Theor Appl Genet (2015) 128:501–516
DOI 10.1007/s00122-014-2448-y

ORIGINAL PAPER

Multi-environment multi-QTL association mapping identifies disease resistance QTL in barley germplasm from Latin America

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Received: 15 October 2014 / Accepted: 17 December 2014 / Published online: 30 December 2014

Abstract

Key message **Multi-environment multi-QTL mixed models were used in a GWAS context to identify QTL for disease resistance. The use of mega-environments aided the interpretation of environment-specific and general QTL.**

Abstract Diseases represent a major constraint for barley (*Hordeum vulgare* L.) production in Latin America. Spot blotch (caused by *Cochliobolus sativus*), stripe rust (caused by *Puccinia striiformis* f.sp. *hordei*) and leaf rust (caused by *Puccinia hordei*) are three of the most important diseases that affect the crop in the region. Since fungicide application is not an economically or environmentally sound solution, the development of durably resistant varieties is a priority for breeding programs. Therefore, new resistance sources are needed. The objective of this work was to detect genomic regions associated with field level plant resistance to spot blotch, stripe rust, and leaf rust in Latin American germplasm. Disease severities measured in multi-environment trials across the Americas and 1,096 SNPs in a population of 360 genotypes were used to identify genomic regions associated with disease resistance. Optimized experimental design and spatial modeling were used in each trial to estimate genotypic means. Genome-Wide Association Mapping (GWAS) in each environment was used to detect Quantitative Trait Loci (QTL). All

Communicated by Xiaoquan Qi.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-014-2448-y) contains supplementary material, which is available to authorized users.

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significant environment-specific QTL were subsequently included in a multi-environment-multi-QTL (MEMQ) model. Geographical origin and inflorescence type were the main determinants of population structure. Spot blotch severity was low to intermediate while leaf and stripe rust severity was high in all environments. Mega-environments were defined by locations for spot blotch and leaf rust. Significant marker-trait associations for spot blotch (9 QTL), leaf (6 QTL) and stripe rust (7 QTL) and both global and environment-specific QTL were detected that will be useful for future breeding efforts.

Abbreviations

AMMI	Additive main effect and multiplicative interaction model
BOPA	Barley oligonucleotide pool assays
CAN_LAN	Lacombe Research Center (Alberta, Canada)
ECU_PIC	"Santa Catalina" Experimental Research Station in Pichincha of the National Agricultural Research Center (INIAP, Ecuador)
ECU_TOL	"Granja Tolilla" Experimental Research Station of the National Agricultural Research Center (INIAP, Ecuador)
GxE	Genotype-by-environment Interaction
GS	Genomic selection
GWAS	Genome-wide association mapping
ME	Mega-environment
MEMQ	Multi-environment multi-QTL model
MEX_TOL	"Toluca" experimental research station of the International Center for Maize and Wheat Improvement (CIMMYT-Toluca, Mexico)
PER_AND	"Andenes" experimental research station of the National Center for Innovation in Agriculture (INIA-Andenes, Cusco, Peru)
PER_COM	"Combapata" experimental research station of the National Center for Innovation in Agriculture (INIA-Combapata, Cusco, Peru)
QEI	QTL-by-environment interaction
QTL	Quantitative trait loci
URU_LE	"La Estanzuela" experimental research station of the National Agricultural Research Institute (INIA-EELE, Colonia, Uruguay)
URU_MC	"Dr. Mario A. Cassinoni" experimental station of the Universidad de la República (UDELAR-EEMAC, Paysandu, Uruguay)

Introduction

Barley (*Hordeum vulgare*, L.) is the fourth most important cereal crop in the world in terms of total production (FAOSTAT 2008; http://faostat.fao.org/site/339/default.aspx, verified 04-21-2014). Fungal diseases are the principal limitation for achieving high grain yields of barley in Latin America (Germán 2007). These diseases affect barley production directly (i.e., reducing grain weight and germination), and indirectly (i.e., reducing photosynthesis, and therefore yield; Nutter et al. 1985). Spot blotch (caused by *Cochliobolus sativus*), stripe rust (caused by *Puccinia striiformis* f.sp. *hordei*) and leaf rust (caused by *Puccinia hordei*) are three of the most important diseases that affect the crop in Latin America (Pereyra 1996). Since fungicide application is not an economically and environmentally sound solution, the development of durable resistant varieties is a high priority for breeding programs. Breeding for resistance would be most effective when based on an extensive catalog of mapped resistance genes/QTL.

Genome-wide association scanning (GWAS) can be used to detect genomic regions associated with target traits (Jannink et al. 2001) and thus provide targets for marker-assisted selection (MAS) in relevant germplasm (Mather et al. 1997). Advantages of GWAS, as compared to bi-parental QTL mapping, include: assessment of genetically diverse germplasm stocks; higher mapping resolution; effective use of historical data; and immediate applicability to cultivar development because the genetic background in which QTL are estimated is directly relevant for plant breeding (Kraakman et al. 2004). A number of reports confirm the utility of GWAS for the improvement of self-pollinated crops, including barley (Kraakman et al. 2004, 2006; Hayes and Szücs 2006; Stracke et al. 2009; Waugh et al. 2009; Roy et al. 2010; Bradbury et al. 2011; von Zitzewitz et al. 2011; Gutierrez et al. 2011). However, very little work has been conducted using data from multi-environment trials (MET) for GWAS.

The complex traits that are targets for GWAS typically show genotype by environment interaction (GxE) (Mathews et al. 2008). Mixed models have been used in balanced populations to detect QTL-by-environment (QEI) effects while modeling the variance–covariance matrix (Piepho 2000; Verbyla et al. 2003; Malosetti et al. 2004; van Eeuwijk et al. 2005; Boer et al. 2007; Mathews et al. 2008). Additionally, QEI may be interpreted within mega-environments (Palomeque et al. 2009). The objective of this work was to detect genomic regions associated with field resistance to spot blotch, stripe rust and leaf rust in Latin American germplasm based on the evaluation of these diseases in multiple environments and explicitly modeling the variance–covariance matrix across environments.

Materials and methods

Plant material and phenotypes

A total of 360 advanced inbred lines and cultivars from national breeding programs and the ICARDA-CIMMYT

program were used. Each genotype was evaluated for disease resistance in several environments (Table 1). However, due to seed availability and missing data generated in the field, not all of the environments contained the 360 lines, and approximately, 280 lines were used in each environment (Tables 2, 3, 4). Standard fertilization and weeding procedures were used in all experiments.

Spot blotch severity was evaluated in eight environments: URU_MC (northern Uruguay) in 2009, 2010, 2011, and 2012; URU_LE (south west Uruguay) in 2009, 2010, and 2011; and CAN (Alberta, Canada) in 2011 (Tables 1, 2). URU_MC consisted of row-plots planted on an augmented unreplicated design with 20 % of repeated systematic checks in 2009 and 2010, and 20 % of repeated checks in an augmented Federer's experimental design (Federer 1961) in 2011 and 2012. Four check-lines were used in these experiments. Two planting dates were used every year to maximize the chances of disease development; in 2009 and 2010 spot blotch symptoms occurred at only one of the planting dates, while in 2011 symptoms were observed, with varying levels of infection at each of the two planting dates. Therefore, data from the latter was analyzed as two distinct environments. Plots were 1 m long and two rows wide (0.20 m between rows). Inoculation was performed by spreading *C. sativus*-inoculated sorghum seed into the plots during stem elongation stage [Zadoks growth stage (ZGS) 31; Zadoks et al. 1974]. Severity of spot blotch infection was evaluated during grain milk stage (ZGS 70, Zadoks et al. 1974) as percentage of diseased area from 0 to 100 % in 2011 and on a 0–9 scale (Saari and Prescott 1975) in 2009, 2010, and 2012. URU_LE consisted of hill-plots planted on an irrigated summer nursery with augmented unreplicated designs with 10 % repeated systematic checks in 2009 and 2010, and 15 % repeated checks in a Federer's experimental design (Federer 1961) in 2011. Two susceptible check-lines were used throughout these experiments. Hill-plots with 15–20 seeds per plot were used. Artificial inoculation with inoculum produced from a mix of eight *C. sativus* monosporic isolates obtained from different years, cultivars, and locations with at least one isolate of known high aggressiveness were used (8×10^3 conidia/mL). At least one spray inoculation was made after stem elongation stage (ZGS 31; Zadoks et al. 1974) and repeated inoculations were performed as needed until flowering stage (ZGS 60, Zadoks et al. 1974). Spot blotch severity was assessed during grain milk stage (ZGS 70, Zadoks et al. 1974) as percentage of diseased leaf area from 0 to 100 %. CAN_LAN consisted of hill-plots planted in augmented resoluble incomplete block designs (Patterson and Williams 1976) with two true replications at Lacombe Research Center, Alberta, Canada in 2011. Hill-plots with 10 seeds per plot were used. Winter wheat grains inoculated with *Cochliobolus sativus* were spread into plots at plant elongation and flowering stages (ZGS 30 and 77; Zadoks et al. 1974). Scoring of disease at first awn and late milky stages (ZGS 49 and 77; Zadoks et al. 1974) were conducted based on the Saari-Prescot 0–9 scale (Saari and Prescott 1975). The maximum of disease severity in two different moments (SEV), as well as the area under the disease progress curve (AUDPC) were used to map QTL in CAN_LAN.

Leaf rust severity was assessed in eight environments: URU_MC 2009, 2010, 2011, and 2012; URU_LE 2009 and 2010; and ECU_PIC (Ecuador) in 2010 and 2011 (Tables 1, 3). At URU_MC leaf rust was scored in the same experimental plots that were used for spot blotch assessment, under natural leaf rust infection. Disease severity was evaluated at milky to dough milky-grain stage (ZGS 71 to 79 Zadoks et al. 1974) when the susceptible checks reached approximately, 90 % infection. At URU_LE leaf rust information was collected from specific experiments with row-plots in augmented unreplicated designs with 10 % repeated systematic checks in 2009 and 15 % repeated checks in a Federer's experimental design (Federer 1961) in 2010. Plots consisted of two, 1 m long rows. *Puccinia hordei* inoculum of race UPh3 (virulent to *Rph3*) was increased on susceptible cultivar Morex in greenhouse conditions. Spreader rows perpendicular to the plots and plots were inoculated with a suspension of UPh3 urediniospores in a non-phytotoxic light mineral oil (Soltrol®, Chevron Phillips Chemical Company LP), from plant elongation to flowering stages (ZGS 30–60, Zadoks et al. 1974). Scoring of disease severity was conducted during milky to dough milky-grain stage (ZGS 71–79 Zadoks et al. 1974) when the susceptible checks reached approximately, 90 % infection, using the Modified Cobb Scale (Peterson et al. 1948). Disease severity corrected by days to anthesis (as estimated by awn appearance) was used to map QTL in URU_LE. At ECU_PIC, augmented unreplicated designs were used in Santa Catalina research station in Pichincha. Two-row plots, 1 m long, were used in 2010 and 2011. Disease severity under natural infection was measured as the percentage of diseased leaf area (from 0 to 100 %) on a plot basis during complete anthesis (ZGS 69; Zadoks et al. 1974).

Stripe rust was evaluated in six environments: ECU_PIC in 2010 and 2011, ECU_TOL (Ecuador) in 2011; MEX_TOL (Mexico) in 2011, and two locations in Peru in 2011 (PER_AND and PER_COM) (Tables 1, 4). The same experimental plots that were used for leaf rust assessment were used in ECU_TOL, where disease severity was measured as the percentage of leaf area affected (from 0 to 100 %), on a plot basis during complete anthesis (ZGS 69; Zadoks et al. 1974). In ECU_PIC, augmented unreplicated designs were used in Granja Tolilla research station. Two-row plots of 1 m long were used under natural infection. Disease severity was assessed as the percentage of diseased leaf area (from 0 to 100 %), on a plot basis during complete

Table 1 Environmental and experimental descriptions of field experiments designed to detect QTL conferring resistance to spot blotch, leaf rust, and stripe rust, using GWAS and a panel of 360 barley accessions

	Location	Country	Year	Lat	Long	Elev. (m)	Cond.	Inf.	Experiment	Plots	Inoc.	Traits
Spot blotch												
URU_MC_09	Paysandu	Uruguay	2009	32°50′S	58°00′W	44	GS	L	UR-AUG 20/4	2R × 1 m	ISS	S10
URU_MC _10	Paysandu	Uruguay	2010	32°50′S	58°00′W	44	GS	L	UR-AUG 20/4	2R × 1 m	ISS	S10
URU_MC _11	Paysandu	Uruguay	2011	32°50′S	58°00′W	44	GS	H	FED-AUG 20/4	2R x 1 m	ISS	S100 (E1, E2)
URU_MC_12	Paysandu	Uruguay	2012	32°50′S	58°00′W	44	GS	M	FED-AUG 20/4	2R × 1 m	NI	S10
URU_LE_09	Colonia	Uruguay	2009	34°20′S	57°40′W	80	ISN	L	UR-AUG 10/2	Hill (15)	I8	S100
URU_LE _10	Colonia	Uruguay	2010	34°20′S	57°40′W	80	ISN	M	UR-AUG 10/2	Hill (15)	I8	S100
URU_LE _11	Colonia	Uruguay	2011	34°20′S	57°40′W	80	ISN	H	FED-AUG 15/2	Hill (15)	I8	S100
CAN_LAN	Alberta	Canada	2011	52°26′S	113°44′W	850	GS	H	RIB-AUG 13/3	Hill (10)	WW	S100, AUDPC
Leaf rust												
URU_MC_09	Paysandu	Uruguay	2009	32°50′S	58°00′W	44	GS	L	UR-AUG 20/4	2R × 1 m	NI	S100
URU_MC _10	Paysandu	Uruguay	2010	32°50′S	58°00′W	44	GS	H	UR-AUG 20/4	2R × 1 m	NI	S100
URU_MC _11	Paysandu	Uruguay	2011	32°50′S	58°00′W	44	GS	H	FED-AUG 20/4	2R × 1 m	NI	S100
URU_MC_12	Paysandu	Uruguay	2012	32°50′S	58°00′W	44	GS	M	FED-AUG 20/4	2R × 1 m	NI	S10
URU_LE _09	Colonia	Uruguay	2009	34°20′S	57°40′W	80	GS	H	UR-AUG 10/2	2R × 1 m	UPh3	S100
URU_LE _10	Colonia	Uruguay	2010	34°20′S	57°40′W	80	GS	H	FED-AUG 15/2	2R × 1 m	UPh3	S100
ECU_PIC_10	Pichincha	Ecuador	2010	0 22′S	78 33′W	3,050	GS	M	UR-AUG 20/2	2R × 1 m	NI	S100
ECU_PIC_11	Pichincha	Ecuador	2011	0 22′S	78 33′W	3,050	GS	H	UR-AUG 20/2	2R × 1 m	NI	S100
Stripe rust												
MEX_TOL_10	Toluca	Mexico	2010	19°17′ N	99°40′ w	2,680	GS	H	UR-AUG 20/2	2R × 2 m	ISol	S100, AUDPC
ECU_PIC_10	Pichincha	Ecuador	2010	0 22′S	78 33′W	3,050	GS	H	UR-AUG 20/2	2R × 1 m	NI	S100
ECU_PIC_11	Pichincha	Ecuador	2011	0 22′S	78 33′W	3,050	GS	H	UR-AUG 20/2	2R × 1 m	NI	S100
ECU_TOL_11	Granja Tolilla	Ecuador	2011	2°00′S	78°43`W	3,230	GS	H	UR-AUG 20/2	2R × 1 m	NI	S100
PER_AND	Andenes	Peru	2010	13°26′S	72°15′ W	3,450	GS	H	FED-AUG 17/8	2 m × 2R	IP	S100, AUDPC
PER_COM	Combapata	Peru	2010	14°60′S	71°25′ W	3,436	GS	H	FED-AUG 17/8	2 m × 2R	IP	S100, AUDPC

Traits: S10, Severity in a scale from 0 to 10; S100, severity in a scale from 0 to 100

Experiment: UR-AUG, augmented unreplicated experiments with systematic checks; FED-AUG, Federer's augmented unreplicated experimental design; RIB-AUG, resoluble incomplete block design with two replications in an augmented checks. The first number indicates the percentage of plots with repeated checks, and the second number indicates de number of check-lines used

Plots: for row-plots the number of rows and the length of the plot in meters is indicated, for hill-plots the number of seeds planted is indicated, hill-plots spacing was 40 cm by 60 cm

Inoculation: ISS, *C. sativus*-inoculated sorghum seed; I, I8 Spray-inoculation with eight *C. sativus* monosporic isolates from different years, cultivars and locations at 8 × 10^3 conidia/ml, inoculation was performed since stem elongation stage (ZGS3.1) at least once. Spot blotch was assessed at grain-milk stage (ZGS 7.0). NI, natural infection; UPh3, inoculation at the plant elongation stage to flowering stage with the predominant race UPh3 (virulent to *Rph3*); IP, infection plots on the border of each block; ISol, inoculation with 1 gram of spores mixed in 1 L of Soltrol® 100 Paraffin solvent. WW, C. sativus-inoculated winter wheat was spread in the experiment on two different dates (ZGS 2.4 and 4.0)

Cond.: *GS* growing season, *ISN* irrigated summer nursery, *Inf.* infection, *L* low, *M* medium, *H* high, *AUDPC* area under the disease progress curve

anthesis (ZGS 69; Zadoks et al. 1974). In MEX_TOL, augmented unreplicated designs with 10 % repeated systematic checks were used. Artificial inoculation was performed by spraying a suspension of 1 gram of spores collected from susceptible checks mixed in 1 liter of Soltrol® (Chevron Phillips Chemical Company LP) 100 Isoparaffin solvent. In Peru, row-plots were used in an augmented Federer's design (Federer 1961) with 17 % repeated checks at both the Andenes (PER_AND) and Combapata (PER_COM) experimental research stations, which belong to INIA. There were eight different checks. Plots were 2 m long and two-rows wide. The Modified Cobb scale (Peterson et al. 1948; Stavely, 1985) was used to assess stripe rust severity for four times at PERU_AND (at 71, 91, 119 and 130 days

Table 2 Summary statistics for spot blotch on selected genotypes: all genotypes selected by allelic states (G selected), phenotypic performance (P selected) or both are shown in the table

	ME1			ME2					ME3	
	URU_LE_09	URU_LE_10	URU_LE_11C	URU_MC_09	URU_MC_10	URU_MC_11_E1	URU_MC_11_E2	URU_MC_12	CAN_SEV	CAN_AUDPC
Summary statistics of population										
Min	5.0	0.5	4.1	0.0	5.0	0.0	5.1	23.0	34.0	59.1
Mean	27.7	12.5	66.6	12.0	28.0	8.0	37.0	44.0	54.0	102.0
Max	65.0	50.0	99.0	6.05	70.0	60.0	77.3	69.0	71.0	132.8
S.E.	13.8	9.9	22.5	11.0	11.0	12.0	14.4	9.0	6.0	12.3
N	249	223	136	261	256	252	272	280	283	283
Summary statistics of selected lines										
Mean G selected ME1	22	7	45	10	30	0	38	50	5	92
Mean P selected ME1	22	10	37	10	30	10	39	50	5	98
Mean G selected ME2	31	13	74	10	30	10	36	40	5	102
Mean P selected ME2	28	13	61	10	20	0	16	40	5	98
Mean G selected ME3	24	10	65	10	30	10	36	40	5	91
Mean P selected ME3	25	10	54	10	20	10	31	40	5	78

Phenotypic performance of genotypes in each environment (grouped by Megaenvironment—ME) is shown

Selection criteria by which the genotype was included is represented as following: G1 for top 10 % of lines selected based on genotypic predictions in ME1, G2 for top 10 % of lines selected based on genotypic predictions in ME2, G3 for top 10 % of lines selected based on genotypic predictions in ME3, P1 for top 10 % of lines selected based on phenotypic predictions in ME1, P2 for top 10 % of lines selected based on phenotypic predictions in ME2, P3 for top 10 % of lines selected based on phenotypic predictions in ME3

after plant emergence) and for three times at PERU_COM (at 82, 96 and 111 days after plant emergence). The maximum disease severity observed over dates (SEV), as well as AUDPC, was used for QTL mapping.

Statistical analysis of field data

Given the disparity in field experimental designs and field heterogeneity, a two-step analysis was performed following Smith et al. (2001). First, field plot data were analyzed individually for each environment using mixed models with the most appropriate model according to the experimental design and spatial modeling. The general model was Federer's (Federer 1961) and the notation follows that of Eckermann et al. (2001) and Verbyla et al. (2003):

$$y_{ijk} = \mu + \beta_i + G_j + \varepsilon_{ijk}$$

where y_{ijk} is the response variable (i.e., severity), μ is the overall mean, β_i is the incomplete-block random effect $\beta_i \sim N(0, \sigma^2_b)$, G_j is the genotypic effect, and ε_{ijk} is the error. The model for G_j is as follows: $G_j = g_i + c_i$, where g_i is the effect of the i-th line with $i = 1, \ldots, n_g$; and c_i represents a fixed effect for the i-th check with $i = n_g + 1, \ldots, n_g + n_c$. In the cases where true replication existed, a term for the complete block was included and incomplete blocks were nested within complete blocks. Additionally, due to the existence of repeated checks within each trial, intra-environmental variance could be modeled and a spatial correction in row and column directions was used with different variance–covariance structures. Spatial models were compared with AIC and BIC; and the most appropriate model in each environment was used to obtain best linear unbiased estimates (BLUE). These analyses were performed in SAS Statistical Software (SAS Institute 2004) with the PROC MIXED procedure.

In the second step of the analysis, the two-way genotype by environment table of BLUE from the single trial analyses (described above) was used in a mixed model to model variance–covariance structure and to detect the most suitable variance–covariance structure for the genotype by environment interaction for each disease. Analyses were performed in SAS Statistical Software (SAS Institute 2004) with the PROC MIXED procedure.

Because different scales were used to measure disease phenotypes (i.e., using a scale or percentage), not all of the residuals from all traits followed a normal distribution. Additionally, some residuals had heterogeneous variances. The appropriate transformations of the original variable were used to fit final models when the untransformed data did not perform properly. Pearson's correlations between pairs of environments were calculated and Mega-Environments (ME) were identified based on additive main effect and multiplicative interaction (AMMI) models. These analyses were performed using R statistical software (R Development Core Team 2005).

Table 3 Summary statistics for leaf rust on selected genotypes: all genotypes selected by allelic states (G selected), phenotypic performance (P selected) or both are shown in the table

	ME1			ME2				
	URU_LE_09	URU_LE_10	URU_MC10	URU_MC_09	URU_MC_E2	URU_MC_12	ECU_10	ECU_11_E1
Summary statistics of population								
Min	0.0	0.0	10.0	0.0	0.0	16.0	0.0	0.0
Mean	53.3	61.5	55.0	2.6	40.2	52.0	19.9	25.2
Max	99.0	99.0	90.0	25.0	93.1	80.0	70.0	90.0
S.E.	27.5	24.6	20.0	4.2	25.5	14.0	17.1	24.5
N	255	289	270	236	267	280	191	155
Summary statistics of selected lines								
Mean G selected ME1	45.4	56.5	47.0	1.4	32.6	46.0	16.5	13.5
Mean P selected ME1	8.0	20.5	24.0	0.8	15.3	52.0	5.2	7.5
Mean G selected ME2	50.8	62.7	48.0	1.6	30.0	47.0	22.6	20.9
Mean P selected ME2	24.1	36.2	38.0	1.2	14.6	49.0	6.8	4.5

Phenotypic performance of genotypes in each environment (grouped by Megaenvironment—ME) is shown

Selection criteria by which the genotype was included is represented as following: G1 for top 10 % of lines selected based on genotypic predictions in ME1, G2 for top 10 % of lines selected based on genotypic predictions in ME2, P1 for top 10 % of lines selected based on phenotypic predictions in ME1, P2 for top 10 % of lines selected based on phenotypic predictions in ME2

Table 4 Summary statistics for stripe rust on selected genotypes: all genotypes selected by allelic states (G selected), phenotypic performance (P selected) or both are shown in the table

	ME1		ME2				ME3
	ECU_10	PER_COM_11_SE	PER_AND_11_SE	MEX	ECU_11_E2	SR_PER_COM_11_AUDPC	SR_PER_AND_11_AUDPC
Summary statistics of population							
Min	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mean	20.8	4.3	34.8	35.2	23.4	64.3	862.7
Max	70.0	80.0	99.0	99.0	90.0	1,320.0	3,980.0
S.E.	17.4	10.7	38.3	36.6	32.7	149.6	994.1
N	205	339	339	339	339	261	309
Summary statistics of selected lines							
Mean G ME1	16.5	5.8	40.3	45.9	36.2	75.2	922.9
Mean P ME1	3.3	1.0	23.9	41.1	35.8	13.3	556.7
Mean G ME2	17.3	3.2	25.5	43.2	21.5	40.2	516.5
Mean P ME2	22.5	1.6	6.9	8.3	5.3	18.7	183.0
Mean G ME3	20.6	4.8	57.8	55.5	32.7	61.6	1,649.7
Mean P ME3	19.5	2.3	1.3	33.4	17.3	25.7	22.3

Phenotypic performance of genotypes in each environment (grouped by megaenvironment—ME) is shown

Selection criteria by which the genotype was included is represented as following: G1 for top 10 % of lines selected based on genotypic predictions in ME1, G2 for top 10 % of lines selected based on genotypic predictions in ME2, G3 for top 10 % of lines selected based on genotypic predictions in ME3, P1 for top 10 % of lines selected based on phenotypic predictions in ME1, P2 for top 10 % of lines selected based on phenotypic predictions in ME2, P3 for top 10 % of lines selected based on phenotypic predictions in ME3

Genotyping

Leaf segments (5–8 cm lengths) from 2-week-old seedlings of each genotype were cut and freeze-dried in 2.0 mL Eppendorf tubes. DNA was then extracted with the DNeasy Plant Mini Kit using the manufactures´ protocol (QIAGEN, Chattsworth, CA, USA). The general quality and quantity of DNA was verified on a 0.8 % agarose gel.

Barley Oligonucleotide Pool Assay-1 (BOPA 1), described in detail by Close et al. (2009) and Szűcs et al. (2009), was used to characterize 1,536 SNPs. The SNP genotyping was done at the University of Minnesota BioMedical Genomics Center (http://www.bmgc.umn.edu/home.html) following the protocols of Illumina's GoldenGate Bead Array Technology (Illumina, San Diego, CA, USA) (Fan et al. 2003a, 2006). To reduce errors in allele calls, all markers and individuals with more than 10 % missing data points were removed. All markers with a minor allele frequency (MAF) lower than 10 % were also excluded. Therefore, 1,096 markers were finally used in this study. The estimated positions of the SNPs are based on the consensus map developed by Muñoz-Amatraín et al. (2011), and are available by downloading the 1.77 version of the barley HarvEST database (http://harvest.ucr.edu; verified 30 April 2014).

Statistical models for association mapping

Multiple mixed models for association mapping were compared using data from a subset of the total number of environments. The most suitable model was then selected and applied to the full set of environments and traits. The general mixed model equation was:

$$\mathbf{Y} = \mathbf{X}\beta + \mathbf{Q}\upsilon + \mathbf{Zu} + \mathbf{e}$$

where $\mathbf{Y}$ is the phenotypic vector, $\mathbf{X}$ is the molecular marker matrix, β is the unknown vector of allele effects to be estimated, $\mathbf{Q}$ is the population structure represented by the scores of the relevant axis of a principal components analysis, υ is the vector of population effects (parameters), $\mathbf{Z}$ is a matrix that relates each measurement to the individual from which it was obtained (an identity matrix in our case), $\mathbf{u}$ is the vector of random background polygenic effects, and $\mathbf{e}$ is the residual errors. Random effects are underlined. We compared the following mainstream models: (1) naïve; a simple test of association (Kruskal–Wallis) with no correction for population structure ($\mathbf{Y} = \mathbf{X}\beta + \mathbf{e}$), (2) fixed; a fixed-effects model using populations structure as fixed covariate ($\mathbf{Y} = \mathbf{X}\beta + \mathbf{Q}\upsilon + \mathbf{e}$), (3) kinship; a mixed model including the coancestry matrix among genotypes as a random effect ($\mathbf{Y} = \mathbf{X}\beta + \underline{\mathbf{Zu}} + \mathbf{e}$ following Parisseaux and Bernardo 2004); (4) Price; a mixed-effects model including population structure but as a random effect ($\mathbf{Y} = \mathbf{X}\beta + \underline{\mathbf{Q}\upsilon} + \mathbf{e}$ following Price et al. 2006 and Malosetti et al. 2007), and (5) Yu; a mixed-effects model including both population structure and coancestry among genotypes ($\mathbf{Y} = \mathbf{X}\beta + \mathbf{Q}\upsilon + \underline{\mathbf{Zu}} + \mathbf{e}$ following Yu et al. 2006a, b). A Principal component analysis (PCA) was used to determine population structure. PCA was included as a random effect in the *Price* model including all significant axes, following Patterson et al. (2006). When used in the *Fixed* or *Yu* model, PCA was included as a fixed effect. In this case, and to avoid overparametrization we used the number of PCA axes until the largest drop in variance explained. These five models were used for the spot blotch and leaf rust data from the following environments: URU_MC_09, URU_MC_10, URU_LE_09, and URU_LE_10. These analyses were performed using R statistical software (R Development Core Team 2005) with modifications of the *emma* package as described by (Kang et al. 2008).

Given the different structure of the fixed effects and the fact that REML estimates were being used, the five models described above using the sub-set of the data were compared by Q–Q plots assuming a uniform distribution of p values under the null-hypothesis of no-QTL (i.e., Schweder and Spjøtvoll plots; Schweder and Spjøtvoll 1982). The most suitable model was used then in the full data set (i.e., all combination of environments and diseases) and results are reported on that model (i.e., the *Price* model, Fig. 1). These analyses were also performed in R statistical software (R Development Core Team 2005) using some modifications of the *emma* package (Kang et al. 2008).

We used a two-step procedure to model QEI and to control for false positives: the first step consisted of a single environment QTL Mapping, followed by a second step of multi-environment multi-QTL model marker selection (MEMQ). The first scan of the genome was conducted with a p value adjustment for multiple testing (i.e., $p < 0.0001$). The final multi-QTL model was fitted with all significant marker-trait associations and modeling the genotype by environment interaction with the covariance structure detected in the previous step (Boer et al. 2007; Stich et al. 2008; Mathews et al. 2008). This model allowed for a global test for each marker as follows:

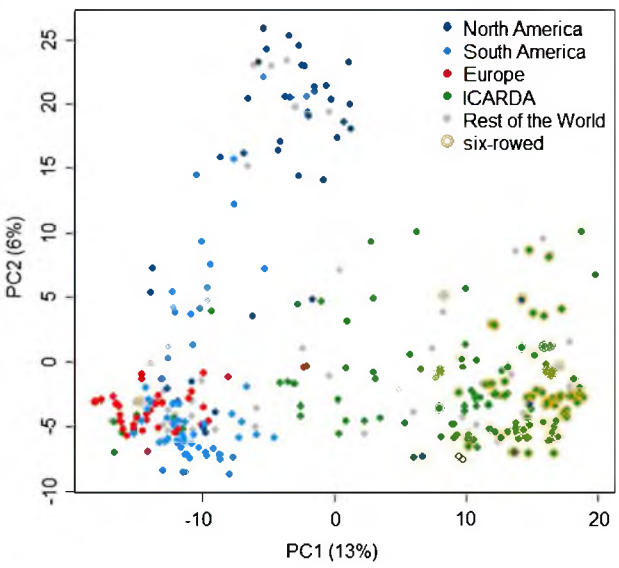

Fig. 1 Principal component analysis using a barley panel of 360 advanced *inbred lines* highlighting the accessions by geographical origin and inflorescence type (i.e., two-row and six-row)

$$y_{ijk} = \mu + x_i\alpha + G_j^* + E_k + x_i\alpha_k + \mathrm{GE}_{jk}^*$$

where y_{ijk} are the BLUE from each environment, μ is the overall mean, $x_i\alpha$ is the i-th QTL main effect, G_j^* is the remaining genotypic effect (not due to the QTL), E_k is the environmental main effect of the k-th environment, $x_i\alpha_k$ is the QEI deviation, and GE_{ik}^* is the remaining GxE effect. A global test was therefore constructed where $x_i\alpha_k{}^* = x_i\alpha + x_i\alpha_k$ is simultaneously tested. In this way, a general test of QTL was performed where specific-environment and global QTL were detected with a Wald F test p value of 0.05 following Malosetti et al. (2007).

Accounting for multiple-comparison is not straightforward in an association mapping panel. When comparing multiple markers one-at-a-time, many comparisons are performed and the chance of getting a false positive increases to the point of having a close to one probability of declaring at least one false positive. Therefore, different strategies have been used to control this problem. They can be grouped in two categories: those that control the number of false positives (Type-I error; i.e., of all the non-significant comparisons, how many are falsely declared positives), and those that control the false discovery rate (i.e., of all the comparisons declared as positives, how many are true-positives; Bernardo 2010). Bonferroni is a Type-I correction that is conservative because not all the tests in an association mapping panel are independent (Benjamini et al. 2001). Another method of the second category is the false-discovery rate proposed by Benjamini and Hochberg (1995). This method is also conservative. Other methods developed strategies to define the number of effective (independent) tests. Among those is a method proposed by Li and Ji (2005) that conducts a Bonferroni-type of adjustment after determining the effective number of tests from an eigenvalue decomposition and using the Tracy–Widom statistic for the distribution of eigenvalues. This method works well for designed population QTL mapping (Mathews et al. 2008), but might not be appropriate for a GWAS population where linkage disequilibrium is caused by physical linkage and other causes like population structure or genetic relatedness. Therefore, we decided to use a liberal p value followed by a MEMQ selection of markers.

The top 10 % of the genotypes were selected based on two criteria: first, individuals were selected based on their genotypic score in each ME; second, individuals were selected based on their phenotypic performance in each ME. Genotypic scores were obtained for each genotype based on the allelic score at the significant marker-trait associations using the Lande and Thompson (1990) equations and the estimated marker effects.

Results

Structure and choice of model for GWAS

The principal determinant of population structure was the geographical origin of the accessions, combined with inflorescence type (e.g., six-row and 2-row; Fig. 1). Six-row ICARDA material is one of the most distinct groups, while the other ICARDA materials are more admixed. Additionally, North and South American materials are distinct, with European accessions grouping with South-American accessions.

There was not a single most appropriate GWAS model for all the traits. A mixed model with population structure either as a random (*Price*, Price et al. 2006) or a fixed (Yu et al. 2006a) effect was the most suitable model for spot blotch (Fig. 2a) for all environments. However, a mixed model without population structure and with coancestry information (*Kinship*, Parisseaux and Bernardo 2004) was the most appropriate model for leaf rust (Fig. 2b). The *Price* (Price et al. 2006) and *Yu* (Yu et al. 2006a) models performed relatively well for leaf rust. In general, fixed effects models performed poorly, while mixed models performed relatively well for all of the environments and traits. Therefore, the *Price* (Price et al. 2006) model was used for all analyses described in this report.

Phenotypes and mega-environments (MEs)

Spot blotch severity was low (with an average up to 15 % and a maximum severity below 70 % at URU_MC_09, and URU_LE_10) to intermediate (with an average between 15 and 30 % and a maximum severity below 70 % at URU_LE_09 and URU_MC_10; Table 2). High severity with an average above 35 % and a maximum severity between 70 and 99 % occurred only in environments URU_LE_11, URU_MC_11, URU_MC_12, and CAN. There were modest correlations (0–0.8) between environments. Three MEs were defined for spot blotch severity (Fig. 3a; Table 2): ME1 included all years for the URU_LE location; ME2 included all years for the URU_MC location; and ME3 included all evaluations for location CAN.

The levels of leaf rust severity were relatively high in all environments, with a minimum average of 3 % at URU_MC_09 and a maximum average of 62 % at URU_LE_10 (Table 3). There were modest to high correlations (0.37 to 0.99) between environments. The highest correlations were between Uruguayan environments (Fig. 3). Two MEs were defined for leaf rust severity (Fig. 3b). ME1 included all years for URU_LE and year 2010 for URU_MC while the ME2 included the remaining locations and years (Fig. 3; Table 3).

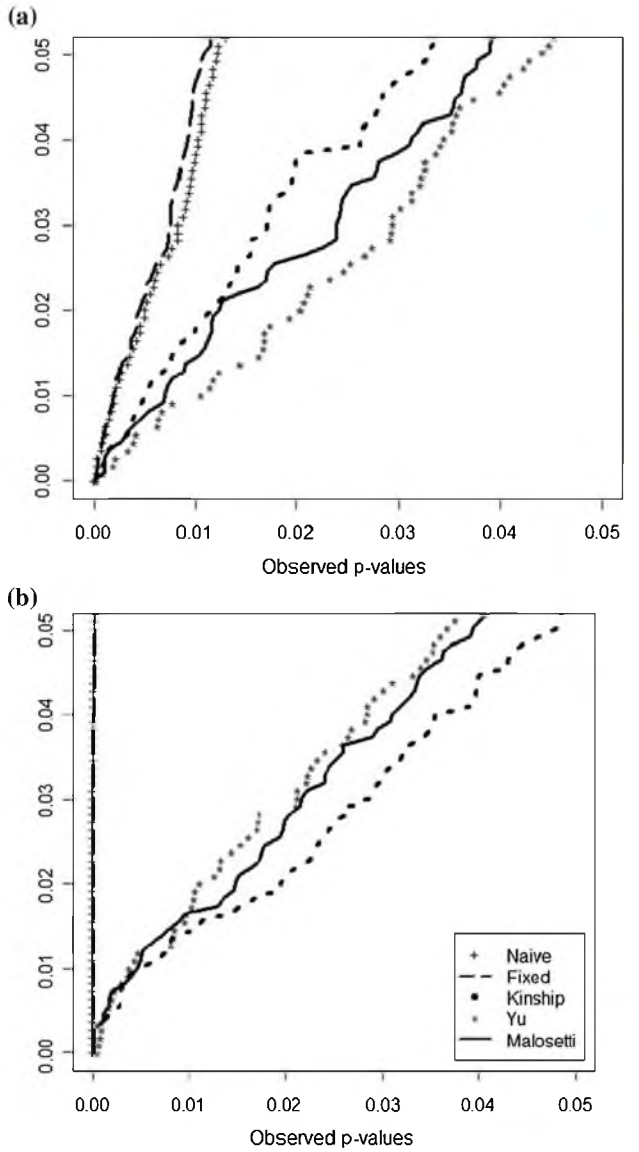

Fig. 2 Cumulative distribution functions (cdf) of *p* values in genome-wide scans for spot blotch (**a**) and leaf rust (**b**) diseases for one of the Uruguayan Locations in 2009 (URU_MC_09)

Stripe rust disease severities were relatively high in most environments with a minimum average of 4 % at PER_COM_11 and a maximum average of 35 % at MEX (Table 4). There were modest to high correlations (0.03–0.98) between environments. Three ME were defined for stripe rust severity and these were not based on locations (Fig. 3c; Table 4).

QTL detection

We detected QTL for resistance to each of the three diseases (Fig. 3). Nine significant marker-trait associations

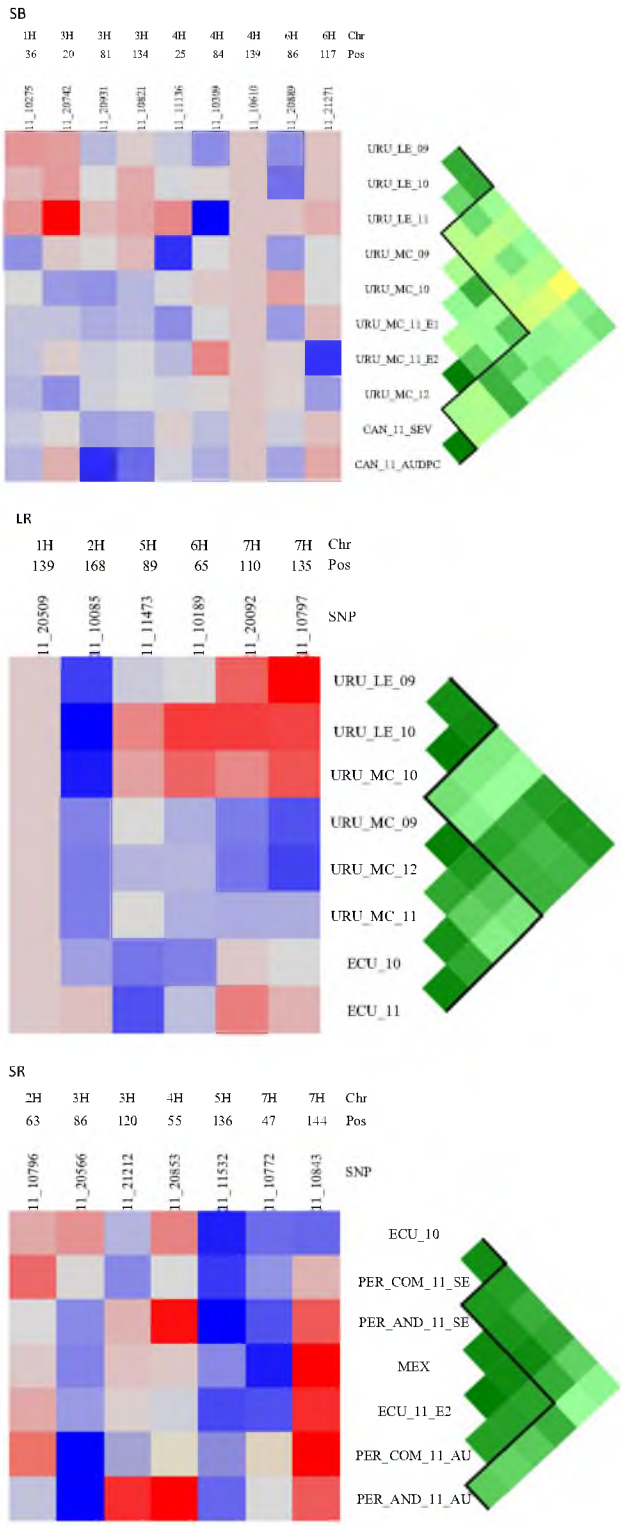

Fig. 3 Marker-trait associations for **a** spot blotch, **b** leaf rust, and **c** stripe rust showing environment-specific and general QTL. *Blue* indicates a large allele substitution effect where *allele 1* indicates smaller values of the trait-value; *red* indicates a large allele substitution effect where *allele 1* indicates large values of the trait-value, and *gray* indicates a small or no allele substitution effect. Correlations across environments and mega-environments are shown on the right of each panel

were found for spot blotch—on chromosomes 1H, 3H, and 6H (Fig. 3a). Six significant marker-trait associations were found for leaf rust—on all chromosomes except 3H and 4H (Fig. 3b). Seven significant marker-trait associations were found for stripe rust—on all chromosomes except 1H and 6H (Fig. 3c). Severity evaluated with the AUDPC yielded the same QTL as severity evaluated with the maximum infection at any point (Fig. 3).

There were both environment-specific and global marker-trait associations, and the former outnumbered the latter (Fig. 3). Only two SNP had global effects; SNP 11_10610 in chromosome 4H at 139 cM for spot blotch (Fig. 3a), and SNP 11_20509 in chromosome 1H at 139 cM for leaf rust (Fig. 3b). No QTL with global effects were detected for stripe rust (Fig. 3c). The effect of SNP 11_10610 was relatively small (a 1.6 % difference in severity) and 15 % of the G-selected individuals carry the positive allele (Fig. 3a, Supplemental Table 1). The effect of the allele substitution for SNP 11_20509 was intermediate (a 2.7 % difference in severity) and nearly half (47 %) of the G selected accessions have the positive allele (Fig. 3b; Supplemental Table 2).

Within the environment-specific associations, some were consistent and showed only magnitude differences between environments. For example, SNP 11_11532 in chromosome 5H at 136 cM and SNP 11_10772 in chromosome 7H at 47 cM had magnitude differences for stripe rust (Fig. 3c). For this disease, two QTL with only magnitude differences were detected across environments (11_20853 and 11_11532) and most G-selected accessions had positive alleles at both QTL (95 and 88 %, respectively; Supplemental Table 3). Additionally, SNP 11_10085 in chromosome 2H at 168 cM had magnitude differences for leaf rust (Fig. 3b).

An example of a change in favorable allele phase across environments is SNP 11_10275 in chromosome 1H at 36 cM for spot blotch, where the favorable allele in environments URU_LE (ME1) was unfavorable in all the other environments (Fig. 3a).Therefore, none of the selected accessions for spot blotch has all positive alleles defined for ME1 or ME2 (Supplemental Table 1). Three accessions (FONT 206, 348, and 351) have positive alleles at the QTL defined for ME2 (Supplemental Table 1). Other examples of change in favorable allele phases across mega-environments are SNP 11_11473, 11_10189, 11_20092, and 11_10797 at chromosomes 5H, 6H, and 7H for leaf rust (Fig. 3b) were favorable alleles for ME1 were different than favorable alleles for ME2. Two accessions have the positive alleles for leaf rust at all QTL for ME1 (FONT 68 and 317, Supplemental Table 2), and two have the positive alleles at all QTL for ME2 (FONT 53 and 300, Supplemental Table 2).

Discussion

Structure and choice of model for GWAS

In most barley GWAS reports based on diverse germplasm panels, the principal determinant of population structure is inflorescence type (two-row vs. six-row) (Muñoz-Amatriaín et al. 2014). We found that the principal determinant of population structure was the inflorescence type and the geographical origin of the accessions. Six-row ICARDA materials form a clearly distinct group. The remaining ICARDA materials are admixed, probably reflecting a broader spectrum of crosses in its development compared with six-row genotypes. Furthermore, European materials are grouped with South-American materials, probably due to shared ancestry (Gutiérrez et al. 2009; Locatelli et al. 2013), while North-American materials are clearly separated from the rest. Diverse origins with small representation were grouped into the rest-of-the-world category and since they do not necessarily share any ancestry they were represented throughout the PCA without being close to any specific origin.

We found that there was not a single model that was most suitable for all traits and environments. Gutierrez et al. (2011), in a study involving malting quality data in barley, compared sixteen different models and found the same pattern: the most suitable models depended on the environment and trait. In this research, we included a model (Price et al. 2006) that has not been used extensively and still arrived at the same general conclusion: most mixed models will identify the principal QTL. The contention is supported by other recent GWAS reports in barley and other crop plants (Cappa et al. 2013).

Phenotypes and mega-environments (MEs)

Evaluating adult plant resistance under field conditions can be challenging. Resistance to some diseases may have a complex genetic basis and may be highly dependent on environmental influences. For these reasons, we measured phenotypes for three economically important diseases using extensive field evaluations, and made every effort to ensure high data quality through the use of appropriate experimental designs for assessing large numbers of unreplicated accessions. Phenotyping is a crucial aspect of QTL detection because poor phenotype data will reduce the ability to detect true QTL (Cooper et al. 2014). However, unmanageable environmental variation is a fact in field-based plant pathology research. Climate, variation in composition and intensity of inoculum, sequential infection, plant maturity and the presence of other diseases may interfere with the targeted disease(s) (Hickey et al. 2011). Controlled environment assessment of disease resistance at the seedling stage can be more highly heritable than adult plant field resistance,

but the former may not be predictive of the latter (Castro et al. 2003). For example, adult-plant resistance to leaf rust in wheat and barley has been shown to involve partial resistance (i.e., additive and/or epistatic effects of multiple genes with minor effects) and may be more durable than seedling resistance (McIntosh 1992; Hong and Singh 1996; Lagudah et al. 2006; Pretorius et al. 2007; Castro et al. 2012). Therefore, field evaluation of disease resistance is still highly relevant even at the cost of decreased power. Based on these considerations, data from multiple environment assessment of adult plant resistance must be carefully reviewed and processed prior to analysis.

QTL: detection, coincidence and novelty

It is challenging to align QTL detected in different germplasm and/or by different methods (e.g., in bi-parental vs. GWAS arrays). However, such an exercise is essential in terms of validation and expanding the catalog of mapped resistance genes and QTL. Of the 22 QTL, we detected for each of the three diseases, 13 were coincident with prior reports, based on the presence of common markers and/or positions on consensus maps (Table 5). Nevertheless, most previous reports have lower resolution than this one, precluding any definite conclusion about identity between QTL. The same does not necessarily apply for the novel QTL we detected, because we did not find any previous reports of resistance QTL in these regions, even when we defined broad cM intervals.

Of the nine QTL detected for spot blotch, three were novel, and these were collocated on chromosomes 4H (cM 25) and 6H (cM 86 and 117). The remaining six QTL were located in genomic regions with previous reports for spot

Table 5 Summary of disease resistance genes and QTL reported in the same genomic regions (located through Varshney et al. 2007; Muñoz-Amatraín et al. 2011) where disease resistance QTL were detected in this report

Spot blotch Marker	Location Chrom.	CM.	Reported name	Mapping population and report	Resistance
11_10275	1H	36	*Rcs-qtl-1H-6-7*	WBDC (Roy et al. 2010), SM (Steffenson et al. 1996)	AP
11_20742	3H	20	*Rcs-qtl-3H-1-2*	CB (Bilgic et al. 2006), VN, TG, NW, WL (Bovill et al. 2010)	AP
11_20931	3H	81	*Rcs-qtl-3H-4-6*	DM (Bilgic et al. 2005), WBDC (Roy et al. 2010)	AP
11_10821	3H	134	*Rcs-qtl-3H-11-12*	SM (Bilgic et al. 2005)	AP
11_11136	4H	25			
11_10309	4H	84	*Rcs-qtl-4H-4-6*	O/H-AB (Yun et al. 2006)	AP
11_10610	4H	139	*Rcs-qtl-4H-10-11*	CB (Bilgic et al. 2006)	Seedl.
11_20889	6H	86			
11_21271	6H	117			
Leaf rust					
11_20509	1H	139			
11_10085	2H	168	*Rphq2*	SV (Jafary et al. 2006), LV (Qi et al. 1998); PS (Liu et al. 2011)	AP
11_11473	5H	89			
11_10189	6H	65	*Rphq3*	LV (Qi et al. 1998), NF (Hickey et al. 2011), BB (Castro et al. 2012)	Seedl/AP
11_20092	7H	110	*Rphq9*	LV (Qi et al.,1998)	AP
11_10797	7H	135	*RphX, Rph3*	CB (Hayes et al. 1996b), SG (Toojinda et al. 2000), S42 (von Korff et al. 2005), BB (Rossi et al. 2006)	AP
Stripe rust					
11_10796	2H	63		SG (Toojinda et al. 2000), BB (Vales et al. 2005)	AP
11_20566	3H	86			
11_21212	3H	120		BB (Vales et al. 2005)	AP
11_20853	4H	55			
11_11532	5H	136			
11_10772	7H	47		BE (Thomas et al. 1995)*	AP
11_10843	7H	144			

BB BCD47 × Baronesse, *BE* Blenheim × E224/3, *CB* Calicuchima-sib × Bowman sib, *DM* Dicktoo × Morex, *LV* L94 × Vada, *NF* ND24260 × Flagship, *NW* ND11231-11 × WI2875-17, *O/H-AB* OU602 × Harrington Advanced Backcross, *PS* Pompadour × Stirling, *S42* Scarlett × ISR42-8 Advanced Backcross, *SM* Steptoe × Morex, *SV* SusPrit × Vada, *TG* TR251 × Gairdner, *VN* VB9524 × ND11231-12, *WBDC* wild barley diversity collection, *WL* WPG84 12-9-2-1 × Lindwall, *AP* adult plant, *Seedl* seedling

* Original report with low resolution

blotch resistance. One of the reports was a seedling resistance QTL: *Rcs-qtl-4H-10-11* on 4H, reported in Cali-sib/Bowman (Bilgic et al. 2006), where we found an adult plant resistance QTL (cM 139). The remaining five adult plant resistance QTL coincident with prior reports were *Rcs-qtl-1H-6-7* on 1H (cM 36) first reported in Steptoe/Morex (Steffenson et al. 1996), *Rcs-qtl-3H-1-2* on 3H (cM 20) first reported in Cali-sib/Bowman (Bilgic et al. 2006), *Rcs-qtl-3H-4-6* on 3H (cM 81) first reported in Dicktoo/Morex (Bilgic et al. 2005), *Rcs-qtl-3H-11-12* on 3H (cM 134) first reported in Steptoe/Morex (Bilgic et al. 2005), and *Rcs-qtl-4H-4-6* on 4H (cM 84) first reported in OU602/Harrington (Yun et al. 2006).

We detected six QTL for leaf rust, two of them were novel and located on chromosomes 1H (cM 139) and 5H (cM 89). The remaining four QTL were coincident with previous reports for adult plant resistance QTL: *Rphq2* on 2H (cM 168), *Rphq3* on 6H (cM 65) and *Rphq9* all reported in L94/Vada (Qi et al. 1998), and *Rph3* on 7H (cM 135) reported in Cali-sib/Bowman (Hayes et al. 1996a).

For stripe rust, seven QTL were detected, four of them novel and located on chromosomes 3H (cM 86), 4H (cM 55), 5H (cM 136) and 7H (cM 144). The three QTL coincident with previously reported QTL (all for adult plant resistance) were on 2H (cM 63) originally reported in Shyri/Galena (Toojinda et al. 2000), on 3H (cM 120) reported in BCD47/Baronesse (Vales et al. 2005) and on 7H (cM 47) reported in Blenheim/E224-3 (Thomas et al. 1995).

Coincidence of QTL detected *de novo* via GWAS of a new germplasm array with prior reports is useful from the standpoint of validation. Determining if the coincident effects represent the effects of novel resistance alleles at known loci, or at tightly linked loci, requires additional and deeper genetic analysis, germplasm development, and careful phenotyping. More obvious candidates for immediate introgression are resistance alleles at novel loci. The effects of these alleles can be validated, and the validated alleles exploited, by developing new germplasm using the resistant accessions with target haplotypes (Supplemental Tables 1, 2, 3). Of particular potential value are the QTL for stripe rust resistance on 5H (cM 136) and 7H (cM 144), which were significant in most environments. In the case of leaf rust, the novel QTL were of limited effect across environments and therefore of less immediate interest for crop improvement. Spot blotch will be the most challenging disease to address, as QTL show environment-specificity and changes in favorable allele state. Perfect biotrophs, such as the rusts, have a more defined host-pathogen relationship than necrotrophs, such as spot blotch. The particular challenges of introgressing QTL alleles for spot blotch resistance were addressed by Castro et al. (2012).

Plant morphology and phenology can play important roles in quantitative adult plant resistance. We found no disease resistance QTL associated with *VRS1*, the gene determining the principal morphological distinction in barley: two-row vs. six-row inflorescence type. One of the most common associations of quantitative resistance is with maturity, measured as heading date. In general, resistance QTL are associated with disease escape since later maturing accessions have lower levels of disease. The marker: trait relationships on 2H (cM 63) and 4H (55 cM) for stripe rust are in the same chromosomal regions as *eps2S* and *eam9* region, respectively (Laurie et al. 1995). We also mapped a heading date QTL coincident with the spot blotch resistance QTL on 4H (cM 25) (data not shown). In the case of leaf rust (URU_LE_09 and URU_LE_10), heading date was used as covariate, therefore removing its effect.

Genotype and QTL by Environment Interaction

QTL main effects, as described in the receding section, are often the drivers of breeding decisions, but for maximum effectiveness QTL should be considered in the context of genotype x environment and QTL x environment interaction. Using mixed models with explicit modeling of GxE allowed us to distinguish between global and environment-specific QTL (Boer et al. 2007; Mathews et al. 2008) and to identify cases of QEI due to cross-over interaction. Most of the QTL detected for stripe rust (i.e., six of seven) had global effects or only magnitude differences across environments, but little cross-over interaction. Only a few QTL detected for leaf rust (i.e., two of six) and spot blotch (one of nine) had global effects. Possible explanations for global QTL include: (1) a consistent pattern of pathogen virulence across environments and therefore consistent patterns of resistance, and/or (2) resistance alleles that are truly non-race specific. By extension, environment-specific QTL would be due to the reverse: different patterns of pathogen virulence across environments and/or race-specificity of resistance. We found that environments with lower levels of disease were always associated with greater GxE but there also where environments with high levels of disease that showed G × E, including PER_AND/PER_COM for stripe rust and CAN/URU_LE for spot blotch. G × E, and environment-specificity, could be due to differences in the pathogen population or the presence of other diseases in the environments. However, these are most likely explained by a combination of plant-pathogen, plant-environment and pathogen-environment interactions (Parlevliet and Zadoks 1977; Niks and Rubiales 2002). An analysis of QEI lays the foundation for judicious selection of markers for implementing molecular breeding strategies and environmental characterization. We provided a guide to interpret results based on their performance within Mega-Environments (i.e., groups of environments within which there is no G × E; Braun et al. 1996). As described in the next section,

we had a couple of cases of a change in favorable allele phase across environments: SNP 11_10275 and 11_20742 for spot blotch resistance, where the favorable allele in environment URU_LE was unfavorable in all the other environments (Fig. 2a). We hypothesize that this effect is due to an uncharacterized aspect of plant morphology and/or phenology that led to reduced disease in one environment and more disease in others. It is also possible that this is an example of extreme race-specificity, where an allele confers resistance to one race and susceptibility to others (Caldwell 1968a; Singh and Bowden 2011). Finally, this could be an example of a repulsion linkage of tightly linked disease resistance loci; there are many examples of this in the literature (Johnson et al. 1995; Tiwari et al. 1998; Toojinda et al. 2000) and is a consequence of genes conferring resistance to different diseases, or different races of the same disease, occurring in physical proximity.

QTL allele deployment and future perspectives

Selecting accessions with resistance alleles that only have global effects will not ensure the maximum effectiveness and durability of resistance. Rather, accessions should have resistance alleles at a maximum number of QTL—both global and environment-specific. In this research, we identified unique germplasm accessions as donors of resistance alleles. For spot blotch, FONT 130 and 284 (two genotypes of ICARDA origin and G-selected for ME1 and P-selected between them for the three ME) have complimentary alleles at the QTL linked to or markers 11_20931, 11_10821, 11_10610, 11_20889, and 11_21271 (Supplemental Table 1). Two ICARDA lines (FONT 348 and 351) and one OSU line (derived from a cross involving ICARDA germplasm, FONT 206) have positive alleles at the QTL defined for ME2. For leaf rust, FONT 121 and 233 (two genotypes from ICARDA, G-selected for both ME, and P-selected for one ME each one) have complimentary allelic states for QTL linked to markers 11_20509, 11_10085, and 11_11473. Finally, for stripe rust the cross of FONT 156 and 165 (one accession selected by INIA-Uruguay and one genotype from ICARDA, both G- and P-selected for ME1) provides the opportunity to pyramid favorable alleles at QTL linked to 11_20566, 11_21212, and 11_10843.

There is a risk that GWAS will not have identified all resistance alleles in the germplasm array since rare, but valuable, alleles may be overlooked due to MAF selection. When a genotypic mean for a class with only few individuals is estimated, its precision is very low, and most likely it would create a false positive. This problem is related to population size; in our case, with a population of 360 individuals, estimating means with 36 individuals or more is reasonable. However, using fewer individuals would decrease the precision considerably. When population sizes are very high (i.e., above 1,000) a lower MAF could be used without compromising precision because the actual number of individuals for each class will still be sufficient. For this reason, GWAS may not always be appropriate for identifying rare alleles. If individuals with the rare alleles could be easily identified phenotypically, then classical biparental (or multi-parental) QTL studies could be conducted to map the QTL. The fact that GWAS may not detect rare alleles should be considered when designing the germplasm panel. There are trade-offs. Genetic diversity is required in the population to be able to map QTL, however, artificially increasing the population size to include extreme individuals will make the task of mapping more difficult (i.e., accounting for population structure could be a challenge). Phenotyping would also become a challenge with increased population size. Other strategies with a combination of GWAS with more balanced populations and nested association mapping have been proposed to increase the power to detect rare variants (Mott and Flint 2002; Meuwissen et al. 2002; Zhu et al. 2008).

If the goal is to accumulate all favorable alleles conferring resistance to multiple diseases, genomic selection (GS) could be used (Meuwissen et al. 2001; Heffner et al. 2009). GS was demonstrated to be superior to GWAS followed by MAS (Bernardo 2008; Lorenz et al. 2011; Heffner et al. 2011). A training population for GS is an excellent tool for GWAS, as the phenotype and genotype data are generated at no additional cost, and the information gained from the GWAS provides a reference point for monitoring changes in allele frequency in response to selection. Additionally, GWAS could lead us to a better understanding of the genetic inheritance of quantitative traits.

Conclusions

We found significant marker-trait associations for leaf rust, spot blotch and stripe rust resistance. Most of the significant associations were detected with the use of different mixed models. Explicit mixed models for QTL by environment interaction were used to fit the final multi-QTL models. This analysis provides a straightforward interpretation of QTL in terms of global vs. environment-specific effects and detection of cross-over interactions. We found nine QTL that were coincident with those previously discovered using bi-parental mapping, thus validating the effectiveness of GWAS. That these resistance QTL were identified in very different germplasm suggests specific regions of the genome could be targeted for intensive characterization to reveal the underlying physical architecture of regions harboring multiple resistance genes. We also detected novel QTL—an encouraging finding that suggests characterization of novel

germplasm is a worthwhile endeavor. GWAS is, therefore, a promising tool for detecting QTL for inclusion in MAS programs and we identify specific accessions that will be useful for pyramiding multiple resistance genes. However, MAS is limited by the number of QTL alleles that can be targeted. Therefore, a combination of GWAS with balanced-population QTL mapping could be effective for trait detection while GS could advance genetic gain.

Author contribution statement LG and AJC designed the experiments. LG, SG, SP, PH, and AJC selected and created the population. SG SP, DF, AL, CAP and AJC conducted disease phenotyping in Uruguay. GO, EN, HA, VG, and RE conducted disease phenotyping in Peru. FC, JH-E, SS, and RS conducted disease phenotyping in Mexico. EEF conducted disease phenotyping in Ecuador. KT conducted disease phenotyping in Canada. LG, NB and AJC conducted statistical analysis. LG and AJC wrote the paper. All authors edited the paper.

Acknowledgments Funding for this project was provided by FONTAGRO (Project FTG 0617-06) and Competitive Grants program for scientific visits of the Comisión Sectorial de Investigación Científica (CSIC), Universidad de la República (UDELAR), Uruguay. The authors wish to express their appreciation for the effort of the technical personnel of all the involved institutions.

Conflict of interest All authors have no conflict of interest. All the experiments conducted under this study comply with the current laws of all the countries in which they were conducted.

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