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STUDY OF BIOCHEMICAL AND MOLECULAR MECHANISMS INVOLVED IN THE REGULATION OF RIPENING IN THE CLIMACTERIC PEACH (*PRUNUS PERSICA* L. BATSCH) FRUIT

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CONTENTS

Summary

Peach (*Prunus persica*[L.] Batsch) is temperate fruit species of economic importance, it ranks second in fruit production in Europe after apple. Peach fruit are classified by different flesh firmness, which strongly influences fruit shelf-life and storage, and ethylene evolution, which determinates the organoleptic quality of the ripening fruit.

Concerning flesh texture, three main distinct peach fruit phenotypes are known, even if not all is understood in terms of their genetic determination and differences in biochemical pathways activated during the final stages of ripening which lead to the definition of phenotypic-specific characteristics. The first two phenotypes, described by Bailey and French, 1932, are the melting (M) and non-melting (NM) flesh. The M texture shows a prominent softening in the final stage of ripening. Variability of firmness is found within this phenotype and Yoshida (1976) distinguished between soft, medium and firm. The NM phenotype (the so called 'canning peach') shows a firm texture when fully ripe that never becomes soft. Rather, it becomes rubbery (for the loss of water from the tissues) during the senescence stage, when most cultivars could also display the development of peculiar off-flavours (Sherman *et al*., 1990). The third flesh texture phenotype was described by Yoshida (1976). He firstly classified a very firm and crispy, 'stony hard' (SH) flesh phenotype as belonging to the M family. However, these fruit never soften and resemble a NM phenotype, becoming rubbery when senescent. A possible fourth flesh texture trait resembles very much the SH flesh in firmness and crispiness, but when fully ripe becomes melting. This flesh texture is found in many recently developed new cultivars (nectarines, as 'Big Top', and standard peaches, e.g. 'Rich Lady' and 'Diamond Princess'; Monet and Bassi, 2008).

Availability of newly established peach cultivars with optimal organoleptic (as for M phenotypes) and durability [as for NM, SH and Slow Melting ('Big Top') phenotypes] characteristics would represent an economic advantage improving shelf-life and economic value of the produce. To do this, and in order to plan proper breeding programmes, a possible strategy may consist in the identification of molecular markers linked to the "flesh texture" phenotype.

In this work, particular attention has been devoted to the development of different characteristics of flesh texture as a function of the structure of the *endo-PG* gene and of fruit ethylene evolution and response. The research work was articulated in four main topics:

1. Chapter I reports the results of some physiological, biochemical and molecular aspects of the Melting of the 'Big Top' Nectarine Fruit. We have characterized the postharvest behavior of 'Big Top' fruit, treated or not with ethylene for five days after harvest (DAH), and compared it with that of '193 Q XXVII 111', a SH peach selection ('Ghiaccio'), and

with that of an accession with very firm flesh, 'D41-62'. *Pp-ACS1* expression, ethylene evolution, endo-PG production and softening characteristics have been evaluated in fruit of the three genotypes and referred to those of ripe melting flesh (M) 'Bolero' at harvest. Like 'Bolero', 'Big Top' fruit did express *Pp-ACS1* and evolve ethylene, but with a 5-d delay during postharvest. *Pp-endo-PG* expression, production of an active endo-PG isoform typical of ripe peach fruits and fruit melting showed a parallel behavior; ethylene treatment further enhanced all the above features. In SH 'Ghiaccio' *Pp-ACS1* expression, ethylene evolution, endo-PG production and softening were virtually absent during the first five DAH in air. 'Ghiaccio' neither expressed *Pp-ACS1* nor evolved ethylene even after ethylene treatment, but responded by accumulating *Pp-endo-PG* transcripts and an active endo-PG protein, thus with consequent flesh melting. A similar behavior was observed in 'D41-62'. Overall, the data confirm the pivotal role of ethylene in the regulation of endo-PG expression and activity, and thus in the determination of peach fruit flesh texture characteristics, and support the evidence that 'Big Top' could be classified as belonging to the 'melting' ('slow melting') phenotype and 'D41-62' to the SH one.

2. Chapter II describes the *EndoPG* locus configuration in different peach genotypes. The work has been undertaken with the aim of ascertaining whether specific mutations (SNPs or InDel polymorphisms, assessed by CAPS or InDel analysis) at the level of the *Pp-endoPG* gene could be used as molecular markers for prediction of fruit phenotype. To do so, a preliminary screening has been conducted on a few established cultivars on the progenies of a few selected crosses identifying, at the level of the *endoPG* gene, the patterns of InDel amplification and CAPS restriction patterns and comparing them with fruit texture (M, NM, SH, Slow Melting) phenotype. The results, albeit preliminary and to be confirmed on a larger number of selection from controlled crosses, seemed to provide satisfactory matching between the molecular results and fruit phenotype**.**

3. Chapter III reports the isolation and preliminary analysis of *Pp-endoPG* promoter region in Melting, Non Melting amd Slow Melting flesh genotypes. The sequences of the 5' upstream region of the *m Pp-endoPG* clone of both NM 'Oro A' and M 'Bolero', of the *M* clone of M 'Bolero and of the *BT* clone of Slow Melting 'Big Top' were obtained. PLACE analysis showed the presence in different positions of the isolated promoter sequences of a few interesting *cis*-acting elements, differently involved in the plant responses to endogenous (phytohormones) and exogenous (light, water stress, etc.) factors suggesting a possible fine regulation of *Pp-endoPG* expression by several factors. Particularly interesting was retrieval of microsatellites, possibly exploitable as molecular markers for the identification of the

fruit phenotype, and of an Ethylene Responsive Element (ERE). The latter one was present in the 5' upstream region of clones *m*-B and *BT* and in *M* clone, while in the 5' upstream region of clone *m*-O a SNP was identified an altered ERE motif. This result may possibly explain the lower expression of *Pp-endoPG* observed in the high ethylene-producing NM cultivar 'Oro A'. Preliminary gel Electrophoresis Mobility Shift Assay (EMSA) gave the indication of a stronger interaction with nuclear protein of the promoter region of clone *m*-B (containing ERE motif) with respect to clone *m*-O. This result, though needing to be confirmed by further experiments, appears consistent with the different expression profiles of the *Pp-endoPG* gene as related to softening behaviour

4. Chapter IV describes the preliminary characterization of some physiological and biochemical traits of fruit of one (BO 95021043) of the selections available at the Di.Pro.Ve., and of their response to treatment, in the postharvest period, with ethylene and/or with its antagonist, 1-methyl-1-cyclo-propene (1-MCP). The results obtained confirmed that these fruit were blocked in the evolution of all the ripening-related parameters. This physiological blockade was accompanied by an overall extremely low and constant degree of expression of a few genes playing a key role in ripening-related process. Lack of endogenous ethylene evolution in SR mutants seemed related to block of the expression of the *Pp-ACO1* gene. SR fruit, though, were able to respond to ethylene treatment as demonstrated by the ethylene-induced slight enhancement of ripening-related parameters. Cleaved Amplified Polymorphis Sequence (CAPS) investigation on the structure of the *Pp-endoPG* gene in the SR BO 92051043 selection indicated the presence of two SNPs (SNP_{390} and SNP_{1310}) common also to 'Big Top', 'Ghiaccio' and 'Yumyeong', possibly indicative of a parentage between these lines.

THESIS INTRODUCTION

1.1 Peach growing: economic and agronomic point of view

Peach [*Prunus persica* (L.) Batsch] is a temperate fruit tree species of economic importance. It ranks second in fruit production in Europe after apple. In 2009 the EU production of peaches and nectarines has been estimated at about 4 million tons, showing a slight increase (+3%) as compared to 2008 (http://www.ismea.it).

Italy is the main producer of peaches in Europe (45% of EU production) and the second in the world after China, followed by U.S.A. (12%). A good share of world peach production is also attributable to Spain and Greece (8%) followed by Turkey, France, Iran, Chile and Egypt (Source: FAO data, 2008). In Italy, peach and nectarine cultivation is spread all over the country, with two main regions of production: Emilia-Romagna in the North and Campania in the South. A minor but valuable production is also present in Piedmont, Veneto and Sicily (Pirazzoli and Regazzi, 2000).

Prunus persica is a diploid (2n = 16) species belonging to the Rosaceae family, which includes a number of economically important fruit tree species, such as apple, pear, almond and cherry. *P. persica* has a medium tree height (up to 8 m); the leaves are lanceolate, glabrous and serrate, broadest near the middle, with a glandular petiole. The flowers are generally pink, but also white or red; the fruit is pubescent or glabrous, fleshy and the mesocarp does not split; the stony endocarp is very deeply pitted, furrowed and lignified (Layne and Bassi, 2008).

A ripe peach fruit is approximately 87% water and contains carbohydrates, organic acids, pigments, phenolics, vitamins, volatiles, antioxidants and trace amounts of proteins and lipids, which make it very attractive to the consumer (Kader and Mitchell 1989; USDA 2003)

1.2. Nutritional quality of fruits

Fruits, nuts and vegetables play a significant role in human nutrition, especially as sources of vitamins [C (ascorbic acid), A, thiamine (B1), niacin (B3), pyridoxine (B6), folic acid or folate (B_9), E], minerals, and dietary fiber (Quebedeaux and Bliss, 1988; Quebedeaux and Eisa, 1990; Craig and Beck, 1999; Wargovich, 2000).

Goldberg *et al*. (2003) reported that the presence of fruits and vegetables in the daily diet is strongly associated with reduced risk for some forms of cancer, heart disease, stroke, and other chronic-degenerative diseases (Prior and Cao, 2000; Tomas-Barberan and Espin, 2001). In fact, fruit and vegetables contain components (phytochemicals) which are strong antioxidants able to control metabolism activation and detoxification/availability of carcinogens, and possibly affecting the course of tumor cell development and growth (Wargovich, 2000).

Peach fruit are rich in macronutrients, such as soluble sugars and organic acids, minerals, dietary fiber, as well as in micronutrients, i.e. antioxidant compounds and, as above stated, vitamins (Rossato *et al*., 2009).

The beneficial nutritional properties of peaches, as of all fruits, are fully developed, and fruit reach their best eating quality, when they are at full ripening and allowed to ripen on the plant (Kader *et al*., 1999).

Changes in nutrient composition occur in the period from harvest to consumption and depend, to a certain degree, on the nature of the nutrient and of the commodity, and on the post harvest handling and storage practices. Most fruits and vegetables are composed of 70-90% water and once separated from their source of water and nutrients (i.e., the mother tree) undergo to stress conditions resulting in moisture loss, higher rates of oxygen consumption, nutrient degradation, with loss of quality paralleled also by potential microbial spoilage.

Nowadays consumers have developed a great awareness of food quality, and give increasing attention to characteristics like taste, flavour and nutritional properties. Some of the most frequent consumer's complaints about peach and nectarine quality are for hard flesh and reduced flavour at consumption, as pointed out by Crisosto (2002) who reported that hard fruit, mealiness, lack of taste, and failure to ripen are the main reasons for low consumption of stone fruit in California (Iglesias and Echeverria, 2009). These problems are caused by harvesting fruit at immature stages in order to avoid produce wastage during shelf-life and allow long distance shipment. Due to these commercial practices, very often the flavour components do not reach acceptable levels determining poor produce characteristics (Crisosto *et al.*, 2001; Etienne *et al*., 2002).

1.3 Fruit ripening

1.3.1 General aspects

Fruit ripening is a unique aspect of plant development with direct implications for large components of the food supply and related areas of human health and nutrition. For this reason, considerable scientific studies have been focused on aspects linked to fruit organogenesis, differentiation, development and maturation, as well as to the genetic, biochemical and physiological basis of the development of fruit organoleptic and nutritional qualities (Giovannoni, 1997; Adams-Phillips *et al*., 2004).

The ripening process is a highly coordinated, genetically programmed, and irreversible phenomenon involving a series of biochemical, physiological and structural changes that have the

biological evolutionary function of making the fruit attractive and palatable to a variety of seed-dispersing organisms ("consumers" in the human consortium). In fleshy fruits, a wide spectrum of biochemical changes such as chlorophyll degradation, biosynthesis of carotenoids, anthocyanins, flavour and aroma components, increased activity of cell wall-degrading enzymes, and, in some fruits, a transient increase in ethylene production are some of the major events occurring during fruit ripening (Prasanna *et al*., 2007).

During fruit ripening, the changes of skin colour are due to the unmasking of carotenoid pigments by degradation of chlorophyll and dismantling of the photosynthetic apparatus. An increase in the levels of carotenoids, such as β-carotene, xantophylls and lycopene, and synthesis of different types of anthocyanins, which accumulate in the vacuole, also occur. In this period the development of flavour and aroma takes place due to the production of a complex mixture of volatile compounds (Lizada, 1993), and the degradation of bitter principles, such as tannins, and related compounds (Tucker, 1993). The development of taste with a general increase in sweetness is the result of increased gluconeogenesis and hydrolysis of polysaccharides, especially starch, and decreased acidity. A proper equilibrium between sugars and organic acids is therefore accomplished at full ripening resulting in a pleasant sugar/acid blend (Lizada, 1993).

Two major classifications of ripening fruits, climacteric and non climacteric, have been utilized to distinguish them on the basis of oxygen consumption and ethylene biosynthesis rates in the phase immediately preceding full ripening ("climacteric"). In climacteric fruit, such as tomato and banana, ripening is accompanied by a peak in respiration and a concomitant burst of ethylene, whereas in non climacteric fruits, such as grapes and citrus, respiration shows no dramatic change and ethylene production remains at a very low level. Therefore, the metabolic changes associated to fruit ripening can include increase in biosynthesis and evolution of the ripening hormone, ethylene (Yang and Hoffman, 1984) and increase in oxygen consumption (Tucker 1993, Negrini *et al.*, 1998). Alteration of cell structure involves changes in cell wall thickness, permeability of plasma membrane, hydration of cell wall, decrease in the structural integrity, and increase in intracellular spaces (Redgwell *et al*., 1997).

The changes at the cell and tissue level finally lead, during fruit ripening, to textural alterations, which are dramatic in climacteric fruits. Flesh firmness is a prime indicator of fruit quality, being a ripening index in fruit such as apples, kiwifruit, stone fruits, mangoes and avocados. Depending upon their inherent composition and nature, different fruits soften at different rates and to different degrees. Fruits such as mango, papaya and banana undergo drastic and extensive textural softening from 'stone hard' stage to 'soft pulpy' stage, whereas apple and citrus do not exhibit such a drastic softening, though they do undergo textural modifications during ripening

(Prasanna *et al.*, 2007). Even though changes in the turgor of mesocarp cells, possibly linked to changes in the balance between storage polysaccharides and soluble sugars with consequent osmotic phenomena as well as to changes in the cuticle water permeability properties (Saladiè *et al*., 2007), have been proposed to be involved in the textural changes of fruit ripening, the main phenomenon responsible for softening in most fruits is generally acknowledged to be the partial breakdown of the fruit cell walls.

Since the process of textural softening is of commercial importance as it directly affects fruit quality, shelf-life and consumer's acceptance, a great deal of research studies on the biochemistry of the cell wall polysaccharides and their degradation and/or rearrangement in ripening fruits is now available in the literature.

1.3.2. The plant cell wall

Primary walls are the major textural component of many plant-derived foods. Fermented fruit products, including wine, contain significant amounts of primary wall polysaccharides (Doco *et al.*, 1997). Primary walls isolated from higher plant tissues and cells are composed predominantly of polysaccharides (up to 90% of the dry weight) together with lesser amounts of structural glycoproteins (2-10%), phenolic esters (<2%), ionically and covalently bound minerals (1-5%) and enzymes.

Primary wall polysaccharides are used commercially as gums, gels and stabilizers. The results of several studies have suggested that primary wall polysaccharides are beneficial to the human health as they have the ability to bind heavy metals, regulate serum cholesterol levels and stimulate the immune system (O' Neill and York, 2003 and references therein). Thus, the structure and organization of primary wall polysaccharides is of interest to the food processing industry and the nutritionist as well as the plant scientist.

1.3.2.i The plant cell wall polysaccharides

The cell walls of angiosperms and gymnosperms are composed of three classes of polysaccharides: cellulose, hemicelluloses (or cross-linking glucans: xyloglucans, (galacto)glucomannans, and xylans) and pectic polysaccharides (homogalacturonans, rhamnogalacturonans, and substituted galacturonans) albeit in different amounts (O' Neill and York, 2003 and references therein).

As far as it concerns the chemical structure, cellulose is composed of (1→4) β-D-glucan chains assembled together with a high degree of polymerization by hydrogen bonds into very long crystalline microfibrils, whose regular arrangement excludes water, so that the glucosidic linkages of internal β-1→4 linked D-glucans are not approachable by enzymes located in the relatively water-rich apoplast.

Xyloglucans are the most abundant hemicellulosic polysaccharides, often comprising 20% of the dry mass of the wall. These polysaccharides possess a (1→4) β-D-glucan backbone similar to cellulose; up to 75% of the backbone residues are branched, bearing β-D-xylose at O6. (Galacto)glucomannans have a backbone composed of regions of (1→4) β-D-glucan and (1→4) β-D-mannan in approximately equal amounts, with some of the β-D-man residues bearing galactose side chains at O6. Xylans, which include arabinoxylans, glucuronoxylans and glucoronoarabinoxylans have a backbone of (1→4) β-D-xylan, with numerous ramifications (O'Neill and York, 2003).

The pectic polysaccharides are homogalacturonans, substituted galacturonans, and rhamnogalacturonans. Homogalacturonan (HG) is a long chain of (1→4)α-D-galacturonic acid in which some of the carboxyl groups are methyl esterified. HG polymers with a high degree of methyl esterification are referred to as 'pectin' whereas HG with low or no methyl esterification is termed 'pectic acid'. HG may account for up to 60% of the pectin in the primary cell wall of dicotyledons; their solubilization and depolymerization appear to play a major role (together with, to a lesser degree, changes in apparent molecular mass of hemicelluloses) in the processes involved in fruit ripening (Ramina *et al.*, 2008 and references therein).

Rhamnogalacturonans are a group of closely related cell wall pectic polysaccharides that contain a backbone of a repeating disaccharide made of alternating α-D-rhamnose and α-D-galacturonic acid residues; substituted galacturonans are a group of polysaccharides that contain a backbone of linear 1,4-linked β-D-galacturonic acid residues differently substituted.

Other important primary wall components are structural proteins, many of which are heavily glycosylated, and numerous enzymes, including those involved in wall metabolism (endo and exoglycanases, methyl and acetyl esterases, and transglycosylases), and enzymes that may generate cross-links between wall components (e.g. peroxidases). Walls also contain proteins referred to as expansins that have been proposed to break hydrogen bonds between xyloglucans and cellulose and thus are believed to regulate wall expansion (O'Neill and York, 2003 and references therein).

1.3.2.ii General features of wall ultrastructural models

Concerning the arrangement of structural components in the cell wall, cellulose microfibrils are coated with and cross-linked together by matrix glycans, and among these the most abundant are xyloglucans that span between adjacent microfibrils linking them together by hydrogen bonds. Glucomannans and glucoronoarabinoxylans also cross-link cellulose microfibrils by hydrogen bonds. The spaces in the cellulose/matrix glycan network are filled with the highly hydrated pectins, forming a network held together by ester bonds between pectin molecules and by ionic calcium network crosslinks between de-methyl-esterified homogalacturonans. Structural proteins may form an additional network.

1.3.2.iii Cell wall reorganization during fruit ripening and softening-related enzymes

As previously stated (see § 1.3.1), the loss of fruit firmness during ripening is believed to result principally from cell wall disassembly and a reduction in cell-cell adhesion following dissolution of the pectinaceous middle lamella (for a review, see Rose *et al*., 2003 and references therein). The structure of cell wall components and the cell wall architecture as a whole are progressively modified; the nature and extent of these changes vary depending on the species. The cell wall structure becomes increasingly hydrated as the cohesion of the pectin gel changes due to pectin hydrolysis, and this affects cell cohesion and fruit texture (Jarvis, 1984). In the apoplast a decrease in pH with a concomitant increase in "free" ion concentration occur determining cell wall loosening and a decline in cell turgor (Brummel and Harpster, 2001).

The regulation of cell wall disassembly is controlled by multiple factors, including the developmental, environmental or hormonal regulation of genes encoding cell wall-modifying proteins which, in turn, provide the basis for a dynamic cell structure able to accommodate changes in cell size or shape or in cell adhesion (Bennett and Labavitch, 2008).

Fruit edible tissues are composed mainly of parenchymatous cells that show little or no lignification and generally have a polysaccharide composition similar to that of type I primary walls, with approximately equal amounts of cellulose and hemicelluloses and a pectin content of 40-60% (Rose *et al*., 2003 and references therein). Fruit softening is probably caused by the cumulative effect of a range of modifications occurring in the networks of polymers which constitute the primary cell wall, which contribute in different ways to loss of firmness and changes in textural qualities. Therefore, the modifications of the cell wall polymers during ripening are complex and involve the coordinated and interdependent action of a range of cell wall-modifying

enzymes and proteins such as cellulase, xyloglucan endotransglycosylase (XET), β-galactosidase, pectin methylesterase (PME), pectate lyase (PL), polygalacturonase (PG), and expansins (EXP) (Brummell and Harpster, 2001). Further variability is brought about, in the different fruit species, by differences in the chemical composition of fruit cell walls (Redgwell *et al.*, 1997; Wakabayashi, 2000) and the nature, timing, and extent of the modifications (solubilization and depolymerization) of cell wall polysaccharides (Rose *et al.*, 1999; Brummell and Harpster, 2001), Thus, the role of individual cell wall-modifying enzymes in fruit softening is also likely to be different in the different fruit species.

Cellulase, often referred to as EGase (endo-1→4-β-D-glucanase), hydrolyzes the glycosidic linkages of (1→4)-β-D-linked glucan chains (cellulose). Plant EGases are encoded by large multigene families (27 predicted members in *Arabidopsis*; Henrissat *et al.,* 2001); ripening-related EGase genes have been described in several species (Brummell *et al.*, 2001), even if studies conducted with transgenic lines of tomato, pepper, and strawberry collectively indicate that EGases alone do not make a substantial contribution to wall disassembly in many ripening fruits (Rose *et al*., 2003 and references therein). A certain degree of hemicellulose depolymerization is a common feature of fruit softening, particularly in the early softening stages, and is believed to contribute significantly to changes in texture (Rose *et al.*, 2003 and references therein). Hemicellulose modification is brought about by enzymes such as EGase and xyloglucan endotransglycosylase-hydrolase (XTH), which comprises xyloglucan endotransglycosylase (XET) and xyloglucan endohydrolase (XEH). XTH carries out a transglycosylation of XyG in which one chain of XyG is cleaved and reattached to the nonreducing terminus of another XyG chain; some XTH, though, appear to function hydrolytically (Carpita and McCann, 2000; Brummel and Harpster, 2001; Rose *et al.,* 2002). Though ripening-related XTH activities or gene expression have been reported in several fruit, to our knowledge no detailed data have yet been published clearly unravelling the significance of XTHs for softening or restructuring of cell wall architecture in ripening fruit (Rose *et al.,* 2003). The enzyme β-galactosidase also appears to play a direct role in fruit softening. One of the most apparent changes in the fine structure of the cell walls of ripening fruit is often a loss of pectic neutral sugars and particularly galactosyl residues, which are likely to occur as side-chains on pectins (Redgwell and Fischer, 2002). The enzyme responsible for their removal is β-D-galactosidase. Seven β-galactosidase genes are expressed during tomato fruit development (Smith and Gross, 2000), six out of these are known to be expressed during ripening and suppression of their mRNAs in transgenic tomato fruits results in 40% increase in fruit firmness. Though the role of galactosyl residues in fruit texture has not yet been fully ascertained, they may act directly to

increase wall strength or indirectly to control the pore size in the pectin matrix, thereby limiting the movement of hydrolytic enzymes (Seymour and Manning, 2002 and references therein).

Despite the plethora of enzymatic activities and non enzymic proteins involved in the overall process of flesh cell wall dismantling during fruit ripening, and on the basis that pectin solubilization and depolimerization are the most pronounced and widely reported changes in the walls of ripening fleshy fruits, closely correlated with softening and cell wall swelling (Rose *et al*., 2003 and references therein), it appears logical to focus the attention on pectin-degrading enzymes.

Pectin-degrading enzymes such as pectin methylesterase (PME), pectate lyase (PL) and polygalacturonase (PG) surely play a major role in fruit softening (Prasanna *et al.*, 2007).

Pectins are believed to be synthesized and deposited into the wall in a highly methylesterified form and to undergo enzyme-mediated demethylation. In tomato, the degree of methyl esterification of galacturonosyl residues in polyuronides declines from levels as high as 90% at a pre-ripe stage to 35% in ripe fruit; similar values were observed in ripening avocado (Harriman, 1990; Wakabayashi *et al.*, 2000). Demethylation of the pectin galacturonosyl residues would result in the generation of carboxylate ions, which can then bind cations such as calcium (Almeida and Huber, 1999). Unesterifed galacturonic acid residues are believed to contribute to the so-called 'egg-box' structure, resulting in the formation of a pectate gel (Pérez *et al.*, 2000).

Changes in the degree of methylation influence pectin charge density and cation binding capacity, apparent molecular size, aggregation, solubility, gelation properties and ultimately function in the wall and middle lamella. Pectin demethylesterification also has significance in facilitating hydrolysis of the pectin backbone by pectate lyases and PG. PME de-esterifies polyuronides by removing methyl groups from the C-6 position of the galacturonic acid residues of pectins. One of the most widely proposed roles for PME is the demethylation of homogalacturonan in ripening fruit, and ripening-related PME activity and gene expression have been described in a range of fruits (Rose *et al.*, 2003 and references therein). PLs catalyse the eliminative cleavage of de-esterified pectin (Carpita and Gibeaut, 1993). The degradation of pectins by PL occurs by a β-elimination reaction in contrast to the hydrolytic mechanism of PG. Plant PLs have been associated with a number of developmental events that involve extensive wall disassembly, including pollen tube penetration into the stylar tissue, tracheary element differentiation, laticifer growth and fruit ripening (Marín-Rodríguez *et al.*, 2002). Ripening-related PLs genes have been identified in several fruit species, and PL-like ESTs have been found at relatively high levels in ripening tomato fruits (Marín-Rodríguez *et al.*, 2002).

Polygalacturonases (PGs) are enzymes that catalyze the hydrolytic cleavage of the β-1,4-D-galacturonan backbone of pectic polysaccharides. They have long been recognized as primary

agents implicated in polyuronide degradation in ripening fruit (Themmen *et al.*, 1982; Huber, 1983, Redgwell and Fischer 2002) and hence involved in the regulation of fruit softening (Brady *et al.*, 1983). PGs occur as a family of genes and the corresponding proteins can act as either endo- or exo-hydrolases (Hadfield and Bennett 1998); however, the endo-acting enzymes are more likely to contribute significantly to pectin depolymerization in ripening fruits. Although endoPG-independent pectin solubilization has been suggested for a range of fruit that undergo ripening-associated cell wall breakdown in the apparent absence of endoPG activity (Redgwell and Fischer, 2002 and references therein), in other species a close relationship between endo-PG and flesh softening has been recognized. In pepper, the soft flesh and deciduous fruit is a dominant trait controlled by the *S* gene encoding PG (Rao and Paran, 2003). In European pear, softening closely parallels endo-PG expression (Hiwasa *et al.,* 2003). In peach, while PME activity appeared to be more closely related with the decrease in flesh firmness in the initiation of softening, a strict relationship between endoPG activity and fruit melting has been demonstrated (Ramina *et al.,* 2008 and references therein).

Tomato fruit PG, perhaps the most thoroughly studied of this class of enzymes, is generally described as comprising three isoforms: PG1, PG 2A and PG 2B. PG 2A and PG2 B are differently glycosylated proteins and represent the catalytic component of PG. PG1 is a heterodimer of either of the PG2 isoforms tightly bound to a protein (β-subunit) whose function has been suggested to be that of altering and restricting the biochemical activity or localization of PG2 in vivo (Rose *et al.,* 2003 and references therein). In ripening fruits, PG mRNA accumulation is ethylene-regulated (Sitrit and Bennett 1998, Hayama *et al.,* 2006a).

A class of cell wall-localized proteins called expansins are associated with numerous tissues and developmental stages of cell undergoing changes in size and shape (Cosgrove, 2000). Expansins appear to operate by disrupting hydrogen bonds between cellulose microfibrils and xyloglucans that bind them to one another in plant cell walls (Whitney *et al.*, 2000). They have also been shown to play an important role in fruit softening (Rose *et al.*, 1997; Anjanasree and Bansal, 2003). The ripening-associated expansins might contribute to cell wall degradation by increasing the accessibility of other cell wall-modifying proteins, such as PG and cellulase, to structurally important cell wall polymers (Rose and Bennett, 1999).

1.4 Ethylene in fruit ripening

The plant hormone ethylene (C_2H_4) is a gaseous molecule that regulates several processes including seed germination, organ senescence, stress response and fruit ripening.

Many years ago it was discovered that two types of fleshy fruits can be recognized, based on the fact that a peak in the rate of respiration can be measured (climacteric fruits) or not (non climacteric fruits) before the visible onset of the ripening process. In climacteric fruits, a peak of ethylene production is normally observed concomitant with climacteric respiration. Therefore, the hormone has been considered as the main signal for the regulation of ripening in these fruits (Abeles *et al.*, 1992).

Studies of ethylene synthesis and mode of action, firstly conducted in *Arabidopsis* also with the aid of *Arabidopsis* mutants, have since long been widened to other species, with particular regard to the fruit-producing ones, due to the pivotal role played by ethylene in the ripening process especially of climacteric fruits. Also in this case, pioneering work on the genetic basis of fruit formation and development has emphasized the model system *Arabidopsis*, whereas investigations of organ expansion, maturity, ripening, shelf-life and nutritional quality have centered on the crop model tomato (Giovannoni, 2004). Tomato, the centerpiece of the Solanaceae family, has emerged as a model of fleshy fruit development, primarily because this is the species for which the genetic and molecular toolkits are most advanced. Well-characterized ripening mutations, short generation time, a long history of physiological, biochemical and molecular investigations related to fruit development and maturation, and interest in the species as an important commodity crop, have fueled considerable effort on understanding ripening in this species (for a review, see Giovannoni, 2007; Lin *et al*., 2009; Bapat *et al*., 2010). Nevertheless, it is important to recognize that other model systems for ripening are fairly widely used. For non climacteric ripening strawberry is a primary model and peach, apple and citrus are developing as strong models for both ripening and genomics analysis of fruit tree species (Giovannoni 2007).

Though ethylene surely plays a key role in fruit ripening, it also important to note that other plant hormones, in addition to ethylene, are likely to influence this developmental process (Beaudoin *et al*., 2000). Of particular importance appears to be the role of auxin; in peach fruits it has been demonstrated that concomitant with the climacteric ethylene production a significant increment of the IAA content takes place in the mesocarp tissues (Miller *et al.*, 1987). Jones *et al.*, 2002) reported in tomato fruits a differential expression of ARF (Auxin Response Factors) and Aux/IAA encoding genes linked to the auxin signalling pathway. More recently, Trainotti *et al.* (2007) showed that in peach many genes involved in biosynthesis and transport and, in particular, the signaling of auxin (receptors, Auxin Response Factors) show an increased expression in the peach mesocarp during ripening, demonstrating the existence of an important cross-talk between auxin and ethylene (Trainotti *et al.*, 2007). In ripening plum fruits, auxin appeared to affect the expression of an ERF gene (El-Sharkawi *et al.*, 2009).

1.4.1 Ethylene synthesis

Two systems for the regulation of ethylene production exist in higher plants. System I, operating in both climacteric and non climacteric fruits as well as in vegetative tissues, has been proposed to be responsible for basal and wound-induced ethylene production, and system II for the upsurge of ethylene production during the ripening of climacteric fruits (Leliévre *et al.*, 1997; Lin *et al*., 2009).

The biochemical features of the ethylene biosynthetic pathway in higher plants are well defined (Yang *et al*., 1984). Ethylene is synthesized from methionine in three steps:

- conversion of methionine to S-adenosyl-L-methionine (SAM) catalyzed by the enzyme SAM synthetase,
- formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM via ACC synthase (ACS) activity,
- conversion of ACC to ethylene, which is catalyzed by ACC oxidase (ACO) (Barry and Giovannoni, 2007).

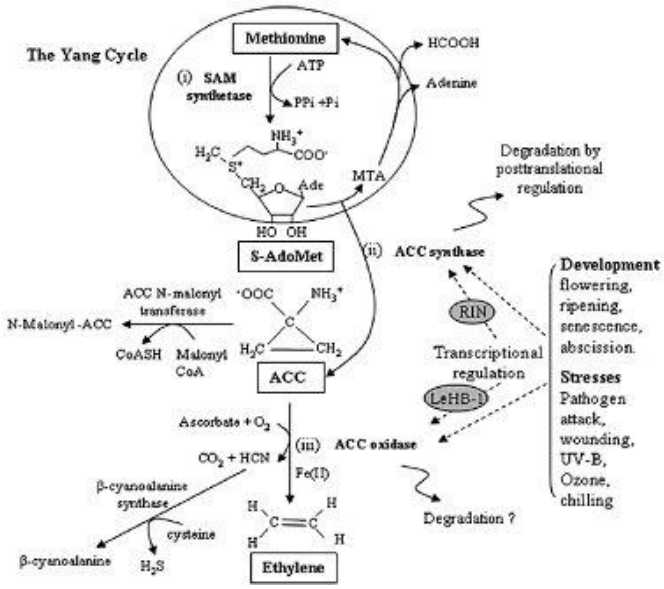

Figure 1. Ethylene biosynthetic pathway. (from Lin *et al.*, 2009).

Both ACS and ACO are encoded by multigene families in most plant species. To date in the *Arabidopsis* genome nine differently regulated *ACS* genes that encode eight functional and one non-functional ACS proteins have been identified, while in tomato eight genes are present (Barry *et al.*, 2000). In pre-climacteric tomato fruits, a few members of the *ACS* and *ACO* gene families (namely, *LeACS1, LeACS3, LeACS6*, *LeACO1* and *LeACO4*) are active and responsible for System I basal ethylene biosynthesis. Transition to System II at ripening is the result of *LeACS6* silencing and

increased expression of *LeACS2*, *LeACS4*, *LeACO1* and *LeACO4* (Barry *et al.*, 2000; Alexander and Grierson, 2002).

1.4.2 Ethylene perception

1.4.2.i The components of the signal transduction cascade

Ethylene (Figure 2) is sensed by a family of receptors that share structural similarity with bacterial two-component histidine kinases (Chang and Stadler, 2001) and have been identified as endoplasmic reticulum (ER)-associated integral membrane proteins with protein kinase activity (Moussatche and Klee, 2004). The receptors are disulfide-linked dimers and ethylene binding is mediated by copper as a co-factor. Genetic and molecular studies indicate that the receptors act as negative regulators of the ethylene signaling pathway (Rodriguez *et al.*, 1999; Tieman *et al.*, 2000; Binder, 2007).

Based on gene and deduced protein structures, the ethylene receptors have been divided into two subfamilies: in *Arabidopsis*, the subfamily I receptors ETR1 and ERS1 show highest similarity to histidine kinases, whereas the subfamily II receptors ETR2, EIN4 and ERS2 appear to have lost most of the amino acids critical for histidine kinase activity and instead possess serine kinase activity (Zhong *et al.*, 2008 and references therein). In tomato six ethylene receptors have been identified: the structures of *LeETR1*, *LeETR2*, *NR* are consistent with subfamily I receptors, while *LeETR4*, *LeETR5* and *LeETR6* can be classified as subfamily II receptors.

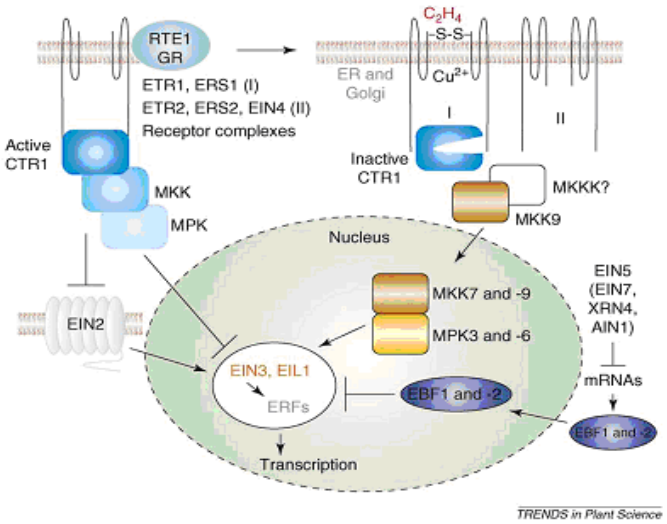

Figure 2. Current general model for the ethylene signal transduction pathway (Yoo *et al.*, 2009).

1.4.2.ii Protein kinase and phosphatase activity in cell signaling: general remarks

Members of the protein kinase superfamily catalyze the reversible transfer of the β-phosphate from ATP to serine, threonine, tyrosine (in animals) or histidine (in plants) amino acid side chains on target proteins (Chevalier and Walker, 2005). Protein kinase activity is counterbalanced by the action of specific protein phosphatases. In most cases phosphorylation modifies target protein activity. One protein kinase molecule can phosphorylate many hundreds of target proteins, thereby greatly amplifying weak signals. Activation of protein kinase has been implicated in cell responses to light, pathogen attack, temperature stress, nutrient deprivation and growth regulators (Trewavas, 2000). Therefore, protein phosphorylation/dephosphorylation may be considered, together with other cellular second-messenger mechanisms, an efficient biological switch.

To date, several hundreds of plant protein kinases have been characterized at molecular level; they have been grouped into families based on their amino acid sequence similarities (Chevalier and Walker, 2005). Besides the ethylene receptors described above, which are attributed to the family of Hys kinases, another important family is that of "Constitutive Triple Response-1 (CTR1)/protein kinase Raf-like" (Gao *et al.*, 2003). CTR1 is the component downstream receptors identified in the ethylene signaling pathway (Figure 3). *CTR1* encodes a putative Raf-like Ser/Thr protein kinase that initiates Mitogen-Activated Protein (MAP)-kinase signaling cascades in mammals. The sequence similarity of CTR1 to known MAPKKKs led to the hypothesis that ethylene signalling may operate through a MAP-kinase cascade (Arora, 2005) that would operate downstream of CTR1 as a positive regulator of the ethylene response (Li and Guo, 2007). Nevertheless, so far no conclusive evidence to support a MAPK cascade operating in the ethylene signal transduction pathway has been presented (Li and Guo, 2007).

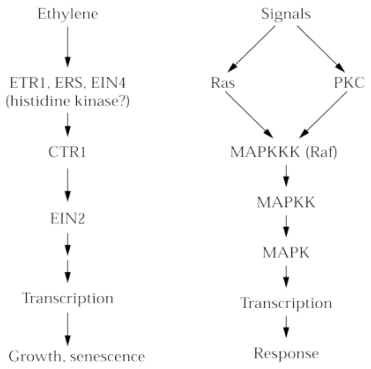

Figure 3. Comparison of ethylene-signaling pathway and animal Ras-signaling pathway. CTR1 has distinct sequence similariy to Raf (MAPKKK). A protein kinase cascade is indicated for transduction of the ethylene signal. In Biochemistry and Molecular Biology of Plants (2000), Buchanan, Gruissem and Jones eds.

The MAPKs belong to the family of Ser/Thr protein kinases and are important components of signal transduction pathways, evolutionarily conserved in all eukaryotes. In plants, MAPKs are regulated by hormones (ABA, IAA, GAs, ethylene), biotic and abiotic stresses and are active in specific cell cycle phases (Soyano *et al.*, 2003 and references therein). MAPKs transduce signals acting in a modular phosphorylation cascade consisting of three members each of which is activated by its MAP kinase (MAPK), MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). The activation, often by phosphorylation, of a MAPKKK is the entry to trigger the MAP kinase cascade, which ends with the phosphorylation of specific targets by a phosphorylated MAPK (Tena *et al*., 2001; Chevalier and Walker, 2005 and references therein).

While only one *CTR* gene apparently exists in *Arabidopsis*, in tomato a family of *CTR1*-like genes has been isolated (Adams-Phillips *et al*., 2004). In particular, *LeCTR2* plays a role in ethylene signalling probably through its interconnections with ETR1-type ethylene receptors of subfamily I (Lin *et al*., 2009). CTR1, which has been found to associate with ER membranes in *Arabidopsis* (Gao *et al.*, 2003) is thought to form a complex with the receptors, at least those of subfamily I, and negatively regulate the downstream ethylene signaling events in absence of the hormone: in fact, it would be inactivated by the receptors when these bind ethylene (Hua and Meyerowitz, 1998).

Two positive regulators act downstream of CTR1: EIN2 (Ethylene Insensitive), which is directly activated by CTR1, and EIN3. Genetic study reveals that EIN2 is an essential component in the ethylene signaling pathway but its biochemical function remains a mystery (Zhu and Guo, 2008), so that it has been defined as “the great unknown” of the ethylene signaling pathway. *EIN2* is the only known gene whose loss of function leads to the complete absence of response to ethylene (Alonso *et al.*, 1995; Zhu *et al.*, 2006). *EIN2* encodes a 1294 amino acid protein with similarity to the Nramp family of metal ion carriers (Alonso *et al.*, 1999), and, based on specific evidences, might represent a common point through which multiple hormone signal transduction

pathways might act (Fujita and Syono, 1996; Beaudoin *et al*., 2000; Ghassemian *et al.*, 2000). EIN2 works upstream of EIN3 and EIL (EIN3-like) family of nuclear localized trans-acting proteins (Guo and Ecker, 2003; Figure 2).

EIN3 is a plant-specific transcription factor and its protein abundance in the nucleus is rapidly induced upon ethylene treatment. In the absence of ethylene signal, EIN3 protein is degraded in a 26S proteasome-dependent manner (Zhu and Guo, 2008 and references therein). To date, four *EIN3-like* genes (*LeEIL1-4*) have been described in tomato (Stepanova and Alonso, 2009). Once in nucleus, EIN3 and EILs bind a conserved motif within the promoters of *ERF1* (Ethylene Responsive Factor) [which, with related genes, forms a subgroup (145 members in *Arabidopsis*) of the large APETALA family encoding DNA-binding proteins, i.e., transcription factors] and activate expression of the corresponding proteins. These ERF1 proteins bind to the GCC-box found in the promoters of ethylene-regulated genes involved in a wide range of developmental processes, responses to environmental challenges and pathogen infection and act by positively or negatively regulating their transcription. Among the ERF1-regulated genes also those involved in cell wall disassembly can be found (Solano *et al*., 1998).

1.4.3. Ethylene synthesis, perception and signaling in peach

Peach is a model organism for Rosaceae, an economically important family that includes fruits and ornamental plants such as apple, pear, strawberry, cherry, almond and rose. It is a model for analysis of conserved ethylene response and ripening mechanisms in fruit tree species. In peach, where the ethylene climacteric peak occurs during the late ripening stage (Tonutti *et al*., 1991), the *ACS* polymorphisms seem crucial in regulating ethylene biosynthesis. Three different members of the *ACS* family have been identified (*Pp-ACS1*, *Pp-ACS2*, *Pp-ACS3*), but only the first one seems associated with ripening-associated ethylene evolution by peach fruit (Mathooko *et al*., 2001; Tatsuki *et al*., 2006).

Regarding the other important component of the ethylene biosynthetic pathway, it has been shown that, in peach, the *ACO* gene belongs to a multigene family made up of at least three members (Ruperti *et al*., 2001). One of these (*Pp-ACO1*) is specifically expressed during fruit ripening and is under developmental control and regulated by ethylene (Tonutti *et al*., 1997). *Pp-ACO2* mRNA is detected in fruit only during early development and is unaffected to ethylene.

Genes similar to *Arabidopsis ETR* and *ERS* are present in the peach genome. Also in peach, the deduced proteins of the two genes contain a sensor domain and a hystidine-kinase domain, in which residues thought to be important for the normal function of ETR and ERS type protein as

ethylene receptors are conserved (Rasori *et al*., 2002). These results indicate that *Pp-ETR1* and *Pp-ERS1* could be putative ethylene receptors with the ability to bind ethylene in peach. One additional ethylene receptor (*Pp-ETR2*) has been identified that, more than *Pp-ERS1*, appears to be induced during the transition from pre-climacteric to climacteric stage (Trainotti *et al*., 2006).

Acting downstream of the receptors is situated *Pp-CTR1*, that interacts directly with the receptor molecules to form a signalling complex (Gao *et al*., 2003). Apart from pear (El-Sharkawy *et al*., 2002) and apple (Dal Cin *et al*., 2005a), no information on this gene is so far available in other fruit of Rosaceae species (Dal Cin *et al*., 2006).

A marked increase in *EIN2-like* transcripts has been detected during the transition from pre-climacteric to early climacteric stage of peach fruit (Trainotti *et al*., 2006). In the same transcriptomic work, 19 ripening-related transcription factors (TFs) belonging to several families as MADS-box, AUX/IAA, bZIP, bHLH, HD, MYB, and AP2 (*APETALA2*, that encodes Ethylene Responsive Elements Binding Proteins) have been shown to be differentially expressed.

1.4.3.i Ethylene inhibitors: the case of 1-methyl-cyclo-propene (1-MCP)

As in several other fields of plant physiology, many of the above cited studies have taken advantage of the use of compounds able to inhibit, with different mechanisms, the action of ethylene. In climacteric fruits, the control of ripening mainly relies on the possibility of affecting (through genetic manipulation, modulation of environmental parameters during storage, or use of specific ethylene inhibitors) ethylene production and/or action. In general, this leads to altered gene transcription pattern and, as a consequence, modified ripening behaviour.

Ethylene can profoundly affect the quality of harvested products. These effects can be beneficial or deleterious depending on the product, its ripening stage, and its desired use (Saltveit, 1999). Endogenous ethylene production is an essential part of ripening of climacteric fruit and probably acts as rheostat for ethylene-dependent processes (Theologis, 1992). Exogenous ethylene application can be used, according to specific local laws, to initiate uniform ripening for fruit such as banana. Most commonly, however, commercial strategies for horticultural products are based on avoiding exposure to ethylene and/or attempting to minimize ethylene production and action during ripening, harvest, storage, transport and handling by temperature and atmosphere control (Watkins, 2002). A new strategy for controlling ethylene production and thus ripening and senescence of climacteric fruit has emerged with the discovery and commercialization of the inhibitor of ethylene perception, 1-methylcyclopropene (1-MCP) (Watkins, 2006). This compound is thought to interact with ethylene receptors thus preventing ethylene-dependent processes (Sisler and Serek, 1997). The

impact of 1-MCP on postharvest science and technology is two-fold. First, it provides the potential to maintain fruit and vegetable quality after harvest. As a matter of fact, by 2005 food use registration for this chemical had been obtained in Argentina, Australia, Austria, Brazil, Canada, Chile, Costa Rica, France, Guatemala and Honduras, Israel, Mexico, the Netherlands, New Zealand, South Africa, Switzerland, Turkey, UK, and the US (Watkins, 2006). Second, 1-MCP provides a powerful tool to gain insight into the fundamental processes that are involved in ripening and senescence.

Dal Cin *et al*. (2006) reported that the effects of 1-MCP are highly variable within the category of climacteric fruit (peaches and apples, in particular), and this has been imputed to different amounts, ratios and regeneration rates of ethylene receptors. Different researches show that 1-MCP can affect ethylene production, respiration, softening, color change, aroma production and the occurrence of physiological disorders with profound effects on shelf-life and taste-life of many fruit produce (Watkins, 2006). As far as it specifically concerns peach fruit, their responses to 1-MCP are affected by concentration and exposure period, and inhibition of fruit ripening appears transitory in all published studies, even if repeated 1-MCP applications help maintain suppression of ripening. In 1-MCP treated peach fruit, flesh firmness is maintained but ethylene evolution is not inhibited and, after a few hours from the end of the treatment period, the ripening processes (included softening) quickly recovers (Mathooko *et al*., 2001; Fan *et al*., 2002; Ziliotto *et al*., 2003; Watkins, 2006 and references therein). Transcriptomic data confirmed that, in 1-MCP-treated fruit, altered transcript accumulation was detected for some genes with a role in ripening-related events such as softening, colour development, and sugar metabolism (Ziliotto *et al.*, 2008).

In spite of these drawbacks, the use of this inhibitor of ethylene action appears useful for studies on the ripening physiology of peach (as well as other) fruit.

1.5. Peach ripening and fruit softening

Peach fruit exhibit a double sigmoid growth pattern, which may be divided into four stages: Stage 1, which includes cell division and cell expansion; Stage 2, during which the endocarp becomes lignified (pit hardening) and fruit growth slows down; Stage 3, when most of the increase in fruit size takes place due to cell enlargement, and Stage 4, characterized by the increase in ethylene evolution and completion of the ripening process. (Figure 2; Tonutti *et al*., 1997).

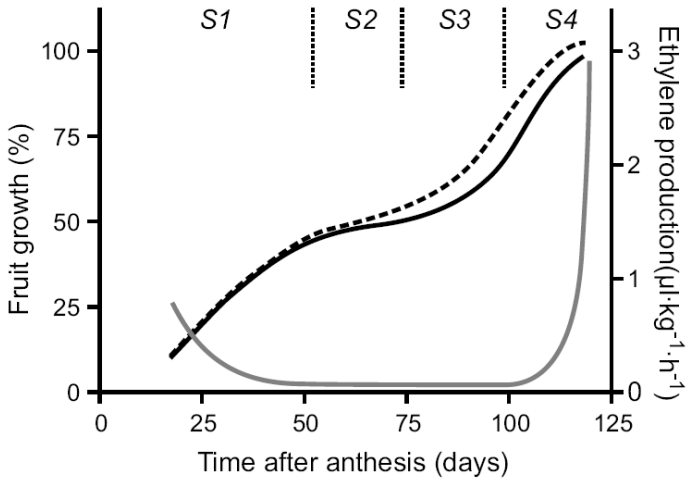

Figure 4. Relative changes in fruit growth of 'Fantasia' nectarine (top unbroken line) and 'Jalousia' (top broken line) peaches. The gray bottom line shows the rates of ethylene production relative to the four stages S1–S4 of fruit development (adapted from Moing *et al.*, 1998 and Tonutti *et al*., 1997).

The main processes defining the peach so-called "ripening syndrome" occur during the last part of fruit development and are genetically controlled. Many of these (accumulation of soluble solids, colour and aroma development and flesh softening) have a direct impact on fruit appearance, composition and consumer's acceptability, while others (ethylene evolution and respiration rates) represent the biochemical/physiological factors which are the basis for the evolution of quality parameters.

Important factors affecting consumer's acceptability of stone fruit are sweetness, acidity and aroma, globally contributing to fruit flavour. The main sugars in peaches are sucrose, glucose and fructose (in proportion roughly 3:1:1; Genard *et al*., 2003) which in ripe fruit comprise 60% of the soluble solids concentration (SSC) and whose relative concentrations influence sweetness (Begheldo, 2008). Acidity is also an essential component of the fruit taste, and is thought to be determined during the early stages of fruit development. In peach the two major organic anions are malate and citrate (present in substantial amounts, to approx. 140 and 120 mM H^+, respectively, which, in high-acid cultivars, tend to decrease during ripening (Ramina *et al.,* 2008). As regards aroma, its composition in ripe fruit varies among peaches and nectarines and in the different cultivars, but the most important contributors are volatile aldehydes, esters, ketones, terpenoids and sulphur-containing compounds (Monet and Bassi, 2008). Unfortunately, peaches and nectarines often can not develop full aroma characteristics due to a too early harvesting and/or to the effects of prolonged low temperature storage. Peaches soften during ripening and this is a very important quality attribute that often dictates shelf life.

Concerning flesh texture, three main distinct peach fruit phenotypes are known, even if not all is understood in terms of their genetic determination and differences in biochemical pathways activated during the final stages of ripening which lead to the definition of phenotypic-specific characteristics. The first two phenotypes, described as early as in the Thirties of the last century (Bailey and French, 1932), are the melting (M) and non-melting (NM) flesh. The M texture shows a prominent softening in the late stage (S4) of ripening. Variability of firmness is found within this phenotype and Yoshida (1976) distinguished between soft, medium and firm. The 'firm' type softens rather slowly, is less susceptible to handling, thus allowing easier management of harvest timing and of grading and shipping operations, and displays a longer shelf life. The NM phenotype (the so called 'canning peach') shows a firm texture when fully ripe that never becomes soft. Rather, it becomes rubbery (for the loss of water from the tissues) during the senescence stage, when most cultivars could also display the development of peculiar off-flavours (Sherman *et al*., 1990). The third flesh texture phenotype was described by Yoshida (1976). He firstly classified a very firm and crispy, 'stony hard' (SH) flesh phenotype as belonging to the M family. However, these fruit never soften and resemble a NM phenotype, becoming rubbery when senescent. A possible fourth flesh texture trait resembles very much the SH flesh in firmness and crispiness, but when fully ripe becomes melting. This flesh texture is found in many recently developed new cultivars (nectarines, as 'Big Top', and standard peaches, e.g. 'Rich Lady' and 'Diamond Princess'; Monet and Bassi, 2008).

As previously stated (§ 1.3), the main phenomenon acknowledged to be responsible for peach fruit softening is the partial breakdown of the fruit cell walls; also in peach the main hydrolases involved in cell wall resctructuring are β-(1,4)-glucanase, β-galactosidase, pectin methyl esterase, pectate lyase and polygalacturonase, which act together with expansins (Brummell, 2006).

The lack of a melting phase in the NM phenotype has been ascribed to either a deletion of an *endoPG* gene or a truncation of a specific *endoPG* mRNA, which causes absence of detectable levels of endoPG protein (Lester *et al*., 1994; Callahan *et al*., 2004). However, Morgutti *et al*. (2006) reported that in the NM cultivar 'Oro A' there was the expression, at low levels, of an *endoPG* isoform which produced a functional transcript, suggesting a transcriptional regulation in the production of *endoPG* in M and NM phenotypes. These data point out that endoPG-mediated pectin modification plays a pivotal role in the later stages of softening and textural changes in melting flesh peaches.

As far as it concerns the characteristics of ethylene evolution by fruit of the different phenotypes, it is worth to be noted that M and NM fruit develop rather high amounts of ethylene at the climacteric peak, between S3 and S4 stage, often more abundantly in NM types (Mignani *et al*.,

2006), and that the only remarkable difference of SH fruit from NM ones is the almost complete absence of ethylene evolution due to the transcriptional suppression of the 1-aminocyclopropane-1-carboxylic acid synthase isogene (*Pp-ACS1*), (Goffreda, 1992; Haji *et al*., 2001, 2003; Tatsuki *et al*., 2006, 2007). Nevertheless, in SH fruits ethylene production can be induced by stress conditions, such as cold-temperature storage (Tatsuki *et al*., 2006; Gamberini 2007; Begheldo 2008). 'Big Top' fruit do evolve ethylene, but in an unpredictable fashion from year to year (Lavilla *et al.*, 2002; Mignani *et al.*, 2006).

Ripe climacteric fruits show wide ranges of ethylene production rates (Knee *et al.*, 1985). In several peach cultivars ethylene evolution begins to increase when fruits have already started to soften and the highest biosynthetic rates occur in the latest stage of ripening, which corresponds to flesh firmness values of 30-40 N. Huge variation exists among cultivars in terms of ethylene evolution rates at ripening (Ventura *et al.*, 2008). Stony Hard peaches are indeed characterized by the absence of both ethylene production at ripening and postharvest softening, and in the 'Fantasia' nectarine cultivar and in some of its progenies a correlation between ethylene evolution and softening rate has been observed. Within these progenies an interesting mutation was found ('Slow Ripening'): fruit remain firm and green and do not exhibit a rise in CO_2 or ethylene production for at least 4 weeks at 20°C (Brecht and Kader, 1984). Despite these observations, which seem to establish a clear positive correlation between fruit ethylene evolution and flesh softening, in M/NM cultivars low levels of ethylene biosynthesis are not necessarily correlated to slower rates of loss of firmness in NM fruits in comparison to the M ones (Brovelli *et al.*, 1999). Therefore, despite the widely recognized knowledge that the expression of the *endoPG* gene, which plays a pivotal role in the melting process in peach fruits, is regulated by ethylene (Hayama *et al.*, 2006), it should be argued that different phenotypic characteristics related to flesh texture in M/NM peach fruit may result from the ability of the tissues to respond to ethylene across a broad concentration range due to some form of signal modulation.

1.6 Peach genetics and breeding

In recent years, the development of high-throughput techniques and new biotechnological approaches covering a broad field of disciplines has opened the Genomic Era.

As far as it concerns the Rosaceae family, the application of molecular technologies has steadily increased and concerted efforts have been undertaken to develop genomic tools (markers, maps, DNA sequences, and identification of Quantitative Trait Loci, QTLs) to be, if possible, rapidly translated into agronomic practice. Some of these have been developed and made available

to researchers. In particular, peach has become the genetic and genomic reference species for *Prunus* due to its small genome (290 Mb; Baird *et al.,* 1994), self-compatibility which allows generation of F_2 progenies, and relatively short intergeneration period (Shulaev *et al*., 2008).

1.6.1. Structural genomics

Initial efforts in the field of structural genomics have been directed toward developing high-density molecular linkage maps to accelerate breeding through MAS (Dirlewanger *et al.,* 2004). Several linkage maps are available today in peach; and in peach × almond F_2 progenies, with the 'Texas' (almond) × 'Early Gold' (peach) linkage map currently considered as the reference map of the *Prunus* genus (Verde *et al*., 2005). Many of the major morphological, quality and agronomic characters with simple Mendelian inheritance and some Quantitative Traits Loci (QTLs, which constitute the bulk of the variability selected during the breeding process in fruit trees as in most cultivated species; Arús *et al*., 2003), most of them polymorphic within the peach genome, have been placed on this consensus map. Moreover, the complete peach genome sequence (peach v1.0) has recently been published (http://www.rosaceae.org/peach/genome). Its analysis will allow the description of the physical nature of the genome and its functional characterization, allowing the detection of the genes that are being turned on or off at any given time depending on endogenous or exogenous factors (Shulaev *et al*., 2008).

1.6.2 Functional genomics

The field of functional genomics is aimed to identify the functions of unassigned genes and use this knowledge to improve economically important agronomic and quality traits in fruit crops. Large-scale Expressed Sequence Tags (EST) collections are being produced from many cDNA libraries of different peach fruit tissues by different institutions: the Clemson University (http://www.genome.clemson.edu/projects/peach/est**),** the Italian ESTree Consortium **(**http://linuxbox.itb.cnr.it/ESTree**)** and**,** more recently, the Programme Genoma Chile **(**http://www.fondef.cl**)** µPEACH 1.0, a 70-mer oligo-microarray, has been developed from EST sequences mainly obtained from cDNA libraries of ripening peach fruits (ESTree Consortium, 2005). This tool has been used to investigate on the molecular events occurring at the transition from pre-climacteric to the early climacteric stage (Trainotti *et al*., 2006). Analysis and annotation of EST frequencies in peach fruit skin and mesocarp in different developmental and ripening stages

and in different cultivars allowed to identify a list of genes putatively involved in the peach aroma production (Vecchietti *et al.*, 2009).

1.6.3 Innovative approaches to peach breeding

The peach breeding industry is one of the most dynamic concerning temperate fruit crops, so that the 20th century has been called "The Golden Age of Peach Breeding" (Fideghelli *et al*., 1998; Byrne 2002). Worldwide, peach breeders supply growers with a large number of improved cultivars able to satisfy, with their diversified characteristics, a wide range of grower's and consumer's demands. Common goals are related to agronomic [extension of the harvest season, control of tree size and architecture, development of resistance to pests (Plum Pox Virus and his vector, the green aphid), powdery mildew, brown rot] and fruit quality-related aspects strictly linked to the consumer's demands (improvement of fruit flavour and aroma and lengthening of the shelf life) (Hancock, 2008). Nevertheless, nowadays peach breeding is required to respond to other serious challenges such as the predictable changes in quality and incidence of biotic and abiotic stresses brought about by climatic changes, and the increased competition, intrinsic to global marketing, among the different peach-growing regions and among peach, as a commodity, and a vast array of other fruits. In general, this will require an even greater commitment that will include exploration of new germplasm through the exploitation of innovative molecular, genomic-based breeding technologies which will empower the traditional ones (outcrossing or inbreeding; Hesse, 1975). These methods, notably molecular markers, promise fast and efficient approaches to cultivar improvement. Early selection with molecular markers (Marker Assisted Selection, MAS) allows an accurate screening of seedlings several years before the characters can be phenotypically evaluated in the field, makes possible the accumulation of different resistance factors in a genotype of interest or shortens the number of generations needed to recover the genotype of the cultivated species after a cross with an exotic genotype or wild species. Overall, the result will be saving of space, and even more importantly, of time, factors particularly important in woody perennials. Obvious targets to the search of markers for early selection are characters that require complex analysis, that cannot be evaluated until the plant has reached the adult stage (such as fruit characters or the self-incompatibility genotype) or those linked to recessive alleles (such as, in peach, hairless skin or round fruit) allowing the identification and selection of heterozygous individuals not expressing the trait at the phenotypic level (Dirlewanger *et al*., 2003).

In peach, MAS may significantly reduce the costs associated with maintaining undesirable trees until maturity (Struik *et al.*, 2005). MAS strategy has been used for pest resistance (Yamamoto and

Hayashi, 2002) and columnar growth habit (Scorza *et al.*, 2002), and it has recently become possible for QTL and single gene studies of fruit quality traits such as sugar and organic acids content (Dirlewanger *et al.*, 2006).

Most modern peach cultivars have originated from those produced by U.S. Government breeding programs of the early 20th Century. These exploited a very limited numbers of parental lines, with the consequence of drastic erosion of the genetic variability of this crop. Moreover, modern peach cultivars are propagated vegetatively, which allows the maintenance of their genetic information, but also makes the breeder's rights more difficult to protect. In this scenario, the high number of existing cultivars and their important economic value has encouraged the development of fast and reliable techniques for peach molecular fingerprinting (Aranzana *et al*., 2003, 2010).

1.6.3.i Genetic aspects of fruit quality

Numerous traits are important to peach breeders as far as it specifically concerns fruit quality. In the fresh-product market, consumers desire a large, well shaped fruit that is flavourful, with a high sugar content and low-to-moderate acidity, and juicy ("melting") flesh. For the processed-product market, other characteristics are appreciated, such as firm flesh, absence of a tip on the pit, no pit cracking, attractive colour (absence of water-soluble anthocyanins) and lack of browning of the flesh (Hancock, 2008).

Unfortunately, peaches and nectarines are highly perishable commodities, with a shelf life of only a few days at room temperature essentially due to the rapid softening (or even melting) of the fleshy tissue after harvest, which makes them easily attackable by spoilage microorganisms.

Flesh firmness, like other characters related to plant growth and architecture, yield, blooming and harvesting times, can be considered as a quantitative trait. It is affected by several biochemical and physiological factors (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.) which, acting in a co-ordinated series of events, lead to flesh softening in the course of ripening (Ferguson and Boyd, 2002; Redgweel and Fischer, 2002). The identification and cloning of single genes of known function involved in (if possible) each step of such network would allow the evaluation of the possible minor or major effects of their polymorphisms on flesh firmness characteristics. As a consequence, the dissection of fruit quality characteristics into a number of elementary components linked to genes of known function ("Candidate Gene" approach) would be extremely useful for the early marked assisted selection of fruit with desirable characteristics. Genes have been identified which control sucrose accumulation,

fruit ripening and carotenoid accumulation (Yen *et al*., 1995; Klann *et al*., 1996; Huh *et al*., 2001). In peach, a few quantitative trait loci (QTLs) involved in fruit quality (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 (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 M trait is also especially desirable because the trait shows only when fruits are mature. Moreover, 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.

As far as it concerns the "melting of the flesh" process, and the related phenotypic trait 'M' or 'NM', it has been reported to be a simple Mendelian one, with the M trait dominant over the NM (Bailey and French, 1932). The M trait is controlled, like the F ('freestone') one, by the *endo-PG* gene (Peace *et al.*, 2005). As already reported, endoPG is a cell wall-metabolizing enzyme involved in fruit softening. Its maximum expression coincides, in peaches and nectarines, with the climacteric peak and the 'melting phase' that is the characteristic of most of the fresh market cultivars (Lester *et al*., 1994; Morgutti *et al.*, 2006).

Genes controlling Mendelian traits, as the M/NM one, where one of the phenotypic extremes typically result from a loss-of-function mutation, are excellent candidates to explain quantitative variation in similar traits, thought to arise from minor sequence differences in such genes (Robertson, 1989). Concerning *endoPG*, smaller changes in the different *endoPG* isogenes have been shown to result in quantitative differences in fruit firmness and texture among peach and nectarine varieties (Peace *et al*., 2007).

1.7 Purpose of the work

Availability of newly established peach cultivars with optimal organoleptic (as for M phenotypes) and durability [as for NM, SH and Slow Melting ('Big Top') phenotypes] characteristics would represent an economic advantage improving shelf-life and economic value of the produce. To do this, and in order to plan proper breeding programmes, a possible strategy may consist in the identification of molecular markers linked to the "flesh texture" phenotype.

The general aim of the Ph.D. activities presented in this thesis was to give a contribute to the overall comprehension of a few biochemical, physiological and molecular aspects of the ripening process of peach fruits directed also toward the identification of molecular factors useful for the improvement of produce quality. Particular interest was devoted to the assessment of the possible existence of differences at the gene level unequivocally related to the flesh texture characteristics possibly exploitable as markers for early selection of fruit phenotype.

The research activities have been conducted with plant material from peach accessions with different flesh texture characteristics (M, NM, SH) and different ethylene evolution ability.

- The first research topic of this Ph.D. thesis has focused on the characterization of the behaviour of fruit of the 'Big Top' cultivar, characterized by a peculiar timing for melting appearance, during postharvest and in response to ethylene treatments, comparing it to the behaviour of a SH ('Ghiaccio') cultivar, of an accession with very firm flesh but not unequivocally characterized ('D41-62') and to the characteristics of a well-known 'melting flesh' cultivar ('Bolero'). The main parameters considered were expression of the *Pp-ACS1* gene, fruit ethylene evolution and expression and levels of endo-PG. The study was been paralleled by molecular investigations aimed to identify *endo-PG* gene polymorphisms between 'Big Top' and the other accessions studied.
- The second research topic has dealt with the analysis of a few peach accessions representing the progenies of the crosses between cultivars with different fruit phenotype. This analysis, aimed to verify the possible relationship between the *endoPG* polymorphisms and fruit phenotype in order to explore the possibility of their use for early genotype selection, has exploited the polymorphisms individuated in the *endoPG* clones (*m, M, SH*) considered. Another goal of this research activity was to analyze these progenies trying to verify whether the *m* and *M endoPG* clones co-segregate.
- The third research topic has focused on the isolation, by Genome Walking techniques, of the promoter regions of the *endoPG m*, *M* and *BT* clones in selected peach accession with NM, M

and slow melting fruit phenotype (‘Oro A’, ‘Bolero’ and ‘Big Top’ respectively), with the aim of individuating polymorphisms and/or putative regulatory sequences possibly related to the different expression of the *endoPG* gene in the different genotypes.

Moreover, with the aim of trying to achieve a better understanding of the possible correlations between ethylene synthesis and perception and the development of different fruit flesh texture characteristics a preliminary molecular, biochemical and physiological characterization of “Slow Ripening” mutants (accession BO 95021043) has also been conducted.

CHAPTER I

MELTING OF THE 'Big Top' NECTARINE FRUIT: SOME PHISIOLOGICAL, BIOCHEMICAL AND MOLECULAR ASPECTS

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Ghiani A., Negrini N., Morgutti S., Baldin F., Nocito F.F., Spinardi A.M.V., Mignani I., Bassi D. and Cocucci M. Melting of the 'Big Top' Nectarine Fruit: Some Physiological, Biochemical and Molecular Aspects

Abstract

The ‘Big Top’ nectarine [*Prunus persica* (L.) Batsch] has peculiar flesh characteristics, resembling, at commercial harvest, the ‘stony hard’ (SH) peach flesh in firmness and crispiness, but becoming melting by a slow pace at full ripening, which gives the fruits appreciable keeping quality.

We have characterized the postharvest behaviour of ‘Big Top’ fruit, treated or not with ethylene for five days after harvest (DAH), and compared it with that of ‘193 Q XXVII 111’, a SH peach selection (‘Ghiaccio’), and with that of an accession with very firm flesh, ‘D41-62’. *Pp-ACS1* expression, ethylene evolution, endo-PG production and softening characteristics have been evaluated in fruit of the three genotypes and referred to those of ripe melting flesh (M) ‘Bolero’ at harvest. Like ‘Bolero’, ‘Big Top’ fruit did express *Pp-ACS1* and evolve ethylene, but with a 5-d delay during postharvest. *Pp-endo-PG* expression, production of an active endo-PG isoform typical of ripe peach fruits and fruit melting showed a parallel behaviour; ethylene treatment further enhanced all the above features. In SH ‘Ghiaccio’ *Pp-ACS1* expression, ethylene evolution, endo-PG production and softening were virtually absent during the first five DAH in air. ‘Ghiaccio’ neither expressed *Pp-ACS1* nor evolved ethylene even after ethylene treatment, but responded by accumulating *Pp-endo-PG* transcripts and an active endo-PG protein, thus with consequent flesh melting. A similar behaviour was observed in ‘D41-62’. The characterization of a ‘Big Top’ *Pp-endo-PG* clone showed several single nucleotide (SNPs) and insertion-deletion (InDels) polymorphisms in comparison to the *M Pp-endo-PG* clone of ‘Bolero’ and substantial identity with the *Pp-endo-PG* clones of ‘D41-62’ and SH ‘Ghiaccio’. In the *Pp-endo-PG* clone of ‘Big Top’ a SNP (base pair 348) peculiar of this genotype and InDels shared by ‘Big Top’, ‘D41-62’ and ‘Ghiaccio’ have been identified, possibly suitable for discriminating among different genotypes on the basis of the *Pp-endo-PG* gene structure and for preliminary assessing whether an accession is genetically related to ‘Big Top’. Overall, the presented data confirm the pivotal role of ethylene in the regulation of endo-PG expression and activity, and thus in the determination of peach fruit flesh texture characteristics, and support the evidence that ‘Big Top’ could be classified as belonging to the ‘melting’ (‘slow melting’) phenotype and ‘D41-62’ to the SH one.

Key words: endo-PG expression and activity, *Pp-endo-PG* gene, ethylene, flesh texture, peach, *Prunus persica*, ripening

Introduction

Two main groups for classification of peach [*Prunus persica* (L.) Batsch] fruit in terms of flesh texture (one of the most important factors determining the consumer's perception of fruit quality; Bruhn, 1994) are melting flesh (M) and non melting flesh (NM). The M texture softens in the last stage of ripening, in correspondence to the peak of ethylene evolution (Tonutti *et al.,* 1996), until a complete melting. The NM phenotype (the canning peaches) shows a firm texture even when fully ripe, softens slowly when overripe but never melts (Bailey and French, 1949; Monet and Bassi, 2008). A third, interesting trait is the so-called 'stony hard' flesh (SH) trait, with almost null ethylene production, crispy fruit flesh and poor softening during ripening (Haji *et al.,* 2003, 2004; Yoshida, 1976), that is considered to be linked to the allelic configuration at a *Hd* locus (Haji *et al.,* 2005). The extremely scarce ethylene production by SH fruit seems due to reduced expression of the gene codifying the 1-aminocyclopropane-1-carboxylic acid synthase 1 (ACS1) of *Prunus persica* (*Pp-ACS1,* Begheldo *et al.,* 2008; Tatsuki *et al.,* 2006, 2007). NM and SH peach fruit have high 'keeping' quality and are expected to become increasingly important in breeding for new fresh market cultivars due to their better postharvest behaviour.

Fruit of the nectarine 'Big Top' have peculiar flesh softening characteristics, resembling the SH flesh in firmness and crispiness at harvest, but melting by a slow pace and developing ethylene, though in unpredictable fashion from year to year (Monet and Bassi, 2008; I. Mignani, personal communication) at full ripening, after a few days of postharvest. This behaviour led a few Authors to consider 'Big Top' fruit as belonging to a SH subgroup (Gamberini, 2007), whereas others, on the basis of phenotypical observations, suggest to categorize them as a truly separate melting sub-group ('melting very firm', MV; Monet and Bassi, 2008).

Flesh softening is a complex phenomenon which involves the sequential activation of several genes whose products act in the definition of cell wall structure (Hayama *et al.,* 2006a; Trainotti *et al.,* 2003); many of them are under the control of ethylene (Cara and Giovannoni, 2008). Among these, a pivotal role in softening is played by endo-polygalacturonase (endo-PG; Brummell *et al.,* 2004; Orr and Brady, 1993; Pressey and Avants, 1978). The NM phenotype of some cultivars appears closely associated to a massive deletion in an *endo-PG* gene, resulting in a complete lack of the expression of the major endo-PG isoform involved in peach fruit softening (Callahan *et al.,* 2004; Lester *et al.,* 1994, 1996). Conversely, in NM 'Oro A' the activity of the same *endo-PG* gene results in weak accumulation of a normal length transcript and little production of endo-PG, suggesting regulation at the transcriptional level (Morgutti *et al.,* 2006). Interestingly, in at least two SH peaches ('Manami' and 'IFF331') both the *endo-PG* transcript accumulation and protein

functionality do not seem altered, as suggested by the observation that ethylene – exogenously applied or endogenously induced by cold stress – promotes *endo-PG* transcription and fruit softening (Haji *et al.*, 2005; Hayama *et al.*, 2006a,b; Begheldo *et al.*, 2008). Thus, results concerning different peach fruit phenotypes seem to suggest that different events may affect the multiple steps along the pathway which leads to endo-PG production and softening. In particular, the amounts of fruit ethylene evolution may not always and necessarily represent the only or the main point involved in the regulation of softening. In fact, NM fruit evolve more ethylene than M ones (Brovelli *et al.*, 1999) but they produce less endo-PG, soften to a lesser extent and do not melt (Morgutti *et al.*, 2006).

'Big Top' fruit have rewarding characteristics for both growers and consumers since they retain flesh firmness on the tree for a longer time than M peaches. This feature makes possible full development of their organoleptic qualities due to high levels of sugar accumulation (Iglesias and Echeverria, 2009) coupled to the low acid trait (Monet, 1979). Occurring of melting after a few days of postharvest enhances these positive flavor traits, making the produce suitable for fresh consumption with a high degree of liking by the consumer.

To further clarify the mechanisms underlying, at different levels, the determination of the peculiar 'Big Top' softening pattern, in the present study we have considered the postharvest behavior in the absence and presence of ethylene treatment, as regards flesh firmness changes, *endo-PG* gene transcription and endo-PG production and activity. The results have been compared to those obtained with SH 'Ghiaccio' fruit and with fruit of an accession ('D41-62') producing very firm fruit, subjected to the same treatments and with physiologically ripe and soft ("ready to eat"; Monet and Bassi, 2008; Cantín *et al.*, 2010) M 'Bolero' fruit.

Firmness (or pace of softening) is an important quality trait to be considered in a breeding program, since it is directly related to susceptibility to mechanical damage during postharvest (Crisosto *et al.*, 2001). In the search of molecular markers for the 'Big Top' fruit phenotype, the analysis has been widened to the *Pp-endo-PG* gene, whose structure in 'Big Top' has been compared to those in M 'Bolero', NM 'Oro A' and SH 'Ghiaccio' and 'Yumyeong' (from which 'Ghiaccio' has been bred by self pollination) accessions, as well as fruit of a selection ('D41-62') with very firm flesh but of uncertain phenotype attribution.

Materials and methods

Plant material. Fruit and leaves were obtained from peach accessions producing fruit with diverse phenotypes, i.e., 'Big Top', 'D41-62' (very firm fruit), 'Yumyeong' and selection '193 Q XXVII 111' ('Ghiaccio' series), derived from self-pollination of 'Yumyeong' (Nicotra *et al.*, 2002) (SH), 'Bolero' (M), and 'Oro A' (NM). The trees were grown under integrated pest management growing operations in a peach collection located at Castel San Pietro (Bologna County, Northern Italy). Fruit were monitored for size and epicarp ground color on the tree during ripening (2007 growing season) and harvested when they could be considered at developmental stages 3-4 (Westwood, 1978). The majority of harvested 'Big Top', 'D41-62' and 'Ghiaccio' fruit, which do not soften on the tree, were harvested when fully developed ("commercially ripe"). The harvested 'Bolero' fruit covered the range from "immature" (full size, no softening, fruit firmness higher than 35 N), to "ready to buy" stage (18-35 N) and physiologically ripe ("ready to eat", less than 18 N) (Cantín *et al.*, 2010).

Ripening parameters and tissue sampling. Immediately after harvest, fruit from each accession were preliminarily classified based on epicarp ground color as a maturity index (Delwiche and Baumgardner, 1985). Epicarp color parameters were measured at two points with no blush with a Minolta Chromameter CR-200 reflectance colorimeter (Minolta Co., Osaka, Japan; Robertson *et al.*, 1990). The Minolta 'a' value was taken as representative of the degree of ripening. Fruit within each class were also weighed and their flesh firmness (Newton, N) was measured after removing a small disc of skin from each side (cheek) of the fruit by an Effegi (Alfonsine, Italy) penetrometer equipped with an 8 mm probe.

Ethylene evolution and ethylene treatments. Immediately after harvest, five whole, healthy fruits of about 150-200 g, whose color and firmness matched the average values (± 10%) recorded at harvest (see above), were placed individually in 1.1-L glass jars and kept in a thermoregulated (20±1 °C) chamber with 95% relative humidity. Ethylene analysis (Dani 3800 gas chromatograph, Dani Co., Cologno Monzese, Italy) was conducted on gas samples (1 mL) collected from the headspace of the jars after 1 h of hermetical closure. Ethylene evolution was monitored every 24 h for 10 days after harvest (DAH).

For ethylene treatments, 10 to 12 fruit were placed in glass containers (total volume ≈ 0.013 m^3) in a thermoregulated (20±1 °C) chamber and flushed (≈ 0.1 m^3 h^{-1}) with humidified air added or not with 100 mL m^{-3} ethylene. The treatments lasted for five days after harvest (DAH), with daily

control of absence of pathogens. At the end of the treatments, fruits were assessed for flesh firmness and pooled mesocarp samples were taken and frozen in liquid N_2. At least five fruit from each treatment were used, in the subsequent 10 DAH, for the evaluation of the time course of endogenous ethylene evolution (see above).

RNA isolation and Northern hybridization analysis. Total RNA was extracted from frozen mesocarp tissue (10 g) according to Loulakakis *et al.* (1996). Northern blot analysis (20 μg RNA) was conducted with [α-^{32}P]dATP-labelled probes (Morgutti *et al.*, 2006). Specific primers for *Pp-endo-PG* (Lester *et al.*, 1994), *Pp-ACS1* (AB044662; Mathooko *et al.*, 2001) and *Pp-ACO1* (AF532976; Moon and Callahan, 2004) probes are reported in Table 1. First strand cDNAs for the genes of interest were synthesized, purified and cloned from 1 μg of total RNA of 'Bolero' soft fruit as previously described (Morgutti *et al.*, 2006). Sequences were determined by Primm srl., Milan, Italy.

Protein extraction and quantitation. Fruit mesocarp was extracted to obtain a fraction enriched in cell wall proteins (≈3 μg of enriched proteins per μL; Morgutti *et al.*, 2006). Protein samples were used in the original state, for nondenaturing polyacrylamide gel electrophoresis (PAGE) with evaluation of in gel polygalacturonase (PG) activity, or desalted with Plus One 2-D Clean-Up Kit (GE Healthcare Europe GmbH, Milan, Italy) for sodium dodecyl sulfate (SDS)-PAGE.

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 srl, Segrate, Italy).

Gel electrophoresis, endo-PG activity staining and Western blotting. Native-PAGE was performed at 4 °C in a MiniProtean™ apparatus (BioRad Laboratories) on 10% polyacrylamide gels (Morgutti *et al.*, 2006). After electrophoresis, gels for PG activity staining were equilibrated twice, for 15 min, in PG assay buffer [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] at 30 °C, incubated for 4 h at 30 °C in 0.2% (w/v) polygalacturonic acid (washed with 80% v/v 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 (Moore and Bennett, 1994).

SDS-PAGE was carried out on 10% polyacrylamide gels according to Schägger and von Jagow (1987) in a MiniProtean™ apparatus (BioRad Laboratories). Before loading, salt-extracted proteins

from the cell walls were denatured in SDS sample buffer (Laemmli, 1970). Molecular weight markers were from Sigma-Aldrich srl., Milan, Italy.

Western analysis was conducted as described in a previous paper (Morgutti *et al.*, 2006). Rabbit anti-endo-PG polyclonal antibodies were raised (Primm) against a synthetic polypeptide ($_2$HN-CREIKLEDVKLTYKN-COOH) constructed on a conserved region of the complete sequence of a Pp-endo-PG obtained from ripe peach fruit (CAA54150; Lester *et al.*, 1994; Morgutti *et al.*, 2006).

Isolation of DNA, cloning of an endo-PG gene and genotyping. Genomic DNA from young leaves (100-150 mg fresh weight) was prepared according to Geuna *et al.* (2004). The *Pp-endo-PG* gene sequence was amplified from genomic DNA with primers designed on the *PRF5 endo-PG* cDNA sequence (Lester *et al.*, 1994; Table 1) at the annealing temperature of 62 °C. Bands obtained from the different accessions were purified, cloned into a pCR®4-Blunt II-TOPO® vector (Invitrogen, S. Giuliano Milanese, Italy) and sequenced (Primm). To discriminate length differences, a region [base pairs (bp) 1455-1892 according to the positions indicated in Fig. 6] of the *Pp-endo-PG* gene comprising the major insertions/deletions (InDels) pointed out by sequencing was amplified with specifically designed primers (FW_{InDel}/R_{InDel}, Table 1). The amplification products were separated on 3% (w/v) agarose gels.

Restriction endonuclease digestion was carried out on selected fragments (1-972 bp) from the *Pp-endo-PG* gene, amplified with proper primers (forward: 5-ATGGCGAACCGTAGAAGCCTCT-3, reverse: 5-CCACAAGCAACGCCTTCTATCC-3). The restriction enzyme *Bst*XI (New England Biolabs, Celbio, Pero, Italy) was used. Reaction products were then separated on 1% (w/v) agarose gels, visualized by ethidium bromide staining and sequenced (Primm).

The PCR 100 bp Low Ladder (Sigma-Aldrich) and the 1 kb Plus DNA Ladder (Invitrogen) were used as molecular markers.

Gene name/gene sequence	Reference/GenBank no.	Primer FW/R
Pp-endo-PG	Lester *et al.*, 1994	5-ATGGCGAACCGTAGAAGCCTCT-3
		5-CTACAAACAACTTGTAGGCTGAAC-3
Pp-ACS1	AB044662	5-ATGGGCTCCTCATCAGCAAC-3
		5-TTAAGTCTTGGCTCGAACGAGAGG-3
Pp-ACO1	AF532976	5-ATGGAGAACTTCCCAATCATCAAC-3
		5-TTAAGCTGTTGCAATTGGACCC-3
Pp-endo-PG$_{InDel}$	DQ659240	5-GTGCCCTGGTCAGGTAAG-3
		5-GGCTAAGCTACGATGAAGTC-3

Table 1. Primers (FW, forward and R, reverse) for Northern analysis of gene expression (*Pp-endo-PG*, *Pp-ACS1*, *Pp-ACO1*) or amplification of selected *Pp-endo-PG* gene sequences (InDel) in peach fruit mesocarp.

Results

Fruit flesh firmness and ethylene evolution: effects of ethylene treatment. The time-course of softening and ethylene evolution during postharvest was studied in 'Big Top', SH 'Ghiaccio' and 'D41-62' fruit, in the absence or in the presence of an ethylene treatment, comparing their behaviour with the features shown by physiologically ripe M 'Bolero' fruit. At harvest, flesh firmness of M 'Bolero' fruit was very low, whereas ripe 'Big Top', 'D41-62' and 'Ghiaccio' fruit showed high flesh firmness. Five days after harvest, 'Big Top' firmness was very low under both air (controls) and ethylene treatment, whereas flesh melting in 'D41-62' and 'Ghiaccio' occurred only under ethylene (Table 2).

	Flesh firmness (N)		
	Harvest	5 DAH	
Accession		Air	Ethylene
'Bolero'	7.9 ± 2.4	-	-
'Big Top'	46.6 ± 5.8	2.5 ± 1.1	3.3 ± 1.2
'D41-62'	40.4 ± 2.2	38.1 ± 7.0	9.4 ± 6.2
'Ghiaccio'	49.6 ± 3.9	37.2 ± 3.6	4.3 ± 2.1

Table 2
Flesh firmness of peach fruit from different accessions at harvest and after 5 d of postharvest in air or under 100 mL m^{-3} ethylene treatment
Data shown are the means ± SD of at least 10 measurements on five different fruits.

Ripe, soft M 'Bolero' fruit evolved ethylene already immediately after harvest (t=0; 9.2 ± 3 μl kg^{-1} fresh weight h^{-1}) and reached the peak of ethylene emission at five DAH, after which ethylene emission progressively decreased (Fig. 1A). At harvest, 'Big Top' did not evolve ethylene. When 'Big Top' fruit were incubated in air, ethylene evolution started at four DAH and increased reaching at nine DAH a peak roughly comparable to that observed at five DAH in M 'Bolero' fruit (Fig. 1B). The evolution of ethylene from 'D41-62' and 'Ghiaccio' fruit was essentially nil (Figs. 1C and 1D).

The evolution of endogenous ethylene was monitored also for the 10 d following the end of the 5-d period in air or ethylene (total 15 DAH; Figs. 1E-1G). As expected, in 'Big Top' control fruit the timing of appearance of the ethylene peak closely corresponded to that observed in the 10-d postharvest period in fruit exposed to air (compare Fig. 1E and Fig. 1B). In the ethylene-treated

'Big Top' fruit, ethylene evolution was lower, possibly because in this condition the climacteric ethylene peak had already occurred during the prior 5-d treatment period (Fig. 1E). In 'D41-62' and 'Ghiaccio' ethylene evolution was almost negligible also after the 5-d ethylene treatment period (compare Figs. 1C-1F and Figs. 1D-1G).

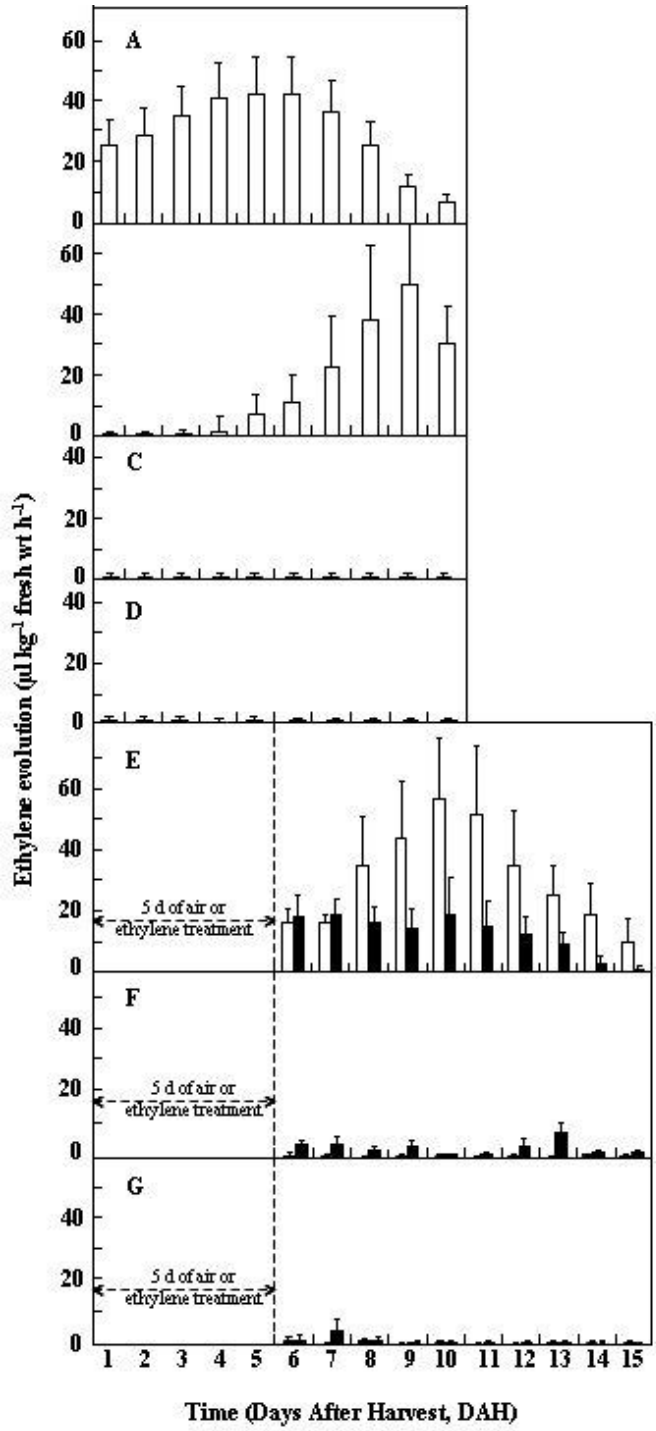

Fig. 1. Ethylene evolution by fruit from 'Bolero' (A), 'Big Top' (B and E), 'D41-62' (C and F) and 'Ghiaccio' (D and G) accessions. A-D: fruit exposed to air monitored for 10 d, from harvest up to 10 DAH). E-G: fruit monitored for 10 d after 5 days in air (open bars) or 100 mL m-3 ethylene (closed bars). The values are the means ± sd of measurements on at least five fruits ranging between 150 and 200 g each. Dotted lines in E-G show end of the 5-d treatments.

Expression of Pp-ACS1 and Pp-ACO1 genes in peach fruit mesocarp. At five DAH, 'Big Top' showed flesh firmness and ethylene emission roughly matching those observed in ripe, soft 'Bolero' fruit at harvest (Table 2, Fig. 1). This result confirmed that 'Bolero' could be taken as representative of typical M fruit and could be used for comparison for the subsequent analyses. Figure 2 shows that in the soft, ethylene-evolving 'Bolero' fruit transcripts of both *Pp-ACS1* and *Pp-ACO1*, isogenes of the ethylene biosynthetic pathway typical of fruit ripening (Mathooko *et al.*, 2001; Moon and Callahan, 2004; Tatsuki *et al.*, 2006). In 'Big Top' the *Pp-ACS1* transcripts were not present at harvest but became clearly detectable at five DAH in control fruit and their levels were higher upon ethylene treatment. No expression was detected for *Pp-ACS1* in 'D41-62' and 'Ghiaccio' in any condition. At harvest in 'Big Top' and 'Ghiaccio' fruit *Pp-ACO1* transcripts were present but in lower amounts than in 'Bolero'. *Pp-ACO1* transcripts were barely detectable in 'D41-62' fruit. At five DAH in air the *Pp-ACO1* transcripts were present in all three accessions and were higher upon ethylene treatment (Fig. 2).

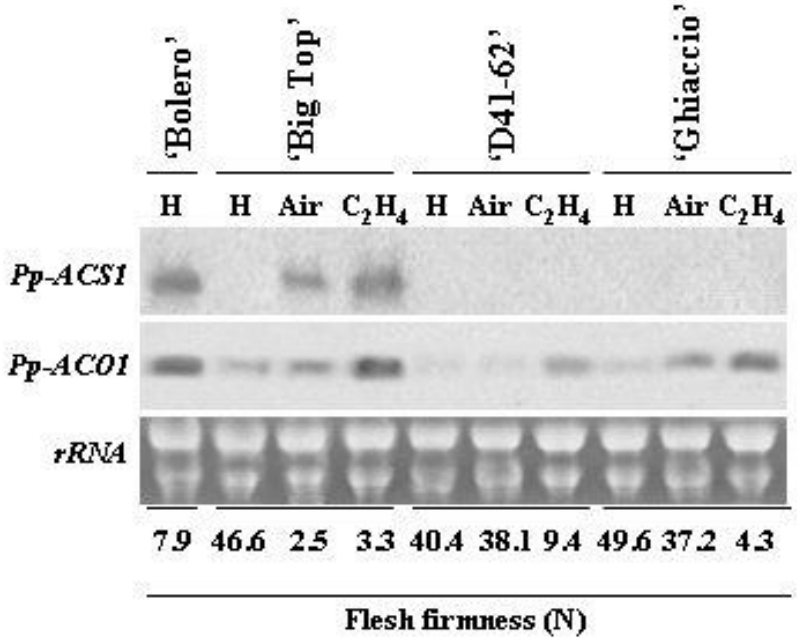

Fig. 2. *Pp-ACS1* (AB044662) and *Pp-ACO1* (AF532976) transcript accumulation, evaluated by Northern analysis, in fruit of 'Bolero', 'Big Top', 'D41-62' and 'Ghiaccio' at different firmness. Samples were obtained immediately after harvest (H) and at five DAH in absence (air) or presence of 100 mL m-3 ethylene (C2H4). The lower panel (rRNA) shows the quantification image of the ethidium-bromide stained RNA gel. Twenty micrograms of RNA was loaded per lane. One representative experiment is shown, from three independent replications.

Endo-PG activity and levels in peach fruit mesocarp. At harvest, endo-PG activity and immunoreaction signal against a native Pp-endo-PG were clearly detectable in soft, ethylene-producing 'Bolero' fruit but essentially undetectable in 'Big Top', 'D41-62' and 'Ghiaccio'. At five DAH in air, in 'Big Top' both endo-PG activity and Pp-endo-PG protein became clearly visible,

whereas no increase was observed in ‘D41-62’ and ‘Ghiaccio’. Ethylene treatment strongly increased endo-PG activity and levels in ‘Big Top’ and induced appearance of both in ‘D41-62’ as well as, to a higher extent, in ‘Ghiaccio’ (Fig. 3). Ethylene, whether endogenously produced (in ‘Bolero’ at harvest and in ‘Big Top’ at five DAH in air) or exogenously applied (in ‘Big Top’, ‘D41-62’ and ‘Ghiaccio’ at five DAH in ethylene), induced appearance of an active endo-PG form different than that recognized by the antibodies used, as indicated by the presence of a topmost band of gel decoloration (Fig. 3A) not coincident with the immunoreaction signal (Fig. 3B).

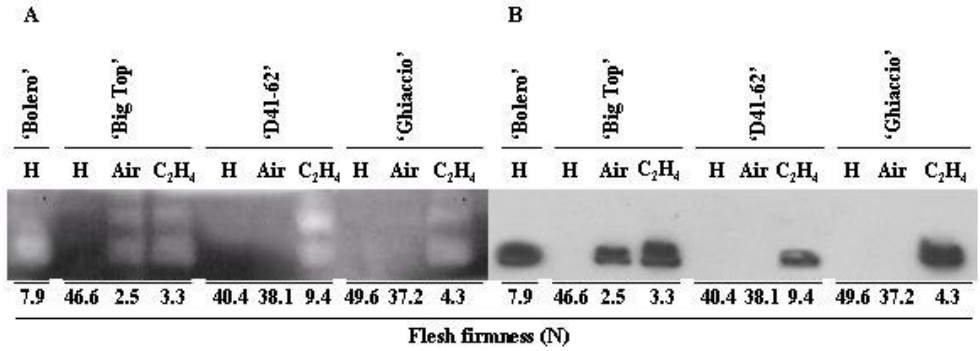

Fig. 3. Endo-PG activity (A) and levels of Pp-endo-PG protein (B) in fruit of ‘Bolero’, ‘Big Top’ and ’Ghiaccio’ at different flesh firmness. Samples were obtained immediately after harvest (H) and at five DAH in absence (air) or presence of 100 mL m^{-3} ethylene (C_2H_4). Both assays were conducted after non-denaturing PAGE of salt-extracted proteins from mesocarp cell walls in gels run in duplicate. Twenty micrograms of protein was loaded per lane. One representative experiment is shown, from three independent replications.

When the proteins from the fruit cell walls were subjected to SDS-PAGE and Western analysis, the immunoreaction signal in the gel zone corresponding to a M_r of ≈ 45 kDa, typical of catalytically active PG forms (Brummell and Harpster, 2001; Lee *et al.*, 1990 and references therein), was clearly visible at harvest in soft ‘Bolero’ fruit but not in any of the other accessions. At five DAH in air, the 45 kDa Pp-endo-PG polypeptide was strongly expressed in ‘Big Top’, while it was almost undetectable in ‘D41-62’ and ‘Ghiaccio’. At five DAH in ethylene, the levels of this polypeptide strongly increased in ‘Big Top’, and became clearly apparent in ‘D41-62’ and ‘Ghiaccio’ (Fig. 4).

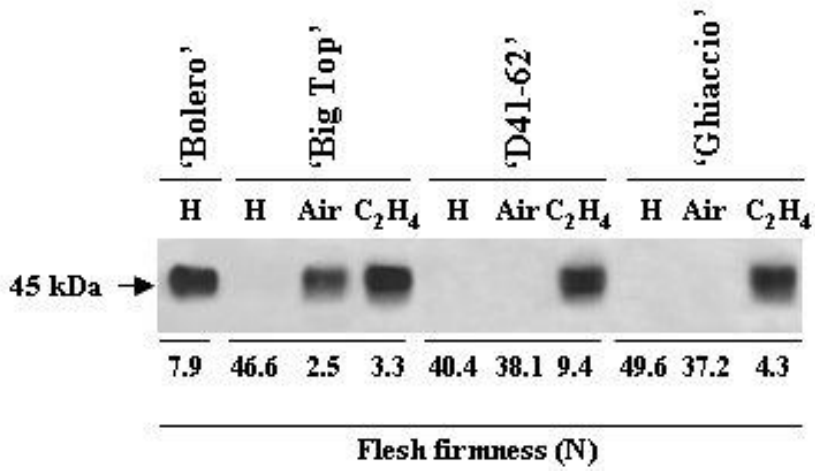

Fig. 4. Endo-PG protein accumulation, evaluated by Western analysis, in fruit of 'Bolero', 'Big Top', 'D41-62' and 'Ghiaccio' at different flesh firmness. Samples were obtained immediately after harvest (H) and at five DAH in absence (Air) or presence of 100 mL m^{-3} ethylene (C_2H_4). Twenty micrograms of protein was loaded per lane. One representative experiment is shown, from three independent replications.

Expression of a Pp-endo-PG gene in peach fruit mesocarp. At harvest, the *Pp-endo-PG* transcripts were very abundant in soft 'Bolero', barely present in 'Big Top' and undetectable in 'D41-62' and 'Ghiaccio'. Transcript accumulation showed an increase at five DAH in control fruit (apparent in 'Big Top' and fainter in 'D41-62' and 'Ghiaccio') and was distinctly promoted by ethylene treatment (Fig. 5).

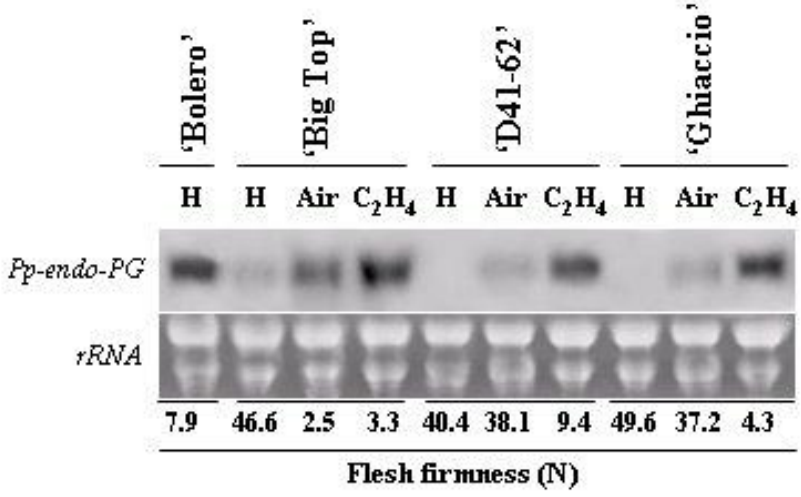

Fig. 5. *Pp-endo-PG* (*PRF5*, Lester et al., 1994) transcript accumulation, evaluated by Northern analysis, in fruit of 'Bolero', 'Big Top', 'D41-62' and 'Ghiaccio' at different flesh firmness. Samples were obtained immediately after harvest (H) and at five DAH in absence (Air) or presence of 100 mL m^{-3} ethylene (C_2H_4). The lower panel (rRNA) shows the quantification image of the ethidium-bromide stained RNA gel. Twenty micrograms of RNA was loaded per lane. One representative experiment is shown, from three independent replications.

Structure of a Pp-endo-PG gene in diverse peach accessions. The molecular features of the *Pp-endo-PG* gene involved in the peach softening process (Callahan *et al.*, 2004; Lester *et al.*, 1996;

Morgutti *et al.,* 2006) were analyzed in the different accessions studied. Genomic DNA was extracted from the leaves of M 'Bolero', 'Big Top', 'D41-62' and SH 'Ghiaccio'. Furthermore, 'Yumyeong' and 'Oro A' were also considered as reference SH and NM genotypes, respectively. Putative *Pp-endo-PG* gene sequences were isolated, sequenced and aligned. Four exons and three introns were deduced from comparison of the genomic sequences with the *Pp-endo-PG* cDNAs previously identified (Morgutti *et al.,* 2006). A single *Pp-endo-PG* clone (*m*, 2238 bp) was isolated in NM 'Oro A', while two clones (*m* and *M*) were isolated in M 'Bolero', consistently with the partial sequence of the same gene (Morgutti *et al.,* 2006). The clone *M* of 'Bolero' showed four deletions in intronic sequences (bp 688-690, 1541-1557, 1756-1772, 1907-1908), one insertion, and 39 SNPs (34 in introns and five in exons) when compared with the clone *m* of 'Oro A', resulting shorter by 37 bp. One (bp 1756-1772) of the two 17-bp intronic deletions of the clone *M* of 'Bolero' was conserved in 'Big Top', 'D41-62','Ghiaccio' and 'Yumyeong'.

The 'Big Top' *Pp-endo-PG* clone showed 62 SNPs and lack of the deletion involving bp 1541-1557 when compared with the clone *M* of 'Bolero', and only five SNPs when compared with the *Pp-endo-PG* clones of 'D41-62', 'Ghiaccio' and 'Yumyeong'. One SNP (bp 348) was peculiar of 'Big Top', and one (bp 1146) of 'Yumyeong', 'Ghiaccio' and 'D41-62' (Fig. 6).

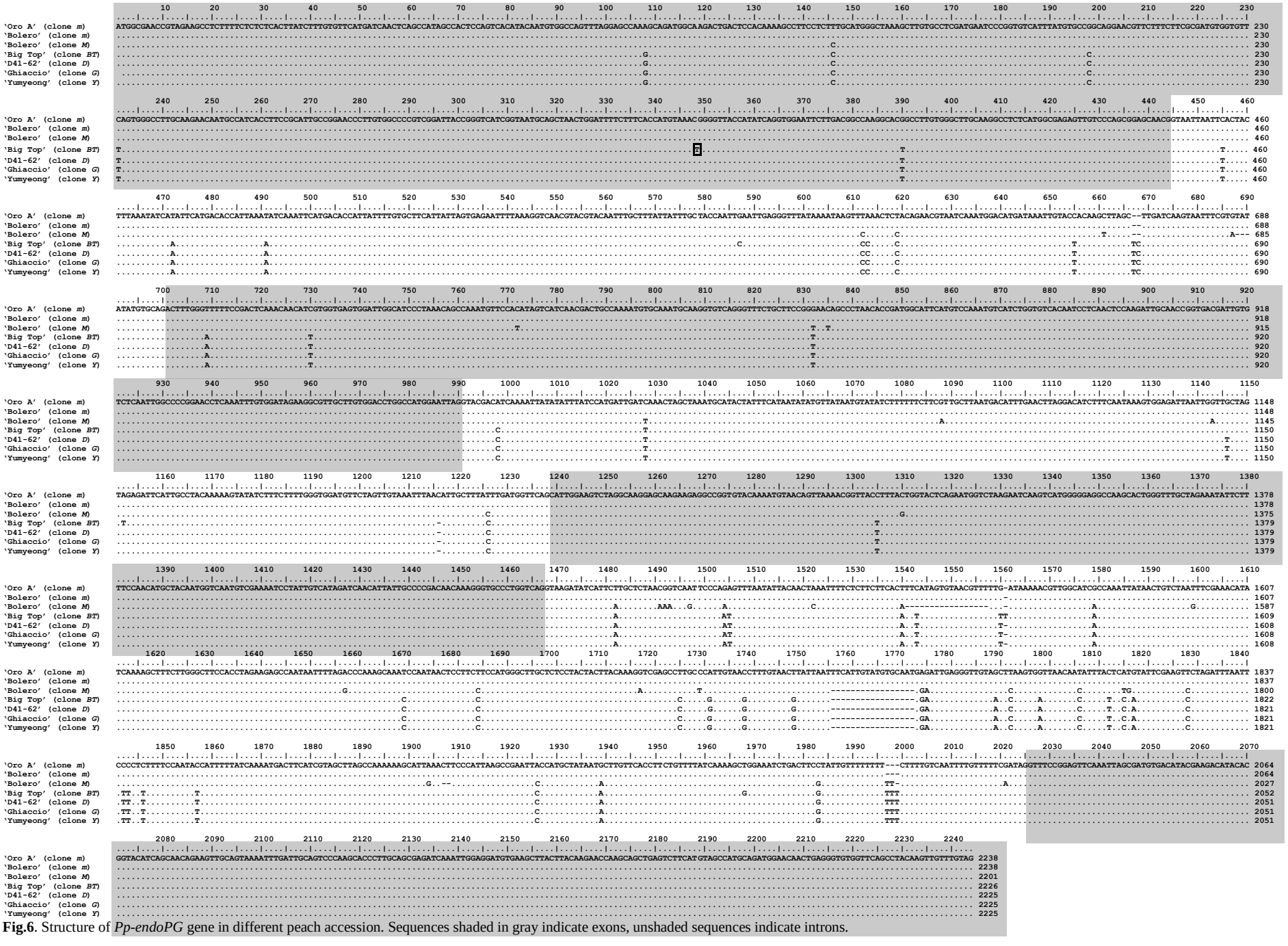

Fig.6. Structure of *Pp-endoPG* gene in different peach accession. Sequences shaded in gray indicate exons, unshaded sequences indicate introns.

Amplification and Cleaved Amplified Polymorphic Sequence (CAPS) analysis of selected Pp-endo-PG sequences from genomic DNA.

Intron 3 of *Pp-endo-PG* showed the most polymorphisms among the accessions considered (Fig. 6). A short (436 bp) sequence comprising these major differences was amplified (see Materials and Methods) from genomic DNA of the six accessions and the products separated in an agarose gel (Fig. 7). 'Oro A' produced a single fragment of ≈ 440 bp, consistent with the length predicted from *in silico* analysis. M 'Bolero' yielded two amplification fragments of ≈ 440 and 400 bp (predicted 436 and 402 bp), while 'Big Top', 'D41-62', 'Ghiaccio' and 'Yumyeong' produced only one fragment of ≈ 420 bp, consistent with the predicted values of 420 bp for 'Big Top' and 419 bp for the other accessions.

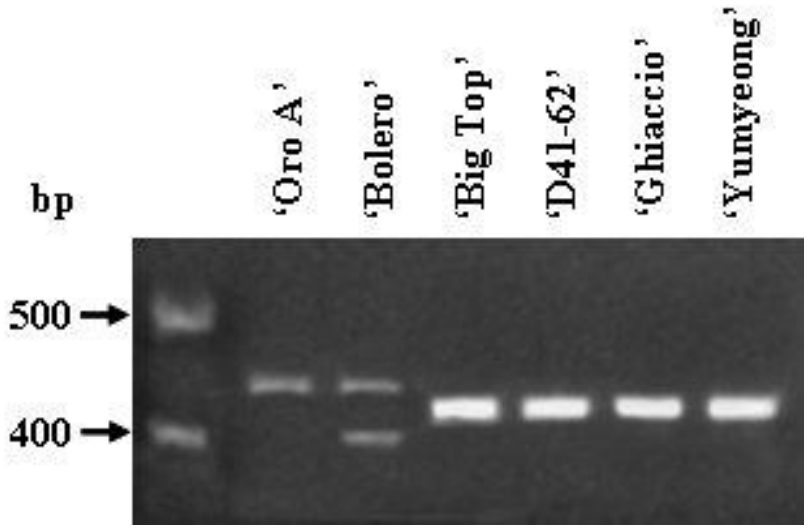

Fig. 7. Amplification of a selected fragment of genomic DNA from leaves of 'Oro A', 'Bolero', 'Big Top', 'D41-62', 'Ghiaccio' and 'Yumyeong' accessions. The selected fragment (bp 1455-1892) of the Pp-endo-PG clone was amplified with proper primers. The lane at the left border shows the positions and lengths (bp) of DNA markers (Sigma PCR 100 bp Low Ladder). Twenty micrograms of DNA was loaded per lane. One representative experiment is shown, from three independent replications.

The peculiar exonic SNP_{348} (C→$\underline{T}_{BigTop}$; Fig. 6) of the whole gene sequence of the 'Big Top' *Pp-endo-PG* clone originated a sequence (CCANNNNN▼NTGG) recognized by the restriction enzyme *Bst*XI. A *Pp-endo-PG* sequence comprising this SNP (bp 1-972) was amplified from genomic DNA and digested with *Bst*XI (Fig. 8). CAPS reaction yielded two bands of ≈ 350 and 600 bp only in 'Big Top', consistent with the lengths calculated for the fragments obtainable by cutting, at the level of SNP_{348}, the selected 972 bp-long DNA sequence (348 and 624 bp, respectively; Fig. 6). *Bst*XI was inactive on all the other accessions.

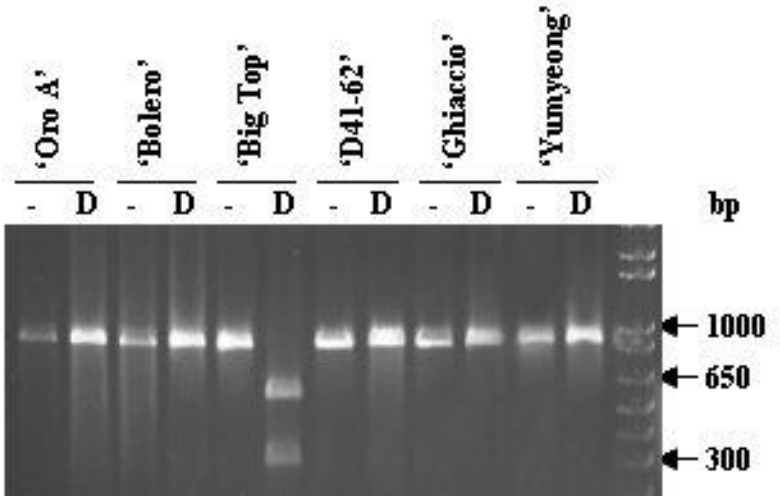

Fig. 8. Cleaved Amplified Polymorphic Sequence (CAPS) restriction patterns of genomic DNAs from leaves of 'Oro A', 'Bolero', 'Big Top', 'D41-62', 'Ghiaccio' and Yumyeong' accessions. Selected genomic sequences of the *Pp-endo-PG* clone (bp 1-972) were amplified with proper primers (Table 1) and digested with *Bst*XI. (-): undigested; (D): *Bst*XI-digested. The lane at the right border refers to the 1 Kb Plus DNA Ladder (Invitrogen). Arrows indicate positions and lengths (bp) of the markers closest to DNA fragments of interest. One representative experiment is shown, from three independent replications.

Discussion

At commercial ripening, 'Big Top' fruit are very similar to the 'stony hard' ones in firmness and crispiness. Nevertheless, our results show that during postharvest the behaviour of 'Big Top' was very different than that of 'D41-62' and SH 'Ghiaccio'. In fact, 'Big Top' evolved ethylene late in postharvest (5 DAH in air; Fig. 1B), with concomitant increased expression of *Pp-ACS1* (Fig. 2), paralleled by increases in *Pp-endo-PG* transcripts (Fig. 5) and in an active endo-PG protein (Figs. 3 and 4), finally leading to fruit melting (Table 2). On the contrary, 'D41-62' and SH 'Ghiaccio' fruit stored in air failed to evolve ethylene apparently as a consequence of a blockade of *Pp-ACS1* expression; this was accompanied by poor *endo-PG* expression and endo-PG production and very limited softening. Nevertheless, the ethylene treatment in postharvest on 'D41-62' and 'Ghiaccio', while showing no effect on the accumulation of *Pp-ACS1* transcripts and a very limited one on ethylene evolution, led to a dramatic increase in endo-PG production and fruit melting (Figs. 1-5). This result is consistent with data showing that in SH peaches very slight *Pp-ACS1* expression occurs only after treatment with the ethylene analogue propylene or upon cold-temperature-induced endogenous ethylene synthesis, with subsequent melting (Haji *et al.*, 2005; Tatsuki *et al.*, 2006; Begheldo *et al.*, 2008). Therefore, the conclusion can be drawn that 'D41-62' belongs to the SH phenotype.

At 5 DAH, 'Big Top' fruit seemed well comparable to melting 'Bolero' fruit harvested at physiological ripening, where the very low flesh firmness was accompanied by high levels of *Pp-ACS1* transcripts and high ethylene evolution, with activation of a *Pp-endo-PG* gene and production of high levels of an active endo-PG protein (Figs. 1-5). These findings give molecular and biochemical support to previous observations (Monet and Bassi, 2008; Lavilla *et al.*, 2002), confirming that 'Big Top' 'slow melting' fruit belong to the melting phenotype, due to delayed *Pp-ACS1* activation and ethylene evolution, which occur only a few days from harvest.

The timing of postharvest ethylene evolution in fruits can vary even within the same species in different cultivars depending on many diverse factors among which morphological traits, such as rind type (as in melon; Ezura and Owino, 2008), fruit developmental stage at harvest (as in peaches; Haji *et al.*, 2004; Lavilla *et al.*, 2002), seed presence/absence (as in avocado; Hershkovitz *et al.*, 2010), all able to affect the expression of members of the *ACS* and *ACO* multifamilies (Bleecker and Kende, 2000; Kende, 1993). A regulatory effect of the tree could also be hypothesized: in fact, most varieties of avocado fail to produce ethylene as long as they are attached to the tree (similar in this respect to 'Big Top' peach), and this behaviour appears essentially due to repression of ACS activity (Sitrit *et al.*, 1986). The endogenous factor/s and

the mechanism/s differently affecting, in M 'Bolero' and in 'Big Top' peaches, the timing of activation of *Pp-ACS1*, that plays a crucial role in the regulation of ethylene evolution during fruit ripening (Mathooko *et al.*, 2001; Tatsuki *et al.*, 2006), are presently unknown.

The mere amounts of fruit ethylene evolution, though, may not necessarily represent the only point of control for ethylene-regulated, endo-PG-dependent softening. Differences may depend on the ability of the fruit tissues to perceive ethylene, transduce its signal, or both (Ghiani *et al.*, 2007; Morgutti *et al.*, 2005), up to the final point of regulating the expression of the target gene(s). Moreover, the recently described possible cross-talk between ethylene and other phytoregulators, such as auxin, in the fruit ripening process further complicates the overall picture (Trainotti *et al.*, 2007; El-Sharkawy *et al.*, 2009; Villarreal *et al.*, 2009).

It is interesting to stress out that in fruit of all the studied accessions ethylene induced the appearance of an active endo-PG, not recognized by the antibodies used in the present work (Fig. 3A and B), that might putatively be ascribed to other Pp-endo-PG forms. In ethylene-treated, soft peach fruit a *PG* transcript has been described (*PpPG1*, AB231902; Murayama *et al.*, 2009) whose deduced protein shares only 36% similarity with that encoded by the *PRF5* sequence (Lester *et al.*, 1994) used as a reference for the development of the anti-endo-PG antibodies used in this study.

Endo-PG plays a pivotal role in the determination of fruit flesh texture characteristics (Inaba, 2007 and references therein). In NM and M peaches, the different softening patterns have been related to specific molecular features of a *Pp-endo-PG* gene expressed in ripe fruit (*M* locus; Callahan *et al.*, 2004; Lester *et al.*, 1996; Morgutti *et al.*, 2006; Peace *et al.*, 2005) or *endo-PG*-associated microsatellites (Peace *et al.*, 2007), independent of the ethylene evolution (Brovelli *et al.*, 1999). On the other hand, SH peaches have been classified as *hdhdM*- and *hdhdmm*, respectively, discriminating the asset at the *endo-PG* locus on the basis of their ability to undergo or not to the melting phase upon ethylene treatment (Haji *et al.*, 2005). On this basis, we considered interesting to investigate also on the molecular features of the 'Big Top' *Pp-endo-PG* gene at the *M* locus and to compare them with those of 'D41-62', of two SH accessions, 'Ghiaccio' (derived from self-pollination of 'Yumyeong') and 'Yumyeoung', and of two reference accessions for NM ('Oro A') and M ('Bolero') phenotypes (Morgutti *et al.*, 2006).

The whole sequence of the 'Big Top' *Pp-endo-PG* clone was very similar to those of the 'D41-62', 'Ghiaccio' and 'Yumyeong' clones, whereas it presented many polymorphisms compared to the 'Bolero' clone *M* (Fig. 6). Analysis of alignment with the recently published peach genome sequence (http://services.appliedgenomics.org/gbrowse/prunus_public/), referred to the doubled haploid peach cultivar 'Lovell') gave consistent results. In fact, the *m Pp-endo-PG* sequences of 'Oro A' and 'Bolero' showed a 100% identity with an *endo-PG* gene located at bp 22650221 on

chromosome 4, while ‘D41-62’, ‘Big Top’, ‘Ghiaccio’ and ‘Yumyeong’ sequences all showed 97% identity. The *M endo-PG* clone of ‘Bolero’ was different, showing identity with the sequence of a different, duplicate *endo-PG* gene, located on the same chromosome 4 but at bp 22684623.

Also the exonic sequences of the ‘Big Top’ *Pp-endo-PG* clone were almost identical to those of ‘D41-62’, ‘Ghiaccio’ and ‘Yumyeong’, with the only exception of the ‘Big Top’ SNP_{348}. Due to the presence of a C replacing a G at position 1310 bp of the *endo-PG* sequence in these accessions, the deduced endo-PG proteins differ from the functional one encoded by the *M Pp-endo-PG* clone of ‘Bolero’ only for a Thr_{269} replacing a Ser_{269}. The ‘Big Top’ SNP_{348}, on the other hand, does not determine any change in the amino acidic sequence of the corresponding deduced protein. The reported experimental biochemical and physiological evidences showing the ability of ‘Big Top’, ‘D41-62’ and ‘Ghiaccio’ fruit to produce an active endo-PG and to melt in response to ethylene, either endogenous or exogenously applied (Fig. 3), are consistent with molecular analysis: the peculiar ‘Big Top’ ‘slow melting’ phenotype seems not ascribable to differences in the *Pp-endo-PG* clone considered.

Nevertheless, the search for molecular markers of the ‘slow melting’ phenotype of ‘Big Top’ allowed the identification in its *Pp-endo-PG* clone of the peculiar exonic SNP_{348} (Figs. 6 and 8). Furthermore, InDel polymorphisms (intron 3) shared by ‘Big Top’, ‘D41-62’, ‘Ghiaccio’ and ‘Yumyeong’ (Figs. 6 and 7) were also found. Insertion or deletion events occurring in introns as well as SNPs are currently used for phylogenetic and parentage analyses (Wei *et al.*, 2006) or identification of cultivar/lines in plants (Shimada *et al.*, 2009). Our preliminary findings, if confirmed on other accessions sharing the fruit ‘slow melting’ phenotype of ‘Big Top’, may represent interesting diagnostic tools to discriminate among different genotypes or in segregating progenies (for molecular assisted breeding) on the basis of the structure of their *Pp-endo-PG* genes or to preliminarily ascertain whether a selection is genetically related to ‘Big Top’. Further analysis on populations segregating for the fruit flesh texture needs to be carried out in order to validate this SNP marker. In conclusion, our data seem to confirm the pivotal role of ethylene in the regulation of endo-PG expression, and thus in the determination of flesh texture characteristics in peach fruit with different ripening phenotype (for reviews, see Bennett and Labavitch, 2008 and Inaba, 2007). The remarkable ability of ‘Big Top’ fruit to develop satisfactory organoleptic qualities while better withstanding the postharvest operations is due to the slow melting process of the flesh. This seems related to corresponding delayed *Pp-ACS1* gene expression and ethylene evolution, with subsequent delay of endo-PG production and melting of the flesh. However, further work is needed to deepen this point.

Acknowledgements

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CHAPTER II

THE *EndoPG* LOCUS CONFIGURATION IN DIFFERENT PEACH GENOTYPES: A POSSIBLE DIAGNOSTIC USE OF THE POLYMORPHISMS INDIVIDUATED IN THE GENE SEQUENCES

Introduction

The overall organoleptic quality of the peach [*Prunus persica* (L.) Batsch] fruit is mainly due to the components of fruit taste (sugars, organic acids, and volatiles) and flesh texture. The latter is one of the most important factors determining the postharvest behaviour of the produce and the consumer's perception of fruit quality (Bruhn, 1994). Peaches are classified on the basis of fruit texture and their ability to undergo a different flesh softening during ripening. In M fruit this process culminates in a marked loss of firmness (melting phase) in the final stages of ripening.

Flesh firmness as a whole can be considered as a quantitative trait affected by several biochemical and physiological factors among which a major role is played by different hydrolytic enzymes/proteins which cooperate in a complex way to modify the structure and composition of the polysaccharides which constitute the cell wall. In particular the melting phase has been linked to a strong increase in the expression of an *endoPG* gene and in the accumulation of an active endoPG protein, which characterizes M fruit (Lester *et al.*, 1994, 1996; Callahan *et al.*, 2004; Morgutti *et al.*, 2006). Melting has long been proposed to be a Mendelian trait (Bailey and French, 1949).

Breeding programs aimed to obtain fruit produce with satisfactory texture qualities (i.e. peaches with high organoleptic properties, like M ones, but still retaining sufficient firmness for handling) are so far using phenotype-based genetic markers whose evaluation for fruit-specific characters is time-consuming. The possibility to dispose of molecular markers, which are segments of DNA representative of the differences at the genome level, could offer numerous advantages over conventional phenotype-based alternatives as they are stable and detectable in all tissues regardless growth, differentiation, development and are not confounded by the environmental, pleiotropic and epistatic effects (Agarwal *et al.*, 2008). With molecular markers, genes of interest can be isolated solely on the basis of their position on the genetic map and it is possible to dissect traits that are controlled by many different factors (quantitative traits) into their individual components (QTL, quantitative trait loci) which can subsequently be molecularly identified. In plant genetic research, molecular markers are also useful for analysis of population structure, to study evolutionary relationships and, in sequenced model species (i.e. *Arabidopsis*), to individuate the genetic structure of individuals at the whole genome level (Ganal *et al.*, 2009).

Different molecular marker techniques can be applied in plant science. Basic marker technique can be classified into two categories: 1) non PCR-based techniques or hybridization techniques, such as in the case of Restriction Fragment Length Polymorphism (RFLP) used to construct physical maps, and 2) PCR-based techniques. These latter can be sequence-non specific, such as in the case of Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment

Length Polymorphism (AFLP) used for gene tagging, or sequence-targeted. With the advent of high-throughput sequencing technology, abundant information on DNA sequences for the genome of many plant species, as well as ESTs of many crop species, have been generated and sequence-specific molecular markers have been designed to correlate DNA sequence information with particular phenotypes (Agarwal *et al.,* 2008). In peach, RFLP, RAPD and AFLP markers have been used to map QTLs controlling fruit quality linked to soluble sugars and organic acids content (Dirlewanger *et al.,* 1999). In *Prunus* the use of microsatellite (Single Sequence Repeat, SSR) markers allowed to increase saturation and coverage of linkage and create reference maps, useful for gene/QTL tagging, whole genome selection and plant breeding applications (Howard *et al.,* 2005; Verde *et al.,* 2005) .

Single Nucleotide Polymorphisms (SNPs) constitute the most abundant molecular markers in the genome and are widely distributed throughout genomes although their occurrence and distribution varies among species. They are usually more prevalent in non-coding regions. Within the coding regions, a SNP could be either synonymous, with effect on mRNA splicing resulting in phenotypic differences, or non-synonymous resulting in an amino acid sequence change. Because of these features SNPs are attractive tools for high-throughput genotyping methods (DNA chips, allele-specific PCR and primer extension approaches) suitable for rapid identification of crop cultivars and construction of ultra high-density genetic maps. However, for many crop plants there are low number of validated SNP markers available (Ganal *et al.,* 2009).

Also Insertion-Deletions (InDels) of nucleotides are polymorphisms which share with SNPs a large abundance in eukaryotic genomes and have been successfully exploited as molecular markers in high-throughput screening. These polymorphisms are mainly located at the intronic sequences and can be functionally critical in view of their potential to influence the binding of transcription factors, the process of alternative splicing, the coding of intronic regulatory elements (i.e.micro-or small nucleolar-RNAs) and non-sense mediated mRNA decay (Guerra Cardoso *et al.,* 2009).

Both SNPs and InDels can modify restriction endonuclease recognition sites in PCR amplicons and cause restriction fragment length polymorphisms which are deciphered by CAPS (Cleaved Amplified Polymorphic Sequence) markers, a technique proved useful for following known mutations in segregating populations and positional based cloning of new genes in plants (Agarwal *et al.,* 2008).

As reported in Chapter 1, SNPs and insertions/deletions polymorphism have been individuated in the different *Pp-endoPG* clones isolated from NM ‘Oro A’, M ‘Bolero’, SH ‘Ghiaccio’, ‘D 4162’, ‘Yumyeong’ and Slow Melting ‘Big Top’ peach genotypes. In order to

evaluate the feasibility to exploit these mutation for peach genotyping and predicting the configuration at *endoPG* locus, molecular markers, such as InDels and CAPS, have been developed from these polymorphisms and used to screen a few peach accessions (well-established cultivars or offspring populations obtained by crossing known parental cultivars) with different fruit flesh softening phenotype.

Materials and methods

Plant material

Plant material was from experimental orchard of the University of Bologna ('Azienda Agricola Zabina' Castel San Pietro, BO) and (Di.Pro.Ve.) and belonged to the peach accession collection of Professor Bassi (DiProVe). Experiments were conducted on leaves of genotypes (well established peach cultivars or offspring population obtained by crossing known peach genotypes) with different characteristics of fruit flesh texture. A schedule of the accession studied is reported below.

Putative phenotype of the accessions			
M (melting) or putative M phenotype	NM (non melting) or putative NM phenotype	Very firm or slow melting phenotype	SH (stony hard)
'Ambra' 'Bolero' 'Contender' 'Glohaven' 'Maycrest' 'Rich Lady' 'Springbelle' 'Springcrest' 'Springred' 'Suncrest' 'Max 7' 96016015, 96016018, 96016023 96016136, 96016165 96016094, 96016208	'Andross' 'Ionia' 'Oro A' 82010054	'Alitop' 'Big Top' 'Ruby Rich' 'Vista Rich' 'Honey Gold' 96028059 96013046 02037001, 02037002, 02037003, 02037004	'Ghiaccio' 'D 41-62' 'Yumyeong' 'Helena Cling' 'Stony Hard'

Offsprings from different crosses	
F2 ('Contender' × 'Ambra') a.i	96016015, 96016018, 96016023, 96016136, 96016165, 96016094, 96016208
'Jungerman' × 'Loadel'	82010054
'Spring Red' × 'Big Top'	96028059
'Bolero' × 'Rich Lady'	96013046
'Maycrest' × 'Vistarich'	02037001, 02037002, 02037003, 02037004
'Ambra' × 'Big Top'	23 seedlings: 02002001- 02002006, 02002008- 02002009, 02002011 - 02002015, 02002017- 02002026
'Max 7' × 'Big Top'	27 seedlings:02004001 – 02004027

Leaves of three haploid accessions ('PV11N', 'PR11N', 'PL31N') were also considered.

Leaves were directly placed in liquid nitrogen and stored at -80° C for subsequent analysis.

Extraction of genomic DNA

Genomic DNA was obtained following the protocol of Geuna *et al*. (2004) with slight modifications.

Peach leaves samples (150 mg) were ground to a fine powder in liquid nitrogen using a mortar and a pestle. Ground powder was mixed with 600 µL of preheated (65° C) extraction buffer (0,2 M Tris-HCl, pH 8.5, 10 mM EDTA, 0,3 M LiCl, 1,5% [v/v] SDS, 1% [w/v] Nonidet P-40, 1 mM DTT). Samples were incubated for 20 min at 65° C and briefly but vigorously shaken every 7–8 min. Cell debris was centrifuge at 15000*g* at 4° C for 20 min. The aqueous solution was sequentially extracted in 1 volume of phenol, 1 volume of phenol–chloroform–isoamyl alcohol (25:24:1) and 1 volume of chloroform, each time shaking to mix thoroughly then centrifuging at 15,000*g* for 20 min. DNA was precipitated by adding 1/10 volume of 3 M NaAc, pH 5.2, and 0.6 volume of 2-propanol, and incubating at 4° C for 1 h. DNA was centrifuge at 15,000*g* at 4° C for 30 min. Pellets were washed with 1 mL of 70% ethanol and air dried. Pellets were resuspended in 100µl of water and transferred to 1.5-mL tubes. Aliquots of 3 µL were analyzed on 1% agarose gels for quality and yield assessment. The agarose gels (1% w/v) was prepared in buffer TBE 1 × low ionic strength, also used as electrophoresis buffer [89 mM Tris, 89 mM H_3BO_3, 2 mM Na_2-EDTA, pH 8.2]. The agarose solution, initially heated to 80° C to allow the dissolution of the polysaccharide and then allowed to cool at room temperature, was added ethidium bromide (0.5 µg/ml), which binds to nucleic acids and shows intense fluorescence red/orange when illuminated by UV light, allowing the view of nucleic acids separated on the gel. The electrophoresis run was conducted by applying 5 mV cm^{-1}. Aliquots of λ genomic DNA and 1 kb DNA Ladder (Invitrogen) were run as standards of concentration and quality, respectively. Quantified DNA was treated with RNase A (10 mg/mL, Invitrogen) to remove any RNA contaminations at 37° C for 1 h and then precipitated as described above.

InDel analysis

To discriminate length differences (InDel polymorphisms), a region (bp 1455-1892) of the *Pp-endo-PG* gene correspondent to Intron 3 and comprising the major InDels was amplified with proper primers *Forward*$_{InDel}$: 5'-GTGCCCTGGTCAGGTAAG-3'; *Reverse*$_{InDel}$: 5'-GGCTAAGCTACGATGAAGTC-3'). The PCR mix contained 20 ng genomic DNA, 0.3 mM dNTPs, 0.3 µM/each InDel Dir and InDel Rev primers, 1×Reaction Buffer GoTaq®, 1 U Go Taq® DNA Polymerase (Promega) and ddH_2O to reach a final volume of 25 µl. The conditions of PCR reaction were: 1 cycle at 94°C for 2 min; 35 cycles: 94°C for 45 min, 62°C for 45 min., 72°C for 1 min.; 1 cycle at 72°C for 30 min.

The amplification products were separated on 3% (w/v) agarose gel and visualized by ethidium bromide. The PCR 100 bp Low Ladder (Sigma-Aldrich) and the 1 kb Plus DNA Ladder (Invitrogen) were used as molecular markers.

Cleaved Amplified Polymorphic Sequence (CAPS) analysis

Appropriate primer pairs were designed to amplify the regions of *endoPG* genes including the SNPs of interest, which determined the polymorphic restriction sites. The amplification reactions, conducted as described for InDel analysis (see above), were followed by the digestion of the polymorphic fragments with proper restriction endonuclease. The reaction was conducted in 15 µl of digestion mix containing 10 µl of amplification products, 2.85 µl of ddH_2O, 1.5 µl of Buffer, 0.15 µl of 10 mg/ml BSA and 0.5 µl of the proper restriction enzyme. Samples were incubated at 37° C for 90 min.

SNP	Primer Pair	Sequence	Fragment length	Endonuclease
At 348 bp ($C_{m,M,SH}\rightarrow T_{BT}$)	$ENDO_{DIR}$ Hd_{Rev}	5'-ATGGCGAACCGTAGAAGCCTCT-3' 5'-CCACAAGCAACGCCTTCTATCC-3'	970 bp	*Bst*XI, Promega
At 390 bp $G_{m,M}\rightarrow T_{SH,BT}$	Hd_{For} Hd_{Rev}	5'-GCCATAGCCACTCCAGTCAC-3' 5'-CCACAAGCAACGCCTTCTATCC-3'	911 bp	*Bts*I, NEB

Reaction products were then separated on 3% (w/v) agarose gels and visualized by ethidium bromide

Results and discussion

As reported in Chapter 1, the different *Pp-endoPG* clones isolated from NM ('Oro A'), M ('Bolero'), Slow Melting ('Big Top') and SH ('Ghiaccio', 'D41-62','Yumyeong') peach genotypes showed a few polymorphisms, some of which peculiar of the different gene variants. Figure 1 summarizes the structure of the *Pp-endoPG* clones and indicates the main polymorphisms characteristic of the different gene variants. In particular three SNPs (SNP_{348} in *BT* clone, SNP_{390} in *SH* and *BT* clones and SNP_{1310} in *m*-O and *m*-B, as well as in *SH* and *BT* clones) were present, which determined polymorphic restriction sites recognized by specific endonucleases. Two more SNPs are shown in the figure, one (SNP_{1146}) peculiar to the *SH* clone and the other (SNP_{146}) peculiar to *m-O* and *m-B* clones. Two deleted sequences (of 17-bp each) in intron 3 (InDel polymorphisms:1541-1557 and 1755-1771 bp) peculiar of *M* clone were identified, one of which (1755-1771 bp) was conserved in *SH* and *BT* clones.

InDels and the SNP_{390} and SNP_{348}, among those determining a polymorphic restriction site, have been exploited to develop markers for the analysis of different peach accessions (well-established cultivars or offspring populations obtained by crossing parental genotypes with known fruit flesh characteristics) in order to gain information on their configuration at the *endo-PG* locus.

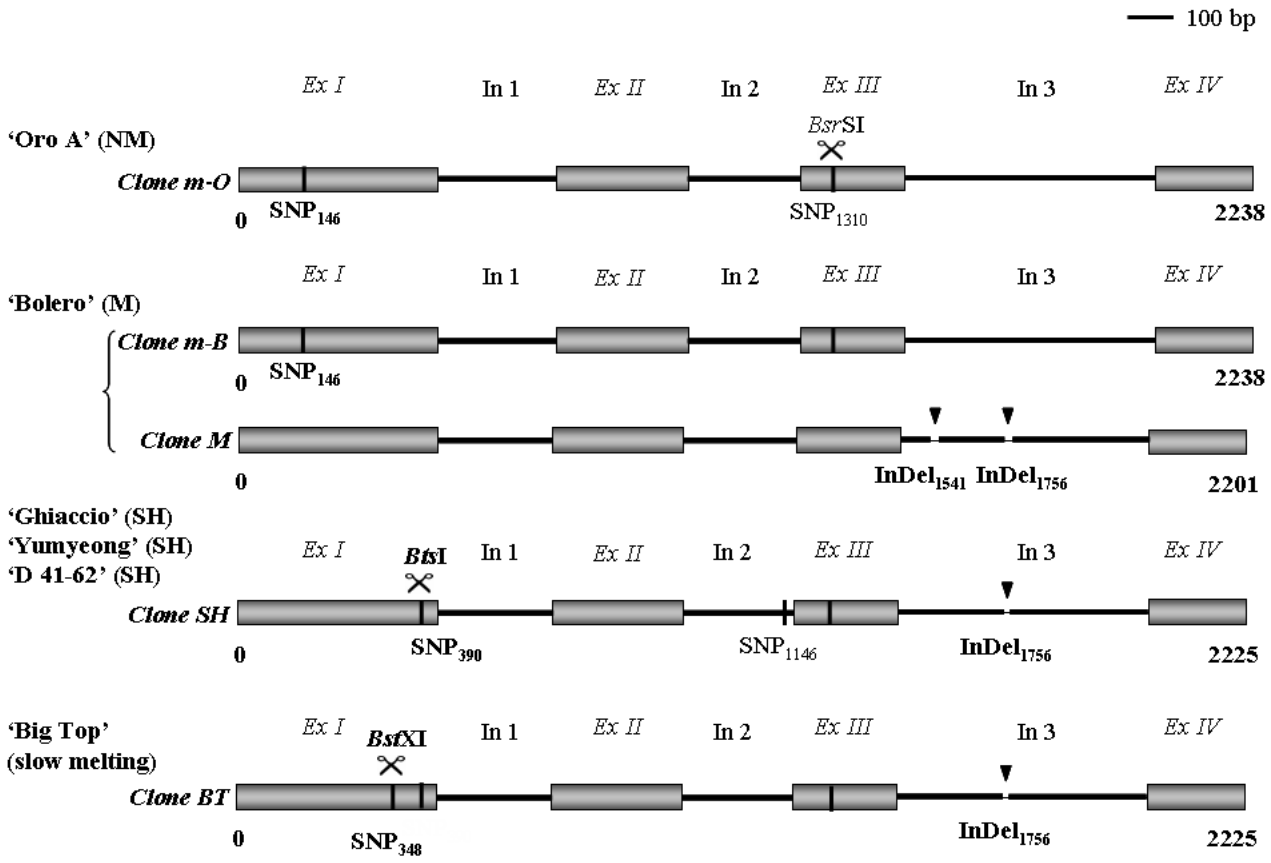


Fig. 1. Schematic diagram showing exon-intron structure of a *Pp-endoPG* gene in different peach accessions as deduced from the sequences of the *Pp-endo-PG* cDNAs from 'Oro A' and 'Bolero' (GenBank DQ340810 and DQ340809, respectively). Exons are indicated by grey boxes and introns by solid lines. Main single nucleotide polymorphisms (SNPs; thin bars) and insertions/deletions (InDels; indicated by inverted arrowheads and breaks of the solid lines) are indicated. The SNP_{146} (C→T_m), SNP_{1310} (G→$C_{m,\ SH,\ BT}$), SNP_{348} (C→T_{BT}), SNP_{390} (G→$T_{SH,\ BT}$) and SNP_{1146} (G→T_{SH}) are indicated. The scissors symbol indicates the restriction sites and the respective endonucleases.

Analysis on different genotypes and offspring accessions obtained by crossing genotypes with different fruit flesh firmness

The main differences in the *Pp-endoPG* gene clones isolated from NM, M, Slow Melting, and SH genotypes were found in intron 3, which showed sequence deletions suitable to develop an InDel marker.

From genomic DNA of the different peach accessions and by the use of $Forward_{InDel}$ and $Reverse_{InDel}$ primers, a selected sequence (bp 1455-1892) of the *Pp-endoPG* gene, relative to the intron 3 region comprising the InDels, was amplified. The amplification of this selected sequence was expected to generate: a single fragment of 447 bp, relative to the *m* clone, in NM 'Oro A'; two fragments of 447 and 413 bp relative to the *m* and *M* clones, respectively, in M 'Bolero'; a fragment of approx. 430 bp relative to the *SH* or *BT* clones, respectively, in SH ('Ghiaccio', 'D 41-62', 'Yumyeong') genotypes and in 'Slow Melting' 'Big Top' (Fig.2 A). The predicted amplification

pattern obtainable by the separation of the fragments on 3% (w/v) agarose gel is also shown (Fig. 2 B).

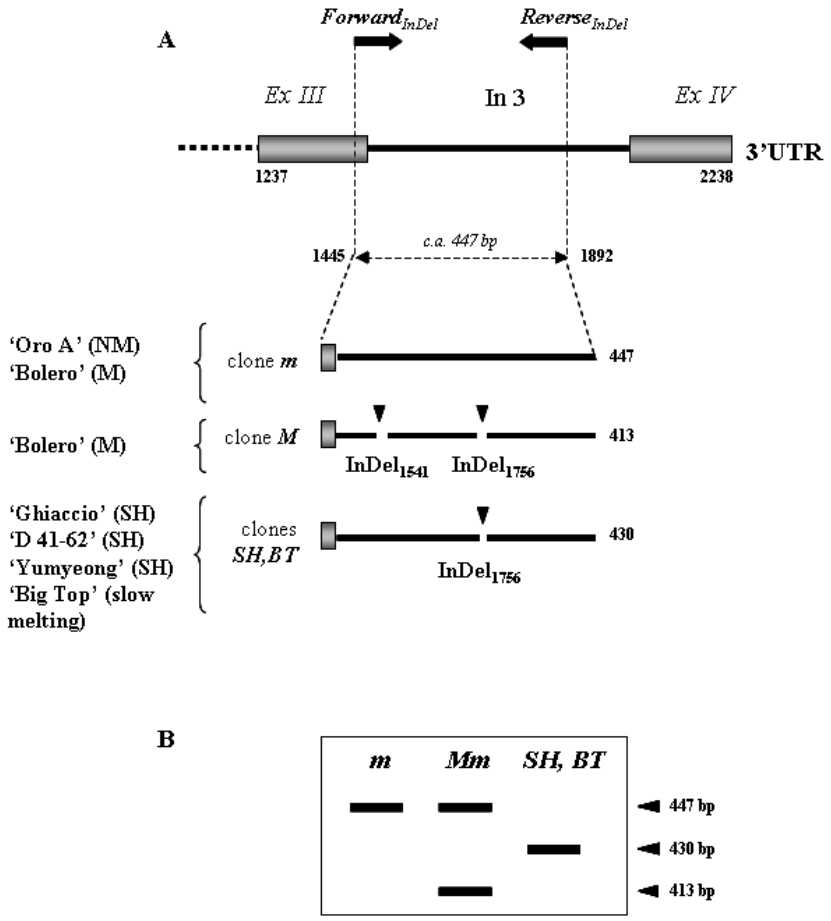


Fig. 2. Scheme of the amplification of a selected sequence of *Pp-endoPG* gene, relative to the intron 3 region comprising the main InDels, and predicted lenghts of the fragments obtainable from different gene clones (A). Scheme of the predicted amplification patterns obtainable from different genotypes (B).

The study was conducted on different well-established genotypes and accessions grouped according to fruit flesh texture characteristics, on the base of previous analyses on fruit phenotype conducted by Prof. Bassi.

Figure 3 shows the results of InDel analysis on: M 'Bolero' and a few putative M genotypes/accessions ['Ambra', 'Contender', 'Maycrest', 'Springbelle', 'Springcrest', 'Springred', 'Suncrest', 'Rich Lady', 'Contender' × 'Ambra' (96016015, 96016018, 96016023, 96016136, 96016165), ('Ambra' × 'Contender') × 'PF2' ('BO 96016094', 'BO 96016208'), 'Glohaven'] (Fig. 3 A); NM 'Oro A' and a few putative NM genotypes/accessions ['Andross', 'Ionia', 'BO 82010054'] (Fig. 3 B); SH ('Yumyeong', 'Ghiaccio', 'D-4162') genotypes and a few genotypes/accessions with fruit which remain firm at ripeness ['D-4162', 'Big Top', 'Yumyeong', 'Honey Gold', 'Ruby Rich', 'Helena Cling', 'Alitop', 'Stoney hard', 'Vistarich', 'BO 96028059', 'BO 96013046', 'Maycrest' × 'Vistarich' (02037001,02037002, 02037003, 02037004)] (Fig. 3 C).

In M ‘Bolero’ (Fig. 3 A) the amplification of a selected sequence of the *Pp-endoPG* gene generated two fragments of about 450 and 410 bp, respectively, consistent with the presence in this genotype of the two *Pp-endoPG* clones *m* and *M*. In the putative M cultivars ‘Contender’, ‘Maycrest, ‘Springbelle’, ‘Springcrest’, ‘Springred’, ‘Glohaven’ and in one (96016136) out of the three offspring accessions from the cross ‘Contender’ × ‘Ambra’ an amplification pattern similar to that of ‘Bolero’ was obtained, suggesting the presence also in these genotypes of both the *m* and *M Pp-endoPG.* In some putative M genotypes, such as ‘Ambra’, ‘Suncrest’, ‘Rich Lady’, and in two (96016015, 96016018) out of the three offspring accessions from the cross ‘Contender’ × ‘Ambra’, as well as in the offspring accessions (‘BO 96016094’ and ’BO 96016208’) from the cross (‘Contender’ × ‘Ambra’) × ‘PF2’, the amplification of the selected *Pp-endoPG* sequence generated an additional fragment of approx. 430 bp suggesting the simultaneous presence in these genotypes of the three *Pp-endoPG* clones: *m*, *M* and *SH* or *BT*.

In NM ‘Oro A’ (Fig. 3 B), consistent with the presence in this genotype of the *m Pp-endoPG* clone, the amplification of the selected sequence generated only one fragment of about 450 bp. Also ‘Andross’ and ‘Ionia’, genotypes and the ‘BO 82010054’ accession appeared to be characterized by the only presence of the *m Pp-endoPG* clone, as indicated by the amplification patterns similar to that of ‘Oro A’.

In SH ‘Ghiaccio’, ‘D-4162’ and ‘Yumyeong’, as well as in Slow Melting ‘Big Top’ (Fig 3 C), the amplification of the selected sequence generated a single fragment of about 430 bp, according to the presence in these genotypes of *SH* or *BT Pp-endoPG* clones, respectively. A similar result was obtained in ‘Helena Cling’ and in three (02037002, 02037003, 02037004) out of the four offspring accessions from the cross ‘Maycrest’ × ‘Vistarich’ suggesting the presence also in these genotypes/accessions of a single variant *SH* or *BT* of the *Pp-endoPG* gene. Unlike the indications obtained by phenotypic investigation, ‘Alitop’ and the offspring accession 02037001 from the cross ‘Maycrest’ × ‘Vistarich’ showed an amplification pattern similar to that obtained in M ‘Bolero’. A few genotypes with firm fruit at ripeness, such as ‘Honey Gold’, ‘Ruby Rich’, ‘Stony hard’, ‘Vistarich’ as well as the accessions (‘Spring Red’ ×’Big Top’) 96028059 and (‘Bolero’ × ‘Rich Lady’) 96013043, showed the presence in the amplification pattern of three fragments (450, 430 and 410 bp) indicating the simultaneous presence in these genotypes/accessions of the *m*, *M* and *SH* or *BT endoPG* clones. In particular, in the case of the two accessions ‘96028059’ and ‘96013043’ this result was coherent with that obtained in the parental genotypes and on the basis of the expected segregation during the cross.

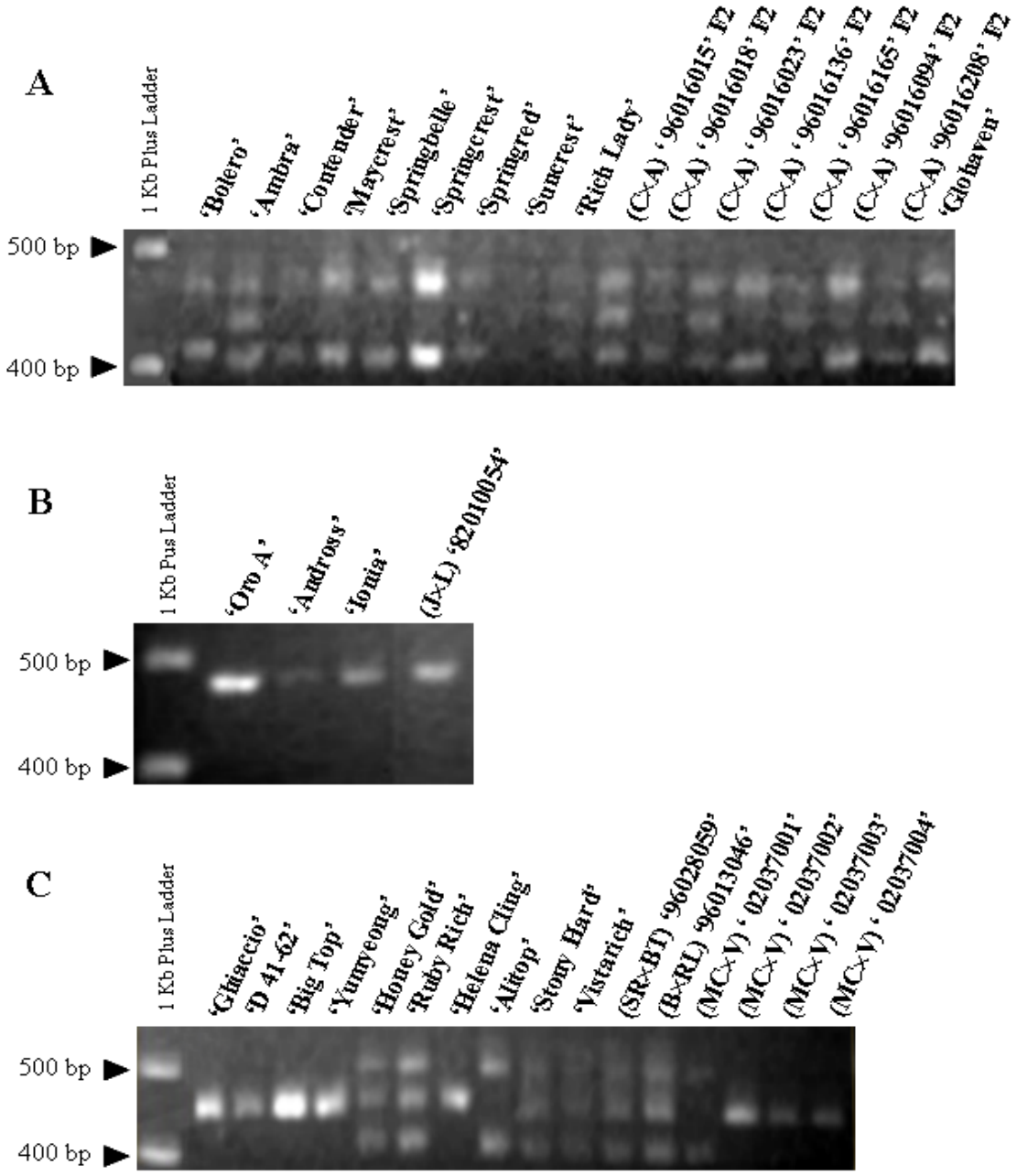


Fig. 3. InDel polymorphism analysis of well established or putative M (A), NM (B) accessions and SH accessions or accessions with firm fruit at ripeness (C). Positions and lengths of DNA markers (1 Kb Plus DNA ladder) are shown on the left. 20 μg of DNA was loaded per lane; the products were separated on 3% (w/v) agarose gels. The results of one representative experiment from three independent replications are shown.

Analysis of offspring populations of 'Ambra' × 'Big Top' and 'Max 7' × 'Big Top' crosses and on haploid accessions.

InDel analysis was also conducted on a number of accessions at our disposal belonging to the offspring populations of 'Ambra' × 'Big Top' and 'Max 7' × 'Big Top' crosses and on three

haploid accessions. The aim of the study was to gain some information which, even if obtained on a small number of individuals among wider populations, could give an insight on the segregation of the different (*m, M, SH or BT) Pp-endoPG* gene variants as a whole and, in particular, on the co-segregation of the *m* and *M* clones. Figure 4 shows the patterns obtained by amplifying a selected *Pp-endoPG* gene sequence from genomic DNA of the parental genotypes 'Ambra' and 'Big Top' and of 23 offspring accessions (02002001-02002006, 02002008 and 02002009, 02002011-02002015, 02002017-02002026) from their cross. In agreement with data reported in Figure 3, the amplification generated in 'Ambra' three fragments of ca 450, 430 and 410 bp, relative to the presence of the *m, M* and *SH* or *BT Pp-endoPG* clones, while in 'Big Top' only one fragment was obtained, relative to the presence of the *BT Pp-endoPG* clone. In the offspring population considered, eight (02002001, 02002006, 02002011, 02002013, 02002019, 02002020, 02002022, 02002025 equivalent to 35%) out of the 23 accessions showed an amplification pattern similar to that of the parental genotype 'Big Top' and 12 (02002002-02002005, 02002008, 02002009, 02002012, 02002014, 02002015, 02002018, 02002024, 02002026 equivalent to 52%) out of the 23 accessions showed an amplification pattern similar to that of the parental genotype 'Ambra', while three (02002017, 02002021, 02002023 equivalent to 13%) out of the 23 accession showed an amplification pattern with two fragments (ca 450 and 410 bp) similar to that of M 'Bolero' genotype which is characterized by the presence of the *m* and *M Pp-endoPG* clones (compare with Fig. 3 A). Also in this condition and according to the data reported in Figure 3, the presence of the *M Pp-endoPG* clone in the amplification patterns was accompanied by the presence of the *m* clone suggesting that *M* could co-segregate with the *m* gene variant. Moreover, in spite of the limited number of the offspring accessions considered, the presence (though in a percentage different from that of 25% as expected by the segregation of a Mendelian trait) of individuals showing an amplification pattern similar to M genotypes suggested the hypothesis that 'Big Top' might have a null *Pp-endoPG* gene variant showing a *BT/–* configuration at the *endoPG* locus .

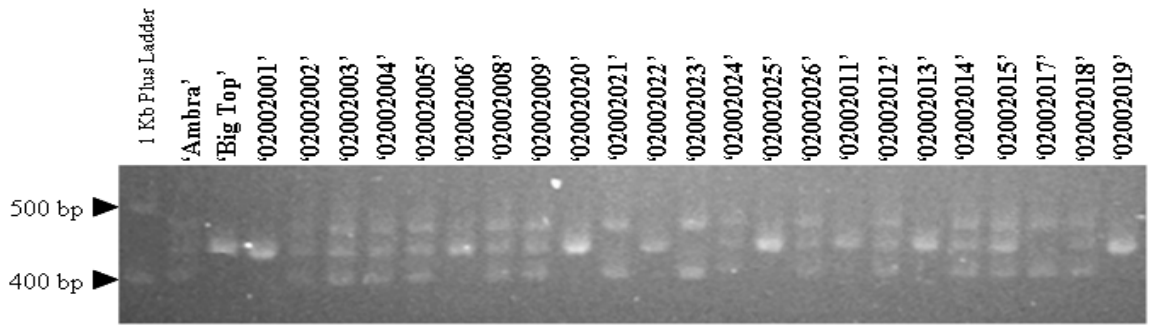


Fig. 4. InDel polymorphisms analysis 'Ambra' × 'Big Top' offspring population. Positions and lengths of DNA markers (1 Kb Plus DNA ladder) are shown on the left. 20 μg of DNA was loaded per lane; the products were separated on 3% (w/v) agarose gels. The results of one representative experiment from three independent replications are shown.

Both hypotheses, suggesting that the *M Pp-endoPG* clone segregated together with the *m* one and that 'Big Top' genotype might be heterozygous at the *endoPG* locus because of the presence of a null variant of *Pp-endoPG* gene, seemed to be confirmed also by the InDel analysis conducted in 27 (02004001-02004027) offspring accessions from 'Max 7' × 'Big Top' cross (Fig. 5). In agreement with the data reported in Figure 3, the amplification of the selected *Pp-endoPG* sequence from genomic DNA generated in 'Max 7' three fragments of ca 450, 430 and 410 bp, relative to the presence of *m*, *M* and *SH* or *BT Pp-endoPG* clones. In the offspring population considered, 13 (02004003, 02004005, 02004010-02004012, 02004014, 02004015, 02004018, 02004020-02004022, 02004026, 02004027 equivalent to 48%) out of the 27 accessions showed an amplification pattern similar to that of the parental genotype 'Big Top' and nine (02004001, 02004004, 02004006, 02004007, 02004016, 02004017, 02004019, 02004023, 02004024 equivalent to 33%) out of the 27 accessions showed an amplification pattern similar to that of the parental genotype 'Max 7', while five (02004002, 02004008, 02004009, 02004013, 02004025 equivalent to 18%) out of the 27 accession showed an amplification pattern with two fragments (ca 450 and 410 bp) similar to that of M 'Bolero' genotype (compare with Fig. 3 A).

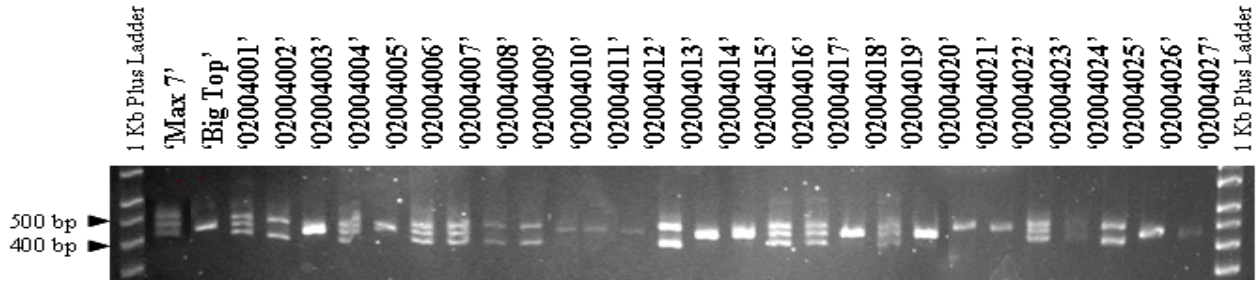


Fig. 5. InDel polymorphism analysis of 'Max 7' × 'Big Top' offspring population. Positions and lengths of DNA markers (1 Kb Plus DNA ladder) are shown on the left. 20 μg of DNA was loaded per lane; the products were separated on 3% (w/v) agarose gels. The results of one representative experiment from three independent replications are shown.

Further results confirming that the *M Pp-endoPG* clone, when present, is always accompanied by the *m* one were obtained by InDel analysis conducted in three haploid accessions ('PV1 1N', 'PR1 1N' and 'PL3 1N'; Fig. 6). 'PV1 1N' and 'PL3 1N' showed amplification patterns characterized by a single fragment of approx. 450 bp, indicative of the presence of the *m Pp-endoPG* clone, while in the amplification pattern of the haploid accession 'PR1 1N' two fragments of ca. 450 and 410 bp were evident, indicative of the simultaneous presence of both the *M* and *m* clones.

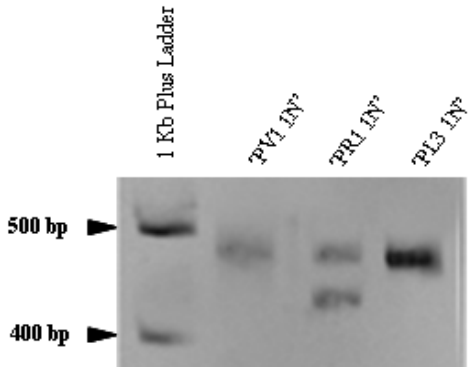


Fig. 6. InDel polymorphism analysis of three haploid peach accessions. Positions and lengths of DNA markers (1 Kb Plus DNA ladder) are shown on the left. 20 μg of DNA was loaded per lane; the products were separated on 3% (w/v) agarose gels. The results of one representative experiment from three independent replications are shown.

Analysis of alignment with the recently published peach genome sequence (International Peach Genome Initiative, 2010), referred to the doubled haploid peach cultivar 'Lovell', indicated that the *m Pp-endoPG* sequences of 'Oro A' and 'Bolero' showed a 100% identity with a sequence (*ppa006839m***)** located at bp 22,650,221 on the peach genome scaffold_4, while identity of the *BT Pp-endoPG* sequence of 'Big Top' and *SH Pp-endoPG* sequences of 'Ghiaccio', 'D 41-62' and

'Yumyeong' was 97%. The *M Pp-endoPG* clone of 'Bolero' showed 100% identity with a duplicate sequence (*ppa006857m*) located on the same scaffold_4 but at bp 22,684,623. In this region of scaffold_ 4 is present a cluster with a duplication in tandem of two *PG* and two *NADP-dependent oxidoreductase* sequences. The results obtained by alignment analysis suggested that the *m*, *SH* and *BT Pp-endoPG* clones are alleles, moreover the evidence that *M* clone is very close to *m* one could account for the low recombination frequency between the two genes leading to their co-segregation.

As far as it concerns the genetic configuration at the *endoPG* locus, our data are in agreement with those obtained by Peace *et al*. (2005).

Peace and co-workers reported that *endoPG* co-segregates completely with the *Melting flesh* trait, linked to the flesh softening characteristics, and *Freestone* trait, linked to pit adherence, and suggested that *endoPG* might controls both traits, as proposed by Lester *et al*. (1996). Peace and coworkers found length polymorphisms in the DNA sequence at the *endoPG* locus and named *F*, *f* and *f1* the alleles individuated. In particular, the authors hypothesized that the original sequence *F°* contained multiple copies of *endoPG* including the F^a sequence (which is suggested to be part of the gene copy controlling the *Freestone* trait) and the F^b sequence (which is suggested to be part of the gene copy controlling the *Melting* trait); the *f* allele resulted from deletion of F^a and mutation of F^b while the *f1* allele resulted from deletion of F^b and mutation of F^a , which could also result in a loss of function. By comparing the sequences of the *Pp-endoPG* clones isolated in the genotypes considered in the present work, and on the basis of the deletion in intron 3, the *M* and *m Pp-endoPG* clones corresponded to the F^b and *f1* alleles, respectively, while the *SH* and *BT Pp-endoPG* clones corresponded to *f* allele. Peace and co-workers indicated also the presence of a null allele possibly arisen from deletion of both F^a and F^b or from deletion of any derived allele.

Cleaved Amplified Polymorphic Sequence (CAPS) analysis

CAPS markers exploit polymorphic restriction sites for mapping purposes. They are co-dominant genetic markers which allow to genotype both chromosomes of a plant, or to highlight different digestion patterns for plants that are homozygous or heterozygous for the parental alleles. The method is based on PCR amplification of genomic DNA regions containing SNPs which determine polymorphic restriction sites recognized by specific endonucleases. The amplification products, once cleaved with the proper restriction enzyme, will produce precise fragments detectable after separation in agarose gel (Konieczny and Ausubel, 1993; Lukowitz *et al*., 2000).

CAPS analysis exploiting SNP_{390} of SH and BT Pp-endoPG clones

In the *SH Pp-endoPG* clone of the SH 'Ghiaccio', 'D 4162' and 'Yumyeong' genotypes, as well as in the *BT Pp-endoPG* clone of the Slow Melting 'Big Top' genotype, a SNP at 390 bp was individuated ($G \rightarrow T_{SH,BT}$) which originated a polymorphic sequence (GCAGTGNN▼) recognized by the *Bts*I enzyme.

The CAPS analysis exploiting SNP_{390} was conducted on genomic DNA of peach genotypes/accessions ('Alitop', 'Honey Gold', 'Ruby Rich', '7-28', 'Vista Rich', 'BO 96013043' and 'BO 96028059') characterized by fruit which remain firm at ripeness. A selected sequence (bp 60-971 equivalent to ≈911 bp) of the *Pp-endoPG* gene, relative to the exon I region comprising the SNP_{390}, was amplified with Hd_{DIR} and Hd_{REV} primers and the amplification products were digested with *Bts*I. The fragments obtained from the different genotypes were then visualized by separation in 3% (w/v) agarose gel.

Figure 7 A shows the amplification scheme of the selected sequence of *Pp-endoPG* gene, with the indication of the cut site and expected length of the fragments obtainable from different *Pp-endoPG* clones by cleavage with *Bts*I enzyme. In Figure 7 B the restriction patterns of different genotypes are reported with the indication of the results obtained by separation of the amplified selected sequences before (–) and after digestion (D).

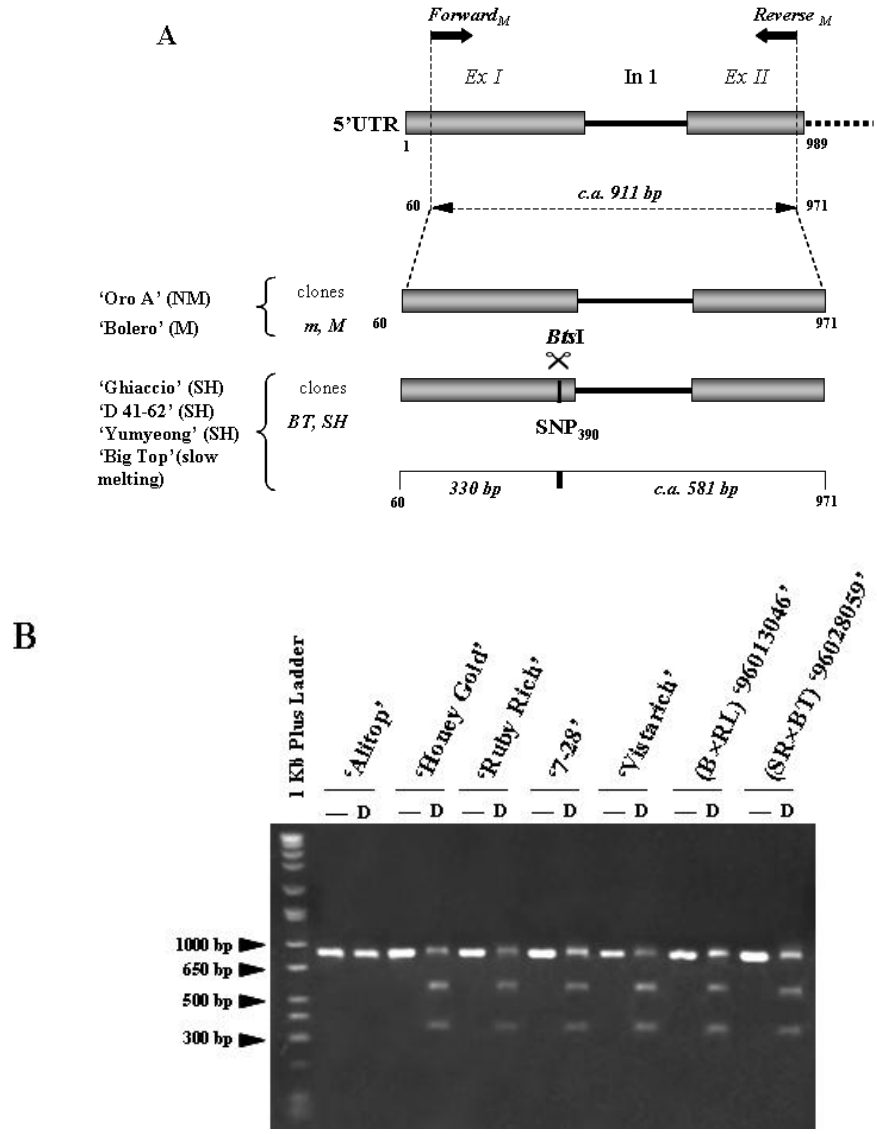


Fig. 7. CAPS analysis exploiting SNP_{390} of the *SH* and *BT Pp-endoPG* clones of SH 'Ghiaccio', 'D 41-62' and 'Yumyeong' and Slow Melting 'Big Top' genotypes. Scheme of the amplification of a selected sequence (≈ 911 bp) of *Pp-endoPG* gene containing the putative polymorphic site and predicted length of the fragments obtainable after cleavage with the *Bts*I enzyme (A). Restriction pattern obtained in genotypes/accessions ('Ali Top', 'Honey Golg', Ruby Rich', '7-28', 'Vista Rich', 'BO 9601346', 'BO 96028059') with firm fruit texture at ripeness. The separation of the amplified selected sequences before (–) and after digestion (D) is indicated (B). Positions and lengths of DNA markers (1 Kb Plus DNA ladder) are shown on the left. 20 μg of DNA was loaded per lane; the products were separated on 3% (w/v) agarose gels. The results of one representative experiment from three independent replications are shown.

In all genotypes considered, with the only exception of 'Ali Top', CAPS reaction yielded three bands, one of ≈900 bp, relative to the undigested selected sequence/s, and two of ≈650 and 350 bp, consistent with the lengths (581 and 330 bp, respectively; Fig. 7 A) predictable for the fragments obtainable by the action of this endonuclease. These results were in agreement with those obtained by InDel analysis and indicated the presence in 'Honey Gold', 'Ruby Rich', '7-28', 'Vista Rich', ('Bolero' × 'Rich Lady') 96013043 and ('Spring Red' ×'Big Top') 96028059 of an *SH* or *BT*

Pp-endoPG clone. The undigested fragment of ≈900 bp was referrable to the *m* and *M Pp-endoPG* clones also present in these genotypes (compare to Fig. 3 C). Moreover, it is interesting to note that the results obtained from the CAPS analysis in the two accessions '96013043' and '96028059' are also coherent with those obtained from InDel analysis in the parental genotypes. The cleaving enzyme was uneffective on 'Alitop' confirming absence of *SH* or *BT Pp-endoPG* clone in this genotype.

CAPS analysis exploiting SNP_{348} of BT Pp-endoPG clone

In the *BT Pp-endoPG* clone of the Slow Melting 'Big Top' genotype a SNP at 348 bp was individuated ($C \rightarrow T_{BT}$) which originated a polymorphic sequence (CCANNNNN▼NTGG) recognized by *Bst*XI enzyme. SNP_{348} was exploited to develop a CAPS marker aimed to individuate the possible presence of the *BT Pp-endoPG* clone in peach accessions with fruit characterized by firm texture at ripeness or, in general, by a 'Big Top'-like phenotype.

The CAPS analysis was conducted on genomic DNA extracted from leaves of NM 'Oro A', ~~MF~~ 'Bolero', Slow Melting 'Big Top', SH 'Ghiaccio', 'D41-62' and 'Yumyeong' as well as from 'Helena Cling', characterized by fruit which remain firm at ripeness. A selected sequence (bp 1-950) of the *Pp-endoPG* gene, relative to the exon I region comprising the SNP_{348}, was amplified with $Endo_{DIR}$ and Hd_{REV} primers and the amplification products were digested with *Bst*XI (Fig. 8 A). The fragments obtained from the different genotypes were then visualized by separation in 3% (w/v) agarose gel (Fig 8 B).

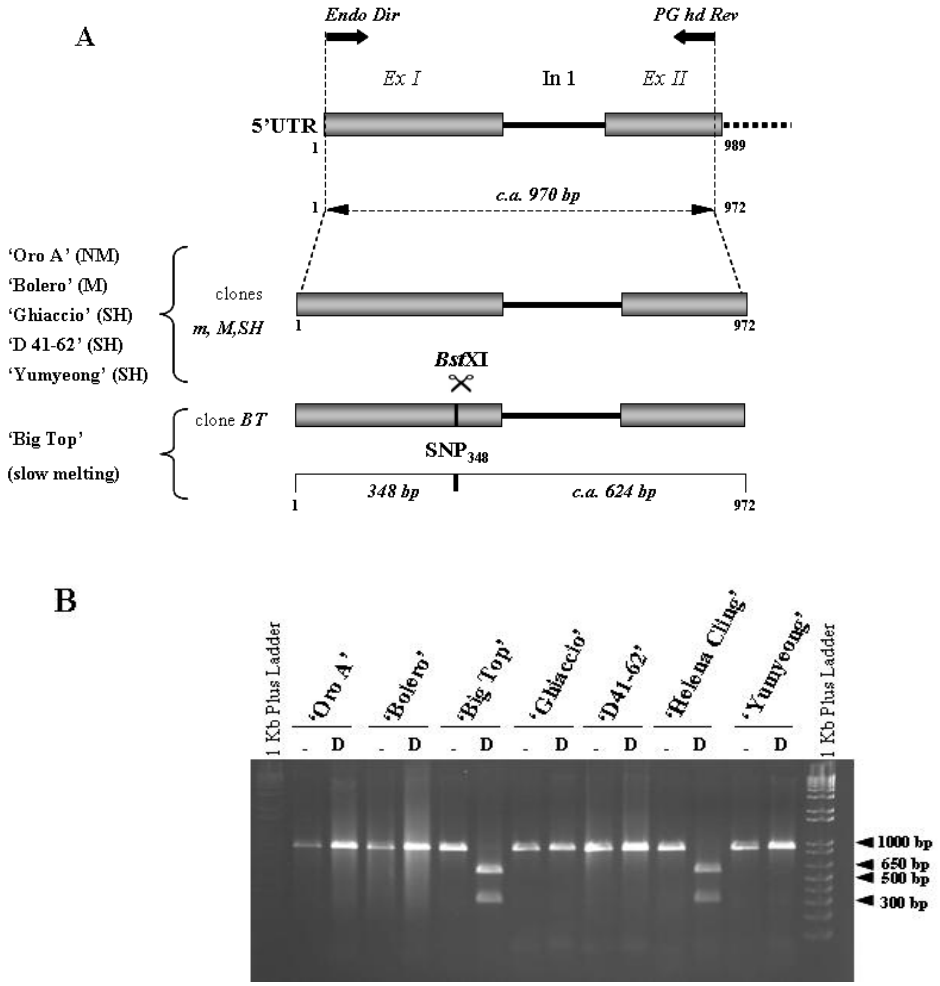


Fig. 8. CAPS analysis exploiting SNP_{348} of *BT Pp-endoPG* clone of the Slow Melting 'Big Top' cultivar. Scheme of the amplification of a selected sequence (≈ 950 bp) of *Pp-endoPG* gene containing the putative polymorphic site and predicted length of the fragments obtainable after cleavage with the *Bst*XI enzyme (A). Restriction patterns obtained in NM 'Oro A', M 'Bolero', Slow Melting 'Big Top', SH 'Ghiaccio', 'D 41-62', 'Yumyeong' genotypes and in 'Helena Cling', genotype with firm fruit texture at ripeness. The separation of the amplified selected sequences before (−) and after digestion (D) is indicated (B). Positions and lengths of DNA markers (1 Kb Plus DNA ladder) are shown on the right. 20 μg of DNA was loaded per lane; the products were separated on 3% (w/v;) agarose gels. The results of one representative experiment from three independent replications are shown.

Figure 8 B shows that in 'Big Top' as well as in 'Helena Cling' CAPS reaction yielded two bands of ≈ 350 and 650 bp, consistent with the lengths (348 and 624 bp, respectively; Fig. 8 A) predictable for the fragments produced by the endonuclease. The cleaving enzyme was uneffective on the M, NM and SH accessions considered. Among the different genotypes with firm fruit at ripeness previously screened for this polymorphism (data not shown), CAPS analysis exploiting SNP_{348} individuated the presence of the *BT Pp-endoPG* clone only in 'Helena Cling'.

SNP_{348} may thus represent a useful diagnostic tool which, by discriminating between *BT* and *SH* clones, allows to gain additional information on the configuration at the *endoPG* locus in different genotypes giving also indications whether a selection is genetically related to 'Big Top'.

In conclusion, the 17-bp-deletions individuated in the sequences of the different *Pp-endoPG* clones in the region relative to intron 3 seem to characterize different gene variants, in agreement with the data in literature (Peace *et al.,* 2005). This polymorphism, as reported for the accessions considered in this study, appears to be in relation with the fruit phenotype and allows to discriminate among M, NM, SH/'Slow Melting'. Two other polymorphisms, SNP_{390} and SNP_{348}, have been described which allow to individuate SH/'Slow Melting' and, in particular, SNP_{348} can discriminate between SH and 'Slow Melting' complementing the information obtainable by insertions/deletions. All these polymorphisms may then be useful for discriminating among genotypes with still unknown phenotypes and seem to represent a powerful tool for Molecular Assisted Selection (MAS) as far as it concerns the genetic improvement of stone fruit quality attributes related to flesh texture.

CHAPTER III

ISOLATION OF *Pp-EndoPG* PROMOTER REGION IN MELTING, NON MELTING AND SLOW MELTING FLESHH GENOTYPES AND PRELIMINARY ANALYSIS

Introduction

Fruit ripening is a complex, genetically programmed process involving increases in respiration and ethylene production, changes in colour, flavour and softening.

The softening process is one of the many physiological events that fall into the more general process of cell separation (Roberts *et al*., 2002). Cell separation occurs whenever the links between cells in a given tissue are loosened as a consequence of the weakening of the cell wall structure. This process involves the expression of different enzymes that irreversibly impair the mechanical properties of the cell wall by acting on polysaccharidic components.

Polygalacturonase (PG) is one of the enzymes involved in cell wall degradation., catalyzing the hydrolytic breakdown of bonds between the galacturonic acid residues constituting polygalacturonans. PGs act as exo- or endo- hydrolase (exoPG or endoPG). ExoPGs catalyze the removal of single galacturonic acid residues from the non-reducing end of polygalacturonic acid, while endoPGs randomly attack the glycosidic bonds within the polymer, hydrolyzing them (Daas *et al*., 2000). PG was first characterized in tomato, a model species for studies on climacteric fleshy fruits (Giovannoni, 2004), and three isoenzymes were identified: PG1, PG2A and PG2B. PG2A and PG2B are single polypeptides and differ from each other primarily in their degree of glycosylation, which leads to the molecular weights of respectively 43 and 45 kDa approximately (Brummell and Harpster, 2001). PG1 is a heterodimer and appears to contain both PG2A and PG2B, combined with a glycoprotein of about 38 kDa (β subunit). PG1 accumulates in the early stages of ripening, while PG2 is the dominant isoform in ripe fruit (Tucker *et al*., 1980).

In tomato, a reduced expression of endoPG, obtained by using antisense technologies, did not dramatically affect fruit softening (Smith *et al*., 1988). Down-regulation of PG seems to prevent over-ripening by prolonging the integrity of the fruit.

In peach, the activities of several enzymes have been shown to increase during softening: exopolygalacturonase (exoPG), endopolygalacturonase (endoPG), endo-1,4-β-mannanase, α-L-arabinofuranosidase and b-galactosidase (Pressey and Avants,1973; Brummell *et al*., 2004); PG has been shown to increase during ripening and the levels of endoPG activity have been reported to differ between Non Melting Flesh (NM) and Melting Flesh (M) types, as well as Stony Hard (SH) ones (Pressey and Avants, 1978, Yoshida, 1976). The reduction in the expression of the *endoPG* gene by genetic manipulation could affect texture since the levels of endoPG transcripts and enzyme activity appear to correlate with flesh texture (Lester *et al*., 1996). The M phenotype is mainly linked to the presence of a ripening related-*endoPG* (Peace *et al*., 2005); M fruit show high amounts of endoPG,

which increase with softening (Karakurt *et al*., 2000; Brummell *et al*., 2004a and references therein).

The NM peach phenotype has been reported to be related to the absence of functional endoPG due to partial or massive deletions of an *endoPG* gene (Callahan *et al*., 2004); nevertheless, Morgutti *et al*. (2006) reported that in NM 'Oro A' fruits a not truncated endoPG cDNA was isolated, which encoded an endoPG protein whose deduced amino acidic sequence is similar to that of M 'Bolero' fruit. The very low levels of accumulation of the endoPG protein in 'Oro A' NM fruit were attributed to low levels of the corresponding transcripts, consistent with the hypothesis of different transcriptional regulation of the *Pp-endoPG* gene.

Ethylene plays a major role in regulating ripening and softening of climacteric fruit (Giovannoni, 2001). Accordingly, the expression of some ripening-related cell wall-associated genes and enzyme activities, including PGs, expansins and endo-1,4-β-glucanases are up-regulated by the phyoregulator (Hiwasa *et al*., 2003, 2004). The absolute amounts of fruit ethylene evolution, though, seem not directly related to the development of texture characteristics of peach fruit: in fact, while SH fruit present almost null ethylene production with crisp and firm fruit which do not lose their firmness for a long period after harvest (Tatsuki *et al*., 2006; Ghiani *et al*., 2010), in NM fruit ethylene evolution was much higher than in M ones, suggesting the possibility of different regulation by the phytoregulator of the expression of the ethylene-responsive genes involved in cell-wall restructuring.

Molecular analysis has been greatly valuable in elucidating nature and function of genes involved in fruit ripening (Giovannoni 2001). To better understand the mechanisms that control the expression of ethylene-responsive genes during ripening, the promoter regions of several of them were isolated and analyzed with the aim of identifying functional regulatory motifs (Cara and Giovannoni, 2008). The characteristic expression patterns of PGs, and particularly so of endoPG, during fruit ripening made the study of the *PG* promoter very attractive for the characterization of gene expression with respect to fruit- and ripening-specific regulation (Montgomery *et al*., 1993; Nicholass *et al*., 1995)

In order to better explain the mechanisms underlying the different expression of the *endoPG* gene in NM and M peach fruit and possibly clarify the apparent paradox between high ethylene evolution in NM fruit and low flesh softening, the structure of the *Pp-endoPG* promoter has been investigated in fruit of reference cultivars for NM ('Oro A'), M ('Bolero') and Slow Melting ('Big Top) flesh texture characteristics and softening patterns.

The study was undertaken also with the aim of identifying, in *Pp-endoPG* promoter the possible presence of sequence differences (SNPs, InDels or microsatellite) useful as molecular markers to distinguish, and early select, peach genotypes with different texture characteristics.

Materials and methods

Plant material and sampling

Plant material was from experimental orchard of the University of Bologna ('Azienda Agricola Zabina' Castel San Pietro, BO) and (Di.Pro.Ve.) and belonged to the peach accession collection of Professor Bassi (DiProVe). Experiments were conducted on leaves of genotypes

Experiments were conducted on peach leaves and fruit of NM 'Oro A', M 'Bolero' and Slow Melting 'Big Top' genotypes.

Sampling of plant material was conducted as described in the sections "Material and Methods" of Chapter 1 and 2.

Isolation of genomic DNA

Genomic DNA was obtained following the protocol of Geuna *et al*. (2004) with slight modifications (for more details see Materials and methods of Chapter 1).

Isolation of RNA

Total RNA was obtained following the protocol of Dal Cin *et al*. (2005).

Synthesis of cDNA

RNA obtained from fruit was treated with Deoxyribonuclease I (DNase I, Amp Grade, Invitrogen), following manufacturer's protocol It was then purified by extraction with phenol:chloroform (3:1), precipitated with one volume of 2-propanol and 1/10 volume of 3 M NaAc at pH 5.2 and, after incubation for 1 h at –80° C, centrifuged at 18,000 *g* for 30 min at 4° C. The pellet was washed with 70% (v/v) ethanol, air dried and resuspended in 50 µL of ddH_2O. RNA obtained was used as template in reverse transcription reaction to obtain the correspondent cDNA in a reaction performed by using 'SuperScript® First-Strand Synthesis System for RT-PCR' (Invitrogen). In order to obtain cDNAs enriched in *endoPG* gene suitable for Genome walking technique, $EndoPG_{8REV}$ (5'–CCGATGACCCGGTAATCCGACGGGGCC–3') was used as Gene Specific Primer (GSP), according to the protocol suggested by the manufacturer.

Isolation of Pp-endoPG promoter sequences by Genome walking technique

Genomic DNA extracted from the leaves of different peach genotypes was used for the construction of DNA libraries, following the protocol described in BD Genome Walker Universal Kit (Clontech) with slight modifications. DNA libraries were used as template for PCR-based DNA walking in two PCR rounds utilizing different primer pairs in order to walk on the genome in the region upstream of the considered *endoPG* gene.

Primer pairs A1 (first PCR: *AP_1* and $EndoPG_{4REV}$) and A2 (second PCR: AP_2 and $EndoPG_{3Rev}$); primer pairs B1 (first PCR: AP_1 and $EndoPG_{3REV}$) and B2 (second PCR: AP_2 and $Prom_{5REV}$); primer pairs C1 (first PCR:AP_1 and $Prom_{5REV}$) and C2 (second PCR: AP_2 and PrO_{4REV}), primer pairs D1 (first PCR: AP_1 and PrO_{4REV})and D2 (second PCR: AP_2 and PrO_{3REV}).

Primer	*Sequence*
AP_1	5’-GTAATACGACTCACTATAGGGC-3’
AP_2	5'–ACTATAGGGCACGCGTGGT–3'
$EndoPG_{3REV}$	5’-CGGGATTCATCGAGGCACAAGCTTTAGCCC-3’
$EndoPG_{4REV}$	5’-CGTTCCTGCCGGCACATAAATGACACCGGG-3’
$Prom_{5REV}$	5’-CTACGGTTCGCCATTTGCGG-3’
PrO_{4REV}	5’-CGTTTTATCCTGTTTCCCACTCC-3’
PrO_{3REV}	5’-CGCTAACAGTGGAAGACAACC-3’

The primary PCR mix contained 1 µL ligated DNA, 0.3 mM dNTPs, 0.3 µM of each primer pair (A1, B1, C1 and D1), 1×Reaction Buffer GoTaq®, 1 U Go Taq® DNA Polymerase (Promega) and ddH_2O to reach a final volume of 25 µl. Secondary PCR was performed in the same final volume with 1 µL of a 50-fold dilution of product obtained from the primary PCR, 0.3 mM dNTPs, 0.3 µM of each primer pair (A2, B2, C2 and D2), 1×Reaction Buffer GoTaq®, 1 U Go Taq® DNA Polymerase (Promega) and ddH_2O to reach a final volume of 25 µl. The PCR conditions for the primary PCR were:

7 cycles	94°C for 25 sec
	72°C for 3 min
32 cycles	94°C for 25 sec
	67°C for 3 min
1 cycle	67°C for 7 min

for the secondary PCR:

5 cycles	94°C for 25 sec
	72°C for 3 min
20 cycles	94°C for 25 sec
	67°C for 3 min
1 cycle	67°C for 7 min

The procedure described above was used also on cDNAs to obtain a cDNA librares useful to isolate the 5'UTR region of the *endoPG* genes.

Cloning and sequencing of PCR products

The PCR products were resolved in a 1% (w/v) agarose gel. After the run, DNA gel slices were eluted and purified using the 'Wizard SV Gel PCR Clean Up System' (Promega), in order to obtain pure DNA for cloning. The purified PCR products were cloned in pGEM T-Easy Vector (Promega) and transformed into *E. coli* TOP10 chemical competent cells, following the manufacturer's protocol. The 'Quick Plasmid Miniprep Kit' (Invitrogen) was used to extract plasmid DNA, which was sequenced by means of the Sanger sequencing method (Sanger *et al*., 1975) at BMR Genomics (Padua, Italy).

PCR Amplification of Multiple Specific Alleles (PAMSA)

Genomic DNA of the different peach genotypes was used for the analysis of the amplification profiles of the region of the *endoPG* gene which contained a Single Nucleotide Polymorphism (SNP) at position 146 (see Chapters 1 and 2). For this analysis three primers were designed (allele-specific primers) with a destabilizing mismatch (**T** and **G** for C) within the five bases at 3′ and a tail at 5' (*cgtcc* for EPGS146T_{FOR}; *attactactagacgg* for EPGS146C_{FOR}) for the

obtainment of PCR products of different length, following the protocol reported by Gaudet *et al*. (2007).

Primer	*Sequence*
$EPGS146T_{REV}$	5'-*cgtcc*GCACAAGCTTTAGCCCAT**A**CAA-3'
$EPGS146C_{REV}$	5'-*attactactagacgg*TGCGGGATAGGCGAATTAG**C**GGTTA-3'
$Endo_{DIR}$	5'-ATGGCGAACCGTAGAAGCCTCT-3'

The PCR reaction was conducted in 12.5 µL total volume with 15 ng DNA,0.3 mM dNTPs, 0.25 µM $EPGS146T_{REV}$, 0.25 µM $EPGS146C_{REV}$, 0.25 µM $Endo_{DIR}$, 1× Reaction Buffer GoTaq®, 1 U Go Taq® DNA Polymerase (Promega) and sterile ddH_2O. The PCR conditions were:

1 cycle	94° C for 3min
35 cycles	94° C for 20 sec
	65.5° C for 45 sec
	72° C for 30 sec
1 cycle	72° C for 4 min

The PCR products were resolved in a 3% (w/v) agarose gel. This procedure proved able to allow, for this particular set of primers, the setting up of the most suitable annealing temperatures on the *m* and *M Pp-endoPG* clones. These clones are in 'pCR®4-Blunt II TOPO® vector' (Invitrogen).

Sequence analysis

The sequences from BMR Genomics were analyzed and aligned by BioEdit. The region upstream of 5'of the *endoPG* gene were analyzed using a databases: PLACE (Higo *et al*., 1999).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear protein extracts were prepared from peach fruit mesocarp as described by Manzara *et al*. (1991) with the modification reported by Montgomery *et al*. (1993). Protein were quantified according to the Bradford method (Bio-Rad Protein assay) using bovine serum albumin as standard. A fragment of 1207 bp in the 5' upstream region of the *Pp-endoPG* gene was amplified from genomic DNA of 'Oro A' and 'Bolero' with $Prom_{3FOR}$ and $Prom_{5REV}$ primers.

Primer pair	*Sequence*
$Prom_{3FOR}$	5’- CGTGGTTGGTGTACCATCC-3’
$Prom_{5REV}$	5’-CTACGGTTCGCCATTTGCGG-3’

PCR was performed in a volume of 25 µL with 1 µL of DNA, 0.3 mM dNTPs, 0.3 µM of both $Prom_{3FOR}$ and $Prom_{5REV}$, 1×Reaction Buffer GoTaq®, 1 U Go Taq® DNA Polymerase ddH_2O in the following conditions:

1 cycle	94° C for 3min
35 cycles	94° C for 30 sec
	58° C for 45 sec
	72° C for 2 min

The PCR products were resolved in a 1% (w/v) agarose gel, purified by ‘Wizard SV Gel and PCR Clean-Up System’ (Promega), and resuspended as described in by Montgomery *et al*. (1993). The mobility shift reaction was conducted, with slight modifications, as described by Montgomery *et al*. (1993) in 20 µL final volume of a reaction mix containing 2 ng of DNA amplified in the PCR reactions plus 2 µg of nuclear protein extract. Electrophoresis was conducted in a 3% (w/v) agarose gel (Hellman and Fried, 2007 and references therein) and DNA was labelled with ethydium bromide and visualized with a UV transilluminator.

Results and discussion

Strategy for the isolation of promoter sequences

In order to analyze promoter sequences of the *Pp-endoPG* clones of NM 'Oro A', M 'Bolero', Slow Melting 'Big Top' genotypes it was necessary to individuate the most appropriate technique for the isolation the 5' upstream region of these gene variants, which previous results had indicated as very similar to each other (see Chapter 2). It is necessary to stress that this work prior to the official delivery of the peach genome (April, 2010).

The isolation of the 5' upstream region of the *Pp-endoPG* clones was conducted by the Genome Walking technique. This method consists in the systematic identification of unknown regions flanking a known DNA sequence. The isolation of such unknown regions can be obtained by screening a genomic or BAC library with known DNA as a probe, see Figure 1 (Siebert *et al*., 1995).

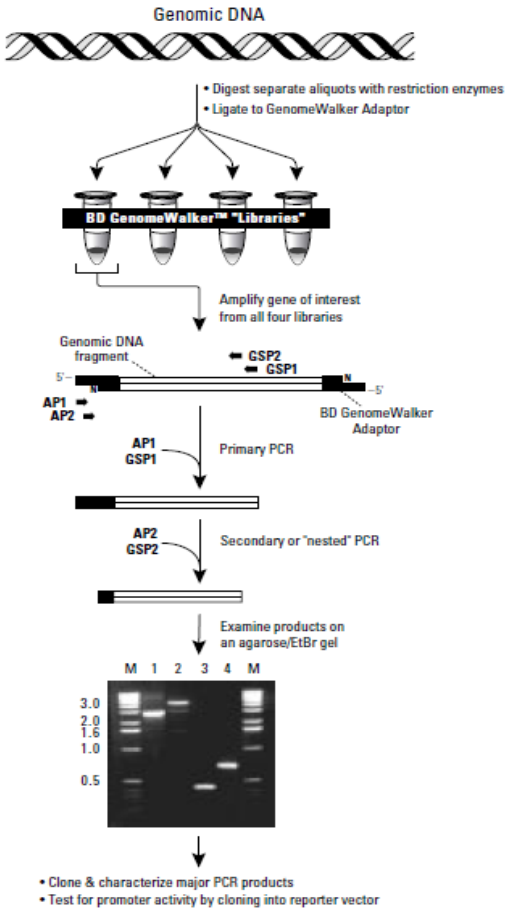


Fig.1. Flow chart of the GenomeWalker™ (Universal Kit, Clontech) Protocol. The gel shows a typical result generated by walking with GenomeWalker human libraries and gene specific primers. Lane 1: *EcoR V* library. Lane 2: *Dra* I library. Lane 3: *Pvu II* library. Lane 4 *Ssp* I library. Lane M: DNA size marker. N: amine group that blocks extension of the 3' end of the adaptor-ligated genomic fragments. AP: adaptor primers. GSP: gene-specific primers.

As reported by Morgutti *et al*. (2006) and in Chapters 1 and 2 of this PhD thesis, in NM 'Oro A' only one clone (*m*) of the *Pp-endoPG* gene was found, while in M 'Bolero' a *Pp-endoPG* clone *m* plus another (*M*), that differs from *m* for some SNPs and two 17-bp deletions, were present. In Slow Melting 'Big Top' only one clone (*BT*) of the *Pp-endoPG* gene was found, which presents a few SNPs and one 17-bp deletion compared to *m* (see Chapters 1 and 2 for more details).

In our case, the screening of genomic libraries allowed the isolation of upstream region of *endoPG* gene clones *m* in 'Oro A', *m* in 'Bolero', and clone *BT* in 'Big Top'. For the heterozygous nature of 'Bolero', the isolation of upstream region of *endoPG* clone *M* provided the use of other techniques, besides Genome walking ones.

Thus, was conducted the isolation of 5'UTR of *endoPG* clone *M* from cDNAs enrich in *endoPG* gene to identify differences between upstream region of *endoPG* clones *m* and *M*, considering that cDNAs derived from soft fruits, where *endoPG* clone *M* is surely present, being the dominant allele of Melting Flesh phenotype (Peace *et al*., 2005).

The identification of differences on 5' UTR sequence from *endoPG* clone *M* allowed to isolate the upstream region of *endoPG* clone *M* on genomic libraries. Besides this, was necessary found another procedure, which discriminated between *endoPG* clones *m* and *M* of 'Bolero' to screen the amplified products obtained from Genome Walking before to send to the sequencing.

To this purpose, the PCR Amplification of Multiple Specific Alleles (PAMSA) technique was used to identify, among the amplified products derived from the upstream region of the *endoPG* gene in M 'Bolero', those related to the clones *m* and *M*. Among the polymorphisms individuated in the *m* and *M* clones possibly useful to discriminate between them (see Figure 2 and compare to Figure 1 of Chapter 2), the SNP at 146 bp downstream ATG in exon I ($\mathbf{T}_m \rightarrow \mathbf{C}_M$) was chosen for being the closest to the start codon (Gaudet *et al*., 2007). For this SNP, a primer set with peculiar characteristics (different "tail" length, see Materials and Methods) was designed following the protocol reported by Gaudet *et al*. (2007).

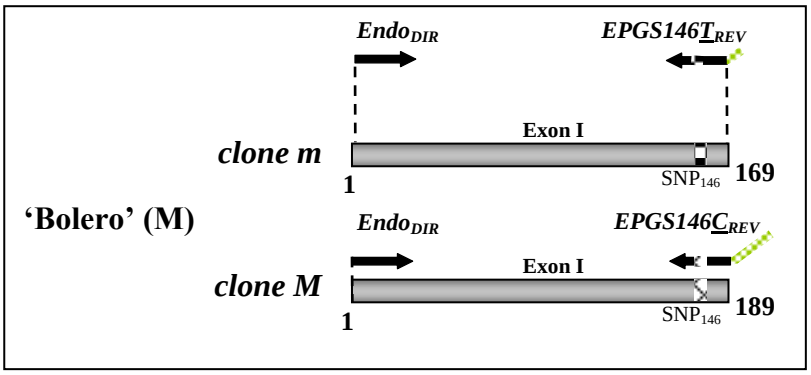


Fig. 2. PAMSA PCR: Amplification scheme of selected fragments in the *Pp-endoPG* gene of the M 'Bolero' exploiting SNP_{146} Use of two clone-specific primers having a tail of different length useful to differentiate the the amplification products of the two clones based on size and polymorphism (SNP_{146}).

The use of such primers, along with the setting-up of the most appropriate annealing temperature for the *m* and *M endoPG* clones (Figure 3A) see Materials and Methods, allowed to discriminate, in agarose gels, among putative *m* and *M endoPG* clones (Figure 3B).

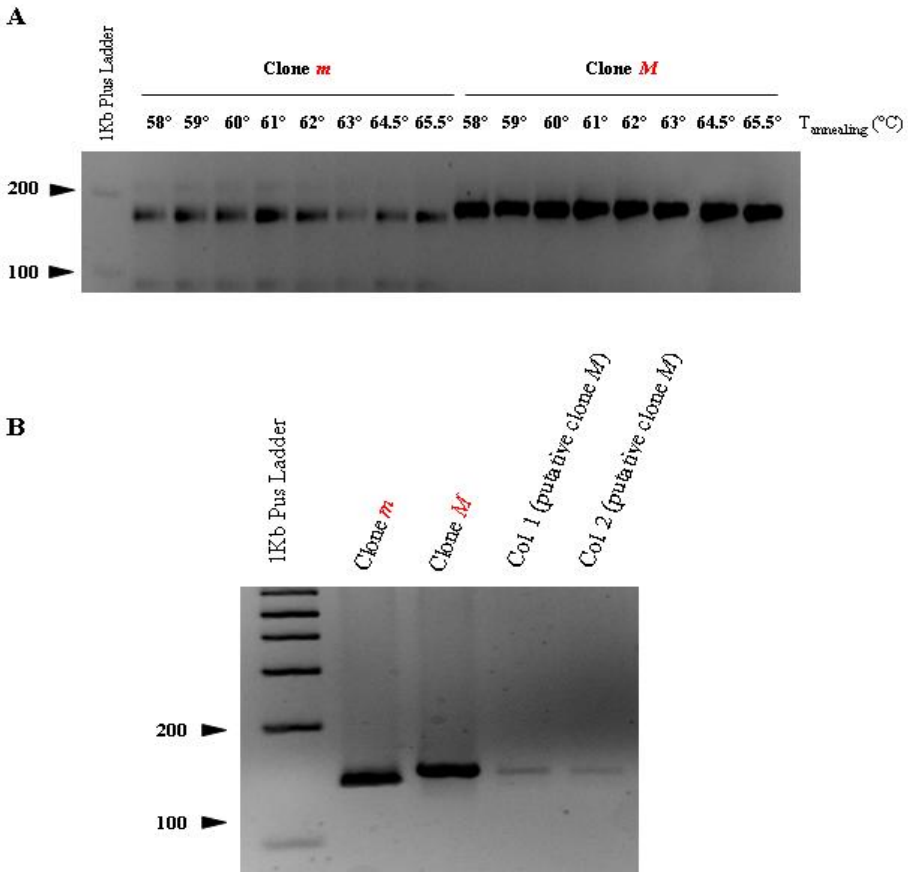
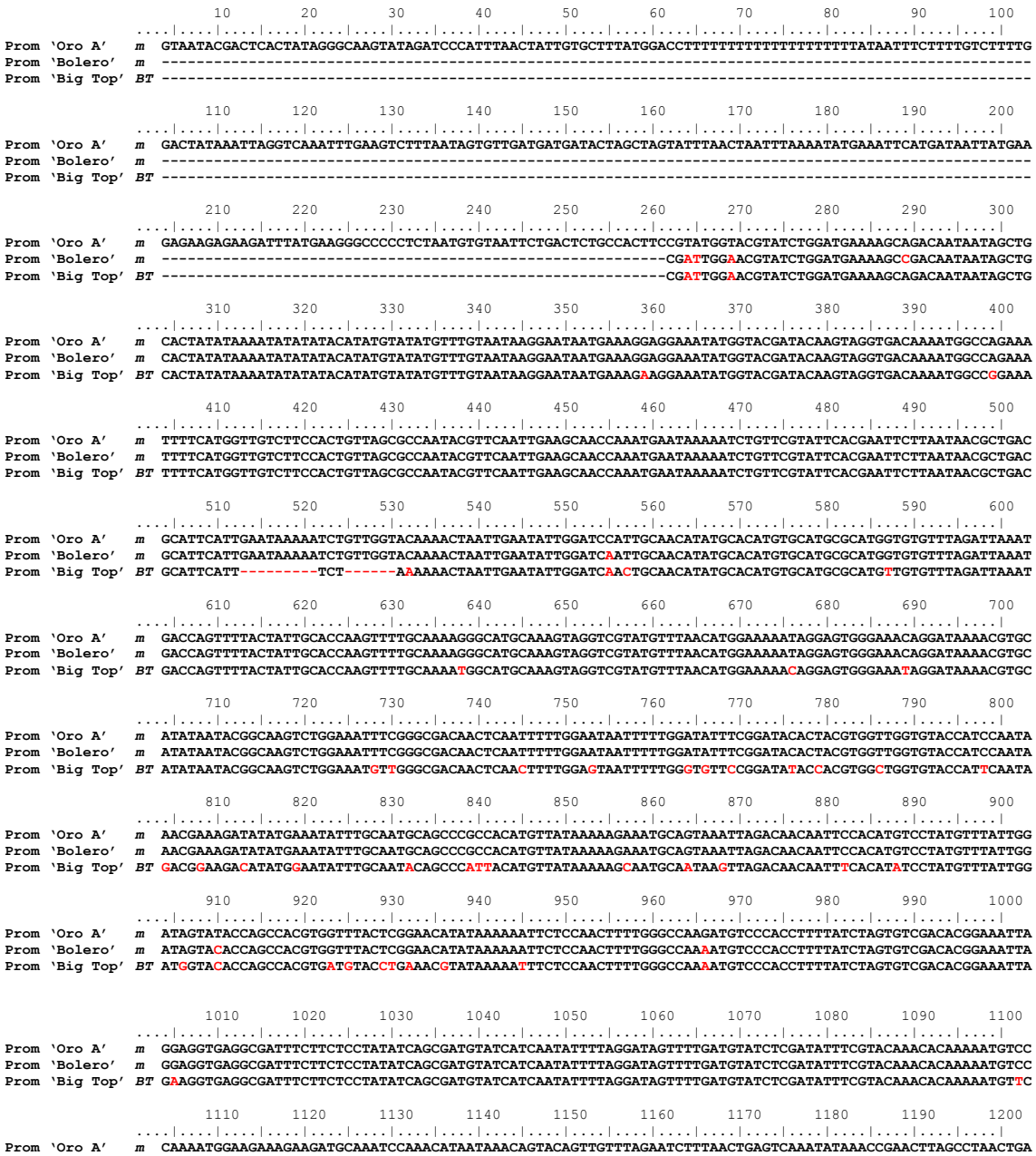


Fig. 3. A: Screening of different annealing temperatures (58°C-65.5°C) on the PAMSA patterns of *m* and *M Pp-endoPG* clones as separated in 3% (w/v) agarose gel. B. Separation pattern in 3% (w/v) agarose gel of the of the products of PAMSA with *m* and *M* clones. One gel representative of three.

Analysis of Pp-endoPG promoter sequences

The isolation strategies adopted allowed to obtain the sequences of the 5' upstream region of the *m Pp-endoPG* clone of both NM 'Oro A' (*m*-O) and M 'Bolero' (*m*-B), of the *M* clone of M 'Bolero' and of the *BT* clone of Slow Melting 'Big Top'. The length of the sequences isolated from the start codon was -1970 bp and -1712 bp, respectively for *m*-O and *m*-B clones, -1712 bp for *M* and -1663 bp for *BT* clones (Fig. 4).

In general, the 5' upstream sequences of the *m* clones of both 'Oro A' and 'Bolero', as well as the 5' upstream sequence of the *BT* clone of 'Big Top', were very similar. The 5' upstream sequence of the clone *m*-B showed the presence of 10 SNPs and two InDels (an insertion of two bases and a deletion of three bases) compared to *m*-O, while the 5' upstream sequence of the clone *BT* showed 64 SNPs and six deletions of different length compared to *m*-O (see Figure 4). Sequence alignment of Figure 5, instead, shows that the 5' upstream region of the *M Pp-endoPG* clone was deeply different than those of the *m* and *BT* clones, sharing with them only the initial 500-bp sequence as from the start codon .


10 20 30 40 50 60 70 80 90 100
Prom 'Oro A' m GTAATACGACTCACTATAGGGCAAGTATAGATCCCATTTAACTATTGTGCTTTATGGACCTTTTTTTTTTTTTTTTTTTTATAATTTCTTTTGTCTTTTG
Prom 'Bolero' m ----------
Prom 'Big Top' BT ----------
110 120 130 140 150 160 170 180 190 200
Prom 'Oro A' m GACTATAAATTAGGTCAAATTTGAAGTCTTTAATAGTGTTGATGATGATACTAGCTAGTATTTAACTAATTTAAAATATGAAATTCATGATAATTATGAA
Prom 'Bolero' m ----------
Prom 'Big Top' BT ----------
210 220 230 240 250 260 270 280 290 300
Prom 'Oro A' m GAGAAGAGAAGATTTATGAAGGGCCCCCTCTAATGTGTAATTCTGACTCTGCCACTTCCGTATGGTACGTATCTGGATGAAAAGCAGACAATAATAGCTG
Prom 'Bolero' m ----------CGATTGGAACGTATCTGGATGAAAAGCCGACAATAATAGCTG
Prom 'Big Top' BT ----------CGATTGGAACGTATCTGGATGAAAAGCAGACAATAATAGCTG
310 320 330 340 350 360 370 380 390 400
Prom 'Oro A' m CACTATATAAAATATATATACATATGTATATGTTTGTAATAAGGAATAATGAAAGGAGGAAATATGGTACGATACAAGTAGGTGACAAAATGGCCAGAAA
Prom 'Bolero' m CACTATATAAAATATATATACATATGTATATGTTTGTAATAAGGAATAATGAAAGGAGGAAATATGGTACGATACAAGTAGGTGACAAAATGGCCAGAAA
Prom 'Big Top' BT CACTATATAAAATATATATACATATGTATATGTTTGTAATAAGGAATAATGAAAGAAGGAAATATGGTACGATACAAGTAGGTGACAAAATGGCCGGAAA
410 420 430 440 450 460 470 480 490 500
Prom 'Oro A' m TTTTCATGGTTGTCTTCCACTGTTAGCGCCAATACGTTCAATTGAAGCAACCAAATGAATAAAAATCTGTTCGTATTCACGAATTCTTAATAACGCTGAC
Prom 'Bolero' m TTTTCATGGTTGTCTTCCACTGTTAGCGCCAATACGTTCAATTGAAGCAACCAAATGAATAAAAATCTGTTCGTATTCACGAATTCTTAATAACGCTGAC
Prom 'Big Top' BT TTTTCATGGTTGTCTTCCACTGTTAGCGCCAATACGTTCAATTGAAGCAACCAAATGAATAAAAATCTGTTCGTATTCACGAATTCTTAATAACGCTGAC
510 520 530 540 550 560 570 580 590 600
Prom 'Oro A' m GCATTCATTGAATAAAAATCTGTTGGTACAAAACTAATTGAATATTGGATCCATTGCAACATATGCACATGTGCATGCGCATGGTGTGTTTAGATTAAAT
Prom 'Bolero' m GCATTCATTGAATAAAAATCTGTTGGTACAAAACTAATTGAATATTGGATCAATTGCAACATATGCACATGTGCATGCGCATGGTGTGTTTAGATTAAAT
Prom 'Big Top' BT GCATTCATT---------TCT------AAAAAACTAATTGAATATTGGATCAACTGCAACATATGCACATGTGCATGCGCATGTTGTGTTTAGATTAAAT
610 620 630 640 650 660 670 680 690 700
Prom 'Oro A' m GACCAGTTTTACTATTGCACCAAGTTTTGCAAAAGGGCATGCAAAGTAGGTCGTATGTTTAACATGGAAAAATAGGAGTGGGAAACAGGATAAAACGTGC
Prom 'Bolero' m GACCAGTTTTACTATTGCACCAAGTTTTGCAAAAGGGCATGCAAAGTAGGTCGTATGTTTAACATGGAAAAATAGGAGTGGGAAACAGGATAAAACGTGC
Prom 'Big Top' BT GACCAGTTTTACTATTGCACCAAGTTTTGCAAAATGGCATGCAAAGTAGGTCGTATGTTTAACATGGAAAAACAGGAGTGGGAAATAGGATAAAACGTGC
710 720 730 740 750 760 770 780 790 800
Prom 'Oro A' m ATATAATACGGCAAGTCTGGAAATTTCGGGCGACAACTCAATTTTTGGAATAATTTTTGGATATTTCGGATACACTACGTGGTTGGTGTACCATCCAATA
Prom 'Bolero' m ATATAATACGGCAAGTCTGGAAATTTCGGGCGACAACTCAATTTTTGGAATAATTTTTGGATATTTCGGATACACTACGTGGTTGGTGTACCATCCAATA
Prom 'Big Top' BT ATATAATACGGCAAGTCTGGAAATGTTGGGCGACAACTCAACTTTTGGAGTAATTTTTGGGTGTTCCGGATATACCACGTGGCTGGTGTACCATTCAATA
810 820 830 840 850 860 870 880 890 900
Prom 'Oro A' m AACGAAAGATATATGAAATATTTGCAATGCAGCCCGCCACATGTTATAAAAAGAAATGCAGTAAATTAGACAACAATTCCACATGTCCTATGTTTATTGG
Prom 'Bolero' m AACGAAAGATATATGAAATATTTGCAATGCAGCCCGCCACATGTTATAAAAAGAAATGCAGTAAATTAGACAACAATTCCACATGTCCTATGTTTATTGG
Prom 'Big Top' BT GACGGAAGACATATGGAATATTTGCAATACAGCCCATTACATGTTATAAAAAGCAATGCAATAAGTTAGACAACAATTTCACATATCCTATGTTTATTGG
910 920 930 940 950 960 970 980 990 1000
Prom 'Oro A' m ATAGTATACCAGCCACGTGGTTTACTCGGAACATATAAAAAATTCTCCAACTTTTGGGCCAAGATGTCCCACCTTTTATCTAGTGTCGACACGGAAATTA
Prom 'Bolero' m ATAGTACCAGCCACGTGGTTTACTCGGAACATATAAAAAATTCTCCAACTTTTGGGCCAAAATGTCCCACCTTTTATCTAGTGTCGACACGGAAATTA
Prom 'Big Top' BT ATGGTACCAGCCACGTGATGTACCTGAAACGTATAAAAATTTCTCCAACTTTTGGGCCAAAATGTCCCACCTTTTATCTAGTGTCGACACGGAAATTA
1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
Prom 'Oro A' m GGAGGTGAGGCGATTTCTTCTCCTATATCAGCGATGTATCATCAATATTTTAGGATAGTTTTGATGTATCTCGATATTTCGTACAAACACAAAAATGTCC
Prom 'Bolero' m GGAGGTGAGGCGATTTCTTCTCCTATATCAGCGATGTATCATCAATATTTTAGGATAGTTTTGATGTATCTCGATATTTCGTACAAACACAAAAATGTCC
Prom 'Big Top' BT GAAGGTGAGGCGATTTCTTCTCCTATATCAGCGATGTATCATCAATATTTTAGGATAGTTTTGATGTATCTCGATATTTCGTACAAACACAAAAATGTTC
1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
Prom 'Oro A' m CAAAATGGAAGAAAGAAGATGCAAATCCAAACATAATAAACAGTACAGTTGTTTAGAATCTTTAACTGAGTCAAATATAAACCGAACTTAGCCTAACTGA

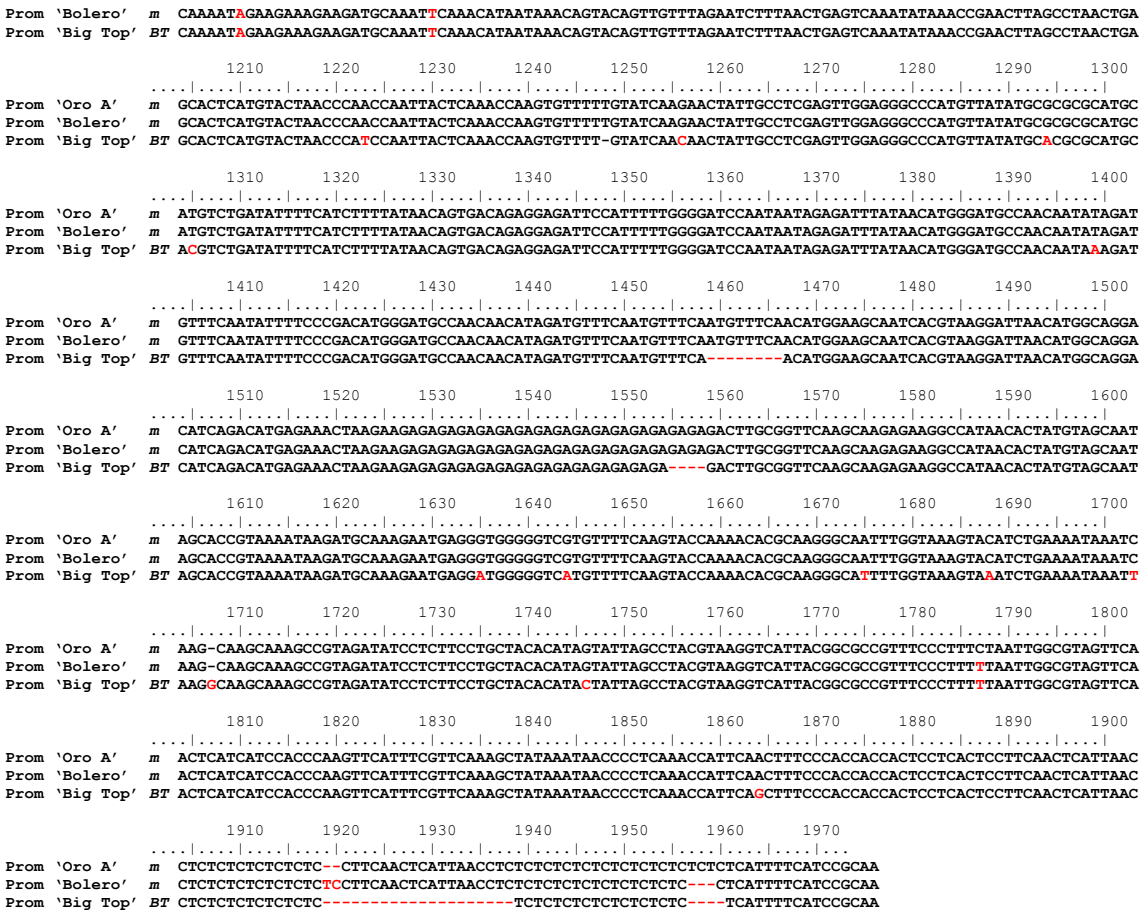


Fig. 4. Sequence alignment of *Pp-endoPG* gene promoter sequences of clones *m*-O ('Oro A'), *m*-B ('Bolero') and *BT* ('Big Top'). In red SNPs and InDels.

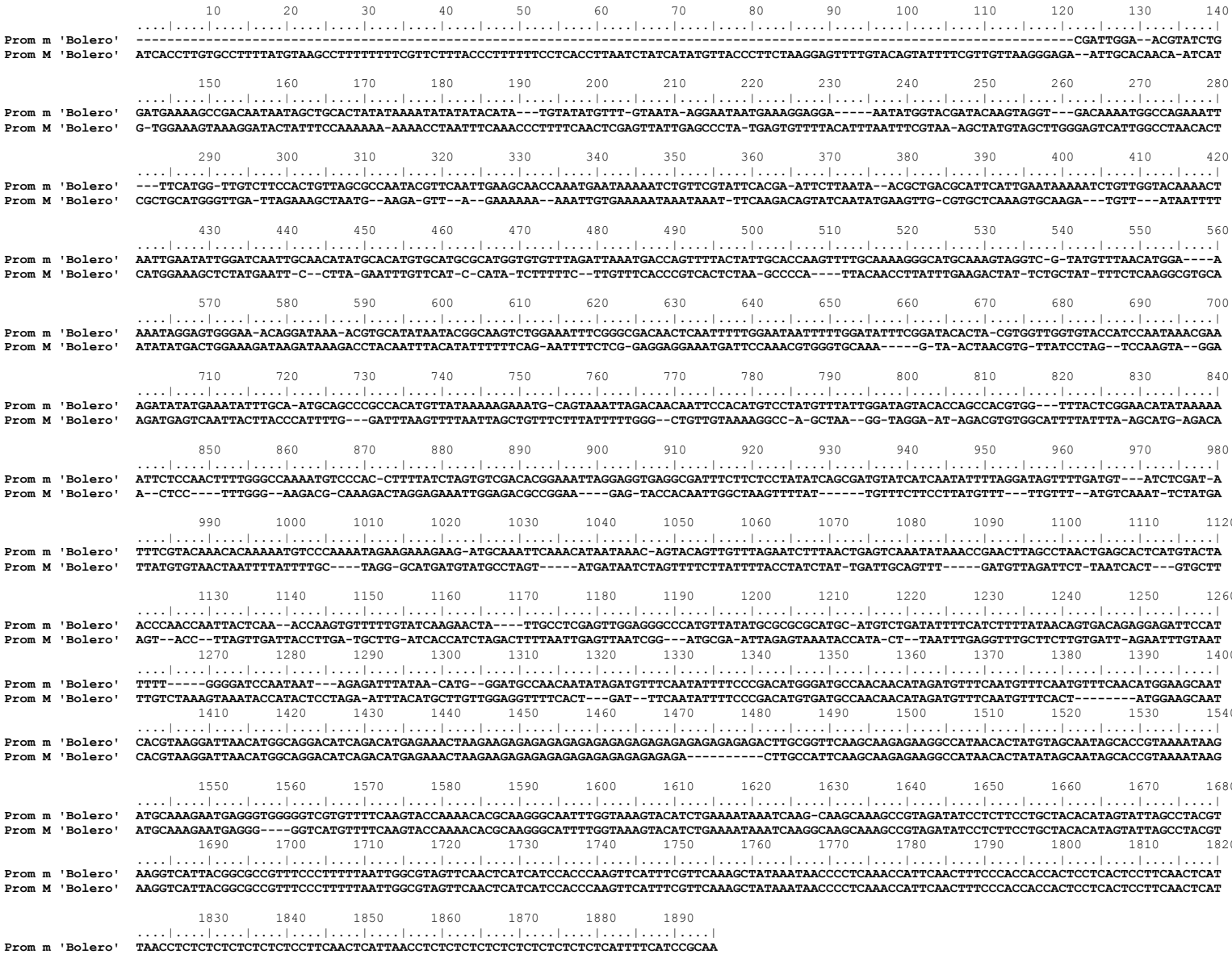


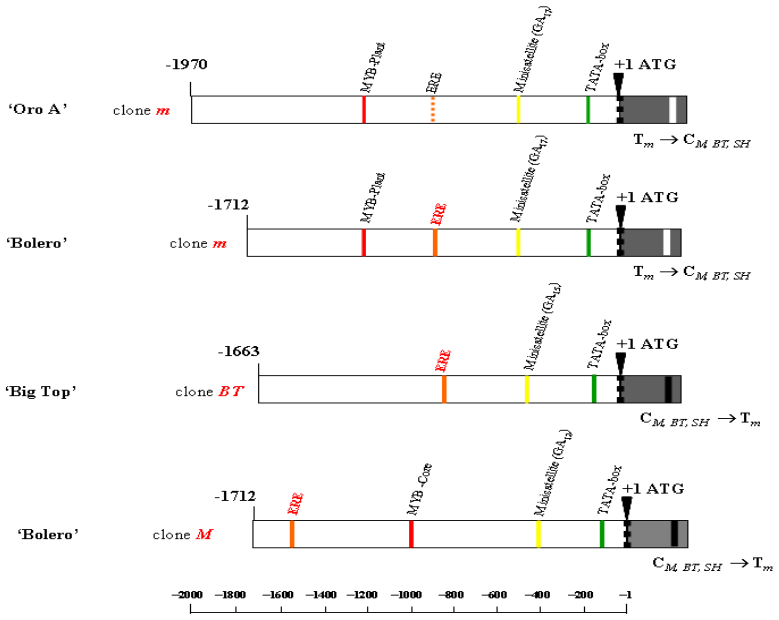
Fig. 5. Sequence alignment of *Pp-endoPG* gene promoter sequences of clones *m*-B and *M* (‘Bolero’).

The sequences similarity of the 5' upstream regions of the *m*-O, *m*-B and *BT Pp-endoPG* clones could suggest a different regulation of the *Pp-endoPG* gene its expression in different genotypes as far as it concerns epigenetic regulation of gene expression which is increasingly recognised as an essential mechanism for the co-ordination of genome activity and, as a consequence, the regulation of many aspects linked to development or response to the environment (Seymour *et al*., 2008).

Epigenetic modifications have deep effects on genome activity (Zhang *et al*., 2008). In eukaryotes, the methylation of cytosine residues in DNA is a common phenomenon (Bird, 1980), that serves as a mechanism to suppress mobile elements (Kato *et al*., 2003) and other nuclear processes such as transcription and recombination (Buard and de Massy, 2007). Evidence about DNA methylation and its effects in the regulation of gene expression has been accumulated by the study of several epigenetic mutants, or epimutants, such as *fwa* (Soppe *et al*., 2000) and *superman* (Jacobsen and Meyerowitz, 1997) in *A. thaliana*. In these epimutants, the affected genes exhibit unusual DNA methylation within promoter regions. In tomato *Colorless Non Ripening* (*Cnr*) mutant fruit the mutation results in absence of fruit pigmentation with a substantial loss of cell-to-cell adhesion (Manning *et al*., 2006). *Cnr* is one of a few well-characterized natural and stably inherited epigenetic mutations. In this mutation, a region of the *Le-SPL Cnr* promoter shows a high degree of methylation which is linked to a suppression of gene expression. In wild-type fruit with *Cnr* phenotype, the promoter contains only few methylated bases or can even show demethylation during development, depending on the cultivar (Seymour *et al*., 2008).

In the present work, the sequences of the 5' upstream region of different *Pp-endoPG* clones (*m-O, m-B, BT, M*) were analyzed by PLACE, a *cis*-acting regulatory element prediction program (Higo *et al*., 1999). In Figure 4 is reported a schematic structure of these sequences with relative lengths and putative *cis*-acting elements found once in a specific position by PLACE (see also Table 1).

A



B

Colour	Motif	Sequence	Putative function	Position	References
	TATA-Box	TATAAAT	Core promoter element of transcription start	-136 (*m*-O, *m*-B) -114 (*BT*) -108 (*M*)	Grace *et al*., 2004
	GAGA Element	$GA_{(n)}$	Microsatellite	-422 (*M*, n=12, *BT*, n=15) -447 (*m*-O, n=17; *m*-B, n=17,)	Espley *et al*., 2009
	ERE Element	AWTTCAAA	Ethylene responsive element	-813 (*BT*) -847 (*m*-B) -1536 (*M*)	Montgomery *et al*. 1993
	MYB-Plant	MACCWAMC	Consensus sequence related to phenylpropanoid metabolism	-1189 (*m*-O) -1190 (*m*-B)	Merali *et al*., 2005
	MYB-Core	CNGTTR	Binding site related to water stress	-1003 (*M*)	Solano *et al*., 1995

Fig. 6. A: Schematic structure of the 5' upstream regions of the *Pp-endo-PG* clones. The promoter and the exonic sequences are represented as white and grey boxes, respectively. Black dotted lines indicate the location of the translation start codon. Black lines within the exon indicate the SNP_{146} (T in *m* and C in *M*, *BT*, *SH Pp-endo-PG* clones). The predicted motifs in the 5' upstream region are indicated with lines of different colors. B: Main regulatory motifs within the 5' upstream regions of the *Pp-endo-P*G gene clones *m* of 'Oro A' and 'Bolero', *BT* of 'Big Top' and *M* of 'Bolero'.

In Table 1 are listed other important *cis*- acting elements found in several positions (Higo *et al*., 1999).

Table 1. Other important regulatory motifs found, using the PLACE software, within the 5' upstream regions of the *Endo-P*G gene clones *m* of 'Oro A' and 'Bolero', *BT* of 'Big Top' and *M* of 'Bolero'.

Motif and sequence	Putative function and literature references	Strand	*m*-'Oro A' (bp)	*m*-'Bolero' (bp)	*BT* 'Big Top' (bp)	*M* 'Bolero' (bp)
ABRE (ACGTG)	*Cis*-acting element involved in ABA responsiveness (Nakashima *et al*., 2006)	+	(695, 777, 915)	(438, 520, 658)	(422, 504, 642)	(601)
		-	(914, 1477)	(657, 1220)	(503,641,1026,1195)	(621, 742,1258)
CAAT	Common *cis*-acting element in promoter and enhancer regions (Yin *et al*., 2008)	+	(289, 430, 439, 739, 796, 825, 874, 1043, 1222, 1359, 1391, 1405, 1446, 1454, 1473, 1597, 1670)	(32, 173, 182, 294,482, 539, 568, 617,786, 965, 1102, 1134, 1148, 1189, 1197, 1216, 1340, 1413)	(31,172, 181, 523, 552, 581, 586, 601, 770, 949, 1085, 1117, 1131, 1172, 1191, 1311)	(131, 353, 518, 552, 654, 828, 1194, 1235, 1254, 1368)
		-	(441, 507, 537, 544, 553, 614, 896, 1258, 1786)	(3, 184, 250, 280,287, 296, 357, 639,1001, 1529)	(3, 183, 264, 271, 341, 623, 984, 1501)	(122,203,260,320, 804,830,844,965,969,1054,1554)
GATA	Motif involved in light-dependent and nitrate-dependent control of transcription (Reyes *et al*., 2004)	+	(147, 189, 371, 689, 760, 769, 808, 900, 1054, 1073, 1307, 1718)	(114, 432, 503, 512, 551, 643, 797, 816, 1050, 1461)	(113,416,496,781, 800,1033,1433)	(151,535,540,935, 1486)
		-	(270, 977, 1026,1037, 1067, 1247, 1720)	(12, 720, 769, 780,810, 990, 1463)	(12, 611, 704, 753, 764, 794, 973, 1435)	(66, 350, 436,627, 960, 1488)
GT1-Consensus (GRWAAW)	Light-responsive element (Ayadi *et al*., 2004)	+	(189, 358 ,666,667, 689, 719, 993, 1676, 1690)	(101, 409, 410, 432, 462, 736, 1419, 1433)	(100, 138,393,394, 408, 416, 446, 720, 1390, 1404)	(311, 325, 540, 587, 935, 1443, 1457)
		-	(400,1310,1409,1957,975,1410)	(143, 718, 053,1152, 1153, 1700)	(142, 702, 1036,1135, 1136, 1650)	(30,40,48,49,102, 157,391,440,502, 564,573,954,1019, 1198,1199)
GT1-Core (GGTTAA)	Light-responsive element (Ayadi *et al*., 2004)	+				
		-	(1895, 1925)	(1638, 1690)		
MYB-Core (CNGTTR)	Binding site related to water stress (Solano *et al*., 1995)	+	(420, 520, 1146)	(163, 263, 899)	(162, 873)	
		-	(1163, 1194, 1324)	(906, 937, 1067)	(278,890, 1050)	
WRKY (TGAC)	*Cis*-acting element involved in gibberellin responsiveness (Zhang *et al*., 2004)	+	(244, 383, 497, 600, 1330)	(126, 240, 343,1073)	(125, 239, 327,1056)	(525)
		-	(114, 1170, 1759)	(913, 1502)	(897, 1352, 1474)	(257,457,652,872, 1405,527)

Sequence analysis detected the presence of a putative *cis*-acting element characteristic of promoter regions, i.e. a TATA-box, in a specific site within the 5' upstream region of the *endoPG* gene clones (-136 bp from the ATG start codon in *m*-O and *m*-B, -114 bp in *BT* and -108 bp in *M*, Fig. 4). Several CAAT-boxes were also found, in different number and different positions in the different clones. Potential regulatory elements associated with hormone-, light- and stress-related responses were also found in the promoter regions. The putative hormone-responsive elements identified included an ABRE (ABA Responsive Element) and a WRKY, *cis*-acting elements involved in the responsiveness to abscisic acid (ABA) and gibberellins, respectively (Nakashima *et al*., 2006; Zhang *et al*., 2004).

The plant hormone ABA not only plays a regulatory role in plant growth and development, seed dormancy, and adaptation of plants to stress conditions, but also induces a pattern of changes similar to ethylene at late stages of fruit development. Some authors considered that ABA had a crucial role, perhaps even more crucial role than that of ethylene, in fruit maturation and senescence (Giovannoni, 2001, 2004; Rodrigo *et al.*, 2003); while WRKY genes have been shown to be involved in biotic (bacterial and fungal diseases) and abiotic (wounding and freezing) stresses, as well as in anthocyanin biosynthesis, senescence, and GA responsiveness (Rushton *et al*., 1995; Zhang *et al*., 2004). The *cis*-acting elements involved in light responses include a GT1-Consensus and a GT1-Core element present in several positions in the promoter regions of all the *Pp-endoPG* clones. In addition, these promoter sequences contain elements related to stress responses, included a MYB-Core element, involved in water stress response: this element is found in the promoter region of the *Pp-endoPG* clone *M* of 'Bolero' at -1003 bp from the start codon, while in several positions in the other promoters . A MYB-Plant element was also found in the promoter region of the *m*-O and *m*-B *Pp-endoPG* clones, at -1189 and -1190 bp from the start codon, respectively.

The presence of this plethora of *cis*-acting elements in the promoter region of the *Pp-endoPG* clones may indicate that expression of the *Pp-endoPG* gene could be subjected to regulation by physiological (hormone) and environmental (light and stress) factors as reported for other ripening-related genes (Rasori *et al*., 2003; Chang *et al*., 2004; Bustamante *et al*., 2009).

In our opinion, the most prominent elements found by PLACE were a microsatellite and an ethylene responsive element (ERE).

A microsatellite was present in the promoter regions of all the *Pp-endoPG* clones. It consisted of simple sequences of repeat units of GA. In *m*-O and *m*-B the microsatellite was found at -447 bp (GA=17), while in *BT* (GA=15) and *M* (GA=12) is was found at -422 bp from the start codon (see Fig.4).

A high rate of microsatellite polymorphisms has been associated with various human pathologies and the heritability of diseases (Goellner *et al*., 1997). Hypermutable minisatellites in promoter sequences have been shown to affect transcriptional regulation in humans (Kennedy *et al*., 1995). In plants, although microsatellites have been used for various evolutionary studies (Sykorova *et al*., 2006) and mapping (Barreneche *et al*., 1998), there is little evidence to date of microsatellite-induced changes in transcriptional regulation. Espley *et al*. (2009) demonstrated that the presence of a microsatellite alters the transcriptional levels of *MYB10* gene. Moreover, early studies on homopurine and homopyrimidine (GA)-(TC) dinucleotide repeat showed that they were able to form triple helices in vitro that have the property to block the synthesis of DNA in a length-dependent manner (Baran *et al*., 1996). Peace and co-workers (2007) found a microsatellite within

the 5' non coding region of peach EST for endoPG as the most polymorphic region of the gene, allowing a detailed examination of endoPG genotypic diversity in *Prunus*.

This microsatellite characteristic could suggest that the different length of microsatellites found in the *m*-O, *m*-B, *BT* and *M Pp-endoPG* clones (17, 17, 15 and 12 units, respectively) could be related to the control of transcriptional regulation of the *Pp-endoPG* gene. As a matter of fact, NM 'Oro A' fruit present low levels of transcripts of the *Pp-endoPG* clone *m* (Morgutti *et al*., 2006), whose promoter had the highest number (17) of repeat units of GA. Such repeat units in *BT* (15) and in *M* (12) were less numerous; this result might be interpreted as consistent with the higher amounts of *Pp*-endoPG transcripts in 'Bolero' and 'Big Top' fruit.

The presence of putative Ethylene Responsive Element (ERE) motifs in the promoter regions of the *Pp-endoPG* clones *m-B* (at -847 bp), *BT* (at -813 bp) and *M* (at -1536 bp), but not in the promoter region of the *Pp-endoPG* clone *m-O* is consistent with literature reports on *endoPG* gene responsiveness to ethylene (Hayama *et al.,* 2006) and may possibly account also for the lower expression of *Pp-endoPG* observed in the high ethylene-producing NM cultivar 'Oro A' (Morgutti et al., 2006). The same ERE element was found in other ripening-related genes and also in genes encoding cell wall-modifying enzymes (Montgomery *et al*., 1993; Chang *et al*., 2007; Bustamante *et al*. 2009).

Electrophoresis Mobility Shift Assay (EMSA)

It is interesting to note that in the promoter region of the *m*-B *Pp-endoPG* clone the ERE is present at -847 bp as from start codon and in the *m*-O clone, at the same position (-847 bp), there is a sequence with a SNP that alters the ethylene binding site (ATCCAAA instead of AWTCAAA). Preliminary studies conducted by gel electrophoresis mobility-shift experiments were performed to determine whether peach fruit sequences, including the ERE binding site, interact specifically with nuclear proteins. In order to preliminarily investigate on this point, M 'Bolero' soft fruit (10 N) have been extracted to obtain a cellular fraction enriched in nuclear proteins. A fragment of the promoter region of the *Pp-endoPG* clones *m*-O and *m*-B which included ERE was amplified from genomic DNA of 'Oro A' and 'Bolero' as described in the scheme of Figure 5A. Both the amplified fragments interacted with nuclear proteins present in the 'Bolero' fruit extract. Figure 5B shows the results of a preliminary gel electrophoresis mobility shift experiment in which *m*-O and *m*-B *Pp-endoPG* promoter fragments were reacted with by the nuclear extract of 'Bolero'. Compared to the run of the *m*-B promoter fragment alone, the run of the *m*-B promoter fragment plus nuclear proteins, which contained the ERE motif, was slowed down to a greater extent than that of the *m*-O

promoter fragment plus nuclear proteins. This result may suggest a higher degree of interaction of the promoter region of clone *m*-B than that of clone *m*-O.

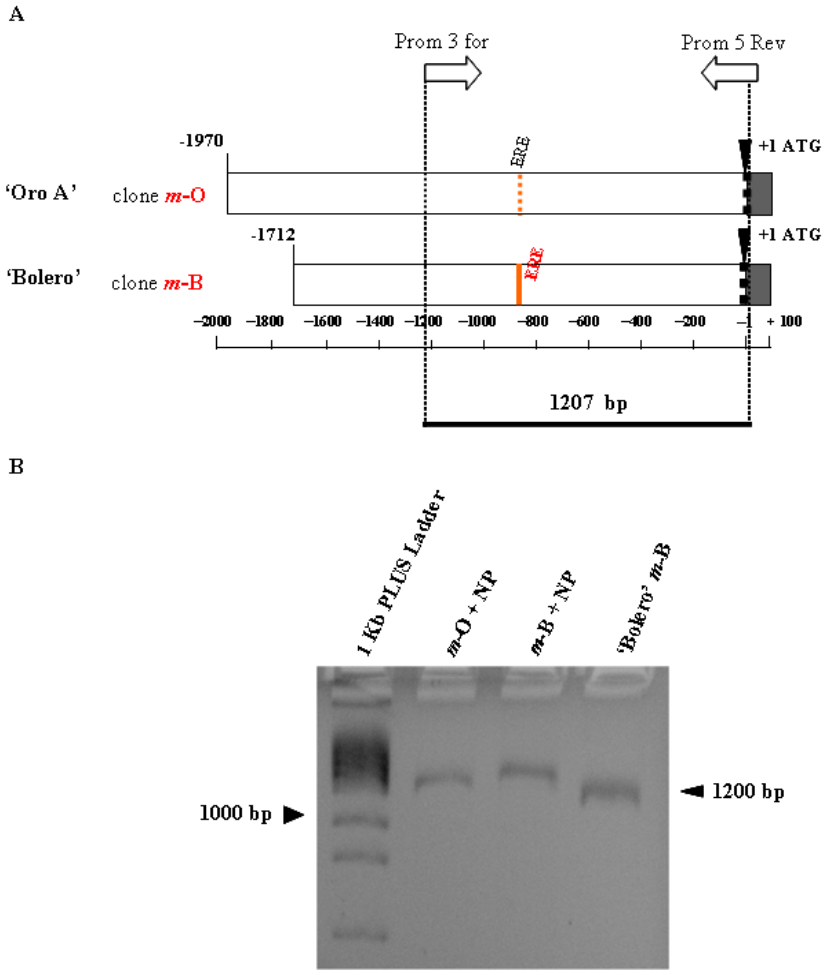


Fig. 7. A: Scheme of the amplification of selected promoter sequences in the Pp-*endoPG* clones of 'Oro A' (*m-O)* and of 'Bolero' (*m-B)* and predicted fragment lengths. B: Gel electrophoresis mobility shift induced by binding of nuclear proteins (NP) from 'Bolero' soft fruit with the 'Oro A' (*m*-O) and 'Bolero' (*m*-B) promoter fragments. In the fourth lane from the left the run of the *m*-B *Pp-endoPG* promoter fragment alone is shown to compare different migration rates.

These preliminary data though needing to be confirmed by further experiments appear consistent with the different expression profiles of the *Pp-endoPG* gene as related to softening behaviour. As a matter of fact, ripe M 'Bolero' and Slow Melting 'Big Top' fruit, present high amounts of *Pp-endoPG* transcripts, parallel to high ethylene evolution (see Chapter 1). These features are consistent with the presence, in the promoter of the *Pp-endoPG* clones *m*-B, *BT* and *M*, of the above highlighted ERE element. On the contrary, NM 'Oro A' fruit show very low accumulation of *Pp-endoPG* transcripts (Morgutti *et al.*, 2006) , even in presence of high amount of ethylene evolution, consistent with absence of any ERE element in the *m*-O *Pp-endoPG* promoter region.

Finally, the identification of SNPs, InDel and microsatellites in the promoter sequences of the *Pp-endoPG* clones seems feasible for the identification of molecular markers useful to screen *Pp-endoPG* clones in peach genotypes with different softening behaviour.

CHAPTER IV

THE BO 950201043 PEACH MUTANT WITH ALTERED FRUIT RIPENING: PRELIMINARY CHARACTERIZATION OF SOME BIOCHEMICAL, PHYSIOLOGICAL AND MOLECULAR TRAITS AND OF FRUIT RESPONSE TO ETHYLENE

Introduction

Ethylene plays a role in many plant developmental processes among which fruit ripening has practical importance to the human diet. In climacteric fruits, an increase in ethylene production is observed before the initiation of ripening and ethylene is a trigger of the ripening process; the ethylene-dependent and -independent pathways coexist to co-ordinate the ripening process. Treatments with inhibitors that block ethylene synthesis or action or manipulation of these processes by natural or induced mutations revealed the essential role of this hormone in regulating fruit ripening (Barry and Giovannoni, 2007 and references therein).

The wide-ranging effects of ethylene, and its easy availability, have with time led to an outburst of research dealing with the ripening of climacteric fruits in general. It was thus possible to understand many molecular details of the ripening process. Nevertheless, although many components of the biosynthesis and signalling pathways are now known, much remains to be learned about the pathways and the complex regulation of proteins involved (Chen *et al*., 2005).

In peach, the ethylene biosynthetic pathway has been studied in detail (Ramina *et al*., 2007), and an increasing body of information is becoming available on ethylene receptors and elements involved in the signal transduction pathway in this fruit species. In peaches the ethylene climacteric at ripening is accompanied by increases in 1-aminocyclopropane-carboxylase (ACC) synthase (*ACS*) and ACC oxidase (*ACO*) transcript accumulation. These genes encode the two key enzymes catalyzing the last steps of the ethylene biosynthetic pathway (Dal Cin *et al*., 2006).

Concerning ethylene perception and signalling, two ethylene receptor genes, *Pp-ETR1* and *Pp-ERS1*, have been isolated in peach showing similar organization to that corresponding genes in *Arabidopsis;* they appear to be induced by ethylene and repressed by 1-methylcyclopropene (1-MCP), an antagonist of ethylene action (Rasori *et al*., 2002). Using transcriptomic approach, Trainotti *et al.* (2006) recently identified one additional ethylene receptors (*Pp-ETR2*) that, more than *Pp-ERS1*, appears to be induced during fruit the transition from pre-climacteric to climacteric stage. The last component of the ethylene receptor family is CTR1, which, like *ETR1* and *ERS1*, acts as a negative regulator of ethylene responses. A decrease in *Pp-CTR1* transcripts accumulation has been observed by Dal Cin *et al*. (2006) in ripening peaches treated with 1-MCP, suggesting that *CTR1* is also ethylene-inducible in this fruit species. EIN2 is the first positive regulator in the ethylene signalling cascade acting downstream from CTR1 (Guo and Ecker, 2003). In peach fruit, an induction of a putative orthologous of *EIN2* was observed during the transition from immature to mature stage (Trainotti *et al*., 2006; Begheldo *et al.*, 2008). Functioning downstream of EIN2 is a multigene family of transcription factors that includes EIN3 and EIN3-like (EIL) proteins (Chen *et*

al., 2005). The EIN3/EIL family is involved in a regulatory cascade and stimulates the transcription of other transcription factors such as Ethylene Responsive Factor (ERF1) (Alonso *et al*., 2003; Fujimoto *et al*., 2000). Even though expression of *ERF*s appears to be regulated not exclusively by ethylene in ripening fruit (Wang *et al.*, 2007), an increasing expression trend of three *ERF*s were observed in Ziliotto *et al*. (2008).

Also the availability of mutants (either natural or induced by genetic transformation) [in tomato, the *Nr* (Wilkinson *et al.*, 1997), *Gr* (Barry *et al.*, 2005; Barry and Giovannoni, 2006) *ACS* (Oeller *et al.*, 1991) and *ACO* (Hamilton *et al.*, 1990)] antisense mutants has helped researchers to understand the mechanisms underlying the ripening process and to confirm the role of ethylene in the regulation of ripening of climacteric fruits. However, the large availability of tomato mutants has also yielded some genetic lines (*nor*, *cnr*, *rin*) whose fruit are unable to ripen even when treated with exogenous ethylene though they do not seem impaired in the hormone signal transduction pathway. The sequence of the *nor* mutant gene has not yet been published but it is has been reported to code for a transcription factor (Adams-Phillips *et al.*, 2004). The mutated *cnr* gene has recently been demonstrated to code for an SBP-box transcription factor where a natural epigenetic mutation has occurred (Manning *et al.*, 2006), while the *RIN* gene has been shown to encode a MADS-box transcription factor (Vrebalov *et al.*, 2002). In other cases, MADS-box encoding genes have been found to be expressed in fleshy fruits although their precise role has not yet been defined (Sung *et al.*, 2000; Boss *et al.*, 2002; Busi *et al.*, 2003; Rosin *et al.*, 2003).

Ripening mutants are present also in other species, but their molecular characterization is more difficult in comparison to tomato for the reduced availability of genomic tools and technical constraints. Considering tree species bearing climacteric fruit, ripening mutants due to disturbance in ethylene physiology have been identified in peach. The Stony Hard (SH) is characterized by lack of ethylene production and firm flesh in mature fruit; the SH trait is controlled by a single recessive gene (*hd*) which is inherited independently of the Melting (M/NM) trait (Yoshida, 1976; Haji *et al*., 2005).

Other interesting ripening mutants in peach have been identified and named 'Slow Ripening'. These mutants were first described in California, USA, in the framework of the Fresno USDA-ARS stone fruit breeding program (Brecht and Kader, 1984). Whereas in commonly grown nectarines fruit ripening is accompanied by changes in colour, firmness, aroma and increased rates of CO_2 and ethylene production, the Slow Ripening mutation described in progenies of 'Fantasia' nectarine hinders completion of ripening. Fruit development is apparently halted before the end of the cell expansion phase (stage III) and the flesh either never softens or softens very slowly, while it keeps a crispy texture. The skin ground colour and flesh are greenish and the flavour is very poor,

despite lower acidity and higher pH and soluble solids (but similar total sugars) than 'Fantasia' (Brecht and Kader, 1984). Ethylene and carbon dioxide production are very low, no aroma is developed and fruit remain firm on the tree even after leaves abscise in autumn. Fruit of this mutant are susceptible to internal breakdown (Brecht *et al*., 1984). After ripening-inducing treatments using propylene gas, fruit eventually become soft and produce ethylene, but maintain poor texture and flavour (Layne and Bassi, 2008). Inheritance of a slow-ripening trait was investigated in segregating progenies of nectarine. Segregation ratios suggested that the trait is controlled by a single and recessive gene (*sr*), whereas 'Fantasia' nectarines are heterozygous (*Sr/sr*) for this trait (Ramming, 1991).

A number of mutants phenotypically resembling the Slow Ripening type ones (BO 95021 series) were obtained also in Italy (prof. D. Bassi, Di.Pro.Ve.). A segregating progeny (BO 95021) was obtained by self-pollination of an F1 plant that was heterozygous for the SR trait. This F1 plant was derived by crossing 'Fantasia' (a normal M flesh nectarine) and BO 7803302 (a SR selection generated by open-pollination of ''Flamekist', another M flesh nectarine that was putatively heterozygous for the SR trait). Several trees of the selfed progeny BO 95021 were obtained. A 3:1 segregation ratio for the SR trait (84 normal ripening genotypes: 23 slow ripening genotypes) was observed in the selfed 'BO 95021' population, supporting the monogenic inheritance of the SR trait (Tataranni *et al.,* 2010). SR-type fruit never attained a normal size (i.e., reduced diameter and weight) nor softened (i.e., a leathery consistency, not able to be analysed using a penetrometer).

The different expression of the softening characteristics may result from the ability of the tissues to respond to ethylene due to some form of signal modulation. Kinase-mediated protein phosphorylation is a common means of signal modulation (Chen *et al.,* 2005). Our previous results indicated that the Ca^{2+}-dependent phosphorylation of a soluble polypeptide (M_r approx. 52 kDa) decreased with ripening in NM and remained constant in M fruits. In SR phenotypes, which neither softened nor produced endoPG, phosphorylation of this polypeptide was not detectable, suggesting that this phenomenon may be involved in the modulation of ethylene signalling and flesh softening (Morgutti *et al.,* 2005). A direct relationship between endoPG expression and ethylene was apparent in SR BO 95021043 fruit. In the SR fruit the lack of ethylene biosynthesis seemed to affect the overall fruit developmental process. In these fruit the effect of exogenous ethylene on the phosphorylation of the 52 kDa soluble polypeptide and induction of *endoPG* transcription could suggest a parallelism between protein kinase activity and ethylene-sensitive events involved in softening (Ghiani *et al.,* 2007).

So, even if in general terms the commercial value of these genotypes appears to be obviously limited because of their poor organoleptic qualities, hard texture even after a ripening

period, and their susceptibility to internal breakdown. Slow Ripening mutants constitute an experimental material useful for gaining a better understanding of the events involved in the regulation of peach fruit development and ripening and of the role of ethylene in this process and, in particular, in softening.

In the present study we have conducted a preliminary characterization of some physiological and biochemical traits of fruit of one (BO 95021 043) of the selections available from the BO 95021 progeny, treated or not in the postharvest period with ethylene or with its antagonist, 1-methyl-1-cyclo-propene (1-MCP). The parameters considered were: a) the evolution in postharvest of flesh firmness changes, total soluble solids contents, colour index changes and ethylene production; b) the levels of expression (transcript accumulation) of a few genes involved in ethylene biosynthesis (*Pp-ACO1* and *Pp-ACS1*), perception/signal transduction (*Pp-ETR2, Pp-ERS1* and *Pp-CTR1*) and cell wall degradation/flesh softening (*Pp-EndoPG* and *Pp-EXP3*). A molecular analysis was also performed on the ethylene-controlled *Pp-endoPG* gene, whose product, endoPG, is a cell wall-polysaccharide- hydrolyzing enzyme known to play a key role in the softening process. In the search of possible differences able to explain the peculiar softening pattern of SR fruit, the structure of the *Pp-endoPG* gene in SR BO 92051 043 has been compared with those of NM 'Oro A', M 'Bolero', Slow Melting 'Big Top', and SH 'Ghiaccio' and 'Yumyeong' *Pp-endoPG* clones.

Materials and methods

Plant material

The plant material came from the Experimental Orchard of the University of Bologna 'Azienda Agricola Zabina' of Castel San Pietro (BO) ('Oro A', 'Bolero', 'Big Top', 'Ghiaccio', 'D-4162' and 'Yumyeong') or from the Azienda Agricola of the University of Milan "Francesco Dotti" (Arcagna, LO) and was kindly made available by Professor Bassi (Di.Pro.Ve.). Peach trees were grown under integrated pest management growing systems.

Experiments used fruit and leaves from different cultivars and selections. In particular, the following have been used:

- Fruit and leaves of the peach mutant selection SR BO 95021 043 F_2 of self pollination of an F1 plant that was heterozygous for the SR trait. This F1 plant was derived by crossing 'Fantasia' (a normal M flesh nectarine) and BO 7803302 (a SR selection generated by open-pollination of 'Flamekist', another M flesh nectarine that was putatively heterozygous for the SR trait).
- Leaves of NM 'Oro A' (open pollination of 'Diamante'), MF 'Bolero' (from 'Cresthaven' × 'Flamecrest'), Slow Melting 'Big Top' (F. Zaiger, Zaiger's Genetics Inc., Modesto, CA), selection 193 Q XXVII 111 ('Ghiaccio' series, derived from self-pollination of 'Yumyeong'; Nicotra *et al.,* 2002) (SH) 'D-4162' and 'Yumyeong'.

Sampling of plant material and determination of fruit epicarp colour, flesh firmness, total soluble solids contents (SSC), colour index and ethylene evolution

Leaves: from all selections, during the growing season (before June), young leaf samples were collected. The samples were directly placed in liquid N_2 and stored at -80° C for subsequent genomic analyses.

Fruit: experiments were conducted on fruit from two growing seasons (2007 and 2009). Fruit were collected in a single date (15 September 2007 and 4 October 2009). Epicarp colour parameters were measured at two different locations from low or no-blush areas by using a Minolta Chromameter CR-200 reflectance colorimeter (Minolta Co., Osaka, Japan; Robertson et al., 1990). The increase in the Minolta a* value, which records the degree of green to red pigmentation, was taken as representative of advancement of fruit development. Fruit within each class were weighed and their flesh firmness (Newton, N) was measured after removing a small disc of skin from each side of the fruit, using a penetrometer (Effegi, Milan, Italy) with an 8 mm probe. At the same time,

samples of juice pressed from each fruit were taken and assessed for SSC using a hand refractometer (N1, Atago Co. Ltd., Tokyo, Japan).

Epicarp and stone were removed and pooled mesocarp samples from fruit of known firmness were snap-frozen in liquid N_2 and stored at -80 °C.

Basal ethylene evolution

Immediately after harvest, five whole, healthy fruits were placed individually in 1.1-L glass jars and kept in a thermoregulated (20±1 °C) chamber with 95% relative humidity. For ethylene analysis (Dani 3800 gas chromatograph, Dani Co., Cologno Monzese, Italy), gas samples (1 mL) were taken every 24 h from the headspace of the jars which had been hermetically closed for 1 h. Ethylene evolution by fruit was monitored for 5-8 days after harvest (DAH).

Ethylene and 1-MCP treatments

Fruit were divided into two homogeneous lots on the basis of fresh weight and placed for 24 h at 20±1°C in air-tight containers (50 L volume) in the presence of air (controls) or 5 ppm 1-MCP (see below). Thereafter, 30 fruit from each treatment were transferred into glass desiccators (total volume ≈ 0.013 m^3) in a thermoregulated (20±1 °C) chamber and flushed (≈ 0.1 m^3 h^{-1}) with humidified air or 100 mL m^{-3} ethylene for the desired additional period.

At the desired intervals a few fruit were assessed for ripening parameters and pooled mesocarp samples were taken and frozen in liquid N_2; other fruit were used for the measurement of ethylene evolution.

1-MCP treatment

1-methylcyclopropene (1-MCP; SmartFresh™) was kindly supplied by AgroFresh Inc. (Rohm & Haas Italia srl., Mozzate) as a powder containing the active principle complexed with cyclodextrins. 1-MCP gas (5 ppm) was generated, following the manufacturer's instructions, by thoroughly dissolving, in a 50-ml flask, 0.4 g of powder in 5 ml of ddH_2O. The flask, hermetically closed with a rubber stopper, was placed on the bottom of the 50-L plastic tank already containing the fruit (60-70 fruit per tank) and quickly opened. The container was then closed immediately. The treatment lasted for 1 d, after which the container was opened under a chemical fume hood and fruit where transferred into the desired experimental conditions (see above). For the controls, the same number of fruit was placed in a tank for 1 d in the absence of 1-MCP.

Extraction of genomic DNA

Genomic DNA was obtained following the protocol of Geuna *et al*. (2004) with slight modifications (for more details see Materials and Methods of Chapter 2).

Cleaved Amplified Polymorphic Sequence (CAPS) analysis

As described in the literature for the analysis by CAPS (Cleaved Amplified Polymorphic Sequences; Lukowitz *et al*., 2000) markers, genomic DNA extracted from leaves of SR BO 92051043 selection was used as template in PCR reactions with proper primer pairs designed to amplify internal regions of *endoPG* gene which included SNPs determining polymorphic restriction sites.

The PCR reaction was performed in 25 µl total volume with 20 ng DNA, 0.3 mM dNTPs, 0.3 µM/each of primer pairs, 1× Reaction Buffer GoTaq®, 1 U Go Taq® DNA Polymerase (Promega) and ddH_2O. PCR conditions were 1 cycle at 94°C for 2 min; 35 cycles: 94°C for 45 min, 62°C for 45 min., 72°C for 1 min.; 1 cycle at 72°C for 2 min.

The amplification reactions were followed by restriction of the fragments obtained. The reaction was conducted at 37°C for 90 min in 15 µl total volume with 10 µl of the amplification products, 2.85 µl of sterile distilled H_2O, 1.5 µl of Buffer (1×), 0.15 µl of BSA (10 mg/ml) and 0.5 µl restriction enzyme.

SNP	Primer Pair	Sequence	Fragment length	Endonuclease
At 390 bp $G_{m,M} \rightarrow T_{SH,BT}$	Hd_{For} Hd_{Rev}	5'-GCCATAGCCACTCCAGTCAC-3' 5'-CCACAAGCAACGCCTTCTATCC-3'	911 bp	*BtsI*, NEB
At 1310 bp $G_{M} \rightarrow C_{m/SH/BigTop}$	MF_NMF_{For} MN_FMF_{Rev}	5'-AGGCGTTGCTTGTGGACCTG-3' 5'-GGCTAAGCTACGATGAAGTC-3'	934 bp	*BsrSI*, Promega

The products generated by amplification PCR and digestion were separated by electrophoresis on agarose gel 3% (w/ v) and visualized, at the end of the reaction, using a UV transilluminator.

RNA extraction

Total RNA was obtained from SR BO 92051043 fruit following the protocol of Dal Cin *et al*. (2005).

Synthesis of cDNA

The RNA extracted was treated with deoxyribonuclease I (DNase I, Amp Grade, Invitrogen), following the manufacturer's protocol, and purified by extraction with phenol–chloroform (3:1). RNA was then precipitated with 1 volume of 2-propanol and 1/10 volume of 3 M NaAc at pH 5.2, incubated for 1 h at –80° C, and centrifuged at 18,000 *g* at 4° C for 30 min. Pellets were washed with 70% (v/v) ethanol, air dried, and resuspended in 50 µL of ddH_2O.

RNA was used as template in reverse transcription reaction in order to obtain the different cDNAs. The reaction was performed by 'SuperScript® VILO cDNA Synthesis Kit' (Invitrogen) following the manufacturer's protocol.

Semiquantitative RT-PCR

The SR BO 92051043 cDNAs obtained from mesocarp fruit were used for semi-quantitative PCR. The PCR was performed in 25 µl with DNA (1 µl), dNTPs (0.3 mM), primer pairs (0.3 µM of each), Reaction Buffer GoTaq® (1×), Go Taq® DNA Polymerase (1 U, Promega) and ddH_2O. The PCR conditions were 1 cycle at 94° C for 3 min; n cycles at 94° C for 25 sec, 60° C for 45 sec, 72° C for 1 min; 1 cycle at 72° C for 2 min. The number of PCR cycles in the linear range of DNA amplification for each gene and designed gene-specific primers were

Gene	Primer pair	Number of cycles
Pp-ACO1	ACO_{IntFOR} 5'-GGAAAGCACCTTCTACTTGCG-3' ACO_{IntREV} 5'-GTGCCAAAAGTTGGTCCATT-3'	33
Pp-ACS1	ACS_{IntFOR} 5'-ATGAAGAGTTTGTGGCAAGG-3' ACS_{IntREV} 5'-TCACACGCCAAAGCACCATC-3'	33
Pp-ERS1	ERS_{FOR} 5'-ATGGATTCCTGTGATTGCATAG-3 ERS_{IntREV} 5'-CCTCATCCCATCACTCATTACC-3'	28
Pp-ETR1	$ETR2_{FOR}$ 5'-TCCTCCCTGGTGGACATC-3' $ETR2_{IntREV}$ 5'-ATCACCTGAAACACCCTTC-3'	33
Pp-CTR1	$CTR1_{FOR}$ 5'-GGCACTGAGGTTGCTGTG-3' $CTR1_{IntREV}$ 5'-CTTCTCATCCAATGCCTCC-3	33
Pp-EndoPG	$ENDO_{DIR}$ 5'-ATGGCGAACCGTAGAAGCCTCT-3' $PG1_{REV}$ 5'-CCCACTGAACACCACATCG-3'	28

Gene	Primer pair	Number of cycles
Pp-EXP3	Exp3-5_{DIR} 5'-TCACTATCAACGGCTTCCG-3' Exp3-1_{REV} 5'-TGCCTCTGACCCTGAAGGAC-3'	28
Pp-18s rRNA	$18SrRNA_F$:5'-TCAGACTGTGAAACTGCGAATGGCTC3' $18SrRNA_{R1}$: 5'-TATTTGAATGATGCGTCGCC-3'	22

The PCR-amplified products were resolved in 1% (w/v) agarose gel.

Northern blotting

20 µg of total RNA extracted from mesocarp fruit were denatured at 55°C for 15 min with 20 µL of formamide, 6 µL of formaldehyde, 4 µL of RNA gel buffer 10 × [0.2 M 3-(N-morpholino) propanesulfonic acid (MOPS), 50 mM Na-Acetate, pH 7, 5 mM EDTA], 2 µL loading buffer 10 × (0.1% w/v xilene cyanol, 1 mM EDTA, 1 mM glycerol, 0.1% w/v bromophenol blue) and ddH_2O up to a final volume of 42 µL. After incubation, samples were fractionated in 2% (w/v) agarose gel in RNA gel buffer 1 ×, with 0.6 M formaldehyde in order to maintain the denaturating condition. The gel runs were conducted at 100 V for 2 h. After the run, gels were washed twice (30 min each) with sterile DEPC H_2O, stained in 10 mM NaOH, 10 mM NaCl, 5 µg mL^{-1} ethidium bromide and finally destained (30 min) in 0.1 M Tris- HCl (pH 7.4). Fractionated RNA was transferred onto Hybond-n membrane (GE Healthcare Europe) by capillary transfer with 10 × standard saline citrate (SSC: 0.15 M NaCl, 15 mM Na-citrate, pH 7) and fixed at 80° C for 2 h. The membrane was pre-hybridized at 65° C for 1 h in a solution containing 2 g dextran-solfate dissolved in 6 mL of SCP buffer 20 × [0.6 M Na_2HPO_4, pH 6, 20 mM EDTA, 2 M NaCl], 20% (w/v) SLS (n-lauril-sarcosin), 15 mg heparin and, to saturate aspecific sites of membrane, 100 µg mL^{-1} Salmon Sperm DNA. The membrane was hybridized overnight at 60°C with ^{32}P[dATP]-labelled *Pp-ACO-1*, *Pp-endoPG* and *Pp-EXP3* probes (see § Preparation of the probe) and subsequently 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 exposed to X-ray film (X-OMAT AR; Kodak) at -80° C, and the film developed according to the manufacturer's recommendations.

Preparation of the probe

The cDNAs obtained from fruit mesocarp tissue were used as template in PCR to amplify *Pp-ACO-1*, *Pp-endoPG* and *Pp-EXP3* sequences used as probes for Northern Blotting.

The primer pairs used were:

Name	Primer pair	Fragment length (bp)
Pp-ACO1	ACO_{FOR} 5'-ATGGAGAACTTCCCAATCATCAAC-3' ACO_{REV} 5'-GGGTCCAATTGCAACAGCTTAA-3'	960
Pp-EXP3	$EXP3N_{FOR}$ 5'-GTCCTTCAGGGTCAGAGGC-3' $EXP3N_{REV}$ 5'-TTTACCCTACTGCCCCTCC-3'	323
Pp-EndoPG	*$Endo_{FOR}$ 5'-ATGGCGAACCGTAGAAGCCTCT-3 *$Endo_{REV}$ 5'-CTACAAACAACTTGTAGGCTGAAC-3'	1200

*(Lester *et al*., 1994)

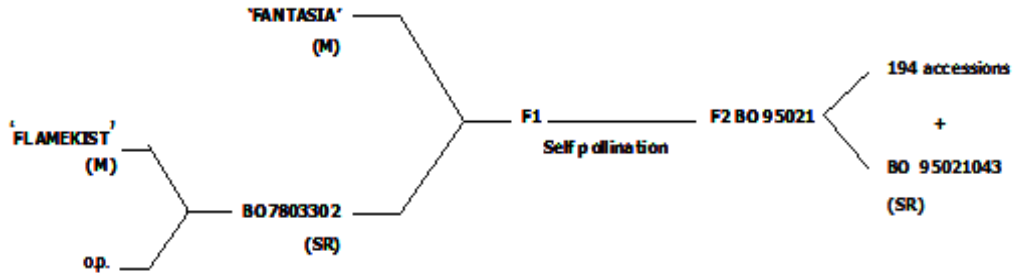
The PCR conditions were 2 min at 95° C, 35 cycles: 94° C for 1 min, 58° C for 40 sec and 1 min at 72° C. The reaction products were analyzed in a 1% (w/v) agarose gel and the bands were purified and cloned in pCR®4-Blunt II TOPO ® vector (Invitrogen). Sequences were determined by BMR Genomics (Padua).

The bands purified were synthesized using 'Multiprime DNA Labelling System' (Amersham) with [α-^{32}P] dATP, follow the manufacturer's conditions. The probes obtained were purified with Sephadex G 75 column and denatured at 100° C for 5 min.

Results and discussion

Effects of ethylene on BO 95021043 fruit ethylene evolution and flesh firmness during postharvest.

The selection used was part of a F_2 progeny obtained from self-pollination of a F1 plant derived by crossing 'Fantasia' and BO 7803302.



Fruit of this selection are small to average: their weight was 75 ± 14 g at harvest, much less than that reported for 'Fantasia' (i.e., 130-140 g).

In order to better characterize the peculiar ripening behaviour of the peach SR BO 95021043 mutant genotype, its fruit were used to conduct different experiments in two growing seasons: 2007 and 2009. Unfortunately, in the 2008 growing season, fruit were badly affected by M*onilinia* infection, so that they did not represent a material suitable for experimentation. The first set of experiments was aimed to evaluate the changes in ethylene evolution and flesh firmness occurring in fruit stored for 6 d after harvest in air or ethylene. The evolution of these parameters was checked for additional 4 d after the end of the treatments (total 10 d after harvest). Figure 1 shows that fruit ethylene evolution increased slightly (albeit the absolute values were extremely low) during the first 10 d of postharvest in air; ethylene was not effective in enhancing it.

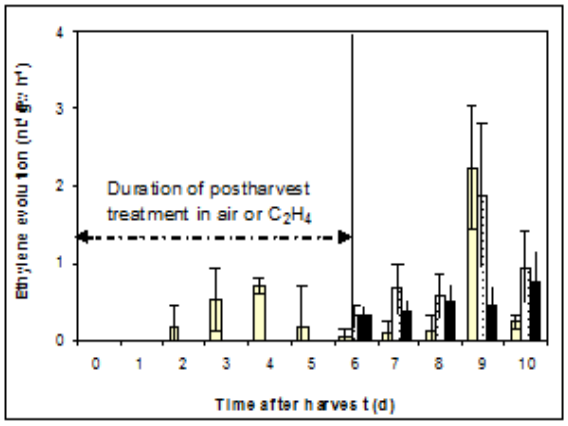


Fig. 1. Ethylene evolution by BO 95021043 fruit during the first 10 d of postharvest. (□): fruit stored at 20°C immediately after harvest; (▨):fruit stored for the first 6 d after harvest under continuous flux of humidified air; (■): fruit stored for the first 6 d after harvest under continuous flux of humidified 100 ppm ethylene. The values are the means ± SD of measurements on at least five fruit.

The changes in selected ripening related parameters (fruit flesh firmness, total soluble solids content and colour index) are reported in Figure 2. Fruit of the BO 95021043 selection were extremely hard at harvest, and their firmness essentially did not change during the considered period of 8 d after harvest. Ethylene induced a slight softening effect after both 6 and 8 d of treatment (Fig. 2A). Soluble solids contents remained essentially unchanged in time, both in the absence and in the presence of ethylene treatment (Fig. 2B), whereas the a* colour index tended to increase slightly in control fruit and slightly more so in the ethylene-treated ones (Fig. 2 C). Concerning the two parameters of ethylene evolution and flesh softening, BO 95021043 fruit seemed to resemble Stony Hard fruit, which neither evolve ethylene nor soften in postharvest but appear to respond to ethylene treatment as far as it concerns flesh softening but not ethylene evolution (Haji *et al.*, 2003; Ghiani *et al.*, 2010).

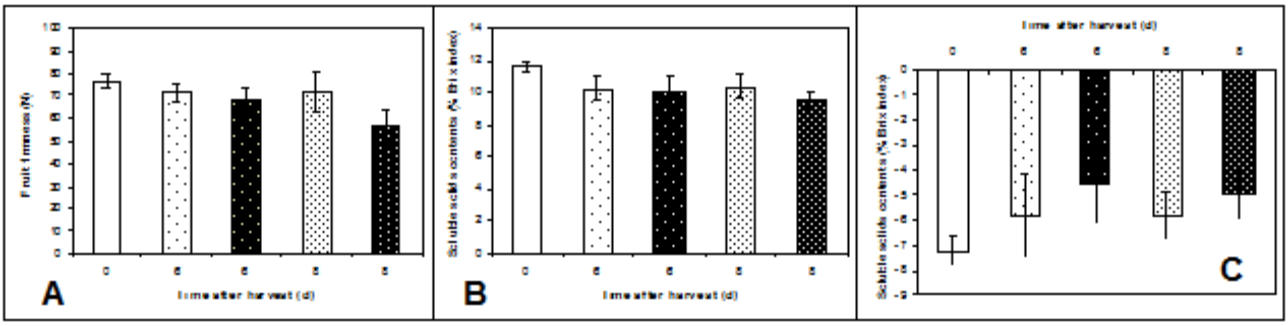


Fig. 2. Changes in ripening-related traits in BO 95021043 fruit after 6 and 8 d of postharvest. A: Fruit flesh firmness; B: Total soluble solids in the juice extracted from fruit mesocarp; C: External ripening-related colour change. (□): fruit assayed for firmness immediately after harvest;; (▨;▨): fruit stored for 6 or 8 d, respectively, at 20°C under continuous flux of humidified air; (▩,▩): fruit stored for 6 or 8 d, respectively, under continuous flux of humidified 100 ppm ethylene. The values are the means ± SD of measurements on at least five fruits.

Effects of ethylene on the expression of Pp-ACO1, Pp-endo-PG and Pp-EXP3 in BO 95021043 fruit mesocarp.

Pp-ACO1 is known to play a key role in ethylene biosynthesis during fruit ripening (Mathooko *et al.*, 2001), and *Pp-endoPG* and *Pp-EXP3* are known to be deeply involved in the process of peach fruit softening (Brummell *et al.*, 2004; Orr and Brady, 1993; Pressey and Avants, 1978, Hayama *et al.*, 2003; Cosgrove, 2003 and references therein). Therefore, we investigated changes in the levels of transcripts of these genes in BO 95021043 fruit mesocarp, stored after harvest in air or ethylene. The results obtained are reported in Figure 3. At harvest, transcripts of all three genes were essentially undetectable, and this situation was unchanged after 8 d of storage in air, concomintant with very slight, not significant (see Figure 2) flesh softening in this condition. The 8-d ethylene treatment, which induced softening of the flesh, induced appearance of transcripts of all three genes, more apparent concerning *Pp-ACO1* and *Pp-endoPG*. Concerning *Pp-ACO1*, the

apparent discrepancy between the positive effect of ethylene treatment on appearance of transcripts and the lack of effect on fruit ethylene evolution may be ascribed to a shift in timing between gene activation and final ethylene production. Moreover, the levels of transcripts of a gene not always nor necessarily correspond to the levels of the active final product of translation, which can also be affected by post-translational modifications. It is interesting to note that previously published data had shown that ethylene treatment on fruit of the same BO 95021043 genotype, though inducing increase in *endo-PG* transcript levels, seemed to have very little effect on fruit flesh softening, as confirmed also by lack of appearance of endoPG protein as visualized by Western blotting (Ghiani *et al.*, 2007). This seeming discrepancy may be explained by comparing the different values of initial fruit flesh firmness, 87 N after 5 d in air in the cited work and 71 N after 8 d in air in the present one, which may suggest a slightly more advanced, even if low, stage of softening possibly coincident with higher sensitivity to ethylene action.

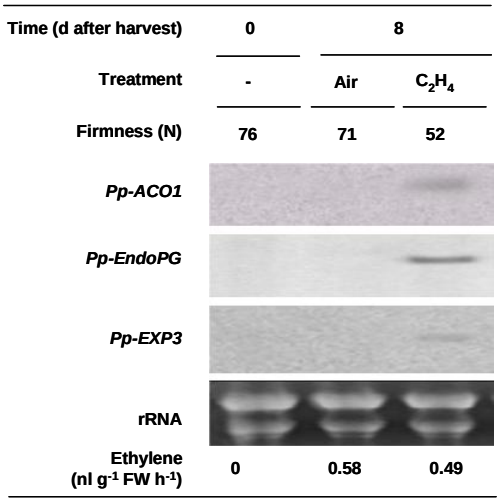


Fig. 3. Northern blot analysis of the changes in the levels of transcripts of *Pp-ACO1*, *Pp-endo-PG* and *Pp-EXP3* in the mesocarp of BO 95021043 peach fruit at harvest and after 8 d of postharvest, in the absence or in the presence of ethylene treatment. The levels of rRNA, visualized by ethidium bromide, are also reported, as well as the values of fruit flesh firmness and ethylene evolution. Twenty micrograms of RNA was loaded per lane. One gel representative of three.

Physiological characterization of the effects of ethylene, in the absence or in the presence of previous treatment with 1-methylcyclopropene (1-MCP), on BO 95021043 fruit

1-methylcyclopropene (1-MCP) is an inhibitor of ethylene perception, as it is thought to prevent ethylene-dependent processes upon interaction with ethylene receptors. 1-MCP presumably binds to a metal in the ethylene receptor, thus competing with ethylene for the receptor, and preventing the latter from binding in treated tissues (Sisler and Serek, 1997, Watkins, 2006).

The mutant fruit phenotype of the BO 95021043 selection used in the present study appeared to be impaired in endogenous ethylene production, probably as a consequence of lack of *Pp-ACO1* transcription, but able to respond to ethylene treatment at least as far as it concerned *Pp-ACO1* and *Pp-endoPG* gene expression (Fig. 3), suggesting functionality of the ethylene perception-transduction systems in fruit of this selection. We looked for further information on the mechanisms involved in the regulation of ripening in this material by subjecting BO 95021043 fruit to ethylene treatment, following or not 1-MCP treatment, in a set of experiments designed as summarized in Figure 4.

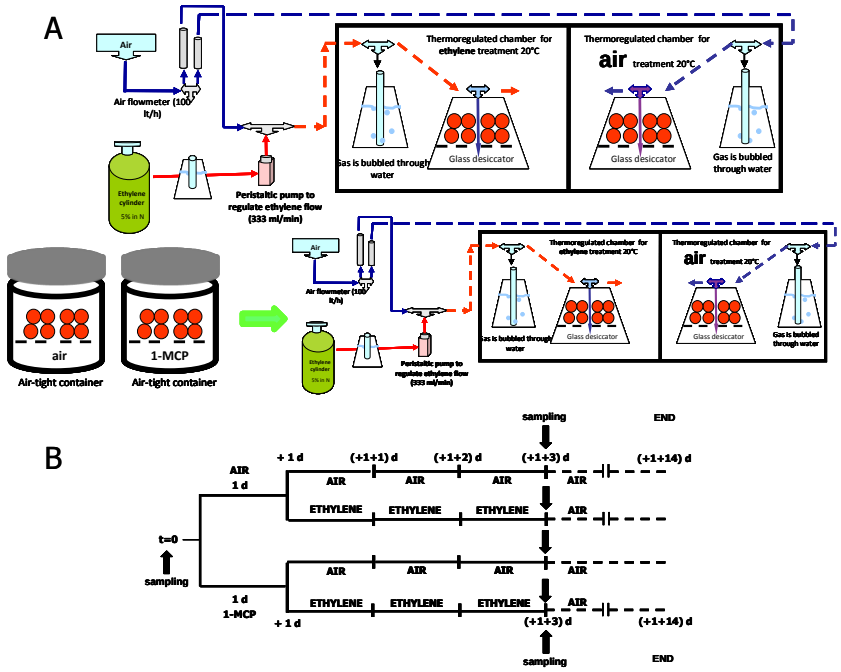


Fig. 4. Experimental design of the treatments of SR BO 95021043 fruit with 1-MCP and/or ethylene. A: Scheme of the experimental device used. B) scheme of sampling times.

Briefly, fruit batches homogeneous for average weight were treated or not for 1 d with 5 ppm 1-MCP, after which each batch was transferred for additional 3 d under a continuous flow of air or ethylene (Fig. 4A). At the desired times, fruit samples were taken, as indicated in Figure 4B. Endogenous ethylene production was monitored for a total of 15 d after harvest (for details, see the Materials and Methods section). Figures 5 describes the changes in flesh firmness, total soluble solids content, and loss of epicarp green colour, in fruit in the different conditions. Although the effects were slight, a general trend could be put in evidence, with ethylene enhancing the development of ripening-related traits and 1-MCP inhibiting the effects of the phytohormone (Fig. 5A-C).

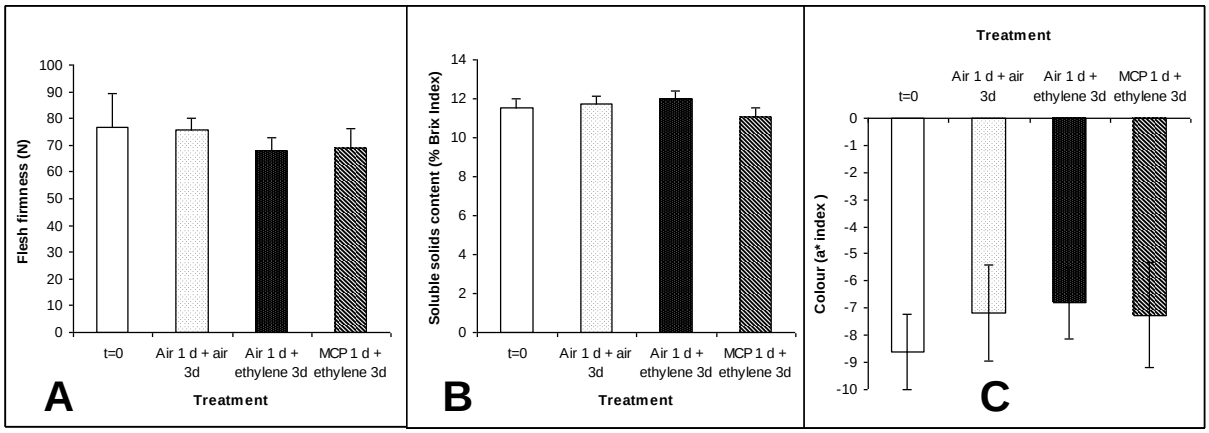


Fig. 5. Effects of ethylene and 1-MCP on ripening-related traits in fruit of the SR BO 95021043 selection (2009 season). A: Fruit flesh firmness; B: Total soluble solids in the juice extracted from fruit mesocarp; C: External ripening-related colour change. Treatments: Air 1 d + air 3 d: fruit stored for 1 d in air in an air tight tank and subsequently transferred for 3 d in continuous flow of humidified 100 ppm ethylene. 1-MCP 1 d + ethylene 3 d: fruit stored 1 d in 5 ppm 1-MCP, in an air tight tank, and subsequently transferred for 3 d under a continuous flow of humidified air 100 ppm ethylene. The values are the means ± SD of measurements on at least five fruits.

The results concerning fruit ethylene evolution are reported in Figure 6. The basal rate of ethylene production by the SR BO 95021043 fruit was extremely low throughout the 15-d period considered (Fig. 6A), consistent with what reported in the literature (Brecht *et al*., 1984). Storage of fruit in an air tight tank, a procedure necessary as a control for 1-MCP-treated fruit, did not alter ethylene production; only a slight, not significant increase was observed in fruit after 1 + 3 d of air treatment (Fig. 6B). The 3-d treatment with ethylene induced an increase in ethylene evolution which, oddly enough, became particularly clear 11 d after ethylene removal (Fig. 6C). Treatment with 1-MCP inhibited the (albeit extremely low) increase in ethylene evolution in air-treated fruit and prevented the ethylene-induced enhancement of endogenous ethylene evolution (Fig. 6D).

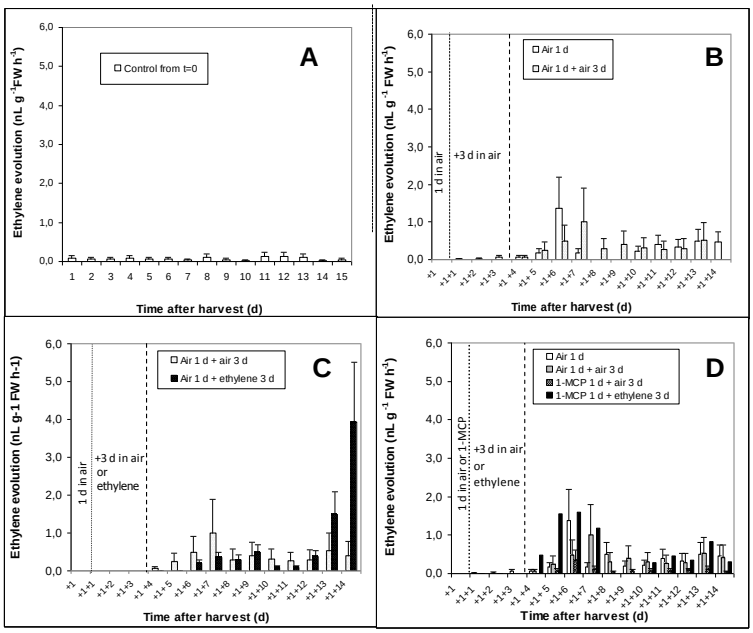


Fig. 6. Ethylene evolution by fruit of the SR BO 95021043 selection. A: fruit exposed to air monitored from harvest up to 15 d after harvest. B: fruit stored for 1 d in air, in an air tight tank (air 1 d) and fruit stored for 1 d in an air tight tank + 3 d in continuous flow of humidified air (air 1 d + air 3 d). C: fruit stored 1 d in air, in an air tight tank, and subsequently transferred for 3 d under a continuous flow of humidified air (air 1 d + air 3 d) or 100 ppm ethylene (air 1 d + ethylene 3 d). D: fruit stored for 1 d in air, in an air tight tank (air 1 d); fruit stored 1 d in air, in an air tight tank, and subsequently transferred for 3 d under a continuous flow of humidified air (air 1 d + air 3 d) or 100 ppm ethylene (air 1 d + ethylene 3 d) and fruit stored 1 d in 5 ppm 1-MCP, in an air tight tank, and subsequently transferred for 3 d under a continuous flow of humidified air (1-MCP 1 d + air 3 d) or 100 ppm ethylene (1-MCP 1 d + ethylene 3 d). The values are the means ± SD of measurements on at least five fruits. Dotted lines in B, C and D show end of the 1-d (·····) or 1 + 3 d (--------) treatments.

Effects of 1-MCP and ethylene treatments on the transcript levels of Pp-ACO1, Pp-endoPG and Pp-EXP3 in BO 95021043 fruit mesocarp

The levels of transcripts of three genes playing important roles in ethylene biosynthesis and flesh softening were investigated in BO 95021043 fruit mesocarp at harvest (t=0) and after treatment or not with for 1 d with 1-MCP followed by a 3-d treatment with ethylene. The results of Northern blot analysis are reported in Figure 7, together with the corresponding values of fruit flesh firmness and ethylene evolution. At harvest and in air, up to the 4-d period of postharvest considered, the levels of *Pp-ACO1* transcripts were negligible, according to almost complete lack of ethylene production, but increased after the 3-d period of exposure to exogenous ethylene. Ethylene proved effective in increasing the levels of *Pp-ACO1* transcripts also after the 1-MCP treatment. Also the levels of *Pp-endoPG* and *Pp-EXP3* transcripts were essentially undetectable in fruit at harvest as well as in those stored in air. Ethylene induced an increase in the levels of transcripts of both genes, more pronounced for *Pp-endoPG* and slighter for *Pp-EXP3*. When ethylene was administered after the 1-d treatment with 1-MCP, its effect was still present as far as it regarded *Pp-EXP3* levels but absent concerning the levels of *Pp-endoPG* transcripts. The data show that fruit of the BO 95021043 mutant were able to respond to ethylene, activating the transcription of all the

three genes considered. 1-MCP proved effective in counteracting the, albeit slow, decrease in flesh firmness observed in fruit stored in air. Ethylene seemed able to revert the inhibitory effect of 1-MCP on flesh firmness decrease but it was unable to induce *Pp-endoPG* gene transcription when it was administered after 1-MCP treatment. Therefore, in these experiments, the very similar values of flesh firmness reached when ethylene was administered after air or 1-MCP seemed independent from the levels of expression of *Pp-endoPG*, whereas in both cases an increase in the levels of *Pp-EXP3* could be detected. This result may be explained keeping in mind that the generally acknowledged role of endoPG is played in melting of peach fruit, a phenomenon that seems not to occur in this mutant material.

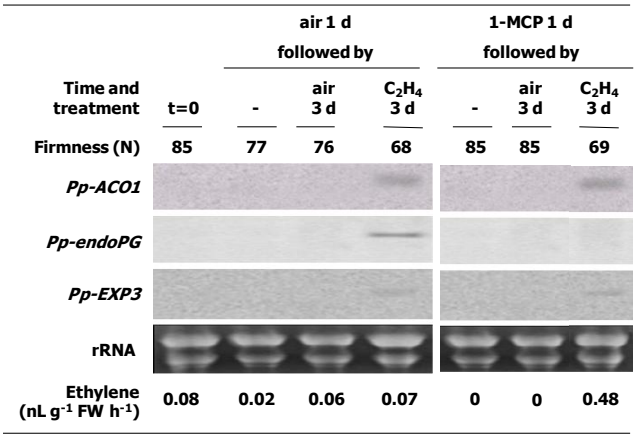


Fig. 7. Northern blot analysis of the changes in the levels of transcripts of *Pp-ACO1*, *Pp-endo-PG* and *Pp-EXP3* in the mesocarp of BO 95021043 peach fruit at harvest (t=0) and treated or not with 1-MCP prior to administration of air or ethylene. The levels of rRNA, visualized by ethidium bromide, are reported, as well as the values of fruit flesh firmness and ethylene evolution. Twenty micrograms of RNA was loaded per lane. One gel representative of three.

The ability of mutant fruit of the SR BO 95021043 selection to respond to ethylene treatment with an increase in the levels of *Pp-endoPG* transcripts represented an additional feature shared with SH fruit, besides the already cited response in terms of flesh softening in the absence of ethylene evolution. The low ethylene evolution could be related to lack of expression of *Pp-ACO1*, the levels of which, on the other hand, were increased in response to exogenous ethylene, similar to what observed for *Pp-ACS1* in SH fruit (Ghiani *et al.*, 2010). *Pp-ACO1* was considered for the present study on the basis of its well known key role in ethylene synthesis (Mathooko *et al.*, 2001).

We hypothesized that the low ethylene evolution by BO 95021043 fruit could be due also to an altered transcriptional regulation of genes involved not only in ethylene biosynthesis, but also in its complex perception/transduction pathway, which finally leads to activation of the autocatalytical System 2 of ethylene production (Inaba, 2007). In order to better clarify this hypothesis, and to confirm the results obtained in the 2007 growing season, new experiments were conducted in 2009 with the same rationale.

Figure 8 shows the results concerning the changes in ripening parameters. Fruit flesh firmness at harvest was very high, showing a value of 80 ± 10.6 N. During postharvest in air, firmness tended to decrease slightly, reaching a value of about 69 N after a total of 7 d of postharvest; ethylene treatment slightly increased this trend. When fruit were treated for 1 d with 1-MCP, the decrease in flesh firmness was approximately equal to that observed in control fruit, but 1-MCP proved able to prevent the effect of ethylene (Fig. 8A). As far as it concerns total SSC, the changes in the values of this parameter were very slight and of difficult interpretation. In fact, albeit a trend to a slight increase could be observed in control fruit, which was slightly enhanced by ethylene, no clear-cut behaviour could be identified concerning the effects of 1-MCP and of the ethylene treatment administered after 1-MCP (Fig. 8B). In all conditions, ethylene evolution was essentially null (data not shown).

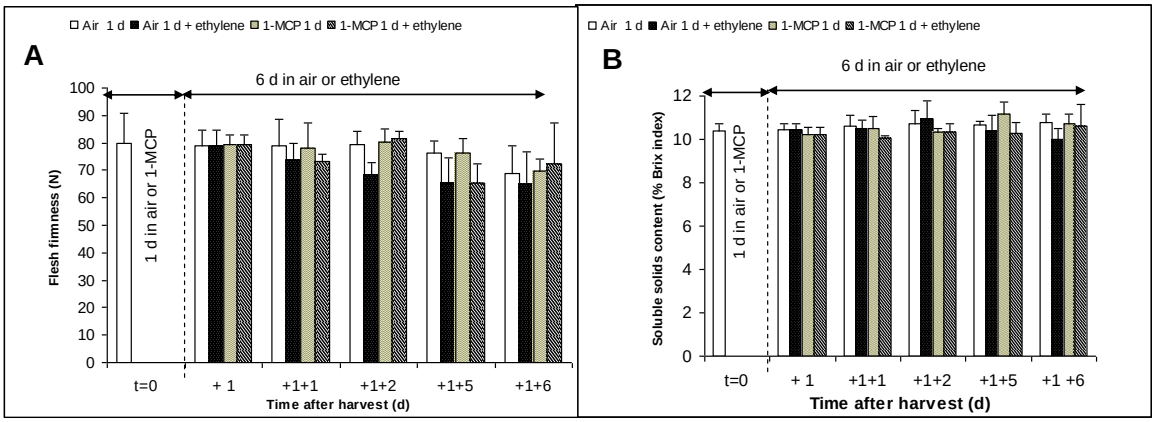


Fig. 8. Effects of ethylene, without and with a pre-treatment with 1-MCP, on ripening-related traits in fruit of the SR BO 95021043 selection (2009 season). A: Evaluation of fruit flesh firmness; B: Total soluble solids in the juice extracted from fruit mesocarp. Fruit were stored for 1 d in air or 5 ppm 1-MCP in an air tight tank and subsequently transferred for additional 6 d in continuous flow of humidified air or 100 ppm ethylene. At the desired time intervals fruit flesh firmness and soluble solids contents were assessed. The values are the means ± SD of measurements on at least five fruits.

After this preliminary phenotypic characterization of the experimental material, the changes in the transcript levels of a few genes involved in ethylene synthesis (*Pp-ACS1* and *Pp-ACO1*), perception and signalling (*Pp-ETR2*, *Pp-ERS1*, *Pp-CTR1*) and final response in terms of flesh softening (*Pp-endoPG*, *Pp-EXP3*) were analyzed in the mesocarp of SR BO 95021043 fruit immediately after harvest (t=0), at the end of the 1-d treatment with air or 1-MCP, and after two additional days in air or ethylene, as indicated in Fig. 9.

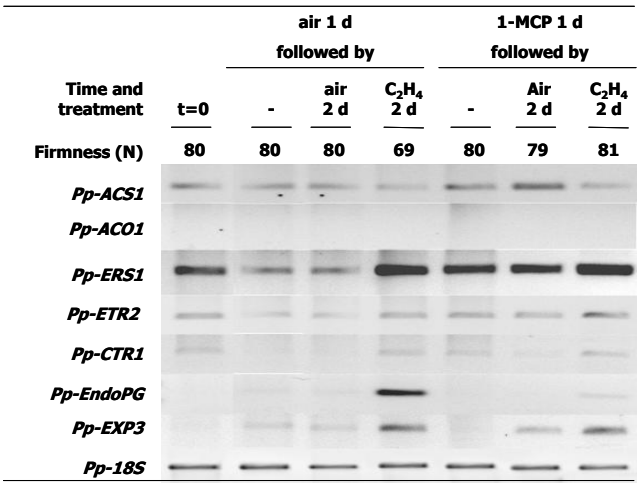


Fig. 9. qRT-PCR analysis of the effects of ethylene, without and with a pretreatment with 1-MCP, on the changes in the levels of transcripts of a few genes involved in ethylene synthesis (*Pp-ACS1* and *Pp-ACO1*), perception and signalling (*Pp-ETR2*, *Pp-ERS1*, *Pp-CTR1*) and final response in terms of flesh softening (*Pp-endoPG*, *Pp-EXP3*) in the mesocarp of BO 95021043 peach fruit. The transcript levels were evaluated, as indicated by the figure captions, at harvest (t=0), at the endo of the 1-d treatment with air or 1-MCP (-) and after two additional days in air or ethylene. The transcript levels of the housekeeping gene *Pp-18s* are also reported, as well as the values of fruit flesh firmness. Twenty micrograms of RNA was loaded per lane. One gel representative of three.

In the mesocarp of control (air-stored) fruit, the levels of transcripts of the genes considered were generally very low and showed very slight changes over the time span considered (a total of 3 d of postharvest), consistent with the features of BO 95021043 fruit, which appear to suffer from a general block of developmental processes. Absence of detectable ethylene evolution (data not shown) was paralleled by lack of *Pp-ACO1* transcripts, whereas *Pp-ACS1* transcripts were present, albeit in low amounts, as well as transcripts of the ethylene receptors *Pp-ETR2* and *Pp-ERS1*.

Transcripts of the negative regulator *Pp-CTR1* were present in low amounts at harvest, and tended to disappear with time. *Pp-endoPG* transcripts were not detectable, but *Pp-EXP3* ones showed a slight tendency to increase with time. Administration of ethylene to fruit stored in air for 1 d had no significant effect on the levels of transcripts of the genes involved in its biosynthesis, whereas the phytoregulator tended to increase the levels of *Pp-ERS1*, *Pp-endoPG* and *Pp-EXP3*, consistent with the observed decrease in flesh firmness in ethylene-treated fruit. Treatment of fruit with 1-MCP appeared to exert essentially no effect on the levels of *Pp-ACS1* and *Pp-ACO1* transcripts.

The observed constitutive expression of *Pp-ETR2* may suggest that this ethylene receptor does not play a major role in regulating hormone signal perception in this peach ripening mutant, different than described in fruit of other peach cultivars (Trainotti *et al*., 2006; Ziliotto *et al*., 2008). *ETR2* is induced at the very early steps of climacteric and displays a ripening-related expression pattern in M nectarines (Trainotti *et al.*, 2006). Moreover, in ripening peaches, a great inductive effect on *ETR2* is yielded by exogenous ethylene treatment (Trainotti *et al.*, 2007). In the BO

95021043 SR fruit mutant the levels of *Pp-ETR2* transcripts were very low and only slightly enhanced by ethylene, consistent with the fruit peculiar ripening pattern, even if it at the moment it appears impossible to establish whether this is a cause or an effect of the impaired ripening mutant phenotype. The accumulation of *Pp-ERS1* transcripts increased after ethylene treatment, consistent with data reported by Rasori *et al.* (2002), but, different to what reported by these authors for ripening M peaches, their levels did not change with time. *Pp-ERS1* transcripts were present at substantial levels also in 1-MCP-treated fruit, consistent with what reported by Dal Cin *et al.* (2006). Concerning the higher levels of *Pp-ETR2* and *Pp-ERS1* observed in 1-MCP treated fruit, it may be hypothesized, assuming that higher transcript levels correspond to higher levels of the corresponding protein that more receptors are synthesized to replace the ones blocked by the ethylene antagonist 1-MCP. Nevertheless, this would not be sufficient to restore the physiological sensitivity to the phytohormone (see also lack of effect of ethylene, when administered after 1-MCP, on fruit flesh softening).

It is interesting to note that 2 d after the end of 1-MCP treatment *Pp-EXP3* transcripts became visible in the mesocarp of air-stored fruit. This result may be interpreted by hypothesizing that the expression of this gene could be in part controlled by ethylene-independent mechanisms; nevertheless, it should be kept in mind that in peach the effects of 1-MCP appear more transitory than in other fruit such as apple (Dal Cin *et al.*, 2006). When 1-MCP-treated fruit were subjected to ethylene treatment, the phytohormone appeared to have contradictory effect. On one hand, it reversed the effects of its antagonist by restoring the levels of *Pp-EXP3*; on the other hand, ethylene was unable to restore *Pp-endoPG* transcription, consistent with its unability to induce the (limited) fruit softening (Fig. 9).

The SR BO 95021043 mutant fruit, that besides other peculiar traits are characterized by lack of ethylene production, appeared to have distinctive features compared with the SH fruit, which also do not produce ethylene. In fact, in ripening SH fruit lack of ethylene production can be clearly related to lack of activation of *Pp-ACS1* transcription (Tatsuki *et al.*, 2006, Ghiani *et al.*, 2010) whereas in the BO 95021043 mutant ones *Pp-ACS1* appeared to be transcribed, even if at a little extent, and *Pp-ACO1* was not. Again, assuming that the levels of transcripts correspond to the levels of the corresponding active protein, lack of ethylene production in these fruit seems therefore ascribable to unpairment of a different step of the ethylene biosynthetic pathway than in SH fruit.

The data taken as whole suggest that the SR BO 95021043 mutant phenotype shares some similarities with SH fruit ('Ghiaccio' cv) as reported in a recent work (Ghiani *et al.*, 2010). In fact, also SH fruit, like the mutant ones considered in the present study, are not able to produce spontaneously ethylene but respond to it in terms of flesh softening and endoPG production.

Nevertheless, their to produce ethylene is ascribable to a block in *Pp-ACS1* transcription, at least in ripening fruit (Tatsuki *et al.*, 2006; Ghiani *et al.*, 2010), whereas BO 95021043 fruit do produce, even in low amounts, *Pp-ACS1* but seem blocked in *Pp-ACO1* transcription.

Amplification and Cleaved Amplified Polymorphic Sequence (CAPS) analysis of selected Pp-endoPG sequences from genomic DNA

The data obtained in 2009 concerning flesh firmness and *Pp-endoPG* transcript levels confirmed the well acknowledged parallelism between endo-PG transcription and flesh softening, which in fruit of the BO 9520143 mutant occurred only after administration of exogenous ethylene and in absence of previous treatment with the ethylene antagonist, 1-MCP. In order to better characterize the SR BO95021043 peach ripening mutant, the configuration of its *endoPG* gene was investigated by use of the CAPS technique. The analysis was conducted on genomic DNA extracted from leaves of BO95021043 and, for comparison, of NM 'Oro A', M 'Bolero', Slow Melting 'Big Top', and of the SH 'Ghiaccio', 'D41-62' and 'Yumyeong' cultivars and selections. By the use of Hd_{FOR} and Hd_{REV} primers (for details, see the Materials and Methods section) an *endoPG* gene fragment of 911 bp (60-971 bp of the complete *Pp-endoPG* gene sequence) was amplifed, and the products obtained from the different peach genotypes were digested with the endonuclease *Bts*I. The SNP generating this CAPS is present at 390 bp of the *Pp-endoPG* clones *SH* or *BT* (see Results Section, Chapter 1 of the present Ph.D. Thesis). The variation in the *Pp-endoPG* sequence is ($G_{m,M} \rightarrow T_{SH,BT}$), which produces a restriction site GCAG<u>T</u>GNN▼ recognized by *Bts*I. Based on this knowledge, we used *Bts*I to challenge an amplified *Pp-endoPG* sequence obtained from BO 95201043, in the attempt of ascertaining whether its configuration at this locus resembled that of a M peach (as could be inferred by the presence, in its lineage, of the M cultivars 'Fantasia' and 'Flamekist') or it had different features, possibly resembling those of a SH genotype.

Figure 10 shows the scheme of amplification of the selected sequence of the *Pp-endoPG* gene and the expected lengths of the digestion fragments obtainable from the different *Pp-endoPG* clones (Fig. 10A), as well as the restriction patterns obtained by CAPS analysis with the use of *Bts*I (Fig. 10B).

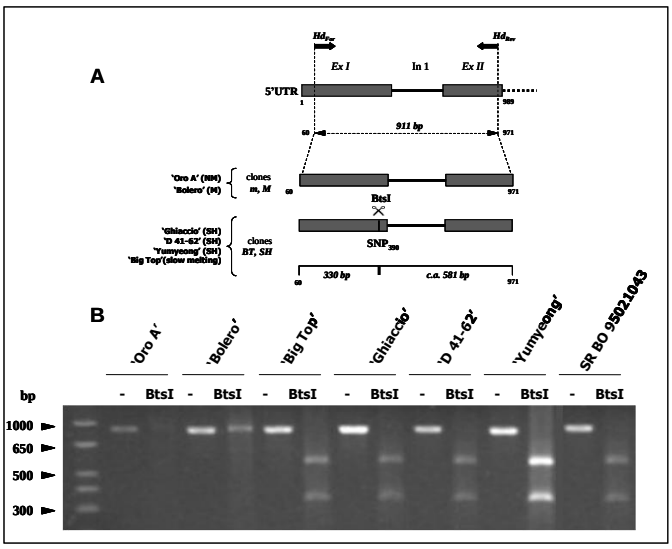


Fig. 10. Cleaved Amplified Polymorphic Sequence (CAPS) restriction patterns of genomic DNA from leaves of 'Oro A', 'Bolero', 'Big Top', 'Ghiaccio', D41-62, 'Yumyeong' and BO 95201043 selections. A: diagrammatic scheme of the amplifcation procedure and of the expected digestion patterns. B: results of *Bts*I endonuclease digestion. (-), undigested; (*BtsI*), *Bts*I-digested. Arrowheads show positions and lengths (bp) of the closest markers to the DNA fragments of interest. One representative experiment is shown, from three independent replications.

With the exception of NM 'Oro A' and M 'Bolero', the amplified genomic sequences of which are not digested by *Bts*I for the absence of the SNP_{390}, all the other selections showed a restriction pattern with two fragments about 600 and 350 bp-long (consistent with the lengths expected on the basis of the SNP_{390} position), characteristic of SH and 'Big Top' genotypes. The *Pp-endoPG* clone of the SR BO 95021043 genotype yielded the same restriction pattern of these clones, suggesting the presence of some SH or 'Big Top' genetic material in its lineage.

A second type of CAPS analysis was conducted on the same samples in the search for other features of the *Pp-endoPG* gene possibly shared between SR BO95021043 and other selections. The analysis exploited the SNP_{1310} (which discriminates between the *M Pp-endoPG* clone, on one side, and the *m* and *SH/BT* clones, on the other side). SNP_{1310}, localized at 1310 bp of the *Pp-endoPG* clone (See Fig. 6, Chapter 1 of the present Ph.D. thesis), is found in the *Pp-endoPG* clone *m* (Morgutti *et al*., 2006) but also in the SH and 'Big Top' *Pp-endoPG* clones. The sequence variation is $G_M \rightarrow C_{m/SH/BigTop}$ producing an additional restriction site A<u>C</u>TGGN▼N recognized by the *BsrSI* enzyme. CAPS analysis on genomic DNA was conducted by amplifying, with the primers MF_NMF_{For} and MF_NMF_{Rev}, a selected sequence of DNA (958-1892 bp) in the *endoPG* gene. The amplification products obtained from the different selections were then digested with *Bsr*SI. Figure 11 shows a diagrammatic scheme of the amplification patterns of the sequence selected in the *Pp-endoPG* gene, with the expected lengths of the digestion fragments in the different genotypes (Fig. 11A) and the restriction profiles obtained by CAPS analysis (Fig. 11B). The amplification of the selected sequence in NM 'Oro A' produced a fragment of approximately 950

bp, and digestion with *Bsr*SI produced two fragments of approx. 550 and 360 bp. The third fragment, originating between the two *Bsr*SI restriction sites, was not visible because, presumably, it was too short (calculated length 48 bp) to be retained in the gel at the agarose concentration used (3% w/v).

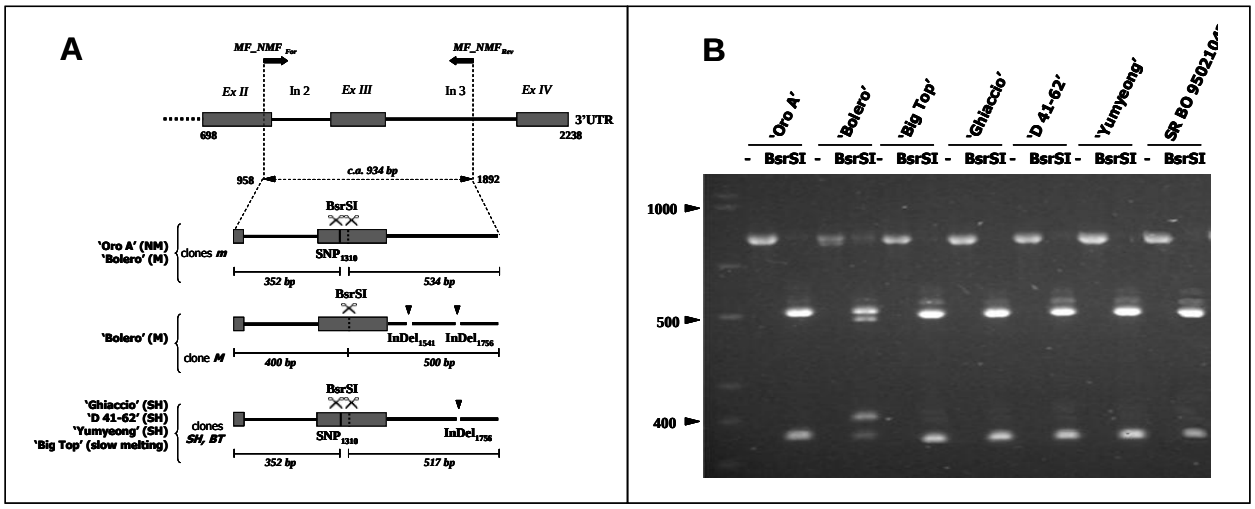


Fig. 11. CAPS restriction patterns of genomic DNAs from leaves of 'Oro A', 'Bolero', 'Big Top', 'Ghiaccio', 'D41-62', 'Yumyeong' and BO 95201043 selections. A: diagrammatic scheme of the amplification procedure and of the expected digestion patterns. B: (-), undigested; (*Bsr*SI), *Bsr*SI -digested. Arrowheads show positions and lengths (bp) of the closest markers to the DNA fragments of interest. One representative experiment is shown, from three independent replications.

Amplification of the same sequence in MF 'Bolero' yielded two products, one of about 950 bp and the other slightly smaller, in agreement with the estimated lengths of 934 and 900 bp of the two *Pp-endoPG* clones *m* and *M*. Consistently, the *Bsr*SI restriction pattern in 'Bolero' presented four fragments of approx. 550, 500, 400 and 360 bp. 'Ghiaccio', 'BigTop', 'D41-62' and 'Yumyeong' showed restriction profiles similar to that of 'Oro A'. Nevertheless, one of the digestion fragments was approximately 530 bp (instead of 550 bp as in 'Oro A'), in agreement with the calculated value of 517 bp due to the presence in the *Pp-endoPG* clones *SH* and 'Big Top', compared to clone *m*, of one characteristic deletion of 17 bp (1756-1772 bp) in intron III (see Figure 6 of Chapter 1). The restriction profile of SR BO 95021043 was similar to that observed in SH genotypes and in 'Big Top', confirming that the *endo-PG* gene of this mutant shares a few characteristics with the SH and 'Big Top' *Pp-endo-PG* clones.

In conclusion, mutant fruit appear affected by a general impairment of develomental process, which is demonstrated by macroscopical (stunted fruit growth, very slight colour change),

biochemical (absence of endogenous ethylene evolution, very slight changes in total soluble solids contents) and molecular (low production of gene transcripts) parameters.

Lack of ethylene production seemed ascribable to lack of transcription of the *Pp-ACO1* gene, with probable consequences also on System 2 of ethylene biosynthesis (Ruperti *et al*., 2001; Mathooko *et al.*, 2001). Under this respect, the behaviour of mutant fruit was different than that of M peach fruit, where *ACO* transcripts strongly accumulate during the last stages of ripening, consistent with the patterns of ethylene evolution and ACO activity (Tonutti *et al.*, 1991), but also than that of SH fruit, which are impaired in *Pp-ACS1* transcription (Ghiani *et al.*, 2010). In the future, it may be interesting to widen the study of BO 95021043 mutant to the molecular characteristics of the *Pp-ACO1* gene, in search for possible mutations at the level of its promoter possibly linked to lack of its expression.

BO 95021043 fruit appeared able to sense the ethylene or respond to it, since they appeared to respond (albeit to a little extent) to treatment with exogenous ethylene by turning the skin color to a less greenish shade (increase of the a* colour index), softening and activating transcription of the *Pp-endoPG* gene. The general features of BO 95021043 mutant fruit, as well as their poor response to ethylene treatment may be related to the essentially constant levels of transcripts of *Pp-ERS1*. In fact, in M peach fruit, the proceeding of ripening is related to the increase in *Pp-ERS1* transcripts levels, whose expression is upregulated by ethylene (Rasori *et al.*, 2002). A similar situation was observed also in the activities of this Ph.D., with higher levels of *Pp-ERS1* transcripts in soft M 'Bolero' fruit (data not shown).

Concerning the genes whose products (cell wall proteins) are known to be involved in fleshy fruit softening and melting, it is interesting to note the presence of *Pp-EXP3* transcripts also in the absence of detectable levels of *Pp-endoPG* transcripts. This result may be interpreted on the basis of the role played by expansins in early fruit softening, while endo-PG and pectin-methyl-esterase (PME) are more deeply involved in the later melting process (Trainotti *et al.*, 2003).

Investigation on the structure of a *Pp-endoPG* clone in BO 9502143 put in evidence the presence of peculiar polymorphisms (SNP_{390} and SNP_{1310}) shared with SH genotypes. Due to the scarce probability of casual occurring of such punctual mutations in the *Pp-endoPG* (as in other) gene(s), the result seems to suggest the presence of some SH akin in the lineage of the BO 9502143 selection.

For the sake of exactness, it should be stressed that BO95021043 fruit showed a not completely unambiguous behaviour in the two growing seasons considered, particularly as far as it concerns the ability of ethylene treatment to induce flesh softening after treatment with the ethylene antagonist, 1-MCP (compare Figs. 5 and 9). A possible explanation for these discrepancies may

reside in the difficulty of identifying, in these fruit which are blocked not only in the ripening process but, in general, in the whole developmental process, matching conditions of physiological status. This possibility is suggested also by the different values of fruit flesh firmness observed at harvest in the two growing seasons considered (2007 and 2009). Such intrinsic differences may also have consequences on the effectiveness of the 1-MCP treatment and/or sensitivity to ethylene action.

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