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ORIGINAL PAPER

The development and mapping of functional markers in *Fragaria* and their transferability and potential for mapping in other genera

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Abstract We have developed 46 primer pairs from exon sequences flanking polymorphic introns of 23 *Fragaria* gene sequences and one *Malus* sequence deposited in the EMBL database. Sequencing of a set of the PCR products amplified with the novel primer pairs in diploid *Fragaria* showed the products to be homologous to the sequences from which the primers were originally designed. By scoring the segregation of the 24 genes in two diploid *Fragaria* progenies FV × FN (*F. vesca* × *F. nubicola* F_2) and 815 × 903BC (*F. vesca* × *F. viridis* BC_1) 29 genetic loci at discrete positions on the seven linkage groups previously characterised could be mapped, bringing to 35 the total number of known function genes mapped in *Fragaria*. Twenty primer pairs, representing 14 genes, amplified a product of the expected size in both *Malus* and *Prunus*. To demonstrate the applicability of these gene-specific loci to comparative mapping in Rosaceae, five markers that displayed clear polymorphism between the parents of a *Malus* and a *Prunus* mapping population were selected. The markers were then scored and mapped in at least one of the two additional progenies.

Introduction

Strawberry (*Fragaria*) belongs to the Rosaceae, one of the most important families of plants horticulturally which also includes fruit crops such as peach, cherry, apple and raspberry, as well as ornamentals such as rose. Strawberry (*Fragaria*), raspberry (*Rubus*) and rose (*Rosa*) belong to the subfamily Rosoideae, diploid forms of which have 14 chromosomes ($2n = 2x = 14$). Peach, cherry and other stone fruits (*Prunus*) belong to the Prunoideae with 16 chromosomes ($2n = 2x = 16$), and apple (*Malus*) and other pome fruit belong to the Maloideae with 34 chromosomes ($2n = 2x = 34$) and are of probable allotetraploid origin. Because of their economic importance, all these crops are the subject of breeding programmes, which are underpinned by structural and functional genomics projects.

In the genus *Fragaria*, an F_2 genetic linkage map has been developed from an interspecific cross between two diploid species *F. vesca* 815 × *F. nubicola* 601 (FV × FN) using 182 transferable markers (Sargent et al. 2006), the majority of which are microsatellites (SSRs). Likewise, well saturated linkage maps of DNA markers have become available for a large number of other rosaceous genera, including *Malus*, initially employing RFLPs (Maliepaard et al. 1998) and subsequently SSRs (Liebhard et al. 2003), and *Prunus*, again at first with RFLPs (Joobeur et al. 1998) and later with SSRs (Aranzana et al. 2003).

RFLPs are relatively laborious to use but have the potential to be transferable between genera; however to date, few attempts have been made to use them for comparative mapping studies in Rosaceae (Dirlewanger et al. 2004) and no RFLPs have been developed for mapping in *Fragaria*. The SSRs employed in the maps of rosaceous genera are highly polymorphic, codominant, easy to score by PCR-based methods and transferable between mapping progenies within and between species (Dirlewanger et al. 2002; Hadonou

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et al. 2004); however, they are generally not transferable between genera. In addition, whilst SSRs are excellent markers for providing a linkage framework, they are primarily developed from non-coding regions of the genome and are generally not known to be tightly linked to genes of known function or to traits of economic importance. Although SSRs have been developed from EST sequences in various rosaceous genera (EST-SSRs; Jung et al. 2004; Bassil et al. 2006; Keniry et al. 2006; Sargent et al. 2006), they are present in less than 10% of rosaceous ESTs screened. Moreover dinucleotide repeats, that are generally the most polymorphic repeat type, are predominantly found in the untranslated regions (UTRs) of genes in Rosaceae (Jung et al. 2004; Newcomb et al. 2006) and thus the sequences flanking them are often not sufficiently well conserved for transferable primers to be designed that would permit comparisons between genera.

In addition to EST-SSRs, there has been recent interest in the mapping of candidate genes in certain rosaceous genera. For example, in *Fragaria* candidate genes for fruit colour from the anthocyanin biosynthesis pathway (Deng and Davis 2001) have been successfully mapped using PCR-based approaches, as have genes for sugar and organic acid content (Etienne et al. 2002) and flowering time (Silva et al. 2005) in *Prunus*. In the studies of Deng and Davis (2001) and Silva et al. (2005), primer pairs were designed where possible from coding regions of genes flanking introns, avoiding UTRs. This yielded polymorphic, informative functional markers that were directly indicative of genes of interest and that were highly transferable between species within genera, as primer pairs were designed from DNA sequence under functional constraint. This approach has also proved successful in studies of other families, such as the Ericaceae (Wei et al. 2005), using EST sequences aligned with *Arabidopsis* gene homologues to predict intron positions.

In this investigation, we set out primarily to increase the number of functional markers genetically mapped in the *Fragaria* genome, using sequence data from *Fragaria* genes and one *Malus* gene deposited in the EMBL database. We designed primer pairs where possible flanking intron sequences from mRNA and genomic DNA sequences, and exploited intron length polymorphisms to map these markers in two diploid *Fragaria* mapping populations. In addition, we tested the potential transferability of the novel markers to species of accessions *Prunus* (Prunoideae) and *Malus* (Maloideae). Many of the primer pairs designed were shown to be highly transferable, amplifying products of the size expected for orthologous genes in the other genera. To demonstrate the utility of such gene loci for comparative mapping, five loci that displayed PCR product length polymorphism between the parents of a *Malus* and a *Prunus* mapping population were selected for further mapping in those genera. Where necessary, primers were redesigned to allow PCR product length polymorphisms to be visualised via electrophoresis. Where heterozygous products were revealed in the parents, the mapping progenies were analysed and the genes were mapped, demonstrating the utility of such markers for comparative mapping in Rosaceae.

Materials and methods

Plant material and DNA extraction

Two diploid *Fragaria* progenies were used to score the segregation of novel gene-specific markers: 94 seedlings of the F_2 reference mapping population FV × FN (Sargent et al. 2004, 2006) derived from an F_1 cross of *F. vesca* 815 × *F. nubicola* 601 and 93 seedlings of the 815 × 903BC BC_1 mapping population (Nier et al. 2006) derived from an F_1 cross of *F. vesca* 815 × *F. viridis* 903. In addition, amplification of novel loci was assessed in two accessions of *Malus* and *Prunus* from which F_1 mapping progenies have been raised at East Malling Research (EMR): *Malus pumila* 'Fiesta' and *Malus* 'Totem' (Fernandez-Fernandez et al. unpublished data); *Prunus avium* 'Napoleon' and *Prunus nipponica* 'F1292' (Bošković and Tobutt 1998). Five loci that were polymorphic in either *Malus* or *Prunus* or both, were also scored in the apple and cherry mapping progenies at EMR: 94 seedlings from the cherry cross 'Napoleon' × 'F1292' (PA × PN) and 88 seedlings from the apple cross 'Fiesta' × 'Totem' (F × T).

DNA was extracted from young leaf tissue using the DNeasy plant miniprep kit (Qiagen) according to the manufacturer's protocol, and diluted to 1–10 ng μl^{-1} for use in PCR.

Marker development

Thirteen full-length genes and 10 mRNA sequences derived from *Fragaria* and one mRNA sequence derived from *Malus* (*Phosphoglyceromutase; PGLM*) were downloaded from the EMBL database (Table 1). The *Fragaria* sequences included two, *Anthocyanidin synthase* and *Dihydroflavonol-4-reductase* (*ANS* and *DFR*), which had been previously mapped in *Fragaria* (Deng and Davis 2001), but which could not be amplified in either FV × FN or 815 × 903BC using previously published primer pairs. Where full-length genomic DNA gene sequences were available, primers

Table 1 Primer sequences for 45 primer pairs designed from 24 gene sequences downloaded from EMBL along with the expected product sizes, EMBL accession numbers of the gene sequences from which they were derived, the nature of the sequences from which the primer pairs were designed (G. flanking introns from genomic DNA; R. from mRNA sequence) and gene function

Gene abbreviation	Accession number	Source	Gene function	Primer name	Forward primer 5' to 3'	Reverse primer 5' to 3'	Expected size
ABP	X91839	G	Auxin-binding protein	EMFxaABP1A	tatctgaggcttccaaatgc	cactgatattcctcaccacagg	300
ACO	AJ851828	G	1-Aminocyclopropane-1-Carboxylate oxidase	EMFxaACO1A	agcaccttctacctcaaacacc	ctcacagaacaagtccaagagc	140
ACO	AJ851828	G	1-Aminocyclopropane-1-Carboxylate oxidase	EMFxaACO1B	gccactccattgttatcaacc	tgctccacactcttgtacttcc	72
AKR	AF039182	R	Aldo-keto reductase superfamily member	EMFxaAKR1	ggttccttcagtaaccctcagc	ctatcgtcaccccatttagtcc	678
ANS	AY695818	G	Anthocyanidin synthase	EMFxaANS	aaggagaagtatgccaatgacc	ctcccttcttctaatcccaagc	385
APX	AF158654	G	Cytosolic ascorbate peroxidase	EMFxaAPX1A	gccaagaggaagctcagagg	gacctcaacagcaacaactcc	498
APX	AF158654	G	Cytosolic ascorbate peroxidase	EMFxaAPX1B	ctggagttgttgctgttgagg	ctttccagcatcaggaagacg	685
APX	AF158654	G	Cytosolic ascorbate peroxidase	EMFxaAPX1C	gtcttcctgatgctggaaagg	agtccagggtccttcaaacc	367
APX	AF158654	G	Cytosolic ascorbate peroxidase	EMFxaAPX1D	gaacggtctgggtttgaagg	agacagggtctgacagaagagc	249
APX	AF158654	G	Cytosolic ascorbate peroxidase	EMFxaAPX1E	gcatatctggtctcgattctgc	cccatagaaacaacacctactgc	334
ARP	X52429	R	Auxin-repressed mRNA	EMFxaARP1	ggttctgctagacaagctttgg	catagacagtgggagagtttgg	290
BG	AY170375	G	Beta-1,3-glucanase	EMFxaBG2	ctgctccttgttctgtttatgc	aaggtaaattgttgccatttcg	488
CAD	AF320110	G	Cinnamyl alcohol dehydrogenase	EMFxaCAD1A	agcaagaacaccccaataagg	acgtctttctctccggtttcc	244
CAD	AF320110	G	Cinnamyl alcohol dehydrogenase	EMFxaCAD1B	gaatgaatggggtttctctacc	gctccctacttctgtaacttcacc	164
CAD	AF320110	G	Cinnamyl alcohol dehydrogenase	EMFxaCAD1C	gaggaggaagctcttaaacacc	gcagaaactgtgtcaatgatcc	209
CAD	AF320110	G	Cinnamyl alcohol dehydrogenase	EMFxaCAD1D	cagagaagcctcttgaacttcc	tctgctgttatgttgtgcttgg	223
CEL-1	AF051346	R/G[b]	Cellulase	EMFnCEL1	tacatcatggttgtgtctgtcc	tccagaaagcctgattctaagg	525
CEL-2	AF054615	R/G[b]	Cellulase	EMFvCEL2	ggtgttcagaccctgttgc	atgcttaccagctttaccttgc	220
DFR	AY575057	G	Dihydroflavonol 4-reductase	FxaDFR1A	ggctcgtcatgagactcctc	tgtggtaactccagcagatgtc	191
DFR	AY575057	G	Dihydroflavonol 4-reductase	FxaDFR1B	caccggagtgtttcatgtcg	aacctccgaactgtctttgc	344
DFR	AY575057	G	Dihydroflavonol 4-reductase	FxaDFR1C	gccggaaagtaaagatgactgg	cttgctcggctagagttttgg	170
DFR	AY575057	G	Dihydroflavonol 4-reductase	FxaDFR1D	cttgtaatcggtccttttctcg	ctaggtgaacgtattggcattg	274
EKO	AY462247	R/G[b]	Ent-kaurene oxidase	EMFnEKO1A	agaagaaacgaattgcttcagg	ttggtccattatcagtgtcc	197
EKO	AY462247	R/G[b]	Ent-kaurene oxidase	EMFnEKO1B	cacatgaagacacccaactagg	aaatctctctggcttccattcc	221
EXP	AF159563	R/G[b]	Expansin	EMFviEXP1A	ctttctatggaggtggtgatgc	ctgcagtgttggttccatacc	243
EXP	AF159563	R/G[b]	Expansin	EMFviEXP1B	ctcagtaccgtgctggtatcg	aaccaagttgaagtaggagtgtcc	236
GAST	AF039183	R	GAST-like gene product	EMFxaGAST1	gacaacatgcttcacttcttgc	ttaaggacacttgcgtttgc	212
LEAFY	AF487165	G	Leafy protein	EMFnLEAFY1	ggtgaaaaatgtcccaccaag	tttttcgcatatctaaacacctc	379
LOX	AJ578035	R/G[b]	Lipoxygenase	EMFnLOX1A	acaagggtccagaatatgaacg	aaacttcacgtggccaaacc	383
LOX	AJ578035	R/G[b]	Lipoxygenase	EMFnLOX1B	aagttaccaaatgggcctacg	actcttcatctgtcctccatgc	242
LOX	AJ578035	R/G[b]	Lipoxygenase	EMFnLOX1C	ggaggacagatgaagagtttgc	ggaggacagatgaagagtttgc	202
LOX	AJ578035	R/G[b]	Lipoxygenase	EMFnLOX1D	aacatgaatggcctcagtgc	ggtggcataggtctttgtcg	247
MET	U81041	R	Metallothionein-like protein	EMFxaMET1	gcctctgagactcttgtcatgg	caggtgcagtagacacacttgc	119
MSR	AJ297967	G	Methionine sulfoxide reductase	EMFxaMSR1	cccatattcggatcttctttcc	tactcctctgccctgtaaaacc	354
MYB	AF401220	R	Transcription factor	EMFxaMYB1	aagacggagacgaccaaagg	aacttgaggatcagccattcc	537
PDC	AF333772	G	Pyruvate decarboxylase	EMFxaPDC1A	ggactaaccggattcttcacc	atcaatcaactcatgtgcatcc	382
PDC	AF333772	G	Pyruvate decarboxylase	EMFxaPDC1B	agcctgtgtatatcagcattgg	gcctctagtccccacttattgc	226
PDC	AF333772	G	Pyruvate decarboxylase	EMFxaPDC1C	agctgaaaccgctgtgattgc	caagagttgctccaactgacc	223
PDC	AF333772	G	Pyruvate decarboxylase	EMFxaPDC1D	ctcaggctgtacctgagaagc	catcgtggacacatcttgagc	246

Table 1 continued

Gene abbreviation	Accession number	Source	Gene function	Primer name	Forward primer 5' to 3'	Reverse primer 5' to 3'	Expected size
PDC	AF333772	G	Pyruvate decarboxylase	EMFxaPDC1E	actacactggactggttgatgc	gtccatttgcagtctctattgc	209
PES	AY324809	G	Pectinesterase	EMFviPES1	gatggttggaccacatttcg	tgaatcctctgccactaacagc	579
PGLM	AJ004915[a]	G	Phosphoglyceromutase	EMFnPGLM1A	gtgatggtggagttcattctcg	ggtcctcttcaagtgtttcagc	262
PGLM	AJ004915[a]	G	Phosphoglyceromutase	EMFnPGLM1B	aagaggaccttgcaaagttacg	gtgtcttgattgcttcaacagc	815
QR	AY158836	G	Quinone oxidoreductase	EMFxaQR1A	cttcccttggctattgaaactg	cacccaaggttctcaagaaatc	304
ZIP	AY805422	G	Zinc transporter protein	EMFxaZIP1A	cggcacagagttatatcaaagg	gcaccacaattcccaactcc	592
ZIP	AY805422	G	Zinc transporter protein	EMFxaZIP1B	ggacttggtggttgcatagc	tgtaagcgagaagaacaacacc	235

[a] Sequence derived from *Malus* × *domestica*. All other sequences derived from *Fragaria* species

[b] PCR products cloned and sequenced to reveal sites of introns. Primers subsequently designed from exon sequences flanking introns

were designed within the exon regions flanking introns; where only mRNA was available, primers were designed within the exon region (avoiding 3′ and 5′ UTRs) to amplify products expected, in the absence of introns, to be between 450 and 650 bp in length. All primer pairs were designed using the software PRIMER 3 (Rozen and Skaletsky 1998). The criteria for design were a T_m of 55–65°C (optimum 60°C), a primer length of 20–24 bp (optimum 22 bp) and a 2 bp GC-clamp at the 5′ end. Primers were named following the nomenclature of Sargent et al. (2003), EM (East Malling) followed by characters denoting the species origin of the sequence from which primer pairs were designed (i.e. Fxa *Fragaria* × *ananassa*) followed by a 3–4 character abbreviation of the gene name (i.e. PGLM), followed by a letter to indicate the intron the primers flanked (A, B, C, etc.).

PCR conditions, product visualization and scoring of segregation in *Fragaria*

All PCRs were performed following the reaction conditions and touchdown protocol described by Sargent et al. (2003), initially in the parents of the FV × FN and 815 × 903BC populations. PCR products generated were separated by electrophoresis through a 300 ml 1.2% TAE agarose gel (110 V for 2.5 h) which was stained with ethidium bromide for 30 min to visualise the products.

For each gene, one primer pair revealing polymorphic genotypes in the parents of one of the two *Fragaria* mapping populations was used to amplify DNA from that progeny. Depending on expected product sizes, segregation of alleles in the progeny was visualised in most cases by electrophoresis through a 1.2% agarose gel (110 V for 2.5 h) and staining in ethidium bromide for 30 min or by electrophoresis through an EL800 Spreadex gel (75 V for 1 h 40 min) and staining with SYBR gold (Invitrogen, USA) for 30 min. Markers for which polymorphism could not be detected after gel electrophoresis were labelled on the forward primer with 6-FAM or NED fluorescent dyes (Applied Biosystems, CA, USA), and the products fractionated by capillary electrophoresis using a 3100 genetic analyser (Applied Biosystems, CA, USA). Data generated were collected and analysed using the GENESCAN and GENOTYPER (Applied Biosystems, CA, USA) software.

Amplification of PCR products in other genera

Primer pairs amplifying PCR products in *Fragaria* were used to amplify DNA from the parents of the East

Malling *Malus* and *Prunus* mapping populations described above. Whether the products were approximately the size expected for homologous gene products was determined by electrophoresis through a 300 ml 1.2% TAE agarose gel (110 V for 2.5 h) and comparison with PCR products generated from *Fragaria* with the same primers.

Cloning and sequencing

The products amplified initially with the primers designed from mRNA sequences contained polymorphic intron sequences. However, because of the large size of the PCR products amplified, segregation of the polymorphism within the diploid *Fragaria* mapping populations could not be scored effectively after electrophoresis; so the products were sequenced to allow new primers to be designed to amplify smaller products. In these instances, the products were cloned into pCR4 vector and transformed into One Shot Chemically Competent *E. coli* cells using the TA TOPO PCR Cloning Kit (Invitrogen) following the manufacturer's protocol. Transformants with inserts of the expected size were identified by electrophoresis through a 1.2% agarose gel (110 V 2.5 h) following PCR with M13 universal primers. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen, CA, USA) and DNA sequence was obtained by sequencing from the M13 forward and reverse primers. Sequences obtained were aligned with the mRNA sequences retrieved from EMBL using MegAlign (DNAStar) and intron sites were identified. New primer pairs flanking introns were then designed within the exon sequences to amplify smaller PCR products and polymorphisms were detected and scored as described above. Sections from the five genes that displayed polymorphisms between the parents of either the *Malus* (F × T) or *Prunus* (PA × PN) mapping populations were cloned and sequenced and where necessary, novel primers flanking the intron polymorphisms within these genes were designed.

Data analysis and map construction

Chi-squared tests of goodness-of-fit to expected segregation ratios of 1:2:1, 3:1 (in FV × FN) and 1:1 (in 815 × 903BC) were carried out for all segregating markers using JOINMAP 3.0 (Van Ooijen and Voorrips 2001). Linkage analysis was then performed with the data of Sargent et al. (2006) and Nier et al. (2006) included, and the novel markers were assimilated into groups for mapping with JoinMap 3.0 with the application of the Kosambi mapping function. Marker positions were determined using a minimum LOD score threshold of 3.0, a recombination fraction threshold of 0.35, ripple value of 1.0, jump threshold of 3.0 and a triplet threshold of 5.0. The data from the two maps were integrated using common markers and the pairwise recombination frequencies for the markers were combined using the '*Combine groups for map integration*' function of JoinMap 3.0. Thus, a single integrated map was produced with all the novel gene loci, which are denoted by a two to four character abbreviation, followed by a number if multiple loci were detected. The maps presented were constructed using MapChart for Windows (Voorrips 2002). Segregation of the five genes selected were scored, where possible, in both the *Prunus* PA × PN and *Malus* F × T mapping progenies, following the procedures described above for *Fragaria* (Clarke et al., unpublished data; Fernandez-Fernandez et al., unpublished data); linkage group determination and marker order on the linkage groups containing this marker were determined using JoinMap 3.0 using the parameters described above.

Results

Primer design and product amplification in *Fragaria*

Thirteen full-length genomic DNA sequences and 11 mRNA sequences representing 24 genes were retrieved from EMBL and primers were designed to flank all introns present in genomic DNA sequences, or to amplify a product of up to 650 bp from mRNA sequences (Table 1). Products generated with six of the primer pairs designed from mRNA sequences were too large (generally over 1,000 bp) to permit detection of polymorphisms via electrophoresis and these were cloned and sequenced to reveal the sites of introns so that further primers could be designed from exons flanking the intron sequences identified. All products cloned and sequenced revealed products homologous to the mRNA sequences from which the primers were initially designed. Two of these sequences (amplified with primer pairs EMFnCEL1 and EMFnCEL2) revealed a single intron, three (amplified with primer pairs EMFnEKO1, EMFviEXP2 and EMFnPGLM1) revealed two introns, whilst the sequence amplified with the EMFnLOX1 primer pair revealed four intron sites. In total, 46 primer pairs were designed to amplify introns within 24 genes having many diverse functions (Table 1). All 46 primer pairs amplified discrete PCR products from the parents of the diploid *Fragaria* mapping populations.

Marker polymorphism and mapping in *Fragaria*

All 24 genes for which primers were designed were polymorphic between the parents of at least one of the two diploid *Fragaria* mapping populations with at least one of the primer pairs designed. Three primer pairs, EMFxaCAD1A and EMFxaBG2 (in FV × FN) and EMFviEXP1A (in 815 × 903BC) amplified more than one locus, with EMFxaBG2 amplifying two loci and EMFxaCAD1A and EMFviEXP1A amplifying three loci each. Table 2 lists the primer pairs used to score segregation at each of the 29 loci in *Fragaria*, the mapping population in which they were mapped and the method of detection, along with the segregation data for each locus and the χ^2 values for goodness-of-fit to the expected 1:2:1, 3:1 (FV × FN) or 1:1 (815 × 903BC) Mendelian segregation ratios.

In total, 17 loci were mapped in FV × FN and the remaining 12, which were monomorphic in FV × FN, were mapped in 815 × 903BC. The 29 loci mapped in the two diploid *Fragaria* mapping populations were evenly distributed throughout the seven linkage groups. Six markers mapped to each of linkage groups (LG) III and VI, four markers mapped to each of LGs II, IV and V, three mapped to LGI and two to LGVII. Figure 1 shows the combined genetic map of FV × FN and 815 × 903BC with the locations of the novel loci.

Transferability to *Malus* and *Prunus*

Twenty-six of the 46 primer pairs designed, representing 18 genes, amplified a PCR product or products of approximately the size expected for a homologous gene in at least one of the two rosaceous genera screened in addition to *Fragaria* (Table 3). In total, 20 primer pairs, representing 14 of the 24 genes, amplified a product of the expected size in all three genera, indicating primer binding sites were conserved across all three rosaceous subfamilies screened.

Amplification, detection of polymorphism and mapping of markers in *Fragaria*, *Malus* and *Prunus*

Primer pairs for the five genes listed in Table 4 amplified discrete, polymorphic PCR products between the parents of the genus for which they were designed. Primer pairs designed for *ANS*, *DFR* and *PGLM* all amplified polymorphic products. However, *ANS* was not heterozygous in either parent of the F_1 PA × PN population and therefore did not segregate. In *Malus*, the primer pairs designed for *ANS* amplified two heterozygous loci that segregated independently in the F × T population. Primer pairs designed for *PGLM* amplified two discrete loci in F × T; however, only one locus segregated and thus the other could not be mapped. Primer pairs for *ACO* and *EKO* were heterozygous and segregated only in the PA × PN population and were therefore not mapped in the F × T population. All segregating loci were scored in the *Malus* and *Prunus* mapping populations and located on the PA × PN and F × T linkage maps. The name of the known-function marker, the population in which it was mapped, segregation data for each locus and the χ^2 values for goodness-of-fit to the expected segregation ratio, along with the linkage group to which it was assigned and the marker to which it was most closely linked are given in Table 5.

Discussion

We have presented 46 novel primer pairs that amplify 24 genes of known function in *Fragaria* designed from mRNA and genomic DNA sequences retrieved from the EMBL database. Through sequencing of the PCR products designed from a number of mRNA sequences, we have shown that the PCR products amplified in *Fragaria* are homologous to the sequences from which they were designed. We have located 29 loci, representing all 24 of these genes, the majority of which are single-copy, on the diploid *Fragaria* genome using the previously published maps of Sargent et al. (2006) and Nier et al. (2006).

Furthermore, we have shown that primer pairs designed for most of the loci mapped amplify similar products in two other genera and subfamilies in Rosaceae, *Malus* and *Prunus*. Five loci that displayed clear polymorphism between the parents of either the *Malus* or *Prunus* mapping population were then scored in those progenies to demonstrate the potential applicability of these *Fragaria* gene-specific markers to comparative mapping between rosaceous subfamilies.

Mapping in *Fragaria*

The 29 loci mapped in this investigation were evenly distributed throughout the seven linkage groups associated with the diploid *Fragaria* genome and no marker clustering, often associated with SSRs (Sargent et al. 2004; 2006; Blair et al. 2003; Li et al. 2003) was observed. The location of these novel loci on the diploid *Fragaria* maps has greatly increased the number of known function genes mapped in the genus to 35. The FV × FN reference map of Sargent et al. (2006) and the previous map of Deng and Davis (2001) each contained just six gene-specific STS markers, of which four were common to both maps.

Table 2 Twenty-nine loci mapped in one of two *Fragaria* mapping populations with the primer pairs used to score segregation and the method of detection, along with the segregation data for each locus and the χ^2 values for goodness-of-fit to the expected 1:2:1, 3:1 (FV × FN) or 1:1 (815 × 903BC) Mendelian segregation ratios

Locus	Primer pair used	Population[a]	Classes	Segregation	χ^2	*df*	Signif.	Visualisation
ABP	EMFxaABP1A	FV × FN	aa:ab:bb	7:42:45	31.8	2	***	Agarose gel electrophoresis
ACO	EMFxaACO1B	FV × FN	aa:ab:bb	23:50:21	0.5	2	–	Fluorescently labelled 6-FAM or NED
AKR	EMFxaAKR1	815 × 903BC	aa:ab	69:24	21.8	1	***	Agarose gel electrophoresis
ANS	EMFxaANS	FV × FN	aa:ab:bb	20:42:32	4.1	2	–	Agarose gel electrophoresis
APX	EMFxaAPX1E	FV × FN	aa:ab:bb	21:51:17	2.2	2	–	Fluorescently labelled 6-FAM or NED
ARP	EMFxaARP1	815 × 903BC	aa:ab	55:38	3.1	1	*	Agarose gel electrophoresis
BG-1	EMFxaBG21	FV × FN	aa:ab:bb	16:51:27	3.3	2	–	Fluorescently labelled 6-FAM or NED
BG-2	EMFxaBG22	FV × FN	aa:b_	16:78	3.2	1	*	Fluorescently labelled 6-FAM or NED
CAD-1	EMFxaCAD1A1	FV × FN	aa:b_	17:76	2.2	1	–	Fluorescently labelled 6-FAM or NED
CAD-2	EMFxaCAD1A2	FV × FN	a_:bb	33:61	5.1	1	**	Fluorescently labelled 6-FAM or NED
CAD-3	EMFxaCAD1A3	FV × FN	aa:b_	23:70	0	1	–	Fluorescently labelled 6-FAM or NED
CEL-1	EMFnCEL1	FV × FN	aa:ab:bb	19:54:21	2.2	2	–	Agarose gel electrophoresis
CEL-2	EMFvCEL2	FV × FN	aa:ab:bb	19:40:35	7.5	2	**	EL800 spreadex gel electrophoresis
DFR	FxaDFR1B	FV × FN	aa:ab:bb	21:51:22	0.7	2	–	Agarose gel electrophoresis
EKO	EMFnEKO1A	FV × FN	aa:ab:bb	6:41:46	35.7	2	***	EL800 spreadex gel electrophoresis
EXP-1	EMFviEXP1A1	815 × 903BC	aa:ab	77:16	40	1	***	Fluorescently labelled 6-FAM or NED
EXP-2	EMFviEXP1A2	815 × 903BC	aa:ab	53:40	1.8	1	–	Fluorescently labelled 6-FAM or NED
EXP-3	EMFviEXP1A3	815 × 903BC	aa:ab	80:13	48.3	1	***	Fluorescently labelled 6-FAM or NED
GAST	EMFxaGAST1	815 × 903BC	aa:ab	54:39	2.4	1	–	Agarose gel electrophoresis
LEAFY	EMFnLEAFY1	815 × 903BC	aa:ab	55:38	3.1	1	*	Agarose gel electrophoresis
LOX	EMFnLOX1A	FV × FN	aa:ab:bb	12:44:34	10.8	2	***	Fluorescently labelled 6-FAM or NED
MET	EMFxaMET1	815 × 903BC	aa:ab	45:48	0.1	1	–	Agarose gel electrophoresis
MSR	EMFxaMSR1	815 × 903BC	aa:ab	53:40	1.8	1	–	Agarose gel electrophoresis
MYB	EMFxaMYB1	815 × 903BC	aa:ab	58:35	5.7	1	**	Agarose gel electrophoresis
PDC	EMFxaPDC1A	815 × 903BC	aa:ab	48:45	0.1	1	–	Agarose gel electrophoresis
PES	EMFviPES1	815 × 903BC	aa:ab	71:21	27.2	1	***	Agarose gel electrophoresis
PGLM	EMFnPGLM1B	FV × FN	aa:ab:bb	31:50:5	18	2	***	Fluorescently labelled 6-FAM or NED
QR	EMFxaQR1A	FV × FN	aa:ab:bb	15:53:25	4	2	–	Fluorescently labelled 6-FAM or NED
ZIP	EMFxaZIP1A	FV × FN	aa:ab:bb	21:54:19	2.2	2	–	Fluorescently labelled 6-FAM or NED

Segregation ratios deviating significantly from the expected ratios ($P \leq 0.05, 0.01, 0.001$) are indicated with *, ** and ***, respectively

[a] Denotes the diploid *Fragaria* population in which the segregation data was generated

df degrees of freedom

In this investigation, we developed new primer pairs for two of the genes, *Anthocyanidin synthase* and *Dihydroflavonol-4-reductase* (*ANS* and *DFR*), first mapped by Deng and Davis (2001) but which could not be amplified in the FV × FN or 815 × 903BC progenies using the primer pairs that they reported. The novel primer pairs presented herein (EMFxaANS and EMFxaDFR1B) amplified polymorphic products in both FV × FN and 815 × 903BC mapping progenies and the linkage groups (LGV and LGII, respectively) and map positions of these genes on our maps is consistent with the findings of Deng and Davis (2001).

Whilst marker order was conserved between the FV × FN *Fragaria* reference map of Sargent et al. (2006) and the 815 × 903BC map of Nier et al. (2006), genetic distances were inconsistent between the two

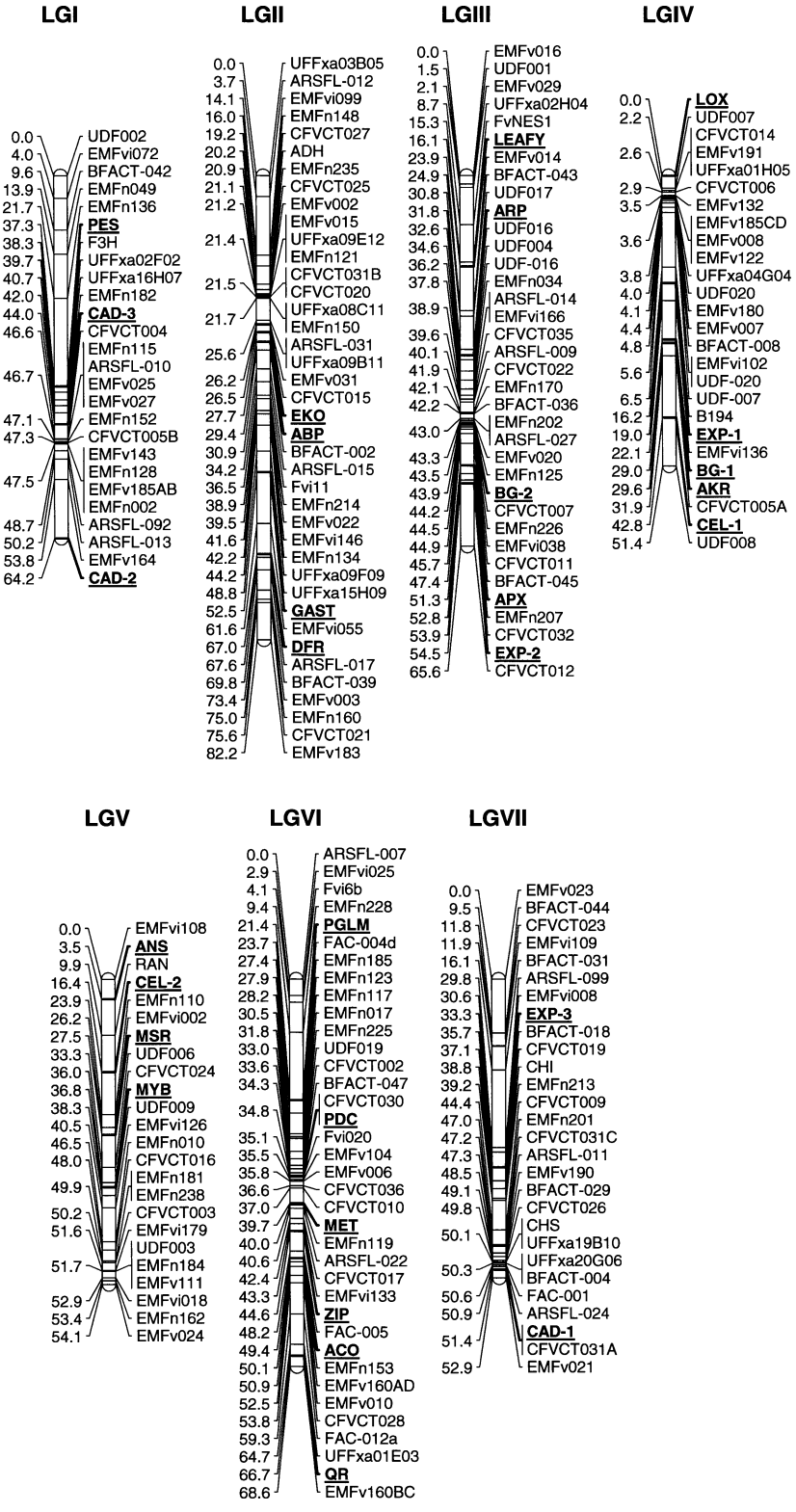

Fig. 1 An integrated genetic map of the diploid *Fragaria* FV × FN and 815 × 903BC progenies showing (underlined in bold) locations of the 29 novel known function loci mapped using primer pairs developed from 24 gene sequences retrieved from EMBL

maps because of the high degree of segregation distortion and the backcross nature of the 815 × 903BC progeny (Nier et al. 2006). Therefore, there are minor differences between the genetic distances of the integrated map presented here and of the FV × FN reference map. The map positions of the novel markers were also calculated on the two *Fragaria* maps independently and these data were used to update the FV × FN and

Table 3 Twenty-six primer pairs, representing 18 genes, from which a PCR product was amplified in at least one of the two rosaceous genera screened in addition to *Fragaria*

Primer name	Amplification	
	Prunus	*Malus*
EMFxaACO1A	Y	x
EMFxaACO1B	Y	Y
EMFxaANS	Y	Y
EMFxaAPX1A	Y	Y
EMFxaAPX1B	Y	Y
EMFxaAPX1C	Y	Y
EMFxaARP1	Y	Y
EMFxaCAD1A	Y	Y
EMFxaCAD1B	Y	x
EMFxaCAD1D	Y	Y
EMFvCEL2	Y	Y
FxaDFR1B	Y	x
EMFnEKO1B	Y	Y
EMFviEXP1A	Y	Y
EMFviEXP1B	Y	Y
EMFnLOX1A	Y	Y
EMFxaMET1	Y	Y
EMFxaMSR1	x	Y
EMFxaMYB1	x	Y
EMFxaPDC1C	Y	Y
EMFxaPDC1E	Y	Y
EMFviPES1	Y	Y
EMFnPGLM1A	Y	Y
EMFnPGLM1B	Y	Y
EMFxaQR1A	x	Y
EMFxaZIP1A	Y	Y

Y—amplification; x—no amplification

815 × 903BC maps on the Genome Database for Rosaceae (GDR) (http://www.genome.clemson.edu/gdr/cmap/).

Amplification in other Rosaceae

For macrosynteny studies, orthologous markers must be mapped in segregating populations to compare genome arrangement between related plant species or genera. The published genetic maps of rosaceous species have employed a number of marker systems, including AFLPs and RFLPs (Joobeur et al. 1998; Maliepaard et al. 1998), but most of the recent rosaceous reference maps have been constructed using microsatellites (SSRs) (Sargent et al. 2006; Aranzana et al. 2003; Liebhard et al. 2003). In *Fragaria* (Hadonou et al. 2004) and *Prunus* (Dirlewanger et al. 2002), the transferability of microsatellites between species within a genus has been well demonstrated, and there are reports of high levels of microsatellite transferability between closely related genera such as *Malus* and *Pyrus* (Yamamoto et al. 2002).

However, transferability of microsatellite loci between more distantly related genera seems to be more problematic. Dirlewanger et al. (2002) reported amplifying microsatellites originally designed from peach (*Prunus persica*) in other members of the Rosaceae (including *Fragaria*), as well as in *Castanea*, *Juglans* and *Vitis*. However, Lewers et al. (2005) reported very low rates of transference of SSR loci between genera in Rosoideae, and Decroocq et al. (2003) reported that apricot SSRs could be transferred only to very closely related species. These latter reports are consistent with our own investigations (Sargent et al., unpublished data) which have shown that, whilst PCR products can sometimes be amplified in *Fragaria* using primer pairs designed from *Malus*, *Prunus* and *Rosa*, in all but a very few cases the banding patterns are complex or monomorphic and products are not of the expected size. Even the SSRs reported by Sargent et al. (2006) which were developed from ESTs (EST-SSRs) were rarely informative in other genera (Sargent et al., unpublished data), most probably indicative of their position, primarily in the 5′ and 3′ UTRs of the genes from which they were retrieved. Thus, alternative markers, such as sequence tagged site (STS) markers, would be more appropriate for the purposes of comparative mapping using PCR-based approaches.

In this investigation, primer pairs were designed from the coding regions of genes of known function which generally display a higher degree of sequence conservation between genera than the non-coding sequences flanking microsatellites and they therefore

Table 4 Genus-specific primer pairs flanking polymorphisms used to map five gene loci in *Prunus* and *Malus* mapping populations

Primer pair name	Locus	Genus	Forward primer 5' to 3'	Reverse primer 5' to 3'
EMFxaACO1B[a]	*ACO*	*Malus*	gccactccattgttatcaacc	tgctccacactcttgtacttcc
EMMdANS1	*ANS*	*Malus*	aaggagaagtatgccaatgacc	gaactttcagtaccttggttgc
EMMdDFRF2	*DFR*	*Malus*	gagtgttccatgttgctacacc	ctcctccacattcacagttcc
EMFxaEKO1B[a]	*EKO*	*Malus*	cacatgaagacacccaactagg	aaatctctctggcttccattcc
EMMpPGLM2	*PGLM*	*Malus*	gtatgtatgtcaccatggatcg	catcctcgttttacaacactcc
EMPpACO1B	*ACO*	*Prunus*	gggaaagcaccttctacttgc	gagctgctctgctaatttctcc
EMPpANS1[a]	*ANS*	*Prunus*	aaggagaagtatgccaatgacc	atcccaacccaagtgacagc
EMPpDFR2	*DFR*	*Prunus*	ccactcctatggattttgagtcc	ctagcaccccatttattgttgg
EMPaEKO1B	*EKO*	*Prunus*	acagtccagctccaatagttcc	gctttcccattgattcttgtcc
EMPpPGLM2	*PGLM*	*Prunus*	aggtgtggatgcacaaattgc	cccatcctcgttttacaactcc

[a] Alleles produced were homozygous in the parental lines screened and therefore were non-segregating

Table 5 Five gene-specific loci mapped in the *Malus* (F × T) and *Prunus* (PA × PN) mapping populations, the segregation data and the χ^2 values for goodness-of-fit to the expected segregation ratios for each locus, along with the linkage groups to which they were assigned and the marker to which they were most closely associated

Mapping population	Locus	Segregation type	Classes	Segregation	χ2	*df*	Signif.	Linkage group	Closest marker
F × T	*ANS1*	Abxab	aa:ab:bb	17:46:21	1.1	2	–	3	CH04D11
F × T	*ANS2*	Aaxab	aa:ab	48:35	2	1	–	6	CH03D07
F × T	*DFR*	Aaxab	aa:ab	38:40	0.1	1	–	15	CH05A02
F × T	*PGLM*	Aaxab	aa:ab	33:43	1.3	1	–	12	CH01D09
PA × PN	*ACO*	Aaxab	aa:ab	40:49	0.9	1	–	3	UDP96-008
PA × PN	*DFR*	Abxcd	ac:ad:bc:bd	28:14:31:17	9.1	3	**	1	CPPCT-34
PA × PN	*EKO*	Aaxab	aa:ab	59:29	10.2	1	***	1	CPPCT-34
PA × PN	*PGLM*	Abxac	aa:ab:ac:bc	37:37:3:3	57.8	3	***	6	UDP98-021

display higher rates of transferability between genera. We have shown that 58% of the genetic loci for which primers were designed could be amplified in all three rosaceous genera surveyed with at least one of the primer pairs available. This level of transferability is considerable higher than for rosaceous SSRs (Decroocq et al. 2003; Lewers et al. 2005) and is comparable to that of RFLP probes transferred between genera in studies of other families such as the Gramineae (Wilson et al. 1999).

Detection of polymorphism, and comparative mapping in the Rosaceae

Comparative mapping studies employing RFLP probes based on cDNA, genomic DNA and ESTs, and isoenzymes have been reported in many plant families including Brassicaceae (Lukens et al. 2003) and Solanaceae (Doganlar et al. 2002) and these have revealed much about genome evolution at the macrosynteny level in those families. Both RFLP probes and isoenzymes have been shown to transfer well between genera (Wilson et al. 1999; Tanksley et al. 1992) and Fulton et al. (2002) reported a set of conserved orthologous (COS) probes from tomato and showed that those with sequences highly conserved between tomato and *Arabidopsis* hybridised to single, or small numbers of, loci in species from a range of dicotyledonous families. However, the use of RFLP probes and isozymes has the disadvantage of being both time-consuming and labour-intensive, and in some cases, multiple loci are detected with a single probe, complicating interpretations as to which loci are orthologous between genera (Krutovsky et al. 2004). Although synteny studies in Rosaceae are still in their infancy, Dirlewanger et al. (2004) reported the comparative mapping of 24 RFLP probes and six isoenzymes in *Prunus* (T × E) and *Malus* ('Prima' × 'Fiesta').

Recently, comparative genetic maps have been successfully constructed in other families, such as Pinaceae (Krutovsky et al. 2004) and Fagaceae (Casasoli et al. 2006) using STS markers such as those we have described here for Rosaceae. As well as being single copy (in diploid genomes) and physically linked to genes of known function, such markers have the advantage of being PCR-based and amenable to high-throughput genotyping using fluorescent dye-labelling and electrophoresis on a semi-automated genotyping platform. This is quicker and more cost-effective than methods of genome analysis based on DNA-hybridisation. It is important, however, when selecting markers for comparative mapping purposes, to be aware of genes which map to multiple loci, such as the *Expansin* (*EXP*) and *Cinnamyl alcohol dehydrogenase* (*CAD*) genes reported here, on account of difficulties in distinguishing between orthologous and paralogous loci amplified using the same PCR primers.

We have demonstrated that a high proportion of the markers developed here from *Fragaria* are transferable to other rosaceous genera and that within those genera, polymorphic products were detected. Five loci that were mapped in *Fragaria* were also mapped in one or both of two further genera representing two other rosaceous sub-families, *Malus* (Maloideae) and *Prunus* (Prunuoideae). *DFR* and *PGLM* were mapped as single loci in both *Malus* and *Prunus*, whilst *ACO* and *EKO* were mapped in *Prunus* as single loci and *ANS* was mapped to two discrete, independently segregating loci in *Malus*.

DFR and *EKO* were both mapped to LGII in FV × FN (*Fragaria*) and were both located on LG1 of PA × PN (*Prunus*). Interestingly, *DFR* mapped to LG15 of the F × T (*Malus*) map, which was the linkage group to which RFLP probe LY37a mapped in the investigation reported by Maliepaard et al. (1998). More recently, Dirlewanger et al. (2004) showed that LY37a mapped to the same region of LG1 in *Prunus* to

which *DFR* and *EKO* located in this investigation. The findings of these previous reports and the data presented herein are thus consistent and indicate that these markers are orthologous loci and that LGII (*Fragaria*), LG15 (*Malus*) and LG1 (*Prunus*) are possible homeologues in the three genera.

In contrast, *ACO* and *PGLM* both mapped to LGVI in FV × FN (*Fragaria*) but segregated independently in PA × PN (*Prunus*), mapping to LG3 and LG6 respectively. *ANS* was located on LGV in FV × FN (*Fragaria*) and mapped to two independently segregating loci in F × T (*Malus*) on LG3 and LG6, two linkage groups not previously reported to be associated with each other. The identification of two loci for *ANS* and also for *PGLM* (one of which was not heterozygous in F × T) is not surprising given the allopolyploid nature of the *Malus* genome (Chevreau and Laurens 1987) and loci such as *ANS* may prove useful in identifying homeologous linkage groups within the *Malus* genome.

Of course, the transferability of markers to other progenies for mapping is not simply a property of the markers themselves, but also depends on the degree to which those progenies segregate. In the same way that two *Fragaria* progenies have been used for the amping described here, it may be that additional *Malus* and *Prunus* progenies will be needed to maximise the opportunities for comparisons of synteny.

Concluding remarks

We have described the development and mapping of 29 functional markers for *Fragaria*, greatly increasing the number of genes of known function mapped in the genus and demonstrating the usefulness of sequence data from public databases into the development of such markers. Many of the markers were transferable to species from other subfamilies of Rosaceae and the mapping of five markers in a *Malus* and/or *Prunus* mapping population has shown the potential of such markers for comparative mapping in the family. Indeed, one of these markers, *DFR*, maps to locations on the *Malus* and *Prunus* linkage maps consistent with the findings of other researchers (Dirlewanger et al. 2004) and the map positions of this, and *EKO* on the *Prunus* and *Fragaria* linkage maps have identified a potentially conserved homeologous section of the genome in all three genera. The development of further markers for comparative mapping not only from loci presented here but also from other previously characterised markers (Etienne et al. 2002; Silva et al. 2005) and also from the wealth of sequence data available in public databases should permit a detailed study of the evolution of genome structure and organisation within Rosaceae.

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