Molecular Breeding (2005) 16: 21–31
DOI 10.1007/s11032-005-0828-3

Endopolygalacturonase: a candidate gene for *Freestone* and *Melting flesh* in peach

C.P. Peace[1,*], C.H. Crisosto[1] and T.M. Gradziel[2]
[1]*Kearney Agricultural Center, Department of Pomology, University of California, Davis, 9240 S Riverbend Ave, Parlier, CA 93648 USA;* [2]*Department of Pomology, University of California, Davis, 1047 Wickson Hall Davis, CA 95616 USA; *Author for correspondence (e-mail: cpeace@uckac.edu; phone: +1-559-646-6576; fax: +1-559-646-6593)*

Received 28 September 2004; accepted in revised form 14 January 2005

Key words: Clingstone, Co-segregation, Fruit softening, Pleiotropy

Abstract

Peach fruit are handled, processed, and marketed according to their stone adhesion and fruit softening type. Uncertainty exists over whether these simply inherited traits are controlled by two linked loci, *Freestone* (*F*) and *Melting flesh* (*M*) or one multi-allelic locus, and whether *M* is controlled by the cell wall degrading enzyme, endopolygalacturonase. From morphological and molecular analysis of two related segregating populations of peach, we conclude that a single locus containing at least one gene for endopolygalacturonase, controls both *F* and *M* with at least three effective alleles. A simple diagnostic PCR test is now available for the three major phenotypes of freestone melting flesh (FMF), clingstone melting flesh (CMF), and clingstone non-melting flesh (CNMF).

Introduction

Numerous simply inherited traits have been described for peach and nectarine (*Prunus persica* [L.] Batsch) (Monet 1989). Of these, *Freestone* and *Melting flesh* provide the basis for classifying fruit into their major forms of consumption: fresh and canned. Freshly consumed fruit tend to be of freestone melting flesh (FMF) and clingstone melting flesh (CMF), and to a lesser extent, clingstone non-melting flesh (CNMF), while fruit for canning is almost exclusively CNMF. Cultivars with consistently freestone non-melting flesh (FNMF) have not been reported (Van Der Heyden et al. 1997).

Adhesion of the endocarp (or stone) to the ripe fruit flesh is controlled by the *Freestone* (*F*) locus, and individual cultivars/selections are classified as either freestone (F-), where the pit freely separates from the flesh, or clingstone (ff), where the pit adheres to the flesh (Bailey and French 1933). However, degrees of adhesion have been observed in peach germplasm, some cultivars are classified as 'semi-freestone' or 'semi-clingstone', and the character can also vary according to seasonal conditions (Bailey and French 1949). This variation may arise from expression of other alleles at the *F* locus or different genes. Nevertheless, clear distinctions between freestone and clingstone progeny usually can be made from testcrosses or selfs of heterozygous (Ff) cultivars. Genetic mapping studies have identified linkage between the *Freestone* locus and DNA markers, with the *F* locus placed near one end of peach linkage group 4 (Dettori et al. 2001).

The *Melting flesh* (*M*) locus controls fruit flesh firmness. Melting flesh genotypes carrying the dominant allele go through a phase of rapid softening in the late stages of fruit ripening (the 'melting phase') that coincides with the climacteric peak (Lester et al. 1996). Fruit of non-melting flesh cultivars, which are homozygous recessive (mm), do not go through this phase and remain relatively firm throughout ripening. Melting flesh cultivars tend to be freestone, while clingstone cultivars tend to be non-melting. This has led to the term 'freestone' being used interchangeably with 'melting', and 'clingstone' with 'non-melting'. However, such terminology can be confusing, since clingstone melting flesh genotypes are also common (Okie 1998).

With FMF and CNMF representing contrasting phenotypes, the occurrence of intermediate CMF progeny in crosses between FMF and CNMF parents has been seen as evidence for *F* and *M* corresponding to two separate, though linked, loci. Bailey and French (1949) examined over 400 progeny in each case of three progeny populations of heterozygous (Ff, Mm) parent cultivars: 'Georgia Belle' selfed, 'Champion' selfed, and F_1 'Georgia Belle'×'Champion'. With 5–10% of the progeny in each population observed as 'recombinant' CMF types, a linkage distance of 15% crossover was calculated between *F* and *M* (Bailey and French 1949). As no FNMF progeny occurred, the authors reasoned that in non-melting flesh fruit (mm), expression of the *F* allele is masked, such that genetically FNMF fruit have the CNMF phenotype.

Recent studies provide no evidence for the two-locus theory. Despite the simple inheritance of *M* and its apparent linkage with *F*, genetic mapping studies in the last decade have not included the *M* locus. As only the *Freestone* locus was mapped when segregation occurred in crosses between FMF and CNMF parents (Dettori et al. 2001; Hayashi and Yamamoto 2002), presumably CMF progeny did not occur. Monet (1989) proposed an alternative hypothesis: of only one locus with three alleles (F/f/f1, F dominant over the others and f1 recessive to the others) controlling the expression of freestone/clingstone and melting/non-melting flesh phenotypes. In this scenario, F- = FMF, ff or ff1 = CMF, and f1/f1 = CNMF (Monet 1989). Under this hypothesis, the CMF progeny observed by Bailey and French (1949) could not arise in selfed populations of diploid 'Georgia Belle' or 'Champion', as three segregating alleles are required for all three phenotypes to occur. Although Monet (1989) did not account for the results of those earlier authors, a possible explanation is that the 'recombinant' progeny were instead the results of unintended outcrossing with a pollenizer carrying the f allele.

The physiological process underlying the freestone and melting flesh traits may be better understood if the controlling genes were known. Potential candidate genes for fruit softening have been sought in many fruit species. Often implicated are genes for enzymes that play a role in cell wall degradation, in particular, endopolygalacturonase (endoPG, EC 3.2.1.15). This enzyme depolymerizes pectin, a major component of cell walls that is extensively disassembled during late stages of fruit softening (Hadfield and Bennett 1998; Brummell and Harpster 2001).

Three separate polygalacturonase (PG) clades were identified by analysis of amino acid sequences from various species (Hadfield and Bennett 1998). Fruit ripening-related endoPGs in melon (*Cucumis melo*) are associated with both Clade A and Clade B, and this appears to be true for other fruit species (Hadfield et al. 1998). Clade C contains only PGs expressed in pollen that are thought to be exopolygalacturonases (Hadfield et al. 1998). In tomato (*Lycopersicon esculentum*), where endoPG has been studied most thoroughly, a particular Clade B form of the enzyme was initially thought to control fruit softening, but is now considered to make only a small contribution to fruit softening (Brummell and Harpster 2001). In *Capsicum*, endoPG (probably Clade B) is responsible for a major and sudden softening phase of certain genotypes (Rao and Paran 2003). In strawberry (*Fragaria ananassa*), a rosaceous crop like peach, the relationship between an endoPG (clade uncertain) and fruit softening is not clear (Redondo-Nevado et al. 2001). Softening of pear fruit (*Pyrus communis*, family Rosaceae) closely parallels endoPG expression, especially that of a Clade A gene (Hiwasa et al. 2003).

Evidence for endoPG control of softening in peach fruit is strong but not conclusive. At least three major endoPG genes have been identified in peach fruit (Lee et al. 1990; Lester et al. 1994, 1996). The melting phase of FMF peach and nectarine fruit ripening is associated with marked

increases in gene expression and enzymatic activity of endoPG (Pressey et al. 1971; Pressey and Avants 1978; Orr and Brady 1993; Lester et al. 1994; Trainotti et al. 2003), whereas CNMF fruit have less ripening-related endoPG expression (Lester et al. 1994) and virtually no endoPG activity (Pressey and Avants 1978). Lester et al. (1994) identified a Clade A endoPG gene that was strongly implicated in control of the melting phase due to its differential expression in FMF, CMF, and CNMF fruit. Lester et al. (1996) detected polymorphism in this gene between melting flesh and non-melting flesh cultivars in the form of a RFLP (restriction fragment length polymorphism). When this RFLP was screened on 20 progeny of a population segregating 1:1 for *Melting flesh*, recombination between *M* and the endoPG gene had apparently occurred for three progeny (15%) (Lester et al. 1996). The authors reconciled this finding by arguing that considerable variation in fruit firmness and texture in the population may have caused incorrect scoring of the melting flesh trait in the field. An alternative explanation is that the polymorphic endoPG gene is genetically linked to the *Melting flesh* locus (with an estimated 15% crossover), but does not control the trait, perhaps controlling *Freestone* instead.

The present study was undertaken to test whether *Freestone* and *Melting flesh* are the same or separate loci, and whether endoPG controls either trait, using PCR tests for the gene and surveying two progeny populations (segregating for both traits) resulting from controlled crossing.

Materials and methods

Plant material

The germplasm tested for inheritance consisted of 284 trees: two cultivars and two progeny populations derived from them. The populations were planted in 1998 and grown adjacently in fields at the Kearney Agricultural Center, Parlier, California. The first population ('Pop-DG') consisted of 70 F_1 progeny of 'Dr. Davis'×'Georgia Belle'. The second population ('Pop-G') of 70 progeny was derived from the selfing of 'Georgia Belle'. 'Georgia Belle' is FMF cultivar, heterozygous for the locus/loci controlling the phenotype. 'Dr. Davis' is a CNMF cultivar, homozygous recessive for the locus/loci. These parent cultivars, growing on their own roots, were present twice each in the field. For both populations, each progeny genotype was also represented twice (with trees growing adjacently in the same row), once on its own roots and once on a common commercial rootstock ('Nemaguard') via bud grafting. Preliminary SSR and RAF marker analysis (see below) identified, in Pop-DG, 18 selfs of 'Dr. Davis' and one outcross (i.e. 'Dr. Davis' crossed with a genotype other than itself or 'Georgia Belle'). The same marker analysis identified, in Pop-G, six outcrosses (i.e. 'Georgia Belle' crossed with some genotype other than itself). 'Dr. Davis' selfs contained only markers present in the 'Dr. Davis' parent, and were lacking any 'Georgia Belle' markers, particularly alleles of codominant SSR markers that were inherited by other progeny. Outcrosses contained several markers or alleles not present in the seed parent or in the intended pollen parent. Population sizes of 'true' progeny were therefore reduced to 51 for Pop-DG and 64 for Pop-G. The 25 'false' progeny remained in the field, but were subsequently analyzed separately. One of the true progeny of Pop-G had not set fruit by 2003, and so could not be assessed morphologically.

Morphological analysis

For each tree of the progeny population germplasm, five fruit were harvested at commercial maturity and allowed to ripen at room temperature (3–4 days). Ripe fruit were used to qualitatively assess stone adhesion (freestone or clingstone) and flesh softening type (melting or non-melting) for each tree, in the 2002 and 2003 fruiting seasons. Segregation for *F* and *M* was expected to be 1:1 in Pop-DG and 3:1 in Pop-G.

DNA extraction

DNA was extracted from leaves of 'Dr. Davis', 'Georgia Belle', and each tree of the two populations by either of two methods. The first was a chloroform extraction-isopropanol precipitation method essentially as described by Foolad et al. (1995). However, instead of cesium chloride–ethidium bromide density gradient centrifugation,

purification steps involved incubation with RNAse (10 mg/ml), followed by extraction with chloroform:isoamyl alcohol (24:1), precipitation with 2/3 volume of isopropyl alcohol, two washes (with a solution of 76% ethanol and 10 mM ammonium acetate), and dissolving in TE.

The second extraction method was identical to the first, except that volumes were scaled down to fit into 1.5 ml microcentrifuge tubes throughout the procedure. For this second method, the plant tissue consisted of 2–6 expanding leaves of 3 cm length or less, ground by hand with disposable plastic pestles in extraction buffer within a separate 0.5 ml tube, which was transferred to 1.5 ml tubes for the 65 °C incubation. In preliminary trials, this scaled-down procedure has proven more time efficient, with adequate yields of DNA obtained for marker systems based on the polymerase chain reaction (PCR) and showing identical marker profiles to the full-scale method. Yield and quality were determined after electrophoresis on 1% agarose gels with comparison against standards (λ *Hind*III digest) of known concentration. Samples were diluted to 20 ng/μl working solutions.

Molecular analysis

As part of a linkage-mapping project for the same peach populations (Peace et al., manuscript in preparation), SSR (simple sequence repeat) and RAF (randomly amplified DNA fingerprinting) marker systems were employed to provide marker profiles that, in the context of the present study, were useful in verifying the parentage of each of the progeny. SSR assays were performed according to Dirlewanger et al. (2002) and RAF assays according to Waldron et al. (2002), using polyacrylamide gels and silver staining (Promega Corporation, Madison WI) for both marker systems. The SSR locus BPPCT-018 (Dirlewanger et al. 2002) identified 'Dr. Davis' selfs in Pop-DG, the SSR locus CPPCT-024 (Aranzana et al. 2003) identified the outcross in Pop-DG, and RAF primers B12 and P04 (Operon Technologies, Inc., Alameda CA) identified outcrosses in Pop-G. Other SSR and RAF primers also confirmed these selfs and outcrosses.

Five sets of primers were designed using Primer3 (Rozen and Skaletsky 1998), to amplify segments of an EST (Contig694, CUGI 2003) having 98% deduced amino acid identity with PRF5 (GenBank accession number X76735), a cDNA encoding an endoPG gene associated with *Melting flesh* in peach (Lester et al. 1994, 1996). Both Contig694 and PRF5 were isolated originally from FMF cultivars ('Loring' and 'Flavorcrest', respectively). The primers and their corresponding amplified fragments were designated as 'endoPG-1', situated at the 5′ end, to 'endoPG-5', situated at the 3′ end of

Table 1. Primer sequences used to amplify segments of a polymorphic endopolygalacturonase gene in peach.

Designation of segment	Forward primer (5′–3′)	Reverse primer (5′–3′)
endoPG-1	CCTTCAACTCATTAACCTCTCTCTC	GGAAGGCTTTTGTGGAGTCA
endoPG-2	TTCCTCTCTGCATGGGCTA	TCCGCTGGGACAACTCTC
endoPG-3	CTCAAACAACATCGTGGTGA	AGCAACGCCTTCTATCCACA
endoPG-4	GAATGGTCTAAGAATCAAGTCATGG	GCTTGGGACTGCAATCAAAT
endoPG-5	GCCTACAAGTTGTTTGTAGAGTGG	CCGGACATAATCTTACAACAGTTC

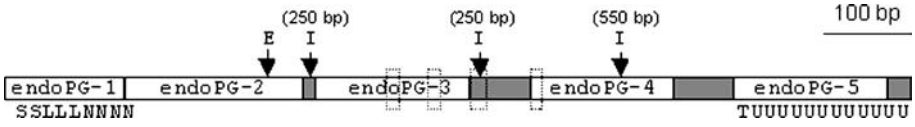

Figure 1. Amplified DNA fragments (endoPG-1 to -5) of an EST encoding a peach endoPG gene. Unshaded regions represent the five fragments of the endoPG gene, while the shaded regions in between were not amplified. Conserved domains associated with endoPG function are shown in the boxed sections with dotted lines. Indicated by arrows are an *Eco*RI restriction site (E) and three intron sites (I) with their approximate lengths. Other letters indicate the locations of a simple sequence repeat (S), the putative leader sequence (L) that includes the start of the coding sequence, the N-terminal sequence (N), and the termination of the coding sequence (T), beyond which lies the 3′ untranslated region of the gene (U).

the gene (Table 1, Figure 1). The positions of the primers were designed so as to scan most of the EST sequence for length polymorphism. The probable locations and sizes of three introns, and the sizes of amplified genomic DNA fragments, were determined by comparing the cDNA sequence of Contig694 with a genomic DNA sequence of endoPG ('clone AC1') for the peach cultivar 'Suncrest' (GenBank accession number AY262754; Callahan et al. 2004). Two of the introns were avoided with the chosen set of primers, while the third intron was located within endoPG-4 (Figure 1). Conserved domains associated with endoPG function were taken from Redondo-Nevado et al. (2001), and corresponded mainly with the 3′ half of endoPG-3 and the 5′ end of endoPG-4 (Figure 1). The gene contains one *Eco*RI restriction site, within endoPG-2 (Figure 1).

PCR reactions were carried out according to Etienne et al. (2002) in 10 µl volumes. Amplification products were separated on 4% polyacrylamide gels with silver staining (Promega Corporation, WI) to detect polymorphisms, and separated on 1% agarose gels with ethidium bromide staining to determine approximate fragment sizes. For initial testing of each endoPG marker, assays were performed on 'Dr. Davis', 'Georgia Belle', and several progeny of Pop-DG including FMF and CNMF types, for a total of 4 FMF and 6 CNMF individuals. Subsequent analyses were performed on all progeny of both populations including false progeny, for a total of 76 FMF, 59 CNMF, and 5 CMF individuals.

Amplification of endoPG-4 and endoPG-5 was performed in the same PCR reaction, using half as much of each primer as for separate reactions. For sequencing of endoPG-4 and endoPG-5, major bands appearing for each allele were excised from the polyacrylamide gel for several individuals carrying that allele and bulked. Excised bands were amplified via the polymerase chain reaction (PCR) in 50 µl volumes, using procedures described by Weaver et al. (1994). Amplification products were separated on 1% agarose gels. The resulting single bands were cut from the agarose gel and the DNA extracted using QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). DNA was re-amplified via PCR in 50 µl volumes, and amplification products purified using the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA), with a final volume of 30 µl. DNA concentration was tested by running 4 µl of the product on a 2% agarose gel. The remaining clean product DNA was used as template for forward and reverse sequencing with an ABI 3700 DNA sequencer at Davis Sequencing (Davis, CA).

Results and discussion

Morphological analysis

For each pair of trees representing the same progeny (same scion genotype), fruit phenotype (FMF, CMF, or CNMF) was identical (as were DNA profiles, confirming their identity). Each tree also had the same phenotype in both years of observation. Within both populations of true progeny, there was complete co-segregation of the freestone and melting flesh traits: all freestone progeny were also melting (i.e. all were FMF), and all clingstone progeny were also non-melting (i.e. CNMF). The segregation ratios fit a pattern of simple Mendelian inheritance, with FMF:CNMF being 29:22 in Pop-DG (1:1) and 45:18 in Pop-G (3:1). In both populations of true progeny, there were no CMF or FNMF progeny. If *Freestone* and *Melting flesh* were separate loci, some CMF and FNMF phenotypes would be expected to occur as recombinants between the loci. Thus, the morphological data supports the hypothesis of Monet (1989) that *F* and *M* are at the same locus, which suggests that 'Dr. Davis' has the allelic combination of f1f1, and that 'Georgia Belle' has Ff1. All 18 'Dr. Davis' selfs had CNMF fruit, as expected. Five of the outcross genotypes (one of 'Dr. Davis' and four of 'Georgia Belle') had CMF fruit, presumably due to expression of an 'f' allele inherited from the unknown pollen parent(s). Such outcrossing with an f-allele pollenizer is the likely explanation for the 5% of progeny being CMF previously reported for a large population derived from selfing 'Georgia Belle' (Bailey and French 1949). The two remaining 'Georgia Belle' outcrosses had FMF fruit, suggesting that 'Georgia Belle' contributed the dominant *F* allele to these progeny.

Molecular analysis

Amplification of various segments of the endoPG gene across the two parent cultivars and several of

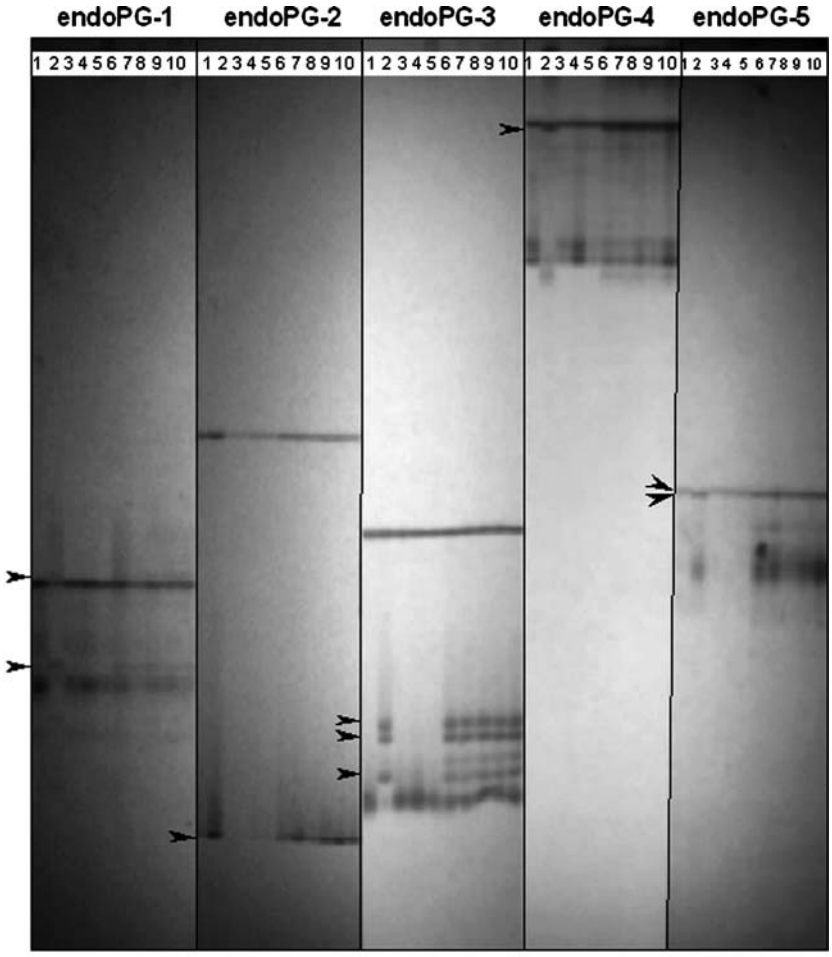

Figure 2. Banding patterns for five PCR markers of endoPG, amplified for peach cultivars 'Dr. Davis' (lane 1) and 'Georgia Belle' (lane 2) and some of their CNMF (lanes 3–5) and FMF (lanes 6–10) F_1 progeny. Arrows indicate the position of major polymorphic bands (though some are hard to see in this reproduction of the stained gel). L = ladder (fragment sizes in bp). [1]Expected fragment length (includes an intron of 554 bp for endoPG-4). [2]Observed length of topmost band on an agarose gel.

their hybrid progeny resulted in one or two intense bands and some fainter bands for each endoPG marker (Figure 2). Polymorphism in major bands was observed for all endoPG markers, with band absence for CNMF individuals for endoPG-1, endoPG-3, and endoPG-4, band intensity polymorphism for endoPG-2, and length polymorphism between FMF and CNMF individuals for endoPG-5 (Figure 2). Other polymorphism between FMF and CNMF was observed in the form of band intensity differences for the fuzzy 'ghost' bands (Figure 2), which may represent PCR artifacts derived from the major bands. Polymorphism for endoPG was therefore completely associated with the qualitative phenotypes of fruit flesh firmness and stone adhesion for this initial set of germplasm.

The endoPG-4 and endoPG-5 markers were subsequently screened (together) on all progeny of both populations. Multiplexing these two endoPG markers was successful, with no 'hybrid' bands observed from inclusion of both primer sets in the one PCR reaction. For both markers and in both populations, there was complete co-segregation of

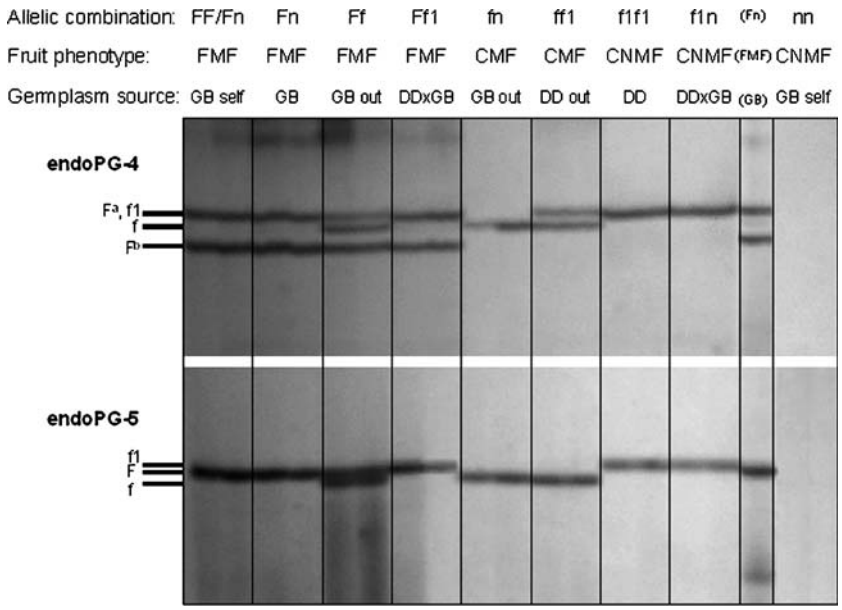

Figure 3. Allelic combinations at the *Freestone–Melting flesh* locus and their associated banding patterns for two endoPG PCR markers, observed in germplasm derived from the peach cultivars 'Georgia Belle' (GB) and 'Dr. Davis' (DD). FMF = freestone melting flesh; CMF = clingstone melting flesh; CNMF = clingstone non-melting flesh; out = outcross.

endoPG polymorphism with the melting flesh and stone adhesion phenotype of each tree, confirming the results of the initial germplasm set. Inheritance analysis was used to determine the endoPG-4 and endoPG-5 banding patterns associated with each *Freestone–Melting flesh* allele and allelic combination. Banding patterns for representatives of each of the available allelic combinations (which were all possible combinations except ff) are shown in Figure 3.

Four alleles of endoPG – F, f, f1, and n (null allele) – were shown to be segregating within this 'Dr. Davis'- and 'Georgia Belle'-derived germplasm. 'Dr. Davis' was confirmed to be f1f1, while 'Georgia Belle' was discovered to be Fn, carrying the null allele instead of the previously suspected f1 allele. No fragments were amplified for the null allele for endoPG-4 and endoPG-5 (nor for the other three endoPG markers, results not shown), such that it was detectable only in homozygous form, i.e., in the CNMF progeny derived from selfing 'Georgia Belle' (Figure 3). The null allele is likely to be the same as that observed for the peach selection 'Fla. 9-20C'. This selection was lacking all three restriction fragments associated with the endoPG gene in other individuals (Lester et al. 1996). The f1 allele of endoPG-4 was not detectable when in combination with the F allele, as the topmost band of the F allele (F^a) occupied the same position as the f1 band (Figure 3). The f1 allele of endoPG-5 was not visible when in combination with the F or f alleles, perhaps due to competition in PCR, which might occur if the f1 allele contains a SNP in either of the primer binding sites (perhaps an extension of the 2 bp deletion that occurs immediately adjacent to the reverse primer binding site for the f1 allele of endoPG-5). Non-detection of certain alleles resulted in the distinction of only six allelic categories (out of a possible ten) from the analysis of endoPG-4 PCR products: FF/Ff1/Fn, Ff, ff/fn, ff1, f1f1/f1n, and nn, though these can be further differentiated by inheritance analysis. For endoPG-5, the ff1 category could not be distinguished from ff/fn.

The fruit phenotypes resulting from each allelic combination are presented in Table 2. In this scheme, which is the same as that of Monet (1989) except that a fourth (null) allele is introduced, F is dominant to all others, f is dominant to f1 and n, and f1 and n are equivalent and recessive to F and f. The f1 allele therefore represents a mutation that nullifies enzyme translation or normal catalytic function, as this allele has the same phenotypic

Table 2. Fruit phenotypes for all possible allelic combinations of four alleles of the *Freestone–Melting flesh* locus observed in germplasm derived from peach cultivars 'Georgia Belle' (GB) and 'Dr. Davis' (DD), according to molecular analysis of an endopolygalacturonase gene. Only the 'ff' combination was not represented in this germplasm. FMF = freestone melting flesh; CMF = clingstone melting flesh; CNMF = clingstone non-melting flesh.

Allelic combination	Fruit phenotype	Example in this study
FF	FMF	25% of GB self progeny
Ff	FMF	One GB outcross
Ff1	FMF	50% of DDxGB progeny
Fn	FMF	GB, 50% of GB self progeny
ff	(CMF)	None available
ff1	CMF	One DD outcross
fn	CMF	Four GB outcrosses
f1f1	CNMF	DD, DD self progeny
f1n	CNMF	50% of DDxGB progeny
nn	CNMF	25% of GB self progeny

effect as complete absence of the gene (the null allele). A single PCR test using either endoPG-4 or endoPG-5 markers can distinguish between the three major phenotypes in peach: FMF = FF/Ff1/Fn and Ff, CMF = ff/fn and ff1, and CNMF = f1f1/f1n and nn (Figure 3 and Table 2). These results are similar to the control of rapid fruit softening by endoPG in *Capsicum annuum*, for which complete co-segregation was observed between an RFLP (presence/absence of a band) and the *Soft flesh* trait (Rao and Paran 2003). RFLP analysis of endoPG (Lester et al. 1996) was similarly limited to two alleles and two phenotypes. In contrast, the PCR test developed here is simpler to perform than RFLP, and can detect four alleles (though there are only three effective alleles as f1 and n have the same consequence) and some of the possible heterozygotes, and identifies three phenotypes.

The *in vivo* expression of the alternative alleles of endoPG in peach appears to match phenotype. Lester et al. (1994) reported that the gene was abundantly expressed during and after the melting phase in FMF fruit (F-), was expressed less in CMF fruit (ff/ff1/fn), and was expressed least for CNMF fruit (f1f1/f1n). Flesh firmness was in the order of FMF < CMF < CNMF (Lester et al. 1994). The transcript was an estimated 250 bp shorter for CNMF fruit than FMF and CMF fruit (Lester et al. 1994). Translation of this aberrant CNMF transcript (f1 allele) apparently does not occur, since Lester et al. (1996) did not detect the endoPG protein in ripe CNMF fruit that was present for ripe FMF and CMF fruit. The lack of significant endoPG activity in CNMF fruit (Pressey and Avants 1978) is consistent with this conclusion. However, gene expression levels, enzyme levels, enzyme activity, and quantitative changes in firmness have not been compared between CNMF individuals with the f1 allele and CNMF individuals homozygous for the null allele.

DNA sequence differences for the F, f, and f1 alleles were in the form of single nucleotide polymorphisms (SNPs) and indels that occurred entirely within non-coding sequences – the intron for endoPG-4 Figure 4), and the 3′ untranslated region for endoPG-5 (results not shown). Coding sequences of endoPG-4 were identical for all alleles of the 'Georgia Belle' and 'Dr. Davis' germplasm and the published sequences of Flavorcrest, Loring, and Suncrest (results not shown). For the endoPG-4 intron, the topmost band of the F allele (F^a) derived from 'Georgia Belle' was identical to that of the f1 allele derived from 'Dr. Davis', and these sequences contained two SNPs compared to Suncrest (at positions 23 and 402 in the clone AC1 sequence, Figure 4). Compared to the F^a, f1, and Suncrest intron sequences, F^b was 34 bp shorter and the f allele was 13 bp shorter (Figure 4), explaining their relative positions after electrophoresis (Figure 3). The F^b band was always present with the F^a band in FMF individuals (Figures 2 and 3), and may represent a duplication of at least part of the gene. Of the 26 SNPs between the introns for F^a and F^b, and the 31 SNPs between F^a and f, 13 were in common between F^b and f (Figure 4). In addition, one of the 17-nucleotide deletions in F^b was also in f, and a T_2 insertion in F^b was a T_3 insertion in f (Figure 4). The similarity with F^b may provide a clue to the origin of the f allele.

Sequence differences that result in altered phenotypes for the f and f1 alleles compared to the F allele presumably occur elsewhere at the locus than in endoPG-4 and endoPG-5. Lester et al. (1996) compared gene fragments of endoPG between CNMF (carrying the f1 allele) and FMF (carrying the F allele and potentially also the f1 or f allele, if heterozygous), using Southern analysis with the restriction enzyme *EcoR*I and a probe covering the gene from the second conserved domain to the 3′ untranslated region (Figure 1). These authors

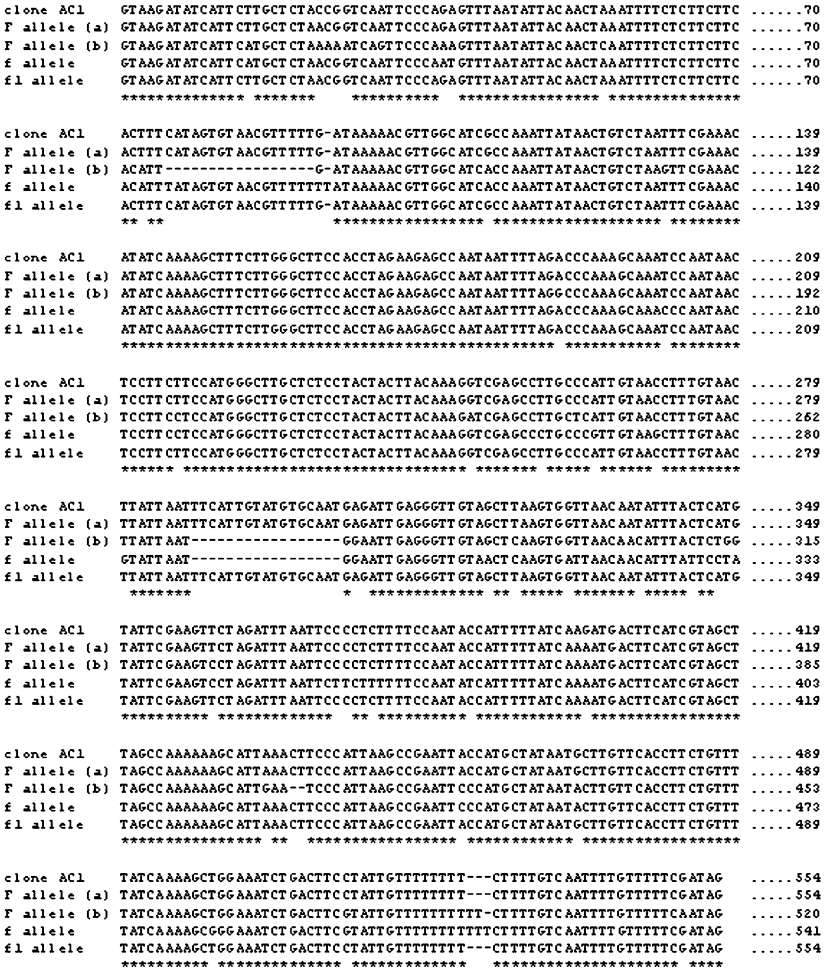

Figure 4. DNA sequence comparison of the intron within endoPG-4, for three alleles of the *Freestone-Melting flesh* locus. Not shown for this endoPG marker are 148 bp of sequence preceding the intron and 94 bp following the intron, which were identical for all sources. Sequences for both of the major bands of the F allele (a = F^a, b = F^b) are included.

reported three major fragments, two or all three of which were missing in CNMF individuals. Such results imply the presence of more than one copy of the gene at the locus, since the probe sequence included no restriction site and should have hybridized with only one fragment, as pointed out by Callahan et al. (2004). Another study using Southern analysis of endoPG, with a longer probe spanning almost the entire gene including the *EcoR*I restriction site, showed at least six fragments in FMF cultivars, one to six being absent in each of three lineages of CNMF cultivars, providing further evidence that multiple copies of endoPG gene exist at the locus (Callahan et al. 2004). Multiple gene copies may be the explanation for the multiple amplified bands of endoPG-1 to -4 (Figure 2), including the F^b band for endoPG-4 (with a lack of multiple bands for endoPG-5 not surprising as this fragment lies almost entirely in the little-conserved 3′ untranslated region).

The origins of each allele can be hypothesized based on endoPG-4 and −5 sequences. First, it is assumed that the original sequence (F^o) contained multiple copies of endoPG, including a gene containing the original endoPG-4 F^a sequence (F^{ao}) and another gene containing the original F^b sequence (F^{bo}). Then the F allele resulted from point mutations in F^{ao} and F^{bo}, the f allele resulted from deletion of F^{ao} and mutation of F^{bo}, and the

f1 allele resulted from deletion of F^{bo} and mutation of F^{ao} (to the point of lost gene function). The null allele could have arisen from deletion of both F^{ao} and F^{bo} in F^{o}, or deletion in any of the derived alleles. This scheme is consistent with the presence of endoPG gene copies being systematically lost in CNMF lineages as suggested by Callahan et al. (2004), with the f allele being just another gene deletion lineage. Coupling this scheme with phenotypes related to each allele leads to the hypothesis that the F^{a} sequence is part of the gene copy controlling *Freestone*, the F^{b} sequence is part of the gene copy controlling *Melting flesh*, the f allele lacks the *Freestone* gene, the f1 allele contains an incomplete version of the *Freestone* gene and lacks the *Melting flesh* gene, and the null allele lacks both genes. Further research is required to determine the extent of gene duplication and identify where the critical mutations occur. From additional sequencing of endoPG, we have observed coding sequence differences between the alleles, particularly in the conserved domains, which may account for phenotypic differences (unpublished data).

Conclusions

Although there is extensive circumstantial evidence in the literature that *Melting flesh* in peach is controlled by endoPG, doubts have remained without absolute correspondence between phenotype and DNA profile in a segregating population. The present study provides that evidence, which is consistent with observations in other studies of a peak of endoPG expression and enzyme activity occurring during the melting phase, late in peach fruit ripening. This study also provides strong evidence that *Melting flesh* and *Freestone* are the same locus, though it is unclear whether the same gene or different copies of endoPG at the locus control the two traits, and whether either is controlled by the gene represented by Contig694/PRF5/AC1. Furthermore, this study shows that various combinations of three effective alleles can explain the three major phenotypes, following the scheme first proposed by Monet (1989). A search for additional allelic variation at this locus is warranted. Other fruit softening phenotypes may be explained by the existence of further alleles, and PCR screening of diverse germplasm for endoPG could implicate novel phenotypes. Screening of further cultivars indicates that the genotype–phenotype scheme is maintained for most peach varieties, and the few exceptions observed appear to be due to further mutations within the alleles described here (unpublished data).

While many other enzymes are likely involved in softening of peach fruit, some being prerequisites for endoPG activity (Brummell and Harpster 2001; Trainotti et al. 2003), endoPG has the most significant effect detected to date. In genotypes where the gene, and hence enzyme, is lacking or impaired, softening is retarded to a very large degree. We suggest that the pleiotropic locus described here be referred to as *Freestone–Melting flesh*. Either of the two gene markers developed (endoPG-4 or endoPG-5) can be used as a 'perfect' (completely linked) PCR marker in marker-assisted selection for distinguishing between FMF, CMF, and CNMF progeny at the seedling stage in peach breeding programs.

Acknowledgements

We thank Dr. F. Bliss for establishing the progeny populations and for useful comments on the manuscript. For assessment of fruit, D. Garner is gratefully acknowledged, as are K. Barckley and J. Williamson for laboratory assistance, and Dr. R. Ahmad for help with DNA sequencing. This research was partially funded by California Tree Fruit Agreement and a UC Discovery Grant (bio01-10203) with the Industry-University Cooperative Research Program.

References

Aranzana M.J., Pineda A., Cosson P., Dirlewanger E., Ascasibar J., Cipriani G., Ryder C.D., Testolin R., Abbott A., King G.J., Iezzoni A.F. and Arus P. 2003. A set of simple-sequence repeat (SSR) markers covering the *Prunus* genome. Theor. Appl. Genet. 106: 819–825.

Bailey J.S. and French A.P. 1933. The inheritance of certain characteristics in the peach. Proc. Amer. Soc. Hortic. Sci. 29: 127–130.

Bailey J.S. and French A.P. 1949. The inheritance of certain fruit and foliage characteristics in the peach. Mass. Agr. Expt. Sta. Bul. 452.

Brummell D.A. and Harpster M.H. 2001. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. Plant Mol. Biol. 47: 311–340.

Callahan A.M., Scorza R., Bassett C., Nickerson M. and Abeles F.B. 2004. Deletions in an endopolygalacturonase gene cluster correlate with non-melting flesh texture in peach. Func. Plant Biol. 31: 159–168.

CUGI. 2003. Clemson University Genomics Institute Peach EST Project. URL http://www.enome.clemson.edu/projects/peach/est/.

Dettori M.T., Quarta R. and Verde I. 2001. A peach linkage map integrating RFLPs, SSRs, RAPDs, and morphological markers. Genome 44: 783–790.

Dirlewanger E., Cosson P., Tavaud M., Aranzana M.J., Poizat C., Zanetto A., Arus P. and Laigret F. 2002. Development of microsatellite markers in peach [*Prunus persica* (L.) Batsch] and their use in genetic diversity analysis in peach and sweet cherry (*Prunus avium* L.). Theor. Appl. Genet. 105: 127–138.

Etienne C., Rothan C., Moing A., Plomion C., Bodenes C., Svanella-Dumas L., Cosson P., Pronier V., Monet R. and Dirlewanger E. 2002. Candidate genes and QTLs for sugar and organic acid content in peach [*Prunus persica*(L.) Batsch]. Theor. Appl. Genet. 105: 145–159.

Foolad M.R., Arulsekar S., Becerra V. and Bliss F.A. 1995. A genetic map of *Prunus* based on an interspecific cross between peach and almond. Theor. Appl. Genet. 91: 262–269.

Hadfield K.A. and Bennett A.B. 1998. Polygalacturonases: many genes in search of a function. Plant Physiol. 117: 337–343.

Hadfield K.A., Rose J.K.C., Yaver D.S., Berka R.M. and Bennett A.B. 1998. Polygalacturonase gene expression in ripe melon fruit supports a role for polygalacturonase in ripening-associated pectin disassembly. Plant Physiol. 117: 363–373.

Hayashi T. and Yamamoto T. 2002. Genome research on peach and pear. J. Plant Biotech. 42: 45–52.

Hiwasa K., Kinugasa Y., Amano S., Hashimoto A., Nakano R., Inaba A. and Kubo Y. 2003. Ethylene is required for both the initiation and progression of softening in pear (*Pyrus communis* L.) fruit. J. Exp. Bot. 54: 771–779.

Lee E., Speirs J., Gray J. and Brady C.J. 1990. Homologies to the tomato endopolygalacturonase gene in the peach genome. Plant Cell Environ. 13: 513–521.

Lester D.R., Sherman W.B. and Atwell B.J. 1996. Endopolygalacturonase and the melting flesh (M) locus in peach. J. Amer. Soc. Hort. Sci. 121: 231–235.

Lester D.R., Speirs J., Orr G. and Brady C.J. 1994. Peach (*Prunus persica*) endopolygalacturonase cDNA isolation and mRNA analysis in melting and nonmelting peach cultivars. Plant Physiol. 105: 225–231.

Monet R. 1989. Peach genetics: past, present and future. Acta Hort. 254: 49–53.

Okie W.R. 1998. Handbook of peach and nectarine varieties: Performance in the southeastern United States and index of names. US Department of Agriculture, Agriculture Handbook No. 714.

Orr G. and Brady C. 1993. Relationship of endopolygalacturonase activity to fruit softening in a freestone peach. Postharvest Biol. Biotech. 3: 121–130.

Pressey R. and Avants J.K. 1978. Difference in polygalacturonase composition of clingstone and freestone peaches. J. Food Sci. 43: 1415–1423.

Pressey R., Hinton D.M. and Avants J.K. 1971. Development of polygalacturonase activity and solubilization of pectin in peaches during ripening. J. Food Sci. 36: 1070–1073.

Rao G.U. and Paran I. 2003. Polygalacturonase: a candidate gene for the soft flesh and deciduous fruit mutation in *Capsicum*. Plant Mol. Biol. 51: 135–141.

Redondo-Nevado J., Moyano E., Medino-Escobar N., Caballero J.L. and Munoz-Blanco J. 2001. A fruit-specific and developmentally regulated endopolygalacturonase gene from strawberry (*Fragaria ananassa* cv. Chandler). J. Exp. Bot. 52: 1941–1945.

Rozen S. and Skaletsky H.J. 1998. Primer3. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3. html.

Trainotti L., Zanin D. and Casadoro G. 2003. A cell wall-oriented genomic approach reveals a new and unexpected complexity of the softening in peaches. J. Exp. Bot. 54: 1821–1832.

Van Der Heyden C.R., Holford P. and Richards G.D. 1997. A new source of peach germplasm containing semi-freestone non-melting flesh types. HortScience 32: 288–289.

Waldron J., Peace C.P., Searle I.R., Furtado A., Wade N., Findlay I., Graham M.W. and Carroll B.J. 2002. Randomly amplified DNA fingerprinting (RAF): a culmination of DNA marker technologies based on arbitrarily-primed PCR amplification. J. Biomed. Biotech. 2: 141–150.

Weaver K.R., Caetano-Anolles G., Gresshoff P.M. and Callahan L.M. 1994. Isolation and cloning of DNA amplification products from silver-stained polyacrylamide gels. Biotechniques 16: 226–227.