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Development and characterization of tomato SSR markers from genomic sequences of anchored BAC clones on chromosome 6

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Abstract Simple sequence repeat motifs are abundant in plant genomes and are commonly used molecular markers in plant breeding. In tomato, currently available genetic maps possess a limited number of simple sequence repeat (SSR) markers that are not evenly distributed in the genome. This situation warrants the need for more SSRs in genomic regions lacking adequate markers. The objective of the study was to develop SSR markers pertaining to chromosome 6 from bacterial artificial chromosome (BAC) sequences available at Solanaceae Genomics Network. A total of 54 SSR primer pairs from 17 BAC clones on chromosome 6 were designed and validated. Polymorphism of these loci was evaluated in a panel of 16 genotypes comprising of *Solanum lycopersicum* and its wild relatives. Genetic diversity analysis based on these markers could distinguish genotypes at species level. Twenty-one SSR markers derived from 13 BAC clones were polymorphic between two closely related tomato accessions, West Virginia 700 and Hawaii 7996 and were mapped using a recombinant inbred line population derived from a cross between these two accessions. The markers were distributed throughout the chromosome spanning a total length of 117.6 cM following the order of the original BAC clones. A major QTL associated with resistance to bacterial wilt was mapped on chromosome 6 at similar location of the reported *Bwr-6* locus. These chromosome 6-specific SSR markers developed in this study are useful tools for cultivar identification, genetic diversity analysis and genetic mapping in tomato.

Keywords Bacterial artificial chromosome · Genetic diversity · Linkage map · Simple sequence repeats · *Solanum lycopersicum*

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Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown and economically important vegetables in the world. Breeding to enhance tomato with higher yield, tolerance to biotic and abiotic stresses, and better nutritional quality is a continuous process that

aims to meet the demands of producers and consumers. Breeding efficiency in tomato has been improved by using molecular markers to tag and transfer useful alleles from germplasm to elite cultivars (reviewed by Foolad 2007). However, there is a lack of sufficient polymorphic markers between closely related tomato species and within cultivars of the same species because the majority of molecular markers were developed based on polymorphisms between domesticated tomato and its wild relatives (Tanksley et al. 1992; Fulton et al. 2002; Frary et al. 2005). This poses difficulty in developing and saturating several species-specific linkage maps. For instance, the mapping populations derived from a cross between West Virginia 700 and Hawaii 7996 have been used to map quantitative trait loci (QTLs) associated with bacterial wilt and late blight, but the low number of polymorphic markers between these two parents has been an obstacle towards fine-mapping and marker-assisted selection (MAS) of these QTLs (Thoquet et al. 1996; Moreau et al. 1998; Wang et al. 2000).

Simple sequence repeat (SSR) markers are often the preferred molecular markers for the purpose of marker-assisted plant breeding when they are available, because the SSR markers possess properties suitable for high-throughput genotyping, such as high reproducibility, co-dominance nature, multi-allelic variation, simplistic assay, low distributing cost and easy automation (Edwards and McCouch 2007). The conventional method for SSR marker development involves construction of genomic libraries and screening them for repeat motifs (Zane et al. 2002). Areshchenkova and Ganal (1999, 2002) used this approach to develop 32 SSR markers for tomato with longer repeats. However, this approach is cumbersome and intensive in terms of cost, time and labor. Alternatively, DNA sequences deposited in public databases provide an easy and economical source for development of SSR markers (Morgante and Olivieri 1993). The tomato genome sequencing project has generated sequences of many bacterial artificial chromosome (BAC) clones which augments the existing genomic resources (Mueller et al. 2009). The search for repeat motifs in genomic sequences and expressed sequence tags (ESTs) of Solanaceae family available in the European Molecular Biology Laboratory (EMBL), GenBank and Solanaceae Genomics Network (SGN) databases enabled to rapidly produce a few hundred SSR markers in tomato (Smulders et al. 1997; He et al. 2003; Frary et al. 2005). Currently, a total of 404 tomato SSR markers have been developed and mapped. Information for 256 and 148 SSR markers is available at SGN and Vegmarks, respectively (http://www.sgn.cornell.edu; http://vegmarks.nivot.affrc.go.jp/). Nevertheless, these SSR markers are not distributed evenly throughout the 12 chromosomes, and tend to cluster around the centromeres (Areshchenkova and Ganal 2002; Ohyama et al. 2009). This phenomenon may be attributed to the fact that a large proportion of SSR markers developed from BAC end sequences were located predominantly in the heterochromatin regions. Most of these SSR markers have not been evaluated for their ability to detect genetic variation within cultivated and closely related tomato species. The number of polymorphic markers is expected to be less for closely related cultivated tomatoes.

The purpose of the present study was (1) to develop substantial SSR markers evenly distributed on chromosome 6; (2) to characterize their utility for genetic diversity assessment in tomato; and (3) to map resistance QTLs associated with bacterial wilt using a recombinant inbred population derived from a cross between two closely related tomatoes West Virginia 700 and Hawaii 7996. Chromosome 6 was chosen because only 15 unevenly distributed SSR markers are currently available on this chromosome. Moreover, a major QTL associated with bacterial wilt caused by *Ralstonia solanacearum* in Hawaii 7996 has been mapped on chromosome 6 (Thoquet et al. 1996; Wang et al. 2000; Carmeille et al. 2006). And several genes and QTLs conferring resistance to various biotic stresses such as bacterial canker (Sandbrink et al. 1995; van Heusden et al. 1999), powdery mildew (Bai et al. 2003), leaf mold (Jones et al. 1993), tomato yellow leaf curl virus (Zamir et al. 1994; Hanson et al. 2000) and root knot nematode (Ammiraju et al. 2003) were mapped to chromosome 6. Additional useful SSR markers on chromosome 6 would facilitate the transfer of these useful alleles in breeding processes.

Materials and methods

Plant materials

A panel of 16 accessions comprising of cultivated tomato and its wild relatives were used for genetic

diversity analysis (Table 1). These accessions represented diverse origins and possessed useful horticultural traits such as resistance to biotic and abiotic stresses. Seeds of these accessions were obtained from Genetic Resources and Seed Unit, AVRDC—The World Vegetable Center (AVRDC), Taiwan. A mapping population comprising of 188 recombinant inbred lines (RILs) (F_9) derived from a cross between West Virginia 700 (*S. pimpinellifolium*) and Hawaii 7996 (*S. lycopersicum*) (Carmeille et al. 2006) was used for genotyping and linkage map construction with SSR markers. Genomic DNA was isolated from fresh young leaves of RILs using GenElute™ plant genomic DNA miniprep kit (Sigma, USA) following the user's instruction manual.

Database search, primer designing, and nomenclature

Eighteen BAC clones on chromosome 6, starting from zero to 101 cM and placed at a distance of approximately 5–10 cM from each other according to SGN were selected for the study. The selected BACs were searched for perfect di-, tri- and tetra nucleotide SSR motifs using simple sequence repeat identification tool downloadable from http://www.gramene.org/db/markers/ssrtool. A minimum number of eight repeat motifs was used as the search criterion. Sixty-one primer pairs were designed from sequences flanking the repeat motifs using PRIMER3.0 (http://www.-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

Table 1 Details of tomato accessions used for genetic diversity analysis

Genotypes/ accessions	Species	Place of development/ collection	Characteristics	Reference
CLN2498E	*S. lycopersicum*	AVRDC, Taiwan	Resistance to tomato yellow leaf curl virus (*Ty2*), bacterial wilt and heat tolerance	AVRDC (2007)
Arka Meghali	*S. lycopersicum*	Indian Institute of Horticultural Research, India	Drought tolerant, suitable for rainfed cultivation	Srinivasa Rao et al. (2000)
CA4	*S. lycopersicum*	Israel	Resistance to tomato yellow leaf curl virus (*Ty3*)	Pers. comm. Dr. P. Hanson, AVRDC
CLN1621L	*S. lycopersicum*	AVRDC, Taiwan	Heat tolerance	Pers. comm. Dr. P. Hanson, AVRDC
FLA456	*S. lycopersicum*	University of Florida, USA	Resistance to tomato yellow leaf curl virus	Pers. comm. Dr. P. Hanson, AVRDC
CL5915-93D4	*S. lycopersicum*	AVRDC, Taiwan	Heat tolerance	Hanson et al. (2002)
Hawaii 7996	*S. lycopersicum*	Hawaii	Resistance to bacterial wilt	Wang et al. (2000)
M-82	*S. lycopersicum*	Israel	Processing tomato line, used in introgression mapping	www.sgn.cornell.edu
T4065	*S. lycopersicum*	USDA, USA	Highly pigmented (*hp*)	Wann (1997)
West Virginia 700	*S. pimpinellifolium*	West Virginia, USA	Late blight resistance (*Ph2*)	Moreau et al. (1998)
LA1579	*S. pimpinellifolium*	Peru	Salt and/or alkali tolerance	http://tgrc.ucdavis.edu/
L3708	*S. pimpinellifolium*	Peru	Late blight resistance	http://tgrc.ucdavis.edu/
LA407	*S. habrochaites*	Ecuador	Arthropod resistance	http://tgrc.ucdavis.edu/
LA1033	*S. habrochaites*	Peru	Late blight resistance	http://tgrc.ucdavis.edu/
LA716	*S. pennellii*	Peru	Salt/Alkali tolerance, drought tolerance, arthropod resistance	http://tgrc.ucdavis.edu/
LA1940	*S. pennellii*	Peru	Arthropod resistance	http://tgrc.ucdavis.edu/

The parameters for primer designing included a product size of 100–300 bp, primer length of 20–25 bp, melting temperature of 54–64°C and a GC content of 40–60%. The primer nomenclature included a serial number with the prefix SLM6 (*S. lycopersicum* microsatellites of chromosome 6). The details of BAC clones, SSR motifs and the designed primers are listed in Table 2.

PCR amplification

PCR amplification of SSRs was performed in a PTC 200 DNA engine thermal cycler (MJ Research, USA). Each 15 µl reaction mixture consisted of 20 ng DNA, 0.3 µM of each forward and reverse primer (Invitrogen, USA), 200 µM of deoxyribonucleotides, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.5 unit of hot start *Taq* DNA polymerase. The temperature profile used for PCR amplification included initial denaturation at 94°C for 10 min, 30 cycles of 94°C for 30 s, 50–55°C for 45 s, 72°C for 45 s, followed by a final extension at 72°C for 7 min. Annealing temperature was adjusted based on the specific requirement of each primer combination. PCR products (3 µl) were analyzed on 6% non-denaturing polyacrylamide gel in 0.5× TBE buffer. After electrophoresis, the gels were stained with 5 µg/ml ethidium bromide and the bands were visualized under UV light using the Alpha Imager 3300 system.

Estimation of polymorphism information content and genetic diversity analysis

Individual SSR alleles observed for the 16 genotypes were scored as present (1) or absent (0) to generate a binary data matrix. Using this data matrix, the polymorphism information content (PIC) of SSR markers and genetic similarity coefficients for genotypes were computed. The PIC value (*Hn*) for each SSR marker was calculated based on the formula, $Hn = 1 - \Sigma pi^2$, where *pi* is the allele frequency for the *i*th allele (Nei 1973). A data matrix was assembled and analyzed using Populations v.1.2.30 (Langella, 1999; http://bioinformatics.org/~tryphon/populations/) and a pairwise distance matrix was generated based on total character difference. The genetic relatedness was analyzed using Unweighted Pair Group Method with Arithmetic Averages (UPGMA). Bootstrap analysis with 1,000 replicates was performed to obtain the confidence of branches of the UPGMA tree. The output dendrogram file was viewed and plotted by TreeView (Page 1996).

Linkage map construction

All the polymorphic SSR markers between West Virginia 700 and Hawaii 7996 were used to genotype the 188 RILs, and an approximate 1:1 ratio with 0.39% expected heterozygosity was tested using chi-square (χ^2) goodness-of-fit analysis. Two publicly available SSR markers SSR47 and SSR350 which showed polymorphism in the mapping population were genotyped and used as anchors on chromosome 6. Linkage analysis was performed using Mapmaker/Exp ver. 3.0b with Kosambi mapping function (Kosambi 1944; Lander et al. 1987). The GROUP command was used to define the tightly linked markers at LOD $\geq$ 6.0 with genetic distance less than 2 cM. In each group of tightly linked markers, the marker with the smallest chi-square statistics for segregation distortion was selected to construct the framework of the linkage map. The ORDER command was used to define linear order of markers. The RIPPLE command was used to confirm marker order at LOD $\geq$ 3.0. The NEAR command was used to mark the most likely mapping position on the framework of the linkage map for the remaining unmapped SSR markers. The heterozygous genotypes in the mapping population were treated as missing value for the linkage analysis. The linkage map was drawn using the Adobe Illustrator CS2 (version 12.0).

Mapping resistance associated with bacterial wilt

The 188 RILs and the two parents were evaluated for their reactions against the *R. solanacearum* strain Pss4 (Phylotype I, race 1, and biovar 3) at seedling stage. The inoculation was conducted following methods described by Wang et al. (2000). Percentages of wilted plants were recorded 28 days after inoculation (dai). For QTL analysis, the percentage data were transformed using arcsin squareroot. QTL detection was conducted using composite interval mapping method with the Windows QTL Cartographer Version 2.5 (Wang et al. 2007). The empirical LOD threshold obtained was 2.7 by computing with permutation test (1,000 permutations; $P = 0.01$).

Table 2 Details of SSR markers derived from BAC sequences specific to chromosome 6 of *Solanum lycopersicum*

BAC clone	Plausible marker match and its map distance in SGN (cM)[a]	SSR marker	Repeat motif	Forward primer	Reverse primer	Expected size (bp)	Tm (°C)[b]	No. of allele	PIC[c]
C06HBa0003K02	TG178 (10.0)	SLM6-3	$(GA)_{12}$	GAAGGGGTTTGGAGCTTTCT	GACAGAACCCGAATTTGGAC	130	55	8 (3)[d]	0.82 (0.59)[d]
		SLM6-4	$(TA)_{15}$	GGGATCATTTGTTGCTGGTT	ACACCAAAGGCTCACAACCT	172	55	9 (4)	0.79 (0.52)
		SLM6-5	$(AT)_{30}$	ATGCACGCAAAGGTTATTCC	AGTCGAAGTTGGCTTGACCA	160	55	10 (5)	0.81 (0.64)
C06HBa0302A23	T0774 (18.0)	SLM6-6	$(TA)_{8}$	CCCGTGTCGAATTCTCCTAA	TCTGCTTCTGCTTCCTCACC	241	55	4 (1)	0.43 (0.00)
		SLM6-7	$(AT)_{22}$	CAATTGAAGATTGGGGCTTT	AGCAGCTCACCTCACGTTTT	236	55	7 (4)	0.74 (0.56)
		SLM6-8	$(TA)_{16}$	AGTCCACGCAGCATCATTTT	GTCGTGGTGGATGGTAGTCA	235	55	1 (1)	0.00 (0.00)
C06HBa0023B17	Fer (25.0)	SLM6-9	$(AT)_{14}$	GCCTTGAGGGGAGTCTTAGG	ACAAGTGCAATGACCAAGCA	287	55	1 (1)	0.00 (0.00)
		SLM6-10	$(TA)_{19}$	ACAGCGTGAGCGAGACAATA	GCATGTAAGGGGAACCTTGA	299	50	6 (2)	0.69 (0.20)
		SLM6-11	$(AT)_{14}$	CTGATGGGGAAGGACTCTTG	TCGTCCTTGACACAGGGTAA	219	55	6 (2)	0.64 (0.20)
		SLM6-12	$(TC)_{12}$	GAGATCACGTTTTTCCTTCCA	GATGGACTATGAAGGAGACTTCG	214	55	4 (3)	0.54 (0.37)
		SLM6-13	$(AT)_{14}$	TCGCCTCTCACGCTTATACA	CGTAAAAGAAACTGGGCATAGG	175	55	5 (3)	0.72 (0.64)
C06HBa0307J13	T0834 (32.0)	SLM6-14	$(AT)_{31}$	TCCGTAATAAGTTGAGGAACCA	TCACAAGAATATTTGCCGTCAT	262	55	9 (4)	0.83 (0.62)
		SLM6-15	$(TA)_{24}$	GGATTTCAGCTGCCTACTGAG	TTCGGAGAACATAATAGGGGTTT	240	55	6 (4)	0.79 (0.67)
		SLM6-16	$(AT)_{25}$	TGGGATATAGTTTGACTGACGA	TCCTCCAATCGCCAAATCTA	274	55	5 (3)	0.69 (0.41)
C06SLe0120K02	NA	SLM6-44	$(TC)_{10}$	ATATACCTCATCGCCGTGGA	GGATCGATTTAACGCACACA	152	55	1 (1)	0.00 (0.00)
		SLM6-47	$(AT)_{25}$	TCCTCCAATCGCCAAATCTA	TGGGATATAGTTTGACTGACGA	274	55	5 (2)	0.65 (0.22)
		SLM6-48	$(AT)_{24}$	TTCGGAGAACATAATAGGGGTTT	GGATTTCAGCTGCCTACTGAG	240	55	5 (3)	0.67 (0.37)
C06SLe0123G17	NA	SLM6-49	$(TA)_{24}$	ATTGATGGATTGGCGTTCTC	AAAATCAGGGGTGAATTTAACG	233	55	4 (3)	0.66 (0.49)
		SLM6-50	$(AG)_{8}$	GCGCATGGTATATGGAGGTT	AGGATGACAGGTTGGTGGAC	247	55	3 (1)	0.32 (0.00)
		SLM6-51	$(AT)_{13}$	TTTGCGCACATTCCAAGTAG	CAGGAGTGACGGATATTGACTG	208	55	4 (3)	0.63 (0.57)
		SLM6-52	$(AT)_{8}(TG)_{10}$	AGGAGTATGCAAGCTGATCTGA	TCAAATGGTGCTCCTTATATTTCA	137	55	4 (2)	0.62 (0.44)
C06HBa0215M16	TM43 (40.0)	SLM6-17	$(TA)_{12}$	TCCTTCAAATCTCCCATCAA	ACGAGCAATTGCAAGGAAAA	186	55	6 (4)	0.75 (0.62)
		SLM6-18	$(AC)_{10}(AT)_{8}$	TCAAATGGTGCTCCTTATATTTCA	AGGAGTATGCAAGCTGATCTGA	137	55	5 (4)	0.72 (0.69)
C06HBa0010E05	T1666 (43.7)	SLM6-19	$(AT)_{10}$	TGGTCCGTACCTCTTTTTCC	TCCGTTGAGGTGAAGAAACA	243	55	2 (1)	0.16 (0.00)
		SLM6-20	$(AT)_{8}$	TGAATCTTTTTGCGTGCTTG	AAGGAGGATGAGCCTGGAAT	269	55	2 (1)	0.24 (0.00)
		SLM6-21	$(TC)_{8}$	AGATCTCCCATTGGTTTGGA	TGTGACACTTGCATCCATCA	209	55	2 (1)	0.26 (0.00)
		SLM6-22	$(AT)_{14}$	TGGTCAAATAGTGATTCTACTGCAT	AGCTCTTAACACTGAGAGGTGAA	284	55	3 (1)	0.43 (0.00)
C06HBa0109C03	TG365 (50.0)	SLM6-53	$(TA)_{25}$	CCCGCAATTTTAATAGTATAACCAA	CGGAATCCATGAATGAGAGC	272	55	3 (1)	0.31 (0.00)
		SLM6-54	$(AT)_{9}$	GCAGAATCCATGAATTAGAGCA	CCAGCTTGAAGGAGGGTGTA	289	55	2 (1)	0.16 (0.00)
C06HBa0066I09	CT204 (54.0)	SLM6-23	$(TA)_{10}$	TGTTGGGTAGTCATTTCAACG	GGATGAGATGGCAACTGGAT	233	55	4 (1)	0.57 (0.00)
		SLM6-24	$(TA)_{9}$	TGCATTGTGTTTTGGCTGTT	ATTCCACTTGCCAAAGGATG	178	55	5 (3)	0.62 (0.37)

Table 2 continued

BAC clone	Plausible marker match and its map distance in SGN (cM)[a]	SSR marker	Repeat motif	Forward primer	Reverse primer	Expected size (bp)	Tm (°C)[b]	No. of allele	PIC[c]
C06HBa0197N20	T1556 (59.0)	SLM6-55	$(AC)_9$	TCCCTTTTGTGCTTCCACTC	TTGCAATTCCAGATTGACCA	203	55	4 (2)	0.48 (0.20)
		SLM6-56	$(AT)_{15}$	TTCAACTTTTCACTTTGGAGCTT	AAATTGGGTTGCCAGAGCTA	144	55	7 (2)	0.70 (0.20)
		SLM6-57	$(TC)_9$	ATGTGGCACTGGTTCACTTG	GGGTGTTGCACTTGTTTGTG	104	55	6 (3)	0.68 (0.37)
		SLM6-58	$(GA)_8$	TGGGTCAGTGGTGAATCTGA	GCGTGAGGCATTTGACTTTT	124	55	4 (2)	0.61 (0.20)
		SLM6-59	$(TG)_9$	GCATGCTATGCACTCCTCAA	CCAGACAATGAACCCAATCC	174	55	4 (2)	0.49 (0.20)
C06HBa0036J15	TG292 (64.0)	SLM6-25	$(ATT)_{19}$	ATGGGCAGGTACTCCATCAA	CATGCACAGCAACATTACCA	223	55	6 (3)	0.78 (0.57)
		SLM6-26	$(AT)_{13}$	AGTGTTCGAGCCAGCTTACC	CGACGGAGTCAAATTGAACA	192	55	4 (2)	0.67 (0.47)
		SLM6-27	$(AT)_9$	CCAAATGATGTGGGATGGTA	CCACTGGACCTCGATTCATT	248	55	2 (2)	0.29 (0.22)
		SLM6-28	$(GGC)_9$	TTAAAGAGATCAATGGAATACGG	GCTGTATGTCGATGGAAACG	128	55	5 (2)	0.62 (0.20)
C06HBa0055E14	T0405 (73.0)	SLM6-29	$(TA)_{16}$	CTCCCTTCATCCGTTGATTT	AGTGAACGGAGAGGAACACAA	228	55	3 (1)	0.42 (0.00)
		SLM6-30	$(TA)_{20}$	TATGGAGCGCAATTATGCAA	CTTCATGGAGGTTAGCCAAG	267	55	5 (3)	0.61 (0.37)
		SLM6-31	$(TA)_8$	GCAACCGATTTAGAGATACGC	ATCCTCCCACATGGCAATAA	298	55	3 (2)	0.41 (0.20)
		SLM6-32	$(AT)_{30}$	CAAGCAATTCGTACCAAGCA	CGACACAATTGAAGGAGGAA	201	55	4 (1)	0.44 (0.00)
C06SLe0129O21	NA	SLM6-60	$(AG)_8$	TGGCAGAGAGAATGTGAAAAGA	TGAACCTAGGTGGAAGCAATG	238	55	1 (1)	0.00 (0.00)
		SLM6-61	$(TA)_{22}(AG)_{17}$	TGAATTTCAAACAGCCTCCTT	CCATCAGTTCAAATCCCAATG	248	55	3 (1)	0.53 (0.00)
C06HBa0169D11	cLEX-2-F13 (85.0)	SLM6-33	$(AT)_9$	CATGTTGGCCAAACAATCTG	GCAAGGGATGCTGTCTTCTT	229	55	4 (2)	0.60 (0.20)
C06SLm0106A20	C2_At1g16870 (92.5)	SLM6-35	$(TA)_{14}$	GTGCAACGCACGTTTTCG	CCGCAAGCTCAACTAAACCT	190	50	2 (1)	0.19 (0.00)
		SLM6-36	$(AG)_{11}$	TGCAAGAAGCACAAGGAGAA	CCCAGCTGATTCTCTTCCAA	155	55	5 (3)	0.64 (0.37)
C06HBa0060A01	C2_At1g20050 (101.0)	SLM6-37	$(TA)_{29}$	TCTTGAGAGGCGGAGTCAGT	ATTTTTGGTGGCTGATCGAC	226	50	6 (2)	0.74 (0.25)
		SLM6-38	$(TA)_{25}$	GCCAAAGGTGTGACCAAAAT	ATGCAAGTCGCAAATCACAA	240	55	7 (3)	0.67 (0.37)
		SLM6-39	$(AT)_{11}$	ATTCTGACACTGGGGTGCTG	AGCCAGCCGTTCTCTAAACA	187	55	5 (1)	0.62 (0.00)
		SLM6-40	$(AT)_{17}$	GGACAGAAACTTGGTCACTGC	TGCAACTTTCTCCCTCAACC	216	50	2 (1)	0.19 (0.00)
		SLM6-41	$(AG)_9$	TCCGGGTAGATCTCAAACCA	AATGTGAGCAGTGCATACCG	154	55	2 (1)	0.27 (0.00)

[a] Based on EXPEN2000 map of tomato in SGN (http://www.sgn.cornell.edu); NA—map position not available; BAC clones anchored on tomato Accessioned Golden Path (AGP) map

[b] Annealing temperature for each SSR primer pair

[c] PIC means polymorphism information content (PIC; *Hn*)

[d] The values in parentheses were calculated using data from the 9 *S. lycopersicum* accessions

Results and discussions

Strategy to develop new SSR markers

Previously developed SSR markers mapped on tomato chromosome 6 were unevenly distributed (http://sgn.cornell.edu/) and showed low polymorphism between closely related tomato species (Frary et al. 2005). To circumvent the issue of uneven distribution, a set of fully sequenced BAC clones uniformly distributed over chromosome 6 were selected for developing new SSR markers. According to SGN, the International Tomato Genome Sequencing Project has released genomic sequences of 157 BAC clones which was equivalent to 56 percentage of euchromatic sequences on chromosome 6 (http://sgn.cornell.edu/). Eighteen BAC clones 5–10 cM apart on the genetic map of chromosome 6 were selected for marker development (Table 2). Among them, 15 BAC clones have been anchored based on RFLP and COS markers. In addition, from each BAC clone, all repeat sequence features with flanking regions amenable for primer designing, were used to develop new SSR markers, in order to ensure that at least one SSR marker from each chosen BAC clone detects polymorphism across closely related genotypes for the use of genetic mapping.

Occurrence of microsatellites in BAC clones

A total of 124 perfect di- and tri-nucleotide SSR motifs were identified from 18 BAC clones belonging to chromosome 6. The number of repeat motifs per BAC clone ranged from 1 to 16. Dinucleotide repeats were predominant followed by trinucleotide repeats. No perfect tetranucleotide repeats were identified. Among the dinucleotide repeats, AT repeat motifs were abundant, depicting a common feature of plant genome (Morgante and Olivieri 1993). Length criterion, represented by number of repeat motifs or nucleotides, is an important factor in the identification of SSRs. Earlier studies showed that adopting a longer length criterion greater than 10 repeat motifs or 20 nucleotides could result in higher level of polymorphism in human genome (Weber 1990). However, recent studies show that the level of polymorphism cannot be reliably predicted from length, emphasizing the utility of short SSRs (Sanwen et al. 2000). In the present study, a length criterion of a minimum of eight repeat motifs was applied to identify SSRs. This represents a lower level of stringency, especially for dinucleotide motifs; a lesser level of stringency has been adopted in other studies as well to minimize the loss of potential markers being identified. Smulders et al. (1997) used six repeat units to identify SSR markers in tomato. Kumpatla and Mukhopadhyay (2005) used a minimum of five repeat units to identify SSR markers in 55 dicotylednous species including tomato, and Portis et al. (2007) included mononucleotide motifs and seven dinucleotide repeats for SSR identification in pepper.

Out of the 124 SSRs identified using the length criterion of eight repeat motifs, 61 SSRs were selected for marker development. The rest were excluded because flanking sequences around these repeat motifs were not amenable for primer designing. The number of SSR primer pairs designed for each BAC clone ranged from 1 to 7. Out of 61 primer pairs screened, seven failed to produce any amplification product, which could be due to unsuitable primer sequences and/or improper PCR conditions resulting in undetectable amounts of amplified products. The remaining 54 primer pairs derived from 17 BAC clone sequences, produced clear banding patterns of DNA fragments with expected sizes. The only exception was SLM6-14, for which the genotype Hawaii 7996 produced a larger fragment size than expected, which could be due to insertions (Fig. 1). These 54 primer pairs were used for diversity estimation and genetic mapping.

Polymorphism of SSR markers

For each of the 54 SSR markers, number of alleles across 16 accessions ranged from 1 (SLM6-8, SLM6-9, SLM6-44, SLM6-60) to 10 (SLM6-5) with an average of 4.35 alleles per loci (Table 2). Examples for the allelic polymorphism are given in Fig. 1. When the estimation of allelic polymorphism was restricted within nine cultivated accessions, the average number of alleles for 54 SSR markers was 2.15 (Table 2). This number was close to the value reported previously (Frary et al. 2005).

Null allele was assigned whenever an amplification product could not be detected for a particular genotype-marker combination. Thirty SSR markers showed null alleles. The frequency of genotypes showing null allele ranged from a minimum of 1 (SLM6-4, SLM6-6, SLM6-23, SLM6-31 and SLM6-38) to a maximum of 7

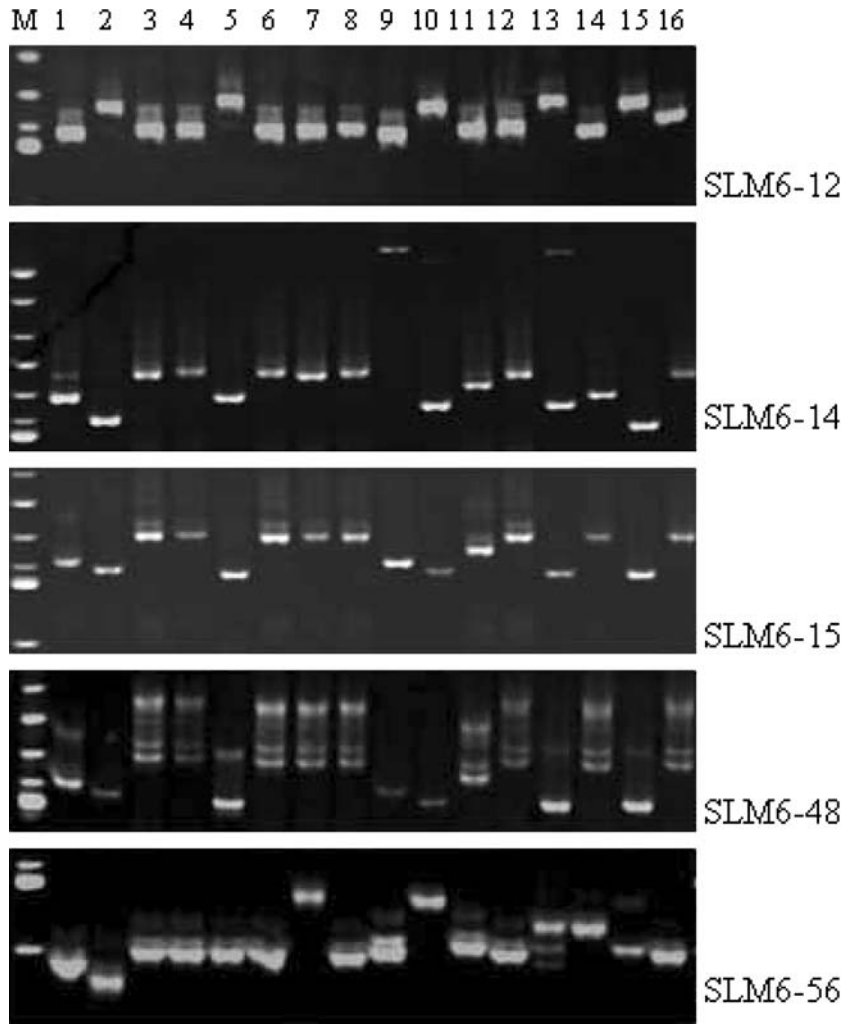

Fig. 1 Allelic distribution of five microsatellite loci across 16 tomato genotypes. M, 25-bp ladder markers; Lane 1, LA1579 (*S. pimpinellifolium*); 2, LA1940 (*S. pennellii*); 3, CLN2498E (*S. lycopersicum*); 4, Arka Meghali (*S. lycopersicum*); 5, CA4 (*S. lycopersicum*); 6, CLN1621L (*S. lycopersicum*); 7, FLA456 (*S. lycopersicum*); 8, CL5915-93-D4 (*S. lycopersicum*); 9, Hawaii 7996 (*S. lycopersicum*); 10, LA716 (*S. pennellii*); 11, West Virginia700 (*S. pimpinellifolium*); 12, M82 (*S. lycopersicum*); 13, LA1033 (*S. habrochaites*); 14, LA3708 (*S. pimpinellifolium*); 15, LA407 (*S. habrochaites*); 16, T4065 (*S. lycopersicum*)

(SLM6-35 and SLM6-40). Null alleles were mostly observed in *S. pennelli* and *S. habrochaites* accessions. Occurrence of null alleles could represent sequence diversity at the primer annealing sites. A higher frequency of null alleles in *S. pennelli* has been reported by Areshchenkova and Ganal (1999, 2002). Frequencies of null alleles were excluded in the calculation of PIC values for each SSR locus.

The PIC values reflected allele diversity and frequency among different accessions. A large variation in PIC values was observed for all the SSR loci tested. The average PIC value was 0.5 and it ranged from zero (SLM6-8, SLM6-9, SLM6-44, SLM6-60) to 0.83 (SLM6-14). Within the nine cultivated accessions, the average PIC value was 0.25 and it ranged from zero to 0.69. No clear relationship could be established between PIC values and the number of repeat units. For example, some shorter SSRs such as SLM6-57 with $(TC)_9$ and SLM6-58 with $(GA)_8$ had PIC values of 0.68 and 0.61 respectively, while some longer SSRs such as SLM6-32 with $(AT)_{30}$ and SLM6-53 with $(TA)_{25}$ had lower PIC values of 0.44 and 0.31 respectively. In a similar study by Smulders et al. (1997), involving seven *S. lycopersicum* cultivars and three wild species *S. pennellii*, *S. peruvianum* and *S. habrochaites*, no clear association of total repeat length with the degree of polymorphism could be deduced between species. This absence of correspondence between PIC and repeat length has been reported in arabidopsis (Bell and Ecker 1994), potato (Milbourne et al. 1998), pepper (Nagy et al. 1998; Sanwen et al. 2000) and common bean (Yu et al. 2000). In contrast,

positive association between the number of repeat units and polymorphism has been observed among the genotypes of *S. lycopersicum* (Smulders et al. 1997; He et al. 2003, Frary et al. 2005). This may be due to a naturally higher frequency of polymorphism exhibited by longer microsatellites within a genetically homogenous group (Smulders et al. 1997).

Genetic diversity analysis

Similarity matrices constructed based on shared allele analysis revealed that the average genetic similarity between genotypes was 0.08 using chromosome 6 specific SSR markers. The dendrogram produced four distinct clusters, one cluster each for *S. lycopersicum*, *S. pimpinellifolium*, *S. habrochaites* and *S. pennellii* accessions (Fig. 2). However, West Virginia 700, despite being an accession of *S. pimpinellifolium* showed relatively lower polymorphism with *S. lycopersicum* compared to other *S. pimpinellifolium* accessions and was separated from the *S. lycopersicum* as well as *S. pimpinellifolium* genotypes in this study. This agrees with previous studies indicating the low polymorphism between West Virginia 700 and Hawaii 7996 (Thoquet et al. 1996; Wang et al. 2000). Our results suggest that SSR markers derived from a single chromosome were sufficient to discriminate the 16 genotypes at the species level. This is due to the rich genetic variability present in wild relatives that render SSR markers to be highly polymorphic at species level. However, within *S. lycopersicum* gene pool, the total genetic diversity is low and only 10% of the tested marker were polymorphic. Among the *S. lycopersicum* genotypes, the lines developed at AVRDC, e.g. CLN1621L, CLN2498E and CLN5915-93D4, shared high similarity and CLN1621L and CL5915-93D4

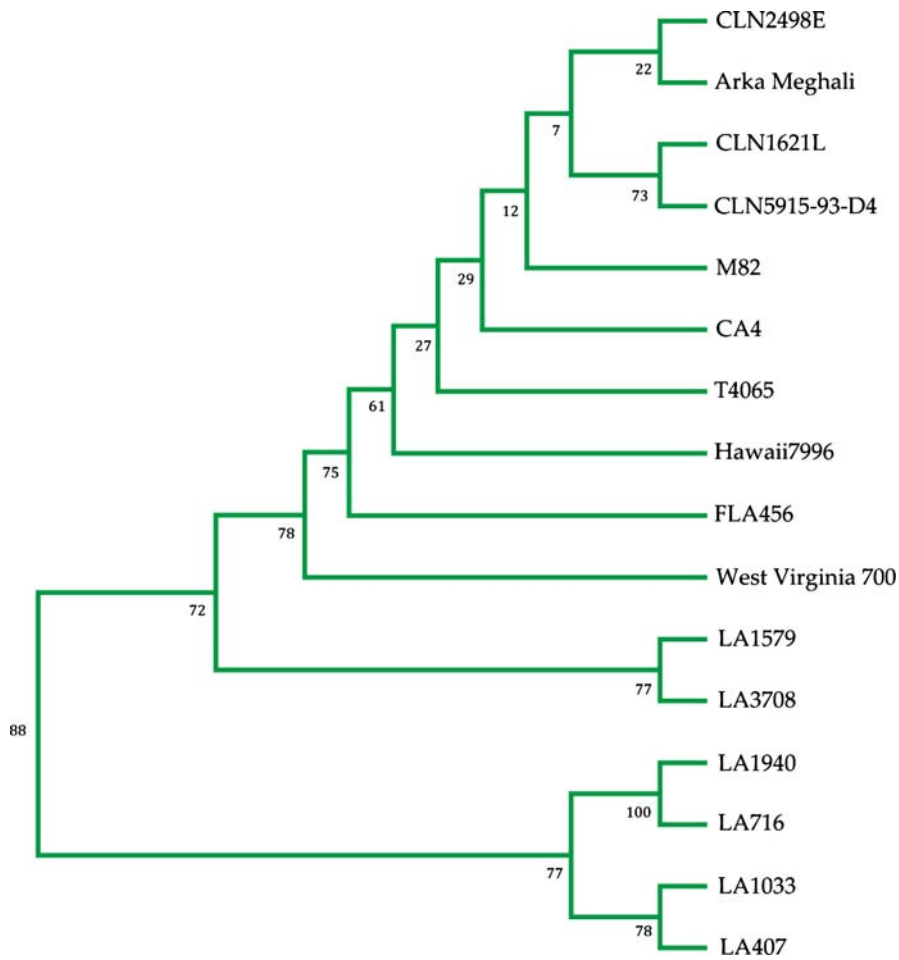

Fig. 2 The topology of the UPGMA dendrogram for individual tomato accessions based on 50 SSR markers. Numbers at nodes indicate bootstrap values (percentage of 1,000 bootstrap re-sampling)

were closely related. This is anticipated as CL5915-93D4 is one of the parents of CLN1621L and also present in the genetic background of CLN2498E. And the introgression of *Ty-2* (a gene derived from *S. habrochaites*) in CLN2498E could make the line genetically more distanced from the other two AVRDC lines. Because the pedigree details were not known for other cultivars, it was not possible to infer their genetic relationship. It was found that as few as 1–2 SSR markers developed in this study were enough to differentiate T4065, CA4, FLA456 and Hawaii 7996 from other *S. lycopersicum* genotypes. Bredemeijer et al. (1998) reported that as few as four SSR markers could differentiate 16 tomato cultivars. In another study by He et al. (2003), use of five SSR loci could effectively differentiate 19 tomato cultivars. Considering the low levels of polymorphism within cultivated tomato, these SSRs are useful in differentiating cultivars and in development of cultivar specific markers.

Linkage map of chromosome 6

Survey of polymorphism between West Virginia 700 and Hawaii 7996 resulted in the identification of 21 polymorphic markers from 54 newly developed SSR markers on chromosome 6. These 21 markers belonged to 13 BAC clones with one or more polymorphic markers representing each BAC clone. Including SSR47 and SSR350, only four out of 23 polymorphic SSR loci on chromosome 6, i.e. SSR47, SLM6-07, SLM6-14, SLM6-48, showed no significant segregation distortion in the F_9 RILs derived from the cross between West Virginia 700 and Hawaii 7996. All the distorted markers showed skewness towards the Hawaii 7996 allele. Such a high level of skewness could be due to the accumulation of distorted alleles in the population with progressive cycles of selfing during the development of RIL. Paran et al. (1995) reported that 73% markers in a RIL population derived from *S. lycopersicum* × *S. cheesmanii* cross showed significant segregation distortion in favor of *S. lycopersicum* alleles. Furthermore, high level of skewed segregation has been observed for markers on chromosome 6 even in other mapping populations involving various interspecific crosses of tomato such as *S. lycopersicum* × *S. pimpinellifolium* (Grandillo and Tanksley 1996; Chen and Foolad 1999; Sharma et al. 2008) and *S. lycopersicum* × *S. hirsutum* (Bernacchi and Tanksley 1997; Zhang et al. 2002). This was mainly attributed to the presence of the self-pruning (*sp*) locus on chromosome 6, near the RFLP marker TG279 (Fulton et al. 1997). According to tomato-EXPEN2000 map (http://www.sgn.cornell.edu), TG279 is close to the BAC clone C06HBa0055E14 (anchored based on T0405) from which the highly distorted markers SLM6-29 and SLM6-30 were derived.

Twenty-one polymorphic SSR markers were placed along with two anchor markers SSR47 and SSR350 in a single linkage group. The genetic map spanned a total length of 117.6 cM (Fig. 3). The order of these markers on the genetic map was in the same order of BAC clones on the high density linkage map of tomato (Table 2). The map distance between markers derived from adjacent BAC clones in the West Virginia 700 × Hawaii 7996 RIL population were comparable

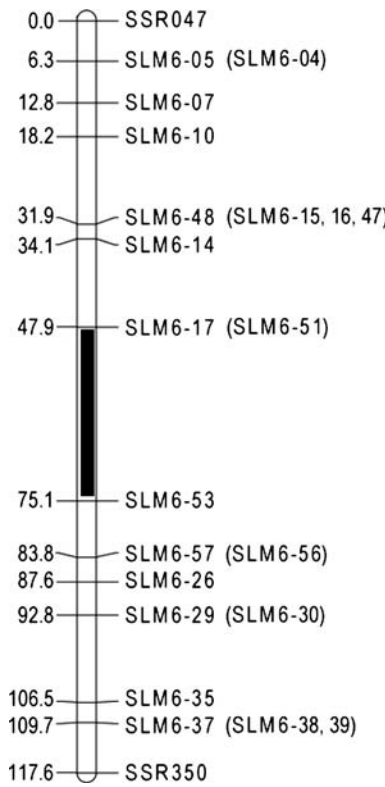

Fig. 3 SSR based linkage map of chromosome 6 based on a RIL population derived from a cross between West Virginia 700 and Hawaii 7996. Markers shown in the parenthesis were tightly linked with the marker indicated on the framework. Map location of the QTL associated with resistance to *R. solanacearum* strain Pss4 was highlighted

to distances between the actual BAC clones anchored on the tomato-EXPEN2000 map except for one marker interval (SLM6-17 to SLM6-53) where an expansion of 27 cM was observed. Such interval specific expansions have been observed in the other *S. lycopersicum* × *S. pimpinellifolium* map (Sharma et al. 2008). The present map with SSR markers derived from BAC clones could provide a good representation of chromosome 6, when integrated with other published SSR markers.

A major QTL associated with resistance to bacterial wilt

Maximum percentage of wilted plants was observed at 28 dai, after inoculating *R. solanacearum* strain Pss4. And distinct reactions were observed between the resistant Hawaii 7996 (19.8% of wilted plants) and susceptible WVa700 (96.9% of wilted plants). The mean percentage of wilted plants among the 188 RILs was 70.4%. Using the linkage map constructed in this study, a major QTL was identified associated with the marker interval SLM6-17–SLM6-53. The estimation of the percentage of phenotypic variation explained by this QTL was 11.2% and the allele from the resistant parent contributed to the resistance phenotype. According to EXPEN2000 map, the location of this QTL is similar with *Bwr-6* reported by Carmeille et al. (2006), which was detected by Wang et al. (2000) as well. *Bwr-6* is linked with TG73 located at 43.3 cM on chromosome 6, and SLM6-17 is located at 47.9 cM. More markers are needed to saturate this interval in order to find a closely linked maker for MAS.

In conclusion, 54 SSR markers specific to chromosome 6 were developed from anchored BAC clone sequences available in the Solanaceae Genomics Network. These SSR markers add to the present repository of molecular markers available for chromosome 6 in tomato and their utility in genetic diversity analysis and mapping studies have been demonstrated in this study.

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