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CURRICULUM AGROBIOTECHNOLOGY

CYCLE: XXV

DISSECTING THE TRANSCRIPTIONAL REGULATORY NETWORK OF SEED AND MESOCARP DEVELOPMENT IN PEACH

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Valerio Zaffalon, 31/01/2014

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Riassunto

Il pesco (*Prunus persica*) è uno dei più importanti alberi da frutto al mondo e la specie modello per le drupacee. Lo sviluppo del frutto di pesco è caratterizzato da un stretto rapporto tra il seme e il pericarpo durante i primi stadi, seguito negli stadi successivi da un disaccoppiamento nello schema di sviluppo dovuto alla lignificazione dell'endocarpo. Le varie cultivar di pesco possono avere dei periodi di sviluppo del frutto dalla lunghezza estremamente variabile, pur avendo un seme che si sviluppa in maniera simile. Per questo, comprendere la relazione tra seme e pericarpo può chiarire il meccanismo che regola lo sviluppo del frutto nel suo complesso. L'approccio transcrittomico è uno strumento potente per analizzare questa relazione, dato che produce un gran numero di informazioni sulla trascrizione di un gran quantitativo di geni in un singolo esperimento.

Il Capitolo II consiste in un articolo pubblicato che descrive l'uso dell'array µPEACH1.0 nello studiare la relazione tra seme e mesocarpo e tra stadi iniziali e finali di sviluppo nella cultivar Fantasia. Campioni di mRNA di pesco sono stati raccolti dagli stadi iniziali e finali dei due organi e ibridizzati sulle 4 806 sonde dell'array µPEACH1.0. I dati trascrittomici ottenuti da questi campioni sono stati quindi confrontati.

Sono stati trovati dei geni marcatori per i quattro stadi di sviluppo del pesco (Stadio S1: divisione ed espansione cellulare nel frutto, S2: lignificazione dell'endocarpo, S3: espansione cellulare nel mesocarpo, S4: maturazione) sia per il mesocarpo che per il seme e la loro espressione confermata con la qRT-PCR: I marcatori stadio-specifici per il mesocarpo sono rispettivamente per S1, S2, S3 e S4: una proteina RD22-like, una serin-carbossipeptidasi, una proteina correlata alla senescenza e una Aux/IAA; mentre per il seme sono, rispettivamente: una proteina trasportatrice di lipidi (LTP1), una proteina correlata alla patogenesi (PR), una prunina e una proteina LATE EMBRYOGENESIS ABUNDANT (LEA). La qRT-PCR ha confermato che questi geni sono marcatori anche in una cultivar precoce (SpringCrest) e in un genotipo a maturazione lenta (*slr*).

Quindi i dati sono stati analizzati con lo strumento HORMONOMETER al fine di misurare indirettamente il quantitativo relativo di ormoni nei vari organi e stadi di sviluppo. E'

emerso che l'auxina, le citochinine e le gibberelline possono essere coinvolte nella segnalazione durante l'inizio dello sviluppo, quando vi è comunicazione tra i due organi.

Il Capitolo III è un articolo non pubblicato nel quale viene descritto come venga utilizzata una nuova piattaforma microarray (µPEACH3.0) nello studiare lo sviluppo del seme e del mesocarpo di pesco. La recente pubblicazione del genoma di pesco ha permesso lo sviluppo di un microarray che copre l'intero genoma, superando così il problema di avere un array che misura l'espressione genica solo di una parte del genoma. Rispetto allo studio descritto nel Capitolo I, anche il numero di campioni è stato incrementato: sono state usate tre repliche biologiche per sei diversi momenti per ciascuno dei due organi, dando così una visuale più vasta sullo sviluppo di questi due tessuti.

L'array µPEACH3.0 ha funzionato bene, dando una correlazione con i dati di qRT-PCR pari a 0.77, un numero simile a quello trovato per altri array. I dati trascrittomici hanno facilmente distinto i due tessuti e i sei campionamenti, come mostrato dall'analisi delle componenti principali. Il 69% delle sonde ha prodotto un segnale significativo in almeno uno dei campioni, ciò nonostante, considerando che il numero di sonde funzionanti decresce se si prende in considerazione un solo tessuto, è probabile che testando il microarray con mRNA proveniente da altri tessuti (come le foglie o le radici) aumenti il numero di segnali significativi provenienti dall'array.

L'analisi globale dell'attività genica è stata indirizzata ai primi stadi di sviluppo. I dati hanno permesso di indentificare parecchi geni coinvolti nei processi del ciclo cellulare che si verificano all'inizio dello sviluppo sia del mesocarpo che del seme. In particolare, è stato trovate che geni della famiglia *TITAN* sono attivi nel seme contenente endosperma. L'analisi dei geni del ciclo cellulare nel mesocarpo ha mostrato l'esistenza di due diversi profili di espressione: mentre i geni relativi alla mitosi erano espressi solo nello stadio S1, i geni della replicazione del DNA hanno mostrato un doppio picco di espressione, in S1 e poi in S3/S4, suggerendo che in questi stadi possono verificarsi eventi di endoreduplicazione. Con l'utilizzo di qRT-PCR, i livelli d'esrpessione di questi geni sono stati testati anche in altre cultivar; i dati ottenuti suggeriscono che nel genotipo *slr* la mancanza di endoreduplicazione possa essere coinvolta nel basso tasso di crescita durante lo stadio S3 di questo genotipo.

Sono stati quindi valutati i profili d'espressione di famiglie di fattori di trascrizione (FT), dato che si ritiene che i fattori di trascrizione siano le proteine con i ruoli più importanti nella regolazione durante lo sviluppo. E' stato trovato che FT delle famiglia SQUAMOSA promoter Binding Protein (SBP) hanno un alto livello di espressione all'inizio dello sviluppo di entrambi gli organi considerati, il quale successivamente diminuisce velocemente. E' stato scoperto che nel seme maturo è indotta la trascrizione di FT di tipo Growth-Regulating Factor (GRF). Questi dati sono stati confermati con l'utilizzo di qRT-PCR in 'SpringCrest' precoce e nel genotipo a lenta maturazione *slr*.

Dato che in altre specie vegetali l'abbondanza dell'mRNA di geni appartenenti a queste famiglie di FT è regolata da microRNA (miRNA) specifici, è stata misurata l'espressione degli omologhi di pesco di questi miRNA. In tre diverse cultivar è stata trovata una correlazione negativa nel contenuto di RNA per le seguenti coppie microRNA/FT: miR156/SBP, miR396/GRF e mir167/ARF8, suggerendo non solo che questi miRNA posseggono la stessa attività un pesco, ma anche che i miRNA sono profondamente coinvolti nella rete regolativa sottostante lo sviluppo del frutto di pesco.

In appendice vi è uno studio pubblicato nel quale viene descritto l'uso di μPEACH3.0 nello studiare gli effetti delle ferite su due cultivar con diversa tolleranza a questo stress. Sono stati utilizzati campioni di RNA estratti da mesocarpi feriti o intatti della cultivar "melting" Glohaven (GH) e della cultivar "slow melting" BigTop (BT). I dati trascrittomici, confermati dall'analisi tramite qRT-PCR, hanno mostrato il coinvolgimento di fattori di trascrizione di tipo WRKY, AP2/ERF, e HSP20 nella risposta di GH alla ferite. Insieme a questi, è stato trovato che nel mesocarpo ferito di GH viene indotta l'espressione anche di geni coinvolti nella risposta agli stress, nel metabolismo della parete cellulare, nella biosintesi dei fenilpropanoidi e triterpenoidi.

Summary

Peach (*Prunus persica*) is one of the most important fleshy fruit crops worldwide and model species for drupe plant species. Peach fruit development is characterized by a tight relationship between seed and pericarp during the early stages, followed in later stages by an uncoupling in the pattern of development due to the lignification of the endocarp. Diverse peach cultivars may have fruit developmental periods of very different length, while having a similar development for the seed. Understanding the relationship between seed and pericarp sheds light onto the mechanism regulating fruit development. Transcriptomic approach is a powerful tool to investigate this relationship, as it gives broad information on the transcription of a large amount of genes in a single experiment.

Chapter II is a published article regarding the use of the µPEACH1.0 array for the understanding of the relationships between seed and mesocarp and between early and late stages of development in the cultivar Fantasia. Peach mRNA samples were taken from early and late developmental stages of the two organs and then hybridized on the 4 806 probes of the µPEACH1.0 array. The transcriptomic data obtained from these samples were then cross-compared.

Marker genes for the four peach developmental stages (S1 stage: fruit cells division and enlargement, S2: lignification of the endocarp, S3: mesocarp cell expansion, S4: ripening) were found for both mesocarp and seed and their expression confirmed by qRT-PCR. Stage- specific markers found for the mesocarp were a RD22-like protein, a serin- carboxypeptidase, a senescence-related protein and an Aux/IAA, for S1, S2, S3 and S4 stages, respectively, while seed markers were a lipid transfer protein(LTP1), a pathogenesis-related (PR) protein, a prunin and Late Embryogenesis Abundant (LEA) protein, for S1, S2, S3 and S4 stages, respectively. By qRT-PCR it was confirmed that these genes act as markers also in an early cultivar (SpringCrest) and a slow ripening genotype (*slr*).

Then, the data were analyzed with the HORMONOMETER tool in order to indirectly measure the relative amounts of hormones in the different organs and developmental

stages. It was found that auxins, cytokinins, and gibberellins may be involved in signaling during the early development, when there is cross-talk between the two organs.

Chapter III is an unpublished article in which it is described how a new microarray platform, µPEACH3.0, was employed to study peach mesocarp and seed development. The recent publication of the peach genome allowed the development of a whole-genome microarray which overcame the problem of having an array assessing gene expression of only one part of the genome. In respect of the study described in Chapter II, also the number of samples were increased: three biological replicates for each of six time-points for each of the two organs were used, giving a larger overview on the development of these two tissues.

The whole genome microarray, µPEACH3.0, performed well, with a correlation with qRT-PCR data of 0.77, a number similar to that found for other arrays. The transcriptomic data easily distinguished the two tissues and the six time-points, as shown by principal component analysis. 69% of the probes gave a significant signal from at least one of the samples. Anyway, considering that the number of functioning probes diminishes if only the samples of one tissue are taken into account, it is probable that testing the microarray with mRNA coming from other tissues (such as leaves or roots) will increase the number of significant signals coming from the array.

Global analysis of gene activity was focused into the early stages of development. Data allowed us to identify several genes involved in cell cycle processes that occur at the onset of both mesocarp and seed development. In particular genes of the *TITAN* family were found to be active in the endosperm containing seed. The analysis of the cell cycle genes in the mesocarp showed the existence of two different patterns of expression: while mitosis related genes were expressed only in stage S1, DNA replication genes showed a double peak of expression, in S1 and then in S3/S4, suggesting that events of endoreduplication may occur in these late stages. By qRT-PCR the expression levels of these genes were tested also in other cultivars, the data obtained suggest that the lack of endoreduplication may be involved in the slow rate of growth in S3 stage of the *slr* genotype.

The patterns of expression of transcription factors (TFs) families were then assessed, as transcription factors are thought to be the proteins with the most important regulatory roles during development. It was found that TFs of the SQUAMOSA promoter Binding Protein (SBP) family have an high expression level at the beginning of the development of both the organs considered, which then quickly decreases. The transcription of Growth Regulating Factors (GRFs) has been discovered to be induced in the mature seed. The data were confirmed by qRT-PCR also in an 'SpringCrest' and the slow ripening genotype *slr*.

Given that the mRNA abundance of genes belonging to these TFs families is regulated by specific microRNAs (miRNAs) in other plant species, the expression of the peach homologues of these miRNAs was measured. In three different cultivars a negative correlation in the RNA abundance was found for the following miRNA/target TF couples: miR156/SBP, miR396/GRF and mir167/ARF8, suggesting not only that these miRNAs have the same activity also in peach, but also that miRNAs are deeply involved in the regulatory network underlying the peach fruit development.

Appendix is a published study in which µPEACH3.0 is used to study the effects of wounding in two peach cultivars with different tolerance to this stress. RNA samples from wounded and unwounded mesocarps of melting cultivar Glohaven (GH) and slow melting cultivar BigTop (BT) were used. Transcriptomic data, confirmed by qRT-PCR analysis, showed the involvement of WRKY, AP2/ERF and HSP20 transcription factors in the GH response to wounding. Along with them, also genes involved in response to stresses, cell wall metabolism, phenilpropanoid and triterpenoid biosynthesis were found to be up regulated in the wounded GH mesocarp.

Chapter I

General Introduction

General introduction

1 The peach fruit

Fruits are the means by which flowering plants (Angiospermae) protect and disseminate their seeds. Fruit plants are cultivated since the origins of agriculture to provide food for humans and domesticated animals. Fruit is botanically described as the development of the flower's ovary. That said, only some botanical fruits are commonly considered "fruits", furthermore, some non ovary-derived organs are also called "fruits" (e.g. pomes). The peach fruit is a fruit both botanically and commonly speaking.

The peach tree was cultivated in China over thirty centuries ago. After seven hundred years, the peach tree is found in Greece, arriving in Europe through Persia (as the scientific name indicates: *Prunus persica*). The peach tree was then exported to Italy during Roman times, and then became a cosmopolitan crop during the age of discovery (15th-17th century).

Two millennia after the appearance of the peach in its territory, Italy is now the biggest European producer of peaches and the second worldwide after China, both in weight and in value (FAO, 2011). In 2011, Italian farms produced 1 660 thousand tons of peaches, valued about 490 million euro (INEA). Among the Italian regions, peaches are produced mainly in Campania (in the countryside of Caserta) and in Emilia-Romagna (next to Forlì and Ravenna). The high production made peach the most studied and the model species for Prunus fruits.

In Italy, the blooming period for peaches is from the end of February to the end of March, depending on the latitude. Instead, harvesting time varies a lot more: from late June to late September depending on the cultivar. This means that the peach development may last from 90 to up to 180 days. On the other hand, storage of peaches is difficult and the fruits must be consumed within a few weeks after harvest. These features make farmers to grow different cultivars of peaches in order to sell fruits all season long.

Peach fruit is botanically classified as a drupe. Other drupes are the fruits of species strictly related to peach, such as almond, plum, apricot and cherry (all belonging to the monophyletic genus *Prunus*), but also of plants as diverse as olive, pistachio or coffee.

Drupe fruit is made of a fleshy tissue (the mesocarp), covered by a skin (the exocarp), which surrounds a hard lignified tissue (the endocarp, commonly known as pit or stone). After the formation of the pit, there is no direct symplastic link between the seed and the rest of the fruit, and apoplastic transport is severely obstructed by the presence of the heavily lignified stone. In addition hormone treatments designed to induce parthenocarpic peach fruit development failed (Stutte and Gage, 1990), showing that some form of communication between the seed and the fruit is necessary.

The fruits of most peach cultivars display a double-sigmoid growth pattern, which may be divided into four growth stages (Tonutti et al., 1997)(Figure 1).
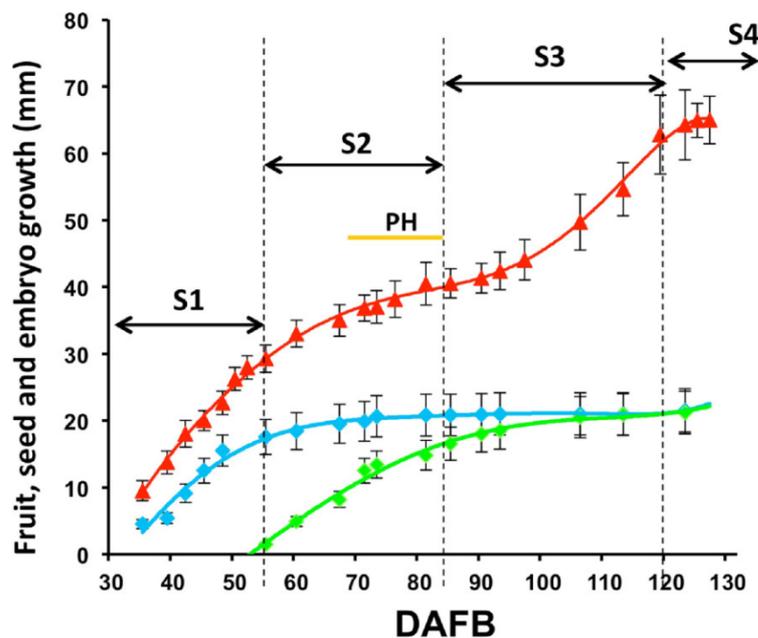
Figure 1. – Fruit growth of peach cultivar 'Fantasia'. (Bonghi et al., 2011). Mesocarp growth shows the typical double sigmoid shape (red) and the four stages are recognizable. Growth of the seed and of the embryo are also shown (blue and green). The embryo starts to be visible only after stage S1 and completely fill the seed before the end of Stage S3. Fruit diameter and seed and embryo lengths were mesaured. DAFB=Days After Full Bloom.

During the first stage (S1), the fruit grows quickly by cell division and expansion. This growth occurs simultaneously in both mesocarp and endocarp, despite the two tissues being anatomically differentiated since anthesis (Arnau et al., 1999): at this time, the

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endocarp already has phenolic vacuolar inclusions (Masia et al., 1992). In addition, during S1 the mesocarp starts to accumulate starch in the chloroplast, while the epicarp is still a single layer of cells (Masia et al., 1992)(Figure 2).
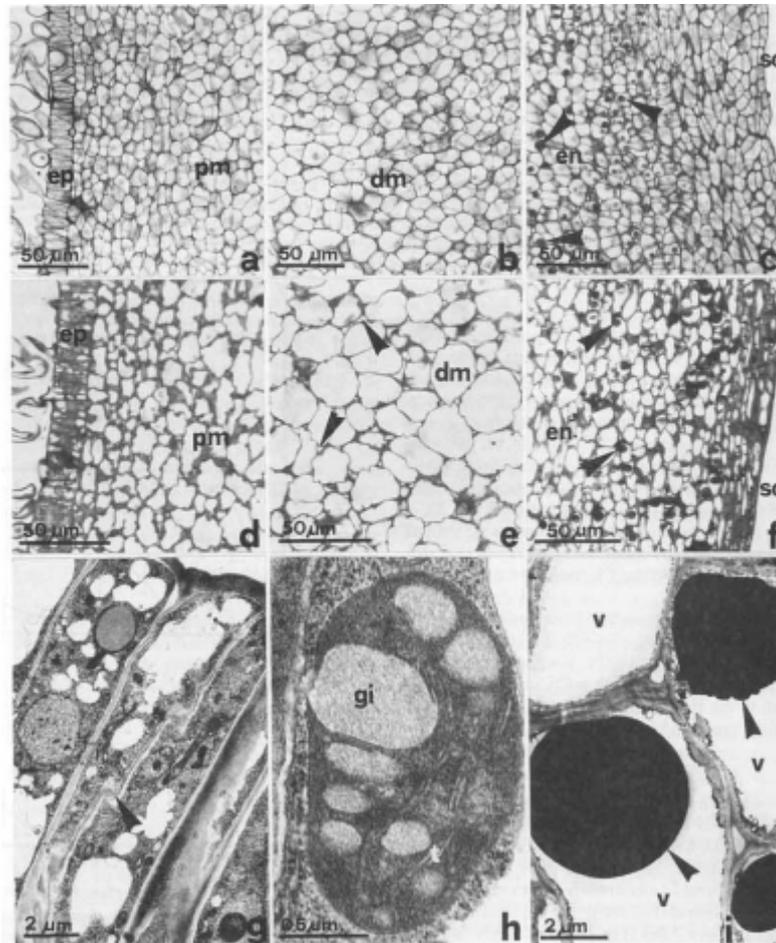
Figure 2. – F Pericarp microscopy during the early stages of growth (Masia et al., 1992). a) b) c) Pericarp at 1 week after full bloom: in all the pericarp, cells undergoing divisions can be seen. In the endocarp, cells already have dark inclusions (arrows); **d) e) f)** Pericarp at 4 weeks after full bloom: epicarp is monolayered and peripheral mesocarp is compact. Conversely, deep mesocarp is starting to enlarge. Endocarp has vacuolar inclusions (arrrows); **g)** anticlinal divisions of the epicarp; **h)** TEM micrograph of mesocarp plastids, which show large granular inclusions, but no starch yet visible; **i)** TEM micrograph of endocarp cells, showing very large vacuolar phenolic inclusions (arrows); ep=epicarp, pm=peripheral mesocarp, dm=deep mesocarp, en=endocarp, sc=seed cavity, t=thylacoids, gi=granular inclusions, v=vacuoles.

The second stage (S2) has a slowed total fruit growth and the endocarp hardens and reaches its finale size, differentiating into the pit. During hardening the endocarp accumulates large amounts of lignin (Ryugo, 1963). Phenylpropanoid and lignin pathway genes are expressed in the endocarp tissue (Dardick et al., 2010), while the phenolic inclusions in the vacuole disappear and the cell walls lignify (Masia et al., 1992).

The third stage (S3) is characterized by a rapid growth due to cell enlargement in the mesocarp, during this time starch disappears and chloroplasts are converted into chromoplasts (Masia et al., 1992).

Finally, in the fourth stage (S4) the fruit enters the ripening and senescence stage. During this period the levels of sucrose synthase increase sharply, resulting in the accumulation of sucrose (Vizzotto et al., 1996; Lombardo et al., 2011). Ripening normally occurs 18 weeks after full bloom, while cultivars with an altered growth pattern also exist. In early cultivars the fruit may complete its development in less than 12 weeks, while in late cultivars peach growth may last up to 24 weeks. In the former case, the lag phase in the S2 stage is shortened, in the latter it is extended.

The stone, which forms during the S2 stage from maternal tissues, can enclose one or two seeds. Seeds are exalbuminous: the endosperm grows in the first stages, cellularizes but it is completely reabsorbed once the fruit enters the S3. Conversely, the embryo develops very slowly initially and speeds up its growth once the fruit has already entered the S2 stage. Remarkably, this is not the case for early cultivars: in these cultivars the embryo is not mature at fruit ripening, and at harvest the seed contains also endosperm. To grow, these embryos have to be rescued by breeders (Bassi and Monet, 2008).

2 Metabolic events that characterize early phases of fruit development

Early phases of pericarp fruit development are characterized by high level of several amino acids, such as Phenyalanine, Proline, Tyrosine, Alanine, and Valine (Lombardo et al., 2011). In this scenario, it is highly probable that amino acids derived from stored proteins in the early immature fruit become the substrates for the phenylpropanoid lignin and for the flavonoid pathways that are induced concomitantly with the deposition of lignin in the stone at S2 (Dardick et al., 2010) (Figure 3).
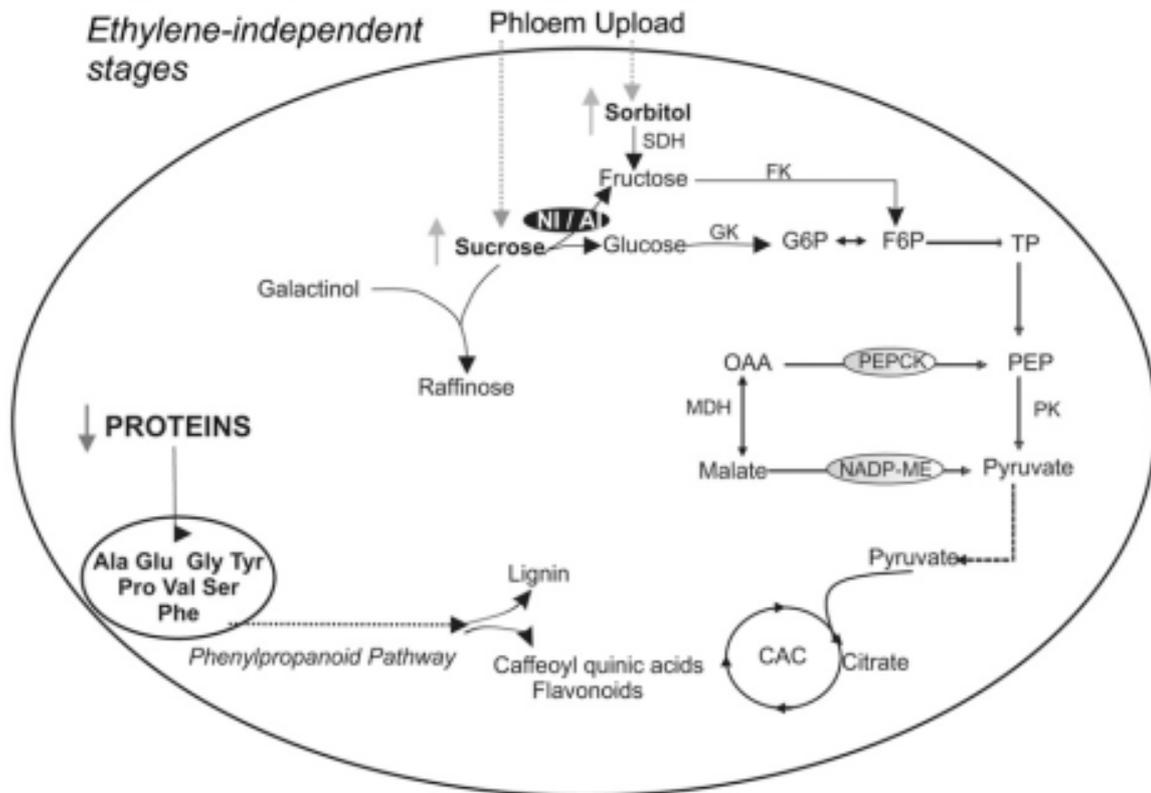
Figure 3. –Simplified scheme of key metabolic processes occurring during early development of Dixiland peach fruit (Lombardo et al, 2011). The key metabolic processes during the development of peach fruit are highlighted. Metabolites indicated with grey up or down arrows increased or decreased, respectively. Invertase (NI/AI) is particularly prominent at early developmental stages. CAC: Citric acid cycle; FK: fructokinase; F6P: Fructose-6-P; GK: glucokinase; G6P: Glucose-6-P; OAA: oxaloacetate; TP: triose phosphate.

With regard to sugars, glucose and fructose were increasing up to S2, while sucrose starts to accumulate from the early S3 phase (Nonis et al., 2007). The rise in sucrose may be due to photosynthate translocation from the leaf, where it is loaded into the phloem in either an apoplastic or a symplastic manner (Moing et al., 1997; Lo Bianco et al., 1999; Nadwodnik and Lohaus, 2008) and is paralleled by the increase of PpNI, a peach neutral invertase gene (Nonis et al., 2007). A marked increase in invertase activity at S2 to S4 would probably favor a high rate of sucrose unloading into the fruit during these stages (Fridman et al., 2004; Lombardo et al., 2011; Nonis et al., 2007).

Considering sugar alcohols, an increase in sorbitol levels in S3 compared with S2 was found, which was not accompanied by changes in SDH levels (Lombardo et al., 2011). Since sorbitol, together with sucrose, is transported from leaves to fruits in Rosaceae, higher levels of this metabolite may be a consequence of greater import due to higher sink strength at this stage of development (Moing et al., 1997; Nadwodnik and Lohaus, 2008). Other sugar alcohols, such as galactinol and raffinose, were highly accumulated both in correspondence with stone formation and seed maturation, suggesting an important role of these compounds in peach fruit development (Lombardo et al., 2011).

The three main organic acids in the peach fruit, citrate, malate, and quinate, have been suggested to undergo continuous accumulation during fruit development for their further respiratory consumption (Moing et al., 1998; Etienne et al., 2002). Lombardo and co-workers observed constant levels of quinate and malate during pericarp development. Malate accumulation can be prevented by the action of NADP-ME and PEPCK, in this context acting as decarboxylating enzymes, that were found highly active during early peach development stage.

The increase of NADP-ME activity at an early stage (S1) has been associated to the request of NADPH for lignin, phenylpropanoid, and flavonoid synthesis during pit hardening (Lombardo et al., 2011).

3 Ripening-related changes at a physiological level

Ripening is a syndrome of fruits at their final stages of development aimed at making them more attractive to animals, which, eating them, scatter the seeds. The events involved in ripening differ in different species, generally, in fleshy fruits, ripening involves the conversion of starch and alcohols into sugars, the softening of the fruit through modifications in the cell wall structure, the synthesis of pigments and aromatic compounds (Giovannoni, 2001)

Fleshy fruits may be broadly divided in two ripening categories: climacteric fruits show a sharp increase of ethylene production and respiration at the beginning of the ripening process, while in non-climacteric fruits these events are not detectable. Among the most studied fruits, tomato, apples and peaches are climacteric fruits, while grape, strawberries, and, interestingly, cherry (a *Prunus* species) are not.

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The increase in ethylene production at ripening in climacteric fruits has been explained through a model which involves the existence of two different ethylene production systems (Barry et al., 2000). System 1 produces the basal levels of ethylene in both vegetative and reproduction organs, conversely, System 2, which is autocatalytic, is activated during specific syndromes, such as ripening and senescence (Alexander and Grierson, 2002.)

Ripening is associated with changes in transcription of a large number of genes. Among regulators of gene expression, an important role is played by transcription factors. In fact, the expression of MADS-box transcription factors, auxin responsive proteins (AUX/IAA), basic leucine zipper domain proteins (bZIP), basic helix-loop-helix proteins (bHLH), homeobox-leucine zipper domain proteins (HD), Myb transcription factors is induced during ripening. Obviously, many ethylene related transcription factors are also particularly expressed (e.g.APETALA2-like proteins/Ethylene Responsive Factors) and, interestingly, along with genes involved in auxin hormone metabolism (biosynthesis, transport and, the signalling) (Trainotti et al., 2007).

Agronomic parameters characterizing ripening include sweetness and acidity (generally negatively correlated), fruit firmness and texture, colouring, aroma development, and ethylene production. In particular, the intercourse between sweetness and acidity are the most important characteristics for consumers preferences, while fruit firmness and texture are involved in fruit storability (Parker et al., 1991).

In peach, sugars are moved from leaves to fruits in the form of sucrose and the sugar alcohol sorbitol, which consist up to 60% of the ripe fruit soluble solid content (SSC) (Ramina et al., 2008). Sucrose translocation is tightly correlated with the increase of fruit dry weight. Monosaccharide, glucose and fructose, accumulate during the S1 stage, then their concentration decreases. On the other hand disaccharide sucrose content increases sharply during the S3 stage, reaching the maximum in the S4 stage: at ripening sucrose consists of about an half of the fruit dry weight (Vizzotto et al., 1996).

The main parameter influencing peach shelf life is the ripening related softening (Sánchez et al., 2012). Softening is reached by the partial degradation of mesocarp cell walls: in particular through pectin hydrolysis , and modifications in cellulose and hemicellulose content. Softening is so characterized by the activity of several different hydrolases,

such as pectinmethylesterases (PMEs), polygalacturonases (PGs), β-(1,4)-glucanases (EGs) and β-galactosidases (β-GALs), and that of other cell wall related proteins such as expansins (EXPs) and pectatolyases (PL)(Brummell et al., 2004).

4 Omics

Omics are a series of fields of biological research, which involve the characterization of a whole set (or at least a large set) of biological molecules which characterize a particular tissue or individual. Genomics is the study of the whole set of genes (the genome) of an individual or of a species. Since it has been discovered that not all the hereditary information is coded into genes, the term has now broadened to include all the DNA (or RNA for some viruses, which don't use DNA to store information), which is transmitted from one generation to the next one. Epigenomics analyzes all the epigenetic modifications of the genome in a specific tissue or developmental stage, in a similar way transcriptomics studies the whole set of transcripts that characterize a tissue or an organ, while proteomics investigates the whole set of proteins, and metabolomics the entire set of metabolites.

Genomic studies in peach have been possible only after the Sanger sequencing completion of the entire peach genome in 2010 by the International Peach Genome Initiative (The International Peach Genome Initiative et al., 2013).

However, transcriptomic studies in peach were first introduced with 200 ESTs coming from three different developmental stages (Hayama et al., 2000). Since then, a lot of transcriptomic studies have been performed on peach using different tools, such as cDNA-AFLP (Ziliotto et al., 2005), ESTs (Trainotti et al., 2003; Vizoso et al., 2009) and microarrays (Trainotti et al., 2006; Ogundiwin et al., 2008). Recently, the first article using RNAseq technology to analyze the peach transcriptome in different tissues has been published (Wang et al., 2013).

Proteomics tools in peach fruit have been mainly used to investigate post-harvest events: from the study of defence and disease (Chan et al., 2007) to the study of mesocarp softening and chilling injury (Nilo et al., 2010). Recently, proteomics have been used also to analyze the lignification of the endocarp, finding that pyruvate dehydrogenase may be involved in the process (Hu et al., 2011). Comprehensive metabolomic analysis of the entire fruit development revealed the profile of concentration of a great variety of

different molecules and singular metabolic programs for peach development such as the identification of amino acids as substrate for phenylpropanoids pathway involved in pit hardening (Lombardo et al., 2011) and the evolution of typical peach aromas (e.g. lactones) (Sánchez et al., 2012).

5 Peach genome

Since the sequencing of the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000), a great variety of plant genomes has been sequenced. In the *Rosaceae* family, the first genome to be sequenced is the genome of apple (*Malus domestica*) (Velasco et al., 2010). Since apple's genome is made of n=17 chromosomes (while other *Rosaceae* species have only n=7 to 9 chromosome), the sequencing easily identified the whole-genome duplication that occurred in the *Pyrae* 50 million years ago. The second species whose genome has been sequenced is a *Rosacea* plant again carrying a false fruit: strawberry (Shulaev et al., 2011). The first *Rosacea* with a true fruit, and member of the *Prunus* genus, whose full-genome sequence has been officially published is the Chinese plum (Zhang et al., 2012). However, the raw sequence of peach genome and basic annotations had already been made available to researchers since 2010. This difference of time between the informal and the official publishing date, allowed many researchers to conduct studies that would have been impossible before. In fact, already in 2011 a first whole-genome study on Single Nucleotide Polymorphism (SNP) in different peach cultivars was published. The genomes of cultivars Dr. Davis, Georgia Belle and of a genotype with a wide range of diversity called F8, were re-sequenced and aligned to the Lovell's genome looking for SNPs. 6 654 SNPs were found among four cultivars (false positives at 0.1%), with an average density of 1 SNP every 40k bases (Ahmad et al., 2011).

The peach genome is made of a diploid series of 8 chromosomes of an estimated size of 265 Mb (Arumuganathan and Earle, 1991). The IPGI sequenced 215.9 Mb organized in eight pseudomolecules corresponding to chromosomes, this totalled to 28,689 transcripts and 27,852 genes, with an average gene density of 12.2 genes per kb. The sizes of the pseudomolecules vary from 18.5 Mb of scaffold 5 to the 46.9 Mb of scaffold 1 (Figure 4). All the information regarding the peach genome is available at the Genome Database for *Rosaceae* (Jung et al., 2008).
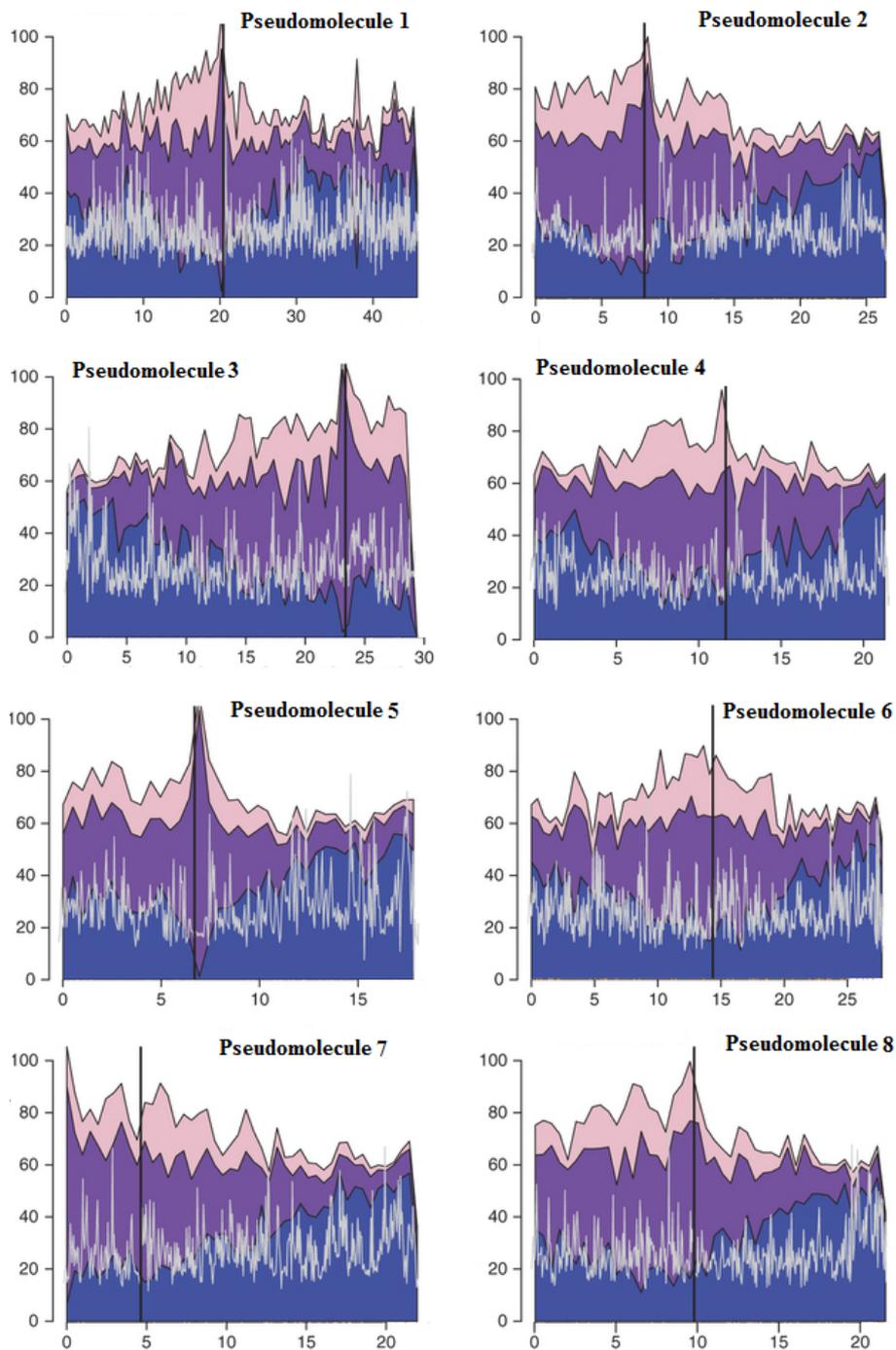
Figure 4. – Plots of the 8 pseudomolecules of the peach genome (IPGI et al., 2013). The approximate positions of centromeres are marked with vertical black bars. The plotted areas show the percentage of the genome (divided in 500-kb long windows) consisting of: type I transposable elements (purple), type II transposable elements (pink) and genes (blue). The gray line shows 100 times the mean r^2 value for all SNPs in 50-kb windows, estimating the linkage disequilibrium. On the X axis are molecules' lengths in Mb).

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The full genome sequence gives a variety of advantages for the analysis of gene expression, or even microRNA expression: primers for Polymerase Chain Reactions (PCRs) can be easily designed, as all the DNA sequences of the genes are available; but it is also useful for transcriptome analyses such as microarrays or RNAseq. The availability of the full genome sequence has removed the need for EST collections such as the Italian ESTree consortium database (Lazzari et al., 2005).

The IPGI re-sequenced another eleven *Prunus persica* cultivars and another four *Prunus* species strictly related to peach, searching for SNPs. Excluding the three most different *Prunus* species, almost one million (996 285) SNPs were found. Unsurprisingly, nucleotide diversity in peach resulted low when compared to wild species like *Medicago truncatula* and wild soybean. But also when compared to widely cultivated fruit tree species such as apple and grape (The International Peach Genome Initiative et al., 2013). This low diversity gives an additional advantage when using sequence-based tools for measuring gene expression in different cultivars (tools such as quantitative Real-Time PCR and microarrays).

Along with genes, 189 conserved miRNA sequences belonging to 57 miRNA families were identified in the genome, with a similar family-size as in the *Arabidopsis* and poplar genomes. This information has been used to identify both chilling responsive miRNAs in leaves (Barakat et al., 2012) and miRNA expressed in response to drought in both leaves and roots (Eldem et al., 2012).

6 Transcriptome analysis of fruit development

Transcriptomic analyses involve the use of tools able to measure the abundance of thousands of different RNA molecules in a tissue at the same time. Overall gene expression is then compared between different treatments making possible to understand the behaviour of classes of genes in different physiological conditions or even identify at glance gene networks involving several genes. Transcriptomic analyses have been used in studying fruit and seed development in a large variety of plants. The first work studying fruit development through transcriptomic tools (a cDNA array) led to the identification of an alcohol acyltransferase involved in flavor biogenesis in the strawberry fruit (Aharoni et al., 2000). Other studies included the development of tomato fruit (Moore et al., 2002: Alba et al., 2005), citrus (Shimada et al., 2005; Cercós et al.,

2006), grape (Waters et al., 2005; Terrier et al., 2005; Deluc et al., 2007; Grimplet et al., 2007), apple (Lee et al., 2007; Janssen et al., 2008; Soglio et al., 2009; Costa et al., 2010), pear (Fonseca et al., 2004).

In Prunus, transcriptomic studies started with µPEACH1.0: the first microarray designed starting from peach sequences. The 4 806 probes of the array were construed using the sequence obtained from Expressed Sequence Tags (ESTs) stored in the ESTree database (Trainotti et al., 2006).

µPEACH1.0 was then used to assess the effects of treatments of hormones on the fruit. The transcriptomic responses to auxin, ethylene, jasmonate and ethylene inhibitor 1-MCP were determined with this array. In particular, the effects of treatments with either auxin or ethylene were compared to the effects of the natural climacteric, shedding light onto the cross-talk between auxin and ethylene related genes during peach mesocarp ripening (Trainotti et al., 2007). µPEACH1.0 analyses helped to demonstrate how treatments with jasmonate may delay ripening, down-regulating ripening associated genes (Ziosi et al., 2008). Peaches at the climacteric treated with ethylene inhibitor 1-MCP produce ethylene but don't lose their firmness, as peach softening at ripening is triggered by ethylene. Treatments with 1-MCP and the use of µPEACH1.0 led to determine which genes are expressed at ripening because of ethylene's action and which genes on the contrary are expressed in response of other stimuli (Ziliotto et al., 2008).

The array was also used to determine differences in expression between two cultivars with different degrees of tolerance to Chilling Injury (CI) (Falara et al., 2011). This array showed its flexibility, when used to measure transcriptome expression at climacteric in apricot (*Prunus armeniaca*) (Manganaris et al., 2011).

Another peach array is the ChillPeach with 4 261 different probes. This array was designed from an EST collection obtained from cold stored peach mesocarps in order to identify genes involved in Chilling Injury (CI). The array identified 399 differentially expressed genes associated with cold treatment. Subsequent qRT-PCR analysis found 10 genes that may be associated to chilling injury tolerance (Ogundiwin et al., 2008).

ChillPeach array was then used mainly in experiments involving biotic or abiotic stresses: it has been employed to study a double infection by *Prunus* Necrotic Ringspot Virus and Peach Latent Mosaic Viroid, and again to study CI in other cultivars (Dagar et al., 2012)

μPEACH2.0 was designed from the same database of μPEACH1.0, but the 4 776 ESTs were selected in order to detect transcripts putatively involved in flavour production. The array was used to compare expression of aroma-related genes in two different cultivars and identified twelve genes implicated in secondary metabolism (esters, norisoprenoids, phenylpropanoids and lactones), whose expression at the climacteric was different between the two cultivars (Pirona et al., 2013). Another *Prunus* microarray was designed starting from 10 641 Prunus mume ESTs to study developmental differences between fruits of *Prunus mume* (Chinese plum) and of *Prunus armeniaca*. 1 418 genes were found differentially expressed at ripening (Li et al., 2012).

The EST-designed microarrays have proven to be useful to shed light onto peach fruit development, but became insufficient, even if combinations of μPEACH1.0 and ChillPeach were used (Dagar et al., 2013). Two different microarrays were used to study at the transcriptomic level the events surrounding the lignification of the endocarp: μPEACH1.0 was used along with a 15 000 features apple microarray to increase the number of genes detected (Dardick et al., 2010). With the publication of the peach genome, the design of a peach whole-genome array, the μPEACH3.0, was possible.

Transcriptomic analysis of seed development started with a study on *Arabidopsis* seed. A small microarray with 2,600 probes was designed from seed-specific ESTs and then used both on *Arabidopsis* and on oilseed rape (*Brassica napus*) seed samples (Girke et al., 2000). A comprehensive profiling of transcription factors involved in *Arabidopsis* seed development was also made (de Folter et al., 2004)(Figure 5). Microarrays to investigate seed growth was then employed in plants whose seed has a dietary value: maize (Lee et al., 2002), rice (Zhu et al., 2003) and soybean (Jones et al., 2010).

Seed transcriptome analyses of peach are more important than in other fleshy fruit as hormone treatments on peach are unable to produce parthenocarpic fruits (Stutte and Gage, 1990), in addition, seeds of some *Prunus* species are edible. Investigations into peach seed transcriptomics may shed light onto *Rosaceae* seed growth in general, as peach seed is easier to manipulate, being quite big in comparison to those of the other Rosaceae.
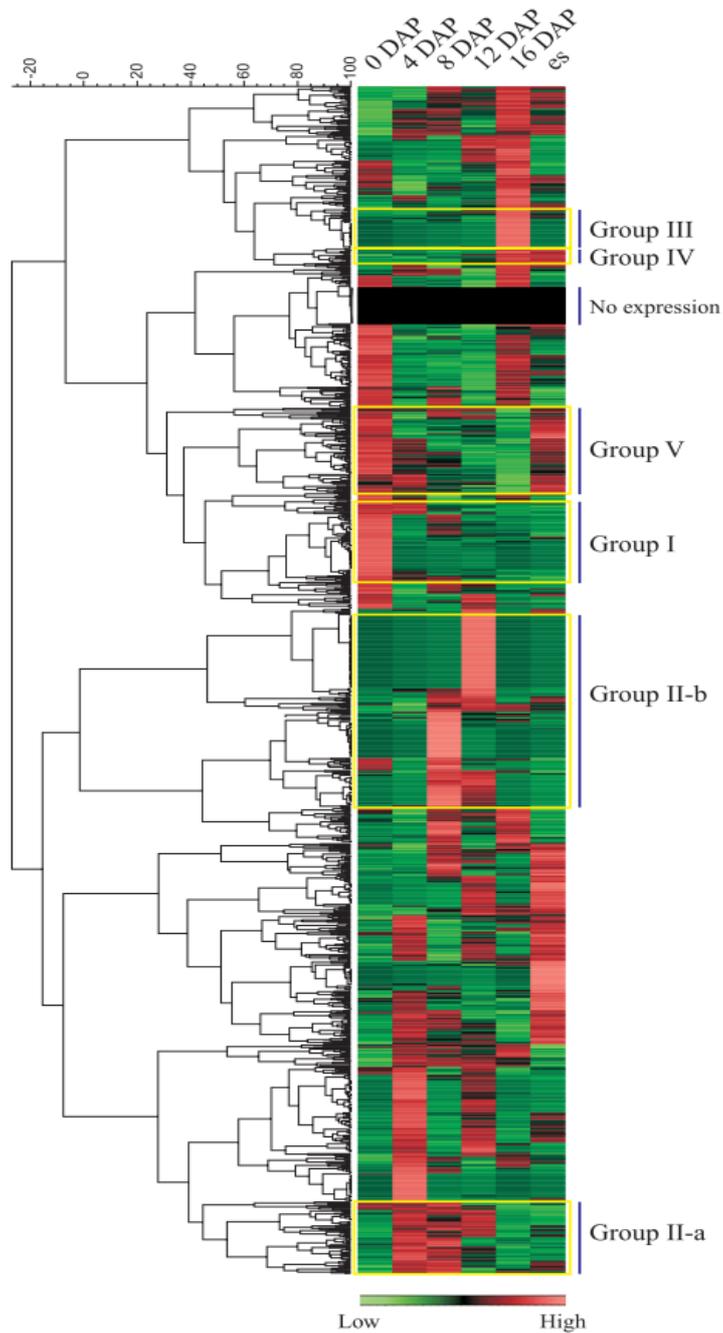
Figure 5. – Expression heat map of TF genes during *Arabidopsis* silique development, ordered by hierarchical clustering (de Folter et al., 2004). TFs group in co-regulated clusters: TFs of pistil development (group I), embryogenesis (group II-a and II-b), seed maturation (group III), fruit maturation (group IV), and fruit development (group V). On the X axis the date of sampling: 0, 4, 8, 12, 16 DAP (days after pollination) and the *empty siliques* (*es*) mutant.

7 The microarray tool

Generally speaking, microarrays are tools used to identify large amounts of nucleic acid molecules with different sequences and quantify them in a single experiment. A variety of probes made of nucleic acid identifies the target sequences by complementary hybridization, while the quantity of the target sequences is measured with the help of fluorescent molecules or chemioluminescent reactions. Microarrays have been widely used to measure gene expression, as the expression of genes, even an entire genome, may be quantified in a single experiment.

Microarrays are technically classified on the basis of a variety of features: 1) the nucleic acid used to make the probes (DNA or RNA), 2) the target nucleic acid (DNA, RNA or RNA-derived cDNA), 3) the method by which the probes are synthetized, 4) the number of probes, 5) the number of technical replicates of the probes, 6) the sizes of the probes, 7) the molecules or the reactions used to signal the probe/target hybridization levels , 8) the devices used to detect the signal molecules or the products of signal reactions, 9) the number of slides that can be measured in the same experiment. In addition to these technical features, there are obviously the sequences of the probes, which gave the biological specificity.

Each one of the technical features exposed above may influence the accuracy and the precision of the experiment: the probes' size, for example, is important to determine their specificity (the longer the more specific) and sensitivity (He et al., 2005), more than one replicate for a probe allows the reduction of false signals, more arrays on the same slide increase the significance of inter-array comparisons and so on. But when it comes to analyze data probably the most important feature is the method used to get the signals from hybridizations: two-channels and one-channel microarrays exist.

Two-channels microarrays use two different fluorophores. Each of the fluorophores is bound to different samples of target RNA, then the two samples of target RNA are poured on the same array and hybridize with the probes. The ratio between the fluorescence intensities produced by the two fluorophores is then used in the subsequent analyses. Two-channel microarray is so preferred when comparing two samples.

One-channel microarrays use a single fluorophore or a single chemioluminescent reaction. There is no need to bind the signal molecule to the RNA sample, and the target/probe

hybridization is measured directly comparing the light intensity of the spots subtracting to it that of the background. When comparing only two samples one-channel microarrays are more expensive, but they are handier when time-courses involving more samples are studied.

8 Analyses of microarray data

A typical pipeline of microarray analyis is drawn in Fig.6. mRNA extraction and array hybridization are depending by plant tissues and array platform design, while for data analysis, even if much software is available, the pipeline is similar for most of the first steps.

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┌─────────────────────────────┐
│      mRNA extraction        │
└─────────────────────────────┘
              │
              ▼
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│     Array hybridization     │
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              │
              ▼
┌─────────────────────────────┐
│          Raw data           │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│      Data normalization     │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│       Statistical tools     │
│  (e.g.differential expression) │
└─────────────────────────────┘
         │            │
         ▼            │
┌──────────────────┐  │
│ Exploratory tools│  │
│ (e.g. clustering)│  │
└──────────────────┘  │
         │            │
         ▼            ▼
┌─────────────────────────────┐
│      Functional analysis    │
│    (e.g. gene enrichments)  │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│   Validation (e.g. qRT-PCR) │
└─────────────────────────────┘
```
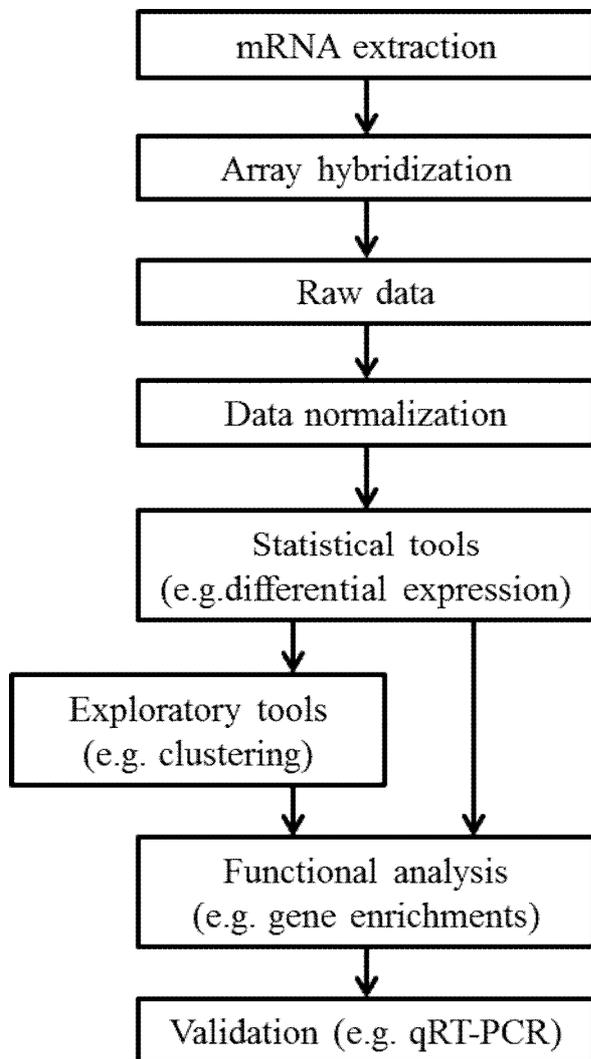
Figure 6. – Pipeline of a tyipical microarray analysis.

8.1 Raw data

Raw data produced by modern arrays are numbers representing the intensities measured by the detector, along with flags representing various parameters useful to understand whether the hybridization occurred correctly. The first operation to do is to eliminate the intensities which are too faint to be significant.

8.2 Data normalization

Normalization is then needed to compare across different arrays. This goal can be achieved in various ways. One of the simplest ways is to use housekeeping genes (an handling similar to that employed for qRT-PCR experiment), but that is unfeasible, as expression of housekeeping genes may slightly vary across samples and little variations may have enormous effects when thousands of genes are involved. Another simple method is to normalize (divide) by the mean or the median intensity of the array. Anyway, one of the best method is that of quantile-normalization as it is independent from the data, quite simple, quick to perform and performs well in reducing bias effects (Bolstad et al., 2003).

8.3 Identification of differentially expressed genes

Differentially expressed genes are identified in various ways, depending on the experimental design. The first naïf method used was to consider differentially expressed those genes whose hybridization intensity ratio was above or below a certain threshold (e.g. more than 2 and less than ½).

The need to consider biological replicates led to the use of more statistically significant methods such as the t-test (Dudoit et al., 2002) or the Significance Analysis of Microarrays (SAM). SAM is stricter than t-test as it gives an estimate of the False Discovery Rate (FDR), namely the ratio of false positives among the genes found differentially expressed (Tusher et al., 2001).

While SAM gives great results for one versus one comparisons, for time-courses experiments different methods are used such as the Analysis of Variance (Park et al., 2003) or more sophisticated liner models (Smyth, 2004).

Anyhow, these methods fail to take into account the different length of the periods between time-points. To this purpose other methods were designed such as Microarray Significant Profiles (Conesa et al., 2006) or EDGE (Storey et al., 2005).

8.4 Cluster analysis

Cluster analysis (clustering) consists in grouping genes on the basis of their expression profiles (a.k.a. patterns). Clustering is not necessary when dealing with one versus one comparisons, as in these cases only two outcomes are possible (up-regulated or down-regulated). Different clustering method has been proposed so far.

Hierarchical Clustering is one of the first methods proposed: genes are considered as leaves of a tree graph, their positions being determined by the pairwise similarity of their expression profiles (Eisen et al., 1998). This method is popular to display the data, but it tells very little about what is occurring.

K-means or K- median Clustering is a non-hierarchical clustering method and was designed to group genes in a pre-determined number of clusters. Genes are then clustered on the basis of their similarity distance to the mean or the median profile of the cluster to which they are assigned. The process is reiterative, genes are moved around the clusters until the total distance is minimized (Soukas et al., 2000). This method is useful because further analyses may be performed on the obtained clusters, unfortunately with this procedure all genes are assigned to clusters, even those genes whose patterns are very different to the mean/median profile of their cluster of assignment.

Quality cluster algorithm (QTC) was developed to overcome this problem: this method is based on the pre-determination of a maximum distance for the genes to belong to the same cluster. There is no need to pre-determine the number of clusters and outlier profiles are simply kept out from all clusters (Heyer et al., 1999). Anyway, QTC is not optimized for time-course experiments.

Short Time-series Expression Miner (STEM) is an algorithm specifically designed for time-course experiments (3 to 8 time-points). The method uses pre-defined patterns to which gene profiles are compared. This allows an easier comparison across different experiments (Ernst and Bar-Joseph, 2006).

Mfuzz is a clustering method using fuzzy logic: instead of assigning specific genes to a specific cluster, it calculates the probability of a gene to belong to a cluster. Fuzzy logic

actually allows genes to belong to none, one or more than one cluster depending on the probability threshold set by the user. The advantage of using this "soft clustering", instead of the "hard clustering" methods, becomes apparent when using subsequent enrichment analyses (Kumar and Futschik, 2007).

Pavlidis template matching is a particular tool to find clusters: the average pattern of the cluster is set by the user, and the algorithm finds all the genes which are less distant from it of a pre-defined threshold. This method is useful when the user wants to find genes having a particular profile, for example to identify genes expressed in two particular samples but not in the others (Pavlidis and Noble, 2001).

8.5 Enrichment analysis

Enrichment analysis determines if a determined set of genes (differentially expressed genes, clusters) is significantly enriched of genes belonging to a particular cluster.

The most used enrichment analysis is Gene Ontology enrichment. The Gene Ontology project assigns to each gene one or more GO terms, those terms describing the gene's molecular functions, or the biological processes into which the gene is involved, or the cellular components into which the encoded protein is located (Ashburner et al., 2000). GO enrichment analysis allows to see if a particular GO term is enriched in a particular set of genes through a Fisher's Exact text. Software products have been developed to do enrichment analysis on a massive basis (i.e. using all the genes of a genome, and all GO terms available), an example is Blast2GO (Conesa et al., 2005).

Enrichment analysis is not limited to GO terms, but every gene classification method with pre-determined terms may be used, accordingly to the purposes of the experiment.

8.6 Integrative platforms for data analysis

Integrative platforms gather together different microarray analysis tools in the same virtual environment. Users are able to deal with microarray data starting from the raw numbers to the final results.

TMEV is a downloadable package with graphical user interface designed by the Institute for Genomic Research. It includes some of the tools that were explained above, from differentially expressed genes to clustering (Saeed et al., 2003).

Babelomics is instead a newer online package, developed by the Centro de Investigación Príncipe Felipe. It consists of some of the same tools as TMEV, but it includes software able to analyze from raw data to functional analysis, including enrichments (Medina et al., 2010).

9 Transcription factors and fruit development

Transcription factors (TFs) are proteins that specifically trigger the transcription of genes. TFs bind DNA in specific promoter sequences and recruit RNA polymerases which produce the mRNA of the transcribed genes. TFs activity is regulated in a variety of ways. Their mRNA abundance is determined in the same manner of other genes: their mRNA transcription is regulated by other TFs and their mRNA quantity may be reduced in a microRNA-dependent manner. Once translated into proteins, TFs activity is then determined in post-translational manners: TFs may be regulated by covalent binding of chemical groups (e.g. by phosphorylation), or by non-covalent interactions with other proteins or ions; TFs may be physically sequestered in specific cellular components, and then released in response to specific signals. Finally TFs may be ubiquitinated and then degraded by the proteasome.

An active signaling pathway is often necessary for TFs activation, signaling pathways include at least a receptor protein, which binds the extra or intracellular signal, and second messenger proteins eliciting the physiological response, which often consists in TFs activation. The activation state of the pool of TFs determine the transcriptomic state of a cell.

Study of transcription factors involved in fruit development have been done in a variety of different plants, but mainly in *Arabidopsis* and tomato. Some particular TFs families seem to be more involved than others in regulating transcription in fruit tissues.

In *Arabidopsis*, many studies had been done on the role of MADS-box TFs at the onset of fruit development (Seymour et al., 2008). A wide analysis of MADS box gene expression during fruit development revealed how some MADS-box genes are related to fruit development (e.g. *AGL47*), others with seed (e.g. *AGL87*), others only with the non-parthenocarpic fruit (e.g. *AGL67*) (de Folter et al., 2004).

MADS box activity has been studied also in other plants. In apple a loss of function mutation in a MADS-box gene (*MdPI*) has been demonstrated to be responsible of the

onset of parthenocarpic fruits (Yao et al., 2001). In tomato a MADS-box gene (*MADS-RIN*) has been found to be necessary for the onset of ripening: a deletion in this gene causes an inhibition of ripening (Vrebalov et al., 2002). In peach another function for MADS-box proteins has been described: the temporal regulation of two genes *PpFRUITFUL* and *PpSHATTERPROOF* may have an effect on the split of peach pit (an event by which the pit is fissured along the suture)(Tani et al., 2007).

Another TFs family involved in fruit development is the AP2/ERF family: in tomato AP2a has been demonstrated to be a regulator of some ripening-associated events, such as the carotenoid and sugar levels (Karlova et al., 2011). In apple instead, two TFs of the same family (*MdAINTEGUMENTA1* and *MdAINTEGUMENTA2*) have been shown to regulate cell proliferation in the cortex well before the climacteric (Dash and Malladi, 2012). A general review of the activity of AP2/ERF and ARF TFs genes during tomato fruit development has been done (Kumar et al., 2011). In *Arabidopsis ARF2* seems to be involved in silique ripening. (Ellis et al., 2005).

10 miRNAs regulating transcription factors during fruit development

microRNAs (miRNAs) are small (19-23 nucleotides), non-coding sequences of RNA involved in transcriptional and post-transcriptional regulation of gene expression. This regulation occurs either by degradation of a gene mRNA or by translational inhibition.

The mechanism of action of miRNAs involves two pre-processing steps in the nucleus and a cleavage step in the cytoplasm. pri-miRNAs (primary miRNAs) are transcribed by the RNA polymerase II, and proteins called CAP-BINDING proteins (CBP) stabilize the small RNA molecules. Then, the pri-miRNA is processed in the nucleus in two steps. In the first step, the pre-RNA is excised from the pri-miRNA by DICER-LIKE 1 (DCL1). Other proteins (HYPONASTIC LEAVES 1 (HYL1) and SERRATE (SER) are involved in this first processing. In the second step, the loop of the pre-mRNA is excised by the same proteic complex, to generate mainly 21-nt long double stranded miRNAs, which are methylated and exported in the cytoplasm. Here, the miRNAs are incorporated by a second protein complex called the RNA-Induced Silencing Complex (RISC), which contains the protein ARGONAUTE 1 (AGO1). In the RISC complex, the miRNA binds to its target mRNA by RNA/RNA complementarity, and the target mRNA is either cleaved or its translation inhibited (Lelandais-Briere et al., 2010) (Figure 7).
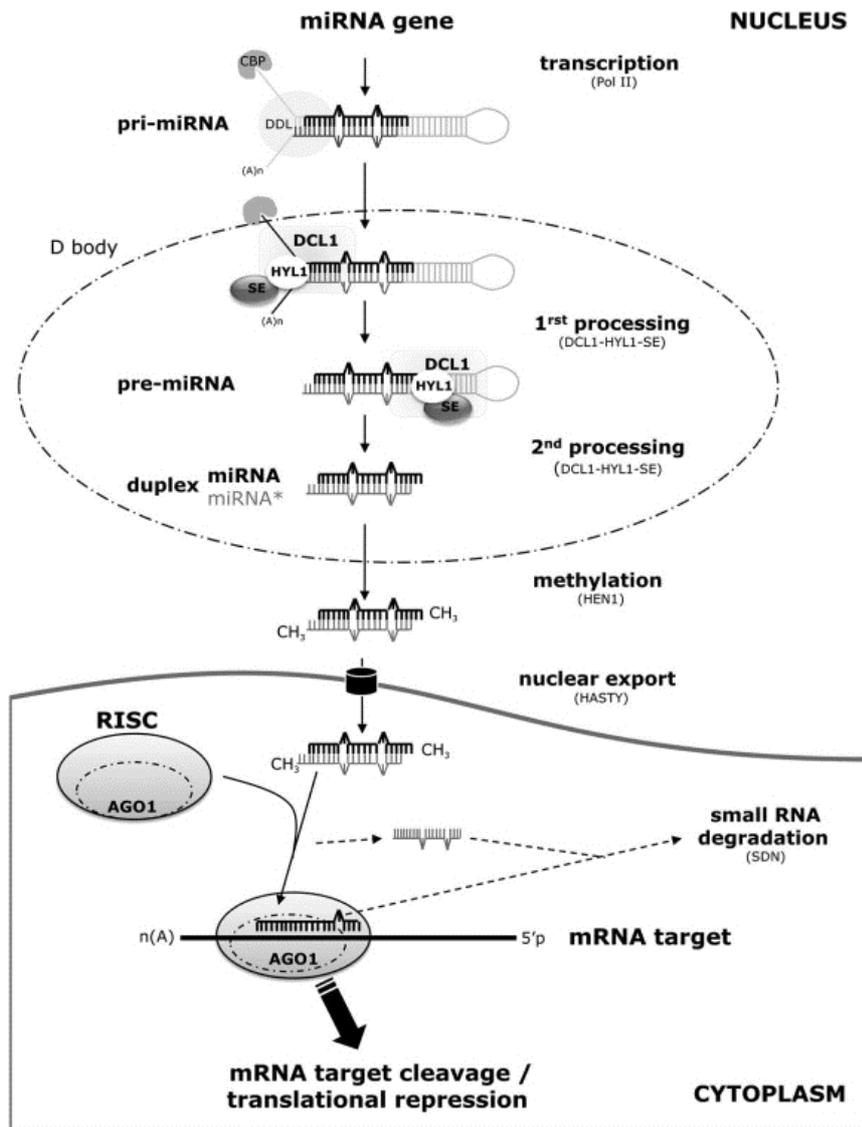
Figure 7. – Scheme of microRNA activity pathway (Lelandais-Briere et al., 2010). In the nucleus, the miRNA gene is transcribed in a primary transcript (pri-miR) and CAP–BINDING PROTEINS (CBP) bind it. The miRNA precursor (pre-miR) is then excised from the pri-miR by DICER-LIKE 1 (DCL1), associated with other dsRNA-binding proteins. The pre-miR is further processed by the same DCL complex (D-body) to generate a 21 nt double stranded miRNA (the miRNA/miRNA* duplex) which is methylated on the 3' ends by HUA ENHANCER1 (HEN1) protein. After the export of the duplex out of the nucleus, the miRNA is preferentially incorporated into the RNA-Induced Silencing Complex (RISC) containing ARGONAUTE1 (AGO1). The miR* is released. Inside the RISC complex, the base pairing of the miRNA and its mRNA target(s) leads to the cleavage and/or the translational repression of the target mRNA.

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The first study designed to investigate miRNAs in fleshy fruits has been done in tomato: nine conserved and twelve novel miRNAs expressed in the fruit were identified (Pilcher et al., 2007). The number of miRNAs in tomato fruit development was soon incremented to 350 small RNAs (even if some of them may be only degradation products), including miR167 (whose target is the AUXIN RESPONSE FACTOR8), miR168 (inhibiting ARGONAUTE1) and miR172 (inhibiting APETALA2) (Itaya et al., 2008). miRNA expression has also been investigated using high throughput screenings. These studies has been performed in a variety of different fleshy fruits: tomato (Moxon et al., 2008; Karlova et al., 2011), strawberry(Xu et al., 2013) and grape (Wang et al., 2014).

In peach, high throughput miRNA analyses were recently used in several tissues, including leaves and dormant buds (Barakat et al., 2012), roots and leaves (Eldem et al., 2012), leaves, stems and floral buds (Luo et al., 2013).

11 Aim of the thesis

The aim of this thesis is to investigate peach fruit and peach seed development mainly using transcriptomic means. This study will try to identify the relationship between these two organs. The study is done on a peach cultivar, the nectarine 'Fantasia', but some analyses have been also extended to other cultivars with a different pattern of development. Understanding the genetic regulation of the various stage of peach development (early growth and ripening), will assist in the selection of new peach varieties. In addition the study of the seed growth, may help in understanding how to produce parthenocarpic peach fruit.

Chapter II is a published article, in which the development of peach fruit and seed is studied using μPEACH1.0 microarray. This study identified markers in both mesocarp and seed in three different genotypes, which may be used to identify developmental stages, and it shed light on the involvement of hormones in the peach fruit development.

Chapter III is a non-published article, in which the development of peach mesocarp and seed has been studied with μPEACH3.0. The use of the latter array increased the number of genes detected from about 4 800 to 29 800 and the number of time-points studied were increased from two to six. The study examines in more detail gene activity during mesocarp and seed development focusing the attention on events occurring at the early developmental phases as cell division growth and endoreduplication processes in

different cultivars. In addition to these genes, the expression patterns of Transcription Factors (TFs) and of their regulators (miRNAs) were described to better define the regulatory mechanisms governing fruit development.

The Appendix is a published article in which μPEACH3.0 array was used to investigate the different expression pattern in wounded and unwounded fruits of two peach cultivars (Glohaven and BigTop) which respond differently to cut injuries.

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Chapter II

A microarray approach to identify genes involved in seed-pericarp cross-talk and development in peach.

A microarray approach to identify genes involved in seed-pericarp cross-talk and development in peach.

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Authors' contributions

CB, LT, ARa devised the study and participated in its design and coordination; FZ conducted the microarray experiments; ARi collected fruit material, measured seed development parameters and performed the validation of microarray data by qRT-PCR; AT performed the validation of microarray data by qRT-PCR; CB, LT, AB, VZ and ARa analyzed the data; CB, AB, LT, ARa and GC wrote the paper. All authors read and approved the final manuscript.

Abstract

BackGround

Field observations and a few physiological studies have demonstrated that peach embryogenesis and fruit development are tightly coupled. In fact, attempts to stimulate parthenocarpic fruit development by means of external tools have failed. Moreover, physiological disturbances during early embryo development lead to seed abortion and fruitlet abscission. Later in embryo development, the interactions between seed and fruit development become less strict. As there is limited genetic and molecular information about seed-pericarp cross-talk and development in peach, a massive gene approach based on the use of the µPEACH1.0 array platform and quantitative real time RT-PCR (qRT-PCR) was used to study this process.

Results

A comparative analysis of the transcription profiles conducted in seed and mesocarp (cv Fantasia) throughout different developmental stages (S1, S2, S3 and S4) evidenced that 455 genes are differentially expressed in seed and fruit. Among differentially expressed genes some were validated as markers in two subsequent years and in three different genotypes. Seed markers were a LTP1 (lipid transfer protein), a PR (pathogenesis-related) protein, a prunin and LEA (Late Embryogenesis Abundant) protein, for S1, S2, S3 and S4, respectively. Mesocarp markers were a RD22-like protein, a serin-carboxypeptidase, a senescence related protein and an Aux/IAA, for S1, S2, S3 and S4, respectively.

The microarray data, analyzed by using the HORMONOMETER platform, allowed the identification of hormone-responsive genes, some of them putatively involved in seed-pericarp crosstalk. Results indicated that auxin, cytokinins, and gibberellins are good candidates, acting either directly (auxin) or indirectly as signals during early development, when the cross-talk is more active and vital for fruit set, whereas abscisic acid and ethylene may be involved later on.

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Conclusions

In this research, genes were identified marking different phases of seed and mesocarp development. The selected genes behaved as good seed markers, while for mesocarp their reliability appeared to be dependent upon developmental and ripening traits. Regarding the cross-talk between seed and pericarp, possible candidate signals were identified among hormones.

Further investigations relying upon the availability of whole genome platforms will allow the enrichment of a marker genes repertoire and the elucidation of players other than hormones that are involved in seed-pericarp cross-talk (i.e. hormone peptides and microRNAs).

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1. Background

Peach fruit development is tightly connected to embryogenesis. Fruit growth displays a double sigmoid pattern in which four stages named S1, S2, S3 and S4 can be distinguished [1]. The early part of S1 is characterized by cell division and enlargement lasting about two weeks, followed by cell enlargement. The slowdown in growth that occurs at S1/S2 transition is followed by endocarp lignification (pit hardening), which lasts for 12-15 days from the middle of S2 to its end. S3 starts with a resumption of growth mainly due to cell enlargement, thus generating the second exponential phase. Maturation is completed by the end of S3 and followed by ripening (S4). The four fruit developmental phases are determined using a mathematical model based on first derivative of the growth curve [1]. Identification of the growth phases is important both for developmental studies and for precision farming. However, the only easily detectable event is the end of pit hardening marking the S2/S3 transition, because the phase length is affected by both genotype (early, middle and late ripening varieties) and environmental cues. A continuous growth model reassessment is therefore required. Accordingly, the identification of developmental phase organ-specific molecular markers would be of great importance for scientific and practical purposes.

Seed development, necessary for fruit set [2], is characterized by a fast endosperm growth that starts immediately after fertilization concurrently with the nucellus re-absorption, and lasts until the beginning of endocarp lignification, when the seed reaches its final size. At the end of pit hardening, seed volume is mainly made up of endosperm and the embryo is at the heart stage. Thereafter, embryo growth resumes and cotyledon development is paralleled by endosperm re-absorption. Seed maturation is characterized by lipids accumulations [3], synthesis of specific late embryogenesis abundant (LEA) proteins and dehydration. Attempts to stimulate parthenocarpic fruit development by hormone applications resulted as being ineffective. Moreover, seed abnormalities at the early stages of development (S1 and S1/S2 transition stages) lead to abortion and fruitlet abscission [4]. Later, (late S2, S3 and S4), the relationships between fruit development and embryogenesis become less strict. This is the case for early ripening varieties characterized by the uncoupling of fruit development and embryogenesis. In fact, at harvest, seed development is still in progress and a long way from maturity. Seed

presence is always necessary to achieve normal fruit development even if embryo development is incomplete [5]. Apart from the above observations, molecular-genetic information on the relationship between fruit and seed development is scarce. Cross-talk between the two organs may involve different components of the signaling network, such as hormones, transcription factors (TFs) and other signaling molecules, playing either direct or indirect roles.

Concerning hormones, parthenocarpic fruit development in some species is induced by applications of auxin or cytokinins (CKs), or gibberellins (GAs), or hormone blends [6]. Molecular approaches have confirmed the role played by hormones, especially auxins [7]. Investigations in *Arabidopsis* identified a mutant, named *fwf* (*fruit without fertilization*), with a normal silique development even in the absence of seeds [8]. Double mutant analysis (*fwf ga1-4, fwf gai, fwf spy, fwf ats*) pointed out that FWF negatively affected GA biosynthesis and GA and auxin signal transduction. The FWF protein may interact with TFs such as Fruitful (FUL) and Aberrant Testa Shape (ATS), members of the MADS-box family, and Scarecrow –SCR- type, which are all involved in cell division [8]. Additional TFs have been identified, some of which are related to hormone action, actively transcribed along peach fruit development and ripening ([9]; [10]). Orthologues of these TFs are also expressed in true (silique and berry) and false (pome and strawberry) fruits, supporting the hypothesis that different fruit types share common regulatory elements [11]. High throughput analysis conducted in *Arabidopsis* showed that some TFs are shared by seed and fruit [12].

Taking this information into account, peach seed and fruit transcriptomes were explored throughout development by means of a massive gene approach based on the use of the µPEACH1.0 array platform and quantitative real time RT-PCR (qRT-PCR). The research identified genes marking organ/tissue developmental phases, as well as candidate signals (hormones and TFs) that may trigger the cross-talk between fruit and seed.

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2. Results

2.1 Seed and fruit growth pattern

Fruit growth analysis was performed on cv Fantasia and assumed as a reference (Figure 1).
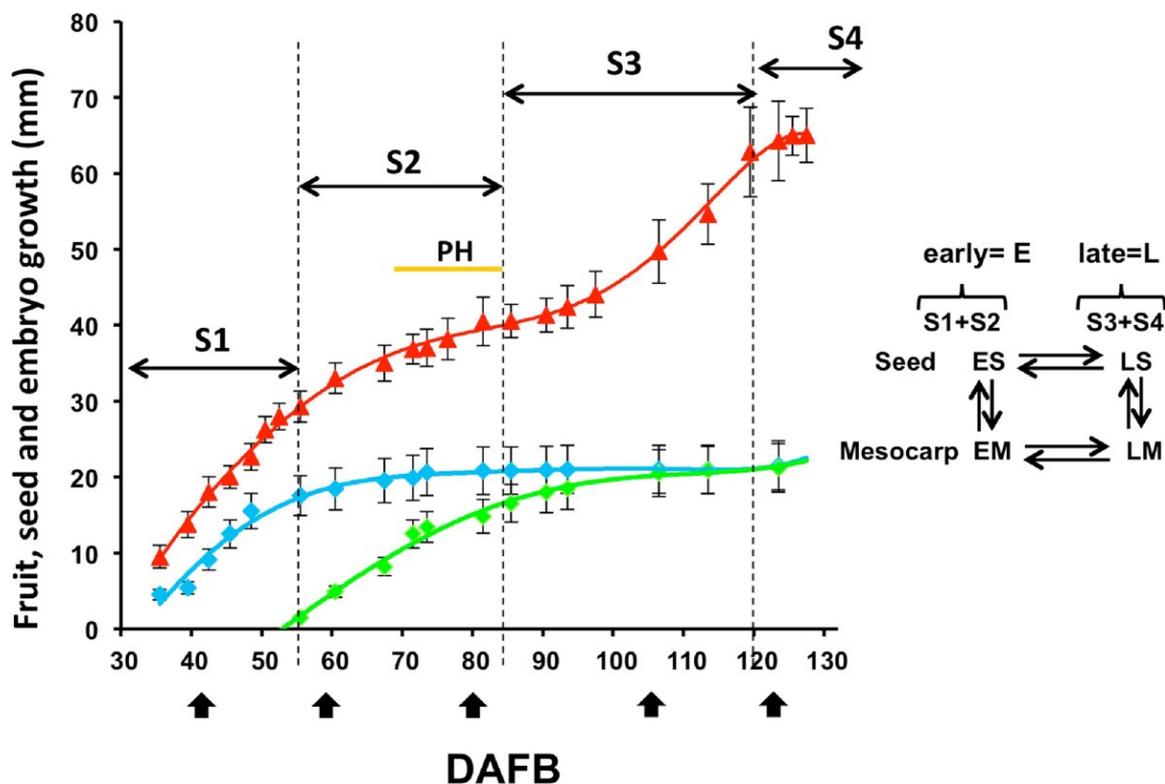
Figure 1. – **Fruit and seed growth pattern (cv Fantasia).** Fruit growth (red) is expressed as cross diameter while length is used for seed (blue) and embryo (green) development. Difference in length between seed and embryo represents endosperm, integuments and nucellus being a minimal part of the seed. Fruit developmental cycle has been divided into 4 main stages (S1 to S4) according to the first derivative of the fruit growth curve. The yellow horizontal line indicates pit hardening. Sampling dates are marked by black arrows. The simple loop microarray experimental design is outlined on the right. For the microarray expression analyses, seed (S) and mesocarp (M) tissues at S1 and S2I, and S3 and S4 were pooled, and defined as early (ES and EM) and late (LS and LM) development, respectively. The comparison has been made between different developmental stages (LS/ES and LM/EM) within the organs and between the two organs (ES/EM and LS/LM) within the developmental stage.

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In this genotype fruit development and ripening are completed in 135-140 days after full bloom (DAFB). Growth dynamics display the typical double sigmoidal pattern in which four developmental stages have been identified according to the first derivative. S1, S2, S3 and S4 lasted for 45, 32, 33 and 17 days, respectively. Pit hardening (PH) started 60 DAFB and was completed by the S2/S3 transition. The seed derives from the fertilized ovule and the initial increase in length (Figure 1) is due to the rapid nuclear division of the endosperm responsible for embryo sac expansion. Endosperm cellularization starts 40 DAFB and is completed by the beginning of PH. The embryo develops very slowly in the early stages (S1 and S2), reaching a length of about 40-60 µm. Later, at the S2/S3 transition, it resumes development reaching its final size by the middle of S3. The morphological completion of development is followed by maturation and desiccation.

2.2 Identification of marker genes

RNAs extracted before (E, early development) and after (L, late development) pit hardening have been used for microarray transcriptome analyses in order to identify genes possibly involved in seed-pericarp cross-talk or useful as organ and developmental phase molecular markers. Data obtained from the microarray analyses were handled either as single comparisons, i.e. late seed vs. early seed (LS/ES), late mesocarp vs. early mesocarp (LM/EM), within each hybridization or by combining the whole set of data, thus also including ES/EM and LS/LM (see Figure 1 insert). The microarray expression data (see Additional file 1), validated by means of qRT-PCR on 29 randomly selected genes, showed a Pearson correlation coefficient ranging, in the four comparisons, from 0.79 to 0.84 (see Additional file 2).

With the single comparison analyses, among the 360 differentially expressed genes within the two organs at early and late development (Figure 2A), 174 and 151 were differentially expressed only in seed (groups A and B) and mesocarp (groups C and D), respectively. Of the seed differentially expressed genes, 108 and 66 were more transcribed at early (group B) and late development (group A), respectively. Four genes, shared by seed and mesocarp, were more actively transcribed at late development (group E), while an additional four showed the opposite trend of expression, being induced in LS and repressed in LM (group H). In addition to the 108 genes more abundantly transcribed in ES (group B), 22 were also expressed in EM (group G), while 5 were

abundant in ES and EM (group F). Among the mesocarp differentially expressed genes, 101 and 50 were more transcribed in EM (group D) and LM (group C), respectively.
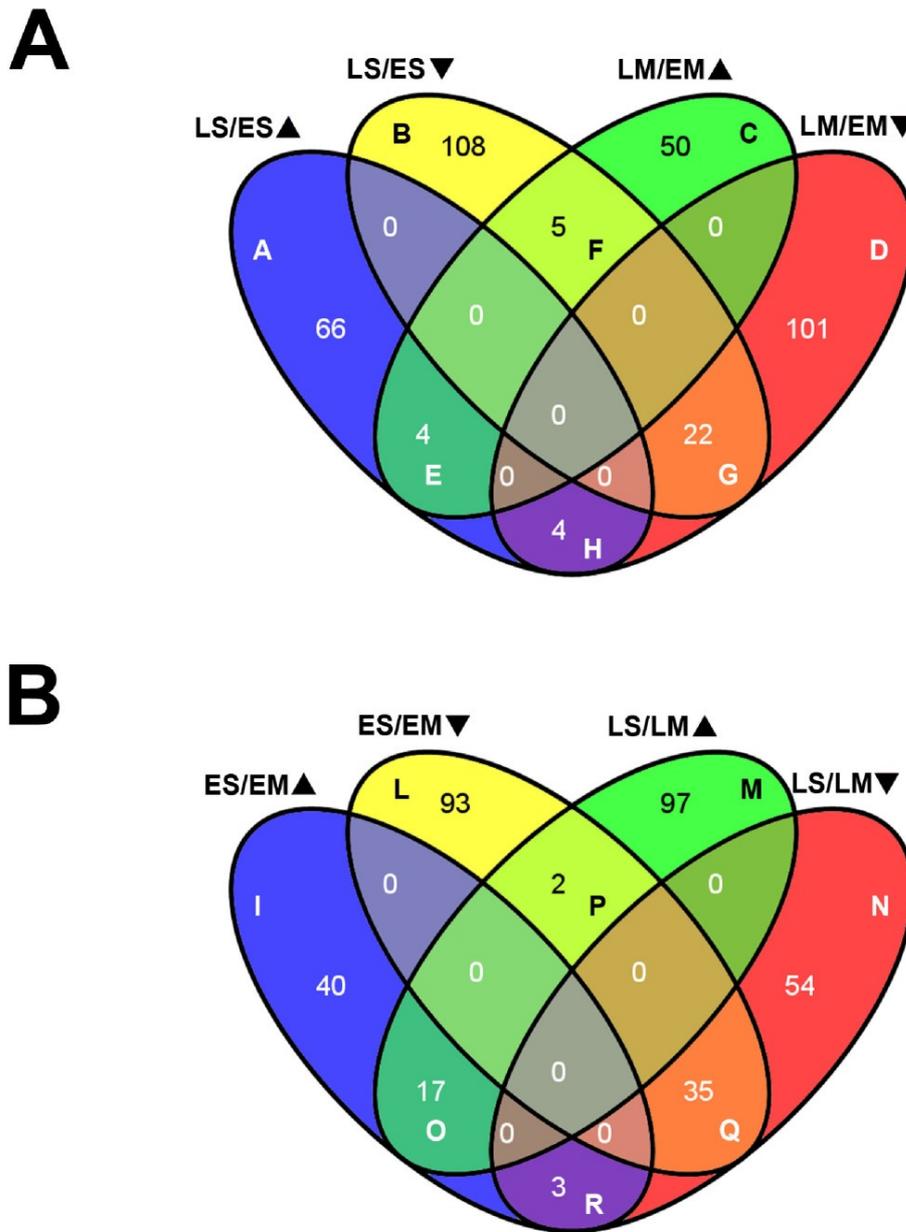
Figure 2. – **Genes differentially expressed according to the developmental stage of the organ**. Venn diagrams were used to visualize genes differentially expressed in the microarray experiments. Comparisons between early (E) and late (L) development (panel A), and seed (S) and mesocarp (M) (panel B), were made by means of a direct comparison approach (LS/ES and LM/EM in A; ES/EM and LS/LM in B). Arrowhead orientation indicates up (▲) and down (▼) regulation. The letters inside the sectors are tags for the identification of the genes listed in Additional file 1.

Taking the comparison between seed and mesocarp (ES/EM and LS/LM) into account, 341 genes were differentially expressed in the two organs (Figure 2B). Among these, 133 and 151 were differentially expressed only at early (groups I and L) and late (groups M and N) development, respectively. Considering the differentially-expressed genes at early development, 40 mRNAs were more abundant in seed (group I) and 93 in mesocarp (group L), while among the late development ones, 97 were more abundant in seed (group M) and 54 in mesocarp (group N). Of the 57 remaining genes, 17 and 35 transcripts were always more abundant in seed (group O) and mesocarp (group Q), respectively, and 5 displayed an opposite pattern, being more (3) or less (2) abundant in ES (group R) and ML (group P). Annotations of genes included in Figure 2 are reported with microarray expression data in Additional file 1.

Based on the above microarray analysis, putative markers were searched to find those that meet the following criteria: a) moderately to highly expressed in only one organ (seed or mesocarp), b) highly expressed/not expressed at specific developmental stage/s (S1 to S4). According to these criteria, 50 potential marker genes, chosen among those differentially expressed in the microarray, were selected and tested by means of qRT-PCR in leaf, flower (data not shown), seed and mesocarp at five developmental stages in cv Fantasia (Figure 3). These detailed expression profiles allowed the identification of eight genes best fulfilling the ideal marker criteria. For seed development, ctg3431, coding for a lipid transfer protein (LTP), ctg1026, coding for a pathogenesis related (PR) protein, ctg1540, coding for a prunin, and ctg3563, coding for a late embryogenesis abundant (LEA) protein, have been chosen as S1, S2, S3 and S4 markers, respectively. Concerning mesocarp development, ctg2909, coding for a RD22-like protein, ctg1751, coding for serine carboxypeptidase, ctg1823, coding for a senescent associated protein, and ctg57, coding for an AUX/IAA protein, have been selected as S1, S2, S3 and S4 markers, respectively (Figure 3). The function as stage markers has been confirmed on the same genotype for an additional growing season (Additional file 3).
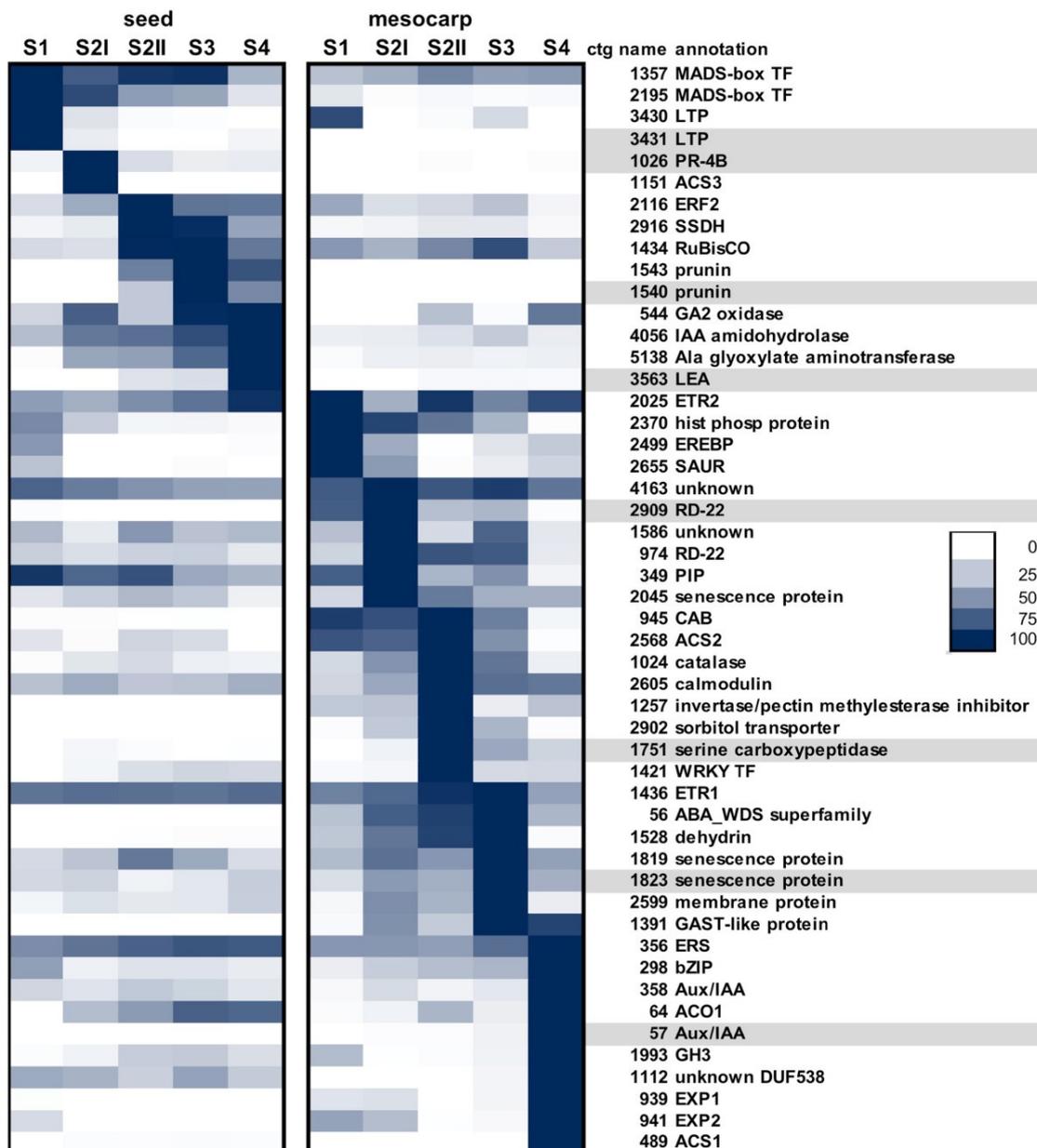
Figure 3. – **Selection of developmental stage and organ specific marker genes.** Identification of putative marker genes was performed by selecting some of those differentially expressed in the microarray analyses and further validated by means of qRT-PCR. This detailed expression profiling allowed the selection of those genes that best fitted the ideal marker characteristics as indicated in the Methods section. Expression profiles of 50 genes were measured in seed and mesocarp at five different developmental stages (S1 to S4). Expression values are related to the highest expression of each gene (0% white, 100% blue). Genes have been manually ordered according to their expression profiles. Grey shading highlights genes selected as markers.

A further validation of the selected genes was performed in two additional genotypes (cv Springcrest and the slow ripening - *slr* - selection) differing for the dynamics of seed and fruit development. In Springcrest, fruit ripening occurred after 86 DAFB (Figure 4A), when seed development was still in progress (Figure 4B). At the end of the growing season (taking cv Fantasia as a reference), *slr* showed a fully developed seed (Figures 4A and B), while the mesocarp development was blocked at stage S3.
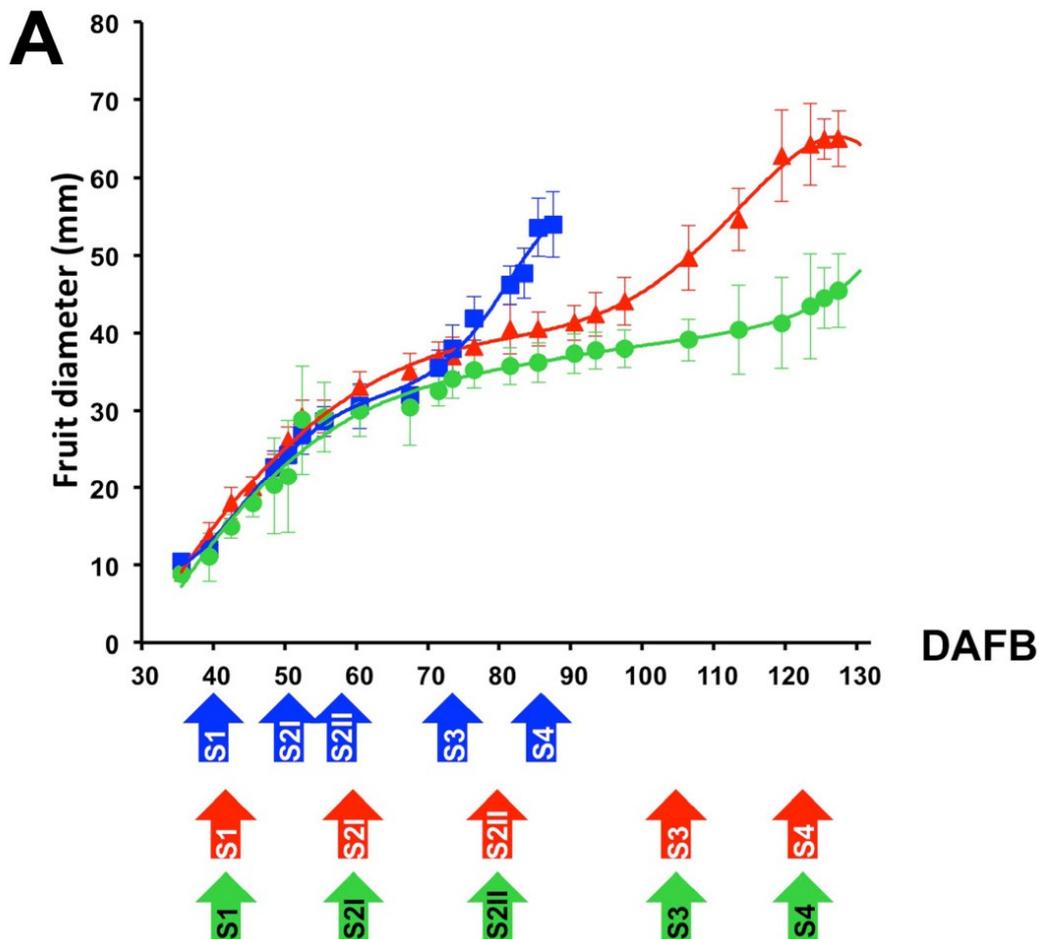
Figure 4 A. – **Dynamics of fruit and seed growth in Fantasia, Springcrest and *slr*.** Fruit growth curves are expressed as cross diameter (mm) for Fantasia (the reference genotype; red triangles), Springcrest (the early ripening genotype; blue squares) and *slr* (the slow ripening genotype; green circles). In the lower part of the panel, the arrowheads indicate the timing of sampling for the 3 cvs and the developmental stage is indicated within each arrow.
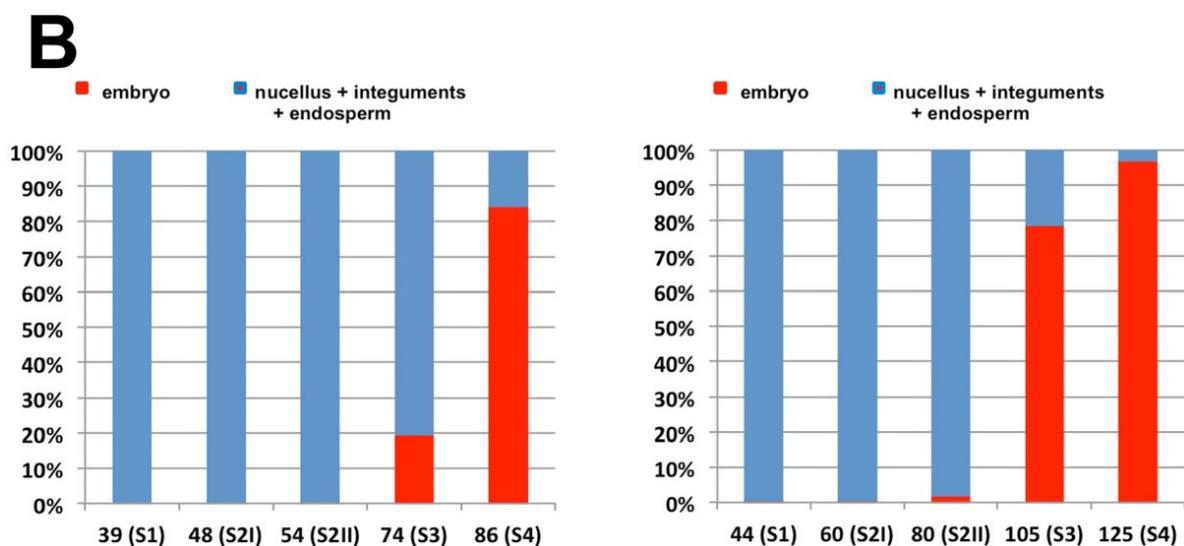
Figure 4 B. – **Dynamics of fruit and seed growth in Fantasia, Springcrest and** *slr*
Dynamics of seed development in Springcrest (left) and Fantasia (right) related to the
fruit developmental stages. Seed development in *slr* is similar to that reported for
Fantasia. Relative abundance of nucellus, integuments and endosperm (blue) and
embryo (red) points out that in Springcrest, at fruit harvest, embryo development is a
long way from maturity, while in *slr*, in spite of the block of fruit ripening, the
completion of embryo development parallels that of Fantasia and the seed is viable.

As regards seed markers, ctg3431, coding for a LTP, clearly marked the S1 stage for both
Fantasia and *slr*, while in Springcrest its expression decreased only at S3 stage (Figure
5A). A PR protein encoding gene, ctg1026, has been selected for the S2 stage. The
highest expression level was found in the seed of cv Fantasia, peaking at early S2 and
decreasing thereafter, as in Springcrest. In *slr*, its expression was broader, being relevant
also at S1 and S2II (87 and 86% of S2I, set as 100%, respectively; Figure 5B). A prunin,
the main seed storage protein in Prunus spp., encoded by ctg1540, is a good marker for
S3 seed development only in Fantasia. In fact, different amounts and kinetics of its
transcript accumulation were observed in the other two genotypes. In Fantasia,
accumulation started between S2I and S2II and increased up to a maximum at S3,
decreasing thereafter, whereas in *slr* and Springcrest transcript accumulation was
delayed, becoming detectable at S3 in the former and S4 in the latter (Figure 5C).
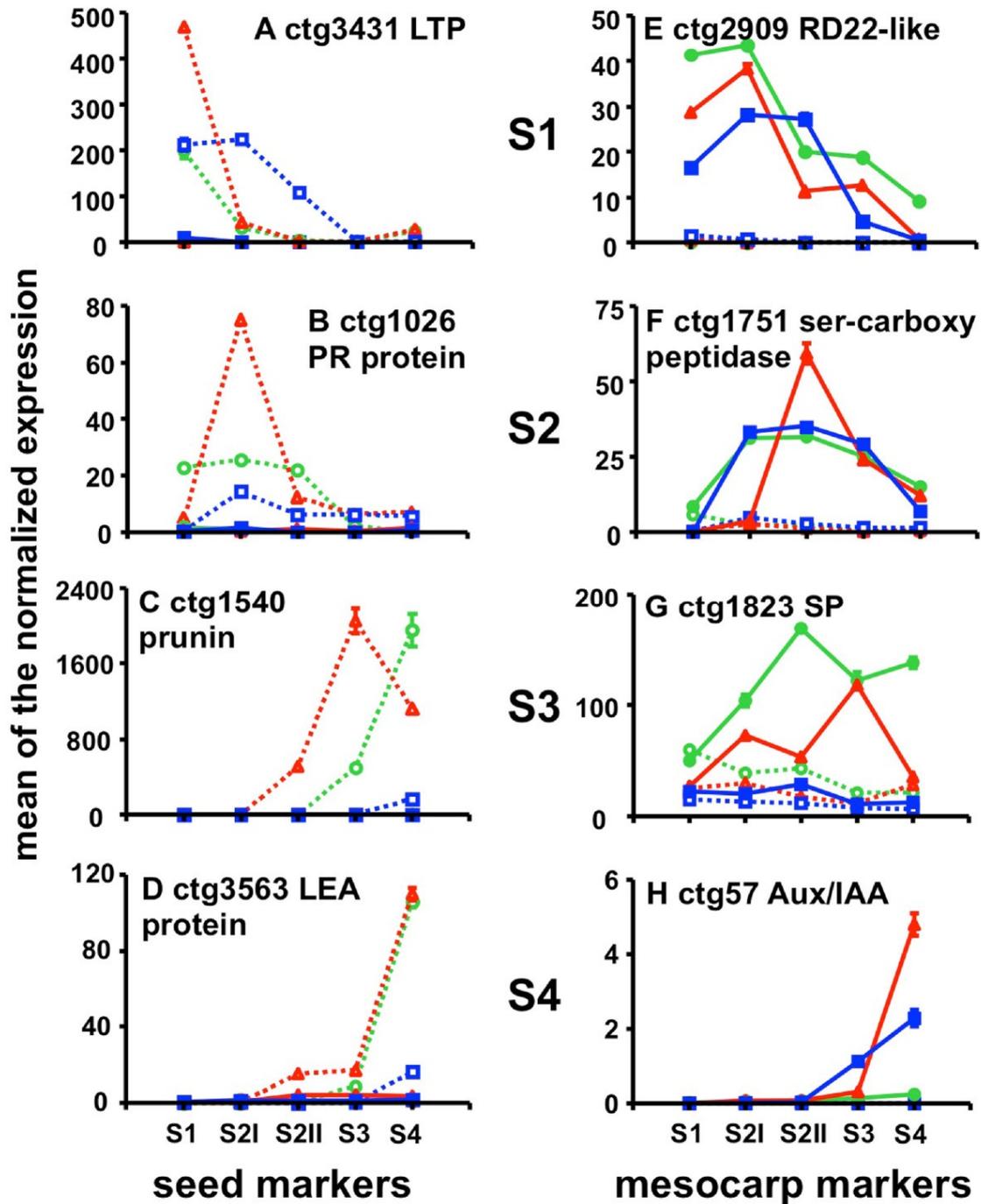
Figure 5. – **Validation of developmental stage and organ specific markers in mesocarp and seed of three genotypes.** Expression pattern, assessed by qRT-PCR, of seed (dashed lines) and mesocarp (solid lines) molecular markers of Fantasia (red triangles), Springcrest (blue squares) and *slr* (green circles), at five developmental stages (S1 to S4). Transcript levels are measured as means of normalized expression ± SEM of three technical replicates.

The expression of the gene encoding a LEA protein (ctg3563) became detectable at S2II in Fantasia and peaked at S4. A similar pattern was observed in *slr*, although the transcript only started to be detectable at S3. In Springcrest, it was detectable only at S4, at levels lower than in the other two genotypes (Figure 5D). The level of expression of the four genes in mesocarp was very low throughout development and comparable in the three cvs (Figures 5A-D).

As regards mesocarp, ctg2909, coding for an RD22-like protein, had maximum expression at S1 and early S2 (i.e. S2I, Figure 5E). In Fantasia and *slr* the expression decreased already at S2II (28% and 32% of the maximum in Fantasia and *slr*, respectively), while in Springcrest its expression was still high (96%) at S2II.

A serine carboxypeptidase (ctg1751) was chosen as a marker for the S2 developmental stage. In Fantasia, the transcript was undetectable at S1, at basal level at S2I, peaked sharply at S2II, and then declined at S3 and S4. Also in the other two varieties the transcript was undetectable at S1, but its expression, already high at S2I, slightly increased at S2II and remained at high levels at S3, decreasing at S4 (Figure 5F). The expression of ctg1823, encoding a senescence related protein, had a maximum in Fantasia at S3 (100%), while expression levels were much lower in the previous and following stages (29 and 9% at S2II and S4, respectively). Although its expression was relatively high (50%) at S2I, it may be considered a good S3 marker. In Springcrest, the expression was generally low at all stages, with a maximum at S2II. In the *slr* genotype, the accumulation of ctg1823 transcripts steadily increased during the early phases up to a maximum at S2II. Although slightly decreasing thereafter, the ctg1823 mRNA was also abundant at S3 and S4 (60 and 74% of S2II, respectively) (Figure 5G). S4 stage is clearly identified by the expression of ctg57, coding for an Aux/IAA protein. In Fantasia, the expression at S3 is about 6% of that measured at S4 and almost undetectable in early phases. In Springcrest its expression is also almost undetectable at S1, S2I and S2II, but at S3 it is already half of that measured at S4. In *slr*, although maximum expression is at S4, the transcripts accumulated at very low levels (5% of Fantasia) (Figure 5H).

In agreement with their being mesocarp markers, all the selected genes are almost undetectable in seed (Figure 5E-H) with the exception of ctg1823 in *slr*.

2.3 Hormones and TFs in seed fruit cross-talk

Hormone-related genes possibly involved in cross-talk between the two organs were identified among those spotted on the microarray based upon the list of hormonal indexes available for *Arabidopsis* ([13]; TAIR website). The portion of hormone responsive genes in *Arabidopsis* ranges between 3.8 and 9.4% of the whole transcriptome (TAIR 10 vers., 27,416 genes), depending on the hormone considered (Additional file 4). For μPEACH1.0 (4,806 targets), the portion of hormone responsive genes parallels that of *Arabidopsis*, ranging from 3.8 to 9.8% with values for each hormone class comparable to those calculated for *Arabidopsis*. An irrelevant bias may therefore be assumed to exist when peach expression data are used for HORMONOMETER analysis [13]. In addition, it could be assumed that the same proportion might be expected if a whole genome array were used.

A heat map was produced by considering the following subsets of genes for each hormone (Figure 6): i) genes involved in signal transduction (ST), ii) hormone-responsive genes (H), iii) genes with hormone-specific responsiveness (SRG), iv) hormone-responsive genes encoding TFs (TFs), and v) genes encoding TFs with hormone-specific responsiveness (sTFs). The subset i) was identified using the classification of *Arabidopsis* orthologs obtained from TAIR GO terms and AHD classification lists (available at http://ahd.cbi.pku.edu.cn/; [14]), and was then analyzed by averaging the log ratios, while the other subsets were used for the HORMONOMETER analyses [13].

Concerning auxin and intra-organ comparisons (LS/ES and LM/EM), a weak activation of ST was observed in LS with respect to ES, paralleled by a partial correlation with the overall reference hormone indexes, whereas a partial anti-correlation was observed when auxin-specific hormone indexes, TF- and specific TF-encoding targets were used. In the mesocarp, a marked up-regulation of ST subset was evidenced in LM, and a good correlation was shown in the same sample both considering the overall hormone indexes and all the other gene subsets. As regards inter-organ comparisons, a decreased transcription of ST elements was always observed in the seed, paralleled by an anti-correlation with the overall hormone indexes at both early (ES/EM) and late (LS/LM) development. However, considering the specific subset, a slight correlation was found in the former comparison, whereas all the results in the latter one were consistent with the

overall HORMONOMETER data. The intra-organ comparison LS/ES indicated a down-regulation of cytokinin (CK) ST elements at late seed development, paralleled by an anti-correlation with both the overall and specific hormone indexes. However, a slight correlation was observed in terms of specific TFs, while all TFs appeared not correlated. Concerning the mesocarp, a lower activation of ST elements in LM than EM was counteracted by a strong correlation with CK indexes. CK-specific genes appeared not correlated, whereas TFs showed a slight correlation, becoming stronger when only the CK-specific TFs were considered. As regards inter-organ comparisons, a low activation of the signal transduction in ES was counteracted by a strong correlation with overall hormone indexes. When the analysis was performed with the other subsets, a significant anti-correlation was observed. Finally, during late seed development, despite the higher activation of ST elements compared to the mesocarp, a general anti-correlation was shown, with the exception of specific TFs, which appeared not correlated.
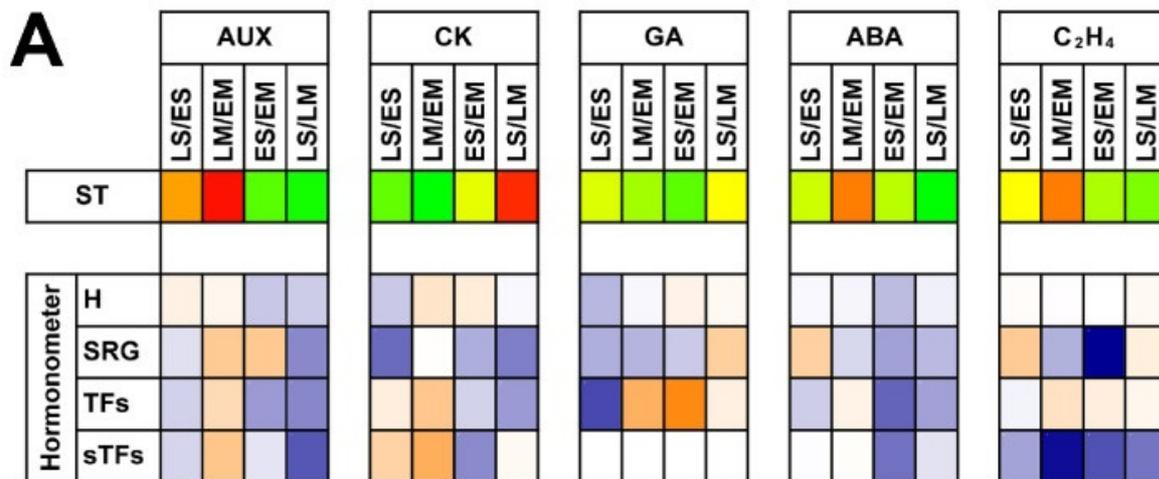
Figure 6A. – **Heat map showing the relationship between the expression of signal transduction and hormone target genes.** The heat map was produced by considering the genes involved in the signal transduction (ST) for auxin (AUX), cytokinin (CK), gibberellic acid (GA), abscissic acid (ABA) and ethylene (C2H4). HORMONOMETER data were grouped into hormone-responsive genes (H), genes with hormone-specific responsiveness (SRG), hormone-responsive genes encoding TFs (TFs), and genes encoding TFs with hormone-specific responsiveness (sTFs). For each hormone, the following comparisons have been analyzed: LS/SE, LM/EM, ES/EM and LS/LM.
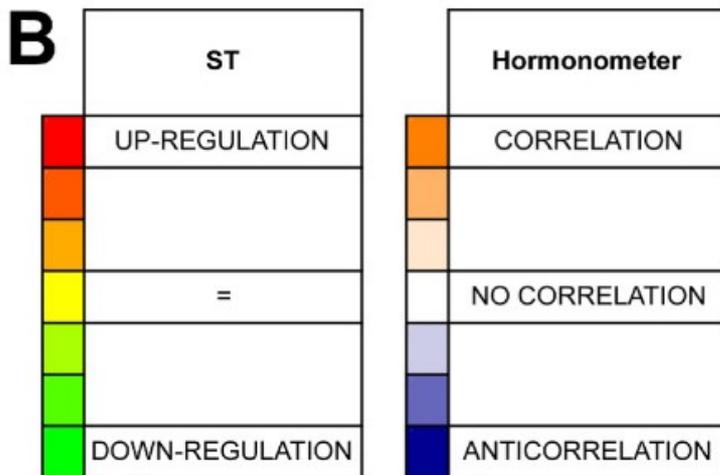
Figure 6B. – **Heat map showing the relationship between the expression of signal transduction and hormone target genes.** Color codes for ST genes and hormone-responsive genes (HORMONOMETER). For ST, red and green represent up- and down-regulation, respectively. In the HORMONOMETER, orange (value = 1), white (value = 0), and blue (value = -1) indicate a complete correlation, no correlation, or anti-correlation, respectively, in terms of direction and intensity of the hormone index with the queried experiment [13].

Considering the gibberellins (GAs)-related expression data, the LS/ES comparison demonstrated a good consistency in signal transduction, and anti-correlation with overall and specific transcriptional indexes, and TFs, except for the GA-specific TFs, that were not correlated. The mesocarp profile was similar except when all TFs were considered, the latter analysis showing a robust correlation. In the ES/EM inter-organ comparison, a depression of the ST pathway in the seed was evidenced. The overall HORMONOMETER analysis showed no correlation with GA hormone indexes, whereas an anti-correlation resulted from the analysis of hormone-specific targets. When all the TFs underwent the HORMONOMETER analysis, a strong correlation was shown, while specific TFs were not correlated. The most significant data pointed out by the LS/LM comparison concerned the analysis of GA-specific indexes, showing a slight correlation.

As regards abscisic acid (ABA) and intra-organ comparisons, in spite of a down-regulation of its ST pathway during late seed development, a correlation was observed in terms of

both overall and ABA-specific indexes. TFs were basically anti-correlated and not correlated, when considered either as a whole or just the specific ones, respectively. In the mesocarp, despite a weak up-regulation of the ST elements found in LM, there was no significant correlation in any of the HORMONOMETER analyses. Moving to inter-organ comparison ES/EM, the down-regulation of signal transduction occurring in ES paralleled an anti-correlation found in all the gene sets. In the LS/LM comparison, similar results were obtained in terms of both signal transduction and HORMONOMETER.

Concerning ethylene, no variation was observed between LS and ES in terms of expression of genes encoding ST elements. In spite of this, a slight correlation was pointed out by both overall and ethylene-specific gene targets. Moreover, TFs were not correlated, while specific TFs were slightly anti-correlated. With the LM/EM comparison, the hormone signaling pathway was up-regulated in LM, paralleled by a partial correlation of TFs. On the other hand, both the hormone specific subsets showed an anti-correlation, stronger in the case of TFs. Both inter-organ comparisons (ES/EM and LS/LM) displayed a down-regulation of the ST pathway in the seed. The HORMONOMETER analyses showed no correlation when all targets and all TFs were considered, and anti-correlation concerning the specific targets and TFs, stronger for the former. Both signal transduction and HORMONOMETER results related to jasmonates, salicylic acid, and brassinosteroids are presented and discussed in Additional file 5.

3. *Discussion*

This research was mainly focused on the relationship between seed and pericarp throughout development, using a mass gene approach by means of the μPEACH1.0 [9]. Although this platform was developed mainly from late development mesocarp cDNAs, hybridization analyses and differential expression profiles assessed for both early developing mesocarp and seed indicate that μPEACH1.0 is also a reliable tool for these transcriptomic investigations.

Concerning marker genes, morphological observations pointed out that the dynamics of seed development in different genotypes is quite synchronous, whereas a wide variation exists in the pericarp, affecting not only the length of the developmental phases but also important traits related to fruit quality, such as the degree of endocarp lignification (cartilaginous endocarp), flesh texture (melting/non-melting), sugar/acid ratio, etc. Accordingly, the singling out of marker genes specific for the same developmental stage is not always unequivocal for all three studied genotypes. Moreover, since seed sampling was referred to the fruit developmental stages (S1, S2, S3 and S4), expression data should be read taking into account the uncoupling that exists between seed and fruit development in Springcrest, an early ripening cultivar.

The ctg3431, marking S1 in the seed, encodes a lipid transfer protein similar to *Arabidopsis* LTP1 [15]. Its gene expression profile in peach is consistent with *Arabidopsis* data, the latter showing that *LTP1*, along with *LTP3*, *LTP4* and *LTP6*, is expressed at high levels during early seed development [16]. The function of this gene as an S1 marker was confirmed in all the genotypes. The delayed decay of transcript accumulation assessed in the seed of cv Springcrest has, in fact, to be related to the acceleration of mesocarp development in this genotype (Figure 4). The ctg1026 (Figure 5B) is similar to a carrot PR which has been related to early embryo development, being expressed in the endosperm and secreted in the apoplast, thus positively regulating embryo fate and patterning [17]. It is interesting to note that in cv Springcrest, the down-regulation of ctg1026 at S3 and S4 occurs at a slower rate than in Fantasia and *slr*, thus confirming the uncoupling of seed and mesocarp development also at the molecular level. The different kinetics observed for the expression of S3 marker, a gene encoding a prunin storage protein (ctg1540, Figure 5C) in *slr* indicates that in this selection, as well as the blocked

development of the mesocarp, some variations in seed storage accumulation may also exist. The apparent delay in transcript accumulation measured in Springcrest is again due, as in the case of ctg3431, to the uncoupled development of seed and pericarp. Ctg3563, encoding a LEA (late embryogenesis abundant) protein, is a very reliable marker of S4, in both Fantasia and *slr*, indicating that the seed can reach a fully matured stage in both genotypes. The very low levels of *LEA* gene expression detected at S4 in Springcrest are consistent with the uncoupling that exists between seed and pericarp maturation in this genotype.

Concerning the mesocarp, ctg2909, marking S1 and S2I, encodes a putative RD22-like protein, whose expression in both *Arabidopsis* and grape is partially under the control of ABA and claimed to be involved in stress responses [18]; [19]. Since the levels of this hormone in peach mesocarp were shown to follow a biphasic pattern with two peaks at S2I and S4 [20], the increasing expression of ctg2909 at early mesocarp development might be related to the level of ABA. However, while the hormone also peaks at S4, the expression levels of this gene did not, thus indicating a dual regulatory mechanism triggering its expression, possibly also under a developmental control as shown in the seed of *Arabidopsis* [19]. The delayed decay of ctg2909 expression observed in Springcrest might be related to the higher growth potential of this early ripening variety documented by the S2 phase length, which is significantly reduced compared to cv Fantasia (Figure 5E). The S2 phase is marked by ctg1751 (Figure 5F), coding for a serine carboxypeptidase (SCP). SCPs are members of the α/β hydrolase family of proteins, claimed to function also as acyltransferases and lyases in the biosynthesis of secondary metabolites [21]. Taking into account that the most important event occurring at S2II is endocarp lignification, an indirect role in this process might be hypothesized for ctg1751. Ctg1823 (Figure 5G) was shown to be a good S3 marker in Fantasia, but not in *slr* and Springcrest. Since this gene encodes a putative senescence-associated protein, a likely failure of the senescence process and/or of its entry phase may be hypothesized in the two genotypes in which mesocarp development is either slowed down or accelerated. Interestingly, when mesocarp development is slowed down, as in *slr*, the peak of expression of ctg1823 is anticipated, whereas in the other case (i.e. in Springcrest), in which mesocarp ripens very rapidly, the peak is almost absent. It may be

speculated that an overly precocious start of senescence would not allow the fruit to shift from maturation to ripening [22], and, vice versa, an acceleration of fruit ripening is achieved if senescence is not initiated. For the S4 phase, a very reliable marker is represented by ctg57 (Figure 5H), coding for an already partially characterized peach Aux/IAA protein [10]. Its expression was shown to increase at early S4, most likely under a developmental control, thereafter decreasing when ethylene climacteric is fully installed. Accordingly, ethylene treatments were shown to reduce the specific transcripts. Besides fully agreeing with previous data, the expression profiles shown here may also represent correlative evidence for a putative functional role. Indeed, no rise of expression was measured in the mesocarp of *slr*, consistent with the block/slowdown of development and ripening. Moreover, in the case of Springcrest, a high ethylene-producing variety [23], the rise in expression of ctg57 is both anticipated, paralleling ripening kinetics, and less pronounced than in Fantasia, in agreement with a negative effect exerted by higher levels of ethylene.

Possible mechanisms involved in seed-pericarp cross-talk should take into account the vascular and cellular connections existing between the two organs. It has been shown that all the maternal tissues of pericarp and seed (integuments) are intensively interconnected (Vizzotto, personal communication), while nucellar tissue is excluded from the plasmodesmata network. This implies that the flux of metabolites, as well as signaling molecules between embryo and fruit, must occur through the apoplast. Taking into account that hormones play a pivotal role in the regulation of seed and fruit development, it has been assumed that they might also be involved in the cross-talk between the two organs. The heat map data (Figure 6) will therefore be discussed taking into account the consistency of the colors in the following main two-by-two comparisons: ST/H, SRG/H, TFs/H, and sTFs/SRG. More specifically, considering the first one (ST/H), consistency of colors may indicate a relationship between the hormone-related response and activation of the corresponding signal transduction pathway. In the second comparison (SRG/H), the same parameter may provide information about the hormone specificity of the transcriptional response, and, at the same time, of the possible cross-talk between hormones. A double comparison (TFs/H, and sTFs/SRG) may allow it to be pointed out if other players besides the TFs are involved in the regulation of the

downstream processes, and if a specific response is mediated by hormone-specific TFs. Auxin, cytokinins, and gibberellins are generally considered to be the most relevant hormones for early seed and fruit development, whereas abscisic acid and ethylene play important roles in seed maturation and fruit ripening. From the point of view of the cross-talk between seed and mesocarp, comparisons should refer to the same developmental stage, i.e. ES/EM and LS/LM. Concerning auxin, the data presented here point out that the specificity of the response to the hormone is higher in ES and LM, although the relationship between the overall HORMONOMETER (H) and ST data indicates that mesocarp is always more sensitive than the seed to the hormone. Taking into account that the presence of a viable seed is required for fruit set and development in peach [2], and that the overexpression of auxin biosynthetic genes in the ovary stimulates the parthenocarpic fruit development in several species [6], it may be hypothesized that the signal produced by the developing seed might be either the auxin itself exported to the fruit, as demonstrated in tomato [24], or a secondary messenger whose target at the fruit level includes a large subset of auxin-responsive genes. This is consistent with both the high specificity of the auxin response shown here in the early developing seed and the higher sensitivity to the hormone displayed by the mesocarp paralleled by a strong hormone response. Among the mesocarp auxin responsive genes, several encode elements regulating transport (ctg2448, ctg2449 and ctg2789 [25] Additional file 1), indicating that auxin movement in this tissue is a relevant process, thus strengthening the hypothesis that auxin produced by the seed may behave as a signal efficiently transported to and within the mesocarp. An Aux/IAA-encoding gene (ctg358) showed an opposite transcription profile in the two organs, being abundant in ES and LM. It has been demonstrated that its tomato orthologue (i.e. LS-IAA9, [26]) acts as a repressor of auxin signaling. Thus, its expression in young organs (low in mesocarp, high in seed) seems to confirm that the hormonal response is not at the synthesis site. Finally, the expression of ctg2655, a SAUR-like IAA responsive gene [27] was found to be higher in mesocarp than in seed (see also Figure 3), thus suggesting a higher auxin level in EM than in ES [28]; [29].

The main process regulated by CKs is cell division, occurring at early development in both seed (endosperm) and mesocarp. In the former, there is an up-regulation of signal

transduction elements, such as ctg2370 coding for a histidine-containing phosphotransfer protein [30] whose transcription is abundant in very young organs (Figure 3). The corresponding substantial activation of hormonal targets, including several CK-specific genes, might differ in the two organs. For example, a cellulose synthase (ctg3673) is activated in EM but not in ES, cytokinesis being an LS event, whereas cyclin D3 (ctg779) was up-regulated in both organs at the early stage. However, this transcriptional response did not just involve CK-specific TFs, implying that other regulatory elements may determine the hormone-specific gene activation. A similar activation of signal transduction elements to that found in the seed is present in the mesocarp at early development. However, the overall and the CK-specific target activation are not correlated to the hormone action, suggesting that CKs may regulate mesocarp cell division at the post-transcriptional level [31], either alone or in cooperation with other phytohormones. Moreover, considering the inter-organ comparison, it is noteworthy that during early development the seed displayed a higher sensitivity to CKs than the mesocarp but a lower specificity of response. The amount of the overall transcriptional response observed in the seed may be due to the involvement of other hormones besides CKs [32]. During late development, an inverse situation was observed compared to the early phases. In fact, the high activation of signal transduction pathways occurring in the seed was uncoupled from the overall transcriptional response, which was even more specific in the mesocarp. The CK-mediated up-regulation of genes encoding sorbitol dehydrogenases (ctg636 and ctg1378, Additional file 1) appears particularly interesting, as this might increase the sink strength of the seed and attract photoassimilates to the entire fruit, which become more competitive in the partitioning process [33].

From a physiological point of view, GAs play a stimulatory role in fruit development, as shown by the ability to induce parthenocarpy in several species [34] when applied in post-bloom phase and/or early development. The initial phases of endosperm and embryo development are usually related to a high level of GAs [35], while seed maturation is paralleled by a decay of free GAs and increase of their conjugates. The HORMONOMETER data confirmed these results both in seed and mesocarp, except for TFs in the latter. In fact, the most relevant transcriptional response occurred during early

development at seed level as pointed out by the ES-specific expression of ctg3431 (Figure 5) encoding an orthologue of the *Arabidopsis* LTP1 (AT2G34580), which is classified as a GA-responsive gene (see at http://genome.weizmann.ac.il/hormonometer) involved in embryo patterning [36]. In the mesocarp, a low correlation was observed between the TF-related transcriptional response and GA action, implying the activation of complex regulatory mechanisms that may play relevant roles in the cross-talk between seed and mesocarp. A possible mode of interaction might be the EM specific expression of a gene coding for a Zinc finger protein (ctg187), whose *Arabidopsis* orthologue (AT2G04240, XERICO) interacts with DELLA proteins, is repressed by GA, and causes ABA accumulation when over-expressed [37]. However, since this transcriptional response lacked specificity, it might be hypothesized that GA action also depends on the interaction with other hormones. It has recently been demonstrated that auxin induced parthenocarpy via GAs in unpollinated tomato ovaries [38]. Furthermore, the peculiar expression profile of ctg1391, encoding a GAST-like protein, orthologue of *Arabidopsis* GASA6 (AT1G74670), in EM is confined to S2 and S4 stages, when cell enlargement is slow (Figure 3). These data are in agreement with the observed inhibition of cell elongation conferred on both *Arabidopsis* seedling and strawberry fruit over-expressing the Fragaria orthologue *FaGAST* [39]. During late development, in spite of the slight correlation existing in terms of GA-specific response, the other gene sets appeared not to be correlated to the hormone action. It may be deduced from this that the role of GA in the cross-talk between seed and mesocarp is negligible during late development.

ABA is known to play an antagonistic role with respect to auxin, GAs and CKs, as observed during fruit development in avocado [40] and tomato [41] [42]. According to the HORMONOMETER, this antagonism was largely confirmed in the seed, the transcriptional response being correlated with higher levels of the hormone in LS compared to ES, also when the ABA-specific subset was considered. In fact, during late seed development, ABA levels are known to increase and GA-related genes such as ctg3430, encoding a LTP-like, are down-regulated (Figure 3 and Additional file 1). This physiological parameter is paralleled by a consistent transcriptional response in which TFs belonging to WRKY (ctg1545), HD (ctg499), Aux/IAA (ctg768), bZIP (ctg 724)

and DREB-like AP2 (ctg 4674) families are involved. Given this interpretation and taking into account that during both early and late development ABA ST pathways and ABA-target responses are more active in the mesocarp, the hormone may play a more relevant role in the development of each organ, rather than in seed-mesocarp cross-talk. In this context, the ABA pool of maternal and zygotic origin may trigger independent transduction pathways.

The well-known role of ethylene in peach ripening [9, 10] was confirmed by the higher level of transcription of its ST elements (ctg4109, ctg244 and 4757 coding for an ETR2-like ethylene receptor and two ERFs, respectively) measured at the mesocarp level during late development. It is worth noting that ethylene-related transcriptional response in the LM/EM comparison resembles that of ABA, most likely because of the significant number of transcriptional targets shared by the two hormones (50 out of 216 ABA- and 235 ethylene- responsive genes). This was not observed in the inter-organ assessments, in which, differently from ABA, a weaker overall transcriptional response was pointed out, with the only exception being ethylene-specific TFs that were more represented in mesocarp. As regards cross-talk, the role of ethylene might be limited to the very early phase of fruit development, as demonstrated in tomato [43]. This might be a consequence of the fact that ethylene acts mainly within the cell where it is synthesized. Also in this case, the hormone of maternal and zygotic origin may activate independent pathways controlling different processes.

4. Conclusions

In this research, genes were identified marking different developmental phases of seed and mesocarp. The reliability of these molecular markers was tested in two subsequent years and a further functional validation was carried out in three different genotypes. In the latter case, data indicate that, while seed markers represent reliable tools in all the tested varieties, in the case of the mesocarp the different developmental and ripening traits of the various genotypes somewhat affect the expression of marker genes, consistently with their putative functions and cv characteristics. The most critical phases, from the point of view of mesocarp marker retrieval, were S2II and S3. This might be related to the high divergence in pericarp development among the different genotypes, as pointed out above. However, this limitation may be partially overcome by using mesocarp markers as a whole, therefore increasing their discriminating power.

As regards the cross-talk between seed and pericarp, possible candidate signals were identified among hormones. In the early phases, when the cross-talk is more vital for fruit set, the candidates are auxin, CKs, and GAs, acting either directly (auxin) or indirectly as signals, whereas ABA and ethylene appear to be involved later on.

Further investigations relying upon the availability of whole genome platforms will allow enrichment of the marker genes repertoire and elucidation of the cross-talk mechanisms between the two organs, taking into account, besides hormones, other players such as hormone peptides and microRNAs.

5. Methods

5.1 Plant materials

Fruit growth analysis was conducted on peach trees of cv Fantasia grown on the experimental farm of the University of Padova (Legnaro), Italy, as described by Tonutti et al., 1997. Fruits from 10 trees were collected at 42, 60, 81, 106 and 123 days after full bloom (DAFB), corresponding to the first exponential growth phase (S1), the onset (S2I) and end (S2II) of pit hardening, second exponential growth phase (S3) and ripening (S4), respectively. For each sampling, mesocarp and seed were excised from 30 fruit, pooled in three biological replicates and then immediately frozen in liquid nitrogen and stored at -80 °C until use. To monitor seed development, seeds were excised from fruit at weekly intervals from late S1 to ripening. Seed and embryo length were measured by stereomicroscopy [44].

Seed and fruit of two additional genotypes (cv Springcrest and selection slow ripening -*slr*) characterized by uncoupling of seed and fruit development, were used for the validation of marker gene functions. In Springcrest, an early ripening cultivar, fruit ripening occurs when seed development is still in progress. In fact, seeds become viable only after in vitro cultivation. *slr* is a selection obtained from a free-pollinated population of Fantasia, characterized by a block of mesocarp development at stage S3 but with a fully developed seed. Sampling of mesocarp and seed was performed throughout fruit development as previously described.

Fruit growth analyses were performed in 2008 and repeated in 2010; the array experiments were performed on samples collected in the former year, whereas expression data were validated by qRT-PCR on samples of both years. Only data related to 2008 are presented and discussed in the paper. Data from 2010 are given in the supplementary material (Additional file 3).

5.2 Transcriptome analysis

For each sampling data, total RNAs were extracted, as described in [45], from each of the three biological replicates of seed and mesocarp and stored at -80 °C for transcriptional analysis.

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To elucidate the interactions between seed and mesocarp, a mass gene approach was followed by using the µPEACH1.0 as described in [10]. Comparisons were made by pooling stage 1 and 2 (named early development, E), and stage 3 and 4 (named late development, L), separately for mesocarp (M) and seed (S), and using a simple loop experimental design (Figure 1).

Data were analyzed using the TM4 software platform [46] as previously described [10]. A SAM (Significance Analysis of Microarrays [47]) analysis was performed to identify significantly differentially expressed genes using a False Discovery rate of 0% (90th%ile). Among these, up and down regulated genes were identified assuming a threshold ratio of expression as log2 higher than 1 and lower than -1, respectively.

To improve the annotation of targets spotted on the µPEACH1.0 platform, all the oligo sequences were blasted against a transcript dataset obtained by assembling 280 000 454 reads (Additional file 7) with the about 90 000 sanger Prunus persica ESTs present in the NCBI database. The 454 reads have been obtained from a normalized library constructed by pooling equal amounts of mesocarp RNA from stages S1, S2, S3 and S4 (GenXPro GmbH, Germany). The 32 162 new contigs present in this new database have been compared to those used to develop the µPEACH1.0 platform and, if longer than the old ones, used for BLAST analysis. Contigs (Additional file 8) were analyzed by BLAST against already classified proteins from *Arabidopsis* (TAIR 10 release) to categorize them by using the GO terms developed by TAIR (http://www.arabidopsis.org/portals/genAnnotation/functional_annotation/ontologies.jsp) for the biological processes ontology. Based on the best BLAST search results and using a cut-off e value of $1*e{-10}$, the peach genes were assigned to the categories according to the most similar *Arabidopsis* genes (Additional file 1).

Differentially expressed genes were visualized with Venn diagrams drawn with Venny [48], clustered according to their expression profiles by using the Quality Threshold Clustering (QTC) coexpression algorithm [49] and grouped in four main charts allowing intra and inter-organ as well as developmental-stage comparisons.

The data discussed in this paper have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE22582 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE22582).

qRT-PCR was performed and the obtained data manipulated as previously described [10]. Briefly, 3 µg of total RNA for each sample, pre-treated with 1.5 units of DNaseI, was converted to cDNAs by means of the "High Capacity cDNA Archive Kit" (Applied Biosystems), which uses random examers as primers. Primer sequences for the selected genes are listed in Additional file 6. Oligonucleotides PpN1for (CCAGGAGAATCGGTGAGCAGAAAA) and PpN1rev (TCGAGGGTGGAGGACTTGAGAATG) annealing to the peach putative transcript ppa009483m, orthologous to *Arabidopsis* AT4G34270, were used to amplify the reference gene. The peach reference gene was selected starting from *Arabidopsis* homologous genes [50], tested for transcript normalization in peach (Tadiello and Trainotti, unpublished results) and chosen to normalize qRT-PCR data because of its superior result compared to the previously used Internal Transcribed Spacer of the ribosomal RNA [10]. Reactions were performed using 10 µL of the "Syber green PCR master mix" (Applied Biosystems), with 0.05 pmoles of each primer, in the "7500" instrument (Applied Biosystems). The obtained CT values were analyzed by means of the "Q-gene" software ([51]), averaging three independently calculated normalized expression values for each sample. Expression values are given as mean of the normalized expression values of the triplicates, calculated according to equation 2 of the "Q-gene" software ([51]). Differences in expression values among probes reflect different quantities of target amounts. Numerical values obtained with these calculations were transformed into graphics or used to build heat maps with MS Excel.

5.3 The HORMONOMETER analysis

The HORMONOMETER (http://genome.weizmann.ac.il/hormonometer/) is a bioinformatic tool for assessing any transcriptome response according to the perspective of similar events occurring upon hormonal activation [13]. A vector-based correlation is calculated by comparing the variation of the transcriptome in a query experiment with an indexed list of pre-calculated transcriptional responses established by published hormone treatments in *Arabidopsis* [52]. Input data, for each gene, consist of the fold change calculated by directly dividing the normalized expression values measured for the two samples to be compared, and the respective P-value of its significance. When the variations detected in the query resemble those of the reference pool related to a certain

hormone, it is assumed that the same hormone may have caused the transcriptional response observed in the query. In the HORMONOMETER output data, the numeral 1 indicates a complete correlation in terms of direction and intensity of the hormone index with the queried experiment, 0 indicates no correlation, and -1 indicates the highest possible anti-correlation for each transcript in the index [13]. Given that input data for each gene derive from a two-by-two comparison (for example, sample A versus sample B), correlation and anti-correlation indicate higher levels of the active hormone that are transduced into a measurable transcriptional response in either sample A or B, respectively, whereas no correlation (the numeral 0) indicates that the levels of the hormone are the same in both samples.

Since HORMONOMETER only accepts *Arabidopsis* data as input along with their corresponding locus name and Affymetrix probe IDs, the putative *Arabidopsis* orthologues of peach genes, obtained by using the best BLAST hit of the updated µPEACH1.0 database against TAIR 10, were used as input data with peach expression values. In addition to the whole set of peach genes, three subsets were submitted to HORMONOMETER: i) genes with hormone-specific responsiveness (i.e. that are not multiple targets of hormones), ii) hormone-responsive genes encoding TFs, and iii) genes encoding TFs with hormone-specific responsiveness.

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Additional material

Additional material associated with this article can be found, in the online version, at http://www.biomedcentral.com/1471-2229/11/107/additional

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Chapter III

Global analysis of gene activity and role of transcription factors during peach mesocarp and seed development

Global analysis of gene activity and role of transcription factors during peach mesocarp and seed development

This research is to be published

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In memory of Angelo Ramina

Authors' contributions

CB, LT, ARa devised the study and participated in its design and coordination; AT conducted the microarray experiments and VZ together ARi collected fruit material, measured seed development parameters and performed the validation of microarray data by qRT-PCR; VZ, CB, LT and ARa analyzed the data; VZ, CB, LT, ARa wrote the paper. All authors read and approved the final manuscript.

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Abstract

In Peach, seed and pericarp have a strict relationship in the early stages of development, which becomes less tight in later stages. In fact, hormones treatments are unable to produce parthenocarpic fruits. On the other hand, while in some cultivars the seed is mature at the fruit ripening, in other early cultivars the seed is not mature and the endosperm is not yet reabsorbed at ripening. Understanding the interactions between seed and fruit will shed light on the different growth behaviours of the various peach cultivars. A new whole-genome microarray tool (µPEACH3.0) has been designed with the purpose of studying this relationship at the transcriptomic level, in order to find similarities and differences in the developmental patterns of these two organs.

The new microarray µPEACH3.0 performed well, a good correlation between array and qRT-PCR data was obtained. 70% of the probes gave a remarkable signal, and it is probable that this ratio will increase when new peach tissues will be tested on the array. The cell cycle genes of mesocarp and seed development have been identified in both tissues. While mitosis-related genes are expressed only in the early stage of mesocarp development, DNA replication genes are expressed also in later stages, suggesting that endoreduplication processes occur in that period. A genome wide analysis of transcription factors (TFs) has been made, which resulted in the identification of TFs of the SQUAMOSA Promoter Binding family expressed in the early mesocarp and seed, and of TFs of the Grwoth Regulating Factors family in the late seed. MicroRNAs that may regulate the expression of these TFs were found and their abundance measured: the data obtained suggest that these microRNAs regulate these TFs also in the peach fruit. These results were confirmed by qRT-PCR in three different genotypes: an early ripening cultivar, a middle ripening one and a slow ripening genotype.

In this study we validated the new peach whole genome microarray µPEACH3.0. The data obtained allowed to identify genes involved in cell cycle regulations and endoreduplication. Moreover. gene expression of TFs involved in seed and mesocarp development was assessed, as well as the abundance of microRNA that may regulated them. Further studies are needed to confirm endoreduplication data, and the array will be used to set up an atlas of the gene expression in the various peach tissues.

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1. Introduction

Peach is the third economically most important fruit of the Rosaceae family worldwide after apple and pear. In addition, thanks also to the release of a high-quality whole genome sequence (The International Peach Genome Initiative et al., 2013), peach is considered a model for functional genomic studies for fruit trees, in particular for those trees whose fruit is a drupe.

Peach fruit is a drupe, a fruit characterized by a lignified endocarp in which the seed completes its development physically separated from the pericarp. Peach fruit, as all those in which the endocarp is lignified, exhibit a double-sigmoid growth curve that can be divided into four stages of development (Tonutti et al., 1997). In the first stage (called S1), the fruit grows quickly by cell division and expansion, the second stage (S2) is characterized by a slowdown in dimensional growth and by the lignification of the endocarp, which hardens around the seed and differentiates into the pit. Since this point on, the seed has no symplastic links with the mesocarp. Then the growth resumes (stage S3), but only by cell expansion in the mesocarp, and finally the fruit ripens after the climacteric (stage S4).

Peach seeds are exalbuminous: the seed is initially filled by the endosperm, then this tissue is reabsorbed by the growing embryo. The seed size increases until the mesocarp's S2 stage, since then the lignification of the endocarp prevents any more growth (Ognjanov et al., 1995; Bonghi et al., 2011).

The relationship between fruit and seed is tight in the early stages as demonstrated by the fact that attempts to induce parthenocarpic peach fruits with hormones failed (Stutte and Gage, 1990). On the contrary, in later stages, the patterns of development of the two organs can be uncoupled as occurs in early cultivars (such as 'SpringCrest') in which the endosperm is not yet completely reabsorbed when the fruit ripens (Bassi and Monet, 2008). The endosperm is totally reabsorbed in cvs classified as middle and late- ripening and therefore the seed development is completed when the mesocarp is fully ripe. In addition to these phenotypes it has been identified a population named *slow ripening* (*slr*) in which the seed reach the maturity while the pericarp development is blocked at S3 stage (Bonghi et al., 2011).

The cross-talk between fruit and seed has been partially analyzed by microarray technology. In that study, using the µPEACH1.0 array platform marker genes for the various developmental stages of seed and mesocarp were identified, along with possible hormone signals between the two organs (Bonghi et al., 2011).

The same array platform, which is made of 4 806 different probes, had already been used in several studies concerning the role of hormones during fruit development alone: the effects of treatments with auxin and ethylene (Trainotti et al., 2007), jasmonates (Ziosi et al., 2008) and the ethylene inhibitor 1-MCP (Ziliotto et al., 2008) were investigated.

Other arrays used to analyze peach development have been designed: ChillPeach is a microarray with 4 261 probes specifically planned to investigate chill damage on harvested peaches (Ogundiwin et al., 2008), while µPEACH2.0 with 4 776 probes was used to identify aroma-related genes (Pirona et al., 2013).

A major drawback of these arrays is that their probes represent only a subset of the whole set of the peach genes, a feature that hinders studies directed to biological events different from those for which the arrays were designed. To overcome this problem, a study intended to analyze endocarp lignification used with success an apple microarray with 15 000 probes, with along with the smaller, but species-specific µPEACH1.0 (Dardick et al., 2010).

Considering the success of microarray technology on dissecting peach development at the molecular level, the necessity of many more probes in the arrays and the pre-publication of the peach genome in 2010, a new whole genome microarray with 29 800 probes called µPEACH3.0 was designed and used in this study. µPEACH3.0 already offered a first whole genome transcriptomic glimpse on a peach tissue, investigating the effects of wounding on two different peach varieties (Tosetti et al., 2014).

Peach ripening syndrome has been already widely studied in many works, including transcriptomic ones: the use of µPEACH1.0 on ripening peaches led to the characterization of a new *ETR* gene along with 19 ripening-related transcription factors belonging to several families (Trainotti et al., 2006), while the System 1 and System 2 *ACO* genes were previously identified (Ruperti et al., 2001) while transcriptional information about the early phases of development are lesser. Morphologically, the early fruit is characterized by both a growing mesocarp and endocarp, despite the two tissues

are already clearly different (Arnau et al., 1999): endocarp cells show phenolic inclusions in the vacuoles, while mesocarp chloroplasts are rich in starch grains.(Masia et al., 1992). The early stages of peach fruit development have not been thoroughly studied at the transcriptomic level, except for the pit hardening syndrome (Dardick et al., 2010), while several studies exist for the tomato and apple.

In tomato, early studies revealed how a protein encoded in the FW2.2 QTL regulates fruit cell division in an organ-specific manner (Cong and Tanksley, 2006), a similar role was found to be played by the protein encoded by the *OVATE* locus (Liu et al., 2002). A first transcriptomic analysis, using an array with about 1200 probes, identified TIP aquaporins as well as auxin-related genes as preferentially expressed in the fleshy and growing locular tissue (Lemaire-Chamley et al., 2005). Cyclins and cyclin-kinases are also involved in the early tomato fruit development: *CDKB1* and *CDKB2* genes are similarly expressed in the these early stages, triggering mitosis, while *CDKA1*, which expression is inhibited by *CDKB*, is expressed later (Czerednik et al., 2012). Whereas CDKB proteins are involved in cell division, CDKA is implicated in the events of endoreduplication, and then in cell expansion (Chevalier et al., 2011). Through a microarray analysis on apple, homologues of *CDKB* genes were found to be expressed during the cell division stage along with a *CKS1* homologue, being CSK1 a protein closely associated with the CDKB (Janssen et al., 2008). qRT-PCRs performed on apple cell-cycle genes confirmed the expression pattern of *CDKB*s and detected an increase of *CDKA* in the period following the cell division stage. Homologues of *DEL1* and *WEE1* were found to be associated with cell production, while homologues of *KRP4* and *KRP5* with the exit of this period (Malladi and Johnson, 2011). A recent study suggests that cell production in apple fruit is triggered by two AINTEGUMENTA protein homologues: ANT1 and ANT2 (Dash and Malladi, 2012). µPEACH3.0 will shed light on how this mechanism works in peach fruit.

Transcription factors have an important role in these processes as well as in the other process of fruit development, as they regulate the transcription of all these genes. In addition, gene expression in fruits may be regulated by smallRNAs such as microRNAs (Seymour et al., 2013).

Specific tanscription factors families were found to be particularly important for fruit development. The ripening syndrome has been thoroughly studied in tomato and involves a series of transcription factors mainly belonging to the MADS-box family. The mutation of the *RIN* gene (a MADS-box of the *SEPALLATA4* clade) inhibits ripening in tomato (Vrebalov et al., 2002), downstream to *RIN* acts *CNR*, another MADS-box, but belonging to the *SQUAMOSA PROMOTER BINDING* family (SBP). Also the homologue of *SHATTERPROOF, TAGL1*, has a role in both tomato ripening (reduction of *TAGL1* mRNA down-regulates *ACS2* expression) and in cell expansion (Vrebalov et al., 2009). Still, the transcription factor triggering the expression of *ACO1*, the gene that produces the climacteric ethylene is a Homeodomain-Leucine zipper Homoeobx protein called LeHB1 (Lin et al., 2008). Downstream to these genes act the apetala2/ethylene responsive factors (AP2/ERF), among them, AP2a negatively regulates tomato ripening (Chung et al., 2010).

MADS-box transcription factors were found to be pivotal in fruit set, In particular those belonging to the *SBP* family, both *SPL9* and *SPL3* are involved in determining floral identity, the former activating the transcription of *SOC1*, the latter the expression of *FUL* (Wang et al., 2009). Interestingly the abundance of the mRNA of both these genes is regulated by miR156 (Rhoades et al., 2002). Strikingly, in *Arabidopsis* leaves, it has been found that *SPL9* in turn regulates the expression of another microRNA mir172, which inhibits the translation of *APETALA2-like* genes. (Chen et al., 2010). Transcription factors and microRNA are strictly entangled in plant regulatory networks.

In *Arabidopsis* seed, a MADS-box heterodimer made of *AGL61* and *AGL80* specifies the central cell of the ovule, and then the endosperm early development (Bemer et al., 2008). A mutation in a bHLH transcription factor, *RETARDED GROWTH OF EMBRYO (RGE1)*, results in a retarded embryo development (Kondou et al., 2008).

The aim of this study, is to broaden the knowledge of the development of both seed and mesocarp development in peach. In fact, a first study on these two tissues has already been performed, but only with two time-points and using a small microarray with 4806 probes (Bonghi et al., 2011). In this study, 6 time-points (two in the S1 and S2 stages, and one for S3 and S4 stages) for each tissue and a whole-genome microarray, μPEACH3.0 are used. In particular the first stages of development of are analyzed in

both organs, and the regulatory networks involving both transcription factors and microRNAs are investigated. The results obtained from the microarray data, were then confirmed by RT-PCR on the early peach cultivar 'SpringCrest', the middle nectarine cultivar 'Fantasia' and the slow ripening nectarine genotype (*slr*).

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2. Materials and methods

2.1 Fruit samples

Fruits of peach cultivars 'Fantasia', 'SpringCrest' and genotype '*slr*' were collected in 2009 and 2011 from trees grown in the experimental farm of the University of Padova in Legnaro (Italy). Peach fruit growth was assessed as described in (Tonutti et al., 1997) and in (Bonghi et al., 2011).

In 2009, 50 fruits from 8 trees of cultivar 'Fantasia' were sampled at 12 time-points (T1-T12). After sample selection based on the expression of developmental markers (Bonghi et al, 2001), only 6 of these were used for the microarray analysis. Samples were collected at 41, 54, 69, 83, 111 and 125 days after full bloom (DAFB), corresponding to:

- T1: the first exponential growth phase (S1),

- T3: the transition between S1 and the phase of pit hardening (S2),

- T5: the S2 phase,

- T7: the lag phase between S2 and the second exponential growth phase (S3),

- T10: the S3 phase,

- T11: the onset of ripening (S4).

At the seed level, these time-points correspond to:

- a seed containing almost exclusively endosperm (T1 and T3),

- a seed containing both endosperm and a growing embryo (T5 and T7),

- a seed including a mature embryo (T10 and T11).

In 2011, 50 fruits from 8 trees of each cultivar were resampled. For the cultivar 'Springcrest' the fruits were collected at 8 different time-points (chronologically corresponding to 'Fantasia' fruit development phases from T1 to T8) covering the entire growth of the fruit, 10 time points were sampled for the cultivar 'Fantasia' (from T1 to T12, excluding samplings at T6 and T8) and additional 2 time-points (T13 and T14) were collected for the slow ripening (*slr*) genotype.

Mesocarps and seeds were excised from fruits at each sampling point and then pooled in 3 biological replicates. Samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

2.2 Microarray features

A new microarray platform, named μPEACH3.0, has recently been developed and it use is here described for the first time. It is based on a custom-made Agilent SurePrint G3 platform with a 8x60k format. The probe selection has been carried out on 30,113 predicted transcripts. The 28,689 officially released transcripts (The International Peach Genome Initiative et al., 2013) have been implemented by adding 1,424 new predictions (data not shown). 454 and Solexa RNAseq experiments have been used to implement the new in silico transcript predictions. Each of the eight arrays on each slide contains the 29,800 peach probes, each printed twice. Each probe has been design to have a Tm of 60 ± 2°C and, at this T, to be specific for a single gene.

2.3 Extraction of RNA and microarray hybridization

RNA was extracted from 36 samples of cultivar 'Fantasia' collected in 2009 (2 tissues, 6 time-points, 3 biological replicates). A CTAB/chloroform method was used as described by (Chang et al., 1993). The chloroform extraction step was repeated five times. 100 ng total RNA from the 36 samples was hybridized on μPEACH3.0 following the manufacturer's instructions.

2.4 Microarray data processing

Microarray data produced by the Agilent microarray scanner were pre-processed deleting from the analysis every spot that was not considered 'well above background' by the Agilent pre-processing software (Agilent Feature Extraction Software). For each probe, intensity data of the two spots were averaged and then quantile-normalized using R library 'preprocessCore' (Bolstad et al., 2003). Moreover, probes that had intensity levels lower than ten-fold the minimum significant threshold in all the replicates were discarded in order to strengthen the analysis.

Data were normalized separately for mesocarp and seed for the differential expression analyses, but together for the PCA analysis. Normalized data were loaded in the TMEV software package (Saeed et al., 2003). Data were logarithmised and samples were analysed using the Principal Component Analysis tool (Raychaudhuri et al., 2000).

2.5 Differential expression and clustering

Differential expressed genes were identified using the EDGE software (Storey et al., 2005) (Leek et al., 2006). Data were logarithmized and then analyzed using the Time Course settings with Natural Cubic as spline type. Every gene with a q-value inferior to 10^{-6} was considered significantly differentially expressed during the timecourse.

Soft clustering was performed with the R library 'mfuzz' (Kumar and Futschik, 2007). In contrast to hard clustering, soft clustering allows genes to belong to none, one or more clusters. The m parameter was set to 1.25 and 6 clusters were found. A threshold of at least 33.3% of affinity was used to determine if a gene was included in a cluster (that means a single gene could not be included in more than 2 clusters).

2.6 Identification of genes involved in cell division and endoreduplication and those coding TFs

Genes related to cell division and endoreduplication were manually identified on the basis of their Uniprot and/or GO terms descriptions. Clustering was performed using hierarchical average linkage clustering tool of the TMEV package.

Transcription factors were manually identified on the reference genome using the annotation of Gene Ontologies produced by the IPGI (The International Peach Genome Initiative et al., 2013). Transcription factors were then assigned to gene families accordingly to the membership of their *Arabidopsis* putative homologues as identified by the IPGI. The TAIR's *Arabidopsis* gene families database was used to assign these homologues to transcription factor families (Lamesch et al., 2012).

2.7 Enrichment analysis

Enrichment analysis was done using the Blast2GO software (Conesa et al., 2005). A two-tailed Fisher's exact test was performed using mfuzz clusters as test-set and the entire peach genome as reference. An FDR lower than 5% was considered significant.

2.8 Semi-quantitative Real time PCR

Semi-quantitative Real Time-PCR (qRT-PCR) was used to validate data and to measure gene expression in cultivars different from 'Fantasia'. Real Time-PCR validation was

performed on the same total RNA samples used for the microarray analysis, while gene expression in the various cultivars was measured in the 2011 samples.

DNase treatment was done using the RQ1 RNase-Free DNase (Promega) following the manufacturer's protocol. The enzyme was eliminated through a phenol/chloroform extraction. RNA was re-suspended in RNase-free water and NanoDrop (Thermo Scientific) was used to quantify the RNA.

cDNA was synthesized using the M-MLV Reverse Transcriptase (Promega) following the manufacturer's protocol. Oligo(dT) was used as a reaction primer, so that polyadenylated mRNA was preferentially amplified.

Table 1 – Primers used in qRT-PCR. The gene ID (from IPGI) and the name of putative *Arabidopsis* homologue (from TAIR) is given.

P. persica ID	TAIR	Forward (5'->3')	Reverse (5'->3')
ppa015949m	CYCD3;1	CTGCCGTTCTTGCTGTGGAT	GGGAACTTGGGTCTCCTCCA
ppa004940m	CYCA2;3	AGGCTGTGCGAACAAATCCA	CAGCAAGCATTGGTCACATCC
ppa024291m	CYCA3;1	CCAAAGCCAAGGCGAAGAAA	AATCATAAATGTCACGAGCATAAGG
ppa009162m	CDKB1;2	CCTCGACACCGATCTCAAGAA	CCCTTGCACAGTTGGTAGAGGA
ppa009392m	CDC2	CGTGGTTTACAAGGCTCGTG	GCCTGACAATGTTGCCATGTT
ppa010514m	KRP3	CATCGGCTTCACCTGTCACC	GCCTCGAAGTCGCCCTAGAA
ppa015604m	WEE1	GCTTGGTGACTTTGGATGTGC	TGGCAACTCCCAAGGAGAAGA
ppa012607m	SPL3	AAGGTCTGCGGTGTTCATGC	ACGCCTCCGACAACTCCTTT
ppa021582m	SPL9	GCTTCCCAATCTGGGTCTCG	TCTGCTGGTTGGTGGAGGAG
ppa023657m	SPL13	TGGGAAATTCAGGCAACAGC	TGCGTGGCTCAAACCAATCT
ppa024293m	GRF7	TTTATTGACGCGTGGTCCAAA	GGTGAGAGCTGCCCACTTGA
ppa021277m	GRF9	CCACTACCACCATTGCTGGAC	TGCTGCTTCCTCTGATCATGC
ppa001069m	ARF8	GCATTCCAAGGCGGTACTGA	TGGAAGCACCAAACGGCATA

qRT-PCR was performed in a volume of 10 µl using Fast SYBR® Green PCR Master Mix (Applied Biosystems), with 300 nM of specific primers (table 1) and 1 µl of a 1:50 dilution of cDNA. The mixture was amplified in a StepOnePlus Real Time machine

(Applied Biosystem) under the following method: an hold of 20 seconds at 95°C to activate the enzyme, 40 cycles constituted by 3 seconds of denaturation at 95°C and 30 seconds of extension at 64°C. At the end of the cycle, the expected melting temperature of amplification products was confirmed through a melt curve step.

Raw data were pre-analyzed by the StepOne Software v 2.0 (Applied Biosystem) to find the threshold cycle of each amplification. Analyses of the qRT-PCR data were done in MS Excel (Microsoft) using a home-modified version of the spread-sheet Q-gene (Simon, 2003).

2.9 miRNA Assay

TaqMan® MicroRNA Assay was used to assess the expression levels of miRNAs in the various samples. To extract total RNA containing miRNAs an extraction procedure slightly different from that previously described was used, The LiCl precipitation step was substituted with a isopropanol precipitation step: the supernatant from the preceding centrifugation step was mixed with a volume of isopropanol and then precipitated with an incubation at -80°C for an hour. Quality and quantity were then measured with both Nanodrop (Thermo Scientific) and with the Small RNA Kit for Bionalyzer (Agilent), quality of total RNA extracted with this method was lower, but quantity of miRNA higher. Quality was high enough to proceed with the following MicroRNA assay steps. Analyses of both the qRT-PCR and the MicroRNA Assay data were done in MS Excel (Microsoft) using home-modified versions of the spread-sheet Q-gene (Simon, 2003).

3. Results

3.1 Fruit growth

In 2009, 'Fantasia' fruit size was assessed during the whole growth process from 41 days after full bloom (DAFB) to harvest time at 137 DAFB. 'Fantasia' peaches grew accordingly to the typical double sigmoid pattern and the four developmental stages (S1, S2, S3, S4) were easily identified. Pit hardening (S2 stage) started between 54 and 62 DAFB and ripening (S4 stage) between 111 and 125 DAFB, a timing similar to that found for the same plants in 2008 (Bonghi et al., 2011). Embryo started to be visible in the seed since 62 DAFB and cotyledons were fully developed at 111 DAFB (Figure 1).
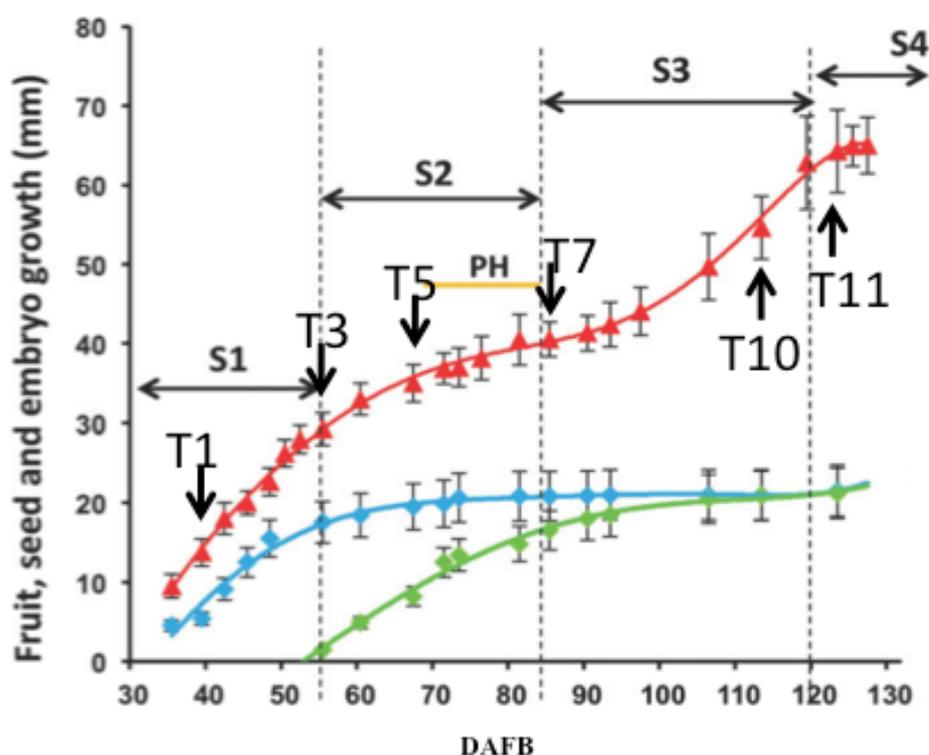

Figure 1. – Fruit growth of 'Fantasia' in 2009. Mesocarp growth shows the typical double sigmoid pattern (red) and the four stages are recognizable. Growth of the seed and of the embryo are also shown (blue and green). The embryo starts to be visible only after T3 and completely fill the seed by T10. The six timepoints that were anaylized by microarray are shown.

In 2011, fruits from 'Fantasia', 'Springcrest' and *slr* were sampled. Fruits of 'Fantasia' were collected at the same stages sampled in 2009, including some more intermediate time-points. Fruits from 'Fantasia' grew normally as in 2011, reaching approximately the same size at 135 DAFB.

Fruits of 'Springcrest' grew quicker than 'Fantasia', as typical for this variety: 'Spingcrest' pit hardening stage (i.e. S2) began one week before 'Fantasia' (between 47 and 54 DAFB) and lasted one week less, 'Springrest' then entered the climacteric phase when 'Fantasia' was moving into S3. At harvest (93 DAFB), 'Springcrest' fruits were about 8 mm smaller than 'Fantasia's' at harvest (137 DAFB). Seeds of 'Springcrest' developed at a similar rate as the seeds of 'Fantasia', in a manner that at fruit harvest endosperm was not already completely reabsorbed (Figure 2).
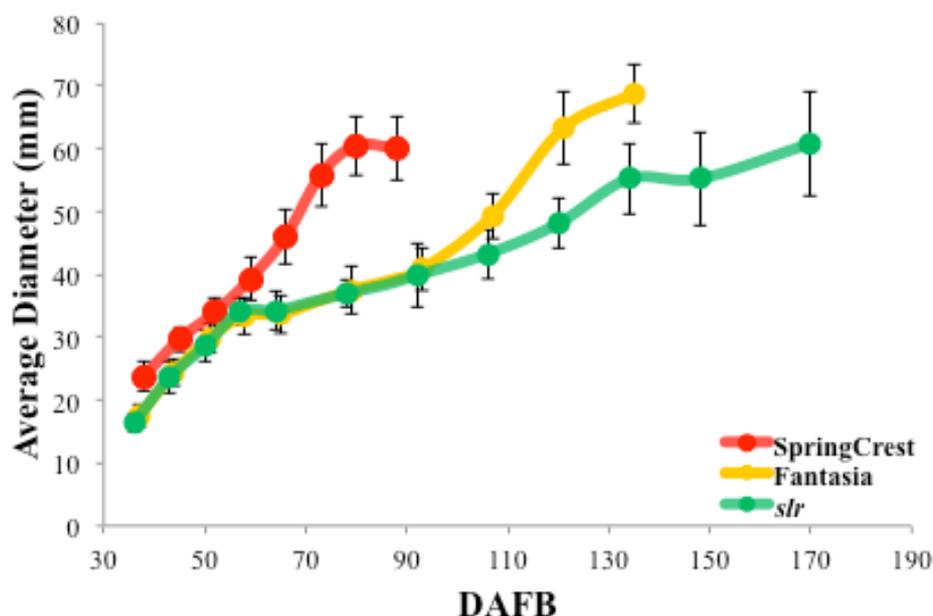
Figure 2. – Fruit growth of 'Fantasia', 'Springrest' and *slr* in 2011. 'Fantasia' follows the typical double sigmoid pattern of growth (yellow). Springrest have a short and almost undistinguishable S2 stage, and ripens well before Fantaisa (red). *slr* doesn't enter the S3 stage and continue to grow slowly (green).

3.2 Microarray data quality

Total RNA was extracted from 2 peach tissues (mesocarp and seed) at 6 time-points in 3 biological replicates (2x6x3 design) for a total of 36 samples. RNA samples were

hybridized on different slides of µPEACH3.0 microarray, each slide containing at least 2 technical replicates.

Data from spots showing non-significant intensities were discarded and data from the technical replicates averaged. The averaged data were quantile-normalized among all the 36 slides, resulting in 20 627 probes showing ten-fold the minimum signal in at least one slide. The array contains 29 800 probes, meaning that 9 173 probes (28.6%) were not used in the PCA analysis.

If only the 18 samples of mesocarp are considered, the number of significant probes diminishes to 17 695. If only seed samples are taken into account, 18 311 probes show a significant signal. Only these probes were used in the tissue-specific analyses.

The comparison between the two gene sets pointed out that 16 303 genes were expressed in both mesocarp and seed, while tissue-specific genes were 1392 for mesocarp and 2 008 for seed, respectively. Among mesocarp-specific genes were included some TFs belonging to MYB (ppa020385m; ppa026640m; ppa008877m; ppa025811m and ppa015973m), AP2/ERF (ppa026499m; ppa012354m and ppa009812m) and ARF (ppa002710m; ppa024354m and ppa001179m) families. The seed-specific gene set included a number of genes encoding for Amygdalin hydrolase isoform AH I (ppa015970m; ppa018404m; ppa019573m; ppa020817m; ppa025067m; ppa025619m; ppa018777m and ppa021869m), an enzyme having prunasin beta-glucosidase activity, for proteins mediating response to ABA (ppa027008m) and GAs (ppa011233m; ppa026043m and ppa013757m), and as well as for Late Embryognesis Abundant proteins (LEA, ppa015214m and ppa012476m).

Microarray data were validated using qRT-PCR: logarithmized expression values of 26 genes were compared to logarithmized microarray intensity signals. The Pearson coefficient was found to be 0.774, confirming a strong correlation between microarray and qRT-PCR data (table S2).

Expression data from the 36 slides were loaded on the TMEV software package and a Principal Component Analysis (PCA) was performed. As expected, PCA showed that the 3 biological replicates of each sampling cluster together, confirming the consistency of the replication (Figure 3).

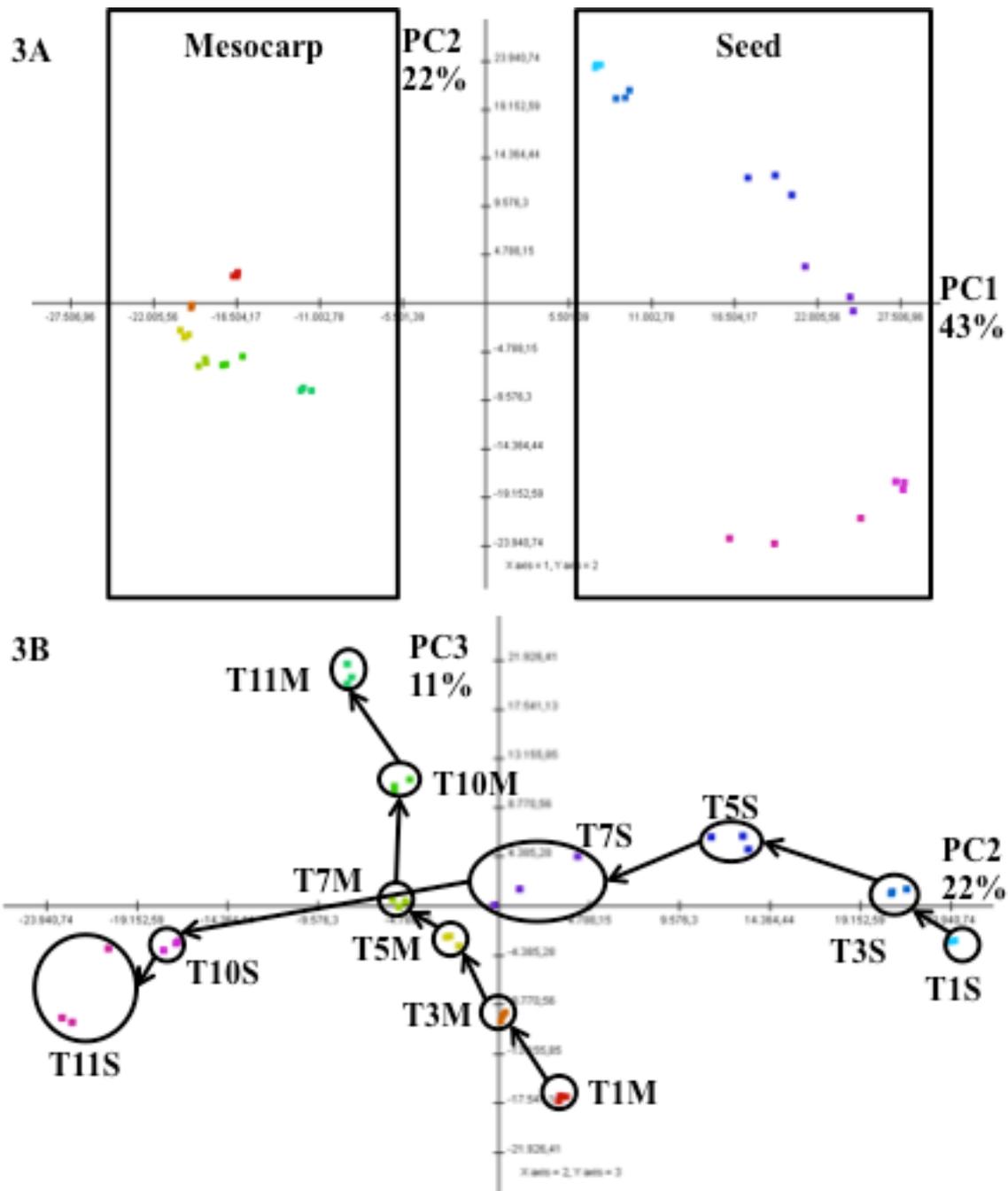**Figure 3. – PCA analysis of the 36 slides of the array**. **A)** Samples segregate accordingly to their tissue of origin: mesocarp samples are on the left, with negative values of PC1, seed samples on the right, with positive values**. B)** PC2 values of seed samples (T1S-T11S) are correlated with the date of sampling (from right to left), as PC3 values of mesocarp samples (T1M-T11M) are (from the bottom to the top).

The first three components describe 76% of the variability observed: PC1 accounts for 42.5% of the variability alone, PC2 for 22.2% and PC3 for 11.3%. PC1 clearly discriminates the samples according to the origin of the tissue: mesocarp samples have negative values, whereas those of the seed samples are positive (Figure 3A). PC2 describes the growth of the seed: early seed samples show higher values, which diminish according to the organ development (Figure 3A and 3B). Finally, PC3 accounts for the development of the mesocarp: its value increases as the fruit grows (Figure 3B).

3.3 Transcriptome changes during mesocarp development

Data coming from the 18 mesocarp samples were quantile-normalized and EDGE software was used to find differentially expressed (DE) genes. 7 933 genes showed differential expression during the time-course. The 'mfuzz' package of R was chosen to cluster DE genes. 'mfuzz' employs a soft-clustering algorithm to group genes. In soft-clustering genes are assigned values which describe the strength of their association to a particular cluster. Depending on the threshold chosen, a single gene may belong to one, none ore more than one cluster. This method effectively diminishes the noise coming from microarray data: if a gene does not follow any of the major pattern of gene expression, it is discarded from analysis, if a gene may keep up with more than one pattern, it is included in all of them (Kumar and Futschik, 2007).

Six gene clusters were identified, each of them showing its maximum expression at different time-points (Figure 4). Genes in cluster CM1 (1,421 genes) have their maximum level at 41 DAFB (S1), then, their expression shows a rapid decrease. Genes in cluster CM2 (1,593) have their maximum expression in the first 60 DAFB, slowly decreasing thereafter. Genes in CM3 (1,094) are maximally expressed at stage S2 (50-80 DAFB), whereas genes in CM4 (985) are associated with stage S3 (80-110 DAFB). CM5 (1,571) gathers genes whose expression slowly increase during development to reach a maximum at the late stages (80-130 DAFB); finally CM6 (1,987) is constituted of genes expressed almost exclusively at ripening in S4 (after 120 DAFB).

A GO enrichment analysis was performed on each of these clusters in order to determine which biological processes characterize each stage of mesocarp development. Because of the great number of terms, only level 4 biological process terms were taken into account in mesocarp GO analyses.

Figure 4. – Mesocarp gene clusters. Patterns of expression of gene clusters found by mfuzz analysis on mesocarp transcriptomic data. Fold Change is measured relative to the average expression level.

In particular, CM1 was particularly enriched of genes involved in the cell cycle process, including the mitotic cell cycle, the fission of organelles, the organization of chromatin and of chromosomes. On the contrary, genes involved in programmed cell death (PCD) were underrepresented (Table 2). Another interesting cluster was CM5, the late mesocarp cluster, which was characterized by the expression of genes engaged in cellular transport, as well as in the metabolism of organic acids (such as amino acids and malic acid) and ketones (amino acids and lipids). Finally, CM6, corresponding to the genes expressed during ripening, showed an enrichment of many different GO terms related to hormone signalling and response, lipid and nucleotide metabolic processes, gene expression and metabolite transport.

Table 2 – GO enrichment analysis of CM1. Mitosis related categories (highlighted) are overrepresented, while programmed cell death is repressed. CM1 pattern is associated with a period of high cell division in the mesocarp**.

GO term	Number of genes in the cluster	Number of genes in the genome	Over/under represented
microtubule-based movement	23	71	over
cell cycle process	25	94	over
mitotic cell cycle	20	64	over
organelle fission	20	68	over
nucleosome organization	17	55	over
chromatin assembly	17	55	over
macromolecular complex assembly	25	153	over
chromosome organization	29	212	over
programmed cell death	*6*	*388*	*under*
response to water deprivation	10	45	over
cellular response to chemical stimulus	39	362	over
response to organic substance	44	434	over
response to water	10	51	over
microtubule-based movement	23	71	over

3.4 Transcriptome changes during seed development

Data from the 18 seed samples were normalized and DE genes were found using EDGE. During the time-course, 11 939 genes changed their expression significantly. These genes were then clustered with R library 'mfuzz'. Six gene clusters with a different expression pattern were obtained (Figure 5).

Genes in clusters CS1 (1 541 genes) and CS2 (2 637) are mainly expressed between 40 and 60 DAFB, during the endosperm cellularization. CS1 differs from CS2 as in the first gene expression decreases soon after the first time-point, while in the latter it stays high also at 54 DAFB and is very low at the end of embryo development. Genes in CS3 (2 770) are associated with the endosperm development and they are expressed between 40 and 90 DAFB, decreasing thereafter. CS4 (1 384) genes have their maximum expression only when the seed contains a cellularized endosperm and the embryo is growing (65-90 DAFB). Finally genes in CS5 (2 824) and CS6 (2 039) are associated with embryo development, with genes in CS5 mainly expressed in the fully-developed

embryo (110-130 DAFB) while those belonging to CS6, besides being co-expressed with those of CS5 at maturation, are also detectable during the early stages of seed development (i.e. 40 and 60 DAFB).
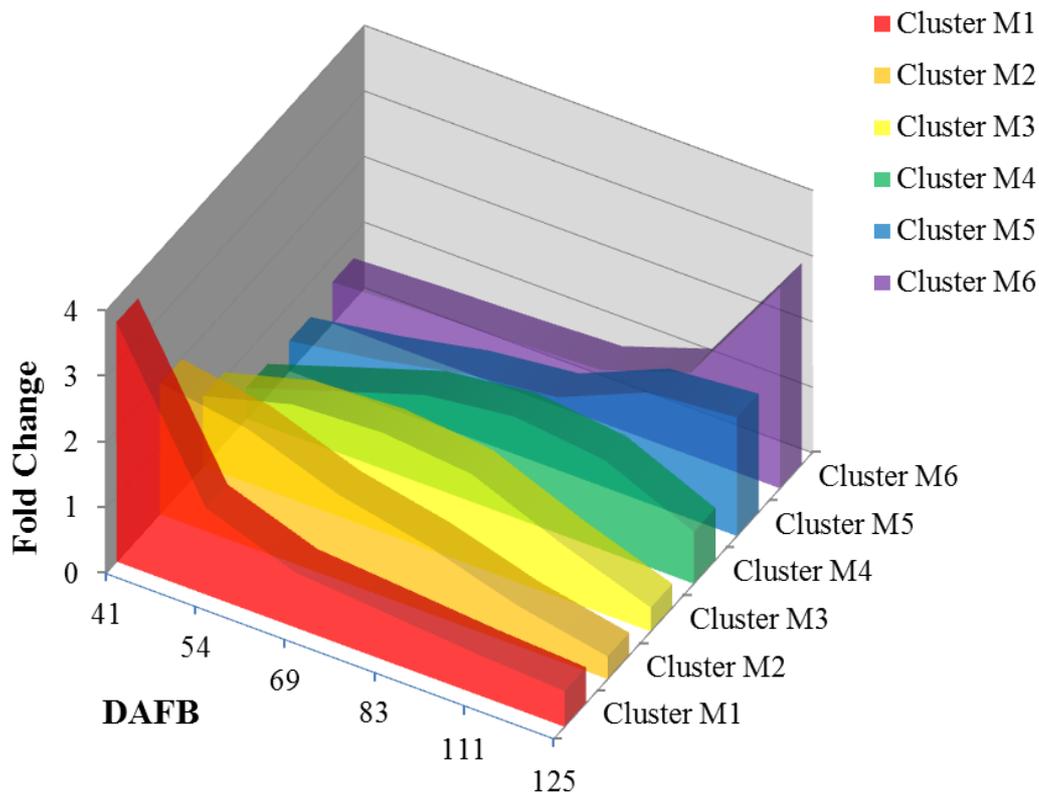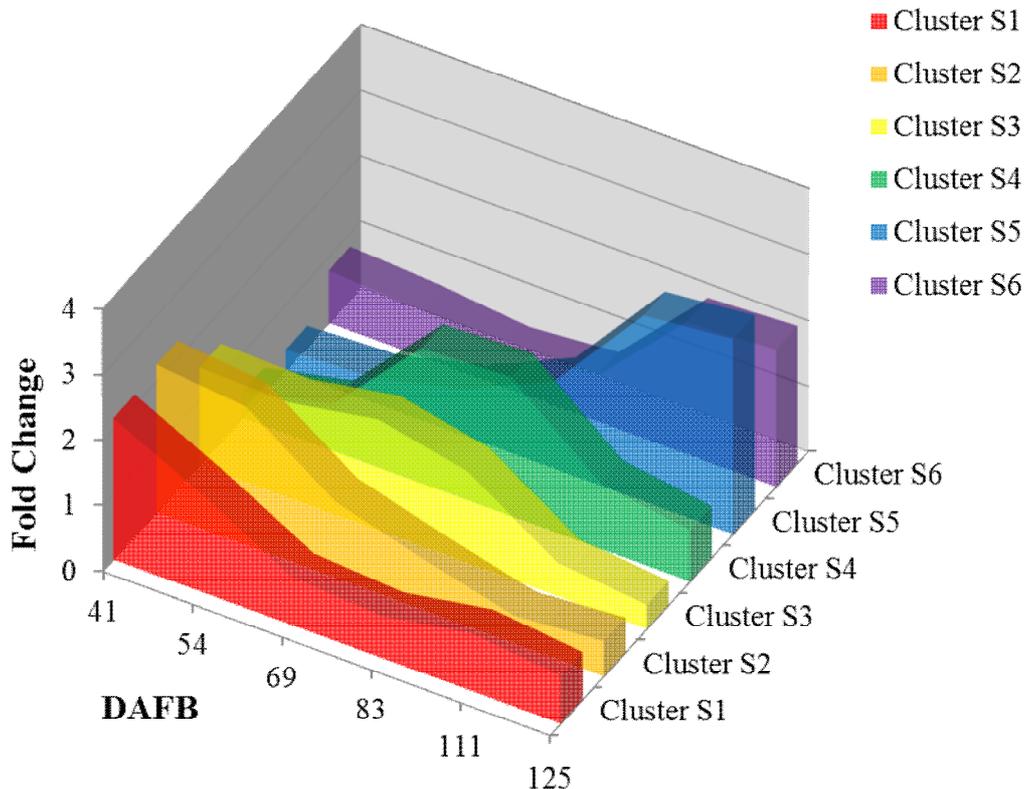

Figure 5. – Seed gene clusters. Patterns of expression of gene clusters found by mfuzz analysis on seed transcriptomic data. Fold Change is measured relative to the average expression level.

A GO enrichment analysis was done on these six clusters. As for the mesocarp, only level 4 GO terms were considered. Among the clusters found, the most interesting is CS3, which is enriched in genes involved in the cell cycle processes, such as the mitotic cell cycle and the organelle fission, and in the organization of the cytoskeleton (Table 3).

CS5 is enriched in genes engaged in hydrolase activity, in particular genes encoding proteases and beta-glucosidases (prunasin beta-glucosidases). In the cluster, genes expressed in response to stresses are underrepresented. Finally CS6 is particularly enriched in genes with peptidase and proteolytic activity.

Table 3 – GO enrichment analysis of CS3. Mitosis related categories (highlighted) are overrepresented. CS3 pattern is associated with a period of high cell division in the seed.

GO term	Number of genes in the cluster	Number of genes in the genome	Over/under represented
microtubule-based movement	37	33	over
cell cycle process	38	52	over
mitotic cell cycle	29	33	over
organelle fission	29	38	over
ribonucleotide binding	426	1876	over
purine nucleotide binding	426	1879	over
phenylpropanoid metabolic process	36	60	over
cellular biosynthetic process	395	1806	over
oxidoreductase activity, acting on CH or CH2 groups	12	8	over
cellular aromatic compound metabolic process	57	158	over
macromolecule biosynthetic process	263	1143	over

3.5 Expression profile of cell cycle genes during mesocarp and seed development

In a previous paper (Bonghi et al., 2011) a transcriptomic description of the seed-mesocarp cross-talk was mainly focused on the events leading to ripening, as the microarray platform (i.e. μPEACH1.0) was biased towards ripening-related, mesocarp-specific genes. Here, having a whole-genome platform, attention was focused on the main biological events of the early fruit development, a period during which the seed-mesocarp relationship is more pronounced, as there are not yet any morphological barriers (i.e. the endocarp) between the two organs.

In the clustering/GO enrichment analysis, it was found that at the transcriptomic level the main biological process happening in the S1 stage of mesocarp growth is cell replication (CM1). Cell replication is an important process also for seed development, even if it takes place in a different period: expression of genes related to replication is maximum at 69 DAFB and repressed at later stages, when cotyledons are fully grown and the endosperm re-absorbed (CS3).

72 genes related to mitosis, DNA replication and endoreduplication were manually selected
and their microarray expression profiles analysed through hierarchical clustering, in
order to see their relationships (Figure 6 and Figure 7).

Figure 6. – Heat map of the array expression of 72 cell cycle related genes. On the
rows, the genes, on the column sthe six time-points. In the mesocarp, mitosis related
genes (red) are expressed mainly in the early stage of development, while DNA
replication genes (yellow) and endoreduplication genes (green) are expressed also in the
late stages of development. The last column's color is for cross-referencing with Figure
7: the same color for the same gene.

At the seed level, the great majority of these genes are expressed at the early stages. It is
particularly evident how most of these genes are highly expressed from 41 to 83 DAFB,
and then switched off at 111 and 125 DAFB, when the embryo is mature.

In the mesocarp, the situation is different: while the genes strictly related to mitosis are expressed at 41 and 54 DAFB (the S1 stage development), the genes related to DNA replication follow a different pattern, showing higher expression at 111 and 125 DAFB (corresponding to S3 and S4), and a minimum at 69 and 83 DAFB (S2 stage). Thus genes related to mitosis are strictly related to the S1 stage in the mesocarp, while DNA replication seems to occur also later, possibly involving events of endoreduplication.
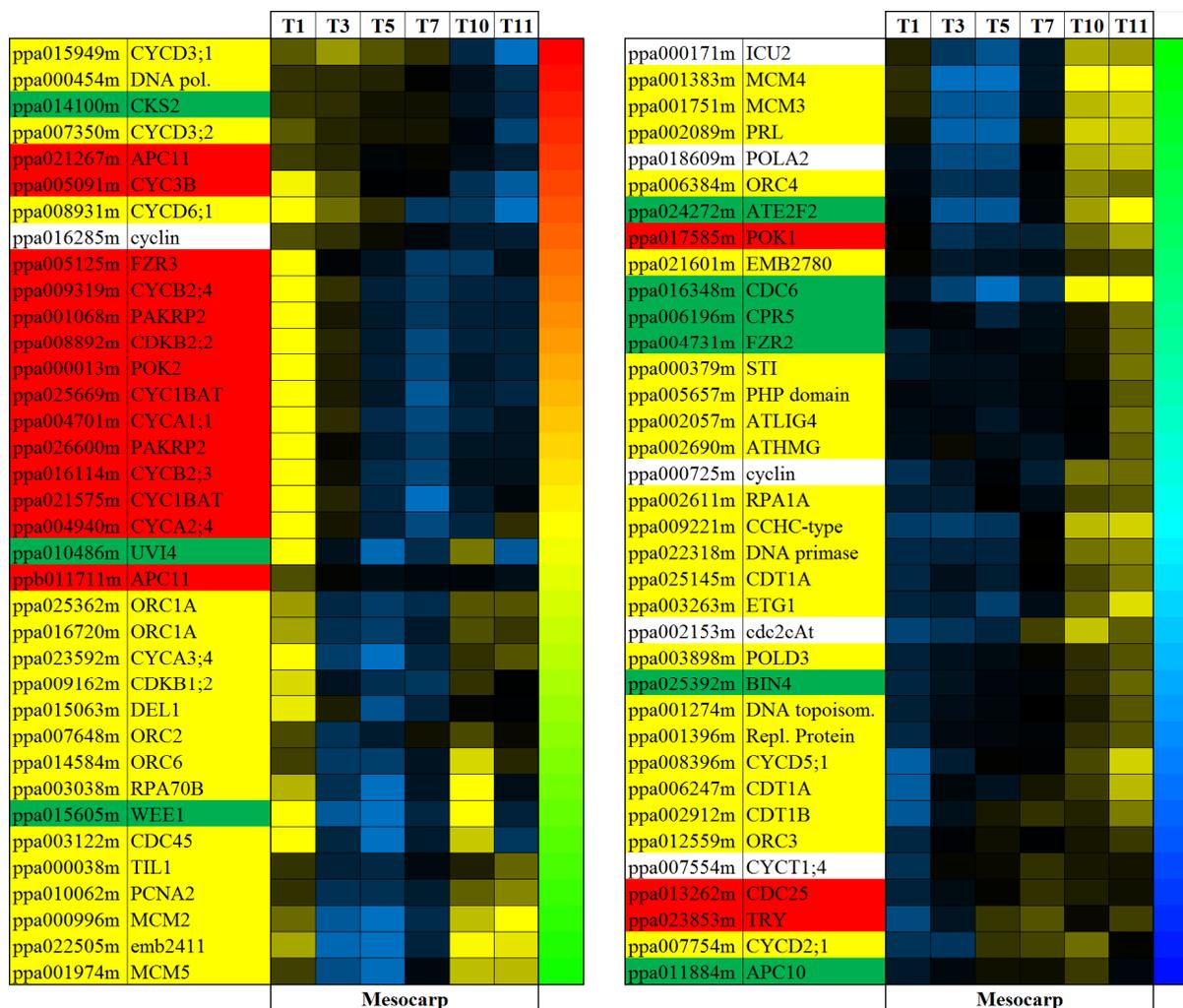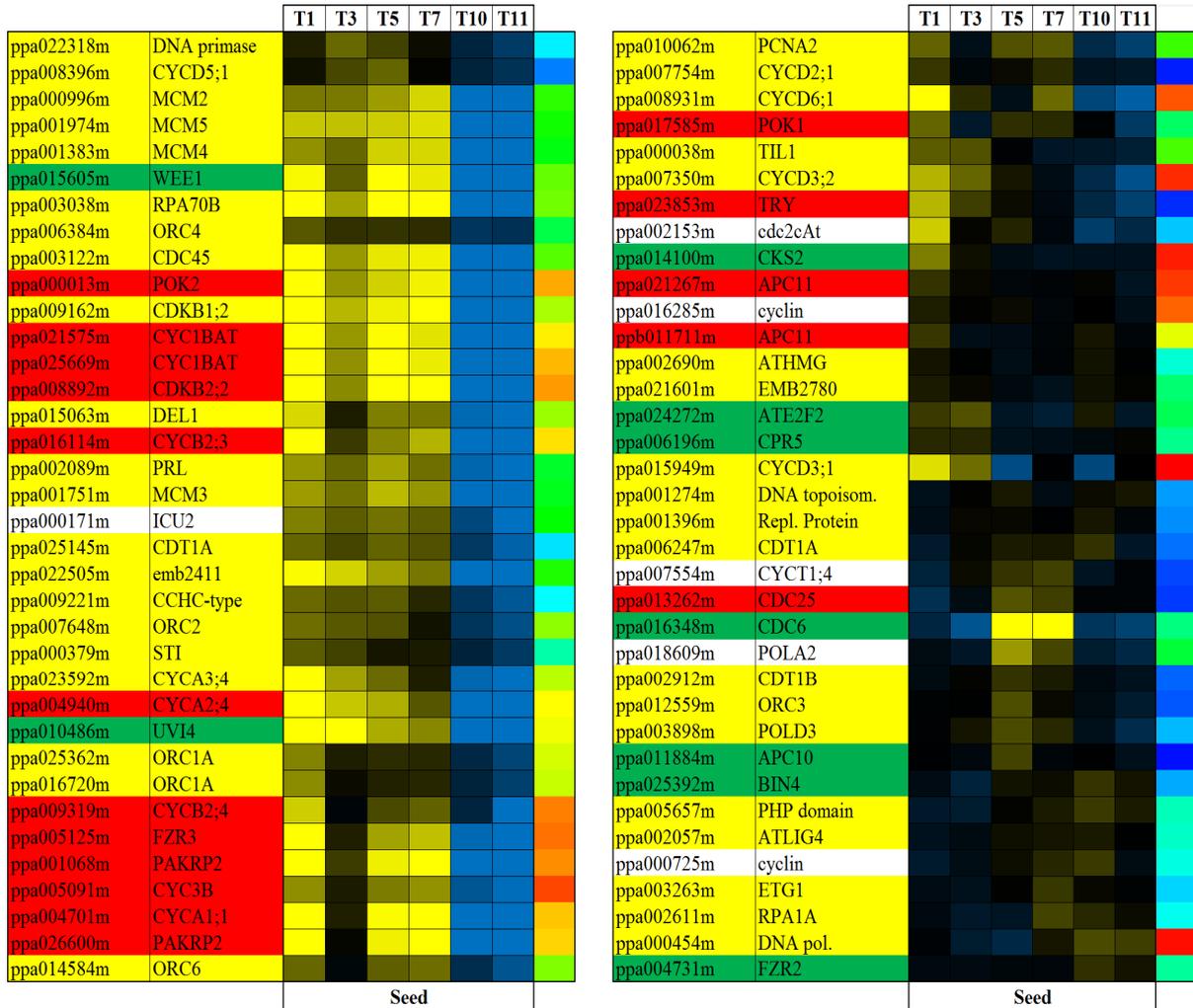

Figure 7. – Heat map of the array expression of 72 cell cycle related genes. On the rows, the genes, on the column sthe six time-points. In the in seed most of these genes are repressed in the latest stages of seed development (mature embryo). The last column's color is for cross-referencing with Figure 6: the same color for the same gene.

Expression of some important genes related to cell cycle regulation (*cyclin D3*, *A2;4* and *A3;4*), DNA replication (*CDKB1;2* and CDC2) and endoreduplication (*KRP3* and

WEE1), was then measured by qRT-PCR in the mesocarp of three cultivars: 'Fantasia', 'SpringCrest' and *slr* (Figure 8) characterized for different kinetics of mesocarp and seed development (Bonghi et al., 2011).

All tested genes are highly expressed in the early stages of mesocarp development in all the three cultivars, and are repressed at the transition between S1 and S2. Thereafter, while in 'SpringCrest' and 'Fantasia' there is a resume of expression at S3 and S4, this is not the case for *slr*. At the transcript level, it seems that in *slr* endoreduplication does not occur or it is less pronounced than in the other two cultivars at later stages.
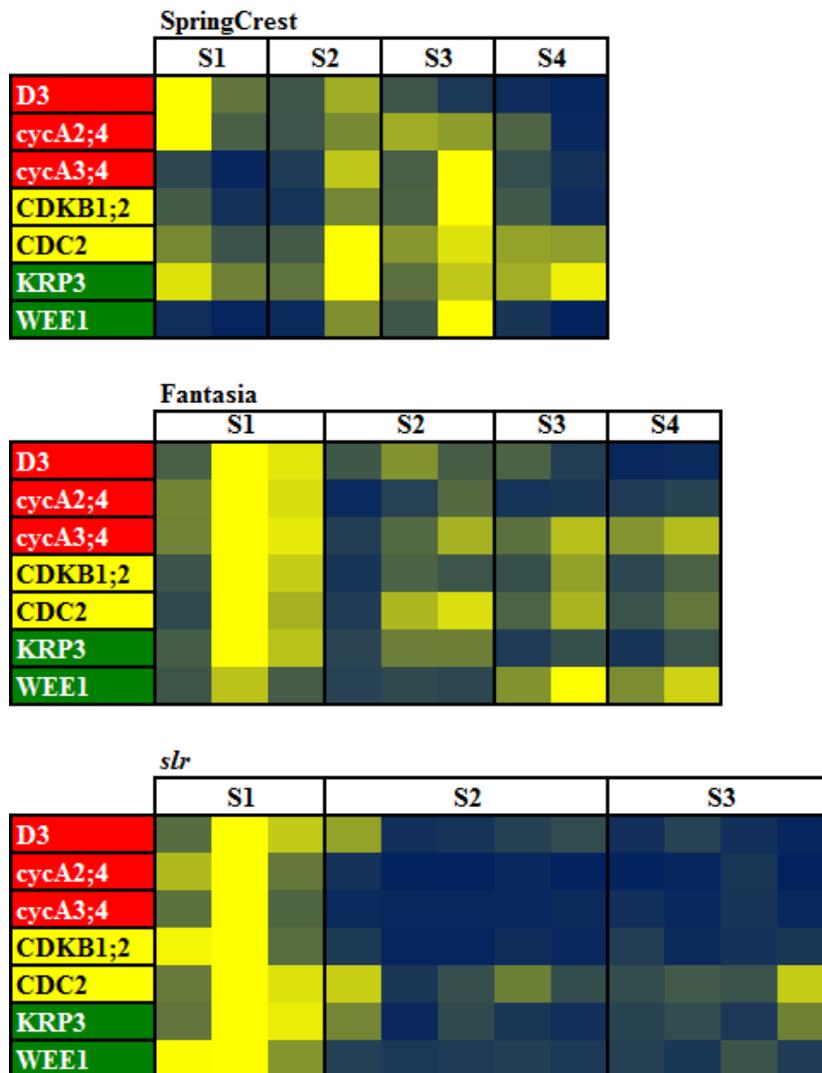
Figure 8. – Heat map of qRT-PCR expression of 7 cell cycle and endoreduplication-related genes in the mesocarp of the three cultivars. The genes are expressed during all the growth period in 'Springcrest' and 'Fantasia', while in *slr*, after S1 the gene expression is repressed. Yellow= maximum expression, Blue= no expression.

In seed the expression of *cyclin A2;4* was at high level only during S1 stage in all cvs and then rapidly decreased, with the exception of 'Fantasia', in which it ceased smoothly up to the end od S1. *Cyclin A3;4* and *WEE1* behaved similarly according to the developmental stage, but differed in the three genotypes. Both peaked at early S1, slowly decreasing to a minimum at the end of S2 and S4 for *cycA3;4* and *WEE1*, respectively, in 'Fantasia'. On the contrary, in 'SpringCrest' both genes had a second strong expression peak at the end of S3, while in *slr* their expression significantly dropped after the first sampling in S1 remaining then constant (Figure 9).
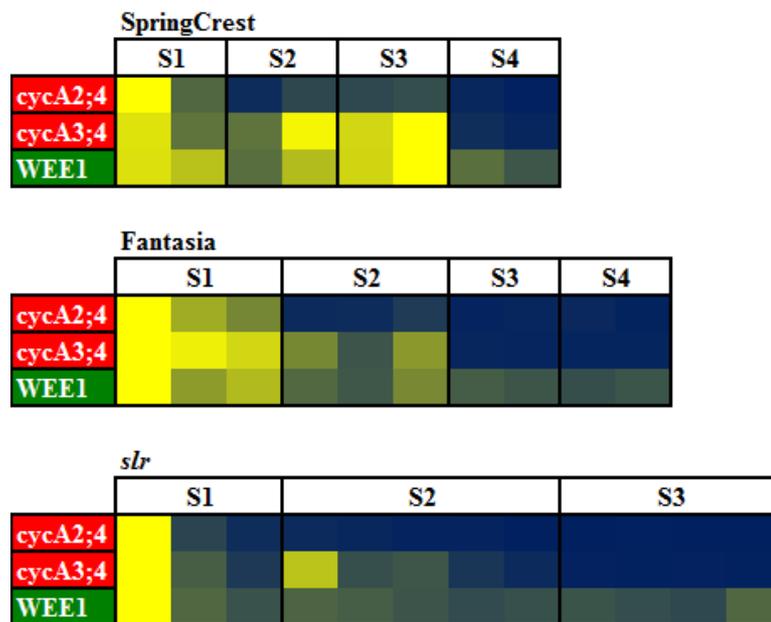
Figure 9. – Heat map of qRT-PCR expression of 3 endoreduplication-related genes in the seed of the three cultivars. Yellow= maximum expression, Blue= no expression.

At the mesocarp level, the situation is different: while the genes strictly related to mitosis are expressed at 41 and 54 DAFB (the S1 stage of peach development), the other genes related to DNA replication follow a different pattern, showing higher expression at 111 and 125 DAFB (corresponding to S3 and S4), and a minimum at 69 and 83 DAFB (S2 stage). Thus genes related to mitosis are strictly related to the S1 stage in the mesocarp, while DNA replication seems to occur also later, possibly involving events of endoreduplication.

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3.6 Identification of TF genes in μPEACH3.0 gene dataset

Out of the 20 627 genes showing ten-fold the minimum signal in at least one slide, 879 were identified as transcription factors on the basis of Gene Ontologies produced by the IPGI (The International Peach Genome Initiative et al., 2013), thus, TFs expressed during mesocarp and seed development account for 4,25% of fruit transcriptome. Putative peach TFs were grouped into families according to the membership of their *Arabidopsis* homologues. Fifty-five families were identified and compared to those registered in PlantTFDB (http://planttfdb.cbi.pku.edu.cn/index.php?sp=Ppe) as TFs for peach (Jin et al., 2014). This comparison showed that in some families the totality of the members were expressed during the development of mesocarp and seed; this happened for ARF (17 out of 17), GRF (10 out of 10) and, nearly, SBP (15 out of 17) families.

Figure 10. – Gene expression of members of the ARF Family in mesocarp and seed. Names of the *Arabidopsis* putative homologues are given along with the peach genome gene ID.

Transcripts corresponding to members of the above cited families were accumulated both in mesocarp and seed, but with qualitatively and quantitatively distinct patterns. For example, two (ppa022314m and ppa002230m) and six (ppa000946m, ppa002617m,

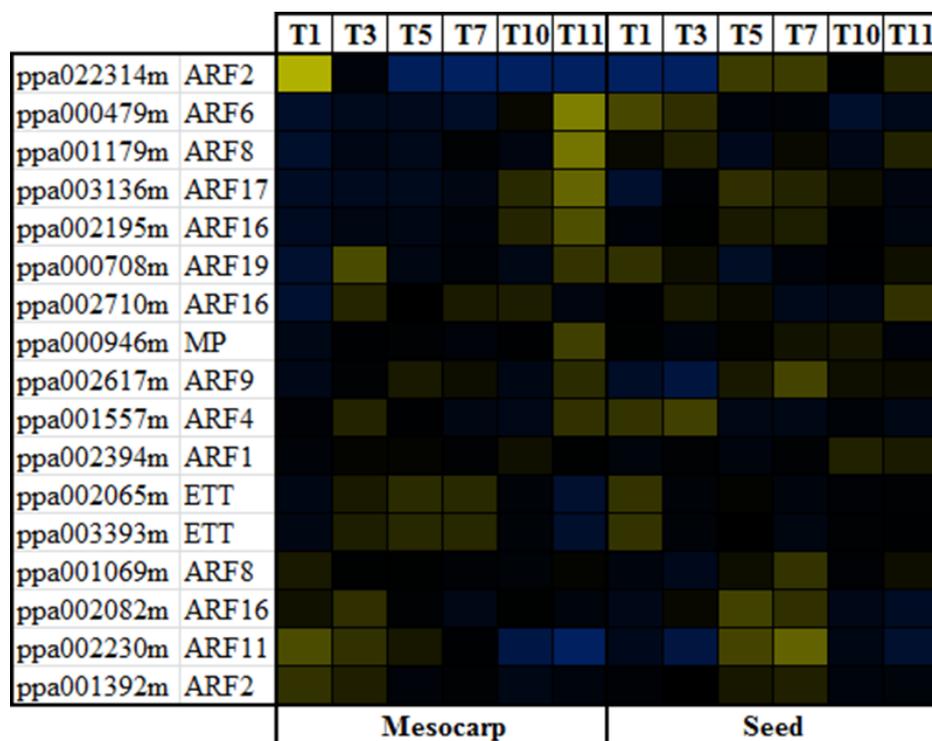ppa002195m, ppa003136m, ppa000479m and ppa001179m) members of the *ARF* family showed the maximum accumulation of their transcripts in the mesocarp at early and ripening developmental phases , respectively, while no significant stage-specific expression was observed in seed (Fig. 10). On the other hand for some families a lower number of members was registered as observed for *MYB* and *MYB*-related (83 out of 176), *ERF* (55 out of 107) and *NAC* (63 out of 115) TFs.

3.7 Expression profiles of transcription factors during mesocarp and seed development

Transcription factors induced or repressed between two consecutive time-points were found using the SAM tool of TMEV package (Table 4).

Table 4 – Transcription factors regulated during development. The number of TFs induced or repressed in each transition between consecutive time-points is given. The TFs induced or repressed were identified with SAM technique.

Mesocarp	T3/T1	T5/T3	T7/T5	T10/T7	T11/T10
Induced	136	54	52	15	74
Repressed	114	83	41	53	53
Seed	**T3/T1**	**T5/T3**	**T7/T5**	**T10/T7**	**T11/T10**
Induced	27	28	3	20	14
Repressed	16	51	0	23	2

In the mesocarp, the highest number of induced and repressed TFs was found between time-points T3/T1 (136 induced and 114 repressed) and between T5/T3 (54 induced and 83 repressed). At T3/T1 transition a *NAC* (ppa009438m) was showing the highest repression remaining at a basal level until the climacteric phase when a sharp increase of its transcripts was observed (data not shown). At ripening (T11/T10) 74 TFs were induced and 53 repressed. Among ripening-related TFs were present members of *NAC* (ppa007828m and ppa007883m) and *MIKC* (ppa010714m and ppa1027139m) families (data not shown). Interestingly, at the transitions between S2 and S1 stages (T5/T3) and between S3 and S2 stages (T10/T11) far more TFs are repressed rather than induced.

In the seed, the amount of induced and repressed TFs is lower than in the mesocarp. The same result has been obtained considering the full genome. The highest amount of

regulated TFs was found at the T5/T3 transition, when the endosperm is absorbed by the growing embryo. Also in this transition, there are more repressed than induced TFs. Among these, some members of the C2H2 family (ppa024496m, ppa024710m and ppa024870m) were those more repressed.

After being grouped into families, an enrichment analysis was done to identify families that were significantly associated with a particular stage. For the mesocarp the S1, S2, S3 and S4 canonical stages were considered, while the development of the seed was tentatively split into four stages: an only-endosperm stage, two growing embryo stages (but with different endosperm/embryo ratios) and a mature embryo stage (Figure 11).

Transcription Factor Families	Mesocarp				Seed			
	T1-T3	T5-T7	T10	T11	T1-T3	T5	T7	T10-T11
AP2-EREBP TFs								orange
ARF TFs							orange	
ARID TFs							orange	
ARR-B TFs						orange		
bHLH TFs		green						
C2C2-Dof TFs	green			orange				
C2C2-YABBY TFs								orange
C2H2 TFs			green				green	
C3H TFs	green		orange					
CCAAT-HAP2 TFs								orange
Dof TFs	green			orange				
EIL TFs	orange							
GRF TFs	orange							orange
Homeobox TFs	green			orange				
MYB TFs			orange					
MYB-Related TFs				orange			orange	
NLP TFs						orange		
SBP TFs	orange				orange			
TCP TFs á					orange			
TGA3-like						orange		
Type I MADS box						orange		
WRKY TFs					green			

Figure 11. – Over and underrepresented TF families in different stages. For each family it is indicated whether its members are over- (orange) or under- (green) represented in the given stages of mesocarp and seed development.

Enrichment analysis showed that TFs belonging to the SQUAMOSA-promoter Binding Protein (SBP) family were particularly expressed during the early development of both mesocarp (S1 stage) and seed (only-endosperm stage). Conversely, the TFs of the Growth Regulation Factor family showed a different pattern of expression in the two organs, being more expressed in the S1 stage of mesocarp development and in the last stage of the seed (mature embryo).

In the mesocarp, another interesting family is the Homeobox Family, whose TFs are over-represented at the S4 stage (Figure 12). In the seed, *NLP* TFs are expressed at the growing-embryo stage (Figure 13).
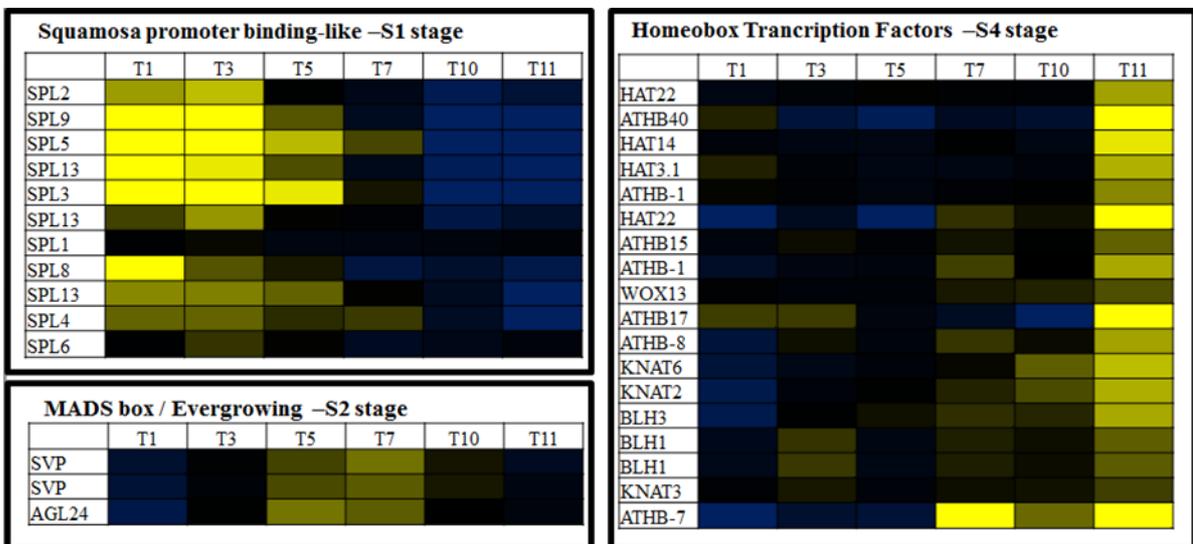

Figure 12. – Gene expression of members of over-represented TF families in the mesocarp. SBP TFs are expressed mainly at the first stages of development (S1: T1-T3), while Homeobox TFs are particularly expressed during ripening (S4-T11). MADS-box of the Evergrowing type are expressed in mesocarp during pit hardening (S2: T5-T7). Gene names of the *Arabidopsis* homologues are used.
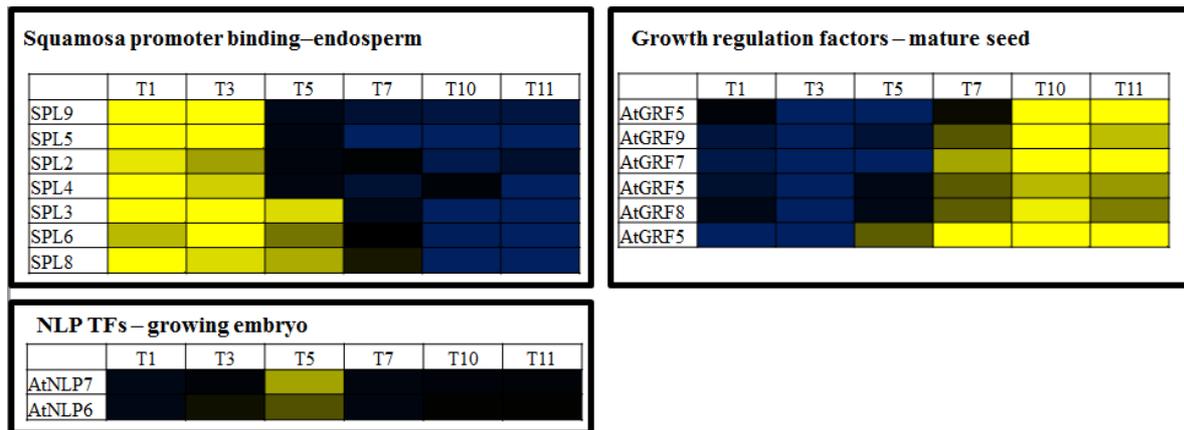
Squamosa promoter binding – endosperm

	T1	T3	T5	T7	T10	T11
SPL9						
SPL5						
SPL2						
SPL4						
SPL3						
SPL6						
SPL8						

Growth regulation factors – mature seed

	T1	T3	T5	T7	T10	T11
AtGRF5						
AtGRF9						
AtGRF7						
AtGRF5						
AtGRF8						
AtGRF5						

NLP TFs – growing embryo

	T1	T3	T5	T7	T10	T11
AtNLP7						
AtNLP6						

Figure 13. – Gene expression of members of over-represented TF families in the seed.
SBP TFs are expressed mainly in the first stages of development (endosperm: T1-T3), conversely *GRF* factors are particularly expressed in the mature seed (T10-T11). *NLP* TFs are expressed during the growing embryo stage (T5). Gene names of the *Arabidopsis* homologues are used.

3.8 Expression profile of Transcription Factors in seed and mesocarp in three cultivars of peach characterized by different kinetics of fruit development

RT-qPCR was used to assess gene expression of some of the TFs whose families were found interesting after enrichment analysis on transcriptomic data.

Among the *SBP* genes, mRNA expression was measured for *SPL3*, *SPL9* and *SPL13* (Figure 14). All these three genes are expressed in the first stages of both mesocarp and seed development in all the three cultivars.

SPL3 shows a higher expression in the mesocarp, rather than in the seed. Interestingly, the peak of expression appears anticipated in 'SpringCrest' and postponed in *slr*, when compared to 'Fantasia'. *SPL9* follows the same pattern of expression of *SLP3*, with the same anticipation/delay of the expression peak. *SPL13* is different, as it is expressed more in the seed than in the mesocarp. In the mesocarp the expression of *SPL13* peaks later than those of *SPL3* and *SPL9*, in the seed there is no single peak, but expression is high at the first stages and then decreases reaching an undetectable level after 90 DAFB: noteworthy, in the 'SpringCrest' cultivar fruit ripens before the expression of this gene may reach zero.

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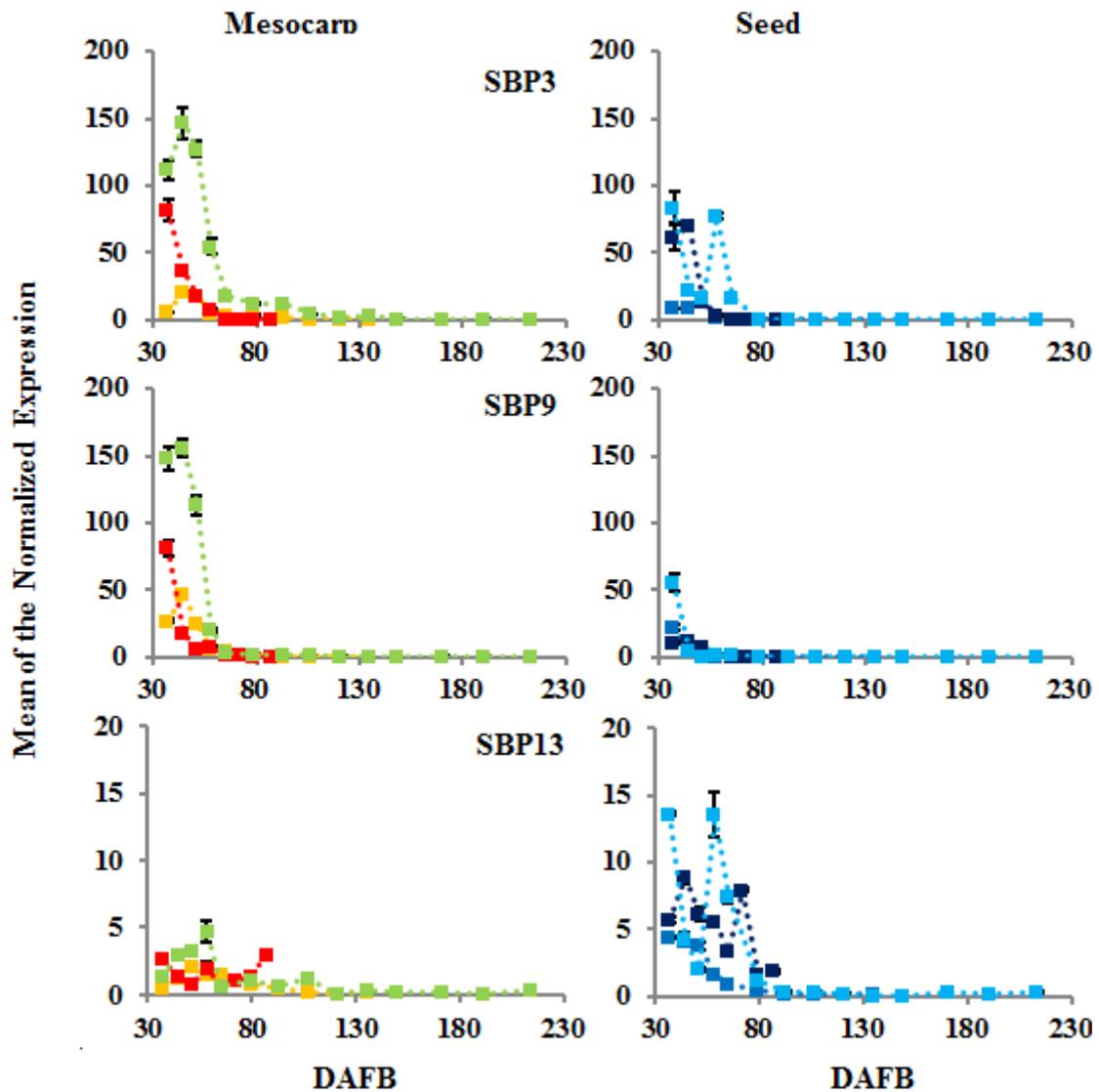
Figure 14. – qRT-PCR gene expression of members of the *SBP* family in different tissues and cultivars. A) *SPL3* **B)** *SPL9* **C)** *SPL13*. Colors: mesocarp: 'Fantasia' (orange), 'SpringCrest' (red), *slr* (green); seed: 'Fantasia' (blue), 'SpringCrest' (dark blue), *slr* (light blue).

Among the *GRF* genes, the expression of *GRF7* and *GRF9* was profiled (Figure 15). Both these genes are expressed only in the seed. *GRF7* is expressed at the end of the time period considered in all the three cultivars, even if in 'SpringCrest' the seed was not fully mature. *GRF9* has a similar pattern, but less sharp, also because the overall expression level of this gene is far lower than *GRF7*'s.

ARF8 is expressed mainly in the mesocarp, in all the three cultivars. In particular its expression is higher in the first stages of development, and then there is a peak at ripening. In 'SpringCrest', the transition from the early stages to ripening is so quick, that *ARF8* expression never drops (Fig. 15).
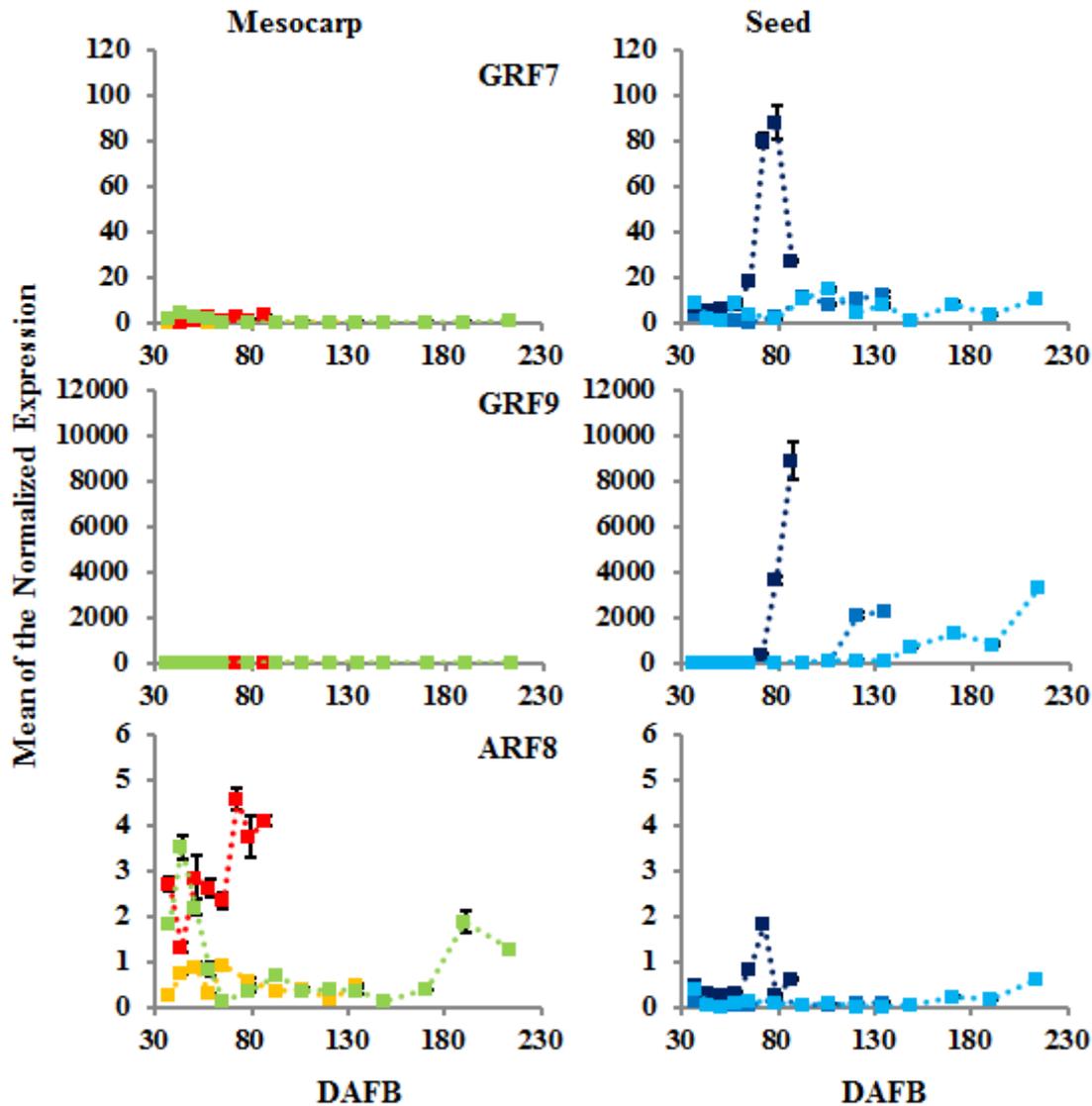
Figure 15. – qRT-PCR gene expression of some Transcription factors in different tissues and cultivars. A) *GRF7* **B)** *GRF9* **C)** *ARF8*. Colors: mesocarp: 'Fantasia' (orange), 'SpringCrest' (red), *slr* (green); seed: 'Fantasia' (blue), 'SpringCrest' (dark blue), *slr* (light blue).

3.9 miRNAs regulate the expression of SBP, GRF and ARF transcription factors

The mRNA abundance of the homologues of some of the TFs previously analysed is known to be controlled by miRNA in *Arabidopsis*. The expression of these particular miRNAs was then checked. *SPL9*'s abundance is known to be regulated by miR156. In peach, in all the three cultivars considered, miR156 is expressed in the last stages of both mesocarp and seed development. *SPL9* shows a symmetrical expression, being expressed at the initial stages of both tissues' development (Figure 16).
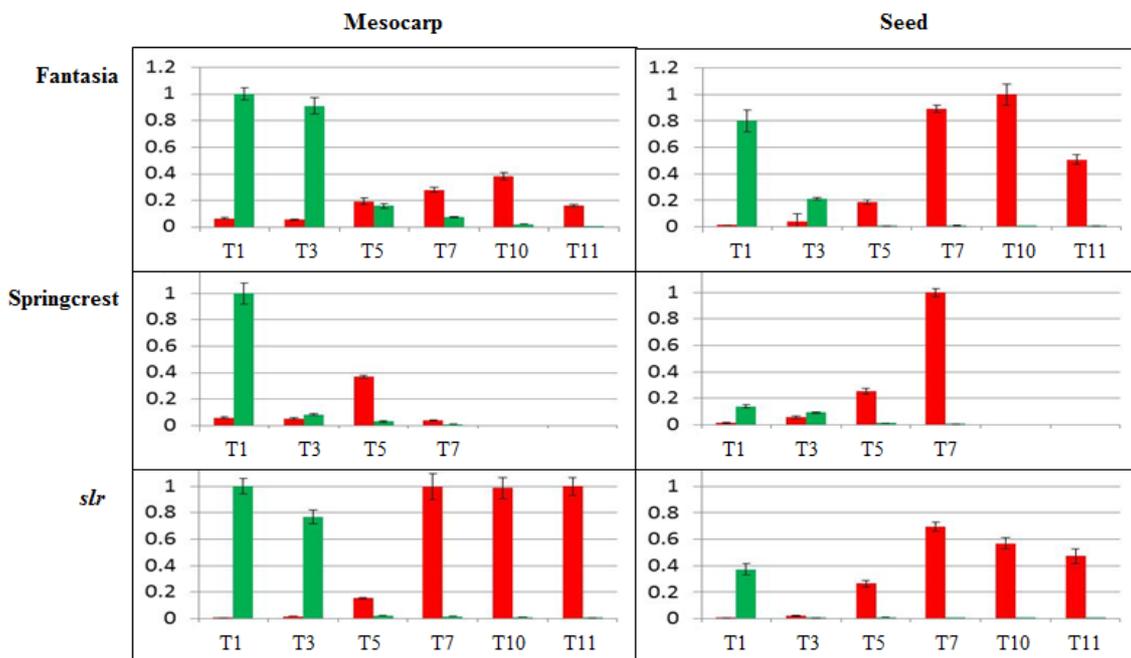
Figure 16. – Gene expression of *SPL9* and miR156. *SPL9* is expressed in the early stages in both mesocarp and seed in the three cultivars (green bars). On the contrary, miR156 is expressed in later stages (red bars).

GRF9 is known to be regulated by miR396's abundance. In the three cultivars considered, miR396 is expressed almost exclusively in the mesocarp, whereas the probable target *GRF9* only in the seed (Figure 17).

Finally, also the expression levels of miR167 and of its target, *ARF8*, were measured. In this case, *ARF8* accumulates in the mesocarp, while the antagonist miRNA in the seed (Figure 18).

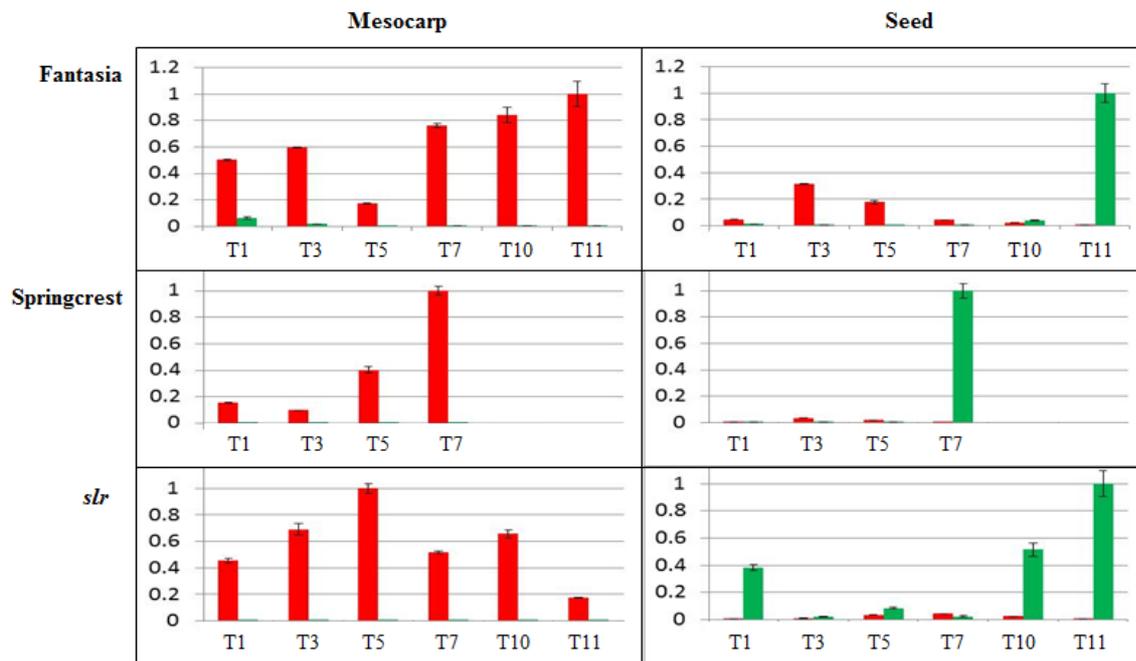**Figure 17. – Gene expression of *GRF7* and miR396.** *GRF7* is expressed in the late stages of seed development in the three cultivars (green bars). On the contrary, miR396 is expressed mainly in the mesocarp (red bars).
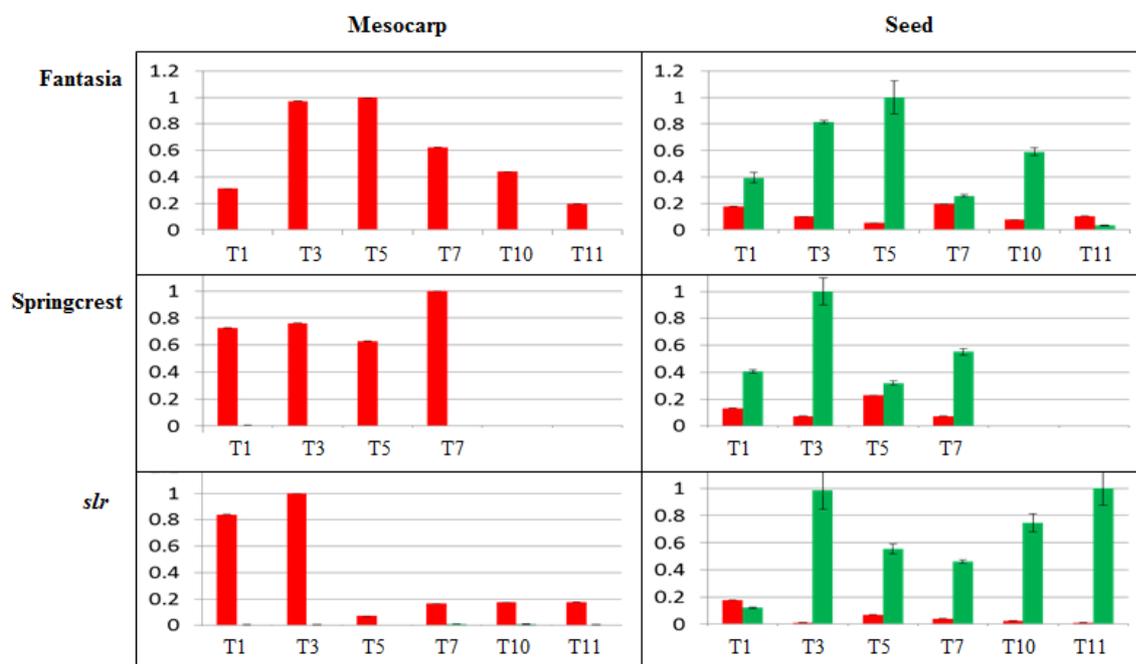

Figure 18. – Gene expression of *ARF8* and miR167. *ARF8* is expressed during seed development (green bars), while miR167 is expressed mainly in the mesocarp (red bars).

4. Discussion

The transcriptomic approach has been widely used in studying fruit and seed development in a variety of plants. Tomato, grape, eggplant, pepper, lemon, apple, cucumber, strawberry are only some of the plants for which one or more transcriptomic analyses of development have been performed (Rahim and Trainotti, 2013). Many of these works have been based on oligonucleotide- based array technologies (Clarke and Zhu, 2006) and, more recently, throughout Next Generation Sequencing (NGS) (Wang et al., 2010).

Compared with microarray, where only difference in expression of the ORFs can be addressed, RNA-seq allows to analyse genome-wide transcription, thus providing additional features such as, analysis of novel transcripts, smRNA, miRNA and alternative splicing events. Nevertheless, microarrays represent a well-established technology and have been widely used in the last decades, leading to availability of extensive information. More than 900 000 published microarray assays are available in repository databases like Gene Expression Omnibus or ArrayExpress and have been shared within the research community. Comparison of microarray experiments from different species targeted to the same developmental process offers the opportunity to compare gene expression patterns for a large number of genes.

Taking into account the availability of microarray data on fruit development and ripening, as well as the advantage of the availability of the whole peach genome sequence produced by the International Peach Genome Initiative (The International Peach Genome Initiative et al., 2013) (available at http:// www.phytozome.net/peach and at http://www.rosaceae.org/species/prunus_persica/genome_v1.0), we developed a new microarray platform, named µPEACH3.0, in which were printed 29 800 probes, designed on the whole peach gene set, plus more than 1 400 home-made gene predictions. This platform was totally unbiased in comparison to µPEACH1.0, the microarray developed starting from an ESTs repertoire originated mainly form fruits sampled at late development (ESTree Consortium, 2005), which was used for the first attempt to study the relationship between mesocarp and seed development (Bonghi et al., 2011).

To increase the knowledge about the development of these two organs, the new full-genome microarray was used and the number of time-points was raised to six.

4.1 µPEACH3.0 quality

µPEACH3.0 is made of 29 800 probes, designed on the whole peach gene set plus more than 1 400 home-made gene predictions (Trainotti et al., 2012). The use of this array on peach mesocarp and seed resulted in 20 627 probes showing a significant signal in at least one of the time-points considered. On the contrary 9 173 probes (30.8%) did not produce a remarkable signal. The absence of a significant signal coming from these probes could be due either to the non-functioning of the probes themselves or to the non-expression of these target genes in these particular peach tissues. In fact, if only the mesocarp is considered, the number of significant probes diminishes to 17 714, which means that 2 913 genes are seed specific. On the other hand, 2 297 probes produced a significant signal only in the mesocarp.The use of other tissues and/or biological conditions, as hormone treatment or pathogen attack, will probably lead to an increase in the number of functioning probes, and decrease the size of gene groups specific for a given tissue, as observed for grapevine, in which organ-specific genes are never more than 500 (Fasoli et al., 2012).

The robustness of the data was confirmed using qRT-PCR. qRT-PCR expression of 26 genes was compared to their expression on microarray and a Pearson correlation of 0.774 was obtained. Similar data were obtained also for µPEACH1.0 (Bonghi et al., 2011). A survey of the literature regarding the use of microarray platforms to profile fruit transcriptome reveals that rarely these correlations are presented with statistical analyses and few authors define the criteria they used to determine acceptable validation of microarray results. Taking into account this bias, correlation values were ranging from 0.68 to 0.95. The latter very high correlation value ($R2=0.95$) was reported by (Matas et al., 2010) working on orange peel cells retrieved by using Laser Miscroscope Dissection (LMD). The use of LMD resulted in a substantial increase in resolution and specificity of the gene expression differences reducing bias due to a mixing of different cells.

Transcriptomic data were able to clearly distinguish the two different organs: the Principal Components Analysis (PCA) discriminated samples on the basis of the tissue of origin at every stage of development. In addition, PCA suggests that seed undergoes deeper modification than mesocarp during development at the transcriptomic level. This result

was not unexpected taking into account that we recorded transcriptome changes occurring in the whole seed without separation among seed coat, endosperm and embryo. During peach seed development, as happen in all exalbuminous seeds, the ratio between the number of endosperm and embryo cells decreases. In fact, the endosperm is re-absorbed by the embryo, as the latter grows within the developing seed and the cotyledons of the embryo become filled with nutrients initially stored in endosperm cells (Ognjanov et al., 1995; Bonghi et al., 2011). This result was confirmed also for the *Arabidopsis* seed, another exalbuminous seed (Le et al., 2010). In this case a transcriptome pairwise analysis carried out in different stages of seed development pointed out a decrease of Pearson correlation coefficients as the pairs of seed developmental stages became more distant to each other developmentally. For example, the average correlation coefficients between globular-embryo (GLOB, endosperm as free nuclei) and cotyledon-embryo (COT, endosperm cellularization) stage and GLOB and postmature embryo (when endosperm is totally re-adsorbed) were 0.87 and 0.41 respectively.

4.2 Global analysis of gene activity during seed and mesocarp development

Gene patterns found for the seed (CS1-CS6) highlight a segmentation in seed development: two clusters (CS1 and CS2) contain genes expressed when the seed is almost entirely made of endosperm, two others (CS5-CS6) contain genes expressed only in the mature embryo, in both cases the only difference being the expression levels between the clusters next to each other.

Note of worthy in CS1 is a number of genes (ppa012054m; ppa000389m; ppa022862m and ppa022240m) showing homology to *Arabidopsis TITAN* family members. Mutation of *TITAN* genes causes an enlargement of endosperm nuclei, a reduction of endosperm cellularization and a defective embryo development (Liu and Meinke, 1998). *TITAN* genes expressed in the endosperm have been claimed into the control of the onset of DNA replication occurring at the first phase of endosperm development (free-nuclei stage) (Berger, 2003). In CS5 and CS6 besides genes encoding for prunins, already identified as seed developmental stage markers (Bonghi et al., 2011), other seed-specific genes are present: some isoform of Amygdalin hydrolase and a gene related to ABA.

The latter, ppa027008m, is belonging to CS5 and showing similarity to *DELAY OF GERMINATION1* (*DOG1*), a gene recently identified as a major regulator of dormancy in *Arabidopsis* thaliana (Nakabayashi et al., 2012). Interestingly, although ABA levels in *dog1* mutants are reduced and GA levels enhanced, Nakabayashi et al., (2012) demonstrated that DOG1 does not regulate dormancy primarily via changes in hormone levels.

In CS3 are included genes related to cell cycle, organelle fission and mitosis; these genes were expressed mainly in the seed containing endosperm and growing embryo, but repressed in the mature embryo.

On the contrary, the six patterns pertaining to the mesocarp (CM1-CM6) constitute a succession of different waves of gene expression miming the growth of the organ itself. Cluster expression profiles change smoothly from one cluster to the following one, suggesting that gene transcription changes regularly during mesocarp development but for the first and the last samples, in which specificity of expression is accentuated. All mesocarp clusters contain genes which have a single peak in expression, either sharp (CM1 and CM6) or broad (CM2 to CM5)

CM1-CM4 contain genes mainly expressed during S1 and S2 growth stages. These genes are less studied in comparison to that related to the peach ripening processes (CM5 and CM6), already deeply analysed by (Trainotti et al., 2006; Trainotti et al., 2007) and (Bonghi et al., 2011), thus we focused our attention on genes involved in early phases of mesocarp development. A proteomic (Hu et al., 2011) study pointed out that early developmental stages of mesocarp are characterized by an increase of enzymes involved in glycolysis and gluconeogenesis and a repression of those related to tricarboxylic acid cycle and carbohydrate metabolism. A more complete picture can be obtained taking into account the metabolomic analysis carried out by Lombardo et al., (2011) that indicates amino acids, in particular aliphatic (Ala and Val) and aromatic (Phe and Tyr), as metabolic markers for the early developmental phases of mesocarp. The increase of these amino acids content, due to the proteolysis of stored protein, has been associated to the requirement of substrate for phenylproipanoids, lignin and flavonoid biosynthesis that are induced concomitantly with the deposition of lignin in the endocarp (Dardick et al., 2010). According to this scenario in CM1, CM2 and CM3 clusters we found GO

terms associated to glycolosys and gluconeogenesis (GO:0006096), polysaccharide catabolism processes (GO:0000272), proteolysis (GO:0004190, GO:0055114, GO:0006508, GO:0004252) and flavonoid metabolism (GO:0016711 and GO:0033772).

However, the main trait of the first phase of peach mesocarp development is the richness in gene associated to cell division growth, especially in CM1 gene set as pointed out by GO enrichment analysis (table 1). Early developmental stages (S1) are characterized by rapid cell divisions in peach (Zanchin et al., 1994), as well as in other fleshy fruits such apple, tomato and cucumber (Lee et al., 2007; Janssen et al., 2008; Mounet et al., 2009; Ando et al., 2012). After that stage, cell cycle ceases as evidenced by the presence of most mitosis-related genes in CM1 cluster (Table 2). This result was expected considering that in peaches the cell division duration is related to the length of whole fruit developmental cycle (shorter in early-ripening cvs and longer in late-ripening ones), but in every case lasts at the end of S1 (Ognjanov et al., 1995). DNA replication genes were showing instead a bimodal pattern of expression with a minimum during the late S2 and early S2 stages (samples TM3 and TM5, respectively). This particular pattern may be related to DNA replication activity without cell division in S3 and S4 stages, a biological process known as endoreduplication (Figure 6).

Endoreduplication cycle or endocycle consists of one or several rounds of DNA synthesis in the absence of mitosis. This alteration of cell cycle often occurs during fruit development as in the case of tomato fruit (Chevalier et al., 2011). During tomato fruit development, clear positive correlations have been established between the mean cell size within the fruit pericarp and the mean ploidy level of various tomato genotypes (Cheniclet et al., 2005). Therefore endoreduplication is a major determinant for the final size of the cell, which can explain the observed gradation in cell size in tomato fruit but contributes also in part to the variation in fruit size.

Peach, together with other few species of the Prunus genus (apricot and plum), is an exception because usually endoreduplication does not occur in species where fruit development lasts for a very long period of time (over 14 weeks) (Bourdon et al., 2010). Our transcriptomic data suggests that this process occurs also in 'Fantasia' peach variety, as genes encoding cell cycle regulatory proteins are differentially expressed in S1 and S3/S4 stages. In order to assess if the endoreduplication process is differentially

modulated in varieties in which fruit development is accelerated as in the early-ripening cv (Springcrest), or slowed as in slow-ripening varieties, some endoreduplication related genes were selected and their expression analysed with qRT-PCR.

Selected genes were members of the cyclin-dependent kinase (*CDKB* and *CDC2*) and of the regulatory cyclin (a D-type Cyclin and two A-type Cyclins, *CyC*) families, which are the main actors in the control of progression within the distinct phases of both the plant canonical cell cycle and the endocycle (Inzé and De Veylder, 2006); moreover, the genes of the orthologues of WEE1 and KRP3 were chosen. Indeed, the former is involved in the phosphorylation/dephosphorylation status of CDK while the latter acts as specific CDK inhibitor (Chevalier et al., 2011). All these genes are highly expressed during S1 and repressed in S2 stages in the 3 cultivars studied, while a resumption of their expression, was observed only in 'Springcrest' and 'Fantasia'. The expression in 'Fantasia' correlates very well with that of several *CYCD* and *CDC2* encoding genes, thus possibly confirming their role as endoreduplication markers also in peach. Note of worthy is that in *slr* the expression of *KRP3* and *WEE1* homologues, two genes claimed to play a regulatory role in tomato fruit endoreduplication (Chevalier et al., 2011), is strongly repressed also during S3 stage, suggesting an absence of endoreduplication. In tomato transgenic plants in which *WEE1* was down-regulated, a clear reduction in the level of endoreduplication in fruit cells was observed (Gonzalez et al., 2007). Endoreduplication has been proposed as a way to fix cell fate (De Veylder et al., 2011) and thus we could consider it as a way to label fruit parenchymal cells that are ready to undergo ripening. Indeed, in 'SpringCrest', whose fruits ripen very quickly, also endoreduplication seems more pronounced. On the contrary, in the slow ripening *slr* genotype, endoreduplication is almost absent. Although we are aware that this conclusion is based only on transcriptomic data and that DNA content needs to be determined, this inability of *slr* may be among the reasons for which it fails to ripe.

Endoreduplication occurs also in seed endosperm and one of the best characterized system is maize (Sabelli et al., 2013). Expression analyses revealed that transcripts for WEE1 strongly accumulate in maize endosperm (Sun et al., 1999). In the seed of 'Fantasia', two peaks of WEE1 transcripts were observed, the first during S1 in correspondence with mitotic activity and the second at the middle of the S2 stage in correspondence of

the free-nuclear endosperm stage and of the beginning of endosperm cellularization (Ognjanov et al., 1995) and with the expression of endoreduplication genes. As for the mesocarp, *WEE1* expression in 'SpringCrest' is higher and lasting longer than in 'Fantasia'. This might reflect an abnormally higher metabolic activity of endosperm cells that, competing with the embryo for nutrients, might cause a delay in its development, thus causing the presence of unripe seed in ripe fruit, typical for this genotype as well as for many early cvs. On the contrary, in *slr WEE1* expression is lower than that of 'Fantasia' also in the seed. Previous reports in the literature (Bonghi et al., 2011 and references therein cited) described *slr* seed development similar to that of 'Fantasia'. Even though there are not macroscopic differences at the anatomical level, the seed is slightly smaller and many genes are differentially expressed, as it is for *WEE1*. Whether endoreduplication is among the mechanisms responsible for the mutant phenotype of *slr* will be evaluated in the next future.

4.3 The role of Transcription factors and microRNAs in the regulation of mesocarp and seed development

A recent paper reports that 1 162 TFs were transcribed in young leaves, flowers at pink stage and fruits collected after 65 days after pollination (Wang et al., 2013). Our data indicated that a part of these (879 TFs) was significantly expressed during mesocarp and seed development of 'Fantasia'. The number is similar to that (821) observed in the developing fruit of tomato (Matas et al., 2011).

Interestingly, some of the fifty-five families identified, in particular ARF, GRF and SBP, were showing almost all their members expressed in peach mesocarp and seed. This result suggests a putative important role in reproductive tissue development for these TF families. The involvement of ARFs and their cognate proteins, termed Aux/IAA, has been largely studied in peach fruit ripening by Trainotti and co-workers (Trainotti et al., 2007). In addition to these, in this work we identified two *ARF* genes showing the highest expression in the early developmental phases of mesocarp (Figure 10). The role for ARFs in the early phases of fleshy fruit development has been recently deeply dissect in tomato (Zouine et al., 2014). In fact, the expression of tomato *ARF*s sharply increases upon pollination/fertilization. Given the role of auxin signaling in the fruit set process (Jong et al., 2009; Devoghalaere et al., 2012), the dynamics of the expression

pattern of tomato ARFs is indicative of their putative involvement in mediating auxin responses during the flower-to-fruit transition. To assign the same role to peach ARFs further investigations are required, but the best candidates are those whose expression has been here described.

The relationship between expression and developmental stages was more stringent for SBP and GRF family members (Figure 12). For 11 out of 15 *SBP* genes the highest expression was observed in the early phases in both mesocarp and seed development (Figure 12). An exhaustive analysis of *SBP* tomato genes expression revealed that a large part of members was ubiquitously and constitutively expressed (from seedling to ripe fruit), while some others were showing an overall more differentiated expression pattern (Salinas et al., 2012). Also in tomato are present *SBP* genes (*SlySBP8a*, *-b*, *SlySBP13* and *SlySBP15*) that are already active in the carpel and then clearly decline or even disappear in the ripe fruit but, differently to that observed for peach *SBPs,* transcripts of *SlySBP12b*, *SlySBP10* and *CNR* were highly accumulated in ripe fruit. Until now few investigations have been performed on the role of *SBP*s genes during early phases of seed development.

All *GRF* members were highly accumulated in the late phases of peach seed development, while they were barely detectable in mesocarp (Figure 13). GRF TFs were well studied in *Arabidopsis* (Kim et al., 2003). Overexpression of *AtGRF1* and *AtGRF2* resulted in larger leaves and cotyledons, assigning their role in the regulation of cell expansion in leaf and cotyledon tissues. The expression pattern of peach *GRFs* might suggest a similar action during embryo development.

In addition to these families, the expression pattern observed for a *NAC* and three C2H2 TFs is interesting. The NAC (ppa009438m) TF showed the highest repression at T3/T1 transition. A similar expression pattern was detected for two *NAC*s that were preferentially expressed during early developmental phases of tomato (Mounet et al., 2009) and cucumber (Ando et al., 2012) fruit. The action of NAC TFs in the early phases of fruit development is less studied in comparison to that played by NOR, a tomato NAC TF, in ripening fruit (Giovannoni, 2007), but published data are suggesting a central role of these TFs also in young fruits. The expression of CH2H2 genes declined at the transition between T3 and T5 in seed. This pattern can be explained taking into

account that an *Arabidopsis* C2H2 gene was strongly expressed in seed up to five days after pollination in correspondence of endosperm endoreduplication (Lu et al., 2012). In addition, the same authors demonstrated that the C2H2 gene silencing via a T-DNA insertional mutation resulted in abortion of 25% of seeds when in heterozygosus state, while the homozygous mutant allele displayed an embryo with a *titan*-like phenotype.

Selected TFs of SBP, GRF and ARF families were tested in genotypes characterized by different mesocarp developmental kinetics (Figure 14-15). The data indicate that in mesocarp the expression of TFs changes accordingly to the developmental kinetic, being the peak of SBPs transcripts earlier in 'Springcrest' than in 'Fantasia' and *slr*. For the seed this relationship is less clear. It is well known that ARF, SBP and GRF are subject to multi-levels post-transcriptional regulation of their expression mainly due to small RNAs (Baucher et al., 2013; Salinas et al., 2012; Zouine et al., 2014). This system of regulation may account for a significant part of the control of TFs expression in developmental processes such as mesocarp and seed development also in peach and it also might explain the differences observed, in terms of TFs expression, in the three tested genotypes. The case of miR156 and its target in peach (*SBP9*) was the clearest. In all tested genotypes miR156 and its target were not only expressed in a complementary fashion, but also in relation to the mesocarp developmental kinetic (i.e. an earlier increase of miR156 was observed in 'Springcrest'). Different patterns of miR156 expression were found in rice genotypes characterized by a different tolerance to low level of nitrogen, demonstrating that expression patterns of miRNAs may vary extensively even between two genotypes of the same species, in this latter case in response to external stimuli (Nischal et al., 2012).

5. Conclusions

μPEACH3.0 is a good and reliable tool to investigate peach gene expression at the transcriptomic level. With more than 20 000 transcripts observed in two organs and six different time-points, this array gave an ample view on the development of the peach fruit at the molecular level, and also lots of data that need to be examined further. The analysis of other tissues with the same tool (leaves, roots, flowers) will allow us to build a general atlas of peach gene expression, such as those available for *Arabidopsis* (Schmid et al., 2005), tobacco (Edwards et al., 2010), soybean (Libault et al., 2010) or grape (Fasoli et al., 2012).

If ripening is the most important biological process in the final stages of peach development, in the first stages the most evident process at transcriptomic level is cell division. Along with cell expansion, cell division determines the final size of a fruit. Microarray data shows how this is also a major process in seed development, despite taking place in a different time period.

Focusing on this aspect of fruit development, and using both array and RT-qPCR data, allowed us to determine that whereas cell division occurs in the first stages, DNA replication continues to take place also in later stages. In particular, for 'Fantasia' DNA replication genes are expressed also in S3 and S4. DNA replication without mitosis is linked with the endoreduplication process, that seems to do not take place during the slow-down of peach growth in S2 stage.

Among the three cultivars studied, only the genotype *slr* does not have a significant expression of selected endoreduplication genes after the S1 stage. The inability of this genotype to grow during the S3 stage, failing to enlarge its cells, may be due to an absence or a reduction in endoreduplication rates. Cytological analyses will be needed to confirm if *slr* cells actually show a lower ploidy number than those of its parent 'Fantasia'. On-going genomic analysis will determine if there are significant mutations on the genes regulating endoreduplication.

A particular attention was given to Transcription Factors (TFs), as they're known to be the key regulators of a cell's transcriptome. Some TF families seem to be particularly active in mesocarp and seed development: SBP TFs are highly expressed in early stages of both organs, while GRFs characterize the mature seed. A negative correlation between

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some TFs and the microRNAs which regulates their homologues in other species has been found also in this tissues, this information needs to be confirmed by other experiments in order to confirm their activity in peach, anyway, these data suggests that the regulatory network of peach fruit development involves also these small RNAs.

The analysis of the data obtained is not complete, and further analyses may be done. The availability of a full genome microarray may allow to set up a peach atlas of expression, as those which are available for other plants species, comprising the transcriptome of also other peach tissues, such as leaves, root and flowers, Or it may be used to assess the transcriptome of fruit and seed development also in other cultivars, which may shed light onto the mechanisms which underlie the phenotypic expression of particular genetic traits.

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Chapter IV

General conclusions

General Conclusions

Peach is considered a model species for studying the growth of drupe fruits in general, its characteristic being a particular relationship between the seed and the pericarp: in early stages of development the two organs are tightly related, then, in later stages, there is an uncoupling in development as the fruit, particularly that of early varieties, may ripe well before the seed is mature. Understanding the relationships between these two organs may shed light onto the different developmental patterns of growth of the fruits of the various cultivars, this in turn may help breeders in marker assisted selections of new cultivars.

For this purposes omic techniques are powerful tools: genomics identify which alleles are related to a phenotypic traits of interests, while transcriptomics identify whether these genes are effectively transcribed, as well as the regulatory networks that link them.

In Chapter II of this thesis, using μPEACH1.0 (a partial genome microarray, covering 4 806 genes), we were able to identify stage specific gene markers, as well as to uncover the role of hormones (in particular auxins) in the cross-talk between seed and mesocarp. Markers' specificity was confirmed in different years and different genotypes through qRT-PCR. Interestingly, we didn't investigate the role of hormones directly (e.g. using hormone treatments or by measuring the production and distribution of hormones in the tissues), but their levels were measured indirectly through microarray transcriptomic data. In fact, the expression data of the great number of genes analyzed were compared to lists of genes known to be responsive to hormones through the HORMONOMETER tool: in this way, starting from the expression levels of the genes, it was possible to determine the relative level of hormones in our experimental conditions. The use of this technique was possible only thanks to the massive amounts of data given by an high-throughput method as the microarray is, because data from few genes can't give meaningful results.

Anyway, even an array with 4,800 probes is not sufficient to give a full overview on the molecular events of fruit and seed development. The sequencing of the whole peach genome carried out by the International Peach Genome Initiative (IPGI) allowed us to finally set up a microarray covering the entire peach genome. This new array, called μPEACH3.0, have 29 800 probes designed from the IPGI's genes and some home-

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identified peach genes. The probes were spotted twice on the array in two technical replicates, which enabled to identify and to eliminate outlier data due to artefacts, which otherwise could have negatively influenced data analysis. 30% of the probes didn't give remarkable signals, which may be due not only to the array design, but also to the fact that only two organs were used in the analyses. On the other hand, we found that the array data were robust, with an high correlation to qRT-PCR measures performed on the same samples.

µPEACH3.0 produced a large amount of data, giving us the possibility to investigate diverse events during both fruit and seed development, as explained in Chapter III. The cell cycle genes underlying fruit and seed growth were identified, incidentally it was found that while mitosis related genes were expressed only in the first stage of mesocarp development, the genes related to DNA replication were actively transcribed also in later stages, suggesting that events of endoreduplication occur in that period. µPEACH3.0 allowed us also to perform a genome-wide analysis of gene expression of transcription factors (TFs), and the important role of some transcription factors families in different stages of development was unveiled, namely the SQUAMOSA Promoter binding proteins at the onset of both fruit and seed development, and the Growth Regulating Factors in the mature seed. As these TFs are known to be regulated by microRNA, the expression of these microRNA was measured; the data effectively suggests that the abundance of TFs mRNAs may be regulated by microRNAs also in peach fruit, extending the regulatory network which was found using microarray data.

Anyway, the analysis of the data obtained from the microarray is not yet finished.

Microarray data can be mapped onto the genome to see whether there are relationships between transcription and the physical positions of genes. This mapping will help to discover "transcription islands": regions of the genome in which transcription is activated in specific periods of peach development, but we will be able also to analyze gene expression in quantitative traits loci, helping to find candidate genes for agronomically important traits.

The µPEACH3.0 may then be used also on other peach tissues, such as flowers, leaves and roots, making available an atlas of gene transcription, such as those that are available for other fruit plants (e. g. tomato, grape). Moreover, it may be used to analyze gene

expression in fruits and seed of other cultivars: while the genomic approach allows to find polymorphisms in the genetic sequences of different cultivars, transcriptomic analysis will be necessary to see the effect of a particular mutation on all the transcriptome of a cell and to find the regulatory network influenced by the mutation.

Finally, recently an article using RNAseq to analyze peach transcriptome has been published. RNAseq technique, whose cost is continuously decreasing, making it more affordable, gives some additional advantages in comparison to the microarray technology. RNAseq avoids the problem of non-functioning probes, which normally affects microarrays, moreover RNAseq may be used also to get transcriptomic informations on microRNAs, which, as we have seen, may be as important as transcription factors in regulating the metabolic state of a cell. Anyway, the statistical techniques used to analyze microarray data may be used in the same way also for data coming from RNAseq experiments, facilitating the integration of data coming from different technical sources.

Appendix

Molecular and biochemical responses to wounding in mesocarp of peach (Prunus persica L. Batsch) ripe fruits

Molecular and biochemical responses to wounding in mesocarp of peach (Prunus persica L. Batsch) ripe fruits

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Authors' contributions

RT, FT and LG carried out the experiments and performed the metabolic, biochemical and molecular (qRT-PCR) analyses. AT and LT performed the microarray hybridizations, VZ and FMG carried out the bioinformatics analyses. PT conceived the experimental trials and together with CB, FMG, LT, and RT analyzed the data, assembled the results and wrote the manuscript. All authors read and approved the final manuscript..

Abstract

The physiological and molecular responses of ripe fruit to wounding were evaluated in two peach (Prunus persica) varieties (Glohaven, GH, melting and BigTop, BT, slow melting nectarine) by comparing mesocarp samples from wedges (as in minimal processing) and whole fruit as the control. Slight differences between the two varieties were detected in terms of ethylene production, whereas total phenol and flavonoid concentration, PPO and POD enzyme activities showed a general increase in wounded GH but not in BT. This was associated with the better appearance of the BT wedges at the end of the experimental period (72h). Microarray (genome-wide μPEACH3.0) analysis revealed that a total number of 2,218 genes were differentially expressed (p<0.01, log2 fold change expression ratio>1 or <-1) in GH 24 hrs after wounding compared to the control. This number was much lower (1,208) in BT. According to the enrichment analysis, cell wall, plasma membrane, response to stress, secondary metabolic processes, oxygen binding were the GO terms categories over-represented among the GH up-regulated genes, whereas plasma membrane and response to endogenous stimulus were the categories over-represented among the down-regulated genes. Only 32 genes showed a common expression trend in the two varieties 24h after wounding, whereas a total of 512 genes (with highly represented Transcription Factors), displayed an opposite behavior. Quantitative RT-PCR analysis confirmed the microarray data for 18 out of a total of 20 genes selected. Specific *WRKY*, *AP2/ERF* and *HSP20* genes were markedly up-regulated in wounded GH, indicating the activation of regulatory and signaling mechanisms probably related to different hormone categories. Compared to BT, the expression of specific genes involved in phenylpropanoid and triterpenoid biosynthetic pathways showed a more pronounced induction in GH, highlighting the difference between the two peach varieties in terms of molecular responses to wounding in the mesocarp tissue.

Keywords: Mechanical stress, Microarray, Minimally processing, Postharvest physiology, Secondary metabolism, Transcription Factors
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1. Introduction

Plants react to stress by activating a number of different mechanisms depending on the genetic background, developmental stage, intensity and the duration of the stress and the type of organ/tissue undergoing the stress. With regard to abiotic stress, in addition to drought, salinity and extreme temperatures, mechanical stress such as physical wounding can occur in the field and, for specific crops (e.g. horticultural produce), also after harvesting. Due to postharvest handling (sorting, storage, transportation, etc.), perishable fruits are at high risk of unintentional mechanical damage (Kays and Paull, 2004). Major (intentional) postharvest sources of wounds include injuries imposed during the preparation of the so-called minimally processed or fresh-cut produce.

Physiological, biochemical and molecular responses to wounding in plants have been described (de Bruxelles and Roberts, 2001; Cheong et al., 2002; Shanker and Venkateswarlu, 2011). Wounding results in metabolic changes which, according to Zhou and Thornburg (1999), are in general aimed at: i) placing mechanical barriers to invading organisms, ii) sealing the wounded tissue, iii) activating defensive compounds against invading organisms, and, iv) recovering from the wound. These changes involve the selective modulation of gene expression. Several wound-related genes have been identified to date and their expression studied (Mitsuda et al., 2007; Koo and Howe, 2009; Trinidade et al., 2011; Leide et al., 2012), also in relation to various hormones such as ethylene, abscisic acid (ABA), and jasmonic acid (JA) (Birkenmeier and Ryan 1998; Delessert et al. 2004; Leide et al., 2011). Several of the genes identified encode signaling molecules. Working on *Arabidopsis*, Cheong et al. (2002) suggest that a cascade of gene regulation is activated after wounding, and that the "early" genes involved encode for regulatory proteins (Transcription Factors, TFs) thus modulating the expression of "late" response genes. Wound-induced TFs such as MYB/Myb-like, WRKY, AP2/ERF have been identified in various crop tissues, such as persimmon (Akagi et al., 2010), tobacco (Hara et al., 2000), and Hevea brasiliensis (Chen et al., 2012b). The "late" response genes encode mainly for effector proteins, including those improving endurance or the recovery of cells from stress or damage, such as heat shock proteins (HSPs), cell wall-modifying enzymes, secondary metabolites and pathogenesis-related (PR) proteins (Cheong et al., 2002).

Compared to other plant organs/tissues, little information is available on the molecular mechanisms of fleshy fruits that actively react to wounding both before and after harvest. In ripening fruits, wounds cause an increase in respiration rate and ethylene production, and generally lead to flesh softening, membrane disruption, browning, accelerated senescence, weight and water losses, and microbial development (Toivonen and Brummell, 2008). An up-regulation of the expression of a phenylalanine ammonia lyase (*PAL*) gene has been reported in the pulp of ripe banana upon wounding where the accumulation of RNAs of different *HSP*s was also observed (Chen et al., 2009). Responses to mechanical stress may change depending on the developmental stage, as observed by Su et al. (2011) in Gala apples, and by Sherf and Kolattukudy (1993) who reported the specific expression of a wound-related anionic peroxidase gene in green, but not red, tomato fruits.

Due to the increasing commercial interest in minimally processed (MP) (or fresh-cut) produce, a better characterization of the effects of mechanical operations (peeling, cutting, slicing, etc.) during minimal processing is needed in order to optimize the protocols and, in particular, to appropriately select the varieties to be used. In fact, the final quality of fresh-cut produce is greatly influenced by many factors during processing and storage, but is also strictly related to the quality of the raw fruit. The selection of the best variety is thus essential. Previous studies on apple, plum, kiwi, tomato and peach confirm that different cultivars show variable responses to mechanical operations (textural deterioration, browning, metabolite concentration, etc.), thus indicating a different stress tolerance (Zhang et al., 2009; Han et al., 2010; Illa et al., 2010; Park et al., 2010; Carbone et al., 2011; Prinsi et al., 2011; Vishwanath et al., 2011; Tosetti et al., 2012; Usenik et al., 2012).

Peach (Prunus persica) is characterized by a wide diversity in terms of geno/phenotypes, in particular considering the ripening behavior and the textural changes of the flesh (Bassi and Monet, 2008). A rapid loss in flesh firmness (melting phase) characterizes the "melting" varieties. This phase is absent in "non-melting" peach genotypes, which have thus been proposed as ideal varieties for fresh-cut preparations (González-Buesa et al., 2011). One of the interesting genotypes for processing/minimal processing is the Stony Hard (SH) (which does not produce ethylene at ripening) together with other varieties,

such as "slow ripening" varieties and BigTop (BT). The latter, defined as "slow melting", is an attractive yellow-fleshed nectarine, which genotypically belongs to the melting category, but shows an altered ripening physiology with a delayed ethylene evolution and a reduced softening rate (Ghiani et al., 2011). Most of the physiological and molecular analyses on peach fruit ripening and postharvest have been carried out on melting varieties thus represnting valuable biological material for physiological studies including those concerning postharvest stress. In order to better describe some of the mechanisms involved in the responses of ripe fruit tissues to wounding, we carried out specific biochemical and transcriptional analyses based on the whole-genome microarray µPEACH3.0 in mesocarp of a melting peach variety (Glohaven) and, for comparative purposes, of BigTop.

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2. Materials and methods

2.1 Plant material and experimental design

Peach (P. persica L. Batsch) fruit of the yellow-fleshed cv. Glohaven (GH, melting) and Big Top (BT, "slow-melting" nectarine) were harvested at flesh firmness values of approximately 30N and immediately transferred to the lab. Forty selected fruits for each variety were used for the experiments. Twenty fruits were stored up to 72h at 4°C, 90-95% RH (control, c) and twenty were used for minimally processing operations (wounded samples, w). From each fruit, eight wedges (mesocarp + epicarp) were isolated using a sharp knife, and then kept at the aforementioned conditions for 72h. For each variety, the biological material was sampled at five time points: at the beginning of the trial (T0) and at 8 (T1), 24 (T2), 48 (T3), and 72 (T4) hours after wounding. For biochemical and molecular analyses, mesocarps of three whole fruits (control, c) and of three wedges (wounded, w) from different fruits were collected at each sampling time and immediately frozen in liquid nitrogen and stored at -80°C. Three biological replicates were collected for each sample.

2.2 Technological, physiological and biochemical assays

Flesh firmness was determined using a digital penetrometer equipped with an 8 mm flat probe (Fruit Texture Analyzer, Model FTAGS-14, Güss, PTY Ltd, Strand, South Africa). Ethylene production was measured by incubating three wedges for each of the three replicates in small jars sealed with an air-tight lid equipped with a rubber stopper. After incubation for 1 hour at room temperature, 1-mL of gas sample was withdrawn and analyzed via GC, as described by Begheldo et al. (2008).

Changes in the color of the mesocarp of the wedges and control fruit were measured using a Minolta CM2500 colorimeter and the ΔE was evaluated. The ΔE was calculated with the formula $\Delta E = \sqrt{(\Delta L*2 + \Delta a*2 + \Delta b*2)}$. For each time point, six replicates/thesis (six individual wedges and two readings for each of the three control fruits) were considered.

The extraction of total polyphenols (TP) and total flavonoids (TFO) was based on a slight modification of the method reported by Du et al. (2009). Fruit tissue (mesocarp, 3 g) was first homogenized in 12 mL of ethanol:acetone (7:3, v/v) at 37°C for 1 h (30). The

extract was filtered using Whatman No. 41 paper and then rinsed with 3 mL of the same extraction solution. The filtrates were collected and stored at -20°C. TP content was determined using the Folin-Ciocalteu assay, according to Dewanto et al. (2002). TP concentration was expressed as gallic acid equivalents (µg GAE/100 g fresh weight) using a calibration curve (50-600 µg mL-1). TFO concentration was determined according to Du et al. (2009) at 506 nm. TFO content was expressed using rutin (mg RE/100 g fresh weight) as a standard (6.25-1000 mg mL-1). Carotenoids were extracted and quantified as indicated in Reyes et al. (2006) starting from 4 g of mesocarp.

Polyphenol oxidase (PPO) and peroxidase (POD) activity determinations were both carried out using the same extract, as described by Loaiza-Velarde et al. (1997) with slight modifications. Tissue (8 g) was homogenized, with the help of purified sand and polyvinylpoly-pyrrolidone (PVPP) 0.1- 0.3% v/v, in 2.5 mL of 50 mM phosphate buffer (pH 6.8). Pellets were separated by centrifugation at 19,000 g at 4°C for 20 min. The supernatant was then collected at 4°C and used for the determinations of both enzymatic activities. PPO activity was determined according to Espin et al. (1997). This assay measures the accumulation of the red-wavelength complex o-quinones-MBTH (3-Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate) followed at 467 nm (ε=22300 M-1cm-1). The reaction mixture contained 50 mM sodium acetate buffer (pH 5.5), 2% DMF (N,N-Dimethylformamide), 3 mM MBTH, 2 mM DHPPA (3,4-Dihydroxyhydrocinnamic acid), 10 µg mL-1 bovine catalase and 5 µL of extract, in a final assay volume of 1 mL. One unit of PPO was defined as the amount of enzyme that produces 1 µmol of MBTH-DHPPA-o-quinone adduct per minute (Espin et al., 1995).

POD was determined according to Rodriguez-Lopez et al. (2000) with some modifications, by measuring the accumulation of the ABTS radical cation (ABTS•+) at 414 nm (ε =31300 M-1cm-1). The reaction mixture for determining POD activity contained 50 mM (pH 4.5), 2 mM ABTS, 2 mM H_2O_2, 0.2 mM tropolone and 2.7 µg of protein of enzymatic extract, in a final assay volume of 1 mL. One unit of POD was defined as the amount of enzyme that produces 1 mol of ABTS •+ per min.

2.3 Gene expression analyses

Total RNA was extracted from freeze-dried mesocarp samples according to Wang and Vodkin (1994). RNA purity and integrity were checked by the Experion automated

station system (Standard-sensitivity RNA analysis kit. BioRad, Berkeley, California). Contaminating DNA was removed using a TURBO DNA-free kit (Ambion, www.ambion.com).

RNA samples extracted from the mesocarp of GH and BT from both control fruit and wedges at T2 were used for microarray analysis. We used a recently developed microarray platform, named μPEACH3.0 (Trainotti et al., 2012). It is based on a custom-made Agilent SurePrint G3 platform with a 8x60k format. The probe selection was carried out on 30,113 predicted transcripts. The 28,689 protein-coding transcripts (The International Peach Genome Initiative, 2013) were implemented by adding 1,424 new predictions (Forcato et al., unpublished data). 454 and Solexa RNAseq experiments were used to implement the new in silico transcript predictions. Each of the eight arrays of the slide contains 29,800 probes, each printed twice. Hybridizations and scanning were carried out following the manufacturer's instructions (available on the Agilent web site). Twenty genes were selected to validate the microarray analyses via quantitative RT-PCR and ten of them analyzed on the whole set of collected samples (T0-T4). Briefly, RNA was reverse transcribed using the High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA). qRT-PCR analysis was carried out using 20 ng of cDNA and iQTM SYBR® Green Supermix (Biorad Laboratories, Hercules,CA), according to the manufacturer's instructions. Expression of Tef2, translation elongation factor 2 (ppa001368m), was used as an internal standard (in our microarray dataset, the gene falls into the lowest quartile of genes sorted by expression variance). Primer pairs (Table S1, Supplementary material) were designed using Primer3 Plus (Untergasser et al., 2007) and quality-checked with Netprimer (http://www.premierbiosoft.com/netprimer/index.html). After the PCR, the consistency of the dissociation curves and the presence of a single amplicon band were checked. Threshold cycles (Ct) were determined using the SDS 1.3 (Applied Biosystem). Data are shown as $2^{\Delta\Delta ct}$, as described in Manganaris et al. (2011). The expression level recorded at T2 by using the microarray platform was confirmed via qRT-PCR for 18 out of 19 genes (no detectable amplification was observed for fructose 1-6 biphosphatase) (Table S2, supplementary material).

2.4 Bioinformatics analysis

Twelve microarrays were analyzed using the R limma package (Smyth, 2005). In detail, raw single channel image files were background-corrected using the normexp method and quantile normalized. All samples passed microarray quality tests performed by Robin (Lohse et al., 2010), and showed a comparable distribution of signal intensity post normalization (Figure S1, supplementary material). A simple hierarchical clustering using the "complete" agglomerative method based on 1-Pearson Correlation Coefficient was performed. The dendrogram (Figure S2, supplementary material) highlighted the third replicate of the treated BigTop class (BT_T1_3) as an outlier, and therefore the analysis was performed without considering this outlier.

Limma eBayes moderated t-tests were then performed on two separate contrasts: BTw vs BTc, and GHw vs GHc. All the R code used to perform the analysis is reported in File S1 (supplementary material). Full results, annotated via the Mercator pipeline for Mapman (Usadel et al., 2009), Uniprot (Apweiler et al., 2004) and Gene Ontology (Ashburner et al., 2000), are reported in Table S3 (supplementary material).

GO enrichment analyses were performed using Fisher's exact test, and a critical False Discovery Rate (FDR) q-value of 0.05 was applied. Blast2GO (Conesa and Götz, 2008) was used to compute enriched GO terms. To summarize GO enrichment results, the enriched GO terms were mapped to a GO Slim annotation, which is a reduced version of the complete annotation with less detailed high-level GO terms, and counting the occurrences (single occurrence option) of GO Slim terms as well as the related lower hierarchy terms using CateGOrizer (Hu et al., 2008).

2.5 Statistical analysis

The statistical analyses of the qRT-PCR data for microarray validation were carried out with Graphpad Prism v5 and CoStat (Bartelett's test and LSD). For firmness, ethylene production, compound content and enzymatic activities, two-way ANOVA analyses were performed using Graphpad Prism v5. Whenever indicated, Mapman ontology term (BIN) over-representation analyses were conducted using a Bonferroni-corrected Fisher's Exact Test as described in Giorgi et al. (2013).

3. Results

Throughout the whole 72-h experimental period, no statistically significant difference (P<0.05) in flesh firmness was observed in wedges in terms of the initial values (T0) of each variety and by comparing the two varieties (Fig. 1A). However, a visual evaluation of the slices at the end of the experiment (T4) clearly highlighted that the overall quality (general appearance, freshness of the cut surfaces) was higher in BT compared to GH. This more pronounced change in visual properties occurring in wounded GH was confirmed by comparing, at T4, the mesocarp of wedges and control fruits, using the parameter ΔE. This index, based on CIELab* values, indicates that the difference between two colors corresponded to 3.72 and 5.46 in BT and GH, respectively.
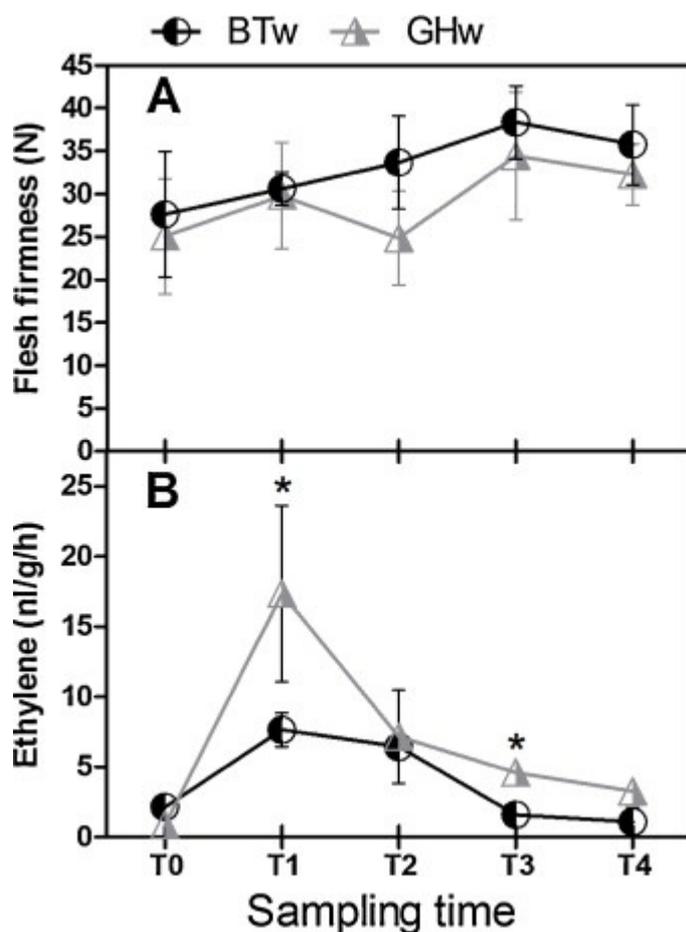

Fig. 1. – Flesh firmness (A) and ethylene biosynthesis (B) of wedges of 'BigTop' (BT) and 'Glohaven' (GH) at the beginning of the trial (T0) and 8 (T1), 24 (T2), 48 (T3), and 72 (T4) h after wounding. Bars indicate ± SD and asterisks significance at $p < 0.05$.

Ethylene evolution showed a similar trend in the wedges of the two varieties, with a peak observed 8h after wounding, followed by a steady reduction throughout the experimental period (Fig. 1B). Statistically significant differences ($P<0.05$) between the two varieties were detetcted at T1 and T3. A different behavior of the two varieties was detected when evaluating biochemical parameters in mesocarp samples of wedges (wounded tissue, w) and of whole fruit (control, c) throughout the 72h of the experiment. In fact, the concentration of total phenols (TP) promptly increased in GHw at T1 and remained high throughout the experiment. In GHc, an increase in TP was observed only at T3 and T4, when no significant difference was detected between the control and wounded samples (Fig. 2A). The TP concentration in BT constantly decreased throughout the experiment with no or limited differences between the BTc and BTw samples (Fig. 2A).
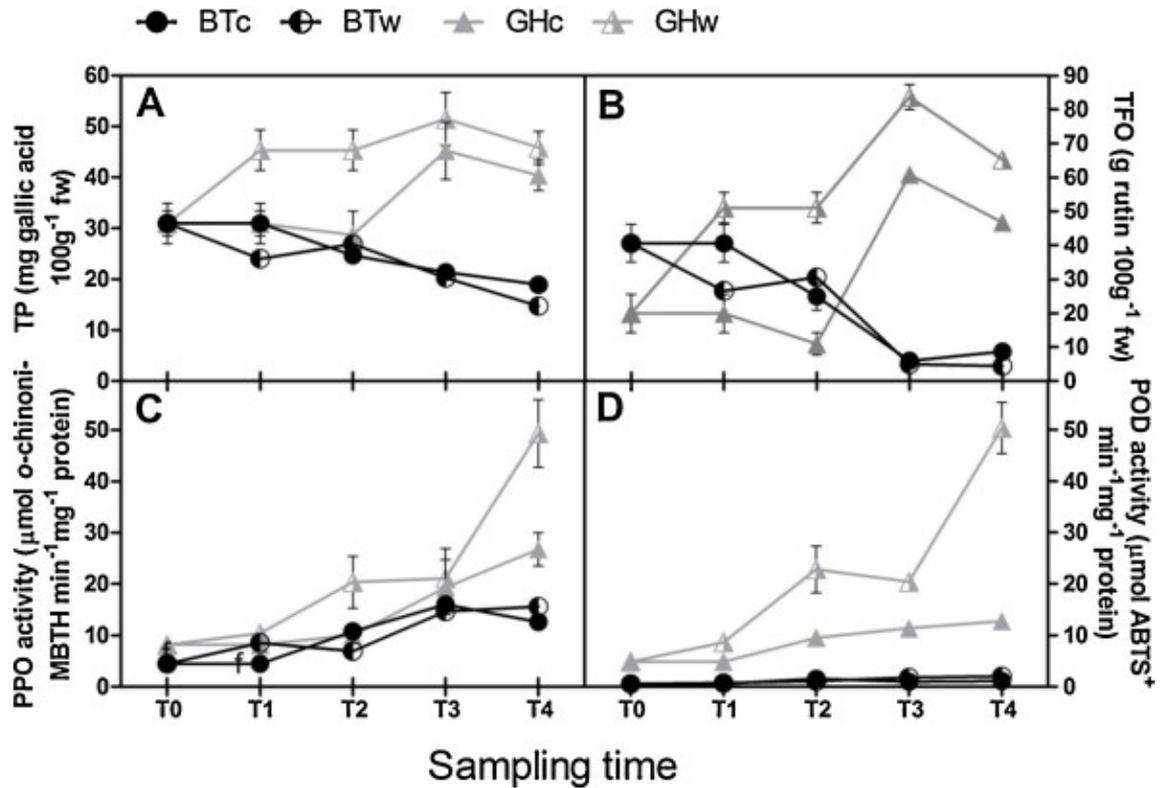

Fig.2. – Total phenol (TP, A) and total flavonoid (TFO, B) concentrations, polyphenol oxidase (PPO, C) and peroxidase (POD, D) activities in mesocarp of wedges (w) and control fruit (c) of 'BigTop' (BT) and 'Glohaven' (GH) at the beginning of the trial (T0), 8 (T1), 24 (T2), 48 (T3), and 72 (T4) h after wounding. Bars indicate ± SD.

A similar trend was observed in both varieties for total flavonoids (TFO, Fig. 2B), with a significant difference between GHw and GHc at T3 and T4. PPO activity showed an increasing trend, more pronounced in GH than in BT, throughout the experiment (Fig. 2C): at T2, PPO activity was higher in GHw than in GHc, whereas no difference was detected between the two BT samples. At T4, a marked increase was observed in GHw, while BT samples still showed no differences. Similarly, POD activity was higher in GH than in BT, where almost no activity was detected in both BTc and BTw (Fig. 2D). Starting from T2, POD activity was significantly higher in GHw than in GHc. Carotenoid content showed different levels in the two varieties (approximately 30 µg β-carotene100 g-1 fw in GH, and approximately 70 µg β-carotene 100 g-1 fw in BT), with no significant changes throughout the experiment in both control and wounded tissues (data not shown). Considering the physiological behavior of GHw and GHc samples, and the difference observed between GH and BT fruits throughout the experimental period, fruit (mesocarp) tissues at T2 were selected for microarray analysis by comparing wounded and control samples using the genome-wide µPEACH3.0 array.

When comparing GHw vs GHc, of the almost 30,000 genes measured by the array, 7,005 were identified as differentially expressed at $p < 0.01$. Of these, 2,920 and 4,085 were up- and down-regulated, respectively. When the comparison was restricted to genes showing log2 expression ratio values higher/lower than +1/-1, the number of up-regulated (1,408) and down-regulated genes (810) changed (Table S3, supplementary material). This dataset was used to carry out an enrichment analysis (Table S4, supplementary material, for the complete analysis). In terms of the up-regulated genes (Table 1a), the GO terms cell wall (GO:0005618) and plasma membrane (GO:0005886), response to stress (GO:0006950) and secondary metabolic processes (GO:0019748), and oxygen binding (GO:0019825) were the most significant of those identified as over-represented in the cellular component (C), molecular function (F), and biological process (P) categories, respectively. As far as down-regulated genes are concerned, the GO terms plasma membrane (GO:0005886) and response to endogenous stimulus (GO:0009719) were over-represented and significant at $p < 10^{-6}$ in the enrichment analysis (Table 1b). Of the genes marked by the GO:0009719 term, those involved in auxin metabolism and action were highly represented.

Table 1. – Enrichment analysis of gene differentially expressed in GHw vs. GHc comparison. Enrichment analysis of GO term, retrieved categorizing genes up- **(A)** and down- **(B)** regulated in 'Glohaven' mesocarp, selected on the basis of p value (<0.01) and FDR (<0.05). GO-ID: the ID number of the GO term. Cat.: GO terms categorization in cellular component (C), molecular function (F) biological processes (P) groups. Term: description of the GO term. FDR: the proportion of false positives was determined by calculating the false discovery rate corresponding to each enrichment score. p value: p value indicating the statistical significance of the difference between the fraction of genes assigned to GO term retrieved and the fraction of all proteins within the background set assigned to the same GO term. Gene count: the number of genes in the differential expressed gene set (test set) assigned to each of GO terms. Exp. gene count: The number of genes was expected for each GO term retrieved in the test set on the basis of its fraction in the background set. O/U: GO terms under or over-represented in the test set.

A

GO-ID	Term	Cat.	FDR	p value	Gene Count	Exp. Gene Count	O/U
GO:0005886	Plasma membrane	C	3.31E-14	6.16E-16	299	178	Over
GO:0009536	Plastid	C	3.09E-04	4.05E-05	95	143	Under
GO:0005856	Cytoskeleton	C	1.89E-03	3.16E-04	8	25	Under
GO:0009579	Thylakoid	C	1.69E-02	5.88E-03	13	27	Under
GO:0016301	Kinase activity	F	7.88E-10	4.77E-11	300	198	Over
GO:0004872	Receptor activity	F	4.79E-08	3.34E-09	209	133	Over
GO:0030246	Carbohydrate binding	F	7.80E-08	5.08E-09	74	32	Over
GO:0003700	Sequence-specific DNA binding transcription factor activity	F	1.06E-04	1.28E-05	100	61	Over

| GO-ID	Term	Cat.	FDR	p-value	Gene Count	Exp. Gene Count	O/U
GO:0003682	Chromatin binding	F	4.23E-03	8.26E-05	1	10	Under
GO:0030528	Transcription regulator activity	F	1.61E-02	5.55E-03	31	51	Under
GO:0008135	Translation factor activity, nucleic acid binding	F	3.13E-02	1.15E-02	11	23	Under
GO:0003677	DNA binding	F	3.88E-02	1.48E-02	225	264	Under
GO:0006259	DNA metabolic process	P	9.07E-17	8.44E-19	18	89	Under
GO:0006464	Cellular protein modification process	P	7.18E-10	3.67E-11	291	191	Over
GO:0009607	Response to biotic stimulus	P	5.22E-06	4.61E-07	59	27	Over
GO:0009875	Pollen-pistil interaction	P	3.14E-04	4.53E-05	30	12	Over
GO:0080167	Response to karrikin	P	1.29E-03	2.04E-04	7	1	Over
GO:0006950	Response to stress	P	1.68E-03	2.74E-04	238	185	Over
GO:0019748	Secondary metabolic process	P	3.13E-03	5.67E-04	51	29	Over
GO:0015979	Photosynthesis	P	5.43E-03	1.09E-03	3	15	Under
GO:0008219	Cell death	P	7.19E-03	1.57E-03	37	61	Under
GO:0016043	Cellular component organization	P	7.24E-03	1.65E-03	88	123	Under
GO:0005975	Carbohydrate metabolic process	P	8.83E-03	2.05E-03	130	97	Over
GO:0006412	Translation	P	1.30E-02	3.38E-03	44	68	Under
GO:0080143	Regulation of amino acid export	P	1.54E-02	5.15E-03	3	1	Over
GO:0006091	Generation of precursor metabolites and energy	P	3.88E-02	1.46E-02	46	31	Over

B

GO-ID	Term	Cat.	FDR	p-value	Gene Count	Exp. Gene Count	O/U
GO:0003682	Chromatin binding	F	4.23E-03	8.26E-05	1	10	Under
GO:0030528	Transcription regulator activity	F	1.61E-02	5.55E-03	31	51	Under
GO:0008135	Translation factor activity, nucleic acid binding	F	3.13E-02	1.15E-02	11	23	Under
GO:0003677	DNA binding	F	3.88E-02	1.48E-02	225	264	Under
GO:0006259	DNA metabolic process	P	9.07E-17	8.44E-19	18	89	Under
GO:0006464	Cellular protein modification process	P	7.18E-10	3.67E-11	291	191	Over
GO:0009607	Response to biotic stimulus	P	5.22E-06	4.61E-07	59	27	Over
GO:0009875	Pollen-pistil interaction	P	3.14E-04	4.53E-05	30	12	Over
GO:0080167	Response to karrikin	P	1.29E-03	2.04E-04	7	1	Over
GO:0006950	Response to stress	P	1.68E-03	2.74E-04	238	185	Over
GO:0019748	Secondary metabolic process	P	3.13E-03	5.67E-04	51	29	Over
GO:0015979	Photosynthesis	P	5.43E-03	1.09E-03	3	15	Under
GO:0008219	Cell death	P	7.19E-03	1.57E-03	37	61	Under
GO:0016043	Cellular component organization	P	7.24E-03	1.65E-03	88	123	Under
GO:0005975	Carbohydrate metabolic process	P	8.83E-03	2.05E-03	130	97	Over
GO:0006412	Translation	P	1.30E-02	3.38E-03	44	68	Under
GO:0080143	Regulation of amino acid export	P	1.54E-02	5.15E-03	3	1	Over
GO:0006091	Generation of precursor metabolites and energy	P	3.88E-02	1.46E-02	46	31	Over

By extending the transcriptome analysis to BT, a clear difference between GH and BT in the molecular responses to mesocarp wounding was evident (Fig. 3). Average expression values showed a slight overall change between wounded and control tissues both in GH (correlation coefficient 0.975, Fig. 3A) and in BT (correlation coefficient 0.987, Fig. 3B). Absolute transcriptional levels were also very similar between BT and GH control samples (correlation coefficient 0.980, Fig. 3C), and significantly lower but still very high (correlation coefficient 0.950, Fig 3D) between BT and GH wound samples.

However, the two varieties showed an opposite tendency in the expressional change of their genes, highlighted by the comparison in the Log2FC shown in Figure 3E. Here, the response to wounding for all genes has a strikingly counter-correlation trend between the two varieties (correlation coefficient -0.431). Indeed, only 4,162 genes were identified as differentially expressed at p<0.01 in BT and of these, 2,180 and 1,982 were up- and down-regulated, respectively. Unlike GH, when the dataset is restricted to genes showing log2 expression ratio values higher/lower than +1/-1, in BT there were no marked changes in the distribution of genes into up- (536) and down- (672) regulated. This resulted in a much lower total number of differentially expressed genes in BT than detected in GH (1,208 vs 2,218) (Table S3, supplementary material).

Fig. 3. – Comparative behavior of gene expression between 'BigTop' and 'Glohaven' peach varieties, shown as scatterplots (each point representing a gene). (A–D) Absolute expression comparisons: (A) between GHc and GHw; (B) between BTc and BTw; (C) between control samples; (D) between wounded samples. The E panel shows a scatterplot between the $\log_2$ FC values obtained from differential expression analysis in 'BigTop' (BTw vs. BTc) and 'Glohaven' (GHw vs. GHc). Above each panel the Pearson correlation coefficient is indicated.
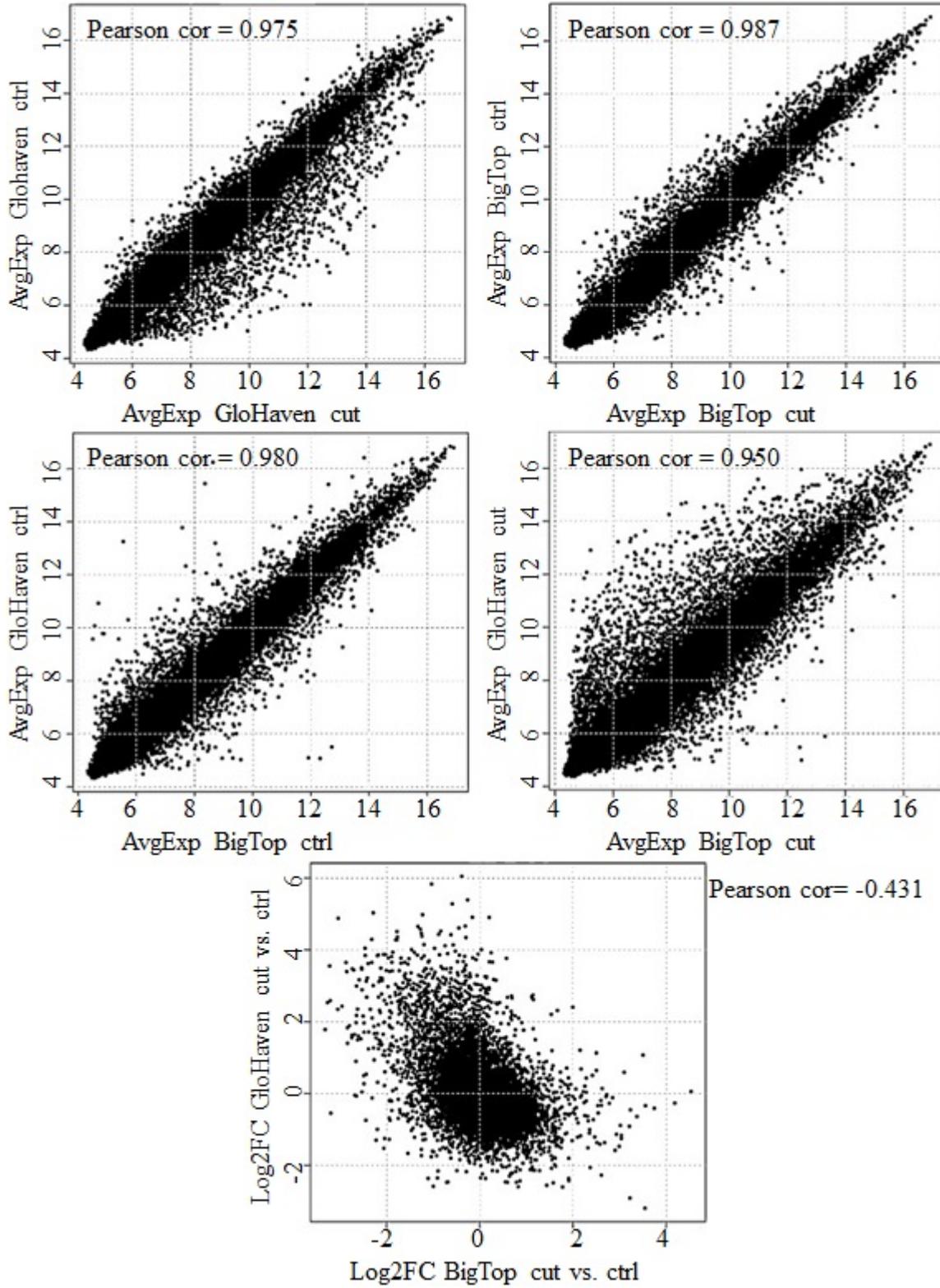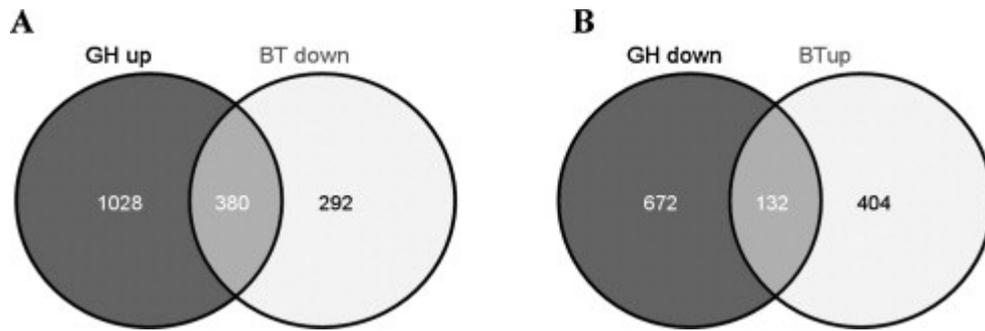
Fig. 4. – Venn diagrams reporting the total number of genes specific and in common when comparing GH up-regulated vs. BT down-regulated (A), and GH down-regulated vs. BT up-regulated (B) microarray genesets.

A comparison between the differentially expressed (w vs c samples) gene sets (Log2 fold change set at ±1) at T2 revealed that only 32 showed a similar expression pattern (i.e. either up- or down-regulated) in the two varieties (Table S5, supplementary material). Two of the up-regulated genes were involved in the regulation of transcription (ppa007919m and ppa011006m) and two in abiotic stress responses (ppa012860m and ppa009649m). The number of common genes increased when comparing the subset showing opposite expression patterns, and was 380 and 132 in GH up- vs BT down-regulated and GH down- vs BT up-regulated genes comparisons, respectively (Fig. 4 and Tab S6, supplementary material).

These data highlight the different behaviors of the two varieties in response to wounding. Of these 512 genes, a high number of transcription factors (TFs) such as *MYB* (ppa007222m, ppa026553m, ppa007823m), *AP2/ERF* (ppa022719m, ppa026499m, ppa012354m, ppa018178m, ppa020605m), *WRKY* (ppa007745m, ppa008566m, ppa007986m, ppa010796m, ppa025013m) and *bHLH* (ppa010972m, ppa017791m, ppa018357m) were present. Compared to the respective controls, members of these TF families were up-regulated at T2 in GHw but down-regulated in BTw. In contrast, members of *HB* (ppa009498m, ppa010647m, ppa011508m) and *AUX/IAA* (ppa011821m) TFs families were down-regulated at T2 in GHw, but up-regulated in BTw.

For ten of the twenty genes used to validate microarray (table S2) a time-course expression analysis on the whole set of samples was performed. Genes were selected among those showing an opposite expression pattern (GH up- vs BT down-regulated) such as the transcription factors (*WRKY* ppa025013m; *AP2/ERF* ppa026499m), a nucleotide binding leucine-reach repeat (*NB-LRR*, ppa000737m) and a squalene epoxidase/monoxygenase, (*SE*, ppa003995m), or a cv-specific differential expression, such as small heat shock protein (*HSP20*, ppa012538m), phenylalanine ammonia lyase (*PAL* ppa002878m), polygalacturonase (*PG* ppa006857m) and pectin methyl esterase, (*PME*, ppa003639m). In addition to these genes, the expression profile of aminocyclopropane carboxylate synthase (*ACS1*, ppa004774m) and aminocyclopropane carboxylate oxidase (*ACO1*, ppa008791m), the key genes of ethylene biosynthesis, was also determined. The microarray analysis showed that *ACS1* and *ACO1* were not differentially expressed at T2 (table S3), but taking into account the role of ethylene in the response to wounding they were included in the time-course expression analysis.

Considering these latter genes, differences in terms of *ACS* expression were detected between wounded and control tissues only in BT at T2 and T4, whereas *ACO* showed an increased expression in GHw in T1-T3 samples, and no significant differences were observed between BTw and BTc throughout the experiment (Figs. 5A and B). *PG* expression did not show any significant differences among the samples, the only exception being T4, when in both BTw and GHw an increased expression was detected (Fig. 5C). *PME* behaved differently and showed a marked up-regulation in GHw starting from T1, when a less pronounced increase in transcript accumulation was also detected in BTw (Fig. 5D).

As far as the genes involved in the secondary metabolism are concerned, *PAL* expression showed a temporary increase in BTw at T1, while in GHw the up-regulation was shifted at T2 (Fig. 5E). SE gene expression promptly increased in GHw at T1, and remained higher than GHc at T2 and T3. In Big Top, an up-regulation of this gene was observed in wounded tissue only starting from T3 (Fig. 5F).

The selected gene encoding *HSP20* showed quite a different expression trend in the two varieties: in fact, it was markedly induced in GHw throughout the experiment, while in BTw a slight transcript accumulation was observed at T3 only (Fig. 5G). An *NB-LRR*

gene displayed a slight up-regulation in GHw at T1; at T3 and, in particular, at T4 this gene was highly expressed in BTw (Fig 5H). Considering two TFs, one *AP2/ERF* gene was up-regulated in GHw at T1 and, in a more pronounced way, at T3 when BTc and BTw samples showed no differences (Fig. 5I). A different behavior between the two varieties was also observed for the selected *WRKY* gene, which showed a dramatic induction in GHw, starting at T1, and reaching the highest expression levels at T3. No difference in terms of *WRKY* gene expression was detected between BTc and BTw (Fig. 5J).

Fig. 4. – Expression analysis (qPCR) of 10 genes in mesocarp of wedges (w) and control fruit (c) of 'BigTop' (BT) and 'Glohaven' (GH) at the beginning of the trial (T0) and 8 (T1), 24 (T2), 48 (T3), and 72 (T4) h after wounding. (A) Aminocyclopropane carboxylate synthase, *ACS1* (ppa004774m); (B) aminocyclopropane carboxylate oxidase, *ACO1* (ppa008791m); (C) polygalacturonase, *PG* (ppa006857m); (D) pectin methyl esterase, *PME* (ppa003639m); (E) phenylalanine ammonia lyase, *PAL* (ppa002878m); (F) squalene epoxidase/monoxygenase, *SE* (ppa003995m); G small heat-shock protein, *HSP20* (ppa012538m); (H) nucleotide binding-leucine-reach repeat, N*B-LRR* (ppa000737m); (I) *AP2/ERF* (ppa026499m); (J) *WRKY* (ppa025013m). Bars indicate ± SD..
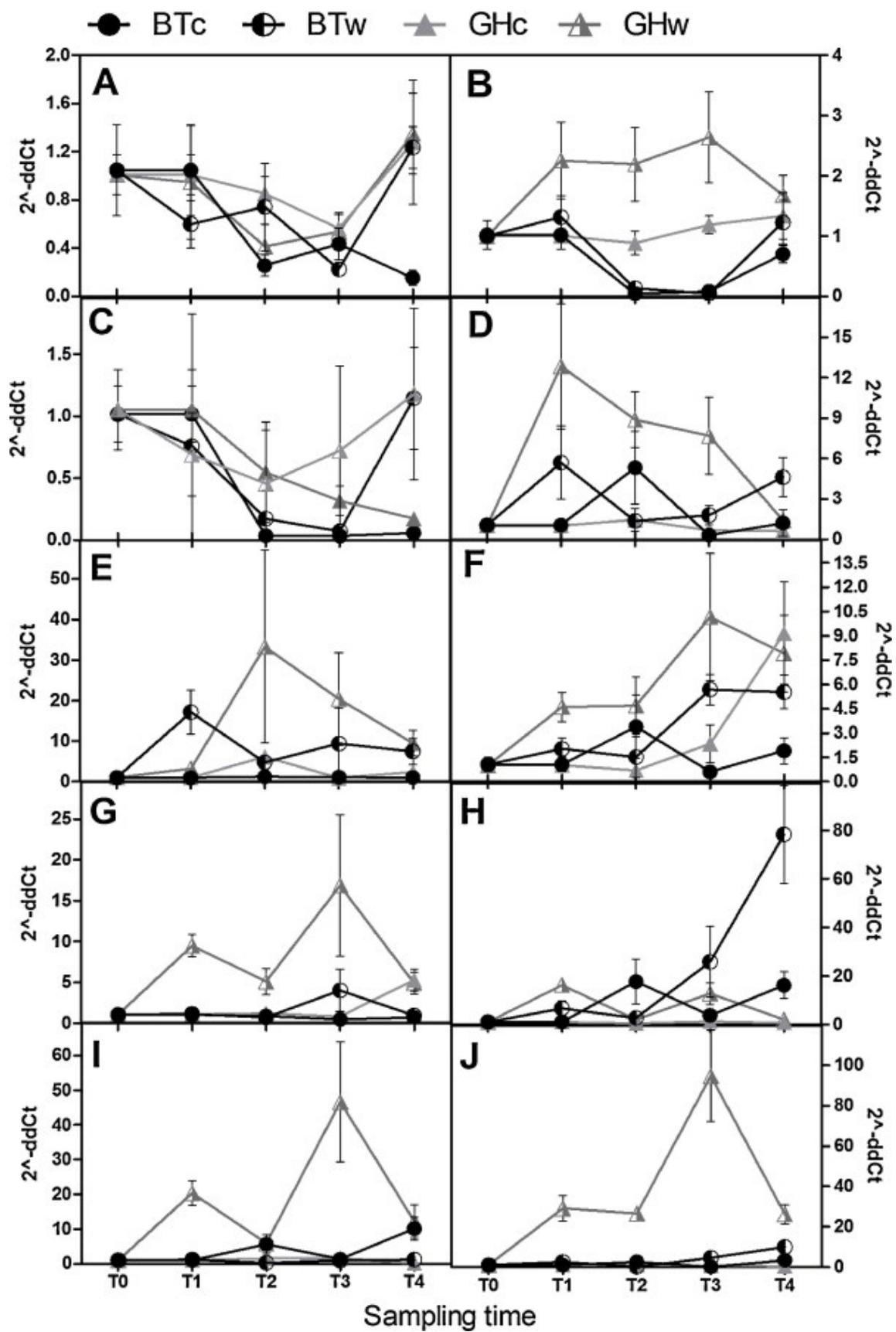
4. Discussion

Responses to wounding have been studied extensively in plants. Hydrogen peroxide responses, the up-regulation of phenylpropanoids, the induction of ethylene biosynthesis and of plant defenses are some of the processes observed in plant tissues subjected to mechanical wounding (Zhou and Thornburg, 1999). In model species and in vegetative tissues, wounding results in the activation of many different genes. The characterization of the genes and the timing of activation enable the various phases following a wound to be identified. Several of the wound-induced genes encode TFs, which are involved in the crosstalk between signaling cascades in the responses to different stresses and are mediators in multiple hormone signaling (Reymond et al., 2000).

Based on the enrichment analysis, responses to stress and secondary metabolic processes were some of the most significant biological processes over-represented in the melting variety Glohaven. Following exposure to stress, such as pathogen attack and wounding, the phenylpropanoid pathway plays an important role in the production of compounds including lignin, flavonoids and phytoalexins. The induction of lignin synthesis and gum deposition in the albedo and flavedo of harvested clementines (Mulas et al., 1996), and accumulation of callose, suberin, tannins and pectic substances, as well as gums and starch in mature pears (Spotts et al., 1998) have been associated with wound-healing responses in fruit. The activation of phenolic metabolic processes appears to be the main response to wounding also in the melting peach used in our trials which revealed an increase in total phenol and flavonoid content, an up-regulation of phenylpropanoid gene expression, and an enhancement in PPO and POD activities. When comparing the two peach varieties and their behavior after the fresh-cut preparation, BigTop maintained a better appearance at the end of the considered period (72h, T4).

Changes in the appearance of fresh-cut produce are due to many unrelated factors, including water loss from the cut surface, microbial colonization and browning (Toivonen and Brummell, 2008). Cut-edge browning reactions have been mainly imputed to the activity of PPO on polyphenols (Martinez and Whitaker, 1995). The better appearance of BT throughout the experimental period was associated with a decrease in total polyphenols and flavonoids (with no differences between the control and wounded samples), no increase in PPO activity and an almost undetectable POD

activity (Fig. 2). González-Buesa et al. (2011) reported that total phenol content decreased during storage in slices of four non-melting clingstone peach varieties, and that PPO activity and browning are correlated.

All these parameters were markedly enhanced in GHw, thus reinforcing the hypothesis that these aspects are linked to changes in the chromatic parameters, which are more pronounced in GH than in BT. *PAL* gene expression generally appeared more induced in GHw than in BTw (Fig. 5E). This might be correlated, as observed in wound pulp tissue of banana (Chen et al., 2009), with the different accumulation of phenolic compounds. In addition, five genes classified under MapMan Bin 16.2.1 (secondary metabolism.phenylpropanoids.lignin biosynthesis) were significantly ($p<0.01$) more expressed (log2 expression ratio >2) in GHw (Table S4, supplementary material). Three genes (ppa007615m, ppa021232m, ppa007627m) putatively encoding cinnamyl alcohol dehydrogenase (CAD) proteins, which are involved in lignin biosynthesis and play a critical role in plant defense against stresses, were highly induced in GHw but not in BTw. A similar behavior was observed for two *PAL* genes (ppa002384m and ppa002878m).

If the lack of (or a reduced) activation of the phenylpropanoid pathway can be considered negative in terms of repair/defense response to mechanical stress in different plant tissues (including immature and developing fruit), this appears not to be the case in the ripe fruit used for MP preparations. Based on our results and those of González-Buesa et al. (2011), it seems that a reduced activation in the phenylpropanoid metabolism represents a key factor in terms of maintaining visual quality and, hence, prolonging the shelf-life of MP peach produce.

Besides the phenylpropanoid pathway, one of the most important secondary metabolic pathways in plants is the isoprenoid biosynthetic pathway which, among others (hormones, carotenoids, etc.), is responsible for the production of sterols and triterpenoids. Triterpenes constitute one of the most important classes of natural products since they exhibit a wide range of biological activities (Vezzaro et al., 2012). In plants they are synthesized from the cyclization of 2,3-oxidosqualene, which is produced from squalene via squalene epoxidase (*SE*). Changes in triterpenoid content and in the modulation of the expression of genes involved in 2,3-oxidosqualene production (such

as *SE*) and cyclization (OSCs, such as β-amyrin synthase, producing one of the most commonly-occurring triterpenes) have been reported in various plant species in response to abiotic stress such as salinity (Basyuni et al. 2012) and drought (Posé et al., 2009). The rapid increase in *SE* gene expression in GHw would seem to indicate that triterpene metabolism is involved in responses to physical wounding in fruit tissue (mesocarp) where, unlike the peel, triterpenoids are not particularly abundant (Szakiel et al., 2012). The expression pattern of the *SE* gene in BTw suggests that this genotype is characterized by a reduced or a delayed activation of wound-related responses. This is also confirmed by the fact that at least three β-amyrin synthase genes (ppa001810m, ppa001817m, ppa001812m) showed, at T2, an opposite expression pattern in the microarray analysis: highly induced (log2 fold change, FC >2) in GHw, and down-regulated (log2 FC <-2) in BTw (Tab S4, supplementary material).

As far as we know, this is the first report showing the putative involvement of triterpenoid biosynthetic genes in response to wounding in plants, in general, and in fruit tissues, in particular. Assuming that the increased expression of *SE* and β-amyrin synthase leads to an increase in triterpenes in wounded peach mesocarp, an interesting aspect to evaluate would be the physiological roles and functions (signaling, antioxidant, antibacterial, antifungal?) played by these secondary compounds

The enrichment analysis highlighted that, in GH, the plasma membrane (GO:0005886) category was over-represented in both the up- and down-regulated gene sets, and the cell wall (GO:0005618) category was over-represented only in the up-regulated gene set. Besides membrane disruption and the effects of cell decompartimentalization, one effect of physical injury in mature fruit tissue is flesh softening (Brecht et al., 2004). In fact, throughout the experiment no significant changes in flesh firmness were detected between the two varieties. This is probably due to the limited sensitivity of the digital penetrometer in measuring changes in this parameter in peach wedges.

Pectolytic enzymes play a crucial role in the processes leading to the loss of flesh firmness in ripening peach fruit (Ramina et al., 2008). Indeed, a high number of genes classified under the MapMan BIN Code 10.6.3 (cell wall degradation.pectate lyase and polygalacturonase) as well as BIN 10.8.01 (cell wall.pectin esterases) were differentially expressed at p<0.01, and this number was higher in GH than in BT. *PME* genes in

particular appeared highly induced in GHw (at least three genes with log2 expression ratio >2). Unlike *PG*, *PME* appeared to be highly induced in GHw starting 8h after wounding, whereas a more limited induction was detected in BTw. (Fig. 5 D). Demethoxylated homogalacturonans can form supramolecular assemblies and/or gels and a substrate for pectin depolymerizing enzymes, associated with texture/viscosity loss (Sila et al. 2009). It is unclear whether these events related to an increase of esterase activity also occur in the mesocarp of peach wedges with an impact on the visual quality of the fresh-cut produce.

The induction of stress-related genes mainly occurs at the transcriptional level, and the modification of the temporal and spatial expression patterns of specific stress-related genes is an important part of the plant stress response. The TFs belonging to the AP2/ERF and WRKY families play a crucial role as mediators in multiple hormone signaling pathways, and in regulating gene expression related to biotic and abiotic stress, including wounding and tissue regeneration (Skibbe et al., 2008; Sena et al., 2009; Asahina et al., 2011, Chen et al., 2012a). Members of the AP2 superfamily may confer tolerance to various stresses by modulating the expression of defense genes. In different plant species, *ERF* genes are responsive to stresses such as salinity, freezing, low oxygen (Zhang et al., 2004; Licausi et al. 2010; Zhang and Huang, 2010), and, in Actinidia deliciosa fruit, a number of *ERFs* have been identified as differentially expressed in response to several postharvest abiotic stresses (Yin et al., 2012).

In our experiments, a high number of genes belonging to the *AP2/EREBP* superfamily were differentially expressed at T2 in both GH and BT. This high number is not surprising considering that a total of 131 *AP2/ERF* genes have been identified in peach and most of them (87.6%) are expressed in mesocarp (Zhang et al. 2012). A marked increase in a specific AP2/ERF mRNA accumulation was detected in wedges of the melting variety throughout the experimental period (Fig. 5K). In addition, based on the microarray analysis, nine *AP2/ERF* genes were up-regulated with a log2 FC >2 in GHw (Table S4, supplementary material). This strongly supports the involvement of members of this TF superfamily in the response to wounding in peach mesocarp of the melting variety.

Interestingly, an opposite behavior was observed in BT, where ten *AP2/ERF* genes appeared to be down-regulated (log2 FC <-2) in wounded mesocarp at T2. The

expression pattern of the *AP2* gene (ppa026499m) analyzed throughout the experiment seems to be only weakly related to the evolution of ethylene and the expression of *ACS* and *ACO* genes. Members of the AP2 superfamily are regulated by different factors and plant hormones, including jasmonic acid, as demonstrated by Asahina et al. (2011) who highlighted that in wounded inflorescence stems of *Arabidopsis*, the expression of *RAP2.6L*, which belongs to the AP2/ERF superfamily, was enhanced upon application of jasmonic acid (JA), and that a lipoxygenase gene (*AtLOX2*), involved in the biosynthesis of JA occurring after wounding (Wasternack and Hause, 2013), showed a superimposable expression profile with *RAP2.6L*.

We did not determine the levels of jasmonic acid in the different samples, and experiments with JA treatments were not performed. However our microarray data revealed that in the wounded tissue of the melting (GH) variety, and unlike BT, a gene similar to AtLOX4 (ppa001085), another member of the LOX family involved in wounding-stimulated JA biosynthesis (Wasternack and Hause, 2013), was significantly up-regulated (log2 FC >2). Changes in JA content in response to mechanical stress, and the possible relationship between JA (and its cross-talk with other hormones) and the expression of AP2/ERF members in response to wounding in fruit tissues would be interesting topics for future research.

JA might also be implicated in the regulatory system controlled by TFs belonging to the WRKY family. A number of *WRKY* genes are induced by wounding treatments. In fact, in Nicotiana attenuata *NaWRKY3* is strongly induced by wounding treatment and regulates expression of JA biosynthesis genes (*LOX, AOS, AOC* and *OPR3*) and JA conjugating genes, thereby increasing the levels of JA (Skibbe et al. 2008). This information along with the marked difference between GHw and BTw in terms of the expression pattern of *WRKY* (Fig. 5I) and *AP2* (Fig. 5J) genes, would suggest a more complex mechanism involving WRKY and AP2 TFs together with JA in the response to wounding in fruit tissue. Perturbations in this mechanism might induce different responses, which could be the case of BT. An additional element supporting the hypothesis of JA involvement in the different responses to wounding in peach mesocarp is the up regulation of JAZ5 (ppa011370), a gene encoding jasmonate ZIM-domain (JAZ) repressor proteins (Ruiz et al. 2013) in GHw but not in BTw (Table S4,

supplementary material). In *Arabidopsis JAZ* gene expression is induced through a complex negative feedback loop mechanism in the presence of JA (Niu et al., 2011).

Different responses to wounding between GH and BT are also represented by the expression pattern of genes encoding HSPs. In *Arabidopsis*, such genes play a wide role in many cellular processes, which may impart a generalized role in tolerance to multiple environmental stress conditions including wounding (Cheong et al., 2002). Indeed, a very high number of genes encoding HSPs (belonging to the stress abiotic.heat, and protein-folding BIN categories) were differentially expressed in both GH and BT but, again, a difference between the two varieties was observed at T2 sampling. In GH at least nine *HSP* genes were highly (log2 FC >2) up-regulated compared to the control, whereas in BT, using the same cut-off parameter (log2 FC >2/<-2), only four down regulated *HSP* genes were in the microarray dataset. The difference observed in the expression pattern of one small (15 to 42 KD according to Trent, 1996) *HSP* gene (*HSP20*) further supports the hypothesis that the two varieties have different physiological reactions to wounding. Small HSPs may have a role in maintaining membrane integrity under stress (Nakamoto and Vigh. 2007), and an increase in both mRNA and protein of small HSPs has been observed in the pulp of fresh-cut banana (Chen et al., 2009). The increase in the expression of *HSP20* detected in GHw but not in BTw throughout our experiments is additional evidence of the different reactions to imposed stress and physiological responses of the two peach accessions used in this trial. It is unclear whether this difference also involves other mechanisms, such as those related to disease resistance responses (e.g. NB-LRR). Considering specifically the expression pattern of the selected *NB-LRR* gene, it is interesting to note that a markedly higher than average SNP diversity is present in the genomes of different peach accessions at the top in the pseudomolecules 2 characterized by a five-fold higher density of genes encoding NB-LRR proteins (The International Peach Genome Initiative, 2013).

5. Conclusions

The mesocarp of ripe peaches responds to wounding by activating biochemical and molecular responses which are in part similar to those observed in other plants/plant tissues, and in part specific to this tissue. Some of the regulatory aspects and elements involved in the stress responses (e.g. AP2/ERF and WRKY TFs) appear to be in common, whereas specific secondary metabolic pathways (e.g. triterpenoids) may be specific to the fruit.

We identified a number of genes differentially expressed in sound and wound tissue, which should be considered as a starting point for future research on the role of different hormones (JA, but also ethylene, ABA and auxins) and their cross-talk in modulating the responses to wounding.

Our comparative approach using a melting variety (Glohaven) as reference and the "slow melting" nectarine BigTop, revealed that in the latter, the delayed ripening also somehow affects the response to wounding. In fact, BigTop seems to be "less reactive" (based on the total number of differentially expressed genes) to wounding and/or has a higher tolerance to the imposed abiotic stress leading to reduced responses (e.g. *PAL* gene expression and phenylpropanoid metabolism) and/or delayed activation of stress-related responses (e.g. *SE* gene expression, triterpenoid metabolism).

Revealing the mechanisms and metabolisms related to these different responses to mechanical stress in the fruit of the two peach varieties, may help to better identify other geno/phenotypes that are less susceptible to postharvest manipulation injuries and that are more suitable for specific preparations such as canning and minimal processing

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2013.12.001.

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