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Fire blight (*Erwinia amylovora*) resistance in apple varieties associated with molecular markers

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Summary: The invasive bacterial disease fire blight, caused by *Erwinia amylovora*, has the potential to destroy fruit tree orchards all over Europe. Effective plant protection methods are lacking in many countries, highlighting the increasing importance placed on identification of germplasm with heritable disease resistance. Recently, a promising QTL (quantitative trait locus) was identified on linkage group 7 in the apple cultivar 'Fiesta' which is derived from 'Cox's Orange Pippin'. In the present study, 144 Swedish and foreign apple cultivars were analysed with the SCAR markers AE10-375 and GE-8019, which flank this QTL. Twenty-nine of the analysed cultivars had both markers, 78 had either AE10-375 or GE-8019, and 37 cultivars did not carry any of the two markers. Seventeen cultivars, 7 with both markers and 10 not having either of the two markers, were then inoculated with the bacterium in a quarantine greenhouse test. Cultivars carrying both DNA markers were significantly less susceptible than cultivars lacking the markers, $P<0.001$. Cultivars that were most resistant had both markers and had 'Cox' in their pedigree. Unrelated cultivars with the markers may still lack the QTL.

Key words: Fire blight, *Erwinia amylovora*, *Malus* x *domestica*, marker-assisted selection

Introduction

Fire blight is a devastating bacterial disease caused by *Erwinia amylovora*, which infects woody plants in the *Rosaceae* family (*Vanneste*, 2000). Fire blight was first seen in North America 200 years ago. It first occurred in the United Kingdom in the late 1950s, and then in the 1960s spread to continental Europe. Among susceptible plant species, apple (*Malus* x *domestica*) is the most important and appreciated fruit crop in temperate regions. Economic losses to apple production were particularly devastating in Europe in 2007 due to high inoculum pressure and favourable warm temperatures during the flowering period (*Duffy* et al., 2007). In Sweden, the first outbreak was found in a pear orchard in 1986 (*Persson*, 1995), but the Scandinavian countries have thus far suffered only minor damage, likely due to cool temperatures during the flowering period. With increasing temperatures and humidity due to global warming, fire blight has the potential to reach devastating proportions also in these areas.

Fire blight assumes two major epidemiological phases. Blossom blight results from infection of flowers during the spring and is the most serious phase in terms of disease spread within and outside orchards. Flower-visiting vectors such as bees can rapidly disseminate the pathogen resulting in epidemics. Foliar blight results from advancement of blossom infections or direct infection due to wounding of foliar tissues (e.g., from hail or pruning damage). Fire blight results in typical symptoms of blackened necrosis, shepherd's crook of shoots and cankers on woody tissue. Warm temperature and humidity favours pathogen growth and forecasting models based on these parameters are reliably used to predict infectious periods during flowering. Fire blight can rapidly spread throughout infected trees and orchards resulting in complete tree/orchard loss. In areas such as Europe, where the pathogen is a quarantine organism, even limited infection can result in losses due to phytosanitary regulations mandating eradication (*Duffy* et al., 2005).

Control options for blossom blight are limited (*Norelli* et al., 2003). The most reliable and effective control for fire blight is application of antibiotics during the flowering period to minimise epidemic spread and infection (*Vanneste*, 2000), however, many countries have severe restrictions or bans on the use of antibiotics in plant agriculture. Biological control using antagonistic bacteria, yeast or bacteriophage has so far provided only moderate and variable control (*Carter & Celetti*, 2006; *Johnson & Stockwell*, 1998). Control options for foliar blight are even more restricted since neither antibiotics nor biocontrol suppress the disease, although the plant growth regulator compound prohexadione-Ca can provide some level of control at the expense of altered tree architecture (*Norelli & Miller*, 2004; *Spinelli* et al,. 2007).

Heritability estimates for field resistance against fire blight have been determined in apple (*Luby* et al., 2002), but no major genes have been identified except for possibly in the wild species *M. robusta* clone 5 (*Peil* et al., 2007).

Cultivar improvement has been attempted through gene transfer (*Borejsza-Wysocka*, 2008; *Flachowsky* et al., 2008), but consumer and grower acceptance is uncertain and so far, no transgenic apple lines have been commercialised. Instead, renewed efforts have been made to identify DNA markers for quantitatively inherited genes that can be applied for marker-assisted selection to streamline the resistance breeding process. A quantitative trait locus (QTL) found on linkage group 7 in the apple cultivar 'Fiesta' (F7) was shown to explain up to 46% of the phenotypic variation in fire blight resistance for 'Prima' x 'Fiesta' F_1 progeny (*Calenge* et al., 2005; *Khan* et al., 2006). The fire blight F7 QTL has been identified in three genetic backgrounds which show high stability of the QTL effect and the reproducibility of the two markers surrounding this QTL (*Khan* et al., 2007). Two SCAR markers, AE10-375 and GE-8019, flanking this QTL have been validated for marker-assisted selection (*Khan* et al., 2007). Cultivars carrying both markers showed significantly more resistance to fire blight than those lacking the markers. This QTL can be tracked back down to 'Cox's Orange Pippin' (*Khan* et al., 2007). 'Cox' descendants carrying the QTL are more likely to show high tolerance compared to descendants not carrying the QTL. Whether the presence of the two markers co-occurs with high tolerance also in unrelated cultivars is not yet determined.

In this study, 144 apple cultivars were analysed with two SCAR markers to identify possible carriers of the QTL-induced fire blight resistance. Seventeen cultivars were then selected for a quarantine-greenhouse fire blight inoculation test.

Materials and Methods

Plant material

A total of 144 cultivars, both Swedish and foreign, were analysed for presence of the two DNA markers. Seven cultivars with both markers and 10 cultivars without either one of them were then selected for a greenhouse fire blight inoculation test. Both of these groups contained some cultivars within the 'Cox' family and some that were presumably unrelated. These were selected based on commercial interest and/or to obtain a high level of genetic diversity.

SCAR analysis

Young leaves were collected in April–May and stored at –80 °C until use. Leaves were powdered with liquid nitrogen in pre-cooled mortars. Genomic DNA was isolated from approximately 100 mg of leaf powder using the Qiagen DNeasy™ Plant Mini Kit (Qiagen AB, Solna) following the manufacturer protocol. The primers AE10-375 and GE-8019 were purchased from Eurofins MWG Operon (Ebersberg, Germany) using the primer sequences described in Khan et al. (2007). The PCR were run as described for SSRs (total volume 15 µl) in Liebhard et al. (2002), on a PX2 Thermal Cycler (Thermo Hybaid, Ulm). The PCR products were run on a 1.8% agarose gel stained with ethidium bromide and then visualised on a UV table. The presence or absence of bands was scored.

Inoculation test

Scion wood of 17 cultivars was collected at Balsgård, Sweden, and sent to the Research Station Agroscope Changins-Wädenswil, Switzerland, for bench-grafting on M9 rootstocks (Table 2). The resulting plants were grown in pots in a greenhouse. Terminal shoots of these plants were inoculated with a highly virulent strain (CFBP1430) of *E. amylovora* (10^7 cfu/ml) following *Khan* et al. (2006). Observation and measurement of the progress and severity of fire blight symptoms were performed at 10 and 20 days after infection. Susceptibility of genotypes was evaluated as percent lesion length of the necrotic regions in the inoculated shoot relative to total shoot length.

Result and Discussion

Out of a total of 144 cultivars tested for the SCAR markers AE10-375 and GE-8019, 29 were carrying both markers, 78 had either AE10-375 or GE-8019 (45 cultivars had only AE10-375 and 33 cultivars had only GE-8019) and 37 cultivars were negative for both markers (*Table 1*). Of the cultivars carrying both markers, one is 'Cox's Orange Pippin' (included as a control) and 16 are known relatives of this cultivar and may have inherited the QTL from this source; 'Aroma', 'Eir', 'Elise', 'Eva-Lotta', 'Holsteiner Cox', 'Idunn', 'Ingrid Marie', 'James Grieve', 'K1016' and 'K1160' (both are Balsgård selections with 'Aroma' as one parent), 'Katinka', 'Katja', 'Kim', 'Queen Cox' (sport of 'Cox's Orange Pippin'), 'Ribston' and 'Rubinola'. This suggests that 12 cultivars may have inherited their AE19-375 and GE-8019 markers elsewhere. However, both the cultivars related to 'Cox's Orange Pippin' and those lacking such relationship may have the two markers on separate chromosomes, and thus may lack the QTL of interest.

The 17 cultivars selected for inoculation tests, were propagated, inoculated and evaluated, with 7 cultivars carrying both markers and 10 lacking the markers. The cultivars were observed and the shoot necrosis for 4–8 shoots per cultivar was measured on a regular basis. The length of the lesion (in cm) of the shoots was directly translated into susceptibility for *E. amylovora*, and a mean value of lesion length (the percent length of the lesion relative to total shoot length, divided by the number of shoots for every cultivar) was then calculated after 10 and 20 days (*Table 2*). The group with the seven cultivars carrying both markers showed the highest tolerance to fire blight, except for the cultivar 'Guldborg' which was the fifth most tolerant cultivar, even though it is lacking both of the markers, and thus also presumably the QTL found on LG7 on 'Fiesta'. Hence the tolerance encountered in 'Guldborg' is, perhaps, brought

Table 1. Cultivars analysed with the SCAR markers AE10-375 and GE-8019 and their geographic origin. + indicates amplification of the marker(s). Cultivars in bold have both markers and therefore potentially carry the QTL for fire blight resistance. * denotes cultivar related to 'Cox's Orange Pippin'.

Variety	Origin	AE10-375	GE-8019
Aila	????	+	
Alexander	Russia		+
Alfa 68	Sweden	+	
Alice*	Sweden		+
Ananas Reinette	Netherlands		+
Annero	Sweden	+	+
Aroma*	Sweden	+	+
Arvidsäpple	Sweden		+
Aspa	Sweden		+
Astrakan, Gyllenkroks	Sweden		+
Astrakan, White	Russia		+
Astrakan, Red	Sweden	+	
Astrakan, Stor Klar	Sweden		+
Belle de Boskoop	Netherlands	+	
Birgit Bonnier*	Sweden	+	
Blenheim Orange	England	+	
Boiken	Germany		+
Borgherre	Germany	+	
Borsdorfer	Germany	+	
Brunnsäpple, Hallands	Sweden	+	
Cellini	England	+	+
Charlamovsky	Russia	+	+
Close	USA	+	+
Cortland	USA		
Cox's Orange Pippin*	England	+	+
Cox Pomona	England		+
Dayton	USA	+	
Delicious, Red	USA	+	
Discovery	England		
Domö Favorit	Sweden		+
Drakenberg	Sweden	+	
Dronning Louise	Denmark	+	+
Druväpple	Europe	+	
Edsele	Sweden		
Eir*	Norway	+	+
Elise*	Netherlands	+	+
Elstar*	Netherlands		+
Eva-Lotta*	Sweden	+	+
Fagerö	Sweden		
Farmors Juläpple	Sweden	+	
Fiholms Ribston	Sweden	+	
Filippa	Denmark		
Flädie	Sweden		
Fredrik*	Sweden		
Frida*	Sweden		
Frösåker	Sweden	+	
Fullerö	Sweden	+	
Förlovningsäpple	Sweden		
Gelber Herbstkalvill	Germany		+
Gelber Richard	Germany		+
Gloster	Germany	+	
Golden Delicious	USA	+	
Golden Pearmain	England		
Granatäpple, Kungsbacka	Sweden		+
Gravensteiner	Europe	+	
Gravensteiner, Red	Europe	+	
Grågylling	Sweden		+
Guldborg	Denmark		
Göteborgs Flickäpple	Sweden	+	
Hanaskog	Sweden		
Hannaäpple	Sweden		+
Hausmütterchen	Germany	+	
Hedenlunda	Sweden	+	

Variety	Origin	AE10-375	GE-8019
Himmelstalund	Sweden	+	
Holländaräpple	Sweden		
Holsteiner Cox*	Germany	+	+
Hugoäpple	Sweden	+	
Idunn*	Norway	+	+
Ingrid Marie*	Denmark	+	+
Ivö	USA/Sweden	+	
James Grieve*	Scottland	+	+
Jonathan	USA	+	
Josefiner	Sweden		
Julyred	USA	+	+
K1016*	Sweden	+	+
K1160*	Sweden	+	+
Kalmar Glasäpple	Sweden		
Katinka*	Norway	+	+
Katja*	Sweden	+	+
Kavlås	Sweden		
Kramforsäpple	Sweden		+
Kim*	Sweden	+	+
Kinnekulle Kantäpple	Sweden	+	
Kingston Black	England	+	
Landskronaäpple	Sweden		+
Larsmässeäpple	Sweden		
Laxton's Superb*	England		
Linda	Canada	+	
Linné's Apple	Sweden		
Lobo	Canada		+
Maglemer	Denmark		
Melon	Germany		
Melon, red	Germany		
Melonkalvill	Sweden		
Meningasker	Sweden		
Mio	Sweden		
Mutsu	Japan	+	
Mälsåker	Sweden		
Nanna*	Norway		
Norrstack	Sweden		
Norrviken	Sweden	+	
Oranie	Sweden		
Oretorp	Sweden	+	
Pigeon	Denmark		+
Prima	USA	+	
Prinsess Apple	Unknown		+
Queen Cox*	England	+	+
Rescue	Canada	+	
Ribston*	England	+	+
Ringstad	Sweden		+
Risäter	Sweden	+	
Rubinola*	Czech Republic	+	+
Rödluvan	Sweden	+	+
Sandbergs Red	Sweden		+
Signe Tillisch	Denmark		+
Silva	Sweden		+
Siv*	Norway	+	
Snövit	Sweden		
Sparreholm	Sweden		+
Spässerud	Sweden		
Stenkyrke	Sweden	+	
Stäringe Karin	Sweden		+
Suislepper	Estonia	+	
Summerred	Canada	+	+
Svanetorp	Europe	+	
Sylvia	Sweden	+	+

Variety	Origin	AE10-375	GE-8019
Särsö	Sweden		
Sävstaholm	Sweden	+	+
Sörmlandsäpple	Sweden	+	
Transparante Blanche	Russia		+
Trogsta	Sweden		
Vallda	Sweden		
Veseäpple	Sweden		
Villands Glasäpple	Sweden	+	
Vista Bella	USA	+	+
Vitgylling	Europe		+
Vittsjö	Sweden		+
Vrams Järnäpple	Sweden	+	+
Värmlands Sötäpple	Sweden		+
Wealthy, Red	USA	+	
Worcester Pearmain	England	+	
Åkerö	Sweden		
Ökna Lökäpple	Sweden		
Ökna Vita Vintergylling	Sweden		

Table 2. Cultivars inoculated with *Erwinia amylovora* CFBP1430 and the number of inoculated shoots for each cultivar. The length of the lesion (cm) for each shoot was measured and a mean value, expressed in percent, was calculated for each cultivar. The cultivars are placed in increasing order of susceptibility after 20 days. Cultivars in bold carry both markers. * denotes cultivar related to 'Cox's Orange Pippin'.

Cultivar	No. of shoots inoculated	Mean lesion length per shoot after 10 days expressed as %	Mean lesion length per shoot after 20 days expressed as %
Rubinola*	7	8	14
Ribston*	8	13	21
Elise*	4	18	27
Cox's Orange Pippin*	7	21	28
Guldborg	6	29	33
Katja*	6	31	38
Close	8	31	42
Annero	8	28	42
Norrstack	7	39	46
Golden Pearmain	7	49	60
Laxton´s Superb*	7	34	62
Nanna*	8	46	65
Filippa	8	50	69
Fredrik*	7	64	72
Spässerud	8	37	78
Frida*	8	66	82
Mio	8	74	89

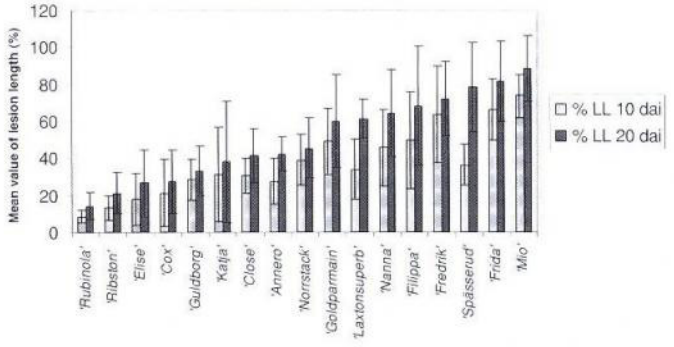

Figure 1. The mean value of percent lesion length (LL) 10 and 20 days after infection (dai). On the axis the cultivars are shown in ascending order of fire blight susceptibility, from left to right. The eight least susceptible cultivars (i.e., 'Rubinola'–'Annero') have both markers except for 'Guldborg', which together with the remainder ('Norrstack'–'Mio') lack both markers. The bars indicate standard error.

about by a different QTL. A Mann-Whitney U-test showed that the two groups of cultivars, i.e., with or without the two markers, differed significantly, U = 67, N1/N2 = 7/10, $P < 0.001$. Many pairwise comparisons among the cultivars were also significant according to an LSD test (*Figure 1*).

Interestingly, within the group of cultivars carrying both markers, the highest resistance was found in 'Cox's Orange Pippin' and other cultivars belonging to the 'Cox' family, with 'Rubinola' being most resistant among these. Disease severity assessments for this cultivar were similar to those of *Khan* et al., (2007) demonstrating the reproducibility and robustness of our bioassay. The two cultivars having the least resistance within this group, 'Close' and 'Annero', have unknown ancestries but this is one indication they may not belong to the 'Cox' family, something that needs to be further evaluated. A higher tolerance against fire blight in cultivars carrying both markers compared to cultivars not carrying the markers was also shown in a previous study (*Khan* et al., 2007). However, presence of the two markers does not necessarily mean that the cultivar is carrying the QTL. The two markers might be located on two different chromosomes, derived from the two different parents, one parent carrying one marker and the other parent carrying the other marker. If a descendant of 'Cox' is positive for both markers, it is also likely to have the QTL. For a non-descendant of 'Cox' which has both markers, the probability that these occur on different chromosomes is considerably higher. Furthermore, for a triploid cultivar, the risk of inheriting markers located on different chromosomes is even higher. Among the cultivars that were inoculated in our study, 'Close', 'Ribston' and 'Norrstack' are triploid, and the first two also have both markers. 'Ribston' is probably the mother of 'Cox's Orange Pippin' and shows high tolerance to fire blight whereas 'Close' is probably unrelated and shows only intermediate levels of tolerance indicating that it may lack the QTL.

In conclusion, the two SCAR markers, AE10-375 and GE-8019, seem to be useful in marker-assisted selection, especially for selecting among cultivars that are 'Cox' descendants. However, the very large number of cultivars having only one of the markers, shows that there is a considerable risk that co-occurrence of the two markers in the same cultivar (especially if the cultivar does not belong to the 'Cox' family) may be unconnected with presence of the QTL. Discovery of further QTLs from other parent sources (*Peil* et al., 2007) and development of markers for combination with those used in this study will improve the screening power for use in germplasm collections.

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