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ORIGINAL PAPER

Aligned genetic linkage maps of apple rootstock cultivar 'JM7' and *Malus sieboldii* 'Sanashi 63' constructed with novel EST-SSRs

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Abstract Identification of markers associated with genes of interest and quantitative trait loci (QTLs), combined with high-density genetic linkage maps, can help reduce labor and costs by enabling marker-assisted selection (MAS). In this study, a dwarfing apple rootstock cultivar 'JM7' (*Malus prunifolia* × *Malus pumila* 'Malling 9') and wild apple *Malus sieboldii* 'Sanashi 63' (section *Sorbomalus*) were used for constructing genetic linkage maps. Here, a species from section *Sorbomalus* was used for the first time as a target species in a genome-wide mapping study. We also developed and mapped 137 novel-expressed sequence tag-simple sequence repeat (EST-SSR) markers. The genetic linkage maps of 'JM7' and 'Sanashi 63' consisted of 415 and 310 loci and spanned 998.0 and 981.8 cM, respectively, comparable to the reference map of *Malus* × *domestica* 'Discovery'. A BLASTN search revealed that all of the EST-SSR sequences used in this study exhibited very high homology to one or more previously characterized apple genome contigs. Although the most homologous contigs of 89 EST-SSRs were located within the same linkage groups (LGs) identified by mapping analysis, the other 48 EST-SSRs were aligned into contigs positioned in different LGs than those identified by mapping. When search criteria were expanded to include the five most homologous contigs of each EST-SSR, at least one of the top five contigs for 15 of these 48 EST-SSRs corresponded to the LG obtained by mapping. The maps of 'JM7' and 'Sanashi 63' may be useful for analyzing important rootstock characteristics and identifying markers for MAS.

Keywords EST-SSR · Genetic linkage map · *Malus sieboldii* · Rootstock

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Introduction

Apple (*Malus* × *domestica* Borkh.) is a major fruit tree in temperate regions, producing 70 million tons of fruit in 2008 according to the Food and Agriculture Organization of the United Nations (http://faostat.fao.org/faostat/). Apple cultivation management methods such as planting density and pruning methods depend on the rootstocks used. Excellent rootstocks derived from *Malus* spp. are essential for commercial apple cultivation because it is necessary to graft

fruiting scions onto rootstocks. Apple rootstock breeding has been conducted worldwide, resulting in the development rootstock cultivars such as the "M" series from the East Malling Research Station, UK (Hatton 1917), the "G" series from Cornell University, USA (Robinson et al. 2003), and the "P" series by Skierniewice, Poland (Zagaja 1980). The National Institute of Fruit Tree Science (NIFTS, Morioka, Japan) developed the "JM" series rootstocks, which change the tree growth habit to dwarf or semi-dwarf and were easy to propagate from hardwood cuttings (Soejima et al. 2010). Since crown gall disease recently became a problem in nurseries, rootstock breeding programs began to introduce genes conferring resistance to crown gall disease into "JM" rootstocks (Moriya et al. 2008). However, evaluations of vigor, ease of propagation, and crown gall resistance require time- and labor-intensive processes for handling many individuals and repeated trait assessments by traditional breeding methods. Identification of markers associated with genes of interest and quantitative trait loci (QTLs), combined with high-density genetic linkage maps, can help to reduce labor and costs by enabling marker-assisted selection (MAS). However, not enough simple sequence repeat (SSR) markers, especially expressed sequence tag (EST)-derived SSR markers, have been developed to allow marker-assisted breeding in apple rootstocks, and very few functional genes have been employed as selection markers.

Despite the broad range of apple species used for rootstocks (Luby 2003), genomics approaches to rootstock breeding have been limited. The genus *Malus* is divided into five sections (*Malus*, *Sorbomalus*, *Eriolobus*, *Choromeles*, and *Docyniopsis*) based on morphological traits and flavonoid similarities (Phipps et al. 1990). Among these, sections *Malus* and *Sorbomalus* have been utilized frequently as rootstocks. However, all of the previously reported genome-wide genetic linkage maps have been constructed using only species belonging to the section *Malus* which includes the fruiting scion apple (*M.* × *domestica*). Researchers have searched for molecular markers associated with genes of interest for rootstocks such as dwarfing (Pilcher et al. 2008), resistance to woolly apple aphid (Bus et al. 2008), and crown gall disease resistance (Moriya et al. 2010). Dwarfing and resistance to woolly apple aphid were studied using genetic linkage maps of F_1 progeny obtained by crossing 'Malling 9' (M.9; *Malus pumila*) with 'Robusta 5' (R.5; *Malus baccata* × *Malus prunifolia*) (Celton et al. 2009). Until now, these were the only genetic maps containing abundant informative markers that had been constructed for rootstock. However, both mapping parents used by Celton et al. (2009) belong to section *Malus*, so genetic information from species outside of section *Malus* has been insufficient. The only genetic linkage maps that have been reported for species outside of section *Malus* were two partial maps constructed using *Malus sieboldii*, which belongs to section *Sorbomalus*. One was for linkage group (LG) 8 of 'Aotea 1' (Bus et al. 2008), and the other was LG 2 of 'Sanashi 63' (Moriya et al. 2010). The LG 2 map of 'Sanashi 63' was used to map the crown gall disease resistance gene *Cg*. Because it has been suggested that both the major gene *Cg* and several QTLs are involved in crown gall disease resistance (Moriya et al. 2010), integrated and high-density genetic linkage maps will be required to conduct more detailed mapping and perform marker-assisted selection (MAS) for resistance.

SSR markers, which can be derived from either genomic or EST sequences, are the most efficient molecular markers with which to construct the landmarks and frameworks of genetic linkage maps. Until now, 298 SSR markers from apple genomic sequences have been developed (Guilford et al. 1997; Gianfranceschi et al. 1998; Hokanson et al. 1998; Liebhard et al. 2002; Hemmat et al. 2003; Silfverberg-Dilworth et al. 2006; Fernandez-Fernandez et al. 2008; Celton et al. 2009). Many SSR markers have exhibited transferability across genera between *Malus* and *Pyrus*, which could help to identify colinearity between *Malus* and *Pyrus*. In particular, EST-based SSRs are located around conserved coding regions, and they are often more transferable between species and genera than are genomic SSRs (Decroocq et al. 2003). Several sequencing projects have generated more than 250 000 ESTs in apple (Newcomb et al. 2006; Naik et al. 2006; Gasic et al. 2009a). Although some of these sequences contain SSRs that have been developed into EST-SSR markers and mapped on linkage maps (Silfverberg-Dilworth et al. 2006; Celton et al. 2009; van Dyk et al. 2010), the number of established EST-SSRs has been limited to about 190.

In this study, we developed 137 novel EST-SSR markers and constructed integrated high-density genetic linkage maps of 'JM7' and 'Sanashi 63'. These maps were compared with the reference maps of the domesticated apples cultivars 'Fiesta' and 'Discovery', and their genome synteny is discussed.

Materials and methods

Plant materials and genomic DNA extraction

An interspecific cross made in 1995 between rootstock cultivar 'JM7' (section *Malus*) and *M. sieboldii* 'Sanashi 63' was used for genome mapping; the mapping population used here consisted of 120 F_1 plants. 'JM7' originated from the cross *M. prunifolia* 'Morioka Seishi' × *M. pumila* 'M.9'. The parents on rootstocks and F_1 progeny on their own roots were grown in the orchard of the Apple Research Station of NIFTS and were cut back to 1-m height each dormant

season. Young leaves of 11-year-old F_1 plants and the parents were harvested in July and stored at −80°C until use. Genomic DNA was isolated according to the method described by Moriya et al. (2010).

Construction of cDNA libraries

Six cDNA libraries were constructed from flower buds, young fruits, mature fruits, and leaves of 'Fuji' and from fruit peels and shoot apices of 'Jonathan' (both *Malus* × *domestica*). Total RNA was extracted using a cetyltrimethylammonium bromide-based method, as described by Kotoda et al. (2000). Messenger RNA was isolated from total RNA using a PolyATract mRNA purification kit (Promega, Madison, WI, USA), and six cDNA libraries were constructed with a Zap-cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA) and Packagene Lambda DNA Packaging System (Promega). After converting each cDNA phage library into a plasmid library by in vivo excision, clones with a cDNA insert were randomly sequenced using an ABI 3730xl automated sequencer (Life Technologies, Carlsbad, CA, USA). A total of 17 774 EST sequences were processed to remove the vector and unreliable sequences using Pregap4 software (Bonfield et al. 1995), and sequences longer than 200 bp were extracted for contig assembly using the Gap4 software program (Bonfield et al. 1995).

After the redundant clones were removed, contig assembly of raw sequences produced 9010 non-redundant sequences. Homology searches within databases of *Arabidopsis* proteins (The Arabidopsis Information Resource (TAIR); http://www.arabidopsis.org/) and contigs from Apple v1.0 genome assembly (Velasco et al. 2010; the Genome Database for Rosaceae (GDR); http://www.rosaceae.org/) were performed using BLASTX and BLASTN, respectively. We used the criterion that E-values under 1E-9 indicated significant homology.

EST-SSR development

The 9010 non-redundant sequences were searched for SSR motifs. Primer pairs were designed based on sequences flanking SSR motifs, which were identified using a search engine available at the GDR homepage. The minimum number of SSR repeats for marker development was set as five repeats for dinucleotide motifs, four repeats for trinucleotide motifs, and three repeats for tetranucleotide motifs. All primer pairs were designed to obtain amplicons ranging between 60 and 350 bp in length.

PCR amplification was performed in a 10-μl solution of 1× *Ex Taq* buffer (Takara, Otsu, Japan), 0.20 mM of dNTPs, 0.4 pmol of each forward primer containing 17 bp (5′-AATACGACTCACTATAG-3′) of tail sequence, 2 pmol of reverse primer containing 7 bp (5′-GTTTCTT-3′) of pigtail sequence, 0.25 U of *TaKaRa Ex Taq* (Takara), and 10 ng of genomic DNA. Amplification was performed as follows: initial denaturation at 94°C for 5 min, followed by ten cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Then, 2 pmol of T7 universal primer (5′-AATACGACTCACTATAG-3′), labeled with fluorescent chemical D2, D3, or D4, was added to each amplification tube, and a second amplification was performed for 30 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min followed by a final extension at 72°C for 7 min. Primer sequences for the EST-SSRs mapped in this study are given in Table 1.

PCR products were separated and detected with a CEQ 8000 Genetic Analyzer (Beckman-Coulter, Inc., Brea, CA, USA). PCR product sizes were determined by using a Size Standard Kit-400 for the CEQ 8000 (Beckman-Coulter).

Analysis of selected landmark SSRs

A total of 275 previously reported SSR markers from two genera of the Rosaceae, *Malus* and *Pyrus* were tested for the F_1 population between 'JM7' and 'Sanashi 63'. Of these, 256 SSRs were derived from *Malus* (Guilford et al. 1997; Gianfranceschi et al. 1998; Liebhard et al. 2002; Hokanson et al. 1998; Silfverberg-Dilworth et al. 2006; Fernandez-Fernandez et al. 2008; Celton et al. 2009; Costa et al. 2008), and 19 SSRs were from *Pyrus* (Yamamoto et al. 2002a, b; Sawamura et al. 2004; Nishitani et al. 2009). After screening for SSR markers showing polymorphism within one or both of the parental cultivars, 166 SSR markers were used for genotyping of the 120 F_1 progeny individuals. SSR-PCR was performed by the method described above or according to Moriya et al. (2011).

Amplified fragment length polymorphism analysis

Amplified fragment length polymorphism (AFLP) analysis was performed using an AFLP Analysis System II kit (Life Technologies), according to the supplier's protocol, except that we used *Eco*RI primers labeled with a fluorescent chemical instead of non-labeled primers. Genomic DNA (200 ng) was digested with restriction enzymes *Mse*I and *Eco*RI; the digested DNA fragments were then ligated to the adapters provided in the kit. Pre-amplification reactions were performed with the supplied pre-amp primer mix. Selective amplification was performed with 16 primer combinations of five Mse primers (KeyGene codes: M48, M50, M59, M61, and M62) and eight Eco primers (KeyGene codes: E31, E32, E33, E34, E39, E40, E41, and E42). Selective primers were synthesized at BEX (Tokyo, Japan). The PCR products were separated and detected using a PRISM 3100 DNA sequencer (Life Technologies). The size of each amplified band was determined using internal-standard DNA (HD400ROX, Life Technologies) and

Table 1 Characteristics of novel EST-SSRs mapped in linkage maps of 'JM7' (J) and/or 'Sanashi 63' (S)

Name	Accession	Forward primer sequence	Reverse primer sequence	Motif	Type of marker	Map position (linkage group)	
						'JM7'	'Sanashi 63'
MEST001	AB627185	agagagcactcttcccacca	tgacgaggtcatcagactgc	(tc)33	sloc[a]	J6	
MEST003	AB627186	ccccgttaacccctcataat	taatggcggctcttcagtct	(ag)29	sloc	J13	S13
MEST006	AB627187	gcattctccgacaccatttt	ttctacatcaccacccctcc	(ag)25	sloc	J5	S5
MEST007	AB627188	ggtgggtatgctgtttggac	ccaagtttctatgcttccgc	(ga)8g(ga)25	sloc	J15	S15
MEST008	AB627189	gattatcattgccacgaccc	ggaaccgtttctgcatttgt	(at)5(ta)25	sloc		S5
MEST009	AB627190	gggcaaaacagaggatttga	tccgatcgaaagaggaagaa	(ag)24	sloc	J16	
MEST011	AB627191	tcaccgttttgatgaagatcc	tccatcttgctcaactcacg	(ct)24	sloc	J12	S12
MEST012	AB627192	acggctttccatttgcatac	cgaccaactctgtaaaaccct	(ag)23	mloc	J2	S2,S7
MEST013	AB627193	gcagcctaatgcgagattgt	cgatgagctcacagcagaat	(ag)23	sloc	J15	S15
MEST014	AB627194	cagtggtgataacctctcctg	ggcattgcctttcattttgt	(ct)22	pres mloc	J9	
MEST016	AB627195	aaacaaaacaaaaggcacgg	taactcatcccctcaccagc	(ag)21	sloc	J11	
MEST017	AB627196	aatagcctctgctgctctcg	ccgttaacccctcgtaatca	(ct)21	sloc	J13	S13
MEST019	AB627197	caaacattcattgccaccag	acttctcttgcctccgcata	(ga)21g(ga)6	mloc	J8,J15	
MEST020	AB627198	caggccattagctccacatt	gccactagcaaagttccctg	(ga)21	sloc	J17	S17
MEST021	AB627199	agttttggtggtttgcaagg	gcccaagactttgtcaccat	(ga)21	sloc	J13	
MEST022	AB627200	ggttggtattgggaccctct	tcccaccaaaggagatgaag	(ga)20	sloc	J15	S15
MEST023	AB627201	aagggtatcattccattgcg	cgatggactaaactgaatggc	(ga)20	sloc	J10	
MEST024	AB627202	tgggatgtcaacctgcagta	aaaacatccctttcggcttt	(ta)20	mloc	J2,J7	S11
MEST026	AB627203	caatctgctcatgttagctgc	gatgcacaacactcccacac	(tc)20	mloc	J15	S8,S15
MEST028	AB627204	attggcattgcttctcacct	tgcaacaacaattcccttca	(ag)19	sloc	J2	S2
MEST029	AB627205	atacaacccacgagtcccaa	cagggaggctaaaatagggc	(ag)19	sloc	J17	S17
MEST031	AB627206	tttcttctctcccaccctcc	ggcatttcttgctcttctgc	(ct)19	sloc		S3
MEST032	AB627207	ttgtttttgatgtaccctgc	ccaaaaatcgaaggaccaaa	(ct)19	sloc	J9	S9
MEST033	AB627208	tcaaaccccactagactcc	caacaaagcagccataagca	(ct)19	sloc	J11	
MEST034	AB627209	aaatcaatgatggaacggga	cgaaggaccaaaaatggaga	(ct)19	sloc	J9	S9
MEST036	AB627210	ttcggtttcggtaccatctc	gacctggcaaccttgtgaat	(ct)18	sloc	J8	
MEST037	AB627211	tcattcgacacttcccaattc	tagcatgcctggtctctcct	(ct)18(ca)6	sloc	J7	S7
MEST038	AB627212	tgcagtgacttgaatttggc	tcagccttctgttcctctctc	(ga)18	sloc		S10
MEST039	AB627213	ttgccactagagagccacac	tgcaacttttgccacatctc	(ga)18	sloc	J9	
MEST041	AB627214	tctcccatctcactttctcca	cgaagatctgcctagccttg	(tc)18	pres mloc	J14	S14
MEST043	AB627215	tgaggagttttatgggccag	gccaaccctttcttcctctt	(ag)17	sloc		S10
MEST044	AB627216	ttcacccaaccgtagcaact	ccctacgatttcgatgagga	(ag)17	sloc		S11
MEST045	AB627217	tggggacattggatggtatt	gttgtcatggagccttggtt	(at)17	sloc	J11	
MEST049	AB627218	ccctcccacttctcgataca	ggatttcccgccatatttct	(tc)17	sloc	J4	
MEST050	AB627219	gattatgaatgtcgtggccc	ccatgatcattcaagaccga	(tg)17	sloc	J15	S15
MEST052	AB627220	cggaccaacctacaaggcta	cacggatctacgactgagca	(ag)16	sloc	J13	S13
MEST054	AB627221	tgtgtgtgtgtgtgtgtgtga	aagatgcgaaccgaagaaga	(ag)16	sloc	J7	S7
MEST056	AB627222	agcttgaacctgtgctagatg	ttggctccctctaaaccgta	(at)16	sloc	J6	
MEST060	AB627223	aagcaaccctccaaaaattc	tggtaagcaagaaagccaca	(ct)16	sloc	J3	
MEST061	AB627224	tcgattcctcaatctctctca	atcggagaaaacccaaatcc	(ct)16	sloc	J8	S8
MEST062	AB627225	caacacttgcccaagaacct	gccattttcagaacagccat	(ct)16	pres mloc	J15	S15
MEST063	AB627226	tcgctgaggtcaaaccttct	ggccattagctccacatcat	(ct)16	sloc	J9	S9
MEST064	AB627227	ttcagtttcaccatccctcc	ggccattagctccacatcat	(ct)16	sloc	J9	
MEST065	AB627228	aaaacccagacggagagaca	ttgtttgccttccaagctct	(ct)16	sloc	J5	S5
MEST066	AB627229	ttggcctctcctctcaaaaa	aaacatcttctgggcctgtg	(ct)16ca(ct)8	sloc	J10	
MEST067	AB627230	attttggtgggcagcaggt	aaaaacccaactctccgctt	(tc)16	sloc	J2	S2

Table 1 (continued)

Name	Accession	Forward primer sequence	Reverse primer sequence	Motif	Type of marker	Map position (linkage group)	
						'JM7'	'Sanashi 63'
MEST069	AB627231	cccgccaagttacaacctaa	cccaaaagaccaatgcttgt	(ct)15	sloc	J17	S17
MEST070	AB627232	ctctaacctacgctgctggg	tgtggacatcaagcttctgc	(ga)15	sloc	J11	
MEST071	AB627233	cccaacaaacttcttctccg	gctttccagctttgcgtaac	(ga)15	sloc	J13	
MEST073	AB627234	ctctgaaactgcgaacacca	taaaccaaaaggctcacgct	(tc)15	sloc	J9	S9
MEST074	AB627235	ttccgtcatcctcaaaggtc	gaggccatcagattttgcat	(tc)15	sloc	J17	
MEST076	AB627236	aagtcccgaaagtgggttct	agacgcagcaaggttagagc	(ag)14	sloc	J3	S3
MEST080	AB627237	gctgcacttcttctgcattg	agttatggcctaatggcacg	(ct)14	pres mloc	J3	S3
MEST082	AB627238	ctacctgagcctcctctccc	ctcggaacggcaagaagtag	(ct)14	sloc	J15	
MEST083	AB627239	tttggggtttcaaagaggtg	ctcaaatctccaaaccctgc	(ga)14	sloc		S7
MEST084	AB627240	caacggttttcattgagcag	gctttcctcaagtcgtcgtc	(ga)14	sloc	J12	S12
MEST086	AB627241	cggaagtggaaagctgaaag	gactgagtttcgcaagaggg	(tc)14	pres mloc	J15	S15
MEST088	AB627242	aaacagcaaaacccagatcg	gggctctataaattccccca	(ag)13	sloc	J13	S13
MEST089	AB627243	tacagatttccgttcccctg	aactccataacgcttcgctc	(ag)13	sloc	J5	S5
MEST091	AB627244	cggatcttttcttgaggtcg	aacagatcgatcgtgaagcc	(ct)13	sloc	J4	
MEST092	AB627245	aaaatgtgtgacacccccat	gagagagagggagtgaaggga	(ct)13	mloc	J15	S11
MEST093	AB627246	tgctttctgcttaggcatctc	agacttctggtgttggggtg	(tc)5(ct)13	sloc	J13	S13
MEST095	AB627247	cctccaaatgctcatccatt	tgcatcaagaggaagcagtg	(ct)13	mloc	J10	S5,S10
MEST096	AB627248	acaatcctccccccaaaactc	cctcctcccccattcatatt	(ct)13	sloc	J17	S17
MEST097	AB627249	ttgatctgggaaaaatttgga	tttcacgctttgcaatgaag	(at)12(ag)25	sloc	J9	S9
MEST098	AB627250	gcaacactgttcttgcggta	ttgatagagagagaatgcggg	(at)11	pres mloc	J16	
MEST099	AB627251	ccctccctccctctctctct	accaccccaatgcaaataat	(cctc)5(ct)13	pres mloc	J1	
MEST100	AB627252	atgtctacggggtttgttgc	tcatatcctttcttgcatttgtt	(tg)12	mloc	J11,J12	
MEST101	AB627253	tgacatacccatctccccat	tgaggagggatttgaaggtg	(tc)12	sloc	J8	S8
MEST102	AB627254	tcccggtctctttctgtctg	attgttgaggcgaagtttgg	(tc)12(ac)10	sloc	J15	S15
MEST105	AB627255	atattgaaggggggcaccaa	ggtgagaaacaaagtgggga	(ta)12	sloc		S2
MEST106	AB627256	catgatgcatggatctgctt	gcattgggagcattatcacc	(ta)12	pres mloc		S8
MEST108	AB627257	aaggaagtgtgtggcactcc	ggctgtatgctgtatgatcagtg	(tc)11(ta)12	pres mloc		S15
MEST109	AB627258	ggtgtaagttgtgtcgccaa	actaacagcaccagcagcag	(ta)12	sloc	J10	
MEST111	AB627259	cacttccagatttccctcca	acaccaaacgaatgaggtcc	(at)12	sloc	J10	S10
MEST112	AB627260	ctcactcaaaccctcccaaa	ctgctcatctcatcagccaa	(tc)8tt(tc)11	mloc	J12,J14	S12,S14
MEST113	AB627261	agagaaaggggggacaaagga	agctcttggatctcggaaca	(ag)12	pres mloc	J1	
MEST117	AB627262	cgaccttgaggcctctgtag	tccccatcaactactccgtc	(ag)12	mloc	J3,J11	S11,S11
MEST118	AB627263	gtggccaccacttgaatgta	ggctactctcgacggctatg	(ag)12	sloc	J11	
MEST119	AB627264	tgcaacaggagaaaaccaga	tccaaatcaagaaactgccc	(at)11	sloc	J17	S17
MEST120	AB627265	agagagctgttttcgcttgg	aatgctgaggcagtaatggg	(ag)11	sloc	J1	
MEST122	AB627266	catcacttgccctccaattt	tgcgacttaatgttgggtga	(at)11	sloc	J7	
MEST123	AB627267	caaaccttcgtcgcct	tgtctttgtctcggcaactg	(ct)11	sloc	J5	S5
MEST124	AB627268	tccagggattacaatttcgg	tgcattggctcaagatgaag	(ga)11	pres mloc	J10	S10
MEST125	AB627269	ccatgagaaggcttggtgtt	cagagccgcattagggttt	(tc)11	sloc		S4
MEST126	AB627270	tgaaaagatggcaggagctt	ccaaagaaatcagaggcgaa	(at)11	sloc	J15	S15
MEST127	AB627271	ggggtgaaggaggtttgaat	acgccagatgagaggctaaa	(ga)11	sloc	J12	
MEST128	AB627272	ttgcattcacttcaccatgc	caagaagatggagatggtcaca	(tc)11	mloc	J15	S8
MEST129	AB627273	gcacgagggagcaaagatt	ttgatcttgcctgggttgat	(ag)11	pres mloc		S1
MEST132	AB627274	taaatcggctgatcactccc	ttggaaaacttccctcctca	(ta)11	sloc	J12	S12
MEST133	AB627275	agctctgaccctcctcaaca	agatttgggctttgaagtcg	(tc)11	sloc	J5	S5
MEST134	AB627276	ccttaaccgactgcctaacc	tggggtgattctctggtttc	(ct)11	sloc	J3	

Table 1 (continued)

Name	Accession	Forward primer sequence	Reverse primer sequence	Motif	Type of marker	Map position (linkage group)	
						'JM7'	'Sanashi 63'
MEST135	AB627277	taggacagattgattccgcc	ggggatttgtcacagcagtt	(ta)11	sloc		S8
MEST136	AB627278	cctctgtacctaaccgcttcc	tcacctgggcagatttaagg	(ct)10	sloc	J16	
MEST138	AB627279	tttctcatggcagcttgatg	tctgcagtctggaagctcaa	(ct)10	mloc	J4,J12	S4,S12
MEST139	AB627280	ggttgttgctgaccctcatt	tacgcttccgtaaaacccac	(ag)10	pres mloc	J13	
MEST140	AB627281	cagcactgtgtgttacggct	atgctgcgtttgtgtgtagg	(tc)10	sloc	J11	
MEST141	AB627282	tttctgagtgttgggttttgg	gaaccccaaaacagagtgga	(ct)10	sloc	J14	
MEST142	AB627283	gttctccttggatcacctcg	caattcagactccctctggc	(at)10	sloc		S2
MEST143	AB627284	ggaagtgggaggggagatag	tcttgctcaaccagcaacac	(ag)10	pres mloc	J1	
MEST144	AB627285	catccaccatggaaagatcc	caggaaatgaaggagggtga	(ag)10	pres mloc	J16	S16
MEST145	AB627286	cgattcgggactcaaaattc	gataccagtgcggaggaaga	(ag)10	sloc		S9
MEST147	AB627287	tccctccctcatcattcatc	gcttgctcttggacttggac	(tc)9	sloc	J15	
MEST148	AB627288	gaggagagaaagtgggaggc	tcctccccaatgtcataaa	(tc)9	sloc		S6
MEST150	AB627289	catcacttgcaagaagccaa	cttgtggtgtgtttcgatgg	(ct)9	sloc	J15	S15
MEST153	AB627290	tttggaagcaaacgagaagc	gggaaactgatttagtgcgg	(tc)9a(at)5	sloc	J13	S13
MEST154	AB627291	ataaaaacgacgtcatcggc	cccgggctagagagagaagt	(tc)9	sloc	J12	S12
MEST155	AB627292	ttttcacagccaaacactgc	gaacaaaggggaagaaaaaca	(ct)8ttt(tc)5	mloc	J2,J15	S2
MEST157	AB627293	gtacggtgacaatggcgaat	tccagaacgataggaatggc	(ct)9	sloc		S1
MEST158	AB627294	gaaagacccgaaacccagat	tagggaaaattggtggcaaa	(ct)9	sloc	J8	
MEST159	AB627295	tgcgtatagctcaaaacaagc	ggcatcgatgaagaagcag	(at)9	sloc	J13	S13
MEST160	AB627296	ttttccctctaaacaacctcca	ttgaaatggaacacgaaaacc	(ct)9ag(tc)5	sloc	J1	S1
MEST161	AB627297	cagagacatcagacagcgga	cagactccaaaacgacgaca	(ag)9	sloc	J6	S6
MEST162	AB627298	agcctctaacctaaccccca	aaaaccgtcaattgctgtcc	(ct)9	sloc	J13	S13
MEST163	AB627299	gttttggagagaagcgaacg	gcttgaacttgctttagccac	(ga)9	sloc	J1	S1
MEST164	AB627300	cattttggtgatccggagag	tcgactgagatggtgagtgc	(ac)9	pres mloc	J17	
MEST165	AB627301	tcacatctgctcttgctgct	ggggaaaccacatgactctc	(ag)9	pres mloc	J15	
MEST168	AB627302	taatggcggctcttcagtct	ccccgttaacccctcataat	(ct)9	sloc	J13	S13
MEST170	AB627303	ttttaccccaaccctaaccc	gactcgggcactgtaattgg	(tctg)6	sloc	J16	
MEST171	AB627304	aaacatgcagacagggaagg	ctgagcagtttgcagctttg	(ggag)6ga(aggg)3	sloc		S14
MEST174	AB627305	cccaaaacccaactcagaaa	aagaatccccaatcccaatc	(gaaa)5	sloc	J13	
MEST176	AB627306	ctaaatccgtcactcccgaa	tgtgactggttttgtgggaa	(ccag)5	pres mloc	J12	
MEST177	AB627307	tttcgaatccgctgctactt	agctcacgtgccttcttgat	(agac)5	sloc		S9
MEST178	AB627308	cccaggggatactctgtgaa	tcgactgcacctgatgagac	(aaag)5	sloc	J15	
MEST182	AB627309	ccagcgagcagtacgga	gattggatggacggagaaga	(gaaa)4	pres mloc	J10	
MEST185	AB627310	agttggaatggctatgtgctc	ggcttcatttgcatttcctg	(tc)11	mloc	J1,J7	S1,S7
MEST187	AB627311	acacacactccctcagaccc	ccagctttctctggacttgg	(catt)4	sloc	J4	S4
MEST188	AB627312	gcacgatcagcagtttttca	tttcctttggtttcggagtg	(agga)4	sloc	J8	
MEST189	AB627313	atagggcaagagctcgacaa	ccacttgtgttctggtcacg	(agaa)4	sloc	J7	
MEST190	AB627314	tttgtgctcagacagatggc	cattgatcatcaccactgcc	(aatt)4	sloc		S9
MEST193	AB627315	attgttcacttggccaccat	tccactgcaaccaacaagag	(tat)10	sloc	J1	
MEST195	AB627316	ctgcctaaacaccaactcacc	gagatcgtcaggggacaaaa	(tcc)10	pres mloc	J12	
MEST196	AB627317	aaaaacaagaagcagcagcc	ttttcgaagtcctcccatct	(ctc)10	sloc	J8	
MEST197	AB627318	gaacccggttttgatcctct	tgacagacactcctccctcc	(gag)9	pres mloc	J17	
MEST198	AB627319	ccctttcagcaaagcaactc	taccataactcgccggagtc	(gca)9	mloc	J2,J15	S13
MEST199	AB627320	gctcacaccactcttcctcc	gttcaagagcctagcggatg	(cct)8	sloc	J1	S1
MEST200	AB627321	acgccttcatccgtatcatc	taaacaaaccaccgaaaccc	(gtt)8	sloc	J9	S9

[a] *sloc* single locus, *mloc* multilocus, *pres mloc* presumed multilocus

GeneScan software. AFLP marker names consist of the names of primers, followed by the size of polymorphic fragment in basepair.

Genotyping of the self-incompatibility locus

Genotypes at the self-incompatibility locus (*S* locus), which is one of the important traits and has been mapped near the bottom of LG 17, were assessed by allele-specific PCR with S_3 and S_9 allele-specific primer pairs (Broothaerts et al. 1995; Janssens et al. 1995). PCR amplification was performed as follows: initial denaturation at 94°C for 5 min, followed by for 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were separated on a 1.5% agarose gel in 0.5× TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light.

Linkage analysis

We used a pseudo-testcross mapping strategy (Grattapaglia and Sederoff 1994) to construct genetic linkage maps of 'JM7' and 'Sanashi 63' with JoinMap v4.0 (van Ooijen 2006). This strategy allowed us to determine an accurate marker order for the polymorphic loci within each of the two parents separately. The regression mapping algorithm and the Kosambi function in JoinMap v4.0 were applied. A minimum LOD score of 8.0 was used to establish the linkage groups. The two parental maps were aligned using the markers segregating within both parents, and the linkage groups were numbered using published apple reference maps (Maliepaard et al. 1998; Liebhard et al. 2003) based on SSR markers in common between the new maps and previously published maps.

Results

Development of EST-SSR markers

Out of the 9010 non-redundant EST sequences evaluated, 170, 9, and 21 EST sequences were selected for primer design for di-, tri-, and tetranucleotide motifs, respectively. PCR products with the desired target size were obtained from 151, 9, and 21 primer pairs for di-, tri-, and tetranucleotide EST-SSRs, respectively. Finally, 137 EST-SSR markers showed scorable heterozygous genotypes for at least one parent, including 119 for di-, 7 for tri-, and 11 for tetranucleotide motifs (Table 1); 102 SSR primer pairs generated amplified fragments from single loci, whereas 35 SSR primer pairs showed multilocus or presumed multilocus amplification.

Functional analysis of EST-SSRs

Characteristics of the EST-SSR sequences were identified by comparisons to *Arabidopsis thaliana* protein sequences in TAIR and the contigs from Apple v1.0 genome assembly. In the BLASTX analysis, the amino acid sequences predicted from 106 of the EST-SSR sequences exhibited significant homology to *A. thaliana* proteins (Supplementary Table 1). Four EST-SSR sequences, MEST039, 140, 187, and 188, exhibited high and significant homology to proteins related to the Myb transcription factor. MEST124 showed high homology to a disease resistance protein of the TIR-NBS-LRR class. In the BLASTN searches of contigs in Apple v1.0 genome assembly, all of the EST-SSR sequences tested in this study showed very high homology to one or more apple genome contigs, with E-values of under 2E-78 (Supplementary Table 2). The most homologous contigs (top hits) of 89 EST-SSRs were located in the same LGs as those identified by mapping analysis (described in detail below), whereas the other 48 EST-SSRs were aligned to contigs in different LGs than those predicted by mapping. When the search criteria were expanded to include the top five most homologous contigs for each EST (data not shown), at least one of the top five contigs for 15 of these 48 ESTs corresponded to the LG obtained in the mapping study.

AFLP analysis

AFLP analysis with 16 combinations of Mse and Eco primers yielded 189 segregating loci, including 125 for 'JM7' and 64 for 'Sanashi 63', with an average value of 7.8 and 4.0 loci per primer combination, respectively. The primer combinations E32M59 and E33M48 generated the greatest number of segregating loci (19), and E41M59 generated the fewest (4).

Genetic linkage maps of 'JM7' and 'Sanashi 63'

SSR markers from several published genetic linkage maps were used to create genetic framework maps for both 'JM7' and 'Sanashi 63'. Out of the 256 apple SSR markers tested, 155 revealed polymorphism in one or both parents and were positioned into framework maps. The other 37 SSRs showed polymorphism within one or both parents but were not used for genotyping of F_1 plants because they were located close to other mapped markers. The remaining 64 SSRs either did not show polymorphism or did not amplify any products. For pear SSRs, 11 of 19 markers could be mapped in one or both parents. One of the pear markers showed polymorphism within one parent but was not utilized for F_1 genotyping because the marker located close to other mapped markers. The remaining seven pear markers

Table 2 Details of the linkage maps of 'JM7' (J) and 'Sanashi 63' (S)

Marker type	Linkage group																	
	J1	J2	J3	J4	J5	J6	J7	J8	J9	J10	J11	J12	J13	J14	J15	J16	J17	Total
Novel EST-SSR (number of loci)	9	6	5	4	5	3	6	7	9	7	8	10	13	3	19	5	8	127
Published SSR (number of loci)	9	12	15	12	15	9	7	6	9	10	12	7	12	11	15	10	9	180
AFLP (number of loci)	10	5	8	6	9	1	5	7	3	5	6	9	5	5	6	10	7	107
Others (STS, cleaved amplified polymorphic sequence and traits) (number of loci)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Total number of marker loci	28	23	28	22	29	13	18	20	21	22	26	26	30	19	40	25	25	415
Length of linkage groups (cM)	72.1	59.6	53.5	84.0	67.8	51.0	51.9	55.0	47.3	61.1	65.8	38.2	53.3	50.9	65.3	69.6	51.8	998.0
Marker density (markers/cM)	0.39	0.39	0.52	0.26	0.43	0.25	0.35	0.36	0.44	0.36	0.40	0.68	0.56	0.37	0.61	0.36	0.48	0.42

Marker type	Linkage group																	
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	Total
Novel EST-SSR (number of loci)	6	6	3	3	7	2	5	7	9	5	5	6	10	3	11	1	5	94
Published SSR (number of loci)	9	16	10	11	12	7	5	9	4	6	10	4	9	9	12	9	12	154
AFLP (number of loci)	0	5	1	3	2	3	4	3	2	2	9	2	2	2	6	9	4	59
Others (STS, cleaved amplified polymorphic sequence, and traits) (number of loci)	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3
Total number of marker loci	15	29	14	17	21	12	14	19	15	13	24	12	21	14	29	19	22	310
Length of linkage groups (cM)	56.6	59.7	54.8	57.5	71.1	59.3	62.1	41.8	58.9	61.8	63.3	24.7	57.8	47.1	90.5	53.5	61.2	981.8
Marker density (markers/cM)	0.27	0.49	0.26	0.30	0.30	0.20	0.23	0.45	0.25	0.21	0.38	0.49	0.36	0.30	0.32	0.36	0.36	0.32

either did not show polymorphism or did not amplify any products.

In total, 137 newly developed EST-SSRs could be positioned on the 'JM7' or 'Sanashi 63' framework maps (Table 2; Fig. 1). Among them, 127 loci detected by 118 markers were mapped in 'JM7' and 94 loci detected by 86 markers were mapped in 'Sanashi 63'.

AFLPs generated from 16 primer combinations were then integrated into these genetic linkage maps. An AFLP was not placed if it made a change in the order of framework SSRs, was more than 20 cM distal to the terminal SSR marker of an LG, or showed severe distorted segregation while neighboring markers did not. In total, 18 AFLP loci for 'JM7' and 6 for 'Sanashi 63' were excluded from the mapping data files. As a result, AFLP analysis yielded 166 mapped loci, including 107 for 'JM7' and 59 for 'Sanashi 63', with an average value of 6.3 and 3.5 loci per primer combination, respectively (Table 2; Fig. 1).

The final genetic linkage maps of 'JM7' and 'Sanashi 63' consisted of 415 and 310 loci and spanned 998.0 and 981.8 cM, respectively (Table 2; Fig. 1). Total marker densities of the maps of 'JM7' and 'Sanashi 63' were 0.42 and 0.32 markers per centimorgan, respectively (Table 2). These two maps could be aligned by using 185 anchor SSR loci. For the map of 'JM7', the length of the LGs ranged from 38.2 to 84.0 cM, with an average length of 58.7 cM. The number of loci per LG in 'JM7' ranged from 13 to 40, with an average of 24.4. For the map of 'Sanashi 63', the length of the LGs ranged from 24.7 to 90.5 cM, with an average length of 57.8 cM. The number of loci per LG ranged from 12 to 29, with an average of 18.2. For both maps, marker density was the highest in LG 12 and the lowest in LG 6. Gaps between markers longer than 20 cM were observed in one or both lines in LGs 1, 4, and 6. The largest gaps between markers were located in LG 6:25.5 cM for 'JM7' and 22.4 cM for 'Sanashi 63', respectively. In both maps, LG 15 contained the most SSR markers and LG 6 had the fewest. Compared with reference maps of 'Discovery' (*M.* × *domestica*; Fig. 1) and 'Fiesta' (*M.* × *domestica*; not shown), new terminal SSR markers were obtained in this study in the lower part of LG 7, the upper part of LG 8, and the upper part of LG 13.

In this study, one published but previously unmapped SSR marker could be mapped, and eight SSR markers were mapped to LGs in addition to those reported in previously published maps. Previously unmapped SSR marker NZ17e6 (Guilford et al. 1997) was mapped in LG 17 of 'Sanashi 63'. SSR markers CH01e12 and CH01c06, both previously reported to be located in LG 8, were also mapped here in LG 1 of 'Sanashi 63'. SSR marker Hi04b12, previously reported to be located in LG 8, was also mapped here in LG 15 of both 'JM7' and 'Sanashi 63'. SSR markers CH03b01 and NZmsEB107305, both reported to be in LG 2, were also mapped here in LG 15 of 'JM7' and LG 7 of both lines, respectively. Similarly, CH05c06, previously reported to be in LG 16, was also mapped here in LG 13 of both 'JM7' and 'Sanashi 63', and CH05g11 and MDAJ761 (AJ000761-SSR), reported to be in LG 14, were also mapped in LG 6 of 'JM7' and/or 'Sanashi 63'.

Three markers previously mapped to only a single locus mapped to several new loci in our mapping population. Hi05g12, previously reported in LG 2, was also mapped here in three new LGs (LG 10, 12, 14), all in 'JM7'. Hi07b02, previously reported in LG 4, was mapped here in three new LGs (LG 7, 13, 17), and NZmsEB177464, previously reported in LG 3, was mapped here in four LGs (LG 3, 10, 11, 17), sometimes in several locations per LG. Three SSR markers, GD103, CH05b06, and Hi04c10, which were previously mapped at two loci, were mapped here at two or more new loci. GD103 revealed two new segregating loci in LGs 4 and 14 in addition to its previously reported locations in LGs 5 and 10; CH05b06 revealed two new segregating loci in LGs 15 and 17 in addition to its previously reported locations in LGs 5, 10, and 16. Hi04c10, previously mapped in LGs 3 and 4, generated five new segregating loci in LGs 2, 3 (a second locus was mapped in 'JM7'), 4 (a second locus was mapped in 'Sanashi 63'), 5, and 13.

Discussion

The genetic linkage map of 'Sanashi 63' is the first genetic linkage map covering all 17 LGs in a species belonging to the section *Sorbomalus*, and it was constructed primarily with informative codominant SSR markers. Although genetic linkage maps of the genus *Malus* have been constructed with SSR markers covering all 17 LGs (Maliepaard et al. 1998; Silfverberg-Dilworth et al. 2006; Fernandez-Fernandez et al. 2008; van Dyk et al. 2010), these maps were intended primarily for the fruiting scion apple (*M.* × *domestica*). The only genetic linkage maps of *Malus* spp. other than *M. domestica* have been those of rootstocks 'M.9' and 'R.5' (Celton et al. 2009). Because 'JM7' is a descendant of 'M.9' and *M. prunifolia*, 'JM7' would be expected to possess a similar genetic background to 'M.9'. However, *M. sieboldii*, to which 'Sanashi 63' belongs, is categorized in section *Sorbomalus* rather than in section *Malus* where *M.* × *domestica* is classified. The section *Sorbomalus* includes *Malus florentina*, *Malus kansuensis*, and *Malus yunnanensis*, and none of which have yet been used in any mapping project.

Overall, a total of 300 SSRs were mapped onto the 'JM7' and 'Sanashi 63' genetic maps, which were then compared with those of 'Discovery' and 'Fiesta' (both *M.* × *domestica*). In most cases, the length of each LG was shorter than

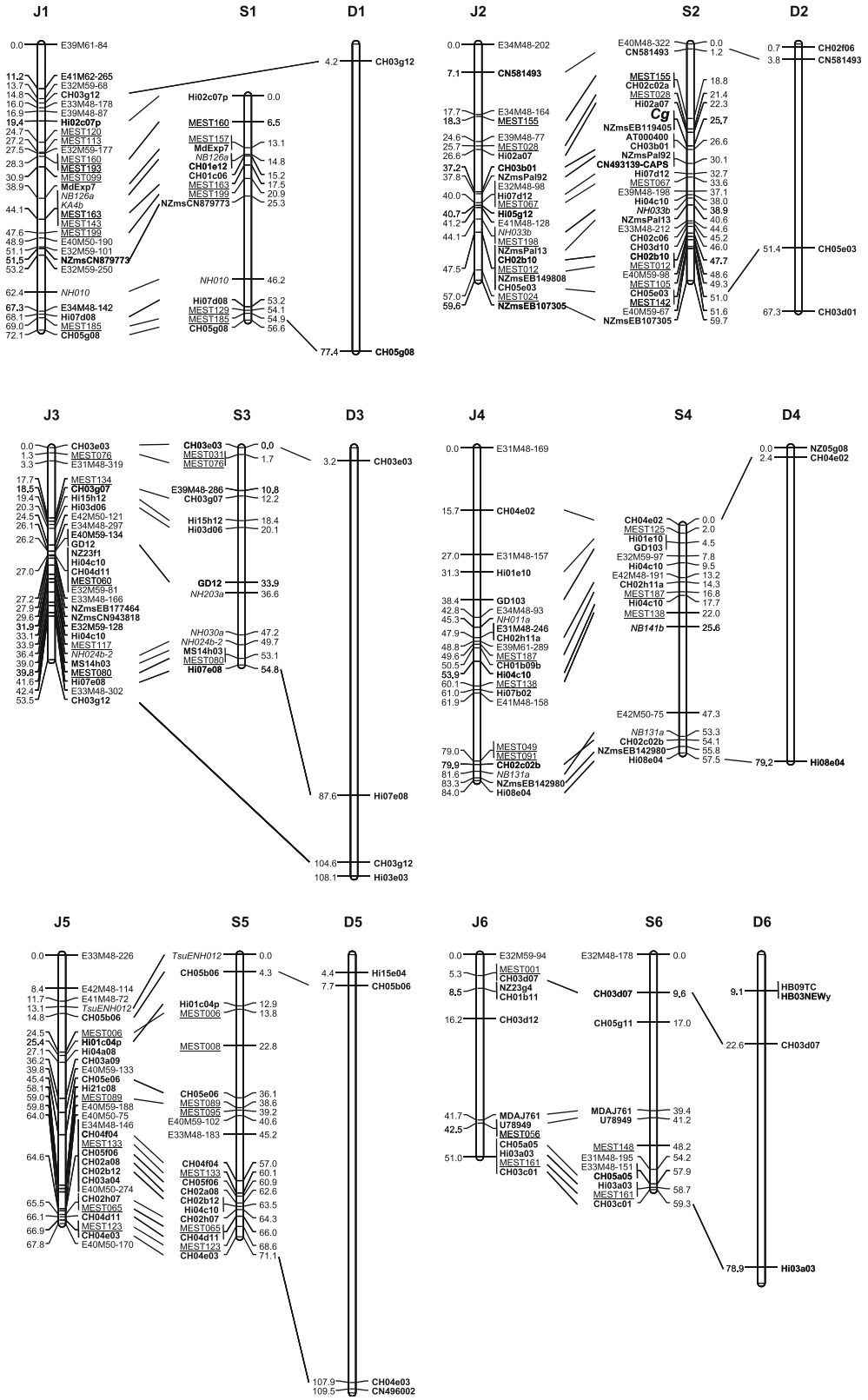

Fig. 1 Genetic maps of 'JM7', 'Sanashi 63', and 'Discovery' (Silfverberg-Dilworth et al. 2006). Linkage groups are designated as J1 to J17 for 'JM7', S1 to S17 for 'Sanashi 63' and D1 to D17 for 'Discovery'. Only terminal SSR markers and anchor SSR markers are indicated in the map of 'Discovery'. Numbering of the linkage group is according to Maliepaard et al. (1998). Genetic distances and marker names are listed next to each linkage group bar. Names of novel apple EST-SSRs are *underlined*. Names of published apple SSRs and pear SSRs are indicated in *bold* and *italic font*, respectively

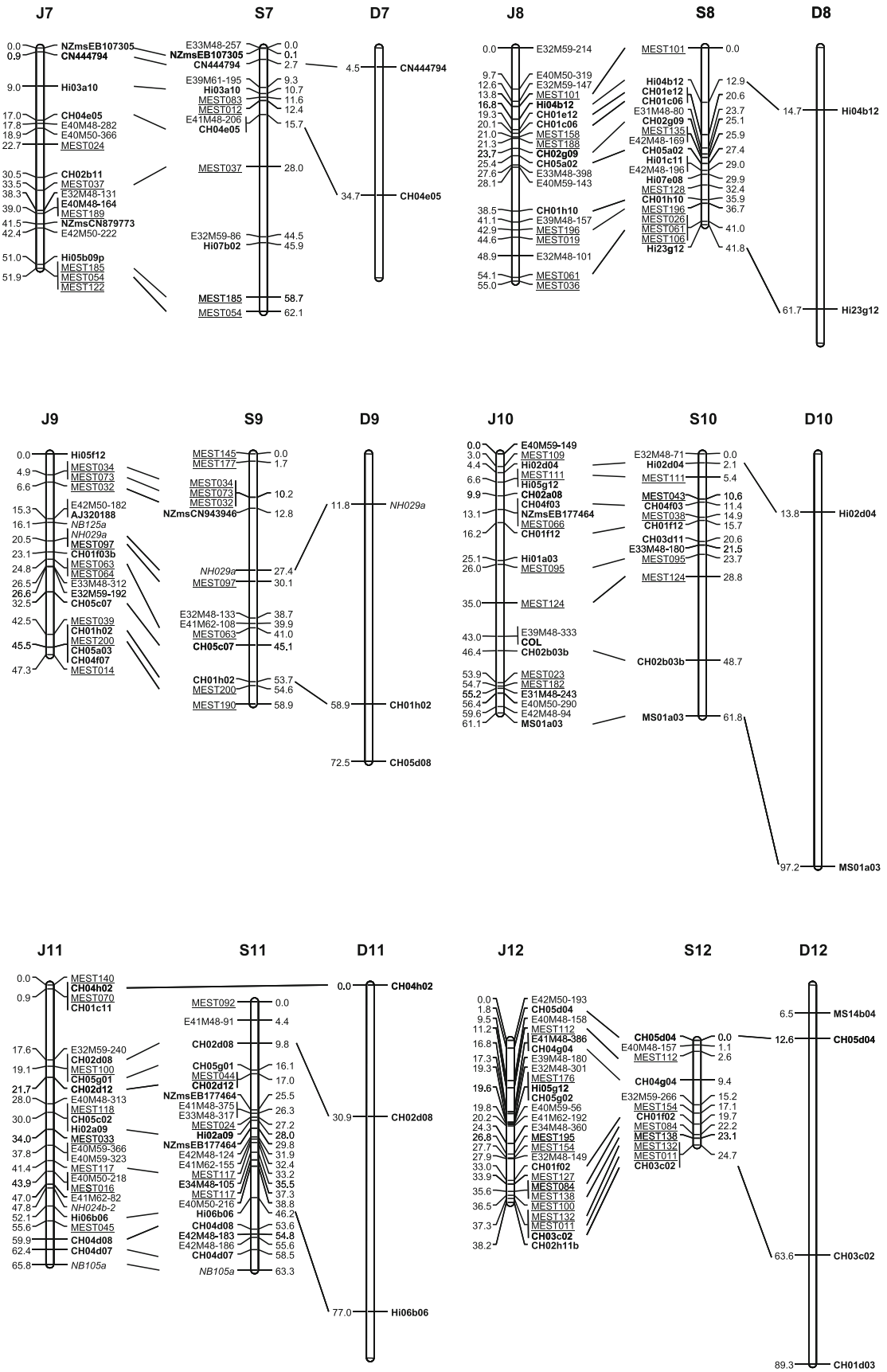

Fig. 1 (continued)

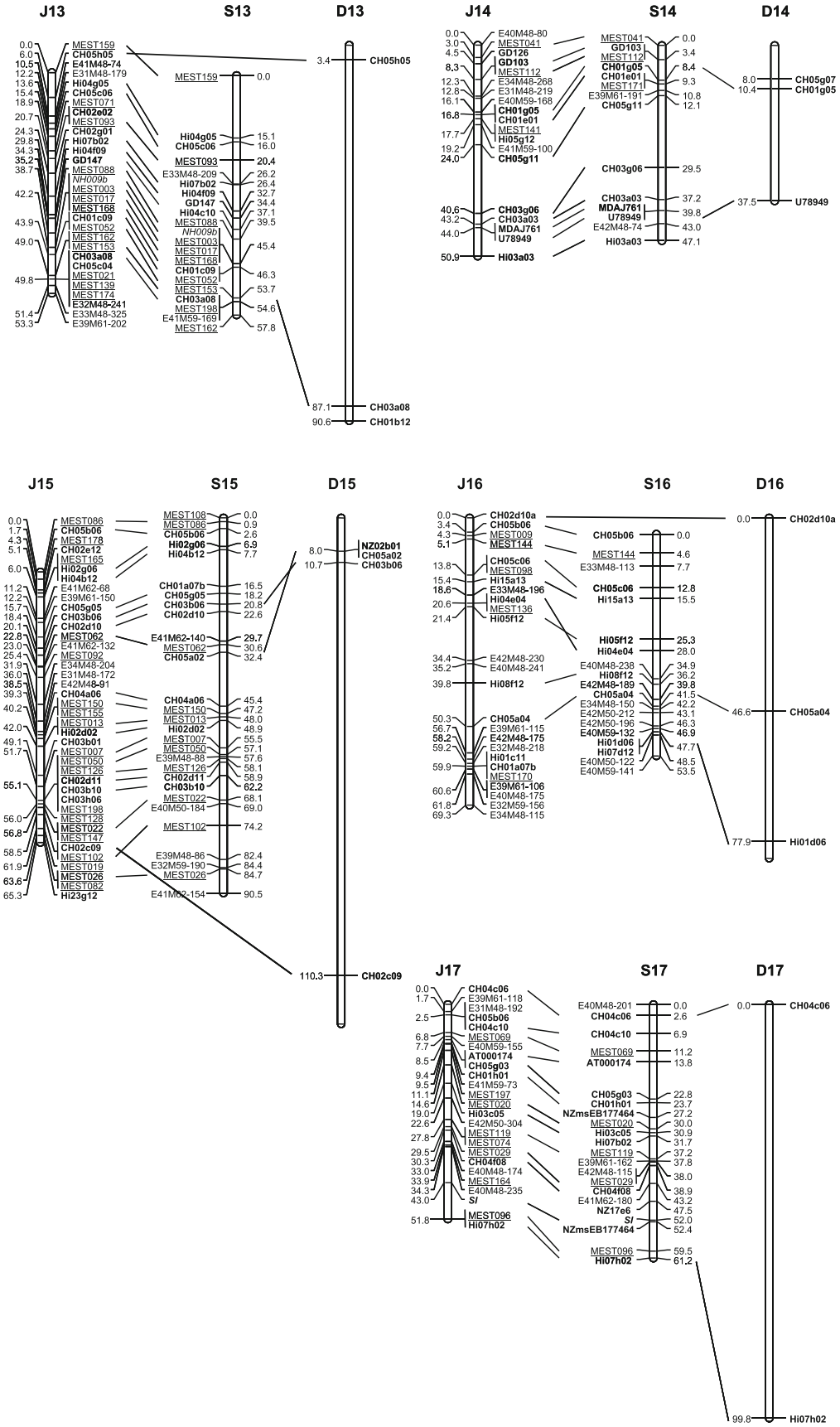

Fig. 1 (continued)

those of the reference maps; however, the differences are not easily explained. Some incomplete regions were observed at the bottom of LG 12, the top of 'Sanashi 63' LG 1, the bottom of 'Sanashi 63' LG 3, the bottom of 'Sanashi 63' LG 9, and the top of 'JM7' LG 11. In these regions, the tested markers could be amplified but did not show polymorphism. Despite the lack of map information in these regions, the genome coverage of the maps of 'JM7' and 'Sanashi 63' is comparable to that of the reference map of 'Discovery' (Silfverberg-Dilworth et al. 2006) because three other regions (LGs 7, 8, and 13) were expanded in this study. The marker density in the maps of 'JM7' (0.42 markers/cM) and 'Sanashi 63' (0.32 markers/cM; Table 2) were markedly higher than those of 'M.9' (0.16 markers/cM) and 'R.5' (0.19 markers/cM). Additionally, the 'JM7' and 'Sanashi 63' maps also showed higher genome coverage than 'M.9' and 'R.5'.

Considering the genetic maps of 'Discovery', 'JM7', and 'Sanashi 63' as representative of domesticated and wild apples in sections *Malus* and *Sorbomalus*, it is possible to compare the genome structures among those apples. The marker order in each of the three maps was almost the same, indicating that macrosynteny is retained among *Malus* spp. Six of the 14 SSRs that mapped in different LGs from previously published maps were located in suggested orthologous LGs (chromosomes) and are presumed to have originated from a genome-wide duplication event (Velasco et al. 2010). Differences between the materials used here and those in previous studies enabled us to map previously unmapped loci. However, eight SSRs were mapped on chromosomes that were inconsistent with the current model of genome-wide duplication in apple. Because most of these SSRs showed three or more amplified bands, these markers may have detected heavily duplicated regions unrelated to genome-wide duplication. SSR marker CH01c06 amplified bands in 'JM7' on LG 8; however, additional bands that mapped to LG 1 was amplified in 'Sanashi 63', possibly suggesting a short block of chromosome rearrangement.

A relatively small number of EST-SSRs have been available until now, and their development is still an important objective. Fewer than 200 EST-SSRs derived from apple were developed and mapped in previous reports (Celton et al. 2009; Silfverberg-Dilworth et al. 2006; van Dyk et al. 2010), whereas approximately 300 genomic SSRs have been reported. An advantage of EST-SSRs is that they are often transferable to other rosaceous crops (Gasic et al. 2009b); thus, development of EST-SSRs in one species can directly benefit genomic studies in another. The EST-SSR markers described here were designed from *M.* × *domestica* sequences because we assumed that these markers would be applicable not only to rootstocks but also to *M.* × *domestica* cultivars. The findings that many of the markers were successfully mapped in 'JM7' or 'Sanashi 63' suggests that these EST-SSRs will be useful within the broad *Malus* collections for accurate and high-throughput genotyping and may be candidates for selection markers for MAS.

The newly developed EST-SSR markers in our study showed higher polymorphism than those reported in previous studies. The proportion of successfully mapped SSR markers was 71% for dinucleotide motifs, whereas 66% was the highest proportion in other reports (van Dyk et al. 2010). Tri- and tetranucleotide motifs also showed higher polymorphism than in previous reports (Celton et al. 2009; van Dyk et al. 2010). These results revealed higher heterozygosity in the genomes of 'JM7' and 'Sanashi 63' than in the cultivated apples for which genetic maps had previously been published. These differences may be explained by our use of interspecific hybrids between wild accessions for genome mapping. Thus, the genetic maps of 'JM7' and 'Sanashi 63' and the mapping populations reported here may be useful to identify the locations of new markers for which the map locations are unknown.

Fourteen of the novel EST-SSRs reported here mapped to multiple loci. The combinations of multiply mapped loci (chromosomes) amplified by one marker were generally consistent with combinations of orthologous chromosomes predicted by a model of genome-wide duplication (Velasco et al. 2010). Markers MEST024 and MEST198 amplified bands corresponding to two loci in 'JM7' and one locus in 'Sanashi 63'. The two loci detected by each of these two markers in 'JM7' were mapped in LGs consistent with genome-wide duplication. However, the map locations for MEST024 and MEST198 in 'Sanashi 63' were different from those of 'JM7' and not readily explained by genome wide duplication, which might suggest a short block of chromosome rearrangement.

When BLASTN searches were performed using the single most homologous contigs in Apple v1.0 genome assembly, 48 EST-SSRs were aligned to contigs in different LGs than those predicted by mapping. When the criteria for BLASTN searches of EST-SSRs were then expanded to include the second- to fifth-most homologous contigs within Apple v1.0 genome assembly, at least one of the top five contigs for 15 of these 48 EST-SSRs corresponded to the LG obtained by mapping, suggesting that these ESTs are members of gene families that are widely scattered in the genome. However, the remaining 33 markers still showed discrepancies between the LG of the most homologous contigs and the LG obtained by mapping. Possible reasons for this could be misassembly of ESTs, gene families consisting of genes possessing similar sequences, and misassembly of contigs in Apple v1.0 genome sequence. These results indicate that BLAST homology searching against Apple v1.0 genome assembly is not always the best approach to estimate the correct map location(s) of a nucleic

acid sequence. Thus, mapping approaches using genetic linkage maps with broad genome coverage are necessary for determining the correct map location(s) of a sequence. Additionally, novel EST-SSRs that were aligned within unanchored metacontigs by BLAST searching were useful in assigning the map positions of those metacontigs within LGs (chromosomes).

The newly constructed maps of 'JM7' and 'Sanashi 63' are expected to provide effective tools for apple rootstock breeding. Apple rootstock breeding has traditionally been conducted by selection of seedlings from wild populations of apples (Webster and Wertheim 2003). Although modern breeders introduced controlled crossing techniques to replace open-pollinated seedling selection; to date, all new rootstocks have been selected using conventional phenotypic evaluation techniques, which are time-consuming and labor-intensive activities. These obstacles can be overcome by using molecular markers linked to important traits. MAS will enable apple breeders to achieve dramatic progress in rootstock breeding programs more quickly and accurately than before. The linkage maps of 'JM7' and 'Sanashi 63' will be worthwhile for analyzing distinctive and important characteristics, such as easy propagation by cutting and resistance to diseases such as crown gall (Moriya et al. 2010) and Valsa canker (Abe et al. 2011). Thus, the genetic maps of 'JM7' and 'Sanashi 63' provide a development platform for assessing inheritance patterns and direct adoption of selection markers for these traits. The major goal of our study was the development of excellent new rootstocks based on introgression of biotic stress resistance into superior rootstock cultivars. The next step to facilitate rootstock breeding by an MAS system is to identify and map loci involved in traits of interest.

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