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Association mapping of leaf rust response in durum wheat

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Abstract Resistance to leaf rust (*Puccinia triticina* Eriks.) is a main objective for durum wheat (*Triticum durum* Desf.) breeding. Association mapping on germplasm collections is now being used as an additional approach for the discovery and validation of major genes/QTLs. In this study, a collection of 164 elite durum wheat accessions suitable for association mapping has been tested for leaf rust response at the seedling stage and under field conditions (adult plant stage). Seedling tests were carried out with 25 selected isolates from durum wheat, bread wheat and triticale, while field experiments were carried out in artificially inoculated plots in Italy and in Mexico. The collection has been profiled with 225 simple sequence repeat (SSR) loci of known map position and a PCR assay targeting *Ppd-A1*. Associations showing highly consistent experiment-wise significances across leaf rust isolates and field trials were mainly detected for the 7BL distal chromosome (chr.) region (harbouring *Lr14* from cultivar Llareta INIA and *QLr.ubo-7B.2* from cultivar Creso) and for two chr. regions located in chrs. 2A and 2B. Additionally, isolate-specific associations and/or associations with smaller effects in the field trials were identified in most of the chromosomes. The chr. 7BL distal region was investigated in detail through haplotyping with 15 SSR markers, revealing that the Creso and Llareta INIA alleles are identical by descent at 6 adjacent SSR loci in the most distal 7BL region spanning 8 cM. Association mapping allowed us to further refine the map location of the *Lr14*/*QLr.ubo-7B.2* resistance gene to the most distal region of the linkage group, tagged by *Xcfa2257.2*, *Xgwm344.2* and *Xwmc10*. The resistant haplotype is present in a number of accessions (ca. 15% of the accessions included in the collection) from the Italian, CIMMYT and ICARDA breeding programmes. Therefore, this chr. 7BL region can be considered as the most important source of resistance to leaf rust currently exploited by durum breeders in the Mediterranean areas. Furthermore, the field trials at the adult plant

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stage allowed us to identify marker associations (e.g. chrs. 2BL and 3BS, proximal regions; chr. 7BS, distal region) which suggest the presence of minor QTLs for slow-rusting resistance.

Keywords Durum wheat · *Puccinia triticina* Eriks · Association mapping · Linkage disequilibrium · Quantitative trait locus · Molecular marker

Introduction

Durum wheat (*Triticum durum* Desf.) is an important cereal crop adapted to the Mediterranean region, where ca. 75% of the worldwide production is concentrated (Morancho 1995; Belaid 2000; Habash et al. 2009). In particular, Italy is one of the most important countries for durum wheat cultivation and the world leader in pasta production (Giunta et al. 2007). In the Mediterranean countries, durum is cultivated under a range of environments characterised by different levels of productivity and constraints, ranging from favourable (temperate regions, with medium to high annual rainfall rate and good crop management) to marginal (drought- and heat-prone areas, often with poor soils and poor crop management) conditions. In spite of its broad adaptation, durum wheat production and kernel quality under the Mediterranean environments are negatively affected by various fungal diseases such as rusts, powdery mildew, septoria leaf blotch, fusarium head blight and root rot (Nachit 2000; Nsarellah et al. 2000; Singh et al. 2005).

Leaf rust (*Puccinia triticina* Erikson = *P. recondita* Roberge ex Desmaz f. sp. *tritici*) is a main fungal disease for durum wheat throughout the entire Mediterranean Basin and other durum growing areas like India, the US and Mexico (Zhang and Knott 1993; Nachit 2000; Singh et al. 2004; Martinez et al. 2005; Amaro et al. 2007). Selection for leaf rust resistance can be facilitated by tagging effective genes with molecular markers that can be subsequently used in breeding programmes to trace and select the useful alleles (via marker-assisted selection: MAS; Varshney and Tuberosa 2007). This notwithstanding, detailed genetic analyses of resistance factors present in *T. durum* and related species have been undertaken only recently (Knott et al. 2005; Herrera-Foessel et al. 2007a, b, 2008a, b, c; Maccaferri et al. 2008a; Gennaro et al. 2009).

Traditionally, the identification of genes/quantitative trait loci (QTLs) for resistance/tolerance to fungal pathogens has been carried out through bi-parental mapping. More recently, the use of association mapping on germplasm collections has been introduced to discover new useful allelic variation through genome-wide scan and/or to validate the effect of genes/QTLs previously discovered by traditional mapping (Rafalski 2002; Flint-Garcia et al. 2003; Gupta et al. 2005; Waugh et al. 2009). In most crops, reference germplasm collections (panels) of diverse accessions have been and/or are being actively assembled and characterised at the molecular and phenotypical levels with the aim of facilitating and standardising association studies. The pre-characterisation of the accessions to be included in the association panels is critical because, differently from experimental bi-parental mapping populations, a common feature of germplasm collections is the presence of non-random, 'background' coancestry among accessions that in some cases can reach notable levels (Flint-Garcia et al. 2005). Since population structure greatly increases the rate of Type I errors (presence of false positives), these mapping tools can be exploited only if germplasm structure is appropriately accounted for (Pritchard and Rosenberg 1999). Recently, specific statistical procedures and analyses have been developed, tested through simulation studies (Yu et al. 2006; Malosetti et al. 2007) and used in real association panels in maize (Crossa et al. 2007; Beló et al. 2008), barley (Cockram et al. 2008) and bread wheat (Breseghello and Sorrells 2006b; Rostoks et al. 2006). The LD decay rate, averaged over the whole genome, is the second main feature of a germplasm collection used for association mapping. This feature, which relates to the resolution or detection capacity of the association analysis, can be highly variable among species (self- *vs.* cross-pollinating species) and, within species, across different germplasm (elite, cultivated germplasm *vs*. landrace selections and non-domesticated relatives).

In durum wheat, germplasm collections suitable for association mapping have already been assembled and characterised by Maccaferri et al. (2005 and 2006) and by Somers et al. (2007). In both cases, the association panels were assembled with accessions (mainly released cultivars) from the cultivated, elite germplasm. The collections, characterised with highly variable simple sequence repeat (SSR) markers of

known map position (Röder et al. 1998; Somers et al. 2004), showed the presence of significant LD among markers at a cM-wide scale, with average LD decay within 5–20 cM, hence suitable for a genome-wide approach even with a limited number of markers.

Resistance to *Puccinia triticina* fungal disease is a valuable target trait for conducting association mapping analysis. In fact, beside its economic importance (Bolton et al. 2008), leaf rust resistance is usually controlled by major genes (conferring race-specific resistances) or, when quantitatively inherited through slow-rusting loci partially reducing the disease infection progress, it is usually characterised by relatively high heritability (Kolmer 1996). Most importantly, slow-rusting loci should provide a higher durability of field resistance (Herrera-Foessel et al. 2006, 2008b; McIntosh 2009).

As compared to more than 50 resistance genes identified in the bread wheat germplasm (McIntosh et al. 2008), only a few major race-specific genes have been mapped in the durum wheat germplasm. Among those, *Lr14/QLr.ubo-7B.2* located in the chromosome 7BL distal region and found in diverse loosely related genetic materials, such as the Chilean cultivar Llareta INIA, the CIMMYT line Somateria (Herrera-Foessel et al. 2008a) and the Italian cultivars Creso and Colosseo (Maccaferri et al. 2008a; Marone et al. 2009). Other characterised genes include *Lr3* (Herrera-Foessel et al. 2007b), *Lr10* (Aguilar-Rincon et al. 2001), *Lr13* (Singh et al. 1992), *Lr23* (from *T. durum* Gaza, McIntosh and Dyck 1975; Nelson et al. 1997) and probably *Lr16* and *Lr17* (Zhang and Knott 1990).

In this study, association mapping was used in a germplasm collection of 164 elite Mediterranean/Mexican durum cultivars challenged by a wide range of isolates to investigate for the presence of significant associations to leaf rust responses at the seedling and at the adult plant stages.

Materials and methods

Plant materials

A collection of 164 durum wheat elite accessions (mainly cvs. and advanced lines) bred in Mediterranean countries (Italy, Morocco, Spain, Syria and Tunisia), the Southwestern USA and Mexico was assembled for conducting association mapping (AM) studies. The accessions included in the collection were chosen from a larger pool of 330 accessions that were collected from various sources and evaluated on a comparative field trial carried out in 2003 in Cadriano, near Bologna, Italy. The choice of the accessions to be included in the AM panel was based on their pedigrees and morpho-physiological scores for traits critical to adaptation, such as plant height and heading date, in order to exclude accessions highly related to each other (e.g. sibs from the same cross, backcross, etc.) and/or with large differences as to heading date, which could have biased the phenotypic evaluation of traits influenced by flowering time. Most of the accessions were semi-dwarf, early to medium heading elite materials released from the early 1970s up to the late 1990s. The collection comprises also 'founder genotypes' widely used as parents in breeding programmes throughout the Mediterranean Basin and at International Centers (CIMMYT and ICARDA). A detailed phenotypic and molecular characterisation of the collection is reported in Maccaferri et al. (2006). In particular, the heading date (average across 15 environments in the Mediterranean Basin) of ca. 90% of the accessions was within 4 days and all of them headed within 7 days (Maccaferri et al. 2009).

To further characterise the molecular haplotypes and the associated phenotypic effects in the chromosome (chr.) region harbouring the *Lr14* gene, the cv. 'Llareta INIA', carrying *Lr14a* (Herrera-Foessel et al. 2008a) and 19 additional advanced breeding lines from the CIMMYT breeding programme were included in the molecular survey.

Leaf rust isolates

The panel of leaf rust isolates that was used to characterise the response of the accessions at the seedling stage included samples collected from durum wheat, bread wheat and triticale. Origin of the isolates from durum wheat are detailed below: (1) four isolates (PSB-01, PSB-14, PSB-13 and PSB-16) were collected from different durum growing areas of Italy; these isolates were provided by the seed company Produttori Sementi Bologna (PSB, Argelato, Italy); (2) two isolates came from the *Puccinia triticina* world collection held at the Cereal Disease Laboratory, St. Paul, MN (Ordoñez and Kolmer 2007) and were collected in Mexico (Mx-14.3) and in

Ethiopia (Eth6.1-1), respectively, and (3) one isolate (LR#Td1649) was from the collection of cereal rust fungi at the Institute for Cereal Crops Improvement, Tel-Aviv University, Israel.

The four Italian PSB isolates, chosen from a collection of 16 PSB isolates based on their virulence and molecular SSR profiles (Mantovani et al. 2009), were classified according to Long and Kolmer (1989) as follows: PSB-01 and PSB-14, race BBBGJ (which is the prevalent race in the 16 PSB Italian isolates; Mantovani et al. 2009); PSB-13, race CBBQQ and PSB-16, race BBBQG.

The isolates from bread wheat and triticale included: (1) one isolate from Israel (LR#Ta1010) and (2) 17 isolates from Central and Northern Europe held at IHAR Radzikov collection (Plant Breeding and Acclimatization Institute, Blonie, Poland).

All these isolates were chosen from larger collections based on their origin and virulence spectrum. In particular, the two isolates from Israel (LR#Td1649 and LR#Ta1010) were characterised, according to Long and Kolmer (1989), as races DBBR and PBBR, respectively. For the 17 isolates supplied by IHAR, five labelled with the acronym NIAB were collected from bread wheat by the National Institute of Agriculture Botany (Cambridge, UK); seven (Pt705, Pt1002, Pt1202, Pt1602, Pt2902, Pt5106 and PtOlivin) were from bread wheat in Poland; one (PtRPA-1) and four (PtZor1, PtZor2, PtWiton1 and PtWiton2) were from triticale in South Africa and Poland, respectively. The complete collection of 164 accessions was evaluated under greenhouse conditions with all the 25 isolates considered herein. Experiments were carried out with two to three replications. The experimental unit consisted of 12 seedlings per accession sown in one of the 28 single cells of a flat tray. One-week old seedlings were inoculated with the single isolates by blowing over the plants 0.1 g/tray of a mixture of talcum and spores (6 to 1 v/v). After inoculation, seedlings were incubated separately in darkness for 24 h at 18°C and 100% relative humidity. Then trays were transferred to the greenhouse in separate transparent plastic chambers and maintained at a temperature ranging from 20–22°C (day) to 16–18°C (night) with 16 h daylight. Leaf rust infection types (ITs) were recorded after ca. 10–12 days, at the two-leaf stage, once the check cultivars reached the maximum level of infection and the number of urediosori did not increase any further. The ITs were recorded using the 0–4 scale of Long and Kolmer (1989) according to the following convention: 0 = immunity, no visible infection, ; = diffuse presence of hypersensitive flecks, no uredinia, 1 = small uredinia surrounded by necrosis, 2 = small or medium uredinia surrounded by chlorosis, X = mesothetic response, with all kind of uredinia present together, 3 = numerous uredinia of moderate size without necrosis or chlorosis, 4 = large uredinia. Larger or smaller uredinia were indicated with the + and − signs. Infection types from 0 to 2 and X were considered as avirulent (resistant response of the plant) while ITs 3 and 4 were considered as virulent.

Field trials

All the materials (183 accessions in total) were evaluated in artificially inoculated field trials carried out in Italy, during 2006 and 2007 in Argelato (Po Valley, 44°39′N, 11°20′E) and in Mexico, during 2006 and 2007, in Ciudad Obregon (27°33′N, 109°09′W) and, during 2008, in El Batan (19°31′N, 98°50′W).

In Italy, the field trials were carried out in the autumn-to-spring crop cycle with sowing in late October and the crop cycle spanning from November to end of June. Leaf rust response scores were recorded from May to June of each year. In Mexico, the field trials were carried out in the winter crop cycle (in 2006 and 2007, field scores recorded from March to April) or in the summer crop cycle (in 2008, field scores recorded from August to September). Field trials were sown in replicated plots arranged in randomised complete block design with three replications; plots consisted of two rows, 2.5 m-long and 0.20 m-apart, spaced 0.60 m from adjacent plots. Two hundred germinating seeds were allotted for each plot.

Susceptible check cultivars were repeated within the experimental blocks to assess the leaf rust infection homogeneity in the field trials. The ordinary cultural practices were applied to fertilize, control weeds and pests and to ensure optimum crop development. No fungicides were applied.

In Italy, the trials were artificially inoculated with a mixture of 16 PSB-isolates of *Puccinia triticina* collected during the 1999–2006 period from

different durum varieties in different Italian locations, representing the main durum wheat-growing areas and usually characterised by high levels of leaf rust epidemics. Six isolates (PSB-01, -05, -06, -07, -09 and -10) were collected in Southern Italy, mainly in the Puglia region, while the others were collected in Northern Italy. Each of the isolates has been maintained on the specific susceptible cultivar initially hosting the pathogen. The procedure to obtain leaf rust inoculum is detailed in Maccaferri et al. (2008a). Briefly, seedlings grown under isolation in mini-tunnels in greenhouse were inoculated at the first-leaf stage with a mixture of talc and spores (6 to 1 ratio; v/v); tunnels were covered for 24 h with a black plastic film (18°C temperature and 100% relative humidity). After removing the black film, temperatures ranged from 20–22°C (day) to 16–18°C (night). Spores were collected 14–16 days after inoculation. Field inoculation was carried out by spraying the plants with water plus 1% Tween 20 (Fluka, Buks, Germany) spore suspension. Three field inoculations were carried out starting from booting stage up to complete flowering (Zadocks scale from 39 to 69); following each inoculation, water was sprayed with sprinklers onto the plants to maintain high moisture and enhance leaf rust spread.

In Mexico the trials were inoculated with a purified single-race inoculum suspended in mineral oil (Sotrol) using the most virulent and dominant races present in this country, i.e., BBG/BN (Singh et al. 2004) in Obregon 2006 and 2007 and BBG/BP in El Batan 2008.

All the genetic materials evaluated in the field trials were scored for reaction to leaf rust by visually estimating the percentage of pustule-infected leaf area (leaf rust susceptibility index: LRS), according to the modified Cobb scale (Peterson et al. 1948); scoring began in each field trial when the reference susceptible cultivars showed a 10% value of infected leaf area across the replicates within blocks. In Argelato, two and three visual scores were recorded in 2006 and in 2007, respectively.

Three and two LRS score surveys were recorded for the field trials carried out in Batan, 2006 and in Obregon, 2008, respectively, while up to six visual scores were recorded in the field trial carried out in Obregon in 2007.

For each field trial, the area under the disease progress curve (AUDPC, Shaner and Finney 1977) was then calculated as follows:

$$\mathrm{AUDPC} = \sum_{i=1}^{n} [(\mathrm{LRS}_i + \mathrm{LRS}_{i+1})/2] \times (t_{i+1} - t_i)$$

where n indicates the number of scores (minimum two and maximum six), LRS indicates the leaf rust susceptibility index, and t the time in days from the first scoring.

For each accession, the relative disease severity index (RDS) in the field has also been obtained referring the AUDPC (for each field trial) to the reference highly rust susceptible cv. Kofa.

Molecular and association analyses

The panel of 164 accessions was profiled at 225 SSR marker loci and at a sequence tagged site targeted to the *Ppd-A1* photoperiod-sensitivity gene (Wilhelm et al. 2009). Genomic DNA was obtained from each accession following the methodology described in Saghai Maroof et al. (1984). A bulk of ca. 25 seeds, from the original pure stock, was sown in growth chamber at 20°C. After 2 weeks, seedling leaves were collected, freeze-dried and ground; DNA was extracted with the C-TAB/chloroform/isopropanol extraction method.

The SSR primers were chosen among the publicly available sets catalogued in the GrainGenes database (http://wheat.pw.usda.gov) as BARC (*Xbarc* marker loci), CFA and CFD and GPW (*Xcfa, Xcfd, Xgpw*), WMC (*Xwmc*) and WMS (*Xgwm*); an additional subset of 61 private genomic WMS primers developed and owned by TraitGenetics (supplied by M. Ganal, TraitGenetics, Gatersleben, Germany) were also considered, 28 of which were profiled on the collection.

In the chr. 7BL distal region known to carry *Lr14* and the leaf rust resistant allele carried by the cultivar Creso and its derivatives (identified as *QLr.ubo-7B* in Maccaferri et al. 2008a), SSR markers from the publicly available data-set were genotyped at a higher density as compared to the other chr. regions. In total, 15 SSR loci were used to profile an interval of 29 cM.

As described in Maccaferri et al. (2008b), a unique thermo-cycling protocol was used for all primer sets and SSR profiles of the accessions were obtained

using the automated LI-COR 4200 IR^2 System (LiCor, Lincoln, NE, USA).

The majority of the SSRs markers considered herein were previously mapped in two intra-specific durum RIL-based linkage maps, from the crosses Kofa × Svevo (K × S, Maccaferri et al. 2008b) and Colosseo × Lloyd (C × L, Mantovani et al. 2008). A combined linkage map was obtained from the two data-sets using the JoinMap v. 4.0 software (Stam 1993; Van Ooijen and Voorrips 2006; http://www.kyazma.nl/index.php/mc.JoinMap/). The corresponding marker order and inter-marker genetic distances were used to report the linkage disequilibrium and association results.

Only non-rare alleles (i.e. with more than 16 counts, corresponding to a frequency ≥ 0.10) were considered for the LD and marker-trait association analyses, thus reducing the false positives and the LD inflation effects which have been frequently associated to the use of rare alleles. Similarly to rare alleles, data-points showing residual allelic heterogeneity within accession were considered as missing data.

For the chr. 7BL distal region where the association analysis pointed out a highly significant association to leaf rust resistance, the pattern of LD decay rate across the region in different mapping materials (i.e. germplasm collections *vs.* recombinant inbred line populations) was investigated using the LD *D'* and r^2 values as a function of the corresponding inter-marker distances. The analysis, carried out with the TASSEL v. 2.0.1. software for LD and association analysis (Bradbury et al. 2007), was performed using (1) the germplasm collection data of all the possible pairs of the 15 SSR markers included in the combined map and (2) the C × L mapping population data of all possible pairs of six SSR and 16 DArT markers.

The genetic structure of the collection has been investigated with a combination of model- and distance-based analyses. A subset of 96 loosely linked and evenly spread SSRs were chosen to investigate the population structure of the collection using the Bayesian model-based clustering method implemented in the software STRUCTURE v. 2.1 (Pritchard et al. 2000; Falush et al. 2003): an optimum number of five hypothetical subgroups were chosen to obtain the Q matrix of membership coefficients of each accession to all subgroups (for details see Maccaferri et al. 2009).

In the distance-based analysis, pairwise genetic similarity values (GS*ij*) were calculated for all possible pairs of accessions using the simple matching coefficient for multi-state markers: a co-ancestry K (kinship) matrix was thus obtained (for details see Maccaferri et al. 2009).

The 226 marker loci, including also *Ppd-A1*, were tested for significance of marker-trait associations under: (1) the fixed general linear model (GLM) including the Q population structure results as covariates, (2) the mixed linear model including the Q population structure results plus the 164 × 164 K kinship matrix. In the GLM analysis, besides the marker-wise association probability values, the experiment-wise association significance probability was obtained based on 1,000 permutations. The following traits were analysed separately: ITs obtained from seedling tests with single isolates and adult plant leaf rust severity score, AUDPC and RDS scores in the field. ITs, assigned according to Long and Kolmer (1989), were expressed using a numeric scale as follows:

$$\text{avirulent phenotypes}: 0 = 0, ; = 0, 1 = 1, 2 = 2, \quad X = 1.5$$

$$\text{virulent phenotypes}: 3 = 3, 4 = 4, + = +0.25, \quad - = -0.25$$

Prior to the association analysis, all data were subjected to square root transformation $(y = \sqrt{x + 0.5})$. The association analysis, similarly to the LD computation, was carried out in the TASSEL v. 2.0.1 software. For each marker significantly associated to the phenotype, R^2 values of the model and of the relevant marker and the least square means of the allelic variants present in the collection were calculated; in case significance was detected for loci with three or more non-rare alleles, the least significant difference between the allele means was calculated.

Results

Leaf rust response at the seedling stage

Twenty-five diverse *Puccinia triticina* isolates from Italy, Northern and Central Europe, Mexico, Ethiopia and Israel were used to characterise the durum germplasm collection for leaf rust response. The

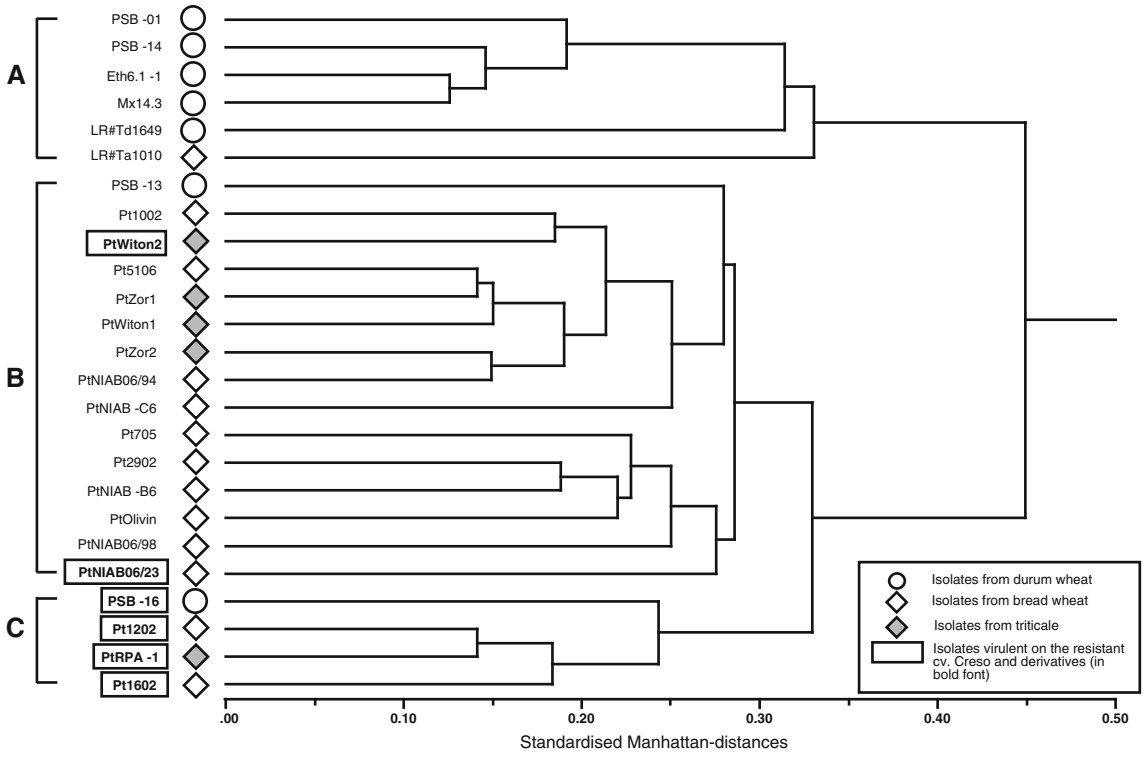


Fig. 1 UPGMA-dendrogram of the 25 leaf rust (*Puccinia triticina* Eriks.) isolates from durum and bread wheat used to characterise the durum germplasm collection. The dendrogram is based on the virulence phenotypes (Infection Types estimated with a 0–4 scale) of the isolates; the distances among isolates have been computed using the standardised Manhattan distances ('city-block' method)

isolates were collected from durum wheat, bread wheat and triticale and were all well characterised as to their virulence spectrum with the reference differential set of near isogenic lines from cv. Thatcher; moreover, some of the above isolates were profiled at molecular level using *Puccinia triticina* SSRs (Mantovani et al. 2009). Thus, the isolate set includes a non-redundant sample of *Puccinia triticina* strains with different virulence characteristics.

The pattern of diversity among isolates, based on the avirulence/virulence infection types of the 164 durum accessions and depicted using UPGMA-cluster analysis, is reported in Fig. 1. The dendrogram shows that the isolates grouped according to their origin and virulence phenotypes into three well-distinct groups: (1) a group of isolates mainly sampled from durum wheat (group 'A') including two Italian (PSB-01 and PSB-14), an Ethiopian (Eth6.1-1), a Mexican (Mx14.3) and two Israeli isolates (LR#Td1649 and LR#Ta1010, with LR#Ta1010 being a bread wheat isolate); (2) a group of 14 bread wheat/triticale isolates of European origin (group 'B') plus an Italian durum wheat isolate (PSB-13) with molecular characteristics close to isolates from bread wheat (Mantovani et al. 2009) and (3) a group of four isolates (group 'C') with the common feature of being virulent on cv. Creso, as well as on several Creso-derivatives and related accessions included in the collection. This latter group included one isolate from durum wheat (PSB-16), two isolates from bread wheat (Pt1202 and Pt1602) and one from triticale (PtRPA-1).

Testing the germplasm collection with the 25 above-mentioned isolates showed that leaf rust resistance at the seedling stage (with ITs equal to 0, 1 or 2) is relatively frequent in the cultivated Mediterranean durum wheat germplasm. In fact, on average across all the tested isolates, 57 and 41% of the cultivars showed a resistant (IT = 0–2) and a highly resistant response (IT = 0 or 1, values indicative of immunity or hypersensitive reaction), respectively (Table 1). As expected, the resistant responses were

Table 1 Distribution of the seedling infection type (IT) response in the collection of 164 elite durum accessions to 25 *Puccinia triticina* isolates collected from *T. durum* or *T. aestivum*. Number and frequencies (%) of the response phenotypes, as evaluated with the 0–4 IT scale, are reported for all the tested isolates. The isolates have been listed according to the cluster analysis classification with three groups (A, B and C) as from Fig. 1. Isolates virulent on the resistant cultivar Creso and derivatives are reported in bold

		Isolate group A						Isolate group B							
		PSB-01	PSB-14	Eth6.1-1	Mx14.3	LR#Td1649	LR#Ta1010	PSB-13	Pt1002	**PtWiton2**	Pt5106	PtZor1	PtWiton1	PtZor2	PtNIAB06/94
		TD[a]	TD	TD	TD	TD	TA	TD	TA	TA	TA	TA	TA	TA	TA
IT 0[b]	(no.)	2	9	15	15	5	21	60	75	74	77	93	90	68	56
IT 1[b]	(no.)	31	11	3	8	7	23	35	8	4	0	5	0	8	8
IT 0–1 subtotal	(no.)	33	20	18	23	12	44	95	83	78	77	98	90	76	64
IT 0–1 subtotal	(%)	20	12	11	14	7	27	58	51	48	47	60	55	46	39
IT 2[c]	(no.)	26	25	20	17	9	22	24	39	24	41	14	27	21	23
IT 0–2 subtotal	(no.)	59	45	38	40	21	66	119	122	102	118	112	117	97	87
IT 0–2 subtotal	(%)	36	27	23	24	13	40	73	74	62	72	68	71	59	53
IT 3[d]		72	52	49	90	9	26	13	41	52	15	5	25	17	32
IT 4[e]		33	0	0	0	133	72	0	0	9	30	46	21	49	44
IT 3–4 subtotal	(no.)	105	52	49	90	142	98	13	41	61	45	51	46	66	76
IT 3–4 subtotal	(%)	64	32	30	55	87	60	8	25	37	27	31	28	40	46
Virulence on Creso/Colosseo		no	no	no	no	no	no	no	no	yes	no	no	no	no	no

Table 1 continued

		Isolate group B							Isolate group C			
		PtNIAB/C6	Pt705	Pt2902	PtNIAB/B6	PtOlivin	PtNIAB06/98	**PtNIAB06/23**	**PSB-16**	**Pt1202**	**PtRPA-1**	**Pt1602**
		TA	TA	TA	TA	TA	TA	TA	TD	TA	TA	TA
IT 0[b]	(no.)	29	100	99	85	115	96	75	6	46	40	53
IT 1[b]	(no.)	42	5	3	0	11	6	11	37	2	6	4
IT 0–1 subtotal	(no.)	71	105	102	85	126	102	86	43	48	46	57
IT 0–1 subtotal	(%)	43	64	62	52	77	62	52	26	29	28	35
IT 2[c]	(no.)	51	41	12	48	20	26	34	24	17	26	38
IT 0–2 subtotal	(no.)	122	146	114	133	146	128	120	67	65	72	95
IT 0–2 subtotal	(%)	74	89	70	81	89	78	73	41	40	44	58
IT 3[d]		8	15	38	16	2	1	21	81	27	41	52
IT 4[e]		31	2	11	13	15	30	21	16	70	50	16
IT 3–4 subtotal	(no.)	39	17	49	29	17	31	42	97	97	91	68
IT 3–4 subtotal	(%)	24	10	30	18	10	19	26	59	59	55	41
Virulence on Creso/ Colosseo		no	no	no	no	no	no	yes	yes	yes	yes	yes

[a] Isolate from *Triticum durum* Desf. (TD) and *Triticum aestivum* L. (TA)

[b] Resistant response: resistance based on immunity or hypersensitivity

[c] Moderately resistant response

[d] Moderately susceptible response

[e] Susceptible response

relatively less frequent when considering the durum wheat isolates (PSB-01, PSB-14, PSB-16, Eth6.1-1, Mx14.3 and LR#Td1649) as compared to the bread wheat isolates. The durum isolates (excluding PSB-13 for its above-mentioned characteristics) showed frequencies of avirulent interactions (resistant response, IT = 0–2) ranging from 13% of the accessions for the highly virulent isolate LR#Td1649 to 41% for PSB-16. The frequency of accessions displaying immune and/or hypersensitive responses (avirulence patterns with IT = 0 or 1) ranged from 7 (LR#Td1649) to 26% (PSB-16) in case of durum wheat isolates and from 27 (LR#Ta1010) to 77% (PtOlivin) in case of bread wheat/triticale isolates. In particular, the bread wheat isolates Pt705, PtNIAB-B6 and PtOlivin were avirulent on more than 80% of the accessions), while, among the durum isolates, only PSB-13, which, as reported above, was grouped with three hexaploid wheat leaf rust isolates, showed avirulent interactions with 73% of the accessions. However, all bread wheat isolates were informative since all of them showed the capability of developing some virulent interactions with the durum accessions.

Figure 2 shows the UPGMA-dendrogram of the accessions based on the phenotypic distances calculated from the seedling IT pattern considering all 25 isolates. This is useful to explore the presence of common IT patterns among accessions which could be indicative of *Lr* genes present in non-rare frequency in this collection of elite durums.

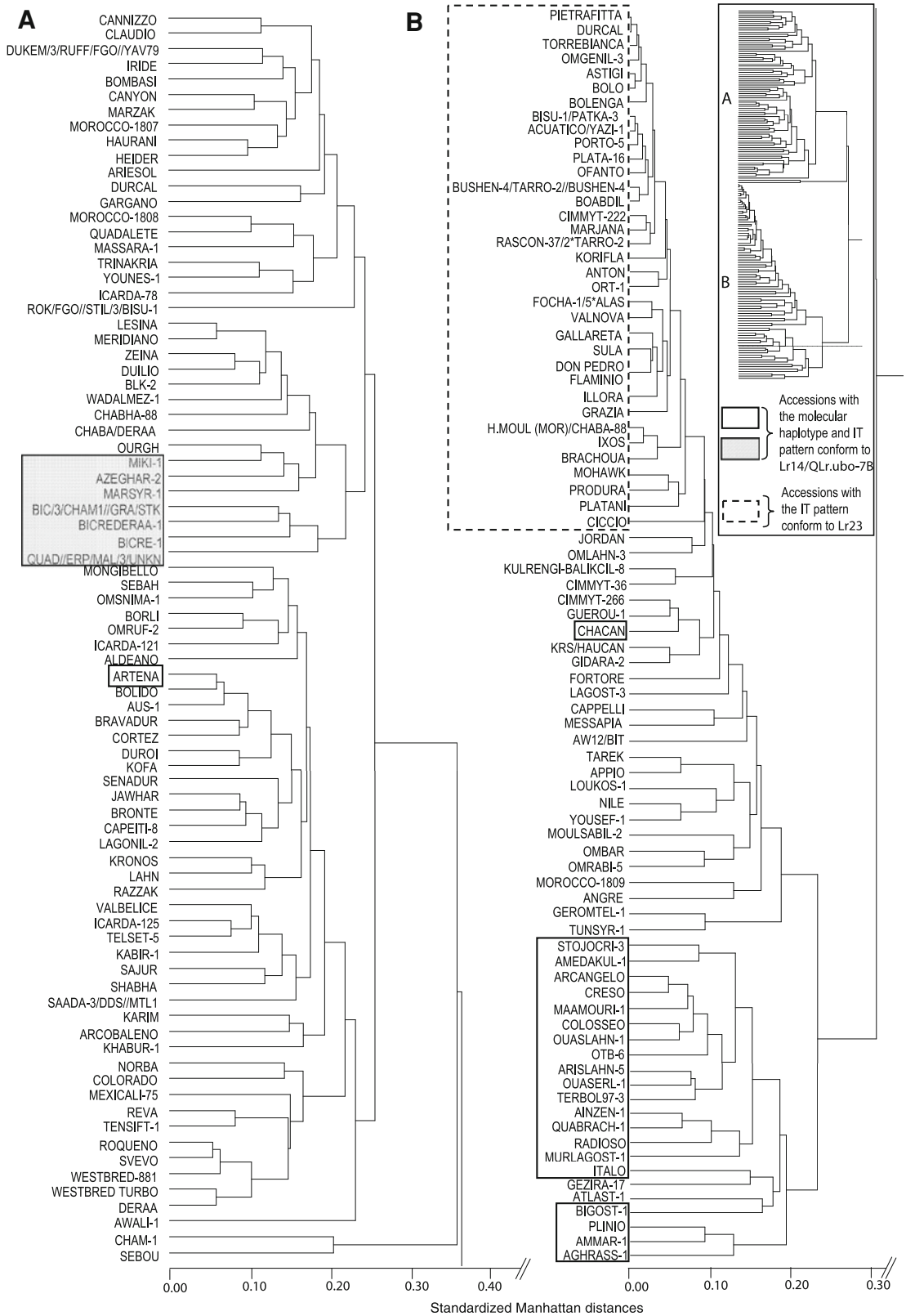
Cultivars Creso and Colosseo (Creso × Mexa's mutant), putatively carrying a resistant *Lr14* allele (Martinez et al. 2007; Maccaferri et al. 2008a; Marone et al. 2009), and a number of accessions related by pedigree to Creso (hereafter reported as 'Creso-derivatives') showed an IT pattern resistant to the majority of isolates, with the exception of six isolates (PSB-16 from durum wheat, Pt1202, Pt1602, PtWiton-2 and PtNIAB06-23 from hexaploid wheat and PtRPA-1 from triticale; see Figs. 1, 2). The durum isolate PSB-16, while being virulent on Thatcher, the susceptible control, showed a mesothetic response (indicative of substantial resistance) on Thatcher-*Lr14a* (Mantovani et al. 2009). On the contrary, the other five bread wheat/triticale isolates were virulent on Thatcher-*Lr14a*. Interestingly, out of the remaining 12 bread wheat isolates avirulent on Creso, nine showed a highly virulent interaction (IT = 4) on Thatcher-*Lr14*a (data not reported).

Fig. 2 UPGMA-dendrogram of the 164 elite durum accessions based on the response phenotypes to 25 leaf rust (*Puccinia triticina*) isolates from durum and bread wheat. The dendrogram is based on the response phenotypes (Infection Types) of the accessions to the isolates; the distances among accessions have been computed using the standardised Manhattan distances ('city-block' method). For sake of clarity, the dendrogram scheme has been subdivided in two portions (**a** and **b**)

The lower portion (B) of the cluster in Fig. 2 shows the presence of a broad sub-cluster of 20 accessions (mostly Italian cultivars and relatively recent ICARDA breeding lines) with IT patterns equal or similar to those of Creso and Colosseo.

Interestingly, the only accessions resistant to the most virulent isolate included in this study, namely LR#Td1649 from Israel (Table 1), are those included in the Creso-group (with the exception of Geromtel-1). Other minor genes in addition to *Lr14*/*QLr.ubo-7B.2* most probably contributed to the phenotypic response of the Creso-derivatives, as shown by the presence of sub-cluster structures within the Creso-group. An IT pattern almost similar to that of Creso has also been observed in the small sub-cluster of Creso-related accessions, from Miki-1 to Quad//Erp/Mal/3/Unk, including the largely used line Bicre (Bittern x Creso, Nachit 2000), positioned in the upper part of Fig. 2. This small sub-cluster is clearly distinct from the larger sub-cluster that includes Creso (lower part of the dendrogram) because the first one showed a susceptible response to isolates Pt1002, PtNIAB06/94, LR#Ta1010 and LR#Td1649, while the second one showed a resistant response. In addition, within the first sub-cluster, cvs. Miki-1, Azeghar-2 and Marsyr-1 showed susceptibility to isolate PSB-01 (to which cv. Creso and derivatives are resistant), while the other four accessions related to Bicre can be differentiated from Creso as well as from Miki-1, Azeghar-2 and Marsyr-1 based on their hypersensitive response to PtNIAB06/98.

Furthermore, a clearly distinct and highly homogeneous pattern common to a relatively high number (35 in total) of accessions which are also interrelated by pedigree (Maccaferri et al. 2005) is evident in the central to lower part of the cluster (accessions from Pietrafitta to Ciccio; part B of Fig. 2). The IT pattern of this group of accessions is clearly distinct based on the common susceptible response to most of the tested durum wheat isolates (PSB-01, PSB-14,


A
CANNIZZO
CLAUDIO
DUKEM/3/RUFF/FGO//YAV79
IRIDE
BOMBASI
CANYON
MARZAK
MOROCCO-1807
HAURANI
HEIDER
ARIESOL
DURCAL
GARGANO
MOROCCO-1808
QUADALETE
MASSARA-1
TRINAKRIA
YOUNES-1
ICARDA-78
ROK/FGO//STIL/3/BISU-1
LESINA
MERIDIANO
ZEINA
DUILIO
BLK-2
WADALMEZ-1
CHABHA-88
CHABA/DERAA
OURGH
MIKI-1
AZEGHAR-2
MARSYR-1
BIC/3/CHAM1//GRA/STK
BICREDERAA-1
BICRE-1
QUAD//ERP/MAL/3/UNKN
MONGIBELLO
SEBAH
OMSNIMA-1
BORLI
OMRUF-2
ICARDA-121
ALDEANO
ARTENA
BOLIDO
AUS-1
BRAVADUR
CORTEZ
DUROI
KOFA
SENADUR
JAWHAR
BRONTE
CAPEITI-8
LAGONIL-2
KRONOS
LAHN
RAZZAK
VALBELICE
ICARDA-125
TELSET-5
KABIR-1
SAJUR
SHABHA
SAADA-3/DDS//MTL1
KARIM
ARCOBALENO
KHABUR-1
NORBA
COLORADO
MEXICALI-75
REVA
TENSIFT-1
ROQUENO
SVEVO
WESTBRED-881
WESTBRED TURBO
DERAA
AWALI-1
CHAM-1
SEBOU
0.00
0.10
0.20
0.30
0.40
B
PIETRAFITTA
DURCAL
TORREBIANCA
OMGENIL-3
ASTIGI
BOLO
BOLENGA
BISU-1/PATKA-3
ACUATICO/YAZI-1
PORTO-5
PLATA-16
OFANTO
BUSHEN-4/TARRO-2//BUSHEN-4
BOABDIL
CIMMYT-222
MARJANA
RASCON-37/2*TARRO-2
KORIFLA
ANTON
ORT-1
FOCHA-1/5*ALAS
VALNOVA
GALLARETA
SULA
DON PEDRO
FLAMINIO
ILLORA
GRAZIA
H.MOUL (MOR)/CHABA-88
IXOS
BRACHOUA
MOHAWK
PRODURA
PLATANI
CICCIO
JORDAN
OMLAHN-3
KULRENGI-BALIKCIL-8
CIMMYT-36
CIMMYT-266
GUEROU-1
CHACAN
KRS/HAUCAN
GIDARA-2
FORTORE
LAGOST-3
CAPPELLI
MESSAPIA
AW12/BIT
TAREK
APPIO
LOUKOS-1
NILE
YOUSEF-1
MOULSABIL-2
OMBAR
OMRABI-5
MOROCCO-1809
ANGRE
GEROMTEL-1
TUNSYR-1
STOJOCRI-3
AMEDAKUL-1
ARCANGELO
CRESO
MAAMOURI-1
COLOSSEO
OUASLAHN-1
OTB-6
ARISLAHN-5
OUASERL-1
TERBOL97-3
AINZEN-1
QUABRACH-1
RADIOSO
MURLAGOST-1
ITALO
GEZIRA-17
ATLAST-1
BIGOST-1
PLINIO
AMMAR-1
AGHRASS-1
0.00
0.10
0.20
0.30
A
B
Accessions with the molecular haplotype and IT pattern conform to Lr14/QLr.ubo-7B
Accessions with the IT pattern conform to Lr23
Standardized Manhattan distances

Mx-14.3 and LR#1649) and on the hypersensitive response to durum isolate PSB-16 (which, notably, is virulent on the *Lr14/QLr.ubo-7B.2* group) and to most of the bread wheat isolates. The accessions grouped in this sub-cluster showed a clear relationship with the Italian breeding lineage tracing back to Valnova (a durum founder derived from the cross between Italian and early CIMMYT germplasm) and to the CIMMYT germplasm related to Gallareta (Altar 84), which is known to carry *Lr23* (Nelson et al. 1997).

With the exception of the Creso-related group, it can be noticed that a few accessions only evidenced IT patterns broadly resistant over several isolates (particularly durum isolates), thus being valuable from a breeding standpoint. In particular, the best performing accessions Atlast-1, Geromtel-1, H. Moul(Mor)/Chaba-88, Krs/Haucan, Mongibello, Morocco-1808, Trinakria, Tunsyr-1 and Yousef-1 can be mentioned for their resistance to multiple isolates at seedling and adult plant stage in the field (detailed IT and field scores available upon request).

Leaf rust response at the adult stage in the field

A number of accessions showed resistance to leaf rust in the field, as estimated based on the relative disease severity index (RDS) obtained referring the AUDPC to the susceptible check Kofa, across all the five field trials. The frequency of accessions that displayed RDS values $\leq$10% was equal to 22% in Argelato-2006, only 4% in Argelato-2007, and from 17 to 19% in the three field trials carried out in Mexico. The frequency of highly susceptible accessions was higher than that of the resistant ones; in fact, frequencies of accessions with RDS values $\geq$80% were equal to 73 and 80% of the total number of accessions for the trials carried out in Argelato in 2006 and 2007, respectively, and equal to 60, 36 and 41% for the trials carried out in Mexico in 2006, 2007 and 2008, respectively (data not shown). Most of the cultivars/lines with resistant response in Italy in 2006 and, to a lesser extent, in 2007 (trials carried out with inoculation of a mixture of 16 Italian leaf rust isolates) confirmed their resistance in the Mexican field trials carried out for 3 years with the Mexican races BBG/BN (2006 and 2007) and BBG/BP (2008). The large majority of the resistant accessions were found to belong to the sub-cluster of the Creso-related accessions (resistant at the seedling stage to isolates PSB-01, PSB-13, PSB-14, susceptible to PSB-16) highlighted in the cluster of Fig. 2. In Argelato 2007, a noticeable drop in the field resistance levels was observed for most of these genotypes, with final RDS values ranging from 20 to 40% as compared to cv. Kofa, the susceptible check. Interestingly, in this trial, only seven genotypes maintained a high resistance score (RDS $<$ 10%) under high disease pressure conditions, three of which (H. Moul (Mor)/Chaba-88, Geromtel-1 and Tunsyr-1) were not grouped into the *Lr14/QLr.ubo-7B.2* sub-cluster; these three genotypes showed also highly resistant responses to the majority of the leaf rust isolates in the seedling tests.

On the contrary, the genotypes included in the large sub-cluster that showed, at seedling stage, susceptibility to PSB-1 and PSB-14 and resistance to PSB-16 were always characterised by medium to high RDS values in all five field trials.

Whole-genome association mapping for leaf rust response in durum wheat

The collection of 164 elite durum accessions was subjected to whole-genome association mapping for leaf rust response at the seedling and adult stages using the profiles at 226 marker loci evenly spread on the 14 durum chromosomes and the complete phenotypic data set.

A primary result of this survey was the presence of highly significant experiment-wise associations to both seedling and field responses in the distal portion of chr. 7BL, where *Lr14* (from Llareta-INIA) and *QLr.ubo-7B.2* (from Creso) have been previously mapped (Herrera-Foessel et al. 2008a; Maccaferri et al. 2008a; Marone et al. 2009). The association results for this chromosome region are presented in the next paragraph.

After excluding the 29 accessions harbouring the Creso-related molecular haplotype at *QLr.ubo-7B.2*, the same data-set was used to test for the presence of associations with genes/QTLs for leaf rust response other than *Lr14/QLr.ubo-7B.2*. Marker- and experiment-wise significant associations were identified in several cases on chrs. 1A, 1B, 2A, 2B, 3B, 5A, 5B, 6B, 7A and 7B (Fig. 3).

Associations can be categorised into three main groups. The first group includes loci associated with significant differences in seedling response (IT) to

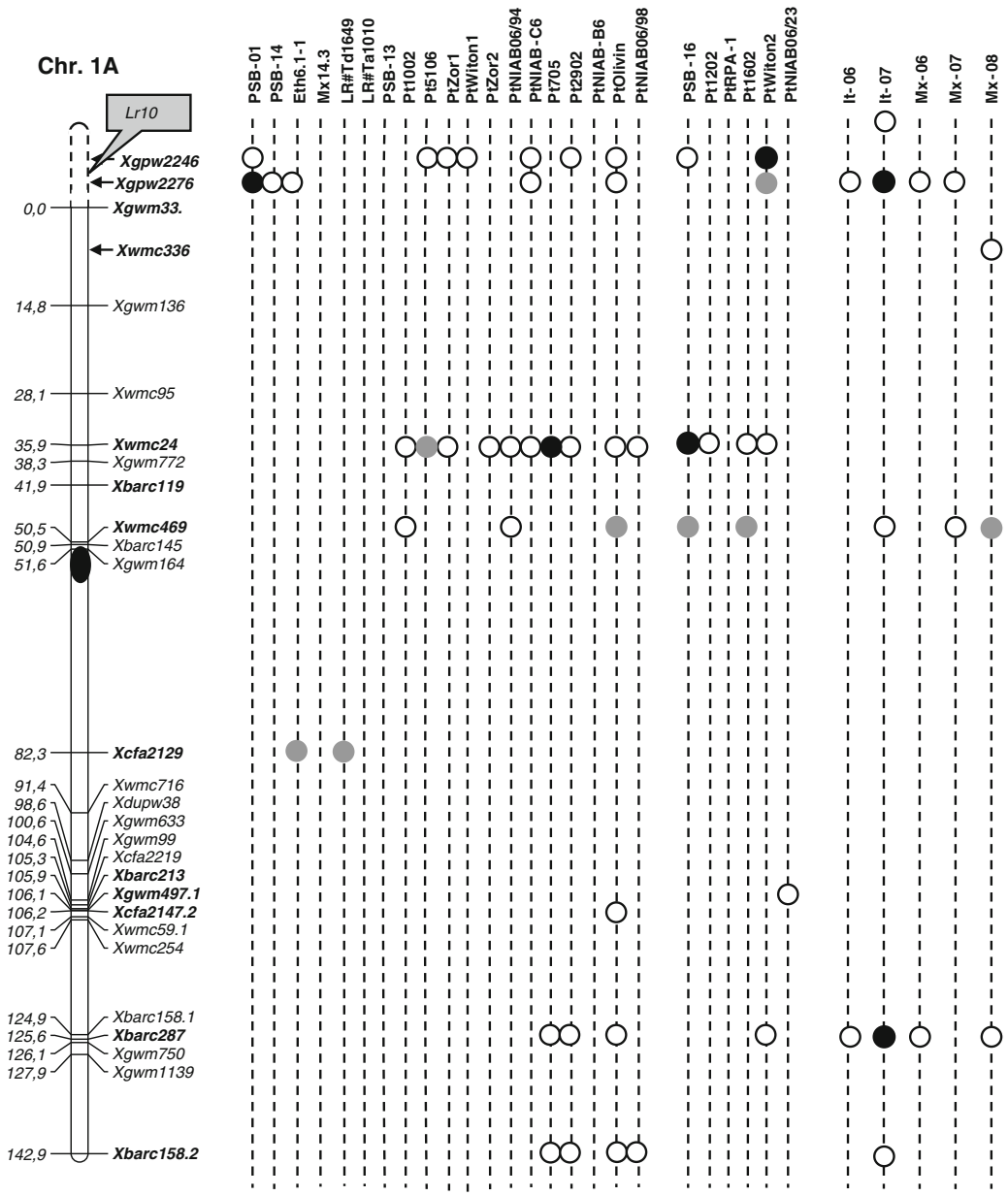


Fig. 3 Chromosome location of the significant marker-trait associations reported on a combined map obtained by merging two intra-specific durum linkage maps (Kofa × Svevo, Maccaferri et al. 2008b, and Colosseo × Lloyd, Mantovani et al. 2008). Markers reported in *bold* have been used to profile the germplasm collection and to perform the association mapping analysis. Marker locations indicated with an *arrow* are as from the literature. The association results are reported for each of the 25 *Puccinia triticina* isolates used in seedling tests and for the five artificially inoculated field trials carried out in Italy (It) and in Mexico (Mx). After the identification of the major effect of the chr. 7BL distal region, any interference caused by the presence of *Lr14*/*QLr.ubo-7BL* was avoided by performing the whole-genome association mapping analysis after excluding the 29 accessions harbouring the Creso-molecular haplotype at *Xcfa2257.2-Xgwm344.2-Xwmc10*. The complete data-set (164 accessions) has been used only for the six isolates (from PSB-16 to PtNIAB06/23) showing high virulence to Creso and derivatives. *White-* and *grey-filled circles* indicate marker-wise significant ($P \leq 0.05$ and $P \leq 0.01$, respectively) associations. *Black filled circles* represent experiment-wise significant associations ($P \leq 0.05$). As to the field trials, the significant marker-trait associations are reported on the *right end* side of the figure. The genetic position of mapped *Lr* genes is reported in the chromosome bars. *Lr* genes assigned to chromosomes but not yet mapped are reported at the bottom of each chromosome

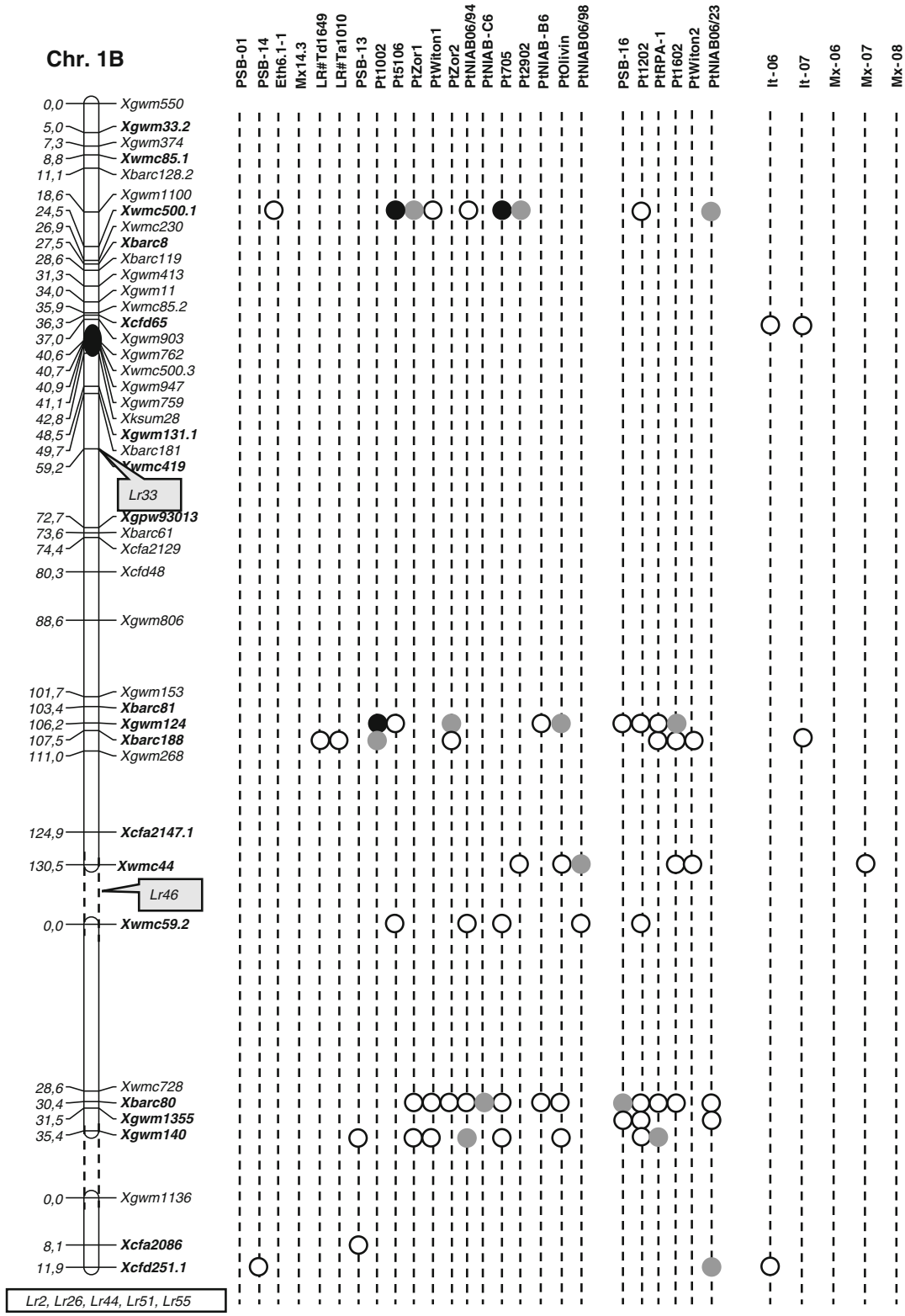


Fig. 3 continued

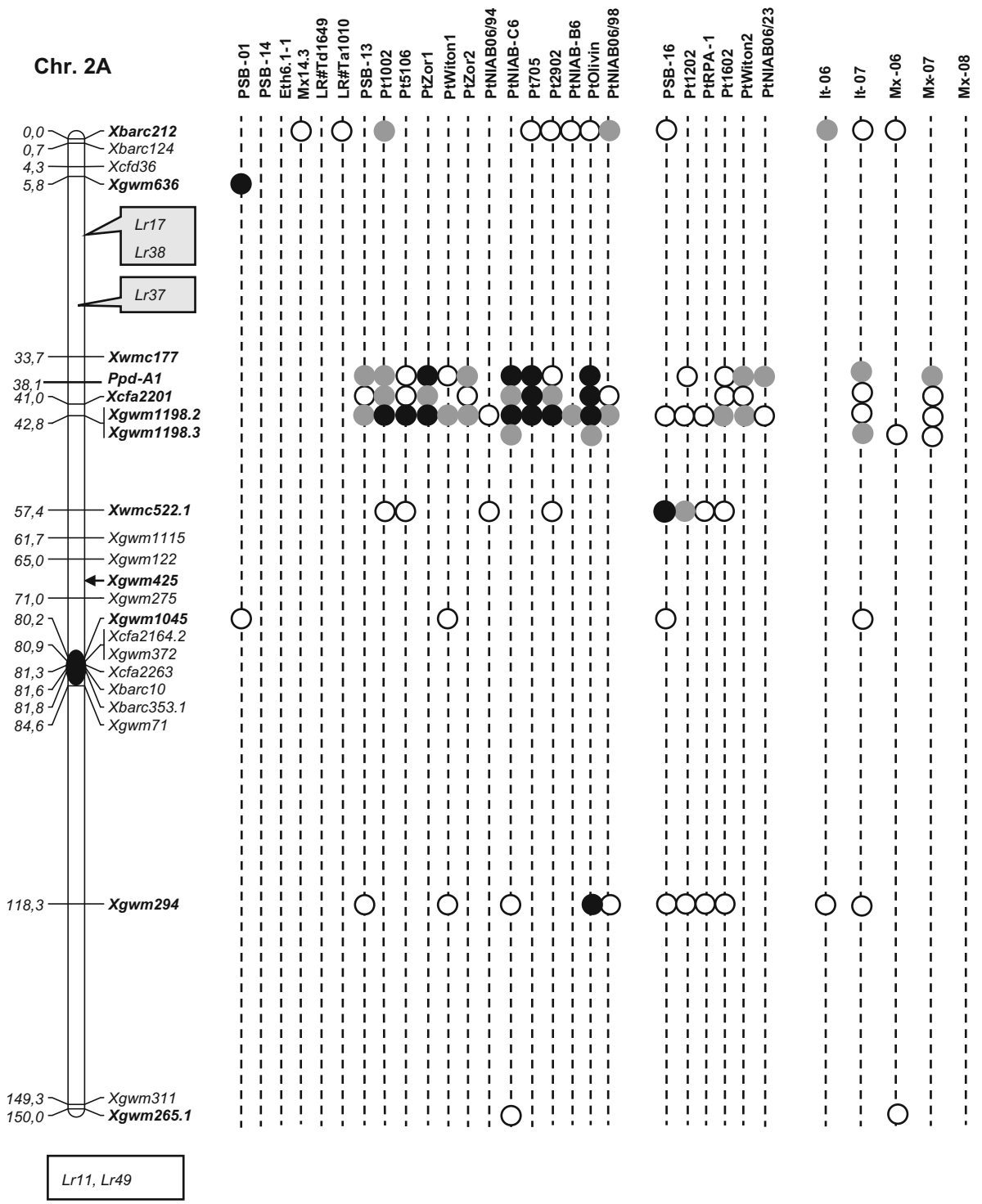
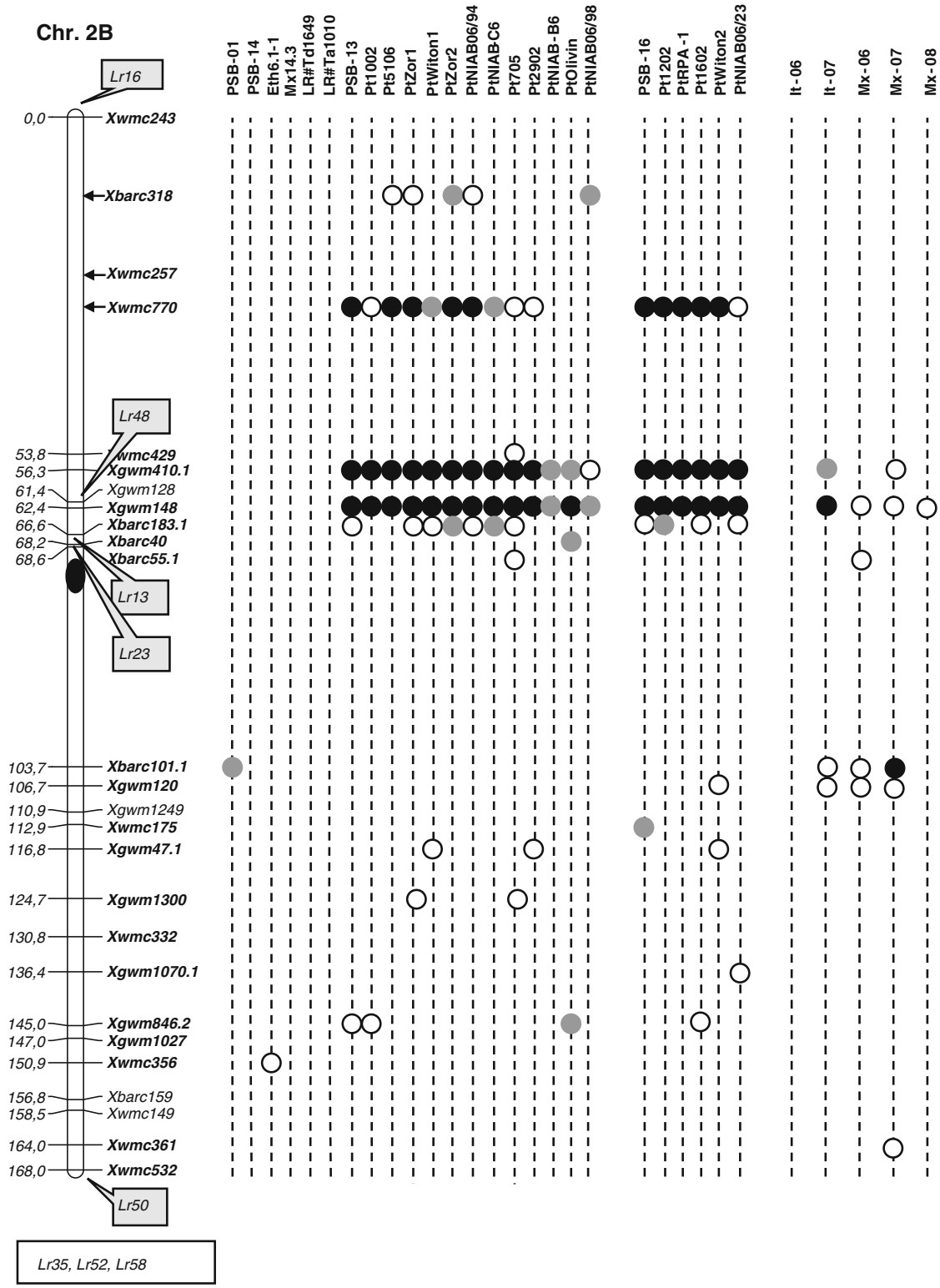


Fig. 3 continued



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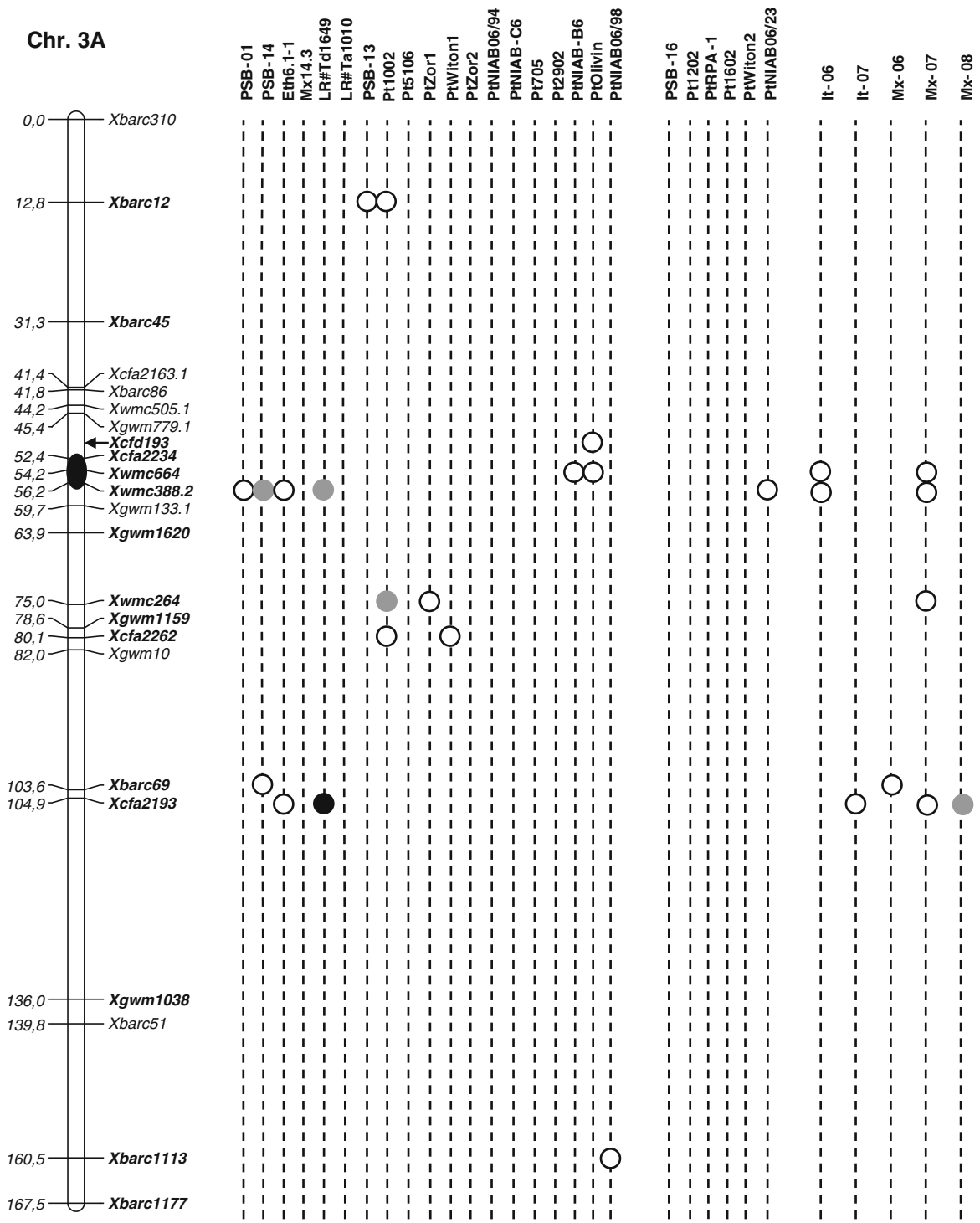
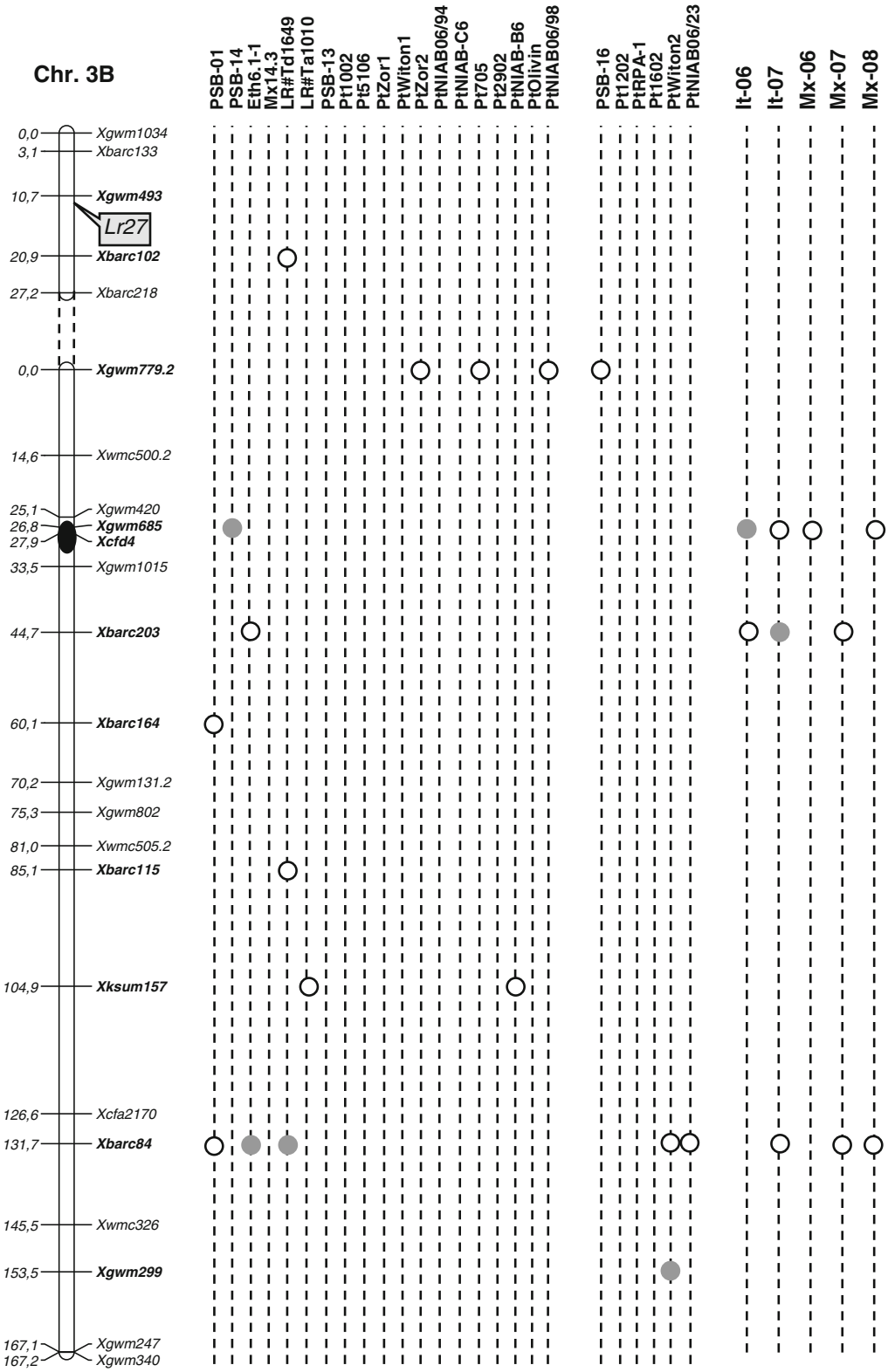


Fig. 3 continued



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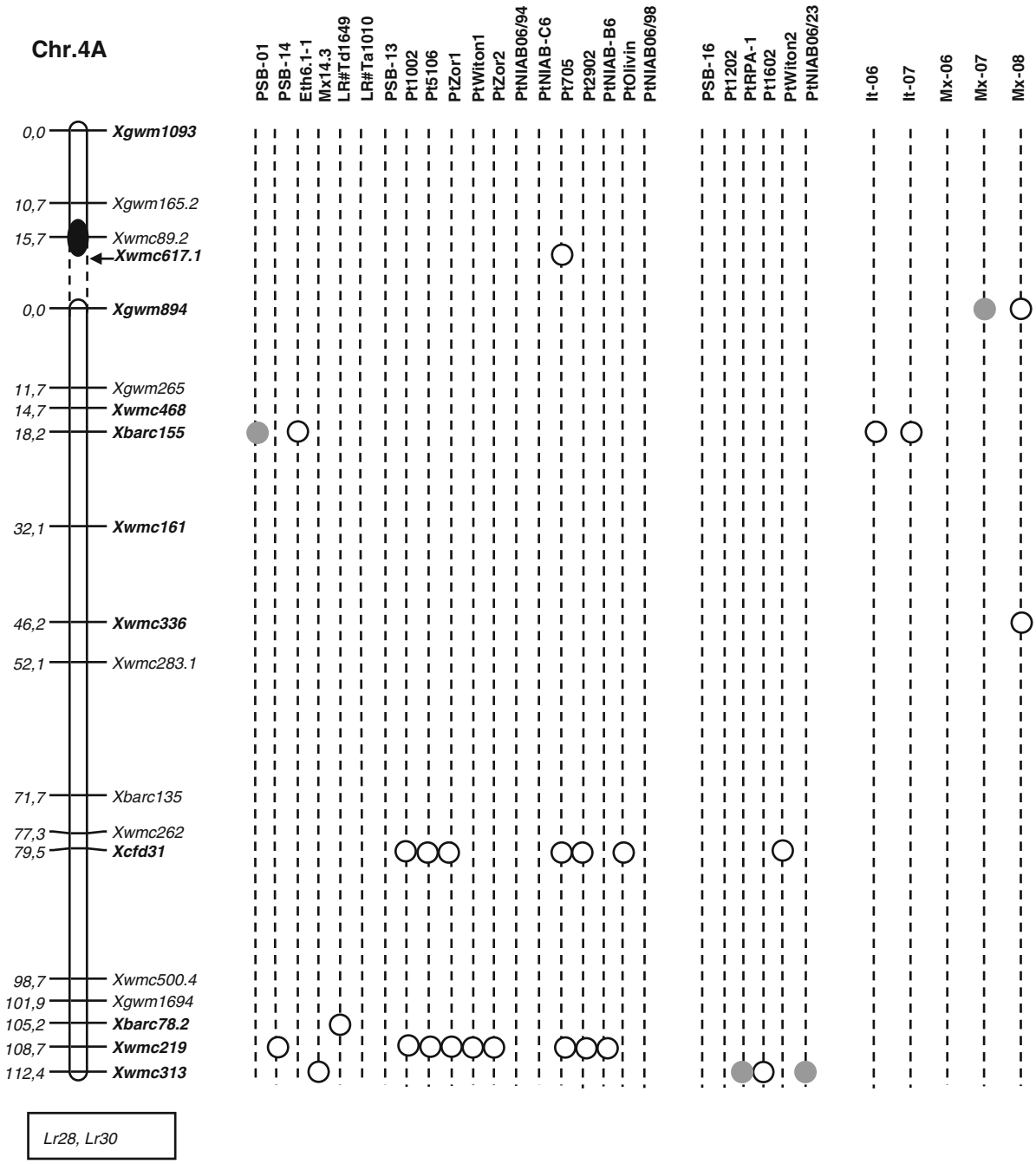


Fig. 3 continued

durum wheat, bread wheat and triticale isolates and also in adult plant response (as from the field trials). The following marker loci are included in this group: *Xgpw2246* and *Xgpw2276* (chr. 1AS, distal region), *Xwmc469* (chr. 1A, centromeric region), *Xbarc212* (chr. 2AS, distal region), the linked loci *Xgwm664* and *Xwmc388.2* (chr. 3A, centromeric region), *Xbarc84* (chr. 3BL, distal region), *Xgwm335* (chr. 5B, centromeric region), *Xwmc621* (chr. 6BL, distal end), *Xwmc323* (chr. 7BS, distal end) and *Xgwm1187* (chr. 7AS, distal region), for which no significant associations with the field response were observed.

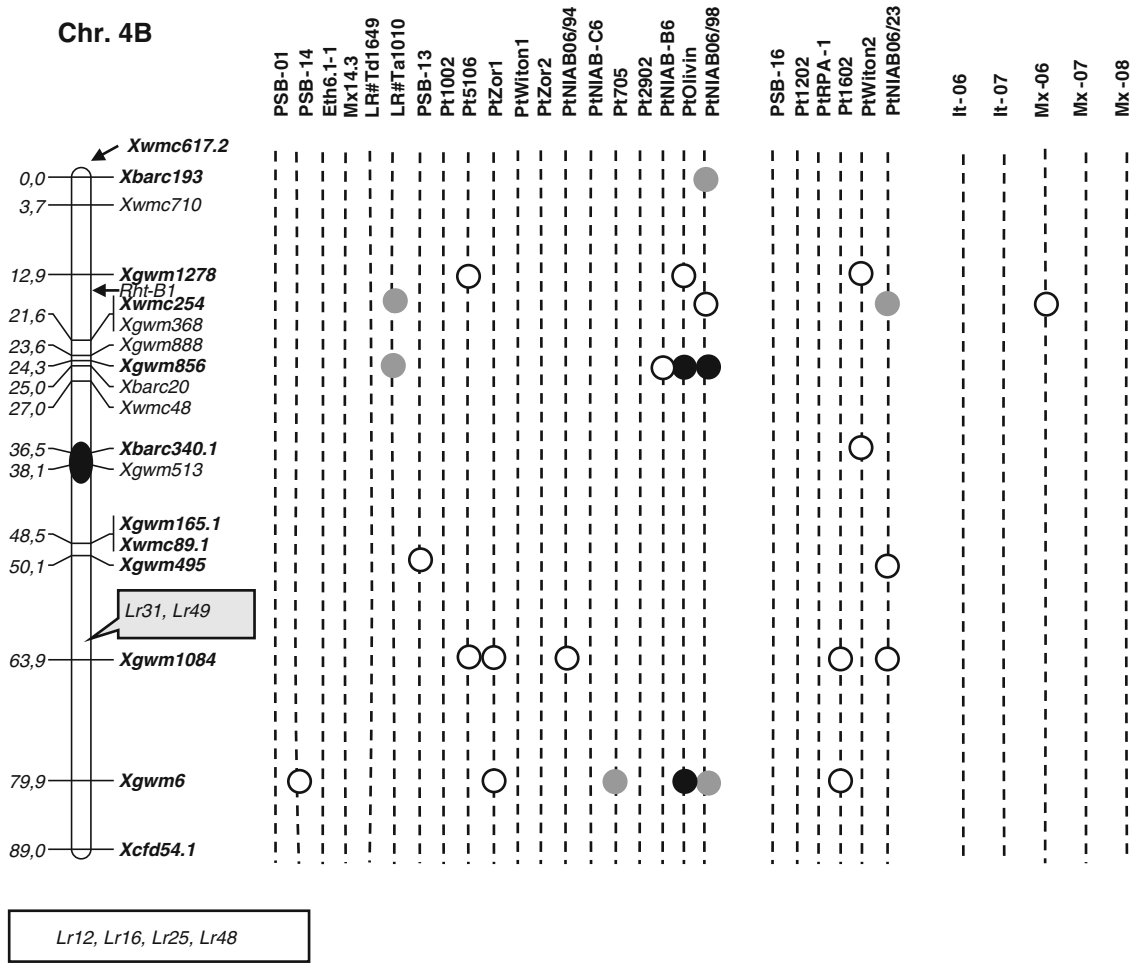
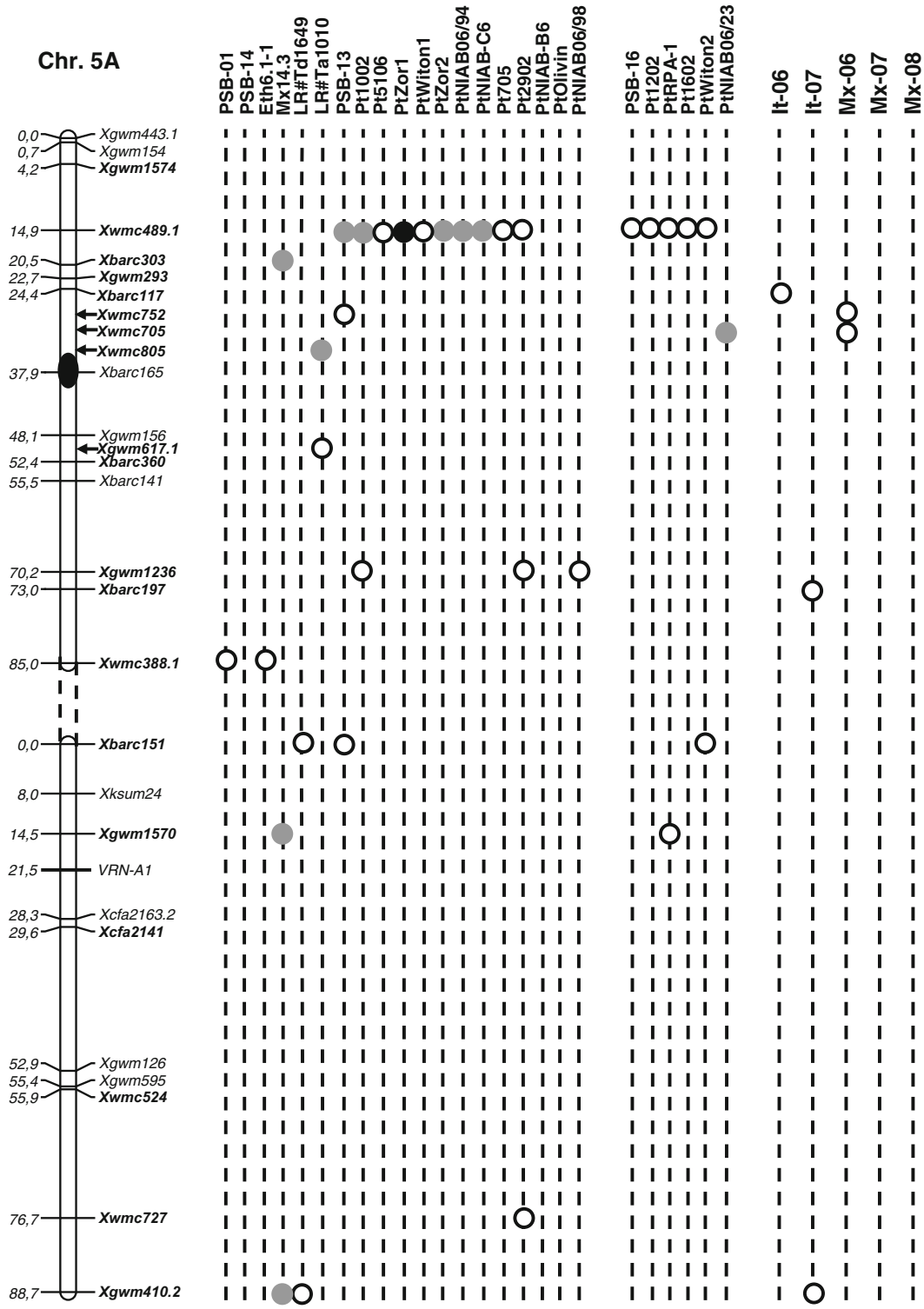


Fig. 3 continued

The second group includes loci associated with differences in seedling IT response only to the bread wheat isolates and to the isolates highly virulent to the accessions carrying the *Lr14*/*QLr.ubo-7B.2* resistance allele; in general, these loci showed no associations with the responses in the field. As to this second group, strong associations were observed for *Xwmc24* (chr. 1A, proximal region), *Xwmc500.1*, *Xgwm124/Xbarc188* and *Xbarc80/Xgwm140* (tagging three different chr. 1B regions), *Xcfa2201* and *Xgwm1198* (chr. AS, close to *Ppd-A1* region), *Xwmc770* and the tightly associated loci *Xgwm410.1/Xgwm148/Xbarc183.1* (chr. 2BS, proximal region), *Xgwm489.1* (chr. 5AS, proximal region), *Xgwm1682* (chr. 6BL, proximal region) and *Xgwm1184* (chr. 7BS, proximal region).

Finally, loci associated with the adult plant response in the field and with low or null effects on the seedling response are included in the third group. In this case, the following loci can be mentioned: *Xbarc287* (chr. 1AL, distal region), the tightly linked *Xbarc101.1/Xgwm120* (chr. 2BL, proximal region), *Xcfa2193* (chr. 3AL, proximal region), *Xgwm685* (chr. 3B, centromeric region), *Xbarc203* (chr. 3BL, proximal region) and *Xgwm894* (chr. 4AL, centromeric region).

In general, except for the chr. 7BL region, highly significant ($P \leq 0.01$) experiment-wise associations were not frequent. However, in Fig. 3, it can be noticed that the chr. 2AS (near *Ppd-A1*) and chr. 2BS (proximal to the centromere) regions were experiment-wise associated with seedling response to the bread wheat isolates and to the durum wheat isolate PSB-16. On the contrary, only weak associations were found for field responses in the present panel.



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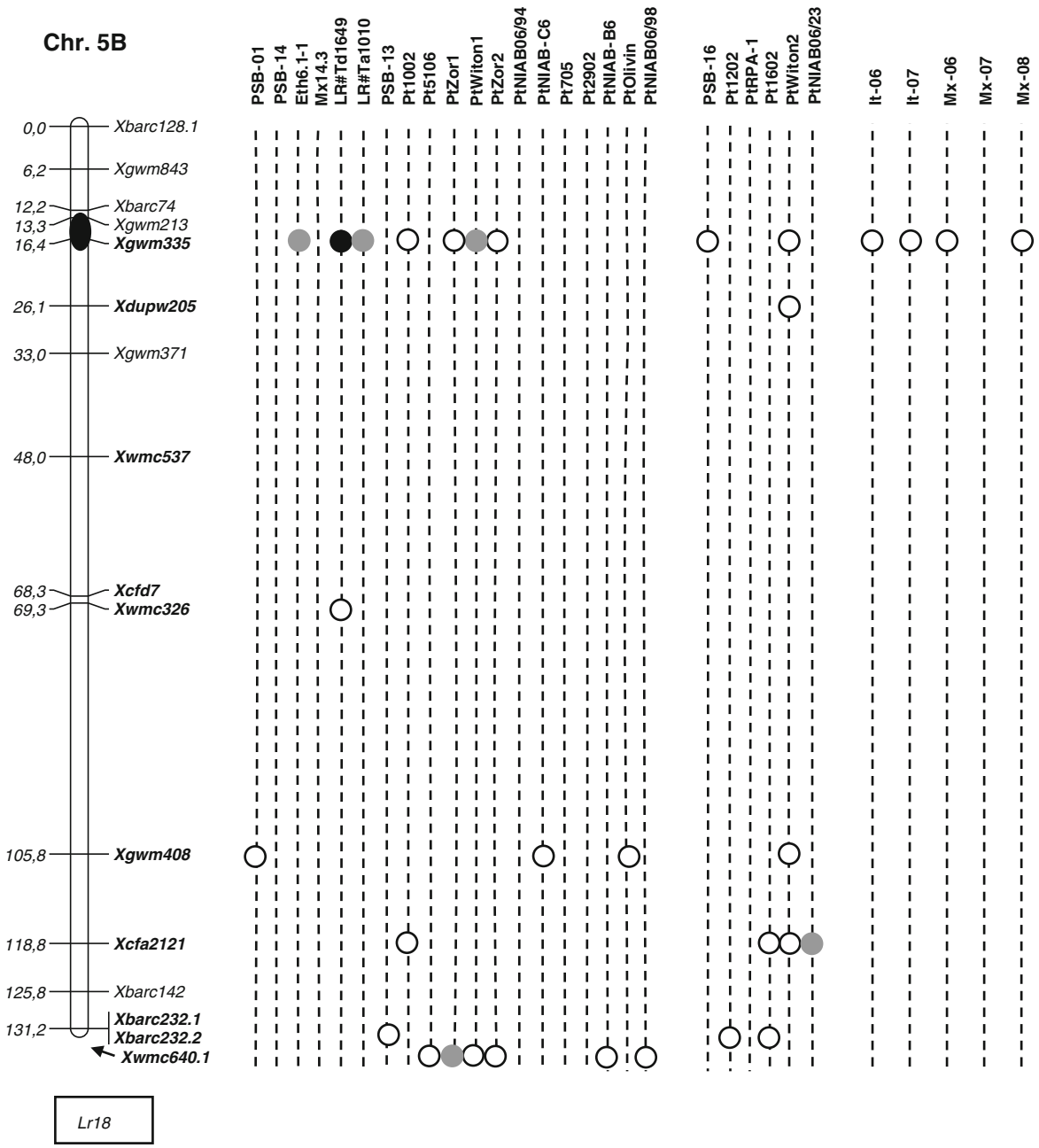

Fig. 3 continued

LD and association mapping at the chr. 7BL region harbouring *Lr14/QLr.ubo-7B.2*

To investigate in detail the pattern of inheritance in the chr. 7BL region harbouring *Lr14* and *QLr.ubo-7B.2*, the same SSR markers used to map *Lr14* (Herrera-Foessel et al. 2008a) and *QLr.ubo-7B.2* (Maccaferri et al. 2008a), with the addition of the distal marker *Xwmc10* and with the exclusion of the DArT markers, were used to profile the durum collection.

In the elite panel herein considered, the LD decay with the genetic distances based on the chr. 7BL region from *Xgwm577* to *Xwmc10* (ca. 30 cM; see Fig. 3) is at least twice longer than that observed in

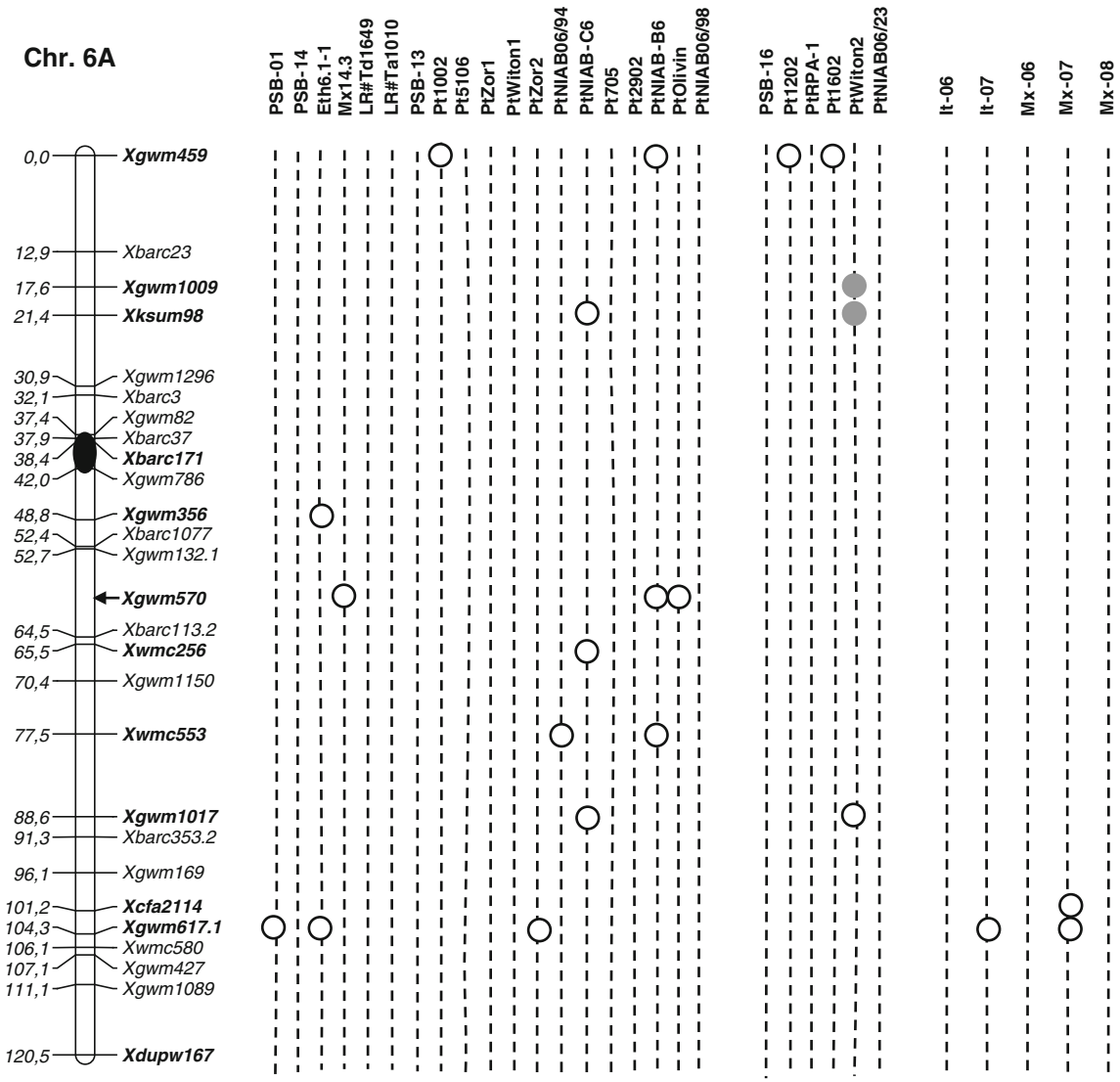

Fig. 3 continued

the C × L population of 176 RILs used to map *QLr.ubo-7B.2* (Maccaferri et al. 2008a). The LD decay patterns of the C × L population and that of the germplasm collection are compared in Fig. 4. This figure shows that in this region the LD values in the RIL population remained above the LD decay thresholds ($r^2 = 0.3$ and $D' = 0.6$), which are usually considered as indicative of the presence of appreciable LD, up to an inter-marker distance of 15–20 cM, while dropping below these values within 5, maximum 10 cM, in the germplasm collection. This provided the opportunity to improve the resolution of genetic mapping as compared to traditional linkage mapping based on RIL populations. The detailed LD pattern in the region (Fig. 5) evidenced that strong pairwise inter-marker LD

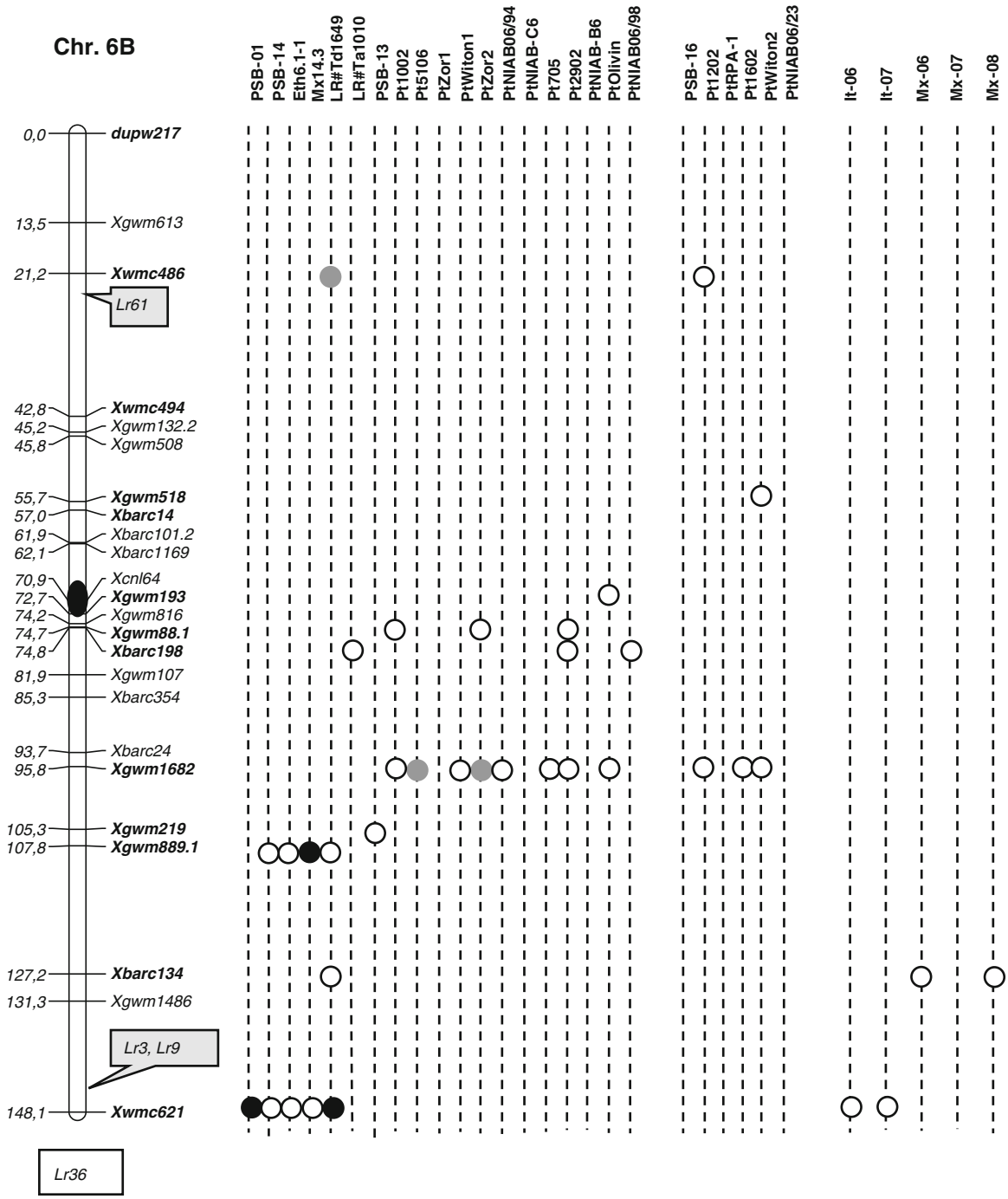

Fig. 3 continued

values, i.e. D' values > 0.6 and r^2 values > 0.30, which are sufficiently high to predict allelic variation at associated functional loci with good accuracy level (Kim et al. 2008), were consistently observed only within intermarker distances equal or less than 5 cM.

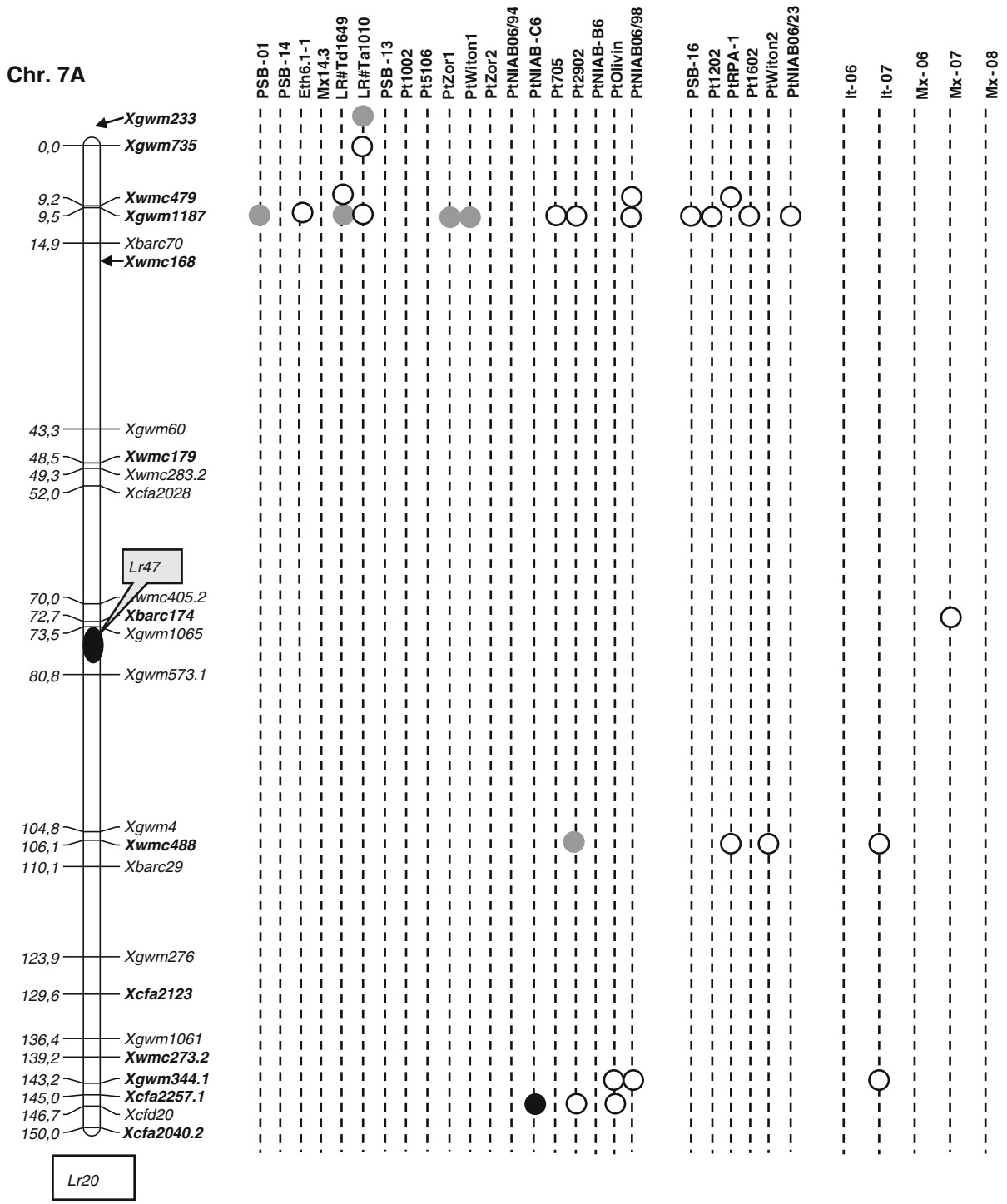

Fig. 3 continued

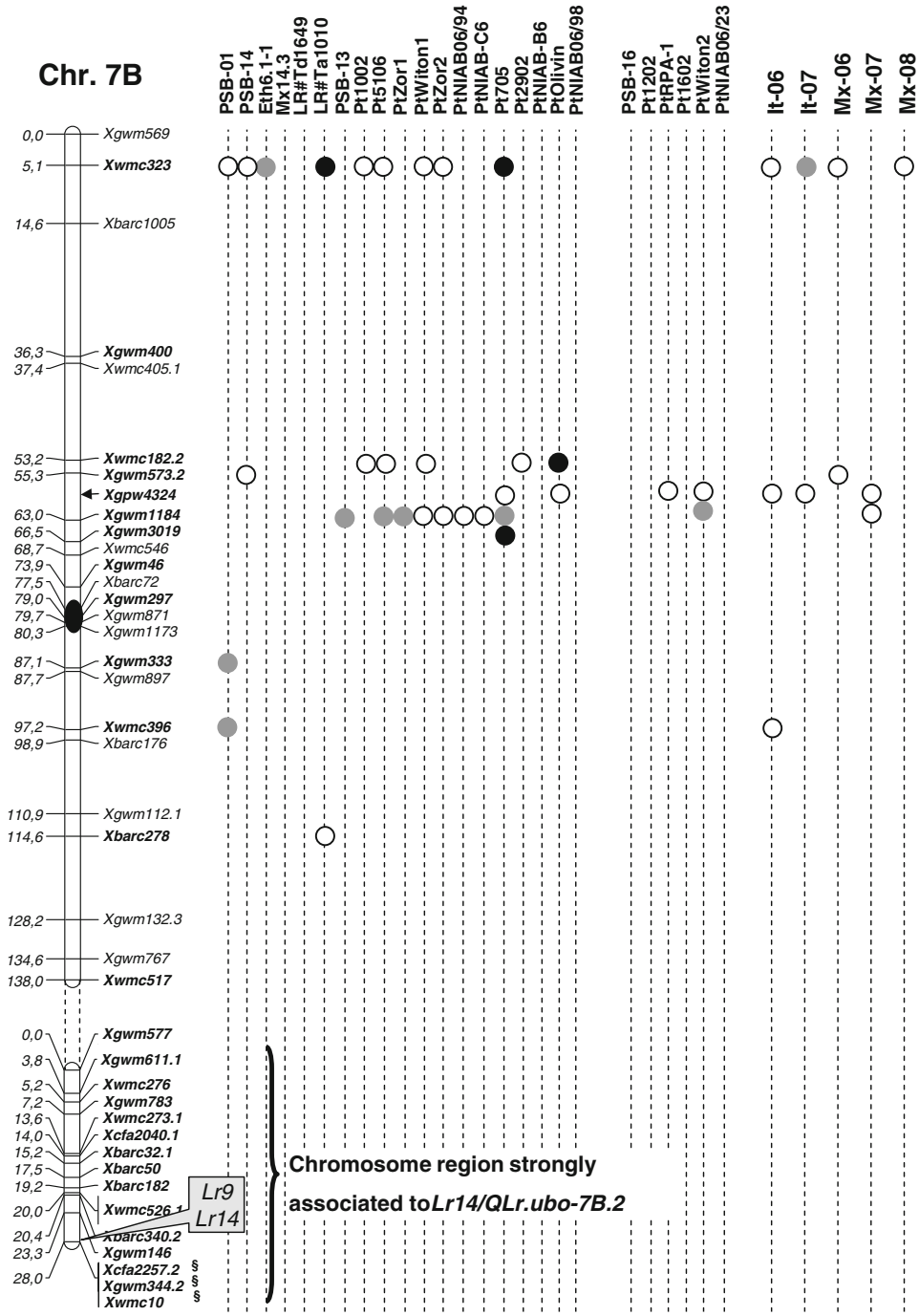
Chr. 7B
PSB-01
PSB-14
Eth6.1-1
Mx14.3
LR#Td1649
LR#Ta1010
PSB-13
Pt1002
Pt5106
PtZor1
PtWiton1
PtZor2
PtNIAB06/94
PtNIAB-C6
Pt705
Pt2902
PtNIAB-B6
PtOlivin
PtNIAB06/98
PSB-16
Pt1202
PtRPA-1
Pt1602
PtWiton2
PtNIAB06/23
It-06
It-07
Mx-06
Mx-07
Mx-08
0,0 Xgwm569
5,1 Xwmc323
14,6 Xbarc1005
36,3 Xgwm400
37,4 Xwmc405.1
53,2 Xwmc182.2
55,3 Xgwm573.2
Xgpw4324
63,0 Xgwm1184
66,5 Xgwm3019
68,7 Xwmc546
73,9 Xgwm46
77,5 Xbarc72
79,0 Xgwm297
79,7 Xgwm871
80,3 Xgwm1173
87,1 Xgwm333
87,7 Xgwm897
97,2 Xwmc396
98,9 Xbarc176
110,9 Xgwm112.1
114,6 Xbarc278
128,2 Xgwm132.3
134,6 Xgwm767
138,0 Xwmc517
0,0 Xgwm577
3,8 Xgwm611.1
5,2 Xwmc276
7,2 Xgwm783
13,6 Xwmc273.1
14,0 Xcfa2040.1
15,2 Xbarc32.1
17,5 Xbarc50
19,2 Xbarc182
20,0 Xwmc526.1
20,4 Xbarc340.2
23,3 Xgwm146
28,0 Xcfa2257.2 §
Xgwm344.2 §
Xwmc10 §
Lr9
Lr14
Chromosome region strongly
associated to Lr14/QLr.ubo-7B.2
§ Marker haplotype most significantly associated to Lr14

Fig. 3

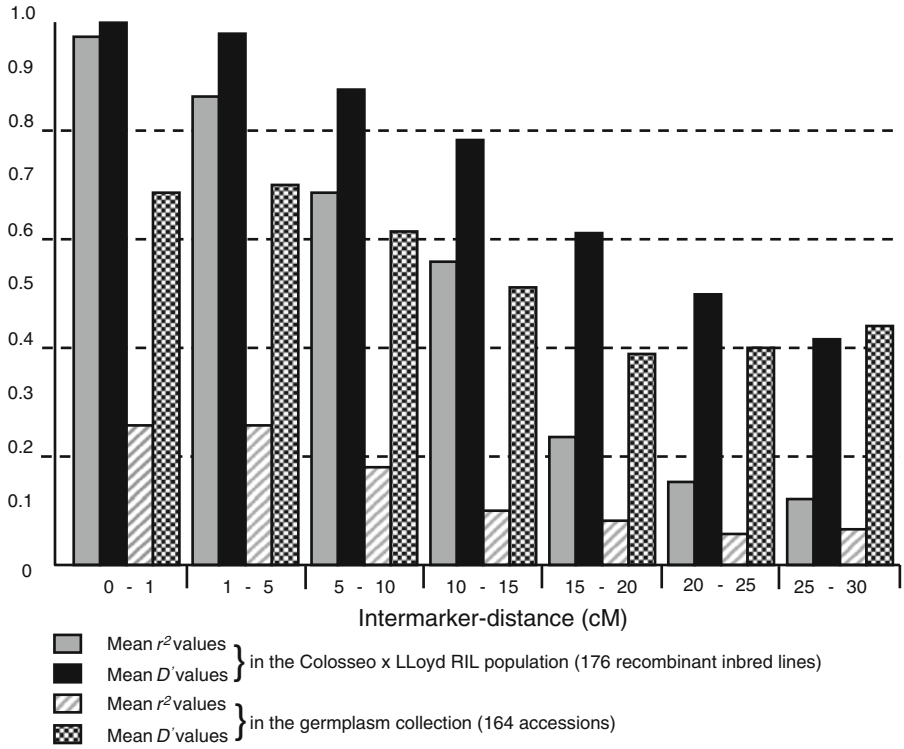

Fig. 4 Rate of LD decay based on the LD r^2 and D' values for the Colosseo × Lloyd RIL population (176 lines) and for the germplasm collection (164 accessions). LD values have been averaged for the seven classes of intermarker distances as reported in the diagram

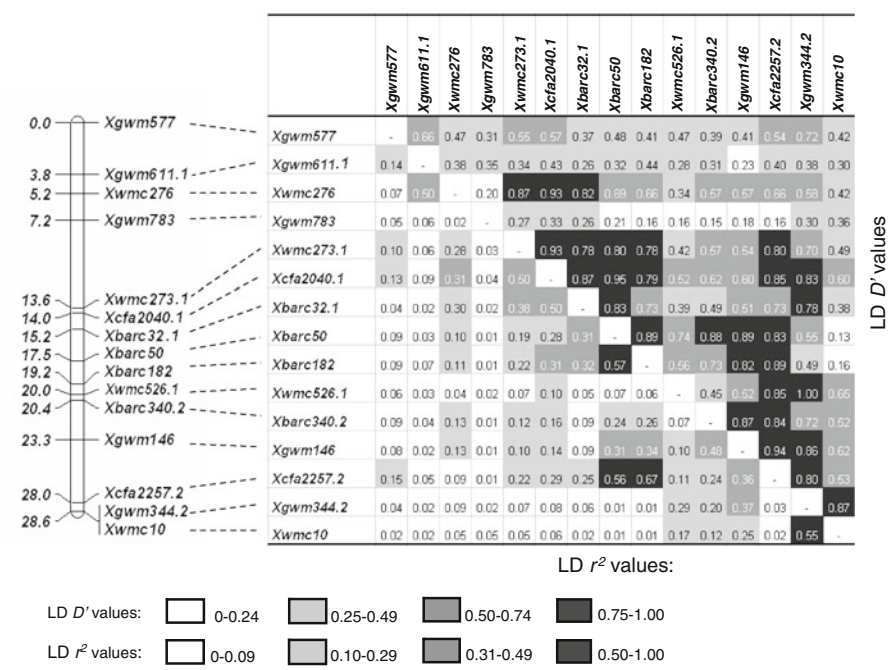

Fig. 5 Combined map of the loci used in the association mapping analysis on the distal region of chr. 7BL, harbouring *Lr14*/*QLr.ubo-7BL*. The LD D' and r^2 values are reported in the Table above and below the diagonal, respectively. The markers on the table have been connected to the map with *dashed lines*. In the table, *grey* shading has been used to differentiate different levels of LD values, with darkness associated to the highest LD levels

Table 2 Results of the single-marker association test based on the seedling infection Type (IT) responses of 164 elite durum accessions for the distal region of chr. 7BL carrying *Lr14*/*QLr.ubo-7B.2*. The germplasm collection was inoculated with 25 *Puccinia triticina* isolates collected from *T. durum* or *T. aestivum*. The results (*F* value and significance level as from general linear model, GLM) herein reported are referred only to those isolates (14 out of 25) that showed a clear avirulent phenotype on cvs. Creso and Colosseo, putatively carrying a resistant allele at *Lr14*, and a strong marker-phenotype association in the *Lr14* region. The map distances, the significance probability level and the *F* test values are reported for each marker

Marker	Map distance (cM)	PSB-01	PSB-14	Eth6.1-1	Mx14.3	LR#Td1649	PSB-13	Pt1002	PtWiton2	Pt5106	PtZor1	PtWiton1	PtZor2	NIAB06/94	NIAB/C6
Xgwm577	0.0	0.9NS	0.7NS	0.8NS	0.7NS	0.6NS	1.0NS	2.9*	0.8NS	2.3NS	1.1NS	0.9NS	0.4NS	0.3NS	0.4NS
Xgwm611.1	3.8	2.8*	3.1*	2.6*	5.7**	3.2*	0.7NS	0.2NS	1.1NS	0.8NS	0.6NS	0.4NS	0.5NS	0.4NS	0.6NS
Xwmc276	5.2	18.9***	9.3***	12.4***	8.7***	26.7***	3.3*	5.3**	5.6**	6.9***	7.2***	7.9***	7.9***	5.2**	3.1*
Xgwm783	7.2	1.1NS	1.9NS	3.1*	5.7**	5.2*	5.2*	2.8*	5.1**	3.5*	2.1NS	2.7*	3.0*	3.1*	1.3NS
Xwmc273.1	13.6	4.1**	3.4*	1.8NS	4.4*	4.4*	2.1NS	3.6*	3.8**	3.6*	4.8**	3.3*	6.2**	3.2*	2.6*
Xcfa2040.1	14.0	1.5NS	1.8NS	1.5NS	4.8**	5.7**	2.0NS	4.3**	4.6**	5.4**	5.9**	5.4***	9.0***	5.7**	2.0NS
Xbarc32.1	15.2	3.2*	1.2NS	2.6NS	2.7*	6.6**	2.2NS	3.1*	3.8*	2.0NS	4.0*	4.1**	4.9**	3.2*	4.5*
Xbarc50	17.5	0.5NS	0.3NS	1.3NS	0.1NS	1.1NS	0.3NS	0.0NS	1.2NS	2.1NS	0.1NS	3.3*	2.3NS	0.0NS	0.5NS
Xbarc182	19.2	2.7NS	0.6NS	1.6NS	0.3NS	2.1NS	0.3NS	1.2NS	0.2NS	0.0NS	0.4NS	0.7NS	0.1NS	0.3NS	0.6NS
Xwmc526.1	20.0	11.9***	7.9**	14.6***	8.9***	21.0***	3.5*	5.7**	4.0*	7.5**	3.6*	3.4*	3.4*	7.6***	4.8*
Xbarc340.2	20.4	22.1***	11.9***	7.4**	7.2***	27.6***	4.4*	2.9*	1.7NS	11.3***	6.9**	5.6**	4.6*	4.9**	2.0NS
Xgwm146	23.3	30.0***	14.3***	17.3***	11.2***	33.1***	6.3***	3.1*	2.6NS	10.7***	7.2***	6.9**	6.2**	7.4***	3.5*
Xcfa2257.2	28.0	3.4*	2.5NS	0.1NS	0.1NS	2.1NS	0.0NS	2.7NS	1.9NS	4.1*	6.9*	3.6**	1.6NS	1.9NS	5.9*
Xgwm344.2	28.6	47.5***	30.0***	43.3***	23.8***	66.6***	12.7***	7.1*	6.2**	21.8***	15.1***	14.5***	13.2***	16.2***	8.5**
Xwmc10	28.6	33.4***	20.2***	32.6***	27.1***	65.4***	10.9**	19.5***	11.5***	30.3***	24.2***	21.5***	34.3***	21.1***	6.6*

NS not significant

*, **, ***: significant at the *P* 0.05, 0.01 and 0.001, respectively

Table 3 Detailed results of the single-marker association test (general linear model, GLM) for the most significant markers (*Xgwm344.2* and *Xwmc10*) and the diagnostic *Xcfa2257.2-Xgwm344.2-Xwmc10* haplotypes in the *Lr14/QLr.ubo-7B.2* region based on the seedling infection Type (IT) responses of 164 elite durum accessions inoculated with 14 *Puccinia triticina* isolates virulent on Creso. Mean values of the allelic/haplotype variants, F and R^2 of the model and of the marker are reported

	Accessions (no.)	*Puccinia triticina* isolates													
		PSB-01	PSB-14	Eth6.1-1	Mx14.3	LR#Td1649	PSB-13	Pt1002	PtWiton2	Pt5106	PtZor1	PtWiton1	PtZor2	NIAB06/94	NIAB/C6
Xgwm344.2															
Allele $\boldsymbol{A_{120}}$	32	2.5	1.7	1.5	1.5	2.3	0.7	0.9	1.1	0.5	0.4	0.5	1.0	1.2	1.2
Allele $\boldsymbol{A}_{\text{null}}$	129	3.2	2.7	2.7	3.0	3.9	1.4	1.4	1.6	1.7	1.7	1.5	2.0	2.2	2.0
F-test		47.4	29.9	43.3	23.8	66.6	12.7	4.1	6.2	21.8	15.1	14.5	13.2	16.2	8.5
R^2 model		43.1	27.3	24.5	15.5	35.6	10.2	11.6	15.7	18.5	13.7	14.2	16.6	19.3	44.7
R^2 marker		17.4	14.2	21.1	13.2	27.8	7.4	4.1	3.4	11.5	8.4	8.1	7.2	8.5	7.5
Xwmc10															
Allele $\boldsymbol{A_{200}}$	41	2.6	1.9	1.8	1.6	2.5	0.8	0.8	1.0	0.6	0.4	0.5	0.8	1.3	1.4
Allele $\boldsymbol{A}_{\text{null}}$	123	3.3	2.7	2.7	3.0	3.9	1.4	1.5	1.7	1.8	1.8	1.6	2.2	2.3	2.0
F-test		33.3	20.2	32.7	27.1	65.4	10.9	19.5	11.5	30.3	24.2	21.5	34.3	21.1	6.6
R^2 model		36.8	22.1	20.1	17.0	35.1	9.2	18.0	17.1	22.3	18.3	17.5	25.6	20.9	90.3
R^2 marker		13.5	10.2	16.7	14.7	27.4	6.4	10.3	6.1	15.2	12.7	11.4	16.5	10.8	6.5
Xcfa2257.2-Xgwm344.2-Xwmc10															
Creso-haplotype	29	2.3	1.5	1.4	1.4	2.1	0.7	0.8	1.0	0.5	0.3	0.4	0.8	1.1	1.2
Recombinant haplotypes	113	3.2	2.7	2.7	3.0	3.9	1.3	1.3	1.5	1.6	1.5	1.5	2.0	2.2	1.8
Non-Creso haplotypes	23	3.3	2.8	2.9	2.6	4.0	1.5	1.6	1.8	2.3	2.4	1.5	2.4	2.3	2.6
F-test		30.0	19.5	25.7	14.2	45.2	5.5	7.7	4.3	13.9	10.6	9.6	11.2	11.4	5.9
R^2 model		45.4	30.5	27.5	17.6	41.6	9.2	15.8	16.4	21.4	16.7	16.5	20.8	22.1	9.6
R^2 marker		20.8	17.5	23.9	15.3	34.0	6.4	8.4	4.7	14.1	11.4	10.3	11.4	11.5	7.0

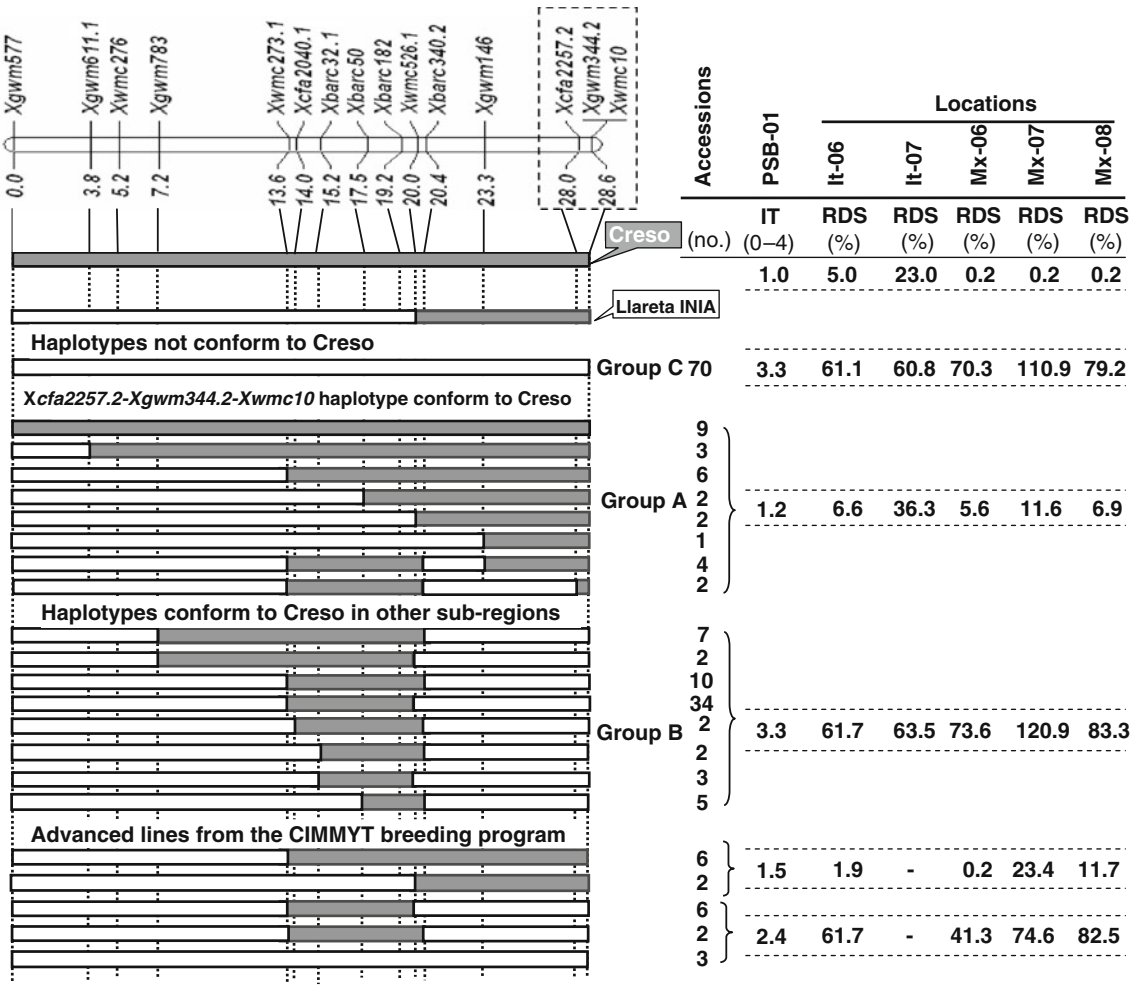

Fig. 6 Graphical genotypes of the haplotypes in the distal region of chr. 7BL, as observed in the germplasm collection of 164 elite accessions and in a set of 19 advanced lines from the CIMMYT breeding programme. Chromosome regions with a minimum of three (or more) adjacent markers showing allelic variants conform to Creso have been considered as 'Creso-related haplotypes' and are indicated with *grey-filled bars*. Chromosome regions unrelated to the Creso haplotype are represented by *white-filled bars*. For each haplotype, the corresponding number of accessions present in the germplasm collection is reported. Haplotypes of the 164 accessions are pooled in three groups: group A includes haplotypes conform to Creso at the most distal sub-region (*Xcfa2257.2-Xgwm344.2-Xwmc10*, putatively carrying *Lr14/QLr.ubo-7BL*), group B includes haplotypes conforming to Creso at sub-regions other than *Xcfa2257.2-Xgwm344.2-Xwmc10*, group C includes haplotypes not conforming to Creso at the distal region of chr. 7BL. Haplotypes of the additional 19 CIMMYT advanced lines are reported in the lower part of the figure. For each haplotype-group, mean values are reported for infection type (IT) at the seedling stage (isolate PSB-01) and relative disease severity index (RDS) in the five field trials carried out in Italy (It) and Mexico (Mx)

The distal region of chr. 7BL (24 cM, starting from *Xwmc276* up to *Xwmc10*) consistently showed highly significant (experiment-wise) associations to IT values of a number of isolates (PSB-01, PSB-14, Eth6.1-1, Mx14.3, LR#Td1649, PSB-13, Pt-1002, PtWiton2, Pt5106, PtZor1, PtWiton1, PtZor2, PtNIAB06/94 and PtNIAB/C06) avirulent on Creso/Colosseo and their derivatives. The remaining isolates LR#Ta1010, Pt705, Pt2902, PtOlivin, PtNIAB06/98 and PtNIAB/B06 showed avirulent interactions with Creso/Colosseo, but not with their derivatives; thus, no consistent experiment-wise association to IT values was detected in the germplasm collection.

Table 4 Detailed results of the single-marker association test (general linear model, GLM) for *Xgwm344.2* and the diagnostic *Xcfa2257.2-Xgwm344.2-Xwmc10* haplotype in the *Lr14/QLr.ubo-7B.2* genetic region based on the adult plant responses of 164 elite durum accessions evaluated in five field trials carried out in Italy (It) and Mexico (Mx) under artificial inoculation with *Puccinia triticina* isolates of different origin. Mean values of the allelic/haplotype variants, F and R^2 of the model and of the marker are reported

	Field trials[1]				
	It-06	It-07	Mx-06	Mx-07	Mx-08
Xgwm344.2					
AUDPC[2]					
Allele A_{120}	58	504	839	2934	707
Allele A_{null}	135	624	1839	4384	1217
F-test	66.9	12.1	72.8	110.4	91.9
R^2 model	43.8	19.8	35.3	55.3	49.3
R^2 marker	24.4	6.3	31.0	32.1	30.4
RDS[3]					
Allele A_{200}	20.1	75.5	17.3	52.2	47.6
Allele A_{null}	46.7	89.5	54.7	111.8	90.8
F-test	71.2	7.8	127.4	151.5	95.7
R^2 model	44.0	12.9	53.0	56.5	48.7
R^2 marker	25.9	4.4	39.4	42.9	32.1
Xcfa2257.2-Xgwm344.2-Xwmc10					
AUDPC					
Creso-haplotype	47	472	605	2694	569
Recombinant haplotypes	129	614	1745	4253	1163
Non-Creso haplotypes	145	660	1856	4589	1136
F-test	39.2	8.7	92.5	69.3	63.1
R^2 model	46.2	22.3	610.7	59.1	55.1
R^2 marker	27.0	8.7	47.2	36.3	36.6
RDS					
Creso-haplotype	15.6	71.4	7.3	38.2	36.6
Recombinant haplotypes	44.5	88.2	51.1	106.3	86.2
Non-Creso haplotypes	48.8	90.6	53.3	108.9	81.3
F-test	42.8	5.6	52.7	109.2	64.1
R^2 model	47.0	14.5	43.1	63.9	54.2
R^2 marker	29.1	6.1	39.0	50.5	37.9

[1] IT-06 and IT-07: trials carried out in Argelato, Italy in 2006 and 2007; Mx-06, Mx-07 and Mx-08: trials carried out in Mexico (Ciudad Obregon in 2006 and 2007 and El Batan in 2008)

[2] Area under the Disease Progress Curve

[3] Relative Disease Severity index

Significance of the marker-trait association tests always peaked on markers *Xgwm344.2* and/or *Xwmc10* (Table 2), which mapped in the most distal portion of the chr. region.

The drop in significance observed at *Xcfa2257.2* can be attributed to the fact that, at this marker, Creso is associated with a null allelic form. Thus, probably, the association analysis based on this single marker was biased by the presence of null alleles other than that tracing back to Creso. The analysis was then conducted based on two- and three-marker haplotypes (Meuwissen and Goddard 2000; Calus et al. 2009): peaks of association were obtained at marker haplotypes *Xwmc10-Xgwm344.2* (data not reported) and/or *Xcfa2257.2-Xgwm344.2-Xwmc10*, with somewhat higher R^2 values than those obtained from the single-marker analysis.

Based on *Xcfa2257.2-Xgwm344.2-Xwmc10*, the haplotype variant homogeneous to Creso predicted the leaf rust resistance phenotype with high accuracy (in terms of success rate) in the seedling tests carried out with the isolates avirulent on Creso/Colosseo (Table 3) and also in the field trials (Table 4). In total, 29 accessions had the *Xcfa2257.2-Xgwm344.2-Xwmc10* haplotype conform to that of Creso. Twenty-eight accessions out of the 29 showing the Creso molecular haplotype were resistant to leaf rust at the seedling and adult plant stages. Cv. Artena was the only accession that, while carrying the Creso haplotype at the three-marker region, showed a highly susceptible response, consistently observed in all the seedling and field trials. The detailed features (including the F-value, the model- and marker-R^2 values and the mean allelic values as from the GLM analysis) of the marker-traits associations for *Xgwm344.2*, *Xwmc10* and for the *Xcfa2257.2-Xgwm344.2-Xwmc10* haplotype are reported in Table 3 (seedling tests) and Table 4 (field trials). As compared to single markers, marker haplotypes improved the prediction of the presence of the resistant *Lr14/QLr.ubo-7B.2* allele in the germplasm accessions through the identification of the shared haplotypes most probably identical by descent to that of Creso.

Considering the distal chr. 7BL region from *Xgwm577* to *Xwmc10*, the unbroken Creso-haplotype present in the 29 accessions extended over a range of distances (Fig. 6, group A). In the direct derivatives of Creso such as Arcangelo, Colosseo, Italo, Plinio and

Radioso (Maccaferri et al. 2003, 2007), the haplotype spanned the entire distal region from *Xgwm577* to *Xwmc10*. Among the ICARDA materials, Bicre (Bittern x Creso) showed a complete Creso haplotype from *Xgwm611.1* to *Xwmc10*, while the Bicre derivatives and other accessions with indirect pedigree relationships to Creso showed a shorter haplotype common to Creso, often with recombination after *Xwmc273.1*. The common feature necessary to be associated to the Creso-IT pattern of resistance was the presence of the shared haplotype at *Xcfa2257.2-Xgwm344.2-Xwmc10*.

A number of accessions (65 in total) displayed haplotype patterns conform to that of Creso in the middle portion (within *Xgwm783* and *Xbarc340.2*) of the distal chr. 7BL region, but these haplotypes were not associated to a resistant response to leaf rust, as indicated by their mean phenotypic values (Fig. 6, group B).

In order to check for the presence of identity by descent (IBD) between Creso and the materials carrying *Lr14a*, cv. Llareta INIA (as previously described by Herrera-Foessel et al. 2008a) and an additional set of 19 advanced lines recently obtained at the CIMMYT breeding programme and somewhat related to Llareta INIA and/or Somateria (both of which carry *Lr14a*) were profiled with the same SSR set. Llareta INIA shared with Creso an unbroken haplotype from *Xwmc526* to *Xwmc10* (six SSR loci spanning 8.6 cM), suggesting that IBD between these two genotypes is likely present in the chr. region that harbours *Lr14*. The additional CIMMYT lines were useful to further confirm that *Lr14* is most probably located in the distal chr. 7BL region; in fact, these genotypes showed a number of haplotypes in the relevant chr. region (see lower part of Fig. 6). Eight lines out of 19 displayed the *Lr14/QLr.ubo-7B.2* haplotype (from *Xwmc273.1*/*Xwmc526*, depending on the line, to *Xwmc10*) and showed a clear resistance pattern as pointed out by the seedling test and the field experiment mean values (Fig. 6). In eight additional lines the graphical genotypes showed the presence of haplotypes shared with Creso only in the proximal portion (comprised between *Xwmc273.1* and *Xbarc182*) of the region, while being unrelated to both Creso and Llareta INIA in the terminal portion of the chr. region; most of them (with the exception of two lines) showed susceptibility to leaf rust; a similar phenotype was also evidenced by the three lines with haplotypes completely different from Creso.

Discussion

Leaf rust response at the seedling stage and adult stage in the field and its association with mapped molecular markers

Extensive surveys and genetic studies for the identification and mapping of leaf rust (*Puccinia triticina* Eriks.) resistance genes in *Triticum durum* have been undertaken only recently (Herrera-Foessel et al. 2005, 2007b, 2008a; Martinez et al. 2007). It is known that the majority of the resistance genes which have been identified and mapped in hexaploid wheat originate from the *T. aestivum* germplasm itself and/or were introgressed from wild relatives and rye (Bolton et al. 2008). This notwithstanding, the durum germplasm and the close relatives *T. dicoccum* and *T. dicoccoides* could harbour potentially useful genes/QTLs for complete, race-specific or partial resistance.

In the present study, we assessed the leaf rust response of a germplasm collection including 164 elite durum wheat accessions chosen from a larger pool of accessions and suitable for association mapping analysis and allele mining (Maccaferri et al. 2006) that are part of a larger germplasm collection. The collection mostly includes durum cultivars or breeding lines adapted to the Mediterranean Basin, the most important region for durum production worldwide. The collection was challenged with leaf rust isolates characterised by a wide virulence spectrum at both seedling stage, under controlled conditions in the greenhouse, and adult plant stage, in several locations under field conditions. These elements make the results of the present study highly relevant to practical durum wheat breeding at the regional and even at the global level.

The seedling response data of the accessions, corresponding to the avirulence/virulence phenotypes of the isolates, have been used to study the pattern of diversity among the 25 *Puccinia triticina* isolates used herein. Cluster analysis of the isolates based on these phenotypic data showed that the response pattern of the isolates overlapped only marginally, suggesting that the phenotypic data-set does not contain redundancy that could bias the interpretation of the results. The clear distinction between the phenotypes of the isolates collected from *T. durum vs.* those collected from *T. aestivum* and triticale was not unexpected, based on previously published pathogenicity studies

(Ordoñez and Kolmer 2007) and our results (Mantovani 2009; Mantovani et al. 2009). Exceptions are *i*) the LR#Ta1010 isolate from Israel, collected from hexaploid wheat but showing a virulence pattern related to that of LR#Td1649, a durum wheat isolate also collected in Israel and correctly included into the group of durum isolates, and *ii*) the Italian durum PSB-13 isolate, with a phenotypic virulence pattern close to those of the hexaploid wheat isolates (results supported by molecular observations based on SSR markers; Mantovani et al. 2009).

The collection includes a number of accessions related to the resistant cultivar Creso, which harbours *QLr.ubo-7B.2* [originally detected by Maccaferri et al. (2008a) and subsequently described also by Marone et al. (2009)], a widely effective major gene for leaf rust resistance assigned to the chr. 7BL distal region where *Lr14* has also been mapped (Herrera-Foessel et al. 2008a). This allowed us to evaluate the leaf rust isolates based on their avirulence/virulence pattern *vs.* the group of Creso-related accessions. Among the durum isolates, only one (the Italian PSB-16) was able to clearly overcome the *Lr14*/*QLr.ubo-7B.2* resistance, while up to five hexaploid wheat isolates from Central and Northern Europe showed clear virulence patterns. This was not unexpected, given that the effectiveness of *Lr14* is known to be low in hexaploid wheat (McIntosh et al. 1995). However, the Creso-derived resistance in durum wheat has lasted since 1974 throughout the Mediterranean Basin from Spain to Italy and Syria (Pasquini and Casulli 1993; Nachit 2000; Martinez et al. 2007) as well as in the North American durum wheat growing areas of Mexico (Amaro et al. 2007). Additionally, the dendrogram of the accessions based on the seedling response showed that a substantial phenotypic diversity for leaf rust response is present within the group of accessions which carry the *Lr14*/*QLr.ubo-7B.2* allele for leaf rust resistance. These results suggest that breeding activities led to the combination of the major *Lr14*/*QLr.ubo-7B.2* gene with other, likely unknown loci effective at the seedling stage (resistance genes and/or suppressors) that contribute to diversify the leaf rust response of the accessions tested herein.

In general, durum wheat accessions carrying at least one hypersensitive resistance gene are relatively frequent in this germplasm collection. This was also observed by Martinez et al. (2007) in a smaller sample of 41 (mostly Spanish) durum wheat cultivars. At least one or two additional major *Lr* genes controlling hypersensitive response, in addition to *Lr14*/*QLr.ubo-7B.2*, are present with high frequency in our germplasm collection (as recognised by avirulence of PSB-16 and other bread wheat isolates). However, even if the map position of these genes has been most probably identified through the present whole-genome association survey on chrs. 2A and 2B, as discussed hereafter, their effectiveness appears limited to the rare PSB-16 durum isolate and to the bread wheat isolates, with null or limited effects on representative durum isolates and, consequently, on leaf rust resistance in the field. Thus these genes alone, while not providing sufficient protection against the durum-specific races, effectively protected durum wheat against most bread wheat races. The further characterisation and identification of these genes may still be interesting for durum wheat breeding, especially in areas where both durum and bread wheat are cultivated and thus co-existing with their corresponding leaf rust races. This is the case of most wheat-growing areas around the Mediterranean Basin. Interestingly, the position of the block of significant markers (*Xgwm410.1, Xgwm148* and *Xbarc183.1*) found in the proximal region of chr. 2BS coincides with that reported for *Lr13, Lr23* and *Lr48,* three known resistance genes (Nelson et al. 1997; Seyfarth et al. 2000; Bansal et al. 2008). It is to underline that *Lr23*, which originated from the *T. durum* Gaza (McIntosh and Dyck 1975), is known to be present in some of the accessions not carrying the resistance haplotype at the chr. 7BL relevant markers, such as Gallareta (= Altar 84) from the CIMMYT breeding programme (Nelson et al. 1997).

More importantly, a few accessions showed resistance to multiple isolates without carrying *Lr14*/*QLr.ubo-7B.2*. Additional multiple-isolate comparative tests with the known bread and durum wheat differential stocks and development of specific bi-parental mapping populations are advisable to further investigate the genetic basis of these effective resistances characterised by rare response patterns in the collection tested in our study.

The field response observations carried out in two to three consecutive years in different

environments (Northern Italy and Mexico) under artificial inoculations with different durum wheat leaf rust races (mixture of durum wheat isolates in Italy and purified BBG/BN or BBG/BP in Mexico) highlighted the effectiveness (and, therefore, the breeding value) of the field resistance conferred by the *Lr14/QLr.ubo-7B.2* gene, as evidenced by the very low AUDPC and RDS values shown by the group of accessions related to Creso, while very few accessions not carrying the *Lr14/QLr.ubo-7B.2* gene exhibited high and consistent (across locations and years) field resistance (i.e. RDS $\leq$ 10%). This finding may appear to be inconsistent with the observations made at the seedling stage, with the PSB-16 isolate being virulent on the *Lr14/QLr.ubo-7B.2* resistant genotypes. It is to note that even the *Lr14/QLr.ubo-7B.2* resistance partially failed to confer satisfactory resistance levels under high disease pressure field conditions as in the case of the trial carried out in Italy (2007), where a mixture of isolates including the PSB-16 isolate was used. However, even under such conditions, a few rare sources of resistance were found to be effective in the germplasm collection. Most probably, the genetics of such valuable resistances could not be mapped via association mapping due to the rare frequency of such allelic variants in the germplasm collection, and thus dedicated bi-parental mapping populations should be developed to further characterise and map these genes.

It has been recently shown that good levels of field resistance can be attained through the accumulation of non-race specific minor genes expressed at adult plant stage by visual selection in segregating populations obtained by crossing lines carrying different minor genes for resistance (Herrera-Foessel et al. 2008c). In this context, the identification of markers with significant, albeit small, effects on field resistance, while not influencing seedling response, may highlight the location of minor resistance genes and possibly their marker-assisted accumulation in breeding programmes. Single markers or small linkage blocks of two to three linked markers evidencing such association patterns were found in the present research (e.g. chr. 1AL near *Xbarc287*, chr. 3B near *Xgwm685*). Further studies, possibly involving sets of near isogenic stocks in diverse genetic backgrounds, are thus required to more appropriately evaluate their association to minor genes.

Further dissection of the major *Lr14/QLr.ubo-7B.2* leaf rust resistance factor and other resistance factors through association mapping

Due to the relevance of the resistance factor identified in the chr. 7BL distal region in different durum wheat genetic backgrounds, e.g. the Italian founder cv. Creso (Martinez et al. 2007) and the Chilean durum Llareta INIA (Herrera-Foessel et al. 2008a), fine mapping of this genetic factor is warranted. In this study, a targeted molecular profiling of the chr. 7BL distal region in a germplasm collection suitable for association mapping has been deployed to further dissect and map the major genetic factor underlying the *Lr14/QLr.ubo-7B.2* resistance. Based on the genetic profiling results of a set of 15 SSR markers spanning the target region at a marker density of ca. 1.8 cM per marker, two main results are discussed hereafter.

In terms of mapping resolution, the use of this set of markers allowed us to more precisely estimate the LD decay rate (and, consequently, the genetic resolution of the mapping study) that characterise the durum germplasm collection herein considered as compared to the genetic resolution that can be attained with a traditional RIL population like the one originally used to map *QLr.ubo-7B.2* (Colosseo $\times$ Lloyd population, Maccaferri et al. 2008a). The observed LD decay rate (decay of LD within 5, maximum 10 cM, at a rate twice as high as that observed in the RIL population) is typical of an elite germplasm collection of a self-pollinated species like wheat (Breseghello and Sorrells 2006b; Maccaferri et al. 2006; Chao et al. 2007; Somers et al. 2007). While the observed LD values should be categorised as belonging to the 'long-range' LD patterns, these values allow further improvement of the resolution of the mapping analysis carried out with traditional bi-parental mapping populations. Controlling for major population structure effects and 'normalising' the panel by excluding highly related entries allowed us to further improve the mapping resolution through association mapping (Breseghello and Sorrells 2006a).

The association results based on both seedling and field data pointed out that the most probable location of the major gene is unequivocally positioned in the most distal fraction of the linkage group, in a ca. 2.5 cM interval tagged by the SSR markers *Xcfa2257.2*, *Xgwm344.2* and *Xwmc10* or distal to them. Therefore, our results strengthened those obtained by the previous

mapping studies while increasing the genetic resolution of *Lr14*/*QLr.ubo-7B.2*.

In terms of haplotype analysis, the study of the chr. 7BL region proved to be very useful to estimate the extent of the haplotype IBD between the founder cultivar Creso, the cultivar Llareta INIA carrying *Lr14*, and the accessions related at various level to Creso considered in our study. Microsatellite markers are known to be very useful in haplotype mapping and IBD prediction (Meuwissen and Goddard 2000; Holland 2007; Maccaferri et al. 2007; Calus et al. 2009). Creso and Llareta INIA shared alleles at six SSR markers spanning 8.6 cM in the region significantly associated to leaf rust resistance. Considering the higher mutation rate and allelic variation of SSR markers as compared to other bi-allelic marker classes (such as RFLPs, AFLPs and, more in general, SNPs), this result could be considered as strong proof of IBD (Grapes et al. 2006). As from the pedigree records, Creso and Llareta INIA share parents from the early CIMMYT breeding programme (first generation of semi-dwarf germplasm developed in the 1960s, tracing back all the way to the source of dwarfing genes, namely the bread wheat Pitic 62, which also carries *Lr 14a*). Furthermore, in our haplotype survey, the analysis based on a three-marker sliding window gave the best results. In fact, checking for the presence of the three-marker haplotype conforming to that of Creso in the most significant region (*Xcfa2257.2*/*Xgwm344.2*/*Xwmc10*) proved to predict with high accuracy (96% of success rate) the leaf rust resistance phenotype at the seedling and adult plant stages in the germplasm collection herein considered.

From a breeding standpoint, the use of haplotype genotyping is generally thought to be more reliable and efficient to track the presence of favourable alleles in germplasm collections and in breeding populations (Hospital and Charcosset 1997; Peleman and van der Voort 2003; Schrag et al. 2009; Villumsen et al. 2009). Once SNP platforms also become available for wheat (Ganal and Röder 2007, Akhunov et al. 2009), haplotyping and marker-assisted selection at the chr. 7BL and other target regions will be greatly facilitated.

Unlike to what stated by McIntosh (2009), the possibility that *QLr.ubo-7B.2* and *Lr14* are the same locus was never dismissed by Maccaferri et al. (2008a). The results of the present molecular comparison between Llareta INIA and Creso strongly support this hypothesis; in fact, sharing of microsatellite alleles at six loci spanning ca. 10 cM strongly suggests identity by descent. However, it is known that resistance loci are hypervariable, leaving open the possibility that different functional alleles could have recently diverged at the *Lr14*/*QLr.ubo-7B.2* locus.

Analogously to what recently found by Marone et al. (2009), i.e. that an isolate from Spain differentiated *Lr14a* from the resistant *QLr.ubo-7B.2* allele carried by Creso (designated as *Lr14c*), also our seedling tests evidenced a differential response between Creso/Colosseo and Thatcher-*Lr14a* with nine bread and one durum wheat isolates. Allelism test is underway to provide further data to unequivocally resolve this issue (K. Ammar et al. personal communication).

Ultimately, the cloning and sequencing of *QLr.ubo-7B.2* and *Lr14* and their regulatory upstream sequences will provide the final clue as to the allelism of these two genetic factors. More importantly, knowing that Creso has been in cultivation since 1974 and still maintains its resistance in most durum wheat areas worldwide, sequencing of *QLr.ubo-7B.2* region may provide very interesting clues as to the molecular basis of the durability of those very few major genes that have lasted so long. To date, only a few major genes for rust (and powdery mildew) resistance have been cloned, mostly through positional cloning approaches. Cloning additional resistance genes should be considered as a major target in wheat disease resistance genetics (Keller et al. 2005, 2007); in fact, besides shedding light on the physiology of plant-pathogen interaction, cloning resistance genes offers great opportunities for more targeted applications, such as the identification of new allelic variants present in diverse germplasm accessions and the exploitation of genetic engineering (Salvi and Tuberosa 2005; Varshney et al. 2007; Krattinger et al. 2009; Qiu et al. 2009). Association mapping studies conducted in durum germplasm collections substantially larger than the one considered herein and possibly including accessions exploring a wider range of genetic diversity, such as unrelated durum landrace-selections and *T. dicoccum* accessions, will improve considerably the genetic resolution at target loci. As an example, association mapping integrated to allele mining at candidate genes and eco-geographical sampling information catalogued at the germplasm repositories is an interesting approach (Focused Identification of Germplasm Strategy,

FIGS), which has been recently applied to another resistance gene (*Pm3*), which has already been positionally cloned (Kaur et al. 2008).

All the information pertaining to *Lr14*/*QLr.ubo-7B.2* presented and discussed above provides a reliable molecular tool to identify the presence of this important resistant factor in breeding materials. However, from a strategic breeding perspective, it is imperative that this tool is used judiciously towards the diversification of the genetic basis of the resistance in durum wheat breeding programmes, rather than towards the perpetuation of a worrying dependence on this factor. As to this last point, it is important to emphasize that one of the main results of the present study involving a wide germplasm collection was the demonstration that most of the resistant genotypes carry the *Lr14*/*QLr.ubo-7B.2* factor. When dealing with a fast-evolving pathogen such as leaf rust, this overdependence on a single major resistance gene is rather worrying. The wide presence of the *Lr14*/*QLr.ubo-7B.2* resistance factor in the durum germplasm can be due to the utilisation in breeding programmes of resistant cultivars often not well characterised in terms of the genetic factor/s underpinning their resistance. Conversely, this could also be explained by the lack of differential lines specifically developed in durum wheat and the paucity of molecular markers tightly linked to the resistance factors. The achievements of recent studies (Herrera-Foessel et al. 2008a; Maccaferri et al. 2008a; Marone et al. 2009) in terms of molecular markers associated with *Lr14a*/*QLr.ubo-7B.2* made it possible to perform, during 2009, the molecular analysis (using a combination of *Xgwm344.2* and *Xgwm146* markers) of leaf-rust resistant advanced breeding lines from the CIMMYT breeding programme. Out of the 1,328 lines that were tested, 1,264 (95%) carried the chromosomal region associated with this genetic factor. The same analysis on a smaller set (119 genotypes) of recently developed breeding lines (all resistant to leaf rust) from the ICARDA programme indicated that 85% of them carried the *Lr14*/*QLr.ubo-7B.2* factor (K. Ammar and S. Dreisigacker, unpublished results). Furthermore, the durability of a major resistance gene, no matter how long it lasted, is no guarantee of future usefulness as clearly demonstrated by the breakdown of the durum resistance of CIMMYT's germplasm to leaf rust in Mexico after being protected for close to 20 years by a single, not yet characterised, major gene (Singh et al. 2004). In light of these results, the enhanced detection capabilities for the *Lr14*/*QLr.ubo-7B.2* factor described herein should be employed by breeders to avoid as much as possible (1) its further deployment as the only protection factor to develop new cultivars and (2) its use in crossing schemes except if combined with other effective resistance genes that can be pyramided via marker-assisted selection.

Conclusions

The characterisation of a germplasm collection of durum wheat suitable for association mapping allowed us to: (1) investigate the genetic variation for leaf rust resistance in a selected reference sample of elite durum germplasm; (2) efficiently map known and new relevant major loci for seedling and adult plant resistance as well as new minor QTLs at the adult stage in the field; (3) further refine the map position of known genes, in particular the region containing *Lr14*/*QLr.ubo-7B.2*, both of which have shown major effects on leaf rust resistance; (4) identify the presence of valuable resistant haplotypes (e.g. at the *Lr14*/*QLr.ubo-7B.2* region) in improved elite accessions by exploiting identity by descent, thus retrieving information useful for breeding activities.

The valuable results obtained for *Lr14*/*QLr.ubo-7B.2* are likely due to the concomitant occurrence in this germplasm collection of two major prerequisites for association mapping analysis: (1) the presence in rather balanced and non-rare frequencies of functional allelic variants with large effects at the targeted region; in fact, through haplotype inspection, it has been shown that the Creso-related haplotype, associated to the resistant allele, is present in the germplasm collection with a relatively high frequency and is widespread in different genetic backgrounds; (2) the relatively low frequency of other resistance genes concomitantly affecting the seedling response to the isolates informative for *Lr14* and, particularly, the adult plant response in the field, a circumstance that facilitated the correct phenotypic classification of the accessions and improved the effectiveness of the marker-phenotype association analysis.

Our association mapping analysis revealed that the major gene/gene cluster present in the *Lr14*/*QLr.ubo-7B.2* region is the most important source of leaf rust resistance currently exploited by durum wheat

breeders, as shown by the presence of the Creso/Llareta haplotype in a number of resistant accessions from the Italian, CIMMYT and ICARDA breeding programmes.

Fine mapping of *QLr.ubo-7B.2* at the sub-cM level is presently underway using a large $F_{2:4}$ mapping population and synteny information. Eventually, this effort may lead to the cloning of *QLr.ubo-7B.2* and the verification of its allelism with *Lr14*.

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