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Endopolygalacturonase Gene Polymorphisms: Asset of the Locus in Different Peach Accessions

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Abstract

Endopolygalacturonase (endoPG) plays a pivotal role in determining peach [*Prunus persica* L. (Batsch)] fruit characteristics. Different *Pp-endoPG* genes or allelic variants have been described, characterized by different polymorphisms: insertions-deletions (InDels) and single nucleotide polymorphisms (SNPs). Eighty-five peach accessions (comprising commercial cultivars, F_1 progenies of selected crosses, and three haploid seedlings) with different flesh softening patterns (Non Melting: NM; Melting: M; Slow Softening: SS; Stony Hard: SH) were screened by exploiting specific polymorphisms, with the aim to characterize their asset at the *endoPG* locus and evaluate a potential relationship with fruit flesh texture phenotype. The results of InDel analysis allowed to distinguish, by a simple genotyping procedure, NM flesh phenotypes from the others. Further information arose from this analysis, showing that two *Pp-endoPG* genes, *i.e.*, *Pp-endoPG*^{m} (*Ppa006839m*), involved in the determination of the Melting/Non Melting trait, and *Pp-endoPG_M* (*Ppa006857m*), involved in the determination of the Clingstone/Freestone trait, always co-segregate, and that SS Big Top possesses a "null" *Pp-endoPG* allele. Cleaved Amplified Polymorphic Sequence (CAPS) analysis allowed to preliminarily discriminate the *Pp-endoPG* variants of the SS and SH accessions considered. The integrated use of the considered polymorphisms in a high number of peach accessions proved useful, by individuating the different gene variants and their combinations, to describe the structure of the *endoPG* locus in different genotypes.

Keywords

CAPS Analysis, InDel/SNP Polymorphisms, *Pp-endoPG*, *Prunus persica*

1. Introduction

Flesh texture and stone adherence are important factors of the overall quality of

the peach [*Prunus persica* (L.) Batsch] fruit, contributing to the consumer's satisfaction, fruit nutritional features, and postharvest behavior [1] [2].

Peaches are classified on the basis of their different flesh softening behavior during ripening. In Melting (M) flesh, a marked and steep loss of firmness ("melting" phase) occurs in the final stages of ripening. The melting process is delayed in Slow Softening (SS) fruit and undetectable in Non Melting (NM) and Stony Hard (SH) ones [2] [3] [4] [5] [6]. The SS phenotype is very interesting, retaining flesh firmness on the tree for longer time than standard M, with full development of sensory qualities, and remarkable keeping quality appreciated by both growers and consumers. The spontaneous occurrence of melting, although a few days later than standard M, allows to group SS fruit into the M phenotype, since the physiological basis of SS is due to a delay in ethylene production [6]. Unfortunately, this phenotype, like the SH one, is of very difficult assessment on the tree when scoring segregating progenies [7].

Fruit flesh texture is affected by biochemical and physiological factors with a major role played by hydrolytic enzymes and other proteins that cooperate to modify the composition and architecture of the cell wall polysaccharides [8] [9]. In particular, peach flesh melting has been related to a strong increase in the expression of *Pp-endopolygalacturonase* (*Pp-endoPG*) and accumulation of an active Pp-endoPG protein. In NM fruit, Pp-endoPG is absent or detected at levels lower than in M ones [5] [10] [11] [12]. Therefore, *Pp-endoPG* is considered as a candidate gene for peach flesh softening behavior [13].

In eukaryotic organisms, Insertion-Deletion (InDel) and Single Nucleotide Polymorphisms (SNPs) represent the most abundant DNA mutations ([14] and references therein) that can modify the amplification patterns of selected DNA sequences as well as the restriction endonuclease recognition sites in PCR amplicons, allowing the development of markers useful for genotyping even in the absence of massive sequencing data ([15] and references therein).

In peach, availability of different molecular markers allowed to gain information on some 760 Quantitative Trait Loci and Mendelian Trait Loci linked to horticultural and physiological traits such as tree development, pest and disease resistance, flowering, ripening, seed and fruit quality [16] [17].

The availability of the peach genome sequence [18] and the development of SNP genotyping resources [19] [20] offer the opportunity to study at the molecular level the development and inheritance of different phenotypic traits, as recently reported for skin blush [21].

A high degree of allelic diversity has been identified in the peach *Pp-endoPG* sequence [13] [22] [23]. We previously identified specific SNPs in the open-reading frame of *Pp-endoPG*, one of which allowed to determine the configuration at the *Melting flesh* locus in the NM (Oro A) and M (Bolero) model cultivars, and in a few M and NM F_1 seedlings from their cross [5] [24]. Moreover, in Bolero (M), Oro A (NM), and in the cultivars Big Top (SS), Yumyeong (SH), and Ghiaccio (SH), representative of different flesh textures,

variants of the *Pp-endoPG* gene characterized by peculiar InDels and SNPs have been individuated. The *Pp-endoPG* locus on chromosome 4 contains two sequences, *i.e.*, F[a]-*Ppa006839m* (GenBank ID: 18781156) and F[b]-*Ppa006857m* (GenBank ID: 18779267), that coincide with *Pp-endoPG*^{m} (GenBank ID: DQ659240.1) and *Pp-endoPG_M* (GenBank ID: DQ659241.1) [6] [18] [23]. F[a]-*Ppa006839m* (*Pp-endoPG*^{m}), the only sequence retrieved in Oro A, shows 97 % identity with its variants *Pp-endoPG*^{SH} (GenBank ID: HQ891822.1 of SH Yumyeong and GenBank ID: HQ891821.1 of SH Ghiaccio) and *Pp-endoPG*^{BT} (GenBank ID: HQ891820.1 of SS Big Top), for the presence in the latter ones of one 17-bp intronic deletion. Moreover, the Big Top-specific *Pp-endoPG*^{BT} sequence shows a peculiar SNP (bp 348) in Exon 1, that does not induce any change in the deduced amino acidic sequence [6]. F[b]-*Ppa006857m* (*Pp-endoPG_M*), present in Bolero together with F[a]-*Ppa006839m*, is by 34 bp shorter than *Pp-endoPG*^{m}, for the presence of one additional 17-bp intronic deletion. On this basis, amplification of a selected (1455-1892 bp) *Pp-endoPG* genomic sequence including these InDels generates fragments specifically referable to F[a]-*Ppa006839m* (*Pp-endoPG*^{m}; 437 bp), to its variants *Pp-endoPG*^{SH} and *Pp-endoPG*^{BT} (both of 420 bp), or to F[b]-*Ppa006857m* (*Pp-endoPG_M*; 403 bp) [6]. F[a]-*Ppa006839m* has recently been proposed as the main responsible for the Melting trait [23].

In the present work the InDel and the CAPS (Cleaved Amplified Polymorphic Sequence) markers developed from the described polymorphisms have been exploited to screen a total of 85 accessions (commercial cultivars, F_1 offsprings from selected crosses, and three haploids) to describe them for the configuration at the *endoPG* locus and evaluate a potential relationship with fruit flesh texture phenotype.

2. Materials and Methods

2.1. Plant Material

The study was conducted on commercial cultivars or on F_1 offsprings of different crosses. Three haploid accessions (P VIN 1 1N from Vineland, P RRL 1 1N from Rutgers Red Leaf, P LOV 3 1N from Lovell) were also considered (**Table 1**).

Plant material was obtained from a peach germplasm collection grown at the Experimental Orchard "Zabina" (Castel San Pietro Terme, Italy; lat. 44˚23'52"N; long. 11˚35'22"E), under the weather and climate conditions detailed in **Table 2**. Trees were grafted on GFF677 rootstock planted at 3.5 × 4.5 m and trained as open vase. Seedlings were planted on their own roots with a spacing of 1 m within and 4 m between rows and trained as slender spindle (one stem with short lateral scaffolds). Pruning was performed yearly and standard cultural practices were applied.

2.2. Fruit Flesh Texture Phenotype Scoring

Flesh texture was scored in the orchard by trained personnel on fruit harvested at commercial ripening (onset of the veraison stage), as identified in each

Table 1. List of the *Prunus persica* accessions used, with fruit flesh texture phenotype, pedigree, estimated length of *endoPG*-derived amplicons, and asset at the *endoPG* locus as hypothesizable by InDel analysis or, where available, reported in the literature. M: Melting; NM: Non Melting; SH: Stony Hard; SS: Slow Softening. NC, not classifiable. OP, open pollination. -, lack of fruit production.

Nr.	Accession	Flesh Phenotype	Pedigree	*EndoPG*		
				Amplicons length (bp)	Locus asset	
					InDel analysis	Literature
1.	Andross	NM	Dix 5A-1 × Fortuna	440	f1/f1; f1/f_{null}	f1/f1; f1/f_{null} [31]
2.	BO 82010554	NM	Jungerman × Loadel	440	f1/f1; f1/f_{null}	-
3.	Ionia	NM	Vivian × Federica	440	f1/f1; f1/f_{null}	-
4.	Oro A	NM	Diamante OP	440	f1/f1; f1/f_{null}	f1/f1 [6] [31]
5.	Ambra	M	Stark Red Gold × Mayfire	440; 420; 410	F/f	-
6.	Bolero	M	Cresthaven × Flamecrest	440; 410	F/F; F/f1; F/f_{null}	F/f1 [6] [31]
7.	Contender	M	Wiblo × {Norman × [Candor × (Summercrest × Redhaven)]}	440; 410	F/F; F/f1; F/f_{null}	F/F [31]
8.	Max 7	M	-	440; 420; 410	F/f	-
9.	Big Top®	SS	-	420	f/f_{null}	f/f; f/f_{null} [31]
10.	Alitop®	SS	(Flavortop × SnowQueen) × Big Top	440; 410	F/f_{null}	-
11.	BO 96016015	M	Contender × Ambra	440; 420; 410	F/f	-
12.	BO 96016018	M	Contender × Ambra	440; 420; 410	F/f	-
13.	BO 96016023	M	Contender × Ambra	440; 420; 410	F/f	-
14.	BO 96016094	M	Contender × Ambra	440; 410	F/F; F/f1; F/f_{null}	-
15.	BO 96016136	M	Contender × Ambra	440; 410	F/F; F/f1; F/f_{null}	-
16.	BO 96016165	M	Contender × Ambra	440; 420; 410	F/f	-
17.	BO 96016208	M	Contender × Ambra	440; 420; 410	F/f	-
18.	BO 96028059	SS	Springred × Big Top	440; 420; 410	F/f	-
19.	BO 02002002	M	Ambra × Big Top	440; 420; 410	F/f	-
20.	BO 02002003	M	Ambra × Big Top	440; 420; 410	F/f	-
21.	BO 02002004	M	Ambra × Big Top	440; 420; 410	F/f	-
22.	BO 02002005	M	Ambra × Big Top	440; 420; 410	F/f	-
23.	BO 02002006	M	Ambra × Big Top	420	f/f; f/f_{null}	-
24.	BO 02002008	M	Ambra × Big Top	440; 420; 410	F/f	-
25.	BO 02002009	M	Ambra × Big Top	440; 420; 410	F/f	-
26.	BO 02002020	M	Ambra × Big Top	420	f/f; f/f_{null}	-
27.	BO 02002023	M	Ambra × Big Top	440; 410	F/f_{null}	-
28.	BO 02002025	M	Ambra × Big Top	420	f/f; f/f_{null}	-
29.	BO 02002026	M	Ambra × Big Top	440; 420; 410	F/f	-
30.	BO 02002001	SS	Ambra × Big Top	420	f/f; f/f_{null}	-
31.	BO 02002011	SS	Ambra × Big Top	420	f/f; f/f_{null}	-
32.	BO 02002012	SS	Ambra × Big Top	440; 420; 410	F/f	-
33.	BO 02002014	SS	Ambra × Big Top	440; 420; 410	F/f	-
34.	BO 02002015	SS	Ambra × Big Top	440; 420; 410	F/f	-
35.	BO 02002017	SS	Ambra × Big Top	440; 410	F/f_{null}	-
36.	BO 02002018	SS	Ambra × Big Top	440; 420; 410	F/f	-
37.	BO 02002019	SS	Ambra × Big Top	420	f/f; f/f_{null}	-
38.	BO 02002021	SS	Ambra × Big Top	440; 410	F/f_{null}	-
39.	BO 02002024	SS	Ambra × Big Top	440; 420; 410	F/f	-

Continued

40.	BO 02002013	NC	Ambra × Big Top	420	f/f; f/f_{null}	-
41.	BO 02002022	NC	Ambra × Big Top	420	f/f; f/f_{null}	-
42.	BO 02004001	M	Max 7 × Big Top	440; 420; 410	F/f	-
43.	BO 02004004	M	Max 7 × Big Top	440; 420; 410	F/f	-
44.	BO 02004006	M	Max 7 × Big Top	440; 420; 410	F/f	-
45.	BO 02004008	M	Max 7 × Big Top	440; 410	F/f_{null}	-
46.	BO 02004015	M	Max 7 × Big Top	420	f/f; f/f_{null}	-
47.	BO 02004016	M	Max 7 × Big Top	440; 420; 410	F/f	-
48.	BO 02004017	M	Max 7 × Big Top	440; 420; 410	F/f	-
49.	BO 02004018	M	Max 7 × Big Top	420	f/f; f/f_{null}	-
50.	BO 02004024	M	Max 7 × Big Top	440; 410	F/f_{null}	-
51.	BO 02004002	SS	Max 7 × Big Top	440; 410	F/f_{null}	-
52.	BO 02004013	SS	Max 7 × Big Top	440; 410	F/f_{null}	-
53.	BO 02004014	SS	Max 7 × Big Top	420	f/f; f/f_{null}	-
54.	BO 02004019	SS	Max 7 × Big Top	440; 420; 410	F/f	-
55.	BO 02004021	SS	Max 7 × Big Top	420	f/f; f/f_{null}	-
56.	BO 02004022	SS	Max 7 × Big Top	420	f/f; f/f_{null}	-
57.	BO 02004023	SS	Max 7 × Big Top	440; 420; 410	F/f	-
58.	BO 02004025	SS	Max 7 × Big Top	440; 410	F/f_{null}	-
59.	BO 02004027	SS	Max 7 × Big Top	420	f/f; f/f_{null}	-
60.	BO 02004026	M	Max 7 × Big Top	420	f/f; f/f_{null}	-
61.	BO 02004003	NC	Max 7 × Big Top	420	f/f; f/f_{null}	-
62.	BO 02004005	NC	Max 7 × Big Top	420	f/f; f/f_{null}	-
63.	BO 02004007	NC	Max 7 × Big Top	440; 420; 410	F/f	-
64.	BO 02004009	NC	Max 7 × Big Top	440; 410	F/f_{null}	-
65.	BO 02004010	NC	Max 7 × Big Top	420	f/f; f/f_{null}	-
66.	BO 02004011	NC	Max 7 × Big Top	420	f/f; f/f_{null}	-
67.	BO 02004012	NC	Max 7 × Big Top	420	f/f; f/f_{null}	-
68.	BO 02004020	NC	Max 7 × Big Top	420	f/f; f/f_{null}	-
69.	Glohaven	M	SH 20 × Kalhaven	440; 410	F/F; F/f1; F/f_{null}	-
70.	Springbelle	M	-	440; 410	F/F; F/f1; F/f_{null}	-
71.	Springcrest	M	FV 89-14 × Springtime	440; 410	F/F; F/f1; F/f_{null}	F/F, F/f1, F/f_{null} [31]
72.	Maycrest	M	Springcrest mutation	440; 410	F/F; F/f1; F/f_{null}	F/F, F/f1, F/f_{null} [31]
73.	Springred	M	Summer Grand OP	440; 410	F/F; F/f1; F/f_{null}	-
74.	7-28	SH	Koyohakuto × Okubo	440; 420; 410	F/f	-
75.	D 41-62	SH	-	420	f/f, f/f_{null}	-
76.	Ghiaccio	SH	193 Q XXVII 111 (Yumyeong self-pollination)	420	f/f, f/f_{null}	-
77.	Yumyeong	SH	Yamato-Wase × Nunome Wase	420	f/f, f/f_{null}	f/f [31]
78.	Helena Cling	SS	-	420	f/f, f/f_{null}	-
79.	BO 96013046	SS	Bolero × Rich Lady	440; 420; 410	F/f	-
80.	Honey Gold	SS	-	440; 420; 410	F/f	-
81.	Ruby Rich	SS	-	440; 420; 410	F/f	F/f [31]
82.	Vista Rich	SS	Rich Lady OP	440; 420; 410	F/f	-
83.	P LOV 3 1N	-	Lovell OP	440	f1	-
84.	P RRL 1 1N	-	Rutgers Red Leaf OP	440; 410	F	-
85.	P VIN 1 1N	-	Vineland OP	440	f1	-

Table 2. Climate and weather conditions recorded in the orchard during the time period of fruit growing season. n. d.: not determined.

Month	Climate and Weather Conditions in the Orchard		
	Average temperature (˚C)	Rainfall (mm)	Relative humidity (%)
March	9.9	101	73.9
April	16.0	19.0	65.3
May	20.1	32.2	60.4
June	22.8	112	64.4
July	26.4	n. d.	41.9
August	23.8	19.6	54.1

cultivar by visual assessment of the skin ground color [2] [25], and sensory determination of approximate firmness.

Assessment of the latter parameter by human inspectors' grading is coherent with instrumented measurements [26], and represents a non-destructive method capable to yield information on the time course of fruit texture evolution.

Discrimination between M and SS phenotypes was based upon sensory scoring of flesh texture changes during 4 d of postharvest at room temperature. Due to stunted growth and low fruit set, in a few F_1 offsprings from selected crosses, fruit were not phenotypically classifiable; haploids also had stunted growth and did not produce fruit at all, so the fruit flesh phenotype remained unascertained.

2.3. Extraction of Genomic DNA

Genomic DNA from young leaves was used as template for the analysis of the amplification profiles based on major InDel polymorphisms in Intron 3 of the *Pp-endoPG* sequence [5] [6], and for CAPS analysis [27] based on the SNP_{348} of the *Pp-endoPG*^{BT} variant of F^{a}-*Ppa006839 m* [6].

Extraction was conducted according to Geuna *et al.* [28]. Frozen leaf samples (100 mg fresh weight) were powdered in liquid N_2 by a mortar and a pestle and mixed with 600 µL of preheated (65˚C) extraction buffer [0.2 M Tris (tris hydroxymethyl aminomethane)-HCl, pH 8.5, 10 mM EDTA (ethylenediaminetetraacetic acid), 0.3 M LiCl, 1.5 % (v/v) SDS (sodium dodecyl sulphate), 1 % (w/v) Nonidet™ P-40, 1 mM DTT (1,4-dithiothreitol)]. Samples were incubated at 65˚C for 20 min and centrifuged (15,000 *g*, 4˚C, 20 min). The aqueous solution was extracted in one volume of (in sequence) phenol, phenol/chloroform/isoamyl alcohol (25/24/1 by volume), and chloroform, with centrifugation (15,000 *g*, 4˚C, 20 min) after each passage. DNA was precipitated by adding 0.1 volume of 3 M Na-acetate, pH 5.2 plus 0.6 volume of 2-propanol, followed by incubation at 4˚C for 1 h and centrifugation (15,000 *g*, 4˚C, 30 min). Pellets were washed with 1 mL of 70% (v/v) ethanol, air-dried and resuspended in 100 µL of double-distilled H_2O. Three microliters of these extracts were analyzed for quality and yield on 1% (w/v) agarose gel in 1 × TBE buffer (89 mM Tris-Borate, 2 mM Na_2-EDTA, pH 8.2 plus 1 µg mL^{-1} ethidium bromide; low ionic strength), also used as elec-

trophoresis buffer. Lambda DNA/Hind III genomic DNA and 1 kb DNA Plus Ladder (Invitrogen Life Technologies, Monza, Italy) were run as standards of concentration and size. Nucleic acids were visualized under UV light. The quantified DNA was treated with 10 mg mL^{-1} RNase A (Invitrogen Life Technologies) at 37˚C for 1 h to remove any RNA contamination and precipitated as described above.

2.4. InDel Analysis

A ~440 bp sequence of the *Pp-endoPG* gene region encompassing a few bases of Exon 3 and a wide sequence of Intron 3 comprising the major InDels [6] was amplified with proper primers (*Pp-endoPG_InDel*$_{For}$: 5'-GTGCCCTGGTCAGGTAAG-3'; *Pp-endoPG_InDel*$_{Rev}$: 5'-GGCTAAGCTACGATGAAGTC-3') in a MyCycler Thermal Cycler (Bio-Rad Laboratories Srl, Segrate, Italy). The PCR mix contained 20 ng genomic DNA, 0.3 mM dNTPs (deoxynucleotides), 0.3 μM of each primer, 1 × GoTaq Reaction Buffer®, 1 U Go Taq® DNA Polymerase (Promega, Segrate, Italy) and double-distilled H_2O to a final volume of 25 μL. The conditions of PCR reaction were: one denaturation cycle (94˚C, 4 min), 35 cycles comprehensive of denaturation (94˚C, 30 s), annealing (62˚C, 30 s) and extension phases (72˚C, 45 s) and one final extension cycle (72˚C, 5 min).

The amplification products were separated on 3% (w/v) agarose gel and visualized (DirectLoad™ 50 bp DNA Step Ladder; Sigma-Aldrich) with ethidium bromide under UV light.

2.5. CAPS Analysis

Primer pairs (*Pp-endoPG*$_{For}$: 5'-ATGGCGAACCGTAGAAGCCTCT-3'; *Pp-endoPG*$^{BT}_{Rev}$: 5'-CCACAAGCAACGCCTTCTATCC-3') were designed to amplify the 1-972 bp region of *Pp-endoPG* including the SNP_{348} of interest [6], which determined the polymorphic restriction site. Amplification was conducted as described above for InDel analysis and was followed by digestion of the polymorphic fragments [1 μg of amplification products, 5 U of *Bst*XI restriction enzyme (Promega), 37˚C, 90 min]. The reaction products, separated on 3% (w/v) agarose gels, were visualized by ethidium bromide (TrackIt™ 1 Kb Plus DNA Ladder; Invitrogen).

3. Results

3.1. InDel Analysis of Different Peach Accessions

Amplification of the cited genomic sequence of *Pp-endoPG* including the InDels of interest confirmed the presence of two amplicons of apparent length of ~440 bp and ~410 bp in Bolero, consistent with the expected lengths of 437 bp and 403 bp for F^a-*Ppa006839m* (*Pp-endoPG*^{m}) and F^b-*Ppa006857m* (*Pp-endoPG_M*) [5] [6].

The M cultivars Contender, Maycrest, Springbelle, Springcrest, Springred, Glohaven, plus two (BO 96016136 and BO 96016094) out of the seven offsprings

from the cross Contender (M) × Ambra (M) showed a Bolero-like amplification pattern with two fragments. In Ambra and in five offsprings from Contender × Ambra (BO 96016015, BO 96016018, BO 96016023, BO 96016165, and BO 96016208) an additional amplicon of ~420 bp was observed (Figure 1).

Figure 2 shows the results obtained in NM, SH, and SS accessions. In the NM Oro A, Andross [29], Ionia, and BO 82010554 (offspring of NM Jungerman × NM Loadel), only one amplicon of ~440 bp was retrieved. In the SH accessions Yumyeong, Ghiaccio, Helena Cling, and D 41-62, as well as in SS Big Top, amplification generated only one fragment of ~420 bp, confirming the presence of the *Pp-endoPG*^{SH}-or *Pp-endoPG*^{BT}-like variants of F^{a}-*Ppa006839m*, respectively [6].

The amplification pattern of SS Alitop was similar to that of Bolero. SH 7-28 and the SS Honey Gold, Ruby Rich, Vista Rich, BO 96028059 (F_1 from M Springred × SS Big Top) and BO 96013046 (F_1 from M Bolero × SS Rich Lady) yielded three amplicons (~440 bp, ~420 bp, and ~410 bp). The two haploids P VIN 1 1N and P LOV 3 1N yielded amplification patterns similar to that of Oro A (a single fragment of ~440 bp), whereas the pattern of P RRL 1 1N was similar to that of Bolero (two fragments of ~440 bp and ~410 bp; Figure 3).

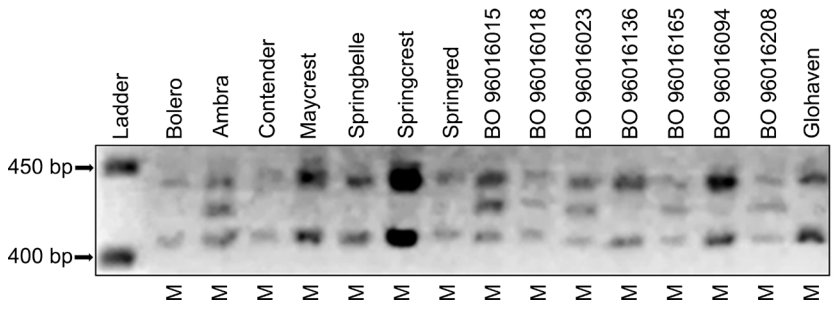

Figure 1. InDel analysis of *Pp-endoPG* in accessions with M fruit flesh texture. Positions and lengths of DNA markers (DirectLoad™ 50 bp DNA Step Ladder; Sigma-Aldrich) are shown on the left. Twenty micrograms DNA were loaded per lane; the amplicons were separated on 3% (w/v) agarose gels. One representative gel from three independent experiments.

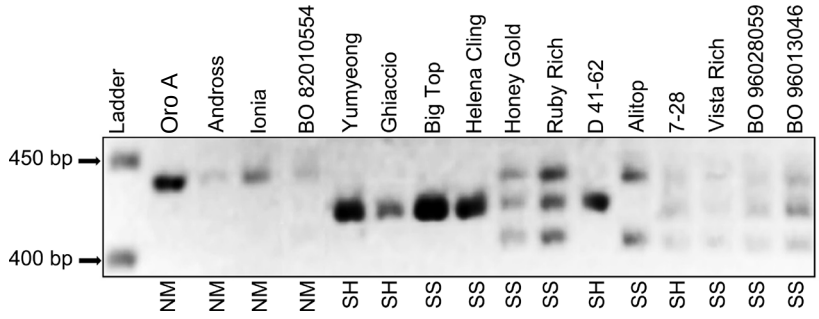

Figure 2. InDel analysis of *Pp-endoPG* in accessions with NM, SH or SS fruit flesh texture. Positions and lengths of DNA markers (DirectLoad™ 50 bp DNA Step Ladder) are shown on the left. Twenty micrograms DNA were loaded per lane; the amplicons were separated on 3% (w/v) agarose gels. One representative gel from three independent experiments.

3.2. Ambra × Big Top and Max 7 × Big Top Crosses: InDel Analysis of F1 Offsprings

Figure 4 shows the amplification patterns obtained in the M Ambra and SS Big Top parents and in 23 F_1 seedlings from their cross (from BO 02002001 to BO 02002006, BO 02002008, BO 02002009, from BO 02002020 to BO 02002026, from BO 02002011 to BO 02002015, from BO 02002017 to BO 02002019). The amplification profiles were similar to that of the Big Top parent (one amplicon of ~420 bp) for eight of them, and to that of the Ambra parent (three amplicons of ~440 bp, ~420 bp, and ~410 bp) for 12. Three accessions showed the same pattern as Bolero (~440 bp and ~410 bp; compare to Figure 1).

Figure 5 shows the amplification profiles obtained in the M Max 7 and SS Big Top parents and in 27 F_1 seedlings from their cross (from BO 02004001 to BO 02004027). In Max 7, as well as in eight offsprings, amplification yielded three fragments (~440 bp, ~420 bp, and ~410 bp). Amplification profiles similar to that of Big Top (one fragment of ~420 bp) were found in 13 seedlings, whereas six seedlings yielded Bolero-like (~440 bp and ~410 bp fragments) patterns (compare to Figure 1).

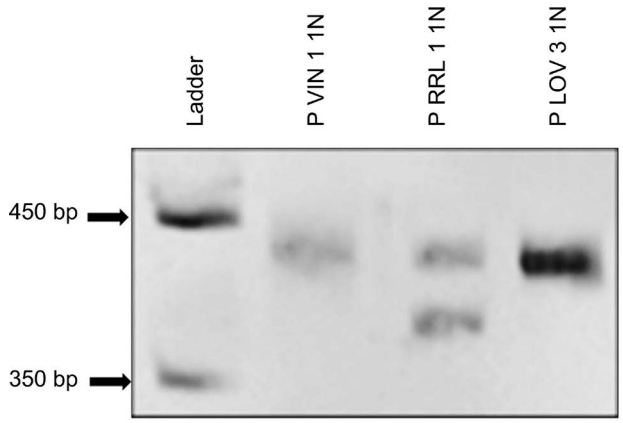

Figure 3. InDel analysis of *Pp-endoPG* in three haploid accessions. Positions and lengths of DNA markers (50 bp DNA Step Ladder; Sigma-Aldrich) are shown on the left. Twenty micrograms DNA were loaded per lane; the amplicons were separated on 3% (w/v) agarose gel. One representative gel from three independent experiments.

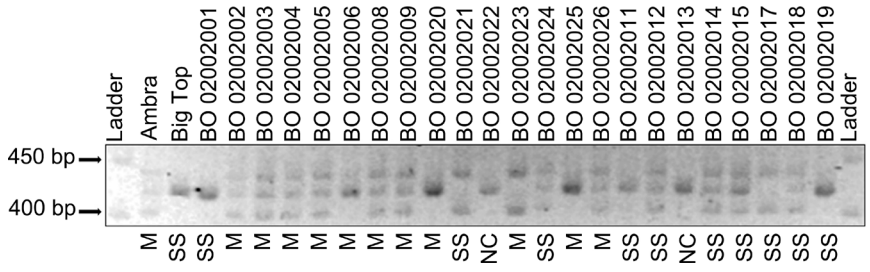

Figure 4. InDel analysis of *Pp-endoPG* in M Ambra, SS Big Top and in 23 F_1 seedlings. Positions and lengths of DNA markers (DirectLoad™ 50 bp DNA Step Ladder) are shown. NC, not classifiable. Twenty micrograms DNA were loaded per lane; amplicons were separated on 3% (w/v) agarose gel. One representative gel from three independent experiments.

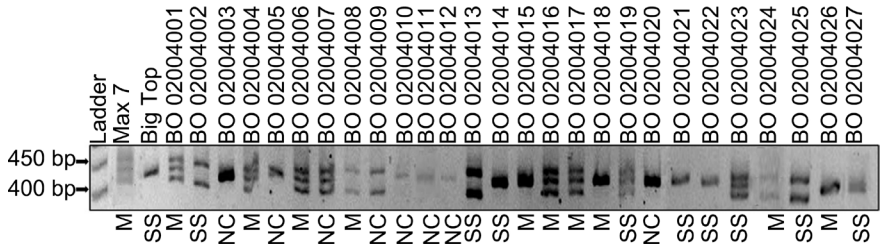

Figure 5. InDel analysis of *Pp-endoPG* in M Max 7, SS Big Top, and in 27 F_1 seedlings. Positions and lengths of DNA markers (DirectLoad™ 50 bp DNA Step Ladder) are shown on the left. NC, not classifiable. Twenty micrograms DNA were loaded per lane; amplicons were separated on 3% (w/v) agarose gel. One representative gel from three independent experiments.

3.3. Cleaved Amplified Polymorphic Sequence (CAPS) Analysis

In the *Pp-endoPG*^{BT} variant of F^{a}-*Ppa006839m* present in Big Top, a peculiar, silent SNP_{348} in Exon 1 originates a polymorphic sequence recognized by the *Bst*XI restriction enzyme (**Figure 6(b)**), suitable for distinguishing between the *Pp-endoPG*^{BT} and *Pp-endoPG*^{SH} variants of F^{a}-*Ppa006839m* (*Pp-endoPG*^{m}) [6].

The SNP_{348} was exploited to broaden CAPS analysis to a few additional cultivars/accessions phenotypically scored as SS (Alitop, Helena Cling, Honey Gold, Ruby Rich, Vista Rich, BO 96028059, BO 96013046) or SH (D 41-62, 7-28) (**Table 1**). *Bst*XI confirmed to be ineffective in Oro A and in Bolero, as it was in all the accessions scored as SH. Concerning the accessions scored as SS, Helena Cling showed, like Big Top, two digestion fragments; Honey Gold, Ruby Rich, Vista Rich, BO 96028059, and BO 96013046 showed two Big Top-like restriction fragments plus an undigested one, whereas *Bst*XI was ineffective in Alitop (**Figure 6(c)**).

4. Discussion

The F allele at the *endoPG* locus on peach chromosome 4 contains two gene sequences, *i.e.* F^{a} (*Ppa006839m*) and F^{b} (*Ppa006857m*), at short distance (32-34 kbp) from each other. Mutations and deletions of these genes determine several allelic variants: F, resulting from F^{a} plus F^{b}; f, resulting from mutation of F^{a} plus complete deletion of F^{b}; f1, resulting from F^{a} plus complete deletion of F^{b}; f_{null}, resulting from deep mutation or complete deletion of both F^{a} and F^{b}. The different alleles F, f, f1, f_{null} at the *endoPG* locus contribute to the fruit phenotype for stone adhesion and flesh texture [4] [18]. In particular, it has been recently suggested that F^{a}-*Ppa006839m* plays a driving role in the determination of the Melting/Non Melting trait, whereas F^{b}-*Ppa006857m* seems involved in the determination of the Clingstone/Freestone trait, although it has been speculated that F^{b}-*Ppa006857m* could exert a pleiotropic effect on flesh melting through a negative feedback control on the transcription of F^{a}-*Ppa006839m* [23].

From reference peach genotypes, peculiar *endoPG* sequences have been isolated. In particular, in NM Oro A has been retrieved a single sequence (*Pp-endoPG*^{m}) coincident with *Ppa006839m*, that does not present any deletion

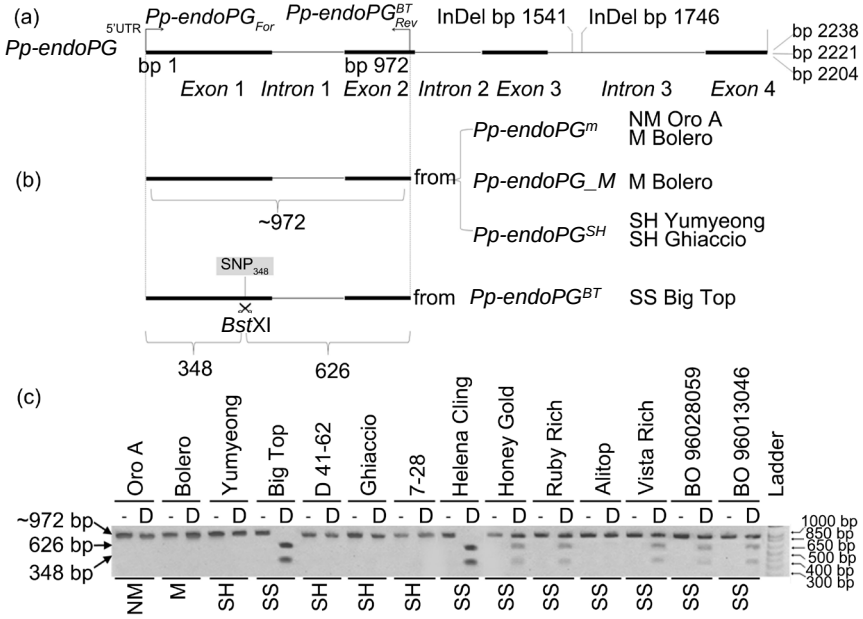

Figure 6. CAPS analysis exploiting the SNP_{348} of the *Pp-endoPG*^{BT} variant of F^{a}-*Ppa006839m*. (a) Schematic representation of the structure of the *Pp-endoPG* gene. Exons and introns are indicated by black solid bars and by solid lines, respectively; positions of the considered InDels of Intron 3 are also indicated. Arrows and vertical dotted lines define the region (1-972 bp) amplified by the primers used. (b) Lengths (~972 bp) of the amplicons obtained from the F^{a}-*Ppa006839m* variants of: NM Oro A and M Bolero (*Pp-endoPG*^{m}), SH Yumyeong and SH Ghiaccio (*Pp-endoPG*^{SH}), and SS Big Top (*Pp-endoPG*^{BT}); position of the peculiar SNP_{348} of the *Pp-endoPG*^{BT} variant and predicted lengths (348 bp and 626 bp) of the fragments obtainable by *Bst*XI digestion (scissors symbol) are also indicated (modified from [6]). The *Pp-endoPG_M* amplicon from F^{b}-*Ppa006857m* of Bolero is also reported. (c) Restriction patterns obtained in different accessions before (-) and after (D) digestion with *Bst*XI. Positions and lengths of DNA markers (1 Kb Plus DNA Ladder, Invitrogen) are shown on the right. Expected lengths (bp) of the undigested amplicons and of the products of *Bst*XI digestion are shown on the left. Twenty micrograms DNA were loaded per lane; 3% (w/v) agarose. One representative gel from three independent experiments.

in Intron 3, as reported also for the F^{a} and f1 *endoPG* selected sequences. The allelic variants (*Pp-endoPG*$^{BT/SH}$) of F^{a}-*Ppa006839m*, isolated from SS Big Top and SH Yumyeong and SH Ghiaccio, present a specific 17-bp deletion in Intron 3, apparently coincident with the deletion of the f sequence. In M Bolero has been isolated a sequence (*Pp-endoPG_M*) coincident with F^{b}-*Ppa006857m* and presenting two 17-bp deletions in Intron 3 as reported for F^{b} [4] [5] [6]. **Figure 7** summarizes the structure of the different alleles at the *endoPG* locus.

Genotype-specific *Pp-endoPG* polymorphisms (InDels and SNPs; [5] [6]) were exploited in the present work as tools to describe 85 peach accessions (**Table 1**). InDel analysis confirmed the co-segregation of F^{a}-*Ppa006839m* (*Pp-endoPG*^{m}) and F^{b}-*Ppa006857m* (*Pp-endoPG_M*). In fact, the longest (~440 bp) amplicon from F^{a}-*Ppa006839m* was always accompanied by the shortest (~410 bp) one from F^{b}-*Ppa006857m*, as indicated by the simultaneous presence of three amplicons in several accessions and of both amplicons in the haploid P RRL 1 1N

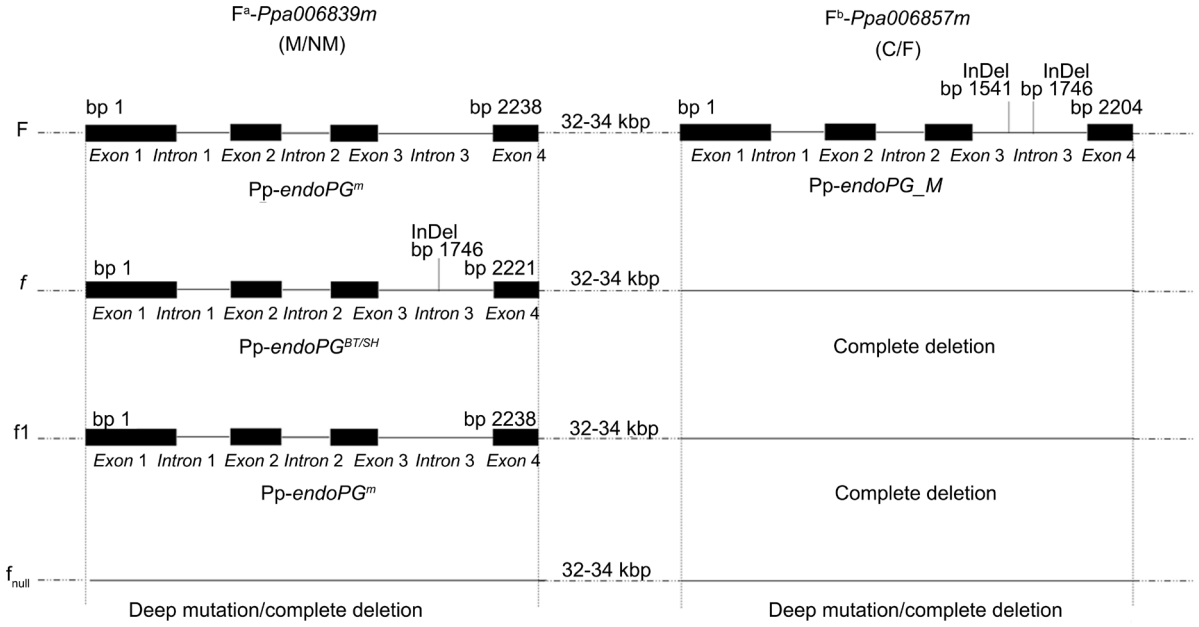

Figure 7. Schematic picture of the structure of the *Pp-endoPG* locus with the different allelic variants and the discussed InDel mutations, as deduced from [4] [5] [6] [18]. C: Clingstone; F: Freestone. Exons are indicated by black solid bars and introns by solid lines.

(**Figures 1-5**). In the doubled haploid P LOV2 2N (from open pollination of Lovell) used for genome sequencing both the F[a]-*Ppa006839m* and the F[b]-*Ppa006857m* sequences are present [18] [23], while only the longest amplicon (~440 bp) was retrieved in the haploid P LOV3 1N (**Figure 3**) suggesting that Lovell is heterozygous (F/f1) at the *endoPG* locus.

The individuated InDel polymorphisms in the *endoPG* sequences seem to distinguish the NM trait from the M one. In all the NM accessions (Oro A, Andross, Ionia, and BO 82010054) only the longest amplicon (~440 bp; **Figure 2**) referable to a F[a]-*Ppa006839m*-like variant is present. This result confirms what reported for NM phenotypes as determined by the presence of the f1 allele, in homozygosis (f1/f1) or in heterozygosis with f_{null} (f1/f_{null}) [23] [30] [31] (**Figure 7**; **Table 1**).

Most (31) of the 64 accessions scored as M or SS (all grouped in a general Melting class [6] [7]) were characterized by presence of all the three amplicons (~440 bp, ~420 bp and ~410 bp), referable respectively to F[a]-*Ppa006839m* (no deletion), its *Pp-endoPG*$^{BT/SH}$-like variants (one 17-bp deletion), and F[b]-*Ppa006857m* (two 17-bp deletions). Eighteen accessions showed a pattern with the two amplicons of ~440 bp and ~410 bp, and 15 showed only the amplicon of ~420 bp (**Figures 1-4**; **Table 1**). From these amplification profiles, it seems reasonable to deduce that the M and SS accessions can present all the three combinations of amplicons.

In particular, the InDel profiles of M Contender, M Maycrest and M Springcrest (**Figure 1**; **Table 1**) appear consistent with their allelic combinations F/F and F/-reported in the literature [30] [31].

The InDel polymorphism, when scored in a cross progeny, appears useful to

deduce the allelic combination at the *endoPG* locus of the parental genotypes, as in the case of Big Top. In fact, a few F_1 individuals of the crosses Ambra × Big Top or Max 7 × Big Top, as well as Alitop [(Flavortop × Snow Queen) × Big Top], lacked the amplicon of ~420 bp (**Figure 2**, **Figure 4** and **Figure 5**). These results can only be explained by hypothesizing that Big Top is heterozygous at the *endoPG* locus for the presence of a "null" allele, in addition to the f allele composed only by the Pp-endoPG^{BT} variant of F^{a}-*Ppa006839m*. The literature proposes for SS Big Top the allelic combinations f/f or f/f_{null} [30] [31]; our results seem to support the latter one (**Figure 4** and **Figure 5**; **Table 1**).

The coherence of the allelic combinations at the *endoPG* locus inferred by the InDel analysis with, where available, those reported in the literature (**Table 1**) suggests that the InDel polymorphism may represent a tool for a simple genotyping of peach accessions. Nevertheless, this polymorphism does not allow to discriminate between the peculiar gene sequences individuated in SS or SH genotypes.

CAPS analysis exploiting the SNP individuated in Exon 1 of the *endoPG* sequence of SS Big Top integrates the InDel results allowing to distinguish between amplicons of ~420 bp derived from the *Pp-endoPG*^{BT}-like variant of F^{a}-*Ppa006839m* and the *Pp-endoPG*^{SH}-like one [6]. All the SS genotypes tested in CAPS analysis proved sensitive to *Bst*XI producing upon cleavage two digestion fragments. In a few accessions, the contemporary presence of an undigested fragment indicated that their allelic asset was heterozygous (F/f; **Figure 6(c)** and **Figure 2**). For Ruby Rich, this asset confirms the functional genotype (F/f) proposed in the literature [30] [31] (**Table 1**). Lack of effect of *Bst*XI in SS Alitop further confirms the inheritance of a possible 'null' *Pp-endoPG* variant from the Big Top parent. Conversely, the SH genotypes tested were not sensitive to *Bst*XI digestion (**Figure 6(c)**).

In the climacteric peach fruit the evolution of texture characteristics is regulated by ethylene. The SH trait (*hdhd*) is related to absence of ethylene production for a mutation in the *Pp-ACS1* gene and altered indole-3-acetic acid levels due to regulation of *PpYUC11* [6] [32] [33] [34]. In SH 7-28, homozygous-recessive for the stony *hard gene* (*hdhd*) [35], InDel analysis shows presence of the ~420 bp amplicon (**Figure 2**) attributable, by CAPS analysis, to a *Pp-endoPG*^{SH}-like sequence (**Figure 6(c)**). The contemporary presence of the other two amplicons allows to assign this accession, as far as it concerns *Pp-endoPG*, to the Stony Hard melting category (*hdhd M-*) consistent to Haji *et al.* [36].

Therefore, it can be concluded that although CAPS analysis of the specific *Pp-endoPG*$^{BT/SH}$ variants seems to allow a preliminary distinction of SS from SH genotypes, conclusive phenotypic attribution of a specific accession will necessarily require the assessment of other activities like fruit ethylene production.

5. Conclusions

The two molecular markers (InDel and CAPS) based upon *Pp-endoPG* poly-

morphisms tested in the present work may represent useful tools for simple genotyping of peach accessions as far as it concerns the characteristics and different allelic combinations at the *endoPG* locus.

In particular, the use of the *Pp-endoPG* InDel marker yields results consistent with the existing knowledge on the polymorphic structure of the *endoPG* locus in peach, and allows to individuate NM accessions. The CAPS marker developed on the basis of the peculiar SNP_{348} of SS Big Top integrates the results of InDel analysis by discriminating the *Pp-endoPG* variants in SS and SH accessions.

Future development of the work may foresee the testing of a broader number of NM, as well as SS and SH accessions, in order to confirm the potential use of the described InDel and CAPS markers in Marker-Assisted Selection peach breeding programs.

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