions between the orthologs were searched in the two model species for the presence of additional genes. The coding sequences of these genes as from the RAP database were used as query against the TaGI database. The resulting full-length wheat cDNAs were then further searched against the Brachybase to confirm the syntenic relationships. The process was then inverted, starting from Brachybase and ending on the RAP. The function of the genes was also predicted on the basis of the annotation (pfam) of the RAP for the corresponding orthologous gene. A similar analysis was also performed with WheatZapper, (http://wge.ndsu.nodak.edu/wheatzapper/) an on-line tool developed at North Dakota State University. The output of this software matched our initial predictions and is reported with minor modifications in Table S2.

The location of the intron/exon boundaries and intron length in the wheat cDNA was predicted through BLASTZ analysis by aligning the wheat cDNA to rice and *Brachypodium* genomic sequences using *CoGe* Blast tool (Comparative Genomics platform: http://www.genomevolution.org/CoGe/CoGeBlast.pl) and the PpETs database (Polyploid Estimated Transcript Server: http://www4.rothamsted.bbsrc.ac.uk/ppets/modperl/index.pl). The edited cDNA sequences were then used for designing primers in the exon sequences to amplify across introns.

UBW marker development

The development of COS markers was undertaken to saturate the *Lr14-Creso* region on the basis of colinearity with rice and *Brachypodium*. Newly identified COS-SNPs were used to generate PCR-based markers which were designated as University of Bologna Wheat (UBW) markers. Two methodologies were used to search for varietal COS-SNPs:

1. A two-step PCR amplification and sequencing approach, with no T-vector cloning required (*ubw15*, *ubw22* and *ubw26*). Primer-Blast tool (available at http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primer pairs amplifying regions containing introns using the edited wheat EST sequences as template. Typically, these primer pairs were common to the homeologous gene sequences. Standard Primer-Blast parameters were maintained except for the primer melting temperatures that were raised to 60–63–65 °C, amplicon length was set at 700–1,300 nucleotides, and GC clamps of minimum two were imposed. DNA of flow-sorted chromosome arms 7AL and 7BL, respectively (Dolezel et al. 2007), was

purified and multiple-displacement-amplified as described in Šimková et al. (2008). Approximately 10 ng of the amplified chromosomal DNA was used as PCR template.

The PCR was run under the following conditions with 1× of GoTaq Flexy Buffer (Promega, Madison, USA), 1.8 mM $MgCl_2$, 0.25 mM dNTPs. 150 nM of each primer, 5 % of DMSO, 0.75 U of GoTaq polymerase (Promega, Madison, USA) using 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C, and a final extension of 10 min at 72 °C. Amplicons were separated on 1.5 % agarose gel and sized using a GeneRuler™ 1 Kb DNA Ladder 100–3,000 bps (Fermentas, Thermo Fisher Scientific, MD, USA) as a standard marker of DNA molecular weight. PCR bands were cut and eluted using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA). Direct sequencing of the eluted products was carried out with the primer pair used for the initial amplification, at PTP (Parco Tecnologico Padano, Lodi, Italy). ChromasPro (Technelysium Pty Ltd) was used to align the sequencing results, remove incorrect or low-quality base calls, and identify chr. 7B-specific HSVs. Primers were hand designed on these HSVs to specifically amplify the chr. 7B segment following these general rules: length of 20 nucleotides, >50 % of GC content, the HSVs were always positioned at the 3′ end, HSVs involving cytosine and guanidine were preferred over adenine and thymine, transitions were preferred, HSVs within exons were favored, trying to maintain maximum overall amplicon size. A potential 7B-specific amplicon was obtained by combining the primers designed on the HSVs with one of the initial sequencing primers. PCR testing was conducted at 55, 60, and 65 °C annealing temperatures at 1.5, 1.8 and 2.0 mM of $MgCl_2$, including gradient PCR, until a clear band was obtained from CS, C and L, but no band in CS N7BT7D. Under these conditions, the C and L bands were eluted and used for the second step of direct sequencing. Single nucleotide polymorphisms (varietal SNPs) were identified and used to design allele-specific primers based on similar rules as those used for HSVs with the following additions: insertion–deletions (INDELs) were preferred over SNPs, multiple SNPs within the primer sequence were favored, independent SNPs were used to design C- and L-specific primers, each C- or L-specific primer was used in combination with the chr. 7B-specific primer designed on the opposite orientation. Where possible a combination of all three primers was used to generate a codominant marker.

1. One-step PCR and sequencing methodology, including T-vector cloning (*ubw14*, *ubw18*, *ubw19*, *ubw31*, *ubw33*, *ubw35* and *ubw44*). A modified version of the general methodology first described in the NSF wheat SNP discovery project "Haplotype Polymorphism in Polyploid Wheats and their Diploid Ancestors" (PI: Jan Dvorack, http://wheat.pw.usda.gov/SNP/internal/protocols.shtml) and then proposed by Schnurbusch et al. (2007) was adopted. Initial conserved primers were designed as described above, but used for direct amplification of DNA from C, L and accession MG29422-1558 of *T. urartu*. Bands were gel-cut, purified and cloned into a subcloning vector (pGEM® T Easy Vector, Promega) and transformed in *E. coli* JM109 competent cells (Promega) by heat shock. For each cloned PCR amplicon, 12 white colonies were picked and amplified by colony PCR from each of the three C, L, and *T. urartu* templates, respectively. Reactions of colonies were performed under standard condition using 1× GoTaq Flexi Buffer (Promega), 3 mM $MgCl_2$, 0.25 mM dNTPs, 250 nM T7-promoter-Forward and SP6-terminator-Reverse universal primers (Sigma) and 1 U of GoTaq DNA Polymerase (Promega). The selected amplicons were purified by ZR-96 DNA Clean-up Kit™ (Zymo Research) and used as template for Sanger sequencing (Macrogen Inc, Seoul, Korea), with the T7-Promoter Forward universal primer. Clones belonging to A and B genome, respectively, were recognized on the basis of strong sequence similarity to the A genome of *T. urartu* using the multiple alignment tools MEGA 5.0.5 (http://www.megasoftware.net) and DNAMAN v. 6 software (Lynnon Corporation). The B genome sequences were then searched for SNPs/INDELs between C and L. As for the previous methodology, three primers were designed, two of which were C- and L-allele specific while one was specific for chr. 7B. The C- and L-specific primers were designed either on two independent SNPs or on the same SNP position for *ubw14*, *ubw18*, *ubw19*, *ubw33* and *ubw35*. The fragment size polymorphism was obtained by adding to the 5′ end of the C-specific primer the M13-tail 21mer sequence (Schuelke 2000). This 21 nucleotide difference in size can be easily detected in 2.5 % agarose. *Ubw31* was developed as a dominant, 7B-specific marker by designing the primer pair directly on the L sequence since it was not possible to obtain the corresponding amplicon in Colosseo (null allele) while *ubw44* was obtained through a High Resolution Melting assay. Forward and reverse primers for *ubw44* were designed to amplify the region containing the single SNP between C and L while providing chr. 7B specificity, as identified by cloning.

Marker development by HRM analysis

Forward and reverse primers spanning every putative SNP were designed for HRM analysis using Primer3 Input

version 0.4.0 tool (available at http://frodo.wi.mit.edu/). The pairs of primers were designed to have a melting temperature between 58 and 61 °C and to produce an expected product size of 50–250 bps. The primers were then analyzed using NetPrimer (http://www.premierbiosoft.com/netprimer/netprimer.html) at Premier Biosoft International to detect any possible secondary structure (i.e., primer dimer, hairpin, palindrome and/or repeats) that could affect the PCR amplification efficiency and therefore the HRM analysis accuracy.

PCR amplifications were performed in 96-well plates (MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL, Applied Biosystems, Foster City, CA) in a total volume of 10 µL using a 7500 Fast Real Time PCR ready instrument (Applied Biosystems). The reaction mixture contained 20 ng wheat genomic DNA, 300 nM forward and reverse primers and 5 µL of MeltDoctorTM HRM Master Mix 2× (Applied Biosystems) with MeltDoctor™ HRM Dye, a stabilized form of the fluorescent SYTO® 9 double-stranded nucleic acid stain developed by Molecular Probes and AmpliTaq Gold® 360 DNA Polymerase (Applied Biosystem). The amplification was achieved by the following PCR protocol: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s followed by annealing/extension for 1 min at the specific annealing temperature, and a final extension at 72 °C for 10 min. Melt/dissociation assays were performed as follows: denaturation 95 °C for 15 s, and 60 °C for 1 min followed by HRM ramping at 60–95 °C with fluorescence data acquisition at 0.025 °C increments (29 fluorescence reads per degree).

Melting curve data were collected using the Expert Mode function within the 7500 Fast SDS v1.4 using the filter 1 only. The melting curves were then analyzed using the HRM Software v2.0 available on the Applied Biosystems 7500 Fast System. Analysis of HRM variants was based on differences in the shape of the melting curves and in T_m values.

The allelic variants were displayed through different HRM plot methods: (1) the aligned melt curves plot (i.e., the melt curves as % melt from 0 to 100 %) over temperature; (2) the derivative melt curves plot that displays the $-(d/dT)$ fluorescence as a function of temperature, the pre- and post-melt regions were reviewed and adjusted to optimize the curve separation and variant calls; (3) the difference plot display in which the data were reported as the difference in fluorescence between the melt curve for a reference sample and the other melt curves. A control genotype was selected as a reference. After the reference selection, the software subtracts the reference curve from the sample curves. The difference plot allowed for more precise visualization of small differences between curves and for identification of outliers.

PCR products analyzed by HRM were purified and cloned using pGEM® T Easy Vector System and transformed in *E. coli* JM109 competent cells (Promega) by heat shock. To validate the HRM results, positive clones were purified, sequenced and analyzed according to the protocol already described herein.

KASPar assay for SNP validation

Samples of genomic DNA of C, L, CS N7BT7D, CS N7A7D and C × L RILs together with the high-quality amplicon sequences obtained as previously described were provided to KBiosciences Company (Hoddesdon, UK) to convert the *ubw14* and *ubw18* markers in KASPar assays by KASP-on-Demand Genotyping service. The KASPar assays were tested on the RILs and the SNPs were validated by the company using KASP Reaction Mix (KBioscience). Complete details on principle and procedure of the assay are available at http://www.kbioscience.co.uk/reagents/KASP_manual.pdf and http://www.kbioscience.co.uk/download/KASP.swf.

Molecular and linkage analysis

A genetic map obtained from the 176 C × L RILs based on SSR and DArT molecular markers was reported in Mantovani et al. (2008). Thirty-five additional molecular markers from the literature were tested following the protocols indicated in the GrainGenes database (http://wheat.pw.usda.gov/GG2/index.shtml) or in publications (Somers et al. 2004; Elouafi and Nachit 2004; Quarrie et al. 2005; Schnurbusch et al. 2007; Mantovani et al. 2008; Xue et al. 2008; Zhang et al. 2008; Herrera-Foessel et al. 2008a). Amplification of UBW markers was performed under standard PCR conditions with 1× GoTaq Flexy Green Buffer (Promega), 1.8 mM $MgCl_2$, 0.25 mM dNTPs, 150 nM of each primer, 1 U of GoTaq DNA Polymerase (Promega) and 100 ng genomic DNA. Thermocycling conditions were as follows: initial denaturation of 5 min at 94 °C followed by 35 cycles of 45 s at 94 °C, 45 s at the specific T_m reported in "Results" and 1 min at 72 °C, followed by a final extension of 10 min at 72 °C. Markers tests for chr. 7B specificity were carried out employing CS and CS N7BT7D and CS N7AT7D as described above. All markers producing a chr. 7B-specific band polymorphic between C and L were then used for genotyping the C × L RILs and the durum panel.

Markers data of the RILs were used as input for the version 1.2.2 of the Carthagene software (de Givry et al. 2005), employing the 'build' command at LOD of 3.0, followed by 'annealing', 'greedy' and 'flips' commands to improve markers order. The resulting high-quality genetic map was then hand-curated and graphically depicted using version 2.2 of MapChart (Vorrips 2002).

Markers' effectiveness for detection of leaf-rust resistance induced by Lr14-Creso in the durum wheat germplasm

The association panel of 164 accessions was used to estimate the efficacy of markers in predicting the leaf-rust response phenotype (markers' breeding value). Detailed information regarding the leaf-rust response, population structure and genetic relationships among accessions are available in Maccaferri et al. (2010, 2011).

The ability to predict the phenotype at the causal locus among lines sampled from the breeding germplasm is a relevant feature for MAS applications. A marker is called 'diagnostic' when it precisely predicts the phenotypic state of a breeding line on the basis of the allelic state at the causal locus.

For each marker, 'predictability' was calculated as the percentage of the durum panel lines where the presence of the C allele (i.e., a Creso-derived allele) at the given molecular marker matched with the resistant leaf rust phenotype, hence the ability to correctly identify accessions carrying the *Lr14-Creso* allele. Predictability was also calculated for the alternative allele as the percentage of the durum panel lines where the presence of the L parental allele was associated to susceptibility. In case of markers with multiple alleles (such as the SSRs), all the accessions with alleles other than the Creso-derived allele were pooled in a unique 'non-Creso' allelic class and checked for matches with the susceptible phenotypes, hence giving the ability to identify breeding lines that do not carry the *Lr14-Creso* allele. Similar analysis was carried out for the selected two-marker haplotypes where the C-derived haplotype was compared to the pool of all the other non-Creso or recombinant haplotypes.

Results

Enrichment of the Lr14-Creso interval

The genetic location of *Lr14a* in durum wheat as it was previously defined by three independent mapping studies carried out by Herrera-Foessel et al. (2008a), Maccaferri et al. (2008) and Marone et al. (2009) is represented in Fig. 1. In this figure, the partial linkage maps of the distal 7BL chr. arm and the markers most closely linked to *Lr14a* (reported as *Lr14-Llareta* and *Lr14-Creso*, respectively) are highlighted. The *Lr14a* location pointed to a region clearly identified on its proximal side by the SSR marker *barc340* while on the distal side the end of the interval was less precisely defined due to scarcity of polymorphic markers. Linkage to *Lr14a* peaked in the close proximity of markers such as *Pst87/Mse67* AFLPs in the Atil C2000 × Llareta-INIA population (Herrera-Foessel et al. 2008a), *wPt-4038* and *wPt-1085* DArT® markers in the Colosseo × Lloyd (C × L) population (Maccaferri et al. 2008) and the *mag4362* STS marker for the Creso × Pedroso population (Marone et al. 2009); these markers were all located distal to SSR marker *gwm344* (Fig. 1).

To further saturate this region, ten previously published wheat chr. 7BL genetic maps spanning the region distal to the landmark SSR loci *gwm577–barc340–gwm146* (Elouafi and Nachit 2004; Somers et al. 2004; Quarrie et al. 2005; Schnurbusch et al. 2007; Herrera-Foessel et al. 2008a, b, c; Mantovani et al. 2008; Xue et al. 2008; Zhang et al. 2008) were hand aligned and searched for molecular markers. In total, 46 markers mapping between *gwm577* and the close proximity of the chr. 7BL telomere were retrieved, including 22 SSRs, 13 EST-derived markers (PSY1, AWW markers and STS markers of the MAG series) and 11 DArT markers.

Among the 46 markers that were retrieved from the literature, four were excluded from further analyses because they were located outside the target interval while eight had already been mapped by Mantovani et al. (2008) and by Maccaferri et al. (2010) in the C × L RILs. The remaining 34 markers, including 18 SSRs and 16 STSs, were investigated for chr. 7B specificity and for polymorphism between the parental lines. At the amplification conditions employed in this study, 21 resulted not to be chr. 7B specific, 10 were chr. specific but monomorphic between C and L and one SSR not previously associated to *Lr14a* (*gpw8090*) and two MAG STSs (*mag1811* and *mag1932*, Xue et al. 2008) were successfully mapped on the 176 RILs, representing only 11 % of the tested markers. In addition, the YP7B-1 STS corresponding to the *PSY-B1* gene, the chr. 7B-homeolog of phytoene synthase 1 (He et al. 2009), was found to be polymorphic in the C × L RIL population. All these four markers mapped within the *Lr14-Creso* interval.

Development of COS-SNP markers from conserved orthologous sets

MAG marker set and YP7B-1 (phytoene synthase, PSY1) are gene-based markers for which the sequence is publicly available. Based on the aligned maps and the two MAG markers mapped in the C × L map, the sequences of these markers were used as anchors to determine the syntenic relationships between the *Triticum durum* chr. 7B (Td7B), *O. sativa* L. (Os) and *B. distachyon* L (Bd) in the *Lr14a* region. The short arm of *Brachypodium* chr. 1 (Bd1S) and the long arm of rice chr. 6 (Os6L) provided the highest colinearity conservation. In this region, Bd1S and Os6L showed very close ancestral relationships, with 13 genes sharing identical genomic order between the two species.

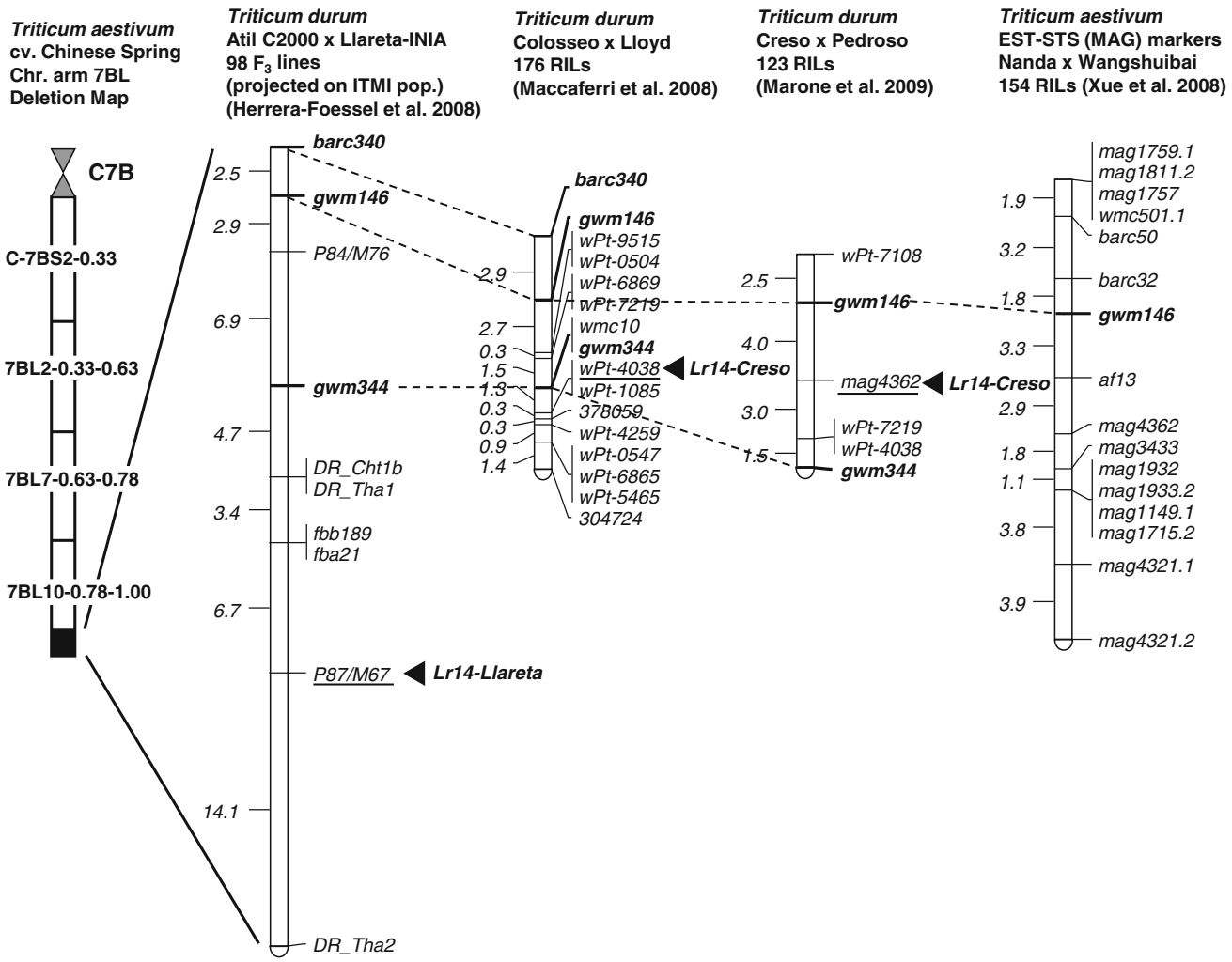


Fig. 1 Genetic maps of *Lr14a* locus on the distal portion of chr. arm 7BL as reported in three durum wheat mapping populations, namely Atil C2000 × Llareta-INIA, Colosseo × Lloyd, Creso × Pedroso. The markers most associated to *Lr14a* are *underlined*. SSR markers that were used as anchors across maps are in *bold* and their positions are connected by *dashed lines*. The most likely mapping position of *Lr14a* is reported with *black-filled arrows*. The map position of *Lr14-Llareta* is reported as projected on the ITMI population, whose marker density is higher than that obtained in the original Atil C2000 × Llareta-INIA cross, as reported by Herrera-Foessel et al. (2008a, b, c). The Colosseo × Lloyd and Creso × Pedroso mapping populations segregate for the same *Lr14a* resistance allele, indicated as *Lr14-Creso*, being Colosseo a direct derivate of Creso. A fourth linkage group from the hexaploid Nanda × Wangshuibai mapping population has been included as a reference for the MAG markers (Xue et al. 2008)

Based on the orthologous genes predicted to map within the *Lr14a* region, we attempted to develop new COS-SNP markers (UBW markers). Two similar strategies based on PCR amplification and sequencing were adopted for marker development. Thirty-three genes were investigated by amplifying and sequencing DNA from the flow-sorted chr. 7A and chr. 7B of Chinese Spring to develop chr. 7B-specific primers. In the first step, the chr. 7B-specific primers were developed for 14 (42 %) out of the 33 loci that were considered. Subsequently, among the ten (30 %) genes that were found to contain SNPs between C and L, robust marker assays were obtained for three (9 %) genes only (*ubw15*, *ubw22* and *ubw26*). *Ubw15* and *ubw22* were developed based on the sequences obtained from the two dominant STS markers *mag1811* and *mag1932*, respectively, that corresponded to COS sets. A total of approx. 23 kb were sequenced to achieve this goal, approx. 10.5 kb from chrs. 7A and 7B, and approx. 12.5 kb from C and L.

Twenty-four genes were investigated by direct amplification, cloning and sequencing of the genomic DNA from C, L and MG29422-1558, a *T. urartu* accession (Figure S1). For each of the sampled genes, at least one PCR amplicon of length comprised in the range of 700–1,200 bp was obtained and cloned. Seven contained reliable SNPs between C and L and were thereafter converted to polymorphic markers that mapped in the *Lr14-Creso* region (*ubw14*, *ubw18*, *ubw19*, *ubw31*, *ubw33*, *ubw35* and *ubw44*) together with the three *ubw* markers developed with the

two-step sequencing approach. The remaining SNPs that were converted to polymorphic markers (*ubw1*, *ubw30* and *ubw32*) mapped on the chr. 7A homeologous region (data not showed). A total of approx. 24.0 kb were cloned and sequenced in the three reference lines to achieve this goal (Figure S1).

Both markers development methods targeted preferentially the variable intron sequences. In this study, the average SNP frequency between C and L was estimated at ca. 1 SNP/1 kb, while the frequency of HSV (polymorphisms between the A and B genomes) was ca. 1 SNP/0.05 kb. Overall, ten markers were developed on the basis of syntenic genes, seven of which were codominant (*ubw14*, *ubw18*, *ubw19*, *ubw22*, *ubw33*, *ubw35* and *ubw44*) and three were dominant (*ubw15*, *ubw26* and *ubw31*) in repulsion, i.e., with the specific PCR amplicon associated to the susceptible parent Lloyd. Table 1 reports the PCR primers and amplification conditions for the developed assays and Fig. 2a and b shows the polymorphic PCR products separated on standard agarose gel electrophoresis.

The SNP tagged by *ubw44* marker was genotyped by developing a HRM assay. A short, 157-bp chr. 7B-specific amplicon that included the *ubw44* SNP was designed and the C and L alleles were discriminated by HRM as shown in Figure S2. The fluorescence difference plot analysis allowed for a robust distinction between the melting features of the two relevant alleles.

Conversion of DArT markers to HRM markers and development of KASPar® assays

The nucleotidic sequences of 13 DArT® markers previously mapped in the target region, available at the Triticarte database (http://www.triticarte.com.au/), were used to design PCR assays. Three of these DArT markers were previously mapped in the C × L population (*wPt-1085*, *wPt-4038* and *wPt-4259*) while the remaining ten DArT markers were mapped in various hexaploid populations (*wPt-0530*, *wPt-0465*, *wPt-0786*, *wPt-0884*, *wPt-1533*, *wPt-4057*, *wPt-4140*, *wPt-4875*, *wPt-7113* and *wPt-7413*). The sequences were assessed for polymorphisms and converted to single locus markers in one-step phase by designing short-amplicon PCR assays (50–250 bp) to be assessed by HRM analysis as described above for *ubw44*.

Thirty primer pairs were designed, amplified on C and L genomic DNA and the PCR products checked for PCR quality on 1.5 % agarose gel electrophoresis, then tested by HRM analysis to detect the presence of SNP and/or INDEL (insertion or deletion) polymorphisms between C and L. Seven primer pairs corresponding to the DArT markers *wPt-1533*, *wPt-4038*, *wPt-4140*, *wPt-0465*, *wPt-0884*, and *wPt-7113* showed high-quality HRM polymorphisms suitable for genetic mapping in the C × L RIL population. Five out of seven assays (*wPt-0465-HRM*, *wPt-1533-HRM*, *wPt-4140-HRM*, *wPt-4038-HRM* and *wPt-7113-HRM*, Table 1) were successfully re-mapped in the *Lr14-Creso* interval. Out of the three DArT® markers already mapped in the region, only *wPt-4038* was converted to an HRM assay capable to efficiently discriminate between the two parental alleles.

Subsequently, the SNP information deployed to design C- and L-specific primers was used to develop KASPar assays for *ubw14* and *ubw18* (Table 2). This platform was then used to genotype C × L RILs and the results were compared to the PCR scores. The two scorings were in perfect agreement ($r^2 = 1.0$), indicating that the KASPar markers can reliably be adopted for durum wheat breeding.

Colinearity within the Lr14a region

The *Lr14a* region, distal of *barc340*, was enriched with one SSR marker (*gpw8090*), one STS marker (YP7B-1, He et al. 2009), ten COS-SNP UBW markers and five DArT® markers converted to locus-specific PCR-based HRM markers. The final map spans 14.1 cM of the distal portion of chr. 7B (Fig. 3), as identified by a total of 36 markers. The 11 gene-derived markers (ten COS-SNPs and YP7B-1) provided ideal anchor points to study the conservation between the *Lr14a* region in wheat and the two grass model species, *Brachypodium* and rice (Fig. 3; Table S2). The *ubw22–ubw35* interval underlining *Lr14a* corresponds to 387.8 kb of Bd1S including 40 predicted genes and to 136.4 kb of rice Os6 (corresponding to 29 predicted genes) plus small and non-precisely colinear portions of Os2 (including six genes), Os11 (including seven genes), Os12 (including two genes) and other genes for a total of 48 predicted genes (Fig. 3; Table 3 and Table S2).

In particular, four predicted genes in a 30 kb region of Bd1 at positions 25,002.6 kb–25,024.3 kb (Bradi1g29450–Bradi1g29427) all matched one single rice gene (Os11g41540) and one single wheat EST (NP9351223). Also, ten other genes from Os6 did not match any genes of *Brachypodium*, even though most of them have an orthologous EST in wheat. Overall, the consolidated information from the two species resulted in the identification of 35 orthologous wheat ESTs. Of the 11 gene-based markers mapped in durum wheat in the target interval, 10 were designed from orthologous genes from Os6 and Bd1, while one marker was designed from a gene with orthologous copies in Os2 and Bd1. Overall, gene order was highly conserved between wheat and *Brachypodium* and, to a lesser extent, between rice and wheat. In general, all the ortholog-derived markers mapped within the interval harboring *Lr14a* as predicted by synteny analysis.

Table 1 Primers and amplification conditions for the allele- and locus-specific PCR markers mapped in the distal region of chr. 7BL harboring *Lr14a*

Wheat marker	Genetic distance[a] (cM)	PCR primers sequence[b] bases (5′–3′)	Annealing temp (°C)	Marker type[c]
wPt-7113-HRM	2.7	F: ACGGCACTGAGCATTGAC	58	HRM
		R: AGAATCCCATGAGACCATACC		
ubw14	3.0	F-C: *agcttgtaaaacgacct***T**TATACTTTTCGGC**A**CAA**G**	60	COS-SNP
		F-L: CT**C**TATACTTTTCGGC**G**CAA**T**		
		R: ATTGTCTCAAAGTAGTACTAGT		
ubw22	4.7	F-C: CCTCCTT**C**AACCCACCCCT**G**	60	COS-SNP
		F-L: CGATATGCTT**G**GCCGAGTTC**AT**		
		R: CCTCCTTCAACCCACCCCTG		
YP7B-1	5.0	F: GCCACAACTTGAATGTGAAAC	60	COS-SNP
		R: ACTTCTTCCATTTGAACCCC		
wPt-4038-HRM	6.9	F: GTTTAAGGTATAACGGCAGTACACT	56	HRM
		R: GTGCAGGGTATAAGTTATACTTATACAGAA		
wPt-4140-HRM	8.0	F: GTAAAAACAGACACGTCGGG	58	HRM
		R: TGCAGTGAAGGAAGGTTCC		
ubw26	9.1	F: GCAGCATTCTGCATCTCTT**T**	58	COS-SNP
		R: GAGGACGTCTTCGAGAGCAT		
ubw15	9.1	F: TGCGTCGATCCTTGC**G**	58	COS-SNP
		R: TGCTAGCTAGAGAGATATCC		
ubw44	9.1	F: ACAAACCCTTGGTTGTCG	59	COS-SNP. HRM
		R: TAAAGTAAGCACTCGGATCG		
ubw18	9.4	F-C: *cacgacgttgtaaaacga*CCTCCTCTCTGATGCAATT**GTATGTC**	60	COS-SNP
		F-L: CAATTGTGTCTGCTTC**A**TCGT**G**		
		R: CCTGGGTTTATTTACTGTCACTAC		
ubw19	9.4	F-C: *cacgacgttgtaaaacga*CTCC**CCGC**CTATGC**T**A**AC**AA**TG**	62	COS-SNP
		F-L: ATTTATCC**TGAT**CTATGC**G**A**GT**AA**CC**		
		R: CATGCACAACATTGCAACCAATGA		
ubw31	9.4	F-L: ATT**C**TT**G**TAAGCTGGATTTT**A**G**T**T**A**	60	COS-SNP
		R: AATCCCATAAACATTTCATACCG		
ubw33	10.5	F: TCAGCAAAATGCCTAGAAGG	60	COS-SNP
		R-C: GATTAGAA**G**ACA**G**TC**G**GC**G**		
		R-L: *cacgacgttgtaaaacga*ATATGTTTCTCCTACATAT**T**TT**C**TAAC		
ubw35	11.1	F-C: ACTATT**AAGTGGTCAAAAAGAATTGT**	58	COS-SNP
		F-L: AAGAGCATAACTGG**C**AACA**A**		
		R: GTGGTGTGATGAACCATTCT		
wPt-1533-HRM	11.4	F: GTCTCCGTGCAGCTACATT	56	HRM
		R: ATCCAGTTTAAATGTTGATGTGA		
wPt-0465-HRM	11.4	F: GTATGCTATAATATCCTCATTACCACA	56	HRM
		R: GAAGTTACTGGCATACTTAATGAAGTT		

[a] Genetic distances as estimated in the C × L RIL population, with marker *barc340* set as the upper limit (0 cM). Genetic distances are in cM

[b] Forward (F) and reverse (R) primers; Creso/Colosseo and Lloyd primer specificities are indicated in the primer names with the 'C' and 'L' acronyms, respectively. Allele-specific bases are indicated in the primer sequences in bold underlined. Oligo tails added to the 5′ end of some of the forward oligonucleotides are reported in italic lower-case font

[c] *HRM* high resolution melting analysis, *COS-SNP* allele-specific PCR

Mendelization of the Lr14a region

Colosseo and Lloyd responded to the *P. triticina* PSB_1–3 isolate with two clearly distinct IT (mesothetic resistant response and susceptibility, as reported in Table 4). The differential response, in combination with the AUDPC values from field observations, was used to predict the allele carried at *Lr14a* by each C × L RIL and the distance

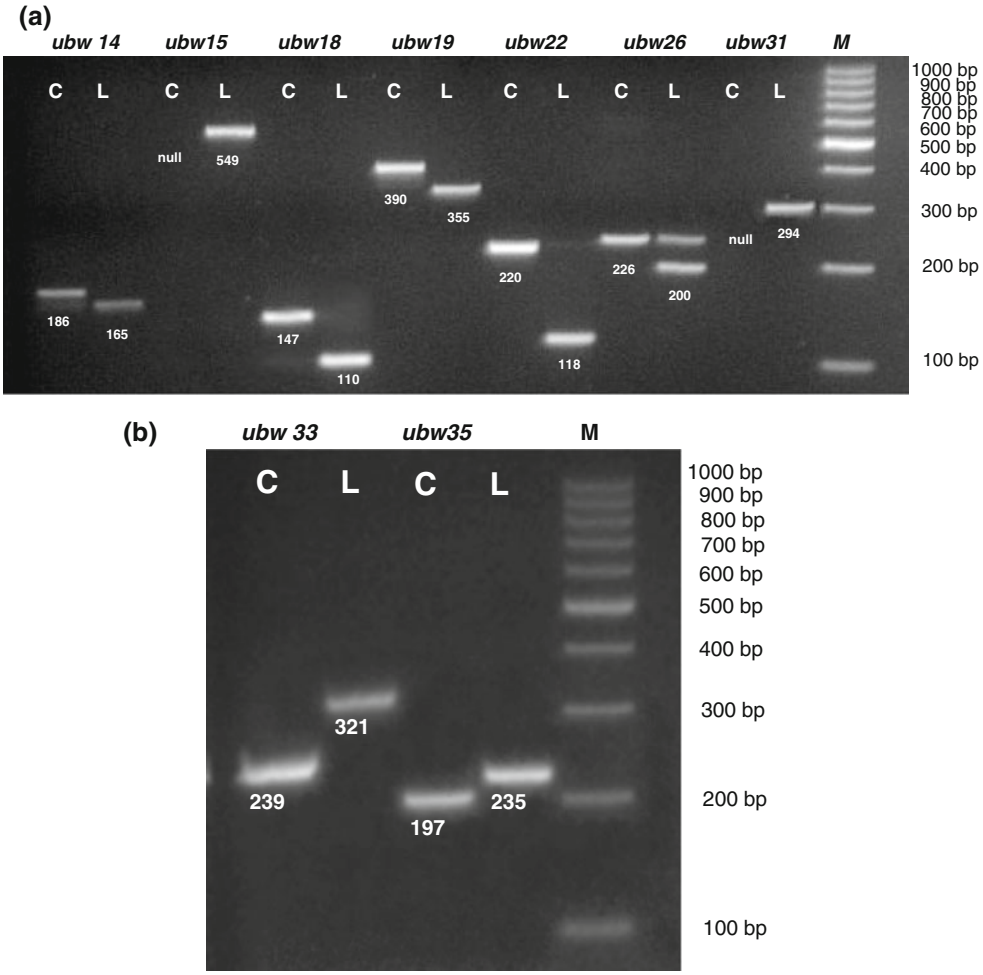


Fig. 2 **a** and **b** PCR products of the chr. 7B-specific COS-SNP *ubw* markers for the two parental lines Colosseo (*C*) and Lloyd (*L*). Allele- and locus-specific PCR products were separated by standard agarose gel electrophoresis. *Ubw15*, *ubw26* and *ubw31* are dominant markers with a single chr. 7B-specific PCR product associated to Lloyd. *Ubw14*, *ubw18*, *ubw22*, *ubw19*, *ubw33* and *ubw35* are codominant assays

Table 2 Primers and amplification conditions for the *ubw14* and *ubw18* KASPar® assays

Wheat marker	PCR primers sequence[a]: bases (5′–3′)	SNP
ubw14 Creso/Colosseo allele-specific primer (FAM)[b]	ACACTAGTACTACTTTGAGACAATTTT**A**	A
ubw14 Lloyd allele-specific primer (VIC)	CTACACTAGTACTACTTTGAGACAATTTT**T**	T
ubw14 common primer	AACAAACTCCAGTGTAAACACCACAGTTT	
ubw18 Creso/Colosseo allele-specific primer (FAM)	GAGCAGGCGCTGCCAGTGG**C**	C
ubw18 Lloyd Allele-specific primer (VIC)	GAGCAGGCGCTGCCAGTGG**T**	T
ubw18 common primer	ACGGGGCTCAGTGGTCCGCTTCCTGGG	

[a] Creso/Colosseo and Lloyd primer specificities are indicated in the primer names with the 'C' and 'L' acronyms, respectively. Allele-specific bases are indicated in the primer sequences in bold underlined

[b] The Creso/Colosseo and Lloyd allele-specific primers have been associated to the FAM and VIC fluorophore dyes, respectively

between the gene itself and the molecular markers mapped in the target interval. *Lr14a* was mapped 1.2 cM distal to the region including *gwm344–wmc10* SSR markers (co-segregating with a series of DArT markers) and 1.1 cM proximal to the series of co-segregating *ubw26–ubw44–ubw15* COS-SNPs, as shown in Fig. 2. The graphical genotypes and the leaf-rust responses of the 19 RILs that showed recombination in the 7BL distal region are shown in Fig. 4. Nine lines (CxL037, 066, 075, 113, 144, 147, 191, 211 and 218) narrowed the *Lr14a* interval to the 3.6 cM region underlined by *gwm344* and *ubw26–ubw44–ubw15*.

Lr14a did not co-segregate with any of the newly developed marker loci and mapped on the proximal side of most of the newly developed COS-SNP markers (from *ubw26* to *ubw35*). However, it was possible to develop two

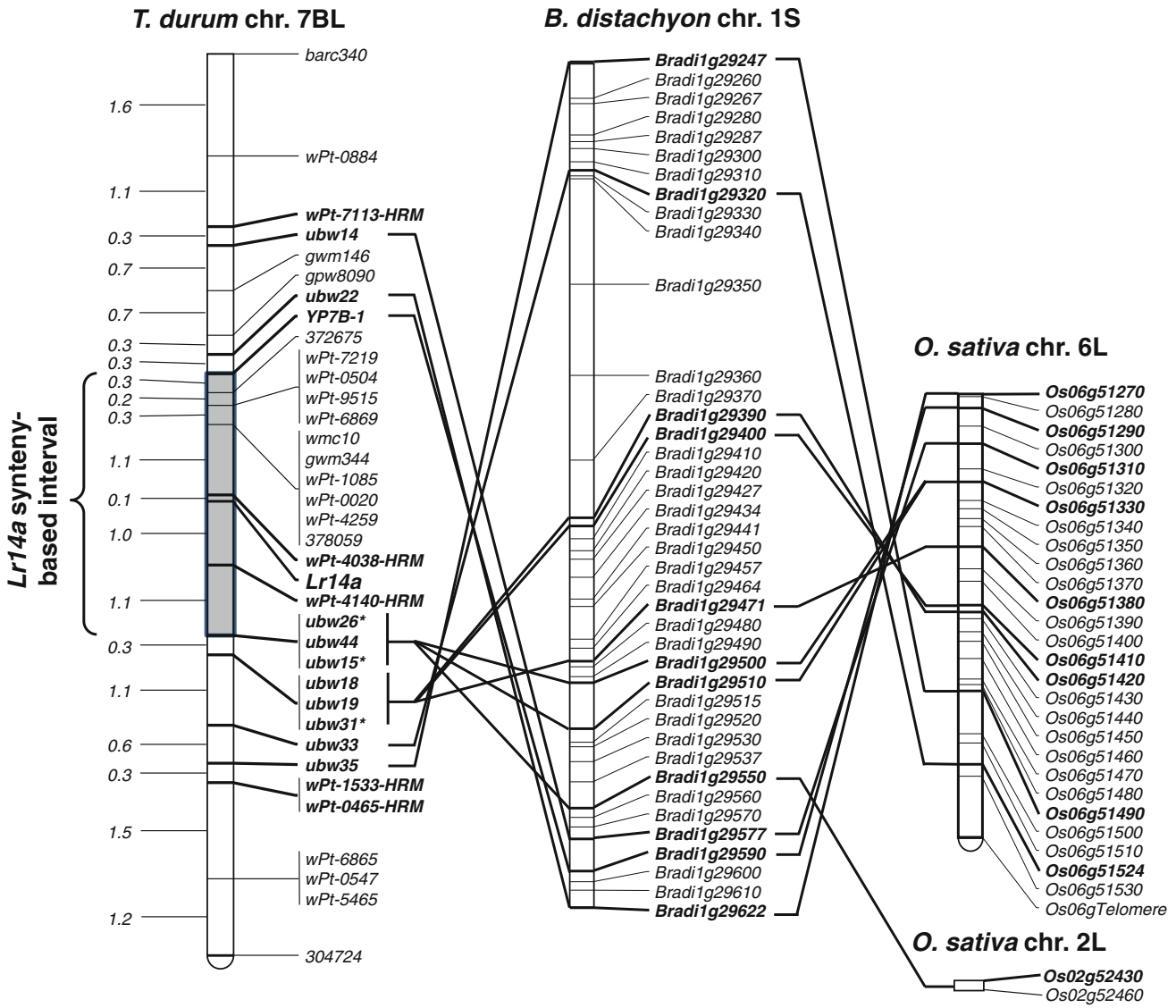


Fig. 3 Saturated genetic map of the chr. 7BL region harboring *Lr-14a* in the Colosseo × Lloyd mapping population and syntenic relationships with *Brachypodium distachyon* chr. 1S and *Oryza sativa* chr. 6L. The conserved orthologs mapped in wheat are indicated in *bold* and their positions across species are connected with *solid lines*. Other orthologous loci found between *Brachypodium* and rice are connected with *dashed lines*. The mapped genomic HRM assays are also reported in *bold*

new locus-specific HRM markers (*wPt-4038-HRM* and *wPt-4140-HRM*) that surrounded *Lr14a*, narrowing its position to a 1.1 cM interval.

Phenotype predictability in the elite durum germplasm

Following the identification of 21 locus-specific PCR-based markers, including five SSRs, their level of association to the leaf-rust response phenotype was investigated in a durum wheat panel of 164 elite lines. The leaf-rust response of these lines, including Creso, Colosseo and several of the Creso derivatives has been reported in Maccaferri et al. (2010).

To estimate the effectiveness of each marker to predict the leaf-rust response phenotype associated to *Lr14-Creso* allele, the adult-plant field-based responses (two environments in Italy and three environments in Mexico) and the IT at the seedling stage were considered. Table 4 reports details on the origin and virulence spectrum of the four single *P. triticina* isolates used for seedling inoculation, as well as the virulence spectrum of the races used for artificial inoculations of the field trials.

The field-based responses and the seedling-based IT values were converted to binomial scores of resistance (R) or susceptibility (S) based on stringent rules (see "Materials and methods"). Field-based responses corresponding to

Table 3 Summary of the wheat-*Brachypodium*-rice collinearity for the conserved orthologous sets (COS) polymorphic between Colosseo and Lloyd and mapped in the chr. 7BL distal region harboring *Lr14a*

Wheat marker	Genetic distance (cM)	*Brachypodium* (ORF)	BLASTn (e value)	Position (start) (kb)	Rice (ORF)	BLASTn (e value)	Position (start) (kb)
ubw14	3.0	Bradi1g29577	1 (e^{-54})	25,130.3	LOC_Os06g51310	3 (e^{-38})	31,069.9
ubw22	4.7	Bradi1g29622	0	25,162.3	LOC_Os06g51270	0	31,047.5
YP7B-1	5.0	Bradi1g29590	4 (e^{-68})	25,145.5	LOC_Os06g51290	1 (e^{-47})	31,054.4
ubw26	9.1	Bradi1g29550	1 (e^{-143})	25,117.2	LOC_Os02g52430	7 (e^{-91})	31,150.3
ubw15	9.1	Bradi1g29510	0	25,080.6	LOC_Os06g51330	6 (e^{-168})	31,087.6
ubw44	9.1	Bradi1g29500	3 (e^{-77})	25,058.9	LOC_Os06g51330	4 (e^{-33})	31,087.6
ubw18	9.4	Bradi1g29464	9 (e^{-62})	25,043.5	LOC_Os06g51380	7 (e^{-56})	31,117.7
ubw19	9.4	Bradi1g29400	3 (e^{-147})	24,987.0	LOC_Os06g51410	2 (e^{-131})	31,144.7
ubw31	9.4	Bradi1g29390	2 (e^{-40})	24,983.3	LOC_Os06g51420	6 (e^{-31})	31,147.1
ubw33	10.5	Bradi1g29320	2 (e^{-48})	24,824.1	LOC_Os06g51524	6 (e^{-31})	31,216.6
ubw35	11.1	Bradi1g29247	4 (e^{-112})	24,774.5	LOC_Os06g51490	3 (e^{-63})	31,183.9

Table 4 Geographical origin and virulence spectrum of the *Puccinia triticina* single spore isolates utilized for the characterization of the C × L mapping population and the association mapping panel at seedling stage and field conditions

P. triticina isolates	Geographical origin	Physiological race[a]	Virulence spectrum	Colosseo (IT)	Lloyd (IT)
Isolates used for the seedling tests					
PSB_1–3	Italy	BBB/GJ (1)	*Lr10*, *14b*, *20*, *33*	; 0	4
Eth6.1-1	Ethiopia	BBB/BB	–[b]	0	3
LR#Td1649	Israel	DBB/R	*LrB*, *2c*, *18*, *20*	1	3
MX14.3	Mexico	BBB/QJ	*LrB*, *10*, *14b*, *20*, *23*, *33*	1	4
Field trials under artificial inoculation					
Mixture of Italian races (BBB/GJ as predominant[c])	Italy-Argelato 2006 and 2007	BBB/GJ	*Lr10*, *14b*, *20*, *33*	–	–
Single race	Mexico-Obregon 2006 and 2007	BBG/BN	*Lr10*, *11*, *22a*, *23*	–	–
Single race	Mexico-El Batan 2008	BBG/BP	*Lr10*, *11*, *12*, *22a*, *23*, *27* + *31*	–	–

[a] Described based on the nomenclature of Long and Kolmer (1989)

[b] Avirulent to all the seedling *Lr* genes tested by Mantovani et al. (2010) using the *Triticum aestivum* Tatcher isolines

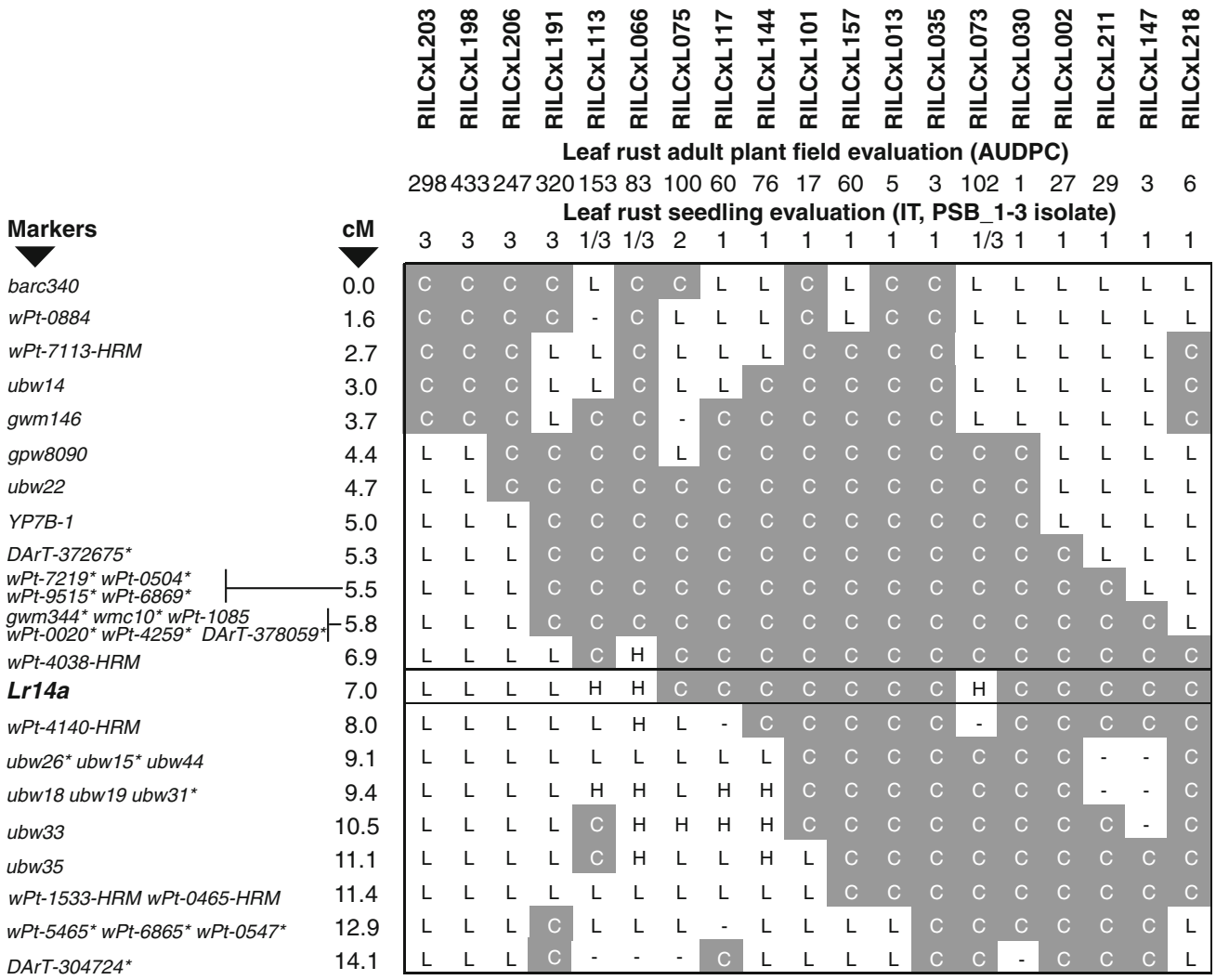
[c] Described in Mantovani et al. (2010)

; Indicates presence of diffuse hypersensitive flecks

intermediate symptom severity responses were not considered for further analysis.

Moreover, due to the good repeatability or results between the two Italian field tests as well as among the three Mexican trials, all reported analyses were carried out based on single response values obtained for each of the two main locations, one that summarized the two Italian environments and the other for the three Mexican environments (see "Materials and methods").

Table 5 reports a summary of the number of durum wheat accessions with clearly resistant, susceptible and intermediate phenotypes observed in the panel as from field and seedling evaluations. Among the adult-plant field-based evaluations, the environmental conditions in Mexico (including the *P. triticina* races used for artificial inoculation) more clearly discriminated between resistant and susceptible lines as compared to the responses observed in Italy. In Mexico, based on the results of over 3 years of trials with the highly virulent races BBG/BN and BBG/BP, most of the accessions (154 over 164) could be unequivocally classified as either clearly resistant (31 accessions as resistant as Creso and its derivatives) or susceptible (123 accessions) while the results obtained in Italy allowed us to unequivocally classify 118 accessions (25 resistant and 93 susceptible) only, with 46 accessions showing an intermediate response. Creso and its direct derivatives (the Italian cvs. Arcangelo, Colosseo, Italo, Plinio and the Syrian cv. Bicre) were consistently classified as resistant throughout all trials (see Table S3).



*: indicates a dominant marker
H: indicates presence of residual heterogeneity in the line

Fig. 4 Colosseo × Lloyd (C × L) RILs with informative recombinations in the distal region of chr. 7BL harboring *Lr14a*. Markers are reported on the *left* based on their map order, the haplotypes of the C × L RILs are reported *horizontally* with their respective area under the disease progress (AUDPC) as evaluated under field conditions and infection type (IT) evaluated with the *Puccinia graminis* isolate PSB-1. Co-segregating markers are grouped by rows

At the seedling stage, the response phenotypes (IT) of the accessions to the four *P. triticina* isolates were less efficient than the field evaluations in discriminating the presence/absence of *Lr14-Creso*. All of these isolates showed avirulent interactions with various *Lr* genes that are present in the accession panel, in addition to *Lr14a* (Table 6). Consequently, the number of resistant accessions at the seedling data was always higher than that observed under field conditions in both Italy and Mexico, with the only exception for the highly virulent isolate Lr#Td1649 from Israel.

The number of accessions that showed a resistant response to the three isolates PSB 1–3, Eth6.1 and Mx14.3 ranged between 59 and 66, that is almost twice as many as those identified in the field evaluations. Conversely, only 22 accessions showed a resistant response with isolate Lr#Td1649. Interestingly, the only accessions resistant to this isolate were those included in the Creso-group (Maccaferri et al. 2010), a finding that strongly suggests the presence of *Lr14-Creso* in these accessions. On the contrary, isolate Eth6.1-1, used as a check, was less virulent than the other isolates (66 resistant accessions); it is interesting to mention that this isolate was expected to be avirulent on all the known *Lr* genes based on the *T. aestivum* Tatcher differential sets (Mantovani et al. 2010).

For each of the PCR-based markers that can track *Lr14-Creso*, the effectiveness in correctly predicting the leaf-rust

Table 5 Number of elite durum wheat accessions from a panel of 164 cultivars and breeding lines of various origins that were classified as either resistant, susceptible or with intermediate response under field conditions with artificial inoculations and at the seedling stage with *P. triticina* isolates selected for capacity to discriminate the presence of *Lr14a*

Leaf-rust response	Adult-plant field conditions		Seedling tests (*P.triticina* isolates)			
	Italy[a]	Mexico[b]	PSB 1–3	Eth6.1	Mx14.3	Lr#Td1649
Resistant[c]	25	31	59	66	61	22
Susceptible[d]	93	123	105	98	103	142
Intermediate or partial[e]	46	10	0	0	0	0

[a] Consensus data from two experiments carried out in Argelato, Italy (2006 and 2007) under the artificial inoculation of 16 representative Italian *P. triticina* isolates

[b] Consensus data from three experiments carried out in Obregon and El Batan, Mexico (2006 through 2008) under the artificial inoculation of two *P. triticina* races (BBG/BN and BBG/BP) prevalent in Mexico and avirulent on *Lr14-Creso*

[c] Resistant adult-plant response: accessions with rAUDPC values included in the rAUDPC range observed for Creso and four of its direct derivatives known to carry *Lr14-Creso* (i.e., Arcangelo, Bicre, Colosseo, Italo, Plinio, Radioso); seedling tests: accessions with infection type (IT) included between 0 and 2 (Long and Kolmer 1989)

[d] Susceptible adult-plant response: accessions with rAUDPC values higher than 50 % (rAUDPC calculated using cv. Kofa as reference for susceptibility); seedling tests: accessions with infection type (IT) equal to 3 or 4 (Long and Kolmer 1989)

[e] Intermediate adult-plant response: accessions with rAUDPC values higher than the maximal values observed among the Creso derivatives but lower than 50 %

response of the 164 elite accessions was calculated as a success rate for the two allelic classes 'Creso-like' and 'non Creso-like' (Table 7). In case of multi-allelic markers such as the SSRs, the 'non Creso-like' allelic class included all the alleles other than that found in Creso and its resistant derivatives.

Effectiveness (defined as *predictability* or *success rate*) of the markers was overall higher for the adult-plant field observations from the Mexican trials as compared to those from the Italian trials and, among the four *P. triticina* isolates, for PSB_1–3 and LrTd#1649 as compared to Eth6.1 and Mx14.3, a result expected on the basis of the averaged responses.

As reported in Table 6, among the markers mapped proximal to *Lr14-Creso*, the two closely linked markers *ubw14* and *gwm146* (4 cM proximal to *Lr14-Creso*) together with the *wPt-4038-HRM* marker (0.1 cM from *Lr14-Creso*) provided the highest predictability for the *Creso-like* allelic class, associated to the resistant phenotype (*Lr14-Creso*). For each of these three markers, the success rate of the *Creso-like* allele in predicting the resistant phenotypes was always higher than 0.80 for the adult-plant field observations in Mexico (range 0.82–0.88) as well as for the PSB_1–3 isolate (range 0.88–0.92); with isolates Eth6.1 and Mx14.3 the success rate was always higher than 0.70 (range 0.73–0.80) while dropping to 0.64–0.72 for the highly virulent Lr#Td1649 isolate. Overall, the predictability of susceptibility for the *non Creso-like* allelic class was high for these three markers as well as for most of the other markers mapping in the region and ranged between 0.88 and 0.95 for the adult-plant responses evaluated in Mexico, 0.75–0.78 for the PSB1–3 isolate and from 0.97 to 1.00 for Lr#Td1649. Good predictability values were also observed for the SSR pair *gwm344–wmc10*, as previously reported (Maccaferri et al. 2010).

On the distal side of *Lr14-Creso*, the HRM-based marker *wPt-4140-HRM* (1.0 cM from *Lr14-Creso*) showed a considerably lower predictability than *wPt-4038-HRM*, with a success rate associated to the *Creso-like* allele not higher than 0.69 across responses. Relatively low predictability values were also observed for most of the high-quality COS-SNP markers that were mapped 2.1–4.1 cM distal of *Lr14-Creso*, with the exception of *ubw15* and *ubw35*. Predictability values for these two markers were equal to 0.64 and 0.69 for the field experiments in Mexico and 0.69 and 0.75, respectively, with the PSB_1–3 isolate. Notably, the allele count for the *Creso-like* variant of these two markers did not exceed the number of 39 accessions for *ubw15* and 33 for *ubw35*, while increasing from 51 to 61 accessions for *ubw33* and *ubw31*, thereby including a number of accessions that were either unrelated to the Creso lineage or characterized by clearly susceptible phenotypes (see Table 5 for comparison).

Based on the adult-plant responses obtained in Mexico, the LD r^2 values between the new marker loci (with '*Creso-like*' and '*non-Creso-like*' allelic states) and the mendelized leaf-rust responses (including only resistant and susceptible responses) from the adult-plant field evaluation was computed and plotted against the markers' map positions (Fig. 5). The highest r^2 values of association with the leaf-rust-resistance phenotype (as induced by *Lr14a*) were those of *ubw14* ($r^2 = 0.63$), *wPt-4038-HRM* ($r^2 = 0.61$), *gwm146* ($r^2 = 0.55$), *ubw35* ($r^2 = 0.54$) and *gwm344* ($r^2 = 0.51$). All the other markers, including the set of eight COS-SNPs that mapped from 2.1 to 4.1 cM distal to *Lr14*, showed r^2 values lower than 0.50.

Table 6 Effectiveness in predicting the leaf-rust response for PCR markers associated to *Lr14a*, as estimated on a panel of 164 elite durum accessions

Marker	Genetic distance[a]	Allele count		Adult-plant field conditions				Seedling tests (*P.triticina* isolates)							
				Italy		Mexico		PSB_1		Eth6.1		Mx14.3		Lr#Td1649	
		C[b]	Non-C[c]	C[d]	Non-C[e]	C	Non-C	C	Non-C	C	Non-C	C	Non-C	C	Non-C
barc340	−6.7	24	131	0.50	0.64	0.80	0.86	0.88	0.73	0.71	0.65	0.75	0.69	0.63	0.95
wPt7133-HRM	−4.0	49	91	0.32	0.69	0.53	0.92	0.61	0.80	0.51	0.65	0.51	0.75	0.41	1.00
ubw14	−3.7	27	130	0.50	0.69	0.85	0.90	0.92	0.78	0.73	0.66	0.73	0.73	0.65	0.98
gwm146	−3.0	28	124	0.50	0.65	0.82	0.88	0.89	0.75	0.75	0.65	0.75	0.71	0.64	0.97
gpw8090	−2.3	39	118	0.34	0.71	0.55	0.93	0.66	0.78	0.55	0.69	0.61	0.74	0.42	0.99
ubw22	−2.0	61	88	0.26	0.68	0.43	0.90	0.52	0.75	0.44	0.61	0.54	0.74	0.33	0.99
YP-7B1	−1.7	40	77	0.38	0.67	0.63	0.90	0.68	0.74	0.63	0.65	0.65	0.71	0.48	0.97
gwm344	−1.2	32	129	0.47	0.67	0.75	0.88	0.81	0.75	0.78	0.70	0.37	0.72	0.59	0.98
wmc10	−1.2	41	121	0.39	0.67	0.63	0.90	0.68	0.75	0.58	0.65	0.63	0.71	0.51	0.99
wPt-4038-HRM	−0.1	26	135	0.56	0.76	0.88	0.88	0.88	0.74	0.80	0.68	0.76	0.71	0.72	0.98
Lr14-Creso	0.0														
wPt-4140-HRM	+1.0	37	108	0.42	0.69	0.64	0.94	0.69	0.77	0.58	0.66	0.69	0.75	0.50	0.99
ubw26	+2.1	55	97	0.28	0.66	0.46	0.89	0.56	0.75	0.41	0.60	0.56	0.73	0.33	0.97
ubw44	+2.1	54	99	0.30	0.68	0.46	0.92	0.61	0.78	0.44	0.62	0.57	0.74	0.37	0.99
ubw15	+2.1	39	118	0.41	0.67	0.64	0.90	0.69	0.65	0.59	0.70	0.59	0.70	0.46	0.97
ubw18	+2.4	54	97	0.30	0.67	0.49	0.92	0.62	0.77	0.45	0.64	0.60	0.75	0.36	0.98
ubw19	+2.4	53	103	0.29	0.69	0.46	0.92	0.60	0.77	0.46	0.61	0.60	0.76	0.36	0.98
ubw31	+2.4	61	96	0.28	0.67	0.44	0.92	0.56	0.77	0.44	0.64	0.51	0.73	0.31	0.98
ubw33	+3.5	51	92	0.30	0.70	0.50	0.95	0.64	0.78	0.48	0.62	0.60	0.77	0.38	0.99
ubw35	+4.1	33	101	0.44	0.69	0.69	0.93	0.75	0.78	0.56	0.60	0.69	0.73	0.56	0.99
wPt-1533-HRM	+4.4	16	128	0.44	0.62	0.81	0.84	0.94	0.72	0.75	0.63	0.88	0.69	0.56	0.91
wPt-0465-HRM	+4.4	52	123	0.33	0.70	0.52	0.92	0.65	0.79	0.50	0.63	0.63	0.76	0.38	0.98

For each marker, efficacy is reported as the success rate (*predictability*) in predicting leaf-rust resistance for the allelic class conforms to *Creso* (C) as well as leaf rust susceptibility for the class of the *non-Creso* alleles, respectively. The phenotypes were obtained from inoculated adult-plant field trials and single-isolate inoculation tests carried out at seedling stage

[a] Genetic distances as estimated in the C × L RIL population, with marker *barc340* set as the upper limit (0 cM). Genetic distances are in cM

[b] Number of accessions harboring the marker allele detected in Creso and in its derivatives known to carry *Lr14-Creso* (allele reported as 'C')

[c] Number of accessions harboring alleles other than 'C' For bi-allelic markers, such as the SNPs, the alternative allele was identical by state to the one carried by Lloyd

[d] Predictability of the 'C' allele. Reported as % of accessions, among those harboring the 'C' allele, with a clearly resistant leaf-rust response at the adult and seedling stages

[e] Predictability of the pool of alleles others than 'C' ('non-C' alleles). Reported as % of accessions among those harboring the 'non-C' alleles, with a clearly susceptible leaf-rust response

Since none of the developed markers showed a predictability value for the *Creso-like* allele reaching 1.00 (i.e., 100 % success rate), combinations of marker pairs bracketing *Lr14a* locus were assessed for predictability. In particular, combinations of *ubw14* and *wPt-4038-HRM* on the proximal side were considered with *ubw18* and *ubw35* on the distal side (Table 7). Overall, the two markers, Creso-like (C–C) haplotype allele was found in 24–26 accessions. The marker predictability of the Creso-like haplotype reached 0.96 for the adult-plant field-based evaluation in Mexico and 0.92 for the seedling IT obtained with the isolate PSB 1–3 (Table 7). Apart from the 26 accessions with the Creso-like haplotype in the *Lr14a* region, a few accessions showed a resistant response consistent across trials (Tables S2 and S3) even though their *Lr14a* haplotype was not conform to Creso.

Discussion

Development of COS-SNP and HRM markers linked to Lr14a

Lr14a is one of the few genes currently targeted by durum wheat programs world wide either for parental line characterization, to control for its presence in the new crosses,

Table 7 Effectiveness in predicting the leaf-rust response for selected two-marker haplotypes associated to *Lr14-Creso* as estimated on a panel of 164 elite durum accessions

Two-marker haplotype	Allele count		Adult-plant field conditions				Seedling tests (*P.triticina* isolates)							
			Italy		Mexico		PSB 1–3		Eth6.1		Mx14.3		Lr#Td1649	
	C–C[a]	Non-C[b]	C–C[c]	Non-C[d]	C–C	Non-C	C–C	Non-C	C–C	Non-C	C–C	Non-C	C–C	Non-C
ubw14–wPt4038	26	138	0.58	0.67	0.96	0.89	0.92	0.74	0.77	0.67	0.77	0.70	0.73	0.98
ubw14–ubw18	25	139	0.56	0.67	0.96	0.88	0.92	0.74	0.76	0.66	0.76	0.69	0.72	0.97
ubw14–ubw35	26	138	0.58	0.66	0.96	0.89	0.92	0.75	0.77	0.67	0.77	0.70	0.73	0.98
wPt4038–ubw18	24	140	0.54	0.66	0.96	0.88	0.92	0.74	0.75	0.66	0.75	0.69	0.75	0.97
wPt4038–ubw35	25	139	0.56	0.67	0.96	0.88	0.92	0.74	0.76	0.66	0.76	0.70	0.76	0.98

For each haplotype, efficacy is reported as the success rate (*predictability*) in predicting leaf-rust resistance for the haplotype allelic class conforms to Creso (Creso allele at both markers defining the haplotype, C–C) and leaf rust susceptibility for the class of the pooled non-Creso haplotype alleles, respectively. Phenotypes obtained from inoculated adult-plant field trials and single-isolate inoculation tests carried out at seedling stage

[a] Number of accessions harboring the Creso marker allele at both markers defining the haplotype (haplotype allele reported as 'C–C')

[b] Number of accessions harboring haplotype alleles other than C–C

[c] Predictability of the 'C–C' haplotype allele. Reported as % of accessions, among those harboring the 'C–C' allele, with a clearly resistant leaf-rust response at the adult and seedling stages

[d] Predictability of the pool of alleles others than 'C–C' ('non-C' alleles). Reported as % of accessions, among those harboring the 'non-C' alleles, with a clearly susceptible leaf-rust response

as well as for MAS, to pyramid it with other genes (Karim Ammar, Diane Mather, Andrea Massi, personal communications). In durum wheat, three independent studies have assigned *Lr14a* to the distal region of chr. bin 7BL10-0.78-1.00 (Herrera-Foessel et al. 2008a, b, c; Maccaferri et al. 2008; Marone et al. 2009). However, these studies did not provide information on the precise location of *Lr14a*, nor did they develop new molecular markers protocols suitable for genotyping of the region and cost-effective MAS.

Currently, large-scale diagnosis of the presence of *Lr14a* and MAS rely on the SSR markers *gwm146* and *gwm344*. These markers are located 4.0 and 1.5 cM proximal from the gene, respectively, with *gwm344* being a dominant marker, a major limitation in MAS applications across a broad range of genetic backgrounds/pedigrees. Accordingly, co-dominant markers that closely linked the target gene, possibly at the sub-cM level, would provide better tools for molecular breeding (Koebner and Summers 2003). Further, the availability of multiple markers close to the target locus would allow for a more accurate haplotype-based screening (haplotype-sharing analysis) which is a more effective approach than single marker-based diagnosis for the precise prediction of the susceptible/resistance status of a plant/breeding line (Meuwissen et al. 2001; Meuwissen and Goddard 2001; Mucha and Wierzbicki 2012; Weng et al. 2012).

Enrichment of molecular markers in wheat is complicated by its allopolyploidy nature, the low polymorphism rate among the cultivated materials, and the lack of a complete sequenced genome (Feuillet et al. 2008; Paux et al. 2011). Consequently, fine mapping in wheat at the sub-cM level requires a considerable investment of time and resources (Keller et al. 2005).

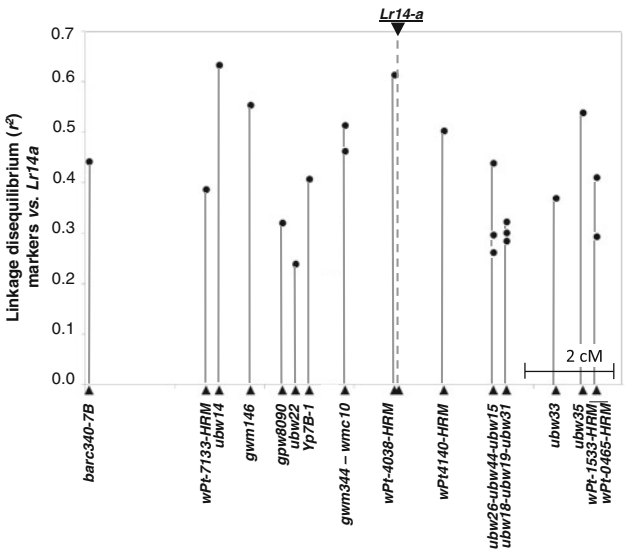


Fig. 5 Linkage disequilibrium plot (r^2 values) of the Mendelized leaf-rust response phenotype versus the allelic states of the markers mapped in the *Lr14-Creso* region. Marker loci are sorted in the *X*-axis of the plot reflecting their relative map order and positions as estimated on the C × L mapping population. For the multi-allelic marker loci (e.g. SSR markers), alleles other than the 'Creso-like' were pooled in a unique class of 'non Creso-like alleles'

In this study, we developed ten new gene-based COS-SNP and five HRM markers spanning an 8.7 cM that includes the *Lr14a* locus, with one HRM marker closely linked (0.1 cM) to the target gene. All the newly developed

assays were carefully designed to provide both the locus- and genome specificity that are required in allopolyploid species such as wheat (You et al. 2009).

A comparative analysis with the sequenced genomes of *Brachypodium* and rice allowed us to develop ten new COS-SNP based markers (UBW series) useful for MAS applications and molecular diagnosis, as well as for the establishment of a gene-based map of the *Lr14a* region, a preliminary step necessary for the eventual positional cloning of this locus. Similar strategies have already been devised to support the fine mapping of valuable loci (Sorrells et al. 2003; Bossolini et al. 2007; Schnurbusch et al. 2007; Bolot et al. 2009; Quraishi et al. 2009), as well as to dissect specific biological pathways (Higgins et al. 2010) in the non-sequenced genome of wheat. More recently, the same strategy has been successfully deployed to fine map various disease resistance genes (Zhang et al. 2010; Burt et al. 2011; Qin et al. 2012; Zhang and Dubcovsky 2011; Xue et al. 2012).

Once the conserved synteny model (at the macro co-linearity level) has been established for the region of interest and the series of wheat orthologs is identified, the corresponding wheat sequences can be used for the development of additional markers. A relatively fast and efficient approach is to develop PCR assays directly from the available EST sequences disregarding the issue of discriminating between the homeologous genomes. This method can be used to design primer pairs for short STS PCR assays that frequently amplify simultaneously all the homeologous sequences. Since the presence of a varietal SNPs can be mostly found in one of the two or three homeologous copies only, these PCR assays can be directly screened for polymorphisms using techniques such as denaturing or non-denaturing polyacrylamide gel electrophoresis, in particular the single strand conformation polymorphism (SSCP) technique (Liu et al. 2006; Quraishi et al. 2009; Qin et al. 2011; Xue et al. 2012). However, these techniques are neither suitable to develop co-dominant markers nor amenable to simple, cost-efficient automated assays as required in most MAS programs. To match these requirements, the homeologous gene copies have to be sequenced and homeolog-specific (based on HSV variants) and allele-specific (based on varietal SNP) primers were designed and combined in a single PCR assay.

We tested two methodologies that have been devised to convert the sequence information from the homeologous gene copies to markers polymorphic between the parental lines Colosseo and Lloyd and amenable to cost-efficient, high-throughput deployment.

The first methodology requires two sequencing steps and the availability of DNA from the flow-sorted chrs. of interests, 7AL and 7BL in our case (Dolezel et al. 2007). Using this methodology, the final success rate reached in developing homeolog- and allele-specific PCR assays (10 %) was considered rather low. This was not due to the lack of polymorphism between the two cultivars, but rather to the difficulty of converting the SNPs into effective polymorphic markers while maintaining the chr. 7B-specificity. Cloning is not required by this approach while two PCR calibration steps and two sequencing steps for each developed marker are still required. However, the initial time-consuming step of identifying HSV is now simplified by the recent release of the wheat chromosomes survey sequences (http://www.wheatgenome.org/; Eversole 2009), that has been obtained by following the same sorting approach and next generation sequencing of the flow-sorted chromosome arms. In fact, in silico comparison between homeolog sequences will allow to directly identify HSVs. Also, all the physical mapping initiatives involving the wheat chromosome sequencing are currently developing HSV-specific primers for their bacterial artificial chr. libraries' fingerprinting (Paux et al. 2008; Eversole 2009), which could then be directly used to identify varietal SNPs by sequencing.

The second approach is a modification of the traditional approach earlier proposed by Dvorak et al. (2006) in the 'Haplotype Polymorphism in Polyploid Wheats and their Diploid Ancestors' project (http://wheat.pw.usda.gov/SNP/new/index.shtml) and by Schnurbusch et al. (2007). This approach requires cloning in *Escherichia coli* and a single sequencing and calibration step. This approach, while being technically more demanding and slightly more expensive than the former one, proved to be very accurate. The use of a diploid wild wheat progenitor (*T. urartu*, A genome) greatly simplified the identification of the A and B genome homeologous copies, as compared to the use of multiple clones from CS N7BT7D and CS N7BT7A as suggested by Schnurbusch et al. (2007). Overall, both methodologies are sufficiently simple and reliable to allow for the development of co-dominant, locus-specific PCR assays amenable to horizontal gel electrophoresis separation techniques. In general, the use of the M-13 tail simplified the allele discrimination phase. Nevertheless, the procedure did not guarantee the 100 % success rate in obtaining co-dominant marker assays (for three genes out of ten it was not possible to develop a functional codominant marker).

Besides the development of locus- and allele-specific assays, for two of the most valuable markers we attempted the conversion to the fluorescent closed-tube KASPar®-based and HRM-based assays. The competitive allele-specific PCR method implemented in the KASPar assay has been recently developed (Nijman et al. 2008) and applied with success to allopolyploids such as wheat (Allen et al. 2011) and cotton (Byers et al. 2012). In wheat, designing the KASPar primers by taking into account both

the target varietal SNP and the adjacent HSV variants guaranteed the highest chances of obtaining informative assays. HRM analysis is also widely used in human clinical laboratories as a high-throughput method in molecular diagnostic. More recently, HRM analysis has been successfully applied to crops for the identification of new sequence variants in EMS-induced mutant populations (Botticella et al. 2011) as well as for SNP discovery and genotyping (Han et al. 2011; Jun and Kang 2012). In our applications, HRM performed equally well for both purposes of genotyping a known SNP (e.g. *ubw44*) as well as for SNP identification in short genomic sequences (DArT-derived markers). Currently, the cost of the single data-point analysis is lower for the KASPar® assay as compared to the HRM. On the contrary, the HRM technique is highly versatile and not subjected to proprietary constrains.

Refining the interval containing Lr14a

Adding 11 gene-derived markers (ten COS-SNPs and one STS tagging *PSY1-7B*) to the C × L map in the target *Lr14* interval distal to *barc340* allowed us to locally explore the degree of synteny conservation between wheat, *Brachypodium* and rice. The *Lr14* location was confirmed to be included in a syntenic interval defined by the two COS markers Bradi1g29622-Os06g51270 (corresponding to marker *ubw22* in durum wheat) and Bradi1g29247-Os06g51490 (marker *ubw35*). From the joined synteny and mapping study, it appeared that while the macrosynteny between the wheat chr. 7BL target region and the corresponding portion of chr. Bradi1S was extensively conserved, the macrosynteny with rice Os6 was not as well conserved as in *Brachypodium*, with synteny breaks over relatively short physical distances, involving regions on chrs Os2 and Os11. One marker (*ubw14*) did not precisely match the syntenic gene order, indicating that re-arrangements ultimately occurred in this region. Still, all the markers developed from the orthologous genes mapped within the wheat interval under study, confirming the validity of synteny analysis as an ideal source for markers development. A similar conclusion was reached by Schnurbusch et al. (2007) when analyzing the *Bo1* region on chr. 7BL, located immediately above (proximal to *barc340*) the one discussed here.

Despite a series of eight adjacent COS genes (from *ubw26* to *ubw35*) mapped in close proximity of *Lr14a*, all of them were positioned on its distal side. Based on the marker order provided by the RILs used in this study, *Lr14a* remained thus confined in a rather wide region (4.1 cM between *YP7B-1* and *ubw26*) where only genomic-based markers—mainly SSR and DArT® markers—were mapped, thus adding further complexity to any eventual positional cloning approach. Notably, in *Brachypodium* this region corresponds to a very small interval of 28 kb harboring four additional genes, including Bradi1g29577, tagged by *ubw14*. However, in wheat *ubw14* was mapped outside to the *Lr14a* above interval, in a non-colinear region that was ca. 2.0 cM proximally located, a result supported by nine recombinant lines. In addition, in rice this target interval (between *YP7B-1* and *ubw26*) corresponded to a region where the synteny with rice chr. Os6 was perturbed by homology to rice Os2 (as in the case of *ubw26* and other nearby genes) and rice Os4. All these observations suggest that, in close proximity of *Lr14a*, both gene content and order in wheat still need to be accurately elucidated. However, the synteny was extensively conserved in the *Lr14a* interval, thus prompting us to carefully scan the predicted orthologous genes for biological functions that could potentially match the rust resistance provided by *Lr14a*. Among these genes, four main candidates could be easily identified: Bradi1g29537 has a *cyclin-L1-1* domain, Bradi1g29530 has a *zinc knuckle* domain, Bradi1g29471 is a *serine carboxypeptidase* homoeolog and Bradi1g29450 is an NBS-LRR disease resistance protein homolog of *Yr10* (Table 5). *Cyclin-L* is one of the several cyclin types present in plants that are mainly involved in the regulation of the cell cycle and division through their association with kinases into cyclin-dependent kinases complexes (CDK) (La et al. 2006). However, their involvement in pre-determined cell death (apoptosis) and regulatory activity of kinase proteins has proposed them as central components in fungal disease resistance in maize and wheat (Maleki et al. 2003; Kump et al. 2011). Similarly, the *zinc knuckle* domain performs numerous functions due to its mRNA-binding activity (Barabino et al. 1997). Among these, it was identified as a critical domain in the *Arabidopsis thaliana* gene *Rpp13* to confer resistance to the fungus *Peronospora parasitica* Pers. ex Fr. (Ding et al. 2007), and as up-regulated in response to the attack of the fungal pathogen *Giberella fujikuroi* S. in *Saccharum officinarum* L. (Lin et al. 2010). *Serine carboxypeptidases* also exert a wide variety of functions related to peptide modifications mainly through acetylation. Recently, this type of protein has shown a key role in the production of antimicrobial compounds for disease resistance in *Avena sativa* (Mugford et al. 2009) and rice (Liu et al. 2008). The majority of disease resistance genes in plants encodes for the combination of nucleotide binding site and leucine-rich repeat domains (NBS-LRR, McHale et al. 2006). In particular, Bradi1g29450 and its three duplicated versions (Table S2) are homologs of *Yr10* which provides resistance to stripe rust (*Puccinia striiformis* f. sp. *tritici*) in wheat (McIntosh et al. 1998; Spielmeyer et al. 2000; Temel et al. 2008); NBS-LRR are also associated to leaf-rust resistance (Spielmeyer et al. 2000; Maleki et al. 2003; Bozkurt et al. 2007).

Further refining the map position of the above-mentioned genes will help us to narrow down the list of potential candidates.

Recently, fine mapping of disease resistance genes in hexaploid wheat with COS markers based on the exploitation of synteny between wheat, *Brachypodium* and rice at 0.1–1.0 cM resolution has been reported by several authors (Zhang et al. 2010; Qin et al. 2011; Quraishi et al. 2011; Somyong et al. 2011; Azhaguvel et al. 2012; Xue et al. 2012). In particular, both Qin et al. (2011) and Xue et al. (2012), using COS markers that were screened with the SSCP technique, were able to fine map the genes of interest down to the 0.1 cM resolution level. However, none of the reported markers was converted to cost-efficient, homeolog- and allele-specific assay, thus delaying their implementation in molecular breeding programs.

Recently, the targeted enrichment of specific genomic regions for high-throughput massively parallel sequencing ('*sequence capture*' technologies) allowed for the direct screening of large COS sets in a two-steps experiment, at a reasonable cost (Saintenac et al. 2011; Winfield et al. 2012). This notwithstanding, further validation and development of gene-specific assay on a SNP by SNP basis remain the final step for marker development.

Suitability of markers associated to Lr14a for breeding

To confirm the usefulness of the markers herein identified, their allelic states were examined on a large panel of durum elite lines. The genotype response to leaf rust infection was tested both at the adult and seedling stages. When single markers were considered, *ubw14*, *gwm146*, *wPt-4038-HRM*, *gwm344*, *ubw15*, *ubw18* and *ubw35* most accurately predicted the leaf-rust response of the germplasm. The remaining SNPs provided a lower predictability as compared to the SSR markers, even though they appeared to map closer to the causal locus than *gwm146* according to the results of bi-parental mapping (Table 3). However, this result was not entirely unexpected given the relatively fast evolutionary dynamics of the causal (effector) locus involved in plant–pathogen interactions (Wicker et al. 2007). The SSR loci, known to be among the fastest evolving DNA sequences (Goldstein et al. 1995; Pollock et al. 1998; Estoup and Cornuet 1999), might have a mutation rate more similar to that of the causal locus as compared to SNPs. Vice versa, in most cases, the occurrence of the SNPs herein identified may have predated the mutation that generated the *Lr14-Creso* allele, a hypothesis reinforced by their relatively high allelic frequencies in the panel of accessions (higher than those observed for the SSRs). Therefore, it is only partially surprising that *ubw14*, while mapping ca. 5 cM away from the *Lr14* region in the C × L population, was one the most predictive markers in the association panel, as compared to the SNP set from *ubw26* to *ubw31* that was mapped closer to *Lr14* (at 2.1 cM). In fact, *ubw14*, together with *wPt-4038-HRM*, *ubw15* and *ubw35*, were the only SNPs for which the allele frequency of the *Creso-like* allele matched with what expected for *Lr14-Creso* allele in the panel. In addition, based on the *Brachypodium* genes order, the gene tagged by *ubw14* corresponded to one of the few orthologs that were located in the shortest interval predicted to harbor *Lr14a*, i.e., between *YP7B-1* and *ubw26*, at the micro-synteny level. Given the high frequency of intra-specific translocation and inversions known to occur in wheat (Badaeva et al. 2007), it is possible that in some lineages of the panel there were local rearrangements, bringing *ubw14* in closer proximity of the *Lr14a* locus, thus explaining its higher predictability when used to screen the germplasm.

The predictability of the resistant leaf-rust response associated to *Lr14-Creso* increased substantially when considering double-marker haplotypes of *Creso-like* alleles at loci flanking *Lr14a*, in particular the combinations *ubw14-ubw18*, *ubw14-ubw35*, as well as *wPt-4038-HRM-ubw35* confirming the known superior performance of haplotypes over single markers diagnosis in tracking identity by descent at the causal loci (Terwilliger 1995; Meuwissen and Goddard 2001).

Conclusions

In an attempt to refine the position of one of the most widely exploited leaf-rust-resistance gene in durum wheat, we have enriched the *Lr14a* region with 15 novel molecular markers that allowed to narrow-down the *Lr14a* interval to a 1.1 cM segment on the distal end of chr. 7BL. The exploitation of the conserved synteny between wheat, *Brachypodium* and rice was instrumental for the development of additional markers, while providing the opportunity to implement effective strategies for gene-based marker development. Among the novel markers, *ubw14*, *ubw18*, *ubw35*and *wPt4038-HRM* provided highly robust, locus-specific assays whose allelic states are easily resolved in standard agarose gel electrophoresis or non-gel-based, high-throughput fluorescent allele detection systems. These four markers were shown to flank *Lr14a*, which allowed for the assembly of molecular assays reliably predicting the presence of the *Lr14-Creso* allele in a broad sample of the cultivated durum wheat. These markers, especially the combinations of *ubw14-ubw18* and *ubw14-ubw35* for standard agarose gel electrophoresis/KaSPAR® assays and *wPt-4038-HRM-ubw35* for high-resolution melting analysis, respectively, are well suited for large-scale gene presence diagnosis and for MAS while providing valuable opportunities toward the fine mapping of *Lr14*.

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