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ETHYLENE AND PEACH FRUIT RIPENING

A functional genomics approach

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Riassunto

Le caratteristiche qualitative dei prodotti frutticoli derivano da fattori genetici, agronomici ed ambientali la cui azione, singolarmente o in modo sinergico, può modulare i processi metabolici tipici delle fasi di pre- e post- raccolta che definiscono e caratterizzano lo sviluppo e la maturazione del frutto. La massimizzazione della produttività, l'ottenimento di frutti di buona pezzatura e di elevata qualità gustativa dovrebbero essere le linee-guida principali seguite dai produttori: in quest' ottica, la scelta del momento della raccolta (e la fase di sviluppo in corrispondenza della quale avviene il distacco dalla pianta madre) è strategica: infatti, frutti raccolti troppo precocemente possono avere un lungo periodo di conservazione ma una bassa qualità organolettica, rispetto a frutti raccolti a maturità più avanzata, in cui la qualità risulta superiore ma la "shelf-life" è ridotta a causa, soprattutto, di una rapida perdita di consistenza. Ciò è vero in modo particolare per pesche e nettarine per le quali la velocità del processo di maturazione e la scarsa attitudine alla conservazione inducono ad effettuare raccolte anticipate e rappresentano i principali ostacoli alla commercializzazione di un prodotto di elevata qualità. La delucidazione dei processi che caratterizzano la sindrome della maturazione e dei meccanismi che sovrintendono questa cruciale fase di sviluppo è il prerequisito per lo sviluppo di adeguate tecniche e strategie di campo e di postraccolta mirate al raggiungimento e al mantenimento di elevati standard qualitativi. Nei frutti climaterici, come le pesche, un fattore chiave nella modulazione della sindrome della maturazione è rappresentato dall'etilene, il quale può regolare e coordinare l'evoluzione di molti processi tipici di questa fase di sviluppo. La comprensione dei meccanismi biosintetici e di trasduzione del segnale dell'etilene ha visto, negli ultimi decenni, enormi progressi grazie soprattutto alla scoperta di specifici inibitori della sintesi o dell'azione dello stesso, e all'utilizzo di mutanti. Un notevole contributo all'ampliamento delle acquisizioni di base è anche giunto dalla messa a punto di nuove tecniche di indagine biomolecolare: gli approcci di genomica funzionale e l'allestimento di potenti strumenti di indagine su larga scala (es. microarray) aprono nuove frontiere di conoscenza relative ai meccanismi che determinano la transizione dallo stadio di frutto immaturo a quello maturo e la regolazione degli eventi metabolici legati alla qualità. Un primo passo verso un approccio di genomica funzionale nello studio della maturazione del frutto di pesco è stato

fatto con la creazione di un repertorio EST (Expressed Sequences Tags) da parte del consorzio italiano ESTree. Da questo repertorio, implementato con sequenze disponibili nei database pubblici, è stato costruito il primo micorarray di pesco (μPEACH 1.0) contenente 4806 oligo. μPEACH 1.0 è stato utilizzato per studiare diversi genotipi di pesco in relazione a trattamenti con etilene: oltre ad una cv di tipo "melting", sono stati utilizzati due mutanti, Slow Ripening (SR) e Stony Hard (SH), nei quali la biosintesi di etilene e (solo per SR) la percezione dell'ormone risultano alterati. Le analisi microarray hanno messo in luce, nei due mutanti, diversi livelli di alterazione a carico del metabolismo dell'etilene. SH, in cui il climaterio etilenico è assente a causa di un blocco della trascrizione di *ACS*, è risultato di interesse anche per studi più puntuali riguardanti l'espressione di elementi della via trasduttiva del segnale dell'etilene in relazione a diversi regimi termici in postraccolta e le connessioni esistenti fra alcuni geni codificanti per proteine di parete e l'etilene. Insieme all'utilizzo di mutanti, anche quello di inibitori che competono per i siti recettoriali dell'etilene può essere un ulteriore strumento per definire il complesso network di segnali ormonali coinvolti nella maturazione. Nettarine trattate con 1-methylcyclopropene (1-MCP), un potente inibitore dell'azione dell'etilene utilizzato su molti prodotti frutticoli per prolungare la vita postraccolta, sono state analizzate in termini di profili di trascrizione nel tentativo di delucidare le cause del limitato effetto dell'1-MCP in questa specie frutticola. I pattern di espressione valutati al termine del periodo di incubazione (24h) e dopo 48h evidenziano un profondo cambiamento della trascrzione di molti geni direttamente coinvolti in importanti processi della maturazione quali il rammollimento, lo sviluppo del colore e il metabolismo degli zuccheri. La variazione dell'espressione di geni codificanti fattori di trascrizione legati all'etilene e di altri coinvolti nel metabolismo dell'auxina confermano l'importanza del "cross-talk" fra diversi ormoni nella regolazione del processo di maturazione dei frutti incluso quello di pesco. Infine, nell'ottica di un approccio di genomica funzionale, tre geni (due di pesco ed uno di pomodoro), che evidenziano espressione differenziale in relazione all'evoluzione della maturazione, sono stati utilizzati in esperimenti di transgenesi in pomodoro.

Summary

Fruit quality traits are the result of genetic, agronomic and environmental factors that, alone or in combination, modulate metabolic processes during both pre- and post-harvest phases and affect fruit development and ripening processes. Productivity, size and organoleptic quality should be the main quality criteria adopted by fruit growers: in this context, harvesting time is crucial. Too early harvested fruit may be stored for a long time but their flavour quality is low, whereas late harvested fruit are of better quality but do not withstand long storage periods and theirs shelf-life is reduced. This is particularly true for peaches and nectarines characterized by a quick ripening evolution and a reduced aptitude to prolonged storage: this induces growers to anticipate harvesting and represents the main constraints for supplying high-quality standard level peaches to the consumers. Elucidating mechanisms and basic processes characterizing ripening and responsible for the evolution of quality parameters is the prerequisite to develop strategies aimed to produce high-quality fruit and to maintain these standards throughout the postharvest chain. In climacteric fruit, including peaches, ethylene is a key factor in coordinating and regulating the evolution of several processes characterizing the ripening syndrome. Thus, studying aspects related to ethylene action has been a challenge during the last few decades. Improvements of the basic knowledge of ethylene physiology also came from the identification of specific inhibitors of its biosynthesis and/or action, and from the use of mutants. The development of highthroughput molecular tools (i.e. microarray) and functional genomics approaches represent a great opportunity for a better understanding of the ripening process and the basic mechanisms governing quality-related metabolisms in fruit. Considering peach, the first step toward a functional approach is represented by the development of an Expressed Sequences Tag (ESTs) repertoire, that, together with other ESTs isolated by other units and available in public databases, allowed to select 4806 oligos, corresponding to an equal number of genes expressed in peach fruit, and construct the first peach microarray (μPEACH 1.0). μPEACH 1.0 has been used to study the effects of exogenous ethylene on different peach genotypes, a “melting flesh ” cv and two ripening mutants, Slow Ripening (SR) and Stony Hard (SH). Microarray analysis revealed marked differences in transcript profiling possibly related to the nature of mutation and differences in ethylene physiology.

SH fruit has also been used for expression analyses of two elements involved in the ethylene signalling pathway. Besides mutants, specific inhibitors of ethylene biosynthesis and/or action represent invaluable tools for elucidating the ethylene role in the ripening process. One of the most powerful inhibitor of ethylene function is 1-methylcyclopropene (1-MCP) that is practically used on different fruit species, but not on peaches, to prolong shelf-life. Using μPEACH 1.0, a large-scale analysis of transcriptome has been performed on nectarine fruit treated with 1-MCP in order to elucidate the molecular mechanisms responsible for the limited effect of the inhibitor on this climacteric fruit species. At the end of the treatment (24h) and 48h hours later, a number of genes involved in quality-related ripening processes (such as softening, sugar metabolism and colour development) appeared to be deeply modified in their expression. Changes in the expression profile of Transcription Factors related to ethylene and auxin action confirmed the importance of "cross-talk" between the two hormones in the modulation of the ripening process in peach. In the context of a functional genomics, three different genes (two from peach and one from tomato), identified following transcriptomics approaches, have been used for transgenic experiments in tomato plants.

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Chapter I

Thesis introduction

Fruit Quality and Ripening Physiology

Peach fruit quality parameters

Fruit, one of the most precious gifts from nature to human beings, are valued because rich in vitamins, mineral salts and sugars, but also for the attractive and inviting flavour. Beautiful in colour, shape, and fragrance, fruit are also important for good health. Fruit are now recognized as indispensable for a balanced diet and consumers are becoming more discriminating in choosing and purchasing the different fruit types available in the market.

Fruit constitute an important part of the Italian diet: as average, Italians consume about 70 Kg of fruit per person per year, and more than 50% is represented by five species: apple (17.6%), orange (15.7%), pear (7.7%), banana (10.5%), and peach (7.1%) (http://www.ismea.it).

In relation to production Italy plays an important role for all these species and in particular for peaches and nectarines due to the about 1.7 million tons produced (FAO, 2006), making Italy the largest producer in Europe (45% of EU production) and the second world producer after China. However, a 5% annual decrease in the period 2000-2004 of domestic peach consumption has been registered (http://www.ismea.it) and Italy is losing part of the European market mainly because of low quality standards due to, in general, anticipated harvesting causing a reduced organoleptic (aroma, taste) traits.

Fruit is characterized during development and ripening by an evolution of the chemico-physical properties defined by a network of processes under genetic control (Giovannoni, 2004). Elucidation of this network is crucial to optimize agronomic practices, define the best harvest time and set up the postharvest strategies necessary to maintain the qualitative traits, to prolong the shelf life and to guarantee a better product to the consumer. Considering in particular peaches (throughout this chapter, unless differently specified, with peach we intend both peach and nectarine fruit), they are characterized by a rapid evolution of ripening parameters and, compared to other climacteric fruit as apples, pears and kiwifruit, a limited storage period. Being highly perishable, peaches must be carefully handled at harvest and during the postharvest phase, when management of temperature storage is essential. Extended storage of peaches, nectarines and other stone fruit can negatively affect fruit quality due to the development of physiological disorders, known as chilling injury (CI) or internal breakdown (Lill *et al.*, 1989; Lurie and Crisosto, 2005). One of the most common disorders is woolliness, which becomes apparent when fruit are

ripened after storage at 2-8 ºC for a period of at least two weeks (Ben Arie and Lavee, 1971; Lill *et al.*, 1989). Thus, most of stone fruit varieties are best stored near freezing temperatures. However, even under ideal conditions, storage life is limited to 2–6 weeks depending on the cultivar. This short storage life provides limited scope for smoothing fluctuations in supply to domestic markets, limits exports by sea freight to nearby markets or requires the use of airfreight for more distant markets.

As above reported, harvesting time is a key factor in determining peach fruit quality traits. Earlier harvesting leads to a worsening of quality: less colour, small size and a bitter-acid taste which is due to relatively low sugar concentrations and a high content of organic acids, polyphenols and aldehydes (Robertson *et al.*, 1988; Horvat *et al.*, 1990). Typical peach flavour compounds such as δ-decalactone, γ-decalactone, linalool and benzaldehyde that contribute to fruit fragrance are low or absent in fruit harvested when immature (Horvat *et al.*, 1990, Visai *et al.*, 1993).

On the other hand, later harvested fruit have higher quality but a shorter storage life compared to early harvested varieties, being more sensitive to pathogens and mechanical damage during transport and handling procedures. Thus, in some cases quality value decreases and physiological disorders appear. The right moment for harvesting results from a compromise between the market demand for high quality fruit and the need to reduce postharvest losses of product during storage and wholesale and retail trade.

Although perishability is the limiting factor in postharvest handling and marketing of peaches and nectarines, final quality and consumer acceptance and some postharvest responses are also affected by pre-harvest factors, such as growing conditions, cultural practices and genetic potential. Various pre-harvest stresses may have significant impact on fruit flavour, weight, general appearance and storage potential (Crisosto *et al.*, 1997).

Flesh firmness, acidity, sugar content, aroma and colour are the major parameters to evaluate, alone or in combination, as the principal index for ripening, and these are the factors that should be considered in research projects aimed to implement peach fruit quality.

In order to be sold in European markets, peaches, as well as other fruit and vegetable products, must be classified according to EU standards that provide consumers with some information about origin and external quality features (namely size and appearance).

According to CEE Reg 3596/90, 1169/93 and 2235/99, peach size should be at a minimum of 56 mm for diameter and 17.5 cm in circumference, and the definition of different commercial categories is mainly based on fruit size.

While there are no European rules regarding qualitative aspects related to flavour, Italy has defined some standards for peaches to be commercialized with the Protected Geographical Indication (IGP) label:

- Size: minimum circumference 17.5 cm;
- Epicarp, shape and weight typical of the variety;
- Sugar content: minimum 9.5° Brix for the early harvesting varieties (up to 30 June); 11.5° Brix for varieties harvested after 1st of July.

This last parameter is of particular importance considering that an increasing interest and concern of consumers is directed toward internal quality aspects as taste, nutritional value and presence of bio-compounds as antioxidants. Both external and internal parameters are the result of the evolution of biological processes taking place during fruit development and differently affected by agronomic and environmental factors before and after harvest.

Quality traits and peach fruit development

The main processes defining the peach ripening syndrome occur during the last part of fruit development and are genetically controlled. Many of these (accumulation of soluble solids, softening and cell wall changes, colour and aroma development) have a direct impact on appearance, composition and consumer acceptability, while others (i.e. ethylene evolution and respiration rate) have mainly a physiological impact, thus indirectly contributing to the evolution of quality parameters.

Peach fruit exhibit a double sigmoid growth pattern. This developmental pattern may be divided into four stages (Tonutti *et al.*, 1991): Stage 1, which includes cell division and cell expansion; Stage 2, during which the endocarp becomes lignified (pit hardening) and fruit growth is slow; Stage 3, when most of the increase in fruit size takes place due to cell enlargement; and Stage 4 is characterised by the increase in ethylene production and the ripening process (Fig. 1). Stage 2 is often not evident in low chill cultivars of peaches and nectarines that have a short fruit development period. Unlike other *Rosaceae* fruit such as

apples and pears, the mesocarp in stone fruit appears homogeneous and neither an inner nor outer cortex can be distinguished. However, regions of the mesocarp appear to be biochemically distinct, as the low temperature storage disorder of peaches and nectarines termed 'leatheriness' is confined to the outer layer of this tissue. In addition, bleeding, a condition resulting from over storage, occurs when anthocyanin pigments appear to spread outwards from the stone but usually they do not reach the outer layer of the mesocarp and the skin (Lurie *et al.*, 2005).

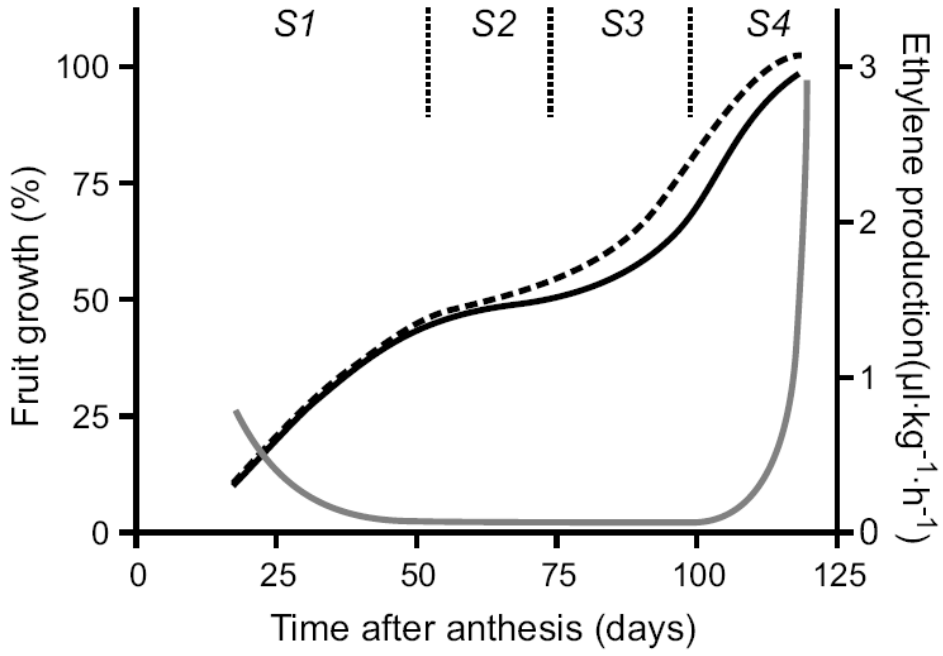

Figure 1 - Relative changes in fruit growth of 'Fantasia' nectarine (top unbroken line) and 'Jalousia' peach (top broken line). The bottom line shows the rates of ethylene production relative to the four stages S1–S4 of fruit development (adapted from Moing *et al.*, 1998 and Tonutti et. al., 1997).

Accumulation of soluble solids

Sweetness and acidity are the most important factors affecting consumer acceptability of stone fruit and they are strictly correlated (Parker *et al.*, 1991); in addition, they contribute with phenolics and carotenoids to fruit quality and nutritional value in terms of modifying colour, taste, aroma and providing health–promoting effects.

Sucrose, glucose and fructose (in proportions of about 3:1:1) are the main sugars in peaches (Génard *et al.*, 2003), and in ripe fruit, they comprise about 60% of the soluble solids

concentration (SSC) as measured with a refractometer. The relative concentrations of these sugars also influence sweetness, as fructose is 2.3 and 1.7 times sweeter than glucose and sucrose, respectively (Kulp *et al.*, 1991). Sucrose and the sugar alcohol sorbitol account for most of the carbon translocated from the leaves to the fruit. Sucrose accumulation closely correlates with increases in fruit dry weight. Glucose and fructose accumulate at a near constant rate during Stage 1 but gradually decrease during the remainder of fruit development. Sucrose accumulates rapidly during Stage 3, reaching a peak in Stage 4 if the fruit are allowed to ripen on the tree, and this sugar accounts for about 50% of the dry weight at ripening. Activities of sucrose hydrolizing enzymes (insoluble acid invertase, soluble acid invertase, neutral invertase and sucrose synthase), high in young fruit, decline sharply with fruit development and ripening concomitantly with accumulation of sucrose (Vizzotto *et al.*, 1996). A correlation between sucrose, glucose and fructose content and the expression of a neutral (cytoplasmic) invertase (*PpNI1*) has been reported by Nonis *et al.* (2007). The glucose to fructose ratio may change from 1:1 during Stage 3 to 1:0.8 during Stage 4 (Souty *et al.*, 1998) showing that glucose is preferentially used for respiration.

Starch may be present in young stone fruit, but unlike other fruit such as apples that convert starch to sugars during ripening, stone fruit do not accumulate further sugars after harvest. Therefore, preharvest factors are strong determinants of sugar accumulation in the fruit. Unfavourable growing conditions, such as low light intensity, uneven light distribution resulting from poor tree training practices, low temperatures, severe water stress, severe nutrient deficiencies and excess crop load are obvious factors that reduce sugar accumulation.

Varieties of peaches can be classified as high, low- or sub-acid types due to major differences in acid metabolism. In ripe 'Fantasia' nectarines (high acid type), the titratable acidity of the juice can reach 400 mM H^+ by Stage 3, but in 'Jalousia', a flat, sub-acid peach, acidity at this stage is only about 40 mM H^+ (Moing *et al.*, 1998). In high acid peaches and nectarines, malate (the most important organic acid in peaches) accumulates during Stages 1 and 2, decreases during Stage 3 but may increase slightly during Stage 4. In contrast, citrate remains relatively low during Stages 1 and 2 but may triple in concentration during Stage 3 and then declines during the latter part of Stage 4 (ripening) (Moing *et al.*, 1998). By comparison, in low acid varieties, the concentration of malate

decreases during Stages 1–4 and citrate remains relatively low, reaching a peak during the middle of Stage 3 before declining (Moing *et al.*, 1998). Although these acids are relatively simple compounds, the processes modulating acid metabolism are complex and involve many enzymes (Etienne *et al.*, 2002), including some responsible for the synthesis and others with transport across the tonoplast and affecting vacuolar accumulation. It was proposed that phosphoenocarboxylase (PEPC) plays a major role in controlling the accumulation of organic acids (Moing *et al.*, 1999). However, mechanisms other than organic acid synthesis may account for differences in acidity between high and low acid peach fruits. The genes involved in organic acid metabolism (mitochondrial citrate synthase, cytosolic NAD-dependent malate dehydrogenase and cytosolic NADP-dependent isocitrate dehydrogenase) show a stronger expression in ripening fruit than during the earlier phase of development, although their expression pattern are not necessarily correlated with changes in organic acid content. Concerning genes involved in storage, the tonoplast proton pump show a biphasic expression pattern more consistent with the pattern of organic acid accumulation and the tonoplast pyrophosphatases are more highly expressed in the fruit of low acid cultivar during the second rapid growth phase (Etienne *et al.*, 2002).

Expression analysis of two peach vacuolar pumps that appear to be more induced in low acid genotype than in normal-acid genotype at a critical stage of citric acid accumulation would be consistent with the hypothesis of an increased leakage of the vacuole in the low acid fruit, leading to an enhanced expression of vacuolar proton pumps to partially compensate for proton leakage (Echeverria *et al.,* 1997). Increased proton leakage out of the vacuole would explain the lower accumulation of organic acids in the peach fruit and possibly the reduced accumulation of sugars (Etienne *et al.*, 2002).

Phenolics and carotenoids are other important components of the SSC, and from a dietary point of view the most important fruit constituents along with fiber. Phenolics contribute to fruit quality and at the same time they are substrates of the classical browning reaction observed in bruised and over-stored fruit and fruit exhibiting chilling injury. The main fruit phenolics (Van buren *et al.*, 1970; Macheix *et al.*, 1990; Tucker *et al.*, 1993), including cinnamic acid derivates, are flavonols and flavans, anthocyanidins and anthocyanins, flavonol glicosides and condensed polyphenols. The levels of these phenolics vary in

relation to the genotype (Gil *et al.*, 2002), and is affected by environmental factors before and after harvest (Gonçalves *et al.*, 2004). The development of specific off flavour during cool storage of some non-melting peach genotypes has been in part related to high concentrations of soluble phenolics including chlorogenic acid (Karakurt *et al.*, 2000). Changes in phenolic compounds can be associated with storage and physical injuries. Browning reactions are the result of the mixing of the natural phenolic substrates (such as chlorogenic acid, p-coumaryl-quinic acid and dimeric flavans) and polyphenoloxidase following loss of cellular compartimentation from physical injuries, chilling injury or senescence (Tucker *et al.*, 1993). The resultant products, which exhibit browning, are generally lower in consumer acceptability. Anthocyanins are also responsible for a postharvest skin disorder of peaches known as "inking". This physiological disorder resulting in discoloration of peel areas is caused when anthocyanins react with iron dissolved in dip tank solutions (Crisosto *et al.*, 2000). Phenolics are also associated with chilling injury in over-stored peaches and nectarines, where they are associated with bleeding and browning in the mesocarp (Uthairatanakij, 2004).

Carotenoids contribute to flesh colour and carotenes, together with xanthophyll, are responsible for the yellow skin and flesh colours in stone fruit. Colour and intensity are strictly related to genotypical differences; for example, non melting peaches have significantly higher total carotenes and xanthophylls than their melting–flesh counterparts (Karakurt et. al 2000; Gil *et al.*, 2002). The amount of carotenoids increases as maturity advances and, in particular, in relation to the evolution of ethylene. A recent paper by Trainotti *et al.* (2006a) revealed that a coordinated increase of the expression of several genes (1-deoxy-D-xilulose 5-phosphate synthase; 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; phytoene synthase; z-carotene desaturase; lycopene b cyclase; b-carotene hydroxylase) involved in carotenoid biosynthesis occurs during the transition from immature to mature stage.

Aroma development

Aroma is an important trait of peaches and nectarines because it affects consumer expectation; however, peaches often do not develop full aroma due to a too early harvesting and/or to the effects of prolonged low temperature storage.

The aroma composition produced by ripe fruit varies among cultivars of peaches and nectarines, both quantitatively and qualitatively. The production of volatile compounds arises from different substrates (i.e. fatty acids, amino acids, phenolics and terpenoids) and the most important products are aldehydes, esters, ketones, terpenoids and sulphur containing compounds. Lactones (γ-decalactone and δ-decalactone) and other products of peroxidation of unsaturated fatty acids are the main products that confer the typical peach aroma (Takeoka *et al.*, 1988). Terpenoids also impart a fruity aroma and may be more important in nectarines than peaches. Increases in benzaldehyde and linalool have also been detected and a good marker for peach aroma is cis-3-hexenylacetate (De Santis *et al.*, 2001). In unripe peaches most of the typical aroma compounds are low or absent, while aldehydes and C6 alcohols (hexanal, trans-2-hexanal, hexanol and trans-2-hexanol) impart green aroma.

Softening and cell wall changes

As all perishable fruit, peaches fruit soften during ripening and this is the major quality attribute that often dictates shelf life. Even though a complex mechanism involving both degradation and synthesis of cell wall material has been proposed (Trainotti *et al.*, 2003), the main change in ripening responsible for softening is the partial breakdown of the fruit cell walls. During ripening the cell wall becomes very hydrous due to the hydrolysis of pectins, and changes in pectin cohesion are responsible for the softening in ripe fruit.

Changes in cellulose and, particularly, hemicelluloses are also involved in loss of flesh firmness. The principal hydrolases involved in peach softening are pectinmethylesterase (PME), polygalacturonase (PG), β-(1,4)-glucanase (EG) and β-galactosidase (β-GAL), which act together with expansins (EXP) and pectatolyase (PL) (Brummell *et al.*, 2004). Hinton and Pressey (1974) found that during peach ripening EG activity increases before any significant change in fruit firmness. Similar results have been obtained by Bonghi *et al.*

(1998) who found that the increase of EG activity, occurring at early softening is due to a pronounced increase in the basic isoform.

At ripening, softening of melting flesh (MF) peaches proceeds in two phases: the initial slow decline in firmness early in ripening followed by a rapid and dramatic softening ('melting') in the later stages (Pressey *et al.*, 1971). The MF phenotype is associated with a large increase in the amount of soluble pectin and progressive pectin depolymerization (Pressey *et al.*, 1971; Dawson *et al.*, 1992; Fishman *et al.*, 1993). An increased gene expression and activity of cell wall-modifying enzymes, in particular of exo- and endo-PG and PME, have been observed in MF peach cultivars (Trainotti *et al.*, 2003; Brummell *et al.*, 2004). In non-melting flesh (NMF) peach, the final melting phase of softening is absent, so fruit remain relatively firm and this is associated to a lack of endo-PG activity resulting in limited pectin depolymerization (Pressey and Avants, 1978; Fishman *et al.*, 1993; Orr and Brady, 1993). The lack of a melting phase in NMF peaches appears to be due either to a deletion of endo-PG genes or to a truncation of specific mRNA, which causes an absence of immunodetectable endo-PG protein (Lester *et al.*, 1994; Callahan *et al.*, 2004). These data point out that endo-PG-mediated pectin modification plays an important role in the later stages of softening and textural changes in MF peach. The strict relationship between PG gene expression and ethylene has been studied in detail in ripening tomato fruit (Sitrit and Bennett, 1998). This relationship appeared to be particularly important also in peaches where the increase of PG transcripts is associated to the melting stage when the ethylene climacteric occurs (Downs *et al.*, 1992). This has been confirmed by Hayama *et al.* (2006a and b) that, working with a Stony Hard (SH) peaches, characterized by the absence of ethylene production during fruit ripening, found that the maintenance of high firmness values is associated to a lack of PG expression and activity and that treatments with exogenous ethylene strongly induce PG resulting in a rapid loss of firmness.

Pectin metabolism is also implicated in the development of chilling injury, leading to the appearance of woolliness. A recurring hypothesis to explain peach woolliness supports the idea that changes in cell wall enzyme activity during cold storage will affect the metabolism of cell wall polysaccharides during the subsequent ripening at warm temperatures (Ben Arie and Lavee, 1971; Buescher and Furmanski, 1978). Woolliness has been attributed to an imbalance between the activity of PG and PME (Ben-Arie and

Sonego, 1980; Zhou *et al.* 2000a; Zhou *et al.*, 2000b; Zhou *et al.*, 2000c; Brummell *et al.*, 2004). Relatively high, PME and low PG activity in chilling-injured fruit leads to an accumulation of demethylesterified pectins which are not subsequently depolymerised.

The role of PLs in peach fruit softening is not clear considering that two members of this multigene family show different expression trend: Trainotti *et al.* (2003) demonstrated that one PL starts to be expressed at the early Stage 3 reaching a maximun at the early Stage S4, and decline thereafter. The second one shows a typical ripening-related pattern with expression that starts at the early Stage S4 and increases in correspondence of the late melting stage. Different transcript accumulation patterns have also been detected for the isolated members of the expansin family. Three expansins, named *Pp-Exp1*, *Pp-Exp2* and *Pp-Exp3* have been isolated from ripe peach fruit (Hayama *et al.*, 2001; 2003): the *Pp-Exp2* RNA is constitutively expressed throughout fruit development but is more abundant in stage III, during exponential growth and maturation. *Pp-Exp1* and *Pp-Exp3* appear up-regulated at the onset of ripening but *Pp-Exp 1* is induced at an earlier stage and *Pp-Exp3* shows a closer association with softening.

Ethylene physiology and fruit ripening

Fruit ripening is a complex syndrome influenced by a number of endogenous and exogenous factors. Ripening-associated events are caused by developmentally and physiologically regulated changes in gene expression which ultimately lead to alterations in colour, texture, flavour and aroma of fruit (Gray *et al.*, 1994). Fruit can be classified into two groups in relation to the presence (climacteric) or absence (nonclimacteric) of increased respiration and synthesis of the gaseous hormone ethylene at ripening. Besides fruit ripening, ethylene affects many diverse processes throughout the lifetime of a plant including seed germination, growth, formation of the apical hook, organ senescence, abscission, gravitropism, and responses to various stresses (Mattoo and Suttle, 1991; Abeles *et al.*, 1992). Much of what is known regarding the mechanisms involved in ethylene metabolism has been realized through studies on the model plant species *Arabidopsis*, despite the dry nature of its fruit (Guo and Ecker, 2004). Nevertheless, these studies have proven extremely useful in advancing ripening research in fleshy fruit species as tomato, that has emerged as the model for the analysis of the fleshy fruit development

and ripening, in part because of available mutants, excellent genetics, routine transformation and numerous molecular and genomics tools (Giovannoni, 2004).

Although ethylene plays a crucial role in regulating the ripening process, in climacteric fruits (such as tomato), it has been proved that both ethylene-dependent and ethylene-independent events co-exist (Lelievre *et al.*, 1997). Considering this, a model has been proposed to explain the basal level of ethylene during the early phases of development and the increase during the climacteric (Barry *et al.*, 2000). According to this model, two systems of ethylene regulation operate in plants. System 1, responsible for producing basal ethylene levels in both vegetative and reproductive tissues, and System 2 operating during the ripening of climacteric fruit and senescence of some petals when ethylene production is autocatalytic (Barry *et al.*, 2000; Alexander and Grierson, 2002). The signal network responsible for the transition between the two systems is still unknown but this transition is perhaps due to the specific factors involved in the development of the fruit and could be explained by the polymorphism of 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS), enzyme responsible for the formation of ACC, the immediate precursor of ethylene (Adams and Yang, 1979). The *Arabidopsis* genome contains nine *ACS* genes that encode eight functional and one non-functional ACS protein differently regulated, while in tomato 8 genes are present (Oetiker *et al.*, 1997; Peck and Kende, 1998; Barry *et al.*, 2000). Molecular genetic studies in *Arabidopsis* (*eto* and *cin* mutants) have provided evidence that the regulation of ACS protein stability also plays a significant role in the control of the ethylene biosynthesis by following two distinct pathway involving phosphorilation and degradation mechanisms (Chae and Kieber, 2005). Both ACS and ACC oxidase (ACO), responsible for the conversion of ACC to ethylene, are encoded by a multigene family in most plant species and, up to now, 4 *ACO* genes have been identified in tomato (Tsuchisaka and Theologis, 2004). In preclimacteric tomato fruit, some members of *ACS* and *ACO* gene families (namely, *LeACS1, LeACS3, LeACS6*, *LeACO1* and *LeACO4*) are active and responsible for System1 basal ethylene biosynthesis. Transition to System2 at ripening is the result of the *LeACS6* silencing and the increased expression of *LeACS2*, *LeACS4*, *LeACO1* and *LeACO4* (Barry *et al.*, 2000; Alexander and Grierson, 2002).

The ACS polymorphism seems to be crucial in regulating ethylene biosynthesis, and also in peach, where the ethylene climacteric occurs during the late ripening stage (Tonutti *et al.*,

1991), three different members of the *ACS* family have been identified (*Pp-ACS1, Pp-ACS2,* and *Pp-ACS).* However, only the first seems to be associated with the ripening-associated ethylene increase (Mathooko *et al.*, 2001; Tatsuki *et al.*, 2006). Recently, it has been shown that the lack of ethylene production in Stony Hard (SH) peaches at ripening is due to a suppression of *Pp-ACS1* transcription (Tatsuki *et al.*, 2006). Interestingly, the same gene appeared to be transcribed in wounded immature, preclimacteric and climacteric SH fruit. This would indicate that one possible mechanism of repression of *Pp-ACS1* mRNA is the interruption of the ripening-related transcriptional activity by some insertion or deletion in the 5'-flanking region of *Pp-ACS1,* which contains a *cis*-regulatory domain of the ripening- related sequences. Another possible explanation is the disruption of a transcrptional factor, specifically activated to induce *Pp-ACS1* mRNA at ripening (Tatsuki *et al.*, 2006). Although SH fruit lack the ethylene climacteric at ripening, they are ethylene sensitive as exogenous ethylene induces the synthesis of volatiles (Hayama *et al.*, 2003) and a rapid loss of firmness (Haji *et al.*, 2005). Hayama *et al.* (2006b) found that the exogenous ethylene-dependent softening of SH 'Manami' fruit correlates with the levels of exoPG and endoPG activities. In addition, an induction of the ripening-related *PpExp3* and accumulation of mRNA for a putative endo-PG an a-L-arabinofuranosidase/b-xylosidase (PpARF/XYL) have been observed following ethylene treatment in SH fruit (Hayama *et al.*, 2003; Tatsuki *et al.*, 2006). These results, obtained using the SH mutant, underline the crucial role of ethylene in the induction of fruit softening (and other ripening-related processes) in peach.

Concerning *ACO*, Ruperti *et al*. (2001) demonstrated that, in peach, *ACO* belongs to a multigene family made up of at least 3 members, one of which (*PpACO1*) is specifically expressed during fruit ripening (Tonutti *et al.*, 1997), is under development control and ethylene-regulated. *Pp-ACO2* mRNA is detected in fruit only during early development and is unaffected by ethylene. Functional analysis (Rasori *et al.*, 2003) showed that within the promoter region of the gene, ethylene responsive elements (EREs) are present in *Pp-ACO1* but not in *Pp-ACO2*. This may account for the different responsiveness to exogenous ethylene of the two genes. In addition, two auxin responsive elements (AUXre), probably responsible for the auxin suppression of the ethylene induction of *Pp-ACO1* gene expression, are present upstream of EREs.

Responses to ethylene are mediated by a family of receptors (Binder 2007, for an updated review on ethylene receptors) (Fig. 2). The number of receptor isoforms varies from species to species. In *Arabidopsis thaliana* where most research has been conducted, there are five receptor isoforms (*ETR1, ETR2, ERS1, ERS2* and *EIN4*) similar to bacterial two-component histidine kinase receptors: all of them can bind ethylene (Chang *et al.*, 2001). However tomato is emerging as another important system to study ethylene receptor function. In tomato there are six receptor isoforms five of which have been tested for ethylene binding and found to bind ethylene with high affinity. Each gene has a distinct expression pattern including a subset strongly induced during ripening (*NEVER RIPE* or *NR* and *LeETR4*) (Payton *et al.*, 1996; Kevany *et al.*, 2007).

Rasori *et al*. (2002) demonstrated that genes very similar to *Arabidopsis* ETR and ERS are present in peach genome. Similarly to what observed in model species, the deduced proteins of the two genes contain a sensor domain and a hystidine-kinase domain, in which residues thought to be important for the normal function of ETR and ERS type protein as ethylene receptors are conserved. These results indicate that *Pp-ETR1* and *Pp-ERS1* could be putative ethylene receptors with the ability to bind ethylene in peach. Quantitative RT-PCR data showed that *Pp-ETR1* and *Pp-ERS1* transcripts are differentially expressed in immature and ripe fruit. *Pp-ETR1* appears to be constitutive and ethylene independent during fruit development and ripening, whereas *Pp-ERS1* transcripts slightly increase during fruit ripening and their expression appears to be somehow induced by ethylene (Rasori *et al.*, 2002). Using a transcriptomics approach, Trainotti *et al.* (2006a) recently identified one additional ethylene receptor (*Pp-ETR2*) that, more than *Pp-ERS1*, appears to be induced during the transition from pre-climacteric to climacteric stage.

Acting downstream of the receptors is a putative MAP-kinase kinase kinase (MAPKKK), termed *CONSTITUTIVE TRIPLE RESPONSE* (CTR1) that interacts directly with receptor molecules to form a signalling complex (Gao *et al.*, 2003) (Fig. 2). In tomato, four *CTR* genes have been identified, three (*LeCTR1, LeCTR3 and LeCTR4*) similar to *CTR1* and one (*LeCTR2*) more similar to pathogen related protein (Adams-Phillips *et al.*, 2004a).

Assuming that tomato ethylene receptors and CTRs interact, as in *Arabidopsis*, the interaction kinetics between the various CTRs and the receptors, in conjunction with the varying ratio of receptors and CTRs encoded by different family members (and for

different tissues and responses), might represent a mechanism for optimizing fidelity of ethylene responses in tomato and other species with multiple CTR genes (Giovannoni, 2004).

Concerning ethylene signalling, epistasis analysis places *ETHYLENE INSENSITIVE 2* (*EIN2*) downstream of *CTR1* in the ethylene signalling pathway (Alonso *et al.*, 1999) (Fig. 2). *EIN2* encodes a protein with similarity to the Nramp family of metal ion carriers, and, based on specific evidences, might represent a common point through which multiple hormone signal transduction pathways might act (Fujita and Syono, 1996; Beaudoin *et al*., 2000; Ghassemian *et al,*. 2000). It is directly activated by CTR1 and operates upstream of EIN3 and the EIL (EIN3-like) family of nuclear localized trans-acting proteins (Guo and Ecker. 2003). Three tomato EIL genes were isolated and shown to be functional redundant (Tieman *et al.*, 2001); a fourth tomato EIL gene (EIL4) exhibiting riepening-induced expression has been cloned (Yokotani *et al.,* 2003).

Ethylene shows an unusual model for signal transduction in plants (Fig. 2): it acts on the receptors and the binding triggers a signal cascade by MAP kinases (MAPK3) until activation of a first class of ethylene transcription factors (such as EIN3 and EIN3-like (EIL) proteins), which bind in a sequence-specific manner to the primary ethylene-response element (*PERE*) of *ERF1*. This is an ethylene inducible gene that belongs to the Ethylene Response Element Binding Protein (EREBP) family of DNA binding proteins (Solano *et al.*, 1998). ERF1 directly activates transcription of a wide variety of ethylene-responsive pathogenesis-related genes or fruit ripening genes by binding to the GCC-box (Giovannoni, 2001).

Additional positive regulators (EIN2, EIN5, EIN6) and Transcription Factors –TFs- (EIN3 and EIL1 ethylene insensitive like) have been reported (Guo and Ecker 2004). EIN3 and EIL1 seem to be required for continued growth inhibition by ethylene in *Arabidopsis* (Binder *et al.,* 2004). This primary ethylene signal transduction chain seems to be common to all ethylene responses and the components involved only respond to ethylene (Guo and Ecker 2004).

A marked increase in *EIN2-like* transcripts has been detected by Trainotti *et al.* (2006a) during the transition from pre-climacteric to early climacteric stage of peach fruit. In the same transcriptomics work, 19 ripening-related TFs belonging to several families as

MADS-box, AUX/IAA, bZIP, bHLH, HD, Myb, and AP2 (*APETALA2* that encodes Ethylene Responsive Elements Binding Proteins) have been shown to be differentially expressed.

Besides ethylene-related TFs, many genes involved in biosynthesis and transport and, in particular, the signalling (receptors, Auxin Response Factors and Aux/IAA) of auxin show an increased expression in the peach mesocarp during ripening (Trainotti *et al.*, 2007). This information supports the idea that also auxins are actively involved in the ripening of peaches. This study also demonstrated the existence of an important cross-talk between auxin and ethylene, with genes in the auxin domain regulated by ethylene and genes in the ethylene domain regulated by auxin (e.g. *ACS, ETR2*).

Studies of ethylene signalling have been initiated in *Arabidopsis* mutants and then on tomato, benefiting from a series of natural mutants in fruit ripening: the main investigations were performed on the *nr*, *rin*, *nor* and *Gr* mutants (Giovannoni, 2007). *nr* mutant has displayed a lesion in an ethylene receptor and this mutation prevents ripening in tomato *via* ethylene insensitivity even when ethylene is applied exogenously (Wilkinson *et al.*, 1997). *rin* (*ripening-inhibitor*) and *nor* (*non-ripening*) are mutants that fail to ripen in response to exogenous ethylene and yet display signs of ethylene sensitivity and signalling, including induction of some ethylene-regulated genes: the genes for both mutations were cloned and they appeared to be implicated in the regulation of gene transcription upstream from the ethylene signalling cascade (Giovannoni *et al.*,1995; Vrebalov *et al.*, 2002). *Gr (Green ripe)* is a mutant in an evolutionarily conserved protein of unknown biochemical function that is associated with ethylene signalling (Barry and Giovannoni, 2006).

Rin, nor and *Cnr* (*colorless non ripening*) loci encode for TFs and thus they provide the first insights into dedicated fruit-specific transcriptional control concerning ripening regulation upstream of ethylene synthesis and response. This non-ethylene mediated aspect of ripening physiology is intriguing because it suggests that both the Rin MADS and Cnr SPB proteins could be candidates for ripening regulators conserved among fruit species (climacteric and non-climacteric) (Giovannoni, 2004). Indeed, the most widely studied non-climacteric ripening system is strawberry, which increases neither respiration nor ethylene production during ripening, and is largely unresponsive to inhibitors of ethylene action such as 1-methylcyclopropene (1-MCP).

Together with the use of model plant species and their mutants, the inhibitors of ethylene biosynthesis and/or action are other important tools to study basic mechanisms of fruit ripening in climacteric fruit. The most important are AVG (Aminoethoxyvinylglycine) that inhibits ethylene biosynthesis, and 1-MCP, an antagonist of ethylene action at receptor level. The former has also been used to study the role of ethylene on plant growth, development, and response to stress (Abeles *et al.*, 1992), bud break (Pereira-Netto, 2001), dry matter partitioning in rice (Mohapatra *et al.*, 2000), fungal pathogenesis (Robison *et al.*, 2001), nodulation in legumes (Mann *et al.*, 2001; van Spronsen *et al.*, 2001) and response to chilling stress (Hong and Gross, 2000), and ripening of different fruit species (Clayton *et al.*, 2000; Wang and Dilley, 2001) including peach (Bregoli *et al.*, 2002; Ziosi *et al.*, 2006). One possible mode of action for AVG could be through its effect on the synthesis of functional proteins. Many proteins are synthesized during the ripening of non-climacteric fruit and during the senescence of vegetables (Grierson, 1987).

1-MCP inhibits ethylene perception in plant tissue by binding to the ethylene receptors, inhibiting the effects of endogenous and exogenous ethylene (Sisler and Serek, 1997). It binds to the copper cofactor of the ethylene receptor but due to steric hindrance, there is no conformational change and the receptors are locked in the active state. The CTR1 protein is thus permanently activated and the EIN2 protein permanently deactivated (Binder and Bleecker 2003; Prange and DeLong 2003).

The effects of 1-MCP application on ripening parameters differ in relation to a number of endogenous and exogenous factors including genotype and ripening stage (Blankenship and Dole, 2003). Different researches show that 1-MCP can affect ethylene production, respiration, softening, colour change, aroma production and the occurrence of physiological disorders with profound effects on shelf- and taste-life of many fruit produce (Watkins, 2006). Since 1-MCP acts as competitor of ethylene for binding sites, its most pronounced effects have been observed in climacteric fruit and those with very specific responses to exogenous ethylene (Huber *et al.*, 2003). However, even within the category of climacteric fruit, the effects of 1-MCP are highly variable and this has been imputed to different amount, ratio and regeneration rate of ethylene receptors as demonstrated in a comparative work with apples and peaches by Dal Cin *et al.* (2006). In apples, ripening is inhibited or delayed for many days and storage prolonged (Fan *et al.*, 2002a; Watkins *et al.*, 2000; Jiang

et al., 2001; Bai *et al.*, 2005). On the other hand, 1-MCP appears to have limited effects on slowing ripening of peaches, in which ethylene production is not inhibited and, after few hours from the end of the incubation period, ripening processes as softening quickly recover (Mathooko *et al.*, 2001; Fan *et al.*, 2002b; Ziliotto *et al.*, 2003). This behaviour makes peaches of particular physiological interest and worthy to be studied more in detail.

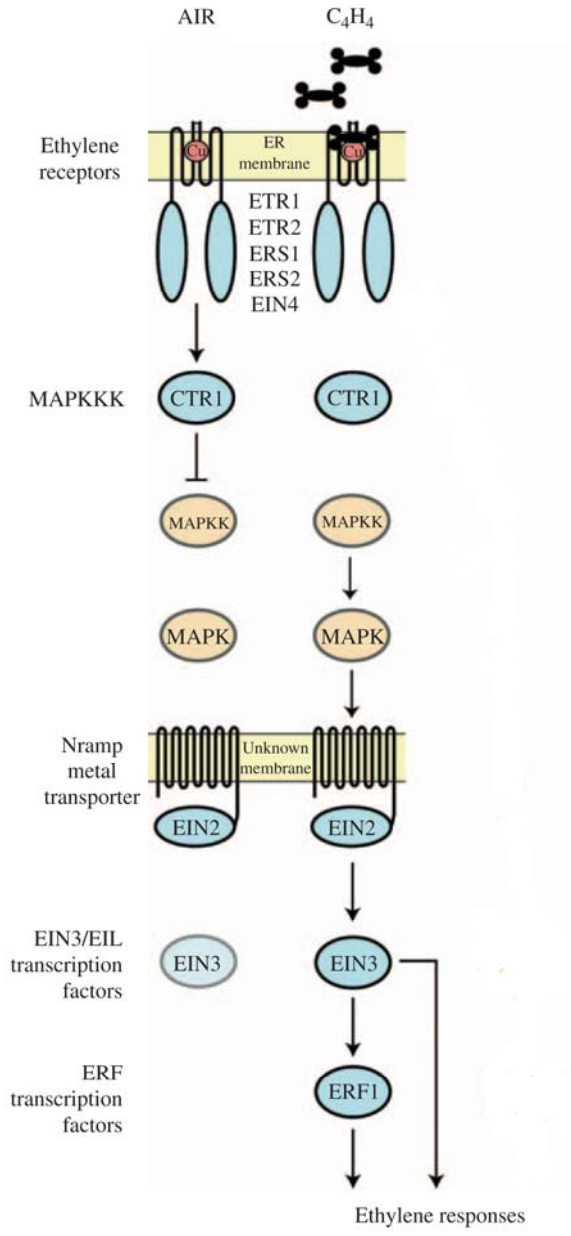

Figure 2 - Model for ethylene signal transduction in *Arabidopsis thaliana* by Chen *et al.* (2005). In air, ethylene receptors maintain CTR1 in an active state that serves to repress ethylene responses. Binding of ethylene inactivates the receptors, thereby inactivating CTR1. As a result, EIN2 is activated and a transcriptional cascade involving the EIN3/EIL and ERF transcription factors is initiated.

Genomics approaches and tools in fruit science

The recent development of high-throughput techniques and new biotechnological approaches covering broad field of disciplines (chemistry, physics, biology, physiology, computer science and robotics) have opened, also in plant research, the Genomics Era, resulting in whole genome and large-scale Expressed Sequence Tags (EST) sequencing projects. Complete genome sequences of dozens of organisms, of which three are plants - *Arabidopsis* (Arabidopsis Genome Initiative, 2000), rice (Goff *et al*., 2005), poplar (Tuskan *et al.*, 2006)- have been launched and completed. Draft sequences are available for grape (The French–Italian Public Consortium for Grapevine Genome Characterization, 2007), the first fruit crop to be sequenced, and in progress for *Medicago truncatula* (Cannon *et al.*, 2005), *Lotus spp.*, tomato and maize. Genomics is aimed to study the organisms' genome and to understand its structure and function: it is traditionally divided into two basic areas, the structural genomics and the functional genomics (also called the post-genomic era). While the former has the goal of describing the physical nature of genomes, the latter is related to the expression of genes and their functional characterization and allows the detection of genes that are turned on or off at any given time depending on endogenous (e.g. development) or exogenous (e.g. environment) factors (Eggen, 2003). Besides RNA, targets of functional genomics studies are also proteins (proteomics) and metabolites (metabolomics). Functional genomics analyses are highly complementary in determining gene functioning: if transcriptional profiling describes gene expression patterns and gene regulatory networks, proteomics provides qualitative and quantitative information about proteins, and metabolomics is aimed to profile the range of metabolite present in the sample at a given time or under certain conditions (Roessner *et al.*, 2001; Rossignol, 2001). This multidisciplinary approach represents the pre-requisite for the development of systems biology (Gutierrez *et al.*, 2005) and brings about a great change in our understanding of the molecular mechanisms of phenotypes including the complex interplay of genetic and environmental factors (Collins *et al.*, 2003).

Structural annotation of genes and gene models relies on abundant experimental evidence such as ESTs and full-length cDNA sequences (FL-cDNA) to improve gene finder output on a genomic sequence. By contrast, functional annotation relies primarily on the use of bioinformatics to determine gene function because experimental evidence that can be used

to assign gene function is available for only a small portion of the genes within a genome. Large EST collections have been produced from many cDNA libraries of different tissues of fruit species (Table 2 in Granell *et al.*, 2007): however, only for few of them [tomato (*Solanum lycopersicon*, Fei *et al.*, 2004), grape (*Vitis* spp., Da Silva *et al.*, 2005; Peng et al., 2007), apple (*Malus domestica*, Newcomb *et al.*, 2006; Park *et al.*, 2006) and citrus (*Citrus* spp., Terol *et al.*, 2007)], analyses have been carried out to study specifically fruit ripening and to identify genes to be likely involved in the process.

Considering peach, three different groups have started specific EST sequencing programmes: the Clemson University group **(**http://www.genome.clemson.edu/projects/peach/est**),** the Italian ESTree consortium **(**http://linuxbox.itb.cnr.it/ESTree**),** and, more recently, the Programa Genoma Chile **(**http://www.fondef.cl**).**

Some results of the ESTree Consortium sequencing programme obtained by the University of Padova - Unit of the Faculty of Agriculture- are reported in Chapter II of the present thesis. Within this project, about 2,500 ESTs have been initially isolated from mesocarp of different peach varieties ("Fantasia", " Redhaven", Oro A" and "Bolero") at two different fruit developmental stages (S3 and S4) (ESTree Consortium, 2005).

ESTs and others tags has allowed the development of hybridization-based approaches as a microarray in which the expression of thousands of genes is simultaneously analyzed at a reasonable cost. The expression level of any gene represented on the array can be deduced from the fluorescence intensity of the corresponding probe, which is recorded by laser scanning. On a whole genome level, microarrays provide a high-throughput platform to measure gene expression and thereby generate functional data for many genes simultaneously. Over the past decade, advances in genomic technologies have resulted in a variety of microarray platforms that are extensively reviewed by Rensink and Buell (2005) and summarized in Table 1. DNA arrays can be used for many different purposes including expression profiling (Schena *et al.*, 1995), identification and genotyping of mutant alleles and DNA marker polymorphisms (Wang *et al.*, 1999), and DNA resequencing (Hacia, 1999). At this moment, access to a large collection of reference expression data from mutants, tissues or treatments can provide a tool for identifying the function of unknown genes (Rensink and Buell, 2005).

Table 1 - Microarray technology: principal type of array platforms that can be used for global gene expression profiling (modified from Rensink and Buell, 2005).

Array platforms
On-slide synthesized arrays: probes are synthesized on the array surface using DNA synthesis chemistry. The activation for oligonucleotide elongation is achieved using a mask (Affymetrix) or maskless (Nimblegen) method. Alternatively, the reagents are delivered to each spot using inkjet technology (Agilent).
Spotted cDNA arrays: clones from a cDNA library are amplified by the polymerase chain reaction (PCR) using generic primers for the vector. PCR products are purified and spotted on glass slides using a robotic arrayer.
Spotted gene-specific sequence tag arrays: unique segment(s) of the gene are amplified from genomic DNA or bacterial artificial chromosome clones using specific primers for each gene. The PCR product s are purified and spotted on glass slides using a robotic arrayer.
Spotted long oligonucleotide arrays: oligonucleotides ranging from 50–70 bases are synthesized for a unique region of the genes of interest. Oligonucleotides are spotted on glass slides using a robotic arrayer.

Analysis of microarray data highlights genes up and down regulated in the specific sample comparison. Generally, these data are subjected to independent validation using other techniques such as quantitative reverse transcription polymerase chain reaction (RT-PCR), and the confirmation rate, at least at the level of recapitulating the observed gene expression pattern, is well over 90% (Quackenbush J., 2003). However, the RT-PCR is estimated to be at least 100 times more sensitive than DNA arrays at detecting transcripts (Horak and Snyder, 2002). For this reason and because all expression profiles produced with array analysis need to be confirmed, the RT-PCR is one of the most common techniques to do it.

For fruit crops, microarray platforms so far developed are mainly home-brewed, permitting robust, reproducible results to be obtained and to focus solely on the biology of interest. cDNA-based arrays, developed in the early days of global transcriptome analysis, have been replaced by those oligos-based that have increased laboratory-to-laboratory reproducibility (Busch and Lohmann, 2007). Following the pioneering work of Aharoni *et al.* (2000) who, using a cDNA microarray, identified a novel alcohol acyltransferase (SAAT) gene responsible for flavor biogenesis in ripening strawberry, some other cDNA-based arrays have been produced and used for transcript profiling during fruit ripening. Alba *et al.* (2005), using TOM1 microarray, identified 869 genes that are differentially expressed in developing tomato pericarp: 37% of these genes are altered in their expression into Nr (Never-ripe) mutants in which sensitivity to ethylene is reduced and ripening inhibited. This approach has shown that C_2H_4, *via* Nr, influences the expression of thousands of genes in green fruit prior to the onset of ripening. In addition, it emerges that this hormone governs morphological and biochemical processes, such as fruit shape (i.e. length-to-width ratio), pericarp thickness, locular development. The Nr lesion also has adverse effects on seed production and/or seed development in mature green fruit, indicating that this locus may be a determinant of fecundity in this species. These results clearly indicate that C_2H_4 action in tomato pericarp is not limited to processes strictly associated with climacteric ripening. Instead, this hormone and receptor govern biochemical, physiological, and developmental processes throughout tomato fruit development, including those that occur prior to ripening. Considering in particular one specific event (carotenoid biosynthesis) linked to important quality parameters (colour and nutraceutical compounds), the microarray analysis indicate that ethylene influences multiple steps in this pathway impacting net and relative accumulation of these compounds. The crucial role of ethylene in modulating gene expression has been also observed in pear fruit by Fonseca *et al.* (2004), who, using a specific cDNA microarray, detected main changes in expression profiles in correspondence to the cessation of growth at maturity and entry into the climacteric phase. Forment *et al.* (2005) developed a cDNA microarray with 6875 putative unigenes from a large Citrus EST collection that has been used to study expression changes during ripening of *Citrus clementina* and thus highlighting key physiological processes such as those concerning citrate utilization (Cercos *et al.*, 2006).

Oligo-based microarrays have been produced for grape (3,175 oligos, Terrier *et al.*, 2005), apple (15,720 oligos, Schaffer *et al.*, 2007) and peach (4,806 oligos, ESTree Consortium, 2005). For the grape platform, 50-mer oligos have been selected in the 3' non-coding region (UTR), thus allowing differential expression profile of isogenes to be distinguished. This approach demonstrated that five isogenes of xyloglucan endotransglycosylase (XET) belong to four clusters characterized by different expression profiles. Berry softening has been confirmed as the earliest sign in ripening induction, thus determining that expression timing of XET isogenes as well as that of other genes encoding cell wall hydrolases is an essential step to dissect the signal network regulating this important event.

The role of ethylene in aroma production of apples has been assessed using a (55-mer) oligo-microarray with samples of untransformed and ACC oxidase antisense "Royal Gala" fruit (Schaffer *et al.*, 2007). This approach allowed the expression profile to be described of a repertoire of 179 candidate genes that might be involved in the production of aroma compounds. Among these only 17 were typically affected by ethylene, suggesting that only certain points of the aroma biosynthesis pathways are regulated by the hormone. Often the first step, and in all pathways the last steps, contained enzymes that were ethylene regulated. This analysis suggests that the initial and final enzymatic steps with the biosynthetic pathways are important transcriptional regulation points for aroma production in apple.

µPEACH 1.0 is an 70-mer oligo-microarray developed starting from EST sequences mainly obtained from cDNA libraries of ripening peach fruits (ESTree Consortium, 2005). This tool has been used to investigate molecular events occurring at the transition from preclimacteric to early climacteric stage (Trainotti *et al.*, 2006a). More than 260 genes resulted induced, while about 100 appeared down-regulated. A coordinated increase of transcripts corresponding to genes involved in carotenoid biosynthesis characterizes the transition from immature to mature stage corresponding to the onset of the ripening-related ethylene increase. Among the differentially transcribed genes, some involved in ethylene biosynthesis, perception and signal transduction, some implicated in cell wall metabolism, and 19 targets encoding regulators of gene expression (TFs) are present. In particular, six members of the Aux/IAA family are highly up-regulated when ripening proceeds: using the same microarray, on mature peaches treated with exogenous ethylene and auxin, Trainotti

et al. (2007) pointed out the importance of the cross-talk between these two hormones at the ripening onset of peach fruit. Considering these results, μPEACH 1.0 appears to be a powerful tool for functional genomics approaches and analyses specifically addressed to better elucidate ethylene physiology in ripening peach fruit.

Thesis Rationale

The general aim of the thesis work was to implement basic knowledge of peach fruit ripening physiology and of the role that ethylene plays in this developmental process of the drupe. The benefits of elucidating the mechanism of fruit ripening of this economically important fruit crop would be numerous for both growers and consumers since better pre- and post-harvest strategies for increasing and maintaining quality could be adopted. In addition, the basic information resulting from the genomics (transcriptomics) approach might represent an important contribution for the scientific community interested in fruit physiology and functional genomics, in particular of the Rosaceae species.

In order to provide a comprehensive report, the dissertation has been organized into six chapters as follows:

- Chapter II describes the work related to the construction of an EST repertoire of ripening (climacteric) peach used for the development of μPEACH 1.0 microarray.
- Chapter III reports the results of specific post-harvest trials on the peach mutant SH in terms of ethylene biosynthesis and signal transduction gene expression
- Chapter IV describes the use of μPEACH 1.0 to elucidate the effects of 1-MCP treatments in nectarines.
- Chapter V describes the use of μPEACH 1.0 to compare transcript profiles of different peach genotypes (including mutants) treated with exogenous ethylene
- Chapter VI describes the preliminary steps of an in-progress functional study of ripening-related genes in tomato.

Chapter II

Molecular and Genetic Aspects of Ripening and Qualitative Traits in Peach and Nectarine Fruits

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Abstract

Expressed Sequence Tags (ESTs) and cDNA AFLP analysis have been used to study gene expression during peach fruit ripening. An EST repertoire (1007 sequences) has been constructed using transcripts in nectarine fruit cv 'Fantasia' at climacteric stage. The EST repertoire was reduced to 696 unigenes: in these 579 (83%) were singletons. EST analysis confirmed that in peach ripe fruit, genes involved in ethylene biosynthesis and cell wall metabolism are highly represented. Three transcription factors (TFs) homologous to SEPALLATA3, atb2, bHLH61 belonging to MADS, bZIP and bHLH families, respectively, have been identified. The abundance of the related transcripts suggests that these TFs play a regulatory role of ripening, as reporter in other fruits species. The EST repertoire has been included in a sequence database assembled by the Italian Consortium for *Prunus* spp genomic study to generate the first peach fruit microarray (μPEACH 1.0). EST analysis has been integrated with cDNA AFLP to study a selection of the nectarine cv 'Fantasia', named *SR*, characterized by a block of ripening. Data pointed out that the *SR* fruit phenotype might be due to disturbance in the onset of senescence

Keywords: EST, cDNA-AFLP, transcription factors, *Prunus persica*

Introduction

Tomato represents the model species for molecular characterization of flesh fruit. An additional model might be represented by peach since its fruit is a different flesh fruit type (drupe vs berry). Moreover, within the Rosaceae family, peach has the following advantages: is a diploid species with 2n=16, its genome is quite small (approximately twice the size of the Arabidopsis genome, Baird *et al.*, 1994), has a relatively short juvenile phase (2-3 years), and cvs with different fruit phenotypes are available. In addition, molecular markers and BAC libraries (Wang *et al.*, 2001; Georgi *et al.*, 2002) are available to facilitate positional cloning.

A systematic study of gene expression requires a complete genome sequencing, but this strategy is costly and time-consuming. An alternative is represented by the ESTs (Expressed Sequence Tags) approach, a partial or total sequencing of cDNAs corresponding to expressed genes in specific organ and at different developmental stages.

In tomato, for example, an EST database including approximately 40,000 sequences derived from fruit at various stages of development has been set up (Giovannoni, 2004). Considering peach fruit, an EST repertoire containing about 10,000 sequences has been developed by Clemson University (http://www.genome.clemson.edu/projects/peach/est/) and research programme aimed to increase peach EST number are currently in progress.

Complementary techniques to the EST approach are based on the differential transcript profiling, as cDNA-AFLP, that combine both features of high specificity and capability of detecting rare transcript tags (Reijans *et al.*, 2003). cDNA-AFLP has been successfully used to isolate genes specifically involved in many plant developmental processes, including fruit ripening (Martelli *et al.*, 2003)

This paper is focused on the analysis of the EST repertoire constructed from climacteric peach fruit cDNA, and on preliminary results, obtained with a cDNA-AFLP approach, on a nectarine selection, named slow ripening (*SR*), derived by free-impollination of cv 'Fantasia' and characterized by a block of ripening

Material and methods

Plant Material

Fruits were harvested from peach trees (cv 'Fantasia' and *SR*), grown at the experimental farm of the University of Padova (Agripolis, Legnaro, Italy) at stages S1, (first exponential growth phase), S2 (pit hardening), S3 (second exponential growth phase) and early and late (climacteric in cv 'Fantasia') S4, as defined by Tonutti *et al.* (1997).

RNA extraction

RNA extraction was carried out as described by Ruperti *et al.* (2001). RNAs have been quantified by spectrophotometer and their integrity evaluated by gel analysis. Transcript analyses were carried out on 10 μg of total RNA as described by Tonutti *et al.* (1997).

cDNA library synthesis

Total RNA extracted from 'Fantasia' mesocarp at climacteric stage was used to purify 5 μg of mRNA using Oligotex® mRNA mini kit (Qiagen) following the manufacturer's protocol. Double stranded cDNA was synthesized using Time saver cDNA kit and Directional

Cloning Toolbox (Amersham Pharmacia Biotech) and as primer a NotI-oligo-dT(18). The cDNAs were cloned directionally into pBS+ (Statagene), which was digested with EcoRI and Not I. Recombinant plasmids were inserted in DHα5 bacteria cells by electroporation.

cDNA selection, sequencing and data processing

cDNAs were selected by PCR using universal primer of M13 phage. Only PCR products longer than 500 bp and without double bands were chosen. Selected cDNAs, have been purified using millipore MONTAGE SEQ 96 following manufacturer's instruction. Sequencing reactions were carried out starting from 5' ends using Big Dye terminator vers. 3.1 (Applied Biosystem) and T7 primer.

Sequencing raw data were pre-processed and clustered using Seqman II software (Lasergene DNASTAR). Pre-processing operations were: 1) end trimming (2 ambiguous bases into window of 50 bases); 2) scan for vector and repeated elements. Clustering setting were: 1) match size higher than 50 bp; 2) 80% as minimum match; 3) sequence length, after trimming, longer than 100 bp.

After clustering ESTs unigenes were subjected to BLASTX algorithm (Altschul *et al.*, 1997), against non-redundant (nr at http://www.ch.embnet.org/software/bBLAST.html) and Arabidopsis proteome databases. Unigenes with BLASTX score higher than e-10 have been annotated as the corresponding Arabidopsis genes categorized by the Munich Information Center for Protein Sequences (MIPS, http://mips.gsf.de/proj/funcatDB/search_main_frame.html).

cDNA AFLP analysis

The first step was the conversion of mRNA into ds cDNA using an oligo-dT primer starting from about 15 μg total RNA, as described by Bachem *et al.* (1996). The cDNAs were digested with two restriction enzymes MseI and EcoRI and ligated to respective adaptor. The pre-amplifications (25 cycles 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s) were performed with the MseI (5'-GATGAGTCCTGAGTAA-3') and EcoRI (5'-GACTGCGTACCAATTC-3') primers without selective nucleotides. One of the primer was end-labeled with [γ-33P]dATP. From a 10-fold dilution of the pre-amplified samples, 5 μl was used for the final selective amplifications using EcoRI with and MseI primers with two and three selective nucleotides, respectively. The PCR conditions were 1 cycle of 94°C for

45 s, 65°C for 30 s, 72°C 60 and 22 cycles of 94°C for 30 s, 55,9°C for 30 s, 72°C 60. The annealing temperature between the second and fourteenth cycles was linked by touchdown phase of decreasing 0.7°C in each cycle. Amplification products were separated on 5% polyacrylamide gels. Gels were dried on 3MM Whatman paper and exposed to Kodak Biomax Films.

Isolation and sequencing of transcript-derived fragments (TDFs)

Bands corresponding to TDFs were cut out from the gel and eluted DNA was re-amplified under the same conditions. Sequence information was obtained by direct sequencing of the re-amplified product with the EcoRI or MseI primers (sequencing reaction condition are described above). The obtained sequences were compared in public available databases (nr and dbEST) by BLASTX and TBLASTX.

Results and discussion

ESTs reportoire analysis

After end-trimming and sequence contamination cleaning (4.5% of sequences), 1007 ESTs, were produced. These sequences ranged between 101 and 1209 bp, with the highest percentage (36%) of cDNA ranging from 500 to 700 bp (Fig. 1). This is due to the short time (30 min) chosen for cDNA synthesis.

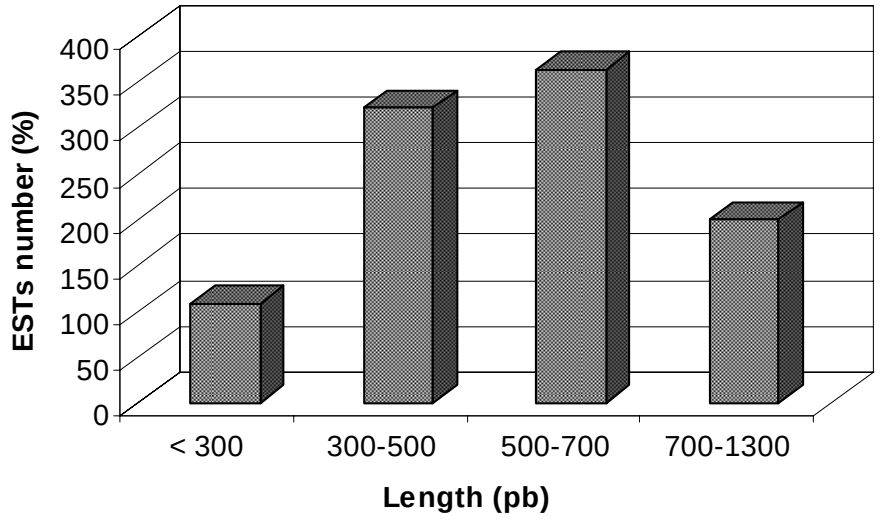

Figure 1 - Distribution of EST repertoire in length (bp) classes.

The EST repertoire was reduced to 696 unigenes in which 579 (83%) were singletons. The remaining sequences (17%), named contigs, were present by a number of sequences ranging from two to fifty. The redundancy of the EST collection (number of ESTs in clusters/total number of ESTs) was calculated to be 42% (Sterky *et al.*, 1998). Therefore, any new sequence obtained from peach mesocarp, has a chance of 42% of being already represented in the repertoire.

ESTs with significant homology (E value <1e-10) were annotated according to BLASTx results (Tab. 1). Each EST was assigned to functional category corresponding to those of the most similar Arabidopsis gene, according to the MIPS role categories (r.c.) (Fig. 2). A total of 54% unigenes did not show any significant Arabidopsis match sequences (e value <1e-10) and thus were considered unclassified. Another 6% matched the Arabidopsis genes annotated as "classification unclear". Thus, only 40% of unigenes were categorized. The largest proportion of functionally assigned unigenes felt into five categories: metabolism (r.c. 1), energy (r.c. 2), protein metabolism (r.c. 4, 12 and 18), cellular organization (r.c. 40, 43 and 70), cellular communication/signal transduction (r.c. 30, 34 and 36). The ESTs included in these categories represented 75% of the assignable unigenes. A similar categorization has been observed for tomato and Arabidopsis EST repertoires (Van der Hoeven *et al.*, 2002).

Genes abundant in ripe mesocarp

Blastx comparison pointed out that the most abundant gene (Contig1) in ripe peach mesocarp (5% of total transcripts) is homologous to a putative allergen of Prunus armeniaca (1.10e-76) (Tab. 1). This putative function is assigned on the basis of homology with a *Hevea brasiliensis* allergen (Hev b5, 7.00e-17). Masia *et al.*, (1992) observed, during peach fruit development, a high secretory activity producing mucilages, associated to vascular bundles. The polypeptide encoded by Contig1 could be a mucilage considering its chemical features and the high accumulation of the related transcript during fruit development (data not shown).

Other abundant genes encode ACC oxidase (3,5%), involved in ethylene biosynthesis, and endo-polygalacturonase (1,9%), Contig170 (homologous to E6 cotton protein, 1.2%) and

Table1 - Highly expressed genes in ripe peach fruit. a) e-value from BLASTX against nonredudant at EMBL; b) *Arabidopsis* gene index number; c) e-value from BLASTX against *Arabidopsis* proteome.

Contig number	GenBank accession number	Gene description	Number of ESTs	% of total ESTs	Putative function	e-value (*a)*	Arabidopsis homologue (*b)*	*At* e-value (*c)*
Contig1	AF134731	Putative allergen protein.[*Prunus armeniaca*]	50	5.0	defense	1.10e-76	no hits found	
Contig60	AF532976	1-aminocyclopropane-1-carboxylate oxidase *[Prunus persica*]	35	3.5	ethylene response	1.00e-144	At1g05010	1.0 e-111
Contig149	AF095577	Endopolygalacturonase.[*Prunus persica*]	19	1.9	cell wall	1.00e-138	At3g59850	1.0 e-101
Contig161	AC006416	Hypothetical protein [*Arabidopsis thaliana*]	12	1.2	unknown	2.10e-40	At1g09310	2.10e-40
Contig170	Q01197	Protein E6.[*Gossypium hirsutum*]	11	1.1	cell wall	8.10e-20	no hits found	
Contig26	AF209908	Hypothetical protein (Fragment).[*Prunus dulcis*]	10	1.0	unknown	1.10e-59	no hits found	
Contig24	P47926	RSI-1 protein precursor (TR132).[*Lycopersicon esculentum*]	10	1.0	unknown	1.10e-42	At3g02885	2.00e-29
Contig63	AB029083	Expansin.[*Prunus persica*]	9	0.9	cell wall	3.10e-39	At1g26770	2.00e-29
Contig164	AX406860	unnamed protein product [*Glycine max*]	8	0.8	unknown	1.10e-36	At5g59845	2.00e-28
Contig184	U93166	Cysteine protease.[*Prunus armeniaca*]	6	0.6	protein biosynthesis/degradation	7.10e-72	At5g60360	2.00e-53
Contig172	AF525402|	SOS2-like protein kinase [*Glycine max*]	6	0.6	signal transduction	5.10e-76	At4g30960	1.00e-59
Contig22	AY466444	ADP-ribosylation factor.[*Medicago sativa*]	6	0.6	metabolism	1.10e-98	At1g10630	1.00e-100
Contig182	AX214375	unnamed protein product [*Glycine max*]	5	0.5	unknown	8.10-36	At1g47960	1.00e-32
Contig180	P17407	21 kDa protein precursor (1.2 protein).[*Daucus carota*]	5	0.5	unknown	3.00e-16	At5g62350	2.00e-14
Contig177		no hits found	5	0.5	unknown		no hits found	
Contig110	AB100869	Plasma membrane intrinsic protein.[*Malus domestica*]	5	0.5	cellular component	1.00e-117	At3g61430	1.0 e-109
Contig28	P13089	Auxin-induced protein AUX28.[*Glycine max*]	5	0.5	hormone response	5.10e-75	At3g04730	4.00e-61
Contig25	AF138266	Papain-like cysteine proteinase isoform III.[*Ipomoea batatas*]	5	0.5	protein biosynthesis/degradation	2.10e-97	At4g39090	6.00e-94
Contig198	Q04960	DnaJ protein homolog [*Cucumis sativus*]	4	0.4	defense stress response	5.10e-83		
Contig118	AF022015	Aux/IAA protein. similar to LeIAA4 [*Lycopersicon esculentum*]	4	0.4	signal transduction	1.0e-50	At2g22670	8.00e-50

Contig number	GenBank accession number	Gene description	Number of ESTs	% of total ESTs	Putative function	e-value (*a*)	Arabidopsis homologue (*b*)	*At* e-value (*c*)
Contig69	AC006416	Hypothetical protein [*Arabidopsis thaliana]*	4	0.4	unknown	7.00e-20	At1g15270	7.00e-20
Contig47	AF435086	GcpE.[*Lycopersicon esculentum*]	4	0.4	secondary metabolism	7.10e-51	At5g60600	4.00e-40
Contig31	AB019230	GTPase activating protein-like.[Arabidopsis thaliana]	4	0.4	transport	3.10e-51	At3g17980	3.10e-51
Contig23	AB050473	Phosphoenolpyruvate carboxykinase (EC 4.1.1.49).[*Flaveria pringlei*]	3	0.3	energy	1.00e-114	At4g37870	1.0 e-112
Contig216	AJ316577	Putative thioredoxin m2.[*Pisum sativum*]	3	0.3	metabolism	6.10e-48	At5g11650	1.00e-37
Contig215		no hits found	3	0.3	unknown		no hits found	
Contig214	AL163814	Lysophospholipase-like protein.[*Arabidopsis thaliana*]	3	0.3	transport	3.10e-35	At3g11780	3.10e-35
Contig213		no hits found	3	0.3	unknown			
Contig212	AC016795	Hypothetical protein [*Arabidopsis thaliana]*	3	0.3	unknown	3.10e-52	At3g11780	3.10e-52
Contig196	AF488594	Putative bHLH61 transcription factor.[*Arabidopsis thaliana*]	3	0.3	transcription factor	2.00e-40	At5g10570	2.00e-40
Contig194	AF532621	bZIP transcription factor similar to ATB2.[*Glycine max*]	3	0.3	transcription factor	1.10e-50	At4g34590	2.00e-36
Contig185	AF516351	Eukaryotic translation initiation factor 5A isoform I [*Hevea brasiliensis]*	3	0.3	protein biosynthesis/degradation	2.10e-77	At1g13950	8.00e-73
Contig89	AY313211	MADS-box protein [*Prunus dulcis*] similar to SEPALLATA3 [*Arabidopsis thaliana*]	3	0.3	transcription factor	5.10e-37	At1g24260	3.00e-18
Contig88		no hits found	3	0.3	unknown		no hits found	
Contig80	P40590|	60S ribosomal protein L34.[*Pisum sativum*]	3	0.3	protein biosynthesis/degradation	6.10e-48	At1g26880	7.00e-39
Contig33		no hits found	3	0.3	unknown		no hits found	
Contig30	CAA56590	S-adenosyl-L-methionine synthetase [*Brassica juncea*]	3	0.3	metabolism	1.00e-27	At1g02500	1.00e-27

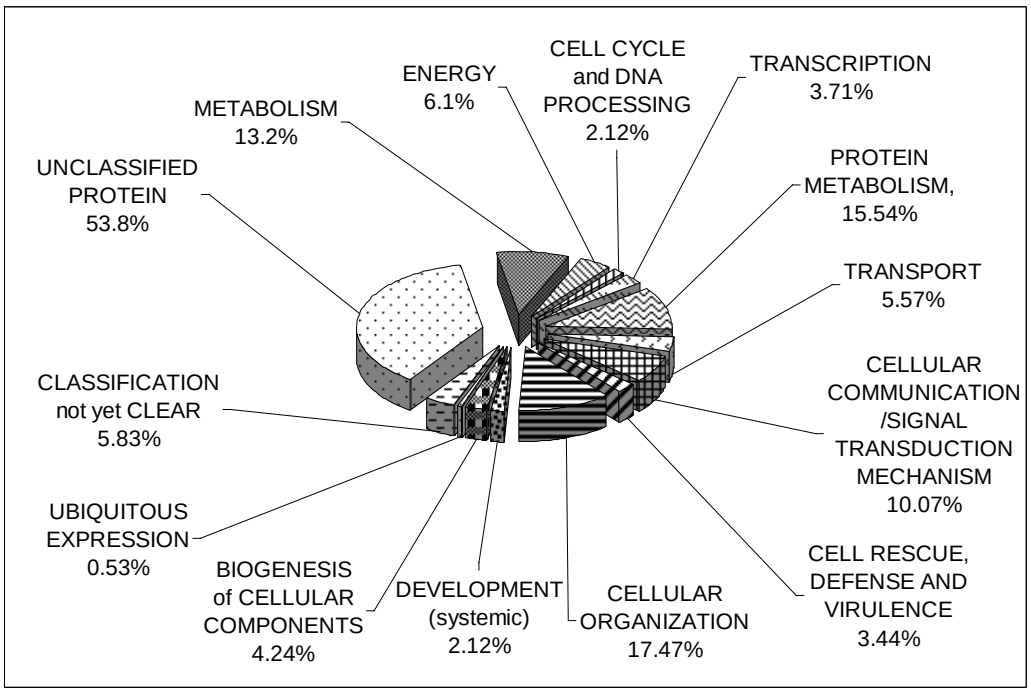

Figure 2 - Peach unigenes putative functions assigned according to MIPS single or assembled role categories (r.c.): metabolism (r.c. 1); energy (r.c. 2); cell cycle and DNA processing (r.c. 10); Transcription (r.c. 11) protein metabolism (r.c. 4, 12, 18); cellular transport (r.c. 20); cellular communication/signal transduction (r.c. 30, 34, 36); cell rescue, defense and virulence (r.c. 32); cellular organization (r.c. 40, 43, 70); development (systemic) (r.c. 41); biogenesis of cellular components (r.c. 42); ubiquitous expression (r.c. 78); classification not yet clear (r.c. 98); unclassified protein (r.c. 99).

expansin (0.9%,), involved in cell wall metabolism. The high expression of these genes at ripening has been previously reported by Ruperti *et al.*, (2001) and Trainotti *et al.* (2003).

Among genes involved in signal transduction and gene regulation, the most represented ones are Contig28 (0,5%) and Contig118 (0,4%), homologous to soybean AUX/IAA28 and tomato LeIAA4, respectively, and three transcription factors (TFs) homologous to the Arabidospis SEPALLATA3 (0.3%, Contig89), bHLH61 (0.3%, Contig194) and atb2 (0.3%, Contig196), belonging to MADS, bHLH (basic helix-loop-helix) and bZIP (basidleucine zipper) families, respectively. The Aux/IAA genes constitute a family of early auxin-responsive genes encoding proteins that, as homo- and heterodimers, are able to regulate auxin response (Reed, 2001). Balbi and Lomax (2003) reported that, in tomato, of the nine Aux/IAA genes expressed at immature stage, only five, including LeIAA4, are actively transcribed at ripening. Among the identified TFs, a putative role in regulation of

grape ripening has been assigned to the SEPALLATA3-like (Boss *et al.*, 2002). ATB2 has been involved in the control of the transport or utilization of sugar in Arabidopsis siliqua (Rook *et al.*, 2003). Expression of bHLH61 has been detected in flowers and siliqua of Arabidopsis (Heim *et al.*, 2003), but its function remains unknown.

Differential gene expression in Fantasia and *SR*

cDNA-AFLP analysis carried out on fruits of cv 'Fantasia' (F) and *SR* selection pointed out the presence of 1385 TDFs (transcript-derived fragments), among which 77 TDFs were isolated and sequenced (7 F and 8 srl in S1, 14 F ans 11 srl in S3, 8 F and 9 srl in early S4, 12 F and 8 *SR* in late S4). Blast results, obtained against nr and dbEST databases, indicated that 12 TDFs (5 for F and 7 for *SR*) have significant homology (e value < 1e-10). Fantasia TDFs are genes mainly related to senescence programme as FI64 and FI69 (a pectinmethylesterase 3 precursor) (Tab. 2). Worthy of note is FI02, homologous to the protein translation factor SUI1 of *Saccharomyces cerevisiae*, detected in Fantasia ripe fruit. SU1 hortologues have been identified in Coffea (CaSUI1, Gaborit *et al.*, 2003) and *Oryza* species (de Pater *et al.*, 1992). Gaborit and co-workers detected a large accumulation of CaSUI1 mRNA in mature coffee beans and suggested that the related protein is necessary to allow an intense transcription and protein synthesis required for coffee seeds germination. Two srl TDFs named FI62 and FI33, selected in early and late S4 stages, respectively, show homology to genes involved in DNA replication and cytokinesis (Menges *et al.*, 2002), events that in normal fruit are completed within 2 weeks AFB (Masia *et al.*, 1992). These data indicate that in srl fruit disturbances in the developmental program related to ripening and senescence occurred.

Table 2 - Selected TDFs identified from cDNA-AFLP analysis differentially expressed (black box) in fruit of 'Fantasia' (F) and *SR*, at different developmental stages as described in material and methods

ID number	BLAST results	GenBank accession number	Size pb	e-value	S1		S3		S4 early		S4 late	
					F.	*SR*	F.	*SR*	F.	*SR*	F.	*SR*
FI02	Protein translation factor SUI1 homolog.[*Salix bakko*]	O48650	344	2e-20							■	
FI07	Pyruvate decarboxylase 1[*Lotus corniculatus*]	AY227204	237	3e-27				■				
FI15	Putative receptor serine/threonine kinase.[*Arabidopsis thaliana*]	AY091006	238	4e-13		■						
FI33	Putative polyprotein- Integrase core domain [*Oryza sativa*]	XP468931	266	1e-16						■		
FI41	NADH dehydrogenase [*Atropa belladonna*]	AJ316582	176	4e-15							■	
FI48	Putative receptor-like protein kinase.[*Oryza sativa*]	AC114983	187	1e-21			■					
FI58	unnamed protein product MTG10.17 [*Arabidopsis thaliana*]	BAB10176	387	1e-13				■				
FI62	MCM protein-like protein.[*Nicotiana tabacum*]	AB032541	232	8e-20								■
FI63	putative auxin response factor [*Arabidopsis thaliana*]	AJ441117.1	236	3e-33		■						
FI64	(SSA-13)Putative senescence-associated protein.[*Pisum sativum*]	AB049723	177	2e-17			■					
FI68	Kinesin-related centromere protein-like.[*Arabidopsis thaliana*]	AB022216	329	2e-44		■						
FI69	(MPE3)Pectinesterase 3precursor.[*Phaseolus vulgaris*]	Q43111	329	1e-23			■					

Conclusions

EST and cDNA AFLP analyses have been used to study gene expression during peach fruit ripening. EST analysis confirmed that genes involved in ethylene biosynthesis and cell wall metabolism are highly represented in peach ripe fruit transcriptome. Some TFs belonging to MADS, bHLH and bZIP families have been identified confirming a role for SEPALLATA3 and atb2 in the fruit ripening regulation, as hypothesized of other species as grape (Boss *et al.*, 2002) and Arabidospis (Rook et al, 2001). In addition, the cDNA-AFLP analysis pointed out that slr fruit phenotype might be determined by disturbances in the onset of senescence.

EST and the cDNA-AFLP approach are complementary, although more exhaustive extensive information on the peach fruit trascriptome relays on microarray analysis. To achieve this goal an Italian consortium, named ESTree, has been established (http://linuxbox.itb.cnr.it/ESTree). The ESTs produced so far by this consortium together with others available in public database (11549 independent entries) have been assembled in a dataset containing 4818, exclusively related to peach fruit development and ripening, independent unigenes. Based on this sequence dataset, 4806 oligo-probes have been synthesized and spotted to generate the first peach microarray (μPEACH 1.0).

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Chapter III

Different Postharvest Conditions Modulate Ripening and Ethylene Biosynthetic and Signal Transduction Pathways in Stony Hard Peaches.

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Abstract

Stony Hard (SH) peaches are characterized, at ripening, by the maintenance of flesh firmness and the lack of ethylene evolution due to a reduced expression of *Pp-ACS1*. In a trial comparing melting flesh (MF, cv. 'Summer Rich') and SH ('IFF331' selection) fruit at two different postharvest temperatures (10 and 20 °C), unexpected behaviour was observed in SH peaches that displayed an increase in ethylene evolution and a decrease in flesh firmness when stored at 10 °C, a temperature regime basically ineffective in delaying ripening in MF fruit. This appeared to be the result of an induction of *Pp-ACS1* transcription, making this genotype of particular interest for studying temperature stress physiology and ethylene-related ripening processes in peaches. Comparative expression analyses of genes involved in cell wall metabolism pointed out the presence of a negative (*Pp-EG4*), positive (*Pp-endoPG*) or no (one member of the *PL* family) relationship with ethylene at ripening. Results clearly showed that the last stage of firmness decrease (melting) only occurs in fruit producing ethylene and is associated with *Pp-endoPG* transcript accumulation. The expression of genes involved in ethylene biosynthesis and signalling pathways has been evaluated via QRT-PCR. *Pp-ACO1* appeared to be induced in SH kept at 10 but not at 20 °C. Transient increases in *Pp-CTR1* and *Pp-EIN2like* gene expression have only been detected at the early stages of ripening in samples producing ethylene, indicating that a causal relationship might exist between ethylene and elements of its transduction pathway during peach fruit ripening.

Keywords: CTR1, EIN2, Ethylene biosynthesis; Ethylene signal transduction; Firmness; Mutants; Postharvest stress; *Prunus persica*.

Introduction

Fruit ripening is a complex genetically-programmed physiological syndrome, defined by concurrent processes not necessarily interrelated from a regulatory point of view in both climacteric and non-climacteric fruit, the former characterized by increases in respiration and ethylene biosynthesis. Both ethylene-dependent and ethylene-independent regulatory mechanisms co-exist (Lelievre *et al.*, 1997; Giovannoni, 2004) and isolation of tomato

mutants has been particular useful for identifying ripening- and ethylene-related processes (Giovanonni, 2007).

Peach (*Prunus persica* L. Batsch) is a climacteric fruit in which the increase in ethylene evolution occurs at an advanced stage of ripening (Tonutti *et al.*, 1991). There is a typical biphasic pattern of loss of firmness in Melting Flesh (MF) peach genotypes: the initial slow rate (softening) is followed by a rapid firmness decrease (melting) in correspondence to the onset of ethylene climacteric (Tonutti *et al.*, 1996). Stony Hard fruit (SH) have crispy flesh at ripening and maintain high firmness values (both on- and off-tree), though they change colour normally and contain high soluble solids (Hayama *et al.*, 2000; Haji *et al.*, 2001, 2004). This behaviour is due to the lack of ethylene evolution and has been attributed to a single recessive gene (*hd*) (Haji *et al.*, 2001, 2005). Working with SH peaches (cv. 'Yumyeong'), Tatsuki *et al.* (2006) showed that ethylene production at ripening is inhibited by a reduced expression of *Pp-ACS1*, a member of the 1- aminocyclopropane-1-carboxylic acid (ACC) synthase gene family, which is responsible for the conversion of S-adenosyl-L-methionine to ACC, the immediate precursor of ethylene (Adams and Yang, 1979). Interestingly, in the same SH genotype, ethylene production increases and *Pp-ACS1* transcripts accumulate in senescing flowers and in wounded immature and mature fruit (Tatsuki *et al.*, 2006). The stony hard trait is inherited independently of the melting/non-melting flesh trait and is epistatic to this trait. When treated with exogenous ethylene, mature SH peaches either ripen to the melting stage or just soften but never melt (Haji *et al.*, 2005). The ethylene-promoted ripening is accompanied by increases in polygalacturonase (PG) gene expression and both endo- and exo-PG activity (Hayama *et al.*, 2006a), but not by *Pp-ACS1* mRNA accumulation (Tatsuki *et al.*, 2006), and autocatalytic ethylene production does not take place. These findings clearly indicate that the *stony hard* locus is related to the regulation of *Pp-ACS1* expression and not to disturbance in the ethylene perception and signal transduction pathways.

The ethylene biosynthesis pathway has been studied in detail in peach (reviewed in Ramina *et al.*, 2007), and an increasing body of information is becoming available on ethylene receptors and elements involved in the signal transduction pathway in this fruit species. Two peach ethylene receptor genes, *Pp-ETR1* and *Pp-ERS1*, have been isolated showing similar organization to that of the corresponding genes in *Arabidopsis* (Rasori *et al.*, 2002).

Unlike *Pp-ETR1*, *Pp-ERS1* appears to be induced by ethylene and repressed by 1-methycyclopropene (1-MCP), an antagonist of ethylene action (Rasori et. al, 2002). Using a genomic approach, Trainotti *et al.* (2006a) identified a new member of the peach *ETR* family, named *Pp-ETR2*, which shows increased expression during the transition from pre-climacteric to climacteric stage. The last part of the ethylene receptor complex is CTR1, which acts as a negative key regulator of ethylene responses. In climacteric fruit, such as tomato (Leclercq *et al.*, 2002) and pear (El-Sharkawy *et al.*, 2003), *CTR1* genes are up-regulated during ripening and specific transcripts accumulate following exogenous application of ethylene. A decrease in *Pp-CTR1* transcript accumulation has been observed by Dal Cin *et al.* (2006) in ripening peaches treated with 1-MCP, suggesting that *CTR1* is also ethylene-inducible in this fruit species. Considering EIN2, the first positive regulator in the ethylene signalling cascade acting downstream from CTR1 (Guo and Ecker, 2004), Zhu *et al.* (2006) observed no changes in *EIN2* transcripts accumulation throughout tomato fruit ripening, whereas Wang *et al.* (2007) reported that an increase in *Le-EIN2* gene expression occurs at the mature green-breaker stages. In peach fruit, an induction of a putative orthologous of *EIN2* was observed during the transition from immature to mature stage (Trainotti *et al.*, 2006a). However, preliminary microarray experiments did not clarify whether *EIN2* transcription is affected by ethylene during peach fruit ripening (Begheldo *et al.*, 2007; Tonutti *et al.*, 2007).

The fact that in SH peaches (cv. 'Yumyeong') *Pp-ACS1* is normally expressed except in ripening fruit has been ascribed to the disruption of a transcriptional factor that is specifically activated to induce *Pp-ACS1* mRNA and/or the presence of inhibitors effective in suppressing *Pp-ACS1* expression at ripening (Tatsuki *et al.*, 2006). Considering this aspect, and the fact that stress conditions (e.g. wounding) are effective in overcoming *Pp-ACS1* inhibition and inducing ethylene production in 'Yumyeong' fruit (Tatsuki *et al.*, 2006), SH peaches represent an interesting model to study factors controlling *Pp-ACS1* transcription, mechanisms modulating its expression, and, using comparative approaches, to better elucidate ethylene physiology in ripening peach fruit.

Materials and methods

Plant material

Peach fruit (*Prunus persica* L. Batsch) of MF cultivar 'Summer Rich' and an SH phenotype 'IFF331' ('Hacuto' x 'New Jersey 256') obtained by the Istituto Sperimentale per la Frutticoltura of Forlì (Italy), were used in the experiments. Fruit were harvested at their commercial maturity stage and, after eliminating defective fruit, they were divided in two different groups (50 fruit each) and stored in ethylene-free air at 20 °C and 10 °C until the end of the experiment.

Flesh firmness (N) was measured after removing a small disc of skin from each side of the fruit, using a penetrometer with a 8 mm probe. Epicarp and endocarp were removed and the mesocarp was frozen in liquid nitrogen and stored at -80 °C until required. Sampling days were selected based on the pattern of ethylene production

Ethylene production

Ethylene biosynthesis was measured on ten individual fruit at varying intervals, as described in Tonutti *et al.* (1991), by enclosing the fruit in 800 ml jars and withdrawing 1 ml of head space gas after 1 h incubation.

Transcript analyses

Total RNA was extracted as reported in Bonghi *et al.* (1998). Northern analysis (10 µg RNA) was performed as described in Ruperti *et al.* (2001), using molecular probes for *pectate lyase* (*PL*) (AJ532967) (Trainotti *et al.*, 2003), *endo-polygalacturonase* (*endo-PG*) (AJ533395) (Trainotti *et al.*, 2003) and *endo-β-1,4-glucanase* (*EGase*) (AJ890497.1) corresponding to *Pp-EG4* (Trainotti *et al.*, 2006b).

Prior to Quantitative Real Time - Polymerase Chain Reaction (QRT-PCR), 30 µg of total RNA was treated with 10 units of RQ1 RNase-Free DNase (Promega) and 1 unit of RNAguard (RNase INHIBITOR) (Amersham) for 30 min, and then purified by phenol-chloroform. One µg of total DNA-free RNA was reverse-transcribed with 200 units of M-MLV Reverse Transcriptase (Promega), 1 unit of RNAguard and 2.5 µM oligo-$dT_{12\text{-}18}$ primer at 37 ^{o}C for 90 min in a final volume of 20 µl, as described in Sambrook *et al.* (1989). The single strand cDNA obtained (100 ng) was subjected to Real-Time PCR in a

final volume of 10 µl containing 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, USA) and specific primers (3 pmol) for *Pp-ACO1, Pp-ACS1, Pp-CTR1, and Pp-EIN2like* (Table 1). Three technical replicates for each sample were run on an ABI 7500 Real Time PCR System Sequence Detection machine (PE Applied Biosystem) programmed to heat for 2 min at 50 °C then 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Fluorescent detection was performed with an extra step of 35 s at temperatures varying in relation to each product's Tm (Table 1). The melting curves were checked for single peaks, and product size was confirmed in an agarose gel. The amplified cDNA fragments were cloned into pBluescript II KS + vector (Fermentas International, Burlington, Canada) and sequenced. For each gene, the PCR Real-Time efficiency was determined by measuring the fluorescence of four serial dilutions of the cDNA template. The expression values were calculated following the mathematical model proposed by Pfaffl (2001), using 25S-5S interspaced sequence as housekeeping.

Table 1. Primers (L:left and R:right) used in the Real-Time PCR mRNA quantification with specific detection step temperature.

Gene	Primer L/R	Detection temperature
R25S-5S	5-TGAATTGCAGAATCCCGTGA-3 5-TGACCTGGGGTCGCGTTGAA-3	85.5 °C
Pp-ACO1	5-GGGAAGTACAAGAGTGTGGAGCACAGAG-3 5-CTTCTCCTCTGCTTCTTTCTCCACCAGTG -3	78 °C
Pp-ACS1	5-ACCGAGACTTGGGATGGAGA-3 5-TGATCAAGCCCTTCACGTTG-3	76.5 °C
Pp-EIN2like	5-ACAGCAGCTGGCGATGTAGC-3 5-GTGAACCAGGACCCTCGTGA-3	77.5 °C
Pp-CTR1	5-GCAAGACTTTCATGCCGAAC-3 5-TATGGACAAGTTTGGGGGCT-3	74.5 °C

Results

MF peaches kept at 20 °C displayed typical ripening behaviour, with an initial stage of slow firmness decrease (softening) followed by the melting stage, leading to firmness values lower than 10 N at 8 days after harvest (DAH) (Fig. 1). If kept at 10 °C, MF peaches showed a similar pattern with slightly higher firmness values than those of control fruit. The melting stage was not delayed in 10 °C MF peaches, indicating that this temperature regime has very limited effects on MF peach ripening. At 20 °C SH peaches showed no change in flesh firmness throughout the postharvest period, whereas, when the temperature was set at 10 °C, they unexpectedly started displaying lower firmness at 10 DAH, reaching values of about 10 N at 15 DAH (Fig. 1).

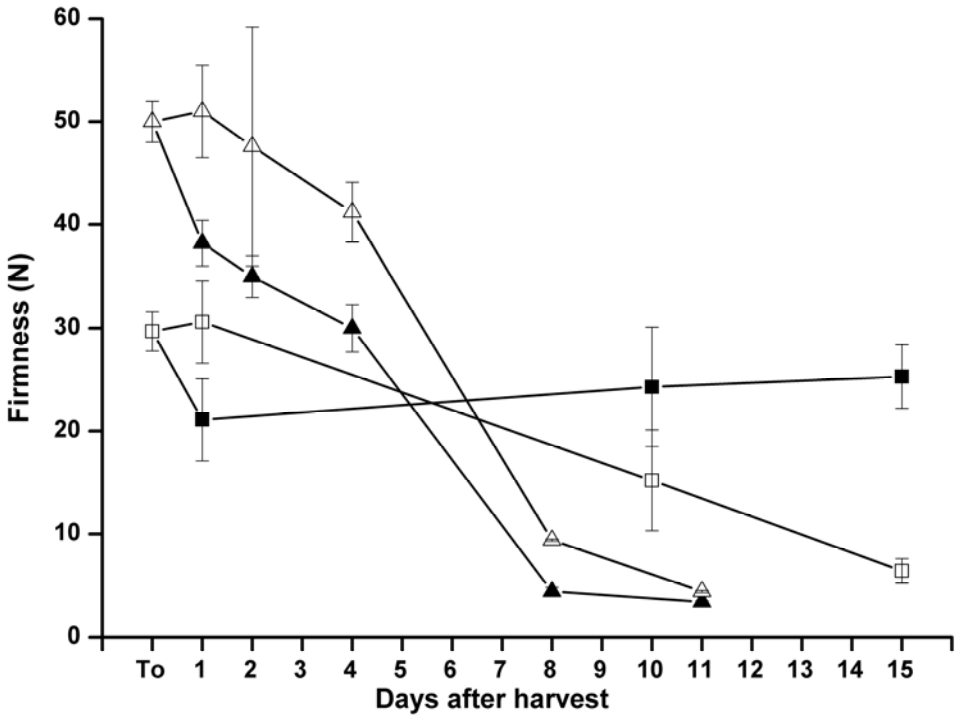


Figure 1 - Flesh firmness changes during postharvest ripening at different temperatures: cv. 'Summer Rich' at 20 °C (▲) and 10 °C (Δ) ; 'IF331' selection at 20 °C (■) and 10 °C (□). Data are means of five replicates ± SE.

In MF peaches the ethylene climacteric rise occurred at the melting stage, when the highest values of ethylene biosynthesis were observed in both samples (Fig. 2). No ethylene evolution was detected throughout the experimental period in SH peaches at 20 °C, while

some evolution of the hormone was registered in SH fruit kept at 10 °C at 10 DAH, followed by massive ethylene production at 15 DAH (Fig. 2)

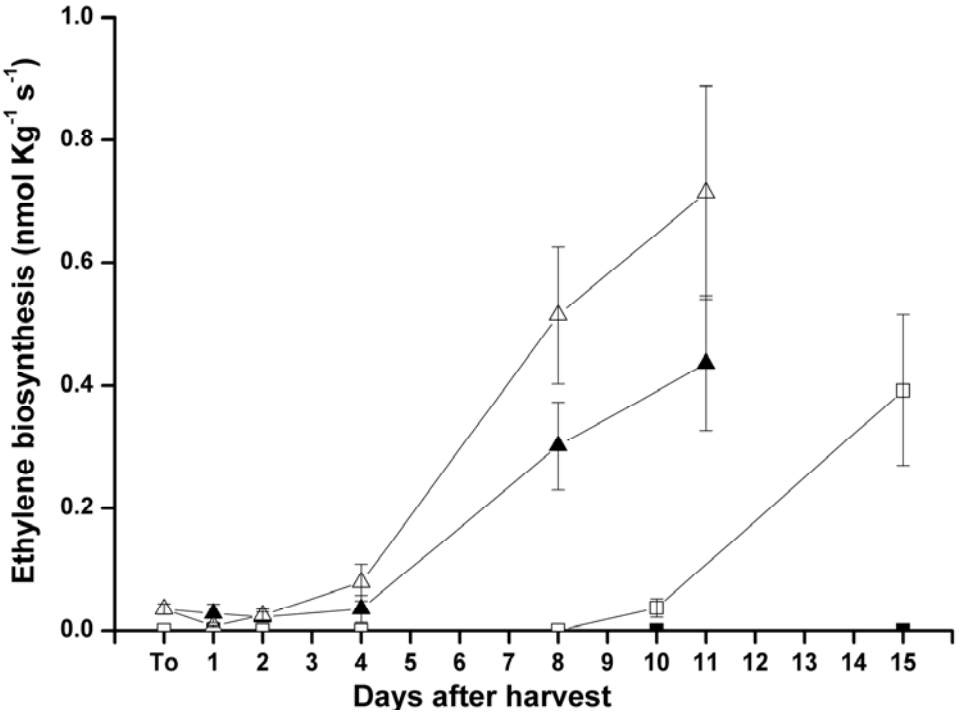


Figure 2 - Ethylene production during postharvest ripening at different temperature conditions: cv. 'Summer Rich' at 20 °C (▲) and 10 °C (Δ) ; cv. 'IF331' at 20 °C (■) and 10 °C (□). Data are means of ten replicates ± SE.

In order to elucidate this unexpected behaviour of SH peaches kept at 10 °C, expression of genes involved in ethylene biosynthesis was analysed by QRT-PCR. In MF peaches, transcript accumulation patterns confirmed that *Pp-ACO1* was induced earlier than *Pp-ACS1* and that the ethylene climacteric was paralleled by a marked increase in transcript accumulation of *Pp-ACO1* (Fig. 4a), while a less pronounced increase in transcript levels of *Pp-ACS1* was recorded at the melting stage (Day 11), when *Pp-ACS1* transcripts appeared to be higher in MF peaches kept at 10 °C than the level observed in 20 °C samples (Fig. 3a). *Pp-ACS1* transcripts were almost undetectable in SH fruit stored at 20 °C whereas, if they were kept at 10 °C, a slight increase in *Pp-ACS1* mRNA was observed 10 DAH and a dramatic accumulation of specific transcripts was apparent after 15 days (Fig. 3b). In SH fruit, *Pp-ACO1* transcripts remained at basal levels if fruit were kept at 20 °C, but not at 10 °C when *Pp-ACO1* resulted as being markedly expressed 10 and 15 DAH (Fig. 4b).

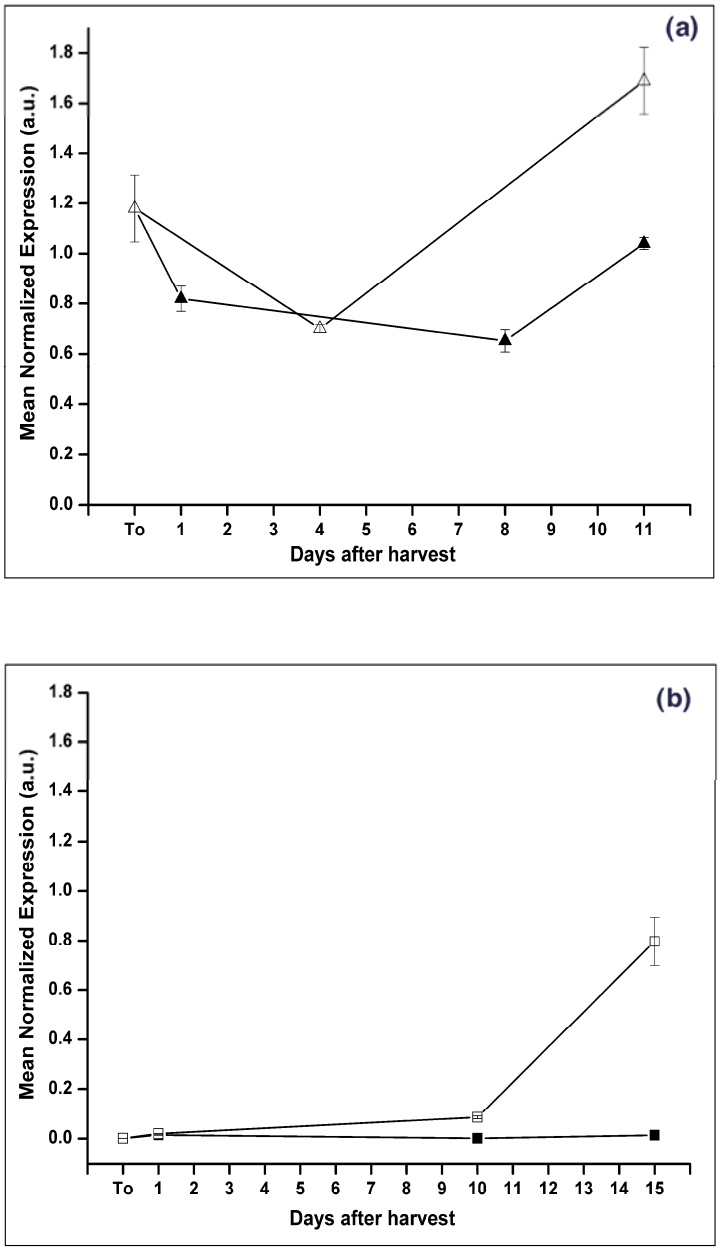


Figure 3 - *Pp-ACS1* transcript accumulation during postharvest ripening: (a) cv. 'Summer Rich' at 20 °C (▲) and 10 °C (Δ); (b) cv. 'IF331' at 20 °C (■) and 10 °C (□).

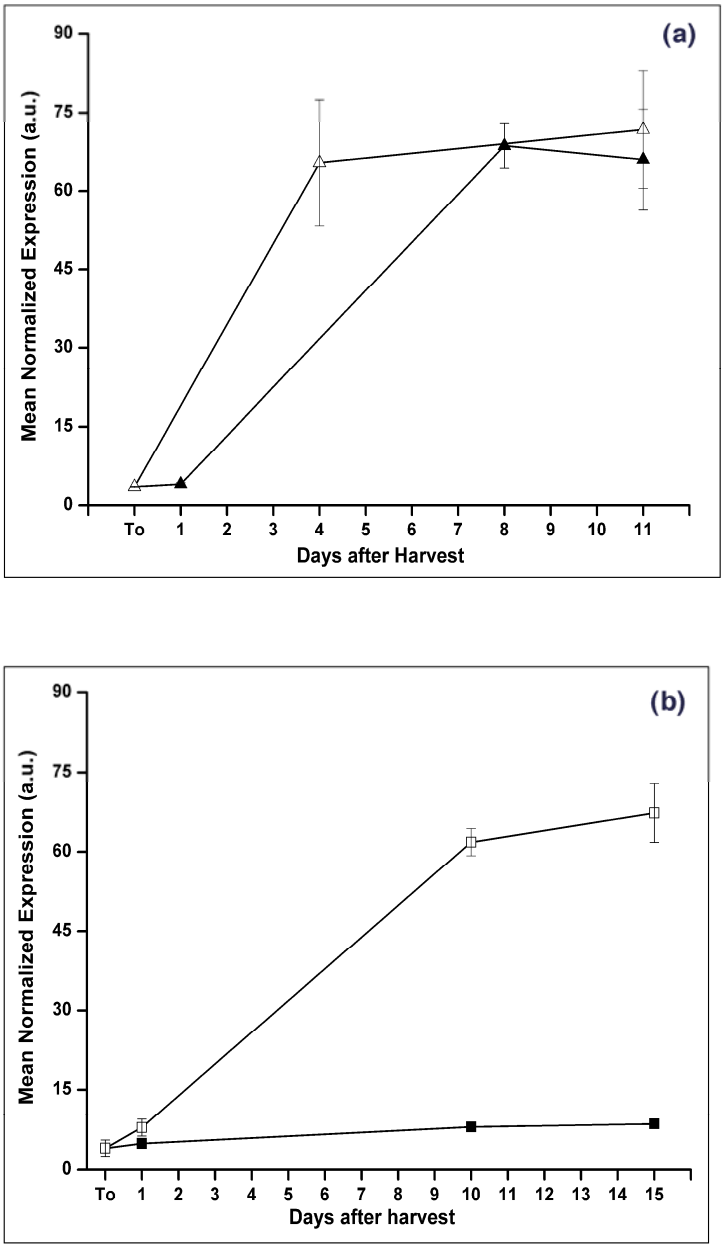


Figure 4 - *Pp-ACO1* transcript accumulation during postharvest ripening: (a) cv. 'Summer Rich' at 20 °C (▲) and 10 °C (Δ); (b) cv. 'IF331' at 20 °C (■) and 10 °C (□).

The nature of the SH mutation and the peculiar ripening behaviour observed in SH fruit kept at 10 °C make this genotype suitable for better elucidating the role of ethylene in the expression of ripening-related genes in peach fruit. Two elements involved in the ethylene

signalling pathway were studied. Regarding *Pp-CTR1*, a transient increase of specific transcripts was present in fruit synthesizing ethylene (both MF samples and SH kept at 10 °C) (Fig. 5a, b) and the highest accumulation levels occurred in correspondence to the onset of ethylene climacteric.

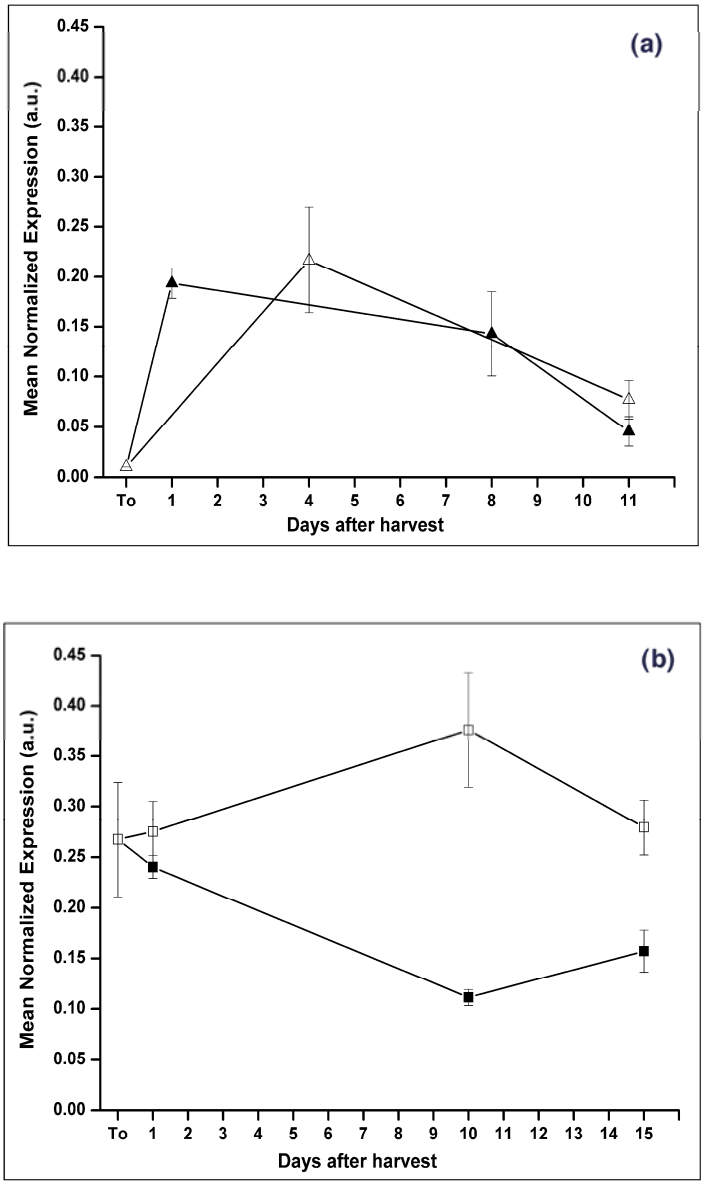
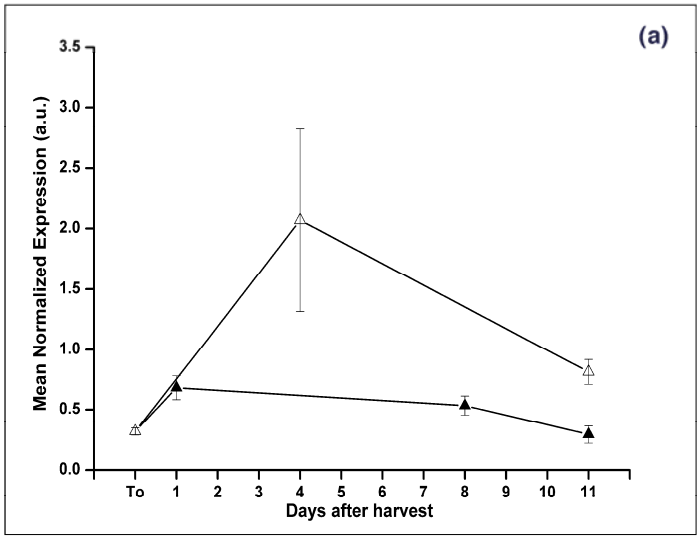


Figure 5 - *Pp-CTR1* transcript accumulation during postharvest ripening: (a) cv. 'Summer Rich' at 20 °C (▲) and 10 °C (Δ); (b) 'IF331'selection at 20 °C (■) and 10 °C (□)

Compared to SH peaches kept at 10 °C, reduced transcript level of *Pp-CTR1* was observed in 20 °C SH samples not producing ethylene (Fig. 5b). Similar results were obtained when *Pp-EIN2-like* transcript accumulation was quantified; again, a transient increase was present only in samples evolving ethylene and not in SH fruit at 20 °C, where a decreasing trend was monitored. (Fig. 6a, b).



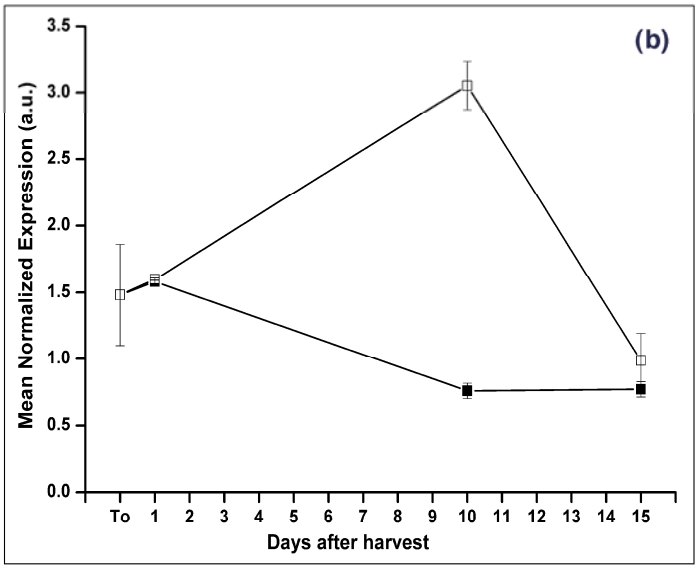


Figure 6 - *Pp-EIN2 like* transcript accumulation during postharvest ripening: (a) cv. 'Summer Rich' at 20 °C (▲) and 10 °C (Δ); (b) 'IF331' selection at 20 °C (■) and 10 °C (□).

Given the different patterns of ethylene biosynthesis and loss of firmness detected in SH peaches kept at two temperature regimes during the postharvest period (Figs. 1 & 2), expression analyses of three cell-wall related genes were performed in SH, as well as in MF peaches for comparison (Fig. 7). *Pp-EG4* expression showed a similar pattern in both MF samples; the highest expression level was detected at harvest, followed by a progressive reduction in transcript accumulation paralleling ethylene evolution. The same expression pattern was observed in 10 °C SH samples but not in 20 °C SH peaches, which maintained high levels of *PpEG4* expression throughout the experiment **(**Fig. 7a**)**.

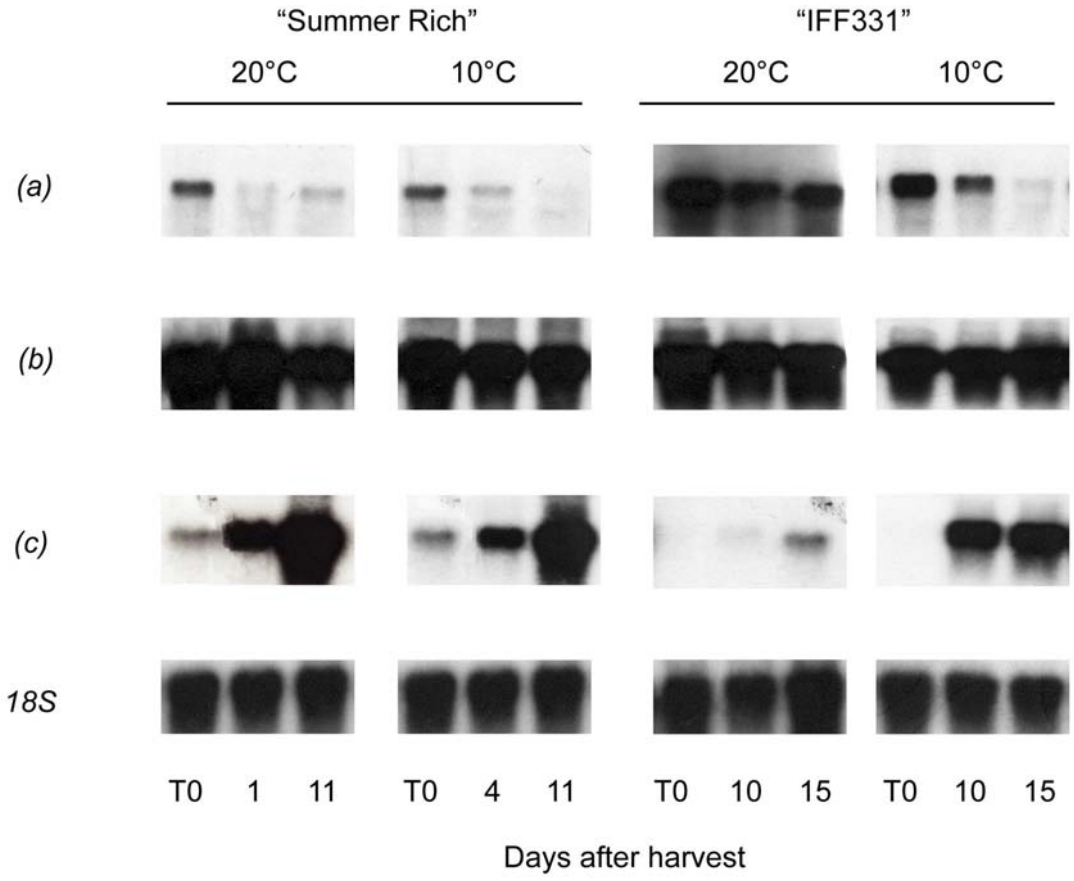


Figure 7 - Endo-β-1,4-glucanase (AJ890497.1) (a), pectate lyase (AJ532967) (b), and endo-polygalacturonase (AJ533395) (c) transcript accumulation, evaluated by northern analysis, in fruit of cv. 'Summer Rich' and cv. 'IF331' during postharvest ripening at 10 and 20 °C. 18S rRNA hybridisation was used to evaluate equal loading.

Analyzing the expression of two genes involved in pectin metabolism, the considered *PL* gene showed no significant changes in both genotypes and postharvest temperature

conditions (Fig. 7b), whereas marked differences were detected in terms of *endo-PG* transcript accumulation (Fig. 7c). In MF peaches the strongest hybridization signal occurred, for both samples, at the melting stage (11 DAH). In SH peaches kept at 20 °C, *endo-PG* transcripts were not detected at harvest and 10 DAH, and a faint hybridization band appeared 15 DAH. When SH peaches were kept at 10 °C, a pronounced activation of *endo-PG* gene transcription was observed at 10 and 15 DAH in correspondence to the ethylene climacteric (Fig. 7c).

Discussion and Conclusions

The different ripening behaviour of Stony Hard compared to Melting Flesh peaches is the lack of ethylene evolution due to a suppression of *Pp-ACS1* (Tatsuki *et al.*, 2006). In the presence of exogenous ethylene, some SH peaches soften normally. This is the result of the induction of ethylene-dependent cell-wall hydrolases, namely PGs, which show increases in both gene expression (Hayama *et al.*, 2006a) and specific activity (Hayama *et al.*, 2006b). Even in the presence of exogenous ethylene, *Pp-ACS1* is not expressed and ethylene does not evolve in ripening SH fruit (Tatsuki *et al.*, 2006). Based on this information, we can exclude that the loss of firmness detected in SH peaches kept at 10 °C for 15 days is the result of the presence of exogenous ethylene, since a marked increase in *Pp-ACS1* transcript, as well as ethylene biosynthesis, was clearly detected starting 10 DAH (Figs. 2 & 3). Similar results were obtained by Gamberini (2007) in 'Yumyeong' SH peaches stored at 4 °C, where a slight increase in ethylene evolution was detected after 5 days and a high production of the hormone following 10 days of storage under the same temperature conditions. Tatsuki *et al.* (2006) clearly showed that *Pp-ACS1* is expressed in ripening 'Yumyeong' peaches in response to wounding: taken together, these results indicate that stress conditions (wounding, low temperature) seems to be effective in overcoming *Pp-ACS1* inhibition that has been ascribed, in SH peaches, to a disruption of a transcriptional factor specifically activated at ripening (Tatsuki *et al.*, 2006). Interestingly, a temperature of 10 °C, basically ineffective in delaying ripening in MF peaches, appears to be perceived as a stress condition and is sufficient to remove *PpACS1* inhibition in SH fruit. Experiments based on different temperature regimes and duration, the definition of the nature of stony hard phenotype, and the elucidation of mechanisms regulating *Pp-ACS1* expression in SH peaches will be of great help in the attempt to identify the stress-induced factor that

promotes *Pp-ACS1* transcription in this phenotype during ripening. The lack of ethylene evolution characterizing SH peaches and their behaviour when exposed to 10 °C for several days make this genotype of particular interest for better elucidating the role of ethylene in regulating expression of different ripening-related genes. Cell wall metabolism during peach fruit ripening is the result of changes in the activity of cell-wall targeted enzymes (Brummell et. al., 2004; Brummell, 2006) encoded by genes differently affected by ethylene (Trainotti *et al.*, 2003). We clearly demonstrate that *PpEG4* is down regulated by ethylene and that its expression is not related to the melting process. The different expression pattern of *endo-PG* in SH fruit synthesizing ethylene (10 °C), or not (20 °C), reconfirms the relationship existing between this hormone, *PG* transcription and melting stage as reported in early work on MF peaches (Downs *et al.*, 1992) and, more recently, on SH peaches that displayed decreasing firmness values following exogenous ethylene treatment (Hayama *et al.*, 2006a and b). The expression of the *PL* gene, with no significant changes in either SH or MF samples throughout the experimental period, would indicate that its role in peach fruit melting is not as crucial as that played by *PG*. Considering results published by Benitez-Burraco *et al.* (2003) and Trainotti *et al.* (2003), we cannot exclude that other *PLs*, showing differential expression, may be active during peach ripening and/or that expression of the *PL* studied in this work is differently modulated according to the genotype.

Some of the genes encoding multiple steps in the ethylene signal pathway are ethylene-inducible at ripening and this suggests a selective advantage for amplifying ethylene signalling machinery during climacteric fruit ripening (Adams-Philips *et al.*, 2004). The differences observed in *Pp-CTR1* and *Pp-EIN2 like* expression pattern in MF and SH ripening peaches reinforce this hypothesis. Marked differences in the mRNA accumulation patterns of the two ethylene response components are present in fruit where ethylene biosynthesis is activated and the climacteric occurs (both MF samples and SH at 10 °C) and fruit (SH at 20 °C) characterized by lack of ethylene evolution. Considering CTR1, a positive relationship between the presence of the ethylene climacteric and a transient accumulation of *Pp-CTR1* transcripts is evident: our data are in agreement with Leclercq *et al.* (2002) who reported that, in tomato, *Le-CTR1* is induced by ethylene during fruit ripening, reinforcing the hypothesis of an important regulatory role played by CTR in

ethylene-dependent events. Similar behaviour has been observed in 'Passe Crassane' pears, where a sharp transient increase in *Pc-CTR1* mRNA occurs during the ethylene climacteric, while in the absence of ethylene evolution or in the presence of 1-MCP, *Pc-CTR1* transcripts fail to accumulate (El-Sharkawy *et al.*, 2003). In *Rosa hybrida* flower opening, two *CTR* genes (*Rh-CTR1* and *Rh-CTR2*) are clearly expressed in an ethylene-dependent manner (Muller *et al.*, 2002; Ma *et al.*, 2006). The increase in *Pp-CTR1* expression in correspondence to the ethylene climacteric rise, together with the up-regulation of (at least) two ethylene receptors, *Pp-ERS1* (Rasori *et al.*, 2002) and *Pp-ETR2* (Trainotti *et al.*, 2006a), support the hypothesis of an important role played by these elements in tempering ethylene response (Klee, 2002). It has been demonstrated that CTR1 acts as negative regulator (Hua and Meyerowitz, 1998), thus a decrease in the expression might be associated with an increase in ethylene sensitivity. In all fruit evolving ethylene (both MF and 10 °C SH samples), *Pp-CTR1* transcript accumulation shows a similar pattern, with a peak at the early stage of ripening, followed by a decrease when ethylene is produced at high levels. As stated above, reduced expression of *CTR1* might be associated with an increase in sensitivity to ethylene. Hayama *et al.* (2006b) showed that, although a dose-response effect is present, exogenous ethylene concentrations as low as 0.1 $\mu L\ L^{-1}$ are enough to accelerate the softening process in 'Manami' SH peaches. Further investigations are needed to demonstrate the possible correlation between the high sensitivity of SH peaches to ethylene and the reduced transcription of *Pp-CTR1*.

LE-EIN2 has been isolated in tomato, and its silencing delayed fruit development and suppressed the ethylene-inducible and ripening-related genes such as *E4* and *PG* (Zhu *et al.*, 2006), indicating that *Le-EIN2* is an essential component of ethylene-signalling during tomato fruit development, although, contrasting data have been published on its expression pattern: Zhu *et al.* (2006) reported that it is constitutively expressed throughout fruit development and ripening, whereas Wang *et al.* (2007) observed an increase in *Le-EIN2* transcripts at mature green and breaker stages, followed by a decrease during advanced ripening. Our results, besides confirming data obtained by Trainotti *et al.* (2006a) that showed an up-regulation of *Pp-EIN2 like* during the transition from immature to mature stage, clearly indicate that a transient increase in *Pp-EIN2 like* expression takes place in pre-climacteric fruit, followed by a sharp decrease in correspondence to the burst of

ethylene production. Treatments of mature-green tomato fruit with exogenously applied ethylene or 1-MCP did not affect *Le-EIN2* expression (Zhu *et al.*, 2006; Wang *et al.*, 2007). Similarly, we observed no changes in *Pp-EIN2 like* expression following 1-MCP treatment in ripening peaches when *Pp-EIN2 like* is highly expressed (Tonutti *et al.*, 2007). In our study, a transient increase of *Pp-EIN2 like* transcript level was detected in correspondence to the ethylene climacteric rise. This behaviour suggests the presence of a positive relationship between ethylene and *Pp-EIN2 like* expression during the early stages of peach fruit ripening. However, we cannot exclude that other factors may be responsible for the different expression pattern of *Pp-EIN2 like*. Alonso *et al.* (1999) pointed out that, in *Arabidopsis*, EIN2 functions in at least two different signalling pathways and is involved in stress responses. If this is also the case for the two peach samples kept at 10 °C remains yet to be elucidated.

In conclusion, we have demonstrated that SH peaches, characterized by the lack of ethylene evolution at ripening, recover the ability to synthesize ethylene when exposed to postharvest temperature stress and this has been exploited to elucidate the role of the hormone in gene expression regulation in ripening peaches. The unexpected behaviour of SH fruit kept at 10 °C after harvest resembles what happens in winter pears that, in order to ripen, require a cold storage period effective in inducing *ACS* transcription, stimulating ethylene evolution and promoting softening (El-Sharkawy *et al.*, 2003). In the absence of cold, ripening does not occur and pears remain firm. One *ACS* gene isoform (*Pc-ACS1a*) appears to be cold- and ethylene-dependent and, in winter pears, it represents the control point in the onset of autocatalytic (system 2) ethylene production (El-Sharkawy *et al.*, 2004). Assuming that similar mechanisms operate in winter pears and SH peaches kept at low temperature and given that both species belong to the same family (*Rosaceae*), a comparative study of SH peach and winter pear *ACS1* promoters together with a large-scale analysis of transcriptome may help to identify (common) factors possibly involved in determining this ripening behaviour. Another future prospect is the elucidation of ripening-related changes in SH phenotypes stored at a range of low temperatures.

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Chapter IV

Transcript Profiling of Ripening Nectarine Fruit Treated with the Inhibitor of Ethylene Action 1-Methylcyclopropene

Abstract

A large scale-analysis of transcriptome has been carried out using μPEACH1.0 microarray on nectarine (*Prunus persica* L. Batsch) fruit treated with 1 μl L^{-1} 1-Methylcyclopropene (1-MCP) for 24h. At the end of the incubation period the ethylene antagonist appeared to be effective in blocking the ripening process since only 9 genes appeared to be differentially expressed (24 h MCP vs fruit at harvest) compared to 90 genes showing up- or down-regulation in control fruit (24h in air vs fruit at harvest). This result has been confirmed by comparing transcriptome of fruit 24h after harvest (24 h MCP vs 24 h in air): 102 genes resulted differentially expressed. The quick recovery of ripening parameters (as softening) in the post-treatment phase is accompanied by a marked change in gene expression pattern: the majority of the genes affected by the chemical at the end of the 24h-treatment showed a marked change in their expression at 72h (48h after the end of the incubation period). A possible explanation of this peculiar behaviour is discussed.

Keywords: Ethylene Biosynthesis, Ethylene Perception, Gene expression, Microarray, Postharvest

Introduction

The control of ripening in climacteric fruit mainly relies on the possibility of affecting ethylene biosynthesis and action. This can be achieved through genetic manipulation, by modulating environmental (temperature, atmosphere composition) parameters during storage, or using specific inhibitors of ethylene biosynthesis/perception. Considering this last option, the antagonist of ethylene action 1-Methylcyclopropene (1-MCP) is widely used for delaying ripening and prolong shelf life of several fruit commodities. 1-MCP is thought to interact with ethylene receptors and thereby prevent ethylene-dependent responses (Sisler and Serek, 1997, 2003). Researches carried out on the effects of 1-MCP have shown that the chemical can reduce ethylene production, respiration, softening, colour change, aroma production and the occurrence of physiological disorders and thus increase storage life of numerous climacteric fruits with treatment efficacy depending on such factors as the concentration of 1-MCP used, storage condition and duration, and maturity of

the fruit before application (Watkins, 2006). Most horticultural commodities that were tested do respond to 1-MCP with the biggest effects found in climacteric fruit and those with very specific responses to exogenous ethylene (Huber *et al.* 2003). However, it is now clear that 1-MCP induce variable responses in different climacteric fruit species and, within the same species, in different varieties (Blankenship and Dole, 2003). This is particularly evident when comparing two important crops such as apples and peaches: following 1-MCP treatment, ethylene production is strongly reduced, ripening is inhibited or delayed for many days and storage prolonged in apples (Watkins *et al.*, 2000; Fan and Mattheis, 2002b; Jiang and Joyce, 2002; Bai *et al.*, 2005). On the other hand, the effects of 1-MCP treatments on peach and nectarine ripening are limited to the incubation period and few hours afterwards when the maintenance of flesh firmness is associated with altered gene expression pattern as observed for polygalacturonase (PG) that appears down-regulated by 1-MCP (Dong *et al.*, 2001; Dal Cin *et al.*, 2005). However, a recovery of ripening parameters occurs within few days after the end of treatment in both peaches and nectarines that display a rapid decrease in firmness values in particular if maintained at room temperature (Mathooko *et al.*, 2001; Fan *et al.*, 2002b; Ziliotto *et al.*, 2003). The physiological and molecular mechanisms underlying this different responses of apples and peaches to 1-MCP are not known. In a comparative work, Dal Cin *et al.* (2006) pointed out that while in apples ethylene biosynthesis is markedly inhibited by 1-MCP, in peaches the evolution of the hormone appears not significantly affected by the chemical. The same authors reported that the different behaviour of the two species in relation to 1-MCP treatment might be due to differences in terms of ratio, expression pattern and/or turn-over of the ethylene receptors. Besides differences in terms of *ETR1* and *ERS1* gene expression, an intriguing aspect is represented by the different expression pattern of ACC synthase that appears down regulated by 1-MCP in apples but not in peaches as also reported by Dong *et al.* (2001) and Mathooko *et al.* (2001) suggesting that the differential effect of 1-MCP on ethylene biosynthesis occurs likely through ACS.

The genomics approach and the development of high throughput technologies for large scale analyses of transcriptome represent powerful tools for unravelling the molecular mechanisms of complex processes as fruit ripening and elucidating, upon the whole, the role and the effects of endogenous and/or exogenous factors. Microarray technology, in

particular, is a powerful technique effective in measuring the level of expression of thousands of genes in a single experiment and is now increasingly used in plant science (Rensink and Buell, 2005) and also for elucidating and studying gene expression pattern and regulation during fruit ripening (Bonghi and Trainotti, 2006). Considering specifically the transition from pre-climacteric to climacteric stage and the role of ethylene during ripening, microarrays analyses have been carried out in pear (Fonseca *et al.*, 2004), tomato (Alba *et al.*, 2005) and peach (Trainotti *et al.*, 2006a): in this last species, differentially expressed genes have been identified throughout ripening and in relation to the onset of ethylene climacteric. In particular, a dramatic up-regulation has been detected for genes encoding transcription factors and enzymes involved in ethylene biosynthesis and action. Trainotti *et al.* (2007), by identifying genes involved in biosynthesis, transport and signaling of auxin showing an increased expression at peach ripening, demonstrated the existence of an important cross-talk between auxin and ethylene, with genes in the auxin domain regulated by ethylene and genes in the ethylene domain regulated by auxin.
Here we report results of a transcriptomic approach undertaken with the aim of elucidating molecular mechanisms and identifying candidate genes responsible for the peculiar behaviour of peach and nectarine fruit following 1-MCP treatment.

Materials and methods

Plant material and experimental design

Nectarine (*Prunus persica* L. Batsch, cv Super Crimson Gold) fruit were harvested at commercial ripening (about 60N flesh firmness) and immediately transferred to the Postharvest Laboratory of the Faculty of Agriculture, University of Padova, Italy, where they were maintained at room temperature (20°C) throughout the experiments. After selection, they were divided in two groups of 40 fruit each: the first group was enclosed in gas-tight glass jars and treated for 24 h with 1 μl L^{-1} of 1-MCP. The second group (control) was enclosed for 24 h in sealed jars of the same volume without 1-MCP. In order to avoid excessive CO_2 accumulation, KOH was added into the jars. At the end of the 24-h incubation period fruit were removed from jars and maintained in air at 20°C for further 48h (72h from harvest). Samplings were performed at the beginning of the experiment (T0), at 24 (24MCP and 24AIR) and 72h (72MCP and 72AIR).

At each sampling date ethylene production of 10 fruit has been determined using a gas chromatograph (DANI 3200) and flesh firmness measured with a penetrometer (TR, Forlì, Italy) equipped with a 6 mm probe. Mesocarp of each fruit was frozen in liquid nitrogen and stored at -80 °C.

RNA extraction and northern analysis

Total RNA was extracted using the protocol described by Ruperti *et al.* (2001). RNA yield and purity was checked by means of UV absorption spectra, whereas RNA integrity was ascertained by means of electrophoresis in agarose gels followed by ethidium bromide staining on the ribonucleic acid.

Microarray preparation and hybridization

The features and the preparation and the hybridization protocols of the peach microarray µPEACH 1.0 are described in Trainotti *et al.* (2006a).

Total RNA (20 µg) from T0, air and 1-MCP treated fruits was converted into target cDNA by reverse transcription using the SuperScript^{TM} Indirect cDNA Labeling System (Invitrogen, USA) following manufacturer instruction. The cDNA labeling was carried out as described by Trainotti *et al.* (2006a).

Data analysis

The microarrays were scanned as described by Trainotti *et al.* (2006a). TM4 (www.tm4.org) package developed at TIGR (www.tigr.org; Saeed *et al.*, 2003) was used to analyse microarray data. The images were processed using the Spotfinder 2.2.3. software by means of the Otsu algorithm. The expression data extracted by Spotfinder were normalized by MIDAS 2.18 using the LOWESS (Locally Weighted Regression Scatter Plot Smoothing; Cleveland, 1979) algorithm with the 'block mode', keeping as reference the Cy3 channel.

After normalization, data from each slide were split in two, by using Microsoft Excel, since each probe is spotted twice on µPEACH1.0. Thereafter, each spot value was considered to be independent.

Normalized split data were loaded in MeV 3.1 and for each comparison (24MCPvsT0, 24 AIRvsT0, 24MCPvsAIR; 72MCPvs24AIR, at least three independent experiments) a 66%

cut-off was imposed to select genes differentially expressed by one-class unpaired SAM (Significance Analysis of Microarrays; Tusher *et al.*, 2001) analysis. Clones with significative changes in expression (threshold ratios, expressed as $\log_2$, higher than 1 and lower than -1 for up- and down-regulation, respectively) were identified at delta values giving a 0% of false discovery rate (FDR).

Quantification of mRNA via RT-PCR

To validate microarray data, transcript accumulation of IAA16, Ethylene Receptor ETR2, Ethylene Binding Factor, Trehalose-6-phosphatase, β carotene hydroxylase genes was evaluated via qRT-PCR using the SYBR Green RT-PCR master mix kit (PE Applied Biosystem) as described by Cecchetti *et al.* (2004). The cDNA single strand used for RT-PCR was obtained as follows: 50 µg of total RNA were treated with 10 units of RQ1 RNase-Free DNAse (Promega) and 1 unit of RNAguard (RNase INHIBITOR, Amersham) for 30 min, then purified by phenol-clorophorm. A 1 µg of total RNA DNA-free reverse-transcribed was incubated with 200 units of M-MLV Reverse Transcriptase (Promega) (Promega), 1 unit of RNAguard (RNase INHIBITOR) and 2.5 µM oligo-dT $_{12\text{-}18}$ as primer at 37 °C for 90 min in a final volume of 20 µl, as described in Sambrook *et al.* (1989). For each sample, three replicates were performed in a 10 µl containing 1 µl of the cDNA, 2X SYBR Green PCR Master mix and specific primers (Tab. 1) according to the manufacturer's instructions. Reaction parameters were: 10 min at 95 °C, 40 cycles including a denaturation for 30 sec at 95 °C, a annealing for 30 sec at 64 °C and an extension for 35 sec at 72 °C; and then an ulterior cycle for the dissociation was composed by 15 sec at 95 °C, 1 min at 60 °C and 15 sec at 95 °C. The amplified cDNA fragments were cloned into pBluescript II KS + vector (Fermentas International, Burlington, Canada) and sequenced. The expression values were calculated following the mathematical model proposed by Livak and Schmittgen (2001) using 18S as housekeeping.

Table1. Primers (L:left and R:right) used in the Real-Time PCR.

Gene	Primer L/R
Catalase	5-GATCCTGTTCGTCATGCTGAGCGCTAC-3
Ctg.1024	5-GCGCCCAAGATCTGTATCTCTCTCCA-3
IAA16	5-CTGGATGTTAGTTGGAGACGTCCCTTGG-3
Ctg.768	5-GTGGCAGAACCTGAGACGTCCTTGGAG -3
Ethylene receptor	5-CTCATGAGGGAGAAGTTGGCCGAGC -3
Ctg.4109	5-GCCTCCTCATCCCATCACTCATTACC-3
βO4Hydroxilase	5-GCACGAGTCTCACCACCGACCCAGAG-3
Ctg.711	5-TCCAAGACCAGCACCAAAACATAGGC-3
TPPA	5-CCGCAATGTAGATGAGAAGAGTTGGCC-3
Ctg.4621	5- GTGACGGCCTTCCCTTTGTCCCAG -3
EREBP	5-CGCATACATGGCTCCAAGGCTTTGCTC-3
Ctg.2757	5-CTGGGACAAAGGGAAGGCCGTCAC-3

Results

At the end of the 1-MCP 24h-incubation period, flesh firmness resulted higher in treated than in control fruit. The difference was maintained 24h after the end of treatment but was barely significative at 48h (72h from harvest) when treated fruit displayed low firmness values (Fig. 1). A similar increasing trend in ethylene biosynthesis has been observed in the two samples throughout the experimental period. No difference was observed at the end of incubation time and 24h afterwards, whereas 1-MCP treated nectarines synthesized more ethylene at 72h (48h from the end of the treatment) (Fig. 1).

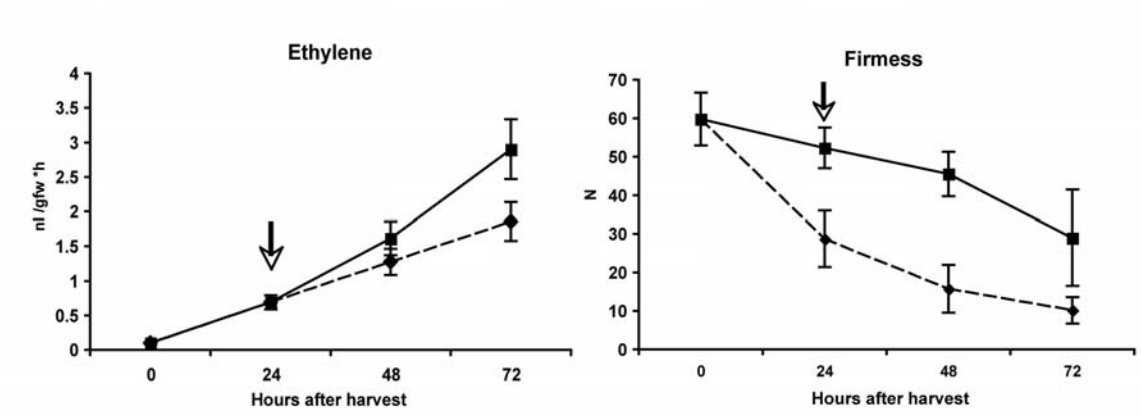

Figure 1 - Ethylene evolution (left panel) and firmness (right panel) in mesocarp of 1-MCP-treated (solid line) and control (dotted line) peach fruits. Arrow indicates the end of the incubation period. Vertical bars represent SD

A first set of microarray hybridizations has been carried out by comparing, using a simple loop/flipped dye design (Fig. 2), T0, 24MCP and 24AIR samples. SAM analysis revealed that in 24AIRvsT0 comparison, a total of 215 targets hybridized whereas 212 were the targets displaying hybridization signals in 24MCPvsT0 and 24MCPvs24AIR comparisons (Fig.2). Data analysis pointed out that the unigenes differentially expressed resulted to be 90 (43 induced and 47 repressed) and 9 (3 induced and 6 repressed) in 24AIRvsT0 and 24MCPvsT0, respectively. In 24MCPvs24AIR comparison, 53 and 49 showed greater and lower transcript accumulation, respectively, in treated fruit. This clearly indicates that the presence of 1-MCP in the atmosphere surrounding nectarine fruit is effective in altering the expression of specific genes resulting in a block of the ripening process. Of the three targets up-regulated by 1-MCP in comparison with samples at harvest (T0), one putatively corresponds to a sucrose synthase gene, and one of the six repressed genes refers to an auxin-induced protein.

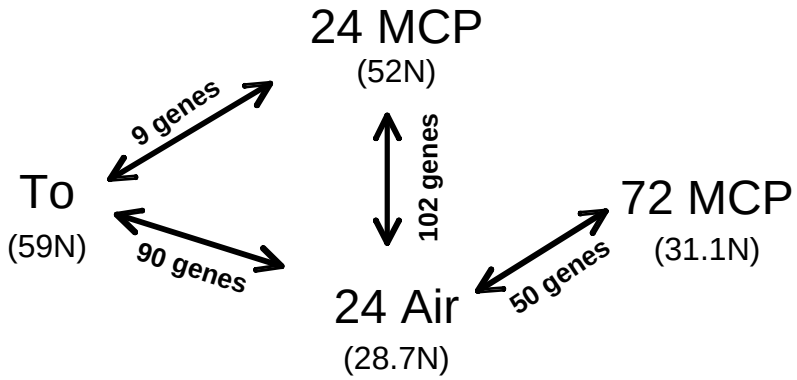

Figure 2 - Experimental design of microarray experiments. In the simple loop, 6 slides (12 technical replicates) were used for the comparison 24MCP/T0 and three (six technical replicates) for 24AIR/T0 and 24 MCP/24 AIR. Dye flip was carried out for each comparison (three for 24MCP/T0 and one for 24AIR/T0 and 24 MCP/24 AIR). For the direct comparison 72MCP/24 AIR, three slides (six replicates) were used including dye flip. For each comparison the number of genes showing differential expression, identified by SAM analysis, is reported. For each sampling date flesh firmness value is reported.

Genes differentially expressed in the comparison 24MCPvs24AIR (reference comparison) are reported in Tables 2 and 3. Considering targets showing greater transcript accumulation in 1-MCP fruit at the end of the incubation period (Tab. 2), three are involved in auxin metabolism (contigs 57, 1741, 2655), and two correspond to genes related to cell wall metabolism (PpEXP2 - ctg 941 and endo-1,4 mannosidase - ctg 1954). Higher transcript levels of contigs 1024 and 5424, corresponding to a catalase and lipoxygenase, respectively, and contigs 1751 and 1759, representing two glucose acyltransferases have been also observed at the end of 1-MCP treatment. Out of the 53 genes more expressed in 1-MCP treated fruit, 51 hybridized also in 24AIRvsT0 comparison microarray, 39 of them (76.5 %) resulting down-regulated (Tab. 2).

Among the 49 targets showing lower transcript accumulation in 24MCPvs24AIR comparison, several cell wall-related genes are present (Tab. 3): PG (ctg 420), a pectin acetylesterase precursor (ctg 1816), a putative pectin methylesterase (ctg 4533), and *PpEXP3* (ctg 676). Genes related to quality parameters as color and flavor development, and sugar metabolism are also included in this list: beta-carotene hydroxylase (ctg 711), omega-6 fatty acid desaturase (ctg 835), pyruvate decarboxylase (ctg 112), sucrose synthase (ctg 61), glucose acyltransferase (ctg 1751), putative invertase inhibitor (ctg 4499), and trehalose-6-phosphate phosphatase, TPPA (ctg 4621). Considering hormone metabolism,

besides two auxin-related genes (IAA16 protein, ctg768, and ARF6, ctg 1991) and nine-cis-epoxycarotenoid dioxygenase2 (ctg 2980), involved in ABA biosynthesis, a number of genes with a role in ethylene production, perception and signal transduction resulted to accumulate lower transcripts in 1-MCP treated fruit: *ACO* (ctg 64), ethylene receptor *ETR2* (ctg 4109), *ERF1* (ctg 3350), *ERF2* (ctg 2116), and *EREBP* (ctg 2757). Two other transcription factors, MADS-box homolog (ctg 1511), and HD-ZIP protein (ctg 2443) were identified. In addition, two targets corresponding to pathogenesis related proteins (ctg 4524 and 1026) and one ripening-related protein (ctg 938) are present. Of the 49 genes showing lower transcript accumulation in fruit at the end of 1-MCP incubation period, 40 hybridized in 24AIRvsT0 comparison microarray, 28 of them (70%) resulting up-regulated (Tab.3).

As reported in fig. 1, after transferring fruit to air at the end of the 24h-incubation period with 1-MCP, a rapid drop of firmness occurred 72h after harvest (48h from the end of 1-MCP treatment). This was accompanied by an increase of ethylene biosynthesis. In order to elucidate at molecular level this behavior, microarray hybridization design has been implemented performing one additional comparison: 72MCP (31.1 N flesh firmness) vs 24AIR (28.7 N flesh firmness). Of the 53 targets showing greater transcript accumulation in 24MCPvs24AIR comparison, 10 targets appeared to be expressed at similar level in 72MCP and 24AIR samples indicating a decreasing transcription activity during the post-treatment phase, whereas 36 targets (68%) still displayed a higher transcript accumulation after 48h from the end of 1-MCP treatment (Tab. 2). Among these targets, catalase (ctg 1024) has been selected to validate, via qRT-PCR the microrray hybridization: as reported in fig. 3A, specific expression analysis confirmed microarray data showing that 1-MCP induces an increased expression of this gene at the end of the incubation period and 48h later.

Considering the 49 targets showing a reduced transcript accumulation at the end of the 24h-treatment period, only 14 still maintained a reduced transcript level in the 72MCPvs24AIR comparison, whereas three targets (ctg 1511, 1512, 3924) showed a greater transcript level in treated fruit and for 26 no difference was observed. This clearly indicates that most of the targets negatively affected by 1-MCP during the 24h-incubation period recover their expression level within 48h after the end of the treatment.

More in detail, *PG* expression showed a recovery trend after 48h in 1-MCP treated fruit. Similarly, putative *PME* (ctg 4533) and pectin acetylesterase precursor (ctg 1816) displayed an increased expression during the 48h post-treatment phase, while *PpEXP3* (ctg 676) still showed a lower transcript accumulation.

Beta-carotene hydroxylase (ctg 711) and omega-6 fatty acid desaturase (ctg 835) showed a marked increase of expression at 48h from the end of the treatment (no difference in 72MCPvs24AIR comparison), whereas Pyruvate decarboxylase (ctg 112), putative invertase inhibitor (ctg 4499), trehalose-6-phosphate phosphatase, TPPA (ctg 4621) and, as a trend, Sucrose synthase (ctg 61) maintained the difference with control, in terms of expression ratio, during the post treatment phase. The two auxin metabolism-related genes (IAA16 protein - ctg 768, and ARF6 – ctg 1991), and the nine-cis-epoxycarotenoid dioxygenase2 (ctg 2980), repressed at the end of the incubation period, showed an increased expression at 48h as well as *ERF2* (ctg2116), and *ETR2* (ctg 4109). Even though *ACO* (ctg 64) displayed a recovery trend of expression, difference between 72MCP and 24AIR remained statistically significative.

MADS-box homolog (ctg 1511), together with two other targets (ctg 1512 encoding for CIP7, and ctg 3924, unknown protein), displaying lower transcript accumulation at the end of the incubation period, showed greater transcript level when comparing 72MCPvs24AIR.

The validation of microarray data via qRT-PCR was performed for contigs 4109 (ethylene receptor ETR2), 2757 (EREBP), 768 (IAA16 protein), 711 (beta-carotene hydroxylase) and 4621 (trehalose-6-phosphate phosphatase, TPPA) (Fig. 3B,C,D,E, F). Specific expression analyses confirmed, for all the considered genes, that a reduced expression, in comparison to control fruit, occurs at the end of the 1-MCP incubation period and, with the exception of contig 2757 (EREBP), an increasing pattern of specific mRNA accumulation is present in control fruit during the 24h period after harvest (24AIRvsT0). Furthermore, qRT-PCR data of these genes demonstrated that, in 1-MCP treated fruit, the reduced expression occurring at the end of the 24h incubation period is followed by a significative recovery of specific transcript accumulation.

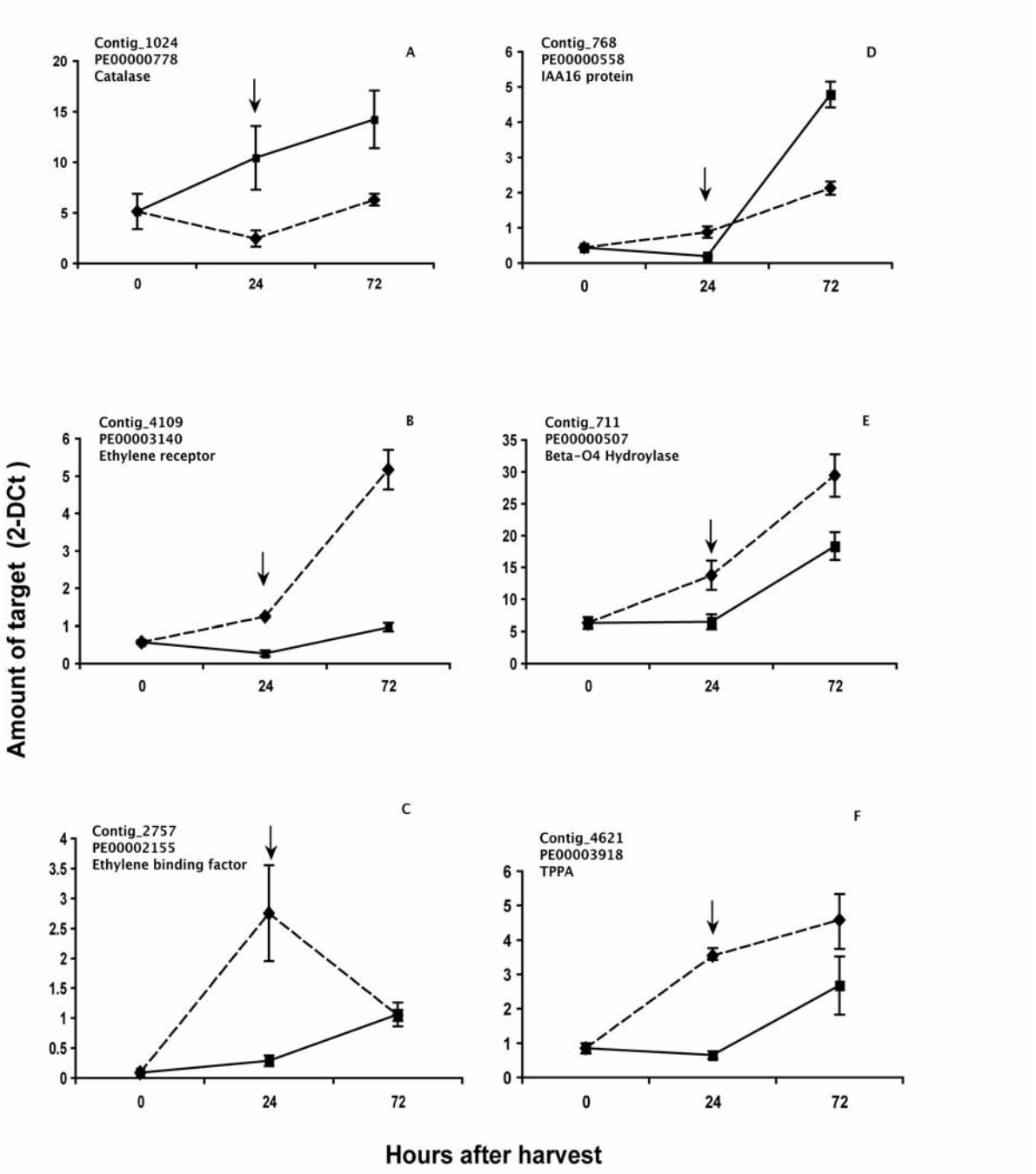

Figure 3 - Relative expression profiles of some peach genes showing lower (A, catalase) and higher (B, *Pp-ETR2like*; C, EREBP; D, IAA16 protein; E, beta carotene hydroxylase; F, TPPA) transcript accumulation in the 24MCPvs24AIR comparison microarray. Continued line indicates 1-MCP treated fruit and dotted line control fruit. Bars represent the standard error of three independent replicates. Arrows indicate the end of 1-MCP treatment.

Table 2. Genes listed in this table have been identified by SAM analysis performed as described in material and methods. Ctg_name is the number of the Contig sequence present in the database and used to synthesize the oligo probes for the μPEACH1.0 microarray. "Oligo ID" is the code assigned to each probe (4806 in total) by the manufacturer (Operon). In the last two columns first column the best BLASTX results and corresponding e-value, respectively, obtained from the comparison of peach sequences against Arabidopsis proteome database is reported. Central columns report the microarray experiment results: 24MCPvs24AIR (reference comparison, all genes showing a $\log_2$ ratio >1) reports [$\log_2$(intensity 24MCP/intensity 24AIR)]; 24AIR/T0 reports $\log_2$ [(intensity 24AIR/intensity T0)]; 72MCP/24AIR reports [$\log_2$(intensity 72MCP/intensity 24AIR)]. Black background color is used to highlight up-regulated genes or those with a higher mRNA accumulation ($\log_2$ ratio ≥1) for the specific comparison; shaded background is used for down-regulated genes or those with a lower mRNA accumulation ($\log_2$ ratio ≤-1) for the specific comparison and white background for genes showing no differential expression ($\log_2$ ratio > -1 and < 1). Empty cells mark targets resulting not significative at SAM analysis

ctg_name	Oligo ID	24MCP/ 24AIR	24AIR/T0	72MCP/ 24AIR	BLAST result	At protein	E-value
1751	PE00001351	2.48608	-2.13461	2.97227	Glucose acyltransferase.[Lycopersicon pennellii]	At1g73270	1E-123
441	PE00000326	2.32187	-2.63905	0.92700	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	At1g62510	6E-24
3563	PE00002895	2.19454	-2.06402	3.47534	T1G11.19 protein [Arabidopsis thaliana]	At1g04560	4E-61
941	PE00000708	2.18339	-2.18919	2.62603	Expansin.(PPEXP2) [Prunus persica]	At1g69530	2E-97
1307	PE00001002	2.12181	-1.12171		nitrate transporter NRT1-2 [Glycine max]	At1g18880	8E-64
134	PE00000090	1.97036	-2.26418	3.39761	no hits found		
1954	PE00001507	1.96532	-2.22593	3.63121	Mannan endo-1,4-beta-mannosidase.[Arabidopsis thaliana]	At5g66460	1E-127
1589	PE00001232	1.74255	-0.86376	1.56672	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (EC 4.1.1.39) [Prunus persica]	AtCg00490	0
2370	PE00001828	1.68071	-2.49635	0.74714	Histidine-containing phosphotransfer protein.[Catharanthus roseus]	At3g21510	3E-48
3203	PE00002559	1.64903	-1.62055	3.27616	Photosystem II 10 kDa polypeptide, chloroplast precursor [Solanum tuberosum]	At1g79040	1E-39
62	PE00000031	1.64220	-1.84389	2.67826	protease-related [Arabidopsis thaliana]	At3g02720	5E-70
2468	PE00001909	1.61848	-1.76022	1.65775	nitrate transporter [Arabidopsis thaliana]	At3g21670	1E-98
1939	PE00001496	1.57743	-1.46425		unknown protein [Arabidopsis thaliana]	At5g44680	1E-47
57	PE00000027	1.57657		1.73867	Auxin-induced protein AUX28.[Glycine max]	At3g04730	8E-73
56	PE00000026	1.53395		1.98408	no hits found		
3293	PE00002645	1.50919	-1.26809	1.73156	homeobox-leucine zipper protein 4 (HAT4) [Arabidopsis thaliana]	At4g16780	4E-09
2123	PE00001639	1.47905	-1.26063	2.18613	CG15019-PA [Drosophila melanogaster]	At2g36540	5.7

ctg_name	Oligo ID	24MCP/ 24AIR	24AIR/T0	72MCP/ 24AIR	BLAST result	At protein	E-value
1993	PE00001536	1.44599	-1.08569	1.32524	auxin-regulated protein GH3 homolog At2g23170 - Arabidopsis thaliana	At2g23170	1E-33
3891	PE00003203	1.41906	-0.91238	1.06074	isoamylase isoform 3 [Solanum tuberosum	At4g09020	2E-74
376	PE00000284	1.39473	-0.81046		receptor protein kinase-related protein [Arabidopsis thaliana]	At4g08850	7E-42
2458	PE00001901	1.38862	-1.27118	1.90194	Putative epimerase/dehydratase [Oryza sativa	At5g28840	0
1024	PE00000778	1.37644	-1.51061	1.07469	Catalase (EC 1.11.1.6).[Prunus persica] (cat2)	At4g35090	0
641	PE00000461	1.35023	-0.86755	1.38843	Malate dehydrogenase (EC1.1.1.40) (Malic enzyme).[Vitis vinifera]	At2g19900	0
1883	PE00001453	1.32938	-1.28195	2.21702	unknown protein [Arabidopsis thaliana]	At4g26850	9E-61
2655	PE00002067	1.32428	-0.60168		auxin-induced protein, putative [Arabidopsis thaliana	At2g21220	3E-44
3228	PE00002583	1.31157	-0.99715	1.24814	ATP synthase beta subunit [Prunus persica	AtCg00480	0
1584	PE00001228	1.30979	-1.09426	2.09853	Vacuolar processing enzyme precursor (EC 3.4.22.-) (VPE).[Citrus sinensis]	At4g32940	1E-160
31	PE00000004	1.30387	-1.50052	0.89153	glyoxalase family protein [Arabidopsis thaliana]	At1g15380	1E-41
1741	PE00001346	1.30031	-0.96260	1.41704	Aux/IAA protein.[Populus tremula x Populus tremuloides]	At2g33310	6E-50
2139	PE00001652	1.29306	-1.55057	2.78917	unknown protein [Arabidopsis thaliana	At2g28305	7E-87
349	PE00000265	1.29091	-1.34388	-0.34335	plasma membrane intrinsic protein 1c [Arabidopsis thaliana]	At2g16850	1E-101
5424	PE00004713	1.25897	-1.43392	2.76681	lipoxygenase [Nicotiana attenuata]	At1g72520	1E-40
5456	PE00004745	1.23616	-1.91927	2.68123	photosystem I reaction center subunit PSI-N precursor (PSI-N)[Arabidopsis thaliana]	At5g64040	3E-45
2585	PE00002010	1.19313	-1.39602	-1.22159	acid phosphatase -related [Arabidopsis thaliana]	At1g04040	1E-65
2741	PE00002144	1.18333	-1.30190	-0.04982	unknown protein [Arabidopsis thaliana]	At5g16550	3E-08
3865	PE00003177	1.17542	-1.32488	2.84985	psbA PSII 32 KDa protein	AtCg00020	0
243	PE00000191	1.17448	-1.09907	1.84518	no hits found		
1515	PE00001179	1.16035	-1.21708	1.42423	Sodium-dicarboxylate cotransporter-like.[Arabidopsis thaliana]	At5g47560	1E-121
4569	PE00003866	1.12247	-1.03102	1.36360	ribosomal protein S3 [Atropa belladonna]	AtCg00800	3E-20
396	PE00000297	1.10311	-1.03986	1.50392	zinc finger (AN1-like) family protein	At1g51200	1E-52
2117	PE00001635	1.09108	-0.54953	-0.14856	2-oxoglutarate-dependent dioxygenase, putative similar to 2A6 (GI:599622) and tomato ethylene synthesis regulatory protein E8	At1g06620	1E-69
4134	PE00003435	1.08444	-1.29689	0.77317	cation diffusion facilitator 8 [Stylosanthes hamata]	At3g58060	5E-59
975	PE00000737	1.08044	-0.91803		FLOWERING LOCUS T protein {Arabidopsis thaliana}	At1g65480	2E-30

ctg_name	Oligo ID	24MCP/24AIR	24AIR/T0	72MCP/24AIR	BLAST result	At protein	E-value
4905	PE00004199	1.07701	-1.09920	1.50747	signal peptidase subunit -related [Arabidopsis thaliana]	At1g15820	3E-64
1495	PE00001162	1.07338	-0.94925	2.15019	calcineurin-like phosphoesterase family [Arabidopsis thaliana]	At1g14700	1E-123
2061	PE00001589	1.06375	-0.59007	1.82264	Granule-bound glycogen synthase (EC 2.4.1.11).[Astragalus membranaceus]	At1g32900	0
3767	PE00003084	1.05047	-0.61235	1.05253	Aspartic protease precursor.[Lycopersicon esculentum]	At1g11910	1E-112
4403	PE00003702	1.04625	-0.33007		no hits found		
1679	PE00001301	1.03395	-0.45560		BRH1 RING finger protein [Arabidopsis thaliana]	At3g61460	2E-28
2081	PE00004801	1.02171	-1.06818	0.66656	unknown protein	At5g41050	2.8
2442	PE00001886	1.01898	-0.76480	1.60584	OSJNBa0060D06.16)OSJNBa0060D06.16 protein.[Oryza sativa]	At5g20900	3E-24
367	PE00000279	1.00756	-0.54879	0.24185	dehydration-responsive family protein similar to early-responsive to dehydration stress ERD3 protein [Arabidopsis thaliana]	At4g10440	3E-95
1759	PE00001356	1.00337	-0.33126	1.36599	glucose acyltransferase [Solanum berthaultii]	At1g33540	2E-69

Table 3. Genes listed in this table have been identified by SAM analysis performed as described in material and methods. Ctg name is the number of the Contig sequence present in the database and used to synthesize the oligo probes for the μPEACH1.0 microarray. "Oligo ID" is the code assigned to each probe (4806 in total) by the manufacturer (Operon). In the last two columns first column the best BLASTX results and corresponding e-value, respectively, obtained from the comparison of peach sequences against Arabidopsis proteome database is reported. Central columns report the microarray experiment results: 24MCPvs24AIR (reference comparison, all genes showing a $\log_2$ ratio >1) reports [$\log_2$(intensity 24MCP/intensity 24AIR)]; 24AIR/T0 reports $\log_2$ [(intensity 24AIR/intensity T0)]; 72MCP/24AIR reports [$\log_2$(intensity 72MCP/intensity 24AIR)]. Black background color is used to highlight up-regulated genes or those with a higher mRNA accumulation ($\log_2$ ratio ≥1) for the specific comparison; shaded background is used for down-regulated genes or those with a lower mRNA accumulation ($\log_2$ ratio ≤-1) for the specific comparison and white background for genes showing no differential expression ($\log_2$ ratio > -1 and < 1). Empty cells mark targets resulting not significative at SAM analysis

Ctg_name	oligo ID	24MCP/24 AIR	24AIR/T0	72MCP/24 AIR	At protein	BLAST result	E-value
420	PE00000317	-4.37352	4.23804	-1.00102	Endopolygalacturonase.[Prunus persica]	At3g59850	1.E-153
1026	PE00000780	-4.29865	3.80277		Pathogenesis-related protein PR-4B precursor.[Nicotiana tabacum]	At3g04720	2.E-53
4499	PE00003797	-3.53222	3.02050	-1.65408	putative invertase inhibitor [Cicer arietinum]	At1g47960	2.E-08
4524	PE00003821	-2.48250	2.01775	-0.82780	pathogenesis-related protein, putative [Arabidopsis thaliana]	At2g14580	1.E-31
938	PE00000706	-2.42560	3.22159	0.09601	Ripening-related protein-like.[Arabidopsis thaliana]	At5g51520	1.E-38
1069	PE00000816	-2.25271	1.17915		Thaumatin-like protein 1 precursor (PpAZ44)	At1g20030	2.E-80
835	PE00000609	-2.15108	1.43694	0.12239	omega-6 fatty acid desaturase [Prunus armeniaca]	At3g12120	2.E-57
1944	PE00001499	-2.14279	1.63277	-4.04068	PS60 protein precursor.[Nicotiana tabacum]	At1g76160	0
64	PE00000033	-2.09306	2.30755	-1.68166	1-aminocyclopropane-1-carboxylate oxidase .[Prunus persica]	At1g05010	1.E-123
560	PE00000424	-2.04428	0.87890	-0.21382	Cystatin-like protein.[Arabidopsis thaliana]	At5g47550	5.E-18
2980	PE00002356	-1.95666	2.10189	-0.20754	nine-cis-epoxycarotenoid dioxygenase2 [Pisum sativum	At1g78390	3.E-14
1511	PE00004799	-1.75300		3.17878	MADS-box homolog Umc1 [Ustilago maydis]	At1g47655	1.E+00
1816	PE00001398	-1.71404			pectin acetylesterase (EC 3.1.1.-) precursor - mung bean	At4g19420	1.E-24
1512	PE00001176	-1.70648		2.92962	No hits found		
4109	PE00003410	-1.65255	1.03576		ethylene receptor [Fragaria x ananassa]	At3g04580	5.E-55
2722	PE00002127	-1.63192		0.10640	probable UDP-glucuronosyltransferase (EC 2.4.1.-) - garden pea	At1g22370	7.E-42
2775	PE00002168	-1.58357	0.76256	-0.22742	Putative serine protease.[Populus euramericana]	At5g67360	6.E-33
1112	PE00000851	-1.57954		-0.57475	unknown protein [Arabidopsis thaliana]	At1g09310	1.E-48
768	PE00000558	-1.55903	1.28230	0.84585	IAA16 protein.[Gossypium hirsutum]\IAA 7 Arabidopsis	At3g23050	1.E-40

Ctg_name	oligo ID	24MCP/24 AIR	24AIR/T0	72MCP/24 AIR	At protein	BLAST result	E-value
2942	PE00002322	-1.54966	0.02843	0.09023	unknown [Arabidopsis thaliana]	At4g13195	2.E-08
2757	PE00002155	-1.53584	0.44052	-1.05855	ethylene-responsive element binding protein [Nicotiana tabacum]	At4g17500	2.E-06
112	PE00000071	-1.52474	1.41591	-1.08092	Pyruvate decarboxylase.[Fragaria ananassa]	At5g01330	1.E-110
4621	PE00003918	-1.50679	1.47776	-0.73047	trehalose-6-phosphate phosphatase (TPPA) [Arabidopsis thaliana	At5g51460	2.E-51
1752	PE00001352	-1.49532	1.52808	-0.13648	Glucose acyltransferase.[Lycopersicon pennellii]	At1g33540	3.E-78
4533	PE00003830	-1.47433	1.96209	-0.06092	putative pectin methylesterase [Oryza sativa (japonica	At1g69940	9.E-20
408	PE00000306	-1.45475	0.99946		pollen specific actin-depolymerizing factor 2 [Nicotiana tabacum]	At4g25590	9.E-47
676	PE00000481	-1.40707	1.74910	-1.46407	expansin [Prunus persica]	At2g28950	1.E-111
3945	PE00003257	-1.38259	0.84016	-1.10814	subtilisin-like proteinase (EC 3.4.21.-) 1 - tomato	At5g67360	2.E-64
1643	PE00001274	-1.37146	1.22335	-0.31456	hsr201 protein, hypersensitivity-related - common tobacco	At5g17540	2.E-62
1447	PE00001122	-1.32957	0.84684	-1.40002	unknown protein [Arabidopsis thaliana]	At5g40450	4.E-09
3592	PE00002920	-1.32527	0.87559	-2.00671	Adenosine 5'-phosphosulfate reductase.[Glycine max]	At4g21990	1.E-176
2443	PE00001887	-1.31164	0.88673	-0.79568	Homeobox-leucine zipper protein HAT22 (HD-ZIP protein 22) [Arabidopsis thaliana	At4g37790	1.E-26
3350	PE00002701	-1.27998	1.25259		ethylene response factor 1 [Lycopersicon esculentum]	At5g07580	7.E-08
3924	PE00003236	-1.26252			unknown protein [Arabidopsis thaliana	At1g24575	2.E-04
4271	PE00003572	-1.25082	1.00408	-0.76758	No hits found		
246	PE00000194	-1.21523	1.21624	-1.45841	P0468B07.6 [Oryza sativa (japonica cultivar-group)]	At3g56710	9.E-04
4772	PE00004067	-1.21231	1.74881	-0.99001	No hits found		
707	PE00000504	-1.15816	2.05203	-1.36610	No hits found		
1991	PE00001534	-1.14539	0.94349	-0.36732	auxin response transcription factor(ARF6) [Arabidopsis thaliana	At1g30330	2.E-39
5374	PE00004663	-1.11498	0.61486	0.72824	translation initiation factor [Triticum aestivum]	At5g54940	3.E-14
3300	PE00002652	-1.11154	0.90651	-0.21474	unknown protein [Arabidopsis thaliana	At4g09150	1.E-47
711	PE00000507	-1.08739	1.24500	-0.28684	beta-carotene hydroxylase [Citrus unshiu]	At4g25700	1.E-104
2403	PE00001854	-1.08101	0.60591	-0.05177	F19K19.6 protein - Arabidopsis thaliana	At1g16650	7.E-47
1978	PE00001525	-1.06880		0.64429	putative serine/threonine protein kinase [Nicotiana tabacum	At1g60940	1.E-65
61	PE00000030	-1.03221	0.96423	-0.93274	Sucrose synthase.[Citrus unshiu]	At5g20830	0
2659	PE00002070	-1.03049		-1.03032	enolase [Ricinus communis	At2g36530	6.E-70
458	PE00000332	-1.01496		0.60204	ate embryogenesis abundant 3 family protein [Arabidopsis thaliana]	At4g02380	1.E-12
2116	PE00001634	-1.00992		-0.54829	ethylene response factor 2 [Lycopersicon esculentum	At2g47520	5.E-25
2424	PE00001871	-1.00896	0.96654	-1.06485	receptor protein kinase-like protein [Capsicum annuum	At3g51550	1.E-45

Discussion and conclusions

The role of ethylene in modulating fruit ripening through the regulation of gene expression is now well established starting from the early works, in the model species tomato, using the antisense technology targeted to inhibit ethylene biosynthesis (Oeller *et al.*, 1991, Picton *et al.*, 1993) up to the recent genomics approaches aimed to describe transcript profiles of tomato mutants altered in their ability to perceive ethylene (Alba *et al.*, 2005). Mutation of the ethylene receptor Nr strongly affects ethylene sensitivity and the inhibition of ripening in this tomato mutant is accompanied by an altered expression of several hundred genes some of them belonging to two main distinct categories: sequence-specific DNA binding proteins and transcriptional coactivators (Alba *et al.*, 2005).

The discovery of 1-MCP as a powerful antagonist of ethylene action has provided exciting opportunities for postharvest scientists to gain insight into the fundamental processes that are involved in fruit ripening: besides commercial applications, the inhibition of ethylene perception by 1-MCP has been used to better describe the ethylene-mediated physiological responses of different fruit types (Watkins, 2006). This, in turn, may allow to understand the biological basis of the variable responses to 1-MCP postharvest applications. A paradigm of different responses to the chemical is represented by apples and peaches: the quick recovery of ripening parameters after 1-MCP treatment, makes the latter species an interesting model to study and characterize at molecular level with the aim of elucidating mechanisms responsible for this peculiar behaviour.

The large-scale analyses of transcriptome performed using μPEACH1.0 clearly indicate that the quick ripening evolution observed after transferring treated peaches to air is not due to a limited effect of 1-MCP during incubation. In fact, at the end of the 24h-treatment period, the maintenance of high firmness values (52.0 N) is accompanied by marked changes in transcript profiling if compared to control fruit (24AIR). Only 9 genes showed significative changes in 24MCPvsT0 microarray analysis compared to 90 targets displaying differential expression during 24h ripening in air (24AIRvsT0). This clearly indicates that the presence of 1-MCP, by altering ethylene perception, induces a block of fruit development and ripening: this physiological effect is confirmed by the 102 genes resulting differentially expressed when comparing 24MCP and 24AIR samples, and the fact that a

number of affected targets correspond to genes with a role in hormone (ethylene, but also auxin and ABA) metabolism and in regulating transcription. Considering that 76.5 and 70% of transcripts displaying greater and lower transcript accumulation, respectively, at the end of the 1-MCP incubation period show an opposite trend in ripening fruit kept in air (24AIRvsT0 comparison), it might be hypothesized that these targets correspond to ripening-related and ethylene-dependent genes.

The maintenance of high firmness values at the end of the incubation period is clearly the result of a down regulation of cell wall-related genes, involved, in particular, in pectin metabolism, as PG. Microarray data on PG confirm specific expression analysis (Ziliotto *et al.*, 2003; Dal Cin *et al.*, 2005), showing that the reduction of PG transcript accumulation at the end of 1-MCP treatment is followed by an expression recovery few hours thereafter, and further emphasize the crucial role played by PG in peach fruit softening as clearly demonstrated in recent works on Stony hard genotypes (Hayama *et al.*, 2006a and b; Begheldo *et al.*, 2008). One of the targets showing higher transcript level in 1-MCP treated fruit is *PpEXP2* and this would confirm that this gene, down-regulated by ethylene, is related to peach fruit growth and, differently from *PpEXP3* (ctg 676) that showed a reduced level of transcript accumulation in 1-MCP treated fruit, is not responsible for softening as reported by Hayama *et al.* (2001; 2003). Catalase appeared among genes with higher transcript level in 1-MCP treated fruit at the end of the incubation period and an increasing expression trend has been confirmed by qRT-PCR analysis. In other fruit species, catalase activity decreases during ripening, as observed in orange (Huang *et al.*, 2007), and increases following 1-MCP treatment, as reported by Fu *et al.* (2007) in Yali pears indicating that some of the changes in enzymatic antioxidant potential occurring at ripening are likely influenced by ethylene (Vilaplana *et al.*, 2006).

When evaluating the list of targets showing lower transcript accumulation at the end of the 1-MCP incubation period, it is interesting to notice that, of the two elements involved in the last ethylene biosynthetic steps, only *ACO* is present whereas *ACS* does not appear to be negatively affected by 1-MCP. In addition, and considering ethylene receptors, only *ETR2*, but not *ETR1* and *ERS1*, appears to accumulate lower transcript levels at the end of the incubation period. These data, confirm results published by Dal Cin *et al.* (2006) showing that *ACS*, *ETR1* and *ERS1* expressions are not negatively affected by 1-MCP in peaches,

and suggest that *ETR2*, induced during the transition pre-climacteric climacteric stage (Trainotti *et al.*, 2006a), seems to play a crucial role in ripening physiology of peaches. This hypothesis is reinforced by the fact that, in ripening peaches, a great inductive effect on ETR2 is yielded by exogenous ethylene treatment (Trainotti *et al.*, 2007) and this is confirmed by qRT-PCR analysis reported in Fig. 3.

As pointed out by Mathooko *et al.* (2001) and Dal Cin *et al.* (2006) *ACS* expression and activity are not down-regulated by 1-MCP. Considering that *ACO* gene expression and activity appear only slightly reduced as reported by our microarray data and by Mathooko *et al.* (2001), the result is that ethylene production is not inhibited by 1-MCP (Fig.1). Considering that in apples, where 1-MCP applications are highly effective in delaying ripening, *ACO* gene expression is, similarly to peaches, only slightly affected by the ethylene antagonist (Dal Cin *et al.*, 2006), the differential effect of 1-MCP on ethylene biosynthesis occurs likely through *ACS*. In a recent paper, Trainotti *et al.* (2007) pointed out that many genes involved in biosynthesis, transport and signalling of auxin have an increased expression in peach mesocarp during ripening and some ripening-related genes, including *ACS* gene are more strongly influenced by exogenous auxin (NAA) than ethylene: this indicates that, besides an independent role played by auxins, an active cross-talk between auxin and ethylene modulates peach ripening This is confirmed by our microarray data showing an effect of 1-MCP on the expression of several auxin metabolism-related genes. If the limited effect of 1-MCP on delaying peach fruit ripening is the result of specific interplay between auxin and ethylene in the modulation of specific genes including *ACS* remains to be elucidated.

The fact (or the hypothesis) that 1-MCP irreversibly binds to the ethylene receptor justifies its effect during the incubation period and this occurs either in systems where ethylene production is blocked (e.g. apple) or not, as in peaches where, at the end of the incubation period the delay of ripening is the result of an altered transcription of specific genes (9 targets differentially expressed in 24MCPvsT0 compared to 90 targets differentially expressed in 24MCPvs24AIR). If ethylene production is not blocked and a continuous production (ethylene-dependent and ethylene-independent) of receptors occurs, a quick recovery of the ripening process takes place. This appears to be the case of peaches where the transcription of ethylene-independent *ETR1* and *ERS1* (Trainotti *et al.*, 2007) is not

affected by 1-MCP (Dal Cin *et al.*, 2006 and our microarray data) and ethylene-dependent ETR2, following the inhibition exerted by 1-MCP, is induced when peaches are transferred to air and the ethylene-related physiological machinery quickly recovers. In fact, the rapid evolution of ripening observed at 72h (48h from the end of 1-MCP treatment) is the result of the recovery of well recognized ethylene-dependent genes as *PG* (Sitrit *et al.* 1998) likely occurring through the induction of transcription factors as the EREBP (ctg 2757) (fig. 3), similar to *Le-EIL1*, that, when overexpressed, specifically induces *PG* transcription in Nr tomato fruit thus partially restoring the wild phenotype (Chen *et al.*, 2004). An other gene, beta-carotene hydroxylase (ctg 711), responsible for the last biosynthetic step of the main pigment of peach flesh, induced at the transition pre-climacteric/climacteric stage (Trainotti *et al.*, 2006a) and repressed at the end of the 1-MCP incubation period, appears to quickly recover during the post-treatment phase. As observed in orange fruit (Rodrigo and Zacarias , 2007), it might be hypothesized that beta-carotene hydroxylase, differently from other genes involved in peach carotenoid biosynthesis, is ethylene sensitive.

As above reported, our data support the hypothesis that peach ripening evolution is under control of an interplay between and auxin: our microarray analysis revealed that, in addition, a possible role might also be played by an interaction between ethylene and ABA, since ctg 2980 (nine-cis-epoxycarotenoid dioxygenase2), involved in ABA synthesis, shows a reduced level of transcript accumulation in 1-MCP treated fruit and a marked expression recovery during the post-treatment phase. Variable mechanisms and modulation of these hormonal interactions might represent the key factor to be further elucidate for understanding the different ripening and postharvest behaviour of climacteric fruit.

Chapter V

Preliminary Characterization of Peach Ripening Mutants by a Transcriptomics Approach

Abstract

Two peach (*Prunus persica* L. Batsch) ripening mutants, a Stony hard (SH) and Slow ripening (SR) genotypes together with a melting flesh nectarine (cv Fantasia) have been used in a large-scale transcriptomics analysis, using μPEACH 1.0 microarray for a comparative physiological study on the role of ethylene in fruit development and a more precise characterization of the two mutants both impaired in producing ethylene at ripening. Analysis of transcript profiling changes occurring during last developmental stages (preclimacteric and climacteric in Fantasia fruit) and in relation to exogenous treatment with the ethylene-analogue propylene allowed to correlate the SH and SR phenotypes to disturbances in ethylene physiology, more pronounced in SR and probably related to other hormones (auxin) in SH.

Keywords: propylene, stony hard, slow ripening, transcription factors.

Introduction

Although multiple hormones, including jasmonates, auxin, and brassinosteroids, have all been implicated in the modulation of fruit ripening (Given *et al.*, 1988; Symons *et al.*, 2006; Trainotti *et al.*, 2007; Ziosi *et al.*, 2008), ethylene is recognized as the key factor that regulates the phenotypic changes occurring at ripening in climacteric fruit. Treatment with inhibitors that block ethylene synthesis or action or the manipulation of these processes by natural or induced mutations revealed the essential role of this hormone in regulating fruit ripening (Barry and Giovannoni, 2007 and references therein). These strategies have been successful applied in tomato where a series of ripening mutants have been well characterized by using positional cloning or by genetic mapping of mutant loci and candidate genes. (http://www.tgrc.ucdavis.edu/, http://www.zamir.sgn.cornell.edu/mutants/). These include pleiotropic ripening mutations, such as *Colorless non-ripening (Cnr), ripening-inhibitor (rin) Never-ripe (Nr), Green-ripe (Gr)* characterized by an altered ripening phenotypes and a disturbance in ethylene biosynthesis, action or signal transduction (Giovannoni 2007). Ripening mutants are present also in other species, but their molecular characterization is more difficult in comparison to tomato for the reduced availability of genomics tools and information and

technical constraints. Considering tree species bearing climacteric fruit, ripening mutants due to disturbance in ethylene physiology have been identified in peach. The Stony hard (SH) is characterized by a lack of ethylene production and firm flesh in mature fruit (Hayama *et al.*, 2000; Haji *et al.*, 2003, 2004). The SH trait is controlled by a single recessive gene (hd) which is inherited independently of the Melting (MF/NMF) trait (Yoshida, 1976; Haji *et al.*, 2005). When the SH is combined with Melting, the fruit reduce their firmness through continuous exposure to ethylene (Hayama *et al.*, 2003; Haji *et al.*, 2005). Therefore, the softening process in SH peaches appears to be blocked by a lack of ethylene, and not by mutations of cell wall-modifying enzymes. A reduced expression of *PpACS1* (ACC synthase) is the cause of lack ethylene production in SH (Tatsuki *et al.*, 2006). Exogenous ethylene induces a rapid loss of firmness (Haji *et al.*, 2003), results of increases in both polygalacturonase (PG) gene expression and enzyme activity (Hayama *et al.*, 2006 a and b). Other ripening mutants in peach have been identified and named "slow ripening" (*SR*). They have been first described by Brecht *et al.* (1984) and are characterized by a block of fruit development at S3 stage (after pit hardening) and the flesh either never softens or softens very slowly, while it keeps a crispy texture. The skin ground color and flesh are greenish and the flavor is very poor, despite lower acidity and higher pH and soluble solids (Brecht *et al.*, 1984). SR fruits do not initiate autocatalytic ethylene production or the associated respiratory climacteric for at least one month after harvest, and their CO_2 and C_2H_4 productions peaks are reduced significantly, when stored at 20° (Brecht and Kader, 1984). Molecular information about these mutants are very poor. It has been reported that the trait is monogenic and recessive (Ramming, 1991) and the gene was described as closely located to the 'nectarine' locus (Chaparro *et al.*, 1994). A mutant phenotypically similar to SR type, named BO80004106 and originated from a free pollination of the cv Fantasia, has been identified at University of Bologna and preliminary analysis revealed a lack of ethylene evolution throughout development.

Considering these features these ripening mutants represent an ideal material to study the role of ethylene in peach fruit ripening also using highthroughput genomic tools as the first peach microarray μPEACH 1.0 (ESTree consortium, 2005).

Materials and methods

Plant material

Nectarine cv Fantasia and the mutant BO8004106 (kindly provided by Prof. Daniele Bassi and named SR hereafter) were grown at experimental farm Faculty of Agriculture of the University of Padova. Fruit growth of SR and Fantasia was determined by weekly measuring of diameter of 100 fruits starting from 33 until 110 days after full bloom (DAFB). The first derivative of the diameter was used to identify the different growth stages. Fantasia fruit were sampled at preclimacteric (S3) and climacteric (S4) stages. Due to the altered growth pattern and physiological processes, S3SR and S4SR fruits were harvested at the same time of Fantasia. SH fruit (cv IFF331, 'Hacuto' x 'New Jersey 256' provided by the Istituto Sperimentale per la Frutticoltura of Forlì, Italy) were harvested in correspondence of firmness values of about 35 N.

Preclimacteric fruit of Fantasia, the corresponding SR samples and SH fruit were treated with the propylene (500 ppm), an ethylene analogous, in humidified air stream (6 l/h) for 48 hr at 20°C.

For all samples ethylene production of 10 fruit has been determined using a gas chromatograph (DANI 3200). Flesh firmness was measured with a penetrometer (TR, Forlì, Italy) equipped with a 6 mm probe.

Mesocarp samples have been frozen in liquid nitrogen and stored at -80 °C.

Determination of Fruit Cell Number

The number of mesocarp cells of SR and Fantasia was estimated throughout fruit development. Measurements were performed according to the method described by Ognjanov *et al.*, (1995) and S1 and S3 stages by scanning electron microscopy. For this analysis, portions of fruit were fixed in 3% glutaraldehyde in 0.1M Cacodylate buffer, pH 7.4 at 4°C overnight. The second fixation was carried out using 1% osmium tetroxide (aqueous) pH 7.4 for 1 hour at room temperature and in a light tight container. Samples were dehydrated and then transferred to critical point drying apparatus for 40 min. Observations were carried out by using a Cambridge Stereoscan 250 at 15 KV available at Centro Universitario Grandi Apparecchiature Scientifiche (C.U.G.A.S.) of the University of Padova

RNA extraction

Total RNA was extracted using the protocol described by Ruperti *et al.* (2001). RNA yield and purity was checked by means of UV absorption spectra, whereas RNA integrity was ascertained by means of electrophoresis in agarose gels followed by ethidium bromide staining on the ribonucleic acid.

Microarray experimental design and hybridization protocol

The features and the preparation and the hybridization protocols of the peach microarray μPEACH 1.0 are described in Trainotti *et al.* (2006a). The experimental design to compare fantasia and SR fruit development was a direct comparison in which transcript profiles of fruit at same (S3Fantasia vs S3SR and S4Fantasia vs S4SR) at different (S3 vs S4 Fantasia and S3SR vs S4SR) developmental stages were analyzed. For the evaluation of propylene treatment, a direct comparison between control fruit (T0, before treatment) and treated fruit (FantasiaP, SRP and S3SHP at the end of 48 h-incubation period). Total RNA (20 μg) from mesocarp of fruit sampled was converted into target cDNA by reverse transcription using the SuperScriptTM Indirect cDNA Labeling System (Invitrogen, USA) following manufacturer instruction. The cDNA labeling was carried out as described by Trainotti *et al.* (2006a).

Data analysis

The microarrays were scanned as described by Trainotti *et al.* (2006a). TM4 (www.tm4.org) package developed at TIGR (www.tigr.org; Saeed *et al.*, 2003) was used to analyze microarray data. The images were processed using the Spotfinder 2.2.3. software by means of the Otsu algorithm. The expression data extracted by Spotfinder were normalized by MIDAS 2.18 using the LOWESS (Locally Weighted Regression Scatter Plot Smoothing; Cleveland, 1979) algorithm with the 'block mode', keeping as reference the Cy3 channel.

After normalization, data from each slide were split in two, by using Microsoft Excel, since each probe is spotted twice on μPEACH1.0. Thereafter, each spot value was considered to be independent.

Normalized split data were loaded in MeV 3.1 and for each comparison (at least three independent experiments) was imposed a 66% cut-off to select the gene set in which to

identify those differentially expressed by one-class unpaired SAM (Significance Analysis of Microarrays; Tusher *et al.*, 2001) analysis. Lists of clones with significant changes in expression (threshold ratios, expressed ad log2, higher than 1 and lower than -1 for up- and down-regulated, respectively) were identified at delta values corresponding to a false discovery rate (FDR) of 0%.

Quantification of mRNA via northern analysis and semiquantitative RT-PCR

To validate microarray data, transcript accumulation was evaluated via northern analysis for ACC oxidase (*ACO*), *PG*, Ctg_1288 (similar to *NtLIM* transcription factor) and Ctg_779 (similar to a cyclin D3 type) and via qRT-PCR for *PpACS1*. Northern analysis (10 µg RNA) was performed as described in Ruperti *et al.* (2001), while semiquantitative RT-PCR on gel was carried out for *PpACS1* using the following primers: forward 5-ACCGAGACTTGGGATGGAGA-3 and reverse 5-TGATCAAGCCCTTCACGTTG-3 (95 °C for 10 min followed by 25 cycles at 94 °C for 1 min, 61 °C for 20 sec , 72 °C for 1 min and a last cycle at 72 °C for 7 min). Three technical replicates for each sample were separated by electrophoresis in a 2% (w/v) agarose gel. For quantification, the integrated intensity of the PCR bands was corrected with the corresponding housekeeping gene (18S) data using the Scientific Imaging Systems software package 1D 3.6 (Kodak, USA)

Results

Fruit development of SR mutant

Comparison between growth curves of SR and Fantasia fruit (Fig. 1) indicates that marked difference in terms of diameter are present after pit hardening, indicated as Stage 2 (S2). In fact after this stage, recognizable for the lower growth rate in Fantasia fruit, SR fruit size had only limited increase showing, at time harvest of Fantasia fruit, a reduction of 29% in terms of diameter.

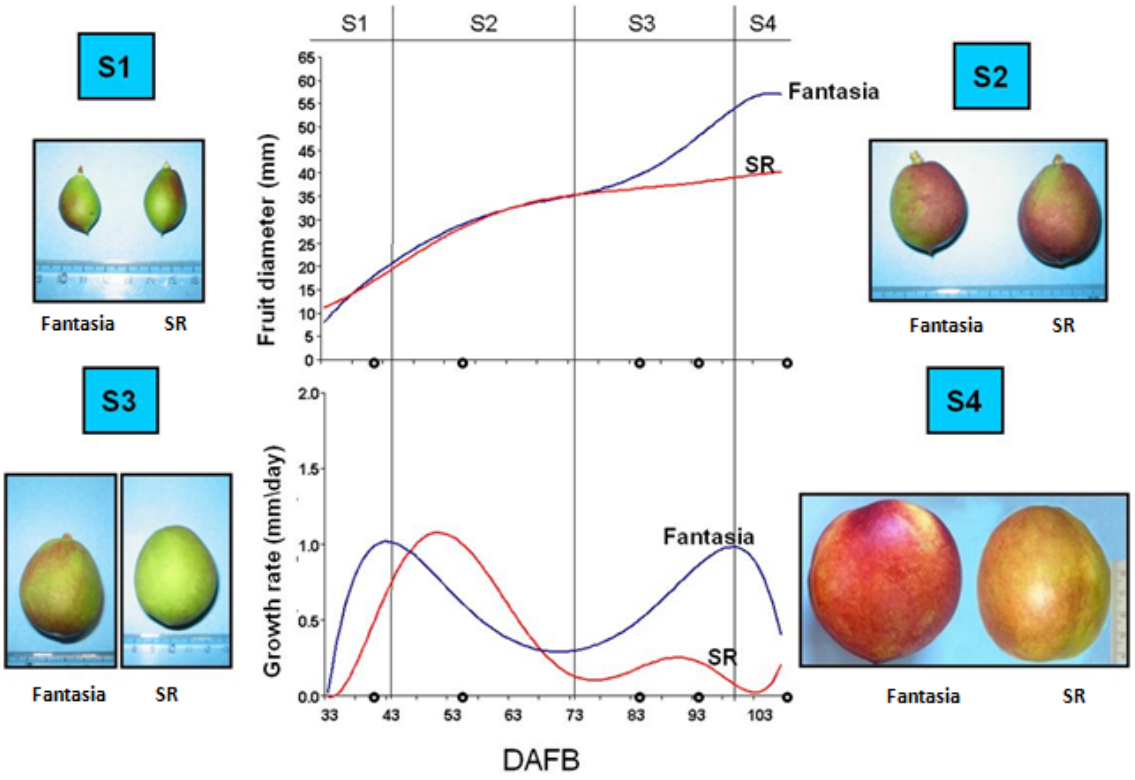

Figure 1 - Fantasia (blu line) and SR (red line) fruit growth curves based on diameter and its first derivative from 30 to 110 days after full bloom (DAFB). S1–S4 represent the four stages of growth up to harvest. Size and external appearance of Fantasia and SR fruit at 39 DAFB (S1), 55 DAFB (S2), 83 DAFB (S3) and 106 DAFB (S4) are shown in pictures.

This reduction is the result of a slower growth rate in S3 (second exponential growth phase due to cell enlargement) in comparison to Fantasia. These data were confirmed by SEM analysis (Fig. 2) that pointed out an increase of 46% in cell size (0.92 μm^2 vs 1.53 μm^2) during the transition from S1 to S3 in SR fruit. During the same period Fantasia fruit cell size showed an increase of 395% (3.95 μm^2 in S3 vs 0.99 μm^2 in S1). This difference was accompanied by a prolonged cell division activity in SR fruit that determined, at the end of S3, a number of cells more than double in comparison to Fantasia fruit ($290*10^6$ for SR vs $130*10^6$ for Fantasia). During the last phase (S4), when ripening processes occur in fantasia fruit, light changes in pigmentation of epicarp (Fig.1) and pulp were observed (see fruit pictures in Fig. 1) in SR fruit. In addition, and differently from Fantasia, SR fruit did not undergo any flesh firmness changes and no ethylene evolution was detected throughout S4 stage of Fantasia (early August) (data not shown).

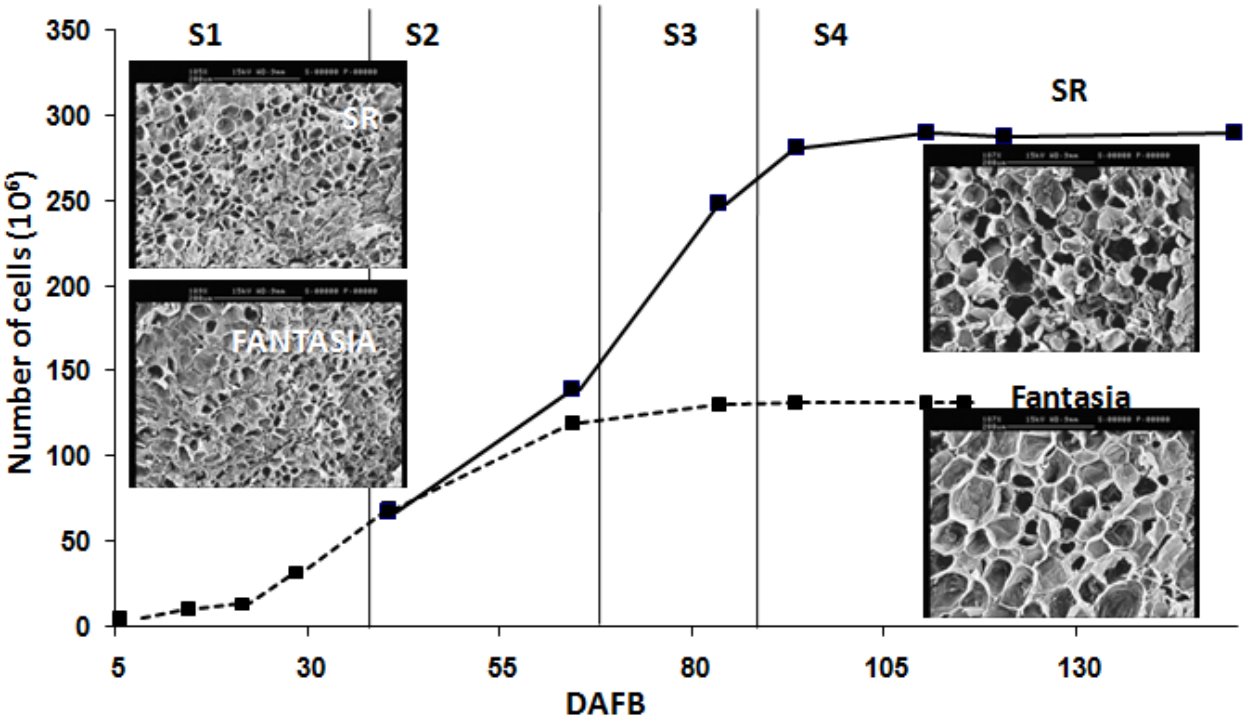

Figure 2 - Dynamics of cell number increase during Fantasia (dotted line) and SR (solid line) fruit growth. Scanning electron microscopy of Fantasia and SR mesocarp at S1 (x189 Fantasia; x 185 SR) and S3 (x185 Fantasia; x185 SR) stages.

These features (no firmness and no ethylene evolution, data no shown) were also registered in the following 8 weeks (up to early October). These differences between Fantasia and SR in terms of ethylene biosynthesis and firmness loss at ripening have been confirmed by expression analysis of *ACS* and *ACO*, involved in ethylene biosynthesis and *PG*, the main enzyme responsible for pectin depolymerization (Fig. 3). In SR fruit the expression of these three genes appeared to be reduced or inhibited throughout the growth cycle and, in particular, in correspondence of the ripening stage of Fantasia fruit characterized by a dramatic up-regulation of all these considered genes.

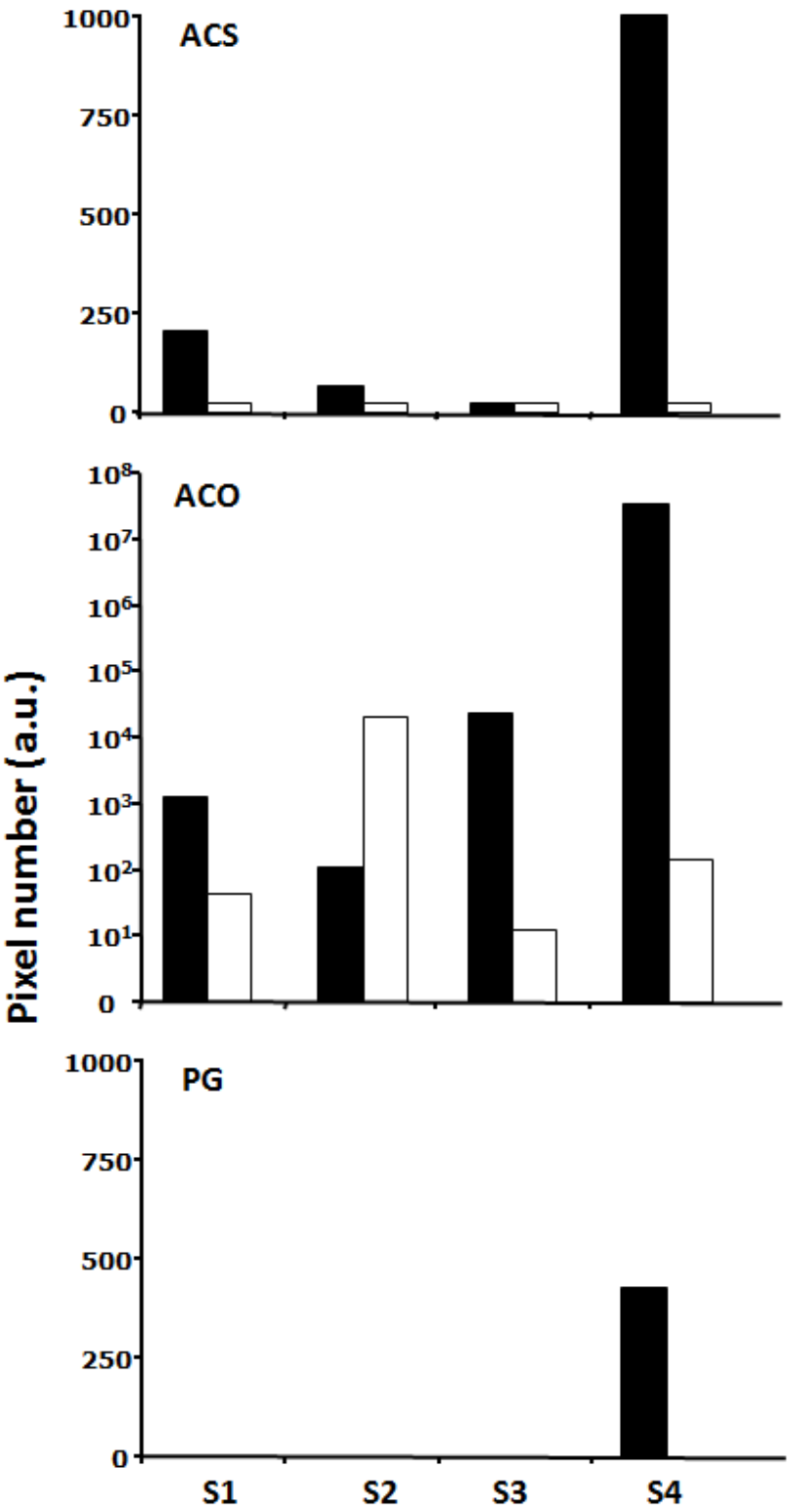

Figure 3 - Accumulation of ACS, ACO and PG transcripts throughout growth cycle in Fantasia (black bars) and SR (withe bars) fruit.

Transcript profiles comparison during the transition from preclimacteric to climacteric stage of Fantasia and in SR fruit

Total of 374 genes were differentially expressed during the transition from preclimacteric (S3, 93DAFB, 0.08 nl/gfw/h) to climacteric (S4, 103 DAFB, 18.6 nl/gfw/h) stage (S3 vs S4 Fantasia) Fantasia fruit, while by comparing SR fruit at the same dates only 54 genes showed changes in their expression (Fig. 4).

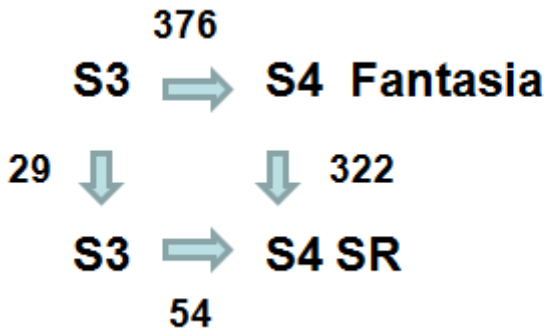

Figure 4 - Scheme of the microarray comparisons in Fantasia and SR fruit sampled in correspondence of preclimacteric (S3) and climacteric (S4) of Fantasia. The number of genes differentially in the specific comparisons are reported.

This difference was confirmed by comparing Fantasia and SR fruits at preclimacteric (S3 Fantasia vs S3 SR) and climacteric (S4 Fantasia vs S4 SR) stages: in fact, in the first comparison only 29 targets appeared to be differentially expressed while, in the second, 322 have been showing significant difference in terms of transcript level (Fig. 4). The majority (73.6%) of the up-regulated genes (195 of 265 genes) during the transition from S3 to S4 in Fantasia fruit resulted unchanged in the S4vsS3 SR comparison, in which only five showed a down-regulation such as Cgt_57 and 4591 encoding protein showing similarity to AUX/IAA (IAA16) protein and EIN2 of Arabidopsis, respectively (Tab. 1). When the expression level of targets resulting up-regulated in S4 vs S3 Fantasia was monitored comparing S4 Fantsia vs S4SR, 195 were more expressed in Fantasia (Tab.1): in particular genes involved in ethylene biosynthesis (ACO Ctg_64 and ACS Ctg_489) and perception and action (ETR2 Ctg_4109, EIN2 Ctg_4591) as well as in cell wall hydrolysis (PG Ctg_420, Pectinmethylesterase PME Ctg_938, Pectate lyase PL Ctg_1200, Expansin, EXP1 Ctg_939) and pulp pigmentation (Zeta carotene desaturase ZDS Ctg_2420 and lycopene β-cyclase Ctg_4052, β-hydroxylase β-OH4 Ctg_711) (Tab. 1).

Table 1 - Results of three microarray experiments. S4 vs S3 Fantasia, S4 vs S3 SR and S4 Fantasia vs S4 SR. This gene list is organized on the basis of the up-regulated genes in the S4 vs S3 Fantasia comparison (third column). The expression value is reported as $\log_2$ ratio of hybridization signals. Black background indicates up-regulation, shaded background indicates down-regulation and white background indicates no differential expression. Empty cells mark targets resulting not significative at SAM analysis. The "Ctg name " refers to the peach contig number in the database used to prepare the oligo probes of the μPEACH1.0 microarray. The "oligo ID" is the code assigned to each probe (4806 in total) by the manufacturer (Operon). At protein, Blastx results and e-value are those obtained by comparing peach sequences against Arabidopsis proteome (Trainotti *et al.*, 2006a).

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
3351	PE00002702	4.32819	0.10826	4.44860	At2g26670	heme oxygenase 1 (HO1) (HY1) identical to plastid heme oxygenase (HY1) [Arabidopsis thaliana] GI:4877362, heme oxygenase 1 [Arabidopsis thaliana] GI:4530591 GB:AF132475; annotation updated per Seth J. Davis at University of Wisconsin-Madison	2E-17
1993	PE00001536	4.23082	0.15804	4.03641	At2g23170	auxin-responsive GH3 family protein similar to auxin-responsive GH3 product [Glycine max] GI:18591; contains Pfam profile PF03321: GH3 auxin-responsive promoter	1E-33
938	PE00000706	3.95341	-0.19357	4.37086	At5g51520	invertase/pectin methylesterase inhibitor family protein low similarity to pectinesterase from Lycopersicon esculentum SP|Q43143, Arabidopsis thaliana SP|Q42534; contains Pfam profile PF04043: Plant invertase/pectin methylesterase inhibitor	1E-38
2895	PE00002280	3.94131	0.01993	4.10687	At2g26670	heme oxygenase 1 (HO1) (HY1) identical to plastid heme oxygenase (HY1) [Arabidopsis thaliana] GI:4877362, heme oxygenase 1 [Arabidopsis thaliana] GI:4530591 GB:AF132475; annotation updated per Seth J. Davis at University of Wisconsin-Madison	3E-69
1200	PE00000916	3.90169	-0.17506	3.91868	At4g24780	pectate lyase family protein similar to pectate lyase GP:14289169 from [Salix gilgiana]	0
64	PE00000033	3.77260	-0.09582	4.85327	At1g05010	1-aminocyclopropane-1-carboxylate oxidase / ACC oxidase / ethylene-forming enzyme (ACO) (EAT1) Identical to 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) gb|X66719 (EAT1). ESTs gb|T43073, gb|T5714, gb|R90435, gb|R44023, gb|AA597926, gb|AI099676, gb|AA650810 and gb|29725 come from this gene	1E-123

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
2980	PE00002356	3.72334		4.90846	At1g78390	9-cis-epoxycarotenoid dioxygenase, putative / neoxanthin cleavage enzyme, putative / carotenoid cleavage dioxygenase, putative similar to 9-cis-epoxycarotenoid dioxygenase [Phaseolus vulgaris][GI:6715257]; similar to neoxanthin cleavage enzyme GI:9857290 from [Vigna unguiculata]	3E-14
651	PE00000466	3.71874		3.82954	At3g21680	expressed protein	1E-09
950	PE00000717	3.70633		3.65920	At5g47650	MutT/nudix family protein similar to Nucleoside diphosphate-linked moiety X motif 6 (Protein GFG) from {Xenopus laevis} SP|P13420, {Homo sapiens} SP|P53370; contains Pfam profile PF00293: NUDIX domain	4E-79
544	PE00000409	3.69070	-0.06661	3.72023	At1g02400	gibberellin 2-oxidase, putative / GA2-oxidase, putative similar to GA2ox2 [GI:4678368]; similar to dioxygenase GI:1666096 from [Marah macrocarpus]; contains PF03171 2OG-Fe(II) oxygenase superfamily domain	2E-15
358	PE00000273	3.56375	-0.80534	3.24603	At5g65670	auxin-responsive protein / indoleacetic acid-induced protein 9 (IAA9) identical to SP|Q38827 {Arabidopsis thaliana}	6E-93
3295	PE00002647	3.49245	0.30162	1.54498	At5g48520	expressed protein similar to unknown protein (gb|AAB97010.1)	9E-26
1101	PE00000844	3.47122	0.11652	3.64481	At5g26600	expressed protein weak similarity to SP|P18549 Isopenicillin N epimerase (EC 5.-.-) {Streptomyces clavuligerus}	1E-161
1292	PE00000988	3.45154	0.31855	3.32754	At5g59845	gibberellin-regulated family protein similar to SP|P27057 GAST1 protein precursor {Lycopersicon esculentum}; contains Pfam profile PF02704: Gibberellin regulated protein	3E-28
3668	PE00002988	3.41059	-0.09945	3.45143	At5g17230	phytoene synthase (PSY) / geranylgeranyl-diphosphate geranylgeranyl transferase identical to GB:L25812; synonymous with geranylgeranyl-diphosphate geranylgeranyl transferase	3E-86
3709	PE00003029	3.33456		3.35019	No hits found		
528	PE00000397	3.31698	0.09079	3.72487	No hits found		
205	PE00000157	3.29917		4.24510	No hits found		
247	PE00000195	3.28573	-0.36966	3.20164	At1g30820	CTP synthase, putative / UTP--ammonia ligase, putative similar to SP|P17812 CTP synthase (EC 6.3.4.2) (UTP--ammonia ligase) {Homo sapiens}; contains Pfam profile PF00117: glutamine amidotransferase class-I; similar to ESTs gb|AA660762, gb|AA220982, dbj|AU008137, gb|AI054783, and gb|AA100804	1E-137
4772	PE00004067	3.27656	0.21654	2.65968	No hits found		
2691	PE00002100	3.19338	0.10648	4.13565	At1g17420	lipoxygenase, putative similar to lipoxygenase gi:1495804 [Solanum tuberosum], gi:1654140 [Lycopersicon esculentum]	2E-22
4560	PE00003857	3.18415	-0.26155	2.24820	No hits found		

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
4304	PE00003605	3.17081	-0.21067	2.10361	At1g17420	lipoxygenase, putative similar to lipoxygenase gi:1495804 [Solanum tuberosum], gi:1654140 [Lycopersicon esculentum]	2E-29
1853	PE00001430	3.16249	0.13510	2.50412	No hits found		
4529	PE00003826	3.14591		3.05071	No hits found		
3509	PE00002849	3.14480		2.59269	At5g54500	quinone reductase, putative similar to 1,4-benzoquinone reductase [Phanerochaete chrysosporium][GI:4454993]; contains flavodoxin domain PF00258	3E-91
67	PE00000035	3.13910		3.32863	At3g22840	chlorophyll A-B binding family protein / early light-induced protein (ELIP) identical to early light-induced protein; ELIP [Arabidopsis thaliana] GI:1872544; contains Pfam profile: PF00504 chlorophyll A-B binding protein; identical to cDNA early light-induced protein GI:1872543	2E-50
508	PE00000377	3.11218	-0.77919	2.49358	At1g26945	expressed protein supported by full length cDNA gi:26453215 from [Arabidopsis thaliana]	1E-29
112	PE00000071	3.10890	-0.25531	3.20104	At5g01330	pyruvate decarboxylase, putative strong similarity to pyruvate decarboxylase 1 [Vitis vinifera] GI:10732644; contains InterPro entry IPR000399: Pyruvate decarboxylase	1E-110
60	PE00000029	3.08837		4.97427	At5g65940	3-hydroxyisobutyryl-coenzyme A hydrolase / CoA-thioester hydrolase (CHY1) identical to gi:8572760; contains Pfam profile PF00388 enoyl-CoA hydratase/isomerase family protein	1E-113
1949	PE00001503	3.05477		2.54162	At2g24820	Rieske [2Fe-2S] domain-containing protein similar to Rieske iron-sulfur protein Tic55 from Pisum sativum [gi:2764524]; contains Pfam PF00355 Rieske [2Fe-2S] domain	1E-152
626	PE00000448	3.04096		2.69798	At4g22920	expressed protein	2E-80
4591	PE00003888	3.01400	-1.00060	2.60388	At5g03280	ethylene-insensitive 2 (EIN2) identical to EIN2 [Arabidopsis thaliana] gi|5231113|gb|AAD41076; member of the natural resistance-associated macrophage protein (NRAMP) metal transporter family, PMID:11500563; metal transport capacity has not been shown, PMID:11500563, PMID:1038174	2.3
1206	PE00000920	2.99754	-0.33688	1.88980	No hits found		
3300	PE00002652	2.98830	0.45750	3.32444	At4g09150	T-complex protein 11 contains Pfam PF05794: T-complex protein 11	1E-47
653	PE00000467	2.97881	0.16023	3.93052	At1g04680	pectate lyase family protein similar to pectate lyase GP:14531296 from [Fragaria x ananassa]	0
826	PE00000605	2.96989	0.62304	2.00782	At5g18650	zinc finger (C3HC4-type RING finger) family protein contains Pfam domain PF00097: Zinc finger, C3HC4 type (RING finger)	1E-143
57	PE00000027	2.90082	-1.24511	2.42151	At3g04730	auxin-responsive protein / indoleacetic acid-induced protein 16 (IAA16) identical to SP|O24407 Auxin-responsive protein IAA16 (Indoleacetic acid-induced protein 16) {Arabidopsis thaliana}	8E-73

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
2721	PE00002126	2.87364		3.46987	At1g22360	UDP-glucoronosyl/UDP-glucosyl transferase family protein contains Pfam profile: PF00201 UDP-glucoronosyl and UDP-glucosyl transferase	1E-109
1752	PE00001352	2.87126	0.28682	2.69820	At1g33540	serine carboxypeptidase S10 family protein similar to GI:8777303 from [Arabidopsis thaliana] (DNA Res. 7 (1), 31-63 (2000))	3E-78
786	PE00000571	2.85228	-0.33000	2.54265	At1g01490	heavy-metal-associated domain-containing protein contains Pfam profile PF00403: Heavy-metal-associated domain	3E-27
707	PE00000504	2.80225	0.25233	2.98150	No hits found		
3721	PE00003041	2.78501	-0.67963	2.55985	At1g73590	auxin efflux carrier protein, putative (PIN1) identical to putative auxin efflux carrier protein; AtPIN1 [Arabidopsis thaliana] GI:4151319; contains Pfam profile PF03547: Auxin Efflux Carrier	2E-81
1068	PE00000815	2.74128	0.23662	2.50782	At3g15540	auxin-responsive protein / indoleacetic acid-induced protein 19 (IAA19) identical to SP|O24409 Auxin-responsive protein IAA19 (Indoleacetic acid-induced protein 19) {Arabidopsis thaliana}	7E-55
69	PE00000037	2.72496	0.18634	2.90863	At2g27510	ferredoxin, putative similar to non-photosynthetic ferredoxin from Citrus sinensis [GI:1360725], Ferredoxin, root R-B2 from Raphanus sativus [SP|P14937]; contains Pfam profile PF00111 2Fe-2S iron-sulfur cluster binding domain	8E-47
3761	PE00003078	2.72197		2.96785	At5g01220	UDP-sulfoquinovose:DAG sulfoquinovosyltransferase / sulfolipid synthase (SQD2) identical to GI:20302857	1E-69
298	PE00000235	2.63986	-0.19647	2.56256	At4g34590	bZIP transcription factor family protein similar to common plant regulatory factor 7 GI:9650828 from [Petroselinum crispum]	5E-36
5227	PE00004518	2.62290		1.66783	No hits found		
780	PE00000565	2.56710	0.06275	2.38943	At3g16150	L-asparaginase, putative / L-asparagine amidohydrolase, putative similar to Swiss-Prot:P30364 L-asparaginase (EC 3.5.1.1) (L-asparagine amidohydrolase) [Lupinus angustifolius]	0.00001
4274	PE00003575	2.56645		1.98171	No hits found		
676	PE00000481	2.55324	-0.10149	3.06205	At2g28950	expansin, putative (EXP6) similar to expansin GI:2828241 from [Brassica napus]; contains Pfam profile PF01357: Pollen allergen	1E-111
3347	PE00002699	2.54721	-0.28674	2.34227	At5g02540	short-chain dehydrogenase/reductase (SDR) family protein contains INTERPRO family IPR002198 Short-chain dehydrogenase/reductase (SDR) superfamily	4E-46
4583	PE00003880	2.50705	0.45559	0.53050	No hits found		
2513	PE00001951	2.49666	-0.02031	1.83982	At2g43710	acyl-[acyl-carrier-protein] desaturase / stearoyl-ACP desaturase (SSI2) identical to gi:15149310; contains Pfam profile PF03405:	2E-91
3434	PE00002784	2.49163	0.24804	1.46848	At4g31130	expressed protein	7E-70

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
2726	PE00002130	2.47413	0.45039	2.36303	At5g66460	(1-4)-beta-mannan endohydrolase, putative similar to (1-4)-beta-mannan endohydrolase [Coffea arabica] GI:10178872; contains Pfam profile PF00150: Cellulase (glycosyl hydrolase family 5)	5E-78
1139	PE00000875	2.43434	-0.97449	1.61810	At5g38650	proteasome maturation factor UMP1 family protein contains Pfam profile PF05348: Proteasome maturation factor UMP1	1E-57
2775	PE00002168	2.40400	0.25648	2.69444	At5g67360	cucumisin-like serine protease (ARA12) Asp48; almost identical to cucumisin-like serine protease (ARA12) GI:3176874 from [Arabidopsis thaliana]	6E-33
1643	PE00001274	2.40189		3.16744	At5g17540	transferase family protein similar to hypersensitivity-related gene product HSR201 - Nicotiana tabacum, EMBL:X95343; contains Pfam transferase family domain PF00248	2E-62
4236	PE00003537	2.40115	-0.19929	3.10447	No hits found		
2420	PE00001867	2.36476	0.03475	2.28902	At3g04870	zeta-carotene desaturase (ZDS1) / carotene 7,8-desaturase identical to SP|Q38893 Zeta-carotene desaturase, chloroplast precursor (EC 1.14.99.30) (Carotene 7,8-desaturase) {Arabidopsis thaliana}	0
81	PE00000047	2.33630	-0.53486	1.26114	At3g17980	C2 domain-containing protein similar to zinc finger and C2 domain protein GI:9957238 from [Arabidopsis thaliana]	9E-73
420	PE00000317	2.33457		5.50328	At3g59850	polygalacturonase, putative / pectinase, putative similar to SP|P48979 Polygalacturonase precursor (EC 3.2.1.15) (PG) (Pectinase) {Prunus persica}; contains PF00295: Glycosyl hydrolases family 28 (polygalacturonases)	1E-153
82	PE00000048	2.33279		1.72104	At1g76160	multi-copper oxidase type I family protein similar to pollen-specific BP10 protein [SP|Q00624][Brassica napus]; contains Pfam profile: PF00394 Multicopper oxidase	0
4342	PE00003643	2.31091	0.00926	1.71152	No hits found		
2467	PE00001908	2.29999	-0.21630	2.82542	At5g61680	pectinesterase family protein contains Pfam profile: PF01095 pectinesterase	7E-45
4394	PE00003693	2.28688	-0.12329	2.08336	At1g15500	chloroplast ADP, ATP carrier protein, putative / ADP, ATP translocase, putative / adenine nucleotide translocase, putative strong similarity to SP|Q39002 Chloroplast ADP,ATP carrier protein 1, chloroplast precursor (ADP/ATP translocase 1) (Adenine nucleotide translocase 1) {Arabidopsis thaliana}; contains Pfam profile PF03219: TLC ATP/ADP transporter	1E-102
61	PE00000030	2.27842	0.24418	3.16217	At5g20830	sucrose synthase / sucrose-UDP glucosyltransferase (SUS1) identical to SP|P49040 Sucrose synthase (EC 2.4.1.13) (Sucrose-UDP glucosyltransferase) {Arabidopsis thaliana}	0
4533	PE00003830	2.27306	0.18879	2.67962	At1g69940	pectinesterase family protein contains Pfam profile: PF01095 pectinesterase	9E-20

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
1347	PE00001038	2.25040	0.10099	1.58882	At4g35300	transporter-related low similarity to hexose transporter [Solanum tuberosum] GI:8347246; contains Pfam profile PF00083: major facilitator superfamily protein	1E-118
407	PE00000305	2.20829	-0.10048	2.12829	At5g60600	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, putative / GcpE family protein similar to GcpE [Plasmodium falciparum] GI:13094969; contains Pfam profile PF04551: GcpE protein; supporting cDNA gi|27462471|gb|AF434673.1	0
2664	PE00002075	2.20686		2.32460	At1g03170	expressed protein	2E-10
771	PE00000560	2.20524		2.51208	At1g04690	potassium channel protein, putative nearly identical to K+ channel protein [Arabidopsis thaliana] GI:1063415; contains Pfam profile PF00248: oxidoreductase, aldo/keto reductase family	5E-94
3611	PE00002937	2.19665	-0.02059	1.97841	At3g23600	dienelactone hydrolase family protein similar to SP|Q9ZT66 Endo-1,3;1,4-beta-D-glucanase precursor (EC 3.2.1.-) {Zea mays}; contains Pfam profile: PF01738 Dienelactone hydrolase family	4E-84
487	PE00000359	2.19081	-0.77181	2.59978	At3g53990	universal stress protein (USP) family protein contains Pfam PF00582: universal stress protein family	7E-63
1	PE00000001	2.18218	0.08308	2.10683	No hits found		
1992	PE00001535	2.17615		2.19866	No hits found		
1398	PE00001083	2.17086	-0.10720	3.15487	At5g17540	transferase family protein similar to hypersensitivity-related gene product HSR201 - Nicotiana tabacum, EMBL:X95343; contains Pfam transferase family domain PF00248	7E-16
1916	PE00001479	2.13637	0.29547	1.22210	At4g23430	short-chain dehydrogenase/reductase (SDR) family protein contains INTERPRO family IPR002198 Short-chain dehydrogenase/reductase (SDR) superfamily; contains Pfam PF00106: oxidoreductase, short chain dehydrogenase/reductase family	7E-79
165	PE00000118	2.12818		3.23438	At5g44060	expressed protein similar to unknown protein (gb|AAD10670.1)	3E-13
5213	PE00004504	2.12333	0.13362	2.54749	At3g10420	sporulation protein-related similar to hypothetical proteins: GB:P51281 [Chloroplast Porphyra purpurea], GB:BAA16982 [Synechocystis sp], GB:P49540 [Odontella sinensis], GB:AAB82669 [Chloroplast Cyanidium caldarium]; similar to stage III sporulation protein AA (GI:18145497) [Clostridium perfringens str. 13]; similar to stage III sporulation protein AA (mutants block sporulation after engulfment) (GI:22777578) [Oceanobacillus iheyensis]	4E-70
1099	PE00000843	2.11703	0.31098	1.88705	At1g14130	2-oxoglutarate-dependent dioxygenase, putative similar to adventitious rooting related oxygenase ARRO-1 from Malus x domestica, gi|3492806; contains Pfam domain PF03171, 2OG-Fe(II) oxygenase superfamily	9E-75

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
1741	PE00001346	2.10505	0.44284	1.77975	At2g33310	auxin-responsive protein / indoleacetic acid-induced protein 13 (IAA13) identical to SP|Q38831 Auxin-responsive protein IAA13 (Indoleacetic acid-induced protein 13) {Arabidopsis thaliana}	6E-50
1472	PE00001145	2.09697	-0.62893	1.49345	No hits found		
243	PE00000191	2.09675	-0.74632	1.82840	No hits found		
1007	PE00000765	2.07988	0.15657	1.48763	At5g19550	aspartate aminotransferase, cytoplasmic isozyme 1 / transaminase A (ASP2) identical to SP|P46645 Aspartate aminotransferase, cytoplasmic isozyme 1 (EC 2.6.1.1) (Transaminase A) {Arabidopsis thaliana}	1E-124
1929	PE00001486	2.07953		1.91827	At5g57630	CBL-interacting protein kinase 21, putative (CIPK21) identical to CBL-interacting protein kinase 21 [Arabidopsis thaliana] gi|14334390|gb|AAK59696	2E-62
1520	PE00001181	2.06898	0.50953	2.10470	At3g22740	homocysteine S-methyltransferase 3 (HMT-3) identical to homocysteine S-methyltransferase HMT-3 [Arabidopsis thaliana] GI:9966515; similar to homocysteine S-methyltransferase AtHMT-2 (GI:6685163) [Arabidopsis thaliana]; similar to selenocysteine methyltransferase GB:P56707 from [Astragalus bisulcatus]	1E-133
5466	PE00004755	2.06789	-0.27326	1.70403	At4g11410	short-chain dehydrogenase/reductase (SDR) family protein contains INTERPRO family IPR002198 Short-chain dehydrogenase/reductase (SDR) superfamily	5E-17
1015	PE00000770	2.06582	-0.08991	1.45776	No hits found		
5160	PE00004452	2.05671	-0.16696	1.45778	At2g46690	auxin-responsive family protein similar to indole-3-acetic acid induced protein ARG7 (SP:P32295) [Phaseolus aureus]	8E-10
835	PE00000609	2.04353	-0.25836	4.66876	At3g12120	omega-6 fatty acid desaturase, endoplasmic reticulum (FAD2) / delta-12 desaturase identical to omega-6 fatty acid desaturase, endoplasmic reticulum (FAD2) [Arabidopsis thaliana	2E-57
1672	PE00001295	2.03053	0.04532	1.37753	At4g34350	LytB family protein contains Pfam profile: PF02401 LytB protein	1E-87
4271	PE00003572	2.02600	0.05859	1.57309	No hits found		
2165	PE00001671	2.01240	-0.13590	1.52780	At4g04020	plastid-lipid associated protein PAP, putative / fibrillin, putative strong similarity to plastid-lipid associated proteins PAP1 GI:14248554, PAP2 GI:14248556 from [Brassica rapa], fibrillin [Brassica napus] GI:4139097; contains Pfam profile PF04755: PAP_fibrillin	4E-93
3592	PE00002920	2.00438	-0.05299	2.65254	At4g21990	5'-adenylylsulfate reductase (APR3) / PAPS reductase homolog (PRH26) identical to 5'-adenylylsulfate reductase [Arabidopsis thaliana]	1E-176
3945	PE00003257	2.00045	-0.07872	1.95631	At5g67360	cucumisin-like serine protease (ARA12) Asp48; almost identical to cucumisin-like serine protease (ARA12) GI:3176874 from [Arabidopsis thaliana]	2E-64

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
399	PE00000299	1.96663	-0.60590	1.43905	At5g40570	surfeit locus protein 2 family protein / SURF2 family protein contains Pfam profile PF05477: Surfeit locus protein 2 (SURF2)	0.0003
489	PE00000361	1.95976		3.25447	At1g01480	1-aminocyclopropane-1-carboxylate synthase 2 / ACC synthase 2 (ACS2) (ACC1) identical to 1-aminocyclopropane-1-carboxylate synthase SP|Q06402 [GI:166578] from [Arabidopsis thaliana]	1E-158
939	PE00000707	1.95794		2.99834	At2g40610	expansin, putative (EXP8) similar to expansin 2 GI:7025493 from [Zinnia elegans]; alpha-expansin gene family, PMID:11641069	2E-97
560	PE00000424	1.95568		1.72366	At5g47550	cysteine protease inhibitor, putative / cystatin, putative similar to SP|P09229 Cysteine proteinase inhibitor-I (Oryzacystatin-I) {Oryza sativa}; contains Pfam profile PF00031: Cystatin domain	5E-18
1094	PE00000838	1.93745	-0.03346	1.78074	At2g19880	ceramide glucosyltransferase, putative similar to ceramide glucosyltransferase (GI:14718995) [Gossypium arboreum]; weak similarity to Ceramide glucosyltransferase (Glucosylceramide synthase) (GCS) (UDP-glucose:N-acylsphingosine D-glucosyltransferase) (GLCT-1) (Swiss-Prot:Q16739) [Homo sapiens]	9E-47
2742	PE00002145	1.92313	-0.28972	1.56300	At3g14690	cytochrome P450, putative similar to GB:Q05047 from [Catharanthus roseus]	3E-74
3687	PE00003007	1.91440	-0.04058	1.39028	At1g63970	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, putative similar to 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase GI:7621712 from [Catharanthus roseus]	1E-07
1743	PE00001347	1.89270	0.35881	1.89714	At5g14780	formate dehydrogenase (FDH) identical to GI:7677266	1E-148
4981	PE00004274	1.89221	-0.16845	1.93021	At3g02630	acyl-[acyl-carrier-protein] desaturase, putative / stearoyl-ACP desaturase, putative similar to Acyl-[acyl-carrier protein] desaturase from Sesamum indicum GI:575942, Cucumis sativus SP|P32061, Ricinus communis SP|P22337; contains Pfam profile PF03405 Fatty acid desaturase	9E-23
3588	PE00002916	1.89059		1.46266	No hits found		
55	PE00000025	1.88557	-1.17506	2.89061	At1g28400	expressed protein similar to E6 (GI:1000090) [Gossypium barbadense]	9E-11
1595	PE00001237	1.86814	-0.23118	1.53984	No hits found		
4757	PE00004052	1.85684	0.01909	1.75543	At3g16770	AP2 domain-containing protein RAP2.3 (RAP2.3) identical to GI:2281631 [Arabidopsis thaliana]; identical to cDNA EBP GI:2190330	0.00002
134	PE00000090	1.85681		2.62296	No hits found		
4052	PE00003358	1.83450	-0.14791	1.44655	At3g10230	lycopene beta cyclase (LYC) identical to lycopene beta cyclase GI:1399183|GB:AAB53337 [Arabidopsis thaliana]	1E-142
42	PE00000013	1.83048	-0.09629	2.43624	At1g04250	auxin-responsive protein / indoleacetic acid-induced protein 17 (IAA17)	9E-49

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
1488	PE00001156	1.83007		0.72942	At3g22540	expressed protein	5E-35
3039	PE00002411	1.82269		1.13407	At2g19880	ceramide glucosyltransferase, putative similar to ceramide glucosyltransferase (GI:14718995) [Gossypium arboreum]; weak similarity to Ceramide glucosyltransferase (Glucosylceramide synthase) (GCS) (UDP-glucose:N-acylsphingosine D-glucosyltransferase) (GLCT-1) (Swiss-Prot:Q16739) [Homo sapiens]	1E-102
5391	PE00004680	1.81732		0.79672	At1g54220	dihydrolipoamide S-acetyltransferase, putative similar to dihydrolipoamide S-acetyltransferase GI:5669871 [Zea mays]; contains Pfam profiles PF00198: 2-oxo acid dehydrogenases acyltransferase (catalytic domain), PF00364: Biotin-requiring enzyme, PF02817: e3 binding domain	0.000009
1548	PE00001201	1.80896	0.36291	1.63278	At5g22460	esterase/lipase/thioesterase family protein low similarity to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase [Rhodococcus sp. RHA1] GI:8978311, SP|Q02104 Lipase 1 precursor (EC 3.1.1.3) (Triacylglycerol lipase) {Psychrobacter immobilis}; contains Interpro entry IPR000379	1E-133
1816	PE00001398	1.80254	-0.05406	2.39950	At4g19420	pectinacetylesterase family protein contains Pfam profile: PF03283 pectinacetylesterase	1E-24
3112	PE00002472	1.79420	-0.08578	1.99037	At5g14780	formate dehydrogenase (FDH) identical to GI:7677266	3E-13
1093	PE00000837	1.78560		1.71461	At5g61640	peptide methionine sulfoxide reductase, putative similar to peptide methionine sulfoxide reductase (msr) [Arabidopsis thaliana] GI:4884033; contains Pfam profile PF01625: Peptide methionine sulfoxide reductase	3E-39
4064	PE00003368	1.77550	-0.47436	1.91412	At5g66910	disease resistance protein (CC-NBS-LRR class), putative domain signature CC-NBS-LRR exists, suggestive of a disease resistance protein.	2E-85
1978	PE00001525	1.77443	0.13414	1.41483	At1g60940	serine/threonine protein kinase, putative similar to serine/threonine-protein kinase ASK1 from [Arabidopsis thaliana], SWISS-PROT:P43291	1E-65
75	PE00000041	1.76869	0.35372	1.00116	At4g40010	serine/threonine protein kinase, putative similar to serine-threonine protein kinase [Triticum aestivum] gi|2055374|gb|AAB58348	1E-115
2135	PE00001648	1.76792	0.06728	1.79529	At3g07880	Rho GDP-dissociation inhibitor family protein similar to SP|P52565 Rho GDP-dissociation inhibitor 1 (Rho GDI 1) (Rho-GDI alpha) {Homo sapiens}; contains Pfam profile PF02115: RHO protein GDP dissociation inhibitor	2E-81
1405	PE00001090	1.76520	-0.17939	1.79290	At4g39090	cysteine proteinase RD19a (RD19A) / thiol protease identical to cysteine proteinase RD19a, thiol protease SP:P43296, GI:435618 from [Arabidopsis thaliana]	1E-157
4351	PE00003651	1.73953	0.03647	1.16104	At1g31812	acyl-CoA binding protein / ACBP identical to acyl-CoA-binding protein (ACBP) [Arabidopsis thaliana] SWISS-PROT:P57752	2E-32

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3132	PE00002492	1.72703	0.37358	1.04350	At1g12230	transaldolase, putative similar to Swiss-Prot:P30148 transaldolase B (EC 2.2.1.2) [Escherichia coli O157:H7]	1E-130
2396	PE00001849	1.70928	-0.25095	2.09025	At4g36010	pathogenesis-related thaumatin family protein similar to receptor serine/threonine kinase PR5K [Arabidopsis thaliana] GI:1235680; contains Pfam profile PF00314: Thaumatin family	1E-20
980	PE00000741	1.70268	-0.16268	1.55815	At1g67300	hexose transporter, putative similar to hexose transporters from Solanum tuberosum [GI:8347246], Nicotiana tabacum [GI:8347244], Arabidopsis thaliana [GI:8347250]; contains Pfam profile PF00083: major facilitator superfamily protein	0.0002
494	PE00000365	1.69833		2.05047	At1g77760	nitrate reductase 1 (NR1) identical to SP|P11832 Nitrate reductase 1 (formerly EC 1.6.6.1) (NR1){Arabidopsis thaliana}	0
65	PE00000034	1.69697	-0.24621	1.28890	At5g60360	cysteine proteinase, putative / AALP protein (AALP) identical to AALP protein GI:7230640 from [Arabidopsis thaliana]; similar to barley aleurain	1E-148
5337	PE00004626	1.69674	0.10699	1.52808	At4g31390	ABC1 family protein contains Pfam domain, PF03109: ABC1 family	1E-89
1399	PE00001084	1.69520	0.22386	1.90826	At2g38630	expressed protein	1E-109
408	PE00000306	1.67688		1.55600	At4g25590	actin-depolymerizing factor, putative strong similarity to pollen specific actin-depolymerizing factor 2 [Nicotiana tabacum] GI:22857914; contains Pfam profile PF00241: Cofilin/tropomyosin-type actin-binding protein	9E-47
2509	PE00001947	1.67242	-0.19481	1.44068	At1g16300	glyceraldehyde 3-phosphate dehydrogenase, cytosolic, putative / NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, putative similar to glyceraldehyde-3-phosphate dehydrogenase [Pinus sylvestris] GI:1100223; contains Pfam profiles PF02800: Glyceraldehyde 3-phosphate dehydrogenase C-terminal domain, PF00044: Glyceraldehyde 3-phosphate dehydrogenase NAD binding domain	4E-83
3437	PE00002787	1.66833	-0.06717	0.68875	No hits found		
2872	PE00002259	1.65891		1.28362	No hits found		
2793	PE00002184	1.65186		0.74572	At4g33565	zinc finger (C3HC4-type RING finger) family protein contains Pfam profile: PF00097 zinc finger, C3HC4 type (RING finger)	4E-25
2849	PE00002237	1.64945	0.03392	1.99184	At5g57050	protein phosphatase 2C ABI2 / PP2C ABI2 / abscisic acid-insensitive 2 (ABI2) identical to SP|O04719 Protein phosphatase 2C ABI2 (EC 3.1.3.16) (PP2C) (Abscisic acid- insensitive 2) {Arabidopsis thaliana}	7E-29
96	PE00000059	1.63324		2.17830	At2g41380	embryo-abundant protein-related similar to embryo-abundant protein [Picea glauca] GI:1350531	6E-75
5470	PE00004759	1.62801	-0.28773	1.00198	At3g60820	20S proteasome beta subunit F1 (PBF1)	7E-08
2448	PE00001891	1.62400		1.66077	At4g30960	CBL-interacting protein kinase 6 (CIPK6) identical to CBL-interacting protein kinase 6 [Arabidopsis thaliana]	3E-34

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1852	PE00001429	1.62281	0.05609	1.25834	At5g26670	pectinacetylesterase, putative similar to pectinacetylesterase precursor GI:1431629 from [Vigna radiata]	1E-141
3780	PE00003095	1.61899		0.56956	At3g17810	dihydroorotate dehydrogenase family protein / dihydroorotate oxidase family protein low similarity to SP|Q12882 Dihydropyrimidine dehydrogenase [NADP+] precursor (EC 1.3.1.2) (DPD) (DHPDHase) (Dihydrouracil dehydrogenase) (Dihydrothymine dehydrogenase) {Homo sapiens}; contains Pfam profile PF01180: Dihydroorotate dehydrogenase	3E-50
4056	PE00003361	1.61540	-0.15118	1.00663	At3g02875	IAA-amino acid hydrolase 1 (ILR1) identical to IAA-amino acid hydrolase 1 (ILR1) [Arabidopsis thaliana] SWISS-PROT:P54968	1E-115
2349	PE00001811	1.60967	-0.20496	2.38042	At3g48990	AMP-dependent synthetase and ligase family protein similar to peroxisomal-coenzyme A synthetase (FAT2) [gi:586339] from Saccharomyces cerevisiae; contains Pfam AMP-binding enzyme domain PF00501; identical to cDNA; identical to cDNA adenosine monophosphate binding protein 3 AMPBP3 (AMPBP3)GI:20799714	2E-44
3017	PE00002391	1.60157	0.31010	1.38695	At3g27925	DegP protease, putative SP:022609; almost identical to DegP protease precursor GB:AF028842 from [Arabidopsis thaliana] (J. Biol. Chem. 273 (12), 7094-7098 (1998))	2E-87
1357	PE00001046	1.60048	0.09067	1.02954	At1g24260	MADS-box protein (AGL9) strongly similar to GB:O22456, MADS-box protein, Location of EST gb|H37053	2E-98
4109	PE00003410	1.59189	0.21818	2.24456	At3g04580	ethylene receptor, putative (EIN4) similar to ethylene receptor GB:AAC31123 [Malus domestica],	5E-55
356	PE00000271	1.59000		1.34633	At2g40940	ethylene response sensor / ethylene-responsive sensor (ERS) [Arabidopsis thaliana] GI:1046225	1E-170
2505	PE00001943	1.58192	-0.36454	1.21108	At5g01220	UDP-sulfoquinovose:DAG sulfoquinovosyltransferase / sulfolipid synthase (SQD2) identical to GI:20302857	2E-58
4438	PE00004792	1.57734		0.57471	No hits found		
2719	PE00002124	1.57544	0.04434	1.21800	At4g12590	expressed protein contains Pfam PF05863: Eukaryotic protein of unknown function (DUF850)	1E-117
1245	PE00000951	1.57479	0.09111	0.63444	At2g20360	expressed protein	2E-72
2429	PE00001875	1.56958		0.92277	At4g30410	expressed protein similar to cDNA bHLH transcription factor (bHLH eta gene) gi:32563007	3E-19
556	PE00000421	1.54933		1.69418	At1g11750	ATP-dependent Clp protease proteolytic subunit (ClpP) identical to ATP-dependent Clp protease proteolytic subunit GI:2827888 from [Arabidopsis thaliana]; contains Pfam profile PF00574: Clp protease; contains TIGRfam profile TIGR00493: ATP-dependent Clp protease, proteolytic subunit ClpP	1E-50

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1348	PE00001039	1.54827	0.02479	1.26328	At3g56240	copper homeostasis factor / copper chaperone (CCH) (ATX1) identical to gi:3168840 Pfam profile PF00403: Heavy-metal-associated domain	6E-25
4575	PE00003872	1.54497	-0.67197	1.21748	At1g07230	phosphoesterase family protein low similarity to SP|P95246 Phospholipase C 2 precursor (EC 3.1.4.3) {Mycobacterium tuberculosis}; contains Pfam profile PF04185: Phosphoesterase family	9E-22
5282	PE00004572	1.53964		0.93223	At2g46540	expressed protein	2E-23
5439	PE00004728	1.52970		1.15750	No hits found		
3604	PE00002931	1.52689	0.08402	0.88566	At5g45410	expressed protein similar to unknown protein (pir||T05524)	1E-86
4524	PE00003821	1.52587	0.46468	1.87129	At2g14580	pathogenesis-related protein, putative similar to SP|P33154 Pathogenesis-related protein 1 precursor (PR-1) {Arabidopsis thaliana}; contains Pfam profile PF00188: SCP-like extracellular protein	1E-31
1067	PE00000814	1.52013	-0.74273	0.69040	At5g18570	GTP1/OBG family protein similar to SP|P20964 Spo0B-associated GTP-binding protein {Bacillus subtilis}; contains Pfam profile PF01018: GTP1/OBG family	3E-53
2366	PE00001824	1.51348	-0.46078	1.43111	At5g50000	protein kinase, putative similar to protein kinase ATMRK1 [Arabidopsis thaliana] gi|2351097|dbj|BAA22079	5E-57
5422	PE00004711	1.51025	-0.19607	1.92618	At3g07130	serine/threonine protein phosphatase family protein contains similarity to purple acid phosphatase [Arabidopsis thaliana] gi|20257489|gb|AAM15914	8E-16
4598	PE00003895	1.50974	0.05014	0.43972	At5g57520	zinc finger (C2H2 type) family protein (ZFP2) identical to zinc finger protein 2 (ZFP2) GI:790674 from [Arabidopsis thaliana]; contains Pfam domain, PF00096: Zinc finger, C2H2 type	2E-16
2085	PE00001607	1.50646	-0.20693	0.78090	At3g11780	MD-2-related lipid recognition domain-containing protein / ML domain-containing protein weak similarity to phosphatidylglycerol/phosphatidylinositol transfer protein [Aspergillus oryzae] GI:10178615; contains Pfam profile PF02221: ML domain	4E-38
3813	PE00003125	1.50361	0.43316	0.30619	No hits found		
3392	PE00002743	1.50181	0.02023	1.17088	At3g24170	glutathione reductase, putative identical to GB:P48641 from [Arabidopsis thaliana]	2E-60
3281	PE00002634	1.50140		1.34524	At1g48030	dihydrolipoamide dehydrogenase 1, mitochondrial / lipoamide dehydrogenase 1 (MTLPD1) identical to GB:AAF34795 [gi:12704696] from [Arabidopsis thaliana]	1E-93
2722	PE00002127	1.49948	0.17959	1.40658	At1g22370	UDP-glucoronosyl/UDP-glucosyl transferase family protein glycosyltransferase family	7E-42
1426	PE00001104	1.49797	-0.21685	0.91500	At3g02090	mitochondrial processing peptidase beta subunit, putative similar to mitochondrial processing peptidase beta subunit, mitochondrial precursor, Beta-MPP [Human] SWISS-PROT:O75439	0

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2000	PE00001540	1.49261	0.34648	-0.06091	No hits found		
727	PE00000522	1.49040	-1.01144	1.47040	At3g01170	expressed protein	9E-38
1526	PE00001186	1.48070	-0.11760	1.49472	At4g21120	amino acid permease family protein similar to cationic amino acid transporter-1 [Rattus norvegicus] GI:1589917; contains Pfam profile PF00324: Amino acid permease	6E-34
1026	PE00000780	1.47239	0.34130	2.10354	At3g04720	hevein-like protein (HEL) identical to SP|P43082 Hevein-like protein precursor {Arabidopsis thaliana}; similar to SP|P09762 Wound-induced protein WIN2 precursor {Solanum tuberosum}; contains Pfam profile PF00187: Chitin recognition protein	2E-53
1113	PE00000852	1.46902	-0.34825	0.70483	At3g13110	serine O-acetyltransferase (SAT-1) identical to serine acetyltransferase (Sat-1) GI:1184048 [Arabidopsis thaliana]	6E-37
62	PE00000031	1.46743	0.50170	0.88543	At3g02720	DJ-1 family protein / protease-related similar to Intracellular Protease [Pyrococcus horikoshii] GI:11513902; contains Pfam profile: PF01965 ThiJ/PfpI family	5E-70
593	PE00000432	1.46046	-0.34066	1.77253	At3g17810	dihydroorotate dehydrogenase family protein / dihydroorotate oxidase family protein low similarity to SP|Q12882 Dihydropyrimidine dehydrogenase [NADP+] precursor (EC 1.3.1.2) (DPD) (DHPDHase) (Dihydrouracil dehydrogenase) (Dihydrothymine dehydrogenase) {Homo sapiens}; contains Pfam profile PF01180: Dihydroorotate dehydrogenase	9E-36
1609	PE00001247	1.45812	0.05311	0.99811	At5g15050	glycosyltransferase family 14 protein / core-2/I-branching enzyme family protein contains Pfam profile: PF02485 Core-2/I-Branching enzyme	1E-44
2713	PE00002119	1.45754	0.16693	0.65674	At3g62980	transport inhibitor response 1 (TIR1) (FBL1) E3 ubiquitin ligase SCF complex F-box subunit; identical to transport inhibitor response 1 GI:2352492 from [Arabidopsis thaliana]	1E-112
745	PE00000538	1.45524	-0.44888	1.20215	No hits found		
1758	PE00001355	1.45436	-0.37096	1.28412	At3g13930	dihydrolipoamide S-acetyltransferase, putative similar to dihydrolipoamide S-acetyltransferase [Zea mays] GI:5669871; contains Pfam profiles PF00198: 2-oxo acid dehydrogenases acyltransferase (catalytic domain), PF00364: Biotin-requiring enzyme, PF02817: e3 binding domain	2E-53
675	PE00000480	1.45360	0.08562	1.16073	At1g66070	translation initiation factor-related similar to Eukaryotic translation initiation factor 3 subunit 1 (eIF-3 alpha) (eIF3 p35) (eIF3j) (Swiss-Prot:O75822) [Homo sapiens]	8E-46
5297	PE00004586	1.45202		1.41533	At1g36390	co-chaperone grpE family protein similar to co-chaperone CGE1 precursor isoform b [Chlamydomonas reinhardtii] GI:15384279; contains Pfam profile PF01025: co-chaperone GrpE	3E-29
711	PE00000507	1.44838		2.09258	At4g25700	beta-carotene hydroxylase identical to GI:1575296	1E-104

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1471	PE00001144	1.44561	0.47358	0.99962	At3g16770	AP2 domain-containing protein RAP2.3 (RAP2.3) identical to GI:2281631 [Arabidopsis thaliana]; identical to cDNA EBP GI:2190330	3E-22
4221	PE00003522	1.43753	0.18947	1.73205	At1g12410	ATP-dependent Clp protease proteolytic subunit (ClpP2) identical to nClpP2 GI:5360589 from [Arabidopsis thaliana]	3E-70
1514	PE00001178	1.43417	-0.39576	2.34025	At5g51460	trehalose-6-phosphate phosphatase (TPPA) identical to trehalose-6-phosphate phosphatase (AtTPPA) [Arabidopsis thaliana] GI:2944178	6E-29
1868	PE00001440	1.42997	-0.06729	1.68861	At1g64380	AP2 domain-containing transcription factor, putative contains Pfam profile: PF00847 AP2 domain	0.00004
1428	PE00001105	1.42893	0.06264	1.39408	At1g47128	cysteine proteinase (RD21A) / thiol protease identical to SP|P43297 Cysteine proteinase RD21A precursor (EC 3.4.22.-) {Arabidopsis thaliana)	1E-174
722	PE00000517	1.42679	0.33858	1.09038	At3g15360	thioredoxin M-type 4, chloroplast (TRX-M4) nearly identical to SP|Q9SEU6 Thioredoxin M-type 4, chloroplast precursor (TRX-M4) {Arabidopsis thaliana}	3E-17
3937	PE00003249	1.42639	-0.51232	1.54119	At1g72770	protein phosphatase 2C P2C-HA / PP2C P2C-HA (P2C-HA) identical to protein phosphatase 2C (AtP2C-HA) GB:AJ003119 [Arabidopsis thaliana] (Plant Mol. Biol. 38 (5), 879-883 (1998))	5E-70
5277	PE00004568	1.42243		1.37008	At3g24740	expressed protein	1E-13
238	PE00000187	1.41894	-0.33720	0.70270	No hits found		
2219	PE00001715	1.40282	-0.16293	1.43005	AtMg00750	orf119 hypothetical protein	4E-15
53	PE00000023	1.39949	-0.35625	0.75630	At1g77400	expressed protein	0.000009
2914	PE00002299	1.39715	-0.07222	0.78855	At1g18640	3-phosphoserine phosphatase (PSP) nearly identical to 3-phosphoserine phosphatase GI:3759177 from [Arabidopsis thaliana]	9E-65
2777	PE00002169	1.39346	0.09017	1.78081	At4g30960	CBL-interacting protein kinase 6 (CIPK6) identical to CBL-interacting protein kinase 6 [Arabidopsis thaliana] gi|9280634|gb|AAF86505	6E-72
843	PE00000616	1.39286		1.02332	At2g26400	acireductone dioxygenase (ARD/ARD') family protein similar to iron-deficiency induced gene [Hordeum vulgare] GI:14522834, SIPL [Homo sapiens] GI:16551383; contains Pfam profile PF03079: ARD/ARD' family	2E-22
2025	PE00001560	1.38923	-0.03148	2.04926	At3g04580	ethylene receptor, putative (EIN4) similar to ethylene receptor GB:AAC31123 [Malus domestica], identical to putative ethylene receptor GB:AAD02485 [Arabidopsis thaliana]; Pfam HMM hit: response regulator receiver domain, signal C terminal domain	1E-40
5039	PE00004332	1.38550		1.23932	At1g28150	expressed protein	5E-13
1400	PE00001085	1.36564	0.05775	0.94636	At3g22110	20S proteasome alpha subunit C (PAC1) (PRC9) identical to GB:AAC32057 from [Arabidopsis thaliana] (Genetics (1998) 149 (2), 677-692); identical to cDNA proteasome subunit prc9 GI:2511583	1E-119

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70	PE00000038	1.36466	0.14095	1.40716	At3g58610	ketol-acid reductoisomerase identical to ketol-acid reductoisomerase, chloroplast precursor (EC 1.1.1.86) (Acetohydroxy-acid reductoisomerase) (Alpha-keto-beta-hydroxylacil reductoisomerase) (Swiss-Prot:Q05758) [Arabidopsis thaliana]	1E-177
4898	PE00004192	1.35901	-0.59693	0.57509	At3g15580	autophagy 8i (APG8i) identical to autophagy 8i [Arabidopsis thaliana] GI:19912167; contains Pfam profile PF02991: Microtubule associated protein 1A/1B, light chain 3; supporting cDNA gi|21636957|gb|AF492760.1|	5E-25
5183	PE00004475	1.34968		0.99392	At5g65780	branched-chain amino acid aminotransferase 5 / branched-chain amino acid transaminase 5 (BCAT5) nearly identical to SP|Q9FYA6 Branched-chain amino acid aminotransferase 5, chloroplast precursor (EC 2.6.1.42) (Atbcat-5) {Arabidopsis thaliana}; contains Pfam profile: PF01063 aminotransferase class IV	1E-99
306	PE00000239	1.34486	-0.03670	0.75054	At1g55265	expressed protein contains Pfam profile PF04398: Protein of unknown function, DUF538	3E-27
4517	PE00003814	1.33124		0.56298	At1g08490	cysteine desulfurase, putative similar to nitrogen fixation protein (nifS) GB:D64004 GI:1001701 from [Synechocystis sp]; contains TIGRFAM TIGR01364: phosphoserine aminotransferase; contains Pfam PF00266: aminotransferase, class V	2E-48
3817	PE00003129	1.32786	-0.53859	0.75026	At3g63460	WD-40 repeat family protein hypothetical protein contains similarity to ec31p [Oryza sativa] gi|13928450|dbj|BAB47154; contains Pfam profile PF00400: WD domain, G-beta repeat	7E-25
475	PE00000348	1.31408		2.30771	At3g02875	IAA-amino acid hydrolase 1 (ILR1) identical to IAA-amino acid hydrolase 1 (ILR1) [Arabidopsis thaliana] SWISS-PROT:P54968	5E-16
1328	PE00001019	1.31329	-0.95448	0.92505	At5g13450	ATP synthase delta chain, mitochondrial, putative / H(+)-transporting two-sector ATPase, delta (OSCP) subunit, putative identical to SP|Q96251; similar to SP|P22778 ATP synthase delta chain, mitochondrial precursor (EC 3.6.3.14) (Oligomycin sensitivity conferral protein) (OSCP) {Ipomoea batatas}; contains Pfam profile PF00213: ATP synthase F1, delta subunit	0.000001
1463	PE00001138	1.31214	-0.14911	1.20389	At1g09970	leucine-rich repeat transmembrane protein kinase, putative Similar to A. thaliana receptor-like protein kinase (gb|RLK5_ARATH). ESTs gb|ATTS0475,gb|ATTS4362 come from this gene isoform contains a TG acceptor site at intron.	1E-123
2942	PE00002322	1.30944		1.93740	At4g13195	expressed protein	2E-08
4006	PE00003318	1.30904	-0.05978	0.77246	At5g65620	peptidase M3 family protein / thimet oligopeptidase family protein similar to SP|P27237	1E-170

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
2036	PE00001566	1.30495		0.88299	At4g04770	ATP-binding-cassette transporter (ABC1) Identical to the protein described in PMID:11156608 and note that sequence was not deposited into GenBank by the authors.	0
3982	PE00003294	1.30406	-0.20856	1.61847	At5g41340	ubiquitin-conjugating enzyme 4 (UBC4) E2; identical to gi:431265, SP:P42748	5E-77
79	PE00000045	1.30200	-0.15261	0.67765	At2g24940	cytochrome b5 domain-containing protein similar to SP|P70580 Membrane associated progesterone receptor component 1 {Rattus norvegicus}; contains Pfam profile PF00173: Heme/Steroid binding domain	1E-41
2120	PE00001637	1.29268		1.18191	At5g47030	ATP synthase delta' chain, mitochondrial identical to SP|Q96252 ATP synthase delta' chain, mitochondrial precursor (EC 3.6.3.14) {Arabidopsis thaliana}; contains Pfam profile PF02823: ATP synthase, Delta/Epsilon chain, beta-sandwich domain	9E-77
1944	PE00001499	1.29220	0.26251	1.96349	At1g76160	multi-copper oxidase type I family protein similar to pollen-specific BP10 protein [SP|Q00624][Brassica napus]; contains Pfam profile: PF00394 Multicopper oxidase	0
538	PE00000405	1.29101	-0.03968	1.17009	At1g66670	ATP-dependent Clp protease proteolytic subunit (ClpP3) identical to ATP-dependent Clp protease (nClpP3) GI:5360591 [Arabidopsis thaliana]	1E-118
3236	PE00002591	1.28566	-0.29179	0.72615	At2g24200	cytosol aminopeptidase identical to cytosol aminopeptidase SP:P30184 from [Arabidopsis thaliana]; contains Pfam profiles: PF00883 cytosol aminopeptidase family catalytic domain, PF02789: cytosol aminopeptidase family N-terminal domain	4E-14
2527	PE00001963	1.25826	0.06253	1.14638	No hits found		
3101	PE00002463	1.25397	0.42040	1.07917	At4g27740	Yippee protein [Homo sapiens] GI:5713281; contains Pfam profile PF03226: Yippee putative zinc-binding protein	6E-36
647	PE00000463	1.25114	-0.16209	0.38309	At5g18670	beta-amylase, putative (BMY3) / 1,4-alpha-D-glucan maltohydrolase, putative almost identical to beta-amylase BMY3 GI:15149457 from [Arabidopsis thaliana]; identical to cDNA putative beta-amylase BMY3 (BMY3) GI:15149456	1E-142
2532	PE00001967	1.24948	-0.05071	1.28141	At4g02590	basic helix-loop-helix (bHLH) family protein similar to A. thaliana putative protein F6I18.110, GenBank accession number 2980768	1E-25
3899	PE00003211	1.24937	-0.20574	0.92436	At1g49670	ARP protein (REF) identical to ARP protein GB:CAA89858 GI:886434 from [Arabidopsis thaliana]; contains Pfam profile PF00107: oxidoreductase, zinc-binding dehydrogenase family	5E-93
3762	PE00003079	1.24109	-0.16333	0.80078	At5g19540	expressed protein	2E-68
1244	PE00000950	1.23912	0.36392	1.12675	At1g49670	ARP protein (REF) identical to ARP protein GB:CAA89858 GI:886434 from [Arabidopsis thaliana]; contains Pfam profile PF00107: oxidoreductase, zinc-binding dehydrogenase family	1E-131

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
1216	PE00000929	1.23649	0.06935	0.41871	At1g66150	leucine-rich repeat protein kinase, putative (TMK1) identical to protein kinase TMK1 gi|166888|gb|AAA32876, SP|P43298 Putative receptor protein kinase TMK1 precursor (EC 2.7.1.-) {Arabidopsis thaliana}	1E-43
1716	PE00001331	1.23609	-0.66956	0.66586	At5g14040	mitochondrial phosphate transporter identical to mitochondrial phosphate transporter GI:3318617 from [Arabidopsis thaliana]	1E-151
670	PE00000475	1.23432	-0.76993	1.32794	At4g27130	eukaryotic translation initiation factor SUI1, putative similar to SP|P32911 Protein translation factor SUI1 {Saccharomyces cerevisiae}; contains Pfam profile PF01253: Translation initiation factor SUI1	1E-55
3138	PE00002498	1.22970	-0.00874	0.90315	At2g36310	inosine-uridine preferring nucleoside hydrolase family protein similar to Chain A, Crystal Structure Of Nucleoside Hydrolase From Leishmania MajorGI:8569431; contains Pfam profile PF01156: Inosine-uridine preferring nucleoside hydrolase	1E-69
3187	PE00002543	1.21902	0.13977	1.80933	No hits found		
1045	PE00000798	1.21848	0.13649	0.80804	At1g16180	TMS membrane family protein / tumour differentially expressed (TDE) family protein contains Pfam domain, PF03348: TMS membrane protein/tumour differentially expressed protein (TDE)	1E-154
197	PE00000149	1.21200	0.23451	0.88076	At1g65980	peroxiredoxin type 2, putative strong similarity to type 2 peroxiredoxin [Brassica rapa subsp. pekinensis] GI:4928472; contains Pfam profile: PF00578 AhpC/TSA (alkyl hydroperoxide reductase and thiol-specific antioxidant) family	1E-74
1768	PE00001363	1.20919	0.52118	0.74578	At4g37870	phosphoenolpyruvate carboxykinase [ATP], putative / PEP carboxykinase, putative / PEPCK, putative similar to phosphoenolpyruvate carboxykinase [Lycopersicon esculentum] GI:16950587, SP|Q9SLZ0 Phosphoenolpyruvate carboxykinase [ATP] (EC 4.1.1.49) (PEP carboxykinase) (Phosphoenolpyruvate carboxylase) (PEPCK) {Zea mays}; contains Pfam profile PF01293: phosphoenolpyruvate carboxykinase	0
2057	PE00001586	1.19739	0.15254	0.75238	At5g14280	DNA-binding storekeeper protein-related contains similarity to storekeeper protein [Solanum tuberosum] gi|14268476|emb|CAC39398; contains PF04504: Protein of unknown function, DUF573	4E-55
84	PE00000049	1.18232		2.03244	At5g43700	auxin-responsive protein / indoleacetic acid-induced protein 4 (IAA4) / auxin-induced protein (AUX2-11) identical to SP|P33077 Auxin-responsive protein IAA4 (Indoleacetic acid-induced protein 4) (Auxin-induced protein AUX2-11) {Arabidopsis thaliana}	7E-54

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
4075	PE00003379	1.17978		0.35351	At4g26270	phosphofructokinase family protein similar to phosphofructokinase [Amycolatopsis methanolica] GI:17432243; contains Pfam profile PF00365: Phosphofructokinase	4E-30
2422	PE00001869	1.17275	-0.56026	1.25791	At4g31990	nearly identical to SP|P46248 Aspartate aminotransferase, chloroplast precursor (EC 2.6.1.1) (Transaminase A) {Arabidopsis thaliana}	1E-134
1320	PE00001015	1.17049	-0.22744	0.13348	At5g13220	expressed protein	2E-18
3487	PE00002831	1.16914		0.57179	At2g01140	fructose-bisphosphate aldolase, putative similar to plastidic aldolase NPALDP1 from Nicotiana paniculata [GI:4827251]; contains Pfam profile PF00274 Fructose-bisphosphate aldolase class-I	0
5307	PE00004596	1.15962	-0.20219	0.45403	At2g17980	sec1 family protein similar to SWISS-PROT:P22213 SLY1 protein [Saccharomyces cerevisiae]; contains Pfam domain, PF00995: Sec1 family	4E-62
2224	PE00001718	1.15157	-0.24923	0.79653	At1g43890	Ras-related GTP-binding protein, putative similar to GTP-binding protein(RAB1Y) GI:1370173 from (Lotus japonicus)	6E-67
3432	PE00002783	1.15104	0.16534	0.65550	At3g05840	shaggy-related protein kinase gamma / ASK-gamma (ASK3) identical to shaggy-related protein kinase gamma SP:P43289 GI:456509 from [Arabidopsis thaliana]	7E-56
1280	PE00000978	1.14820	-1.82374	0.43143	No hits found		
3903	PE00003215	1.14336	-0.48327	-0.16991	At1g76680	12-oxophytodienoate reductase (OPR1) identical to 12-oxophytodienoate reductase OPR1 GB:AAC78440 [Arabidopsis thaliana]	7E-80
2850	PE00002238	1.13372	0.17645	0.10621	At2g32240	expressed protein contains Pfam profile: PF04508 viral A-type inclusion protein repeat	2E-30
3695	PE00003015	1.11742	0.21812	0.64766	At3g48890	cytochrome b5 domain-containing protein similar to SP|O00264 Membrane associated progesterone receptor component (mPR) {Homo sapiens}; contains Pfam profile PF00173: Heme/Steroid binding domain	1E-58
3220	PE00002575	1.11105	-0.45109	0.80430	At3g23600	dienelactone hydrolase family protein similar to SP|Q9ZT66 Endo-1,3;1,4-beta-D-glucanase precursor (EC 3.2.1.-) {Zea mays}; contains Pfam profile: PF01738 Dienelactone hydrolase family	4E-47
403	PE00000302	1.10705	-0.14334	0.80301	At5g06720	peroxidase, putative identical to peroxidase [Arabidopsis thaliana] gi|1491617|emb|CAA68212	3E-84
1020	PE00000774	1.09735		1.21605	At5g20820	auxin-responsive protein-related similar to auxin-induced protein TGSAUR21 (GI:10185818) Tulipa gesneriana]	2E-19
682	PE00000484	1.09444		0.46487	At5g65020	annexin 2 (ANN2) identical to annexin (AnnAt2) [Arabidopsis thaliana] GI:4959108	1E-112
3624	PE00002948	1.09172	-0.06185	0.29622	At4g34490	cyclase-associated protein (cap1) identical to cyclase-associated protein (cap1) GI:3169136 from [Arabidopsis thaliana]	2E-57
1615	PE00001252	1.08860	-0.36640	0.87778	At3g47810	calcineurin-like phosphoesterase family protein contains Pfam profile: PF00149 calcineurin-like phosphoesterase	2E-85

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
2724	PE00002129	1.08671	-0.36687	0.50291	At3g11530	vacuolar protein sorting 55 family protein / VPS55 family protein contains Pfam domain PF04133: Vacuolar protein sorting 55	3E-59
4571	PE00003868	1.08664	-0.41782	0.68268	At3g08610	expressed protein	3E-23
1462	PE00001137	1.07866	-0.01073	1.09346	At5g64460	expressed protein	1E-114
1013	PE00000769	1.06889	0.35519	0.13117	No hits found		
3890	PE00003202	1.06804	-0.03407	0.51589	At4g14880	cysteine synthase / O-acetylserine (thiol)-lyase / O-acetylserine sulfhydrylase (OAS1) nearly identical to SP|P47998 Cysteine synthase (EC 4.2.99.8) (O-acetylserine sulfhydrylase) (O-acetylserine (Thiol)-lyase) {Arabidopsis thaliana}; identical to cDNA O-acetylserine lyase (At.OAS.5-8) GI:6983573	6E-83
4662	PE00003959	1.06328	-0.58956	0.18704	At3g13550	ubiquitin-conjugating enzyme (COP10) identical to ubiquitin-conjugating enzyme COP10 [Arabidopsis thaliana] GI:20065779; contains Pfam profile PF00179: Ubiquitin-conjugating enzyme	7E-22
4623	PE00003920	1.05243		0.22132	At1g77670	aminotransferase class I and II family protein similar to kynurenine aminotransferase /glutamine transaminase K GI:1030066 [Rattus norvegicus]	1E-102
3170	PE00002527	1.02704		-0.25040	At1g60690	aldo/keto reductase family protein contains Pfam profile PF00248: oxidoreductase, aldo/keto reductase family	3E-76
2525	PE00001961	1.01220	0.25608	0.51589	At2g33040	ATP synthase gamma chain, mitochondrial (ATPC) identical to SP|Q96250 ATP synthase gamma chain, mitochondrial precursor (EC 3.6.3.14) {Arabidopsis thaliana}; contains Pfam profile: PF00231 ATP synthase	1E-122
5324	PE00004613	1.00134		0.58326	At2g18600	RUB1-conjugating enzyme, putative strong similarity to gi:6635457 RUB1 conjugating enzyme [Arabidopsis thaliana]; contains Pfam profile PF00179: Ubiquitin-conjugating enzyme	4E-79

Table 2 – Results of three microarray experiments. S4 vs S3 Fantasia, S4 vs S3 SR and S4 Fantasia vs S4 SR. This gene list is organized on the basis of the down-regulated genes in the S4 vs S3 Fantasia comparison (third column). The expression value is reported as $\log_2$ ratio of hybridization signals. Shaded background indicates down-regulation, black background indicates up-regulation and white background indicates no differential expression. Empty cells mark targets resulting not significative at SAM analysis. The "Ctg name " refers to the peach contig number in the database used to prepare the oligo probes of the µPEACH1.0 microarray. The "oligo ID" is the code assigned to each probe (4806 in total) by the manufacturer (Operon). At protein, Blastx results and e-value are those obtained by comparing peach sequences against Arabidopsis proteome (Trainotti *et al.*, 2006a).

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
4518	PE00003815	-2.65785	0.48238	-2.48872	No hits found		
1372	PE00001059	-2.62242	-0.25534	-3.30228	At5g51550	phosphate-responsive 1 family protein similar to phi-1 (phosphate-induced gene) [Nicotiana tabacum] GI:3759184; contains Pfam profile PF04674: Phosphate-induced protein 1 conserved region	1E-140
2089	PE00001610	-2.57611	0.28364	-1.75598	At5g54160	quercetin 3-O-methyltransferase 1 / flavonol 3-O-methyltransferase 1 / caffeic acid/5-hydroxyferulic acid O-methyltransferase (OMT1) identical to O-methyltransferase 1 [Arabidopsis thaliana][GI:2781394], SP|Q9FK25 Quercetin 3-O-methyltransferase 1 (EC 2.1.1.76) (AtOMT1) (Flavonol 3-O-methyltransferase 1) (Caffeic acid/5-hydroxyferulic acid O-methyltransferase) {Arabidopsis thaliana}	2E-73
1764	PE00001359	-2.51772	-0.30342	-2.31667	No hits found		
4121	PE00003422	-2.51235	0.65920	-2.33837	No hits found		
3043	PE00002415	-2.48770	1.27339	-1.94605	At5g20280	sucrose-phosphate synthase, putative similar to sucrose-phosphate synthase (EC 2.4.1.14) isoform 1 - Citrus unshiu, EMBL:AB005023	2E-90
1588	PE00001231	-2.46590		-2.49408	At5g06780	emsy N terminus domain-containing protein / ENT domain-containing protein contains Pfam profile PF03735: ENT domain	3E-49
4836	PE00004130	-2.45658		-1.28875	At5g60800	heavy-metal-associated domain-containing protein similar to farnesylated protein ATFP3 [GI:4097547]; contains Pfam profile PF00403: Heavy-metal-associated domain	5E-22
31	PE00000004	-2.42710	-0.70876	-2.37566	At1g15380	lactoylglutathione lyase family protein / glyoxalase I family proteincontains glyoxalasefamily protein domain Pfam:PF00903	1E-41

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
2727	PE00002131	-2.35626	-0.34108	-1.57219	At1g55510	2-oxoisovalerate dehydrogenase, putative / 3-methyl-2-oxobutanoate dehydrogenase, putative / branched-chain alpha-keto acid dehydrogenase E1 beta subunit, putative strong similarity to branched chain alpha-keto acid dehydrogenase E1 beta subunit [Arabidopsis thaliana] GI:7021286; contains Pfam profiles PF02779: Transketolase, pyridine binding domain, PF02780: Transketolase, C-terminal domain	2E-57
119	PE00000077	-2.34730	0.57613	-2.96884	At3g18280	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein similar to TED4 [Zinnia elegans] GI:493721; contains Pfam protease inhibitor/seed storage/LTP family domain PF00234	9E-24
1288	PE00000984	-2.31390	0.08261	-2.59894	At1g10200	transcription factor Ntlim1 [Nicotiana tabacum]	1E-89
4322	PE00003623	-2.29488	1.53035	-1.39452	At4g02280	sucrose synthase, putative / sucrose-UDP glucosyltransferase, putative strong similarity to sucrose synthase GI:6682841 from [Citrus unshiu]	5E-47
4949	PE00004242	-2.29022	0.00680	-1.51953	At3g10740	glycosyl hydrolase family protein 51 similar to arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II from GI:13398414 [Hordeum vulgare]	1E-29
2486	PE00001927	-2.27407	0.06566	-2.02948	At2g03500	myb family transcription factor contains Pfam profile: PF00249 myb-like DNA-binding domain	6E-23
4364	PE00003664	-2.26018	0.55674	-1.96797	At3g52090	DNA-directed RNA polymerase II 13.6 kDa subunit (RPB13.6) identical to SP|Q38859 DNA-directed RNA polymerase II 13.6 kDa polypeptide (EC 2.7.7.6) {Arabidopsis thaliana}	1E-19
1410	PE00001091	-2.25662	-0.24856	-1.76362	No hits found		
2471	PE00001912	-2.20397	1.10659	-1.77211	At2g45820	DNA-binding protein, putative identical to DNA-binding protein gi|601843|gb|AAA57124 [Arabidopsis thaliana];	5E-31
3195	PE00002551	-2.15645	0.01895	-3.49490	At3g13460	expressed protein contains Pfam profile PF04146: YT521-B-like family	2E-45
3064	PE00002432	-2.15250	0.44133	-2.47570	At4g39860	expressed protein	1E-15
1373	PE00001060	-2.14222	-0.44972	-2.68590	No hits found		
3742	PE00003060	-2.07194	-0.19344	-2.87530	At3g13460	expressed protein contains Pfam profile PF04146: YT521-B-like family	1E-88
1931	PE00001488	-2.06985	0.11649	-1.79501	At1g30230	elongation factor 1-beta / EF-1-beta identical to SP|P48006 Elongation factor 1-beta (EF-1-beta) {Arabidopsis thaliana}	3E-41
974	PE00000736	-2.00948	0.76262	-1.96158	At5g25610	dehydration-responsive protein (RD22) identical to SP|Q08298 Dehydration-responsive protein RD22 precursor {Arabidopsis thaliana}	1E-55
525	PE00000394	-1.95013	-0.04374	-1.60685	At1g50010	tubulin alpha-2/alpha-4 chain (TUA2) identical to tubulin alpha-2/alpha-4 chain SP|P29510 GB:P29510 from [Arabidopsis thaliana]	0

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
2902	PE00002287	-1.93030	-0.25489	-1.59549	At3g18830	mannitol transporter, putative similar to mannitol transporter [Apium graveolens var. dulce] GI:12004316; contains Pfam profile PF00083: major facilitator superfamily protein	1E-113
4042	PE00004789	-1.92867	0.37486	-0.40681	No hits found		
605	PE00000435	-1.90208	-0.02426	-2.49211	At5g04170	calcium-binding EF hand family protein low similarity to peflin [Homo sapiens] GI:6015440; contains INTERPRO:IPR002048 calcium-binding EF-hand domain	6E-79
2891	PE00002277	-1.89398	0.92975	-2.75516	At3g26510	octicosapeptide/Phox/Bem1p (PB1) domain-containing protein contains Pfam profile PF00564: PB1 domain	2E-33
2592	PE00002014	-1.88813	0.17660	-2.08909	No hits found		
3821	PE00003133	-1.82754	0.40187	-1.68703	At1g09020	protein kinase, putative similar to protein kinase AKINbetagamma-2 [Zea mays] GI:11139548, AKINbetagamma-1 [Zea mays] GI:11139546; contains Pfam profile PF00571: CBS domain	2E-27
2823	PE00002212	-1.81836	-0.35309	-1.17193	At2g31980	cysteine proteinase inhibitor-related contains similarity to extracellular insoluble cystatin GI:2204077 from [Daucus carota]	1E-20
3382	PE00002733	-1.81678	-0.22213	-1.45452	At1g53190	zinc finger (C3HC4-type RING finger) family protein similar to RING-H2 finger protein RHG1a GI:3822225 from [Arabidopsis thaliana]; contains Pfam profile PF00097: Zinc finger, C3HC4 type (RING finger)	4E-30
3714	PE00003034	-1.80938	0.39914	-0.83371	At1g12900	glyceraldehyde 3-phosphate dehydrogenase, chloroplast, putative / NADP-dependent glyceraldehydephosphate dehydrogenase, putative similar to SP|P25856 Glyceraldehyde 3-phosphate dehydrogenase A, chloroplast precursor (EC 1.2.1.13) (NADP-dependent glyceraldehydephosphate dehydrogenase subunit A) {Arabidopsis thaliana}; contains Pfam profiles PF02800: Glyceraldehyde 3-phosphate dehydrogenase C-terminal domain, PF00044: Glyceraldehyde 3-phosphate dehydrogenase NAD binding domain	1E-80
732	PE00000527	-1.80534	0.23624	-1.49568	No hits found		
822	PE00000602	-1.78183	-0.05692	-1.45859	No hits found		
704	PE00000502	-1.76327	-0.22155	-0.85877	At5g42570	expressed protein low similarity to SP|P51572 B-cell receptor-associated protein 31 {Homo sapiens}	4E-59
4525	PE00003822	-1.75811	-0.17528	-0.61719	At1g68410	protein phosphatase 2C-related / PP2C-related similar to protein phosphatase-2C GB:AAC36697 from [Mesembryanthemum crystallinum]	0.00001
3308	PE00002660	-1.75028	0.62996	-0.24983	At5g40200	DegP protease, putative contains similarity to DegP2 protease GI:13172275 from [Arabidopsis thaliana]	1E-109

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
1128	PE00000864	-1.74482	0.59900	-0.98934	At1g44130	nucellin protein, putative similar to nucellin GI:2290202 from [Hordeum vulgare]	2E-66
734	PE00000529	-1.71135	-0.12020	-2.08579	At1g28330	dormancy-associated protein, putative (DRM1) identical to dormancy-associated protein [Arabidopsis thaliana] GI:2995990; similar to dormancy-associated protein GI:2605887 from [Pisum sativum]; contains Pfam profile PF05564: Dormancy/auxin associated protein	1E-26
2430	PE00001876	-1.70870	-0.06095	-0.64172	No hits found		
618	PE00000443	-1.70144	-0.46049	-0.78859	At5g51190	AP2 domain-containing transcription factor, putative contains similarity to ethylene responsive element binding factor	2E-28
2758	PE00002156	-1.70116	-0.00522	-0.55297	At2g32700	WD-40 repeat family protein contains 7 WD-40 repeats ; similar to LEUNIG (GP:11141605)[Arabidopsis thaliana]	2E-40
412	PE00000310	-1.67854	1.24657	-1.99036	At5g01600	ferritin 1 (FER1) identical to ferritin [Arabidopsis thaliana] GI:1246401, GI:8163920	8E-60
1974	PE00001521	-1.66271	0.06232	-1.93491	At3g25520	60S ribosomal protein L5 similar to 60S ribosomal protein L5 GB:P49625 from [Oryza sativa]	4E-97
3312	PE00002664	-1.65015	0.34788	-1.49094	At2g28380	double-stranded RNA-binding domain (DsRBD)-containing protein contains Pfam profile PF00035: Double-stranded RNA binding motif	3E-64
5382	PE00004671	-1.63843		-2.61009	At2g39970	peroxisomal membrane protein (PMP36) identical to 36kDa-peroxisomal membrane protein (PMP36) GI:15146342 from [Arabidopsis thaliana]	4E-43
5000	PE00004293	-1.62652	0.19842	-1.22234	At4g03510	zinc finger (C3HC4-type RING finger) family protein (RMA1) identical to RING zinc finger protein RMA1 gi:3164222	2E-18
5287	PE00004577	-1.61566	-0.64657	-2.44511	At2g39570	ACT domain-containing protein contains Pfam ACT domain PF01842	2E-24
2809	PE00002200	-1.61418	-0.17449	-0.98796	At5g55390	hydroxyproline-rich glycoprotein family protein	9E-85
4207	PE00003508	-1.61233	-0.12668	-1.33398	At3g49660	transducin family protein / WD-40 repeat family protein beta-transducin, Schizosaccharomyces pombe, EMBL:CAA17803	5E-36
5374	PE00004663	-1.60945	0.22966	-0.52370	At5g54940	eukaryotic translation initiation factor SUI1, putative similar to SP|P32911 Protein translation factor SUI1 {Saccharomyces cerevisiae}; contains Pfam profile PF01253: Translation initiation factor SUI1	3E-14
441	PE00000326	-1.58233	-0.31692	-1.27913	At1g62510	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein contains Pfam protease inhibitor/seed storage/LTP family domain PF00234	6E-24
515	PE00000384	-1.57660	-0.20420	-0.57312	At3g14240	subtilase family protein contains similarity to SBT1 GI:1771160 from [Lycopersicon esculentum]	4E-48
3449	PE00002798	-1.55516	-0.15566	-0.30418	At3g08030	expressed protein contains Pfam profile PF04862: Protein of unknown function, DUF642	1E-141

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
4758	PE00004053	-1.55398	0.35319	-1.46857	At2g16230	glycosyl hydrolase family 17 protein similar to elicitor inducible chitinase Nt-SubE76 GI:11071974 from [Nicotiana tabacum]	2E-34
2474	PE00001915	-1.54798	-0.06513	-1.55392	At5g22650	expressed protein non-consensus AT donor splice site at exon 3, AC acceptor splice site at exon 4;	1E-13
765	PE00000556	-1.54598		0.15865	At3g10190	calmodulin, putative similar to calmodulin NtCaM13 [Nicotiana tabacum] GI:14625425, calmodulin GB:AAA34015 [Glycine max]; contains INTERPRO:IPR002048 calcium-binding EF-hand domain	9E-26
3511	PE00002851	-1.53629	-0.01029	-1.22017	At4g22760	pentatricopeptide (PPR) repeat-containing protein contains Pfam profile PF01535: PPR repeat	1E-35
1444	PE00001120	-1.53261	-0.01696	-0.89524	At5g46860	syntaxin 22 (SYP22) (VAM3) identical to GP|8809669| syntaxin related protein AtVam3p [Arabidopsis thaliana]	2E-32
3228	PE00002583	-1.51918	0.37781	-1.13714	AtCg00480	atpB ATPase beta subunit	0
4569	PE00003866	-1.49149	-0.00947	-1.31851	AtCg00800	rps3 ribosomal protein S3	3E-20
4678	PE00003975	-1.49129	-0.30276	-1.30667	At4g31940	cytochrome P450, putative cytochrome P450 monooxygenase, Pisum sativum, PATCHX:G894153	2E-41
1566	PE00001214	-1.48445	0.30922	-0.74262	At4g13340	leucine-rich repeat family protein / extensin family protein similar to extensin-like protein [Lycopersicon esculentum] gi|5917664|gb|AAD55979; contains leucine-rich repeats, Pfam:PF00560; contains proline rich extensin domains, INTERPRO:IPR002965	0.0000006
1664	PE00001287	-1.40933	-0.16972	-0.60613	At1g65820	microsomal glutathione s-transferase, putative similar to MGST3_HUMAN SP:O14880	3E-46
3193	PE00002549	-1.39115	0.22471	-1.22288	At3g60400	mitochondrial transcription termination factor-related / mTERF-related contains Pfam profile PF02536: mTERF	1E-52
4759	PE00004054	-1.37237	0.90451	-1.00813	At1g69840	band 7 family protein strong similarity to hypersensitive-induced response protein [Zea mays] GI:7716466; contains Pfam profile PF01145: SPFH domain / Band 7 family	1E-43
4161	PE00003462	-1.36981	0.30463	-0.89334	At2g34770	fatty acid hydroxylase (FAH1) identical to fatty acid hydroxylase Fah1p GB:AF021804 GI:2736147 from [Arabidopsis thaliana]	1E-73
2753	PE00002152	-1.36002		-0.21002	At4g29830	transducin family protein / WD-40 repeat family protein contains 7 WD-40 repeats (PF00400); G protein beta subunit-like protein, Schistosoma mansoni, gb:U30261	1E-33
2765	PE00002161	-1.34904	0.10078	-1.07134	At5g62390	calmodulin-binding family protein contains IQ calmodulin-binding motif, Pfam:PF00612	1E-39
5303	PE00004592	-1.32125	0.18650	-0.48580	No hits found		
1589	PE00001232	-1.31384	0.82840	-0.67928	AtCg00490	rbcL large subunit of riblose-1,5-bisphosphate carboxylase/oxygenase	0
4306	PE00003607	-1.31107	-0.39368	-0.56618	At2g36830	major intrinsic family protein / MIP family protein contains Pfam profile: MIP PF00230	2E-47

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
1054	PE00000805	-1.29986	-0.12114	-0.15014	At3g01470	homeobox-leucine zipper protein 5 (HAT5) / HD-ZIP protein 5 / HD-ZIP protein (HB-1) identical to homeobox-leucine zipper protein HAT5 (HD-ZIP protein 5) (HD-ZIP protein ATHB-1) GB:Q02283 [Arabidopsis thaliana]	3E-33
5225	PE00004516	-1.29812	0.17242	-0.41232	At5g51540	peptidase M3 family protein / thimet oligopeptidase family protein low similarity to SP|Q99797 Mitochondrial intermediate peptidase, mitochondrial precursor (EC 3.4.24.59) {Homo sapiens}; contains Pfam profile PF01432: Peptidase family M3	2E-38
2488	PE00001929	-1.29282	-0.18729	-0.22176	At4g38580	heavy-metal-associated domain-containing protein / copper chaperone (CCH)-related low similarity to copper homeostasis factor [PMID:9701579][GI:3168840]; nearly identical to farnesylated protein TFP6 [GI:4097553]; contains Heavy-metal-associated domain PF00403	2E-70
2130	PE00001645	-1.28511	0.24802	-0.46700	At3g54900	CAX-interacting protein 1 (CAXIP1) identical to cDNA CAXIP1 protein (CAXIP1) GI:27752304, CAXIP1 protein [Arabidopsis thaliana] GI:27752305	8E-55
4031	PE00003342	-1.26951	0.11256	-1.47379	At1g74780	nodulin family protein similar to nodulin-like protein [Arabidopsis thaliana] GI:3329368, nodule-specific protein Nlj70 [Lotus japonicus] GI:3329366	3E-80
196	PE00000148	-1.24025	0.75608	0.02658	At5g56550	expressed protein	1E-11
748	PE00000540	-1.23620	0.21581	-0.62764	No hits found		
4674	PE00003971	-1.23305	0.86369	-0.00165	At4g39780	AP2 domain-containing transcription factor, putative similar to AP2 domain containing protein RAP2.4,Arabidopsis thaliana	0.0000002
1696	PE00001316	-1.19721	0.50730	-0.81392	At5g38830	tRNA synthetase class I (C) family protein similar to SP|Q06752 Cysteinyl-tRNA synthetase (EC 6.1.1.16) (Cysteine--tRNA ligase) (CysRS) {Bacillus subtilis}; contains Pfam profile PF01406: tRNA synthetases class I (C)	3E-48
3538	PE00002873	-1.19472	-0.27698	-0.25454	At4g11220	reticulon family protein (RTNLB2) similar to SP|Q64548 Reticulon 1 (Neuroendocrine-specific protein) {Rattus norvegicus}; contains Pfam profile PF02453: Reticulon	2E-68
779	PE00004780	-1.18995		-1.65100	At3g50070	cyclin D3 [Malus x domestica	9E-31
1673	PE00001296	-1.18835	0.73002	-0.52645	No hits found		
1397	PE00001082	-1.17488	0.17746	-0.54172	At1g55900	NLI interacting factor (NIF) family protein contains Pfam profile PF03031: NLI interacting factor	2E-47
4392	PE00003691	-1.17027	-0.14602	-1.18559	No hits found		
929	PE00000698	-1.16524	0.05396	-0.38825	At5g10860	CBS domain-containing protein contains Pfam profile PF00571: CBS domain	2E-65
115	PE00000074	-1.16045	-0.28077	-0.81103	At1g68300	universal stress protein (USP) family protein similar to ER6 protein [Lycopersicon esculentum]	3E-38

ctg name	Oligo_ID	log S4/S3 Fantasia	logS4/S3 SR	log S4Fantasia/ S4SR	At protein	BLAST results	E-value
3547	PE00002880	-1.14913	0.28095	-0.13040	No hits found		
1511	PE00004799	-1.10995	-0.28022	0.06709	No hits found		
2017	PE00001553	-1.10788	-0.14858	-1.35794	At3g27090	expressed protein similar to gda-1 [Pisum sativum] GI:2765418	1E-118
59	PE00000028	-1.10775	-0.20951	-0.25069	At4g08685	pollen Ole e 1 allergen and extensin family protein contains Pfam domain, PF01190: Pollen proteins Ole e I family	3E-54
1882	PE00001452	-1.10191	-0.04282	-0.27658	No hits found		
3515	PE00002855	-1.08786	-0.66446	-1.43224	At3g21190	expressed protein contains Pfam PF03138: Plant protein family. The function of this family of plant proteins is unknown;	1E-117
3823	PE00003135	-1.08725	0.20707	-1.28656	No hits found		
499	PE00000369	-1.08371	0.02994	-1.18592	At2g22430	homeobox-leucine zipper protein 6 (HB-6) / HD-ZIP transcription factor 6 identical to homeobox-leucine zipper protein ATHB-6 (HD-ZIP protein ATHB-6) (SP:P46668) [Arabidopsis thaliana]	1E-20
1512	PE00001176	-1.08310	-0.08330	0.48764	No hits found		
2009	PE00001545	-1.08017	0.16137	-1.32806	At3g55750	60S ribosomal protein L35a (RPL35aD) ribosomal protein L35a.e.c15, Saccharomyces cerevisiae, PIR:S44069	2E-20
99	PE00000061	-1.07484	0.06609	-0.62595	At1g67360	rubber elongation factor (REF) family protein contains Pfam profile: PF05755 rubber elongation factor protein (REF)	3E-57
215	PE00000165	-1.07334		-0.69229	At1g20693	high mobility group protein beta1 (HMGbeta1) / HMG protein beta1 nearly identical to HMG protein (HMGbeta1) [Arabidopsis thaliana] GI:2832359	1E-31
3025	PE00002397	-1.06891	0.51965	-0.44428	At5g42340	armadillo/beta-catenin repeat family protein / U-box domain-containing protein low similarity to immediate-early	1E-82
1188	PE00000909	-1.04043	0.01567	-0.85230	At3g44110	DNAJ heat shock protein, putative (J3) identical to AtJ3 [Arabidopsis thaliana]	1E-173
853	PE00000626	-1.03562	-0.25212	-1.33544	At1g22410	2-dehydro-3-deoxyphosphoheptonate aldolase, putative [Nicotiana tabacum],	1E-39
941	PE00000708	-1.02679	-0.20772	-0.93671	At1g69530	expansin, putative (EXP1) identical to expansin (At-EXP1) [Arabidopsis thaliana] GI:1041702; alpha-expansin gene family, PMID:11641069	2E-97
1242	PE00000948	-1.01654	0.88320	-0.07956	No hits found		
3505	PE00002847	-1.01274	0.74043	-1.05191	At2g18730	diacylglycerol kinase, putative contains INTERPRO domain, IPR001206, DAG-kinase catalytic domain	1E-12
3663	PE00002984	-1.01240	-0.25604	0.52874	At5g58490	cinnamoyl-CoA reductase family similar to cinnamoyl-CoA reductase from Pinus taeda [GI:17978649], Eucalyptus gunnii [GI:2058311]	3E-80

Considering the 109 genes down-regulated genes identified during the transition from S3 to S4 in Fantasia fruit (Tab. 2), the comparison of the corresponding stages in SR pointed out that most of them appeared to be unchanged while four genes showed an opposite trend (Tab. 2). Of these 109 genes, sixty-two appeared to be more expressed in SR than in Fantasia when comparing fruit sampled at the same time (S4 stage of Fantasia) (tab. 2). Three of them are worthy of note: Ctg_499, is to a transcription factor (TF) belonging to HD-ZIP family that in sunflower induces a delay of senescence (Manavella *et al.*, 2006); Ctg_779 showing similarity to a cyclin D3 that is actively involved in cell proliferation phase of some fruits (Kvarnheden *et al.*, 2000), and Ctg_1288 similar to a NtLIM, a TF regulating the biosynthetic pathway of phenylpropanoids in tobacco (Rogers and Campbell, 2003). For Ctgs_779 and 1288 validation of microarray data has been performed via northern analysis. Fig. 5 shows that both genes resulted more expressed in SR than in Fantasia fruit in all the four stages of development.

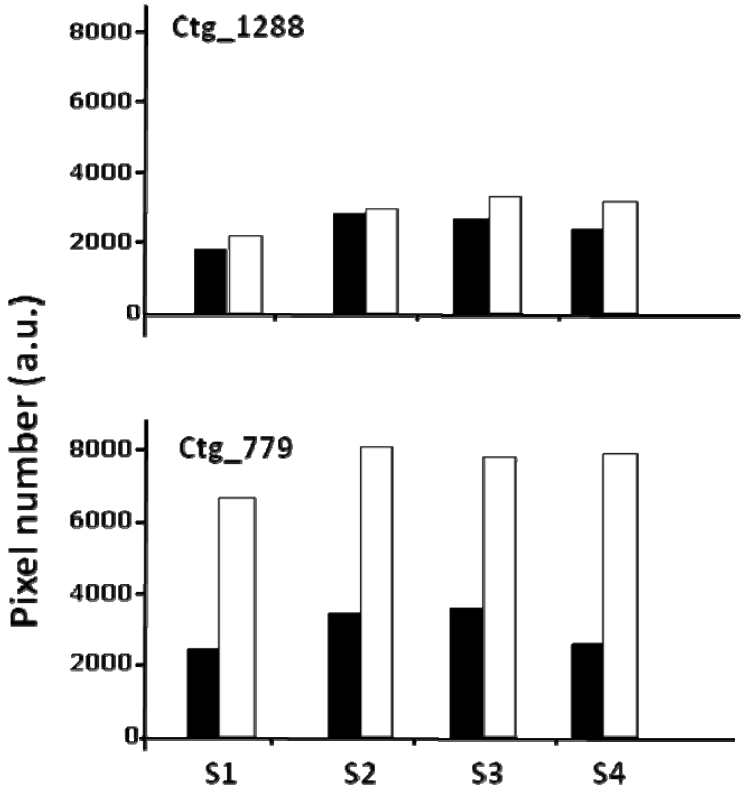

Figure 5 -Accumulation of Ctg_1228 (similar to *NtLIM*) and Ctg_779 (simiar to cyclin D3 type) transcripts throughout growth cycle in Fantasia (black bars) and SR (white bars) fruit.

For comparative purposes, expression ratio data of gene involved in ethylene physiology have been plotted together (Fig. 6) what emerges from the global analysis of these data is that SR fruit are characterized by a disturbance of ethylene biosynthesis resulting in (or accompanied by) an altered expression of genes involved the perception and signal transduction pathways (e.g. Ctg_4109, ETR2, Ctg_4591, EIN2 and Ctg_3350, ERF5).

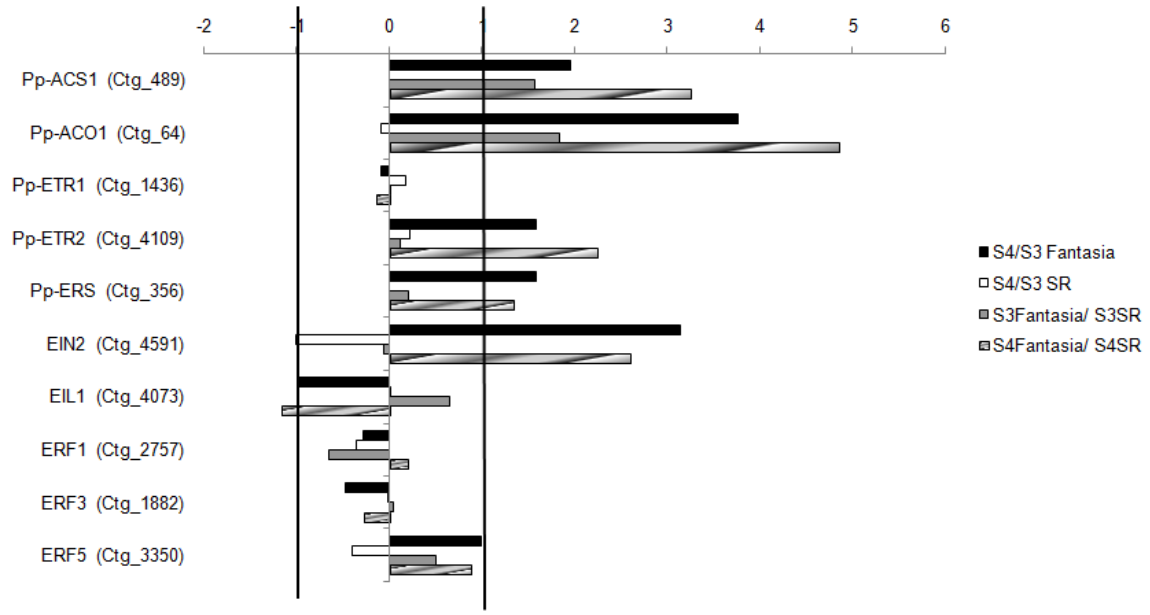

Figure 6 - Hybridization intensity ratio, in four different microarray comparisons of probes corresponding to genes involved in ethylene biosynthesis, perception and signal transduction spotted on µPEACH 1.0. The ratio threshold value has been arbitrarily fixed in -1 and 1.

Effect of ethylene-analogous propylene treatments on transcript profiles of Fantasia, SR and SH fruit.

Propylene treatment at preclimacteric stage induced a significant stimulation of ethylene biosynthesis in Fantasia and SR and not in SH (Tab. 3). Considering flesh firmness an accelerated softening process was observed in Fantasia and SH, but not in SR (Tab.3). These results suggest that mutations responsible for altered phenotype in SR and SH are related to different aspects of ethylene biosynthesis and/or action. In order to confirm this hypothesis microarray analyses were performed in propylene treated fruit by comparing transcriptome before and the end of 48h propylene treatment.

Table 3 -Ethylene biosynthesis and firmness value in Fantasia, SR and SH fruit at the beginning of treatment (T0), and after 48h in propylene and in air (control).

	Genotypes								
	Fantasia			SR			SH		
Parameters	T0	air	propylene	T0	air	propylene	T0	air	propylene
ethylene (nl/g/h)	0.08	0.15	**0.49**	0	0	**0.46**	0	0	**0**
firmness (N)	50	46	**8.5**	50	50	**49**	35	35	**6.9**

Propylene up-regulated 85, 17 and 54 genes in Fantasia and SR fruits, respectively (Fig. 7). In the up-regulated set only five genes were in common shared for all three genotypes. These genes, corresponding to Ctgs_ 1026, 1069, 1515, 2429, 4499, are similar to the pathogenesis-related protein PR-4B precursor, the thaumatin-like protein 1 precursor (PpAZ44), the Sodium-dicarboxylate co-transporter-like, an unknown protein and a putative invertase inhibitor, respectively (Tab. 4).

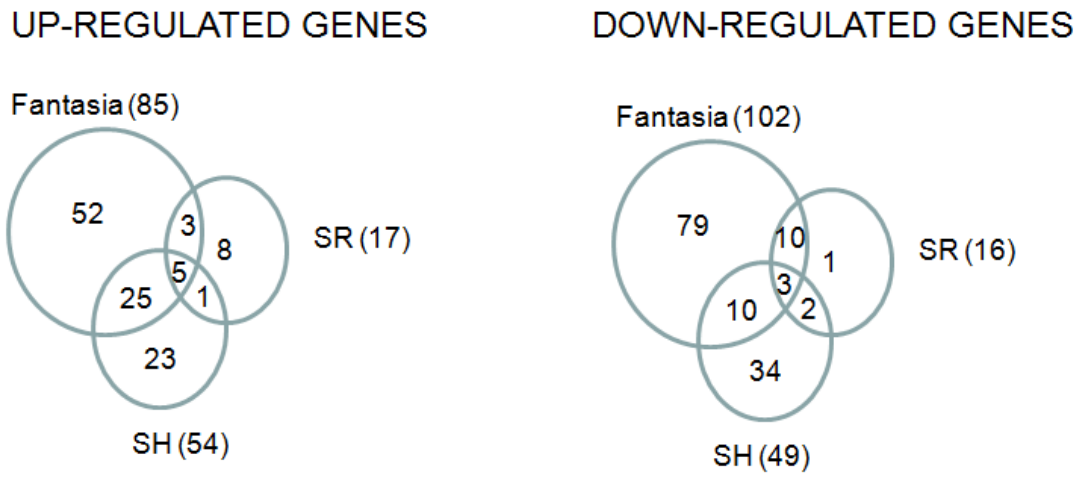

Figure 7 - The Venn diagram reports the number of induced and repressed genes in Fantasia, SR, and SH fruit fruit treated with propylene (48h) in comparison to fruit before the treatment (time zero). In the overlapping areas the number of genes induced and repressed in common are reported.

Considering only the comparison between Fantasia and SH the number of genes arose to 30. This set included ACO (Ctg_64), PG (Ctg_420), endo-1,4-beta-D-glucanase (Ctg_2196), Pp-EXP3 (Ctg_676), a PIN1-like protein (Ctg_3721), nine-cis-epoxycarotenoid dioxygenase2 (NCED, Ctg_2980). All these genes have been identified as

ripening-related in peach (Trainotti *et al.*, 2006a) indicating that in SH exogenous application of ethylene effectively induces the ripening syndrome. Only three (two Pectate lyases Ctg_1200 and 653, and an unknown protein Ctg_826) and one (an unknown proteins Ctg_458) up-regulated genes were shared by SR with Fantasia and SH, respectively. A large set of genes (52) was induced only in Fantasia. This includes four genes encoding for AUX/IAA proteins (Ctg_42, 57, 84, 1068) indicating that in the two mutants the relationship between IAA and ethylene is probably impaired (Tab. 4).

Propylene treatment negatively affected a total of 102 and 16 genes in Fantasia and SR fruit, respectively. In SH, the down-regulated set was composed by 49 genes. Only three genes corresponding to Ctgs_1528, 2909 and 4838 were common to all genotypes and they encode proteins having similarity to a dehydrin, an unknown protein and a nodulin-related protein, respectively (Tab. 4). Among those (10) shared between Fantasia and SR, no genes with function related to ripening or ethylene already identified were present. A catalase (Ctg_1024) was included in those down-regulated by ethylene and common to Fantasia and SH fruit. In Fantasia fruit, propylene specifically down-regulated 79 genes including some involved in oxidative stress (Fig. 7, Tab 4).

Table 4 - Results of three microarray experiments. Propylene treated vs control fruit in Fantasia, SR and SH genotypes. The expression value is reported as log_2 ratio of hybridization signals. Black background indicates up-regulation, shaded background indicates down-regulation and white background indicates no differential expression. Empty cells mark targets resulting not significative at SAM analysis. The "Ctg name " refers to the peach contig number in the database used to prepare the oligo probes of the μPEACH1.0 microarray. The "oligo ID" is the code assigned to each probe (4806 in total) by the manufacturer (Operon). At protein, Blastx results and e-value are those obtained by comparing peach sequences against Arabidopsis proteome (Trainotti *et al.*, 2006a).

ctg_name	Oligo ID	log Prop/T0 Fantasia	log Prop/T0 SR	log Prop/T0 SH	At protein	BLAST result	E-value
1026	PE00000780	3.66207	3.56281	4.23467	At3g04720	Pathogenesis-related protein PR-4B precursor.[Nicotia tabacum]	2E-53
1069	PE00000816	3.71066	5.19154	2.56706	At1g20030	Thaumatin-like protein 1 precursor (PpAZ44)	2E-80
1515	PE00001179	1.04676	1.00974	1.38875	At5g47560	Sodium-dicarboxylate cotransporter-like.[Arabidopsis thalia]	1E-121
2429	PE00001875	1.33998	1.33998	1.43103	At4g30410	unknown protein [Arabidopsis thalia]	3E-19
4499	PE00003797	3.35854	1.60301	3.68984	At1g47960	putative invertase inhibitor [Cicer arietinum]	2E-08
653	PE00000467	3.02104	1.09872	0.97600	At1g04680	Pectate lyase (Fragment).[Fragaria assa]	0
826	PE00000605	1.44568	1.80971	-0.47894	At5g18650	unknown protein [Arabidopsis thalia]	1E-143
1200	PE00000916	2.55212	1.78857	-0.39923	At4g24780	Pectate lyase.[Salix gilgia]	0
64	PE00000033	2.22569	0.38249	2.22926	At1g05010	1-aminocyclopropane-1-carboxylate oxidase	1E-123
68	PE00000036	1.31001	0.14004	1.16657	At1g02500	S-adenosylmethionine synthetase.[Litchi chinensis]	0
247	PE00000195	2.62225	-0.36522	1.23765	At1g30820	CTP synthase (CTP-synthetase, putative).[Arabidopsis thalia]	1E-137
298	PE00000235	2.62531	-0.24076	3.22262	At4g34590	bZIP transcription factor ATB2.[Glycine max]	5E-36
362	PE00000275	1.82947	0.65272	1.32336	At4g19120	dehydration-induced protein, putative [Arabidopsis thalia]	0
420	PE00000317	2.93304	-0.59070	3.07871	At3g59850	Endopolygalacturose.[Prunus persica]	1E-153
636	PE00000458	1.53175	-0.42436	1.31723	At5g51970	D-dependent sorbitol dehydrogese.[Prunus persica]	1E-95
651	PE00000466	1.01195	0.41194	1.23617	At3g21680	Hypothetical 20.4 kDa protein (Emb|CAB85509.1).[Arabidopsis thalia]	1E-09
676	PE00000481	3.21344	0.36479	1.34125	At2g28950	expansin [Prunus persica]	1E-111
771	PE00000560	1.02600	0.59237	1.08941	At1g04690	probable potassium channel beta chain KB1 - potato	5E-94
835	PE00000609	1.01205	0.08835	2.09611	At3g12120	omega-6 fatty acid desaturase [Prunus armeniaca]	2E-57

ctg_name	Oligo ID	log Prop/T0 Fantasia	log Prop/T0 SR	log Prop/T0 SH	At protein	BLAST result	E-value
938	PE00000706	2.04544	-0.07473	5.06567	At5g51520	Ripening-related protein-like.[Arabidopsis thalia]	1E-38
1112	PE00000851	1.36690	-0.52564	1.88877	At1g09310	unknown protein [Arabidopsis thalia]	1E-48
1415	PE00001094	1.50789		1.11974	At2g33840	probable tyrosine-tR ligase (EC 6.1.1.1) - common tobacco	1E-158
1752	PE00001352	1.73634	0.05317	1.35215	At1g33540	Glucose acyltransferase.[Lycopersicon pennellii]	3E-78
2196	PE00001697	1.67778	0.43586	1.13617	At1g64390	endo-1,4-beta-D-glucase [Pyrus communis]	1E-110
2839	PE00002227	1.53285	0.75796	1.20080		no hits found	
2895	PE00002280	1.00111	0.18683	1.13883	At2g26670	heme oxygese 1 [Lycopersicon esculentum	3E-69
2980	PE00002356	1.57000	-0.91510	1.20856	At1g78390	nine-cis-epoxycarotenoid dioxygese2 [Pisum sativum	3E-14
3039	PE00002411	1.80552	0.51449	1.15208	At2g19880	ceramide glucosyltransferase [Gossypium arboreum	1E-102
3709	PE00003029	1.04136	-0.03934	2.20756		no hits found	
3721	PE00003041	1.34725		1.34679	At1g73590	PIN1-like auxin transport protein.[Populus tremula x Populus tremuloides]	2E-81
4524	PE00003821	1.38173	0.76676	2.38143	At2g14580	pathogenesis-related protein, putative [Arabidopsis thalia]	1E-31
5104	PE00004397	2.06289		1.00748		no hits found	
4981	PE00004274	1.34916	0.56589	1.52892	At3g02630	no hits found	9E-23
358	PE00000273	1.42764	0.03638	0.52932	At5g65670	Aux/IAA protein.[Vitis vinifera]	6E-93
1	PE00000001	2.39220	0.85196	0.67387		no hits found	
42	PE00000013	1.03663	-0.13759	0.98965	At1g04250	IAA16 protein.[Gossypium hirsutum]	9E-49
55	PE00000025	2.22385	0.24848	0.27952	At1g28400	fiber protein E6 (clone CKE6-4A) - upland cotton	9E-11
57	PE00000027	1.05958	-0.79877	-0.41252	At3g04730	Auxin-induced protein AUX28.[Glycine max]	8E-73
61	PE00000030	1.55307	0.14264	0.77999	At5g20830	Sucrose synthase.[Citrus unshiu]	0
84	PE00000049	1.56344			At5g43700	Auxin-induced protein 22D (Indole-3-acetic acid induced protein ARG13).[Phaseolus aureus]	7E-54
124	PE00000082	1.01831	-0.48837	-0.37197	At1g05850	class1 chitise [Pisum sativum]	1E-143
243	PE00000191	1.49352		-0.07707		no hits found	
312	PE00000243	1.10387	-0.15977	0.83416	At2g21740	At2g21740 protein.[Arabidopsis thalia]	3E-25
638	PE00000459	1.09842	0.11362	-0.39647		no hits found	
648	PE00000464	2.15981		0.37250	At1g15760	Jp18.[Poncirus trifoliata]	3E-48
711	PE00000507	1.31480	-0.02621	0.15009	At4g25700	beta-carotene hydroxylase [Citrus unshiu]	1E-104
792	PE00000575	1.29688	0.44595	0.32521	At1g78300	14-3-3 protein.[Populus x canescens]	1E-123
939	PE00000707	2.89172	0.12762	0.95991	At2g40610	Expansin.(PPEXP1) [Prunus persica]	2E-97
981	PE00000742	1.28389				no hits found	
983	PE00000744	1.28649		0.32326	At3g57040	Response regulator 6.[Zea mays]	1E-55

ctg_name	Oligo ID	log Prop/T0 Fantasia	log Prop/T0 SR	log Prop/T0 SH	At protein	BLAST result	E-value
1019	PE00000773	1.16869	0.16540			no hits found	
1044	PE00000797	1.68870		0.11877	At2g46330	hypothetical protein At2g46330 [imported] - Arabidopsis thalia	1E-11
1068	PE00000815	1.90671	-0.10741	0.41235	At3g15540	Auxin-induced protein AUX22.[Glycine max]	7E-55
1094	PE00000838	1.89034	0.35726	0.84827	At2g19880	At2g19880/F6F22.9 [Arabidopsis thalia]	9E-47
1099	PE00000843	1.00631	0.51306	0.41842	At1g14130	oxygese ARRO-1, 2-oxoacid dependent - [Malus domestica]	9E-75
1101	PE00000844	1.37287	0.05426	0.61428	At5g26600	unknown protein [Arabidopsis thalia]	1E-161
1292	PE00000988	1.47326	-0.18619	-0.51552	At5g59845	skin-1 [Solanum tuberosum]	3E-28
1347	PE00001038	1.50656	0.54272	0.83406	At4g35300	Putative sugar transporter.[Oryza sativa]	1E-118
1460	PE00001135	1.11312		0.53418	At4g24040	Trehalase 1 GMTRE1.[Glycine max]	1E-113
1603	PE00001242	1.36915	0.90900	0.45813	At3g06840	glucosidase [Bacillus halodurans]	4.E-04
1774	PE00001368	1.02053	0.33741	-0.14456	At5g62890	permease 1 - like protein [Arabidopsis thalia]	0
1821	PE00001403	1.41196	-0.19907	-0.29656	At5g61440	thioredoxin oriza	3E-07
1852	PE00001429	1.19524	0.63572	0.20438	At5g26670	pecticetylesterase, putative [Arabidopsis thalia]	1E-141
1853	PE00001430	1.24420		0.53943		no hits found	
2139	PE00001652	1.25976	0.27313	0.00450	At2g28305	unknown protein [Arabidopsis thalia	7E-87
2179	PE00001683	1.10945	-0.53540	-0.10973	At3g06880	transducin / WD-40 repeat protein family [Arabidopsis thalia]	6E-59
2200	PE00001701	1.19488	0.29591	0.22739		no hits found	
2218	PE00001714	1.07939		-0.39427		no hits found	
2349	PE00001811	1.31985	0.56742	0.84760	At3g48990	OSJNBa0088H09.2 [Oryza sativa (japonica cultivar-group)]	2E-44
2396	PE00001849	1.03736	0.45899	0.42865	At4g36010	thaumatin family [Arabidopsis thalia]	1E-20
2513	PE00001951	1.06112	0.17263	0.04171	At2g43710	acyl-[acyl-carrier-protein] desaturase (stearoyl-ACP desaturase) [Arabidopsis thalia	2E-91
2719	PE00002124	1.54104	0.65259	0.82161	At4g12590	unknown protein [Arabidopsis thalia]	1E-117
2742	PE00002145	1.11994	-0.85476	0.23708	At3g14690	Putative cytochrome P450 protein.[Arabidopsis thalia]	3E-74
2775	PE00002168	1.11730	0.94660	0.70249	At5g67360	Putative serine protease.[Populus euramerica]	6E-33
3105	PE00002467	1.37361	0.33709	0.82421	At5g26667	predicted protein [Arabidopsis thalia	6E-90
3303	PE00002655	1.68078		0.11187		no hits found	
3351	PE00002702	1.00864	0.46782	0.88604	At2g26670	heme oxygese 1 (HO1) [Arabidopsis	2E-17
3449	PE00002798	1.69052	0.48911	-0.45541	At3g08030	unknown protein [Arabidopsis thalia]	1E-141
3452	PE00002800	1.07368		-0.42091		no hits found	

ctg_name	Oligo ID	log Prop/T0 Fantasia	log Prop/T0 SR	log Prop/T0 SH	At protein	BLAST result	E-value
3551	PE00002884	2.06452		0.61338	At5g51460	Trehalose-6-phosphate phosphatase.[Arabidopsis thalia]	2E-67
3620	PE00002944	1.11630	0.06897	0.79637	At4g16120	COBRA-like protein 7 precursor.[Arabidopsis thalia]	1E-71
3945	PE00003257	1.20669	0.41163	0.69106	At5g67360	subtilisin-like proteise (EC 3.4.21.-) 1 - tomato	2E-64
4235	PE00003536	1.01751	0.39401	0.44107	At2g26830	choline kise -related [Arabidopsis thalia	9E-55
4560	PE00003857	1.26282	-0.82619	0.23414		no hits found	
4621	PE00003918	1.77812		0.56271	At5g51460	trehalose-6-phosphate phosphatase (TPPA) [Arabidopsis thalia	2E-51
458	PE00000332	0.66223	2.47380	1.80233	At4g02380	hypothetical protein T14P8.2 - Arabidopsis thalia	1E-12
1528	PE00001188	-2.05987	-1.66819	-1.24877	At3g50970	Dehydrin.[Prunus persica]	7E-11
2909	PE00002294	-2.83718	-1.13052	-2.15031		no hits found	
4838	PE00004132	-1.36669	-2.14047	-1.34677	At2g16660	nodulin-related protein [Arabidopsis thalia]	1E-12
56	PE00000026	-1.50399	-1.11969	-0.03552		no hits found	
349	PE00000265	-2.01109	-1.11054	-0.65516	At2g16850	plasma membrane intrinsic protein 1c [Arabidopsis thalia]	1E-101
441	PE00000326	-1.23799	-1.32821	0.25905	At1g62510	HyPRP.[Fragaria assa]	6E-24
853	PE00000626	-1.25086	-1.15608	-0.27532	At1g22410	Phospho-2-dehydro-3-deoxyheptote aldolase 2, chloroplastprecursor (Phospho-2-keto-3-deoxyheptote aldolase 2) (DAHP synthetase 2) (3-deoxy-D-arabino-heptulosote 7-phosphate synthase 2	1E-39
945	PE00000712	-2.32187	-1.40917	-0.72890	At2g05100	Light harvesting chlorophyll A/B binding protein.[Prunus persica]	1E-117
971	PE00000734	-2.49469	-1.16955	-0.77962	At5g25610	Dehydration-responsive protein RD22 (Fragment).[Prunus persica]	3E-57
2370	PE00001828	-2.04879	-1.78155	-0.61510	At3g21510	Histidine-containing phosphotransfer protein.[Catharanthus roseus]	3E-48
2788	PE00002180	-1.24607	-1.50225	0.73426	At3g22120	proline rich protein - apple tree (fragment	2E-09
3007	PE00002382	-1.34418	-1.41156	-0.12538	At4g25650	Rieske [2Fe-2S] domain-containing protein [Arabidopsis thalia	8E-74
3872	PE00003184	-1.26013	-1.03879	-0.16098	At2g36390	1,4-alpha-glucan branching enzyme [Solanum tuberosum]	5E-10
115	PE00000074	-1.28765	0.04179	-1.28765	At1g68300	Hypothetical protein (At1g68300).[Arabidopsis thalia]	3E-38
285	PE00000226	-2.08534	-0.55508	-1.04816	At1g70850	major latex-like protein [Prunus persica	2E-43
412	PE00000310	-1.32923	-0.71195	-1.11089	At5g01600	Ferritin 1, chloroplast precursor.[Brassica pus]	8E-60
1024	PE00000778	-1.44669	0.44922	-1.09060	At4g35090	Catalase (EC 1.11.1.6).[Prunus persica] (cat2)	0
1300	PE00000995	-1.07735	-0.38585	-1.17546	At1g19530	unknown protein [Oryza sativa]	7E-08

ctg_name	Oligo ID	log Prop/T0 Fantasia	log Prop/T0 SR	log Prop/T0 SH	At protein	BLAST result	E-value
2471	PE00001912	-1.56116	-0.53846	-1.09310	At2g45820	Remorin (Putative D-binding protein) (Putative remorin protein).[Arabidopsis thalia]	5E-31
2741	PE00002144	-1.04651	-0.39585	-1.02039	At5g16550	unknown protein [Arabidopsis thalia]	3E-08
3228	PE00002583	-1.20479	-0.45279	-1.52362	AtCg00480	ATP synthase beta subunit [Prunus persica	0
3865	PE00003177	-1.22883	-0.82621	-1.86029	AtCg00020	no hits found	0
4905	PE00004199	-1.19666	-0.59010	-1.01870	At1g15820	sigl peptidase subunit -related [Arabidopsis thalia]	3E-64
308	PE00000240	-1.14309	0.94352	1.08254	At5g11650	Lysophospholipase-like protein.[Arabidopsis thalia]	1E-126
3430	PE00002781	-1.75937	-0.39642	2.38756	At5g59320	Nonspecific lipid-transfer protein 1 precursor (LTP 1).[Prunus dulcis]	3E-32
941	PE00000708	-1.53379	-0.32971	-0.70698	At1g69530	Expansin.(PPEXP2) [Prunus persica]	2E-97
85	PE00000050	-1.08351	-0.15476	-0.08141	At4g33000	calcineurin B-like protein (fragment) [Arabidopsis thalia]	8E-70
99	PE00000061	-1.39594	-0.41161	-0.91092	At1g67360	stress related protein -related [Arabidopsis thalia]	3E-57
111	PE00000070	-1.33339	-0.89210	-0.37156	At3g02555	unknown protein [Arabidopsis thalia]	2E-18
119	PE00000077	-1.44828	-0.98028	-0.36518	At3g18280	Nonspecific lipid-transfer protein 2 (LTP 2).[Prunus armeniaca]	9E-24
186	PE00000138	-1.04091	-0.34442	-0.35857		no hits found	
187	PE00000139	-1.93408	-0.05053	-0.38892	At2g04240	zinc finger (C3HC4-type RING finger) protein family [Arabidopsis thalia	2E-10
246	PE00000194	-1.23811	0.77428	0.40412	At3g56710	P0468B07.6 [Oryza sativa (japonica cultivar-group)]	9.E-04
350	PE00000266	-2.80815	-0.18947	0.03472	At3g53420	plasma membrane intrinsic protein 2-1 [Pyrus communis]	4E-73
402	PE00000301	-1.25009	-0.60467	-0.29945	At5g46860	Syntaxin.[Glycine max]	3E-92
640	PE00000460	-2.91141	0.63473	-0.60982	At4g25810	Xyloglucan endotransglycosylase.[Malus domestica]	1E-112
710	PE00000506	-1.52146	-0.29796	-0.08192	At3g27210	unknown protein [Arabidopsis thalia]	1E-17
898	PE00000670	-1.09234	-0.71241	-0.35412	At5g19450	calcium-dependent protein kise [Fragaria x assa	3E-38
946	PE00000713	-1.19527	-0.68333	-0.29015		no hits found	
973	**PE00000735**	**-1.39080**		**0.19986**	**At5g25610**	**Dehydration-responsive protein RD22 (Fragment).[Prunus persica]**	**6E-85**
974	PE00000736	-1.18815	-0.08940	-0.52120	At5g25610	Dehydration-induced protein RD22-like protein.[Gossypium hirsutum]	1E-55
986	PE00000747	-1.05201	0.32680	0.28599	At3g43810	Calmodulin 1.[Medicago truncatula]	2E-81
1005	PE00000763	-1.03175	0.33165	-0.78961	At2g43840	glycosyltransferase family [Arabidopsis thalia]	8E-55
1022	PE00000776	-1.40550	-0.16062	0.03748	At2g44110	seven transmembrane MLO protein family (MLO15) [Arabidopsis thalia]	1E-27

ctg_name	Oligo ID	log Prop/T0 Fantasia	log Prop/T0 SR	log Prop/T0 SH	At protein	BLAST result	E-value
1046	PE00000799	-1.20501	-0.16355	-0.27855	At3g63010	unknown protein [Arabidopsis thalia]	4E-53
1064	PE00000812	-2.33759	-0.53162	0.12300	At4g02280	sucrose synthase [Citrus unshiu]	1E-100
1083	PE00000827	-1.10021	0.87512	0.09212		no hits found	
1113	PE00000852	-1.35313	0.37217	-0.68305	At3g13110	serine acetyltransferase 1 [Nicotia tabacum]	6E-37
1115	PE00000854	-1.16254	-0.34386	0.21338	At2g36690	Putative giberellin beta-hydroxylase.[Arabidopsis thalia]	2E-44
1128	PE00000864	-1.33554	-0.33511	0.31051	At1g44130	nucellin protein, putative [Arabidopsis thalia]	2E-66
1184	PE00000906	-1.13235	-0.22038	-0.16982	At5g23040	unknown protein [Arabidopsis thalia]	1E-87
1257	PE00000959	-3.23641	-0.65751	0.37220	At5g62360	unknown [Arabidopsis thalia]	7E-54
1296	PE00000992	-1.24336	0.21412	-0.13552	At3g03870	unknown protein [Arabidopsis thalia]	5E-23
1307	PE00001002	-1.99797	-0.05441	-0.55080	At1g18880	nitrate transporter NRT1-2 [Glycine max]	8E-64
1512	PE00001176	-1.25036	-0.68323	-0.44164		no hits found	
1622	PE00001258	-1.12340	-0.36742	-0.27097	At4g23890	unknown protein [Arabidopsis thalia]	2E-46
1679	PE00001301	-1.23158	-0.29399		At3g61460	BRH1 RING finger protein [Arabidopsis thalia	2E-28
1711	PE00001326	-1.01622	-0.73912	-0.58942	At5g37770	B1147A04.10 [Oryza sativa (japonica cultivar-group)]	4.E-06
1751	PE00001351	-1.64664	0.73481	-0.99231	At1g73270	Glucose acyltransferase.[Lycopersicon pennellii]	1E-123
1816	PE00001398	-1.01022	0.23943	-0.21639	At4g19420	pectin acetylesterase (EC 3.1.1.-) precursor - mung bean	1E-24
1907	PE00001473	-1.23375	-0.06019	-0.20368	At1g30760	FAD-linked oxidoreductase family [Arabidopsis thalia	1E-73
1974	PE00001521	-1.16333	-0.75094	0.02962	At3g25520	ribosomal protein, putative [Arabidopsis thalia	4E-97
2068	PE00001594	-1.10649		-0.23332	At1g64450	B1060H01.30 [Oryza sativa (japonica cultivar-group	2.E-05
2088	PE00001609	-1.27817	-0.63588	0.25807	At1g01060	circadian clock associated1 [Mesembryanthemum crystallinum	4E-13
2103	PE00001623	-1.90252	-0.90024	-0.75191	At2g15890	unknown protein [Arabidopsis thalia	1E-47
2117	PE00001635	-1.35086	0.11616	-0.81841	At1g06620	Hypothetical 41.8 kDa protein (Fragment).[Prunus armeniaca]	1E-69
2183	PE00001687	-1.15192	-0.37952	-0.12904	At5g25360	unknown protein [Arabidopsis thalia	1E-52
2225	PE00001719	-1.92687	-0.27143	0.18419	At5g06320	harpin inducing protein [Nicotia tabacum]	5E-52
2250	PE00001741	-1.92043	-0.02789	0.25086	At4g28050	hypothetical protein [Arabidopsis thalia]	2E-84
2395	PE00001848	-1.03076	0.19145	0.49400		no hits found	
2458	PE00001901	-1.32542	-0.98600	-0.08340	At5g28840	Putative epimerase/dehydratase [Oryza sativa	0
2489	PE00001930	-1.53197	-0.14952		At3g26510	octicosapeptide/Phox/Bem1p (PB1) domain-containing protein [Arabidopsis thalia	3E-39

ctg_name	Oligo ID	log Prop/T0 Fantasia	log Prop/T0 SR	log Prop/T0 SH	At protein	BLAST result	E-value
2504	PE00001942	-1.01153	-0.52257		At2g41250	unknown protein [Oryza sativa (japonica cultivar-group)]	1E-101
2616	PE00002032	-1.24454	0.02382	-0.26708	At3g48700	unknown protein [Arabidopsis thalia	6E-55
2666	PE00002077	-2.07685	-0.93483		At1g64660	methionine/cystathionine gamma lyase -related [Arabidopsis	3E-64
2902	PE00002287	-1.63115	-0.38306	-0.04500	At3g18830	Putative sorbitol transporter.[Prunus cerasus]	1E-113
2956	PE00002336	-1.58640	-0.83873	0.06408	At5g11580	P0042A10.5 protein.[Oryza sativa]	3E-86
3059	PE00002427	-1.00159	-0.14715	-0.58383	At4g01150	unknown protein [Arabidopsis thalia	2E-52
3117	PE00002477	-1.00730	0.41803	-0.15085	At1g01470	putative desiccation protectant protein [Pyrus communis	4E-34
3220	PE00002575	-1.20479	-0.45279	0.41848	At3g23600	unknown protein [Arabidopsis thalia	4E-47
3338	PE00002690	-1.58654	0.15685	-0.17267	At3g61890	homeobox-leucine zipper protein ATHB-12 (HD-Zip transcription factor Athb-12) [Arabidopsis	5E-32
3441	PE00002791	-1.74982	-0.21976	-0.45515	At2g45400	2'-hydroxy isoflavone/dihydroflavonol reductase homolog (Fragment).[Glycine max]	1E-68
3563	PE00002895	-1.08266	-0.60161	-0.59784	At1g04560	T1G11.19 protein (Hypothetical 19.8 kDa protein).[Arabidopsis thalia]	4E-61
3702	PE00003022	-1.64342	-0.03966	-0.63459	At4g25650	Rieske [2Fe-2S] domain-containing protein [Arabidopsis thalia	1E-31
3778	PE00003093	-1.18052	-0.19808	-0.15197	At4g15440	fatty acid hydroperoxide lyase [Psidium guajava	6E-29
3927	PE00003239	-1.93151	-0.70239	-0.24244		no hits found	
4094	PE00003395	-1.09825	-0.13893	-0.12797	At3g18830	putative sorbitol transporter [Prunus cerasus]	1E-47
4322	PE00003623	-1.42538	-0.41774	0.02159	At4g02280	sucrose synthase 1 [Pyrus pyrifolia	5E-47
4330	PE00003631	-1.30532	-0.13402	-0.17173	At3g55610	VuP5CS [Vig unguiculata]	8E-81
4424	PE00003723	-1.22158	0.29261	-0.54974	At1g12780	UDP-glucose 4-epimerase (Galactowaldese) (UDP-galactose4-epimerase)	2E-62
4471	PE00003769	-1.26513	-0.88658	-0.69554		no hits found	
4645	PE00003942	-1.12812	-0.54396	-0.97448	At2g37660	unknown protein [Arabidopsis thalia]	1E-102
4668	PE00003965	-1.20548	0.12682	-0.72909	At5g05390	diphenol oxidase	5E-21
4759	PE00004054	-1.05748	0.38006	-0.61072	At1g69840	hypothetical protein [Cicer arietinum]	1E-43
4796	PE00004091	-1.62590	-0.26941			no hits found	
4911	PE00004205	-1.72309	0.11125		At4g26590	isp4 like protein [Arabidopsis thalia]	5E-66
5000	PE00004293	-1.21087	0.21593	-0.17281	At4g03510	putative RING protein [Populus x canescens]	2E-18
5016	PE00004309	-1.44069	-0.80697	-0.16329	At2g36390	1,4-alpha-glucan branching enzyme [Solanum tuberosum]	1E-29

ctg_name	Oligo ID	log Prop/T0 Fantasia	log Prop/T0 SR	log Prop/T0 SH	At protein	BLAST result	E-value
5049	PE00004342	-1.06735	-0.52226		At1g01060	LHY protein [Phaseolus vulgaris] transcription factor in circadian expression	3E-08
5303	PE00004592	-1.42181	-0.03331	0.56233		no hits found	
5374	PE00004663	-1.74839	-0.05082	-0.18086	At5g54940	translation initiation factor [Triticum aestivum]	3E-14
5424	PE00004713	-1.71094	-0.50983	-0.26311	At1g72520	lipoxygese [Nicotia attenuata]	1E-40
219	PE00000169		-1.28015	-1.28015	At3g24350	syntaxin of plants SYP32 [Arabidopsis thalia	3E-38
352	PE00000268	-0.72279	-2.05405	-3.62258	At2g45180	hybrid proline-rich protein [Catharanthus roseus]	3E-33
5471	PE00004760	0.16326	-1.72569	-0.20473	At2g15290	unknown protein [Arabidopsis thalia]	3E-34
30	PE00000003	-0.40780	0.40800	-1.36779	At4g22880	Leucoanthocyanidin dioxygese (EC 1.14.11.19) (LDOX) (Leucocyanidin oxygese) (Leucoanthocyanidin hydroxylase) (Anthocyanidin synthase).[Malus domestica]	1E-135
63	PE00000032	-0.82896	0.14566	-1.01484	At1g10630	ADP-ribosylation factor [Vig unguiculata]	1E-100
77	PE00000043	-0.41967	-0.79137	-1.26097	At1g19180	T29M8.5 protein (Hypothetical 27.6 kDa protein) (At1g19180/T29M8_5).[Arabidopsis thalia]	2E-42
324	PE00000252		-0.36889	-1.22043	At1g63240	OSJNBb0033G08.18 [Oryza sativa (japonica cultivar	2E-10
605	PE00000435	-0.42250	-0.18928	-1.18831	At5g04170	calcium-binding EF-hand family protein [Arabidopsis thalia]	6E-79
1107	PE00000846	-0.32015	0.01616	-1.30062	At2g21660	Putative glycine rich protein.[Rumex obtusifolius]	2E-35
1372	PE00001059	0.02920	-0.31381	-1.02090	At5g51550	AT5g51550/K17N15_10.[Arabidopsis thalia]	1E-140
1410	PE00001091	0.22794	-0.10672	-1.38227		no hits found	
1583	PE00001227	-0.64249	-0.60049	-1.08497	At1g62710	Vacuolar processing enzyme precursor (EC 3.4.22.-) (VPE).[Glycine max]	0
1647	PE00001277	-0.33842	-0.64183	-1.53825		no hits found	
1777	PE00001370	-0.88639	0.06967	-1.90648		no hits found	
1896	PE00001463	-0.84656	-0.32339	-1.17857		no hits found	
2045	PE00001575	-0.17173	-0.61630	-1.01083	At5g20700	senescence-associated protein -related [Arabidopsis thalia	1.E-04
2161	PE00001668	-0.96740	-0.49814	-1.09096	At4g27000	Putative D binding protein.[Arabidopsis thalia]	2E-81
2505	PE00001943	-0.59700	0.02978	-1.09384	At5g01220	UDP-sulfoquinovose:DAG sulfoquinovosyltransferase (sulfolipid synthase) (SQD2) [Arabidopsis thalia	2E-58
2727	PE00002131	-0.06386	-0.18811	-1.12524	At1g55510	branched-chain alpha-keto acid decarboxylase E1 beta subunit [Arabidopsis	2E-57
2823	PE00002212	-0.06821	-0.30912	-1.35941	At2g31980	cystatin [Malus x domestica	1E-20

ctg_name	Oligo ID	log Prop/T0 Fantasia	log Prop/T0 SR	log Prop/T0 SH	At protein	BLAST result	E-value
3033	PE00002405	-0.29315		-1.14466	At1g06550	3-hydroxyisobutyryl-coenzyme A hydrolase (CoA-thioester hydrolase) family [Arabidopsis thalia	7E-34
3065	PE00002433	-0.99302	0.70375	-1.65132	At5g59720	Heat shock protein 17.4.[Quercus suber]	6E-51
3067	PE00002434	-0.45757	-0.71773	-1.49960	At2g29500	small heat shock protein - soybean	2E-29
3072	PE00002439		-0.18176	-2.62570	At5g59720	heat shock protein [Daucus carota	8E-40
3188	PE00002544	-0.24537	0.76595	-1.02319	At1g30360	Unknown protein [Arabidopsis thalia	1E-34
3203	PE00002559	-0.84906	-0.64678	-1.28539	At1g79040	Photosystem II 10 kDa polypeptide, chloroplast precursor (Lightinducible tissue-specific ST-LS1 protein [Solanum tuberosum]	1E-39
3713	PE00003033	-0.36425	-0.04130	-1.57135	At2g23090	Hypothetical 8.7 kDa protein.[Cicer arietinum]	6E-33
3739	PE00003057	-0.87136	-0.13755	-3.12173	At2g46240	At2g46240 protein.[Arabidopsis thalia]	4E-11
3961	PE00003273	-0.35268	0.06517	-1.17498	At5g64140	shikimate kise homolog F23K16.170 - Arabidopsis thalia	4E-19
4207	PE00003508	-0.54399	-0.44943	-1.15820	At3g49660	transducin / WD-40 repeat protein family [Arabidopsis thalia]	5E-36
4515	PE00003812			-1.06211	At3g46940	dUTP pyrophosphatase-related protein [Arabidopsis thalia]	7E-22
4518	PE00003815	-0.31581	-0.58488	-1.01474		no hits found	
4569	PE00003866	-0.76506	-0.73358	-1.31621	AtCg00800	ribosomal protein S3 [Atropa belladon]	3E-20
4731	PE00004026	0.62855		-1.35074	At2g21790	ribonucleotide reductase [Nicotia tabacum	8E-81
4947	PE00004240	-0.25929	-0.77329	-1.89105		no hits found	
5287	PE00004577		-0.45110	-1.33937	At2g39570	ACT domain-containing protein [Arabidopsis thalia]	2E-24
2081	PE00004801	-0.38158	0.21249	-1.17649		no hits found	
1031	PE00000785		2.31225		At1g02640	Putative beta-D-xylosidase (PpAz152)	1E-174
1440	PE00001116	-0.83629	1.24277	-0.52660		no hits found	
1702	PE00001321	-0.02085	1.99901	-0.50434	At1g24020	Major allergen Pru ar 1.[Prunus armeniaca]	2E-07
3340	PE00002692		2.00883	0.14866	At3g54420	class IV endochitise [Vitis vinifera	2E-32
3771	PE00003087	-0.26870	1.15205	0.18252	At5g20250	glycosyl hydrolase family 36 [Arabidopsis thalia	3E-55
4674	PE00003971	0.46903	1.21672	0.17326	At4g39780	AP2 domain transcription factor, putative [Arabidopsis thalia	2E-07
4741	PE00004036	0.47991	1.05992	0.31433	At5g54240	unknown protein [Arabidopsis thalia	3E-74
5068	PE00004361	0.10108	1.10842	-0.07010	At3g22890	ATP sulfurylase [Brassica juncea]	6E-43
67	PE00000035	0.59117		1.52992	At3g22840	Putative early light induced protein (Fragment).[Arachis hypogaea]	2E-50
82	PE00000048	0.94625	-0.25805	1.71410	At1g76160	PS60 protein precursor.[Nicotia tabacum]	0

ctg_name	Oligo ID	log Prop/T0 Fantasia	log Prop/T0 SR	log Prop/T0 SH	At protein	BLAST result	E-value
205	PE00000157	-0.21405	0.90110	1.32902		no hits found	
459	PE00000333		-0.10059	1.40699	At5g57960	GTP binding protein-related [Arabidopsis thalia]	1.E-05
560	PE00000424	0.53797	0.79865	1.25250	At5g47550	Cystatin-like protein.[Arabidopsis thalia]	5E-18
745	PE00000538	0.88028	0.30857	1.36283		no hits found	
1206	PE00000920	0.30906	-0.02154	1.12095		no hits found	
1216	PE00000929	0.62582	-0.00119	1.25895	At1g66150	OSJNBa0070M12.3 [Oryza sativa (japonica cultivar-group)]	1E-43
1429	PE00001106	0.51807	0.97737	1.41988	At4g36880	Cysteine proteise precursor.[Phaseolus vulgaris]	1E-117
1472	PE00001145	0.41420	-0.03028	1.24508		no hits found	
2420	PE00001867	0.99540	0.02427	1.29354	At3g04870	Putative zeta-carotene desaturase (EC 1.14.99.30).[Helianthus annuus]	0
2527	PE00001963	0.29352	0.16638	1.05756		no hits found	
2757	PE00002155		0.17072	2.72574	At4g17500	ethylene-responsive element binding protein [Nicotia tabacum]	2.E-06
3350	PE00002701	-0.12933	0.91433	2.00925	At5g07580	ethylene response factor 1 [Lycopersicon esculentum]	7E-08
3429	PE00002780	-0.15552	0.14736	1.36443	At2g20370	unknown protein [Arabidopsis thalia	4E-99
3523	PE00002862	-0.25401	-0.10921	1.21022	At3g10985	F9F8.20 protein.[Arabidopsis thalia]	9E-27
4376	PE00003675	0.95430	0.73114	1.13022	At4g16120	GPI-anchored protein [Arabidopsis thalia]	1E-106
4729	PE00004024	0.38323	-0.17737	1.41600		no hits found	
4757	PE00004052	0.50209	0.53902	1.16768	At3g16770	AP2 domain transcription factor RAP2.3 [Arabidopsis thalia	2.E-05
5160	PE00004452	0.14710	0.42217	1.06033	At2g46690	auxin-induced (indole-3-acetic acid induced) protein family [Arabidopsis thalia	8E-10
5244	PE00004535	0.37267		1.03136	At4g03260	putative protein phosphatase regulatory subunit [Arabidopsis thalia]	2E-79

Discussion and conclusions

In climacteric fruit including peach, ethylene is considered to be a trigger of fruit-ripening events. Nevertheless, in ripe peaches ethylene peak occurs very late (Tonutti *et al.*, 1996) when some typical ripening-related processes, as firmness loss, are already initiated. In addition and differently from other climacteric fruits 1-Methylcyclopropene has only effect in delay peach fruit ripening (Rasori *et al.*, 2002; Dal Cin *et al.*, 2006; Begheldo *et al.*, unpublished). Based on these observations peach fruit appear to be an interesting model for studying ethylene physiology and the role of the gaseous hormone in the fruit development. Results reported in the present paper demonstrate that differently from SH, where the ethylene inhibition is due to a mutation involving *Pp-ACS1* gene (Tatsuki *et al.*, 2006) the phenotype of SR appear to be the result of a profound alteration of mechanism regulating growth and development. This is evident the reduced size of the SR fruit and the impaired developmental cycle not allowing the fruit to ripen. This block is associated with the low transcript accumulation of genes ethylene- and ripening- related as ACO (Ctg_64) and PG (Ctg_420) that resulted 29 and 45 times lower than those measured in Fantasia at S4 stage, respectively. The hyperproliferation of cells could be explained considering that among genes more expressed in SR fruit is present the Ctg_779 that encodes a cyclin D 3 type, a gene involved in cell division process. A slowing-down of plant growth and an alteration of leaf tissues differentiation accompanied by a very high number of cells has been reported for *Arabidopsis* by Dewitte *et al.*, (2003) when a cyclin D3 gene (*AtCyCD3, 1*) resulted over-expressed. In addition to Ctg_779, other two transcripts related to cell division, identified by using a cDNA AFLP approach, are more expressed in SR (Ziliotto *et al.*, 2005) supporting the hypothesis of a distrurbance in the regulation of growth processes occurring in this mutant. Beside the protein encoded by Ctg_779, other two candidates as responsible for altered SR fruit phenotype are two transcription factors belonging to LIM (Ctg_1200) and HD-ZIP (Ctg_499) families that show the same expression profile. In transgenic plant of tobacco, the down-regulation of *NtLIM* factor induced a lower transcription of genes involved in biosynthetic pathway of phenylpropanoids as phenylalanine ammonia-lyase (*PAL*), hydroxycinnamate: CoA ligase (*4CL*), and in particular, hydroxycinnamyl alcohol dehydrogenase (*CAD*) (Kawaoka *et al.*, 2000). Different expression level of *CAD* have been associated to different pulp firmness in

strawberry: compared to the cv Holyday (hard pulp) in cv Gorella (soft pulp) lower *CAD* transcripts accumulation has been detected (Salentijn *et al.*, 2003). In *Helianthus annuus* the expression of an hortologue (*Hahb-4*) of Ctg_499 had a major repressive effect on genes related to ethylene synthesis, such as *ACO* and *SAM*, and on genes related to ethylene signalling, such as *ERF2* and *ERF5* (Manavella *et al.*, 2006). In SR fruit, beside *ACO*, a lower expression was observed also for *ERF5*. In addition *Arabidopsis* plants overexpressing *Hahb-4* were less sensitive to external ethylene, entered in the senescence pathway later and did not shown the typical triple response (Manavella *et al.*, 2006). Also in SR fruit propylene treatment was scarcely effective since only 33 genes were differentially expressed at the end of incubation period and, among these, those related to ethylene biosynthesis and action, cell wall hydrolysis or pulp pigmentation were not present. A very limited effect of exogenous treatment with propylene on SR fruit types was reported by Brecht and Kader (1984), that did not observe changes in flesh color and only a tendency to enhance ethylene biosynthesis and softening after four weeks of continuous exposure to propylene. This is in agreement with the fact that the stimulatory propylene effect on gene transcription was restricted to genes highly sensitive to ethylene in peach ripening fruit as two pathogenesis related proteins (Ctg_1069 and 1026) that appeared to be the most repressed by the application of 1-MCP (Begheldo *et al.*, unpublished). These genes belong to the set common to the three analyzed genotypes indicating that exogenous ethylene is able to exert a positive action on them independently to the genetic background. The fact that in SH the number of genes affected by the hormone treatment is 103 against 33 in SR indicates that in the former, differently from the latter, some ethylene-related processes can be activated. The most evident is firmness loss (-81% at the end of treatment) and is in agreement with the up-regulation of *PG* (Ctg_420), *PpEXP3* (Ctg_676) and an endo-1,4-beta-D-glucanase (Ctg_2196). The increase in expression of these genes has been reported for another SH type fruit treated with ethylene (cv Manami) by Hayama *et al.*, (2006a). Besides these data, microarray analysis revealed that in SH, but not in SR, the treatment induced a strong expression of Ctg_2757, encoding a protein similar to an Ethylene Responsive Element binding protein (EREBP). In *Nr* tomato fruit its hortologue (*Le-EIL1*), when overexpressed, specifically induces *PG* transcription and a partial restoring of the wild phenotype (Chen *et al.*, 2004). In SH ethylene treatment induced the

expression of Ctg_2980 that encodes a protein similar to NCED, involved in ABA biosynthesis and actively transcribed during fruit ripening (Rodrigo *et al.* 2003) suggesting that a relationship between ethylene and ABA is operating in this genotype differently from SR. On the other hand, these two genotypes showed the same expression profile for genes involved in the cross-talk between auxin and ethylene. In fact, most the AUX/IAA genes (Ctg_42, 57, 84, 1068) affected by ethylene in Fantasia were included among those unchanged in SH and SR. Considering that Trainotti *et al.*, (2007) demonstrated that *ACS* is strongly induced by 1-naphthalene acetic acid (NAA) in ripening peaches, an analogous of auxin, and that in SH *ACS* transcription is impaired by a specific TF (Tatsuki *et al.*, 2006), the expression data herein reported suggest a possible implication of auxin in the determining the SH phenotype.

Chapter VI

Early steps for functional analysis of three Transcription Factors related to peach fruit development and ripening

Introduction

Transcription factors (TFs) are sequence specific DNA-binding proteins that are able of activating and/or repressing transcription (Riechmann *et al.*, 2000). They are largely responsible for the selectivity in gene regulation, and are often expressed in a tissue-specific, developmental-stage-specific, or stimuli-dependent manner. They play an important role in plant development (Long and Benfey, 2006) and belong to a number of families acting in a complex and interplaying network (Fig.1). Considering fruit development, TFs have been extensively studied in non-fleshy fruit as *Arabidospsis* siliqua (de Folter *et al.*, 2004) and, to a lesser extent in the model species for fleshy fruit tomato (Giovannoni 2001; 2004). Only few information is available on TFs function in non model fleshy fruit because of the limited number of functional genomics studies and, in the case of fruit tree species, for technical constraints as the well-known difficulties in the regeneration of transformed plantlets. However, the number of functional genomics approaches in fruit tree species is rapidly increasing as demonstrated by the recent development of large collections of ESTs, physical and functional maps, and expression databases based on transcriptome analysis carried out using different techniques and tools including microarray. Considering peach, a large fruit EST repertoire is now publicly available (http://www.itb.cnr.it/estree) and the first microarray, named μPEACH 1.0, has been recently developed (ESTree Consortium, 2005). The use of these tools to investigate the ripening process in peach (chapters II and V of the present thesis), allowed to isolate ripening related TFs and three of them have been chosen for a functional analysis in a heterologous system. The first (*PpNOR-like*) has been isolated from a cDNA library produced from S4 peach mesocarp tissues (Ziliotto *et al.*, 2005); the second (*PpLIM-like*) has been identified by using μPEACH 1.0 in the comparison of S3 and S4 stage of Fantasia cv and its "slow ripening" (SR) selection. The last TF (*LeAGL62-like*) has been found out by comparison of transcription profiles of apple, peach, grape and tomato ripe fruit using TOM1 microarray (http://bti.cornell.edu/CGEP/CGEP.html) (Ziliotto *et al.*, 2004).

PpNOR-like is a sequence similar to *LeNOR*, a TF belonging to the NAC family (NAM, ATAF1,2, CUC2) (Moore *et al.*, 2002). Mutation of this gene is responsible for the *nor* (*non-ripening*) tomato phenotype, a recessive mutant that fails to produce autocatalytic ethylene, to ripen even in presence of exogenous ethylene. The strongest evidence for non-

ethylene-mediated ripening control of *NOR* comes from the analysis of gene expression in fruit (Moore *et al.*, 2002; Yokotani *et al.*, 2004). These results have been interpreted to indicate that higher order regulatory constraints are placed on climacteric fruit maturation in addition to general ethylene biosynthesis and signalling. Such type of mechanisms could include fruit-specific regulation of certain subsets of ethylene-regulated genes or regulatory mechanisms that operate separately and in addition to ethylene.

PpLIM-like is similar to the *NtLIM*, transcription factor belonging to LIM family in tobacco and involved in the cell wall lignification (Kawaoka and Ebinuma, 2001). The Ntlim1 protein has two LIM domains that are cysteine-rich polypeptides composed of two special zinc fingers separated by a two-amino acid spacer. The LIM domain may function as an interface for protein-protein interaction (Crawford *et al.*, 1992; Sadler *et al.*, 1992) with a Pal-box binding specificity. The AC-rich motif, Pal-box, is an important cis-acting element regulating gene expression in phenylpropanoid biosynthesis. (Zhong and Ye, 2007). This transcription factor appeared to be more expressed in Slow ripening mutant SR than in wild type (nectarine melting flesh cv Fantasia) fruit (Chapter V).

The last, *LeAGL62-like,* is a tomato MADS box (MCM1, AGAMOUS, DEFICIENS, SRF) (Riechmann and Meyerowitz, 1997; Theissen *et al.*, 2000) similar to *AGL62* of *Arabidopsis thaliana*. It has been isolated in cross species TOM1 microarray hybridizations and appeared up-regulated during ripening only in fleshy climacteric fruit (apple, tomato and peach), but not in fleshy a non climacteric fruit (grape berries) and dry fruit (*Arabidopsis thaliana*) (Ziliotto *et al.*, 2004).

As functional genomics approach and in order to clarify their possible role in fruit ripening as controller of developmental traits in fruits independently of the considered species, in co-operation with Dr. J.J. Giovannoni (Boyce Thompson Institute-Cornell University, Ithaca-NY), the following strategies have been adopted:

- Complementation of the *nor* tomato mutant with *PpNOR-like.*
- Over-expression of approach of *PpLIM-like* in tomato wild type.
- Knockout of *LeAGL62* via RNAi technique in tomato wild type

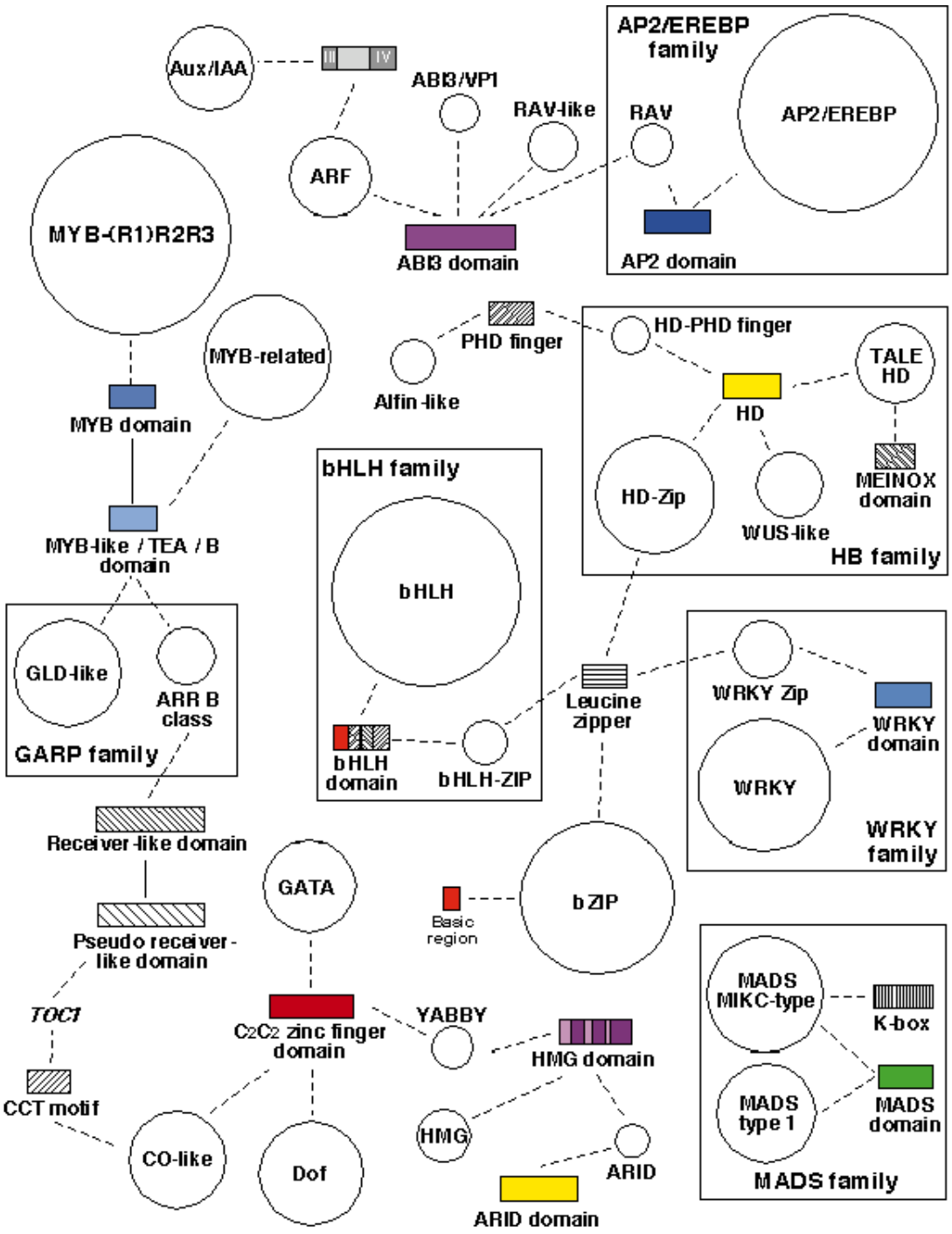

Figure 1 - Transcription factors net in plant: relationships and domain shuffling among the different *Arabidopsis* transcription factor families. Gene families are represented by circles, whose size is proportional to the number of members in the family. Domains that have been shuffled, and that therefore 'connect' different groups of transcription factors are indicated with rectangles, whose size is also proportional to the length of the domain. DNA binding domains are colored; other domains (usually protein-protein interaction domains) are shown with hatched patterns. Dashed lines indicate that a given domain is a characteristic of the family or subfamily that it is connected to. Gene names are written in italics. (from Riechmann *et al.*, 2000).

Material and methods

Plant material

Seeds from *nor* mutant and wild-type tomato (*Lycopersicum esculentum* Mill. cv Ailsa Craig) have been sowed in to produce plantlets to be used in transgene experiments (Frary *et al.*, 1987).

Plantlets were grown in a growth chamber and, after transformation, in a greenhouse under standard condition at Boyce Thompson Institute-Cornell University (Ithaca-NY).

DNA Constructs

For the overexpression of *PpLIM-like*, the PCR product containing the full open reading frame was obtained by amplifying the cDNA clone PPLea0021C04 (supplied from Clemson University, South Carolina) using the PpLIMSmaI and PpLIMSalI primers (Tab.1). The PCR product contains *PpLIM-like* DNA sequence ending with SmaI and SalI restriction sites. The PCR product was digested and gel-purified using QIAGEN spin column (QIAGEN). The gel purified digested PCR product was subcloned into pBTEX (Frederick *et al.*, 1998) in which the strong and constitutive 35S promoter of *Cauliflower mosaic virus* drives its expression (Fig. 2).

Following the same procedure, in order to complement the *nor* tomato mutant, *PpNOR-like* DNA was obtained by amplifying cDNA clone (AJ704829.1) using the primers PpNORXhoI and PpNORBglI (Tab.1). Digestion was conducted using XhoI and BglII restriction enzymes to insert the product into SLJ4K1 (Jones *et al.*, 1992) replacing GUS gene from the vector. EcoRI and SmaI digestion was performed to transfer the construct into the binary vector pBIN19 (Frisch *et al.*, 1995) (Fig.2).

In the third approach, in order to generate a *LeAGL62-like* RNAi construct, the partial cDNA clone (P74 clone; www.sgn.cornell.com) was amplified using LeAGL62Knockout primers (Tab.1). The primers contain *LeAGL62-like* gene-specific sequence with the addition of flanking attB1 and attB2 sequences used for Gateway site specific recombination. The 267 bp 3'UTR product with attB and attB2 sequences at either ends was gel purified (QIAGEN column) and subcloned into PHELLSGATE 2 vector (Wesley *et al.*, 2001) by site-specific recombination using the Gateway BP Clonase Enzyme Mix according to the manufacturer's instructions (Invitrogen). During this reaction the 5' ccdB

unit in the vector was replaced with the sense orientation PCR product and the 3' ccdB unit with an antisense orientation PCR product (Fig.2).

Complete sense (*PpNOR-like* and *Pp-LIM-like*) and RNAi (*LeAGL62-like*) constructs were transformed into *E.coli* DH 10B cells and the resulting plasmid was isolated and confirmed by using the endonuclease reaction (Tab.1) to release the insert and by DNA sequencing of the whole insert in pBTEX, PBIN19 and PHELLSGATE 2. The RNAi inserts were confirmed by digestion and by by sequencing using insert-specific vector primers.

One confirmed clone for each construct was electroporated into *Agrobacterium tumefaciens* strain LBA 4404 (Gibco BRL, MD). The resulting *Agrobacterium t*. was transferred into wild type and *nor* mutant tomato plants (*Solanum lycopersicon* cv. Ailsa Craig).

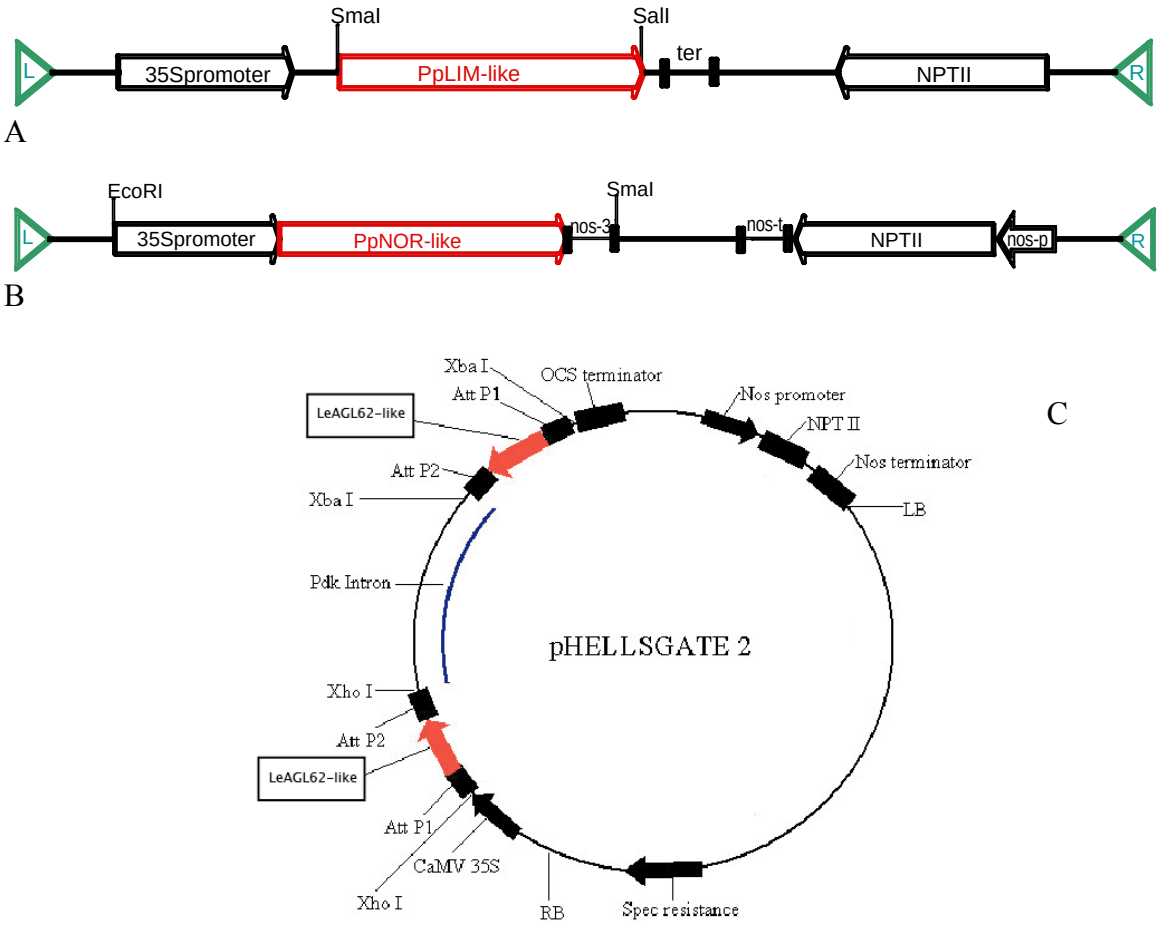

Figure 2- *PpLIM-like* (A), *PpNOR-like* (B) and *LeAGL62-like* (C) constructs. NPTII provides selection of transformants on kanamycin. Nopaline synthase promoter and terminator are represented by Nos-pro and Nos-ter while the right and left border of the Ti plasmid is denoted by RB and LB, respectively.

Plant transformation

For each construct 200 cotyledon explants from tomato wt or *nor* mutant AC have been transformed following the protocol proposed by Frary and Earle (1996). Shooting and rooting in selective media have been carried out following the same protocol in a growth chamber at 25°C and 16 hours photoperiod.

Tomato DNA isolation

Young expanding leaf tissue (1-2 g) from individual T0 plantlets was collected and total DNA was extracted as follows. The tissue was homogenised with 270 µl of DNA extraction buffer (0.35 Sorbitol, 0.1M Tris-base and 5mM EDTA) and incubate for 20 minutes at 65°C. After incubation 700 µl of chloroform:octanol (24:1) was added and the mix was centrifuged at 8000 rpm for 15 minutes. Supernatant was carefully transferred in a new tube with 700 µl of RT isopropanol and inverted until DNA precipitated. DNA was spin down for 10 minutes at maximum speed, washed with 60% ethanol, drayed and dissolved in 50 µl of TE buffer.

Confirmation of transgenic plants

Transgenic plants were confirmed by PCR of genomic DNA using the primers from 35S promoter (5'-CAAGAAGTACATTCTCGGAACAAA-3' and 5'-GAAGAGAGACCCAAGCAGTCAAT-3').

T1 generation plants

For each construct 10 positive T0 independents lines and one negative line have been recovered and grown in greenhouse. Select transformed T0 plants have been kept for seeds collection (T1).

T1 independent lines have been grown for each construct (4 lines for *PpLIM-like* and *PpAGL62-like*, and 2 lines for *PpNOR-like*). Total DNA extraction and PCR analysis were carried out in order to confirm the transgene presence, using 35S primers and specific transgene primers (Tab.1). Positive lines have been kept for phenotype characterisation.

Table 1- Primers sequence used to clone cDNA sequence into the vector and to screen transformed plants.

Primer name	Primer sequence	Amplified region
PpLIMSmaI	5'tttttcccggggtgtgaacaggaagaagtcatggca 3'	45-732
PpLIMSalI	5'tttttgtcgaccatctcattgtagcattcacaacct 3'	
PpNORXhoI	5'acgactcgaggtaatatgtgcttggctgcc 3'	4-1185
PpNORBglII	5'ataaagatctccatacaccaaccaaaccat 3'	
LeAGL62 Knockout	5'ggggacaagtttgtacaaaaaagcaggcttgataaatg gttcttcttcaatgg 3'	604-869
LeAGL62 Knockout	5'ggggaccactttgtacaagaaagctgggtcaagaagat gttaatgcaagaatcag 3'	
PpLIMspecific	5'ttgaagggacaccaaaaattg 3'	243-606
PpLIMspecific	5'atctcggcagcaacttctct 3'	
PpNORspec.	5'tttggaaggtgcaggttcat 3'	367-748
PpNORspec.	5'cgacgtcagggtattggaag 3'	
LeAGL62spec	5'tgataaatggttcttcttcaatgg 3'	604-869
LeAGL62spec	5'caagaagatgttaatgcaagaatcag 3'	
PpNOR3'UTR	5'aagactctatggaggacatgatgg 3'	658-1180
PpNOR3'UTR	5'gagagagcaagttggcaatagaag 3'	

Total RNA extraction and northern analysis

Tomato leaves and pericarp from different developmental stages [breaker fruits (BB), and 10 day after breaker (BB+10)] were collected from tomato (*Solanum lycopersicon* cv. Ailsa craig) plants. Leaves and pericarp tissues were immediately frozen in liquid nitrogen and homogenized with a mortar and pestle for RNA extraction. Total RNA was extracted as follows: homogenized leaf or fruit tissue (1-2g) was added to 10 ml of 80°C extraction buffer (100 mM Tris-HCl (pH 8.0), 25mM EDTA, 2% CTAB, 2%PVP, 2M NaCl and

0.5g/l Spermidine. The extraction mixture was vortexed prior to addition of 10 ml chloroform/isoamyl alcohol (24:1), vortexed again and centrifuged at RT at 13000 rpm for 25 minutes. Following centrifugation, the aqueous phase was removed and added to 10 ml of chloroform/isoamyl alcohol (24:1) and centrifuged at RT at 13000 rpm for 15 minutes. Following the centrifugation the aqueous phase was removed and RNA was precipitated with 4ml 10M LiCl overnigth. The precipitated pellet was washed with 70% ethanol and air-dried. The dried pellet was re-suspended in DEPC H_2O.

Total RNA (20μg) was denatured at 65°C for 15min with denaturing solution consisting of 25% (v/v) formamide, 8.25% (v/v) formaldehyde (pH 7.0), 20mM sodium phosphate buffer (pH 6.5), and 5mM EDTA (pH 8.0). Following denaturation, total RNA was fractionated through 1% (w/v) agarose gels containing 8% (v/v) formaldehyde. Gels were blotted onto Hybond N nylon membrane (Amersham-Pharmacia Biotech, Uppsala) according to the manufacturer's instructions. Filters were baked at 80°C for 2 h.

Probes for *Le-NOR* and *PpNOR-like* were obtained by PCR amplification of the corresponding cDNA clones using gene specific primers in the 3'UTR (Tab.1). PCR products were column purified and random hexamer labeled with ^{32}P. The filters were pre-hybridized at 42 °C overnight in a solution of 5X SSC, 0.5% (w/v) SDS, 5mM Na-P (pH 7.5), 5X Denhardt, and 0.1 mg/ml denatured salmon sperm DNA. Hybridization with heat-denatured probes was carried out at 42 °C overnight in 5X SSC, 0.5% (w/v) SDS, 5mM Na-P (pH 7.5), and 5X Denhardt's solution. After hybridization, filters were washed at 42 °C first in 2X SSC, 0.1% (w/v) SDS, then in 1X SSC, 0.05% (w/v) SDS, and finally in 0.5X SSC, 0.025% (w/v) SDS, for 20 min each time and exposed to X-ray film (Kodak).

Results and discussion

T0 plant regeneration

Plant regeneration after transformation (from explants until root formation) required a period of about 14 weeks. After this period a total number of 10 T0 plantlets for each construct was successfully obtained (Fig.3).

One *PpNOR:35S* line bore little red-orange little tomatoes suggesting a possible partial complementation. These fruit contained only few seeds, thus a limited T1 plants could be obtained.

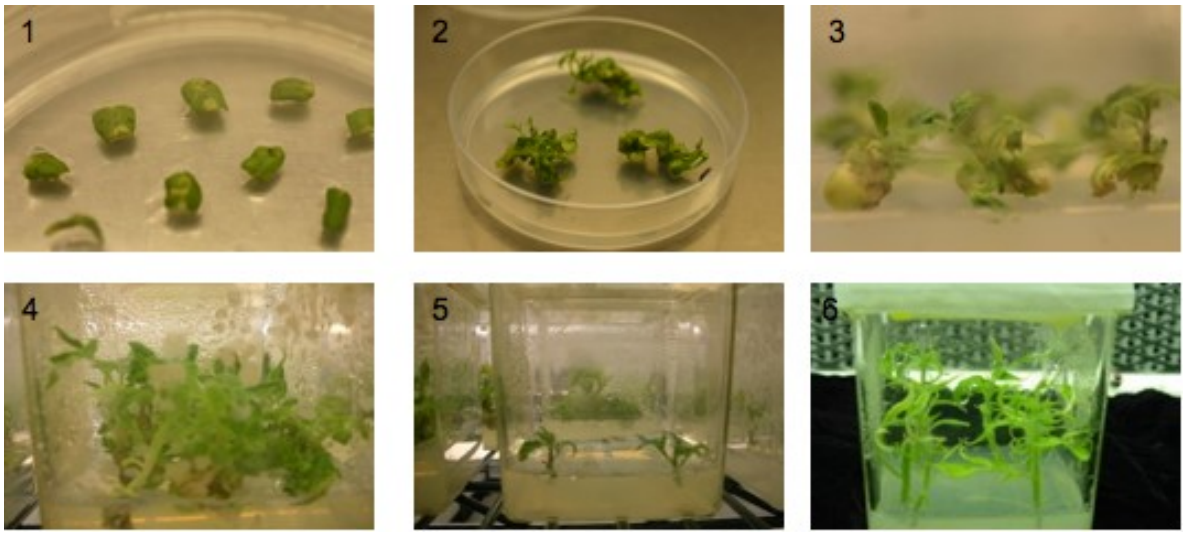

Figure 3 - Regeneration of transformed tomato Ailsa Craig plantlets. 1.Explants (6 days after transformation), 2. Callus (3 weeks after transformation), 3. and 4. Shooting explants (5 and 11 weeks after transformation, respectively), 4. and 5. Rooting shoots (13 and 21 weeks after transformation, respectively).

T1 plant genotyping

Genomic PCR with 35S and specific primers have been used to verify transgenic T1 plants. Ten plants were obtained for each of the four *PpLIM-like:35S* lines. Genomic PCR confirmed that transformant plants (five for each line) were recovered from all T1 lines. They didn't show any apparent altered phenotype in comparison to wild type (Fig. 4), except for two independent lines both showing curlier and thicker leaves. (Fig. 4). These

plants didn't yield fruit but only flowers with the exception of one plant bearing three small fruit

Four LeAGL62RNAi lines (10 plants for each) have been sowed. Two lines showed a unusual phenotype without flowers, but these plants didn't contain the transgene (Fig.4) Only two positive lines were recovered from T1 generation and the phenotype was no different from wild type.

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Figure 4. Transformant plants of *PpLIM-like*:35S line with two different phenotypes (1, 2 with leaf particular on 5); trasformant plant of *LeAGL62-like* RNAi line (3) and non-trasformant plant (4). From positive lines of both constructs some representative fruits at braker stage (6, *PpLIM-like* line and 8, *LeAGL62-like* line) and at 10 days after breaker (7, *PpLIM-like* line and 9, *LeAGL62-like* line) are reported.

Two *PpNOR-like:35S* lines have been sowed and only one was positive to the transgene test. This line (three plants) was derived from the T0 showing a possible partial complementation. For only two of them the presence of the transgene was detected (Fig.5, lanes 1 and 2).

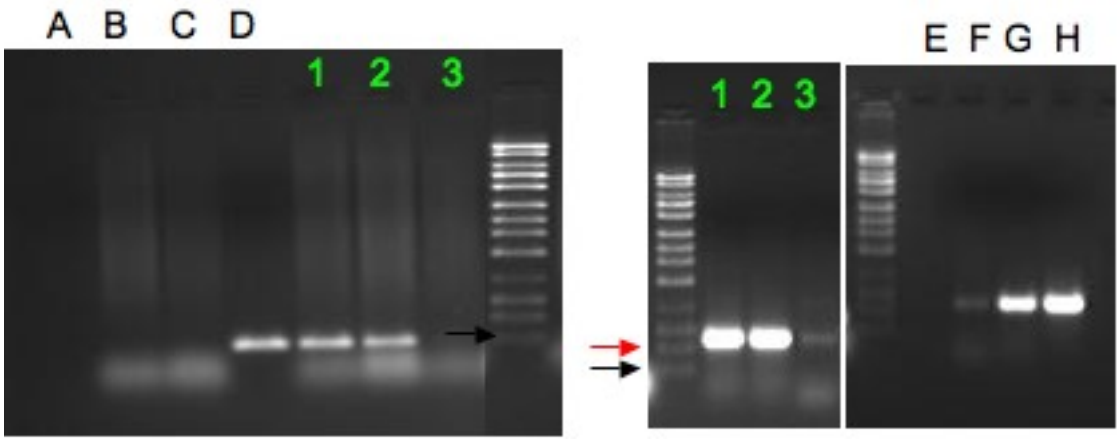

Figure 5- Genomic PCR on T1 lines *PpNOR-like:*35S. In the panel 1, amplification with PpNOR specific primers shows a presence of a band into two plants of the same line. Number 3plant was considered as negative control. Same results were obtained with 35S primers amplification as displayed in panel 2. Negative and positive controls for each amplification were performed (A/E Blank; B/F Ailsa Craig +/+; C Ailsa Craig *nor*; G Ailsa Craig transgenic plant; D/H PpNOR-like plasmid. Black arrow indicates 300bp and red indicates 500bp.

In one of these two plants (plant1), the typical *nor* phenotype with green-yellow and never ripening fruit was observed (Fig.6a); in the second (plant2), a partially complemented phenotype was observed (Fig. 6b). The third plant (plant3), in which the presence of the transgene was not detected showed the typical *nor* phenotype (negative control) (Fig. 6c).

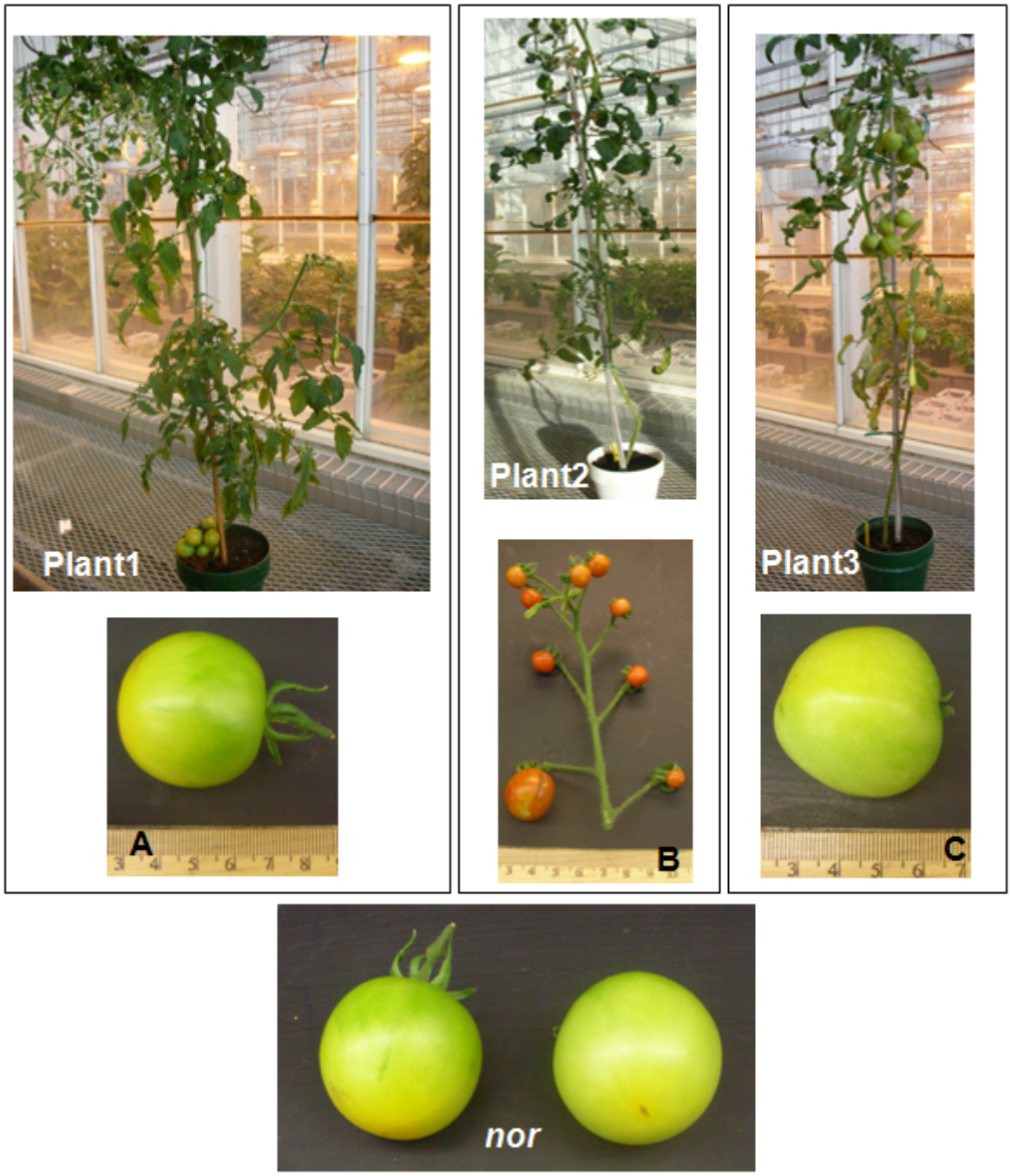

Figure 6- *PpNOR-like*:35S transformant plants and fruit. Three plants of the same line bearing fruits showing no complementation (plant1 and its fruit in A; plant3 and its fruit in C) and partial complementation (plant2 and its fruit in B) to when compared to the *nor* fruit phenotype (at the bottom).

Considering that only transformant lines carrying *Pp-NOR* showed altered phenotypes molecular investigations have been performed only for these plants. In these plants and the wild type Ailsa Craig (AC++) the level of transcripts corresponding to tomato NOR (*Le-NOR*, Fig. 7A) and peach NOR (*Pp-NOR*, Fig. 7B) was determined in leaf and fruit at 35 DAFB, corresponding to the breaker stage of AC++. For *Le-NOR* no hybridization was observed in leaf tissues and its accumulation in the fruit of three plants was similar and lower than that detected in AC++ (Fig. 7A). For *Pp-NOR*, the lack of ectopic expression in leaf tissues suggests that the transgene could be silenced. This could explain the fact that a faint signal was detected only in the orange fruit of the line showing a partial complementation (Fig. 7B, lane F2).

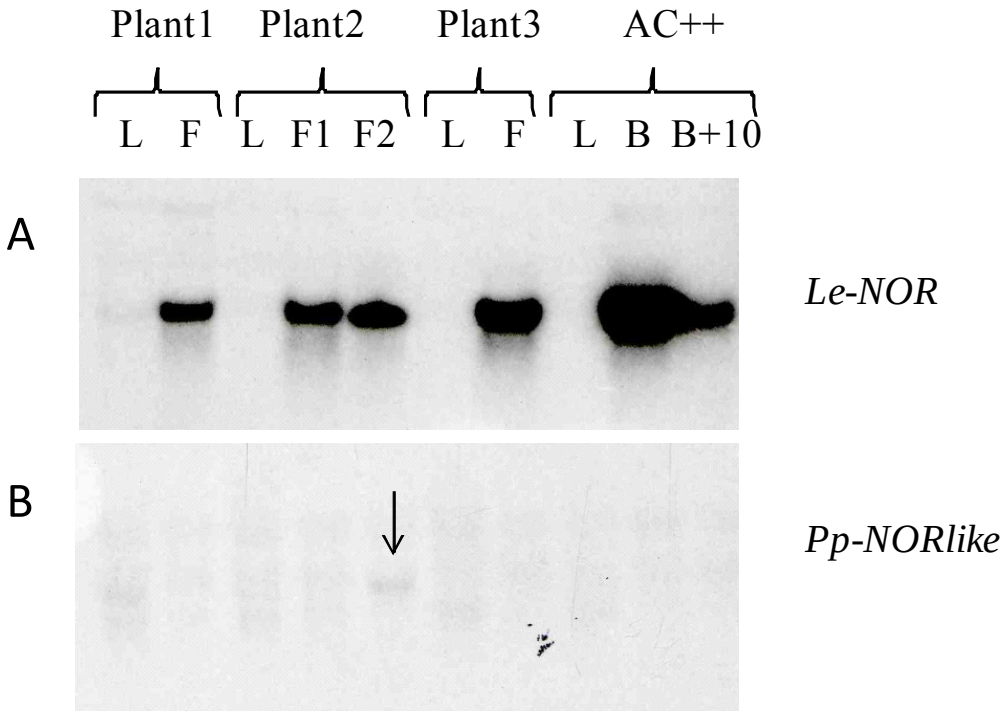

Figure 7 – Transcript accumulation of *Le-NOR* (panel A) and *Pp-NORlike* (panel B) in transformant (1,2 and 3) and Ailsa Craig (AC++) plants. L indicates leaf tissues, F indicates fruit, B fruit at breaker stage, and B+10 fruit at 10 days after breaker stage. Arrow indicates the faint hybridization signal of *PpNORlike* in fruit of Plant2 showing an altered ripening phenotype compared to *nor* plants.

These preliminary results are promising for further analyses aimed to ascertain whether *Pp-NORlike* is the hortologue of *Le-NOR* and to elucidate its role in ripening physiology of climacteric fruit

Concluding remarks

The recent development of high-throughput techniques and new biotechnological approaches covering broad field of disciplines (chemistry, physics, biology, physiology, computer science, robotics) have opened, also in plant research, the Genomics Era. In the last decade, comprehensive genomics approaches have been developed for many different plants, including some species bearing fleshy fruit of commercial interest. Functional genomics is aimed to study the expression of genes and to define their function and is based on complementary analyses: if transcriptional profiling describes gene expression patterns and gene regulatory networks, proteomics provides qualitative and quantitative information about proteins, and metabolomics is aimed to profile the range of metabolite present in the sample at a given time or under certain conditions. Using such approaches and techniques, alone or in combination, a great body of information is now available for tomato, the model species for fruit metabolism and ripening studies: a better insight of the mechanisms regulating specific quality-related pathways and the role of endogenous factor (as the plant hormone ethylene) is the result of the application of this integrated studies. Marked differences (in terms of quality parameters, physiology, structure) are however present among fruit types: thus, specific functional genomics protocols and approaches must be developed also for non-model species as in the case of peach. Results reported in this thesis demonstrate that the development of genomics tools as the EST database and the construction of the first fruit-specific microarray (μPEACH 1.0) revealed of paramount importance and great help in better elucidating the ripening physiology of peaches and the role played in this process by ethylene. In this context, collection and analysis of data obtained from transcriptome analysis of wild-type and ripening mutants together with the evaluation (at molecular level) of the effects of the ethylene action inhibitor (1-MCP) has allowed to better describe specific physiological traits and identify new genes and mechanisms involved in the evolution of ripening, the acquisition of quality traits, and possibly responsible for the postharvest behaviour of this important stone fruit species. Results reported here might also be useful for comparative studies within the *Rosaceae* family. The transcriptomics approach used in this thesis work must be considered as a

starting point of a wider strategy: optimization of field practices and storage (and transport) conditions depends upon a better understanding of basic processes and the complex mechanisms that link genotypes to phenotypes and responsible for the quality traits and evolution. This must be performed through the integration of genomics data sets resulting from the application of transcript and protein abundance, metabolite accumulation and metabolic flux analyses

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