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Pathogen Spotlight

Genetics and Genomics of Resistance

Quantitative Trait Locus Mapping for Fire Blight Resistance in an F_2 Population of *Malus fusca* MAL0045 Uncovers Novel Resistance Loci

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Abstract

Several fire blight resistance loci in *Malus* genotypes map on different linkage groups (LGs) representing chromosomes of the domesticated apple. Prior genetics studies primarily focused on F_1 populations. A strong resistance quantitative trait locus (QTL) explained up to 66% of phenotypic variance in an F_1 progeny derived from crossing the highly resistant wild apple genotype *Malus fusca* MAL0045 and the highly susceptible apple cultivar 'Idared', which was previously mapped on LG10 (*Mfu10*) of MAL0045. Strains of the causative bacterial pathogen *Erwinia amylovora*, notably those that show a single nucleotide polymorphism in the *avrRpt2*$_{EA}$ effector protein sequence at position 156 (e.g., Ea3049), are more virulent and overcome some known fire blight resistance donors and their QTLs. However, MAL0045 is resistant to Ea3049 and *Mfu10* is not overcome, but most of the F_1 progeny were highly susceptible to this strain. This phenomenon led to the assumption that other putative resistance factors not segregating in the F_1 progeny might be present in the genome of MAL0045. Here, we crossed F_1 progeny together to obtain 135 F_2 individuals. Facilitated by genotyping-by-sequencing and phenotypic assessments, we identified and mapped two novel resistance QTLs in these F_2 individuals on LGs 4 and 15, which were not identified in the F_1. To our knowledge, these are the first resistance QTLs mapped in F_2 progeny in *Malus*. In addition, we report that neither MAL0045 nor *Mfu10* is broken down by a highly aggressive U.S. strain, LA635, after analyses in the original F_1 individuals.

Keywords: *Erwinia amylovora*, LA635, MAL0045, *Mfu10*, *Mfu15*, *Mfu4*

The bacterial disease fire blight is caused by *Erwinia amylovora* (Burrill) Winslow et al. (1920), leading to huge economic losses in pome fruit orchards (Peil et al. 2021). Besides affecting the genus *Malus*, to which the domesticated apple belongs, the pathogen also affects other genera in the Rosaceae family of plants, for example, *Pyrus* (i.e., pears) (Peil et al. 2009). To cause fire blight, the pathogen deposits effector proteins into host cells using the type III secretion system (T3SS) aided by other chaperons (Oh and Beer 2005; Yuan et al. 2020). *E. amylovora* adopts biotrophic and necrotrophic lifestyles by invading living hosts and overwintering in dead tissues (Sobiczewski et al. 2017). Primary infection occurs in flowers, leading to blossom blight, which results in the reduction of crop yield (Peil et al. 2009). The migration of bacterial cells down to the shoot destroys the annual wood that bears the fruit spurs for the following season, with the possibility of tree death once cells reach large trunks or limbs of the tree. Rootstock infection can also lead to death of the tree (Norelli et al. 2003; Peil et al. 2009, 2019). Management of fire blight epidemics is difficult, as most apple cultivars available in the market are susceptible to fire blight (Peil et al. 2021). The use of antibiotics can mitigate the devastation of fire blight epidemics (Beckerman and Sundin 2016) especially against blossom blight (McManus et al. 2002) but not against shoot and rootstock phases of the disease (Johnson and Temple 2013). However, heavy dependency on antibiotics has led to the evolution of antibiotic-resistant strains of *E. amylovora* (McGhee et al. 2011; McManus et al. 2002; Russo et al. 2008). In Europe, phytosanitary measures including pruning of affected plant parts and complete destruction of affected trees are recommended, as the use of antibiotics is banned (Peil et al. 2021). In addition, fire blight was designated as a quarantine disease in Europe for many years but is now being treated as a regulated non-quarantine pest (Peil et al. 2021).

The future of fire blight epidemic prevention lies in the development and use of resistant cultivars. However, breeding of new resistant apple cultivars is challenging for many reasons, including the long juvenile phase of apple (Hanke et al. 2020) and the fact that strong fire blight resistance is found in wild apple genotypes with tiny and astringent fruits (Durel et al. 2009; Emeriewen et al. 2014, 2017a; Peil et al. 2007, 2019). Thus, their introgression will require several pseudo-backcrosses to eliminate linkage drag (Peil et al. 2021). Furthermore, an effective breeding strategy relies on the knowledge of *Malus* host−*E. amylovora* interactions, especially as it pertains to effector-triggered resistance/susceptibility (Emeriewen et al. 2019; Vogt et al. 2013). There are wild apple genotypes that are resistant to particular types of *E. amylovora* strains, which possess cysteine amino acid (C-allele) at position 156 in the *avrRpt2*$_{EA}$ effector protein sequence (Vogt et al. 2013), for example, *M.* ×*robusta* 5 (Mr5) (Peil et al. 2007), *M. floribunda* 821 and the ornamental cultivar 'Evereste' (Durel et al. 2009), *M. fusca* accession MAL0045, and *M.* ×*arnoldiana* accession MAL0004 (Emeriewen et al. 2014, 2021). However, *E. amylovora* strains, which do not possess cysteine but instead serine amino acid (S-allele) at the same position (Vogt et al. 2013), can overcome the resistance of the aforementioned genotypes (Peil et al. 2011; Wöhner et al. 2014, 2018) save MAL0045 (Emeriewen et al. 2015) and MAL0004 (Emeriewen et al. 2017a). In addition, *E. amylovora* mutant strains were shown to overcome the resistance of Mr5 (Vogt et al. 2013), *M. floribunda* 821, and 'Evereste' (Wöhner et al. 2018).

Nonetheless, biparental F_1 populations that segregate for fire blight resistance/susceptibility were established by crossing the

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aforementioned wild apple genotypes with different apple cultivars or rootstock cultivars, which facilitated quantitative trait locus (QTL) mapping of their respective resistance to different linkage groups (LGs). A strong fire blight resistance QTL explaining around 80% of phenotypic variance in an F_1 population of the apple cultivar 'Idared' × Mr5 cross is located on LG3 (Peil et al. 2007, 2019). Similarly, QTLs that map in close proximity at the distal end of LG12 were identified in F_1 populations established between rootstock cultivar 'MM106' × 'Evereste', as well as the apple cultivar 'Golden Delicious' × *M. floribunda* 821 (Durel et al. 2009). Emeriewen et al. (2017a) also reported a strong QTL in an F_1 progeny derived from a cross of 'Idared' × MAL0004 on LG12 (Emeriewen et al. 2017a). The LG12 QTLs all explained more than 50% of phenotypic variance in the respective F_1 populations. In the same vein, the fire blight resistance QTL of *M. fusca* MAL0045 was mapped on LG10 and explained up to 66% of the phenotypic variance in a MAL0045 × 'Idared' F_1 population (Emeriewen et al. 2014). It is noteworthy that whereas the aforementioned QTLs could be detected with *E. amylovora* strains that bear the C-allele (Vogt et al. 2013), only the fire blight resistance QTLs of *M. fusca* MAL0045 on LG10 and MAL0004 on LG12 have also been identified with a strain, Ea3049, which possesses the S-allele (Emeriewen et al. 2015, 2017a). Peil et al. (2011) reported that Ea3049 breaks down the Mr5 QTL on LG3, and Emeriewen et al. (2023a) showed that the Mr5 QTL on LG7 is not dependent on the cysteine/serine single nucleotide polymorphism (SNP). However, it has not been reported yet whether this particular strain or any other S-allele strain breaks down the QTLs of *M. floribunda* 821 and 'Evereste' on LG12.

MAL0045 is highly resistant to both C-allele (Ea222) and S-allele (Ea3049) strains of *E. amylovora*, with no disease symptoms observed with the former (Emeriewen et al. 2014) and only 1.5% disease severity observed with the latter strain (Emeriewen et al. 2015). Although the major QTL on LG10 of MAL0045 was still detected using Ea3049 (Emeriewen et al. 2015), a mean disease necrosis of 62.4% was observed for the F_1 progeny in comparison with 13.6% observed with Ea222 (Emeriewen et al. 2014) in three different years of trial. The strong resistance of MAL0045 but the high susceptibility of its progeny and the fact that *Mfu10* explains only 66% of the phenotypic variation led us to assume that there are other putative resistance factors in MAL0045, which are not segregating in the F_1 progeny. To test this hypothesis, we established F_2 progeny by crossing individuals of the F_1 progeny. These individuals were evaluated genotypically and phenotypically, which allowed for the identification of novel fire blight resistance QTLs on LG4 and LG15.

Materials and Methods

Plant materials and DNA extraction

We previously established F_1 individuals by crossing MAL0045 with 'Idared', leading to two subpopulations called 05210 (Emeriewen et al. 2014) and 09260 (Emeriewen et al. 2020). To establish the F_2 populations, we crossed F_1 individuals from the 05210 population as follows: 05210-046 × 05210-062, leading to 36 individuals designated as the 19213 population; and 05210-165 × 05210-062, leading to 99 individuals designated as the 19214 population. Although 05210-046 and 05210-062 express strong fire blight resistant phenotypes, 05210-165 is susceptible to the disease. Thus, the 19213 and 19214 F_2 individuals share a common parent in 05210-062 and common grandparents (Fig. 1).

DNA was extracted from the grandparents, parents, and F_2 individuals using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. DNA was thereafter quantified, diluted, and stored at −20°C until required for PCR amplification. In addition, young leaves collected from the F_2 population were lyophilized and sent to the company Data2Bio, LLC (Ames, Iowa, U.S.A.) for DNA extraction and genotyping-by-sequencing (GBS) analyses.

Phenotypic evaluation

The F_2 individuals were phenotyped with the C-allele strain Ea222 and the S-allele strain Ea3049. Artificial shoot inoculation and evaluation were performed as described by Emeriewen et al. (2018). Briefly, scions of each individual were grafted on rootstock M111 in the greenhouse. Replicates of each individual ranged from 6 to 10. Greenhouse conditions were 25 to 27°C (day), 20°C (night), and 85% humidity. Only plants that grew up to 25 cm were phenotyped by artificial inoculation. Inoculation was performed by cutting the two youngest leaves with a pair of scissors dipped into inoculum at a concentration of 10^9 CFU/ml. Twenty-eight days postinoculation (dpi), fire blight lesion length (cm) was measured and converted to percent lesion length (PLL) by dividing the necrotic shoot by the total shoot length and multiplying by 100. The parents and grandparents were included as controls.

Additionally, we phenotyped the 05210 and 09260 F_1 individuals with a highly aggressive U.S. strain, LA635, using the aforementioned protocol (Emeriewen et al. 2018).

GBS

GBS analysis was performed to identify de novo SNP markers for the 135 F_2 individuals. Performed by Data2Bio, LLC, the tunable-GBS technology (tGBS) procedure, which was conducted with the restriction enzyme Bsp1286I (Ott et al. 2017), was carried out on these individuals as previously descried for the F_1 individuals (Emeriewen et al. 2020). Samples were sequenced using the Illumina HiSeq X instrument, and the generated individual sequence reads were scanned for regions of low-quality sequence, defined as having a PHRED quality score of 15 or less. The SNP calling bioinformatics pipeline used is available at https://schnablelab.plantgenomics.iastate.edu/software/123SNP/ (Emeriewen et al. 2020). Quality trimmed reads were aligned to the *Malus domestica* GDDH13 v1.1 reference genome (Daccord et al. 2017) using GSNAP (Wu and Nacu 2010), and only reads that aligned uniquely were used for subsequent analyses.

Uniquely aligned reads were used for SNP discovery. Polymorphisms at potential SNP sites were used to determine putative homozygous and heterozygous SNPs using the criteria detailed in Emeriewen et al. (2020). However, in contrast to Emeriewen et al. (2020), where the generated SNPs were further filtered to define a specific minimum call rate of 70% (MCR70) across all samples, in the current study, SNPs were further filtered to define a specific minimum call rate of 50% across all samples (MCR50). The positions of the identified SNPs on the GDDH13 genome are included in their nomenclature.

Microsatellite application and development

Simple sequence repeats (SSRs) in the genetic map of the F_1 individuals (Emeriewen et al. 2014, 2020) were used to genotype the F_2 progeny. In addition, SSRs were developed from the GDDH13 reference sequence (Daccord et al. 2017) for linkage groups 4 and 15. SSR motifs within the region of interest were manually searched.

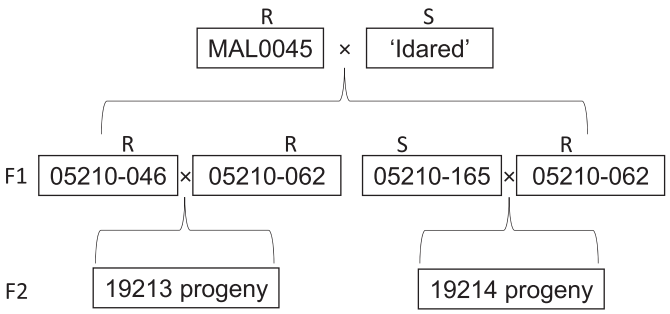


Fig. 1. Illustration of the pedigree of the F_2 individuals. R = resistant, S = susceptible.

Primer pairs flanking SSR motifs were designed using Primer3 version 0.4.0 (Rozen and Skaletsky 2000) and tested for polymorphism on the F_2 progeny by PCR. A polymorphism test was performed according to Schuelke (2000). The PCR protocol for polymorphism test and SSR genotyping for polymorphic SSRs and already mapped SSRs were the same, and as previously described (Emeriewen et al. 2014) using the Type-It kit (Qiagen) according to the manufacturer's protocol in a 10-µl volume. PCR conditions were 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. The PCR products were diluted 1:200, and 1 µl of the dilution was mixed with 8.95 µl of HiDi formamide (Applied Biosystems) and 0.05 µl of Liz 600 size standard (Applied Biosystems) in a total volume of 10 µl, denatured in a thermocycler at 94°C for 5 min and thereafter analyzed on an ABI 3500xL Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Darmstadt, Germany). Subsequently, the SSR fragments were analyzed using GeneMapper software version 6 (Thermo Fisher Scientific).

Construction of genetic linkage maps

Genetic maps of the susceptible parent, 05210-165, and the common resistant parent, 05210-062, were developed using the generated SNP and the SSR data for the 19214 population. Here, only SNP markers that are polymorphic in either or both of the F_2 parents, with fewer than five missing data, were used for mapping. Mapping was performed using the software JoinMap 4.0 (Van Ooijen 2006) at a logarithm of odds (LOD) threshold of 8.0 to 12.0. Linkage groups were created using the Kosambi function on JoinMap 4.0. Groups were assigned to the corresponding chromosomes already assigned to the SNPs following their unique alignment to the various chromosomes on the reference genome. The physical positions of the SNPs identified in the F_2 population on the GDDH13 genome are included in their nomenclature. The positions of the SSRs previously mapped on the genetic map of the F_1 individuals, and those specifically developed from certain chromosomes on the reference genome, were further used as quality control for the assignment of linkage groups. Comparisons of genetic maps was performed with MapChart software version 2.32 (Voorrips 2002).

Genotype-phenotype association and QTL (interval) mapping analyses

Marker data for the 99 F_2 individuals of the 19214 cross were combined with their phenotypic data to determine genotype-phenotype correlation by Kruskal-Wallis analysis, which is a non-parametric equivalent of the one-way analysis of variance, ranking all individuals in terms of their quantitative trait and classifying them according to their marker genotype. Kruskal-Wallis analyses and interval mapping were performed using MapQTL version 5 (Van Ooijen 2004). Permutation tests to determine the genome-wide or chromosome significance of detected QTLs were calculated automatically using the same software program. We used MapChart software (Voorrips 2002) to align the generated maps and QTLs. Similarly, we used the phenotypic data of the 05210 and 09260 F_1 individuals for LA635, as well as the map data from Emeriewen et al. (2020), to perform genotype-phenotype analyses and interval mapping with the F_1 map data (Emeriewen et al. 2020). QTL intervals were determined using the 1 LOD and 2 LOD interval as described in Voorrips (2002).

Statistical analysis

SAS (SAS Institute, Cary, NC, U.S.A.) GLIMMIX (generalized linear mixed model) analysis was performed to determine the significance of the respective effects of the detected loci using the phenotypic values (PLL) of each of the individuals as well as the alleles of the most significantly correlated molecular marker for each QTL. Pearson's correlation/linear regression of phenotypic data for Ea222 and Ea3049 evaluated was calculated using SAS Enterprise Guide 4.3.

Results

Crosses and phenotypic evaluation of the F_2 individuals

Thirty-six individuals originated from the resistant F_1 × resistant F_1 cross (19213), whereas 99 individuals originated from the susceptible F_1 × resistant F_1 cross (19214). Therefore, both crosses, which had the resistant F_1, 05210-062, as a common male parent led to 135 F_2 individuals in total. The individuals were inoculated with Ea222 and Ea3049, which differ in aggressiveness/virulence because of the C156S SNP in the bacterial effector avrRpt2$_{EA}$ (Vogt et al. 2013). Due to the grafted scion growth rate, it was possible to phenotype 31 individuals of the 19213 cross and 92 individuals of 19214 cross—123 in total, with Ea222. Similarly, 33 and 87 individuals respectively from both crosses, 120 in total, were phenotyped with Ea3049. The results showed that although the mean PLL of the 123 individuals inoculated with Ea222 was 22%, the mean PLL of the 120 individuals inoculated with Ea3049 was 46%. A PLL of less than 1% was observed for 31 individuals with Ea222 in comparison with only 13 individuals with Ea3049. Figure 2A shows the distribution frequency of the inoculated individuals for the two different strains. The correlation and simple linear regression calculated for 115 individuals, which possessed phenotypic data for both strains, is presented in Figure 2B and shows a correlation (multiple r) of 0.75 and a coefficient of determination (r^2) of 0.56. The regression statistics are shown in Supplementary File S1. The results of the F_2 parents agreed with previous results, as the resistant parents, 05210-046 and 05210-062, showed zero disease symptoms for both strains, whereas PLLs of 28 and 62% were recorded with Ea222 and Ea3049, respectively, for the susceptible parent, 05210-165.

In addition, the phenotypic results of the F_1 individual (i.e., 05210 and 09260) crosses with LA635 were similar to what was reported in Emeriewen et al. (2015) for the highly aggressive Canadian strain Ea3049. Of 107 F_1 individuals inoculated with LA635, 68 individuals recorded above 50% lesion length, with 62.7% calculated as the overall mean lesion length. Although a mean PLL of 4.5% was calculated for MAL0045 with LA635, 100% was calculated for all replicates of the susceptible parent 'Idared'. The distribution frequency of PLL of the F_1 individuals with LA635 is presented in Supplementary Figure S1.

tGBS SNP identification

From the 05210-046 × 05210-062 F_2 individuals, the total SNPs identified were 172,935. Upon the application of more stringent criteria (e.g., minimum percentage of call data, minimum and/or maximum heterozygosity rates, and/or minimum minor allele frequencies) and minimum call rate (MCR) per SNP of 50% or less, the final number reduced to 112,343 SNPs (termed MCR50 SNPs). For the 05210-165 × 05210-062 F_2 individuals, the total SNPs identified were 162,709, which reduced to 93,000 MCR50 SNPs following stringent criteria application. The physical positions of the SNPs identified in the F_2 population on the GDDH13 genome are included in their nomenclature.

Microsatellite application

Thirty-six SSR markers, which are located on different linkage groups in the *M. fusca* MAL0045 genetic map, were tested on the F_2 individuals. Of these, 30 SSRs were polymorphic in the F_2 individuals. In addition, following preliminary mapping with only SNP data, LG4 and LG15 were identified as potentially harboring fire blight resistance QTLs in the F_2 population. Therefore, we used the positions of SNP markers that are linked to the potential loci to determine the corresponding positions on the respective chromosomes in the GDDH13 genome (Daccord et al. 2017). For LG4, the region of interest was between 22 and 33 Mbp, whereas for LG15, the region was between 40 and 47 Mbp. Twelve primer pairs surrounding SSR motifs were designed for each region of interest. Of these, nine SSRs were polymorphic in the F_2 individuals—five

on LG4 and four on LG15. Table 1 shows the polymorphic SSR markers and the allele sizes.

Marker data analyses and genetic mapping

We applied criteria that are more stringent for the analyses of SNP data prior to mapping. SNPs with more than one missing data point in the F_2 individuals were excluded from mapping analyses. Furthermore, only SNPs heterozygous in one or both F_2 parents can be used for mapping; hence, homozygous SNPs were also discarded. As a result, 1,975 SNPs and 39 SSRs were used to create the genetic map of 05210-062, with 2,014 markers in total (Supplementary File S2). Similarly, 1,977 markers comprising 1,940 SNPs and 37 SSRs were uploaded onto JoinMap for the creation of the genetic map of 05210-165 (Supplementary File S3). Of the 2,014 and 1,977 markers employed for mapping the respective genetic maps of 05210-062 and 05210-165, the majority (i.e., 1,831 markers) were polymorphic in both F_2 parents. It was not logical to create a genetic map of the other resistant F_2 parent (i.e., 05210-046). Because 19213 consists of only 36 individuals, no genetic map was created for parent 05210-046. Instead, these 36 individuals were added to the 99 individuals of the 19214 cross for the creation of an integrated map of their common parent, 05210-062, using markers polymorphic in this particular parent. A total of 1,505 markers comprising 9 SSRs and 1,496 SNPs were employed for the integrated map (data not shown).

Table 2 shows the characteristics of the genetic maps of the respective F_2 parents for the 19214 population. Mapping analyses excluded ungrouped and identical loci; hence, the final parental map of 05210-165 comprised 1,055 markers in a total length of 1,096.9 cM spread across 17 linkage groups. The linkage groups with the highest and least number of markers were LG15 and LG5, respectively. For the parental map of 05210-062, the total number of mapped markers was 1,070 with a total length of 1,130.6 cM. In addition, LG9 and LG13 possessed the highest and least number of markers, respectively. Two groups, LG12 and LG16, were the same for the parental maps of 05210-165 and 05210-062. The order of the SNP markers in the maps mostly corresponded with their physical position in the genome. Furthermore, the positions of the SSRs, which served as anchor markers and quality control, were in agreement with their previously published groups (Emeriewen et al. 2020). However, no SSR marker mapped on LG1, LG2, and LG5.

Genotype-phenotype association analyses and interval mapping

We used the respective F_2 parental map data for the 19214 population and the phenotypic data of these F_2 individuals for genotype-phenotype association (Kruskal-Wallis) and QTL analyses (interval mapping). Kruskal-Wallis analyses showed that markers on LG10, LG4, and LG15 correlated significantly with Ea222 phenotypic data of the F_2 individuals. Markers on LG10 showed

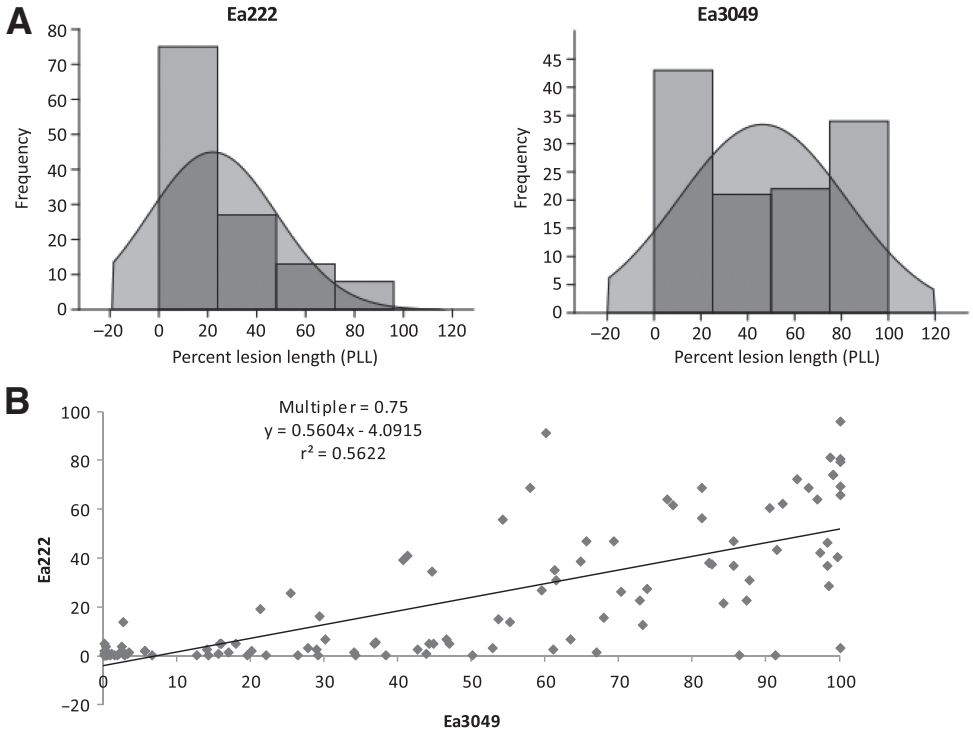


Fig. 2. A, Distribution frequency of phenotypic evaluation of the F_2 individuals with Ea222 and Ea3049 and **B,** the correlation/regression analyses between both phenotypic evaluations. Ea222 possesses the C-allele of the avrRpt2$_{EA}$ at position 156 of the amino acid sequence, and Ea3049 possesses the S-allele at the same position. Thus, both strains differ in their virulence on the F_2 individuals.

TABLE 1. Simple sequence repeat (SSR) markers developed from chromosomes 4 and 15 on the GDDH13 genome including the allele sizes of the three generations

SSR	Forward primer	Reverse primer	Allele sizes (bp)[a]				
			Malus fusca	Idared	05210-046	05210-062	05210-165
Chr4_R5_24/24M5	GAAACTCGTGCCTCCTCTCT	GACGACGATGGACATTGCTC	216, 218	214, 220	214, 218	216, 220	218, 220
Chr4_R13_27/27M1	CGGCCATGTTATTCCTTCCC	TCTAAGTAGCTGATCCGCCA	225	259, 265	225, 259	225, 266	225, 266
Chr4_R13_27/27M2	CTTAACCCACCCAACGTTCG	AGCCGGAGAGAAACAACAGA	231	211, 233	231, 233	211	211
Chr4_R16_28/28M3	GCGACATGGTTCGTTTCATT	CCGTCCCATATTTGACATCC	171, 177	160, 179	177, 179	160, 171	160, 177
Chr4_R25_32/32M5	GAGAAAAGTGGTGCCGCTAC	ACGCCTTGCTAAAAGTTCCA	195	190, 214	190, 195	195, 214	195, 214
Chr15_R40/40Ma	GCTTATGGACGAGTACAGCC	CTAATTGAACTCTCGCGGCC	154	166, 230	154, 166	154, 166	154, 166
Chr15_R41/41Ma	AACAACAGGAATGTGGGTGC	GGGAGGAGGCTCTATGTCAC	177	181, 191	177, 191	177, 191	177, 191
Chr15_R447Ma	CTCTCAGTTCCGCGTTTTGA	GCCCTGCCAACAAAACAAAC	177, 191	208	177, 208	191, 208	191, 208
Chr15_R45M	CGGCTTTCTAGTTCGAACGG	CGCATACCTGTACATCCCGT	225	236, 238	225, 236	225, 236	225, 236

[a] Allele sizes are according to Schuelke (2000).

the strongest correlation, followed by those on LG4 and then LG15. Three QTLs of significant LOD scores, each on the aforementioned groups, were detected with Ea222. Table 3 shows the characteristics of the identified QTLs. Of the three QTLs detected with Ea222, only the QTLs on LG10 and LG4 exceeded the genome-wide threshold of an LOD of 3.9 and their respective chromosome-wide thresholds (3.3 and 3.2, respectively) to be significant at a P value of 0.05 (Table 3). The QTL on LG15 only reached the chromosome-wide threshold for a significance level of $P = 0.05$. Whereas the QTL on LG10 explained 60.6% of the phenotypic variance in the 19214 F_2 individuals, the QTLs on LG4 and LG 15 explained 21.9 and 15% of the phenotypic variance, respectively, at a significance level of $P = 0.05$. Figure 3 shows the regions of the linkage groups harboring the identified QTLs.

Furthermore, whereas the markers on LG10 maintained a strong correlation with Ea3049 phenotypic data, the significance of the markers on LG4 and LG15 reduced drastically. Thus, only the QTL on LG10 retained significance on both genome-wide and chromosome levels of $P = 0.05$ and explained 61.8% of the phenotypic variance (Table 3). The marker with the highest LOD score changed with Ea3049; however, the region of the QTL remained the same (Fig. 3). An effect on LG4 with Ea3049 was found, although it did not rise to the significance level of $P = 0.05$. No effect was found on LG15 with Ea3049. Table 4 shows the mean PLL of individuals of the various segregation types for markers with the highest LOD scores for both strains.

Furthermore, we used the MAL0045 F_1 map data (Emeriewen et al. 2020) and the phenotypic data of the F_1 individuals with LA635 for marker-phenotype analysis and interval mapping. With this strain, only markers on LG10 correlated significantly with fire blight resistance in MAL0045 F_1 individuals. A QTL with an LOD of 21, well above the genome-wide and chromosome-wide significance, was detected on LG10 explaining 63.2% of the phenotypic variance (data not shown). The QTL, which maps below the SSR CH03d11, is in the same region in the genetic map of MAL0045 F_1 individuals as reported for previous strains (Emeriewen et al. 2014, 2015, 2020). A comparison of the QTL regions on LG10 of MAL0045 (i.e., F_1 genetic map) (Emeriewen et al. 2020) and 05210-062 (i.e., F_2 genetic map with 19214) is presented in Figure 4.

TABLE 2. Characteristics of the parental linkage maps created for the F_2 individuals using the 19214 population

	05210-165		05210-062	
Linkage group (LG)	Length (cM)	Number of markers	Length (cM)	Number of markers
LG1	55.758	64	58.372	63
LG2	64.764	53	57.932	52
LG3	82.247	71	78.437	75
LG4	66.871	77	66.342	76
LG5	58.981	42	63.730	55
LG6	52.444	44	54.823	50
LG7	80.017	73	70.177	80
LG8	50.709	48	67.431	42
LG9	53.046	75	52.510	83
LG10	72.025	71	75.520	75
LG11	73.509	62	81.069	56
LG12	53.155[a]	55[a]	53.155[a]	55[a]
LG13	63.038	44	57.487	41
LG14	53.244	55	70.172	59
LG15	84.286	88	88.621	81
LG16	65.607[a]	67[a]	65.607[a]	67[a]
LG17	67.216	66	69.189	60
Total	1,096.917	1,055	1,130.574	1,070

[a] Maps were the same for both 05210-165 and 05210-062 parental maps.

TABLE 3. Summary of quantitative trait locus (QTL) characteristics identified on linkage groups (LGs) 10, 4, and 15 on the 05210-062 parental map with the 19214 population

Strain and QTL parameters[a]	LG10	LG4	LG15
Ea222			
GW-LOD threshold	3.9	3.9	3.9
Chr-LOD threshold	3.3	3.2	3.1
Marker	Chr10_33337254	Chr04_26882435	Chr15_R447Ma
LOD	18.59	4.9	3.3
% Explained	60.6%	21.9%	15%
Map position	53.65 cM	47.45 cM	75.24 cM
Ea3049			
GW-LOD threshold	3.6	3.6	3.6
Chr-LOD threshold	3.3	3.1	3.1
Marker	Chr10_33882294	Chr04_30516210	–
LOD	18.10	2.26[b]	–
% Explained	61.8%	11.9%	–
Map position	55.81 cM	58.12 cM	–

[a] GW-LOD = genome-wide logarithm of odds, Chr-LOD = chromosome-wide logarithm of odds, and % Explained = phenotypic variation explained.
[b] Not significant at $P = 0.05$.

Discussion

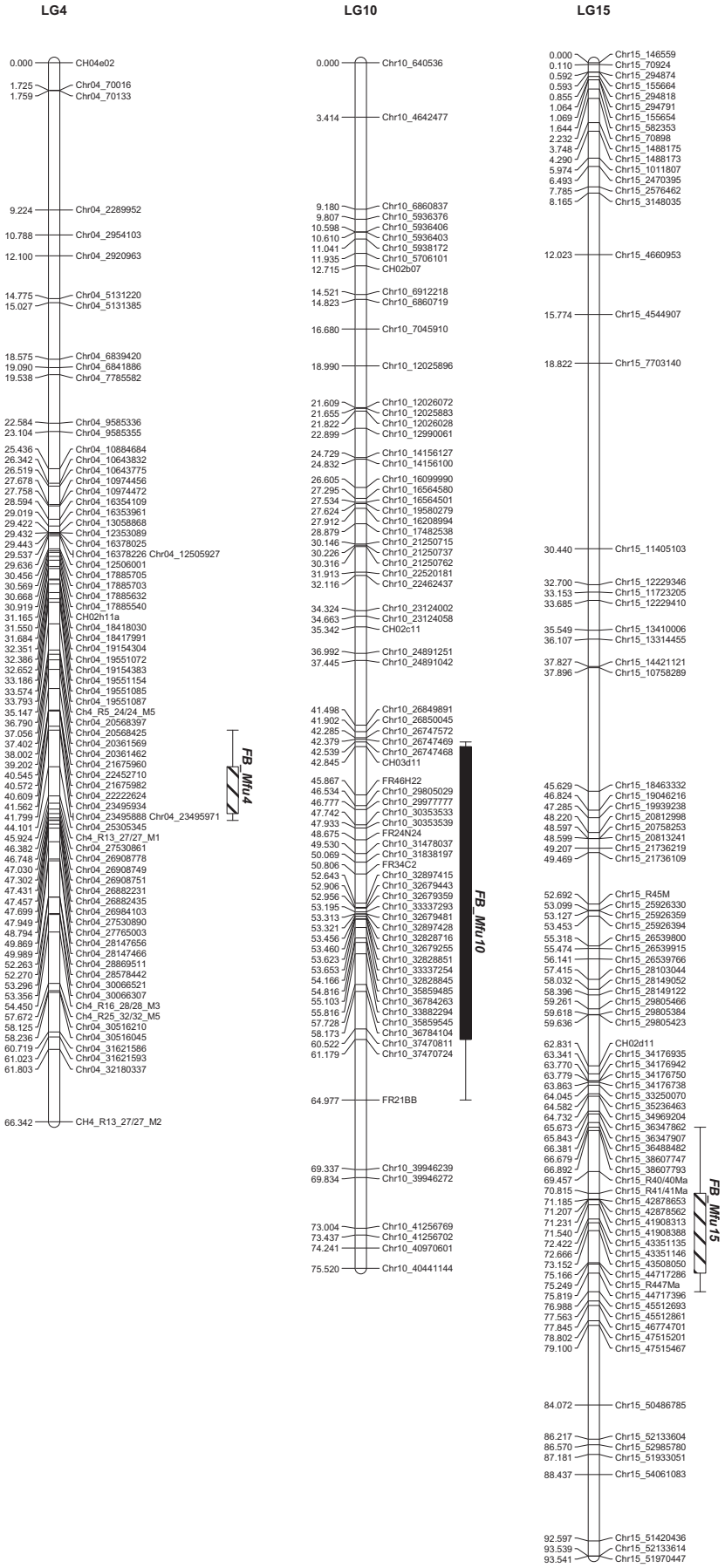
Fire blight resistance in *Malus* is strain-dependent, resulting in the breakdown of identified resistance donors by highly aggressive strains of the pathogen (Emeriewen et al. 2019; Peil et al. 2011; Vogt et al. 2013). Previously, only one major QTL that explained 66% of the phenotypic variance in the F_1 progeny of MAL0045 was mapped on LG10 (*Mfu10*) following phenotypic analysis with a C-allele strain, Ea222 (Emeriewen et al. 2014). *Mfu10* explained 41% of phenotypic variance with an S-allele strain, Ea3049, in the same F_1 progeny (Emeriewen et al. 2015). This S-allele strain is responsible for the breakdown of known resistance donors (Vogt et al. 2013; Wöhner et al. 2018). Although MAL0045 itself and *Mfu10* were not broken down by Ea3049, it caused a dramatic increase of the percentage of disease necrosis in the F_1 progeny of MAL0045. Thus, we assumed that other resistance factors in the genome of MAL0045 must contribute to its fire blight resistance and that such factors are not segregating in the F_1 individuals. Ea3049 was comparatively more aggressive on the F_2 individuals than Ea222 in this study, in a similar trend also observed in the F_1 individuals (Emeriewen et al. 2014, 2015). This aggressiveness is attributed to the SNP at position 156 of the amino acid sequence of the avrRpt2$_{EA}$ effector (Vogt et al. 2013). Nevertheless, it is clear that the QTL on LG10 of the genetic map of the F_2 individuals in the current study is *Mfu10*, inherited from MAL0045 and previously identified in the

TABLE 4. Mean percent lesion length (PLL) of individuals of the various segregation types for markers with the highest logarithm of odds (LOD) score of the quantitative trait loci (QTLs)

Strain	Marker with highest LOD score	Mean PLL of allele categories[a]
Ea222	Chr10_33337254	$nn = 47.0\%$
		$np = 4.5\%$
	Chr04_26882435	$hh = 51.2\%$
		$hk = 19.7\%$
		$kk = 16.8\%$
	Chr15_R447Ma	$hh = 32.4\%$
		$hk = 17.4\%$
		$kk = 40.9\%$
Ea3049	Chr10_33882294	$hh = 77.0\%$
		$hk = 53.9\%$
		$kk = 16.7\%$
	Chr04_30516210	$hh = 45.3\%$
		$hk = 44.9\%$
		$kk = 73.5\%$

[a] $nn \times np$ segregation = marker heterozygous in the paternal parent; $hk \times hk$ segregation = marker heterozygous in both parents (two alleles).

Fig. 3. Positions of quantitative trait loci (QTLs) on linkage groups (LGs) 4, 10, and 15 in F_2 individuals of MAL0045 phenotyped with Ea222. QTLs are shown in the parental map of 05210-062 with the 19214 population. The black bar represents the *FB_Mfu10* interval on LG10 (same region for Ea222 and Ea3049), and the interval of the newly detected QTLs is represented by the diagonally striped bars. The physical positions of the F_2 single nucleotide polymorphisms on the GDDH13 genome are included in their nomenclature (right of the linkage map). Values on the left side of the linkage map are genetic distances (cM).



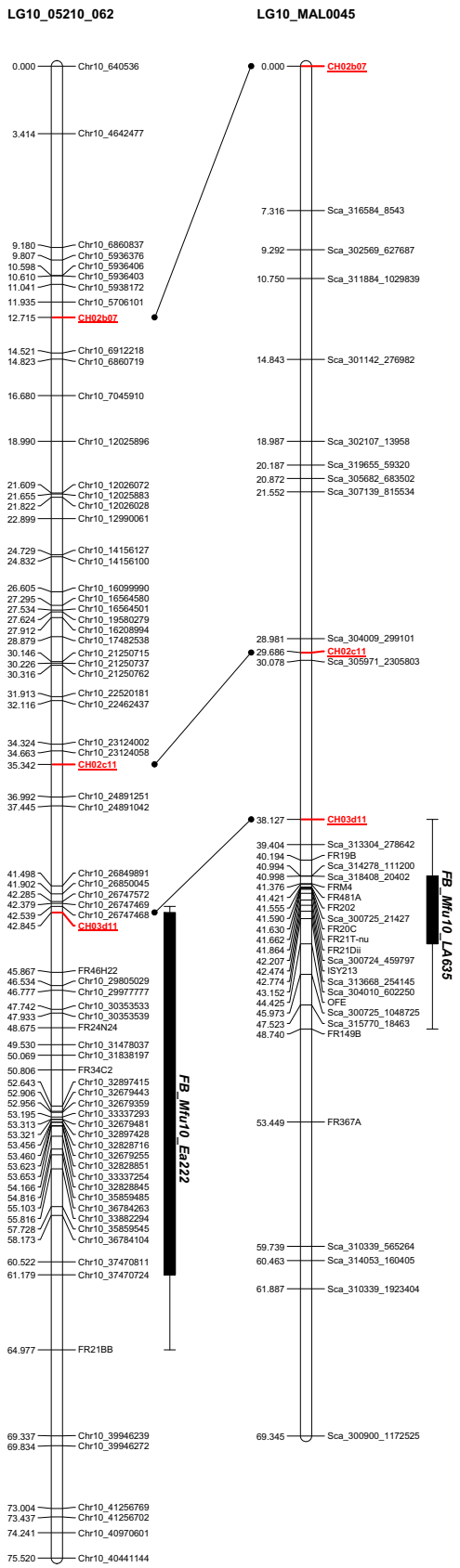


Fig. 4. Comparison of the positions of quantitative trait loci (QTLs) on LG10 in the F_1 and F_2 genetic maps show that *FB_Mfu10* is situated below the simple sequence repeat (SSR) marker CH03d11 in the MAL0045 genome. SSR markers shared by both maps are shown in red. QTLs are shown in the MAL0045 genetic map (Emeriewen et al. 2020) and the genetic map of 05210-062 for 19214 population developed in this study.

F_1 individuals (Emeriewen et al. 2014). The position of *FB_Mfu10* in both the F_1 and F_2 genetic maps is in agreement with the physical position on the GDDH13 genome reported by Emeriewen et al. (2018). Interestingly, the QTL retained a strong LOD value with Ea3049, and the difference in PLL between F_2 individuals inheriting the resistance allele and susceptible allele was 60% (Table 4), in stark contrast to the F_1 individuals, where this difference was just 36% (Emeriewen et al. 2015). These results confirm that the major QTL on LG10 is the result of a dominant resistance gene (Emeriewen et al. 2018, 2022), and it is not broken down by mutations in the avrRpt2$_{EA}$ effector (Emeriewen et al. 2017b, 2023b). Furthermore, Wöhner et al. (2018) reported 14.1% disease necrosis on MAL0045 with the S-allele strain LA635, leading to the suspicion that this strain could be more virulent on MAL0045 and *Mfu10*. However, the current study indicates that MAL0045 was only minimally affected, with 4.5% disease necrosis, and the strong effect of *Mfu10* with this strain dispels such suspicion, thus confirming the strong fire blight resistance of this wild apple species.

The other two QTLs identified on LGs 4 and 15 in this study are novel, confirming our assumption that other contributory resistance factors are present in the genome of MAL0045, which did not segregate in the F_1 progeny, presumably due to their homozygosity. Furthermore, *Mfu4* and *Mfu15* explained 21.9 and 15% of the phenotypic variance, respectively, among these F_2 individuals with Ea222, thus making them less effective in comparison with *Mfu10*, which explained 60.6%. Nonetheless, individuals with the homozygous *hh* allele for the marker with the highest LOD for *Mfu4* possessed 34% less mean disease necrosis than individuals with the homozygous *kk* allele at this locus. In the case of *Mfu15*, individuals with the heterozygous *hk* allele for the marker with the highest LOD possessed 23% less mean disease necrosis than individuals with the homozygous *kk* allele. Although the significance level of *Mfu4* with Ea3049 reduced, 28% mean disease necrosis was the difference between individuals with the homozygous *kk* allele and those with the homozygous *hh* allele of the marker with the highest LOD score at this locus for this strain. Taken together, these effects are significant for determining and selecting fire blight resistant individuals using molecular markers linked to these loci.

To date, no fire blight resistance QTLs have been reported on LG4 (chromosome 4) of *Malus*. However, there are reports of two fire blight QTLs on LG15 (Durel et al. 2009; Khan et al. 2013). Durel et al. (2009) reported a fire blight QTL on LG15 of the ornamental cultivar 'Evereste' (E15) in an F_1 progeny derived from a cross with the rootstock 'MM106'. E15 explained up to 6.9% of the phenotypic variance in this population (Durel et al. 2009). Similarly, in a 'Co-op 16' × 'Co-op 17' population, Khan et al. (2013) reported a QTL for fire blight resistance on LG15 that explained 17.4% of the phenotypic variation. Whereas the position of the QTL in the 'Co-op 16' × 'Co-op 17' map could not be determined, the QTL on LG15 of 'Evereste' is located close to the SSR marker Hi04c05 (Durel et al. 2009). The map of LG15 in the current study does not have any common marker with that of E15. However, *Mfu15* maps below the apple SSR marker CH02d11 in the lower part of LG15. Conversely, the closest marker to E15, Hi04c05, maps 24 cM above CH02d11 in the genetic map of the apple cultivar 'Discovery' (Liebhard et al. 2002). This suggests that *Mfu15* and E15 are different.

In conclusion, to our knowledge, this is the first report of mapping fire blight resistance loci using the F_2 progeny of a strong resistance donor. This is an important strategy to map resistance factors, which do not segregate in the F_1 progeny. Here, we elucidated the fire blight resistance inherited from the crabapple, *M. fusca*, confirmed the major locus *FB_Mfu10*, and mapped two novel strain-specific loci on LGs 4 and 15. It is worthwhile to select individuals carrying a combination of the favorable alleles of the fire blight resistance loci of MAL0045 in pre-breeding programs. For this, tightly linked SSR markers for these QTLs were developed and are available for application. Finally, the results presented herein support the role of MAL0045 toward achieving durable fire blight resistance

in *Malus* by pyramiding with other resistance donors, which are already overcome by more aggressive strains of the pathogen.

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