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"Nihil sige magno vita labore dedit mortalibus"

(Orazio, *Satire*, I, 9, 59)

"Rem tene; verba sequentur"

(Catone il Censore)

"Sono fin troppo consapevole del fatto che si vive in un'epoca in cui solo gli ottusi sono presi sul serio, e io vivo nel terrore di non essere frainteso"

(Oscar Wilde)

Contents

Abstract

Softening is a process which accompanies ripening and post-harvest life of fruits. The process is mainly in charge of enzymatic action on fruit tissues, in particular affecting cell wall polysaccharides. In peaches and nectarines, climacteric fruits, the process and enzyme action is mainly coordinated by the hormone ethylene. Enzyme action is quite complicated, since many enzymes and substrates are involved during the process, and the same enzyme can act in different moments of the process itself. Besides, the hormone role is complex, and ethylene biosynthesis and signaling is still far to be completely known, and many dark areas still remain.

Almost uniquely among fruits, in peach is possible to identify three different phenotypes. The most common, called "melting" (MF), is characterized by a rapid softening phase and a discrete ethylene emission. Melting flesh fruits are commonly used for fresh consumption. On the contrary, a second texture type, called "non melting" for it retains its firmness for a long time after harvest, exist. The "non melting" (NMF) type is mostly used for canning, since its consistency make fruits suitable to stand heat treatments occurring in transformation processes. A third flesh type exist, called "stony hard". This phenotype, which is characterized by an almost undetectable ethylene production, retain its firmness for longer than any other, without any appreciable sensory difference. For breeding purposes this trait is quite relevant, since introduction of this trait into "high-performing" cultivars could severely improve their shelf life. However, stony hard trait is almost unknown so far, and its objective identification is still quite difficult. Traditional systems, such as texture and ethylene measurement, have proved to have a low efficiency, and other systems, such as ACC expression (using RNA analysis) has proved to be quite complicated and time consuming. Besides, all of them require fruits for the analysis, hence their use for MAS is reduced. With the exception of Endo-PG marker, able to identify between MF

and NMF, no molecular markers for flesh texture are available so far, and none is suitable for "stony hard" phenotype.

In this work, the research of a molecular marker capable of detecting the stony hard trait has been researched. The RAF protocol has been used to achieve this result, adopting a BSA approach. Many markers have been investigated and a pool of effective markers have been found and discussed.

Moreover, many attempts of studying the softening process have been done. Over 40 accessions, belonging to the University of Bologna, have been investigated for the main genes involved in enzymatic action and ethylene signaling and perception. The candidate genes approach has been used, and an almost complete allelic database for those cultivar has been made available. In order to ease the research of a marker and the allelic investigation, the accessions analyzed have been characterized for the main biochemical parameters, following ethylene and firmness evolution in a ten days period after harvest. A theoretical explanation of the biochemical basis determining "stony hard" trait has been provided.

Chapter 1

Introduction.

Peach tree (*Prunus persica* Batsch) originated in China, where is considered as the symbol of immortality. Its flowers have been celebrated by poets, painters and singers since its introduction, with many differences according to the varying image associated with peach in the different cultures: in the ancient Egypt, peaches were considered sacred to Arpocates, God of silence and childhood. From the Far East the peach reached on the wheels of merchants caravans the Persian lands, where the fruit took its name ("Persica" comes from "Persia"). After Alexander the Great (King of Macedonian Empire since 336 B.C.) and his conquests in the middle east, peach trees spread in the Mediterranean basin, reaching Rome in the I century A.D., where peach cultivation has been recorded, painted and described.

1.1 Peaches and Market.

Peach is an important commodity: FAO statistics estimated the worldwide production in 2003 at about 15,353,000 tonnes, most of which concentrated in Asia (mainly China). Europe can count on about 30% of the total production, where Mediterranean Countries are responsible of almost 90% of the total [99, 38].

Growers often feel the urgency of selling their goods in periods when the market is capable of absorbing the whole production, racing for better prices (in particular for early productions). More often, however, such urgency leads

growers to feed the market with fruits that are qualitatively unsatisfactory because not mature yet. Such a product cannot be suitable to satisfy consumer's needs and requests. Consumers, on the other side, have nowadays developed a greater awareness of the concept of quality, not referred only to the appearance of the fruit but also to sensory aspects and nutraceutical factors [70].

On this basis, growers and marketers behaviour can easily generate a negative feedback, that can be enlightened in two different, but consequential, moments. In the short period, non-conformity with consumer expectations leads to an unavoidable disaffection of the consumer to peach consumption. Consumers, then, tend to turn their attention to other fruits. Considering the possibility of untying market availability from seasons, both for greenhouses and importation of products from other Countries, the choice of alternative products to peaches appears to be more sensitive -and ruinous- than a few years ago. In a long-term perspective, this behaviour can have even more serious consequences for peach industry. Great market chains, although still not completely holding the reins of the marketing of orchard commodities, are increasing year after year their role in product distribution [36].

Consumer disaffection to peaches may lead to a decrease of peach request in supermarkets and, as a direct consequence, to the formation of unsold production and the lowering of prices. In the same way, a policy that is not paying attention to quality traits leads to an erosion of market quotes in foreign countries, as happened in France and Germany, where Italian exports have recorded a decrease in the previous years: in the EU balance of peach exports, Italian relevance shifted from almost 70% to less than 55% in just four years [36]. A wise management of quality is the key to fix the problem and keep peach industry at good levels. This aim can be reached with a good management of existing resources (pest management, growers know-how, harvesting techniques, storage systems, etc.) and with the developing of new peach cultivars more capable to satisfy market requests.

As known, peaches can have two main uses: for fresh consumption and canning. Processes of peaches can vary from canning of whole peaches or slices (with syrups), juices, dried or candies.

Peaches and nectarines are mostly used for the fresh market, while non-melting flesh peaches are usually running on the canning path. However, this is just a raw division, since in some countries (like southern Italy, or Spain) those peaches are eaten as fresh as any other peach or nectarine.

What is really important for the canning is the texture of the peach, rather

than any other aspect: transformation is usually a series of mechanical and high-temperature processes that are almost likely damaging the flesh before the end, altering the aspect of the fruit (bruises, browned areas, etc.) and the aroma. That is particularly relevant for canning purposes, where the fruit, peeled, should preserve unspoilt the flesh appearance. That is the reason for the use of non-melting texture peaches for the canning industry: those peaches are characterized by a flesh which retains its high firmness even through the heat treatments. A more detailed description of flesh types will be treated below (see 1.1.4).

1.1.1 Peach botany.

Peach (*Prunus persica* L.) belongs to the order of *Rosales*, family of *Rosceae*, tribe of *Amigdaleae*, section of *Prunoidee*. The Peach, on an agronomic, marketing and processing point of view can be divided into three main varietal groups, according to the fruit characteristics (skin fuzz and flesh texture):

- ➢ Peaches, with fuzzy skin, mainly used for fresh consumption;
- ➢ Non-melting flesh peaches, fuzzy skin, with characteristic pit adherence of the flesh and hard texture, mainly for canning purposes;
- ➢ Nectarines, with smooth, fuzzless skin, for fresh market.

The root system is quite developed, most on the more superficial layer of the soil, very branched and rich of lentils.

The aerial system is usually not very large, depending on the vigor of the variety. Branches show a typical green-red pigmentation in the first year, then turning into brown color and scaly appearance, since the second year. One year old branches can be divided into "wood branches" if they bear only vegetative buds, often quite vigorous, and "mixed branches", usually less vigorous than the former, bearing both flower and vegetative buds. Different tree growth habit exists, differing from the insertion angle of the branches and the internode lenght, traits under monofactorial genetic control (see figures 1.1 on the following page and 1.2 on page 13).

Leaves are lance-shaped, with a brighter green on the upper page. The stem is characterized by the presence of glands, formations made of secretory

../Nuova cartella/habitus_edited.JPG

Figure 1.1: Peach tree habits: ST = standard; SD = semi-dwarf; SP = open; UP = upright; PI = columnar (pillar); WE = weeping (reworked from [99]).

tissues that can be globous or kidney-shaped. There can be one ore more on the same stem, or the leaf can completely lack of them. The absence of this organs is strictly related to an higher susceptibility to powdery mildew (*Sphaeroteca pannosa* (Wallr.) var. 'Persicae').

Buds, alone or grouped by three, are at the armpit of the leaf. Usually, the central is the vegetative, while the laterals are the flower ones. Each bud develops just one flower.

The flower is characterized by a pentameric symmetry. The corolla, dialypetalous, display a color varying from pure white to bright red (monogenic traits), but pink is the most common. It can have various sizes, and two shapes are found: showy (large and open petals) and non-showy (little petals, bowed toward the center of the flower). There can be petaloidy, often associated to late flowering, that is interesting for breeding for ornamental purposes. The flower has 20-30 stamens, white, bearing reddish anthers. The female part of the flower is made of a lonely pistil, whose ovary has two ovules, but only one is usually fertilized. The calyx, gamosepalous, is inserted below the ovary. Inner calyx color is related to fruit flesh color: in yellow flesh cultivars the calyx is yellow-orange; in white-fleshed fruits the color is green-yellow. Anthesys starts before the sprouting of shoots. There's no correlation between flowering time and ripening time.

The fruit is a drupe, usually globous shaped, but a great degree of polymorphism is detectable in the specie. The fruit has a suture, which runs from the calyx pit to the apex of the fruit. The apex, as the suture, can be sunken, flat or outcropping, rounded or pointed. Skin ground color may show many colours, according to the flesh colour. Ground color can be hidden by a red overcolor (due to the accumulation of anthocyans), whose extension on the fruit surface and hue brightness depends on the cultivar (genetic factor) and on the growing conditions (environmental factors, i.e.: a good exposition to the sunlight increases the extension and brightness).

Flesh colour can be white or yellow. The former can vary from totally white to a more greenish hue; the latter can assume any nuance from pale yellow to a

bright, orange colour-like. However, independently from its color, the flesh can show anthocyanins build up in the vacuols, which may have even a considerable degree. This red pigments accumulation can be uniformly diffused in the flesh ("blood peaches": 'Sanguigna', 'Topazio' cultivars) or localized in some areas: under the skin ('Merrill Gem'), close to the pit ('Elberta'), in veins and strays in the flesh ('Redcap', 'Fairhaven').

The pit is woody, of various dimensions and colors (from red to dark brown) depending on the cultivar. Shape is elongate. The outer surface is furrowed, and the depth of this trait may vary considerably among the cultivars, but it is not useful for characterization. The flesh can be freestone or clingstone, depending on the adhesion of the flesh to the pit. The freestone trait displays a pit that is almost completely separated from the flesh, the clingstone has the flesh that is strongly connected to the stone. Many degrees of adherence may be detected in peaches and nectarines. It is not rare the pit fissure: in the upper part of the pit, close to the stem, there may be a cleft (that may be even visible from outside), that compromises the seed germination more or less irreparably: the cleft is a easy passage for parasites and pathogens, that can easily attack the seed.

1.1.2 Peach Breeding: state of the art and perspectives.

Breeding has been accomplished in an empiric manner until the dawn of 1750, in England, where the first records of cultivars obtained from intervarietal crossing can be found. Still, the "mechanics" of breeding ignored the rules that drove the breeding process.

Only at the beginning of the 19^{th} century, with Mendel's formulation of The Three Laws of Genetics, breeding could finally arose to the role of a real science. From there on, great results have been reached worldwide, even if a leading role must be reserved for USDA and Universities of California (Davis), Michigan, Massachusetts, etc, where many programs enlightened the mechanisms controlling the heritability of many traits. Many new cultivars have been introduced since. Not to be forgotten also the activities performed in other countries, such as Canada, France and Italy.

Peach can stand many cycles of self-pollination without inbreed problems, and therefore it's the fruit tree specie that has the highest degree of homozygous. Besides, peach juvenility is quite short compared to other species: seedlings can

produce fruits in the second-third year. As result, the inbreeding index for peaches is quite high [78]. However, peach breeders have yet a considerable number of genetic resources, whose importance is still growing: it is now common opinion that an high inbreed can be a serious problem for it can lead to bad influence on yield, disease resistance and flower fertility [99].

So far, most of breeders releases come from controlled crosses (around 50% of the total), open pollination (15-20% circa) or bud mutation (5%). Yet, for many releases it is impossible to clearly define the parental origin in the crossing lines: it is estimated that 12% of canning peaches and about 30% of nectarines cultivars comes from unknown sources[35].

1.1.2.1 Peach Genetics and Genomics.

Peach is one of the species whose genetic control is better known. One of the main reason is that peach genome is quite little, compared to other species: it has only 8 chromosomes (2n=16), not affected by polyploidy. Moreover, peach is not affected by self-incompatibility, even if male sterility may be present. Seedlings that bear this latter are obviously discarded from breeding programs, considering that in normal conditions more than 90% of the fruits on the same tree originates from self-pollination. Moreover, many traits of monogenic inheritance are known (see below: 1.1).

More recently, many researchers have added insights into the biomolecular bases underneath the expression of many traits, and some markers have been identified and mapped to facilitate the MAS (marker assisted selection), enhancing the efficiency of breeding.

For breeding purposes, traits can be divided into two main groups, based on their genetic heritability:

A) Qualitative traits. Traits are controlled by one or a few genes. They're also called "Mendelian" and are located on a single locus of the chromosome. The effect of allelic substitution is relevant, while the environmental effect on their expression is almost irrelevant. Many monogenic traits have been described so far.

A summary of known monogenic traits is reported in below (figures 1.2 on the facing page, 1.3 on the next page and 1.4 on the facing page).

B) Quantitative traits. Those traits are defined with measurement (and for this reason are also known as "metric"). They are controlled by many genes, and the level of such expression depends on specific interactions (e.g. additive

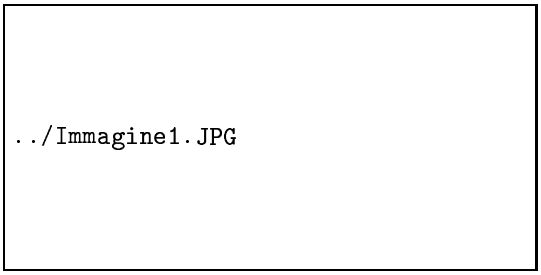

Figure 1.2: Monogenic traits described in peach (reworked from [99]).

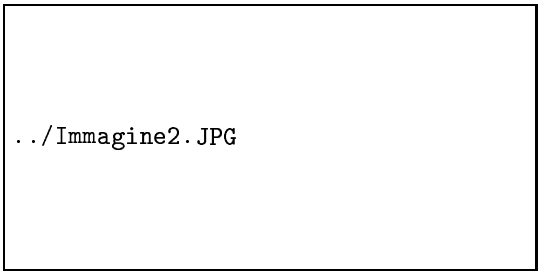

Figure 1.3: Monogenic traits described in peach (continued) (reworked from [99]).

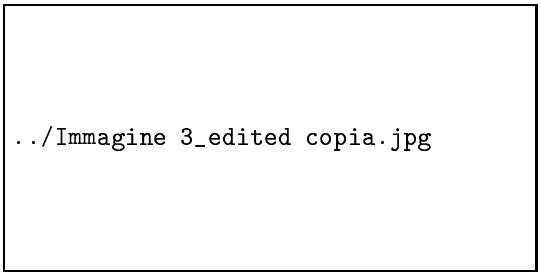

Figure 1.4: Monogenic traits described in peach (continued) (reworked from [99]).

and epistatic effects). In such a polygenic system, trait expression is slightly affected by the substitution of one or few alleles, but the environmental effect could be substanzial. This influence strongly dependss on the trait. Because of the relative high number of genes involved in their control, heritability of such traits is low. Since they're controlled by many genes, the alleles can be present on different chromosomes. A segregating population for these traits assumes a continuous distribution of the phenotypes. Breeding for this traits is quite time consuming. Most of the traits that are of horticultural (field) interest (yield, disease resistance, chill requirement, etc.) fall in this class. For a successful breeding of these traits it is required to choose parents with high phenotype value.

DNA regions in which a quantitative trait is most fully expressed is called QTL, quantitative trait loci. Genomic maps have been made to locate QTLs and candidate genes controlling those traits, and physical chromosome maps have been constructed [99]. Of many notable traits nowadays is known the position on physical maps: around 13 for powdery mildew resistance [34]; 2 for leaf curl, located in LG 3 and 6; 24 for blooming date, fruit size and shape, skin color, sugar and acid content [85]. So far, 31 major genes have been positioned on specific linkage groups (LG) of the eight chromosomes [34, 8]. Based on this data, a reference map for *Prunus* has been drafted: more than 500 codominant markers (mainly RFLPs and SSRs) have been positioned, with an average distance of 0.92 cMs, have been positioned on an almond x peach population ('Texas' x 'Earlygold': TxE map)[99]. Still, there are many "dark areas" poorly covered by markers, requiring further definition.

An important boost to these efforts has recently evolved from international programs from USA, Italy and Chile. The former, called genome Database for Rosaceae (GDR) at Clemson University, is designed to complete the characterization of species belonging to *Prunus* genus by developing RFLP and SSR markers on the TxE map. So far, more than 3,800 putative genes (over 2,000 in BACs, bacterial artificial chromosomes), and many insights have been provided, since one of the main goals of this project is the complete mapping of the peach genome. Italian project, called ESTree Center, is a joint effort between Milan University an other research centers for genomic studies on stone fruit trees, with peach as the model specie. Its goal is the complete sequencing of peach genome, and so far it has provided more than 8,000 ESTs (expressed sequences target) sequences. The latter is a consortium based effort to explore peach functional genomic, mostly for studying peach internal breakdown (chilling injury):

about 50,000 ESTs have been sequenced so far [99].

1.1.2.2 Peach breeding state of the Art.

It is said that the 20th century could be called "The Golden Age of Peach Breeding" for it saw the development of thousands of new cultivars and a remarkable enhancement of many fruit traits, such as size, blush extension, flesh-to-pit ratio, etc. [99]. Most of the novel releases of peaches and nectarines come from the private sector, whilst most of the breeding for canning peaches has been accomplished by public institutions [35]. A rapid glance at the main programs worldwide shows that the goals in nowadays programs are the followings [99]:

Environment. Environmental adaptability, which is headed to an upgraded cold resistance, especially important for Countries like Poland, Canada and Russia, high (to avoid frost damage) or low (to extend peach to warm latitudes) chill requirement [67, 41].

Seasonality. Extending seasonality introducing early-, late- and very late-ripening cultivars to gain a six-month calendar, since peach germplasm possess also winter genotypes that could be used for breeding.

Ornamental. Although not a priority for European programs, ornamental breeding is quite important in Countries like China and Japan, whose germplasm has many sources to work with. Moreover, such germplasm can be a source for useful traits like the ones for biotic and abiotic resistance.

Rootstock. All the goals are related to soil adaptability, although with differences between countries related to the peculiarity of their soils and biotic threats (such as nematodes or bacterial diseases like *Xanthomonas* and *Pseudomonas*). Another branch of research is oriented toward the management of vigor, enhancing it through crossing with other *Prunus* species (*P. davidiana* or *P. dulcis*), or reducing it, by crosses with *P. cerasifera*.

Growth habit. Trees that could be easily managed without recurring to ladders or machinery can lead easily to a dropping of management costs (pruning, picking, etc.). Since no good dwarfing rootstocks are currently available, researches are focused on breeding new cultivars whose growth habit is more likely to be fitting to high-density orchards. Some researches

are aiming to obtain dwarf trees (like in Romania), whilst others are breeding for weeping (like in France), or pillar (as in Italian and American programs) growth habit [69, 84].

Quality. Enhancing fruit quality traits related not only to appearance (like shape, color, firmness, shelf-life, aroma, etc.) and recovering traits typical of old, local cultivars (as in Europe). Many programs have focused on enhancing the nutritional or nutraceutical traits of the fruit, increasing fibres, vitamins and aromatic compound content. See 1.1.3 section for more details.

Diseases. Disease resistance is one of the most pursued goal. One of the greatest hopes is the introduction of the Sharka (PPV) resistance, but despite all the efforts, no source of resistance has been detected in peach germplasm, and tolerance alone cannot be considered as a success since tolerant plants can act as virus incubator. A possible alternative could be the resistance trait, since PPV is inoculated by the insect. *P. davidiana* is a source of multiple resistance (PPV, powdery mildew, etc.) and it has been hybridized to peach to introdce the resistance to the Sharka virus[80, 111]. Since no source is found in peach for PPV resistance, some efforts have been made toward introduction of resistance genes via genetic engineering, whose limiting factor is recalcitrance of peach to regenerate the entire plant by in vitro techniques [82]. Other, not less important, goals are the introduction of resistance to fungi (like powdery mildew, brown rot, leaf curl, etc.), low susceptibility to green aphid and codling moth and to bacterial diseases and nematodes.

1.1.3 Breeding for quality traits.

Since the end of the II WW the importance and the concept itself of "quality" have dramatically evolved for fruit production. At the very beginning, quality was considered luxury: Europe was kneed by war, and was aiming only to allow people to have access to food style, so that fruit quality was almost ignored. Only in the '70s quality begun to assume an increasing role (becoming a market tool), finally becoming -at the end of the '80s- a discriminant factor for market, a surplus value to the fruit itself. Nowadays, the concept of food quality is probably the other dish of the balance (Besides the prize) in consumer choice, an

increased attention that growers, dealers and breeders must perceive in planning their job.

Definition of quality is a problem that have been studied by many, but it is still without a common answer. A "compromise" definition could be that food quality is *"the correspondence of the food characteristics to precise standards"*. This definition postulates:

- ➢ for each product shall be codified quality standards, and those should possibly be structured in a way that allows a quick and easy identification of "quality levels";
- ➢ parameters used for quality definition should be as much objectively measurable as possible.

However, appears immediately evident that such definition, for foods -and consequently for fruits- is quite difficult, due to the complexity of the factors that take part in the play. Besides, quality subjective component is burdened by social and cultural factors, that may differ significantly from area to area.

Aiming to meet consumer expectancy, producers and sellers adopted a wide spectrum of tools aimed to create a "quality system" through the whole pipeline (from producers to consumers) suited to guarantee -as much as possible- an unaltered quality of fruit production. In such systems every operation should be carried on assuring that every factor that -directly or indirectly- have an influence on quality is not going to take part in its determination casually, but in a coordinated and controlled way in order to ensure the maximum of quality and its keeping.

In general terms, quality can be divided into two main groups of characteristics: "PRIMARY" and "SECONDARIES". The former are directly related to the product: color, shape, flavor, attractiveness, etc., but also everything regarding nutraceutical aspects and responds to laws (i.e. pesticide residuals). The latter are tied to the "landscape" where the product is: the way it's sold on the marketplace, the ease of purchasing it, the relationship between consumer and produce (traditional fruit or new release, typical of the area or coming from other Countries, etc.), the source or the processes that has occurred to the fruit along the pipeline (biological, integrated or traditional production, post-harvest treatments, etc.).

Peach quality, for the different types, is strongly affected by many factors, among which the commercial destination. The same traits that are in high regard for a peach for fresh market are often not requested for canning peaches.

This is the case of canning peaches, where overcolor is not considered of any importance, since the fruits are peeled; viceversa, for those peaches is more relevant the uniformity of flesh color and the resistance to browning caused by termic treatments.

Peach primary quality traits can be divided into two more categories: "**extrinsic**" (size, color, shape, etc.) and "**intrinsic**" (handling resistance, shelf-life, flavor, etc.). Considering this classification, however, must be taken into account that is quite impossible to classify quality traits into well defined categories, since each component interacts, more or less directly, with the others. A complete dissertation about this aspects is quite impossible, since for almost every Country there is a specific "fruit fashon". Here we'll discuss about the main quality aspects considered for the european market.

1.1.3.1 Extrinsic traits.

This category includes those traits that are immediately perceived by consumers. For this reason, those traits are subject to a great variability, depending upon the periodical (and, often, unexpected) consumer's variation of fancy. Most of the times, to this group of attributes belong quantitative traits.

Appearance. Skin color is mainly determined by carotenoids, with a particular relevance of the β-carotene [112, 62, 33]. In a ripe peach carotenoids are about 30 mg/kg of dried weight. Ground color should be yellow or orange-yellow in yellow-flesh peaches, while in white-fleshed ones should tend to a clear white color, without the greenish hue. Changing from green to yellow is due mainly to degradation of the skin chlorophyll [90]. Fuzziness of the skin is a quality trait whose relevance is often shifting. generally speaking, a small amount of hair is considered a valuable trait, because if too developed tends to give a dull hue to the skin, and is almost disliked when the consumer has to eat the fruit. Moreover, skin should be without any rusty zone or bruises, and be as smoother as possible, without bumps or digs.

Overcolor is the trait that is mostly responsible for attractiveness, and a fruit with a good commercial value can't have a broad extension of it [41]. Ideally, the whole fruit should be covered by overcolor, masking completely the ground color, that should appear only around the stem. To the expression of this trait contribute anthocians, but those -contrary to carotenoids- are quite sensible to heat and are undergoing to denaturing processes is the peach sustain termical

treatments [112]. The brighter the overcolor, the more will be appreciated the fruit. Lacking of fuzziness, nectarines have usually a brighter overcolor than peaches. Non-melting flesh peaches have significantly higher total carotenes (althought considered that arotenoids content decreases during the postharvest phase) and xanthophylls than their melting-flesh counterparts [66].

Flesh color is not as much important as the skin one, but generally speaking is much more appreciated the uniformity of color, without marbling, especially if too intense. More tolerated are marbling around the pit in peaches and nectarines for fresh consumption; canning peaches require, however, no traces of anthocianines, since heat treatments used for pasteurization cause pigmentation to oxidize, leading to a dark-brown hue of the flesh.

Size and shape. Fruit morphology is extremely various, shifting from elongate to flat. However, both for peaches and nectarines, the most appreciated form is the rounded one or slightly elongated [41]. A flat or doughnut shaped peach (i.e. 'Peento') has long been known in China. Although they were known to breeders, little interest has been shown in this shape in Western countries until recently: shape was irregular and the fruit were susceptible to severe splitting at the calyx end. Recently flat peaches and nectarines have been bred and are nowadays largely free of these defects. Flat peaches are a source of genes for higher sugar levels and low acidity ("honey peaches", see 1.1.3.2 on page 22). Suture should be not outcropping too much: the best appearance is judged the one with a flat or even slightly burrowed. Most consumers appreciate an apex which is flat or just visible.

About size, quality standards is quite variable, depending also on the ripening season. A fruit is considered having a good size if weights more than 120 g [26]. More liked are fruits whose weight is more than 150 g, and consumers usually reward such fruits with higher prices paid. However, fruit sized more than 200 g are considered having a lower quality: fruits are too big to see -and eat as well- and often with a less flavoured (due to the elution of sugars and volatiles in the flesh). The size is also correlated to shelf-life: the smaller the fruit, the lower the probability of mealiness from chill damages (even if stored in controlled atmosphere or low-temperature storage) [32, 30, 123].

1.1.3.2 Intrinsic traits.

In this category can be found most of the traits involved in fruit resistance to handling and in conservation. For that reasons, they're not immediately appreciated by the consumers but by producers and distributors. Belonging to this group of traits are also those that contribute to gustative quality (flavor and aroma).

Handling resistance. Resistance to handling is related to the sensibility of the skin and the first layers of flesh cells under the skin to hurts and bumps [41]. Browning consequential to such traumas is cause by oxidation of poliphenols contained in the cell sap of flesh and skin: at cell level, any trauma leads to the rupture of the cell walls and membranes, exposing those compounds to the atmosphere, allowing the oxidation reaction.

Although the probability of this are almost the same for yellow- and white-flesh fruits, the phenomenon is much more evident (and problematic) in peaches and nectarines with a light-colored skin and white flesh.

Shelf-life. Is the characteristic that allows the fruit to keep a good level of firmness through time, retarding the loosening of tissues. Many are the factors affect this trait, which is particularly important both for distributors and consumers since a prolonged shelf-live means that the fruit will keep almost unaltered its mechanic and sensory characteristics.

Storability. Skin integrity and health state of the fruit are heavly inciding on this trait. Bruises and batters, in addition to all the problems related to the appearance of the fruit, are a way throgh diseases can attack the fruit, compromising irreparably and swiftly the characteristics. Moreover, oxidation it's a reaction which is not stopped with cold storage, and goes on alonthe the post-harvest life of the fruit.

Pit adherence is strongly related to the keeping of flesh juyciness for a long time after harvest: freestone peaches have a lower retainment of juices than clingstones, and this seems to be due to tha absence of an "empty space" between the pit and the flesh, leading to a slower loss of water from the tissues [15, 16]. Water loss is variable depending on the cultivars [31, 42].

Sensorial traits. When ripe, peach fruit is made for less than 90% of water. Soluble solids can vary around 10-13% of the total, whilst organic acids

can be present around 0.5-0.7% and nitrogenous compounds for about 1% (see table 1.1).

Compound	Amount
Water (%)	90.7
Proteins (g)	0.8
Fats (g)	0.1
Amids (g)	2.1
Soluble sugars (%)	10-13
Fibers (%)	31-36
Calories (Kcal)	27
Sodium (mg)	3
Potassium (mg)	260
iron (mg)	0.4
Calcium (mg)	4
Phosphor	20
Thiamine (mg)	0.01
Riboflavin (mg)	0.03
Niacin (mg)	0.5
Vitamin A (μg)	27
Vitamin C (mg)	4

Table 1.1: Peach flesh average composition (reworked from [26]).

Many factors are involved in flavor expression, but sugar and acid contents (and the ratio of the two) are the ones with the greatest relevance [31, 42, 44, 43]. Being so graved by personal taste, a definition of quality is hard to define for this trait. Below will be treated each of the aspects that are undergoing to this main subject.

Aroma. Aroma is determined by volatile components produced by peaches in concentrations that can be perceived by the human nose. Aroma compounds arise from several different substrates including fatty acids, amino acids, phenolics and terpenoids account for most of the important flavour volatiles in fruits.

Typical ripe peach aroma is mainly due to the presence of many aldheydes, esters, ketones, lactones and terpenoids (responsible for the "flowery smell"), sulphur-containing compounds, acetaldehyde, ethyl alcohol and ethylene [117]. Lactones, benzaldehyde and other volatile compounds decrease during ripening process, while others tend to increase during the post-harvest life of the fruit [113]. Major compounds forming peach aroma are reported in table 1.2 .

LACTONS	ALCOHOLS	ALDEHYDES
γ-exalacton	hexanol	Exanal
γ-heptalacton	trans-2-exanol	trans-2-exanal
γ-octalacton	cis-3-exanol	benzaldehyde
γ-nonalacton	**TERPENOIDS**	**ESTERS**
γ-decalacton	α-terpinen	Esil-acetate
γ-dodecalacton	γ-terpinen	Trans-2-esil-acetate
δ-decalacton	terpinolen	
δ-dodecalacton	linaiol	

Table 1.2: Main volatile compounds identified in peach (reworked from [117]).

Sugars. Commonly, peaches are associated to an idea of sweetness. In the total of sugars concurring to this characteristic it is possible to highlight some differences both in chemical species and sweetening power. All sugars come from the conversion of sucrose and sobithol (both of them deriving from photosynthetic carbon) in the different forms. In the same tree, fruits can have a quite varying amount of those sugars , depending on many factors interacting each other, like position of the fruit on the tree and branches, fruit density, sun exposition, etc.. Accumulation of sugars is tightly bound to the third phase of fruit growth (see paragraph 1.1.5.1). In particular, an high growth intensity can lead to a lower accumulation of non-reducing sugars [44].

Sucrose, glucose, fructose and sorbithol are the main sugar species that can be found in peaches, followed by xilose, xilithol, mannose, maltose and inositole, that are present in very low amounts compared to the formers [4]. However, for sweetness perception the relative importance of those compounds is very different. Ordered by a decreasing sweetness, we have: fructose, sucrose, glucose, sorbithol and inosithole [45, 37, 118].

Sugar quantity in flesh is commonly expressed through the measurement of solid soluble refractometric (SSR), by a refractometer expressed in °Brix. This value, for quality purposes, varies depending the fruit type. For nectarines with "low acid" trait [42], an SSR value of 10-12 °Brix can be considered as the threshold below which the flavor is to be considered as insufficient, while for peaches with the same trait the threshold is lower. Some studies have defined as a good sugar content for peaches an SSR value between 8.5 and 13.0 °Brix [4]. Noticeable is a characteristic behaviour of white-fleshed peaches (and nectarines) compared to the yellow-fleshed ones. Even if there are no differences for total quantity of sugars, in the white-fleshed ones the glucose and fructose ratio on

the total is higher than in the yellow ones [92]. This could easy explain the reason for a sweeter flavor of those peaches.

Acids. Acid perception and so the preference toward different levels of acidity is varying with the individual taste of costumers [32]. Peach fruit main acids are malic and citric, that can reach in the flesh concentrations of 14 and 12 meq/100g of flesh respectively [44]. Other acids, like succinic and quinic have a lesser importance, both in quantitative and qualitative terms.

Among those, the one that is more responsible for the perception of tart flavor is the malic acid, followed by the others. An higher concentration of citric acid is often related to a big diameter of the fruitlet at the moment of thinning; a low concentration and an higher amount of malic acid are positively related to an higher and earlier ethylene fruit emission [44]. An optimum value for acid content is not easy to define. Therefore, it is preferred to define their optimum in ratio with sugar content. This ratio, that is really varying among different cultivars, is considered at its best when between 7.8 and 8.5, with an organic acid concentration in the flesh of 1.0-1.6 g/100g of flesh fresh weight [4].

To be highlighted is the "low acid" trait (see figure 1.4 on page 13). In low acid phenotypes the amount of malic and citric acid is poor, below 4-5 meq/100g of flesh fresh weight. This trait enhance the sweetness of sugars, conferring to the fruit a flavor quite different from the normal fruits. On this trait is supposed to have a major role the concentration of quinic acid: more than 30% in those phenotypes, against a 20% of normal fruits) [42, 115]. The relevance of this trait is discussed, since European consumers seem not to like such kind of peaches, while in the far east this trait is much more appreciated. Anyway, there's no way to detect this trait by morphological markers, only through analytical ways or by taste [32].

Tannins and phenols. Tannins are important because they are responsable of fruit astringency, phenomenon due to capacity of those compounds to bind and precipitate salivar proteins. Decrease of astringency, and conseguential increase of fruit edibility are related to a change in quantity and typilogy of tannins. Tannins are localized mainly in big cells of the mesocarp [100]. An increased dimension of condensed polymers (phenoles and polyphenoles) formed from tannins is responsible of the loss of astringency, because such compound lack of the ability to bind proteins. Deposition of tannins monomery (chatechins, epicathechins and anthocyanidines) begins in the frist steps of fruit develope-

ment, with a peak between the frist and the second developing phase; phenoles formation increases immediately and sensibly in the following phase. For a good quality peach tannins content should be as lower as possible.[113].

Anti-oxidant activity of phenolic compounds is closely related to their molecular structure [91], in particularly the B-ring hydroxylation level and glycosylation extent. Glycosilated forms are normally characterized by a lower anti-oxidant activity compared to the non-glycosilated ones. Flavanols, which are glycosylated forms of quercitin and kampherol are the most abundant phenolics in peach and other stone fruits [7, 59]. Myricetin has been proposed as a phenolic marker for peaches since this flavonol is not present in other fruit species [39]. The phenolic profile appears to be consistent among varieties, although concentration of each compound may vary in different cultivars and fruit tissues (higher in zones clos to the pit and skin). Cyanidin and glycosylated quercitine are present mainly in the epicarp, while cinnamic acids (including chlorogenic and neo-chlorogenic acids) are the most distributed in skin and flesh.

Vitamins. One of the most important aspect in determining nutritional value of a fruit is undoubtedly bound to vitamin C (ascorbic acid) content. The higher the amount in the peach, the higher the quality of the fruit, although peach fruits are scarsely provided of this vitamin compared to other fruits. Ascorbic acid content in the flesh is rather variable, but in the average it is present around 7-10 mg/100 g of fresh weight [70].

Carotenoids are converted to vitamin A after ingestion. Yellow flesh peaches are considered as good source of provitamin A-carotenoids, mainly β-carotene and β-cryptoxanthin [48]. Carotenoids are unstable when exposed to low pH, oxygen and light [**?**].

Nowadays, many programs are focusing on the increase of the content of this compounds (see paragraph "breeding for quality" on page 16).

Fibers. Complex carbohydrates in fruits (such as cellulose, hemicellulose, and pectins, see 1.1.5.2.2 on page 31), which are not digested by humans, include dietary fiber [98]. Fiber consumption, mainly the soluble forms, decreases plasma cholesterol and glycaemic response. Recent studies reported that total dietary fiber constitutes about 31-36 % of dry matter in peach concentrates with insoluble fiber being the major fraction (20-24%) [49].

Melting and non-melting flesh peach types differ in pectin content and solubility, and this influences fruit softening [66, 83]. These differences may be

of interest from a nutritional point of view, because of the well recognized role of fiber in regulating intestine function, peristaltic movement and, as a consequence, the hunger and/or satiety sensation.

1.1.4 Peach flesh firmness and texture.

Flesh firmness can be considered as a quantitative trait, being affected by several biochemical and physiological factors, such as differences in the levels of apoplastic Ca^{2+}, regulation of cell turgor in relation to plasma membrane functionality and levels of intracellular osmolytes, changes in cell wall architecture (flesh texture), etc. The identification and cloning of genes of known function (i.e. genes involved in cell wall loosening) allow evaluation of the possible effects of their polymorphism/s on flesh firmness variability [77]. Large varietal differences in the softening and maturation characteristics of peach fruit exist, and therefore great phenotypic differences can be described.

For peach fruits it is possible to identify at least three different flesh texture types depending on softening process, that will be exposed below.

1.1.4.1 Melting Flesh texture (MF).

Since this trait is controlled by one gene ("F locus", also responsible for pit adherence, see 1.1 on page 12), melting flesh is expressed whenever the *"F"* allele is dominant: *F/-* or *F/n* (melting freestone) and *f/f*, *f/f1* or *f/n* (melting clingstone). A recent study demonstrated that endopolygalacturonase gene is responsible of the expression of flesh texture and pit adherence [81]. Evolution of ripening is fast, with a noticeable flesh softening, due to an high content of water-soluble pectins and a low concentration of insoluble ones [35]. Also Ca^{++} bound to the cell walls is quite low [5, 119]. Melting fruits are characterized by a noticeable production of ethylene during ripening accompanied with rapid loss of firmness in a few days after picking, whose speed depend strongly on the cultivar[5, 55], in the late phase of ripening ("melting phase") that coincides with the climacteric peak [81, 68].

Within this flesh type a great degree of variability exist regarding flesh texture, from very soft to very firm. Generally, soft melting flesh peaches are characterized by a rapid loss of firmness, that is medium-poor when the fruit is full ripe, and an ethylene production between 5 and 25 ppm/kg/h. They show also a very poor quantity of insoluble pectins compared to very firm phenotypes.

The "very firm" type has an unique behaviour: when fully ripe shows an high degree of firmness, and a very high production of ethylene, often over 65 ppm/kg/h, and an higher insoluble pectin content [5]. No extensive studies have been carried on so far, hence this relation could be loose or just accidental.

Most of peach and nectarine cultivars intended to the fresh consumption are belonging to this category.

1.1.4.2 Non-melting Flesh texture (NMF).

Non-melting fruits are not undergoing to the rapid softening seen for melting ones and remain relatively firm throughout ripening, without showing the "melting phase". Even when ripe, consistency is rubbery. Biochemically, it is characterized by a cell wall water-soluble pectins and Ca^{2+} concentrations higher than the previous phenotype [5, 17]. The difference between melting and non-melting seems to be due to the presence/absence of enzymes involved in softening, particularly endopolygalacturonase [56, 21, 15, 68]. Ethylene production is usually higher than in the melting phenotypes, ranging from 10 to 25 ppm/kg/h (sometimes also exceeding 50 ppm/kg/h), with a sudden growth [14].

Overall, the changes in endo-PG levels and endo-PG expression in NMF and MF peach fruits during softening were been extensively studied [77, 74]. The difference in softening between melting and non melting cultivars was previously attributed to the presence of both endo- and exo-polygalacturonase in melting cultivars, whereas non melting cultivars had only endopolygalacturonase [83, 55]. Non-melting flesh phenotype have an allelic combination of *f1/f1*, *f1/n* or *n/n* [81].

Cultivars intended for canning belong to this category [15, 17].

1.1.4.3 "Stony hard" Flesh texture (SH).

Stony hard texture is characterized by the almost complete absence of ethylene (less than 10 ppm/kg/h) production and post harvest softening in ripe fruit [56, 42, 5]. Therefore, stony hard fruits retain their firmness for long time after harvest, keeping a crispy rather than "rubbery" texture as in non melting phenotype. Cells contain a higher Ca^{2+}than melting phenotypes, but lower than non melting ones, and a pectin formula similar to the melting very firm one [5]. Stony hard is believed to be the result of a mutation in ethylene production [57, 55].

Within the stony hard texture is possible to identify two distinct behaviour: a"*cripsy type*" where ethylene emission is almost zero toward the end of fruit life and a loss of firmness almost undetectable, as in 'Yumyeong' [55]; a "*melting type*", that shows a small peak of ethylene emission at the latest phases of softening (but less than 10 ppm/kg*h) and a melting phase at the very end of the fruit life, as in 'Big Top' nectarine.

Stony hard flesh is determined by the recessive omozygosis at "*Hd*" locus (*hd/hd*). Although "stony hard" flesh produces little or no ethylene, fruits are sensitive to exogenous ethylene. Recent works demonstrated that "stony hard" fruits, if treated with ethylene, show a softening process that can follow both the melting- and the non melting-path, depending on the allelic combination of the "*F*" locus, since the "stony hard" gene is epistatic to the former [56]. In other words, if the allelic combination is *F/-*, *f/f*, *f/f1* or *f/n*, and is treated with exogenous ethylene, although "stony hard" fruits will soften with a pattern typical of melting fruits (see 1.1.4.1 above); Besides, if the allelic combination at "*F*" locus is *f1/f1*, *f1/n* or *n/n*, stony hard fruits will undergo to the same softening process previously seen for non-melting fruits (see 1.1.4.2).

It is supposed that lack of large amounts of ethylene production at the end of the ripening ripening stage might suppress the cell-wall degrading enzymes that would be otherwise stimulated by the hormone [105]. However, the stony hard mechanisms are still almost unknown: little biochemical explanation has been given so far, and many dark areas still remain to work on.

In some far-east cultivars (i.e. 'Hakuto' and 'Yumyeong') seems to exist a genic association between stony hard and pit adherence. Most of the stony hard peaches show the "low acid" trait [42]. There is no linkage between these two Mendelian traits, but when the low acid trait is lacking, the "stony hard" flesh is very acidic.

Table 1.3 on the next page reports a summary of the main differences between those phenotypes.

For sensorial aspects, among those three phenotypes listed above there are little differences [58, 55]. Moreover, no effective tools for flesh type discrimination have been developed so far. There is need for a method capable of detecting precsely the texture phenotype for melting flesh ("firm", "very firm", etc.) and to distinguish between "stony hard" and non melting flesh without recurring to ethylene measurement.

Moreover, the only ethylene production seems not to be an objective tool, since it is too strictly related to many factors that can skew the result (ripening

Flesh Type	Pectin content		Ca^{2+}content	Ethylene (ppm/kg/h)	Firmness
	Water-soluble	Non-water soluble			
MF (firm)	High	Poor	+	10-25	Medium-poor
MF (very firm)	High	High	+++	>10, often >65	High
Stony hard	High	High	++	<10	Very high
NMF	Very high	High	+++	>10, often >25	Very high

Table 1.3: Peach flesh genotypes (reworked from [99]). MF = Melting flesh; NMF = Non-melting flesh.

evaluation, sanitary state, etc). Nowadays, the only molecular marker available for detecting melting- from non melting-flesh have been developed using endopolygalacturonase gene [81].

1.1.5 Peach ripening and softening.

Peach fruit development follows a typical "three phases model", a double-sigmoid curve characteristic of all the stonefruits. The first phase, that follows anthesys, has a variable lenght (four to five weeks), characterized by a low diametric growth of the fruitlet and an intense cell multiplication (cytokinesis). The second phase shows a noticeable reduction of cell division, due to the lignification of the endocarp. Lignification requires intense metabolic efforts, explaining the reduced fruit growing rate. This phase is particularly marked in late-ripening cultivars: early ripening ones, often, have a very short lignification phase, driving to an incomplete lignification that can cause pit cracking. The third phase is characterized by cell stretching, that is the expansion of cells due to metabolic products in the sap, that will determinate final fruit size. This phase is characterized by a noticeable growing speed reprise, toward the ripening of the fruit and a parallel increase of the water requirement of the tree [23].

1.1.5.1 Fruit ripening.

Ripening can be considered as a syndrome, that is a series of biochemical and physiological processes that drive the fruit to the final stage of its development, making it suitable to human consumption or seed dispersal through animals that feed on it. This syndrome, on the producer point of view, constitutes the fundamental -but non conclusive- stage of fruit life [46].

Fruit ripening is a complex, genetically programmed process that culminates in dramatic changes of color, texture, flavor and aroma of fruit flesh [2]. Ripen-

ing brings the fruit to assume some macroscopic traits: starch stored in vacuoles is converted into soluble, simple sugars (increasing fruit sweetness); chlorophyll lysis and pigments and thocyans syntesis drives to a tonig from the green color typical of immature fruits to the one of the ripe fruit; acids are used as a substrate for respiratory processes; aromatic volatile substances are developed; etc.

Usually, ripening proceeds not in an uniform way in the tissues of the same fruit: usually, the process moves from the area surrounding the endocarp toward the skin, and from the peduncle toward the calyx area. On the basis of such variations are important modifications at cell level, complex and hardly analyzable separately. Ripening is also influenced by many other factors, like exposition to sunlight, fruit load on the branch and position of the single fruit in the tree (i.e., fruits on the tree top are usually early ripening). The most characteristic trait of ripening is, however, the softening of flesh tissues. This phenomenon is caused by a certain numbers of factors:

1. Cell wall degradation.

2. Starch degradation: in some fruits starch represents, when unripe, a noticeable percentage of fresh weight (such as bananas). In peach fruits, however, this process is not relevant.

3. Loosening of cell turgor due to dehydration (non-physiological process, associated to post-harvest phase).

Of the three factors, the most important one is the last, cell wall degradation. Fruits are categorized as climacteric (i.e. tomatoes, avocados, bananas, peaches, apples) and non-climacteric (strawberries, grapes, citrus fruits, etc.) based on ripening process, in particular ethylene and respiration production.

Climacteric fruits. fruits belonging to this class are characterized by a noticeable increase of the respiration rate and biosynthesis of ethylene during ripening. Climacteric fruits require, for the coordination of the factors involved in ripening and the accomplishment of the maturative syndrome, the presence of ethylene. In those fruits the increase of ethylene emission usually precedes the CO_2 one (but literature reports also examples of climacteric fruit behaving oppositely, such as mango). Marked differences have also been observed in relation to the duration of development: the shorter the developmental cycle , the higher and more pronounced the respiration rate at the climacteric [116].

In climacteric fruits, as peaches and nectarines, ethylene and endo-polygalacturonase (endo-PG) are in close relationship with flesh softening. however, ripening and softening processes differ greatly between species, even among genotypes of the same specie (i.e. peach), and ethylene-dependent as well as ethylene independent regulatory mechanisms co-exist [60]. The central role of ethylene in climacteric fruit ripening is confirmed by the evidence that ripening syndrome can be accelerated or reduced through somministration of exogenous ethylene or the inhibition of endogenous ethylene production [9].

NON-CLIMACTERIC FRUITS. Non-climacteric fruits do not require ethylene to accomplish ripening precedes, even if some species (such as citrus fruits) can be influenced by ethylene exogenous [61, 47]. Recently, however, studies conduced on grapes and strawberries revealed that this fruit too are influenced by somministration of exogenous ethylene [108, 25]. Hence, the distinction between climacteric and non-climacteric fruits is less clear-cut than before.

A schematic representation of ripening in relation with the two factors influencing climacteric and non-climacteric ripening is evident in the figures 1.5: it is evident how in the ripening stage the former show a remarkable increase of ethylene and CO_2 production. However climacteric peaks are highly variable and range from 15 to 80 ml CO_2/Kg/h according to genotypes including yellow vs white flesh, melting vs non-melting, clingstone vs freestone [14, 116].

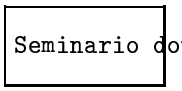

Figure 1.5: Climacteric and non-climacteric evolution of ethylene and CO_2 emission.

1.1.5.2 Cell wall.

Plant cells are coated by a rigid, fibrous wall that is called "cell wall". This structure has many functions: rigidity and mechanical resistance (structural support, shape and dimension determination); protection from dehydration, pathogens and environmental stresses; reserve of metallic ions and carbohydrates; contribution to signaling transduction and adhesion between adjacent cells. In cross-section, cell wall is formed by three concentric layers, differing for the composition, structure and function: lamella, primary wall and secondary wall (1.6 on the facing page).

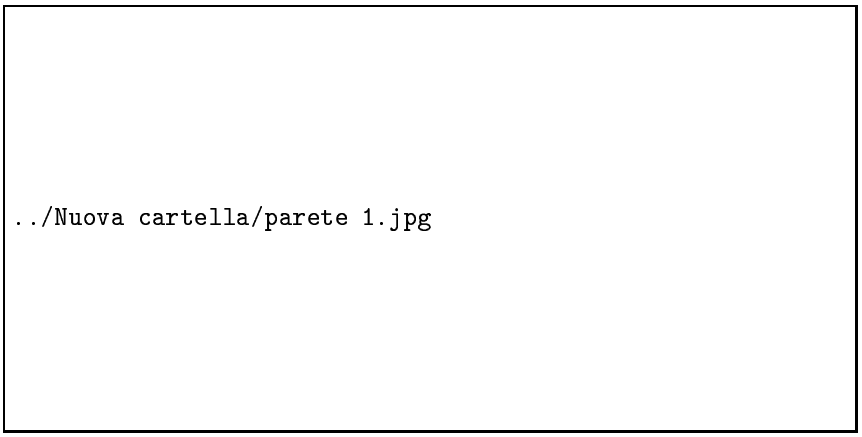

Figure 1.6: Cross-section of a vegetal cell ([122]).

1.1.5.2.1 Lamella is the outern layer of the cell wall. It is made by a thin pectin layer. Thanks to the adheseveness nature of polysaccharides that compose it, it is shared by contiguos cells and has the role to keep them glued aven after the differentiation. Calcium ions are responsible of the stability of bounds between lamellas of adjacent cells: Ca^{2+} administered as anti-senescence in post-harvest treatments accumulates in this area, rendering bounds stronger and reducing flesh softening [20].

1.1.5.2.2 Primary wall is made of microfibrils of cellulose immersed in a matrix composed of two classes of polysaccharides: hemicellulose and pectins.

Cellulose is a linear molecule (made of glucose monomers linked by β-1,4-glucosidic bonds) organized in microfibrils, in chains made of two molecules each, kept tied togheter by crossed H-bonds, originating a highly ordered structure [93, 29, 71] : figure 1.7 on the next page. To the cell wall are associated also many structural proteins (whose typology and quantity vary depending on the tissue, development stage, external stimuli, etc.) and enzymes (taht can have many functions, from cell wall etension to pathogen interaction) [29].

Hemicellulose is made by a miscellaneous group of non-crystal glicanes (see figure 1.8 on the following page) associated to cellulose by non-covalent bonds, long polymers of mono-, di- and tri-saccharides (xilose, galactose and fucose) bound by β-1,4 glucosidic bonds [93, 29]. Those chains tie toghether

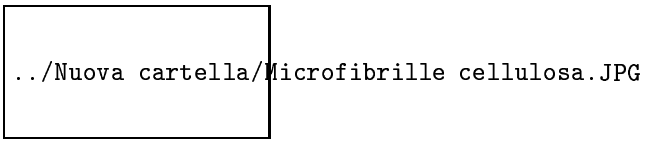

Figure 1.7: Cellulose structure (reworked from [122]).

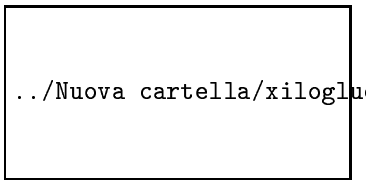

Figure 1.8: Xiloglucane molecules.

many cellulose microfibrils, forbidding their movement.

Pectins are the matrix where all the others components are immersed (figure 1.9). Those polysaccharides are the most soluble of cell walls, and are made mainly of galacturonic acid molecules bound by β-1,4-glucosidic bonds (but other monosaccharides, such as ramnose, can be present), often tied toghether through bivalent ions (i.e. Ca^{2+} and Mg^{2+}), see figure 1.10. Each ion binds and salifies two carboxilic grupos belonging to different chains. From this derives a reticular structure, gelatinous and highly adhesive toghether [71].

Figure 1.9: Undifferentiated cell wall representation.

1.1.5.2.3 **Secondary wall** is leaning against the inner face of primary wall, and is gradually thickened in centripetal direction. In the various concentric layers that are deposed microfibrills are parallel to each other. this structure presents a greater percentage of cellulose and a lower amount of non-cellulose polysaccharides compared to primary wall. Depending on the differentiation that occurred to the cell, secondary wall can contain molecules that are conferring it different properties: cutin and suberin (impermeability); lignin (mechan-

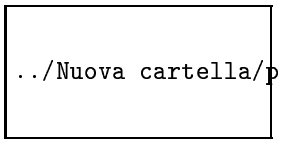

Figure 1.10: Pectin structure (reworked from [122]).

ical resistance); mineral salts (increased wall hardness) [71].

Continuity between cytoplasms of adjoining cells of a same tissue is assured by plasmodesmatas, prolongations of cytoplasm that cross cell walls through structures called punctation, coated with cell membranes.

During ripening, cell wall undergoes important changes that take the fruit to assume a lower firmness and, in the more advanced steps of ripening, a literal deliquescence. Some changes related to ripening are universal (like the de-polymerization of the glicanic matrix), while others work in different ways or are completely absent in other species (i.e., there's no loss of galactose in watermelons and plums) [18]. Those modifications are operated by a complex array of degrading enzymes and through important changes of the structure of cell wall polysaccharides, many of which are directly influenced by ethylene presence.

1.1.5.3 Ethylene: biosynthesis, perception and transduction of the signal.

Ethylene (C_2H_4) is a gaseous hormone, biologically active at very low concentrations. The highest ethylene production occur in senescent tissues or ripening fruits, but all the tissues of a plant can synthesize it. Being a gas, its permeability through cell membranes is really high. In this way, diffusion throughout plant tissues (independently from the phenomenon that triggered the emission) is extremely fast, capillary and especially uniform, differently from any other hormones, that must be translocated through xylem and phloem.

Hormone action is complex, and varies depending on tissue or organ, development stage and interaction with other regulatory factors. In the plant, in addition to the role in ripening process, ethylene performs also numerous other functions, related both to vegetative phase (leaf and fruit abscission, epinasty, plantulas accretion, regulation of seeds and buds dormancy, etc.) and response to biotic and abiotic stresses (wounds, pathogens attacks, etc.) [97]. Ethylene biosynthesis and action might be involved in the regulation of carotenoid biosyn-

thesis during ripening, since 1-MCP treatments applied at a preclimacteric stage reduce the level of pigments [75]. With a few exceptions, all the tissues that are wounded or mechanically disturbed, increase sensibly (within 25-30 minutes) their ethylene production. After this burst, ethylene concentrations go back to normal values. Also adverse environmental conditions (relatively to oxygen, temperature and light) influence positively ethylene production: stresses change cell membrane proprieties, and these variations seem to be responsible of the synthesis induction. Ethylene activity increases at the oxygen concentration raising, and diminish as CO_2 saturates the environment. Many inshights on ethylene importance for ripening of fleshy fruits and of its pathway have been obtained studying tomato mutants [101].

The processes whose regulation depend on ethylene coexist both in climacteric and non-climacteric fruits [2]. In peach it seems that ethylene doesn't have an important role in fruit ripening without softening, because both in stony hard and non-melting flesh peaches color changes normally, flavors are normally expressed and SSR content is at high levels [105].

Ethylene biosynthesis. Methionine is the ethylene precursory. On methionine acts a S-adenosyl-methionine transferase that transforms the compound into S-adenosyl-methionine (AdoMET), that is an intermediate shared between biosynthesis of ethylene and polyamines. AdoMET, through Aminocyclopropane-carboxylic-sinthetase (ACS) action, originates methyl-thioadenosyne (that is converted to L-methionine and re-leaeded in the circle) and 1-Aminocyclopropane-1-Carboxylic Acid (ACC) [121].

ACC, under the action of ACC-oxidase (ACO), is oxidated to Ethylene (C_2H_4), HCN and CO_2 . ACC can be conjugated with Glutathione or Malonate, through the enzymatic way, producing MACC (Malonil-ACC) or GACC (Glutation-ACC), that can constitute acumulation forms for the plant in case of stresses. However, conjucation is energetically unfavourable, can happen only if ACO is saturated or absent [103] (see figure 1.11).

During vegetative stage, the limiting factor of ethylene production is ACS: in vegetative tissues ACS is deactivated by AdoMET, so occours ex-novo synthesis of the enzyme in order to obtain new ACC [114]. ACS activity is not influenced, in this stage, by exogenous ethylene: induction of this enzyne is hence depending on the fruit development stage [6]. During ripening, on the other hand, the ethylene signal trasduction key-enzyme is ACO, codified by a multigenic family (in peach two are known: PpACO-1 and PpACO2) [96, 106].

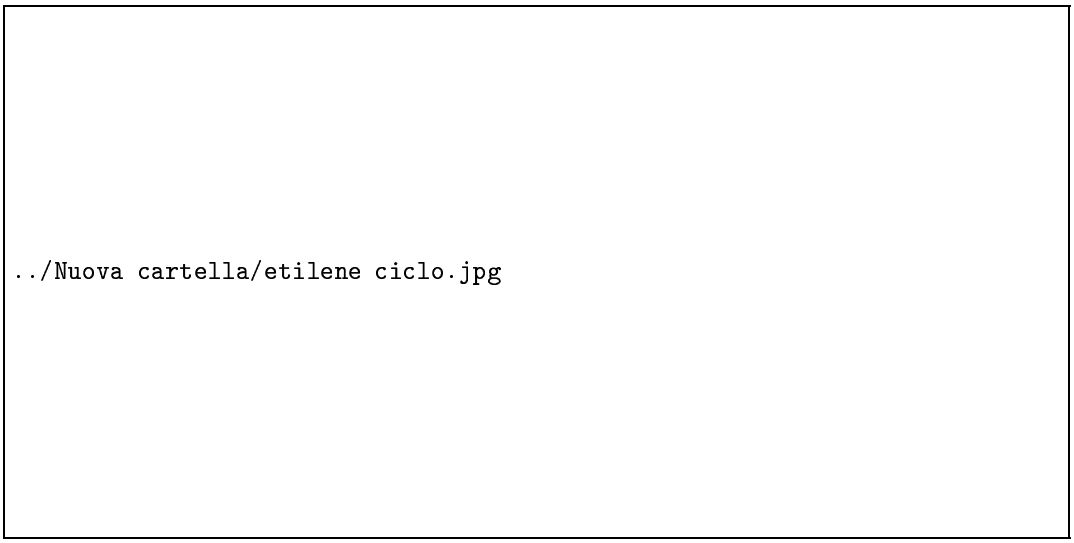

Figure 1.11: Ethylene biosynthesis (from [103]).

Ethylene signal pathway. During fruit development, until the onset of ripening process, ethylene inner concentration is lower than 1 μl/l. This minimum level is defined as "***System 1***" , responsible of the basal level of ethylene during growth, insensitive to exogenous ethylene and self-inhibiting endogenous ethylene production [6].

At the last stage of ripening, however, ethylene amount increases sensitively, up to a thousand times compared to unripe fruits (with different speeds depending on species and varieties), reaching a peack (ethylenic climacteric) around the latest phases of ripening. In this phase, the hormone is characterized by an auto catalytic action ("***System 2***") , hence influenced by exogenous ethylene ("positive feedback" system).

Transition between system 1 and system 2 is supposedly determined by a variation of flesh tissue sensibility to ethylene. The reason seems to be related to the transcriptional factor RIN, firstly studied in tomato [46]). LeMADS-RIN is a molecule that activates the transcription of genes responsible of ACS synthesis in fruit flesh cells, in particular ACS 4 [47, 6]. RIN expression is induced at the beginning of ripening, without being influenced in a substantial way by ethylene: this indicates that RIN gene is regulated by other factors [46]. System 1 (through action of ACS 1 and 6, responsibles of the basal level of ethylene production and involved in other phisiologica processes, such as wound response and senescence)

produces discrete amounts of ethylene. In the transitory phase, RIN protein activates ACS 4 expression. ACS 4 activation, together with to ACS 1 activity, entails a noticeable increase of ethylene production. This increase stimulates activation of ACS 2, initiating the self-catalytic phase (System 2). Besides, ethylene increase inhibits ("negative feedback") ACS 6 and 1 activity [6].

The number of receptors involved in ethylenic signal perception vary depending on the specie (for example, six receptors have been identified in tomato, five in Arabidopsis) [1, 87, 88].

In peach, ethylene signal perception and its first transduction are regulated by three genes, ERS 1 (*Ethylene Responsive Sensor*), ETR 1 (*EThylene Responsive*) and CTR 1 (*Constitutive Triple Response*), the latter acting as negative regulator on hormone action (see figure 1.12). Perception requires presence of Cu^{2+} strictly bound to the receptor-protein: receptors without this ion cannot bind the hormone, determining insensitiveness to ehtylene (no signal transmission, see figure 1.13 on the facing page). Transfer of the signal during transduction happens through a succession of phosphorilations of the proteins encoded by the above-mentioned genes [24]. These proteins are positioned on the endoplasmatic reticulum, can be structurally divided in three "domains": a "sensor" domain, where the ethylene binds the sensor (hydrophobic bond); a "kinase" domain, protein amino-terminal region, highly omologous with Histidine-kinasis, constituted of five sub-domains that are building the catalytic center of the molecula, where the phosphorilation happens; a "receiver" domain, caboxi-terminal region of the receptor where the phosphate is transferreed from the previous domain [24, 63].

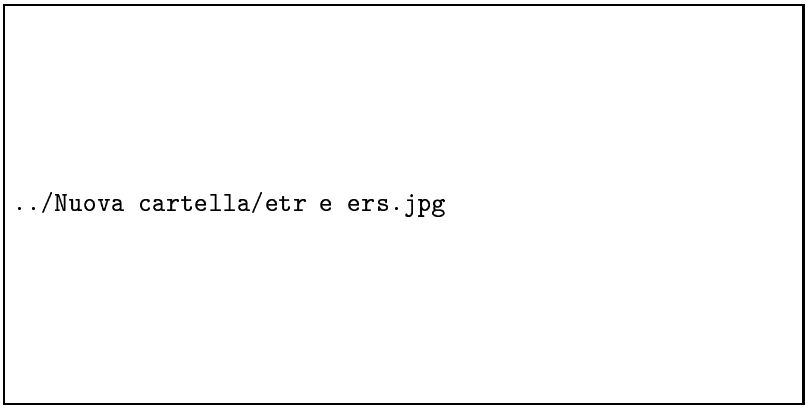

Figure 1.12: Peach ETR and ERS sensors (from [88]).

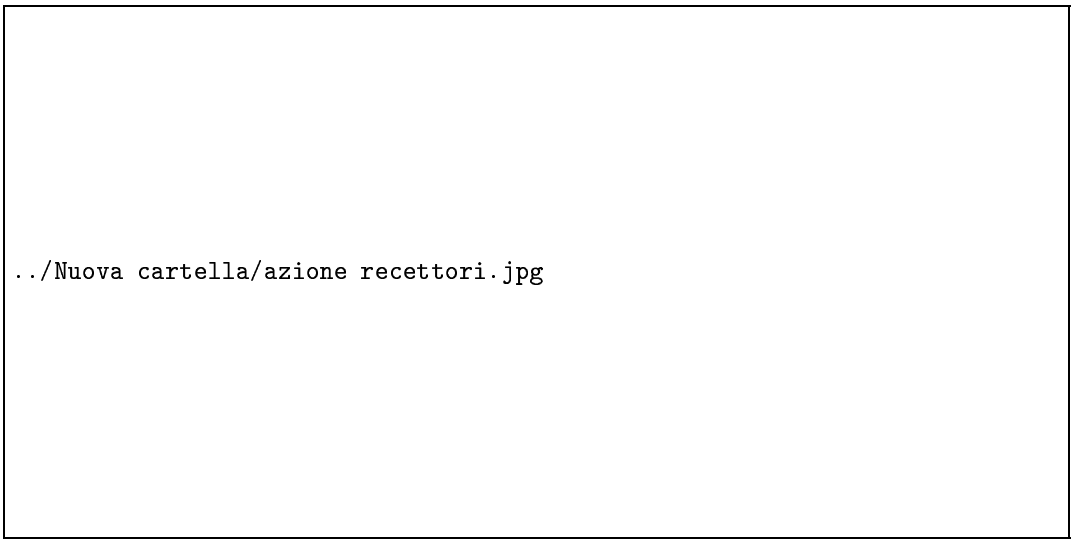

Figure 1.13: Ethylene receptors: hormone perception and transmission of the signal (from [50]).

The former two genes, belonging to multigenic families, are rather similar (ERS 1 lacks of the receptive domani present in ETR 1), but they differ in an essential way in the ethylene response: ETR 1 is indipendent from the ethylenic signal and is ubiquitary; vice versa, ERS 1 is tissue-specific and ethylene-dependent [88, 10].

CTR 1 has a repressor-type action mechanism. In absence of ethylene, it is phosphorilated, activating a cascade action idicated as MAPK or MAPKKK (*Mitogen Activated Protein Kinase-Kinase-Kinase*, see figure 1.14 on the next page), whose effect consists in the inactivation of the genes responsibles of the next transduction of the ethylene signal [46, 2, 10].

After CTR 1 action is inactivated by ethylene (see 1.1.5.3 on page 39), the ethylene signal is allowed to reach EIN genes, *Ethylene INsensitive* (EIN 2 and EIN 3 in particular, but EIN 5 and EIN 6 also). EIN 2 is the only known gene whose loss of function drives to the complete absence of response to ethylene. The N-terminal region of EIN 2, in fact, acts as a ethylenic signal sensor of the previous components (ERS and ETR genes), while the C-terminal region is responsible of trasduction of it to the following components (EIN 3 and EIL). Studies on *Arabidopsis* and *Petunia* demonstrated that in this gene C-terminal region is particulary conserved, and that in *Petunia* gene expression is variably regulated in different tissues [102]. In Arabidopsis, EIN 2 regulates the accumu-

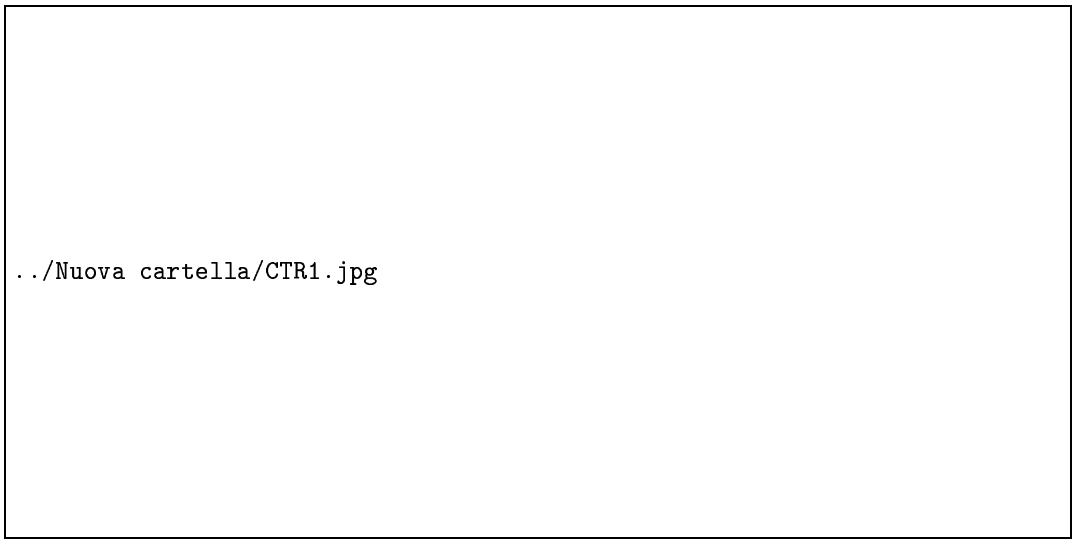

Figure 1.14: CTR 1 action (from [63]).

lation of EIN 3 protein. Biochemical function of EIN 2 in transductional terms is still not completely known. EIN 2, associated to nuclear membrane, acts on the transductional chain as intermediary between the hormone and the molecule codified by EIN 3, its immediate successor. Togheter with EIN 3, also other genes (EIL, *EIN3-Like*) are activated, driving to the synthesis of proteins with analogous action to EIN 3. EIN 3 encoded protein is characterized by a rapid turnover (about 20-30 minutes): the molecule is attacked by proteases (Ubiquitines), whose activity is mediated by two proteins, EFB 1 and EFB 2. Those molecules bind EIN 3 protein, making it susceptible to enzymatic degradation and stopping, in this way, signal transduction.[51].

EIN 3 and EIL are transcriptional factors that regulate expression of their immediate followers in the methabolic pathway, the ERF genes (*Ethylene Response Factors*), whose proteins regulate ethylenic stimuli expression [50]. Signal transduction happens through binding of transcriptional factors EIN 3 and ERF genes (see figure 1.16 on page 40).

ERF genes codify an EREBP protein (*Ethylene Responsive Element Binding Protein*) that binds GCC-boxes of genes directly determining the response to ethylene (Endo-PG gene, for example): see figure 1.17 [50, 103].

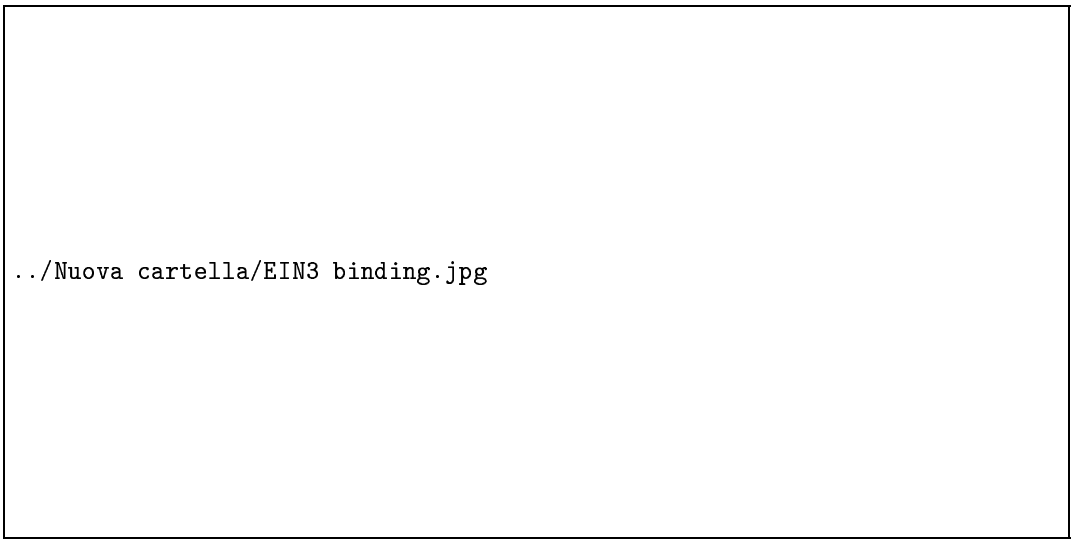

Figure 1.15: EIN 3 degradation (from [51]).

Ethylene perception. Ethylene receptors bind, in a more or less irreversible way, hormone molecules, self-inactivating. Hence, receptors are supposed to act as "negative regulators": ethylene effects on ripening begin to appear as soon as a certain number of receptors is inactivated. Once the receptors binds the hormone, they are no more able to repress expression of ethylene-induced genes: so, synthesis of new receptors is necessary to repress ethylene action. The apparent contrast between receptors overexpression and progression of ripening syndrome is explainable considering that during ripening the amount of synthesizes ethylene is overabundant the tissue capacity to synthesize enough receptors to arrest the effects [64]. Suppression of some of the genes responsible for receptor synthesis does not completely prevent ripening evolution, suggesting a certain degree of redundancy between different receptors [64, 63, 47].

ERS 1 and ETR 1 are physically associated to CTR 1 molecule through the kinasic domain: ethylene sensors form a sort of pincer that holds CTR 1 in place. When ethylene binds the sensors, they change their spatial conformation, releasing CTR 1 protein in the cytosol. Binding of hormone and receptor, through following phosphorilation reaction, modifies CTR 1 conformation, inhibiting or sensibly reducing its phosphorilation and kinasic activity, hence preventing its repressive action [86]. The same work demonstrated that in ETR-like ethylene receptors the suppression of the receiver domain in not enough for ethylene sig-

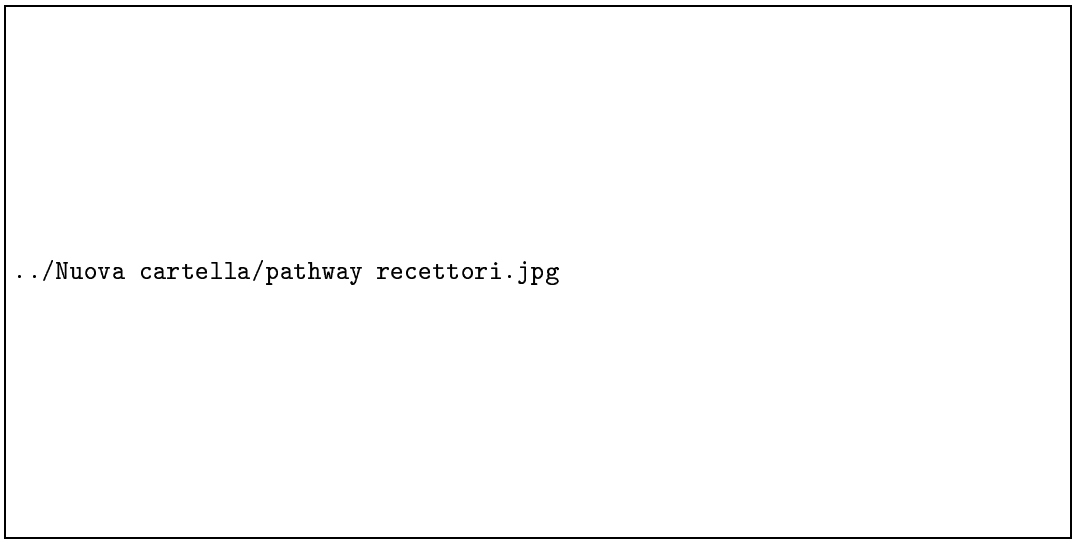

Figure 1.16: Ethylene receptors pathway (from [103]).

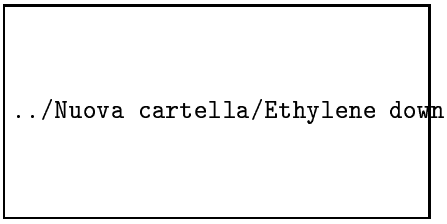

Figure 1.17: Ethylene pathway: from perception to expression (from [50]).

nal repression: ERS 1 lacks of it, still exerting an analogous activity to ETR 1.

CTR 1 loss of function allows signal transduction to EIN 3. Phosphorilation of EIN 3 (as response to ethylene transduction) alters the protein conformation, making the molecule insensitive to degradation enzymes or enhancing its resistance to proteolitic action [51]. Thanks to this post-transductional mechanism, plant is able to finely tune ethylene response: the hormone stops EIN 3 degradation ensuring a rapid response, avoiding the necessity of synthesizing new proteins.

Some chemicals are used in post-harvest fruit management, to slow down or inhibit ethylene action on tissues, sensibly increasing storability. Poliamines, 1-MCP (1-methilcyclopropane) and AVG (aminovinylglycine) are able to inhibit

ripening process. 1-MCP is a competitor for ethylene binding sites, while AVG inhibits ethylene synthesis by preventing ACS action. Preharvest application of AVG has been shown to delay ripening and to allow further increase in fruit size [89]. Softening was retarded in AVG treated fruit ripened at 20°C soon after harvest whereas AVG treated fruit ripened after one week of storage at 1.0 -1.5°C softened as untreated fruit.

1.1.5.4 Main enzymes involved in peach softening.

The difference between fruit cell relaxation during growing phase and during softening phase is determined by the coordination ot the degradative enzymes pool in coordination with the ones responsible of cell-wall polymers synthesis. During the growing phase, the process is mediated by auxins, while during flesh softening the enzymes coordination is mainly in charge to ethylene.

Flesh softening mainly occurs by cell wall degradative enzymes action. Those proteins can act with a primary role, directly disassembling the polymers of the walls, or indirectly "preparing" cell walls to the action of the degrading enzymes. The result of this synergetic action is the relaxing of the cell wall, that in fruits in an advanced ripening stage is highlighted by a noticeable increase of mesocarp intercellular spaces. Studies on mutants (especially conduced on tomato) demonstrated that suppression of a single enzyme involved in ripening is not enough to determine a blockage of the process, even if different degrees of delay are evident [47, 19].

Main enzymes involved in peach softening process are discussed below.

Expansin. This enzyme is not much present in cell walls, but its concentration increases noticeably during ripening. Expansin atc breaking non-covalent bonds (H bridges) between hemicellulose and cellulose, allowing sliding between each other (with a so called "inchworm progress"). H bonds are formed again immediatly after enzyme action, although in other positions: hence, expansin action (contrary to all the other enzymes) has a reversible action: is more correct, in fact, defining its action as "spatial re-arrangement" (see figure 1.18).

Moreover, lacking of any cell turgor, expansin action is irrilevant, since the loosening itself is irrilevant: in order to have cell relaxation/elongation is necessary that fibers are tensioned by the pressure applied by turgor to allow a sensible sliding of ficrofibrils. This enzyme is poorly movable along fibrils, preventing in this way movement of this proteins to adjacent cell where they could

alter normal accretion processes. This behavior allows, eventually, a selective growth of cell wall during accretion phase in specific directions. On the other hand, this enzyme's action allows a easier access to the substrates for catalytic enzymes during ripening [27, 28, 94].

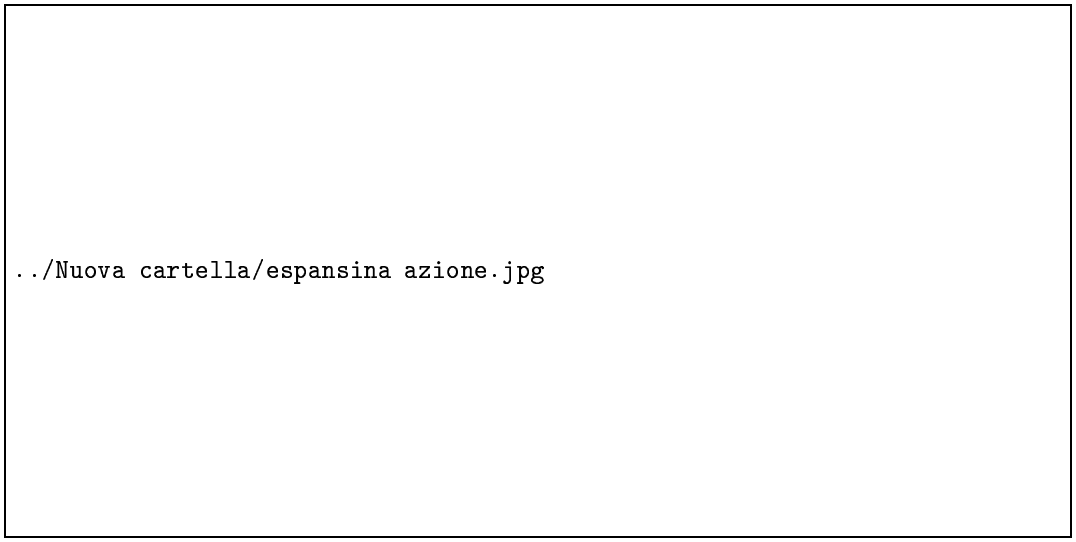

Figure 1.18: Expansin action: a) before action; b) enzyme binding cellulose and hemicellulose; c) new arrangement of fibrils (from [47]).

This enzyme is encoded by a multigenic family, and many expansin types are known. In peach there are at least three expansine identified and characterized (PpExp1, PpExp 2 and PpExp 3), but only the last one seems to have an expression pattern related to ripening progress [53, 54], as in apricot [73]. Ripening related expansins tend to accumulate at the beginning of ethylene auto-cathalytic phase, and are positively influenced by the hormone [109].

All expansins have in common the primary structure, constituted of two coding regions separated by two introns [27, 28]. Tertiary structure (see figure 1.19 on the facing page) seems to be made of three domains:

1. Peptide-signal, that drives the newly syntethised protein to the secretory system of the endoplasmatic reticulus (Golgi apparatus), and that is lost when the protein access it by enzymatic action.

2. Catalytic domain, that performs the scission of H-bridges between cellulose and emicellulose. This domain, even if lacking of a glucanasic activity, is homologous with a member of the family 45 of glucanases [27, 28].

3. Binding-domain, at the C-terminal end, that has the function of binding the protein to the cell wall polysaccharides limiting the enzyme movement.

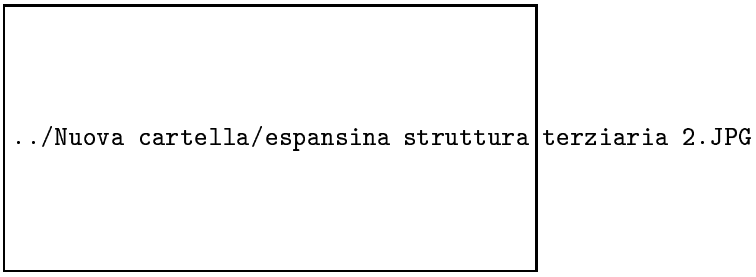

Figure 1.19: Expansin tertiary structure (from [47]).

Eso-glicanase (glicosidase). This enzyme acts removing single glucosidic residuals from polysaccharides chains belonging to cell walls, even if with a lower effectiveness compared to other enzymes. Their importance is in the loosening of the fibril reticulum [94, 95]:

- ➢ removing lateral glicosidc chains from the polymers (exposing them to the degrading action of other enzymes);
- ➢ modifying xiloglucane affinity with cellulase microfibrils (alteration of stability).

Xiluglocane endo-transglicolase (XET). This enzyme catalyzes the breaking of Xiloglucane (hemicellulose) molecule β-1,4 glucosidic bonds. The enzyme removes a monomer from the chain, forming a stable complex. The complex splits spontaneously giving up the monomer to another xiloglucane chain (transglicosilation). If another Xiloglucane acceptor molecule is lacking, the enzyme catalyzes an hydrolysis reaction yielding the glicosidic residual to a water molecule (this reaction is, however, unfavorable).

The enzyme has a double role: during the growing phase, the main role is merging in the cell wall molecules of newly synthesized xiloglucane; during ripening, it plays a part in cell wall reticulum loosening and rearranging.

Endo-β-1,4-Glucanase (Cellulase or EGase). The enzyme acts with an hydrolysis of the β-1,4-glucosidic bonds of hemicellulose polymers, determining

the loosening of the reticulum of primary wall [11, 110]. EGase acts probably on side regions of non-crystalline hemicellulose and xiloglucane as substrates. Its actions drives to a loosening of the cell wall polymers.

Cellulose is mainly involved in the initial phases of softening, before any other significant change in fruit firmness (it anticipates polygalacturonase action). Ethylene stimulates noticeably EGase action before climacteric stage [11]. However, its action seems to be inhibited when endogenous ethylene concentration increases [109]. The presence of different EG isoforms in peach suggests that a multigenic family is present as observed in other species. Three cDNA clones have been isolated from peach leaf and fruit abscission zone RNA: the three clones showed a high degree of divergence and different expression patterns [110], suggesting that the enzyme might be codified by a multigenic family.

β-galactosidase (pectin-lyases). Pectin-lyases are enzymes that cut β-galactosilic terminal residuals from pectins, determining cell walls β-galactanes degradation (figure 1.20 on the next page). Three isoforms of this enzyme were described, whose activity varies depending on development stage. Loss of galactose determines a loosening of the wall structure in the first phases of ripening. Hence, the enzyme acts in an indirect way on softening, increasing cell wall porousness, allowing a better access to substrates for the other enzymes involved in softening [12]. Pectin-lyases activity is enhanced by ethylene [109].

Pectin-methilesterase (PME). PMEs catalyze de-esterification of pectins removing methyl groups from C 6 position of galacturonic acid residuals (see figure 1.20 on the facing page). This process changes cell wall pH and electric charge, making pectins more susceptible to polygalacturonase action [22, 47].

In tomato, PMEs belonging to two different isoforms have been described: a first group specific of fruit tissues and a second ubiquitous in all tissues. Suppression with anti-sense RNA of one of those two isoforms has proved to be insufficient to inhibit ripening syndrome, demonstrating that the enzyme has mainly a preparatory action for polygalacturonases [72]. PME is expressed before complete ripening, and its activity is negatively influenced by ethylene as soon as ripening begins [46]. However, some Authors report that enzyme activity is positively affected by the hormone [109].

Polygalacturonase (PG). PGs are the enzyme directly involved in cell wall pectin degradation. Polygalacturonases catalyze hydrolysis of pectins galactur-

Figure 1.20: PME and Pectine-lyase action (reworked from [122]).

onic binds, but only after the preventive action of other enzymes such as PMEs, that remove lateral chains from pectines that are highly methyl-esterificated. However, PGs activity itself is not enough to determine softening, since suppression of this enzyme does not halt ripening and softening [47]. Two classes of PGs can be found (figure 1.21 on the next page):

- ➢ Endo-Polygalacturonases, that randomly cut polygalacturones inside the pectin chains.
- ➢ Exo-Polygalacturonases, that remove single terminal residuals of poygalactuorones chains.

The activity of this enzymes increases slowly during the softening process, but they raise considerably only when the fruit is already quite soft, as in the final stages of ripening and during post-harvest. The enzyme is active at very

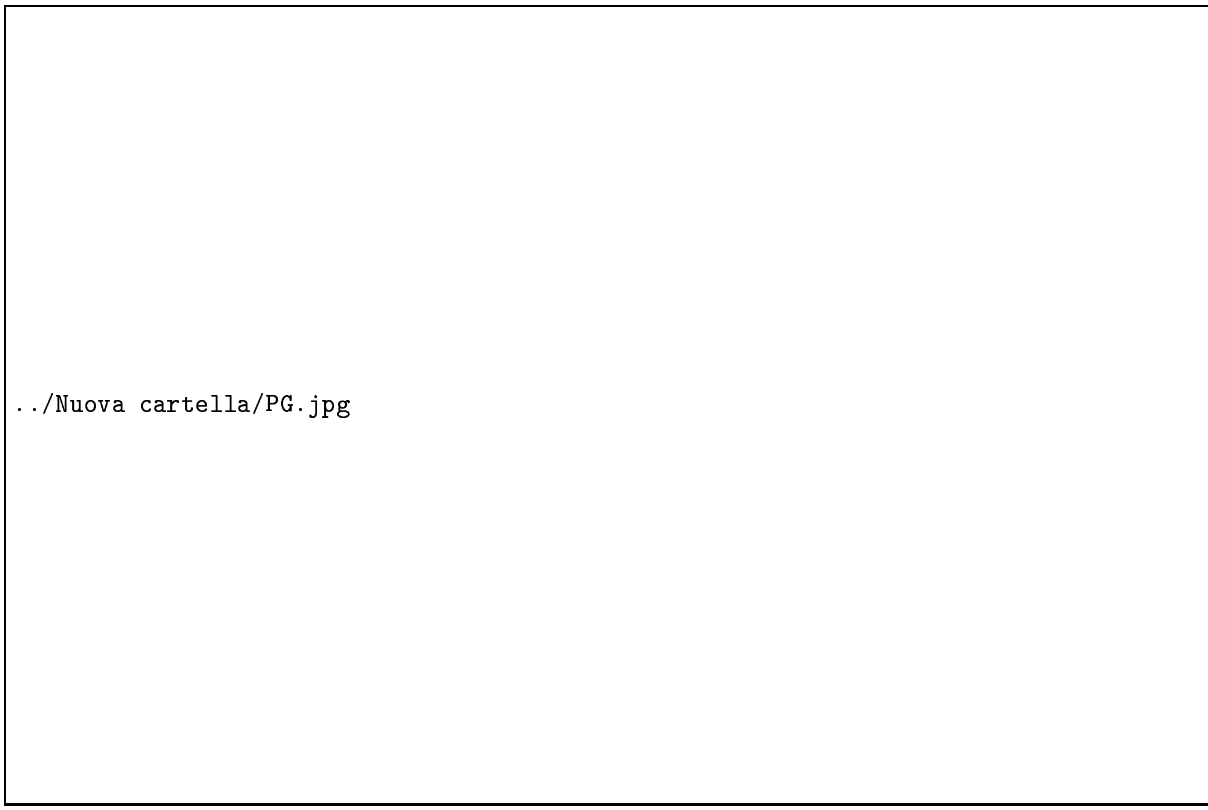

Figure 1.21: Polygalacturonase action (reworked from [122]).

low concentrations.

Of the two forms, Endo-PGs are more influenced by ethylene concentration, therefore more related to ripening than Exo-PGs [109]. Polygalacturonase is negatively influenced by low temperatures [79]. This behave seems to be the basis for the physiological disorder known as "peach mealiness". This disorder appears during cold storage (below 8°C): the fruit assumes a dry appearance, brown, flavorless and unable to release much juice. The reason for this can be related to an abnormal metabolism of pectins: PME activity -that is not influenced by temperature- releases many de-estherificated pectins, with an high molecular weight, that are able to "trap" water molecules in a gel, while PGs (affected by low temperatures) cannot hydrolyze those compounds[13, 72, 79].

PGs are part of a multigenic family, whose isoforms are differentially expressed depending on the tissue. The different isoforms show low levels of

homology in the same species, but different PG with same function show an high similarity among different species [52]. Endo-PG gene has been suggested as a candidate gene for peach flesh texture (melting and non-melting) and pit adherence [81].

Previous works demonstrated the participation in softening of more enzymes, like α-Arabinosidase and endo-1,4-β-mannanase. The former acts on arabinose polymers, dismantling them from larger chains and allowing a better action for cell wall specific degrading enzymes; for the latter, however, its substrate is still unknown [13].

1.1.6 Molecular markers.

Given the poor level of knowledge of the genome in Prunus species, the efforts needed to bring them to the level of other important crops has been noticeable, particularly in the last decade. Results are now emerging, some important tools (markers, maps, DNA sequences, quantitative trait loci) have been developed and made available to researches, and applications at the breeding program level have been started. Prunus, in particular, has experienced a quite fast growth in these years [34].

The dissection of fruit quality into a number of elementary components linked to genes of known function would be extremely useful for the early marker-assisted selection of fruit with desirable characteristics [77].

"Molecular marker" is defined any molecule (protein, enzyme, polyphenols, etc.) that is expressed in a discriminant way in different genotypes, so that it may be used as a marker of a given genotype/trait. The more useful markers are DNA sequences, that allow a much more accurate characterization than metabolites ,that are primary or secondary products of DNA expression (then influenced by environmental factors).

Markers could be used in breeding programs for "assisted selection" or MAS (marker assisted selection), because they allow detection of a trait independently from its phenotypic expression and are unaffected by the environment. A greater opportunity for increase MAS efficiency will depend on further knowledge of the molecular bases of the traits [76]. Markers can be detected both for qualitative and quantitative traits. The latter, anyway, are unfortunately more complicated due to the poly factorial control: it is quite difficult (if not impossible) determine the number of genes involved in trait control and to evaluate the contribution of the single on the whole phenotypic expression. For this reason, markers

for polygenic traits are defined QTLs, quantitative trait loci, expressed as the probability that a candidate gene to a given trait could be in the considered chromosome segment. The more precise the phenotypic characterization of the trait is, the greater will result the correlation with the QTL.

Molecular markers (of many sources) are also used to draw genetic maps (schematic representations of gene position on chromosomes), precious tools for breeding programs. In peach many maps are already existing. The most important map for peach (and *Prunus* in general) is the 'Texas' (almond) x 'Earlygold' (peach) F_2 progeny map (TxE), issued from an USA project [34]. On this map 562 markers have been already positioned, mostly RFLPs (restriction fragment length polymorphisms) and SSRs (simple sequence repetitions), but also sequence-tagged sites and isozymes. This map detected eight linkage groups (G1 to G8), with a total distance of 591 centimorgans (cM) [34]. The probability of a crossing-over between two associated traits has been chosen as measurement of their genetic distance: 1% of recombination between two markers corresponds to 1 cM (centimorgan) on a genetic map. No direct correlation between distance in cMs and effective numbers of base pairs between two markers exists.

Chapter 2

Purposes of the work.

To date, no simple marker is available to discriminate among peach flesh types (texture). Pectin analysis and cell wall Ca^{2+} content are time consuming. Ethylene emission measurement is influenced a lot by many external factors, such as harvest time (i.e. fruits can be picked too ripe or not at complete ripening), health of the fruits (wounded or diseased fruits have an altered ethylene emission). Quantification of ACO expression through RNA analysis are still time consuming and expensive.

All the mentioned methods have another big fault, though: they should be run on fruit, and this reduces their usefulness due to the need to have fruiting trees before assessing the presence/absence of the flesh trait.

So far, only one molecular marker has been developed for assessing flesh type [81]: it is perfectly suited to discriminate between melting and non-melting flesh. This marker is able to detect allelic profile of endo-polygalacturonase ("Endo-PG") gene: depending on the allele set it is possible to determine belonging of an accession to one flesh type or the other (see 1.1.4).

The main goal of this work is to find a set of molecular markers able to distinguish the "stony-hard" from melting and non-melting flesh. An hypothesis on the molecular basis determining stony hard flesh will also be discussed.

Another aim of this work is to characterize the allelic composition of a large number of cultivars and selections for the main genes involved in peach ripening and softening process.

Chapter 3

Materials and Methods.

3.1 Phenotypic characterization.

3.1.1 Plant material.

Analysis have been carried on fruits of 34 cultivars and selections, distributed between the main flesh phenotypes: 12 melting flesh (among them, 5 described as melting very-firm), 9 non melting and 12 stony hard. Furthermore,a "slow ripening" selection, BO83007016 (reported by [3]) has been chosen because fruits are unable to reach a complete ripening, hence interesting for studies regarding the ripening and softening processes. See table 3.2 on the following page for the complete list of the cultivars/selections sampled.

Each plant whose fruits have been used for following analysis was trained as free vase (2.0 x 3.0 m), in experimental fields of University of Bologna, Italy. 'Oro A' fruits, instead, came from Istituto Sperimentale di Frutticoltura of Rome (Italy), since the plant in the germplasm collection was still too young to bear enough fruits for experimentation.

Fruits have been harvested when full ripe ("phisiological ripening"), assessing the right picking time on field evaluation (ground color and firmness). For each cultivar/selection an average of 30 fruits have been harvested and stored for analysis (see below 3.1.3 on page 54 and 3.1.2 on page 53). Only perfect fruits were collected.

Fruits were collected and stored at 4°C, removing them from cold storage 24 hours before the analysis to allow the fruit to resume normal physiological processes. However, a parallel trial has been conduced on a restricted pool

of cultivars (See table 3.1). For each of them, two sets of samples have been collected and stored differentially: one set in cold storage (4°C) and the other at room temperature (about 25°). Data collected showed, at the end of the first year, that the fruits of some cultivars had a different ethylene emission pattern in the two storage conditions. Therefore, in the following year the fruits were kept only at room temperature (about 25°C).

CULTIVAR	Flesh type
Vistarich	MVF
Bolero	MF
Yumyeong	SH
D 41-62	SH
Frederica	NMF

Table 3.1: subset of accessions investigated for ethylene emission at different storage temperatures. MVF = melting flesh, very firm; MF = melting flesh; SH = stony hard flesh; NMF = non-melting flesh.

Accession	***Flesh type***	***Accession***	***Flesh type***
Bolero*	MF	Oro A	NMF
Glohaven	MF	Romea	NMF
Maygrand	MF	Salkaja	NMF
Redhaven Bianca	MF	Yu Bay	NMF
Rosa del West	MF	BO83007016	SR
RR 53-272*	MF	D 41-62	SH
Crizia	MVF	Ghiaccio	SH
May Crest	MVF	Grezzano	SH
Springbelle*	MVF	Jing Yu	SH
Spring Crest*	MVF	Helena Cling	SH
Vistarich	MVF	Honey Gold	SH
Ambra	NMF	Maria Dolce	SH
Anita	NMF	NJ 307	SH
Carson	NMF	Rich Lady	SH
Frederica	NMF	Stoney Hard	SH
Jonia	NMF	White Lady	SH
Lamone	NMF	Yumyeong	SH

Table 3.2: Accessions analyzed. Classification is precedent to biochemical analisys. MF = Melting flesh; NMF = Non melting flesh; MVF = Melting very firm flesh ; SH = Stony Hard; SR = Slow Ripening. (*) Etherozygous for the non-melting flesh allele.

3.1.2 Flesh type characterization.

Two parameters have been considered for flesh characterization: fruit flesh firmness and ethylene production. All the analysis have been carried on at the Dipartimento di Colture Arboree at the University of Bologna.

Fruits have been sampled at harvest and five and ten days after harvesting, with the aim to monitor the evolution of firmness and ethylene. Every analysis was carried on an average of 8 fruits representative of the ripening stage of the fruit sample. Ethylene emission was sampled always on the same 8 fruits from harvest to day 10, only replacing the rotten fruits, while for firmness assessment eight different fruits have been used, except for the last measurement, when firmness was taken after ethylene measurement.

For ethylene masurement, fruits were closed into a glass holder, with an airtight lid, for one hour, allowing the gas to fill the inside atmosphere. A gas sample was then taken using a 10 ml syringe for injection. A DANY 86.10 HT gascromatographer, equipped with a CARBOPACK QS 80-100 MESH 2 mm x 6 x 4 m (RT for ethylene of 1.2minutes) column and a FID (flame ionization detector). Operative conditions were: 80°C for sample run, 150°C at injection and 180°C the detector temperature. Each fruit has been sampled three times.

Fruit firmness has been measured using a flesh tester (Stalter digital penetrometer), checking both cheeks of the fruit after peeling an adequate area of the skin. An 8 mm diameter tip has been used for the test, and firmness expressed in kg/cm^2. The lowest value of the two has been considered as the most representative of the effective ripening stage of the fruit.

ACCESSION	FLESH TYPE
Bolero	MF
BO83007016	SR
Frederica	NMF
Vistarich	MVF
Yumyeong	SH

Table 3.3: Subset of accessions used for primary evaluation of the method. MF = Melting flesh; NMF = Non melting flesh; MVF = Melting very firm flesh ; SH = Stony Hard; SR = Slow Ripening.

In the first year, measurements have been carried only on a subset of accessions representative of the different flesh textures (see table 3.3), to tune the system and assess the possibility of using ethylene emission and flesh softening to distinguish between flesh types. On the following year, the same analysis has

been extended to the whole set of cultivars. Data have been used to divide into flesh groups the cultivars and selections for further analysis.

3.1.3 Fruit quality evaluation.

At harvest, a sample of 8 fruits out of the 30 has been used. Each cultivar and selection has been evaluated for the main quality traits. Sugar content (°Brix, determined by an Atago PR 100 digital refractometer), titratable acidity, fruit size and weight, overcolor extension (in percentage, by visual assessment) shade ("L", "a" and "b" values, by a Minolta colorimeter), skin type (fuzzy or glabrous), flesh color, pit adherence (clingstone, freestone or semi-freestone) have been determined.

3.1.4 DNA sampling.

During the summer of 2004 and 2005 leaves samples have been collected for DNA extraction, and then stored at -80°C until processing. A total of 44 cultivars and selections have been sampled in both years. See table 3.4 on the next page for the complete list of accessions sampled.

For DNA extraction, the protocol for mechanical grinding with a RETSCH MM 300 tissue grinder has been followed. Three sub-samples have been obtained from each sample and quantified using a NANODROP ND-1000 spectrophotometer. One of the sub-samples has been used to prepare the working solutions (with a DNA concentration of 20 ng/μl).

See annex 6 on page 115 for the adopted extraction protocol.

3.1.5 Allelic characterization.

The candidate genes (CG) strategy for the main traits involved in ripening and softening has been followed using primers specifically designed. All the analysis have been carried on in Kearney Agricultural Center (Parlier, CA), University of California, under C. Peace and C. Crisosto supervision.

A subset of eight cultivars and selection have been chosen as representative of the whole set (see table 3.5 on page 56). CGs primers have been tested in order to check wether any polymorphism was present before extending the analysis to the whole set of accessions.

Accession	flesh type	Accession	flesh type
Ambra	MF	Lamone	NMF
Bolero	MF	Maria Dorata	NMF
Glohaven	MF	Oro A	NMF
Maria Bianca	MF	RR 53-272	NMF
Maygrand	MF	Romea	NMF
Redhaven Bianca	MF	Salkaja	NMF
Red Valley	MF	Yu Bay	NMF
Rosa del West	MF	BO 83007016	SR
Supercrimson G.	MF	Big Top	SH
Crizia	MVF	D 41-62	SH
Diamond Princess	MVF	Ghiaccio	SH
Elegant Lady	MVF	Grezzano	SH
May Crest	MVF	Helena Cling	SH
Springbelle	MVF	Honey Gold	SH
Spring Crest	MVF	Jing Yu	SH
Vistarich	MVF	Maria Dolce	SH
Andross	NMF	NJ 307	SH
Anita	NMF	Rich Lady	SH
Babygold 9	NMF	Ruby Rich	SH
Carson	NMF	Stoney Hard	SH
Frederica	NMF	White Lady	SH
Jonia	NMF	Yumyeong	SH

Table 3.4: Accessions used for DNA extraction. MF = Melting flesh; NMF = Non melting flesh; MVF = Melting flesh, very firm; SH = Stony Hard; SR = Slow Ripening.

3.1.5.1 Plant material and candidate genes.

Since the aim of this work was the investigation about fruit ripening and softening, researches have focused on those genes related to enzymes involved in cell wall disassembling and genes involved in ethylene biosynthesis and expression. Primers (most of them already available at Kearney Station and new primers specifically designed) were related to these two main groups of genes.

Genes investigated (and relative primer sequences) are reported in tables 3.6 on page 57, 3.7 on page 58 and 3.8 on page 59.

3.1.5.2 Conditions of analysis.

Every sample has been prepared using 10 μl of solution composed of 7.0 μl of sterile H_2O, 1 μl of PCR Promega buffer, 0.5 μl of $MgCl_2$ (Promega, 25

Accession	Flesh type
Bolero	MF
Maygrand	MF
Crizia	MVF
Frederica	NMF
Oro A	NMF
BO83007016	SR
NJ 307	SH
Ghiaccio	SH

Table 3.5: Subset of accessions checked for the polymorphism test. MF = Melting flesh; NMF = Non melting flesh; MVF = Melting very firm flesh; SH = Stony Hard; SR = Slow Ripening.

nM), 0.2 μl of dNTPs (10 mM), 0.1 μl of Taq, 0.2 μl of primer mix (forward and reverse) and 1.0 μl of DNA (20 ng/μl) as template. Conditions for PCR reactions were: a 90°C cycle for 5', followed by 30 cycles at 72°C for 1'30" and a cycle of 5' at 72°C. PCR products have been run on a polyacrilamide gel and visualized using a Silver Staining Promega kit, following procedures indicated by the producer.

3.1.6 Stony hard molecular marker.

3.1.6.1 Genetic material.

For the detection of a possible "stony hard trait" marker the RAF (Randomly Amplified DNA Fingerprinting) technique has been used [120]. This technique is based on primers with a very short sequence (about 10 bp) that bind randomly to the DNA, determining amplification of many fragments, in a way analogous to AFLP technique.

Four groups of accessions have been created. Two bulks were composed of cultivars showing the stony hard phenotype (named "SH A" and "SH B" bulks); the other two were built with melting- and non-melting-flesh cultivars ("NOR A" and "NOR B"). Two more bulks of stony hard and non-stony hard accessions were added, using material available at Kearney Laboratories ("SH C" and "NOR C" respectively). Single bulks have been assembled reducing as much as possible the difference among the accessions to the only flesh-type trait in order to reduce the interpretation of the polymorphisms between bulks. Bulk compositions are reported in table 3.9 on page 60.

Each RAF primer has been individually tested on the bulks. When a poly-

Primer	Gene	Sequences
PME 1	Pectinmethylesterase	FW: AGGGCCTCCTCTGTTCTGTT
		RV: GAGATGAGGGGCAACTTGAG
PME 2	Pectinmethylesterase	FW: TGCCGAATATGGTATTCTTAGC
		RV: TTATGGATGGGTGCATCAGA
PME 3	Pectinmethylesterase	FW: TGTTGCTCTTCGAGTTGGTG
		RV: GCCACTGAACCCTACCTGAA
PME 5	Pectinmethylesterase	FW: AGAAGATTCTGGTCGACGTGT
		RV: CCCTATCGTCACACAACGTG
PME 6	Pectinmethylesterase	FW: TCGACCACCTAGACCCAACT
		RV: GCAGTGAGCAAAGCAGGAAT
PME 7	Pectinmethylesterase	FW: CATATCTGGGTAGGCCTTGG
		RV: TGACTCCACTGTGAACTTGG
Man 1	Endo-1,4-β-mannase	FW: ACACCTACCAAAGAGACTTGCTC
		RV: TATCGTACCCATCACCGAAC
Gal 1	β-Galactosidase	FW: TGGTAGCTCGCCAATTCTTC
		RV: GTCCCATTGTTGAACCCAGT
Gal 2	β-Galactosidase	FW: CCTTTTCCGTTGATTGAACC
		RV: TGTCAACTGTTCCCGTTTGA
Gal 3	β-Galactosidase	FW: TCCATTAAATCTTGGCTCTTCC
		RV: GTGCTTTCGATCCCAACATT
Pel 1	Pectate-lyase	FW: TGACCTATTGCGAGGGACAT
		RV: CAGGCCGAGGTTGTAATCAT
Pel 2	Pectate-lyase	FW: TGTTTTTCTCTCTGCTCTCTGC
		RV: CGCCAAGAAAGCACCAGTA
EGase	Endo-β-1,4-Glucanase	FW: TGGCCTTCAGAAGTACAGGAA
		RV: TCAAAGGATTCTCTCCCAACA
PAE 1	Putative pectinacetylesterase	FW: TCTCTGGGTGTTCAGCTGGT
		RV: AGGATGCGGGTAGGTTCTTT
UGT	UDP-glucose transferase	FW: TGAGGAAGAAAGCCCTTGAA
		RV: TCGGCAAACGGTACATATTTT

Table 3.6: Candidate genes involved in enzymatic hydrolisation of cell walls (continue).

(continue)

Primer	Gene	Sequences
Exp 1	Expansin	FW: ACCTCACACTTAGCCATTGCT
		RV: TAGAATTGACCGCCCGAAAA
Exp 2	Expansin	FW: GCGCAGGGTGATTTGTATATG
		RV: TCAAAGATATTAATGTGCATTTTGC
Exp 2b	Expansin	FW: GCTGCCAGTGCAGTCAATAG
		RV: ATGTGGCATGACCACCTTC
Exp 3	Expansin	FW: TGTTTCTGCTTGCCTTGCCT
		RV: TCTTCCCGGTGAATGTTTGA
Exp 4	Expansin	FW: GCGGATCGCATTTTTGTACT
		RV: GCTGACGGGTATTTTCAGGA
Exp 5	Expansin	FW: GCAGGAGAAGGGGAGGTATC
		RV: CCTTGAAAGAGAGGCTTTGG
Ara	α-arabinosidase	FW: CAACCAAGGCAAGAAGGAAG
		RV: AAGACAGACCCTTTACCAGCA
PG 4	Polygalacturonase	FW: AAGGGTTCAACAGGAGCAGA
		RV: GGTGATCGCTCAAACCATTT
PG 5	Polygalacturonase	FW: GCCTCTTGTTCCAGTCTTGC
		RV: ACAAACCACGAGGGAGAATC
GT 1	Glucosyl-transferase	FW: GTGCAGGATGTTTGGAAGGT
		RV: CCGTCACTGACAAGCCTCTA
Endo-PG 1	Endo-polygalacturonase	FW: CCTTCAACTCATTAACCTCTCTCTC
		RV: GGAAGGCTTTTGTGGAGTCA
Endo-PG 6	Endo-polygalacturonase	FW: CGGGGTTACCATATCAGGTG
		RV: TTAGGGATGCCAATCCACTC
Unk 3	unknown protein upregulated by ethylene[109]	FW: AGTTCAACCCATACCCTTTGT
		RV: GGGCTAAGGGGTTTGCTTAT
Unk 2	unknown protein upregulated by ethylene[109]	FW: TCCTATTCTGTTCCCCGAGA
		RV: TTTGCCAAGCATAATTGGTT

Table 3.7: Candidate genes involved in enzymatic hydrolisation of cell walls.

Primer	Gene	Sequences
SamDC 1	S-adenosylmethionine Decarboxylase	FW: TTGTGAACATTGAGCAGCAG
		RV: AGTGGGACACGAATTGGAAC
SAMS 1	S-adenosylmethionine Sinthetase	FW: CTCTCTCCTTGGCTGTAAAATC
		RV: CAATGTGTATGTGGGTCCTG
SAMM	SAM-2-demethylmenaquinone methyltransferase-like protein	FW: GACCCCGCTCTCTCTCTCTC
		RV: GCACCCGTAGATCACCACTT
ACO 1	1-aminocyclopropane-1-carboxylic acid oxidase	FW: GCTGCATTGAAATTAACAACACTT
		RV: AAGAAGCCCCAGTTCTCACA
ACO 2	1-aminocyclopropane-1-carboxylic acid oxidase	FW: GTGCCCTCTCTATTGCTTGG
		RV: TGCTGCTTCAGGACCACAT
ACO 3	1-aminocyclopropane-1-carboxylic acid oxidase	FW: TCTTCAGGGTCAGTCAGGGTA
		RV: TTTATAGGCGCGAAGGGAGT
ACS 1	1-aminocyclopropane-1-carboxylic acid synthase	FW: TGTACGCCAAGGGAAGAAAG
		RV: TCTCATTTAAACTGACCACCCTTA
ACS 2	1-aminocyclopropane-1-carboxylic acid synthase	FW: ACTTTGCGCGAGAGAAGAAC
		RV: GGAGTAGATAACACCCGCCTTA
ER 1	Ethylene Receptor	FW: AAGGGATTGCCAATGAGCTA
		RV: GAAGACAGTTTCCATTACCTGTGA
ER 2	Ethylene Receptor	FW: GGACTTTATGCCTCCACGAA
		RV: CAATTGCTTGAGAAATAGGGAA
ER 3	Ethylene Receptor	FW: ATCCATACCAACCCTCATGC
		RV: AGGGACTCTTTGTGCACTGG
CTR 1	Constitutive Triple Response	FW: TCCCGT TTAAAGGCAAACAC
		RV: AAATCACGTGGAATCTCAAGC
ERP	Ethylene Responsive Factor	FW: CCCTCTTTCTCTCTCCCTCTC
		RV: CCATCTGAGAAGCGCAAAAG
EREBP	Ehtylene Responsive Element Binding Protein	FW: ATGAAATGGTTGAGGCCAAG
		RV: ATCTCTGCAGCCCACTTACC

Table 3.8: Candidate genes involved in ethylene signalling.

SH A BULK	FLESH TYPE	NOR A BULK	FLESH TYPE
Helena Cling	SH	Rosa del West	MF
Yumyeong	SH	Bolero	MF
Maria Dolce	SH	Glohaven	MF
Ruby Rich	SH	Elegant Lady	MVF
White Lady	SH	Lamone	NMF
D 41-62	SH	Yu Bay	NMF
Rich Lady	SH	Oro A	NMF
SH B BULK	**FLESH TYPE**	**NOR B BULK**	**FLESH TYPE**
NJ 307	SH	Redhaven Bianca	MF
Ghiaccio	SH	Maygrand	MF
Honey Gold	SH	Supercrimson G.	MF
Grezzano	SH	Crizia	MVF
Jing Yu	SH	Frederica	NMF
Stoney Hard	SH	Salkaja	NMF
Big Top	SH	Jonia	NMF
SH C BULK	**FLESH TYPE**	**NOR C BULK**	**FLESH TYPE**
Yumyeong	SH	Hakuto	MF
7-28	SH	Indian Free	MF
Red Bird Cling	SH	Chinese Cling	NMF
BY 86-1079	SH	BY 98-4649	MF
BY 89-P2787	SH	BY 99-1343	MF
BY 93-P4371	SH	BY 99-1644	MF

Table 3.9: Composition of bulks used for RAF analysis.

morphism, the primer has been tested on each single accession to verify the reliability of the marker. In order to map the candidate marker on a *Prunus* map, each polymorphic marker has been tested on the "TxE" map BIN set (developed by the University of Clemson) and on 'Dr. Davis' and 'Georgia Belle', cultivars used as parents by the U. C. Davis peach map. Each primer was called with an abbreviation composed of a letter and a cardinal number. List of markers tested is reported in 3.10 on the facing page.

3.1.6.2 Condition of analysis.

Every sample has been prepared using 10 μl of solution composed of 2.65 μl of sterile H_2O, 1 μl of 10X RAF buffer, 0.2 μl of dNTPs (10 mM), 0.15 μl of Taq (STOFFEL), 5.0 μl of primer and 1.0 μl of DNA (20 ng/μl) as template. Conditions for PCR reactions were: one cycle at 95°C for 5'; 30 cycles of 94°C for 30", 57 °C for 1', 56°C for 1', 55°C for 1', 54°C for 1' and 53°C for 1'; one

PRIMER CATEGORY	PRIMER NUMBER
A	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20
B	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20
C	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20
D	1
E	2, 8, 20
P	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15
X	1, 4
W	6
V	1, 12, 18
Y	1, 3, 9, 18

Table 3.10: RAF primers used to obtain a molecular marker for stony hard trait.

cycle at 72°C for 5'. PCR products have been run on a polyacrilamide gel and visualized using a silver staining PROMEGA KIT, following procedures indicated by the producer.

Chapter 4

Results and discussion.

4.1 Phenotipic characterization.

4.1.1 Fruit quality evaluation.

Data collected about the cultivars analyzed are reported on page 117.

It is immediately evident the great variability existing among the cultivars analyzed.

A great variability has been noticed, as expected, for SSR values, that are swinging between the minimum recorded for 'Crizia' ("melting very firm") , 8.24°Brix, and the maximum value of 13.35°Brix (in 'NJ 305', stony hard).

Given the high value of acidity (9.99 mg of malic acid, against an average of 5.80 mg for other melting flesh cultivars) and the high firmness values (5.87 kg/cm^2,compared to an average of 1.82 kg/cm^2) recorded for 'Bolero', has been evident that fruits of this cultivar were harvested too early, when physiological ripening was not yet completed. This was consistent with the low ethylene values recorded for this fruits and discussed below.

A great deal of variability has been recorded for overcolor value, that ranges from the complete absence registered for 'Ghiaccio sel. 193 QXVII 111' and 'Oro A' to the almost complete coverage of the fruit that was typical of 'Diamond Princess' and 'Big Top'. However, while 'Oro A' fruits were completely yellow (ground color), 'Ghiaccio sel. 193 QXVII 111' were still completely green. Similar variability is evident for fruit weight, spacing from the 61 g of 'Maygrand' to the almost 265 g of 'Elegant Lady'.

4.1.2 Comparison between cold and room temperature storage.

Data recorded in 2004 and 2005 showed a different ethylene emission (table 4.1).

		4°C storage			25°C storage		
Accession	Flesh Type	Harvest	5 days	10 days	Harvest	5 days	10 days
Vistarich	MVF	3.03	3.70	13.25	20.25	37.99	61.67
Bolero	MF	1.02	1.62	4.35	5.30	9.92	13.40
Yumyeong	SH	0.05	0.55	16.56	0.00	0.00	0.00
D 41-62	SH	1.53	2.38	8.08	2.27	3.05	5.30
Frederica	NMF	3.47	6.46	7.92	12.38	31.73	40.70

Table 4.1: Ethylene emission (in ppm/kg/h) at different storage conditions at harvest and after 5 and 10 days.

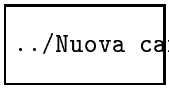

Figure 4.1: Ethylene emission in different storage conditions.

It is evident that storage conditions distort in a more or less significant way hormone emission (see figure 4.1). For some of them ('Vistarich', 'Frederica' and 'Bolero') cold storage reduced sensibly ethylene biosynthesis compared to room temperature. 'Bolero' fruits, however, showed a less marked influence. For 'Yumyeong' and 'D 41-62', on the other side, ethylene emission after cold storage was quite higher than if stored at 25°C, 'D 41-62' at room temperature showed a value is surprisingly high if compared to the ones stored at room temperature. It would seem that stony hard cultivars are stimulated in ethylene emission if stored at about 0°C, but since the number of accessions investigated is low, this association could be due to reasons completely independent from the texture of the fruits.

4.1.3 Phenotype characterization

4.1.3.1 Fruit softening.

Data of softening, ethylene emission and weight loss for all the cultivars analyzed are reported in the attached table (see on page 117).

General remarks. Considering the average values of those traits recorded for each flesh phenotype, it has been possible to draw some conclusions.

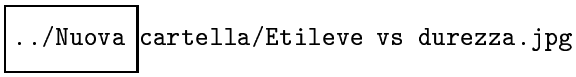

Figure 4.2: Comparison between ethylene emission (curves) and firmness (columns) from harvest to day 10. MF = melting flesh; NMF = non-melting flesh; MVF = melting flesh, very firm; SH = stony hard flesh.

Ethylene vs firmness. Analyzing firmness and ethylene emission (see figure 4.2)is immediately evident that flesh phenotypes acted as expected from each type.

Melting cultivars registered a brisk increase of hormone emitted (7.9 to more than 40 ppm/kg/h), while firmness was rapidly decreasing from the initial evaluation (average value of about 2.4 kg/cm^2) to the last measurement (less than 0. 4 kg/cm^2). This rapid change in fruit firmness and ethylene emission is consistent with the typical behavior of melting fruits and with data reported in literature.

The same happened with the "melting, very firm" phenotype: although starting from an higher value for both factors compared to the previous type, ethylene increased, with an analogous ratio, though with a flexion between day 5 and day 10 (figure 4.2), accompanied by a parallel decrease of firmness. Since this one is just a subclass of the melting flesh phenotype, it was expected to undergo to a melting process analogous to the one discussed before, with the ethylene emission reaching values even higher than in non-melting peaches.

This latter, considering the average value of the cultivars analyzed, showed a rapid increase in ethylene emission between harvest (at that moment the average value was already high, more than 17 ppm/kg/h) and day 5 (about 37 ppm/kg/h), when reached a *"plateau"* that was maintained until the end of experiment almost unaltered. Besides, firmness decreased, but after an initial sensible reduction (from 2.9 to 2.2 kg/cm^2), fruits retained their consistency till the end of their post-harvest life. Considering that those data derived from the average value of fruits of many different cultivars, hence suffering of a wide range of initial values, they are consistent with literature reported in this work.

Data recorded for "stony hard" accessions reported an ethylene emissions that was very low compared to all the others, with no sensible differences be-

tween harvest and 5 days records, and just a small increase after 10 days (3.2, 3.5 and 6.5 ppm/kg/h respectively). Although deriving from an average value, this pattern resembled the one previously described in this text when it was discussed the stony hard flesh phenotype. Reasons of this late burst of ethylene will be discussed below. By side, hormone emission wasn't accompanied by a parallel flesh softening. As expected, firmness was retained from harvest to flesh rot almost unaltered. Firmness changed from 3.5 to 2.8 kg/cm^2(-0.5 kg/cm^2) in 10 days, differently from all the other groups. This was consistent too with stony hard trait.

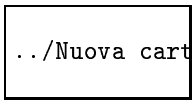

Figure 4.3: Comparison between ethylene emission (dotted lines) and weight loss (continuous lines) from harvest to day 10. MF = melting flesh; NMF = non-melting flesh; MVF = melting flesh, very firm; SH = stony hard.

Ethylene vs weight. With the progression of post-harvest life fruits lost weight because of the dehydration of tissues: water was lost after transpiration and respiration progresses, driving fruit skin to become coarse. As ethylene increases, as symptom of cell activity, weight decreases.

However, it must be noticed that in stony hard fruits, where ethylene emission is rather low (biochemical activity is independent from level of ethylene emission) the loss of weight was greater than in all the others. The average value moved from 224 to 173 g in five days (-51 g, about 10 g/day), close to a weight loss of more than 40% in ten days. In the same period, the other phenotypes lost an average of 3.0 and 4.6 g/day in melting and "melting very firm" groups respectively (losing 17% and 23% of the initial weight), and 4.4 g/day in non-melting cultivars one (20% of weight loss).

No explanation was provided for this higher weight loss, but it might be considered an index of an intense methabolic activity, since in stony hard fruits ripening processes occur as in any other flesh type (with the obvious exception of softening).

Firmness vs weight. Progression of softening was accompanied by loss of fruit weight (see figure 4.4). No particular relationship was pointed out considering the way these two processes acted together, although the rapid loss

../Nuova cartella/Durezza vs peso.jpg

Figure 4.4: Comparison between firmness (dotted lines) and weight loss (continuous lines) from harvest to day 10. MF = melting flesh; NMF = non-melting flesh; MVF = melting flesh, very firm; SH = stony hard.

of weight of stony hard individuals discussed above was not accompanied by a reduction of firmness as straight as in all the other flesh groups.

As general remark, firmness and weight decreased with different speed in each flesh group. Melting cultivars were characterized, on a ten days period, by a loss of about 4.07 g/day of weight and 0.2 kg/cm^2/day. Similarly, the very firm lost in the same period an average of 3.37 g/day of weight and 0.19 kg/cm^2/day of firmness. It appeared immediately evident how those two groups behaved in the same way: melting flesh types were indeed characterized by a rapid loss of firmness accompanied by a progressive loss of water and weight. However, considering the first 5 days from harvest, if firmness decreased with no significant differences (-0.3 kg/cm^2/day for melting very firm flesh and -0.32 for melting flesh), weight loss was different in those two groups, as seen before.

On the other hand, non-melting cultivars were reporting a little reduction of firmness (-0.11 kg/cm^2/day) and average loss of weight of about 2.7 g/day. Compared with data collected in the first 5 days (and discussed above), weight loss registered a difference in its ratio: 4.4 g/day in the first 5 days and 1.2 g/day in the second half of experiment.

Differently, in "stony hard fruits" the almost irrelevant reduction in firmness value along the ten days of storage (-0.07 kg/cm^2/day) was accompanied by a sensible reduction of weight: as average value, more than 7.3 g/day.

Selection 'BO86007016'. 'BO86007016' fruits had a very high firmness at harvest, that remained longer than any other accession (see table 4.2 on the next page and figure 4.5 on the following page).

Fruits of this accession had a very reduced flesh softening: from an initial value at harvest of 10.15 kg/cm^2 they reached after ten days a value of 7.72 kg/cm^2, less than 2.5 kg/cm^2/day. Although as ratio it is analogous to the one recorded for melting flesh fruits, fruits retained their high firmness until rot.

When ethylene emission was measured, fruits produced just a little quantity,

	HARVEST	DAY 5	DAY 10
Ethylene (ppm/kg/h)	0.02	0.04	0.07
Firmness (kg/cm^2)	10.15	8.17	7.72
Weight (g)	147.02	139.26	125.17

Table 4.2: Selection 'BO86007016' evolution of the three considered parameters.

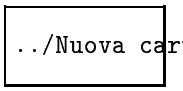

Figure 4.5: Evolution of ethylene, firmness and fruit weight in selection 'BO007016' at harvest, after 5 days and 10 days of storage: a) Ethylene emission vs firmness; b) Firmness vs weight.

similarly to "stony hard" cultivars of the "'Yumyeong' phenotype". However, it has not been possible to include this cultivar among the "stony hard", since no sign of ripening evolution has been recorded: as reported in literature this selection is a result of a mutation that inhibits ripening.

Fruits showed also reduced loss of weight: in the first 5 days fruits passed from the initial value of about 147 g to 139.36 g (-0.6%), and in the following five to little more than 125 g (-11%), with an average weight loss of about 2.19 g/day (with a reduction of the initial weight of -15% in ten days). Compared to the flesh types previously treated, this one was the lowest ratio found. Since ripening processes in this selection are inhibited, this weight loss was probably due to bare respiration of tissues.

Flesh groups. In order to detect accession wrongly attributed to a flesh phenotype, data concerning each of this trait have been crossed. This allowed to remove a few of them from a flesh group, avoiding further problems. Below each flesh group will be discussed separately from the other.

Weight loss. No significant differences were pointed out: all the accessions, regardless the phenotypic class, showed a progressive loss of fresh wight due to dehydration of tissues. Results are reported in figure 4.6.

The intensity of this process was different in the cultivars. 'D 41-62' and 'White Lady', belonging to "stony hard" flesh type, had a dramatic decrease of their weight in the first five days of sampling, that tended to become slower and gradual after that point.

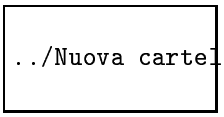

Figure 4.6: Weight loss: a) melting flesh cultivars; b) melting flesh, very firm cultivars; c) non-melting flesh cultivars; d) stony hard flesh cultivars.

Stony hard fruits, however, had a more rapid loss of weight compared to all the others, with the exception of 'Diamond Princess' cultivar, which showed a very rapid loss. Fruits of this cultivar had a very short post-harvest life indeed, since they were completely "melted" just after 5 days from harvest.

Ethylene. Data collected were consistent, for most of the cultivars investigated, with the behavior expected from the preliminary classification. Each flesh type group will be discussed below:

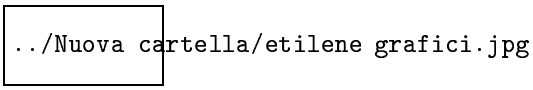

Figure 4.7: Ethylene emission (in ppm/kg/h): a) melting flesh cultivars; b) melting flesh, very firm cultivars; c) non-melting flesh cultivars.

Melting flesh. Accessions could be divided into two main groups, according the evolution of ethylene emission (see figure 4.7). A first group, including 'Rosa del West', 'Glohaven' and 'Redhaven Bianca', was characterized by a burst of ethylene emission in the first five days after harvest in all the accessions and a less marked release in the following days.

The second group, made of all the remaining cultivars, showed a constantly increase of ethylene, with a sensible increase in the latest 5 days of analysis, although with different behavior: 'Maria Bianca' and 'Maygrand', for example, showed an evident increase of emission at 10 days after harvest, about 100% more the value recorded after 5 days (80.5 ppm/kg/h vs 36.9 recorded in 'Maria Bianca' and 71.1 ppm/kg/h vs 36.2 for 'Maygrand').

All the accessions previously described as "melting flesh' cultivars had an ethylene emission consistent with the model proposed for this flesh type, although some of them at harvest showed an ethylene level lower than 10 ppm/kg/h. That could be attributed to the variability within the fruits of the sample, where some

of them could have reached an insufficient ripening level. However, the rapid and constant increase of emission, that reached significantly high levels in a few days is an evidence that such fruits, and the rapid softening ratio (see below) recorded, demonstrates that classification was correct.

Melting flesh, very firm. Accessions previously classified in this group had ethylene emission consistent with levels reported previously when discussed flesh types (1.3 on page 28). With the exception of 'Springcrest', whose ethylene emission was constantly around 46 ppm/kg/h throughout the whole analysis, all the accession had an increase in hormone emission (see figure 4.7). Generally, ethylene levels were over 25 ppm/kg/h since harvest. 'Spingrest', considering the high value of ethylene emission and the low firmness at harvest, is probably a "melting, very firm" cultivar whose harvesting has been done when the fruits were too ripe compared to the optimum moment for picking.

However, single cultivars showed emission patterns quite different to each other. 'Crizia' was characterized by a very high level of emission, slightly increasing in the first 5 days, reaching a maximum of emission that last until the end of the experiment. Similarly did 'Springbelle' fruits, even if this cultivar has been characterized by a dramatic increase in ethylene release (from 23.6 registered at harvest to 59.6 ppm/kg/h) in the first period of post-harvest life. Differently were the values recorded for 'Vistarich' cultivar, where ethylene increased more in the second half of the analysis than in the first 5 days: from 29.23 at harvest to 41.03 ppm/kg/h at five days (+11,8 ppm/kg/h), to 58.34 ppm at 10 days (+17,3 ppm/kg/h).

The other two accessions, 'Diamond Princess' and 'Elegant Lady' previously defined as "very firm" had to be reconsidered. Although fruits had a firmness higher than others of this phenotype, ethylene profile was not consistent with the classification suggested (lower than 30 ppm/kg/h at its peak). This dichotomy between ethylene emission and firmness is a further evidence that classification of melting flesh into a few subclasses is difficult and most of the times arbitrary, since many of this "borderline" cultivars can be found.

Non-melting flesh. Ethylene emission was high since harvest (over 10 ppm/kg/h) in all accessions (see on figure 4.7). Cultivars of this group showed two different patterns of hormone release. A first group, formed by 'Anita', 'Romea' and 'Maria Dorata', was characterized by an increase of ethylene emission from day 0 to day 5, followed by a decrease in the following 5 days. The

second group reached an upper limit after 5 days, maintaining emission almost constant until the last day of analysis. Apart from this two groups is 'Oro A', whose hormone release has always been over 50 ppm/kg/ h till the beginning and has grown slowly but constantly from day 0 to day 10.

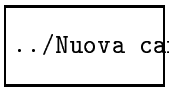

Figure 4.8: Ethylene emission (in ppm/kg/h) in stony hard flesh cultivars. 'Yumyeong' is put in the graph to compare data.

Stony hard flesh. As supposed, ethylene emission was at harvest lower than 10 ppm/kg/h. Cultivars grouped in this class could be divided into two subclasses, based on the ethylene emission pattern (see figure 4.8).

One group, formed by 'Ghiaccio sel. 193 QXVII 111' and 'NJ 307' did not emitted sensible amounts of hormone, remaining to 0 ppm throughout the whole analysis. Those two accessions were hence attributed to the "Yumyeong type" group, as seen before. The other group was formed by 'White Lady', 'Big Top' and 'D 41-62', and characterized by a discrete amount of ethylene emitted by fruits: hormone was present in small quantities since harvest (although lower than 10 ppm), reaching this threshold only after 10 days of storage in 'White Lady' and 'Big Top'. This behavior made those three cultivars fit the second subclasses of stony hard flesh (see paragraph 1.1.4.3). Some of the higher levels of recorded for ethylene in those cultivars might be due to undetectable latent infections of brown rot.

In a intermediate situation is 'Ruby Rich' cultivar: this one produces discrete quantities of hormone throughout the whole development, never higher than 7 ppm/kg/h. Is not possible to fit it into one of the two classes discussed before, further evidence of the difficulty of a schematic classification of subclasses for each flesh type.

Fruit softening. As expected, cultivars had softening patterns fitting with discussed models. Each flesh group will be discussed below:

Melting flesh. From an initial value, after 5 days firmness decreases sensibly, reaching very low levels after 10 days from harvest (see figure 4.9). For example, 'Bolero' fruits have been harvested at a firmness of 5.9 kg/cm^2 and,

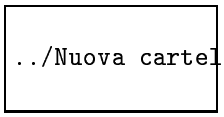

Figure 4.9: Firmness evolution: a) melting flesh cultivars; b) melting flesh, very firm cultivars; c) non-melting flesh cultivars; d) stony hard flesh cultivars.

after 5 days, it was decreased to 1.7 kg/cm^2, while in the same period 'Maygrand' fruits recorded 1.74 and 0.99 kg/cm^2 respectively. It must be pointed out that 'Bolero' has been previously classified as "firm melting flesh" cultivar for its good firmness compared to others. 'Maygrand', on the other hand, although being harvested at a low firmness value demonstrated the lowest softening ratio compared to all the other accessions analyzed.

All the accessions, however, after ten days of storage had reached firmness values lower than 1 kg/cm^2, making difficult to establish the firmness value with ease, since for that time most of the fruits were rot or almost completely melted.

Considering the ethylene output, for the following analysis presented in this work all the accessions collected in this group have been considered as melting, without trying any further sub-classification.

Melting flesh, very firm. As seen for the previous group, firmness decreased dramatically soon after 5 days of storage and reached very low levels at the end of the analysis. This proved to be consistent with expectations, since this kind of flesh is just a subclass of the more general "melting flesh" type. The softening process follows the pattern seen before, with the difference that fruits recorded higher firmness values at harvest.

With the exception of 'Springcrest', all the cultivars followed a "two step" softening, with a first, sudden softening between harvest and day 5 and a less dramatic one between that point and day 10. For two of them, 'Vistarich' and 'Elegant Lady' the first step has been particularly brisk, registering a variation from 4.14 and 3.68 kg/cm^2 respectively to 1.43 and 1.60 kg/cm^2 . 'Springcrest', differently from any other, has a regular progress from harvest to day 10, with no significant difference between the two parts of the curve in the graph.

As seen when discussed about ethylene, 'Diamond Princess' completely melted and rot before day 10, so the data is missing. Fruits lost more than 1.3 kg/cm^2 in the first 5 days, and the definitive collapse of texture happened in two days, underlying the dramatic loss of firmness that is typical of melting flesh fruits.

Non-melting flesh. Non-melting fruits demonstrated to have a loss of firmness less evident than the previous two types. Although firmness measurement by itself is not completely able to render the rubbery consistency of the flesh. When fruits started to rot, loss of firmness was still poor.

Softening pattern was characterized of a progressive loss of firmness, but the dramatic loss seen in the previous two classes is not present, consistently with the retaining of firmness even in the latest phases of post-harvest life that is characteristic of this phenotype.

Two accessions had a softening that wasn't consistent with the expected one: 'RR 53-272' and 'Romea'. The latter highlighted a pattern that was more similar to a melting flesh one: fruits lost their initial firmness of 3.6 kg/cm^2 to a value after 5 days of 1.9 kg/cm^2(-1.7 kg/cm^2), too much if compared to the loss recorded for other non-melting fruits (ranging from a minimum of -0,5 kg/cm^{2}in 'Tebana' to a maximum of -0.8 kg/cm^2 in 'Jonia').

'RR 53-272', on the other hand, recorded at harvest too low values of firmness to be considered a proper non-melting cultivar. Ethylene emission however were consistent with those of a non-melting cultivar.

Since this characterization was had mainly the purpose to avoid mis-classification of accessions for stony hard molecular marker research, we decided to keep those two away from the pool of cultivars that would have been used fort that purpose.

Stony hard flesh. As expected, cultivars included in this class had fruits that retained their firmness throughout the whole duration of analysis. Loss of firmness was limited: between -0.6 ('Big Top') and -0.8 kg/cm^2('Ghiaccio sel. 193 QXVII 111') between harvest and day 10 (see figure 4.9).

'Big Top', in particular, was characterized by a retinue of firmness (3.6 kg/cm^2) almost unaltered from harvest to day 5, that reached a value of 2.8 kg/cm^2 only by the end pf the experiment, at once with the ethylene biosynthesis peak.

On the other side, 'Ruby Rich' registered a progressive loss of firmness, ranging from 2.3 kg/cm^{2}at harvest to 1.4 kg/cm^2 at day 10. This softening was gradual, completely different from the brisk one of melting cultivar. The low level of ethylene emitted, on the other side, is not enough to classify this cultivar as a non-melting one. Although its ambiguous behavior, 'Ruby Rich' has been included in the stony hard pool of cultivars.

4.2 Allelic characterization.

Preliminary analysis with sample subset allowed to screen over the whole primer set, detecting which ones were more likely to have a great degree on polymorphisms. An example of how candidate genes analysis worked is reported in figure 4.10.

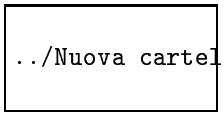

Figure 4.10: Example of candidate gene analysis: a) multiplexed primer output on the subset of cultivar for preliminary analysis; b) non-multiplexed primer output of cultivar subset; c) example of extended analysis on the whole set of cultivars.

In this way, of the whole array of 41 different primers for CGs that have been investigated, only 15 have been proved to be polymorphic. On the basis of collected data has been possible to explore allelic variability, for genes of interest, present in cultivars and selection examined. Those genes are listed in table 6.3 on page 118.

4.2.1 Allelic variability for assessed genes.

As a general remark, given the large variability found in this work, there is a great abundance of allelic forms in peach germplasm, although that the number was quite low compared to the huge number of cultivars released in the past years.

ACO 3.

Many alleles have been detected for this gene, and are listed below:

- 120 bp: only in five accesssions, always paired with the 150 bp allele;
- 150 bp: in homozygosity in 7 accessions and in heterozygosity in 5 more;
- 200 bp allele, detected only in 'Glohaven' in heterozygosity with the 244 bp allele.
- 220 bp: detected only in 3 accessions ('Springcrest', 'Ghiaccio sel. 193 QXVII 111' and 'Frederica'), always in heterozygosity with the 268 bp allele;

- 236 bp: only in 'Maria Dolce' and in homozygosity;
- 244 bp: this allele is present in many combinations:
  - "244/256" in 'Carson' and 'Yu Bay';
  - "244/268" in 'Helena Cling';
  - "244/200" in 'Lamone';
  - "244/244" in 'Ambra'.
- 256 bp: in heterozygosity with 244 bp allele and in homozygosity in 'D 41-62'.
- "*null*" allele, detected only in 'BO 86007016'.

This great abundance of allelic forms is not accompanied by different segregation for flesh type, thus making impossible their use as reliable marker. This may be explained by considering *ACO 3* primer amplifying an intron region of ACO gene, not directly responsible of gene activity. This hypothesis would explain the lack of any polymorphisms in other tested ACO primers, that are amplifying coding regions, and therefore are more conserved in peach genome.

Ara.

Two alleles have been found, 378 bp and 380 bp. Accessions were equally divided between combinations "380/378" (14 cultivars), "380/380" (11 cultivars) and "378/378" (8 cultivars). 'Oro A' is the only one showing no amplification for this primer, suggesting that the cultivar might have a "*null*" allele for this gene.

EGase.

Three alleles have been found: 1020 bp (in 15 cultivars); 1000 bp, only in 'Ghiaccio sel. 193 QXVII 111' and in heterozygosity with 1020 bp allele; a "*null*" allele, in all the remaining ones. This null allele doesn't seem to be related to any particular flesh phenotype, suggesting that the designed primer is unable to amplify the genomic region of EGase, either because it is not EGase-specific (hence, base pairing is difficult and incomplete, driving to a less efficient amplification) or its sequence amplifies only a specific part of the gene sequence that is not present in the cultivars showing the so called "null" allele.

EndoPG 1 and 6.

Those genes acted together in identifying "*F*" alleles, hence are reported in the table with the unique caption "EndoPG". Eight different alleles have been identified: $F_{205}$and F_{231}, dominant alleles; f_{209}, f_{211}, f_{229}, $f1_{null}$, $f1_{201}$, recessive alleles. Using the primer output has been possible to verify the flesh type of accessions, according to Peace's work [81], where combination of this alleles has been described. Among those, an allelic form that wasn't described before has been found in 'Salkaja', f_{215}, that will be discussed in a more detailed way below, when treating 'Salkaja' cultivar.

EndoPG 1 and 6 markers test on 'RR 53-272' and 'Romea' cultivars (see 4.1.3.1) proved that those two are not non-melting cultivars. Considering the rapid loss of firmness, and the high levels of ethylene emission, however, it is possible to classify those two as "melting flesh, very firm" cultivars. In 'Romea' this has been supported also considering the acidity value recorded for this cultivar with firmness values (see 4.1.1), whose high values were symptoms of an anticipate harvest.

Using the marker, 'Crizia' appeared to be a non-melting cultivar and not a "melting very firm" as appeared from the biochemical analysis. Softening profile and ethylene emissions, anyway, were comparable to the ones recorded for accessions of the melting very firm group. This might be due to either two reasons. Loss of firmness can't be considered as an unmistakeable index for assessing flesh phenotype, and ethylene emission (as reported before) is not an objective index, hence 'Crizia' has been wrongly classified. It might also be that a faint amplification of bands in the polyacrilamide gel masked the real presence of the "F" bands, suggesting that the allelic profile was the reported "$f1_{(null)}/f1_{(null)}$".

EREBP.

For this gene, three alleles have been detected: 996 bp, mostly in homozygosity; 998 bp, only in 'Yu Bay' in homozygous; 1000 bp, in 'Salkaja', only in heterozygosity with the most common allele 996 bp. The existence of so little variability suggest that the EREBP gene is very conserved in peach genome, for its role in ethylene signal transmission. Supporting this hypothesis is that no particular effect of the two isoforms has been measured in ripening fruits. The differences in the gene sequence may be present in the introns, so that EREBP activity is not affected.

GT 1.

Three alleles have been found for this gene: 200 bp, only in 'Yumyeong' and 'Ghiaccio sel. 193 QXVII 111's; 204 bp, in homozygosity in all accessions, with the exception of the previous two and 'Salkaja', that showed the third allele, 208 bp. It suggests that GT gene is not belonging to a multigenic family and is strongly conserved in peach genome.

Pel 2.

Seven different allelic form have been found: 160 bp, 194 bp, 200 bp, 204 bp, 208 bp, 218 bp and 224 bp. Most of the accessions were showing "200/204" (15 out of the total) allelic set and "204/204" (9 out of the whole sample) set. No relation between *Pel 2* alleles and flesh type could be found.

Many of, but not all, the stony hard accessions showed the 224 bp allele compared to the others. However, is not possible to consider this allelic difference as a marker for stony hard trait.

PG 4.

Several alleles have been detected: 792 bp (in 'Salkaja' only),796 bp, 798 bp, 800 bp, 802 bp. Most of the accessions showed the "800/798/796" polymorphism (16 accessions out of 34). This can possibly be due to a doubling of the PG gene, so that one form is homozygous and the other is heterozygous, thus resulting in three alleles detected. Since the *PG 4* primer wasn't designed to specifically detect endo- or exo-PG, it may also be that the primer is suitable to amplify both forms.

Other five accessions showed the "802/800/798/796" polymorphism, suggesting a double-etherozygosis for PG genes. However, no relation between PG allele set and Endo-PG alleles has been found, hence strenghtening the ipothesys of a doubling of an exo-PG gene. This is particularly true considering 'Yu Bay' cultivar, that has no alleles for *Endo-PG* (since is a non-melting cultivar), but has the "802/800/798/796" polymorphism for *PG 4* primer.

All the remaining cultivars have proved to be or homozygous for one allele only (five were "798/798" and five were " 800/800") or heterozygous ("800/796").

PG 5.

Two allelic forms have been found, of 380 bp and 378 bp. The latter has never shown to be homozygous. Three cultivars ('Rosa del West', 'Springcrest' and 'Vistarich', all melting flesh cultivars) did not show any amplification. This may suggest that those cultivars are lacking this enzyme form, but since no differential softening ratio has been observed among those cultivars and all the other melting flesh ones, this suggests that other PG forms should be present with an effect comparable to *PG 5*.

***PME* family.**

Six different primers have been tested, and four showed segregation.

PME 1. Showed three alleles: 380 bp (only in 'Salkaja'), 396 bp and 398 bp. Most of the accessions analyzed are homozygous for "396/396" polymorphism, but seven of the stony hard showed the "398/398" allele set. It is probable that those cultivars share common ancestors, since are all from the Far-East (hence, the "398" allele could come from Chinese or Korean germplasm) or obtained from crossing with oriental parents. Through self-pollination cycles, the latter could have achieved an homozygous configuration as their oriental forefathers. In this way, *PME 1* 396 bp allele could be used as a marker for studies on phylogenesis.

PME 5. Five alleles (896 bp, 898 bp, 899 bp, 900 bp and 902 bp) have been detected. Many of the accessions are homozygous for "902/902" (11 out of the total) or "898/898" alleles (7 cultivars). Many of, but not all, the stony hard cultivars have shown the 898 bp allele.

PME 6. Showed three alleles (150 bp, 250 bp and 252 bp). All the accessions had the 250 bp allele, with the exception of 'Salkaja' (150 bp) and 'Honey Gold' and 'Springbelle', that are the only two showing the 252 bp allele.

PME 7. Three alleles were found: 960 bp (only in 'Salkaja'), 970 bp and 980 bp. Those alleles are spreed among the accessions, thus making impossible any grouping by the flesh type.

Unk 3.

Three different allelic forms have been detected: 248 bp, 250 bp and a "*null*" allele. Most of the cultivars were homozygous for a 250 bp allele. Five showed a "2650/248" polymorphism ('BO83007016', 'Yumyeong', 'Frederica', 'Honey Gold' and 'Oro A'), while 7 were showing the "*null*" allelic form. Since no relationship between *Unk 3* and the flesh type could be detected, no specific hypothesis could be formulated about the allelic function.

4.2.2 Particular allelic profiles.

Even if all the accessions have been analyzed for the main genes involved in softening process, it was not possible to find any allele specifically linked to the flesh type. The only exception is Endo-PG marker, that has proved rather precise in determining melting and non-melting flesh types. according to Haji *et al.* ([57]), using this primer allowed to screen stony hard cultivars and verify which typology of flesh they have, independently from the "hd" allele masking effect. All of stony hard accessions have shown to have melting flesh fruits.

Among stony hard cultivars, 'Ghiaccio sel. 193 QXVII 111' and 'Yumeyeong' allelic profile has to be pointed out. The two cultivars showed an almost identical profile for genes of interest. 'Ghiaccio sel. 193 QXVII 111' originates from a self-pollination of 'Yumyeong', and such heritage is enlightened by this allelic analysis. This behavior is particularly evident considering *GT 1* primer: both are the only ones to show the 200 bp allele. Exceptions are for two primers, *EGase* and *Unk 3*: for the former, 'Ghiaccio sel. 193 QXVII 111' exhibited a second allele, at 1000 bp, while for *Unk 3* the cultivar has showed to be homozygous for 250 bp allele, whereas 'Yumyeong' has the 248 bp allele too.

'Salkaja' (non melting flesh) exhibited a particular allelic set, different from all other accesssions. The most possible explanation for this particular behavior is that 'Salkaja' could have introgressed genetic material from other species. It is probable that this cultivars comes from a crossing between a peach and a peach x almond hybrid[1]. This hypothesis is supported by some particular alleles, specific to almond, in particular regarding Endo-PG alleles (Peace, personal communication). 'Salkaja' had a novel allele, 205 bp, undetected before in *P. persica.* Moreover, for *PME 1*, *6* and *7* this cultivar showed a unique

[1] 'Salkaja' is a local selection from Guatemala. As almost all the germplasm accessions from Central and South America, its origins must be tracked down to Spain. It can either be that the crossing with almond x peach hybrid happened after the introduction in America or even before.

configuration. For the *PME 1*, the 380 bp allele; *PME 6* primer showed a band at 150 bp. For *PME 7* showed the 960 bp allele. This configuration could be either due to a different sequence in gene responsible for pectin-metylesterase synthesis or in different PME isoforms. This cultivars differed for more primers: *EREBP* (1000 bp allele) and *PG 4* (792 bp allele), *Endo-PG 1* ($f_{(215)}$) and *GT 1* (208 bp allele) too, probably due to almond ancestry. Furthermore, 'Salkaja' is homozygous for *PME 5* (double 899 bp allele), *Pel 2* (double 200 bp allele) and *Unk 3* (double 248 bp allele) No different behavior due to flesh type could be associated to this uniqueness in the allelic profile, indicating that different allelic forms are not always likely to affect in a significant way gene activity.

Using Endo-PG marker has been possible to determine that 'BO 86007016' selection ("slow ripening"[2]) is potentially a melting flesh phenotype. Although further analysis would be necessary to prove it (such as exposition to exogenous ethylene in controlled conditions) is possible to draw some hypothesis on its inability to accomplish ripening and softening. Since other tested ACO primers did not show polymorphisms, this phenotype could be determined by a mutation of gene responsible of *ACO* activity: 'BO 86007016', contrary to any other tested accessions, did not show amplification band for those primer.

As considered when discussing *ACO 3* variability, 'BO 86007016' ACO gene could have had a mutation that led to a termination sequence in the intron region, that would therefore arrest polymerase activity and, conseguently, ACO expression. This hypothesis is supported also by biochemical analysis of this slow ripening phenotype: during post-harvest life, those fruit did not develope any ethylene and, differently from stony hard fruits, did not show any symptom, although slow, referable to softening: fruits slowly rot without reaching maturity.

4.3 Stony hard marker.

In order to obtain a molecular marker for stony hard trait, 90 RAF primers have been tested on the bulks, for a total of about 9000 markers. Out of the whole, only four of them have been proved more or less related to the presence/absence of the "*hd*" trait in selected cultivars.

[2] 'BO 86007016' selection is unable to reach a complete ripening. The fruit, in the latest stages of development, is still bright green, with just blushes of pale red overcolor on the exposed cheek. Flesh is white, clingstone. Pit itself is likely to break when trying to remove it from the flesh.

Through RAF analysis, three primers have shown polymorphisms and have been tested on the whole array of accessions: "**P11**", "**B12**" and "**Y09**". A fourth, "**B03**", shown a polymorphism that could not be detected in the extended analysis: after the initial amplification in bulks, the primer did not operated the amplification when used for single cultivars and selections. Since during analysis the primer was depleted, such a problem could be imputed to a wrong primer sequence. Wheter the cause, the primer showed a polymorphism that could be potentially used as a "marker" for the researched trait: further analysis will be carried on to set matter straight on this.

Primers that demonstrated to be polymorphic are reported in table 4.3.

PRIMER	SEQUENCE
B12	CCTTGACGCA
P03	CTGATACGCC
P11	AACGCGTCGG
Y09	AGCAGCGCAC

Table 4.3: RAF primers and sequences.

P11: five polymorphisms have been detected for this primer (see figure 4.11):

../Nuova cartella/P11 generale con scritte.jpg

Figure 4.11: polyacrilamide gel reporting P11 primer output. The two main polymprphic bands are highlighetd. "*****" stands for 'Dr. Davis' (to the left) and 'Georgia Belle' (to the right) parentals used for mapping purposes.

P11$_{(930)}$: codominant marker, with an apparently low ability to discriminate stony hard phenotype. The marker shows a positive response to phenotype in about 40% of cases (see figure 4.4). As appears from the polyacrilamide gel, "B" (lower band) and "AB" (presence of both upper and lower band) combinations appear to be related, although faintly, with stony hard trait.

P11$_{(790)}$**:** non-codominant marker. band presence seems not to be related to stony hard trait.

PHENOTYPE	CORRESPONDENTS	NON-CORRESPONDENTS
STONY HARD	7 (9)	3
NON-STONY HARD	4 (5)	10

Table 4.4: correspondence between phenotype and $P11_{(930)}$ marker. Value between brackets indicates the number of individuals showing a codominant band.

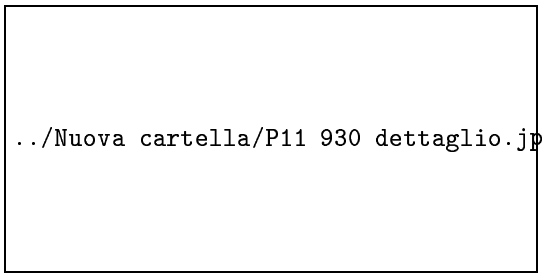

Figure 4.12: detail of $P11_{(930)}$ polymorphism, showing the codominance of the marker.

$P11_{(650)}$: non-codominant marker. Polymorphic, but not associated with stony hard gene. Maps on TxE map in position 6:25.

$P11_{(440)}$: non-codominant marker. Polymorphic, but unrelated to stony hard trait.

$P11_{(360)}$: codominant marker. The lower band alone or the concurrent presence of both bands seem to be associated to "*hd/hd*". In particular, in 'Ghiaccio sel. 193 QXVII 111' and 'Yumyeong' cultivars only the lowest band is present. The upper band alone seems to be related to the presence of "*Hd*" allele (see figures 4.12 and 4.14 on the next page). It is particular the behaviour of 'D 41-62' cultivar: althought it has the lower set of band, it is evident a duplication of the band, about 6 bp lower. No differences in softening have been detected, hence this unique "double band" is not related to an higher intensity of stony hard trait expression. This anomaly has been found only for this marker. $P11_{(360)}$efficiency as marker is narrow, because it seems to be able to distinguish between "normal" types and stony hard ones for just 50% of samples (see 4.5 on the facing page). On TxE this marker maps in 4:63, same group of Endo-PG marker [81].

PHENOTYPE	CORRESPONDENTS	NON-CORRESPONDENTS
STONY HARD	4 (12)	3
NON-STONY HARD	13 (5)	2

Table 4.5: correspondence between phenotype and $P11_{(360)}$ marker. Value between brackets indicates the number of individuals showing a codominant band.

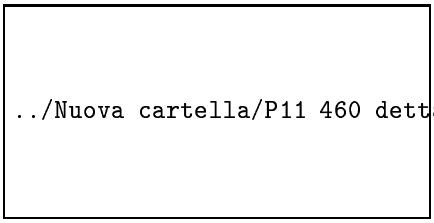

Figure 4.13: detail of $P11_{(360)}$ marker.

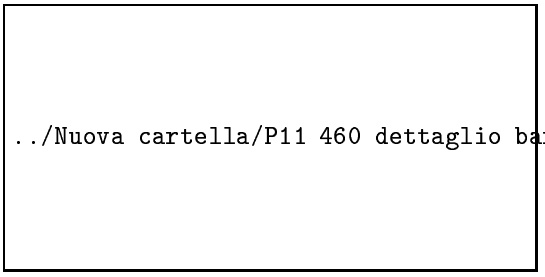

Figure 4.14: detail of $P11_{(360)}$ marker: codominance is evident. "*" indicates 'D 41-62' sample.

***B12*:**

only two polymorphisms have been detected with this primer, but one of those has proved to be the most effective as stony hard marker. The polymorphic ones are listed below.

$B12_{(680)}$: non-codominant marker (see figures 4.15 on the next page and 4.16 on the following page). Individuals that are phenotypically stony hard exhibited a band at 680 bp, that was missing in melting and non-melting flesh ones. Efficiency of this primer as stony hard marker is quite high, as shown in table 4.6 on the next page. However, mapping the primer has proved to be impossible both on TxE and 'Dr. Davis' x 'Georgia Belle' maps, since none of the individual used for mapping purposes had the

band seen for "stony hard" accessions. Hence, it is impossible to associate the trait to any known gene lying in the surroundings.

PHENOTYPE	CORRESPONDENTS	NON-CORRESPONDENTS
STONY HARD	14	3
NON-STONY HARD	12	3

Table 4.6: correspondence between phenotype and $B12_{(680)}$ marker.

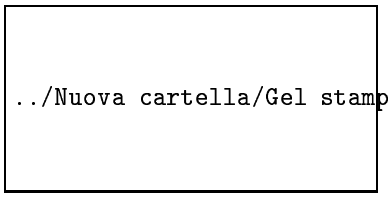

Figure 4.15: B12 primer output. The arrow on the left indicates the main polymorphism related to "stony hard" trait. The "*" locates 'Slakaja' cultivar on the gel.

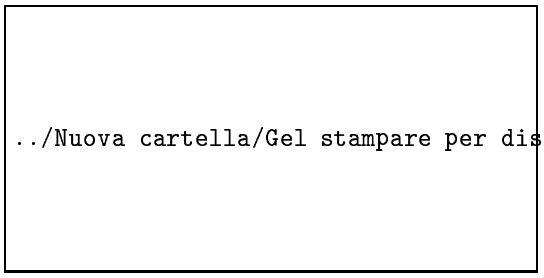

Figure 4.16: B12 output detail.

As seen for allelic characterization, 'Salkaja' shows here a different behaviour compared to all the other accessions. This cultivar has a few bands more than any other (figure 4.15). The most important thing is that none of them is positioned in the 680 bp area, hence allowing to classify the cultivar as non-stony hard, as phenotypically is. However, it is evident that they are coupled, so that the band at about 676 is a duplication of 686 band, and 658 bp band is paired with one of the two at about 668 bp. Also this phenomenon is also attributable to the almond heritage of this cultivar.

To prove the association betweeen B12 marker and "stony hard" trait, the same B12 primer has been tested on a collection of 36 individuals (33 F_2 off-

springs, the two parentals and the F_1) belonging to the collection of the I.S.F. (Roma, Italy), that kindly allowed to use the material. Such a population has been obtained crossing a stony hard individual with a melting flesh one. Since the I.S.F. is conducing similar researches on stony hard trait, the name of the parentals and seedlings has not been diffused, and no data is shown here.

Out of the total, 30 seedlings had the band that is supposed to be linked to the recessive allele. The melting flesh parental was without any band, while the F_1 was showing it. According to this theory, supposing that melting parental would be "*Hd/Hd*" (since no band was detected) and the stony hard parent was "*hd/hd*" (presence of the band), the theoretical segregation rates would be 1:2:1 ("*Hd/Hd*", "*Hd/hd*" and "*hd/hd*" respectively). Thence, 3 individuals out of 4 ("*Hd/hd*" and "*hd/hd*" combinations) would show the 680 bp band, while only one should be without.

What previously said would be correct if the analyzed population was complete, that means that the samples analyzed were the whole offspring of the self-pollination of F_1. However, in our specific case the accession analized were not the whole offspring, but a subsample composed by an equal number of "stony hard" and melting phenotypes. The χ^2 test conduced on observed and expected values demonstrated that the subsample was not a real segregant population (see table 4.7).

	BAND PRESENCE	BAND ABSENCE
EXPECTED VALUES	24	8
OBSERVED VALUES	30	2

Table 4.7: 680 bp band presence observed and expected frequencies for $B12_{(680)}$ marker.

Considering the primer output it is supposed that B12 primer is able not just to identify stony hard phenotypes, but any bearer of the recessive allele "*hd*": it drives to the assessment that the band should be present both for "*Hd/hd*" (melting and non melting phenotypes) and "*hd/hd*" genotypes (stony hard phenotype).

However, B12 as stony hard marker could allow the statment that none of the three peach parentals used for mapping purposed ('Earlygold', Dr. Davis' and 'Georgia Belle') had in their genome the recessive allele, hence making impossible to map this marker on those maps because no segregation could be possible.

B12$_{(290)}$: codominant marker, polymorphic but not associated to "*hd*" allele.

***Y09*:** non codominant marker. Four different bands have been detected, scored with a number depending on their position: "1" for 228 bp band; "2" for 226 bp band; "3" for 220 bp band and "4" for 216 bp one. Band 1 was present in many of the accessions, but no relationship with the flesh type could be traced since it was present in all accessions. A significant polymorphism was detected only for band "3" (see figure 4.17).

Stony hard individuals showed this band, always combined either with band "2" or "4" (only in one case, 'NJ 307', band "3" was paired with band "1"), while non-stony hard samples were presenting only combinations of the other "2" and "4" (see figure 4.18). Marker efficency was higher than B12, as reported in table 4.8.

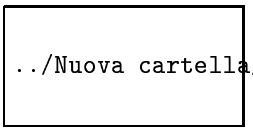

Figure 4.17: Y09 output. "a" (on the left) shows the first attempt and the results obtained, while "b" (right) shows the mapping of the marker on "TxE" and a repetition of the test on some samples.

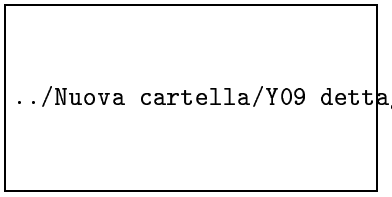

Figure 4.18: a (left): Y09 bands detail; b (top right): detail of "BIN set": from left to right, T, E, H, 5 (TxE), 12 (TxE), 23 (TxE), 30 (TxE), 34 (TxE), 83 (TxE).

PHENOTYPE	CORRESPONDENTS	NON-CORRESPONDENTS
STONY HARD	16	4
NON-STONY HARD	13	6

Table 4.8: correspondence between phenotype and Y09$_{(210)}$ marker.

As seen for B12 marker (see 4.3 on page 83), some of the non-"stony hard"

accessions where showing the band that has been supposed to be associated to stony hard trait. In particular, 'Hakuto', 'BY 99-1343' and ' BY 98-4649', melting flesh cultivars, where scored as "2,3", "2,3" and "2,3,(4)" (this could be easly due to a contamination of the well with part of the sample from the adjoining one: in the next gel, the band number 2 was absent and "3" band more evident -data not shown-) bands respectively (see figure 4.18 on the preceding page). With B12 marker the same three samples showed an analogous output (680 bp band), since in all of them Y09 could be able to detect the presence of the recessive allele "*hd*". Theoretically, as seen for B12, Y09 could be able to detect the presence of "*hd*" allele. So, the same marker was tested on a subsample of I.S.F. individuals (data not shown) to verify the hypotesis.

Data collected in this second test showed that the efficency of Y09 in detecting the "stony hard" trait was lower than expected (see table **??** on page ??). That was probably due to an greater distance between "hd" locus and Y09 marker than in B12. hence, the probability of having a recombination is significatively higher, determining a relevant number of "misreadded" genotypes.

In 'BY 93-P4371' (SH selection) accession, on the other hand, both with B12 and Y09 markers resulted to be without any band associated to stony hard trait. Even if it could be due to a low efficency of both markers in detecting the presence of the trait, it drives to the hypotesis that this individual has been wrongly classified as stony hard by previous analysis (since the sample was coming from U.C. Davis collection, no phenotypical data about flesh characterization is available).

Using the "BIN set" has been possible to determine that this marker maps in 5:46 on TxE map, far from Endo-PG marker area and from ACS mapping region.

***P03*:** as stated before, for this primer we have only preliminary data. However, three different polymorphisms have been detected (see figure 4.19):

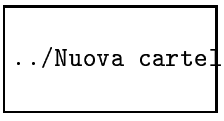

Figure 4.19: P03 marker output. a) 1000 bp and 660 bp polymorphisms; b) 300 bp polymorphism; c) a detail of 660 bp polymorphism extended to the complete set of bulks available ("Dr. D" and "G. Belle" labels stand for 'Dr. Davis' and 'Georgia Belle' cultivars respectively).

$P03_{(1000)}$: non-codominant marker. The "stony hard" bulks were characterized by a low band at about 1000 bp that was absent in the non-"stony hard" ones.

$P03_{(660)}$: non-codominant marker: in the cluster of bands at 660 bp, "SH" bulks had one more band, about 2 bp higher, than "NOR" bulks. In the figure 4.19, the "c" section shows a band, although faint, in the "NOR B" bulk which should not be present. This could either be due to the pollution of the bulk's well with the "SH B" bulk material or the incomplete efficiency of the marker in assessing the flesh type, revealing a "false positive" output. A more detailed analysis, carried on the whole set of samples, would have proved which of the two explanations would have been true. 'Dr. Davis' and 'Georgia Belle' samples were added to try a preliminary mapping of the marker on the relative map developed by U.C. Davis, but since this particular polymorphism as proved to be non-codominant, mapping was impossible (both parentals, non-stony hard cultivars, where lacking the band).

$P03_{(300)}$: codominant marker: stony hard groups were showing a broad band at 296 bp and another one, fainter, at 300 bp, while all the other bulks where showing the opposite situation (fainter the lower and broad the higher).

The potential of P03 as marker for stony hard trait is high, since it should work both as a codominant ($P03_{(300)}$marker) and non-codominant ($P03_{(1000)}$and $P03_{(660)}$) marker, hence potentially giving a more detailed characterization of the singular cultivars. A whole array of possibilities can arise. Should the non-codominat markers work as seen and supposed for B12, detecting the recessive allele, as it seems to be for the output similar to B12 (absence of the band in "NOR" groups, and presence in "SH" ones), it could be used to detect bearers of this allelic form. On the other side, the presence of a codominant marker could not reasonably be able to reveal the genotype of the sample (i.e. "Hd/Hd", "Hd/hd" and "hd/hd") directly from the DNA. So far, is possible to define the ouput of the $P03_{(300)}$marker in that sense: if the "double 300 bp band" could be masked by the presence of etherozygous individuals using bulks, stony hard individuals, that have both alleles in recessive form, should show immediatly a "double 296 bp band".

4.4 Biochemical basis for the "stony hard" trait.

Although no experimental evidence has been obtained so far, could be interesting to try to have an explanation to the "stony hard" trait on the molecular basis. This may be possible combining the knowledge of what happens in softening process and stony-hard behavior.

In order to understand better how it could work we need to go back to ethylene pathway (see 1.1.5.3 on page 35). Haji *et al.* ([57]) reported that ACS is responsible for the stony hard behavior, in particular for lack or low activity of ACC synthase. In a recent work, ACS 1 mRNA was not induced during ripening stage, and ethylene production was inhibited [105]. Researches hypotized that this could be due to a mutation (deletion or insertion of bases in flanking regions) of ACS gene [104].

Since ACS activity is suppressed or severely reduced, little or no ACC can be produced, hence inhibiting ethylene biosynthesis. This seems to be consistent with no emission of the hormone from stony hard fruits (e.g. 'Yumyeong' and the "crispy type").

However, not all the stony hard fruits are lacking ethylene (see 1.1.4.3 on page 26), and ACS 1 mRNA is induced and expressed in wounded and senescent tissues [105]. Besides, no significant differences in ACS alleles have been detected in this work (see section 4.2) between "stony hard" and the other phenotypes, although the mutation suggested as explanation for "stony hard" trait could be punctual. Moreover, none of the tested possible markers linked to stony hard trait mapped in any genomic region close to ACS. Re-discussing the hypothesis that explains stony hard trait as a mutation of ACS gene is hence allowed.

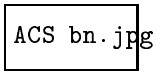

Figure 4.20: passage from system 1 to system 2 in climacteric fruits.

As studied in tomato (see figure4.20), during *System 1* phase only ACS 1 and 6 are working, producing subtle amounts of ACC, responsible of the basal emission of ethylene in fruits, which is not sufficient to stimulate fruit softening. During the transition phase, RIN transcriptional factor (belonging to the big family of MADS-box transcriptional factors) triggers ACS 4 activity, causing an increase in ACC (therefore, in ethylene too). This increase stimulates ACS 2 expression, that inhibits ACS 1 and ACS 6 and massively enhances ACC

synthesis, which brings to a sensible increase in ethylene production. A recent work reported also that MADS-box-like transcriptional factors are induced in ripening peach fruits [107], as happens in non-climacteric fruits [40].

A mutation of the RIN gene could be supposed to lead to a severe reduction or complete inhibition of its expression. The effect of an altered RIN activity is the suppression of ACS 4, that leads to the impossibility to repress ACS 1 and ACS 6 and to activate ACS 2. Since their activity is not suppressed, ACS 1 and ACS 6 produce ACC throughout the whole ripening and softening. This amount of ethylene is not enough to saturate completely ethylene receptors, triggering the fruit normal softening (determined by the "*F*" alleles set), but the little ethylene produced may be sufficient to induce ripening preocesses (change of color, degradation of acids, increase of sugar content, etc.) without having the flesh softening [105]. Hence, recessive "*hd*" allele would be responsible for the mutated RIN sequence that drives to the suppression or reduction of the transcriptional factor activity during ripening.

An ethylene production of less than 10 ppm/kg/h could be attributable to the activity of ACS 1 and ACS 6. Eventually, ACS 1 and 6 activity could determine in the latest phases of ripening an accumulation of enough ACC products to trigger ACS 2, that could explain the ethylene emission in the "melting type" (i.e. 'Big Top') stony hard fruits and their late softening (which would require more abundant ethylene). Late ACS 2 expression can repress ACS 1 activity, according to [105]. A schematic representation of this altered pathway can be see in figure 4.21.

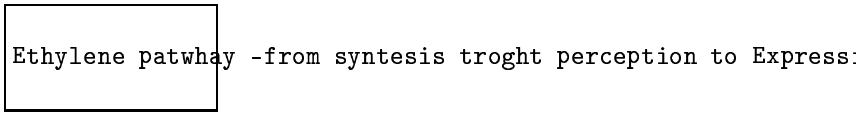

Figure 4.21: Proposed model for the "stony hard" trait.

Stony hard fruits are acting almost as non-climacteric fruits, because of a possible mutation of the RIN gene: this mutation could lead to the inactivation of the gene or to a severe reduction of RIN expression, hence inhibiting ACS 4 triggering. As non-climacteric fruits, indeed, only *System 1* is carrying on the ripening process [61], that is hence not relying only on ethylene influence, even though it can be influenced.

However, since stony hard peaches belong to a climacteric specie, they are influenced by exogenous ethylene treatments. As reported by Haji ([56, 57]),

stony hard fruits soften as melting- or non-melting flesh fruits, depending on their genotype. "Melting - stony hard" fruits (*F/-, hd/hd*) show an increase in ethylene response, with a normal Endo-PG activity that leads to the melting phase; "non melting - stony hard" fruits (*f1/f1, hd/hd*), on the other hand, will show the same softening typical of non-melting peaches, due to the lack of Endo-PG activity, and exogenous ethylene is replacing RIN activity in those fruits, triggering ACS 2 and allowing the "normal" ripening.

Chapter 5

Conclusions.

This work has approached many points. First, numerous accessions, belonging to the main flesh phenotypes have been characterized for their phenotypic traits (SSR, weight, acididy, etc.). All those have been investigated for the main aspects related to softening process occurring to the fruit. Ethylene emission, softening evolution and weight loss have been measured and discussed, allowing a more precise classification of those cultivars into four well defined classes. That was done for a double purpose: firstly, avoiding any "contamination" of the material used for the allelic characterization and the research of a molecular marker for stony hard trait; second, to have a complete characterization of a part of the germplasm collection available at University of Bologna. A re-classification of some cultivars (such as 'Romea' and 'RR 53-272') has been dne, in order to correct a previous wrong categorization. Moreover, many of the data collected in this way have been used to explain particular behaviour in the following parts of this work.

This work has also developed allelic profiles for the main genes involved in ripening and softening process of peach fruits. Despite the initial goals, no re-grouping of cultivars/selection depending on allelic profile could be done, since no relationship between flesh type and allelic profile could be traced. As result, the differences between stony hard, melting- and non melting- flesh can't be attributed to a different allelic set for the investigated genes. A wide database of alleles has been made available for future works and for breeding purposes, allowing researchers to predict the genetic profile for those genes in seedlings deriving from controlled crossings. Moreover, such a database could ease the detection of unknown parentals in offspring showing a different behavior com-

pared to the others of the same lot. Besides, some particular allelic profile has been highlighted, and an explanation of particular softening processes has been provided using the allelic configuration.

The main goal of this work has been achieved, since a pool of molecular markers for stony hard trait has been made available for further works. Of the four markers detected, one (P11, in particular $P11_{(360)}$and $P11_{(930)}$) has a low efficency and hence cannot be used directly as marker for diagnostic purposes, but is still a valuable tool if used in combination with the others.

B12 and Y09 are the most powerful markers tested. The latter, non codominant marker, still needs to be tested on a wider segregant population, since its efficiency in detecting stony hard trait is, even if high, still susceptible to a certain percentage of errors. Its interest is lying in the known position on the "TxE" map: given the possibility to test it on a wide segregant population, it could be possible to track the closest gene to the marker, which is most probable to be the one responsible for the stony hard trait, and sequence it.

The former, on the other hand, is the most effective marker developed so far. Its efficiency in detecting the recessive allele is high (over 80% of positive results): this allows the primer to be used for two functions. To date, one of the more incident cause of errors in researches on stony hard trait is the wrong attribution of a fruit to a specific texture phenotype. On one hand, B12 is an excellent system to remove doubts about attribution of an accession to the texture phenotype, especially if combined with more "traditional" methods (i.e. ethylene emission measurement) to go past the *empasse* caused by the genotypes "*Hd/hd*", phenotypically MF or NMF but showing the "stony hard" band. On the other side, B12 is a powerful tool for breeding purposes. Its ability to detect the recessive "*hd*" allele allows the marker to be used for MAS selection, isolating the bearers and planning further crossings. Despite all the attempts, mapping B12 on "TxE" and "Dr. Davis x Georgia Belle" maps has proved to be impossible, for the nature of the marker itself: "*hd*" is revealed by the presence of a band only, and no bands could be detected in mapping individuals. Moreover, none of them has an oriental ancestor that could have brought the allele in their genome, so in order to map B12 is necessary to create a map on a stony hard segregating population or anchor the marker to another whose position on a map is well known.

The third one, P03, even if not fully developed, is a good marker. This one has shown to have three different polymorphisms ($P03_{(1000)}$, $P03_{(660)}$, and $P03_{(300)}$), two of them detecting stony hard individuals by presence/absence of

a band and the third showing co-dominance. Should it prove to be as efficient as B12, such a primer showing three polymorphisms would be a formidable tool. Moreover, diffrently from B12 marker, the presence of a segregating band also for non stony hard individuals (as in $P03_{(300)}$) would make the mapping possible on "TxE" with an higher precision and reliability, allowing sequencing of the region and a better comprehension of the genetic basis regulating the "stony hard" trait.

Furthermore, a theoretical explanation of molecular basis of stony hard trait has been given. According to this theory, a mutation of peach RIN transcriptional factor would be the cause of "stony hard" phenotype. As result, severe reduction or inhibition of RIN activity would be caused, driving to the inactivation of *ACS 2*. The absence of massive productions of hormone (typical of melting and non-melting fruits) registered in stony hard accessions would derive from this inactivation. Therefore, the enzyme coordination throughout ripening would be drive only by the little ethylene produced by *System 1.* (i.e. *ACS 1* and *ACS 6*). In other words, "stony hard" peaches ripe as non-climacteric fruits.

Chapter 6

Future opportunities.

This work opens many possibilities of research.

The phenotipic characterization of the fruit texture of so many accessions might be extendend to the germplasm collection of Bologna University, in order to have a better classification of the available material for further works. Besides, a good opportunity would be the extention of the measurement of ethylene to a more complex experiment. From Haji's works ([55, 57, 58, 56]) we know that stony hard fruits treated with exogenous ethilene are undergoing to normal softening processes depending on their "*F*" allelic status. The same accessions could be measured for their ethylene emission in controlled conditions (i.e., after propilene treatments and emission at normal conditions), hence having a complete profile of softening process in "stony hard" and tracing a sort of "additive effect" to firmness retaining confered by the "*hd*" allele.

More interesting are the opportunities given by markers developed for stony hard trait. To find the map position of B12 would be a good problem to deal with: a mapping population obtained from a cross with a stony hard parental could be used to test the primer and map it on this one. After that, to link the primer on "TxE" one would be easier. Y09 marker is still to be improved, extending the test to a whole population, since the preliminary analysis were carried on a limited subset of individuals. More work will be required to test P03 marker: initially, testing it on the whole set of cultivars and selection composing the bulks tested; then extending it to a segregating population (as did for B12). If proved as an useful tool, mapping P03 on the main maps available for peach would be the immediate and obvious further step. Besides, a whole universe of RAF primers could be tested, with the aim to obtain better markers for

this trait. Since only 90 primers have been used in this work, more than 400 could still be further tested for this goal: the possibilities of finding an excellent marker are still quite high.

Finally, the explanation given for stony hard molecular basis is still lacking of a severe experimental proof. A good way to proceed with this goal is testing primers specifically designed for peach RIN, in order to identify any allelic difference between stony hard and the other flesh phenotypes. Should exist differences in that sense, sequencing stony hard RIN would give an explanation for "stony hard" particular behavior. Moreover, focusing research on RIN expression during softening in cultivars or seedlings with different flesh types through RNA analysis would be a powerful tool to demonstrate the theory.

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ENCLOSURES

DNA extraction protocol

1. Grind 0.05 g of liofilized tissue in a 2.0 ml Eppendorf vial with a pinch of silicon carbide into the mixer mill plates. Set the mill to 29 hits/second for 3'. Revert plates and repeat grinding.

2. Add 900 μl of pre-heated 60°C extraction buffer, vortex and incubate at 60°C for 30', with occasional inversions.

3. Add an equal volume of isoamilic dichloromethane (24:1) and emulsion for 10'.

4. Centrifuge for 5' at 5000 rpm. Collect supernatant and transfer it to a new 2.0 ml Eppendorf vial.

5. Add 10 μl of RNase and incubate in oven (set to 37°C) for 30'.

6. Repeat points 4 to 6.

7. Precipitate supernatant adding 550 μl of cold Isopropanole (stored at -20°C in freezer). Gently invert tuber 5-6 times to have a better DNA condensation and store at 0°C for 20'.

8. Centrifuge samples at 5000 rpm for 5'. On the vial bottom will form a white pellet.

9. Re-suspend pellet with TE (300 μl) and NaCl 5M (200 μl), mixing gently. Then add 2 volumes of Ethanol 100% to precipitate DNA.

10. Centrifuge (5000 rpm for 5') and remove supernatant.

11. Wash pellet in 500 μl of Washing Buffer (ethanol 76% and Na-acetate 0.2 M). Remove washing buffer.

12. Dry pellets with a speed-vacuum (4') or at open air (fume hood) and re-suspend in 100 μl of sterile water.

TABLES

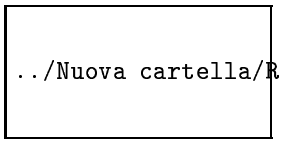

Figure 6.1: Resumptive table of biochemical analisys recorded for each accessions. MF = melting flesh; NMF = non-melting flesh; MVF = melting flesh, very firm; SH = stony hard flesh; SR = slow ripening. Cultivars are grouped according the classification adopted before biochemical characterization.

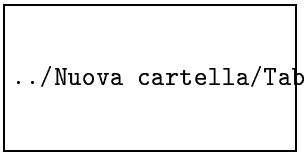

Figure 6.2: Resumptive table of firmness (in kg/cm^2), ethylene emission (in ppm/kg/h) and weight (in g) recorded for analyzed accessions. MF = melting flesh; NMF = non-melting flesh; MVF = melting flesh, very firm; SH = stony hard flesh. Cultivars are grouped according the classification adopted before biochemical characterization.

Figure 6.3: Table of alleles. For each candidate gene (CG) are listed the alleles individuated. Except for EndoPG, the number indicates the weight (in bp) of the band as scored on the polyacrilamide gel. EndoPG report directly the allele name (as in [81]).