

Advanced strategies for breeding fire blight resistant high quality apples

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Abstract

Fire blight caused by *Erwinia amylovora* is a major disease in apple production. Marker-assisted selection (MAS) is applied at the Research Station Agroscope Changins-Wädenswil (ACW) to develop new fire blight tolerant apple varieties with marketable fruit quality. The selection of varieties that have a tolerance to this disease is especially desirable in organic fruit growing. MAS facilitates the early selection of parents for further crosses and of novel cultivars carrying not only markers for fire blight tolerance, but also for resistances towards apple scab and powdery mildew. Selected progeny plants of 'Milwa' x 'Enterprise' and 'FAW 8099' x 'Enterprise' were tested for the presence of the molecular markers AE10-375 and GE-8019, flanking a quantitative trait locus (QTL) for enhanced fire blight resistance identified in the apple cultivar 'Fiesta'. In order to validate the MAS results for fire blight resistance, selected plants were subsequently inoculated with *E. amylovora* in the quarantine glasshouse for shoot lesion length evaluation. Offspring of the cross 'Milwa' x 'Enterprise' carrying the markers flanking the resistance QTL were on average significantly less susceptible than offspring lacking the markers. This supports the utility of these markers in MAS.

Keywords: apple breeding, fire blight, marker-assisted selection, multiple disease resistance

Introduction

Fire blight, caused by the bacterium *Erwinia amylovora*, is a major disease in apple production. The bacteria infect blossoms, fruits, vegetative shoots, woody tissues, and rootstock crowns in several species of the *Rosaceae* family. Only few tools are available to control the disease, which has made it difficult to stop or slow the progress of fire blight epidemics (Norelli *et al.*, 2003). Fire blight management strategies focus on the reduction of inoculum in the orchard and the use of antimicrobial treatments to prevent infection. However, the application of antibiotic is not a desired and sustainable control method and is not applicable to organic fruit growing (Kellerhals *et al.*, 2009a). In contrast, the strategy of reducing the host's susceptibility to fire blight infection is especially valuable for organic fruit growing and fruit growing in general.

The selection of new fire blight tolerant apple varieties with good agronomic performance and multiple disease resistance is a current objective of apple breeding at ACW. The most advanced and promising breeding material and commercial varieties, old varieties or wild species, with known low susceptibility towards fire blight and/or other desired characteristics are used as parents. Breeding strategies include classical and molecular selection techniques and are continuously adapted to new methods and technologies. Marker-assisted selection (MAS) is an established procedure in apple breeding to confirm the presence of the desired genes (Kellerhals *et al.*, 2009b). It also allows an early and

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facilitated selection of seedlings and parents for further crosses. To achieve multiple disease resistance not only towards fire blight, but also towards apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*), different resistances can be combined in one genotype by performing crosses. By the application of molecular markers seedlings with the desired compositions of alleles can be selected. Some of these traits are determined by major genes such as scab (*Rvi6*, *Rvi2*, *Rvi4*, *Rvi11* respectively *Vf*, *Vh2*, *Vh4*, *Vbj* and other scab resistance genes) (Gessler *et al.*, 2006; Patocchi *et al.*, 2009) and mildew (*PI1*, *PI2*, *PI4* and *PIw* mildew resistance genes) (Markussen *et al.*, 1995; Seglias & Gessler, 1997; James & Evans, 2004). For resistance to fire blight no major, qualitative gene has been identified to date (Durel *et al.*, 2009). The tolerance to fire blight is mainly based on QTLs. In large germplasm evaluations many sources of resistance against this disease have been identified. Nevertheless only few genetic markers are available and can be applied in MAS for fire blight tolerance. A major QTL for fire blight resistance, identified in the cultivar 'Fiesta', is responsible for approximately 35-40% of the phenotypic variation (Calenge *et al.*, 2005; Kahn *et al.*, 2006). This QTL maps on linkage group 7 and is flanked by the SCAR (sequence-characterized amplified region) markers AE10-375 and GE-8019 (Kahn *et al.*, 2006; Kahn *et al.*, 2007). The FBF7 QTL traces back to 'Cox's Orange Pippin' (Kahn *et al.*, 2007). Due to its broad-sense heritability (Calenge *et al.*, 2005; Kahn *et al.*, 2006) and the presence in commercial cultivars with large fruit and acceptable quality it can be readily transferred into new apple cultivars. A further major QTL was identified in 'Robusta 5' (Peil *et al.*, 2007a; Peil *et al.*, 2007b; Peil *et al.*, 2008). This strong QTL explained 67%-83% of the phenotypic variation, and in each family tested about 20-25% of the seedlings remained disease-free (Peil *et al.*, 2008). Another strong QTL explaining 50-70% of phenotypic variation was identified in 'Evereste' (Durel *et al.*, 2009). The QTLs from 'Robusta 5' and 'Evereste' in contrast to the QTL from 'Fiesta' confer (near) immunity but have been found in small-fruited crab apples with low fruit quality. Therefore, they would require several generations of backcrossing before a new commercial cultivar carrying this resistance could be obtained.

Material and Methods

Plant material

Progeny 'Milwa' x 'Enterprise'

The cross 'Milwa' x 'Enterprise' was established in 2006. 'Enterprise' carries the scab resistance gene *Rvi6* (respectively *Vf*) and the markers flanking the FBF7 QTL. Branches of the mother trees were bagged and flowers manually pollinated with the pollen of the father tree at balloon stage and re-bagged until petal fall. Fruits resulting from the crosses were harvested in autumn and seeds extracted and subsequently stratified in humid sand at 2°C for 2 months. The seedlings were raised in a glasshouse, individually labelled and a leaf was collected from each seedling for molecular analyses.

Progeny 'FAW 8099' x 'Enterprise'

The cross 'FAW 8099' x 'Enterprise' was established in 2002. 'FAW 8099' carries the scab resistance gene *Rvi6* (respectively *Vf*) and the mildew resistance gene *PI2*. The seedlings (n=409) grown in 2003 were phenotypically scored in the glasshouse for scab symptoms after artificial inoculation according to Chevalier *et al.* (1991).

Marker analyses

MAS was applied at different stages to identify plants carrying the desired resistance genes. Young leaf samples were collected and DNA extraction was performed using the Qiagen Multiplex Kit (Qiagen, Basel, Switzerland) according to Frey *et al.* (2004). Subsequently, using the liquid handling robot epMotion 5075 (Eppendorf), multiplex PCR's

with fluorescently labelled primers were assembled according to Patocchi *et al.* (2009), using the primers indicated below. Fragment analysis was carried out on a 3130xl Genetic Analyzer (Applied Biosystems) and data analysis was done with GeneMapper™ Software (Applied Biosystems).

Progeny 'Milwa' x 'Enterprise'

The seedlings (n=251) raised in 2007 were genetically analysed for the microsatellite marker (simple sequence repeat, SSR) CHVf1 linked to the *Rvi6* scab resistance (Vinatzer *et al.*, 2004) and the two SCAR markers AE10-375 and GE-8019 (Kahn *et al.*, 2007). Twenty cultivars positive for CHVf1, 10 of which were positive for both SCAR markers and 10, which were negative for both SCAR markers, were subsequently selected for a greenhouse fire blight inoculation test in 2009.

Progeny 'FAW 8099' x 'Enterprise'

The seedlings were analysed for the SSR marker CHVf1 linked to the *Rvi6* scab resistance and the SSR marker CH04H02 linked to the *PI2* mildew resistance locus (Seglias *et al.*, 1997). In 2007 and 2008, the fruit quality of the plants carrying both markers was evaluated. Nine genotypes with encouraging fruit quality were selected for a greenhouse fire blight inoculation test in 2009 and for genetic analyses of the markers AE10-375 and GE-8019.

Inoculation test

The fire blight inoculation test was conducted in the quarantine glasshouse for the selected progeny plants 'Milwa' x 'Enterprise' in April 2009 and 'FAW 8099' x 'Enterprise' in June 2009. Scion wood was grafted on M9 rootstock. Twelve trees per selection were planted in plastic deep-pots 60 from Stuewe & Sons (Corvallis, US) with a length of 35.5 cm and diameter of 7 cm. 'Gala' was included as a susceptible control. 'Rewena' and 'Enterprise' were included as tolerant controls. Prior to inoculation the plants were grown in a greenhouse for four to five weeks where one shoot per plant was raised. For each selection, up to ten randomised replicate shoots, with a minimum length of 12 cm, were inoculated at the shoot tip with a syringe containing an *E. amylovora* solution of 10^9 cfu/ml strain FAW 610. The length of necrotic lesion (cm) was measured 1, 2 and 3 weeks after inoculation [WAI].

Statistical analysis

Statistical analysis was performed using SPSS. The susceptibility of the genotypes was estimated by calculating the percent lesion length (PLL = lesion length (cm) at each time point (i.e. 1, 2 and 3 WAI) divided by the shoot length (cm; measured at 1 WAI) in percent) (Norelli *et al.*, 1984). Data were checked for normal distribution, and outliers among replications of each genotype were detected using Grubb's test (Grubbs, 1969). As outliers could not be explained by error in measuring or other obvious reason they were not excluded in the following calculations. Mean and standard error of PLL were calculated for each time point. The group of progeny plants of 'Milwa' x 'Enterprise' carrying both AE-10-375 and GE-8019 were compared to the group not carrying the markers for PLL3 using a Mann-Whitney U-test to confirm a statistical difference between the two groups of plants.

Results

'Milwa' x 'Enterprise'

The genotypes of the cross 'Milwa' x 'Enterprise' were grouped on the basis of the presence or absence of the SCAR markers AE10-375 and GE-8019 (Figure 1). Figure 1 illustrates the variability within the groups and the overall higher tolerance of the group with the markers to fire blight. A Mann-Whitney U-test showed that the two groups differed significantly, $U = 17$, $N1/N2 = 10/10$, $Z = -2.495$, $P = 0.0055$ (1-tailed).

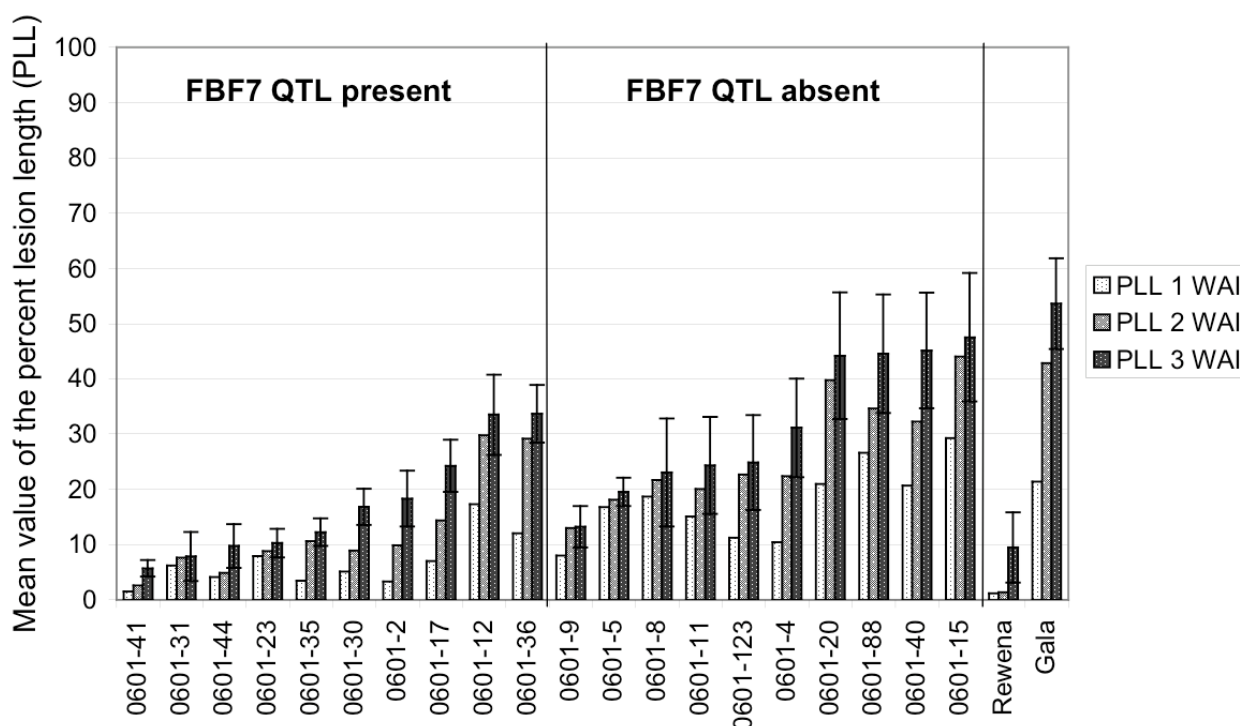


Figure 1: Mean percent lesion length (PLL) 1, 2 and 3 weeks after infection (WAI) for 'Milwa' x 'Enterprise' progeny plants. On the x-axis the genotypes are grouped for the presence and absence of the markers flanking the FBF7 QTL and in ascending order of fire blight susceptibility. 'Rewena' (tolerant) and 'Gala' (susceptible) were control cultivars. The bars indicate standard error.

'FAW 8099' x 'Enterprise'

Among the nine genotypes of the cross 'FAW 8099' x 'Enterprise' tested for the presence or absence of AE10-375 and GE-8019, four were carrying both markers, two only GE-8019 and three had none of them (Table 1). The selected genotypes of the cross 'FAW 8099' x 'Enterprise' for the fire blight test, based on molecular marker analyses, are predicted to carry genes for scab (*Vf*) and mildew resistance (*PI2*) from the markers CHVf1 and CH04H02 which makes them multiple disease resistant.

Table 1: Parents and progeny plants of the cross 'FAW 8099' x 'Enterprise' analysed with the SSR markers CHVf1 and CH04H02, and the SCAR markers AE-10-375 and GE-8019. + indicates desired amplification and - absence of the marker(s). Genotypes in bold have both markers AE-10-375 and GE-8019 and therefore are carrying the QTL for fire blight resistance.

Genotype	<i>Vf</i> CHVf1.	<i>PI2</i> CH04H02	AE10-375	GE-8019
FAW 8099	+	+	+	+
Enterprise	+	-	+	+
FAW 17041	+	+	-	+
FAW 17044	+	+	+	+
FAW 17045	+	+	-	-
FAW 17046	+	+	+	+
FAW 17053	+	+	+	+
FAW 17055	+	+	-	-
FAW 17057	+	+	-	+
FAW 17059	+	+	-	-
FAW 17061	+	+	+	+

Figure 2 illustrates the high level of variation between progeny plants. 'FAW 17044' and 'FAW 17041' demonstrated low susceptibility towards fire blight at a comparable level to the tolerant control 'Enterprise'. In contrast, 'FAW 17055' showed high levels of susceptibility towards fire blight comparable to the susceptible control 'Gala'.

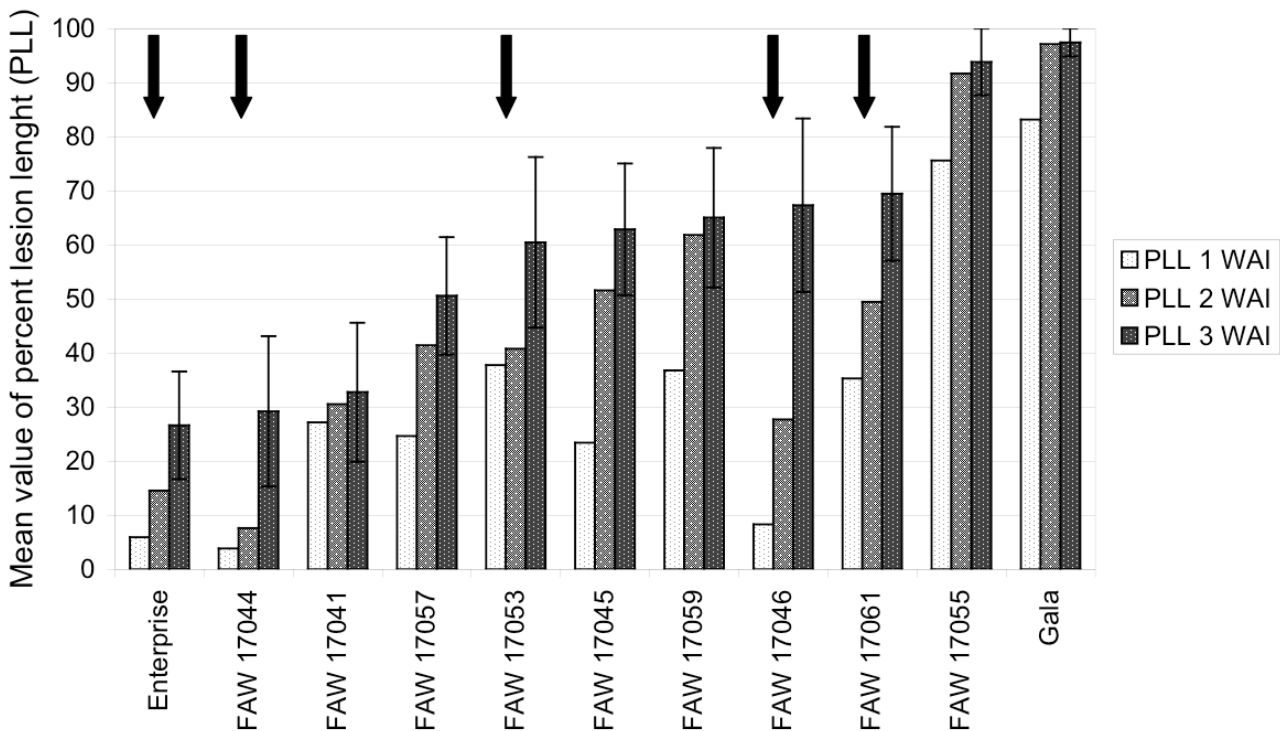


Figure 2: The mean value of percent lesion length (PLL) 1, 2 and 3 weeks after infection (WAI) for progeny plants of the cross 'FAW 8099' x 'Enterprise'. On the x-axis the genotypes and the control cultivars 'Enterprise' (tolerant) and 'Gala' (susceptible) were displayed in ascending order of fire blight susceptibility. The arrows indicate genotypes carrying both markers associated to the FBF7 QTL. 'FAW 17041' and 'FAW 17057' have only marker GE-8019, but are likely to carry the QTL for fire blight resistance. The bars indicate standard error.

Discussion

This study has shown that genotypes carrying the markers AE10-375 and GE-8019 have on average a higher tolerance against fire blight compared to genotypes not carrying the markers. Previous studies came to the same conclusion (Kahn *et al.*, 2007; Sehic *et al.*, 2009). The higher average tolerance against fire blight in genotypes carrying AE10-375 and GE-8019 supports their usefulness for MAS. An advantage of the FBL7 QTL is its presence in large-fruited cultivars with commercial fruit quality that can readily be used for breeding new varieties. However, it confers only a moderate, partial resistance (Calenge *et al.*, 2005; Kahn *et al.*, 2006; Kahn *et al.*, 2007).

The detection of fire blight resistant seedlings can be further improved by introducing additional resistance QTLs from other sources. One strategy is to introduce QTLs for high fire blight resistance from crab apples such as 'Robusta 5' and 'Evereste'. Due to the small fruit size and low fruit quality several generations of backcrossing are required before a new variety will be ready commercially.

Genetic markers can reduce the cost of breeding new varieties due to early selection. The number of plants and the time for evaluation can be reduced and varieties, even with combined resistances, can become commercially available sooner. Genotypes such as 'FAW 17044' with multiple resistance genes and low susceptibility to fire blight are interesting as breeding parents or potential cultivars. 'ACW 14959' and 'ACW 14995' are 2

advanced selections carrying the FBF7 resistance QTL towards fire blight and the Vf scab resistance. They originate from a cross of 'Topaz' x 'Fuji' and display good fire blight tolerance and interesting fruit quality features.

Fire blight resistance evaluations under controlled conditions have been shown to correlate well with field resistance (Quamme *et al.*, 1976). Many factors, such as host and environmental conditions, affect disease establishment and development (van der Zwet & Keil, 1979; van der Zwet & Beer, 1991; Brisset & Paulin, 2006). But even with inoculation conditions and techniques being as uniform as possible, the findings of fire blight resistance studies have proven to be variable to a certain extent. Variability in climatic conditions or growth stage of the shoots can influence the symptoms. Furthermore, shoot inoculation tests provide information about the shoot susceptibility but not the susceptibility of the floral organs. Flower and shoot resistances are often correlated, but there also are many occasions where this is not the case (Le Lezec *et al.*, 1987). Therefore, further testing under natural field conditions is required to confirm the susceptibility of new varieties to fire blight.

Acknowledgements

This research was supported by the Swiss Federal Office of Agriculture project 'ZUEFOS' (breeding of fire blight tolerant fruit varieties). The authors thank Rolf Blapp for the preparation of plant material, Claudia Good and Jürgen Krauss for technical support and Markus Kalisch and Lukas Rosinus (Seminar für Statistik, ETH Zürich) for valuable suggestions on statistical analysis and Fiona Walsh for proof-reading.

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