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Fire blight resistance of the wild apple species *Malus fusca*

Dissertationen aus dem Julius Kühn-Institut

Julius Kühn-Institut
Bundesforschungsinstitut für Kulturpflanzen

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Fire blight resistance of the wild apple species *Malus fusca*

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XXVII CYCLE

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Dedicated to my lovely wife, Oluwaseun Atinuke Emeriewen; my parents, Rev Bernard Mevayabore and Kate Isioma Emeriewen; and my parents- in-law; Rev Bankole and Adefolake Ojumu

'Although the fig tree shall not blossom, neither shall fruits be in the vines…Yet I will rejoice in the LORD…The LORD God is my strength….' Habakkuk 3:17-19

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Abstract

Erwinia amylovora is the pathogen responsible for inciting fire blight - the most dreaded bacterial disease of apple (*Malus* × *domestica*) and other members of the Rosaceae family. The disease is very destructive as is difficult to control. Even though fire blight was first observed over two centuries ago in America, no sustainable control measure is known till date. Disease management practices such as pruning of affected tissues as well as the application of copper and antibiotics, for example, streptomycin, are to minimize the population of *E. amylovora* in an orchard. However, the use of streptomycin is not allowed in many European countries as a consequence of its environmental risks and the issue of raising antibiotic resistant *E. amylovora* populations. Therefore, natural resistance is thought to be the most sustainable approach to manage fire blight. Genetic resistance has been investigated in *Malus* leading to the detection of several quantitative trait loci (QTLs) in apple cultivars and apple wild species accessions. Nevertheless, only one functionally characterized fire blight resistance gene has been isolated till date. This situation, coupled with the proof that resistance is strain specific, reinforces the need to detect more donors that could be used to establish durable resistance against fire blight. For this reason, the works described in this thesis aimed at investigating fire blight resistance in another apple wild species – *Malus fusca*. Different accessions of *M. fusca* were phenotyped in JKI, Dresden, Germany to ascertain the accession with a high resistance level. Accession MAL0045, found as having a very high resistance level was then crossed with the very susceptible apple cultivar 'Idared' to establish a segregating F1 population of 134 individuals. To facilitate the development of a genetic map of *M. fusca*, molecular markers such as DArT (Diversity Arrays Technology), SNPs (Single Nucleotide Polymorphisms) and SSRs (Simple Sequence Repeats) were developed, sourced, tested and polymorphic ones applied to the 134 individuals and then mapped. The phenotypic and genotypic data were employed for QTL analyses which resulted in the identification of a major quantitative trait locus which is located on linkage group 10 (LG10) of the apple genome and could explain about 66 % of the phenotypic variation; the second highest effect of all QTLs previously detected in *Malus*. Furthermore, this thesis also describes the stability and validation of the *M. fusca* fire blight resistance locus (*Mfu10*) after another phenotypic evaluation of the F1 population with a highly virulent *E. amylovora* isolate Ea3049 originating from Canada. Moreover, the fine mapping of the resistance region was undertaken via chromosome walking approach with the development of closely linked SSR markers suitable for marker assisted selection (MAS). For this purpose, the population was substantially increased to 1,336 individuals from an additional cross of *M. fusca* × 'Idared' and a reciprocal cross of 'Idared' × *M. fusca*. Genotyping of the whole population allowed for the identification of individuals showing recombination events within the interval of the QTL region. Phenotyping of recombinant individuals ensured that the exact position of the QTL was well defined. The first steps towards uncovering the underlying gene(s) responsible for the resistance of fire blight in *M. fusca* have been achieved with the development and screening of a *M. fusca* bacterial artificial chromosome (BAC) library with SSR markers closely linked to *Mfu10* and the identification of some BAC clones in the QTL interval. This is the first report of a major quantitative trait locus for resistance to fire blight in this wild relative of apple. The implications of the results obtained in these research works in respect to breeding for resistance against the very destructive fire blight disease of *Malus* are discussed extensively.

Riassunto

E*rwinia amylovora* è l'agente patogeno responsabile del colpo di fuoco batterico - la malattia batterica più temuta in melo (*Malus* × *domestica*) e dagli altri membri della famiglia delle Rosaceae. La malattia è molto distruttiva è difficile da controllare. Anche se il colpo di fuoco è stato osservato per la prima volta oltre due secoli fa in America, nessuna misura di controllo sostenibile è nota fino ad ora. Le pratiche di gestione delle malattie come la potatura dei tessuti colpiti, nonché l'applicazione di rame e antibiotici (per esempio, streptomicina) servono a minimizzare la popolazione di *E. amylovora* in un frutteto. Tuttavia, l'uso della streptomicina non è consentito in molti paesi europei come conseguenza dei suoi rischi ambientali e per il problema dell'incremento di resistenza nelle popolazioni di *E. amylovora* agli antibiotici. Pertanto, la resistenza naturale è pensata come l'approccio più sostenibile per gestire il colpo di fuoco. La resistenza genetica è stata studiata in *Malus* ha portato alla individuazione di diversi loci di caratteri quantitativi (QTL) in cultivar di melo e specie selvatiche. Tuttavia, solo un gene di resistenza è stato caratterizzato dal punto di vista funzionale ed isolato fino ad oggi. Questa situazione, insieme con il fatto che la resistenza è ceppo specifica, rafforza la necessità di individuare più donatori che potrebbero essere utilizzati per stabilire la resistenza durevole contro il fuoco batterico. Per questo motivo, i lavori descritti in questa tesi sono rivolti allo studio della resistenza al colpo di fuoco batterico in altre specie selvatiche di melo - *Malus fusca*. Diverse accessioni di *M. fusca* sono state fenotipizzate in JKI, Dresda, Germania per accertare l'accessione con un alto livello di resistenza. L'accessione MAL0045, la quale mostra un livello molto elevato di resistenza, è stata incrociata con la cultivar di melo molto sensibile 'Idared' ed è stata creata una popolazione F1 segregante di 134 individui. Per facilitare lo sviluppo di una mappa genetica di *M. fusca*, marcatori molecolari, come DArT (Diversity Array Technology), SNPs (Single Nucleotide Polymorphism) e SSR (Simple Sequence Repeats) sono stati sviluppati, testati e quelli polimorfici applicati al 134 individui e quindi mappati. I dati fenotipici e genotipici sono stati impiegati per le analisi QTL essi hanno portato alla individuazione di un importante locus quantitativo che si trova sul gruppo linkage 10 (LG10) del genoma del melo e potrebbe spiegare circa il 66% della variazione fenotipica; il secondo effetto più alto di tutti i QTL in precedenza rilevati in *Malus*. Inoltre, questa tesi descrive anche la stabilità e la convalida del locus (*Mfu10*) in *M. fusca* per la resistenza al fuoco batterico dopo un'altra valutazione fenotipica della popolazione F1 con un ceppo altamente virulento di *E. amylovora* Ea3049 proveniente dal Canada. Inoltre, la mappatura fine della regione di resistenza è stata effettuata tramite approccio "chromosome walking" con lo sviluppo di marcatori SSR strettamente connessi adatti alla selezione assistita da marcatori (MAS). A tal fine, la popolazione è sostanzialmente aumentata a 1.336 individui da un'incrocio supplementare di *M. fusca* × 'Idared' e un'incrocio reciproco di 'Idared' × *M. fusca*. La genotipizzazione di tutta la popolazione ha permesso l'identificazione di individui che mostrano eventi di ricombinazione entro l'intervallo della regione QTL. La fenotipizzazione di individui ricombinanti ha permesso di confermare che la posizione esatta del QTL era ben definita. I primi passi verso la scoperta del gene (geni) responsabile per la resistenza del colpo di fuoco batterico in *M. fusca* sono stati raggiunti con lo sviluppo e lo screening di una collezione di cromosomi batterici artificiali (BAC) di *M. fusca* con marcatori SSR strettamente connessi a *Mfu10*, portando all'individuazione di alcuni cloni BAC nell'intervallo QTL. Questo è il primo risultato riportato che individua un locus quantitativo per la resistenza al colpo di fuoco batterico in questi parenti selvatici di melo. Le implicazioni dei risultati ottenuti in questo lavoro di ricerca in materia di incroci per la resistenza contro il colpo di fuoco batterico (malattia molto distruttiva di *Malus*) verranno discussi ampiamente.

Chapter One

General Introduction

Origin and economic importance of apple (*Malus* × *domestica* Borkh.)

The domesticated apple (*Malus* × *domestica* Borkh.) is one of the most important and widely cultivated perennial fruit crops of the cold and temperate parts of the world (Zohary and Hopf, 2000). The apple tree belongs to the genus *Malus* in the subfamily Spiraeoideae of Rosaceae family. Other genera, for example, *Pyrus*, *Cotoneaster*, and *Pyracantha* also belong to this family (Potter *et al*. 2002; 2007). The genus *Malus* comprises about 55 species, however; between eight and 79 species have been recognized (Harris *et al.* 2002). The origin of apple is believed to be in the Tien Shan Mountains in central Asia (between Kazakhstan and China), where its wild ancestor believed to be *Malus sieversii* still grows till date, with humans and animals acting as distribution vehicles which carried it to regions of the Middle East, Europe and North America (USDA). The assumption that *M. sieversii* and its close relatives were the progenitors of the domesticated apple according to Vavilov (1926) is because they produce similar fruits to the domesticated apple. Molecular analysis later backed up this claim leading to concrete suggestions that *M. sieversii* is, in fact, the progenitor of the domesticated apple (Robinson *et al*. 2001; Harris *et al*. 2002).

Apple production amongst other economically important fruit crops worldwide is ranked fourth behind *Citrus*, grape and banana. The biggest global markets for fresh apple fruits are China, the European Union, and the United States. World Apple Report (2009) first indicated the production of apple exceeding 60 million tons. The World apple production for 2011/2012 was then estimated to be 65.23 million tons. This was a 4 % increase from 2010 (USDA). China produces more than half of all apples around the globe with 35 million tons of global production. Other countries with relatively large apple production include Russia, Turkey, Chile, India and Iran. The top 10 apple producing countries is depicted in a map shown in Figure 1.1. Some apple cultivars well known in the global market include ‘Golden Delicious’, ‘Delicious’, ‘Cox’s Orange Pippin’, ‘Rome Beauty’, ‘Granny Smith’, ‘McIntosh’, ‘Jonathan’,

'Fuji' and 'Gala' (Janick *et al*. 1996; Gardiner *et al*. 2007). Apple fruits could be consumed fresh or processed into juices, sauces, slices and even cider beer. A species of *Malus* known as crab apples are cultivated for ornamental purposes.

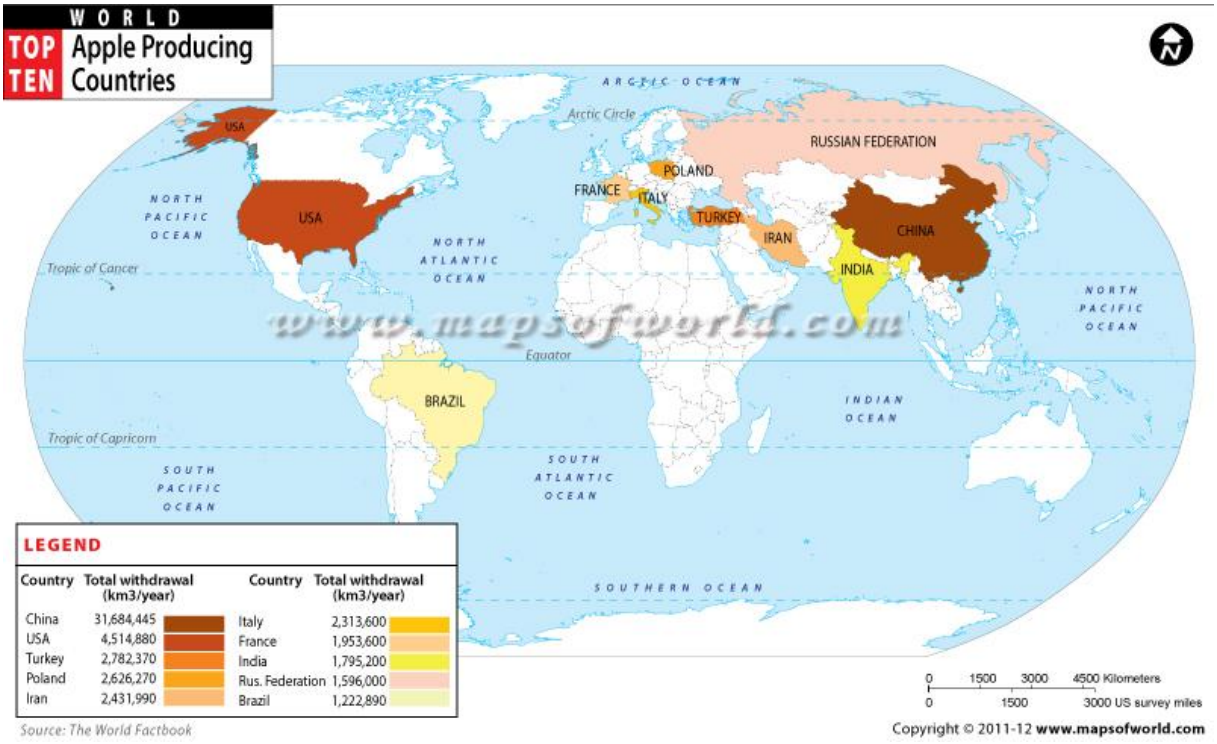

Figure 1.1 World top 10 apple producing countries. Source: The World Factbook available at www.mapsofworld.com

M. fusca, the Pacific crab apple (Figure 1.2) used in the research works described in this thesis, is a small deciduous shrub naturally occurring on mesic habitats along the Pacific coast of North America, specifically from the northern part of California to the Kenai Peninsula in Alaska (Viereck and Little 1986; Routson *et al*. 2012). It falls genetically within the group of species that are native to central Asia based on AFLP analysis (Qian *et al*. 2006) and nuclear ribosomal and chloroplast DNA (Robinson *et al*. 2001). The branches bear sharp thorn-like spur-shoots, and areas of older bark have deep fissures. Its flowers – white or light pink, are

easily noticeable in bloom time, appearing in a cluster of flowers in the end of the branches. Pacific crab apple fruits can serve as food, the leaves and bark for medicinal purposes and the wood for construction. The tree can be utilized as rootstock for domesticated apple trees in waterlogged areas. *M. fusca* is also a major source of fire blight resistance (Flachowsky *et al*. 2011; Emeriewen *et al*. 2014).

Figure 1.2 A *M. fusca* tree (**a**) growing in the orchard of JKI, Dresden (**b**) the oblong shape of the fruits.

Fire blight – the most dreadful apple bacterial disease

The Gram-negative enterobacterium, *Erwinia amylovora* (Burrill) Winslow *et al*., is the pathogen responsible for the very destructive and most dreadful bacterial disease affecting apple production (Malnoy *et al*. 2012). It also affects plant members of some genera of the Rosaceae family including *Pyrus* and some important ornamental species such as *Crataegus, Cotoneaster, Prunus,* and *Pyracantha*. The first incidence of fire blight was reported in1780 in an orchard of apples and pears in Hudson Valley, New York, USA. Fire blight is now a global threat to pome fruit production since it has now been reported in at least 40 countries across North America, Europe, the Middle East and New Zealand (Bonn and Van der Zwet 2000); and all popular apple cultivars are susceptible to the disease (Peil *et al*. 2009). In Europe, it is a quarantine disease listed under the A2 regulation of the European Plant Protection Organization (EPPO). Past epidemics have resulted in severe economic losses in pome fruit production worldwide. In Germany costs of US$1.6 million were reported in Southern Germany in 2007 as a result of fire blight epidemics (Scheer 2009); with US$9 million reported in Switzerland between 1997 and year 2000 (Hasler *et al*. 2002). An estimated annual cost of US$100 million has been reported in the United States due to fire blight epidemics (Norelli *et al*. 2003).

The most effective strategy to control the disease available to apple growers is the use of antibiotics such as streptomycin or copper derived compounds. The application of streptomycin antibiotics by apple growers in the USA amounts to approximately US$2.8 million per year (Gianessi *et al*. 2002). However, evolution of new *E. amylovora* streptomycin-resistant strains has been reported in some countries including the United States, Canada, Israel and New Zealand (McManus *et al*. 2002; McGhee *et al*. 2011). As a consequence, the application of streptomycin sprays is strictly regulated in Germany (Peil *et al*. 2009). It is completely banned in many other European countries. Biological control

strategies involving the use of antagonistic bacteria such as *Pseudomonas agglomerans* and *Pseudomonas fluorescens* (Johnson and Stockwell 2000; Mercier and Lindow 2001; Vanneste *et al*. 2002) have been tried with encouraging results, although consistency has been difficult to obtain. Such inconsistency and the challenges of registering biological control agents are factors inhibiting the wide use of biological control measures. On the other hand, biological control measures could be inefficient if not ineffective in seasons with weather conditions which favour disease manifestation (Johnson and Stockwell 1998). Sanitary practices and pruning of infected tissues, though more labor intensive but less expensive compared to quarantine and preventive measures, have a potential to contribute to the spread of the pathogen through wounded plant tissues if not properly executed (McManus *et al*. 2002; Khan *et al*. 2012). Therefore, the most feasible strategy to manage the incidences of fire blight would be the planting of stable fire blight resistant cultivars (Peil *et al*. 2007). Donor sources of resistance exist among several wild apple species (Aldwinkle *et al*. 1999; Peil *et al*. 2009; Vogt *et al*. 2013). Breeding programs have been established with the primary aim of breeding for fire blight resistance. Consequently, attempts have been made to uncover genomic regions which play important role in resistance and hence several quantitative trait loci (QTLs) have been identified in different genetic backgrounds (Calenge *et al*. 2005; Khan *et al*. 2006; Peil *et al*. 2007; Durel *et al*. 2009; LeRoux *et al*. 2010; Emeriewen *et al*. 2014; Wöhner *et al*. 2014). These QTLs confer different levels of resistances to fire blight depending upon the virulence of pathogen strain used for inoculation. The QTL with the highest effect in terms of phenotypic variation explained is that of *Malus* ×*robusta* 5 (Mr5) which explained 80 % of phenotypic variation (Peil *et al*. 2007); and confirmed in two other independent mapping populations with consistent high phenotypic variation explained (67-83 %; Peil *et al*. 2008a; Gardiner *et al*. 2012). However, the resistance of Mr5 has been broken down by more virulent strains of *E. amylovora* (Peil *et al*. 2011; Vogt *et al*. 2013; Wöhner *et al*. 2014); and thus justifies resistance breeding aimed at pyramiding the promising fire blight resistance QTLs

into apple cultivars since most of these cultivars available in the market are highly susceptible.

Disease cycle of *Erwinia amylovora* – an uninvited guest

Erwinia amylovora affects about 200 species in 40 genera including the subfamily Spiroideae. Distinct phases of the disease are known including blossom blight, shoot blight and rootstock blight (Vanneste 2000; Norelli *et al*. 2003; Peil *et al*. 2009). Typical symptoms of fire blight infected-tissues include necrosis, shepherds crook and bacterial ooze released from tissues, with tissues appearing as though they were scotched by fire (Figure 1.3)

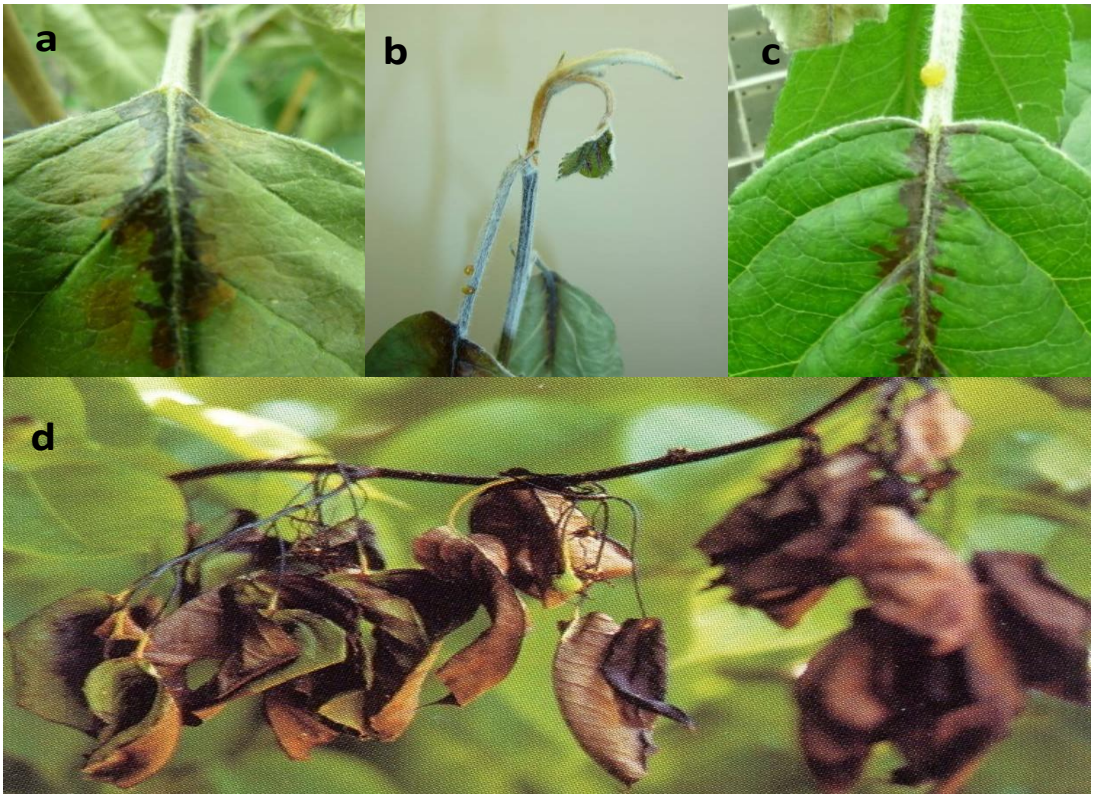

Figure 1.3 Typical symptoms of fire blight (**a**) progressive necrosis (**b**) shepherd's crook (**c**) ooze production (**d**) leaves appearing as though they were scotched by fire

The cells of the pathogen overwinter in cankers and from it emerges the bacterium as ooze at the commencement of the spring season with hot temperatures (Malnoy *et al*. 2012). Ooze

produced from cankers comprises bacteria cells in a polysaccharide matrix and are attractive to insects such as flies which are capable of disseminating bacterial cells to stigmas. Growth of cells in stigmas depends on temperature, with optimal growth rates occurring between 21 ^{o}C and 27 ^{o}C (Malnoy *et al*. 2012). With the assistance of moisture and dew, bacterial cells migrates downwards the stigma resulting in infection and blossom blight symptoms (Figure 1.4). Shoot blight occurs after *E. amylovora* cells rapidly migrate through host tissues leading to ooze production providing inoculum for shoot infection. Shoot infection occurs most readily through wounds on actively growing shoots (Vanneste 2000; Malnoy *et al*. 2012). Rootstock blight occurs following downward internal migration of *E. amylovora* cells from the scions which directly infect rootstock suckers and wounds (Momol *et al*. 1998). The disease life cycle is shown in Figure 1.4.

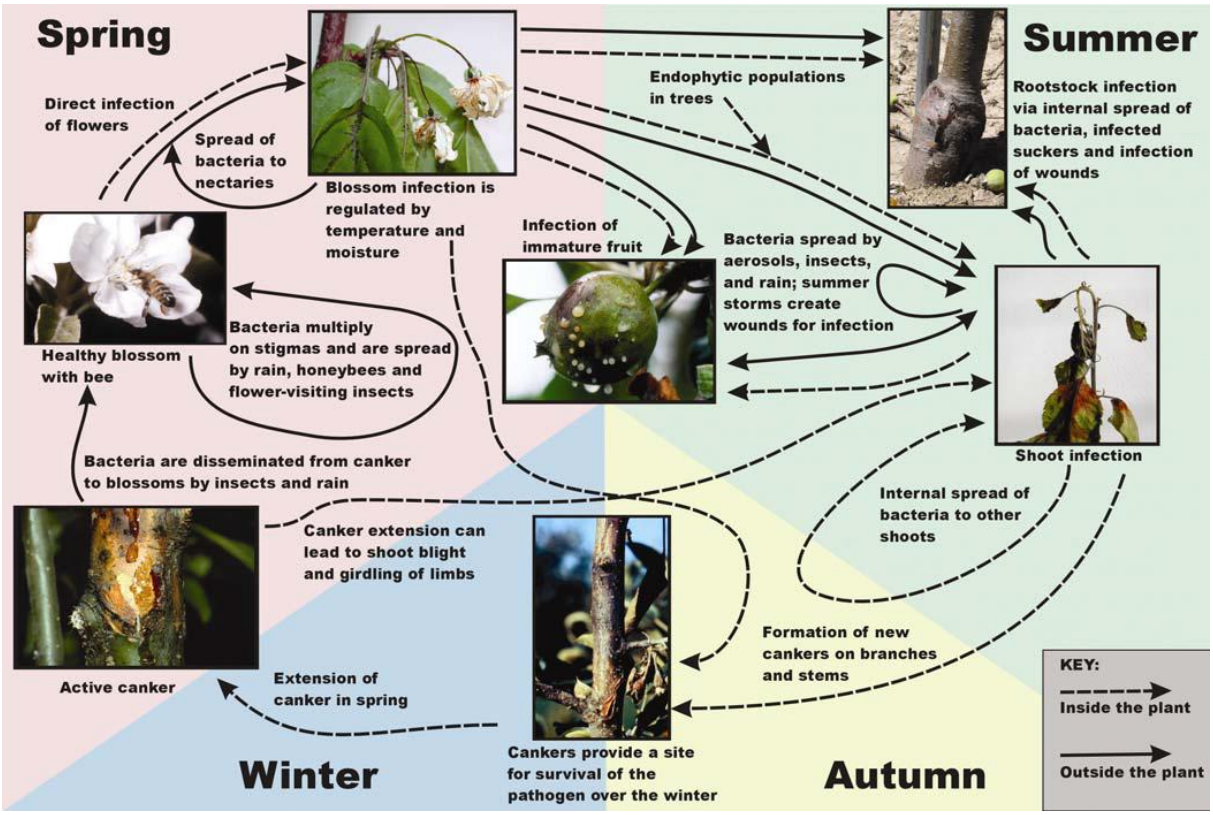

Figure 1.4 Disease cycle of fire blight caused by the bacterium *Erwinia amylovora*. Dashed lines represent movement of bacteria and spread of disease within the plant, and solid lines represent movement of bacteria outside the plant. Source: Norelli *et al*. (2003)

Molecular mechanism of *Erwinia amylovora* pathogenicity and host resistance

Since the inception of fire blight epidemics till date, many genes have been hypothesized as being involved in the ability of *E. amylovora* to cause infection in host plants with several genes characterized (Oh and Beer 2005; Khan *et al*. 2012). *E. amylovora* posseses the Type III pathogenicity requiring the type 3 *hrp* protein (*hrp*-T3SS); the type 3 effector (T3E) DspA/E, and the exopolysaccharide amylovoran (*ams*) to cause infection (Oh and Beer 2005; Khan *et al*. 2012; Malnoy *et al*. 2012). On the other hand, host plants rely on quantitative resistance genes which respond or interact with pathogen effectors to trigger defense mechanism (Khan *et al*. 2012). The mechanisms of pathogen attack and host defense is depicted in Figure 1.5. The molecular basis of host-pathogen interactions have been reviewed extensively (Oh and Beer 2005; Khan *et al*. 2012; Malnoy *et al*. 2012).

The Type III secretion pathway is encoded by a cluster of hypersensitive response and pathogenicity genes (*hrp* genes). The ability of *Erwinia amylovora* to cause fire blight in susceptible hosts and to elicit the hypersensitive response (HR) is controlled by these genes. The mutation of some genes could annul the pathogenicity effect but retained the hypersensitive response (HR) in tobacco plants and thus these mutated genes were termed *dsp* (*disease specific*) genes (Barny *et al*. 1990). The hrp/dsp gene cluster together with several other characterized effector proteins and regions constitute the so-called pathogenicity island (PAI). PAIs have been identified in several bacterial pathogens (Schmidt *et al*. 2004). Notable among *Erwinia amylovora* strains is the sequenced and characterized PAI of strain Ea321 (Oh *et al*. 2005), reviewed by Oh and Beer (2005) shown in the Figure 1.6.

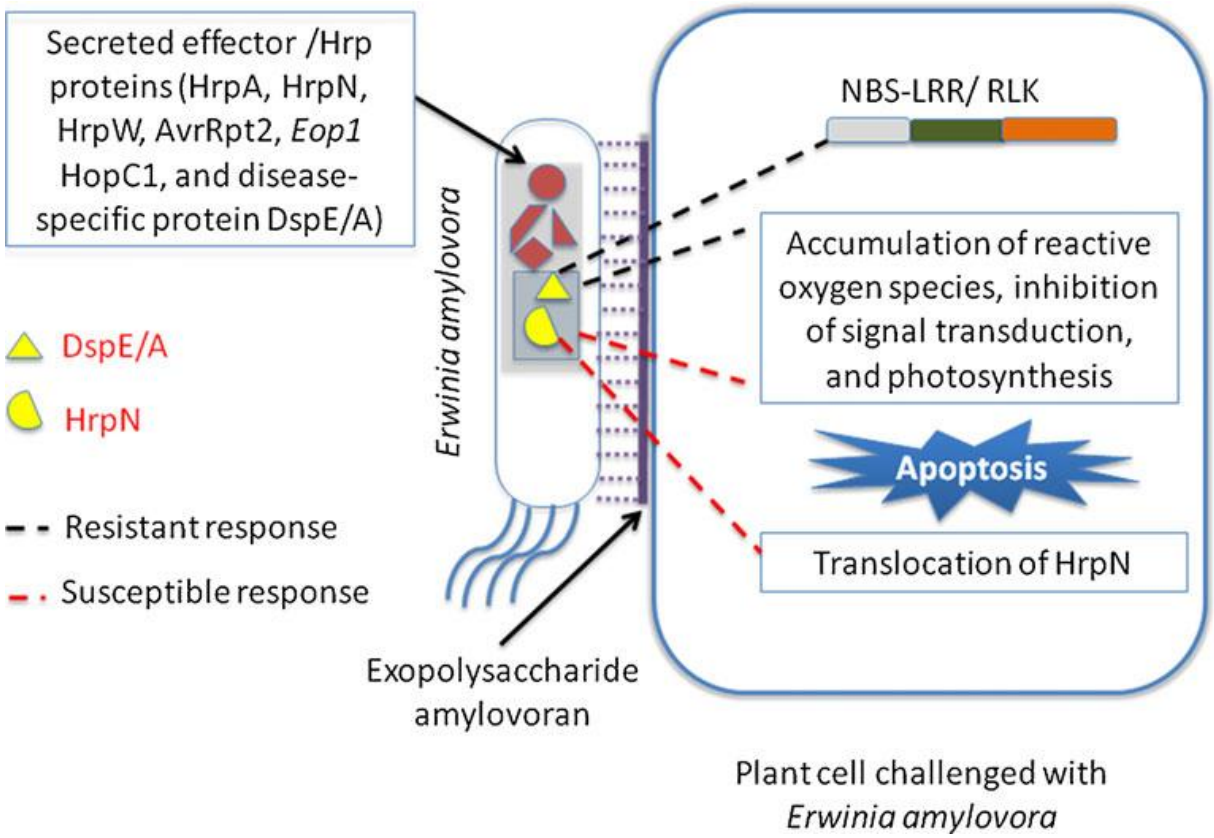

Figure 1.5 Schematic diagram of a type III secretion system illustrating interactions of a bacterial cell of *Erwinia amylovora* with a plant host cell. Source: Khan *et al*. (2012)

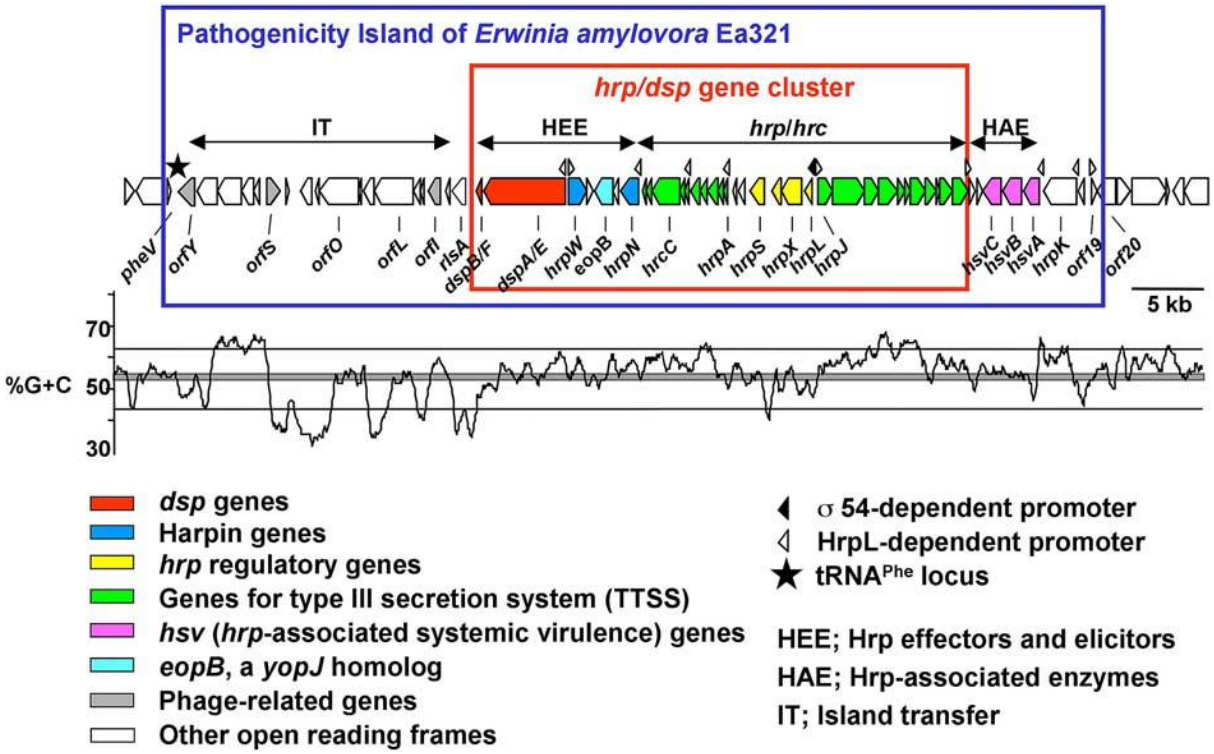

Figure 1.6 The Hrp pathogenicity island of *E. amylovora* strain Ea321. It consists of four DNA regions: the hrp/hrc region, the HEE region, the HAE region, and the IT region. The hrp/dsp gene cluster includes the hrp/hrc region and the HEE region. The genes having significant functions or homology with other significant genes are color-coded as indicated. The % G + C graph is the result of a sliding window of 500 nucleotides. Source: Oh and Beer (2005)

Fire blight resistance breeding in *Malus*

In recent years, breeding for fire blight resistance in apple has become an important objective since it seems to be the only sustainable approach to control fire blight epidemics. Before now, priority emphasis has been on breeding for fruit quality; and although it was not of high priority in many international breeding programs, the development of fire blight resistant cultivars was still an objective, for example, in the United States, Canada, New Zealand and Germany (Peil *et al*. 2009). It is challenging to combine breeding for resistance against pathogens and at the same time maintaining commercial fruit quality because resistance breeding against the major pathogens of apple such as fire blight, scab and powdery mildew require raising a population, thus making the process of apple breeding cumbersome (Gardiner *et al*. 2007). Furthermore, donors of such resistant traits are mostly wild apple species with less appealing fruit quality such as *M. fusca*, *M. baccata* and *M. ×robusta* (Aldwinkle and Van der Zwet 1979; Peil *et al*. 2009). Moreover, it takes a long time to establish pre-breeding material from several pseudo-backcrosses which is major prerequisite in the introduction of resistances from wild species. Accelerated classical breeding approach will therefore depend on our understanding of the genetics of the disease, biotechnology as well as genetic engineering (Peil *et al*. 2009). Thus, breeding methods have been investigated aimed towards shortening the long juvenile phases of apple seedlings using chemical and physical strategies (Meilan 1997) and transgenic approaches (Hanke *et al*. 2007; Flachowsky *et al*. 2009).

The crossing of cultivars of the domesticated apple (*M. domestica* Borkh.) bearing some degree of resistance with other cultivars that are tolerant or even susceptible but with excellent fruit quality, thereby obtaining a new cultivar with higher level of resistance, are possible (Fischer and Richter 1999). This suggests that other possibilities exit by which fire blight resistance could be introduced into popular cultivars available in the market other than

the long and cumbersome process involving decades of pseudo-backcrosses. Breeding using apple scions have been popular in breeding programs. Apple genotypes which have consistently shown low susceptibility to fire blight over the years are selected for use as parents in such programs (Kellerhals *et al*. 2011). Scions of the resultant progeny seedlings, grafted onto rootstocks, are then selected for fire blight resistance by inoculation with *E. amylovora* strains. Inoculations are performed at an early stage of the seedlings. This is the case in Dresden-Pillnitz, Germany, where breeding of resistant apple is aimed at production of cultivars with high quality and multiple resistances to both biotic and abiotic stresses (Peil *et al*. 2008b). Re®-cultivars have been developed with combined resistances to fire blight, scab and powdery mildew. And currently, the resistance breeding in Dresden-Pillnitz is focused on pyramiding of the resistance genes of apple scab as well as those of powdery mildew while also attempting to elucidate the resistance mechanisms for fire blight. Therefore classical screening methods are combined with molecular marker techniques.

The last decade has seen the emergence of the use of molecular markers for marker-assisted breeding/selection (MAB; MAS). This approach first requires the identification of genomic regions also known as quantitative trait locus or loci (QTLs) contributing to fire blight resistance. The first QTL conferring resistance to fire blight was reported by Calenge *et al*. (2005). The QTL (F7 QTL) which is located on linkage group (LG) 7 of the cultivar 'Fiesta' and explains 34.3 – 46.6 % of phenotypic variation was identified in a population derived from a cross of 'Fiesta' and 'Discovery'. Khan *et al*. (2006) while calculating the area under disease progress curve (AUDPC), confirmed the F7 QTL in a different set of individuals from the cross 'Fiesta' × 'Discovery'. Khan *et al*. (2007) transformed the RAPD markers bracketing this QTL into SCAR markers, AE10-375 and GE-8019, and also designed an SSR marker specific for the region. These molecular markers considered promising proved to be so since Baumgartner *et al*. (2010) reported their use for the breeding program of Agroscope

(ACW Switzerland). Furthermore, Le Roux *et al*. (2010) identified a minor QTL on chromosome 10 of the commercial apple variety 'Florina' (FLO10) which explained 17.9 and 15.3 % of phenotypic variation at two time points (PLL1 and PLL2) respectively. Peil *et al*. (2007) identified a QTL on the proximal part of LG3 of the genotype *M.* ×*robusta* 5, between two SSR markers CH03g07 and CH03e03 which explained about 80% of the phenotypic variation. Fahrentrapp *et al*. (2013) then reported the enrichment of the QTL region with SSR markers. Durel *et al*. (2009) reported two QTLs mapping closely on LG12 of the apple genotypes 'Evereste' and *M. floribunda* clone 821, explaining 53 and 40 % respectively of phenotypic variation. An overview of the positions of QTLs for resistance to fire blight in Malus is presented in Figure 1.7.

Following QTL mapping approach which determines genomic regions contributing to fire blight resistance, identification of candidate genes and positional cloning of the underlying gene(s) could be undertaken. Subsequently, two probable fire blight resistance genes showing homology with the *Pto/Prf* complex in tomato were reported in the apple cultivar 'Evereste' (Parravicini *et al*. 2011). Furthermore, the association of putative resistance gene markers with the fire blight resistance QTL of Mr5 was reported by Gardiner *et al*. (2012) with the hypothesis that several genes that play different roles in fire blight resistance could be located in the upper region of linkage group 3 of *M.* ×*robusta* 5. In this same region of Mr5, Fahrentrapp *et al*. (2013) identified an NBS-LRR candidate gene, *FB_MR5*, for fire blight resistance. *FB_MR5* is the only functionally characterised gene conferring resistance to fire blight. Broggini *et al*. (2014) transferred *FB_MR5* into the genome of the apple cultivar 'Gala' via genetic engineering and confirmed it to be the resistance determinant of Mr5 since transformed lines possessed the same gene-for-gene (host-pathogen) interaction as does Mr5 and *E. amylovora* (Vogt *et al*. 2013).

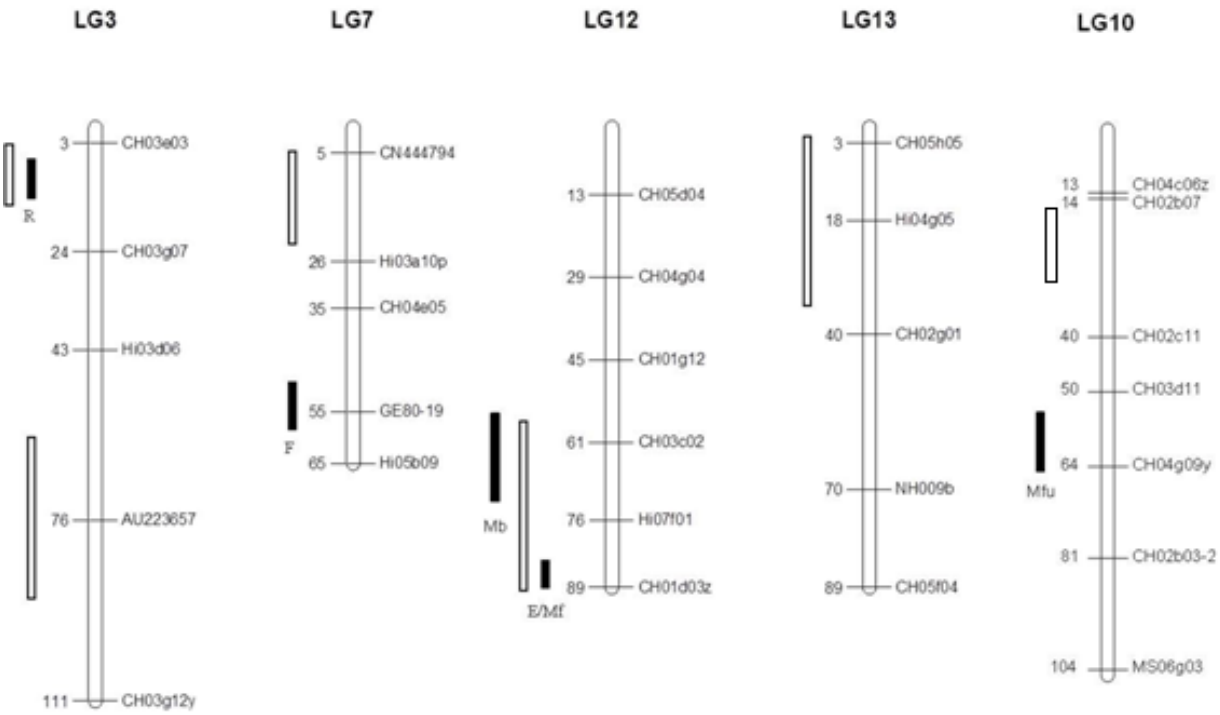

Figure 1.7 Position of quantitative trait loci (QTL) for resistance to fire blight. Black bars represent major QTL (R- *M*. ×*robusta* 5, F- Fiesta, Mb- *M*. *baccata*, E/Mf- Evereste, *M*. *floribunda*, Mfu- *M*. *fusca*). White bars represent minor QTL. Source: Peil *et al*. (2014)

Scope and aim of thesis

The domesticated apple remains an economically important fruit crop globally. Its perennial nature implies that the apple tree is exposed to long periods in the environment which promotes development and mutation of pathogens. Fire blight, caused by the Gram-negative bacterium, *E. amylovora*, remains the most destructive bacterial disease affecting apple production worldwide. Apple growers mostly rely on the use of antibiotic treatment to control the disease. However, this strategy is strictly regulated if not completely banned in many European countries. Breeding of resistant apple cultivar therefore is thought to be the most feasible and sustainable strategy to control the menace of fire blight. Sources of resistance have been described in apple wild species accessions. Consequently, quantitative trait loci have been reported in some *Malus* wild species accessions such as Mr5 (Peil *et al*. 2007) and *M. floribunda* clone 821 (Durel *et al*. 2009). The breaking of such a promising resistance QTL of Mr5 by *E. amylovora* strains differing by just a single nucleotide polymorphism (Vogt *et al*. 2013; Wöhner *et al*. 2014), supports the case for pyramiding of the promising resistance QTLs identified in *Malus spp* in order to attain durable resistance. It is therefore useful to investigate genetic resistance to fire blight in more wild species accessions. The major aims of the works presented in this thesis are to firstly, identify, then isolate and characterize resistance to fire blight from another wild species, *M. fusca*. To achieve these aims, specific target objectives were set to include:

1. Raising a population from a cross of *M. fusca* × 'Idared'
2. Development of molecular markers
3. Establishment of a linkage map
4. Mapping of fire blight resistance
5. Fine mapping of resistance region via genome walking approach
6. Chromosome landing

In the research paper presented as Chapter Two in this thesis, the major resistance QTL against fire blight from the wild species accession, *M. fusca*, is described. The whole processes leading to its identification, as well as the implications of these results, are discussed extensively in the chapter. In Chapter Three, the stability of *Mfu10* after another phenotypic evaluation with a highly virulent *E. amylovora* isolate Ea3049, originating from Canada is reported.

The development of closely linked molecular markers suited for marker-assisted selection (MAS), for fine mapping the resistant region of *M. fusca* through genome walking approach, is reported in the paper presented as Chapter Four. The chapter also discusses chromosome landing approach undertaken, involving the construction of a *M. fusca* bacterial artificial chromosome (BAC) library and screening with molecular markers closely linked to the fire blight resistance QTL. Chapter Five summarizes the works presented in this thesis. The implication of these results is discussed extensively.

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Chapter Two

Identification of a major quantitative trait locus for resistance to fire blight in the wild apple species *Malus fusca*

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Identification of a major quantitative trait locus for resistance to fire blight in the wild apple species *Malus fusca*

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Abstract

Fire blight, caused by the Gram-negative bacterium, *Erwinia amylovora*, is the most important bacterial disease affecting apple (*Malus* × *domestica*) and pear (*Pyrus communis*) productions. The use of antibiotics treatment, though effective to some degree, is forbidden or strictly regulated in many European countries, and hence an alternative means of control is essential. The planting of fire blight-resistant cultivars seems to be a highly feasible strategy. In this study, we explored a segregating population derived from a cross between the apple wild species *Malus fusca* and the *Malus* × *domestica* cultivar 'Idared'. F_1 progenies used for mapping were artificially inoculated with *Erwinia amylovora* strain Ea222_JKI at a concentration of 10^9 cfu/ml in three different years. The averages of percentage lesion length (PLL) of all replicates of each genotype were used as numerical traits for statistical analysis. A Kruskal-Wallis analysis was used to determine marker-phenotype association and revealed a linkage group with Diversity Arrays Technology (DArT) markers significantly linked with fire blight. After locating the positions of the DArT markers on the 'Golden Delicious' genome, simple sequence repeat (SSR) markers were developed from chromosome 10 to replace the DArT markers and to determine the quantitative trait locus (QTL) region. Multiple QTL mapping (MQM) revealed a strong QTL (*Mf10*) on linkage group 10 of *M. fusca* explaining about 65.6 % of the phenotypic variation. This is the first report on a fire blight resistance QTL of *M. fusca*.

Keywords: *Malus fusca*, fire blight, *Erwinia amylovora*, QTL mapping, DArT markers, SSR markers

Introduction

Fire blight (FB), a devastating necrogenic disease, caused by the Gram-negative bacterium, *Erwinia amylovora* (Burrill) Winslow *et al*., is the most important bacterial disease affecting pome fruit and several other members of the Rosaceae family (Malnoy *et al*. 2012). Since its discovery in America about 220 years ago, the number of reported cases of the disease has continually increased (Vanneste 2000), and has been reported in at least 40 countries across North America, Europe, the Middle East and New Zealand (Bonn and Van der Zwet 2000). The spread of the disease has been attributed to human activity, most notably, through long distance shipments of either bud wood or trees (Peil *et al*. 2009). The pathogen infects the host primarily through natural openings in the flowers or wounds on vegetative tissues. *E. amylovora* growth hugely depends on favourable temperature ranging from 21^{o}C to 27^{o}C for maximal growth (Malnoy *et al*. 2012). Migration of bacterial cells to openings where infection occurs is enabled by moisture from rainfall or heavy dew.

Fire blight infects blossoms, fruits, stems, leaves, woody branches and rootstocks crowns thereby causing blossom blight, shoot blight and rootstock blight (Peil *et al*. 2009) with affected areas appearing to have been scorched by fire. Once established in the host, the bacteria multiplies rapidly and progresses into intercellular spaces of the parenchyma tissue, leading to progressive necrosis of infected tissue and ooze production, which are two characteristic symptoms of the disease (Thomson 2000). Due to the accumulation of the bacteria in the xylem tissue, distal plant parts become blighted leading to their death as a result of the blockage of water inflow (Oh and Beer, 2005) and it is possible for necrosis to extend to the entire tree within a single growing season in highly susceptible genotypes. There are in-depth reviews of the pathogen and host interactions (Peil *et al*. 2009; Malnoy *et al*. 2012; Khan *et al*. 2012).

The control measures for fire blight include quarantine, eradication and the use of antibiotics (McManus *et al*. 2002; Norelli *et al*. 2003). The difficulties encountered by the numerous attempts to eradicate or minimise the progress of FB epidemics are a result of limited availability of management tools to control the disease as well as the diversity of tissues susceptible to infection (Norelli *et al*. 2003). Antibiotics like streptomycin and the use of heavy metals such as copper-derived chemical compounds have proven to partly control the disease (Calenge *et al*. 2005); however, their application is banned for ecological reasons in many countries. While this is a justifiable consideration, they are also not completely successful in the eradication of FB due to the difficulty in forecasting the disease as a result of its epidemiological tendencies (Parravicini *et al*. 2011). Moreover, there is the issue of the rapid selection of antibiotic-resistant populations as a result of antibiotics use and consequently, streptomycin-resistant strains of the causal organism, *E. amylovora*, have been isolated in several pome fruit production orchards worldwide (Jones and Schnabel, 2000). There have been reports of some successes achieved with the use of bio control measures (Johnson and Stockwell, 1998) and with the use of plant growth regulators (Rademacher and Kober 2003), but to date no reliable treatments are available. However, given the ecological effects of antibiotic or copper based applications, the most feasible alternative control measure would be the use of fire blight resistant cultivars.

Genetic resistance has been shown to play an important role in the management of fire blight. In the genus *Malus*, a large variability of resistance to the disease has been observed (Aldwinckle *et al*. 1976), and several have been identified as potential sources of FB resistance including *M. robusta*, *M. atrosanguinea*, *M. sublobata*, *M. prunifolia* and *M. fusca* (Aldwinckle and Beer, 1979). Some accessions of these wild *Malus* species have been utilised as sources of FB resistance. Fire blight resistance in general is thought to be quantitatively controlled (Korban *et al*. 1988). Several quantitative trait loci (QTLs) for resistance were

identified (Calenge *et al*. 2005; Khan *et al*. 2006; Peil *et al*. 2007; Durel *et al*. 2009; Le Roux *et al*. 2010), and exhibit different levels of resistance to the disease. Calenge *et al*. (2005) identified a major QTL for fire blight resistance in the cultivar 'Fiesta' on linkage group (LG) 7 which explained 34.3 – 46.6 % of phenotypic variation. Khan *et al*. (2006) confirmed the same QTL in a different set of individuals from the cross 'Fiesta' × 'Discovery', but did not detect minor QTLs which Calenge *et al*. (2005) had reported. Peil *et al*. (2007) identified a QTL on LG3 of the genotype *M.* ×*robusta* 5 which explained about 80% of the phenotypic variation, and Durel *et al*. (2009) reported that apple genotypes 'Evereste' and *M. floribunda* clone 821 showed high resistance to FB. Furthermore, Le Roux *et al*. (2010) reported the identification of two significant QTLs in the commercial apple variety 'Florina' which explained 17.9% and 15.3% of phenotypic variation, respectively. Recently, the identification of serine/threonine kinase and nucleotide-binding site-leucine-rich repeat (NBS-LRR) genes was reported in the apple cultivar 'Evereste' (Parravicini *et al*. 2011). Of the eight genes in the cluster identified by this group, two were probable fire blight resistance genes showing homology with the *Pto/Prf* complex in tomato. More recently, Gardiner *et al*. (2012) reported the association of putative resistance gene markers with the fire blight resistance QTL in *M.* ×*robusta* 5. These authors suggested that the upper region of linkage group 3 of *M.* ×*robusta* 5 contains several genes which play varying vital roles in fire blight resistance depending upon pathogen virulence and other factors. Furthermore, Fahrentrapp *et al*. (2012) identified an NBS-LRR candidate gene for fire blight resistance, *FB_MR5* in *M.* ×*robusta* 5 in the same region. Additionally, a gene-for-gene interaction between *M.* ×*robusta* 5 and *E. amylovora* was proposed by Vogt *et al*. (2013).

A QTL mapping approach can identify regions of the chromosomes controlling quantitative traits. This approach has been successfully used to identify several agronomically important traits including disease resistance in different plant species. The availability of genetic linkage

maps is an essential prerequisite to map QTLs. Several apple genetic linkage maps including the maps of Hemmat *et al*. (1994), Conner *et al*. (1997), Maliepaard *et al*. (1998), Liebhard *et al*. (2002), Silfverberg-Dilworth *et al*. (2006), Han *et al*. (2011) using molecular markers such as RAPD (Random Amplified Polymorphic DNA), SSR (Simple Sequence Repeats), SNPs (Single Nucleotide Polymorphisms) or DArT (Diversity Arrays Technology) markers have been established. Furthermore, the completion of the apple reference genome (Velasco *et al*. 2010) has thrown more insight into genetic mapping studies of this extensively studied species. In this study, we explored a segregating population for resistance to fire blight inherited from the wild relative of apple, *M. fusca*, through QTL analysis. It appeared that resistance to FB in *M. fusca* is explained by a major QTL localised on LG10 which explained up to 65.6 % of the phenotypic variation.

Materials and Methods

Plant materials

The mapping population derived from a cross between the FB-resistant *M. fusca* accession MAL0045 and the FB-susceptible apple (*M.* × *domestica*) cultivar 'Idared' resulting in an F_1 progeny of 134 individuals. The progeny was grown in the greenhouse at the Julius Kühn-Institut (JKI), Germany and later transferred to the orchard.

Artificial shoot inoculation and evaluation of disease resistance

For inoculation experiments, scions of both parents and progenies were grafted on rootstock M9. *Erwinia amylovora* strain Ea222_JKI was used for inoculation at a concentration of 10^9 cfu/ml. Inoculation was performed in the green house in 2006, 2007 and 2012 as described by Peil *et al*. (2007). Up to 10 replicates of each individual were inoculated every year. Lesion

length (cm) was measured 28 days post inoculation (dpi). Incidence of fire blight was determined as percentage lesion length (PLL): length of necrotic shoot divided by total shoot length multiplied by 100.

DNA isolation

DNA was isolated from young lyophilized leaves using the CTAB method according to Doyle and Doyle (1987). DNA was quantified and diluted to a concentration of 20ng/µl and stored in -20^{o}C until required for amplification of markers.

Marker development and application

DArT markers

A new library of DArT clones was constructed using the same method of complexity reduction as described in Schouten *et al*. (2012). *Pst*I/*Alu*I restriction enzymes were used to digest genomic DNA of *M. fusca* accession MAL0045 and amplified restriction fragments cloned as described by Kilian *et al*. (2012). Clones were generated and inserts amplified from the cloning vector according to Kilian *et al*. (2012). The inserts (in 384-well plate format) were arrayed together with 3,072 markers polymorphic in cultivated apples and rootstock materials and with 1,536 clones from *M. sieboldii* library developed for another project. A total of 7,680 clones were printed on SuperChip poly-L-lysine slides (Thermo Scientific) using a MicroGrid arrayer (Genomics Solutions) in full replication.

Both parents and 92 progenies were assayed using methods described above for library construction. Genomic representations were labelled with fluorescent dyes (Cy3 and Cy5). Labelled targets were then hybridized to DArT arrays (of composition described above) for 16 h at 62 °C in a water bath. Slides were processed as described by Kilian *et al*. (2012) and scanned using Tecan LS300 scanner (Tecan Group Ltd, Männedorf, Switzerland) generating three images per array: one image scanned at 488 nm for reference signal measuring the

amount of DNA within the spot based on hybridisation signal of FAM-labelled fragment of a TOPO vector multiple cloning site fragment, and two images for "target" signal measurement. Signal intensities were extracted from images using DArTsoft 7.4.7 software (http://www.diversityarrays.com/software.html). DArTsoft was also used to convert signal intensities to presence/absence (binary) scores used in the downstream analysis. Both DArT assays and DArTsoft analysis were performed at DArT P/L in Canberra, Australia.

SNP markers

A SNP array (SNPlex™) had previously been designed by Micheletti *et al*. (2011) which identified approximately 1,400 SNPs in the 'Golden Delicious' genomic contigs (Velasco *et al*. 2010). Similarly, 270 SNPs were entered into a SNPlex automated multi-step pipeline as described by Micheletti *et al*. (2011). The SNPlex™ genotyping assays (Tobler *et al*. 2005) were performed on 1 µl (between 45 and 225 ng) of fragmented GenomiPhi amplified DNA on both parents and 114 progeny, according to the manufacturer's instruction. All other procedures of sample preparation and data analysis were as reported in Micheletti *et al*. (2011); this involved running the samples on a 373 × l DNA analyser (Applied Biosystems Inc.), analysing the data using Gene Mapper v.4.0 software (Applied Biosystems Inc.) and performing genotype analysis using the SNPlex_Rules_3730 method according to the manufacturer's default settings.

SSR markers

Previously published SSRs (Liebhard *et al*. 2002; Silfverberg-Dilworth *et al*. 2006) were chosen according to their location on the 'Fiesta' × 'Discovery' reference genetic map to cover all linkage groups. Additionally, SSR markers were developed using the 'Golden Delicious' reference genome (Velasco *et al*. 2010) to replace DArT markers linked to the resistance QTL. SSRs were selected based on dinucleotide and trinucleotide repeats. Primer

pairs flanking the SSR sequences were designed using the Primer3 program (Rozen and Skaletsky 2000). SSRs were tested for polymorphism on both parents and a subset of six progenies of the population either by conventional PCR method using GeneAmp PCR reagents (Applied Biosystems, Italy) or by multiplex PCR using the Qiagen Type-It Kit (Qiagen, Hilden, Germany). SSRs polymorphic in *M. fusca* were thereafter applied to the whole mapping population. Polymorphism was tested according to Schuelke (2000). Conventional PCR was performed in a total volume of 10.2 µl consisting of 1 × PCR buffer (GeneAmp), 2.4 mM $MgCl_2$, 0.05 mM dNTPs, 0.75 U AmpliTaq Gold DNA polymerase, 2 mM SSR primer mix (forward and reverse primers) and 40 ng genomic DNA with the following profile: 94 ^{o}C for 10 min, followed by 32 cycles of 94 ^{o}C for 30 s, 60 ^{o}C for 1 min, 72 ^{o}C for 1 min, and an extension at 72 ^{o}C for 5 min. Multiplex PCR was performed using the Type-It kit according to the manufacturers protocol with up to three microsatellites per PCR in a total volume of 10 µl. PCR conditions were 95 ^{o}C for 5 min, followed by 30 cycles of 95 ^{o}C for 1 min, 60 ^{o}C for 1 min 30 s, 72 ^{o}C for 30 s, and an extension at 60 ^{o}C for 30 min.

PCR fragments were analyzed either on 3730xl DNA Analyzer (Applied Biosystems, Vienna, Austria) for which SSR primers were labelled with NED, FAM or HEX, or on a CEQ 2000XL DNA sequencer (Beckman Coulter, Germany) for which primers were labelled with BMN-5, BMN-6 or DY751. Sample preparations for the two analyzers were as follows: for the 3730xl DNA Analyzer, PCR products were diluted 1:20 and 0.5 µl of this dilution was mixed with 9.8 µl of formamide (Applied Biosystems) and 0.2 µl of 500 Liz GeneScan^{TM} size standard (Applied Biosystems). Samples were denatured for 3 min at 95 ^{o}C and placed on ice before loading on the analyzer. When using the Beckman Coulter analyzer, PCR products were diluted 1:100 using sterile water and 2 µl of this dilution was mixed with 29.9 µl of sample loading solution (Beckman Coulter) and 0.1 µl of size standard.

DArT markers sequencing

DArT markers significantly linked to fire blight resistance and others on the same group, 13 in total, were reamplified from single inserts of the DArT clones by PCR using the primers T7 (AATACGACTCACTATAG) and M13R (CAGGAAACAGCTATGAC). The PCR was performed in a 25-µl volume consisting of 1 × Dream Taq buffer (Thermo Scientific, Leon-Rot, Germany), 0.2 mM dNTPs, 1 µM each of forward and reverse primers, 0.5 U Dream Taq DNA polymerase (Thermo Scientific, Leon-Rot, Germany) and 1 µl of template. Running conditions of PCR were: 95 ^{o}C for 4 min, 57 ^{o}C for 30 s, 72 ^{o}C for 1 min, followed by 35 cycles of 94 ^{o}C for 35 s, 52 ^{o}C for 35 s, 72 ^{o}C for 1 min, and an extension at 72 ^{o}C for 7 min. Purification of PCR products was performed using the MSB® Spin PCRapace kit (Invitek GmbH, Berlin; Germany) according to the manufacturer's protocol. Sequencing of the amplification products was conducted by Eurofins MWG Operon. The alignment of the resulting sequences was performed using the 'Golden Delicious' reference genome.

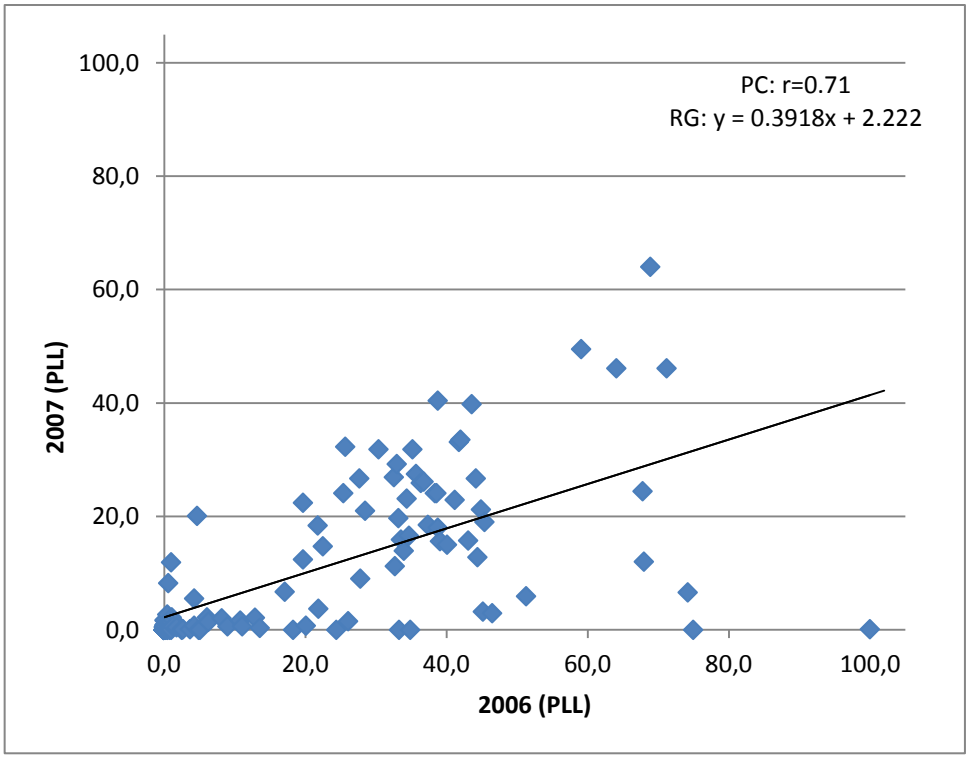
Statistical analyses

Pearson's correlations of phenotypic data evaluated in three different years were calculated using SAS Enterprise Guide 4.3 (SAS Institute Inc, Cary, NY). To determine marker-phenotype association, a Kruskal-Wallis analysis - a non-parametric equivalent of the one-way ANOVA, ranking all individuals in terms of their quantitative trait while at the same time classifying them according to their marker genotype (Van Ooijen and Maliepaard 1996) - was performed. JOINMAP® 3.0 (Van Ooijen and Voorips 2001) was used for mapping at an LOD threshold of 5.0. Linkage groups were assigned by comparing results with location of SSRs in the reference genetic linkage map of apple (Silfverberg-Dilworth *et. al*. 2006). Interval mapping and multiple QTL mapping (MQM) were done with MapQTL® 5 (Van Ooijen 2004).

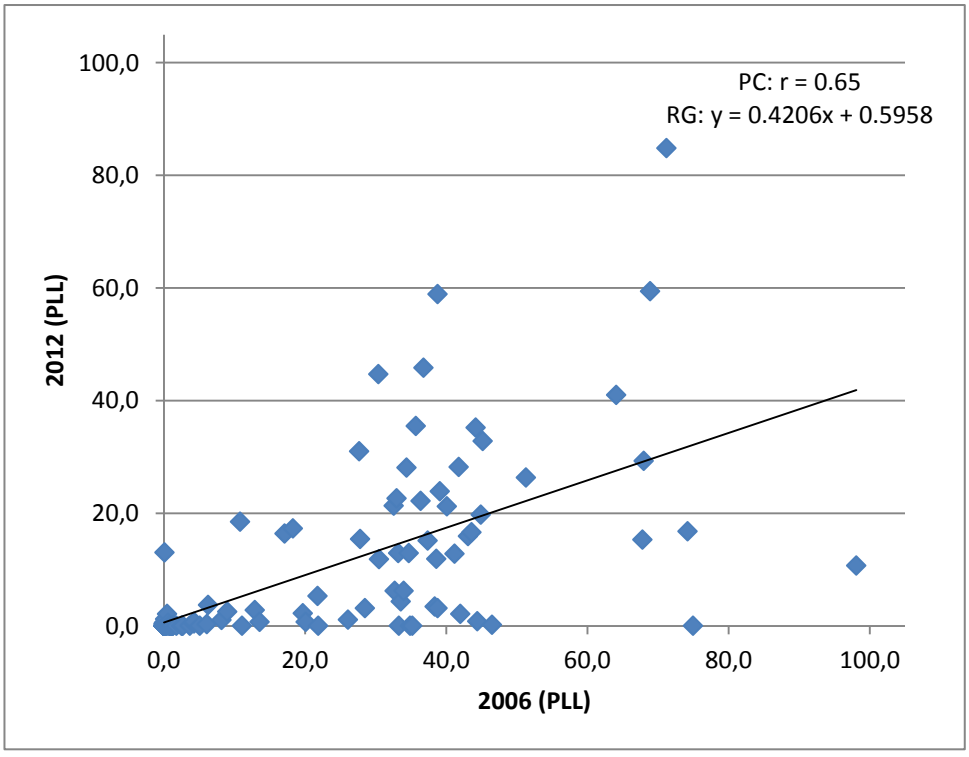
Results

Phenotypic evaluation

A total of 134 progeny were screened in the green house for resistance/susceptibility to fire blight. Progenies were phenotyped in 2006, 2007 and 2012. Phenotypic data from the three years of trials showed that in 2007 and 2012 the overall average percentage lesion length (PLLs), 9.4 and 9.0 % respectively, were relatively low compared to 2006 with 22.6 %. The average lesion length of all replicates of all individuals was 4.7 cm, equivalent to 14.4 %. Figures 1a–c shows the relationship of PLL of each individual between the years and the respective correlation for all progenies. The strongest correlation (r = 0.77) could be observed between the phenotypic data surveyed in 2007 and 2012, the two years showing very similar average PLL. In 2006, 18 plants showed no necrosis while in 2007 and 2012, 28 and 51 plants, respectively, showed no necrosis. No disease symptoms were observed in all three years on eight progenies. The averages of all replicates of each individual over the three years of trialling were used for the final interval mapping analysis, whilst the averages of all replicates of one individual from one year of trials were used to determine the Kruskal-Wallis values and LOD scores for each year of phenotyping.


PC: r=0.71
RG: y = 0.3918x + 2.222
2007 (PLL)
2006 (PLL)
100,0
80,0
60,0
40,0
20,0
0,0
0,0
20,0
40,0
60,0
80,0
100,0

a


PC: r = 0.65
RG: y = 0.4206x + 0.5958
2012 (PLL)
2006 (PLL)
100,0
80,0
60,0
40,0
20,0
0,0
0,0
20,0
40,0
60,0
80,0
100,0

b

c

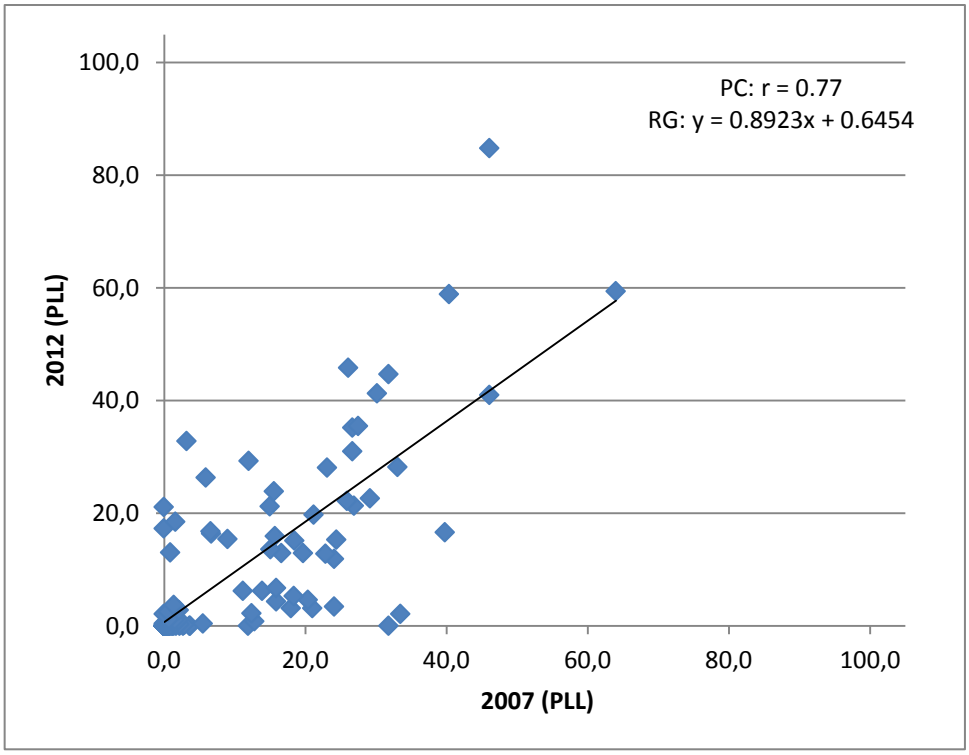


Figure 1 Pairwise comparison of PLL of *M. fusca* × 'Idared' progenies of 3 years of inoculation **a** 2006 and 2007; **b** 2006 and 2012; **c** 2007 and 2012. *PLL* percentage lesion length, *PC* Pearson's correlation coefficient, *RG* regression line function

Marker development

DArT markers

A total of 3,072 clones were generated and inserts amplified from the cloning vector as described by Kilian *et al*. (2012). Together with 3,072 markers polymorphic in cultivated apples and rootstock materials and with 1,536 clones from *M. sieboldii* library, an array of 7,680 clones was established on SuperChip poly-L-lysine slides (Thermo Scientific).

The *M. fusca* × 'Idared' progeny were hybridised to the dedicated DArT array using apple-optimised complexity reduction procedure as described by Schouten *et al*. (2012). When the images produced were processed using DArTsoft under standard marker quality thresholds, 1,080 DArT markers were identified. Of the aforementioned DArT markers 351 (32.4 %) were polymorphic in the FB-resistant parent *M. fusca*, 603 (55.7 %) in the FB-susceptible parent 'Idared', 97 (8.9 %) in both parents and 29 (2.7 %) were not polymorphic in either parent.

DArT marker sequencing and SSR marker development

An initial Kruskal-Wallis analysis revealed one linkage group containing DArT-markers significantly linked to fire blight resistance. The analysis was performed with the phenotypic data from 2006 and 2007 inoculations. Most markers showed highly significant *K* values for both years, with DArT markers 971000, 970854, and 970840 possessing the highest *K* values (Table 1).

To determine the corresponding linkage group, 13 out of 18 DArT markers forming the group were sequenced and the sequences aligned to the 'Golden Delicious' genome. The alignment of the resulting sequences showed that while nine DArT markers aligned to LG10 of 'Golden Delicious', two aligned to LG11, one aligned to linkage group 9 and another aligned to LG15

(Table 1). Two other DArT markers, whose sequences were provided by Diversity Arrays Technology, also aligned to LG10.

SSR markers were developed using the 'Golden Delicious' sequence as template. Primers for 23 SSR motifs in total were designed from contigs adjacent to the contig where sequences of the two DArT markers which were most significantly linked with fire blight resistance, 971000 and 970840, were localised on LG10 of 'Golden Delicious'. Of the 23 SSRs, two were polymorphic in both parents, four in only *M. fusca*, seven in only 'Idared' and ten were monomorphic or amplified no fragment. Primer sequences and allele sizes of the six SSRs polymorphic in *M. fusca* are listed in Table 2.

Table 1 Initial Kruskal-Wallis analysis of the LG showing significant correlation to fire blight and alignment of DArT marker sequences to the 'Golden Delicious' pseudo-chromosomes

DArT Markers	Position	*K*[a]		LG GD[b]
	(cM)	2006	2007	
972352	0	2.9*	8.2****	10
442947[c]	0.02	2.9*	8.4****	10
443272[c]	0.02	2.9*	8.3****	10
970891	0.02	2.9*	8.3****	10
901573	0.03	3.0*	8.2****	
969611	12.01	3.0*	10.1****	
971614	12.05	3.3*	10.1****	10
971449	12.45	32.9*******	45.1*******	10
970425	12.49	33.9*******	45.9*******	10
970518	12.49	33.9*******	45.9*******	10
971227	12.85	33.8*******	47.3*******	9
969555	13.7	27.8*******	38.1*******	15
971000	22.71	44.2*******	52.6*******	10
970854	22.95	46.7*******	54.7*******	
970840	23.45	39.9*******	48.9*******	10
970048	49.11	9.6****	15.8*******	11
969599	49.12	8.8****	15.4*******	11
971659	64.43	1.7	--	10

[a]Value of Kruskal-Wallis analysis (significance levels: * = 0.1, **** = 0.005, ******* = 0.0001)

[b]LG GD: pseudo-chromosome (linkage group) of the 'Golden Delicious' genome

[c]Sequences delivered by Diversity Arrays Technology.

Table 2 SSR markers developed from contigs of pseudo-chromosome 10 of the 'Golden Delicious' genome

SSR	Forward	Reverse	Allele sizes (bp)		LG
			M.fusca	Idared	
FR481A	CGGAAGCAAAAAGGAACAAA	GCCGTAGTCTTCGCTCTCAC	177[a], 188	--	10
FRM4	GGGTTTGGTGGAGTGTCAAT	AAAGGCAGATCTGGTGATGC	156, 166	--	10
FR149B	TGTGACAACAGTGACGTGGA	TGCAAGTTTGACCATTGACC	95, 97	111, 113	10
FR367A	TCCCCCAACACAAAATATGC	AAAGGGTCGAGCAGATCGTA	159, null	170, 190	10
FR536B	AGAAACATGCAGTATTTAGAGGAAC	TCCTCTAGGATAATGTTTCCGATT	166, 168	--	16
FR274A	TCGGTACGGGAATCTGTCTC	GGAAAAGGCAGTGAAGAAAGG	244, 246[b]	--	3

LG linkage group

[a] Inherited together with 184-bp fragment

[b] Size with elongated primer (Schuelke 2000)

Genetic mapping and association of markers to fire blight

An array of 270 SNP markers applied to both parents and 114 progeny showed that only five SNPs, representing 1.8 % were heterozygous in *M. fusca* and 82 SNPs, representing 30.4 %, were heterozygous in 'Idared'. Fifty-two already-published SSRs (Liebhard *et. al*. 2002; Silfverberg-Dilworth *et. al*.2006) distributed over the genome, were polymorphic in the population. Of these, 34 were polymorphic in the two parents, six in only *M. fusca* and 12 in only 'Idared'. Together with the six SSRs developed from the published sequence of LG10 of the 'Golden Delicious', 46 SSRs were used for mapping. Additionally, 258 high quality DArT markers of the 448 DArTs polymorphic in *M. fusca* were useful for mapping. In total, 309 markers representing 315 loci were used for mapping. Analysis with JoinMap resulted in 29 linkage groups with at least three markers each. With the exception of LG6, 20 linkage groups could be assigned to the remaining 16 linkage groups of apple by corresponding SSR and SNP markers, comprising a total length of 889 cM consisting of 213 loci. Genetic map of *M. fusca* is given in Appendix 1 (Published as supplementary document).

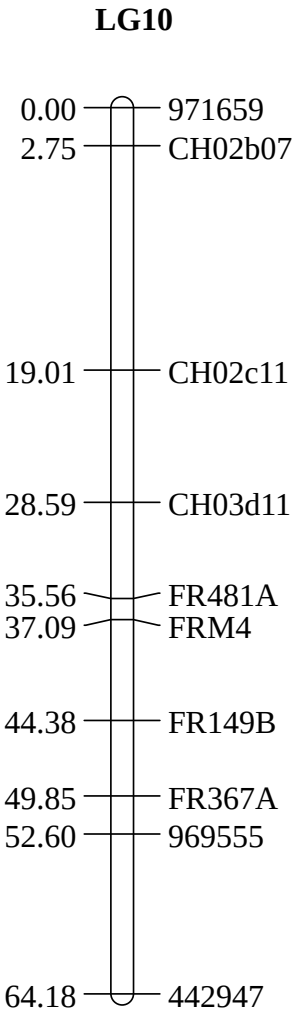


Figure 2 Genetic map of linkage group 10 of *M. fusca* shown after discarding redundant and DArT markers in between SSRs. The genetic distance is given in cM.

Since DArT markers were applied to only 92 progenies and SSR markers were developed to surround the region of interest, a genetic map was constructed containing only SSR markers and the DArT markers at the top and the bottom of LG10. All DArTs in between SSRs were eliminated (Figure 2). Thereafter, a Kruskal-Wallis analysis was performed using the averages of percentage lesion length of the replicates of each genotype for each year of inoculation and for all three years together as numerical traits. Results showed two newly developed SSR markers, FR481A and FRM4, to have the highest significance correlating with fire blight resistance with the highest *K* values in every year (Table 3). Compared with the initial Kruskal–Wallis analysis, the top *K* value increased from 54.7 (Table 1) to > 90 when using the data of all three years together. Progenies possessing the 177-bp allele of FR481A showed an average necrosis rate of 1.96 %, whereas plants expressing the 188-bp allele showed an average necrosis rate of 28.32 %. Similarly, progenies with the 156-bp allele of FRM4 had an average necrosis rate of 2.29 %, compared to 27.87 % average necrosis rate when expressing the 166-bp allele. In correspondence to Kruskal-Wallis analysis, SSR markers FR481A and FRM4 showed the highest association with the QTL as determined by the LOD values obtained after interval mapping using MapQTL® (Van Ooijen 2004) for all three years and the average of all years of inoculation (Table 3). Furthermore, MQM mapping performed with MapQTL® with marker FR481A set as co-factor revealed the presence of a strong QTL on LG10 of *M. fusca* (*Mfu*10) explaining about 65.6 % of the phenotypic variation at a LOD of 31.0 (Figure 3) and a LOD threshold of 5.8.

Table 3 Kruskal-Wallis and LOD values of the interval mapping using the final map of LG10 of *M. fusca* for 3 years of inoculation and for the average of all replicates of each genotype of all 3 years

	2006		2007		2012		2006, 2007, 2012	
Locus	*K*[a]	LOD[b]	*K*	LOD	*K*	LOD	*K*	LOD
971659	3.828	2.27	4.880	4.32	12.605	10.28	6.465	4.17
CH02b07	7.527	2.86	15.062	4.08	24.126*	9.84	17.482*	4.95
CH02c11	42.472*	12.63	45.157*	12.87	40.812*	8.09	51.594*	15.90
CH03d11	56.527*	18.13	68.972*	20.96	64.621*	11.19	73.031*	21.24
FR481A	71.932*	25.22	83.975*	27.77	78.027*	14.92	92.437*	30.98
FRM4	68.404*	23.80	80.992*	26.66	71.428*	13.54	84.084*	27.96
FR149B	54.643*	16.11	69.674*	19.63	59.317*	10.19	68.982*	20.56
FR367A	41.081*	10.62	64.707*	18.85	51.301*	9.86	54.802*	16.01
969555	27.498*	10.66	33.769*	17.53	29.182*	9.23	33.034*	15.17
442947	4.703	11.70	6.523	16.45	6.574	11.88	4.457	13.72

[a] Value of Kruskal-Wallis analysis (*significance level: $p = 0.0001$)

[b] *LOD* logarithm of the odds

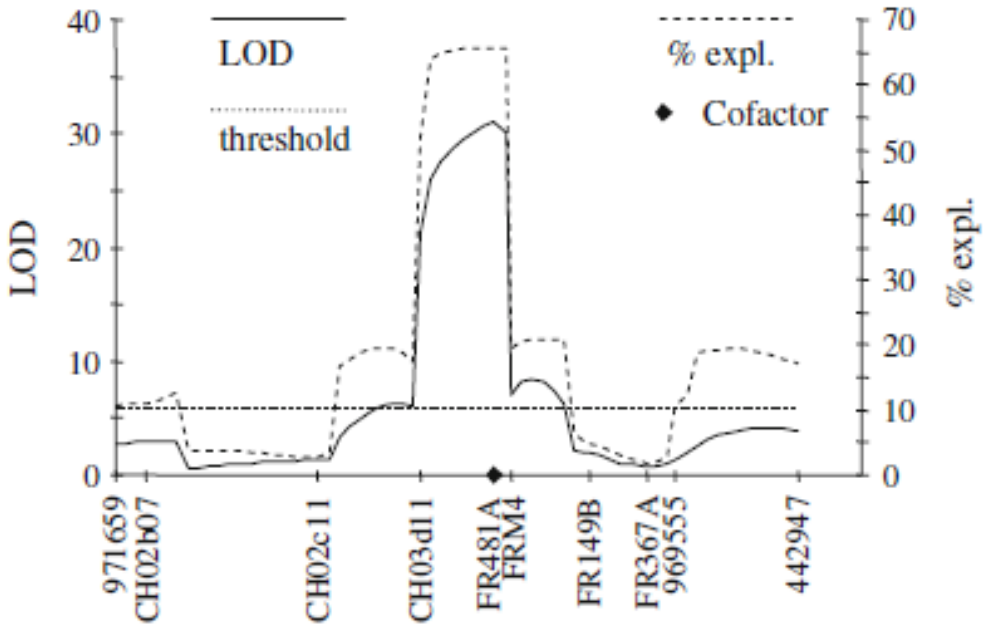


Figure 3 LOD score plot, threshold and percentage phenotypic variation explained (*% expl*.) of the necrosis trait along LG10 of *Malus fusca* determined by MQM mapping with marker FR481A set as co-factor

Discussions

Phenotypic evaluation

A reliable phenotypic evaluation is the most critical aspect in determining QTLs. This is especially true in the case of fire blight, where great variation in the response of replicates to the pathogen could be observed. In the current study, the final degree of tissue area affected was recorded at a time (28 dpi) when the progress of the disease has mostly ceased (Kleinhempel *et al*. 1984; Peil *et al*. 2007). In other studies, disease progress was measured at 7 and 14 dpi (Durel *et al*. 2009; Le Roux *et al*. 2010; Khan *et al*. 2013). Khan *et al*. (2006) determined the area under disease progress curve (AUDPC) by measuring necrosis length at 6, 13, 20 and 27 dpi. The average percentage lesion lengths recorded in the present study in the trial years 2007 and 2012, 9.4 and 9.0 %, respectively, although similar, were significantly lower than 22.6 % - the average PLL calculated in 2006. The reason for this significant difference in average PLL values could not be exactly ascertained as the strain, the concentration of inoculum (10^9 cfu/ml) and inoculation procedure remained the same throughout the years of trial. It is, however, probable that differences in the time of inoculation in the respective years as well as environmental conditions which could affect greenhouse conditions, may have affected *E. amylovora* infestation in the 3 years of trial. The recently published review of Hua (2013) on the modulation of plant immunity by light, circadian rhythm and temperature gives an insight on this assumption.

To our knowledge, this is the first report on mapping of fire blight resistance using the results of 3 years of phenotyping. However, in reporting the confirmation of the fire blight QTL on LG3 of the wild species accession *M.* ×*robusta* 5, Peil *et al*. (2008) recorded almost identical averages of 34.7 and 32.2 % lesion length in 2005 and 2006 respectively. The average PLL of all replicates of the entire three years of phenotyping, 14.4 %, is significantly lower than the

32.9 % average shoot blight reported by Peil *et al*. (2008) for both years of inoculation. The assay of *E. amylovora* used in this study is the same as reported by Peil *et al*. (2007; 2008) with respect to strain and concentration (Ea222; 10^9cfu/ml) and method of disease assessment. Nevertheless, although the values of phenotyping vary between the three years of observation, the results obtained by Kruskal-Wallis analysis and interval mapping always determined the same position of the QTL.

However, the comparison of averages of PLL is difficult even if the same population, inoculation procedures and disease assessment are used, as can be seen for the differences in the 3 years of phenotyping in this study. Furthermore, previous studies reported the use of different bacterial strains for inoculation, for example, CFBP 1430 (Calenge *et al*. 2005; Durel *et al*. 2009; Le Roux *et al*. 2010); Ea610 (Khan *et al*. 2006) and Ea273 (Khan *et al*. 2013), and moreover, the different populations used for these various studies imply that the resistant donors are different.

Molecular markers used for genetic mapping

Recently, DArT, a generic, hybridisation-based, cost-effective fingerprinting method, has been developed (Jaccoud *et al*. 2001). It overcomes some of the known limitations of currently available marker technologies. Initially developed for rice, DArT markers have now been applied to other plant species including barley (Wenzel *et al*. 2004), wheat (Akbari *et al*. 2006; White *et al*. 2008), cassava (Xia *et al*. 2005), *Arabidopsis* (Wittenberg *et al*. 2005), pigeon pea (Yang *et al*. 2006), sorghum (Mace *et al*. 2008), oat (Tinker *et al*. 2009), apple (Soriano *et al*. 2009; Schouten *et al*. 2012) and tobacco (Lu *et al*. 2013). This technology has been developed and utilised for nearly 100 organisms. The present study further demonstrates the performance of DArT markers as a high-throughput genotyping technology in the wild apple species *Malus fusca*. A total of 7,680 clones were established on SuperChip poly-L-lysine slides and images were processed using DArTsoft software as earlier stated, which

resulted in the identification of 1,080 candidate polymorphic DArT markers. This relatively high frequency of polymorphism (14.1 %) in a biparental cross can be attributed to the development of a dedicated array encompassing markers derived from the wild parent. Nevertheless, Schouten *et al*. (2012) reported a similar number of candidate polymorphic markers (i.e. 16 %) for apple with the same complexity reduction method, whereas the *Pst*I/*Bst*NI complexity reduction method resulted in only 10 % candidate polymorphic markers (Schouten *et al*. 2012). Of the aforementioned DArT markers, 29 markers (2.6 %) were not polymorphic in either of the two parents of the progeny and could not be used for mapping.

Of 270 SNPs applied to the mapping population, 183 were not heterozygous in both parents representing 67.7 %. Only five SNP markers were heterozygous in *M. fusca* and 82 SNP markers were heterozygous in 'Idared' – a cultivar of the domesticated apple. This represents 1.8 % transferability from 'Golden Delicious' (*M.* × *domestica*) to *M. fusca* and 30 % transferability to the *M.* × *domestica* cultivar 'Idared', respectively. The transferability of 30 % to 'Idared' falls within the lower range of 25.7 - 67.8 % reported by Micheletti *et al*. (2011) for the transferability of 'Golden Delicious' SNPs (T_{SNP}) to apple cultivars and is in accordance to the value of 25.7 % T_{SNP} found for 'Wagener' (Micheletti *et al*. 2011), a parent of 'Idared'. These authors however, obtained much lower T_{SNP} for the wild apple species *M. sieversii* (mean of 6.4 %) and *M. sylvestris* (mean of 6.1 %), both members of the series *Pumilae* like *M.* × *domestica*. Therefore, the 1.8 % transferability of SNPs to *M. fusca* is in accordance to the greater distance of these wild apple species to 'Golden Delicious'. Twenty-three SSRs have been developed from genomic contigs of 'Golden Delicious' (Velasco *et al*. 2010). These markers were obtained by searching for SSR motifs from the sequences of various contigs immediately surrounding the contig where DArT markers significantly linked to fire blight resistance were localised on chromosome 10 of 'Golden Delicious'. The same

approach was successfully used by Fahrentrapp *et al*. (2013) for the SSR enrichment around the major fire blight QTL on LG3 of *M.* ×*robusta* 5. Six of the 23 SSR developed in this study were polymorphic in *M. fusca* and nine were polymorphic in 'Idared', including the two SSRs polymorphic in both parents. Compared to the transferability of SNPs from 'Golden Delicious' to *M. fusca* (1.8 %) we observed a very high transferability of 26.1 % of SSRs, suggesting that this approach is suitable for the marker enrichment in a given region. Furthermore, regardless of the approach used in their development, SSR markers ought to be relatively simple to use, reproducible and exchangeable between laboratories (Jones *et al*. 1997).

Genetic mapping and QTL analysis

To facilitate the construction of a *M. fusca* genetic map, DArT markers and SNPs polymorphic in *M. fusca* were employed. The application of SSRs permits the reliable identification and orientation of homologous linkage groups among cultivars (Liebhard *et. al*. 2002), hence these markers were sourced and applied in an attempt to standardise the map. A total of 20 linkage groups spanning 889 cM, comprising 209 markers, representing 213 loci, could be constructed. A relatively high number of DArT markers showed identical segregation patterns, hence clustered when mapped and thus were classified as redundant markers. Similarly, redundancy of DArT markers (54 %) was observed by Schouten *et al*. (2012) when mapping in a population derived from 'Prima' × 'Fiesta' using DArT markers. Since the presence of such markers do not provide any further genetic information and lead to statistical overweighting of the particular region (Schouten *et al*. 2012), only one from each cluster of redundant markers was retained and the others discarded in the present study. The elimination of DArT markers between SSR markers shortened the final map of LG10 by 0.63 cM. In contrast, no clustering was observed in other markers such as SNPs and SSRs.

An initial Kruskal-Wallis analysis identified a linkage group with DArT markers showing significant association with fire blight resistance. After sequencing 13 of these DArT markers and aligning the sequences to the 'Golden Delicious' genome, LG10 could be assigned as the corresponding chromosome, although three DArTs aligned with the highest *E* value to other linkage groups. A possible reason is the existence of similar sequences in different regions of the apple genome. To confirm LG10 as the corresponding chromosome, SSR markers, CH02b07, CH02c11 and CH03d11 mapping on LG10 (Liebhard *et al*. 2002), and six SSR markers developed from contigs of LG10 of the 'Golden Delicious' genome, adjacent to the position of DArTs showing the highest correlation with fire blight, were applied to construct a better map of *M. fusca* LG10. Joinmap created a linkage group with 17 of the DArTs and all three SSRs from Liebhard *et al.* (2002), together with all SSRs developed but two. These two SSRs mapped on LGs 3 and 16. In a map construction approach using the *Malus Infinium* whole-genome genotyping array, Antanaviciute *et al.* (2012) found that 13.7 % of all mapped markers conflicted with their predicted regions in the 'Golden Delicious' genome. These authors suggested a misplacement of contigs during assembly of the apple genome or the existence of paralogous genomic regions.

Previously, major QTLs were reported for the apple wild species *M.* ×*robusta* 5 on LG3 (Peil *et al.* 2007) and *M. floribunda* 821 on LG12 (Mf821; Durel *et al*. 2009), the ornamental apple cultivar 'Evereste' on LG12 (Durel *et al*. 2009) and the apple cultivar 'Fiesta' on LG7 (Calenge *et al*. 2005; Khan *et al*. 2006) explaining up to 80, 40, 53 and 46 % of the phenotypic variation, respectively. Besides the QTL for 'Florina' on LG10 (*FLO10*; Le Roux *et al*. 2010), which could explain up to 17.9 % of the phenotypic variation, some minor QTLs have been detected (Calenge *et al*. 2005, Durel *et al*. 2009, Le Roux *et al*. 2010, Peil *et al*. 2011). *Mfu10*, also located on LG10 explains around 66 % of the phenotypic variation, the second highest only behind that of Mr5. Whereas *FLO10* is located in the upper part of LG10

above CH03d11; *Mfu10* is in the region below CH03d11. Therefore, it is expected that both loci are independent.

All major QTLs mentioned above belong to different donors, suggesting that different mechanisms of resistance could be involved, and thus could be pyramided to establish a more durable resistance to fire blight. Exceptions may be the fire blight QTLs from 'Evereste' and Mf821 located closely together on LG12 (Durel *et al.* 2009). Durel *et al.* (2009) proposed that there could be only one resistance gene underlying the QTL and that the genes are allelic or that two distinct, closely linked, resistance genes are present. Pyramiding of different mechanisms is strongly recommended to obtain durable resistance because it was shown, at least for *M.* ×*robusta* 5, that resistance is strain specific and can be overcome (Norelli and Aldwinckle 1986, Peil *et al.* 2011, Vogt *et al*. 2013). Vogt *et al*. (2013) were able to demonstrate that resistance of the accession of *M. fusca* used in this study is not overcome by the avrRpt2$_{EA}$ deletion mutant ZYRKD3-1 breaking resistance of Mr5. This is also true for the highly virulent *E. amylovora* strain Ea3049 (data not shown), proving that different mechanisms underlie the resistances of Mr5 and *M. fusca*. Since less is known of the mechanisms of fire blight resistance of Mf821 and 'Evereste', at least pyramiding of QTLs of Mr5 and *M. fusca* makes sense. To gain more insight in specific QTL-strain interaction, the donors used for introgression of fire blight resistance should be phenotyped with *E. amylovora* strains differing in virulence factors. This strategy can be used to identify QTLs worthy of pyramiding.

The introduction of resistances from wild species in to cultivated apples requires several pseudo-backcrosses to establish advanced pre-breeding material, which takes a long time. Nevertheless, it is important to establish pre-breeding material with different fire blight resistances. Two different strategies are being applied for the introduction of fire blight resistance of *M. fusca*: the use of the fast breeding system to shorten generation cycles which

allows around one generation per year (Flachowsky *et al*. 2011), and conventional breeding. Furthermore, crosses have been performed to pyramid fire blight resistance QTLs of Mr5 and *M. fusca* to prove their interaction.

The QTL reported in this paper does not co-localise with QTLs for fire blight resistance identified in previous studies and has the second strongest effect on fire blight resistance. This is the first report of a fire blight resistance QTL of *M. fusca* and could contribute to pyramided, durable resistance to fire blight. SSR markers surrounding *Mfu10* can be used in marker-assisted selection for pyramiding. In conclusion, it is essential to validate *Mfu10* using strains differing in virulence factors to elucidate the resistance mechanism.

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Chapter Three

The fire blight resistance QTL of *Malus fusca* (*Mfu10*) is affected but not broken down by the highly virulent Canadian *Erwinia amylovora* strain E2002A

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The fire blight resistance QTL of *Malus fusca* (*Mfu10*) is affected but not broken down by the highly virulent Canadian *Erwinia amylovora* strain E2002A

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Abstract

Recently, *Mfu10* - the major QTL for resistance to fire blight was reported on linkage group 10 of *Malus fusca* which could explain about 66 % of the phenotypic variation in a population artificially inoculated with *E. amylovora* strain Ea222_JKI. In the meantime, it had been reported that the resistance of the fire blight QTL of *Malus* ×*robusta* 5 (Mr5), which could explain 80 % of phenotypic variation is strain specific and is completely overcome by the highly virulent strain Ea3049 originating from Canada (E2002A). In contrast to Mr5, the *M. fusca* donor of *Mfu10* is only very slightly affected by this highly virulent strain. In this short communication, we prove that this particular strain is not able to overcome the resistance of *M. fusca* but affects *Mfu10* strongly. The same F1 progenies, derived from a cross between the resistant *M. fusca* and the susceptible apple cultivar, Idared, used to detect *Mfu10*, was used for phenotyping with Ea3049. Although the mean shoot necrosis of all progenies was 62.4 %, marker-phenotype association determined by Kruskal-Wallis analysis showed that markers on LG10 correlate significantly to resistance levels with SSR markers FR481A and FRM4 having the highest *K* values of 37.1 and 36.7, respectively. Interval mapping and multiple QTL mapping (MQM) performed with MAP-QTL®, showed that *Mfu10* could still be detected on the same position on LG10, but explaining only 41.2 % of phenotypic variation. The implications of these results are discussed.

Keywords: *Mfu10*, *Malus fusca*, *Erwinia amylovora*, fire blight resistance, QTL mapping

Erwinia amylovora (Burrill) Winslow *et al*. has been known to incite fire blight which is regarded as the most destructive bacterial disease of *Malus*, for over two centuries, yet no sustainable control measure is available till date. The most promising strategy to defeat the disease could be the development of cultivars with durable resistance against the pathogen. Nevertheless, this has proven difficult to accomplish due to the fact that resistance is strain-dependent. There is therefore a need to identify donors that are resistant to very highly virulent isolates of *E. amylovora*. In the genus *Malus*, donors bearing major or minor quantitative trait loci (QTL) have been identified using different *E. amylovora* strains (Calenge *et al*. 2005; Khan *et al*. 2006; Peil *et al*. 2007; Durel *et al*. 2009; Le Roux *et al*. 2010; Emeriewen *et al*. 2014; Wöhner *et al*. 2014).

This report will focus on the major QTL identified in *M. fusca* (*Mfu10*) in comparison to the major QTL of *M.* ×*robusta* 5 (Mr5). While the Mr5 QTL is located on linkage group (LG) 3 and explained up to 80 % of phenotypic variation (Peil *et al*. 2007), *Mfu10* is located on LG10 and explained about 66 % of phenotypic variation of the trait necrosis (Emeriewen *et al*. 2014). It is important to note that while both QTLs were detected using different segregating populations, the same *E. amylovora* isolate Ea222_JKI was used for phenotyping. Although the Mr5 QTL has the highest effect of all QTLs previously described, it has been overcome by the highly virulent Canadian strain Ea3049 and the QTL on LG3 breaks down (Peil *et al*. 2011; Wöhner *et al*. 2014). In this short communication, we report the stability of *Mfu10* after phenotypic evaluation of the original segregating population using the highly virulent Canadian strain Ea3049. The results presented here highlight the importance and usefulness of *Mfu10* in establishing durable resistance against the very destructive fire blight disease of *Malus*.

Initially, a progeny population of 134 individuals was derived from a cross between the wild apple species accession *M. fusca* (MAL0045) and the apple cultivar, Idared (Emeriewen *et al*. 2014). For phenotypic studies, up to nine replicates each of the same 134 progeny individuals and the parents *M. fusca* and Idared were grafted onto rootstock M9. Plants were grown in the greenhouse and actively growing shoots with a minimum length of 25 cm were inoculated by incising the two youngest leaves with a pair of scissors dipped in bacterial suspension. Artificial inoculation was performed using the Canadian strain E2002A (strain numbers in different collections: Ea3049, Ea395, CFBP 3049, CUCPB 265 – according to the denomination at JKI this strain will be named as Ea3049 in this paper) at a concentration of 10^9 cfu/ml. Four weeks after inoculation, percentage lesion length (PLL) was determined as part of the necrotic shoot on total shoot length. Results of phenotypic evaluation showed that whereas an average of 1.5 % lesion length was recorded for the resistant parent, *M. fusca*, an average of 92 % was recorded for the susceptible parent, Idared. The mean PLL recorded for all 134 progeny was 62.4 % with a median of 69 %. The standard deviation for the replicates of one genotype ranged from 0.0 to 43.3. 100 % average lesion length was observed for eight progenies with 2 % recorded as the lowest PLL for only one progeny. In total, above 50 % lesion length was recorded for 75 progenies.

In Figure 1, a pairwise comparison of the PLLs obtained after inoculation of the progenies with *E. amylovora* strains Ea222_JKI (data from Emeriewen *et al*. 2014) and Ea3049 are presented. The highest PLL observed with Ea222_JKI was 75 % whereas that of Ea3049 was 100 %. A Pearson's correlation of $r = 0.62$ was calculated using SAS Enterprise Guide 4.3 (SAS Institute Inc, Cary, NY, USA) for the phenotypic values of the progenies after inoculation with both strains. The averages of all replicates of each genotype were used as numerical traits to determine marker-phenotype association and to perform interval mapping and multiple QTL mapping (MQM) using MAP-QTL® 5 (Van Ooijen 2004). The linkage map

of *M. fusca* developed by Emeriewen *et al*. (2014) was used as template for MAP-QTL® 5 (Van Ooijen 2004). Kruskal-Wallis analysis revealed that only markers mapping on LG10 were significantly correlated with the resistance levels. SSR markers FR481A and FRM4 possessed the highest *K* values of 37.1 and 36.7, respectively, in this group (Table 1). The differences of PLL determined for the alleles in coupling and in repulsion are listed in Table 1. The greatest difference, i.e. 36.2 %, was observed for markers CH03d11, FR481A and FRM4. Multiple QTL mapping revealed only one QTL with a significant LOD-score. This QTL, being at the same position on LG10 of *M. fusca* like *Mfu10* (Emeriewen *et al*. 2014), could explain about 41.2 % of the phenotypic variation at a logarithm of the odd (LOD) of 11.9 (Figure 2). No minor QTL could be detected on other linkage groups.

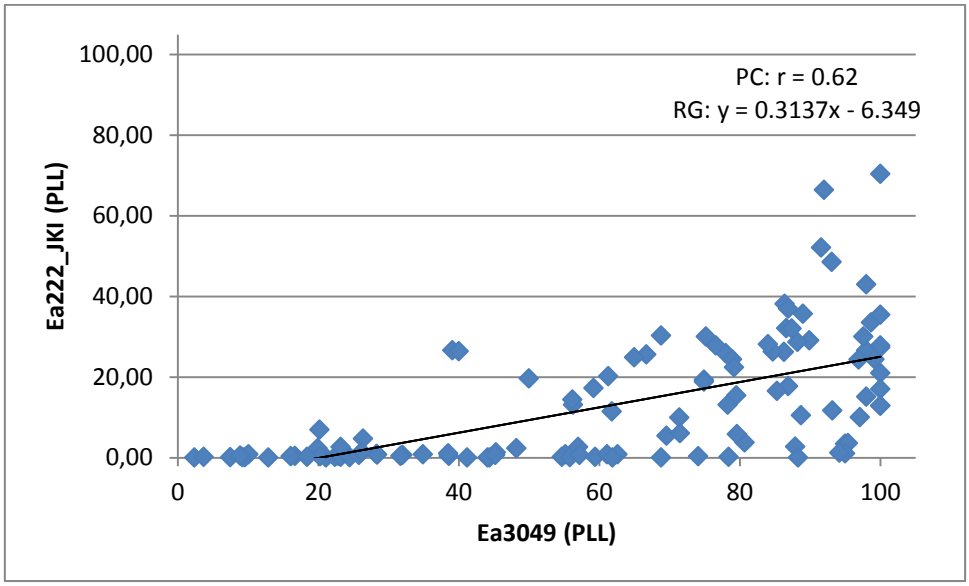

Figure 1 Pairwise comparison of PLL of *M. fusca* × 'Idared' progenies after inoculation with *E. amylovora* strains Ea3049 and Ea222_JKI

PLL: percentage lesion length; PC: Pearson's correlation coefficient; RG: regression line function

Table 1 Kruskal-Wallis analysis of linkage group 10 of *Malus fusca* after inoculation with Ea3049

Locus	Position	*K*	PLL of alleles in:	
			coupling	repulsion
971659	0	4.702**	54.3	71.7
CH02b07	2.75	11.174*****	51.7	73.7
CH02c11	19.01	24.799*******	47.5	79.2
CH03d11	28.59	35.804*******	46.0	82.2
FR481A	35.56	37.109*******	46.8	83.0
FRM4	37.09	36.745*******	47.1	83.3
FR149B	44.38	32.805*******	47.1	80.9
FR367A	49.85	25.336*******	50.7	79.9
969555	52.60	10.583****	52.1	76.5
442947	64.18	0.076	-	-

K Value of Kruskal-Wallis analysis (significance levels: ** = 0.05, **** = 0.005, ******* = 0.0001)

Locus and map position as in Emeriewen *et al*. (2014); PLL: percentage lesion length

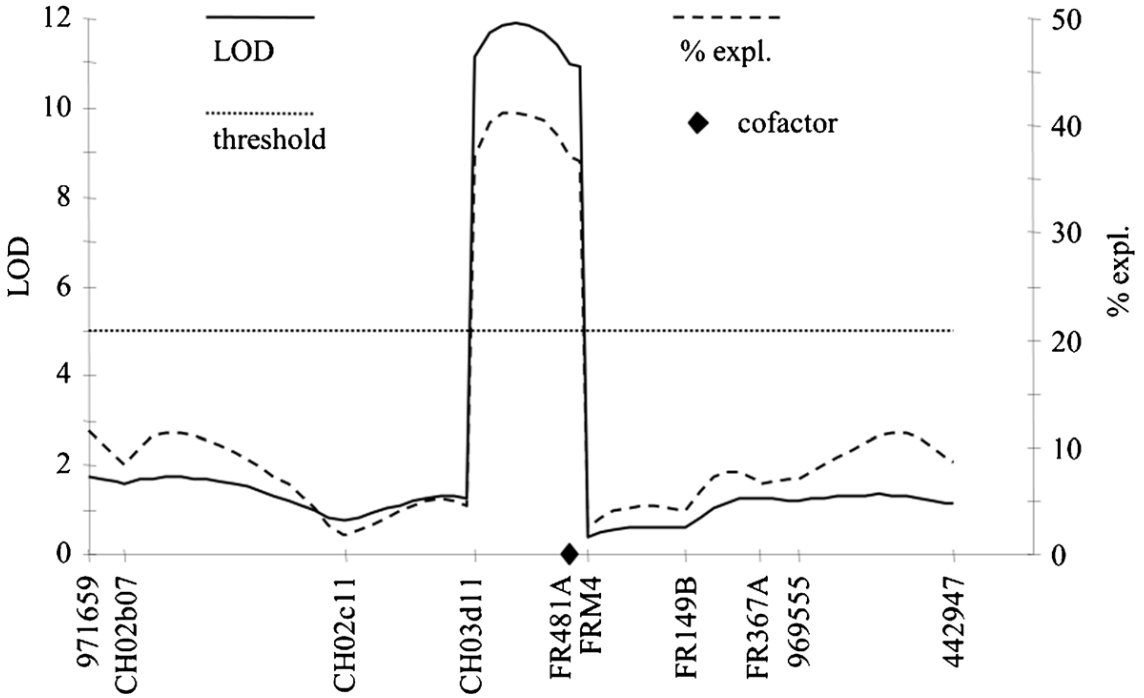

Figure 2 LOD score plot, threshold and percentage phenotypic variation explained (*% expl*.) of the necrosis trait along LG10 of *M. fusca* determined by MQM with marker FR481A set as cofactor. *Mfu10* explained 41.2 % of phenotypic variation after inoculation with the very highly virulent *E. amylovora* strain Ea3049.

Natural resistance to *Erwinia amylovora* is a desirable alternative strategy for the sustainable management of fire blight. Resistance QTLs have been described in wild apple species accessions including *M.* ×*robusta* 5 (Mr5; Peil *et al*. 2007; 2008), *M. floribunda* clone 821 and the ornamental cultivar, Evereste (Durel *et al*. 2009), and recently *M. fusca* (Emeriewen *et al*. 2014); explaining 80, 40, 53 and 66 % of phenotypic variation respectively. Resistance to fire blight has been reported to be strain-specific for at least some accessions (Norelli and Aldwinkle 1986). Recently, Vogt *et al*. (2013) described how just a single nucleotide polymorphism (SNP) in the $avrRpt2_{EA}$ effector of *E. amylovora* was responsible for virulence and avirulence on Mr5 by means of substituting Cysteine (C) amino acid to Serine (S) at position 156 of the $avrRpt2_{EA}$ protein sequence. Consequently, the resistance QTL of Mr5 located on LG3 was overcome by the highly virulent S-allele carrying *E. amylovora* strain Ea3049 (Peil *et al*. 2011; Wöhner *et al*. 2014). The QTL of Mr5 explaining up to 80 % of phenotypic variation was detected using strain Ea222 (Peil *et al*. 2007), bearing the C-allele (Vogt *et al*. 2013), the same strain used to identify the resistance QTL of *M. fusca* (Emeriewen *et al*. 2014.). The function of the resistance gene, *FB_MR5* (Fahrentrapp *et al*. 2013), underlying the Mr5 QTL was recently proven by Broggini *et al*. (2014). Since *FB_MR5* and *Mfu10* are located on different linkage groups, a different mechanism was assumed. This assumption was confirmed by Vogt *et al*. (2013) when, in describing the gene-for-gene relationship between Mr5 and *E. amylovora*, reported that the same *M. fusca* accession (MAL0045) used to detect *Mfu10* was resistant to Ea3049. To gain more insight, the progeny used to identify *Mfu10* was inoculated with the highly virulent Ea3049 strain in order to ascertain whether the resistance QTL is stable in the progeny.

The phenotypic evaluation after inoculation with Ea3049 revealed a much higher average PLL (62.4 %) on the progeny compared to the results of Emeriewen *et al*. (2014) where averages of 9.4, 9.0 and 22.6 % were reported respectively in the trial years of 2006, 2007 and

2012 but using the C-allele strain Ea222_JKI. Wöhner *et al*. (2014) measured an even higher mean PLL (80.4 %) in a population of Idared × Mr5 after inoculation with Ea3049. However, these authors recorded a PLL correlation of 0.31 between Ea3049 and Ea222_JKI used for inoculations in their study. This is significantly lower than the correlation (0.62) calculated in this study between the S-allele strain Ea3049 and the C-allele strain Ea222_JKI. Kruskal-Wallis analysis and interval mapping performed using the *M. fusca* map (Emeriewen *et al*. 2014) as template and the phenotypic data obtained after inoculation with *E. amylovora* strain Ea3049, revealed only markers on LG10 correlating significantly with resistance levels. Multiple QTL mapping was then performed using SSR marker FR481A as cofactor. The major QTL on LG10 (*Mfu10*) could be confirmed although the phenotypic variation explained by the QTL decreased from 65.6 % at a LOD of 31.0 (Emeriewen *et al*. 2014) to 41.2 % at a LOD of 11.9 (Figure 2). The position of the QTL remained the same as previously reported with SSR markers FR481A and FRM4 appearing underneath the LOD plot and bracketed by two other SSR markers; CH03d11 to the right and FR149B to the left. This is different from the situation in Mr5 as the major QTL on LG3 of Mr5 completely broke down after inoculation with Ea3049 (Peil *et al*. 2011; Wöhner *et al*. 2014). Furthermore, Wöhner *et al*. (2014) instead detected minor effect QTLs on linkage groups 5, 7, 11 and 14 of Mr5. No other minor QTLs could be detected in this study. Nevertheless, there is a discrepancy between the behaviour of *M. fusca* and the *M. fusca* × Idared progeny after inoculation with the highly virulent *E. amylovora* strain Ea3049. Although the resistant parent - *M. fusca* is only minimally affected after inoculation with Ea3049, the progenies showed a high average PLL, indicating that other genetic factors are contributing to resistance of *M. fusca* to Ea3049. But nevertheless, progenies which inherited the alleles in coupling to *Mfu10* showed in average a 36 % lesser PLL than the progenies with the alleles in repulsion.

The results presented here clearly shows that the highly virulent Ea3049 strain is not able to breakdown the resistance of *Mfu10*, although it proved to be a more virulent strain as a higher level of necrosis could be observed and recorded for the progeny leading to the decrease in the phenotypic variation explained by the QTL. These results confirm previous assumptions that different mechanisms underline the resistance QTLs of *M. fusca* and *M.* ×*robusta* 5.

It further gives support to our suggestion that it is worthy pyramiding the resistance QTLs of *M. fusca* and Mr5 so as to obtain a more durable resistance; however, genotypes have to be selected showing the lowest PLL after inoculation with Ea3049.

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Chapter Four

Fine mapping of the *Malus fusca* fire blight resistance locus (*Mfu10*)

Manuscript to be submitted as Emeriewen *et al*. 'Fine mapping of the *Malus fusca* fire blight resistance locus (*Mfu10*)

Abstract

Breeding of cultivars that are resistant to the very destructive fire blight disease is an important aim in most European apple breeding programs. Since most popular apple cultivars are highly susceptible to the disease, some wild apple accessions have been explored for resistance resulting in the detection of major and minor quantitative trait loci in several wild species accessions including *Malus fusca*. It has been previously reported that the resistance to fire blight in *M. fusca* is explained by a major quantitative trait locus located on chromosome 10 (*Mfu10*) of the apple genome which explained about 66 % of the phenotypic variation. Here we report the development and saturation of the QTL region with SSR markers in order to determine more precisely the location of *Mfu10*. The *M. fusca* × Idared progeny population was substantially increased to 1,336 progeny individuals and the recombinants determined by applying the two closest SSRs surrounding *Mfu10* were phenotyped by inoculation with *Erwinia amylovora* strain Ea222_JKI at a concentration of 10^9 cfu/ml. Sixteen markers were developed and mapped in the given interval using the 'Golden Delicious' and the *M. fusca* genome sequences as templates. Linkage mapping showed that the region of interest has been reduced to 0.47 cM. Further, QTL mapping performed demonstrated that the percentage variation explained by *Mfu10* increased from 66 to about 77 % with newly developed SSR markers significantly correlating and associating with the QTL. SSR markers are currently being used for marker assisted breeding/selection. The work reported here paves the way for the map based cloning of the underlying resistance gene of *M. fusca*.

Keywords: Fire blight, *Malus fusca*, *Mfu10*, fine mapping, SSR markers, recombinant individuals

Introduction

The most serious threat to apple production is fire blight, considered as the most dreadful bacterial disease affecting its production. Incited by the bacterial pathogen, *Erwinia amylovora* (Burrill) Winslow *et al*., as many as forty six countries have reported the incidence of fire blight (Bonn and Van der Zwet 2000) in apple (*Malus* × *domestica*) and pear (*Pyrus communis*) orchards causing huge losses to production. The pathogen like other Gram-negatives, possesses the type III secretion (T3SS) pathogenicity island (PAI) which is encoded by a cluster of hypersensitive response and pathogenicity (*hrp*) genes, that deposits effector proteins into the host and controls the ability of the pathogen to cause disease in susceptible host plants (Khan *et al*. 2012). *E. amylovora* enters the hosts through natural openings like nectarthodes or wounded tissues aided by ants, flies or rain (Thomson 2000) and can migrate into the stem down to the roots (Bogs *et al*. 1998). Primary infection occurs during flowering and depending on the degree of susceptibility of a cultivar, could spread within the entire tree in a single growing season. Once established in an orchard, fire blight can cause severe damages of trees leading to economic losses. Fire blight has proven over the years to be very difficult to manage. Some preventative control measures employed by pome fruit growers include rigid pruning of affected tissues and the use of antibiotic sprays. However, till date, no sustainable control measure exits. Since commercial apple cultivars differ in susceptibility to fire blight infection, best results of the disease management is obtained in cultivars with decreased susceptibility (Malnoy *et al*. 2012).

Natural immunity to *Erwinia amylovora* is a desirable trait and could prove to be a reliable strategy for the sustainable management of the disease. Nonetheless, most commercial apple cultivars are susceptible to the disease and hence several breeding programs have been established aimed at introgressing fire blight resistance into commercial apple cultivars from wild relatives. Genetic resistant donors which confer resistance to fire blight by possessing

minor or major quantitative trait locus (QTL) have been described in wild apple species accessions and apple cultivars (Calenge *et al*. 2005; Khan *et al*. 2006; Peil *et al*. 2007; Durel *et al*. 2009; Le Roux *et al*. 2010; Emeriewen *et al*. 2014; Wöhner *et al*. 2014). The strongest QTLs reported in wild apple species are located on linkage group 3 (LG3) of *Malus ×robusta* 5 (Mr5; Peil *et al*. 2007; 2008) and LG10 of *Malus fusca* (Emeriewen *et al*. 2014) explaining 80 and 66 % respectively of the phenotypic variation to the pathogen isolate Ea222. Resistance to fire blight is strain-specific (Norelli and Aldwinkle 1986). Peil *et al*. (2011) described the break-down of the major QTL on LG3 of Mr5 and the presence of a minor QTL on LG5 after inoculation with the highly virulent Canadian strain Ea3049. Besides, Wöhner *et al*. (2014) added more markers to the Mr5 linkage map and could identify additional minor QTLs on LGs 7, 11 and 14 of Mr5 after inoculations with Ea3049, ZYRKD3-1 and ZYRKD3-1 (pZYR2-415) while confirming the breakdown of the major QTL on LG3. The isolates that broke down the resistance of Mr5 all possessed the S-allele of the *avrRpt2*$_{EA}$ effector of *E. amylovora* (Vogt *et al*. 2013), in contrast to the C-allele isolate Ea222 which was used to detect the Mr5 QTL in the first instance.

During host-plant interactions, pathogenic effectors, for example, avirulence (Avr) protein such as type III effector protein avrRpt2$_{EA}$ (Vogt *et al*. 2013) play critical roles via the type III secretion system (T3SS) in regulating plant susceptibility, stimulating growth and circulating the pathogen (Khan *et al*. 2012; Malnoy *et al*. 2012). Moreover, Vogt *et al*. (2013) while proposing a gene-for-gene relationship between Mr5 and *E. amylovora*, demonstrated that just a single nucleotide polymorphism (SNP) in the *avrRpt2*$_{EA}$ effector of *E. amylovora* was responsible for virulence and avirulence on Mr5 by means of substituting Cysteine (C) amino acid to Serine (S) at position 156 of the *avrRpt2*$_{EA}$ protein sequence. These results were supported by the fact that the *avrRpt2*$_{EA}$ deletion mutant ZYRKD3-1 (Zhao *et al*. 2006) also breaks the resistance of Mr5 whereas the wild type does not (Vogt *et al*. 2013). The

breakdown of resistance of a major QTL as Mr5 by just a single SNP reinforces the essence of breeding aimed at pyramiding different fire blight resistance QTLs in apple cultivars (Emeriewen *et al*. 2014). The *M. fusca* QTL on LG10 (*Mfu10*) can play a critical role in pyramided resistance. The stability of *Mfu10* was proven and its importance highlighted when it did not break down, after inoculation with the resistance breaking isolate Ea3049; even though the QTL was clearly affected since the phenotypic variation explained after inoculation with Ea3049 was 41.2 % (Emeriewen *et al*. 2015) in contrast to 66 % using the isolate Ea222 (Emeriewen *et al*. 2014).

Regardless of the reported breakdown of the resistance QTL of Mr5, the most substantial progress in terms of elucidating resistance mechanisms against fire blight in the genus *Malus* has been achieved with this wild apple clone. In addition to the gene-for-gene interaction between Mr5 and *E. amylovora* (Vogt *et al.* 2013), a candidate gene for fire blight resistance in *Malus* ×*robusta* 5 was described by Fahrentrapp *et al*. (2013). The gene, *FB_MR5*, an NBS-LRR gene, was hypothesized to monitor RIN4 from Mr5, a homolog to RIN4 of *Arabidopsis thaliana* (Fahrentrapp *et al*. 2013). These authors suggested a decoy/guard model of *FB_MR5*, RIN4 and avrRpt2$_{EA}$ acting in a similar manner like the RPS2, RIN4 and avrRPT2 model in the host-pathogen system *Arabidopsis thaliana-Pseudomonas syringae*. Furthermore, *FB_MR5* is the first, and currently the only functionally characterised fire blight resistance gene of apple (Broggini *et al*. 2014). In recent past, Parravicini *et al*. (2011) identified *in silico* two genes as the most probable fire blight resistance genes from the ornamental apple cv. 'Evereste' in a cluster of eight resistance gene analogs, seven Ser/Thr kinases and one CC-NBS-LRR. Both genes showed homology with the *Pto/Prf* complex in tomato (Parravicini *et al*. 2011), which confers resistance to *Pseudomonas syringae*. However, no functional confirmation of one of these genes conferring resistance to fire blight has been reported till date.

The saturation of a given resistance locus of interest with molecular markers is an important aspect towards uncovering the genes responsible for any agronomic trait. This critical step was successfully undertaken prior to the identification and subsequent characterization of *FB_MR5* (Fahrentrapp *et al*. 2013; Broggini *et al*. 2014). The development of such markers as SSRs (simple sequence repeats) linked to a particular resistance locus is equally important for marker assisted selection/breeding (MAS; MAB) prior to the introgression of traits into related cultivars. For example, the development of new apple cultivars that are resistant to diseases (e.g. fire blight and apple scab) relies on the use of MAS. The accessibility of such molecular markers linked with quality QTLs will enhance comparative mapping studies as well as studies aimed at testing for their validity and stability in different populations (Khan *et al*. 2013). Robust genomics tools are now available that enhance the identification of putative resistance genes and development of molecular markers required for marker-assisted selection/breeding (Velasco *et al*. 2010).

The introgression of traits of fire blight resistance into apple, using classical systems, is particularly difficult due to the polygenic nature of genetic resistance mechanisms (Khan *et al*. 2012). Moreover, classical apple breeding approach, with the aim of introgressing traits like fire blight resistance into commercial cultivars is time consuming since it requires several pseudo-backcrosses to establish advance pre-breeding material (Emeriewen *et al*. 2014); and also due to the long juvenile phases that require several decades to attain the desired characteristics. A feasible alternative is the introduction of resistance genes into apple cultivars by means of genetic modification. With the progress in apple research, the first resistance genes originating from apple have been identified, transformed in to apple cultivars and functionally confirmed. This includes the two scab resistance genes *Rvi6* (Belfanti *et al.* 2004) and *Rvi15* (Schouten *et al*. 2014) and the fire blight resistance gene *FB_MR5* (Broggini *et al*. 2014). The next step in apple transformation has been achieved with the establishment

of cisgenic ‘Gala’. The cisgenic ‘Gala’ contains the scab resistance gene *Rvi6* under the control of its own regulatory sequences with the selection marker excised by a chemically inducible *Cre/loxP* recombinase system (Vanblaere *et al*. 2011; 2014). A heat inducible Flp/*FRT* recombinase system for the elimination of the marker gene has been recently established (Herzog *et al*. 2012) and optimized (Würdig *et al*. 2013) in apple. The recombinant DNA-technology delivers not only a method for functional verification of candidate genes but also a tool for the improvement of established cultivars. The cisgenic approach is favoured because it could generate resistant cultivars with advantages to the environment and producer and should not raise as much concern as transgenic apples to the consumers (Broggini *et al*. 2013).

Here, we pave the way towards the identification of the underlying gene(s) by fine mapping the region of the fire blight resistance QTL located on LG10 of *Malus fusca* (Emeriewen *et al.* 2014) using chromosome walking approach. The region of interest of the resistance locus was enriched with SSR markers developed using the ‘Golden Delicious’ (GD) reference genome and an annotated *M. fusca* sequence and applied on a larger population of individuals derived from a cross between *M. fusca* and the susceptible apple cultivar ‘Idared’. This allowed for the identification of recombinant individuals whose phenotypic evaluation ensured that the exact position of the QTL was well defined. It is imperative to identify an alternative and supplement gene to the Mr5 fire blight resistance gene. This is particularly necessary since the resistance of *FB_MR5* is strain specific with a different resistance mechanism to *Mfu10*.

Materials and methods

Plant material and DNA extraction

The initial mapping population derived from a cross between the fire blight resistant *Malus fusca* and the susceptible *M.* × *domestica* cultivar 'Idared' as previously reported by Emeriewen *et al*. (2014) was substantially increased to a total of 1,336 individuals (Table 1). Besides the initial mapping population where cetyltrimethylammonium bromide (CTAB) DNA extraction procedure was used (Doyle and Doyle 1987), DNA was extracted by adding a piece of leaf (4 mm diameter) into 50 µl extraction solution (Sigma Aldrich, Hamburg, Germany) followed by heating in a thermo cycler for 10 min at 95 ^{o}C and then adding 50 µl of Extract-N-Amp plant dilution solution (Sigma Aldrich, Hamburg, Germany) before removing the leaf material. DNA was diluted 1: 5 and stored in -20^{o}C until required for PCR.

Table 1 Three populations used in this study with a total number of 1,336 progeny individuals

Population name	Cross	Number of individuals	Recombinants between CH03d11 and FR149B
05210	*Malus fusca* × Idared	134	17
12228	Idared × *Malus fusca*	205	26
12229	*Malus fusca* × Idared	997	103
		1336	145

Identification of recombinant individuals

The 12228 and 12229 populations, 1,202 individuals in total (Table 1), were screened with four of the SSR markers located on LG10 of *M. fusca*; firstly with CH03d11and FR149B in order to identify individuals showing recombination events in the interval between CH03d11 (28.59 cM) and FR149B (44.38 cM), and additionally with FR481A and FRM4 (Emeriewen *et al*. 2014) to precise their linkage to resistance. Application of these markers was performed using a PCR approach in a volume of 10.2 µl comprising 1 × PCR buffer (GeneAmp, Applied Biosystems, Monza, Italy), 2.4 mM $MgCl_2$, 0.05 mM dNTPs, 0.75 U AmpliTaq Gold DNA polymerase (GeneAmp, Applied Biosystems, Monza, Italy), 2 mM SSR primer mix (forward and reverse primers) with the following profile: 94 ^{o}C for 10 min, followed by 30 cycles of 94 ^{o}C for 30 s, 60 ^{o}C for 1 min and 72 ^{o}C for 1 min, and an extension of 72 ^{o}C for 5 min. PCR fragments were analysed on a 3730xl DNA analyser (Applied Biosystems, Vienna, Austria).

Fire blight phenotypic evaluation of recombinant individuals

Recombinant individuals identified after genotyping the entire 12228 and 12229 populations with markers FRM4, FR481A, FR149B and CH03d11 were phenotypically evaluated. Up to 10 replicates of each recombinant individual were grafted onto M9 rootstock and grown in the greenhouse before being transferred to the quarantine greenhouse where artificial *E. amylovora* inoculations were carried out with the parents; *M. fusca* and 'Idared' as controls. Inoculation was performed by incising the two youngest leaves with a pair of scissors dipped into *E. amylovora* inoculum strain Ea222_JKI at a concentration of 10^9 cfu/ml (Peil *et al*. 2007; Emeriewen *et al*. 2014). Shoot length and lesion length of replicates of each genotype were measured 28 days post inoculation (dpi), transformed into percentage lesion length per shoot (PLL) as initially described by Peil *et al*. (2007), and then averaged. All inoculations were carried out at the Julius Kühn-Institut, Quedlingburg, Germany.

SSR marker development, application and fine mapping of the resistance locus

SSR markers FRM4 and FR481A mapping on chromosome 10 of *M. fusca* were the closest and most significantly linked with *Mfu10*, (Emeriewen *et al*. 2014). In order to saturate the region close to the markers, new SSRs were developed from contigs surrounding the position of both SSR markers. After précising the interval of *Mfu10*, SSR markers were developed from contigs of 'Golden Delicious' positioned in this interval. Further, a single *M. fusca* BAC clone detected with FR481A was isolated, sequenced and was used as template for the development of new SSRs. In general, primer pairs flanking SSR sequences in the contigs were developed using Primer 3 (Rozen and Skaletsky 2000). To test for polymorphism, developed microsatellites were first tested on both parents and a subset of six progenies of the 05210 population, three of which inherited the 156 allele of FRM4, in coupling with resistance, and the other three inheriting the 166 allele, in repulsion to resistance. Polymorphism was tested according to Schuelke (2000). When polymorphic and segregating

properly among the six selected individuals, forward primers of the SSR markers (Table 2) were ordered with labelled dyes; BMN-5, BMN-6 or DY751 to enable multiplexing of primers and tested on the entire 05210 population, analysed and then mapped, in order to ascertain their position on LG10 before screening on the recombinant individuals of the 12228 and 12229 populations. Multiplex PCR was performed using the Type-It-Kit (Qiagen, Hilden, Germany) in a 10µl volume. PCR conditions were the same as previously described by Emeriewen *et al*. (2014). Amplified PCR fragments were diluted 1:100 and analysed on the CEQ 2000XL Genetic Analysis System (Beckman Coulter, Germany).

Table 2 SSR markers developed from the 'Golden Delicious' Pseudo-chromosome 10 (Velasco *et al*. 2010) to fine map the QTL region of *Mfu10*

SSR marker name	Forward primer	Reverse primer	Allele size (bp) *Malus fusca*	Allele size (bp) Idared
FR19B	GCTATACAGCTACAGCAAGCAGA	GCATGGAATCTTTTTATTCCCTTA	201[a], 213	210, 222
FR20C	AGTATGGGGTGACATGCAGA	CCCTCTCTCTTCCCCTCATC	162, 164	168[b]
FR20D	CCTTGCTTGCATTATCTCAGC	AAATGTCGGCAAGTCCACTC	123, 125	149[b]
FR22A	CGGGAACAAAACCAAGAAGA	TCCAATGTTGCAAAAGCAAA	210, 212	218[b]
FR22Ai	GGCCATCCACTGTCTTCTGT	CGGCCTCTTGCCATATCTTA	228, 235	224[b]
FR202	CCTCCAACAATTCACCAACC	TTGTCGCCATAGTTGCTCAG	201[a], 222	178[b]
FR132	GGCACAAGACTGACATGAATC	GACCACGAACTTGATGAGCA	210[a], 241	212[b]
FR210	TATTTCTGTGCCCGCCTTCT	GCTTCAAGGGCACGATGT	185[a], 191	212, 214
OFE	AAGGCCGAGGTTGGTAAGAC	TGGTTGCATTCACTTTGAGG	185, 191[a]	176, 196
FR21Dii	GAGGTAGGGTGGGGTTGATT	ACTTTGCGCCATGTTGATAA	Null, 141	164[b]
FR21BB	GCTCGATCTGGTGGTGATTG	CAAGGAAAACGTGGCCATCA	199, 205	--
FR21T-nu	AACCTCGAATGTTGCTGCTC	ATAAGGGACAGGGCATGAGG	137, 181	--
FR342i	GCAAGCCCTGTAAATGCAAC	CAAATCAGATACATGGGCGGA	210, 214	--
YO_FC1**	CCGTTGCCTTTACGAACACT	GAGCAGAGCAGAGAGAGTGGA	244[a], 248	203[b]
V_BORE	AGTAAACTCGATCCCCACGA	CCCTGTGCTCCTGACATACC	190[a], 197*	--
ISY213	CCATCAGGTACTGCAAAGCA	AGTACCAAGCAACTGATGCAA	233, 238[a]	190, 196

[a] Size with elongated primer (Schuelke 2000)

[b] Monomorphic in 'Idared'

*Inherited together with 283-bp fragment

**Developed from the *M. fusca* BAC clone Mfcl_FR481A

Statistical analyses

Phenotypic data were transformed into binary data marker as described by Durel *et al*. (2009). The prepared genotypic and phenotypic binary marker data were mapped with JOINMAP 3.0 (Van Ooijen and Voorips 2001) using the mapping function of Kosambi with a logarithm of the odd (LOD) threshold of 5.0 for grouping. Kruskal-Wallis analysis was performed to determine marker-phenotype association followed by interval mapping and multiple QTL mapping (MQM) using MAP-QTL (Van Ooijen and Maliepaard 1996).

***Malus fusca* BAC library construction, screening and clone sequencing**

The *M. fusca* bacterial artificial chromosome (BAC) library was produced using high molecular weight genomic DNA prepared at Amplicon Express (Pullman, Washington, USA) as described by Tao *et al*. (2002). DNA was partially digested with *Hind*III, size selected and ligated into pCC1BAC® vector (Epicentre, Madison, USA) and transformed into DH10B *E. coli* cells. Clones were arrayed on 384-well plates with LB media and frozen. The BAC library screening was performed in two separate rounds of PCR on extracted DNA from independently grown, separately pooled BAC clones using SSR primers of FR481A, FRM4, FR22Ai, FR22A, FR20D and FR20Dii. The first round of the PCR was performed on 14 Superpools containing all BAC clones in the *M. fusca* library. The second round of PCR was then performed on the respective matrix pools to determine plate, row and column; each position representing single BAC clones, reacting positively to the applied SSR. PCR for the two rounds of screening were performed as described above depending on the DNA-sequencer used to analyse the PCR fragments; CEQ 2000XL Genetic Analysis System (Beckman Coulter, Germany) or 3730xl DNA analyser (Applied Biosystems, Vienna, Austria). BAC clones were cultured in LB medium overnight at 37 ^{o}C. Plasmid DNA was thereafter extracted from clone cultures using NucleoBond® Xtra MIDI Plasmid DNA Purification Kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's

protocol. The BAC clone identified with FR481A was sequenced by the sequencing platform of the Edmund Mach Foundation, Italy, using 454-pyro-sequencing. Assembly of the derived sequences was performed using Mira software available at (http://www.chevreux.org/projects_mira.html).

Results

Identification of recombinant individuals

In order to identify progeny individuals showing recombination events between CH03d11 and FR149B, an interval of 15.79 cM, both markers were applied on the 12228 and 12229 populations, 1,202 progeny individuals in total. Genotyping resulted in the identification of 25 recombinant individuals from the 12228 population, 103 from the 12229 population in addition to the 17 identified in the original mapping (05210) population (Table 1). In total, 145 recombinants could be identified from the entire population of 1,336 individuals. Twelve individuals which possessed different alleles as the expected parental alleles were discarded from further analyses. The 145 recombinants formed the basis for subsequent analyses.

Phenotypic evaluation of recombinant individuals

107 recombinant individuals were phenotyped together with the parents *M. fusca* and 'Idared' as controls by artificial inoculation with *E. amylovora* isolate Ea222_JKI. Results showed that whereas the resistant parent *M. fusca* showed no sign of lesion in all replicates, an average percentage lesion length (PLL) of 64 % was recorded for the susceptible parent - 'Idared' (Figure 1a). Figure 1b shows the distribution of the susceptibility/resistance level of the phenotyped recombinants. A zero PLL was observed in six recombinant individuals whereas no 100 % PLL was recorded in all 107 recombinants. The highest PLL recorded was 83 %. Averagely, the PLL recorded for all 107 recombinant individuals phenotyped was 18.3 % with a median of 11 %.

Figure 1a Virulence and resistance levels of *E. amylovora* strain Ea222_JKI on both parents – Idared and *M. fusca* respectively. *M. fusca* showed no signs of necrosis in all replicates as shown in plants pictured 28 dpi.

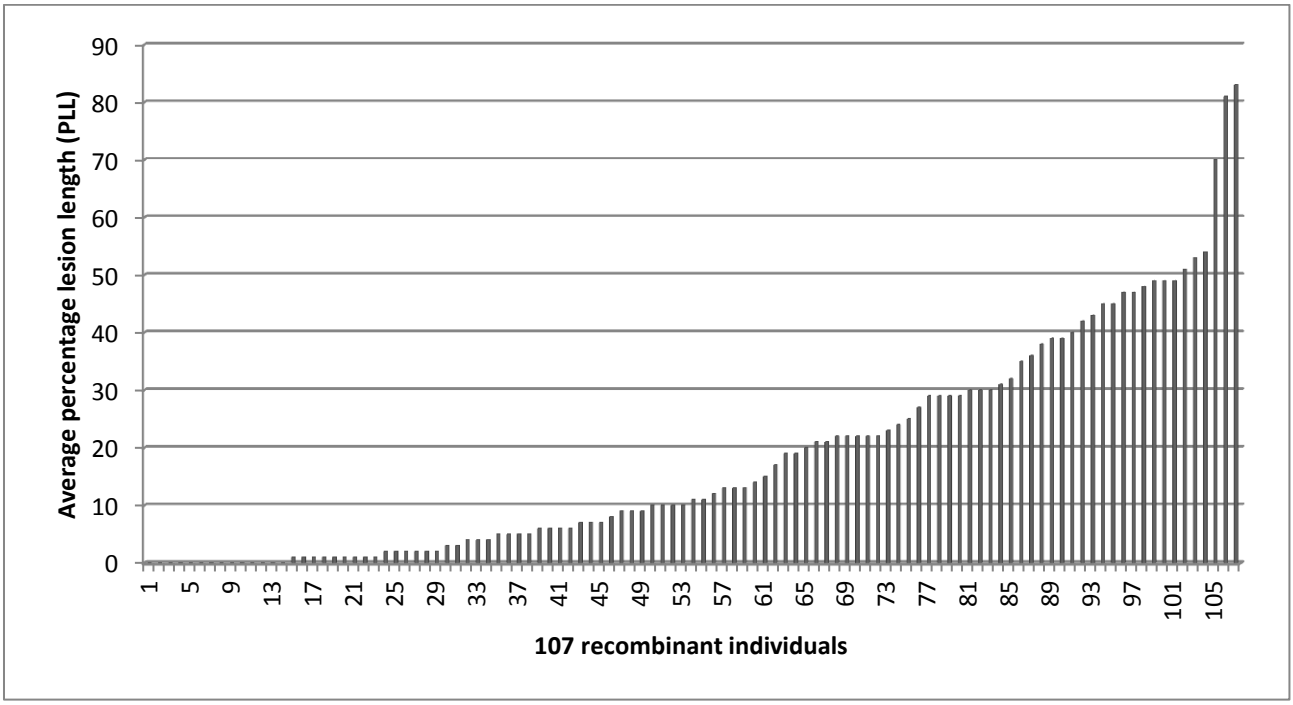

Figure 1b Distribution of recombinant individuals showing their different levels of resistance/susceptibility to fire blight. Individuals are ordered according to percentage necrosis (PLL).

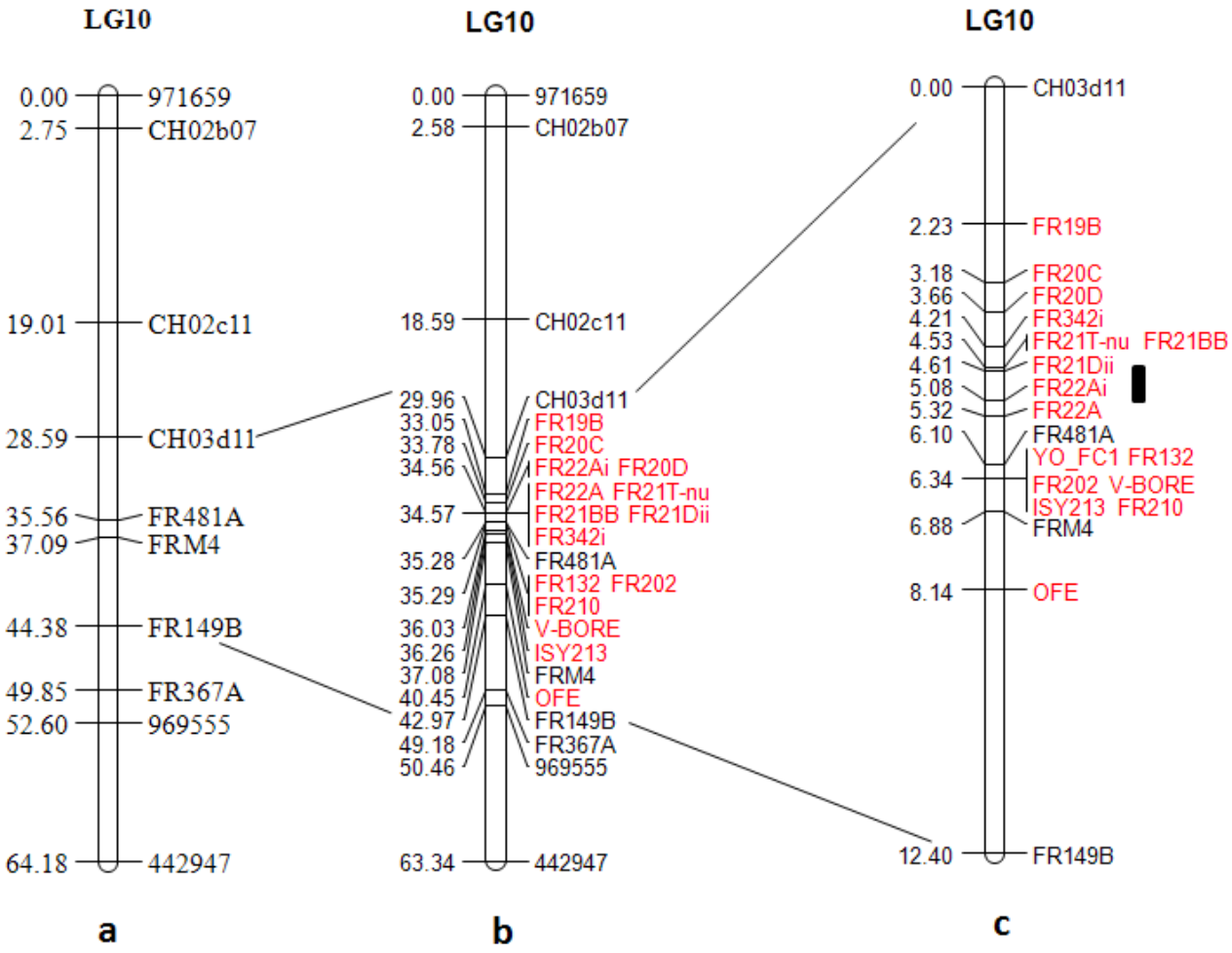

Figure 2 Linkage group 10 of *M. fusca* (**a**) the initial map developed using 134 individuals Emeriewen *et al*. (2014) (**b**) enrichment with new closely linked SSR markers (highlighted in red) in the QTL interval between CH03d11 and FR149B (**c**) the mapping of only the interval between markers CH03d11 and FR149B using all three population listed in Table 1.

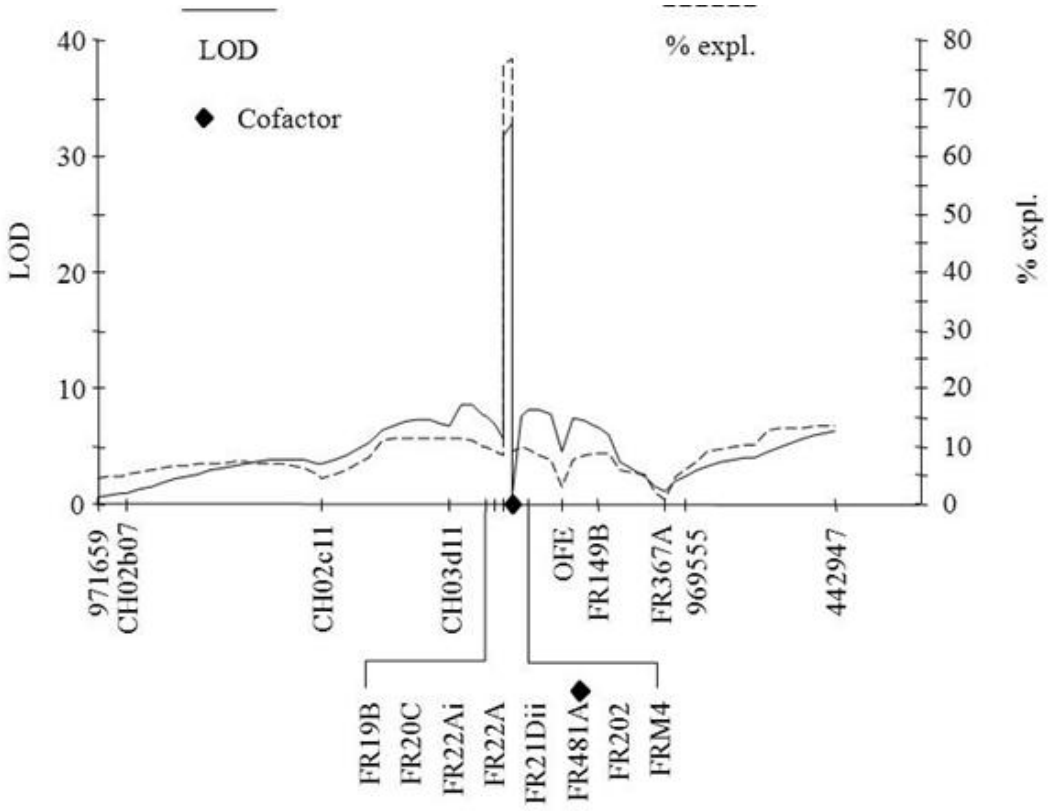

Figure 3 LOD score plot, threshold and percentage phenotypic variation explained (*% expl*.) of the necrosis trait along LG10 of *M. fusca* determined by MQM with marker FR481A set as cofactor. QTL explained about 77 % of phenotypic variation due to the substantial increase in population and new closely linked SSR markers

SSR marker development, application and fine mapping of the resistance locus

Emeriewen *et al.* (2014) reported that SSR markers FR481A and FRM4 mapped on 35.56 cM and 37.09 cM respectively on linkage group 10 (LG10) of *M. fusca* and are significantly correlated with fire blight resistance appearing underneath the peak of the LOD plot. Fifteen polymorphic SSR markers developed from the corresponding pseudo-chromosome 10 of the 'Golden Delicious' genome, and additionally, one SSR marker from the BAC clone sequence of Mfcl_FR481A (Table 2) could be mapped. Figure 2a shows the map of *M. fusca* LG10 map as developed by Emeriewen *et al.* (2014) using population 05210. All sixteen markers were first applied to that population and mapped (Figure 2b). One SSR marker, YO_FC1 was discarded, because it distorted the order of the other markers on LG10. Ten SSR markers clustered with FR22A, FR21T-nu, FR21BB, FR21Dii, and FR342i together, FR22Ai and FR20D together, FR132, FR202 and FR210 together. Multiple QTL mapping of resistance to fire blight using the map with additional 15 SSRs (Figure 2b) and FR481A as cofactor resulted in an increase of the phenotypic variation explained by *Mfu10* from 66 to about 77 % (Figure 3).

After ascertaining the linkage of the SSR markers to LG10 of *M*. fusca on population 05210, linkage mapping was thereafter performed using all the SSRs mapping between CH03d11 and FR149B on all individuals (Table 1). Figure 2c shows only the interval between CH03d11 and FR149B using all individuals. The region, now designated as the region of interest (roi), spans 13.01 cM, 2.78 cM less than originally reported with 0.95 cM and 0.47 cM as mean and median distances respectively between these markers.

To determine the position of the gene(s) underlying *Mfu10*, phenotypic data of the recombinants and the deduced phenotypic data of non-recombinant individuals were transformed to binary data. Genetic mapping using these data revealed the position of the resistance locus (*FB_Mfu10*) between SSR markers FR22Ai and FR21Dii (Figure 4) spanning an interval of 0.59 cM between both markers. Six recombinants defined this interval (Figure

4). Two of the recombinants in this interval were resistant and the other 4 were susceptible. The phenotype and genotype of one recombinant close to that region did not match the estimated position of the resistance gene(s).

Mfu × ID	ID × Mfu	Mfu × ID	ID × Mfu	Mfu × ID	Mfu × ID	Mfu × ID	Mfu × ID	Mfu × ID	Mfu × ID	Cross
12229-402	12228-101	12229-99	12228-173	12229-996	12229-525	12229-500	12229-475	12229-32	12229-436	Plant number
R	R	R	R	S	S	S	S	S	S	CH03d11
R	R	R	R	S	S	S	S	S	S	FR19B
R	R	R	R	S	S	S	S	S	S	FR20C
R	R	R	R	S	S	S	S	S	S	FR20D
S	R	R	R	S	S	S	S	S	S	FR342i
S	S	R	R	S	S	S	S	S	R	FR21T-nu
S	S	R	R	S	S	S	S	S	R	FR21BB
S	S	R	R	S	S	S	S	R	R	FR21Dii
S	S	R	R	S	S	S	S	R	R	*FB_Mfu10*
S	S	S	S	R	R	R	R	R	R	FR22Ai
S	S	S	S	R	R	R	R	R	R	FR22A
S	S	S	S	R	R	R	R	R	R	FR481A
S	S	S	S	R	R	R	R	R	R	FR202
S	S	S	S	R	R	R	R	R	R	FR132
S	S	S	S	R	R	R	R	R	R	FR210
S	S	S	S	R	R	R	R	R	R	ISY213
S	S	S	S	R	R	R	R	R	R	V-BORE
S	S	S	S	R	R	R	R	R	R	YO_FC1
S	S	S	S	R	R	R	R	R	R	FRM4
S	S	S	S	R	R	R	R	R	R	OFE
S	S	S	S	R	R	R	R	R	R	FR149B

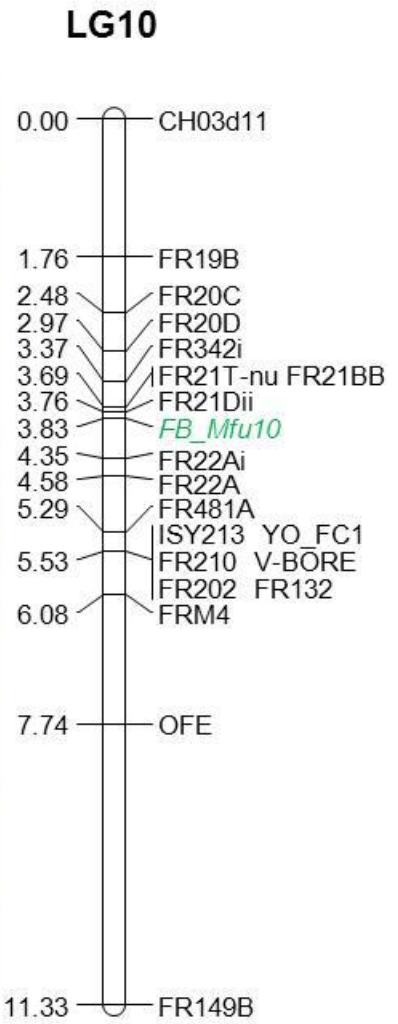

Figure 4 Single gene mapping using genotypic data of the whole population, phenotypic data of recombinants and deduced phenotypic data of non-recombinant individuals. R and S represent alleles in coupling and repulsion respectively to the fire blight locus *FB_Mfu10*.

Discussion

In the past, the devastating effect of fire blight epidemics on apple orchards in Europe has been enormous. It remains the most dreadful bacterial disease in apple production. Due to the strict regulation or complete ban of the use of antibiotics treatment for the management of the disease in most European countries, breeding programs primarily aimed at developing new resistant apple cultivars as well as studies with the target objective of investigating resistance to fire blight have emerged within the past decade. Consequently, several resistance donors possessing major or minor QTLs for resistance to fire blight in apple cultivars and wild apple species accessions have been so far reported (Calenge *et al*. 2005; Khan *et al*. 2006; Peil *et al*. 2007; Durel *et al*. 2009; Le Roux *et al*. 2010; Emeriewen *et al*. 2014; Wöhner *et al*. 2014). The most promising resistance QTLs are those inherited from wild apple species like *M.* ×*robusta* 5 (Mr5; Peil *et al*. 2007) and *M. fusca* (Emeriewen *et al*. 2014) which could explain 80 and 66 % of the phenotypic variation respectively. From the former, the isolation of a candidate gene, *FB_MR5*, has been described (Fahrentrapp *et al*. 2013), and functionally characterised by transformation into the genome of the apple cultivar, 'Gala' (Broggini *et al*. 2014). Nevertheless, the resistance of Mr5 and the responsible QTL has been broken-down by the highly virulent Canadian *E. amylovora* strain Ea3049 and the $avrRpt2_{EA}$ deletion mutant strains, ZYRKD3-1 and ZYRKD3-1 (p2YR2-415) (Peil *et al*. 2011; Vogt *et al*. 2013; Wöhner *et al*. 2014). However, Vogt *et al*. (2013) were able to demonstrate that these strains of *E. amylovora* could not break the resistance of the *M. fusca* accession (MAL0045) used in the current study. This was confirmed by Emeriewen *et al*. (2015) who showed that the resistance QTL (*Mfu10*) of *M. fusca* (accession MAL0045) was affected but did not break down after inoculation with Ea3049. These authors reported the decrease of the phenotypic variation explained by *Mfu10* from 66 to 41 % after phenotyping with Ea3049. This is a clear indication

that the resistance mechanisms of Mr5 and *M. fusca* are in fact different, and could contribute to pyramided durable resistance against this devastating disease.

It was therefore imperative to fine map the fire blight resistance locus of *M. fusca* which is a step closer towards elucidating the resistance mechanism of this highly resistant wild species. To achieve this, firstly, the progeny population size was substantially increased (Table 1). The increase in population size is one of the major prerequisite in fine mapping any given resistance locus. This increases the chances of recombination events in chromosomal regions of interest and thus allows for a high resolution mapping and precisely locating the QTL after genotyping with molecular markers; which is critical to map-based cloning. This approach was successfully used in *Malus* for the identification of two scab resistance genes, *Rvi6* (Belfanti *et al.* 2004) and *Rvi15* (Galli *et al*. 2010a; b) and the fire blight resistance gene, *FB_MR5* (Fahrentrapp *et al*. 2013). Parravicini *et al*. (2011) could identify a region containing a cluster of eight resistance gene analogs linked to fire blight resistance in the apple ornamental cultivar 'Evereste'. However, none of these hypothesized genes have been functionally proven till date. Consequently, the substantial increase of the population size, in addition to genotyping the population with SSR markers, led to the identification of 145 recombinants in a 15.79 cM interval between CH03d11 and FR149B from the 3 populations used in the current study. This equates to 9.1 recombinants per cM. Nevertheless, Parravicini *et al*. (2011) identified 103 recombinant individuals in a significantly smaller interval, 3.91 cM, which equates to 26.3 recombinants per cM, of the resistance region of 'Evereste' between the markers Hi23d11y_E and M35TA_256s_E, from a significantly larger population of 2,703 progeny individuals. Since only recombinant individuals within a given region of interest provide vital information in resolving the map position of a resistance locus (Fahrentrapp *et al*. 2013), these recombinants formed the basis for phenotypic evaluation and genotyping with newly developed SSR markers. The results of the phenotypic evaluation of

the resistant and susceptible parents clearly confirmed their resistant and susceptible status. The average PLLs of zero and 64 recorded for *M. fusca* and 'Idared', respectively, were similar to values of *M.* ×*robusta* 5 (0 %) and 'Idared' (52 %) reported by Fahrentrapp *et al*. (2013) for the mapping of the resistance locus of Mr5. These authors also reported lesions of phenotyped recombinant individuals of the cross 'Idared' × Mr5 ranging from 0 to 77 %, which is similar to the range of 0 to 83 % obtained for the recombinants in the present study.

Fine mapping of the fire blight resistance locus

Previously, a major quantitative trait locus (*Mfu10*) was identified in *M. fusca* which explained about 66 % of the phenotypic variation (Emeriewen *et al*. 2014). These authors demonstrated that *Mfu10* is localised in the region of chromosome 10 below SSR marker CH03d11, on the apple genome. Therefore, to further narrow the size of the resistance region and to accurately estimate the exact location of *Mfu10*, 15 polymorphic SSR markers which were developed from contigs of the 'Golden Delicious' genome corresponding to the chromosome of interest (Velasco *et al*. 2010), and additionally one SSR marker from the BAC clone Mfcl_FR481A, were mapped. Linkage mapping was first performed using only the initial mapping population (05210; Table 1) comprising 134 individuals (Emeriewen *et al*. 2014) to ascertain the positions of the markers on LG10 of *M. fusca*. The inclusion of SSR marker, YO_FC1, developed from the assembled sequence of the BAC clone Mfcl_FR481A, significantly disrupted the initial order of markers on LG10 and thus was excluded for the final mapping using the initial mapping population. In comparison to the initially reported map of LG10 of *M. fusca*, the map using only the 134 initial mapping individuals now comprised the three DArTs and 7 SSR markers previously reported (Figure 2a; Emeriewen *et al*. 2014), and additionally, 15 SSRs, with a total length of 63.34 cM (Figure 2b) instead of 64.18 cM. The interval between markers Ch03d11 and FR149B thus decreases from 15.70 to 13.01 cM in the new map of *M. fusca* LG10. Interestingly, marker YO_FC1 did not

significantly distort the marker positions when mapping only the interval between CH03d11 and FR149B using all individuals (Figure 2c). Since the distance between markers FRM4 and FR149B was 7.29 cM, SSR marker OFE was developed to reduce this distance.

These new closely linked markers were used to perform marker-phenotype association by means of Kruskal-Wallis analysis and were shown to possess similar *K* values as markers FRM4 and FR481A (Emeriewen *et al*. 2014), confirming that they are highly associated with fire blight resistance. Furthermore, interval mapping and multiple QTL mapping (MQM) indicated that the position of *Mfu10* is defined by the interval of FR481A and FR20D below SSR marker CH03d11, thus confirming the hypothesis of Emeriewen *et al*. (2014). The accuracy of phenotypic and genotypic data of closely linked markers is critical towards positional cloning. Following the confirmation and position of *Mfu10*, phenotypic data of the recombinants were transformed into binary marker data as Durel *et al*. (2009) described. The phenotypes of non-recombinant individuals were deduced based on the assumption that an individual is phenotypically resistant or susceptible if it inherits the genetic alleles in coupling or repulsion. This facilitated single gene mapping of the position of the fire blight (*FB_Mfu10*) resistance locus between SSR markers FR22Ai and FR21Dii, the later closer to the resistance locus by 0.07 cM. Both SSRs flanking the resistance locus are 0.59 cM apart corresponding to approximately 450 kb on the 'Golden Delicious' genome. In previous studies, Parravicini *et al*. (2011) reported a distance of 0.18 cM between markers ChfbE01 and ChfbE08 flanking the resistance QTL of 'Evereste' with another marker, ChfbE02-7, co-localising with the locus. Also, Fahrentrapp *et al*. (2013) showed that markers FEM14/FEM47 and rp16k15 flanked the resistance locus of Mr5 (*FB_Mr5*) and were 0.23 cM apart. Furthermore, the screening of the *M. fusca* bacterial artificial chromosome (BAC) library with the SSR markers flanking *Mfu10* as well as tightly linked surrounding markers has allowed for the identification of some resistant and susceptible BAC clones spanning

about half of the region of interest (data not shown). The assembled sequence of one of the BAC clones (Mfcl_FR481A) was used as template for the development of marker YO_FC1. All previous studies on the isolation of scab and fire blight resistance genes in *Malus* relied on the availability of BAC libraries containing large inserts of genomic DNA. Such libraries were then screened using tightly linked molecular markers of the trait with the aim of physically reducing the region containing the resistance locus. Subsequently, one BAC which spans the region or a contig of BACs spanning the region is sequenced and annotated sequences used for gene prediction analyses.

This report therefore is the first step towards the isolation of the underlying resistance genes of *M. fusca*. The isolation of functional genes underlying QTLs allow for better understanding of the quantitative trait. The *Mfu10* underlying gene is particularly of interest since till date only one fire blight resistance gene, *FB_MR5* (Farentrapp *et al* 2013) has been functionally proven (Broggini *et al*. 2014), although this resistance has succumbed to more virulent isolates of *E. amylovora*. Therefore it is essential for future breeding strategies to identify, isolate and functionally characterize additional fire blight resistance genes. This will allow for the breeding of new cultivars as well as the development of cisgenic cultivars with pyramided fire blight resistance genes in order to establish more durable resistance. It is therefore expected that the resistance gene(s) underlying *Mfu10* will provide resistance to the resistance breaking *E. amylovora* isolates of *FB_MR5* since *Mfu10* is not overcome by the highly virulent Canadian isolate Ea3049 (Emeriewen *et al*. 2015).

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Chapter Five

General Discussions and Conclusions

General Discussion and Conclusions

The domesticated apple (*Malus* × *domestica* Borkh.) is an important fruit crop in Europe and other temperate regions of the world. Its continuous optimum production is an important contribution towards global food security. However, production of apples is under serious threat by fire blight which has now been known for over two centuries as a devastating bacterial disease on pome fruits. Caused by the Gram-negative enterobacterium, *Erwinia amylovora* (Burrill) Winslow *et al*., fire blight is dreaded by apple growers more than any other apple disease. The pathogen overwinters in cankers and during warm temperatures, colonizes the surfaces of buds and barks, flowers, leaves and rootstock crowns causing blight (Peil *et al*. 2009). The sudden appearance of blight, followed by its quick migration can cause significant damages in a matter of days and huge economic losses on a larger scale within a single growing season.

Fire blight is not impossible to control. It is critical to know where the bacteria are most likely to be found in an orchard and how best to get rid of them. Disease management tactics such as quarantine, eradication of affected tissues, the use of bio-control agents and application of copper and antibiotics have all been used to reduce the population of *E. amylovora* in orchards (Johnson and Stockwell, 1998; McManus *et al*. 2002; Norelli *et al*. 2003). The best results have been achieved with the application of streptomycin on flowers, since the primary point of entry is through the nectaries. Nevertheless, the concerns of raising *E. amylovora* strains that are resistant to streptomycin is real, and as such have been isolated in pome fruit orchards worldwide (Jones and Schnabel, 2000). Hence, streptomycin use is completely banned if not strictly regulated in many European countries. Therefore, best results of management of the disease are achieved in cultivars with reduced susceptibility (Malnoy *et al*. 2012). The most promising sustainable alternative measure could be the development of durable resistant cultivars.

The development of new resistant cultivars will rely on the introgression of natural resistance against fire blight obtained from wild *Malus* species. Wild *Malus* accessions, hybrids or ornamental crab apples have been shown to carry higher resistance levels than cultivated apple cultivars (Aldwinckle *et al*. 1976; 1999). Consequently, quantitative trait loci (QTLs) have been detected hitherto in wild *Malus* spp. namely: *M*. ×*robusta* 5 (Peil *et al*. 2007), *M*. *floribunda* clone 821 (Durel *et al*. 2009), the ornamental cultivar Evereste (Durel *et al*. 2009), which explained 80, 40 and 53 % of phenotypic variation, respectively. These QTLs were detected using the classical QTL mapping approach in bi-parental populations. So far, only the gene underlying the *M*. ×*robusta* 5 (Mr5) QTL, *FB_MR5* (Fahrentrapp *et al*. 2012) has been functionally proven (Broggini *et al*. 2014).

The research works described in this thesis were aimed at investigating fire blight resistance in *Malus fusca*. Firstly, five different *M*. *fusca* accessions namely: MAL0045, MAL0200, MAL0289, MAL0357, MAL0768 were artificially inoculated with *E*. *amylovora* strain Ea222_JKI. The lowest percentage lesion length was obtained in accession MAL0045 (data not shown). To establish a bi-parental population, *M*. *fusca* accession MAL0045 was crossed with the very susceptible apple cultivar Idared, resulting in 134 F1 progeny individuals. Since phenotyping is a very critical step in mapping QTLs in bi-parental populations, phenotypic evaluation using the strain Ea222_JKI (Peil *et al*. 2007) was performed on the 134 *M*. *fusca* × Idared progenies in three different years; 2006, 2007 and 2012.

For phenotyping, scions of each individual genotype were grafted onto rootstock M9 and replicated up to 10 times. One major problem observed was stunted growth of plants in the greenhouse. The reason for this was as a result of death of the rootstock on which scions were grafted. In some cases, scions died but rootstock survived. In these cases, plants were discarded. Hence, only plants that grew up to 25 cm were phenotyped and consequently not all genotypes could be replicated 10 times. Phenotyping was performed by incising the two

youngest leaves with a pair of scissors dipped into bacterial suspension. Disease incidence was recorded 28 days post inoculation (28 dpi) when the progress of disease had ceased (Kleinhempel *et al*. 1984; Peil *et al*. 2007). In fire blight phenotypic evaluation, great variations in response of replicates to the pathogen were usually observed. For this reason, and since a reliable phenotypic evaluation is the most critical aspect in detecting QTLs (Emeriewen *et al*. 2014), phenotyping was carried out in three years to reduce the margin of errors to the barest minimum. This is the first report of QTL analyses in a bi-parental population where data of three years of phenotypic evaluation was used.

Furthermore, a prerequisite for mapping QTLs in any genus is the development of a genetic linkage map using molecular markers. This is especially important since molecular markers play an integral role in the selection of pre-breeding materials for fire blight resistance. Therefore, DArTs, SNPs and SSRs formed the basis of the *Malus fusca* genetic map as described in the paper presented as Chapter Two of this thesis. Summarily, 20 linkage groups which spanned a total of 889 cM, comprising 209 markers, representing 213 loci, could be constructed (Appendices A). Quantitative trait locus analyses performed using the phenotypic data of the 134 F1 individuals and the *M. fusca* map as template, resulted in the detection of a major QTL on LG10 which could explain about 66 % of the phenotypic variation. The *M. fusca* resistance QTL (*Mfu10*) confers the second highest effect of all QTLs previously described in *Malus*, only behind that of *Malus* ×*robusta* 5 which explained up to 80 % of the phenotypic variation (Peil *et al*. 2007; 2008). The closest molecular markers to *Mfu10* are SSR markers FR481A and FRM4 mapping at 35.56 cM and 37.09 cM, respectively. These two SSRs are bracketed by two other SSRs CH03d11 (28.59 cM) and FR149B (44.38 cM), and are all suitable for marker assisted selection (MAS).

The validation of strain specificity of QTLs is important as differential virulence of *E. amylovora* isolates have been reported (Norelli *et al*. 1984; Norelli and Aldwinckle 1986; Peil

et al. 2011; Wöhner *et al*. 2014). In fact, Peil *et al*. (2011) and Wöhner *et al*. (2014) reported the breakdown of the Mr5 major QTL located on LG3 by the highly virulent Canadian strain Ea3049. Although, *FB_MR5* (Fahrentrapp *et al*. 2013) is the only functionally proven fire blight resistance gene of *Malus* (Broggini *et al*. 2014), its breakdown by just a single SNP (Vogt *et al*. 2013), supports the argument to validate strain specificity as well as reiterates the need to identify more resistance donors that will contribute to the establishment of durable resistance by means of pyramiding. Therefore in this context, strain specificity validity test was carried out to determine the stability of *Mfu10* using the 'resistance-breaking' highly virulent *E*. *amylovora* isolate Ea3049 which is indigenous to Canada. The methods and results are presented in Chapter Three. Briefly however, this particular strain of *E*. *amylovora* could not break down the resistance of *Mfu10*. However, the resistance level was strongly affected by Ea3049 as *Mfu10* which explained about 66 % of phenotypic variation after inoculation with Ea222_JKI, only could explain about 41 % of phenotypic variation after inoculation with Ea3049. Nevertheless, this is in contrast to the Mr5 QTL which completely broke down after inoculation with Ea3049. This is strong evidence that *Mfu10* is stable and useful for the establishment of durable resistance. It was recommended in the paper presented as Chapter Three, that it is worthwhile to pyramid the Mr5 QTL and *Mfu10*.

In Chapter Four, the first steps towards the map-based cloning of the *Mfu10* underlying gene(s), following the confirmation of the stability of the QTL are presented. The region of the resistance locus of *M. fusca*, located on LG10 within the 15.79 cM interval between the SSR markers CH03d11 and FR149B (Emeriewen *et al*. 2014), was saturated with 16 newly developed closely linked SSR markers using chromosome walking approach. For this purpose, the population size was increased from 134 to 1,336 F1 individuals. Genotypic analysis using closely linked SSRs allowed for the identification of 145 progeny showing recombination events within the 15.79 cM interval. Phenotypic evaluation of these 145

recombinants using the *E. amylovora* strain Ea222_JKI, together with genotypic data of closely linked SSR markers ensured that the resistance locus was well defined, reducing the interval to 0.59 between the flanking SSR markers FR22Ai and FR21Dii, corresponding to about 450 kb on the 'Golden Delicious' reference genome (Velasco *et al*. 2010). Single gene analysis using phenotypic values transformed into binary values as described by Durel *et al*. (2009) and adopted successfully by Fahrentrapp *et al*. (2012), mapped and confirmed the resistance locus between the flanking SSRs FR22Ai and FR21Dii. This 0.59 interval was defined by six recombinant individuals; two resistant and four susceptible. Furthermore, a *M. fusca* bacterial artificial chromosome (BAC) library has been developed. Some BAC clones which hybridize to closely linked SSRs have been isolated.

The differential responses of the *M. fusca* resistance QTL to two differentially virulent strains of *E. amylovora* indicate gene-for-gene interactions. Moreover, the fact that the resistant donor – *M. fusca* (MAL0045) is only minimally affected by the highly virulent Ea3049 strain whereas the *M. fusca* × 'Idared' progeny is strongly affected, suggests that other factors and not just an underlying gene play a role in the resistance of *M. fusca*. Therefore, besides the isolation of the gene(s) which underlie the *M. fusca* resistance locus, future work on this research should include the investigation of other possible factors that contribute to the resistance of *Mfu10*. Research proposals which will address these questions are currently being designed at the Julius Kühn-Institut (JKI), Pillnitz, Dresden, Germany. The wild apple species, *Malus fusca* offers promising prospects for the fight to defeat the very destructive fire blight disease by means of breeding new resistant apple cultivars. This thesis will not be the last report of results in this regard.

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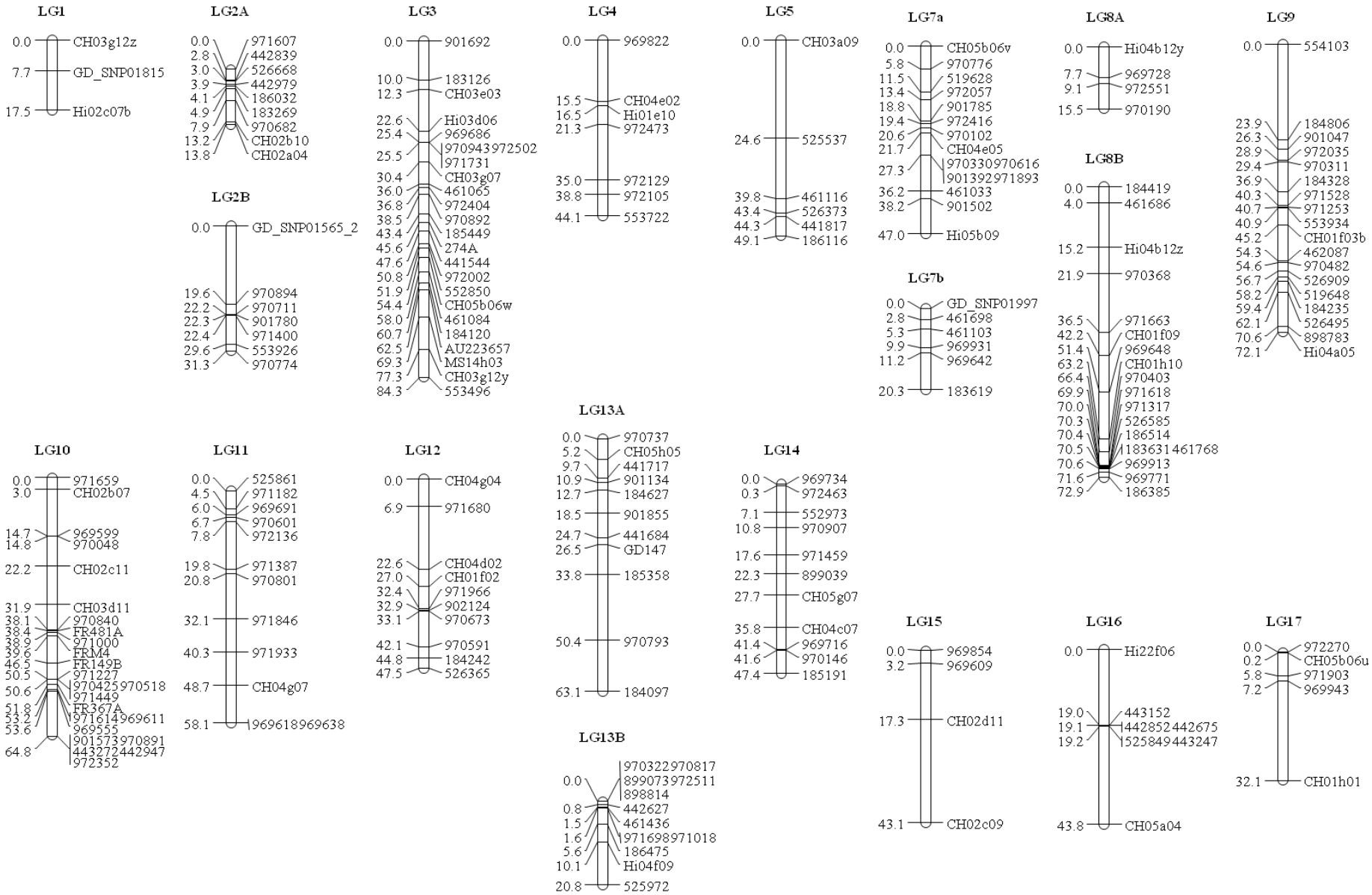
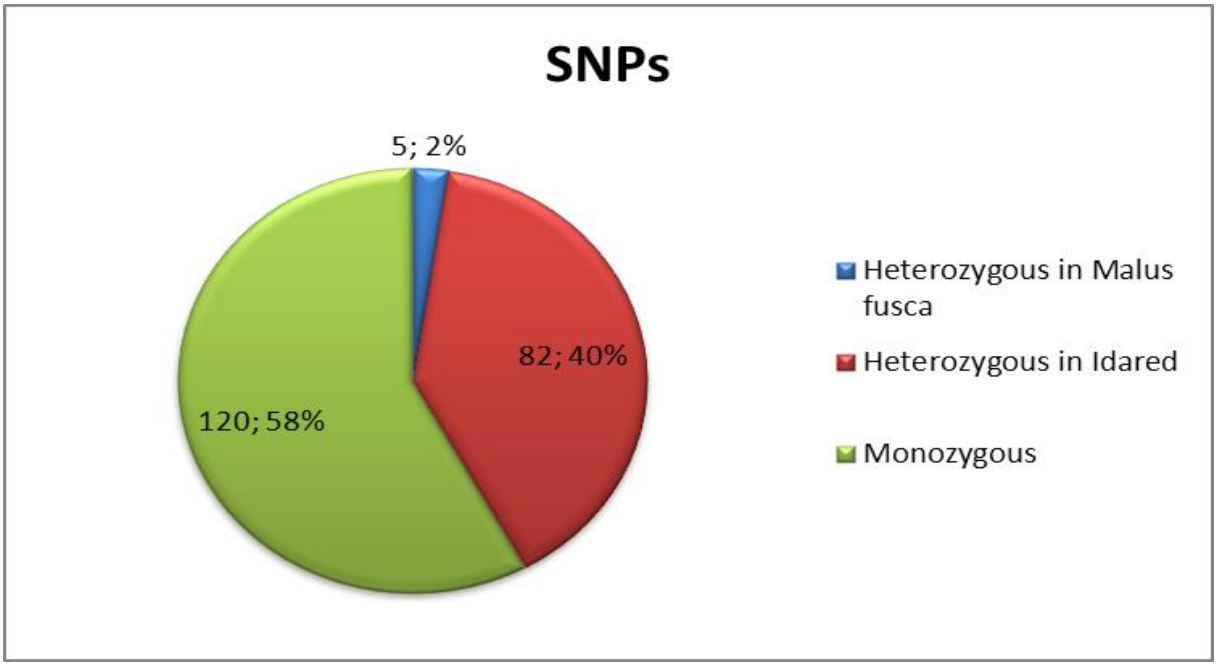


Appendix A: Figure S1: Genetic map of *Malus fusca.* (Emeriewen *et al*. 2014)

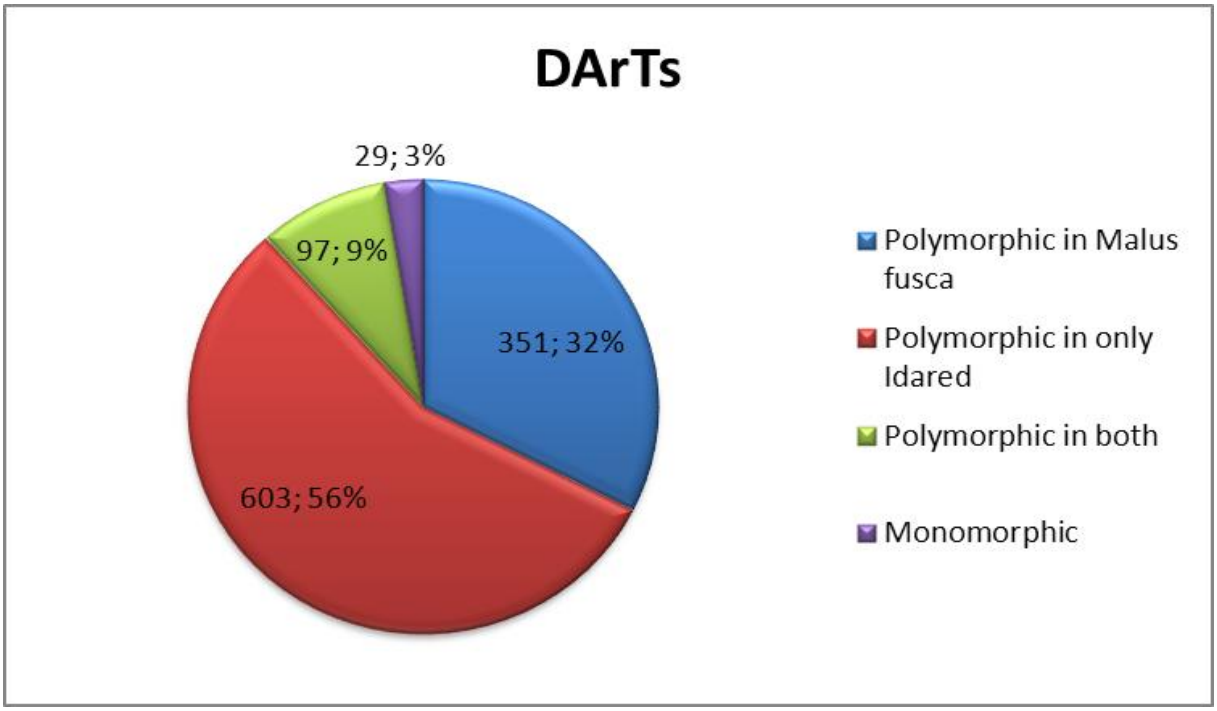
Appendix B: Results of SNP markers application.

As reported in Chapter 2 (Emeriewen *et al*. 2014), transferability of 'Golden Delicious' SNPs to *Malus fusca* was very poor. A better transferability was observed in the susceptible parent 'Idared' – a cultivar of the domesticated apple like 'Golden Delicious'.

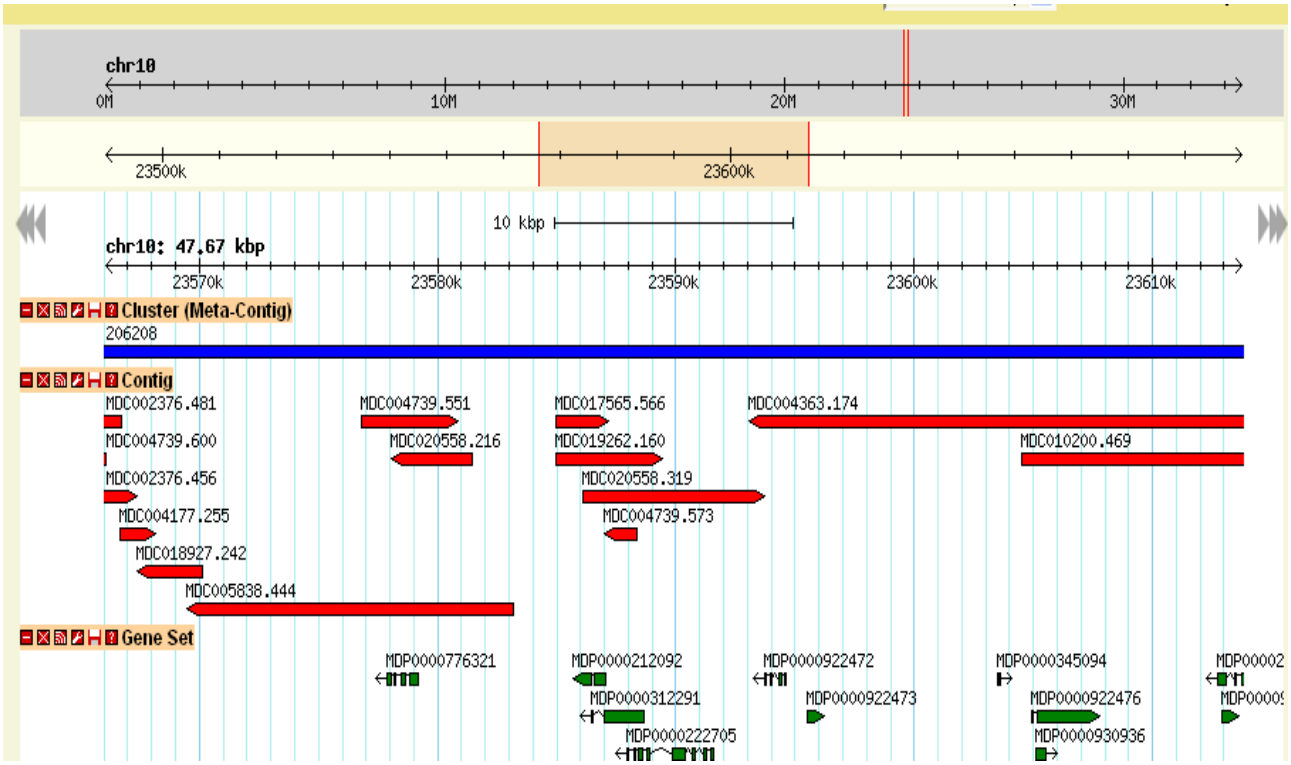


Appendix C: Result of DArT markers development.

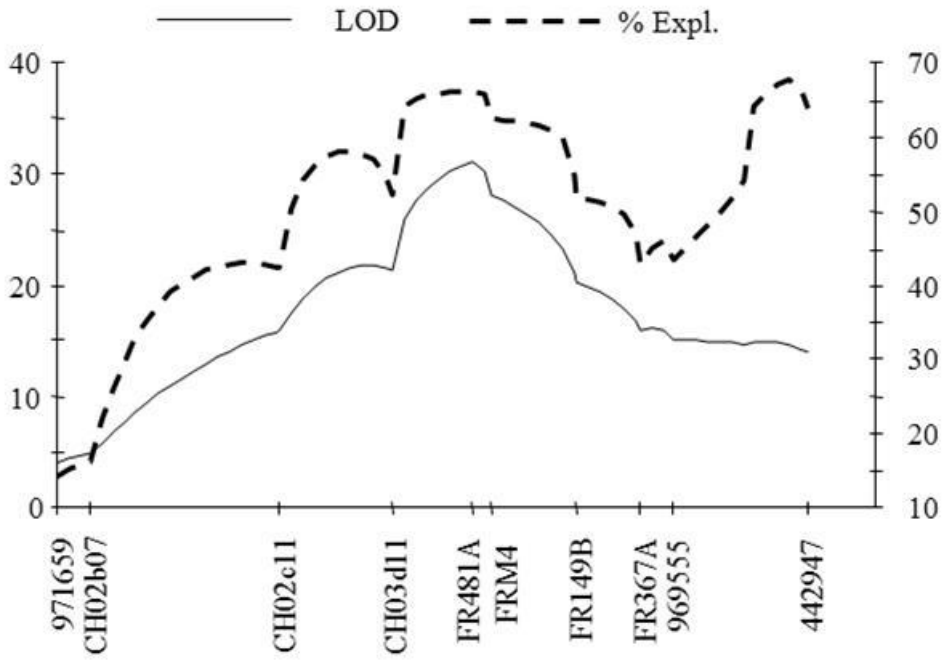
Since SNPs approach failed due to poor transferability, a library of DArT clones was constructed leading to the identification of 1080 DArTs.



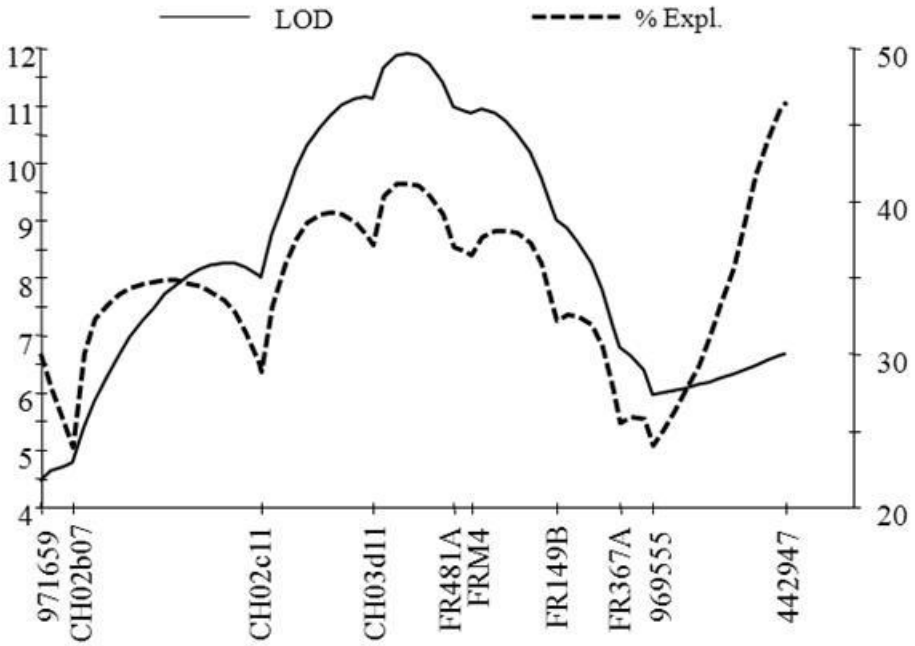
Appendix D: Screenshot of 'Golden Delicious' contigs where DArT markers correlating significantly with fire blight resistance aligned on chromosome 10 of the apple genome. This was the first indication that linkage group 10 was the chromosome of interest as reported in Chapter Two.



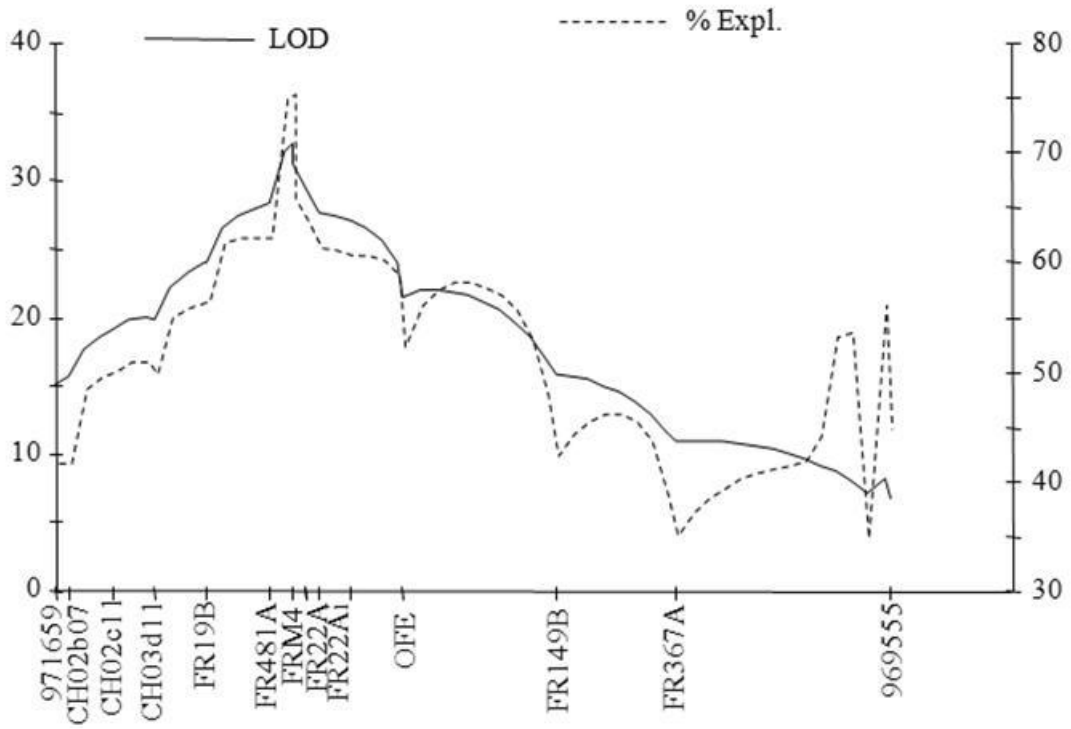
Appendix E: LOD score plot, threshold and percentage explaining about 66 % of the phenotypic variation (% *expl*.) of the trait necrosis along LG10 of *Malus fusca* determined by interval mapping after inoculation with Ea222_JKI



Appendix F: LOD score plot, threshold and percentage explaining 41.2 % of the phenotypic variation (% *expl*.) of the trait necrosis along LG10 of *Malus fusca* determined by interval mapping after inoculation with Ea3049



Appendix G: LOD score plot, threshold and percentage explaining about 77 % of the phenotypic variation (% *expl*.) of the trait necrosis along LG10 of *Malus fusca* determined by interval mapping after inoculation with Ea222_JKI

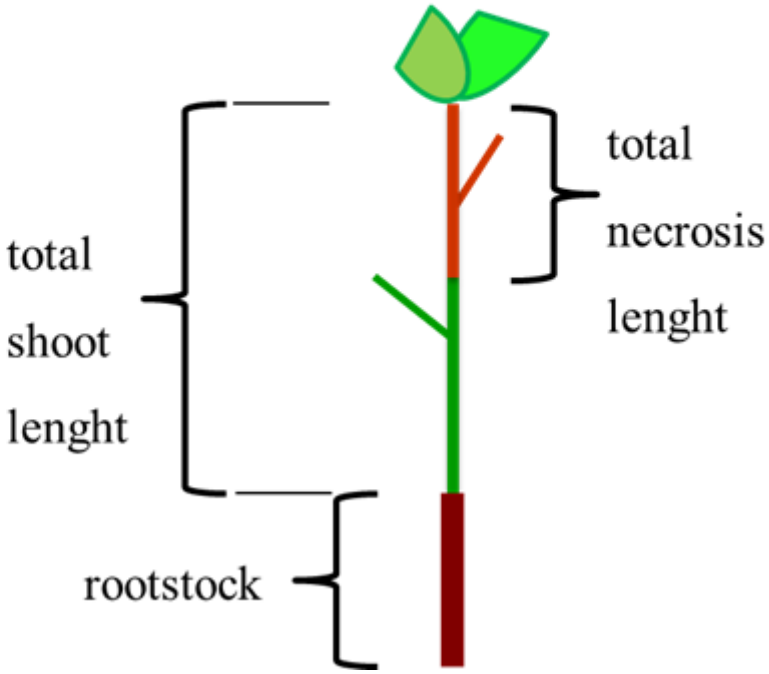


Appendix H: 1,202 F1 progeny individuals from the crosses of *Malus fusca* × 'Idared' and 'Idared' × *Malus fusca* growing in the greenhouse. These were established to add to the initial 134 mapping individuals in a fine mapping approach.

Appendix I: Phenotyping of recombinant individuals. Picture shows typical fire blight symptoms observed on recombinants in the greenhouse after inoculation with Ea222_JKI

Appendix J: Illustration of phenotypic evaluation 28 days post inoculation. % necrosis = Percentage lesion length (PLL)

$$\% \text{ necrosis} = \frac{\text{total necrosis length}}{\text{total shoot length}} \times 100$$



Curriculum Vitae

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PhD Title: 'Fire blight resistance of the wild apple species *Malus fusca*'

Supervisors: Dr. Andreas Peil (JKI); Dr. Mickael Malnoy (FEM); Prof. Nicola Pecchioni (Modena)

2009-2010: Master of Science degree from the University of Nottingham, United Kingdom.

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Publication Activities

Emeriewen O, Richter K, Kilian A, Zini E, Hanke M-V, Malnoy M, Peil A **(2014)** Identification of a major quantitative trait locus for resistance to fire blight in the wild apple species *Malus fusca*. *Molecular Breeding* 34 (2): 407-419

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Peil A, **Emeriewen O**, Richter K, Wöhner T, Malnoy M, Hanke M-V, Flachowsky H **(2014)** Comparative genetic mapping of resistance to fire blight in *Malus* sp. *Journal für Kulturpflanzen* 66 (12): 409-416

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International conferences

Oral presentations

Emeriewen O, Ritcher K, Kilian A, Hanke MV, Malnoy M, Peil A **(2013)** Evidence of a major QTL for fire blight resistance in the apple wild species *Malus fusca.* Oral presentation at the 13th ISHS International Fire Blight Workshop, Zurich, Switzerland

Emeriewen O, Ritcher K, Kilian A, Hanke MV, Malnoy M, Peil A **(2013)** DArT and SSR markers are linked to a major QTL for resistance to fire blight in the apple wild species *Malus fusca*. Oral presentation at the 3rd ISHS Molecular Markers in Horticulture International Symposium, Riva del Garda. Italy

Poster presentations

Emeriewen O, Zini E, Kilian A, Malnoy M, Peil A **(2012)** Functional characterization of fire blight resistance in *Malus fusca* fine mapping, cloning and characterization of fire blight resistance genes from *Malus fusca*. Poster presentation at 6th International Rosaceous Genomics Conference (RGC6), Mezzocorona (TN).

Ofere Emeriewen, Klaus Richter, Magda-Viola Hanke, Mickael Malnoy **(2014)** Resistance of *Mfu10* is not broken down by the highly virulent Canadian Erwinia amylovora strain Ea3049. Poster presentation at the 1st International Workshop on the Molecular Basis of Fire Blight, Bolzano, Italy.


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