Development of molecular markers linked to the 'Fiesta' linkage group 7 major QTL for fire blight resistance and their application for marker-assisted selection

Muhammad A. Khan, Charles-Eric Durel, Brion Duffy, Damien Drouet, Markus Kellerhals, Cesare Gessler, and Andrea Patocchi

Abstract: A fire blight resistance QTL explaining 34.3%–46.6% of the phenotypic variation was recently identified on linkage group 7 of apple cultivar 'Fiesta' (F7). However, markers flanking this QTL were AFLP and RAPD markers unsuitable for marker-assisted selection (MAS). Two RAPD markers bracketing the QTL have been transformed into SCAR (sequence-characterized amplified region) markers, and an SSR marker specific for the region was developed. Pedigree analysis of 'Fiesta' with these markers enabled tracking of the F7 QTL allele back to 'Cox's Orange Pippin'. Stability of the effect of this QTL allele in different backgrounds was analyzed by inoculating progeny plants of a cross between 'Milwa', a susceptible cultivar, and '1217', a moderately resistant cultivar, and a set of cultivars that carry or lack the allele conferring increased fire blight resistance. Progenies and cultivars that carried both markers were significantly more resistant than those that did not carry both markers, indicating high stability of the F7 QTL allele in different backgrounds. This stability and the availability of reproducible markers bracketing the QTL make this locus promising for use in MAS.

Key words: Malus × *domestica*, *Erwinia amylovora*, quantitative resistance, quantitative trait locus.

Résumé : Un QTL de résistance au feu bactérien expliquant entre 34,3 % et 46,6 % de la variation phénotypique a été récemment identifié sur le groupe de liaison 7 du pommier chez le cultivar 'Fiesta' (F7). Cependant, les marqueurs bordant ce QTL étaient des AFLP et des RAPD impropres à la sélection assistée. Deux marqueurs RAPD flanquant le QTL ont été transformés en marqueurs SCAR et un marqueur SSR spécifique à cette région a été développé. Une analyse du pedigree de 'Fiesta' à l'aide de ces marqueurs a permis d'attribuer l'origine de l'allèle QTL F7 au cultivar 'Cox's Orange Pippin'. La stabilité de l'effet de cet allèle QTL au sein de différents fonds génétiques a été analysée en inoculant la descendance d'un croisement entre 'Milwa', un cultivar sensible, et '1217', un cultivar moyennement résistant, ainsi qu'un ensemble de cultivars porteurs ou dépourvus de l'allèle conférant une résistance accrue au feu bactérien. Les descendants de ce croisement et les cultivars qui étaient positifs pour les deux marqueurs étaient significativement plus résistants que ceux qui étaient négatifs pour ces marqueurs, ce qui indique une grande stabilité de l'allèle QTL F7 au sein de différents fonds génétiques. Cette stabilité et la disponibilité de marqueurs reproductibles bordant ce QTL sont prometteurs en vue de son exploitation en sélection assistée.

Mots-clés : Malus × *domestica*, *Erwinia amylovora*, résistance quantitative, locus d'un caractère quantitatif.

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Introduction

Fire blight, the most damaging bacterial disease of apples and pears, is caused by *Erwinia amylovora*. Fire blight control measures are inefficient and the use of antibiotics is banned in several countries. However, several heterologous genes (i.e., not from *Malus* spp.) have been used to produce fire blight resistant cultivars (reviewed in Gessler and Patocchi 2006). As in many plant diseases, the resistance against fire blight is quantitative in nature and has been reported in both wild and cultivated *Malus* species as well as *Pyrus* species (Brisset et al. 2002; Dondini et al. 2004; Durel et al. 2004). Studies have been performed to identify the quantitative trait loci (QTLs) linked to fire blight resistance in pear

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(Dondini et al. 2004) and apple (Calenge et al. 2005; Khan et al. 2006). Calenge et al. (2005) identified a major QTL linked to fire blight resistance that explained 34.3%–46.6% of the phenotypic variation. This QTL was identified on linkage group 7 of the apple cultivar 'Fiesta' (F7) at the same genetic position in 2 different genetic backgrounds, i.e., 'Fiesta' × 'Discovery' (F × D) and 'Prima' × 'Fiesta' (P × F). Four minor QTLs were also identified in this study, one on linkage group 3 of 'Fiesta' and one on linkage group 3 of 'Prima' using F × D and P × F crosses, respectively, and one on each of linkage group 12 and linkage group 13 of 'Discovery' in an F × D cross. In both sets of progenies, the maximum-likelihood position (QTL peak) of the QTL on F7 was close to the common RAPD marker GE80-19-0550 (about 1 cM in P × F and 4–6 cM in F × D). The simple sequence repeat (SSR) marker closest to GE80-19-0550 is CH04e05, which is 25 cM from the RAPD marker in the P × F cross and 31 cM from the RAPD marker in the F × D cross (Calenge et al. 2005). Khan et al. (2006) independently reported the identification of a QTL linked to fire blight resistance on F7 in a second set of seedlings derived from a 'Fiesta' × 'Discovery' cross (F × D-CH) but grown in Switzerland and inoculated with a Swiss *E. amylovora* strain. The range of phenotypic variability explained by the F7 QTL in this study was similar to that reported by Calenge et al. (2005), being 37.5%–38.6%. The marker at the QTL peak is an amplified fragment length polymorphism (AFLP) marker, E37M40-0400, and the markers flanking the QTL peak are 2 RAPD markers, AE10-400 and B07-1700, which are 4 cM from E37M40-0400 on either side. The SSR marker CH04e05 was also mapped in this cross at 23 cM from E37M40-0400. Because the same SSR was used in both studies it was estimated that the QTLs identified in these studies are probably the same (Khan et al. 2006).

Markers linked to a complex trait can be used for the early selection of seedlings with desirable characters to reduce the number of plants kept in nurseries and fields (Dirlewanger et al. 2004). Markers linked to many important traits, especially apple scab (reviewed in Erdin et al. 2006; Gessler et al. 2006) and mildew resistance (Evans and James 2003; James et al. 2004), have been developed and are being used in apple breeding programs (Dirlewanger et al. 2004). However, most of these traits are monogenic.

Many studies have pointed out (Jung et al. 1999) that prior to using marker-assisted selection (MAS) to select for a QTL allele in a breeding program, it is important to test the stability and efficiency of the QTL allele in different genetic backgrounds and economically important cultivars. Beavis et al. (1991) warned against drawing conclusions based on a single genetic background. Besides the stability in different genetic backgrounds, the effectiveness and usefulness of MAS depends on the distance and type of the markers. The F7 QTL is currently the most promising of the available fire blight resistance QTLs for use in MAS (Calenge et al. 2005). Until now, only RAPD and AFLP markers have been located close to the F7 QTL, and both types of markers are not suitable for use in MAS (Hernández et al. 1999) because of their dominant nature and difficult reproducibility across different laboratories and conditions. The objectives of this study were to (*i*) develop markers flanking the F7 QTL suitable for MAS, (*ii*) test the effectiveness of this QTL in different backgrounds, and (*iii*) propose a protocol for using the markers developed for MAS to select for fire blight resistance in apple seedlings.

Materials and methods

Plant material

Newly developed markers were mapped on the reference map of apple (Liebhard et al. 2003) using 251 'Fiesta' × 'Discovery' (F × D-CH) progeny plants grown in an experimental orchard at Agroscope Changins-Wädenswil (ACW). Consistency of the F7 QTL in an additional genetic background was tested using progenies of a 'Milwa' × '1217' cross. A cross between 'Milwa' (susceptible to fire blight) and '1217' (moderately resistant to fire blight) was made in 2003; this cross consisted of 171 progeny plants and was grown in an orchard at ACW. 'Milwa' lacks the F7 QTL, while '1217' is a progeny of the cross F × D-CH, which carries the F7 QTL. Thirty-one cultivars (see Table 3) were used for validating the F7 QTL through cultivar analysis. Budwood of the cultivars was obtained from INRA, Angers, France.

DNA extraction

The DNA of 251 F × D-CH progeny (Liebhard et al. 2003) was used in this study. Young leaves of 171 3-year-old 'Milwa' × '1217' progeny plants as well as young leaves from shoots of 1-month-old grafted trees of the 31 selected cultivars were collected and lyophilized. DNA was extracted from lyophilized leaves according to Koller et al. (2000), gel quantified, and diluted to 1 ng/μL.

Development and mapping of markers

Development of sequence-characterized amplified region (SCAR) marker AE10-375

The PCR protocol used Operon Technologies primer AE10 (5′-CTGAAGCGC-3′) to test 6 F × D-CH progeny plants along with the parents as described for RAPD analysis by Koller et al. (1994). The PCR products were separated on a 1.5% agarose in 0.5× Tris-borate-EDTA (TBE) gel, stained with ethidium bromide, and photographed. The banding pattern obtained was compared with the original banding pattern used to map the RAPD marker AE10-400. The polymorphic band was excised from the gel and purified according to the Promega PCR purification kit protocol (Promega, Madison, Wisconsin).

The purified fragment was ligated into the TA Cloning® Kit pCR®2.1 vector and chemically transformed into One Shot® TOP10F′ chemically competent *E. coli* cells (Invitrogen, Basel, Switzerland), following the supplier's instructions. Positive colonies were picked with sterile tips, suspended in 50 μL H_2O, and denatured for 5 min at 94 °C; 5 μL was used as template for PCR. The size of the insert was verified by PCR with M13 forward and reverse primers and by comparison with the peqGOLD 100 bp DNA Ladder Plus (PeqLab, Erlangen, Germany). Reaction and cycler conditions for specific PCR were as described by Liebhard et al. (2002) for MS-SSRs. Amplified product was separated on a 1.5% agarose in 0.5× TBE gel, stained with ethidium bromide, and photographed. The band was excised from the

gel, purified using the Promega gel cleanup system following the manufacturer's protocol, and gel quantified. The purified PCR product was subsequently sequenced from both ends with the BigDye® kit (PE Applied Biosystems, Foster City, California) and run on an ABI PRISM® 3100 DNA sequencing system (PE Applied Biosystems). The original AE10 primer sequence was extended from 10 base pairs (bp) to 18 bp (forward primer) and 22 bp (reverse primer). The resulting forward and reverse primers for SCAR marker AE10-375 were purchased from Microsynth AG (Balgach, Switzerland).

Development of SCAR marker GE-8019

PCR was performed with GENSET oligo GE-80-19 (5′-ACGCCCTGGC-3′) on 20 'Prima' × 'Fiesta' (P × F) progeny plants along with the parents. The following PCR conditions were used: 0.8% formamide, 1× Red Taq buffer (10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, 0.01% gelatin), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP, 4 μmol/L of primer, 1 U of Red Taq polymerase (Sigma-Aldrich, Lyon, France), and 50 ng of genomic DNA in a final volume of 25 μL. The PCR products were separated on a 1% agarose in 0.5× TBE gel, stained with ethidium bromide, and photographed. The banding pattern obtained was compared with the original pattern used to map the RAPD marker GE80-19-0550 (Maliepaard et al. 1998). The corresponding polymorphic band was excised from the gel for both 'Fiesta' and an individual P × F progeny ('J19') and purified according to the Maestro Life Science purification kit protocol (Millipore, Billerica, Massachusetts).

The purified fragments were ligated into the pGEM®-T Easy cloning vector (Promega) and heat-shock transformed into chemically competent *E. coli* cells. Positive colonies were picked with sterile tips, suspended in 30 μL H_2O, and denatured for 5 min at 94 °C; 1 μL (~0.1–1 ng) was used as template for PCR. The size of the insert was verified by PCR with T7 forward and SP6 reverse primers and by comparison with the Eurogentec DNA SmartLadder (Seraing, Belgium). Reaction conditions for specific PCR (15 μL) were as follows: 1× Red Taq buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP, 0.067 μmol/L each primer, 0.5 U of Red Taq polymerase (Sigma-Aldrich), and 0.1 ng of DNA. Amplification conditions were as follows: 94 °C for 5 min followed by 35 cycles of 1 min at 94 °C, 1 min 30 s at 50 °C, and 1 min 30 s at 72 °C, ending with a 15 min extension at 72 °C. Amplified product was separated on a 1% agarose in 0.5× TBE gel, stained with ethidium bromide, and photographed. Plasmid DNA was extracted by standard alkaline lysis from 2 positive colonies deriving from each of the genotypes 'Fiesta' and 'J19'. The purified plasmid DNA products were sequenced from both T7 and SP6 ends by Genome Express (Meylan, France). The complete sequences deriving from 'Fiesta' and 'J19' clones were 99% identical. Two specific primers were designed from the 'J19' sequence using the software Primer3 (http://frodo.wi.mit.edu/primer3/). The forward and reverse primers for SCAR marker GE-8019 were purchased from Sigma Aldrich.

Mapping of SCAR markers

PCR with SCAR primers was performed as described by Liebhard et al. (2002) for SSRs in 15 μL volumes in a PerkinElmer GeneAmp 9600 PCR System. Both SCAR markers, AE10-375 and GE-8019, were screened over 251 F × D-CH progeny plants. Amplified products were run on a 1% agarose in 0.5× TBE gel, stained with ethidium bromide, and photographed. The presence or absence of bands was scored and data were added to the data file of Liebhard et al. (2003). Map position was calculated using the software JoinMap3.0 (Van Ooijen and Voorrips 2001).

Table 1. Sequence (5′–3′) of primers degenerate at either the 5′ or the 3′ end containing microsatellite motifs that were used to search for microsatellite repeats in 'Florina' BAC clone 59M8.

Primer name	Sequence (5′–3′) of primer
CT	CTCTCTCTCTCTCTWWT
GT	GTGTGTGTGTGTGTYC
ACA	BDBACAACAACAACAACA
CAA	CAACAACAACAACAA
CAG	CAGCAGCAGCAGCAG
CCA	DDCCACCACCACCACCA
CGA	DHBCGACGACGACGACGA
GATA	VSSGATAGATAGATAGATAGATA

Development and mapping of region-specific SSR

Two different subsets of the 'Florina' bacterial artificial chromosome (BAC) library (Vinatzer et al. 1998) filters (12 288 clones for each set) were screened using the primers for AE10-375 and GE-8019 as probes to "fish out" BAC clones containing AE10-375 and GE-8019 sequences. Preparation of the probes, hybridization, washing of the filters, and exposure were performed as described by Patocchi et al. (1999*a*). Extraction of BAC plasmids of positive clones was as described by Patocchi et al. (1999*b*). Extracts were diluted to 1:100 and used as template in PCR using primers with microsatellite motifs anchored at either the 3′ or the 5′ end in all combinations (Table 1). The same PCR conditions were used as described previously for amplification of SSRs. PCR products were run on a 1% agarose in 0.5× TBE gel, and amplicons were excised and purified using the Promega gel cleanup system, following the manufacturer's protocol, gel quantified, and used for direct sequencing with the corresponding degenerate primers. Sequences were compared with GenBank sequences for homology using the BLAST program (NCBI, Bethesda, Maryland). In cases where no microsatellite repeats were found by direct sequencing of PCR product, specific primers were designed and purchased from Microsynth AG for sequencing directly from the BAC plasmid. Once SSR motifs were identified, primers were designed based on the sequences flanking the repeat using Primer3 (Rozen and Skaletsky 2000) and purchased from Microsynth AG. SSR reactions were performed and scored as described in Silfverberg-Dilworth et al. (2006) and SSR markers were mapped as described for SCAR markers.

Pedigree analysis of 'Fiesta' with SCAR and SSR markers

The known pedigree of 'Fiesta' is 'Cox's Orange Pippin' ('Ribston Pippin' × O.P.) × 'Idared' ('Jonathan' × 'Wagener') (see Fig. 2) (Götz and Silbereisen 1989; Bošković and Tobutt 1999). These cultivars (except 'Wagener' and

the unknown parent of 'Cox's Orange Pippin') were genotyped with the 2 SCAR markers, AE10-375 and GE-8019, and with 2 SSR markers, MS06c09 and Hi05b09, located in the neighborhood of the F7 QTL on linkage group 7 (Maliepaard et al. 1998; Silfverberg-Dilworth et al. 2006) to follow the inheritance of the QTL across 3 generations.

Validation of F7 QTL

Validation of F7 QTL in a different genetic background

AE10-375 and GE-8019 were tested on the entire population of 'Milwa' × '1217'. Subsequently, 50 progeny plants of 'Milwa' × '1217' were selected — 25 plants positive for both markers and 25 plants without the markers. Six replications for each of the 50 progeny plants of 'Milwa' × '1217' were chip-grafted on virus-free M.9 T337 rootstocks. Plants were grown in the greenhouse facility for 45 days and then moved to the quarantine greenhouse at ACW. Inoculation was performed using the reference strain of *E. amylovora* CFBP 1430. Plants with a minimum shoot length of 9.0 cm were inoculated as described in Khan et al. (2006). Lesion length (cm) was measured at 3 time points after inoculation (i.e., 6, 14, and 21 days after inoculation [DAI]).

Validation of F7 QTL through cultivar analysis

For further validation of the F7 QTL, cultivars were selected based on pedigree information, i.e., with 'Cox's Orange Pippin' in the pedigree, as this had been determined to be the source of the F7 QTL. The AE10-375, GE-8019, and CH-F7-Fb1 markers were tested on 31 cultivars. Grafting, tree growth, inoculation, and disease scoring conditions were as described above for 'Milwa' × '1217' plants.

Statistical analysis

Statistical analysis was performed using SYSTAT software (SPSS Inc. 2000). Percent lesion length (PLL) was calculated by dividing the lesion length (cm) at each time point (i.e., 6, 14, and 21 DAI) by the shoot length (cm; measured at 6 DAI) (Norelli et al. 1984); PLL at 6, 14, and 21 DAI is referred to as PLL1, PLL2, and PLL3 following Khan et al. (2006). Data were checked for normal distribution, and outliers among replications of each plant were detected using Grubbs' test (Grubbs 1969). Mean and standard deviation of PLL were calculated for each progeny and cultivar separately at each time point. Progenies of 'Milwa' × '1217' were grouped based on the presence or absence of both AE10-375 and GE-8019 for average PLL1, PLL2, and PLL3 separately, and analysis of variance was performed between the groups.

Results

Development and mapping of markers

Development of SCAR markers

Two dominant SCAR markers have been developed from the 2 RAPD markers AE10-400 and GE80-19-0550. The exact size of the amplification product of RAPD marker AE10-400 was calculated from the sequence as 375 bp and accordingly the SCAR marker was named AE10-375. AE10-375 showed a very clear polymorphism between 'Fiesta' and 'Discovery' characterized by the presence and absence of the fragment, respectively.

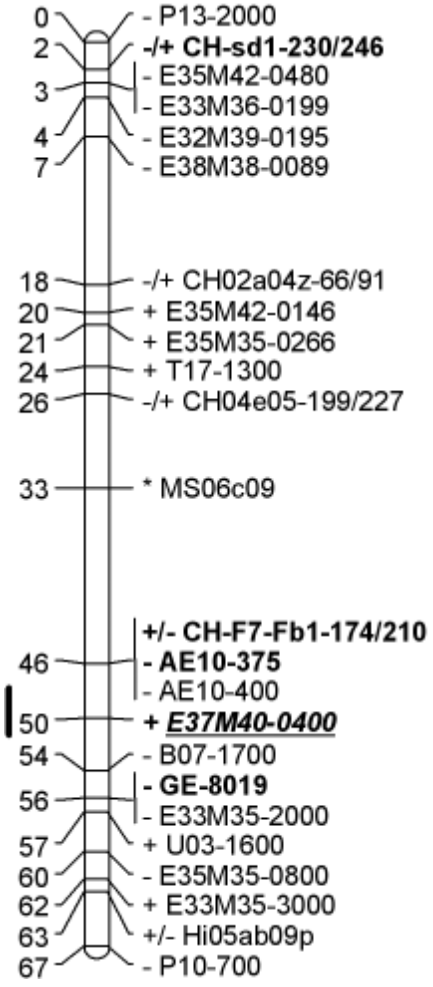


Fig. 1. Linkage map of 'Fiesta' for chromosome 7. Newly developed SCAR markers, AE10-375 and GE-8019, and 2 SSR markers, CH-F7-Fb1 and CH-Sd1, are shown in bold. The marker at the peak of the F7 QTL (E37M40-0400) is shown in underlined bold italic and the black bar indicates the 2-LOD support interval for the position of the F7 QTL (Khan et al. 2006). Linkage phase information is provided for markers as – or +, or both. *The position of MS06c09 is approximate, estimated after Calenge et al. (2005).

The exact size of the amplification product of SCAR marker GE-8019 was calculated from the sequence as 397 bp. When the primer for GE-8019 markers was tested on 'Fiesta' and 'Discovery', it amplified a product of the expected size from the first cultivar; however, the same amplicon was also obtained (faint band) from the second cultivar. This enabled the discarding of reactions in which PCR had failed, i.e., where there was no band.

Mapping of SCAR markers

AE10-375 mapped to the same position as RAPD marker AE10-400 (Fig. 1), 4 cM from the marker at the F7 QTL peak (E37M40-400). By scoring stronger and weaker amplification intensity as presence and absence of the band, respectively, it was possible to map GE-8019 in the Liebhard et al. (2003) cross (F × D-CH). Marker GE-8019 mapped at

Table 2. Primer sequence, annealing temperature (°C), and product size of the developed molecular markers linked to the F7 QTL and associated with the *Sd*1 locus.

Molecular marker	Sequence (5′–3′) of primers	Annealing temperature (°C)	Product size (bp)
AE10-375	CTGAAGCGCACGTTCTCC CTGAAGCGCATCATTTCTGATAG	60	375
GE-8019	TTGAGACCGATTTTCGTGTG TCTCTCCCAGAGCTTCATTGT	60	397
CH-F7-Fb1	AGCCAGATCACATGTTTTCATC ACAACGGCCACCAGTTTATC	60	174[a,b], 210[a]
CH-Sd1	TGCGTATCCAACTCATTCTCC GCCATAAAGGAGGTCGAATTTAC	63	230[a], 242[b], 246[a], 256[b]

[a]Alleles of 'Fiesta'.
[b]Alleles of 'Discovery'.

6 cM from the AFLP marker E37M40-400 (Fig. 1). Thus, the SCAR markers AE10-375 and GE-8019 bracket the F7 QTL, 10 cM apart from each other and 4 and 6 cM from the AFLP marker E37M40-400, respectively.

Development and mapping of SSR associated with SCAR markers

Radioactively labeled probes for both AE10-375 and GE-8019 were used to identify BAC clones of a 'Florina' BAC library carrying these markers. The AE10-375 probe hybridized to BAC clone 59M8, while the GE-8019 probe hybridized to hundreds of clones, indicating the presence of a highly repetitive sequence. We therefore decided not to develop an SSR from the region of SCAR marker GE-8019. PCRs with primers containing SSRs anchored at the 5′ or 3′ ends in all possible combinations and with the plasmid of BAC clone 59M8 as template allowed identification of 2 primer combinations, each generating 2 amplicons. The first combination of primers (primers ACA and CCA) produced 2 amplification products of approximately 2.5 kb and 500 bp. Sequencing of the 500 bp amplicon allowed the identification of a composed $(A)_{13}$ and $(AT)_{15}$ repeat. Primers were designed on the flanking sequence of this repeat. The SSR marker was named CH-F7-Fb1. CH-F7-Fb1 was polymorphic between 'Fiesta' and 'Discovery'. 'Fiesta' yielded amplicons of 210 bp and 174 bp, while 'Discovery' yielded only the 174 bp amplicon (Table 2). This polymorphism (amplification of the 210 bp amplicon) was also easily scorable on an agarose gel. Marker CH-F7-Fb1 was mapped in the F × D-CH cross and was found to share the position of SCAR marker AE10-375.

The primer combination CAA and CCA amplified 2 bands, one approximately 2.0 kb and the other 500 bp. The 2.0 kb band was sequenced using primer CCA. Specific primers were designed and used to further extend the sequence by sequencing the BAC clone directly. The sequence obtained with these primers was compared with the sequence data in GenBank. It showed 95% and 94% identity with 2 different parts of the sequence of a 'Florina' BAC clone (49N23) from *Malus* linkage group 7 associated with the aphid resistance locus *Sd*-1 (Cevik and King 2002). Six different microsatellites were found in the sequence of 'Florina' BAC clone 49N23. Primers flanking the microsatellites were designed and used to test for polymorphism between 'Fiesta' and 'Discovery'. The SSR named CH-Sd1, containing 12 CT repeats (Table 2), showed polymorphism between 'Fiesta' and 'Discovery'. The primers for this SSR amplified 2 alleles for each of 'Fiesta' and 'Discovery': 230 and 246 bp and 242 and 256 bp, respectively. Marker CH-Sd1 was mapped in the F × D-CH cross at the top of linkage group 7 of 'Fiesta' (Fig. 1).

Pedigree analysis of 'Fiesta' and specificity of the developed markers

SCAR marker GE-8019 and allele '144' for SSR Hi05b09 were traced from 'Fiesta' to'Ribston Pippin' (Fig. 2). However, SCAR marker AE10-375 was present in both of the maternal grandparents (i.e., 'Ribston Pippin' and 'Jonathan') of 'Fiesta'. SSR MS06c09 was mapped in the cross 'Prima' × 'Fiesta' 13 cM above GE-8019 (Calenge et al. 2005). The same genotype (108-116) was scored for this SSR for both 'Cox's Orange Pippin' and 'Idared'. Since the genotype of the father of 'Cox's Orange Pippin' is unknown, it is not possible to infer a single solid solution for the 2 phases of 'Cox's Orange Pippin'. This means that the favorable F7 QTL allele carried by 'Cox's Orange Pippin' could derive from either 'Ribston Pippin' or the unknown father of 'Cox's Orange Pippin'.

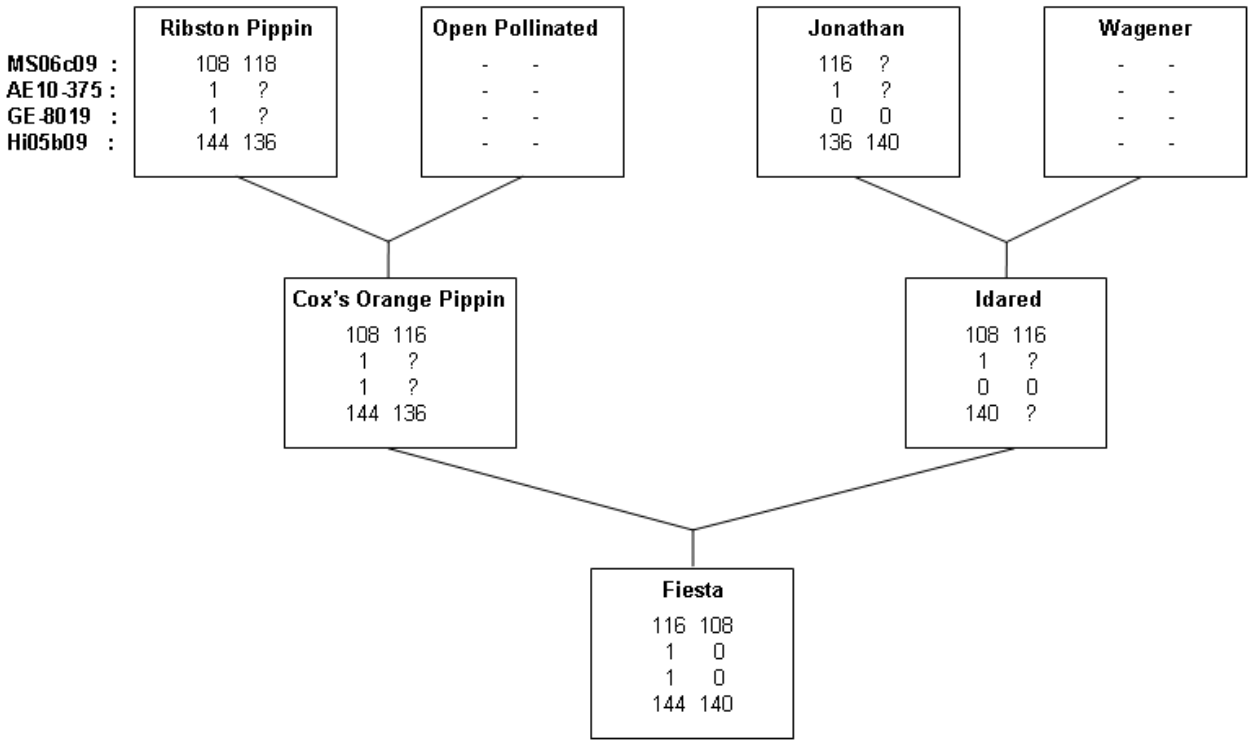
SCAR markers AE10-375 and GE-8019 and SSR marker CH-F7-Fb1 were screened over 31 cultivars (Table 3). Marker analysis showed that 12 cultivars were positive for GE-8019 and all of them have 'Cox's Orange Pippin' in their pedigree, pointing to high specificity of GE-8019 for the QTL allele inherited from 'Cox's Orange Pippin'. All plants tested that carried SCAR marker AE10-375 also carried allele 210 bp of CH-F7-Fb1. SCAR marker AE10-375 and allele 210 bp of CH-F7-Fb1 are not specific for the QTL allele inherited from 'Cox's Orange Pippin', as 11 cultivars carrying AE10-375 as well as allele 210 bp of CH-F7-Fb1 do not have 'Cox's Orange Pippin' in their pedigree (Table 3). When CH-F7-Fb1 was screened over the 31 cultivars, a total of 8 alleles were amplified, with sizes of 168, 174, 180, 184, 186, 207, 210, and 218 bp.

Validation of F7 QTL

Validation of F7 QTL using 'Milwa' × '1217' progeny

Parent '1217', a progeny of the F × D-CH cross, has both SCAR markers (AE10-375 and GE-8019), while 'Milwa' does not have the markers. Symptoms of fire blight infection

Fig. 2. Pedigree of the cultivar 'Fiesta' with genotypes of parents and grandparents for SCAR markers AE10-375 and GE-8019 and for SSR markers MS06c09 and Hi05b09.



at each of 3 time points (PLL1, PLL2, PLL3) were much higher in 'Milwa' than in '1217' (Fig. 3). Of 25 progeny plants of 'Milwa' × '1217' with AE10-375 and GE-8019 markers and 25 progeny plants without these markers, 22 and 21 progeny plants, respectively, produced sufficient plant material for inoculation. Progeny plants with both SCAR markers had significantly ($p < 1 \times 10^{-11}$) lower fire blight infection at each of the 3 time points than those that did not have both markers (Fig. 3). In successive analyses of variance, the presence/absence of both SCAR markers accounted for 34% to 50% of the total phenotypic variation (R^2) at the 3 time points. Parent '1217' showed average fire blight infection values similar to those of the progeny plants lacking both SCAR markers, but mean PLL values for both '1217' and 'Milwa' were based on data from only 3 replications and thus were not very accurate.

Phenotypic and genotypic analysis of cultivars

Of 31 cultivars screened, the most resistant cultivars were 'Reanda' (PLL3 7% ± 4%) and 'Remo' (8% ± 6%), while the most susceptible cultivars were 'Durello di Forli' (100% ± 0%), 'Idared' (100% ± 0%), 'Otava' (100% ± 0%), and 'Elstar' (100% ± 0%) (Table 3). Eleven cultivars were predicted to carry the F7 QTL, since they were positive for both AE10-375 and GE-8019, although the presence of both markers is not definite proof of the presence of the F7 QTL because AE10-375 was also present in a number of cultivars unrelated to 'Fiesta'. In some of these 11 cultivars that carry both markers, a crossing-over could have occurred between GE-8019 and the F7 QTL in the GE-8019 donor parent, while AE10-375 could derive from the alternative parent. Twenty cultivars were predicted not to carry the F7 QTL. Eight cultivars did not carry either of the SCAR markers, 11 cultivars had only AE10-375, and 1 cultivar ('Elstar') carried only GE-8019 (Table 3). Again, crossing-over may be responsible for false predictions of the absence of the F7 QTL.

The average PLL3 of the 11 cultivars expected to carry the F7 QTL (35% ± 20%) was significantly ($p = 0.040$) lower than that of the 20 cultivars not expected to carry the F7 QTL (64% ± 29%). The percentage of total phenotypic variation explained by the presence of both SCAR markers was approximately 41%. Of 14 cultivars having 'Cox's Orange Pippin' in their pedigree, the 11 cultivars carrying both SCAR markers had significantly ($p = 0.044$) lower fire blight infection than the 2 cultivars, 'Otava' (100% ± 0%) and 'Santana' (72% ± 37%), not carrying the markers (Fig. 4). 'Elstar' was very susceptible (100% ± 0%) despite the presence of marker GE-8019 (but the absence of AE10-375 suggests a putative recombination between GE-8019 and the F7 QTL).

Discussion

Development of markers

Two SCAR markers and one SSR marker flanking the F7 QTL were successfully developed and mapped. The RAPD markers AE10-400 and GE80-19-0550 were converted into SCAR markers AE10-375 and GE-8019, respectively. The F7 QTL peak is located between the 2 SCAR markers, which have been mapped 10 cM from each other (Fig. 1). The region-specific SSR (CH-F7-Fb1) developed from the sequence of 'Florina' BAC clone 59M8 is not highly polymorphic, as primers for this SSR amplified only 2 alleles in the F × D-CH population. Moreover, primers for CH-F7-Fb1 identified only 8 alleles in 31 cultivars screened, with some

Table 3. Ranking of cultivars in descending order of fire blight resistance based on percent lesion length 3 weeks after inoculation (PLL3).

Cultivar	PLL3	SD	*n*	CH-F7-Fb1	AE10-375	GE-8019
Reanda*	6.7	4.0	5	1	1	1
Remo*	7.6	6.1	4	1	1	1
Priscilla	11.2	7.3	4	0	0	0
Kidd's Orange Red*	14.9	11.3	6	1	1	1
Novaeasygro	17.8	16.0	4	1	1	0
Starking Delicious	23.0	8.0	5	1	1	0
Rubinola*	31.2	24.2	6	1	1	1
James Grieve*	31.3	4.3	4	1	1	1
Fiesta*	34.6	21.7	6	1	1	1
Cox's Orange Pippin*	35.3	26.4	4	1	1	1
Rubinette*	40.7	32.5	6	1	1	1
Florina	41.1	31.7	6	1	1	0
Prima	41.9	13.9	2	1	1	0
Belle de Boskoop	43.1	19.6	6	1	1	0
Pinova*	48.0	27.8	5	1	1	1
Granny Smith	52.6	10.6	2	1	1	0
Reinette Grise du Canada	52.8	43.1	3	1	1	0
Topaz*	56.9	23.6	5	1	1	1
McIntosh	57.6	33.7	6	0	0	0
Fuji	61.8	20.6	2	1	1	0
Santana*	71.8	36.7	4	0	0	0
Golden Delicious	73.6	36.0	4	1	1	0
Reglindis*	74.1	22.8	5	1	1	1
Braeburn	74.9	20.7	3		0	0
Gloster 69	78.1	21.8	6	1	1	0
Reine des Reinettes	78.4	21.4	6	0	0	0
Durello di Forli	100.0	0.0	5	0	0	0
Idared	100.0	0.0	5	0	0	0
Otava*	100.0	0.0	4	0	0	0
Jonagold	100.0	0.0	2	1	1	0
Elstar*	100.0	0.0	4	0	0	1
1217	23.4	19.9	3	1	1	1
Milwa	72.8	38.2	3	0	0	0

Note: *n*, number of replications. 1 indicates amplification and 0 indicates no amplification for AE10-375, GE-8019, and CH-F7-Fb1 allele 210 bp in coupling with the F7 QTL.

*Cultivars with 'Cox's Orange Pippin' in their pedigree.

cultivars, such as 'Cox's Orange Pippin' and 'Rubinette', carrying 4 (174, 184, 207, and 210) and 3 alleles (168, 180, and 210 bp), respectively. This indicates that CH-F7-Fb1 is a multi-locus SSR; the second locus was not mapped because it did not segregate in the 'Fiesta' × 'Discovery' population. Liebhard et al. (2002, 2003) mapped 2 SSRs, CH02a04z and CH04e05, on linkage group 7 of 'Fiesta' and 'Discovery'. However, Silfverberg-Dilworth et al. (2006) could map only a single SSR marker (Hi05b09) in the distal part of linkage group 7 (Fig. 1). This may be an indication of reduced heterozygosity in this region among the homologous chromosomes of both 'Fiesta' and 'Discovery'.

There was high sequence identity (94% and 95%) between the sequence (500 bp) of an amplicon of 'Florina' BAC clone 59M8 found to be derived from the distal part of linkage group 7 and 2 different parts of a sequence of 'Florina' BAC clone 49N23 from the region of the *Sd*-1 locus (Cevik and King 2002). SSR CH-Sd1 was developed from the sequence of 'Florina' BAC clone 49N23, enabling mapping of the *Sd*-1 locus in the proximal region of linkage group 7 of 'Fiesta' on the apple reference map (Fig. 1). This indicated the presence of a repeated sequence on the top and bottom parts of linkage group 7. Mapping of SSR CH-Sd1 added another common marker in our F × D-CH linkage group 7, which, along with other common markers, will help to align different *Malus* linkage group 7 maps.

Comparison between fire blight resistance QTLs

Mapping of the SCAR marker GE-8019 derived from the RAPD marker of Calenge et al. (2005) on the F × D-CH map allowed comparison of the position of the F7 QTL identified by Khan et al. (2006) and the major fire blight resistance QTL identified by Calenge et al. (2005). GE-8019 was mapped close (6 cM) to the marker at the peak of the F7 QTL (E37M40-0400), which indicates that the fire blight resistance QTLs identified in both studies are probably the same. We therefore name this fire blight resistance locus identified first in 'Fiesta' on linkage group 7 as "FBF7".

Stability of the FBF7 QTL in different genetic backgrounds

The pedigree analysis of 'Fiesta' with markers AE10-375 and GE-8019 bracketing the FBF7 QTL enabled tracing of the QTL back to 'Cox's Orange Pippin'. The SCAR markers

Fig. 3. Fire blight severity assessment for 'Milwa' × '1217' progeny plants inoculated with the reference strain *E. amylovora* CFBP1430. Percent lesion length (PLL) was assessed 1 (PLL1), 2 (PLL2), and 3 weeks (PLL3) after inoculation. Progenies and parents ('1217' and 'Milwa') are divided into 2 groups according to the presence or absence of both AE10-375 and GE-8019. Error bars represent the standard deviation. Mean PLL values for 'Milwa' and '1217' are based on data from 3 replications.

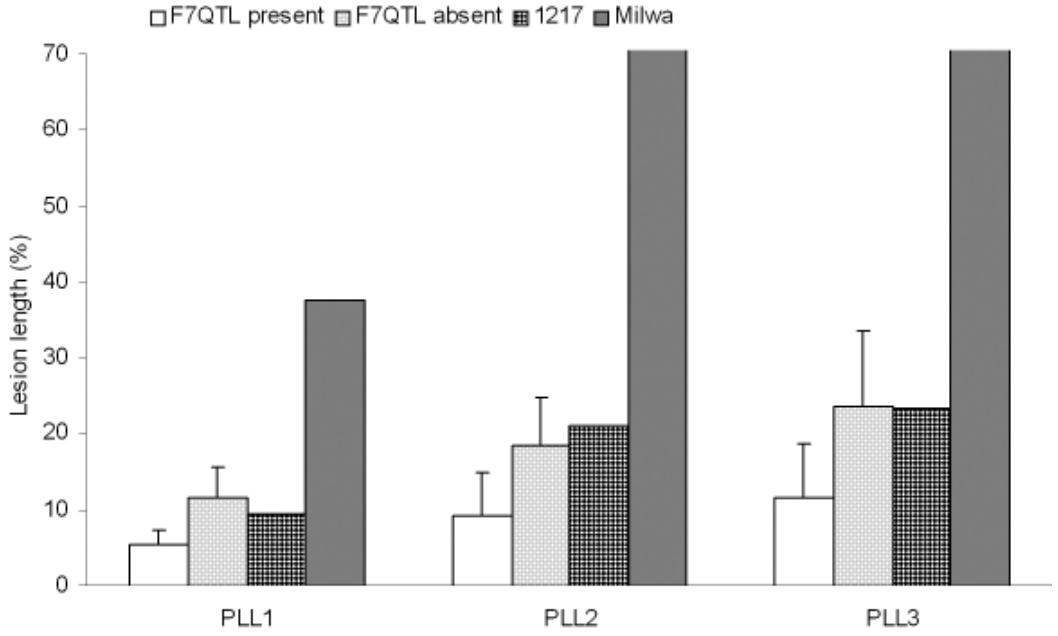
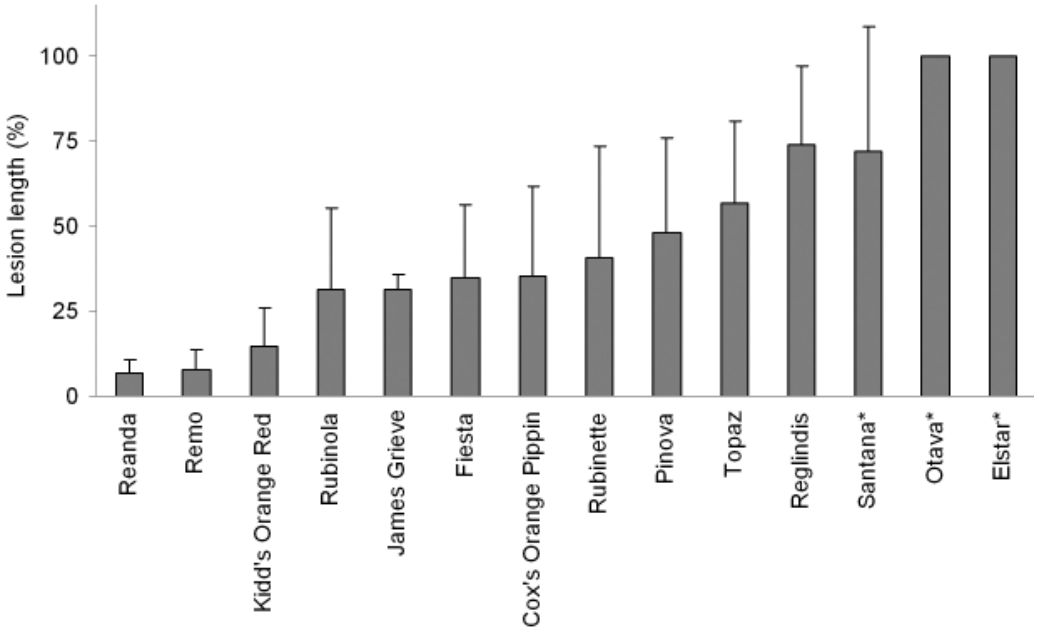


Fig. 4. Fire blight severity assessment for apple cultivars with 'Cox's Orange Pippin' in their pedigree, inoculated with the reference strain of *E. amylovora* CFBP 1430. PLL3 is shown on the *y*-axis and error bars represent the standard deviation. *Cultivars that do not yield amplification products for SCAR marker alleles associated with the fire blight resistance locus FBF7.



also enabled postulation of the presence of the QTL in the 14 cultivars known to have 'Cox's Orange Pippin' in their pedigree. The significantly higher level of fire blight resistance in the 11 cultivars expected to carry the favorable FBF7 QTL allele cannot be attributed solely to the presence of this QTL. Indeed, the set of cultivars used in the present study may not be representative of all the cultivars deriving (or not) from 'Cox's Orange Pippin'. Additional resistance factors probably segregate within the cultivars involved and may have resulted in over- or underestimation of the effect of the FBF7 QTL in the set of cultivars tested here. 'Priscilla', 'Novaeasygro', and 'Starking Delicious' are some examples of cultivars missing or probably missing the favorable FBF7 QTL allele but exhibiting high fire blight resistance (Table 3). Nevertheless, despite a limited, possibly biased, set of cultivars, a significant effect of the pres-

ence/absence of both SCAR markers was detected, indicating a probable stable effect of the QTL in different genetic backgrounds.

The average fire blight resistance of the cultivars having 'Cox's Orange Pippin' in their pedigree and carrying both SCAR markers was statistically ($p = 0.044$) higher than that of the 2 cultivars, 'Otava' and 'Santana', that also have 'Cox's Orange Pippin' in their pedigree but do not carry the markers. During the breeding process, 'Otava' and 'Santana' or one of their ancestors did not inherit the allele of FBF7 QTL conferring increased resistance to fire blight. 'Elstar' has 'Cox's Orange Pippin' in its pedigree and also carries GE-8019 but lacks AE10-375. Moreover, it is highly susceptible (100% ± 0%) to fire blight. This indicates that 'Elstar' might have lost the FBF7 QTL owing to a recombination event between GE-8019 and the QTL.

Our results also showed that the progeny plants of 'Milwa' × '1217', which carried both SCAR markers, had significantly ($p < 1 \times 10^{-11}$) lower fire blight infection at 3 time points (PLL1, PLL2, PLL3) than those that did not carry both markers. Interestingly, the R^2 value associated with the presence/absence of both SCAR markers (i.e., presence/absence of the favorable QTL allele) was very similar to the R^2 values obtained for the same QTL by Calenge et al. (2005) and Khan et al. (2006). The FBF7 QTL has now been identified in 3 different genetic backgrounds, namely, 'Fiesta' × 'Discovery' (Calenge et al. 2005; Khan et al. 2006), 'Prima' × 'Fiesta' (Calenge et al. 2005), and 'Milwa' × '1217'. This indicates the high stability of the QTL effect in different backgrounds.

Marker-assisted selection

In our study, greenhouse inoculations allowed identification of cultivars 'Reanda', 'Remo', 'Priscilla', 'Kidd's Orange Red', 'Novaeasygro', 'Starking Delicious', 'Rubinola', 'James Grieve', 'Fiesta', and 'Cox's Orange Pippin' as possible sources of fire blight resistance. It is possible to select tolerant cultivars using phenotypic results; however, selection within progeny based entirely on phenotypic results is labor-intensive and time-consuming, since several replicates are necessary to properly evaluate the fire blight resistance of genotypes and appropriate quarantine facilities are required in many regions. Consequently, early phenotypic selection is rarely used for fire blight resistance in current apple breeding programs worldwide (Laurens 1999). Among the 10 most resistant cultivars in this study, 7 have 'Cox's Orange Pippin' in their pedigree and carried both SCAR markers (Table 3), which indicates the putatively strong effect of this allele of the FBF7 QTL. The probability of double recombination events between SCAR markers and the FBF7 QTL is extremely unlikely (2.4×10^{-3}), which increases the reproducibility and effectiveness of the FBF7 QTL for MAS. Co-segregation of allele 210 bp of CH-F7-Fb1 and AE10-375 suggests that either of the markers could be used in combination with GE-8019, depending on the availability of facilities. Moreover, the presence of SSRs CH-F7-Fb1 and Hi05b09, which are approximately 17 cM from each other and 4 and 13 cM from the QTL peak, respectively, provide another way to select for the FBF7 QTL. The choice between SSRs (CH-F7-Fb1 and Hi05b09) and SCAR markers (AE10-375 and GE-8019) depends on the availability of facilities and which markers are polymorphic between the parents in the breeding programme, but in choosing to use SSRs one should also consider the higher chance (5.2×10^{-3}) of losing the FBF7 QTL owing to the broader marker bracket and low level of polymorphism of both SSRs.

Conclusion

Consistent, explained phenotypic variation (30%–50%), stability across different genetic backgrounds, and availability of reproducible markers bracketing the FBF7 QTL make this locus promising for use in MAS. In our study, possible fire blight resistant cultivars were identified. We suggest selecting cultivars that have 'Cox's Orange Pippin' in the pedigree and confirming the presence of the FBF7 QTL with the 2 flanking SCAR markers (AE10-375 and GE-8019). One should use both SCAR markers to maximize the chance of selecting seedlings that do have increased fire blight resistance. Implementation of MAS could lead to improved resistance to fire blight in apples through plant breeding. For more reliable and accurate MAS, it will be important to test the levels of resistance or susceptibility in both the greenhouse and the field and then compare these results with marker as well as pedigree information.

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