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A Recombinant Inbred Line Population of Tomato and its Genetic Map Constructed Based on a *Solanum lycopersicum* × *S. pimpinellifolium* Cross

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Abstract

A recombinant inbred line (RIL) population of tomato was developed from a cross between a tomato breeding line (NC 84173) and an accession (LA 0722) of the tomato wild species *S. pimpinellifolium*. NC 84173 is a horticulturally-superior, multiple-disease resistant inbred line that has been used as a parent in production of several commercial tomato hybrid cultivars. LA 0722 is a self-compatible, inbred accession, which was previously identified as a genetic source for fruit quality, disease resistance, and abiotic stress tolerance. The RIL population is in the F_9 generation and consists of 145 lines. A genetic linkage map of the population was developed with 191 molecular markers, including 129 RFLPs and 62 RGAs. The genetic map spans 1505 cM of the 12 tomato chromosomes with an average inter-marker distance of 7.9 cM. The RFLP markers were chosen from the high-density map of tomato, previously developed based on a *S. lycopersicum* × *S. pennellii* F_2 population. The RGA markers were derived using degenerate oligonucleotide primers designed based on conserved leucine-rich repeat (LRR),

nucleotide binding site (NBS), and serine/threonine protein kinase (PtoKin) domains of known resistance genes (R genes). Many RGAs were clustered, a characteristic of many R gene families. Some RGAs mapped to chromosomal locations where known R genes and QRLs were previously mapped to, suggesting potential evolutionary relationship with RGAs and R genes. The RIL population is segregating for numerous desirable characteristics and together with its genetic map can be utilized for identification, characterization and exploitation of important genes or QTLs in LA 0722 and for introgression of useful traits into the cultivated tomato via marker-assisted breeding.

Keywords: disease resistance, genetic markers, molecular map, permanent genetic population, recombinant inbred lines (RILs), restriction fragment length polymorphism (RFLP), resistance gene analogs (RGAs)

1. Introduction

The use of early filial or backcross populations for genetic studies may have several disadvantages, including limitations in population duplication for repeating experiments in time or space, high level of heterozygosity and heterogeneity and thus variation from sample to sample, and elevated linkage disequilibrium resulting in detection of false linkages in genetic mapping studies. In contrast, the use of permanent (immortal) segregating populations such as recombinant inbred lines (RILs), doubled haploids (DHs), backcross inbred lines (BILs) or introgression lines (ILs) can be advantageous due to several reasons, including 1) presence of high level of homozygosity and the ability to regenerate population without changing its genetic composition, 2) opportunity to repeat experiments in time or space and under different environmental conditions, 3) accurate separation and estimation of genetic and environmental effects on trait expression, 4) increase in trait heritability by reducing environmental variation via repeated experiments, 5) reliable gene/QTL mapping for traits segregating in the population, and 6) reliable expression analysis across treatments and environmental conditions. Among the aforementioned populations, RILs are generally more desirable due to the presence of greater genetic variation in the population (i.e., exhibiting segregation for all traits differed between the two parents), greater recombination representation in the population, and greater ability to test for epistatic interaction effects. RILs are particularly useful for genetic mapping studies due to 1) presence of low level of linkage disequilibrium, allowing high resolution mapping, 2) absence or limited heterozygosity, allowing efficient use of dominant markers, 3) reduced genetic and environmental background noises, allowing efficient QTL validation, and 4) stable genotypes, allowing precise quantification of G × E interaction effects associated with QTLs.

RILs can be developed in both self- and cross-pollinated plant species, though they are more commonly developed in self-pollinated species. The most common protocol for developing RILs is by generation advancement of F_2 progeny (usually

derived from a cross between two inbred lines) via self-breeding and single-seed descent breeding approach until homozygosity or near homozygosity is reached [11]. RIL populations have been developed and used for numerous studies in many plant species, including agronomic and vegetable crops as well as model plants such as *Arabidopsis*. In tomato (*Solanum lycopersicum* L.), several RIL populations have been developed and used extensively in genetic and breeding research. The first RIL population of tomato, including 97 F_8 lines, was developed from a cross between a *S. lycopersicum* processing tomato line (UC 204B) and an accession (LA 0483) of tomato wild species *S. cheesmaniae* (L. Riley) Fosberg [75]. This population and its genetic map were subsequently used for various studies, including mapping of QTLs for fruit weight, soluble solids content, seed weight and plant morphological characteristics [76]. The second RIL population of tomato was constructed based on an intraspecific cross between tomato inbred lines Cervil (*S. lycopersicum) and* Levovil (*S. lycopersicum* var. *cerasiforme* [84]. This population, which included 153 RI lines, was used to compare the efficiency of RFLP, RAPD and AFLP markers for developing genetic maps in tomato [84] and to investigate genetic basis of several fruit quality characteristics [12]. Later a 77-line RIL population with a 107-marker genetic map was developed from a cross between tomato breeding NC 23E-2 and *S. pimpinellifolium* L. accession L3708 (aka LA 1269 and PI 365957) [41]. This population has had limited utility due to its small size and limited number of genetic markers. Subsequently, two F_6 RIL populations were developed from crosses between a wild form of the cultivated tomato species, *S. lycopersicum* f. sp. *cerasiforme* L., and either an accession of *S. pimpinellifolium* or an accession of *S. cheesmaniae* [97]. The limited number of genetic markers used in their maps, presence of big marker gaps in various chromosomes (e.g. some chromosomes with only a few markers), and the use of internationally-unknown parental genotypes and genetic markers greatly limited utility of these populations. More recently, a RIL population of tomato (188 lines) and its genetic map (with 361 markers) was developed from a cross between *Solanum lycopersicum* line H7996 (resistant to bacterial wilt caused by *Ralstonia solanacearum*) and *S. pimpinellifolium* accession WVa700 (susceptible to bacterial wilt) [94]; this populations has been used mainly for genetic characterization of bacterial wilt resistance in tomato [98]. However, development of additional RIL populations of tomato using internationally known, accessible and superior genotypes is necessary and would be useful for tomato breeding purposes as well as basic genetic research.

Among the wild species of tomato, the red-fruited *S. pimpinellifolium* is the most closely related and the only species from which natural introgression into the cultigen has been documented [68]. In addition, extensive genetic introgression from *S. pimpinellifolium* into the cultivated tomato has been made through deliberate plant breeding [43]. Accessions within *S. pimpinellifolium* are highly self-compatible and bi-directionally cross compatible with the cultivated tomato. Furthermore, they have much fewer undesirable characteristics compared to the more distantly related wild species of tomato, making them more desirable for to-

mato crop improvement. Therefore, RIL populations developed from crosses between *S. pimpinellifolium* and the cultivated tomato species will have great utilities for both basic and applied tomato research.

To assess the potential utility of accessions within *S. pimpinellifolium* for breeding purposes, previously we conducted evaluation of ~300 accessions within this species for numerous desirable characteristics, including disease resistance, abiotic stress tolerance, and fruit quality. This research resulted in the identification of several accessions with one or more desirable characteristics, including resistance to early blight (EB) caused by *Alternaria solani* [31] (Foolad MR, unpublished data), resistance to late blight (LB) caused by *Phytophthora infestans* [34], tolerance to salt- [30] (Foolad MR, unpublished data), cold- [58] (Foolad MR, unpublished data) and drought-stress (Foolad MR, unpublished data), and fruit quality [48] (Foolad MR, unpublished data). Subsequently, a few of these accessions were used to investigate the genetic basis of several desirable characteristics, including salt tolerance [27, 29], cold tolerance [28], drought tolerance [35], EB resistance [2, 26, 32], LB resistance [65, 66], and fruit quality traits such as high fruit lycopene content [3, 16, 51]. Many of these studies were conducted using early filial or backcross populations. Development of advanced segregating populations (such as RILs) could facilitate effective genetic characterization and exploitation of desirable traits in *S. pimpinellifolium* accessions.

Recently we developed and reported an F_{10} RIL population from a cross between *S. pimpinellifolium* accession LA 2093 and tomato line NC EBR-1 [4]. Accession LA 2093 was identified with numerous desirable horticultural characteristics, including high fruit quality and disease resistance. Subsequently, this RIL population and its genetic map was used to characterize the genetic basis of various fruit quality characteristics such as high fruit lycopene content [3, 51] and resistance to tomato early blight [2, 26]. During our studies, we also identified another *S. pimpinellifolium* accession (LA 0722) with numerous desirable horticultural characteristics. Subsequently, we developed early backcross populations from crosses between LA 0722 and a tomato breeding line (NC 84173) and constructed a genetic linkage map [15], which were used for numerous genetic studies such as mapping of QTLs for fruit quality traits [16], cold tolerance [28], salt tolerance [27, 29], and drought tolerance [33, 35]. LA 0722 was also identified with various desirable fruit quality characteristics by other researchers [82]. To facilitate further characterization and exploitation of the full genetic potential of LA 0722, we developed an F_9-RIL population from a cross between this accession and tomato breeding line NC 84173 and constructed a medium-density genetic linkage of the population using RFLP and RGA (resistance gene analog) markers. Here we report this RIL population and its genetic map, which could be used to facilitate genetic characterization and exploitation of desirable traits in LA 0722. We also have compared the chromosomal locations of the RGAs with locations of known tomato resistance genes and quantitative resistance loci (QRLs).

2. Material and Methods

Plant materials

Hybridizations were made between *S. lycopersicum* breeding line NC 84173 and *S. pimpinellifolium* accession LA 0722 to produce F_1 progeny. NC 84173 is a horticulturally superior, multiple-disease resistant fresh-market tomato breeding line that has been used as a parent for production of several commercial tomato F_1 hybrids [38, 39]. LA 0722 is a self-compatible, inbred accession which readily hybridizes with *S. lycopersicum* and is a rich source of genes for desirable characteristics, including abiotic stress tolerance, high fruit quality and disease resistance [16, 27, 29-31]. A single F_1 hybrid plant was self-fertilized to produce F_2 progeny. Approximately 200 random F_2 plants were used to develop a recombinant inbred line (RIL) population by self-breeding and a single-seed-descent breeding approach. During the course of population advancement, several lines were lost and 145 lines reached F_9 generation, which constituted the RIL population and were used for linkage map construction.

RFLP analysis

Nuclear DNA was extracted from leaf tissue of each of the parental lines and 145 F_9 RILs, treated with RNase, and digested with 5 restriction enzymes, including *DraI*, *EcoRI*, *EcoRV*, *HindIII* and *XbaI*, according to the manufacture's instruction (Amersham Pharmacia Biotech, NJ, USA). Agarose gel electrophoresis, Southern blotting, hybridization and autoradiography were carried out as described elsewhere [4]. The RFLP probes included 113 random genomic (TG) or cDNA (CD or CT) clones of tomato obtained from S.D. Tanksley, Cornell University, Ithaca, NY USA; nine germination related cDNA clones of tomato (denoted as C, CEL and KJB) obtained from K.J. Bradford, University of California, Davis, CA USA; and 2 cDNA clones of potassium transport-related genes of tomato or potato obtained from L. Kochian, U.S. Plant, Soil and Nutrition Laboratory, USDA-ARS, Cornell University, Ithaca, NY USA. The RFLP clones from Cornell University were chosen based on their map positions on the high-density map of tomato [81] so to provide a good coverage of the genome. A total of 124 clones resulted in production of 129 polymorphic RFLP markers.

RGA analysis

Degenerate oligonucleotide primers were previously designed based on the conserved leucine-rich repeat (*LRR*), nucleotide binding site (*NBS*) and *serine/threonine* protein kinase (*PtoKin*) motifs of several known resistance genes (R genes) from different plant species, including *Arabidopsis*, rice, tomato, tobacco, flax, and wheat [17, 57, 60, 89, 100]. Eighteen primers (Table 1) were chosen for this study and used in 10 different primer-pair combinations for PCR amplification. Some of the primers were chosen to be degenerate at the redundant

third position (3' end) in codons in order to cover a range of possible sequences encoding the motifs, and thus to increase the efficiency of polymerase chain reaction (PCR) amplification, as described elsewhere [89, 100]. Only one pair of primers was used for each PCR amplification. Standard PCR conditions were applied to a 25 µl reaction volume consisting of 300 µM each of dATP, dCTP, dGTP and dTTP, 5 mM of $MgCl_2$, one unit of *Taq* DNA polymerase, 2.5 µl of 10X buffer (PCR Core System I; Promega, Madison, WI), 2 µM of each primer, and 40 ng of genomic DNA. For the control reaction, the DNA template was substituted by sterile nuclease-free water (ddH_2O) to ensure that there was no contamination. The PCR reaction was overlaid with mineral oil and carried out in a Perkin Elmer DNA Thermal Cycler 480, programmed for 4 min at 94 °C for an initial denaturation, and 36 cycles of 1 min at 94 °C, 1 min at 50 °C and 1.5 min at 72 °C, followed by a final 7 min extension at 72 °C. Following PCR amplification, a 12 µl of loading solution (10M Urea and 0.08% xylene cyanole) was added to the 25-µl reaction volume, heated at 95 °C for 5 min to denature the amplified DNA, and immediately put on ice. Denaturing polyacrylamide gel electrophoresis (PAGE) was used to separate the amplification products. A denaturing gel (7M urea, 6% polyacrylamide) was prepared in a sequencing gel apparatus (420 × 330 × 0.4 mm; Fisher Biotech, Springfield, NJ) using Bind- and Repel-Silane (Promega). After polymerization, the gel was pre-run in 1X TBE buffer for 30 min at 40 W (~1400 V) to reach a gel temperature of 50 °C. Twelve µl of loading buffer (10M urea and 0.08% xylene cyanole) was added to each 25-µl amplified DNA sample and the mixture was denatured at 95 °C for 5 min and immediately put on ice. After cleaning the gel loading area, a 0.4-mm-thick shark comb (Fisher Biotech) was inserted into the gel. Subsequently, 7 µl of each PCR-amplified sample was loaded. Each gel accommodated 60 DNA samples and three DNA size markers (1 Kb, 100 bp, 50 bp; for locating corresponding RGA bands in different gels). The gel was run at 35 W (~1350 V) for 3.5-4 h.

After electrophoresis, the gel, fixed to the Bind-Silane surface of one glass plate, was silver-stained following the manufacturer's protocol (Promega). The gel was air dried at room temperature overnight and stored in darkness for future scoring and scanning. Following gel electrophoresis and staining, polymorphic and monomorphic bands were observed. A total of 50 polymorphic bands with scorable segregation in the RIL population were directly recorded from the polyacrylamide gels. Furthermore, 52 strong monomorphic bands, resulting from different primer pairs, were isolated from dried gels by fine-needle scratching of the surface and re-amplified using the same primers and PCR conditions. The new PCR products were used as probes to hybridize the Southern membranes developed for RFLP analysis. Of these probes, 12 detected polymorphism between the two parents, which were used to score 12 RGA-RFLP markers in the RIL population. Thus, a total of 62 RGA markers were successfully scored and mapped onto the 12 tomato chromosomes.

Table 1. Oligonucleotide primers designed based on the conserved amino acid sequences within the *LRR*, *NBS* and *Pto* protein domains encoded by various R genes

Group	Primers	Sequences (5'- 3')	Design Basis	References
LRR	XLRR-for. XLRR-rev.	CCGTTGGACAGGAAGGAG CCCATAGACCGGACTGTT	LRR domain of the rice *Xa21* gene conferring resistance to *Xanthomonas campestris pv oryzae*	(Chen et al., 1998)
	CLRR-for. CLRR-rev.	TTTTCGTGTTCAACGACG TAACGTCTATCGACTTCT	LRR domain of the tomato *Cf-9* gene conferring resistance to *Cladosporium fulvum*	
	NLRR for. NLRR rev.	TAGGGCCTCTTGCATCGT TATAAAAAGTGCCGGACT	LRR domain of the tomato *N* gene conferring resistance to *Cladosporium fulvum*	
NBS	ANo.-2 ANo.-3	TATAGCGGCCGCIARIGC IARIGGIARNCC ATATGCGGCCGCGGIGGIG TIGGIAARACNAC	Conserved P-loop and hydrophobic NBS regions of the *N* and *RPS2* genes from tobacco and *Arabidopsis* respectively	(Speulman et al., 1998)
	NBS-for. NBS-rev.	GGAATGGGNGGNGTNGG NAARAC YCTAGTTGTRAYDATDA YYYTRC	Conserved peptide sequence of the two NBS domains present in the *N* and *RPS2* genes of tobacco and *Arabidopsis* respectively	(Yu et al., 1996)
	S-1 AS-1 S-2 AS-3	GGTGGGGTTGGGAAGAC AACG CAACGCTAGTGGCAATCC GGIGGIGTIGGIAAIACIAC IAGIGCIAGIGGIAGICC	Hydrophobic domain and P-loop of conserved NBS from the *Arabidopsis N* and *RPS2* genes and the flax *L6* gene conferring resistance to rust	(Leister et al., 1996) (Mago et al., 1999)
PtoKin	Ptokin-1 Ptokin-2 Ptokin-3 Ptokin-4	GCATTGGAACAAGGTGAA AGGGGGACCACCACGTAG TAGTTCGGACGTTTACAT AGTGTCTTGTAGGGTATC	Serine/threonine protein kinase domain of the tomato Pto gene conferring resistance to the bacterial pathogen *Pseudomonas*. *syringae* pv tomato	(Chen et al., 1998)

*Code for mixed bases: Y=C/T, N=A/G/C/T, R=A/G and D=A/G/T

To determine RGA fragment size, PAGE polymorphic and monopolymorphic fragments were excised from the dried polyacrylamide gel and re-amplified. The amplified products and DNA size markers (1 Kb, 100 bp, and 50 bp) were run on a 1.0% agarose gel, stained with ethidium bromide, and photographed.

Statistical and linkage analyses

Segregation of the 191 marker loci (129 RFLPs and 62 RGAs) in the RIL population was examined for deviation from the expected Mendelian genotypic ratio of 1:1, using chi-square (χ^2) goodness-of-fit analysis and QGENE computer program [70]. Multipoint linkage analysis of the genetic markers was performed using the MAPMAKER program v. 3.0 [55] and a genetic linkage map was constructed using Kosambi mapping function [53]. The distribution of percentage of the *S. lycopersicum* (L) genome and percentage heterozygosity in the RIL population were estimated using the computer program QGENE [70].

3. Results

Development of the RIL population

A RIL population of tomato in F_9 generation was developed from a cross between tomato (*S. lycopersicum*) breeding line NC 84173 and accession LA 0722 of the tomato wild species *S. pimpinellifolium* using self-breeding and a single-seed-decent breeding approach. This RIL population consists of 145 lines and is segregating for numerous desirable characteristics derived from its two parents, including disease resistance, abiotic stress tolerance, and fruit quality traits. A genetic linkage map of the RIL population was also developed to facilitate the use of the population for basic and applied genetic and breeding studies.

Marker segregation

Of the 191 markers scored in the RIL population, 41 (~21%) exhibited significant deviation from the expected Mendelian genotypic ratio of 1:1 at $P < 0.01$ (Table 2). Of these, 28 markers, located on chromosomes 1, 3, 4, 7, 9 and 10 were distorted in favor of *S. pimpinellifolium* homozygote and 13 markers on chromosomes 2, 5 and 8 were distorted in favor of *S. lycopersicum* homozygote.

The magnitude and location of segregation distortions observed in this population were generally similar to those previously reported in other interspecific populations of tomato (discussed below). Of the 41 markers that exhibited skewed segregation, 19 were RFLPs (~15% of all RFLP markers used) and 22 were RGAs (~35% of all RGA markers used). The higher level skewed segregation observed in RGA markers could be attributed to the dominant nature of these markers, as discussed below.

Table 2. Significant deviations from the expected Mendelian 1:1 ratio in two homozygous classes in the *Solanum lycopersicum* × *S. pimpinellifolium* RIL population (*L = lycopersicum* allele, *Pm = pimpinellifolium* allele)

Chr.	Marker	*L/L*	*L/Pm*	*Pm/Pm*	*L/L:Pm/Pm*	χ^{2*}
1	XLRR_110	50	0	91	0.55	11.92
	CT55a	55	0	87	0.63	7.21
	S13_190	55	0	88	0.63	7.62
	S13_390	53	0	88	0.60	8.69
	S13_200	48	0	94	0.51	14.90
	AN23_220	50	0	94	0.53	13.44
	TG125	49	6	90	0.54	12.09
	PK34_320	45	0	92	0.49	16.12
	S11_220	44	0	100	0.44	21.78
	AN23_100	26	0	64	0.41	16.04
	AN23_200	44	0	77	0.57	9.00
	TG70	49	12	80	0.61	7.45
	CT132	48	4	93	0.52	14.36
	TG273	50	5	88	0.57	10.46
2	S23_500	92	0	48	1.92	13.83
	S11_125	100	0	44	2.27	21.78
	SA2_300	85	9	49	1.73	9.67
	TG608	86	10	47	1.83	11.44
	CT205	81	13	50	1.62	7.34
3	TG132	51	11	82	0.62	7.23
	CT85	45	13	87	0.52	13.36
	TG242	47	7	91	0.52	14.03
4	TG272	48	16	80	0.60	8.00
	TG163	25	2	117	0.21	59.61
5	TG96A	87	4	51	1.71	9.39
	TG318	86	5	54	1.59	9.39
	PK12_100	88	0	53	1.66	8.69
	CT118A	83	12	50	1.66	8.19
7	TG113A	52	11	82	0.63	6.72
	CT52	50	12	83	0.60	8.19
	S11_75	53	0	90	0.59	9.57
	PK34_500	51	0	86	0.59	8.94
	PK34_800	50	1	83	0.60	8.19
	NLRR_48	52	0	90	0.58	10.17
8	S11_350	97	0	43	2.26	20.83
	PK12_150	99	0	42	2.36	23.04
	S11_80	95	0	49	1.94	14.69
	C21B	92	7	44	2.09	16.94
9	S11_200	53	2	90	0.59	9.57
10	PK34_150	48	0	91	0.53	13.30
	TG241	54	1	90	0.60	9.00

All χ^2 values significant at $P < 0.01$.

Genomic composition of the RI lines

The 145 F_9 RILs were examined for genome composition using the 129 RFLP and 62 RGA markers. Genome composition of individual RILs ranged from 22.5% to 72.5% with an average of 48.9% from the *S. lycopersicum* (*L*) parent (Fig. 1A). Further, at each co-dominant RFLP locus, the RI lines were scored as either *S. lycopersicum* homozygous (*LL*), *S. pimpinellifolium* homozygous (*PmPm*), or heterozygous (*LPm*). At each dominant RGA locus, the RI lines were scored as either homozygous *LL* or homozygous *PmPm*. On average, RI lines were homozygous *LL* for 46.8% of their marker loci (ranging from 17.4% to 70.2% across the lines) and homozygous *PmPm* for 49.1% of their marker alleles, suggesting similar contribution from both parents in the RIL population. The percentage heterozyosity (*LPm*) across all RFLP markers ranged from 0 to ~13%, with an average residual heterozygosity of 4.2% (Fig. 1B). This average was greater than the expected heterozygosity (0.4%) for an F_9 generation, but it was similar to or lower than what has been reported in other RIL populations of tomato (discussed below).

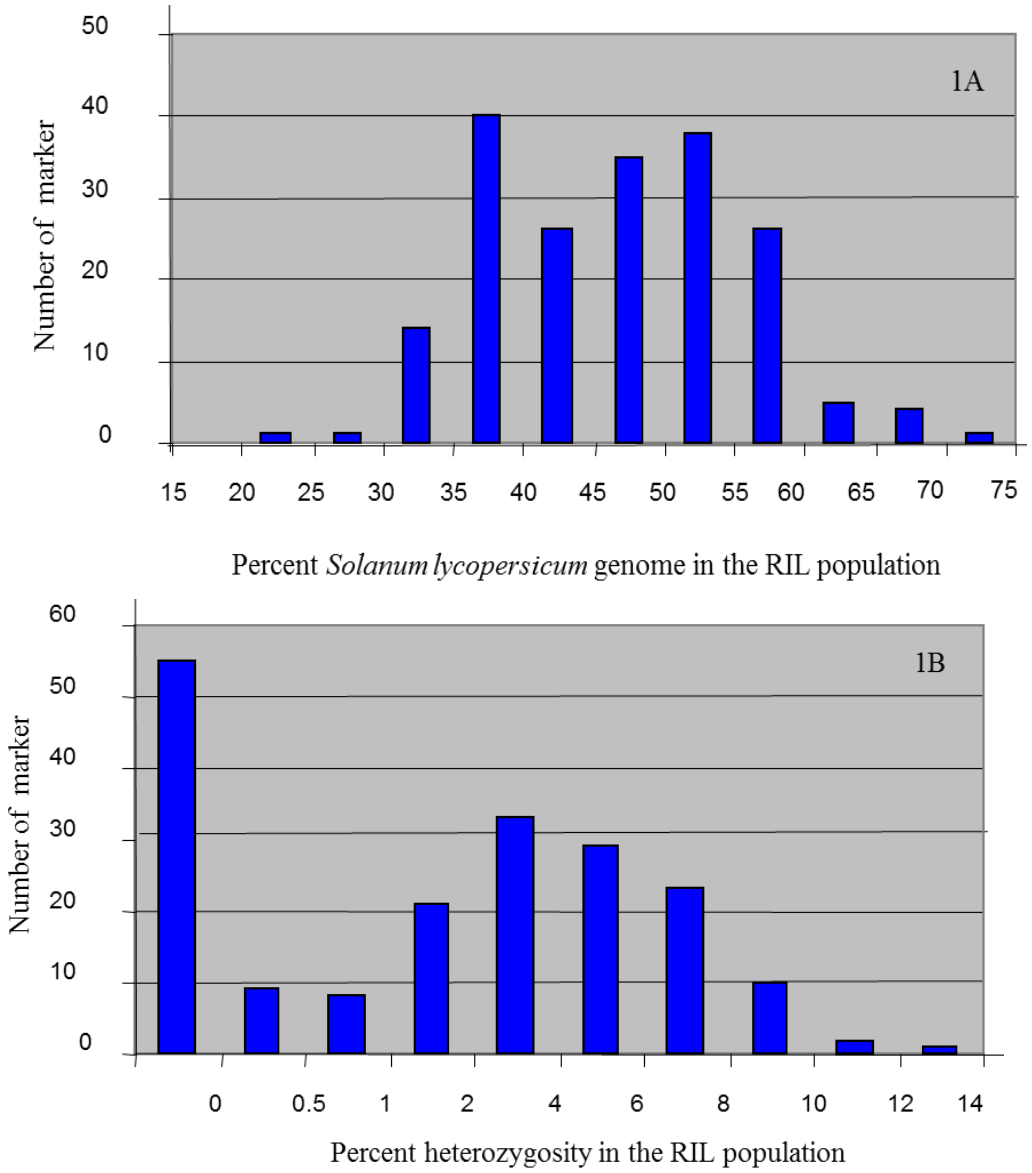
RFLP markers and marker polymorphism

RFLP markers were mainly chosen from the high-density RFLP map of tomato, constructed based on a *S. lycopersicum* × *S. pennellii* cross [81]. Of the markers surveyed, ~40% exhibited polymorphism between NC 84173 and LA 0722. A low level of RFLP polymorphism was expected because *S. pimpinellifolium* is phylogenetically much closer to the cultivated tomato than is *S. pennellii* [68].

RGA markers and marker polymorphism

Ten degenerate oligonucleotide primer-pair combinations (Table 1) were used for PCR amplifications, which resulted in a total of 335 scorable RGA fragments. Each primer pair produced between 10 (for primer pair S-2/AS-3) and 66 fragments (for primer pair PtoKin-1/PtoKin-2) (Table 3). Of the total of 335 fragments, 50 were polymorphic between the parents and were scorable in the RIL population. However, there were great differences among primer pairs in producing polymorphic markers, ranging from 0 (for CLRR-for./ CLRR-rev. and NBS-for./ NBS-rev.) to 10 (for S-1/ AS-1), with an average of 5 markers per primer pair (Table 3). The polymorphic rate across primer pairs ranged from 0% (for CLRR-for./ CLRR-rev. and NBS-for./ NBS-rev.) to 50% of the fragments (for S-2/ AS-3). The fragment size for the polymorphic markers ranged from ~48 bp to ~800 bp, with an average of 304 bp. To assess the utility of the monomorphic fragments, 52 strong fragments from the various primer pairs were excised from the dried acrylamide gel, re-amplified, labeled with ^{32}P and used as probes for Southern blot analysis. Of these, 12 detected polymorphism as RFLP markers, bringing the total number of useful RGA markers to 62.

Fig. 1. Distribution of percent *Solanum lycopersicum* parent genome (1A) and percent heterozygosity (1B) in the RIL population (n = 145), estimated based on 191 molecular markers.



Construction of the genetic linkage map

A genetic linkage map (referred to as *L* × *Pm*-R map) was constructed based on 145 F_9 RILs and 191 genetic markers (129 RFLPs and 62 RGAs). The map spanned 1,505 cM of the 12 tomato chromosomes, with an average marker distance of 7.9 cM (Fig. 2). The number of markers per chromosome ranged from 7 (chr. 11) to 25 (chr. 1), with an average of 16. Due to a low level of RFLP polymorphism between the two species, a few regions of the genome contained marker intervals larger than 20 cM (Fig. 2). For example, of the RFLP markers surveyed from the high-density RFLP map of tomato [81] only ~40% exhibited po-

lymorphism between NC 84173 and LA 0722. The genetic length of each chromosome ranged from 91 cM (chr. 11) to 182 cM (chr. 1) (Table 4), generally consistent with other reported RFLP linkage maps of tomato (see below).

Table 3. List of RGA primer combinations and total number of PCR amplified fragments, number of polymorphic markers and rate for polymorphism for each primer pair

Primer pair	Total fragment	Polymorphic fragment	Polymorphic rate (%)
ANo-2/ ANo-3	19	6	31.6
CLRR-for./ CLRR-rev.	30	0	0.0
NLRR-for./ NLRR-rev.	27	4	14.8
NBS-for./ NBS-rev.	40	0	0.0
Ptokin-1/ Ptokin-2	66	5	7.6
Ptokin-3/ Ptokin-4	36	8	22.2
S-1/ S-1	31	10	32.3
S-1/ S-3	39	9	23.1
S-2/ S-3	10	5	50.0
XLRR-for./ XLRR-rev.	37	3	8.1
Total	335	50	
Average	33.5	5	15.8

Comparison of the RIL map with a BC_1 map of the same cross

Previously, we developed a linkage map of tomato based on a BC_1 population of the same (NC 84173 × LA 0722) cross (referred to as *E* × *Pm*-1 map) [15]. The *E* × *Pm*-1 map was constructed based on 119 BC_1 individuals and 151 RFLP markers. The present map (*E* × *Pm*-R) included 123 of the same RFLP markers in addition to 6 other RFLP and 62 RGA markers. In general, the linear order of the markers was the same in the two maps with the exception of a few tightly-linked markers on chromosomes 2, 4, 7, 10 and 12. Furthermore, as expected, the RIL map was generally expanded (with a total length of 1505 cM) compared to the BC_1 map (1186 cM). For all but chromosomes 2 and 9, chromosome lengths were greater in the RIL map than in the BC_1 map (Table 4). Such expansion was presumably due to greater chances of recombination between markers during 8 generations of self-breeding and population advancement to F_9 generation.

Comparison of the RIL map with other linkage maps of tomato

The present map (*L* × *Pm*-R) was compared with two other *S. lycopersicum* × *S. pimpinellifolium* maps, including *L* × *Pm*-2 [42] and *L* × *Pm*-3 [87], and with the high-density *S. lycopersicum* × *S. pennellii* (*L* × *P*) map of tomato [81]. The *L* × *Pm*-2 was constructed based on a BC_1 population of a cross between a processing tomato cultivar (M82-1-7; recurrent parent) and *S. pimpinellifolium* accession LA-

1589 using 120 markers. The *L* × *Pm*-3 was constructed based on an F_2 population of a cross between tomato line NC EBR-1 and *S. pimpinellifolium* accession LA 2093 using 115 RFLP, 94 EST and 41 RGA markers. The high density *L* × *P* map was constructed based on an F_2 population of a cross between tomato cultivar VF36-*Tm2*^{a} and *S. pennellii* accession LA 0716 using over 1000 markers. In general, the order of the markers in the present map was similar to the other maps except in a few cases where tightly linked markers switched positions. Table 4 displays the length of each of the 12 chromosomes and the total length in different linkage maps as well as the ratio of chromosome lengths for pairwise comparisons across the maps. The total length of the current map (1505 cM) was larger than the total length of each of the other 4 tomato linkage maps.

Fig. 2. A genetic linkage map of tomato constructed based on an F_9-RIL population of a cross between a tomato (*Solanum lycopersicum*) breeding line (NC 84173) and an accession (LA 0722) of the tomato wild species *S. pimpinellifolium*. The map comprises 191 molecular markers, including 129 RFLP (black font) and 62 RGA markers (red font). The names of the markers are shown at the right and the map distances between markers (in cM based on Kosambi function) are shown at the left of the chromosomes. The names of RGA markers were derived from the names of the corresponding primers (see Table 1 for the list of primers) and the fragment size. The approximate chromosomal locations of disease-resistance genes (R genes) and quantitative resistance loci (QRLs), as inferred from other published researches, are shown in parentheses to the right of chromosomes. The descriptions of the R genes and QRLs are as follows: *Asc*: resistance to Alternaria stem canker (*Alternaria* alternata f. sp. *lycopersici*) [67]; *Bw* (*1-5)* or *Rrs* (*3-12*): QLRs for resistance to bacterial wilt (*Ralstonia solanacearum*) [61, 93]; *Cf* (*1-9, ECP2*): resistance to leaf mould (*Cladosporium fulvum*) [45, 56, 92]; *Cmr*: cucumber mosaic virus [90]; *Fen*: sensitivity to herbicide fenthion [64]; *Frl*: resistance to Fusarium crown and root rot (*Fusarium oxysporum f. sp. radicis-lycopersici*) [95]; *Hero*: resistance to potato cyst namatode (*Globodera rostochiensis*) [37]; *I* (*I, 1, 2, 2C, 3*): resistance to different races of Fusarium wilt (*Fusarium oxysporum f. sp. lycopersici*) [74, 86, 88]; *Lv*: resistance to powdery mildew (*Leveuillula taurica*) [19]; *Meu-1*: resistance to potato aphid [50, 83]; *Mi* (*Mi, 1, 2, 3, 9)*: resistance to root knot nematodes (*Meloidogyne* spp.) [1, 50, 99]; *Ol* (*1, 2, 3*): resistance to powdery mildew (*Oidium lycopersicum*) [6]; *Ph* (*1, 2, 3*): resistance to late blight (*Phytophthora infestans*) in tomato [20, 69, 80]; *Pot-1*: resistance to potyvirus [77]; *Pto* and *Prf*: resistance to bacterial speck (*Pseudomonase syringae* pv *tomato*) [63, 85]; *Py-1*: resistance to corky root rot (*Pyrenochaeta lycopersici*) [24]; *Rcm* (*1-10*): QRLs for resistance to bacterial canker (*Clavibacter michiganensis*) [96]; *Rrs* (*3-12*) or *Bw* (*1-5)*: QLRs for resistance to bacterial wilt (*Ralstonia solanacearum*) [21, 61, 93]; *Rx* (*1, 2, 3, 4*): resistance to bacterial spot (*Xanthomonas campestris*) [5, 7]; *Sm*: resistance to *Stemphilium* [8]; *Sw-5*: resistance to tomato spotted wilt virus [10]; *Tm-1* and *Tm-2*^{a}: resistance to tobacco mosaic virus [73, 95]; *Ty* (*1, 2, 3*): resistance to tomato yellow leaf curl virus [13, 47]; *Ve*: resistance to *Verticillium dahliae* [23].

Figure 2

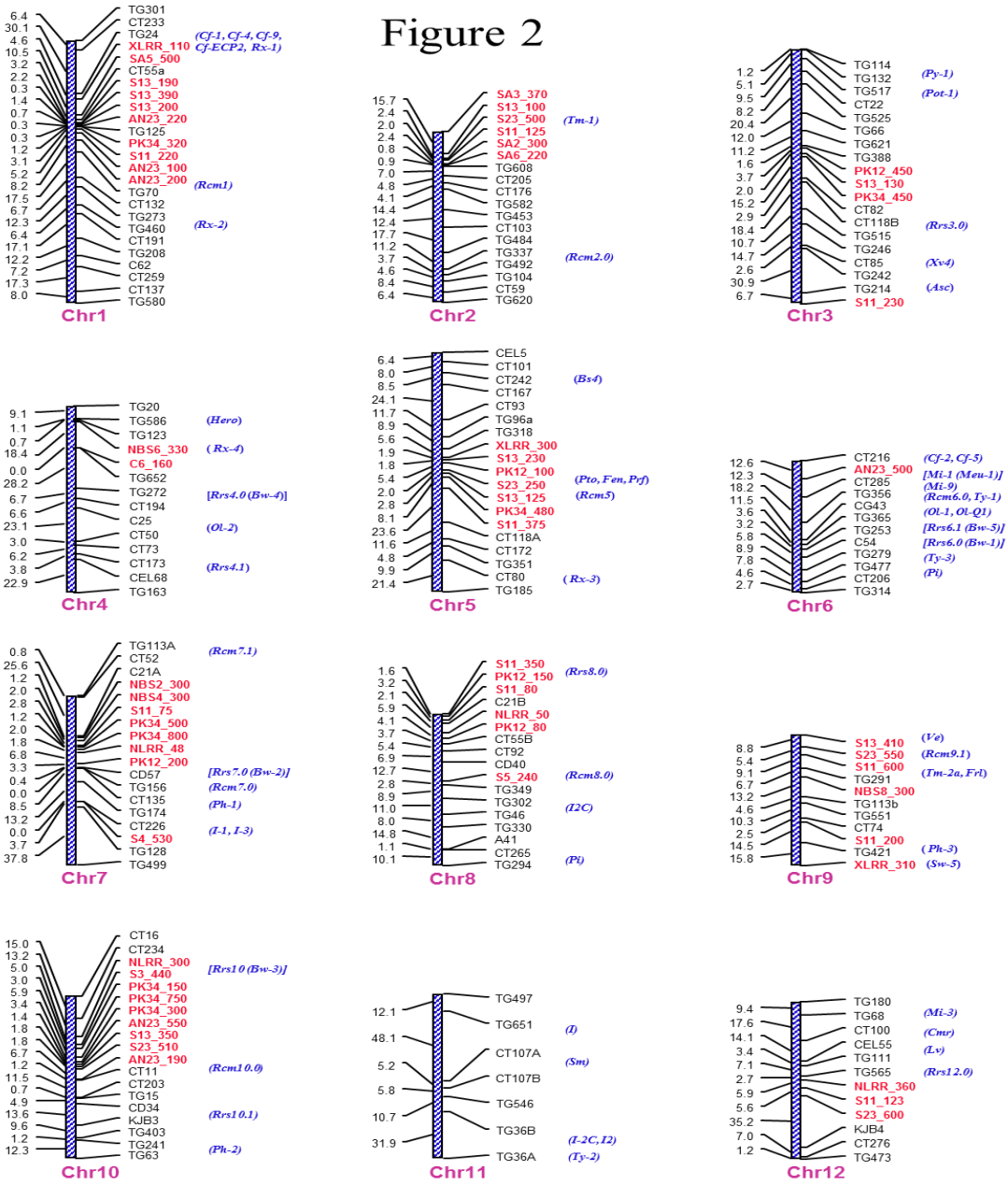

Chr1
TG301 CT233 TG24 XLRR_110 SA5_500 CT55a S13_190 S13_390 S13_200 AN23_220 TG125 PK34_320 S11_220 AN23_100 AN23_200 TG70 CT132 TG273 TG460 CT191 TG208 C62 CT259 CT137 TG580
(Cf-1, Cf-4, Cf-9, Cf-ECP2, Rx-1)
(Rcm1)
(Rx-2)
Chr2
SA3_370 S13_100 S23_500 S11_125 SA2_300 SA6_220 TG608 CT205 CT176 TG582 TG453 CT103 TG484 TG337 TG492 TG104 CT59 TG620
(Tm-1)
(Rcm2.0)
Chr3
TG114 TG132 TG517 CT22 TG525 TG66 TG621 TG388 PK12_450 S13_130 PK34_450 CT82 CT118B TG515 TG246 CT85 TG242 TG214 S11_230
(Py-1)
(Pot-1)
(Rrs3.0)
(Xv4)
(Asc)
Chr4
TG20 TG586 TG123 NBS6_330 C6_160 TG652 TG272 CT194 C25 CT50 CT73 CT173 CEL68 TG163
(Hero)
(Rx-4)
[Rrs4.0 (Bw-4)]
(Ol-2)
(Rrs4.1)
Chr5
CEL5 CT101 CT242 CT167 CT93 TG96a TG318 XLRR_300 S13_230 PK12_100 S23_250 S13_125 PK34_480 S11_375 CT118A CT172 TG351 CT80 TG185
(Bs4)
(Pto, Fen, Prf)
(Rcm5)
(Rx-3)
Chr6
CT216 AN23_500 CT285 TG356 CG43 TG365 TG253 C54 TG279 TG477 CT206 TG314
(Cf-2, Cf-5)
[Mi-1 (Meu-1)]
(Mi-9)
(Rcm6.0, Ty-1)
(Ol-1, Ol-Q1)
[Rrs6.1 (Bw-5)]
[Rrs6.0 (Bw-1)]
(Ty-3)
(Pi)
Chr7
TG113A CT52 C21A NBS2_300 NBS4_300 S11_75 PK34_500 PK34_800 NLRR_48 PK12_200 CD57 TG156 CT135 TG174 CT226 S4_530 TG128 TG499
(Rcm7.1)
[Rrs7.0 (Bw-2)]
(Rcm7.0)
(Ph-1)
(I-1, I-3)
Chr8
S11_350 PK12_150 S11_80 C21B NLRR_50 PK12_80 CT55B CT92 CD40 S5_240 TG349 TG302 TG46 TG330 A41 CT265 TG294
(Rrs8.0)
(Rcm8.0)
(I2C)
(Pi)
Chr9
S13_410 S23_550 S11_600 TG291 NBS8_300 TG113b TG551 CT74 S11_200 TG421 XLRR_310
(Ve)
(Rcm9.1)
(Tm-2a, Frl)
(Ph-3)
(Sw-5)
Chr10
CT16 CT234 NLRR_300 S3_440 PK34_150 PK34_750 PK34_300 AN23_550 S13_350 S23_510 AN23_190 CT11 CT203 TG15 CD34 KJB3 TG403 TG241 TG63
[Rrs10 (Bw-3)]
(Rcm10.0)
(Rrs10.1)
(Ph-2)
Chr11
TG497 TG651 CT107A CT107B TG546 TG36B TG36A
(I)
(Sm)
(I-2C, I2)
(Ty-2)
Chr12
TG180 TG68 CT100 CEL55 TG111 TG565 NLRR_360 S11_123 S23_600 KJB4 CT276 TG473
(Mi-3)
(Cmr)
(Lv)
(Rrs12.0)

Table 4. Comparison of the present map (*L* × *Pm*-R) with other tomato maps for chromosome lengths based on orthologous markers

Linkage map[a]	Chromosome length (cM)													
	1	2	3	4	5	6	7	8	9	10	11	12	Average	Total
L × *Pm*-R	182.4	118.9	177.0	129.8	166.5	91.2	111.1	102.3	90.9	112.2	113.8	109.2	125.4	1505.3
L × *Pm*-1	129.7	121.9	133.8	108.0	94.1	82.8	91.3	64.4	104.8	84.9	78.2	92.6	98.9	1186.5
L × *Pm*-R / *L* × *Pm*-1	1.4	1.0	1.3	1.2	1.8	1.1	1.2	1.6	0.9	1.3	1.5	1.2	1.3	
L × *Pm*-2	149.6	93.8	116.6	63.7	108.2	85.2	98.5	57.6	104.2	101.5	93.1	105.2	98.1	1177.2
L × *Pm*-R / *L* × *Pm*-2	1.2	1.3	1.5	2.0	1.5	1.1	1.1	1.8	0.9	1.1	1.2	1.0	1.3	
L × *Pm*-3	102.9	92.6	85.3	72.2	70.6	74.6	69.8	86.6	96.1	80.6	88.3	83.4	83.6	1003.0
L × *Pm*-R / *L* × *Pm*-3	1.8	1.3	2.1	1.8	2.3	1.2	1.6	1.2	0.9	1.4	1.3	1.3	1.5	
L × P	133.5	124.2	126.1	124.8	97.4	101.9	91.6	94.9	111.0	90.1	88.0	93.1	106.4	1276.6
L × *Pm*-R / *L* × P	1.4	1.0	1.4	1.0	1.7	0.9	1.2	1.1	0.8	1.2	1.3	1.2	1.2	
Average length	139.6	110.3	127.8	99.7	107.4	87.1	92.5	81.2	101.4	93.9	92.3	96.7	102.5	1229.7
Average ratio	1.45	1.1	1.6	1.5	1.8	1.1	1.3	1.4	0.9	1.2	1.3	1.2	1.3	

[a] *L* × *Pm*-R = *S. lycopersicum* (NC84173) × *S. pimpinellifolium* (LA722) F_9 RILs map (the present map)
L × *Pm*-1 = *S. lycopersicum* (NC84173) × *S. pimpinellifolium* (LA722) BC_1 map (Chen and Foolad, 1999)
L × *Pm*-2 = *S. lycopersicum* (M82-1-7) × *S. pimpinellifolium* (LA1589) BC_1 map (Grandillo and Tanksley, 1996)
L × *Pm*-3 = *S. lycopersicum* (NCEBR-1) × *S. pimpinellifolium* (LA2093) F_2 map (Sharma et al., 2008)
L × P = *S. lycopersicum* (VF36-*Tm*2[a]) × *S. pennellii* (LA716) F_2 map (Tanksley et al., 1992)

As alluded to before, the expansion in the E × *Pm*-R map was most likely due to several generations of self-breeding and recombination to produce the RIL population.

4. Discussion

The RIL population presented here consists of 145 inbred lines in F_9 generation, which segregate for numerous horticultural traits derived from its two parents, including plant type (determinate *vs*. indeterminate), fruit characteristics (e.g., size, shape, soluble solids content, shelf life), disease resistance (e.g. fusarium wilt and verticillum wilt resistance derived from NC 84173 and early blight resistance derived from LA 0722), and abiotic stress tolerance (e.g. salt, cold and drought tolerance derived from LA 0722).

This RIL population along with its genetic map will facilitate further genetic characterization and breeding exploitation of the desirable traits segregating in this population. The permanent nature of a RIL population allows its reproduction and use by numerous researchers, under different conditions, and for studying all traits segregating in the population. This is particularly useful for investigating complex traits, such as early blight resistance, which expressions are often influenced by several to many genes and numerous environmental factors. The use of RILs in trials replicated in time or space allows repeated measurements of a complex trait, improving the quality of phenotypic data, and thus the correspondence between phenotype and genotype. In addition, a RIL population is the result of several generations of genetic recombination, providing greater chances for linkage break and separation of linked genes and genetic markers. According to Haldane [46] the chance of recombination between tightly linked genes in a RIL population is, on average, twice that in an F_2 or a BC_1 population; this allows a more accurate estimation of map distances in a RIL populations [11]. Furthermore, the decrease in heterozygosity and increase in the frequency of homozygous loci allow for increased differences between lines, making dominant markers more informative than they are in early filial or backcross populations where the frequency of heterozygosity is high. There are numerous other advantages to using RIL populations, as mentioned in the Introduction and discussed elsewhere [11, 75].

Observation of skewed segregation for ~21% of the markers in the RIL population was not unexpected. Similar deviations were previously reported in other interspecific populations of tomato, as discussed below. However, in comparison, more skewed segregation was observed in this RIL population than in a BC_1 population of the same cross (~10%) previously developed [15]. This difference could be due to several reasons. First, the use of dominant RGA markers in the RIL map, as opposed to the use of only co-dominant RFLP markers in the BC_1 map. In the RIL population, a greater percentage of RGA markers (~35%) exhibited skewed segregation compared to RFLP markers (~15%), which could be

due to reduced accuracy in scoring dominant markers. The moderately high level of residual heterozygosity (4.2%) remained in the RIL population could have affected segregation and scoring of the RGA markers. The second reason for the higher percentage of skewed segregation in the RIL population could be the occurrence of unwanted selections during population development. The F_9 RIL population was the result of 8 generations of self-breeding and progeny advancement, during which several lines were lost due to various reasons. Third, as expected, a higher level of skewness is generally observed in interspecific filial than interspecific backcross population of tomato, as discussed below.

Skewed segregation has been observed in most interspecific populations of tomato, with the extent of skewness often being greater in wider crosses (e.g. *S. lycopersicum* × *S. pennellii* and *S. lycopersicum* × *S. habrochaites*) than crosses between closely-related species (e.g. *S. lycopersicum* × *S. pimpinellifolium* or *S. lycopersicum* × *S. cheesmaniae*), and generally higher in filial (average 50% distortion) than in backcross populations (average 34%). For example, skewed segregation was 80% in a *S. lycopersicum* × *S. pennellii* F_2 population [22], 75% in a *S. lycopersicum* × *S. cheesmaniae* RIL population [75], 51% in a *S. lycopersicum* × *S. cheesmaniae* F_2 population [78], and 16% and 30%, respectively, in an F_2 [87] and a RIL population [4] of a *S. lycopersicum* × *S. pimpinellifolium* cross. In comparison, skewed segregation was 68% in a *S. lycopersicum* × *S. chmielewskii* BC_1 population [79], 62% in one [101] and 20% in another *S. lycopersicum* × *S. habrochaites* BC_1 population [9], and 8% in one [42] and 10% in another *S. lycopersicum* × *S. pimpinellifolium* BC_1 population [15]. Overall, the *S. lycopersicum* × *S. pimpinellifolium* populations exhibited less skewed segregation than other interspecific populations of tomato, consistent with the close phylogenetic relationship between *S. lycopersicum* and *S. pimpinellifolium*. However, skewed segregations in interspecific populations of tomato have been attributed to causes such as self-incompatibility, unilateral incongruity, gametophytic selection, zygotic and viability selection in segregating populations, and environment effects [9, 18, 25].

Knowledge of the genome composition of a population is important as it facilitates identification of individuals (or lines) with desirable genetic background for further studies. For example, in a recent study, for developing near-isogenic lines (NILs) and fine mapping of a major fruit lycopene QTL that was previously identified in an accession of *S. pimpinellifolium*, we started the backcross breeding program using a *S. lycopersicum* × *S. pimpinellifolium* F_{10} RI line as the donor parent; this line contained the QTL of interest and only ~30% background genome from the *S. pimpinellifolium* parent. Subsequently, only two generations of backcrossing and marker-assisted selection (MAS) were required to fine-map the QTL within about 1 cM [51]. In the present study, on average the two parents contributed equally to the genomic composition of the RIL population, with 48.9% from the *S. lycopersicum* and 51.1% from the *S. pimpinel-*

lifolium parent. However, on individual RIL basis, the percentage of genome from the *S. lycopersicum* parent varied from 22.5% to 72.5% (Fig. 1). These observations indicate that some RILs had significantly more contribution from one or the other parent than the population average. Such knowledge would be helpful when selecting lines for specific purposes, such as breeding for a particular trait or fine mapping of a gene or a QTL.

The residual heterozygosity in this RIL population (4.2%) was greater than the theoretical 0.4% expected in an F_9 generation of a cross between two inbred lines. Higher than expected levels of heterozygosity in RIL populations have been reported in different plant species, including tomato [75], maize [11], rice [36], barley [62], alfalfa [49], pearl millet [59] and soybean [52]. The higher than expected level of heterozygosity could be due to various reasons, including heterozygous advantage, possible outcrossing during generation advancement, and unintentional selections against plants with low fertility. For example, in the present study, during the process of developing the RILs, some lines produced few or no fruit leading to their elimination. However, the presence of 4.2% heterozygosity in this RIL population did not affect map construction as temporary exclusion of heterozygous loci from the analysis did not affect marker orders or map distances (data not shown).

Linkage analysis indicated that the 62 RGA markers were distributed throughout the genome, mapping to 11 of the 12 tomato chromosomes (Fig. 2). On each of chromosomes 1, 2, 3, 4, 5, 7, 8, 9, 10 and 12, two or more RGA loci, which were amplified by the same or different primer pairs, mapped to the same or nearby positions (Fig. 2). This observation indicates that degenerate primers may initiate amplification of closely-linked RGA loci, which might be members of the same or different gene families. Similar RGA clustering was previously noted in tomato [101] and other plant species [54], and is similar to clustering reported for R genes in different plant species [44, 102]. While such clustering may indicate that the mapped R genes or RGAs might be members of the same gene families, clustering on a genetic linkage map does not necessarily imply physical clustering of genes or RGAs on a chromosome [14].

Tomato chromosomes contain distal and pericentromeric heterochromatin regions where recombination is greatly repressed. Despite clustering of RGAs in some regions on the present map, they could be physically apart, depending on their physical locations on the chromosome. With the availability of genome sequence of tomato [91], sequence investigation of RGAs may lead to the identification of their actual physical locations. RGA markers were assessed for their positional association with known tomato R genes and major quantitative resistance loci (QRLs). The chromosomal positions of R genes and QRLs were inferred from previously-published maps, as displayed and described in Fig. 2. Most positions were inferred based on linkage to reference RFLP markers and thus should be considered best approximations. Several RGA loci were seemingly

mapped to genomic regions that were known to contain tomato resistance loci, including regions on chromosomes 1, 2, 5, 6, 8, 9, 10 and 12 (Fig. 2). This co-localization suggests possible involvement of RGAs with disease resistance, and that RGAs may be good candidates for finding new resistance genes. However, mapping of candidate RGAs in populations that are segregating for the co-localized resistance genes or QRLs, and more importantly their cloning and molecular characterization, are necessary before any functional relationship could be established. Similar co-localizations were previously reported in other plant species and a few functional relationships were established in plant species such as soybean, *Arabidopsis*, rice, barley, wheat, potato, maize and apple [40, 71].

However, in a previous study, cloning and sequence analysis of RGAs that were mapped in a *S. lycopersicum* × *S. habrochaites* population of tomato indicated that only about 16% of RGA fragments were related to plant disease resistance or defense-response genes [72]. Further studies would be needed to determine whether the RGAs mapped in this study have any roles in plant disease resistance.

Conclusions

A new RIL population of tomato consisting of 145 lines, developed from a cross between a *S. lycopersicum* inbred line and a *S. pimpinllifolium* accession, and it genetic map, based on 191 RFLP and RGA markers, is introduced. The RIL population segregates for numerous desirable characteristics, including fruit quality, disease resistance, and abiotic stress tolerance, and together with its genetic map they will facilitate genetic characterization and marker-assisted exploitation of such traits. The graphical genotypes of individual RILs will allow identification of desirable lines for further research. Seed of this RIL population can be obtained from Penn State for further genetic and breeding studies.

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