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SHORT COMMUNICATION

The fire blight resistance QTL on LG7 of *Malus* ×*robusta* 5 is not dependent on the avrRpt2$_{EA}$ 156 S/C amino acid switch

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Abstract
Malus ×*robusta* 5, which has been the subject of extensive fire blight resistance research over time, is highly resistant or susceptible to the fire blight-causative bacterial pathogen, *Erwinia amylovora* – depending on the strain. *M.* ×*robusta* 5 has been crossed with susceptible apple cultivars and rootstocks, and inoculated with several *E. amylovora* strains in order to study the genetics and mechanism of its fire blight resistance and susceptibility. A strong resistance QTL was first mapped on linkage group 3 (LG3) of *M.* ×*robusta* 5 using an F1 progeny derived from a cross with the apple cultivar 'Idared' in Germany. This QTL was confirmed in two other F1 populations derived from crossing *M.* ×*robusta* 5 with the rootstock 'Malling 9' in New Zealand, and with 'Ottawa 3' in the USA. A second QTL on LG7 was detected in the 'Idared' × *M.* ×*robusta* 5 population but only with strains that break the LG3 QTL. However, in the US population of 'Ottawa 3' × *M.* ×*robusta* 5, the LG7 QTL was detected regardless of strain-specificity, unlike in the New Zealand population of 'Malling 9' × *M.* ×*robusta* 5 where the LG7 QTL was not also detected. Here, we report the detection of the LG7 QTL in a different 'Malling 9' × *M.* ×*robusta* 5 population in Germany, and confirm the independence of the LG7 locus to *E. amylovora* strains.

Keywords *Erwinia amylovora* · Mr5 · Cysteine-serine SNP · Strain-specificity · LG7 resistance locus

The most destructive bacterial disease of the genus *Malus*, i.e. the domesticated apple (*Malus* × *domestica* Borkh.) and its wild relatives – usually referred to as crabapples (Fiala 1994), is fire blight, caused by *Erwinia amylovora* (Burrill) (Winslow et al. 1920). A variability of resistance/susceptibility to fire blight is found in *Malus*, and largely depends on the strain of the pathogen (Vogt et al. 2013; Emeriewen et al. 2019). Whilst most cultivars of the domesticated apple are highly susceptible to fire blight, a few wild apple genotypes possess strong resistance (Peil et al. 2021). Resistance breeding is generally thought to be the most eco-friendly solution to fire blight epidemics, especially in Europe where the use of antibiotics is forbidden. Hence, enormous efforts have been invested into breeding research over the last two decades to understand the genetics of susceptibility and resistance to *E. amylovora* and to identify *Malus* resistance donors (Emeriewen et al. 2019; Peil et al. 2021). These studies, amongst other things, involved raising biparental populations and artificially inoculating mainly scions of grafted *Malus* plants although the primary infection site of *E. amylovora* in the orchard is through the flowers (Norelli et al. 2003). The reason for mainly inoculating grafted scions in the greenhouse is the long juvenile period of apple (Hanke et al. 2020). In addition, artificial inoculation of flowers in the field is much more time consuming and strictly restricted to only very few approved sites. Nonetheless, data obtained were sufficient to identify resistance donors, and also reliable for quantitative trait locus (QTL) analyses leading to the identification of QTLs in some apple cultivars (Calenge et al. 2005; LeRoux et al. 2010; Desnoues et al. 2018; van

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de Weg et al. 2018; Kostick et al. 2021) and in wild apple genotypes, regardless of whether grafted scions or flowers were inoculated (Peil et al. 2007, 2019; Durel et al. 2009; Emeriewen et al. 2014, 2017a, b), as well as identifying fire blight resistance candidate genes in *Malus* (Parravicini et al. 2011; Fahrentrapp et al. 2013; Emeriewen et al. 2018, 2021, 2022).

The crabapple, *Malus* ×*robusta* 5, is known in Germany by its accession number MAL0991 as Mr5 and in the USA by its accession number PI588825 as 'R5' (Wöhner et al. 2014b). It is the model genotype for fire blight resistance research as significant progress has been achieved in understanding the genetics of its resistance and interaction with *E. amylovora*. Firstly, the strongest fire blight resistance QTL in *Malus* explaining 80% of phenotypic variance in an 'Idared' × Mr5 F1 progeny established at the Institute of Breeding Research on Fruit Crops, Germany, was identified on linkage group 3 (LG3) of Mr5 (Peil et al. 2007). This QTL was confirmed in two other genetic backgrounds using F1 progeny of 'Malling 9' (M.9) × 'R5' in New Zealand, and 'Ottawa3' ('O3') × 'R5' in USA (Gardiner et al. 2012). Peil et al. (2011) then reported that this major QTL on LG3 was completely broken down after inoculation of the same 'Idared' × Mr5 F1 progeny with a highly virulent Canadian strain, Ea3049. Wöhner et al. (2014a) later confirmed this break down and reported that a few minor QTLs on LGs 5, 7, 11 and 14 were instead identified with Ea3049. Vogt et al. (2013) showed that a single nucleotide polymorphism (SNP) in *E. amylovora* effector avrRpt2$_{EA}$ amino acid sequence whereby cysteine (C-allele) changes to serine (S-allele) at position 156, as well as the complete knockout of *avrRpt2*$_{EA}$ was responsible for the breakdown of Mr5 fire blight resistance and the corresponding QTL on LG3, thus establishing the first gene-for-gene interaction between a *Malus* genotype and *E. amylovora*. Wild type strains that broke down the LG3 QTL of Mr5 thus possessed the S-allele. In contrast to Mr5 LG3 QTL, the LG7 QTL was detected only with the S-allele strain in the 'Idared' × Mr5 F1 progeny, which suggests that the LG7 QTL is dependent on the avrRpt2$_{EA}$ SNP (Wöhner et al. 2014a). However, this was not the case with the 'O3' × 'R5' F1 progeny. As previously reported in Gardiner et al. (2012), the QTL on LG7 was evident and significant with both Ea273 (C-allele strain) and E2002a (S-allele strain; synonym of Ea3049) (Vogt et al. 2013).

Here, we report the identification of Mr5 LG7 QTL with a C-allele strain in Germany for the first time in an M.9 × Mr5 F1 progeny. This QTL was not identified in an F1 progeny with both parents in New Zealand (Gardiner et al. 2012), thus making this discovery interesting. We also provide interaction analyses on the QTLs of Mr5 using the 'O3' × 'R5' population established in the US (Gardiner et al. 2012).

A recent cross between the fire blight susceptible rootstock M.9 and Mr5 in Germany resulted in 138 F1 progeny individuals. It was possible to carry out two independent phenotypic evaluations in 60% of this F1 individuals. Artificial inoculation was performed on individuals growing on their own roots, and which were at least 25 cm in height, using *E. amylovora* strain Ea222 – carrying the C-allele of the effector avrRpt2$_{EA}$ (Vogt et al. 2013). Inoculation was done by cutting the youngest leaves with a pair of scissors dipped into inoculum of 10^9 cfu/ml concentration. Disease incidence i.e. length of shoot necrosis was measured 28 days post inoculation (dpi) and converted to percent lesion length (PLL) by dividing the length of necrotic shoot by the total shoot length and multiplied by 100 as described elsewhere (Peil et al. 2019). Five replicates of each individual were artificially inoculated for the respective phenotypic evaluations. The PLL of individuals resulting from the two independent inoculation experiments were subjected to Pearson's correlation analysis. Furthermore, the M.9 × Mr5 population was genotyped-by-sequencing (GBS) in order to generate *de novo* SNP markers (Reim et al. 2023). The procedure, parameters and filtering employed for GBS analyses on the population are similar to the procedures previously reported for another *Malus* wild species – *M. fusca* (Emeriewen et al. 2020). In addition, microsatellite (SSR) markers were applied to the population to serve as anchor markers. Both SNP and SSR markers were used to develop a genetic map for the M.9 × Mr5 population using the Kosambi function in JoinMap® software (Ooijen 2006, 2018). Genotype–phenotype association analyses and quantitative trait locus (QTL) mapping were performed using Kruskal-Wallis analysis and Interval mapping modules on MapQTL 5 software (Ooijen 2004). Permutation test to determine significance of QTL was calculated using MapQTL 5.

A Pearson's pairwise correlation of the PLLs obtained for the independent inoculation experiments of the progenies is presented in Fig. 1a, and shows strong correlation coefficient and coefficient of determination ($r = 0.92$; $r^2 = 0.84$). The average PLL of the inoculated individuals for the two independent experiments were 14.1% and 15.6%, with 79% and 67% recorded respectively as the highest PLLs. Several individuals in both experiments showed no disease symptoms indicating strong resistance to this strain like Mr5. The distribution of the average PLLs of both independent inoculation experiments of the individuals is shown in Fig. 1b. Genotypic data of the 138 F1 individuals were used for genetic mapping. The genetic maps constructed for M.9 and Mr5 using GBS-derived *de novo* SNP markers and SSR anchor markers comprised of 17 linkage groups each with 2139 loci and 2989 loci for the parental maps of M.9 and

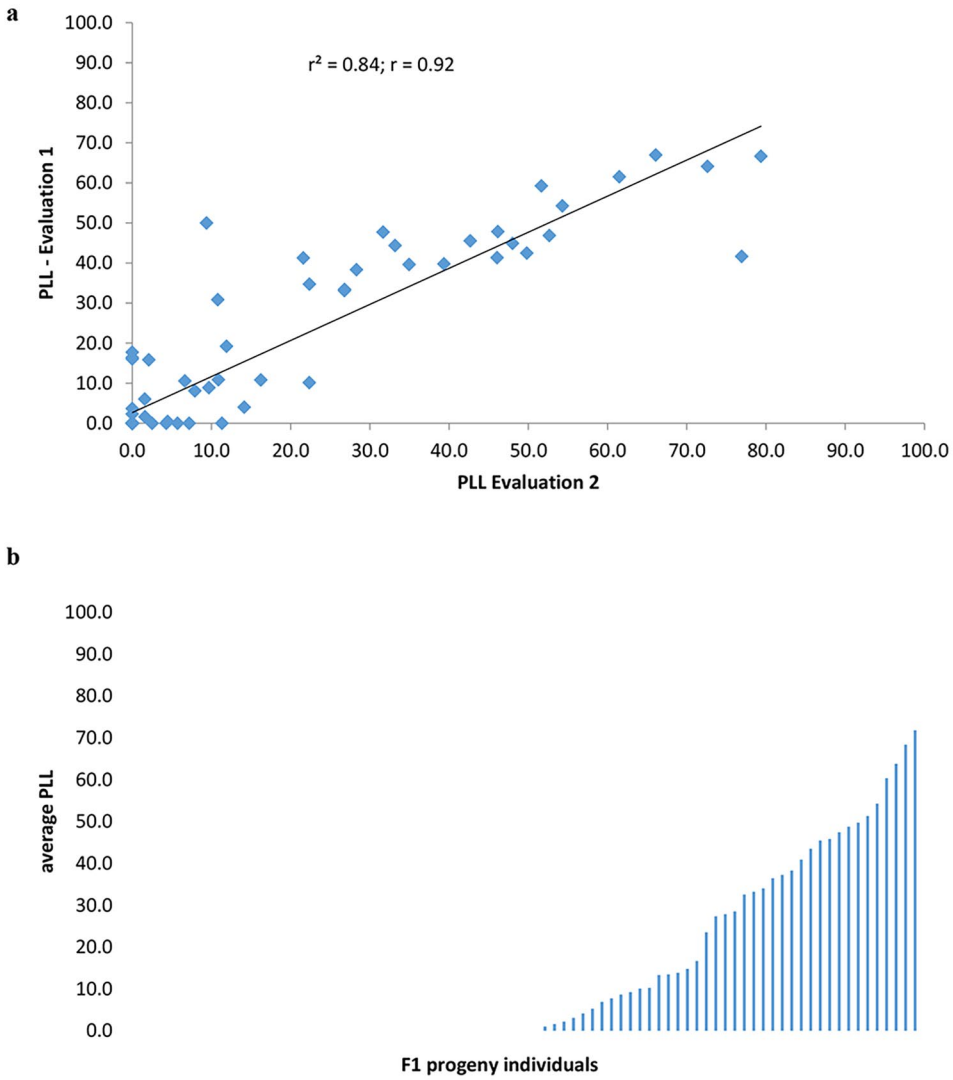

Fig. 1 (**a**) Pearson's pairwise comparison of two independent phenotypic evaluations of M.9 × Mr5 progeny inoculated with Ea222 carrying the C-allele of $avrRpt2_{EA}$ at position 156 in the amino acid sequence. A strong correlation coefficient and coefficient of determination (r = 0.92; $r^2 = 0.84$) is shown. (**b**) Distribution of F1 progeny of M.9 × Mr5 using the average PLL for both phenotypic evaluations with Ea222. PLL = percent lesion length

Mr5, respectively. Whilst the M.9 map spanned 1255.17 cM with an average length of 73.83 cM, the Mr5 map spanned 1097.69 cM with an average length of 64.57 cM (results not shown). The average PLL of the replicates of each progeny for both experiments and the map data of each parent were used for genotype – phenotype association analysis and for QTL mapping. Kruskal-Wallis analysis with the genetic map of the susceptible parent i.e. M.9, did not show any significance between mapped loci and fire blight phenotypic data of the F1 progeny. In contrast, markers on LG3 and LG7 of Mr5 correlated significantly with fire blight resistance levels in the F1 progeny. Table 1 shows markers with the highest significance in both LGs including the differences of PLL calculated for the progenies possessing alleles of the markers linked to resistance and susceptibility. Markers on LG3 of Mr5 showed the highest correlation with phenotypic data. With significance set at a LOD of 4.3 with a confidence level of 95% following permutation test, two QTLs were detected on LGs 3 and 7. The LOD plot for LG3 (not shown) positions the QTL at the top region similar to previous reports (Peil et al. 2007; Fahrentrapp et al. 2013). The QTL on LG7 is situated between markers EMPc117 at 35 cM and SNP_50982 at 51 cM (Fig. 2). This is a similar region to the LG7 QTL reported in 'O3' × 'R5' population (Gardiner et al. 2012). The Mr5 genetic map used in this study and that of Gardiner et al. (2012) have two SSR markers in common namely NZmsCN943067 and Hi05b09. Interestingly, the results of both studies are in agreement, that the LG7 QTL is in the region between both aforementioned SSR markers with NZmsCN943067 not showing any significant correlation with the QTL in this study (Fig. 2) and in Gardiner et al. (2012).

With respect to the'O3' × 'R5' population, strain specificity was also suggested by Fazio et al. (2008) since there was a high overall level of infection in the population after inoculation with E2002a (i.e. Ea3049) compared to

Table 1 Most significant markers correlating with fire blight resistance following Kruskal-Wallis analysis for linkage groups 3 and 7 of *Malus* ×*robusta* 5. The analyses was performed using MapQTL software using the map data and phenotypic data of the progeny derived from M.9 × *M.* ×*robusta* 5 cross following inoculation with Ea222

Marker	LG	Position (cM)	*K-value*	PLLs of plants with	
				Sus-alleles	Res-alleles
CH03e03[a]	LG3	1.11	40.0*****	28.7	3.5
Fem18b[a]	LG3	3.11	40.6*****	35.3	0.9
SNP_49046	LG7	42.06	11.3***	24.2	4.7
SNP_49045	LG7	42.27	12.1***	24.2	4.2
SNP_39454	LG7	48.87	13.0***	23.1	3.9
SNP_50982	LG7	51.22	12.1***	22.1	4.3

K Value of Kruskal-Wallis analysis (significance levels: *=0.05, ***=0.005, *****=0.0001); *PLL* percentage lesion length; sus-alleles (alleles for susceptibility); Res-alleles (alleles for resistance)

[a] Fire blight resistance gene *FB_MR5* underlying the QTL of LG3 is located between these two markers

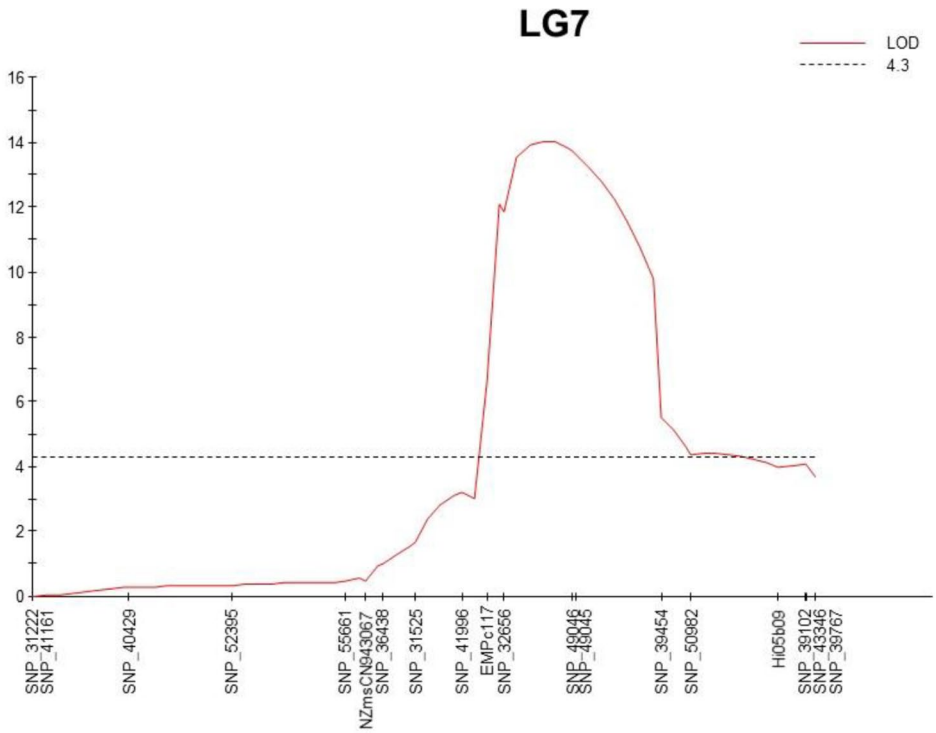

Fig. 2 LOD score plot of interval mapping (IM) of the necrosis trait along LG7 of Mr5 following genetic and phenotypic analyses of the M9 × Mr5 F1 progeny. The QTL interval is between the SNP markers SNP_41996 and SNP_39454. Red line = LOD, dotted line = LOD threshold

Ea273. A strain specific reaction in related apple rootstocks was detected when four strains of *E. amylovora* (E4001a, E2002a, E2017p and Ea273) were used to inoculate 24 apple rootstocks (Fazio et al. 2006) also showing that E4001a and E2002a, both S-allele strains (Vogt et al. 2013), as the most aggressive strains. Similar field experiments on segregating populations and advanced apple rootstock selections confirmed the nature of the strain by genotype interaction derived from 'R5' (Fazio, unpublished data) and QTLs. Further analysis of the same data reported in Gardiner et al. (2012) using an enhanced map of the 'O3' × 'R5' population with additional SNP markers from the 20 K Infinium® SNP array (Illumina Inc.) (Bianco et al. 2014) confirmed the LG3 and LG7 QTLs for Ea273, E4001a and E2002a strains.

We used an analysis of variance (Standard Least Squares method using JMP Pro 16 software) that included CH03e03 (LG3) and SNP_FB_0716018 (LG7) and their interaction to test the significance and independence of the loci for all strains tested on the 'O3' × 'R5' progeny (Table 2). The ANOVA revealed that both loci are highly significant. However, their interaction was significant only for Ea273 and E4001a but not for E2002a. The interaction plots in Fig. 3a-c showing the juxtaposition of each locus effect against the other revealed that the LG3 *d* allele from Mr5 is providing resistance to the progeny and the homozygous LG7 *hh* is providing resistance to the progeny. Both 'O3' and Mr5 are heterozygous at this locus; 'O3' is derived from

Table 2 Summaries of independent analyses of variance (ANOVA) for each strain tested using the most significant markers from the 'O3' × 'R5' population analysis representing QTLs for linkage group 3 (CH03e03) and linkage group 7 (SNP_FB_0716018) and their interaction

*Significance at < 0.05 level; DF: degree of freedom

ANOVA	Source	DF	Sum of Squares	F Ratio	Prob > F
Strain Ea273	CH03e03	3	0.50811882	8.79	< 0.0001*
	SNP_FB_0716018	2	0.24875908	6.46	0.0021*
	CH03e03×SNP_FB_0716018	6	0.31190816	2.70	0.0163*
Strain E2002a	CH03e03	3	1.2003852	4.62	0.0040*
	SNP_FB_0716018	2	2.0541843	11.8	< 0.0001*
	CH03e03×SNP_FB_0716018	6	0.5711301	1.10	0.3645
Strain E4001a	CH03e03	3	1.5674941	7.74	< 0.0001*
	SNP_FB_0716018	2	2.5206431	18.6	< 0.0001*
	CH03e03×SNP_FB_0716018	6	1.5095337	3.72	0.0018*

an interspecific cross between M.9 and a type of crabapple that could be sharing similar alleles as Mr5.

This is the first report of the detection of Mr5 LG7 QTL in an M.9 × Mr5 progeny, and with an *E. amylovora* strain that expresses cysteine amino acid at position 156 of the avrRpt2$_{EA}$ effector protein. Although an M.9 × 'R5' progeny was initially investigated in New Zealand by artificial inoculation and QTL mapping analyses, only the LG3 QTL was detected (Gardiner et al. 2012) as was also the case with an 'Idared'× Mr5 progeny (Gardiner et al. 2012; Peil et al. 2007). Gardiner et al. (2012) applied six LG3 SSR markers to ascertain the identity of the three different *M.* ×*robusta* 5 accessions, and reported that the US accession differed from the German and New Zealand accessions in alleles of one SSR marker suggesting a different clonal variant. However, twelve SSRs across eleven linkage groups applied by Wöhner et al. (2014b) showed the German and US accessions to have the same fingerprints, therefore, the genetic identity of both accessions have to be examined in more detail. Nevertheless, the Mr5 from the Germany germplasm used in the current study is the same as previously reported (Peil et al. 2007; Gardiner et al. 2012; Wöhner et al. 2014a). Moreover, with the same 'Idared'× Mr5 progeny established in Germany, it was shown that the QTLs of Mr5 were dependent on the single nucleotide polymorphism in the avrRpt2$_{EA}$ effector protein (Vogt et al. 2013) because with this population, the LG3 QTL could be detected with strains carrying the C-allele of avrRpt2$_{EA}$ and not with the S-allele strains (Peil et al. 2007; Wöhner et al. 2014a), whereas the LG7 QTL could be detected with strains carrying the S-allele of avrRpt2$_{EA}$ and not with the C-allele strains (Peil et al. 2011; Wöhner et al. 2014a). Thus, this is the first time the LG7 QTL is detected in a genetic map of the German Mr5 with a C-allele strain. Nevertheless, the results obtained from inoculation of the M.9 × Mr5 population differed substantially from the results obtained from the 'Idared' × Mr5 population (Peil et al. 2007) with the same *E. amylovora* strain: the mean PLL was around 35%, which is around 20% higher than in M.9 × Mr5, and the artificial inoculation in the greenhouse was performed on grafted scions instead of replicates on their own roots. The observed differences could be due to a lower virulence of the strain, even after passage and re-isolation through a plant, the phenotyping of only 60% of the progeny, any genetic influence of M.9 or the inoculation of plants on their own roots. Evidence for the reliability of the QTL on LG7 after phenotyping with a C-allele strain is the resulting data from 'O3' × 'R5' population. Furthermore, a reason for the failure to detect the QTL on LG7 in the 'Idared' × Mr5 population could be the lack of the corresponding allele in 'Idared' to get homozygosity on that locus. This is because in the 'O3' × 'R5' population, the homozygous LG7 *hh* allele is providing resistance to the progeny. M.9 is in the pedigree of 'O3' and could contribute to the homozygosity in the M.9 × Mr5 progeny and therefore, conferring resistance to C-allele strains, whereas the effect of the heterozygous LG7 QTL is masked by the strong effect of the LG3 QTL and the significance of the Mr5 LG7 alleles seems to intensify with the virulence of the strain.

Taken together, the crabapple *Malus* ×*robusta* 5, which has a strong fire blight resistance QTL on LG3, has another fire blight resistance QTL on LG7. This LG7 QTL is independent of the QTL on LG3 and is not affected by the avrRpt2$_{EA}$ 156 S/C amino acid switch.

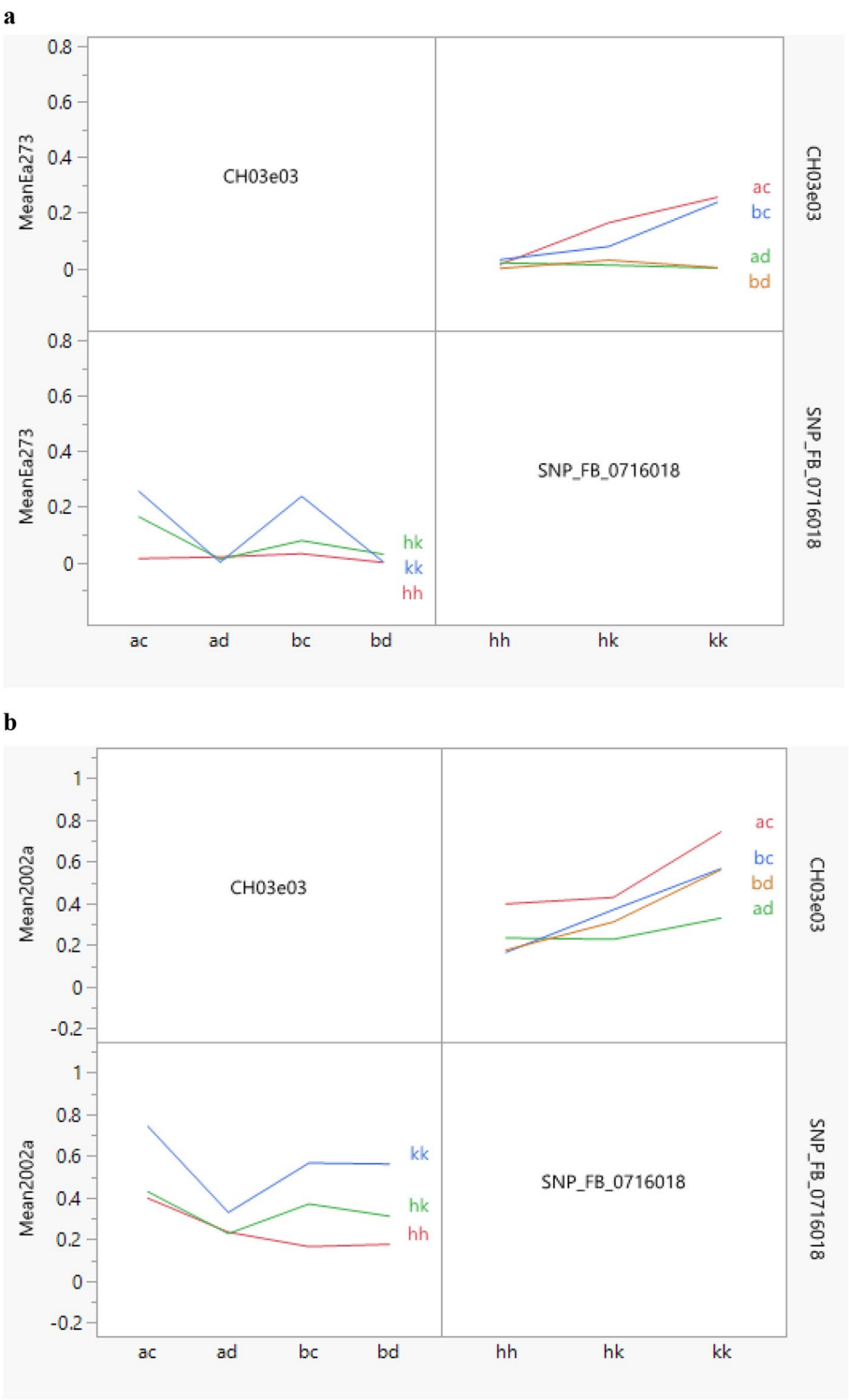

Fig. 3 Interaction plots of LG3 and LG7 loci for strains Ea273 (**a**), E2002a (**b**) and E4001a (**c**). The segregating allele convention is the same as the Joinmap/MapQTL software indicating a tetra allelic segregation (*ac*, *ab*, *bc*, *bd*) for CH03e03 and a bi-allelic double heterozygote segregation (*hh*, *hk*, *kk*) for SNP_FB_0716018. The interaction plots show the juxtaposition of CH03e03 for LG3 fire blight resistance locus and SNP_FB_0716018 for the LG7 fire blight resistance locus. The plots show that the LG3 *d* allele from Mr5 is providing resistance to the progeny and the homozygous LG7 *hh* is providing resistance to the progeny. Progeny inheriting the aforementioned alleles of both loci display less necrosis/disease compared to the progeny inheriting the other alleles e.g. *bc* and *ac* for CH03e03 and *kk* and *hk* for SNP_FB_0716018. The means in this figure correspond to the ANOVA in Table 2 performed with the individuals of the 'O3' × 'R5' population in separate, independent experiments

Authors contribution OFE, AP, HF, TW, SR, GF, HA for concept, SR and GF established populations, KR, GF, HA for artificial inoculation of populations, SR, OFE, GF for genetic analyses. OFE, AP and GF interpreted data and prepared the manuscript, all authors read and approved the manuscript.

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Declarations

Consent for publication We confirm that all authors read and approved this manuscript for publication.

Competing interests The authors' declare no competing interests.

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