# Identification of a major QTL for *Xanthomonas arboricola* pv. *pruni* resistance in apricot

**D. Socquet-Juglard · B. Duffy · J. F. Pothier · D. Christen · C. Gessler · A. Patocchi**

**Abstract** *Xanthomonas arboricola* pv. *pruni* causes bacterial spot of stone fruit resulting in severe yield losses in apricot production systems. Present on all continents, the pathogen is regulated in Europe as a quarantine organism. Host resistance is an important component of integrated pest management; however, little work has been done describing resistance against *X. arboricola* pv. *pruni*. In this study, an apricot population derived from the cross "Harostar" × "Rouge de Mauves" was used to construct two parental genetic maps and to perform a quantitative trait locus analysis of resistance to *X. arboricola* pv. *pruni*. A population of 101 F1 individuals was inoculated twice for two consecutive years in a quarantine greenhouse with a mixture of bacterial strains, and disease incidence and resistance index data were collected. A major QTL for disease incidence and resistance index accounting respectively for 53 % (LOD score of 15.43) and 46 % (LOD score of 12.26) of the phenotypic variation was identified at the same position on linkage group 5 of "Rouge de Mauves." Microsatellite marker UDAp-452 co-segregated with the resistance, and two flanking microsatellites, namely BPPCT037 and BPPCT038A, were identified. When dividing the population according to the alleles of UDAp-452, the subgroup with unfavorable allele had a disease incidence of 32.6 % whereas the group with favorable allele had a disease incidence of 21 %, leading to a reduction of 35.6 % in disease incidence. This study is a first step towards the marker-assisted breeding of new apricot varieties with an increased tolerance to *X. arboricola* pv. *pruni*.

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## Introduction

Bacterial spot of stone fruit caused by *Xanthomonas arboricola* pv. *pruni* is one of the major diseases of peach, apricot, plum, and almond (du Plessis 1988; Pothier et al. 2011a). It produces necrotic lesions on leaves, which may lead to severe defoliations of the trees. Fruit lesions appear as water-soaked spots becoming brown on the surface (Civerolo and Hatting 1993). Cankers on twigs can be observed, especially on plum (Garcin et al. 2005). *X. arboricola* pv. *pruni* has been identified on all continents since its first description in the USA by Smith (1903). In Europe, severe outbreaks occur in France, Italy, Bulgaria, Romania, and Ukraine (Anonymous 2006). In Switzerland, it was first detected on apricot in 2005 in canton Valais (Pothier et al. 2010).

The bacteria overwinter in buds and cankers or in leaf scars (Zaccardelli et al. 1998; Battilani et al. 1999). Spring temperatures higher than 20 °C, heavy winds, and a high humidity for at least 3 days are the most favorable

conditions for bacteria multiplication and for obtaining severe infections (Battilani et al. 1999). Late infections can be also observed in the fall (Garcin et al. 2005). In neglected peach orchards, *X. arboricola* pv. *pruni* can damage 25 % to 75 % of the fruits (Dunegan 1932; Pothier et al. 2011b).

In Europe, phytosanitary measures to avoid the spread of the pathogen and the application of copper sprays are currently the two strategies used to control *X. arboricola* pv. *pruni*, but the efficacy of copper sprays is hindered by environmental problems due to copper residues and by leaf damages it causes (Ritchie 1995). In the USA, oxytetracycline is used to control the disease but has a limited efficacy (Ritchie 1995).

Host resistance has been studied in peach, plum, and apricot (Keil and Fogle 1974; Werner et al. 1986; Topp and Sherman 1990; Layne 1991; Layne and Hunter 2003; Garcin et al. 2005). Resistance screenings among the cultivars have been conducted in the field and showed variation in susceptibility to the disease. Symptoms were observed also on the more resistant varieties, therefore indicating that complete resistance seems to not exist (Garcin et al. 2005). These results indicate that the resistance might be controlled in a quantitative manner. Although breeding for quantitative resistance would be more challenging than in the case of a qualitative resistance, quantitative resistance still represents an interesting potential for breeding of more resistant cultivars.

*Prunus* genetic maps have been constructed in recent years based on combinations of random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and simple sequence repeat (SSR) markers (Hurtado et al. 2002; Vilanova et al. 2003; Dondini et al. 2007). The *Prunus* reference map has been constructed from a cross between almond ("Texas") and peach ("Earlygold"; T × E) and is saturated with RFLPs, SSRs, isoenzymes, and sequence-tagged sites (Joobeur et al. 1998; Dirlewanger et al. 2004a; Howad et al. 2005). The high level of synteny reported in *Prunus* allows map comparison and the transfer of markers among diploid ($2n=16$) *Prunus* species (Dirlewanger et al. 2004a, b; Lambert et al. 2004; Olmstead et al. 2008). The very recent availability of the peach physical map (Zhebentyayeva et al. 2008) and of the genome sequence (International Peach Genome Initiative: Peach Genome v1.0. 2010 http://www.rosaceae.org/peach/genome) permit the development of markers for saturation of very specific regions and for the cloning of genes.

Quantitative trait loci for resistance against several pathogens and pests have been mapped in *Prunus*. Resistance against sharka in peach, apricot, and *Prunus davidiana* has been extensively investigated and a major QTL has been found on linkage group (LG) 1 (Lambert et al. 2004; Decroocq et al. 2005; Sicard et al. 2008; Soriano et al. 2008; Dondini et al. 2010; Vera Ruiz et al. 2011). Other resistance QTLs from *P. davidiana* have been detected for powdery mildew in peach × *P. davidiana* crosses on LG1, LG2, LG3, LG5, LG6, and LG8 (Dirlewanger et al. 1996; Foulongne et al. 2003) and for leaf curl caused by *Taphrina deformans* in a peach × *P. davidiana* cross on six linkage groups (Viruel et al. 1998). Resistance genes against root-knot nematode were identified and validated with molecular markers on LG7 in plum and almond and on LG2 in peach (Claverie et al. 2004; Dirlewanger et al. 2004b; Esmenjaud et al. 2009; van Ghelder et al. 2010).

To date, despite regular yield losses in peach and apricot productions due to *X. arboricola* pv. *pruni*, host resistance against bacterial spot has not been intensively studied and no major gene or QTL has been mapped. The objectives of this study were to identify quantitative trait loci controlling *X. arboricola* pv. *pruni* resistance using AFLP- and SSR-based maps of the apricot population, "Harostar" × "Rouge de Mauves", and to identify SSR markers linked to the QTLs for further use in a marker-assisted selection program.

## Material and methods

### Plant material

A cross between the Canadian cultivar "Harostar" and the French cultivar "Rouge de Mauves" provided 101 F1 plants and was used as a mapping population. "Harostar" is reported to have a good resistance level against *X. arboricola* pv. *pruni* (Layne and Hunter 2003) whereas resistance level of "Rouge de Mauves" was unknown. Plants were grown in an orchard in Conthey, Switzerland. Five replicates per F1 and ten replicates from each parent were grafted on the plum "Saint-Julien 655-2" rootstock, resulting in a total plant number of 525. All plants were kept in a cold chamber at 2 °C for short-term storage. Plants were then potted and grown under greenhouse conditions at 23 °C, 60 % humidity for 1 week before being transferred in a quarantine greenhouse. Inoculations were performed over 2 years (2010 and 2011) on the same population; plants were kept in the quarantine greenhouse over winter. In 2011, 94 plants across 50 genotypes that had died during the previous year were replaced with newly grafted plants on the same "Saint-Julien 655-2" rootstock.

### Bacterial strains

In the first year, four strains of *X. arboricola* pv. *pruni* were used in a mix. Two of them were collected in Switzerland (Valais), one strain in 2005 (XA1.29) and the second in 2007 (XA1.51) (Pothier et al. 2010). The two others were the pathotype strain NCPPB 416 and the genome sequenced strain CFBP 5530 (Pothier et al. 2011a; 2011c). In the second year, a third Swiss strain, XA1.15 (Pothier et al.

2010), was added to the mix. The genetic diversity of *X. arboricola* pv. *pruni* being low (Boudon et al. 2005), strains were chosen for their diversity in origins and for their different years of isolation on different hosts in the attempt to inoculate the plants with an inoculum with a high genetic diversity (Pothier et al. 2011a). For long-term storage, strains were kept in 40 % glycerol at −80 °C.

Inoculation

Inoculum was prepared as described by Socquet-Juglard et al. (2012) and the inoculation procedure was modified as following: prior to inoculation, plants were put in dark conditions, 23 °C and 90 % humidity for 24 h for an optimal stomata opening (pre-conditioning); the whole plant material was randomly divided into three blocks in the same quarantine greenhouse chamber and inoculation took place during 3 days. To avoid possible block effects as observed in the 2010 inoculation, the pre-conditioning step was not performed in the 2011 inoculation and all plants were inoculated on the same day. Actively growing shoots (10 to 20 cm long) were immersed in the bacterial suspension, and gently agitated for about 5 s until leaf surfaces were fully wetted on both sides (Topp and Sherman 1995). In 2010, shoots of some plants were too long so they could not be entirely inoculated. In those precise cases, not inoculated leaves were marked and were not taken into account in the scorings. In autumn 2010, plants were cut back and in 2011, all leaves from young new shoots were inoculated. Following inoculation, climate conditions were set to 23 °C in the day, 18 °C in the night, and 85 % humidity.

Evaluation of disease resistance

Trees were assessed at 42 days post inoculation (dpi). The number of inoculated leaves per plant showing symptoms was counted and was expressed as disease incidence in percent (DI10 and DI11). During the third assessment in 2010 and in 2011, the percentage of damage of each inoculated leaf was assessed and used to calculate the tree resistance index (RI10 and RI11; adapted from Le Lézec et al. 1997) according to the following formula:

$$RI = [(n_1 \times 1) + (n_2 \times 2) + (n_3 \times 3) + (n_4 \times 4) + (n_5 \times 5)]/N$$

where $n_1$ is the number of symptomless leaves, $n_2$ is the number of leaves presenting 1 % to 25 % damage, $n_3$ 26 % to 50 %, $n_4$ 51 % to 75 %, $n_5$ 76 % to 100 % damage, and $N$ is the total number of inoculated leaves per plant.

DNA isolation

Young leaves were collected in May from orchard plants, frozen in liquid nitrogen, and stored at −80 °C. Leaf samples were lyophilized for 48 h. Genomic DNA was extracted using the QIAGEN DNeasy Plant Mini kit (Qiagen, Hilden, Germany). DNA quantity and quality was assessed with a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) before being used for both AFLP and SSR marker analysis.

SSR and AFLP analysis

One hundred and four SSR markers developed from apricot, peach, cherry and almond (Table 1) were selected to

**Table 1** Characteristics and origin of microsatellite (SSR) markers series used for the construction of the "Harostar" (Ha) and "Rouge de Mauves" (RM) linkage maps

| Marker designation | Species | Tested | Amplified | Polymorphic | Mapped Ha | Mapped RM | Common SSRs | Reference |
|---|---|---|---|---|---|---|---|---|
| AMPA | *P. armeniaca* | 15 | 15 | 11 | 6 | 6 | 4 | Hagen et al. (2004) |
| Aprigms | *P. armeniaca* | 4 | 3 | 3 | 0 | 3 | 0 | Vilanova et al. (2006) |
| BPPCT | *P. persica* | 11 | 9 | 7 | 5 | 5 | 3 | Dirlewanger et al. (2002) |
| CH-PP | *P. persica* | 2 | 1 | 1 | 1 | 1 | 1 | This study |
| CPPCT | *P. persica* | 3 | 2 | 2 | 1 | 0 | 0 | Aranzana et al. (2002) |
| CPSCT | *P. salicina* | 1 | 1 | 0 | 0 | 0 | 0 | Mnejja et al. (2004) |
| MA | *P. persica* | 1 | 1 | 1 | 1 | 1 | 1 | Yamamoto et al. (2002) |
| Pac | *P. armeniaca* | 5 | 5 | 4 | 3 | 3 | 2 | Decrooq et al. (2003) |
| PaCITA | *P. armeniaca* | 12 | 12 | 11 | 9 | 7 | 6 | Lopes et al. (2002) |
| Pchgms | *P. persica* | 1 | 1 | 1 | 1 | 1 | 1 | Sosinski et al. (2000) |
| PS | *P. cerasus* | 1 | 1 | 0 | 0 | 0 | 0 | Sosinski et al. (2000) |
| UDAp | *P. armeniaca* | 44 | 42 | 38 | 34 | 24 | 21 | Messina et al. (2004) |
| UDP | *P. persica* | 4 | 2 | 2 | 2 | 2 | 2 | Cipriani et al. (1999); Testolin et al. (2000) |
| | Total | 104 | 95 | 81 | 63 | 53 | 41 | |

regularly span the whole genome, according to the genetic map of Dondini et al. (2007) and the *Prunus* reference map T × E (Joobeur et al. 1998; Howad et al. 2005). To cover the top of LG7, an additional marker was developed from the Genome Database for Rosaceae (GDR) with the following primer sequences: CH-PP-01: F5′ GTCACGTTCAAAGTCCTGC 3′ and R5′ CAGAATCAGCTCCTGGTA 3′. All markers were first screened for polymorphism within and between the two parents by testing the two parents and five progeny plants. Two different methods for PCR amplification were used. One set of markers was amplified with FAM or HEX dye-labeled forward primers. In this case, PCR conditions were 94 °C for 15 min, 35 cycles of 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 75 s; and a final extension step of 72 °C for 5 min. For the second set, markers were amplified using M13-fam, SP6-hex, T7-atto565, and E31-atto550 universal primers as described by Schuelke (2000). In this case, 8 cycles of 94 °C (30 s), 53 °C (45 s), and 72 °C (45 s), and a final extension of 10 min followed the 35 cycles previously described for the markers of the first set. PCR amplifications were performed in 10 μl reaction mixture containing 5 μl Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 1 μl Q-solution, 10 nM of primer mix, 10 ng genomic DNA, and 2 μl dH$_2$O.

AFLP analysis was performed using protocol described by Vos et al. (1995) and modified by Habera et al. (2004) for fluorescent labeling, using the 6-FAM dye. The combination of the restriction enzymes *Eco*RI and *Mse*I was used. Five *Eco*RI+2-*Mse*I+2 primer combinations (Table 2) were tested for reproducibility on 10 individuals; because they amplified more than 25 polymorphic fragments per combination, they were used to screen the rest of the progeny. All polymorphic bands in the range 50–500 bp were scored visually for presence or absence. AFLP markers were named in function of the length of the fragment obtained in addition to the name of the primer combination used.

AFLP and SSR fragments were diluted in dH$_2$O (1:100) and mixed with 8.8 μl of formamide and 0.2 μl GeneScan 500 LIZ size standard (Applied Biosystems, Foster City, California, USA). Samples were denatured for 5 min at 95 °C and immediately cooled at −20 °C for 3 min. Amplified PCR products were separated on an ABI 3130 sequencer (Applied Biosystems) and scored using the software GeneMapper version 4.1 (Applied Biosystems).

Linkage mapping and QTL analysis

Linkage analysis was performed using JoinMap 4.0 software (van Ooijen 2006). The pseudo-test cross strategy was chosen using JoinMap CP (cross pollinating function) to obtain two separate parental maps. Linkage groups were established using a minimum log of odds (LOD) of 5.0 for parent "Harostar" and 4.0 for parent "Rouge de Mauves"; and a maximum recombination fraction of 0.40. Kosambi's mapping function was used to calculate map distances. Markers with distorted segregation, determined by the $\chi^2$ analysis, were first included in the analysis; if those markers created severe conflicts with the segregation pattern of markers located in the same area, they were excluded. Nomenclature and orientation of all linkage groups were based on the maps published by Dondini et al. (2007) and on the *Prunus* reference map (T × E, Joobeur et al. 1998).

Kruskal–Wallis (KW) tests and interval mapping (IM) analysis, as well as multiple-QTL analysis (MQM), were performed with MapQTL 5.0 (van Ooijen 2004). A permutation test (1,000 permutations) was performed to calculate the appropriate LOD score thresholds. A 5 % genome-wide error rate was chosen and all values above each trait threshold were considered significant.

Statistical analysis

In 2010, inoculation was performed on three consecutive days leading to three blocks of plants, thus data produced in 2010 were checked for progeny × inoculation date effect. In 2011, inoculation occurred on the same day; however, new plants were added to replace the dead ones so data were verified for progeny × plant age effect. In order to remove the inoculation date effects of the first year and the plant age effect of the second year, but also to allow comparison between traits over

**Table 2** AFLP combinations tested and number of amplicons mapped in the apricot parents "Harostar" (Ha) and "Rouge de Mauves" (RM)

| Primer combination | Primer designation[a] | Fragments detected | Polymorphic fragments in Ha | Polymorphic fragments in RM | Total polymorphic fragments | Total mapped fragments |
|---|---|---|---|---|---|---|
| EAG-MAC | E13-M12-x | 161 | 16 | 11 | 27 | 25 |
| EAG-MAG | E13-M13-x | 162 | 18 | 14 | 32 | 30 |
| EAC-MAC | E12-M12-x | 147 | 14 | 11 | 25 | 23 |
| EAC-MAG | E12-M13-x | 157 | 13 | 17 | 30 | 24 |
| EAG-MCT | E13-M18-x | 136 | 28 | 10 | 38 | 34 |
| Total | | 763 | 89 | 63 | 152 | 136 |
| Average | | 152.6 | 17.8 | 12.6 | 30.2 | 27.4 |

[a] -x designates the size of the polymorphic band

2 years and to combine datasets from both years, a standardization procedure was performed for each plant in both years datasets following the formula:

$$X_{std} = (X - Xm)/\text{sd}$$

where in 2010 $X_{std}$ is the standardized value of the plant, $X_m$ is the mean of the values collected for a specific inoculation date to which plant $X$ belongs to, and sd is the standard deviation of the value of this subset; while in 2011 $X_{std}$ is the standardized value of the plant, $X_m$ is the mean of the values of the "old" or "new" plants to which $X$ belongs to, and sd is the standard deviation of the value of this subset. Outliers were checked before combining datasets of both years using Jackknife distances for each genotype and removed. The distributions of each variable were finally tested for normality. Broad-sense heritability was estimated using the following formula:

$$h^2 = \sigma_g^2 / \left[ \sigma_g^2 + (\sigma_e^2/n) \right]$$

where $\sigma_g^2$ is genetic variance, $\sigma_e^2$ is environmental variance, and $n$ is the mean number of replicates per genotype (Foulongne et al. 2003; Calenge et al. 2004). Frequency distributions, segregation models, one way ANOVA, and Student's $t$ tests were calculated for each assessment using software JMP®v.8.0 (SAS Institute Inc, Carey, NC, USA).

Candidate gene search

Based on the peach genome sequence predicted genes list available at http://www.rosaceae.org/peach/genome, all genes located on scaffold 5 between markers BPPCT037 and BPPCT038A were selected and a search for genes involved in disease resistance was manually performed by inspection of the genome annotations. A special interest was given to disease resistance genes, but also to receptor-like kinases containing leucin-rich repeats (LRR) because they are known to be involved in tomato resistance to *X. campestris* pv. *vesicatoria* (Mayrose et al. 2004) and in rice resistance to *X. oryzae* pv. *oryzae* (Song et al. 1995; Xiang et al. 2006). Kinases could also play a role in soybean resistance to *X. axonopodis* pv. *glycines* (Kim et al. 2011) and in cassava resistance to *X. axonopodis* pv. *manihotis* (Perez-Quintero et al. 2012). Sequences of each gene were then analyzed with BLASTX (http://blast.ncbi.nlm.nih.gov) in order to obtain updated annotations.

## Results

Phenotyping *X. arboricola* pv. *pruni* resistance

In 2010, first symptoms appeared at 1 week post-inoculation and were recognizable as primarily circular yellow spots;

the first necrosis appeared 1 week later. Severe defoliations occurring in the inoculated part of the shoots were observed for the most susceptible genotypes at 42 dpi. In 2011, grayish zones spanning large parts of basal leaves were observed at 1 week post-inoculation; these zones became necrotic and most heavily affected leaves dropped during the time of the experiment. At 42 days post inoculation, disease incidence varied from 11.8 % to 85.3 % in 2010 and from 3.6 % to 82.7 % in 2011 (data not shown).

The ANOVA performed to test the effects of the date of inoculation the first year and the effect of the age of the plant (grafted in 2010 or grafted in 2011) during the second year revealed significant differences among those blocks for both disease incidence and resistance index, and therefore, data were standardized. When dividing the block of plants grafted in 2010 according to genotypes with one to several dead plants, or according to genotypes without any dead plant, no significant difference of the average values was obtained between these two sub-groups (Fig. 1).

Correlation coefficients between the two different standardized resistance traits were high ($P<0.001$, Table 3) in each of the 2 years; a correlation for the same trait per genotype between the two consecutive years could be also observed ($P<0.05$, Table 3). When combining datasets of the two years, both parents had in all cases higher values than the F1 average, showing transgressive segregations in the progeny (Fig. 2).
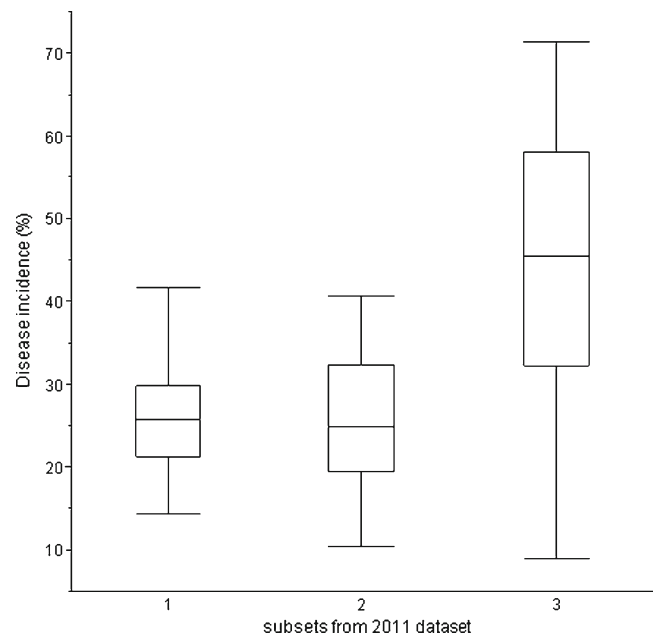


**Fig. 1** Effects of the age of the plant on disease incidence in 2011. *1* mean disease incidence value of the genotypes for which no plant died in 2010 (plants grafted in 2010); *2* mean disease incidence value of the genotypes for which at least one plant died in 2010 (plants grafted in 2010); *3* mean disease incidence value of the plants of the same genotypes as in *2*, but grafted in 2011. *1* and *2* were not significantly different by Student's t test ($P<0.05$) but both were significantly different from *3*

**Table 3** Pearson correlation coefficients between all *Xanthomonas arboricola* pv. *pruni* resistance traits in the "Harostar" × "Rouge de Mauves" progeny

|         | DIstd   | DI10std | DI11std | RIstd   | RI10std |
|---------|---------|---------|---------|---------|---------|
| DIstd   |         |         |         |         |         |
| DI10std | 0.69**  |         |         |         |         |
| DI11std | 0.79**  | 0.21*   |         |         |         |
| RIstd   | 0.91**  | 0.61**  | 0.74**  |         |         |
| RI10std | 0.67**  | 0.85**  | 0.22*   | 0.75**  |         |
| RI11std | 0.73**  | 0.15    | 0.89**  | 0.80**  | 0.23*   |

Years are indicated in names as *10* or *11*; RIstd and DIstd are values obtained with both datasets combined

*DI* disease incidence, *RI* resistance index, *std* standardized data

*$P<0.05$; **$P<0.001$

Construction of linkage maps

Five AFLP primer combinations and 104 SSR markers were tested for polymorphism to construct the two parental linkage maps (Tables 1 and 2). The five AFLP combinations gave a total of 763 fragments; 152 were polymorphic and 136 of them were mapped. Eighty-nine AFLP fragments were polymorphic in "Harostar", 5 of them remained ungrouped and 3 were excluded because they created tensions in the maps; 63 were polymorphic in "Rouge de Mauves", 4 of them remained ungrouped and 4 were excluded (Table 2). Ninety-four (90.4 %) SSR markers produced amplicons; of those, 14 (14.9 %) were monomorphic for both parents and 3 were not used because they had an "hk × hk" segregation type. Two SSR markers (PaCITA11 in "Rouge de Mauves" and AMPA123b for "Harostar") were excluded because they created tensions in the maps. Forty-one SSR markers were shared between the maps of the 2 parents "Harostar" and "Rouge de Mauves", 22 were mapped only in "Harostar" while 12 were mapped only in "Rouge de Mauves". Five microsatellite markers were multilocus, three of them,

UDAp-428 (Ha_LG2), UDAp-433 (Ha_LG6), and UDAp-424 (RM_LG7), mapping both loci on the same linkage group on a very close genetic distance. UDAp-471 amplified one locus on LG8 in "Rouge de Mauves" and one locus on LG7 in "Harostar". UDAp-424 mapped only one locus in "Harostar" (LG7), and UDAp-433 mapped only one locus in "Rouge de Mauves" (LG6). Finally, PaCITA10 was multilocus only in "Harostar" (LG3 and 4).

The number of marker loci per linkage group ranged from 11 to 20 in "Harostar" and from 3 to 21 for "Rouge de Mauves". The average distances between two consecutive loci were 4.9 cM for "Harostar" and 6.9 cM for "Rouge de Mauves". The "Harostar" linkage map of 553.6 cM in total was composed by the expected 8 linkage groups (Fig. S1). The two largest gaps (24 and 26 cM) that could not be filled with markers were located on linkage groups 1 and 7. Concerning the genetic map of "Rouge de Mauves", it was composed of nine linkage groups and its total map length was 684 cM (Fig. S2). The additional group is due to the splitting of LG2 between markers UDAPp-428 and UDAp-457. None of the 10 SSR markers tested to try to fill this gap were polymorphic for this parent. Two gaps of 30 and 34 cM were observed on linkage groups 1 and 7.

QTL analysis

Broad-sense heritability values ranged from 0.96 for the disease incidence to 0.97 for the resistance index obtained with combined datasets. The genome-wide LOD thresholds calculated by the permutation tests ranged from 2.4 to 2.5 (data not shown) and therefore all QTLs with a LOD score higher than 2.5 were considered as significant ($P<0.05$). The MQM analysis did not lead to the detection of other QTLs, hence only results of Kruskal–Wallis and QTLs found by interval mapping are presented.

The Kruskal–Wallis test identified three markers of "Rouge de Mauves" linked ($P<0.005$) to both traits disease
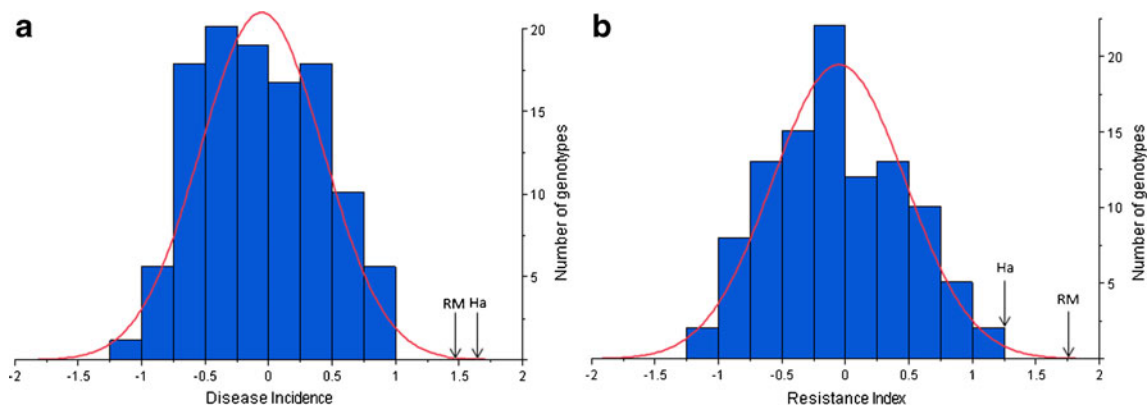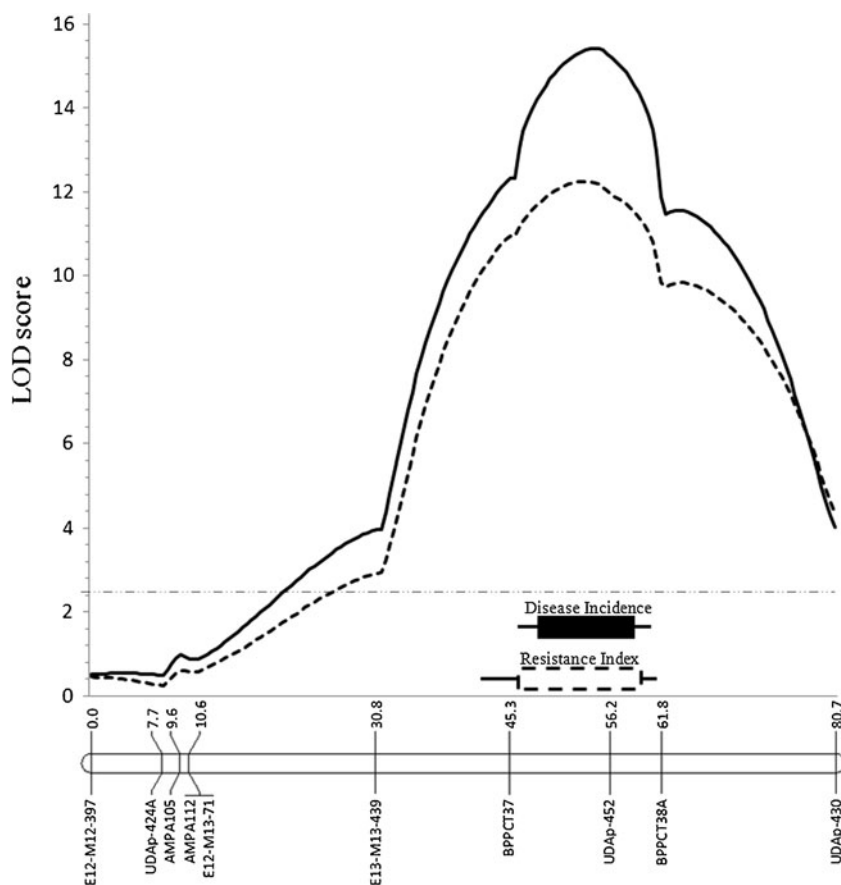


**Fig. 2** Distribution in the 101 F1 progeny of **a** the disease incidence and **b** the resistance index with standardized data of both years combined at 42 days post inoculation. Parental values (*Ha* for "Harostar" and *RM* for "Rouge de Mauves") are indicated by *arrows*

incidence and resistance index on linkage group 1 (LG1; BPPCT011), LG5 (UDAp-452), and LG7 (Ma10a). Marker UDAp-446 on LG3 was only linked to resistance index. Two QTLs were identified by interval mapping in the same region of linkage group 5 of "Rouge de Mauves" for disease incidence (LOD score of 15.4 and 53.6 % of phenotypic variation explained, PVE) and for the resistance index (LOD 12.3, and 46.2 % of the PVE; Fig. 3 and Table 4). The peak of the QTL for both traits is between the SSR markers BPPCT037 and BPPCT038A with the marker UDAp-452 being basically at the peak position. The QTL peak had a maximum likelihood position at 52.8 cM for the resistance index and at 54.3 cM for the disease incidence. These two QTLs found with the pooled data were also detected by using separately the 2010 and 2011 datasets (data not shown). No other significant QTL (at LOD 2.5 or higher) was detected on any other linkage group of "Rouge de Mauves".

For "Harostar", the Kruskal–Wallis identified only marker E12-M13-278 on LG8 as significantly linked to both resistance traits, the other markers were either linked to disease incidence (PaCITA7 on LG1, E13-M13-380 on LG2 and UDP98-412 on LG6) or to resistance index (UDAp-446 on LG3 and UDAp-430 on LG5). No significant QTL was detected in "Harostar" by interval mapping.

To check for the allelic effects of the QTL obtained, we considered only the subset of the disease incidence obtained for the old plants in 2011; in order to avoid age effect which would require a standardization and would render more difficult the interpretation of the results, all data obtained from the new plants added in 2011 were excluded. A significant difference was observed ($P<0.0001$) between the untransformed means of disease incidence (Fig. 4a) and resistance index (Fig. 4b) for the two subpopulations obtained when dividing for the allelic effects of the SSR marker UDAp-452. The disease incidence was 21 % for the subgroup of genotypes possessing the favorable allele, whereas for the subgroup with the negative allele the disease incidence was 32.6 %, leading to a reduction of 35.6 % in disease incidence for the subgroup possessing the favorable allele. Concerning the resistance index, mean values obtained for the subgroup with the favorable allele was 1.3 and the subgroup possessing the negative allele had a mean value of 1.48, showing a reduction of 37.5 % of susceptibility for the subgroup possessing the favorable allele.

Candidate genes search

A total of 448 genes were found between markers BPPCT037 and BPPCT038A, and 6 of these, encoding for



Fig. 3 Location of QTLs on LG5 involved in resistance against *Xanthomonas arboricola* pv. *pruni* in "Rouge de Mauves" by combining datasets of both years. *Dotted horizontal line* represents the genome-wide threshold (LOD of 2.5); *solid curve* shows the QTL for disease incidence and *dotted curve* shows the QTL for resistance index. Confidence intervals of 2-LOD are represented with *lines*. *Boxes* represent a 1-LOD confidence interval. *Box with solid line* represents the QTL for disease incidence, and *dotted box* represents the QTL for resistance index

**Table 4** Summary of the QTLs for resistance against *Xanthomonas arboricola* pv. *pruni* detected in the progeny "Harostar" × "Rouge de Mauves" by Kruskal–Wallis test and interval mapping

| Trait | LG[a] | Position | Marker[b] | KW[c] | LOD[d] | PVE[e] | Allele size[f] | | Allele effect[f] | |
|-------|-------|----------|-----------|-------|--------|--------|------|------|------|------|
| | | | | | | | 0 | 1 | 0 | 1 |
| DIstd | Ha1 | 74.17 | PaCITA7 | 8.67** | – | – | 196 | 213 | 0.13 | −0.23 |
| DIstd | RM1 | 135.3 | BPPCT011 | 8.77** | – | – | 0 | 196 | 0.09 | −0.29 |
| DIstd | Ha2 | 25.12 | E13-M13-380 | 10.08** | – | – | – | – | −0.24 | 0.16 |
| DIstd | RM5 | 54.3 | UDAp-452 | 52.34*** | 15.43 | 53.6 | 174 | 184 | 0.21 | −0.50 |
| DIstd | Ha6 | 48.99 | UDP98-412 | 8.26** | – | – | 136 | 140 | −0.22 | 0.10 |
| DIstd | RM7 | 24.64 | Ma10a | 7.62* | – | – | 124 | 126 | 0.11 | −0.22 |
| DIstd | Ha8 | 12.96 | E12-M13-278 | 10.13** | – | – | – | – | −0.36 | 0.08 |
| RIstd | RM1 | 135.3 | BPPCT011 | 8.59* | – | – | 0 | 196 | 0.09 | −0.28 |
| RIstd | Ha3 | 10.11 | UDAp-446 | 10.15** | – | – | 135 | 168 | −0.26 | 0.14 |
| RIstd | RM3 | 29.35 | UDAp-446 | 7.52* | – | – | 135 | 154 | −0.20 | 0.14 |
| RIstd | Ha5 | 53.42 | UDAp-430 | 7.03* | – | – | 178 | 200 | −0.17 | 0.11 |
| RIstd | RM5 | 52.8 | UDAp-452 | 43.98*** | 12.26 | 46.2 | 174 | 184 | 0.21 | −0.49 |
| RIstd | RM7 | 24.64 | Ma10a | 9.98** | – | – | 124 | 126 | 0.15 | −0.30 |
| RIstd | Ha8 | 12.96 | E12-M13-278 | 7.02* | – | – | – | – | −0.30 | 0.04 |

DI and RI: QTLs detected for the disease incidence and resistance index using a combination of standardized datasets from both years

[a] Parental map (*RM* "Rouge de Mauves", *Ha* "Harostar") and linkage group on which marker was linked to resistance

[b] Molecular marker closest to the peak of each QTL

[c] Kruskal–Wallis score; *P* values are presented (*$P<0.005$; **$P<0.001$; ***$P<0.0001$)

[d] LOD (logarithm of odds ratio) score determined by interval mapping

[e] Phenotypic variance explained by the peak of the LOD score in %

[f] Allele sizes and their respective mean phenotypic score of progeny carrying the *0* or *1* allele. Favorable allele is the lowest value

receptor-like protein kinases, LRR proteins or disease resistance proteins (Table 5), could be involved in primary sources of resistance. Two genes similar to disease resistance protein At1g58400 and another one to the F-box/LRR-repeat protein At5g63520 have large introns (more than 1,000 bp) and are therefore present twice in Table 5.

**Fig. 4** **a**, **b** Allelic effects of the disease incidence (**a**) and resistance index (**b**) for "Rouge de Mauves" at marker locus UDAp-452 with untransformed values obtained from the old plants in 2011. Favorable alleles are represented by a *1* and unfavorable alleles are represented by a *0*. Standard errors are represented. Means sharing a letter were not significantly different using a *t* test ($P<0.05$)
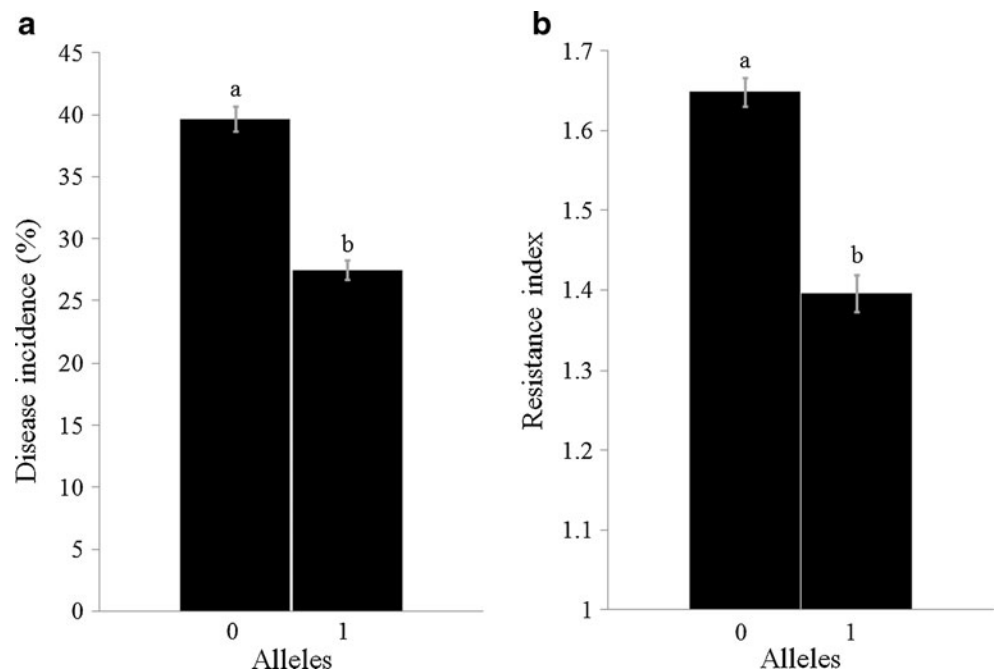
**Table 5** Candidate genes involved in putative resistance between markers BPPCT037 and BPPCT038A of LG5 in peach with their best BLASTX alignments and putative functions of the genes based on Genbank annotations

| Peach genome locus tag | Transcript start | Transcript stop | Genbank accession | Organism | Most similar protein sequence | Total score | Query coverage | E value | Maximum identity |
|---|---|---|---|---|---|---|---|---|---|
| ppa002786m | 14,204,987 | 14,206,985 | XP_002276125 | *Vitis vinifera* | LRR receptor-like serine/threonine/tyrosine-protein kinase SOBIR1 | 840 | 94 % | 0 | 71 % |
| ppa000993m | 14,614,860 | 14,617,720 | XP_002278672 | *V. vinifera* | LRR receptor-like serine/threonine-protein kinase GSO1 | 1285 | 99 % | 0 | 73 % |
| ppa001911m | 13,804,474 | 13,807,656 | XP_002279697 | *V. vinifera* | Probable inactive LRR receptor-like protein kinase At3g03770-like | 785 | 75 % | $3e^{-159}$ | 81 % |
| ppa017731m | 14,474,974 | 14,476,131 | XP_002278135 | *V. vinifera* | Putative disease resistance protein At1g58400-like | 417 | 96 % | $2e^{-137}$ | 64 % |
| ppa023827m | 14,472,683 | 14,473,351 | XP_002278135 | *V. vinifera* | Putative disease resistance protein At1g58400-like | 246 | 95 % | $7e^{-74}$ | 64 % |
| ppa015025m | 12,471,703 | 12,473,070 | XP_002276590 | *V. vinifera* | Probable disease resistance protein At5g66900 isoform 1 | 164 | 80 % | $7e^{-41}$ | 36 % |
| ppa004403m | 13,602,452 | 13,606,441 | XP_003523154 | *Glycine max* | F-box/LRR-repeat protein At5g63520-like | 610 | 40 % | $4e^{-32}$ | 74 % |
| ppa019969m | 13,598,585 | 13,601,215 | XP_002283895 | *V. vinifera* | F-box/LRR-repeat protein At5g63520-like | 254 | 47 % | $2e^{-20}$ | 41 % |

## Discussion

### Phenotypic screenings

Resistance to bacterial leaf spot of "Harostar" has been described as "good" in field trials (Layne and Hunter 2003), equaling the ones of "Veecot", "Haroblush", and "Harogem" cultivars. Under controlled conditions, in both years "Harostar" showed very few necroses per leaf although most of the leaves were symptomatic. As a consequence, its disease incidence was higher than the average observed in the progeny plants, equaling scores obtained for the second parent. No information about the resistance level to bacterial spot of "Rouge de Mauves" was available at the beginning of the project. The results of our inoculations showed that both parents were moderately susceptible under greenhouse conditions. This small phenotypic difference obtained between the parents was not expected, but a transgressive segregation was observed and skewed towards resistance, indicating that alleles from both parents are contributing to an increase of resistance against the pathogen. Student's *t* tests conducted with the genotypes for which one to several plants died in 2010 showed that the increase in resistance observed on the older plants in 2011 in comparison to the new plants was due to a plant age effect rather than from a genetically explained susceptibility, and that

plants which died in 2010 died for other reasons than the susceptibility to the disease (Fig. 1). Standardization of the datasets was consequently a necessary step to combine the set of older plants with the new ones. Differences that have occurred between both experiments, such as the addition of a new *X. arboricola* pv. *pruni* strain into the inoculum mix, or the differences in pre-conditioning did not significantly changed the disease reactions of the plants (Table 3) and permitted to pool the datasets obtained from both years, allowing a more precise QTL detection.

### Linkage analysis and map construction

The use of multiplex and megaplex PCRs for SSR analysis as previously reported for apple, barley, wheat, apricot, and cherry (Patocchi et al. 2008; Hayden et al. 2008; Campoy et al. 2010) permitted to efficiently screen many markers in a limited time and at a reduced price. In addition, the use of fluorescent labeling and the combination of E primer and M primer with only two selective nucleotides instead of three for the AFLP analysis has been particularly efficient and permitted to obtain on average 30 polymorphic fragments per combination, whereas with the use of polyacrylamide gels and primer combinations of three selective nucleotides for one primer and two for the second primer, Vilanova et al. (2003) obtained about eight polymorphic bands per

combination and Lalli et al. (2008) obtained nine polymorphic bands.

The genetic linkage map of "Harostar" had the expected 8 linkage groups; whereas the linkage map of "Rouge de Mauves" had one additional linkage group that could be assigned to LG2 (Figs. S1 and S2). Despite the ten microsatellite markers used to try to fill this gap and the relatively high number of AFLPs mapped, we could not join the two fragments into a single chromosome. Failures to obtain the eight linkage groups have been previously reported in *Prunus* (Dirlewanger et al. 2006; Illa et al. 2009; Eduardo et al. 2011). An explanation could be a large homozygous region on the LG2 of "Rouge de Mauves" either derived from selfing or from the use of the same cultivar in the breeding process of this parent. However, both hypotheses cannot be tested since the pedigree of "Rouge de Mauves" is unclear.

Four markers mapped in new regions, three of them were never reported as multilocus: PaCITA10 which mapped on LG3 and LG4, UDAp-433 which mapped twice on LG6 and UDAp-471 which mapped on LG7 and LG8. The fourth SSR, UDAp-486, is normally known to map on LG6, but it has been found in our case only on the bottom of LG4 of the "Harostar" map. This could be another multilocus marker, as one peak was present in both parents but did not segregate in our population. Total lengths of our maps (553 cM for "Harostar"; 684 cM for "Rouge de Mauves") are in the average of those reported in apricot (504 cM for "Lito" and 620 cM for "BO81604311", Dondini et al. 2007).

QTL analysis

Although resistance traits were normally distributed and that several regions on both parents were found to be linked with the resistance traits by Kruskal–Wallis, indicating a polygenic control of the trait, only one significant QTL involved in disease resistance was found by both Kruskal–Wallis and interval mapping tests in "Rouge de Mauves". Mapped on LG5, this QTL accounted for 53 % of the phenotypic variation explained for the disease incidence and for 46 % of the phenotypic variation explained for the resistance index. The peak of the QTL is located very close to marker UDAp-452, in a region downstream the minor QTL involved in sharka resistance (Lambert et al. 2007) and upstream a major QTL involved in powdery mildew resistance (Foulongne et al. 2003; Lalli et al. 2005). Several possibilities could explain why we did not find any significant QTL by interval mapping in "Harostar", such as a too small size of the population, which did not permit to have a statistical analysis powerful enough to identify QTLs having a small contribution to the variation of the traits. Other possibilities for a lack of detection of QTLs could be the interaction between QTLs or the genetic background of the parents

(e.g., epistasis) or a too small difference in effect of two alleles of a QTL. The use of a mix of bacterial strains in the inoculum might also have hindered the detection of strain-specific QTLs to the advantage of a strain non-specific QTL. Finally, the QTL on LG5 was found on a moderate susceptible parent, but this phenomenon is not uncommon and has been reported in different plant species (Pilet et al. 1998; Wang et al. 2000; Calenge et al. 2004).

Perspectives for marker-assisted selection (MAS)

Accounting for both disease incidence and severity, resistance index was thought to be the trait that could represent best the difference between susceptibility and resistance against *X. arboricola* pv. *pruni*. Therefore in resistance breeding, QTLs revealed by this trait should be the most interesting. A reduction of both factors is expected to reduce the negative impact of the disease from an epidemiological point of view, resulting in a lower economic damage. However both traits resistance index and disease incidence were highly correlated and led to the detection of a QTL on the same locus, although the QTL for resistance index was less precise with a larger LOD confidence interval. This may be due to the fact that resistance index is based on a visual score of the damage on the leaves, which may lead to a bias although extra care was taken during evaluations, in contrary to the disease incidence.

Our results highlight a region on LG5 of "Rouge de Mauves" accounting for 53 % of the phenotypic variation explained by the disease incidence QTL with SSR marker UDAp-452 being near the peak position. Its two flanking markers BPPCT037 and BPPCT038A have a high polymorphism in both cherry and peach (Dirlewanger et al. 2002), which increase the chance of being polymorphic in different backgrounds. The differences that occurred between the experiments conducted in 2 years did not lead to significant changes, even with the addition of a new bacterial strain, showing that this region is stable. Therefore the proposed markers upon validation in a different background can be used in a MAS program in which breeders could be interested in both a reduced disease incidence and a reduction of the damage on leaves.

Understanding gene functions and molecular mechanisms underlying the resistance is of importance. The release of the peach genome sequence (International Peach Genome Initiative: Peach Genome v1.0. 2010 http://www.rosaceae.org/peach/genome) permitted to perform a candidate gene search between the two flanking markers BPPCT037 and BPPCT038A. Kinases are known to play an important role in plant defense response and have been identified in different pathosystems involving different *Xanthomonas* species (Song et al. 1995; Mayrose et al. 2004; Kim et al. 2011; Perez-Quintero et al. 2012).

Between the two flanking markers three putative genes encoding LRR receptor-like serine/threonine kinases were found using the peach genome annotations; in addition, two genes encoding putative disease resistance proteins and one encoding a putative F-box/LRR-repeat protein could be found in this region. Although peach and apricot genomes are highly synthenic and co-linear (Dondini et al. 2007; Illa et al. 2009; Illa et al. 2011), these results have to be taken with care since differences such as insertions, deletions, or inversions between both genomes cannot be excluded. Another approach to identify target genes involved in disease resistance could be the use of transcriptomic tools available such as microarrays or RNA-seq (Martínez-Gómez et al. 2011) and to consider pathogen virulence factors as related to plant interactions (Hajri et al. 2012).

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