MILDEW LOCUS O Mutation Does Not Affect Resistance to Grain Infections with *Fusarium* spp. and *Ramularia collo-cygni*

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ABSTRACT

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MILDEW LOCUS O defines a major susceptibility gene for powdery mildew, and recessive *mlo* resistance alleles are widely used in breeding for powdery mildew resistance in spring barley. Barley powdery mildew resistance, which is conferred by *mlo* genes, is considered to be costly in terms of spontaneous defense reactions and enhanced susceptibility to

cell-death-inducing pathogens. We assessed fungal infestation of barley (*Hordeum vulgare*) grain by measuring fungal DNA after natural infection with *Fusarium* spp. and *Ramularia collo-cygni* or after inoculation with *Fusarium* spp. in the field. Powdery-mildew-resistant *mlo5* genotypes did not show enhanced *Fusarium* spp. or *R. collo-cygni* DNA content of grain over four consecutive years. Data add to our understanding of pleiotropic effects of *mlo*-mediated powdery mildew resistance and contributes to the discussion of whether or not application of barley *mlo* mutations may support pathogenesis of cell-death-inducing fungal pathogens under field conditions.

Durable disease resistance is, in addition to yield, one of the most important traits in plant breeding (Summers and Brown 2013). However, there are reports about a possible trade-off between disease resistance and yield (Purrington 2000). Genetic resistance to biotrophic pathogens might come with pleiotropism with regard to enhanced susceptibility to hemibiotrophs or necrotrophs. A prominent example for pleiotropism is the *MILDEW LOCUS O* (*mlo*) powdery mildew (*Blumeria graminis* f. sp. *hordei*) resistance locus in barley (*Hordeum vulgare*). The *MLO* wild-type (WT) allele codes for a negative regulator of plant defense (Büschges et al. 1997), whereas loss-of-function mutations in *MLO* implement recessively inherited, monogenic, race-nonspecific, and durable resistance against barley powdery mildew (Jørgensen 1992) by reducing fungal penetration success (Stolzenburg et al. 1984).

In addition to this benefit, *mlo* resistance is known to come along with detrimental effects in terms of yield (Kjær et al. 1990). The spontaneous occurrence of necrotic leaf lesions on *mlo* mutants results in reduced grain number per stem (Thomas et al. 1998) and thousand-kernel weight (Hackett et al. 2001). Because this phenomenon was identified as a pleiotropic effect (Jørgensen 1992), breeders were able to produce high-yielding powdery-mildew-resistant barley *mlo* varieties by rearranging genes in other parts of the genome (Brown 2002).

Other trade-offs were documented for *mlo* resistance in barley with regard to susceptibility to nonbiotrophic pathogens. Most of the published data suggest increased susceptibility of *mlo* mutants to hemibiotrophic and necrotrophic fungi. Detached-leaf assays with plants of near-isogenic backcross (BC) lines revealed greater susceptibility of genotypes carrying a recessive *mlo* gene compared with WT individuals when inoculated with the hemibiotrophic rice blast pathogen *Magnaporthe oryzae* (Jarosch et al. 1999) or when infiltrated with toxic culture filtrates of the barley leaf spot blotch

fungus *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*) (Kumar et al. 2001). Jansen et al. (2005) conducted experiments in which detached barley spikes and caryopses were inoculated with *Fusarium graminearum*, resulting in increased susceptibility of *mlo* mutants when compared with *MLO* WT. Both, *B. sorokiniana* and *F. graminearum*, are often considered to be necrotrophic, because they produce host-nonspecific toxins. However, *B. sorokiniana* can also penetrate living host cells (Schäfer et al. 2004) and *F. graminearum* can grow symptomless between cells or between the cuticle and the cell wall (Brown et al. 2010; Linkmeyer et al. 2013). Makepeace et al. (2008) obtained conflicting data for the *Ramularia* (*Ramularia collo-cygni*) leaf spot disease (RLS) when inoculating barley seedlings under different light regimes. RLS has a long period of latency. The pathogen behaves like an endophyte until it switches to a necrotrophic lifestyle, which is likely triggered by host developmental cues (Havis et al. 2015). Pallas *MLO* WT plants were more heavily diseased in conditions without near-ultraviolet light than plants of its near-isogenic BC line, containing the *mlo5* resistance gene, which represents a functional null allele (Büschges et al. 1997). This was reversed in conditions with near ultraviolet light. However, a previous study, visually assessing the diseased leaf areas, showed reduced rather than enhanced susceptibility of *mlo* genotypes to RLS in the field (Makepeace et al. 2007). Barley *mlo* lines also show enhanced abiotic flecking (Brown and Rant 2013; Makepeace et al. 2007), which can be difficult to distinguish from RLS symptoms. Other field trials with regard to RLS resulted in enhanced RLS leaf symptom scores for genotypes containing recessive *mlo* alleles compared with plants with the WT *MLO* alleles (Pinnschmidt and Sindberg 2009). This was confirmed and further supported with the use of near-isogenic *mlo* BC lines in controlled environments (McGrann et al. 2014). Apart from those experiments, which specifically addressed the role of *mlo*, data from field trials are lacking regarding susceptibility of *mlo* mutant genotypes to pathogens other than powdery mildew. Studies with intact *mlo* plants instead of excised plant organs and experiments with quantification of present fungal biomass or DNA appear underrepresented. Measurements of fungal DNA appear to be an appropriate tool to assess infestation rates, particularly when symptoms are absent or unspecific. For instance, symptoms can

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either be unspecific, as for *Fusarium* spp. on barley (Tekauz et al. 2000), or difficult to quantify by visual methods, as for *R. collo-cygni* (Havis et al. 2015).

Fusarium head blight (FHB) of small grain cereals such as barley is caused by a complex of *Fusarium* spp. In spite of an international predominance of *F. graminearum*, *F. culmorum*, and *F. avenaceum* (Parry et al. 1995), the structure of pathogen spectra can differ between countries and include also *F. sporotrichioides*, *F. tricinctum* (Bottalico and Perrone 2002), *F. langsethiae* (Torp and Nirenberg 2004), and others. The damage caused by FHB is multifactorial and has relevance for barley comparable with that for wheat. On the one hand, FHB is capable of causing yield reduction (McMullen et al. 1997). Although yield damage potential on wheat appears more severe than on barley, reports from the 1990s documented appreciable yield losses in Northern Great Plains' barley production (Windels 2000). On the other hand, quality reductions with regard to baking, brewing, and seed features result from *Fusarium* infections (Parry et al. 1995). In addition, several *Fusarium* spp. have the potential to produce a range of mycotoxins (Bottalico and Perrone 2002) with negative effects on human and animal health (Bennett and Klich 2003). Consequently, yield and quality losses result in direct and secondary economic damages. In the period of 1993 to 1998, barley producers in North Dakota, South Dakota, and Minnesota had to register a total net revenue loss caused by FHB of \$406 million (Windels 2000). Production and price losses for barley producers in North Dakota added up to 25.7% in the period of 1998 to 2000 (Nganje et al. 2002).

FHB is known as a largely soilborne disease (Parry et al. 1995). *Fusarium* pathogens have the capability to overwinter and grow saprophytically on harvest residues (Jenkinson and Parry 1994). Plant infection may occur when cereal seed are sown into infested soil. This could lead to *Fusarium* seedling blight and *Fusarium* foot rot. At advanced stages of the vegetation period, airborne inoculum and rain splash can infect the spikes of plants and cause FHB. As an alternative to harvest residues, *Fusarium* spp.-infested grains as seed material can transmit the pathogen into the next season (Parry et al. 1995).

R. collo-cygni is likely distributed by airborne conidia released from typical swan-neck-shaped conidiophores (Havis et al. 2015). In addition, seed transmission is considered to be an important inoculum source with regard to epidemiology and perceived as a challenge for disease control and quarantine (Havis et al. 2011). RLS on barley has been an increasing problem since the end of the 1990s and may affect other cereal crops (Walters et al. 2008). RLS leads to a loss of green leaf area by causing reddish-brown lesions. Barley yield reductions caused by RLS can add up to 40% (Oxley and Havis 2004).

Under German production conditions, RLS causes high yield losses but FHB only moderately reduces yield in spring barley. However, both *Fusarium* spp. and *R. collo-cygni* can be seed transmitted and threaten malting quality of spring barley grain (Havis et al. 2015; Windels 2000).

Because *mlo* resistance in barley is known to have pleiotropic effects on the susceptibility toward necrotrophic and hemibiotrophic pathogens, the present study investigates whether *mlo* powdery mildew resistance enhances infestation of barley kernels with *Fusarium* spp. and *R. collo-cygni* DNA in spring barley grain when produced under natural environment conditions.

MATERIALS AND METHODS

Field experiment. Fundamentals. A quadrennial field experiment (2010–2013) was carried out including various spring barley varieties and *Fusarium* inoculation treatments (Supplementary Table S1). Because of the naturally high disease pressure in this region additional inoculation with *R. collo-cygni* was not necessary. The single field trials were carried out in Freising (Germany, 450 m above sea level, average temperature 7.7°C, average precipitation

814 mm, silt loam) by using microplots (double rows, 80 cm long, 17-cm row spacing, 30-cm plot spacing, 40 seeds/plot). Plant protection, growth regulation, and fertilization were carried out according to regional standard application rates. Matured plants (growth stage [GS] 92 to 95) were manually harvested and dried for 7 to 14 days in paper bags. Threshing took place with a single-spike threshers. Until further processing, generated grain material was stored in paper bags at room temperature.

Varieties or lines. The field trial included spring barley 'Ingrid' and 'Pallas' and their corresponding near-isogenic, powdery-mildew-resistant BC lines, BC Ingrid-*mlo5* and BC Pallas-*mlo5*. The tested lines of both Ingrid and Pallas were considered to be susceptible to FHB and RLS because symptoms of both diseases could be observed in preliminary tests.

Inoculation. For a facilitated inoculation handling, the trial setup provided a split-plot randomized complete block design, each block consisting of three to six replications of every variety or line. Corresponding blocks were spray inoculated with spore suspensions of *F. culmorum* (2010 to 2013), *F. graminearum* (2013), *F. avenaceum* (2010 and 2013), *F. tricinctum* (2013), *F. langsethiae* (2013), and *F. sporotrichioides* (2010 and 2013). A control block remained noninoculated. Spore densities reached from 1×10^6 and 1×10^7 conidia/m² (2013) through 3.6×10^7 conidia/m² (2010 and 2011) up to 7.5×10^7 conidia/m² (2012). The application was carried out twice within 7 days in the time period of mid- to late anthesis of plants (GS 65 to 69) using a backpack sprayer (pressure: 2.1 bar, nozzle type: 110/04).

Preparation of fungal material and inoculum suspensions. Single-spore isolates of *F. culmorum* (Fc002 and Fc03), *F. graminearum* (Fg07), *F. avenaceum* (Fa001 and Fa002), *F. tricinctum* (Ft001 and Ft01b), *F. langsethiae* (Fl001 and Fl002), and *F. sporotrichioides* (Fs002 and Fs01) were acquired, propagated, and processed to inoculum suspensions as described by Linkmeyer et al. (2013). All isolates derived from barley grain grown in Germany (Fc002, Fg07, Fa001, Fa002, Ft01b, Fl001, Fl002, Fs002, and Fs01), Denmark (Ft001), or Poland (Fc03).

Measurement of pathogen infestation. Isolation of genomic DNA from barley grain. Of the total yield (approximately 60 to 100 g/microplot), a grain subsample of 50 g per microplot was ground to fine powder using a laboratory mill. Isolation of DNA from barley grain material followed recommendations of the European Community Reference Laboratories (Joint Research Centre 2007) for the isolation of maize DNA, including some modifications described by Linkmeyer et al. (2013).

Quantification of genomic DNA in barley grain. To ascertain DNA amounts of *Fusarium* spp. in grain deriving from inoculated and control plots, a quantitative polymerase chain reaction (qPCR) protocol for fungal and barley DNA was accomplished on the basis of Nicolaisen et al. (2009) adopted by Linkmeyer et al. (2013). Amplification of DNA was carried out in a total volume of 20 µl containing 10 µl of 2× Maxima SYBR Green qPCR Master mix (Fermentas, St. Leon-Rot, Germany), forward and reverse primer at 300 nmol/liter each, 10 µg of bovine serum albumin, and 100 ng of genomic DNA. PCR amplification was performed in duplicates in an MX3000P Cycler (Stratagene, Santa Clara, CA). The thermal profile started with an initial step at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Subsequent melting curve measurement was performed at 55 to 95°C. In addition to specific primers for *Fusarium* spp. (FgramB379fwd/FgramB411rev, FculC561fwd/FculC614rev, FspoA18 fwd/FspoA85rev, FlangA29fwd/FlangA95rev, Fave574fwd/Fave627rev, and Ftri573fwd/Ftri630rev) a barley DNA assay was included for normalization (Hor1f/Hor2r). An external standard calibration was carried out to accomplish absolute quantification of barley and *Fusarium* DNA. For that purpose, dilution series (100, 10, 1, 0.1, and 0.01 ng of DNA) of pure fungal and barley DNA were generated and included in the qPCR assay. *Fusarium* DNA was diluted in fungal DNA-free barley DNA at 20 ng/µl to mimic matrix effects in DNA samples extracted from

barley grain material. Individual qPCR assays were verified for reproducibility, specificity, and sensitivity in our lab according to Nicolaisen et al. (2009) (Linkmeyer 2012).

Quantification of *R. collo-cygni* DNA in grain deriving from noninoculated control plots occurred by using the Taq-Man qPCR protocol of Taylor et al. (2010), including some modifications: PCR assays were performed in a total volume of 25 µl containing 1× iQ Supermix (Bio-Rad, Hercules, CA), 400 nmol⁻¹ forward and reverse primer (RamF6/RamR6), 150 nmol⁻¹ *Ramularia* probe (FAM; Ram6), 5 µl of DNA template (20 ng µl⁻¹), and PCR-grade water to make up the final volume. To confirm the reproducibility, PCR amplification was performed in duplicates using a MX3000P Cyclor (Stratagene). Nuclease-free water was utilized for negative (no template) control.

The thermal profile started with an initial step 10 min at 95°C and was followed by 48 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 20 s. A final extension step of 95°C for 1 min completed the profile. Absolute quantification of *R. collo-cygni* DNA was performed by external standard calibration. Therefore, dilution series of DNA from *R. collo-cygni* culture were generated (0.128, 0.64, 3.2, 16, 80, 400, 2,000, and 10,000 pg) and served as standard rows. Reproducibility, specificity, and sensitivity of the qPCR assay were reproduced in our lab according to Taylor et al. (2010).

Definition of inoculation success. To measure the inoculation success, DNA amounts of corresponding *Fusarium* spp. gave detailed information about the achieved infestation levels. Inoculation success was defined as significant increase ($P \leq 0.05$) of DNA amount in inoculated plots compared with noninoculated control plots.

Statistics. Generated data were collected and prepared with Microsoft Excel 2010. Statistical operations were carried out with IBM SPSS 21 statistics. Analyses of variance using the Tukey B test ($P \leq 0.05$) were conducted to compare means of multiple data sets.

RESULTS

To test the influence of the *mlo5* gene on *Fusarium* infection, *Fusarium* pathogen pressure was artificially increased by

inoculation with spore suspensions. Quantification of fungal biomass by qPCR analysis was used to measure fungal infection and inoculation success, because symptom formation on barley is unspecific for *Fusarium* spp. and *R. collo-cygni* infection (see below) on barley spikes is difficult to visually assess.

In 2010, we spray inoculated with spore suspensions of *F. culmorum*, *F. avenaceum*, and *F. sporotrichioides*, which produce different types of mycotoxins, in the field. All three inocula significantly and strongly enhanced fungal DNA content of the respective *Fusarium* spp. in harvested grain (Table 1). In 2013, we additionally included inoculum of *F. graminearum*, *F. tricinctum*, and *F. langsethiae* to test species frequently appearing and considered as important barley pathogens. However, inoculation success was detectable in 2013 only for *F. culmorum*, *F. sporotrichioides*, and *F. graminearum* but not for *F. avenaceum*, *F. tricinctum*, and *F. langsethiae* (Table 2).

Influence of *mlo5* on *Fusarium* infections. We compared *Fusarium* DNA amounts in barley grain of parent *MLO* Pallas or Ingrid and BC *mlo5* lines grown in naturally infected control plots and after spray inoculation in the field. This displayed higher natural inoculum pressures in 2010 than in 2013, as reflected by *Fusarium* DNA contents. In 2010, the Pallas background appeared slightly more susceptible to natural grain infestation by *Fusarium* spp. than the Ingrid background (Table 1). However, at the lower natural disease pressure in 2013, this difference was not visible any more (Table 2). Without artificial inoculation in the field, BC *mlo5* lines of Pallas or Ingrid were not significantly more or less infested with *Fusarium* spp. than WT *MLO* parent lines (Tables 1 and 2; Supplementary Table S3).

After spray inoculation in the field, BC *mlo5* lines appeared generally less infested with single *Fusarium* spp. in 2010. This effect was consistent in tendency and seen in both genetic backgrounds. It was statistically significant for *F. culmorum* between WT *MLO* lines and BC *mlo5* lines regardless of the Pallas or Ingrid background (Table 1). For *F. avenaceum*, enhanced infestation of BC *mlo5* genotype was statistically significant only in the background of Ingrid. For *F. sporotrichioides*, no statistically significant difference in fungal DNA in grain of *MLO* versus BC *mlo5* genotypes was observed. The tendency of reduced susceptibility of BC *mlo5* lines to grain

TABLE 1. *Fusarium* spp. DNA in grain of spring barley genotypes in 2010^z

Inoculum	<i>Fusarium</i> spp. DNA in pg/ng of barley DNA							
	Ingrid		BC Ingrid- <i>mlo5</i>		Pallas		BC Pallas- <i>mlo5</i>	
	Cont	Inoc	Cont	Inoc	Cont	Inoc	Cont	Inoc
<i>Fusarium culmorum</i>	0.66 a	118.03 c	1.95 a	61.86 b	2.79 a	117.33 c	3.10 a	68.59 b
<i>F. avenaceum</i>	1.71 a	34.68 c	2.84 a	19.42 b	3.75 a	43.63 c	4.01 a	34.01 c
<i>F. sporotrichioides</i>	0.24 a	32.82 b	0.24 a	23.64 b	0.11 a	31.52 b	0.47 a	33.12 b

^z Quantitative polymerase chain reaction analysis: DNA amounts are indicated as picograms of *Fusarium* spp. DNA per nanogram of barley DNA. BC = backcross, Cont = control, and Inoc = inoculated. Statistically significant differences in DNA amounts in rows are marked by different letters according to analyses of variance results. Inoculation success was defined as significant increase ($P \leq 0.05$) of DNA amounts in inoculated plots compared with noninoculated control plots; $n = 6$.

TABLE 2. *Fusarium* spp. DNA in grain of spring barley genotypes in 2013^z

Inoculum	<i>Fusarium</i> spp. DNA in pg/ng of barley DNA							
	Ingrid		BC Ingrid- <i>mlo5</i>		Pallas		BC Pallas- <i>mlo5</i>	
	Cont	Inoc	Cont	Inoc	Cont	Inoc	Cont	Inoc
<i>Fusarium culmorum</i>	0.01 a	1.07 c	0.01 a	0.67 bc	0.01 a	0.80 bc	0.02 a	0.50 b
<i>F. graminearum</i>	0.03 a	0.51 b	0.04 a	0.29 ab	0.04 a	0.39 b	0.05 a	0.26 ab
<i>F. avenaceum</i>	0.05 a	0.09 a	0.04 a	0.18 a	0.05 a	0.11 a	0.11 a	0.11 a
<i>F. tricinctum</i>	0.07 a	0.01 a	0.12 a	0.03 a	0.01 a	0.01 a	0.02 a	0.12 a
<i>F. langsethiae</i>	0.85 a	1.54 a	1.17 a	1.32 a	1.07 a	0.78 a	0.85 a	0.72 a
<i>F. sporotrichioides</i>	0.00 a	0.79 c	0.01 a	0.55 bc	0.01 a	0.41 abc	0.00 a	0.24 ab

^z Quantitative polymerase chain reaction analysis: DNA amounts are indicated as picograms of *Fusarium* spp. DNA per nanogram of barley DNA. BC = backcross, Cont = control, and Inoc = inoculated. Statistically significant differences in DNA amounts in rows are marked by different letters according to analyses of variance results. Inoculation success was defined as significant increase ($P \leq 0.05$) of DNA amounts in inoculated plots compared with noninoculated control plots; $n = 6$.

infestation following inoculation with *Fusarium* spp. in the field was visible in 2013, too. However, this effect was not statistically significant, likely due to the lower inoculation success (Table 2). In 2011 and 2012, we inoculated with *F. culmorum* only. This again resulted, by trend in 2011 and significantly in 2012, in lower fungal DNA content in grain of BC *mlo5* lines when compared with WT *MLO* lines.

In summary, there was no *MLO* genotype-dependent difference in *Fusarium* spp. DNA content of grain without inoculation. By contrast, BC *mlo5* lines were less field susceptible to grain infestation following inoculation with single *Fusarium* spp.

Influence of *mlo* resistance on the impact of *R. collo-cygni*. We further quantified *R. collo-cygni* DNA in barley grain deriving from noninoculated field plots over four consecutive years (2010 to 2013). A comparison of different trial years showed the highest *R. collo-cygni* infestation in 2012 (682.47 pg/ng of total DNA), followed by 2010 (256.11 pg/ng of total DNA) and 2011 (173.28 pg/ng of total DNA). In 2013, we determined the lowest infestation rate (86.80 pg/ng of total DNA). No consistent difference in infestation levels could be observed over the years between the WT *MLO* Pallas and Ingrid. When we compared WT *MLO* genotypes of Ingrid and Pallas with their near-isogenic BC *mlo5* lines, we detected no significant differences in *R. collo-cygni* infestation in any year of trial (Table 3). However, *R. collo-cygni* DNA content was consistently lower in the BC Ingrid-*mlo5* line compared with the Ingrid WT line. No such consistent tendency was observed in the Pallas background.

In summary, quantification of *R. collo-cygni* DNA in naturally infected barley grain did not detect significant differences in grain infestation that could be explained by the allele present at the *MLO* locus.

DISCUSSION

Our data suggest that powdery-mildew-resistant *mlo5* spring barley lines do not show enhanced field susceptibility toward grain infestation by partially seed-transmitted necrotrophic or hemibiotrophic ascomycete fungi of the *Fusarium* genus or *R. collo-cygni*. On the contrary, tested *mlo5* genotypes appeared similar or in several cases less infested with corresponding *Fusarium* pathogens than the respective near-isogenic WT lines. Although environmental factors, such as humidity and precipitation, might have influenced the natural infection conditions over the years (Meteorological Network of the Bavarian State Research Center for Agriculture 2014, Supplementary Table S2), data show a rather protective *mlo5* effect for *F. culmorum* infestation over 4 years with inoculation. *F. culmorum* is relevant for European spring barley production (Nielsen et al. 2011) and, in our opinion, the most reliable leads to inoculation success in the field (Linkmeyer et al. 2013). For other *Fusarium* spp. (*F. avenaceum*, *F. sporotrichioides*, *F. graminearum*, *F. tricinctum*, and *F. langsethiae*), data from 1 or 2 years of trials supported the idea that *mlo5* genotypes are at least not more susceptible to *Fusarium* grain infestation. This also suggests that the type of toxin produced by individual *Fusarium* spp. is possibly not decisive for the infection success on *mlo5* barley genotypes.

Previous studies have demonstrated enhanced susceptibility of *mlo5* genotypes to *M. oryzae* (Jarosch et al. 1999), *F. graminearum* (Jansen et al. 2005) and toxic culture filtrates of *B. sorokiniana* (Kumar et al. 2001). These studies were carried out under controlled environmental conditions, with high inoculum pressure and partially with excised plant organs. Our present results suggest that *Fusarium* spp. and *R. collo-cygni* grain infestation as one final outcome of a seasonal host-parasite interaction is not affected by a trade-off with *mlo5*-mediated powdery mildew resistance under field conditions. As grain infestation is highly relevant for malting quality and symptom formation is unspecific for *Fusarium* spp. and *R. collo-cygni* infection on barley spikes, quantification of corresponding fungal DNA was chosen as appropriate tool for assessing infestation.

Little information is available about the influence of the *MLO* genotype on spike infections in barley. Jansen et al. (2005) demonstrated a clear difference in progressive penetration success of *F. graminearum* into barley *MLO* or *mlo5* aleurone and endosperm of isolated caryopses (Jansen et al. 2005). Beside a decomposition of corresponding cells, large lesions were identified in the hypodermis of BC Ingrid-*mlo5* and BC Pallas-*mlo5*. Higher susceptibility of BC *mlo5* lines to lesion formation was independent on the ability of the fungus to produce trichothecenes. Our data do not support enhanced *Fusarium* susceptibility of the same BC *mlo5* lines to grain infestation in the field. No significant differences in *F. graminearum* DNA amounts were detected for different *MLO* genotypes of Ingrid and Pallas in 2013 (Table 2). Rather less *F. graminearum* DNA was detected in BC *mlo5* lines. In particular in 2010 grain infestation by several other *Fusarium* spp. was even lower in BC *mlo5* lines than in WT *MLO* lines after inoculation. Experimental conditions might have caused differences in the interaction outcome between the study of Jansen et al. (2005) and the present study. In vitro inoculation of barley-isolated caryopses occurred after removing glumes, lemma, and palea and by direct application of macroconidia on sterilized tissue (Jansen et al. 2005). Under field trial conditions, fungal spores had to overcome several barriers before a successful infection of caryopses might take place. Therefore, higher susceptibility of *mlo5* caryopses to fungal penetration might not manifest when considering intact plants. This questions the relevance of enhanced *mlo5* tissue susceptibility to necrotization under high *Fusarium* spp. pressure for field conditions and, hence, for decision making in plant breeding and integrated disease management. However, our data do not exclude the possibility that *mlo5* genotypes might show enhanced susceptibility to *Fusarium* spp. under environmental conditions that did not take place during our time frame of observation. It is further important to note that *mlo5*-mediated powdery mildew resistance is sensitive to abiotic stress conditions such as release from soil water shortage and heat (Schwarzbach 2001). Therefore, we cannot exclude the possibility that environment-genotype interactions have masked possible negative *mlo5* effects on basal *Fusarium* resistance. However, in four consecutive years with inoculations in the field, BC *mlo5* lines were consistently less susceptible to *F. culmorum*. We made similar observations for *Fusarium* spp. other than *F. graminearum* or *F. culmorum* which produce toxins; namely, the trichothecene A producer *F. sporotrichioides* or the enniatin and beauvericin producer *F. avenaceum*. Therefore, we conclude that the use of *mlo* alleles in spring barley breeding and disease management likely does not come with a general trade-off in regard to *Fusarium* grain infections.

McGrann et al. (2014) demonstrated enhanced susceptibility of diverse *mlo* mutant genotypes to RLS symptoms under natural infection conditions. The *mlo* effects on RLS symptoms were further confirmed under controlled conditions. However, previous triennial field trial studies did not observe similar effects but rather found reduced susceptibility to Rhynchosporium leaf spots and RLS (Makepeace et al. 2007). Influence of environmental factors on genotypes differing at the *MLO* locus was considered as a possible

TABLE 3. *Ramularia collo-cygni* DNA in grain of noninoculated spring barley genotypes^z

Year	<i>R. collo-cygni</i> DNA in pg/ng of total DNA			
	Ingrid	BC Ingrid- <i>mlo5</i>	Pallas	BC Pallas- <i>mlo5</i>
2010	228.77 a	127.20 a	231.47 a	394.03 a
2011	220.10 a	195.83 a	153.17 a	124.00 a
2012	779.19 a	414.31 a	798.08 a	738.28 a
2013	107.48 a	71.98 a	57.37 a	110.37 a

^z Quantitative polymerase chain reaction analysis: DNA amounts are indicated as picograms per nanogram of total DNA. Analyses of variance calculation could not detect any significant difference in DNA amounts between the wild type and backcross (BC) *mlo5* lines. Years: 2010, *n* = 2 (BC Ingrid-*mlo5*) and *n* = 3 (others); 2011, *n* = 3; 2012, *n* = 6; and 2013, *n* = 6.

reason for conflicting results. No information is available on whether fungal biomass or DNA content correlated with RLS symptoms on diverse *MLO* genotypes under field conditions. In addition to the perception of *R. collo-cygni* as a leaf pathogen, the fungus has the potential to infect spikes (Matusinsky et al. 2011; Oxley and Havis 2004). With regard to this, the present study has not detected an effect of *mlo* on *R. collo-cygni* grain infestation. In spite of annual variability in natural infection levels, we did not find significant differences between WT lines and BC *mlo5* genotypes of Ingrid and Pallas. However, there are several possible inoculum sources for *R. collo-cygni* (Frei et al. 2007) and, thus, our data cannot be generalized in terms of susceptibility of *mlo* mutant genotypes to *R. collo-cygni*. Tissue-specific differences may well be responsible for the lack of *mlo* impact on grain infestation by *R. collo-cygni*. Nevertheless, seed transmission is considered to be an important method of distribution for *R. collo-cygni* (Havis et al. 2014) and our data suggest that there is no general pleiotropic negative effect of *mlo*-mediated powdery mildew resistance on seed contamination with *R. collo-cygni*.

It was discussed that *mlo* mutants might show a generally reduced basal resistance to cell-death-inducing pathogens (Jansen et al. 2005; Jarosch et al. 1999; Kumar et al. 2001). Although fungi appear to penetrate deeper into tissue of *mlo5* mutant than WT *MLO* genotypes (Jansen et al. 2005; Jarosch et al. 1999), no quantification of fungal biomass on intact plants was carried out. Similar studies with *Arabidopsis thaliana* demonstrated that powdery-mildew-resistant *mlo* mutants developed more disease symptoms when inoculated with necrotrophic pathogens. However, disease symptom development did not consistently correlate with enhanced fungal biomass (Consonni et al. 2006). Enhanced susceptibility of *mlo5* mutants to *B. sorokiniana* was more reproducible when injecting toxic fungal culture filtrates than when inoculating with actual spore suspensions (Kumar et al. 2001). Together, this raises the question of whether *mlo5* mutants are indeed more susceptible to fungal necrotrophs or hemibiotrophs or, rather, develop more symptoms when challenged by cell-death-inducing agents. Virtually no information is available that would help in answering this question, in particular when considering field studies (Brown and Rant 2013). Loss-of-function *mlo* mutants do not only show powdery mildew resistance but also spontaneous defense responses, necrotic *mlo* spots, early senescence, and enhanced expression of defense genes, regardless of whether they are inoculated with biotrophic powdery mildew or with hemibiotrophic pathogens (Jarosch et al. 2003; Peterhänsel et al. 1997; Piffanelli et al. 2002; Schultheiss et al. 2003; Wolter et al. 1993). Therefore, it appears possible that inconsistent results on susceptibility of *mlo* mutant genotypes to hemibiotrophs results from *mlo*-mediated enhanced defense potential on the one hand, which might affect the pathogen during a biotrophic phase; and, on the other hand, a reduced threshold of *mlo* mutants for undergoing cell death when confronted with fungal toxin effectors during a necrotrophic phase. This gets even more complex considering that cell-death-inducing fungi can pursue a wide range of individual infection strategies (Horbach et al. 2011) and change trophism (Lee and Rose 2010) and relation to their host plant (Newton et al. 2010) within their life cycle. In particular, it may explain the differences between the studies of different necrotrophic pathogens in field and laboratory experiments in the regard to *mlo* resistance. Certainly, *mlo* resistance is a valuable tool to study differences in host–parasite interaction of different hemibiotrophic and necrotrophic cereal pathogens to further elucidate similarities and differences between biotrophic and nonbiotrophic pathogens in host–parasite interaction.

Genetic loss of susceptibility is considered to be a valuable tool in plant breeding but might come with a trade-off (Brown and Rant 2013; Hückelhoven et al. 2013). Loss of WT *MLO* function leads to powdery mildew resistance in many plant species (Acevedo-Garcia et al. 2014). Most recently, genome editing of hexaploid bread wheat succeeded in generation of powdery-mildew-resistant *mlo*

mutant wheat (Wang et al. 2014). Possible implementation of *mlo* resistance into wheat breeding programs has to consider *mlo* effects on basal resistance to *Fusarium* spp., *Magnaporthe* spp., and *R. collo-cygni*. Based on our data, an increased field susceptibility of *mlo* mutant genotypes to grain infestation by *Fusarium* spp. and *R. collo-cygni* grain infection cannot be declared. However, the outcome of an interaction of *mlo* mutants with fungal agents might change depending on environmental impacts or plant organ- or plant age-dependent factors. We recommend a reconsideration of the *mlo* mutant effect on field susceptibility to cell-death-inducing pathogens.

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