Breeding apples to withstand infection pressure by fire blight and other diseases

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Abstract

Commercial cultivars, old varieties and wild species with known resistance against fire blight, caused by the bacterium Erwinia amylovora*, were selected as breeding parents. Due to strong fire blight quantitative trait loci (QTLs), the crab apples 'Robusta 5' and 'Evereste' confer virtual immunity against the disease. However, these crab apples lack the characteristics of good fruit quality. In a multilevel phenotypic and molecular selection process, progeny plants were selected based on additional breeding objectives such as agronomic tree features and scab and mildew resistance.*

'Ladina' is a newly named variety and 'ACW 14995', and 'ACW 11303' are advanced selections with interesting fruit quality. They all carry Rvi6 (Vf) scab resistance and have a low susceptibility against fire blight based on shoot and flower inoculation tests in the greenhouse. Latent E. amylovora *presence was analysed in shoots of plants grown in the greenhouse without visible disease symptoms after artificial inoculation by using quantitative polymerase chain reaction (qPCR). The development of apple cultivars with marketable fruit quality and low susceptibility to* E. amylovora *and other major apple diseases is especially valuable for organic growing and fruit growing in general.*

Keywords: *Malus x domestica*, *Erwinia amylovora*, multiple disease resistance, marker-assisted selection, plant inoculation

Introduction

Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow *et al.*, is one of the most important plant diseases in pome fruit production. Recently, the disease has become a considerable economic problem in high-density orchards. Bacteria infect blossoms, fruit, vegetative shoots, and rootstock crowns. During flowering under optimal conditions *E. amylovora* is actively distributed through pollinating insects. After fast multiplication on stigma and migration to the nectarthodes (Spinelli *et al*., 2005) bacteria invade the floral cluster as well as petiols and migrate further through the stem to the roots (Bogs *et al.,* 1998).

Planting of resistant cultivars is potentially the most promising disease control strategy (Peil *et al*., 2009). However, classical apple breeding of resistant cultivars with high fruit quality is a costly and time consuming process due to the long juvenile stage (Kellerhals *et al*., 2008). The most promising breeding parents are selected based on literature, phenotypic or molecular testing results. Different sources of QTLs against fire blight are known. In cultivars such as 'Enterprise', 'Rewena', 'Free Redstar' and 'Fiesta' the susceptibility towards fire blight is reduced compared to the susceptible cultivar 'Gala Galaxy' (Fischer & Richter, 1999; Calenge *et al*., 2005; Khan *et al*., 2006; Sobiczewski *et al*., 2006). The QTL of 'Fiesta' (*FBF7*) is flanked by the SCAR (sequence-characterized amplified region) markers AE10-375 and GE-8019 (Khan *et al*., 2006; Khan *et al*., 2007). In 'Robusta 5' and 'Evereste' strong fire blight QTLs (*FB_MR5* and *Fb_E*, respectively)

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have been found (Peil *et al.,* 2007; Peil *et al*., 2008; Durel *et al.,* 2009; Fahnentrapp et al., 2011). Due to low fruit size and poor quality of these crab apples the QTL cannot be directly introduced while breeding for marketable fruit quality. This leads to pseudo-backcrosses with qualitative cultivars over several generations to eliminate most of the negative fruit traits of these wild apples.

For the development of new disease resistant apple varieties, progeny plants are selected based on the breeding objectives for multiple disease resistance, tree features and fruit quality. By performing crosses multiple disease resistances against fire blight (*FR*), apple scab (*Venturia inaequalis*; *SR*) and powdery mildew (*Podosphaera leucotricha, MR*) can be combined. For a more sustainable and stable situation, polygenic resistances and resistance pyramids are developed, combining several resistance genes or QTLs for the same disease. For example the scab resistance *Rvi6* (= *Vf*) of *Malus floribunda* 821 can be combined with *Rvi4* (= *Vh4*) of the Russian Seedling R12740-7A. Disease resistance can be determined with molecular markers or phenotypic inoculation tests. Marker-assisted selection (MAS) is an established procedure to confirm the presence of the desired resistance genes (Kellerhals *et al*., 2009) and has the advantage, that it can be applied at an early seedling stage. In phenotypic tests fire blight susceptibility can be investigated with shoot and blossom inoculations. Real Time PCR is a fast and sensitive method to quantify the bacteria within plant tissue (Higuchi *et al*., 1993). Voegele *et al.* (2010) have shown that *E. amylovora* can even be present in tissue without visible disease symptoms.

This paper highlights results of the project ZUEFOS, breeding fire blight resistant fruit varieties. We describe breeding activities including phenotyping and molecular selections and present new cultivars with low susceptibility to fire blight, good fruit quality and tree features.

Material and Methods

Plant material

Parents for crosses were chosen based on literature, phenotypic and molecular testing results.

Pollen of the selected father trees was collected at balloon stage and dried. Branches of mother trees were bagged, flowers manually pollinated and re-bagged until petal fall. From the harvested fruits, seeds were extracted and stratified in humid sand for 2 months at 2°C. The crosses of ‘58/06’ x ‘ACW 11301’ and ‘01/05’ x ‘ACW 11301’ were established in 2010. ‘58/06’ and ‘01/05’ both originate from a cross of ‘Resi’ x ‘Julia’ obtained by a ZUEFOS project partner, with low fire blight susceptibility and carrying the *FBF7* resistance QTL. ‘ACW 11301’ is an advanced selection with low fire blight susceptibility carrying *Rvi4* and *Rvi6* scab resistances.

‘Ladina’ (= ‘ACW 14959’), a newly named variety, ‘ACW 14995’, and ‘ACW 11303’ are advanced selections with interesting fruit quality. ‘Ladina’ and ‘ACW 14995’ originate from a cross of ‘Topaz’ x ‘Fuji’, ‘ACW 11303’ from a cross of ‘ACW 6104’ (‘Arlet’ x ‘Gloster’) x ‘Rewena’. All of them carry *Rvi6* scab resistance, ‘ACW 11303’ carries additional *Rvi4* scab resistance and ‘Ladina’ and ‘ACW 14995’ the *FBF7* fire blight resistance QTL. All three selections were investigated upon their fire blight susceptibility using inoculation tests.

Scab inoculation

After germination, seedlings were raised in a greenhouse. After artificial scab inoculation plants were scored for scab symptoms according to Chevalier *et al*. (1991). A control progeny with scab susceptible parents was included, in 2011 ‘Scifresh’ x ‘La Flamboyante’.

Seedlings rated in classes 0 to 3b were considered as scab resistant. Seedlings with high susceptibility and heavy sporulation (class 4) were excluded from further analyses.

Marker analyses

For MAS, phenotypically scab resistant seedlings of the progeny populations '58/06' x 'ACW 11301' (n = 61) and '01/05' x 'ACW 11301' (n = 42) were individually labelled as well as leaf samples collected for molecular analysis. DNA was extracted according to Frey *et al.* (2004), followed by multiplex PCRs with fluorescently labelled primers assembled according to Patocchi *et al.* (2009). Seedlings were genetically analysed for the two SCAR markers AE10-375 and GE-8019 (Khan *et al*., 2007) and for the two microsatellite markers, (simple sequence repeat, SSR) CH02C02a linked to the *Rvi4* scab resistance and CHVf1 linked to the *Rvi6* scab resistance. Fragment analysis was carried out on a 3130xl Genetic Analyzer (Applied Biosystems) and data were analysed using GeneMapper^{TM} v.4.1 Software (Applied Biosystems).

Fire blight inoculation tests of shoots and flowers

Fire blight inoculation tests of shoots and flowers were conducted in a quarantine greenhouse. 'Gala Galaxy' was included as a susceptible control, 'Enterprise' as tolerant control. Prior to inoculation plants were grown for four to six weeks in a greenhouse.

For shoot inoculations scion wood was grafted on M9 rootstock for twelve replicate trees per genotype. One or two shoot(s) per plant were inoculated at the tip with a syringe containing an *E. amylovora* suspension of 10^9 cfu/ml Swiss strain FAW610 (Rezzonico & Duffy, 2007). The length of necrotic lesion (cm) was measured 7, 14 and 21 days after inoculation. Susceptibility of the genotypes was estimated by calculating the percent lesion length (PLL (%) = lesion length (cm) divided by shoot length (cm)).

For flower inoculations perennial wood was grafted on M9 rootstock. 'Enterprise' was included as three year old trees grafted on M9. One day old floral clusters were reduced to three flowers per cluster and pollinated. The subsequent day flowers were injected with an *E. amylovora* solution of 10^8 cfu/ml strain FAW610 in PBS (200-250 µm, i.e. 10^7 cfu/ml per flower). Infection symptoms were scored 4, 7, 14 and 28 days after inoculation.

qPCR analyses

Thirteen weeks after shoot inoculation three random shoots including new secondary shoots and rootstock of 'Ladina', 'ACW 14995' and 'Enterprise', and eight weeks after inoculation three random shoots of 'Gala Galaxy' were cut into 5 cm long pieces (starting from the shoot tip) and analysed with Real Time PCR according to Voegele *et al.* (2010).

Results

In 2008, 2009, 2010 and 2011, between 3,500 and 11,000 seeds were produced each year for the project ZUEFOS. Selected examples of fire blight relevant crosses are shown in Table 1.

In the scab inoculation test, 268 out of 433 '58/06' x 'ACW 11301' and 273 out of 465 '01/05' x 'ACW 11301' progeny plants were rated as scab resistant (Fig. 1). In the control progeny 'Scifresh' x 'La Flamboyante' 9 out of 53 plants were rated as scab resistant.

Scab resistant seedlings of all progenies were selected for tree features and powdery mildew resistance in the first autumn. From the crosses of 2008, 2009 and 2010 17.7% of the plants were selected for further investigation of fruit and trees, and grafted on 'M27' rootstock with 'Golden Delicious' as interstem. These selection steps allowed for the reduction of 10,744 primal seedlings to 1,000 grafted plants.

The results of the marker analyses of scab resistant seedlings are shown in Table 2. Eleven (18%) of the progeny seedlings '58/06' x 'ACW 13001' and 13 (34%) of the progeny seedlings '01/05' x 'ACW 11301' analysed at the molecular level carry markers for *FBF7* as well as the markers for *Rvi4* and *Rvi6*.

Table 1: Examples of fire blight relevant crosses and progeny size in years 2008, 2009 and 2010.

Year	**Mother (parents; resistances)**	**Father (parents; resistances)**	**Progeny size**
2008	ACW 11303 (ACW 6104 x Rewena; *FR*, *Rvi4*, *Rvi6*, *MR*)	Evereste (*Fb_E*, *Rvi6*)	39
2008	La Flamboyante – Mairac®	DA02. 2.7 (Idared x *Malus robusta* 5; *FB_MR5*, *Rvi4*)	196
2008	Milwa – Junami®	Free Redstar (*FR*, *Rvi6*)	539
2009	Ariane (*Rvi6*)	Ladina (= ACW 14959; Topaz x Fuji; *FBF7*, *Rvi6*)	1383
2010	58/06 (Resi x Julia; *FBF7*)	ACW 11301 (ACW 6104 x Rewena; *FR*, *Rvi4*, *Rvi6*)	310
2010	01/05 (Resi x Julia; *FBF7*)	ACW 11301 (ACW 6104 x Rewena; *FR*, *Rvi4*, *Rvi6*)	308

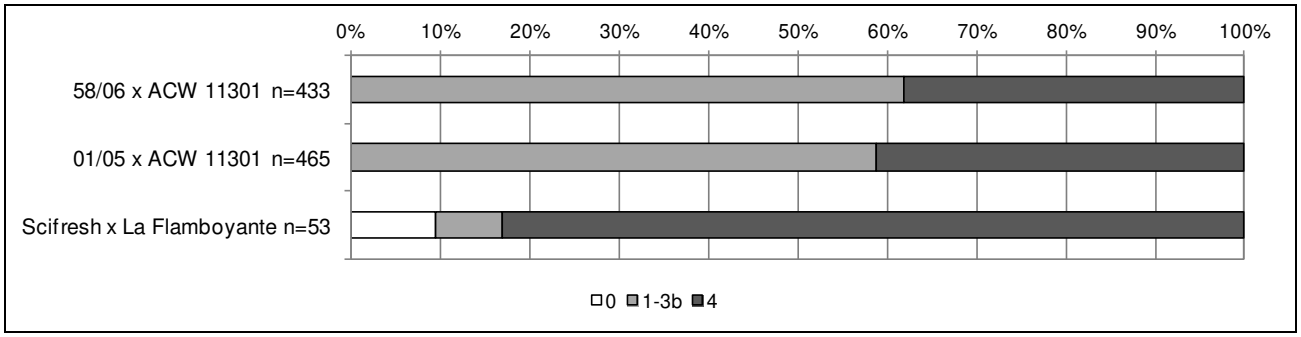

Figure 1: Scab screening of selected crosses 2010. Seedlings rated in classes 0 to 3b were considered as scab resistant and seedlings in class 4 with heavy sporulation as highly susceptible.

Table 2: Molecular analyses of phenotypically scab resistant progeny seedlings '58/06' x 'ACW 11301' (n=60) and '01/05' x 'ACW 11301' (n=38). Percentage of observed and from segregation expected progeny population carrying the markers for *FBF7*, *Rvi4*, *Rvi6* resistance.

58/06 (*FBF7*) x ACW 11301 (*Rvi4*, *Rvi6*)			
Resistances	*FBF7*	*Rvi4*	*Rvi6*
Observed (n)	26	30	53
Observed (%)	43	49	87
Expected (%)	50	50	50
01/05 (*FBF7*) x ACW 11301 (*Rvi4*, *Rvi6*)			
Resistances	*FBF7*	*Rvi4*	*Rvi6*
Observed (n)	29	21	35
Observed (%)	76%	55%	92%
Expected (%)	50%	50%	50%

The results of the tested advanced selections 'Ladina', 'ACW 14995' and 'ACW 11303' revealed some differences in susceptibility towards *E. amylovora* between the different years (Fig. 2). Control cultivar 'Enterprise' was as expected very low susceptible with a mean of 12.9% percent lesion length relative to 'Gala Galaxy'. 'Ladina' was in the same range as 'Enterprise' with a mean of 14.2% percent lesion length. 'ACW 11303' and 'ACW

14995' reached a mean percent lesion length relative to 'Gala Galaxy' of 33.6% and 37.1%, respectively.

Figure 3 illustrates the levels of fire blight susceptibility after flower or shoot inoculation. The highest infection symptoms were observed with 'Gala Galaxy' with 61.7% of bourse shoots or shoots with fire blight symptoms four weeks after flower inoculation and 87.0% of total shoot length three weeks after shoot infection. 'Ladina', 'ACW 11303' and 'ACW 14995' were less susceptible with 10.0%, 20.0% and 32.7% of bourse shoots or shoots with fire blight symptoms four weeks after flower inoculation and 12.6%, 19.7% and 13.5% of total shoot length three weeks after shoot infection. Control cultivar 'Enterprise' is very low susceptible with 2.9% of bourse shoots or shoots with fire blight symptoms four weeks after flower inoculation and 5.9% of total shoot length three weeks after shoot infection.

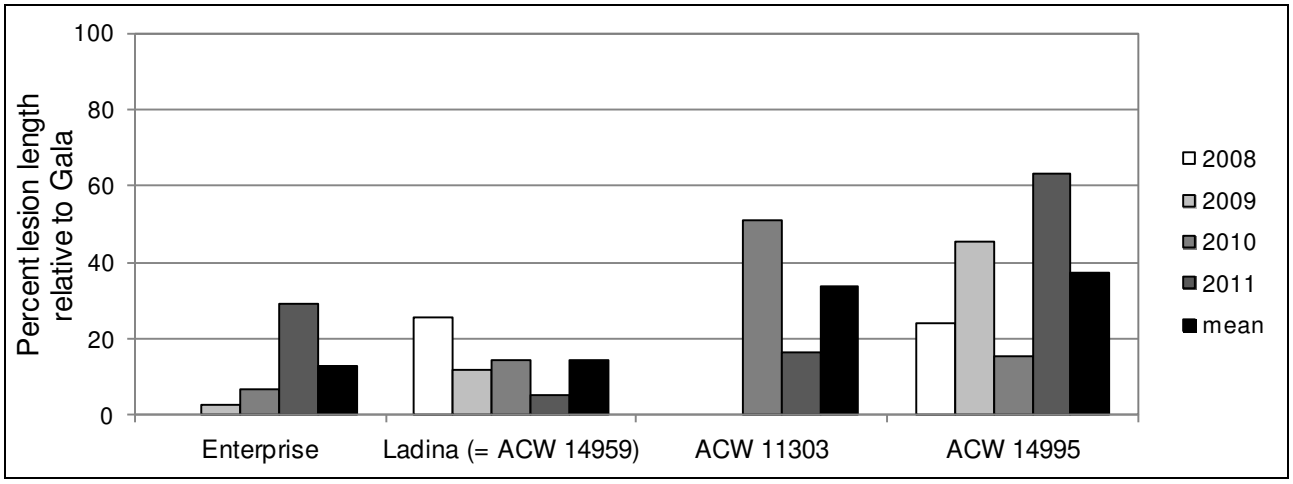

Figure 2: Mean lesion length in percent of total shoot length relative to 'Gala' for advanced selections 3 weeks after shoot infection in years 2008 to 2011 compared to 'Enterprise' (tolerant control).

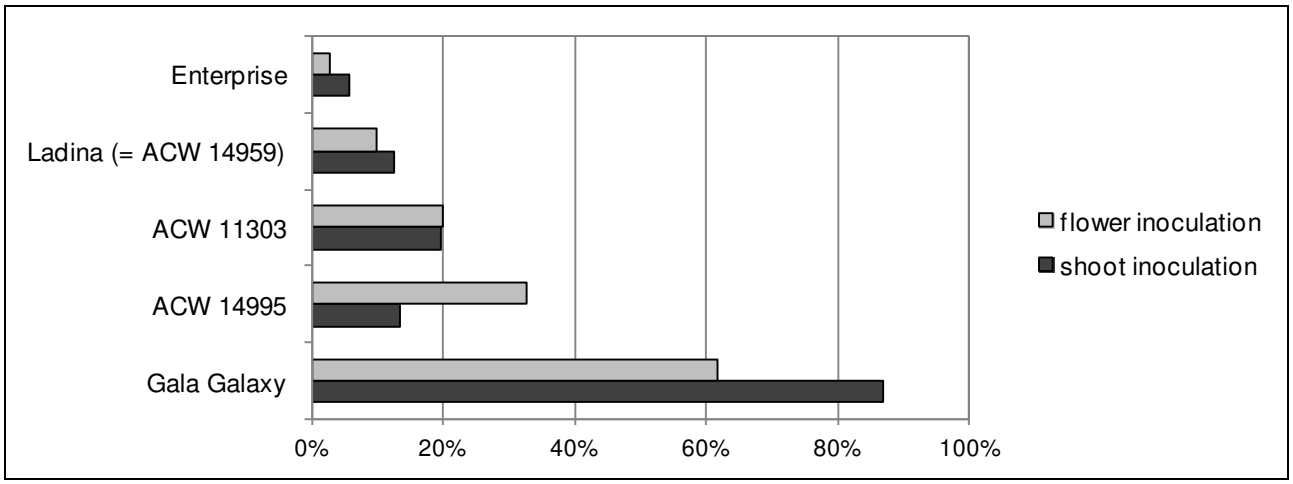

Figure 3: Fire blight symptoms after flower or shoot inoculations for advanced selections compared to 'Enterprise' (tolerant control) and 'Gala Galaxy' (susceptible control). The upper bar shows the percentage of bourse shoots or shoots with fire blight symptoms 4 weeks after flower inoculation in year 2011. The mean lesion length in percent of total shoot length 3 weeks after inoculation in year 2010 is shown in the lower bar.

Real Time PCR results of shoots after artificial fire blight inoculation show that significant amounts of bacteria, up to 10^8 cells per gram of tissue, were detected in samples without

visible symptoms. Spread of bacteria down the shoot and to the rootstock seems to proceed faster than upwards towards new shoots. In symptomatic tissue of 'Gala' shoots highest amounts of bacteria with up to 10^{12} cells per gram of tissue were measured. In new shoots and rootstocks only minor differences between cultivars were found.

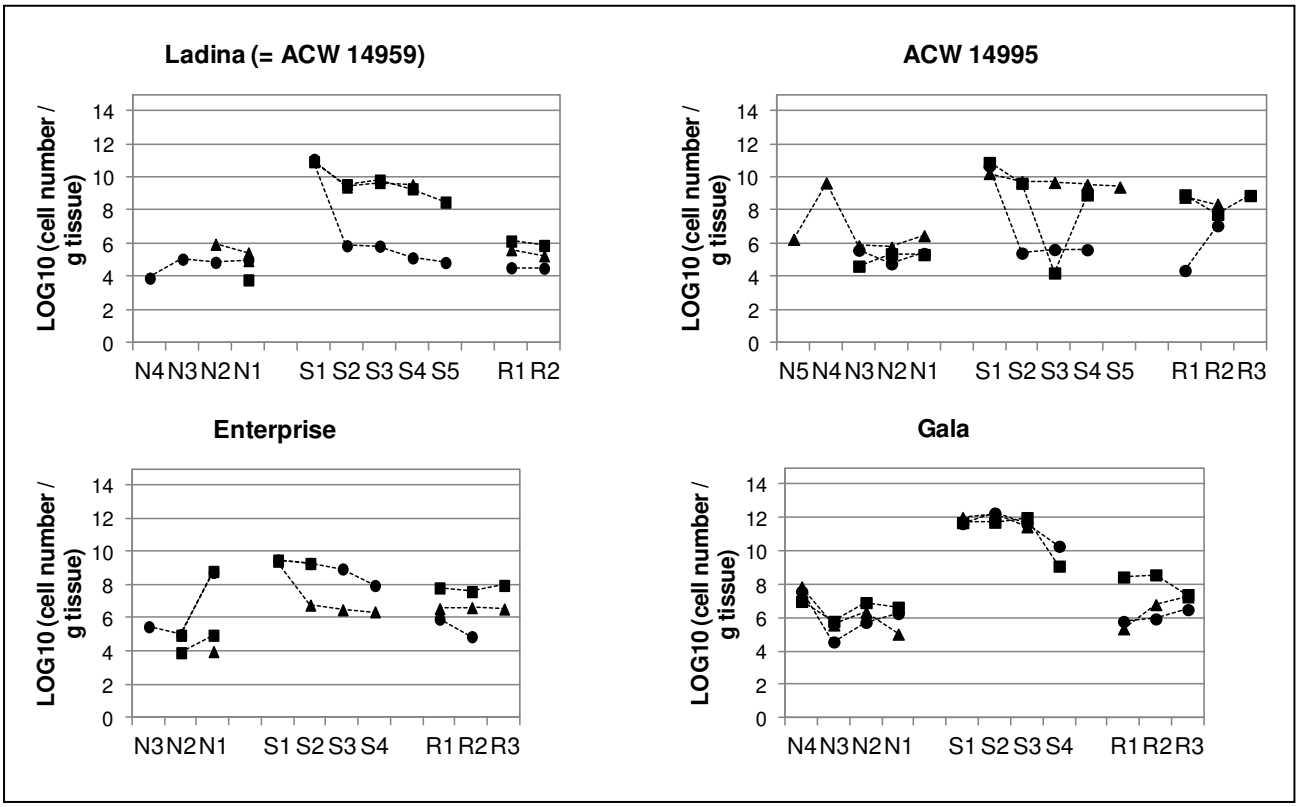

Figure 4: Spread of *E. amylovora* after artificial shoot inoculation in new secondary shoots (N), shoots (S) and rootstock (R).

Discussion

In this paper we show that crosses within the project ZUEFOS lead to a wider genetic diversity pool in respect to fire blight resistance in the ACW breeding program. By scab inoculation, molecular analyses, evaluation of tree features and powdery mildew the seedling number was considerably reduced. Fruit quality and tree features of selected plants, as well as fire blight susceptibility with different inoculation tests will be further evaluated within the next years.

Screenings with molecular markers for the *FBF7* QTL can provide some information about susceptibility to fire blight but the association between marker presence and tolerance is not absolute (Nybom *et al*., 2011). Seedlings with fire blight resistance of 'Evereste' or 'Robusta 5' need four to five generations of pseudo-backcrossing due to the small fruit size and poor fruit quality of the parental resistance donors. In 2012 we expect plants of the third pseudo-backcross-generation with *FB_MR5* resistance and first flowers in second generation with *Fb_E* resistance. Within the F_2 seedling progeny plants, while using a set of molecular markers covering the whole genome, some individuals might be identified with a high proportion of genome inherited from the high quality parents (Volz *et al*., 2009). Discovery of further QTLs from other parent sources (Peil *et al*., 2007) and development of markers will improve germplasm screenings and enable the selection of plants with pyramided fire blight resistance QTLs for durable resistance.

The newly named variety ‘Ladina’ carries *Rvi6* scab resistance and was low susceptible in fire blight inoculation tests of both shoots and flowers. Fire blight resistance evaluations under controlled conditions have been shown to correlate well with field resistance (Quamme *et al*., 1976). Our results have shown, that flower and shoot resistances are often correlated, but there are occasions reported where this is not the case (Le Lezec *et al.,* 1987). However, even in asymptomatic tissue, *E. amylovora* was detected. Such latent infections could explain fire blight infections in orchards where no fire blight had ever been seen before (Vanneste & Eden-Green, 2000). Further testing of advanced selections under field conditions is planned from 2012 onward.

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