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New broad-spectrum resistance to septoria tritici blotch derived from synthetic hexaploid wheat

S. Mahmod Tabib Ghaffary · Justin D. Faris · Timothy L. Friesen ·
Richard G. F. Visser · Theo A. J. van der Lee ·
Olivier Robert · Gert H. J. Kema

Abstract Septoria tritici blotch (STB), caused by the ascomycete *Mycosphaerella graminicola*, is one of the most devastating foliar diseases of wheat. We screened five synthetic hexaploid wheats (SHs), 13 wheat varieties that represent the differential set of cultivars and two susceptible checks with a global set of 20 isolates and discovered exceptionally broad STB resistance in SHs. Subsequent development and analyses of recombinant inbred lines (RILs) from a cross between the SH M3 and the highly susceptible bread wheat cv. Kulm revealed two novel resistance loci on chromosomes 3D and 5A. The 3D resistance was expressed in the seedling and adult plant stages, and it controlled necrosis (*N*) and pycnidia (*P*) development as well as the latency periods of these parameters. This locus, which is closely linked to the microsatellite marker *Xgwm494*, was tentatively designated *Stb16q* and explained from 41 to 71% of the phenotypic variation at seedling stage and 28–31% in mature plants. The resistance locus on chromosome 5A was specifically expressed in the adult plant stage, associated with SSR marker *Xhbg247*, explained 12–32% of the variation in disease, was designated *Stb17*, and is the first unambiguously identified and named QTL for adult plant resistance to *M. graminicola*. Our results confirm that common wheat progenitors might be a rich source of new *Stb* resistance genes/QTLs that can be deployed in commercial breeding programs.

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S. M. Tabib Ghaffary · T. A. J. van der Lee · G. H. J. Kema (✉)
Plant Research International, Biointeractions and Plant Health,
P.O. Box 16, 6700 AA Wageningen, The Netherlands
e-mail: gert.kema@wur.nl

S. M. Tabib Ghaffary · R. G. F. Visser ·
T. A. J. van der Lee · G. H. J. Kema
Graduate School of Experimental Plant Sciences,
Wageningen University, Wageningen, The Netherlands

Present Address:
S. M. Tabib Ghaffary
Safiabad Agricultural Research Centre,
P.O. Box 333, Dezfoul, Iran

J. D. Faris · T. L. Friesen
Northern Crop Science Laboratory, USDA-ARS Cereal Crops
Research Unit, 1307 18th Street North, Fargo,
ND 58102-2765, USA

R. G. F. Visser
Department of Plant Breeding, Wageningen University,
P.O. Box 386, 6700 AJ Wageningen, The Netherlands

O. Robert
Bioplante, Florimond Desprez, BP41,
59242 Cappelle-en-Pévèle, France

Introduction

Since early history, wheat (*Triticum aestivum* L., $2n = 6\times = 42$, AABBDD) was a main source of food and feed. It is the oldest and has been the most widely grown crop since 10,000–8,000 BC (Heun et al. 1997; Luo et al. 2007; Nesbitt and Samuel 1998). Due to its importance and increasing demand, it is a key commodity to eradicate global hunger not only by ensuring sufficient production to feed a world population that will grow by 50% and reach 9 billion by 2050, but also by guaranteeing access to food (FAO 2010). Still, in 2010 annual bread wheat production is projected to decline and diseases play a significant role in such reductions (USDA 2010). In Western Europe, which is among the largest wheat production areas, septoria tritici blotch (STB) caused by the ascomycete *Mycosphaerella graminicola* (Fuckel) J. Schröt is the most recurrent and

important wheat disease. STB is also a major disease in the Americas, Central and West Asia, and particularly on durum wheat in North Africa. STB can cause yield losses that typically range from 10–15%, but under conducive weather conditions, losses can easily exceed 50%, particularly in low-input agriculture where disease management is frequently suboptimal (Duveiller et al. 2007; Eyal 1999; King et al. 1983).

In intensive wheat production areas, disease management is often accomplished by fungicide applications and the deployment of resistant wheat cultivars (Goodwin 2007; Lehoczki-Krsjak et al. 2010). STB is the major target of the agrochemical industry that has Western Europe as its prime market (Jorgensen 2008), but fungicide applications are not always timely, environmentally sound or economically viable (Paveley et al. 1997). Under conditions favorable for disease, 2–12 fungicide applications are required to control STB (Burke and Dunne 2008), and the costs easily reach approximately 150 Euro per hectare (Beest et al. 2009). Most importantly, fungicide efficacy towards STB is hampered by the development of fungicide resistant strains of the pathogen (Fraaije et al. 2005; Mavroeidi and Shaw 2005; Stergiopoulos et al. 2003). Therefore, host resistance is an important component of effective disease management strategies for commercial wheat production.

To date, 15 major resistance genes, *Stb1–Stb15*, have been identified and characterized, but compared to yellow rust, leaf rust, stem rust and powdery mildew—with 73, 89, 61 and 95 mapped resistance genes, respectively—this number is limited. Moreover, the majority of these genes have narrow spectra of specificity towards *M. graminicola* isolates that represent current field populations in major wheat producing areas, and this limits their use (Arraiano and Brown 2006; Chartrain et al. 2005b). Furthermore, *M. graminicola* is a heterothallic filamentous fungus with multiple sexual cycles during the growing season that defines its complex genetic population structure and influences disease management (Chen and McDonald 1996; Kema et al. 1996c; McDonald et al. 1996). The wheat–*M. graminicola* pathosystem complies with the gene-for-gene hypothesis where a pathogen effector interacts with a host target (Brading et al. 2002). Hence, the selection pressure that new *Stb* genes may exert on natural *M. graminicola* populations calls for responsible deployment strategies and a continuous effort to unveil key genes that control this disease (Cowger et al. 2000; Linde et al. 2002; Zhang et al. 2001).

Modern wheat improvement programs and wheat domestication processes resulted in narrow diversity of wheat germplasm (Christiansen et al. 2002; Raman et al. 2010). For this reason, wild wheat progenitors have been considered potential sources for the recovery of genetic diversity (Dreisigacker et al. 2008; Ortiz et al. 2008;

Warburton et al. 2006; Zhang et al. 2006). The production of synthetic hexaploid (SH) wheats goes back to the 1940s but is recently considered a strategic approach to exploit germplasm of wild wheat progenitors in commercial breeding programs (Mizuno et al. 2010; van Ginkel and Ogbonnaya 2007; Warburton et al. 2006; Xie and Nevo 2008; Yang et al. 2009). SHs are produced by crossing tetraploid wheat (*T. turgidum* L., $2n = 4\times = 28$, A and B genomes) with diploid goatgrass (*Aegilops tauschii* Coss., $2n = 2\times = 14$, DD genomes) followed by chromosome doubling of the F_1 hybrid. The resulting synthesized hexaploids provide a rich source of genetic variation and can be readily hybridized with elite bread wheat cultivars and germplasm. Breeders have exploited these sources for resistance to a wide range of biotic and abiotic stresses (Adhikari et al. 2003; Arraiano et al. 2001b; Assefa and Fehrmann 1998, 2000, 2004; Berzonsky et al. 2004; Cakmak et al. 1999; Genc and McDonald 2004; Gororo et al. 2001; Konik-Rose et al. 2009; Lage et al. 2003, 2004; Lage and Trethowan 2008; Mujeeb-Kazi et al. 2001a, b; Sotelo et al. 2009; Xu et al. 2004, 2006). Here, we further investigate the potential of SHs and derived breeding lines as sources of resistance to *M. graminicola* in commercial resistance breeding programs.

Materials and methods

Mycosphaerella graminicola isolates, plant materials and experimental design

A global panel of 20 *M. graminicola* isolates (Table 1) was assembled and used to characterize the response of mapped *Stb* genes and compare their resistance spectrum with uncharacterized resistance to STB in SHs and derived breeding lines. A set of 20 wheat accessions comprising 13 *M. graminicola* differential cultivars, five SHs, and the susceptible checks cv. Taichung 29 and the hard red spring wheat cv. Kulm (Table 2), was tested in a triplicate seedling experiment.

F_1 and F_2 plants, and an $F_{6:7}$ population of recombinant inbred lines (RILs) developed by single-seed descent were produced from a cross between the SH M3 and cv. Kulm. M3 (W-7976) was developed at CIMMYT by A. Mujeeb-Kazi and has the pedigree Cando/R143//Mexi'S'/3/*Ae. tauschii* (C122), whereas cv. Kulm was developed at North Dakota State University, Fargo, ND. The hexaploid wheat cv. Chinese Spring (CS) and CS chromosome 5A deletion lines 5AS-1, 5AS-3, 5AL-10, 5AL-12, and 5AL-17 (Endo and Gill 1996) were used to locate chromosome 5A markers to deletion bins.

The various wheat accessions were grown in VQB $7 \times 7 \times 8$ cm TEKU® plastic pots with ten linearly sown

Table 1 The original hosts and origin of the global panel of *Mycosphaerella graminicola* isolates used in the present study

Isolate nr	Origin		
	Country	Location	Year of collection
IPO94218	Canada	Saskatoon	1994
IPO00003	USA	Colusa	2000
IPO00005	USA	Colusa	2000
IPO90006	Mexico	Toluca	1990
IPO90015	Peru	Unknown	1990
IPO87016	Uruguay	Dolores	1987
IPO86068	Argentina	Balcarce	1986
IPO99015	Argentina	Unknown	1999
IPO89011	Netherlands	Barendrecht	1989
IPO92004	Portugal	Casa Velhas	1992
IPO95054	Algeria	Berrahal	1995
IPO92034	Algeria	Guelma	1992
IPO88018	Ethiopia	Holetta	1988
IPO88004	Ethiopia	Kulumsa	1988
IPO95036	Syria	Minbeg	1995
IPO86013	Turkey	Adana	1986
IPO02166	Iran	Dezful, Safi Abad	2002
IPO02159	Iran	Gorgan, AqQaleh	2002
IPO95052[a]	Algeria	Berrahal	1995
IPO86022[a]	Turkey	Altinova	1986

[a] All isolates are bread wheat isolates except IPO95052 and IPO86022, which are durum adapted isolates

seeds per pot. RILs were planted in 5.5 × 5 cm round Jiffy® pots with three seeds per pot using a steam-sterilized peat/sand mixture. All plants were grown in a controlled greenhouse compartment with a 16/8 h light/dark cycle supplemented with son-T Agro 400 W lamps (Hortilux, Boca Raton, Florida, USA). Pre-inoculation temperature and relative humidity (RH) were 18/16°C (day/night rhythm) and 70% RH, while post-inoculation temperature and RH were 22/21°C and ≥85% RH, respectively. Plants were grown in an alpha lattice experimental design with pots as experimental units that were randomly arranged for each isolate-replication combination on separate parallel tables in the greenhouse compartment.

Inoculation procedures

Pre-cultures of each isolate (Table 1) were prepared in an autoclaved 100 ml Erlenmeyer flask containing 50 ml yeast-glucose (YG) liquid medium (30 g glucose, 10 g yeast per liter demineralized water). The flasks were inoculated using a small piece of frozen isolate mycelium maintained at −80°C and were placed in an incubated rotary shaker (Innova 4430, New Brunswick Scientific, USA) set at 125 rpm and 18°C for 5–6 days. These pre-cultures were then used to inoculate three 250 ml Erlenmeyer flasks containing 100 ml YG media per isolate that were incubated under the afore-mentioned conditions to provide enough inoculum for the

seedling inoculation assays at growth stage (GS) 11 (Zadoks et al. 1974). The inoculum concentration was adjusted to 10^7 spores/ml in a total volume of 40 ml for a set of 18 plastic pots or 24 Jiffy® pots and was supplemented with two drops of Tween 20 (MERCK®, Nottingham, UK). The screening of the 20 wheat accessions as seedlings was conducted using the collection of 20 isolates (Table 1). Seedlings of the entire RIL population were initially tested with *M. graminicola* isolates IPO92004, IPO92034, IPO94218 and IPO88018, and the results of these pre-screening experiments were used to select the most appropriate isolates (IPO94218 and IPO88018) for the second and third replications that were also used to screen F_1 and F_2 seedlings.

Adult plant screening of the RILs and parents was carried out in a greenhouse experiment with three replications using *M. graminicola* isolate IPO88018 (0.6×10^6 spores/ml) at GS 47–58.

Data collection and analysis

Wheat germplasm

Disease severity was evaluated 21 days after inoculation by estimating the percentage necrosis (N) and pycnidia (P) on the inoculated first leaves (GS 11–12) (Zadoks et al. 1974) in the seedling assays. Data were transformed to the logit scale for statistical analysis using residual maximum

Table 2 Hexaploid wheat germplasm that was tested with a global panel of 20 *Mycosphaerella graminicola* isolates to determine potentially new genes for resistance to septoria tritici blotch

Line	Growth habit	Origin	*Stb* gene	References
Bulgaria 88	W	Bulgaria	*Stb1* (5BL) + *Stb6*	(Adhikari et al. 2004c; Chartrain et al. 2005b)
Veranopolis	S	Brazil	*Stb2* (3BS) + *Stb6*	(Adhikari et al. 2004b; Chartrain et al. 2005b)
Israel 493	S	Israel	*Stb3* (7AS) + *Stb6*	(Adhikari et al. 2004b; Chartrain et al. 2005b)
Tadinia	S	USA	*Stb4* (7DS) + *Stb6*	(Adhikari et al. 2004a; Chartrain et al. 2005b; Somasco et al. 1996)
Cs Synthetic (6×)7D	S	China/USA	*Stb5* (7DS) + *Stb6*	(Arraiano et al. 2001b)
Shafir	S	Israel	*Stb6* (3AS)	(Brading et al. 2002)
Estanzuela Federal	S	Uruguay	*Stb7* (4AL)	(McCartney et al. 2003)
M6 synthetic (W-7984)	W	USA	*Stb8* (7BL)	(Adhikari et al. 2003)
Courtot	W	France	*Stb9* (2BL)	(Chartrain et al. 2009)
Kavkaz-K4500	F	CIMMYT	*Stb10* (1D) + *Stb12* (4AL) + *Stb6* + *Stb7*	(Chartrain et al. 2005a)
TE9111	S	Portugal	*Stb11* (1BS) + *Stb6* + *Stb7*	(Chartrain et al. 2005c)
Salamouni	S	Canada	*Stb13* (7BL) + *Stb14* (3BS)	http://wheat.pw.usda.gov/ggpages/awn/53/Textfile/WGC.html
Arina	W	Switzerland	*Stb15* (6AS) + *Stb6*	(Arraiano et al. 2007; Chartrain et al. 2005b)
Kulm	S	USA	*Susceptible parent*	
M3 synthetic (W-7976)	S	USA	*Stb16q* (3DL) + *Stb17* (5AL)	This study
Nogal synthetic	W	France	*Unknown*	
FD 2054.3 synthetic	W	France	*Unknown*	
TA4152-19 synthetic	S	USA	*Unknown*	
TA4152-37 synthetic	S	USA	*Unknown*	
Taichung 29	S	Japan	*Susceptible check*	

S spring type, *W* winter type, *F* facultative

likelihood (REML) variance component analysis (Genstat 13th edition, VSN International Ltd, Hemel Hempstead, UK). Significant differences were determined using the least significant difference (LSD) of back-transformed N and P values. Logit transformed data analysis resulted in minor changes between observed and processed data to cope with zero scores of N or P.

RILs

A total of 96 RILs were evaluated in the pre-screening (first replication) and 103 RILs in the second and third replications. Disease severity on the seedlings was evaluated 23 days post inoculation (dpi) by scoring N and P on the primary leaves. Latency periods (*NLP* and *PLP*: days between inoculation and first N and P appearance) were also determined in the second and third replications of the seedling assays. Adult plant responses—total leaf area covered with sporulating STB lesions—were scored on the flag leaves (F) or the second leaf layer (F-1) at 21 and 28 dpi. Bartlett's χ^2 test was employed to evaluate the homogeneity of replication error variances and calculated

using the Excel formula option. Data homogeneous across replications were subsequently averaged and used for QTL analysis (Chu et al. 2010; Friesen et al. 2009).

Molecular mapping in the RIL population

DNA was extracted from M3, cv. Kulm and the RILs as described in Faris et al. (2000). A total of 609 microsatellite (simple sequence repeat; SSR) primer pairs were tested on M3 and cv. Kulm to reveal polymorphisms. The microsatellite primers were derived from the following sets: GWM (Roder et al. 1998), WMC (Somers et al. 2004), HBG, HBD, HBE (Torada et al. 2006), CFA, CFD (Sourdille et al. 2004), BARC (Song et al. 2005), and FCP (Faris et al. 2010; Reddy et al. 2008; Zhang et al. 2009). Methods for PCR, polyacrylamide gel electrophoresis, and fragment visualization were as described in Lu et al. (2006). Primer pairs revealing polymorphism between M3 and cv. Kulm were subsequently used to genotype the 103 RILs.

A total of 284 of the 609 (47%) primer sets revealed polymorphisms and detected 349 marker loci (1.2 loci per

primer set). Linkage analysis of the 349 loci was conducted using Mapmaker (Lander et al. 1987) for Macintosh and the Kosambi mapping function (Kosambi 1944) as described in Liu et al. (2005).

QTL analysis

Linkage maps consisting of 296 markers giving the most complete genome coverage were used to detect genomic regions associated with phenotypic means. Composite interval mapping (CIM) was performed using the computer program QGene (Joehanes and Nelson 2008). A permutation test with 1,000 permutations was conducted to determine that a critical LOD threshold of 4.7 in this population yields an experiment-wise significance level of 0.05.

Genotype to phenotype discrepancy

Analysis of the allelic marker segregation and concurrent phenotypic data of the RILs enabled us to study genotype to phenotype discrepancies with respect to STB resistance. We used all observed disease parameters (N, P, NLP and PLP) and distributed the RILs in statistically significantly different ($\chi^2_{1:1}$) groups. Subsequently, marker segregation was superimposed on these data to determine sliding windows of lower to upper limits of the aforementioned disease parameters for each isolate to determine the threshold values for segregation analyses. At a later stage 11 individual RILs (KM7, KM8, KM14, KM15, KM20, KM21, KM32, KM41, KM63, KM73 and KM88) were screened with the entire panel of M. graminicola isolates to confirm broad efficacy of the identified resistance loci.

Results

Wheat germplasm screen

All control inoculations resulted in excellent disease development enabling precise phenotyping of wheat germplasm and the Kulm/M3 RIL population. None of the differential cultivars was completely resistant to the global M. graminicola panel, whereas all SHs, including M3, were widely resistant to the entire set of isolates (Table 3). The number of identified Stb genes in each differential cultivar (Table 2) positively correlated with broader efficacy (r = 0.75, P < 0.01; N = 13, df = 11) indicating that accumulation of Stb genes is a valid resistance breeding strategy. In contrast, the SHs showed a significantly different pattern for they were resistant to all M. graminicola isolates (Fig. 1; Table 3). We therefore focused further analyses on the cv. Kulm/M3 RIL population. The parental lines differed significantly for N (values for cv. Kulm and

M3 ranging from 2.2 to 91.8 and 1.1 to 6.8, respectively) and P (values for cv. Kulm and M3 ranging from 0 to 37.5 and 0, respectively) over the 20 isolates (Fig. 1). This enabled the selection of isolates IPO94218, IPO92004, IPO88018 and IPO92034 for further analysis.

Mapping

The 349 microsatellite markers were assembled into linkage groups representing the 21 hexaploid wheat chromosomes and spanned a genetic distance of 2,465 cM. Only chromosomes 3D and 5A were associated with STB resistance and these will be shown here, details of map construction and analysis will be published elsewhere. The genetic map of chromosome 3D in the cv. Kulm/M3 RIL population consisted of 27 markers spanning a genetic distance of 67.9 cM and included a cluster of 18 co-segregating markers near the distal end of the long arm (Fig. 3). Comparison with the 3D deletion-based physical map indicated that this suppressed recombination occurred across much of the long arm of chromosome 3D (Fig. 4). Closer evaluation of the 3D marker profiles indicated that most were codominant, and hence, there was no indication of a large deletion on chromosome 3D in either M3 or cv. Kulm.

The linkage map of chromosome 5A consisted of 13 markers spanning 125.4 cM (Fig. 3). Of the markers mapped to 5A in the cv. Kulm/M3 population, only Xbarc180, Xcfa2250, Xbarc141, Xgwm617, Xgwm595, and Xgwm291 were previously located on the deletion-based physical map (Sourdille et al. 2004). Therefore, we tested markers Xhbd160, Xhbg247, Xhbg219, Xbarc232, Xhbd150, and Xwmc524 on the 5A deletion lines to determine their locations on the physical map. Comparison of the cv. Kulm/M3 5A genetic map with the 5A physical map indicated that the genetic linkage map of 5A developed in the cv. Kulm/M3 population accounted for most of the chromosome (Fig. 4).

Phenotyping and QTL analyses

RIL screening

We produced 103 cv. Kulm/M3 RILs and 96 were inoculated with M. graminicola isolates IPO94218, IPO92004, IPO88018 and IPO92034 in the first replication (Fig. 2). The results of this experiment indicated that segregation ratios of P fit 1:1 ratios for M. graminicola isolates IPO92004, IPO88018 and IPO92034, suggesting segregation of a single genetic factor. The result with IPO94218, however, indicated that more genes could be involved. We, therefore, continued analyses for the second and third replications with M. graminicola isolates IPO88018 and

Table 3 Phenotypic responses of wheat cultivars and synthetic hexaploids or derivatives to a global panel of 20 *Mycosphaerella graminicola* isolates. Significant differences are based on Least Significant Differences of back transformed logit values of *P*

Cultivar	*Mycosphaerella graminicola* isolates																		Durum wheat	
	Bread wheat																			
	IPO 94218	IPO 00003	IPO 00005	IPO 90006	IPO 90015	IPO 87016	IPO 86068	IPO 99015	IPO 89011	IPO 92004	IPO 92034	IPO 95054	IPO 88004	IPO 88018	IPO 95036	IPO 86013	IPO 02166	IPO 02159	IPO 86022	IPO 95052
Bulgaria	0	**6**	0	0	0	**10**	**8**	0	**4**	0	1	1	**3**	**6**	**24**	**13**	**13**	**4**	0	0
Veranopolis	22	1	2.3	0	38	4	1	1	37.6	13	0	0	**5**	**3**	**33**	**35**	**31**	0	**5**	1
Israel 493	1	1	1	0	0	3	1	0	**5**	3	24	1	0	1	**6**	17	4	1	0	0
Tadinia	0	1	1	0	1	35	2	**10**	**10**	**10**	1	**6**	**11**	**3**	**15**	**17**	**13**	**6**	0	0
Cs/synthetic (6×)7D	25	**3**	1	0	1	2	1	1	0	1	**6**	**2**	**4**	1	**12**	**12**	**16**	1	**2**	**2**
Shafir	24	**21**	13	45	41	60	**4**	22	9	15	45	15	24	22	23	24	12	1	0	0
Estanzuela Federal	4	**31**	3	42	3	1	10	32	30	16	21	1	45	33	6	26	43	16	0	0
W-7984	26	**20**	7	8	6	12	**8**	19	6	11	35	28	17	3	58	35	26	7	0	0
Courtot	1	**52**	1	30	45	5	3	55	1	18	46	13	4	1	1	1	18	0	0	0
Kavkaz-K4500	1	**11**	0	23	2	0	2	21	6	8	2	0	19	2	1	0	0	0	0	0
TE9111	0	**10**	0	0	0	0	1	0	13	4	4	0	1	1	0	2	0	1	0	0
Salamouni	0	1	1	1	4	30	**8**	7	7	1	20	2	21	1	5	31	24	10	0	0
Arina	0	**6**	0	0	5	2	2	0	6	3	1	0	0	1	1	0	1	0	0	0
Kulm	37.5	**23**	16	7	21	13	**31**	19	22	22	26	5	1	16	31	33	0	1	0	0
M3	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Nogal	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
FHD 2054.3	0	1	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0
TA4152-19	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
TA4152-37	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Taichung 29	42	63	15	45	39	55	15	41	48	24	45	31	3	24	66	32	43	11	1	0

Not significantly different from $P = 0\%$ ($P < 0.05$) has been given in italic

Not significantly different from maximal P value ($P < 0.05$) has been given in bolditalic

$2.3 < P < 37.6$ significantly different from either $P = 0\%$ or maximal P value ($P < 0.05$) has been given in bold
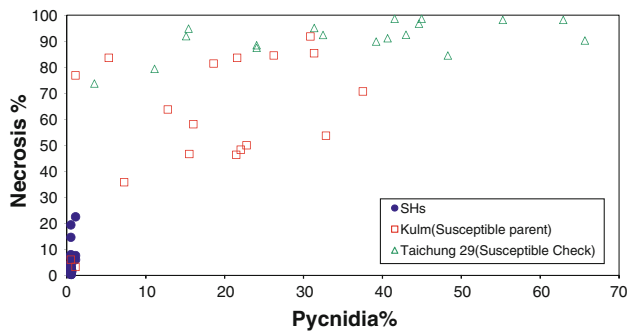
Fig. 1 Scatter plot of *N* and *P* values of SHs and the cvs. Kulm and Taichung 29 after inoculation with 18 bread wheat *Mycosphaerella graminicola* isolates

IPO94218. Ranking of the RILs for *N* and *P* showed highly significant correlations for *N* and *P*, indicating that the same genetic factor(s) could control resistance to these isolates (Table 4).

QTL analyses

Seedling resistance

QTL analysis using CIM indicated that, for both *M. graminicola* isolates IPO88018 and IPO94218, markers located on the long arm of chromosome 3D were significantly associated with *N*, *P*, *NLP*, and *PLP* in seedlings (Table 5; Fig. 3). The QTLs peaked at position 58.0 cM between SSR loci *Xwmc494* and *Xbarc125* for each trait (Figs. 3, 4), and resistance effects were derived from M3. LOD values were highly significant ranging from 11.7 to 22.3 for the phenotypes caused by isolate IPO94218 and 19.0–27.0 for those caused by isolate IPO88018 (Fig. 3; Table 5). The QTL explained from 41 to 64% of the phenotypic variation for the disease caused by isolate IPO94218, and 58–71% of the variation for disease caused by isolate IPO88018.

Adult plant resistance

QTL analysis of adult plant reactions to *M. graminicola* isolate IPO88018 indicated that the resistance locus on 3DL identified at the seedling stage, was also significantly associated with resistance at both the 21 and 28 dpi readings (Fig. 3; Tables 6, 7). The QTL peaked at the same cM position as for the seedling data for both isolates and had LOD values of 7.2 and 8.4 for the 21 and 28 dpi readings, respectively. The locus explained 28% of the variation in STB at 21 dpi, which increased to 31% at 28 dpi. In addition to the resistance locus on 3DL, an additional QTL associated with adult plant resistance derived from M3 was identified on the long arm of chromosome 5A (Fig. 3). The

5AL QTL had a LOD value of 3 and explained 12% of the variation at 21 dpi, but had stronger effects at 28 dpi with an LOD of 8.9, explaining 32% of the variation (Table 6). The 5AL QTL was flanked by SSR loci *Xgwm617* and *Xhbg247*, and it peaked approximately 3.1 cM proximal to *Xhbg247* (Fig. 3). Comparisons between the genetic and physical maps indicated that this QTL was located in the deletion bin defined by the breakpoints in deletion lines 5AL-10 and 5AL-17, which is in the distal half of 5AL (Figs. 4, 5). Comparative RIL (KM7, KM20, KM41 and KM73) genotyping/phenotyping showed that the presence of the 5AL locus in KM41 specifically incited resistance to isolate IPO88018 in adults plants, but KM41 was susceptible in the seedling stage similar to KM73 that lacks the 3DL as well as the 5AL QTL (Table 7).

F_1 and F_2 screening

Four F_1 plants were inoculated with *M. graminicola* isolate IPO88018 and showed only minor tip leaf necrosis and no pycnidia formation at 21 dpi (data not shown). Thirty-two and 28 F_2 plants were then inoculated with *M. graminicola* isolates IPO88018 and IPO94218, respectively. Segregation ratios (resistant:susceptible) for *N* and *P* did not significantly differ from the expected 3:1 (Table 8), suggesting the inheritance of a single dominant gene.

Genotyping versus phenotyping discrepancies

Analyses of the phenotypic and genotypic data indicated that lines with the *Xwmc494* allele from M3 had *P* values that ranged from 0 to 5 and *N* values from 0 to 30, with averages over both isolates of 1 and 15, respectively. On the contrary, RILs carrying the cv. Kulm allele for *Xwmc494* had values that ranged from 8 to 70 *P* and 37–100 *N*, and averaged over both isolates of 30 and 80, respectively.

Despite the indications for a single locus inheritance, the observed recombination suppression on chromosome 3D could also mask several genes at the 3D locus. We, therefore, tested RILs KM7, KM8, KM14, KM15, KM20, KM21, KM32, KM41, KM63, KM73 and KM88 with the entire panel of isolates (Table 1) to confirm either broad susceptibility or resistance with the presence of the Kulm or M3 alleles of the flanking *Xwmc494* and *Xbarc125* SSR loci, respectively (Table 9). The phenotypes of the majority of RILs was as expected either broadly resistant (KM7 and KM20 with M3 alleles of the flanking markers) or susceptible (KM15, KM41, KM21, KM63 and KM73 with M3 alleles of the flanking markers), although KM63 was unexpectedly resistant to isolate IPO86068. This could have been an incidental escape as Kulm itself also showed some variation compared to the earlier screen (Table 3).

Fig. 2 Pre-screening results (*P*) of the cv. Kulm/M3 RIL population with four *Mycosphaerella graminicola* isolates. Box **a** significantly deviates from a 1:1 ratio, whereas boxes **b–d** have segregation ratios that are not significantly different from 1:1 (based on χ^2 test; P = 0.05)
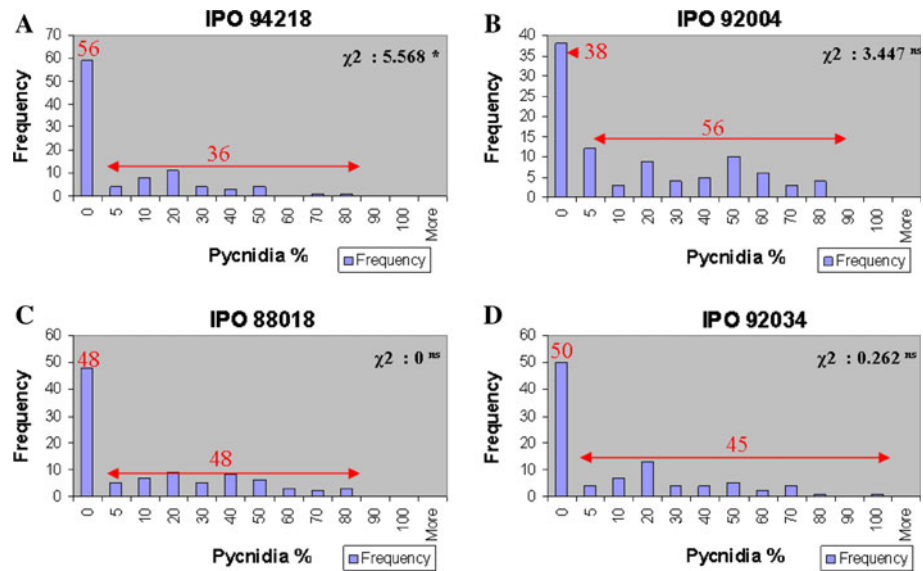
Table 4 Correlation coefficients between ranked *P* and *N* values of 86 (96 − 10 missing values for some isolates) cv. Kulm/M3 RILs after inoculations with four *Mycosphaerella graminicola* isolates

		IPO88018 *N*	IPO92004	IPO94218	IPO92034
IPO88018	*P*		0.77***	0.62*** (0.83***)[a]	0.68***
IPO92004		0.68***		0.59***	0.61***
IPO94218		0.58*** (0.83***)[a]	0.56***		0.53***
IPO92034		0.70***	0.61***	0.62***	

[a] Correlation coefficient of the second and third replication between IPO88018 and IPO94218

*** Significant at P = 0.001

Table 5 LOD and R^2 values for *Stb16q* associated with broad-spectrum seedling resistance to *Mycosphaerella graminicola* in the recombinant inbred population derived from the cross between cv. Kulm and M3

| Dataset	*Stb16q*	
	LOD	R^2
Isolate IPO88018		
% *N* average	27.0	0.71
% *P* average	19.0	0.58
NLP average	20.7	0.61
PLP average	22.8	0.64
Isolate IPO94218		
% *N* average	22.3	0.64
% *P* average	11.7	0.41
NLP average	16.9	0.55
PLP average	18.9	0.59

However, RILs KM8, KM32, KM88 and KM14, which also have the M3 alleles of the flanking markers, clearly differed from the parental phenotypes by showing specificity to isolate panel.

Discussion

Here we report two new STB resistance genes that were derived from the SH wheat line M3. Segregation and QTL analyses as well as genetic and physical mapping suggested that a single locus on chromosome 3D derived from M3 conferred resistance to all STB disease parameters in the seedling stage in the cv. Kulm/M3 RIL population. Although (1) no additional QTLs were significantly associated with any of the seedling phenotypes caused by either isolate in genome-wide scans, (2) none of the known *Stb* genes were mapped on chromosome 3D and, (3) the 3D QTL was highly significant and explained a large portion of the phenotypic variation, we cannot unequivocally conclude on single gene inheritance due to the substantial recombination suppression along the long arm of chromosome 3D, which is not due to a large deletion. However, it is possible that a large inversion exists in 3D of one of the parents, which could be the cause of the extreme suppression of recombination on 3DL. Due to the highly suppressed recombination along chromosome arm 3DL, comparison with the physical map of 3D yielded little

Fig. 3 LOD profiles of detected QTLs associated with resistance to *Mycosphaerella graminicola* isolates IPO94218 and IPO88018 on chromosomes 3DL in the seedling as well as 3DL and 5AL using IPO88018 in the adult plant stage. The *black bar* represents the centromere position
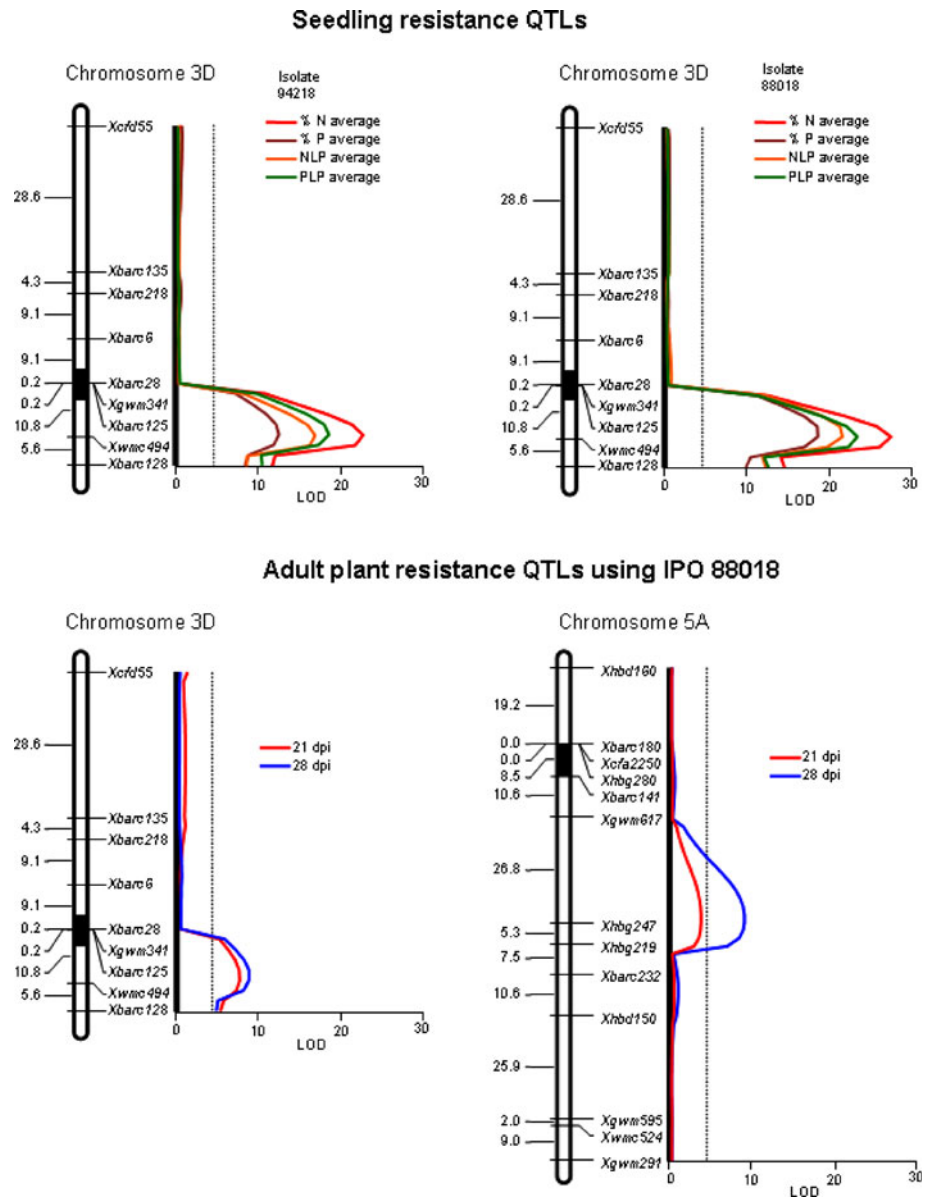

additional information. We therefore propose to tentatively designate this locus *Stb16q* in accordance with a suggestion in a recent community wide discussion on *Stb* nomenclature to add 'q' for cases where presented data do not unequivocally show that a single gene underlies a detected QTL. Indeed additional inoculation studies with 11 selected RILs using the full isolate panel showed that RILs KM8, KM32, KM88 and KM14 showed a differential pattern that cannot be explained by a single gene. Nevertheless, the results indicate that *Stb16q* lies on the long arm of chromosome 3D and that it was derived from the *Ae. tauschii* accession C122, which was the donor of the D-genome chromosomes in M3. Zwart et al. (2010) reported a SH derived QTL with multiple unrelated functions including STB resistance on chromosome 3D, but the

LOD scores were relatively low and STB resistance was only tested with a single non-characterized *M. graminicola* isolate. Our study showed that *Stb16q* had an unusually broad efficacy in the seedling stage as shown by the resistance to the global panel of isolates, and is also expressed in adult plants.

In addition we determined a QTL on chromosome 5AL that does not confer resistance to STB in seedlings, but specifically in adult plants. None of the previously characterized *Stb* genes was mapped on chromosome 5A (Arraiano et al. 2007; Chartrain et al. 2009; Goodwin 2007) and we, therefore, conclude that this QTL represents a novel gene for STB resistance. At 28 dpi is showed a highly significant LOD that explained a substantial percentage (32%) of the observed STB variation compared

Fig. 4 Comparison of the Chinese Spring chromosome 3D and 5A deletion-based physical maps with the 3D and 5A genetic linkage maps developed in the cv. Kulm/M3 population. Deletion breakpoints are indicated to the *left* of the physical maps and bin-located markers are shown along the *right*. On the linkage maps, cM distances are shown along the *left* and markers along the *right*. The QTL regions associated with STB resistance are indicated by the *red lines*
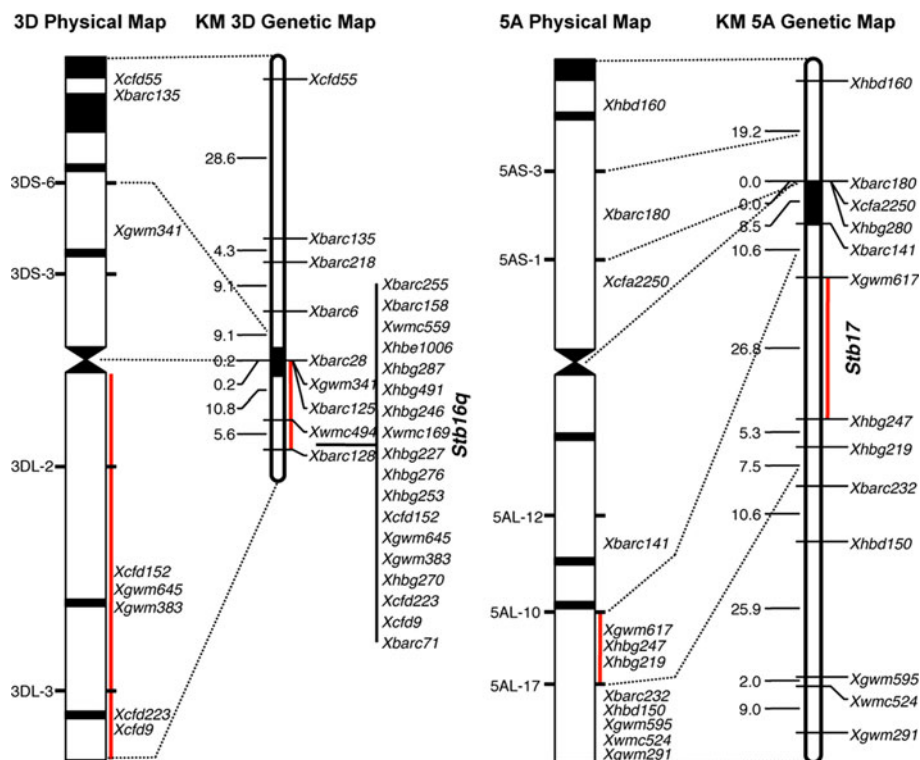
Table 6 LOD and R^2 values for *Stb16q* and *Stb17* associated with adult plant resistance to *Mycosphaerella graminicola* isolate IPO88018 in the recombinant inbred population derived from the cross between cv. Kulm and M3

Gene/Chromosome arm	Marker interval	Position (cM)	Resistance source	LOD (21 dpi/28 dpi)	R^2 (21 dpi/28 dpi)	Additive effect (21 dpi/28 dpi)
Stb16q/3DL	*Xbarc125–Xbarc128*	58.0	M3	7.2/8.4	0.28/0.31	7.4/11.9
Stb17/5AL	*Xgwm617–Xhbg247*	62.0	M3	3.0/8.9	0.12/0.32	4.5/12.3

Table 7 Comparative seedling and adult plant stage phenotyping of four Kulm/M3 recombinant inbred lines with or without the mapped *Stb16q* and *Stb17* loci

RILs	Mapped loci		Phenotyping		
			Seedlings		Adult plants (21 dpi)
	Stb16q	*Stb17*	88018	94218	88018
KM20	+	+	0	0	2
KM7	+	−	0	0	15
KM41	−	+	45	45	3
KM73	−	−	52	32	45

with the 21 dpi observations. This is not surprising since disease development in adult plants usually takes slightly longer, particularly under greenhouse conditions. A recent community wide discussion on *Stb* nomenclature suggested that *Stb* genes should at least explain 50% of the observed

STB variation before a number could be assigned to such a locus. We currently consider this as an unjustified criterion for gene designation as we recently showed that the percentage of explained variation strongly depends on the applied isolates (Tabib Ghaffary et al. 2011). Hence, we designate the 5AL locus as *Stb17*, which originated from the tetraploid durum wheat line used in the development of M3. Previous experiments conducted to compare seedling and adult plant STB resistance suggested the occurrence of specific seedling resistance loci, but no specific adult plant resistance genes were identified (Kema and van Silfhout 1997). All previously reported *Stb* genes are effective in the seedling and adult plant stage as indicated in the present study with *Stb16q*. Evidently, the unequivocal identification of specific adult plant resistance loci can only come from comparative seedling/adult plant mapping studies using the same populations. The majority of analyses has been performed in either of both stage and cannot conclude on the occurrence of specific adult plant
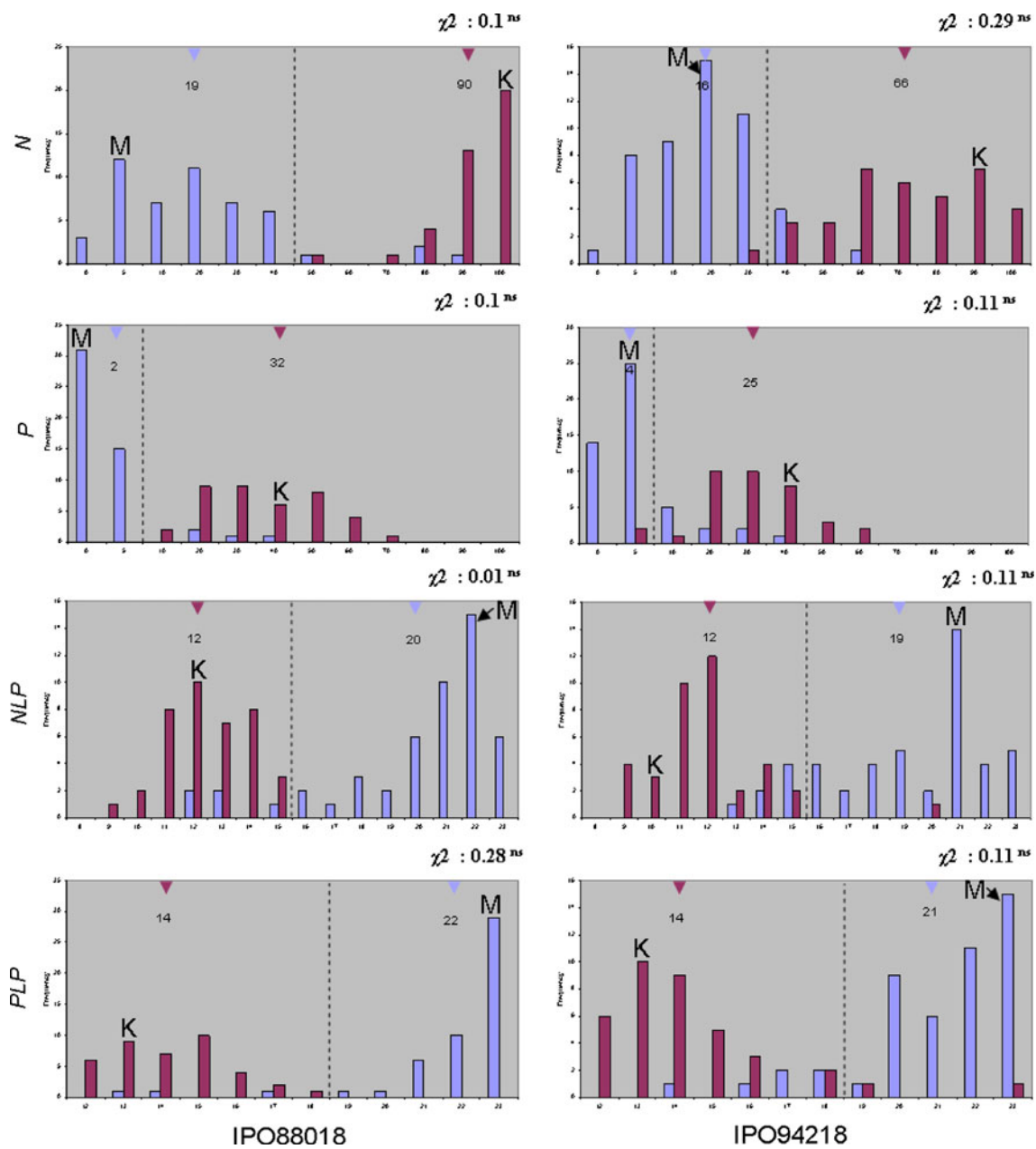
Fig. 5 Segregation for *N, P, NLP* and *PLP* in the cv. Kulm/M3 RIL population inoculated with *M. graminicola* isolates IPO88018 and IPO94218 overlaid with allelic segregation of the *Xwmc494* SSR marker which is linked to *Stb16q*. 'M' and 'K' indicate parental bin-values. *Blue* and *purple triangles* indicate average values of RILS with 'M' and 'K' alleles, respectively. The *vertical dashed line* is the $\chi^2_{1:1}$ validated threshold position between resistant and susceptible RILs

Table 8 Segregation analysis of the cv. Kulm/M3 F_2 population after inoculation with two *Mycosphaerella graminicola* isolates

[a] χ^2 for single gene segregation according to a 3R:1S ratio where R stands for resistance and S for susceptible

Isolates	Criteria	Number of plants with (+) and without (−) symptoms		χ^2 (P = 0.05)[a]
		−	+	
IPO 88018	*N*	22	10	0.67[ns]
	P	28	4	2.67[ns]
IPO 94218	*N*	19	9	0.76[ns]
	P	23	5	0.76[ns]

Table 9 Phenotyping of 11 Kulm/M3 recombinant inbred lines with the entire panel of isolates to test for coinciding broad susceptibility or resistance with the presence of the Kulm (K) or M3 (M) alleles of the flanking Xwmc494 and Xbarc125 SSR loci of Stb16q, respectively

RILs and cultivars	Flanking SSR loci of Stb16q		Mycosphaerella graminicola isolates									
	Xwmc494	Xbarc125	IPO94218	IPO00003	IPO00005	IPO90006	IPO90015	IPO87016	IPO86068	IPO99015	IPO89011	IPO92004
M3	M	M	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
KM 20	M	M	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
KM 7	M	M	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
KM 8	M	M	*5*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	**20**
KM 32	M	M	*0*	*0*	*0*	*0*	*0*	*5*	*5*	*5*	*0*	*0*
KM 88	M	M	**20**	**10**	*0*	**20**	*0*	**35**	*5*	**35**	*0*	**25**
KM 14	M	M	**25**	*0*	*5*	*0*	*0*	*5*	*0*	*0*	*0*	**15**
KM 15	K	K	**40**	**70**	**75**	**75**	**60**	**80**	**70**	**80**	**30**	**30**
KM 41	K	K	**35**	**55**	**50**	**50**	**80**	**90**	**45**	**30**	**15**	**60**
KM 21	K	K	**25**	**35**	**60**	**50**	**80**	**60**	**45**	**50**	**40**	**25**
KM 63	K	K	**55**	**95**	**30**	**50**	**50**	**80**	*5*	**70**	**55**	**50**
KM 73	K	K	**25**	**50**	**80**	**25**	**35**	**50**	**20**	**30**	**40**	**60**
Kulm	K	K	**25**	**50**	**75**	**30**	**75**	**30**	**80**	**30**	**60**	**40**
Taichung 29	check	check	**50**	**100**	**50**	**45**	**80**	**40**	**85**	**90**	**80**	**40**

RILs and cultivars	Flanking SSR loci of Stb16q		Mycosphaerella graminicola isolates									
	Xwmc494	Xbarc125	IPO95054	IPO92034	IPO88018	IPO88004	IPO95036	IPO86013	IPO02166	IPO02159	IPO95052	IPO86022
M3	M	M	*5*	*0*	*5*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
KM 20	M	M	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
KM 7	M	M	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
KM 8	M	M	*0*	*0*	**10**	*0*	*5*	*0*	*0*	*0*	*0*	*0*
KM 32	M	M	**50**	*0*	**10**	*0*	*5*	*0*	*0*	*0*	*0*	*5*
KM 88	M	M	**55**	**45**	**40**	**10**	**20**	**15**	**15**	*0*	*0*	*5*
KM 14	M	M	*0*	*5*	*5*	*0*	**10**	*0*	*0*	*0*	*0*	*0*
KM 15	K	K	**70**	**60**	**45**	**50**	**100**	**75**	**60**	**60**	*0*	*0*
KM 41	K	K	**25**	**40**	**50**	**80**	**50**	**50**	**40**	**40**	*0*	*0*
KM 21	K	K	**45**	**60**	**25**	**20**	**70**	**35**	**35**	**30**	*0*	*0*
KM 63	K	K	**30**	**80**	**50**	**65**	**75**	**70**	**70**	**50**	*0*	*0*
KM 73	K	K	**40**	**30**	**10**	**30**	**100**	**70**	**70**	**50**	*5*	*0*
Kulm	K	K	**75**	**50**	**20**	*0*	**100**	**10**	**10**	**60**	*0*	*0*
Taichung 29	check	check	**70**	**40**	**85**	**40**	**100**	**100**	**100**	**50**	*0*	*5*

R ≤ 5% pycnidia has been given in italic

5% pycnidia < S has been given in bold

resistance (Adhikari et al. 2003, 2004a, c; Chartrain et al. 2005a, b, c, 2009; Risser et al. 2011). Three studies used seedling and adult plant data in mapping analyses. Arraiano et al. (2001b) mapped *Stb5* in seedling and adult plant experiments. In the study of Simon et al. (2004) none of the scored parameters resulted in a significant QTL except for AUDPC (QStb.ipk-7B) that mapped on the same position as *Stb8*, which is also expressed in seedlings (Table 3). Finally, Simon et al. (2010) mapped resistance in seedlings and adult plants on chromosome 7D, but only interval mapping suggested that the adult plant locus was apart from *Stb4* or *Stb5* that map on the same chromosome. Moreover, the adult plant data only included limited necrosis scores, no LOD values of the identified QTLs were provided and none of them were named. We, therefore, claim that *Stb17* is the first gene for adult plant resistance to *M. graminicola* since the 5A QTL is specifically expressed in adult plants after inoculation with isolate IPO88018 and chromosome 5 has not yet been associated with resistance (the efficacy of *Stb17* to a wider set of isolates has to be determined and we cannot exclude that it might be expressed in seedlings with other isolates). This complies with what is classically described as adult plant resistance, which is very common to other cereal diseases such as the rusts and has been associated with temperature sensitivity and other abiotic environmental factors (McIntosh et al. 1995), and we adopt that interpretation here.

Interestingly, the response of M3 to the global panel of *M. graminicola* isolates was very similar to those of the other tested SHs. The broad resistance spectrum of *Stb16q* might be due to the apparent dichotomy of host specificity in the wheat-*M. graminicola* pathosystem. Kema et al. (1996a, b) summarized and extended these observations and showed that *M. graminicola* isolates are in general either pathogenic on bread wheat or durum wheat. Recently, Wittenberg et al. (2009) and Ware Sarrah (2006) showed that genetic recombination during sexual reproduction in *M. graminicola* easily results in progeny with altered cultivar and host specificity. However, tetraploid wheats are in general resistant to *M. graminicola* isolates derived from bread wheat and vice versa. This was confirmed in the current experiments because neither of the durum wheat-derived isolates IPO86022 and IPO95052 were virulent on any of the tested bread wheat accessions including the susceptible parent cv. Kulm and the susceptible check cv. Taichung 29. Therefore, a SH is expected to be resistant to such bread wheat derived *M. graminicola* isolates unless the D genome component affects the expression of resistance, which has been shown for rust diseases (Kerber and Green 1980, Kema et al. 1995). Assefa and Fehrmann (1998) also documented broad-spectrum resistance to *M. graminicola* (99% of 194 accessions) in seven *Aegilops* species, while only 8, 11, 16

and 24% of this collection was resistant to stem rust, leaf rust, eyespot and powdery mildew, respectively. Similar broad spectrum resistance was observed in phenotypic screens of the diploid wheat *T. monococcum*, which led to the identification of the resistance locus *TmStb1* and the linked microsatellite locus *Xbarc174* on chromosome 7A[m] (Jing et al. 2008). Because SHs effectively combine the genomes of tetraploid and diploid wheat progenitors and relatives (Mujeeb-Kazi et al. 1996; Yang et al. 2009), they may carry a reservoir of novel genes for resistance to *M. graminicola*. Despite the value of the genes that we discovered, exposure to *M. graminicola* populations may potentially enable the fungus to adapt and circumvent them (Wittenberg et al. 2009; Ware Sarrah 2006; McDonald and Linde 2002a, b; Linde et al. 2002; Zhan et al. 2007). Hence, their commercial deployment should take these observations into consideration to maximize their efficacy under practical conditions.

To date, there has been no report of mapping host QTLs associated with life strategy parameters such as latency period and the lesion development rate of *M. graminicola*. Here, we characterized classical (*N* and *P*) and new parameters (*NLP, PLP*) to investigate whether a major STB resistance gene also controls underlying pathogenicity factors, which is relevant, as resistance to STB is characterized by the absence of the hypersensitive response (HR) (Kema et al. 1996d). Interestingly, all the analyzed parameters mapped to the *Stb16q* locus. In the absence of the HR, resistance is achieved by reducing the development of fungal biomass, which may occur by reducing infection rates. Such partial, or 'horizontal', resistance has been observed in some cereal rust interactions (Aghnoum and Niks 2010; Marcel et al. 2008). One of the best-known 'slow rusting' loci is the *Lr34/Yr18/Pm38* complex (Singh et al. 2007), which confers partial resistance to stripe rust, leaf rust and powdery mildew. Molecular cloning of the *Lr34/Yr18/Pm38* locus indicated that it is a unique functional ABC transporter (Krattinger et al. 2009; Lagudah et al. 2009). On the contrary, genes that confer complete, or 'vertical', resistance to pathogens with biotrophic lifestyles and susceptibility to necrotrophic pathogens usually harbor NBS and LRR domains (Bent and Mackey 2007; Jones and Dangl 2006; McDowell and Simon 2006; Lorang et al. 2007; Nagy and Bennetzen 2008; Faris et al. 2010). *Tsn1*, a gene controlling sensitivity to a host-selective toxin produced by the necrotrophic fungal pathogens *Stagonospora nodorum* and *Pyrenophora tritici-repentis* has resistance gene-like features including protein kinase and NBS-LRR domains (Faris et al. 2010). Interestingly, *S. nodorum*, *P. tritici-repentis* and *M. graminicola* are close relatives and belong to the Dothideomycete class of fungi. However, nothing is currently known about the molecular characteristics of *Stb* resistance genes. Therefore, the wide

efficacy of the *Stb16q* locus and the abovementioned findings call not only for further deciphering and understanding of the resistance mechanism exerted by these new genes for resistance to STB, but also for unveiling their molecular structure.

Because the *M. graminicola*-wheat pathosystem is characterized by the absence of an HR, resistance and susceptibility are currently usually expressed on a quantitative scale. However, symptom expression is strongly affected by environmental fluctuations and hence repeatability of experiments might be low (Arraiano et al. 2001a; Bearchell et al. 2005; Czembor et al. 2010; Kema et al. 1996a). Early reports determined an arbitrary threshold of resistance and susceptibility by using a 0–5 scoring scale (Rosielle 1972) that was more qualitative than quantitative. Later, applications of complex statistics were used to turn qualitative data into qualitative determinants (Eyal and Levy 1987; Eyal et al. 1985; Yechilevich-Auster et al. 1983). Eventually, Kema et al. (1996a, b) used quantitative data in cluster analyses based on interaction components of analyses of variance to group isolates and cultivars with similar responses and hypothesized that *N* and *P* were controlled by different genetic factors in the fungal genome. This was later corroborated by formal fungal genetics (Kema et al. 2000, 2002; Wittenberg et al. 2009; Ware Sarrah 2006). Adhikari et al. (2003, 2004a, b, c) used a modified 0–5 scale, which considered pycnidia percentage and density, for the mapping of several *Stb* genes, but phenotypic classifications were not matched with allelic segregations of the associated markers. A detached leaf assessment method also has been established for the characterization and mapping of some *Stb* genes (Arraiano et al. 2001a; Chartrain et al. 2005a, c, 2009). Essentially, all these phenotyping assays address the phenotyping versus genotyping problem (Dowell et al. 2010). Here we had the opportunity to study phenotype/genotype variation in more detail using the allelic information of all RILs along with all observed disease assessment parameters. As *Stb16q* controls all the observed disease parameters for a global panel of unrelated *M. graminicola* isolates, the phenotypes of RILs with alternative parental alleles at the *Xwmc494* locus are of interest. RILs with the *Xwmc494* allele of M3 showed 0–5 *P* and 0–30 *N* values, whereas RILs with cv. Kulm allele showed 8–70 *P* and 37–100 *N* values. We do not know the origin of these sliding disease parameter windows, but we cannot exclude phenotyping errors due to environmental fluctuations, despite the accordance of all replications. We can exclude genotyping errors and recombination events between the *Xwmc494* marker and *Stb16q* as possible sources of error because the results indicate significant recombination suppression in this region evidenced by the fact that 18 SSR markers that co-segregated at a single locus on the genetic map were

distributed across 3DL on the deletion-based physical map. However, unknown genetic modifiers could also play an important role in genotype to phenotype variation in wheat. What counts, however, is that despite the presence of *Stb16q*, resistant plants may develop up to 5% *P* and 30% *N*, which is close to the lowest values for plants lacking *Stb16q*, which had values as low as 8% *P* and 37% *N*. The application is that the distinguishing threshold between resistance and susceptibility in a given population should not be taken arbitrarily, as indicated previously (Adhikari et al. 2003, 2004b; Chartrain et al. 2005b), and ought to be based on appropriate genotype versus phenotype analyses.

In conclusion, the present results show that *Stb16q* and *Stb17* are valuable new resistance loci that can be easily deployed in national and international marker-assisted resistance breeding programs. However, *M. graminicola* is classified as a high to moderate risk pathogen due to its multiple asexual and sexual cycles per year and its effective spore dissemination mechanism (McDonald and Linde 2002a, b), which enabled the fungus to circumvent *Stb* genes deployed in commercial wheat (Linde et al. 2002; Wittenberg et al. 2009; Zhan et al. 2007). We, therefore, discourage using *Stb16q* or *Stb17* as single genes, but rather suggest pyramiding strategies with other STB resistance genes in order to maximize their commercial life span.

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