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Genome-wide association mapping of genetic factors controlling Septoria tritici blotch resistance and their associations with plant height and heading date in wheat

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Abstract Septoria tritici blotch (STB), caused by the ascomycete fungus *Zymoseptoria tritici* (also known as *Mycosphaerella graminicola*), is one of the most devastating foliar wheat diseases worldwide. Host resistance is the most effective strategy for management of the disease. A factor that complicates the determination of resistance is its reported interaction with heading date (Hd) and plant height (Ph). In this study, we report findings from a genome-wide association study of resistance to STB in a world-wide collection of 96 wheat accessions. The collection was evaluated under conditions of artificial infection for seedling and adult plant STB resistance, Hd and Ph in field trials. Marker-trait associations (MTAs) were detected using a mixed linear model. STB disease severities showed significant phenotypic variation. In total, 73 MTAs involving STB resistance were detected. The chromosomal locations of some of them were similar to known *Stb* genes or quantitative trait loci; whereas others were detected in new genomic regions. The field experiment showed evidence of genetic association between STB resistance and Hd, but only for a few genotypes. This was corroborated at the molecular level, where a total of eight genomic regions associated with STB resistance were located in similar positions to MTAs for Hd. New genomic regions associated with STB resistance found here could be useful in wheat breeding aimed at controlling STB after validation in relevant genetic backgrounds

Keywords DArT · *Triticum aestivum* · *Zymoseptoria tritici*

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Introduction

Septoria tritici blotch (STB), caused by the ascomycete fungus *Zymoseptoria tritici* (also known as *Mycosphaerella graminicola*), is one of the most devastating foliar wheat diseases (King et al. 1983). It is considered one of the top two or three diseases in

most wheat-growing regions worldwide, including Europe, North America, South America and Australia (Eyal et al. 1987). Control of STB relies to a large extent on the use of fungicides. In Europe, more than 70% of the fungicides applied to wheat are used to control this disease (Goodwin 2007). The excessive use of fungicides to control the disease has led to resistance and/or reduced sensitivity against the widely used chemical groups such as the strobilurins, triazoles and most recently succinate dehydrogenase inhibitors (SDHIs) (Cools and Fraaije 2008; Torriani et al. 2009; Dooley et al. 2016). As a result, the identification and use of resistance genes is the most effective, economical and environmentally safe strategy for successful management of the disease. As described by Brown et al. (2015) resistance to STB may be isolate-specific or quantitative, polygenic and isolate-nonspecific. Specific interactions between wheat cultivars and *Z. tritici* isolates may occur in both seedling tests and under field conditions (Arraiano et al. 2001).

During the last decade, 21 major resistance genes (*Stb* genes) were identified and characterized (McIntosh et al. 2013). Nevertheless, this number is limited mainly because most of them exhibit isolate-specific resistance, thus limiting their use (Arraiano and Browm 2006). The pathogen population is highly diverse (Goodwin et al. 2011) because *Z. tritici* undergoes several sexual cycles during the wheat-growing season (Suffert and Sache 2011). Genetic recombination increases the likelihood of emergence of new variants and the risk of adaptation to resistance genes deployed in the host population. This emphasizes the need to identify new genes and to combine them in the same genetic background. Such combinations could improve the level and durability of resistance under field conditions, a strategy that has sometimes been effective in controlling other crop diseases (Mundt 2014). As well as reports on mapping single *Stb* genes there are several recent studies on mapping quantitative trait loci (QTL) (Brown et al. 2015). Complete and partial resistance to STB may be also combined; there are several quantitative studies that reveal the presence of general and specific combining ability of resistance to STB (Van Ginkel and Scharen 1987; Simón and Cordo 1998).

Many STB resistance factors have been identified in classical bi-parental mapping populations. Although linkage analysis in bi-parental crosses has been successful in identifying QTL associated with traits of agronomic importance the approach has major disadvantages. It demands considerable time and cost in the development of segregating populations, besides being able to identify the actual resistance alleles in the two parents (Adhikari et al. 2011). Association mapping (AM) or linkage disequilibrium mapping is an alternative method of QTL detection that allows identification of direct associations between genotype and phenotype. Numerous marker-trait associations (MTAs) in wheat were detected in earlier GWAS studies exploiting DArT marker technology (Crossa et al. 2007; Adhikari et al. 2011; Neumann et al. 2011).

One of the most confounding factors in determining resistance to STB is the reported correlation between resistance, plant height (Ph), and heading date (Hd) (Simón et al. 2005). Many phenotypic studies assumed that this relationship was due to genetic linkage (Rosielle and Brown 1979; Eyal 1981; Baltazar et al. 1990). This was confirmed in genetic studies by the co-localization of QTL for STB resistance and Hd loci as well as Ph QTL (Risser et al. 2011; Goudemand et al. 2013; Kollers et al. 2013). However, in these studies disease development was measured on the same day for all cultivars. According to Arama et al. (1999) and Simón et al. (2004, 2005) disease severity should be measured at the same stage of development in order to compare the resistance of a group of cultivars and its correlation with earliness and height. Covariance analysis was proposed by Van Beuningen and Kohli (1990) and Simón et al. (2004) to identify resistant genotypes independently of Hd and Ph. Otherwise, differences in disease levels between earlier and later, or shorter and taller, cultivars may be due to differences in leaf age as well as to differences in the duration of exposure of leaves to the disease. Phenotypic studies that consider this aspect (Arama et al. 1999; Simón et al. 2005) conclude that this correlation is due to epidemiological or environmental factors rather than genetic linkage. However, the presence of genetic linkage may depend on the genetic material.

The goals of the current study were to (i) assess seedling and adult stage STB responses of a core collection of 96 winter wheat accessions of diverse origin, (ii) identify MTAs for resistance to *Z. tritici* expressed as necrosis percentage (NP) and pycnidial coverage (PC) through GWAS, (iii) compare the results with the chromosomal locations of known *Stb* resistance

genes and QTL, and (iv) determine phenotypic and genetic relationships between STB response, Ph and Hd.

Materials and methods

Plant materials

In this study, a core collection of 96 winter wheat accessions (mainly cultivars and advanced breeding lines) from 20 countries across five continents was used as an AM population (Table 1). These genotypes were carefully targeted from a larger collection maintained at the Institute of Field and Vegetable Crops (Novi Sad, Serbia), on the basis of contrasting phenotypic expression of traits of agronomic importance, including those tested in this study (Kobiljski et al. 2002; Quarrie et al. 2003). The collection included a number of important "founder genotypes" widely used as parents in breeding programs across the world.

Experimental design and phenotypic trait evaluation

Seedling stage

Two field experiments were carried out at the J. Hirschhorn Experimental Station, Faculty of Agricultural and Forestry Sciences, National University of La Plata, Argentina during 2012 and 2013 under artificial inoculation in a split–split–plot design with two blocks. Main plots were years (2012 and 2013) and subplots were inoculations with two isolates (FALP0112 and FALP0212) within each year. The sub-subplots were 98 genotypes (two Argentinean spring wheat cultivars: BioINTA 3005 and Buck SY 110, susceptible to *Z. tritici* used as controls, and the 96 wheat accessions). Because many genotypes had winter growth habit seeds were germinated in Petri dishes and vernalized for 3 weeks at 4–8 °C in a growth chamber before planting in the field. The experiments 1 and 2 were sown on 21 June 2012 and 31 July 2013, respectively. Ten to 15 seedlings of each genotype were planted in each row. Plots were 0.5 m single rows separated by one row of oat to reduce interplot interference; rows were 0.2 m apart. Weather data (monthly precipitation, relative humidity, and minimum, maximum and mean temperature) were recorded at a Davis Meteorological Station situated 300 m from the experiments.

The entire collection was inoculated with *Z. tritici* isolates FALP0112 and FALP0212 from the Argentinean locations Pla and Nueve de Julio, respectively. Inoculum was prepared by aseptically scraping sporulating colonies with a scalpel and suspending the conidia in deionized water. The conidial suspension was adjusted to 5×10^6 spores mL^{-1} using a Newbauer counting chamber. Tween 20 (Polysorbate 20) was added at 0.5 mL L^{-1}. The plant material was inoculated at growth stage (GS) 12 (Zadoks et al. 1974) until runoff with a manually operated sprayer, which was calibrated at a rate of 10 L 100 m^{-2}. After inoculation, plants were kept moist by sprinkling water several times a day over a 3 days period to maintain humid conditions necessary for disease development. In order to estimate seedling resistance, disease severity was assessed 28–30 days after the first inoculation at the same time for all cultivars by estimating the NP and PC on the inoculated three first leaves of seven plants of every plot for each replication. Evaluations started when STB was well expressed in the two susceptible Argentinean cultivars used as a control.

Adult plant stage

Three field experiments (1, 2 and 3), sown on 21 June 2012, 14 June and 31 July 2013, respectively, under artificial infection with the above isolates were conducted at the J. Hirschhorn Experimental Station. The experiments were performed as described above with inoculations being made at tillering (GS 23). For evaluation of adult-stage response disease severities were assessed 30 d after flag leaf emergence by estimating the NP and PC on the three upper leaves (flag leaf, flag leaf-1, flag leaf-2) of seven plants of every plot for each replication. Average responses of the three leaves of each plot were calculated for both growth stages.

Ph was recorded as the average of 10–15 plants per plot, measured in centimeters from the soil surface to the tip of the spike. Days to heading were recorded as the number of days from planting until 50% spike emergence.

Phenotypic data analysis

The phenotypic data collected and calculated across trials (NP, PC, Ph and Hd) were analyzed by combined analysis of variance (ANOVA) for split–

Table 1 Name, growth type and origin of the 96 wheat genotypes in the germplasm set, along with their sub-group Q, defined by STRUCTURE analysis (assigned to a sub-group if probability > 0.5) (Kobiljski et al. 2002)

Genotype	Origin	Growth type	Q	Genotype	Origin	Growth type	Q
Magnif 41	ARG	Winter	1	Florida	USA	Winter	1
Cook	AUS	Spring	1	Hays 2	USA	Winter	1
Kite	AUS	Spring	1	Helios	USA	Winter	1
Min. Dwarf	AUS	Spring	1	Holly E	USA	Winter	1
Timson	AUS	Winter	1	Hope	USA	Winter	1
Triple Dirk B	AUS	Spring	1	INTRO 615	USA	Winter	1
Triple Dirk S	AUS	Spring	1	Lr 10	USA	Winter	1
Rusalka	BUL	Winter	1	Norin 10/Brev.14	USA	Winter	1
Lambriego Inia	CHL	Winter	1	Phoemix	USA	Winter	1
Al-Kan-Tzao	CHN	Spring	1	Puerd./Loras	USA	Winter	1
Ching-Chang 6	CHN	Spring	1	Purd.38120	USA	Winter	1
Peking 11	CHN	Facultative	1	Purd.5392	USA	Winter	1
Ana	CRO	Winter	1	Red Coat	USA	Winter	1
ZG 1011	CRO	Winter	1	Semilla Eligulata	USA	Winter	1
ZG 987/3	CRO	Winter	1	UC 65680	USA	Spring	1
ZG K 3/82	CRO	Winter	1	Vel	USA	Winter	1
ZG K 238/82	CRO	Winter	1	WWMCB 2	USA	Spring	1
ZG K T 159/82	CRO	Winter	1	Ivanka	SER	Winter	1
Cappelle Desprez	FRA	Winter	1	Mina	SER	Winter	1
Durin	FRA	Winter	1	NS 22/92	SER	Winter	1
Avalon	GBR	Winter	1	NS 559	SER	Winter	1
Brigand	GBR	Winter	1	NS 602	SER	Winter	1
Highbury	GBR	Spring	1	NS 63-24	SER	Winter	1
TJB 990-15	GBR	Winter	1	NS 66/92	SER	Winter	1
Bankut 1205	HUN	Winter	1	NS 79/90	SER	Winter	1
L-1	HUN	Winter	1	Renesansa	SER	Winter	1
Szegedi 768	HUN	Winter	1	Sava	SER	Winter	1
Hira	IND	Spring	1	Slavija	SER	Winter	1
Sonolika	IND	Spring	1	Gala	ARG	Winter	2
Suwon 92	IND	Winter	1	Triple Dirk B (bulk)	AUS	Spring	2
UPI 301	IND	Spring	1	BCD 1302/83	MDA	Winter	2
Acciaio	ITA	Facultative	1	Cajeme 71	MEX	Spring	2
Ai-bian	JPN	Spring	1	Bezostaja 1	RUS	Winter	2
Norin 10	JPN	Winter	1	Centurk	USA	Winter	2
Saitama 27	JPN	Spring	1	Lr 12	USA	Winter	2
Tr. Compactum	LV	Winter	1	Tr. Sphaerococcum	USA	Winter	2
Inia 66	MEX	Spring	1	L 1/91	SER	Winter	2
Mex. 120	MEX	Spring	1	L 1A/91	SER	Winter	2
Mex. 17 bb	MEX	Winter	1	Nizija	SER	Winter	2
Mex. 3	MEX	Spring	1	Nov. Crvena	SER	Winter	2
S. Cerros	MEX	Spring	1	Nova banatka	SER	Winter	2
Vireo “S”	MEX	Winter	1	NS 33/90	SER	Winter	2
F 4 4687	ROM	Winter	1	NS 46/90	SER	Winter	2
Donska polupat.	RUS	Winter	1	NS 55-25	SER	Winter	2

Table 1 continued

Genotype	Origin	Growth type	Q	Genotype	Origin	Growth type	Q
Tibet Dwarf	TIB	Spring	1	NS 74/95	SER	Facultative	2
Tom Thumb	TIB	Winter	1	PKB Krupna	SER	Winter	2
Mironovska 808	UKR	Winter	1	Pobeda	SER	Winter	2
Benni Multifloret	USA	Winter	1	Sofija	SER	Winter	2

ARG Argentina, *AUS* Australia, *BGR* Bulgaria, *CHL* Chile, *CHN* China, *CRO* Croatia, *FRA* France, *GBR* Great Britain, *HUN* Hungary, *IND* India, *ITA* Italy, *JPN* Japan, *MDA* Moldova, *MEX* Mexico, *ROM* Romania, *RUS* Russia, *SRB* Serbia, *TIB* Tibet region of China, *UKR* Ukraine, *USA* United States of America

split–plot design in Genstat 12 Ed (VSN International 2009). Heritability (h^2) was calculated as the ratio of genotypic to phenotypic variance: $h^2 = \sigma_g^2 / \left(\sigma_g^2 + \sigma_{ge}^2/E + \sigma^2/ER\right)$. Where σ_g^2 denotes the genotypic variance, σ_{ge}^2 the genotype × environment interaction variance and σ^2 the error variance. E and R are the number of environments and blocks, respectively. On the other hand, multiple linear regression analyses, considering disease severity (NP and PC) as the dependent variable and Hd and Ph as the independent variables were performed to determine the relationship and percentage of variation in severity accounting for the morpho-physiological traits.

Genotyping, population structure and linkage disequilibrium analysis

DArT technology was provided by Triticarte Pty Ltd (Canberra, Australia; http://www.triticarte.com.au), a whole-genome profiling service laboratory. Each of the 874 polymorphic markers was named using the prefix ''wPt'' followed by a unique numerical identifier. Thirty-nine DArT markers with <5% allele frequencies were excluded from all analyses. The integrated map of DArT markers developed by Crossa et al. (2007) was used to assign 525 trait-associated markers to chromosome arms. The chromosomal locations of previously unmapped markers were provided by Triticarte. The methodology and results concerning population structure and DL of this population were described earlier (Neumann et al. 2011). ANOVA was performed for all phenotypic traits using population subgroups to determine the significance of the confounding effect of population structure on the phenotypic traits.

Marker-trait association (MTA) analysis

For MTA analysis, each trait was represented by the means of the two blocks in each experiment and analyses were performed separately for every experiment. Associations between the markers and each trait were calculated using a mixed linear model (MLM) based on both Q-matrix and kinship-matrix, suggested by Yu et al. (2006), with the software program TASSEL 2.1. The Q-matrix was derived from STRUCTURE and kinship-matrix was calculated in TASSEL. The efficient mixed model analysis (Kang et al. 2008) was chosen to reduce computing time and the MLM parameters were left at the default settings from TASSEL. In all cases, MTAs were considered significant when the P value was < 0.05 and highly significant at $P < 0.01$. Consistency through at least two experiments was taken into account as an additional criterion to reduce the risk of false MTAs. Maps and significantly associated regions with each of the traits were represented with the MapChart version 2.3 software (Voorrips 2002). Genetic distances are provided in centimorgans (cM).

Results

Weather conditions

Considering the whole growing season (June to December), trends of temperature and relative humidity were similar for both years (2012 and 2013) (Fig. 1a, b). In contrast, the rainfall pattern and total precipitation varied considerably between years. Total precipitation in 2012 was 687.6 mm and in 2013, 414.1 mm compared to the regional mean of

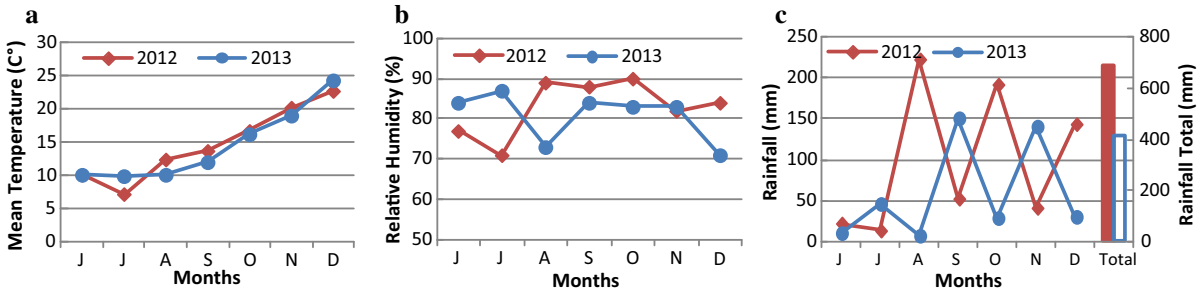

Fig. 1 Weather data for two growing season (2012 and 2013): **a** monthly mean temperature; **b** monthly mean humidity (%); and **c** monthly mean and total rainfall

519.5 mm. The most important difference in monthly rainfall was for August, where precipitation was much higher in 2012 (222.8 mm) compared to 2013 (7.2 mm) and therefore more conducive to disease development (Fig. 1c).

Phenotypic data

Analysis of variance revealed highly significant differences ($P < 0.001$) among genotypes regarding response to STB, Hd and Ph in the combined data analysis across experiments (Table 2). NP and PC were highly significant ($P < 0.001$) at both the seedling and adult stages, for genotypes, experiments × genotypes, isolates × genotypes and experiments × isolates × genotypes interactions. There were also significant differences ($P < 0.001$) between genotypes and experiments × genotypes in days to Hd and Ph. Moreover, there were significant differences for Hd ($P < 0.001$) but not for Ph ($P = 0.49$). Some lines showed a differential response at both the seedling (Fig. 2a, b) and adult (Fig. 2c, d) stages. The level of resistance of some genotypes also differed between seedlings and adult plants (Fig. 2e, f) for PC and NP, respectively.

The resistance components (NP and PC) at the seedling and adult stages showed only moderate correlations across experiments (Fig. S1). This was due to the considerable variation in rainfall between 2012 and 2013, and consistently reflected in the disease severity values. Consequently, mean values of NP and PC were higher in 2012 than in 2013. Heritability was 0.40 and 0.76 for NP and PC at seedling stage, whereas at adult stage it was 0.75 and 0.78 for each resistance component, respectively. Finally, Hd and Ph as expected, had higher heritabilities of 0.95 and 0.98, respectively.

Regarding the two structural groups previously determined in this mapping population by Neumann et al. (2011) there were significant differences for

Table 2 Mean squares for the combined analysis of variance of NP and PC caused by *Z. tritici* at seedling and adult stage for the AUDPC, Hd and Ph in 96 wheat accessions in field experiments

Source of variation	df	Seedlings		Adult stage		Hd	Ph
		NP	PC	NP	PC		
Experiments (E)	2	9.4***	304.6	27095.2***	5343***	120859.8***	918.4
Isolates (I)	1	444.2*	388.4	2336***	10800***	15	35.4
E × I	2	34.4	1696.5*	1135.3***	439.8***	71.9	124.9
Genotypes (G)	97	276***	63.3***	2572.7***	592***	1551.3***	4960.8***
E × G	194	164.5***	15.3***	635***	127.8***	82.3***	99.3***
I × G	97	101.9***	13.4***	170.5***	144.2***	8.4	17.8
E × I × G	194	103.5***	26.8***	242.2***	65.7***	8.3	21
Error	582	1.4	1.6	7	0.5	7.7	20.4

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

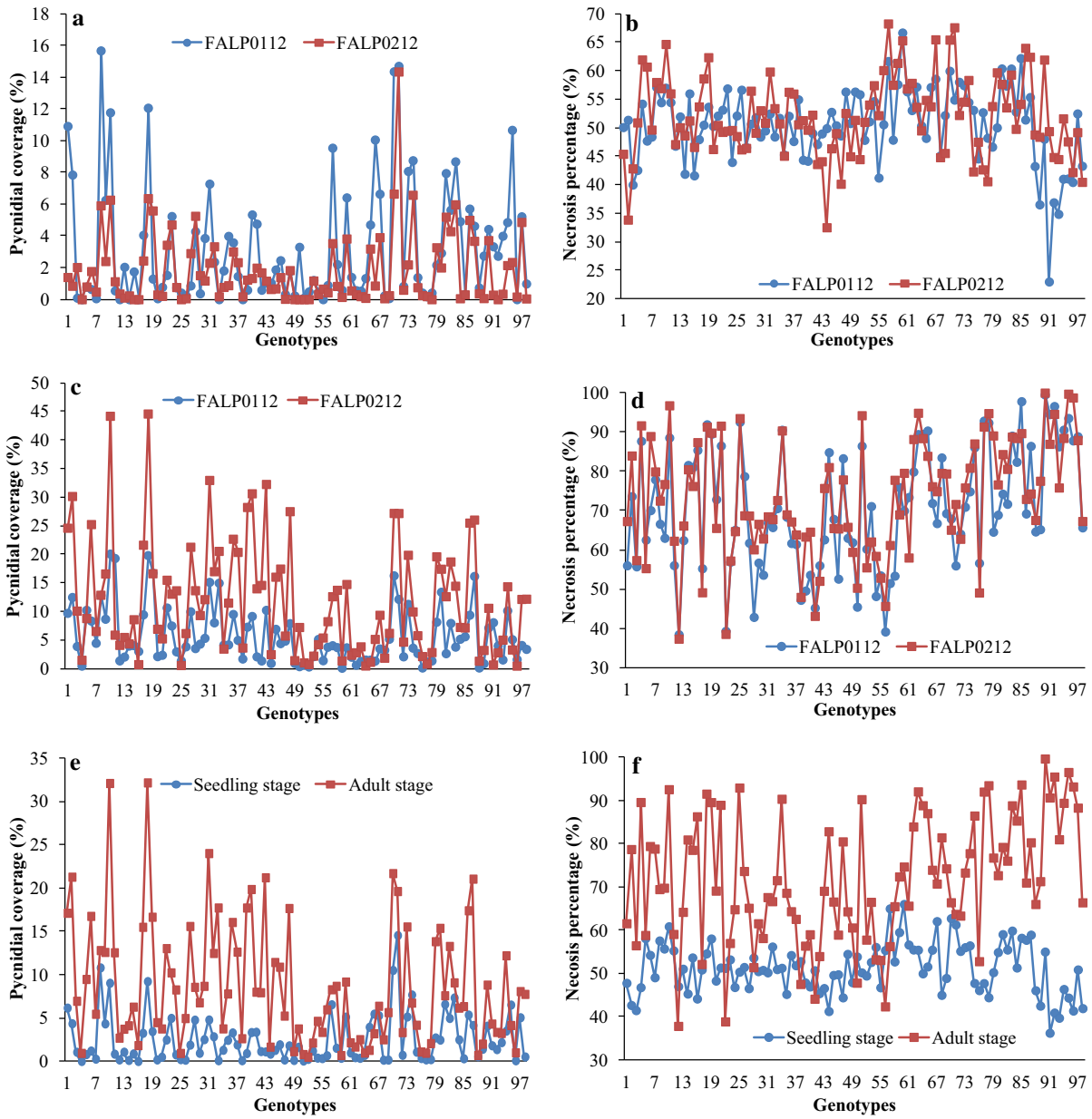

Fig. 2 Response of the mapping population to STB expressed as necrosis percentage (NP) and pycnidial coverage (PC) against isolates FALP0112 and FALP0212 at the seedling (**a** and **b**) and adult (**c** and **d**) stages, and between seedlings and adult plants (**e** and **f**)

STB response expressed as PC and NP at the seedling and adult stages ($P < 0.01$) and Hd ($P < 0.01$). Considering PC at both stages, for example, group Q1 comprising genotypes of diverse origin was more resistant with a mean of 2.40 and 7.76% compared to Q2, which contained mainly Serbian elite lines and cultivars, with means of 2.96 and 11.78%, respectively, for the averages of isolates and experiments. At both stages, the more resistant genotypes in Q2 originated mainly from countries like Tibet, USA, Ukraine and Great Britain. The average Hd of genotypes within group Q1 was about three days earlier than genotypes in group Q2.

Marker-trait association (MTA) analysis

Considering all assessed traits 100 MTAs involving 78 DArT markers were identified. Sixty-one markers were associated with a single trait and were therefore called trait-specific markers; whereas the others involving two or more traits were considered multi-trait markers. For a given trait two associated markers were considered as belonging to the same associated locus/region when they were separated by a distance of less than 2 cM or shared at least one associated group and were less than 5 cM apart. In this way, association analysis revealed several genomic regions conferring resistance to STB among the collection of 96 mainly winter wheat accessions.

Thirty-eight MTAs involving 30 DArT markers were significantly associated with STB response of seedlings. Eight and 10 of them were effective against the FALP0112 isolate, whereas six and 14 were effective against isolate FALP0212 for NP and PC, respectively. Seven markers were associated with both isolates. The 30 DArT markers corresponded to 26 distinct genetic loci/regions on chromosomes 1A (two), 1B (two), 2A (one), 2B (three), 2D (one), 3A (one), 3B (two), 3D (one), 4B (two), 5A (one), 5B (one), 6A (three), 6B (two) and 7A (four). The *P* values of significant MTAs and phenotypic variations explained by DArT markers across experiments are presented in Table 3.

Thirty-five MTAs (32 DArT markers) in 24 genomic regions were associated with STB response at the adult plant stage. These regions involved 14 chromosomes: 1B (two), 1D (one), 2A (one), 2B (three), 2D (one), 3A (two), 3B (one), 4A (one), 4B (one), 5D (two), 6A (two), 6B (one), 7A (four), and 7B (three). Fourteen and five markers were effective against FALP0112, whereas seven and 10 were effective against FALP0212 for variables NP and PC, respectively. Among all significant markers three were common for both isolates. *P* values of significant MTAs and phenotypic variations explained by the DArT markers across of the experiments are presented in Table 4. Markers significant for PC at the adult stage, including wPt7094, wPt8168, wPt7062, wPt3883, and wPt7734 located on chromosomes 1B (position 11.9 cM), 1B (44.7), 4B (109.8), 7A (88.8), and 7A (89.6), respectively, were also significant at the seedling stage.

Twelve MTAs (11 markers) were found for Ph on chromosomes 1B (one), 2B (one), 3A (two), 4A (one), 6A (one), 6B (two), 7A (two), and 7B (two). Nine markers were associated only with this trait, whereas the other three were involved in other trait-associations (two with Hd and one with STB response). For Hd 18 MTAs were located on chromosomes 1A (one), 1B (two), 2B (three), 3B (three), 4B (one), 5D (two), 6A (two), and 6B (four). Seven were associated only with Hd, nine with STB response, and two with Ph (Table 5).

Relationships between STB resistance, Hd and Ph

At both the seedling and adult stages multiple linear regression analysis between PC as the dependent variable and Ph and Hd as independent variables, yielded significant R^2 values for most isolates and experiments. In general, regression coefficients of Hd were negative, significant and very consistent. By contrast coefficients for Ph were rarely significant and inconsistent. Multiple linear regression analysis using NP as the dependent variable and Hd and Ph as independent variables was also significant for most isolates and experiments at both growth stages. Regression coefficients for Hd were also significant, but the relationship was negative for seedling assays and positive for adult plant tests. Again, when NP was used as the dependent variable, regression coefficients for Ph were inconsistent. For the average of all experiments and isolates, models including both independent variables accounted for 11.95 and 6.35% and 8.8 and 32.3% of the variation in PC and NP, in seedlings and adult plant stage, respectively (Table 6).

In addition, genetic linkage among markers significantly associated with STB resistance, Hd and Ph was determined following the classification proposed by Maccaferri et al. (2005), who defined four classes of marker pairs: 1—tight linkage (distance < 10 cM); class 2—moderate linkage (10–20 cM); class 3—loose linkage (20–50 cM); and class 4—independence (>50 cM). Considering both growth stages, a total of eight genomic regions (one at the seedling, two at the adult, and five at both growth stages) on 1BS (two), 2BS, 3BS, 4BS, 5DS and 6AS and three genomic regions (one at the seedling, one at the adult and one at both growth stages) on 1BS, 3AS and 6AS respectively) associated with STB resistance were found in a similar position and close linkage with markers associated with Hd and Ph, respectively (Fig. 3).

Table 3 Marker-trait associations detected in both experiments for seedling STB response to isolates FALP0112 and FALP0212 expressed as NP and PC using 96 wheat accessions

Trait	Marker	Chr	Pos	Experiment 1		Experiment 2	
				p MLM	R^2	*p MLM*	R^2
STB NP FALP0112	wPt4765	1A	6.3	7.5E−03	0.0864	1.1E−02	0.0789
	wPt7030	1A	23.6	1.0E−02	0.0806	6.7E−03	0.0890
	wPt0694	2B	94.3	1.2E−02	0.0787	4.7E−02	0.0396
	wPt6854	3A	11.7	3.4E−02	0.0672	3.9E−02	0.0640
	wPt7341	3B	17.1	4.9E−02	0.064	4.9E−02	0.0460
	wPt8855	3B	17.1	3.1E−02	0.0648	4.1E−02	0.0424
	wPt2748	3B	44.0	3.1E−02	0.0648	4.1E−02	0.0424
	wPt6116	6B	171.4	4.2E−02	0.0493	5.2E−03	0.1070
STB NP FALP0212	wPt8168	1B	44.7	2.4E−02	0.0542	2.2E−02	0.0579
	wPt6240	1B	45.6	1.9E−03	0.0972	4.3E−02	0.0589
	wPt7306	2A	59.6	5.0E−03	0.0895	4.1E−02	0.0593
	wPt4144	2D	5.3	4.9E−02	0.0422	1.8E−02	0.0797
	wPt8650	4B/7A	7.8/67.7	4.2E−02	0.0337	3.2E−02	0.0649
	wPt3924	5A	62.6	3.0E−03	0.0855	7.4E−02	0.0428
STB PC FALP0112	wPt8455	1A	27.1	4.8E−03	0.1010	6.9E−03	0.1370
	wPt4029	1A	28.8	2.6E−03	0.1060	9.4E−03	0.1280
	wPt8267	1B	11.6	1.6E−02	0.0883	4.2E−02	0.0518
	wPt7094	1B	12.0	3.4E−02	0.0622	3.9E−02	0.0559
	wPt8168	1B	44.7	3.1E−02	0.0660	6.6E−03	0.1810
	wPt2106	2B	22.8	5.9E−02	0.0638	2.5E−02	0.0838
	wPt0049	2B	116.0	5.0E−02	0.0457	2.1E−02	0.0542
	wPt8650	4B/7A	7.8/67.7	7.5E−03	0.0923	3.6E−02	0.0589
	wPt3457	5B	92.3	1.6E−02	0.0829	1.1E−02	0.0871
	wPt7763	7A	222.6	2.9E−02	0.0740	3.9E−02	0.1040
STB PC FALP0212	wPt8455	1A	27.1	4.3E−03	0.1300	1.1E−02	0.0959
	wPt4029	1A	28.8	6.7E−03	0.1040	1.0E−02	0.0925
	wPt8267	1B	11.6	4.9E−02	0.0503	3.3E−02	0.0669
	wPt8168	1B	44.7	1.2E−03	0.1480	2.7E−02	0.0845
	wPt3451	1B	45.6	4.7E−02	0.0513	1.4E−02	0.0815
	wPt6240	1B	45.6	4.0E−03	0.1190	4.2E−02	0.0525
	wPt2757	3D	6.0	3.3E−03	0.1150	3.1E−02	0.0651
	wPt7062	4B	109.9	2.2E−02	0.0743	4.9E−02	0.0416
	wPt8650	4B/7A	7.8/67.7	4.7E−02	0.0436	2.1E−02	0.0822
	wPt0832	6A	3.9	1.3E−02	0.1010	3.7E−02	0.0575
	wPt7599	6A/6B	56.5/7.2	6.5E−04	0.1540	2.7E−02	0.0637
	wPt3883	7A	88.8	2.1E−02	0.0840	4.2E−02	0.0511
	wPt7734	7A	89.6	2.2E−02	0.0831	3.6E−02	0.0594
	wPt4555	7D	97.1	2.6E−03	0.1150	2.3E−02	0.0726

Chr chromosome, *Pos* marker position in the linkage map

Discussion

We report the results of a GWAS aimed at identifying genomic regions conditioning resistance to STB, Hd and Ph in a core collection of 96 mainly winter wheat accessions, employing 525 polymorphic DArT markers. Considerable phenotypic variation was observed in all measured traits across the 96 accessions. This allowed to study of the genetic basis of phenotypic variation under a wide range of environmental

Table 4 Marker-trait associations significant through at least two experiments for adult plant STB resistance expressed as NP and PC of FALP0112 and FALP0212 isolates in 96 wheat accessions

Trait	Marker	Chr	Pos	Experiment 1		Experiment 2		Experiment 3	
				p MLM	R^2	*p MLM*	R^2	*p MLM*	R^2
STB NP FALP0112	wPt0003	2A	6.07	4.9E−02	0.0237	6.2E−04	0.0644	5.3E−03	0.0678
	wPt0615	2B	69.67	ns	0.0056	1.1E−02	0.0568	2.7E−02	0.0431
	wPt0714	3A	18.47	3.6E−02	0.0750	ns	0.0258	1.2E−02	0.0786
	wPt9432	3B	44.03	1.9E−02	0.0513	ns	0.0071	1.1E−02	0.0773
	wPt9510	3B	44.4	3.9E−02	0.0497	ns	0.0043	2.4E−02	0.0714
	wPt1400	5D	0	ns	0.0302	6.3E−03	0.0843	4.0E−03	0.0732
	wPt2856	5D	17.6	4.4E−02	0.0660	ns	0.0003	2.3E−02	0.0648
	wPt3524	6A	25.68	2.0E−02	0.1542	ns	0.0027	4.3E−02	0.0896
	wPt7027	6A	26.71	1.2E−02	0.1691	ns	0.0120	3.7E−03	0.1162
	wPt0689	6A/7B	25/225	1.5E−02	0.1601	ns	0.0027	2.3E−02	0.0991
	wPt3376	6B	60	3.2E−02	0.0714	5.E−02	0.0585	5.0E−02	0.0421
	wPt6034	7A	57.83	4.3E−03	0.1038	3.6E−03	0.1133	1.2E−02	0.0659
	wPt7653	7B	45.88	3.4E−02	0.0476	3.8E−02	0.0461	1.9E−03	0.1243
STB NP FALP0212	wPt0003	2A	6.07	ns	0.0006	1.2E−03	0.0461	2.6E−03	0.0494
	wPt0950	2B	31.27	5.0E−02	0.0425	4.0E−02	0.0917	4.0E−02	0.0374
	wPt0615	2B	63.67	ns	0.0367	5.5E−03	0.0691	2.7E−03	0.0756
	wPt0714	3A	18.47	1.5E−02	0.0630	ns	0.0516	ns	0.0479
	wPt4725	3A	21.03	3.6E−03	0.0897	4.2E−03	0.0484	1.1E−02	0.0596
	wPt8479	4A	56.85	7.7E−03	0.0706	3.9E−02	0.0542	ns	0.0359
	wPt1853	7B	30.65	ns	0.0191	2.4E−02	0.1085	3.6E−02	0.0680
STB PC FALP0112	wPt8168	1B	44.74	1.0E−02	0.0782	3.2E−03	0.0837	ns	0.0119
	wPt9380	1D	49.22	4.0E−02	0.0598	3.4E−04	0.1166	ns	0.0000
	wPt5503	1D	49.85	4.0E−03	0.0851	1.7E−03	0.0896	ns	0.0001
	wPt6003	2D	4.53	2.5E−02	0.0528	ns	0.0119	3.8E−02	0.0520
	wPt4229	6A	135.9	ns	0.0141	2.3E−02	0.0642	3.2E−02	0.0419
STB PC FALP0212	wPt7094	1B	11.95	4.6E−02	0.0559	2.3E−02	0.0480	ns	0.0015
	wPt0413	1D	49.19	4.3E−02	0.0529	9.0E−03	0.0656	ns	0.0011
	wPt4916	2B	17.67	1.9E−02	0.0734	3.8E−02	0.0418	ns	0.0066
	wPt7062	4B	109.87	2.4E−02	0.0422	4.1E−02	0.0424	ns	0.0080
	wPt3965	6A	37.83	2.4E−02	0.0656	6.8E−03	0.0707	6.0E−03	0.0593
	wPt7623	6A	38.01	ns	0.0318	2.3E−02	0.052	3.1E−03	0.0640
	wPt3572	7A	74.52	ns	0.0513	1.5E−02	0.0596	4.0E−02	0.0436
	wPt3883	7A	88.82	1.2E−02	0.0767	2.1E−02	0.0517	4.3E−01	0.0064
	wPt7734	7A	89.57	1.2E−02	0.0811	1.5E−02	0.0568	ns	0.0094
	wPt8920	7B	12.43	1.5E−02	0.0404	5.0E−02	0.0395	2.4E−03	0.0587

Chr chromosome, *Pos* marker position in the linkage map

conditions. Regarding STB severity, the higher levels observed in 2012 compared to 2013 were attributed to more favorable environmental conditions for disease development. The main source of *Z. tritici* inoculum during the spring and summer are pycnidiospores (Eriksen and Munk 2003; Suffert and Sache 2011). The pycnidiospores are spread from the base and disperse upwards in the crop canopy through rain

Table 5 Marker-trait associations significant through at least two experiments for plant height and heading date in 96 wheat accessions

Trait	Marker	Chr	Pos	Experiment 1		Experiment 2		Experiment 3	
				p MLM	R^2	*p* MLM	R^2	*p* MLM	R^2
Plant height	wPt8168	1B	44.7	4.0E−02	0.0625	4.5E−02	0.0397	2.7E−02	0.0695
	wPt0408	2B	68.3	3.0E−03	0.0798	2.0E−04	0.1181	2.0E−03	0.0855
	wPt1562	3A	19.2	4.4E−02	0.1395	4.1E−02	0.1565	1.7E−02	0.1664
	wPt1688	3A	41.9	9.0E−04	0.1372	1.8E−03	0.1280	5.0E−03	0.1229
	wPt2151	4A	183.6	1.1E−03	0.1470	8.2E−03	0.1120	1.4E−02	0.1139
	wPt0959	6A/6B	57/14	2.4E−02	0.0828	ns	0.0521	2.0E−02	0.1129
	wPt1048	6B	118.0	2.0E−02	0.0958	1.5E−02	0.1041	3.9E−02	0.0881
	wPt0433	7A	129.3	1.4E−02	0.1610	3.2E−03	0.1745	9.2E−03	0.1488
	wPt6495	7A	222.5	3.6E−02	0.1264	ns	0.0800	3.9E−02	0.1009
	wPt1553	7B	91.2	2.7E−02	0.0555	ns	0.0407	3.9E−02	0.0534
	wPt6320	7B	137.9	4.2E−02	0.1106	2.0E−02	0.1254	4.9E−02	0.1074
Heading date	wPt5374	1A/2B	2/52	ns	0.0862	1.9E−02	0.1119	3.1E−02	0.1017
	wPt1328	1B	11.6	1.5E−02	0.0508	1.5E−02	0.0574	3.8E−02	0.0376
	wPt6240	1B	45.5	1.3E−02	0.1677	1.1E−02	0.1622	1.6E−02	0.1348
	wPt0335	2B	75.9	1.3E−02	0.1211	2.0E−02	0.0971	2.6E−02	0.0916
	wPt9736	2B	95.7	2.5E−02	0.0506	1.8E−02	0.0450	1.4E−02	0.0637
	wPt9432	3B	44.0	3.9E−02	0.0705	8.4E−03	0.0970	2.9E−02	0.0773
	wPt9510	3B	44.4	4.0E−02	0.0771	4.6E−03	0.1091	4.0E−02	0.0814
	wPt3921	3B	74.9	2.9E−02	0.0962	ns	0.0302	1.9E−02	0.0813
	wPt6209	4B	110.5	6.1E−03	0.0129	6.4E−03	0.0946	1.7E−03	0.1521
	wPt1400	5D	0	2.5E−02	0.0392	4.4E−03	0.0553	2.9E−03	0.0572
	wPt2856	5D	17.6	2.4E−03	0.1073	2.6E−02	0.0743	1.5E−02	0.0892
	wPt7063	6A	77.7	6.0E−03	0.1942	1.4E−02	0.1333	7.7E−03	0.1535
	wPt0959	6A/6B	57/14	3.6E−02	0.1108	4.0E−02	0.0833	1.7E−02	0.1171
	wPt7954	6B	47.5	3.0E−02	0.1112	6.4E−03	0.1234	4.5E−02	0.0995
	wPt1241	6B	62.5	ns	0.0410	2.2E−02	0.0834	4.2E−02	0.0529
	wPt1541	6B	180.9	1.9E−02	0.1404	ns	0.0743	4.8E−02	0.1047

Chr chromosome, *Pos* marker position in the linkage map

splash. Significant differences between the two subgroups generated by STRUCTURE were also observed for STB response at the seedling and adult stages and for Ph. These results are in agreement with Edae et al. (2014), who reported significant effects of population structure in winter wheat on Ph and stem-related traits.

Moderately high heritabilities of combined and separate seedling and adult responses were observed for the variables NP and PC, ranging from 0.40 to 0.79, due to resistance genes that were effective across environments. Thus, repeatable and reliable phenotypic data for GWAS were obtained. Moderately high heritabilities of STB response were also reported in other studies (Goudemand et al. 2013; Kollers et al. 2013; Simón and Cordo 1998) indicating that resistance to STB can be improved by selection.

Specific interactions among wheat cultivars and *Z. tritici* isolates may occur in combined, and separate seedling and adult-plant stage tests (Arraiano et al. 2001; Simón et al. 2005). In our study, correlations between seedling and adult plants for PC showed moderate values for both isolates, indicating that there were genotypes with combined seedling and adult

Table 6 Multiple linear regression for PC and NP caused by two isolates of *Z. tritici* as dependent variables and with Hd and Ph as independent variables on the panel of 96 winter accessions at the seedling stage and adult plant in field experiments

Growth stage		Parameter (*P* value)							
		FALP0112				FALP0212			
		Constant	Hd	Hg	R^2	Constant	Hd	Hg	R^2
Exp 1									
PN	Seedling	106.80***	−0.12**	−0.06	0.08*	112.32***	−0.20**	0.00	0.11*
	Adult	−32.16*	0.85***	0.04	0.44***	34.35*	0.32*	0.08	0.10**
PC	Seedling	19.85***	−0.17**	0.09	0.10**	4.95***	−0.04**	0.01	0.08*
	Adult	7.68	0.01	−0.01	0.00	49.51***	−0.25*	−0.01	0.06*
Exp 2									
PN	Seedling	19.26*	−0.09	0.05	0.03	26.05*	−0.18	0.09	0.04
	Adult	−4.76	0.59**	0.25**	0.18***	−28.73	0.89***	0.18	0.21***
PC	Seedling	7.22***	−0.08***	0.02*	0.17***	11.74***	−0.13***	0.04*	0.12***
	Adult	9.88	−0.01	−0.04	0.02	36.53**	−0.22	−0.06	0.04
Exp 3									
PN	Adult	−101.38***	1.26***	−0.00	0.58***	−58.34***	0.97***	−0.00	0.40***
PC	Adult	24.04***	−0.13***	−0.03*	0.26***	39.68***	−0.20**	−0.08	0.14***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

stage resistance and others displaying resistance only at specific growth stages. Simón et al. (2005) proposed that selection at both stages might be done to achieve acceptable levels of resistance for the entire growing period. Additionally, several genotypes in the core collection showed similar reactions to both isolates. Although more isolates should be tested this suggests that non-isolate-specific horizontal resistance may be also present. This type of resistance is an important genetic resource for the improvement of wheat against STB as reported in earlier studies (Jlibene et al. 1994; Simón and Cordo 1998).

Despite the high economic importance of STB only 21 major resistance genes have been named (McIntosh et al. 2013). However, many QTL for partial resistance have been reported, and these have greater significance for breeding (Brown et al. 2015). Here we report a total of 73 MTAs related to 57 DArT markers significantly associated with STB resistance. The 53 DArT markers were distributed over 42 chromosomal regions on 18 of the 21 chromosomes. Among these regions, 18 and 16 were specifically associated with resistance to STB at the seedling and adult stages. The remaining 8 regions showed DArT markers simultaneously associated with resistance at both growth stages. These results are consistent with phenotypic performance and indicate that resistance at both the seedling and adult stages is controlled by some common regions but also by different regions.

Of all the chromosomal regions associated with STB response those located on 1BS, 1DS, 2BL, 3AS, 3BS, 5AL, 6AS, 7AS and 7BL, were identified at similar regions to known genes *Stb11/Stb2, Stb10, Stb9, Stb6, Stb14, Stb17, Stb15, Stb3 and Stb8,* respectively (Brown et al. 2015). However, further work is needed to confirm whether the genomic regions found here correspond to known *Stb* genes. It would be important to use DArT markers found in our study for marker-assisted selection (MAS). As far as we know no *Stb* gene is located on chromosome arms 1AS, 1BL, 2AS, 2BS, 3BL 2DS, 4AS, 4BS/L, 5BL, 5DS, 6AL, 6BS/L, 7AL and 7BS where genomic regions associated with STB response were found in the present population. This infers that new sources of STB resistance were detected in our study.

In addition to identified genomic regions suggestive of *Stb* genes DArT markers wPt7030 (1AS), wPt5672 (2BS), wPt8650 (4BS), wPt7062 (4BL), and wPt7777 located in the same position as wPt1856 were previously reported as significant for STB by Risser et al.

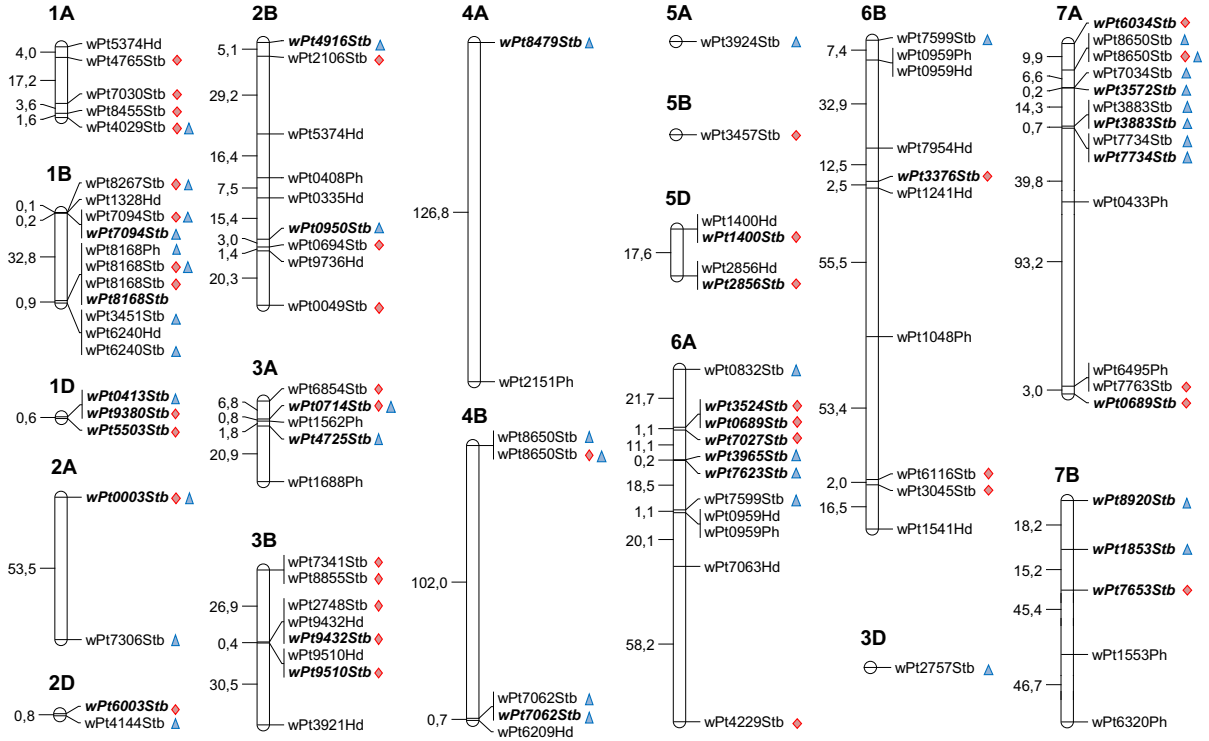

Fig. 3 Genetic map including marker-trait associations (MTAs) identified for measured phenotypic traits. Markers in *bold* represent MTAs detected at the seedling stage. Underlined and not underlined markers correspond to PC and NP, respectively. Symbols *diamond* and *triangle* represent isolates FALP0112 and FALP0212, respectively. Genetic distances between markers are in centimorgans (cM)

(2011) and Goudemand et al. (2013). On the other hand, the genomic regions on chromosomes 1AS (markers wPt5374 and wPt4765), 1BS (markers wPt1328 and wPt7094) and 6AL (markers wPt0959 and wPt4229) were reported as conferring resistance to tan spot (caused by *Pyrenophora tritici-repentis*), leaf rust (*Puccinia triticina*) and yellow rust (*Puccinia striiformis*) (Crossa et al. 2007; Singh et al. 2015). Broad-spectrum resistance (BSR) has been widely reported in wheat, including detection in non-adapted germplasm with resistance to multiple diseases and clustering of resistance genes in distinct genomic regions (Gurung et al. 2009). These results are also in line with those reported by Miedaner et al. (2012, 2013) and Zwart et al. (2010), who found genomic regions conferring BSR to Fusarium head blight (*Gibberella zeae*), tan spot, STB and leaf rust. Stacking QTL that affect response to traits of interest from different chromosome regions into one background is a challenging and time consuming task in plant breeding. The use of multi-trait markers in MAS may increase QTL pyramiding efficiency (Edae et al. 2014).

Regarding the relationship between STB resistance and traits Hd and Ph there was a significant negative association between earliness and STB response expressed as PC, a finding that agrees with previous results (Risser et al. 2011; Goudemand et al. 2013). Although not always significant NP also showed a negative trend with earliness. Ph by contrast displayed no consistent relationship between variables (NP and PC) and experiments. These results are in agreement with Simón et al. (2004), who only found associations between susceptibility and reduced Ph in very short wheats, indicating that moderately short wheats are not necessarily more susceptible to STB. At seedling all genotypes were inoculated and scored at the same growth stage and at the same time and therefore disease development was subjected to the same weather conditions. We assume that the relationship found between earliness and STB infection at the

seedling stage is due to a genetic association between the two traits. However, the low correlation values could be attributed to only a few genotypes (e.g. Tibet Dwarf, Tom Thumb, Brigand, Ana, Mexico 3, Helios, Hira) that showed this association, contrary to many others where this relationship did not occur. In the latter group, for example, the genotypes Lambriego Inia and Tr. Compactum with moderate-long growth cycles (131 and 134 d to Hd) showed higher values (56.62 and 3.95%, respectively) for NP and PC than the averages (51.23 and 2.53%) for isolates and experiments. Genotypes like Triple Dirk 'S' and Ai-Bian with moderately short growth cycles (103 and 105 d to Hd) showed lower values (41.43 and 0.6%) for NP and PC, respectively, than the average (51.23 and 2.53%) for both isolates and experiments.

PC maintained a negative relationship with Hd at the adult stage, but this was not always significant. All genotypes were scored at the same growth stage (30 days after flag leaf emergence) but at different dates. Weather conditions (temperature, precipitation and humidity) during the period of exposure of the leaves to the pathogen obviously varied within and between experiments. Temperatures were higher for later genotypes (20.75, 19.04 and 19.68 °C) than for earlier ones (17.9, 14.34 and 17.62 °C, respectively) in all three experiments. For PC, an optimum temperature of 25 °C was reported (Hess and Shaner 1987). Hence, temperatures were more favorable for the expression of the disease in later genotypes than in earlier ones, but in general, this was not reflected in the final levels of PC. Conversely, humidity was slightly lower for later genotypes (82.05, 82.91 and 79.66%) than for earlier ones (88.63; 84.68 and 81.89%) in the three experiments, respectively. Finally, precipitation was higher for the later (2.82, 4.48 and 3.24 mm) than for the earlier (5.49, 1.51 and 2.46 mm) genotypes in two of the three experiments. These results assume the presence of genetic association, which is supported by the fact that the relationship between earliness and STB infection expressed as PC was always negative despite the fluctuations in the weather conditions. In contrast, there was a significant and positive association between earliness and NP. Apparently, this could be due to the greater values of radiation and temperature to which later genotypes were exposed. This could have generated higher levels of senescence due to other causes.

Interestingly, at both stages, all the genotypes that were significant for the association between Hd and PC belonged to sub-group 1 (Q1), except one (NS 46/90) that belonged to sub-group 2 (Q2). This finding as well as low r values indicated that presence or absence of genetic linkage between Hd and STB resistance depended on genotype. Thus, as reported by Simón et al. (2004) some STB resistance factors are subject to this complication (association between resistance and Hd or Ph) but are not. Hence it should be possible to improve the STB resistance independently of Hd.

The genetic associations between STB resistance and Hd were confirmed by the presence of genetic linkage or co-localization of markers associated with both traits. Eight genomic regions associated with STB resistance were located in similar positions to Hd MTAs. One region for seedling stage STB resistance, two for adult stage response and five identified at both stages were in similar positions to genes for Hd. Associations of resistance genes on chromosomes 2BL and 3BL and Hd found in this study were also reported by Risser et al. (2011). This indicates that the genetic association between STB response and Hd not only depends on the evaluated material, but also the growth stage.

In conclusion, this study identified new germplasm with moderate to high levels of STB resistance to two pathogen isolates. Some genomic locations seem to be new whereas others were at similar positions to known *Stb* genes and QTL. Confirmation of these results is required and it would be important to use DArT markers found in our study for MAS. The STB resistances found in our study involve several loci, each explaining only a small part of the total phenotypic variance. Conventional selection of QTLs with small effects on phenotypic variation could be improved in a breeding population through cycles of MAS for multiple QTLs. Although there were significant associations of STB resistance and Hd this applied to only relatively few genotypes.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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