UNIVERSITY OF PADOVA

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PHD IN CROP SCIENCE

CYCLE XXVIII

A systems biology approach to shed light on apple fruit development

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Declaration

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January 31st, 2016 Francesca Populin

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Riassunto

Le ricerche illustrate nella presente tesi di dottorato si collocano nell'ambito del progetto "TranscrApple" (www.transcrapple.com), finanziato dalla Provincia Autonoma di Trento (PAT) nell'ambito del bando Grandi Progetti 2012. Gli obiettivi generali del progetto, che in larga parte si accomunano a quelli della presente tesi, prevedono di caratterizzare, nella maniera più ampia possibile con le tecnologie attualmente disponibili, gli eventi transcrizionali, compresi quelli relativi agli *small RNA* (non affrontati nella presente tesi), metabolici, su un subset di metaboliti primari e secondari, e ormonali, tramite un approccio di *hormone profiling*, che si verificano durante lo sviluppo della mela.

La presente tesi è organizzata in diversi capitoli, riflettendo la logica sperimentale e temporale effettivamente seguita per sviluppare le ricerche. Lo scopo principale del lavoro qui presentato è quello non solo di fornire una panoramica di informazioni su trascritti, ormoni e metaboliti e le loro variazioni durante lo sviluppo del frutto, ma anche di proporre delle soluzioni tecniche e sperimentali per poter collocare le informazioni acquisite in una piattaforma integrativa, secondo la logica della *systems biology*. Nelle specie modello, tutto ciò è fortemente facilitato dall'ampia disponibilità di *tool* bioinformatici pronti all'uso ma non sufficientemente flessibili per poter essere adattati ad altre specie. Tuttavia, soprattutto per quanto riguarda soprattutto le specie arboree da frutto, questo tipo di approccio è ancora lontano dell'essere definito e standardizzato.

Il Capitolo 1 introduce l'argomento "sviluppo della mela" in relazione all'adozione del melo come specie arborea modello, in misura sempre più crescente soprattutto nell'ultimo decennio grazie anche alla disponibilità della sequenza del genoma. Dopo un *excursus* sulle principali e più recenti acquisizioni relative allo sviluppo del frutto del melo, vengono discusse alcune delle principali criticità e lacune, sia dal punto di vista tecnologico che scientifico, che impediscono una visione completa degli eventi regolativi che coordinano lo sviluppo e la crescita della mela, anche in relazioni ai principali parametri qualitativi e produttivi.

Nel Capitolo 2 si inizia ad entrare nel merito delle ricerche, illustrando la fase preparativa di ricerca e validazione multipla (tra cultivar diverse e annate diverse) di marcatori trascrizionali delle fasi di sviluppo del frutto indirizzate alla corretta selezione di campioni rappresentativi in serie temporali raccolte in annate differenti. Sono stati identificati, validati ed utilizzati

diversi marcatori fra quelli proposti in letteratura, consentendo la selezione dei campioni di cv Golden Delicious (qui usata come modello) da utilizzare nelle successive fasi di caratterizzazione trascrizionale e metabolomica condotte nelle tesi.

Nel Capitolo 3 si affronta il primo importante studio dei profili ormonali durante lo sviluppo del frutto. I risultati acquisiti hanno consentito non solo di acquisire dati relativi alla maggior parte degli ormoni da poter utilizzare in ricerche future, ma anche di chiarire, confermare e/o ipotizzare delle interazioni ormonali in funzione dello stadio di sviluppo o della transizione tra stadi diversi. La peculiarità di questo studio consiste nell'aver ottenuto, per la prima volta nel melo, dati quantitativi di un set importante di ormoni a partire dagli stessi campioni.

Il Capitolo 4 affronta invece la questione relativa ai metaboliti e alle loro variazioni nel corso dell'intero sviluppo del frutto. Viene in questo modo fornita una visione complessiva di come variano le diverse classi di metaboliti (principalmente zuccheri, acidi organici, amminoacidi e polifenoli) durante lo sviluppo. Anche in questo caso i dati sono stati acquisiti dagli stessi campioni utilizzati negli altri capitoli per le altre tipologie di analisi e potranno essere impiegati in ricerche successive, ad esempio, in una logica integrativa, insieme a dati trascrittomici di diversa natura, epigenetici, ecc.

Nel Capitolo 5, finalmente, la tesi si addentra fra mille difficoltà tecniche, poi in parte superate, nella giungla della cosiddetta *systems biology*, fornendo un esempio di come i dati ormonali possono essere valorizzati attraverso la loro integrazione con i dati trascrittomici ottenuti tramite RNAseq a partire dagli stessi campioni.

Summary

The research carried out and discussed in the present dissertation is positioned within the "TranscrApple" project (www.transcrapple.com), funded by the Provincia Autonoma di Trento (PAT) within the call "Grandi Progetti 2012". The general objectives of this project, a significant part of which overlap with the present thesis, deal with the characterization, as wide as possible with the currently available technologies, of the transcriptional events, including those related to small RNAs (not discussed in the present dissertation), the metabolic changes, on a subset of primary and secondary metabolites, and hormones' cross-talk, through a hormone profiling approach, occurring during apple fruit development.

The present thesis is organized in different chapters, mirroring the experimental and temporal rationale effectively pursued to develop the research herein described. The main objective of the present work deals not only with providing an overview of transcripts, metabolites and hormones and their variations during fruit development, but also with the setting up of technical and experimental solutions aimed at using the achieved information within an integrative platform, according to a "systems biology view". In model species, all this kind of studies are extremely easier, thanks to the availability of ready-to-use bioinformatics tools that are not flexible enough to be used in other species. However, especially in tree crops, this approach is still far from being defined and standardized.

Chapter 1 introduces the theme "apple fruit development", discussing the adoption of apple as a model system that, in the last decade, acquired great importance in terms of research among the fruit species thanks to the availability of its sequenced genome. After a brief introduction on the fundamental information available about the apple fruit growth, taking into account the technological and scientific points of view, few difficulties and gaps that hamper the achievement of a complete overview of the regulatory events coordinating the development and growth of the apple, are discussed in relation to the main quantitative and qualitative parameters characterizing the apple fruit production.

Chapter 2 enters into the apple fruit research area; the preliminary phase and the multiple validations (concerning different cultivars and seasons) of transcriptional markers during the main apple developmental stages were shown to be fundamental for choosing, on the base of the expression profiles of these genes, the most representative samples, among those collected also in different seasons. Several markers have been identified, validated and

employed, among those available from literature, allowing the selection of samples of cv Golden Delicious (herein considered as model) to be used for the subsequent transcriptional and metabolomic characterization carried out in the present research.

Chapter 3 deals with the hormonal profiling survey carried out along the apple fruit development for the first time in this species. The results have allowed not only the achievement of brand new data related to the major hormonal classes, to be employed for further researches, but also the clarification and/or confirmation of new hormonal interactions connected to the fruit development stage or the transition between stages. Moreover, the relevance of this study consists in having achieved, for the first time in apple, quantitative data of an important set of hormones concurrently on the same samples.

Chapter 4 concerns the survey on the metabolites and their variations during the apple fruit development. A complete overview of the changes of the different classes of metabolites (mainly sugars, organic acids, aminoacids and polyphenols) is given during the apple developmental cycle. The acquired data have been derived from the same samples already analyzed in the previous chapters, and will be integrated with data of diverse nature, such as the RNAseq.

Chapter 5 of the present thesis comes into the "system biology" area, initially among several technical difficulties, then partially solved, and gives an example of an alternative interpretation of the hormonal data put within a correlative network along with the RNAseq results achieved on the same samples.

Chapter 1 - Introduction

Apple (*Malus domestica* L. Borkh) as an emerging model for fruit development

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Abstract

Apple research has undergone great improvements in the last years, in both quantitative and qualitative terms. A huge amount of data are now available, especially as far as the early development and the ripening phase are concerned. Moreover, the recent release of the apple genome sequence is significantly speeding up research, allowing on one hand to shed light on the most critical aspects of fruit development with almost immediate practical implications and, on the other hand, to identify new molecular markers that will improve the future breeding programs. In this context, apple is being increasingly considered as a model for fruit development studies, although many gaps still exist in apple research. These gaps are being filled by coupling the next generation high-throughput technologies with new physiological approaches, aimed at achieving both new basic knowledge and innovative tools to improve the final quality of the fruit. In this review, the available information on the regulatory aspects of apple fruit development will be reported and discussed in the light of the future perspectives of apple research.

Keywords

Early development, fruitlet abscission, maturation, ripening

Introduction

Apple (*Malus* x *domestica* L. Borkh), with its wide diversity of climatic adaptation, has become the most widely planted tree fruit of the temperate zone and one of the most widely cultivated in the world, covering about 5 millions hectares and with a production of more than 75 millions tonnes (FAOSTAT, 2011). Over the last decades, the researchers' attention on this species has grown exponentially, as shown by the increasing number of scientific publications dealing not only with different aspects of apple cultivation, post-harvest management, and fruit consumption, but also with its basic biology (source: PUBMED; **Figure 1**). This growing interest has led to significant achievements not only in practical and applied terms, thus greatly improving the quality of the final product, but also from the point of view of the basic research, taking apple as a model species for studying pome fruit development and many other related processes.

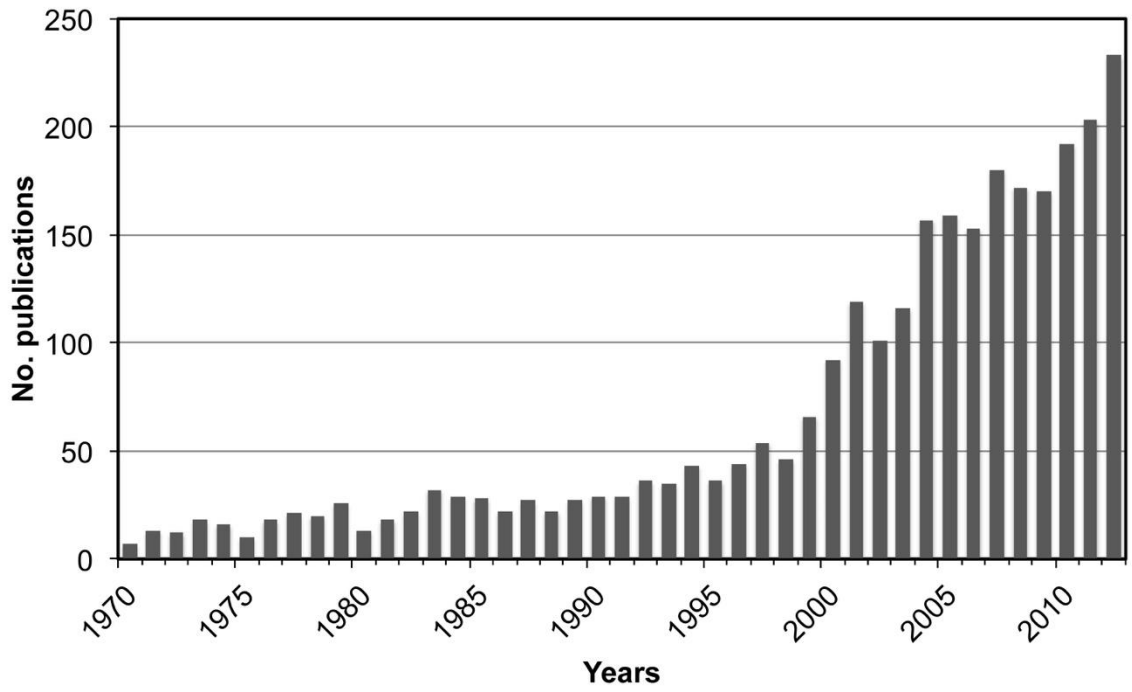

Figure 1 - Number of scientific articles, published in years from 1970 to 2012, dealing with different aspects of apple cultivation, biology, postharvest, etc. (Source: Pubmed).

A key event in apple research was unquestionably the recent release of its genome sequence (Velasco et al. 2010), which represents a fundamental tool for the scientific community to finally improve the quality of the research carried out on this species, allowing to adopt experimental and analytical approaches (i.e. RNA-Seq technologies) previously unfeasible.

Although this powerful tool was not available to the public before 2010, massive genomic approaches such as EST sequencing and microarrays, and targeted molecular techniques (i.e. real-time PCR), along with classical biochemical methodologies, allowed to shed light, at least in part, on the physiology of some basic processes dealing with apple fruit development, ripening and global quality, thus further strengthening a number of research lines that are currently being pursued by several researchers around the world.

By focusing just on the fruit developmental cycle and thus ruling out pre-fertilization and 'late' post-harvest aspects (*i.e.* long-term storage), apple research may be grouped into two main lines: i) early fruit development, and ii) late fruit development. The former would include all the topics dealing with fruit set, the cell division growth phase, the so-called 'correlative inhibitions' (*i.e.* source-sink relationships), and fruitlet abscission, while the latter would mainly deal with fruit maturation and ripening, and all the aspects related to global fruit quality and consumption (*i.e.* aromas, nutritional properties, etc.). It becomes immediately evident that the intermediate developmental stages, i.e. those spanning between the end of the cell division phase and fruit maturation, have not been well investigated, apart from few exceptions (Janssen et al. 2008), taking advantage of the most advanced genomic tool. This would denote a relevant gap in apple research that must be filled with future project initiatives.

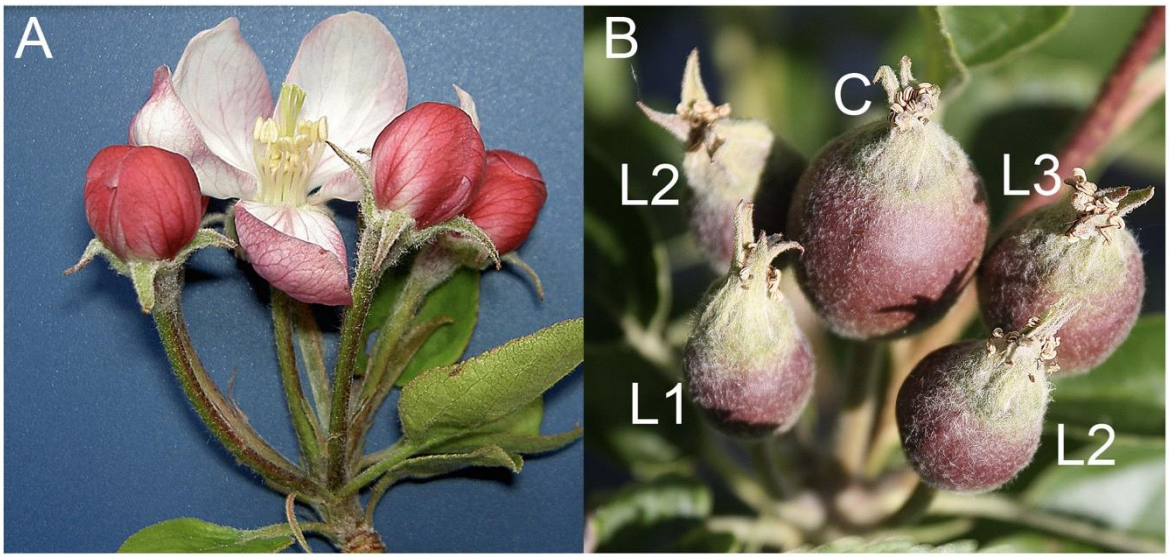

Figure 2 - A. The apple corymb (cv Golden Delicious) with the central flower at bloom, whose anticipation with respect to the lateral flowers is clearly visible. B. Apple cluster with a clear hierarchy, as indicated by letters and progressive numbers (C = central fruitlet; L1 = small lateral fruitlets; L2 = medium lateral fruitlets; L3 = big lateral fruitlets). Adapted from Botton et al. (2011).

The adoption of apple as a model is mainly due to some specific morpho-physiological features (more details are given in the next sections). In general, apple represents an interesting model system to study the molecular mechanisms regulating early fruit development and fruit developmental plasticity in response to endogenous and environmental signals, as it develops fruit clusters, the corymbs, with a clear gradient of correlative dominance related to the position and size of the fruit (**Figure 2**: Botton et al. 2011, Eccher et al. 2013). Besides displaying a wide phenotypic variability, this feature can be also affected by several chemical treatments (i.e. the fruit thinners), thus generating a huge number of case studies, that are very useful for physiological and molecular assessments focused, for example, on fruit set, fruitlet abscission, and source-sink interactions. On the other hand, apple is an interesting model species also for studies focused on fruit ripening and postharvest physiology, the latter not being discussed in detail in the present review. The fact that apple is a climacteric fruit (i.e. it shows a peak of ethylene biosynthesis concurrent with a respiration burst during the last developmental stages) along with the availability, of physical and chemical tools, such as temperature, controlled atmosphere, and ethylene action inhibitors, able to modify the progression of the ripening syndrome, are the key requirements for apple to be considered a model.

In the present review, a brief description of the most recent results related to apple fruit development and ripening will be given, along with a discussion of the most critical aspects and future perspectives.

Taxonomy and botany

Cultivated apple is a deciduous, rarely evergreen tree or shrub, belonging to the Maleae tribe (synonym Pyrae) of the Maloideae subfamily, within the Rosaceae family. Differently from other Rosaceae species, it has a distinctive basic haploid chromosome number of $x = 17$, probably due to allopolyploidization between species belonging to Spiraeoideae ($x = 9$) and Amygdaleoideae ($x = 8$), although a within-lineage polyploidization event has also been hypothesized (Evans & Campbell 2002). Polyploid apple varieties of 3*x*, 4*x*, or 5*x* are also cultivated, most of which are apomictic.

The flowering habits of domestic apple is determined by two kinds of buds, vegetative and mixed. Apple inflorescence is determinate, in general with 5 flowers at the base of which are several lateral vegetative buds. Flower buds are borne on shoots or short spurs at terminal

position, with some exception in 1-year shoots which may carry flowers derived from lateral buds. Flower initiation usually occurs during early summer and may be inhibited by nearby developing fruitlets resulting in biennial bearing.

The apple flower is epigynous and hermaphroditic. Every flower has a syncarpous gynoecium, with the ovary surrounded by non-ovarian tissue that will develop to form a pseudocarpic fruit (also called 'false' or 'pome' fruit; Rohrer et al. 1991), whose structure includes an epidermis and a cortex, the latter representing the flesh of the pome. Flowers have a variable number of stamens (between 9-20) distributed in 3 whorls and two outer whorls of 5 petals and 5 sepals. The variation of the number of stamens in different cultivars is related to polyploidy, being tetraploid flowers the ones with the lowest number of stamens. Each stamen is composed of a filament and an anther with two pollen sacs. The female part is formed by a single ovary divided in 5 fused carpels or locules, each of them bearing commonly two ovules (that can be up to four) (Pratt 1988). Nevertheless, lateral flowers compared to the central ones are more variable in the number of differentiated ovules. Every ovary ends in 5 styles united by the base ending in a wet-type stigma (Cresti 1980).

Germination of pollen and growth of the pollen tube through the transmitting tissue of the gynoecium and towards ovules are the main steps leading to fecundation. The growth of the pollen tube is controlled by compatibility reactions and temperature. It has been described that pollen tube can reach the ovary within two days (Haasbrook et al. 1967, Stott 1972, Pratt 1988). After anthesis the ovules inside a flower are receptive for pollination for up to 11-12 days, but it has been observed that after 6 days of anthesis, pollination does not lead to fruit development (Child 1966). Polyploidy also affects the maturation of embryo sacs in ovules leading to a different length of the receptive period after anthesis. In diploid cultivars the embryo sacs are mature at flower opening, whereas in triploid cultivars it has been observed that maturity is reached 2-3 days after anthesis and ovaries can be fecundated for a longer time. This could be a mechanism of compensation for the higher sterility of triploid versus diploid cultivars. The pollination period is also affected by biennial bearing, being longer during fruit productive years.

After pollination the seeds start to develop inside the ovaries. A few hours after fertilization the endosperms start a series of synchronized mitotic divisions taking place from the central part of the sac towards the margins. There is a resting period after these divisions when some

nuclear fusions occur and finally cell walls enclosing these nuclei are synthesized, starting at the micropylar end towards the chalaza. The endosperm will be absorbed by the developing embryo as it grows, being just a surrounding layer when the embryo reaches maturity.

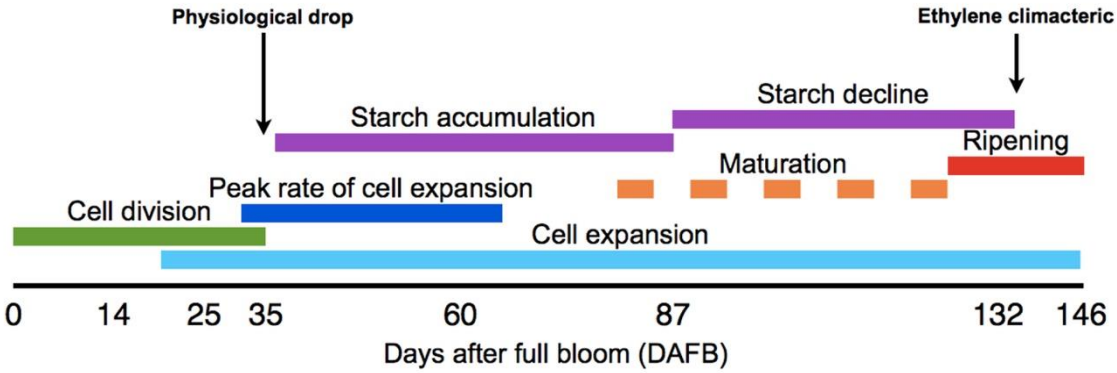

Figure 3 - Diagram of fruit development showing the timing of major physiological events in cv Royal Gala, adapted and modified from Janssen et al. (2008). Maturation is shown as dashed orange, as the exact timing of this phase has not been clarified yet.

Early fruit development

From a physiological point of view, apple fruit development may be divided into four partially overlapping stages: cell division, cell expansion, maturation and ripening (Dong et al. 1997, Janssen et al. 2008) (**Figure 3**). The relative progression of each developmental stage is under the control of a complex network of both endogenous and exogenous factors. Among the former, hormones play a pivotal role, although their specific function and intensive cross-talk still need to be investigated, especially at the seed level, where they are mainly synthesized. Seed and seed development are thus fundamental in the regulation of apple fruit growth (Gillaspy et al. 1993), because they ensure a satisfactory hormone production and regulate the flow of assimilates and nutrients (i.e. fruit's sink strength; Crane 1969, Treharne et al. 1985, Brenner & Cheikh 1995). They affect fruit size and shape, which definitely depend on seed number and distribution within the core tissue (Brittain 1933, Brittain & Eidt 1933, Free 1993, Brault & de Oliveira 1995, Keulemans et al. 1996). The development of a viable seed has been in part characterized at transcriptional level in apple by following the expression of MADS-box genes (Sung & An 1997, Yao et al. 1999, Sung et al. 2000), that are the key players of the endogenous control of apple fruit development. For example, a recessive mutation of a MADS-box transcription factor, *MdPI* (Yao et al.

2001), stimulates parthenocarpy, proving that at least the onset of fruit development is even under a strict genetic regulation.

During the early developmental stages, fruit, nucellus and endosperm grow rapidly mainly due to cell divisions, whereas embryos have a much slower development. This phase, whose length is substantially stable among the different varieties, starts just after fertilization and ends at about 25-30 days after petal fall (DAPF). Thereafter the endosperm becomes cellularized and the embryo speeds its development to reach maturity. During this phase, whose length differs among the cultivars (Luckwill 1948), the fruit enlarges exclusively due to cell expansion.

To deepen the knowledge on the early phases of fruit development, the expression of genes involved in key processes, such as photosynthesis, protein synthesis, cell cycle, energy, metabolism and defense, has been investigated by means of the microarray technology (Lee et al. 2007, Janssen et al. 2008, Soglio et al. 2009, Soria-Guerra et al. 2011). During cell division, the control on cell cycle and energy supply is essential and a perturbation affecting both may have great negative impacts on fruit quality or even trigger fruitlet abscission. The control of cell cycle during the cell division phase is fundamental in affecting the final fruit size. Moreover, a close relationship between cell number and size has been widely observed (Smith 1950, Bain & Robertson 1951, Skene 1966, Westwood et al. 1967). Among apple varieties, a significant difference in fruit size has been observed depending on the length of both the cell division and expansion stages (Harada et al. 2005) and on events perturbing the natural cell cycle progression (Malladi & Hirst 2010). Several cell cycle genes have been shown to be over-expressed during the early developmental stages (Janssen et al. 2008, Malladi & Jonhson 2011), along with *MdANT1* and *MdANT2*, homologues of the *AINTEGUMENTA* gene coding for an APETALA-2 transcription factor (Dash & Malladi 2012). A down-regulation of all the above genes and an up-regulation of *MdKRP4* and *MdKRP5*, negative regulators of cell cycle, have been detected during the transition from cell division to cell expansion (Malladi & Jonhson 2011). Additionally, a suppression of *SEPALLATA1/2*-like genes, *MADS8* and *MADS9*, involved in cell division and expansion of the hypanthium tissues, was shown to cause a great reduction of apple fruit size (Ireland et al. 2013). The latter is known to be affected also by auxin, whose exogenous applications at relatively low concentrations positively affect cell expansion concurrently with the up-

regulation of the *ARF106* gene (Devoghalaere et al. 2012), mapped within QTL for fruit weight on linkage group 15 (Liebhard et al. 2003). The natural levels of auxin within the fruit cortex increase during the cell division phase, peak at early cell expansion, and then decrease thereafter throughout the late cell expansion stage, maturation and ripening (Devoghalaere et al. 2012). As far as the nutritional state is concerned, sugars are needed to supply the required energy to the developing fruitlets, beside all other growing organs, and are known to function as signaling molecules. Sorbitol and sucrose are the main sugars translocated within the phloem saps and are unloaded in the fruitlet tissues via apoplast (Zhang et al. 2004). During early fruit development, their metabolism is highly regulated at transcriptional level in both cortex and seed tissue (Nosarzewski & Arcbold 2007, Li et al. 2012). The sink strength of the fruit in attracting nutrients is tightly related to internal factors such as hormones and seed developmental status (Bangerth 2000, Avanzi et al. 1988, Forino et al. 2000). Smaller fruitlets, characterized by minor auxin content and lower number of viable seeds, and thus considered weaker sinks, are less competitive compared to larger fruits and other developing organs. The tree, unable to meet the nutritional requirement of all the developing organs, economizes the resources by regulating its crop load throughout a "physiological drop", during which weaker fruitlets are released (Bangerth 2000). This process, also known as abscission of young fruitlets, is triggered during the cell division stage, but occurs later on after about two weeks (**Figure 3)**, thus indirectly ensuring a return of flowering in the following year (Dennis et al. 1999, Foster et al. 2003). In most cultivars, however, this is not sufficient to avoid biennial bearing habits and ensure a proper fruit quality in relation to crop load, thus requiring the application of practices such as "fruit thinning", able to improve the final size and quality of the remaining fruits. Trees can be thinned manually, with high impacts in terms of cost and low efficiency, mechanically, requiring suitable tree forms and specific pruning, or chemically, although often with relevant environmental impacts. Among all the chemical thinners, benzyladenine (BA), a cytokinin-like compound, is widely employed in ordinary orchard practice. Its effect relies on exacerbating the existing nutritional competitions between shoot and fruitlets (Bubàn 2000, Quinlan & Preston 1971), by stimulating cell division at the level of the vegetative organs, thus requiring an additional support in terms of assimilates. Apple represents an extraordinary model for studying abscission because it is characterized by correlative inhibitions among fruitlets belonging

either to the same or different corymbs, and between fruits and shoots (Bangerth 2000). Preliminary studies on BA effect have been carried out in cv Golden delicious (Dal Cin et al. 2007, 2009a, 2009b) in order to isolate key abscission-related genes. The role of ethylene as abscission marker in abscising fruitlets has been observed (Angeli et al. 2002, Dal Cin et al. 2005) along with its ability in affecting MADS gene expression (Dal Cin & Ramina 2011). Nevertheless, a recent massive transcriptomic and metabolic dissection of abscission induction has been carried out in cortex and seed tissues of abscising versus non-abscising fruitlets (Botton et al. 2011) allowing to shed light not only on BA-induced abscission, but also on the molecular/metabolic pathways likely involved in the regulation of early apple fruit development, including the natural path to abscission (**Figure 4**).

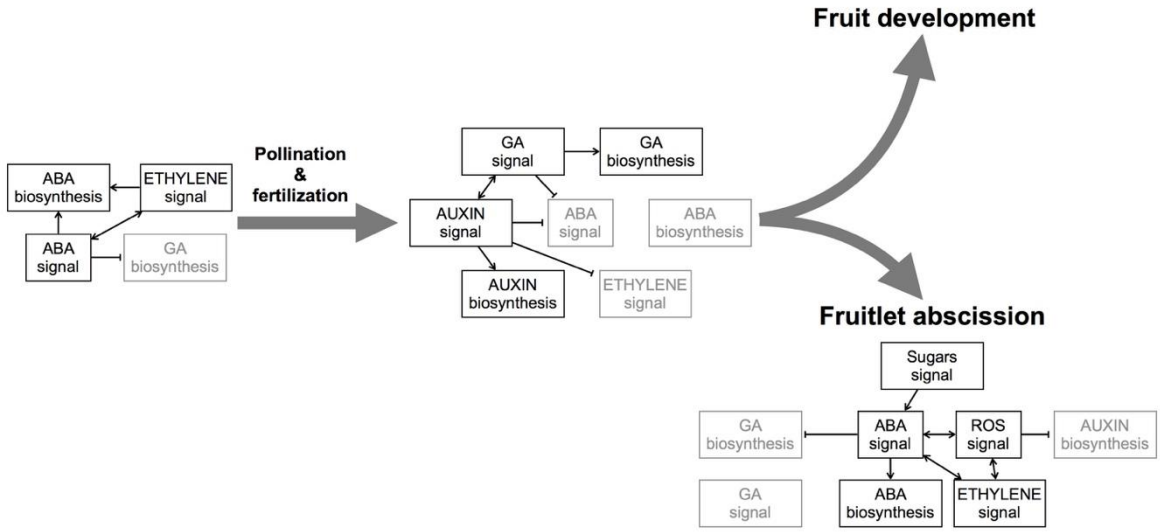

Figure 4 - Schematic representation of the most important signalling pathways involved in the determinism of fruit's destiny during early developmental phases. Grey-shaded boxes represent down-regulated/inactive pathways. Conventional arrowheads are used to show positive and negative interactions.

A working model was prompted and further developed by Eccher et al. (2013), according to which sugars are involved as primary signaling molecules in establishing the nutritional shortage in cortex. Sucrose and trehalose seem to play a relevant role in this context, as proven also by the up-regulation of the related genes. Consequently to the persisting action of sugar signaling, abscisic acid and ethylene, whose biosynthesis are stimulated along with the expression of key genes such as *MdACO1* and *MdACS5B* for ethylene and *MdNCED1* for abscisic acid, respectively, in concert with other secondary non-hormonal signaling, embodied by isoprene (Eccher et al. 2013) and ROS, transmit the abscission signal to the

seed. Here, a block of embryogenesis, as indicated by the down-regulation of *MdMADS10* expression, followed by a reduction of auxin biosynthesis occur. The polar auxin transport within abscission zone decreases and abscission starts (Roberts et al. 2000, Patterson 2001, Bonghi et al. 2000). Within this context a shift of ethylene biosynthesis, similar to that occurring from system 1 to 2 during ripening, was also hypothesized (Botton et al. 2011). Fruitlet abscission studies allowed also to point out what happens during development of persistent fruits, which largely overlaps the hormonal mechanisms found in tomato and the regulation of the related genes (Gillaspy et al. 1993, Dong et al. 1998, Giovannoni 2004, Wang et al. 2005, Goetz et al. 2006, Vriezen et al. 2008, de Jong et al. 2009). Briefly, hormone-related transcriptomic signatures assessed in the cortex of abscising fruitlets resemble those claimed to be responsible for the negative feedback regulation occurring before pollination and fertilization, and preventing fruit set in tomato (Vriezen et al. 2008). In fact, ABA and ethylene signalling are strongly up-regulated concurrently with a down-regulation of gibberellin signalling specifically in fruits induced to abscise. On the contrary, a down-regulation of the former signalling pathways counteracted by an up-regulation of the latter were observed in persisting fruitlets, indicating that the interplay among these three hormones is essential for early fruit development to proceed.

After fruitlet drop, the remaining fruitlets continue to develop and thus enter the cell expansion stage. This phase is characterized by a strong water uptake, as testified by the up-regulation of the plasma membrane intrinsic protein gene *MdPIP1* (Hu et al. 2003), coupled with transport of sugars and others solutes into the vacuoles (Coombe 1976), causing the increase in fruit volume, and by an accumulation of starch up to the maturation stage (Janssen et al. 2008, Li et al. 2012).

Late fruit development and ripening

Fruit maturation and ripening are the last important steps of fruit development during which apple reaches its full size, acquires the competence to ripen even without the need of the mother plant, and finally ripens. Despite its importance, maturation is still poorly characterized whereas ripening has been widely dissected at all “omics” levels focusing on the numerous physiological, biochemical and structural alterations occurring in both cortex and skin, whose regulation is under the control of both external, such as light and

temperature, and internal signals, such as hormones and genetic regulators (Costa et al. 2010a, Gapper et al. 2013).

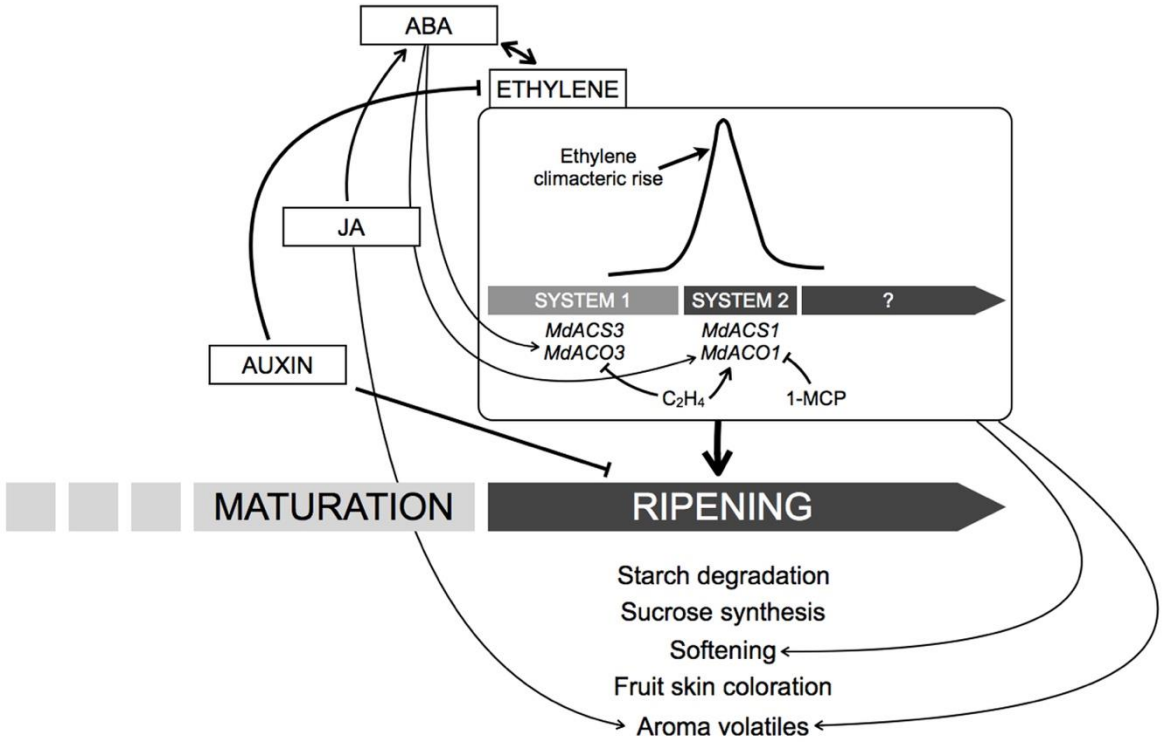

Figure 5 - The main hormonal pathways and interactions controlling the progression of the ripening syndrome in apple (C_2H_4 = ethylene; 1-MCP = 1-methylcyclopropene).

As far as hormones are concerned (**Figure 5**), ethylene is extremely important in controlling many processes of the ripening syndrome (Seymour et al. 2013, Gapper et al. 2013) and its increasing biosynthesis coupled to a rapid enhancement of respiration rate, called "climacteric rise" (**Figure 3**), characterizes the onset of ripening in apple, as well as in other climacteric fruit. For these reasons numerous studies were focused on the regulation of ethylene biosynthesis, perception and transduction pathways during ripening. Ethylene biosynthesis is under the control of two systems: system 1, active in all vegetative tissues including non-ripening fruits, accounts for the low and self-inhibited hormone production, whereas system 2 for the high and self-stimulated ethylene biosynthesis (Gapper et al. 2013, Tan et al. 2013). Ethylene is synthesized from S-adenosyl-L-methionine (SAM) by two key enzymes, ACC synthase (ACS) and ACC oxidase (ACO), both encoded by multi-gene families with complex expression patterns. During apple fruit ripening only expression of *MdACS1* and *MdACS3* (Wakasa et al. 2006, Wiersma et al. 2007), *MdACO1* and *MdACO3*

(Yang et al. 2013) is detected. MdACS3 and MdACO3 catalyze ethylene biosynthesis in system 1 while MdACS1 and MdACO1 in system 2 (Wang et al. 2009, Yang et al. 2013, Tan et al. 2013). Wang et al. (2009) showed even that expression levels of *MdACS3* and *MdACO3* decrease while *MdACS1* and *MdACO1* increase during ripening, thus proving the existence of a feedback at transcriptional level during the shift between the two systems. Additionally Yang et al. (2013) demonstrated that an exogenous ethylene treatment is able to repress the expression of *MdACS3* and *MdACO3*, whereas 1-Methylcyclopropene (1-MCP, an inhibitor of ethylene perception) applications do not affect their transcription. Nevertheless, other results revealed an up regulation of *MdACS3* upon 1-MCP treatments (Costa et al. 2010a, Varanasi et al. 2011). On the contrary, *MdACS1* and *MdACO1* are up-regulated by ethylene, down-regulated by 1-MCP (Yang et al. 2013) and positively regulated also by n-propyldihydrojasmonate (PDJ), a synthetic jasmonate-like compound (Kondo et al. 2009). As far as ethylene perception and transduction pathways are concerned, five ethylene receptors (*MdETR1*, *MdETR2*, *MdETR5*, *MdERS1* and *MdERS2*) (Yang et al. 2013), *MdCTR1* (Dal Cin et al. 2008, Wiersma et al. 2007, Yang et al. 2013), one EIN3-like gene, *MdEIL4* (Yokotani et al. 2003, Huang et al. 2010, Yang et al. 2013), and two ethylene responsive factors, *MdERF1* and *MdERF2* (Yang et al. 2013), are expressed during the ripening stage. Ethylene and 1-MCP affect positively and negatively, respectively, the transcriptional levels of all the above genes, except for *ERF2* whose expression is unaffected by the chemical (Yang et al. 2013). Taken as a whole, these data indicate that the ethylene network is fundamental for ripening progression. Part of this network is constitutively active during ripening, whereas another set of genes is regulated coordinately upon the shift from system 1 to 2, concurrently with the climacteric rise.

The regulatory role of ethylene is very important in determining the advancement of many ripening-related processes, thus affecting the final quality of the apple. Johnston et al. (2009) showed that initial changes in acidity and starch degradation, typically occurring during the transition between maturation and ripening, are less dependent on ethylene but highly sensitive to this hormone, whereas late ripening events, such as softening and volatile production, are strictly regulated by ethylene and almost insensitive to low concentrations of the hormone.

Apple sugar content depends at a large extent on starch degradation and sucrose synthesis, whose activity may rely upon genotype-specific traits. Moreover, internal ethylene concentration (IEC) was shown to be uncorrelated with the ability of 1-MCP application of delaying starch degradation, thus proving that ethylene may have only a partial control of starch content (Thammawong & Arakawa 2007). It was also demonstrated that starch degradation and sucrose synthesis are both developmentally regulated at transcriptional level (Brookfield et al. 1997, Janssen et al. 2008, Li et al. 2012). Genes encoding for starch degrading enzymes, such as α- and β-amylases (Janssen et al. 2008), and sucrose-phosphate synthases (Li et al. 2012) were show to be up-regulated during late maturation/early ripening, concurrently with genes for sucrose transporters and fructose tonoplast monosaccharide transporters (Li et al. 2012), accounting accumulation of these hexoses in the vacuole. In addition, Ireland et al. (2013) reported that two *SEPALLATA1/2*-like genes could be involved in the control of starch degradation as developmental regulators acting upstream of the ethylene pathway. In fact, in the antisense-suppressed MADS8as-9 apple line, the ripening syndrome is blocked such as in ACO1as lines in which ethylene biosynthesis is inhibited (Schaffer et al. 2007). An exogenous ethylene application to MADS8as-9 line is able to partially stimulate ripening, even though starch degradation and other late ripening processes still remain uncompleted (Ireland et al. 2013).

Softening, a late ripening event, involves the disassemblement of the cell wall at pectin and hemicellulose level, causing a water loss and, consequently, a lack of turgor that negatively affects apple fruit shelf-life. Many genes encoding for cell-wall degrading enzymes, such as xyloglucan endotransglucosylase (MdXTH), expansin (MdEXP) and pectate lyase (MdPL), are mainly involved in this process. Recent studies carried out in two apple cultivars, Honeycrips and McIntosh, characterized by low and high degrees of softening, respectively, have shown that cell wall metabolism genes (*MdPG*, *MdEXP2*, *MdPL*) well correlate with softening features. In fact, in Honeycrisp apples, these genes are expressed at lower levels than in McIntosh, despite these cultivars share comparable IECs (Harb et al. 2012). As far as polygalacturonase (PG) genes are concerned, the role of *MdPG1* in softening and its ethylene dependent expression has been widely investigated (Li et al. 2010, Costa et al. 2010b, Atkinson et al. 2012, Harb et al. 2012, Longhi et al. 2013). In the last decade, apple breeding programs have been looking for new marker genes in order to select accessions characterized

by low IEC and softening to obtain new cultivars with high firmness and longer shelf-life. Costa et al. (2005) and other authors (Zhu & Barritt 2008) performed allelic genotyping of *MdACS*1 and *MdACO1* in diverse cultivars with divergent softening degrees. Results allowed to point out that low softening is directly related to a homozygosity state of these genes and therefore divergences at genetic level could be the reason for which different cultivars display significant differences in softening.

The fruit skin color is the result of flavonoids, such as anthocyanins, flavonols and proanthocyanidins, and carotenoids production occurring during the whole developmental cycle; both compounds are important antioxidants with beneficial effects for human health (Diretto et al. 2007). Flavonoids are phenolic compounds and account for the red-purple-blue color of fruits, whereas carotenoids are isoprenoid compounds and are responsible for yellow-orange-red coloration (Tanaka et al. 2008). A controversial role was pointed out for ethylene in flavonoids production. Kondo et al. (1991) showed that the rise of IEC is paralleled by a positive accumulation of anthocyanins, and ethylene may directly affect their biosynthesis. Other results pointed out that accumulation of these compounds occurs as a response to developmental stimuli (Saure 1990). Recently, Whale & Singh (2007) confirmed that IEC was significantly associated with total anthocyanin content but not with other flavonoids, such as catechin and epicatechin, the latter displaying a developmentally regulated accumulation as well. *MdDFR*, encoding a dyhidroflavonol reductase, *MdUFGT*, coding for a UDP-glucose:flavonoid 3-O-glucosyltransferase, and other three anthocyanin biosynthesis genes are developmentally regulated and their expression is positively correlated with anthocyanin levels (Honda et al. 2002). Several studies are being carried out on anthocyanin biosynthesis regulation, which led, especially in tomato (Al-sane et al. 2011), to the identification of key-genes whose expression is under the control of specific endogenous and/or exogenous factors. Several transcription factors affecting flavonoid gene expression are also under the control of abiotic factor, such as temperature and light. For example, low temperatures stimulate the transcription of a gene coding for MdbHLH3, a bHLH transcription factor, which regulates the expression of *MdDFR*, *MdUFGT*, and *MdMYB1*, thus resulting in anthocyanin accumulation and fruit coloration (Xie et al. 2012). The same genes display tissue-specific expression patterns: *MdMYB1* and *MdMYBA* are expressed in the epidermis (Allan et al. 2008, Lin-Wang et al. 2010), *MdMYB110a_JP* in the

cortex (Umemura et al. 2013), whereas *MdMYB10* in both tissues (Chagnè et al. 2007, Espley et al. 2007, Umemura et al. 2013).

Concerning carotenoids, genes involved in their biosynthesis, such as *phytoene desaturase* (*PDS*), *ζ-carotene isomerase* (ZISO), *carotene isomerase* (CRTISO) and *lycopene ε-cyclase* (*LCY-ε*) are up-regulated and correlated with carotenoid content (Amphoman-Dwamena et al. 2012). So far, research focused on hormonal and environmental factors regulating carotenogenesis is still missing in apple, despite some pieces of information are available about light and heat stress induction of carotenoids biosynthesis (Felicetti et al. 2009), as found in tomato (Liu et al. 2004).

The principal components of the apple fruit aroma are volatile esters, counting for more than 80% of the total (Dirinck et al. 1989). Among acetate esters, hexyl acetate, butyl acetate and 2-methylbutyl acetate are crucial for the characteristic aroma (Dunemann et al. 2012) and their concentration is highly correlated with ethylene production (Defilippi et al. 2005), suggesting a putative ethylene-dependent regulation of their biosynthesis (Schaffer et al. 2007). In apple transgenic lines with suppressed ethylene biosynthesis, total ester production is inhibited by 70% (Dandekar et al. 2004), along with the enzymatic activity of MdAAT, an alcohol acyl transferase catalyzing the final step of ester biosynthesis. The enzymatic activities of MdADH, an alcohol dehydrogenase, and MdLOX, a lipoxygenase, are instead unaffected (Defilippi et al. 2005). Different studies confirmed that expression of different *MdATT* genes is ethylene regulated and, in addition, highlighted that, within the *MdLOX* and *MdCXE* (carboxylesterase) multi-gene families, only some specific members are ethylene-dependent (Schaffer et al. 2007, Souleyre et al. 2011).

Besides its regulatory role during apple ripening, ethylene affects also the pre-harvest fruit abscission (PFA) process. PFA is closely related to ethylene levels even if this hormone does not participate in the onset of this process (Dal Cin et al. 2008) but rather follows than precedes this event. Sun et al. (2009), in order to assess the role of ethylene during PFA in wild and domesticated apple cultivars differing in IEC, analyzed the sequence of *MdACS1* involved in climacteric ethylene production. Results demonstrated that *MdACS1-1/1* allelotype is determinant for PFA, while *MdACS1-2/2* is related to non-abscission traits. A transcriptomic approach has also been adopted to characterize PFA in two cultivars, Golden Delicious and Fuji, displaying high and low abscission potential, respectively (Li et al. 2010).

Genes involved in ethylene biosynthesis (*MdACS5*, *MdACO1*) and signaling (*MdETR2*, *MdERS2*), and cell wall degradation (*MdPG2*, *MdEG1*) are more up-regulated in Golden Delicious than in Fuji, thus demonstrating a putative involvement of these genes in PFA. Treatments with the synthetic auxins 1-naphtylacetic acid (NAA) and 2,4-dichlorophenoxy-propionic acid (2,4-DP), and with the ethylene biosynthesis blocker L-aminoethoxyvinylglycine (AVG) showed a reduction of PFA, a delayed ripening (Yildiz et al. 2012) and a shift of ethylene-related gene expression (Dal Cin et al. 2008, Kondo et al. 2009). The reduction of ethylene biosynthesis exerted by AVG application would keep the abscission zone inactive, thus delaying PFA, as confirmed by Dal Cin et al. (2008).

The role of ethylene as a master regulator of the ripening syndrome in apple is unquestionable. Other hormones, however, may also play important roles, either as positive or negative actors, either alone or cross-talking with ethylene itself (**Figure 5**). So far, the crosstalk between ABA and ethylene has been examined considering the role of ABA in stimulating ethylene biosynthesis (Liebermann et al. 1977, Vendrell et al. 1989, Chen & Zhang 2000). At the end of the maturation stage, an exogenous application of ABA induces the increase of both ACS and ACO protein levels and ethylene production (Lara & Vendrell 2000). Kondo et al (1991) showed that ABA content increases paralleling ethylene and ACC content, and Setha et al. (2004) observed a decrease of both xanthoxin (XAN) and dihydrophaseic acid (DPA) content, an ABA precursor and its major metabolite, respectively. Based upon these data, it has been hypothesized a positive feedback regulation of ethylene, which may stimulate ABA biosynthesis at the level of XAN conversion to ABA, even though this mechanism is still unclear. Another hormone that may participate to the onset of ripening is jasmonic acid. Applications of PDJ at pre-ripening are able to stimulate the increase in ABA content later on (Kondo et al. 2000), thus promoting the transcription of *MdACS1* and *MdACO1*, which would enhance ethylene biosynthesis within 24 hours after the treatment (Kondo et al. 2009). In Summerred apples, the application of exogenous methyl jasmonate (MJ) is also able to positively affect other ripening-related changes, such as ester, alcohol, and acetic acid production (Fan et al. 1997).

Oppositely to the role played by ethylene, ABA and jasmonate, auxin display a substantial inhibitory role towards ripening. Schaffer et al. (2013) showed that a down regulation of auxin biosynthesis genes is a prerequisite for apple ripening to occur. Devoghalaere et al.

(2012) reported that two *GH3-like* genes, *GH3.1* and *GH3.202*, involved in auxin conjugation, are up-regulated during the onset of ripening, in agreement with the need to lower auxin content. In addition, it was also demonstrated that in MADS8as-9 apple lines that are unable to ripen, auxin biosynthesis and conjugation genes display an up and down regulation, respectively, with respect to the wild type (Schaffer et al. 2013).

Gaps and future perspectives

Apple research has undergone great improvements in the last decade, not only in quantitative terms, as previously pointed out, but also from a qualitative point of view. The high quality of results will have both short-term practical impacts on the final quality of the commodity and medium/long-term implications in apple breeding programs, thanks to the availability of molecular markers, which will hopefully speed up the release of new genotypes with superior traits (resistances, storability, taste, hypo-allergenicity, etc.). Despite the increasing interest on this fruit species and the important results being achieved by different research groups around the world, the 'apple community' should improve and extend its way of disseminating and/or exchanging results. As an example, there is no international symposium focused on apple, such as for many other Rosaceae species. This may represent an obstacle for apple scientists who want to exchange and update their knowledge, or even establish new research collaborative networks with other international institutions.

From a scientific point of view, apple physiology and genetics have many knowledge gaps, mainly due to a concentration of research efforts just on the early and late (ripening) developmental stages, although for obvious reasons. The intermediate phases, that are known to be important, have not been investigated at a comparable detail. In order to have a complete view of the regulatory networks controlling apple fruit development, it will be necessary to investigate more in detail the molecular events occurring during the expansion phase and, especially, the maturation phase, the latter being crucial for a successful transition to the ripening stage. What kind of 'molecular' competences does the apple fruit acquire during the maturation phase? Which are the modulators of this process? Can this process be tuned by exogenous treatments? A lot of work has to be done, not only by using the available 'omics' technology but, especially, by combining the most advanced techniques to classical physiological approaches, relying on observations on the tree in the orchard.

Another relevant gap deals with the intra-fruit signals that regulate the early development and, especially, the interactions occurring between the seeds and the cortex. What kind of signals do the seeds and the cortex exchange during early development, having the ability to decide the final fruit size? Only hormones? What about other mobile signals? What about, for example, small RNAs? Do they play relevant roles or are they in some way involved as intra-fruit signals? Also in this case, recent advances in high-throughput genomic techniques and in cell biology (i.e. high-processing robotized station for hystochemical analyses, new probes for *in situ* hybridizations, etc.) may give a significant help.

A further 'black hole' in apple research is represented by the signal that triggers fruitlet abscission. Its nature is still under investigation, but the signal is far from being identified. Shedding light on this topic may have relevant impacts in terms of both basic knowledge and practical applications. Despite apple being a good model for studying immature fruitlet abscission, the experimental limitations of this tree species still represent an obstacle for accelerating research on this topic. Within this context, the availability of self-thinning genotypes is progressively filling the experimental gaps.

Considering on one hand all the gaps that are still present in apple research, at least those dealing with its fruit development, and on the other hand the great availability of 'omics' technology and the high quality of the available body of information, a conclusion can be drawn: the future of apple research is undoubtedly full of challenges.

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Chapter 2

Selection of molecular markers of apple fruit developmental stages and samples' characterization

Highlights

Transcriptional markers were identified and validated, and can be efficiently used to discriminate cortex samples collected at different developmental stages in different years.

Keywords

Molecular markers, developmental stages, cell division, cell expansion, maturation, ripening, developmental transitions.

Introduction

From a physiological point of view, apple fruit development may be divided into four partially overlapping stages: cell division, cell expansion, maturation and ripening (Dong et al., 1997; Janssen et al., 2008), as shown in Figure 3 of Chapter 1.

In some circumstances, for example during years with extreme climatic conditions or as a consequence of extreme events occurring during the most critical developmental stages, the relative duration of each stage may vary. Therefore, the biological validation of different types of data collected over different years, especially if spanning the whole developmental cycle, may be easily biased if a careful selection of sample to be compared is not performed. In order to overcome these effects, the employment of molecular markers (i.e. metabolites or genes), whose amounts or expression profiles are related to a particular stage or transition between stages, could be essential to discern which samples, collected in different seasons, locations or other experimental conditions, are comparable from others that are not. Therefore, it is recommended to establish a method or a pipeline to analyse the expression profiles of these molecular markers on fruit samples before running expensive analyses. Hence, the availability of several published data on the characterization of the transcriptional events occurring during the apple fruit development (Janseen et al., 2008; Soglio et al., 2009; Soria-Guerra et al., 2011) represents only a starting point, since those information was achieved in different cultivars and need further validations.

The early and the late stages are the two key stages that have been widely examined: the length of the cell division phase affects the size of the fruit whereas maturation and ripening represent the stages in which several changes occur bringing the fruits to become edible. The role of the hormones and sugar-signalling network is undoubtedly the most relevant during these stages and some hormone- and sugar-related genes could therefore be retained good markers. The uncontested role of auxin and ethylene during the cell division and ripening stages, respectively, have been already proved, therefore some of their biosynthetic or hormone-responsive genes may be employed as markers. An auxin transporter belonging to the PIN family, *PIN3*, is characteristically expressed at cell division (Devoghalaere et al., 2012), while one of its transcription factors, *ARF13*, during cell expansion and maturation (Devoghalaere et al., 2012). Some ethylene-related genes such as *ACS1*, *ACO1* and *ERS1* (Wiersma et al., 2007; Varanasi et al., 2011), and *PG1* (Atkinson et al., 2012; Costa et al.,

2010) are mainly expressed at ripening. As far as the sugar-related genes are concerned, Li et al. (2012) characterized the expression profiles of several genes involved in sugar metabolism and accumulation during apple fruit growth and, among them few coding for a sugar transporter (*TMT1*), a sucrose phosphate synthase (*SPS6*), a sucrose dehydrogenase (*SDH1*), and a fructose kinase (FK1) showed a maturation- and ripening-characteristic profile that could be easily highlighted. In addition, a *neutral invertase 3* (*NINV3*) (Li et al., 2012) showed a specific profile characterizing both cell division and cell expansion, whereas an *expansin* (EXP) the cell expansion only (Atkinson et al., 1998): this latter stage is still less characterized and less information is available. Besides the role of hormones and sugars, during cell division several other genes are claimed to regulate this stage such as cyclin-dependent-kinases (*CKB1;2*) (Malladi and Jonhson, 2011), transcription factors (*ANT1* and *ANT2*) (Dash and Malladi, 2012) and a *SEPALLATA* gene (*MADS118*) (Ireland et al., 2013). The end of cell division is characterized by the decrease of expression of the above genes and the increase of negative cell cycle regulators, such as *KRP5* (Malladi and Jonhson, 2011).

Table 1 - List of candidate markers (Gene ID of Rosaceae database) selected among the reported references for the characterization of the apple fruit developmental stages (CD=cell division, CE=cell expansion, M = maturation and R = ripening).

Gene	Gene ID	Stage	Reference
PIN3	MDP0000156440	CD	Devoghalaere et al. 2012
ANT1	MDP0000175309	CD	Dash and Malladi, 2011
ANT2	MDP0000190889	CD	Dash and Malladi, 2011
MADS118	MDP0000220008	CD	Ireland et al. 2013
CKB1;2	MDP0000223519	CD	Malladi and Jonhson, 2011
NINV3	MDP0000596702	CD,CE	Li et al. 2012
KRP5	MDP0000258414	CE	Malladi and Jonhson, 2011
EXP	MDP0000681724	CE	Atkinson et al. 1998
ARF13	MDP0000412781	CE, M	Devoghalaere et al. 2012
SDH1	MDP0000305455	M, R	Li et al. 2012
TMT1	MDP0000381084	M, R	Li et al. 2012
FK1	MDP0000173131	R	Li et al. 2012
SPS6	MDP0000288684	R	Li et al. 2012
PG1	MDP0000326734	R	Atkinson et al. 2012
ACS1	MDP0000370791	R	Varanasi et al. 2011
ACO1	MDP0000195885	R	Wiersma et al. 2007
ERS1	MDP0000288468	R	Wiersma et al. 2007

Aim of the research

The present study aims at selecting some genes (Table 1) among those previously identified in other molecular studies on apple, and characterizing their expression profiles during the

apple fruit development in the cv Golden Delicious, in order to confirm or discard their role as candidate markers characteristic of a stage or a transition between stages. Moreover, the designated candidates will be used to select the 2014 samples to be analysed with omics approaches in the next chapters of the present dissertation.

Materials and methods

Plant material (2010 and 2014)

Preliminary experiments and samplings were conducted in the 2010 season on 7 year-old apple trees of cv Golden Delicious/M9 trained with standard horticultural practices at the experimental farm ”Maso Maiano” (Cles, Trento, Italy) of the Istituto Agrario San Michele all’Adige, Edmund Mach Foundation (FEM-IASMA, Trento, Italy). For each apple tree, fruit load was manually normalized according to the trunk section area. A randomized block design was adopted, with three blocks, each including five trees. Each block represented a biological replicate. Cortex of each biological replicate was collected from a suitable number of king fruits, according to their size, and immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses. During the early developmental stages the sample included cortex and skin tissues, whereas as soon as the fruitlet started to rapidly expand the cortex was separated from the peel.

The sample collection started from the early days of the fruitlet development and ended at the fruit ripening, during which fruits were collected close to the commercial harvest date up to when ripening occurred “*in planta*”. The sampling was performed according to the following time course: 18, 19, 20, 21, 61, 87, 113, 147 and 172 days after petal fall (DAPF).

A denser sampling was performed in 2014 on 9 years-old apples trees of cv Golden delicious/M9 grown with a slender spindle form and trained with standard horticultural practices, with no hormonal treatments, at the experimental field “Piovi” (Roverè della Luna, Trento, Italy; see Figure 8 of Chapter 3) of the Istituto Agrario San Michele all’Adige, Edmund Mach Foundation (Trento, Italy). For each apple tree, fruit load was manually normalized according to the trunk section area (TSA), up to a value of 6 fruits/cm^2. A randomized block design was adopted, with three blocks, each including five trees.

Samples collection started when flowers were still closed but well developed (unpollinated flowers), to proceed up to fruit ripening *in planta*, according to the following time course

(expressed in days after full bloom, DAFB): -3, 4, 13, 20, 31, 39, 53, 67, 88, 103, 118, 124, 132, 139, 146, 157, and 172. At the beginning samples included the whole receptacle (i.e. with epidermal tissue), whereas from 20 DAFB only the cortical tissue was collected. Plant material was immediately frozen in liquid nitrogen and stored at -80°C. All samples were collected during the morning, from 9 pm within maximum 2 hours.

Fruit growth measurements

In the preliminary experiments of 2010, the cross-diameter of at least ten fruits, tagged on ten trees randomly chosen within the orchard, was measured at each time point of the sampling for setting up the fruit growth curve.

In 2014, fruit cross diameter of 120 fruits was measured by using a manual caliper and recorded weekly from full bloom up to the last sampling date.

Table 2 - List of the primers for the qPCR analyses. (*= Gene ID of NCBI database)

Gene	Gene ID	Primer F	Primer R
PIN3	MDP0000156440	CGATGGTGGCAAAGACCTA	CTCATCATAATCTTTGTGGAGAGC
ANT1	MDP0000175309	GCAGCTCCAGAGAAGGAAGC	GGCAGCAAAGAGAGGCAACT
ANT2	MDP0000190889	AGAATGTGCAGTGGCAGTCA	CGTCCACCTATGCCTTGTGA
MADS118	MDP0000220008	AGCAGCAAAGAGCTTGAATCG	ACGTGTTCATTTCGCTGAAGG
CKB1;2	MDP0000223519	CTCAAGAGCTACACGCACGA	GTGGAGTAGTGAGCCGAACC
NINV3	MDP0000596702	GGTACTTGGTAGCGAAGATGATGT	AGCAAGTCCACGAATTTGATCT
KRP5	MDP0000258414	GCGGATGAGAGTGAACAGGT	CTCCGACACATTCACTACCG
EXP	MDP0000681724	TGTGGGTACGGCAACCTCT	CGTTGTTTGGTGGACAGAAA
ARF13	MDP0000412781	CAAGATCCGCCATCCTTTATC	GTGACCTGCTGACCGAACTG
SDH1	MDP0000305455	ATAGAGGAAGTTGGGAGTGAGGT	CTGATTTGCCAGACAACCATT
TMT1	MDP0000381084	TCGTCTATTTCTGCGTCTTTGTC	CCCAATCCAGTACACCAAGG
FK1	MDP0000173131	TTGACACAACTGGTGCTGGT	GGAATGGCTCCTCTTTCTGTT
SPS6	MDP0000288684	AGGTTCTGTTGAGTATGGCAGTGAG	GTGCTTCAAGTGCCGCTGAGA
PG1	MDP0000326734	TCCTTCATACACGGACACCA	TCAGCACCATTCCCTTTAGC
ACS1	MDP0000370791	ACACCTTTGAAGCCGAAATG	GCATTGCCAAGTCCAGAGTTC
ACO1	MDP0000195885	CAGTCGGATGGGACCAGAA	GCTTGGAATTTCAGGCCAGA
ERS1	MDP0000288468	CAACTAGGGATATGCGAC	CACTGGCATCCAAAGACTTC
18s	DQ341382.1*	GTTACTTTTAGGACTCCGCC	TTCCTTTAAGTTTCAGCCTTG

RNA extraction and cDNA synthesis

Total RNA was extracted following the method of Ruperti et al. (2001), with a few adaptations as Botton et al. (2009 and 2011): 0.6 g of fruitlet was extracted in 10 ml of extracting buffer whereas 6 g of fruit cortex in 15 ml. Total RNA was quantified with the

NanoDrop 2000c (Thermo Scientific, Waltham, MA) and its integrity checked by running 1 μg in a 1% agarose stained with SYBR® Safe (Life Technologies, Carlsbad, CA).
cDNA was synthesized with the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, Carlsbad, CA) from 200 ng of DNA-free total RNA in a final volume of 40 μL, according to the instructions provided by the manufacturer. The reaction was performed in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA).

Real-time PCR

Real-time PCR relative quantification was performed in triplicate on three biological replicates, as described by Botton et al. (2011). The nucleotide sequences of the primers for both the target and reference genes are reported in Table 2. Data were acquired, elaborated and exported with the StepOne Software v2.1 (Applied Biosystems, Foster City, CA), whereas all the final calculations were made with the automated Excel spreadsheet Q-Gene designed by Simon (2003), using the modifications of the delta Ct method suggested by Pfaffl (2001).
Gene expression values were normalized to *Md18S* (Table 2). Expression levels were then reported as arbitrary units (A.U.) of Mean Normalized Expression, calculated using equation 2 of the Q-Gene spreadsheet.

Results and discussion

Validation of candidate markers in Golden Delicious 2010 samples

The fruit growth kinetics (Figure 1) of year 2010 was clearly characterized by three peculiar phases: an initial exponential phase (until about 30 DAPF), overlapping the entire cell division and the initial cell expansion stage, followed by a constantly increasing fruit diameter (from about 30 to 110 DAPF), corresponding to the cell expansion phase, and a final plateau phase (from about 110 to 172 DAPF), in which only small differences in fruit size were observed. In this last step, both fruit maturation and ripening take place, the fruit turns from immature to mature (maturation) and acquires the competence to respond to some specific hormonal stimuli (Lelièvre et al. 1997) that are responsible for major physical and chemical changes in the fruit, such those related to colour and flavour.

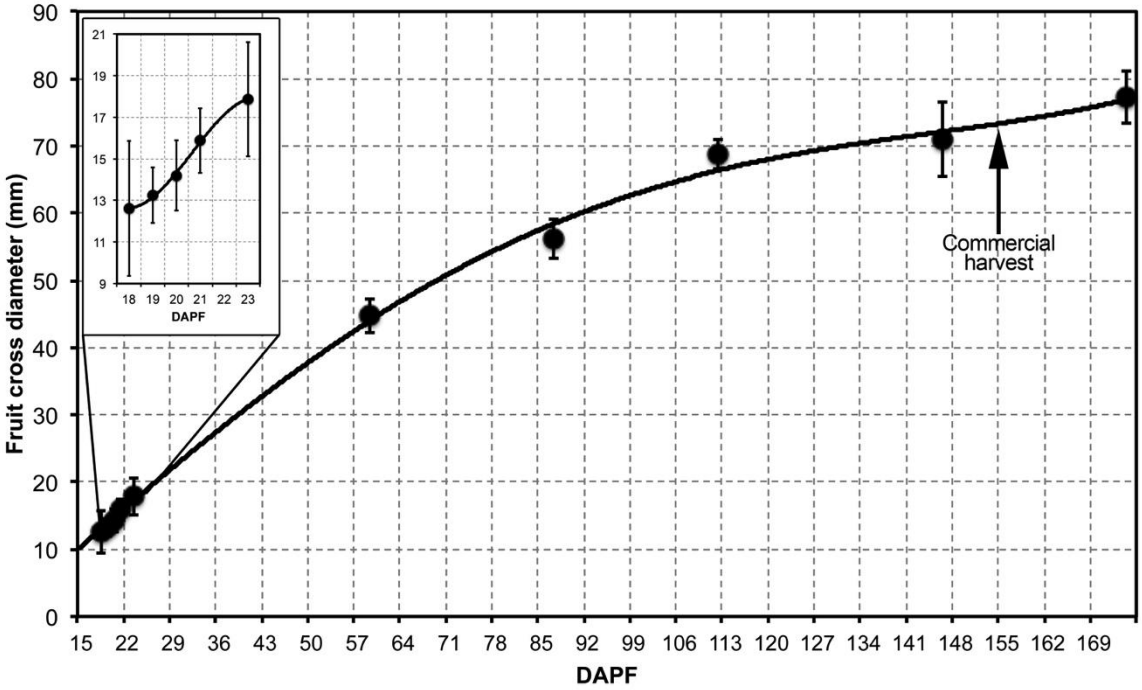

Figure 1 - The fruit growth kinetics obtained by measuring fruit cross diameter from at least 10 fruits of cv Golden Delicious per time point. An inset was included (top left) to give a more detailed view of the early developmental stages. Commercial harvest is also indicated. (CD = cell division, CE = cell expansion, M = maturation, R = ripening)

The characterization of apple fruit development and growth may not rely only on physical parameters, such as the fruit cross-diameter, but on more precise surveys concerning the specific, but invisible, changes occurring at the metabolic and transcriptional levels. The employment of molecular markers could be a good option that may overcome the effect on the regular fruit growth of climatic and extreme events. For this reason, five putative markers for cell division stage (*PIN3*, *ANT1*, *ANT2*, *MADS118* and *CKB1;2*), one for cell division/cell expansion transition (*NINV3*), two for the cell expansion (*KRP5* and *EXP*), one for the cell expansion/maturation (*ARF13*), two for maturation/ripening (*SDH1* and *TMT1*) and six for the ripening (*FK1*, *SPS6*, *ACS1*, *ACO1*, *ERS1* and *PG1*) were analysed in order to assess the power of each marker in discerning the stage it is involved in.

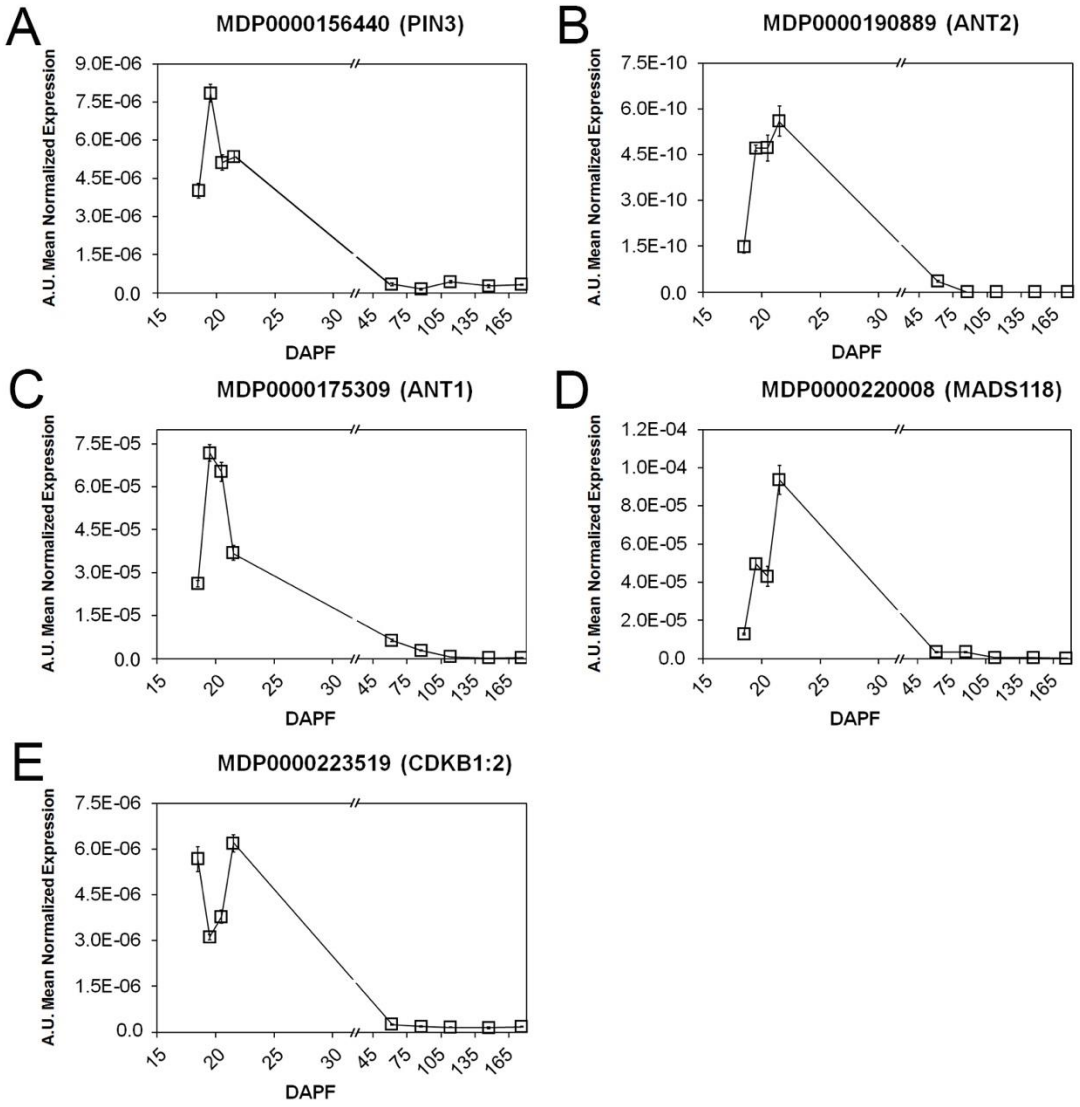

Figure 2 - qPCR gene expression data of the five marker genes for the cell division stage. The gene IDs and names are reported within each chart. Bars represent standard error.

Marking cell division - Concerning the cell division stage, the qPCR data obtained for the five putative markers for the whole fruit development (Figure 2) highlighted that all the genes were up-regulated during cell division (until 21 DAPF), afterwards a sharp decrease of their trends to a basal level was evident. Focusing on the trend of the expression profiles, the genes could be divided in two groups: *PIN3* and *ANT1* may be involved in the early cell division events, as their expression peaked at 19 DAPF, whereas *ANT2*, *MADS118* and *CKB1;2* may be involved later on, as they showed to be highly expressed at 21 DAPF. The qPCR profiles

confirmed a likely role of these genes in marking the cell division stage as long as their expression levels are high.

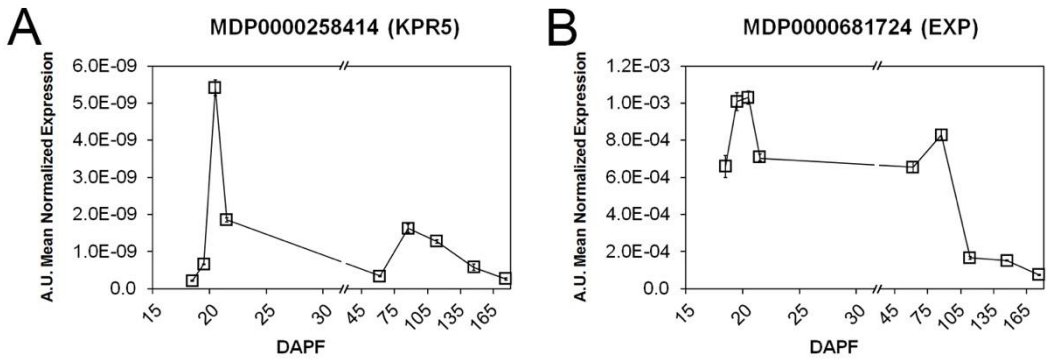

Figure 3 - qPCR data expressions of the putative markers for the cell expansion stage. The gene IDs and names are reported within each chart. Bars represent standard error.

Marking transition from cell division to expansion - The late cell division stage may include two main events: i) the cessation of the cell cycle, and ii) the rearrangement of cell wall composition that allows the cell to expand in volume. The role of both some negative regulators of the cell cycle and the expansins has already been widely described (Malladi and Jonhson, 2011; Atkinson et al., 1998). The gene *KRP5*, coding for a negative regulator of the cell cycle and *EXP*, encoding an expansin, were taken into account to mark a boundary between cell division and cell expansion stages. The qPCR data (Figure 3) showed an up-regulation of *KRP5* during the late cell division stage (20 DAPF) followed by a decrease of expression until 61 DAPF. Afterwards, a slight increase in expression at 87 DAPF was detected, followed by a slow decrease to basal levels. The 61 DAPF could represent the time point in which the cell division is concluded and cell expansion starts. Hence, this gene may be considered a good molecular marker for the transition between these two stages.

Considering the result obtained for the *EXP* gene (Figure 3), its expression is high in both cell division and expansion stage: in fact, as shown by the Figure 3 of the Chapter 1, the cell expansion event starts at, and partially overlaps with, the late cell division stage. *EXP* gene expression peaked at 20-21 DAPF and again at 87 DAPF, indicating that the gene is active in both stages. After the latter date it followed a constant decreasing trend. These results indicate that this gene is not able to mark univocally any particular developmental stage.

Marking late cell expansion - From a physiological point of view, during the apple fruit development, the changes in the transport and metabolism of the sugars (Li et al., 2012) have

been widely investigated, especially those concerning the enzymatic reactions that convert the main sugars, sucrose and sorbitol, into glucose and fructose, and the events claimed to regulate the transport of these elementary sugars. Sucrose is converted to glucose and fructose by both cell wall (CWIN) and neutral (NINV) invertases, whereas the sorbitol is converted into fructose by sorbitol dehydrogenases (SDH). The fructose can be transformed to fructose-6-phosphate by fructokinase (FK), and can be combined to UDPG to resynthesize sorbitol by the sucrose-phosphate-synthase (SPS) (Li et al., 2012). Among all the genes coding for the invertase enzymes, *NINV3* (Figure 4) showed a decreasing expression trend from the early cell division to the late cell expansion stage when its level was shown to be basal and unchanged through the maturation and ripening stages, in cv Greensleeves (Li et al., 2012). In cv Golden Delicious, the qPCR data showed several differences: an initial up and down trend, likely erratic, detected until 87 DAPF was followed by a decrease in expression until 147 DAPF, just before the commercial harvest. Since the expression of this gene was still high around 130 DAPF instead of reaching a steady level around 110 DAPF, corresponding to the late cell expansion, the *NINV3* was discarded from being a good marker.

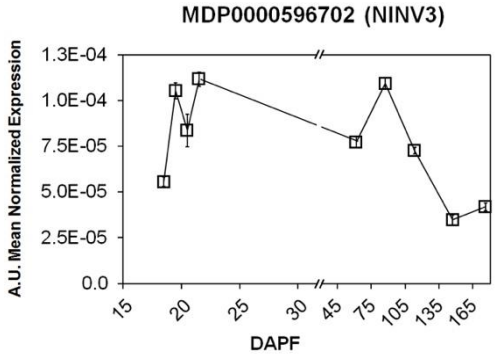

Figure 4 - qPCR gene expression data for the *neutral invertase 3* (*NINV3*). The gene ID and name is reported within the chart. Bars represent standard error.

Marking the maturation-ripening transition - As far as the maturation and ripening stages are concerned, a *tonoplast monosaccharide transport 1* (*TMT1*) and a *sorbitol dehydrogenase 1* (*SDH1*) genes have been identified as putative markers. Considering the early fruit stages (Figure 5), the expression of *TMT1* did not change, whereas the expression of *SDH1* reached a peak at 21 DAPF, *i.e.* late cell division, and then decreased at 61 DAPF. Both genes shared the same expression trend from 61 DAPF to the ripening "*in planta*": their expression peaked at 113 DAPF, decreased at 147 DAPF, and then increased again at 176 DAPF. The same

patterns were observed in cv Greensleeves (Li et al., 2012), confirming that these genes can be regarded as reliable maturation-ripening markers.

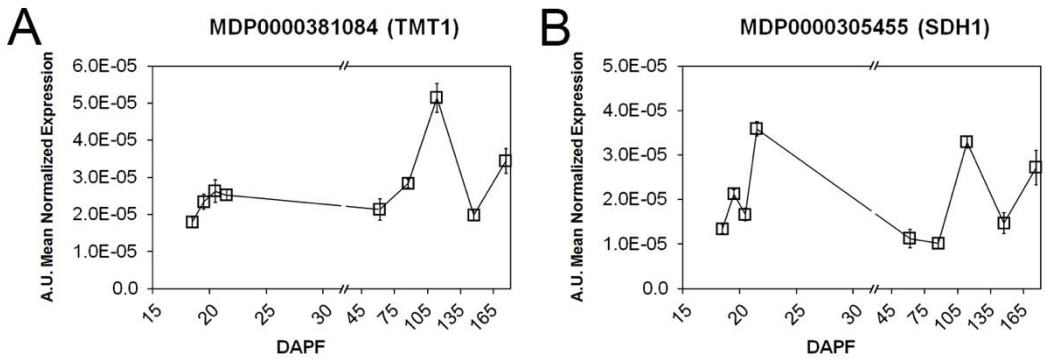

Figure 5 - qPCR gene expression data for the *tonoplast membrane transporter* 1 (*TMT1*) and *sorbitol dehydrogenase 1* (*SDH1*). The gene IDs and names are reported within each chart. Bars represent standard error.

FK1 and *SPS6* have been tagged as markers for the maturation and ripening stages. Their expression profiles in Golden Delicious were similar to *SDH1* (Figure 6), peaking at 21 and decreasing at 61 DAPF, during the transition from cell division to cell expansion. Regarding the late stages of development, both genes were positively regulated at 113 DAPF, then a decreased at 147 to increase again at 176 DAPF. The increasing expression of both genes observed at 113 DAPF could be due to rearrangements among the sugar components, causing a decrease in the acid content in the cortex and ensuring the conversion of the apple into an edible commodity, just before the commercial harvest. Hence, these genes were confirmed markers for the late developmental stages. Nevertheless, they could be employed to characterize the transition between cell division and expansion.

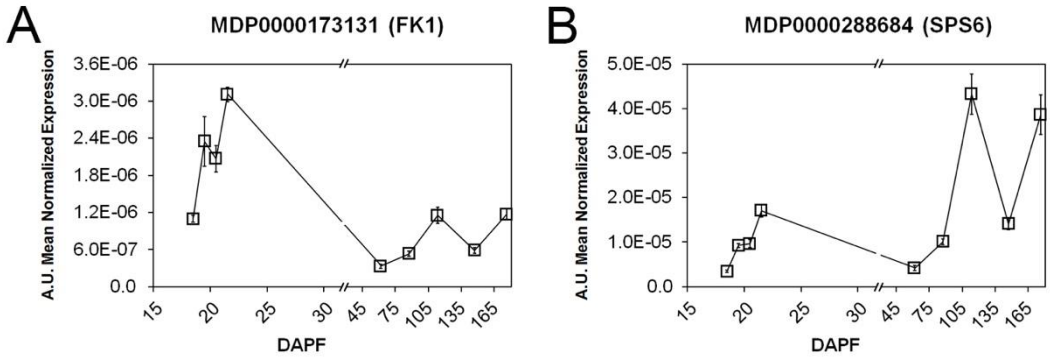

Figure 6 - qPCR gene expression data for the *fructose kinase 1* (*FK1*) and *sucrose phosphate synthese 6* (*SPS6*). The gene IDs and names are reported within each chart. Bars represent standard error.

Taking into account the role exerted by hormones during apple fruit development, several metabolic and hormone-responsive genes have been considered as putative markers for the middle and late stages.

The transition between cell expansion and maturation is still less investigated than early fruit set and ripening, therefore few information is available making the research of putative gene markers arduous. A gene coding for an auxin responsive factor, *ARF13*, was analysed and the qPCR (Figure 7) data confirmed it as a marker for this stage: its expression peaked at 87 DAPF, at cell expansion, and afterwards decreased until 147 DAPF. Before it reached its lowest levels of expression, maturation is supposed to start. The fruit could be considered "mature" when it becomes competent to sense the action of ethylene, but a prerequisite of this change is a decrease of auxin (Devoghalaere et al., 2012). Consistently, the expression profiles of *ARF13* could reflect the changes of activity of this hormone: cell expansion is characterized by high auxin activity that decreases at the end of this stage allowing the maturation to occur.

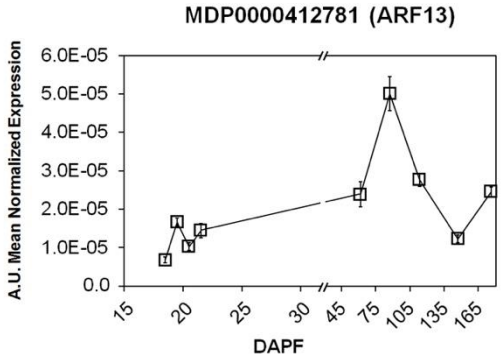

Figure 7 - qPCR gene expression data of the auxin responsive factor, ARF13, is showed. The gene ID and name is reported within the chart. Bars represent standard error.

The maturation and ripening stages could be traced by analysing the expression profiles of ethylene-related genes, such as those involved in the biosynthesis (*ACS1* and *ACO1*) and perception (*ERS1*) pathway, or ethylene-responsive genes (i.e. *PG1*) (Figure 8). The qPCR data of *ACS1*, *ACO1*, and *PG1* revealed closely similar trends of expression along fruit development. A peculiar up-regulation of these ethylene-related genes was pointed out at 113 DAPF, soon followed by a prompt down-regulation and the well-known and expected up-regulation at ripening. The peak of transcription rate observed at 113 DAPF is likely unrelated to development and was probably due to a sudden and unusual environmental stress

(particular weather conditions or extreme events), sensed by the fruit as an imposed abiotic stress, which could have stimulated the transcription of ethylene-related genes. Further investigations are still in progress to recover and assess the climatic data registered by the meteo station located in the orchard. In any case, the second expected peak indicates that these genes are good and reliable ripening markers.

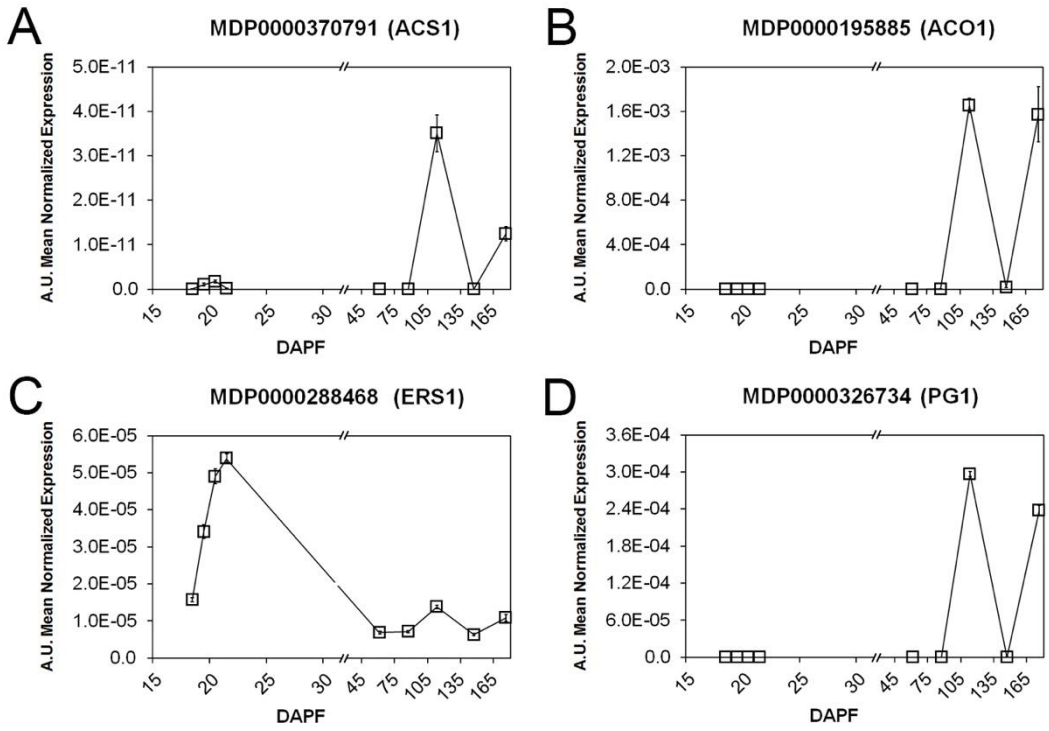

Figure 8 - qPCR gene expression data for three ethylene-related genes (*ACS1*, *ACO1* and *ERS1*) and one ethylene-responsive gene (*PG1*). The gene IDs and names are reported within each chart. Bars represent standard error.

Fruit growth kinetics in 2014

The growth kinetics (Figure 9) obtained during the sampling of 2014 season resulted to be very informative, as it was more complete and denser than in 2010, spanning from the early development events (*i.e.* before fertilization) until ripening "*in planta*". As observed before for 2010 data, three patterns can be clearly distinguished, this time following a well defined simple sigmoidal pattern: i) a first exponential phase, that lasted until 40 DAFB, characterizing the cell division stage during which the fruit grew very quickly, ii) a subsequent phase, marked by a constant fruit enlargement (cell expansion) until 130 DAFB, and iii) the last developmental phases during which fruit growth was significantly reduced.

In general, these data are comparable to those of the 2010 season, confirming that among different seasons the basic pattern of fruit growth remained quite unchanged.

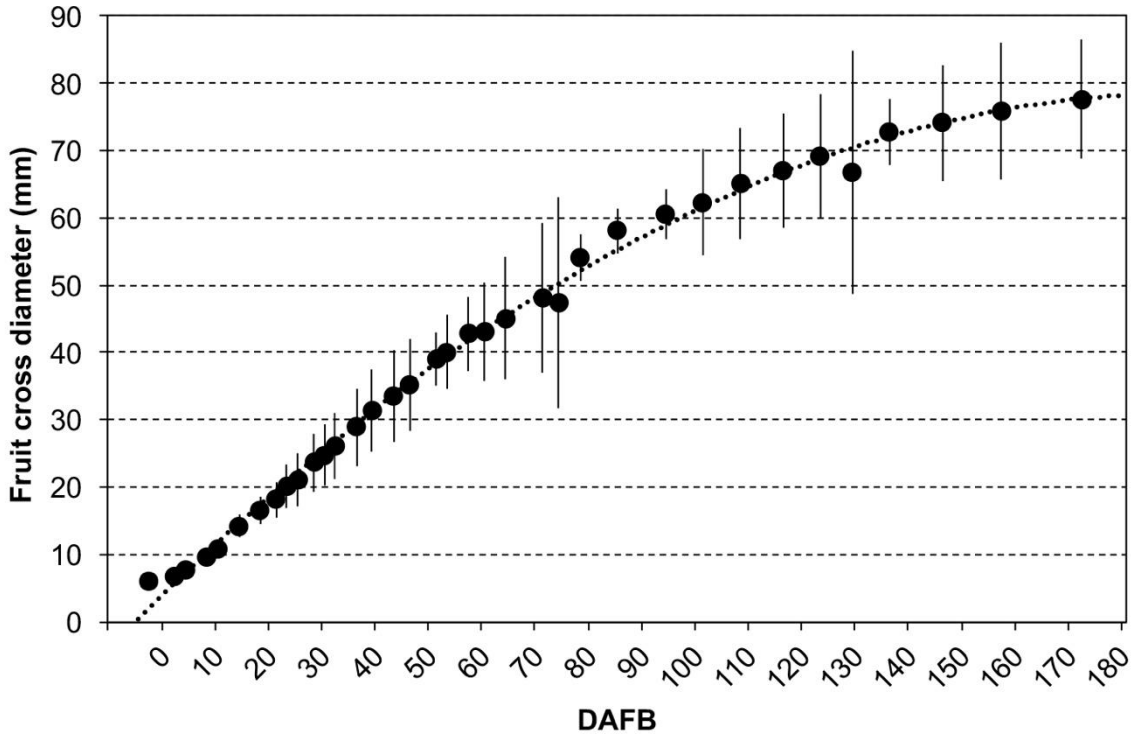

Figure 9 - Fruit growth kinetics obtained by measuring fruit cross diameter from at 120 fruits of cv Golden Delicious per time point (year 2014). Bars represent standard deviation

Marker-assisted selection of 2014 samples

Among the fifteen developmental marker genes, nine were selected and analysed in the cortex of fruits of the 2014 season: two marking cell division (*PIN3* and *MADS118*) and cell expansion (*EXP*) stages, one the cell-expansion/maturation transition (*ARF13*), two the maturation/ripening transition (*SDH1* and *TMT1*), and three the ripening stage (*FK1*, *ACO1* and *SPS6*). Cortex of the fruitlets collected at 13 DAFB was not included in the analyses since it was already known to represent the early cell division stage.

Concerning the full cell division stage, *PIN3* and *MADS118* displayed closely similar patterns (Figure 10): their expression levels were high during the cell division stage and then decreased at 53 DAFB. *PIN3* expression sharply declined already at 31 DAFB as its function is known to be exerted at early cell division, whereas *MADS118* expression was still high at 39 DAFB and then rapidly decreased. Thereafter, their transcript levels remained substantially unchanged along the whole fruit development, except for *PIN3*, which

displayed a peak at 146 DAFB as probably involved in the transport of the auxin in the late growth phase anticipating the ripening events.

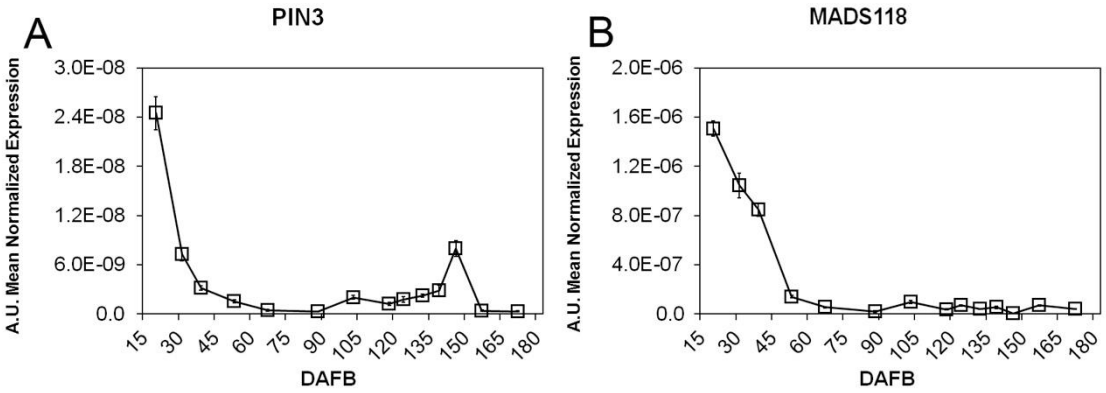

Figure 10 - qPCR gene expression data of *PIN3* and *MADS118* on samples of year 2014. The gene names are reported within each chart. Bars represent standard error.

The cell enlargement phase was analysed by monitoring the *EXP* gene (Figure 11): its expression was up-regulated in two different moment of fruit growth: a first peak was evident at the end of the cell division at 53 DAFB and a second one at 118 DAFB, at late cell expansion. At 124 DAFB the cell expansion stage was almost concluded and the expression of this gene reached a basal level and remained substantially unchanged through the rest of the developmental cycle.

The expression of *ARF13* (Figure 11) increased from 67 to 118 DAFB, during the cell expansion phase, and from 124 to 132 DAFB. Then it decreased at *139* DAFB and then raised at the late developmental phase. Therefore, between 132 and 146 DAFB the fruit should acquire the "competence to ripen" and turn into "mature", followed by an increase in the auxin transcription factor expression, as shown in the profile, which could play a role in stimulating the ACC synthase expression and therefore increasing the internal concentration of ethylene.

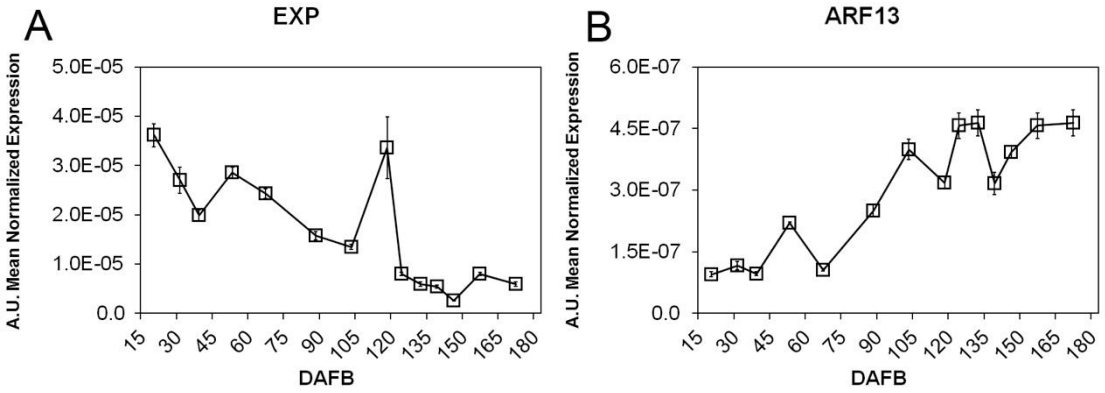

Figure 11 - qPCR gene expression data of *EXP* and *ARF13* on samples of year 2014. The gene names are reported within each chart. Bars represent standard error.

As far as the maturation-ripening transition is concerned, transcript accumulation of both *TMT1* and *SDH1* showed an increment at 139 and 146 DAFB, respectively, as putatively involved in the early and late maturation events, but the *TMT1* expression still remained high while *SDH1* was down-regulated at 157 DAFB, remaining unchanged thereafter (Figure 12). Again, the profile of *TMT1* revealed that, between 132 and 146, a change from cell expansion to maturation occurred. Therefore within this time frame the fruits should have turned from an immature to a mature state.

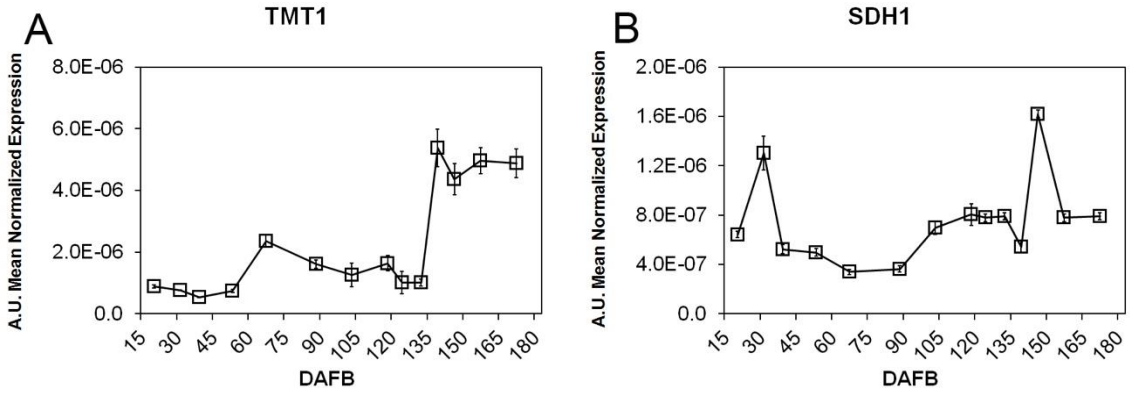

Figure 12 - qPCR gene expression data of *TMT1* and *SDH1* on samples of year 2014. The gene names are reported within each chart. Bars represent standard error.

An ethylene biosynthesis-related (*ACO1*) and two sugar metabolism-related (*FK1* and *SPS6*) genes were selected as ripening markers (Figure 13). *ACO1* and *SPS6* displayed the same trend of expression, which is characterized by a peak at 157 DAFB, whereas the *FK1* seemed unrelated to the other two genes, since its expression peaked at 146 DAFB. The latter gene

may be more likely involved in the transition between maturation and ripening, while the others may be related only to the ripening-related events.

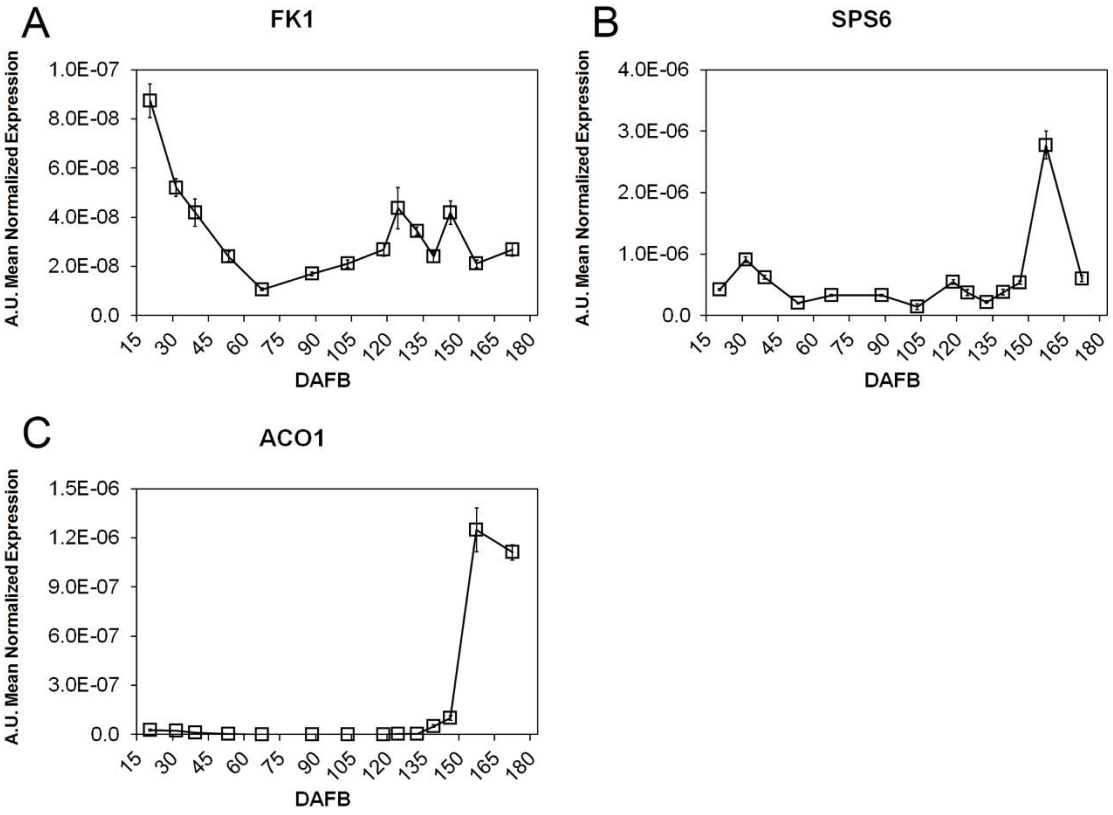

Figure 13 - qPCR gene expression data of *FK1*, *SPS6* and *ACO1* on samples of year 2014. The gene names are reported within each chart. Bars represent standard error.

On the basis of the evidences retrieved by investigating the expression profiles of these developmental markers, the most representative samples can be selected (Table 3). The sample collected at 13 DAFB was already considered an important point for the early cell division (ECD) stage, since its transcriptional inventory may resemble those events occurring in the cortex soon after fertilization is achieved. The sample at 20 DAFB was suitable for representing the cell division (CD) stage, whereas samples at 53 DAFB the late cell division (LCD)/early cell expansion (ECE) transition. For the cell expansion (CE) stage the sample at 88 DAFB was selected, whereas for the maturation and ripening stages four samples were chosen as follow: fruits at 132 DAFB represented the immature (IM) stage, during which the fruit was unable to ripen if picked from the plant, whereas the sample at 146 DAFB embodied the mature (M) stage, as it was prompt to ripen. The samples collected at 157 DAFB

corresponded to the commercial harvest (CH) stage, and the last one at 172 DAFB to the ripening "*in planta*" (R) stage.

Table 3 – Samples selected for the omic analyses. For each sample, the time of sampling, a name, a description of the developmental stage and an additional code are given.

Sampling time (DAFB)[1]	Sample name	Description of the developmental stage	Sample code[2]
-3	T0	pre full bloom	PFB
4	T1	full bloom	FB
13	T2	early cell division	ECD
20	T3	cell division	CD
53	T4	late cell division/early cell expansion	LCD/ECE
88	T5	cell expansion	CE
132	T6	late cell expansion/immature	LCE/IMMATURE
146	T7	mature (pre-climacteric)	MATURE
157	T8	commercial harvest	CH
172	T9	ripening	PH

1) Full bloom is equal to 0 DAFB.
2) As referred to fruit development.

Conclusions

In the recent years, the apple fruit development has to deal with climatic changes and the appearing of unwanted extreme events that may affect the regular proceeding of its growth. Concerning the scientific research, the increasing need to validate transcriptional data in different years and, therefore, sampling fruit tissues in various seasons is hampered by the above issues. The employment of molecular markers, for improving the capability of discerning the different stages of fruit development in samples collected in different years may guarantee that the results obtained are not biased by the developmental stage, at least not significantly.

Figure 14 – Fruits collected from 109 to 148 DAFB and allowed to ripen detached from the tree.

A practical confirmation of these results was given by a small pilot experiment carried out in 2014, consisting in harvesting 10 fruits from the trees of the experimental orchard in the period spanning from 109 up to 148 DAFB (Figure 14). Those fruits were then allowed to ripen on a bench at room temperature, in order to check if they were able to ripen detached from the mother tree and, therefore, which of them was "mature". A visual examination of the fruits allowed to observe that up to 130 DAFB fruit were not able to ripen, whereas the samples at 137 DAFB showed colour and textural changes (data not shown) typical of the ripening syndrome. Fruit harvested up to 115 DAFB underwent senescence (mainly wilting) without showing the typical traits of ripening fruits. These simple observations, which were not found anywhere in literature, allowed to confirm that samples at 132 and 146 DAFB, that were chosen using the molecular markers, were representative of two actually different developmental stages (immature and mature, respectively).

In this chapter the detection of the expression profiles of several putative markers, which have been showed to characterize the four major developmental stages, revealed the power of this method. In fact, among all the seventeen markers tested in the cortex of cv Golden Delicious, only the *neutral invertase* (*NINV3*) gene was discarded from the final list. Still, besides the role of the genes in tagging one stage or a transition time point, the results so far obtained gave new insight into the role of three genes, *FK1*, *SPS6* and *SDH1*, which have been previously identified as maturation and ripening markers. The present results indicate that all those genes were found to mark also the transition from cell division to cell expansion. In addition, a surprising results obtained by analysing the ethylene-related genes revealed that during the apple fruit development an abiotic stress may have occurred (without any apparent consequence) and this unwanted event, perceived by the fruit, caused an increase in ethylene production. Therefore, the choice of marker of the last developmental phases must be careful and should include also genes other that those directly related with ethylene. Such genes must be identified.

Again, in this chapter, a more detailed sampling program carried out in 2014, during which fifteen samples of cortex were collected, was employed in order to validate the expression profile of nine genes (*PIN3*, *MADS118*, *EXP*, *ARF13*, *TMT1*, *SDH1*, *FK1*, *SPS6* and *ACO1*), among all the development markers so far characterized. Differences and similarities were underlined between 2010 and 2014. Nevertheless, the fruit growth kinetics displayed a

similar trend in both years, although the expansion and the maturation stage showed unexpected features. In 2010, the expansion stage finished around 115 DAFB, whereas in 2014 around 130 DAFB, and these data where validated by analysing the gene expression of *EXP*. At the end of development, however, the mean cross diameter of the fruits was closely similar, indicating a substantial conservative strategy adopted by development in order to achieve the same objective, *i.e.* fruit ripening.

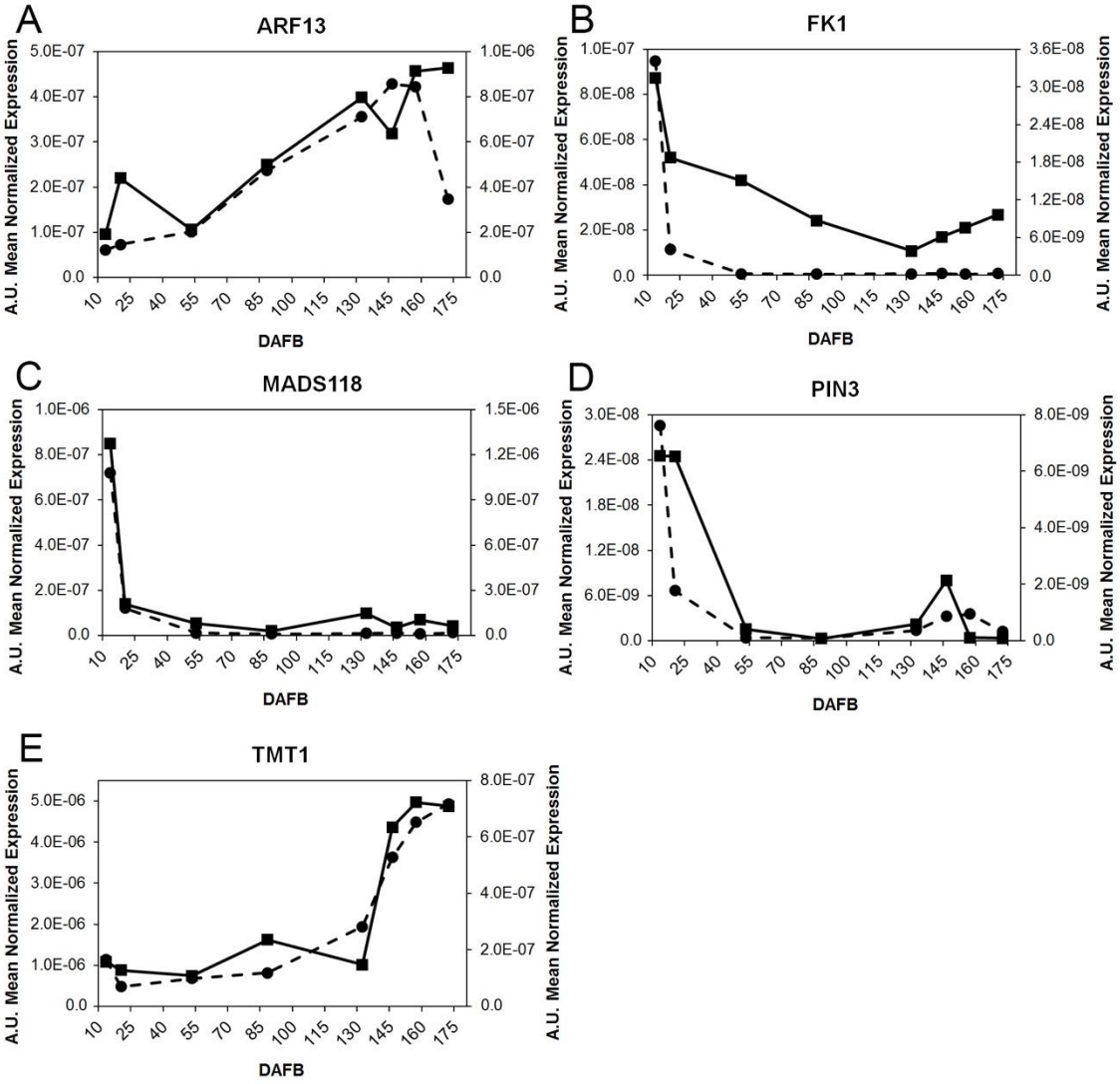

Figure 15 - Validation of marker genes on samples of year 2014 (continuous line) and 2015 (dashed lines). The gene names are reported within each chart. Bars represent standard error.

Finally, the markers validated and selected as "reliable" for 2014 samples were further validated in a different time series of samples collected in the following year (2015), confirming once again their reliability (Figure 15).

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Chapter 3

Quantification of the main hormones during apple (*Malus domestica* L. Borkh) fruit development

Highlights

The hormonal variations pointed out during apple fruit development depict a very interesting view of the cross-talk among hormones and their roles during each developmental phase and at transitions between different stages.

Keywords

Hormones' profiling, cross-talk, gibberellins, cytokinins, auxin, ethylene, abscisic acid, biosynthetic precursors.

Introduction

Endogenous signals: the plant hormones

Developmental processes and responses of the tree to environmental stimuli are regulated by the interaction and cross-talk of both endogenous and exogenous signals, which modulate all the genetic and metabolic aspects of the tree physiology. Concerning the endogenous signals, hormones play a prominent role. Present at very low levels (ng/g of fresh weight), the five "classical" plant hormones are the auxins (mainly IAA, indole-3-acetic acid), gibberellins (GAs), cytokinins (CKs), abscisic acid (ABA), and ethylene, to which the so called "plant growth regulators" (i.e. brassinosteroids, polyamines, jasmonates, salicylic acid, and strigolactones) should be added (Figure 1). Actually, some of the latters have often shown such important effects that they must be fully included in just one great category of plant hormones.

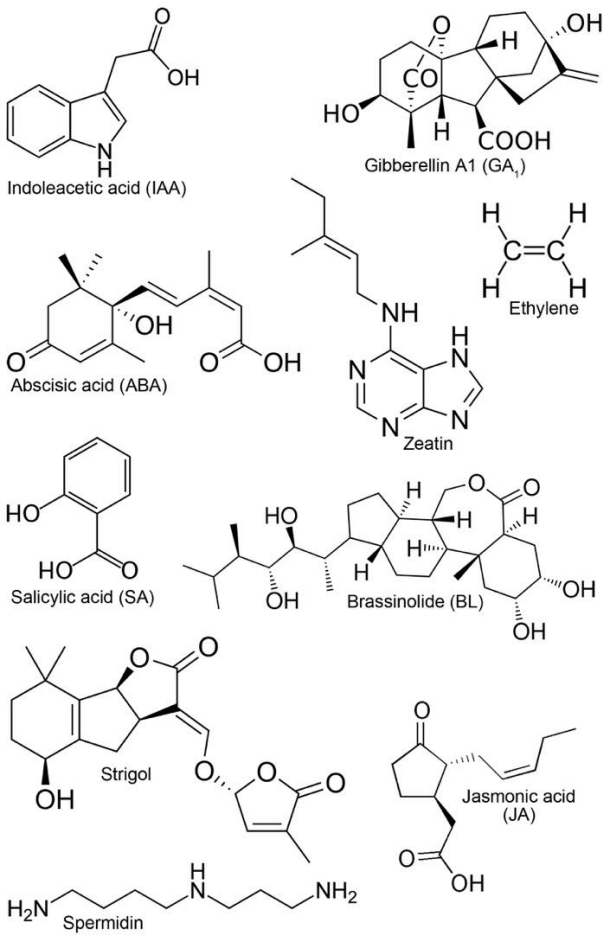

Figure 1 - Chemical structure of plant hormones and the so-called "growth regulators".

Although several processes are regulated uniquely, or at least prevalently, by a single class of hormones, most of them display a pleiotropic behaviour and are involved in the regulation of the entire plant ontogenesis, even by sharing part of their transduction pathways. In general, IAA controls cell division and expansion, apical dominance, tropisms, stem elongation, cambium activity and rhyzogenesis (Figure 2).

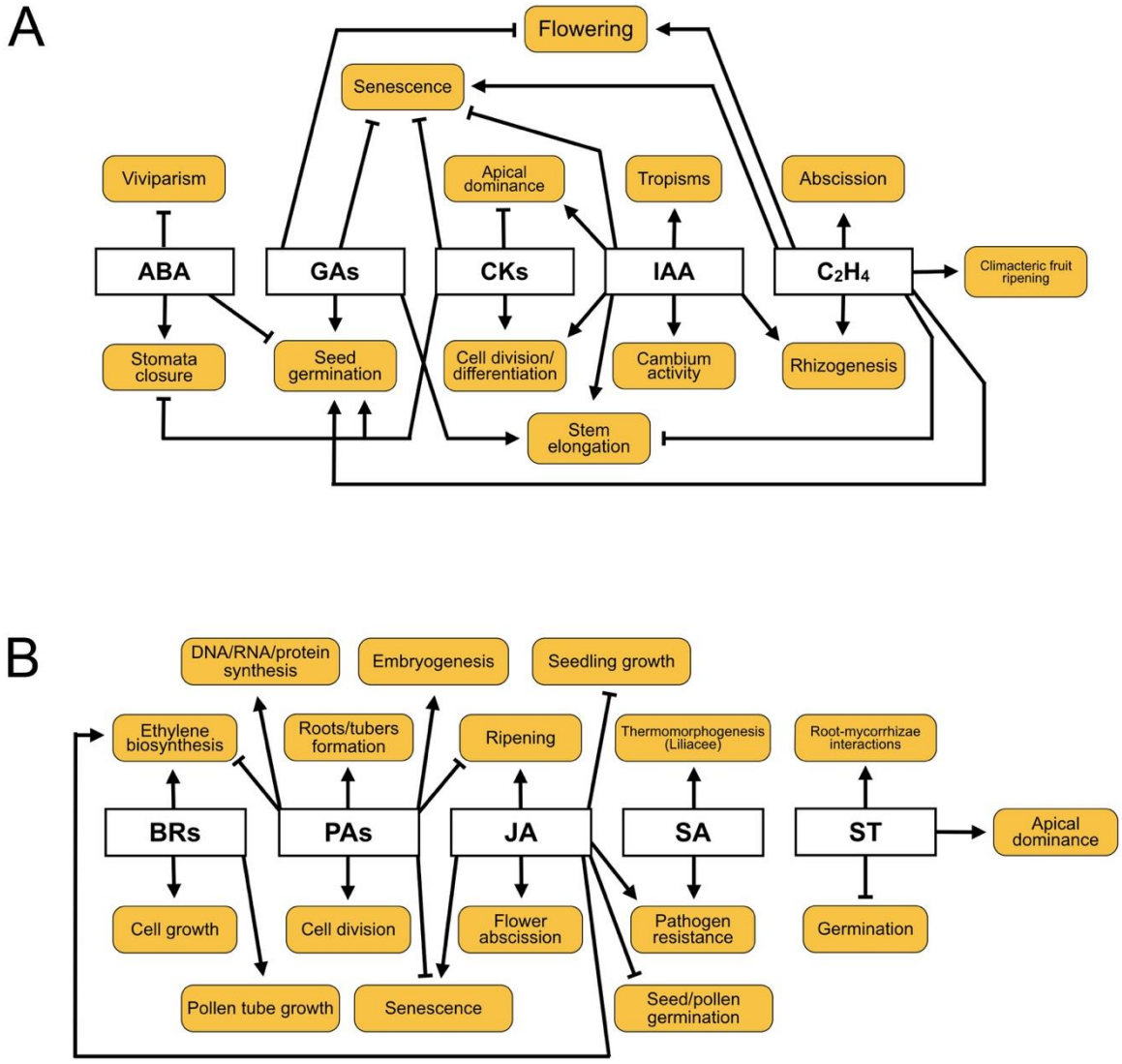

Figure 2 - Main physiological processes regulated by hormones (A) and growth regulators (B). Only the most important effects are reported (ABA, abscisic acid; GAs, gibberellins; CKs, cytokinins; IAA, indoleacetic acid; C_2H_4, ethylene; BRs, brassinosteroids; PAs, polyamines; JAs, jasmonates; SA, salycilic acid; STs, strigolactones). (modified from *Botton et al., 2007. Italus Hortus 14(1):24-36*).

This hormone is mostly synthetized in actively growing organs from tryptophan via indole-3-pyrivate and indole-3-acetaldehyde, but it may derive also from the hydrolysis of IAA-

glycoside conjugates (Figure 3). Moreover, tryptophan-deficient mutants of maize and Arabidopsis allowed to point out that an additional tryptophan-independent pathway may also exist.

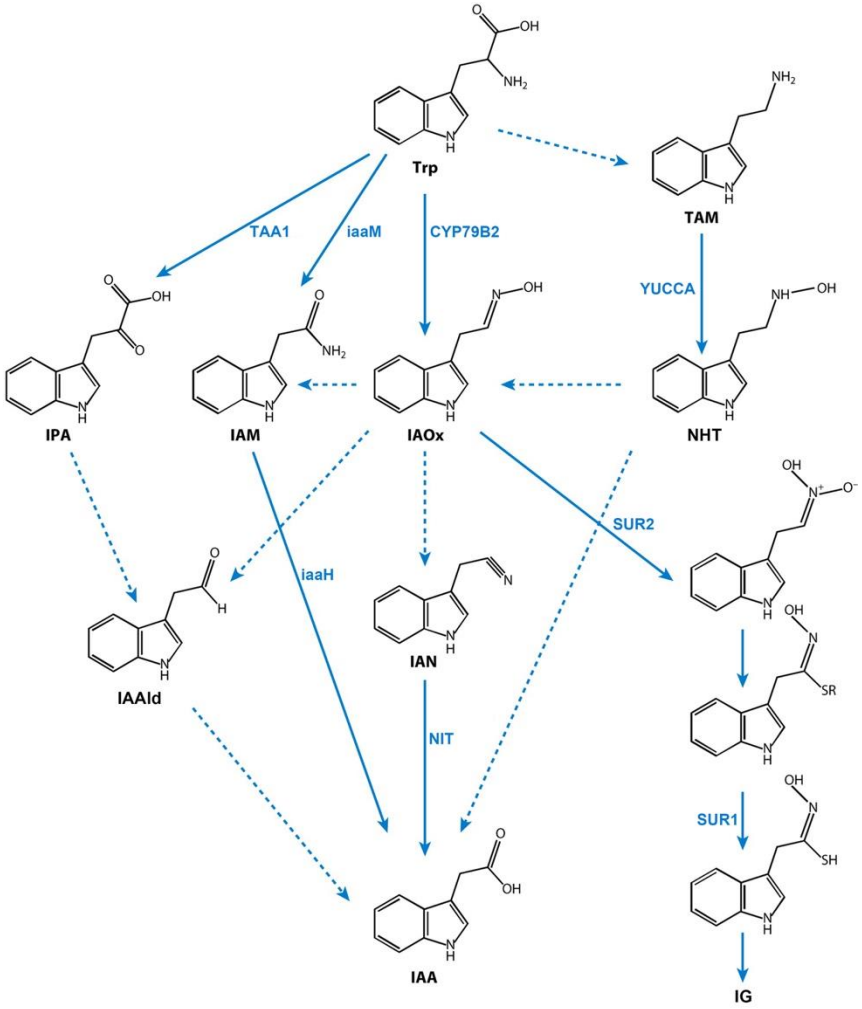

Figure 3 - Trp-dependent auxin biosynthesis pathways. Solid arrows: genes responsible for the steps have been identified in plants or microorganisms. Dashed arrows: proposed steps, but genes for the steps have not been conclusively determined. Abbreviations: Trp = tryptophan; TAM = triptamine; IPA = indole-3-piruvate; IAM = indole-3-acetamide; NHT = *N*-hydroxyl triptamine; IAOx = indole-3-acetaldoxime; IAN = indole-3-acetonitrile; IAAld = indole-3-acetaldehyde; IG = indolic glucosinolate; IAA = indole-3-acetic acid; TAA1 = Triptophano Aminotransferase of Arabidopsis 1; CYP79B2 = cytochrome P450 monooxygenase; YUCCA = flavin monooxygenase; NIT = nitrilase; iaaM = triptophan-2-monooxygenase; iaaH = hydrolase; SUR1 (SUPERROOT 1)= cystein-liase; SUR2 (SUPERROOT 2)= cytochrome P450 monooxygenase). *Modified from Zhao, 2010. Annu. Rev. Plant Biol. 61:49-64.*

Particularly interesting are the molecular mechanisms regulating polar auxin transport (PAT), as strictly related to cell polarization and differentiation. Although part of the auxins is rapidly distributed by mass non-polar transport through the phloem (5-20 cm/h), PAT

represents a unique mechanism in plants and is relevant not only from a biological point of view, but also for its possible practical applications.

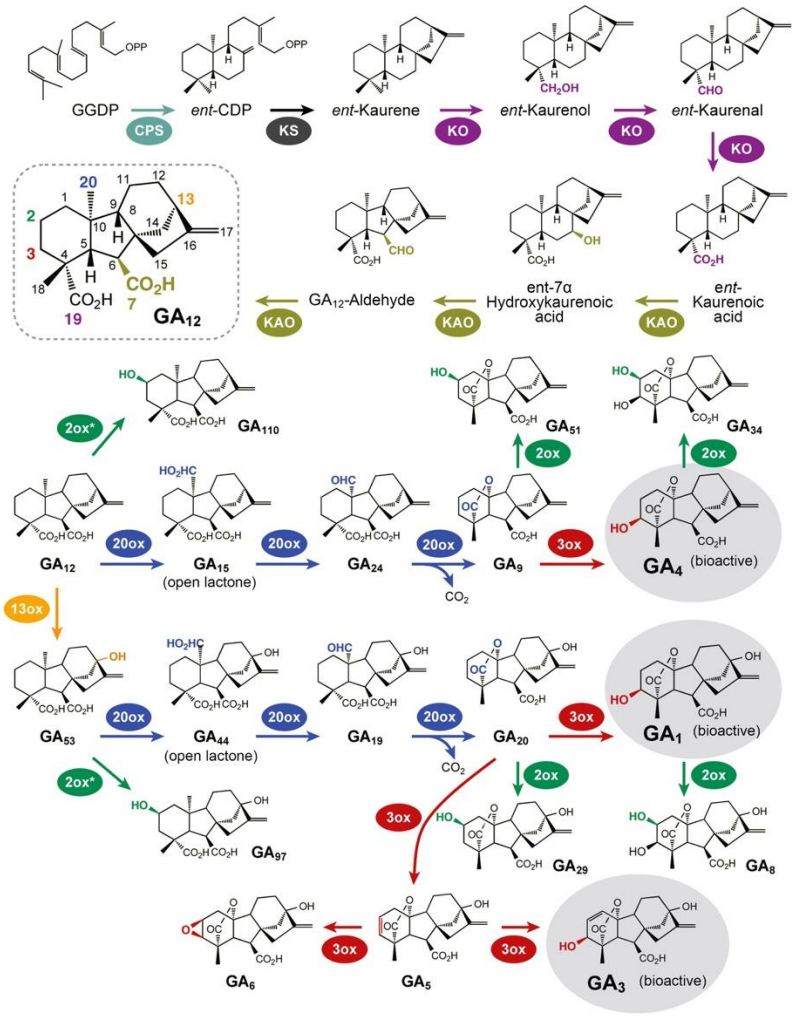

Figure 4 - GA biosynthesis pathways and deactivation by GA2-oxidase in plants. Bioactive GAs found in a wide variety of plant species (highlighted grey) are shown. GA5 and GA6 may also function as bioactive hormones. In each metabolic reaction, the modification is highlighted in colour. GA7 (13-nonhydroxy GA3) is biosynthesized from GA9 in a similar pathway to the synthesis of GA3 from GA20, but is not shown in this figure. 2ox, GA2-oxidase (Class I and II); 2ox*, GA2-oxidase (Class III); 3ox, GA3-oxidase; 13ox, GA13-oxidase; 20ox, GA20-oxidase; GGDP, geranylgeranyl diphosphate; ent-CDP, ent-copalyl diphosphate; CPS, ent-copalyl diphosphate synthase; KS, ent-kaurene synthase; KO, ent-kaurene oxidase; KAO, ent-kaurenoic acid oxidase. (From *Yamaguchi, 2008. Annu. Rev. Plant Biol. 59:225-51*).

GAs stimulate seed germination and stem elongation, while either delaying or inhibiting flowering and leaf/fruit senescence (Figure 2A). GAs are diterpenic acids synthetized from isopentenyl-pyrophosphate (IPP), after its conversion to geranylgeranyl pyrophosphate (GGPP) and the following cyclization to ent-kaurene (Figure 4), the latter reaction being catalysed by two cyclases localized within the plastid. Thereafter, *ent*-kaurene is oxidized to

ent-kaurenoic acid, GA_{12}-aldehyde and, finally, GA_{12}, by means of ER-localized cytochrome-P450-monooxygenases and cytoplasmic dioxygenases. GA_{12} is the first gibberellin of the pathway (biologically inactive) from which all the C20 and C19 GAs derive. Cloning the genes encoding the enzymes of the GAs pathway allowed to shed light on the feedback mechanisms that regulate the biosynthesis of bioactive GAs, that are the β-carboxylation on C-7 and β-hydroxylation on C-13 and C-3. Bioactive GAs may also be deactivated through β-hydroxylation on C-2, glycosylation or formation of ketoderivatives.

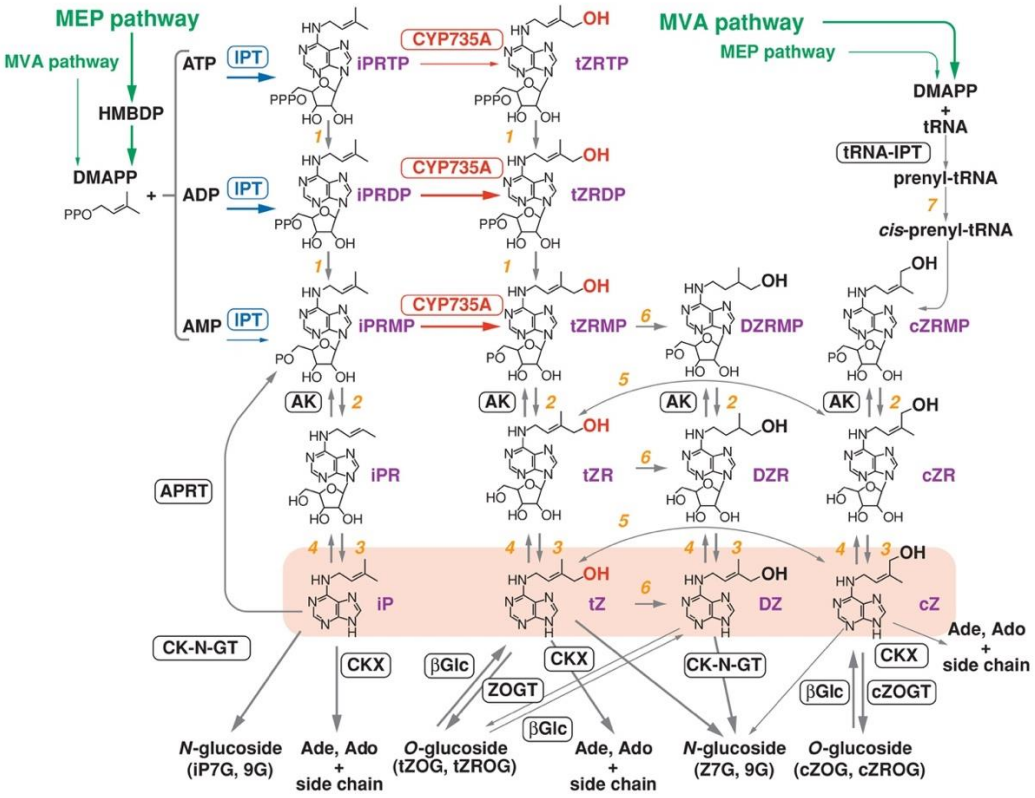

Figure 5 - Cytokinin (CK) biosynthesis in Arabidopsis. Isoprenoid side chains of N^6-(Δ^2-isopentenyl)adenine (iP) and trans-zeatin (tZ) predominantly originate from the methylerythritol phosphate (MEP) pathway, whereas a large fraction of the cis-zeatin (cZ) side chain is derived from the mevalonate (MVA) pathway (green arrows). Plant adenosine phosphate-isopentenyltransferases (IPTs) preferably utilize ATP or ADP as isoprenoid acceptors to form iPRTP and iPRDP, respectively (blue arrows). Dephosphorylation of iPRTP and iPRDP by phosphatase, phosphorylation of iPR by adenosine kinase (AK), and conjugation of phosphoribosyl moieties to iP by adenine phosphoribosyltransferase (APRT) create the metabolic pool of iPRMP and iPRDP. APRT utilizes not only iP but also other CK nucleobases. The CK nucleotides are converted into the corresponding tZ-nucleotides by CYP735A (red arrows). iP, tZ, and the nucleosides can be catabolized by CKX to adenine (Ade) or adenosine (Ado). cZ and tZ can be enzymatically interconverted by zeatin cis-trans isomerase (5). tZ can be reversibly converted to the O-glucoside by zeatin O-glucosyltransferase (ZOGT) and β-glucosidase (βGlc). CK nucleobases also can be converted to the N-glucoside by CK N-glucosyltransferase (CK-N-GT). The width of the arrowheads and lines in the green, blue, and red arrows indicates the strength of metabolic flow. Flows indicated by black arrows are not well characterized to date. tZRDP, tZR 5′-diphosphate; tZRTP, tZR 5′-triphosphate; 2, 5′-ribonucleotide phosphohydrolase; 3, adenosine nucleosidase; 4, purine nucleoside phosphorylase; 6, zeatin reductase; 7, CK cis-hydroxylase (from Sakakibara, 2006).

CKs, along with auxins, promote cell division and differentiation, delay leaf senescence, are involved in the control of apical dominance, and seem to act as a long-distance signal of nitrogen availability for root and canopy development (Figure 2A). The first enzyme of CK biosynthesis is the isopentenyltransferase (IPT), producing isopentenyladenosine-5'-monophosphate (iPMP) starting from adenosine-5'-phosphate (ATP, ADP, AMP) and dimethylallyl-pyrophosphate (DMAPP) (Figure 5). Isopentenyladenine, dihydrozeatin and zeatin, the latter being the most active cytokinin, are then synthetized from iPMP. In Arabidopsis, seven IPT genes were identified, that are differentially localized in the plastid, mytochondrion, and cytosol. CK may be further metabolized either through hydrogenation, hydroxylation, glycosylation or deletion of the lateral chain, or glycosylation or alanination of the purine ring.

ABA is generally associated to drought tolerance, soppression of viviparism, and stomata closure induced caused by water stress (Figure 2A). As for GAs, fungi can synthetize ABA from farnesyl-pyrophosphate, a C15 precursor of the terpenoid pathway. In plants, ABA is produced from 9'-*cis*-neoxanthin (C40), first oxydized to xanthoxin (C15) and then converted to ABA-aldehyde and ABA. ABA can be metabolized to phaseic acid (PA), dihydrophaseic acid (DPA), or DPA-glycoside (Figure 6). Despite the broad availabilty of ABA biosynthesis mutants, the genes of the ABA pathway have not been fully characterized yet.

Ethylene affects stem and root growth, flower development, fruit ripening, leaf senescence and organ abscission (Figure 2.2A). Methionine is the precursor of ethylene and is converted first to S-adenosylmethionine (SAM) by means of SAM-synthase, and then to 1-aminocyclopropane carboxylic acid (ACC) and 5'-methylthioadenosine (MTA) by means of ACC-synthase. The final step of ethylene biosynthesis consists in the oxidation of ACC to ethylene catalyzed by ACC-oxidase (ACO). MTA is regenerated to methionine through the Yang Cycle (Figure 7). ACS and ACO enzymes are encoded by multigenic families whose members have been in part characterized also in several tree species (mainly apple and peach). The polymorphism found among the different ACS and ACO genes was used to shed light on the two systems of biosynthesis found in plants: system 1, responsible for basal level of ethylene, and system 2, on which ethylene itself is autocatalytic.

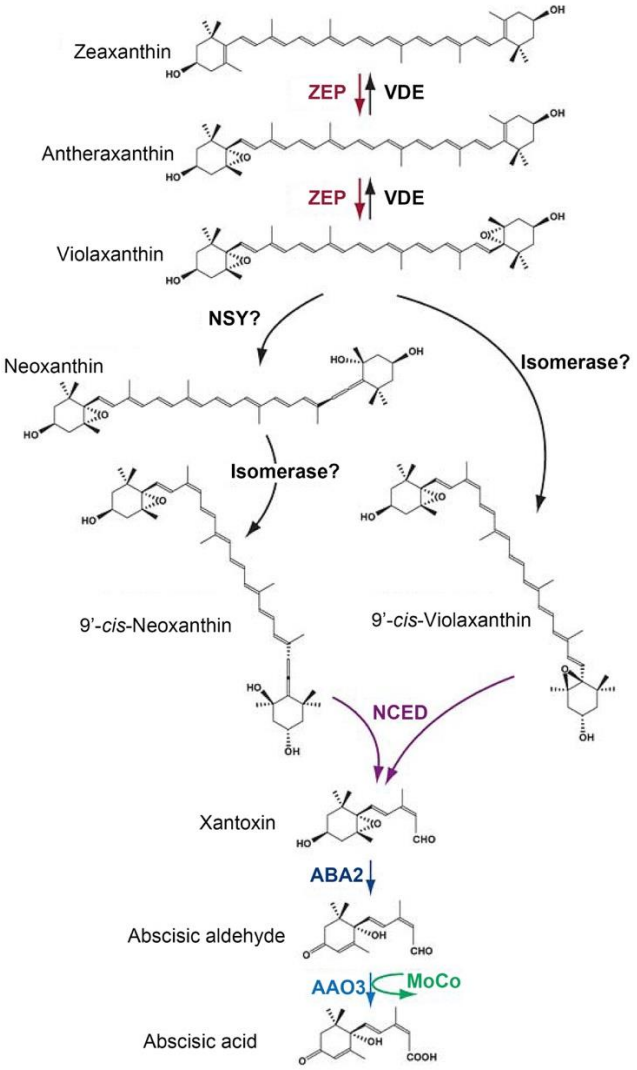

Figure 6 - ABA biosynthetic pathway. Synthesis of violaxanthin is catalyzed by zeaxanthin epoxidase (ZEP). A reverse reaction occurs in chloroplasts in high light conditions catalysed by violaxanthin de-epoxidase (VDE). The formation of cis-isomers of violaxanthin and neoxanthin may require two enzymes, a neoxanthin synthase (NSY) and an isomerase. Cleavage of cis-xanthophylls is catalysed by a family of 9-cis-epoxycarotenoid dioxygenases (NCED). Xanthoxin is then converted by a short-chain alcohol dehydrogenase (ABA2) into abscisic aldehyde, which is oxidized into ABA by an abscisic aldehyde oxidase (AAO3). AAO3 protein contains a molydenum cofactor activated by a MoCo sulfurase (from Nambara and Marion-Poll, 2005).

Brassinosteroids (BRs) stimulate cell division and expansion, stress tolerance, vascular system differentiation, leaf development and photomorphogenesis (Figure 2B). BR synthesis or perception mutants show severe alterations of growth as well as reduced fertility. Brassinolide is the most active BR, and is synthetized from campesterol. Polyamines (PAs) are involved in DNA, RNA and protein synthesis, and in several other growth and developmental processes (Figure 2B). Putrescine (Pu), spermidine (Sd) and spermin (Sm)

are synthetized from arginine and ornithine by adding an aminopropylic group derived from SAM. For this reason, there may be some competition between PAs and ethylene biosynthesis, particularly under SAM limitation. PAs metabolism involves oxidation and conjugation to *p*-coumaric, ferulic, and caffeic acid.

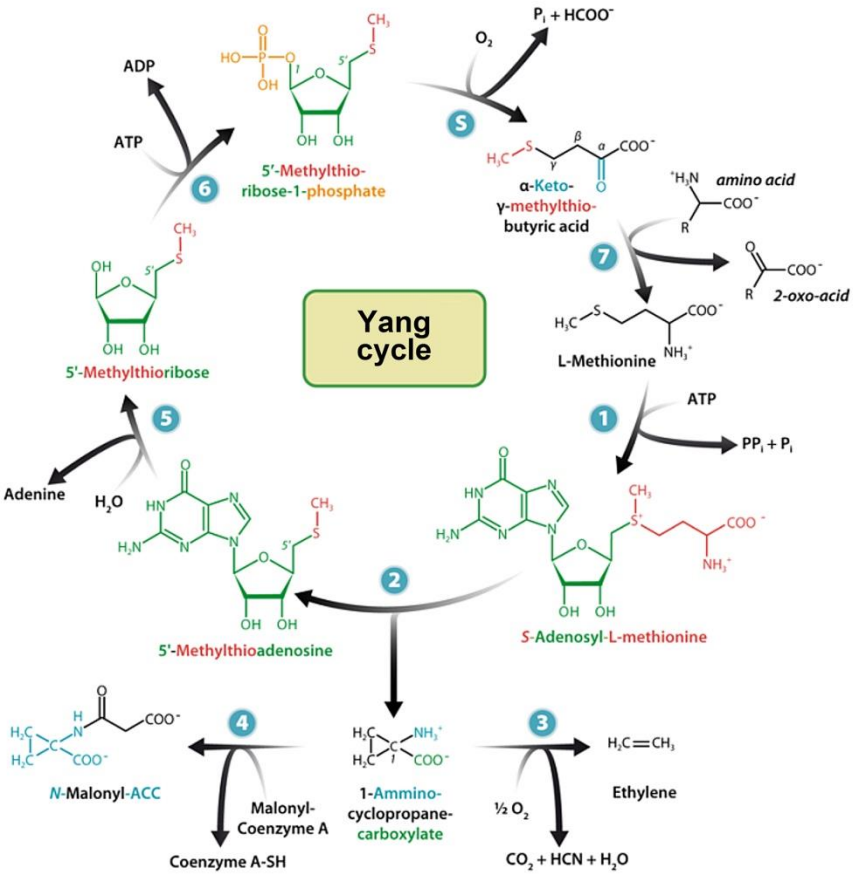

Figure 7 - Ethylene biosynthesis and Yang's Cycle. SAM formation is catalysed by SAM synthase (1) from methionine and ATP. ACC is the intermediate precursor of ethylene. Conversion of SAM to ACC + MTA by ACC synthase (ACS; 2) represents a rate-limiting step. MTA recycling allows to maintain a constant level of methionine even under high ethylene production. ACC malonylation to malonyl-ACC deprives ethylene biosynthesis from ACC. ACC oxydase (ACO; 3) catalyses the final step of ethylene biosynthesis, using ACC as substrate and generating carbon dioxide and cyanuric acid as secondary products. (4, ACC *N*-malonyl transferase; 5, MTA nucleosidase; 6, MTR kinase; 7, transaminase; S, spontaneous reaction). (image taken from *Wikipedia, http://en.wikipedia.org/wiki/ File:Yang-cycle.png)*.

Jasmonates (JAs) are associated to disease resistance, inhibition of seed/pollen germination, seedling growth, and may stimulate flower abscission and fruit ripening (Figure 2.2B). JAs derive from α-linolenic acid through the key limiting action of allene oxyde synthase (AOS), which catalyses the conversion of 13(S)-hydroperossilinolenic acid into 12,13(S)-epossilinolenic acid. Jasmonic acid can be metabolized through its hydroxylation or

conjugation to either glucose or aminoacids. Salicylic acid (SA) is involved in pathogen resistance mechamisms and thermogenesis (Figure 2B). It is synthetized from *trans*-cynnamic acid through a branch of the phenylpropanoid pathway and metabolized to SA-glycoside and glycosylester of 2,5-dehydrobenzoic acid. Finally, strigolactones regulate the plant-mycorrizal interactions and control seed germination and axillary meristem development, playing an important role in apical dominance, thus determining the plant's architecture. Strigolactones are 4-ring lactoterpenes derived from carotenoids metabolism. Their biosynthesis is controlled by the RAMOSUS (RMS) enzymes, whose products regulate apical dominance by interacting with IAA and CK signalling.

The importance of hormones for apple growers

Apple fruit is an important commodity and represents an economically relevant crop for Italy. Therefore, an increasing attention is being paid to the environmental impact of apple cultivation, its increasing production costs, and the improvement of the final quality of the products, in order to be more competitive, especially for the European market, and respond to the increasing demand of environmentally-friendly agriculture. Each of these issues relies upon the specific features of apple fruit developmental cycle, which is characterised by key stages requiring specific exogenous treatments and agronomic practices aimed to reducing losses and increasing quality. During the apple fruit development there are two main critical steps that affect the productivity and the final quality of the commodity, the first during early stages of fruit development that are characterized by the physiological fruit drop, and the last stages in which the ripening process occurs, eventually preceded by a pre-harvest fruit abscission (PFA). In this scenario, treatments with several bioregulators (with both hormones, natural or synthetic, and molecules that inhibit the endogenous hormones biosynthesis or perception) may play an important role by perturbing the normal hormonal endogenous status and are frequently used to controls numerous physiological processes. In fact, for example, the physiological fruit drop is a natural process in which the trees release fruitlets in order to balance the resources between the vegetative and reproductive organs. An appropriate fruit load is a desirable trait mainly for two reasons, first to guarantee the flowering return in the following year, and thus avoid the biennial bearing, second the number of fruits *per* tree may affect the size and the final quality of the product. For some cultivars, the physiological fruit drop is not able alone to assure an optimal fruit load to

achieve an acceptable quality of the final products, and thinning is thus required. Trees can be thinned either manually, with high impacts in terms of cost and low efficiency, mechanically, requiring suitable tree forms and specific pruning, or chemically, although often with relevant environmental impacts. Among the chemical thinners, benzyladenine (BA), a cytokinin-like compound, is widely employed in ordinary orchard practice. Its effect relies on exacerbating the existing nutritional competitions between shoot and fruitlets (Bubàn and Lakatos, 2000; Quinlan and Preston 1971), by stimulating cell division at the level of the vegetative organs, thus requiring an additional support in terms of assimilates (Eccher et al., 2014 and 2015). Other commercial compounds that are used as thinning agents are Ethephon®, that is converted to ethylene by the plant, reducing the number of flowers and thus the final number of fruit *per* tree, and 1-naphthaleneacetic acid (NAA), a synthetic auxin. Other treatments at the early stages are done with the commercial compound Promalin®, a BA and $GA_{4/7}$ mixture, that improves the final size of the product stimulating cell division. In the last developmental stages, some treatments are also useful to improve the final quality and reduce fruit loss. For example, pre-harvest treatment with ReTain®, L-aminoethoxyvinilglycine (AVG), and/or with NAA can delay PFA, maturity, and improve fruit size and the natural coloration of red varieties. Moreover, methyl-jasmonate treatments could improve ester volatile production. The effect exerted by bioregulators depends on their capability to alter the normal hormonal endogenous status and the mechanisms of actions of the different phytohormones. It is noteworthy that, in order to use these molecules in the appropriate and most efficient way, a thorough knowledge of both the biosynthetic pathway of phytohormones and their mechanism of action is necessary. In fact, a hormone can regulate different physiological processes and an exogenous application can produce different effects in relation to the developmental stages of the fruit in which the treatment is applied. A clear example is given by auxin treatments (i.e. NAA) that may have indeed opposite effects on fruitlet abscission at a distance of few days. Despite of the important results obtained up to now with these compounds in the field, it is necessary to improve the knowledge on fruit development and growth, the related physiological processes, and hormonal cross-talk, in order to set up new strategies that link together the necessity of an environmental friendly agriculture, a safe and healthy fruit, and an other economical sustainability of the

Aim of the research

Only few studies report the quantification of endogenous hormones in apple fruit. Moreover, available information is usually restricted to the most characterized hormones, such as ethylene, auxin and ABA, and limited to a particular developmental phase. Taking this into account and considering the importance of hormones within the context of apple fruit development, both from a basic research point of view and for the relevant applied aspects, a specific subproject was addressed to a detailed hormone profiling during the whole developmental cycle. To this aim, a simultaneous quantification of several hormones/precursors was pursued in order to give an overall view of the changes of hormones' levels occurring during the whole apple fruit developmental cycle in relation to the specific phases.

Materials and methods

Experimental setup and plant materials

Experiments were conducted in 2014 on 9 years-old apples trees of cv Golden delicious/M9 grown with a slender spindle form and trained with standard horticultural practices, with no hormonal treatments, at the experimental field "Piovi" (Roverè della Luna, Trento, Italy; Figure 8) of the Istituto Agrario San Michele all'Adige, Edmund Mach Foundation (Trento, Italy).

For each apple tree, fruit load was manually normalized according to the trunk section area (TSA), up to a value of 6 fruits/cm^2. A randomized block design was adopted, with three blocks, each including five trees.

Fruit cross diameter was measured weekly from full bloom up to the last sampling date, by using a manual caliper and recording diameters of 120 fruits.

Samples collection started when flowers were still closed but well developed (unpollinated flowers), to proceed up to fruit ripening *in planta*, according to the following time course (expressed in days after full bloom, DAFB): -3, 4, 13, 20, 31, 39, 53, 67, 88, 103, 118, 124, 132, 139, 146, 157, and 172. At the beginning samples included the whole receptacle (i.e. with epidermal tissue), whereas from 20 DAFB only the cortical tissue was collected. Plant material was immediately frozen in liquid nitrogen and stored at -80°C. All samples were collected during the morning, from 9 pm within maximum 2 hours.

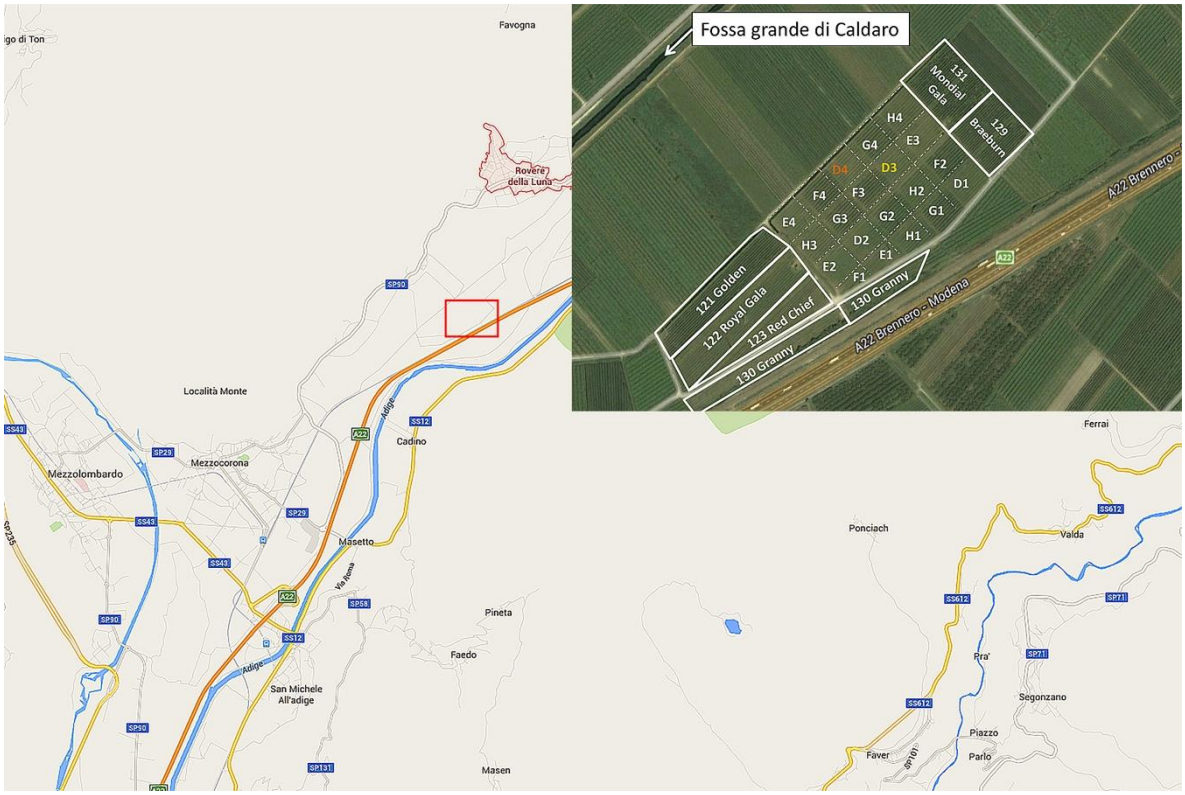

Figure 8 – Map showing the location Roverè della Luna and the exact place where experiments were carried out (red rectangle) (taken from Google Maps). Detailed map of the experimental field "Piovi" (top right) with plots D3 (yellow) and D4 (orange) that were used to select the trees for the experiments (modified from Google Maps).

Sample selection

A restricted number of samples (Table 1) was selected for all the hormonal measurements according to their representativeness of a particular developmental phase, taking into account a full coverage of the fruit developmental cycle as a whole. The whole procedure is described in Chapter 2.

Hormones' measurements

Hormonal analysis was carried out on 6 biological replicates of 10 selected samples, covering the whole developmental cycle, with the only exception of the sample at -3 and 4 DAFB for which only three biological replicates were available. Nineteen compounds, namely abscisic acid (ABA), bioactive gibberellins (GA_1, GA_3, GA_4 and GA_7) and some of their precursors (GA_9, GA_{19}, GA_{20} and GA_{24}), bioactive cytokinins (zeatin, Z; dihydrozeatin, DZ; 2-isopentenyladenine, iP), CKs precursors (isopentenyladenosine, iPR; zeatin riboside, ZR; dihydrozeatin riboside, DZR), jasmonic acid (JA), salicylic acid (SA), indoleacetic acid

(IAA), and the ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (ACC), were quantified with liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UPLC/ESI-MS-MS), as reported in Müller and Munnè-Bosch (2011), with minor modifications. For all samples, 100 mg of frozen material was extracted with methanol:isopropanol:glacial acetic acid mixtures, 50:49:1 (v/v/v) + 1% of Butylhydroxytoluene (BHT) as an antioxidant.

Headspace ethylene accumulation was measured on fifteen fruits with a gas-cromatograph Thermo-Finnigan Focus GC equipped with a FID detector and a capillary column MEGA PS264 (length 50 m, thickness 0.32 mm).

Table 4 – Samples selected for hormone profiling. For each sample, the time of sampling, a name, a description of the developmental stage and an additional code are given.

Sampling time (DAFB)[1]	Sample name	Description of the developmental stage	Sample code[2]
-3	T0	pre full bloom	PFB
4	T1	full bloom	FB
13	T2	early cell division	ECD
20	T3	cell division	CD
53	T4	late cell division/early cell expansion	LCD/ECE
88	T5	cell expansion	CE
132	T6	late cell expansion/immature	LCE/IMMATURE
146	T7	mature (pre-climacteric)	MATURE
157	T8	commercial harvest	CH
172	T9	ripening	PH

1) Full bloom is equal to 0 DAFB.
2) As referred to fruit development.

Statistical analyses

All multiple comparison statistics were calculated using the R software version 3.2.2 (www.r-project.org/). In detail, normality was verified with Shapiro-Wilk test, homoscedasticity was verified with Bartlett's and/or nonparametric Levene's test, and differences among samples were verified with either ANOVA (normality and homogeneous variances) or Welch's one-way ANOVA (normality and non-homogeneous variances) followed by post hoc LSD or Waller-Duncan test, respectively, and with Kruskal-Wallis (non-normality and homogeneous variances) or Friedman test (non-normality and nonhomogeneous variances). For all statistics a *P*-value threshold of 0.05 was adopted.

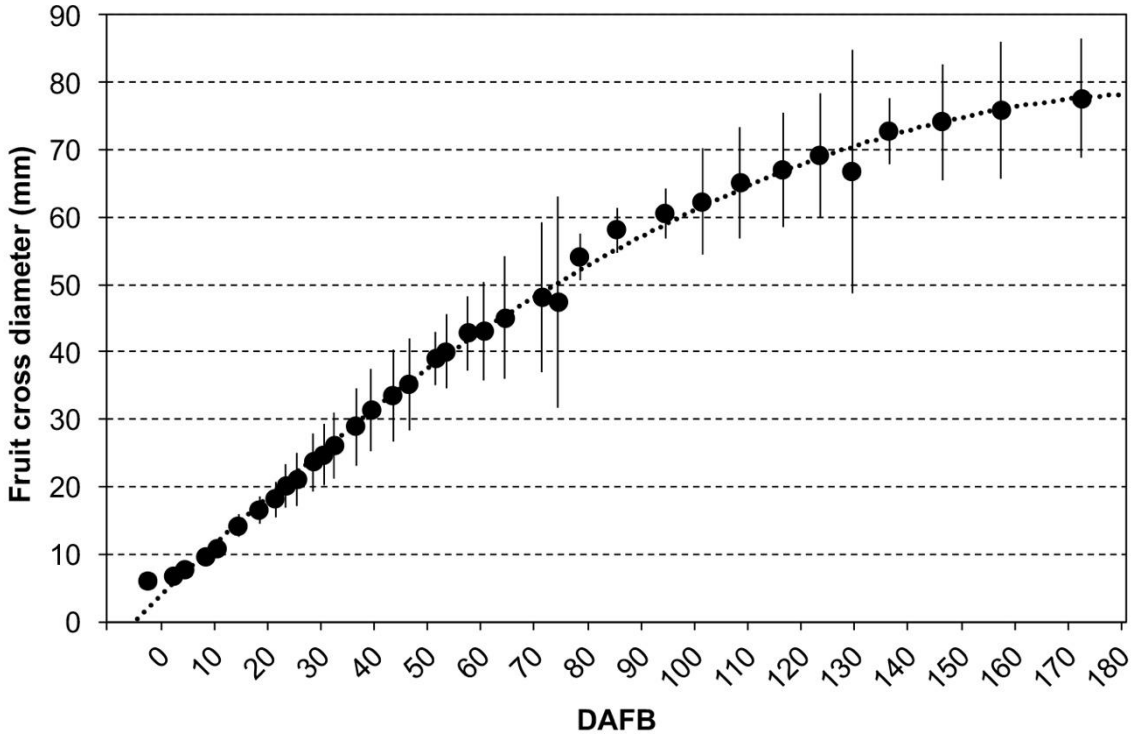

Figure 9 – Fruit growth kinetics obtained by measuring fruit cross diameter from at 120 fruits of cv Golden Delicious per time point (year 2014). Bars represent standard deviation.

Results and discussion

Fruit growth kinetics

Fruit growth kinetics (Figure 9) substantially paralleled the results obtained in 2011 (see Chapter 2, Figure 1). The characteristic single sigmoidal trend was displayed, with typical features such as: 1) the rapid growth observed at early stages due to cell division, 2) the constant growth due to cell expansion, and 3) the progressive plateau observed at the end of fruit development through ripening and senescence.

Quantification of endogenous hormones

Gibberellins

The levels of active GAs during fruit development are shown in Figure 10. It is notable that a wide range of concentrations was measured, ranging from 10^2 ng/g for GA_4 to 10^{-1} ng/g for GA_7, thus indicating possible different roles of the different GAs during fruit development.

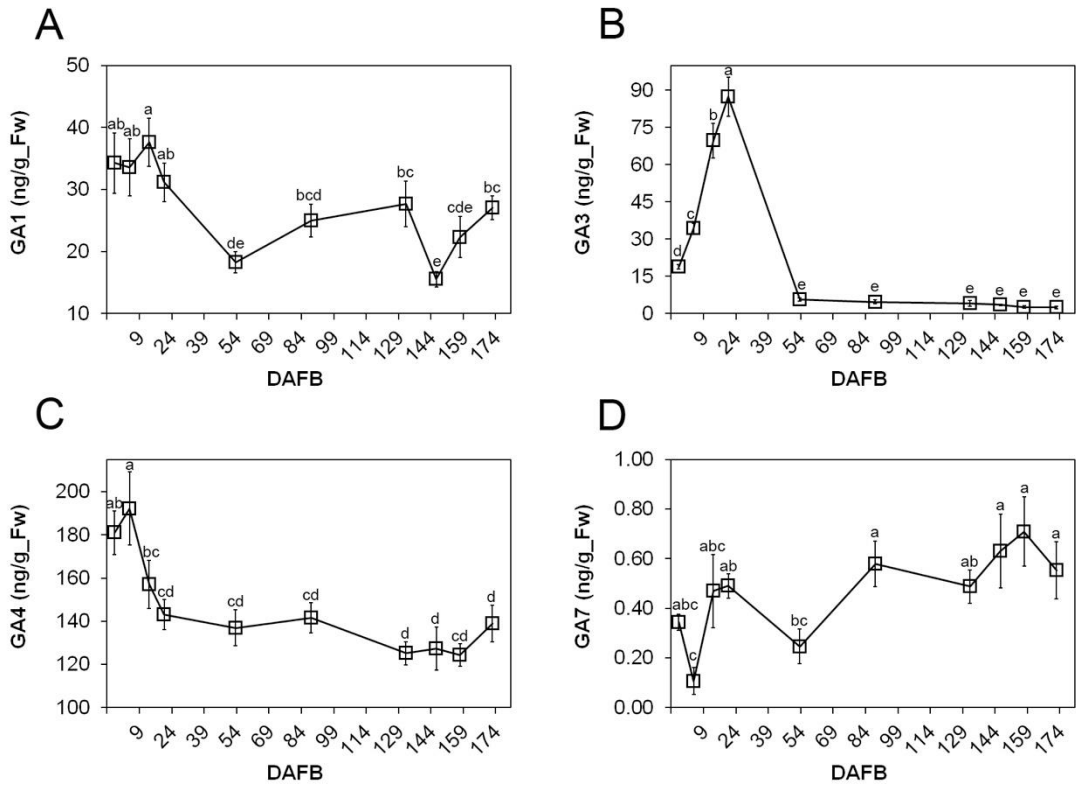

Figure 10 – Endogenous concentration of bioactive gibberellins expressed as nanograms per gram of fresh weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom.

The GA_1 showed an oscillatory trend (Figure 10A). Its levels were high in the first samples with a peak at 13 DAFB, decrease significantly up to 53 DAFB and, then, increased again until the immature stage (132 DAFB) When the fruit underwent maturation (132-146 DAFB), its amount dropped rapidly, to finally increased during fruit ripening. These results may ascribe a possible role of this GA immediately after fertilization occurred, but not during cell division, when its level dropped. However, the main correlation was reported with cell enlargement (53-132 DAFB), when it increased again, while its sudden drop when the fruit is undergoing maturation and the rapid increase during ripening may indicate a pivotal role also in these stages.

Concerning GA_3 content (Figure 10B), its levels rapidly increased during the full cell division phase, up to 20 DAFB, and then immediately dropped down, becoming almost undetectable along all the other stages. These results clearly suggest that GA_3 is deeply involved during

early fruit development, possibly playing the role that was recently claimed for GAs by Botton et al. (2011).

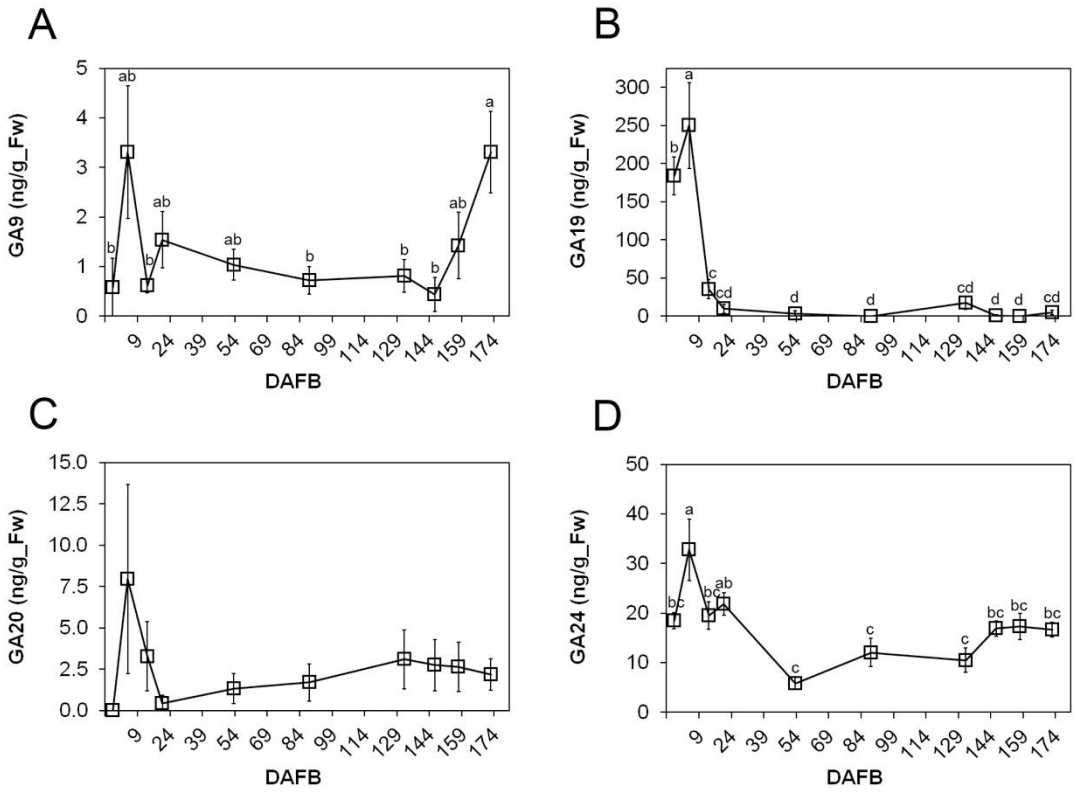

Figure 11 - Endogenous concentration of selected gibberellins' precursors expressed as nanograms per gram of fresh weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom.

Generally, the GA_4 was often shown to be the most active and abundant gibberellin in plants (Yamaguchi, 2008). Consistently, its concentration in the receptacles was the highest among all samples and GAs, especially at advanced full bloom, followed by a clear decrease at early fruit development, *i.e.* fruit set (Figure 10C). Then, its levels in the apple fruit remained high and stable (with respect to the other gibberellins) throughout whole development. These results may indicate a constitutive role of this GA in the maintenance of basal physiological process that are essential along all fruit development and that may not be directly related to fruit growth.

Finally, GA_7 concentration (Figure 10D) was very low (10^{-1}ng/g FW) with respect the other GAs. Its pattern was quite erratic, although tendentially increasing during whole development. Based upon these results, it is difficult to ascribe a role to this GA as related to the different fruit developmental stages.

Regarding the four GAs precursors (Figure 11), a common pattern was reported at early fruit development, with a peak at full bloom followed by a general decrease. Thereafter, GA_9 and GA_{24}, precursors of both GA_4 and GA_7, showed an increasing trend that corresponded to the ripening *in planta*, coherently with the trends of the active forms. GA_{19} rapidly decreased at 13 DAFB and remained at very low levels thereafter. Similarly, GA_{20}, although with no statistically significant variation.

ABA, JA, SA, ACC and ethylene

ABA levels (Figure 12A) started to decrease immediately after fertilization (4-13 DAFB) and then, more rapidly, up to 53 DAFB, thus fitting the model proposed for other fruits, such as tomato (Vriezen et al., 2008), and for apple itself (Botton et al., 2011). Thereafter, the levels of this hormone remained very low up to the late cell expansion, and at 146 DAFB started to gradually increase up to the final stage. This trend is clearly visible, although with no statistical significance.

Regarding the ethylene precursor ACC (Figure 12B), after the very high initial levels measured in non-pollinate flowers, its amount decreased rapidly during early development and then showed a small, but statistically significant, peak at 53 DAFB, at the transition between cell division and cell enlargement. Thereafter, its concentration decreased again and remained constant up to the mature stage. In the last two time points, corresponding to the ripening stage, ACC concentration increased significantly, as expected. It is worthy to note that in the last two samples (*i.e.* 157 and 172 DAFB) the ethylene precursor concentration is likely to be inversely correlated with ethylene biosynthesis, due to a a rapid conversion of ACC into the gaseous hormone. For the timecourse shown in the present research, expression levels of the markers shown in Chapter 2, especially *MdACO1*, along with the ethylene measurements made on fruits of the last three samples (Figure 13) indicate that the transition between system 1, in which ethylene has a negative feedback regulation on its own biosynthesis, to system 2, in which the hormone positively induces his own production (Tan et al., 2013), is likely to have occurred between 146 and 157 DAFB. According to these data, the samples collected at 146 DAFB can be labelled as "pre-climacteric", with a very low and almost undetectable ethylene production, while the last samples (at 172 DAFB) as "post-climacteric", with an ethylene biosynthesis significantly decreased with respect to the

previous date; the sample at 157 DAFB can be either climacteric or, in any case, around that, with system 2 fully activated and a very high ethylene biosynthetic rate.

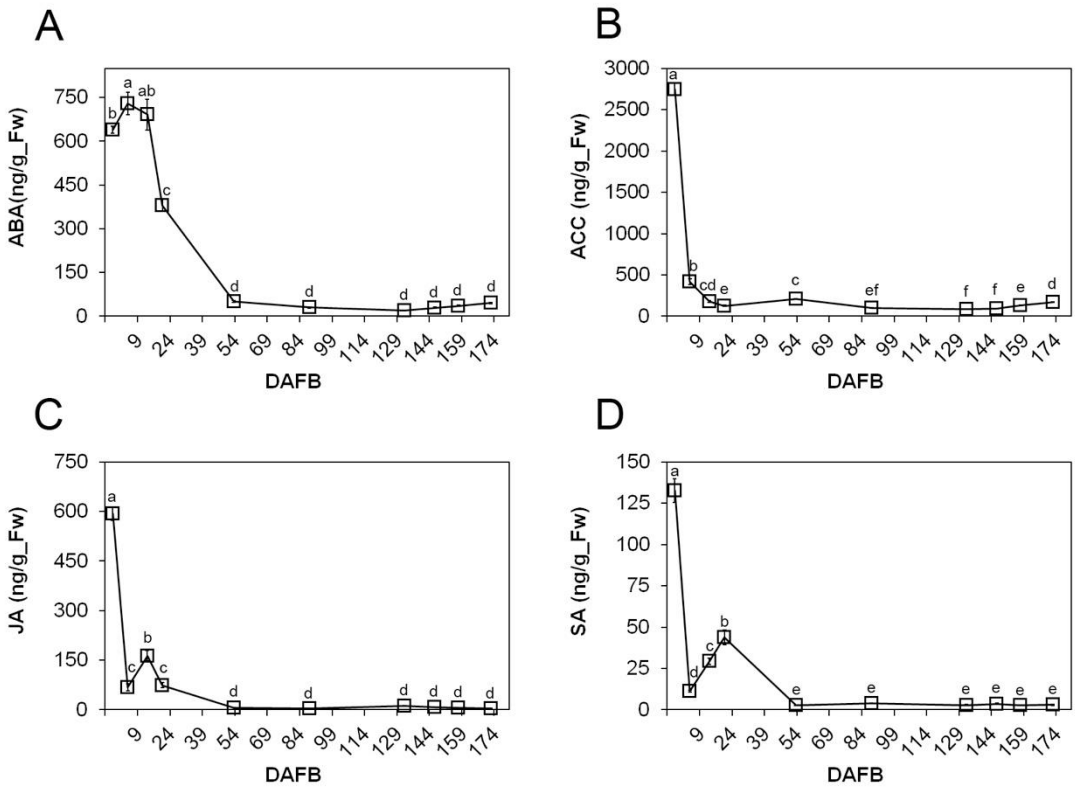

Figure 12 - Endogenous concentration of abscisic acid (ABA; A), 1-amino-cyclopropane-1-carboxylic acid (ACC; B), jasmonic acid (JA; C), and salicylic acid (SA; D), expressed as nanograms per gram of fresh weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom.

JA showed an accumulation pattern very close to that of ACC (Figure 13C), except for the sample at 13 DAFB (early cell division), which showed a significant peak, and the constant low levels observed in this case up to the end of the timecourse.

SA (Figure 13D) showed a similar pattern, but the increasing trend observed after the second date lasted up to 20 DAFB. Concerning SA, it is worthy to note that its levels dropped just at the transition between cell division and cell expansion. A role of SA in inhibiting the latter process in several species, but not in apple, has already been pointed out (Rivas-San Vicente and Plasencia, 2011) and can be extremely interesting also within the context of apple fruit development.

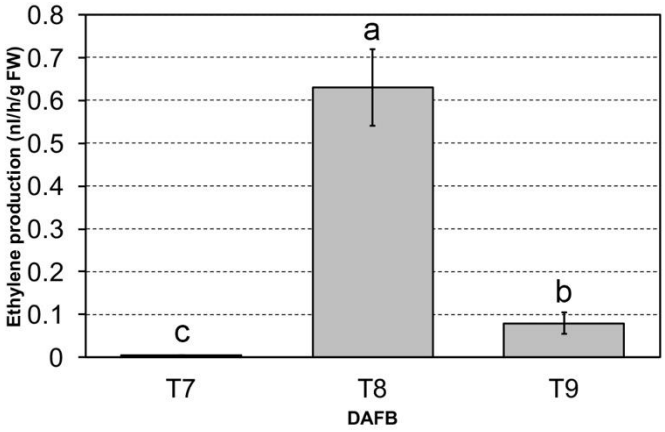

Figure 13 – Ethylene biosynthesis measured in fruits of T7, T8, and T9 (see Table 1 for sample names and a detailed description), collected at 146, 157, and 172 DAFB, respectively. Bars represent standard deviation. Different letters distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom.

Cytokinins

Quantification of active cytokinins and some of their glycosylated precursors is shown in Figure 14. In general, these compounds are present at very low amounts with respect to those previously shown. The levels of the glycosylated precursors iPR, ZR, and DZR was always higher than the active cytokinins derived from their conversion and with similar accumulation patterns, i.e. with the highest values in flowers' receptacle (-3 DAFB, before full bloom and fertilization), followed by a rapid decrease thereafter up to 20 DAFB.

The active forms showed instead different behaviours. While iP was highly correlated to its precursor iPR during the whole timecourse, zeatin showed a significant peak at 20 DAFB, possibly consistent with the concurrent drop of its precursor ZR, followed by a drop to constant levels at 53 DAFB. In this phase, zeatin may be the principal CK involved in the regulation of cell division, during which its biosynthesis is likely to be specifically controlled at both genetic and enzymatic level. Following this stage, its levels dropped down and the cells started to expand under the control of other hormones. DZ levels were erratic and did not differ significantly from a statistical point of view.

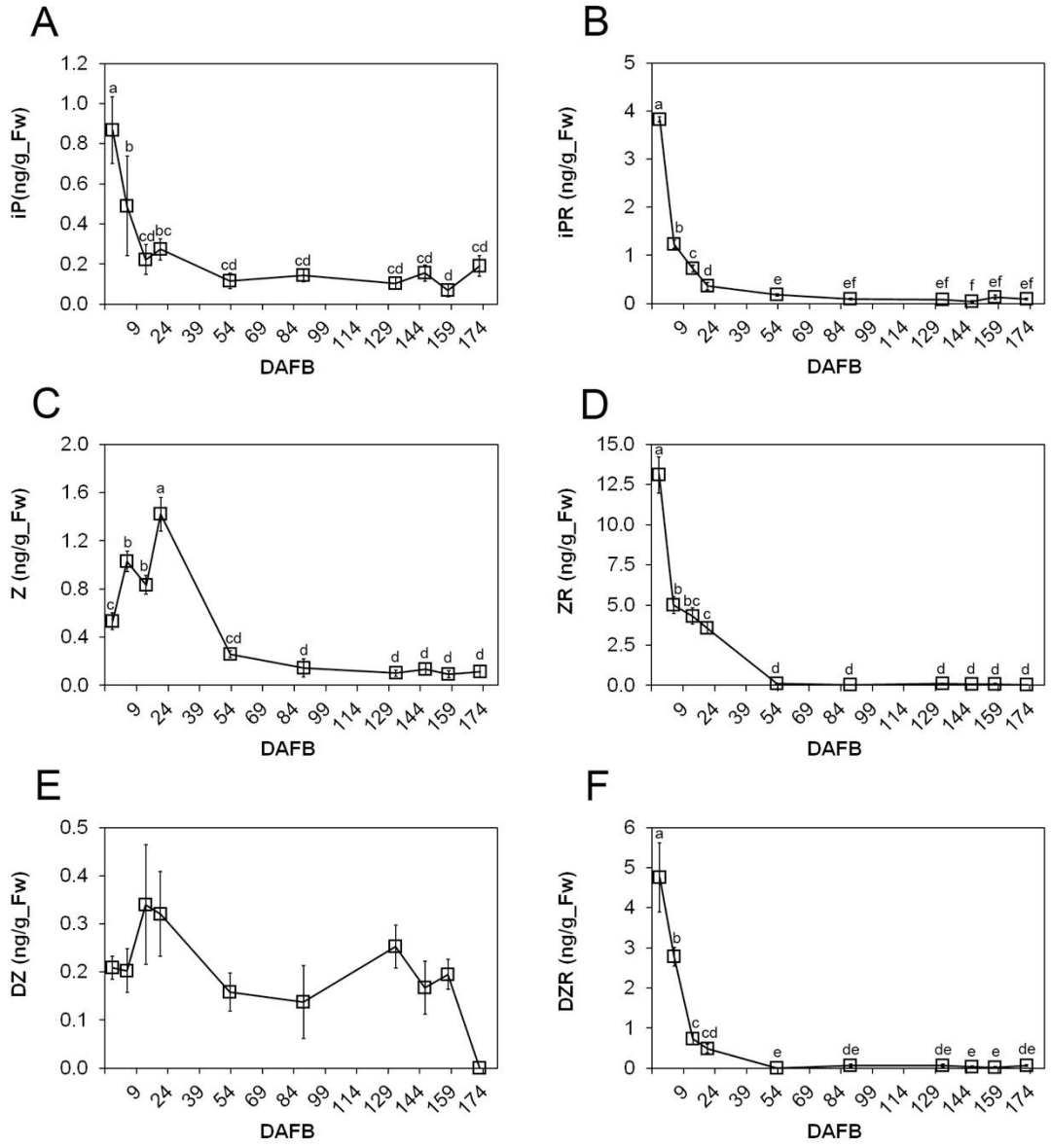

Figure 14 – Endogenous concentrations of active cytokinins (2-isopentenyladenine – iP, A; zeatin - Z, C; dihydrozeatin - DZ, E) and glycosylated precursors (isopentenyladenosine - iPR, B; zeatin riboside - ZR, D; dihydrozeatin riboside – DZR, F) expressed as nanograms per gram of fresh weight of fruit cortex. Bars represent standard error. Different letters, where present, distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom.

Indol-3-acetic acid

Quantification of IAA was hindered by some oxidation phenomena occurring during the preparation of the first three samples (-3, 4, and 13 DAFB). Therefore, despite the use of butylhydroxytoluene as an antioxidant, an accurate quantification was not possible at these developmental stages and a higher standard error was reported. In spite of this, for the remaining time points no oxidation problems occurred and the quantification was very

reliable. Indoleacetic acid showed a peak at the cell division stage (20 DAFB), then decreased thereafter up to 53 DAFB remaining at very low levels throughout the cell expansion phase. A significant increase was observed in correspondence of fruit maturation (146 DAFB), at the pre-climacteric phase, followed by a drop and a new increase in the last sample. As reported for other fruits (Trainotti et al., 2007; Böttcher et al., 2010) and also shown in apple (Schaffer et al., 2012), an increase of auxin often occurs before the onset of fruit ripening, prior to the enhancement of ethylene biosynthesis and the transition to system 2 of its biosynthesis.

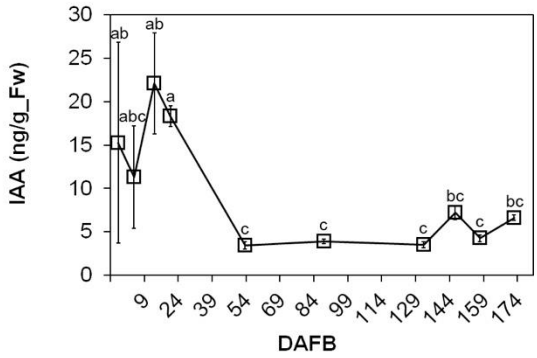

Figure 15 - Endogenous concentration of Indol-3-acetic acid (IAA) expressed as nanograms per gram of fresh weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom.

Conclusions

The hormone profiling herein described allowed to give an overview of the variations in the levels of the most important hormones during apple fruit development and their possible involvement at each stage and, possibly, transitions between stages. A principal component analysis (PCA) was performed to summarize these results (Figure 16), but the first two principal components were able to explain only 58.58% of the total variance. It is worthy to note that, according to the PCA, the role of the hormones becomes indispensable just after fertilization occurs, as evidenced by the differences between the first sampling date and the following stages. This, however, does not exclude or minimize the role of hormones during flower development, in part already studied and known (Chandler, 2011), but the hormonal perturbations occurring after full bloom are so significantly correlated with fruit set that this moment represents, undoubtedly, the most interesting one.

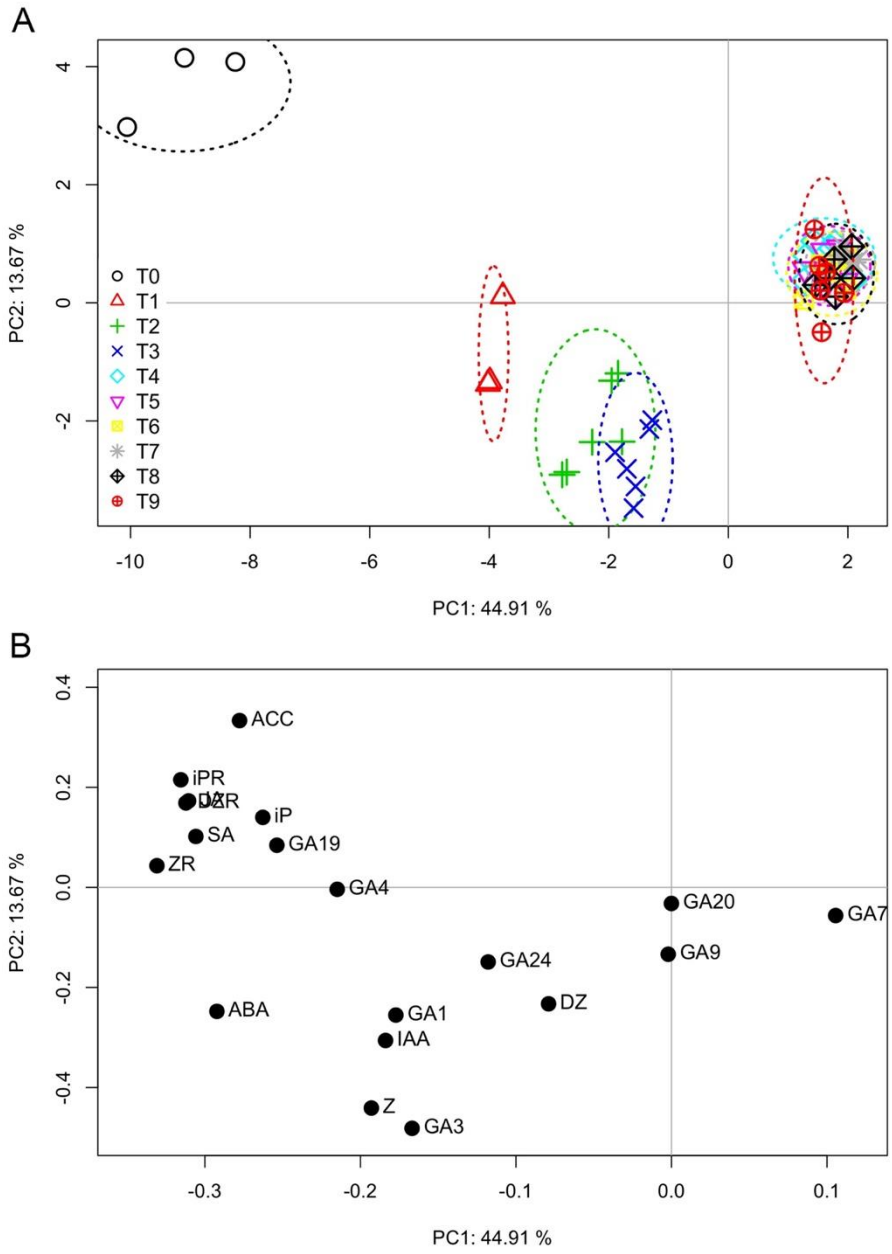

Figure 16 - Principal component analysis (PCA) of hormones measured on different biological replicates of apple fruit cortex sampled throughout development (from T0 up to T9; see Table 1 of Chapter 3 for sample codes). (A) PCA scores plot of the first two principal components (PC1 and PC2) explaining 58.58% of the total variance. (B) PCA loadings plot (see Table 4 for acronyms).

Considering the low amount of variance explained by the first two principal components, it is not surprising the PCA was not able to separate samples after the T3 (20 DAFB). Also for this reason, an "empirical" model was also built to focus on the most important hormonal players (Figure 17) during apple fruit development. However, the most important achievement of this research is related not only to the quantification data themselves, but

especially to the fact that the levels of all those hormonal compounds and some of their precursors were, for the first time in a fleshy fruit, precisely a pome, measured at the same time in the same samples. In literature, many reports describe the variation of the levels of several plant hormones in the fruits (Eccher et al., 2013; Srivastava and Handa, 2005; Zaharah et al., 2012; McAtee et al., 2013) but all those studies were carried out on different species, genotypes and environments, with various technologies, and often on one hormone by one.

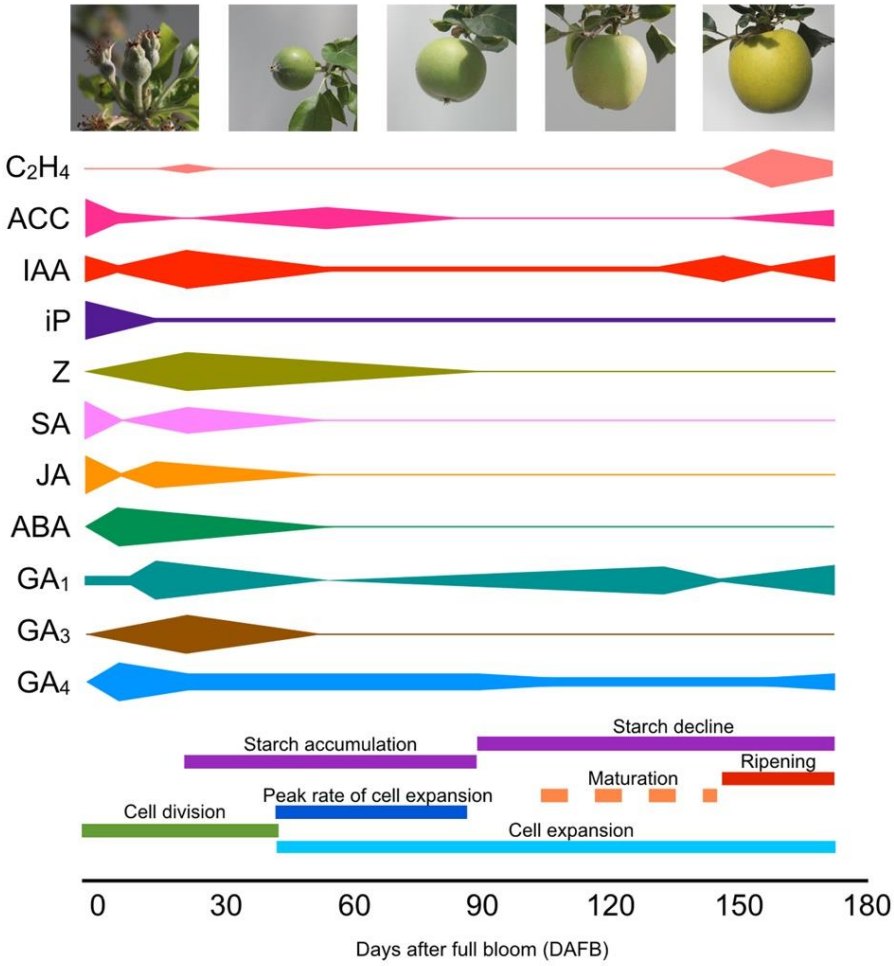

Figure 17 – Model representing the relevance of some selected hormones with respect to their quantified levels and the different stages of apple fruit development in the cv Golden Delicious. Quantification data were those achieved in the present research, except for those of ethylene at early stages, which were inferred from Botton et al. (2011) and Eccher et al. (2015). DAFB, days after full bloom.

According to the data herein presented, it may become possible to hypothesize new synergisms, and antagonisms among the different hormones, mainly at early and late development, and also a constitutive role of some of them during the intermediate

developmental stages, usually underestimated by researchers. Of course, some of these interactions and cross-talks were already known or, at least, hypothesized. For example, the antagonisms between ABA and gibberellins was already known and several evidence, also from a molecular point of view, was already provided for apple in the last few years, although in the context of fruitlet abscission (Botton et al., 2011). The mode of interaction between these two hormones is not only confirmed, but the hormone profiling allowed us also to identify the specific gibberellin, which is GA_3, most likely involved in such antagonism. The levels of this gibberellin increased by almost six fold during early development.

In general, the patterns of accumulation of the different gibberellins are extremely fascinating and likely ascribe to this class of hormones a pivotal role in apple fruit development. Data pointed out also a likely constitutive role for GA_4. The latter, in fact, is the active hormone present in the fruit at the highest levels during the intermediate developmental stages. An interesting and specific role can be also hypothesized for GA_1, which peaked not only at the cell division phase, but also during maturation and at the end of ripening (i.e. senescence). The first two peaks of this hormone are extremely interesting, as they appear to be related to critical stages of fruit development and are also in part consistent with known roles of gibberellins, *i.e.* in regulating cell division (Achard et al., 2009). A possible involvement of GA_1 in the maturation process is even more intriguing, although more specific investigations must be addressed to this aim. On the other hand, the increase of GA_1 during ripening is quite peculiar. Although gibberellins are known to generally repress processes related to senescence (Noodén and Leopold, 1988), as demonstrated with respect to strawberry, tomato, and banana fruit ripening (Martinez et al., 1994; Dostal and Leopold, 1967; Vendrell, 1970), a maturation/ripening-promoting role was shown in apple cv "Honeycrips" fruits (Schmidt et al., 2008). Worthy to note is also the synergism between IAA and GA_1, clearly pointed out in the PCA loading plot (Figure 16B) where both were almost overlapping. Taken as a whole, these data open the way to the development of field treatments specifically addressed to specific processes by using the respective gibberellin.

A specific involvement of zeatin in the cell division phase was herein shown, being its levels well correlated to the very early stages of fruit development.

Besides ABA, gibberellins and cytokinins, other hormones showed very interesting patterns, in some cases with multiple potential roles during the developmental cycle. This is the case

for IAA, ethylene and, indirectly, its precursor ACC. IAA content peaked both at early and late development, in both cases with a likely cross talk with ethylene, whose levels were consistent with those of ACC, being the latter available to be converted to ethylene in advance with respect to the peak of the gaseous hormone.

Last but not least, also JA and SA showed an intriguing behaviour. The peak of the former at early apple fruit development was already shown by Kondo et al. (2000) and is herein confirmed. Concerning the latter, instead, the peak displayed at the early stages, prior to cell expansion, has never been demonstrated in apple. The role of SA in repressing cell expansion is known (Scott et al., 2004; Xia et al., 2009) and some treatments with SA on apple were already performed with significant effects in terms of delays of fruit development (Youn et al., 2004). However, in order to demonstrate a role for this hormone not only on a correlative basis, a specific study is needed.

Summarizing, the hormone profiling performed during apple fruit development was mainly useful to identify: i) specific roles for the different bioactive gibberellins with potential application on the field, and ii) a potentially relevant role for SA in determining the size of the fruit by affecting cell expansion. In addition, these data can be integrated with different datasets (i.e. RNA-seq), in order to identify: i) specific transcriptional targets, ii) transcriptional indexes with highly significant and potentially predictive correlation with hormone levels, and iii) the functional meaning of the different hormones (i.e. the extension of the related transcriptional network).

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Chapter 4

Quantification of the main classes of metabolites during apple (*Malus domestica* L. Borkh) fruit development

Highlights

The different classes of metabolites show diverse variations according to their nature (primary or secondary), with some of them, *i.e.* the phenolics, displaying common patterns during apple fruit development.

Keywords

Metabolomics, sugars, sugar alcohols, amino acids, organic acids, phenolics.

Introduction

Apple (*Malus domestica* L. Borkh) is one of the most economically and diffuse tree crop in the world and produces fruits representing an important source of nutrients, non-nutrients, antioxidants, carbohydrates, and fiber that are essentials for the human diet and health (Biedrzycka and Amarowicz, 2008; Gerhauser, 2008; Boyer and Liu, 2004), such as phenolic compounds, sugars, alcohol sugars, organic acids and amino acids, which determine a large part of the final nutritional quality of the product.

The balance between sugars and organic acids contributes to the flavor and taste of apple, whereas the phenolic content is the most important determinant of the healthy properties of apples (Francini and Sebastiani, 2013). Regarding the primary metabolites, carbohydrates are involved in the energy metabolisms and are essential during fruit growth and development. In addition, the phosphorylated forms of sucrose, fructose and glucose, are known to act as signalling molecules regulating genes involved in several plant growth and developmental processes (Mishra et al., 2009; Cho and Yoo, 2011). The soluble sugars accumulation during fruit development is strictly related to the sweetness at harvest. In apple, sorbitol and sucrose are produced in photosynthetic leaves and then translocated through the phloem to the fruit, where the 70-80% of the total carbon flux is converted to fructose from sorbitol and glucose. For this reason, fructose is the most abundant sugar in the apple fruit (Li et al., 2012). Starch accumulation starts during the cell expansion phase and its content decreases with the progression of development up to the ripening (Li et al., 2012; Zhang et al., 2010; Berüter, 2004). Among the organic acids, malic acid was shown to be the most abundant in apple, especially during the early stages when it accumulates at high levels. Then, its concentration decreases with fruit development and at ripening it represents the major substrate for respiration (Berüter, 2004). About the amino acids, they are fundamental blocks for proteins construction and source of carbon and nitrogen for plant cell (Hildebrandt et al., 2015). They are also involved in other important pathways, i.e. aspartate, glycine and glutamine are precursors of nucleotide, and phenylalanine is involved in the phenylpropanoids biosynthesis, which may be quite relevant in the fruit (Hildebrandt et al., 2015; Cheynier et al., 2013; Henry-Kirk et al., 2012). Methionine derives from aspartate and is an immediate precursor of S-adenosylmethionine involved in the ethylene biosynthesis (Hildebrandt et al., 2015; Ravanel et al., 1998). Among the secondary metabolites, phenols are important for the

final quality of fruit products and for human health, as they include some strong antioxidant and anticancer molecules (Boyer and Lui, 2004). In apple pulp, six classes of phenols are present that can be divided in flavanols (monomeric, i.e. catechins, and oligomers i.e. procyanidins also known as tannins), flavonols (i.e. quercitrin), hydroxycinnamic acids (i.e. chlorogenic acid) and dihydrochalcones (i.e. phloridzin). All of them are synthetized by the phenylpropanoids pathway and their composition and concentration are characteristic of each apple cultivars, with significant changes along fruit development (Verdu et al., 2013; Wu et al., 2006).

It has widely been demonstrated that fruit development and ripening are characterized by a number of dynamic processes in which a complex series of molecular and biochemical changes are involved (Lombardo et al., 2011). A large amount of the metabolic studies produced up to now are mainly focused on the metabolites characterization of the fruit at ripening or during the post-harvest phase (Wu et al., 2006) or related to the quality of its products, such as apple juice or cider (Verdu et al., 2013). Only few metabolic studies reported the quantification of the main classes of metabolites during whole apple fruit development. Zhang et al. (2010) reported a comprehensive study about the quantifications of the main class of metabolites such as sugars, organic acids, amino acids and phenolic compounds along cv Honeycrisp fruit development. Another study was conducted on young and ripen fruits of cv Gala, in which the levels of ascorbic acid and the activity of the ascorbate-recycling enzymes were determined (Li et al., 2008).

Aim of the research

In this study, the main classes of metabolites were quantified along apple fruit development in cv Golden delicious, in order to give an overview of their variations and provide data to be used for integrative studies on apple fruit metabolism and its regulation.

Materiali e metodi

Experimental setup and plant materials

Experiments were conducted in 2014 on 9 years-old apples trees of cv Golden delicious/M9 grown with a slender spindle form and trained with standard horticultural practices, with no

hormonal treatments, at the experimental field "Piovi" (Roverè della Luna, Trento, Italy) of the Istituto Agrario San Michele all'Adige, Edmund Mach Foundation (Trento, Italy).

For each apple tree, fruit load was manually normalized according to the trunk section area (TSA), up to a value of 6 fruits/cm^2. A randomized block design was adopted, with three blocks, each including five trees.

Fruit cross diameter was measured weekly from full bloom up to the last sampling date, by using a manual caliper and recording diameters of 120 fruits.

Samples collection started when flowers were still closed but well developed (unpollinated flowers), to proceed up to fruit ripening *in planta*, according to the following time course (expressed in days after full bloom, DAFB): -3, 4, 13, 20, 31, 39, 53, 67, 88, 103, 118, 124, 132, 139, 146, 157, and 172. At the beginning samples included the whole receptacle (i.e. with epidermal tissue), whereas from 20 DAFB only the cortical tissue was collected. Plant material was immediately frozen in liquid nitrogen and stored at -80°C. All samples were collected during the morning, from 9 pm within maximum 2 hours.

Sample selection

Samples were the same as in the hormone profiling (see Chapter 3, Table 1), with the only exception of the flowers' receptacles. The whole procedure of sample selection is described in Chapter 2.

Metabolomic analyses

Metabolites were quantified on 5 biological replicates of 8 selected samples, covering the whole developmental cycle, with the only exception of the sample at 13 DAFB for which only three biological replicates were available. The major classes of polar metabolites (sugars, amino acids and organic acids) were identified and quantified by ^{1}H-NMR using 30 mg of freeze-dried cortex tissue as described by Mounet et al. (2007) and Biais et al. (2009). Polar compounds were extracted using a hot ethanol/water series with the final addition of an EDTA sodium salt solution to enhance the resolution and quantification of organic acids (Moing et al., 2011). Samples were analysed using a Bruker 500 MHz Avance spectrometer (Wissembourg, France) with BBI 5 mm and BBFO 5mm Bruker probes.

Identification and quantification of phenolic compounds was carried out with HPLC coupled with UV detector, as described by Verdu et al. (2013). The quantity of freeze-dried tissue for

this analysis was chosen in accordance with the stage of fruit development and the relative abundance of polyphenols content, as follows: 10 milligrams for the early stages (13 and 20 DAFB), 20 for 53 DAFB, 40 for 88 DAFB, 60 for 132 DAFB, and 80 for the last stages (146 to 172 DAFB).

Starch quantification

Starch content was determined enzymatically, as described by Hendricks et al. (2003) with minor modifications, after ethanolic extraction from 30 mg of freezed-dried apple powder. Supernatants were analyzed by ^{1}H-NMR and pellets freeze-dried and resuspended in 3 mL of 100 mM NaOH. The homogenate was incubated at 95°C for 30 min then mixed with 600 µL of neutralization buffer (500 mM HCl, NaOH-Acetate 100 mM pH 4.9). A volume of 750 µL of starch degradation solution (50 mM NaOH-Acetate pH 4.9, 140 U.ml-1 amyloglucosidase, 10000 U.mL-1) was added to the mixture. Reaction mixtures were incubated at 37°C for 24 h. After centrifugation, the glucose released in supernatant was assayed using the same method as described by Hendricks et al. (2003).

Statistical analyses

Descriptive statistics, principal component analysis (PCA), hierarchical clustering and all common statistical analyses (*i.e.* Pearson's correlation coefficients and significance) were performed with the R software version 3.2.2 (www.r-project.org/) using its standard packages plus some specific packages for plotting graphics. All multiple comparison statistics were calculated using the same software. In detail, normality was verified with Shapiro-Wilk test, homoscedasticity was verified with Bartlett's and/or nonparametric Levene's test, and differences among samples were verified with either ANOVA (normality and homogeneous variances) or Welch's one-way ANOVA (normality and non-homogeneous variances) followed by post hoc LSD or Waller-Duncan test, respectively, and with Kruskal-Wallis (non-normality and homogeneous variances) or Friedman test (non-normality and nonhomogeneous variances). For all statistics a *P*-value threshold of 0.05 was adopted.

Results and discussion

Graphic representation of metabolomic analyses

To obtain an overview of the levels of the main classes of metabolites during apple fruit development, main polar compounds, polyphenols and starch were quantified. Figures 1 and 2 represent the scatter plots between the concentrations expressed per dry weigh (DW) and fresh weigh (FW), for polar metabolites and polyphenols, respectively. The coefficients of determination indicate that the linear model can well approximate the observed data.

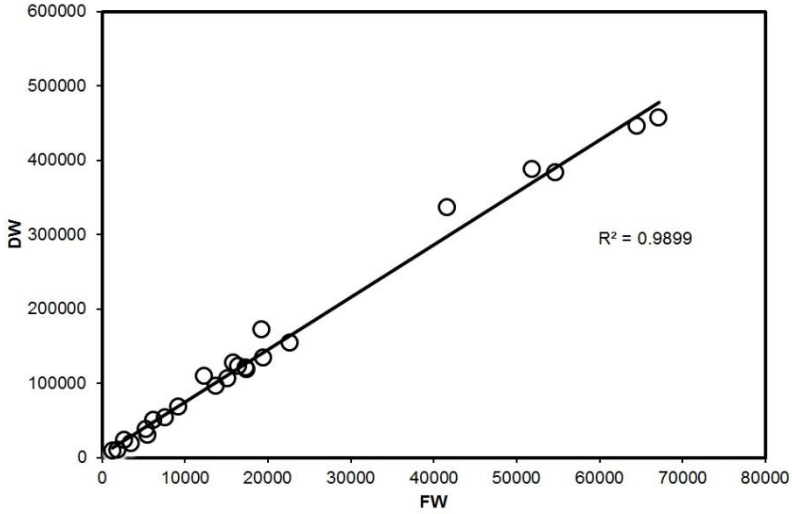

Figure 1 - Scatter plot chart between the two variables FW (x) and DW (y) for the polar compounds. (R^2 = R-square coefficient or coefficient of determination).

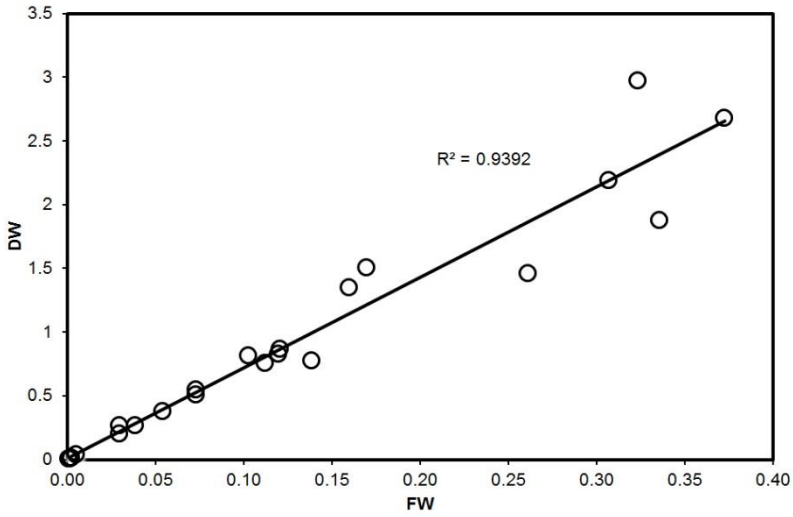

Figure 2 - Scatter plot between the two variables FW (x) and DW (y) for polyphenols. (R^2 = R-square coefficient or coefficient of determination).

In addition, the Pearson's coefficients were calculated (ρ = 0.999 and ρ = 0.991 for polar compounds and polyphenols, respectively, with a *P*-value < 0.001), strengthening the strong linear and positive correlation. Based upon these calculations, the metabolites' concentrations were herein expressed in terms of µg (or mg) per gram of dry weight (g DW).

Starch evolution

The starch content was determined enzymatically and the concentration was expressed in equivalent of glucose mg/g of dry weight apple material. Figure 3 shows starch evolution along the apple fruit development. Levels were very low at the first two stages, then rapidly increased and peaked at 88 DAFB (corresponding to the cell expansion phase), and progressively decreased up to ripening (172 DAFB). This results are fully in agreement with previous studies carried out in other apple cultivars (Li et al., 2012; Zhang et al., 2010; Berüter, 2004).

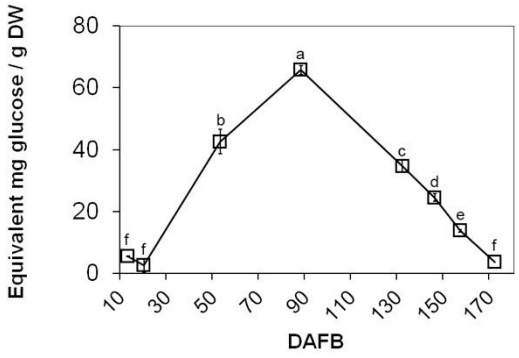

Figure 3 – Starch evolution along apple fruit development. Concentration is expressed as milligrams of glucose equivalent per gram of dry weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom.

Polar metabolites

A targeted analysis approach was used to identify and quantify the polar metabolites through ^{1}H-NMR. The main classes of compounds analyzed with this technology were sugars (both soluble and alcohol sugars), amino acids and organic acids. Twenty-nine compounds were quantified, out of which twenty were correctly identified, three were generally classified as phenolic derivatives, and six were unknown compounds (complete list in Table 1). Eighteen compounds were present at all the time points, nine were present only at ECD (13 DAFB) and CD (20 DAFB) stages, and only two compounds were exclusive of the others.

Table 1 – List of compounds identified and quantified with both ^{1}H-NMR and HPLC-UV techniques.

Class	Compound	Abbreviation	Analytical method
Sugars and alcohol sugars	glucose	Glu	^{1}H-NMR
	sucrose	Sac	
	fructose	Fru	
	xylose	Xyl	
	galactose	Gal	
	inositol	Ino	
Organic acids	citrate	Cit	
	malate	Mal	
	quinate	Qui	
	succinate	Suc	
Amino acids	alanine	Ala	
	asparagine	Ans	
	aspartate	Asp	
	glutamate	Glu	
	isoleucine	Ile	
	valine	Val	
	phenylalanine	Phe	
	tyrosine	Tyr	
Amino derivate	choline	Chol	
Unknown compounds	unkM7,07	/	
	unkM6,21	/	
	unkM6,07	/	
	unkD5,67	/	
	unkM5,20	/	
	unkM0,87	/	
Phenolic derivates	chloro1	Chlo1	
	chloro2	Chlo2	
	chloro3	Chlo3	
Hydroxycinnamic acids	chlorogenate	Chlo	HPLC-UV
	4-*p*-coumaroylquinic	PCQ	
	5-caffeoylquinic acid	CQA	
Flavanols	catechin	CAT	
	epicatechin	EC	
	procyanidin	B1	
	procyanidin	B2	
	procyanidin	B5	
Flavonols	avicularin	AVI	
	hyperoside	HYP	
	isoquercitrin	iQCI	
	quercitrin	QCI	
	reynoutrin	REY	
Dihydrochalcones	phloridzin	PLZ	
	phloretin xyloglucoside	XPL	

Sugars - Five sugars and one alcohol sugar (inositol) were identified and quantified (Figure 4 and, as a modelled overview, Figure 5). The most abundant sugar was fructose, that was

on average three fold more concentrated than glucose and sucrose along apple fruit development. This result is in agreement with what reported in literature, indicating that the 80% of the carbon flux towards the apple fruit is converted in fructose (Li et al., 2012; Zhang et al., 2010; Wu et al., 2006; Berüter, 2004). As expected, most sugars showed their highest levels during the last developmental stages (157 and 172 DAFB), corresponding to the ripening phase (Figure 4A, B and D), except for xylose that had its highest value earlier at 13 DAFB and then drastically decreased at low levels up to ripening (Figure 4E).

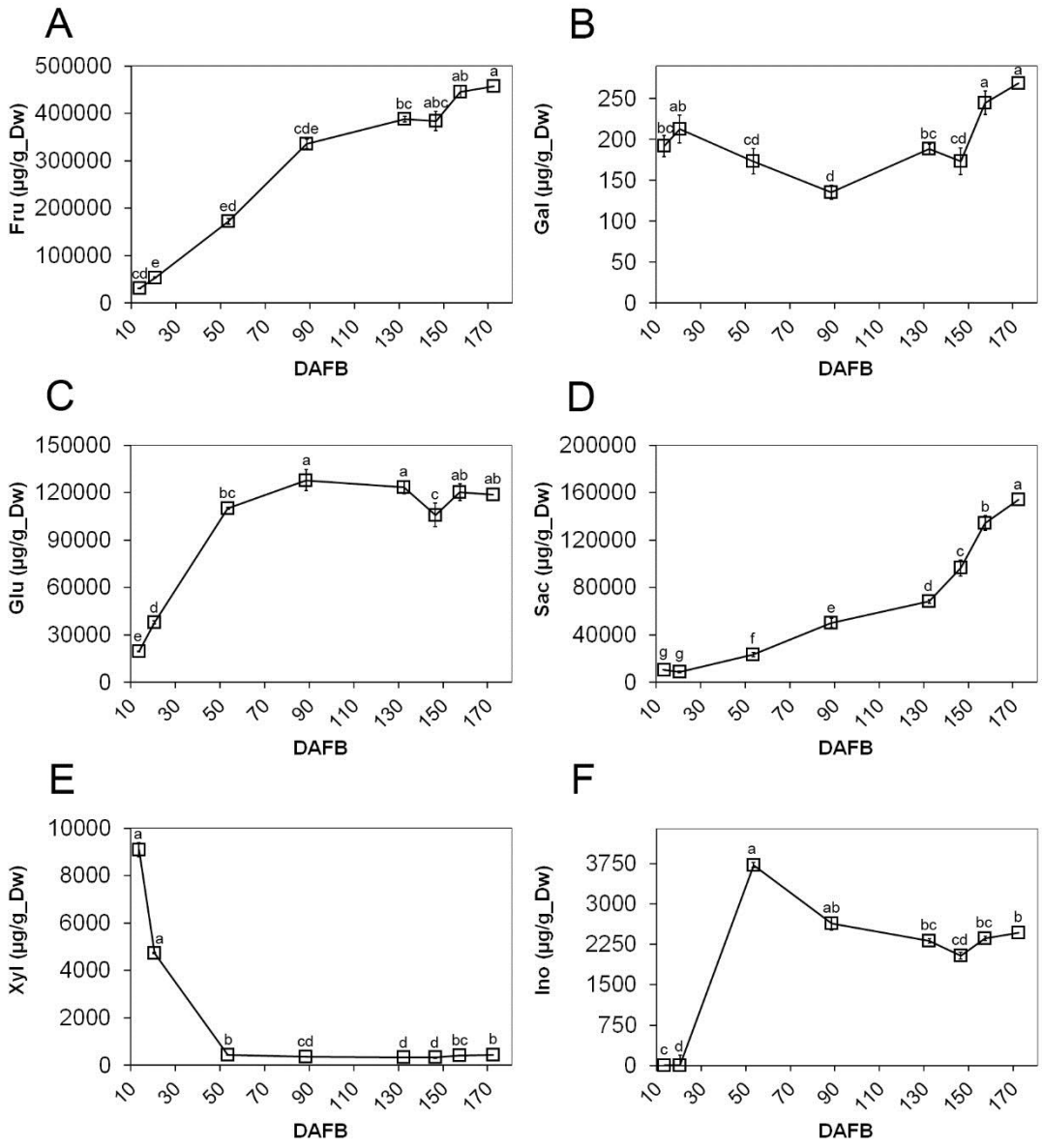

Figure 4 – Concentration of sugars and alcohol sugar during apple fruit development, expressed as micrograms per gram of dry weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom. Acronyms are explained in Table 1.

Fructose and sucrose content grew gradually along the time course, consistently with what previously reported by Li et al. (2012) and Zhang et al. (2010), although in different cultivars (Figure 4A, C and D). On the other hand, the glucose content showed a different pattern, with an exponential trend up to 132 DAFB and a slight decrease of its concentration at 146 DAFB that corresponded to the mature phase (Figure 4C). This result is partially in agreement with what reported in previous studies in which glucose showed a peak around late cell division/early cell expansion, decreased during the intermediate phases, and then rose up at ripening (Li et al., 2012; Zhang et al., 2020; Berüter, 2004). This difference could be explained by the fact that these studies were conducted in cultivars with a shorter developmental cycle (120-140 DAFB) with respect the cv Golden delicious (170 DAFB). Therefore, since glucose content is known to be affected also by environmental factors, such as temperature especially during the pre-harvest phase (Yamada et al., 1994), the prolonged developmental cycle of the cv Golden delicious, spanning through the whole summer, may determine this different behavior. Galactose levels were three orders of magnitude lower than the other sugars, decreasing from 20 DAFB up to 88 DAFB (cell expansion stage), then increasing again up to a peak in the last two phases (Figure 4B). About alcohol sugar concentration, inositol was undetectable in the first two stages, then peaked at the transition between cell division and cell expansion (53 DAFB), and remained quite constant from 132 DAFB up to the end of the developmental cycle (Figure 4E).

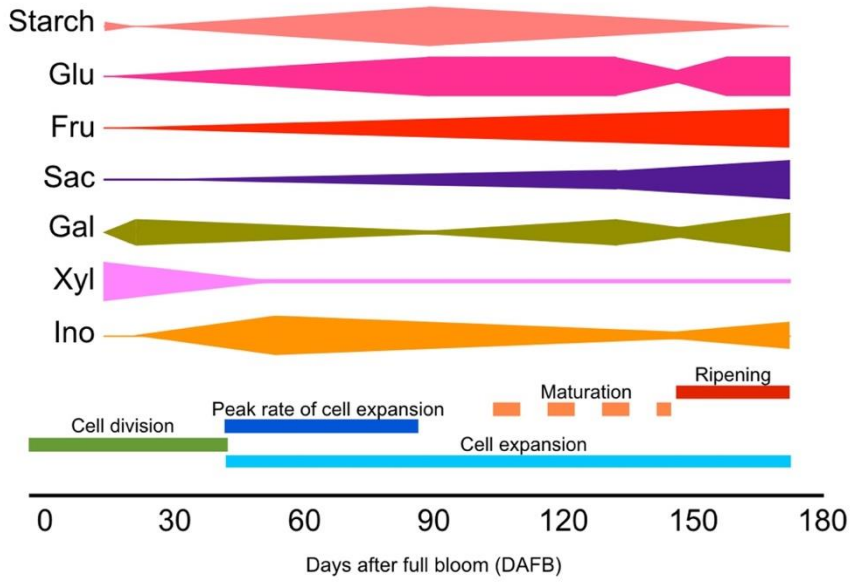

Figure 5 – A modelled overview of the changes in starch, sugars and sugar alcohols content during apple fruit development. See Table 1 for acronyms. DAFB, days after full bloom.

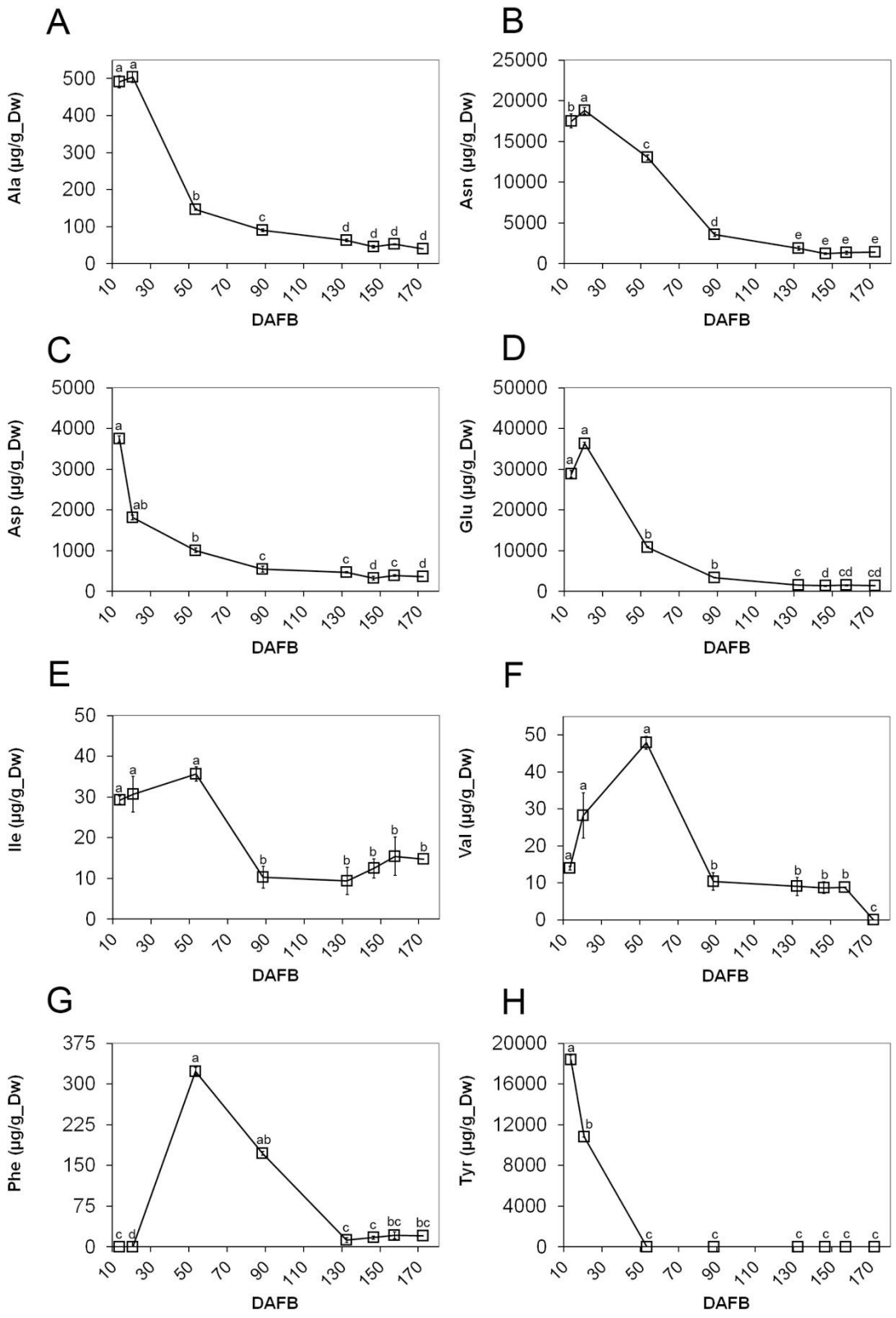

Figure 6 – Concentration of amino acids during apple fruit development expressed as micrograms per gram of dry weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with P ≤ 0.05. DAFB, days after full bloom. For acronyms see Table 1.

Amino acids - During apple fruit development it was possible to identify and quantify a total of eight amino acids, all of which showed higher levels during early fruit development followed by a progressive decrease throughout the later stages (Figure 6). In detail, alanine, asparagine and glutamate peaked at 20 DAFB and then decreased gradually up to 132 DAFB, keeping constantly low levels up to ripening (Figure 6A, B, and D).

Both aspartate and tyrosine levels decreased from the first sample, although with different patterns. While the former showed a progressive decreasing trend up to the end of fruit development, the latter dropped down at 53 DAFB and remained undetectable thereafter (Figure 6C and H). Finally, isoleucine, valine, and phenylalanine peaked all at 53 DAFB, but then displayed different behaviors. In particular, valine was not detectable at the last sampling date, while phenylalanine was not measurable both at 13 and 20 DAFB (Figure 6E, F, and G). All these results are consistent with the previous data obtained in cv Honeycrisp by Zhang et al. (2010).

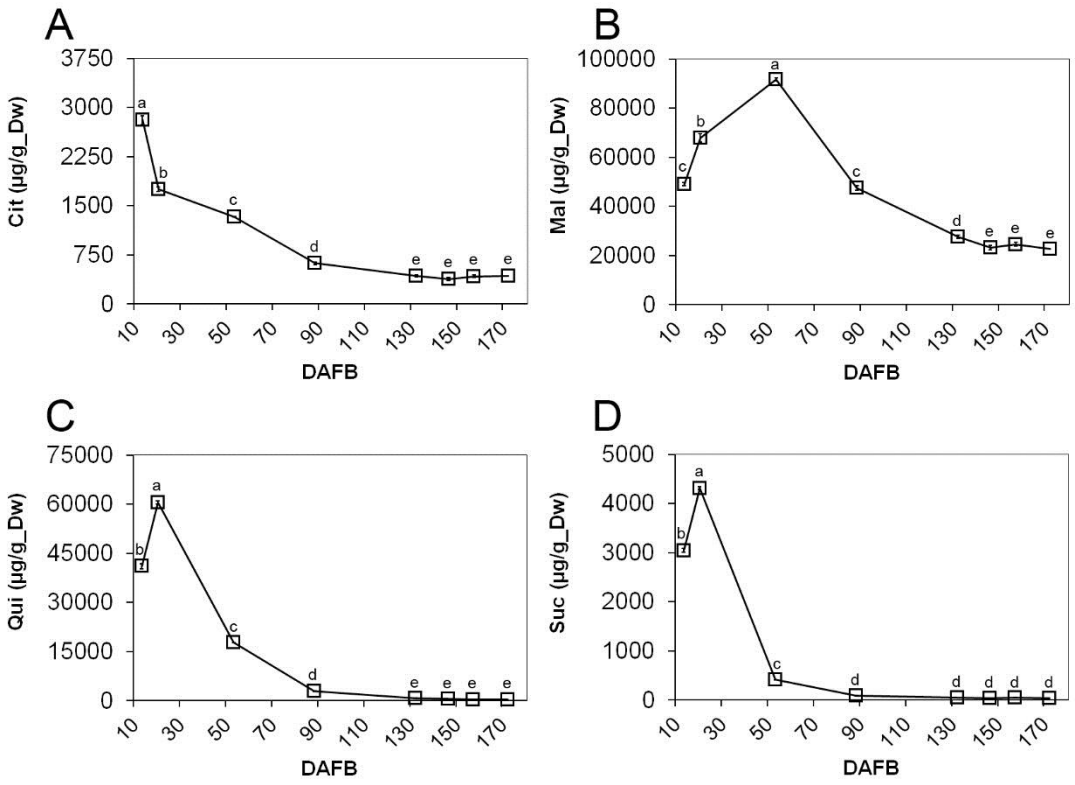

Figure 7 – Organic acids concentration belong the fruit development expressed as micrograms per gram of dry weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with P ≤ 0.05. DAFB, days after full bloom. Acronyms are explained in Table 1.

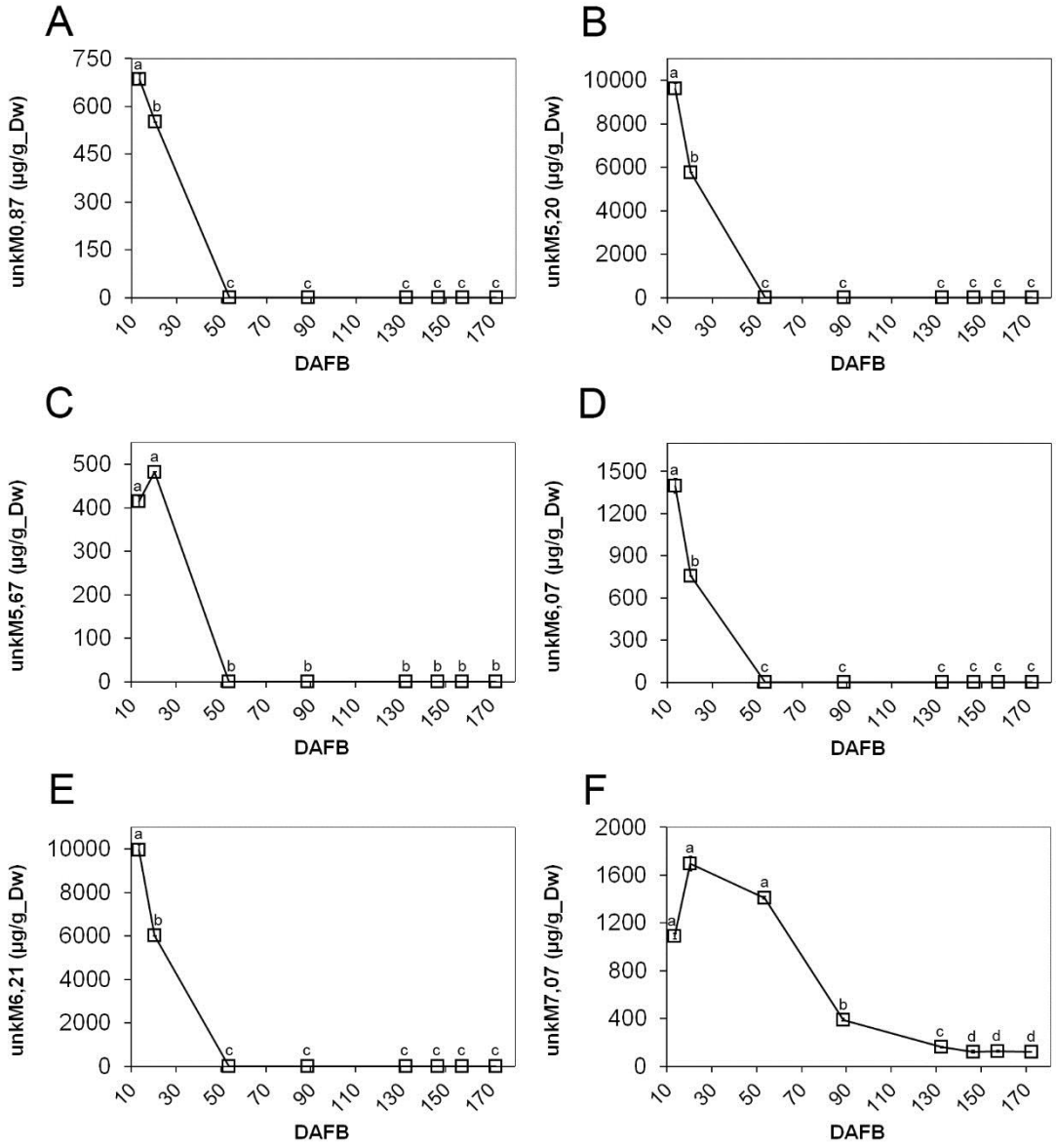

Figure 8 – Concentration of unknown compounds expressed as micrograms per gram of dry weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom. Acronyms are explained in Table 1.

Organic acids - Regarding the organic acids (Figure 7), four compounds were quantified with ^{1}H-NMR, among which malic acid was the most abundant, followed by quinate, 10^{2} order of magnitude higher than citrate, the latter showing the lowest concentration as already reported in other cultivars (Zhang et al., 2010; Petkovsek et al., 2007; Wu et al., 2007). The malic acid content gradually increased from 13 DAFB to 53 DAFB, decreased up to 132 DAFB and then remained constant up to the ripening phase (Figure 7B). The developmental pattern of malic acid was closely similar to those reported for other apple cultivars (Berüter,

2004). Regarding the citric acid concentration, levels were high at 13 DAFB than slowly decreased, and stayed at a basal level from 146 DAFB up to 172 DAFB (Figure 7A). Quinate and succinate peaked at 20 DAFB, corresponding to the cell division phase, then rapidly decreased at 53 DAFB and remained constant from 88 DAFB up to the last stages (Figure 7C and D). These results are consistent with those by Zhang et al. (2010) in cv Honeycrisp.

Unknown compounds - Six different unknown compounds were detected and discriminated according to their molecular weight. Five were exclusively found in samples of the first two stages (Figure 8A, B, C, D and E), while "unkM7.07" was detectable at all the developmental phases (Figure 8F). The "unkM0.87" promptly decreased from 13 to 53 DAFB, when its concentration is non detectable. "UnkM5.20", "unkM6.07" and "unkM6.21" showed similar trends, while "unkD5.67" peaked at 20 DAFB and then sharply decreased up to zero at 53 DAFB. "UnkM7.07" was the only compound to be present along all the developmental phases, peaking at 20 DAFB, decreasing gradually up to 132 DAFB (*i.e.* the immature phase), and finally remaining at a constant level.

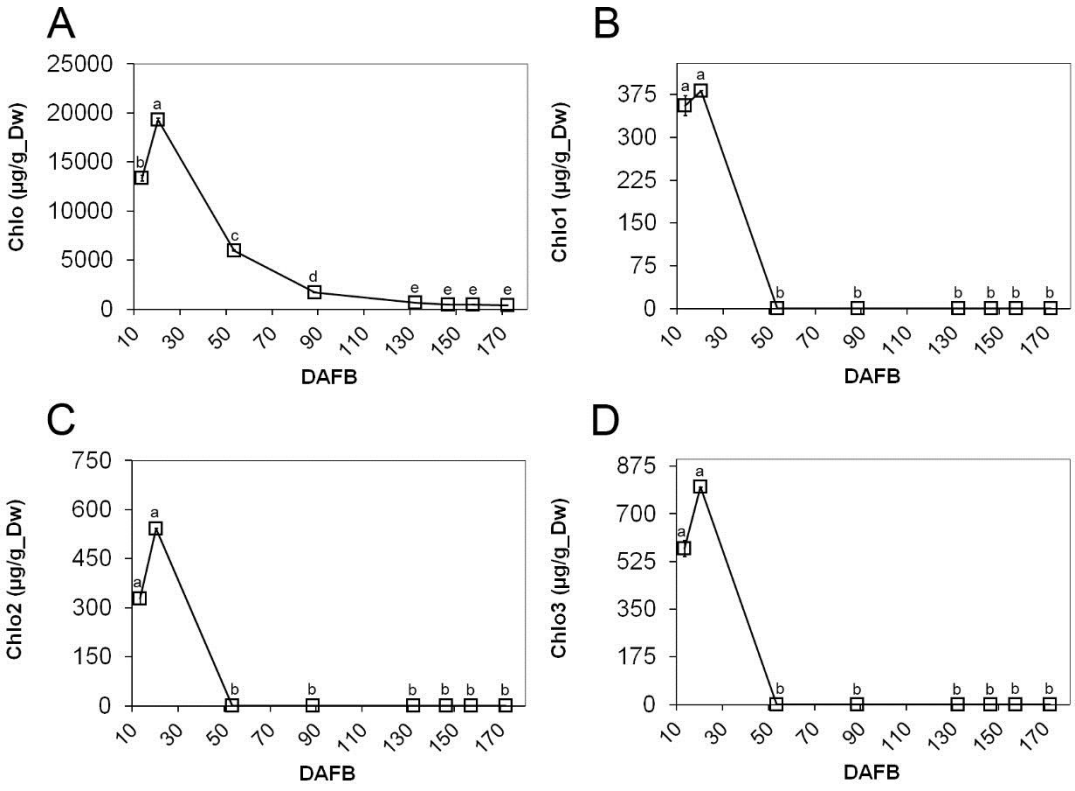

Figure 9 – Levels of chlorogenic acid (A) and phenolic-like compounds (B, C, D) expressed as micrograms per gram of dry weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom. Acronyms are explained in Table 1.

Chlorogenic acid, phenolic and amino derivatives - Chlorogenic acid was the only phenol quantified and identified with ^{1}H-NMR method and with a reliable concentration (Figure 9A). This compound peaked at 20 DAFB and gradually decreased throughout fruit development, as also reported by Zhang et al. (2010). In addition, three phenolic-like compounds were quantified, although the extraction protocol used would not be suitable to fully preserve phenolics. These phenolic-like compounds were detected only in the first two stages with a peak at 20 DAFB (Figure 9B, C and D). Finally, the amino derivative choline peaked at 20 DAFB and then gradually decreased during cell expansion phases. From 132 DAFB up to ripening, its concentration remained constant and at low levels (Figure 10).

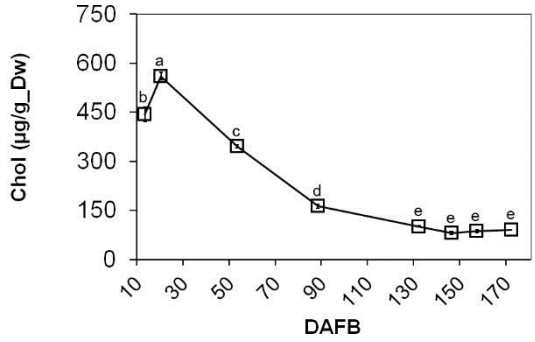

Figure 10 – Level of choline (Chol) during apple fruit development. Bars represent standard error. Different letters distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom.

Phenols quantification

To identify and quantify polyphenols in the fruit cortex, the HPLC-UV technique was used. Fourteen compounds were identified along fruit development belonging to four different classes of phenols: flavonols, flavanols, dihydrochalcones, and hydroxycinnamic acids (Table 1).

Flavanols - For this class of polyphenols, five compounds were identified and quantified: three procyanidins (B1, B2, and B5), catechin and epicatechin (Figure 11). In general, all of them showed a peak at 20 DAFB, during full cell division phase, then gradually decreased through development up to the ripening stage (172 DAFB), with the only exception of catechin. This flavanol, in fact, pointed out a slight but significant increase at ripening, keeping its concentration above the detection limit up the the last sampling date. These results are consistent with the data reported by Zhang et al. (2010). In particular, The most abundant

flavanol in the cv Golden delicious was epicatechin, followed by procyanidin B2, both directly responsible for the astringent sensation resulting from their complexation with salivary proteins (Podsędek et al., 2000).

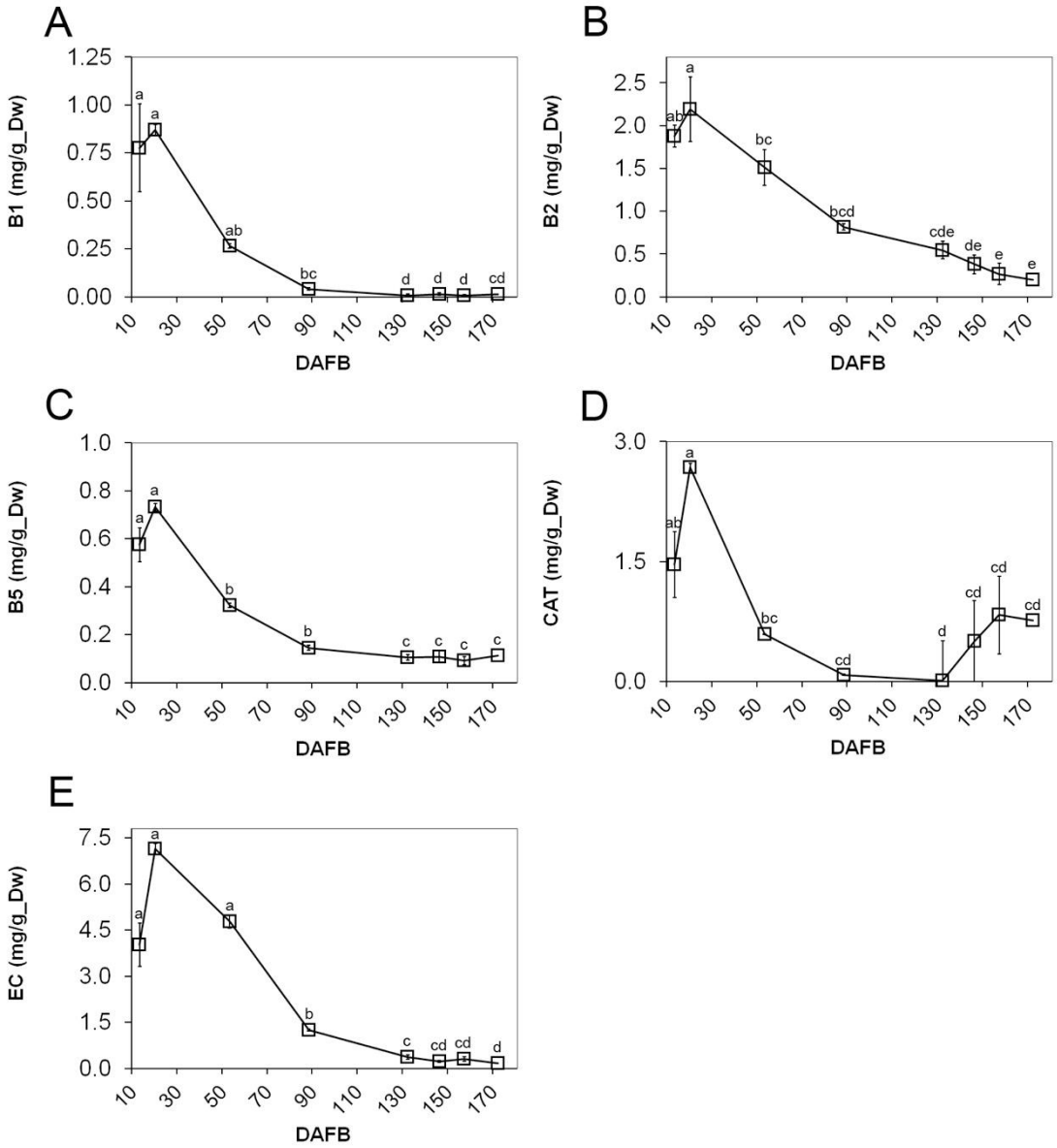

Figure 11 – Flavanols concentration along apple fruit development expressed as milligrams per gram of dry weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with P ≤ 0.05. DAFB, days after full bloom.

Flavonols - Five flavonols were identified and quantified that are avicularin, hyperoside, isoquercitrin and reynoutrin. All showed their highest levels around 13 and 20 DAFB, then their concentration immediately dropped down and stayed at very low levels up to the

ripening (Figure 12A, B, C, and E). HYP and iQCI levels were not detectable from cell expansion (88 DAFB) up to the last developmental stage (172 DAFB). Regarding quercitrin, its maximum level was reached at 13 DAFB and then exponentially decreased along development (Figure 12D). Also these data confirm results previously obtained by Zhang et al. (2010).

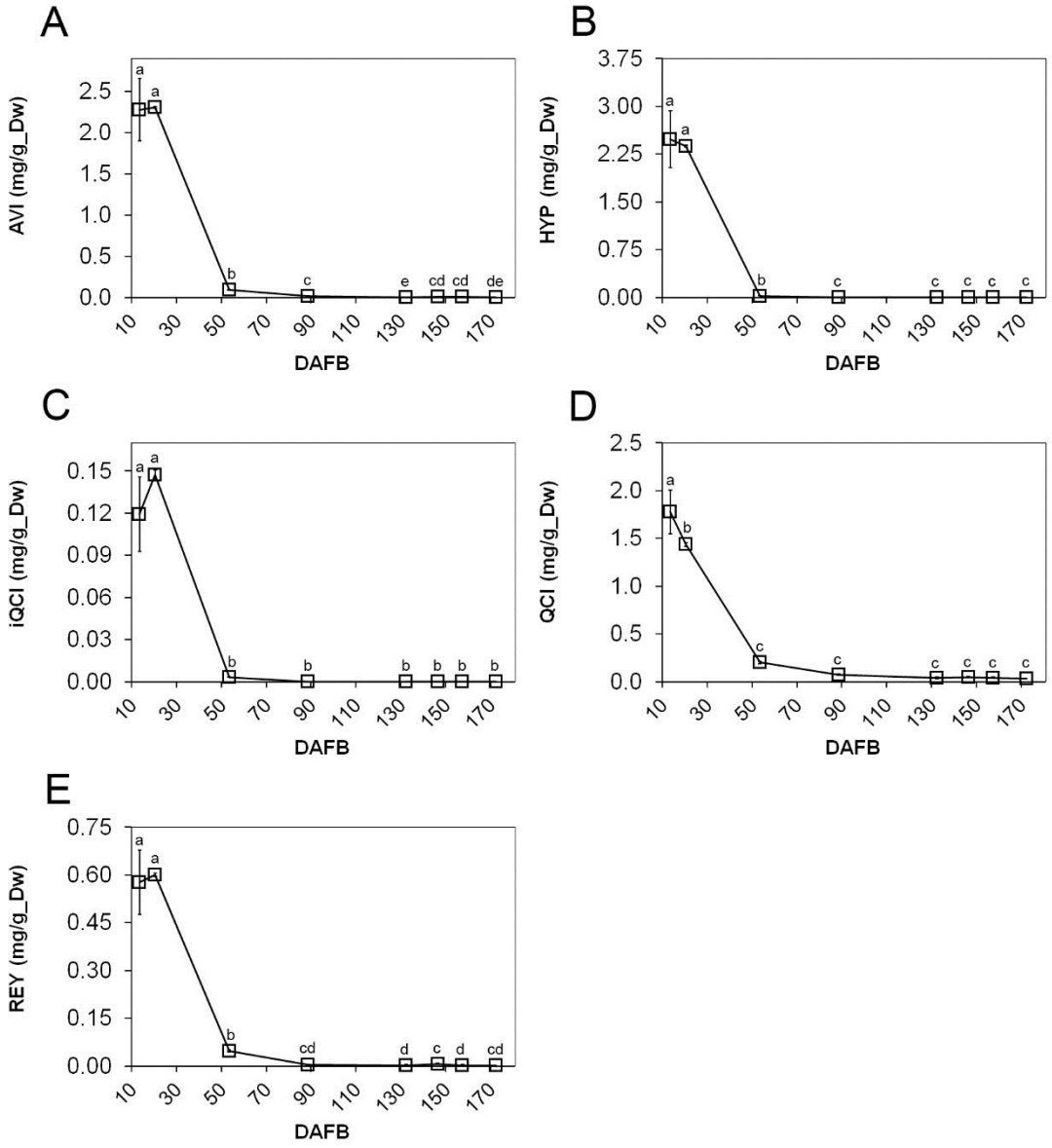

Figure 12 – Flavonols concentration during apple fruit development, expressed as milligrams per gram of dry weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with P ≤ 0.05. DAFB, days after full bloom.

Hydroxycinnamic acids and dihydrocalcones - Two hydroxycinnamic acids (4-*p*-coumaroylquinic acid and neochlorogenic acid) and two dihydrocalcones (phloridzin and

phloretin xyloglucoside) were identified and quantified (Figure 13). While the hydroxycinnamic acids peaked at 20 DAFB and rapidly decreased along fruit development (Figure 13A and B), PLZ, the most abundant phenol herein quantified, rapidly decreased from 13 DAFB to 55 DAFB and stayed at low levels during the remaining phases of development (Figure 13C). Finally, XPL constantly decreased from 13 DAFB up to the late developmental stages (Figure 13D).

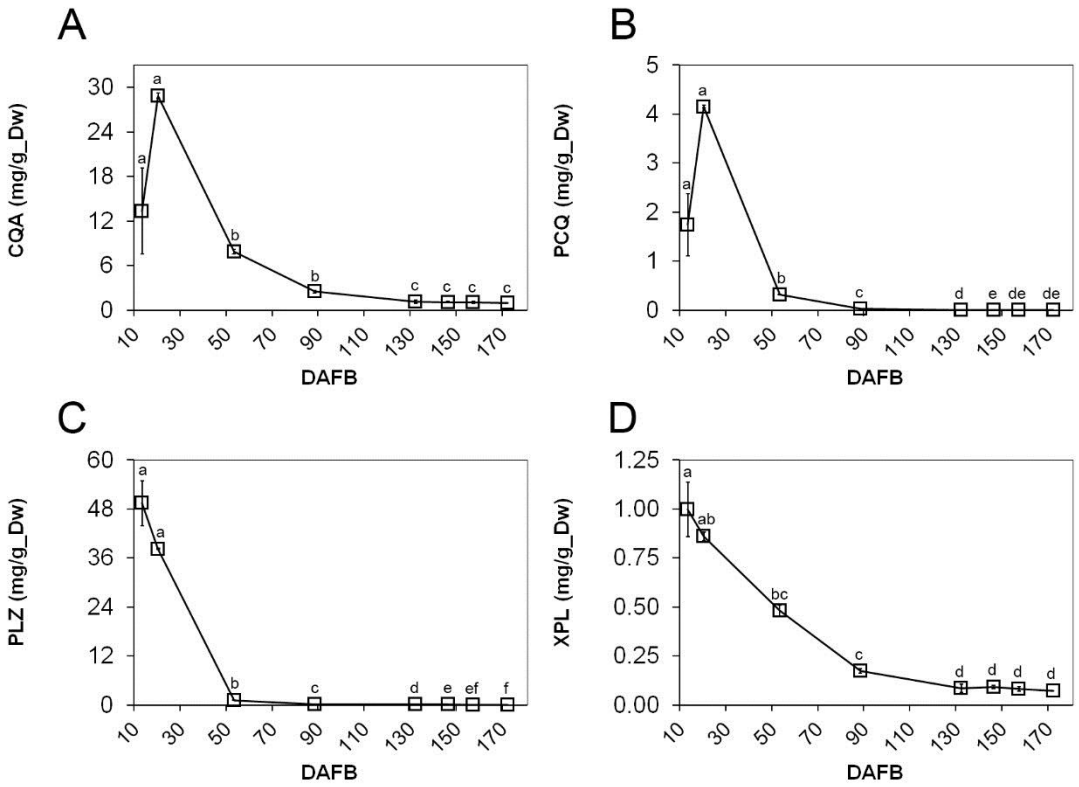

Figure 13 – (A, B) Hydroxycinnamic acids and (C, D) dihydrocalcones concentration during apple fruit development, expressed as milligrams per gram of dry weight of fruit cortex. Bars represent standard error. Different letters distinguish statistically significant differences with $P \leq 0.05$. DAFB, days after full bloom.

Conclusions

In order to obtain an overview of the primary and secondary metabolic changes along apple fruit development, a principal component analysis (PCA) was performed (Figure 14). It is notable that PC1 explained the 76.28% of the total variance observed in metabolites and was able to discriminate mainly between the early stages (from 13 up to 53 DAFB) and the late ones (from 88 up to 172 DAFB). The second component (PC2) explained the 11.58% of the variance and was shown to discriminate mainly the samples T4 (53 DAFB) and T5 (88

DAFB) from the rest. The loading plot (Figure 14B) indicates that the early stages were richer in amino acids, organic acids, and phenols, whereas the late stages were mainly characterized by the soluble sugars. Catechin represented an exception, as it showed a clear, although lowly significant, increase during the last stages.

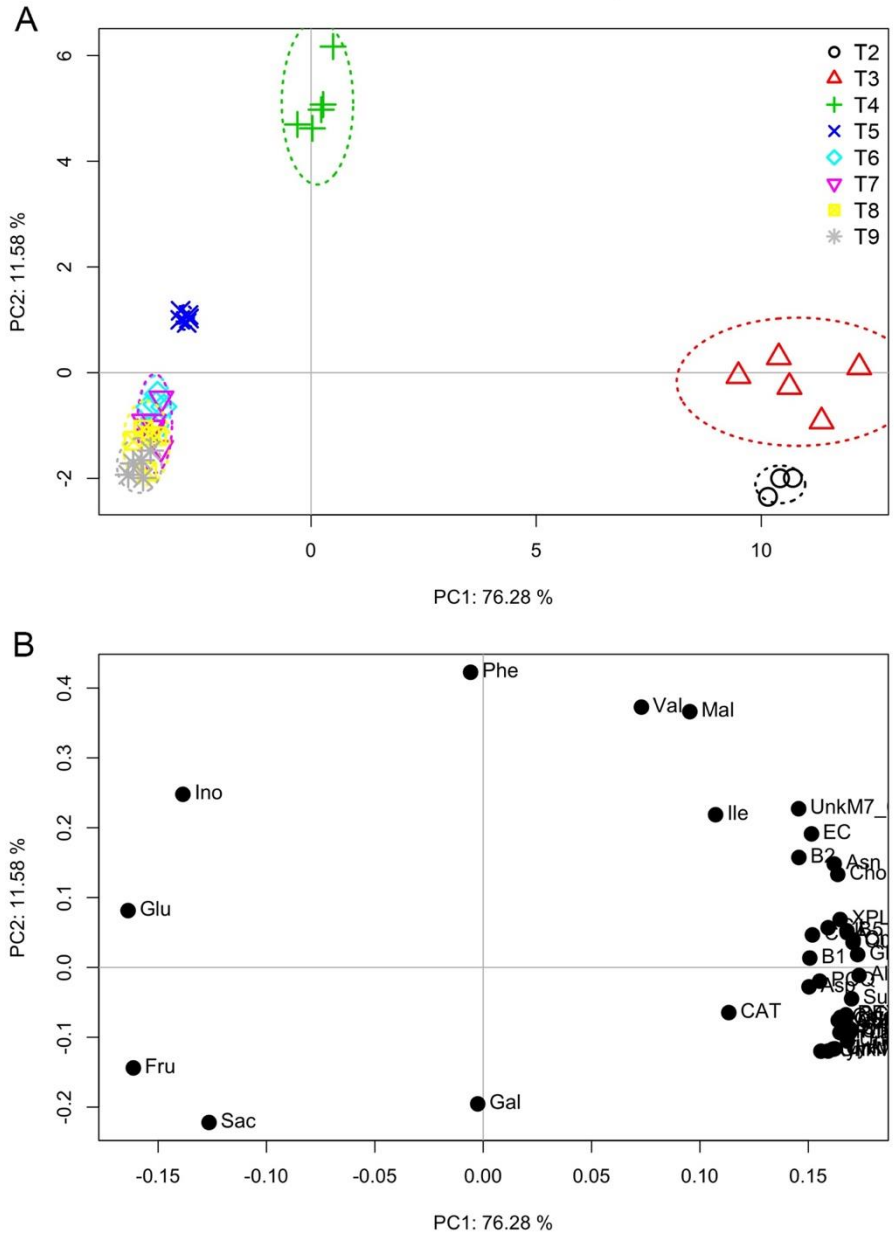

Figure 14 – Principal component analysis (PCA) of metabolites, measured by ^{1}H-NMR and HPLC-UV on different biological replicates of apple fruit cortex sampled throughout development (from T2 up to T9; see Table 1 of Chapter 3 for sample codes). (A) PCA scores plot of the first two principal components (PC1 and PC2) explaining 87.86% of the total variance. (B) PCA loadings plot (see Table 1 of this chapter for acronyms).

Among the metabolites, galactose and starch are extremely interesting, because they were the only compounds showing clear turning points at intermediate development, both at 88 DAFB. Among all the compounds analysed during fruit development in the present dissertation, only some hormones showed significant variations at intermediate development besides the two metabolites. Therefore, particularly in the case of galactose, a functional meaning can be hypothesized, considering also that significant variations of this sugars already found in tomato at ripening (Kim et al., 1991) and its long time known ripening-promoting effect in the same fruit (Gross, 1985). Some molecular information is also available concerning the regulatory role of galactose, which needs to be validated also in the apple fruit (Smith and Gross, 2000). In fact, only few information is available for apple, in particular concerning the β-galactosidase activity during the last developmental stages (Bartley, 1977; Ross et al., 1994).

Such as in the case of the hormones, the metabolite profiling performed during apple fruit development was mainly useful to: i) give an overall description of metabolic changes occurring in cv Golden Delicious, herein taken as a model genotype, and ii) identify metabolites showing variations in developmental stages other than the ripening phase. In addition, these data can be integrated with different datasets (i.e. RNA-seq), in order to identify: i) transcript encoding regulatory elements of a particular metabolism, ii) transcriptional indexes with highly significant and potentially predictive correlation with metabolite levels, and iii) the possible functional meaning of some metabolites (i.e. the sugars).

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Chapter 5

Correlative networks as a tool to integrate hormone profiling and RNAseq data

Highlights

Correlative networks represent a reliable tool to integrate data coming from different analytical approaches, such as RNA-seq and metabolomics.

Keywords

Correlative networks, Cytoscape, nodes, edges, hormones, metabolites, transcripts.

Introduction

Systems biology is a biology-based inter-disciplinary field that makes use of holistic approaches to understand complex dynamics of biological systems and their interactions. Nowadays, the advances in high-throughput technologies permit to obtain a large amount of data from different "omic" disciplines, such as transcriptomics, metabolomics and proteomics. These data can be analysed either alone or combined using mathematical and computational models to generate interaction networks based upon different types and degree of relationships, such as a simple correlations measured by Pearson coefficient (Saito and Matsuda, 2010; Fukushima and Kusano, 2014).

The general objective of systems biology is to predict the outcomes due to perturbing conditions, such as environmental changes, biotic/abiotic stress, developmental transitions, different treatments or diverse genotypes (Gapper et al., 2014). Moreover, it can also help to predict unknown gene functions through the so-called gene co-expression analysis, widely used in Arabidopsis and other model plants (Usadel et al., 2009; Yonekura-Sakakibara et al., 2013; Gehan et al., 2015). This approach is based on the postulate that if two or more biological entities are strongly correlated or respond in a similar way to different environmental or cellular circumstances, they are functionally related (Tohge et al., 2015). Important to note is that not always these approaches reveal a causal relationship among genes and are not able to distinguish direct or indirect associations between molecules or cellular components (Fukushima and Kusano, 2014). Therefore, it is essential to validate the results of co-expression analysis with detailed *ad hoc* measurement, additional experiments and comparison with existing information (Tohge et al., 2015). Systems biology is a hypothesis-driven science useful for experimental design and represent a starting point for further analysis (Chuang et al., 2010).

Some integrative studies were performed on tomato (*Solanum lycopersicum*) as a model plant for fleshy fruit development and ripening processes. For example, a systemic analysis approach was used to integrated microarray gene expression measures and metabolic data in the ethylene receptor *Never-ripe* (*Nr*) mutant (Alba et al., 2005). In this study, 37% of genes were shown to be affected by the *Nr* mutation with respect to the number of genes that are differential expressed in the wild type. Moreover, in the mutant, there are alterations in the fruit morphology and metabolic processes related to the role of ethylene during the ripening

syndrome, such as ascorbate accumulation and carotenoids biosynthesis. Osorio et al. (2011) adopted an integrative approach to study three ripening-related mutants, *nonripening* (*nor*), *ripening inhibitor* (*rin*) and *Nr*, at the transcriptomic, proteomic and metabolomic levels along fruit development and ripening. This study revealed several aspects related to the metabolic control during ripening. Specific groups of metabolites, such as sugars, organic acids and cell wall-related metabolites showed a strong correlation with ripening related transcripts and highlighted their importance during this process in tomato.

A comparative integrative analysis study was conducted on climacteric (tomato) and non-climacteric (pepper) fruits and revealed both common behaviours and differences during the ripening syndrome. Despite of the fact that both these species shown similar signal components related to ethylene response and perception, pepper fruit showed a different regulation pattern, in which the ethylene biosynthesis genes were not induced during ripening whereas the genes downstream of ethylene signals, i.e. carotenoids biosynthesis and cell wall metabolism, were induced like in tomato fruit. This could be due to either a different sensitivity to the hormone or other ethylene-independent regulators (Osorio et al., 2012).

These studies put in evidence that the ripening process is a complex developmental program in which metabolic pathways and transcriptional regulation are deeply involved and interconnected. Despite the importance of this late event that directly affects the final organoleptic characteristics of the products, the final quality of the commodity is affected at different extents also by other developmental transitions occurring earlier during fruit development, such as the shift from cell division to cell expansion or the less known transition occurring during the so called 'fruit maturation' (Eccher et al., 2015).

However, there are many gaps regarding the regulatory network, the hormone interactions and the metabolic control along fruit development. Systems biology could provide a pivotal help in filling these gaps and new information about these processes that can drive research to the 'next step'.

Aim of this research

This part of the thesis was aimed at setting-up a bioinformatics pipeline for the integration of "omic" data coming from diverse types of analysis, such as RNA-seq, metabolite and hormone profiling, by using the correlative network approach.

Materials and methods

Experimental setup and plant materials

Experiments were carried out in 2014 on 9 years-old apples trees of cv Golden delicious/M9 grown with a slender spindle form and trained with standard horticultural practices, with no hormonal treatments, at the experimental field "Piovi" (Roverè della Luna, Trento, Italy) of the Istituto Agrario San Michele all'Adige, Edmund Mach Foundation (Trento, Italy).

For each apple tree, fruit load was manually normalized according to the trunk section area (TSA), up to a value of 6 fruits/cm^2. A randomized block design was adopted, with three blocks, each including five trees.

Fruit cross diameter was measured weekly from full bloom up to the last sampling date, by using a manual caliper and recording diameters of 120 fruits.

Samples collection started when flowers were still closed but well developed (unpollinated flowers), to proceed up to fruit ripening *in planta*, according to the following time course (expressed in days after full bloom, DAFB): -3, 4, 13, 20, 31, 39, 53, 67, 88, 103, 118, 124, 132, 139, 146, 157, and 172. At the beginning samples included the whole receptacle (i.e. with epidermal tissue), whereas from 20 DAFB only the cortical tissue was collected. Plant material was immediately frozen in liquid nitrogen and stored at -80°C. All samples were collected during the morning, from 9 pm within maximum 2 hours.

Sample selection

Samples were the same as in the metabolite profiling (see Chapter 4). The whole procedure of sample selection is described in Chapter 2.

RNA extraction

Total RNA was extracted following the method of Ruperti et al. (2001), with few adaptations as Botton et al. (2009 and 2011): 0.02 g of receptacle and 0.6 g of fruitlet tissues were extracted in 1 and 10 ml of extracting buffer, respectively, whereas 6 g of fruit cortex in 15 ml. RNA was quantified with the NanoDrop 2000c (Thermo Scientific, Waltham, MA) and its integrity checked by running 1 μg in a 1% agarose stained with SYBR® Safe (Life Technologies, Carlsbad, CA).

RNA-seq library construction and sequencing

RNA integrity was checked with Agilent RNA Nano Kit, following manufacture's instruction, using the Agilent 2011 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The recommended RIN (RNA Integrity Number) for proceeding with the RNA library preparation was greater than 6 for all the samples. One microgram of total RNA was employed in the TruSeq Stranded Total RNA Lybrary prep kit LT (low-throughput) (Illumina, Inc., San Diego, CA), which included the removal of unwanted cytoplasmic, mitochondrial, and chloroplast ribosomal RNA prior sequencing, following manufacturer's instruction. During the amplification step of the fragmented strand that included the ligation of the adapter, an RNA adapter index was chosen for each library, thus permitting a multiplex sequencing analysis of several libraries, having different indexes, in a single lane. The quality control of the RNA library was performed with Agilent DNA 1000 kit using the Agilent 2011 Bioanalyzer (Agilent Technologies, Santa Clara, CA), following manufacture's instruction. Afterwards, the quantification of the RNA library was checked by means of Qubit 2.0 DNA HS kit (Thermo Fisher Scientific Inc., Waltham, MA). In order to have an equal representation of each RNA library, all the libraries were diluted in a multiplex mix to a final concentration of 20 nM. For sequencing, a dilution to 2 nM was performed for each multiplex mix. The sequencing run was carried out on a HiSeq2000 sequencer (Illumina, Inc., San Diego, CA) at the CIBIO NGS platform (University of Trento) (http://web.unitn.it/en/cibio/21325/next-generation-sequencing-ngs) following the manufacture's instruction. The 8-lane flowcell employed in the analysis allowed an output of 270-300 Gigabase (http://www.illumina.com/systems/hiseq_2500_1500/performance_specifications.htm) with a total read number of 3 billions in the paired end sequencing.

Reads mapping and counting

Reads were trimmed, filtered, quality-checked and, finally, mapped to a new version of the apple transcriptome, still unreleased to the public and made available within the project TranscrApple (www.transcrapple.com). The software Bowtie2 was used for mapping, with default parameters and no mismatch allowed. A count matrix was finally generated.

For the following applications, the RPKM values were calculated as follows:

$$RPKM = nR / (gL / 1000 * tNR / 1,000,000)$$

with nR = number of reads mapped to a gene sequence, gL = length of the gene sequence, and tNR = total number of mapped reads of a sample.

Statistical analyses

Pearson's correlations indexes and significance were calculated with the R software version 3.2.2 (www.r-project.org/).

Raw counts data were normalized for GC content using the same software with the package EDASeq (Risso et al., 2011) and the normalization method 'full; full'.

Pairwise differential expression analyses were performed with the package DESeq (Anders and Huber, 2010) by dividing the samples into three partially overlapping subsets: the "early" subset included samples from T2 to T5, the "intermediate" one comprised those from T4 to T7, and the "late" subset the samples from T6 to T9. Significance levels were established at the following thresholds: FDR-corrected *P*-value ≤ 0.01 and $\log_2$ (fold change) ≥ 1. For each subset, a list including only the genes with at least one significant differential expression was assembled to be used for building correlative networks.

cDNA synthesis and real-time PCR

cDNA was synthesized with the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, Carlsbad, CA) from 200 ng of DNA-free total RNA in a final volume of 40 µL, according to the instructions provided by the manufacturer. The reaction was performed in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA).

Real-time PCR relative quantification was performed in triplicate on three biological replicates, as described by Botton et al. (2011). The nucleotide sequences of the primers for both the target and reference genes are reported in Table 2 of Chapter 2. Data were acquired, elaborated and exported with the StepOne Software v2.1 (Applied Biosystems, Foster City, CA), whereas all the final calculations were made with the automated Excel spreadsheet Q-Gene designed by Simon (2003), using the modifications of the delta Ct method suggested by Pfaffl (2001). Gene expression values were normalized to *Md18S*. Expression levels were then reported as arbitrary units (A.U.) of Mean Normalized Expression, calculated using equation 2 of the Q-Gene spreadsheet.

Correlative networks

Correlative networks were built starting from the quantification data of metabolites, hormones, and transcripts (only the significant ones) and using software Cytoscape v3.2.1 (www.cytoscape.org) with the Expression Correlation plugin v1.1.0 (www.baderlab.org/Software/ExpressionCorrelation). The following thresholds were used to filter the networks: -0.99 ≥ Pearson ≥ 0.99. The network was then filtered by viewing only the nodes corresponding to hormones, metabolites, and transcripts that were directly connected to either of them. Edges were bundled (number of handles = 3; spring constant = 0.003; compatibility threshold = 0.3; maximum iterations = 10000) and the edge-weighted spring embedded algorithm was used for the network layout. Enrichment analysis was performed with the Cytoscape plugin BinGO v2.4.4 using annotations of the apple genome that are publicly available (https://phytozome.jgi.doe.gov). All analyses were performed using an Apple iMac equipped with an Intel Core i7 3.5GHz and 32GBytes of RAM memory.

Results and discussion

Validation of RNAseq results

In order to confirm the reliability of the RNAseq transcriptomic data, a selection of the qPCR results achieved for the marker genes (*FK1*, *MADS118*, *SPS6*, *SDH1* and *TMT1*; see Chapter 2) in 2014 samples were compared with data derived from the deep sequencing of the transcriptomes, calculated as RPKM (Reads per kilo base per million mapped reads). The comparisons are shown in Figure 1. For each gene, a Pearson's coefficient was calculated between the RNAseq and qPCR results. The range of values went from 0.696, for *FK1*, up to 0.996, for *SPS6* gene, all with *P* values ≤ 0.05. Generally, the RNAseq data followed the same trends of the qPCR with no significant variations. These results confirm the reliability of the RNAseq data.

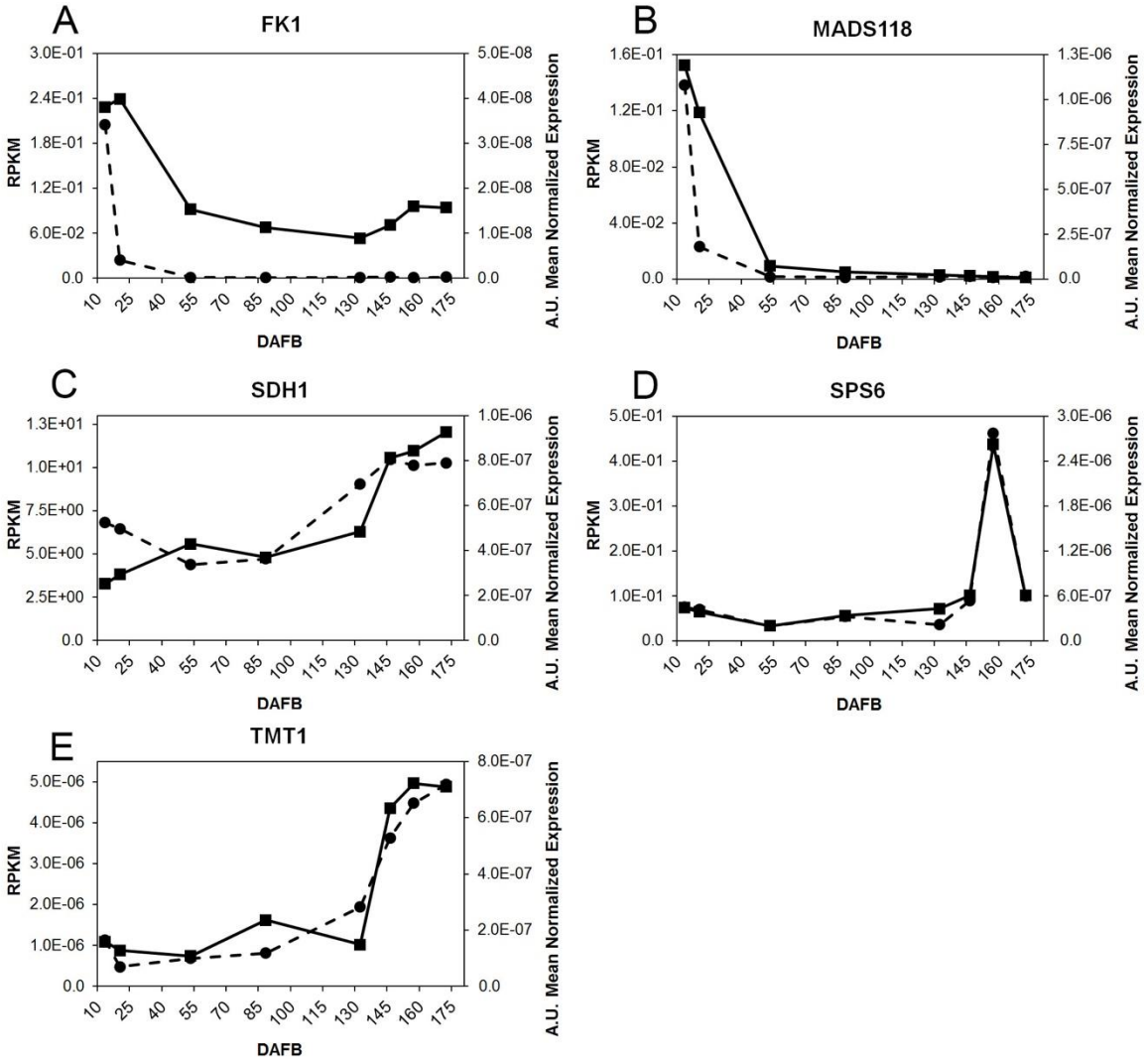

Figure 1 - Comparisons between RNAseq data, reported as RPKM (continuous line), and qPCR, reported as Arbitrary Units of Mean Normalized Expression (dashed line).

Correlative networks

First of all, some technical considerations were done before setting up the pipeline for network construction. Preliminary trials were carried out, tempting to assemble a network including the values of all transcripts, metabolites and hormones and all the samples at the same time. This procedure, however, was characterized by a very high demand of RAM memory, above the 32 Gigabytes available. In order to overcome this technical problem, the dataset was split into three parts, as described above. A second choice was made, concerning the network structure. Only the neighbor nodes of either hormones or metabolites were kept in the network, in order to simplify the view.

Table 1 – Summary of the main features of the three subnetworks.

Features		Subnetworks		
		Early	**Intermediate**	**Late**
Nodes	Hormones (19)	17	15	19
	Metabolites (44)	7	2	5
	Transcripts[a]	1,788 (11,519)	416 (8,686)	1,258 (4,699)
	Total no. nodes	1,812	433	1,282
Edges		1,798	419	1,264

a) The total number of transcripts' data used for correlation calculations is given between parentheses.

"Early" network - The "early" network (Figure 2) covered the cell division and expansion phases, from 13 up to 88 DAFB. The input dataset consisted of 11,519 DEGs (differentially expressed genes), obtained from the pairwise comparisons between the four time points, 19 hormones (HRs), and 44 metabolites (30 polar metabolites and 14 phenols), previously quantified and discussed respectively in Chapters 3 and 4. The total number of central nodes, used as guides, is 24, divided into 17 hormones and 7 metabolites (6 polar metabolites and 1 phenols), while the interactions are 1,798, of which 1,788 are with transcripts and the other between hormones and/or metabolites (Table 1). In this preliminary analysis, the attention was focused on the interactions among HRs and transcripts, the latter being either direct or indirect targets of the former or just sharing a co-regulated behaviour. Among the hormones or their precursors, the most connected with transcripts were, in the order, ZR (342 transcripts), DZ (272), GA_3 (111), ABA (108), and GA_{24} (103). The main "early" network included 11 subnetworks, or modules, three of which, i.e. the widest, showed multiple interactions among transcripts and different nodes. In other words, some transcripts interconnect different metabolites or hormones/precursors. Among these cases, the most interesting are the modules IAA-SA-$GA_{3\text{-}24}$ (Figure 2B) and ABA-JA (Figure 2C). Concerning the former, besides the well known interactions existing between auxin and gibberellins (Björklund et al., 2007; Depuydt and Hardtke, 2011; Weiss and Ori, 2007), the concurrent involvement of the salicylic acid in the same correlative network is someway surprising, since most of the information available in literature ascribes a controversial role to the interactions between these two hormones. In fact, while in responses to biotic stress IAA and SA may display some synergism (Zhang et al., 2007), in other contexts, such as the abiotic stress, the two hormones showed antagonistic behaviours (Iglesias et al., 2011).

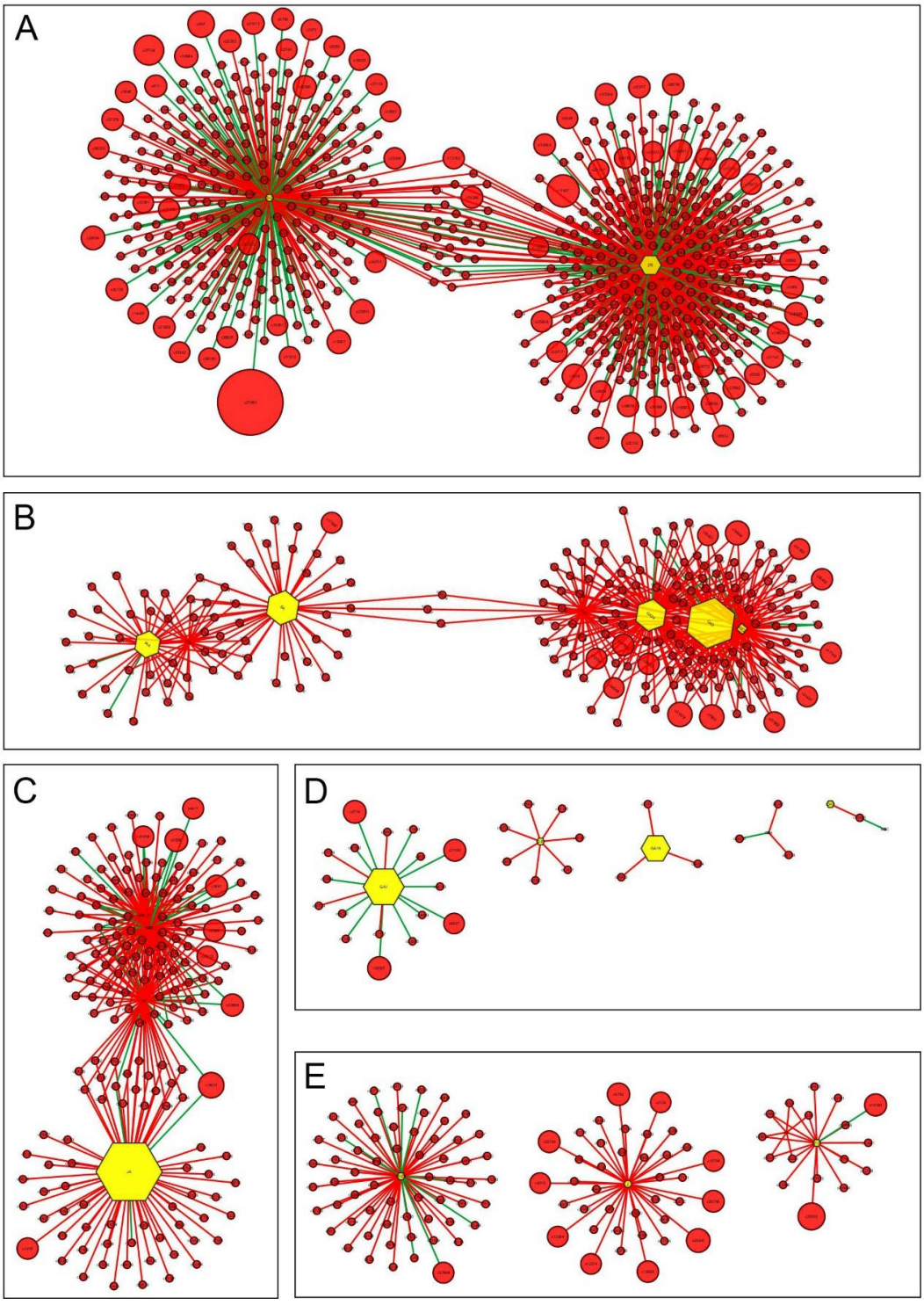

Figure 2 – "Early" network overview. (A) ZR, left, and DZ, right. (B) IAA-SA, left, and GA_3-GA_{24}, right. (C) ABA, top, and JA, bottom. (D) from left, GA_1, iP, GA_{19}, GA_4, GA_7-ACC. (E) from left DZR, Z and iPR. Nodes are characterized by different colors and shapes: hormones are yellow hexagons, metabolites are blue squares, and transcripts are red circles. The size of each node reflects the average quantitative data in the four time points of the network. Edges' length corresponds to the strength of the Pearson's coefficient. Red and green colors mark direct and indirect correlations, respectively.

Concerning, instead, the second module, *i.e.* ABA-JA, a cross-talk between these two hormones has already been described before, although not in the context of fruit development. In fact, both jasmonates and ABA were shown to trigger, in a synergic manner, stomatal closure (Munemasa et al., 2011) and the response is conserved among various plant species including *Arabidopsis thaliana* (Suhita et al., 2004), *Hordeum vulgare* (Tsonev et al., 1998), *Commelina benghalensis* (Raghavendra and Reddy, 1987), *Vicia faba* (Xin et al., 2005), *Nicotiana glauca* (Suhita et al., 2003), *Paphiopedilum Supersuk* and *Paphiopedilum tonsum* (Gehring et al., 1997). On the other hand, some antagonistic interactions were also demonstrated (Robert-Seilaniantz et al., 2011), which most likely fit the findings herein described, with opposite trends of variations observed for the two hormones (see Figure 17, Chapter 3).

Table 2 – Summary of the main interactions between the guide nodes (hormones or precursors) and the other node types included in the "early" network (*=biologically active hormones).

Hormones or precursors (HRs)	No. interactions		
	Genes	Metabolites	HRs
IAA*	36	1	-
DZ*	272	-	-
DZR	63	-	-
Z*	38	-	-
ZR	342	1	-
iP*	7	-	-
iPR	17	1	-
GA_1*	17	-	-
GA_3*	111	1	1
GA_4*	3	-	-
GA_7*	1	-	-
GA_{19}	3	-	-
GA_{24}	103	2	1
ABA*	108	2	-
ACC	1	-	-
JA*	64	-	-
SA*	43	1	-

Assuming that the relevance of a hormone may be inferred also by assessing the extent of its transcriptional response and that transcript whose levels are correlated with the amount of active hormones may represent such response, cytokinins, gibberellins, and ABA, with 317, 132, and 108 interconnected transcripts, respectively, resulted to be pivotal during early fruit development. The role of ABA and gibberellins and their antagonism during early fruit

development was widely discussed in several plant model systems, such as tomato (Vriezen et al., 2008) and apple itself, in the latter case within the context of fruitlet abscission (Botton et al., 2011; Eccher et al., 2013).

Table 3 – Enrichment analysis of the terms associated to nodes of the "early" network.

Biological process	
Go term	***P*-value**
cellular component movement	1.04E-03
microtubule-based movement	1.04E-03
fatty acid biosynthetic process	1.65E-03
DNA metabolic process	1.67E-03
nitrogen compound transport	2.01E-03
monocarboxylic acid biosynthetic process	2.53E-03
microtubule-based process	2.97E-03
chromosome organization	3.44E-03
cellular macromolecular complex assembly	4.21E-03
chromatin assembly	5.39E-03
nucleosome assembly	5.39E-03
DNA packaging	5.39E-03
nucleosome organization	5.39E-03
protein-DNA complex subunit organization	5.39E-03
protein-DNA complex assembly	5.39E-03
macromolecular complex assembly	5.61E-03
DNA replication	5.88E-03
cell cycle	6.16E-03
chromatin assembly or disassembly	6.17E-03
fatty acid metabolic process	6.54E-03
regulation of DNA replication	7.46E-03
cellular component biogenesis	7.57E-03
monocarboxylic acid metabolic process	9.73E-03
Molecular function	
Go term	***P*-value**
microtubule motor activity	8.73E-04
exonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters	1.22E-03
motor activity	1.86E-03
hydrolase activity	2.96E-03
hydroxymethylbilane synthase activity	3.58E-03
GDP-dissociation inhibitor activity	7.00E-03
Rho GDP-dissociation inhibitor activity	7.00E-03
ATPase activity	7.77E-03
nucleoside-triphosphatase activity	8.94E-03
Cellular component	
Go term	***P*-value**
protein-DNA complex	2.17E-03
chromosomal part	2.40E-03
chromosome	4.00E-03
nucleosome	4.72E-03
chromatin	4.72E-03
DNA packaging complex	4.72E-03
DNA bending complex	4.72E-03
nucleus	7.64E-03

An enrichment analysis of the whole network was also performed for the three gene ontologies "biological process", "molecular function" and "cellular component" (Table 3). Results show a high proportion of genes significantly enriched (P-value ≤ 0.01) with terms

such as “cellular component movement” and “microtubule-based movement”, “fatty acid biosynthetic process”, “DNA metabolic process”, “nitrogen compound transport”, “monocarboxylic acid biosynthetic process” and “chromosome organization” for the “biological process” ontology. Concerning the molecular functions, the terms enriched were “microtubule motor activity”, “exonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters”, “hydrolase activity”, “GDP-dissociation inhibitor activity”, “Rho GDP-dissociation inhibitor activity”, “ATPase activity” and “nucleoside-triphosphatase activity”. Regarding the cellular component ontology most genes were enriched with “protein-DNA complex”, “chromosomal part”, “chromosome”, “nucleosome”, “chromatin”, “DNA packaging complex”, “DNA bending complex” and “nucleus”.

Taken as a whole, these results reflect the complexity of the molecular and physiological processes occurring during early fruit development, and the central role of the hormones involved.

Table 4 - Summary of the main interactions between the guide nodes (hormones or precursors) and the other node types included in the “intermediate” network (*=biologically active hormones).

Hormones or precursors (HRs)	**No. interactions**	
	Genes	**Metabolites**
IAA*	21	-
DZ*	1	-
DZR*	3	-
iP*	1	-
iPR*	25	-
Z*	16	-
ZR*	1	-
GA_1*	1	-
GA_3*	78	-
GA_4*	2	-
GA_7*	1	-
GA_9*	6	-
GA_{20}*	16	-
ABA*	9	-
ACC*	97	2

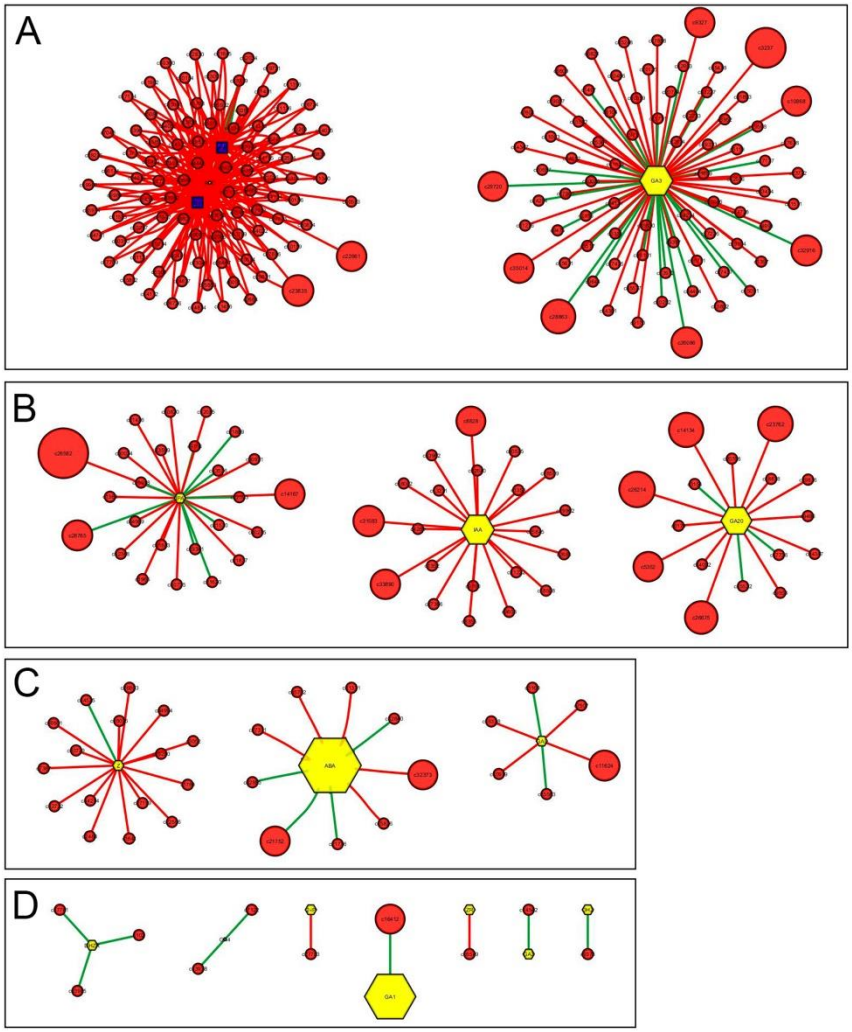

Figure 3 - "Intermediate" network overview. (A) ACC, GA_3. (B) iPR, IAA, GA_{20}. (C) Z, ABA, GA_9. (D) DZR, GA_4, iP, GA_1, ZR, GA_7, DZ. Nodes are characterized by different colors and shapes: hormones are yellow hexagons, metabolites are blue squares, and transcripts are red circles. The size of each node reflects the average quantitative data in the four time points of the network. Edges' length corresponds to the strength of the Pearson's coefficient. Red and green colors mark direct and indirect correlations, respectively.

"Intermediate" network - The "intermediate" network (Figure 3) covered the cell expansion and the maturation phases, from 53 up to 146 DAFB. The input dataset consisted of 8,686 DEGs (Table 1), obtained as previously described. Seventeen guide nodes were included in the correlative network, divided in 15 hormones and 2 metabolites (both are phenols). The interactions are 419, 416 of which are with transcripts and the other between hormones and/or metabolites (Table 4). Among hormones and their precursors, the most connected with transcripts were ACC (97), and GA_3 (78). This network included 15 modules and, with respect to the other networks (Figure 2 and 4), the total number of interactions was

lower and each guide node was connected with an exclusive subset of transcripts without any multiple interaction among sub networks. Except for the ACC network in which there are multiple interactions among the hormones, the phenols and some shared genes. This could reflect a physiological steady state in which hormones are only partially involved in the maintenance of the basal processes (only few changes in terms of concentration occurred). It is likely that other signalling molecules could play regulatory roles during these developmental stages. Only the generic terms "DNA-binding" was enriched in this network, with a *P*- value ≤ 0.01.

Table 5 - Summary of the main interactions between the guide nodes (hormones or precursors) and the other node types included in the "late" network (*=biologically active hormones).

Hormones or precursors (HRs)	No. interactions		
	Genes	Metabolites	HRs
IAA*	29	-	-
DZ*	127	-	-
DZR	7	-	-
iP*	81	1	-
iPR	4	-	-
Z*	3	-	-
ZR	128	-	-
GA_1*	1	-	-
GA_3*	85	2	-
GA_4*	80	-	-
GA_7*	19	-	-
GA_9	22	-	-
GA_{19}	48	-	1
GA_{20}	131	-	-
GA_{24}	116	-	1
ABA*	139	2	-
ACC	66	-	-
JA*	172	-	-
SA*	2	-	-

"Late" network - The "late" network covered the maturation and the ripening phases, from 132 up to 172 DAFB. The input dataset consisted of 4,699 DEGs (Table 1). The total number of central guide nodes is 19, while interactions are 1,264, among which 1,258 are with transcripts (see Table 5 for a detailed description). Despite the lower numbers of DEGs used as input, compared with the other two networks, in this case the highest percentage of initial transcripts (27%) was included.

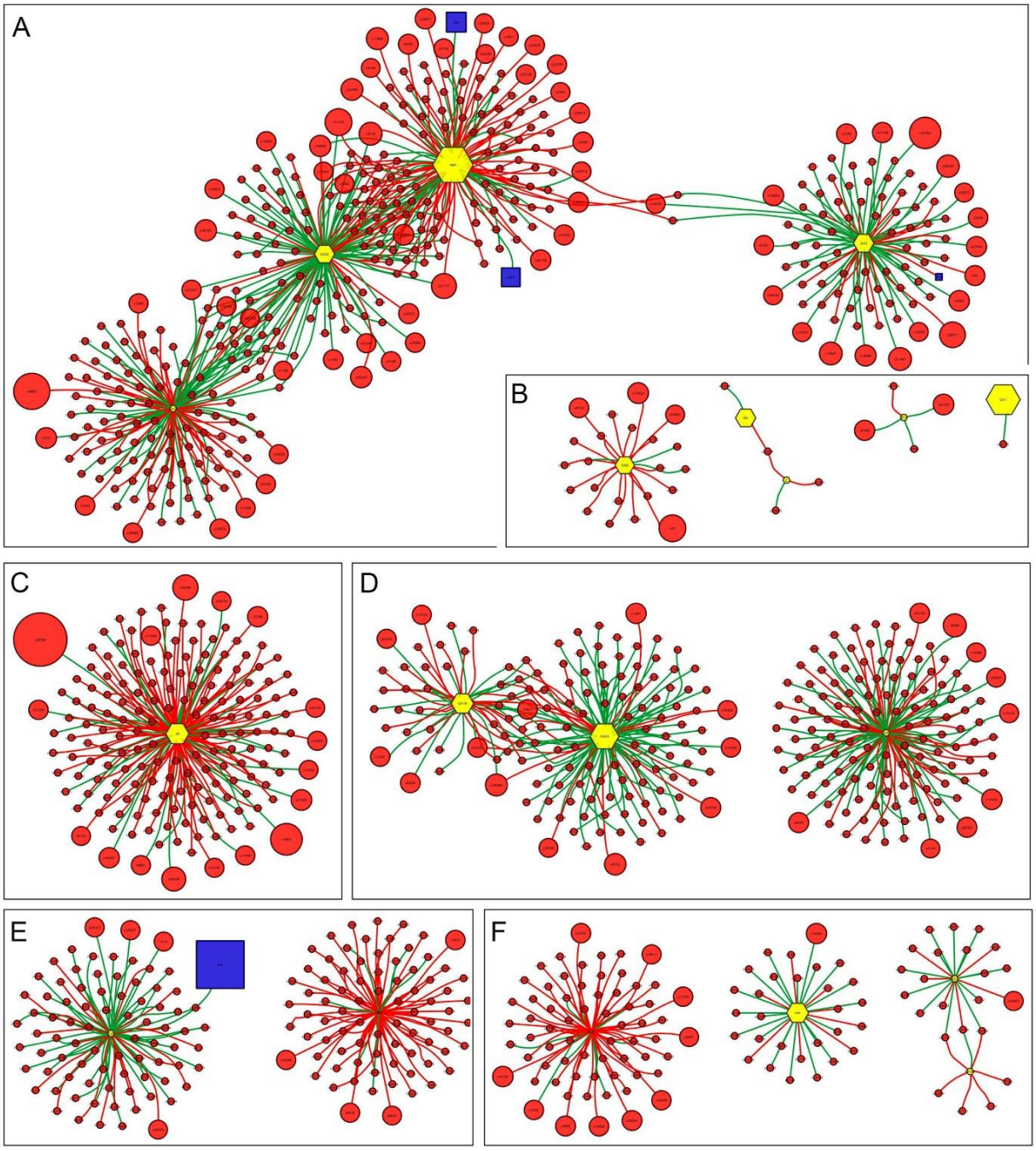

Figure 4 - "Late" network overview. (A) DZ-GA_{20}-ABA-GA_3. (B) GA_9, SA-Z, iPR, GA_1. (C) JA. (D) GA_{19}-GA_{24}, ZR. (E) iP, GA_4. (F) ACC, IAA, GA_7-DZR. Nodes are characterized by different colors and shapes: hormones are yellow hexagons, metabolites are blue squares, and transcripts are red circles. The size of each node reflects the average quantitative data in the four time points of the network. Edges' length corresponds to the strength of the Pearson's coefficient. Red and green colors mark direct and indirect correlations, respectively.

Among the hormones and their precursors, the most connected with transcripts were JA (172), ABA (139), GA_{20} (131), ZR (128), DZ (127), and GA_{24} (116) (Table 5). Fourteen modules are contained in the "late" network, four of which showed transcripts that are shared

between different guide nodes. One of the modules included DZ, ABA, and GA_3 (Figure 4A), although only DZ showed some variations during the last developmental phases. This result was quite unexpected and would deserve more attention in order to understand the real power of this approach. Nonetheless, the importance of ABA during the last developmental phases, especially at ripening, has already been widely investigated (Leng et al., 2014) and some confirmations were recently found also in apple (Falchi et al., 2013), although without any mechanistic information. Therefore, the network module including ABA must be further studied in order to investigate the possible role of the correlated transcripts, the exact time of their involvement, and the specific processes of ripening in which they play a pivotal role. It is noteworthy that starch is the metabolite directly linked to ABA in the same module, since this would indicate that within the nodes of this subnetwork, several important genes may be found that are involved in the ripening phase.

No term belonging to the "biological process" ontology was significantly enriched in the "late" network, whereas for the other ontologies high proportions of genes from the categories "beta-galactosidase activity", "galactosidase activity", "hydrolase activity", "hydrolyzing O-glycosyl compounds", "hydrolase activity, acting on glycosyl bonds", and "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen" and "beta-galactosidase complex" were found.

Conclusions

In this study, for the first time in apple, metabolomic, transcriptomic and hormone profiles data obtained from the same set of samples covering all apple fruit development were merged together following an integrative approach. Although this study represents just a starting point for further analysis, the correlative networks revealed putative key regulators that could have an important role during apple fruit development and highlighted possible cross-talks among hormones, metabolites and transcripts showing a very high and significant correlation.

A likely cross-talk between IAA and SA, already reported in the context of different processes and diverse species (Zhang et al., 2007; Iglesias et al., 2011), was pointed out, and an ABA-JA cross-talk, previously documented also in this case in different physiological contexts (Munemasa et al., 2011; Suhita et al., 2004; Tsonev et al., 1998; Raghavendra and

Reddy, 1987; Xin et al., 2005; Suhita et al., 2003; Gehring et al., 1997; Robert-Seilaniantz et al., 2011), was observed at the early stages.

This approach has definitely put in evidence the complexity of the developmental program, especially during the early and the ripening phases, whereas during the intermediate stages the hormones' involvement seems to be different and most likely related to the maintenance of basal physiological processes. During this phases, "Mister Development" seems to make use of different players (sugars?) to control its prosecution integrating both the internal and external stimuli to "prime" the transition to maturation, which, in turn, opens the way to ripening. One of the most interesting candidate player is the starch (and its related metabolic pathways), whose levels started to decrease just in the middle of development, when nothing, hormonally speaking, seems to occur. Of course, this is a new hypothesis that needs to be better investigated with a targeted experimental approach. Thus, the following step is to deeply understand which transcripts are related to hormones and metabolites, with a special attention on those showing multiple interactions.

Finally, these preliminary results could help us to understand the function of non-annotated genes, add new information about the targets of hormones' regulation during each developmental phase. Last bout not least, the set of genes with very high and significant correlations with hormones may be used to set up a predictive model for hormone levels in the apple fruit, similarly to the Hormonometer tool developed in Arabidopsis (Volodarsky et al., 2009).

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