



Detection and breakdown of the fire blight resistance QTL of *Malus floribunda* 821 on linkage group 12

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Abstract

Most cultivars of the domesticated apple (*Malus domestica* Borkh.) are susceptible to fire blight, caused by the bacterium, *Erwinia amylovora*. Although some moderately resistance QTL have been identified in apple, the major QTL have been found in wild apple species. Resistance to fire blight in *Malus* is, however, strain-dependent with proven gene-for-gene relationships. The QTL of three wild *Malus* species, namely *Malus floribunda* 821 (Mf821), the ornamental cultivar ‘Evereste’, and *Malus ×arnoldiana* accession MAL0004, have been mapped to a similar region at the distal end of linkage group 12 (LG12). A previous study provided evidence that the resistance mechanisms of these three sources could differ, as an Eop1 effector mutant strain (Ea1189Δeop1) of the pathogen was shown to overcome the resistance of Mf821 and ‘Evereste’, but not that of MAL0004. Nevertheless, although MAL0004 is not overcome by Ea1189Δeop1, its QTL on LG12 was completely broken down by this mutant strain. In this study, the LG12 resistance locus of Mf821 was investigated using a segregating F₁ population. The progenies were genotyped with SSR markers, and the data were used to develop a genetic linkage map for Mf821. The F₁ progeny was inoculated with a wild-type strain, Ea222, and the mutant Ea1189Δeop1 strain. Through QTL analysis, we detected *FB_Mf821* on LG12 with Ea222, and for the first time, we report the complete breakdown of this locus by Ea1189Δeop1. We therefore present the strongest evidence to date of a gene-for-gene interaction between the Eop1 effector of *E. amylovora* and *FB_Mf821* on LG12.

Keywords *Erwinia amylovora* · Bacterial effectors · Eop1 · Gene-for-gene · QTL mapping

Introduction

Erwinia amylovora (Burrill, 1883) Winslow et al. (1920), a gram-negative rod-shaped bacterium belonging to the *Enterobacteriaceae* family, continues to be a devastating pathogen affecting several species in the Rosaceae family, thereby causing fire blight disease. The pathogen utilizes

the type III secretion system (T3SS) to deliver five major effectors—AvrRpt2_{Ea}, DspE/A, Eop1, Eop3, and HopPto-C_{Ea}—into plant cells to promote infection (Zhao et al. 2005; Nissinen et al. 2006; Pester et al. 2012). The bacterium’s host range includes economically important species such as apple (*Malus*) and pear (*Pyrus*) (Peil et al. 2009, 2021). Fire blight disease is erratic due to variations in the severity of yearly outbreaks, influenced by several factors such as temperature and humidity (Peil et al. 2009). The adverse effects of fire blight have been recorded since the late 18th century, with initial sightings in New York on apples, pears, and quince (Puławska et al. 2023). Within 50 years, the disease had spread to Canada, New Zealand, Mexico, and Bermuda (van der Zwet 2002). It soon reached England, leading to further rapid spread to Western Europe and the Middle East (van der Zwet 2002). Currently, fire blight has been reported in most pome-fruit-producing countries worldwide, including China (Sun et al. 2023) and the Republic of Korea (Myung et al. 2016).

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The disease develops after *E. amylovora* enters a host through natural openings, such as flowers or through wounds. Upon initial contact with a host, *E. amylovora* goes through an initial epiphytic stage, colonizing the primary point of entry, the flower, accumulating on the stigma before moving down to the hypanthium (Thomson 2000; Slack et al. 2022). After sufficient growth, it begins to rapidly move intercellularly and through vascular tissue, causing various disease symptoms (Vanneste and Eden-Green 2000). Infection of different plant organs results in distinct phases of fire blight, namely blossom blight, shoot blight, fruit blight, and rootstock blight (Thomson and Gouk 2003; Malnoy et al. 2012). Infected organs wilt or turn brown/black as they rot. Necrotic tissue produces bacterial ooze composed of bacterial cells and the exopolysaccharides levan and amylovan, facilitating further spread of the disease to uninfected parts that are dispersed by splashing during rainfall, wind, and by pollinators such as bees (Slack and Sundin 2017; Zeng et al. 2021). The economic impact of fire blight outbreaks ranges from yield losses in orchards and increased management costs to disruption of international trade, amounting to several hundred million dollars in losses globally (Douglas 2006; Rodoni et al. 2006). However, the overall economic impact of fire blight is difficult to estimate as low impacts are rarely reported (Vanneste 2000). *Erwinia amylovora* is ranked as one of the top 10 phytopathogenic bacteria due to its economic importance (Mansfield et al. 2012).

The most effective control method against fire blight is the application of antibiotics such as streptomycin and oxytetracycline, which achieves a significant effect against the blossom blight stage of infection (Norelli et al. 2003; Stockwell et al. 2008; Stockwell and Duffy 2012). However, there are fewer means for effective control against the shoot blight stage of infection (Rezzonico et al. 2024). Moreover, resistance to antibiotics has been reported for streptomycin (Sundin and Wang 2018) and for oxytetracycline (Sundin et al. 2023). Hence, previous and recent occurrences (Manulis et al. 1998; Jones and Schnabel 2000) justify the prohibition of antibiotics in many countries and in Europe; however, in the USA, antibiotics are approved and continue to be integral for fire blight control (Donley 2019). The development of biological control methods followed the increased demand for sustainable management methods of fire blight (Johnson and Stockwell 2000). Antagonistic bacteria belonging to the genera *Bacillus*, *Pantoea*, and *Pseudomonas* are the active ingredients of several marketed biological control agents (BCA) such as Bloomtime Biological™, BlightBan C9-1® and BlightBan A506®, whereas fungi such as *Aureobasidium pullulans* and *Papiliotrema terrestris* are the active ingredients in Blossom Protect® and YSY, respectively (Zeng et al. 2023, 2024). While the results of the application of biopesticides are promising, the level of control achieved

is lower and less consistent compared to chemicals and antibiotics (Vanneste et al. 2001; Stockwell et al. 2010; Temple et al. 2019; Kunz et al. 2023).

Host resistance to *E. amylovora* is one approach to combat fire blight epidemics, although most apple (*Malus domestica*) cultivars are more or less susceptible to the pathogen (Kostick et al. 2019; Peil et al. 2021). However, some wild crabapples show strong levels of resistance to *E. amylovora* (Emeriewen et al. 2019). Research and development aimed at fire blight resistance are useful for enhancing breeding objectives to develop resistant cultivars. Consequently, a handful of quantitative trait loci (QTLs) with potential for breeding applications have been identified in moderately susceptible apple cultivars and highly resistant crabapples (Emeriewen et al. 2019). A moderate effect QTL explaining 34–46% of the phenotypic variation was identified by Calenge et al. (2005) on linkage group (LG) 7 (*FB_F7*) in ‘Fiesta’, and confirmed in Khan et al. (2006). ‘Enterprise’ and other cultivars carrying *FB_F7* or its alleles demonstrate reduced susceptibility or moderate resistance (Sehic et al. 2009). Minor QTLs *FLO5* and *FLO10*, explaining 10–18% of phenotypic variation, were detected in ‘Florina’ (Le Roux et al. 2010). In an accession of the primary progenitor of the domesticated apple, *M. sieversii* accession KAZ 95 17–14, a moderate QTL was identified on LG7 (*Msv_FB7*) explaining 48–53% (Tegtmeier et al. 2023), which might be the same as *FB_F7*. In wild apple species, major QTLs were described, for example, in *M. ×robusta* 5 (Mr5) on LG3, which explained 80% of phenotypic variation that was attributed to a single gene, *FB_MR5*, underlying the QTL region (Peil et al. 2007; Fahrretrapp et al. 2012). Similarly, a major QTL on LG10 explaining 65% of phenotypic variation was identified by Emeriewen et al. (2014) in the crabapple *M. fusca*. Of the major resistance donors, only in *M. fusca* is a gene-for-gene relationship not yet found, demonstrating a broad-spectrum resistance against different strains of *E. amylovora* (Emeriewen et al. 2015, 2020; Wöhner et al. 2018). The fact that most major resistance donors are overcome highlights the strain specificity of resistance in *Malus*.

This research paper is based on the fire blight resistance previously mapped in a similar position on LG12 in three *Malus* species (Durel et al. 2009; Emeriewen et al. 2017). Whilst Durel et al. (2009) mapped two major co-localizing QTLs on the distal end of LG12, explaining 40–50% of phenotypic variation in *M. floribunda* 821 (Mf821) and in the ornamental cultivar ‘Evereste’, Emeriewen et al. (2017) mapped a major QTL in the same region in *M. ×arnoldiana* accession MAL0004. It has long been undetermined whether these three resistances are allelic or the same, especially as fine mapping studies revealed similar kinds of serine-threonine candidate genes underlying the QTL regions

of ‘Evereste’ and MAL0004 (Parravicini et al. 2011; Emeriewen et al. 2021). The use of bacterial strains in which bacterial effectors are deleted has been instrumental in similar studies for dissecting their roles in the pathogenicity of *E. amylovora* and durability of resistance sources (Zhao et al. 2005, 2006; Vogt et al. 2013; Wöhner et al. 2018). In the meantime, it was determined that the resistance mechanisms of the aforementioned LG12 resistance donors could be different, as an Eop1 effector mutant strain of *E. amylovora*, Ea1189Δeop1, elicits a peculiar response amongst them (Wöhner et al. 2018). Whereas the wild-type strain Ea1189 did not affect all three sources, significant disease symptoms were induced by Ea1189Δeop1 in both Mf821 and ‘Evereste’, suggesting gene-for-gene interactions in both species (Wöhner et al. 2018). Furthermore, the breakdown of the resistance donor, Mf821, hints at the possibility of a corresponding breakdown of its locus. In contrast, although Ea1189Δeop1 did not overcome the resistance of MAL0004, it led to the breakdown of the QTL on LG12 (Emeriewen et al. 2024). The pattern of donor and locus breakdown was first observed in Mr5, which is broken down, along with its LG3 QTL, by SNP variants and a deletion mutant of AvrRpt2_{Ea} (Vogt et al. 2013; Wöhner et al. 2014).

On this premise, this study represents the first attempt to investigate the resistance locus of Mf821 on LG12 by analysing a segregating population established with the donor parent, both genotypically and phenotypically, using two strains of *E. amylovora*: wild-type Ea222 and deletion mutant Ea1189Δeop1. Our aim is to primarily identify and confirm the resistance locus *FB_Mf821* and subsequently investigate any relationship with the effector Eop1 by QTL analysis. In addition, we set out to determine the common alleles shared by the three species within the identified resistance regions.

Materials and methods

F₁ mapping population and DNA isolation

A mapping population, designated as ‘18219’, was derived from a cross between the susceptible *M. domestica* cv. ‘Idared’ and *M. floribunda* 821 (Mf821). The cross resulted in 126 F₁ individuals that are being maintained as trees in the experimental orchard of the Julius Kühn-Institute in Dresden-Pillnitz. During the spring of 2023, young leaves were harvested from the F₁ population and both parents for DNA extraction. DNA was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The concentration of DNA was measured using a NanoDrop Microvolume

Spectrophotometer (Thermo Fisher Scientific, Germany) and diluted to 10–20 ng/μL for PCR amplification.

Grafting, artificial shoot inoculation, and disease assessment

Both parents and the F₁ population were grafted on rootstock M111 and grown under the following greenhouse conditions: 25–27 °C during the day, 20 °C at night, and 85% air humidity, with normal day and night lighting conditions. Two strains of *E. amylovora*, Ea222 (wild-type) and Ea1189Δeop1 (mutant), were used for artificial shoot inoculation as described elsewhere (Emeriewen et al. 2024). The F₁ population was inoculated with Ea1189Δeop1 in two consecutive years, but only in one year with Ea222. A bacterial suspension of the respective strains at a concentration of 10⁹ cfu/ml was prepared, and a pair of scissors was dipped into this before making incisions into the youngest leaves of the shoots. For the respective inoculation experiments, we aimed to use 10 replicates per genotype, but this was not always possible, as only shoots that grew up to 25 cm were inoculated. We inoculated 113 and 123 F₁ individuals with Ea1189Δeop1 in the two consecutive years, respectively, and 122 individuals with Ea222 in the single-year experiment. Disease incidence was measured 28 days post inoculation (28 dpi) and converted to percent lesion length (PLL) as the necrotic region of the shoot against the total shoot length multiplied by 100 as described previously (Peil et al. 2007; Emeriewen et al. 2015).

Marker sourcing and genotyping

Simple sequence repeats (SSR) markers spanning all 17 linkage groups (Table S1) of *Malus* were sourced from HIDRAS (High Disease Resistant Apples for a Sustainable Agriculture) and published linkage maps (Parravicini et al. 2011; Emeriewen et al. 2017, 2023). Most forward primers were labelled with fluorescent dyes at the 5’-end; however, for those without fluorescent dyes, the M13 tag (TGTAACACGACGGCCAGT) was added to the forward primer according to Schuelke (2000). SSR primers were first tested for polymorphism on the ‘18219’ population using both parents and six F₁ individuals. Only polymorphic markers were selected for further genotyping analyses. To apply the SSR markers for polymorphism test and the subsequent genotyping analyses, PCR reactions were conducted using the Type-It Microsatellite PCR kit (Qiagen, Germany) in a 10 μL reaction volume containing 10–20 ng genomic DNA under the following conditions: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min 30 s, and 72 °C for 30 s and an extension at 60 °C for 30 min. One μL of a 1:200 diluted PCR product was added to 8.95 μL

Hi-Di™ Formamide (Applied Biosystems) and 0.05 µL Liz 600 size standard (Applied Biosystems) and denatured at 95 °C for 5 min. The samples were analysed by loading into the ABI 3500xL Genetic Analyzer, and the results of fragment analysis were viewed on GeneMapper™ Software 6 (Applied Biosystems).

SSR marker development for LG12

Six SSR markers were designed for linkage group 12 (LG12) from the Golden Delicious Double Haploid Genome (GDDH13 v1.1) (Daccord et al. 2017). SSR motifs within the region bordered by CH04g04 and CH03c02 markers were selected, and flanking primers were developed using Primer3 (Untergasser et al. 2012). The uniqueness and specificity of designed primers were determined by aligning primer sequences to the GDDH13 reference genome (Daccord et al. 2017) using BLAST on the Genome Database for Rosaceae (Johnson et al. 2008). SSR primers are presented in Table 1.

Construction of an SSR-based genetic map for Mf821

A genetic map was constructed for Mf821 using the SSR data of the 126 F₁ individuals. SSR alleles (in bp) for each polymorphic marker were converted to genotype codes for a cross-pollinated population and assigned to each locus for every individual. Data was imputed into JoinMap 4 (Van Ooijen 2006). Loci were grouped based on independence LOD scores between 5 and 10, using a regression mapping algorithm with Kosambi's function to calculate map distances. The constructed map was visualized in MapChart (Voorrips 2002). Quality control of the map order and linkage group assignment was performed using published *Malus* genetic maps as a reference (Liebhard et al. 2003; Silfverberg-Dilworth et al. 2006).

QTL mapping, genotype-phenotype association, and statistical analysis

Phenotypic data from disease assessment in the form of PLL values were used together with the SSR marker data of the F₁ population for genotype-phenotype correlation analysis

and for QTL mapping. Significant genotype-phenotype associations were determined by a Kruskal-Wallis analysis—a nonparametric equivalent of ANOVA (Van Ooijen 2004). Interval mapping (IM) was performed using MapQTL 5 Software (Van Ooijen 2004). Logarithm of the odds (LOD) score threshold, which determines the significance of the detected QTLs, was calculated using a permutation test in MapQTL 5 at alpha values of $p < 0.05$. To evaluate the effects of putative QTLs on necrosis length in the progeny, the Wilcoxon test was employed to compare the mean PLL values of F₁ individuals possessing an allele from markers associated with resistance. For this analysis, the progenies were divided into two groups: individuals carrying the resistance allele and those without it.

Genotyping donors of LG12 resistance with markers tightly linked to resistance regions

We genotyped Mf821, 'Evereste', and MAL0004 with markers from LGs 12 and 15 to determine allele sizes amplified by the donors of LG12 fire blight resistance. Whereas LG12 is the linkage group containing their major QTL (Durel et al. 2009; Emeriewen et al. 2017), our preliminary studies indicated putative minor QTL on LG15; hence, markers from both linkage groups were used for this analysis.

Results

Artificial inoculation and phenotypic assessment

In the first year of the inoculation experiment with Ea1189Δeop1, we inoculated and assessed 113 progenies and both parents. Unfortunately, we could only generate a single replicate of Mf821, which showed no disease symptoms compared to an average of 31.4 PLL calculated for nine replicates of 'Idared'. The overall mean and median PLL for the 113 progenies were 24.4 and 12.4, respectively, with 13 individuals showing complete resistance (PLL=0) and two individuals being completely blighted (PLL=100). The percentage of the progeny showing higher susceptibility than 'Idared' was 28.3%.

In the second year of the inoculation experiment with Ea1189Δeop1, we inoculated 123 progenies and eight and

Table 1 Polymorphic simple sequence repeat (SSR) markers developed from chromosome 12 of the *M. domestica* GDDH13 reference genome (Daccord et al. 2017)

Marker	Forward sequence	Reverse sequence	Alleles	
			Mf821	Idared
TM3D2Y_101	AAGCAAGACTCGGGGGTTAC	CGCAAGTCACGTACGAGTTG	187; --	241; --
TM3D2Y_103	TGTTTGGAGTCAATCAACCAA	AAATCAGCTTGCTCCTGTCC	191; --	226; --
TM3D2Y_153	GGTTATGTTTGATGCATTGAA	TGCACAACATAGAAGAAGCAAAA	249; 261	192; 220
TM3D2Y_202	GGCACGGTTTAGTAGCCATT	TGCAGAGGCAACAATCACAT	153; 224	178; 236

seven replicates of Mf821 and ‘Idared’, respectively. The average PLL calculated for Mf821 and ‘Idared’ was 23.2 and 51.0, respectively. The mean and median PLL for the 123 progenies were 55.6 and 55.9, respectively. In this same year of inoculation trials, we inoculated 122 progenies and seven replicates of both parents with Ea222. In this experiment, the average PLL calculated for Mf821 and ‘Idared’ was 1.6 and 42.8, respectively. The mean and median PLL for the 122 progenies inoculated with Ea222 were 17.3 and 13.2, respectively. The descriptive statistics and distributions of the PLL of the population for the three experiments are shown in Table 2 and Fig. 1.

SSR marker application

We tested a total of 190 SSR markers and applied 117 (61.6% polymorphism rate) that were polymorphic in either both parents or in only Mf821 to the entire ‘18219’ progeny. Markers that were polymorphic in only ‘Idared’ were discarded because we aimed to generate a genetic map of the resistance donor. Some SSRs were multi-allelic and hence could map on more than one linkage group. The polymorphic SSR markers developed on LG12, including their respective allele sizes, are presented in Table 1.

Construction of an SSR-based genetic map for Mf821

The genetic linkage map of the resistance donor Mf821 was constructed using the genotypic data of 126 F₁ individuals of the ‘18219’ population. The map consisted of 117 markers, ordered into 17 linkage groups (Figure S1), with an average of six markers per linkage group. The characteristics of the genetic map are presented in Table 3. LG12 had the highest number of markers, and LG6 had the lowest number of markers. The total length of the Mf821 genetic map was 838.73 cM. The map length of the linkage groups ranged from 9 to 71 cM. Marker order was assessed using *M. domestica* reference genetic maps (Liebhard et al. 2003; Silfverberg-Dilworth et al. 2006) and integrated maps on High-quality Disease Resistant Apples for a Sustainable Agriculture SSR database (HiDRAS website). The comparison showed a good level of collinearity in marker

order. Further, the LG4 markers Ch4_R13_27_27, Ch4_R18_29_29, and Ch4_R25_32_32 developed by Emeriewen et al. (2023) for QTL analysis in *M. fusca* MAL0045, which are not in the HiDRAS website, mapped on LG4 in the Mf821 map and in a similar order as previously reported.

We focused more on LG12 (Fig. 2) since it harbours the QTL of Mf821, ‘Evereste’, and MAL0004 (Durel et al. 2009; Emeriewen et al. 2017). The markers CHFbE02 and CHFbE08 developed by Parravicini et al. (2011) during fine mapping of *Fb_E* in ‘Evereste’ mapped on the distal end of LG12. Similarly, markers FRMb103x, FRMb197, FRMb199, FRMb251 and FRMb533 developed by Emeriewen et al. (2017) in a QTL mapping study of MAL0004 also mapped on the lower end of LG12 (Fig. 2).

QTL mapping using different strains

We used the individual phenotypic and genotypic data of the ‘18219’ population for QTL mapping, including marker-phenotype correlation analysis. Results of the Kruskal-Wallis analyses with data from the three independent inoculation experiments are shown in Table 4. For the first inoculation experiment with Ea1189Δeop1, there was clearly no significant association between the phenotypes and markers, which map at the distal end of LG12, previously implicated with the fire blight resistance of LG12. In fact, only three markers (CH02d11, Hi02f06, and CH03b10) that mapped to a region on LG15 showed some significance (K -value=20.4–24.5). For the second inoculation experiment with Ea1189Δeop1, the significance of the aforementioned markers on LG15 dropped (K =10.5–12.9), with no other markers within a genetic region on the 17 linkage groups showing any significant association with the phenotypes in the Kruskal-Wallis analysis. In contrast, there was a significant correlation between markers at the distal end of LG12 when using the phenotypic data of Ea222 for Kruskal-Wallis analysis, which ranged from K =36.3 for FRMb251 to K =59.5 for FRMb199. The SSR markers that have been previously mapped to the fire blight region of ‘Evereste’ and MAL0004 are highly associated with the resistance phenotype on LG12 (Table 4).

Similarly, interval mapping detected a corresponding minor QTL (LOD=5.95) only in the same region on LG15 with the first inoculation experiment data of Ea1189Δeop1 (Figure S2). The genome-wide threshold for LOD significance of the QTL was determined by a permutation test to be a LOD of 5.0; hence, this QTL slightly exceeded the genome-wide threshold. The LG15 QTL explained 27.9% of the phenotypic variation. With the second inoculation experiment data of Ea1189Δeop1, the QTL on LG15, now having a LOD of 2.9, was, however, not significant at the genome-wide level as the calculated threshold was 4.1. A

Table 2 Summary of F₁ population ‘18219’ disease assessment from independent inoculations using Ea1189Δeop1 and Ea222 strains

Strain	Ea1189Δeop1		Ea222
	Year 1	Year 2	Single Year
No. of progeny	112	123	122
Mean PLL	24.4	55.6	17.3
Median PLL	12.4	55.9	13.2
Asymptomatic progeny	13	0	24
Fully blighted progeny	2	1	0

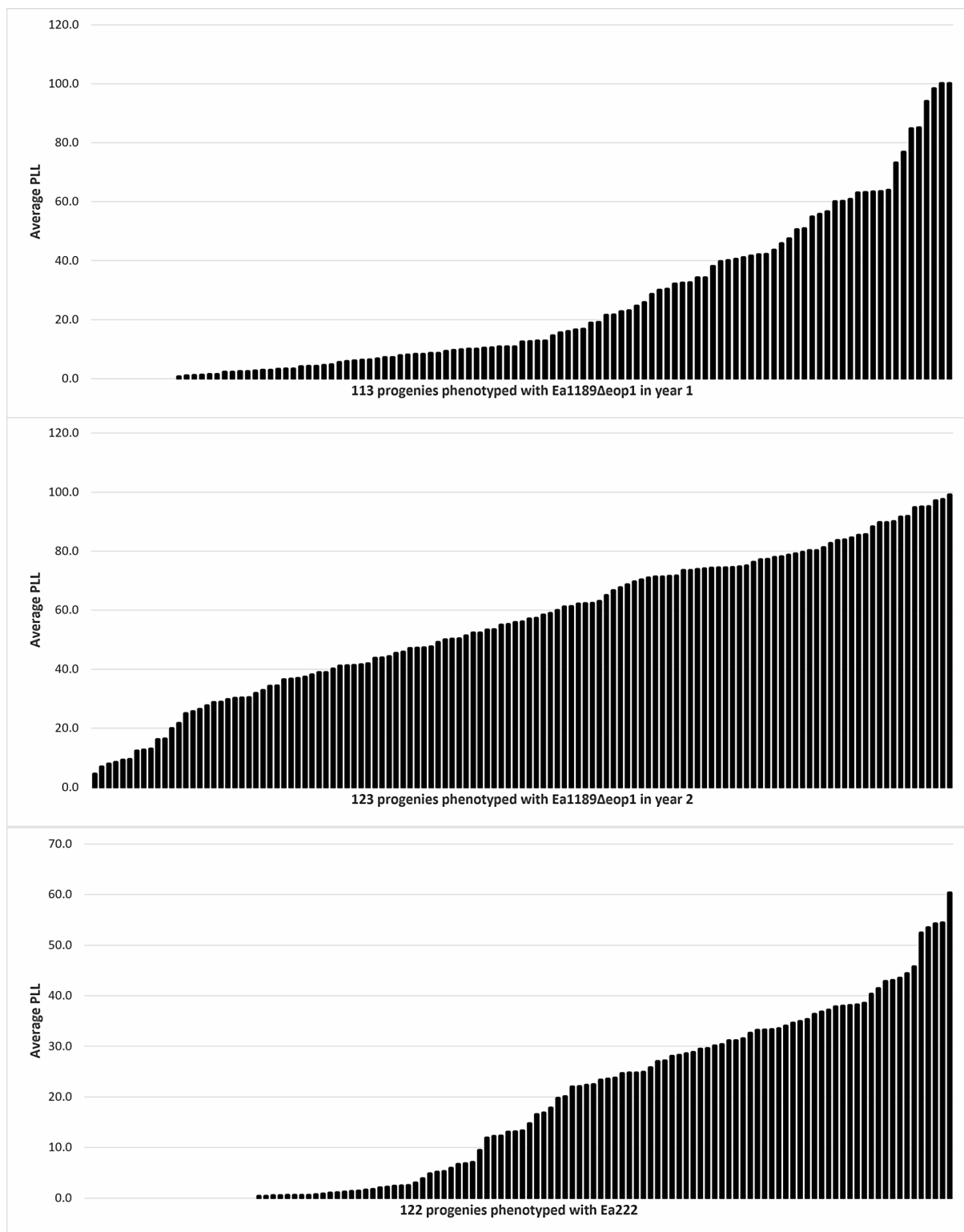


Fig. 1 Distribution of the '18219' population according to mean PLL values from three independent disease assessments using Ea1189Δeop1 and Ea222 strains

Table 3 Characteristics of the genetic linkage map with the number of SSR markers and map length (cM) per chromosome of the resistance donor *Malus floribunda* 821

LG	No. of Markers	Map length cM	<i>Malus</i> Chromosome
1	4	43.65	Chr1
2	6	62.71	Chr2
3	5	52.56	Chr3
4	8	54.16	Chr4
5	5	57.0	Chr5
6	3	9.31	Chr6
7	7	52.21	Chr7
8	8	50.79	Chr8
9	7	40.89	Chr9
10	7	40.31	Chr10
11	8	71.71	Chr11
12	15	54.58	Chr12
13	7	54.98	Chr13
14	4	52.7	Chr14
15	11	48.35	Chr15
16	6	46.72	Chr16
17	6	46.1	Chr17
Total	117	838.73	

major QTL with a LOD of 20.76, which explained 61.4% of the phenotypic variation, was detected at the distal end of LG12 with the inoculation experiment of Ea222 (Fig. 2). The QTL exceeded the genome-wide threshold (LOD=4.8) and is tightly linked with the markers, which previously mapped to the fire blight region of ‘Evereste’ and MAL0004. The LOD score plot showing the QTL detected with Ea222, including the allele classes of F_1 progenies for CHFbE08, and its breakdown by Ea1189 Δ cop1 is presented in Fig. 3.

Genotyping donors of LG12 resistance with markers tightly linked to resistance regions

The allele sizes amplified by Mf821, ‘Evereste’, and MAL0004 with SSR markers linked to QTL regions of LG12 and LG15 are shown in Table 5. All three donors amplified the same resistance alleles for markers FRMb31M27a (211 bp), FRMb32M27 (143 bp), and FRMb32M21 (276 bp) linked to *FB_Mar12*, the fire blight resistance locus of MAL0004 (Emeriewen et al. 2021), and ChFbE01 (247 bp) and ChFbE02 (231 bp) linked to *FB_E*, the resistance locus of ‘Evereste’ (Parravicini et al. 2011). Whereas FRMb32M21 co-segregated with *FB_Mar12* (Emeriewen et al. 2021), ChFbE02 co-segregated with *FB_E* (Parravicini et al. 2011). Similarly, the donors amplified the same resistance alleles of FRMb197 (219 bp), one of the markers that map at the distal end on LG12 of MAL0004 (Emeriewen et al. 2017, 2021). However, there are clear recombinations between the three donors with markers that map above the resistance region on LG12, e.g., CH04g04 and CH03c02.

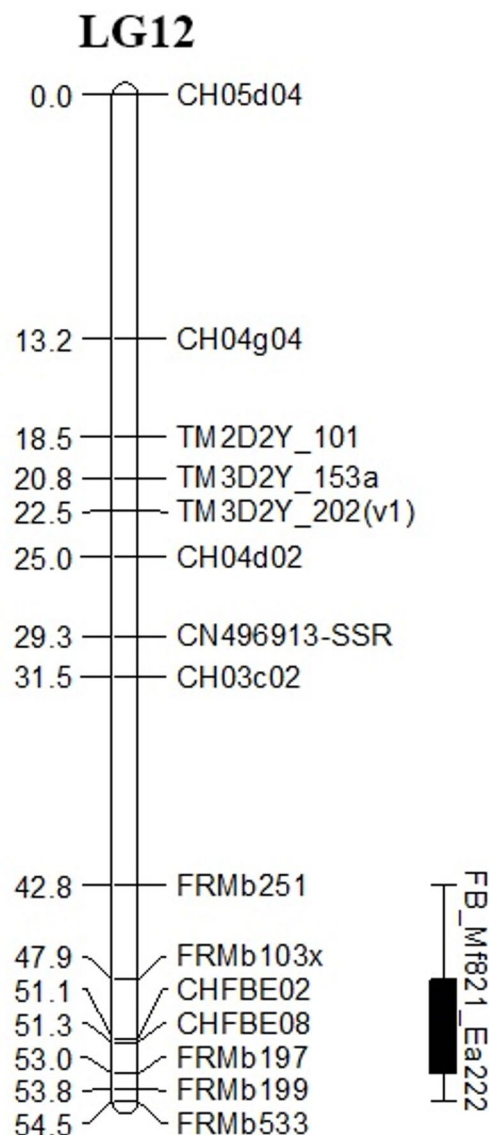


Fig. 2 Linkage group 12 of *Malus floribunda* 821, including distal SSR markers previously mapped to the fire blight region of ‘Evereste’ and MAL0004, and the QTL position represented with the black bar. Lines represent the 1-LOD and 2-LOD intervals

For markers that map within the minor QTL region on LG15, at least one allele of each of the four SSR markers was amplified in MAL0004 and Mf821, in contrast to ‘Evereste’, which did not share any allele with them.

Discussion

Plant genetic resistance to diseases, especially those of a monogenic nature, is often limited by the evolution of the pathogen, which can result in the breakdown of resistance (Quenouille et al. 2013). This has also been demonstrated for resistance to fire blight in *Malus*, where a point mutation

Table 4 Kruskal-Wallis analysis of LG12 and LG15 of *M. floribunda* 821 using two-year data of inoculation with Ea1189 Δeop1 and single-year data of inoculation with Ea222

Position (cM)	Locus	Ea222		Ea1189Δeop1 – Year 1		Ea1189Δeop1 – Year 2	
		K*	Signif.	K*	Signif.	K*	Signif.
LG12							
0	CH05d04	4.7	-	14.2	****	6.7	*
13.21	CH04g04	2.5	-	6.8	***	7.3	***
18.46	TM2D2Y_101	6.1	**	4.8	**	5.2	**
20.77	TM3D2Y_153a	9.3	****	3.7	*	3.5	*
22.49	TM3D2Y_202(v1)	13.2	****	4.3	-	3.5	-
24.96	CH04d02	12.9	****	4.8	-	3.3	-
29.29	CN496913-SSR	16.6	*****	3.2	*	0.8	-
31.50	CH03c02	20.1	*****	5.1	-	2.2	-
42.76	FRMb251	36.3	*****	1.2	-	0.4	-
47.93	FRMb103x	51.6	*****	2.5	-	0.0	-
51.09	CHFBE02	48.7	*****	2.7	*	0.1	-
51.34	CHFBE08	59.1	*****	2.0	-	0.2	-
53.01	FRMb197	56.1	*****	1.1	-	0.8	-
53.82	FRMB199	59.5	*****	1.1	-	0.6	-
54.48	FRMb533	54.7	*****	0.8	-	0.4	-
LG15							
0	CH02e12	1.3	-	0.3	-	0.8	-
7.09	Hi02g06	0.9	-	0.3	-	0.2	-
14.47	CH03b06	3.7	-	3.4	-	5.3	-
19.47	Hi03g06	6.8	*	3.9	-	2.4	-
26.44	Hi04c05	0.9	-	7.5	***	6.6	***
28.07	CH02d11	5.9	-	20.4	*****	10.5	**
31.92	Hi01c06	0.6	-	8.1	****	3.5	*
33.11	CH03b10	6.8	*	24.5	*****	12.9	*****
33.12	Hi02f06	7.2	*	20.9	*****	11.0	**
44.00	CH01d08	7.6	*	9.9	**	10.1	**
48.35	CH02c09	4.4	-	16.2	****	0.8	-

K* Value of Kruskal-Wallis analysis (Significance levels: * = 0.1, ** = 0.05, *** = 0.01, **** = 0.005, ***** = 0.0005, **** = 0.0001)

at position 156 in the amino acid sequence of the avrRp-t2_{EA} effector of *E. amylovora* leads to the breakdown of the resistance of *M. ×robusta* 5 (Mr5) (Peil et al. 2011; Vogt et al. 2013). The use of mutant strains of *E. amylovora* has, over the years, proven to be an effective approach to identify putative gene-for-gene relationships in the *E. amylovora* and *Malus* pathosystems (Vogt et al. 2013; Broggini et al. 2014; Wöhner et al. 2018; Emeriewen et al. 2024). A knock-out mutant strain of avrRp-t2_{EA} effector induced fire blight disease symptoms in the otherwise highly resistant Mr5, completely broke down its resistance QTL mapped on LG3 (Wöhner et al. 2014), and also broke down the NLR gene, *FB_MR5*, which was found to underlie the QTL region (Broggini et al. 2014).

There are not many donors of strong fire blight resistance in *Malus* (Emeriewen et al. 2019). However, the donors of fire blight resistance loci mapped to the distal end of LG12 (Durel et al. 2009; Emeriewen et al. 2017), as well as their relationship with, and role of the effector Eop1 in pathogenesis, is intriguing, but not fully understood. Wöhner et

al. (2018) reported that only two LG12 resistance donors, Mf821 and ‘Evereste’, elicited disease symptoms upon challenge with an Ea1189Δeop1 mutant strain, with the other donor, MAL0004, exhibiting resistance to this strain. Emeriewen et al. (2024) confirmed that Mf821 was susceptible, and MAL0004 was resistant to Ea1189Δeop1. However, although MAL0004 was resistant, the QTL on LG12 was completely broken down by Ea1189Δeop1 (Emeriewen et al. 2024). Similar types of resistance candidate genes were already reported in the resistance region of ‘Evereste’, *FB_E*, from BAC clone sequences (Parravicini et al. 2011) and from the MAL0004 region, *FB_Mar12* (Emeriewen et al. 2021), by aligning tightly linked markers to the *M. baccata* draft genome (Chen et al. 2019). Furthermore, *M. ×arnoldiana* is a hybrid of *M. floribunda* and *M. baccata* (Fiala 1994), while ‘Evereste’ has Mf821 as an ancestral parent in its pedigree (Durel et al. 2009). Taken together, the foregoing information gives rise to several critical questions, some of which were previously highlighted by Zeng et al. (2024) namely: (i) would the QTL of Mf821

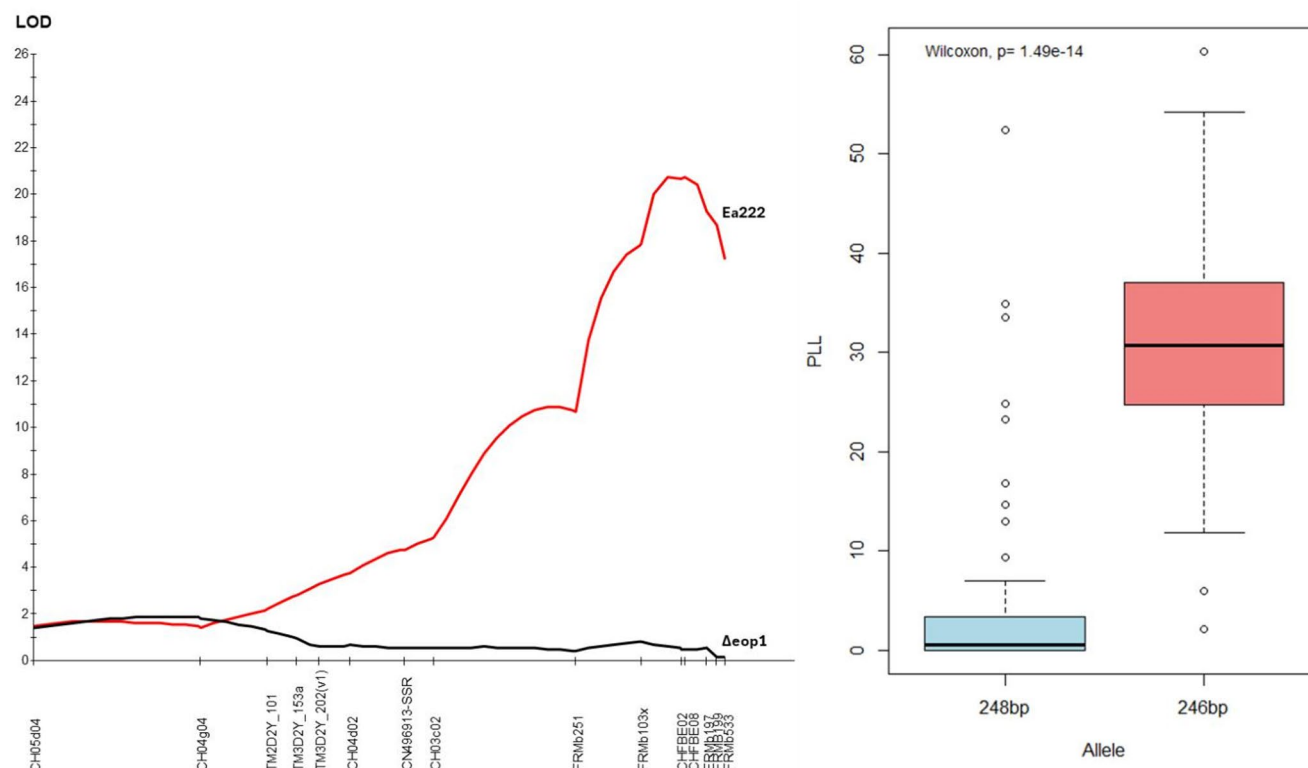


Fig. 3 Logarithm of the odds (LOD) score plot of LG12 for mean PLL following phenotyping of the ‘18219’ mapping population with Ea222 and Ea1189Δeop1 (**left**). The LG12 QTL is detected with the strain Ea222 but not with the mutant strain Ea1189Δeop1. Box plot repre-

senting fire blight resistance and susceptibility in individuals following inoculation with Ea222 according to alleles inherited at the CHFbE08 locus: 248 bp (coupling) and 246 bp (repulsion) (**right**)

Table 5 SSR alleles sizes amplified by *M. floribunda* 821, *M. ×arnoldiana* (MAL0004) and ‘Evereste’ in the QTL regions on LG12 and LG15

Markers on:	Alleles (bp) amplified by:		
LG12	Mf821	MAL0004	Evereste
CH04g04	166, 174	166, 172	170, 176
CH03c02	111, 137	117, 137	111, 123
+FRMb31M27a	211 , 249	211	198, 211
+FRMb32M21	276	276 , 297	276
+FRMb32M27	131, 143	114, 143	143
*ChFbE01	241, 247	247	241, 247
*ChFbE02	231	85, 231	85, 231
*ChFbE08	246, 248	246, 248	241, 248
+FRMb197	219 , 253	219	219 , 226
LG15			
CH02d11	124, 136	121, 136	134, 147
CH03b10	103, 117	103, 113	90, 98
CH02c09	230, 250	250	226, 254
CH01d08	237, 241	241, 273	239, 245

Allele sizes in bold are linked to resistance. *Markers tightly linked to *FB_E* (Parravicini et al. 2011; +Markers tightly linked to *FB_Mar12* (Emeriewen et al. 2021). bp; base pair

and ‘Evereste’ be overcome by Ea1189Δeop1, (ii) if so, are the QTL mapped in the three wild accessions allelic or the same, (iii) are they controlled by the same resistance genes, (iv) where is the resistance originating from?

Here, we report for the first time that the fire blight resistance of Mf821, together with its QTL on LG12, is broken down by the Ea1189Δeop1 mutant following genetic studies using a segregating population derived by crossing ‘Idared’ and Mf821. The segregating population ‘18219’ was challenged in three independent experiments, twice with Ea1189Δeop1 and once with a wild-type strain Ea222. The strain Ea222 was previously used to identify the resistance locus of MAL0004 on LG12 (Emeriewen et al. 2017), as well as other fire blight studies (Vogt et al. 2013; Wöhner et al. 2014; Peil et al. 2007, 2019). Disease severity was higher in the second year than in the first year with Ea1189Δeop1 inoculation; however, the moderate correlation observed between the years shows that host responses remained relatively consistent. In contrast, correlations between Ea1189Δeop1 and Ea222 were lower, and the reduced mean PLL observed for Ea222 indicates that Ea1189Δeop1 is more virulent on the ‘18219’ population (results not shown). A higher number of individuals were completely resistant (PLL=0) from Ea222 inoculation than in any of the Ea1189Δeop1 experiments. Similarly, Mf821 exhibited higher necrosis following inoculation with Ea1189Δeop1 than with Ea222, confirming the findings of Wöhner et al. (2018) and Emeriewen et al. (2024) regarding the susceptibility of Mf821 to the Ea1189Δeop1 mutant.

Furthermore, using the phenotypic data from a single-year inoculation experiment with the wild-type strain Ea222, we found significant marker-trait associations on the distal end of LG12 and subsequently detected a major QTL in the same region, explaining 61.4% of the phenotypic variation. We therefore confirm the presence of a fire blight QTL of Mf821 on LG12, first reported by Durel et al. (2009), albeit in a population with a different genetic background. The QTL co-localizes and shares similar alleles with *Fb_E* and *FB_Mar12* (Emeriewen et al. 2017, 2021). The failure to detect this QTL upon challenge with Ea1189Δeop1 in two different years of inoculation is strong evidence of its breakdown by Ea1189Δeop1 mutant and a putative gene-for-gene interaction with the genes controlling the locus, which is a similar situation reported for the locus of MAL0004 (Emeriewen et al. 2024). Given that all three donors of LG12 resistance, Mf821, MAL0004, and ‘Evereste’, share the same resistance alleles for markers co-segregating with the fine-mapped loci of MAL0004 and ‘Evereste’ (Parravicini et al. 2011; Emeriewen et al. 2021), is strong evidence that these resistance donors might have the same resistance genes and mechanism at the distal end of LG12. The shared ancestry suggests that the LG12 resistance allele may represent the same ancestral allele; in other words, the QTL may be identical by descent (IBD), inherited from a common origin. Thus, it is also highly plausible that the resistance QTL and the underlying genes of ‘Evereste’ will be broken down by Ea1189Δeop1. Interestingly, other accessions of *M. floribunda*, *M. baccata*, and *M. prunifolia* from our genebank amplified the same resistance alleles on LG12 (results not shown). The aforementioned species are speculated to contribute to the ancestry of the three LG12 fire blight resistance donors. Nevertheless, the question of the origin of the fire blight resistance mapped on LG12 of *Malus* remains.

In addition, we detected a year-dependent, strain-specific minor QTL on LG15 of Mf821 explaining 27.9% of phenotypic variation. This QTL could only be detected using phenotypic data from the initial inoculation with Ea1189Δeop1. Following the second inoculation with Ea1189Δeop1, we observed a loss of significance of the QTL, corresponding with the increased mean and median PLL observed, suggesting an instability of the locus. Nevertheless, individuals inheriting the 117 bp allele at the CH03b10 locus (highest *K* value) showed significantly less necrosis (16%) than individuals inheriting the 102 bp allele. In their study, Durel et al. (2009) also reported a second resistance locus on LG15 of ‘Evereste’ mapping nearest to the marker Hi04c05, in a population derived from ‘MM106 × ‘Evereste’. It is noteworthy that Durel et al. (2009) did not detect the LG15 QTL in Mf821 in the population derived from ‘Golden Delicious’ × Mf821. Our marker analysis with the markers mapping within the LG15 QTL region revealed that Mf821 and ‘Evereste’ do not share any common alleles in

the LG15 QTL region, suggesting that the two QTLs may be distinct.

In conclusion, our findings provide the first evidence of the breakdown of the fire blight resistance locus of Mf821 and its associated R-genes that suggest a gene-for-gene interaction between Mf821 and Eop1 effector of *E. amylovora*. We also reinforce the strain-specific nature of fire blight resistance and underscore the importance of challenging resistance donors with different mutant strains of the pathogen. Our results also support previous assumptions that the fire blight resistance QTL of the three donors could be the same, as they all share the same resistance haplotype, and their respective locus is overcome by the same effector mutant, at least for MAL0004 and Mf821. While the nature of the interaction between Eop1 and LG12 resistance genes is unclear, the effector’s sequence and structural similarity with Hopz3 and HopZ1a suggests a potential association with kinase resistance elements (Lewis et al. 2013; Ma and Ma 2016; Tomar et al. 2023). Fine mapping studies in ‘Evereste’ and MAL0004 showed homologous candidate genes encoding serine/threonine kinases, making such an interaction plausible and strongly suggesting that the same genes underly *FB_Mf821* (Parravicini et al. 2011; Emeriewen et al. 2021). Future work should therefore focus on direct interaction studies between Eop1 and the proposed candidate genes and the resistance proteins within the LG12 fire blight resistance regions to pinpoint the single R-gene or cluster of R-genes within the locus that controls LG12 fire blight resistance, and confirm the gene-for-gene interactions. When identified, such a gene(s) could be overexpressed in a susceptible apple background, with the transgenic lines subsequently challenged with the wild-type and Ea1189Δeop1 mutant strain to confirm the monogenic-resistance function of the gene. The clarification of *Malus* LG12 fire blight resistance will play an important role in achieving durable resistance via informed breeding decisions regarding donors for pyramiding crosses.

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Author contributions AP, OFE, HF, and TWW conceived the research. AP and TWW performed crosses to establish the F1 population. HZ inoculated and phenotyped the F1 population. TSM and OFE genotyped the F1 population, analysed the results, and created the genetic map. DS genotyped the donors with markers and analysed the results. OFE and AP supervised the study. TSM and OFE wrote the initial manuscript draft. All authors contributed to the revision and approved the final draft of the manuscript.

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Data availability The datasets used during the current study are presented in the manuscript. Data in other formats are available from the corresponding author upon reasonable request.

Declarations

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