

# Fine mapping and analysis of a candidate gene in tomato accession PI128216 conferring hypersensitive resistance to bacterial spot race T3

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Received: 24 April 2011 / Accepted: 7 October 2011 / Published online: 22 October 2011  
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**Abstract** Bacterial spot caused by *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri* is one of the most destructive diseases in tomatoes (*Solanum lycopersicum* L.) growing in tropical and subtropical regions. Exploring resistance genes from diverse germplasm and incorporating them into cultivated varieties are critical for controlling this disease. The *S. pimpinellifolium* accession PI128216 was reported to carry the *Rx4* gene on chromosome 11 conferring hypersensitivity and field resistance to race T3. To facilitate the use of marker-assisted selection in breeding and map-based cloning of the gene, an F<sub>2</sub> population derived from a cross between the susceptible variety OH88119 and the resistant accession PI128216 was created for fine mapping of the *Rx4* gene. Using 18 markers developed through various approaches, we mapped the gene to a 45.1-kb region between two markers pcc17 and pcc14 on chromosome 11. A NBS-LRR class of resistance gene was identified as the candidate for the *Rx4* gene based on annotation results from the International Tomato Annotation Group. Comparison of the genomic DNA sequences of the *Rx4* alleles in PI128216 and OH88119 revealed a 6-bp insertion/deletion (InDel) and eight SNPs. The InDel marker was successfully used to

distinguish resistance and susceptibility in 12 tomato lines. These results will facilitate cloning the *Rx4* gene and provide a useful tool for marker-assisted selection of this gene in tomato breeding programs.

## Introduction

Bacterial spot caused by *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri* (Jones et al. 2004) occurs for many areas of the world wherever tomatoes (*Solanum lycopersicum* L.) are grown and environmental conditions are favorable for disease development. The disease is of great economic importance because it causes severe yield loss and fruit quality reduction (Jones et al. 1991; Zhao et al. 2004; Cui et al. 2005). Five races designated T1 through T5 (T1 in *X. euvesicatoria*, T2 in *X. vesicatoria*, T3, T4, and T5 in *X. perforans*) have been defined by their virulence on tomato plants (Jones et al. 2000, 2005; Astua-Monge et al. 2000a). Due to the existence of multiple sources of inoculum, lack of efficacy of commonly applied chemicals and development of resistance to these chemicals in bacterial populations (Yang and Francis 2007; Stall et al. 2009), control of the disease using chemicals has not been effective once epidemics start. The use of host resistance would be a more efficient, economical and environmentally friendly approach to control the disease.

Resistance to bacterial spot in tomato has been defined as hypersensitive resistance (HR) and field resistance. Several tomato lines that develop a typical HR upon bacterial attack have been identified (Scott and Jones 1986; Scott et al. 1997; Astua-Monge et al. 2000b). Unimproved breeding line Hawaii7998 developed HR to race T1 strains expressing the *avrRxv* gene (Whalen et al. 1993).

Communicated by J. Yan.

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Three non-dominant genes, *rx1* and *rx2* on each arm of chromosome 1 and *rx3* on chromosome 5 acted independently, while three additional regions on chromosomes 3, 9 and 11 carrying susceptible alleles appeared to modify the HR in this line (Yu et al. 1995). The HR to race T3 found in an unimproved breeding line Hawaii7981 and two *S. pimpinellifolium* accessions PI126932 and PI128216 was mediated by the interaction between *avrXv3* and a single gene *Xv3* (Minsavage et al. 1996; Scott et al. 1996; Stall et al. 2009). A later study designated the gene in PI128216 as *Rx4* (Robbins et al. 2009). Both *Xv3* and *Rx4* were mapped to the same region on chromosome 11 (Robbins et al. 2009; Wang et al. 2011). The hypersensitivity to race T4 in *S. pennellii* accession LA716 was mediated by interaction between *avrXv4* and a single gene *Xv4*, which was mapped to chromosome 3 (Astua-Monge et al. 2000b). However, the map position of *Xv4* has not been confirmed and may be inaccurate (Stall et al. 2009). A bacterial avirulence gene *avrBs4* activates HR in most *S. lycopersicum* germplasm. Recognition of *avrBs4* was controlled by a single dominant gene *Bs4* (Ballvora et al. 2001). Although the *Bs4* gene has been cloned from tomato, it only confers resistance to bacterial strains that cause bacterial spot on pepper not on tomato (Stall et al. 2009).

Sources for field resistance to bacterial spot have also been identified in several tomato lines. Hawaii7998 was considered as the most reliable source of resistance to race T1 (Jones and Scott 1986). Two QTLs have been identified in this line. The locus *Rx3* on chromosome 5 explained 41% of total phenotypic variation, and a locus on chromosome 4 associated with susceptibility in Hawaii7998 explained 11% of total phenotypic variation (Yang et al. 2005b). The *Rx3* locus had a dominant effect on the field resistance while HR was controlled by the recessive locus *rx3*. It remains unclear whether they are the same gene with different functions or they are different genes on the same chromosomal region. Hawaii7981, PI126932 and PI128216 also showed partial resistance to race T3 in the field (Scott et al. 1995). Resistance in Hawaii7981 was conditioned by the *Xv3* gene and some modifiers (Scott et al. 2001), while resistance in PI128216 was controlled by a single gene *Rx4* (Robbins et al. 2009). *S. lycopersicum* var. *cerasiforme* accession PI114490 showed field resistance but no HR to races T1–T4 (Scott et al. 1995, 2003; Yang et al. 2005a; Hutton et al. 2010). Resistance to races T2 and T3 were controlled by two to three genes, respectively (Scott et al. 2003), while resistance to race T4 was controlled by several QTLs and a locus on chromosome 11 explained 29.4% of total phenotypic variation (Hutton et al. 2010).

The first occurrence of tomato bacterial spot in China was reported in 1940s (Yang et al. 2007). Due to the success of quarantine, the disease did not become a serious problem before the 1990s. The outbreak of the disease in

many regions was first reported in 1991 (Sun et al. 1991). Since then, the disease spread quickly and caused severe yield losses throughout the country (Yang et al. 2007). Identification and characterization of isolates from five provinces suggested that two races T1 and T3 existed in China, and race T3 was dominant (Sun et al. 1999). However, none of the commercial cultivars currently growing in China were resistant to these two races (Zhang et al. 2009). Therefore, herein we characterized resistance to race T3.

The gene *Rx4* in PI128216 has been mapped to an interval of 22.1 cM between markers SL20181 and SL10027 on chromosome 11. The closest marker SL20181 is 3.3 cM away from the gene (Robbins et al. 2009). It remains a risk in using the marker to select lines with the *Rx4* gene and to pyramid the gene with other resistance genes. Identification of molecular markers more tightly linked to the gene is necessary. In the present study, we mapped the *Rx4* gene to a 45.1-kb region on chromosome 11. Furthermore, we identified a candidate gene associated with HR to race T3 in this region. This achievement is a major step toward the ultimate goal of cloning the *Rx4* gene to elucidate the mechanisms involved in hypersensitivity to bacterial spot race T3 in tomato.

## Materials and methods

### Plant materials and mapping population

*Solanum pimpinellifolium* accession PI128216 has been reported to contain the *Rx4* gene conferring HR to tomato bacterial spot race T3 (Robbins et al. 2009). OH88119 is an elite processing tomato parent (Berry et al. 1995; Francis et al. 2002) but susceptible to bacterial spot race T3 (Robbins et al. 2009; Zhang et al. 2009). A cross was made between OH88119 and PI128216 to develop an F<sub>2</sub> population and subsequent generations for fine mapping of the *Rx4* gene.

Tomato seeds of two parents, F<sub>1</sub>, F<sub>2</sub> and F<sub>2:3</sub> were sown in 288 Square Plug Tray Deep (Taizhou Longji Yuanyi Cailiao Co., Ltd, Zhejiang, China) filled with a mixture of peat and vermiculite (3:1) in a protected greenhouse. Seedlings were transplanted to the same greenhouse 50 days after seeding.

### Inoculum preparation and inoculation

The *X. perforans* race T3 strain Xv829 used in the study was kindly provided by Dr. Jeffery B. Jones at the University of Florida. The bacteria were grown on yeast, dextrose, and calcium carbonate (YDC) agar medium (Lelliot and Stead 1987) at 28°C for 48 to 72 h. Bacterial

cells were washed from the agar plates with sterile double-distilled water (ddH<sub>2</sub>O) and the suspension was adjusted to approximately  $3 \times 10^8$  CFU/ml ( $A_{600} = 0.15$ ). One hour prior to inoculation, the plants were misted with water. Inoculations were performed by leaf infiltration (Yang and Francis 2005) on at least three leaflets per plant. The bacterial suspension was infiltrated through the abaxial side of fully expanded leaflets using a 5-ml syringe without a needle until the infiltration area reached approximately 1.5 cm in diameter. Control plants were infiltrated with ddH<sub>2</sub>O. The inoculated plants were kept in the protected greenhouse at 22–28°C and misted with water twice per day (9:00 a.m. and 5:00 p.m.) after infiltration to increase humidity. Ten days after infiltration, all inoculated leaves were removed and the second infiltration was conducted using the same approach as described above.

#### Disease scoring

The HR response was visually inspected using the method described by Robbins et al. (2009). Individual plant showing a clear response (confluent necrosis) on at least one leaflet was scored as HR, while plants without response were scored as non-HR (susceptibility). If an F<sub>2</sub> plant was scored as an HR in one inoculation and a susceptible response in the other inoculation, seeds of the plant were saved and 20 F<sub>2,3</sub> plants were evaluated for response to the bacteria. A few F<sub>2</sub> plants with ambiguous responses were excluded from the statistical analysis.

#### Molecular marker analysis and gene mapping

Young leaves were collected from F<sub>2</sub> individuals and ten plants for each parent. Genomic DNA was isolated using the modified CTAB method (Kabelka et al. 2002). A total of 18 markers were used for mapping of the *Rx4* gene and selection of recombinants in this study (Table 1). Five of them were adopted from the literature (References in Table 1). Based on the map position of *Rx4* on chromosome 11 (Robbins et al. 2009), 13 new markers including simple sequence repeat (SSR), single nucleotide polymorphisms (SNP), and insertion/deletions (InDel) in the region between markers SL20181 and SL10027 were developed by either comparing genomic DNA sequences between Heinz1706 (SL2.31sc03786) and LA1589 obtained from Sol Genomics Network (SGN) (<http://solgenomics.net>) or comparing the sequences of PCR products amplified from PI128216 and OH88119 using primers of known markers from SGN. The scaffold SL2.31sc03876 was determined by searching the sequences of markers SL20181, cLEC-24-C3, and C2\_At1g07960 from tomato whole genome shotgun sequences (SGN). Polymorphisms of SNP markers were detected as cleaved amplified polymorphisms (CAP)

(Yang et al. 2004) or dCAP (Neff et al. 1998). Polymorphisms of SSR and InDel markers were detected using the methods described in Shen et al. (2011).

Two steps were adopted to determine the position of *Rx4* gene on chromosome 11. To narrow down the region containing the *Rx4* gene, a linkage map was first created by analyzing a subset of the F<sub>2</sub> population consisting 187 individuals with eight markers (SL20181, L19498, EP1552, cLEC-24-C3, C2\_At1g07960, TAO1, C2\_At2g28490, and SL10027) in the region between markers SL20181 and SL10027. After the gene was located in a narrowed region, seven new markers (C2\_At3g11840, cLEF-51-G9, cLEF-10-O11, Solcap36510, pcc8, pcc12, and pcc14) were developed and used to genotype the whole F<sub>2</sub> population. Three more markers (pcc15, pcc16, and pcc17) were used to characterize the recombinant individuals. All linkage analysis was conducted using both JOINMAP3.0 (Van Ooijen and Voorrips 2001) and MAPMAKER/EXP3.0 (Lander et al. 1987).

#### Candidate gene identification and semi-quantitative RT-PCR analysis

cDNA sequences of candidate genes between markers pcc14 and pcc17 in the region of scaffold SL2.31sc03786 were obtained by searching the genomic DNA sequence against the ITAG Release 2.31 Predicted CDS provided in SGN. Predicted functions of these genes were also obtained from SGN by searching cDNA sequences against ITAG Release predicted proteins (SL2.31). Primers (Table 2) were designed for each annotated gene in the region and used for semi-quantitative RT-PCR analysis.

Total RNA was isolated from leaves of PI128216, Hawaii7981 and OH88119 plants infiltrated with  $3 \times 10^8$  CFU/ml suspension of tomato bacterial spot race T3 strain Xv829 or mock-infiltration with ddH<sub>2</sub>O. Since the locus Xv3 conferring HR to race T3 in Hawaii7981 is also mapped to the same region chromosome 11 (Wang et al. 2011), this line was included in this study for comparison analysis. Leaf samples were collected from two plants per genotype at 0.5, 1, 3, 6, 9, 12, 24, and 48 h post-infiltration. Total RNA was extracted using TRIZOL Reagent (Invitrogen, CA, USA) following the method described by the manufacturer. Single-stranded cDNA was synthesized with Oligo (dT) primer using Reverse Transcriptase M-MLV (TaKaRa, Dalian, Liaoning, China) following the manufacturer's instructions. The different cDNA samples were adjusted to equal concentrations by running a PCR primer specific for *actin* for calibration. PCRs were conducted with an initial denaturation step at 94°C for 3 min, followed by 22 cycles of 30 s at 94°C, 30 s at 58°C, a 30 s extension at 72°C, and terminated by a final extension at 72°C for 3 min.

**Table 1** Molecular marker information for mapping of *Rx4* in *Solanum pimpinellifolium* conferring hypersensitive resistance to bacterial spot race T3 (*Xanthomonas perforans*)

Marker name	Marker type	Primer sequence (5'–3')	Annealing temperature	Cycles	Restriction enzyme	Product size (bp)		Reference
						PI128216	OH88119	
SL20181	SNP	f: GGAGGAAGCTTTCATCTGA r: ATGCTCCAGGCTACCTACA	56	38	<i>SfcI</i>	117/115	232	Robbins et al. (2009)
L19498	InDel	f: CGGTCTATCAGGGAGTGAA r: GCAAGCGATGAAATTGGATT	45	38	–	211	216	This study
EPI552	SSR	f: AACTTACCACACCAAGGGC r: ACACAAGAGGAGGTGGTGA	56	38	–	120	124	This study
cLET-10-O11	InDel	f: ACAAGCCGCATCACCTATT r: CCTCGGGGTACAGAACTTT	52	38	–	342	354	This study
cLEF-51-G9	InDel	f: ACTTGAGCCGAGGTCTTTC r: CAGTAGCAGGTGCTCCATGA	58	38	–	179	176	This study
Sotcap36510	SNP	f: ACCGTTGAAATGTTGGAAAG r: AGGTGGCTTGGTTGATTACG	56	38	<i>RsaI</i>	141	52/89	This study
C2_At3g11840	InDel	f: TAGTCAACGCGGAAAAGGT r: CCAACTTCTCCATGCACCT	56	38	–	211	205	This study
pecc8	InDel	f: TTAAGGCATGCAATGGATCA r: TGTAAACCAACATGAAACCATCA	54	38	–	221	209	This study
cLEC-24-C3	dCAPS	f: CAGACTGGAGAGTCAAAGGT r: CCTTGTGATAATCTGCAAGTTGTTAA	54	38	<i>HpaI</i>	216	196/20	Wang et al. (2011)
pecc17	InDel	f: TTTTGGAAACCACAACCACA r: GCAATCCATGGTTGGAAAAC	52	38	–	300	289	This study
pecc16	InDel	f: AGTAGGGAGTTGGGGCTTA r: ACGCCATAGTCCCAAACAAG	56	38	–	143	139	This study
pecc15	InDel	f: TGTTTGGGATGCATCTATGG r: TTCCAGCCTCATTCAGAAAA	54	38	–	268	264	This study
pecc12	InDel	f: TCCACATCAAATGCGTTTCT r: TTCCAATCCTTCCATTTCCG	52	38	–	113	119	This study
pecc14	InDel	f: TTTTCCCGCCATCTAGAAA r: GGAATCATATATCCGACGAAATCA	52	38	–	257	238	This study
C2_At1g07960	dCAPS	f: GTAGTGCATGAAATGTGGCTTG r: CAAAATCATCCAAAAGTGTCCAGATT	54	38	<i>HinfI</i>	200	180	Wang et al. (2011)
TAO1	CAPS	f: GGGCTCTAATCCGIGCTTCA r: GGTGGAGGATCGGGTTTGTTC	58	38	<i>RsaI</i>	109/275/ 518	109/275/232/ 290	Staniaszek et al. (2007)
C2_At2g28490	dCAPS	f: GGAATGCAATTGTATCTCCCTGC r: CCCTAGGATTAGAAGAAACTCACAAGTTA	54	38	<i>HpaI</i>	162	140	This study
SL10027	SNP	f: GATCCAGTTTCCCTCTTACCG r: CCATTAGAGCCAAGACGCTC	60	38	<i>SfcI</i>	736	542/194	Robbins et al. (2009)

**Table 2** The six annotated genes in the *Rx4* region of tomato chromosome 11 and information for semi-quantitative RT-PCR and isolation of the *Rx4* gene

Gene/marker name	ITAG release 2 predicted proteins (SL2.31)	Primer sequence (5'–3')	Annealing temperature	Cycles	PCR product size (bp)
For semi-quantitative RT-PCR					
Solyc11g068970.1.1	Aluminum-activated malate transporter	f: TCCTCAAGTGAAGGCCAGAT r: AGATCTTCACCAGCCCACAC	56	28	195
Solyc11g068980.1.1	Mitochondrial import inner membrane translocase subunit	f: GGATGCCTACTGTGGAGGAA r: GCAGCAGAGAAAACCAAACC	56	28	253
Solyc11g068990.1.1	Methyltransferase-16, putative	f: ACAGGCAGCAGAGTTGGACT r: TGGATGCTTGGATGTTGGTA	54	28	285
Solyc11g069000.1.1	T-complex protein 1 subunit beta	f: TCAACAGGCAGAGGACACAG r: TGAAGTTGTCCCATCACCAA	54	28	144
Solyc11g069010.1.1	Alpha-L-fucosidase 1	f: GGGATAGGCATGAGCATTGT r: TCCATATCCTTCTCGCCATC	56	28	145
Solyc11g069020.1.1	Disease resistance protein	f: TGATGCAACTCCATCCAAAA r: TAATCGATGAACGCCAACAA	52	28	239
<i>actin</i>		f: GTCCTCTTCCAGCCATCCA r: ACCACTGAGCACAATGTTACCG	58	28	126
For genomic DNA amplification					
Rx4 g		f: TCACTTGCTAAGACAGCCACT r: CTGCACACGACACGCTTAAT	56	38	2,831

Semi-quantitative RT-PCR was conducted in a 20- $\mu$ l reaction volume. Each reaction consisted of 10  $\mu$ M Tris-HCl (pH 9.0 at room temperature), 50  $\mu$ M KCl, 1.5  $\mu$ M MgCl<sub>2</sub>, 50  $\mu$ M each of dNTP, 0.3  $\mu$ M each primer, 2  $\mu$ l cDNA template, and 1 unit of Taq DNA polymerase (Takara). Reactions were heated at 94°C for 3 min followed by 28 cycles of 30 s at 94°C, 30 s at specific annealing temperatures (Table 2), and a 30 s extension at 72°C. Final reactions were extended at 72°C for 5 min. Amplification was performed in a programmable thermal controller (PTC-100; MJ Research, Inc., Watertown, MA, USA). The PCR products were separated on 1% agarose gel, stained with ethidium bromide, and photographed using a GIAS-4400 Gel Documentation System (Beony Science and Technology Co., Beijing, China).

#### Sequence analysis

Genomic DNA sequences of the candidate gene were determined through sequencing PCR products amplified from genomic DNA isolated from PI128216, Hawaii7981 and OH88119. Primers (Table 2) were designed based on the sequences of the candidate gene in genome sequences of LA1589 and Heinz1706. PCR was conducted using the method described in Yang et al. (2004) using Takara LA Taq DNA polymerase (Takara). The PCR products were separated on 1% agarose gel, purified using Agarose Gel Purification Kit (Biomed, Beijing, China), cloned into

pMD19-T vector (Takara) and sequenced at Sunbiotech (Beijing, China). Coding regions were determined by searching the genomic DNA sequence against the ITAG Release 2.31 Predicted CD (SGN). The sequences were analyzed using DNAMAN software (Lynnon Corporation, Quebec, Canada).

#### Marker-trait association analysis

To verify the association between the candidate gene and hypersensitive resistance, 12 tomato lines including five *S. pimpinellifolium* accessions (LA1269, LA1589, PI128216, PI365914, and LA2804), one *S. lycopersicum* var. *cerasiforme* accession PI114490, and six *S. lycopersicum* cultivars (Moneymaker, Rio Grande, OH88119, Liger 87-5, Baiguoqiangfeng, and Zhongshu 5) were subject to HR testing as described above and genotyping with the InDel marker pcc12, which was derived from the candidate gene.

## Results

#### Response of parental, F<sub>1</sub> and F<sub>2</sub> plants to tomato bacterial spot race T3

All individuals from PI128216 and the F<sub>1</sub> showed HR to race T3 within 24 h after infiltration, while OH88119 plants showed water-soaked lesion 4 days after infiltration.



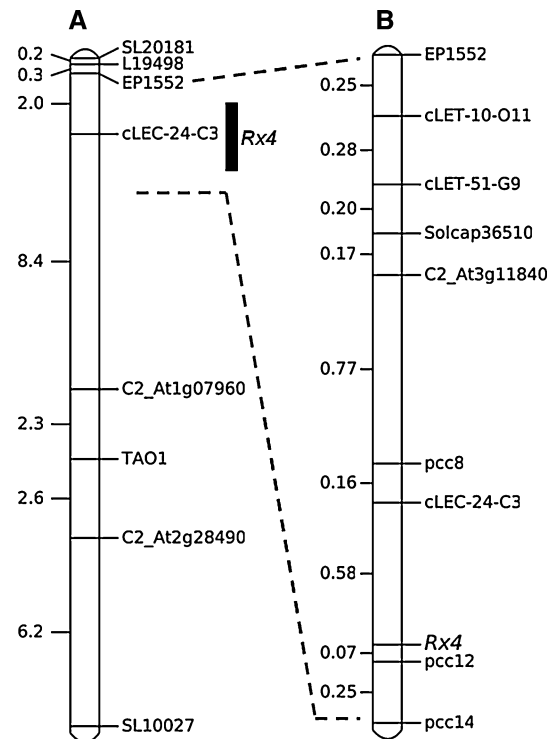
In the  $F_2$  population, clear differences between HR and water-soaked lesions were observed. Approximately 3% of the individuals had different responses between two infiltrations. Most of them could be classified into either HR or susceptible to the strain by re-evaluating the  $F_{2:3}$  plants. Thus, a total of 1,116  $F_2$  individuals were used for final data analysis, of which 852 showed HR to the strain and 264 were susceptible. These data indicated that a single dominant gene in PI128216 ( $\chi^2 = 1.0753$ ) conferred HR to tomato bacterial race T3, which was consistent with the observation of Robbins et al. (2009).

#### Fine mapping of *Rx4*

Based on the phenotypic and genotypic data for the subset of 187 individuals in the  $F_2$  population and eight markers, linkage maps for the chromosomal region between markers SL20181 and SL10027 were created by JOINMAP3.0 and MAPMAKER/EXP3.0. Markers were in the same order with similar genetic distance on the two maps. However, the *Rx4* gene was located between markers EP1552 and cLEC-24-C3 on the map created by JOINMAP3.0, while it could be placed at either side of the marker cLEC-24-C3 on the map created by MAPMAKER/EXP3.0. Considering the small size of the mapping population, the linkage map created by MAPMAKER/EXP3.0 with a black bar indicating the potential position of the *Rx4* gene is presented in the current study (Fig. 1a). The overall genetic distance for the region was 22.0 cM, which was almost the same (22.1 cM) as in Robbins et al. (2009). New markers developed for the region between markers EP1552 and C2\_At1g07960 were used to genotype the whole  $F_2$  population of 1116 individuals. The *Rx4* gene was then mapped to a 0.90-cM chromosomal region between markers cLEC-24-C3 and pcc14. The marker pcc12 was the closest to the *Rx4* gene with a genetic distance of 0.07 cM (Fig. 1b). Five recombinants were identified in this region. Analysis of these recombinants using three additional markers pcc15, pcc16, and pcc17 suggested that the *Rx4* gene was in the region between markers pcc17 and pcc14 (Fig. 2a).

#### Candidate gene identification and expression

The region between markers pcc14 and pcc17 in the scaffold SL2.31sc03876 was approximately 45.1 kb. Based on ITAG Release 2.31 Predicted CDS, six genes were identified in this region (Table 2; Fig. 2b). Semi-quantitative RT-PCR analysis revealed that five of them expressed equally in both inoculated and mock-inoculated plants of PI128216, Hawaii7981 and OH88119 (data not shown). However, one gene *Solyc11g069020.1.1* coding a disease resistance protein differentially expressed in resistant and



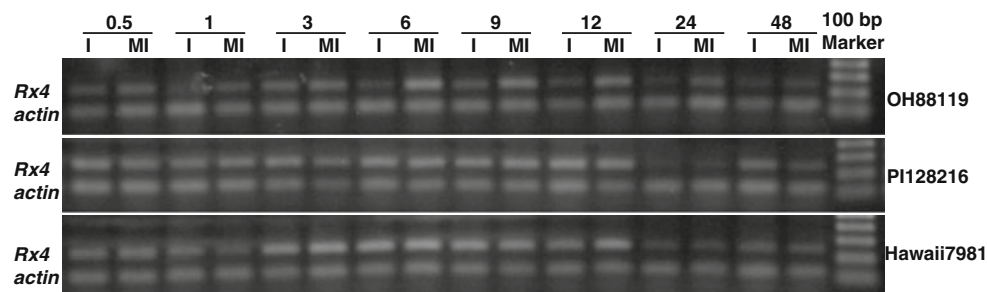
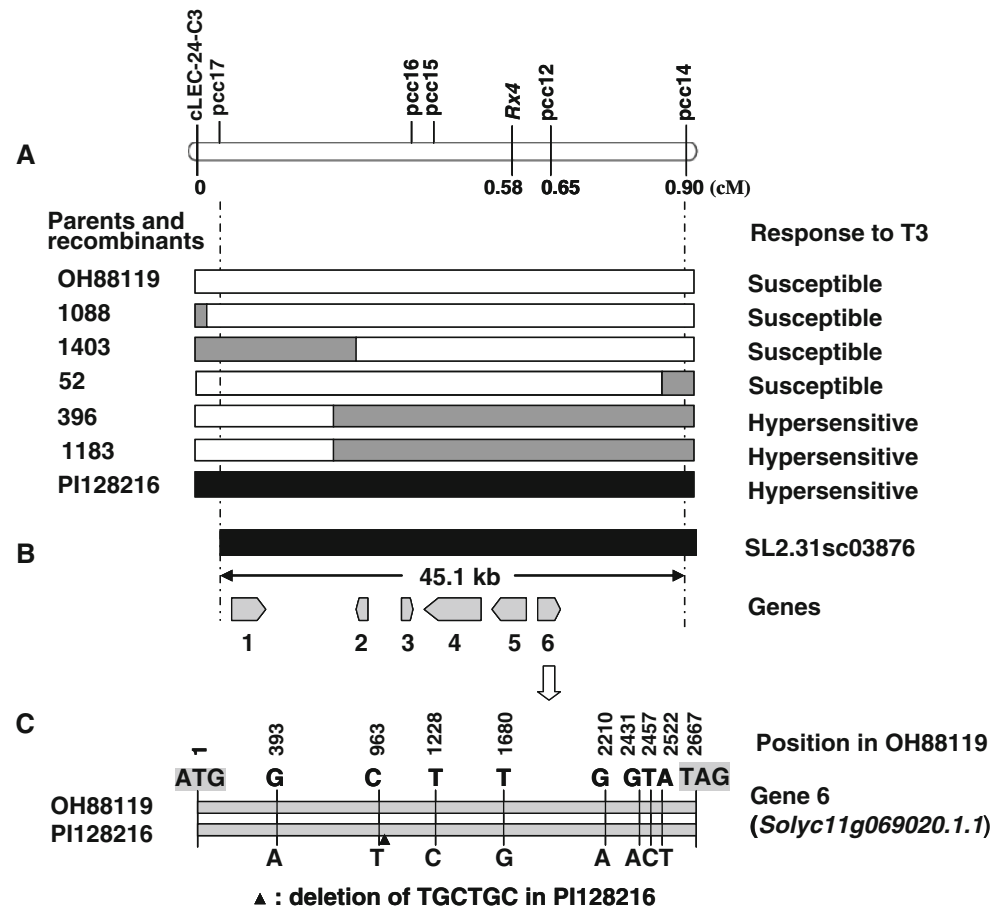
**Fig. 1** Map position of *Rx4* in *Solanum pimpinellifolium* accession PI128216 conferring hypersensitive resistance to tomato bacterial spot race T3 (*Xanthomonas perforans*) on chromosome 11 of tomato. Map distances (centimorgans) between each marker are on the left with marker names on the right. **a** Linkage map based on eight markers and 187  $F_2$  individuals derived from the cross between the susceptible variety OH88119 and resistant accession PI128216. **b** Linkage map based on nine markers and 1,116  $F_2$  individuals derived from the cross between the susceptible variety OH88119 and PI128216

susceptible lines when the plants were inoculated with race T3 strain. The expression of the gene increased from 0.5 to 12 h after infiltration in leaves of PI128216 and Hawaii7981 plants infiltrated with bacteria or mock-infiltrated with ddH<sub>2</sub>O, and then decreased. The same pattern of the gene expression was observed in leaves of OH88119 plants mock-infiltrated with ddH<sub>2</sub>O. However, the expression of the gene kept at a low level at all time points in OH88119 plants when the bacterium was presented (Fig. 3).

#### Isolation of DNA sequence for the candidate gene

Genomic DNA sequence for the candidate gene was 2,661 bp in PI128216 (Genbank accession no. JF743045) and Hawaii7981 (Genbank accession no. JF743044), and 2,667 bp in OH88119 (Genbank accession no. JF743043) from the start codon ATG to the stop codon TAG. No difference was observed between PI128216 and Hawaii7981 at the nucleotide level. However, a 6-bp InDel

**Fig. 2** Molecular marker genotypes and phenotypes of the recombinants and candidate gene analysis. **a** Molecular marker genotypes and phenotypes of the recombinants. The *black*, *white* and *gray bars* denote the marker genotypes of PI128216, OH88119 homozygotes and heterozygotes, respectively. **b** Candidate region of the *Rx4* gene and the annotated genes in *Solanum lycopersicum* cultivar Heinz1706 from <http://solgenomics.net>. The *Rx4* gene was narrowed down to a 45.1-kb region in the scaffold SL2.31sc03876. There were six annotated genes in this region. **c** Sequence comparison between OH88119 and PI128216 on the gene 6 coding region. Two SNPs at sites 963 and 2457 were synonymous mutation, and the remains were non-synonymous mutation. A 6-bp (TGCTGC) deletion was observed in PI128216 allele



**Fig. 3** Semi-quantitative RT-PCR analysis of the candidate gene expression in leaves of tomato line PI128216, Hawaii7981 and OH88119 at various time points after inoculation of bacterial spot race T3 strain Xv829 (*I*) or mock-inoculation with ddH<sub>2</sub>O (*MI*). The

amplification of the actin was used as a control to show that approximately equal amounts of total RNA had been used in the RT-PCR analysis

and eight SNPs were detected between sequences of PI128216 and OH88119 (Fig. 2c). Predicted cDNA analysis suggested that no intron existed in the gene. Therefore, the sequence encoded 886 amino acids in PI128216 and Hawaii7981, and 888 amino acids in OH88119. The 6-bp deletion in PI128216 caused the deletion of two amino acids. Six of eight SNPs caused non-synonymous substitution between PI128216 and OH88119, while two SNPs at nucleotide sites 963 bp (T/C) and 2,457 bp (C/T) resulted in synonymous substitutions.

Association of the candidate gene with hypersensitive resistance in 12 tomato lines

Of the five *S. pimpinellifolium* accessions, LA1589, PI128216 and LA2804 showed HR to race T3, while LA1269 and PI365914 were susceptible to race T3. All six *S. lycopersicum* lines including OH88119 were susceptible to race T3. The sizes of PCR products amplified by the marker pcc12 were the same in LA1589 and LA2804 as in PI128216, and the PCR product sizes were the same in all



**Fig. 4** Images of polyacrylamide gel electrophoresis for separating PCR fragments amplified from 12 tomato lines using the marker *pcc12*. Lines PA1589, PI128216 and LA2804 showed hypersensitive resistance to tomato bacterial spot race T3 (*X. perforans*), while the remains were susceptible

susceptible lines as in OH88119 (Fig. 4). These results confirmed that marker *pcc12* derived from the candidate gene was associated with HR to race T3.

## Discussion

Combining high resolution map and genome sequence information can facilitate the process of gene identification and cloning. In the present study, we mapped the *Rx4* gene to a 0.9-cM region on chromosome 11. The *Rx4* gene was 0.07 cM away from the marker *pcc12*. With the tomato whole genome shotgun sequence, we identified a candidate for the *Rx4* gene and found that the marker *pcc12* was actually derived from the sequence of the candidate gene. Since a single misclassified progeny would result in a linkage estimate of 0.09 cM for the population size of 1,116 individuals, we carefully checked the genotypes and phenotypes for all individuals. All progenies showing HR to race T3 strain of tomato bacterial spot had the PI128216 allele homozygous or heterozygous, while all susceptible progenies had the OH88119 allele for the marker *pcc12*. The linkage map constructed using only three markers *pcc14*, *pcc12* and *cLEC-24-C3* as well as the phenotypic data put the *Rx4* gene exactly at the *pcc12* marker site (data not shown) suggesting that the *Rx4* gene should always co-segregate with the marker *pcc12*.

Interactions between tomato plant resistance genes and bacterial spot pathogen avirulence genes have been well characterized (Whalen et al. 1993; Minsavage et al. 1996; Astua-Monge et al. 2000a, b). However, all these studies focused on isolating avirulence genes from the pathogen and analyzing their functions on inciting the hypersensitivity in tomato plants. The progress on isolation of genes for resistance to bacterial spot in tomato plants has been slow. Of the six resistance genes/QTLs (*rx1*, *rx2*, *rx3*, *Xv3/Rx4*, *Xv4*, and *Bs4*) mapped to several chromosomal regions (Yu et al. 1995; Ballvora et al. 2001; Robbins et al. 2009; Wang et al. 2011), only the *Bs4* gene for HR to

bacterial strains causing the disease on pepper (Ballvora et al. 2001) has been fine-mapped. We here mapped the *Rx4* gene to a 45.1-kb region and identified a candidate gene, which is a member of NBS-LRR class of resistance genes. The expression of the gene was higher in PI128216 and Hawaii7981 plants than in OH88119 plants inoculated with the bacterium, suggesting that the gene might be responsible for hypersensitivity in resistant lines. The results obtained here will facilitate the process of cloning the gene and understanding the mechanism of resistance to bacterial spot race T3 in tomato.

A number of tomato lines including Hawaii7981, PI128216, PI126932, Hawaii7998, PI114490, PI155372, PI340905-S and PI126428 have been reported to have hypersensitivity and/or field resistance to bacterial spot race T3 (Scott et al. 1995). The HR is controlled by a common locus in PI126932 and PI128216, but is different from the locus in Hawaii7981 (Hutton 2008). However, a recent study suggests that *Xv3* in Hawaii7981 and *Rx4* in PI128216 controlling HR and field resistance to race T3 might be allelic (Wang et al. 2011). *Xv3* is an incompletely dominant gene where the homozygous HR plants had confluent necrosis at 24 h and the heterozygotes at 48 h (Scott et al. 1996; Wang et al. 2011), while *Rx4* is a dominant gene for HR (Robbins et al. 2009). The F<sub>1</sub> plants of OH88119 x PI128216 showing HR at 24 h after inoculation in this study also supported a dominant gene model. By isolating and comparing genomic DNA for *Rx4* alleles in Hawaii7981 and PI128216, we did not detect any difference at nucleotide level. Gene expression detected by semi-quantitative RT-PCR also showed the same pattern in two lines, suggesting the different performances of HR at heterozygous resistance gene alleles between Hawaii7981 and PI128216 might not be due to the gene itself. Currently, we suspected that the variation of HR in two sources might be caused by the precision of determining a rapid HR and an intermediate response in different laboratories. Indeed, Hawaii7981 showed dominance in other experiments (Samuel Hutton, personnel communication). Since plants heterozygous for *Xv3* from Hawaii7981 were not included in this study, it is therefore not possible to directly compare the performances of *Xv3* and *Rx4* to race T3.

Breeding for resistance to bacterial spot in tomato using field and greenhouse selection has met with limited success due to the existence of multiple races of the pathogen, the lack of easy method to screen plants for resistance and the quantitative inheritance of resistance. Although some resistant varieties have been reported in India (Kavitha and Umesh 2008), resistance has not yet been an effectively deployed tool for the prevention and management of the disease in most production regions. Identification of molecular markers linked to the resistance gene and the application of marker-assisted selection (MAS) offer an



opportunity to pyramid resistance in a breeding line or hybrid. With the molecular marker Rx3-L1 tightly linked to *Rx3* locus, selection of desirable individuals carrying the gene has been conducted on an accelerated time-scale (Yang et al. 2005b; Zhang et al. 2009). In this study, we discovered a 6-bp InDel in nucleotide sequences of the *Rx4* alleles between resistant and susceptible lines. The marker pcc12 detecting the InDel will be useful to pyramid this gene with other resistance gene (e.g. *Rx3*) to develop lines or hybrids with resistance to multiple races of bacterial spot in tomato.

**Acknowledgments** The work was supported by National Natural Science Foundation of China (30972003), the Project Sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry, and the Program for New Century Excellent Talents in University (NCET-08-0531).

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