Phenotypic and genetic analysis of the *Triticum monococcum–Mycosphaerella graminicola* interaction

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Summary

• Here, the aim was to understand the cellular and genetic basis of the *Triticum monococcum–Mycosphaerella graminicola* interaction.
• Testing for 5 yr under UK field conditions revealed that all 24 *T. monococcum* accessions exposed to a high level of natural inocula were fully resistant to *M. graminicola*. When the accessions were individually inoculated in the glasshouse using an attached leaf seeding assay and nine previously characterized *M. graminicola* isolates, fungal sporulation was observed in only three of the 216 interactions examined. Microscopic analyses revealed that *M. graminicola* infection was arrested at four different stages post-stomatal entry. When the inoculated leaves were detached 30 d post inoculation and incubated at 100% humidity, abundant asexual sporulation occurred within 5 d in a further 61 interactions.
• An F_2 mapping population generated from a cross between *T. monococcum* accession MDR002 (susceptible) and MDR043 (resistant) was inoculated with the *M. graminicola* isolate IPO323. Both resistance and *in planta* fungal growth were found to be controlled by a single genetic locus designated as *TmStb1* which was linked to the microsatellite locus *Xbarc174* on chromosome 7A^m.
• Exploitation of *T. monococcum* may provide new sources of resistance to septoria tritici blotch disease.

Key words: compatible and incompatible disease interactions, *Mycosphaerella graminicola*, resistance gene mapping, scanning electron microscopy, septoria tritici blotch, *TmStb1*, *Triticum aestivum*, *Triticum monococcum*.

Introduction

The ascomycete fungus *Mycosphaerella graminicola* is the causal agent of septoria tritici blotch disease of wheat. Significant yield losses caused by septoria tritici blotch have been documented in the world's major wheat production areas since the late 1980s (Eyal & Levy, 1987; Loughman & Thomas, 1992; Jorgensen *et al.*, 1996; Cowger *et al.*, 2000; Chungu *et al.*, 2001; Hardwick *et al.*, 2001). Currently, the control of this disease relies heavily on the use of fungicides. Nonetheless, the development of fungicide resistance in *M. graminicola*

populations makes this control less efficient (Fraaije *et al.*, 2005, 2007).

Breeding for resistance is an efficient, economical and environmentally friendly approach to control *M. graminicola* epidemics. Resistance of common hexaploid wheat (*Triticum aestivum*; $2n = 6x = 42$, AABBDD) to *M. graminicola* can be either isolate nonspecific or isolate-specific. Isolate nonspecific resistance is partial, polygenic (i.e. controlled by quantitative trait loci (QTL)) and effective against all *M. graminicola* isolates (Zhang *et al.*, 2001; Chartrain *et al.*, 2004b; Simon *et al.*, 2004). By contrast, isolate-specific resistance is near complete

Table 1 Major genes and quantitative trait loci conferring resistance to *Mycosphaerella graminicola* identified in hexaploid wheat

Locus	Chromosomal location	Linked markers	Reference
Stb1	5BL	*Xbarc74, Xgwm335*	Adhikari *et al*. (2004c)
Stb2	3BS	*Xgwm389, Xgwm533.1, Xbarc133, Xbarc75, Xgwm493*	Adhikari *et al*. (2004a)
Stb3	6DS	*Xgdm132*	Adhikari *et al*. (2004a)
Stb4	7DS	*Xgwm111*	Adhikari *et al*. (2004b)
Stb5	7DS	*Xgwm44, Rc3, Xgwm111, Xgwm437, Xgwm121*	Arraiano *et al*. (2001b)
Stb6	3AS	*Xgwm369, Xwmc11*	Brading *et al*. (2002)
Stb7	4AL	*Xgwm160, Xwmc219, Xwmc313*	McCartney *et al*. (2002)
Stb8	7BL	*Xgwm146, Xgwm577, Xgwm611*	Adhikari *et al*. (2003)
Stb9	2B		Chartrain *et al*. (2004a)
Stb10	1D	*Xgwm848*	Chartrain *et al*. (2005a)
Stb11	1BS	*Xbarc8, Xbarc137*	Chartrain *et al*. (2005c)
Stb12	4AL	*Xwmc219, Xwmc313*	Chartrain *et al*. (2005a)
Stb15	6AS	*Xpsr563a, Xpsr904*	Arraiano *et al*. (2007)
Stbx	1D, 2D and 6B (seedlings), 3D and 7B (adult)		Simon *et al*. (2004)
QStb.risø-2B	2BL	*Xwmc175a–Xwmc175b* (16 cM)	Eriksen *et al*. (2003)
QStb.risø-3A.1	3AS	*Xgwm369* (2 cM)	Eriksen *et al*. (2003)
QStb.risø-3A.2	3AS	*Xwmc489–Xwmc505* (5 cM)	Eriksen *et al*. (2003)
QStb.risø-3B	3BL	M62/P38-373	Eriksen *et al*. (2003)
QStb.risø-6B.1	6B	M48/P32-112	Eriksen *et al*. (2003)
QStb.risø-6B.2	6B	*Xwmc397–Xwmc341* (3 cM)	Eriksen *et al*. (2003)
QStb.psr-6B-1	6B	*Xwgm133*	Chartrain *et al*. (2004b)
QStb.risø-7B	7B	M49/P38-229–M49/P11-229 (22 cM)	Eriksen *et al*. (2003)

against avirulent genotypes of the pathogen (Somasco *et al.*, 1996; Arraiano *et al.*, 2001b; McCartney *et al.*, 2002) and follows the classic 'gene-for-gene' relationship (Brading *et al.*, 2002). To date, thirteen *Stb* genes and several QTL conferring resistance to *M. graminicola* in seedlings and/or adult plants have been identified in a range of hexaploid wheat varieties, and their chromosomal locations determined (Table 1). However, the current inventory of *Stb* genes in hexaploid wheat has so far failed to provide adequate and durable protection because the efficacy of individual resistance genes against natural *M. graminicola* populations is low. For example, in detached leaf tests using a high fungal inoculum concentration up to a 20% leaf area under the disease progress curve was observed even in the most resistant wheat breeding line known in Europe (i.e. TE9111), which harbours *Stb11, Stb6* and probably *Stb7* as well as several QTL (Chartrain *et al.*, 2005c). Furthermore, deployment of available *Stb* genes can lead to selection pressure on *M. graminicola* populations, which may result in a rapid development of virulence to individual or particular combinations of the resistance genes (Cowger *et al.*, 2000). The emergence of fungal isolates harbouring mutations in avirulence genes matching plant isolate-specific resistance genes could rapidly lead to the break-down of resistance. Thus, expanding the repertoire of available resistance genes and identifying those where the loss of the matching avirulence genes confers a fitness penalty on the pathogen (Leach *et al.*, 2001) is important for disease control.

Wild relatives of crop species have frequently proven to be an excellent source of novel resistance to various pathogens. *Triticum monococcum* L. ($2n = 2x = 14$, A^{m}A^m), commonly known as the einkorn wheat, is a diploid relative of hexaploid wheat. *T. monococcum* was domesticated from a small group of wild *Triticum boeoticum* in the Karacadag Mountains region of south-eastern Turkey and was widely cultivated in the Fertile Crescent at the beginning of human farming activities (Heun *et al.*, 1997; Salamini *et al.*, 2002). However, the production of *T. monococcum* was abandoned before the Bronze Age owing to the rise of emmer and common wheat, and therefore einkorn wheat has rarely entered into modern wheat breeding programmes (Dvorak *et al.*, 1993; Dubcovsky *et al.*, 1995; Baum & Bailey, 2004). Thus, the A^m genome is underrepresented in the current elite wheat germplasm. Our recent studies indicate that *T. monococcum* possesses many useful traits that can be utilized for hexaploid wheat genetic improvement (Jing *et al.*, 2007; http://www.WGIN.org.uk). Resistance to *M. graminicola* has not been sufficiently explored in *T. monococcum*. To date, only a few studies have been reported and provide conflicting results. In one of the studies (Yechilevich-Auster *et al.*, 1983), 20 of 22 *T. boeoticum* accessions were found to be resistant when infected with nine isolates of *M. graminicola*. In a second study (McKendry & Henke, 1994), all of the 13 *T. monococcum* accessions tested were found to be susceptible. This discrepancy may be caused by differences in the einkorn wheat subspecies and accessions selected, fungal isolates used, or differences in

inoculation methods. However, the most likely cause is that two different scoring systems were used. In the initial study, resistance was defined as < 30% pycnidia coverage of the leaf area, whereas in the later study only the area of leaf necrosis was scored without considering the degree of pycnidia development. A more recent study showed that two *T. monococcum* accessions were resistant to an American *M. graminicola* isolate (Singh *et al.*, 2006).

In this study we characterized in detail the interaction between *T. monococcum* and *M. graminicola*. We aimed to address the following questions: (1) Does effective resistance to *M. graminicola* exist in *T. monococcum*? (2) How and where in the fungal infection cycle is growth arrested? (3) Is the nature of resistance polygenic or monogenic? (4) If the resistance is monogenic, is the effect isolate-specific or isolate nonspecific? (5) Are the genetic loci controlling resistance in *T. monococcum* located in chromosomal regions syntenic to those containing *Stb* loci or major *M. graminicola* resistance QTL in durum and hexaploid wheat?

Material and Methods

Plant materials

The twenty-four *T. monococcum* accessions were selected for this study because of their known high genetic diversity and resistance to many other diseases (Jing *et al.*, 2007; see the Supporting Information, Table S1). The hexaploid wheat varieties Riband and Hereward were used in various experiments for comparison.

Field trials

During the period 2004–2008 eight field trials were undertaken at different sites on the Rothamsted Research Farm, UK. The sites were chosen based on the incidence of high natural disease pressure. All the experiments were conducted in randomized complete blocks of five replicates, generated using the statistical package GENSTAT for Windows, 10th edition (VSN International, Hemel Hempstead, UK). A single plot consisted of three rows of 50 cm length. A 50-cm path of either bare soil or sown with the nonhost species barley was used to separate each plot. Forty-five seeds per accession, 15 per row, were sown in individual plots in late October. In the 2005–2006 field trial disease pressure was also artificially enhanced by spray-inoculating the 24 *T. monococcum* accessions at growth stage GS 69 with a mixture of four *M. graminicola* (Fückel) Schröter in Cohn. (Anamorph *Septoria tritici* Roberger in Desmaz.) isolates collected from hexaploid wheat varieties in the same growing season. The four isolates (RG001, RG002, RG003 and RG004) had been stored in the Rothamsted isolate collection. Each plot was sprayed with 25 ml of a fungal spore suspension (4×10^6 spores ml^{-1}) on 16 June and again on 22 June, 2006. The disease incidence and severity were assessed monthly from seedling stages onwards by measuring the leaf area covered by necrotic lesions bearing pycnidia.

Glasshouse experiments and *M. graminicola* isolates

For experiments conducted under glasshouse conditions, seeds were germinated in Rothamsted standard compost mix in 40×30 cm trays, typically 30–40 seeds in a row. The compost mix was supplied by Petersfield Products (Cosby, Leicester, UK) and consisted of 75% medium grade peat, 12% screened sterilized loam, 3% medium grade vermiculite, 10% Grit, Osmocote Exact (3.5 kg m^{-3}) from Scotts UK professional (Ipswich, UK), PG mix (0.5 kg m^{-3}) from Hydro Agri (UK) Ltd (Immingham, UK) and ~3 kg m^{-3} Lime to adjust the pH to 5.5–6.0. The seedlings were grown in glasshouse compartments as previously described (Keon *et al.*, 2007). Eight bread wheat isolates of *M. graminicola* from five countries and a durum wheat isolate were chosen for this study as previous studies had shown that these could be used to identify specific resistances in hexaploid wheat (Table 2). The isolates were kept as glycerol stocks at −80°C and grown on yeast and potato dextrose agar (YPDA) solid media at 15°C for 7–10 d before spores were harvested (Keon *et al.*, 2005).

Disease and resistance screening using an attached leaf assay

The interactions of the 24 *T. monococcum* accessions and nine *M. graminicola* isolates were all studied using an attached leaf assay. The second leaves of 21-d-old seedlings were fastened to a plastic platform using double-sided tape. The inoculum concentration of *M. graminicola* isolates was adjusted to 10^7 spores ml^{-1} using deionized water containing 0.01% Tween-20. Various methods were used for inoculation depending on the experiments. In the resistance screening experiment, the spore suspension was inoculated onto the attached leaves using cotton swabs; for the cytological experiment, the spore suspension was spray-inoculated using a fine nozzle sprayer. The plants were then covered with transparent boxes to retain high humidity for the first 72 h. After removal of the covering boxes, plants were kept for daily assessment of the development of macroscopic symptoms and pycnidia formation for 4–5 wk until the attached leaves senesced. In each experiment, at least four replicate leaves of each accession–isolate combination under consideration were examined. Owing to space limitations, inoculations were done in batches and each batch contained hexaploid wheat variety Riband as a control. A *T. monococcum* accession was considered susceptible only when lesions bearing pycnidia were observed on the inoculated leaves. The screen was repeated at least three times for each accession–isolate combination.

Table 2 *Mycosphaerella graminicola* isolates used in this study

Isolate	Mating type	Origin	Resistant bread cultivars	Effective *Stb* gene(s)	References
IPO87019	MAT1-1	Uruguay	TE 911, Catbird, Kavkaz-K4500 L.6.A.4	*Stb7*	McCartney *et al.* (2003); Chartrain *et al.* (2005c)
IPO88004	MAT1-1	Ethiopia	Senat, Israel 493, Veranopolis, Chaucer, Olaf, Riband, Longbow, Baldus	*Stb15*	Arraiano *et al.* (2007)
IPO89011	MAT1-2	Netherlands	Tonic, Courtot	*Stb9*	Chartrain *et al.* (2004a)
IPO94269	MAT1-2	Netherlands	Gene, Mentana, Frontana	*Stb5, Stb10*	Arraiano *et al.* (2001a); Chartrain *et al.* (2005a)
IPO92006	MAT1-2	Portugal	Chaucer	?*	Chartrain *et al.* (2004b)
IPO001	MAT1-1	Netherlands	Equinox, Reaper	?*	Chartrain *et al.* (2004b)
IPO90012	MAT1-1	Mexico	Olaf, TE 911, Veranopolis, Israel 493	*Stb11*	Chartrain *et al.* (2005c)
IPO323	MAT1-1	Netherlands	Numerous cultivars	*Stb6*	Brading *et al.* (2002); Chartrain *et al.* (2005b)
IPO95052	MAT1-2	Durum wheat from Algeria	hexaploid wheat	?*	

*The corresponding resistance genes have not been identified.

Sporulation induction experiments

The inoculated leaves were detached from plants at the end of an experiment (30 d post inoculation (dpi)) when completely senesced. Leaves were briefly surface cleaned by immersing in 1% (v : v) sodium hypochlorite solution prepared using commercial bleach for 5 min and then washed with sterile water three times. Each leaf was individually transferred into a 15-ml centrifuge tube containing a piece of cotton wool saturated with sterile water. The tubes were then sealed and kept at 15°C for 5 d. Different surface cirrus formation and sporulation phenotypes were observed under a Leica MZFL111 light microscope (Cambridge, UK). The images were captured with a Leica Digital Camera DC300F with IM50 Software.

Scanning electron microscopy (SEM) and cryofracture

Eight accession–isolate combinations were selected for SEM observations. The *T. monococcum* accessions MDR002, MDR037, MDR043 and MDR308 were independently inoculated with *M. graminicola* isolates IPO323 or IPO89011. Leaves were spray-inoculated with a spore suspension adjusted to 10^7 spores ml^{-1} using deionized water containing 0.01% Tween-20. Between 3 and 30 dpi, the inoculated leaf segments were taken at 3 d intervals for SEM examination of fungal surface growth, development and penetration events. At each time-point and for each accession–isolation interaction, four leaf segments each containing *c.* 20 stomata apertures were used. The leaf specimens were glued to metal holders and quench-frozen in liquid nitrogen slush and transferred under vacuum to a Cryo-SEM preparation chamber (Gatan Alto 2100; Gatan UK, Abingdon, UK). Samples were then fractured, etched by sublimation at 85°C for 2 min, sputter coated with gold and examined at 5–15 kV in a JEOL JSM-6360 LV scanning electron microscope.

Polymerase chain reaction (PCR) diagnosis and quantification of fungal biomass

DNA extraction and quantification for fungal and plant samples and PCR conditions followed previously described methods (Fraaije *et al.*, 2005). The primers used were to detect the *ß-tubulin* gene and the G143A mutation in the *cytochrome b* gene, which confers resistance to strobilurin (QoI) fungicides in *M. graminicola*.

Genetic analysis

Accessions MDR002 and MDR043 were used as the female and male parent, respectively, to generate a mapping population for differential interactions with *M. graminicola* isolate IPO323. The authenticity of the resulting F_1 seeds was verified by comparing the electrophoretic profiles of seed storage proteins with those of parental lines (Jing *et al.*, 2007; data not shown). For segregation analyses 68 F_2 progeny were grown in the glasshouse and the second leaves of the 21-d-old seedlings were infiltrated with 0.5 ml of a spore suspension adjusted to 10^7 spores ml^{-1} of *M. graminicola* isolate IPO323 using a PLASTIPAK syringe (Becton Dickinson, Oxford, UK). Earlier replicated experiments involving various contrasting inter-actions had revealed that identical sporulation/no sporulation phenotypes were obtained using the cotton swab and infiltration methods. However, the latter did not require the use of high-humidity conditions post-inoculation. The inoculated leaves were harvested 21 dpi for visual scoring of the presence and absence of pycnidia and then used for subsequent DNA isolation and fungal biomass quantification using a quantitative PCR method (Fraaije *et al.*, 2005). The young fourth and fifth leaves from each F_2 progeny were harvested for genomic DNA isolation and genotyping using 45 polymorphic microsatellite markers (Jing *et al.*, 2007).

The microsatellite markers were selected from the Graingenes database (http://wheat.pw.usda.gov/GG2/index.shtml). The microsatellite marker and fungal biomass data were plotted into JOINMAP 4 and MAPQTL 5 for linkage and mapping analyses (van Ooijen, 2004, 2006).

Results

Triticum monococcum exhibited high level resistance to *M. graminicola* under UK wheat production conditions

Over the period 2004–2008, the 24 *T. monococcum* accessions and two hexaploid wheat varieties were evaluated in eight field experiments done on the Rothamsted Research farm (Supporting Information, Fig. S1a). In each field experiment, plants of the hexaploid wheat varieties (Riband or Hereward) developed typical STB disease symptoms. By late winter 100% of plants had at least one leaf covered with necrotic lesions bearing pycnidia and subsequently the incidence and severity of the disease symptoms increased (Fig. S1b). This is indicative of a high level of natural disease pressure. By contrast, no visible disease symptoms were ever found on the green leaves of *T. monococcum* (Fig. S1c). In the 2005–2006 field trial, in which the disease pressure was artificially enhanced, small necrotic patches were observed on a few *T. monococcum* accessions within 3 wk. However, no pycnidia formation occurred. In one case small brown lesions containing immature pycnidia were identified on one partially senescent leaf of accession MDR002 in February in the 2004–2005 trial year. This was found 2 wk after the entire trial had been covered with approx. 5 cm of snow for 5 d. The *M. graminicola* isolate recovered from the immature pycnidia, was single-spored and then used to inoculate the susceptible bread wheat variety Riband in the glasshouse conditions. Typical leaf lesions with abundant pycnidia development occurred within 14 dpi (data not shown). A PCR diagnosis revealed that this isolate harboured the G143A mutation in the *cytochrome b* gene which confers resistance to strobilurin fungicides (Fig. S1d). These data suggest that the *M. graminicola* isolate recovered from the *T. monococcum* accession in February 2005 originated from a hexaploid wheat donor plant.

In summary, the diploid wheat *T. monococcum* possesses a high level of resistance to *M. graminicola* under UK field conditions.

Accession–isolate specific interactions in the *T. monococcum*–*M. graminicola* pathosystem

The field results raised the possibility that *T. monococcum* is a nonhost for *M. graminicola*. To test this hypothesis, we used the established attached leaf assay (Keon *et al.*, 2007) and examined a total of 216 interactions between 24 *T. monococcum* accessions and nine well-characterized *M. graminicola* isolates

as well as one control compatible interaction involving the hexaploid wheat Riband and the isolate IPO323 (Fig. 1a). The length of latent period to the onset of visible symptoms and the production of oozing pycnidia were observed. Resistant responses and incompatible interactions were defined as inhibition of sporulation, whereas the formation of pycnidiospores indicated a susceptible host response and a compatible interaction.

The hexaploid wheat variety Riband, when inoculated with IPO323, consistently developed large chlorotic lesions by 11–12 dpi and abundant pycnidia production occurred by day 14 (Fig. 1b). Pycnidia were absent in 213 of the 216 interactions examined and were considered to be incompatible interactions. Among the accession–isolate combinations where incompatible interaction phenotypes were observed, some *T. monococcum* accessions started showing macroscopic symptoms exemplified by flecking and/or visible yellowing either at a similar time to Riband *c*. 11–12 dpi (Fig. 1c), or from 15 d onwards (Fig. 1d). Others remained green for over 30 dpi (Fig. 1e). Only three compatible interactions were observed: MDR002 vs IPO89011, MDR002 vs IPO323 and MDR037 vs IPO323 (Fig. 1f). However, pycnidiation only occurred after 21 dpi and thus lagged significantly behind pycnidia formation in the hexaploid wheat control. These results indicate that *T. monococcum* is a host species for *M. graminicola*, but fungal development was slower in *T. monococcum* than in hexaploid wheat.

The sporulation induction assay confirms the high level of resistance in *T. monococcum*

This assay was devised to explore the resistance phenotypes post natural leaf senescence (Fig. 1g). Table 3 summarizes the sporulation induction results for the 216 accession–isolate interactions. Under these high-humidity conditions, abundant production of pycnidia with cirrhi was evident in the susceptible bread wheat variety Riband as well as the three identified compatible *T. monococcum* accession–isolate combinations (Fig. 1h). In the remaining accession–isolate interactions examined, which inhibited *in planta* sporulation, four different phenotypes were observed. Phenotype I (*n* = 61, 28%) was characterized by pycnidia production and cirrhus formation after damp-chamber incubation (Fig. 1i). In phenotype II (*n* = 38, 18%) no fully matured pycnidia were found, instead pycnidia with ostiole-like structures, but without cirrhi or pycnidiospores, formed and occupied nearly all the stomata (Fig. 1j). Phenotype III (*n* = 43, 20%) was characterized by the lack of pycnidia development and fungal growth was arrested within the stomatal cavity, as manifested by the stomata blackening possibly caused by fungal melanization in the substomatal cavity (Fig. 1k). Phenotype IV (*n* = 71, 34%) was similar to Phenotype III but with a lower frequency of blackening of stomatal cavity (Fig. 1l). As shown in Table 3, the durum wheat isolate IPO95052 and the hexaploid wheat
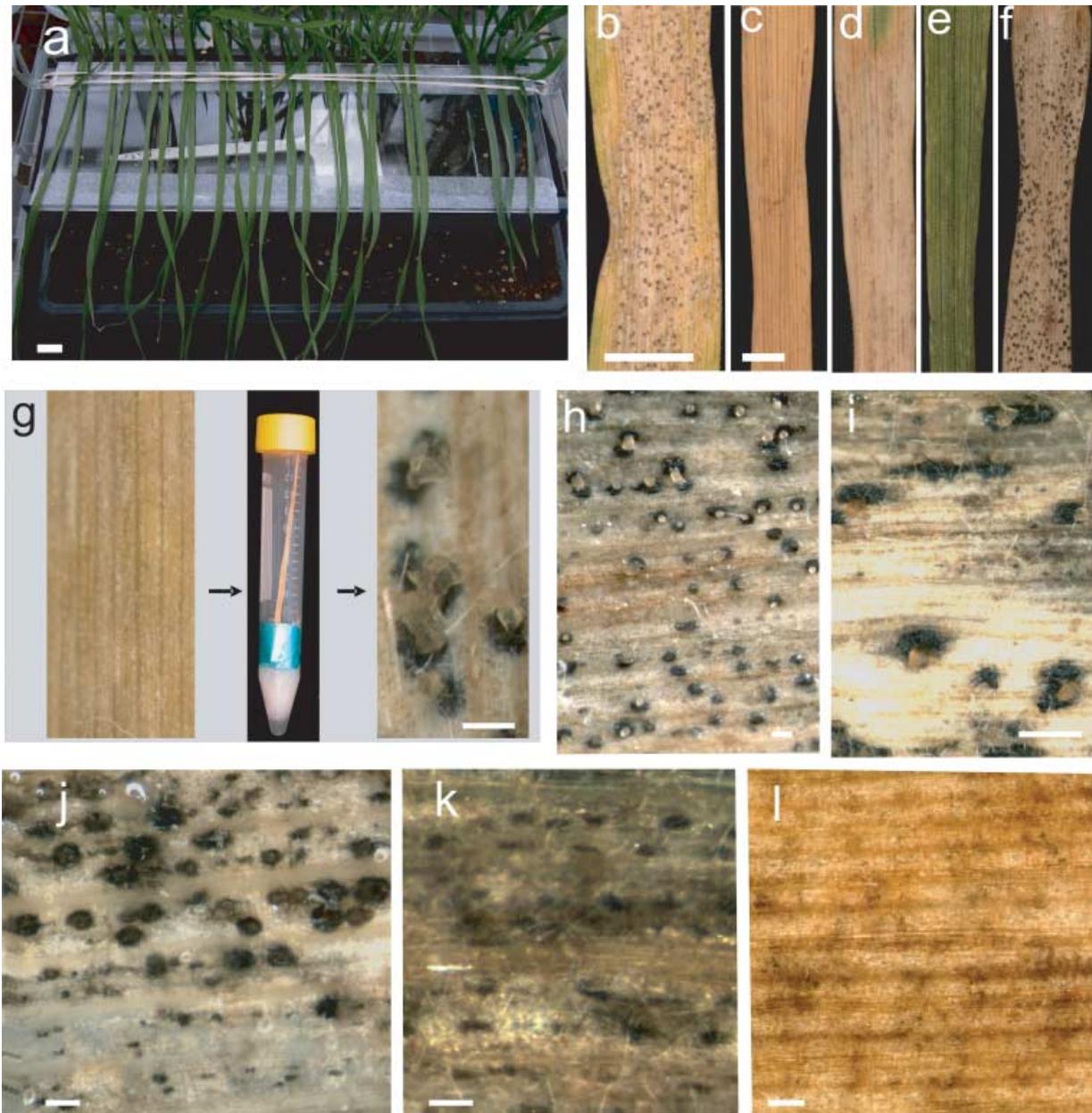
Fig. 1 Examination of resistance and susceptibility of *Triticum monococcum* accessions to nine *Mycosphaerella graminicola* isolates using the attached leaf bioassay under glasshouse conditions and subsequently in the detached leaf sporulation induction assay. (a–f) Representative photographs show the setup of the attached leaf assay and the development of macroscopic symptoms in hexaploid wheat and *T. monococcum*. (a) The setup for the attached leaf assay. (b–f) Representative leaf segments showing the development of macroscopic symptoms in different accession–isolate combinations. (b) Susceptible hexaploid wheat variety Riband at 14 days post inoculation (dpi) with *M. graminicola* isolate IPO323; (c) *T. monococcum* accession MDR043 at 14 dpi with isolate IPO89011; (d) *T. monococcum* accession MDR037 at 17 dpi with isolate IPO89011; (e) *T. monococcum* accession MDR243 at 30 dpi with isolate IPO94269; (f) *T. monococcum* accession MDR002 at 21 dpi with isolate IPO323. Abundant *in planta* sporulation of *M. graminicola* is evident only in (b) and (f). Bars: (a,b), 5 mm; (c), 2 mm. Photographs (c–f) were taken at the same magnification. (g–l) Sporulation induction within a damp-chamber system for detached leaves of *T. monococcum* accessions previously inoculated with different *M. graminicola* isolates using the attached leaf assay. (g) The damp-chamber sporulation induction assay: (left) a representative inoculated leaf segment cut at 30 dpi with no visible pycnidia; (middle) incubation of a leaf segment in a damp chamber; (right) evidence of pycnidia with cirrhi formation 5 d post incubation. (h–l) Representative leaf segments showing various sporulation phenotypes post incubation in the damp-chambers for 5 d. (h) *T. aestivum* 'Riband' vs IPO89011 with abundant cirrhi and pycnidiospores; sporulation type S in Table 3 (i) *T. monococcum* accession MDR037 vs isolate IPO89011 shows low-density pycnidia with occasional cirrhi, sporulation type I; (j) *T. monococcum* MDR045 vs IPO323 showing immature pycnidia with ostioles, sporulation type II; (k) *T. monococcum* MDR045 vs IPO89011 showing blackening of stomatal cavity, sporulation type III; and (l) *T. monococcum* MDR308 vs IPO89011 showing no formation of pycnidia, sporulation type IV. Bars: (g–l), 50 µm.

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Table 3 Induction of sporulation in various *Triticum monococcum* accession–*Mycosphaerella graminicola* isolate interactions*

Accession	IPO87019	IPO88004	IPO89011	IPO94269	IPO92006	IPO001	IPO90012	IPO323	IPO95052
MDR001	III	III	IV	IV	III	II	IV	II	IV
MDR002	IV	IV	S_{Tm}	III	I	IV	I	S_{Tm}	III
MDR024	II	I	I	III	II	I	II	II	I
MDR031	III	III	II	IV	I	II	IV	III	I
MDR034	I	IV	IV	I	II	IV	IV	II	IV
MDR035	III	I	II	IV	I	II	IV	IV	III
MDR037	III	III	I	I	IV	I	II	S_{Tm}	II
MDR038	IV	I	I	IV	III	IV	II	IV	III
MDR040	II	I	IV	III	II	II	IV	IV	III
MDR043	IV	I	II	I	IV	III	IV	I	IV
MDR044	I	IV	III	IV	II	I	IV	I	I
MDR045	III	IV	III	I	IV	IV	IV	II	II
MDR046	I	III	III	IV	II	I	IV	I	IV
MDR047	III	III	IV	III	II	II	II	III	II
MDR048	I	II	I	III	IV	II	IV	I	I
MDR049	II	I	IV	III	III	I	II	I	II
MDR050	II	I	II	I	II	I	IV	I	I
MDR218	IV	IV	III	I	I	IV	IV	IV	I
MDR234	IV	I	III	IV	IV	III	IV	IV	I
MDR243	III	I	III	I	III	III	IV	IV	II
MDR236	IV	IV	IV	IV	IV	IV	IV	III	I
MDR258	IV	I	IV	IV	I	I	III	II	I
MDR303	I	IV	III	I	I	I	I	II	I
MDR308	IV	IV	III	IV	II	III	I	IV	I
Riband	S	S	S	S	S	S	S	S	S

*Infected leaf tissue was detached after natural senescence and was used for sporulation induction, as shown in Fig. 1g. Sporulation Types I–IV developed no pycnidia under glasshouse high disease pressure conditions.
S, Susceptible response, abundant mature pycnidia with cirrhi.
S_{Tm}, Susceptible response, delayed development of mature pycnidia with cirrhi.
I, High stomata infection, pycnidia with cirrhi containing pycnidiospores.
II, High stomata infection, pycnidia with ostiole but no cirrhi.
III, High stomata infection, stomatal blackening, no pycnidia.
IV, Very low stomata infection, occasional black stomata, no pycnidia.

isolate IPO88004 originated from Ethiopia most frequently induced Phenotype I. By contrast, isolate IPO90012 from Mexico only induced Phenotype I in three of the 24 accessions. Interestingly, the efficacy of resistance varied between the *T. monococcum* accessions tested. For example, Phenotype I was absent in accessions MDR001 and MDR047. Furthermore, for accession MDR236 seven of the nine isolates only resulted in sporulation induction phenotype IV in which no pycnidia development occurred.

Collectively, these observations indicate that pycnidia development had been arrested at different stages and that the efficacy of this resistance differed amongst the *T. monococcum* accessions.

Infection by *M. graminicola* is arrested at different stages after stomatal entry in resistant *T. monococcum* accessions

To gain a better understanding of the mechanism of resistance at the cellular level, a cytological study of the infection process on attached leaves was carried out using four *T. monococcum* accessions MDR002, MDR037, MDR043 and MDR308 inoculated with *M. graminicola* isolate IPO89011 or IPO323. These represented different macroscopic outcomes of the interaction (Fig. 1). The SEM micrographs of leaf surfaces showed that pycnidiospore germination and hyphal growth was evident at 4 dpi in all eight interactions (Fig. 2a).

The SEM cryofracture analysis of the susceptible wheat variety Riband revealed intensive colonization of the substomatal cavity by *M. graminicola* (Fig. 2b). In *T. monococcum*, four different patterns of *M. graminicola* hyphal growth were observed post stomatal entry. Pattern A was represented by the compatible interactions between MDR002 vs IPO89011, MDR002 vs IPO323 and MDR037 vs IPO323, in which abundant hyphae were present in the substomatal cavities and sporulation occurred by day 23 (Fig. 2c). Pattern A, therefore, resembled the infection process in the susceptible hexaploid wheat Riband. For pattern B (MDR037 vs IPO89011 and MDR043 vs IPO323), fungal hyphae were found in-between collapsed
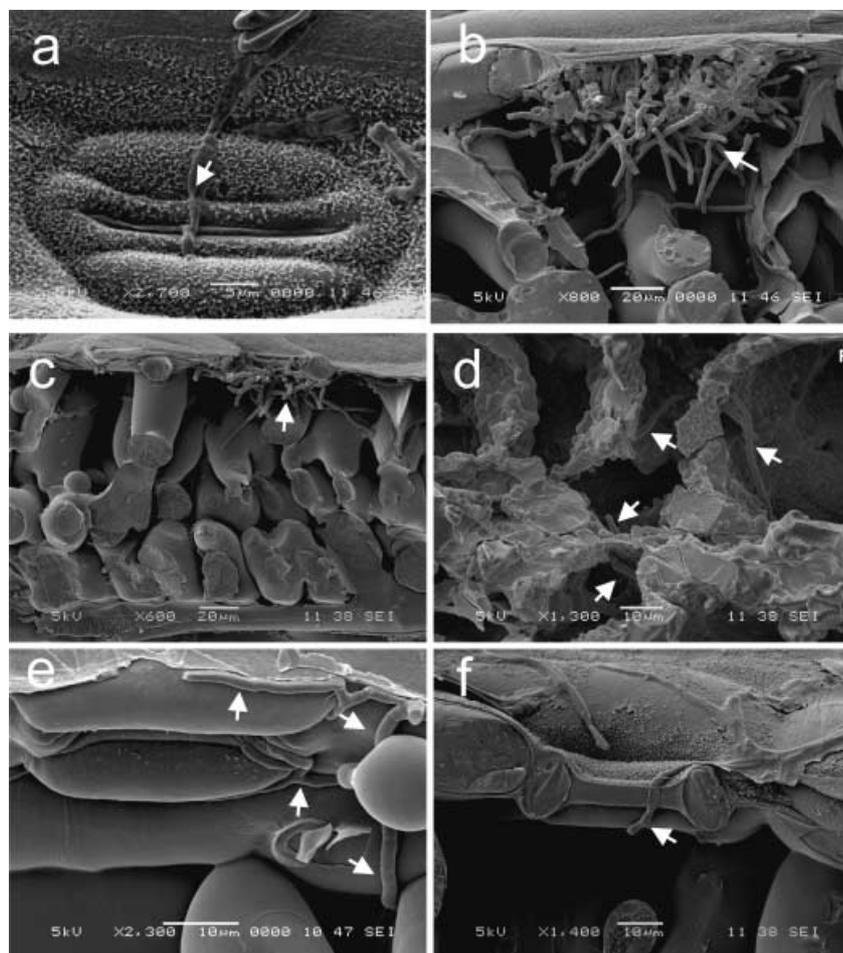
Fig. 2 Scanning electron microscopy (SEM) and cryofracture analysis of the leaf infections of *Triticum monococcum* by *Mycosphaerella graminicola*. (a) Micrographs reveal hyphal growth on leaf surface of *T. monococcum* at 4 days post inoculation (dpi). (b–f) Cryofracture micrographs of the five post-penetration hyphal growth patterns identified. (b) Interaction between wheat variety Riband vs IPO323 at 11 dpi; (c) interaction between *T. monococcum* MDR037–IPO323 at 17 dpi, growth pattern A; (d) interaction between *T. monococcum* MDR043 vs IPO323 at 22 dpi, growth pattern B; (e) interaction between *T. monococcum* MDR045 vs IPO89011 at 10 dpi, growth pattern C; (f) interaction between *T. monococcum* MDR308 vs IPO323 at 13 dpi, growth pattern D. See the text for details on each growth pattern. White arrows indicate fungal hyphae.

mesophyll cells but colonization of substomatal cavities and pycnidia formation did not occur (Fig. 2d). Therefore, the spore induction assay indicated that resistance in these interactions results in a fungistatic state of the pathogen (Table 3). In Pattern C (MDR043–IPO89011), hyphal growth was restricted to the intercellular space adjacent to the mesophyll cells surrounding substomatal cavities (Fig. 2e), and the sporulation induction assay revealed that only immature pycnidia had formed. Pattern D (MDR308 vs IPO323 and vs IPO89011) was characterized by the arrest of hyphal growth immediately post-stomatal entry, and hyphae were absent in the substomatal cavity (Fig. 2f). Collectively, these cytological analyses indicate that *M. graminicola* hyphal growth is arrested at four different infection stages post-stomatal penetration in *T. monococcum*.

The sporulation induction assay reveals that fungal development and pycnidiation can take place in infected leaves of *T. monococcum* under favourable environmental conditions. This can even occur in the severely restricted accession–isolate interactions types B and C, as identified by SEM. Thus, the fungal hyphae were just fungistatically constrained.

Identification of a genetic locus in *T. monococcum* conferring isolate specific resistance to *M. graminicola* isolate IPO323

The identification of specificity in the *T. monococcum–M. graminicola* interaction prompted us to study the genetic basis of resistance. Six F_1 seedlings resulted from a cross between MDR002 and MDR043 were infiltrated with a *M. graminicola* IPO323 spore suspension to examine the inheritance of resistance. At 10 dpi, fungal sporulation was evident in the infiltrated leaf areas of the susceptible accession MDR002, whereas no fungal sporulation occurred in MDR043 and the F_1 progeny (Fig. 3a). These results indicate that the resistance is inherited as a dominant or semidominant trait. To further understand the genetic basis of resistance, 68 F_2 lines were infected and the presence and/or absence of pycnidia scored. The 68 F_2 lines were scored as 55 resistant (no pycnidia) and 13 susceptible (bearing pycnidia). This segregation fits into a 3 : 1 ratio ($\chi^2 = 1.25$, $P = 0.263$). Furthermore, fungal biomass in the infected leaves of the F_2 lines was quantified using a quantitative PCR method (Fraaije *et al.*, 2005) and it was found that the quantitative data followed a binomial distribution
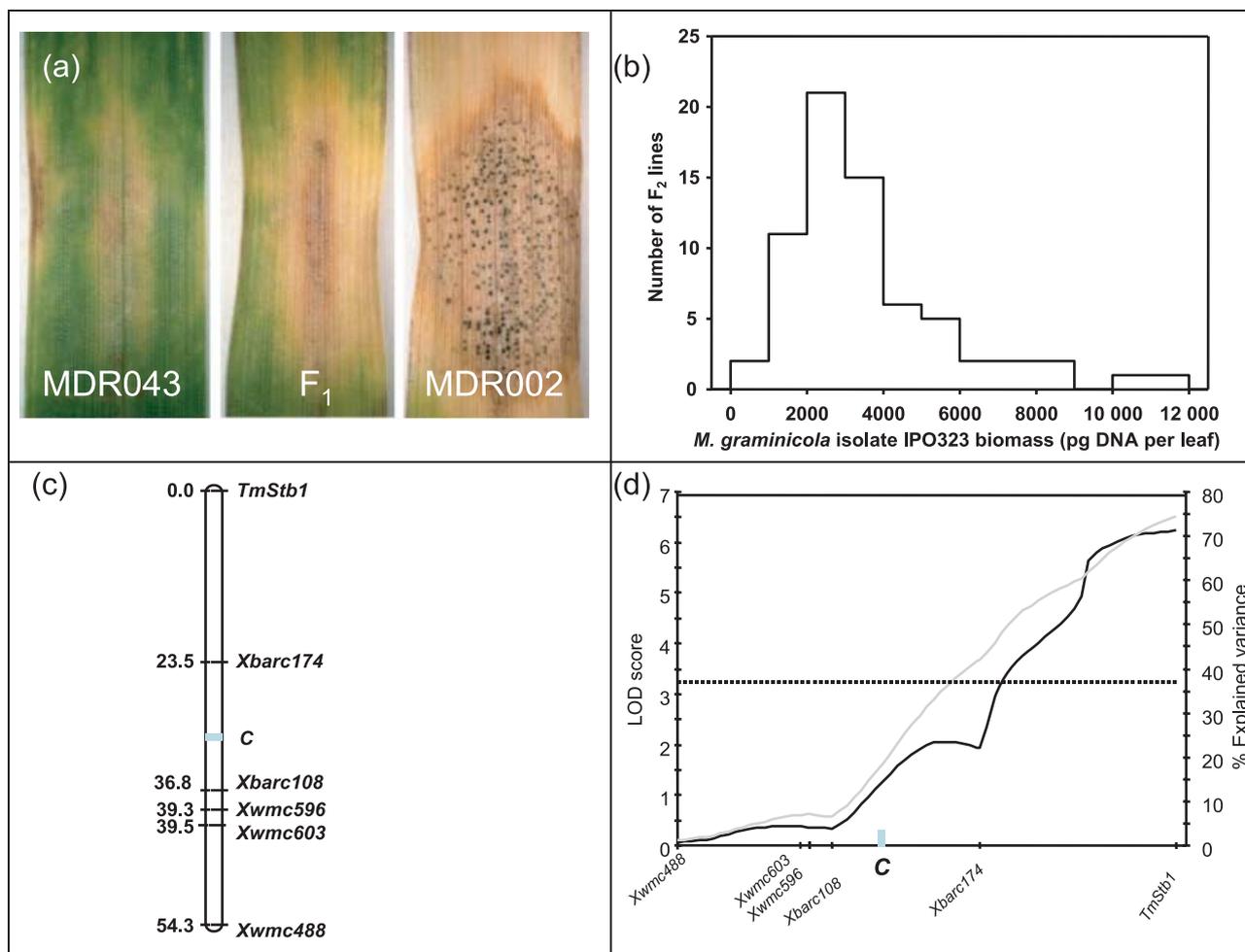
Fig. 3 Genetic analysis of resistance to *Mycosphaerella graminicola* isolate IPO323 using the *Triticum monococcum* mapping population derived from a cross between MDR043 (resistant) and MDR002 (susceptible). (a) Photographs show the isolate IPO323-inoculated leaf phenotypes of *T. monococcum* accessions MDR043, MD002 and F_1 progeny at 16 days post inoculation (dpi). (b) Histogram of fungal biomass obtained from inoculated leaves on plants belonging to 68 F_2 lines. (c) Chromosomal location of the resistance locus *TmStb1* in *T. monococcum*. The microsatellite markers in the linkage group and their relative positions to *TmStb1* are indicated. The possible position of the centromere is indicated by a grey bar labelled *C*. (d) LOD scans (grey line) for MQM analysis for chromosome 7A^m where *TmStb1* conferring resistance against *Mycosphaerella graminicola* isolate IPO323 was identified. The curve of percentage explained phenotype variance (black line) is also shown. The dotted line at LOD 3.3 represents 5% significance threshold.

(Fig. 3b). These results indicated that the resistance to IPO323 in MDR043 is controlled by a single genetic locus. This locus is designated as *TmStb1*.

To map the chromosomal location of the *TmStb1* locus, 68 F_2 progeny were genotyped with 45 microsatellite markers that exhibited polymorphism between the parental accessions MDR002 and MDR043. The visual scoring data were combined with the marker data for linkage analysis using JOINMAP 4 computing package. By setting the independence LOD as the grouping parameter and the Kosambi's mapping function in the regressing mapping algorithm, the *TmStb1* locus was found to form a linkage group with microsatellite loci *Xwmc488*, *Xwmc603*, *Xwmc596*, *Xbarc108* and *Xbarc174* at a LOD score of 6 (Fig. 3c). These markers are known to map

to chromosome 7A in a hexaploid wheat (Somers *et al.*, 2004). *TmStb1* maps at 23.5 cM distal to the microsatellite locus *Xbarc174*. To further confirm the initial linkage analysis results, the quantitative PCR fungal biomass data were analysed for linkage with microsatellite markers by using MAPQTL 5. Marker trait regression using Kruskal–Wallis function detected that *TmStb1* was highly significantly linked to *Xbarc174* with a Kruskal–Wallis statistic (K) value of 20.9 at significance level of 0.0001. Further multiple QTL mapping (MQM) analysis indicated that the log-likelihood of *TmStb1* was 6.3 and explained 70.7% of the phenotypic variance in mean logit-scores (Fig. 3d). This is consistent with the analysis of the visual scoring data which indicates the resistance to *M. graminicola* isolate IPO323 is controlled by a single locus

in *T. monococcum* accession MDR043. The five microsatellite markers linked to *TmStb1* exhibited a very high colinearity between *T. monococcum* and *T. aestivum* (Somers *et al.*, 2004, Fig. 3c). Inferred from the hexaploid wheat microsatellite consensus map, *Xwmc488*, *Xwmc603*, *Xwmc596* and *Xbarc108* are located on the long arm of chromosome 7A^m in *T. monococcum*, whereas *Xbarc174* is on the short arm of chromosome 7A^m. Therefore, *TmStb1* is a newly described genetic locus and the first one on chromosome 7A^m conferring resistance to *M. graminicola* in *T. monococcum*.

Discussion

The interaction between *T. monococcum* and *M. graminicola* has been investigated in detail. All the *T. monococcum* accessions examined maintained resistance to a high level of natural *M. graminicola* inocula over five successive UK field seasons. The interactions between 24 *T. monococcum* accessions and nine *M. graminicola* isolates were studied under glasshouse conditions that generated high disease pressure and favourable infection conditions. The majority of the interactions (98.6%) were incompatible with only three compatible interactions observed. Cytological studies indicated that resistance was achieved by post-stomatal entry inhibition of fungal development. When the inoculated leaves from resistant *T. monococcum* accessions senesced and were placed in a sporulation induction assay, in a further 28% of the interactions fungal sporulation was observed. This indicated that resistance in *T. monococcum* rendered *M. graminicola* fungistatic at different stages of plant infection. A single genetic locus *TmStb1* was found to confer resistance to *M. graminicola* isolate IPO323 in *T. monococcum*.

Efficiency of resistance to *M. graminicola* in *T. monococcum*

Mycosphaerella graminicola can germinate and initiate hyphal growth on essentially almost every surface (Duncan & Howard, 2000). Colonization of substomatal cavities and the intercellular space between mesophyll cells as well as the formation of mature pycnidia in the substomatal cavities and subsequent extrusion of pycnidiospore-bearing cirrhi are the key steps in completing a successful infection cycle (Kema *et al.*, 1996). Field experiments conducted over four successive growing seasons showed that all *T. monococcum* accessions did not produce lesions bearing mature pycnidia. Even in a glasshouse with very high disease pressure and favourable infection conditions just three compatible interactions were observed (1.4%), indicating that *T. monococcum* is very efficient in arresting *M. graminicola* infections.

Our cytological observations showed that resistance of *T. monococcum* to *M. graminicola* does not operate by inhibiting the initial growth and development of *M. graminicola* on the leaf surface (Kema *et al.*, 1996). These results confirm the notion that *M. graminicola* initiates germination and hyphal growth on any surface (Duncan & Howard, 2000). Resistance in *T. monococcum* blocked *M. graminicola* at three post-stomatal entry stages which correlated with the sporulation induction phenotypes. The *T. monococcum* accessions that tolerated more hyphal growth in substomatal cavities and intercellular spaces frequently displayed phenotypes I and II with the production of mature pycnidia, cirrhi and pycnidiospores in the sporulation induction assay (Table 3). By contrast, phenotypes III and IV mostly appeared on *T. monococcum* accessions that severely restricted post-penetration hyphal growth of *M. graminicola*. Hence, post-stomatal entry arrest appears to be the major cellular event conferring resistance to *M. graminicola* in *T. monococcum*. The sporulation induction assay revealed that in the majority of the *T. monococcum* incompatible interactions *M. graminicola* was fungistatically contained, which was also observed in resistant hexaploid wheat cultivars (unpublished).

Genetic basis of resistance to *M. graminicola* in *T. monococcum*

To date, resistance in hexaploid wheat has been shown to be controlled by isolate-specific *Stb* genes and isolate nonspecific QTL. No formal genetic tests on resistance to *M. graminicola* had previously been reported for *T. monococcum*. An earlier study assumed a 'gene-for-gene' mode of inheritance for resistance operating in *T. boeoticum* and seven hypothetical resistance genes were proposed (Yechilevich-Auster *et al.*, 1983). The 5 yr of field evaluation indicated that the resistance in *T. monococcum* was effective against UK *M. graminicola* populations. Often, such a high level of resistance to multiple isolates of a pathogen species is associated with nonhost resistance, which provides defence against infection and colonization by the majority of microbes (Thordal-Christensen, 2003; Holub & Cooper, 2004; Jones & Takemoto, 2004). However, this phenotype is unlikely in *T. monococcum*–*M. graminicola* interactions because full susceptibility was observed in three accession–isolate interactions under high disease pressure and favourable infection conditions. Furthermore, a unique epidermal cell death phenotype was observed in barley, a known nonhost of *M. graminicola*, but was never observed in either hexaploid wheat or *T. monococcum* (H. C. Jing, unpublished).

Using the existence of compatible and incompatible interactions between *T. monococcum* accessions and *M. graminicola* isolates, the resistance of the *T. monococcum* accession MDR043 to *M. graminicola* isolate IPO323, revealed by the complete inhibition of sporulation, was found to be controlled by a single genetic locus *TmStb1* rather than by multiple small-effect QTL. In hexaploid wheat resistance to IPO323 was shown to be controlled by a genetic locus *Stb6* that resides on the short arm of chromosome 3A (Brading *et al.*, 2002). *TmStb1* is linked to microsatellite markers mapped to chromosome 7A in hexaploid wheat (Somers *et al.*, 2004). None of the previously reported *Stb* loci and resistance QTL have

been mapped to this chromosomal arm (Table 1). Therefore, identification of *TmStb1* has pinpointed another chromosomal region in the wheat genome, which has evolved to influence the outcome of the wheat–*M. graminicola* interaction. Interestingly, *Stb4* and *Stb5*, which recognize, respectively, an American field isolate (IN95-Lafayette-1196-WW-1-4 and a Dutch isolate IPO94269, have been localized to homoeologous regions on 7D in hexaploid wheat (Arraiano *et al.*, 2001b; Adhikari *et al.*, 2004b).

Acknowledgements

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Examination of resistance of *Triticum monococcum* to natural isolates of *Mycosphaerella graminicola* under field conditions.

Table S1 *Triticum monococcum* accessions used in this study

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