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**A trade off between \( \textit{mlo} \) resistance to powdery mildew and increased susceptibility of barley to a newly important disease, \textit{Ramularia leaf spot}**

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**Abstract**

\textit{Ramularia leaf spot} (RLS), caused by the fungus \textit{Ramularia collo-cygni}, is a serious, recently emerged disease of barley in Europe and other temperate regions. This study investigated the trade off between strong resistance to powdery mildew conferred by \( \textit{mlo} \) mutant alleles and increased susceptibility to RLS. In field trials and seedling tests, the presence of \( \textit{mlo} \) alleles increased severity of RLS. Genetic analysis of a doubled-haploid population identified one quantitative trait locus for susceptibility to RLS, colocalizing with the \( \textit{mlo-11} \) allele for mildew resistance. The effect of \( \textit{mlo-11} \) on RLS severity was environmentally sensitive. Analysis of near-isogenic lines of different \( \textit{mlo} \) mutations in various genetic backgrounds confirmed that \( \textit{mlo} \) alleles increased RLS severity in seedlings and adult plants. For \( \textit{mlo} \) resistance to mildew to be fully effective, the genes \( \textit{ROR1} \) and \( \textit{ROR2} \) are required. RLS symptoms were significantly reduced on \( \textit{mlo-5 ror} \) double mutants but fungal DNA levels remained as high as in \( \textit{mlo-5} \) single mutants, implying that \( \textit{ror} \) alleles modify the transition of the fungus from endophytism to necrotrophy. These results indicate that the widespread use of \( \textit{mlo} \) resistance to control mildew may have inadvertently stimulated the emergence of RLS as a major disease of barley.

**Key words:** Biotrophic pathogens, \textit{Blumeria graminis}, disease resistance, \textit{Hordeum vulgare}, necrotrophic pathogens, plant breeding, \textit{Ramularia collo-cygni}, ROR genes.

**Introduction**

Breeders of crop species need to select plants with adequate resistance to all significant diseases in the region for which seed of new cultivars is to be sold. They must also combine disease resistance with other commercially important traits, such as yield (Summers and Brown, 2013). Durable disease resistance can contribute to stability of yield despite annual variation in biotic and abiotic stresses and thus promote economic and environmentally sustainable food production. If a gene increases resistance to an important disease but has an undesirable impact on other traits, understanding the trade offs between these positive and negative effects can give insights into the gene’s function and inform rational decisions about the likely value of the gene for plant breeding (Brown and Rant, 2013). Here, this work reports that \( \textit{mlo} \) resistance to powdery mildew of barley, one of the most successful and widely used durable resistances to an important crop disease, has the detrimental effect of increasing susceptibility to another disease, \textit{Ramularia leaf spot}, which has become important over the period that \( \textit{mlo} \) has been widely deployed in cultivars.

A major success in plant breeding for disease resistance is the broad-spectrum, durable control of powdery mildew (\textit{Blumeria graminis f. sp. hordei}) of barley conferred by recessive alleles of
**Mlo.** Wild-type *Mlo* encodes a seven transmembrane-domain protein that interacts with calmodulin to negatively regulate plant defence and promote susceptibility to powdery mildew fungi in both monocot and dicot plants (Büschges et al., 1997; Kim et al., 2002; Consonni et al., 2006). Loss-of-function mutations in *Mlo* result in broad-spectrum resistance to mildew by restricting fungal penetration (Piffanelli et al., 2002). Mutations in *Mlo* have provided resistance against *B. graminis* f. sp. hordei for nearly 40 years (Jørgensen et al., 1992). The most commonly used alleles in agriculture are *mlo-11*, from an Ethiopian landrace, which is now used in about half of all European spring barley cultivars, and, in a few cultivars, *mlo-9*, an artificial mutant (Jørgensen et al., 1992; Dreiseitl, 2012; http://www.crpmb.org/mlo/). The *mlo-11* mutation results in aberrant transcription and thus a greatly reduced level of the MLO protein (Piffanelli et al., 2004). *mlo*-mediated resistance to *B. graminis* f. sp. hordei is compromised by mutations to two genes designated ROR1 or ROR2 (Required for mlo Resistance; Freialdenhoven et al., 1996).

There are agronomic costs associated with the use of *mlo* alleles in barley cultivars. Leaves of lines carrying *mlo* mutations typically suffer from apparently spontaneous necrotic spotting (Wolter et al., 1993). This results in a yield penalty, the size of which is correlated with the amount of spotting, which implies that it should be possible to select *mlo* varieties in which the yield penalty is mitigated by re-sortment of other, unidentified genes (Kjaer et al., 1990). The second cost of using *mlo* mutations in spring barley is an enhanced susceptibility to several facultative pathogens, including *Magnaporthe oryzae* (blast; Jarosch et al., 1999), *Cochliobolus sativus* (spot blotch; Kumar et al., 2001), and *Fusarium graminearum* (head blight; Jansen et al., 2005). Makepeace (2006) reported increased *Pyrenophora teres* (net blotch) in *mlo* plants although Jansen et al. (2007) reported no significant effect on this disease. By contrast, *mlo* may somewhat reduce susceptibility to *Rhynchosporium commune* (Makepeace, 2006; Makepeace et al., 2007). Why mutations in *Mlo* increase susceptibility to most facultative pathogens tested is not yet understood.

Emerging pathogens are a constant threat to many crops and pose novel disease resistance problems to plant breeding programmes. Barley is the fourth most important cereal in terms of global production, across temperate regions in both the northern and southern hemispheres. Ramularia leaf spot (RLS), caused by the ascomycete fungus *Ramularia collo-cygni*, has been increasing in importance as a pathogen of this crop plant since the late 1990s (Walters et al., 2008). *R. collo-cygni* is seed-borne and is also dispersed by spores (Schützendübel et al., 2008; Havis et al., 2011). Symptoms of RLS consist of characteristic reddish-brown rectangular lesions that are visible on both sides of the leaf and are often surrounded by a chlorotic halo. The disease is typically observed late in the growing season and can result in direct yield losses of up to 40% (Oxley and Havis, 2004) as well as greater losses in the market for malting because of higher screenings. There is a long latent period following *R. collo-cygni* infection before symptoms develop, implying that this fungus may be an endophyte that under certain conditions undergoes a transition to become a necrotrophic pathogen (Salamati and Reitan, 2006; Newton et al., 2010; Havis et al., 2014). The reasons why RLS is increasing in importance are not known. Changes in agricultural practices and reduced fungicide sensitivity (Fountaine and Fraajie, 2009) may be involved in the rise of RLS in Europe and it has been suggested that changes in climate may have increased its prevalence (Roos et al., 2011; West et al., 2012).

The enhanced susceptibility to facultative fungal pathogens of plants containing mutations in *Mlo* (Jarosch et al., 1999; Kumar et al., 2001; Jansen et al., 2005), the widespread use of spring barley varieties with *mlo* mildew resistance (http://www.crpmb.org/mlo/) and the emergence of RLS as an important new facultative disease of barley led to the hypothesis that *mlo* alleles may be implicated in the increased prevalence of RLS in Europe (Makepeace et al., 2007). There have been contrasting reports about the effect of *mlo* mutations on the severity of RLS. In a series of field trials, adult plants of lines containing *mlo* mutations had lower RLS scores than near-isogenic lines (NILs) with the wild-type *Mlo* allele. In experiments using artificial inoculation of seedlings in controlled environment conditions, however, plants with the *mlo-5* allele developed more RLS symptoms than *Mlo*+ NILs, especially when the plants were exposed to high light intensity before inoculation (Brown and Makepeace, 2009). In inoculated field trials, commercial spring barley varieties with an *mlo* allele were on average more susceptible to RLS than *Mlo*+ varieties, with all the most susceptible varieties having an *mlo* allele and all the least susceptible having *Mlo*+ (Pinnschmidt and Sindberg, 2009); these results are consistent with the seedling experiments of Brown and Makepeace (2009). It is therefore possible that *mlo* mildew resistance may play an important role in the aetiology of RLS, a disease which has become a major threat to barley production in temperate regions. The evidence for such a relationship, however, is currently ambiguous.

This paper reports a genetic analysis of resistance to RLS, showing that *mlo* mutant alleles enhance susceptibility to RLS in spring barley in an interaction which appears to be environmentally dependent. Furthermore, experiments using barley lines with mutations in *ROR* genes indicate that the enhanced susceptibility to RLS is functionally linked to *mlo*-mediated mildew resistance. This implies that there is a trade off between resistance and susceptibility to these two diseases which depends on genetic background and environmental conditions. The data presented here emphasize the risk that susceptibility to previously unknown plant diseases can arise from plant breeding strategies that rely on the successful use of single major resistance genes to control one disease in elite crop varieties.

**Materials and methods**

**Plant material**

The genetics of resistance to RLS were investigated using two doubled-haploid (DH) populations developed from crosses between the spring barley cultivars Power × Braemar (POBR, 196 lines) and Decanter × Cocktail (DECO, 144 lines). In field trials, Power typically develops little RLS whilst Braemar is highly susceptible (Oxley et al., 2008; Pinnschmidt and Sindberg, 2009; Oxley and Havis,
Decanter and Cocktail have been described as resistant and susceptible, respectively, to RLS (Oxley and Havis, 2010). Decanter, however, is susceptible to RLS at the seedling stage (Makepeace et al., 2008) and allows high R. collo-cygni DNA levels to build up despite limited symptom development in field conditions (Oxley and Havis, 2010). Both crosses allowed assessment of the effect of mlo on RLS development as Braemar and Decanter carry the mutant mlo-11 allele while Power and Cocktail have the wild-type allele, Mlo.

In addition, mlo and Mlo" NILs were used to investigate the role of mlo alleles in susceptibility to R. collo-cygni. The backcross lines carrying mlo-1, mlo-3, mlo-5, and mlo-9 mutations in cv. Ingrid, mlo-1 in cv. Haisa, mlo-3 in cv. Malteria Heda, and mlo-5 in cv. Pallas (line P22) as well as mlo-5+ror-1 (line A89) and mlo-5+ror-2 (line A44) double mutants in the cv. Ingrid background have been described previously (Jorgensen, 1976; Kolster et al., 1986; Freidalenhoven et al., 1996).

Ramularia collo-cygni isolate and culture medium

R. collo-cygni Rcc09B4 collected from Bush Estate, Midlothian, Scotland in 2009 by Dr Neil Havis (Scotland’s Rural College, Scotland) was used in all disease experiments. Fungal cultures were maintained as previously described (Makepeace et al., 2008). Rcc09B4 liquid cultures were prepared as described by Peraldi et al. (2014).

Ramularia collo-cygni inoculated seedling bioassays

Seeds were sown in Levinton F2 compost media (Scotts Professional, Ipswik, UK) in a controlled environment room (Sanyo) with a 16/8 light dark cycle (220 μmol m⁻² s⁻¹ fluorescent lighting, 18/12 °C). In each of three replicate experiments, six seeds each from 196 lines of the POBR DH population plus the parents Power and Braemar were sown in rows of 3×3×5 cm (P60) pots. In separate experiments, 10 seeds of each of the barley near-isogenic mlo backcross lines and the Ingrid mlo-5+ror double-mutant lines were sown into individual 8×8×10 cm pots. Rcc09B4 inoculum was prepared and plants inoculated with a slurry of hyphal fragments using an airbrush sprayer as described by Peraldi et al. (2014), developing the method of Makepeace et al. (2008). Plants were then placed under plastic covers to maintain high relative humidity (80–100%) in the dark for 48h, then under fluorescent lighting at 220 μmol m⁻² s⁻¹ for the duration of the experiment. Plants were watered as required to keep the potting medium moist but not saturated. To delay the onset of senescence of the prophyl, new leaf growth was cut from the plants every 2 or 3 days. Disease was scored as the percentage area of the prophyll covered with RLS lesions. Leaves were scored three to six times between 1 and 4 weeks after inoculation and the area under the disease progress curve (AUDPC; Shaner and Finney, 1977) was calculated. Pathology data for the mlo NILs and ror mutants was collected from separate experiments. Data on the mlo backcross lines was collected from three independent experiments and for the ror mutants from five experiments.

Field trials of Ramularia leaf spot

Six field trials were conducted during 2012 at Irlbach, Mallersdorf and Moosburg an der Isar, Bavaria, Germany, and Bush (Midlothian), Glenrothes, and Perth, Scotland. Four replicates of each population were trailed in each country, with at least one complete replicate of each population at each site. Partial replication of lines within sites provided control of environmental variation. As well as the parents of each cross, 188 and 144 DH lines of the POBR and DECO populations, respectively, were tested. Field trial management, including cultivation, fertilizer, insecticide, and herbicide, was according to standard practice at each site. Where required, powdery mildew was controlled by spraying with Cyflamid (0.5 l ha⁻¹), which does not affect RLS, and other diseases with a strobilurin (quinine-outside inhibitor) fungicide, to which R. collo-cygni is now resistant (Fountain and Fraaije, 2009).

At each site, RLS was scored as percentage leaf area covered with disease symptoms on the highest leaf layer on which symptoms were fully developed but which had not yet senesced. At sites in Germany, this was the flag (topmost) leaf, but at Irlbach, scores were obtained for the flag leaf and the second leaf layer separately. RLS was scored on the second leaf layer at sites in Scotland. Plants in Germany were at growth stage (Tottman and Makepeace, 1979) 71–85 when assessed and those in Scotland at growth stage 67–72. RLS levels on the DECO population at Moosburg were very low and so this trial was not scored.

Several spotting syndromes affect barley (Oxley et al., 2010). RLS symptoms were scored following Makepeace et al. (2008); see also Oxley et al. (2010, photographs on pp 9–16). Briefly, RLS was distinguished from other symptoms by the four Rs: small lesions which are rectangular, reddish-brown in colour, surrounded by a ring of chlorosis, and, as mature lesions, extend right through the leaf between the adaxial and abaxial surfaces. The spotting form of net blotch (P. teres var. maculata), with which RLS can be confused (Oxley et al., 2010, p 50), was not observed in these field trials.

Adult plant polytunnel trial

Adult plants of Ingrid, Haisa, Malteria Heda, and Pallas and mlo NILs of these cultivars were tested for susceptibility to RLS in a polytunnel trial. The experiment was planted in four blocks with three replicate plants of each line in each block in a randomized block design. Plants were inoculated with one of two doses of Rcc09B4 inoculum (2.5 or 10%, v/v, liquid culture in water supplemented with 0.01%, v/v, Tween 20), and at one of two times, growth stage 32 or growth stage 65 (i.e. before or after heading). Inoculum was applied using a CP3 knapsack sprayer (Cooper Pegler, Villefranche-sur-Saône, France). RLS severity was scored after flowering (growth stage 67–72) as described for the field trials.

R. collo-cygni DNA quantification

In planta R. collo-cygni DNA was quantified by quantitative PCR (Taylor et al., 2010) in DNA extracts from leaves of plants inoculated with Rcc09B4, sampled on the last scoring date in each experiment. Genomic DNA was extracted using a DNeoasy Plant Mini Kit (Qiagen) following the manufacturer’s instructions and amplified in a CFX96 thermocycler (Bio-Rad) as previously described (Taylor et al., 2010). DNA was quantified by comparison to a standard curve of known R. collo-cygni genomic DNA concentrations. Measurements were made from five independent samples of each line collected from three independent experiments.

B. graminis f. sp. hordei inoculation experiments

Six seeds of each of the POBR DH lines plus their parents were sown in F2 compost in P60 trays and placed on outdoor benches at the John Innes Centre to allow natural infection by B. graminis f. sp. hordei. Plants were scored for mildew at growth stage 36 using the 0–4 infection-type scale (Moseman et al., 1965). In practice, because only scores of 0 or 4 on the infection-type scale were recorded, they were converted to 0 and 1, respectively, to indicate absence or presence of mildew. Two independent experiments were done, which produced identical scores for all lines.

Genotyping

The POBR and DECO DH populations, namely all 196 POBR lines and 144 DECO lines, were genotyped with the cultivar-optimized subset of 384 single-nucleotide polymorphism markers (Moragues et al., 2010). Two additional PCR markers that distinguished between the wild-type MLO allele and the introgressed chromosome segment bearing mlo-11 were added to confirm the MLO status of each line in the POBR population (Piffanelli et al., 2004; Reinstadler et al., 2010). Marker 11_20119 was used as a diagnostic marker for mlo-11 in the DECO DH population. A genetic map of each cross was produced using the programme JoinMap version 3.0 (van Ooijen and Voorrips, 2001). Markers were formed into one or more groups within known
chromosomal locations using a log-likelihood (LOD) threshold 
(details in Results). A map of the markers in each group was then 
estimated using the following parameters: minimum LOD to detect 
linkage 1.0, maximum recombination fraction 0.4, maximum LOD 
jump to retain marker in map 5.0. After the addition of each marker, 
the map was ‘ripped’ by optimizing the order of triplets of mapped 
markers. The Kosambi map function was used to transform recombi-
nation fractions to map distances. All chromosomes were mapped in 
the first round of the JoinMap method, indicating that there was little 
or no conflict in the map positions of linked markers.

**Statistical analysis**

In all experiments, disease data were analysed as AUDPC as a percent-
age of the maximum possible AUDPC (maxAUDPC). Data were logit-
transformed to normalize the variance of errors and make the errors 
independent of expected values. An empirical logit transformation 
(Collett, 2002), log([AUDPC+0.25]/(maxAUDPC−AUDPC+0.25)), 
was used to avoid an undefined transformed value when AUDPC=0 
or 100, although the latter result did not occur in these experiments.

Seeding disease data were analysed by linear mixed model-
ing. To estimate the effect of Mlo alleles, the fixed effects were Experiment*Gmo genotype, the random effects were Line and Tray 
nested within (f) experiment, and the residual random term was Row 
within Tray. To estimate lines’ mean scores for quantitative trait 
locus (QTL) analysis, the fixed model was Experiment*Line and the 
random model Experiment/Tray/Row.

To analyse data from the Mlo NILs and the Ingrid mlo-5+or 
experiments, Experiment and Line were fixed effects and Pot the random 
effect. The significance of differences between lines or other factors, 
here and in other experiments, was tested by Fisher’s protected least 
significant difference. Data on amounts of log-transformed amounts 
of R. collo-cygni DNA in seedlings were analysed by general linear 
modelling. The model used was Line + Experiment.

Although all field trials were laid out as two incomplete blocks, 
the arrangement of blocks was not relevant to spatial variation at 
most trial sites. Predicted means for each line were estimated sepa-
arately in each trial.

Post-hoc analysis of spatial layout was assessed in a model which 
fitted the two major spatial axes of the trial layout then the blocks. 
Blocks and directional axes were dropped successively from the 
model if they were not statistically significant (P>0.05) in a general 
linear model. The effect of Line at each site was estimated in a model 
with relevant spatial variables+Line as fixed effects and Plot as the 
random effect. The effect of Mlo allele was estimated using spatial 
variables + Mlo as fixed effects and Line + Plot as random effects.

In the inoculated polytunnel trial of the NILs as adult plants, 
treatment factors + Line were fixed effects and Replicate the ran-
dom effect.

Where required, the significance of differences in unplanned com-
parisons of different factors was tested by Fisher’s protected least 
significant difference. All statistical analysis was conducted using 
Genstat version 15 (Payne et al., 2009).

**QTL analysis**

Predicted means of logit-transformed percentage RLS for each line 
were used to identify and map QTL for susceptibility to RLS, using 
the programme MapQTL 5 (van Ooijen, 2004). Data from each popu-
lation (POBR or DECO) in each trial, including seedling experiments 
on POBR, were analysed separately. For each dataset, automatic 
cofactor selection with a significance test probability (P) of 0.02 was 
run, first for each linkage group separately then iteratively for the 
whole genome, as described in the MapQTL 5 manual. Once a final 
set of cofactors was selected, QTLs were located by multiple QTL 
mapping. The significance of mapped QTLs was determined by a per-
mutation test (Churchill and Doerge, 1994). To test the prior hypo-
thesis of an association between Mlo alleles and severity of RLS, the 
significance threshold for chromosome 4H was used. The significance 
of other QTLs was tested by the higher, genome-wide P-value.

**Results**

**Ramularia leaf spot in field trials and seedling experiments**

Mean RLS severity on each line of both DH populations had an 
approximately continuous distribution, both within trials and 
across the series of trials. The range of mean RLS severity 
was larger for the POBR population (Fig. 1a) than for DECO 
(Fig. 1b), reflecting a substantially greater difference between 
Power (12%) and Braemar (53%) than between Decanter 
(18%) and Cocktail (24%). The Mlo allele had a large, significant 
effect on RLS scores in the POBR population (P<0.001), 
with a mean score for the Mlo+ lines of 22.6% (95% confidence 
interval, CI, 21.3–24.0%) and 35.3% (95% CI 33.5–37.3%) for the 
mlo-11 lines. There was a small but significant interaction 
between the Mlo allele and the field trial site (P<0.001); while 
there was significantly more RLS on mlo-11 lines at all six sites, 
this effect was stronger in the trials in Germany than in 
Scotland (Fig. 2a). In the DECO population, the Mlo allele did not 
have a significant effect on RLS scores in the series of trials as a 
whole (P=0.6) but there was a significant interaction with trial site (P=0.002). At both sites scored in Germany, mlo-11 
lines had significantly more RLS than Mlo+ lines, whereas at all 
three sites in Scotland, the mlo-11 lines had less RLS than Mlo+ 
but the differences were not significant (P>0.1 in each case, 
Fig. 2b). These results imply that both the environment and the 
genetic background influence the effect of Mlo alleles on RLS.

The POBR population was also tested for responses to R. 
collo-cygni at the seeding stage. As in the field trials, RLS 
severity in seedlings of the POBR population had an approximately 
continuous distribution (Fig. 1c). Disease severity varied 
between experiments (P<0.001) but in all three, the more-
resistant parent Power had less RLS than Braemar. The presence 
of the wild-type Mlo+ allele significantly reduced RLS 
levels in the seedling assays (P<0.001), a trend that was con-
sistent across experiments (P=0.09, Fig. 2c). The mean of the 
AUDPC of RLS was 6.4% (95% CI 5.8–7.0%) for Mlo+ and 
7.4% (95% CI 6.8–8.1%) for mlo-11.

**QTL analysis of RLS severity**

Of the 384 single-nucleotide polymorphism markers tested, 
125 and 127 were polymorphic between the parents of the 
POBR and DECO DH populations, respectively, and 122 
and 120 markers were mapped in these populations. With 
one exception, linkage groups were formed at a threshold of 
LOD2.4. In the POBR population (Supplementary Fig. 
S1, available at JXB online), there was a spurious linkage 
between two markers, one previously assigned to chromo-
some 2H and the other to 6H, so those groups were separated 
at LOD26. Chromosomes 1H and 2H were mapped in two 
linkage groups each. One marker, previously assigned to chro-
mosome 4H, had strongly distorted segregation and was not 
included in the map while two other markers, 11_21353 and 
11_21504, were omitted because, while they had previously 
been mapped to the middle of chromosome 4, in POBR they 
were closely linked to each other but not to other markers on 
chromosome 4. As expected, the two Mlo-specific markers 
cosegregated and mapped to the distal end of the long arm of
Effect of *mlo* mildew resistance on susceptibility to Ramularia leaf spot

Fig. 1. Histograms of the mean Ramularia leaf spot disease levels in field trials of the doubled-haploid (DH) populations: Power×Braemar (a), Decanter×Cocktail (b), and seedlings of Power×Braemar (c). Arrows indicate the means for the parents of each population. lai, leaf area infected.
A single QTL for RLS severity in field trials was closely linked to the Mlo locus in the POBR population, with increased resistance contributed by the allele from Power, which has the Mlo* allele for mildew susceptibility. This QTL was significant (P<0.05) at five of the six trial sites with narrow-sense heritability (equivalent to the percentage genetic variation in disease severity explained by the QTL) between 4 and 37% (Table 1). The Mlo locus had a greater effect on RLS at the sites in Germany than in Scotland (Table 1 and Fig. 2a). No QTL for RLS severity associated with the BOPA1 marker 11_20119, closely linked to Mlo, was identified in the DECO population at any field trial site, which is consistent with the smaller and less consistent effect of Mlo alleles on RLS in this population (Fig. 2b). Analysis of the seedling data also identified a QTL for RLS severity at the Mlo locus. Although mlo-11 lines had a greater mean RLS score in all three experiments, the QTL was only significant (P<0.05) in one experiment, in which the narrow-sense heritability associated with mlo-11 was 6% (Table 1).

No other QTL were detected above the critical LOD threshold in either the field trials or seedling experiments when RLS data for all the progeny of the POBR population were analysed. Data for Mlo* and mlo-11 lines were analysed separately in POBR, in case the effect of Mlo was epistatic to that of other genes. Although some QTL effects were significant at individual sites, none were replicated at more than one site. It is quite likely that their identification was a type I statistical error (false positive). The absence of consistent QTLs in the subdivided data implies that no epistatic effect on RLS of other genes interacting with Mlo alleles on RLS in this population (Fig. 2b). Analysis of the seedling data also identified a QTL for RLS severity at the Mlo locus. Although mlo-11 lines had a greater mean RLS score in all three experiments, the QTL was only significant (P<0.05) in one experiment, in which the narrow-sense heritability associated with mlo-11 was 6% (Table 1).

To test the function of mlo-11 on conferring almost-complete immunity to powdery mildew, the POBR DH population was screened for resistance to natural infection by B. graminis f. sp. hordei. Two QTL for mildew resistance were identified. One, on chromosome 4H, coincided with the mlo-11 allele in Braemar and accounted for 42% of genetic variation in mildew (Table 2). The other, controlling 13% of variation, was on chromosome 1H between markers 11_10332 and 11_10775, which flank the multiallelic Mla gene controlling gene-for-gene resistance to mildew. As mlo-11 confers strong resistance to

![Fig. 2](image-url)
Effect of mlo mildew resistance on susceptibility to Ramularia leaf spot

Log-likelihood (LOD) scores were calculated by multiple QTL mapping (details in text) of logit-transformed percentage data on leaf area affected by Ramularia leaf spot (RLS). LOD are shown for four markers at the distal end of chromosome arm 4HL; no QTL were detected in other regions of the genome. RLS: seedling, maximum area under the disease progress curve; field trials, leaf covered with RLS. Additive effect calculated on logit scale; $h^2$, narrow-sense heritability.

### Table 1. QTL analysis of Ramularia leaf spot scores in a population of F1 doubled-haploid progeny of the spring barley cultivars Power×Braemar

Log-likelihood (LOD) scores were calculated by multiple QTL mapping (details in text) of logit-transformed percentage data on leaf area affected by Ramularia leaf spot (RLS). LOD are shown for four markers at the distal end of chromosome arm 4HL; no QTL were detected in other regions of the genome. RLS: seedling, maximum area under the disease progress curve; field trials, leaf covered with RLS. Additive effect calculated on logit scale; $h^2$, narrow-sense heritability.

| Site            | LOD (position on 4H, cM) | Critical LOD* | RLS for allelic class (%) | Additive effect | $h^2$ (%) |
|-----------------|--------------------------|---------------|---------------------------|-----------------|-----------|
|                 | 11_10751 (100.5)         |               |                           |                 |           |
| Seedling 1      | 0.3                      | 6.2           | 0.0                       | 1.6             | 0.095     |
| Seedling 2      | 0.3                      | 10.5          | 8.9                       | 1.5             | 0.075     |
| Seedling 3      | 0.4                      | 11_10332      | 14.1                      | 10.9            | 0.36      |
| Germany         | 0.3                      | 11_10775      | 14.1                      | 10.4            | 0.36      |
| Scotland        | 0.1                      | 11_10030      | 16_10262                  | 16.2            | 0.2       |
| Perth           | 0.0                      |               |                           |                 |           |
|                 | 11_20732 (102.1)         | 62.5          | 0.2                       | 0.6             | 0.2       |
|                 | 11_20119 (113.1)         | 102.0         | 2.2                       | 1.8             | 0.96      |
|                 | MLO (115.7)              | 114.8         | 22.8                      | 2_0119          | 0.096     |

*P≤0.05 from a permutation test (van Ooijen, 2004).

### Table 2. QTL analysis of powdery mildew scores in a population of F1 doubled-haploid progeny of the spring barley cultivars Power×Braemar

Log-likelihood (LOD) scores were calculated by multiple QTL mapping of data on the presence or absence of mildew on barley seedlings exposed to natural inoculum. LOD are shown for relevant markers around the two QTL detected on chromosomes 1H and 4H. $h^2$: narrow-sense heritability.

| Chromosome | Marker | Position (cM) | LOD* | Mean score for allelic class | $h^2$ (%) |
|------------|--------|---------------|------|-----------------------------|-----------|
|            |        |               |      | Power                       | Braemar   |
| All lines  | 1H     | 11_21226      | 6.2  | 0.0                         | 1.0       |
|            | 11_10332 | 10.5          | 10.5 | 8.9                         |           |
|            | 11_10775 | 14.1          | 10.2 | 13.0                        |           |
|            | 11_10030 | 16.2          | 0.2  |                             |           |
|            | 4H     | 11_10262      | 62.5 | 0.6                         | 0.05      |
|            | 11_10751 | 102.0         | 12.2 | 41.6                        |           |
|            | 11_20732 | 103.5         | 1.1  |                             |           |
|            | 11_20119 | 114.8         | 22.8 |                             |           |
|            | MLO    | 117.3         | 26.4 |                             |           |
| MLO+ lines | 1H     | 11_21226      | 6.2  | 0.2                         | 0.03      |
|            | 11_10332 | 10.5          | 10.5 | 39.1                        |           |
|            | 11_10775 | 14.1          | 10.9 |                             |           |
|            | 11_10030 | 16.2          | 0.4  |                             |           |

*Genome-wide critical value of LOD for unplanned tests = 2.7 ($P \leq 0.05$, permutation test: van Ooijen, 2004).

mildew and is thus epistatic to other mildew-resistance genes, the QTL analysis was repeated for the MLO+ lines only; the QTL on chromosome 1 was now the only one identified and controlled 39% of genetic variation (Table 2). This implies that Braemar has an unidentified allele of Mla effective against the local population of B. graminis f. sp. hordei.

### Table 2. QTL analysis of powdery mildew scores in a population of F1 doubled-haploid progeny of the spring barley cultivars Power×Braemar

Log-likelihood (LOD) scores were calculated by multiple QTL mapping of data on the presence or absence of mildew on barley seedlings exposed to natural inoculum. LOD are shown for relevant markers around the two QTL detected on chromosomes 1H and 4H. $h^2$: narrow-sense heritability.

| Chromosome | Marker | Position (cM) | LOD* | Mean score for allelic class | $h^2$ (%) |
|------------|--------|---------------|------|-----------------------------|-----------|
|            |        |               |      | Power                       | Braemar   |
| All lines  | 1H     | 11_21226      | 6.2  | 0.0                         | 1.0       |
|            | 11_10332 | 10.5          | 10.5 | 8.9                         |           |
|            | 11_10775 | 14.1          | 10.2 | 13.0                        |           |
|            | 11_10030 | 16.2          | 0.2  |                             |           |
|            | 4H     | 11_10262      | 62.5 | 0.6                         | 0.05      |
|            | 11_10751 | 102.0         | 12.2 | 41.6                        |           |
|            | 11_20732 | 103.5         | 1.1  |                             |           |
|            | 11_20119 | 114.8         | 22.8 |                             |           |
|            | MLO    | 117.3         | 26.4 |                             |           |
| MLO+ lines | 1H     | 11_21226      | 6.2  | 0.2                         | 0.03      |
|            | 11_10332 | 10.5          | 10.5 | 39.1                        |           |
|            | 11_10775 | 14.1          | 10.9 |                             |           |
|            | 11_10030 | 16.2          | 0.4  |                             |           |

*Genome-wide critical value of LOD for unplanned tests = 2.7 ($P \leq 0.05$, permutation test: van Ooijen, 2004).

Ramularia leaf spot in Mlo near-isogenic lines

In seedlings of NILs inoculated with R. collo-cygni isolate Rcc09B4, the presence of mutant mlo alleles was associated with significantly greater RLS in all the genetic backgrounds tested compared to mother lines with the wild-type
The presence of the mlo mutant alleles also significantly increased RLS symptoms on inoculated adult plants compared to their respective wild-type lines ($P<0.001$; Fig 3b). There was also an effect of the timing and dose of inoculation on development of RLS in the adult-plant experiment, with greater disease on plants inoculated with the higher dose after heading than in the other treatments ($P<0.01$). There were significant differences in RLS levels between seedlings ($P<0.01$) and adult plants ($P<0.001$) of the Ingrid NILs with different mlo mutant alleles but these differences were not consistent between the two plant growth stages (Fig. 3).

Susceptibility to RLS was tested in NILs of cv. Ingrid with the mlo-5 allele in which susceptibility to mildew has been partially restored by mutations at the ROR1 or ROR2 loci (Freialdenhoven et al., 1996). Seedlings with the mlo-5+ror1-2 or mlo-5+ror2 genotypes had significantly less RLS symptoms than near-isogenic IngridBCmlo-5 plants with wild-type ROR1 and ROR2 ($P<0.001$, Fig. 4a and b). The mlo-5+ror1-2 genotype had less disease than the mlo-5+ror2 genotype (Fig. 4b). In inoculated adult plants, RLS symptoms on mlo-5+ror1-2 plants were reduced approximately to the level on cv. Ingrid (Fig. 3b).

R. collo-cygni DNA levels were measured by quantitative PCR in leaves of Ingrid, IngridBCmlo-5, and the two mlo-5+ror genotypes at the final scoring date of each experiment to test if RLS symptoms were correlated with fungal biomass. Fungal DNA levels were higher in IngridBCmlo-5 than in Ingrid ($P<0.001$, Fig. 4c), consistent with RLS symptoms in these two genotypes (Fig. 4b). R. collo-cygni DNA levels in mlo-5+ror1-2 and mlo-5+ror2 plants were not reduced compared to IngridBCmlo-5 (Fig. 4c) despite the reduced disease development and lower final RLS levels in these mutants (Fig. 4b and d). R. collo-cygni DNA levels were similar between IngridBCmlo-5 and mlo-5+ror2 but were significantly higher in mlo-5+ror1-2 ($P<0.05$, Fig. 4c).

### Discussion

These results show that recessive loss-of-function alleles of Mlo, which confer resistance to powdery mildew (Büschges et al., 1997), greatly increase susceptibility of barley to Ramularia leaf spot caused by R. collo-cygni. This effect is apparent in controlled, inoculated experiments on near-isogenic lines in which different mlo alleles are present in various genetic backgrounds (Fig. 3). It is also associated with the introgression of mlo-11 in barley populations in naturally infected field trials (Fig. 2a and Table 1) but the effect of mlo-11 on increasing susceptibility to RLS depends on the genetic background (Fig. 2a and b) and the environment (compare sites within Fig. 2a and b).

The effect of mlo-11 on increasing RLS in the POBR population was strong with a QTL for RLS mapping to the Mlo locus (Table 1). Although the single-nucleotide polymorphism markers 5_1_20119, which is closely linked to mlo-11 (Table 1) was associated with higher RLS in the DECO population at the sites in Germany (but not Scotland), the effect was smaller than for POBR and there was no significant QTL effect at that locus (note that the significance test in QTL mapping is more conservative than the $F$-test in linear mixed models).

The conclusion that the effect of mlo-11 on RLS depends on environmental conditions is consistent with previous results. Abiotic stress such as exposure to high light intensity increases RLS symptoms (Makepeace et al., 2008; Peraldi et al., 2014) and this response is enhanced by the presence of
Effect of mlo mildew resistance on susceptibility to Ramularia leaf spot

The result that, in inoculated adult plants, all mlo alleles tested in diverse genetic backgrounds were consistently associated with increased RLS (Fig. 3) is in striking contrast to Makepeace et al. (2007), who found that, in the same lines, mlo alleles were associated with somewhat lower RLS symptoms in trials in Scotland and Ireland. Two major differences between the present results and those of Makepeace et al. (2007) are that the polytunnel inevitably provides environmental conditions which are unnatural and may be stressful and that levels of RLS were considerably greater in the polytunnel. Either or both factors may have led to the contrasting results.

Developing elite crop varieties that maintain high yields across different environments is an important goal for plant breeding. Durable disease resistance helps to increase the stability and predictability of yield in the face of diverse pathogens. While many durable resistances are oligogenic or polygenic (St Clair, 2010), those controlled by single genes with large effects are particularly easy to select and are therefore attractive for breeding. mlo resistance to powdery mildew is an outstanding example of such a gene for durable resistance as it has been effective since its introduction into spring barley cultivars in the 1970s (Jørgensen et al., 1992), especially since the early 1990s (Thomas et al., 1998; Dreiseitl, 2012). Biotrophic parasites such as mildew fungi are closely adapted to their hosts’ physiology so a mutation which involves almost complete loss of susceptibility to such a pathogen might be expected to have substantial pleiotropic effects on the plant.
mlo mutations incur agronomic costs caused by spontaneous leaf spotting reflected in a yield penalty (Kjaer et al., 1990; Thomas et al., 1998) but even so, more than half of all northern European spring cultivars now have an mlo mildew resistance allele (Dreiseitl, 2012; http://www.crpmb.org/mlo/). mlo alleles also elevate susceptibility to several hemibiotrophic fungi in laboratory experiments (Jarosch et al., 1999; Kumar et al., 2001; Jansen et al., 2005), although these studies did not investigate the implications of increased susceptibility for crop management.

This paper demonstrates that loss-of-function and reduced-function mutants of Mlo are associated with increased susceptibility to a facultative disease in field conditions. Spot blotch (C. sativus) and blast (M. oryzae) are significant pathogens in the warm tropics, where powdery mildew is rare and mlo mutations are not used in breeding. RLS, by contrast, has become an important challenge to economic production of barley in many regions in temperate latitudes over the last 15 years, including Europe, New Zealand, and the Southern Cone of South America (Walters et al., 2008), all places where mlo alleles, particularly mlo-11, have been important in breeding for durable resistance to mildew in spring barley. The implication of the results presented here is that, along with climatic and agronomic factors (Oxley and Havis, 2010; Roos et al., 2011; West et al., 2012), widespread use of mlo alleles in spring barley cultivars may have contributed to the epidemic of RLS. Indeed, given that the effect of mlo on RLS depends on environmental conditions, it is conceivable that the widespread use of mlo-11 may have interacted with changes in agronomy and the climate to stimulate the RLS epidemic.

The effect of mlo alleles on increasing RLS may be mediated by perturbation of reactive oxygen species (ROS). RLS is a late season disease with symptoms typically occurring after ear emergence (Oxley and Havis, 2004), implying that the fungus maintains a relatively benign association with its host until one or more factors trigger a transition from endophyte to necrotrophic pathogen. Some endophytes become pathogenic in response to specific environmental and host physiological stimuli (Schulz and Boyle, 2005; Kogel et al., 2006). In the field, the onset of RLS symptoms is preceded by a general decline in the host antioxidant system, while leaf senescence may promote the development of disease (Schützendübel et al., 2008). As the host antioxidant system declines, the plant undergoes oxidative stress as levels of ROS increase. In experimental conditions, RLS symptom development is promoted by environmental stresses such as high light (Makepeace et al., 2007; Brown and Makepeace, 2009) which increase ROS levels, promoting oxidative stress and leaf senescence (Biswal and Biswal, 1984; Mullineaux et al., 2006).

The effect of mlo alleles on RLS may relate to leaf senescence, ROS levels, or both, consistent with the results of Schützendübel et al. (2008). mlo mutations do not affect the onset of leaf senescence but they accelerate the rate at which senescence progresses once it has been initiated (Piffanelli et al., 2002). ROS production and the onset of host cell death are also altered in mlo plants (Peterhänsel et al., 1997; Piffanelli et al., 2002) and mlo resistance to B. graminis f. sp. hordei is associated with an H2O2 burst beneath the site of attempted penetration. Accelerated leaf senescence in mlo plants may trigger more rapid transition of R. collo-cygni from endophyte to necrotroph, which may be further enhanced by environmental conditions that induce oxidative stress in the plant. Enhanced mesophyll cell death has been suggested as the reason why mlo mutants are more susceptible to the hemibiotrophic pathogens M. oryzae and Bipolaris sorokianiana (Jarosch et al., 1999; Kumar et al., 2001) although restricted cell death in a barley gain-of-function DELL A mutant increased RLS symptom development whereas the loss-of-function mutant with enhanced cell death was more resistant to this disease (Saville et al., 2012). These results may not be inconsistent with each other because restriction of host cell death during endophytic growth of R. collo-cygni may promote leaf colonization whereas enhanced mesophyll cell death once the fungus is established may increase RLS symptom development. The latter effect may be responsible for increased RLS in mlo plants.

mlo-mediated resistance to B. graminis f. sp. hordei is a prepenetration response preventing fungal entry into the attacked epidermal cell (Piffanelli et al., 2002), which requires the genes ROR1 and ROR2 to be functional. Whereas the susceptibility of mlo-5+ror1-2 and mlo-5+ror2 lines to mildew is partially restored compared to mlo-5 ROR1 ROR2 lines (Freialdenhoven et al., 1996), the opposite effect on RLS was detected (Fig. 4), implying that mlo-dependent enhanced susceptibility to the necrotroph R. collo-cygni may operate by the same pathway as mlo-mediated resistance to the biotrophic B. graminis f. sp. hordei. While the ror mutants reduced the development of RLS symptoms, they did not reduce the amount of the R. collo-cygni fungus in barley leaves (Fig. 4c), implying that the ROR genes affect transition from the endophytic phase of the fungal life cycle to necrotrophy but not the growth of the fungus itself. The peroxide burst in response to B. graminis f. sp. hordei is enhanced in mlo mutants and extends into the mesophyll resulting in cell death (Peterhänsel et al., 1997; Piffanelli et al., 2002). Mutation of either ROR1 or ROR2 diminishes this peroxide burst but both mlo-5 ror mutant genotypes accumulate more H2O2 in the mesophyll and experience more mesophyll cell death than in mlo-5 ROR1 ROR2 (Huckelhoven et al., 2000; Piffanelli et al., 2002). H2O2 accumulation may also be involved in the pathogenesis of Bipolaris sorokianiana on barley (Kumar et al., 2001). A hypothesis arising from the work reported here, therefore, is that ROS, especially peroxide ions, stimulate the endophyte–necrotroph transition in R. collo-cygni but do not affect growth of the fungus itself in planta. The effect of mlo on RLS may be mediated directly by ROS as signalling molecules or indirectly via accelerated leaf senescence.

mlo mutations may promote susceptibility to different hemibiotrophic and necrotrophic pathogens by at least partly different mechanisms. As in RLS (Fig. 4c), enhanced development of blast symptoms in mlo plants is associated with enhanced growth of M. oryzae (Jarosch et al., 1999) but mlo-promoted development of M. oryzae was not altered in the mlo-5+ror1-2 mutant, unlike RLS (Fig. 4c) and B. sorokianiana culture filtrate (Jarosch et al., 1999; Kumar et al., 2001).
The results here indicate that plant breeders should be able to combine mlo-11 mildew resistance and polygenic partial resistance to RLS in spring barley cultivars. There was substantial genetic variation in susceptibility to RLS in both Mlo+ and mlo-11 lines in both crosses (Figs. 1 and 2) although no other QTL controlling significant proportions of resistance to RLS was identified (Table 1). A method of selecting reliably for reduced susceptibility, breeders should be able to accumulate polygenes to control RLS in barley cultivars carrying mlo-11 and there is considerable variation in RLS susceptibility amongst the highly mildew-resistant (mainly mlo-11) varieties on the UK Spring barley Recommended List (http://www.hgca.com/content.template/23/0/Varieties/WaltersSelectionofcultivars/). This could be investigated genetically in crosses between mlo-11 cultivars such as Braemar and Decanter, which differ greatly in susceptibility to RLS. Genome-wide association studies could assist the search for genes which control RLS in mlo cultivars. Care would need to be taken to trial these populations in diverse environments to account for the environmental sensitivity of the mlo effect on RLS.

The emergence of Ramularia leaf spot as a major new disease of barley in temperate regions, including much of Europe since the mid-1990s (Walters et al., 2008) may have been stimulated by the mlo mildew resistance in spring barley in combination with agricultural and meteorological factors. In laboratory experiments, mlo alleles have also been associated with susceptibility to two other economically significant diseases, Fusarium head blight (Jansen et al., 2005) and net blotch (P. teres; Makepeace, 2006). The availability of mlo mutations to control mildew has benefitted barley production but, as there are effective alternative methods of controlling mildew, including several systemic fungicides (Walters et al., 2012) and breeding for durable, partial resistance (St Clair, 2010), it is possible that the costs of using mlo alleles have outweighed their benefits. It may be possible, however, to restore the value of mlo in breeding by combining it with partial resistance to RLS and, if necessary, net blotch and Fusarium head blight. The existence of substantial genetic variation in RLS severity, over and above the susceptibility conferred by mlo-11 (Fig. 2), implies that there is scope for selection of cultivars which combine strong mlo resistance to mildew with adequate resistance to RLS.

Supplementary material

Supplementary data are available at JXB online.

Supplementary Fig. S1. Genetic map of the Power×Braemar doubled-haploid population

Supplementary Fig. S2. Genetic map of the Decanter×Cocktail doubled-haploid population

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