Development and linkage mapping of E-STS and RGA markers for functional gene homologues in apple

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Abstract: Linkage maps developed from known-function genes can be valuable in the candidate gene mapping approach. A set of 121 expressed sequence tagged site (E-STS) primer pairs were tested on a framework genetic linkage map of apple (*Malus* × *domestica* Borkh.) constructed using simple sequence repeats (SSRs) and randomly amplified polymorphic DNA (RAPD) markers. These known-function gene markers, E-STSs, were supplemented by markers for resistance gene analogues (RGAs), designed based on conserved motifs in all characterized resistance genes isolated from plant species. A total of 229 markers, including 46 apple E-STSs, 8 RGAs, 85 SSRs from apple and peach, and 88 RAPDs, were assigned to 17 linkage groups covering 832 cM of the apple genome, based on 52 individuals originating from the cross 'Antonovka debnicka' (Q12-4) × 'Summerred'. Clusters of E-STS and RGA loci were located in linkage groups previously identified to carry resistance genes, some of which confer resistance to apple scab disease caused by *Venturia inaequalis* (Cke.) Wint.

Key words: apple scab, EST, *Malus*, RAPD, SSR.

Résumé : Des cartes génétiques élaborées à l'aide de gènes de fonction connue sont utiles dans le cadre de stratégies de clonage par gène candidat. Cent vingt et une paires d'amorces amplifiant des étiquettes de gènes exprimés (E-STS) ont été utilisées pour situer ces gènes sur une carte de référence chez le pommier (*Malus* x *domestica* Borkh.), laquelle était constituée de microsatellites (SSR) et de marqueurs RAPD. Ces marqueurs pour des gènes de fonction connue ont été bonifiés à l'aide de marqueurs pour des analogues de gène de résistance (RGA) conçus sur la base motifs conservés chez tous les gènes de résistance provenant de plantes. Au total, 229 marqueurs dont 46 E-STS et 8 RGA de pommier, 85 SSR de pommier ou de pêcher et 88 RAPD ont été assignés à 17 groupes de liaison couvrant 832 cM en analysant 52 progénitures du croisement entre 'Antonovka debnicka' (Q12-4) × 'Summerred'. Des groupes de locus E-STS et RGA ont été trouvés sur des groupes de liaison déjà connus comme étant porteurs de gènes de résistance, certains conférant la résistance à la tavelure causée par le *Venturia inaequalis* (Cke.) Wint.

Mots clés : tavelure du pommier, EST, *Malus*, RAPD, SSR.

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Introduction

Apple (*Malus* × *domestica* Borkh.) is one of the most important tree fruit crops grown in North America and around the world, and belongs to the family Rosaceae. This family includes several other genera, accounting for most of the important deciduous temperate fruit crops, including pear (*Pyrus communis* L. and *P. serotina* L.), stone fruits (*Prunus*), such as peach (*Prunus persica* (L.) Batsch), cherry (*P. avium* L. and *P. cerasus* L.), plum (*P. domestica* L. and *P. salacina* Lindell), apricot (*P. armeniaca* L.), and almond (*P. amygdalus* Batsch) as well as valuable ornamental plants, such as roses (*Rosa*) and hawthorn (*Crataegus*).

Codominant simple sequence repeat (SSR) markers available in apple have been reported to be useful in map alignment and are transferable between mapping populations (Gianfranceschi et al. 1998; Liebhard et al. 2002, 2003*b*). SSRs can be used to produce framework maps for other apple populations, with average marker distances of 15–25 cM. Apple linkage maps published so far are composed mainly of restriction fragment length polymorphisms (RFLPs), isozymes, random amplified polymorphic DNAs (RAPDs),

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amplified fragment length polymorphisms (AFLPs), and SSRs (Hemmat et al. 1994, 2003; Conner et al. 1997; Seglias and Gessler 1997; Maliepaard et al. 1998; Liebhard et al. 2002, 2003*b*; Kenis and Keulemans 2005). Dominant markers, such as RAPDs, can be used for map alignment if they are heterozygous in both parents, but their transferability to other maps is limited. Liebhard et al. (2002) have positioned 115 SSR markers on a genetic linkage map of the cross 'Fiesta' × 'Discovery'. All 17 linkage groups, corresponding to the 17 chromosomes of apple, have been identified. In a later study using the same population, a total of 840 molecular markers, including AFLPs, RAPDs, SSRs, and sequence-characterized amplified regions (SCARs), have been used for constructing the 2 parental maps 'Fiesta' and 'Discovery', spanning 1140 cM and 1450 cM, respectively (Liebhard et al. 2003*b*). This is by far the most saturated linkage map for the apple genome.

Recently, the nucleotide binding site (NBS) profiling method has been used to identify and map resistance gene analogs (RGAs) in apple, thus enabling the amplification and mapping of genetic markers anchored in the conserved NBS encoding domain identified in all characterized plant disease resistance genes to date (Calenge et al. 2005). The structural similarity of cloned disease resistance (R) genes offers opportunities for isolating RGAs from different plant species using PCR. Compared with arbitrary markers, the RGA scanning technique is highly reproducible due to the use of longer primers. Moreover, amplified products may be correlated with biological functions. Recent reports of the successful use of these RGAs to identify markers closely linked to resistance genes in various plants (Leister et al. 1996; Shen et al. 1998; Hayes and Maroof 2000; Rajesh et al. 2002) have prompted us to use these markers in apple. We have designed primers based on conserved sequences present in all characterized resistance genes isolated from various plant species (Rajesh et al. 2002) to add this new class of markers to the existing apple linkage map. R genes are known to be clustered on chromosomes, and clusters of apple scab (*Venturia inaequalis* (Cke.) Wint.) resistance genes are located on linkage group 2 of the apple genome (Bus et al. 2004, 2005*a*). A cluster of 4 resistance paralogs (*Vfa1*, *Vfa2, Vfa3*, and *Vfa4*) has been identified at the *Vf* locus, and these paralogs are predicted to encode proteins characterized by extracellular leucine-rich repeats (LRRs) and transmembrane (TM) domains (Xu and Korban 2002). Previously, Vinatzer et al. (2001) reported on the identification of a cluster of receptor-like genes with homology to the *Cladosporium fulvum* (Cf) resistance gene family of tomato on BAC clones derived from the *Vf* scab resistance locus.

The sequence-tagged site (STS) approach is an alternative to using SSRs for developing sequence-specific PCR markers that share all characteristics of SSR markers. Recently, large numbers of expressed sequence tags (ESTs) have been developed in many species, including apple (Korban et al. 2005). These ESTs are mostly anonymous, in that their functions and map locations have not been characterized. More often, maps are based on PCR-based markers, such as RAPDs, AFLPs, and SSRs, which target noncoding regions, but markers developed from known-function genes, such as EST-derived STSs (E-STSs) and RGAs can complement those studies by revealing the location, and possibly the structure, of gene-rich regions in the genome. Additionally, for a trait of interest, markers can be preselected based on the biochemical and physiological properties of the gene of interest with which they are associated (Chee et al. 2004). Maps developed from known-function genes may be particularly valuable in a candidate gene mapping approach, thereby facilitating the dissection of complex traits (Pflieger et al. 2001). Studies aimed at characterizing genes involved in resistance against pathogens in apple are limited (Belfanti et al. 2004).

In the present study, we have used markers from published apple maps to construct a framework anchor map for a mapping population of 'Antonovka debnicka' (Q12-4) × 'Summerred' (A×S). The objectives include the following: (*i*) develop a framework map for the A×S population using available SSRs and RAPDs; (*ii*) map new RGA loci using primers based on conserved sequences of common, characterized R genes; and (*iii*) develop PCR-based E-STSs, and use these to map EST loci. E-STSs were directly from genes transcribed in apple tissues, obtained from cDNAs. RGAs target conserved motifs within putative genes, and in addition, are not specific to apple. Forty-six E-STS markers and 8 RGA markers have been mapped on the SSR–RAPD anchored map of the A×S population to reveal their chromosomal location in the apple genome.

Materials and methods

Plant material and DNA isolation

The Russian apple cultivar 'Antonovka', along with its subclones and (or) variants, are known to have either monogenic or polygenic resistance to apple scab (Quamme et al. 2003) and cold hardiness (Khanizadeh and Cousineau 1998). A cross between 'Antonovka debnicka' (Q12-4) and 'Summerred' was made in 1998 (Quamme et al. 2003). The cross resulted in a population of 91 individuals. However, some individuals died as a result of fire blight or powdery mildew infections, resulting in a final usable progeny of 52 individuals.

Total DNA was extracted from young leaves (200 mg dry weight) collected from seedlings growing in the field, using the DNeasy® plant Maxi prep kit (Qiagen, Mississauga, Ont.) following the method described by the manufacturer. The concentration of DNA was measured visually by running samples on an agarose gel against DNA of known concentration.

Molecular markers

We screened a total of 169 SSRs from apple and 41 SSRs from peach (Table 1) using our mapping population. PCR was performed in a My Cycler ™ thermal cycler (BioRad, Hercules, Calif.). PCR conditions for the various groups of SSRs were as described by Liebhard et al. (2002) for CH and MS apple SSRs, as described by Guilford et al. (1997) for New Zealand (NZ) apple SSRs, as described by Hokanson et al. (1998) for golden delicious (GD) apple SSRs, and as described by Dirlewanger et al. (2002) for Bordeaux *Prunus persica* microsatellites isolated from library enriched in CT repeats (BPPCT) peach SSRs. PCR products were separated by electrophoresis using 2.5%–3.0% Metaphor agarose® (Mandel Scientific, Guelph, Ont.) in 0.5×

Table 1. Sources of various SSR markers used.

Prefix	Reference	Origin	No. of markers	No. of (%) polymorphic loci
CH	Liebhard et al. 2002	Apple	140	61 (43)
NZ	Guilford et al. 1997	Apple	14	5 (35)
MS	Liebhard et al. 2002	Apple	7	3 (43)
GD	Hokanson et al. 1998	Apple	8	4 (50)
BPPCT	Dirlewanger et al. 2002	Peach	41	4 (9)

Note: NZ, New Zealand; GD, golden delicious; BPPCT, Bordeaux *Prunus persica* microsatellites isolated from library enriched in CT repeats.

TBE buffer (45 mmol/L Tris–borate, 1 mmol/L EDTA), stained with ethidium bromide (0.8 mg/mL), and visualized using UV light. This permitted a 2% resolution, which is comparable with that of polyacrylamide gels (4% to 8%).

RAPD markers from an apple map (Hemmat et al. 1994) were used in this analysis. PCR cycles included an initial denaturation cycle of 2 min at 94 °C, 39 cycles of 1 min at 94 °C, 2 min at 35 °C, 2 min at 72 °C, and a final extension cycle of 8 min at 72 °C. Amplification products were separated on a 2% agarose (Invitrogen, Burlington, Ont.) – 0.5× TBE gel, and visualized on a UV transilluminator after staining with ethidium bromide.

Twenty-four pairs of RGA primers (Rajesh et al. 2002) were used. Each PCR reaction was performed in a 25 μL total volume consisting of 0.2 mmol/L of each of dATP, dCTP, dGTP, and dTTP; 5 mmol/L of $MgCl_2$; 1 U *Taq* DNA polymerase (Invitrogen), 2.4 ng/μL each forward and reverse primers; 30 ng template DNA; 2.5 μL of *Taq* polymerase buffer (Invitrogen). The PCR conditions were as follows: 5 min at 94 °C for initial denaturation, 45 cycles consisting of 1 min at 94 °C, 1 min at 45 °C, and 2 min at 72 °C, followed by a final 7 min extension at 72 °C. PCR products were separated on a 2.5% Metaphor® agarose – 0.5× TBE gel and visualized under UV light after staining with ethidium bromide.

DNA sequences from an apple unigene set (26 880 singletons and contigs) were used as a source of EST markers. The apple unigenes were assembled from EST sequences derived from both primary and normalized cDNA libraries, developed from various developmental stages of leaf (Mdlv), bud (Mddb), flower (Mdfw), shoot (Mdst), and fruit (Mdfr/Mdfrt) of apple cv. 'GoldRush' (S.S.Korban, manuscript in preparation). A web-based application for data management and viewing, Expressed Sequence Tag Information Management and Annotation (ESTIMA; Kumar et al. 2004), developed at the University of Illinois at Urbana-Champaign (UIUC), was used to identify DNA sequences. The analysis was carried out on apple EST data, available as of November 2004. Unique apple sequences (121, numbered UIUC-1 to UIUC-125) were compiled and a pair of oligonucleotide primers was designed for each sequence using the Vector NTI software (Invitrogen) with the following criteria: 45–65 °C melting temperature, 40%–60% G+C content, 18–25 bp primer length, and 100–300 bp amplicon size. A complete list of primers is available at the apple ESTIMA website (http://titan.biotec.uiuc.edu/apple/resources.shtml). PCR was carried out in a 25 μL solution containing either 50 or 25 ng genomic DNA template, 5.5 *p*mol of each primer, 2.5 mmol/L $MgCl_2$, 0.125 mmol/L each dNTP, 10× reaction buffer, and 1 U *Taq* polymerase (Invitrogen). A list of E-STS primer pairs that produced polymorphic loci mapped in our population, along with their corresponding T_m and putative associated resistance function is shown in Table 2. The typical cycling conditions for PCR were 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, primer specific annealing temperature for 1 min, and 72 °C for 1.2 min. Following the last cycle, reactions were incubated at 72 °C for 6 min before cooling them down to 10 °C. PCR products were separated on 2.5% Metaphor® agarose – 0.5× TBE gels containing ethidium bromide, and viewed under UV light.

Genetic mapping

Fifty-two apple individuals were used to perform segregation analysis using Joinmap 3.0 software (Stam 1993; Stam and Van Ooijen 1995). A CP type data set, designed for a mapping population that is the result of a cross between 2 heterozygous diploid parents for which no known linkage phase was available (Van Ooijen and Voorrips 2001), was set up for the A×S population. To evaluate the segregation ratio, the χ^2 goodness-of-fit test was performed using the locus genotype frequency command. Markers showing a distorted segregation ratio ($p < 0.05$) were added to the map at the end, but initially, linkage groups were established using a minimum of 5.0 threshold LOD, using the LOD grouping command. Once groups were determined, the map was calculated using a maximum recombination fraction (θ) of 0.3, and a minimum LOD score of 1.0 with a threshold of 5.0 for removal of loci with jumps in goodness of fit. The framework obtained was then fixed using the fixed-order command. Those markers that were not placed along the framework map were finally added using a minimum LOD score of 3.0 and a θ of 0.4. The Kosambi mapping function was used to convert recombination units into genetic distances.

Results and discussion

Genetic linkage map

The map constructed in this study is organized into 17 linkage groups (LGs) covering 832 cM. It is composed of 227 loci, including 85 SSRs, 88 RAPDs, 8 RGAs, and 46 E-STS (Fig. 1). Ninety-five additional markers (42 SSRs, 32 RAPDs, 3 RGAs, and 18 E-STS) remained unlinked and were discarded. LGs were numbered according to the nomenclature adopted by Liebhard et al. (2002, 2003*b*) because of the SSR loci held in common. LG1 and LG3 are small LGs composed of 3 loci, and the SSR CH03g12 amplified fragments of different sizes on both these LGs (CH03g12z and CH03g12y, respectively). The average distance between markers is 3.6 cM, after accounting for all

Table 2. Primer pair sequences for known-function genes based on EST sequences from the apple ESTIMA database at UIUC.*

Primer name	ESTIMA nomenclature of EST contig or singleton†	5′→3′ primer sequence	
		Forward primer	Reverse primer
UIUC-2	Apple_0902.219.C1.Contig350	TTGAAACCTAATCATCATCC (52)	AATTGTCGAATCATTTCCAG (55)
UIUC-3	Apple_0902.798.C1.Contig1191	TGTCACAATTCAATTACAACCAAGC (62)	GCTGTCAGCTTTCCAATTCA (59)
UIUC-4	Apple_0902.800.C1.Contig1194	ACTTACAGGTTTTTTCAGGATTCAC (59)	ATTTTGTCAGAGCCTTGGAC (57)
UIUC-7	Apple_0902.1565.C1.Contig2153	CATTAAACACAATCACGATGCA (59)	AGAACGTATTTCAATTCCTCCC (59)
UIUC-9	Apple_0902.2198.C1.Contig2868	TGAAAACTTGTACAGGATAATGGTC (59)	TTTCTTCTGGAGGAGTGATGAG (59)
UIUC-10	Apple_0902.2198.C1.Contig2958	TACACCAACAGAAACTATGCTACAG (58)	AGAGAACAGAAGCATCAGCCTAT (59)
UIUC-11	Apple_0902.2429.C2.Contig3146	GTATTTTGATCCATATGCTGTCG (59)	AAGACACCCGCTTGAATTTT (59)
UIUC-19	Apple_0902.4130.C1.Contig4978	GACGTTGAGGACCGTGAGAC (61)	TCTTTACTTAACGGCGGTGG (60)
UIUC-27	Apple_0902.4699.C1.Contig5556	CAAACTTAAAAGCACACTTCTTGAC (59)	TTCTTGCGACGCTAAATCTC (58)
UIUC-29	Apple_0902.4775.C1.Contig5633	CATCTTTCGACATGTATAACAATGG (60)	TTGTTGTTGATGATGTGGACAA (60)
UIUC-34	Apple_0902.6098.C1.Contig6988	TACCACAGTTATATTACAAATCCGG (58)	ATCAGCGATTGCGAGAAATT (60)
UIUC-40	Apple_0902.7440.C1.Contig8276	ACAAACAAATTGCTGGAACG (59)	TGACGACATGAATGTTGATGTG (60)
UIUC-43	Apple_0902.7653.C1.Contig8481	TTTGCCAAAGATATTCATCACC (59)	AGTTATTGCTCCCAGCTTCAT (58)
UIUC-52	Mddb5003n19.y1	ACGTCCTCGCTCTTCTTTTC (59)	CCACTCGCCAAAGTTAGTATTT (58)
UIUC-62	Mddb5019m02.y1	TCCTTTGCGAGCTCTCTGTC (61)	TGAACGAAGACAGGTAGAGGAA (59)
UIUC-66	Mddb5023i18.y1	ATAAAGAAGCAGGCTGGTACAG (58)	TTACGAACCATCGGACTTCA (59)
UIUC-75	Mdfrt3045d02.y1	TCTCTTCCGAGGGATTTCTTT (59)	AAGAGGGGAATGAACTGCTTAT (58)
UIUC-85	Mdfw2007i04.y1	TGGAATCACTAGGATAAGTAGGTGT (57)	TGGAAGGCCACTTGCATAAT (60)
UIUC-89	Mdfw2013c15.y1	AACTTGTCTAGGAATCAATTGACTG (58)	AAGATCAATGAGCCGCAAAC (60)
UIUC-91	Mdfw2015k02.y1	CCAATTGTAGTGACTGTCATTGC (60)	TGATTCGCTTTGCAAGTTTC (59)
UIUC-93	Mdfw2018d19.y1	ATGGAGCATGAGTTCAAGTGAC (59)	CAGAGTTACAACTTGGACCCAG (59)
UIUC-98	Mdfw2024i03.y1	ACCTAGAAGAATGGCCTGGG (60)	CCGTAGCTGTCGTAACTTTCC (59)
UIUC-99	Mdfw2024m05.y1	ACCTTAAATAATAGAGATGCAAGCG (59)	GGTATCTGACAAGTATCTGGGACA (59)
UIUC-104	Mdfw2029k20.y1	GGCCTCACAGCACTCCAAAG (63)	GCCCTCACGCCCATATTTTA (62)
UIUC-107	Mdfw2034i14.y1	AATCTGTTGGAGCTAACTTTGGA (59)	AGGGCGTGAATTTCATATACAC (58)
UIUC-108	Mdfw2040e02.y1	AAACAAAGAGTTTGGGCAAGG (60)	TTTGAAGGACTGCGATCTCG (61)
UIUC-111	Mdfw2042e17.y1	ACTTAACCTACAACGTTGCAAAAG (59)	TTAACCGCTGTTCAACCGAT (61)
UIUC-117	Mdfw2056e06.y1	CAAAGAAGCATATTGTGGTGC (58)	AAACTACAATCGAAACCCTTGA (58)
UIUC-120	Mdfw2064a22.y1	CAATCTCACCGGAAGCATTC (61)	AGCCATTGTTTCCGGAGTAA (60)

*Prepared from apple unigene data available as of November 2004.
†Source cDNA library for singleton sequences: mdfw, flower; mdst, shoot; mddb, bud; mdfr/mdfrt, fruit.
‡Base pairs.
§Polymorphisms associated with 'Antonovka debnicka' (Q12-4) are printed in bold.
‖Putative functions of EST sequences obtained by BLASTx similarity searches against *Arabidopsis* and rice proteins (NCBI accession numbers in parentheses) of EST contig or singleton in base pairs.

markers, with distances ranging from 2.0 cM in LG2 and LG11 (with 14 and 31 loci, respectively) to 8 cM in LG3 (with 3 loci). Among other marker-rich linkage groups are LG12 with 34 markers (an average of 2.4 cM), LG14 with 20 markers (an average of 2.2 cM), and LG15 with 25 markers (an average of 3.3 cM). LG15 with 84 cM and LG12 with 83 cM are the largest linkage groups. While gaps larger than 10 cM are located on all linkage groups, except for linkage groups 2 and 10 through 14, the largest gap of 22 cM is located in LG7. The small size of the linkage groups is consistent with an earlier report by Kenis and Keulemans (2005), indicating that the map size per haploid genome is rather small in Rosaceae. The average marker density of 3.6 cM is within the range of those reported earlier for apple: 2.6 –2.9 cM by Liebhard et al. (2003*b*), and 4.3–5.1 cM by both Conner et al. (1997) and Maliepaard et al. (1998).

Fifty-two loci (22.7%), showing distorted segregation, were distributed in all linkage groups, except for LG8, which did not deviate from the expected Mendelian ratio (Fig. 1). The percentage of markers showing distorted segregation is higher than that reported in peach (15% in Lu et al. (1998) and 18.5% in Dettori et al. (2001)) and apricot (11% in Hurtado et al. (2002)), but lower than that observed in peach × almond maps (37% in Foolad et al. (1995), 46% in Joobeur et al. (1998), and 45% in Bliss et al. (2002)). Clusters of distorted loci were also reported on apple linkage groups 3, 5, 6, 10, 13, and 14 by Liebhard et al. (2003*b*). Although segregation distortions were distributed on all linkage groups in our map, LG 2, 7, 11, 12, 14, and 15 had clusters of distorted loci (Fig. 1). The presence of segregation distortion in this study is consistent with reports in other crops and is assumed to be the result of sublethal genes present on those chromosomal regions (Liebhard et al. 2003*b*). As clustering of distorted loci could be influenced by the genetic load, as well as by the phenotype 'low viability' (Liebhard et al. 2003*b*), the observed distortion was likely dependent on the parental genotypes used in this study.

SSR framework map enriched with RAPD markers

A molecular map with a backbone consisting of codominant markers, such as RFLPs or SSRs, is deemed

Calculated size[‡]	Obtained size range (polymorphic band size[‡§])	Mapped linkage group[§]	Putative functions of ESTs[‖]
1010	100–1053 (105**, 1053**)	11**, 12**	NBS-LRR (NM_112306)
720	180–900 (**810**, 890)	**10**, 14	TIR- NBS-LRR (NM_124291)
796	150–900 (**590**)	**11**	TIR-NBS-LRR (NM_124271)
891	120–895 (**390 895**)	**17**	CC-NBS-LRR (NM_103079)
718	150–900 (**325**)	**2**	CC-NBS-LRR (NM_120554)
1317	400–800 (**390**)	**12**	TIR-NBS-LRR (NM_114308)
1121	150–1500 (**710**)	**4**	TIR-NBS-LRR (NM_105942)
628	180–1000 (**190**, 700)	7, **11**	LRR (NM_124353)
654	150–600 (**180**)	**2**	LRR (NM_120776)
748	150–1400 (**680**, 1350)	7, **10**	TIR-NBS-LRR (NM_102480)
1028	**710**	11	TIR-NBS-LRR (NM_114335)
690	550–950 (**650, 750, 950**)	**12**, **14**, **17**	TIR-NBS-LRR (NM_105080)
716	170–1400 (250, 950)	8, 12	TIR-NBS-LRR (NM_123893)
419	300–1500 (975**, 1450**)	**12**, 13	TIR-NBS-LRR (NM_123337)
557	200–800 (**450**)	**10**	LRR-RI (NM_105551)
578	150–950 (**610, 950**)	**8, 11**	TIR-NBS-LRR (NM_121774)
387	300–1200(**450**, 1100)	**2**, 11	TIR-NBS-LRR (NM_123051)
415	190–700 (190)	14	TIR-NBS-LRR (NM_114316)
493	350–700 (420, 500, 550)	2, 5, 12	TIR-NBS-LRR (NM_123995)
394	300–700 (300)	15	LRR (NM_195473)
505	300–1050(**1050**)	**15**	LRR (NM_120797)
587	350–1000(150, 325)	15, 16	LRR-RI (NM_103083)
544	450–1500(**750**)	**16**	CC-NBS-LRR (NM_104215)
480	400–900 (590)	7	CC-NBS-LRR (NM_124238)
468	250–900 (**250**, **775**)	**12**, **16**	CC-NBS-LRR (NM_104635)
418	450–1100(**480**, 800)	14, **16**	TIR-NBS-LRR (NM_124021)
610	400–1800 (490**, 1800**)	4, **15**	TIR-NBS-LRR (NM_117271)
555	375–1000 (**650**)	**12**	TIR-NBS-LRR (NM_102479)
492	600–1500 (**1300**)	**12**	LRR (NM_195473)

ideal because it is reproducible, transferable to other progeny, and can then be enriched with other markers (Liebhard et al. 2003*b*). A total of 210 SSRs from different sources were evaluated for polymorphisms using the 2 parents (Table 1). Of 169 apple SSR markers used in this study, 73 produced polymorphic patterns (6 producing more than 1 mapped loci), yielding a total of 81 segregating loci in our mapping population (Fig. 1). Twelve previously unmapped SSRs, including CH01b07, CH01b09b, CH02b11, CH02e12, CH03g06, CH03h06, CH04d08, CH04f03, CH05g01, CH05g02, CH05g05, and CH05h12, were integrated in our map, and 3 SSRs (CH01f12, CH01h10, and CH02a10) mapped onto linkage groups (Fig. 1) that were different than those originally reported (Liebhard et al. 2002, 2003*b*). Two of five polymorphic NZ apple SSR loci (Guilford et al. 1997), NZ04f3 and NZ26c6, were integrated into the present map, but were not previously mapped by Liebhard et al. (2002, 2003*b*). Sixty-one (43%) of the CH apple SSRs, 3 (43%) of the 'MS' apple SSRs, and four (50%) of the GD apple SSRs produced polymorphic patterns. Four (9%) of the BPPCT markers from peach produced polymorphic loci, thus raising the number of segregating SSR loci to 85, covering all 17 linkage groups (Fig. 1). Of these, 40 loci (47%) segregated codominantly. Sixteen markers deviated significantly from the expected segregation ratios (3:1 and 1:2:1). SSR loci were distributed uniformly on all linkage groups, ranging from 1 marker on each of LG1 and LG16 to 12 markers on LG11.

Earlier studies suggested a high level of synteny between apple (*Malus*) and *Prunus* maps (Dirlewanger et al. 2004). However, our effort to establish homologies with peach were unsuccessful, as only 4 peach markers mapped onto our apple population (Fig. 1), although 31 of the 41 markers amplified PCR products in apple, since the rest were not polymorphic. Of the 4 peach SSRs segregating in our population, only 1 (BPPCT 018) was mapped onto LG5, and it was not previously reported to identify polymorphisms in apple (Dirlewanger et al. 2002). The remaining 3 mapped onto LGs 12, 5, and 15 (Fig. 1).

Sixty-six RAPD markers from the apple map (Hemmat et al. 1994) were used to enhance the SSR framework map. Forty primers (60%) produced polymorphic patterns com-

Fig. 1. Genetic linkage map of the apple progeny 'Antonovka debnicka' (Q12-4) × 'Summerred' showing the position of E-STS and RGA loci. Linkage groups are numbered from LG1 to LG17. The group assignments in the present map were based on marker distribution in a previous apple SSR map (Liebhard et al. 2002). E-STSs, RGAs, peach SSRs, and apple SSRs not mapped in previous publications are printed in bold. Segregation distortion is indicated by means of significance level p of the χ^2 test as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; *****, $p < 0.0005$; ******, $p < 0.0001$. The E-STS loci are prefixed as UIUC. Apple SSR marker names prefixed with CH, MS, or NZ are described by Liebhard et al. (2002); apple SSR primers prefixed with GD were reported by Hokanson et al. (1998); peach microsatellite markers prefixed with BPPCT were reported by Dirlewanger et al. (2002); prefixes for RGA markers Pto-Kin, XLRR-INV, S2/AS3-INV, XLRR, and NLRR-INV were adopted from Rajesh et al. (2002); RAPD primers prefixed with P, S, and T were reported by Hemmat et al. (1994). Markers amplified by the same primer pair, but mapping at different loci, are given a z, y, x, or w suffix.

prising 88 loci covering all 17 linkage groups. Multiple loci were produced by 29 of these primers. Dominant segregation ratios were prevalent in all loci scored with Joinmap 3.0 segregation type codes of lmxll and nnxnp for a 1:1 ratio (locus heterozygous in either parent), and hkxhk for 1:3 or 3:1 ratios (locus heterozygous in both parents; Van Ooijen and Voorrips 2001). Those markers that were faint or not reproducible were not used for mapping.

Resistance gene analogues

In most cases, the amplification of apple genomic DNA with 24 RGA primer pairs (Rajesh et al. 2002) resulted in several amplification products on 2.5% Metaphor® agarose gels. Twelve primer pairs produced polymorphic patterns; however, only 5 primer pairs identified 8 loci that mapped on 5 different linkage groups (Fig. 1). The primer pair XLRR-INV1–XLRR-INV2 produced 3 loci mapping on LG 5 (a 450 bp product, XLRR-INV-y), LG11 (a 600 bp product, XLRR-INV-z), and LG12 (a 350 bp product, XLRR-INV-x). The primer pair Pto-kin3–Pto-kin4 produced 2 loci, a 450 bp fragment mapped on LG2 (Pto-kin-x) and a 600 bp fragment on LG15 (Pto-kin-y). The polymorphic locus amplified by primer pair S2-INV–AS3-INV was mapped on LG11 (a 410 bp product, S2–AS3-INV), and those identified by primer pairs NLRR-INV1–NLRR-INV2 and XLRR For–XLRR Rev, NLRR-INV (a 310 bp product) and XLRR (a 210 bp product), respectively, mapped to LG12 (Fig. 1).

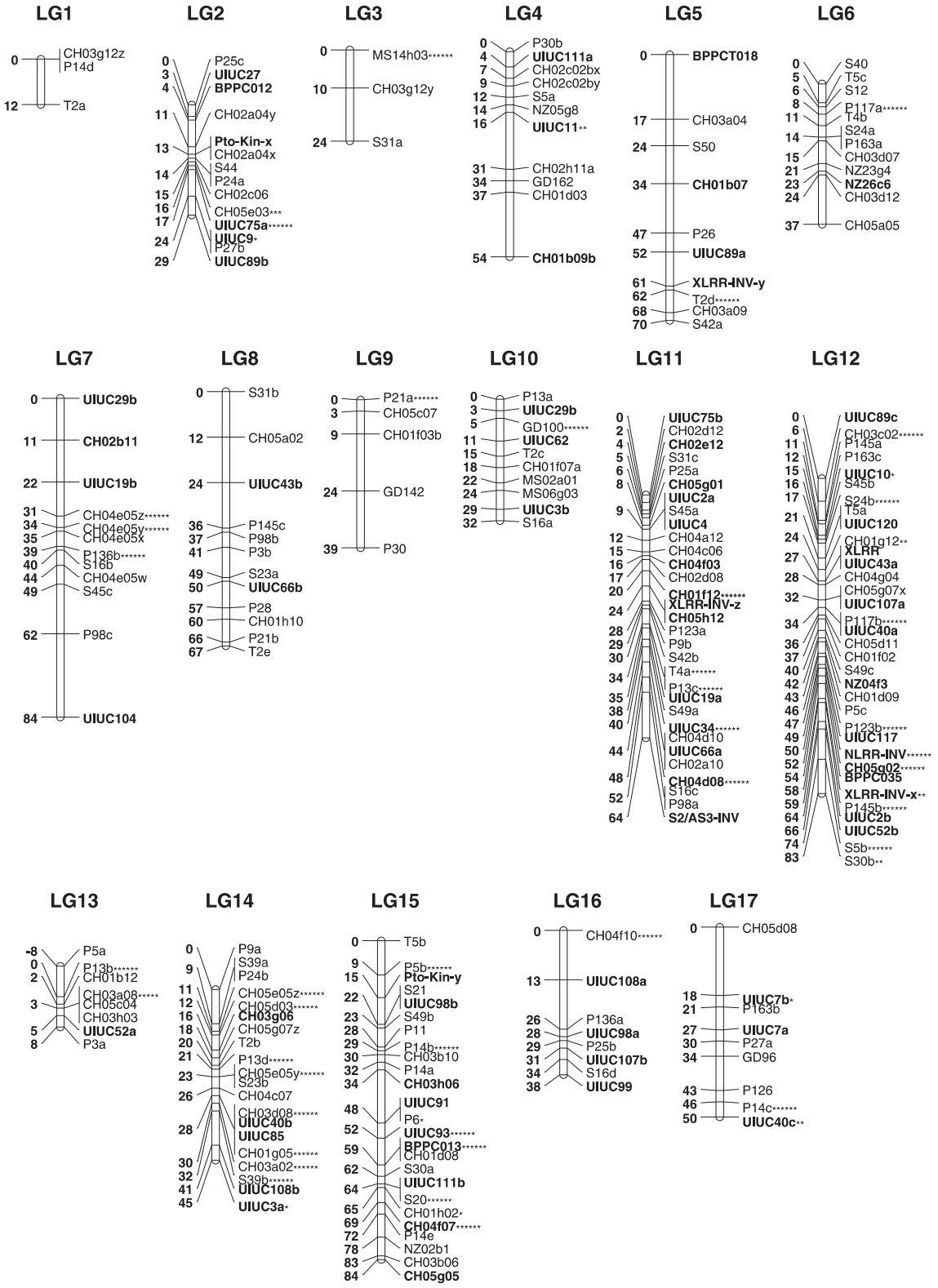
While the number of amplified bands varies among RGA primer pairs, there was no obvious relationship between the group of primers used and the level of polymorphism detected. A significant advantage of RGAs over arbitrary DNA markers is that RGAs represent potentially functional genes. Although not all amplified products are functional disease resistance genes, they all contain conserved sequences representing LRR, kinase, and (or) NBS domains (Chen et al. 1998). Eleven distinct families of RGAs with the characteristic NBS were identified in both wild and cultivated apples using PCR approaches with degenerate primers based on 2 conserved motifs of known NBS-LRR resistance genes (Lee et al. 2003). There is a high probability that amplification products are involved in signal-transduction pathways in plants. Detected polymorphisms in plants may result from mutations due to duplications, frame shifts, and deletions in and (or) between repetitive sequences present within conserved domains. Regardless of the molecular basis for the observed polymorphisms, amplified products may represent candidate genes for disease resistance or other important signal-transduction processes in plants. In brief, RGA markers offer a number of advantages for evaluating resistant germplasm compared with existing molecular markers. The RGA markers serve as both candidate genes for cellular recognition and informative markers for inferring genetic relationships between germplasm accessions.

Mapping of E-STS markers

E-STS-PCR primers were developed from 121 EST contigs and singleton sequences that showed high similarity to genes involved in disease resistance (http://titan.biotec.uiuc.edu/apple/resources.shtml). A total of 111 E-STS primer pairs amplified apple DNA. However, only 28 were found to produce polymorphic profiles between the parents 'Antonovka debnicka' (Q12-4) and 'Summerred' (Table 2). Of these, 15 produced multiple polymorphic loci, thus culminating in a total number of 46 loci segregating in our mapping population. The primer pairs UIUC-40, UIUC-89, and UIUC-111 each produced 3 loci, while 12 of the primer pairs produced 2 loci. The remaining pairs produced single polymorphic loci. Multiple loci produced from a certain E-STS primer pair mapped to different linkage groups in most cases, except for UIUC-7, which produced 2 loci that mapped on LG17. UIUC E-STS loci were distributed on all LGs except for LGs 1, 3, 6, and 9 (Fig. 1).

Previously, Gardiner et al. (2003) established that ESTs often clustered around R genes of different classes within a region of several cM in the apple genome. In this study, RGA and E-STS loci were mapped on all linkage groups, except for LG3 and LG6. It has been reported that the SSR marker CH02d12 on LG11 mapped close to a QTL associated with the scab resistance gene *Vg* (Liebhard et al. 2003*a*) and to a powdery mildew resistance gene, also located on LG11 (Seglias and Gessler 1997). In this study, 5 E-STS and 2 RGA loci were mapped on LG11. E-STS loci UIUC-75b and UIUC-2a flanked CH02d12 at 2 and 7 cM, respectively (Fig. 1). At least 4 scab resistance genes have previously been mapped on LG2, including *Vbj*, *Vh8,* and *Vr/Vh2* (Gygax et al. 2004; Bus et al. 2004, 2005*a*, 2005*b*; Patocchi et al. 2004). Our mapping of a single RGA and 3 E-STS loci to LG2, combined with an earlier report of 3 RGA loci found on LG2 (Baldi et al. 2004), suggest that LG2 is an interesting candidate for further study. Taken together, these results suggested the presence of gene-rich regions on LG2, some of which might be involved in scab resistance.

In this study, PCR amplification results indicate that extensive nucleotide divergence may have occurred among apple cultivars to hinder at least some primer pairs from amplifying genomic DNA, since 10 E-STS primers failed to amplify DNA from 'Antonovka debnicka' (Q12-4), 'Summerred', or any of their progeny. The presence of



introns (discussed below) may also contribute to difficulties in amplification of some sequences. Improper PCR optimization and (or) poor EST sequence quality, from which primers are designed, are additional possible causes of failure of amplification. Some E-STS primers generated multiple PCR products that mapped to different linkage groups. This may be attributed in part to the fact that some E-STS primers were designed using the consensus sequence from a contig consisting of several EST sequences, which in turn could be derived from different members of a gene family that are differentially expressed and (or) spliced in different tissues and are located on various LGs.

A comparison between the observed and expected amplicon sizes for primer pairs amplifying a single DNA fragment revealed some discrepancies. All polymorphic amplicons generated from 10 E-STS primer pairs were larger in size than expected from the EST sequence they were designed from. However, these primer pairs often produced multiple products, some of which were actually smaller than the size calculated from the original EST sequence. Of the 46 loci mapped and based on EST sequences, 16 amplicons were smaller than expected (Table 2). All 19 primer pairs producing a single PCR product amplified a DNA fragment that was smaller than that predicted from the EST sequence, and none, except for UIUC-34, were polymorphic in our population. While amplified products of sizes larger than the corresponding EST sequences might contain introns, amplicons smaller than predicted are harder to explain. One possibility is that such amplicons are derived from nonorthologous gene loci with similar flanking sequences. These amplicons likely contain pseudogenes or paralogs of the targeted genes (Senchina et al. 2003). Nevertheless, the origin of these sequences was most likely nonhomologous loci and similar to those nontarget sequences amplified in wheat and barley when STS primer pairs were transferred across species (Roder et al. 1995). These results suggest that not all products from EST-based primers are amplified from the fragments of functional genes. Therefore, when primers are designed from a consensus sequence representing several aligned EST sequences, it is advisable to compare the obtained amplicon size with its expected DNA sequence length. Also, verification by sequencing, or at least by restriction pattern analysis, would be needed for any amplicon different from the expected size.

EST primer pairs have been reported to produce amplicons from genomic DNA with as many as 4 introns and individual intron sizes ranging from 77 to 611 bp in length (Chee et al. 2004). Furthermore, introns harbour higher nucleotide substitution rates than exons (Chee et al. 2004). Therefore, future efforts to develop STS markers based on either anonymous or known-function genes should target gene regions with introns to increase the probability of detecting polymorphisms (Iwata et al. 2001). Recent data suggest that intron position and size may be conserved among plants and animals in orthologous genes, especially those that share high levels of amino acid homology (Fedorov et al. 2002). Thus, it is desirable to compare EST sequences with genomic DNA sequences from the same or other species when selecting EST sequences for PCR primer development. This strategy may well be feasible, because the number of public, well characterized genes, as well as complete genome sequences of plant species (i.e., *Arabidopsis* and rice), continues to increase. EST-derived SSRs may offer other means for mapping apple ESTs using PCR amplification, as demonstrated in both cotton (Chee et al. 2004) and wheat (Yu et al. 2004). Alternatively, perhaps single-stranded conformational polymorphisms (McCallum et al. 2001; Schneider et al. 2002) or other mutation-scanning techniques may offer more practical means for mapping sequence-specific PCR markers. Clearly, a single-nucleotide polymorphism based assay is preferred (Henikoff and Comai 2003) to permit a high degree of automation (Edwards and Mogg 2001). The low polymorphism rate would be partly compensated for by the higher return in genetic information these EST markers provide. Although the main goal of this study was to document the feasibility of developing known-function gene markers through ESTs from public databases, with a specific emphasis on disease resistance genes, this strategy could be readily adapted to target genes in a specific biosynthetic pathway (Schneider et al. 2002), or those implicated in phenotypic variation (Giroux et al. 2000; Beecher et al. 2002). The use of known-function gene markers, such as E-STSs and RGAs, greatly enhances the use of a genetic map because it facilitates the transition from genetic linkage analysis to a candidate-gene mapping approach for dissecting complex traits.

In conclusion, a framework map for the A×S population using SSR and RAPD markers was developed. RGA loci amplified by primers for conserved sequences of characterized R genes were placed along this map. We also developed E-STSs primers from ESTs for known-function genes potentially involved in disease resistance, and these were also placed on the map. Several RGA and E-STS were mapped in close vicinity on different linkage groups. PCR-based markers targeting known-function genes offered a valuable means of locating genes that were not yet represented on the genetic map. For example, the ESTIMA database contained several listings of stress-related ESTs that could be used to characterize the winter hardiness phenotype present in 'Antonovka'.

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