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ORIGINAL PAPER

Genetic mapping of two QTL from the wild tomato *Solanum pimpinellifolium* L. controlling resistance against two-spotted spider mite (*Tetranychus urticae* Koch)

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Abstract A novel source of resistance to two-spotted spider mite (*Tetranychus urticae* Koch) was found in *Solanum pimpinellifolium* L. accession TO-937 and thereby a potential source of desirable traits that could be introduced into new tomato varieties. This resistance was found to be controlled by a major locus modulated by minor loci of unknown location in the genome of this wild tomato. We first applied a bulked segregant analysis (BSA) approach in an F_4 population as a method for rapidly identifying a genomic region of 17 cM on chromosome 2, flanked by two simple sequence repeat markers, harboring *Rtu2.1*, one of the major QTL involved in the spider mite resistance. A population of 169 recombinant inbred lines was also evaluated for spider mite infestation and a highly saturated genetic map was developed from this population. QTL mapping corroborated that chromosome 2 harbored the *Rtu2.1* QTL in the same region that our previous BSA findings pointed out, but an even more robust QTL was found in the telomeric region of this chromosome. This QTL, we termed *Rtu2.2*, had a LOD score of 15.43 and accounted for more than 30 % of the variance of two-spotted spider mite resistance. Several candidate genes involved in trichome formation, synthesis of trichomes exudates and plant defense signaling have been sequenced. However, either the lack of polymorphisms between the parental lines or their map position, away from the QTL, led to their rejection as candidate genes responsible for the two-spotted spider mite resistance. The *Rtu2* QTL not only serve as a valuable target for marker-assisted selection of new spider mite-resistant tomato varieties, but also as a starting point for a better understanding of the molecular genetic functions underlying the resistance to this pest.

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Introduction

All commercial varieties of tomato, *Solanum lycopersicum* L., are susceptible to two-spotted spider mite, *Tetranychus urticae* Koch, a serious tomato pest in temperate regions. The spider mites suck the contents of the plant cells, causing tiny pale spots or scars where the epidermal cells have been destroyed. Later stages of the infestation entail chlorosis, defoliation and even plant death, which contribute to a significant crop loss (Tomczyk and Kropczynska 1985). Even in less severe cases, spider mites feeding on the fruits originate scars that depreciate the value of the tomato production (Fig. 1). Control strategies such as the application of chemicals or other agricultural practices are, in most of the cases, partially effective or completely ineffective as two-spotted spider mites have become resistant to several of these agents due to the species' high reproductive potential and extremely short life-cycle, which combined with frequent acaricide applications, facilitates its resistance build-up (Van Leeuwen et al. 2005). Failures in the chemical control of *T. urticae* caused by the occurrence of new resistances to diverse

compounds, such as dicofol, chlorfenapyr, fenbutatin, bifenthrin and more recently to organophosphates have been reported in several countries (Van Leeuwen et al. 2005; Van Leeuwen and Tirry 2007; Jahangir et al. 2010). Moreover, trends in modern pest management include lowering pesticide use, not only to reduce costs, but also to minimize environmental impact. Although two-spotted spider mite-resistant *S. lycopersicum* accessions have not yet been identified, growing resistant cultivars should be one of the best solutions to minimize the problems caused by this pest.

Potential sources of resistance to spider mite have been identified within wild related species of tomato such as *S. pennellii* Correl (Saeidi et al. 2007), *S. habrochaites* S. Knapp and D.M. Spooner (Snyder et al. 2005) and *S. habrochaites* f. glabratum C.H. Müll. (Carter and Snyder 1985; Chatzivasileiadis and Sabelis 1997). The density of type IV and VI glandular trichomes and the presence of volatile compounds in trichome gland secretions have been described as factors mediating this resistance in some accessions of *S. habrochaites* (Guo et al. 1993) and *S. pennellii* (Blauth et al. 1998; Maluf et al. 2010; Saeidi et al. 2007). Unfortunately, crosses between cultivated tomato and these wild relatives often result in a progeny bearing many undesirable traits inherited from the wild parent, such as small and hairy-green fruits and the presence of trichomes other than those conferring resistance. Therefore, although *S. pennellii* and *S. habrochaites* are cross-compatible with cultivated tomato species (Rick 1979), as far as we know, no spider mite-resistant cultivar has been developed for commercial use from these wild species as donors of the resistance.

We found a novel source of resistance to the two-spotted spider mite in *S. pimpinellifolium* L. accession TO-937 (Fernandez-Muñoz et al. 2000). The advantages of accession TO-937 as a source of genetic resistance, compared with those described in other wild species, are that *S. pimpinellifolium* is a red-fruited species and it is very closely related to the cultivated tomato, the small size of *S. pimpinellifolium* fruits being its only undesirable characteristic. On the other hand, first results about the inheritance of resistance to the spider mite in S. *pimpinellifolium* TO-937, based on the analysis of F_2 populations, indicated that a major locus modulated by minor loci controls this trait (Fernandez-Muñoz et al. 2003) although spider mite-resistance has been repeatedly reported as polygenic in the other three taxa (Blauth et al. 1998, 1999; Saeidi et al. 2007). In addition, recent studies have uncovered the role played by acylsucrose accumulation and type IV glandular trichome density in the resistance of TO-937 to the two-spotted spider mite (Alba et al. 2009).

Whereas introducing disease resistance genes from wild relatives into cultivated tomato plants is becoming a routine procedure in breeding programs, introgressing arthropod or other pest resistances into cultivated tomato has been proved difficult and little or no progress has been reported. Keeping uniform conditions of infestation, together with linkage drag due to the genetic control of these resistances are the major impediments of tomato breeding for pest resistance, and therefore genetic progress has been quite limited (Foolad 2007). Such problems also affect the development of tomato breeding lines for resistance to two-spotted spider mite, their evaluation being a time-consuming, labor intensive and expensive process (Fernandez-Muñoz et al. 2000). Studies designed to determine the number and chromosomal location of the loci contributing to spider mite resistance are needed to diminish the breeding effort. The use of molecular markers and marker-assisted selection (MAS) techniques can facilitate introgression of this resistance. With this aim, using a BSA approach, we have identified two simple sequence repeat (SSRs) markers on chromosome 2 linked to resistance to the spider mite in an F_4 segregating population from an *S. lycopersicum* cv. Moneymaker × *S. pimpinellifolium* TO-937 cross. A quantitative analysis of the trait carried out in a recombinant inbred line (RIL) population derived from this cross has confirmed chromosome 2 as the chromosome harboring QTL significantly associated with the inheritance of spider mite-resistance in TO-937. In addition, the utility of the markers flanking the resistance QTL in MAS tomato breeding programs as well as the implication of candidate genes located in the same genomic region than the QTL are discussed.

Materials and methods

Plant material

An interspecific cross between *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium* accession TO-937 was made to produce F_1 seeds. Seeds derived from a single F_1 plant were sown to obtain an F_2 population. In order to reduce the inherent environmental variability associated with plant–insect interactions, two more advanced segregating populations were generated by single seed descent (SSD), an F_4 population and a set of RILs when the F_8 generation was reached. Seeds from the two parental genotypes, the F_1 hybrids, and six plants of each the F_4 or the F_8 families were sown to perform an evaluation of the spider mite attack in two different experiments. In each experiment, seeds were first individually sown in pots (60 ml) containing 15 % plant-nutrient loaded zeolite and 85 % coconut fiber substrate. Plantlets were grown in a glasshouse until they reached the 3–5 leaf stage. At this stage, 18 *S. pimpinellifolium* resistant parental plants, 18 F_1

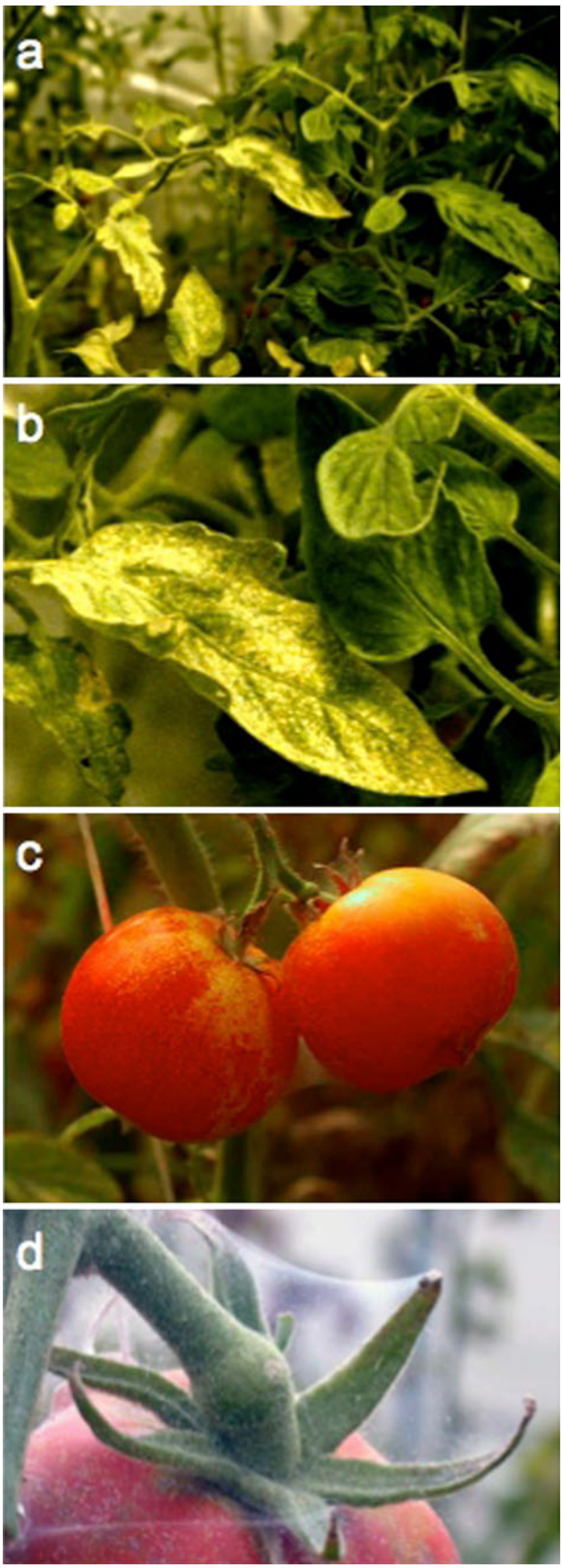

Fig. 1 Spider mite infestation symptoms in tomato plants. Infested ► cv. Moneymaker plants show chlorosis (*left* in **a** and **b**) whereas wild tomato accession TO-937 resistant plants remain green and healthy (*right* in **a** and **b**). Fruits of infested plants show characteristic brown scars that depreciate them (**c**). Severely infested plant showing silky web in sepals (**d**)

plants and the segregating plants were transplanted to soil in a plastic-house following a random distribution in which all these plants were surrounded by control susceptible plants of cv. Moneymaker as we previously described (Fernandez-Muñoz et al. 2003). The transplants were placed 0.4 m apart in a row whereas distance between rows was 1 m. Plants were watered at 3- to 4-day intervals and fed with soluble fertilizer once a week.

Spider mite infestation and resistance evaluation

Adult females of *T. urticae* were obtained from colonies reared on French bean plants (cv. F-15, Semillas Fitó, Barcelona, Spain) inside a growth chamber at 18–25 °C, 50 % RH and L16:D8. The colony originated in 1999 from mites collected on *Ricinus communis* L., *Convolvulus arvensis* L., and *Lavatera* spp. plants, located in the Experimental Station La Mayora-CSIC, Málaga, Spain. This population has repeatedly shown its ability to infest and grow on tomato plants.

Resistance to spider mite was estimated in two different assays by means of a test based on an experimental design to avoid overestimation of pest resistance and conducted in commercial greenhouse cultivation conditions (Fernandez-Muñoz et al. 2000). The first experiment included plants from the two parental lines, *S. lycopersicum* cv. Moneymaker and S. *pimpinellifolium*, the F_1 hybrid, as well as a subset of the 169 available F_4 lines. In the second experiment, all the 169 F_8 (RIL) lines were evaluated, together with 18 plants from the resistant parental line and the 18 F_1 hybrid plants. In order to reduce individual, environmental and methodological variability, six plants of each of the F_4 and F_8 lines, surrounded by control susceptible parental plants, were evaluated. The spider mite attack evaluation followed the protocol optimized by Fernandez-Muñoz et al. (2003). According to it, a previous controlled infestation was performed on plantlets in the 10- to 15-leaf stage and a second infestation was done 2 weeks later. Both infestations were done by placing on the shoot apex of each evaluated plant highly webbed pieces of French bean plants infested with more than 20 living *T. urticae* adult individuals and many nymphs, larvae and eggs. Evaluation of the degree of the two-spotted spider mite attack started 6 weeks after the first infestation. Inspection with a magnifying glass of the characteristic symptoms of damage, the number of mites and presence of silky webs was made on

all the leaves of four canopy heights of the tomato plants: basal (leaves 1 to 5), medium-basal (leaves 6 to 10), medium-apical (leaf 11 to 15) and apical (leaf 16 and above). On each canopy height, a spider mite attack index (SMAI) ranging from 0 to 5 was given considering 0 when no mites were found and 5 when severe leaf damage and large populations of spider mite causing intense webbing was observed (Fernandez-Muñoz et al. 2003). A mean SMAI value was assigned to each plant by calculating the average of the four canopy heights SMAI values per plant. More than 1,000 plants of the tomato susceptible parent (cv. Moneymaker) were analyzed, all of them being severely infested. However, their SMAI index was assessed from 18 plants chosen at random because this was the number of plants analyzed in the resistant parental line and the F_1 progeny.

DNA isolation and marker and sequence analysis

Leaf material from parents and progenies was collected and quickly frozen in liquid nitrogen. Leaves were ground using a Retsch MM301 mixer mill shaker at maximum speed. DNA was isolated from 100 mg of leaf tissue using DNeasy Plant Mini Kit. Total DNA was quantified by comparison with DNA standards after electrophoresis in 0.8 % agarose gels in 1xTBE (Tris–borate-EDTA). Bulks for the BSA analysis were generated by mixing equal amount of DNA, as previously described (Michelmore et al. 1991), from eight spider mite-resistant F_4 plants (SMAI value ranged from 0 to 0.5) and from eight pest-susceptible F_4 plants (SMAI value ranged form 3.0 to 3.9). Sets of single-nucleotide polymorphism (SNP), SSR and insertion/deletion (InDel) markers were obtained from the Solanaceae Genomics Network (http://solgenomics.net) and the Tomato Mapping Resource Database (http://tomatomap.net/). Another set of microsatellite markers was selected from published results (Areshchenkova and Ganal 1999; Smulders et al. 1997). Marker analysis of DNA form *S. lycopersicum*, *S. pimpinellifolium*, F_1, F_4 or the RIL population was done according to the standard PCR protocols and one of each primer pair was labeled with an Applied Biosystems fluorescent label. SSR and InDel loci were amplified in a 10-µl reaction mixture containing 10 ng of template DNA, 50 ng of each primer, 100 µM of dNTPs, 1.5 mM of $MgCl_2$, 0.2 unit of RedTaq DNA polymerase and 1× Taq buffer supplied with the enzyme (Sigma-Aldrich). PCR was performed in an Eppendorf Mastercycler ep gradient S. The samples were denatured at 94 °C for 3 min, followed by 30 cycles consisting of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min. SSR and Indel fragments were resolved using a fluorescent dye (FAM, VIC, NED, or PET), and the PCR products were separated by capillary electrophoresis using a DNA sequencer (ABI PRISM® 3130 XL Genetic Analyser, Applied Biosystems, USA). An internal size marker, GeneScan 500 LIZ (35–500 bp; Applied Biosystems), was added, allowing the co-loading of different labeled reactions. Data regarding selectively amplified DNA fragments were analyzed with GeneMapper® Software 3.7 (Applied Biosystems).

DNA sequencing of amplified PCR fragments was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the instructions provided by the manufacturer. Nucleotide sequences were obtained after capillary electrophoresis on ABI PRISM® 3130 XL Genetic Analyser.

The allelic discrimination of SNP genotyping products by high-resolution melting (HRM) analysis was achieved by detecting the difference in melting temperature between the different PCR fragments amplified by each allele. Genomic DNA (10 ng) was mixed with 5 µl of HotShot Mastermix (Taq DNA Polymerase, anti-Taq monoclonal antibodies in 2× reaction buffer with 400 µM dNTP and Stabiliser), 1 µl LCGreen™ Plus+ (Idaho Technology Inc.), 1 µl of 10 mM primers and completed to 10 µl with MQ water according to manufacturer recommendations (Clent Life Science, UK). The HRM PCRs were performed in FramStar 96-well plates (4titude, UK) under the following conditions: 94 °C, 10 min; 45 cycles of two steps, 94 °C, 30 s, 60 °C, 30 s; a denaturation step of 30 s at 94 °C and renaturation by cooling to 30 °C. Reactions were analyzed and genotyped using a LightScanner® System (Idaho Technology Inc., USA) following the protocols described in Montgomery et al. (2007).

QTL analysis

Marker data were analyzed and a genetic linkage map was calculated with Joinmap 4.0 (Van Ooijen 2006). A logarithm of odds ratio (LOD) of 5.0 and a maximum recombination fraction of 0.5 were established to consider significant linkage, and genetic map construction was performed using Kosambi mapping function (Kosambi 1944) with the following JoinMap parameters: Rec = 0.4, LOD = 1.0, and Jump = 5.

QTL were calculated using the Multiple QTL Mapping procedure (MQM; Jansen and Stam 1994; F_7 dataset) included in MapQTL 5.0 software (Van Ooijen 2004). For MQM analysis, the markers located closest to the QTL peaks were selected as cofactors by the automatic cofactor selection procedure and a genome-wide significance threshold 3.6 ($P < 0.01$) was calculated for the trait using the permutation test of MapQTL. The genetic linkage map was drawn using MapChart 2.2 software (Voorrips 2002). Additionally, the inclusive composite interval mapping (ICIM) approach was applied by means of the QTL IciMapping 2.2 software (Li et al. 2007). For the analysis of

digenic QTL interactions, the two-dimensional ICIM (ICIM-EPI) mapping method of the latter software was used.

Results

Identification of SSR markers linked to spider mite resistance

Preliminary results on the inheritance of spider mite resistance in *S. pimpinellifolium* accession TO-937 were obtained from the analysis of an F_2 segregating population no longer available because infested plants did not yield fruits, and they indicated that a dominant major gene was involved in this agronomical trait (Fernandez-Muñoz et al. 2000). We generated a novel F_2 and, by SSD, a more advanced F_4 segregating population from the original *S. lycopersicum* cv. Moneymaker × *S. pimpinellifolium* accession TO-937 so each genotype could be evaluated by examining several descendants. In a first assay, 51 F_4 lines were screened for spider mite resistance determining the average SMAI value of six descendent plants of each line. The SMAI value of the analyzed F_4 lines ranged from 0 to 3.8, values quite similar to the resistant parental line *S. pimpinellifolium* TO-937 (observed SMAI value 0) and susceptible *S. lycopersicum* cv. Moneymaker (average SMAI value 3.9), respectively.

A BSA approach was designed as a method for identifying molecular markers linked to any major locus responsible for resistance to the spider mite. DNA from eight spider mite-resistant F_4 lines (scored 0–0.5 according to SMAI scale) was pooled to build a resistant bulk and DNA from eight pest-susceptible F_4 lines (scored 2.9–3.8 according to SMAI scale) was pooled to make a susceptible bulk. Molecular markers available in the published tomato genetic map (Fulton et al. 2002) were screened to identify polymorphic markers between the parental lines. For the purpose of covering as many genomic regions as possible, we selected 116 markers, all of them SSRs evenly distributed throughout the tomato chromosomes. The markers covered 600 cM and showed 5.1 cM as the average genetic distance between them. Parental lines *S. lycopersicum* and *S. pimpinellifolium*, the F_1 and both bulks were genotyped for these SSR markers. Although most of the SSR markers segregated independently from the pest resistance, the BSA analysis and the further genotyping indicated that a region of 20 cM on chromosome 2, flanked by markers SSR605 and SSR50, might harbor some of the major loci involved in the tomato resistance to spider mite. Interestingly, the SSR50 marker cosegregated with two-spotted spider mite resistance in the analyzed F_4 plants indicating the usefulness of this marker for MAS of the pest resistance.

Resistance to spider mite assay in an F8-RIL population

With a view of not only to confirm that the genomic region of chromosome 2 identified in the BSA approach was involved in spider mite resistance, but also to reduce the inherent environmental variability associated with plant–insect interactions, we generated a RIL population by selfing the progeny of individual members of the F_4 until the F_8 generation was reached. RIL lines were evaluated for two-spotted spider mite resistance as described in "Material and methods". The result of the analysis of at least six descendant plants per RIL was the average SMAI assigned to the lines. The mean SMAI in the RIL population was 1.14, with values ranging from 0 to 5 (Fig. 2). The Shapiro–Wilk test showed a non-normal distribution of phenotypic values ($P < 0.05$), indicating that the inheritance of the resistance does not fit a polygenic inheritance model. Asymmetric distribution toward the resistant parent was observed confirming the dominant nature of the resistance to two spider-mite of *S. pimpinellifolium* TO-937 accession. All *S. lycopersicum* cv. Moneymaker analyzed plants showed severe mite damages (SMAI = 3.55 ± 0.62) while plants of the resistant TO-937 parent were characterized by a very low SMAI value (0.12 ± 0.17), reflecting that the plants' canopies were free of spider mite reproduction. Some spider mite feeding symptoms were observed in F_1 plants, but their SMAI value (1.50 ± 0.16) demonstrated a level of resistance similar to that of *S. pimpinellifolium* resistant parent. This result, together with the asymmetrical trend showed by the phenotypic distribution of the SMAI index in the RIL lines, indicated that a major genetic locus may be involved in the inheritance of resistance to the two-

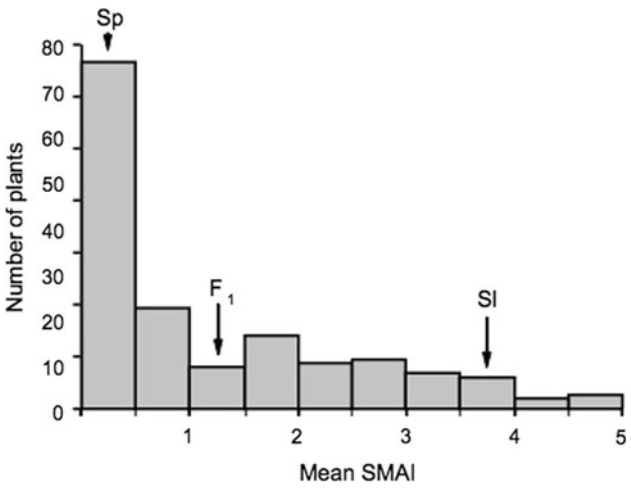

Fig. 2 Phenotypic variation of the spider mite attack index (SMAI) in a RIL population developed from the cross of *S. lycopersicum* cv. Moneymaker × *S. pimpinellifolium* acc. TO-937. The average SMAI value of the parental lines *S. lycopersicum* (Sl) and *S. pimpinellifolium* (Sp) as well as the progeny F_1 are indicated by *arrows*

spotted spider mite which agrees with previous results reported (Fernandez-Muñoz et al. 2003).

QTL analysis for spider mite resistance

In order to identify all the genomic regions implicated with the inheritance of the spider mite resistance in the RIL population, a genetic map of 244 markers (119 SSRs, 112 SNPs and 13 InDels) was generated with this population. Details of the new map will be published elsewhere. Interval mapping and MQM approach of the pest resistance were carried out based on the newly developed *S. lycopersicum* × *S. pimpinellifolium* genetic map. Only two QTL with a LOD score above the genome-wide confidence threshold of 3.6 ($P < 0.01$) were identified, both located on chromosome 2 and named *Rtu2.1* and *Rtu2.2* (Resistance to *T. urticae* QTL on Chromosome 2). *Rtu2.1* was located between markers SSR605 and SSR50, coinciding with the genomic region identified during the BSA analysis, whereas *Rtu2.2* was located between markers TG140 and CT24. Each QTL covered a region of approximately 10 cM and together accounted for nearly 40 % of the total phenotypic variation of this trait. With the purpose of increasing the density of markers located on the genomic regions where the two QTL were found, eight additional markers were selected from the solgenomics database, genotyped in the RIL population and included in a new version of the map. The new QTL analysis performed with the 252 marker map confirmed the locations of *Rtu2.1* and *Rtu2.2* (Fig. 3), although the new markers narrowed the genomic region containing both QTL. The position of *Rtu2.1* (LOD score of 4.57) was then defined between the markers SSR605 and cLEF41. The total phenotypic variance accounted for this QTL was 8 %. The new markers added to chromosome 2 also pinpointed the location of *Rtu2.2* (LOD score of 15.43) between SGN4 and SGN2 markers in an interval of 6.7 cM located 3.6 cM distally from *fw2.2* a major QTL for fruit size. *Rtu2.2* was responsible for explaining the 31.6 % of the total phenotypic variation of the resistance trait (Fig. 3).

The distorted phenotypic segregation with an excess of resistant plants could be explained by the presence of possible epistatic interactions between *Rtu2.1* and *Rtu2.2*. For this reason, an additional analysis using the IciMapping 2.2 software was performed. However, no significant epistatic interactions between *Rtu2.1* and *Rtu2.2* were detected, thus suggesting that these two genomic regions on chromosome 2 participate independently in the genetic control of the resistance to spider mite in the TO-937 wild tomato accession. In order to confirm this hypothesis, plants homozygous for each allele combination of the two QTL were selected and their average SMAI index was calculated (Fig. 4). Plant homozygous for the *S. lycopersicum* allele of both QTL

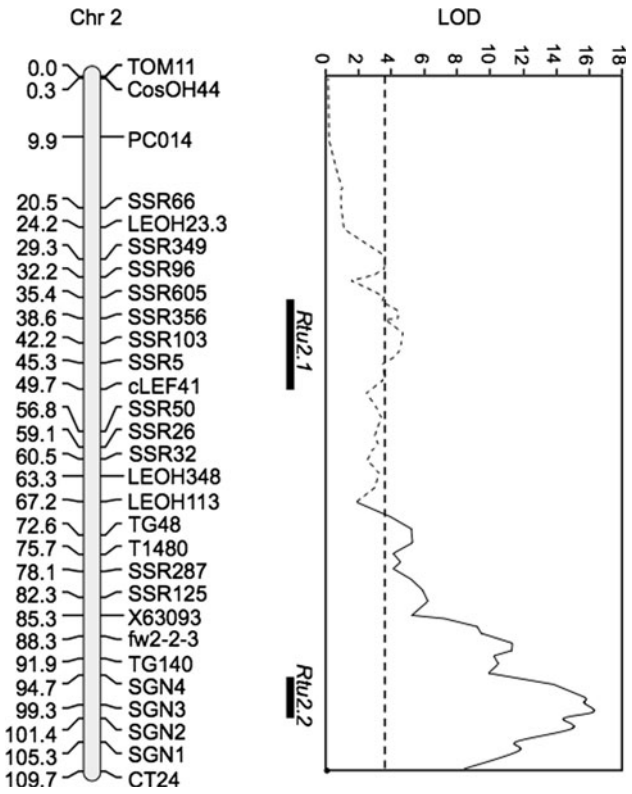

Fig. 3 Chromosomal locations of QTL *Rtu2.1* and *Rtu2.2* involved in the tomato resistance to *T. urticae*. On the *left*, the tomato chromosome 2 linkage map with distances among markers in cM is showed, whereas on the right the *broken horizontal line* represents the LOD value for the permutation test. *Vertical bars* represent the 2-LOD interval of QTL according to the MQM mapping using *Rtu2.1* (*dashed line*) or *Rtu2.1* (*continuous line*) as cofactor

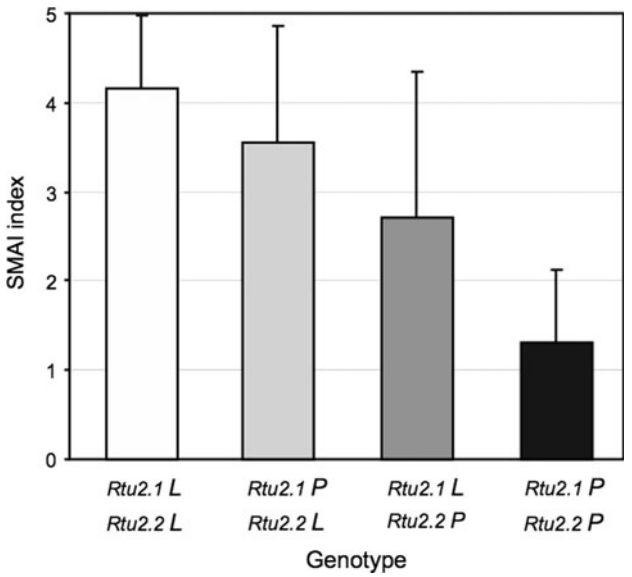

Fig. 4 Contribution of *Rtu2.1* and *Rtu2.2* to the pest resistance. The average (*bars*) and standard deviation (*lines above bars*) of RIL lines homozygous for the *S. lycopersicum* (L) or the *S. pimpinellifolium* (P) allele of each QTL are showed

showed an average SMAI index of 4.17 (SD = 0.81). Plants homozygous for the resistant allele of *Rtu2.1* and homozygous for the *S. lycopersicum* allele of *Rtu2.2* showed an average SMAI index of 3.56 (SD = 1.31), whereas plants homozygous for the *S. lycopersicum* allele of *Rtu2.1* and homozygous resistant form *Rtu2.2* showed and average SMAI index of 2.72 (SD = 1.62). And finally, plant homozygous for the *S. pimpinellifolium* alleles of both resistance QTL showed an average SMAI index of 1.31 (SD = 0.81), indicating that although both QTL seem to contribute to the spider mite resistance, there seems to be some synergistic interaction between them.

Discussion

The resistance to spider mite found in TO-937 is a genetically controlled natural defense mechanism of the plant against *T. urticae* that the pest has not been able to overcome, probably because it is not based in just one single molecule. The significance of our results lies in the fact that acaricide resistances continue to be a major problem in the control of this pest since the early 1950s, when the first serious failure in the chemical control of spider mites occurred and resistance to organophosphates was detected in greenhouse populations in the United States and Europe. Over the past decades, *T. urticae* has developed resistance to almost every chemical used for its control (Van Leeuwen and Tirry 2007; Jahangir et al. 2010).

First results about the inheritance of the resistance to spider mite of TO-937 indicated that a small number of genes were involved in the resistance on the basis of the analysis of an F_2 and BC1 populations (Fernandez-Muñoz et al. 2000). However, problems related to negative interplot interference, due to the high frequency of resistant genotypes observed in that assay, hindered the precise calculation of the number of genes responsible for this trait. Later, we improved the pest resistance test by interspersing susceptible tomato plants between the tested ones and results obtained indicated that pest resistance might be controlled by a single dominant major locus although it could be additionally modulated by unknown minor loci (Fernandez-Muñoz et al. 2003). We have confirmed these previous results indicating that resistance to spider mite in TO-937 should be governed by a major locus, the *Rtu2.2* QTL located in the telomere of chromosome 2. Given that we used more advanced segregating populations, our results should be considered more robust than those previously reported. The F_4 and the RIL population we have used in this work have some advantages over the F_2 segregating populations employed in preceding works, among others, the higher number of recombination events occurring during the construction of the RIL, which provides greater chances for linkage breakdown and separation of linked markers. On average, the chance of recombination between tightly linked genes in a RIL population is twice as in an F_2 and BC1 populations and even more frequent for less linked genes (Burr and Burr 1991), thus allowing a more accurate estimation of map distances. Concerning our results, the higher rate of recombination in the RILs used in our study has made possible to identify two QTL located in chromosome 2, *Rtu2.1* and *Rtu2.2*, linked to the resistance to spider mite, which could have been masked as only one locus when an F_2 population was previously analyzed (Fernandez-Muñoz et al. 2003).

The segregation of chromosome 2 in the RIL population deviated from the expected Mendelian ratio in the RIL population. Skewness has been described in multiple studies and is generally believed to be related to the presence of genes involved in reproductive behavior such as pollen, gamete and (or) zygote viability (Bernacchi and Tanksley 1997; Paterson et al. 1990, 1991; Stamova and Chetelat 2000). The chromosomal region surrounding the SSR26 marker displayed a highly distorted segregation in this study (65 LL, 18 LP and 83 PP genotypes, $\chi^2 = 136.05$, being L and P alleles from *S. lycopersicum* and *S. pimpinellifolium*, respectively), which is a characteristic of this tomato genome region previously reported by Villalta et al. (2005) in RIL lines descendant from *S. lycopersicum* crossed with a different *S. pimpinellifolium* accession. The presence of a self-incompatibility locus on chromosome 2 has been suggested as an explanation of this phenomenon reported in several studies (Bernacchi and Tanksley 1997; Monforte and Tanksley 2000; Paterson et al. 1990). This skewed segregation of chromosome 2 genomic regions containing *Rtu2.1* and *Rtu2.2* toward the wild species allele could also be responsible for the high number of resistant RIL lines we have found (Fig. 2).

We have identified novel QTL involved in the resistance to one of the most serious tomato pests, two-spotted spider mite. This finding could mark the starting point for isolating the possible candidate genes involved in this trait. The disclosure of our results coincides with the release of the tomato genome and the pre-release of the whole genome shotgun assembly for *S. pimpinellifolium* accession LA1589 (The Tomato Genome Consortium, 2012). The accessibility of a well-annotated *S. pimpinellifolium* genome will greatly facilitate the detection and characterization of the genes responsible for the resistance to spider mite in the wild tomato. One of the questions to be answered is how many genes may contain each *Rtu2* QTL to control the resistance to two-spotted spider mite. In this respect, the tomato mutant *odorless-2* (*od-2*) has been found to be defective in its resistance to several herbivore insects (Kang et al. 2010). The *od-2* mutation, located in chromosome 11, disrupts the production of several

compounds synthesized in type VI glandular trichomes. However, *od-2* trichomes produce normal levels of acylsugars, glycoalkaloids and other compounds of the jasmonate (JA) pathway demonstrating that a single gene can control the level of several compounds in highly specialized cells like glandular trichomes. In our case, it is impossible to predict if each *Rtu2* QTL will contain just one gene or several genes involved in the inheritance of the resistance.

Concerning the mechanism underlying the resistance to the two-spotted spider mite in *S. pimpinellifolium* TO-937, recent studies indicate that it was due to the acylsucrose exudates arisen from type IV trichomes (Alba et al. 2009). These authors found a negative correlation between type IV trichome density and spider mite attack and other related traits such as repellence, mortality, and reduced oviposition. Density of type IV trichomes and acylsugar secretion had been previously involved in the resistance to two-spotted spider mite in other wild tomato such as *S. pennellii* (Maluf et al. 2010; Resende et al. 2002; Saeidi et al. 2007). Currently, an alternative strategy to map insect resistance QTL for breeding these complex traits is choosing biochemical and/or physical traits associated with the resistance. In that regard, the first study about the inheritance of type IV trichome density was published by Lemke and Mutschler (1984) who concluded that two dominant unlinked genes might be controlling their presence on *S. pennellii*. Later on, the same group carried out a QTL analysis based on a *S. lycopersicum* × *S. pennellii* cross, and identified several QTL associated with traits related to glandular trichome and acylsugar levels (Blauth et al. 1998, 1999). Two of these QTL, one associated with type IV trichome density (QTL 2A) and the other one related to acylsugar accumulation (QTL 2B), were identified on chromosome 2. Unfortunately, the genetic map used by these authors showed a very low marker density, particularly in chromosome 2, where only five codominant markers and one anonymous RAPD were located. However, the fact that these two QTL are linked to TG14, a marker located in the EXPEN2000 map (http://solgenomics.net), 3 and 1 cM away from SSR103 and SSR5, respectively, may indicate that *Rtu2.1* harbors some genes related to trichome development or acylsugar biosynthesis orthologous to those found in *S. pennelli* resistance QTL 2A and 2B. Interestingly, the *S. pennellii* chromosome 2 does not seem to harbor other QTL involved in trichome density or acylsugars levels because the SSR marker TG167 did not show linkage to any of those pest resistance traits mapped in *S. pennellii* (Blauth et al. 1998, 1999), but this marker is located near the telomeric position where we have located *Rtu2.2* in *S. pimpinellifolium* TO-937. Although little is known about the genetic network controlling tomato multicellular trichome formation and density, fine mapping of the *woolly* (*Woo*) gene controlling multicellular trichome formation and embryo lethality recently published have located this gene in chromosome 2 (Yang et al. 2011). Sequences alignment of the published genes surrounding the *woo* mutation (Yang et al. 2011) demonstrates the *Woo* gene is located between SSR50 and SSR26, indicating that this gene is not responsible of the TO-937 resistance to spider mite and suggesting that chromosome 2 of tomato contains several loci implicated in trichome formation.

Glandular, secreting trichomes have been widely studied as the source of compound from their exudates, which confer aroma and flavor besides to their role in plant–insect interaction. Recently, new -omics approaches have been employed to identify key enzymes of the secondary metabolism of type VI trichomes (Schilmiller et al. 2010), the most abundant tomato glandular trichomes. These new approaches identified genes highly expressed in trichomes and those located in chromosome 2 should be considered as candidate genes implicated in the resistance conferred by *Rtu2.1* and *Rtu2.2*. Unfortunately, of the 30 EST identified as highly expressed in type VI trichomes by massive cDNA sequencing (Schilmiller et al. 2010), only the *Pectin methylesterase* gene was located in chromosome 2 flanked by SSR50 and SSR32 markers in a position between *Rtu2.1* and *Rtu2.2*, indicating that these genes highly expressed in type VI trichomes seem not be involved in type IV trichome-based TO-937 resistance to two-spotted spider mite. Even recently identified and characterized genes implicated in acylsugar production in *S. pennellii* and *Nicotiana benthamiana* (Slocombe et al. 2008), which could be considered the first candidates implicated in *S. pimpinellifolium* TO-937 two-spotted spider mite resistance, are not located in the same genomic region as QTL *Rtu2*, suggesting that this genotype may contain novel genes implicated in the formation of type IV trichomes and in the synthesis of acylsugars in the glandular cells which compose these specialized plant structures. Interestingly, although *S. pimpinellifolium* is described as a tomato species harboring only type VI glandular trichomes (Simmons and Gurr 2005; McDowell et al. 2011) we have found such trichomes in three out seven *S. pimpinellifolium* accessions from La Mayora Experimental Station germplasm bank (Fernandez-Muñoz et al. 2003). Therefore, the phylogenetical origin of type IV glandular trichomes present in some accessions of *S. pimpinellifolium*, like TO-937, is not known.

When plants are attacked, defense proteins act as natural defenses, inhibiting digestive enzymes in the gut of some herbivorous insects (Gatehouse 2002). The expression of defense genes is activated by the JA family of signaling molecules via the octadecanoid pathway (Koo and Howe 2009). One of the signaling molecules from the octadecanoid pathway is an allene oxide cyclase enzyme (AOC) and

the gene encoding this protein has been located on chromosome 2. It is specifically positioned between the RFLP markers TG463 and CT232 (Stenzel et al. 2003), and therefore it should be located between markers SSR32 and TG48 in the genomic region between *Rtu2.1* and *Rtu2.2*. In addition, the comparison of the coding sequences of the *AOC* gene in *S. lycopersicum* and *S. pimpinellifolium* TO-937 did not reveal any polymorphism (data not shown), which should exclude this gene as candidate for conferring resistance to spider mite of TO-937. Another gene that has been related to the wound response in tomato is *LeCOI*, the tomato homolog of *CORONATINE-INSENSITIVE1* (*COI1*), an Arabidopsis gene coding for an F-box protein that is required for jasmonic acid signaling processes associated with resistance to two-spotted spider mite in tomato. *LeCOI* controls the expression of early wound-response genes and silencing this gene in a Micro-Tom background severely compromises the level of natural resistance to spider mite that is inherent in tomato plants (Li et al. 2004). Anyway, this level of inherent resistance to two-spotted spider mite in tomato is considered as susceptibility when we apply the SMAI value in our evaluations, which demonstrates the high level of resistance conferred by *Rtu2* QTL. Moreover, *LeCOI* might not be related to spider mite resistance in TO-937 because it is located on chromosome 5. All these observations would indicate that genes conferring resistance in TO-937 are not related to those involved in wound-induced responses triggered by the molecules of the JA pathway.

The QTL identified in this study provide an excellent source of resistance that could be introgressed into commercial cultivars. Because of the close phylogenetic relationships between *S. pimpinellifolium* and *S. lycopersicum*, there is little to no difficulty in initial crosses or in subsequent generations of pre-breeding and breeding activities. It should be noted that the infestation pressure in the greenhouse evaluations was extremely favorable for the pest while this pressure in commercial greenhouses is lower and less favorable for pest invasion. On the other hand, the QTL linked to spider mite resistance have been located on chromosome 2, where *fw2.2*, the major QTL for fruit size, had been located (Alpert et al. 1995; Frary et al. 2000). Despite of the linkage found between *Rtu2.2* and *fw2.2*, the inclusion of the latter in the genetic map here described made possible the design of molecular marker-assisted breeding programs to select for *Rtu2.2* alleles of *S. pimpinellifolium* and simultaneously for *fw2.2* alleles of 'Moneymaker', therefore overcoming the major disadvantage of TO-937 that is the linkage of spider mite resistance alleles to small fruit size alleles. Our results indicate that each resistance QTL contribute to the spider mite resistance (Fig. 4), although both QTL are necessary to reach a highly resistant phenotype. Our finding that plants homozygous for the resistance alleles of *Rtu2.1* and *Rtu2.2* showed an average SMAI index similar to that of the F_1 hybrid plants indicates that it's not necessary for an homozygous condition to reach a pest resistance phenotype.

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