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ORIGINAL PAPER

Map position and functional allelic diversity of *Md-Exp7*, a new putative expansin gene associated with fruit softening in apple (*Malus* × *domestica* Borkh.) and pear (*Pyrus communis*)

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Abstract Fruit ripening can be considered as a complex set of biochemical and physiological changes occurring at the end of the developmental stage. Ripe fruit texture notably affects overall quality and consumer appreciation. Excessive softening limits shelf-life and storability, thereby increasing disease susceptibility and economic loss. Fruit softening is a process due to the depolymerisation of different polysaccharide classes, an event controlled by a synergic and coordinated action of several enzymes among which expansins play a fundamental role. To date, six expansin genes are known to be expressed during apple fruit ontogeny, from full bloom up to fruit ripening. We identified a novel expansin apple homolog (*Md-Exp7*) sharing high sequence similarity with specific-ripening expansin genes of other crops. A functional marker (Md-Exp7$_{SSR}$) based on an SSR motif located within the untranslated region of the gene was developed and mapped on Linkage Group 1 of the apple and pear genomes in a region where one major apple QTL for fruit firmness had been previously identified. The allelic composition of 31 apple varieties for the SSR marker was associated with differences in fruit softening.

Keywords Expansin gene · Candidate gene · Apple ripening

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Introduction

During maturation and ripening, fruit undergo several physiological and biochemical changes which determine the organoleptic quality traits associated with consumer preferences. Among these complex processes, softening is one of the most evident alterations affecting general fruit sensory characteristics. Excessive softening not only makes fruit less attractive to consumers, but also increases costs for shipping and storage because of higher pathogen susceptibility (Brummell and Harpster 2001; Seymour et al. 2002). The softening process includes complex rearrangements in cell-wall architecture (Powell et al. 2003; Brummell and Harpster 2001; Rose et al. 1998) due to enzymatic polysaccharide hydrolisation. Several hydrolases are involved and act on the covalent bond that links together different classes of polysaccharides (Brummell et al. 1999; Cosgrove 2000a, b; Giovannoni 2001). However, it has been shown that the known cell-wall enzymes (polygalacturonase, endo-1,4-β-glucanase, pectin-methylesterases, pectate lyases and glycosidases) cannot fully explain the softening process (Brummell and Harpster 2001). Expansins, a novel class of proteins, have been proposed as the major enzymatic agent in cell-wall

remodelling (Cosgrove 2000b) and are thought to be involved in the disruption of the non-covalent bonds between the hemicellulose matrix and the cellulose microfibril (Cosgrove 1997), thus exposing the structural polymer to the action of other cell-wall enzymes. Recently, a two-step fruit softening model has been defined in which expansin acts in the early stage and the latter is regulated by hydrolases like polygalacturonases (Rose et al. 1997; Powell et al. 2003).

Most important agronomic traits are quantitatively inherited, being controlled by several genes. Studies carried out on apple-segregating populations have identified important QTLs for firmness and flesh texture on linkage groups LG01, LG08, LG10, LG15 and LG16 (King et al. 2000; Maliepaard et al. 2001; Seymour et al. 2002). Novel insight into fruit-firmness control in apple has recently been presented via a candidate-gene approach investigating the effects of *Md-ACS1* and *Md-ACO1*, two genes involved in ethylene production during the ripening stage, on fruit softening (Harada et al. 2000; Oraguzie et al. 2004, 2007; Costa et al. 2005). *Md-ACO1* was mapped on LG10 within the 5% confidence interval border of an important QTL for fruit firmness, genetically linking ethylene production to the rate of apple softening (Costa et al. 2005). We identified a new expansin homolog in apple following a candidate-gene approach and determined its map position and the functional association between Md-Exp7$_{SSR}$'s allelic variability and differences in fruit softening.

Materials and methods

Phenotyping

To examine whether the allelic variation of Md-Exp7$_{SSR}$ marker is associated with differences in firmness, previous firmness data of 152 out of the 154 seedlings of the cross Prima × Fiesta (King et al. 2000; Maliepaard et al. 2001) were associated with our current genetic data. In addition, 31 apple cultivars covering the harvest calendar from August 13 to October 2 in northern Italy (Table 1) were genotyped with the Md-Exp7$_{SSR}$ marker and phenotyped for firmness. Fruit samples were harvested at the starch value of 7 (on a 1 to 10 scale, where 10 corresponds to the complete starch hydrolysis). Firmness was measured at harvest and after 2 months of cold storage (2–4°C) with an Effegi penetrometer (11.2-mm probe). At each date, at least ten apple fruits were assessed at the two opposite peeled sides. The difference in firmness between these two dates generated the softening values.

In pear, 41 individuals of the Passe Crassane × Harrow Sweet mapping population were phenotyped. Fruits were harvested at a firmness value of 6 kg cm^{-2} (according to the general harvest index used for pear); firmness data were taken as in the apple samples.

Identification of Md-Exp7 expansin apple homologous

Several heterologous specific-ripening expansin gene sequences were selected from the NCBI database: *Lycopersicon esculentum—Le-Exp1* (U82123); *Fragaria* × *ananassa—Fa-Exp1* (AF163812), *Fa-Exp2* (AF159563), *Fa-Exp5* (AF226702); *Prunus armenica—Pa-Exp1* (U93167), *Pa-Exp2* (AF038815); *Prunus avium—Pav-Exp1* (AF297521), *Pav-Exp2* (AF297522) and *Prunus cerasus—Pc-Exp1* (AF350936), *Pc-Exp2* (AF350937) and *Pc-Exp4* (AF350939). All available apple expansin partial sequences were selected from the NCBI database (from AB099925 to AB099930). ClustalW (http://www.ebi.ac.uk/clustalw) sequence multialignment carried out on the selected sequences allowed the identification of the most conserved regions on which two primers were designed:

Md-Exp (sense): CAAAAGTTTGTGGCCGTGACAA,
Md-Exp (antisense): TGATGCCTCTGGAACTATGG.

This pair was tested on gDNA of the cultivar 'Mondial Gala', which was isolated from leaves according to Doyle and Doyle (1989).

PCR reaction was performed in a final volume of 25 µl: one cycle at 94°C for 120 s, 35 cycles at 52°C for 45 s, 72°C for 120 s, 94°C for 30 s; one cycle at 52°C for 45 s and a final extension of 10 min at 72°C. Amplification reaction was performed with a PTC-200 Peltier Thermal Cycler (MJ Research) using 50 ng of gDNA, 0.25 mM of each dNTP, 1 mM $MgCl_2$, 0.2 µM of each primer, 2.5 µl 10× PCR buffer and 1 U of Taq polymerase (Amersham Pharmacia). PCR products were separated on a 5% poly-acrylamide gel. The amplicon was cloned in *E. coli* DH5α strain via pGEM T-Vector SystemI (Promega). Sequencing was performed by MWG Biotech (http://www.mwg-biotech.com). This sequence served as starting point for a genome walking procedure towards the upstream gene region (Fig. 1).

Genome walking

To explore the upstream region of the apple expansin amplicon, genome walking was performed after Devic et al. (1997), where 2.5 µg of gDNA of the apple cultivar Mondial Gala was digested overnight at 37°C with 80 U of three rare cutting restriction enzymes: *Dra*I, *Pvu*II and *Ksp*I. In a final equimolar concentration of 25 µM, 10 µl of digested gDNA was mixed with 4 µl of adapters, 4 µl of 5× RL buffer, 1 µl ATP (10 mM) and 2 µl T4 DNA ligase (5 U/µl). PCR walking reaction was performed using High Fidelity Taq polymerase (Roche) to guarantee amplification

Table 1 Harvest date, firmness value at harvest and after two months' cold storage, softening value and allelic constitution of the three functional markers (Md-Exp7, Md-ACS1, Md-ACO1) for 31 apple cultivars

Cultivar	Harvest date	Firmness harvest (kg cm^{-2})	Firmness storage (kg cm^{-2})	Softening (kg cm^{-2})	Md-ACS1	Md-ACO1
Md-Exp7$_{SSR}$-198/202						
Gloster	11 Sept.	7.28	5.31	1.97	2/2	1/2
Idared	15 Sept.	5.78	5.76	0.00		1/2
Mutsu	02 Oct.	7.32	6.88	0.44	1/2	2/2
Granny Smith	02 Oct.	7.93	7.37	0.56	1/2	2/2
Md-Exp7$_{SSR}$-202/202						
Pinova	26 Aug.	6.85	6.63	0.22	2/2	1/1
Fuji	02 Oct.	7.58	7.21	0.37	2/2	1/1
Pilot	26 Aug.	7.90	7.26	0.64	2/2	1/1
Elstar	26 Aug.	6.22	4.01	2.21	2/2	1/1
Elan	26 Aug.	6.55	3.89	2.66	2/2	1/1
Rubin	22 Aug.	6.75	4.18	2.57	2/2	1/1
Gala	13 Aug.	8.47	5.97	2.5	2/2	1/2
Starking D.	03 Sept.	7.52	6.44	1.08	1/2	1/1
Braeburn	15 Sept.	8.79	8.55	0.24	1/2	1/2
E210-80	28 Aug.	7.70	7.23	0.47	1/2	1/2
Fiesta	13 Aug.	9.27	7.91	1.36	1/2	1/2
E210-198	28 Aug.	7.92	6.45	1.47	1/2	1/2
Joanathan	10 Sept.	7.68	5.46	2.22	1/2	1/2
Jonamac	28 Aug.	6.67	4.42	2.25	1/2	1/2
Priscilla	11 Sept.	6.48	3.20	3.28	1/2	1/2
Red Rome	02 Oct.	9.74	6.77	2.97	1/2	2/2
Golden D.	10 Sept.	7.84	5.07	2.77	1/2	2/2
Rome Beauty	22 Sept.	9.78	7.25	2.53	1/2	2/2
Lord L.	10 Sept.	5.47	2.40	3.07	1/2	2/2
Ben Davis	08 Oct.	7.84	7.38	0.46	1/1	2/2
Durello di Forlì	25 Sept.	11.03	10.07	0.96	1/1	2/2
McIntosh	19 Aug.	7.51	4.16	3.35	1/1	2/2
Md-Exp7 $_{SSR}$-202/214						
Topaz	10 Sept.	8.35	7.30	1.05	2/2	2/2
Florina	10 Sept.	8.15	6.64	1.51	1/2	2/2
Prima	13 Aug.	9.24	5.34	3.90	1/2	2/2
Saturn	03 Sept.	6.30	3.92	2.38	1/1	2/2
Akane	19 Aug.	7.88	5.56	2.32	1/1	2/2

The varieties are grouped according to their Md-Exp7$_{SSR}$, Md-ACS1, and Md-ACO1 genotype. Allele 1 of Md-ACO1 and allele 2 of Md-ACS1 code for high ethylene production, and their respective alleles 2 and 1 for low ethylene production (Costa et al. 2005).

of long fragments. Three nested PCRs were performed consecutively with oligonucleotide complementary to the 5′ upstream region of *Md-Exp* fragment (Fig. 1):

Md-Exp-1PW: TATACAGATTTCCGTAGCCACAGGCC,
Md-Exp-2PW: GATGTGGGGTTTTGAAATTAGAGGGTC,
Md-Exp-3PW: ATTCAGGGGGGTGAAATATCTTGAGC, and an antisense primer for the specific adapter, as reported in Devic et al. 1997.

The structure of the sequence assembled was analysed in silico using the browser http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi.

Development of the Md-Exp7$_{SSR}$ marker

Md-Exp7 showed a CT repeat. Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to identify the best primer pair combination for the segments flanking the SSR stretch (Fig. 1):

Md-Exp7$_{SSR}$ (sense): CATAGAAGGTGGCATGAGCA,
Md-Exp7$_{SSR}$ (antisense): TTTCTCCTCACACCCAAACC.

The SSR marker was amplified as follows: one cycle at 94°C for 150 s, 32 cycles at 60°C for 45 s, 72°C for 60 s and 94°C for 30 s, one last cycle at 60°C for 45 s and 72°C

Fig. 1 The *Md-Exp7* sequence of Mondial Gala showing in *bold* the microsatellite $(CT)_{12}$ motif. Positions of the primers used for the first amplification, the SSR marker and for PCR walking are underlined

CGCCGGGGGGGGTTAAACATAAAAAGTTGTAATATAGTGTGTGTGTATCAAATACAAAATTGAGGGCCTC
TCTAGGGTAGGGACCCTAGACGGTCCGTCTATTTTAGCTATCTTCAAGGCCGGCCTCGAGAGTATTAAAT
AATTAAACGAGCAAATTAATAAATCGAGGTTCAATCGTGGGCTACTATTCTTTCAAGCATCCTACTCTTT
TTCGTTCAATTGAAGAGAACCAAACCCCTTATTCTCCTCAATGCACCAAGATCATAATTTTAGACGTACA
ATCCTCACCCTAAAACTTGTTAAAGGGAATTAACCAAAAGGGCATTTCCGTCAATTGAGAATAAAAAAAG
AGTTGAACGGAATTACCGCCAAAAAAAAACCTTCAACTACTCGTGGGAGACCAAAGCCGCATTAATCAAG
GTGATGATTTTGTCACCTTTTGACAATGTTTCTCTGCAACTTTGTTCATAAATAGCACTCCACCCCTCTG
CTTTCTCCTCACACCCAAACCCTCCAACTCAAAAGCCCCAT**CTCTCTCTCTCTCTCTCTCTCTCT**AGAAA
GCAATGGCCGCCATGGCTCCTCTGCTCTGCATTGCTTCTCTCATGGCACTGGCCAGTACCATTGTAGTCG
AGGCCAGAATTCCTGGTGTTTACACTGGCGGAGCGTGGCAGGATGCTCATGCCACCTTCTATGGCGGCAG
TGATGCCTCTGGAACTATGGGTAAGCTCAAGATATTTCACCCCCCTGAATTTTTTAAGCATAATATAACA
TAAAGTCCATTTCTTTTGGATTTAGACCCTCTAATTTCAAAACCCCACATCAAAATTTCCCAAAATCTCT
CAAAATATTACAACAAAAACTAAAAGTTCATGCCTTTTGATTGTCTCTGTGTATATACAGGAGGGGCCTG
TGGCTACGGAAATCTGTATAGCCGGGGCTACGGAGTGAACACTGGCGGCGCTGAGCACGGCACTCTTCAA
CAACGGTTTGAGTTGTGGAGCTTGCTTCGAACTCAAGTGCGCAAATCAGCCTCAGTGGTGCAAATCTGGC
AGTCCCTCCATTTTTGTCACGGCCACAAACTTTTGCCCTCCAAACTTCGCCCAGCCCAGCGACGATGGCG
GCTGGTGCAACCCTCCTCGCACCCACTTCGATCTCGCCATGCCCATGTCCTCAAGATCGCCGAGTACCGC
GCCGGCATGTCCTGTTTCTTACCGCCGTGAGCACAGCACTCTCTCTTCTTTATACTCTGTTTCCGTTATA
TTTGGTCGTTGTAATGCCAAATGACTAAATGCCTTTACTGTGGTATAGTGCCATGC

for 10 min. The reaction mix was identical as that for the identification of the *Md-Exp7* gene.

Mapping procedure

$Md\text{-}Exp7_{SSR}$ mapping in apple was performed on the molecular marker linkage map developed on Prima × Fiesta progeny (Maliepaard et al. 1998) upgraded by an increase of 88% in marker number and 18% in length (Van de Weg et al. unpublished). $Md\text{-}Exp7_{SSR}$ was genotyped on 144 out of the 154 progeny seedlings. Genomic position of this marker was also determined in pear, genotyping the 99 seedlings of the Passe Crassane × Harrow Sweet genetic map (Dondini et al. 2004). Linkage analysis for both mapping populations was performed with the software JoinMap® 3.0 (Van Ooijen and Voorrips 2001) using the Kosambi mapping function. Markers were grouped into 17 linkage groups at a LOD value of 4, and their order was determined with a recombination threshold of 0.45. Visual representations of the marker maps were created with the software MapChart (Voorrips 2001).

QTL Analysis

QTL analysis on the Prima × Fiesta phenotypic data set was performed with MapQTL® 5.0 software (Van Ooijen 2004). Maximum Likelihood-based interval mapping was performed to examine the occurrence of new QTL due to the extension of the Prima × Fiesta linkage map. Initial cofactors were selected from regions where the LOD was greater than 3.0, choosing the marker nearest to each QTL peak. Subsequent rMQM (restricted multiple QTL mapping) function was used to further precise the likelihood and position of a putative QTL, as this analysis corrects for disturbing effects of other QTLs due to unbalanced representation of their QTL-alleles. Next, MQM analysis was performed to examine the putative presence of closely linked QTL. Significance thresholds for the LOD values were calculated by genome-wide permutation tests using 1,000 permutations. Tests for putative interactions among any two loci were performed by dropping one interaction term at a time from the full model, including all additive terms and all possible two-way interactions using the GLM facilities of GenStat (http://www.genstat.co.uk).

Allele mining

Each of the 31 apple cultivars and 41 pear seedlings were genotyped with $Md\text{-}Exp7_{SSR}$ and also with the two functional markers related to the two enzymes involved in the ethylene pathway: Md-ACS1 (*1-aminocyclopropane-1-carboxylate synthase*) and Md-ACO1 (*1-aminocyclopropane-1-carboxylate oxidase*), identified by Sunako et al. 1999; Harada et al. 2000 and Costa et al. 2005. We tested these markers because of their known association between allelotypes and apple fruit softening (Oraguzie et al. 2004; 2007; Costa et al. 2005). Significance of differences among apple and pear genotype classes was calculated using one way ANOVA followed by Newman–Keuls test. A $p<0.05$ was required for statistical significance.

Results

Characterisation of the Md-Exp7 gene

The first expansin fragment identified (384 bp) showed no useful polymorphism between apple cultivars. To extend the sequence discovery in the upstream region, Mondial Gala genome walking exploration was performed using *DraI* digestion enzyme. A final fragment of 1,316 bp (Fig. 1) was obtained that revealed the following structure: 5′UTR (1–563 nt), 1st exon (564–864 nt), 1st intron (865–960 nt), 2nd exon (961–1,311 nt) and a partial portion of

the 2nd intron (1,311-end). In silico analysis of the resulting expansin protein (BLASTx algorithm) gave the similarities (UniProt database) reported in Table 2. Protein similarity was the highest with *Pc-Exp7*, which also gave the highest nucleotide similarity with our sequence (DQ072009), showing an identity value of 94%. One relatively large repetitive stretch of $(CT)_n$ (12*x*) was found in the 5′ untranslated end of our sequence, as in *Pc-Exp7*.

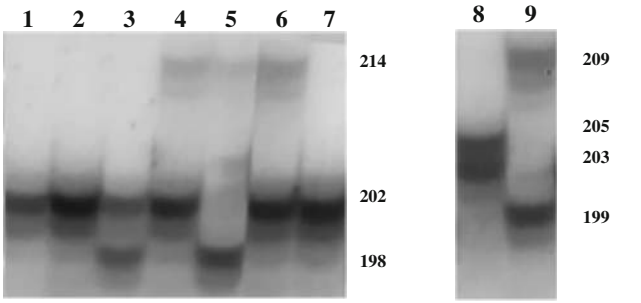
Variability of the SSR marker Md-Exp7$_{SSR}$

The SSR marker we developed for the CT repeat was tested on 31 apple cultivars and showed three alleles of 198, 202 and 214 bp (Fig. 2a). In pear, this SSR gave four alleles in the two mapping parents of 199, 203, 205 and 209 bp (Fig. 2b). Apparently, all apple alleles had an even number of nucleotides and all pear sequences an odd number. All these marker alleles were cloned and sequenced (Fig. 3), and their alignment explained the systematic 1 bp difference between the currently identified apple and pear sequences by an insert of a single C at position 41 in pear. The SSR pattern of all four apple cultivars and one of Passe Crassane (pear) were perfect, while those of Harrow Sweet were imperfect $(TC)_nTG(TC)_2$. The pear sequences differed consistently from those of apple by five specific SNPs at positions 29 (C/T), 34 (G/A), 83 (C/T), 135 (C/G), and 193 (A/G). The apple sequences were much more conserved, being completely identical except for the number of CT repeats; the pear sequences were slightly less conserved, being polymorphic at four positions (29th, 134th, 150th and 172nd nt; Fig. 3).

Md-Exp7 mapping and association with QTLs

Despite the considerable extension of the Prima × Fiesta map, no new QTLs were identified in addition to the three previously reported by King et al. (2000) and extensively described by Maliepaard et al. (2001). These QTLs, with a LOD value greater than 4.5, were located in the LG01, LG08 and LG10, and explained, respectively, 19%, 16% and 22% of the total variance. Two additional putative QTLs were also found on LG03 (LOD of 3.6) and LG15 (LOD of 3.5) which explained, respectively, 17% and 10% of the total variance (King et al. 2000; Maliepaard et al. 2001). Md-Exp7$_{SSR}$ marker was polymorphic in Prima (202/214) but not in Fiesta (202/202), thereby allowing its mapping on linkage group 1 of Prima at 8.9 cM to the *Vf* gene (Fig. 4), and within the two-LOD interval of the QTL on LG01. This QTL region is likely to contain just one QTL according to a MQM analysis, as one marker from this region could explain almost all the phenotypic variation (Maliepaard et al. 2001; Fig. 5c). The 214 allele of Prima is associated with a high- and the 202 allele with a relatively low firmness.

We phenotyped and genotyped a set of 31 apple cultivars to further examine the association of *Md-Exp7* with fruit firmness and to get an impression about the function associated with marker allele 198, which was present in some of the apple cultivars tested (Table 1) but not in Prima × Fiesta. The cultivars were grouped into three classes according to their Md-Exp7$_{SSR}$ allelic constitution. Softening of the individual cultivars varied between 0 and 4 kg cm^{-2} after 2 months of cold storage. The three classes showed significant differences in softening ($P<0.05$; Fig. 6a); changing from 0.62 kg cm^{-2} for Md-Exp7$_{SSR}$-198/202, to the other two classes of 1.8 kg cm^{-2} for Md-Exp7$_{SSR}$-202/202 and 2.26 kg cm^{-2} for Md-Exp7$_{SSR}$-202/214, respectively. These results suggest that the 198 marker allele is associated with the best and the 214 allele with the worst firmness retention; allele 202 was associated with an intermediate softening rate.



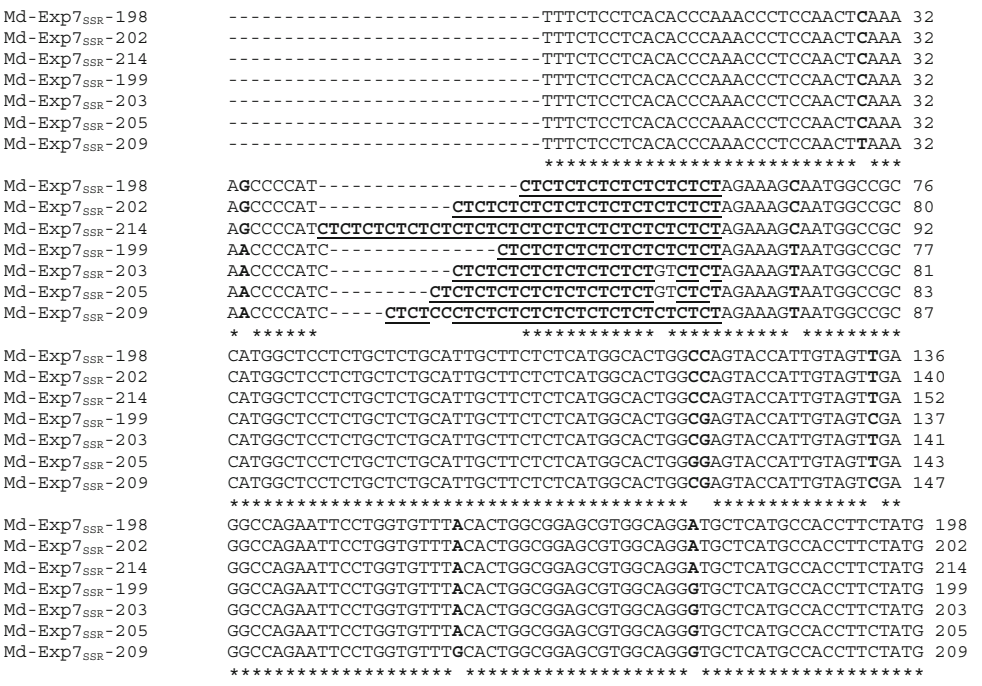
a apple genotypes **b** pear genotypes

Fig. 2 Visualised PCR products and allele sizes (bp) of the Md-Exp7$_{SSR}$ marker of seven apple (**a**) and two pear (**b**) cultivars: *1*:Fuji (202/202), *2*:Mondial Gala (202/202), *3*:Granny Smith (202/198), *4*:Florina (202/214), *5*:Delorina (214/198), *6*:Prima (214/202), *7*:Fiesta (202/202), *8*:Harrow Sweet (205/203), *9*:Passe Crassane (209/199)

Table 2 Expansin genes with their % similarity and *E* value to the amino acid sequence of *Md-Exp7*

UniProt, accession number	% Similarity	*E* value
Pc-Exp7 (Q84L75)	87.5	$3.2e^{-30}$
Md-Exp6 (Q76HY6)	86.2	$3.5e^{-29}$
Fa-Exp6 (Q93XH6)	78.02	$2.2e^{-28}$
Le-Exp1 (O04359)	70.33	$2.1e^{-24}$
Fa-Exp5 (Q93XH7)	67	$1.3e^{-21}$

E value (expectation value): is the probability that the alignment might occur by chance; the lower the value, the more significant the alignment. % similarity: score reflecting the degree of similarity between the compared sequences



Apple alleles
Md-Exp7$_{SSR}$-198: $(CT)_9$
Md-Exp7$_{SSR}$-202: $(CT)_{12}$
Md-Exp7$_{SSR}$-214: $(CT)_{18}$

Pear alleles
Md-Exp7$_{SSR}$-199: $(CT)_{10}$
Md-Exp7$_{SSR}$-203: $(CT)_9$+TG+$(CT)_2$
Md-Exp7$_{SSR}$-205: $(CT)_{10}$+TG+$(CT)_2$
Md-Exp7$_{SSR}$-209: $(CT)_{2+12}$

Fig. 3 Sequences of three apple Md-Exp7$_{SSR}$ marker alleles (198, 202, 214) and four pear alleles (199, 203, 205, 209). *Bold text* indicates microsatellite sequences and SNPs. *Box* indicates microsatellite polymorphisms

Synteny between apple and pear

Our apple Md-Exp7$_{SSR}$ marker also amplified in pear (Fig. 2), giving similar partial sequences (Fig. 3) and mapping on a homologous position on LG01 in both parental cultivars of the pear mapping population (Dondini et al. 2004, Fig. 4). It also proved to be associated with softening (Fig. 6b) in the pear progeny, as alleles 199 and 205 were associated with minimum softening, and alleles 203 and 209 with increased softening (Fig. 6b).

Md-ACS1, Md-ACO1 and their relationship with Md-Exp7

Md-ACS1 and Md-ACO1 are two candidate genes that are known to be associated with fruit softening in apple (Harada et al. 2000; Oraguzie et al. 2004 and Costa et al. 2005).

Both functional markers were mapped in Prima × Fiesta population (Costa et al. 2005). More precisely Md-ACO1 co-localised with a major QTL for fruit firmness on LG10 (King et al. 2000; Maliepaard et al. 2001), while Md-ACS1 was genetically positioned on LG15, but distant from the previously identified QTL (King et al. 2000; Maliepaard et al. 2001).

We genotyped all 31 apple cultivars with the two functional markers (Table 1). On average, Md-ACO1 had a larger effect than Md-ACS1, as genotypes homozygous for the Md-ACO1 allele for low ethylene production (allele 2) lose 60% less firmness than the genotypes with a homozygous allele configuration for high ethylene production (allele 1); this reduction was only 17% for Md-ACS1 (Fig. 7). Similarly, Md-Exp7$_{SSR}$ showed a softening reduction of 72.6% (Fig. 6a) changing from the unfavourable allele combination (202/214) to the favourable combination (198/202). Md-ACO1 and Md-Exp7 are thus the most effective markers in explaining the observed variability in apple fruit softening, and in Prima × Fiesta they worked independently (P=0.98 for interaction). Also,

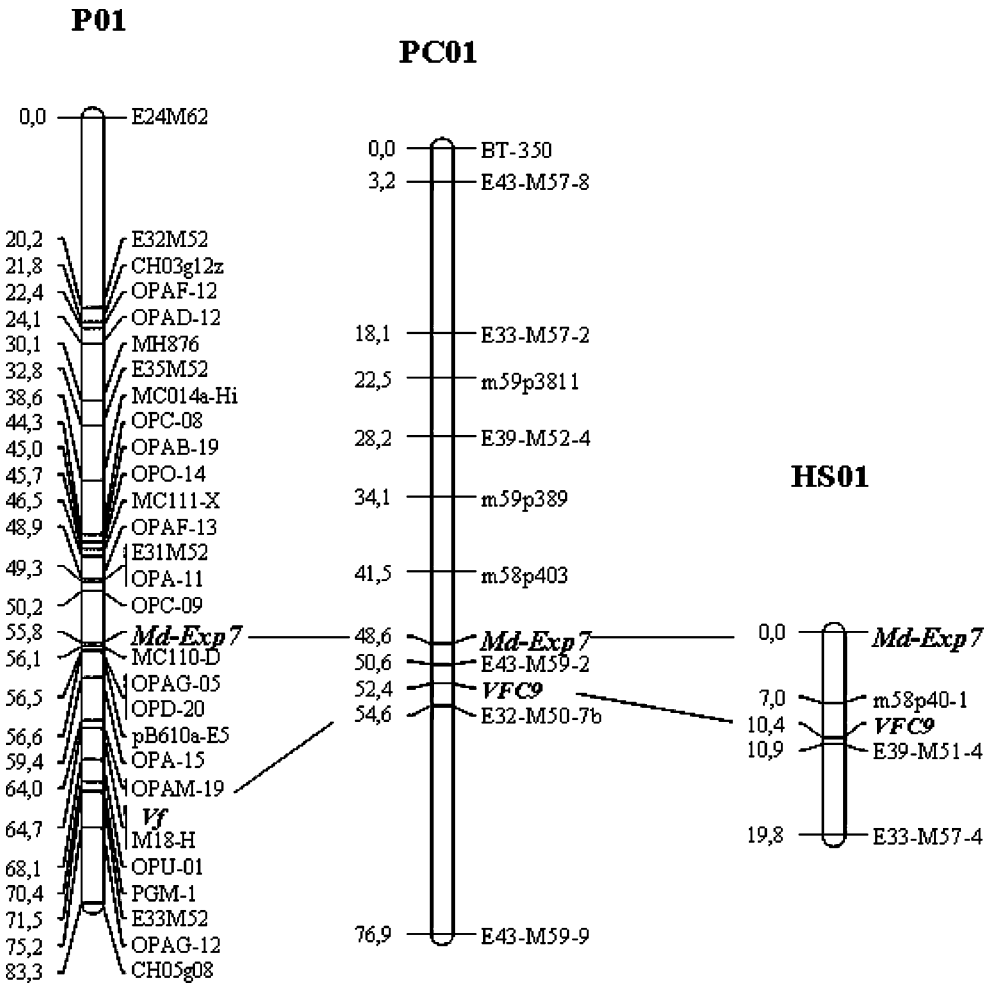


Fig. 4 Map position of *Md-Exp7* on linkage group1 of the apple cultivar Prima (*P*), and of the pear cultivars Passe Crassane (*PC*) and Harrow Sweet (*HS*)

Md-ACS1 did not show any epistatic effects with Md-Exp7 nor with Md-ACO1, and the suit of cultivars did not allow tests on interactions due to the too low numbers of individuals for seven out of the nine possible genotype classes, having just 0–3 seedlings.

Discussion

Md-Exp7 functional marker

Here, we report the identification of the new apple expansin *Md-Exp7*.

The best sequence similarity (94%) was found with *Pc-Exp7* (*Pyrus communis*—AB093034), a pear expansin expressed during the ripening of young fruit (Hiwasa et al. 2003). *Md-Exp7* also showed a high nucleotide similarity to *Fa-Exp1* (AF163812) and *Fa-Exp5* (AF226702), both involved in strawberry ripening (Harrison et al. 2001). The sequence of *Md-Exp7* also presented the same structures as found in other expansin genes, with two introns at conserved positions (Li et al. 2002). Expansin belongs to a wide gene family that is diverged in three main groups (Li et al. 2002), of which the α-expansins are responsible for cell-wall loosening in dicots. While in *Arabidopsis*, 26 α-expansins have been identified; to date, only six are known in apple and are expressed during fruit ontogeny (Wakasa et al. 2003). *Pc-Exp7* (Hiwasa et al. 2003) and the novel *Md-Exp7* are phylogenetically grouped close to *Le-Exp1* (Fig. 8), a tomato expansin gene that is fundamental for fruit softening (Rose et al. 1997).

Association of Md-Exp7 with softening

Md-Exp7 is located within the interval of a known major QTL for fruit firmness identified by King et al. (2000) and extensively analysed by Maliepaard et al. (2001). Despite the LOD-CURVES for two QTL peaks, Maliepaard et al. (2001) clearly demonstrated that the data only support the presence of just a single QTL. *Md-Exp7* was selected as a candidate gene for softening based on its biological function. Its location within a QTL for firmness further supports its status as candidate gene. The favourable marker allele of Prima (214) is linked in coupling phase with the *Vf allele* for resistance to apple scab which derived from the

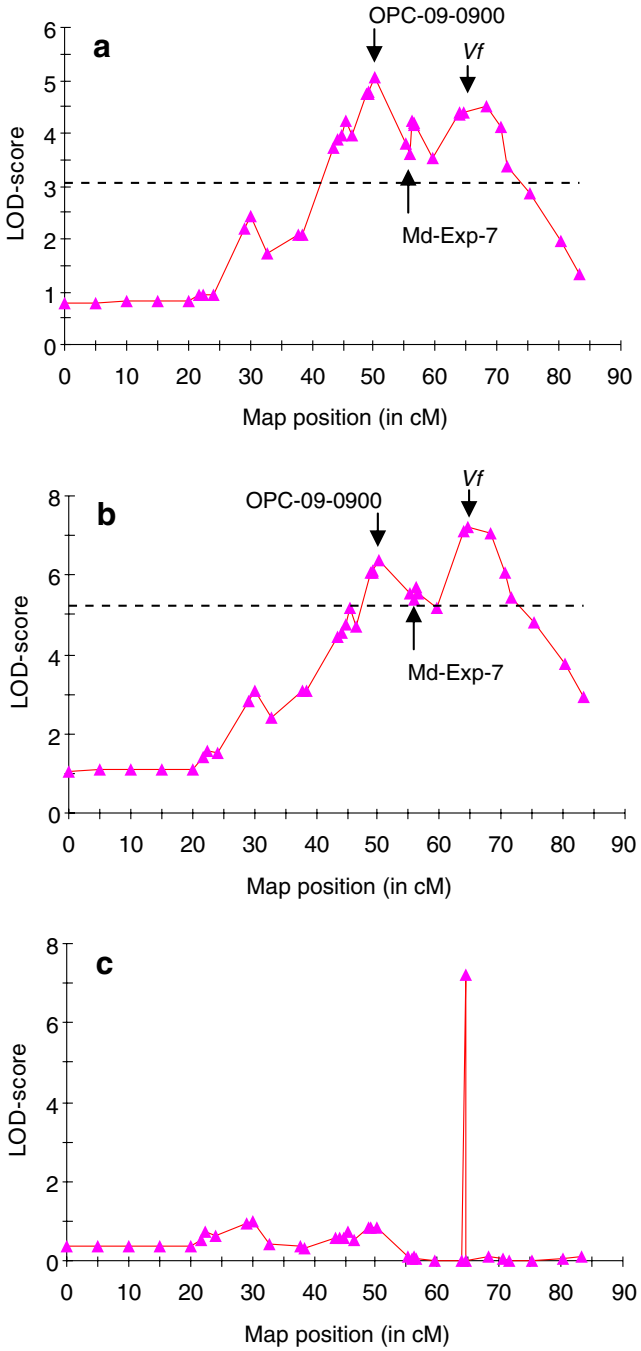


Fig. 5 QTL-LOD profiles for fruit firmness on LG01 of *Prima* resulting from interval (**a**), rMQM (**b**) and MQM (**c**) mapping. Cofactors needed for the rMQM and MQM mapping (see "Materials and methods") were located in the previously identified QTL regions (Maliepaard et al. 2001). For the rMQM analysis, these were OPC-09-900 of linkage group P1, OPC08-0790 of F10, and Ly37a of F15. In the MQM analysis, OPC-09-0900 was replaced by *Vf* due to the results of the rMQM analysis. *Dotted lines* indicate the two-LOD thresholds

wild species *M. floribunda* (Hough et al. 1953). Considering the extent of linkage drag that is still present in Prima (King et al. 1999), Prima's 214 allele of *Md-Exp7* should also be derived from *M. floribunda*. Further validation on the role of *Md-Exp7* may be obtained by data generated in the HiDRAS project (Gianfranceschi and Soglio 2004), where more than 600 *Vf*-possessing seedlings, deriving from 13 crosses, have been phenotyped for firmness and softening. The approximately 130 (=number of individuals×interval size (22 cm)/100) seedlings that show recombination within the current 2-LOD QTL interval should allow a considerable narrowing down of this interval.

Role of the microsatellite (CT) repeat

Within the *Md-Exp7* sequence, we detected a simple sequence repeat (SSR) in the 5′ untranslated region, for which we developed an SSR marker. The association analysis with the suite of 31 apple cultivars suggested that softening is correlated with the CT repeat number: the longer the Md-Exp7$_{SSR}$ marker fragment, the stronger the softening.

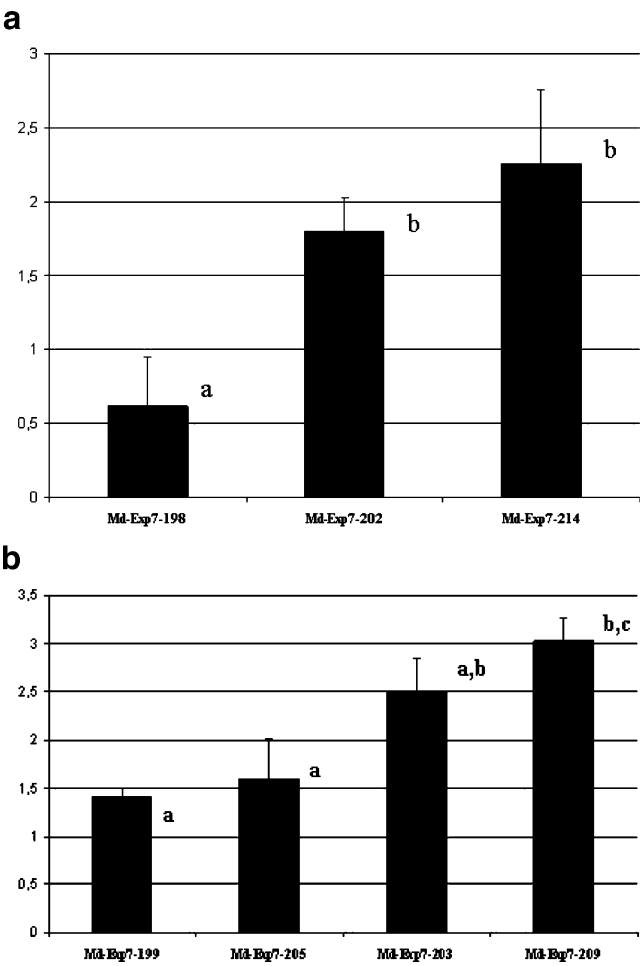


Fig. 6 Average softening (kg cm^{-2}) associated with different alleles of the marker Md-Exp7$_{SSR}$ as observed in 31 apple varieties (**a**) and in a pear mapping population (**b**). *Bars* represent the standard error. Groups with different subscripts (**a**, **b**) differ significantly ($p<0.05$, ANOVA followed by Newman–Keuls test)

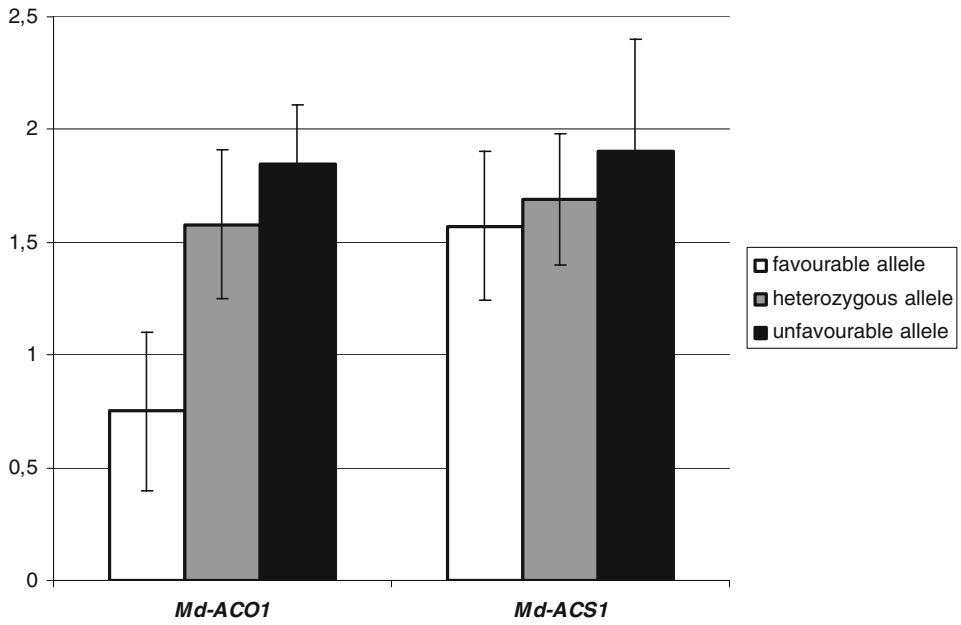


Fig. 7 Average of loss of firmness (kg cm^{-2}) of the three possible *Md-ACO1* and of the three possible *Md-ACS1* genotypes for the 31 apple cultivars. *Bars* represent the standard error

Favourable allele: *Md-ACS1-1/1, Md-ACO1-2/2.*
Heterozygous allele: *Md-ACS1-1/2, Md-ACO1-1/2.*
Unfavourable allele: *Md-ACS1-2/2, Md-ACO1-1/1.*

A similar observation was obtained also in rice, where a $(CT)_n$ microsatellite discovered in the 5′ untranslated region of the *waxy* locus explained most of the apparent amylase content variation (Bao et al. 2002), suggesting this as a good candidate for improving the grain's quality.

Analysis of the complete genome of *Arabidopsis* revealed that SSRs are mostly located in the 5′ untranslated region (Zhang et al. 2004; Zhang et al. 2006), where they occurred approximately three-fold more frequently than in other genomic fraction, and it has been estimated that these microsatellites are under a strong positive selection (Morgante et al. 2002).

In *Arabidopsis*, (CT) repeats are one of the most abundantly occurring simple sequence repeats. They are proposed to act as *cis*-regulatory elements that can be recognised by transcription factors (Zhang et al. 2006). Microsatellite repeats in the promoter can affect also its activation (Fujimori et al. 2003). They may interfere with transcription activity, influencing the interaction with regulatory proteins by changing the distance between transcriptional factors binding sites (Li et al. 2004). They may also modify the DNA secondary structure, which can cause quantitative variation in the transcriptional activity due to the structure of different alleles (Iglesias et al. 2004).

For *Md-Exp7*, it is not clear yet whether the variation of CT repeats or other critical polymorphisms (e.g. at the coding level leading to different proteins or within the introns) are responsible for the observed variation in fruit softening.

Expansin synteny between apple and pear

Apple and pear belong to the same *Rosaceae* sub-family and have a common basic chromosome number (x=17). Recent mapping studies have shown a high synteny level between apple and pear: SSR markers developed in apple map on homologous positions in pear and vice versa (Yamamoto et al. 2004; Dondini et al. 2004; Pierantoni et al. 2004; Silfverberg-Dilworth et al. 2006). Syntenic position of homologous functional genes have been reported for the self-incompatibility S-locus (Yamamoto et al. 2002) and scab resistance genes (*Vf*, King et al. 1998; *VhK*, Yamamoto et al. 2004) on LG01. To these results, we can now add the homologous position of the Md-$Exp7_{SSR}$ marker, showing for the first time, the mapping of an expansin gene in apple and pear. The pear amplicons of the Md-$Exp7_{SSR}$ marker showed 91–96% sequence similarity to *Pc-Exp7*. As we obtained only partial sequences of limited length, our data do not allow conclusions about the identity of the pear amplicons, i.e. whether they belong to *Pc-Exp7* or to a new expansin in pear.

The effect of Md-Exp7, Md-ACO1 and Md-ACS1 on fruit softening

Md-ACS1 and *Md-ACO1* are two genes involved in the ethylene pathway that have been associated with softening of fresh and/or cold-stored apples (Harada et al. 2000; Oraguzie et al. 2004, 2007; Costa et al. 2005). While the

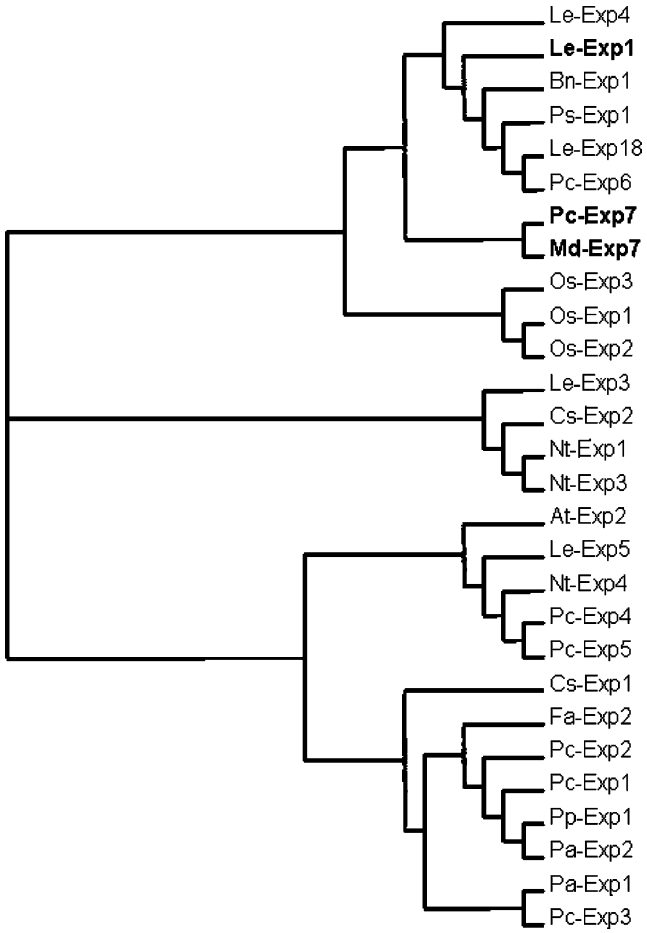


Fig. 8 Expansin genes in phylogenetic representation. The tree was obtained by aligning the nucleotide sequences of 28 expansin genes from different species with ClustalW. Phylogenetic tree was visualised with TreeView. In *bold* are our apple *Md-Exp7*, the pear *Pc-Exp7* and *Le-Exp1* of tomato

effect of *Md-ACS1* is well documented in these studies, and although it had a larger effect on ethylene production and softening than *Md-ACO1* (Costa et al. 2005), no effect could be detected in Prima × Fiesta, nor in the suite of 31 apple cultivars. The reason for this is not clear. Possibly, *Md-ACS1* acts only in certain gene combinations, due to which it will be hard to detect as a QTL by classical QTL mapping approaches used in MapQTL. The polymorphism from which the Md-ACS1 marker is based is a retroposon-like sequence insertion in the promoter region (Sunako et al. 1999). Due to the type and location of the polymorphism, it was readily assumed that this polymorphism was responsible for the observed variation in *Md-ACS1*-associated softening.

Our data suggest a higher impact of *Md-Exp7* and *Md-ACO1* in the control of the loss of firmness in apple. In our observation, the difference between the unfavourable and the favourable allele of *Md-ACS1* cause a difference of only 17% in fruit softening. This variation rose up to 60% and 72.6% in the case of *Md-ACO1* and *Md-Exp7*, respectively. This is in agreement with our QTL mapping carried out in Prima × Fiesta, where these two genes are mapped in two main QTLs for firmness: LG01 and LG10.

Breeding

Firmness and shelf-life during storage is a main worldwide breeding goal. Phenotypic evaluation is only possible in a late stage of the selection process as sufficient numbers of fruit became available, and phenotyping for softening is a laborious and time-consuming process. Pre-selection by molecular markers at a young seedling stage may therefore increase considerably the breeding efficiency for this trait. Marker-assisted breeding requires a clear insight into the genetic basis of a trait. Thus, various critical genes have been identified, such as *Md-ACS1* and *Md-ACO1*, which are responsible for the two final steps in the ethylene pathway. Our study now shows that the expansin *Md-Exp7* may be involved in this trait determination, as it co-localises with a major QTL for firmness and softening. Although considerable progress has been made in the identification of critical genes and genomic regions, the genetic picture is still far from complete. It is, for instance, not clear yet why cultivars like Ben Davis can have an excellent firmness retention with an *ACS-ACO* genotype for high ethylene production, while other cultivars with a favourable genotype for low ethylene production have poor retention (Rubin, Elan; Table 1). Both cases occur within the same *Md-Exp7* marker genotype. Consequently, at least one other, as yet unidentified, gene is also critical, which could be a gene for the QTL on the top of linkage group 15, as found in Prima × Fiesta (King et al. 2001, Maliepaard et al. 2001), or *Md-Exp3*, which is the first identified expansin to be associated with softening in apple (Wakasa et al. 2003) but whose map position is still unknown. Alternatively, different associations could exist between the size of one of the markers and the functionality effecting polymorphism. Due to this incomplete knowledge, one must still be cautious in applying these markers extensively in marker-assisted seedling selection, although they already may be useful to breeders as support in the parental selection for crossing. They can now take into account that *Md-Exp7* mapped on the same region where sub-lethal genes and a recessive gene for dwarfing are also located (Gao & Van de Weg 2006), which may affect the proportion of favourable offspring, especially since the Md-Exp7$_{SSR}$-214 allele for low softening of cultivars like Prima is not only associated with the favourable dominant *Vf* allele for scab resistance, but also with the unfavourable recessive *sl1* allele for sub-lethality.

Thus, the new apple expansin gene *Md-Exp7*'s co-localisation with a known major QTL for fruit firmness, its association with fruit softening over a set of apple

cultivars and its sequence similarity and functional synteny with its pear orthologue indicate that it is an important candidate gene for dissecting the genetic variability of fruit softening. Our study therefore enhances the importance of the candidate-gene approach in identifying genes that may be the genetic basis of horticulturally important traits and functional markers for assisted breeding.

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