Quiero dedicar este esfuerzo a cuatro mujeres maravillosas cuyas decisiones inspiradas por su amor altruista han influenciado positivamente mi vida
Margarita Bermudez, Esperanza García, Caroline Kuffner y Zdenka Snirch
A mis hermanos Italo e Iván García-Libreros en gratitud.

Genetic mapping and characterization of resistance factors in apple (*Malus sp*.) against fire blight

Dissertation

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Contents

Zusammenfassung

Feuerbrand, verursacht durch das Bakterium *Erwinia amylovora,* ist die wichtigste Krankheit von Apfel. Alle Kultursorten sind in unterschiedlichem Ausmaß anfällig. Quantitative Resistenz wurde in Wildäpfeln beschrieben und bietet vielversprechende Ansätze für die Resistenzzüchtung. In dieser Dissertation soll der genetische Hintergrund von Feuerbrandresistenz in Apfel untersucht werden.

Als Grundlage diente eine Population von 140 Nachkommen einer Kreuzung der anfälligen Sorte 'Idared' mit dem resistenten Wildapfel '*Malus x robusta* 5' (Mr5). Anhand von Amplified Sequence Length Polymorphisms (AFLP) und Simple Sequence Repeats (SSR) wurden Kopplungskarten für 'Idared' und Mr5 erstellt. Die 'Idared'-Karte umfasste 1062 cM mit einer Dichte von 6.6 cM/Marker, die Mr5-Karte 1012 cM mit einer Dichte von 5.2 cM/Marker.

Aufgrund der Ko-Segregation zwischen SSR Markern und phenotypischer Resistenz in der Population 'Idared x Mr5' wurde ein Quantitative Trait Locus (QTL) auf der Kopplungsgruppe 3 (LG3) von Mr5 identifiziert. Dieser erklärte in Feuerbrand-Inokulationsversuchen 2005 und 2006 75% der Resistenzvariation und wurde von Forschungspartnern auch in einer 'Mr5 x Malling9' Population nachgewiesen.

Anhand der beschriebenen Genkarten und der Population 'Idared x Mr5' wurde außerdem der Marker AT20-SCAR für das Mehltau-Resistenzgen *Pl1* auf LG12 lokalisiert. In weiterer Folge wurden die SSRs der LG12 verwendet, um die genaue Position von *Pl1* anhand einer zweiten, auf Mehltauresistenz getesteten, Kartierungspopulation zu bestimmen.

Mittels Genexpressionsanalyse wurde *Pl-Mal,* ein putatives Proteinaseinhibitor-gen, identifiziert. Bei Feuerbrandinfektion ist die Expression von *Pl-Mal* in 'Idared' stark reduziert, was auf eine wichtige Rolle in der Immunantwort hindeutet. Ein Single Nucleotide Polymorphism marker (SNP) wurde für *Pl-Mal* entwickelt und

auf LG3 von Mr5 kartiert. Mit dieser Arbeit wurde ein Grundstein für die Lokalisierung der Resistenzressourcen von Mr5 gelegt.

Summary

Fire blight caused by the bacterium *Erwinia amylovora* is the most important disease of apple. Quantitative resistance has been reported for wild apples, offering promising perspectives for resistance breeding. The aim of this research was to elucidate the genetic basis of fire blight resistance in apple.

A population of 140 progenies originating from a cross between the highly susceptible cultivar 'Idared' and the resistant wild apple '*Malus x robusta* 5' (Mr5) was the base of this work. Amplified Fragment Length Polymorphisms (AFLP) and Simple Sequence Repeats (SSR) were analyzed and linkage maps were constructed for 'Idared' and Mr5, spanning 1062 cM with a density of 6.6 cM/maker and 1012 cM with a density of 5.2 cM/maker, respectively.

Co-segregation of certain SSR markers and phenotypic fire blight resistance in the 'Idared x Mr5' population identified a quantitative trait locus (QTL) in linkage group 3 (LG3) of Mr5. This QTL explained 75% of the resistance variation in 2005 and 2006 after inoculation of the population with *E. amlyovora,* and was also confirmed in the genetic background 'Mr5 x Malling9' by research partners.

Furthermore the 'Idared x Mr5' population and the derived linkage map permitted localization of the AT20-SCAR marker, which is linked to the mildew resistance gene *Pl1*, on LG12. The SSR markers of LG12 could then be employed to determine the precise position of *Pl1* by screening a second mapping population, for which mildew resistance data was available.

Finally, expression analysis revealed the pronounced down-regulation of a putative protease inhibitor gene (*Pl-Mal*) in 'Idared' subsequent to fire blight infection, suggesting a central role in defense reactions. The linkage maps of 'Idared' and Mr5 and the 'Idared x Mr5' population were used to map a Single Nucleotide Polymorphism marker (SNP) for the *Pl-Mal* gene on LG3. With this thesis a solid background for the localization of the resistance resources in Mr5 is provided.

1 Introduction

1.1 Apple

1.1.1 Origin and history

Cultivated apple (*Malus* × *domestica* Borkh.) belongs to the genus *Malus* of the subfamily *Maloideae* and family *Rosaceae.* This subfamily is thought to have evolved from the hybridization of a *Spiraeoidae* (*x* = 9) and a *Prunoidae* (*x* = 8) ancestors resulting in the basic haploid number of *x* = 17 for the Pomoidae. (Challice 1981, Lespinasse *et al.* 1999, Korban and Skirvin, 1984). Most *Malus* species are diploids (2*n* = 34), but few are triploids, tetraploids and some species show variable levels of ploidy (Way *et al.* 1990). The estimate of nuclear DNA content per nucleus is 1.54 - 1.65 pg/ 2C among the diploid apple cultivars, and the average genome size calculated for apple is nearly 743-796 Mbp/haploid (Arumuganathan and Earle, 1991).

The ancestor of the cultivated apple remains unknown. The main progenitor of the domestic apple is considered to be *Malus sieversii*, which grows wild in the Heavenly Mountain (Tien Shan) at the boundaries between western China and the former Soviet Union to the edge of the Caspian Sea (Morgan and Richard 1993, Forsline and Aldwinckle 2002). Central Asia is the center of origin and the area with the greatest apple diversity.

The apple was transported by humans in to the Middle East, Europe and North America. Research expeditions in Central Asia have shown that *M. sieversii* is endemic from the Republic of Kazakhstan (Harris *et al.* 2002 Hokanson *et al.* 1998, Luby *et al.* 2001).

1.1.2 Importance and production worldwide

Apple is after grapes, bananas, and oranges the fourth biggest fruit crop produced globally in the world. More than 50 million tons of apples are produced worldwide annually (www.faostat.fao.org). Apples can grow in different climates, but they are best adapted to the cool temperature. Apples present a rather more northern range than many other tree fruits, due to relatively late blooming and cold hardiness. World apple production has risen in recent years (Table 1). However experts expect a significant increase in production and consumption in the coming years mainly in Asia due to the nascent economies surrounding China and economic evolution in China itself that contributes to a considerable increase of apple consumption in Asia. Youngbin (2006) reported that China is the largest producer of apples, the USA second, followed by apple production in the European Union (with Poland, Italy and France being the largest producers).

Area	2000	2005	2010*	2015*
Europe	14.104	12.394	13.739	14.245
North America	5.671	5.187	5.487	5.521
Asia	23.638	28.383	29.788	32.718
Southern Hemisphere	4.301	4.978	5.795	6.330
World	59.199	63.489	68.441	72.820

Table 1 Trends in world apple production (× 1.000 tons).Source: World Apple Report, 2006. *Forecast.

The principal market for most apples produced around the word is for domestic fresh use. There are other market options for apples processed into forms like slices, pie fillings, dried apples, apple sauce, and apple juice; optionally apple could be fermented to produce cider, vinegar, and apple wine. The old saying “an apple a day keeps the Doctor away” truly expresses the nutritional value of this fruit. Table 2 presents the information regarding to the nutrition value of this fruit.

Nutrient	Per 100 g	Mineral	Per 100 g	Vitamins	Per 100 g
Energy	52 kcal	Calcium, Ca	6.0 mg	Vitamin C	4.6 mg
Water	85.56 g	Iron, Fe	0.12 mg	Vitamin B-1	0.017 mg
Protein	0.26 g	Magnesium, Mg	5.0 mg	Vitamin B-2	0.026 mg
Total Lipid	0.17 g	Phosphorus, P	11.0 mg	Vitamin B-3	0.091 mg
Carbohydrate	13.81 g	Potassium, K	107.0 mg	Vitamin B-5	0.061 mg
Total Sugar	10.39	Sodium, Na	1.0 mg	Vitamin B-6	0.041 mg

Table 2. Nutrient values and weights per 100 grams for edible portion of raw apples, *with skin*; based on data from USDA National Nutrient Database http://www.ars.usda.gov/main/site_main.htm?modecode=12-35-45-00.

1.1.3 Botany and breeding

Apple is a perennial woody plant and presents a long juvenile period ranging from 5 to 7 years. Apple is self-incompatible and highly heterozygous, which results in very diverse progeny with only a few of them being a major improvement on the parents. Most of the characters are under polygenic control, low efficiency in genetic improvement of breeding lines together with a long juvenile period make breeding in this perennial woody crop a slow and expensive process (Bringhurst, 1983, Khan 2007, Maliepaard *et al.* 1998, Oraguzie *et al.* 2004). In nature, apple pollination is carried out by insects and wind, in agricultural systems; it is propagated by humans through asexual methods such as grafting and budding.

Fruit quality, key factor is flavor and disease resistance are the most important challenges for breeding in apple. Adaptation to climatic conditions is also of interest for the countries located in the marginal regions either in Nordic or subtropical areas (Janick *et al.* 1996). The different breeding programs are

working on development of new cultivars for the fresh market emphasizing in appearance and eating quality that meet consumer demands. Selection criteria for external quality mostly concern to skin color, the size and shape of the fruit, while internal quality is preponderantly determined by flesh texture, firmness, juiciness, and sugar acidity content. Flavor is also a very important trait but difficult to assess. The judgment of fruit quality is subjective and depends on the people and their countries. Breeding programs in China, Japan, Brazil and India are interested on sweet-lasting fruits like “Fuji” “Gala” or “Red Delicious”. People from northern countries prefer more acid fruits like “Elstar” “Jonagold” or “Braeburn” (Gardiner *et al.* 2007). In apple breeding two cultivars with interesting characteristics are crossed to develop a large full-sib family, and later the progeny is screened for the traits of interest. Since the parents in the cross are usually heterozygous, F_1 progeny segregate for an abundant number of traits and each cross leads to genetically unique seedlings, which will be different from the female parent, the male parent and each other (Gessler and Patocchi, 2007, Khan 2007).

1.1.4 Pests and diseases

Apple is susceptible to diseases caused by fungi, bacteria, and virus. These pathogens produce significant losses in yearly apple production. Many insects and pests are potential threats to apple trees. Different aphids e.g., rosy apple aphid, wooly apple aphid, the complex of green apple aphid and spirea aphid are agents originators of severe problems to apple stem, fruits, leaves and flowers in many orchards (Qubbaj, *et al.* 2005, Khan, 2007) The most serious pest of apple is the codling moth, *Cydia pomonella* (L). It harms the fruit by both internally and externally. The oriental fruit moth, *Grapholitha molesta* Busck, is another pest which attacks the fruit and young terminal shoots of the tree.

Viruses or virus-like agents cause over 50 identified diseases of apple. Some of these diseases produce a great economic impact because they are associated with fruit deformities or severe tree decline and even death. The degree of distress depends on the rootstock, scion selection, pathogen isolate and climate. Some examples of apple virus diseases are apple mosaic caused by apple mosaic ilarvirus, apple chlorotic leaf spot caused by apple chlorotic leaf-spot trichovirus, apple union necrosis caused by apple ring spot nepovirus (van Oosten, 1983).

Fungi are the group of plant pathogens that cause the majority of apple diseases. Pathogenic fungi can produce the following symptoms: root rots, leaf spots, leaf blights, blossom blights, fruit decay, fruit spots, defoliation and trunk, branch and twig cankers. Most of the pathogenic fungi for apple belong to the taxonomic groups *Basidiomycetes* and *Acomycetes*. These fungi generally reproduce by spores that are dispersed by air currents or splashing water. Some examples of apple diseases caused by fungi are apple scab caused by *Venturia inaequalis*, powdery mildew caused by *Podosphaera leucotricha* (MacHardy, 1996, Gianfranceschi *et al.* 1996 Calenge *et al*. 2004, Calenge *et al*. 2006)

There are many diseases caused by bacteria in apple, in areas where these bacterial pathogens either are not established or are rare, bacterial diseases are often avoided by planting pathogen-free nursery stock (Montesinos *et al.* 1999). Where the diseases are well established the management includes orchard sanitation, wound minimization the use resistant rootstocks and cultivars. Some of the apple diseases and their bacteria agent are blister spot caused by *Pseudomonas syringae* pv. *papulans*, crown gall caused by *Agrobacterium tumefaciens*, Hairy root caused by *Agrobacterium rhizogenes*, fire blight caused by *Erwinia amylovora* which is the most important apple disease caused by bacteria. (Vanneste, 2000).

1.2 Fire blight

Fire blight is the most important concern in all countries where apples are cultivated even those that are currently free of this disease. When fire blight becomes epidemic, it can harm seriously the apple tree and generate a big loss in nurseries and orchards, even leading to orchard removal. Strict measurements like quarantines and restrictions are maintained in countries where there has been no fire blight outbreak. In high-risk areas, fire blight is limiting the planting of some highly susceptible apple cultivars and rootstocks. The name of the disease addresses the issue that the affected plant parts looked as they would be consumed by fire (Montesinos *et al.* 2000, Khan 2007, Vanneste 2000).

1.2.1 History of the spread and damage

1.2.1.1 Worldwide

Erwinia amylovora was the first bacterial plant pathogen described. Fire blight was reported for the first time in 1780 in New York State. Since its first discovery in approximately 135 years it was found all over USA and soon spread to Canada, México and Guatemala. The fist report from fire blight in Asia came from Japan in 1903 and later on in 1919 it was also found in New Zealand, in Australia and South Korea in 1997 and 1999 respectively.

In Europe it is present since the 1950s. Fire blight has been reported in 40 countries of the world from all continents and could be present in many more countries not yet observed or reported. (Bonn and Van der Zwet, 2000). The spread of the disease is mainly through human activity i.e., the long distance shipment of bud or trees. Fire blight epidemics are more severe in warm and humid regions than in cooler and dry areas (Van der Zwet 2000, Vanneste, 2000) (Figure 1).

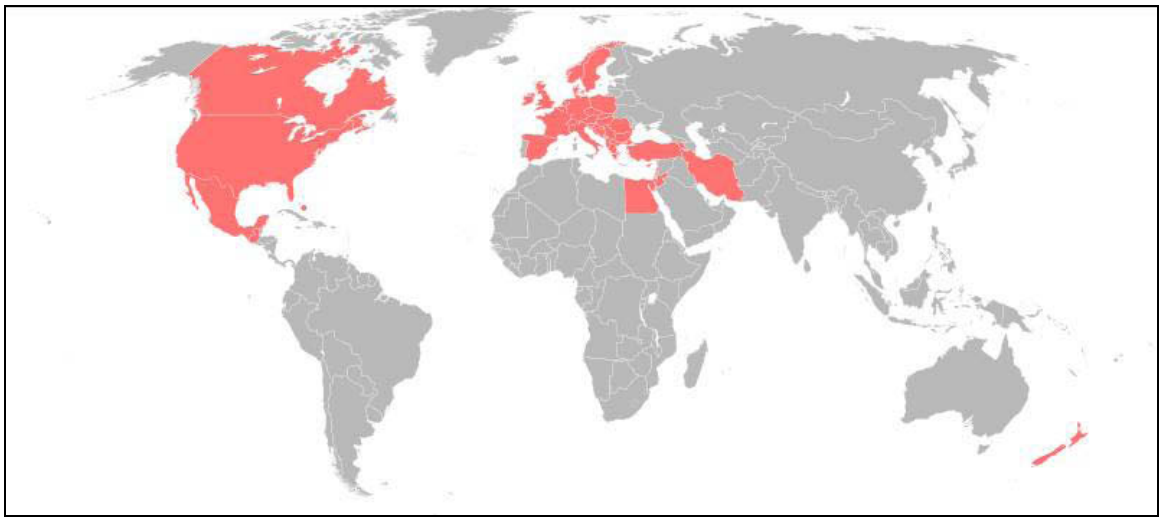


Figure 1.

Fire blight world distribution 2006 in red colour. Map from Österreichische Agentur für Gesundheit und Ernahrungssicherheit AGES Website.

1.2.1.2 Austria

Fire blight was identified for the first time in Austria in 1993 on a few *Cotoneaster salicifolius* plants in Vorarlberg close to the German border (Keck, 1996). Over the following years especially after 1998 an increase in outbreaks of fire blight and stepwise migration from west to east has been observed (Keck *et al.* 2004). Austria as a whole was considered as a fire blight protected zone in the European Union until 2001. Currently, the far-eastern and south-eastern regions, where the main pome-fruit areas (Steiermark) are located, remain a protected zone (see Figure 2).

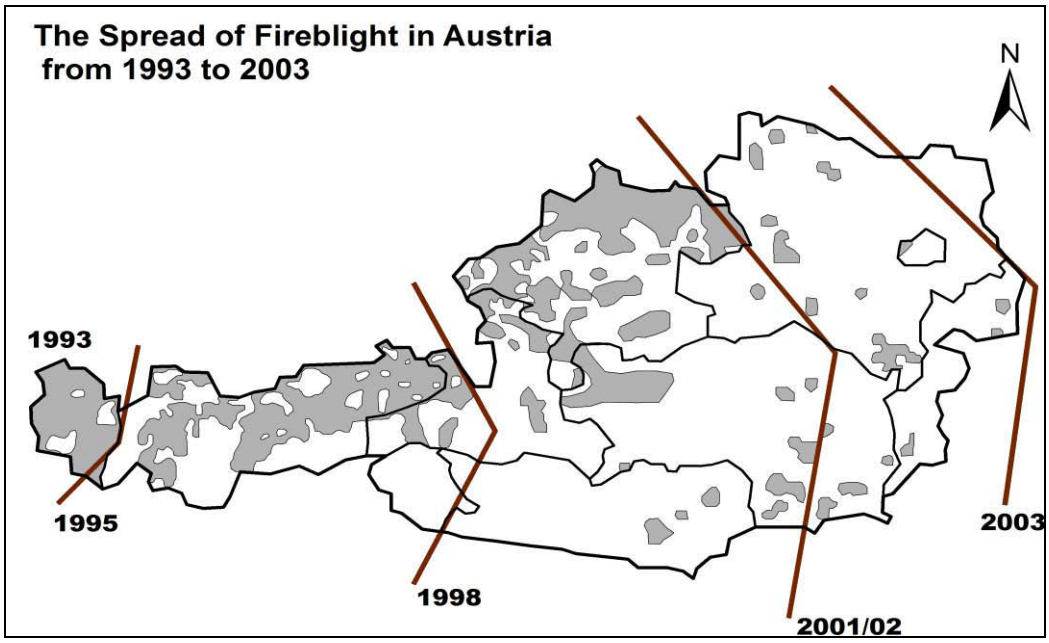


Figure 2

Spread of fire blight in Austria from 1993 to 2003. (Keck, 2004)

1.2.2 The disease

1.2.2.1 The bacterium Erwinia amylovora

The genus *Erwinia*, which owes its name to the memory of the plant pathologist Erwin F. Smith, was originally grouped in the *Enterobacteriaceae* associated with plants, sharing the characteristics of being Gram-negative, bacillary shape, non-sporulating and motile bacteria (Brenner, 1984). This definition led to the classification of the organisms of this genus into four groups (Dye, 1968, Dye 1969a ,b, c) "*amylovora"* for pathogen that cause wilting and necrosis, *"carotovora*" for causing soft rot, "*herbicola*" for saprophytes bacteria and the last group for atypical *erwinias*. With the advent of new molecular techniques the taxonomic studies later disagreed with this previous classification. The current classification based on comparison of sequences of 16S ribosomal DNA allowed the grouping of species of *Erwinia* strains into four phylogenetic groups/genera: the true *Erwinia* which contains *E. amylovora E. persicinus*, *E. psidii*, *E. rhapontici*, *Pantoea agglomerans E. tracheiphila*. They produce necrosis and

wilting symptoms. The Genus *Pectobacterium* characterized by pectinolytic activity includes the "*carotovora"* group *P.cactidium*, *P. carotovorum*, *P. chrysanthemi* and *P. cypripedii.* The Genus *Brenneria* grouping the species erwinias *B. alni*, *B. nigrifluens*, *B. paradisiaca*, *B. quercina*, *B. rubrifaciens.* They produce generally canker with exudates. The Genus *Pantoea* includes *P. ananatis* and *P. stewartii* opportunistic plant, animals and human pathogens. (Samson *et al.* 2005)

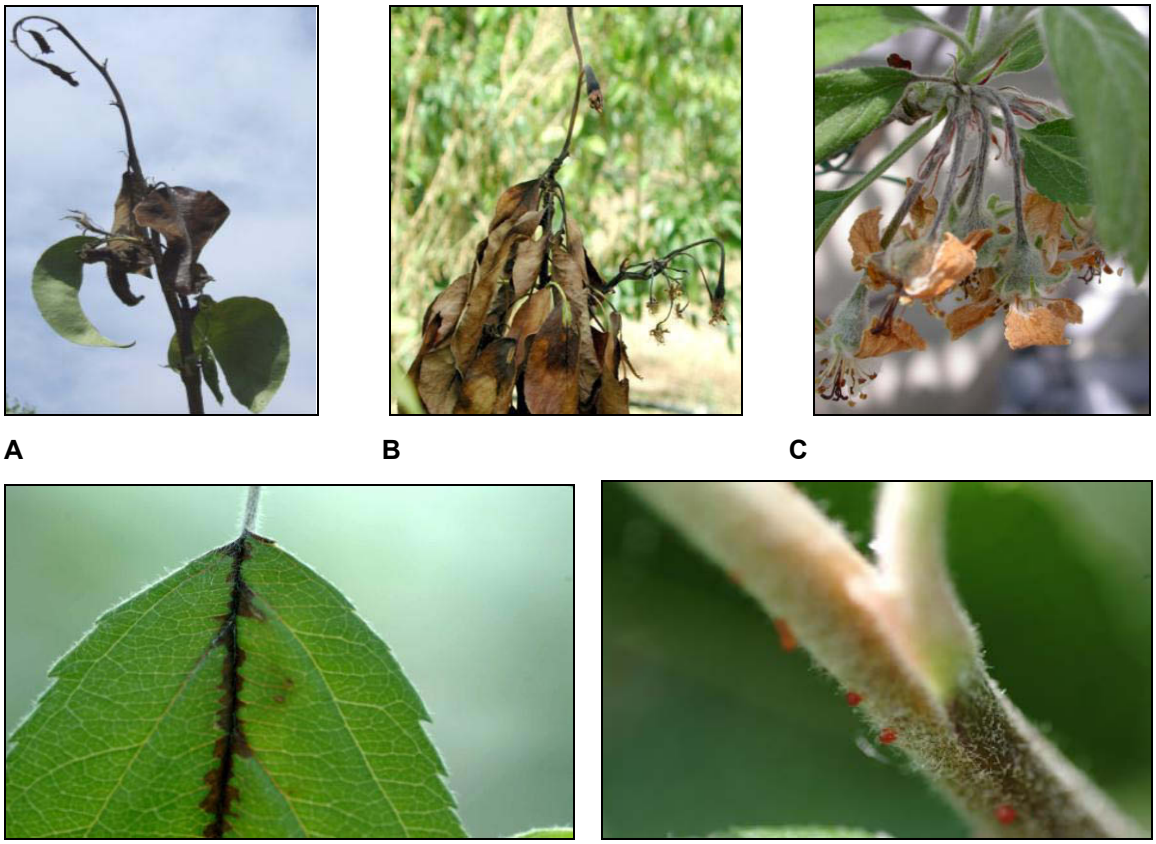
According to Holt *et al*. (1994), the differentiation between species of *Erwinia* relies on a few positive cultural physiological characteristics. These typical features are weak anaerobic growth, mucous growth, production of acetoin (in shaken culture) liquefaction of gelatin, motility. For growing in minimal medium, *E. amylovora* requires unconditionally nicotinic acid.

1.2.2.2 Hosts and symptoms

Since its first observation in 1780 in New York in Pome (Winslow *et al.* 1920) fire blight has been described in more than 200 plant species from 40 genera all belonging to the family Rosaceae (Thibault and Le Lezec, 1990, van der Zwet and Keil, 1979). However, most of the affected species belong to the subfamily *Maloidae* in particular the fruit trees such as *Pyrus* (pear) *Malus* (apple), *Cydonia* (quince) and *Eriobotrya* (loquat). In the category of ornamental and wild plants *Crataegus* (hawthorn*), Chaenomeles*, *Cotoneaster*, *Photinia*, *Pyracantha* (Firethorn), *Sorbus* (Rowan) and *Stranvaesia* (Balduque *et al.* 1996, van der Zwet and Beer 1995, Cambra *et al.* 1996) are included.

The first symptoms of fire blight can be found in flowers or young necrotic fruits (Balduque *et al.* 1998, Cambra *et al.* 2002) (Figure 3). Subsequently, the infection is progressing very fast, reaching first the peduncle and later on the base of the branches and finally colonizing the whole plant (van der Zwet and Beer, 1995, Montesinos and Lopez, 2000, Paulin *et al*. 1973). Blossom blight symptoms affects the entire blossom cluster, which wilts and dies, turning to

brown color in apple and black in pear (Figure 3B and C). On the leaves the first symptom is the necrosis of the middle rib (see Figure 3D). If the weather conditions are favorable for the pathogen development, conglomerated of bacterial ooze can be observed on the blossoms (Figure 3E). The infection spreads from the blossom cluster into the portions of the supporting limb. Young shoots in active growth are very susceptible to this disease, their wilt causes a loss of stiffness, the curled feature called "shepherd's crook" symptom (Figure 3A) (Cambra *et al.* 2004).



A **B** **C**

D **E**

Figure 3.

Typical fire blight symptoms in apple. A. Shepherd´s hook, B. and C Infected blossom. D infected leaf E. Infected shoot with bacterial Ooze. Picture A kindly supported by Marco-Noales and M Ordax. Pictures: B, C, D, E with friendly permission of Ulrike Persen from AGES.

1.2.2.3 Disease cycle

The bacterium overwinters in the bark of cankers and limbs infected during the previous season, these cankers are the most likely source of primary inoculums. Warm and moist weather are favorable conditions for the fire blight infection. In early spring the disease cycle starts when the temperature rises above 18ºC. The bacteria at canker margins begin to multiply rapidly and produce sticky yellow white ooze. This ooze contains bacteria and exopolysaccharides (EPS) (Geider, 2000). These exopolysaccharides support the bacteria in the colonization and propagation process. From the flowers, the bacteria is very efficiently distributed by wind, rain many casual insect visitors like bees (pollinating insects rapidly move the pathogen to other flowers) and birds (Figure 4).

Once the blossoms are infected, the bacteria can quickly disseminate into shoots and branches, colonizing interior plant tissues. At this point of the disease development, blossom infections become visible as “strikes” or dying shoots. Infected shoots are sources of *Erwinia amylovora*. Secondary infections can continue to occur throughout the growing season. The bacteria can enter the host through wounds and natural openings like lenticels (Aldwinckle and Beer, 1979)

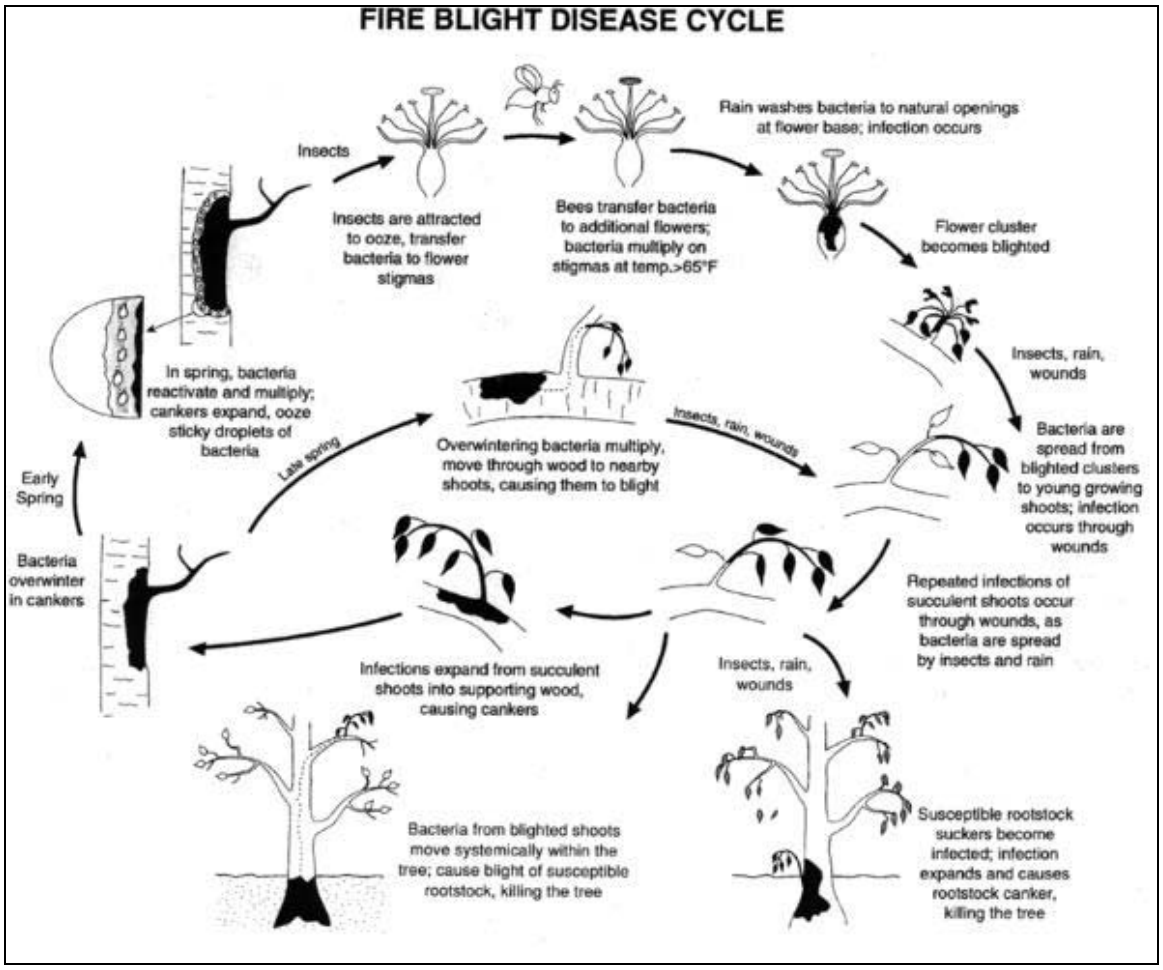


Figure 4

Fire blight disease cycle. Chart taken from www.nysipm.cornell.edu/factssheets/

1.3 Current management strategies

Most strategies are focusing on the reduction of the amount of inoculum available in the orchard and the use of antimicrobial treatments to prevent infection. Therefore pruning, eradication of infected plants, prevention measures, application of chemicals and biological control agents in combination are currently applied (Paulin, 2000).

1.3.1 Phytosanitary measures

Phytosanitaries measures are aiming to reduce primary inoculums in the orchard by removing remaining cankers during winter pruning. Once the infection is in the orchard, pruning of infected parts of the plants is suggested. Due to the endophytic nature of the bacterium, overwintering in the bark or root tissue and a secondary infection may occur. Thereofore sometimes the eradication of the whole tree is necessary.

1.3.2 Chemical control

Antibiotics such as Streptomycin have been recognized to be particularly effective chemicals to protect plants from fire blight. Other chemical compounds used against fire blight are oxytetracycline, kasugamycin, flumequin, fosetyl-AL and oxolinic acid. However antibiotic applications raise health concern for agricultures and consumers. Moreover continuous usage of Streptomycin has already led to the emergence of streptomycin resistant *E. amylovora* strains (McManus *et al.* 2002).

In addition copper compounds such as copper sulphate, copper hydroxide, copper oxide and copper oxychloride are used as a preventive measurement. They provide a hostile environment over the bark and bud surfaces of the trees preventing the bacteria from getting established. However copper accumulates in soil and has adverse effects on earthworm activity and microbial biomass (Komarek *et al.* 2010).

The plant growth regulator prohexadione-calcium (Apogee) is also used to reduce the occurrence and severity of fire blight shoot infection by controlling the shoot growth.

Generally, the application of chemicals against fire blight is rather a preventive than curative measurement. Therefore research for effective fire blight control strategies is of enormous importance.

1.3.3 Biological control

Biocontrol is generally a promising approach, and can be an alternative to antibiotics in particular during the blossom phase. For effective biocontrol of the pathogen, the stigmatic surface of a blossom is the principal tissue that the bacterial antagonist must colonize. In this way the antagonistic microorganisms can suppress first the establishment and growth of *E. amylovora* on stigmatic surfaces. This reduces the probability of floral infection and inhibit the pathogen dispersion to other blossoms. Only few bacterial antagonists have been found to be effective against fire blight. One is *Pseudomonas fluourescens* strain 506 (PfA506) available commercially for biological control of fire blight (previously Blight Ban A 506, now Plant Health Technologies Boise ID). In experiments it suppresses growth of *E. amylovora* on blossoms if it is applied 72 hours before the inoculation with the pathogen, but does not when it is co-inoculated with the pathogen. PfA506 colonize the same places on stigmas as *E. amylovora* and utilizes nutrients required for the growing of the pathogen (Paulin *et al*. 1999).

Another antagonist *Erwinia herbicola* strain C9-1 (reclassified as *Pantoea agglomerans* EhC9-1) produces two antibiotics called hebicolins O and I, which are inhibitory to strains of *Erwinia amylovora*. *Bacillus subtilis* stain BD170 was found effective in reducing blossom blight under both natural and artificial pathogen inoculation conditions. The biological control can be integrated with the conventional chemical management strategies and phytosanitary measures.

1.4 Strategies to increase fire blight resistance in cultivars of *Malus x domestica*

1.4.1 Aims and strategies of resistance breeding

As the available methods are insufficient for reliable fire blight control, there is great interest in the development of fire blight resistant apple varieties. Apart from quality aspects regarding fruit shape and taste, pest and disease resistance are the second major objectives in apple breeding (Khan 2007, Alston and Spiegel-Roy, 1985, Brown, 2012). Several members of the genus *Malus* are naturally resistant to fire blight, and numerous international fruit breeding projects aim at the integration of these resistance traits into novel apple varieties. Modern resistance breeding relies on marker assisted selection (MAS).

MAS benefits from progress in plant genetic research and has much higher public acceptance than genetic engineering (Khan *et al.* 2012). MAS involves screening of seedling populations for DNA recognition sequences (DNA markers) to predict favorable phenotypes and to select individuals carrying the desired alleles (Kellerhals *et al.* 2009). Considering the long juvenile period of apple this approach is time saving, and in contrast to phenotypic scoring it allows to distinguish homozygous from heterozygous individuals for a specific trait. With respect to disease resistance, MAS helps to avoid screening experiments with dangerous pathogens. Finally MAS facilitates breeding towards genotypes with multiple resistances (pyramiding of resistance traits). Pyramiding is favorable since single resistance mechanisms can be broken by pathogen evolution (Joshi and Nayak 2012). Unraveling the genetic basis of fire blight resistance and the development of resistance specific markers is crucial for efficient breeding of resistant apples (Khan *et al.* 2012).

1.4.2 Natural fire blight resistance in *Malus*

Members of the genus *Malus* vary strongly in their susceptibility to fire blight. Several wild *Malus* species including *Malus robusta*, *Malus fusca, Malus sublobata*, *Malus atrosanguinea* and *Malus prunifolia* have been identified as immune against this disease (Khan *et al.* 2012). Moreover a high level of resistance has been observed in the ornamental *Malus* × *domestica* cultivar 'Evereste' (Durel *et al.* 2009, Paravicini *et al.* 2011). All current *Malus* × *domestica* crop cultivars are susceptible to fire blight, however certain varieties such as 'Fiesta', 'Florina' and 'Nova Easygro' show reduced sensitivity (Calenge *et al.* 2005, Khan *et al.* 2007, Le Roux *et al.* 2010, Khan *et al.* 2012).

Presently, little is known about the genetic determinism of fire blight resistance in *Malus*. Specific pathogen resistance in plants frequently relies on a gene-for-gene relationship, where hosts expressing a resistance gene (R-gene) are immune against pathogens carrying a corresponding avirulence gene (Avr-gene). Such major resistance genes have been identified in apple for scab resistance (James and Evans, 2004) and mildew resistance (Gessler *et al.* 2006). However, so far no R-genes matching Avr genes of *E. amlyovora* have been described in any plant host (Durel *et al.* 2009). Fire blight resistance in apples has been reported to be a quantitative trait, i.e. controlled by several genes (Lespinasse and Aldwinckle, 2000). Many interesting agronomic traits of plants, such as fruit quality, adaptability and resistance to certain diseases, are of quantitative nature (Liebhard *et al*. 2003b). The genetic loci that harbor the sets of genes collectively controlling the expression of such traits are called quantitative trait loci (QTL)

1.5 Mapping and identification of resistance genes in apple

1.5.1. Principle

Chromosomal localization (mapping) of genetic determinants is a common first approach for the elucidation of the molecular basis of complex genetic traits. In diploid self-incompatible species such as apple, this requires crossing of two varieties presenting the trait of interest in contrasting form. Based on the segregation of DNA markers in the resulting progeny population, genetic linkage maps for the parent varieties can be constructed. Linkage maps present the individual chromosomes as linkage groups (LG) and predict the linear arrangement of the applied markers on each chromosome. Finally, co-segregation of individual DNA markers with the trait of interest in the progeny population is statistically analyzed, to determine near which marker the gene or QTL of interest is located (Celton *et al.* 2009, Fernandéz-Fernandéz *et al.* 2012). The linkage map should be densely covered with molecular markers in order to maximize the probability of identifying a QTL (Gessler *et al*. 2006, Khan 2007). If DNA markers sufficiently close to the locus of interest are available, the corresponding chromosome region can be cloned and sequenced for gene identification (Paravicini *et al.* 2011, Brown *et al.* 2012).

The availability of molecular markers and genetic linkage maps has allowed the identification of major genes as well as quantitative trait loci. Quantitative traits like height, leaf size, number of flowers, sugar content of fruit and fruit acidity, stem diameter (Liebhard *et al.* 2003a) and for aroma compounds (Dunemann *et al*. 2009) have been mapped successfully. Moreover QTL analysis has proven to be useful for mapping genes for resistance to mildew (Stankiewicz-Kosyl *et al.* 2005, Calenge *et al.* 2006, Dunemann *et al.* 2007) and apple scab (Durel *et al* 2003, Liebhard *et al*. 2003b, Calenge *et al*. 2004, Schouten and Jacobsen, 2008).

1.5 2 DNA markers

DNA markers are detected variations at DNA level that distinguish species or individuals. The marker sites are specific regions in the genome called marker loci showing variation in the population investigated and supply landmarks in the genome. This variation may be identified as a presence or absence of the particular DNA fragment, fragment length variation, or nucleotide difference. With the advent of polymerase chain reaction (PCR) (Mullis and Faloona, 1987, Saiki *et al.* 1985) different applications for molecular markers based on the PCR technology were developed. PCR allowed tracing polymorphisms at DNA level in a versatile and cheap way (Fluch, 2010). Today there is a variety of marker systems available, including RAPDs, SSRs, AFLPs, RFLPs and SNPs.

1.5.2.1 RFLP

Restriction Fragment Length Polymorphism (RFLP) was developed before the advent of the PCR-era (Soller and Beckmann, 1983, Fluch 2010). Most of the studies in systematics, evolution, phylogeney, and map construction for different crops species were originally based on RFLPs markers. The DNA polymorphisms is detected by hybridizing a chemically/radioactively labelled DNA probe determined for a specific locus to a total genomic DNA which was digested by restriction endonucleases and separated on a gel to produce a Southern blot (Paterson *et al*. 1988). The digested DNA is hybridized with the labelled DNA resulting in differential DNA profile. This technique is too laborious for large scale crop improvement programs (Fluch, 2010, Pupilli *et al.* 2000). However, RFLPs have been characterized and applied for mapping in a broad number of rosaceous genera, including *Prunus* (Dirlewanger *et al.* 2004) and *Malus* (Maliepaard *et al*. 1998).

1.5.2.2 RAPDs

The first maps of apple and pear were based on random amplification of polymorphic DNA (RAPDs). The RAPD reaction is a PCR done with arbitrary short primers of 8-12 nucleotides. RAPDs have been used for phylogenetic studies due to the advantage of being readily employed and requiring small amounts of genomic DNA (Oliveira *et al*. 1999, Teng *et al*. 2001, 2002)

1.5.2.3 AFLPs

AFLP stands for amplified fragment length polymorphism: This dominant marker system is an exceedingly sensitive method for identifying polymorphism (Vos *et al*. 1995). AFLP uses restriction enzymes to cut genomic DNA, followed by ligation of complementary double stranded adaptors to the end of the restrictions fragments. A subset of the restriction fragments are then amplified using two primers complementary to the adaptor and restriction site fragments. AFLP has some advantages over the RAPD technology, like a higher number of loci analyzed and a better reproducibility. AFLPs have been used for phylogenetic studies in pear and apple (Monte-Corvo *et al.* 2000).

1.5.2.4 SSRs or microsatellites

Simple sequence repeats (SSR) or microsatellites are polymorphic loci present in nuclear DNA, they are made of repeating units or motifs of 2-5 base pairs length. Microsatellites are neutral, codominant and show high degree of polymorphism and may be used as high throughput markers (Weber and May, 1989). SSR analysis is a very convenient DNA fingerprinting method compared to other molecular markers due to its codominant inheritance, large number of alleles per locus, abundance in genomes and small amount of DNA needed for the analysis. They have wide-ranging applications like evaluation of genetic diversity (Morgante *et al.* 2002) cultivar identification and construction of genetic linkage maps. SSRs have become the marker of preference for linkage mapping in apple (Celton *et al*, 2009, Gasic *et al.* 2009, Fernandéz-Fernandéz *et al.* 2012).

1.5.2.5 SNPs

Single nucleotide polymorphism (SNP) is a DNA sequence variation that happens when a single nucleotide in the genome differs between members of the species or between homologue chromosomes in an individual. SNPs are found in the entire genome in coding regions, noncoding regions of genes or in the intergenic regions between genes. SNPs identified in expressed regions are particularly interesting because they can cause changes in the function and structure of the protein encoded by this gene, or the change in the amino acid sequence can remain undetected due to degeneracy of the genetic code. They are suitable for gene mapping studies (Fluch, 2010). An example of developing a SNP markers from a candidate gene for red flesh and foliage colour in apple has recently been reported by Chagné *et al*. (2007). It has been postulated that SNP markers will be the most efficient tool for comprehensive genetic studies in the future.

1.5.2.6. Usage of dominant and codominant marker systems

AFLPs, and RAPDs are dominant markers. They allow simultaneous analysis of many loci per experiment, and a previous knowledge of the genome sequence is not required. Dominant markers indicate presence or absence of the detected fragment, but the emitted signal can not be directly correlated with the presence or absence of a specific allelic variant. The parental origin of the polymorphism remains unknown. In contrast codominant markers, such as RFLPs, SSRs allow the analysis of just one locus per experiment, but enable the tracing of the parental contribution. Allelic variations of the considered locus can be easily detected. They are more informative but for their development a prior knowledge of the genome sequence is necessary (Fluch, 2010). Dominant markers can only be used for map alignment purposes if these markers are heterozygous for one specific locus in both parents and their reproducibility and transferability to other maps is limited. Codominant markers are generally appropriate for map alignment and highly transferable between mapping populations

1.5.3 Linkage maps

The earliest generation of genetic maps of apple was developed in USA mainly based on RAPDs (Hemmat *et al*. 1994, Conner *et al*. 1997) and isoenzyme markers. European researchers created through an international initiative a genetic map based mostly on RFLPs and a small number SSRs (Maliepaard *et al*. 1998). In the last decade SSRs have become the marker of preference for apple linkage mapping. Over 500 SSR makers have been developed (Guilford *et al.* 1997, Giafranceschi *et al.* 1998, Hokanson *et al.* 1998, Liebhard *et al.* 2002, Hemmat *et al.* 2003, Vinatzer *et al*. 2004, Silfverberg-Dilworth *et al*. 2006, Celton *et al.* 2009, Gasic *et al.* 2009, van Dyk *et al.* 2010) and are available on a website created in the framework of the European Union funded research project HiDRAS (http://www.hidras.unimi.it/).

Until now linkage maps from over 50 apple crosses have been produced (Brown, 2012), including 'Prima' x 'Fiesta' (Maliepaard *et al.* 1998, Liebhard *et al.* 2003a), 'Breaburn' x 'Telamon' (Kenis and Keulemans, 2005), 'Fiesta' x 'Discovery' (Silfverberg-Dilworth *et al.* 2006), 'Fiesta' x 'Totem' (Fernandéz-Fernandéz *et al.* 2008) and the recent rootstock cross 'Malling 9' x *Malus robusta* 5' (Celton *et al.* 2009). Despite the abundance of robust sequence-characterized markers available for *Malus*, their distribution across the genome is not even and many linkage maps still contain areas of low marker coverage (Fernandez-Fernandez *et al.* 2008).

In the past, the development of novel SSR markers relied on screening of BAC libraries (Vinatzer *et al.* 2004) or on sequence analysis of EST databases (Celton *et al.* 2009). In addition AFLP analysis has been used for filling the gaps in SSR-based linkage maps (Liebhard *et al.* 2003a). The apple genome sequence, which has recently become available, offers numerous opportunities for improved marker development and mapping.

1.5.4 Genome sequencing

In 2010 Velasco and coworkers have published the entire genome sequence of *Malus* x *domestica* cultivar 'Golden Delicious'. Total contig length of the assembled sequencing reads was 603.9 Mb, equating 81% of the estimated size of the *Malus* genome. The availability of this reference genome sequence is of immense benefit for future genetic investigations in *Malus*. First, it can be anchored to existing linkage maps to provide an interface between the genetic data generated for a specific population and the underlying genome sequence data (Fernandez-Fernandez *et al.* 2012). Thus it will permit rapid identification of candidate genes located in the QTLs identified through linkage mapping. Secondly, assembly of the two 'Golden Delicious' haplotypes has identified over 10^6 SNPs, as a virtually unlimited source of novel DNA markers. With this information saturation of poorly covered regions in genetic linkage maps high density mapping of QTL intervals and can be tackled efficiently (Fernandez-Fernandez *et al.* 2012). Finally the genome sequence can be screened *in-silico* for candidate genes of various traits, including disease resistance.

1.5.5 QTLs for fire blight resistance

For the identification of QTLs related to fire blight resistance, apple progenies derived from parents with different susceptibility levels, are inoculated with *E. amylovora* in controlled greenhouse experiments. Variation in the severeness of infection is compared to the segregation of DNA markers in the progeny population. Seven such crossing experiments have been carried out to date and have identified resistance related QTLs in linkage groups 3, 5, 7, 9, 10, 12, 13 and 15 (Table3).

Population size	QTL donor parent	LG	Peak (cM)	PVE (%)	References
'Prima'×'Fiesta'					
144	Prima	3	53.8	5.1	Calenge *et al*. (2005)
	Fiesta	7	46.7	43.2	
	Prima	3	53.8	7.5	
	Fiesta	7	46.7	46.6	
'Fiesta'×'Discovery'					
188	Fiesta	3	0	4.4	
	Fiesta	7	52.7	42.6	
	Discovery	12	62.3	5.4	
	Discover	13	10.2	7.9	
	Fiesta	3	0	4.9	
	Fiesta	7	50.7	34.3	
'Fiesta'×'Discovery'					
86	Fiesta	7	50.1	38–39	Khan *et al*. (2006,07)
'MM106'×'Evereste'					
194	Evereste	12	54.2	56.5	Durel *et al*. (2009)
	Evereste	15	40.3	6.2	
	Evereste	12	54.2	57.2	
	Evereste	15	40.3	6.9	
'Golden Delicious'×'M. floribunda 821'					
125	M. floribunda	12	23.3	39.5	
	M. floribunda	12	23.3	40.4	
'Florina'×'Nova Easygro'					
120	Florina	10	30.9	16–18	Le Roux *et al*. (2010)
	Florina	5	34.8	6–7	
	Nova	5	14.9	7.9	
	Nova	9	37.5	7.3	
	Nova	10	56.9	–	
	Florina	10	30.9	13–15	
	Florina	5	34.8	9 / 10	
	Nova	5	0	7.2	
	Nova	9	37.5	6.6	
'Idared'×'Malus×robusta 5'					
146	M.robusta 5	3	48.2–58.9	75	Peil *et al.* (2007, 08 i.e. chapters 2 and 3 in this thesis)

Table 3. Fire blight QTLs identified in apple (simplified from Khan *et al.* 2012)
Abbreviations used in the table: linkage group (LG), position of QTL, phenotypic variation explained (PVE)

In most cases one QTL with strong association to phenotypic resistance and 1-3 additional QTLs with weak effects have been identified. The strong QTLs identified in different *Malus* species and varieties are located on different chromosomes, indicating the existence of several fire blight resistance mechanisms in apple (Khan *et al.* 2012). Crossing of the relatively resistant *M* x *domestica* cultivar 'Fiesta' with either 'Prima' or 'Discovery' identified a QTL on LG 7 of Fiesta (F7), explaining about 40% of the resistance variation (Calenge *et al.* 2005, Khan *et al.* 2006). The independent detection of F7 in different genetic backgrounds and by different authors validates the stability and reliability of this locus. Pedigree analysis with specific sequence characterized amplified region (SCAR) markers tracked the QTL F7 back to the ancestral apple Cox's orange Pippin (Khan *et al.* 2007). However F7 has also been detected in apples not related to this ancestor and it's presence in different cultivars does not infallibly predict resistance (Nybom *et al.* 2012). Recently, two resistance QTLs have been detected on LG5 and LG10 of the cultivar 'Florina', explaining respectively 10% and 16% of the resistance variation in a population derived from the cross 'Florina' x 'Nova Easygro' (Le Roux *et al.* 2010).

Genetic loci with stronger relation to resistance have been detected in crossing experiments with wild or crap apples. Durel *et al.* (2009) detected a fire blight resistance QTL at the bottom of LG12 of the resistant ornamental crap apple 'Evereste' as well as in the resistant wild apple *M. floribunda* 821. This QTL explained 57% of resistance variation in a 'Evereste' x 'MM106' progeny, and 40% of the variation in a '*M. floribunda* 821' x 'Golden delicious' progeny population, respectively. The chromosomal region, where this QTL is located carries four major scab and powdery mildew resistance genes (Khan *et al.* 2012). Chromosome landing and sequencing of the QTL on chromosome 12 of 'Evereste' identified a fire blight resistance candidate gene, showing homology to the Pto/Prf complex, conferring resistance to Pseudomonas syringae in tomato (Paravicini *et al.* 2011).

Finally this thesis will deal with the discovery of a fire blight resistance locus on LG3 of the apple rootstock *Malus x robusta* 5, which explains 75% of the phenotypic variation in a *M.x robusta* 5 x 'Idared' and may carry a major fire blight resistance gene.

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2 Objectives

The presented thesis was carried out within the frame of the BMLFUW and Bund-Bundesländer funded research project „Molecular and genetic mechanisms of fire blight resistance" in which the Julius Kühn Institute in Dresden-Pillnitz (Prof. Dr. Hanke, Dr. Peil and Dr. Flachowsky), the Technical University of Vienna (Prof. Dr. Stich) and the Austrian Institute of Technology collaborated to develop approaches for fire blight control. One of the most promising strategies is breeding for increased resistance in apple. This requires knowledge on the poorly understood genetic basis of this trait. Therefore the specific aim of this thesis was to identify genetic determinants of fire blight resistance in the wild apple clone *Malus x robusta* 5, which is immune against the most aggressive strains of *Erwinia amylovora*, the causative fire blight agent.

The first step was the analysis of AFLPs and SSRs markers in a progeny population derived from a cross of the highly susceptible cultivar 'Idared' with '*M.x robusta* 5'. Based on the segregation of these markers, saturated parental linkage maps were constructed as a framework for the localization of genetic loci involved in fire blight resistance (**Section 3.1.**).

The second objective was the localization of fire blight resistance determinants within this linkage map. **Section 3.2.** presents phenotypic fire blight resistance data of the 'Idared x *M.x robusta* 5' mapping population which revealed co-segregation of individual DNA markers with phenotypic resistance and enabled identification and mapping of a major fire blight quantitative trait locus (QTL).

The detection of QTLs is sensitive to environmental conditions and to the genetic background. Therefore the presence of the identified fire blight resistance locus in *M x robusta* 5 was confirmed in a second year of observation of 'Idared x *M.x*

robusta 5' progeny and verified with the mapping population 'Malling 9 x *M.x robusta* 5' (**Section 3.3**).

Resistance breeding aims at the development of plants that are resistant to multiple diseases, and *M. robusta* is also an interesting source for resistance to fungal pathogens such as mildew. Knowledge on the chromosomal location of the different resistance genes is essential and appropriate DNA markers are required to screen breeding populations for individuals with multiple resistances. The suitability of the genetic map as a backbone for localizing markers linked to other resistance traits is exposed at the example of a SCAR marker for the mildew resistance gene *Pl1* in **section 3. 4**.

Apart from the identification of resistance genes, also information on expression of putative functional genes involved in the basic host defense reaction is valuable for breeding. **Section 3.5**. reports on the identification and mapping of a putative protease inhibitor gene (*Pl-Mal*), which was significantly downregulated in 'Idared' upon infection with *E. amylovora*.

The **final chapter** provides general conclusions drawn from the results of this thesis and an outlook on future research on fire blight resistance in apple.

3 Results

3.1 Genetic linkage maps of the apple cultivar Idared (*Malus* × *domestica* Borkh.) and the fire blight resistant wild species clone *Malus x robusta* 5 based on AFLP and microsatellite markers

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Abstract

Genetic linkage maps of the fire blight resistant '*Malus x robusta* 5' (Mr5) and the susceptible *Malus x domestica* Borkh. cultivar 'Idared' are presented, as a base for QTL analysis of fire blight resistance in apple. 72 SSR primer pairs and 24 AFLP primer pairs were selected to screen 80 polymorphic SSR loci and 213 polymorphic AFLP loci in 140 progenies derived from the cross 'Idared x Mr5'. Two separate parental linkage maps were calculated from the segregation patterns of these 293 markers. The 'Idared' map comprised 160 markers and spanned a genetic distance of 1062 cM. The 'Mr5' map comprised 194 markers and spanned 1012 cM. In both maps the 17 linkage groups of the apple genome could be identified based on the presence of SSR markers. However two linkage groups in each map remained fragmented and need to be saturated by introduction of additional markers. The maps presented in this chapter have been successfully used for mapping a fire blight resistance QTL and a mildew resistance gene of 'Mr5' as well as a putative proteinase inhibitor protein gene involved in the fire blight response of 'Idared' (Sections 3.2, 3.3, 3.4 and 3.5 of this thesis).

Key words: Apple, Genetic linkage map, Mapping, QTL, AFLP, SSR.

Introduction

The cultivated apple (*Malus* × *domestica* Borkh.) is the most important temperate fruit species (FAO, 2005) and breeding of novel varieties with superior agricultural traits is of great agronomic importance. Particularly the development of disease resistant cultivars can reduce the risk of harvest losses and at the same time improve the environmental situation in apple cropping areas due to reduced pesticide demand (Kellerhals 2009a). The efficiency of apple breeding can be greatly enhanced by marker assisted selection (MAS), i.e. the use of DNA markers to screen seedling populations for presence of desired traits (Kellerhals *et al.* 2009b).

The construction of genetic linkage maps, based on marker segregation in progeny populations derived from parents differing in a trait of interest, enables the identification of DNA markers linked to this trait. Moreover genetic linkage maps give information on the overall genome structure of an organism and allow localization and identification of the genes conferring specific traits (Paterson 1990). Importantly, genetic linkage maps are suitable for the identification of both major genes and quantitative trait loci (QTL) (Ferreira and Grattapaglia 1995). QTLs are genomic loci harboring sets of genes which are collectively involved in the control of a given trait. Many agronomic traits of plants, such as fruit quality, adaptability and resistance to certain diseases, are of quantitative nature (Liebhard *et al.* 2003a). In apple research genetic linkage maps have supported localization of QTLs for various biometric characteristics (Liebhard *et al.* 2003b), aroma compounds (Dunemann *et al*. 2009), self incompatibility (Fernandez-Fernandez *et al.* 2012) and of genes for resistance to mildew (Stankiewicz-Kosyl *et al.* 2005, Calenge *et al.* 2006) and apple scab (Durel *et al* 2003, Liebhard *et al*. 2003a, Calenge *et al*. 2004, Schouten and Jacobsen, 2008).

Various DNA marker systems are available for the construction of linkage maps. Microsatellites, or simple sequence repeats (SSRs) have become the marker of preference for linkage mapping in apple (Fernandéz-Fernandéz *et al.* 2012). SSRs are abundant and uniformly distributed across plant genomes (Morgante *et al.* 2002). The

high degree of polymorphism of these co-dominant markers allows detection of many different alleles per locus (Weber and May, 1989). Moreover SSRs are generally conserved in *Malus*. Therefore they can be transferred between different cultivars and species and allow map alignment (Guilford *et al.* 1997, Liebhard *et al.* 2002). Over 500 *Malus* SSR markers have been described and are available for map construction and comparison (Guilford *et al.* 1997, Giafranceschi *et al.* 1998, Hokanson *et al.* 1998, Liebhard *et al.* 2002, Hemmat *et al.* 2003, Vinatzer *et al*. 2004, Silfverberg-Dilworth *et al*. 2006, Celton *et al.* 2009, Gasic *et al.* 2009, Van Dyk *et al.* 2010).

However, to date several areas of the genome remain insufficiently covered in SSR based maps (Fernandez-Fernandez *et al.* 2008). The amplified fragment length polymorphism (AFLP) technique is very suitable for enhancing saturation of SSR-based linkage maps. AFLP does not require sequence knowledge and generates a large number of reliable dominant markers, widely distributed across the genome with relative low input of labor and time (Vos *et al.* 1995). Combination of SSRs and AFLP markers has proven very efficient to create high density linkage maps for several cultivars of *Malus x domestica* Borkh. (Liebhard *et al.* 2003b, Kenis and Keulemans, 2005).

In this work the construction of two genetic linkage maps derived from a cross between the *Malus x domestica* Borkh. cultivar 'Idared' and the wild species clone '*Malus x robusta* 5' (Mr5) based on SSR and AFLP markers is described. 'Idared' is a tasty and economically important cultivar, but susceptible to the fire blight disease caused by the bacterium *Ervinia amylovora*. Mr5 is a vigorous rootstock, producing fruits without commercial interest, but resistant to several pests and diseases including the most aggressive strains of *E. amylovora* collected in Germany (Richter *et al.* 2005). The genetic determinism of fire blight resistance in plants is still largely unknown. Therefore the maps presented here were prepared as a basis for localization (QTL analysis) of fire blight resistance in *Malus*, and for the development of appropriate DNA markers for the respective resistance locus or loci.

Materials and methods

Plant material

A cross between 'Idared' and '*Malus x robusta* 5' (Mr5, Acc APF0409) denoted as crossing (I x *Mr*-5) was carried out in 2003 at the Julius Kühn-Institut (JKI) in Germany. Mr5 was the pollen parent in this cross. The resulting progeny No. 04208 consisted of 280 individuals, 140 of which were used as mapping population in this study.

DNA isolation

Genomic DNA was isolated in 2006 from young leaf material (approximately 0.1 g) using the DNeasy plant miniprep kit (Qiagen, Hilden, Germany). The quantity and quality of DNA was controlled by agarose-gel electrophoresis (1% agarose). For SSR analysis the DNA was diluted to a concentration of 10 ng/µl.

SSR analysis and SSR nomenclature

Initially a set 101 apple SSRs from published apple linkage maps (Liebhard *et al*. 2002, Silfverberg-Dilworth *et al*. 2006, Vinatzer *et al*. 2004) was tested for polymorphism in the parents 'Idared' and 'Mr5' and in five progenies. Seventy four polymorphic SSRs were identified and analyzed in the total mapping population (Table 1). Multiplex PCRs with six primer pairs were performed using the QIAGEN Multiplex PCR Kit (QIAGEN, Hilden, Germany) and NED, FAM and HEX as fluorescent labels. Each label was used for two different microsatellites differing in fragment size. Composition of PCR reaction mixes and cycling conditions have been described previously (Peil *et al.* 2007). For detection, 0.5 µl PCR product were amended with 0.3 µl GeneScan 500 XL size standard (Applied Biosystems Forster City, CA) and 10 µl Hi-Di formamide (Applied Biosystems), denatured for 5 min at 95 °C and run on a 3100 Genetic Analyser automated sequencer (Applied Biosystems Inc., Foster City, CA) using a 22 cm capillary with the settings: injection voltage of 6 kV, injection time 3 sec, run voltage 15 kV and run time 720 sec.

AFLP analysis and AFLP nomenclature

AFLP analysis was performed according to the method of Vos *et al*. (1995), with some modifications. Digestion, either with *Eco*RI and *Mse*I or with *Pst*I and *Mse*I (New England Biolabs, Ipswich, MA) was performed in one step together with ligation. Restriction ligation (RL) reactions in1x RL-buffer (New England Biolabs) were carried out in 40 µl volumes containing 5 units of each enzyme, 0.5 mM ATP and 100 ng of template DNA. RLs were incubated overnight at 37°C. Depending on the enzymes used for restriction, either primers EcoRI01 and MseI or Pst00 and MseI were used for preamplification. Five µl of RL product were used as template in 20 ul preamplification reactions containing 1xPCR polymerase buffer, 2.25 ng of each preamplification primer, 200µM of each dNTP and 2 units of DyNAzyme polymerase (Finnzymes, Fischer Scientific, Vantaa, Finland). Preamplification products were diluted 10 times with TE buffer and 2ul of these dilutions were added as templates to selective amplification reactions. Selective amplifications were carried out in 20 µl volumes containing 1xPCR Polymerase buffer, 3 mM MCl_2 200 µM of the two selective primers (either Eco and Mse of Pst and Mse) and 0.6units of FIREpol polymerase (Solis BioDyne,Tartu, Estonia). Detection was carried out in the 3100 Genetic Analyser automated sequencer as described for SSR analysis.

A total of 94 AFLP primer combinations were tested on 'Idared', 'Mr5' and 5 'I × *Mr*-5' individuals to select primer combinations which amplify the most clear and unambiguous polymorphic fragments. Scoring of AFLP markers was based upon presence or absence of the amplification peak (dominant scoring) and carried out in GENESCAN and GENOTYPER. AFLP markers were designated according to standard AFLP nomenclature (http://wheat.pw.usda.gov/ggpages/keygeneAFLPs.html) based on the name of the two restriction enzymes used and a number code for the selective nucleotides (Table 2). In the linkage map, designations of AFLP loci were extended by and the size of the local fragment (Figure 1).

Map construction

All markers were separated into three groups: (1) those showing only alleles of the female parent 'Idared', (2) those exhibiting only alleles of the male resistant parent '*M.x robusta 5*' and (3) those presenting alleles of both parents. Markers of types 1 and 3 were used to construct a genetic linkage map for 'Idared' while markers of types 2 and 3 were used to create a genetic linkage map for 'Mr5'.

All data analysis was performed using the JoinMap v 4.0 software (Van Ooijen and Vorrips, 2001). Markers with missing data for more than half of the scored 'I × *Mr*-5' genotypes were excluded. The remaining markers were tested for segregation distortion using the chi-square test, before they were used for mapping. Subsequently, linkage grouping of markers was compared at various LOD thresholds (from 2 to 10) to evaluate the stability of the resulting linkage groups. Finally an LOD threshold of 7.0 was chosen to calculate the linkage groups for the parental linkage maps. To determine the order of markers within linkage groups, the JoinMap parameters were set as follows Rec = 0.4, LOD = 7.0, Jump = 5. Exclusion criteria for individual markers were defined as follows: markers inhibiting map calculations or the establishment of the marker order, and markers presenting a severe segregation distortion (a=0.05) while markers in the immediate vicinity did not were to be excluded. Map distances were calculated in centimorgans (cM) using Konsambi´s function. The final version of the genetic maps was drawn using the Map Chart 2.1 Software (Voorrips 2002). Linkage groups were assigned by comparison of the observed SSR marker placement to that in the apple map published by Silfverberg-Dilworth *et al*. (2006).

Results

SSR Markers

Out of the tested 101 SSR primer combinations 72 produced polymorphic fragments in the 'I × *Mr*-5' population. Seven of these were multilocus SSRs so that 80 SSR loci were amplified. The 80 polymorphic SSR loci were distributed across all seventeen linkage groups of the apple genome (Table 1). In total 236 different alleles were obtained from

the 80 loci. 141 alleles were amplified from ‘Idared’, 137 alleles from Mr5. 42 alleles were shared between both parents (Table 1). In four SSRs (2%) distorted segregation (chi square test, $p < 0.05$) was observed.

Number	SSR	Idared	*Malus x robusta* 5	Linkage group	Reference
1	CH03g12a,b*	149/167, 179/195	147/147, 173/195	1, 3	Liebhard *et al*. 2002
2	CH05g08	179/182	176/176	1	Liebhard *et al*. 2002
3	CH-Vf1	138/172	134/156	1	Vinatzer *et al*. 2004
4	CH02c02a	168/176	183/183	2	Liebhard *et al*. 2002
5	CH02c06	240/245	245/250	2	Liebhard *et al*. 2002
6	CH02f06	148/157	141/146	2	Liebhard *et al*. 2002
7	CH03d01	103/109	105/105	2	Liebhard *et al*. 2002
8	CH03d10	163/169	163/175	2	Liebhard *et al*. 2002
9	CH05e03	171/183	161/161	2	Liebhard *et al*. 2002
10	AU223657	127/146	121/146	3	Silfverberg *et al*. 2006
11	CH03e03	205/205	183/205	3	Liebhard *et al*. 2002
12	CH03g07	217/226	226/231	3	Liebhard *et al*. 2002
13	Hi03d06	114/141	119/143	3	Silfverberg *et al*. 2006
14	MS14H03	111/136	115/136	3	Liebhard *et al*. 2002
15	CH02c02b	108/108	108/110	4	Liebhard *et al*. 2002
16	CH02h11a	102/119	101/124	4	Liebhard *et al*. 2002
17	CH01d03z	134/134	134/156	4	Liebhard et al. 2002
18	CH05b06a, b*	171/177, 199/219	177/197, 199/199	5, 16	Liebhard *et al*. 2002
19	CH03a04	119/161	103/111	5	Liebhard *et al*. 2002
20	CH03a09	120/138	118/128	5	Liebhard et al. 2002
21	CH04e03	186/198	167/186	5	Liebhard et al. 2002
22	CH05e06	150/150	123/135	5	Liebhard *et al*. 2002
23	CH05f06	174/178	176/181	5	Liebhard et al. 2002
24	Hi04a08p	212/244	216/224	5	Silfverberg *et al*. 2006
25	Hi04d02p	175/205	220/220	5	Silfverberg *et al*. 2006
26	CH05a05a,b*	198/229, 0/83	216/0, 0/0	6, 6	Liebhard *et al*. 2002
27	CH02d12a,b,c,d*	0/102, 0/117, 0/119, 0/121	0/0, 0/117, 0/119, 0/121	6, 6, 6, 6	Liebhard *et al*. 2002
28	CH03d07	199/203	168/189	6	Liebhard *et al*. 2002
29	Hi03a10	215/239	0/0	6	Liebhard *et al*. 2002
30	Hi07N06	181/206	175/179	6	Liebhard et al. 2002
31	CH04e05	173/225	180/180	7	Liebhard *et al*. 2002
32	CH03A08	183/183	169/172	7	Liebhard *et al*. 2002
33	Hi05b09	136/136	130/147	7	Silfverberg *et al*. 2006
34	CH05a02	126/131	111/121	8	Liebhard *et al*. 2002
35	CH01c06	159/159	146/159	8	Liebhard *et al*. 2002

Table 1: Polymorphic SSR loci in 'Idared' and Mr5. The SSR alleles are sized in basepairs.

*multilocus SSRs

Number	SSR	Idared	*Malus x robusta* 5	Linkage group	Reference
36	CH02g09a, b*	108/132, 186/186	108/114, 161/161	8, 8	Liebhard *et al*. 2002
37	Hi23g12a, b*	237/243, 220/220	243/243, 222/228	8, 8	Silfverberg et al. 2006
38	CH01f03b	160/174	174/174	9	Liebhard *et al*. 2002
39	CH05c07	137/137	133/145	9	Liebhard et al. 2002
40	CN444542	128/149	149/149	9	Silfverberg et al. 2006
41	Hi05e07	211/226	190/226	9	Silfverberg *et al*. 2006
42	CH02a10	175/175	157/165	10	Liebhard *et al*. 2002
43	CH02b07	106/106	93/108	10	Liebhard et al. 2002
44	CH02c11	228/234	203/217	10	Liebhard et al. 2002
45	CH03d11	119/123	104/106	10	Liebhard *et al*. 2002
46	CH02d08	223/227	210/212	11	Liebhard *et al*. 2002
47	CH04a12	181/184	179/179	11	Liebhard *et al*. 2002
48	CH04g07	147/170	164/168	11	Liebhard *et al*. 2002
49	CH01f02	181/206	175/179	12	Liebhard *et al*. 2002
50	CH01g12	105/180	105/105	12	Liebhard *et al*. 2002
51	CH03c02	123/131	117/125	12	Liebhard *et al*. 2002
52	CH04d02	120/148	122/148	12	Liebhard *et al*. 2002
53	Hi07f01	202/202	195/197	12	Silfverberg *et al*. 2006
54	MdFT_1_2	241/244	244/0	12	Unpublished data
55	MdFT_3_4	256/256	249/24	12	Unpublished data
56	CH03h03a, b*	75/75, 116/166	70/74, 91/91	13, 13	Liebhard et al. 2002
58	CH05c04	125/140	170/190	13	Liebhard *et al*. 2002
59	CH05f04	162/162	153/158	13	Liebhard *et al*. 2002
60	CH03a02	118/154	158/180	14	Liebhard *et al*. 2002
61	CH04c07	109/111	106/108	14	Liebhard et al. 2002
62	CH04f06	175/182	184/184	14	Liebhard et al. 2002
63	CH05e05	151/151	0/0	14	Liebhard *et al*. 2002
64	CH05g11	234/244	246/246	14	Liebhard et al. 2002
65	MDAJ761	240/240	250/260	14	Silfverberg *et al*. 2006
66	CH01d08	269/291	280/282	15	Liebhard et al. 2002
67	CH03b10	102/118	94/110	15	Liebhard *et al*. 2002
68	NZ02b01	226/235	0/0	15	Liebhard et al. 2002
69	CH02a03	149/154	132/149	16	Liebhard *et al*. 2002
70	CH04c06	181/186	184/184	17	Liebhard *et al*. 2002
71	CH02g04	0/0	173/206	17	Liebhard *et al*. 2002
72	CH05g03	160/182	142/188	17	Liebhard *et al*. 2002

Table 1 (continued): Polymorphic SSR loci in 'Idared' and Mr5. The SSR alleles are sized in basepairs.

*multilocus SSRs

AFLP markers

Out of 94 AFLP primer combinations tested, 24 combinations were selected for mapping (Table2). The remaining combinations produced either weak peaks or monomorphic fragments. Together, the 24 selected primer combinations yielded 823 fragments, 213 of which were polymorphic in the mapping population. Thus on average each primer combination contributed 34.3 total and 8.9 polymorphic fragments, and the AFLP polymorphism rate between 'Idared' and Mr5 was 26%. Of the 213 polymorphic fragments 67 were detected in 'Idared', 93 in 'Mr5' and 53 in both parents (3:1 segregation indicating heterozygous presence in both parents).

Number	**Marker (primer combination)**	**Total Fragments**	**Polymorphic fragments**			
			Idared	***Mr* 5**	**Both**	**Total**
1	E33M32	26	1	1	1	3
2	E40M33	28	4	5	1	10
3	E40M49	29	1	3	3	7
4	E40M50	27	4	1	2	7
5	E41M32	29	0	4	1	5
6	E41M33	28	2	4	2	8
7	E41M49	27	1	3	2	6
8	P12M32	37	3	4	0	7
9	P12M38	36	1	4	3	8
10	P12M39	37	7	4	5	16
11	P12M40	34	4	3	4	11
12	P12M48	39	3	4	2	9
13	P12M49	38	2	6	1	9
14	P14M37	32	2	1	1	4
15	P14M48	37	7	3	2	12
16	P14M49	40	3	3	2	8
17	PAGM32	36	4	6	3	13
18	PAGM37	20	0	2	4	6
19	PAGM38	39	1	9	2	12
20	PAGM42	40	4	6	2	12
21	PAGM48	43	3	8	2	13
22	PAGM49	44	5	4	3	12
23	PAGM58	32	1	2	4	7
24	PAGM60	45	4	3	1	8
		823	**67**	**93**	**53**	**213**

Table 2 AFLP-Markers. Number of total and polymorphic fragments

Map construction

In total 80 SSR loci and 213 AFLP loci, genotyped over 140 progenies, were used to construct parental maps for 'Idared' and Mr5 (Figure 1). None of the polymorphic markers met the exclusion criteria detailed above (Materials and methods section). Thus no exclusions were necessary. Even the four SSRs with distorted segregation patterns could be retained in the dataset, as they were located together on LG5. However, nine valid polymorphic microsatellite loci and 27 AFLP loci could not be mapped by JoinMap. Placement of all remaining markers required only a single calculation round. Key information on the two maps is summarized in Table 3.

	Idared	*Malus* × *robusta* 5
Number of LGs	21	20
Number of LGs with SSRs	19	20
LGs not assigned	2	0
Number of loci	160	194
Total length (cM)	1062	1012
Marker densitiy (cM/marker)	6.6	5.2

Table 3.

Description of the 'Idared' and '*Malus x robusta* 5' maps.

The genetic linkage map of 'Idared' consisted of 160 markers (68 SSRs and 92 AFLPs) linked in 21 linkage groups. Based on SSR markers 19 of these linkage groups could be assigned to the 17 chromosomes of apple, with LG6 and LG17 being split into two parts. The remaining 2 linkage groups could not be assigned to LGs of published apple maps, due to lack of SSRs, and were designated "LG A" and "LG B". The total map covered a genetic distance of 1062 cM with an average marker density of 6.6 cM/marker. The length of the individual linkage groups varied from 12 cM to 96 cM (Table 4) and the largest gap was observed on LG15 (28.9 cM).

For 'Mr5' 194 markers (66 SSRs and 128 AFLPs) coalesced to 20 linkage groups that could be assigned to the 17 chromosomes of apple according to SSRs. LG6 and LG7 remained split into two and three parts respectively (Figure 1). The map spanned 1012 cM of the apple genome with a density of 5.2 cM/marker and with linkage group lengths

varying between 2 cM and 100 cM. In Mr5 the largest gap was located on LG17 of (28.4 cM) (Table 4).

Linkage group Idared	Size cM	No of markers	Marker density (cM/marker)
1	65	6	13.00
2	96	15	6.86
3	66	11	6.60
4	51	10	5.67
5	69	18	4.06
6	46+25[a]	9+2 [a]	5.2, 12.7 [a]
7	62	9	7.75
8	55	6	11.00
9	62	9	7.75
10	78	9	9.75
11	47	9	5.88
12	48	11	4.80
13	50	7	8.33
14	66	12	6.00
15	29	2	29.00
16	88	8	12.57
17	34+24 [a]	3+5 [a]	11.3, 4.8 [a]
A	12	2	12.00
B	17	2	17.00

Linkage Group *Mr*-5	Size cM	No of markers	Average marker interval (cM)
1	76	8	10.86
2	99	20	5.21
3	65	20	3.42
4	54	15	3.86
5	67	16	4.47
6	51+2+25 [a]	14+2+2 [a]	3.7, 1.3, 12.6 [a]
7	56+11 [a]	6+2 [a]	5.8, 9.4 [a]
8	23	3	11.50
9	60	12	5.45
10	75	15	5.36
11	54	10	6.00
12	72	14	5.54
13	13	4	4.33
14	50	15	3.57
15	28	3	14.00
16	72	10	8.00
17	61	6	12.20

Table 4.

Length and marker density of the individual linkage groups.

[a] LG6 and LG17 of Idared and LG6 and LG17 of the Mr5 map were split

Finally the two parental linkage maps were aligned based on 97 common markers comprising 63 common microsatellites and 34 common AFLPs (Figure 1). Pairwise alignment was successful for all assigned linkage groups. Only the two unassigned linkage groups A and B of the Idared map did not share any markers with linkage groups of Mr5.

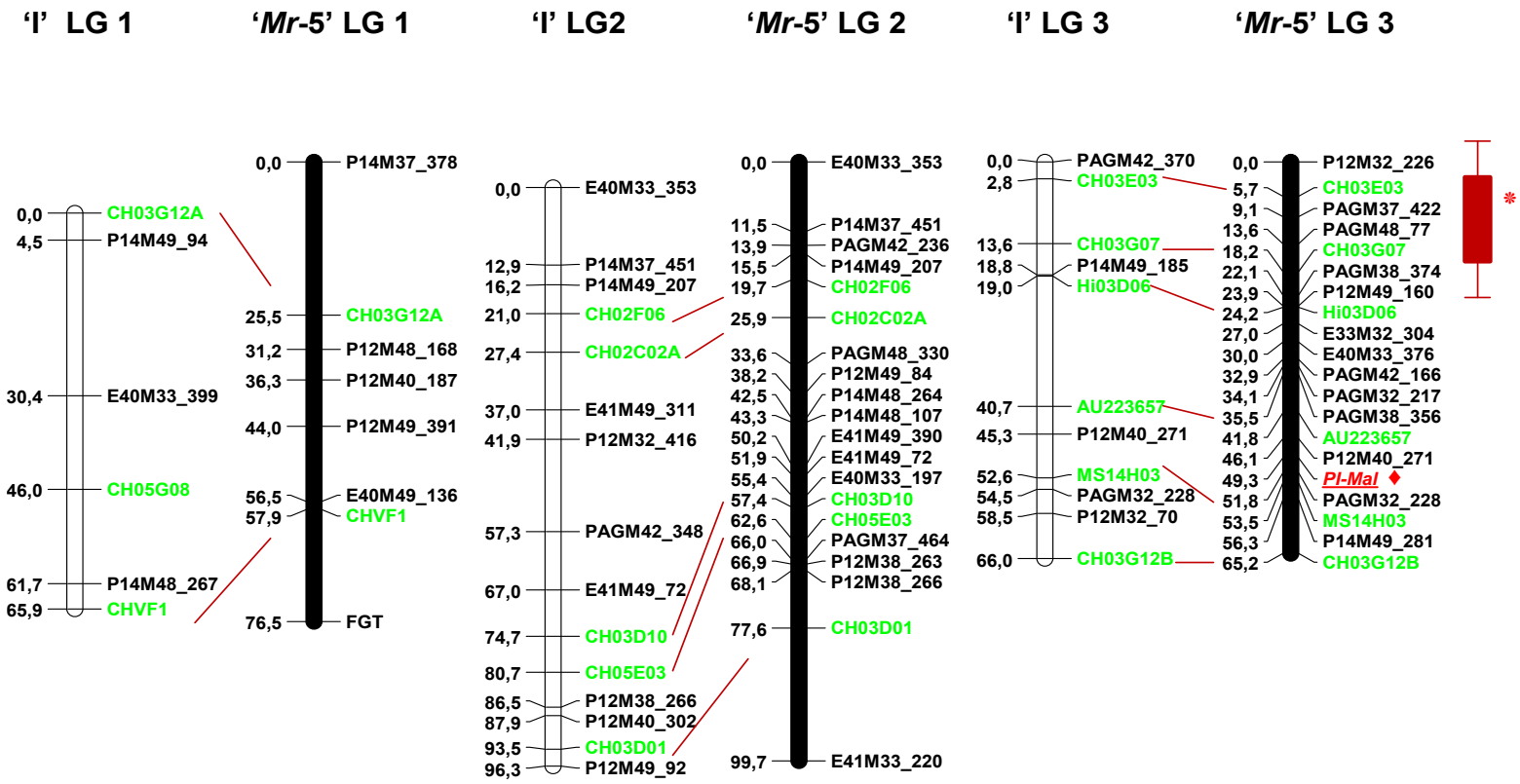

Figure 1. Genetic linkage map of Idared and *Malus x robusta* 5. Numbering of the linkage groups according to Silfverberg-Dilworth *et al*. (2006). SSRs are indicated in green, AFLPs are indicated in black, gene specific markers that are described in later sections of this thesis are marked in red.

Position of the fire blight resistance QTL described in Sections 3.2 and 3.3

♦SNP marker for the putative protease inhibitor gene *PI-Mal* (see Section 3.5).

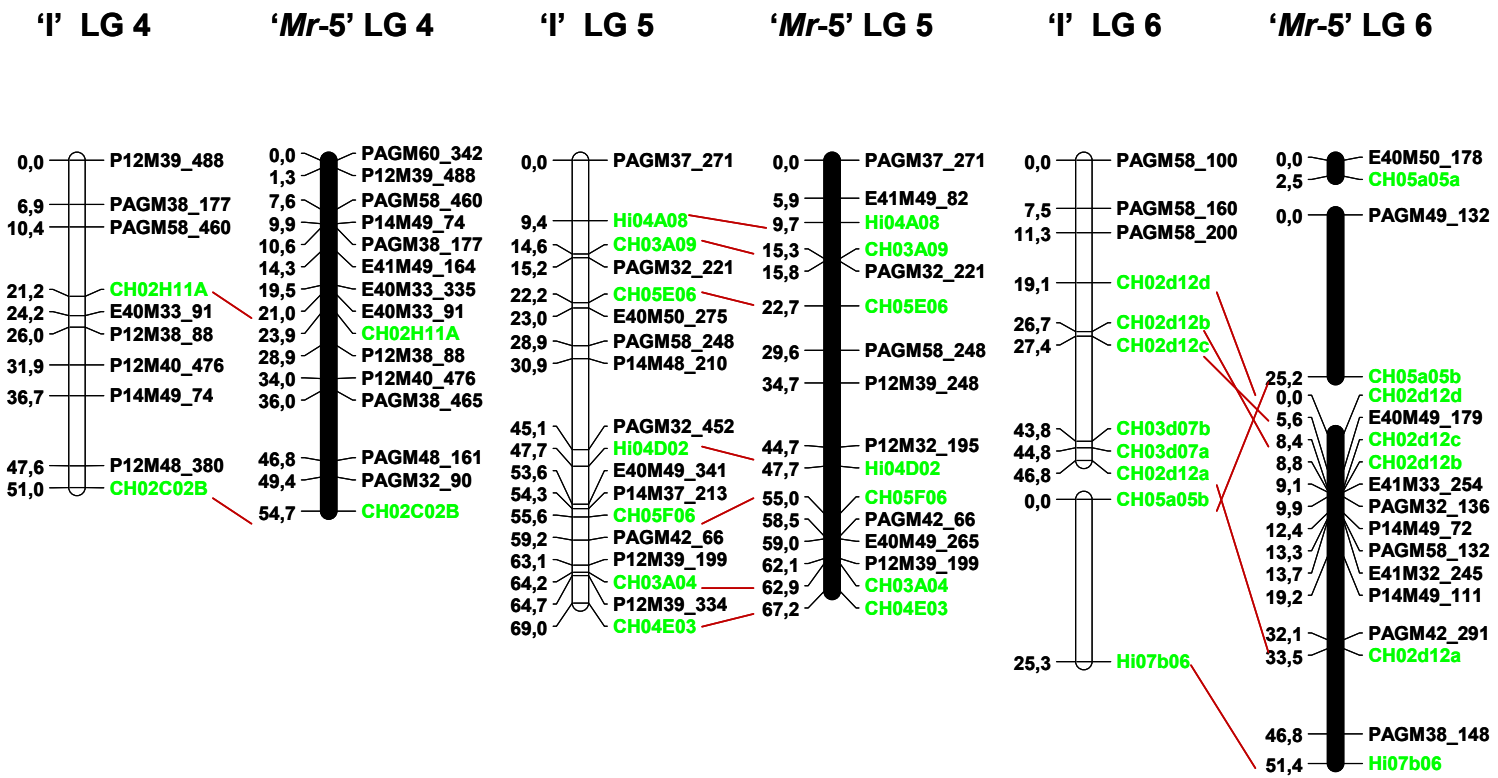

Figure 1 (continued)**.** Genetic linkage map of Idared and *Malus x robusta* 5. Numbering of the linkage groups according to Silfverberg-Dilworth *et al*. (2006). SSRs are indicated in green, AFLPs are indicated in black.

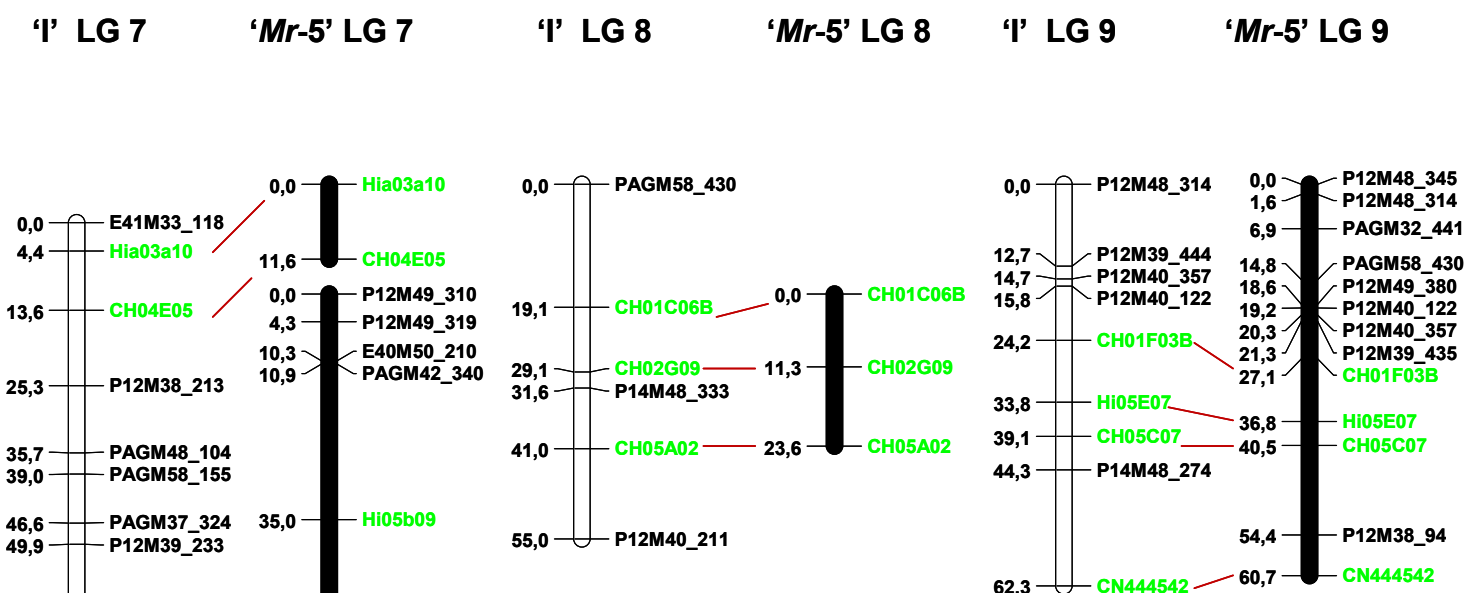

Figure 1 (continued)**.** Genetic linkage map of Idared and *Malus x robusta* 5. Numbering of the linkage groups according to Silfverberg-Dilworth *et al*. (2006). SSRs are indicated in green, AFLPs are indicated in black.

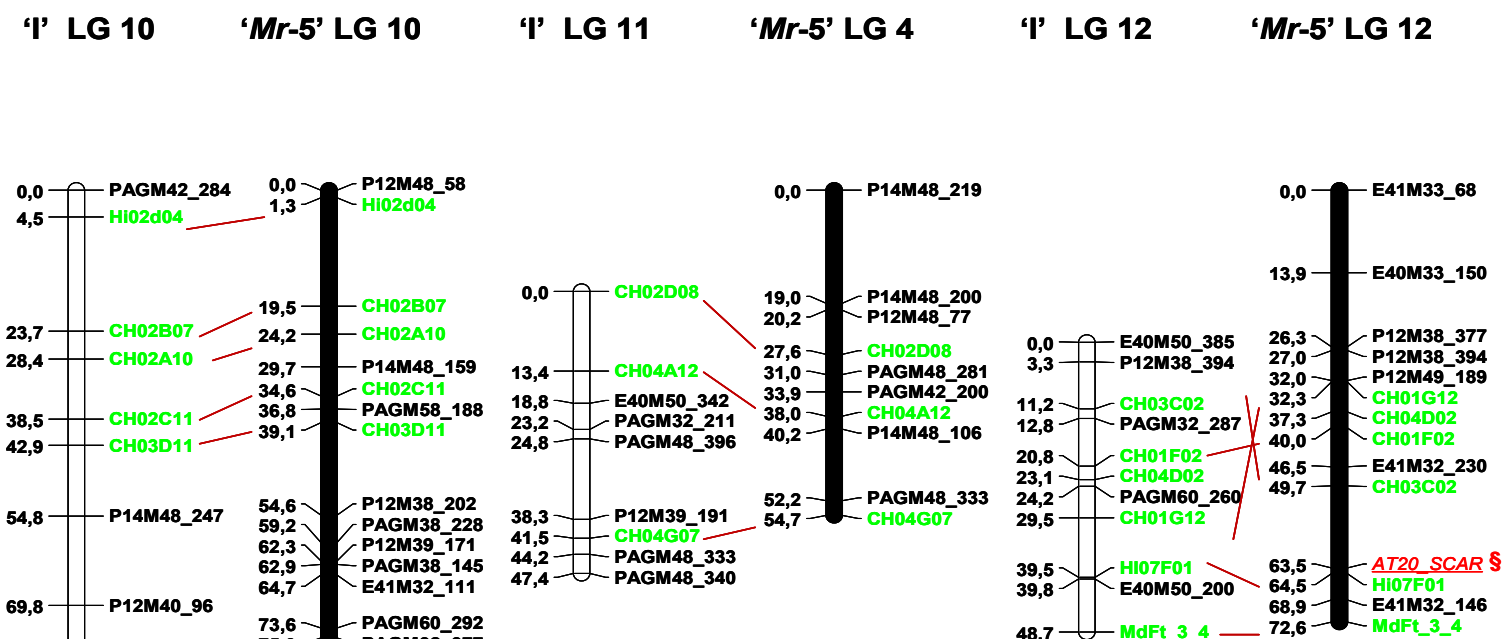

Figure 1 (continued)**.** Genetic linkage map of Idared and *Malus robusta* 5. Numbering of the linkage groups according to Silfverberg-Dilworth *et al*. (2006). SSRs are indicated in green, AFLPs are indicated in black, gene specific markers that are described in later sections of this thesis are marked in red.

§SCAR marker for the mildew resistance gene *Pl1* (see Section 3.4).

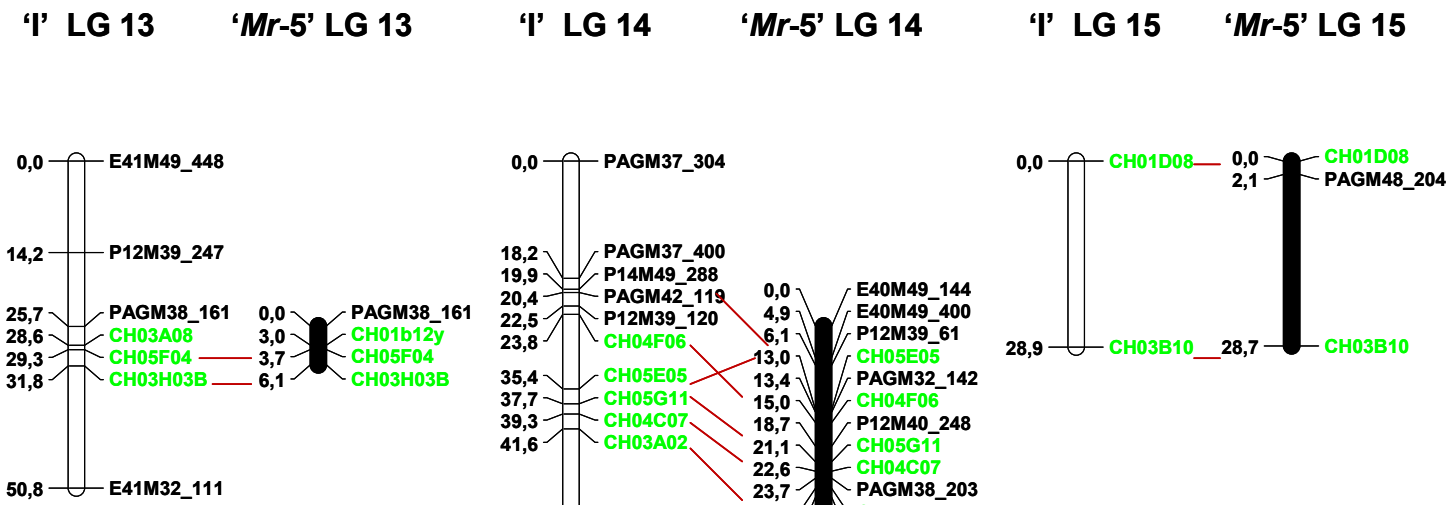

Figure 1 (continued). Genetic linkage map of Idared and *Malus robusta* 5. Numbering of the linkage groups according to Silfverberg-Dilworth *et al*. (2006). SSRs are indicated in green, AFLPs are indicated in black.

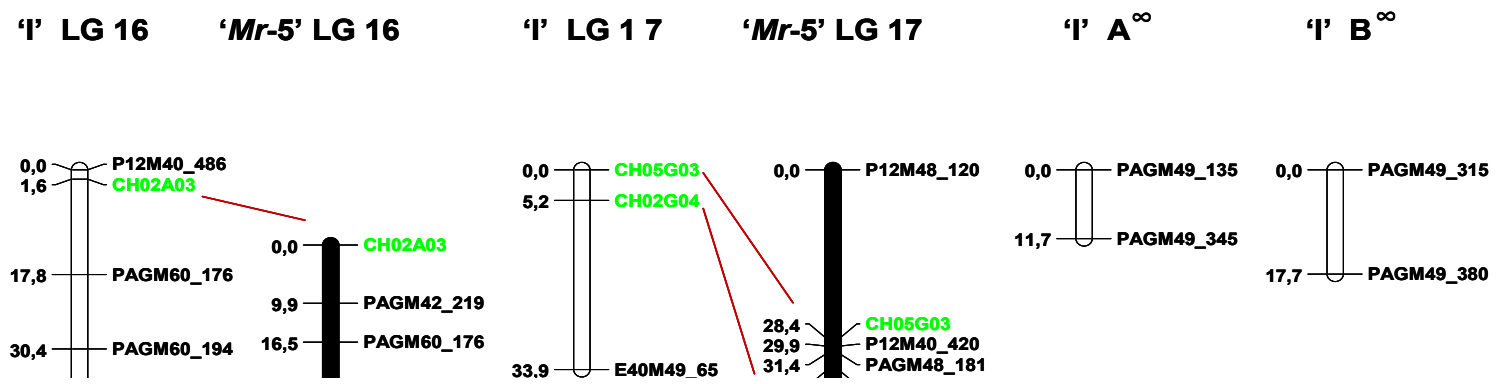

Figure 1 (continued)**.** Genetic linkage map of Idared and *Malus robusta* 5. Numbering of the linkage groups according to Silfverberg-Dilworth *et al*. (2006). SSRs are indicated in green, AFLPs are indicated in black.

∞ Idared - Linkage groups A and B did not contain SSRs and could therefore not be assigned to one of the 17 LGs of the apple genome.

Discussion

SSRs and AFLPs have been analysed in 140 ‘I x Mr5’ progenies, to create parental linkage maps for the fire blight resistant wild apple *M.x robusta* 5 and the susceptible cultivar ‘Idared’, as a basis for QTL mapping of fire blight resistance in apple.

The availability of over 300 SSRs in the continuously updated reference map ‘Fiesta’ x ‘Discovery’ (Silfverberg-Dilworth *et al.* 2006, www.hidras.unimi.it) enabled straightforward selection of 101 primer combinations for SSRs distributed across all 17 apple linkage groups. When applied to the ‘I x Mr5’ population, 72 of these primer combinations yielded polymorphic fragments from 80 genetic loci. 54 of these loci have been sufficient to roughly reconstruct 14 of the 17 apple linkage groups (Peil *et al.* 2007). In the present study we introduced 36 new SSR loci and employed the common strategy of AFLP-based map saturation (Liebhart *et al.* 2002, Kenis and Keulemans, 2005, Le Roux *et al.* 2010). Although 70% of the tested AFLP primer combinations produced either weak or monomorphic profiles, 24 suitable combinations were identified within short time. The 24 selected AFLP primer combinations defined 213 additional marker loci for the parental maps and enabled the computation of all 17 expected linkage groups. The observed SSR-based polymorphism rate (72%) was higher than previously documented values (Celton *et al.* 2009) whereas the degree of AFLP- polymorphism (26%) was comparable to that reported by Kenis and Keulemans (2005). High levels of polymorphism are a general characteristic of *Rosaceae*.

Combined mapping of SSRs and AFLPs produced a linkage map with 160 marker loci spanning a total distance of 1062 cM for ‘Idared’ and a linkage map with 194 loci spanning 1012 cM for Mr5. The two maps had 97 markers in common, which allowed alignment of homologous linkage groups. The order of

the shared SSR markers along each linkage group was generally consistent between 'Idared' and Mr5, confirming the quality of the two independent linkage calculations. The only two exceptions to this consistency were the switch between CH02d12 and CH05a05b on LG6 and the disparate arrangement of CH01F12, CH04D12, CH01F02, CH03C02 on LG12. CH02d12 and CH05a05 were both multilocus SSRs with a high incidence of null alleles concealing segregation patterns. The observed discrepancies in LG12 were most likely due to the relatively high proportion of missing values for the respective loci. Comparison to the reference map 'Fiesta' x 'Discovery' (www.hidras.unimi.it) revealed that Mr5 displayed the true order of markers on LG12, and generally confirmed the correct arrangement of the SSR markers in the 'Idared' x 'Mr5' map.

The observation that the SSRs with distorted segregation clustered in one linkage group (LG5) suggests that this genomic area may carry genes affecting viability and may therefore be subjected to direct (gametophytic) selection. Previous studies on apple have reported segregation distortions of marker sets mainly on LG10, LG15 and near the self-incompatibility locus on LG17 (Kenis and Keulemans, 2005, Fernandez-Fernandez *et al.* 2012).

The overall marker density was one marker every 6.6cM for 'Idared' and one marker every 5.2cM for Mr5. The gaps on several linkage groups did not exceed 10cM, which has proven ideal for efficient QTL mapping (Piepho *et al.* 2000). However the distribution of markers was not uniformous across the linkage maps, which may to some extent reflect the occurrence of recombination hot spots and cold spots in the genome (Collard *et al.* 2005). On the other hand it is evident, that addition of new markers could significantly improve our linkage map in several areas. Particularly the fragmentation of individual linkage groups and the fact that 9 SSRs and 27 AFLPs could not be mapped due to lack of neighboring markers indicated local undersaturation. Fragmentation of linkage groups regularly occurs in newly constructed genetic maps (Liebhard *et al.* 2002,

Le Roux *et al.* 2010), and as long as the fragments carry SSRs from reference maps, correct assignment is possible. Still, splits may hamper QTL detection in the respective areas.

Relaxation of the linkage calculation parameters may have enabled placement of the above mentioned unmapped markers and closure of the splits in our map. An LOD threshold of 7.0 was chosen, which was relatively stringent compared to the settings used by other authors (Celton *et al.* 2009, Le Roux *et al.* 2010), to minimize the risk of marker misplacement. The strategy was to extend and further resolve linkage groups of interest rather by increasing the number of markers than by decreasing stringency. In its current state, the map displays a total length and a marker density comparable to recently published apple maps, which have been successfully used for QTL analysis (Celton *et al.* 2009, Le Roux *et al.* 2010). Moreover, the observation that the length of the individual linkage groups did not necessarily reflect the number of markers per group signalizes, that parts of the map are approaching saturation.

While there is no reference map available for 'Idared', Mr5 has been previously mapped based on a cross with 'Malling 9' (Celton *et al.* 2009). Although these authors combined SSRs with SNPs, RAPDs and SCARs, and their Mr5 map has only 25 SSRs in common with ours, the two maps are similar in several respects. First of all total length was 1087 cM for the Mr5 map of Celton and coworkers (2009) and 1012 cM for our Mr5 map. Secondly overall marker density was exactly 5.2 cM/marker in both maps. And thirdly, the length and coverage of most individual linkage groups corresponded well. Only LG1 and LG2 were considerably longer in our map, and LG3, LG10 and LG14 were much better covered. On the other hand our LG13 and LG15 were shorter and our LG8 was both shorter and less covered than the corresponding LGs in the map of Celton *et al*. (2009). Comparison to the latest version of the reference SSR-map 'Fiesta' x 'Discovery' (www.hidras.unimi.at) confirmed, that additional SSR markers are located beyond the terminal SSRs of these linkage groups in our map.

Unfortunately none of the AFLP markers had captured these regions. In several other linkage groups however, AFLPs successfully extended linkage group length, particularly in LG16, which carried a single SSR, but spanned 89 cM and 72 cM in Idared and Mr5, respectively, matching the standard of the most recently released apple maps (Fernandez-Fernandez *et al.* 2012, www.hidras.unimi.at).

The primary purpose of the presented maps was to provide a framework for localizing QTLs controlling fire blight resistance. Major fire blight QTLs have been previously identified on LG7 of 'Fiesta' (Calenge *et al.* 2005, Khan *et al.* 2006), on LG12 of 'Evereste' and *M. floribunda* (Durel *et al.* 2009, Paravicini *et al.* 2011) and on LG10 of 'Florina' (Le Roux *et al.* 2010). In the presented Mr5 map LG7 was unfortunately split between the SSRs CH04E05 and Hi5b09, which define the region, where the fire blight QTL was located in 'Fiesta' (Calenge *et al*. 2005). In contrast, LG12 and LG10 were intact and showed satisfying lengths and marker densities. LG12 included the SSR Hi07f01, which was significantly linked to fire blight resistance in 'Evereste' and '*M. floribuda*' (Durel *et al.* 2009). LG10 carried the SSRs CH202A10 and CH02B07 which were significantly linked to fire blight resistance in 'Florina' and bracketed the resistance locus (Le Roux *et al.* 2010). Therefore it can be assumed, that the presented map is suitable for testing the presence of the previously documented fire blight resistance loci on LG12 and LG10 in Mr5. Furthermore it is of particular importance, that additional markers are integrated to close the gap in LG7. Five of the tested SSRs for LG7 could not be mapped, because 'Idared' and Mr5 were homozygous at these loci. Fortunately, the genome sequence of 'Golden Delicious' has recently become available (Velasco *et al.* 2010), and greatly facilitates targeted marker development for poorly covered regions in linkage maps (Fernandez-Fernandez *et al.* 2012).

The 'Idared' x Mr5 map has already allowed the identification of a major fire blight resistance QTL on LG3 of Mr5 (Sections 3.2 and 3.3) Thereafter efforts were

made towards the saturation of LG3 with all SSRs available in the Hidras database. As a result a high resolution genetic background for further fire blight resistance studies in Mr5 is presented here. Moreover the presented map has enabled localization of the apple powdery mildew resistance gene *PL1* and an associated candidate resistance gene on LG12 (Section 3.4). Finally the presented map provided the framework for mapping the *PI-Mal* gene on LG3, which encodes a putative protease inhibitor protein playing a central role in fire blight infection response of 'Idared' (Section 3.5). It can be assumed that the presented linkage map will be valuable for further exploration of genetic loci controlling favorable traits of the important and tasty cultivar 'Idared' and the resistant and 'robust' rootstock *Malus x robusta* 5.

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3.2 Strong evidence for a fire blight resistance gene of *Malus robusta* located on linkage group 3

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Tania Garcia Libreros isolated the apple DNA and together with Andreas Peil tested SSR markers, screened polymorphic SSRs in the mapping population, assigned mapping codes, calculated the linkage map and performed QTL analysis.

Abstract

Fire blight (FB), caused by the Gram-negative bacterium *Erwinia amylovora* is a dangerous disease on pome fruit, including apple. The FB-susceptible cultivar 'Idared' was crossed with the resistant wild species clone *Malus* × *robusta* 5. A segregating population of 146 progeny has been tested by artificial shoot inoculation for susceptibility to FB. Progeny were infected from 0% to 100% of the shoot length. To identify chromosomal regions or loci responsible for resistance to FB of *Malus* × *robusta* 5, a set of microsatellite markers (simple sequence repeat, SSRs) was chosen covering all linkage groups of apple. Up to eight different microsatellites were bulked to one multiplex PCR using four different labels and a fifth label for a size standard. Fifty-nine microsatellite markers out of 72 SSRs were polymorphic. Fifty-four of 66 loci detected could be mapped and were useful for the detection of related resistant loci. Alleles of microsatellites Hi03d06, CH03g07 and CH03e03 originating from the resistant donor *M. robusta* were associated with resistance to *Erwinia amylovora*. Up to eighty percent of the phenotypic variation could be explained by the interval spanned by SSRs CH03g07 and CH03e03, indicating the presence of a major resistance gene. All three microsatellites are located on the distal part of linkage group 3, spanning 15 cM. The SSR marker CH03e03 can be regarded as diagnostic marker for FB resistance. Only seven progeny expressing allele b (184 bp) of CH03e03 showed blighted shoot lengths of more than 30% and only nine progeny lacking allele b showed blighted shoot lengths of <30%. By setting a threshold of 30% shoot necrosis for resistance to FB, the 146 individuals segregate into 71 susceptible and 75 resistant plants, and resistance to FB maps 9 cM away from marker CH03e03.

Key words: *Malus* × *domestica* — *Malus* × *robusta* 5 — fire blight resistance — genome scan — multiplex polymerase chain reaction — mapping

Introduction

Fire blight (FB), caused by the Gram-negative bacterium *Erwinia amylovora* (Burrill) Winslow *et al*., is a dangerous disease on pome fruit, including apple, pear, quince and ornamentals like *Cotoneaster, Crategus* and *Mespilus*. First reports on this bacterial disease in America date back 200 years. Fire blight spread from North America to the British Isles (in 1951) and then to continental Europe (in 1961). In Germany, the disease was detected for the first time in 1971 and, following an epidemic-like dissemination of the bacterium in 1985, heavy losses in apple orchards were noted in South and Southwest Germany in 1993 and 1994 (Fischer and Richter 1999, Richter 1999). The disease moved eastwards and a severe outbreak of FB was noted in the pome fruit orchards of the Institute of Fruit Breeding in Dresden-Pillnitz at 2003 (Peil *et al*. 2004).

Fire blight can infect flowers, fruits, vegetative shoots, woody tissues and rootstock crowns, causing blossom blight, shoot blight and rootstock blight. Due to the limited number of management tools available to control the disease and the diversity of tissues susceptible to infection it is difficult to stop or slow the progress of FB epidemics (Norelli *et al*. 2003). *Erwinia amylovora* is able to migrate rapidly inside the plant tissues from the top of the tree to the roots, leaving behind tissues that will rapidly necrose (Vanneste and Eden-Green 2000). Besides necrosis, water soaking, wilting, shepherd's crooks and ooze are symptoms of FB. Bacteria can be spread from the bacterial ooze by ants, flies, rain or hail (Thomson 2000). The most specific control of the disease can be achieved with antibiotics, but these are being increasingly banned for ecological considerations. An appropriate alternative to current control strategies based on ionic copper or biological antagonists would be stably resistant cultivars. Among the genetic resources in the genus *Malus*, a broad range of resistance levels can be found (Aldwinkle *et al*. 1976). Some wild species have been used as sources for FB resistance but the genetics of FB resistance are not well known. Investigating the inheritance of resistance, Gardener *et al*. (1980) proposed that

in the accessions *Malus* × *robusta* 5 and *Malus* × *sublobata* cv. 'Novole', resistance may be determined by dominant genes, whereas Korban *et al*. (1988), analysing 16 progeny of controlled crosses of scab-resistant and scab-susceptible cultivars, found evidence that FB resistance is quantitatively controlled. Recently, a major QTL on linkage group 7 of the cultivar 'Fiesta' and several minor QTLs on linkage groups 3, 12 and 13 have been reported for the two interrelated crossprogeny, 'Fiesta' × 'Discovery' and 'Prima' × 'Fiesta' (Calenge *et al*. 2005).

A cross-progeny of the cv. 'Idared' and the FB-resistant accession *Malus* × *robusta* 5 was developed to detect regions within the genome responsible for FB resistance of the wild species accession. *Malus* × *robusta* 5 has displayed durable resistance for 14 years against the three most aggressive isolates of *E. amylovora* collected in Germany each year for inoculation (Richter *et al*. 2005), but nevertheless some *E. amylovora* strains can overcome the resistance of *Malus* × *robusta* (Paulin and Lespinasse 1990).

A rapid genome scanning approach using simple sequence repeat (SSR or microsatellite) markers was chosen, utilizing the abundant information available from genetic mapping studies in apple (Maliepaard *et al*. 1998, Hemmat *et al*. 1994, 2003, Liebhard *et al*. 2002, Silfverberg-Dilworth *et al*. 2006). Based on positive experience with marker-multiplexing techniques in genetic mapping or cultivar differentiation [see Ponce *et al*. (1999) in Arabidopsis, Beekman *et al*. (2001) in human, Tang *et al*. (2003) in sunflower and Patocchi *et al*. (2005) in apple], up to eight markers within a single PCR have been successfully multiplexed. Mapping of the SSRs and subsequent QTL mapping revealed a major QTL for FB resistance in *Malus* × *robusta* 5 explaining up to 80% of the phenotypic variation.

Materials and Methods

Plant materials:

The FB-susceptible cultivar ‘Idared’ of apple *Malus* × *robusta* 5 was crossed in 2003 with the FB-resistant wild species *Malus* × *robusta* 5, Acc. APF 0409. The resulting progeny No. 04208 was grown up in a greenhouse in 2004 and used for mapping.

Artificial shoot inoculation:

In an assay similar to that of Kleinhempel *et al*. (1984), shoots of 146 progeny, from four to 12 graftings on rootstock M9 for each individual, were inoculated in 2005 byi incising the tips of the two upper leaves with scissors that had been dipped in a suspension of *E. amylovora*, strain 222 (109 cfu/ml). Only shoots with a minimum length of 25 cm were inoculated. The graftings needed around 4 weeks for growing in spring without additional light. *Erwinia amylovora* strain 222 was chosen for its strong virulence to the susceptible parent ‘Idared’. The inoculated plants were kept in the greenhouse at 25–27 °C (day), 20 °C (night) and 85% air humidity. Length of necrosis of each grafting was measured with a ruler 28 days postinoculation (dpi). The length of necrotic shoot tissue relative to total shoot length averaged over all replicates was recorded.

Multiplex PCR:

DNA of parents and progeny was isolated according to Doyle and Doyle (1987) and diluted to a concentration of 10 ng/µl. SSRs used for mapping were chosen from published genetic linkage maps for apple (Liebhard *et al*. 2002, Silfverberg-Dilworth *et al*. 2006) to cover all 17 linkage groups of *Malus*. Multiplex PCR was performed using the QIAGEN Multiplex PCR Kit (QIAGEN, Hilden, Germany) using the Q-Solution protocol with slight modifications. The reaction was done in a 10 µl volume instead of 50 µl and only 10 ng DNA was taken. Primer mixes contained six to eight different microsatellites. They were composed of three and

four different labels and with two microsatellites with different fragment sizes for each label, respectively.

Microsatellites for multiplexes with six primer pairs were labelled with NED, FAM and HEX and for multiplexes with eight different microsatellites with FAM, NED, PET and VIC. Primers labelled with FAM or HEX were obtained from VBC-Genomics (Vienna, Austria) and primers labelled with NED, PET or VIC from Applied Biosystems (Vienna, Austria). As size standards, Gene Scan^{TM} – 500 LIZ® Size Standard for multiplexes with eight primer pairs and Gene Scan^{TM} – 500 ROX® Size Standard (Applied Biosystems, Vienna, Austria) for multiplexes with six primer pairs were used. Polymerase chain reactions were diluted 1 : 100 and 1 µl of diluted PCR was mixed with 10 µl of HiDi^{TM} Formamide (Applied Biosystems, Vienna, Austria) and 0.3 µl size standard. After denaturation for 3 min at 95 °C and quick chilling on ice, samples were analysed on an Abimed 3100 Genetic Analyser (Applied Biosystems, Vienna, Austria) using the following conditions, i.e. 3 s injection time and 490 s retention time. Data were analysed using programs GENESCAN and GENOTYPER (Applied Biosystems, Vienna, Austria).

Statistical analyses:

Mapping was performed using JoinMap 3.0 (Van Ooijen and Voorrips 2001) at an LOD threshold of 5.0 for grouping. Linkage groups were assigned by comparison of results obtained with location of SSRs in the genetic linkage map of apple published by Silfverberg-Dilworth *et al*. (2006). Interval mapping was performed using MAP-QTL 4.0 (Van Ooijen and Maliepaard 1996). The mean values of percentage of necrotic shoot for each genotype were used as numerical traits. Marker phenotype association was performed with MAP-QTL 4.0 (Van Ooijen and Maliepaard 1996) using the Kruskal – Wallis analysis. The Kruskal – Wallis test can be regarded as the nonparametric equivalent to the one-way analysis of variance, ranking all individuals according to the quantitative trait, while it

classifies them according to their marker genotype (Van Ooijen and Maliepaard 1996).

Results

Resistance screening

Figure 1 shows the frequency distribution of FB severity for 146 individuals of the 'Idared' × *Malus* × *robusta* 5 population, ordered from resistant to susceptible. No disease was observed on 31 individuals, whereas 17 genotypes developed an average of more than 80% of shoot tissue affected. About one-half of the population assayed (75 progeny) had <30% shoot tissue blighted. On average 35% of each shoot was blighted.

Multiplex PCR

Seventy-eight SSRs distributed over the 17 linkage groups of apple were tested for polymorphisms. Among the 61 polymorphic SSRs, two did not produce fragments in multiplex PCR. The 59 polymorphic SSRs amplified 181 different alleles representing 66 loci, i.e. 2.7 alleles per locus. Among both parents 18 alleles were shared, the cultivar 'Idared' contributed 99 alleles and *Malus* × *robusta* 100 alleles.

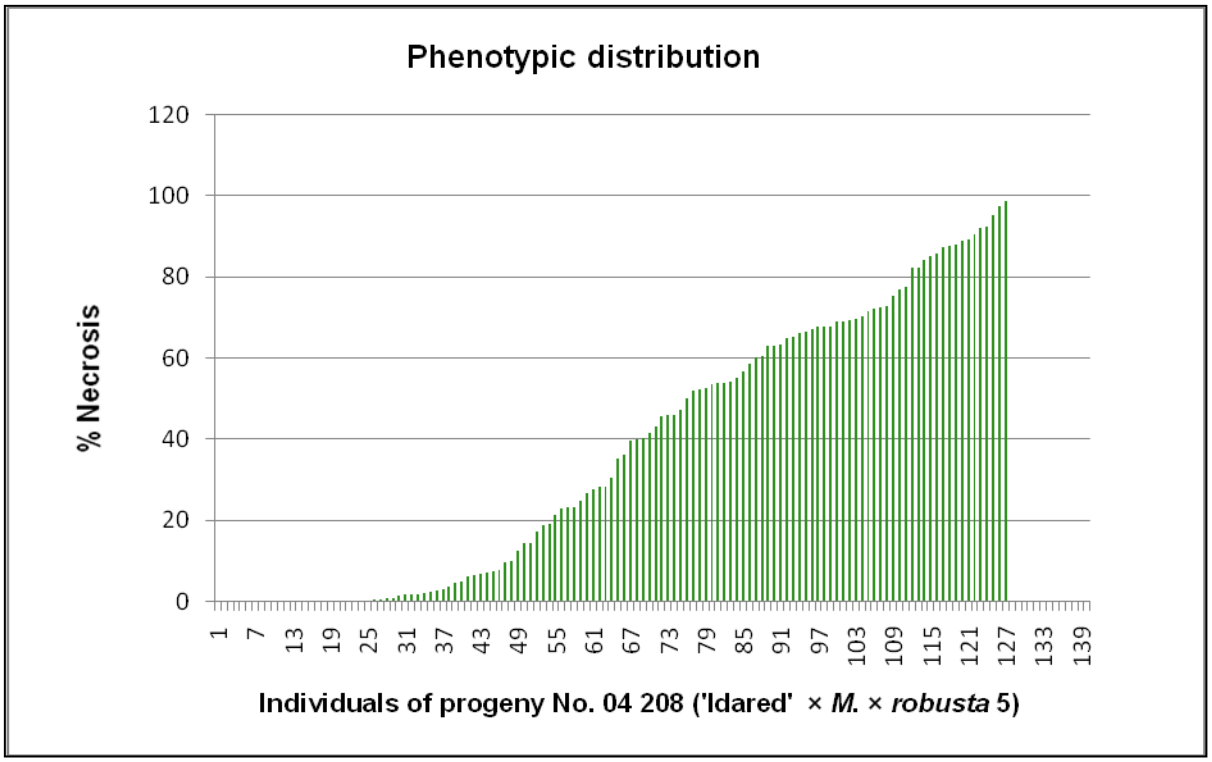

Fig. 1. Distribution of progeny of population 04208 for their resistance to fire blight. Fire blight resistance was recorded as percentage of shoot length blighted to total shoot length (necrosis). The seedlings are ordered by percentage of necrosis.

Mapping and fire blight marker association

In total, 54 loci that were amplified by 51 microsatellite markers could be mapped to 14 tentative consensus chromosomes of apple. Twelve loci could not be linked to any other loci. The order of most markers on the 14 linkage groups was according to their order on apple chromosomes as described by Silfverberg-Dilworth *et al*. (2006). A Kruskal–Wallis analysis revealed highly significant association of the resistance level with three SSRs located on linkage group 3 (Table 1). No other markers, including the SSRs which could not be mapped, were found to be significantly associated with the resistance level as determined by the assay of disease severity.

The Kruskal–Wallis analysis compared the mean values of necrosis of all progeny with the same allele combination with the mean value of necrosis of the other allele combinations of a SSR marker and determined if the mean values were significantly different. Progeny containing allele *c* of Hi03d06 were infected with FB at a mean level of 17%, progeny without allele *c* showed a mean necrosis level of 51.5%. If allele *c* of SSR CH03g07 was present in the progeny, the mean length of shoot blighted was 15.6 and increased to 54.5% if allele *c* was not present.

All marker alleles associated with FB resistance, designated as *c* Hi03d06, *c* CH03g07 and *b* CH03e03, were inherited by *Malus* × *robusta* 5 (Table 1). The average disease severity of all individuals carrying allele *b* CH03e03 (184 bp size) was 11% and 28 of 31 individuals that remained unaffected carry this allele. In contrast, progeny without allele *b* CH03e03, showed on average 60% shoot blighted. Only seven individuals possessing this allele developed more than 30% diseased shoot tissue and only eight plants without this allele were <30% blighted. One putative locus conferring resistance to FB appears to be located near one end of this chromosome, because the association with the distal marker CH03e03 was most significant (Table 1). Mapping of FB for all individuals with <30% shoot blighted as being resistant and more than 30% as susceptible resulted in a locus for FB resistance 9 cM below CH03e03 on linkage group 3 (Fig. 2). The recombination frequencies and LOD scores for all markers of linkage group 3 are presented in Table 2.

Figure 3 shows the result of interval mapping for linkage group 3. Up to 80% of the phenotypic variation can be explained by the interval of markers CH03g07 and CH03e03.

Discussion

Resistance screening

On average, 35% of each shoot from population 04208 studied here was blighted. This value is comparable with the unpublished results from artificial inoculation of apple seedlings of 'Idared' × *M. prunifolia* (31%) and 'Idared' × *Malus* × *floribunda* (38%), whereas progeny of 'Idared' × *M. atrosanguinea* exhibited a higher average severity of 45.2% and progeny of 'Idared' × *M. baccata* showed much less severity of 16.1%. Data obtained from progeny of 'Idared' and different wild species indicate the existence of different resistance mechanisms.

The assay of FB severity used in this study differs from that of Calenge *et al*. (2005) with respect to inoculum concentration (109 instead of 107 cfu/ml used by Calenge *et al*. 2005) and method of disease recording. These authors measured the shoot length blighted, if necroses reached the stem, after 7 and 14 dpi. In the present study, the final degree of tissue area affected was recorded at a time (28 dpi) when the disease progress has already ceased (Kleinhempel *et al*. 1984).

Table 1. Kruskal–Wallis analysis of linkage group 3 of population No. 04208 for the trait severity to fire blight infection

SSRs on linkage group 3	Map distance (cM)	K1 (df)	Genotype of SSRs			Number of Progeny	Mean severity to fire blight
			'Idared'	*M.* × *robusta* 5	Progeny		
					ac	30	0.35
					ac	37	0.39
CH03g12y	0.0	0.629 (3)	ab	ac	ba	39	0.36
					bc	35	0.31
					aa	35	0.46
					ac	35	0.24
AU223657	21.2	9.199 (3) **	ab	ac	ba	41	0.41
					bc	34	0.28
					ac	33	0.18
					ad	38	0.50
Hi03d06	42.6	38.397 (3) ******	ab	cd	bc	38	0.16
					bd	37	0.53
					aa	36	0.52
					ac	34	0.14
CH03g07	48.2	49.409 (3)******	ab	ac	ba	37	0.57
					bc	38	0.17
					aa	72	0.60
CH03e03	58.9	74.448 (1) ******	a-	ab	ab	74	0.11

[1]K: value of Kruskal–Wallis analysis (significance levels: **0.1, *******0.0001); df: degree of freedom; genotype of SSRs: allele combination of the respective SSR of the parents and the progeny; number of progeny: number of progeny with the same allele combination for the respective SSR; mean of severity to fire blight: mean of severity to fire blight of all progeny with the same allele combination of the respective SSR; SSR,simple sequence repeat.

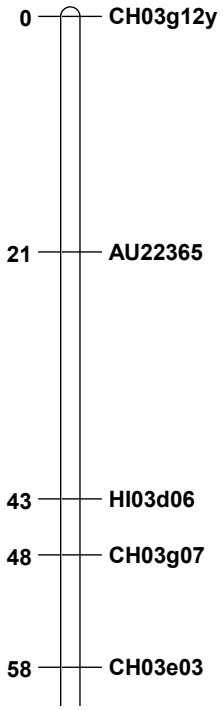

Fig. 2: Genetic map of linkage group 3 of the population 'Idared' × *M. robusta* 5 including a locus for fire blight resistance. Genotypes with a severity of more than 30% shoot blighted were determined as susceptible and with <30% as resistant. The genetic distance is given in cM

Table 2. Recombination frequencies and LOD scores of all markers located on linkage group 3

Marker	CH03g12y	AU223657	Hi03d06	CH03g07	CH03e03
AU223657	0.1993[1] 21.80[2]				
Hi03d06	0.3624[1] 3.28[2]	0.2026[1] 24.68[2]			
CH03g07	0.3986[1] 1.71[2]	0.2452[1] 17.43	0.05561 59.96		
CH03e03	0.4631[1] 0.29[2]	0.294[1] 4.93[2]	0.1623[1] 15.57[2]	0.1046[1] 22.56[2]	
FB	0.4894[1] 0.00[2]	0.3379[1] 2.14[2]	0.2192[1] 9.54[2]	0.1655[1] 14.15[2]	0.1027[1] 22.96[2]

[1]Recombination frequency (Kosambi).

[2]LOD score.

FB, fire blight resistance.

Calenge *et al*. (2005) investigated the rate of disease progress for two populations of apple crosses. On average, an increase of disease progress from 2.6 at 7 dpi to 5.3 cm at 14 dpi was found in 'Prima' × 'Fiesta', and from 3.8 to 6.6 cm in 'Fiesta' × 'Discovery'. The different procedures do not allow the comparison of the average severity of blighted shoots for the populations examined. Nevertheless, the mapping of loci responsible for resistance to FB is independent of the method used for measurements of FB severity.

Bogs *et al*. (2004) tried to associate colonization of apple leaves with GFP-labelled *E. amylovora* with visual disease ratings on plants, based on the progression of the necrotic zone of inoculated shoots in order to describe the virulence of the pathogen. For six of seven *E. amylovora* strains, they observed a relationship between migration in the leaf and virulence and noted differences in

virulence. In this study, only one strain of *Erwinia*, highly virulent to one parent, was used to determine the susceptibility of the progeny, but experience of 14 years of inoculation of *Malus* × *robusta* 5 with mixtures of *E. amylovora* indicate that inoculation with other strains would result in similar segregation patterns, because *Malus* × *robusta* 5 was resistant to almost all strains.

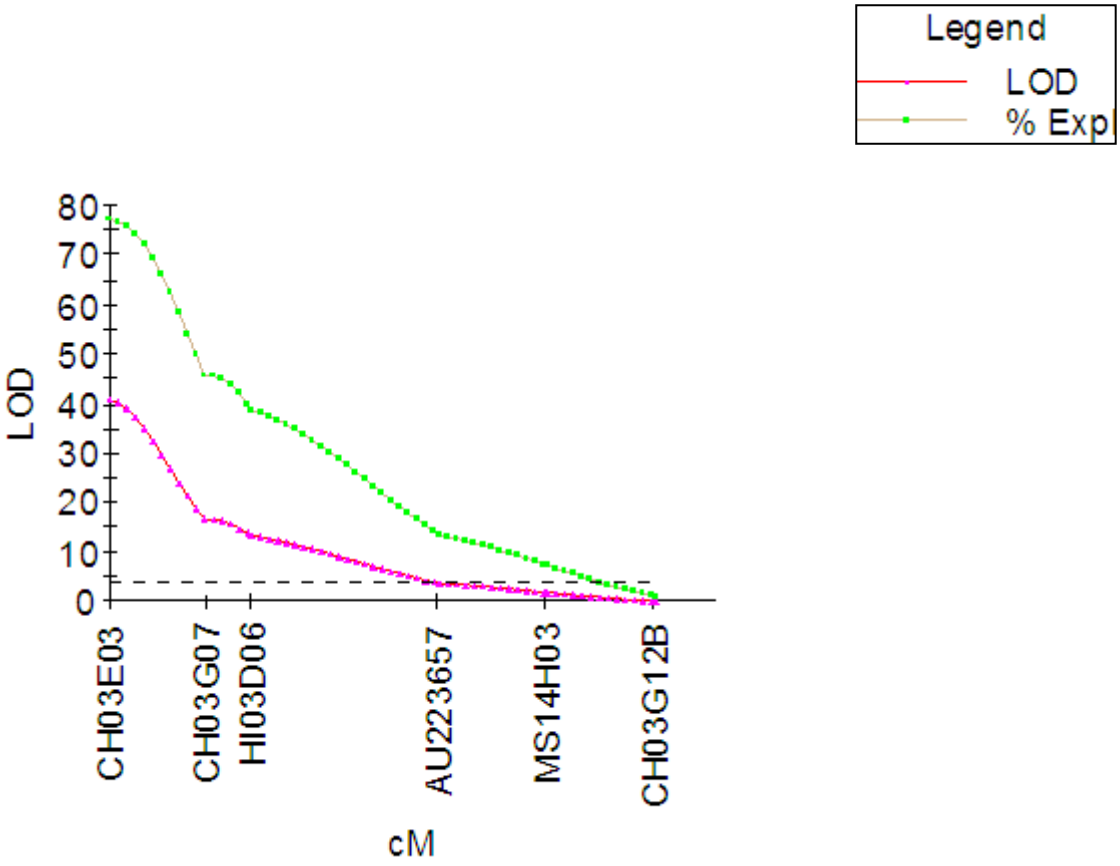

Fig. 3. LOD score values (LOD) and percentage explaining (%exp) the phenotypic variation of the trait necrosis along linkage group 3 of population No. 04208, 'Idared' × *Malus* × *robusta* 5, on which a QTL for resistance to fire blight has been identified (LOD score: >25) according to the order of simple sequence repeats from linkage group 3

Multiplex PCR

Microsatellites distributed over the 17 linkage groups of apple were applied in multiplex PCRs for genome scanning to detect loci correlated with FB resistance rapidly. More than 80% of SSRs were polymorphic. This rate of informative markers exceeds that found by Patocchi *et al*. (2005) who used 51 SSRs for a genome scanning approach to detect markers for scab resistance in a cross of two apple cultivars. Only 27 (i.e. 49%) of them were informative, the rest were being uninformative or even not producing a PCR product. Two SSRs failed in amplification and 22 SSRs were of no use. Presumably, the great genetic distance of the parents of population No. 04208, 'Idared' and *Malus* × *robusta* 5 could be the reason for the higher level of informative markers in population No. 04208.

The use of multiplex PCR is very economical, saving a lot of time and money. Up to eight SSRs in one PCR were multiplexed for this analysis. By using four different florescence dyes, two SSRs with different sizes for each label had to be determined. Due to the dye and the specific SSR the peak levels were different. Preferential amplification of SSRs was not investigated. As the number of alleles is limited in a progeny it was no problem to assign the fragments produced by the progeny. The number of SSRs combined to multiplexes depends upon the purpose of the experiment. For high through put genetic mapping in *Arabidopsis thaliana*, Ponce *et al*. (1999) showed the possibility of bulking 21 different SSRs, using three dyes. This high-throughput approach has been used by Quesada *et al*. (2000) and Robles and Micol (2001) to map mutant loci or to determine QTLs (Robles *et al*. 2001) in *Arabidopsis.* Tang *et al*. (2003) established a set of 13 multiplexes, each consisting of six SSRs called a standard genotyping set for genome-wide scans in *Helianthus annus*. Multiplexes of up to 12 SSRs were applied in cultivated rice to evaluate genetic diversity of ecotypes and isozyme groups (Blair *et al*. 2002). A total of 563 alleles were detected by a multiplex containing 10 SSRs applied to 72 rice accessions, the SSRs were labelled with three different dyes (Blair *et al*. 2002). An estimation of genetic diversity is

possible, of course, but the assignment of a specific fragment to a specific SSR seems to be impossible.

One of the PCR markers (CH-Vf1) chosen for rapid detection of chromosomal regions responsible for FB resistance of *Malus* × *robusta* was isolated from a BAC contig carrying the *Vf* resistance gene of *M. floribunda* 821 (Vinatzer *et al*. 2004). The allele of 159 bp in size, is reported to be in coupling with *Vf.* The alleles amplified by CH-Vf1 in *Malus* × *robusta* 5 were 134 and 156 bp long and 138 and 172 bp in size for 'Idared', indicating that *Vf* is not present in either of them. Vinatzer *et al*. (2004) determined the allele sizes for *Malus* × *robusta* 5 as 137 and 157 bp, which is slightly different from the sizes determined in this study. But nevertheless, Ch-Vf1 in population 04208 is linked to other SSRs of linkage group 1 as published for *Vf* (Maliepaard *et al*. 1998).

Knight and Alston (1968) reported that *Malus* × *robusta* carries the dominant powdery mildew resistance gene *Pl1* and Markussen *et al*. (1995) developed a SCAR AT20 for *Pl1*. This SCAR was tested on population No. 04208 additionally, to determine the corresponding linkage group. AT20 SCAR mapped on linkage group 12, showing the same segregation pattern as SSR Hi07F01 (data not presented). The location of *Pl1* on linkage group 12 was approved by Dunemann *et al*. (2007).

Mapping and fire blight marker association

A Kruskal–Wallis analysis revealed the significance of markers of linkage group 3 being associated with necrosis length, i.e. resistance to *E. amylovora*. No other markers were found to be significantly correlated with FB resistance. This result was confirmed by interval mapping. The interval of SSRs showing the highest Kruskal–Wallis values, CH03e03 and CH03g07, could explain up to 80% of the phenotypic variation. This is different from results published recently by Calenge *et al*. (2005) and Khan *et al*. (2006) concerning QTL mapping of FB in apple. Whereas Calenge *et al*. (2005) used two populations for mapping (i.e. 'Fiesta' ×

‘Discovery’ and ‘Prima’ × ‘Fiesta’), Khan *et al*. (2006) used one progeny (i.e. ‘Fiesta’ × ‘Discovery’). Calenge *et al*. (2005) described a major QTL explaining 34.3–46.6% of phenotypic variation on linkage group 7 of ‘Fiesta’ found in both progeny at the same position and Khan *et al*. (2006) confirmed this QTL on linkage group 7 on ‘Fiesta’. Whereas Calenge *et al*. (2005) could identify additional QTLs on linkage group 3 (‘Fiesta’), 12 (‘Discovery’) and 13 (‘Discovery’) for ‘Fiesta’ × ‘Discovery’ and on linkage group 3 (‘Prima’) for ‘Prima’ × ‘Fiesta’, Khan *et al*. (2006) could not confirm the additional QTLs for ‘Discovery’ or ‘Fiesta’. QTLs identified by Calenge *et al*. (2005) on linkage group 3 of ‘Fiesta’ and ‘Prima’ were not at the same position of the chromosome and had likelihood peaks which were around 40 cM apart. Two of the markers located on linkage group 3 ‘Fiesta’ of population ‘Fiesta’ × ‘Discovery’ were also used for this analysis, i.e. CH03g12y and CH03g07. The minor QTL and both SSRs on linkage group 3 of ‘Fiesta’ are in the following order: CH03g12y, CH03g07, minor QTL, and the distance of the SSRs was determined to be 47 cM (Calenge *et al*. 2005). This is in accordance with the data presented in this study. The distance found between both markers is about 48 cM and the order of the SSRs and the major QTL is the same. But nevertheless, whereas Calenge *et al*. (2005) detected the major QTL for resistance to FB on linkage group 7 of ‘Fiesta’ and only a minor QTL on linkage group 3, the major QTL of *Malus* × *robusta* 5 is located on linkage group 3. Probably different mechanisms are present in apples acting against FB, but maybe the locus on linkage group 3 displays different alleles conferring different levels of resistance.

Because 80% of the phenotypic variation could be explained by the interval on linkage group 3, a major resistance gene was assumed. This accords with a hypothesis of Gardener *et al*. (1980) who proposed a dominant resistance gene conferring resistance to FB in *Malus* × *robusta* 5. Mapping of this dominant resistance gene, determined a locus for FB resistance (FB) 9 cM apart from CH03e03 at the distal end of linkage group 3. This is in accordance with the recombination frequencies of SSRs and resistance to FB on linkage group 3. The

locus for fire blight resistance, FB, has to be located in the order CH03g07, CH03e03, FB with FB as a marker determining the end of that linkage group.

A recombination frequency of only 10.3% was found between the resistance locus and SSR CH03e03 and, therefore, CH03e03 can be regarded as a diagnostic marker for resistance to FB. Taking into account results obtained by Calenge *et al*. (2005) and Khan *et al*. (2006) this marker at least will be useful for FB resistance inherited by *Malus* × *robusta* 5. This is the first report presenting a molecular marker which could be used for marker-assisted selection for FB resistance.

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3.3 Confirmation of the Fire Blight QTL of *Malus* × *robusta* 5 on Linkage Group 3

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Author contributions

Tania Garcia Libreros isolated DNA and together with Andreas Peil tested SSR markers, screened polymorphic SSRs in the 'Idared' × R5 population, assigned mapping codes, calculated the linkage map and performed QTL analysis.

Abstract

Recently, a major QTL for fire blight resistance was identified in the wild species accession *Malus* × *robusta* 5 (R5) based on a single phenotypic evaluation of a progeny of 154 seedlings derived from a cross with 'Idared'. In this paper, we confirm the presence of this QTL in a repeated inoculation of the 'Idared' family in Germany as well as in a M.9 × R5 family in New Zealand. Linkage maps were constructed for each family and susceptibility to *Erwinia amylovora* was determined by artificial shoot inoculation of scions grafted onto rootstocks in the greenhouse in 2005 and 2006 in Germany and 2006 in New Zealand. In 2005, interval mapping of susceptibility resulted in the identification of a QTL on linkage group 3 (LG3) of apple between microsatellites Ch03E03 and Ch03G07 explaining up to 79% of phenotypic variation. The QTL was confirmed in the replicate inoculation in the 'Idared' × R5 family explaining up to 67% of the phenotypic variation with an average of 75% for both years and 83% in a New Zealand M.9 × R5 family. The collective results of the German and New Zealand populations confirm the presence of a significant QTL for fire blight resistance on LG3 of R5 under different phenotyping conditions. The interpretation of this QTL as a major fire blight resistance locus when regarding resistance as a dominant trait will be discussed.

Keywords: *Erwinia amylovora*, fire blight resistance, mapping

Introduction

Fire blight, caused by the gram negative bacterium *Erwinia amylovora*, can be a devastating disease causing dramatic losses in pome fruit growing, as was the case in 2007 in some regions of Europe. The pathogen can cause blossom blight, shoot blight and rootstock blight. *E. amylovora* is able to move rapidly inside the plant tissue from the top of the tree to the roots. Typical symptoms are the shepherd's crook, bacterial ooze, necrosis and wilting. Bacterial ooze is important for the distribution of bacteria as it can be spread by ants, flies, rain or hail and lead to new infections.

Different strategies can be used to control the disease. The most readily used one is the application of antibiotics, but these are increasingly banned due to ecological and pathogen resistance considerations. A promising strategy for fire blight control is the breeding of resistant cultivars. For the efficient breeding of such cultivars, an understanding of the genetics of the plant's fire blight resistance is essential. Recently, several fire blight QTLs have been identified in large-fruited apple accessions, with a major QTL located on linkage group 7 (LG7) of 'Fiesta' (Calenge *et al*., 2005; Khan *et al*., 2006). Another major QTL from 'Robusta 5' (R5) was preliminarily mapped to LG3 based only on one year of data (Peil *et al*., 2007). Since the expression of quantitative trait loci are usually sensitive to both phenotyping and environmental conditions, we repeated the phenotyping of the R5 mapping population (Peil *et al*., 2007) in a second season and phenotyped a M.9 × R5 family in New Zealand.

Matherial and Methods

Plant Material and Phenotypic Evaluation

In Germany, the 'Idared' × R5 family consisting of 154 progeny (Peil *et al*., 2007) was phenotyped for the second time; while in New Zealand, 119 plants from a 'Malling9' (M.9) × R5 family consisting of 146 progeny were phenotyped for the first time. The latter progeny was developed in 1999 and maintained as stoolbeds

at the Hawke's Bay Research Centre of Hort Research. The number of replications varied from one to twelve plants per progeny for the 'Idared' family and from four to five, with many plants having two shoots, for the M.9 family. The 'Idared' family was inoculated in 2005 and 2006 with German *E. amylovora* strain Ea222 and the M.9 family inoculated in 2006 with New Zealand strain 11176 (International Collection of Microorganisms from Plants (ICMP), Manaaki Whenua Landcare Research, Auckland, New Zealand) at a bacteria concentration of 109 cfu/ml. Phenotypic evaluation of susceptibility to fire blight ofprogenies of both populations was performed according to Peil *et al*. (2007).

Genetic Markers and Maps

1. 'Idared'× R5 population. For the mapping and assignment of linkage groups, see Peil *et al*. (2007). QTL mapping was performed using interval mapping together with the MQM analysis of MapQTL® 4 (Van Ooijen and Maliepaard, 1996). Co-factors for MQM analysis were determined with MapQTL® 5 (Van Ooijen, 2004).

2. M.9 × R5 population. Leaf material was harvested from stoolbeds and DNA was isolated according to Gardiner *et al*. (1996). Eighty-six markers (77 SSRs, 5 SCARs, 3 SNPs and 1 RAPD) spanning the M.9 genome with an average distance between them of 15 cM and 85 markers (78 SSRs, 4 SCARs and 3 SNPs) spanning the R5 genome with an average distance between them of 14 cM were used to construct the genetic maps. Assignment of markers to map positions was performed using JoinMap® 3.0 (Van Ooijen and Voorrips, 2001) and a LOD score of 4 was used for grouping. The framework maps were aligned with published maps using common markers, and marker order and distances were checked for discrepancies. QTL analysis was performed independently for M.9 and R5 using data collected from 91 individuals of the M.9 × R5 population. QTL mapping was performed using simple interval mapping (SIM) together with the approximate multiple QTL model (MQM) analysis of MapQTL® 5 (Van Ooijen, 2004).To select co-factors for MQM analysis, markers which displayed a LOD score superior to the LOD score threshold determined by permutation test

(1,000 permutations) were selected. An estimation of the total variance explained by the marker with the highest LOD score was given by MapQTL® 5.

Results and Discussion

The different environments, the different susceptible parents nor the different *E. amylovora* strains used for inoculation appeared to have a major influence on the range and distribution of susceptibility (Fig. 1). An abundant number of seedlings showing no necrosis and seedlings nearly total necrotic were detected in both progenies. On comparing both years of inoculation of the 'Idared' × R5 population, a good concordance of the results was obtained, with many progeny showing no symptoms in either year (Fig. 1a). The heritability of resistance to fire blight in the 'Idared' × R5 population was 0.76 and 0.71 in 2005 and 2006, respectively, and 0.73 for both years. Nevertheless, some progeny showed quite contrasting necrosis over the two years (Table 1), which in some cases could be explained by the low number of replicates. However, only a few seedlings showed differences of more than 30% necrosis between 2005 and 2006 (Table 1). The mean of %necrosis for the population 'Idared' × R5 were 34.7% and 32.2% for 2005 and 2006, respectively, and 32.9% for both years. Khan *et al*. (2006) determined a mean of%necrosis for a 'Fiesta' × 'Discovery' population of 25.7%. The mean of this population is higher than the mean values for each parent: the %necrosis for 'Fiesta' was 19.0% and 'Discovery' was 21.5%. In comparison, 'Idared' (91.0%) and R5 (0.4%) displayed a very high contrast in average %necrosis in 2005 after inoculation with a mixture of three virulent *E. amylovora* strains in 2005. Results from Calenge *et al*. (2005), who inoculated a 'Prima' × 'Fiesta' and a 'Fiesta' × 'Discovery' family are not directly comparable because only the length of the necrosis was measured.

The major QTL at the top of LG3 of R5 reported previously (Peil *et al*., 2007) was confirmed in both families. It maps between the markers Ch03e03 and Ch03g07 (Fig. 2). The QTL explained up to 79% (LOD=37.1) and 67% (LOD=28.6) of the

phenotypic variation in the 'Idared' family in 2005 and 2006, respectively, with an average of 75% (LOD=39.4). It explained up to 83% (LOD=22.5) in the New Zealand M.9 × R5 family. This was the only region of the R5 genome to exhibit a QTL peak above the threshold level. Previously a major QTL for fire blight on LG7 of 'Fiesta' was confirmed in two different populations (Calenge *et al*., 2005) and in a different environment (Khan *et al*., 2006); this major QTL contributes from 34.3 up to 46.6% of the phenotypic variation.

Since the single QTL explains the majority of the phenotypic variation, it can also be regarded as a dominant major effect gene. Assuming that the gene allows some minor disease development in some genetic backgrounds, the fire blight resistance in R5 can be considered to be under monogenic control but that some additional QTLs moderate the susceptibility of the susceptible seedlings. Considering all progeny with less than 30% necrosis as resistant and all other progeny as susceptible resulted in a R:S segregation ratio of 1:1 (Peil *et al*., 2007), thus resistance to fire blight could be mapped as a monogenic trait (Fig. 3). Further research is being performed to identify additional putative QTLs influencing susceptibility at a lower intensity than the LG3 QTL region does.

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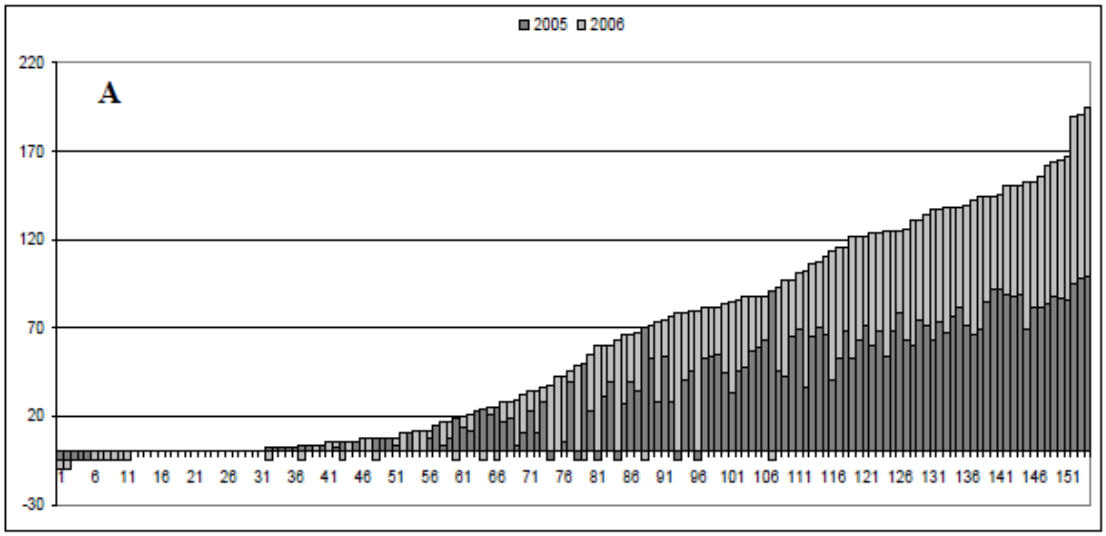
Tables

Table 1. Progeny of 'Idared' × 'Robusta 5' showing more than 30% difference in the % necrosis in the fire blight screenings of 2005 and 2006.

	2005			2006		
		% necrosis			% necrosis	
Clone	rep[1]	Ø	Range	rep[1]	Ø	Range
1	11	40.0	0-91.9	3	5.8	1.3-16.2
10	8	63.1	42.9-76.6	5	24.6	0-69.7
29	5	92.3	85.7-100	4	51.5	21.1-71.7
32	7	54	27.5-78	7	20.6	1.4-53.3
35	6	0.8	0-2.0	1	40.6	40.6
39	9	65.2	38.9-100	10	31.6	3.1-84.6
40	8	92.3	54.1-100	10	52.5	35.1-77.5
100	9	69.0	40.9-95.6	3	32.3	0-61.0
106	8	52.6	4.8-89.2	4	17.5	0-37.9
114	4	77.6	64.1-90.5	7	46.7	27.8-70.2
123	11	4.9	0-12.0	6	38.3	0-100
143	4	69.8	57.1-78.6	3	36.5	21.0-55.8
157	6	40.6	0-88.1	2	71.6	70.3-72.9

[1]rep = number of replicates

Figures



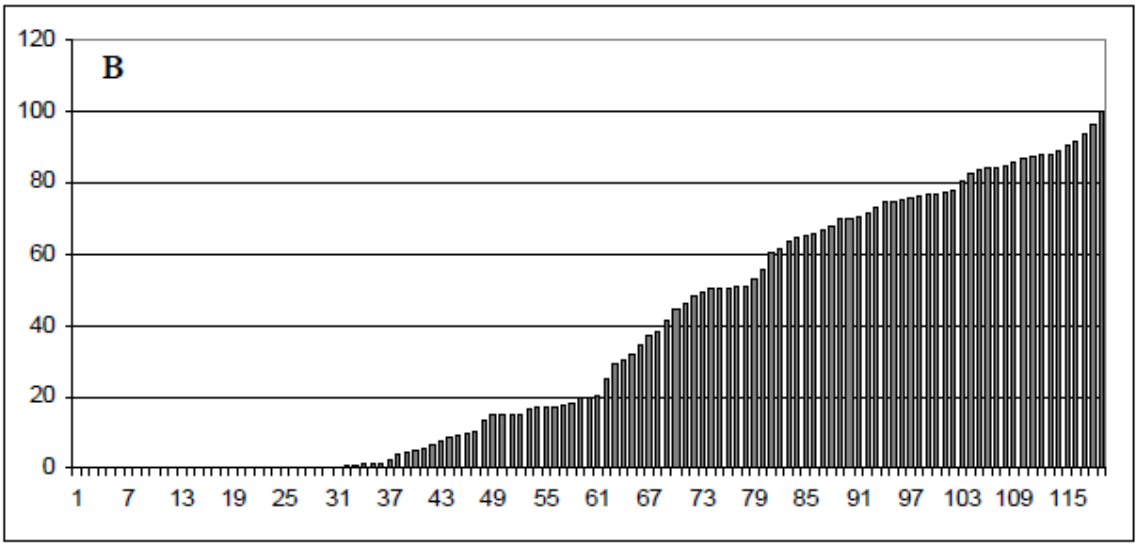


Fig. 1. Distribution of progeny susceptibility to fire blight ordered by %necrosis (y-axis). a) Seedlings from cross of 'Idared' × R5, cumulative necrosis of screenings in both 2005 and 2006; b) Seedlings of M.9 × R5, screening 2006. (In panel 'a', bars <0%= not tested).

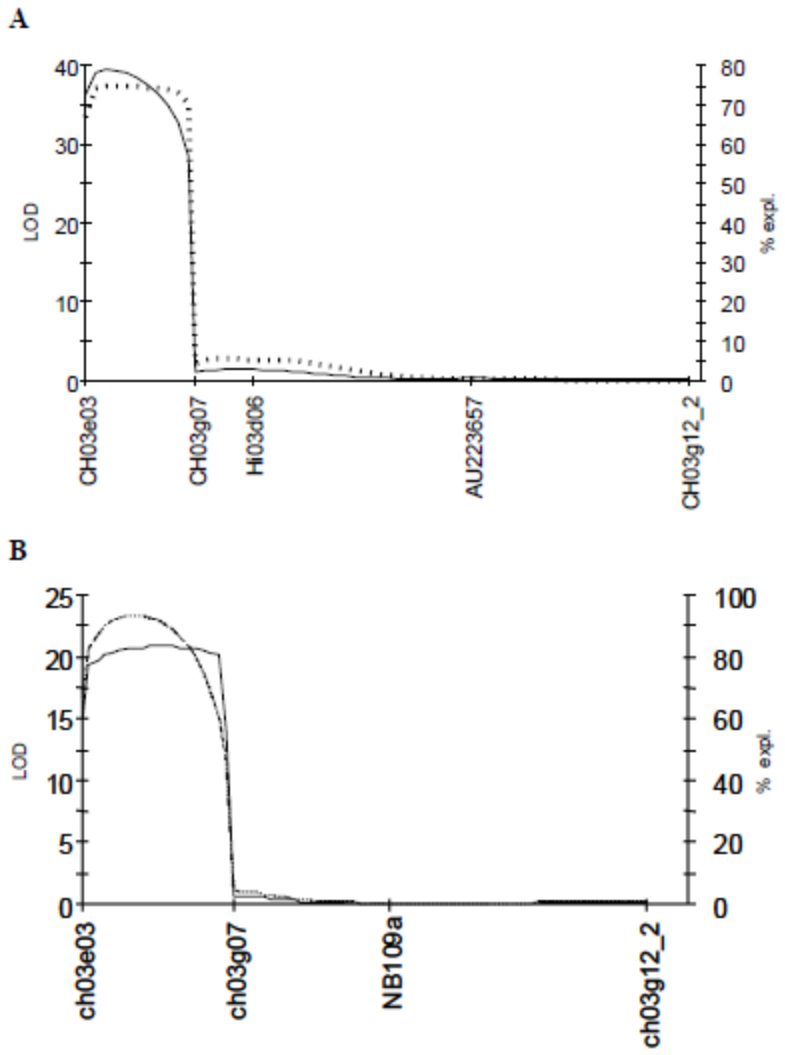


Fig. 2.Interval mapping of LG 3 using MQM analysis. A) ‘Idared’ × R5, average of trait necrosis determined 2005 and 2006; B) M.9 × R5, trait necrosis determined 2006. LOD score: dashed line, % explaining: filled line.

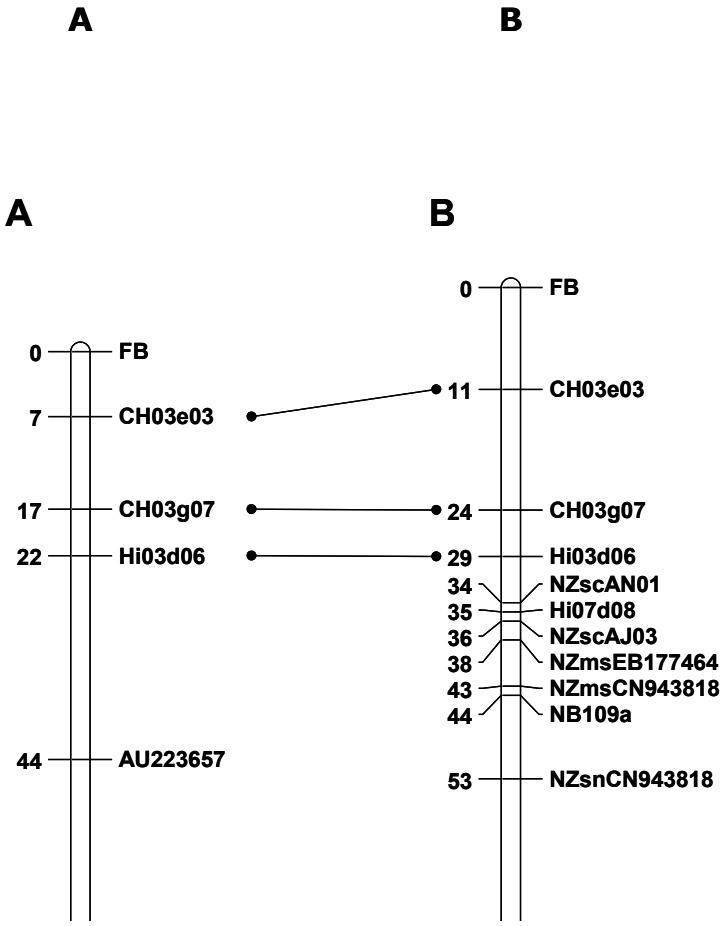


Fig. 3. Mapping of fire blight (FB) resistance to linkage group 3 of 'Robusta 5' when all progenies with less than 30% necrosis where regarded as resistant and all other progenies as susceptible. A) 'Idared' × R5; B) M.9 × R5.

3.4 Mapping of the apple powdery mildew resistance gene *Pl1* and its genetic association with an NBS-LRR candidate resistance gene

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Author contributions

Tania Garcia Libreros carried out the DNA isolation and together with Andreas Peil analyzed the SCAR marker AT20 in the mapping population 04208 and determined the linkage group carrying this marker.

Abstract

Molecular markers for the major apple powdery mildew resistance gene *Pl1* were identified and are presently used in marker-assisted selection in apple breeding. However, the precise map position of the *Pl1* gene in the apple genome was not known. The objectives of this investigation were the identification of the *Malus* linkage group (LG) carrying the *Pl1* locus, mapping of the resistance gene by simple sequence repeat (SSR) markers, and the analysis of genetic associations between the *Pl1* gene and the numerous NBS-LRR resistance gene candidates already mapped in the apple genome. A two-step linkage mapping was used, based on two different apple families. The identification of LG 12 carrying *Pl1* was performed indirectly by mapping the SCAR marker AT20 in an apple progeny for which there was a core genetic map but no mildew data available. Then, the position of *Pl1* on LG 12 was determined by SSR markers in a second population which has been scored for mildew over 6 years in a greenhouse and in the field. The SSR Hi07f01, previously mapped on LG 12 [Tree Genet. Genomes, 2 (2006), 202] cosegregated with AT20 and was closely linked (~1 cM) to the *Pl1* gene. The TIR-NBS-LRR resistance gene analogue 15G11 mapped by the SSCP technique was also closely linked to the *Pl1* resistance locus and might be a candidate for *Pl1* itself, a second powdery mildew major resistance gene (Pld, [Theor. Appl. Genet., 110 (2004), 175]), or two scab resistance genes (Vg, [IOBC/WPRS Bull., 23 (2000), 245]; Vb, [Genome, 49 (2006), 1238]) which all seem to be located in a common R gene cluster at the distal end of apple LG 12.

Key words: *Malus* spec. — *Podosphaera leucotricha* — disease resistance — *Pl1* gene mapping — DNA markers — resistance gene analogue — marker-assisted selection

Introduction

Powdery mildew, caused by the obligate biotrophic ascomycete fungus *Podosphaera leucotricha*, is one of the main fungal diseases in commercial apple production. High amounts of fungicides are applied to reduce the damage caused by the pathogen. The development of apple cultivars displaying durable resistance to *P. leucotricha* is one of the major aims in apple resistance-breeding worldwide. However, at present, the majority of apple cultivars of world-wide economic importance are still susceptible. Several sources of resistance are known. Major genes such as *Pl1* from *Malus robusta* and *Pl2* from *Malus zumi (*Knight and Alston 1968) have been used widely in apple-breeding programmes to include mildew resistance. Other major genes that are used with increasing importance are *Plw* from the ornamental crab apple 'White Angel' (Gallot *et al*. 1985), *Pld* from the D12 clone (Visser and Verhaegh 1979) and *Plmis* from 'Mildew Immune Seedling' (Dayton 1977). In addition, there are several other *Malus* species known to be resistant, as well as a number of apple cultivars displaying variations of mildew tolerance. The existence of different physiological races of *P. leucotricha* has been demonstrated by pathogenicity tests based on fungal isolates (Krieghoff 1995, Lesemann *et al*. 2004, Urbanietz and Dunemann 2005) as well as under natural conditions in the orchard (Caffier and Laurens 2005). Therefore, the putative race specificity of powdery mildew resistance genes has to be considered. Because it has been recognized that durable powdery mildew resistance can probably most efficiently be achieved by pyramiding different monogenic resistances, several molecular marker approaches have been performed in the past. Easy-to-use SCAR or simple sequence repeat (SSR) markers are available for the five resistance sources mentioned above. A first SCAR marker (AT20-450) was developed for *Pl1* (Markussen *et al*. 1995), followed by the N18-SCAR for a major gene involved in *Pl2* resistance (Seglias and Gessler 1997). Several *Plmis* markers were identified (Gardiner *et al*. 2003) as well as SSR and SCAR markers for *Plw* and *Pld* (Evans and James 2003, James *et al*. 2004). With the exception of *Pl1*, the position of

the other powdery mildew resistance genes in the *Malus* genome is known. The availability of several saturated apple linkage maps sharing a high number of common SSRs (Liebhard *et al*. 2003, Silfverberg-Dilworth *et al*. 2006) has facilitated the assignment of the resistance genes to a specific linkage group (LG). *Plw* is located at the top of LG 8 (James and Evans 2004), whereas *Pld* was mapped at the bottom end of LG 12 (James *et al*. 2004). The *Pl2* and *Plmis* major genes were both reported to be linked to the N18-SCAR (Gardiner *et al*. 2003) which is on LG 11 (Seglias and Gessler 1997).

Regarding the *Pl1* resistance from *M. robusta*, several SCAR and Amplified fragment length polymorphism (AFLP) markers have been identified (Markussen *et al*. 1995, Dunemann *et al*. 2004) but there were no attempts in the past to assign the markers to a specific apple LG. Initially, marker assisted selection (MAS) using the *Pl1* markers was reported to be unreliable at different apple-breeding stations. For example, in a report from Switzerland (Kellerhals *et al*. 2000), it was pointed out that the SCAR marker AT20-450 did not correlate with the powdery mildew infection data. Also, in the Hort- Research (New Zealand) breeding programme, there was no correlation between the AT20-SCAR and the mildew field resistance (Dunemann *et al*. 2004). However, within the German apple breeding germplasm, a good correspondence between phenotypical and marker data was found over the years which is still in agreement with the hypothesis that *Pl1* is controlled by a single major gene which is acting together with a second (precursor) gene (Dunemann *et al*. 2004). The two gene-model together with knowledge of the genetic background of the *Malus* material used for *Pl1* resistance-breeding at different research stations around the world does explain the different levels of success in MAS. As the *Pl1* resistance still proved to be useful for resistance pyramiding strategies, it was necessary to identify the map position of this major gene. The more different resistance factors (scab, mildew, fire blight) that can be accumulated in a single apple genotype, the more important is the knowledge about the location of the genes within the genome. As the example of *Pl2/Plmis* (Gardiner *et al*. 2003) demonstrates, the possibility of

using the same markers for molecular selection does exist, provided that specific marker alleles were detected.

Another good reason for attempting to determine the map position of *Pl1* is the availability of a large number of mapped candidate resistance genes for *Malus*. Because plant-resistance genes are known to be clustered in the genome (Hulbert *et al*. 2001, Leister 2004) they can serve as tightly linked molecular markers for another gene of the same cluster, or even as a starting point for a map-based cloning approach. More than 100 resistance gene analogues (RGA) of the NBS-LRR (nucleotide binding site/leucin rich repeat) class of plant resistance genes have been identified by PCR-based approaches and mapped for the most part in the *Malus* genome (Thiermann 2002, Lee *et al*. 2003, Baldi *et al*. 2004, Calenge *et al*. 2005). Although the first fungal resistance genes isolated from apple, conferring resistance to apple scab (*Venturia inaequalis*), do not belong to this major resistance gene class but to the receptor-like protein (RLP) class (Vinatzer *et al*. 2001, Xu and Korban 2002), it is possible that other fungal pathogens are controlled by NBS-LRR genes. The powdery mildew resistance genes *Mla6* from barley (Haltermann *et al*. 2001) and *Pm3* from wheat (Srichumpa *et al*. 2005) are both encoding coiled-coil NBS-LRR proteins.

In this paper, the identification of the *Malus* LG carrying the *Pl1* locus, its mapping by SSR markers, and the tight linkage to a NBS-LRR candidate resistance gene are reported.

Materials and Methods

Plant materials and powdery mildew assessments:

Two apple populations were used for a two-step *Pl1* mapping approach. The first population (04/208) was produced in 2003 by crossing the mildew susceptible cultivar ‘Idared’ with an accession of *Malus robusta* (Robusta 5). Some 300-resulting seedlings have not yet been planted in the field and not yet scored for powdery mildew resistance, but they have been used for analysing fire blight

resistance (Peil *et al*. 2007). This family was used for the first step (identification of the LG) by placing the *Pl1*-SCAR AT20 in a core genetic map consisting of 55 SSR loci (Peil *et al*. 2007). The verification of the assumed *Pl1* position and further mapping using the AT20-SCAR marker, SSR markers and a RGA-derived marker was performed in the second population (99/2) for which six sets of mildew-resistance data were available from previous years. The family 99/2, with about 400 individuals, was produced in 1998 by crossing 'Idared' with the *Pl1*-resistant genotype 78/18-4, which derived from MAL59/1 (*M. robusta o.p*.) over the intermediate breeding clone A142/5. The 99/2 seedlings were planted in a foil greenhouse and scored for mildew infection under high natural infection pressure in the years 1999, 2000 and 2001. Then the trees were planted in the field and screened for natural infection for another three seasons. The mildew symptoms in the greenhouse were scored using a scoring scheme from 0 to 5: 0 (no symptoms), 1 (very slightly infected), 2 (slightly infected), 3 (moderately infected), 4 (severely infected) and 5 (completely infected). In the field, a 3-step scoring scale was used: 1 (no mildew), 2 (slight infection on leaves, occasionally on shoots), 3 (moderate to heavy infection on leaves and shoots).

SCAR marker and SSR analyses:

Apple DNA of population 04/208 was extracted from young leaves using the DNeasy Plant Mini-Kit (Qiagen, Hilden, Germany) whereas for progeny 99/2 a miniprep protocol adapted from Doyle and Doyle (1987) was used for genomic DNA preparation. The AT20-SCAR marker study was performed with the PCR primers AT20-for (5´-atcagccccacatgaatctcatacc) and AT20-rev (5´-acatcagccctcaaagatgagaagt) (Markussen *et al*. 1995). PCR amplification of the specific SCAR fragment was performed using 20 ng of genomic DNA in 25 µl volume containing 50 mM Tris– HCl pH 8.8, 16 mM $(NH_4)2SO_4$, 0.1% Tween 20, 1.5 mM $MgCl_2$, 100 $_{\mu M}$ of each dNTP, 200 nM of each primer and 1.2 U Taq polymerase (Invitek, Berlin, Germany). Amplification conditions for the SCAR marker were as follows: one cycle of 5 min at 94 °C, 35 cycles of 94 °C 1 min, 60

°C 2 min, 72 °C 2 min, followed by a final elongation step of 94 °C, 1 min; 60 °C, 2 min; 72 °C, 10 min.

For LG identification in progeny 04/208, a set of 78 chosen SSRs covering all 17 apple LGs (Liebhard *et al*. 2002, 2003, Silfverberg- Dilworth *et al*. 2006) was screened for genetic association with the AT20-SCAR. The SSR analyses were performed on an ABI 3100 by multiplexing six-to-eight SSRs (Peil *et al*. 2007).

For *Pl1* mapping in population 99/2, the following SSRs located in the lower half of LG 12 were chosen after the SSR scanning approach: CH01g12, CH01f02, CH03c02, CH01d03 and Hi07f01 (Liebhard *et al*. 2003, Silfverberg-Dilworth *et al*. 2006). SSR marker analysis was carried out according to the PCR conditions described by Liebhard *et al*. (2002). SSR allele identification was made on a dual-laser automated DNA sequencer (model 4200L-2; LI-COR, Lincoln, NE, USA).

RGA mapping:

Identification of *Malus* NBS-LRR RGA and their mapping in an apple progeny derived from a cross between 'Discovery' and 'Prima' was described by Thiermann (2002). Briefly, multiple combinations of degenerate primers designed from motifs in the nucleotide binding sites (NBS) of group 1- (TIR, toll interleukin receptor-like region) and group 2- (CC, coiled coil domain) type NBS-LRR genes (Leister *et al*. 1996, Pan *et al*. 2000) were used to amplify specific RGA sequences in the moderate powdery mildew resistant apple cultivar 'Discovery' and in *M. robusta*. About 50 clones with high sequence similarity to NBS-LRR-type resistance genes were used for specific PCR primer design. About 40 RGAs were finally mapped in the 'Discovery'× 'Prima' (D×P) map by using the SSCP technique (Thiermann 2002).

The NBS-LRR candidate gene RGA 15G11 located on LG12 in the D x P map was analysed for polymorphisms suitable for mapping in the progeny 99/2 as

follows: a 480 bp-fragment was amplified by using the 15G11-specific PCR primers (15G11 primer sequence information is available on request) and cleaved with the restriction enzyme *Taq*I (Fermentas, St Leon-Rot, Germany) into three smaller fragments. Four microlitres of the cleaved PCR products were denatured together with 4 ll of 95% formamide-containing buffer and separated for SSCP detection on a 0.5x non-denaturing MDE gel (Mutation Detection Enhancement; Cambrex, Rockland, ME, USA) using a manual sequencing apparatus (Model S2001; Invitrogen, Carlsbad, CA, USA) at constant voltage (1.300 V) at room temperature. After electrophoresis, the gel was silver-stained with the DNA Silver Staining kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's protocol.

Linkage analysis:

Linkage analysis was performed on 154 selected individuals of population 04/208. For progeny 99/2, two separate maps Mapping of the apple powdery mildew resistance gene *Pl1* 477 were constructed using different subsets of this family. For mapping the *Pl1* greenhouse data (locus designation *Pl1-Gh*), 161 plants were chosen, which showed similar mildew resistance reactions over the 3 years of scoring. For mapping based on mildew field-scoring data (locus designation *Pl1-F*) linkage analysis was performed on 125 clearly resistant (score 1) and susceptible (score 3) plants. Maps of LG 12 were constructed using JoinMap 3.0 (Van Ooijen and Voorrips 2001) applying the Kosambi mapping function. The graphical presentation of the maps was generated with the program MapChart 2.1 (Voorrips 2002).

Results

Mildew assessments and confirmation of AT20-SCAR

The results of powdery mildew scoring over 3 years in a foil greenhouse, as well as in the field, are shown in Table 1. When for the greenhouse data the scoring classes 0 and 1 were regarded to contain the resistant individuals and the

classes 2–5 the susceptible plants, in only one of the three seasons (2001) was the expected 1:1-segregation observed. In the other 2 years, an excess of non-infected seedlings was observed. Nevertheless, for mapping the *Pl1* resistance factor, the hypothesis of a single major gene was maintained. For linkage mapping, 161 genotypes of a subpopulation (designated as 99/2-Gh) were considered that were clearly resistant/susceptible in each of 3 years. Mildew scoring in the field was strongly influenced by the climatic conditions. In the year after planting (2003) due to extremely high summer temperatures the mildew infestation was generally low. If the segregations displayed in Table 1 were defined only on the basis of the extreme scoring classes (1 and 3, respectively), in 2005 a 1:1-segregation was observed. A subpopulation named as 99/2-F consisting of 125 individuals (110 of them also present in 99/2-Gh) with consistent mildew scores over the years was used for linkage mapping.

A molecular marker assessment was carried out in 233 individuals of progeny 99/2 using the co-dominant SCAR AT20-450 to verify the linkage of this marker to *Pl1* in this population (Fig. 2). The relationship between the phenotype data and the AT20 marker data was evident (Table 2). If the limit between the resistant and susceptible is put between classes 1 and 2, a segregation of 128 (res):105 (susc) was obtained in the year 1999. Out of the 128 resistant plants, only 10 did not possess the AT20 marker (Table 2). The calculated recombination frequency for loci *Pl1* and AT20 is 7.3% in 1999 and 10.3% in 2000. The relationship between the field data and the AT20 marker was initially more difficult to confirm. However, considering only the highly susceptible plants, as for example the 55 individuals scored with 3 in 2003 (Table 1), only in four plants (7.3%) was the AT20 marker present (not shown).

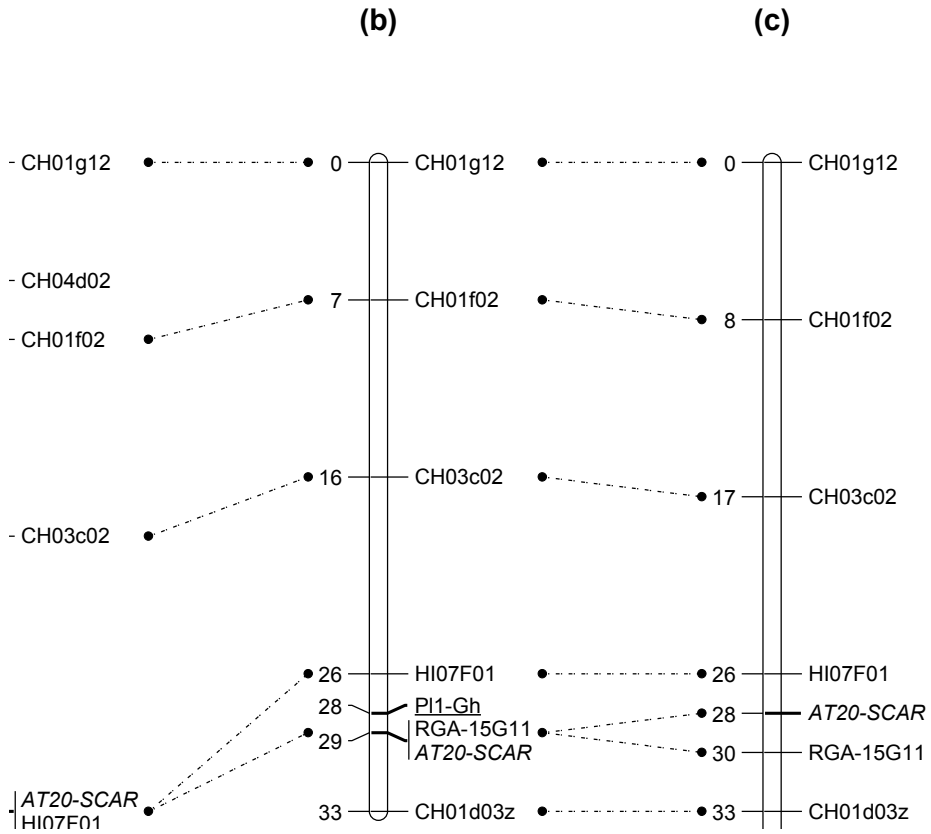

Fig. 1: Genetic maps for the *PI1*-carrying linkage group LG 12 created for (a) 154 individuals of population 04/208 ('Idared'× Robusta 5), (b) 161 individuals of population 99/2-Gh ('Idared'× 78/18-4, mildew scored in a greenhouse, locus *PI1-Gh*) and (c) 125 individuals of population 99/2-F ('Idared'× 78/18-4, mildew scored in the field, locus *PI1-F*). within the 161 plants (Fig. 1b). For the P*I1* field data (locus *PI1-F*, Fig. 1c), the calculated position of the resistance gene moved to the proximal end of the LG.

PI1 linkage mapping and RGA analysis

As a result of SSR marker segregation analysis and mapping of the AT20-SCAR in the population 04/208, a co-segregation of AT20 with the SSR Hi07f01 was found (Fig. 1). The SCAR AT20 was also linked to the SSRs CH03c02, CH01f02, CH04d02 and CH01g12, all located on the lower half of the apple LG 12 (Fig. 1a). The order of SSRs and their genetic distance was in agreement with the

published map information (Liebhard *et al*. 2003, Silfverberg-Dilworth *et al*. 2006). Based on this finding, the SSRs Hi07f01, CH03c02, CH01f02 and CH01g12 together with an additional SSR (CH01d03z) from the bottom end of LG 12 were chosen for *Pl1* mapping in the subpopulations 99/2-Gh and 99/2-F. CH01g12 segregated for four alleles, one of them being a null allele, SSRs CH03c02 and CH01f02 showed a segregation of three alleles, and the two remaining SSRs showed a simple RAPD-like segregation (not shown). The co-dominant AT20-SCAR segregated in progeny 99/2 also with only one allele representing the *Pl1* marker (Fig. 2). As shown in Fig. 1, the AT20 marker in this population also mapped at the expected position. In the case of the *Pl1*-*Gh* locus, a close distance of about 1.8 cM was calculated which represents the number of three recombinants.

Year	Greenhouse/field	Number of plants			
		Total	Resitant (greenhouse: Classes 0 and 1; field: class 1)	Susceptible (greenhouse: Classes 2-5; field: class 3)	X^2 (1:1)
1999	Greenhouse	404	235	169	10.70**
2000	Greenhouse	404	257	147	29.95***
2001	Greenhouse	404	189	215	1.67
2003	Field	241	186	55	71.21***
2004	Field	239	156	83	22.30***
2005	Field	202	109	93	1.27

Table 1: Segregation for powdery mildew resistance/susceptibility in progeny 99/2; classification of greenhouse data based on all individuals, classification of field data based on subpopulations with the scores 1 and 3.

The RGA 15G11 representing a 480-bp sized part of the NBS of a typical plant TIR-NBS-LRR gene amplified by a degenerate primer strategy was mapped together with *Pl1* and AT20 in the same region of LG 12 (Fig. 1). To detect polymorphisms for mapping in progeny 99/2, several restriction enzymes with four-base recognition sites were used to cleave the 480-bp PCR fragment obtained with the specific 15G11 primers into smaller fragments suited for most effective SSCP analysis. Both the enzyme combination *Alu*I/*Rsa*I and the enzyme *Taq*I produced SSCP markers that could be scored in the progeny (Fig. 3). As both SSCP markers were present in the same genotypes, only the *Taq*I-produced SSCP marker was analyzed for mapping. The 15G11 SSCP marker

was present in the resistant parent 78/18-4 and in the susceptible 99/2 individuals indicating linkage in repulsion phase (Fig. 3). No recombination occurred in the subpopulation 99/2-Gh between the RGA 15G11 and AT20 SCAR. The genetic distance between the RGA locus and *Pl1-Gh* was only 1.2 cM (two recombinants). These findings indicate the location of *Pl1* and RGA 15G11 in a common resistance gene cluster on LG 12.

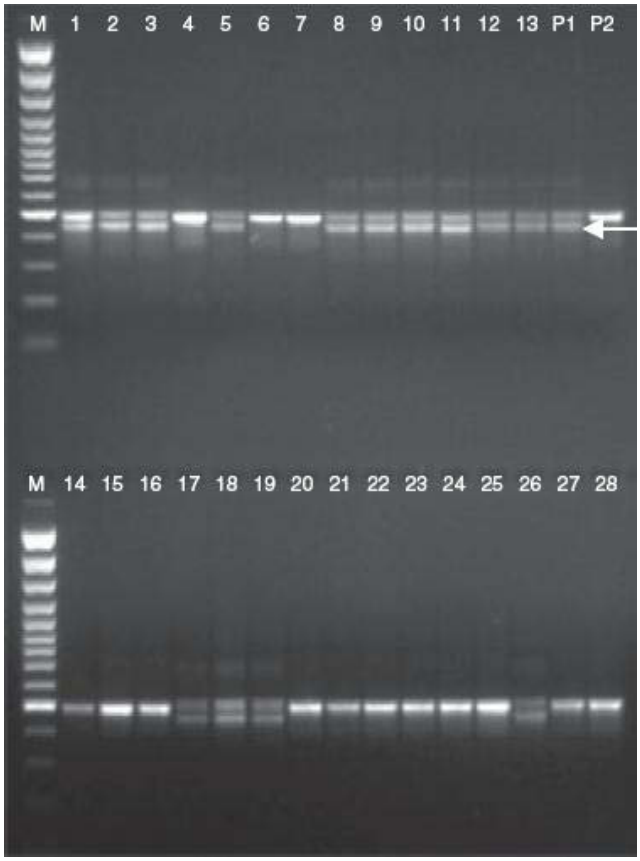

Fig. 2: PCR amplification patterns obtained with the AT20-SCAR primers for 28 individuals (1–28) of population 99/2 ('Idared' × 78/18- 4) and the parental genotypes 78/18-4 (P1) and ('Idared' × (P2); M-Size marker; arrow – 450 bp SCAR fragment.

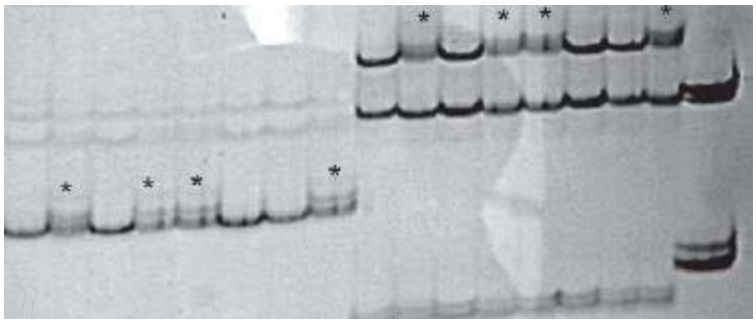

Fig. 3: SSCP patterns of PCR fragment 15G11 digested with *Taq*I (left) and *Alu*I/*Rsa*I (right); P1 – parent ('Idared' (susceptible), P2 – parent 78/18-4 (resistant), 1 to 6 – individuals of progeny 99/2, plants 1, 4, 5 are resistant, plants 2, 3, 6 are susceptible; polymorphic SSCP bands are marked by an asterisk; M – size standard.

Discussion

Two-step linkage mapping was used for identification of the position of the apple powdery mildew resistance gene *Pl1*. Although several molecular markers for MAS have been developed for *Pl1* in the past by bulked-segregant analyses (Markussen *et al*. 1995, Dunemann *et al*. 2004), the map position of this gene was not known. An apple linkage map constructed on the basis of a population derived from a cross between 'Discovery' and 'Prima' could not be used for mapping of the AT20-SCAR and the other *Pl1* markers. Very probably the *Pl1* marker fragments represent DNA segments specific for the genome of *M. robusta* which is the original resistance gene donor. As another *M. robusta*-derived population (04/208), which is presently used for fire blight resistance gene mapping, segregated for the AT20 marker, this marker could be placed in a core genetic map constructed for this family by a set of selected SSRs.

The AT20-SCAR co-segregated with the SSR Hi07f01 which is located at the distal end of LG 12 (Silfverberg- Dilworth *et al*. 2006) indicating that *Pl1* also maps in this region of the apple genome. Confirmation of the *Pl1* map position

was performed in a second progeny (99/2) which has been characterized phenotypically over the past years in the greenhouse and in the field under a high natural powdery mildew infection pressure. A *Malus* TIR-NBS-LRR candidate resistance gene (15G11) which has been mapped in the D × P map in the same region of LG 12 (Thiermann 2002) was shown to be linked with the *Pl1* locus in family 99/2.

	AT20-SCAR	Number of plants/percentage						
Year	marker presence	Class 0 resistant (%)	Class 1 Resistant (%)	Class 2 susceptible (%)	Class 3 susceptible (%)	Class 4 Susceptible (%)	Class 5 Susceptible (%)	Total
1999	Present	98 (78.4)	20 (16.0)	3 (2.4)	2 (1.6)	0 (0)	2 (1.6)	125 (100)
	Absent	7 (6.5)	3 (2.8)	15 (13.9)	68 (62.9)	11 (10.2)	4 (3.7)	108 (100)
2000	Present	111 (88.8)	8 (6.4)	1 (0.8)	3 (2.4)	2 (1.6)	0 (0)	125 (100)
	Absent	13 (12.0)	5 (4.6)	6 (5.6)	58 (53.7)	20 (18.5)	6 (5.6)	108 (100)

Table 2: Relationship between presence of the AT20-SCAR marker and powdery mildew data in 2 years of greenhouse assessments (class 0: no mildew infection, class 5: severe mildew infection); classes 0 and 1 contain the resistant plants, classes 2–5 the susceptible ones

The position of the *Pl1* locus together with the AT20-SCAR and the RGA 15G11 at the bottom part of apple LG 12 indicate that *Pl1* might be a further gene of an important resistance gene cluster located in this region of the apple genome. Several major genes for powdery mildew and apple scab as well as QTLs for both diseases have previously been mapped in this region: *Pld* as a major powdery mildew resistance gene (James *et al*. 2004), a stable QTL for mildew resistance from the apple clone U211 (Stankiewicz-Kosyl *et al*. 2005), the scab resistance gene *Vg* conferring resistance to scab race 7 (Durel *et al*. 2000), and, as a recent result, the *Vb* major scab resistance gene (Erdin *et al*. 2006). The comparison of the positions of the SSRs CH01g12, CH01f02, CH03c02 and CH01d03 in the *Pl1* maps and in the *Vg* map published by Calenge *et al*. (2004) indicates that *Pl1* and *Vg* could be tightly linked. The distance of *Pl1* from CH03c02 is between 12 cM (locus *Pl1-Gh*) and 19 cM (locus *Pl1-F*), whereas *Vg* is linked to CH03c02 with a genetic distance of 18 cM (Calenge *et al*. 2004). The

position of *Pld* relative to SSR CH03c02 is 8 cM and to CH01g12 is in the same order (35 cM) as in the *Pl1* maps and in the *Vg* map (James *et al*. 2004). The resistance gene analogue 15G11 seemed to be co-localized with *Pl1*. Whether this RGA is a candidate for *Pl1* itself, or for other genes such as *Pld* or *Vg*, can not be answered at this stage. RT-PCR expression studies showed an obviously constitutive expression of this gene in resistant genotypes but also in some mildew-susceptible cultivars (data not shown). Further work is underway to investigate if the RGA 15G11 is an expressed pseudogene, a candidate for *Pl1*, or one of the neighbouring resistance genes.

The prominent resistance gene cluster on LG 12 is not the only one in the apple genome. At least three other hot spots consisting of major scab and mildew resistance genes, QTLs and RGAs are known on LGs 2, 8 and 17. For example, the cluster in the upper part of LG 2 contains major scab resistance genes such as *Vr1 (*Boudichevskaia *et al*. 2006), *Vr2* (Patocchi *et al*. 2004) and *Vh4* (Bus *et al*. 2005) together with stable mildew QTLs (Calenge and Durel 2006) and several NBS-LRR RGA (Baldi *et al*. 2004, Calenge *et al*. 2005). For more details on co-localization of NBS-LRR candidate genes and loci for scab and mildew resistance in the apple genome, refer to the paper of Calenge *et al*. (2005).

Podosphaera leucotricha is an obligate parasite and does not grow on artificial media. The existence of races has been demonstrated (Caffier and Laurens 2005, Urbanietz and Dunemann 2005), but in contrast to apple scab (*Venturia inaequalis*), race specific pathogenicity tests are not easily to apply to larger genetic mapping experiments. Evaluation of powdery mildew resistance by using a natural inoculum is strongly influenced by environmental conditions and further factors. Seedlings showing no sign of powdery mildew infection after greenhouse testing have been observed to be susceptible in the field (Korban and Dayton 1983). This phenomenon could also be observed in some cases in the greenhouse and field tests here over the 6 years of mildew scoring in family 99/2, but the opposite reaction was also found quite often. This may be attributed to

the presence of different races of the mildew fungus, the higher inoculum dose in the greenhouse, the physiological status of the apple trees or the changing climatic conditions being more favourable in the greenhouse. Regarding the race spectrum, it is probable from the molecular studies on the pathogen (Urbanietz and Dunemann 2005) that in the greenhouse experiment only a single or a few genetically similar races were present, whereas outside in the field a more complex race spectrum occurred. To consider this situation, the greenhouse and field scoring data were separated and separate linkage maps were constructed which finally proved to be comparable regarding the location of the *Pl1* locus. Nevertheless, the simple division of the seedlings into resistant and susceptible groups harbors the risk of wrongly classifying some of the plants with intermediate resistance reactions.

Pl1 mapping was facilitated by the availability of the SCAR AT20, which is being used now for MAS successfully in other *Pl1*-based mildew resistance breeding programs too (Kellerhals *et al*. 2004). However, it should be noted that the *Pl1* resistance was overcome in race-specific powdery mildew tests (Lesemann and Dunemann 2005) and that the genetics of *Pl1* resistance is not yet fully understood. Even if the value of *Pl1* for future breeding purposes is restricted, this gene might be an interesting target for the isolation of a first *Malus* powdery mildew resistance gene and may help to better understand the action of powdery mildew resistance genes in the *Rosaceae* family.

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3.5 Differential response of a gene encoding a Kunitz-type protease inhibitor in fire blight-susceptible and resistant apples

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Abstract

The fire blight disease, caused by the bacterium *Erwinia amylovora*, of trees from the *Maloideae* subfamily of the *Rosaceae*, is a serious economic threat and has spurred research for genetic resistance. We made and screened a normalized cDNA library from infected and non-infected individuals of two apple species with contrasting response to the disease. The cultivar Idared (*Malus* x *domestica*) was highly susceptible to fire blight, whereas the clonal accession Mr-P of the crabapple *Malus* × *robusta* (Carrière) Rehder (a cross of *M. baccata* × *M. prunifolia*, Syn.: *Malus microcarpa* var. *robusta* Carrière) var. *persicifolia* was known to possess high levels of resistance to the disease. One gene encoding a putative Kunitz-type protease inhibitor protein, denominated as *PI-Mal* (Protease Inhibitor-Malus) was outstanding as its expression levels significantly decreased in Idared but not in Mr-P following inoculation with the fire blight pathogen. We genetically mapped the *PI-Mal* locus to chromosome 3 of apple. The *PI-Mal* gene and its conspicuous pattern of expression are discussed in the context of identifying potential markers to select for fire blight resistance in breeding programs of apple.

Keywords: Apple, fire blight disease, *Erwinia amylovora*, *Malus*, *M*. x *robusta*, crabapple, Gene expression profile, Protease inhibitor gene, *PI-Mal*

Introduction

Erwinia amylovora fire blight causes substantial economic losses in pomefruit including apple production. The highly infectious bacterium is spread during blooming and enters the host tissue through the flowers. Infections become visible by necroses in twigs which die and obtain a “burnt” morphology. Other ways of invasion take advantage of wounds and rootstocks (Peil *et al.* 2009). With the exception of the increasingly banned control by antibiotics the disease can only be stopped by rigorous removal of infested wood and often entire trees and even plantings. Therefore, genetic resistance is a viable, economic alternative. Because of the disease’s quantitative appearance—parts of the trees become necrotic and not all trees show similar degrees of infestation—it has been believed that only quantitatively inherited resistance can be found among the germplasm of the *Maloideae* subfamily (Peil *et al.* 2009). In contrast, the existence of qualitatively inherited major-gene resistance has been put forward only recently as a conclusion from a genetic mapping study by Peil *et al.* (2007) of quantitative trait loci (QTL) in cross progenies segregating for resistance. Several such major QTLs that can be tested for the presence of major genes were described by Calenge *et al.* (2005), Khan *et al.* (2006), Peil *et al.* (2007), and Durel *et al.* (2009). Selections of *M*. x *robusta*, the crabapple, a cultivated relative of the common apple, have been in use as rootstocks (Qu *et al.* 2005) as they retard growth and are resistant to many diseases. The specific source of resistance reported in Peil *et al.* (2007) is a clonal accession recognized by Gardner *et al.* (1980) as a progenitor of strong fire blight resistance that has been widely distributed as *M*. *robusta* selection No. 5 (also known as “R5” or “robusta 5”, we refer to it as Mr-5). It is a good candidate to harbor an effective major, dominant resistance gene for fire blight. We employed the Idared x Mr-5 mapping population described in Peil et al. (2007) for genetic mapping of the *Pl-Mal locus*. Further we have used a similar clonal accession of *Malus* × *robusta* (Carrière) Rehder (a cross of *M. baccata* × *M. prunifolia*, synonym: *Malus microcarpa* var. *robusta* Carrière) var. *persicifolia*, referred to as Mr-P to make an ordered normalized subtraction library of cDNA representing genes that are expressed

during the response of the stem tissue to inoculation by *E. amylovora*. Mr-P possesses a fire blight resistance phenotype akin that of Mr-5. Good candidate genes of the clonal accession Mr-P with highly reproducible, clear-cut increased or reduced expression levels were rarely found. A detailed description of this is given in Milčevičová *et al.* (2010). However, variants of genes with disease-associated expression were isolated from the susceptible apple cultivar Idared which was also used for a cDNA library. Therefore, this report is on a putative protease inhibitor gene that was gradually, but significantly and reproducibly down-regulated during the fire blight disease in cv. Idared.

Materials and methods

In-vitro plantlets of the *M.* x *domestica* Borkh. cv. Idared susceptible to fire blight, and the resistant *M.* x *robusta* (Carrière) Rehder var. *persicifolia* accession Mr-P were grown on MS medium containing 3% sorbitol. Six-week-old, 4-cm-long green, woody stems of the plantlets were toothpick-inoculated with *E. amylovora* strain 295/93 (~10^8 cfu/ml from overnight cultures in liquid King's B medium) and kept at 24+-2°C. Healthy control plants of the same cultivars were toothpick-inoculated with sterile King's B medium. Shoot tips of 1 cm length directly above the inoculation site were collected by immediate immersion in liquid nitrogen at 6, 24, 48, and 72 h (0.25, 1, 2, 3 days) after inoculation. Total RNA was isolated by the method of Chang *et al.* (1993) from 3 replicates, each composed of 5 shoot tips. Suppressive subtraction cDNA libraries were produced with the PCR-select cDNA subtraction kit (Clontech) on RNA of the inoculated plants as a reporter and control plants as a driver, for both cultivars. Ninety six clones of these libraries were sequenced (AGOWA, Berlin, Germany) and submitted to Genebank (NCBI, expressed sequence tag accessions FK938840-953). Of these, the expression patterns of FK938848, a putative Kunitz-type protease inhibitor gene fragment (partially similar to ACS92521.1 from *Populus* spp.; 90% coverage, 52% identity at 2e-33) with marginal similarity to miraculin-like

sequences (as an example, XP_002525873 from *Ricinus communis*; 52% identity at 86% sequence coverage, 2e-29) were investigated. We applied realtime reverse-transcription polymerase chain reaction (RT-PCR) on an iCycler device (BIO-RAD) with the iQ TM SYBR Green Supermix (BIO-RAD) and primers RT-PI-Mal-1 (5´-CAGTCTCACCCACCTTCCAT-3´) and RT-PI-Mal-2 (5´-TTTTGTTGAGGGGGAAACTG-3´), together with primers 5´-TCTTTGTGAAAACCCTCACC-3´ and 5´-ATCCCTTCCTGGTCCTGGAT-3´ (CX023931) for ubiquitin as a calibrator. For genetic mapping, a 140-progeny biparental Idared x Mr-5 cross population, phenotyped for quantitative response to fire blight (Peil *et al.* 2007), was used to map the *PI-Mal* locus (see below, Results and Discussion). A polymorphic part of the locus was isolated from genomic DNA of both parental cultivars with the primers PI-Mal-for; 5'-TCAACCCTAGCCCAAACATC-3' and PI-Mal-rev; 5'-CTGGTGTTGCCTGCTTTGTA-3', cloned and sequenced to detect single nucleotide polymorphisms. In turn, two allele-specific primers, ASP-PI-Mal-A and ASP-PI-Mal-G (5´-GGGCGTTTCACTTTGRACAA/G-3´) with RT-PI-Mal-1 as a complementary primer, were applied to all 140 mapping progeny and their parents. Amplicons were visualized on standard agarose gels and the locus mapped in JoinMap (van Ooijen and Voorrips 2001), with the Kosambi mapping function and a logarithm of the odds (LOD) score of 8 for marker grouping. Assignment of the consensus chromosomes (genetic linkage groups) of apple was made based on the nomenclature proposed in Liebhard *et al.* (2002) and Silfverberg-Dilworth *et al.* (2006).

Results and discussion

In-vitro plantlets of cv. Idared quickly developed dark-brown lesions on their stems and leaf petioles upon inoculation with the bacterium. In contrast, no disease symptoms were macroscopically visible on Mr-P plantlets until 4 days after inoculation (DAI) when the experiment was finished. These patterns

corresponded well to the infection response of trees in the field. Whereas Idared trees develop necrotized twigs and become severely affected by the disease, only very small lesions as signs of reduced spread of the bacterium within Mr-P, similar to those reported from Mr-5 (Gardner *et al*. 1980) have been observed (AP, unpublished observation). Our cDNA libraries contained to a great part putative genes that were detected also by Norelli *et al.* (2009) and Baldo *et al.* (2010) in studies on gene expression under the influence of fire blight disease (not shown). Candidate genes with differential expression associated with the resistant accession, Mr-P, were described in Milčevičová *et al.* (2010). Among 96 sequenced cDNA fragments, a single clone was encountered that was not covered by any of the above studies. This clone had partial similarity to two cDNAs from apple, CV084148 and CO576696, which resemble putative Kunitz-type protease inhibitors with marginal similarity to Miraculin (Major and Constabel 2008) from poplar and other plants (Figure 1).

Figure 1. Amino acid sequence fragment of a putative Kunitz-type protease inhibitor (*PI-Mal*) from apple cultivars Idared (*M*. x *domestica*; susceptible to fire blight) and Mr-P (*M*. x *robusta* var. *persicifolia*, resistant). Alignment with best-matching Genebank accessions (identified by tBLASTx) encoding putative protease inhibitors (XP_002265535.1; *Vitis*, ACS92522.1; *Populus*, and ACU13608.1; *Glycine max*), state, September, 2010. The large frame indicates

the Kunitz motif, and a disulfide bridge could be formed in the mature protein between the two framed C residues.

Using the parents of the Idared x Mr-5 mapping population (Peil *et al*. 2007), a polymorphic 417-bp fragment containing 17 single nucleotide polymorphisms (SNPs; not shown) was amplified from the genomic locus that was tentatively labeled *PI-Mal*, with the PI-Mal-for/-rev primers. In turn, with the two allele-specific primers, ASP-PI-Mal-A and ASP-PI-Mal-G, 207-bp single-allele fragments were amplified from genomic DNA. Whereas the A allele was uniformly amplified from all 140 Idared x Mr-5 cross progenies, the G allele originating in Mr-5 segregated 1:1 and could thus be used in genetic mapping. Consequently, the *PI-Mal* gene locus was mapped near the framework map's MS14H03 locus on chromosome 3 of the resistant parent, *M*. x *robusta* Mr-5 (Fig. 2).

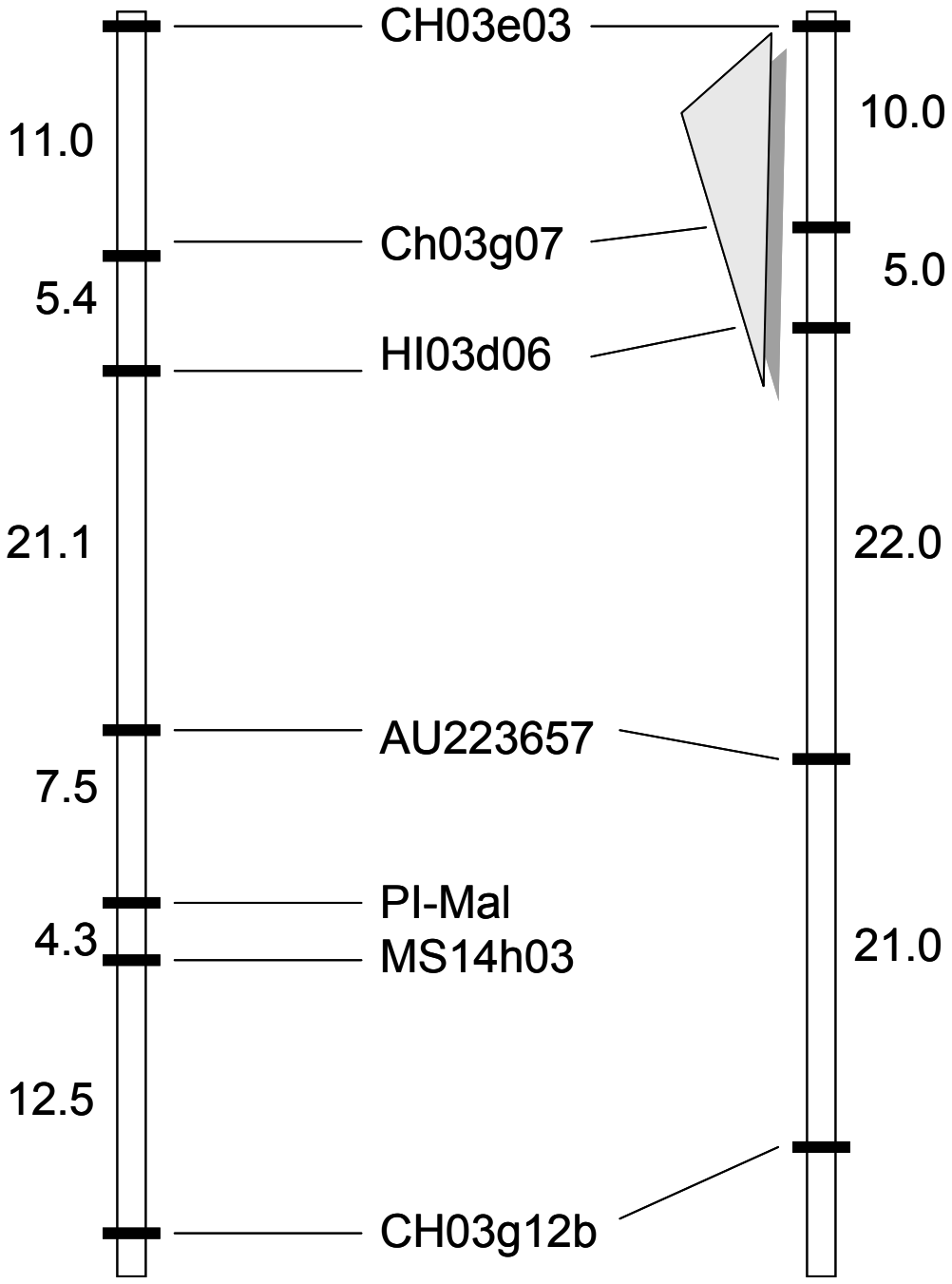

Figure 2. Genetic map of the *M. x robusta* cv. Mr-5 consensus chromosome 3 ("linkage group 3") including the *PI-Mal* locus (left). The map was generated with 140 individuals and the parents of the Idared x Mr-5 population described in Peil *et al*. (2007). The corresponding part of the Peil *et al*. (2007) map including a major locus for fire blight resistance of Mr-5 (triangle representing the locus as a quantitative trait), is shown at the right side. The *PI-Mal* locus was positioned in

the interval between AU223657 and MS14H03, opposite to the chromosome arm carrying the resistance locus. Genetic distances between adjacent markers are indicated in centi Morgan.

RT-PCR on a time series of diseased and healthy samples revealed that *PI-Mal* from the susceptible cv. Idared was significantly and consistently throughout all replicates, down-regulated by days 2 and 3 after inoculation ($P<0.001$; Figure 3). In Mr-5, a minimal down-regulation at 2 days after infection was observed that immediately rose back to levels indistinguishable from those of the controls inoculated with King's B medium. While Kunitz-type protease inhibitors act on several different types of proteases and are implicated in defense against insect pests (Schuler *et al.* 1998), these protease inhibitors also have been encountered frequently among transcriptionally activated genes during stress and development of plant disease. The causal mechanisms may be related to disease response-specific protein turnover during the active disease process within affected host cells. Due to their great biological efficiency and substrate specificity (Xia 2004, Habib and Fazili 2007) it is conceivable that even slightly altered expression rates similar to those observed by us could exert a considerable effect on the actual protein pool of the cell.

For *Arabidopsis thaliana*, Li *et al.* (2008) reported a serine protease (Kunitz trypsin) inhibitor gene, *AtKTI1*, also sharing partial similarity with a miraculin homolog and a drought repressed gene from the same plant, whose expression is induced late in response to bacterial and fungal elicitors. Silencing of *AtKTI1* led to enhanced resistance to the virulent pathogen *Erwinia carotovora* subsp. *carotovora* SCC1, while overexpression of *AtKTI1* resulted in higher susceptibility towards this pathogen. Therefore, AtKTI1 may represent a modulator of resistance and susceptibility in plant–pathogen interactions. We compared our *PI-Mal* fragment to the complete *AtKTI1* sequence (At1g73260). Thirty *Genebank*

entries that shared partial identity with both sequences/predicted proteins (via tBLASTx) were among the first 100 entries with similarity to either *PI-MaI* or *AtKTI1*, nine of these identified as protease inhibitor- or miraculin-type genes. However, the similarity of *PI-MaI* to any of these accessions was consistently lower than the similarity to *AtKTI1*, indicating that *PI-MaI* might represent an ortholog of the gene from *Arabidopsis* that can be involved in modulating susceptibility, or attenuating the disease severity, of apple to bacterial pathogens.

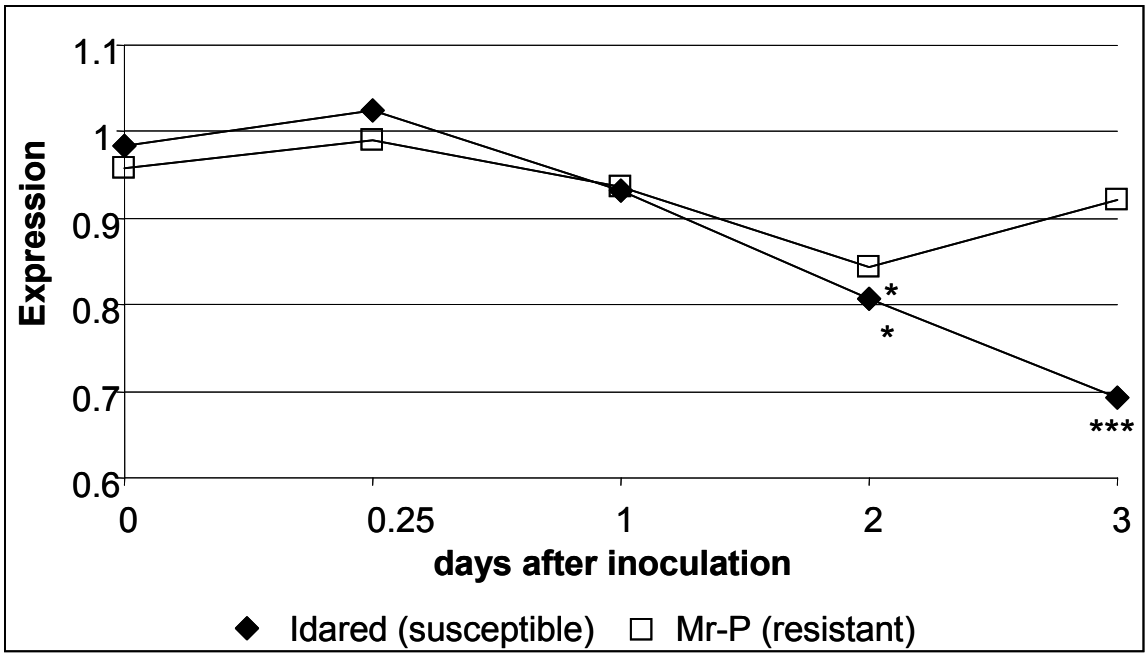

Figure 3. Levels of *PI-MaI* transcription throughout the course of 3 days after inoculation with *E. amylovora* strain 295/93. Expression levels relative to corresponding controls inoculated with sterile King's B medium. Values were standardized against ubiquitin A. Asterisks indicate significant relative expression of disease-treated vs. control samples determined with the permutation test implemented in the REST program (Pfaffl et al. 2002). *; P<0.05, ***; P<0.001).

The *PI-MaI* locus was invisible in earlier experiments on the Idared x Mr-5 population with the aim of mapping resistance (Peil *et al.* 2007), and this is

expected as the phenotype screenings applied to segregating populations are designed to record levels of resistance. A phenotype of susceptibility is not complementary to a phenotype of resistance as molecular and physiological mechanisms of susceptibility may be acting independent of mechanisms conferring resistance. Only in the absence of effective major-gene resistance the modulation of susceptibility by potential genetic elements that can act as gradual enhancers of susceptibility will become visible and detectable by the phenotype of disease. The differential expression of *PI-MaI* occurred late during the onset of the fire blight disease within a susceptible genotype, whereas expression remained nearly constant within a resistant genotype.

In breeding, positive and negative selection schemes can be equally applied to isolate desired recombinants. While positive selection is directly calling on individuals possessing the desired trait, individuals devoid of the trait are detected and eliminated in schemes of negative selection. The pattern of response caused by *PI-MaI* could be directly used as a marker in negative selection for fire blight resistance of recombinant apple offspring. We observed that the transcription of *PI-MaI* is reduced during development of the disease in the susceptible cultivar Idared. The pattern of *PI-MaI* expression will be measured in more susceptible and resistant apple cultivars of different and also similar genetic background including Idared x Mr-5 cross progenies. When the association of disease with reduced transcription of *PI-MaI* is confirmed it could be used to select and eliminate those recombinants that show this response and are therefore highly likely to be susceptible to fire blight. Complementarily, positive selection for offspring maintaining unaltered expression levels would directly target potential resisters. For the development of allele-based DNA markers of susceptibility/resistance related to the *PI-MaI* locus, additional research is necessary to detect and isolate alleles at those unknown genetic loci that are responsible for the differential transcription of *PI-MaI*.

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4 General conclusions

‘Idared’ and ‘*Malus x robusta* 5’ -a highly susceptible apple cultivar and a virtually immune wild apple- have provided a suitable background for genetic analysis of fire blight resistance in *Malus*. Genotyping of 293 SSR and AFLP markers in 140 progenies derived from these two parents allowed the construction of parental linkage maps sufficiently saturated for efficient QTL mapping. Phenotypic data obtained from the same progeny population enabled the localization of a major QTL for fire blight resistance in linkage group 3 of ‘*M.x robusta* 5’. The presence this locus was verified in a second year of observation and confirmed in the mapping population ‘Malling 9 x *M.x robusta* 5’.

In parallel, other authors have identified fire blight resistance QTLs in other apple species and cultivars, which are located on different linkage groups, suggesting the existence of several resistance mechanisms in *Malus*. The QTL on LG3 of ‘*M. x robusta* 5’ explained 75% of the phenotypic variation, more than any other fire blight resistance QTL identified to date. This gave rise to the hypothesis, that fire blight resistance, which is generally supposed to be a quantitative trait, may be controlled by a major gene in ‘*M. x robusta* 5’.

Furthermore, the population ‘Idared x *M.x robusta* 5’ and the derived linkage map was suitable for mapping a major gene conferring resistance to powdery mildew, which is apart from fire blight one of the most devastating diseases in apple, on LG12. SSH-based gene expression data from ‘Idared’ and ‘*M.x robusta’* revealed that apart from segregating the resistance QTL, these two apples differentially expressed a putative protease inhibitor gene (*PI-Mal*). A SNP marker could be developed for *PI-Mal* and was mapped on LG3 of ‘*M.x robusta* 5’. We are confident that the presented linkage maps will be valuable for further exploration of genetic loci controlling favorable traits of the popular scion cultivar ‘Idared’ and the viable rootstock ‘*M.x robusta* 5’.

Combined use of different marker systems, including SSRs, AFLPs, SCARs and SNPs has allowed the construction of genetic linkage maps as well as the localization of specific disease related loci. As applicable information for marker assisted breeding in apple, this thesis has revealed tight linkage between fire blight resistance and the SSR CH03e03, and provides the SNP marker PI-Mal-G for the defense-related gene *PI-Mal*. These markers, which have served for mapping, can be used for the selection of potentially fire blight resistant individuals as parents for breeding immune apple varieties.

This study was initiated in the pre-genomic era of apple research, i.e. before the release of the apple genome sequence and before the development of high throughput-sequencing technologies. At that time sequence information on the gaps between the markers of genetic linkage maps was not accessible. The primary challenge consisted in identifying sufficient numbers of polymorphic markers to compute and cover the 17 linkage groups of apple. The number of markers as well as the number of individuals that could be screened within a study was limited to the scale of few hundreds. QTLs were mapped to intervals of several cM and further resolution was difficult to achieve.

Genome sequencing of 'Golden Delicious' has given access to the sequence between the DNA markers of genetic linkage maps. QTL intervals can now be mined *in silico* for open reading frames and other genetic elements. An important step in future fire blight resistance research will be the *in silico* examination of the QTL interval on LG3, that has been identified in this thesis. Assembly of the two 'Golden Delicious' haplotypes has identified over 10^6 SNPs, as a virtually unlimited source of novel DNA markers. With this information, high density mapping of QTL intervals, such as the distal end of LG3 can easily be accomplished. Moreover high-throughput screening systems have been developed for analyzing thousands of markers in large mapping populations. Therefore it will soon be possible to resolve the fire blight resistance locus of '*M.x robusta 5'* into individual reading frames and to answer the question on whether it

contains a major resistance gene. Finally, annotation of the apple genome sequence has identified 992 resistance-related genes, opening up untold perspectives for breeding of disease resistant apple cultivars.

Curriculum Vitae

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Work Experience

2011 -2012 Internship at the United Nations Office on Drugs and Crime, UNODC Expert Analysts, Laboratory and Scientific Section LSS

2008 – 2010 Research Scientist The project was a joint initiative between the Institute of Fluid Mechanics and Heat Transfer the Graz University of Technology in Graz (TU-Graz) and The Coca-Cola Company in Graz, Austria

2005 – 2008. Visiting Scientist, Austrian Institute of Technology (AIT) (former Austrian Research Centers). Seibersdorf, Austria**.** Research work for Ph.D. thesis: "Genetic mapping and characterization of resistance factors in apple (*Malu*s sp.) against Fire Blight".

2003 - 2004. Associated Researcher, Joint Molecular Biology Laboratory of the Corporacion Autónoma Regional del Cauca (CRC), Colombia and Alexander von Humboldt Institute, CIAT, Cali, Colombia.

2001 - 2002. B.Sc. research and thesis, Biotechnology Research Unit of the International Centre for Tropical Agriculture (CIAT, its Spanish acronym) and Universidad del Valle. Cali, Colombia.

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