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ORIGINAL ARTICLE

# Development of *mlo*-based resistance in tetraploid wheat against wheat powdery mildew

**Christina R. Ingvardsen[1] · Julio A. Massange-Sánchez[1,2] · Finn Borum[3] · Cristobal Uauy[4] · Per L. Gregersen[1]**

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## Abstract

Powdery mildew is a severe disease in wheat. In barley, durable resistance exists, based on non-functionality of the *Mlo* gene. As a model to analyse the effects of mutagenesis in the homoeologous *Mlo* genes of wheat, we developed *mlo*-based powdery mildew resistance in tetraploid durum wheat. To obtain *Mlo* mutations, we screened a TILLING population developed in tetraploid wheat "Kronos" for which the captured exome sequence of > 1500 lines is available. This resulted in 23 mutants for *Mlo-A1* and 26 non-redundant mutants for *Mlo-B1*. Two *Mlo-A1* and four *Mlo-B1* mutants were crossed to obtain eight $F_2$ mutant lines that showed a range of phenotypes from susceptibility to full resistance. Pot experiments under semi-field conditions confirmed the resistance levels for six of the mutants without any signs of adverse pleiotropic effects. Resistance ranking was similar across six powdery mildew isolates, indicating no isolate specificity of the *mlo*-based resistance. The effect of mutations in the *Mlo-B1* gene was stronger than in the *Mlo-A1* gene, probably reflecting differences in wild-type *Mlo* gene expression levels. Strong partial resistance effects were observed with single *mlo-B1* mutations hence, revealing a dosage effect of *mlo* mutant alleles. Two of the four *mlo-B1* mutations (W163* and P335L) were very strong; however, the highest combined effect was observed with the MloA-P335S/MloB-P335L combination, suggesting that non-functional, but full-length Mlo proteins might have the strongest effect compared with nonsense mutations. Our results show that *mlo*-based resistance might offer possibilities to introduce durable protection in tetraploid wheat against powdery mildew.

## Introduction

The recessive mutation of the *Mlo* gene in barley (*Hordeum vulgare* L.) has proved to be an effective and long-lasting source for resistance ("Mlo resistance") against attacks by the powdery mildew pathogen *Blumeria graminis* DC Speer f.sp. *hordei* Marchal (Jørgensen 1992). Hence, *mlo* mutations occur in a high proportion of barley cultivars and advanced breeding lines in Northern Europe and Scandinavia (Bengtsson et al. 2017). The *Mlo* gene encodes an integral membrane protein with seven transmembrane domains, and it belongs to a small gene family across both mono- and dicot plants (Büschges et al. 1997; Devoto et al. 2003). Similarly to the barley *Mlo* gene, there is now experimental evidence that mutations in one or more of *Mlo* gene orthologues may protect a range of plants against infection by powdery mildew fungi (Kusch and Panstruga 2017).

Even though the molecular function of the Mlo protein has still not been established, the generally accepted hypothesis for the role of the wild-type Mlo protein is that it attenuates general defence responses in the epidermal cells towards powdery mildew infection attempts. Hence, the *mlo* mutations cause a stronger and earlier response in barley compared with wild-type genes (Zierold et al. 2005). This leads to fast formation of large callose-containing papillae in the host cell wall at the fungal penetration site in barley leaves (Skou 1982; Skou et al. 1984; Stolzenburg et al. 1984), which apparently is sufficient to prevent the mildew pathogen from successful establishment. Exceptions are infection

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✉ Per L. Gregersen
per.gregersen@mbg.au.dk

[1] Department of Molecular Biology and Genetics, Science and Technology, Aarhus University, Slagelse, Denmark

[2] CINVESTAV-IPN, Irapuato, Guanajuato, Mexico

[3] Sejet Plant Breeding, Horsens, Denmark

[4] John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK

attempts into subsidiary cells of stomata from which powdery mildew colonies indeed can be initiated even in *mlo* mutant lines with high levels of resistance (Jørgensen and Mortensen 1977).

Since powdery mildew is an important disease also in wheat, caused by *Blumeria graminis* DC Speer *f. sp. tritici* Marchal, there has been a number of studies addressing the possibility of generating *mlo*-based resistance in wheat similar to the barley *mlo*-based resistance. Hexaploid wheat possesses three homoeologous *Mlo* genes that are very similar to the orthologous barley *Mlo* gene. Elliott et al. (2002) showed that the wheat orthologues could complement the *mlo* mutation in barley when expressed transiently in epidermal cells. Furthermore, Várallyay et al. (2012) showed that concomitant virus-induced silencing of the three wheat *Mlo* genes in wheat resulted in increased powdery mildew resistance. More recently, Wang et al. (2014) showed that simultaneous knockout of the three wheat *Mlo* homoeologues by genome editing techniques gave resistance towards powdery mildew infection. Moreover, Acevedo et al. (2017) obtained partial *mlo*-based resistance in hexaploid wheat through a TILLING screening for *mlo* mutations and subsequent crossings to obtain triple mutations in all of the three *Mlo* homoeologues. Hence, there is a firm basis for exploitation of *mlo*-based resistance towards powdery mildew in wheat, although the allopolyploidy of wheat makes the generation of mutated plants complicated, since mutations are required in all of the homoeologues, *Mlo-A1*, *-B1*, and *–D1* in hexaploid wheat, and *Mlo-A1* and *-B1* in tetraploid wheat.

The history of the barley Mlo resistance shows that it is pertinent to have several sources of this resistance (Jørgensen 1992), in particular due to the possibility of adverse pleiotropic effects associated with *mlo*-based resistance. In barley, adverse pleiotropic effects are mainly related to an increased formation of necrotic spotting on the leaves, which may cause yield reductions (Kjær et al. 1990). Another suggested side effect of the *mlo* mutation is increased susceptibility to some necrotrophic leaf pathogens, in particular *Ramularia collo-cygni* (McGrann et al. 2014). Thus, in the context of pleiotropic effects, a complete knockout of the two or three wheat *Mlo* homoeologues may not be the best solution, and only empiric testing of different combinations of "weak" or "strong" mutations may point to optimal levels of *mlo*-based resistance in order to hamper adverse side effects (Kusch and Panstruga 2017).

Even though powdery mildew appears to be a disease problem primarily in hexaploid common wheat under temperate humid climates (Bennett 1984), e.g. in Northern Europe, resistance against this disease is also important for the breeding of tetraploid durum wheat, *Triticum turgidum* L. spp. *durum*, in the Mediterranean area (Ben-David et al. 2014). Here, disease severity can be augmented in areas with irrigation (Bennett 1984). During winters under normal Mediterranean conditions powdery mildew attacks can also be associated with yield decreases in durum wheat (Cerón and Martel 2003). Hence, there is a need to develop powdery mildew resistant cultivars also in tetraploid durum wheat, and this could be based on the highly effective *mlo*-based resistance, as we aim to do in this work.

The screening for recessive mutations in allopolyploid wheat is not straight forward, since dominant wild-type gene(s) in the unaffected homoeologues can mask the phenotypic effects of homozygous recessive mutations following self-pollination (Borrill et al. 2015; Uauy et al. 2017). Hence, the screening for mutations must be based on genotyping. In order to screen for *mlo* mutations in the present work, we took advantage of a TILLING (targeting induced local lesions in genome) population developed in tetraploid (AABB) durum wheat "Kronos", for which the captured exome sequence of $>1500$ lines have been sequenced (Krasileva et al. 2017). Thus, genotyping of all mutations in this population was already performed, and we only needed to make an in silico screening to find the right mutations. Following identification of *mlo* mutations in the database (www.wheat-tilling.com/), we combined mutations from the A and B genomes by crossing and obtained double homozygous mutations. We investigated the different levels of powdery mildew resistance provided by different mutant alleles and obtained one combination of A and B genome mutations with a very high resistance level.

## Materials and methods

### Plant material

The EMS (ethyl methanesulfonate)-mutagenized population of tetraploid durum wheat "Kronos" was developed by University of California Davis (Uauy et al. 2009) and subsequently exome sequenced (Krasileva et al. 2017). Kronos lines mutated in *Mlo-A1* and *Mlo-B1* genes were identified by in silico screening of the wheat TILLING database (http://www.wheat-tilling.com/). Genomic DNA and cDNA sequences (Genbank: KF009556.1, AF384144.1, GQ397361.1) of hexaploid *Mlo-A1* and *Mlo-B1* genes were used as templates for the search of Kronos mutant lines. The latest RefSeqv1.1 gene names for the two genes are: TraesCS5A02G494800 (*Mlo-A1*) and TraesCS4B02G322700 (*Mlo-B1*). Seeds of Kronos mutant lines carrying mutations in *Mlo-A1* and *Mlo-B1* genes were ordered from the SeedStor Germplasm Resources Unit (https://www.seedstor.ac.uk/shopping-cart-tilling.php). The single nucleotide polymorphisms (SNPs) of selected mutant lines were confirmed by sequencing of PCR products spanning the putative mutations.

Six Kronos homozygous mutant plants for *Mlo-A1* (2 lines) and *Mlo-B1* (4 lines) genes were used in crosses to generate all eight possible $F_1$ combinations. $F_1$ Kronos heterozygous mutant plants were self-pollinated to generate $F_2$ populations. Following genotyping, double homozygous $F_2$ mutant plants for the eight combinations were self-pollinated to produce enough $F_3$ seeds for further experiments (see below).

## Fungal material

Five powdery mildew isolates, RO 18, Ri 120, H 8/6, Ha. 3, and Revn/10, were kindly provided by Department of Agroecology, Aarhus University (Hovmøller 1989, MS Hovmøller, personal communication), while one isolate was collected from the fields at Sejet Plant Breeding (Horsens, Denmark) and named Sej-1. All isolates were maintained on hexaploid wheat "Anja".

## KASP and HRM analysis

The Kompetitive Allele-Specific PCR (KASP™) genotyping analysis and High-Resolution Melting (HRM) analysis were performed to determine Kronos mutant plant segregation, using a ViiA™ 7 Real-Time PCR System (Darmstadt, Germany). Four out the six genetic variations in nucleotide sequences (MloA-H232Y, MloA-P335S, MloB-P464S and MloB-P335L) were identified by KASP™ genotyping assay (Online Resource 1: Table S1). DNA extracted with the Extract-N-AmpTM Plant PCR Kit (Sigma-Aldrich, St. Louis, MO, USA) was diluted 20 times in water, and 2.5 µl was combined with 2.5 µl reaction mix and 0.07 µl primer mix to give 5 µl reaction volume in 384-well plates. KASP™ thermal cycling conditions were set according to manufacturer recommendations (65–57 °C touchdown protocol), using 31 cycles. (https://www.lgcgroup.com/LGCGroup/media/PDFs/Products/Genotyping/KASP-thermal-cycling-conditions-all-protocols.pdf). Extra runs of the plate were required in some cases, using the recycling protocol with four cycles. HRM markers were used for the MloB-W163* and MloB-P200L mutations. Specific primers flanking the SNPs are listed in Online Resource 1 (Table S2). The reaction volume was 10 µl in 384-well plates. Each 10 µl reaction contained 5 µl MeltDoctor HRM Mastermix, 2.5 µl DNA (as above), 0.6 µl forward primer (5 µM), 0.6 µl reverse primer (5 µM), and 1.3 µl deionized water. PCR conditions were slightly modified from manufacturer recommendations (Catalog # 4453724 from https://www.thermofisher.com), as we ran 45 cycles with an annealing/extension temperature of 64° for 50 s. The data analysis was performed using the licence ViiA™ 7 Software High-Resolution Melt Module v1.2.

## Detached leaf resistance test

From each $F_1$ combination, 150–200 $F_2$ Kronos mutant plants were sown. DNA was extracted from the primary leaf (12 days) with the Extract-N-AmpTM Plant PCR kit. KASP and HRM markers were used to determine Kronos mutant plant segregation (see above). Tests of powdery mildew resistance were performed with the Revn/10 isolate for seven different genotypes (AABB, aaBB, AAbb, AaBb, aaBb, Aabb and aabb) in the eight combinations of mutations. For this, 2.5 cm pieces of third leaves were excised and put on square petri dishes (120 × 120 × 17 mm, GR-688102, Greiner) containing 0.5% water–Phytoagar with 25 mg/L benzimidazole (senescence inhibitor) with the abaxial side up. Then, plants previously infected with Revn/10 powdery mildew spores were shaken on top of a cylindrical settling tower (107 × 35 cm) allowing spores to settle on the detached leaves. Following inoculation, the number of spores was counted to standardize the amount of spores across the eight $F_1$ combinations.

A subset of the leaves was left for 6–7 days for documentation of macroscopic symptoms. The remaining leaf pieces (4–7 per genotype) were collected after 72 h to count successful infection attempts. The leaves were cleared for chlorophyll using a solution of ethanol/acetic acid (3:1, vol/vol) for 2 times 24 h. After rehydration in water for 24 h, the leaves were stored in lactoglycerol (lactic acid/glycerol/distilled water, 1:1:1, vol/vol/vol). All steps before staining were performed on filter paper in six-well cell culture plates in order not to wash off the powdery mildew spores from the leaves. The cleared leaves were stained in a 0.25% (in ethanol) Coomassie Brilliant Blue R250 solution for 2 min, rinsed in water and mounted in 28% glycerol on microscope slides. Successful infection attempts, i.e. visible micro-colonies with secondary hyphal growth, were counted in 1.2 mm strips on each side of the midrib along the whole leaf piece using a Zeiss Axioplan 2 imaging microscope. The experiment was repeated on the fourth leaf to confirm the results.

## Semi-field tests

$F_3$ plants of six mutant combinations were selected for cultivation under outdoor semi-field conditions (roofed area with drip irrigation). Ten seeds per combination of single (AAbb and aaBB) or double (aabb) homozygous plants were sown in 5 L pots and placed in the semi-field facility. The pots were randomized in three groups across the semi-field area. After seedling emergence, up to five plants were kept in each pot per combination. Kronos wild-type plants were sown as controls. Four-week-old plants (BBCH 29–30) were scored for naturally occurring powdery mildew infection, and monitored for any pleiotropic spot formation or early senescence. After scoring for powdery mildew resistance,

plants were treated with fungicide (metrafenone, Flexity, BASF) to ensure seed setting in all genotypes. Re-infection by powdery mildew was assessed on 7-week-old plants (BBCH-scale 51). Final height, measured from soil level to the lower end of the spike, was measured on the tallest tiller of each plant. All data were obtained using triplicates of each combination ($n = 15$).

### Seedling screening

$F_3$ plants with the eight mutation combinations were tested against six different powdery mildew isolates at seedling stage. Four plants per combination and Kronos wild-type were grown in pots (8 × 8 × 8 cm). After 2 weeks, leaves were inoculated with RO 18, Ri 120, H 8/6, Ha. 3, Revn/10 or Sej-1 isolates. Ten days after infection, plants were scored for disease level on a scale from 0 to 4 (using Kronos wild-type infected plants as reference) for each isolate. Range of infection: very few pustules (0), less than 10% (1), 10–30% (2), 30–60% (3), 60–100% (4). In some cases, the scoring label 0(4) was applied, as used by Lundqvist et al. (1991) to indicate very high levels of resistance, but with few normal pustules developing.

### Papilla study

Plants from wild-type and highly resistant plants (MloA-P335S/MloB-P335L) were sown in pots. After emergence, the second leaf was bent over a plastic rack, fixed with surgical tape with the abaxial side up, and inoculated with the powdery mildew isolate Revn/10. Leaf pieces (2.5 cm) were harvested 24 and 48 h after inoculation. One leaf per genotype was left to confirm susceptibility/resistance. The harvested leaves were cleared for chlorophyll and stored in lactoglycerol as described above. The cleared leaves were stained in a 0.25% (in ethanol) Coomassie Brilliant Blue R250 solution for 2 min and rinsed briefly in water. The leaves were rinsed 3 times in buffer (0.15 M $K_2HPO_4$, pH 9.5) and stained in Aniline Blue (0.005% Aniline Blue in 0.15 M $K_2HPO_4$, pH 9.5) for 30 min. After three final rinses in buffer, the leaves were mounted in 28% glycerol on microscope slides and analysed with a laser scanning microscope (Olympus FV 1000, excitation wavelength of violet laser of 405 nm, using green pseudocolour for fluorescence emission, 535–635 nm). Measurement of the papilla diameter was made in the z-plane where fluorescence was maximal. Papilla sizes were counted on 9–10 leaves per genotype/time, with 4–13 measurements per leaf (mean 11.6 per leaf). Transmitted light imaging (excitation wavelength of violet laser of 405 nm) was used for overlays of fungal structures with fluorescence images.

### In silico gene expression analysis

The transcript abundance from *Mlo-A1* and *Mlo-B1* genes in Kronos plants was investigated using an RNA-Seq database (Pearce et al. (2015); https://wheat.pw.usda.gov/WheatExp/), from which a time course of early senescence from Kronos flag leaves at heading date (HD) and 12 and 22 days after anthesis (DAA) was extracted.

### Statistical analysis

Statistical analysis of number of micro-colonies, plant height, and papilla size was performed using basic tools of R version 3.4.1 (R Core Team 2017), in particular using the lme, anova, and HSDTukey functions. Analysis of counts of micro-colonies for Fig. 2 was done by negative binomial regression following the example by Mangiafico (2016). One genotype with only zero values posed a special problem in this analysis, which was addressed by introducing a single value of 1 for one sample.

## Results

### Identification of *mlo* mutations in Kronos

In order to identify mutations in the *Mlo* genes of tetraploid Kronos wheat lines, the database at www.wheat-tilling.com were searched by Blast using wheat *Mlo* DNA sequences as input. This resulted in a list of 23 and 26 non-redundant database entries showing non-synonymous mutations in exons of *Mlo-A1* and *Mlo-B1*, respectively (Online resource 1, Table S3). All of the mutations were single nucleotide changes, specifically EMS-type G/C → A/T transitions as anticipated from the general analysis of the Kronos mutant population (Krasileva et al. 2017). For a few of the mutations, seeds were not available from SeedStor.ac.uk. Furthermore, seeds from a few of available lines did not germinate well or did not produce spikes amenable for crossings. Mutations from the remaining available lines were assessed based on their SIFT (Sorting Intolerant From Tolerant) score, where low scores indicate a high degree of intolerant mutation effects on the encoded proteins (Sim et al. 2012). The threshold for including mutations was a SIFT score ≤ 0.05. In addition, the mutations were compared to mutations at the same amino acid position in barley, for which effective resistance was previously shown (Reinstadler et al. 2010). The amino acid sequence of the wheat Mlo shifted one amino acid compared with the barley Mlo, due to an insertion of one amino acid in exon 1 of the former. The two homoeologous Kronos Mlo proteins show 96% amino acid identity to each other and 89% identity to the barley Mlo protein. The Kronos Mlo proteins are 100% identical to

available hexaploid *Mlo*-A1 and *Mlo*-B1 protein sequences at the amino acid level. The screenings and assessments ended up in selection of two Kronos lines with mutation in *Mlo-A1* and four with mutation in *Mlo-B1* (Table 1).

Both mutations in *Mlo-A1* (Table 2) had a SIFT score of 0.00 and were in amino acid positions (232 and 335) with corresponding effective mutations in barley, although the amino acid exchanges were different (Reinstadler et al. 2010). The *Mlo-A1* mutations were localized in exon 6 and 9, respectively, and in the second and third cytoplasmic loops of the Mlo protein, both suggested to be critical for conferring powdery mildew susceptibility by the Mlo protein (Reinstadler et al. 2010). The *Mlo-B1* mutations (Table 2) included one nonsense mutation in the second cytoplasmic loop (position 163, exon 4), and three missense mutations in exon 5 (position 200, second cytoplasmic loop), exon 9 (position 335, third cytoplasmic loop), and exon 11 (position 464, cytoplasmic C-terminus). Two of the *Mlo-B1* mutations, in exon 4 and 9, had equivalent mutations in barley, in particular the mutation in exon 9 showing the same amino acid exchange as the effective *mlo-29* mutation in barley (Reinstadler et al. 2010). The P3353L mutation in exon 9 is also the same mutation as the strongest *mlo* mutation found by Acevedo-Garcia et al. (2017) in hexaploid wheat.

PCR fragments covering the exon of the mutations in the genome were Sanger sequenced, which confirmed the identity of the six mutations. Based on mutation genotypes, primers for KASP markers were designed for the mutations (Online resource 1: Table S1); however, for two of them, MloB-W163* and MloB-P200L, the separation of wild-type and mutants did not work properly with KASP markers. Hence, HRM markers, which turned out to work better, were used instead (Online resource 1: Table S2).

## Crossings of mutant lines

Crossings between *Mlo-A1* and *Mlo-B1* mutants were performed (Online Resource 1: Table S4), resulting in eight combinations of mutations that were used for assessments of powdery mildew resistance. The heterozygous $F_1$ offspring plants from the crosses were self-pollinated, which resulted in harvest of sufficient amounts of $F_2$ seeds from all crosses to perform a genotyping screen for the occurrence of combinations for wild-type and mutant alleles in the two *Mlo* loci. For further studies on the resistance levels obtained with the mutations, seven different combinations of wild-type and mutant alleles were used (see Fig. 1): the homozygous wild-type or mutant alleles in both loci (AABB and aabb); homozygous alleles for one of the mutations (AAbb and aaBB); triple mutant alleles (aaBb and Aabb); and heterozygous alleles for both loci (AaBb). Selected plants with these, or some of these, genotypes were assessed for resistance towards powdery mildew infection using three different methods: a detached leaf test on phytoagar with visual assessment and counting of successful infection attempts; a semi-field test with plants in pots in an outdoor area with natural infection; and infection of seedlings with six different isolates of *B. graminis* f.sp. *tritici*.

**Table 1** Results of in silico screening for *mlo* mutations in tetraploid Kronos wheat at http://www.wheat-tilling.com, with number of identified and obtained *mlo* mutants

| | Mlo-containing contigs | Mutant lines | Effect of mutation | | | SIFT | Crossings | |
|---|---|---|---|---|---|---|---|---|
| | | | Nonsense | Missense | Splice region | Intolerant | Available | Used |
| *Mlo-A1* | 7 | 61 | 1 | 21 | 1 | 10 | 5 | 2 |
| *Mlo-B1* | 4 | 215 | 3 | 24 | 1 | 16 | 6 | 4 |

*SIFT* sorting intolerant from tolerant. Intolerant: $p \leq 0.05$

**Table 2** Selected Kronos *mlo* mutant lines, used in crossings to obtain double mutant lines

| Name | Kronos line | Genome | Exon | SNP | | Amino acid change | SIFT score | Described barley mutation |
|---|---|---|---|---|---|---|---|---|
| | | | | WT | Mutant | | | |
| MloA-H232Y | Kronos3487 | A | AE6 | C | T | H232Y | 0 | H231L = *mlo-35* |
| MloA-P335S | Kronos2879 | A | AE9 | C | T | P335S | 0 | P334L = *mlo*-29 |
| MloB-W163* | Kronos2526 | B | BE4 | G | A | W163* | – | W162R = *mlo-1* |
| MloB-P200L | Kronos2876 | B | BE5 | C | T | P200L | 0 | |
| MloB-P335L | Kronos2381 | B | BE9 | C | T | P335L | 0 | P334 L = *mlo*-29 |
| MloB-P464S | Kronos2333 | B | BE11 | C | T | P464S | 0.05 | |

*SIFT* sorting intolerant from tolerant

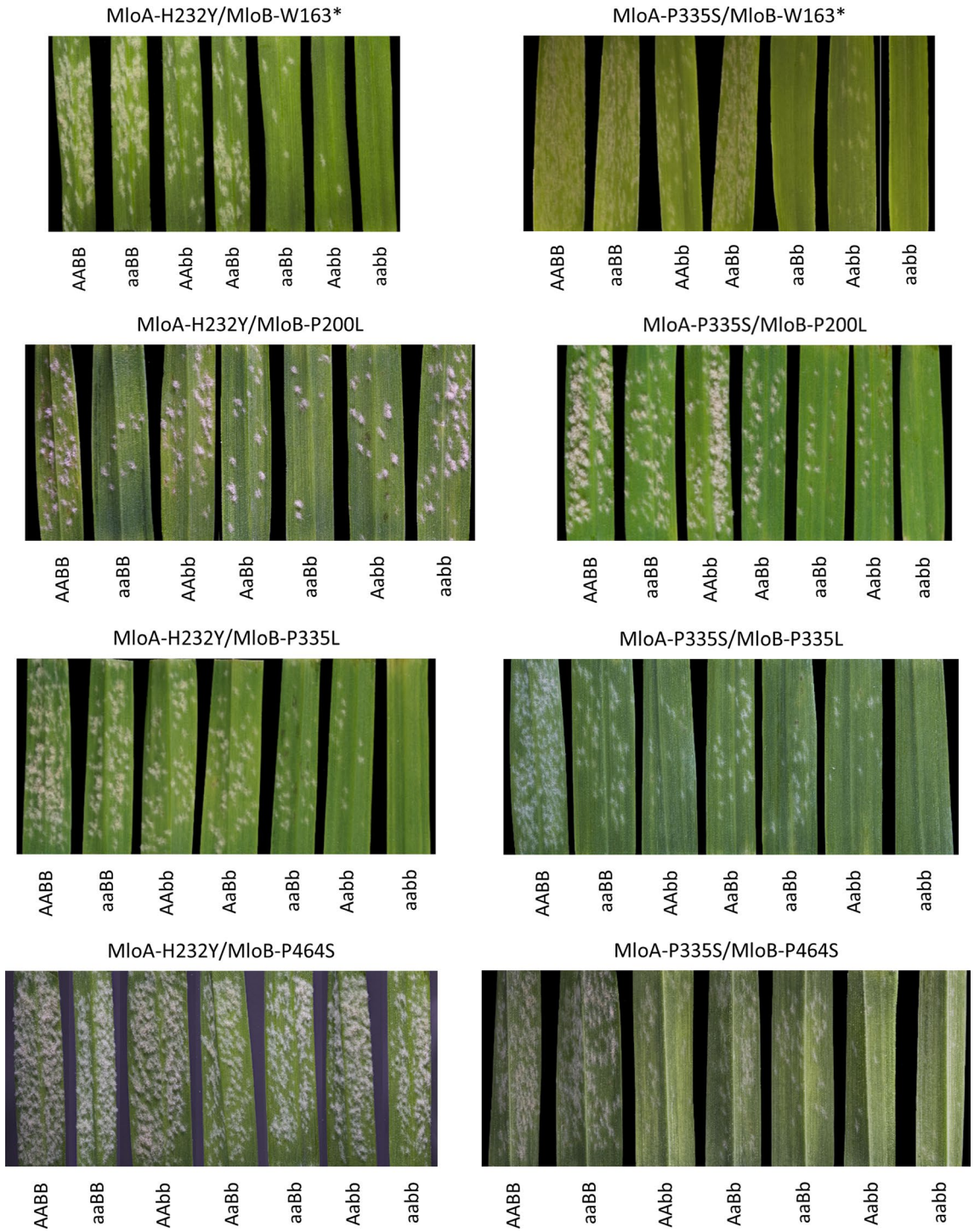


**Fig. 1** Detached leaf test of resistance level of the eight Kronos *mlo* mutant lines by visual assessment of disease development. For each line, disease development with the Revn/10 powdery mildew isolate was observed across seven genotypes: AABB, aaBB, AAbb, AaBb, aaBb, Aabb, and aabb. The overall assessments of the individual lines, based on the aabb genotype, are listed in Table 3 as full resistance, partial resistance or susceptibility

## Disease tests on detached leaves

Leaf segments from the third or fourth leaf of seedlings were cut and placed on Phytoagar for inoculation with powdery mildew in a settling tower. A spore density of approx. 120 spores/mm$^2$ was aimed at; however, the resulting number of pustules on the leaves varied considerably, due to inevitable variations in spore viability and incubation conditions. Following 6–7 days of incubation, the disease level was assessed by ranking the double homozygous mutant plants as susceptible, partially resistant or fully resistant. A "fully resistant" assessment reflected no or only a few scattered pustules on the leaf pieces from the homozygous mutant for both *Mlo* loci (aabb). Only one combination of mutations, MloA-H232Y/MloB-P464S, turned out to be fully susceptible, whereas the remaining seven combinations all showed some degree of resistance. However, only four of them showed full resistance with no or very few mildew pustules on the detached leaves of the double homozygous mutant. The other three combinations showed a reduced number of mildew pustules in the double homozygous mutant compared with the wild-type and, hence, were classified as partially resistant (Fig. 1 and Table 3). Based on the visual assessment, a preliminary conclusion is that the exon 11 mutation MloB-P464S had no effect on the resistance level, and that the effect of MloA-P335S was stronger than that of MloA-H232Y, since the former showed an effect even in combination with the MloB-P464S mutation.

In order to assess the effects of the individual mutations, images of the seven homo- and heterozygotic allele combinations were aligned as shown by representative images in Fig. 1. From this visual assessment, gradients of disease development across the allele combinations were evident in mutant combinations that showed resistance in the double homozygous mutants, i.e. all but MloA-H232Y/MloB-P464S. This indicated that the individual alleles had a dosage effect on the resistance level, and, hence, that mutations in all alleles were not required to obtain at least some degree of resistance. However, full resistance in the detached leaf test, i.e. with no development of or very few pustules on the detached leaves, was only observed in four double homozygous mutants as listed in Table 3, and of them only combination MloA-P335S/MloB-P335L showed complete absence of pustules across all tested leaf pieces.

In order to study the effects of the individual mutations in more detail, microscopic counting of successful infection attempts by the powdery mildew fungus (micro-colonies) was performed 72 h after inoculation (Fig. 2). Replicate leaves for these counting experiments were obtained from the same inoculation experiment as used for the images in Fig. 1. Thus, also in this case, the number of inoculated and germinating spores was subject to considerable and inevitable fluctuations among the different samples, and in this case also apparently due to variability in microenvironments across leaves in the same inoculation event. However, the countings in general reflected the assessments made on macroscopic pustules. Thus, three combinations assessed above as fully resistant (MloA-H232Y/MloB-W163*; MloA-P335S/MloB-W163*; and MloA-P335S/MloB-P335L) showed only very few micro-colonies for the double homozygous mutant; in fact, for MloA-P335S/MloB-P335L, there were none. For all of these three combinations of mutations, it was clear that the effect of the *mlo-B1* allele was stronger than that of the *mlo-A1* allele when comparing the wild-type AABB genotype with either aaBB or AAbb genotypes. However, it was also apparent from comparing AAbb with aabb that the *mloA* alleles were still needed to confer full resistance. The different strengths of the two *mlo-A1* mutations, as described above for the susceptible combination, were evident from comparing the double mutant combinations of MloA-H232Y/MloB-P335L and MloA-P335S/MloB-P335L. The former showed a few micro-colonies, whereas the latter was completely without any micro-colonies (Fig. 2).

Despite considerable variation across the different specimens examined, a clear picture was obtained with a satisfactory number of germinating spores and micro-colonies for the highly resistant combinations. The MloA-H232Y/MloB-P200L posed a special situation in that only a very few micro-colonies had formed 72 h after inoculation. This was observed in repeated experiments, and we speculate whether this was due to a delay in fungal development in this situation, maybe because of some other mutations in the specific mutant lines. At the macroscopic level, this

**Table 3** Resistance levels of wheat lines with the eight combinations of mutations based on visual assessment of disease development in two different experiments: a detached leaf experiment with infection of leaf pieces on Phytoagar with the Revn/10 powdery mildew isolate, and a pot experiment under semi-field conditions with natural infections

| Mutations | | Phytoagar, Revn/10 | Semi-field, natural infection |
|---|---|---|---|
| A genome | B genome | | |
| MloA-H232Y | MloB-W163* | R | Partial R |
| MloA-P335S | MloB-W163* | R | R |
| MloA-H232Y | MloB-P200L | Partial R | – |
| MloA-P335S | MloB-P200L | Partial R | S |
| MloA-H232Y | MloB-P335L | R | Partial R |
| MloA-P335S | MloB-P335L | R | R |
| MloA-H232Y | MloB-P464S | S | – |
| MloA-P335S | MloB-P464S | Partial R | S |

In the semi-field experiment, only six of the eight mutant lines were represented. R: full resistance with no or only very few powdery mildew pustules developing; S: full susceptibility at the level of wild-type plants; Partial R: intermediate development of disease

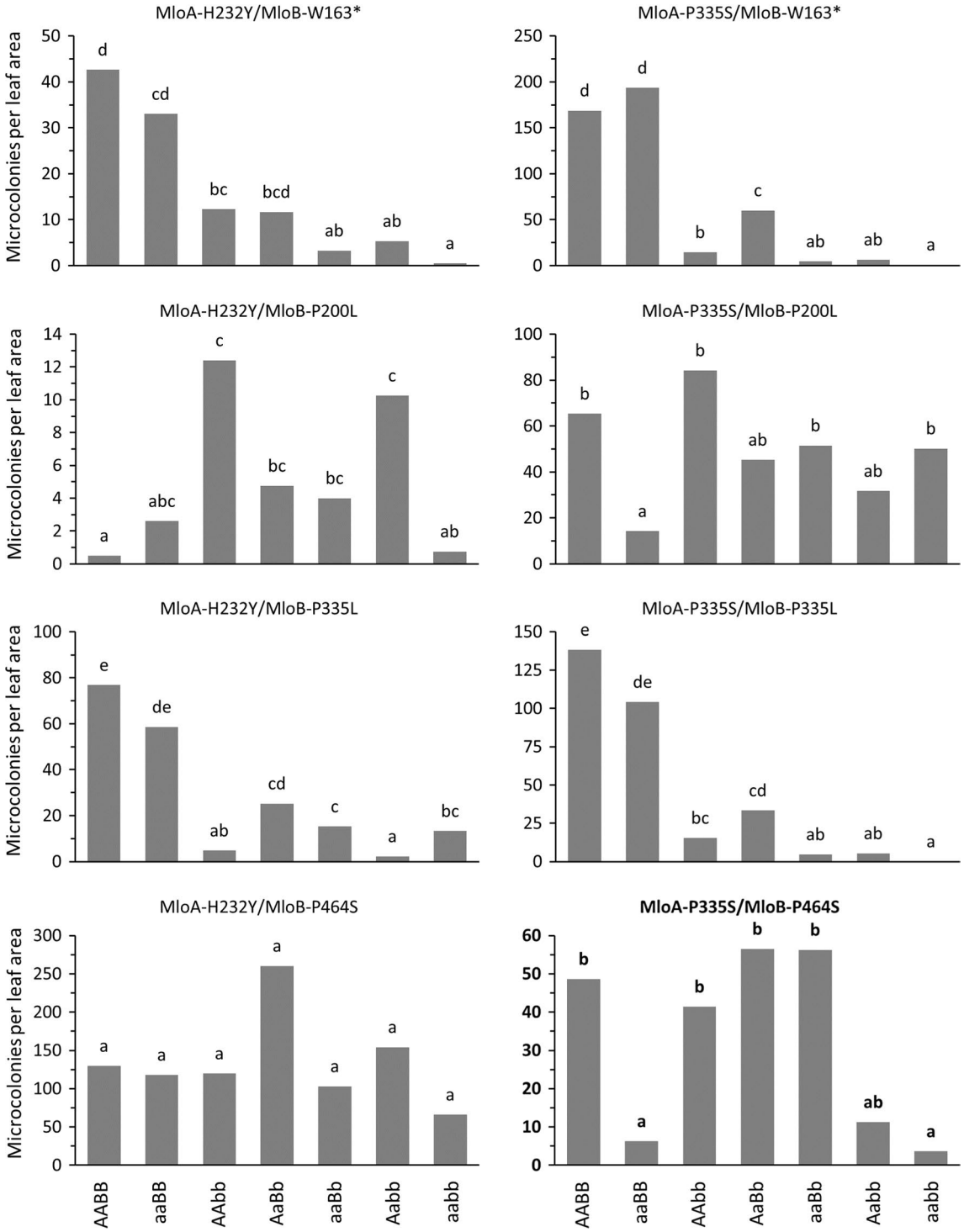


**Fig. 2** Countings of micro-colonies at 72 h after inoculation on leaf pieces from the same experiment as in Fig. 1. Leaf area is a 1.2 mm strip on each side of the midrib along the whole 2.5-cm-long leaf piece. Genotypes that do not share lower case letters above the histograms are significantly different according to Tukey pairwise comparisons

combination showed a similar level of pustule development as the other mutant combinations (Fig. 1).

Overall, the detached leaf tests showed the full range of resistance levels in double homozygous mutant plants for the *Mlo-A1* and *Mlo-B1* loci, from highly susceptible to fully resistant leaf pieces. Hence, both strong and weak mutations with respect to effects on resistance level were present in the material. Mutations in both *Mlo-A1* and *Mlo-B1*

were required for full resistance, but the *mlo-B1* mutations appeared to have stronger effects than *mlo-A1* mutations, with clear effects on resistance level also without any *mlo-A1* mutation (dosage effect). Two of the four *mlo-B1* mutations tested showed strong effects: unsurprisingly the nonsense mutation MloB-W163* was one of them, but intriguingly the strongest effect was obtained with the MloB-P335L mutation in combination with a *mlo-A1* mutation affecting the same amino acid in the Mlo protein (MloA-P335S), although the change in amino acid was different.

## Semi-field test: natural infection and possible pleiotropic effects

During the summer of 2017, six of the double homozygous mutant lines, with available seeds, were grown outdoor under Danish summer conditions in triplicated pots in a covered semi-field area with drip irrigation, along with single homozygous mutant lines and wild-type plants. The aim was to test for powdery mildew resistance following natural infection and to monitor the general phenotype of the plants with respect to possible pleiotropic effects. The plants grew and developed well under these conditions until complete maturity. The selected wild-type plants showed high levels of powdery mildew disease originating in natural infection, which reflected the high degree of susceptibility of the wild-type Kronos variety. Based on disease level, the mutant plants (4-week old) were again assessed as either susceptible, partially resistant or fully resistant (Table 3). Following assessment, the plants were treated with fungicides to suppress disease development and secure proper plant development. Re-infection on 7-week-old plants showed the same pattern of disease levels across the lines; however, with a tendency that partially resistant lines showed slightly less disease. Altogether, the ranking in assessment in general followed the assessments already observed in the detached leaf test; however, with a tendency that the plants developed more disease. Thus, two mutant lines, MloA-P335S/MloB-P200L and MloA-P335S/MloB-P464S, which were assessed as partially resistant in the detached leaf test, were assessed here as fully susceptible with heavy development of disease. Furthermore, the different effects of the two *mlo-A1* mutations became rather clear here, based on their combinations with the MloB-W163* mutation: MloA-P335S/MloB-W163* was assessed as fully resistant, whereas MloA-H232Y/MloB-W163* only showed partial resistance with some development of disease. In addition to P335S/MloB-W163*, only the MloA-P335S/MloB-P335L combination was assessed as fully resistant.

The plants were monitored throughout the whole growth period for their general phenotype, with respect to morphological features such as height, tillering, colours, etc. (data not shown), and in particular to any development of necrotic leaf spots, which was reported previously for barley plants with *mlo* mutations. No apparent differences in any of these features were observed across resistant and susceptible plants throughout the whole growth period. Both wild-type and mutant plants all looked healthy and reached full maturity with normal seed setting under these semi-field conditions.

## Seedlings inoculated with different isolates

For the detached leaf tests, only one isolate of *B. graminis* f.s. *tritici* was used (Revn/10). In order to test for any possible isolate specificity of the resistance observed in the mutant lines, an experiment with inoculation of seedling plants with six different isolates differing in origin was performed. Disease development on double homozygous mutant lines for *Mlo-A1* and *-B* was visually assessed, relatively to the wild-type plants, and the results are shown in Table 4. A simple five-step scoring scale, 0–4, was used, and in some case the scoring label 0(4) was applied (see materials and methods). In general, high inoculum densities

**Table 4** Disease development on plants with the eight combinations of mutations following inoculation with six different powdery mildew isolates

| Mutations | | Powdery mildew isolate | | | | | |
|---|---|---|---|---|---|---|---|
| A genome | B genome | Revn/10 | H 8/6 | Sej-1 | Ri 120 | RO 18 | Ha. 3 |
| MloA-H232Y | MloB-W163* | 2 | 2 | 3 | 2 | 3 | 2 |
| MloA-P335S | MloB-W163* | 1 | 1 | 1 | 3 | 2 | 2 |
| MloA-H232Y | MloB-P200L | 4 | 4 | 4 | 4 | 4 | 4 |
| MloA-P335S | MloB-P200L | 4 | 4 | 4 | 4 | 4 | 4 |
| MloA-H232Y | MloB-P335L | 2 | 3 | 2 | 3 | 4 | 3 |
| MloA-P335S | MloB-P335L | 0 (4) | 0 (4) | 1 | 1 | 1 | 1 |
| MloA-H232Y | MloB-P464S | 4 | 4 | 4 | 4 | 4 | 4 |
| MloA-P335S | MloB-P464S | 4 | 4 | 4 | 4 | 4 | 4 |

Range of infection, compared with wild-type plants: very few pustules (0), less than 10% (1), 10–30% (2), 30–60% (3), 60–100% (4). In some cases, the scoring label 0(4) was applied, as used by Lundqvist et al. (1991)

were used in these experiments and the spore viability of the different isolates also turned out to differ. Hence, it was difficult to reach the same level of disease in wild-type plants across the different isolates, with confluent/"saturated" pustule densities in some cases. However, in general, the assessments followed similar patterns across the isolates and, thus, indicated no isolate specificity of the resistance in the mutant lines. We interpret the scoring of 1 in the highly resistant line MloA-P335S/MloB-P335L for four of the six isolates as a result of the high inoculum density used (see discussion below).

## Papilla size measurements

Wild-type and highly resistant plants (line MloA-P335S/MloB-P335L) were inoculated with the powdery mildew isolate Revn/10, and after 24 and 48 h, leaf pieces were harvested to study the infection structures. On susceptible plants, the major part of penetration events are compatible, i.e. leading to formation of a haustorium and secondary hyphal growth (See Online Resource 1: Fig. S1). However, a minor part of penetration events will, even on susceptible plants, be unsuccessful (incompatible interactions), which will result in an enlarged cell wall apposition (papilla) and a circular area in the cell wall below the penetration site known to UV fluoresce following staining with aniline blue. This is indicative for the presence of callose (Aist et al. 1988). The *mlo* resistance in barley is known to be associated with enlarged papillae (Skou et al. 1984). Hence, in our tetraploid wheat plants, we compared the size of papillae formed in highly resistant plants with papillae in incompatible interactions from susceptible wild-type plants. Examples of papillae are shown in Online Resource 1 (Fig. S1). At both 24 and 48 h after inoculation, the papillae in the resistant plants were significantly larger than in susceptible plants, on average 44% (Fig. 3). In addition, the intensity of the fluorescence of the papillae was clearly stronger in the resistant plants as compared with susceptible plants, as also indicated by the images in Online Resource 1 (Fig. S1). We did not quantify the intensity, but the difference was evident across all investigated leaf specimens.

## Expression analysis of the *Mlo* genes

In order to relate the differential effects of mutations in *Mlo-A1* and *Mlo-B1* to gene expression levels, the transcript abundance for the *Mlo-A1* and *Mlo-B1* genes in Kronos cultivar was investigated using available RNA-Seq data (Pearce et al. 2015; https://wheat.pw.usda.gov/WheatExp/). A time course of early senescence from flag leaves at heading date (HD), 12 and 22 days after anthesis (DAA) showed similar patterns of expression for *Mlo-A1* and *Mlo-B1* genes, with maximal expression at 12 DAA (Fig. 4). Furthermore, the *Mlo-B1* gene was more highly expressed than *Mlo-A1* gene at all three stages; 13 times higher expression at HD and 12 DAA, and 17 times higher expression at 22 DAA (Fig. 4).

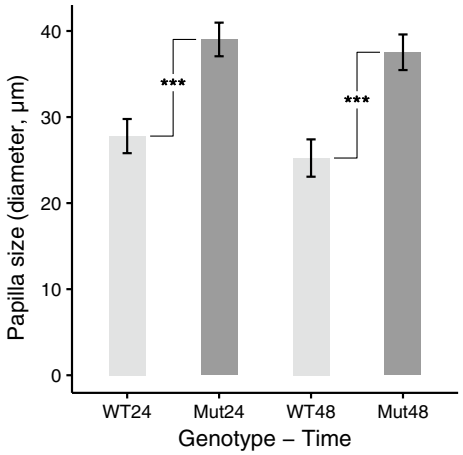


**Fig. 3** Papilla size (diameter) measured at fungal penetration sites in powdery mildew inoculated leaves of the highly resistant mutant line MloA-P335S/MloB-P335L and wild-type Kronos plants. In the wild-type plants, only papillae in unsuccessful (incompatible) interactions were measured. Papillae were observed as circular UV-fluorescing formations in the epidermal cell wall following aniline blue staining of cleared leaves. WT24/WT48: Wild-type plant at 24 and 48 h after inoculation. Mut24/Mut48: *mlo*-resistant line 24 and 48 h after inoculation. Asterisks indicate significance of the pairwise comparison of papilla size at 24 and 48 h, respectively (***$p < 0.001$), using a nested anova analysis

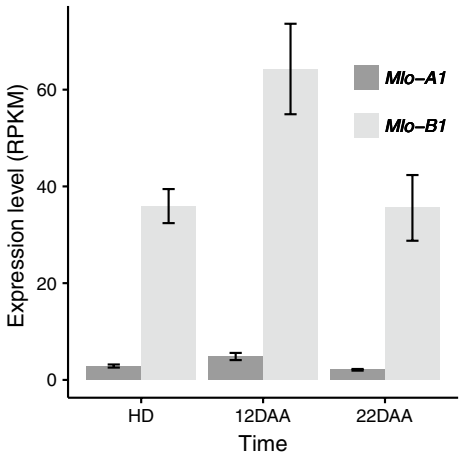


**Fig. 4** Expression level, i.e. transcript abundance, of *Mlo-A1* and *Mlo-B1* genes in flag leaves of Kronos wheat at three different developmental stages: heading date (HD), 12 and 22 days after anthesis (DAA). Error bars show the standard errors. Expression data were derived from a publicly available RNA-Seq database for wheat gene expression (Pearce et al. 2015; https://wheat.pw.usda.gov/WheatExp/)

## Discussion

In this study, we used an available mutant collection of the tetraploid wheat variety Kronos (Krasileva et al. 2017) to find mutations in the *Mlo* genes of both the A and B subgenomes. This collection of mutants, characterized by sequencing of the exome-captured DNA from > 1500 EMS-mutated Kronos lines, provides efficient access to mutations in wheat via the associated database (www.wheat-tilling.com and *Ensembl*Plants). In total, we found 51 non-synonymous *Mlo* mutations (23 in *Mlo-A1* and 28 (26 non-redundant) in *Mlo-B1*). Practical circumstances, e.g. seed availability and germination ability, reduced the number of mutant lines for further studies. In addition, the random nature of the EMS-induced mutations warrants an initial evaluation of the different mutations with respect to their effects, based on the observed changes in the encoded protein, before proceeding to detailed studies of mutant lines. Nonsense mutations that introduce stop codons in the encoded proteins are expected to have strong influence on the phenotype, whereas the effect of missense mutations leading to amino acid changes might have varying effects. A preliminary indication of the effect of a mutation can be obtained from the SIFT score, a popular method to estimate the tolerance of a protein for specific mutations, e.g. an amino acid change, based on an algorithm that tests for evolutionary conservation of protein motifs (Sim et al. 2012). We used this method here to select six *Mlo* mutations (two mutations in *Mlo-A1* and four in *Mlo-B1*) with apparent intolerant amino acid changes, before entering into elaborate crossings of mutations and testing of their effects on resistance levels. In the end, we obtained effective mutations in this way; however, we also conclude that some mutations with low SIFT score, MloB-P200L and MloB-P464S, did not have a strong effect on the powdery mildew resistance level. Hence, in this case of the *Mlo* genes, the SIFT score can only work as a guideline for selection of strong mutations. As discussed by Reinstadler et al. (2010) for barley *Mlo* mutations, the efficiency of *Mlo* mutations may be related to the position in the protein, and mutations affecting the second and third cytoplasmic loop of the protein appear to be the most effective.

Of the six *Mlo* mutations studied here, we observed that the two *mlo-A1* mutations had less effects on resistance compared with the *mlo-B1* mutations. We relate this to differences in expression level of the *Mlo* genes, since the *Mlo-A1* appears to be expressed at a lower level than the *Mlo-B1* in Kronos (Fig. 4). A low expression of the *Mlo-A1* gene was also observed by Acevedo-Garcia et al. (2017) in hexaploid wheat, when compared with expression levels of the *Mlo-B1* and *Mlo-D1* genes. However, mutation in the *Mlo-A1* gene is still required in tetraploid Kronos wheat in order to obtain a high degree of resistance in combination with an effective *Mlo-B1* mutation. We observed that the MloA-P335S mutation is particularly effective for this, whereas the MloA-H232Y mutation had a weaker effect and did not contribute to obtain full resistance even with the highly effective MloB-P335L mutation.

Based on the different resistance phenotypes of the eight crosses, this study identifies three highly effective mutations, MloB-P335L, MloB-W163*, and MloA-P335S, in tetraploid wheat. The MloA-H232Y mutation also gives rise to resistance; however, this *Mlo-A1* mutation is definitely weaker in its effects than MloA-P335S. It is not surprising that the MloB-W163* shows high impact on the resistance level, since we must expect the encoded mutated protein to be non-functional. It is, however, more intriguing that the highest level of resistance was observed with the combination of the two proline-335 missense mutations. The P3353L mutation is the same mutation as the strongest *mlo* mutation found by Acevedo-Garcia et al. (2017) in hexaploid wheat. Since the MloA-P335S/MloB-P335L combination showed a higher level of resistance than the MloA-P335S/MloB-W163* combination, we can conclude that MloB-P335L is stronger in its effect than MloA-P335S, even though the comparison is somewhat obscured by the differences in expression levels from the two genes. We speculate whether the combination of two mutations in the same amino acid position, the P335, may have an extraordinary combined effect on the resistance phenotype, because this proline residue is one of the invariant residues across the MLO protein family and, hence, probably of high importance for structure/function of the protein (Elliott et al. 2005).

No measurements of the Mlo protein levels (functional or non-functional) were made in this study; however, the truncated and ostensibly non-functional protein arising from the MloB-W163* mutation is probably removed during processing of the protein before it reaches its destination in the plasma membrane. This could either be through ER-associated degradation of the protein (Müller et al. 2005) or already at the mRNA stage through the mRNA decay system (Nyikó et al. 2013). In the absence of the Mlo-B1 protein, resistance of plants with the MloA-P335S/MloB-W163* mutation combination is primarily determined by the functionality of the MloA-P335S protein, which we can conclude has been at least partially compromised by the mutation. The Mlo protein of the barley mutant, *mlo-29*, which is mutated in the equivalent position 334, appears to be a stable protein (Müller et al. 2005). Thus, our results suggest that the P335L mutation is completely non-functional, since the effect of MloA-P335S/MloB-P335L is stronger than MloA-P335S/MloB-W163*. If the MloB-P335L protein is present, but non-functional, it might disguise any residual effects of the lowly expressed MloA-P335S protein.

Our data support the finding by Acevedo-Garcia et al. (2017) that there is a dosage effect of the *mlo* mutations in polyploidy wheat. This is obvious from the observed gradients of pustule density across the different resistant genotypes in Fig. 1. However, it is most clearly seen in the MloA-P335S/MloB-P335L combination, when comparing development of micro-colonies for the aaBB, aaBb, and aabb genotypes, showing increasing levels of resistance (Fig. 2). A dosage effect has not been reported for *mlo* mutations in the diploid barley species, probably because resistance as a standard has been monitored in homozygous $F_2$ generations, and not studied in detail in heterozygous plants. In a study of QTLs for powdery mildew resistance in a Nordic spring barley panel, Bengtsson et al. (2017) detected the *Mlo* locus as one of the several QTLs for powdery mildew resistance. Similarly, but in the more complex alloploidy situation, we speculate that dosage effects of unknown *mlo* mutations might give rise to QTLs for quantitative powdery mildew resistance in wheat.

Overall, the three different ways that we tested resistance levels of the mutant lines showed consistency in ranking of the lines and definitely identified one highly resistant line, MloA-P335S/MloB-P335L. However, they also showed that the revealed level of resistance might differ considerably, from the complete resistance in the detached leaf tests with no observed powdery mildew pustules to a considerable number of pustules developing in the seedling test for the most resistant line, MloA-P335S/MloB-P335L. We believe that these differences mainly relate to the amount of inoculum used in the different tests in combination with the nature of the *mlo* resistance. Thus, in barley, it has been known for a long time, that rare, but successful establishment of powdery mildew colonies can be initiated from stomatal subsidiary cells in *mlo*-resistant plants (Jørgensen and Mortensen 1977), apparently due to some kind of induced susceptibility in the surrounding epidermal cells following the initial successful infection. This gives rise to the 0(4) scoring of the *mlo* phenotype (Lundqvist et al. 1991), since even highly resistant lines can develop scattered isolated pustules of normal size. This is particularly evident if high amounts of inoculation spores are used, as given in Table 4. Jørgensen and Mortensen (1977) noted that this phenomenon in fact might lead to erroneous classification of effective *mlo* mutants in barley as susceptible, if very large numbers of conidia are applied as inoculum during the resistance testing.

Plant/leaf age could be another reason for the observation of different levels of *mlo*-based resistance in the same mutant line (Ge et al. 2016). It is difficult to compare age across different experimental set-ups; thus, the only situation where this issue was reflected in our experiments was in the semi-field tests where re-infection of older plants following fungicide treatment showed slightly stronger resistance in the partially resistant lines. Ge et al. (2016) suggest that different levels of *mlo*-based resistance in barley are associated with different expression levels from the *Mlo* gene. We only used publicly available gene expression data here to discuss the differential effects of the *mlo-A* and *mlo-B* homoeologues on the resistance level. A detailed time-course study on the correlation between resistance level and gene expression is a complex task in the tetraploid wheat species and, hence, it was outside the scope of this work.

In *mlo*-resistant barley plants, the resistance is known to be associated with the formation of large papillae below the fungal penetration site (Skou et al. 1984). We showed here the same to be the case in our *mlo*-resistant tetraploid wheat plants (Fig. 3). Hence, the mechanisms of the *mlo*-based resistance across barley and wheat appear to be the same. Apart from the very rare cases of successful infection in stomatal subsidiary cells, we did not observe successful penetration events across the leaf specimens from the strongly resistant plants that were investigated in this study. In all cases, the fungal penetration attempt was stopped in a large, strongly UV-fluorescing papilla.

Even with the highly resistant line, MloA-P335S/MloB-P335L, we did not at any time throughout the testing of lines observe any apparent pleiotropic effects from the *mlo* mutations; most convincingly, in the semi-field pot experiments, where the double homozygous mutant plants grew along with wild-types and single homozygous mutant plants under normal Danish summer weather conditions. The mutant plants looked healthy and matured as expected, comparable to wild types. More experiments under different natural conditions are, however, needed in order to conclude firmly that no pleiotropic effects, e.g. in the form of necrosis as known from barley (Jørgensen 1992) or increased susceptibility to necrotrophic pathogens as suggested in barley (McGrann et al. 2014), are accompanying the *mlo* mutations in our material. Nevertheless, our conclusion so far is that it is possible to achieve high levels of powdery mildew resistance in tetraploid wheat by combining *mlo-A1* and *mlo-B1* mutations, and that this resistance might offer possibilities to introduce durable protection in tetraploid wheat crops against attacks by the powdery mildew pathogen. We furthermore conclude that knockout mutations, giving rise to truncated proteins (e.g. as the W163* mutation), are not necessarily the best mutations to aim for. Our results showing the highest effect with the MloA-P335S/MloB-P335L combination of mutations suggest that non-functional, but full-length Mlo proteins might have the strongest effect on resistance levels.

**Author contribution statement** Planning of project was done by CRI, FB, CU, PLG; experimental work was done by CRI, JAM-S, FB; writing manuscript was done by CRI, JAM-S, FB, CU, PLG.

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## Compliance with ethical standards

**Conflict of interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

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