Changes in endopolygalacturonase levels and characterization of a putative *endo-PG* gene during fruit softening in peach genotypes with nonmelting and melting flesh fruit phenotypes

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Summary

- The changes in endopolygalacturonase (endo-PG) levels and *endo-PG* expression in nonmelting flesh (NMF) and melting flesh (MF) peach fruits (*Prunus persica*) during softening were studied. The *endo-PG* gene was analysed to identify polymorphisms exploitable for early marker-assisted selection (MAS) of flesh texture.
- The role of endo-PG in softening was assessed by western and northern blotting and by biochemical analyses. Polymorphisms in the *endo-PG* gene were revealed by reverse transcription–polymerase chain reaction (RT–PCR) and sequencing.
- An endo-PG protein was detected in both NMF and MF fruits. The levels of this endo-PG protein were higher and increased with softening in MF fruits, but remained lower and were constant in NMF fruits. The different levels of endo-PG appeared to be caused by the differential expression of an *endo-PG* gene, whose open-reading frame (ORF) showed five single nucleotide polymorphisms (SNPs) in NMF 'Oro A' compared with MF 'Bolero'. One of these SNPs allowed us to determine the allelic configuration at the *melting flesh* (*M*) locus and also seemed to be exploitable for early MAS in other NMF/MF phenotypes.
- The NMF phenotype does not seem to be caused by a large deletion of the *endo-PG* gene.

Key words: cleaved amplified polymorphic sequence (CAPS), endopolygalacturonase (endo-PG) levels, *endo-PG* expression, marker-assisted selection (MAS), nonmelting flesh/melting flesh phenotype, *Prunus persica*, single nucleotide polymorphisms (SNPs).

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Introduction

Overall organoleptic quality of the peach (*Prunus persica*) fruit is mainly caused by the components of fruit taste (sugars, organic acids and volatiles) and flesh texture. The latter is one of the most important factors determining the consumer's perception of peach fruit quality (Bruhn, 1994). Peaches are classified as either melting flesh (MF) or nonmelting flesh (NMF) on the basis of fruit texture. Decrease in fruit firmness occurs during ripening of both phenotypes, but is more pronounced in MF fruits, where marked loss of firmness ('melting') occurs in the final stages of ripening (Brovelli *et al.*, 1998). For this reason, if MF peaches are left to ripen on the tree to achieve maximum eating quality, they are very easily damaged during handling and shipping. Some current breeding programmes are developing NMF peaches for the fresh market which can fully ripen on the tree and still retain sufficient firmness to allow satisfactory handling and shelf-life.

Peach trees must be grown for at least 3 yr before they bear fruit, which makes breeding time-consuming, especially for

fruit-specific characters. The dissection of fruit quality into a number of elementary components linked to genes of known function would be extremely useful for the early marker-assisted selection of fruit with desirable characteristics. Genes have been identified which control sucrose accumulation and fruit ripening in tomato (Yen *et al.*, 1995; Klann *et al.*, 1996) and carotenoid accumulation in red pepper (Huh *et al.*, 2001). In peach, a few quantitative trait loci (QTLs) involved in fruit quality, such as size, titratable acidity, levels of malic and citric acids, levels of soluble sugars and soluble solids, have been identified (Abbott *et al.*, 1998; Dirlewanger *et al.*, 1998, 1999; Quarta *et al.*, 2000). A candidate gene–QTL co-location, involving a cDNA encoding a tonoplast H^+-pyrophosphatase which energizes solute accumulation into the vacuole and QTLs for sucrose and soluble solid content, has been described recently (Etienne *et al.*, 2002).

Likewise, identification of a single (or a few) genetic traits unequivocally linked to the process of flesh softening would be of great benefit. A genetic marker for the MF trait is also especially desirable because the trait shows only when fruits are mature.

Flesh firmness can be considered as a quantitative trait, being affected by several biochemical and physiological factors, such as differences in the levels of apoplastic Ca^{2+}, regulation of cell turgor in relation to plasma membrane functionality and levels of intracellular osmolytes, changes in cell wall architecture, etc. The identification and cloning of genes of known function (i.e. genes involved in cell wall loosening) would allow evaluation of the possible effects of their polymorphism/s on flesh firmness variability.

Cell wall changes associated with the softening process, including modifications of the structure and composition of the constituent polysaccharides, have been related to the expression of a number of hydrolases and transglycosylases (reviewed in Fischer & Bennett, 1991). The activities of several hydrolytic enzymes, such as polygalacturonases (PGs), pectinmethylesterases, endo-1,4-β-glucanases, endo-1,4-β-mannanase, α-arabinosidase and β-galactosidase (Hatfield & Nevins, 1986; Bonghi *et al.*, 1998; Brummell *et al.*, 2004) have been shown to increase during fruit ripening. The role of PG in fruit ripening has been studied extensively (reviewed in Hadfield & Bennett, 1998). In peach, Pressey & Avants (1973) showed that endo-PG (EC 3.2.1.15) and exo-PG (EC 3.2.1.67) were present in ripe fruit and that endo-PG activity increased during ripening, particularly in MF peaches (Orr & Brady, 1993). The expression of the *endo-PG* gene was very abundant in ripe MF fruits, whereas in NMF peaches only a deleted mRNA was detectable (Lester *et al.*, 1994). The NMF phenotype was ascribed to a partial or a complete deletion of the *endo-PG* gene (Lester *et al.*, 1996; Callahan *et al.*, 2004) and no PG protein was detected in the NMF peaches studied, whereas it was detected in MF phenotypes (Lester *et al.*, 1996). In other species, a close relationship between endo-PG and flesh softening has also been recognized. In pepper, the soft flesh and deciduous fruit is a dominant trait controlled by the *S* gene encoding PG (Rao & Paran, 2003). In European pear, softening closely parallels *endo-PG* expression (Hiwasa *et al.*, 2003).

In the present study we examined the changes during ripening and flesh softening in the expression of an *endo-PG* gene, accumulation of endo-PG protein and degrees of endo-PG activity in the flesh of two parents ('Oro A', with the NMF phenotype, and 'Bolero', with the MF phenotype) and of two of their offspring. An analysis of the structure of the *endo-PG* gene has also been conducted in these and other NMF/MF accessions in order to identify polymorphisms that are potentially exploitable for the early marker-assisted selection of flesh firmness.

Materials and Methods

Plant material

Peach (*P. persica* L. Batsch) accessions grown at the Experimental Orchard of the Arboriculture, Viticulture, Forestry and Landscape Department of the University of Bologna (Italy) were used. Fruits from different NMF and MF accessions were monitored on the tree during ripening. The NMF/MF phenotype was assessed by sensory evaluation at the eating-ripe stage. 'Oro A' (NMF) and 'Bolero' (MF) were assessed together with their offspring 'BO 96014125' (NMF) and 'BO 96014137' (MF). Other accessions considered were 'Andross', 'Jonia' and 'BO 82010054' for the NMF phenotype and 'Springcrest', 'Springbelle' and 'Maycrest' for the MF phenotype. For each accession, all fruits were harvested at the same time, when the very first fruits on the tree were physiologically ripe. All fruits could be considered to be in stages 3–4 (Westwood, 1978) and covered the range from immature (full size fruit, no softening) to ripe (at the climacteric peak, with a fruit firmness of 20–30 N) and over-ripe (< 10 N).

Ripening parameters and tissue sampling

Immediately after harvest, all of the fruits from each accession were divided into ripening classes, based on epicarp ground colour as a maturity index (Delwiche & Baumgardner, 1985). Epicarp colour parameters were measured at two different locations in the equatorial zone or from low or no-blush areas by using a Minolta Chromameter CR-200 reflectance colorimeter (Minolta Co., Osaka, Japan) (Robertson *et al.*, 1990). For a preliminary classification of the fruits, the Minolta 'a' value, which records the degree of green-to-red pigmentation, was taken as being representative of the degree of ripening.

Flesh firmness measurements were performed by a hand penetrometer (Effegi, Milan, Italy) with an 8-mm flat probe at two equidistant locations in the equatorial region of each fruit after epicarp removal. Data were expressed in Newtons.

The epicarp and stone were removed and mesocarp samples from single fruits of known firmness were snap-frozen in liquid

N_2 and stored at −80°C. All subsequent analyses were conducted on single fruits.

Protein extraction from mesocarp cell walls

Protein extraction from mesocarp cell walls was performed as described by Moore & Bennett (1994), with minor modifications. Mesocarp tissue (15 g) was crushed to powder with liquid N_2 and 10% (w/w) polyvinylpolypyrrolidone (PVPP) in a mortar. Four volumes of ice-cold low-salt extraction buffer [10 mM Na-acetate, pH 5.5, 20 mM β-mercaptoethanol (β-ME), 2 mM phenylmethylsulfonylfluoride (PMSF)] were added. The thawed slurry was centrifuged at 10 000 ***g*** for 20 min at 4°C and the pellet washed with the same ice-cold buffer [four volumes on the basis of fresh weight (FW)]. After centrifugation, the pellet was resuspended in one volume per gram FW of high-salt extraction buffer (40 mM Na-acetate buffer, pH 5.5, 1.5 M NaCl, 20 mM β-ME, 2 mM PMSF) and stirred overnight at 4°C. The slurries were then centrifuged (10 000 ***g***, 30 min, 4°C) after which the supernatants (high-salt extracts) were filtered through Miracloth (Calbiochem, Milan, Italy) and through a 45 μm polyvinylidene difluoride (PVDF) membrane and then spun in a microconcentrator (Centricon Plus-20; Millipore, Billerica, MA, USA), with a total of two volumes of the same buffer without NaCl added at intervals to reduce the salt concentration. The final average protein concentration recovered in the cell wall extracts was approx. 3 μg of protein per μl. Suspensions were used either as such for nondenaturing polyacrylamide gel electrophoresis (PAGE) and determination of PG activity or treated with Plus One 2-D Clean-Up Kit (GE Healthcare Europe GmbH, Milan, Italy) for protein sodium dodecyl sulfate (SDS)–PAGE and immunoblotting.

Protein determination

Protein content was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard (Micro-Bio-Rad Protein Assay; Bio-Rad Laboratories, Segrate, Italy).

Nondenaturing PAGE and activity staining

Nondenaturing PAGE was performed at 4°C in a MiniProtean™ apparatus (Bio-Rad Laboratories) and in 10% polyacrylamide gels, as described by Moore & Bennett (1994). After electrophoresis, gels for activity staining were equilibrated twice, for 15 min, in 10 mM Na-acetate buffer containing 1 mM 1,2-di(2-aminoethoxy) ethane-*N,N,N′,N′*-tetra-acetic acid (EGTA), pH 4.0 (PG assay buffer) at 30°C, incubated for 4 h at 30°C in 0.2% (w/v) polygalacturonic acid (washed with 80% ethanol before use), pH 4.0, and finally transferred into the same assay buffer for an additional 20 h. Gels were then briefly rinsed with water before staining for 10 min in 0.5% (w/v) methylene blue and destaining with water.

SDS–PAGE

Salt-extracted proteins from mesocarp cell walls were denatured in SDS sample buffer (Laemmli, 1970) and then analysed by tricine-SDS–PAGE (10% total acrylamide/bis-acrylamide concentration; Schägger & von Jagow, 1987) in an SE 600 Ruby Standard Dual Cooled Vertical Unit (Amersham Biosciences); gels were silver stained according to Heukeshoven & Dernick (1985). Molecular weight markers were from Sigma-Aldrich (Sigma-Aldrich s.r.l., Milan, Italy).

Anti-endo-PG polyclonal antibodies

Database query [accession no. (acc. no.) CAA54150] allowed us to identify a conserved region (residues 355–369: CREIKLEDVKLTYKN) in the complete sequence of an endo-PG of ripe peach fruits (Lester *et al.*, 1994). This was used by the Primm Company (Milan, Italy) to synthesize the corresponding $_2$HN-CREIKLEDVKLTYKN-COOH polypeptide, which was subsequently conjugated with the immunogenic peptide keyhole limpet haemocyanin (KLH) and inoculated in rabbit to produce antibodies. Antiserum was collected after 48 d and tested for antibody titre.

Immunoprecipitation

Salt-extracted proteins from mesocarp cell walls were added with the anti-endo-PG rabbit antiserum (1 : 5 total protein ratio), incubated on ice with gentle agitation for 1 h and immunoprecipitated by the addition of protein A–Sepharose (Sigma-Aldrich), activated according to the manufacturer's instructions. After an additional 1 h on ice with gentle agitation, immune complexes were removed by centrifugation (16 000 ***g***, 1 min at 4°C).

Western blotting

Proteins were electroblotted for immunodetection onto PVDF membrane in a Multiphor II Nova-Blot (GE Healthcare Europe) apparatus. Nondenaturing gels were preincubated in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS)-NaOH buffer, pH 11.0, containing 10% (v/v) methanol and 0.1% (w/v) SDS, three times, for 10 min each, before electroblotting. Protein transfer was carried out at 4°C (nondenaturing gels) or at room temperature (SDS–PAGE) at 0.8 mA cm^{-2} in the same CAPS-NaOH buffer without SDS. After blocking [for 2 h, in PBS-Tween (PBS-T; 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% (v/v) Tween-20) containing 3% (w/v) nonfat dry milk powder], membranes were incubated overnight at 4°C with the anti-endo-PG antiserum (1 : 3000 dilution in PBS-T). Blots were washed in PBS-T, incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody and then treated with chemiluminescent reagents (GE Healthcare Europe ECL Western Blotting Detection Kit) before exposure to Hyperfilm ECL (GE Healthcare Europe).

In vitro assay of endo-PG activity

Aliquots of the protein suspensions obtained by saline extraction of mesocarp cell walls followed by concentration (see the section on protein extraction from mesocarp cell walls) were used to determine *in vitro* endo-PG activity (Pressey & Avants, 1973; Gross, 1982).

RNA isolation and cloning of *endo-PG* cDNA

Total RNA was extracted from frozen mesocarp tissue (10 g) according to Loulakakis *et al.* (1996). First-strand cDNA was synthesized from 1 μg of total RNA using Superscript II Reverse Transcriptase (Invitrogen, San Giuliano, Italy) primed with oligo(dT)$_{12–18}$ according to the manufacturer's instructions. *Endo-PG* sequences were amplified by the polymerase chain reaction (PCR; 2 min of initial denaturation at 95°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 40 s and a 1-min extension at 72°C) using two primers from the PRF5 *endo-PG* cDNA sequence (Lester *et al.*, 1994, forward: 5′-ATGGCGAACCGTAGAAGCCTCT-3′; reverse: 5′-CTACAAACAACTTGTAGGCTGAAC-3′). Reaction products were analysed in a 1.5% (w/v) agarose gel. A single band (of approx. 1200 bp) was purified and cloned into a pCR®4-Blunt II-TOPO® vector (Invitrogen). Sequences were determined by the Primm Company.

Northern analysis

Twenty micrograms of total RNA from fruit mesocarp were denatured and fractionated on a 1% formamide/formaldehyde denaturing gel. Fractionated RNA was transferred onto Hybond-N membrane (GE Healthcare Europe) by capillary transfer with 10× standard saline citrate (SSC; 1× : 0.15 M NaCl, 15 mM Na-citrate, pH 7.0). The RNA was fixed at 80°C for 2 h. The membrane was hybridized with ^{32}P[dATP]-labelled *endo-PG* probe (see the 'RNA isolation and cloning of *endo-PG* cDNA' section) synthesized using the PRIME-A-GENE® labelling system (Promega, Milan, Italy) at 60°C overnight and then washed three times at room temperature (20 min, 0.1 × SSC, 1% (w/v) (SDS) followed by two additional washes at 65°C. The blot was then exposed to X-ray film (X-OMAT AR; Kodak, Rochester, NY, USA) at −80°C, and the film developed according to the manufacturer's recommendations.

Isolation of DNA and genotyping

Total genomic DNA from young leaves (100–150 mg FW) was prepared according to Geuna *et al.* (2004). DNAs were used as template for amplification with two primers, designed after alignment of the *endo-PG* cDNA clone with the partial genomic sequence of the peach *endo-PG AC1* clone (acc. no.: AY262754; forward: 5′-AGGCGTTGCTTGTGGACCTG-3′; reverse: 5′-CTCGCTGCAAGGGTGCTTGGGAC-3′) at the annealing temperature of 60°C. Restriction endonuclease digestion was carried out on the amplification products using the restriction enzyme *Bsr*SI (Promega). The restriction enzyme mix (10 μl), containing 2 μl of restriction enzyme buffer and 15 U of enzyme, was added to each tube and incubated for 3 h at 65°C. Fragments were then separated on 2% (w/v) agarose gels, visualized by ethidium bromide staining and sequenced (Primm).

Chemicals

All chemicals, unless stated otherwise, were purchased from Sigma-Aldrich.

Results

Peach fruit flesh firmness during ripening

All fruits from each accession were collected on a single date and sorted into classes on the basis of epicarp colour. Within each genotype, increasing 'a' values (degree of green-to-red pigmentation, data not shown) were taken as representative of increased ripening. Fruit firmness decreased with ripening. In 'Oro A' and 'BO 96014125', softening was less than in 'Bolero' and 'BO 96014137'. For the two accessions ('Oro A', 'BO 96014125') that had been classified as NMF in the field, the firmness values of the softest fruits was 25 ± 3 N. In the accessions classified as MF ('Bolero', 'BO 96014137'), the firmness of the softest fruits was 10 ± 2 N.

Levels of endo-PG polypeptide in fruit mesocarp

In order to assess whether the polyclonal antibodies produced (see the Materials and Methods) recognized the corresponding functional endo-PG protein, proteins were salt-extracted from a cell wall-enriched fraction from the mesocarp of a 'Bolero' fruit (MF phenotype) at the lowest firmness (12 N), separated by nondenaturing electrophoresis and assayed for both immunoreaction and endo-PG activity in a duplicate gel. The position of the cross-reaction band after nondenaturing electrophoresis (Fig. 1a, lane 1) closely matched that of the corresponding band of activity staining (Fig. 1b, lane 3), indicating that the antibodies against the synthetic polypeptide recognized a protein of the fruit cell walls, and that this protein had endo-PG activity. Pre-immune serum did not detect the protein (data not shown). The result was confirmed by the disappearance of both immunoreaction and endo-PG activity following removal of the antigen (endo-PG) from the protein extracts by immunoprecipitation with protein A–Sepharose (Fig. 1a,b, lanes 2 and 4, respectively).

Proteins were then extracted from the cell wall-enriched fraction of the flesh of single fruits of decreasing firmness from each accession, separated by SDS–PAGE and western blotted using anti-endo-PG antibodies as the probe. Figure 1(c) shows that the anti-endo-PG antibodies reacted with a polypeptide

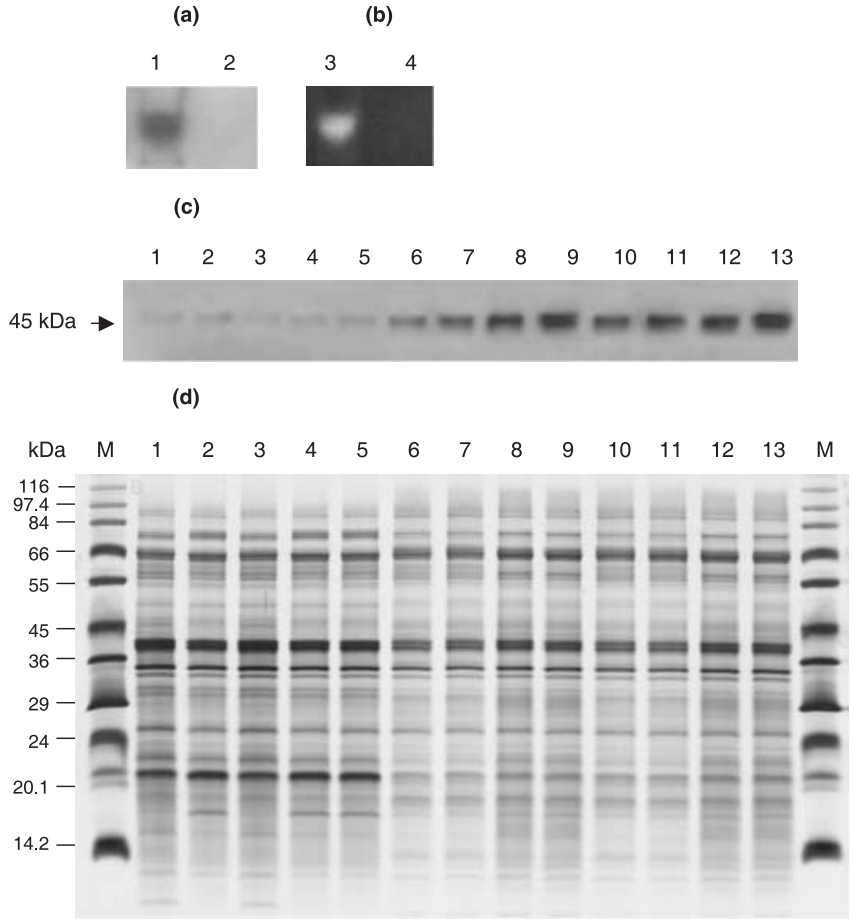

Fig. 1 Levels of endopolygalacturonase (endo-PG) in peach (*Prunus persica*) fruits with different nonmelting flesh (NMF)/melting flesh (MF) phenotypes. (a) Antibody validation. Western blot analysis was conducted after nondenaturing polyacrylamide gel electrophoresis (PAGE) of salt-extracted proteins from mesocarp cell walls of a soft (12 N) 'Bolero' fruit. The blot was reacted with the polyclonal antibodies raised against a conserved sequence of ripe peach endo-PG and visualized by chemiluminescence (see the Materials and Methods). (b) Activity staining was conducted in a duplicate gel of that shown in (a), as described in the Materials and Methods. Lanes 1 and 3, 20 μg of protein; lanes 2 and 4, after immunoprecipitation of the same initial protein amount with protein A–Sepharose. The results of one experiment, representative of three, are shown. (c) Immunoblotting of endo-PG polypeptide after sodium dodecyl sulphate (SDS)–PAGE of proteins from the mesocarp cell walls of peach fruits with different NMF/MF phenotypes. Lane 1, 'Oro A' at 46 N; lane 2, 'Oro A' at 34 N; lane 3, 'BO 96014125' at 49 N; lane 4, 'BO 96014125' at 32 N; lane 5, 'BO 96014125' at 28 N; lane 6, 'Bolero' at 54 N; lane 7, 'Bolero' at 47 N; lane 8, 'Bolero' at 28 N; lane 9, 'Bolero' at 12 N; lane 10, 'BO 96014137' at 46 N; lane 11, 'BO 96014137' at 39 N; lane 12, 'BO 96014137' at 20 N; and lane 13, 'BO 96014137' at 6.9 N. (d) Silver stain image of a duplicate gel of that electroblotted for (c). Lane M, molecular mass markers; lanes 1–13, as detailed for (c). Loading (20 μg protein per lane) was equal in (c) and (d); the results of one experiment, representative of three, are shown.

band of approx. 45 kDa, a value consistent with the molecular mass values reported for catalytically active PG forms (Lee *et al.*, 1990 and references therein; Brummell & Harpster, 2001). The endo-PG polypeptide was present in both NMF and MF fruits, but at different levels compared with the equal protein loading of each lane (Fig. 1d). In all NMF fruits screened, the endo-PG polypeptide levels were very low and essentially independent of the value of flesh firmness (Fig. 1c, lanes 1–5), while in MF fruits the levels were higher, clearly detectable even in fruits with the firmest flesh, and increased markedly as flesh firmness decreased (Fig. 1c, lanes 6–13). Nevertheless, no quantitative correlation between the values of flesh firmness and the amounts of the polypeptide were demonstrated. Fruits with essentially the same value of flesh firmness (*c.* 46 N) showed barely detectable accumulation of the endo-PG polypeptide in NMF 'Oro A' (Fig. 1c, lane 1) and much higher accumulation in MF 'Bolero' (Fig. 1c, lane 7) and 'BO 96014137' (Fig. 1c, lane 10). The same was true at 28 N flesh firmness for NMF 'BO 96014125' (Fig. 1c, lane 5) and MF 'Bolero' (Fig. 1c, lane 8). In addition, even within the same phenotypic group, no quantitative correlation was observed: in MF fruits, 'Bolero', at 47 N flesh firmness (Fig. 1c, lane 7), showed lower levels of the endo-PG polypeptide than 'BO 96014137' at 46 N (Fig. 1c, lane 10).

In vitro endo-PG activities of protein extracts from the cell walls of the same fruits, already tested for production of the

endo-PG polypeptide, which showed maximum and minimum values of flesh firmness, ranged from an average value of *c.* 600 pmol reducing groups released per μg of protein in 24 h in NMF 'Oro A' and 'BO 96014125', to *c.* 1200 pmol reducing groups released per μg of protein in 24 h in MF 'Bolero' and 'BO 96014137', consistent with the higher levels of endo-PG polypeptide of MF fruits (Fig. 1c) and confirming data previously reported in the literature (Pressey & Avants, 1978; Orr & Brady, 1993; Brummell *et al.*, 2004).

Isolation and sequencing of endo-PG cDNA clones

In order to clarify the nature of the differences between NMF and MF fruit phenotypes with regard to the accumulation of the endo-PG polypeptide, regulation involving mRNA levels was investigated.

RNAs were extracted from the mesocarp of NMF 'Oro A' or MF 'Bolero' fruits at their maximum flesh softening (34 N and 12 N, respectively); by reverse transcription (RT)–PCR with primers (see the Materials and Methods) designed on the peach PRF5 *endo-PG* cDNA (Lester *et al.*, 1994), two cDNA fragments, one per each parental accession, were isolated (GenBank acc. no. DQ340810 for 'Oro A' and DQ340809 for 'Bolero', respectively). Molecular analysis (sequencing, alignment and comparison; Fig. 2) showed that both cDNA clones from 'Oro A' and 'Bolero' fruits were 1182 bp long and corresponded to the complete PRF5 open reading frame (ORF) of the *endo-PG* gene from the MF peach cultivar 'Flavorcrest' (Lester *et al.*, 1994, GenBank acc. no. X76735). The ORF from MF 'Bolero' fruits presented seven single nucleotide polymorphisms (SNPs, at nucleotide positions 71, 76, 413, 533, 568, 569 and 1066) compared with the *endo-PG* sequence of MF 'Flavorcrest'. All of these SNPs induce a change in amino acid composition ($Ser_{Fla}24$ to $Thr_{Bol}24$, $Leu_{Fla}26$ to $Val_{Bol}26$, $Cys_{Fla}138$ to $Ser_{Bol}138$, $Phe_{Fla}178$ to $Cys_{Bol}178$, $Arg_{Fla}190$ to $Ala_{Bol}190$, $Arg_{Fla}356$ to $Ser_{Bol}356$; Fig. 3). The ORF sequence from NMF 'Oro A' fruits, compared with that of MF 'Bolero', showed five SNPs at nucleotide positions 146, 516, 576, 579 and 806 (Fig. 2). Three (positions 516, 576, 579) did not induce any changes in the encoded amino acid, while the SNPs at positions 146 and 806 induced substitutions, respectively, of $Ser_{Bol}49$ to $Phe_{Oro}49$ and $Ser_{Bol}269$ to $Thr_{Oro}269$. In particular, the $Thr_{Oro}269$ residue can also be found in the polypeptidic sequence deduced from the *endo-PG* ORF of NMF fruits 'Feicheng' (NCBI acc. no. AF095577; Fig. 3).

The general comparison of the deduced amino acid sequences of the endo-PG protein of MF 'Bolero' and NMF 'Oro A' fruits with those of MF 'Flavorcrest' and NMF 'Feicheng' fruits confirms the presence of features widely shared among other eukaryotic PGs [i.e. four conserved domains, nine conserved Cys residues (55, 81, 135, 143, 221, 238, 318, 349, 355), a highly conserved His residue (position 242) in a Gly-rich region corresponding to domain III assigned to a catalytic function in all PGs sequenced, and a Tyr residue (position 311) strictly conserved and essential for activity (Hadfield & Bennett, 1998; Hadfield *et al.*, 1998; Redondo-Nevado *et al.*, 2001)].

Expression of an endo-PG gene in peach fruit mesocarp

Our results show that an endo-PG polypeptide was present in the flesh of both NMF 'Oro A' and MF 'Bolero' fruits (Fig. 1c), suggesting that in both phenotypes an *endo-PG* gene was expressed and translated, and produced at least one form of endo-PG recoverable (even if in lesser amounts in NMF than in MF fruits) in protein extracts obtained from the mesocarp cell walls. This hypothesis was strengthened by the data on the recovery of complete and only slightly different *endo-PG* cDNA clones from ripe fruits from both 'Oro A' and 'Bolero' genotypes (Fig. 2).

RNAs produced in NMF 'Oro A' and MF 'Bolero' fruits with progressively decreasing values of flesh firmness (46 N and 34 N for 'Oro A'; 54 N, 47 N, 28 N and 12 N for 'Bolero') were extracted and analysed by northern blotting, using the ^{32}P-labelled *endo-PG* cDNA, obtained from ripe 'Bolero' fruits, as a probe. Figure 4 shows that the *endo-PG* transcript was produced in both NMF 'Oro A' and MF 'Bolero' fruits. In 'Oro A', *endo-PG* mRNAs were absent in the firmest fruits and were produced to a small extent only in the softest fruits (Fig. 4, lanes 5, 6), whereas in 'Bolero' their levels tended to be higher and markedly increased with flesh softening (Fig. 4, lanes 1–4).

These data are consistent with those concerning the endo-PG protein levels (as detected by western blots, Fig. 1c), indicating that in the NMF 'Oro A' genotype an *endo-PG* gene is also present and is expressed with the production of an endo-PG protein.

Molecular and CAPS analysis of an endo-PG fragment from genomic DNA

Out of the five SNPs which characterized the *endo-PG* ORF in the NMF 'Oro A' accession, the one located at position 806 ($G_{Bol}\rightarrow\underline{C}_{Oro}$) generates a restriction site in the sequence A$\underline{C}$TG GN recognized by the enzyme *Bst*SI. The same restriction region was also found 48 bp downstream (nucleotides 853–858) in the ORFs of both 'Oro A' and 'Bolero' (Fig. 2). The polymorphism at position 806 of the *endo-PG* ORF sequence allowed us to develop a molecular marker with the CAPS (Koniekzny & Ausubel, 1993) procedure in order to obtain accession-specific digestion patterns of the peach *endo-PG* gene considered.

The *endo-PG* ORF sequences from 'Oro A' and 'Bolero' were aligned with the genomic sequence of the peach *endo-PG AC1* clone (acc. no. AY262754; Callahan *et al.* 2004), which represents the largest fragment of genomic sequence considered allelic to PRF5, available to date in GenBank. This allowed us to design primers useful for amplifying, in the genotypes of

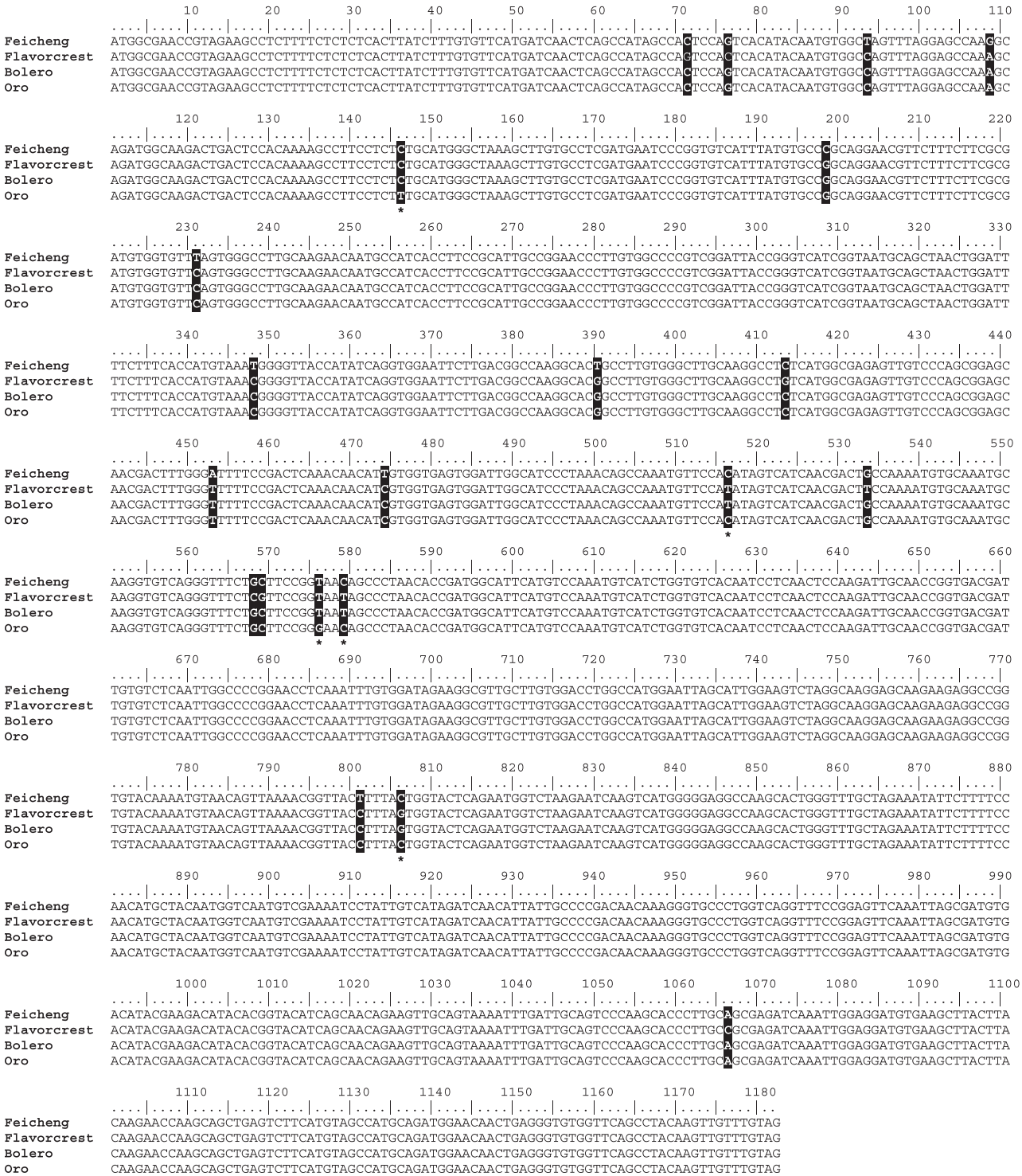

Fig. 2 Comparison of the nucleotide sequences of the peach (*Prunus persica*) *endopolygalacturonase* (*endo-PG*) open-reading frames (ORFs) from nonmelting flesh (NMF) 'Feicheng' (accession no. AF095577), melting flesh (MF) 'Flavorcrest' (accession no. X76735), MF 'Bolero' and NMF 'Oro A' fruits. The 'Bolero' and 'Oro A' sequences were obtained from the *endo-PG* cDNA clones from ripe fruits. The reverse contrast marks the presence of single nucleotide polymorphisms (SNPs); asterisks mark SNPs between 'Bolero' and 'Oro A'.

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                10        20        30        40        50        60
          ....|....|....|....|....|....|....|....|....|....●....|
Feicheng  MANRRSLFSLSLIFVFMINSAIATPVTYNVASLGAKADGKTDSTKAFLSAWAKACASMNP 60
Flavorcrest MANRRSLFSLSLIFVFMINSAIASPLTYNVASLGAKADGKTDSTKAFLSAWAKACASMNP 60
Bolero    MANRRSLFSLSLIFVFMINSAIATPVTYNVASLGAKADGKTDSTKAFLSAWAKACASMNP 60
OroA      MANRRSLFSLSLIFVFMINSAIATPVTYNVASLGAKADGKTDSTKAFLFAWAKACASMNP 60

                70        80        90       100       110       120
          ....|....|....|....|●...|....|....|....|....|....|....|....|
Feicheng  GVIYVPAGTFFLRDVVFSGPCKNNAITFRIAGTLVAPSDYRVIGNAANWIFFHHVNGVTI 120
Flavorcrest GVIYVPAGTFFLRDVVFSGPCKNNAITFRIAGTLVAPSDYRVIGNAANWIFFHHVNGVTI 120
Bolero    GVIYVPAGTFFLRDVVFSGPCKNNAITFRIAGTLVAPSDYRVIGNAANWIFFHHVNGVTI 120
OroA      GVIYVPAGTFFLRDVVFSGPCKNNAITFRIAGTLVAPSDYRVIGNAANWIFFHHVNGVTI 120

               130       140       150       160       170       180
          ....|....|....●....|..●.|....|....|....|....|....|..●.|
Feicheng  SGGILDGQGTALWACKASHGESCPSGATTLGFSDSNNIVVSGLASLNSQMFHIVINDCQN 180
Flavorcrest SGGILDGQGTALWACKACHGESCPSGATTLGFSDSNNIVVSGLASLNSQMFHIVINDFQN 180
Bolero    SGGILDGQGTALWACKASHGESCPSGATTLGFSDSNNIVVSGLASLNSQMFHIVINDCQN 180
OroA      SGGILDGQGTALWACKASHGESCPSGATTLGFSDSNNIVVSGLASLNSQMFHIVINDCQN 180

               190       200       210       220       230       240
          ....|....|....|....|....|....|....|....|●...|....|....|..●.|
Feicheng  VQMQGVRVSASGNSPNTDGIHVQMSSGVTILNSKIATGDDCVSIGPGTSNLWIEGVACGP 240
Flavorcrest VQMQGVRVSRSGNSPNTDGIHVQMSSGVTILNSKIATGDDCVSIGPGTSNLWIEGVACGP 240
Bolero    VQMQGVRVSASGNSPNTDGIHVQMSSGVTILNSKIATGDDCVSIGPGTSNLWIEGVACGP 240
OroA      VQMQGVRVSASGNSPNTDGIHVQMSSGVTILNSKIATGDDCVSIGPGTSNLWIEGVACGP 240

               250       260       270       280       290       300
          ....|....|....|....|....|....|....|....|....|....|....|....|
Feicheng  GHGISIGSLGKEQEEAGVQNVTVKTVTFTGTQNGLRIKSWGRPSTGFARNILFQHATMVN 300
Flavorcrest GHGISIGSLGKEQEEAGVQNVTVKTVTFSGTQNGLRIKSWGRPSTGFARNILFQHATMVN 300
Bolero    GHGISIGSLGKEQEEAGVQNVTVKTVTFSGTQNGLRIKSWGRPSTGFARNILFQHATMVN 300
OroA      GHGISIGSLGKEQEEAGVQNVTVKTVTFTGTQNGLRIKSWGRPSTGFARNILFQHATMVN 300
           *
               310       320       330       340       350       360
          ....|....|.●..|..●.|....|....|....|....|...●|....●....|
Feicheng  VENPIVIDQHYCPDNKGCPGQVSGVQISDVTYEDIHGTSATEVAVKFDCSPKHPCSEIKL 360
Flavorcrest VENPIVIDQHYCPDNKGCPGQVSGVQISDVTYEDIHGTSATEVAVKFDCSPKHPCREIKL 360
Bolero    VENPIVIDQHYCPDNKGCPGQVSGVQISDVTYEDIHGTSATEVAVKFDCSPKHPCSEIKL 360
OroA      VENPIVIDQHYCPDNKGCPGQVSGVQISDVTYEDIHGTSATEVAVKFDCSPKHPCSEIKL 360
             *
               370       380       390
          ....|....|....|●...|....|....|...
Feicheng  EDVKLTYKNQAAESSCSHADGTTEGVVQPTSCL 393
Flavorcrest EDVKLTYKNQAAESSCSHADGTTEGVVQPTSCL 393
Bolero    EDVKLTYKNQAAESSCSHADGTTEGVVQPTSCL 393
OroA      EDVKLTYKNQAAESSCSHADGTTEGVVQPTSCL 393
```

Fig. 3 Amino acid sequence comparison of the predicted peach (*Prunus persica*) endopolygalacturonase (endo-PG) proteins, as deduced from the cDNA sequences of nonmelting flesh (NMF) 'Feicheng', melting flesh (MF) 'Flavorcrest', MF 'Bolero' and NMF 'Oro A' fruits reported in Fig. 2. Conserved domains with other fruit polygalacturonases are underlined. Dots indicate conserved cysteine residues. Asterisks mark highly conserved His and Tyr residues essential for activity (Redondo-Nevado *et al*., 2001). Bold letters indicate differences in amino acids between 'Flavorcrest' (accession no. CAA54150) and 'Bolero'; the reverse contrast indicates differences in amino acids between 'Bolero' and 'Oro A'.

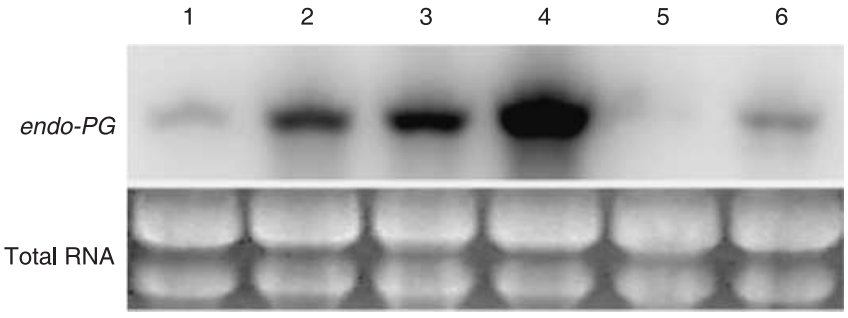

Fig. 4 Expression analysis of an *endopolygalacturonase* (*endo-PG*) gene in nonmelting flesh (NMF) 'Oro A' and melting flesh (MF) 'Bolero' peach (*Prunus persica*) fruits with decreasing flesh firmness. Upper panel: hybridization with the *endo-PG* cDNA clone from soft MF 'Bolero' fruits. Lane 1, MF 'Bolero' at 54 N; lane 2, MF 'Bolero' at 47 N; lane 3, MF 'Bolero' at 28 N; lane 4, MF 'Bolero' at 12 N; lane 5, NMF 'Oro A' at 46 N; and lane 6, NMF 'Oro A' at 34 N. The lower panel (total RNA) shows the quantification image of the ethidium bromide-stained RNA gel. Twenty micrograms of RNA was loaded per lane; the results of one experiment, representative of three, are shown.

interest, genomic fragments (nucleotide residues 869–2043 relative to the *AC1* sequence; see the Materials and Methods) comprising the SNP $G_{Bol} \rightarrow \underline{C}_{Oro}$ found at position 806 of the cDNA clones, as well as two intronic sequences. Genomic DNA was then extracted from the leaves of NMF 'Oro A' and MF 'Bolero', and the selected fragment was subsequently amplified, cloned and sequenced. The results (Fig. 5) showed that all of the 'Oro A' clones yielded a single amplification

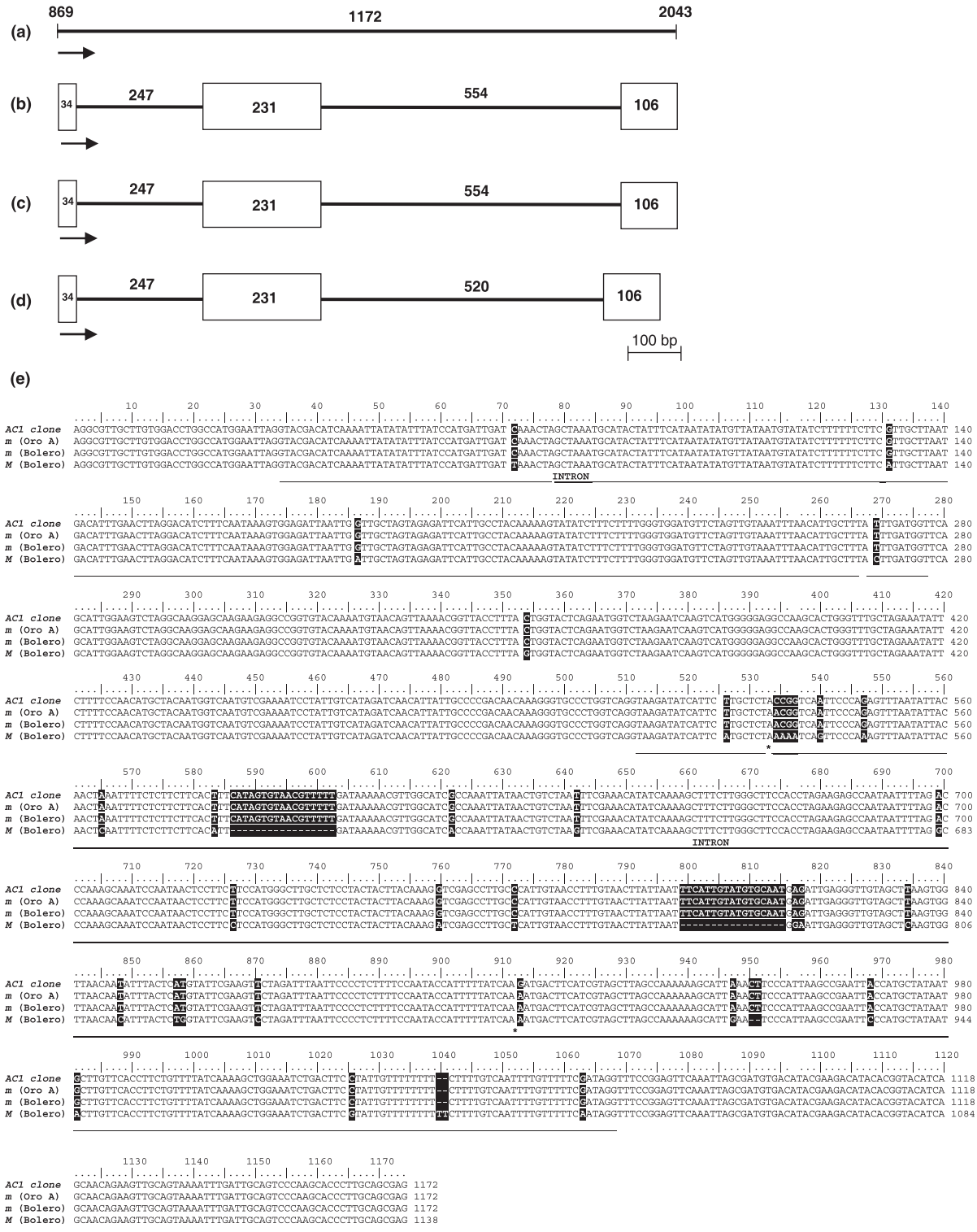

Fig. 5 Structure of selected fragments of an *endopolygalacturonase* (*endo-PG*) gene in peach (*Prunus persica*). (a) Position (bp) of the selected fragment within the peach *AC1 endo-PG* clone (accession no. AY262754). (b) Genomic structure of the *AC1* clone. (c) Genomic structure of the *m* clone. (d) Genomic structure of the *M* clone. (e) Nucleotide sequence alignment of portions (nucleotide residues 869–2043) of the *endo-PG AC1*, the *m* [nonmelting flesh (NMF) 'Oro A' and melting flesh (MF) 'Bolero'], and the *M* (MF 'Bolero') clones containing the major sequence differences. In (b), (c) and (d), open bars and lines indicate exons and introns, respectively. Numbers indicate lengths (bp) of each sequence. Arrows indicates the direction of the gene. In (e), sequence alignment was performed using the BIOEDIT program. Reverse contrast marks the presence of single nucleotide polymorphisms (SNPs), deletions and insertions; asterisks mark SNPs between *AC1* and *m*. The selected DNA fragments were obtained from genomic DNA from NMF 'Oro A' and MF 'Bolero' accessions after amplification with the appropriate primers (see the Materials and Methods).

product (*m*) of the predicted length of 1172 bp. However, two amplification products were recovered from the 'Bolero' clones, one of which was identical to that of 'Oro A' (*m*), whereas the other (*M*) showed a few differences, involving almost exclusively the intronic sequences. In particular, the *M* fragment showed 31 SNPs, two main deletions of 17 bp each (positions 586–602 and 798–814), a 2-bp deletion (positions 949–950) and a 2-bp insertion (positions 1039–1040). The *M* fragment was thus shorter by 34 bp than the *m* fragment (Fig. 5).

The *Bsr*SI-recognizable SNP $G_{Bol} \rightarrow \underline{C}_{Oro}$ at position 806 of the cDNA clones (Fig. 2) was found at position 353 of the selected genomic *endo-PG* sequence in the *m* allelic configuration typical of 'Oro A' which was also present in the 'Bolero' genotype. Forty-eight base pairs downstream, the second *Bsr*SI-specific restriction site, found in both the *m* and *M* allelic configurations, was present (Fig. 5). CAPS analysis was performed on genomic DNA extracted from leaves of the two parents (NMF 'Oro A' and MF 'Bolero') and of the two offspring, NMF 'BO 96014125' and MF 'BO 96014137'. Figure 6 shows that the undigested DNA fragment from both of the NMF accessions considered ('Oro A' and 'BO 96014125') produced a single band (Fig. 6, lanes 2 and 4), with estimated length (approx. 1200 bp) consistent with that (1172 bp) expected from *in silico* analysis. Restriction with *Bsr*SI produced two bands with estimated sizes of approx. 770- and 350 bp, respectively (Fig. 6, lanes 3 and 5). In both of the MF accessions 'Bolero' and 'BO 96014137', the undigested DNA fragment produced two bands with only slightly different estimated lengths (Fig. 6, lanes 6 and 8), while restriction with *Bsr*SI produced four bands, of estimated lengths 770-, 730-, 400- and 350 bp (Fig. 6, lanes 7 and 9), confirming a different allelic configuration of the relevant *endo-PG* gene in NMF and MF accessions.

The same CAPS analysis was repeated on six additional peach genotypes with either NMF ('Andross', 'Jonia', 'BO 82010054') or MF ('Springcrest', 'Springbelle', 'Maycrest') fruit phenotypes. Figure 7 shows that the restriction patterns of the selected *endo-PG* gene sequence which characterized the NMF and MF accessions described in Fig. 6 were maintained.

Discussion

The MF trait in peach fruit has been known for many years, the first literature report being by Bailey & French (1949). Since then, many studies have been conducted to elucidate its biochemical and molecular basis. NMF peaches are reported to contain only exo-PG activity, while MF peaches contain both exo- and endo-PG activities. Moreover, ripe MF fruits present much higher endo-PG activity than NMF fruits (Pressey & Avants, 1973, 1978). These biochemical findings were supported by results obtained from northern analysis showing that in the NMF peach 'Carolyn' transcription of the *endo-PG* gene occurred as well as in MF phenotypes, but with an RNA transcript of approx. 250 bp shorter, combined with failure in production of the endo-PG protein, as shown by immunoblotting (Lester *et al.*, 1994, 1996). In another NMF cultivar, 'Fla.9–20C', Southern analysis showed complete absence of *endo-PG*-related bands, suggesting deletion of a portion of the gene (Lester *et al.*, 1996). Consistent results were also obtained for eight other NMF cultivars, where greatly reduced or undetectable levels of *endo-PG* PRF5-related PG mRNA were found (Callahan *et al.*, 2004). These data were interpreted as probably reflecting the presence of a sequence

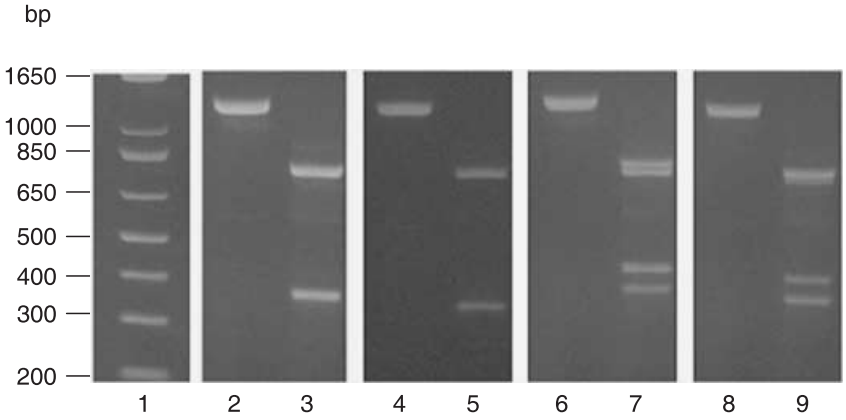

Fig. 6 Cleaved amplified polymorphic sequence (CAPS) restriction patterns of genomic DNA from leaves of peach (*Prunus persica*) accessions with nonmelting flesh (NMF) ('Oro A', 'BO 96014125') or melting flesh (MF) ('Bolero', 'BO 96014137') fruit phenotypes. Selected genomic DNA fragments of the *AC1 endopolygalacturonase* (*endo-PG*) clone, obtained as described in the Materials and Methods, were restricted with *Bsr*SI. Lane 1, 1-kb DNA ladder; lane 2, undigested *endo-PG* gene sequence from 'Oro A'; lane 3, *Bsr*SI-digested *endo-PG* gene fragments from 'Oro A'; lane 4, same as lane 2, from 'BO 96014125'; lane 5, *Bsr*SI-digested *endo-PG* gene fragments from 'BO 96014125'; lane 6, same as lane 2, from 'Bolero'; lane 7, *Bsr*SI-digested *endo-PG* gene fragments from 'Bolero'; lane 8, same as lane 2, from 'BO 96014137'; and lane 9, *Bsr*SI-digested *endo-PG* gene fragments from 'BO 96014137'. Twenty micrograms of DNA was loaded per lane; the results of one experiment, representative of three, are shown.

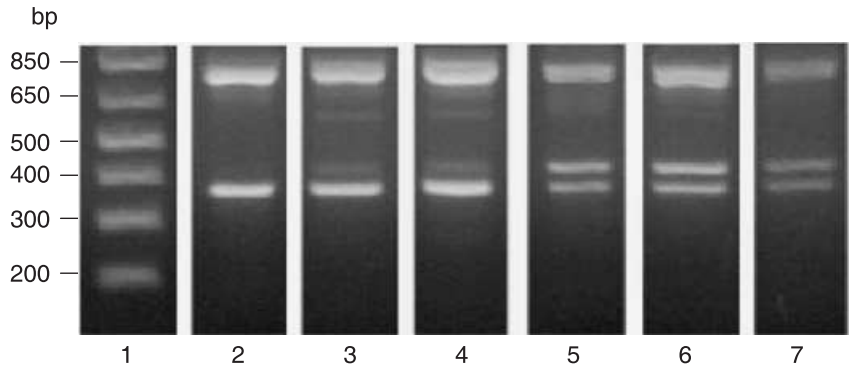

Fig. 7 Cleaved amplified polymorphic sequence (CAPS) restriction patterns of genomic DNA from leaves of peach (*Prunus persica*) accessions with nonmelting flesh (NMF) ('Andross', 'Jonia' and 'BO 82010054') or melting flesh (MF) ('Springcrest', 'Springbelle' and 'Maycrest') fruits. Selected genomic DNA fragments of the *AC1 endopolygalacturonase* (*endo-PG*) clone, obtained as described in the Materials and Methods, were restricted with *Bsr*SI. Lane 1, 1-kb DNA ladder; lane 2, *Bsr*SI-digested *endo-PG* gene fragments from 'Andross'; lane 3, same as lane 2, from 'Jonia'; lane 4, same as lane 2, from 'BO 82010054'; lane 5, same as lane 2, from 'Springcrest'; lane 6, same as lane 2, from 'Springbelle'; and lane 7, same as lane 2, from 'Maycrest'. Twenty micrograms of DNA was loaded per lane; the results of one experiment, representative of three, are shown.

aberration affecting translation and/or production of an active form of the enzyme in NMF peaches.

The work described here shows that an *endo-PG* gene, corresponding to that expressed in ripe peach fruits (acc. no. X76735) was present and expressed, albeit at different extents, in both groups of NMF and MF fruits assessed (Figs 2, 4). Moreover, the same polyclonal antibodies raised against a conserved sequence of an endo-PG form of ripe peaches recognized, among the proteins extracted from the cell walls of ripe MF 'Bolero' fruits, a protein with endo-PG activity (Fig. 1a,b), and in both NMF and MF fruits, a polypeptide (Fig. 1c), whose molecular mass (approx. 45 kDa) closely matched that reported for mature endo-PGs (Lee *et al.*, 1990 and references therein; Brummell & Harpster, 2001). These findings give sound circumstantial evidence that a corresponding endo-PG polypeptide was produced and delivered into the mesocarp cell walls (Fig. 1c).

The lower proportion, in NMF as compared with MF fruits, of ripe-fruit endo-PG polypeptide (in relation to the total proteins extractable in saline conditions from the cell walls; Fig. 1c) was consistent with the different levels of ripe-fruit *endo-PG* mRNA observed in the NMF and MF fruits assessed (lower in NMF 'Oro A', higher and increasing with softening in MF 'Bolero') (Fig. 4), suggesting a regulation of endo-PG production at the transcriptional level, in relation to both phenotype and degree of softening. Nevertheless, some other type of regulation at translational or post-translational levels cannot be excluded, as suggested for the production of the active form of tomato fruit PG (DellaPenna & Bennett, 1988).

In vitro specific endo-PG activities (expressed on a per-protein basis) were generally higher (by approx. 100%) in MF than in NMF fruits, consistent with the lower degree of flesh softening of NMF fruits as compared with MF fruits. Nevertheless, the presence of other endo-PG isoforms, not specifically involved in the process of ripening-related flesh softening, cannot be excluded, as suggested by the measurable amounts of *in vitro* endo-PG activity found also in 'Oro A' and 'BO 96014125' NMF fruits, where the levels of the endo-PG polypeptide typical of ripe, soft fruits were extremely low (Fig. 1c, lanes 1–5). A higher proportion, in MF fruits, of the endo-PG isoform, specifically involved in ripening-related fruit softening and recognized by the antibodies used, may also explain the higher levels of endo-PG polypeptide observed in firmer (54 N) MF 'Bolero' fruits as compared with softer (46 N) NMF 'Oro A' fruits (Fig. 1c, lanes 6 and 1, respectively). Early synthesis and secretion into the cell wall of the endo-PG protein seems thus to precede, in MF fruits, the actual flesh softening, in agreement with the general understanding that this complex phenomenon is a result of the ordered and sequential expression of many genes involved in the modification of the cell wall structure (Trainotti *et al.*, 2003).

The NMF 'Oro A' did not show any large differences at the level of the *endo-PG* transcript considered, as shown by the identical lengths (1182 bp) of the *endo-PG* cDNA clones obtained from either 'Oro A' or 'Bolero' fruits. Nevertheless, the 'Oro A' *endo-PG* cDNA clone showed five SNPs compared with that of 'Bolero' (Fig. 2). Three (nucleotide positions 516, 576, and 579) did not induce any change in the deduced amino acid sequence (Fig. 3), whereas the SNP at position 806 bp induced the substitution of a Ser residue (MF 'Bolero') with Thr (NMF 'Oro A') at position 269 of the deduced amino acid sequence of the protein. This change is conservative in terms of amino acid properties. However, the SNP at nucleotide position 146 induced a change in position 49 of the amino acid sequence, from Ser ('Bolero') to Phe ('Oro A'). It cannot be excluded that this change may alter the properties of the endo-PG protein.

Further investigation, at the molecular level (sequencing and CAPS analysis), of the selected *endo-PG* gene fragments

from NMF and MF accessions, gave interesting information. The genomic DNA fragments obtained from 'Oro A' and 'Bolero' by amplification of the selected *endo-PG* gene sequence comprising the nucleotide residue mutated in 'Oro A' (C_{Oro}) as compared with 'Bolero' (G_{Bol}), were of one type only (*m*) in 'Oro A', whereas in 'Bolero' there were two different types of fragments (*m* and *M*; Fig. 5), indicating homozygosis (*m*/*m*) at the *M* locus for 'Oro A' and heterozygosis (*m*/*M*) for 'Bolero'. The heterozygous nature at the *M* locus of 'Bolero' was then confirmed in a progeny raised by self-pollination (D. Bassi, unpublished). The *m* fragment (present in both 'Oro A' and 'Bolero'), with the *Bsr*SI-restrictable SNP at position 353 bp, presented two restriction sites, expected to produce three fragments of, respectively, 767-, 357-, and 48 bp. The *M* fragment (only one *Bsr*SI restriction site), present in one copy in 'Bolero', was expected to produce two fragments, of 733- and 405 bp, respectively, because of the shorter (by 34 bp) length of the original DNA sequence. For the sake of clarity, the schemes depicted in Fig. 8 illustrate the expected lengths of the restriction fragments (Fig. 8a) as well as the expected gel-separation patterns (Fig. 8b) for homozygous (*m*/*m*; *M*/*M*) or heterozygous (*m*/*M*) allelic configurations, respectively. In both 'Oro A' and 'BO 96014125' only two bands (corresponding to DNA fragments of approx. 770- and 350 bp, respectively) could be observed, confirming the presence of two identical *m* alleles (*m*/*m*) in the NMF accessions. In the MF accessions 'Bolero' and 'BO 96014137', four bands were detected after digestion with *Bsr*SI: two bands (approx. 770- and 350 bp) apparently originated from the *m* allele, and two (approx. 730- and 400 bp) originated from the *M* allele (Fig. 6). Considering that a 48-bp DNA fragment is surely too small to be retained in the 2% agarose gel used, the results are in good agreement with those expected from Fig. 8, and are consistent with data recently obtained in peach accessions derived from 'Dr Davis' or/and 'Georgia Belle' cultivars, showing that DNA sequence differences for different *endo-PG* alleles in NMF/MF phenotypes are in the form of SNPs and indels involving an intronic sequence. In particular, the F^b intronic sequence of the *endo-PG* gene, which is hypothesized to characterize the MF phenotype in 'Dr Davis' × 'Georgia Belle' progeny, shows the same loss of two 17-bp sequences (Peace *et al.*, 2005) as that reported in the present work for the *M* allele in the MF germplasm (Figs 6 and 7). This result may suggest a possible regulatory role of the considered intronic sequence (Lorković *et al.*, 2000) for the expression of the *endo-PG* gene during fruit ripening.

Also interesting is the finding that the SNP observed in the NMF 'Oro A' *endo-PG* ORF at 806 bp (Fig. 2) is also conserved in another NMF cultivar, the chinese 'Feicheng' (Ma *et al.*, 1999, acc. no. AF095577). On the basis of the results reported in the literature about different NMF peach germplasm, it appears very likely that mutations in the *endo-PG* gene, causing absence of expression of the endo-PG protein, could be of more than one type, and that the NMF

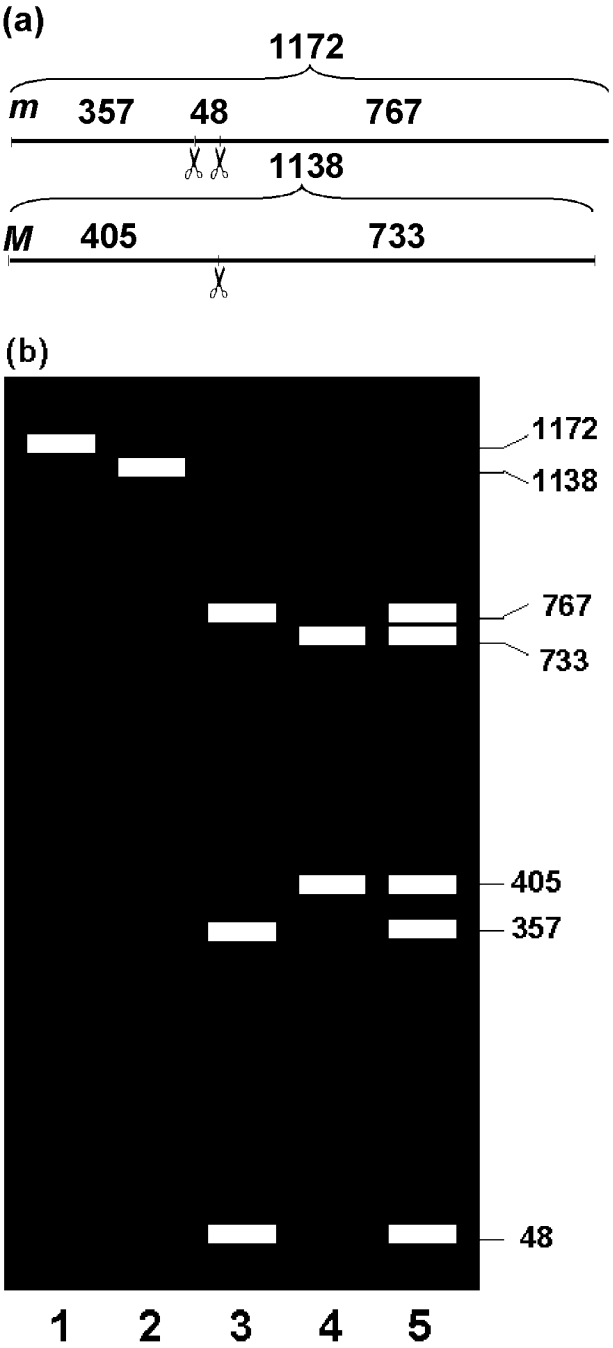

Fig. 8 Restriction fragments expected from the digestion, by *Bsr*SI, of a selected sequence [1172 bp; nucleotides 869–2043 of the *AC1 endopolygalacturonase* (*endo-PG*) clone from peach (*Prunus persica*)] of the peach *endo-PG* genomic sequences of *m* and *M* clones. (a) Numbers above the curly brackets indicate total length (bp) of the selected *m* and *M endo-PG* gene fragments, respectively. Numbers above the lines indicate the expected lengths (bp) of the restriction fragments obtained after digestion with *Bsr*SI of the *m* and *M endo-PG* clone fragments. (b) Expected gel-separation patterns of the DNA fragments shown in (a). Lane 1, undigested *m endo-PG* clone fragment; lane 2, undigested *M endo-PG* clone fragment; lane 3, *Bsr*SI-digestion pattern for the *m*/*m* allelic configuration; lane 4, *Bsr*SI-digestion pattern for the *M*/*M* allelic configuration; and lane 5, *Bsr*SI-digestion pattern for the *m*/*M* allelic configuration.

trait has arisen from more than one source differently distributed in the different growing areas (Lester *et al.*, 1996; Callahan *et al.*, 2004). However, the results concerning the NMF/MF peach accessions studied in the present work (Figs 6, 7) seem to indicate that the same polymorphism in the same position of the *m* allele of the *endo-PG* gene is conserved among different accessions with NMF phenotype.

This seems to represent satisfactory preliminary evidence for the possible use of this SNP to develop a molecular marker useful for predicting the fruit phenotype in newly established peach accessions. The same SNP may also be exploited for identifying the genetic asset at the *melting flesh* (*M*) locus in a progeny homozygous and heterozygous for this locus.

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