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Expression profiling in developing peach seed and mesocarp as affected by growth regulators

Karina Beatriz Ruiz Carrasco

Coordinatore Dottorato
Luca Corelli Grappadelli

Relatore
Patrizia Torrigiani

Co-Relatore
Claudio Bonghi

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To Laura:

thanks for bringing me back!

ABSTRACT

Jasmonates (JAs) and spermidine (Sd) influence fruit (and seed) development and ripening. In order to unravel their effects in peach fruit, at the molecular level, field applications of methyl jasmonate (MJ) and propyl dihydrojasmonate (PDJ), and Sd were performed at an early developmental stage (late S1). At commercial harvest, JA-treated fruit were less ripe than controls. Realtime RT-PCR analyses confirmed a down-regulation of ethylene biosynthetic, perception and signaling genes, and flesh softening-related genes. The expression of cell wall-related genes, of a sugar-transporter gene and hormone-related transcript levels was also affected by JAs. Seeds from JA-treated fruit showed a shift in the expression of developmental marker genes suggesting that the developmental program was probably slowed down, in agreement with the contention that JAs divert resources from growth to defense. JAs also affected phenolic content and biosynthetic gene expression in the mesocarp. Levels of hydroxycinnamic acids, as well as those of flavan-3-ols, were enhanced, mainly by MJ, in S2. Transcript levels of phenylpropanoid pathway genes were up-regulated by MJ, in agreement with phenolic content. Sd-treated fruits at harvest showed reduced ethylene production and flesh softening. Sd induced a short-term and long-term response patterns in endogenous polyamines. At ripening the up-regulation of the ethylene biosynthetic genes was dramatically counteracted by Sd, leading to a down-regulation of softening-related genes. Hormone-related gene expression was also altered both in the short- and long-term. Gene expression analyses suggest that Sd interfered with fruit development/ripening by interacting with multiple hormonal pathways and that fruit developmental marker gene expression was shifted ahead in accord with a developmental slowing down. 24-Epibrassinolide was applied to Flaminia peaches under field conditions early (S1) or later (S3) during development. Preliminary results showed that, at harvest, treated fruit tended to be larger and less mature though quality parameters did not change relative to controls.

CONTENTS

INTRODUCTION

1. Seed and fruit development

1.1 General aspects of flower and fruit

Fruits are confined to the Angiosperms. The botanical definition of fruit is a mature ovary. Embryonic development in many Angiosperms occurs concomitantly with the development of the ovary into this specialized organ, the fruit, which provides a suitable environment for seed maturation and often a mechanism for the dispersal of mature seeds, as Darwin observed (reviewed by Seymour et al. 2008).

The first Angiosperm fruits appear in the fossil record in the Cretaceous period. Early fruits were dry with separate carpels (enclosures of seed within ovary) that probably split down one side to release their seeds. Fruit with fused carpels only appear in the middle Cretaceous, some 97 million years ago. Drupes and berries, the classic fleshy fruits, first appeared in the late Cretaceous or early Tertiary. Dry fruits can either be indehiscent or non-dehiscent, and fleshy fruits are either “true fruits” (deriving from carpels) or “false fruits” that include parts associated with accessory structures (Figure 1).

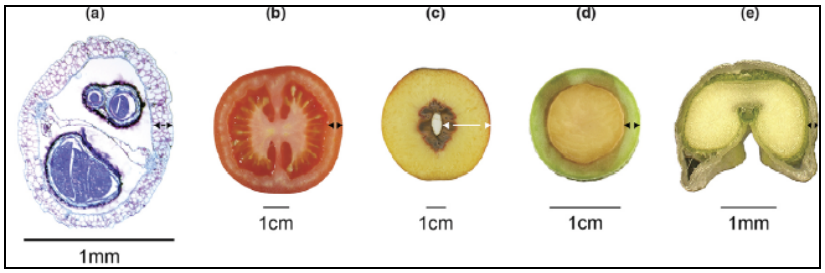

Figure 1. Cross-section of different fruits showing large differences in lignification or fleshiness of tissues. a) transverse section of *Arabidopsis* silique, b) tomato; c) nectarine; d)acorn; e)wheat grain. Tissues considered to be mature fruit pericarp are delineated by arrows. (From Seymour et al. 2008)

Plants have evolved a wide array of strategies for seed protection and dispersal. Among these, *Prunus* species including cherry (*P. cerasus, P. avium*), peach (*P. persica*), plum (*P. domestica, P. salicina*), apricot (*P. armeniaca*) and almond (*P. dulcis*) have developed a unique adaptation where the seed is encased by an extremely hard wood-like carapace called the stone. The stone is formed through lignification of the endocarp layer, a feature that defines a broader class of fruits called drupes. Mango (*Mangifera indica*), olive (*Olea europaea*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), blackberries (*Rubus* spp.) and

pistachio (*Pistacia vera*) are all examples of drupes highlighting their diversity and agricultural importance.

So, the biochemistry, physiology, and structure of the reproductive organ (seed and fruit) is developmentally altered to influence appearance, texture, flavor, and aroma in ways designed to attract seed-dispersing organisms (Figure 1) (Adams-Phillips et al. 2004).

1.2 Flower-fruit relations

An emerging theme from recent discoveries in the past two to seven years is that genes controlling fruit development and ripening are closely related to regulatory factors involved in modulating the development of floral organs.

Pioneering work on the genetic basis of fruit formation and development has emphasized the model system *Arabidopsis* (Dinneny et al. 2005), whereas investigations of organ expansion, maturity, ripening, shelf-life and nutritional quality have centered on the crop model tomato (*Solanum lycopersicum*) (Giovannoni, 2007).

The development of flowers and fruits is a complex set of molecular circuits that share as key players various members of the MADS box family of transcription factors (TF) (Seymour et al. 2008). Studies in the model plant *Arabidopsis* have revealed that a combination of activities of the AGAMOUS (AG) and SEPALLATA (SEP) genes is required to bring about carpel development. The SEP family genes are not found in Gymnosperms and are thought to have played a crucial role in the origin of floral structures. The expression of these genes appears conserved throughout the Angiosperm lineage, including monocots. A gene duplication event led to the AG and the related PLENA lineages, the latter including the SHATTERPROOF (SHP) genes. Genes belonging to the SQUAMOSA class of MADS box TFs include APETALA1 (AP1) and FRUITFULL (FUL) are functionally redundant in specifying floral meristems.

Many of the carpel development genes appear to have additional functions in fruit development and ripening beyond those required for flowering in both dry and fleshy fruit types. In most plants, early fruit development can be divided into three phases. Different types of fruit display variations of this general developmental program (Gillaspy et al. 1993).

Phase I: Ovary Development, Fertilization, and Fruit Set

The earliest phase involves the development of the ovary and the decision to abort or to proceed with further cell division and fruit development, which is generally referred to as fruit set.

Phase II: Cell Division, Seed Formation, and Early Embryo Development

In the second phase, fruit growth is due primarily to cell division.

Phase III: Cell Expansion and Embryo Maturation

The third phase begins after cell division ceases. During this phase, fruit growth continues, mostly by cell expansion, until the fruit reaches its final size. This growth phase is the most visible and physiologically most significant because of the strong sink activity exerted by the expanding cells.

Alba and collaborators (2005) propose that fruit development occurs in five stages, including organogenesis, expansion, maturation, ripening and senescence, being the ripening an aspect of development that is unique to fruit and that is initiated after seed maturation has been completed (Gillaspy et al. 1993) and represents the terminal stage of development in which the matured seeds are released.

1.3 *Arabidopsis thaliana* as model for developmental regulation of ripening in dry fruits

Genetic studies on the dry fruits of *Arabidopsis* have revealed the high level molecular circuits controlling the development and dehiscence of siliques. Briefly, the region-specific expression of the FUL and SHP MADS-box genes is determined by the expression of a range of other TFs and leads to normal valve and valve-margin development in the silique (Lewis et al. 2006). INDEHISCENT (IND), a bHLH TFs direct the differentiation of the valve margin into the separation and lignified layers. Lignification of the endocarp layer in the silique requires the activities of IND, SHP, ALC and FUL. Genetic analysis has shown that IND, SHP and ALCATRAZ (ALC) are part of a network controlling pod shatter. Uncovered networks appear to be conserved in other dry fruit types. Additionally, recent work has revealed a set of 15 TFs involved in pericarp development from profiling of both normal and parthenocarpic 'empty' siliques in *Arabidopsis* (de Folter et al. 2004).

1.4 Tomato model to understand fleshy fruit development and ripening.

Fossil and molecular evidence suggests that fleshy fruits developed from dry forms (Knapp 2002). It is therefore not unreasonable to speculate that some of the regulatory genes involved in the development and ripening of dry types have been conserved in the evolution of fleshiness. This may seem unlikely at first glance since there is no analogous process to dehiscence in fleshy fruits, but the valve tissue of the *Arabidopsis* silique is analogous to the pericarp of the tomato. Furthermore, lignified endocarp layers occur in both siliques and fleshy fruits such as drupes, for example peaches and nectarines (Figure 1). However, in contrast to dry fruits, fleshy fruit development leads to the formation of a juicy, expanded, and generally sweet and colored fruit (Coombe, 1976).

Even if research on flesh fruit is moving ahead very fast, much less is known about the regulatory networks controlling fruit development and ripening (Seymour et al. 2008).
Tomato, the centerpiece of the Solanaceae family, has emerged as a model of fleshy fruit development, primarily because this is the species for which the genetic and molecular toolkits are most advanced. Short time generation, extensive germplasm collections, well-characterized mutant stocks, high-density genetic maps, immortalized mapping populations, efficient transient and stable transformation, deep expressed sequence tag (EST) resources, microarrays and an ongoing genome sequencing effort all contribute to the utility of this experimental system (Mounet et al. 2009).
In tomato flowers, the ovary wall consists of undifferentiated parenchyma cells, vascular bundles, and inner and outer epidermal cell layers. Ongoing the development, fleshy fruits increase in size by both cell division and cell expansion; commonly undergoing marked changes in texture, colour and flavour during ripening.

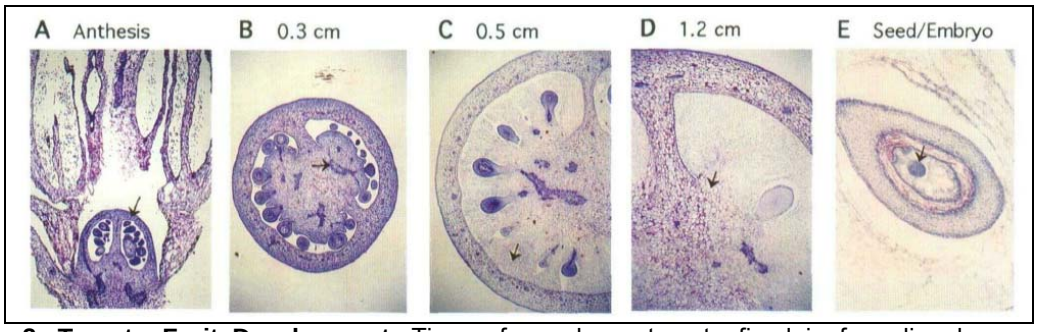

Figure 2. **Tomato Fruit Development.** Tissue from cherry tomato fixed in formalin plus acetic acid, embedded in paraffin, cut into 10-um sections, and stained with toluidine blue. (A) Longitudinal section through the ovary within the flower at anthesis. Arrow indicates the pericarp. (B) Cross-section of a fruit 0.3 cm in diameter. Arrow points to vascular tissue within the placenta. (C) Cross-section of a fruit 0.5 cm in diameter. Arrow indicates the presence of locular tissue, which has differentiated from the placenta. (D) Part of a cross-section through *a* fruit 1.2 cm in diameter. Arrow points to the gradient zone of differentiation between placenta and locular tissue. (E) Cross-section through a developing seed from a fruit 1.2 cm in diameter. Arrow points to the developing embryo within the seed. (From Gillaspy et al. 1993).

During fruit development, the ovary wall becomes the pericarp, which consists of three distinct layers: the endocarp, mesocarp, and exocarp. The septa of the carpels divide the ovary and fruit into two or more locules. An elongated axial placenta, to which the seeds are attached, is highly parenchymous and later gives rise to the tissue that fills the locular cavity. A distinct concentric vascular system radiates through the pericarp but is more diffuse in other parts of the fruit (Figure 2). The pericarp is covered on the outside by a thin cuticle that thickens as the fruit ages. The skin of the pericarp further consists of an epidermal layer and three to four layers of collenchymous tissue. The outer epidermal cells contain little to no starch and no stomata, but the inner pericarp cells contain many starch grains. In tomato, the cells that contribute to most of the carpel and fruit pericarp

are large and vacuolated, and they are morphologically similar to leaf palisade cells. They contain most of the chloroplasts that give the developing fruit its green appearance. Cells in the outer and inner epidermal layer are small and have fewer chloroplasts. Thus, the carpel can be viewed as a modified leaf that has folded into a tubular structure that encloses the ovules. The fusion of two or more carpels in fruits such as tomato results in complex morphological structure in which it is difficult to discern the ontogenical relationships of cells in the fusion zones. Cells in developing fruit often contain photosynthetically active chloroplasts and express nuclear and plastid genes for photosynthetic proteins. This is consistent with the ontogenic relationship between cells in leaf and fruit (reviewed by Gillaspy et al. 1993).

Studies on non-ripening mutants of tomato have hitherto revealed unsuspected regulators of ripening in fleshy fruit (Figure 3). When mutated, the gene at the ripening inhibitor (*rin*) locus abolishes normal ripening, the fruit remaining firm and unripe for extended periods. The *rin* gene is a SEP4 MADS-box TF. Another fruit mutation, Colourless non-ripening (*Cnr*) in tomato is the result of the silencing of a SQUAMOSA Promoter Binding Protein (SBPbox) gene, LeSPL-CNR. The promoter of TDR4, a SQUAMOSA family MADS-box gene is a probable target of the LeSPL-CNR gene product, and TDR4 expression is repressed in the *Cnr* mutant (Cara and Giovannoni, 2008). The *Arabidopsis* transcription factor most similar in sequence to TDR4 is FUL, the gene that restricts valve-margin development in siliques. SBP-box genes and their putative MADS-box promoter targets appear to regulate fruit tissue development in both dicots and monocots (Seymour et al. 2008).

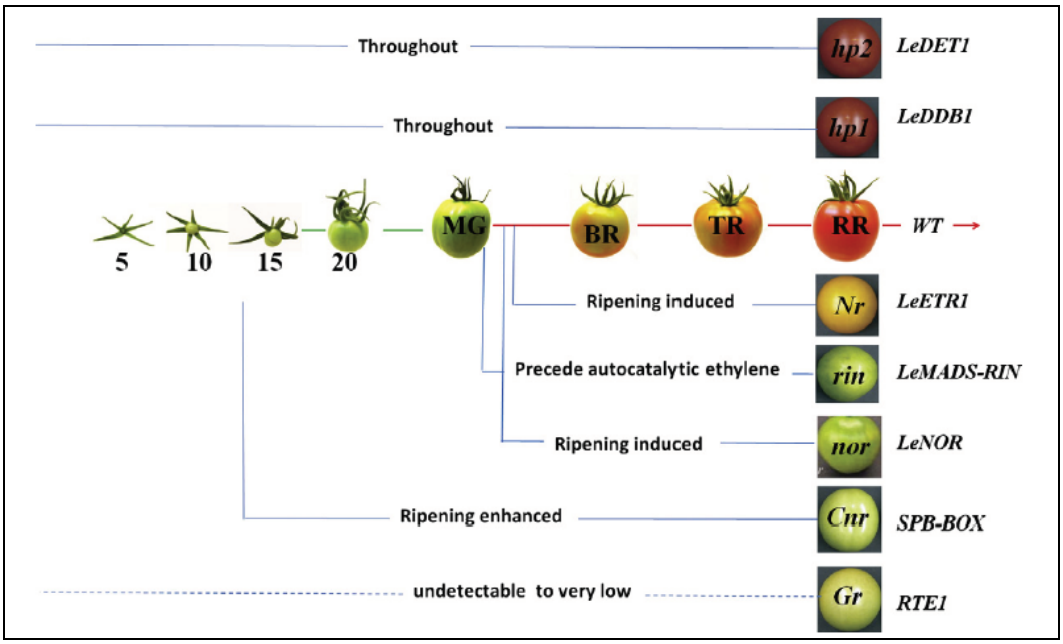

Figure 3. Temporal and developmental regulation of ripening and composition-altering genes in the wild-type fruits from young to fully mature and ripening (BR to RR) stages of tomato fruits. Typical phenotypes of various altered ripening tomato mutants at a physiological stage comparable to BR +10 days WT fruits. The lines to the left of each mutant fruit represent the stages when the WT fruit exhibit expression of ripening and the composition-altering genes. The broken line indicates that *RTE1* expresses at very low level throughout development and ripening of the WT fruit. 5, 10, 15, and 20 represent days after anthesis and MG, BR, TR, and RR represent mature green, breaker, turning, and red ripe, respectively. Abbreviations: hp1, high pigment 1 (DDB1, UV-damaged DNA binding); hp2, high pigment 2 (SlDET1, deetiolated 1); *Nr*, never ripe (SlETR1, ethylene response 1); *rin*, ripening inhibitor (SlRIN-MADS, RIN-MADS box-transcription factor); *nor*, non-ripening (nor, nonripening); *Cnr*, Colorless non ripening (SlSPB-Cnr, squamosa promoter binding protein); and *Gr*, green ripe (RTE1, reversion to ethylene sensitivity 1). Except for the *Gr* mutation that activated expression of RTE1 in Gr mutant, all other shown mutations resulted in loss of functional expression of their respective genes. (From Handa et al. 2012).

1.5 Prunus persica emerges as model of fleshy fruit development and ripening in the stony fruits.

Prunus persica, the subject of study in this thesis belongs to Rosaceae. This family comprised over 100 genera and 3,000 species, like almond, apple, plum, peach, pear, raspberry, sour cherry, sweet cherry, and strawberry. It is the third most economically important plant family in temperate regions (Dirlewanger et al. 2002). Currently, the best-developed model species for Rosaceae include apple (*Malus domestica*), peach (*Prunus persica*), and diploid strawberry (*Fragaria vesca*) (Shulaev et al. 2008).

Rosaceous fruits are consumed in multiple forms, including fresh, dried, juice, and processed products. Rosaceae fruits are also a major human dietary source of phytochemicals and compounds that could potentially yield health and disease-fighting advantages as well as antioxidants and/or cancer-inhibiting compounds that have been identified in these fruits (Boudet, 2007).

Traditionally, new traits for improvement of crop plants have been primarily derived from their related wild species. However, genomics and bioinformatics advances over the past

decade have provided new options for identifying useful compounds in plants and for manipulating encoding genes responsible for their production. A further challenge is to maintain crop quality following harvest while avoiding loss due to chilling injury disorders, decay, chemical contamination, and over-ripening. Thus, postharvest and consumer valued qualities and traits serve as ideal targets for crop product improvement.

Peach is the genetic and genomic reference species for *Prunus* because of its high economic value, self-compatibility allowing for development of F2 progenies, availability of homozygous doubled haploids, and possibility of shortening its juvenile period to 1 to 2 years following planting. However, peach transformation remains inefficient (Abbott et al. 2008)

1.6 Peach fruit development and ripening

Prunus genus has characteristic stone fruits or drupes in which seeds are encased in a hard, lignified endocarp (the stone), and the edible portion is a juicy mesocarp. Agriculturally important stone fruit species include *P. persica* (peach, nectarine), *P. domestica* (European or prune plum), *P. salicina* (Japanese plum), *P. cerasus* (sour cherry), *P. avium* (sweet cherry), *P. armeniaca* (apricot), and *P. amygdalus* (almond).

Peach pericarp is analogous to the valve tissue in *Arabidopsis* siliques where FUL and SHP orthologues are expressed. Expression studies suggest that temporal regulation of potential peach FUL and SHP orthologues may be involved in modulating the properties of the lignified endocarp (Seymour et al. 2008; Dardick et al. 2010).

The fruit development is strictly connected to embryogenesis in peach, moreover, in this specie, seed development is also necessary for fruit set. In the seed, development is characterized by a fast growth of endosperm that starts immediately after fertilization concurrently with the nucellus re-absorption, and lasts until the beginning of endocarp lignification, when the seed reaches its final size. At the end of pit hardening, seed volume is mainly made up of endosperm and the embryo is at the heart stage. Thereafter, embryo growth resumes and cotyledon development is paralleled by the endosperm re-absorption. Seed maturation is characterized by lipid accumulation (Ognjanov et al. 1995) synthesis of specific late embryogenesis abundant (LEA) proteins and dehydration. Seed abnormalities at the early stages of development (S1 and S1/S2 transition stages of fruit development) bring about abortion and fruitlet abscission (Stutte and Gage, 1990). Seed presence is always necessary to achieve normal fruit development even though embryo development is incomplete (Bonghi et al. 2011)

Later on (late S2, S3 and S4), the relationships between fruit development and embryogenesis become less strict. This is the case of early ripening varieties characterized by the uncoupling of fruit development and embryogenesis. In fact, at harvest, seed development is still in progress and far away from maturity (Bassi and Monet, 2008)

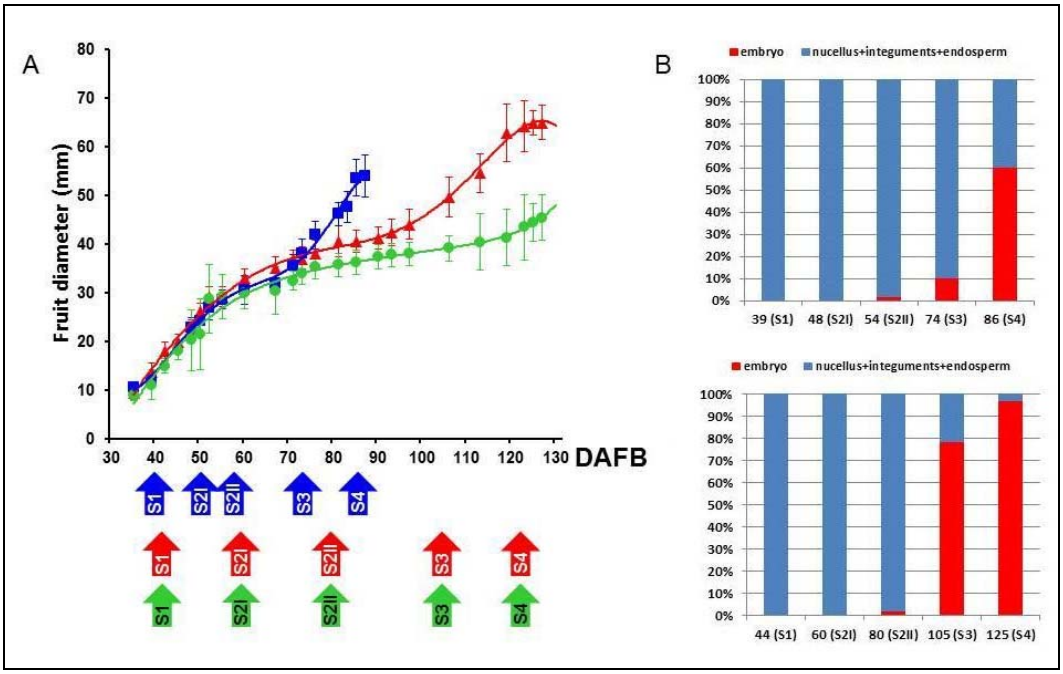

Figure 4. Dynamics of fruit and seed growth in Fantasia, Springcrest and slr. A) Fruit growth curves are expressed as cross diameter (mm) for Fantasia (the reference genotype; red triangles), Springcrest (the early ripening genotype; blue squares) and slr (the slow ripening genotype; green circles). In the lower panel, the developmental stage for the 3 cvs is indicated within each arrow. B) Dynamics of seed development in Springcrest (upper) and Fantasia (lower) related to the fruit developmental stages. Seed development in slr is similar to that reported for Fantasia. Relative abundance of nucellus, integuments and endosperm (blue) and embryo (red) points out that in Springcrest, at fruit harvest, embryo development is a long way from maturity, while in slr, in spite of the block of fruit ripening, the completion of embryo development parallels that of Fantasia and the seed is viable. (From Bonghi et al. 2011)

Fruit growth displays a double sigmoid pattern in which four stages named S1, S2, S3 and S4 can be distinguished (Tonutti et al. 1997). The early part of S1 is characterized by cell division and enlargement lasting about two weeks, followed by cell enlargement. The growth slowing down that occurs at S1/S2 transition is followed by endocarp lignification (pit hardening), which starts at the middle of S2 and is completed by the end of S2 lasting for 12-15 days. S3 starts with a resumption of growth mainly due to cell enlargement, thus generating the second exponential phase. By the end of S3, maturation is completed and followed by ripening (S4). The four fruit developmental phases are determined using a mathematical model based on first derivative of the growth curve. The identification of the growth phases is important for both developmental studies and for precision farming. However, the only easily detectable event is the end of pit hardening marking the S2/S3

transition, because the phase length is affected by both genotype (early, middle and late ripening varieties) and environmental cues, requiring a continuous reassessment of growth model.

One fact to take carefully into account is seed-fruit development and ripening depends on the genotypes. In Springcrest, fruit ripening occurred after 86 DAFB (Figure 4A), when seed development was still in progress (Figure4B). At the end of the growing season (taking cv Fantasia as a reference), slr (slow ripening genotype) showed a fully developed seed (4A and 4B), while the mesocarp development was blocked at stage S3.

Cross-talk between seed and fruit organs may involve different components of the signaling network playing either direct or indirect roles, such as hormones, transcription factors (TFs), and other signaling molecules (reviewed by Bonghi et al. 2011).

2. Hormonal control of fruit development

Fruit ripening is a highly co-ordinated, genetically programmed, and irreversible process that once started cannot be arrested; however it can be modulated in variety of fruit by several externally applied procedures like chemicals, phytohormones and more recently, by molecular biology tools (reviewed by Payasi and Sanwal, 2010)

The phytohormones are a collection of trace amount growth regulators that participate in almost every developmental process, including embryogenesis, seed germination, vegetative growth, fruit ripening and senescence. Auxin, abscisic acid (ABA), cytokinin, gibberellins (GA) and ethylene are well-known phytohormones as “classic-five”, and several new members, such as brassinosteroids (BR), jasmonic acid (JA) and salicylic acid (SA), have been identified over the past decades. In addition, several products of secondary metabolism such as nitric oxide, peptides and strigolactones are considered to act as phytohormones in plant growth and development. The chemical structures of phytohormones are quite simple, but their action and physiological effects are very complex. In general, a hormone response proceeds through the following successive steps: initiate signaling by binding to its receptor, transduce the signal by the downstream molecular cascade, and output the signal by metabolic and cellular changes. Genes involved in the phytohormones action usually recruit feedback regulation at multiple levels to coordinate plant growth and development, as the cases of GA, BR and auxin actions (Xiong et al. 2009).

Gillaspy et al. 1993, propose an integrative model about the main phytohormones involved in tomato fruit development and ripening (Figure 5). This model is being constantly

updating by new discovers but still remains a good support to understand fruit development.

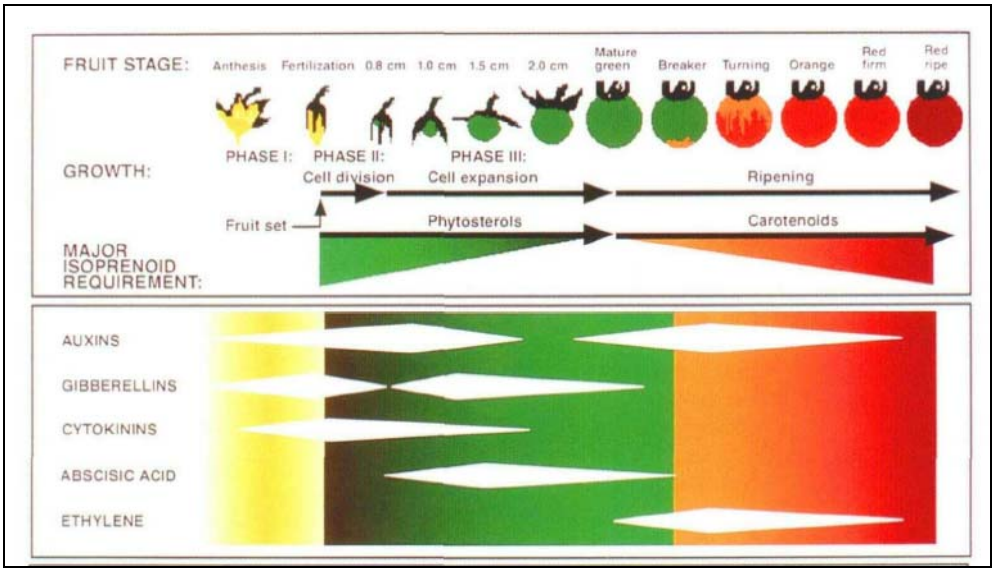

Figure5. Hormones changes during fruit development using tomato as model for fleshy fruits. (From Gillaspy et al. 1993)

2.1 Ethylene: a major player in the fruit ripening

Fruit species are classically defined physiologically on the basis of the presence (climacteric) or absence (non-climacteric) of increased respiration and synthesis of the gaseous hormone ethylene at the onset of ripening (Lelievre et al. 1997). Although the specific role of climacteric respiration in fruit ripening remains unclear, the recruitment of ethylene as a coordinator of ripening in climacteric species likely serves to facilitate rapid and coordinated ripening. It is well known, however, that ethylene alone is not sufficient for ripening and that a developmental ‘‘competence’’ to respond to ethylene must be achieved (Giovannoni, 2001). Consequently, immature fruit typically do not ripen in response to exogenous ethylene. On the other hand, the control of the onset of ripening in non-climacteric fruit is poorly understood, and suggestions of a control by sugar sensing and hormones such as abscisic acid (ABA) have been put forth (Gambetta et al. 2010). Examples of common climacteric fruits that require ethylene for ripening include tomato, apple, banana, and most stone fruits (Figure 6), whereas non-climacteric fruits include grape, citrus, and strawberry. Interestingly, climacteric fruit span a wide range of angiosperm evolution, including both dicots (e.g. tomato) and monocots (e.g. banana). Tomato fruit has been used extensively as a model system to understand ripening in climacteric fruit and strawberry as a model to investigate ripening in non-climacteric fruits (Zhang et al. 2010).

Two distinct ethylene biosynthesis systems have been described in plants. System 1 corresponds to low ethylene production in the pre-climacteric period of climacteric fruit and is present throughout the development and ripening of non-climacteric fruit. System 1 functions during normal growth and development and during stress responses whereas system 2 operates during floral senescence and fruit ripening. System 2 refers to an auto-stimulated massive ethylene production, called 'autocatalytic synthesis' and is specific to climacteric fruit. Therefore, the major differences related to ethylene between climacteric and non-climacteric fruit are the presence or absence of autocatalytic ethylene production (Bapat et al. 2010).

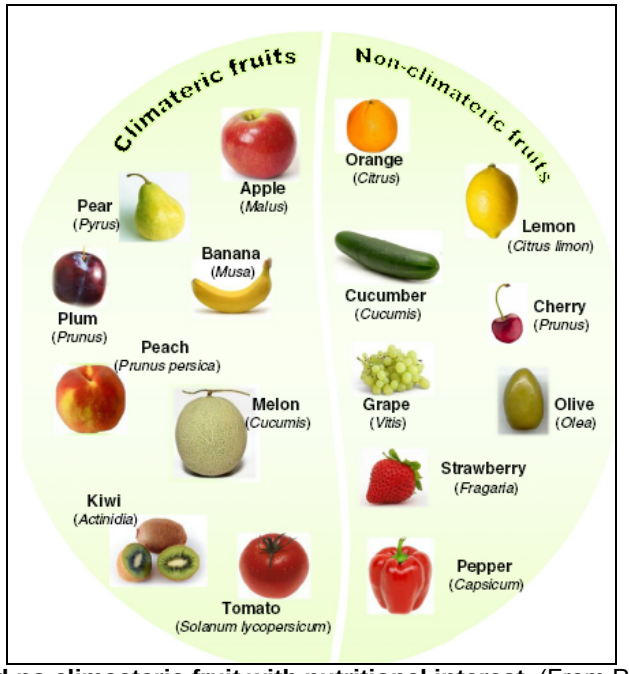

Figure 6. Climateric and no climacteric fruit with nutritional interest. (From Palma et al. 2011)

ACS and ACO belong to a family of genes that have been characterized from tomato, melon, apple, banana, pear, kiwifruit, peach, and persimmon (Cara and Giovannoni, 2008; Lin et al. 2009; Bapat et al. 2010). The members in each gene family are differentially expressed during development and in response to environmental cues. Nine ACS and five ACO genes were identified in tomato. Among them, four ACS (SlACS1A, SlACS2, SlACS4, and SlACS6) and three ACO (SlACO1, SlACO3, and SlACO4) members are differentially expressed during tomato fruit development. SlACS6 is predominantly expressed in the green fruit and is associated with ethylene production during the early stages of fruit development. SlACS1A is also expressed in green fruit but at lower levels than SlACS6. SlACS1A and SlACS4 are induced alongside ripening transition and

proposed to be responsible for the induction of SlACS2, a gene implicated in the autocatalytic production of ethylene during the robust ripening process. The autocatalytic ethylene production may exert negative feedback inhibition on early ethylene production by reducing the expression of SlACS1A and SlACS6 (Barry et al. 2000).

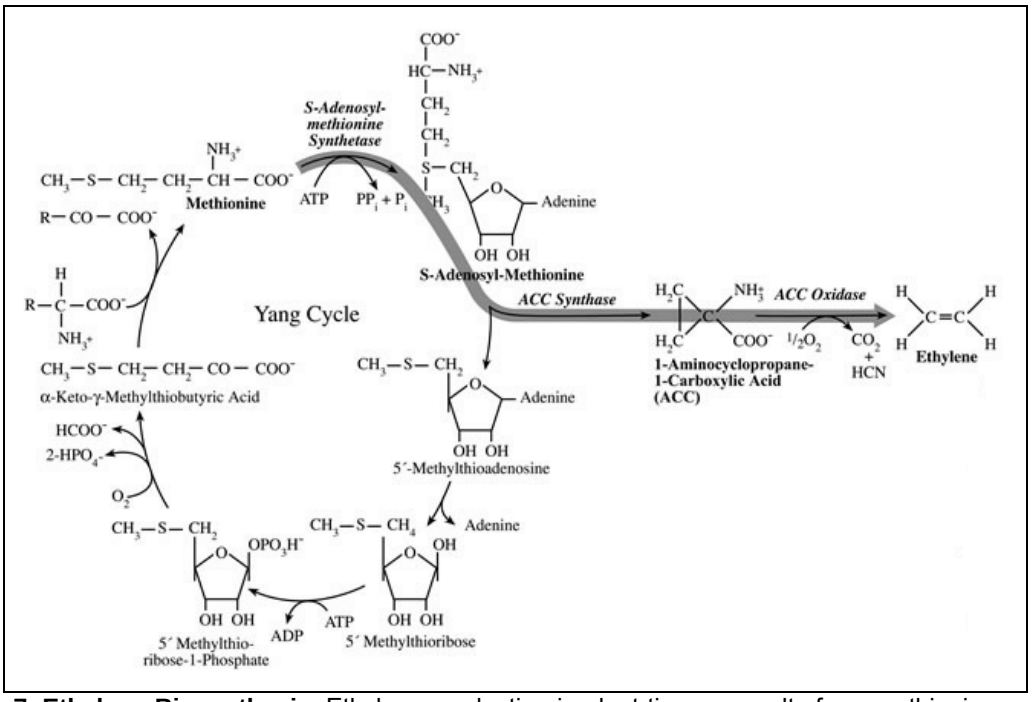

Figure 7. **Ethylene Biosynthesis**. Ethylene production in plant tissues results from methionine metabolism (Yang cycle). The rate-limiting steps in fruit ethylene synthesis include the conversion of S-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) via ACC synthase (ACS) and the subsequent metabolism of ACC to ethylene by ACC oxidase (ACO). In addition to ACC, ACS forms 5'-methylthioadenosine (MTA) which is utilized to form more methionine via the Yang cycle (Pech et al. 2010). The formation of ACC and its conversion to ethylene are the two committed steps in the hormone synthesis as the Yang cycle maintains high levels of biosynthesis even under low methionine availability. ACS, and in some cases ACO, are generally considered the rate-limiting steps in ethylene production in fruit (Cara and Giovannoni, 2008).

Among the ACOs, SlACO1 and SlACO4 are expressed throughout fruit development, but their expression increases dramatically at the onset of ripening and remains elevated thereafter. The ripening-associated induction of these ACOs is ethylene dependent, since their increased expression is impaired by treatment with 1-methylcyclopropene (1-MCP), an ethylene perception inhibitor (Mattoo and Suttle, 1991). SlACO3 is expressed during fruit maturation and is transiently induced at the breaker stage of fruit ripening (reviewed by Handa et al. 2012).

Ethylene is perceived by receptors and the functional ethylene receptors are disulfide-linked dimers and need copper ions to bind ethylene. In *Arabidopsis*, ethylene receptors are encoded by a multi-gene family (ETRs). Five ETRs (ETR1, ETR2, ERS1, ERS2, and EIN4) genes have been characterized. The tomato ETR family contains six members

(SlETR1, SlETR2 and SlETR3, also called NR; SlETR4, SlETR5 and SlETR6). Although they show significant divergence in primary sequence, all bind to ethylene (Zhang et al. 2010). Tomato ETR1 is constitutively expressed in vegetative and reproductive tissues (Zhou et al. 1996).

Ethylene induces the degradation of its own receptor likely by the 26S proteasome-dependent pathway (Kevany et al. 2007). These results are consistent with a model in which receptor levels modulate timing of the onset of fruit ripening by measuring cumulative ethylene exposure. This provides an explanation as to why immature fruits ripen early after exposure to exogenous ethylene. Conversely, altering the ethylene-dependent signal transduction networks has a considerable effect on fruit ripening and associated changes, including fruit color, aroma, sugar, and organic acids (Giovannoni, 2007).

Ethylene response factors (ERFs), also called ethylene-responsive element binding proteins (EREBPs), are plant transcriptional regulators that mediate ethylene response likely via binding to a conserved motif AGCCGCC (GCC-box) located in the promoter region of ethylene-regulated genes. In tomato, five ERFs (SlERF1, SlERF2, SlERF3, SlERF4, and SlERF3b) show binding capability to GCC-box elements. These genes exhibit differential expression patterns during plant development and fruit ripening. SlERF2 and SlERF3b transcripts accumulate during fruit ripening (Montgomery et al. 1993).

In tomato, three pleiotropic non ripening mutants (Figure 3), ripening-inhibitor (*rin*), non-ripening (*nor*), and Colorless non-ripening (*Cnr*), have been described in which virtually all aspects of the ripening process are inhibited, including ethylene synthesis, increased respiration, carotenoid accumulation, softening, and aroma production. Although ethylene synthesis is blocked in these mutants, studies using *rin* and *nor* fruits have indicated that they retain the capacity to synthesize wound ethylene, indicating that the mutations are not simply the result of a general block in ethylene synthesis. Similarly, exogenous ethylene does not restore ripening in these mutants, although ethylene-regulated gene expression can be partially restored, indicating that *rin*, *nor*, and *Cnr* fruits do not display ethylene insensitivity (Yokotani et al. 2004). Together these data suggest that *rin*, *nor*, and *Cnr* act upstream of ethylene in the ripening cascade and determine the competency of the fruit to ripen.

2.2 Auxins and their role in the fruit ripening

In comparison with ethylene, very little is known about the role of other hormones in fruit development.

The hormone auxin has long been known to be involved in pollination, fruit set and ripening and its role has been extensively investigated in several fruits (Deytieux-Belleau et al. 2007).

For early stages of fruit development, auxin, gibberellins and brassinosteroids are believed to act as major regulatory signals (Balbi and Lomax, 2003; Montoya et al. 2005). Moreover, auxin signalling appears to be a prerequisite for enlargement of cells in tomato fruits. This is probably mediated partly through interaction with gibberellin biosynthesis (Gillaspy et al. 1993). An intense cross talk between auxin and ethylene has been suggested for regulating early fruit development and the onset of ripening (Jones et al. 2002; Balbi and Lomax, 2003). Ethylene production in response to auxin treatment has been reported for a number of plant tissues (Bleecker and Kende, 2000). The stimulation in ethylene synthesis could be thought as the action of auxin on the expression of the key enzyme, ACS (Abel and Theologies, 1996). Therefore, under such conditions, any effect of auxin on the ripening process would be indirect and mediated by ethylene.

Two auxin biosynthesis pathways have been proposed. One is dependent on the precursor tryptophan (Trp) and the other is Trp-independent.

The Trp-dependent pathway includes:

(1) IAM pathway: conversion of tryptophan (Trp) to indole-3-acetamide (IAM).

(2) IPA pathway: conversion of Trp to indole-3-pyruvic acid (IPA) by the action of the tryptophan aminotransferase genes.

(3) TAM pathway: conversion of Trp to tryptamine (TAM) probably due to a tryptamine decarboxylase.

(4) IAOx pathway: the indole-3-acetaldoximine (IAOx) pathway has been proposed to occur only in cruciferous species such as *Arabidopsis*, in which glucosinolate secondary metabolism occurs.

Studies of the orange pericarp mutant, which fails to make tryptophan (because of a deficiency in tryptophan synthase b, which converts indole to tryptophan) but still makes auxin, provided the first evidence that tryptophan-independent biosynthesis occurs in maize.

Tryptophan-independent auxin biosynthesis also occurs in *Arabidopsis*. When auxin is not made from tryptophan it is thought to be made via indole-3-glycerol phosphate or indole (Woodward and Bartel, 2005).

Much of the auxin found in the plant is stored as conjugates to amino acids or sugars. For example, in maize, Zm IAGLU protein conjugates IAA to glucose (Ludwig-Muller et al. 2005) while OsGH3-like genes, conjugates IAA to amino acids in rice (Jain et al. 2006).

Regards to PIN1, an auxin efflux facilitator carrier, three homologs of the PIN1 have been reported in maize and rice. A gene encoding a putative PIN1 (Paponov et al. 2005) is also found to be expressed in the peach mesocarp particularly during ripening process. However, the expression of this gene appears to be significantly increased by ethylene rather than auxin. This supports the existence of a cross-talk between auxin and ethylene in regulation of ripening, besides the independent roles played by each hormone (Trainotti et al. 2007).

The auxin signaling pathway is mediated by regulatory proteins belonging to the auxin/indole-3-acetic acid (Aux/IAA) and auxin response factor (ARF) families of transcription factors. In *Arabidopsis*, 25 Aux/IAA genes have been found and 31 in rice, 24 of which are regulated by auxin (reviewed by McSteen, 2010). A gene family of ARF has been described in rice with 25 OsARF genes (Wang et al. 2007). ARFs and Aux/IAA encoding genes have been reported in tomato fruits and data suggest that auxin may be part of the mechanism that control the ripening of climacteric fruits (Jones *et al.* 2002).

Several genes, encoding TIR1 genes, coding for auxin receptors, have been isolated from peach mesocarp (Trainotti et al. 2007). Many of these genes, which are involved in auxin biosynthesis, transport and signaling (receptors, ARF, Aux/IAA encoding genes) display increased expressions in the mesocarp during ripening.

In climacteric peach fruit it has been shown that, concomitant with ethylene production, increases in the amount of auxin can also be measured (Miller et al. 1987). In ripening fruits, increases in auxin content have also been reported to parallel those in climacteric ethylene production (Gillaspy et al. 1993). Auxin promotes the enlargement of mesocarp disc as well as ripening processes, such as softening and anthocyanin formation in peach fruit (Ohmiya, 2000). The effect of auxin in fruit ripening is dependent on the penetration of auxin into the bulk of fruit tissue. The effect of high concentrations of auxin is to induce early ethylene production, which in turn initiates ripening.

Finally, regarding postharvest application of auxin, it has been observed that IAA stimulates ethylene synthesis, but prevents the climacteric rise in respiration (Payasi and Sanwal, 2010).

2.3 Cytochinins

Kinetin is the main cytochinin. It is known to prevent senescence by arresting protein and chlorophyll degradations and also acts as a senescence retardant in fruits particularly in peel. Infiltration of kinetin into fresh banana slices enhances the ethylene production and respiration, but other ripening changes in banana fruit slices are delayed, particularly degreening of peel (Rao and Chundawat, 1986). Treatment of apple fruits with cytokinin and cytokinin-like compounds produces firmer fruits before and after storage (Elfving and Lougheed, 1994).

Cytokinin application stimulates ethylene production in post-climacteric avocado fruit. This pattern could be related to the aging effect of ethylene; with isopentenyl adenine (IPA) application it is possible to retard aging effects in ripening fruit as occurs in leaf tissue (Payasi and Sanwal, 2010).

A given physiological process can be regulated by different phytohormones through the control of expression of a common set of downstream genes. For example, Cyclin D3 (CYCD3) expression, which is involved in the control of the cell cycle, can be induced by cytokinins. Brassinosteroids (BR) are also able to induce the expression of CYCD3 and even to replace the function of cytokinin in maintaining the cell division (Hu et al. 2000). A work on the interdependence of auxin and BR signaling has well demonstrated that the complex cross-talk among phytohormones can also be achieved by coordinated regulation of a common set of downstream genes (Hardtke, 2007).

2.4 Gibberellins

Gibberellins also act as senescence retardant. Gibberellic acid (GA) delays fruit maturation and ripening. The peach fruit pressure-infiltrated with GA had reduced ethylene emission and respiration rate, which reflects a delay of the ripening process (Martinez-Romero et al. 2000). The GA treatment delays fruit ripening in whole banana (Payasi et al. 2004). Infiltrating GA in banana fruit affected the triggering of starch breakdown and sucrose synthesis. GA plays an important role in the delay of enzyme activities, especially of the cell wall, carotene synthesis and degradation of chlorophyll in banana (Rossetto et al. 2004). The GA treatment of kakis fruit retards ripening as well as the decrease of pulp firmness, titratable acidity, chlorophyll and phenolic content, ethylene production, the accumulation of carotenoids, and the change in external color (Ferri et al. 2004). Treatment of mangoes with GA leads to delay in ripening and starch degradation (Singh et al. 2007). The GA treatment also delays ripening and increases the shelf life of sapota fruits (Sudha et al. 2007).

2.5 Abscisic acid

Abscisic acid (ABA) acts as an important regulator of natural ripening of fruits (Payasi et al. 2010). ABA stimulates ethylene biosynthesis and shortens the time required for ripening initiation in mangoes, tomato and facilitates initiation and progress in the sequence of ethylene-mediated ripening events in banana. ABA is thought to have an important role in promoting climacteric ethylene production in apple (Lara and Vendrell, 2000) and therefore may indirectly induce apple softening.

ABA specific-binding proteins have been characterized in the flesh of developing apple fruit and it is hypothesized that these binding proteins may be putative ABA-receptors that mediate ABA signals during fruit development (Zhang et al. 2001). The possible role of ABA in controlling grape berry ripening has been suggested (Deytieux-Belleau et al. 2007).

ABA content is very low in unripe fruit but increases during the process of fruit ripening in both climacteric and non climacteric fruits. At present, a relationship between ABA and ethylene during ripening and senescence was indicated in the tomato fruit (Martinez-Madrid et al. 1996), peach and grapefruit. The potential contribution of ABA in the induction of fruit ripening was analyzed in relation to ethylene and the results demonstrated: (1) PpNCED1 and VvNCED1 initiated ABA biosynthesis at the beginning of fruit ripening in peach and grape, respectively, (2) ABA accumulation preceded the climacteric rise in ethylene production, (3) exogenous ABA stimulated ethylene production and accelerated fruit ripening possibly via the regulation of ACS and ACO gene expression, (4) inhibition of ABA synthesis by fluridone or NDGA suppressed ethylene production and delayed fruit ripening and (5) ethylene plays a important role in the later stage of fruit ripening. Together, these evidences indicate that ABA may be a trigger hormonal stimulus inducing ethylene production and consequently initiating the ripening process (Zhang et al. 2009).

2.6 Jasmonates

Jasmonates (JA) which include the jasmonic acid methyl ester (MeJA), JA amino acid conjugates, JA metabolites and their octadecanoid precursors, belong to a broad class of chemicals, called the oxylipins. JA are ubiquitous plant hormones best known for their role in responses to biotic and abiotic stresses (Wasternack, 2007) but also responsible for the regulation of several physiological processes related to plant development including secondary metabolism, reproductive processes and fruit development, and senescence.

JA biosynthesis originates from polyunsaturated fatty acids in chloroplast membranes via multiple steps in the octadecanoid pathway (Figure 8).

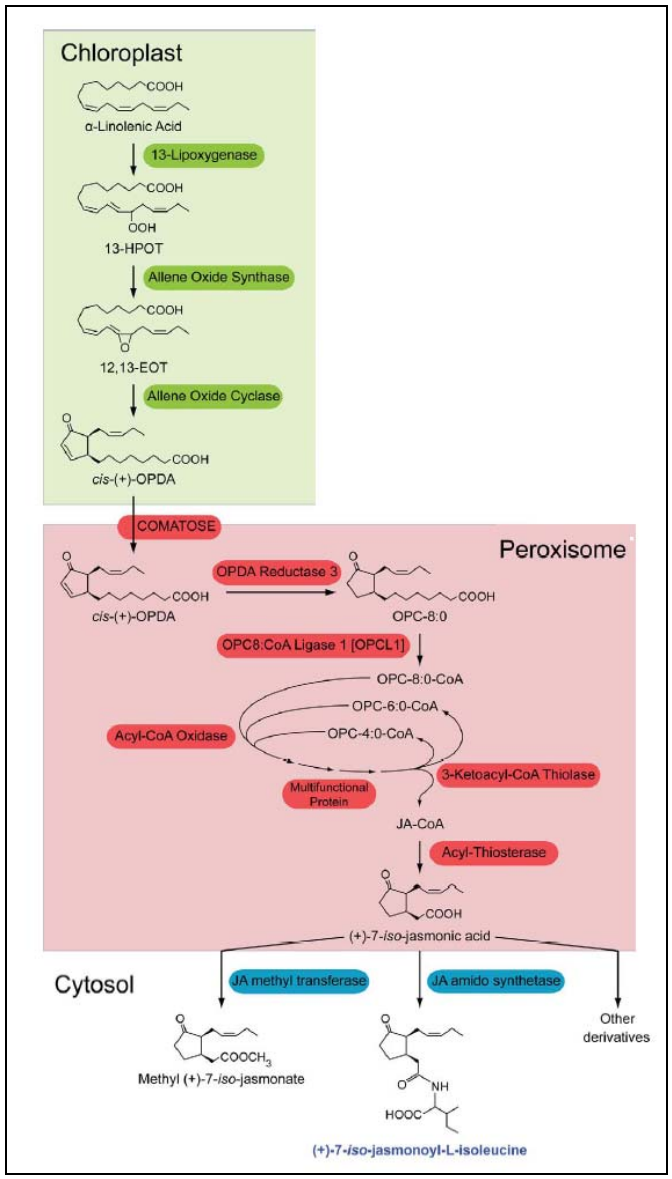

Figure 8. **Jasmonate biosynthesis** starts from α-linolenic (α-LA) acid which is found in great extent in plastidial membranes. From there, α-LA is suggested to be released by the action of phospholipases (LIP) like A_2 and A_1 DAD1 [19]. The subsequent oxygenation of aα-LA is catalyzed by 13-lipoxygenase (LOX) [1]. The resulting 13(S)-hydroperoxy-9(Z),11(E),15(Z)- octadecatrienoic acid (13-HPOT), is further dehydrated with the help of Allene Oxide Synthase (AOS), and this is the first step in the JA biosynthesis [29, 30], providing the unstable intermediate 12,13-epoxy-9(Z),11,15(Z)-octadecatrienoic acid (12,13-EOT). Allene Oxide Cyclase [14, 32] catalyzes the conversion 12,13-EOT to 12- oxo-10,15(Z)-phytodienoic acid (OPDA). OPDA is then transferred from the chloroplast to the peroxisome where it is further metabolized by oxo-phytodienoic acid reductase [28], yielding 3-oxo-2(20(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0). Due to radiotracer experiments [36], it is generally agreed that OPC-8:0 undergoes three consecutive cycles of β-oxidation which results in the production of bioactive JA, i.e. (þ)-7-iso-JA. Once formed, JA may go through different metabolic conversions originating distinct jasmonates. Picture modified from Acosta and Farmer, 2010.

A main route of JA metabolism includes the amide-linked conjugation of the carboxyl group to isoleucine (Ile) and other aminoacids to yield JA-Ile. This last class of reactions has proven to be central to JA hormone signaling and in *Arabidopsis* JA-Ile is synthesized by an enzyme encoded by the JAR1 gene (Suza and Staswick, 2008).

Transcriptional profiling experiments identified a set of JASMONATE ZIM-DOMAIN (JAZ) genes that are rapidly and strongly induced by JA. Characterization of the JAZ proteins revealed that they act as repressors of JA-induced genes. Their discovery led directly to the recognition of JA-Ile as the highly active form of the hormone in promoting the COI1-JAZ1 interaction in a dose-dependent manner, whereas other JAs, including JA, methyl-

JA, and OPDA are inactive and the identification of the receptor as the CORONATINE INSENSITIVE1 (COI1) F-box protein that directs ubiquitination of JAZ proteins by the Skp/Cullin/F-box (SCF) COI1 complex, and the recognition of MYC2 as one of the transcription factors regulated by the JAZ proteins (reviewed by Acosta and Farmer 2010). The mechanism of JA-Ile hormone action has striking parallels to auxin perception and signaling. COI1 is a close phylogenetic relative of the auxin receptors, TIR1, and the AUXIN F-BOX (AFB) proteins.

All genes encoding enzymes of JA biosynthesis are JA-inducible (Wasternack, 2006), and promoters analyzed so far increase their activity upon JA treatment. This has led to the suggestion that JA biosynthesis is regulated by a positive feedback.

The growing importance of JAs and their impact on horticultural crop protection from environmental stress, postharvest decay and handling has been recently reviewed (Rohwer and Erwin 2008). In fruit, JAs assume particular relevance since they induce, when exogenously supplied, secondary metabolite biosynthesis as a stress response, and this often translates in an improvement of fruit quality and nutritional properties. For instance, JAs are able to stimulate β-carotene and anthocyanin biosynthesis, and volatile emission in apple (Kondo et al. 2005; Rudell and Mattheis 2008), flavonoid accumulation and antioxidant activity in blackberry (Wang et al. 2008), and resveratrol biosynthesis in grape (Vezzulli et al. 2007). Nevertheless, reciprocal relationships between JAs and ethylene are not well established. In particular, during fruit ripening, contradictory results have been reported, and the effects of JAs on ethylene seem to strongly depend, besides fruit species and cultivar, upon physiological stage of application (Kondo et al. 2007; Ziosi et al. 2008).

2.7 Brassinosteroids

Brassinosteroids (BRs) are necessary for normal plant growth and development (Clouse and Sasse, 1998). There is evidence indicating that BRs are involved in the regulation of cell elongation, cell division, leaf bending, reproductive and vascular development, membrane polarization, proton pump, responses to light, modulation of stress, and senescence (recently reviewed by Clouse 2011).

The BL, 24-epibrassinolide, is believed to be the most powerful of the BRs. BRs are found in a variety of plant tissues throughout the plant kingdom. Their hormonal activity in plants was only discovered in 1970 by Mitchell and collaborator in plant tissues. However, levels of the bioactive BRs, castasterone (CS) and brassinolide (BL) differ greatly in different parts of the plant, in a pattern that is similar in a wide range of plant species. For instance,

the highest levels of bioactive BRs generally occur in reproductive organs such as pollen, seeds, and fruits (0.2–3.5 ng g^{-1} FW). This is consistent with the important roles proposed for BRs in processes such as fruit development and ripening. In contrast, bioactive BR levels in vegetative (shoot) tissues are generally much lower (0.12– 2 ng g^{-1} FW), whilst the lowest levels of bioactive BRs are found in the roots (Clouse 2011).

However, unlike some other plant hormones, it appears that BR levels are not regulated by the long-distance transport of these compounds around the plant. It appears that BRs are transported over short distances, since they are synthesized inside the cell but perceived on the cell's exterior. It seems reasonable to suggest that the transport of BRs out of the BR-producing cell does not stop at the exterior surface of that cell, but continues on to the surfaces of neighbouring cells (which themselves are exporting BRs), thereby ensuring that a relatively large number of cells within the tissue perceive the same concentration of bioactive BRs. This would result in a coordinated BR response across the cells concerned. Presumably, the presence of the receptor on the exterior of the cell facilitates this co-ordinated response (Symons et al. 2008).

BRI1 is the major BR receptor in higher plants (Figure 9). Mutations of the BRI1 orthologs in tomato, rice, and pea cause BR-insensitive phenotypes. There are three close homologs of BRI1 in *Arabidopsis* and at least two of them, BRL1 and BRL3, bind to BR with high affinity (Clouse et al. 2011).

The availability of a wide range of synthetic methods for the production of both natural BRs and their synthetic analogues with high biological activity, together with the very low concentrations needed for the manifestation of their effect on plants, make BRs logical candidates for the agricultural application. Exogenously applied BRs have long been known to increase growth and yield in many economically useful plant species like in cereals, leguminous crops, rapeseed and cotton.

In Cucumber (*Cucumis sativus* L.) parthenocarpic growth was induced by exogenous BRs but inhibited by the inhibition of BR synthesis. BRs triggered active cell division associated with increased transcripts of cell cycle-related genes, especially that of cyclin D3 genes. These findings indicate that BRs play a regulatory role in early fruit development of cucumber plants (Fu et al. 2008).

Some data suggest that exogenous BRs may promote ripening (via increases in ethylene levels) in tomato, a climacteric fruit (Vardhini and Rao, 2002), and also in non-climateric fruit like grape (Symons et al. 2006). Pattern of gene expression and plant hormone levels throughout grape (cv Cabernet Sauvignon) berry development indicates that BR levels may influence the process of berry ripening. Furthermore the manipulation of BR levels via

the application of exogenous BR and a BR biosynthesis inhibitor can significantly promote or delay berry ripening.

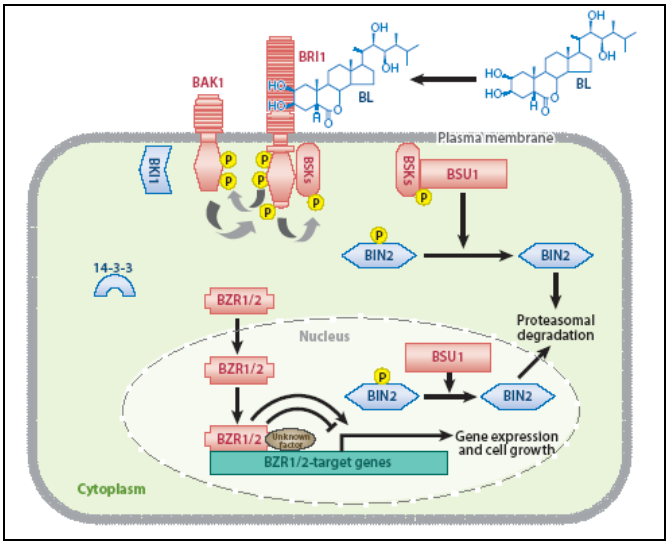

Figure 9. BRs signal transduction cascade includes BR perception by the BRI1 receptor kinase at the cell surface, activation of BRI1/BAK1 kinase complex by transphosphorylation, subsequent phosphorylation of the BSK kinases, activation of the BSU1 phosphatase, dephosphorylation and inactivation of the BIN2 kinase, and accumulation of unphosphorylated BZR transcription factors in the nucleus. Active BZR1 and BZR2/BES1 bind to genomic DNA to regulate BR-target gene expression, thereby modulating growth and development of plants (From Kim and Wang, 2010).

2.8 Polyamines

Polyamines (PAs) constitute a class of ubiquitous plant growth regulators, characterized by their polycationic nature and their binding ability with biological macromolecules aimed at stabilizing them and favouring their function; in fact, PAs are known to play a positive role in plant growth and differentiation and in stress/defence responses (Kusano et al. 2008).

PA levels change whenever cells change their physiological status upon perception of an endogenous (hormonal) or exogenous (environmental) stimulus. Indeed, during fruit development and ripening, endogenous PA concentrations undergo dramatic changes, the maximum levels occurring during the early growth stages (cell division phase) and the minimum levels at ripening (reviewed by Torrigiani et al. 2008); in climacteric fruit such as peach, pear and others, minimum PA levels coincide with climacteric ethylene production. Moreover, SAM concentration, the common precursor of PAs and ethylene pathway (Figure 10) exceeds that needed for PAs and ethylene synthesis so a competition between both pathways for SAM is unlikely.

Pionering studies of Costa and Bagni (1983) demonstrated that PA applications at flowering resulted in substantial increases in fruit set and final yield at harvest. Afterwards,

many other fruit species have been treated with PAs, mainly in postharvest, showing, almost invariably, that these compounds exert a beneficial effect on postharvest decay and on fruit shelf-life (Valero et al. 2002; Liu et al. 2006).

Pu, Sd and Sm at different concentrations were applied by spraying to peach trees (Redhaven) at mid-S3 (pre-harvest) in order to check *in planta*, under field conditions, what the impact of these molecules on ethylene production was, and what effects these growth regulators exerted on peach fruit ripening and quality (Bregoli et al. 2002). Later, the study was extended to nectarines (Stark Red Gold) and included other fruit developmental stages, each corresponding to different endogenous PA concentrations in the mesocarp (Ziosi et al. 2003; Ziosi et al. 2006a). Thus, PA treatments at S1, mid-S3 and late-S3 stages were performed showing that early and late applications all lead to a delay in ripening (Torrigiani et al. 2012 in press).

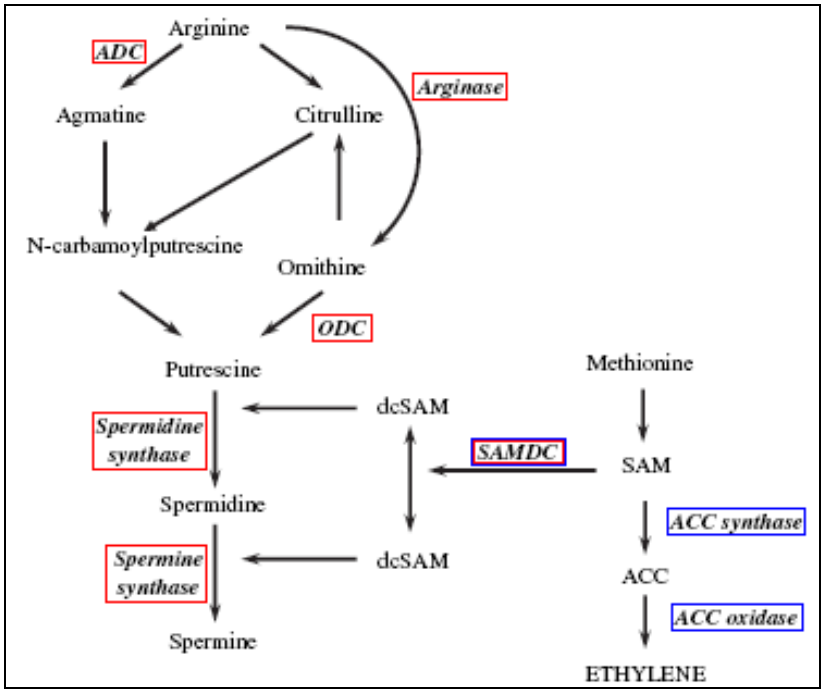

Figure 10. Biosynthesis of Polyamines. The diamine precursor of the higher PAs spermidine (Sd) and spermine (Sm) is putrescine (Pu). Pu is synthesized indirectly from arginine via arginine decarboxylase (ADC) and/or directly from ornithine via ornithine decarboxylase (ODC). S-adenosylmethionine (SAM), which is also precursor for ethylene biosynthesis, is decarboxylated by SAM decarboxylase (SAMDC); decarboxylated SAM furnishes the aminopropyl groups needed for Sd and Sm biosynthesis from Pu and Sd via Sd (SPDS) and Sm synthase (SPMS), respectively. Besides their biosynthetic enzymes, cellular PA levels are also regulated by the action of di- and polyamine oxidases and by conjugation to hydroxycinnamic acids.

In Redhaven peaches, PA application prior to the start of ripening (19 d before harvest, mid-S3) strongly reduced or even nullified ethylene production, and the extent of ethylene inhibition seemed to depend upon the type and concentration of the PA applied: Sd was more efficient than Pu and Sm. PA-treated fruit also showed a lower SSC. In Stark Red

Gold nectarine, field application (mid-S3, 28 d before harvest) of both Pu and Sd strongly reduced ethylene production, though generally less than in the previous case, confirming previous observations with Redhaven peaches (Torrigiani et al. 2004); however, in this case, effects appeared independent of PA concentration or type. For both mid- and late-S3 treatments, data on ethylene production in nectarine were corroborated by gene expression analysis; results showed that the expression of ethylene biosynthetic genes PpACS and/or PpACO1 was down-regulated at harvest in PA-treated mesocarp; moreover, PAs also affected the expression of ethylene receptors genes PpETR1 and PpERS1.

In planta, late PAs application led to a weaker the effect on ripening. This may be the result of the fact that, when the molecular processes which underlie ripening are too far advanced, they cannot be effectively counteracted and no dose-dependent responses were ever observed in PA-treated fruit (Ziosi 2006a).

Pu infiltrated in ripe peaches increased firmness (FF) thereby delaying the ripening process (Martinez-Romero et al. 2000). Equally, in damaged plum fruits, application of Pu increased FF, accompanied by an increase in the acid-insoluble conjugated PAs. In Redhaven peach insoluble conjugated PAs occur, in nectarine, they were not detected during fruit ripening (Ziosi et al. 2006a). Thus, at least in nectarines, the control of firmness seems to be unrelated to the cross-linkages between PA conjugates and cell walls constituents. On the contrary, it is likely that the control of FF exerted by PAs is mediated by ethylene, which plays a predominant role in this phenomenon by acting on PG activity and gene expression (Bonghi and Trainotti, 2006).

The relationship between PAs and fruit quality has been approached by genetic engineering in tomato giving rise to interesting results. Transgenic tomato overexpressing a yeast SAMDC gene and over-accumulating Sd and Sm have been shown to accumulate more lycopene and to have prolonged vine life (Metha et al. 2002). Transcript profiling has shown changes in gene expression consistent with the role of PAs as stress protecting and antisenescence effectors (Srivastava et al. 2007). Interestingly, engineered accumulation of higher PAs in tomato restored metabolic activity even at late stages of fruit ripening, reviving cellular programs underlying N:C signalling, energy and glucose metabolism (Mattoo and Handa, 2008).

Endogenous PA concentration is scarcely affected by exogenous PAs aplication. This is a positive aspect, especially considering that high levels of PAs in the fruit mesocarp could be an undesirable side-effect for consumption under particular regimes (Bardócz, 1995). All these data contributes to the notion that these naturally occurring substances can be a

useful tool for controlling fruit ripening in the field arises. No dose-dependent responses are in general observed, suggesting that the lesser PA concentrations may be suitable (Torrigiani et al. 2008).

3. Biochemical and physiological processes that define a ripen fruit.

Fleshy fruits undergo a ripening process in which a wide spectrum of biochemical changes occurs. The specific biochemical programs resulting in the ripening syndrome vary among species but typically changes involved increased respiration, chlorophyll degradation, biosynthesis of carotenoids, anthocyanins, essential oils, and flavor and aroma components, increased activity of cell wall-degrading enzymes that consequently augment susceptibility to pathogen attack, and a transient increase in ethylene production (Prasanna et al. 2007). All these changes lead to the development of a soft and edible ripe fruit with desirable quality attributes.

While the fruit ripening process is obviously important, there is also a growing body of evidence that supports the key role of early fruit development for the acquisition of several fruit quality traits, including the accumulation of sugars and organic acids (Carrari and Fernie, 2006), the determination of cell wall and texture characteristics, and the cuticle biosynthesis. In the growing fruit, these processes mainly take place during the cell expansion phase, which sustains fruit growth by allowing a large increase in fruit cell volume linked with membrane and cell wall/synthesis and the concomitant accumulation of water, mineral ions, and metabolites in the vacuoles, thereby conferring its fleshy characteristics to the fruit. Increasing evidence links the enlargement of fruit cells with an increase in nuclear ploidy level, but the way in which fruit growth and associated metabolic changes are regulated and coordinated largely remains an open question (Mounet et al. 2009).

3.1 Ripening–related aspects focused on peach fruit

Neri et al. (1996), propose the minimal parameters related to quality traits than peach fruit should possess at harvest. These parameters include FF no more than 45N, positive “a” values and SSC not less than 12%.

Change in ground colour is a useful maturity index because is strongly correlated with changes in other important ripening parameters such as SSC, flesh firmness and content of volatile compounds (Ramina et al. 2008).

3.2 Respiration

Peach, defined as a climacteric fruit, increase its own respiration (CO_2/kg per h) during ripening according to growth stage (Tonutti et al. 1991), that is high during the Stage I of

fruit development when it is growing by cell division, decrease during stage II and III, rising again in the end of stage III to finally reach the climateric peak tipical of the stage IV. The climacteric peaks are depending on genotype and melting vs non melting flesh (Brovelli et al. 1999).

3.3 Softening

Ripening-associated fruit softening involves modifications to the polysaccharide and protein components of the primary cell wall and middle lamella, resulting in a loosening of the structure. The process of fruit textural changes is very complex and many protein families along with water relations and free radicals contribute to it. Developmental regulation of cell wall stabilizing and depolymerizing proteins as well as protein glycosylation has been demonstrated during fruit ripening. These include polygalacturonase (PG), pectin methylesterases (PMEs), β-galactosidase, β-mannanase, xyloglucan xyloglucosyltransferase/endohydrolase (XET), pectate lyase (PL), endo-β-1,4-glucanases (EGases, cellulases), expansins, α-Man, and β-Hex. Because of the economic significance of fruit textural changes, there is a significant interest in understanding the biochemical mechanisms regulating this process (recently reviewed by Handa et al. 2012). In peaches, both PG and EG, tend to be absent in mature green fruit but their activities become measurable only with the onset of ripening and increase strongly in climacteric stage. Moreover, EG is mainly involve in the initial phase of peach fruit softening, before the action of PME and PG. Expansins are protein to contribute to tomato fruit softening and also has been related to fruit firmness losses in peach (Hayama et al. 2003). Three expansins Pp EXP1, Pp EXP2 and Pp EXP2 were detected exclusively in fruit. Pp EXP2 is expressed during all fruit development but is more abundant in the stage III, during exponential growth and maturation. Pp EXP1 and Pp EXP3 are upregulated at the onset of ripening but Pp EXP1 is induced in an earlier stage. However Pp EXP3 shows the closer association to softening. In tomato, simultaneous downregulation of SlEXP1 and SlPG resulted in fruit that retained firmer texture and maintained cellular integrity for a longer period compared to parental wild-type fruit (Powell et al. 2003).

3.4 Sugar metabolism and organic acid content

Acidity is an esencial component in the fruit taste that is determined during the early stages of fruit development. In peaches and the most of the fruits the two major organic acid compounds are malate and citrate and tend to decrease during ripening. The Phosphoenolpurivate carboxilase (PEPC) plays an important role in controlling the fruit organic acid metabolism.

Sweetness is a trait that concern acceptation from consumers. Sucrose, glucose and fructose are the main sugars in peaches (Genard et al. 2003) and are in a ratio 3:1:1 in high eating quality peaches. The sugars represents more than 60% of the soluble solids concentration (SSC) as measured with a refractometer and there is a general agreement that SSC of at least 12% is required to ensure peaches consumption, however acidity can modify flavor perception, so, acid/sugar ratio also defines fruit flavor.

Carbon metabolism is strictly correlated with the pathways of secondary metabolite synthesis, such as pigments, vitamins and volatiles. Acids, alcohols, aldehydes, esters and lactones, responsible for fruit aroma, and terpens, that are also precursors of lycopene and carotenoids, are derived from fatty acid degradation.

In higher plants, sucrose is the main form of transport carbohydrate. Sucrose use in sink tissues depends on sugar transport from apoplast, through cytosol, to vacuoles (Nguyen-Quoc and Foyer, 2001). In this process, sucrose is degraded in glucose and fructose by sucrose synthase (SuSy) or by invertases (Koch, 2004), and resynthesized by SuSy or sucrose phosphate synthase (SPS).

In peach fruits, glucose and fructose are predominant in the early stages of development; then their concentration decreases, while sucrose concentration exponentially increases until it becomes the principal carbohydrate in mature fruits and sorbitol remains low during the whole growth period (Vizzotto et al. 1996). Nonis et al. (2007) studied the expression of a gene encoding a neutral invertase (NI) isolated from peach (PpNI1) in relation to sucrose metabolism and mesocarp development in two genotypes (cv. Springcrest and cv. Redhaven) differing for fruit growth, and sugar accumulation dynamics. The gene was differentially regulated during development and showed a correlation with sucrose, and glucose and fructose mesocarp contents.

3.5 Synthesis of pigments and volatiles compounds

In most fruits, as ripening advances, a progressive exocarp and mesocarp de-greening occurs due to the conversion of chloroplast in cromoplast. Thylakoids, starch deposits and chlorophyll degrade progressively and are substituted by carotenoids and anthocyanins, which are synthesized from isopentenil diphosphate) and through the phenylpropanoid pathway, respectively (Winkel-Shirley, 2001).

This aspect of fruit maturation is important not only from a commercial point of view, because fruit color is widely appreciated by consumers, but also from a health and nutritional point of view as carotenoids and flavonoids are molecules with elevated antioxidant power (Sajilata et al. 2008). Quantitative and qualitative profiles of phenolic

compounds vary greatly between species and also are affected by several factors, such as light quality and quantity, temperature, post-harvest storage conditions, genotype and fruit ripening stage (Tomás-Barberán and Espín, 2001). In tomato peel the main flavonoid accumulated is naringenin chalcon and to a lesser extent quercetin-rutinoside (Verhoeyen et al. 2002), pome fruits are characterized by a high production of catechin, procyanidine and cinnamic acids (Nicolas et al. 1994). Peach and nectarines are rich in cinnamic acid, catechin, epicatechin and procyanidine (Andreotti et al. 2008).

Phenolic compounds are plant secondary metabolites, characterized by at least an aromatic ring with one or more hydroxylated substistuents. They include a large variety of chemical structures, such as benzoic acids, hydroxycinnamic acids, flavonoids, isoflavonoids, stilbenes, lignans, coumarins, tannins, phenolamides and dihydrochalcones. These secondary metabolites are widely distributed in plant kingdom, where they play crucial roles in the interactions between plants and their environment, conferring plant mechanical support, serving as attractants for pollinators and other beneficial organisms and participating in plant hormone signalling and in several mechanisms of defence against biotic and abiotic stresses (Harborne and Williams, 2000).

The most evident function of phenolic compounds in plant is their contribute to flowers, fruit, stems, leaves and roots colouration, due to the accumulation of anthocyanin pigments belonging to the class of flavonoids. The most common anthocyanins in higher plants are: delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin (Tanaka et al. 2008). These phenolic compounds can confer colourations ranging from red to purple and blue, which serve as attractants for pollinators in flowers and for herbivores for seeds dispersion in fruit. In addition, other flavonoids and phenolics, such as flavones, flavonols and cinnamic acids, have been reported as co-pigments able that stabilizes and enhance the colour of anthocyanin by molecular interactions.

Anthocyanins are synthesized by a branch of the flavonoid pathway (Figure 11), which is composed of a sequence of enzymatic steps, starting with phenylalanine ammonia lyase (PAL), followed by chalcone synthase (CHS) for the synthesis of naringenin chalcone, chalcone isomerase (CHI) for the conversion of naringenin chalcone to naringenin, flavanone 3'-hydroxylase (F3'H) and flavonoid 3-hydroxylase (F3'H) for the subsequent hydroxylations of naringenin, NADPH-dependent dihydroflavonol reductase (DFR) for the production of leucoanthocyanidins, leucoanthocyanidin dioxygenase (LDOX) for the conversion of leucoanthocyanidins to anthocyanidins, and UDP-Glc: flavonoid 3-Oglucosyltransferase (UFGT) for the generation of glycosylated anthocyanidins (Winkel-Shirley, 2001; Tanaka et al. 2008).

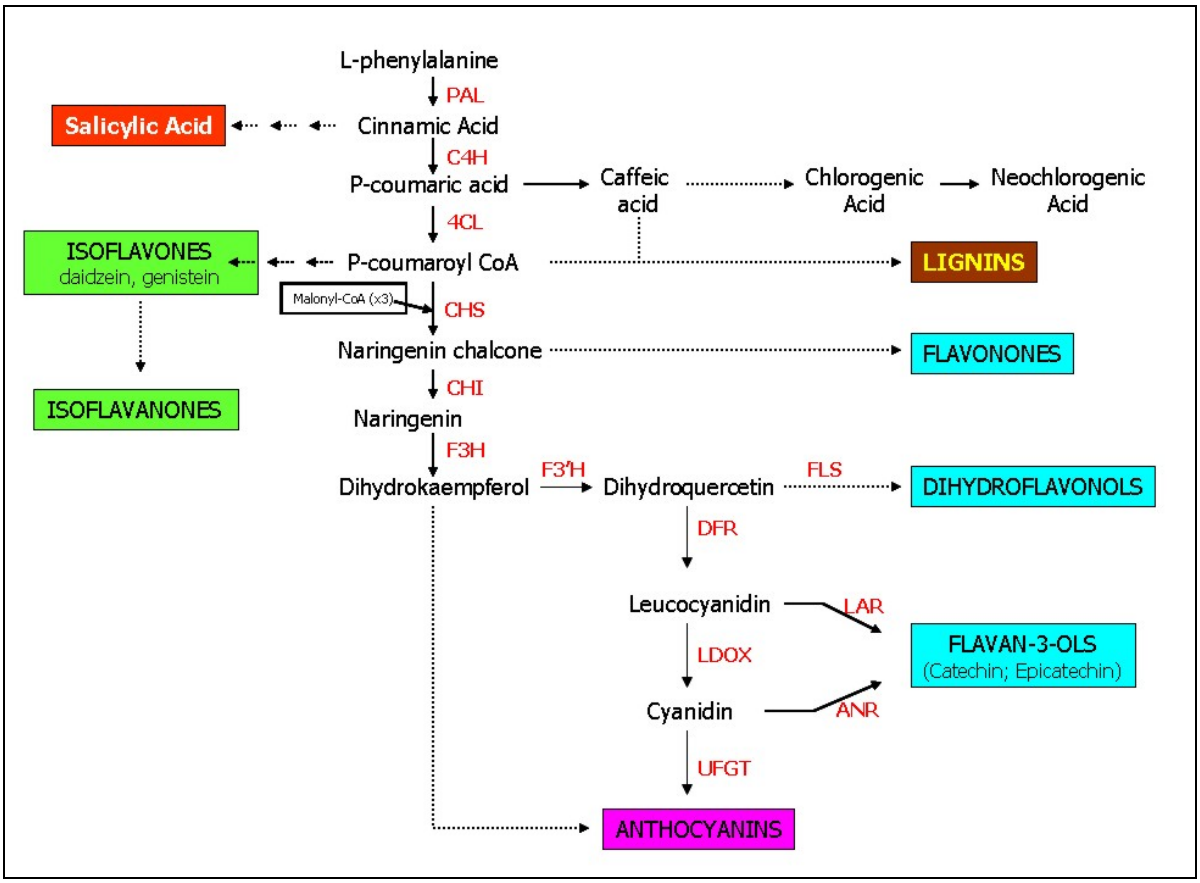

Figure11. Phenylpropanoid biosynthetic pathway involving anthocyanins and flavonoids. In red: biosynthetic enzymes. PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; ANR, anthocyanidin reductase; UFGT, UDP-glucose:flavonoid 3-O-glucosyltransferase.

The regulation of anthocyanin biosynthesis involves numerous regulatory factors that control the expression of different anthocyanin biosynthetic genes in *Arabidopsis*. It has been shown previously that the *Arabidopsis* WD-repeat/ Mybs/ bHLH complex including transcription factors PAP1 (Myb75), PAP2 (Myb90), EGL3, GL3, and TTG1 predominantly regulate the expression of the "late" anthocyanin biosynthetic genes including DFR, LDOX, and UFGT over the expression of the "early" anthocyanin biosynthetic genes such as PAL, CHS, CHI, and F3H (Gonzalez et al. 2008). Some WD-repeat independent Mybs have been demonstrated to be regulators for the early gene expression, such as Myb11, Myb12, and Myb111 (Stracke et al. 2007).

Physiological and molecular evidence reveals that (i) JA-induced anthocyanin accumulation is primarily via up-regulation of the late anthocyanin biosynthetic genes DFR, LDOX, and UFGT, and COI1 is essential for the JA-induced expression of these anthocyanin biosynthetic "late" genes; (ii) the expression of anthocyanin biosynthetic

regulators, PAP1, PAP2, and GL3 is significantly induced by JA, and COI1 is required for JA-induced PAP1, PAP2, and GL3 transcription. It is speculated that COI1 regulates the expression of the transcription factors, including PAP1, PAP2, and GL3, which mediates the “late” anthocyanin biosynthetic genes DFR, LDOX, and UFGT, and thereby modulates JA induced anthocyanin biosynthesis in *Arabidopsis* (Shan et al. 2009).

The primary pigment responsible for red colouration in peaches and nectarines is cyanidin and, in particular, cyanidin 3-glucoside, whereas catechin, the main flavan-3-ol detected in both skin and flesh, is a major influence on fruit flavour. Therefore, these classes of phenolic compounds provide essential cultivar differentiation for consumers and represent important factors for marketability. Moreover, anthocyanin and flavan-3-ols, together with cinnamic acids and flavonols, contribute to the antioxidant potential and health-promoting properties of these fruit (Andreotti et al. 2008).

The production of volatile compounds arises from several differents substrates including fatty acids, aminoacids, phenolics and terpenoids (Dudareva et al. 2004). Aldehydes, esters, ketones, terpenoids and sulfur-containing compounds account for most of the important flavour volatiles in fruits. Fruits also produce acetaldehyde and ethanol by alcoholic fermentation during ripening (Pesis, 2005). The quantity and quality of volatiles produced during ripening is characteristic of the fruit species. In peach and nectarine, a hundred of volatile compounds that contribute to fruit aroma have been identified. Esters, aldehydes and C_6 alcohols, like hexanal, trans-2 hexanal, hexanol, trans-2-hexanol, are present in unripe fruits, while, during ripening, their concentration decreases concomitant to an increase in γ- and δ-decalactones, linalool and benzaldehyde that confer the typical peach aroma (Ramina et al. 2008).

OBJECTIVES

The growing competitiveness of Europeans peach producers, mainly with Spain and other manufacturers and market speculation has led to a crisis situation in the Italian peach breeding that make difficult even recover costs of production (Coldiretti, 2011). To overcome this situation, peach-industry experts suggest that farmer efforts should lead to production reorganization and distribution chain, with an increase operator's investment and the development of new markets. Other suggestions are to obtain a fruit based on the acquisition of technological innovations that improve the quality and shelf-life from many points of view (Della Casa, 2005). Scientific research and information campaigns by the Health Ministries and FAO have raised awareness by consumers of the risks associated with an unbalanced diet and the importance of the contribution of fiber, minerals, vitamins and substances antioxidants contained in fruits.

In this context, the concept of fruit quality has been greatly expanded. Parameters like size became so relevant like ease of manipulation of the fruit, absence of defects, sanitation, nutritional and organoleptic characteristics (Shewfelt, 1999). Most of these parameters depend strictly from the time of harvest of the fruit from the mother plant; in fact, when the biochemical processes that encompass fruit development and ripening are not completed or occur in an unusual way led to a fruit not fully evolve in their organoleptic characteristics. Peaches and nectarines are climacteric fruit characterized by fast development and quality decay (Lill et al. 1989). For this reason, production process tends to anticipate the fruit harvesting to marketing, through the use of low temperatures and/or modified atmosphere to avoid fruit metabolism-associated damages (Pesis, 2005).

A deeper knowledge about biochemical and molecular mechanisms that regulate the peach fruit ripening is basic to prepare strategies to control this process in pre-and post-harvest to arrive to consumers with a product with the best organoleptic and nutritional characteristics and health benefits (Abbott et al. 2009).

The role of ethylene in the regulation of climacteric fruit ripening gained much attention and has been extensively studied. In recent years, the attention of researchers has focused on the role played by growth regulators and genetic mechanisms controlling the fruit ripening (Giovannoni, 2007), deepening in their interconnections to coordinate this process. In contrast, less attention is paid to early fruit development that have been revealing a key role for the acquisition of several fruit quality traits, including the accumulation of sugars and organic acids (Carrari et al. 2006), the determination of cell

wall and texture characteristics (Chaïb et al. 2007), and the cuticle biosynthesis (Mintz-Oron et al. 2008).

Jasmonates (JAs), in particular jasmonic acid (JA) and methyl-jasmonate (MJ) are cyclopentanone compounds, synthesized from linolenic acid, which play a crucial role in the response to biotic and abiotic processes and are involved in various developmental processes including differentiation of floral organs and growth and maturation of the fruit (Wasternack 2007).

On the other hand, the aliphatic polyamines putrescine (Pu), spermidine (Sd) and spermine (Sm) are considered as positive growth regulators and stress-responsive molecules; they may act as cellular signals in the cross talk with hormonal pathways including ethylene, auxin and ABA. In pear plants, overexpression of Sd synthase confers multiple abiotic stress tolerance by altering PA contents and increasing antioxidant properties (Wen et al. 2008) and also, PAs have been repeatedly shown to interfere with fruit set, development and ripening in several species including peach by modulating these processes (Torrigiani et al. 2008).

The phytohormones brassinosteroids (BRs) are widely distributed in the plant kingdom and regarded as “hormones of the 21st Century” (Clouse and Sasse, 1998). They are involved in cell expansion, cell division, membrane properties, vegetative growth, reproductive biology, senescence, seed germination and stress responses (recently reviewed in Clouse 2011). They have shown to improve fruit growth and promote ripening in some plant species.

The aim of this research thesis is to investigate the effects of the above cited natural growth substances that display quite different physiologic features, as deduced by early (S1) applications under field conditions to peach fruit. Fruit quality traits were determined at harvest and transcriptional profiles of several hormones- cell wall- related and developmental marker genes in the mesocarp and/or seed were evaluated as follows.

I. To better understand the physiological role of JAs during fruit/seed growth and ripening, MJ and the synthetic analogue PDJ were applied to young (late S1) Stark Red Gold nectarines. Fruit samples were harvested at time intervals until ripening and seed and mesocarp separated. In control and treated fruit, flesh firmness (FF) and soluble solids concentration (SSC) were assessed and ripeness was established by the I_{AD}. The pattern of transcript levels of several hormone-, cell wall- and defence- related genes was determined in the seed and mesocarp, separately; the expression of developmental marker genes was evaluated in the seed. Moreover, JA

effects on biosynthetic gene expression and accumulation of phenolic compounds were analysed.

II. To gain a deeper insight into the molecular bases of the ripening control exerted by PAs when applied to Stark Red Gold nectarines under field conditions at an early (S1) developmental stage, the effects of spermidine (Spd) on ethylene production and fruit quality, and ethylene-, cell wall-, auxin- related and developmental marker gene expression were studied during development and ripening in the mesocarp.

III. Early and late exogenous field applications to Flaminia peaches of 24-epibrassinolide were performed and their effects on fruit growth, quality traits and extent of ripening during fruit development and ripening were evaluated.

RESULTS

4. Jasmonate application to young peach fruits interferes with expression profiles of ethylene-, cell wall-, defense- and other hormone-related genes in the seed and mesocarp during fruit development and ripening

4.1 Introduction

Jasmonic acid, its volatile ester methyl jasmonate (MJ), and other derivatives, collectively known as jasmonates (JAs), are ubiquitous signalling molecules which mediate plant responses to environmental stress and also play a role during developmental processes, including root growth, seed germination, pollen development fruit development and ripening (Peña-Cortés et al. 2005; Wasternack 2007).

JAs are synthesized via a series of steps starting from α-linolenic acid in the octadecanoid pathway. Allene oxide synthase (AOS) is the first specific enzyme and the major control point of their biosynthetic pathway. AOS is up-regulated in response to wounding and treatments with JAs in leaves of *Arabidopsis*, tomato and tobacco (Kubigsteltig et al. 1999; Howe et al. 2000; Ziegler et al. 2000) indicating that a positive feedback regulation in JA biosynthesis occurs, leading to an amplification of the JA signal (Laudert and Weiler 1998). Indeed, genome-wide transcript profiling has provided insight into the pleiotropic control exerted by JAs on plant development and survival (Memelink 2009; Pauwels et al. 2009). Consequently, JA treatment leads to dramatic transcriptional alteration in most plant tissues analyzed. In particular, up-regulation of JA-responsive genes leads to the synthesis of JA-induced proteins which include enzymes of JA biosynthesis and secondary metabolism, anti-nutritional proteins, pathogenesis-related (PR) and stress-protective proteins, and proteins involved in cell wall metabolism (Wasternak 2007).

JAs interact with other plant hormones through the intersection with their signaling pathways, and this complex cross-communication might help fine-tune JA biosynthesis and signaling. JA and ethylene signal transduction pathways act synergistically or antagonistically in a variety of responses. For example, the ERF1 transcription factor functions as a positive regulator of both pathways (Lorenzo et al. 2003). On the contrary, the two hormones act as antagonists in modulating ozone-induced cell death, and in the control of peach fruit ripening (Overmyer et al. 2003; Ziosi et al. 2008).

It has been observed in several fruit species that endogenous JA levels are elevated during the early stages of fruit development, when cell division occurs, and then gradually

decrease (Fan et al. 1997, 1998; Kondo et al. 2000, 2004; Kondo and Fukuda 2001; Torrigiani et al. 2012). In climacteric species, such as apple and peach, JA concentration rises in the fruit mesocarp during ripening (Fan et al. 1997; Kondo et al. 2000; Ziosi et al. 2008). When exogenously applied to fruit, JAs stimulate the synthesis of secondary metabolites, improve fruit quality and antioxidant activity, and enhance disease resistance during storage (Rower and Erwin 2008). These findings lend support to the relevance of horticultural applications of JAs. In peach fruit, it has been demonstrated that when exogenously applied under field conditions, at late developmental stages (S3/S4) to peaches and nectarines, MJ and the synthetic analog propyl dihydrojasmonate (PDJ) delay the accumulation of several ripening-related gene transcripts, thereby causing a delay in fruit ripening (Ziosi et al. 2008a; 2009; Soto et al. 2010); a transcriptome microarray analysis revealed a differential expression of cell wall and stress/defense-related genes in a possible trade-off between growth and defense (Ziosi et al. 2008a). Conversely, relatively little is known about the role of JAs during seed development. JAs inhibit germination of dormant seeds and their levels in late stages of seed development are higher than shortly after anthesis (Creelman and Mullet, 1995). A transgenic approach has shown that MJ may inhibit/slow down regular seed growth (Cipollini, 2010).

In peach, fruit growth occurs in four stages (S1–S4) with a sigmoid pattern (Tonutti et al. 1991). There is a tight relationship between mesocarp and seed/embryo development until the end of pit hardening (S2–S3); later on, this relationship becomes less strict. In the early stages of fruit development, the endosperm grows quickly while embryo development is very slow, while from S2-S3 onwards, the embryo begins to develop rapidly to reach maximum size and fill the entire ovule, while the endosperm is re-absorbed (Ognjanov et al. 1995). The maximum size and entire ovule filling by the embryo is reached about 100 to 105 days after full bloom in Suncrest nectarine (Ognjanov et al. 1995) and 125 dAFB in Fantasia nectarine (Bonghi et al. 2011). Seed development, dormancy and germination are characterized by the differential involvement of phytohormones as also revealed by an extensive hormonal profiling in *Arabidopsis* (Finkelstein 2004; Kanno et al. 2010). In particular, during peach seed development, ethylene production is higher than in the mesocarp, and shows a different pattern in different cultivars (Bonghi et al. 1997). A microarray approach has led to identification of genes, in particular developmental marker genes, involved in seed-pericarp cross-talk and development in peach fruit (Bonghi et al. 2011).

In order to better investigate the molecular basis of the developmental and ripening control exerted by JAs in fruit mesocarp and seed, the aim of the present work was to clarify

whether, when applied to young peach fruit at an early (S1), rather than a late (S3/S4; Ziosi et al. 2008), developmental stage, and under field conditions, MJ and its synthetic analog PDJ were still able to influence fruit development and ripening. To this aim, maturity and quality traits were assessed during ripening, and transcript levels of the following genes were monitored in controls and JA-treated seed and mesocarp until harvest: i) ethylene biosynthetic (ACS1 and ACO1) and signaling genes (ETR1, ETR2; ERF2); ii) cell wall- (PG, EXPs) and sugar-related (SOT) genes. Since JAs elicit defense/stress responses and cross talk with the other hormones, the effect of MJ/PDJ treatment on transcript levels of a number of hormone- (AOS, NCED, IAA-AH) and defense-related (CAT, SSADH) genes was also analyzed. Moreover, transcriptional effects of JAs on some seed developmental markers, as previously established by a microarray analysis (Bonghi et al. 2011), were evaluated.

4.2 Materials and Methods

4.2.1 Plant material and experimental design

The trial was carried out on 8-years old peach (Prunus persica L. Batsch cv. 'Stark Red Gold', nectarine) trees (4 per treatment) grown at the experimental farm of the University of Bologna, Italy. Four branches per plant, homogeneous for size and fruit load (3-4 fruits per branch) were selected for the experiments. For each treatment, 16 branches were sprayed with 0.80 mM MJ or 0.44 mM PDJ (Nippon Zeon Co. Tokyo, Japan). Both compounds were dissolved in an aqueous solution containing surfactant and ethanol (150 mL per branch) as described by Ziosi et al. (2008a). Control branches only received an aqueous solution containing the same concentration of surfactant and ethanol. The double sigmoid growth pattern of peach fruit was established and the four growth stages S1-S4 determined (Figure 1) as described by Torrigiani et al. (2004). MJ and PDJ were applied 59 days after full bloom (dAFB; late S1) and samples of 10 control and 10 treated fruit were collected 0 (S1), 1 (S1/S2), 7 (S2I), 16 (S2II), 52 (S3), 70 (S4) days after treatments and subdivided in mesocarp and seed; 70 days after treatment coincided with harvest (130 dAFB, flesh firmness 40 N). At ripening, the Index of Absorbance Difference (IAD, Ziosi et al. 2008b) and quality traits were determined on the whole fruit, while for real-time RT-PCR analyses fruit mesocarp and seed samples were stored at -80°C until use.

4.2.2 Ethylene and fruit quality traits determination

The index of difference of absorbance (I_{AD}; Ziosi et al. 2008b) was measured in one hundred fruit. Flesh firmness (FF) was measured using a pressure tester (EFFE.GI,

Ravenna, Italy), and soluble solids concentration (SSC) was measured with an Atago digital refractometer (Optolab, Modena, Italy), as previously described by Bregoli et al. (2002).

4.2.3 Quantitative Reverse Transcription- Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from tissue samples according to Chang et al. (1993). RNA yield and purity were checked by means of UV absorption spectra, whereas RNA integrity was determined by electrophoresis in agarose gel. DNA was removed using the TURBO DNA-free™ (Applied Biosystems, Foster City, CA, USA) from 10 µg aliquot of total RNA. The first-strand cDNA was synthesized from 6 µg of the DNase I-treated RNA by means of the High-Capacity cDNA Kit (Applied Biosystems), using random primers.

Real-time RT-PCR was performed in a reaction mixture final volume of 25 µl containing 9 ng of cDNA, 5 pmol of each primer, and 12.5 µl of the Fast SYBR Green PCR master mix (Applied Biosystems), according to the manufacturer's instructions. The oligonucleotides DZ79 (5'-TGACCTGGGGTCGCGTTGAA- 3', sense) and DZ81 (5'-TGAATTGCAGAATCCCGTGA- 3' antisense), annealing to the internal transcribed spacer (ITS) of the rRNA, were used to amplify the reference gene (Trainotti et al. 2007) with peach samples. All the primer sequences utilized are listed in Trainotti et al. (2007) and Appendix 1. PCRs were carried out with the StepOnePlus™ 7500 Fast (Applied Biosystems) for 2 min at 95°C and then for 40 cycles as follows: 95°C for 15 s, 60°C for 15 s, and 65°C for 34 s. The obtained CT values were analyzed by means of the Q-gene software by averaging three independently calculated normalized expression values for each sample. Expression values are given as the mean of the normalized expression values of the triplicates, calculated according to equation 2 of the Q-gene software (Muller et al. 2002).

4.2.4 Statistical analysis

For real time RT-PCR (n=3) differences between control and treated samples were compared using GLM (general linear model) analysis with Tukey as posthoc test (StatSoft Statistics v.8 software).

4.3 Results

4.3.1 Early JA treatment impairs ripening

Peach fruit growth was followed during S1-S4 development until commercial harvest (39 to 130 days after full bloom (Figure 1).

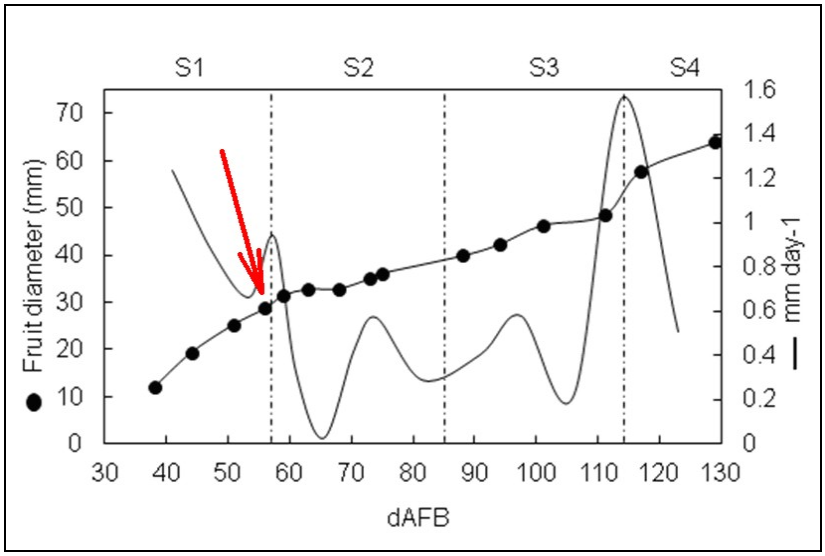

Figure 1. Growth curve of 'Stark Red Gold' nectarine based on diameter (filled circles) and its first derivative (continuous line) from 39 to 130 days after full bloom (dAFB). S1– S4 represent the four stages of growth up to harvest. Time of PDJ and MJ treatments is indicated by the red arrow. Data represent the means (n=10).

Early treatments (S1/S2) with MJ and PDJ on peach fruit branches led to changes in fruit ripeness. At harvest (70 d after treatment), fruit ripeness assessed by I_{AD} measurements, which directly correlates with ethylene production (Ziosi et al. 2008b), indicated that among the less ripe fruit (I_{AD}>0.9) there was an higher percentage of JA-treated fruit, while among the riper fruit (I_{AD} 0.6-0.3) there was a higher percentage of control fruit (Figure 2). Flesh firmness was retained and SSC concentration was lower in JA-treated fruit at harvest (data not shown) further confirming that there was a ripening delay.

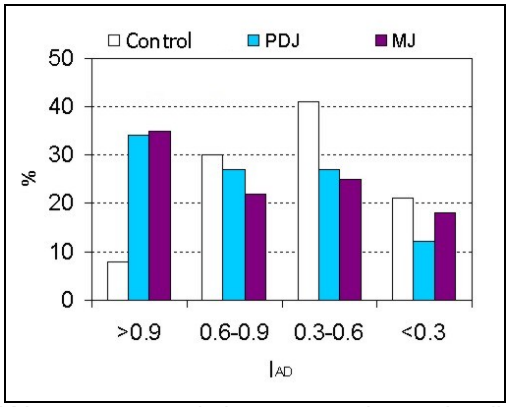

Figure 2. Effect of PDJ and MJ treatments, relative to controls, on the distribution of ripen fruit measured with the DA-meter (I_{AD}) at 130 days after bloom.

4.3.2 JAs interfere with the expression of ethylene biosynthetic and perception genes

Mesocarp - As far as ethylene biosynthesis and perception are concerned, ACS1 transcript levels were hardly detectable during fruit development; only at ripening PpACS1 gene expression increased abruptly in control fruit; both MJ and PDJ dramatically inhibited this rise by about 90% (Figure 3G). ACO1 transcript amount did not change significantly during S1-S2 stages, slightly increased in S3 and dramatically increased at harvest; also in this case MJ and PDJ inhibited the ACO1 transcript rise in S3 and in S4 (about 60%) (Figure 3H). The expression of the ethylene receptor ETR1 did not change during the considered period; only MJ enhanced its transcript levels in S2II and both MJ and PDJ inhibited it in S3 (Figure 3I). ETR2 gene expression tended to decrease during development showing a peak of transcript accumulation at the S1/S2 transition (Figure 3J). Both MJ and PDJ inhibited ETR2 gene expression one day after treatment (S1/S2) while at harvest they enhanced transcript levels, especially MJ (about 30-fold). In control fruit ERF2 mRNA amount peaked at the S1/S2 transition and then decreased until harvest. MJ and PDJ dramatically inhibited ERF2 transcript accumulation at S1/S2 (Figure 3K).

Seed - ACS1 mRNA levels decreased throughout the considered period and JAs did not affect it (Figure 3A). Initially, PpACO1 transcript amount did not change (S1-S2I) then increased up to day 52 (S3 phase) to almost disappear at harvest (Figure 3B). PDJ upregulated ACO1 in S2I and both MJ and PDJ reduced by 60-70% ACO1 gene expression in S3. Both ACS1 and ACO1 were less expressed in the seed than in the mesocarp. ETR1 gene expression did not change during seed development; PDJ upregulated ETR1 in S2I and both JAs downregulated ETR1 in S3 (Figure 3C). ETR2 gene expression peaked at S1/S2 transition and then decreased throughout seed development (Figure 3D); JAs did not substantially alter this pattern except in S3 when they inhibited ETR2 gene expression (Figure 3D). ERF2 mRNA amount exhibited a first peak in S1/S2 and a second at S2II (Figure 3E). Both JAs inhibited peak ERF2 accumulation throughout.

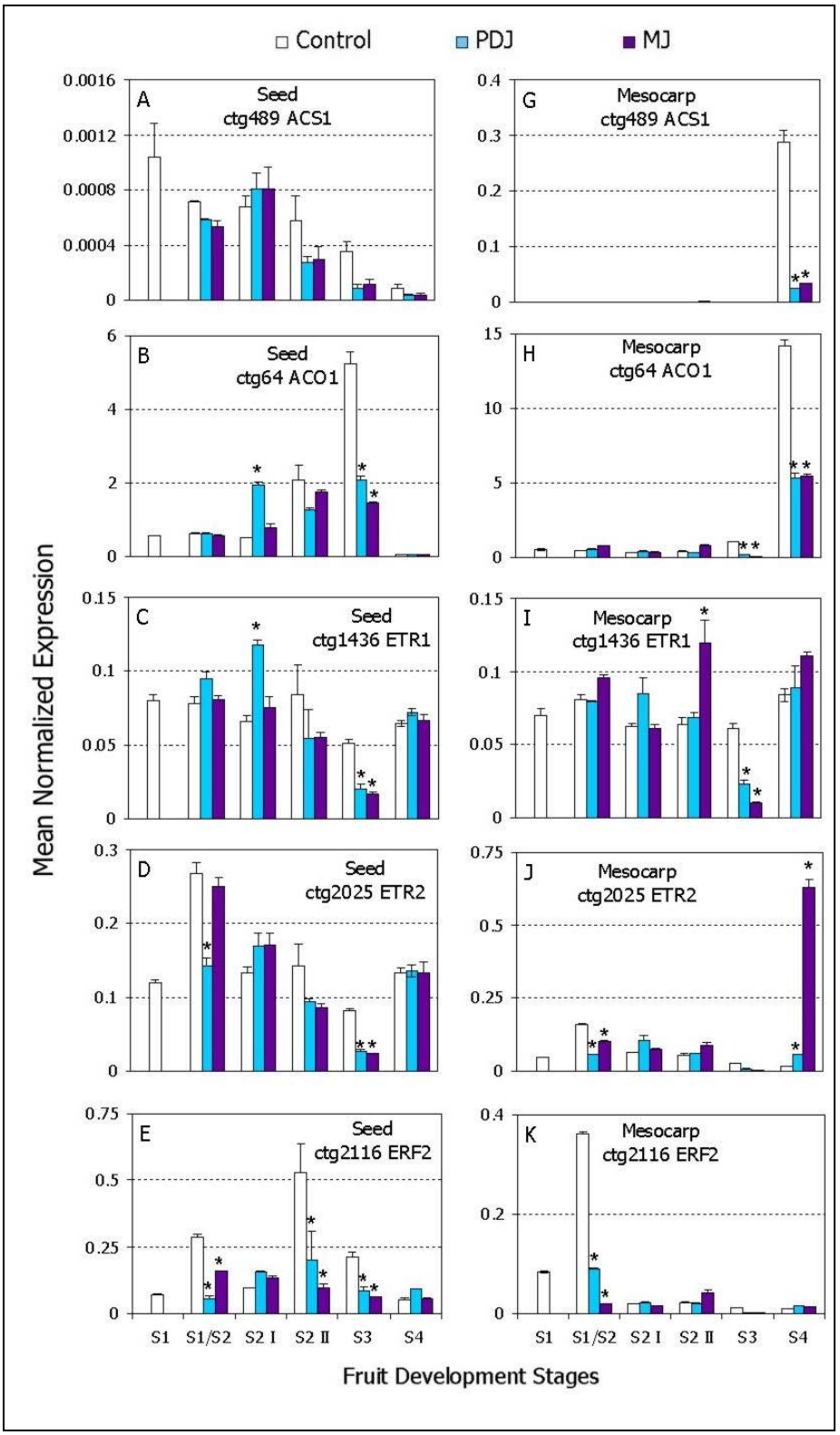

Figure 3. Expression profiling by real-time RT-PCR of ethylene biosynthetic and signalling genes during the S1-S4 stages of peach fruit development and ripening. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). ACS1 (A), ACO1 (B), ETR1 (C), ETR2 (D) and ERF2 (E) in the seed and ACS1 (G), ACO1 (H), ETR1 (I), ETR2 (J) and ERF2 (K) in the mesocarp. Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * $P<0.05$.

4.3.3 JAs affect the expression of cell wall-related genes

Mesocarp - PG expression was undetectable during fruit development but dramatically rose at harvest (Figure 4E); MJ and PDJ almost totally inhibited PG transcript accumulation. EXP1, EXP2 and EXP3 transcript amount gradually increased during development and ripening (Figure 4F, G and H, respectively); PDJ enhanced EXP1 gene expression in S2I and inhibited it in S4 while MJ only enhanced it in S4. With regard to EXP2, PDJ enhanced transcript accumulation in S2I and in S4 while decreased it in S3. MJ showed a similar behaviour but its stimulating effect was detectable already one day after treatment. Only MJ increased EXP3 gene expression starting from S2II and especially in S4 (about 6-fold).

Seed - PG was expressed at low levels during seed development and its transcript peaked at S3 (Figure 4A); MJ down-regulated PG starting from S2II (by about 80%) while PDJ only in S3 (by about 80%). EXP1 and EXP2 transcript amount was the highest at S1 and then decreased; both JAs upregulated EXP1 in S1/S2 (Figure 4B). JAs did not significantly affect EXP2 pattern (Figure 4C). EXP3 peaked in S2II; in this case only MJ enhanced transcript accumulation in S2I (Figure 4D). Transcript accumulation of these wall-related genes in the seed is much lower than in the mesocarp.

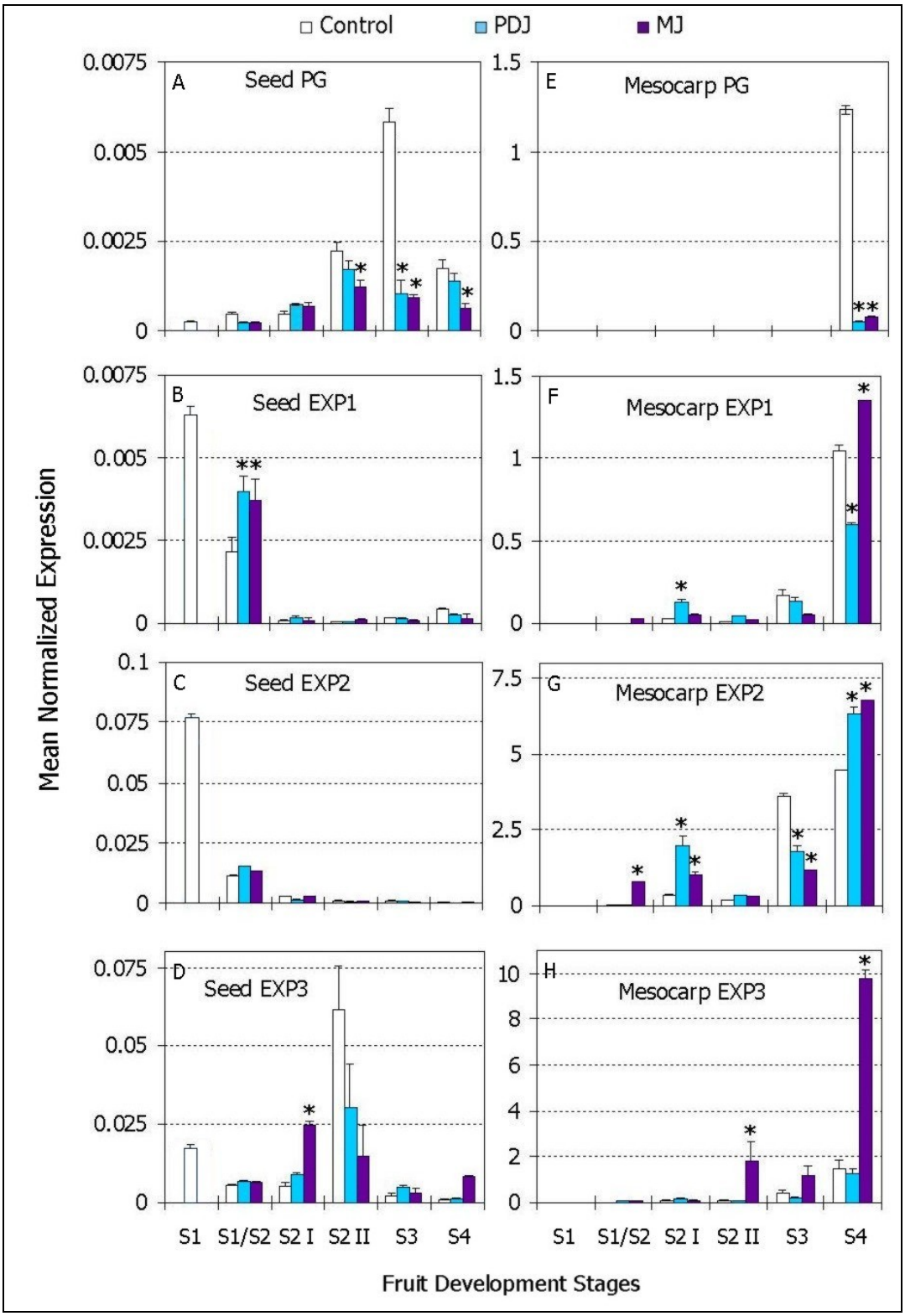

Figure 4. Expression profiling by real-time RT-PCR of cell wall-related genes during the S1-S4 stages of peach fruit development and ripening. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). PG (A), EXP1 (B), EXP2 (C) and EXP3 (D) in seed and PG (E), EXP1 (F), EXP2 (G) and EXP3 (H) in mesocarp tissue. Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * $P<0.05$.

4.3.4 JAs influence transcript accumulation of hormone -related genes

Mesocarp - Transcript amount of the JA synthesis key enzyme AOS1 decreased during fruit development up to harvest. While PDJ did not significantly altered this pattern MJ transiently enhanced AOS transcript accumulation at S2II (Figure 5C). NCED transcript was hardly detectable during fruit growth and increased abruptly at harvest. Both PDJ and MJ dramatically reduced transcript accumulation, by 90 and 80%, respectively (Figure 5D).

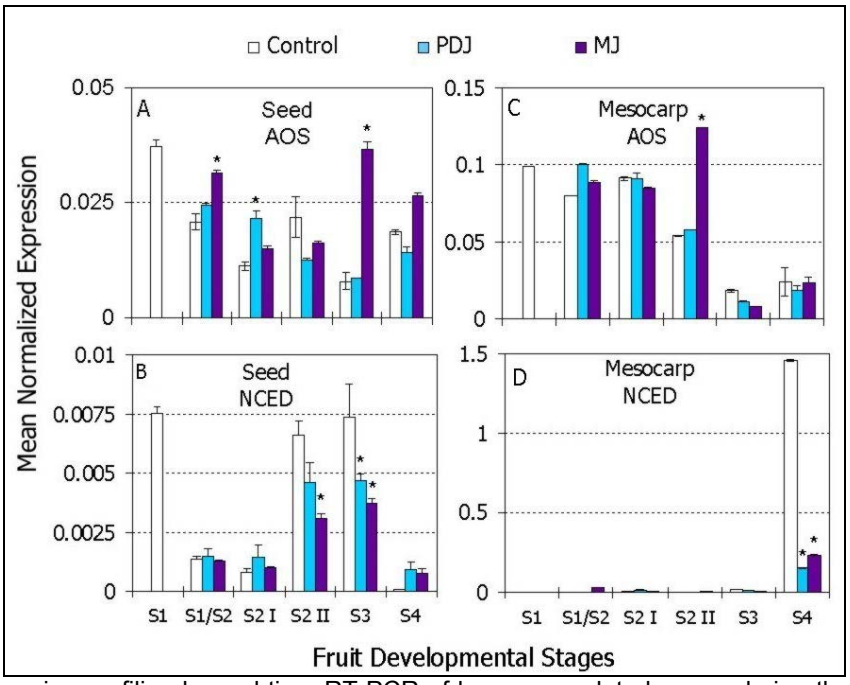

Figure 5. Expression profiling by real-time RT-PCR of hormone -related genes during the S1-S4 stages of peach fruit development and ripening. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). AOS and NCED expression in the seed (A and B) and mesocarp (C and D) respectively. Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * P<0.05.

IAA amidohydrolase (IAA-AH, ctg 4056) in the mesocarp fluctuated during the mesocarp development and only MJ increased significantly its transcript accumulation in S2 II (Figure 6C). About the other IAA-AH, ctg 4705, transcript accumulation increased during fruit growth. Only MJ had an upregulating effect in S2 II (Figure 6D).

Seed - AOS1 transcript fluctuated during seed development; MJ altered this pattern in S1/S2 and in S3 (about 4-fold) by enhancing transcript accumulation (Figure 5A) while PDJ increase AOS transcript accumulation in S2I. NCED was scarcely expressed and its transcript amount showed oscillations; JAs significantly down-regulated gene expression at S2II (only MJ) and S3 (Figure 5B). IAA-AH (ctg 4056 and ctg 4705) mRNA amount increased up to S3 and then decreased (Figure 6A and 6B). PDJ and MJ tended to

abolish the S3 peak. In S4 only PDJ upregulated ctg 4056 causing a shift ahead in gene expression (Figure 6A).

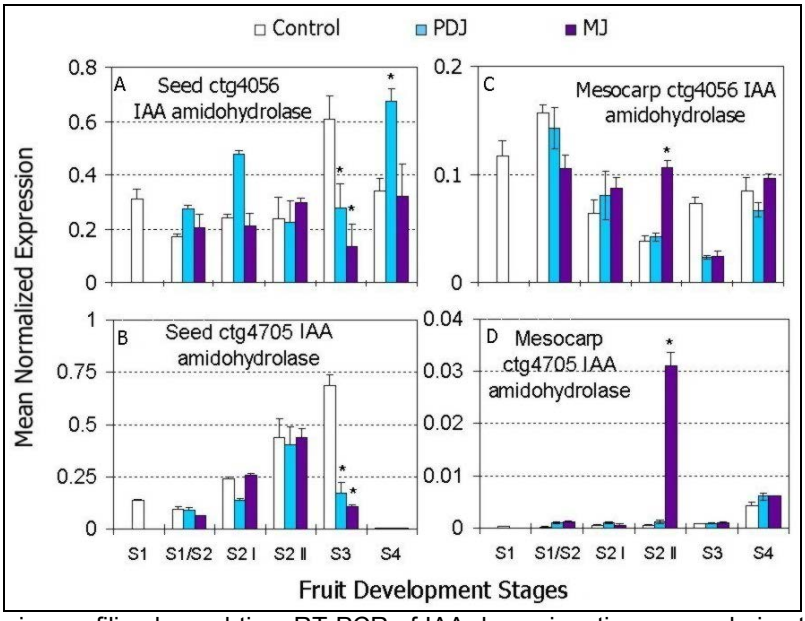

Figure 6. Expression profiling by real-time RT-PCR of IAA de-conjugating genes during the S1-S4 stages of peach fruit development and ripening. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). Expression of IAA amidohydrolases (ctg4056 and ctg4705) in seed (A and B) and mesocarp (C and D), respectively. Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * P<0.05.

4.3.5 JAs impair transcript accumulation of sugar-related and defence-related genes

Mesocarp - In control fruit sorbitol transporter (SOT, ctg 2902) expression increased in S2I and remained constant until S3, then decreased at harvest (Figure 7B).

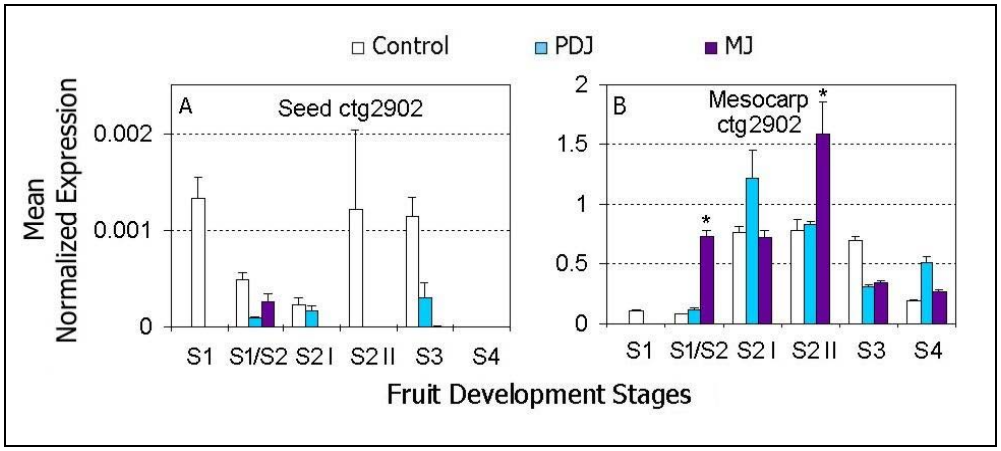

Figure 7. Expression profiling by real-time RT-PCR of sugar transport-related genes during the S1-S4 stages of peach fruit development and ripening. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). Ctg 2902 SOT (A) and (B), seed and mesocarp respectively. Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * P<0.05.

MJ enhanced SOT transcript accumulation one day after treatment and at S2II while PDJ had no effect (Figure 7B). Catalase (CAT, ctg 1024) transcript levels in the mesocarp gradually decreased during development; MJ reduced transcript accumulation in at S1/S2 while PDJ enhanced it in S1/S2 and S2I (Figure 8B).

Seed - SOT is expressed at low levels throughout and JAs did not affect its transcript accumulation (Figure 7A). CAT gene expression increased during seed development; it was only transiently inhibited by both JAs (Figure 8A).

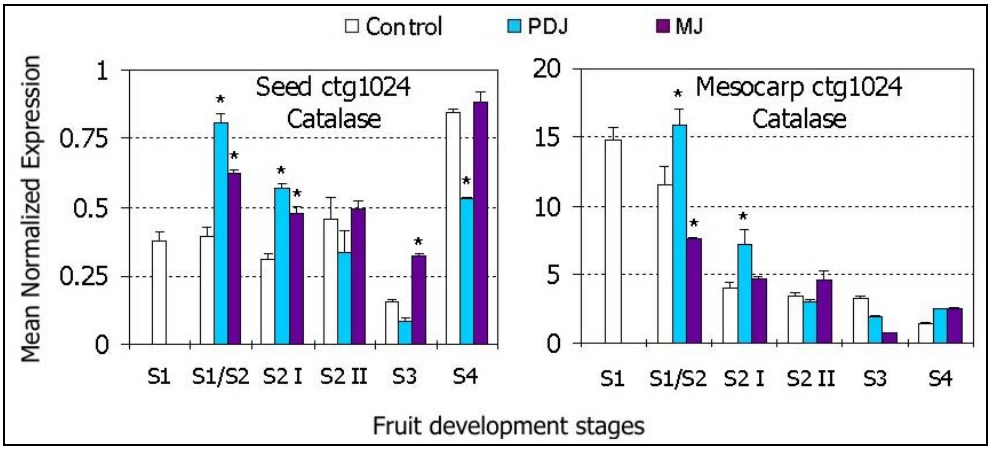

Figure 8. Expression profiling by real-time RT-PCR of *CAT* (ctg 1024) a defence-related gene during the S1-S4 stages of peach fruit development and ripening. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB) (A) in seed and (B) mesocarp. Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * P<0.05.

4.3.6 JAs affect transcript accumulation of developmental marker genes in the seed

A pathogenesis-related protein (PRP, cgt 1026) was only expressed during the first seed developmental phases (S1/S2) and was first stimulated by PDJ in S2I, and then repressed by both JAs in S2II (Figure 9A). Both PRU transcripts (PRU, ctg 1540 and 1543) accumulated dramatically at the end of seed development and peaked in S3; in the latter, both JAs repressed PRU transcript accumulation while in S4 they up-regulated transcript (Figure 9B and 9C, respectively). SSADH expression (ctg 2916) was maximum in S3 when both JAs inhibited transcript accumulation. MJ up-regulate SSADH in S4 (Figure 9D). A late embryogenesis abundant (LEA) protein (ctg 3563) was highly expressed in S4 and JAs significant down-regulated LEA transcript accumulation in S4 (Figure 9E).

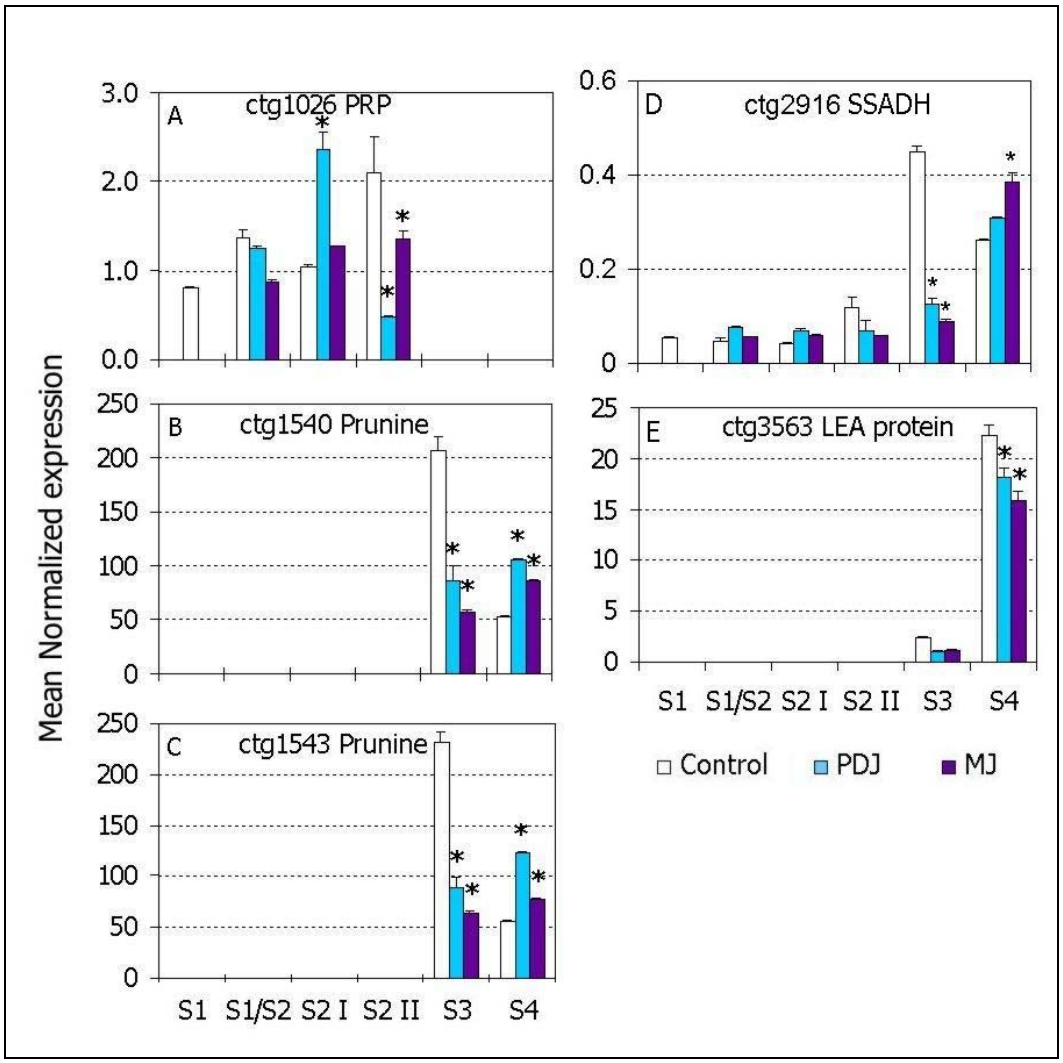

Figure 9. Expression profiling by real-time RT-PCR of marker genes in the seed during the S1-S4 stages of peach fruit development and ripening. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). PRP (A), ctg 1540 (B) and ctg 1543 (C) prunines, SSADH (D) and *LEA* protein (D). Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * P<0.05.

4.4 Discussion

Present results show that exogenous JAs, applied to young fruits at an early developmental stage (late S1), interfere with the expression of ethylene-, cell wall-, hormone-and defence-related genes in both seed and mesocarp leading to a slowing down of ripening as also revealed by I_{AD}. Transcriptional JA effects in the mesocarp and seed will be discussed separately.

4.4.1 JAs impair gene expression in the mesocarp

4.4.1.1 Fruit are less mature and major ripening-related genes are downregulated

Fruit growth monitoring in JA-treated fruit did not reveal any change in fruit in fresh weight, firmness or diameter, but, at harvest, treated fruits are less mature, as revealed by I_{AD}, and this suggests that they produce less ethylene than control fruit. Ziosi et al. (2008b) reported, in fact, that by correlating the I_{AD} with the changes in ethylene emission, peaches and nectarines could be sorted as belonging to a given maturation class. The JA-induced slowing down of ripening is in agreement with previous reports dealing with MJ and/or PDJ treatments performed at much later (S3 and S4) developmental stages. In all these cases fruits resulted less ripe, firmer and less sweet (Ziosi et al. 2008a; 2009; Soto et al. 2010).

Several results concerning JA effects on ripening-related parameters have been reported; in fact, whereas β-carotene, anthocyanin accumulation and volatile emission are generally stimulated in JA-treated fruit (Perez et al. 1993; Kondo et al. 2005; Rudell et al. 2005), other ripening related parameters such as fruit FF and SSC may be unaltered or differentially affected (González-Aguilar et al. 2004; Kondo et al. 2005; Ziosi et al. 2008a). In nectarines it is confirmed that, also when applied early, JAs induce a slowing down of ripening probably by mainly impairing ethylene production.

The analysis of the expression pattern of several ripening-related genes confirms the above hypothesis. Indeed, the inhibition of ethylene production as suggested by the I_{AD} is supported by the reduced expression in S4 fruit of the main ethylene biosynthetic genes ACS1 and ACO1 which are strongly induced during ripening (Trainotti et al. 2006; Bonghi et al. 2011). Contrary to peach, in preclimacteric apples and pears PDJ enhanced the expression of ethylene biosynthetic genes (Kondo et al. 2005; Kondo et al. 2007) and in tomato and strawberry producing low ethylene levels ethylene metabolism was enhanced by MJ (Yu et al. 2009). This led to the idea that when ethylene production is basal (system 1 ethylene production) JAs accelerate ripening. However, present and previous data (Ziosi et al. 2008a; 2009; Soto 2011) indicate that in peach, in both the short- and the long-term, JA delay ripening by reducing ACS1/ACO1 expression and ethylene production, and

suggest that the effect may be species-specific. Transcript levels of both ethylene perception genes were altered indicating that ethylene signalling was affected early during fruit development (ETR2) or late (ETR1/ETR2) following JA treatment. This is confirmed by the early alteration of the ERF2 gene expression. The stimulation of the expression of the ethylene receptor ETR2 could be in relation with the decrease in ethylene production since ethylene receptors negatively regulate ethylene signal transduction (Guo and Ecker, 2004). Modulations of ethylene perception and signalling were also observed in both Redhaven and Stark Red Gold with late JA application under field conditions (Ziosi et al. 2008a; Ziosi et al. 2009; Soto, 2011).

Although flesh firmness was not affected, as measured by a pressure tester, transcript levels of cell wall-related genes were influenced. In particular, the dramatic reduction in PG mRNA amount in JA-treated fruits suggests that, actually, in these fruits softening was impaired relative to controls according with previous findings (Ziosi et al. 2008a; Soto, 2011). PGs are expressed in various plant tissues, and are involved in diverse developmental processes especially those involving cell separation. Firmness is an important quality trait and its extent is closely associated to ripening. Softening is a complex process characterized by the sequential action of several cell wall hydrolases including PGs and EXPs. Endo-PG transcript amount (and activity), responsible for pectin modification and textural changes in melting flesh peach, greatly increase during softening and prior to the beginning of ripening and was also shown to positively respond to ethylene (Trainotti et al. 2003; Trainotti et al. 2006b). Moreover, firmness retention in non-melting cultivars is associated with lack of PG activity (Brummel et al. 2004). Thus, inhibition of PG transcript accumulation in JA-treated fruit at ripening probably accounts for firmness retention and ripening delay.

Modulation of EXP gene expression during the considered period also indicates that alterations in cell wall architecture occurat ripening in treated fruits. Expression of specific EXPs, which belong to a large multigene family, is associated with numerous aspects of plant development. EXPs promote, *in vitro*, extension of plant cell walls by disrupting hydrogen bonding at the cellulose/hemicellulose interface, thereby relaxing an important constraint to turgor driven cell expansion, though no detectable enzymatic activity was found (McQueen-Mason and Cosgrove, 1994). EXP action is probably crucial for coordinated cell-wall disassembly in both growing vegetative tissues and ripening fruit (Rose and Bennett 1999). The pattern of EXP transcript amount presently observed in control mesocarp was in accord with previous work (Hayama et al. 2003) and with a transcriptome analysis which showed that EXP1, 2 and 3 are up-regulated at the S3-S4

transition of the fruit growth (Trainotti et al. 2006). Present results show that all EXPs were generally, early or late, up-regulated by JAs. Thus, while counteracting softening and ethylene production; JAs also increase EXP transcript accumulation. This suggests that EXPs may not only be involved in cell wall loosening but in other aspects of cell wall re-arrangement during softening. Upregulation of EXP1 and 2 transcript levels were also reported in JA-treated and spermidine-treated S3 nectarines, respectively, which produced less ethylene than controls (Ziosi et al. 2008a; present thesis).

SSC concentration did not vary in JA-treated fruits relative to controls. However, the modulation of SOT gene expression suggests that sugar levels were also impaired in ripe fruits. The acyclic polyol sorbitol is a primary photosynthetic product and the principal photosynthetic transport substance in many economically important members of the family Rosaceace including peach. In sour cherry (*Prunus cerasus*) fruit tissues, that contain large quantities of sorbitol, PcSOT1 is expressed throughout fruit development, but especially when growth and sorbitol accumulation rates are the highest (Gao et al. 2005). Sorbitol concentration correlates with the expression of the SOT gene and decreases when sucrose becomes the most abundant sugar. Once unloaded into sink tissues, sorbitol seems to be readily converted into fructose and glucose by sorbitol dehydrogenase and sorbitol oxidase, respectively. Present data show that SOT (ctg 2902) gene expression is the highest in S2/S3 according to Bonghi et al. (2011); MJ enhanced SOT expression suggesting a transient increase in sorbitol levels. Although SSC does not change in JA-treated fruit, it can not be excluded that increased sorbitol levels could negatively affect final sucrose concentration at ripening.

4.4.1.2 Hormone- and defence related genes are differentially orchestrated by JAs

AOS, a cytochrome P450 of the CYP74A family, is the first specific enzyme and the major control point of the JA biosynthetic pathway (Haga and Lino 2004). AOS message is up-regulated in response to wounding and treatments with JAs in leaves of *Arabidopsis*, tomato, and tobacco (Howe et al. 2000) indicating that a positive feedback regulation in JA biosynthesis occurs, leading to an amplification of the hormone signal. AOS gene expression is developmentally regulated in peach seed and mesocarp (Torrigiani et al. 2012). In control nectarines, AOS transcript levels decreased until ripening in agreement with previous findings and with the pattern of jasmonic acid levels (Torrigiani et al. 2012). The AOS upregulation by MJ in late S2, though transient, is in line with the known positive feed-back regulation of the AOS enzyme (Kubisteltig et al. 1999) and may be associated with an increase in JA production in treated fruit supporting the hypothesis that increased JA levels impair peach fruit ripening. In fact, in a previous study, in JA-treated nectarines

in planta, an increase in AOS transcript levels was observed concomitant with an increase in endogenous jasmonic acid concentration 1 day after treatment (Ziosi et al. 2008a).

NCED transcript levels increased abruptly at ripening in control fruit. JAs strongly counteracted the rise in NCED gene expression again suggesting a slowing down of ripening. In peach, NCED has been found to initiate ABA biosynthesis and this preceded the climacteric ethylene production (Zhang et al. 2009a; Zhang et al. 2009b); exogenous ABA stimulated ethylene production and accelerated fruit ripening. Inhibition of NCED gene expression, which is already detectable in S3, may correlate with a delay in initiation of ripening process.

Transcript levels of IAA-AH genes (ctg 4056 and 4705), responsible for active IAA releasing from conjugates with amino acids, peptides or sugars (Bartel and Fink 1995) were quite low in the mesocarp; their upregulation in S2II suggests an interference with auxin metabolism. Auxin actively participates not only in fruit growth processes but also in coordinating the ripening syndrome (Trainotti et al. 2007). Results are in accord with the intense cross talk between MJ and auxin, reported in Redhaven peach treated with MJ in S3 (Soto 2011). In the latter work the expression of several genes involved in auxin biosynthesis, conjugation, transport and signalling was affected leading to a slowing down of auxin metabolism concurrent with that of ethylene and consequently of ripening.

JAs are able to induce the expression of numerous defence genes in plant organs including fruit (Wasternack 2007, Ziosi et al. 2008). CATs are predominantly located in the peroxisomes and are important enzymes scavenging H_2O_2. Besides elimination of peroxisomal H_2O_2 production, the scavenging action of CAT also appears critical for maintaining redox balance during oxidative stress and is indispensable for stress defence. CAT2 and 3 activity declined in leaves with the progression of senescence, resulting in a decrease of the antioxidative capacity of the cells and probably creating a signal for the cells to promote senescence (Dat et al. 2000). Present results show that CAT (ctg 1024) expression declines during fruit development in accord with previous results (Bonghi et al. 2011). The early and transient up-regulation of PDJ of CAT in the mesocarp is in line with the elicitation of defence genes by JAs.

4.4.2 Changes in gene expression are also induced by JAs in the seed

Besides those analyzed in the mesocarp, other genes thought to be developmental marker (Bonghi et al. 2011) were screened in the seed.

4.4.2.1 Hormone- and defence related-transcript levels are altered

The downregulation by JAs of the ethylene biosynthetic genes, which were scarcely expressed in the seed, confirm their their inhibitory effect on ethylene production also in the seed. In middle-ripening peach cvs. such as Stark Red Gold, seed ethylene production remains at basal levels throughout development (Bonghi et al. 1997) in accord with the low extent of ACS1 expression presently observed; however, the alteration of ACO1 expression together with that of ethylene receptors and ERF2, already detectable one day after JA application, suggests that JAs rapidly reached the seed, as also demonstrated in MJ-treated detached S1 nectarines (Torrigiani et al. 2012), and exerted an effect there. It has been reported, in fact, that exogenous MJ can be transformed into jasmonic acid and translocated through the plant (Sato et al. 2009).

Cell wall-related genes were also analyzed in the seed where, however, they display a quite low expression. EXPs were scarcely affected by JAs while PG was strongly down-regulated in S3. Few is known about the involvement of these genes during seed development. While EXP1 and 2 are expressed early when endosperm develop and accumulate reserves, EXP3 and PG peak at S2II and S3, respectively, when embryo begins to develop (S2II) and fill almost the entire seed (S3) suggesting a differential involvement of these genes in the building of new cell walls.

An AOS transcript was detected in the seed whose amount fluctuated throughout. In *Arabidopsis* jasmonic acid and its active conjugate JA-isoleucine (JA-Ile) decrease during the first phase of seed growth; JA-Ile levels rise again during the second developmental phase until maturation in both the embryo and envelope (Kanno et al. 2010) suggesting a developmental regulation. In peach seed, AOS1 expression, which is known to positively correlate with JA levels (Wasternak 2007), was found to be differentially expressed in the embryo and cotyledons according to the different function and fate of the two seed components (Torrigiani et al. 2012). Up-regulation by JAs of AOS1 transcript levels in S3/S4 suggests a possible increase in endogenous JA levels. This could negatively interfere with the normal progression of seed development as shown by a transgenic approach (Cipollini 2010). It is known in fact that JAs elicit defence gene expression and proteins and that diverts resources from growth to defence (Schmidt and Baldwin 2006) as also observed in field treated nectarines (Ziosi et al. 2008a).

NCED expression peaked in S2/S3; this rise was counteracted by both JAs suggesting a negative interference with ABA accumulation in the seed. ABA plays crucial role during seed development and maturation. In *Arabidopsis* the accumulation of storage compounds is associated with an increase in ABA content so that in the final stage of

seed development the seed (embryo) acquire desiccation tolerance and become quiescent (Kanno et al. 2010). The timing of ABA accumulation in *Arabidopsis* seed is in agreement with the presently observed enhanced transcript levels of NCED in S2II/S3, period in which embryo develops fast and undergoes dormancy (Bonghi et al. 2011). The impairment of NCED gene expression by JAs may suggests a delay in the initiation of dormancy by ABA.

Both IAA-AH (ctg 4056 and 4705) examined in the seed peaked in S3; only PDJ shifted the former (ctg 4056) peak to S4 while both JAs down-regulated both contig expression in S3. This suggests an impairment of auxin release from conjugates that could negatively reflect on the levels of free auxin and thus on seed development. In *Arabidopsis* IAA accumulated to high levels in silique from early to mid-seed development then decreased in both envelope and seed, concomitant with the increase in ABA levels (Kanno et al. 2010). In nectarine seed, it appears that a late peak in the release of IAA from conjugates occurs at S3, which is a stage of fast embryo growth. The negative interference of JAs with IAA-AH gene expression suggests an impairment of embryo development and could account for a slowing down of this process.

CAT was less expressed in the seed than in the mesocarp, peaked at maturity and was early (one day after treatment) up-regulated by both JAs probably as a defence response to JAs. CAT1 is the isoform mainly involved in the removal of H_2O_2 from glyoxysomes and highly abundant in seeds and young seedlings consistent with its antioxidant role (Dat et al. 2000).

SSADH can accumulate in plants under stress, and react with DNA, oxidize membrane lipids, modify proteins or influence the transcription of stress-related genes, thereby causing cellular and developmental problems (Deewatthanawong et al. 2010). Consequently, enzymes and metabolic pathways that transform these compounds are probably essential for maintaining plant health. Downregulation of SSADH in the seed could be explained in this light.

4.4.2.2 Seed marker gene expression is shifted

Some developmental marker gene expression (Bonghi et al. 2011) was affected by JAs in the seed: a PRP (ctg 1026), two prunins (PRUs; ctg 1540 and 1543), the main seed storage proteins in *Prunus* spp with allergenic properties (Botton et al. 2009) and a late embryogenesis abundant protein (LEA, ctg 3563). PRP (ctg1026), marker of S2I in Fantasia and of S2II in Stark Red Gold, was first up-regulated by PDJ, possibly as a defence response; both PRUs that mark S3 stage, were first down-regulated and then

upregulated. This suggests a delay/shifting of the rise of gene expression and may account for a maturation slowing down of the seed.

Seed maturation is characterized by synthesis of specific late embryogenesis abundant (LEA) proteins correlated with dehydration (Finkelstein et al. 2002). The expression of a gene encoding a LEA protein became detectable at S2II in Fantasia nectarines and peaked at S4 (Bonghi et al. 2011) as presently observed in Stark Red Gold. Thus LEA is a very reliable marker of S4, in both Fantasia and Stark Red Gold, indicating that the seed (embryo) can reach a fully mature stage in both genotypes. The very low levels of LEA gene expression detected at S4 in Springcrest are consistent with the uncoupling that exists between seed and pericarp maturation in this genotype (Bonghi et al. 2011). The inhibitory effect of LEA expression by JAs would suggest a delay of maturation.

5. Biosynthetic gene expression and accumulation of phenolic compounds in the mesocarp are transiently affected by jasmonate application to young peach fruits

5.1 Introduction

Phenylpropanoids are a diverse group of compounds derived from phenylalanine which is an end product of the shikimate pathway. The latter pathway also gives rise to the aromatic-amino acids tyrosine and tryptophan (Tzin and Galili, 2010). From phenylalanine, the pathway leads to molecules such as flavonoids, coumarins, hydroxycinnamic acid conjugates, lignines and alkaloids (Vogt, 2010) collectively regarded as secondary metabolites. Phenylpropanoids are involved in plant defense and structural support, and appear to be indispensable to the survival of the plant.

Several studies demonstrate the efficacy of polyphenolic antioxidants from fruits and vegetables to reduce neuron death and their connection to the control of specific events related to atherosclerotic lesions. Polyphenols could be also involved in the prevention of cardiovascular diseases and soy isoflavonoids may be effective in preventing osteoporosis (Boudet, 2007).

Phenolic compound pathway is composed of a sequence of enzymatic steps (Figure 1) including phenylalanine lyase (PAL) for the synthesis of cinnamic acid, chalcone synthase (CHS) for the synthesis of naringenin chalcone, chalcone isomerase (CHI) for the conversion of naringenin chalcone to naringenin, flavanone 3-hydroxylase (F3H) and flavonoid 3'-hydroxylase (F3'H) for the subsequent hydroxylations of naringenin, NADPH-dependent dihydroflavonol reductase (DFR) for the production of leucoanthocyanidins, leucoanthocyanidin dioxygenase (LDOX) for the conversion of leucoanthocyanidins to anthocyanidins, and UDP-Glc:flavonoid 3-O-glucosyltransferase (UFGT) for the generation of glycosylated anthocyanidins (Fraser and Chapple, 2011).

The regulation of anthocyanin/flavonoid biosynthesis involves numerous regulatory factors that control the expression of different anthocyanin biosynthetic genes in *Arabidopsis*. In *Arabidopsis*, transcription factors PAP1 (Myb75), PAP2 (Myb90), EGL3, GL3, and TTG1 predominantly regulate the expression of the 'late' anthocyanin biosynthetic genes including DFR, LDOX, and UF3GT over the expression of the 'early' anthocyanin biosynthetic genes such as PAL, CHS, CHI, and F3'H (González et al. 2008).

Accumulation of flavonoids in plants is stimulated by diverse developmental signals, sugar and environmental stresses including UV light, high-intensity light, temperature, pathogen infection, wounding, drought, and nutrient deficiency (reviewed by Ferrer et al. 2008).

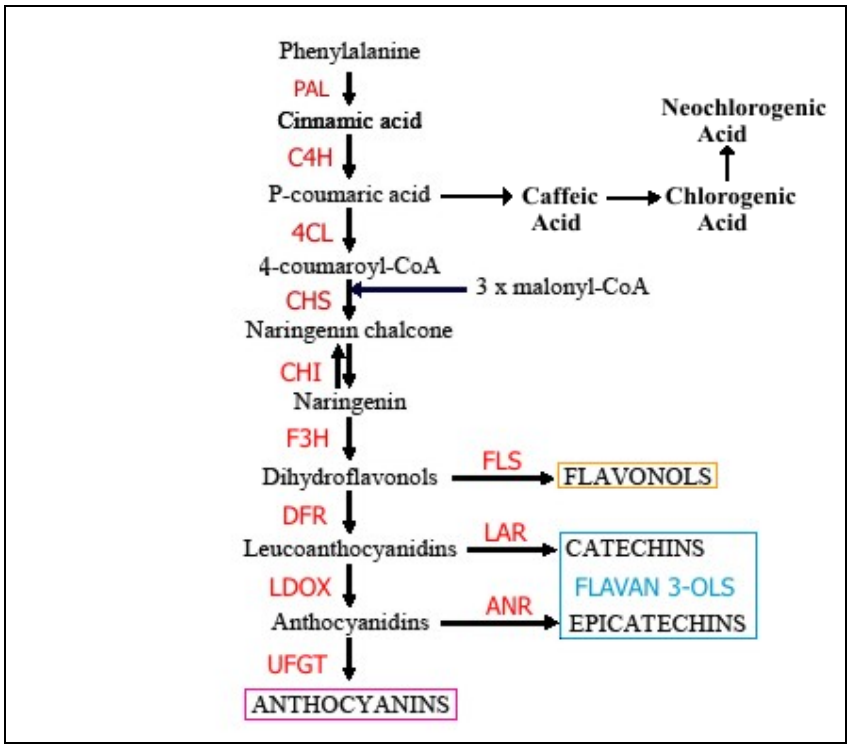

Figure 1. Phenylpropanoid biosynthetic pathway involving antocianins and flavonoids (integrated from Pelletier et al. 1997 and Winkel-Shirley (2002)). In red: biosynthetic enzymes. PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; ANR, anthocyanidin reductase; UFGT, UDP-glucose:flavonoid 3-O-glucosyltransferase.

Plant hormones are also involved in the regulation of flavonoid accumulation. Cytokinins have been shown to induce accumulation of anthocyanins in *Arabidopsis*, to modulate expression of CHS and DFR probably at the transcriptional level, and to regulate PAL1 and CHI post-transcriptionally (Deikman and Hammer, 1995). Gibberellins (GAs) have been shown to be required for the induction of anthocyanin gene transcription and for the accumulation of the pigment in the developing corolla in *Arabidopsis* (Weiss et al. 1995). Jasmonates (JAs) exogenously applied in pre-harvest to mature apple fruit enhance skin red color without negatively interfering with fruit quality traits (Zioisi et al. 2007). N-propyl dihydrojasmonate (PDJ), a synthetic analogous of jasmonates, increased abscisic acid (ABA) and anthocyanin content of apples (Kondo et al. 2000).

Several studies about phenolic composition in peaches indicated the class of cinnamic acid compounds as the main phenolics present in the mesocarp during the development (Andreotti et al. 2008; Ravaglia et al. 2010). Fruit phenolic compounds are subject to

quantitative/metabolic changes until fruit complete the entire cycle (development, senescence processes/ripening and death); moreover, they are influenced by abiotic and biotic stresses and field management practices, and environment (Andreotti et al. 2010; Ravaglia et al. 2010).

JAs, which include jasmonic acid and its cyclopentane precursors as well as cyclopentenones (Reymond and Farmer, 1998), are synthesized from the octadecanoid/hexadecanoid pathways and widely distributed throughout the plant kingdom. They mediate plant responses to stress, wounding, insect attack, pathogen infection, and UV damage (Wasternack, 2007), and also regulate many other plant developmental processes including fruit ripening (Browse, 2009). JAs have also been shown to enhance pigmentation in plants and fruit (Tamari et al. 1995; Ziosi et al. 2008c).

To better investigate the molecular basis of the developmental and ripening control exerted by JAs in fruit mesocarp, the aim of the present work was to clarify whether, when applied to young peach fruit at an early (S1) developmental stage, and under field conditions, MJ and its synthetic analog PDJ were able to influence the phenolic biosynthetic pathway gene expression and the accumulation of phenolic compounds. To this aim, maturity and quality traits were assessed during fruit ripening (see section 4.3.1), phenolic profiles and transcript levels of the following genes were monitored in controls and JA-treated mesocarp until harvest: PAL, CHS, CHI, F3'H, DFR, LAR, LDOX, ANR, UFGT and the transcription factors bHLH and MYB.

5.2 Materials and methods

5.2.1 Plant material

The trial was carried out on 8-years old peach (*Prunus persica* var. *laevis* Grey. cv. 'Stark Red Gold', nectarine) trees (4 per treatment) grown at the experimental farm of the University of Bologna, Italy. Four branches per plant, homogeneous for size and fruit load (3-4 fruits per branch) were selected for the experiments. For each treatment, 16 branches were sprayed with 0.80 mM MJ or 0.44 mM PDJ (Nippon Zeon Co. Tokyo, Japan). Both compounds were dissolved in an aqueous solution containing surfactant and ethanol (150 mL per branch) as described by Ziosi et al. (2008a). Control branches only received an aqueous solution containing the same concentration of surfactant and ethanol. The double sigmoid growth pattern of peach fruit was established and the four growth stages S1-S4 determined (Figure 2) as described by Torrigiani et al. (2004). MJ and PDJ were applied 59 days after full bloom (dAFB; late S1) and samples of 10 control and 10 treated fruit were collected 0 (S1), 1 (S1/S2), 7 (S2I), 16 (S2II), 52 (S3), 70 (S4) days after treatments;

70 days after treatment coincided with harvest (130 dAFB, flesh firmness 40 N). At ripening, the Index of Absorbance Difference (IAD, Ziosi et al. 2008b) and quality traits were determined on the whole fruit as previously described (section 4.2.2). The quantification and determination of phenolic profiles and real-time qRT-PCR analysis were made only in the mesocarp tissue.

5.2.2 Identification and quantification of phenolic compounds

Phenolic compounds were extracted from freeze-dried flesh fruit samples. Two independent extractions were made by a method described by Andreotti et al. (2008). About 100 mg of samples were ground to a fine homogeneous powder and then extracted in methanol (100% v/v) containing 6-methoxy-flavone (0.025 mg mL^{-1} in methanol) as an internal standard. Phenolic extracts were analysed by a Waters HPLC system with a Photodiode Array Detector (Waters 2996) and a reverse-phase SupelcosilTM LC-18 HPLC column (15 cm long, 4 mm internal diameter and octadecyl silane particles of 5 µm diameter) using a methanol:H_20 mobile phase gradient.

Each phenolic compound was identified through comparison of the retention time values and UV spectra (detected between 210 and 560 nm wavelength) with authentic standards. Phenolic compound concentrations, expressed in mg g^{-1} dry weight (DW), were calculated from calibration curves obtained with the corresponding external standards. Standards for qualitative and quantitative determinations were purchased from Sigma-Aldrich (St Louis, MO, USA), whereas methanol for liquid chromatography and phosphoric acid were purchased from Carlo Erba (Milan, Italy).

5.2.3 Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from fresh mesocarp tissue samples according to Chang et al. (1993). RNA yield and purity were checked by means of UV absorption spectra, whereas RNA integrity was determined by electrophoresis in agarose gel. DNA was removed from 10 µg aliquots of total RNA using the TURBO DNA-free™ (Applied Biosystems, Foster City, CA, USA). The first-strand cDNA was synthesized from 3 µg of the DNaseI-treated RNA by means of the High-Capacity cDNA Kit (Applied Biosystems), using random primers. Real-time RT-PCR was performed in a reaction mixture, final volume 25 µl, containing 9 ng of cDNA, 5 pmol of each primer, and 12.5 µl of the Fast SYBR Green PCR master mix (Applied Biosystems), according to the manufacturer's instructions. Oligonucleotides PpN1 (5'- CCAGGAGAATCGGTGAGCAGAAAA- 3', sense) and PpN1 (5'- TCGAGGGTGGAGGACTTGAGAATG- 3' antisense), annealing to the peach putative transcript ppa009483 m, orthologous to *Arabidopsis* AT4G34270 were used to amplify the

internal standard with peach samples. The analyzed flavonoids biosynthetic enzymes and transcript factor primer sequences are listed Appendix 1. Quantitative RT-PCRs were carried out with the StepOnePlus™ 7500 Fast (Applied Biosystems) for 2 min at 95 °C and then for 40 cycles as follows: 95 °C for 15 s, 60 °C for 15 s, and 65 °C for 34 s. For each gene, a standard curve was generated using a cDNA serial dilution. The obtained CT values from each gene expressed were analyzed with the Q-gene software by averaging three independently calculated normalized expression values for each sample. Expression values are given as the mean of the normalized expression values of the triplicates, calculated according to equation 2 of the Q-gene software (Muller et al. 2002).

5.2.4 Statistical analysis

Phenols data quantified by HPLC (2 biological replicates, n=3 each; only results from the second experiment were used for graphs and statistical analysis) from treated and control mesocarp samples were statistically analyzed using a GLM (general linear model) analysis with LSD as posthoc test. Quantitative RT PCR (n=3) differences between control and treated samples were compared using GLM (general linear model) analysis with Tukey as posthoc test (StatSoft Statistics v.8 software).

5.3 Results

Peach fruit growth was followed during S1-S4 development until commercial harvest (Figure 1) and fruit ripeness assessed by I_{AD} as previously described (section 4.3.1).

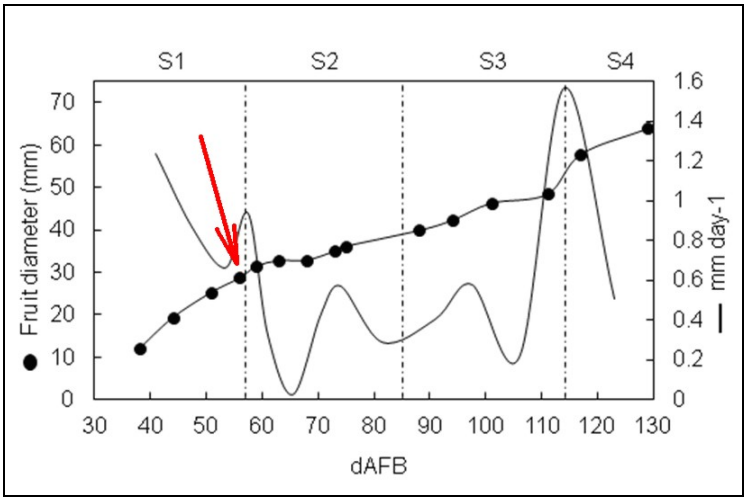

Figure 2. Growth curve of 'Stark Red Gold' nectarine based on diameter (filled circles) and its first derivative (continuous line) from 39 to 130 days after full bloom (dAFB). S1– S4 represent the four stages of growth up to harvest. Time of PDJ and MJ treatments is indicated by the red arrow. Data represent the means (n=10).

5.3.1 JAs transiently modify flesh phenolic composition

The most abundant phenolic compounds detected in the flesh of treated and untreated Stark Red Gold fruits are chlorogenic acid and neochlorogenic acid (belonging to cinnamic acid class) and catechin (a flavan-3-ol) (Figure 3); p-coumaric and caffeic acid, and epicatechin were also identified. In the methanol-extract there are several unidentified compounds classified like unknown which were not taken into account for calculation of total phenolic content.

Coumaric acid content reached the highest levels in S2I and then decreased until ripening when it was not present any longer (S4) (Figure 3A). In S2I stage PDJ significantly decreased while MJ increased its accumulation. In S2II both compounds increased p-coumaric acid accumulation over the control.

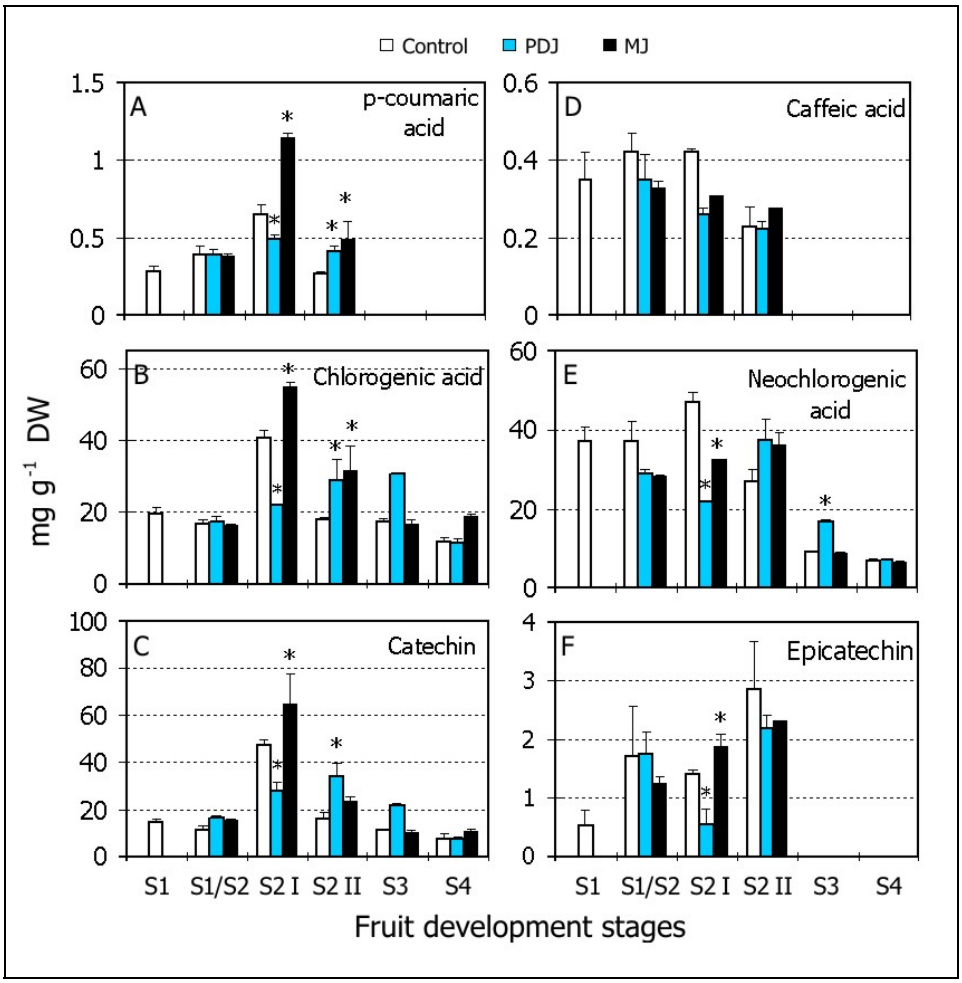

Figure 3. Profile of main phenolics compound in the flavonoids biosynthetic pathway during the S1-S4 stages of peach mesocarp development and ripening determined and quantified by HPLC. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). p-Coumaric acid (A), Chlorogenic acid (B), Catechin(C), Caffeic acid (D) Neochlorogenic acid (E) and Epicatechin (F). Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with LSD as posthoc test; * $P<0.05$.

Caffeic acid was detectable until S2II and was not affected by treatment in any developmental stages of the mesocarp (Figure 3D). Chlorogenic and neochlorogenic acids were highly accumulated in the mesocarp (up to about 40 mg g^{-1} DW respectively) peaking in S2I; thereafter they decreased until ripening (Figure 3B and 3E, respectively). Treatment with PDJ substantially decreased chlorogenic acid content in S2I (to about 50%) while in S2II and S3 enhanced it. Regards to MJ effects, it displayed a positive effect on chlorogenic acid accumulation in S2I and S2II (Figura 2B). About neochlorogenic acid accumulation, both PDJ and MJ had a depleting effect in S2I stage, while in S3 only PDJ treatments increased neochlorogenic acid content. Catechin (Figure 3C) and epicatechin (Figure 3F) are compounds that belong to the flavan-3-ol category. Catechin was detected throughout development in control mesocarp, reached a peak of accumulation in S2I and decreased thereafter up to ripening. PDJ had an inhibitory effect in S2I but in S2II it was able to enhance catechin content. MJ only increased catechin amount in S2I. Epicatechin amount was significantly reduced by PDJ and increased by MJ only in S2I.

Regards to the total content of phenolic compounds, in control fruits (Figure 4), a peak of accumulation occurred in S2I after that, a decrease during development until ripening was observed. PDJ diminished phenol content only in S2I but enhanced it in S2II and S3, while MJ increased phenol content only in S2II.

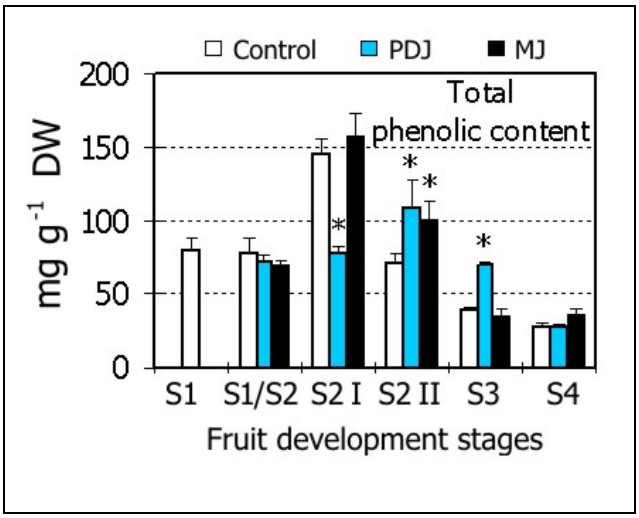

Figure 4. Content of total phenolic compounds identified during the S1-S4 stages of peach mesocarp development and ripening; they were determined and quantified by HPLC. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with LSD as posthoc test; * $P<0.05$.

5.3.2 JAs alter the expression phenol biosynthetic genes and transcription factors

Phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI) and flavanone 3-hydroxylase (F3'H) are considered early genes in the flavonoid pathway (Figure 5).

PAL is involved in the first step of flavonoid biosynthesis. In control mesocarp, transcript accumulation reached its maximum in S2I and then decreased until the end of fruit development (Figure 5A). Already one day after treatment, in S1/S2, PDJ was able to strongly up-regulate PAL transcription (about 3-fold); in S2I both JAs enhanced PAL (1.6 and 2.3-fold, respectively).

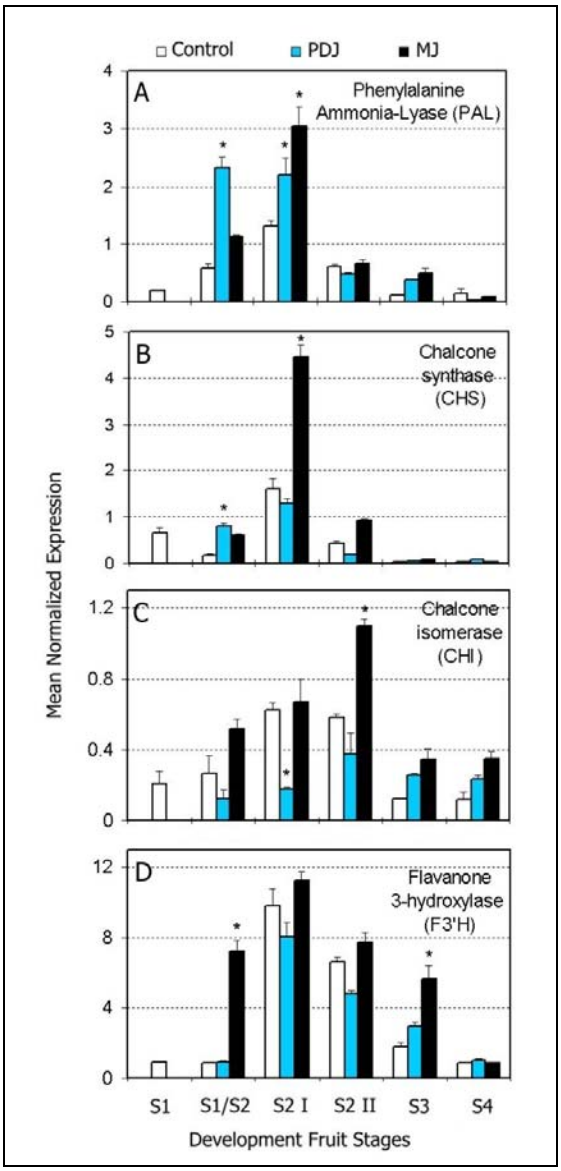

Figure 5. Expression profiles of genes (PAL, CHS, CHI and F3'H) early involved in the flavonoid pathway during the S1-S4 stages of peach mesocarp development and ripening as determined and quantified by real time RT-PCR. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * P<0.05.

Transcript levels of CHS were mainly detected from S1 to S2II peaking in S2I (Figure 5B). Transcript accumulation was up-regulated by PDJ in S1/S2, one day after treatment, while MJ induced a highly significant increase (more than 2-fold) in S2I. Regards CHI (Figure 5C), transcript amount was less abundant than CHS and was present during all fruit stages monitored. PDJ Inhibited CHI transcript accumulation in S2I while MJ up-regulated CHI transcription in S2II. F3'H displayed a peak of expression in S2I, decreasing thereafter until ripening (Figure 5D). In fruit treated with PDJ no differences relative to controls were found. MJ was able to strongly increase F3'H transcript levels one day after treatment (7-fold) and again in S3, by about 2-fold.

As late genes in the flavonoid pathway dihydroflavonol-4-reductase (DFR), leucoanthocyanidin reductase (LAR), leucoanthocyanidin dioxygenase (LDOX), anthocyanidin reductase (ANR) and UDP-flavonoid-glucosyltransferase (UFGT) were taken into account (Figures 6 and 7).

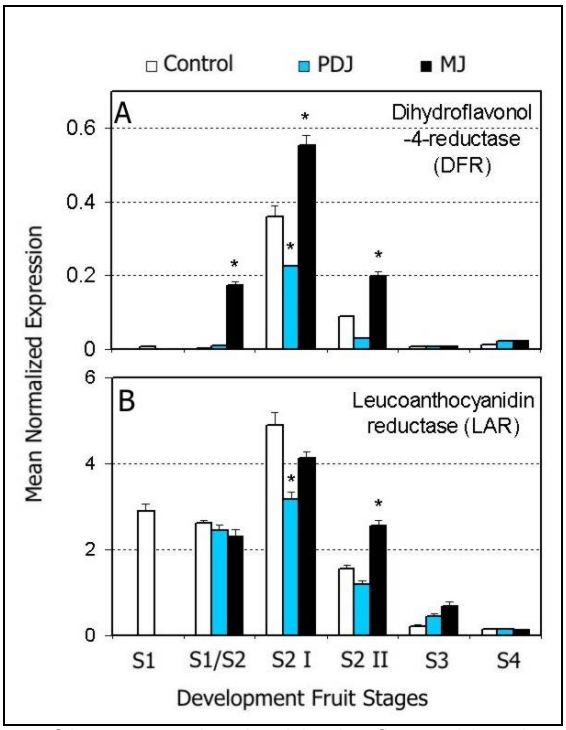

Figure 6. Expression profiles of late genes involved in the flavonoid pathway during the S1-S4 stages of peach mesocarp development and ripening as determined and quantified by real time RT-PCR. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). DFR (A); LAR (B). Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * $P<0.05$.

In control fruit DFR transcript showed a peak of accumulation in S2I and then decreased for the rest of fruit development (Figure 6A). JA-treated fruit displayed a peak in DFR transcript in the same stage (S2 I) as control. PDJ down-regulated DFR transcript amount in S2I while MJ up-regulated it in S1-S2 especially one day after treatment.

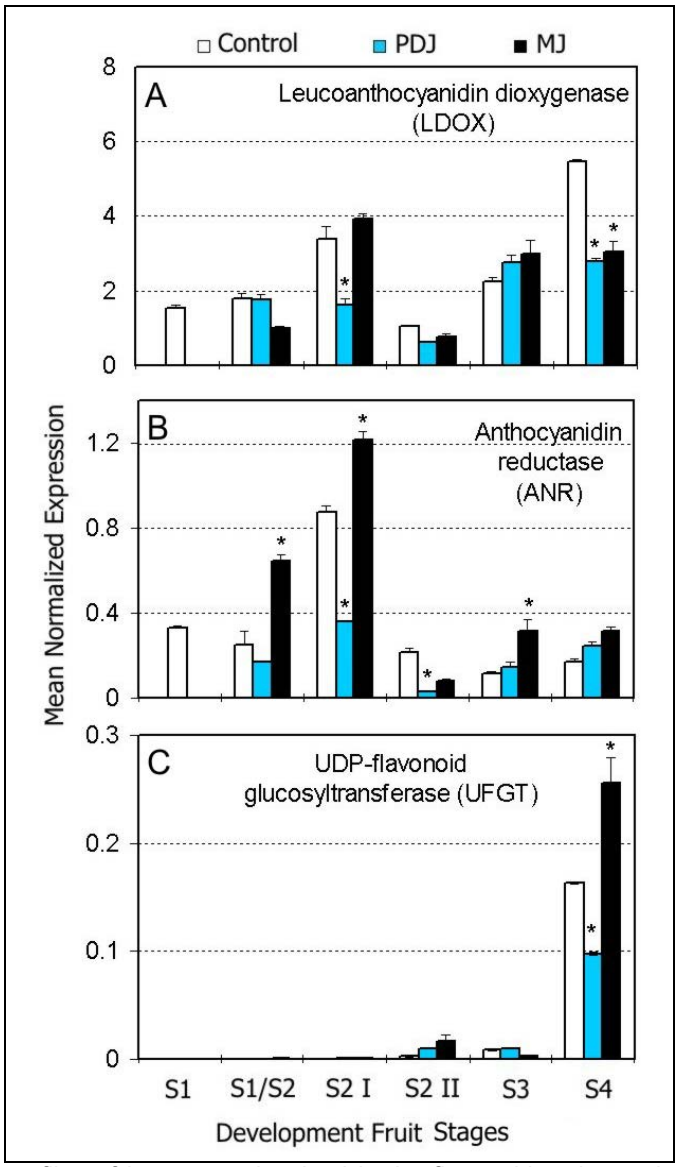

Figure 7. Expression profiles of late genes involved in the flavonoid pathway during the S1-S4 stages of peach mesocarp development and ripening determined and quantified by Real time qPCR. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). LDOX (A), ANR (B) and UFGT (C). Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * $P<0.05$.

About LAR (Figure 6B), which is directly involved in the synthesis of catechin and epicatechin, in control mesocarp, it is highly expressed in the early stages of fruit development and showed a peak of expression in S2I, then diminishing until ripening. PDJ decreased LAR transcript amount only in S2I while MJ up-regulated it in S2II.

In control mesocarp LDOX transcript accumulation was detectable during all development and peaked in S4 (Figure 7A). PDJ was able to down-regulate LDOX transcript in S2I and

S4 (about 50 % in both stages). Also MJ impaired LDOX transcript amount but only in S4 by about 50%.

ANR transcript levels were measurable during all fruit development stages and peaked in S2I (Figure 7B). PDJ and MJ did not alter this pattern. However, PDJ decreased ANR transcript amount in S2 while MJ enhanced ANR transcription throughout in S1-S3. UFGT was mainly expressed in S4 in control and treated fruit (Figure 7C). At this stage, PDJ was able to decrease transcript content by about 45% while MJ enhanced it by about 40%.

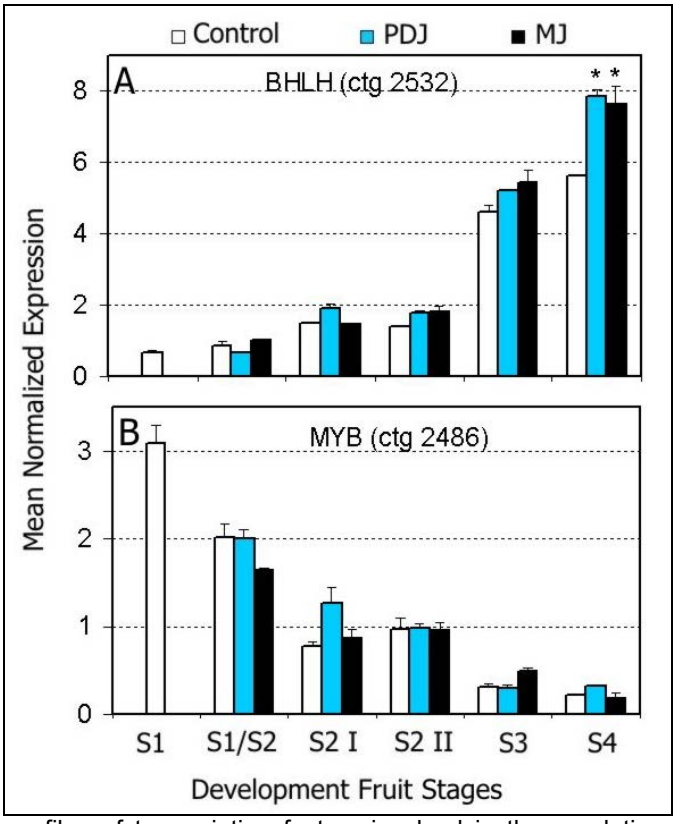

Figure 8. Expression profiles of transcription factors involved in the regulation of biosynthetic genes of flavonoid pathway during the S1-S4 stages of peach mesocarp development and ripening determined and quantified by real time RT-PCR. Fruits were treated or not (Control) with PDJ or MJ under field conditions in S1 stage (59 dAFB). bHLH type (ctg 2532) (A) and MYB type (ctg 2486) (B). Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * P<0.05.

Transcription factors bHLB type (ctg 2532) and MYB type (ctg 2486) were detectable during all fruit development (Figure 8A and 8B, respectively). bHLH displayed an increasing pattern until ripening in control and treated fruit; PDJ and MJ were able to up-regulate bHLH transcript (about 35%) compared with controls only in S4. About the MYB tested here, in controls, it was observed a decreasing pattern of accumulation during fruit development; treatments did not affect this pattern.

5.4 Discussion

5.4.1 JAs enhance phenolic accumulation

In all plant organs phenolic compound accumulation depends on development, abiotic and biotic stresses, as well as agronomic techniques and management applied to crops. When the fruit is close to ripening and harvest, phenolic compounds accumulate in the peel more than in the mesocarp.

Fruit and vegetables rich in anthocyanins (e.g. strawberry, raspberry and red plum) demonstrate the highest antioxidant activities, followed by those rich in flavonones (e.g. orange and grapefruit) and flavonols (e.g. onion, leek, spinach and green cabbage), while the “hydroxycinnamate rich fruits” (e.g. apple, tomato, pear and peach) exhibit lower antioxidant activities (Boudet, 2007). Peach fruit flesh accumulates phenolics especially at early stages of development and their concentration decreases during development (Andreotti et al. 2008). Peach mesocarp is an important source of chlorogenic acid. This hydroxycinnamic acid derivative is considered a powerful antioxidant compound. Present results show that chlorogenic acid, neochlorogenic acid and catechin are the most abundant phenolic compounds in the mesocarp of Stark Red Gold nectarine, confirming results recently reported by Ravaglia (2010). Epicatechin, coumaric acid and caffeic acid were also present but at lower levels during all development.

Under normal growth conditions, in nectarines, the peak of accumulation of phenolic compounds occurs in S2I, relatively early during mesocarp development (Ravaglia, 2010). JAs does not affect phenolic content one day after treatment. In fact the first positive effects are observed 7 days (S2I) and last until 16 days (S2II) after both treatments, while, at ripening, control and JA-treated fruit display a similar phenolic accumulation pattern either monitored by single compound or by total content. Thus, JAs, with the exception of PDJ in some cases, are able to increase the amount of phenols in a developmental stage in which phenols exhibit a peak of accumulation thus maintaining a high phenolic content in a wider window of time during development. In this way fruit could be better protected by the accumulation of these compounds during an early developmental phase; this could have positive reflections in terms of defense on the subsequent fruit growth though total phenolic content and fruit quality traits (fruit weight, SSC and firmness) were unaffected at ripening. JA enhancement of phenolic levels is in line with the contention that they are major elicitors of defence response.

Results also show that early PDJ and MJ applications exert differential effects on phenolic compound accumulation. While in S2I PDJ impair the concentration of almost all

compounds identified, MJ enhance it. In S2II both JAs are able to increase phenolic content and this trend is maintained until S3 only by PDJ. This different behavior, which was previously observed (Ziosi et al. 2008a) could be imputed to the fact that, different from MJ, PDJ is synthetic and is not metabolized to other JAs. PDJ in fact does not affect AOS transcript levels at all (present thesis). On the other hand, MJ can be transformed back to jasmonic acid and re-entry the biosynthetic pathway (Wasternack, 2007).

It is known that JAs interfere with the synthesis of various classes of phenolic compounds. For instance, JAs can enhance anthocyanin content of fruits and vegetables. When treated with JAs, *V. vinifera* cell cultures show a rise in anthocyanin accumulation (Curtin et al. 2003; Zhang et al. 2002), and, in particular, MJ increases anthocyanin amount in apple peel (Rudell et al. 2002; Kondo et al. 2001; Rudell and Mattheis, 2008), *A. thaliana* (Jung, 2004) and peach shoots (Saniewski et al. 1998). At high concentrations JAs enhance peach skin color formation (Janoudi and Flore, 2003). Pre-harvest treatment of *R. idaeus* and *R. occidentalis* L. fruits with MJ enhances anthocyanin, phenolic, anti-oxidant, and flavonoid contents (Wang and Zheng, 2005). MJ-treated strawberry fruit also has greater anthocyanin and phenolic contents after 12 d storage (Ayala-Zavala et al. 2005). Meng et al. (2009), however, report a postharvest reduction by JAs in peach flesh phenolic content.

Some anthocyanins have chemopreventative or other health-related properties and the antioxidant properties of flavonoids are well known (Boudet, 2007). The higher phenolic content of JA-treated nectarines may increase the healthfulness and nutraceutic properties of the product for the benefit of the consumers.

5.4.2 JAs enhance phenolic biosynthetic gene expression

All biosynthetic genes tested display a clear peak of transcript accumulation in S2I in controls, except UFGT. A similar expression pattern was also observed in the mesocarp of Stark Red Gold nectarine by Ravaglia (2010). The expression of anthocyanin biosynthetic genes in peach mesocarp seems to be highly coordinated during fruit development. In peach fruits, glucose and fructose are predominant in the early stages of development; then their content decreases, while sucrose exponentially increases until it becomes the principal carbohydrate in mature fruits (Vizzotto et al. 1996). Since sugars accumulate during fruit development, it is tempting to speculate that sugars modulate the expression of anthocyanin biosynthetic genes. In *Arabidopsis*, about early genes of phenolic pathway, glucose is more effective in increasing PAL, 4CL, CHS, CHI expression while fructose increase C4H and F3'H transcription. Sucrose, but neither glucose nor fructose, increases

the mRNA level of late anthocyanin biosynthetic genes DFR, LDOX, UF3GT and triggers an increased anthocyanin synthesis (Solfanelli et al. 2006) and the expression of grape DFR (Gollop et al. 2002). The up-regulation by MJ of SOT expression (present thesis) could increase sorbitol availability and this reflects in higher glucose/fructose concentration and the latter influence the presently observed phenolic accumulation.

JAs have an immediate effect in modifying gene transcript pattern of phenolic biosynthetic enzymes. In fact, PDJ treatment up-regulates early biosynthetic PAL and CHS genes (one day after treatment). This effect is observed also with MJ but the increase is detected later (7 days after treatment). On late genes in the phenolic biosynthesic pathway, PDJ preferably has a down-regulation effect on transcript accumulation while MJ tend to up-regulate them. This could be due to the transformation of MJ, but not of PDJ, in other active compounds.

UFGT is the sole gene expressed almost exclusively in S4. This gene can be enhanced by several stimuli (Xie and Zheng, 2011). MJ, but not PDJ, up-regulates UFGT in S4 peach mesocarp. Ethylene and ABA are also able to stimulate UFGT accumulation in grape berries (Umphon et al. 2007).

PAL plays a pivotal role in phenolic synthesis and thousands of reports emphasize the correlation between increases in the corresponding PAL gene/protein expression/activity and increases in phenolic compounds in response to different stimuli (reviewed by Boudet, 2007). Induction of PAL and downstream enzymes of the phenylpropanoid pathway is associated with viral-induced necrosis in tobacco and suppression of PAL compromises systemic resistance in tobacco plants infected with tobacco mosaic virus (TMV). The role of PAL in the defense against pathogen attack is well established. Besides protection from different abiotic stresses, stimulation of the phenolic pathway may also have positive reflection on biotic stress resistance (Naoumkina et al. 2008).

Transcription factors (TFs) are involved in the regulation of transcription processes and are classified based on their DNA-binding motifs. In fruit trees as well other plant species, transcription of structural genes of anthocyanin biosynthesis is strictly regulated by complexes of MYB TFs, basic helix-loop-helix (bHLH) TFs and WD-repeat proteins (MYB-bHLH-WD40: MBW). In most studies, MYB action needs bHLH members to increase anthocyanins content in plant (recently reviewed by Xie and Zheng, 2011).

In this work we report the pattern of two different bHLH (ctg 2532) and MYB (ctg 2486) TFs. These two contigs were reported by Trainotti et al. (2006) in the context of an investigation on transcriptome changes during the transition of peach fruit from the preclimateric to climacteric phase. Ctg 2532 is homologous to members of *Arabidopsis*

basic helix-loop-helix (bHLH) family, and in climacteric fruit it is up-regulated while ctg 2486, with a Myb domain, is identified among the down-regulated TFs.

In this work, a differential pattern of TF transcript accumulation is displayed during peach fruit development in accord with previous work (Trainotti et al. 2006). While bHLH expression increases during fruit development, the MYB transcript tested here decreases until ripening. Only bHLH TF is up-regulated by both JAs at ripening (S4). In fact, bHLH is highly represented among the MJ-induced genes (Naoumkina et al. 2008). It is hypothesized that bHLH (ctg 2532) is regulated by both developmental stimuli and JAs in peach fruit.

5.4.3 The pattern of phenolic compounds correlates with biosynthetic gene expression

The single phenolic accumulation pattern display a good correlation with the phenylpropanoid biosynthetic genes tested as observed by Ravaglia (2010). This is the case for coumaric acid/chlorogenic acid/neochlorogenic acid and the expression of PAL, and for catechin/epicatechin and CHS/DFR/LAR/ANR gene expression. LAR and ANR, involved in the last steps to achieve flavan-3-ol compound biosynthesis are differentially affected by JAs. In S2I PDJ impairs both LAR and ANR transcript accumulations while MJ is able to up-regulate ANR expression in agreement with catechin/epicatechin contents.

In peach fruit phenolic content and their biosynthetic genes could be differentially regulated in response to developmental stimuli, as described in grape, apple and bilberry (Xie and Zheng, 2011) but also in response to JAs. In particular, UFGT in peach fruit appears regulated independently of the other flavonoid genes, as observed in grape berry skin, where UFGT represents a key checkpoint to anthocyanin biosynthesis (Kobayashi et al. 2002).

6. Spermidine application to young developing peach fruits leads to a slowing down of ripening by impairing ripening-related ethylene and auxin metabolism and signaling

6.1 Introduction

Although the *Arabidopsis* silique and the tomato berry are regarded as models for dry and fleshy fruits, respectively, peach is now emerging as a model species for the drupe type of fruit. In fact, there is an increasing body of information regarding transcriptome and proteome changes occurring during fruit development and ripening in this species (Trainotti et al. 2003; Trainotti et al. 2006; Trainotti et al. 2007; Bonghi and Trainotti 2006; Ziliotto et al. 2008; Ziosi et al. 2008; Dardick et al. 2010; Bonghi et al. 2011; Ghiani et al. 2011) and, recently, the full genome sequence has been published (Sosinski et al. 2010). With this information and with these tools it has become possible to dissect some of the regulatory mechanisms activated during the ripening process, most of which related to hormones. It is well known that gibberellins, cytokinins and auxins play a major role in the early phases of fruit development, while abscisic acid (ABA) and ethylene are mainly involved in the maturation/ripening phase (Payasi and Sanval 2010; Bonghi et al. 2011). In peach, as in other climacteric fruit, ethylene is considered the main signal in the regulation of ripening. The hormone is required for the onset and progression of this developmental process (Giovannoni et al. 2004); thus, the manipulation of ethylene production and/or action is the main tool for controlling the ripening kinetics. This can be achieved through genetic manipulation, by modulating environmental parameters (e.g. temperature, air composition) during storage, by using specific ethylene antagonists, such as 1-methylcyclopropene (1-MCP), or by supplying plant hormones. The impact of these strategies has been studied at the transcriptional level in tomato (Tiwaria and Paliyath 2011), apple (Costa et al. 2010) and peach (Trainotti et al. 2006; Ziliotto et al. 2008). Results pointed out that genes, including transcription factors, involved in ethylene biosynthesis, perception, and signal transduction were deeply affected by 1-MCP, although in peach, different from tomato and apple, rapid recovery of a fully functioning “ethylene machinery” was observed (Dal Cin et al. 2006; Ziliotto et al. 2008). As regards auxin, exogenous application can stimulate climacteric ethylene synthesis (system 2) through 1-aminocyclopropane carboxylate synthase (ACS) up-regulation (Abel and Teologis 1996; Kondo et al. 2009). The genes involved in auxin biosynthesis, transport,

and signaling that show an increased expression at ripening in peach mesocarp have been identified, and the occurrence of a cross-talk between auxin and ethylene, with genes in the auxin domain regulated by ethylene, and genes in the ethylene domain regulated by auxin, reported (Trainotti et al. 2007)

The anti-senescence biogenic polyamines (PAs) putrescine (Pu), spermidine (Sd) and spermine (Sm) are involved in plant growth processes including embryo development, flowering, fruit set, seed production, fruit ripening, and senescence (Nambeesan et al. 2008). In plants, blocking PA biosynthesis leads to lethal phenotypes (Ge et al. 2006) and over-expression of PA biosynthetic genes in transgenic plants generally leads to an improved stress tolerance (Alcazar et al. 2010). PAs are worthy of note because they may act as cellular signals in the intricate cross-talk between hormonal pathways, including ethylene, auxin and ABA (Alcazar et al. 2010; Cui et al. 2010; Parra-Lobato and Gomez-Jimenez 2011). They share the precursor S-adenosyl methionine (SAM) with ethylene and the fact that PAs and ethylene, with their antithetic roles, may compete for this common precursor has been considered though there is evidence that, actually, this is not the case (Nambeesan et al. 2008). PAs have been repeatedly shown to interfere with fruit set, development and ripening in several species including peach (Mehta et al. 2002; Liu et al. 2006; Torrigiani et al. 2008). In the latter, exogenous applications of Pu, Sd or Sm at late developmental stages (S3, S3/S4) were shown to differentially affect ethylene synthesis and fruit quality traits, and to change the expression pattern of ethylene and PA biosynthetic genes leading to a ripening delay (Bregoli et al. 2002; Torrigiani et al. 2004; Ziosi et al. 2006; Bregoli et al. 2006). Transgenic tomato fruits over-expressing SAM decarboxylase (SAMDC), the rate-limiting enzyme which provides the substrate for Sd/Sm biosynthesis, accumulate the higher PAs, Sd and Sm, produce more lycopene, and exhibit a longer vine life and increased shelf-life, while spermidine synthase (SPDS) over-expressing tomato shows reduced shriveling and delayed decay symptoms (Mehta et al. 2002; Nambeesan et al. 2010). Indeed, transcriptomic, proteomic and metabolic profiling studies have yielded significant information on the cellular processes positively impacted by genetic manipulation of PA synthesis in tomato (Mattoo and Handa 2008). The fact that individual biogenic amines, such as Sd, have a defined action in plant biology, and differentially affect growth and development has also been underlined (Handa and Mattoo 2010).

In order to gain a deeper insight into the molecular basis of the ripening control exerted by PAs, the aim of the present work was to clarify whether, when applied to peach fruit at an early (S1), rather than a late (S3/S4), developmental stage, and under field conditions, the

triamine Sd, the most biologically active PA (Hanfrey et al. 2002; Imai et al. 2004), was still able to influence fruit development and ripening. To this aim, ethylene production and quality traits were measured during ripening, and transcript levels of developmental marker genes as deduced from previous array analyses (Bonghi et al. 2011) were determined in fruit following Sd application. In parallel, the expression profiles of ethylene biosynthesis (ACS1 and ACO1) and perception (ETR1, ETR2) genes, of the ethylene-related transcription factor ERF2, and of cell wall-related genes (PG, PME inhibitor and EXP2) was monitored in controls and Sd-treated fruit until harvest. Moreover, since auxin has been shown to be positively involved in the ripening process, and since PAs may enhance auxin levels (Altman 1989), the effect of Sd treatment on transcript levels of a number of auxin metabolism and signalling genes was also analyzed together with the expression of other hormone-related genes.

6.2 Materials and methods

6.2.1 Plant material and experimental design

The trial was carried out on 8-year old peach (*Prunus persica* var. laevis Gray; cv. 'Stark Red Gold' nectarine) trees (7 per treatment) grown at the experimental farm of the University of Bologna, Italy. Four branches per plant, homogeneous for size and fruit load (3-4 fruits per branch) were selected for the experiments. Branches were sprayed with an aqueous solution of 1 mM Sd (150 mL per branch) as described by Ziosi et al. (2006). Control branches only received distilled water. The double sigmoid growth pattern of the fruit was established on the basis of fruit diameter, and then the four growth stages S1-S4 (characterized by different growth rates by cell expansion) determined (Figure 1) as described by Torrigiani et al. (2004). Sd was applied to young fruit (about 25 mm diameter) 41 days after full bloom (dAFB; late S1). Samples of 10 control and 10 treated fruit were collected at 42 (S1), 47 (S1/S2), 85 (S2/S3), 105 (S3) 115 (S3/S4), 119 (S4I), 125 (S4II, commercial harvest) dAFB, i.e. 1, 6, 44, 64, 74, 78 and 84 days after treatment. Ethylene production and fruit quality traits were determined on the whole fruit starting from 115 dAFB. At each sampling time, mesocarp pieces, excluding the innermost part, from 10 control and Sd-treated fruits were freeze-dried and stored at -80°C for biochemical and molecular analyses.

6.2.2 Ethylene and fruit quality traits determination

Ethylene production was measured by placing the whole detached fruit in a 1-L jar sealed with an air-tight lid equipped with a rubber stopper, and left at room temperature for 1 h. A

10 mL gas sample was taken and injected into a Dani HT 86.01 (Dani, Milan, Italy) packed-gas chromatograph (Bregoli et al. 2002). Flesh firmness (FF) was measured using a pressure tester (EFFE.GI, Ravenna, Italy), and soluble solids concentration (SSC) was measured with an Atago digital refractometer (Optolab, Modena, Italy), as previously described by Bregoli et al. (2002).

6.2.3 HPLC determination of polyamines

Three aliquots (about 100 mg each) of freeze-dried mesocarp pieces from 10 randomly selected control and treated fruit were powdered in liquid nitrogen, extracted in 4% perchloric acid (PCA) and centrifuged at 20,000 x g for 30 min at 4°C. The pellets were washed twice by re-suspension in PCA, re-centrifuged, and resuspended in the original volume of PCA. Aliquots (0.3 mL) of the resuspended pellet (PCA-insoluble fraction) and of the supernatant (PCA-soluble fraction) were subjected to acidic hydrolysis (6 N HCl at 110°C overnight) in order to release PAs from their PCA-insoluble and -soluble conjugates, respectively. Aliquots (0.2 mL) of the supernatant, containing free PAs, and of the hydrolysed supernatant and resuspended pellet were dansylated, extracted in toluene, and analyzed by HPLC as previously described (Torrigiani et al. 2004). PA levels were analyzed in two biological replicates which gave similar results, and only one set of data is shown.

6.2.4 Quantitative qRT-PCR analysis

Total RNA was extracted from freeze-dried mesocarp samples according to Chang et al. (1993). RNA yield and purity were checked by means of UV absorption spectra, whereas RNA integrity was determined by electrophoresis in agarose gel. DNA was removed from 10 µg aliquots of total RNA using the TURBO DNA-free™ (Applied Biosystems, Foster City, CA, USA). The first-strand cDNA was synthesized from 3 µg of the DNaseI-treated RNA by means of the High-Capacity cDNA Kit (Applied Biosystems), using random primers. Real-time RT-PCR was performed in a reaction mixture, final volume 25 µl, containing 9 ng of cDNA, 5 pmol of each primer, and 12.5 µL of the Fast SYBR Green PCR master mix (Applied Biosystems), according to the manufacturer's instructions. The oligonucleotides DZ79 and DZ81 annealing to the Internal Transcribed Spacer (ITS) of the rRNA, were used to amplify the reference gene (Trainotti et al. 2007). The ethylene-, auxin-, cell wall-related and marker genes, and the GA2ox and NCED primer sequences are listed in Appendix S1 in Supporting Information. PCRs were carried out with the StepOnePlus™ 7500 Fast (Applied Biosystems) for 2 min at 95 °C and then for 40 cycles as follows: 95 °C for 15 s, 60 °C for 15 s, and 65 °C for 34 s. The obtained CT values were

analyzed by means of the Q-gene software by averaging three independently calculated normalized expression values for each sample. Expression values are given as the mean±SE of the normalized expression values of the triplicates, calculated according to equation 2 of the Q-gene software (Muller et al. 2002). As CT values have been calculated using the same parameters for all genes, differences in expression values among probes reflect different quantities of target amounts. Quantitative RT-PCR analyses were performed on two biological replicates with similar results. Only one set of data was shown.

6.2.5 Statistical analysis

Data on ethylene production and quality parameters represent the means±SE (n=10) and were analyzed by analysis of variance (ANOVA) procedures using the SAS Statistical Software (SAS Institute, Cary, NC, USA) and were separated, between controls and treatments, and among treatments, using Duncan's multiple range test at the 5% level. For real time RT-PCR (n=3) and PA levels (n=4-6) differences between control and treated samples were compared using Student's t -test.

6.3 Results

6.3.1 Spermidine affects ripening-related parameters, endogenous PA levels, and the expression of developmental marker genes

The fruit growth of 'Stark Red Gold' nectarine was followed from 30 to 125 days after full bloom (dAFB, Figure 1).

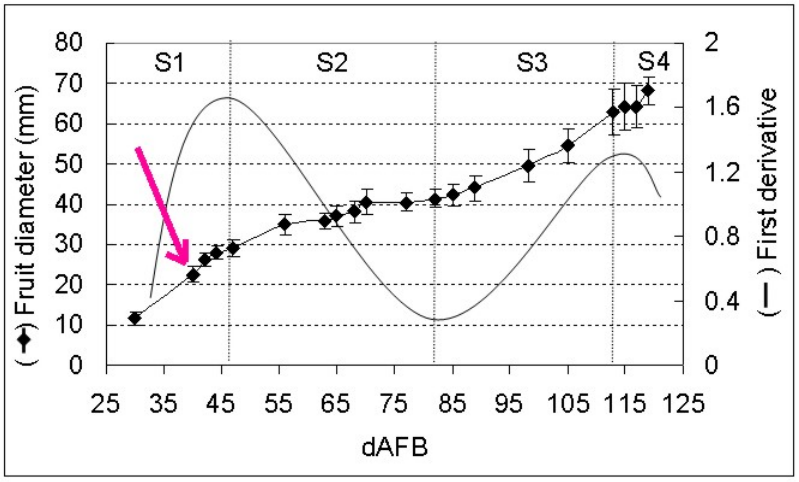

Figure1. Growth curve of 'Stark Red Gold' nectarine based on diameter (filled circles) and its first derivative (continuous line) from 30 to 125 days after full bloom (dAFB). S1– S4 represent the four stages of growth up to harvest. Time of Sd treatment is indicated by a pink arrow. Data represent the means (n=10).

The Sd treatment performed at the S1 developmental stage (41 dAFB) had no significant effects on SSC and nectarine fruit weight (data not shown) throughout development up to the ripening stage. Nevertheless, ethylene production and FF measured prior to and during ripening (S3/S4 up to S4II) revealed a long-term (about three months after treatment) effect of the exogenously applied PA. In both control and Sd-treated fruit, ethylene production became detectable at the S3/S4 transition and increased at later ripening stages (S4I, S4II, Figure 2A); at all three sampling times, Sd significantly reduced ethylene production (by 74, 55 and 59%, respectively) relative to controls. The gradual reduction of FF observed in control fruit was significantly counteracted by Sd; in particular, at commercial harvest (125 dAFB i.e. 84 days after treatment), FF was about twice that of controls (Figure 2B). However, SSC was not significantly affected by treatment with the PA (Figure 2C).

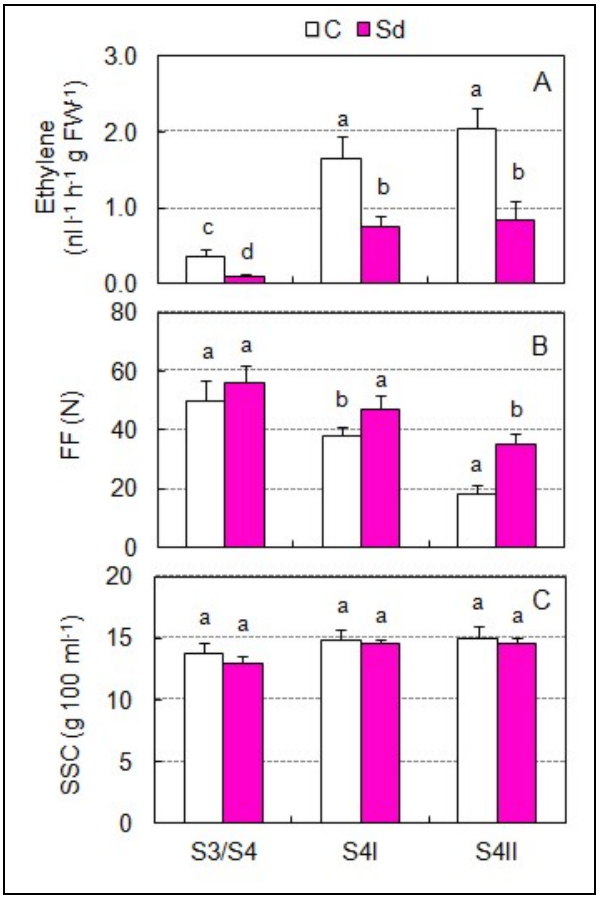

Figure 2. Pattern of ethylene production (A), flesh firmness (FF, B) and soluble solids concentration (SSC, C) in ripening peach fruit treated or not (C) with 1 mM Sd under field conditions in S1 stage (41 dAFB). Data are the mean ± SE (n=10). Different letters indicate significant differences at $P < 0.05$.

Free and conjugated PA levels were determined during fruit development and ripening in control and Sd-treated fruit. In controls, free Pu concentration did not show any particular

trend during the considered period, while free Sd and Sm levels decreased throughout (Table 1).

Table 1. Free polyamine concentration in developing mesocarp of 'Stark Red Gold' nectarine treated or not (control) with spermidine (Sd) 1 mM 41 dAFB (S1) under field conditions. C, control, Pu, putrescine; Sm, spermine. Data are the means ± SD (n=4-6). Asterisks represent significant differences at P<0.05 between control and treated fruit.

Fruit stage	Pu nmol g^{-1} FW		Sd nmol g^{-1} FW		Sm nmol g^{-1} FW	
	C	Sd	C	Sd	C	Sd
S1	64.7±25.0	**166.0±9.12***	107.4±18.4	**140.4±14.6***	16.9±2.12	**27.0±4.74***
S1/S2	88.4±17.1	90.5±7.65	62.8±16.3	79.7±4.57	12.7±3.21	18.1±2.70*
S2/S3	81.5±20.2	69.5±8.95	74.0±8.91	60.7±4.74	14.1±2.93	11.9±4.00
S3	109.2±11.5	125.6±16.4	51.9±8.67	62.0±9.33	6.58±2.68	**11.2±0.23***
S3/S4	71.3±6.23	82.0±9.20	30.6±1.44	40.1±2.50	6.13±1.51	4.50±0.90
S4I	111.4±9.21	**140.4±5.1***	47.9±3.86	**34.1±4.75***	6.2±1.66	4.18±0.89
S4II	75.0±11.0	87.7±12.9	32.9±5.00	**43.6±4.05***	2.50±0.400	**3.35±0.14***

In Sd-treated fruit, free Pu and Sm levels were enhanced (1.3- to 2.6-fold) at day 1 (S1) after treatment, but already after 6 days (S1/S2) they returned to control levels. A significant increase in Pu, Sd and Sm amount (about 1.3-fold) relative to controls was also detected at ripening (S4I or S4II). Soluble conjugated PA content decreased throughout development in control fruit; however, Sd treatment induced a significant increase in Pu (up to 3.4-fold), Sd (up to 1.5-fold) and Sm (about 5-fold) levels starting from S3-S4 (Table 2).

Table 2. PCA-soluble conjugated polyamine concentration in developing mesocarp of 'Stark Red Gold' nectarine treated or not (control) with spermidine (Sd) 1 mM 41 dAFB (S1) under field conditions. C, control, Pu, putrescine; Sm, spermine. Data are the means ± SD (n=4-6). Asterisks represent significant differences at P<0.05 between control and treated fruit.

	Pu nmol g^{-1} FW		Sd nmol g^{-1} FW		Sm nmol g^{-1} FW	
Stage	C	Sd	C	Sd	C	Sd
S1	230.4±19.7	217.7±29.9	337.4±40.1	349.0±4.99	64.8±8.09	54.4±7.57
S1/S2	162.0±8.34	**120.9±5.23***	302.7±20.2	250.1±12.8	40.4±4.68	33.8±2.99
S2/S3	199.8±25.1	**141.6±16.2***	189.9±14.2	217.3±33.2	24.0±1.44	29.0±0.27
S3	109.5±2046	104.7±3.44	163.6±9.87	149.4±2.40	9.87±1.03	7.97±3.30
S3/S4	75.6±9.04	**150.5±10.2***	81.6±12.2	**110.2±5.0***	7.41±1.26	8.31±2.00
S4I	134.5±13.7	**273.9±3.19***	91.2±4.51	**128.5±14.2***	2.92±0.12	**15.1±1.83***
S4II	70.2±11.0	**241.4±20.1***	65.3±4.33	**100.4±4.34***	1.20±0.21	**6.01±1.37***

In control fruit, insoluble conjugated PA content, which was lower than that of the other forms, also decreased from S1 up to harvest; in Sd-treated fruit a modest but significant

accumulation of insoluble conjugated Pu, Sd and Sm (35, 27 and 38%, respectively) was observed after one day and at S4II for Sd (1.6-fold; Table 3).

Table 3. PCA-insoluble conjugated polyamine concentration in developing mesocarp of 'Stark Red Gold' nectarine treated or not (control) with spermidine (Sd) 1 mM 41 dAFB (S1) under field conditions. C, control, Pu, putrescine; Sm, spermine. Data are the means ± SD (n=4-6). Asterisks represent significant differences at P<0.05 between control and treated fruit.

	Pu nmol g^{-1} FW		Sd nmol g^{-1} FW		Sm nmol g^{-1} FW	
Stage	C	Sd	C	Sd	C	Sd
S1	25.6±1.67	**39.5±3.53***	29.5±6.78	**40.8±0.46***	4.24±0.40	**6.73±0.17***
S1/S2	39.8±8.72	35.6±0.61	34.1±1.77	32.4±1.97	6.49±0.23	6.10±0.64
S2/S3	30.1±3.91	25.2±1.07	23.4±4.90	18.1±3.62	3.77±0.55	2.99±0.13
S3	19.6±1.34	20.5±1.45	11.8±0.52	15.6±0.37	1.77±0.30	2.28±0.41
S3/S4	16.1±1.38	18.2±0.25	10.8±1.72	12.4±0.30	2.07±0.33	1.80±0.40
S4I	16.2±1.56	20.1±2.83	8.57±2.64	11.8±2.81	0	0
S4II	12.4±0.30	14.1±1.16	3.50±0.45	**5.71±0.79***	0	0

To assess at the molecular level the effects of the Sd treatment on the progression of fruit development, the expression of genes either known to be modulated by PAs (Stes et al. 2011) or previously identified as developmental markers (Bonghi et al. 2011) were evaluated (Figure 3).

A cyclin D3 gene (CYCD3, ctg779), whose maximum expression occurred at the S1/S2 transition, was transiently up-regulated one day after treatment (Figure 3A); thereafter, its mRNA level was comparable to that of control fruit. Genes coding for a senescence protein (ctg1823) and for an Aux/IAA protein (ctg57) were used as S3 and S4 markers, respectively.

The peak in ctg1823 expression was delayed from S3/S4 to S4I in Sd-treated fruit (Figure 3B). Similarly, the increase in expression of ctg57 was also slowed down (Figure 3C), with levels found at S4II in treated samples being comparable to those measured at S4I in controls.

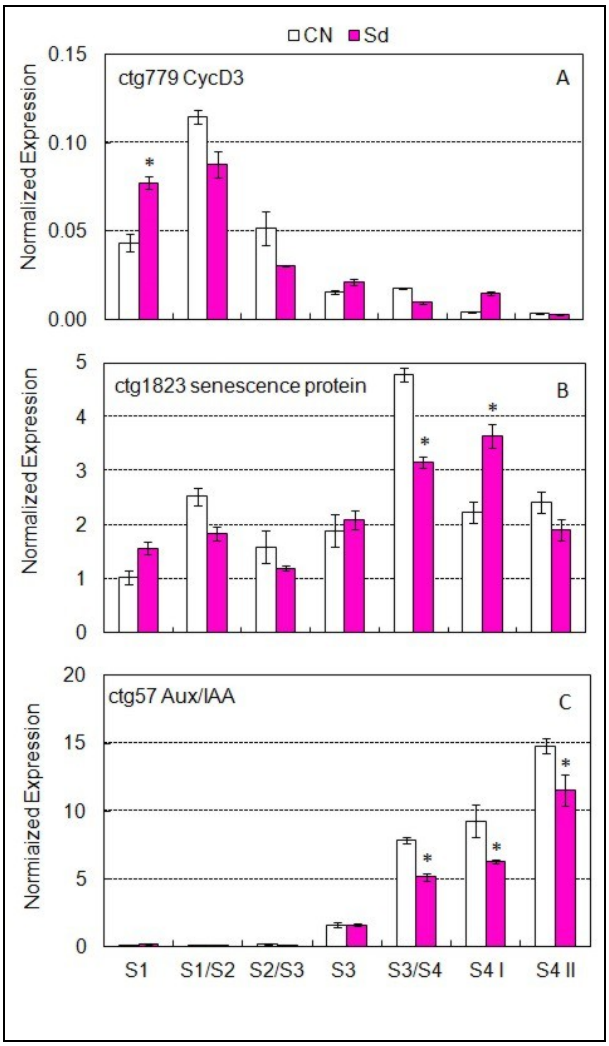

Figure 3. Expression profiling by real-time RT-PCR of CycD3 (A), senescence protein (B) and Aux/IAA (C) genes during peach fruit development and ripening in Sd-treated (Sd) and control (C) fruit. Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using Student's t test; * P<0.05.

6.3.2 Ethylene- and cell-wall-related gene expression is strongly affected by Sd

Taking into account that early Sd application impairs climacteric ethylene evolution (Figure 2A), the expression profile of the main genes involved in ethylene biosynthesis and signaling was analyzed during fruit growth. ACS1 and ACO1 transcripts began to accumulate at the S3/S4 transition, concomitant with the onset of ethylene production, and their amount increased abruptly during S4 (Figure 4A, B).

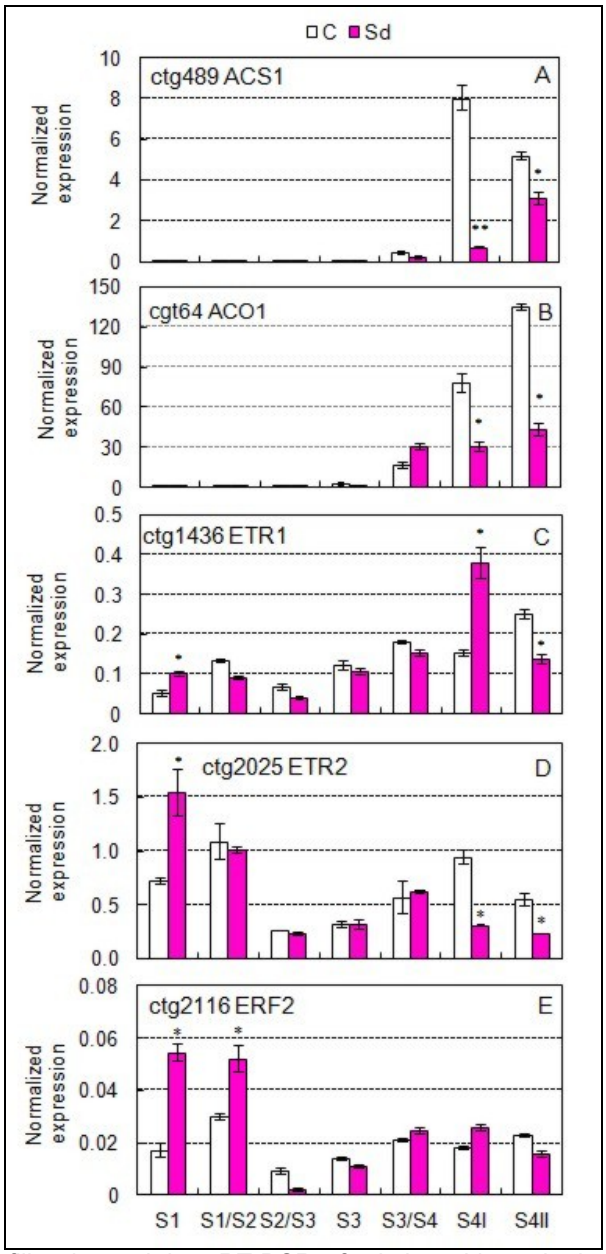

Figure 4. Expression profiling by real-time RT-PCR of ethylene biosynthetic and signaling genes during peach fruit development and ripening in Sd-treated (Sd) and control (C) fruit. *ACS1* (A), *ACO1* (B), *ETR1* (C), *ETR2* (D) and *ERF2* (E). Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using Student's t test; * P<0.05, ** P<0.01.

In Sd-treated fruit this increase was strongly counteracted, with transcript levels reduced by as much as 90% for *ACS1* and 70% for *ACO1* relative to controls at S4I and S4II, respectively.

The expression of ethylene receptor genes ETR1 and ETR2 was differentially affected by Sd treatment (Figure 4C, D). While ETR1 expression was almost constitutive, thus confirming previous data (Rasori et al. 2002; Trainotti et al. 2007), and stimulated by Sd

treatment in S1 and S4I, ETR2 transcript amount was enhanced in Sd-treated fruit soon after application (S1) but down-regulated in S4. Similar to ETR2, ERF2 (Ethylene Response Factor 2) was also up-regulated by Sd, but only on days 1 and 6 after the treatment (Figure 4E).

Given the strong effect on firmness retention exerted by Sd treatment (Figure 2B), it was reasonable to assume that the expression of some cell wall-related genes, such as those encoding for an endo-polygalacturonase (PG), a pectin-methylesterase (PME) inhibitor (PMEI) and an expansin (EXP2) (Brummel et al. 2004), might also be modulated by the PA. As expected, the increase in PG gene expression (ctg420), encoding the main enzyme responsible for textural changes and flesh softening in melting peaches (Trainotti et al. 2003; Ghiani et al. 2011), was dramatically inhibited by Sd treatment (Figure 5A). Similarly, the ripening-induced accumulation of ctg938 transcripts, a gene coding for a PMEI, was also strongly reduced in Sd-treated S4 fruit (Figure 5B). On the contrary, the rise in expression of the EXP2 gene (ctg941), occurring in the pre-climacteric phase (i.e. the S3/S4 transition), was delayed (to S4I) and strongly enhanced by the Sd treatment (Figure 5C).

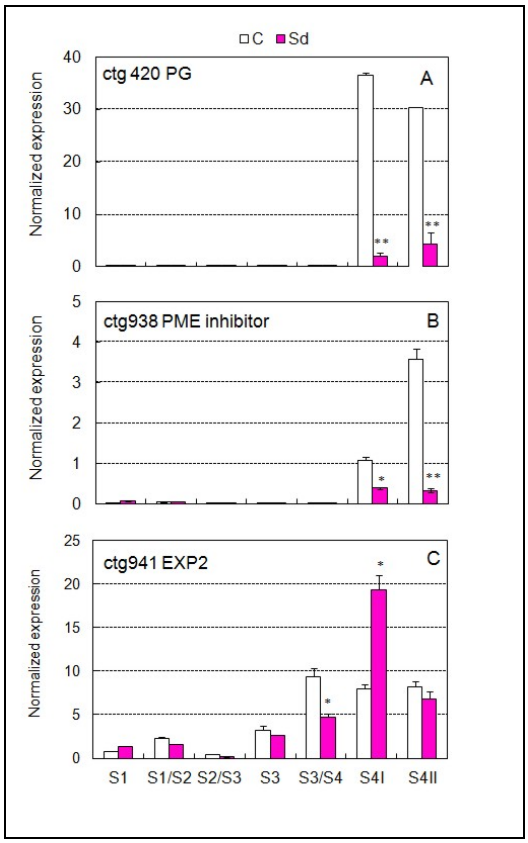

Figure 5. Expression profiling by real-time RT-PCR of cell wall-related genes during peach fruit development and ripening in Sd-treated (Sd) and control (C) fruit. PG (A), PMEI (B) and EXP2 (C). Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using Student's t test; * P<0.05, ** P<0.01.

6.3.3 The expression of several hormone-related genes is altered by Sd

Some genes involved in auxin synthesis, conjugation or signalling, which have been reported to be ripening-associated and whose expression increases concomitant with climacteric ethylene production (Trainotti et al. 2007), were analyzed. The expression pattern of two genes involved in early steps of IAA biosynthesis, the first encoding a tryptophan synthase β subunit (TRPB, ctg3371), and the second an indol-3-glycerol phosphate synthase (IGPS, ctg3575), involved in the tryptophan-dependent and independent IAA biosynthetic pathway, respectively (Woodward and Bartel 2005), revealed that a clear inductive effect by Sd occurred only for TRPB one day after treatment (Figure 6A, B).

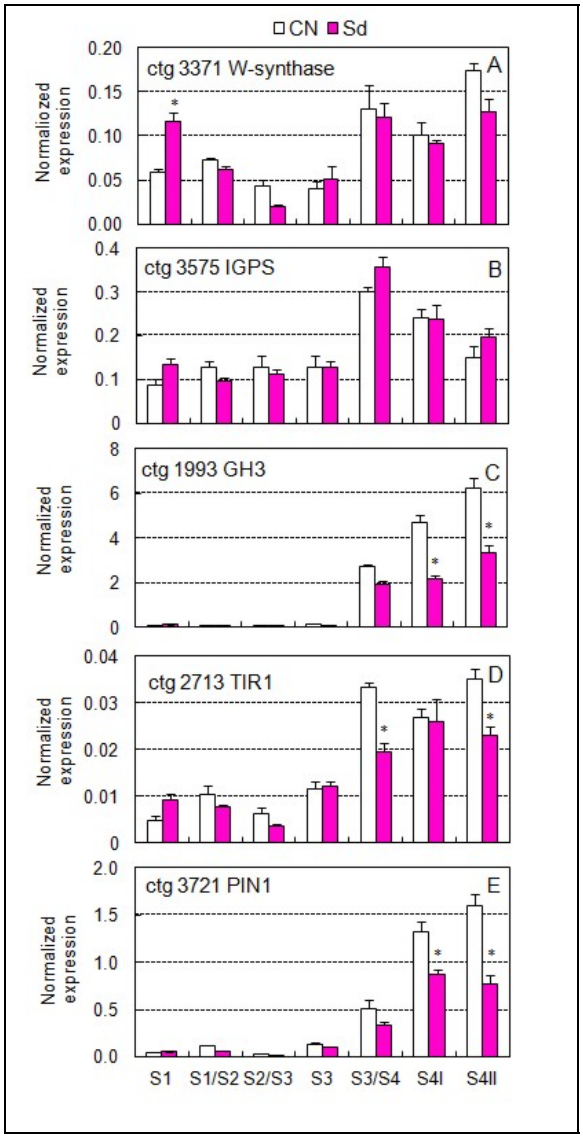

Figure 6. Expression profiling by real-time RT-PCR of auxin metabolism genes during peach fruit development and ripening in Sd-treated (Sd) and control (C) fruit. Wsynth (A), IGPS (B), TIR1 (C), GH3 (D) and PIN1 (E). Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using Student's t test; * $P<0.05$.

The effect of the PA on the accumulation of GH3 (IAA-amino acid synthase; ctg1993) and PIN1 (ctg3721) transcripts was an increasing inhibition at ripening (Figure 6 C, E); the former codes for a member of a gene family that conjugates amino acids to IAA and the latter for an auxin efflux facilitator, and both are known to display a ripening-related expression pattern (Trainotti et al. 2007; Bonghi et al. 2011). Sd application also inhibited the accumulation of a TIR1 (transport inhibitor response, ctg2713) mRNA coding for an auxin receptor (Dharmasiri et al. 2005) at S3/S4 and S4II relative to controls (Figure 6D).
Other hormone-related genes, shown to be highly expressed during fruit ripening (Bonghi et al. 2011), were also examined. The transcript levels of GA2 oxidase (GA2-ox, ctg544), a gene encoding the major gibberellin (GA) catabolic enzyme, dramatically increased at ripening, and this increase was strongly down-regulated by Sd (Figure 7A). Similarly, the gene encoding a 9-*cis*-epoxycarotenoid dioxygenase (NCED, ctg2980), the key enzyme in the ABA biosynthetic pathway, was strongly down-regulated by Sd at ripening (Figure 7B).

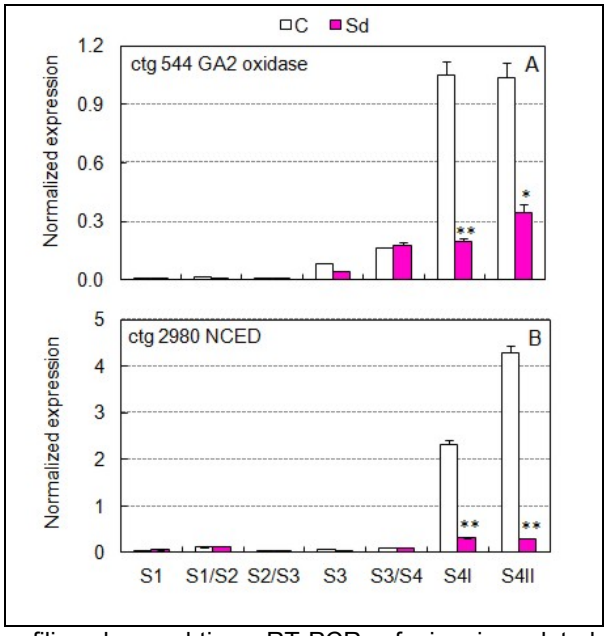

Figure 7. Expression profiling by real-time RT-PCR of ripening-related genes during peach fruit development and ripening in Sd-treated (Sd) and control (C) fruit. GA2-ox (A), NCED (B). Data are the mean ± SE of three replicates. Statistical significance between treatments and related controls was analyzed using Student's t test; * P<0.05, ** P<0.01.

6.4 Discussion

Present results show that exogenous Sd, applied at an early stage of fruit development (41 dAFB, late S1), slowed down ripening as revealed by agronomical parameters and changes in the expression of developmental marker and ripening-related genes. Sd effects were detectable in two distinct phases: very soon after treatment (one day) as well

as later, i.e. at ripening (S4I-S4II). Short-term (one day after treatment) and long-term (74-84 days after treatment) effects will be discussed separately.

6.4.1 Short-term effects of spermidine treatment

Noticeable changes in free and conjugated PA concentrations accompany fruit development and ripening in peach as well as in other fruit, and have been previously discussed (Kushad 1998; Bregoli et al. 2002; Ziosi et al. 2003; Liu et al. 2006; Ziosi et al. 2006; Nambeesan et al. 2010). In particular, free PA levels decrease in the course of fruit growth/development, insofar as the highest concentrations are associated to the cell divisions characterizing early fruit growth, and still present in S1 (Zanchin et al. 1994); conversely, the lowest concentrations are found at the ripening stage. Present results show that Sd application led to free Pu and Sm accumulation relative to controls one day after treatment; Sd did not accumulate significantly since its accumulation is tightly regulated (Hanfrey et al. 2002), as confirmed by the fact that in tomato over-expressing Sd synthase (SPDS) Sd levels at most doubled (Neily et al. 2011). Apparently, in Sd-treated nectarines, the excess Sd was converted to Sm or back to Pu; back conversion of Sd to Pu has been already described in plants and it is probably due to the action of a polyamine oxidase, as reported in *Arabidopsis* (Moschou et al. 2008). In Sd-treated fruit, in the short term, part of the over-accumulated free PAs were also converted to the insoluble conjugated forms (about 30-40% increased levels) possibly as part of a mechanism aimed at regulating their concentration (Edreva et al. 2007). The putative 'rejuvenating' effect of Sd is presently attested by the fact that the expression of a gene involved in cell division (i.e. CYCD3) was early enhanced while that of genes associated to late phases of fruit development and ripening, e.g. those encoding a senescence protein (ctg1823) and an Aux/IAA protein (ctg57; Bonghi et al. 2011), was delayed. There is some evidence indeed that PAs are able to induce the expression of cyclin genes and thus to prolong the duration of the cell cycle by maintaining cells in S1 and G2 phases (Jang et al. 2006); in particular Pu, whose amount was almost 3-fold higher in Sd-treated fruit, has been shown to enhance CYCD3 expression in *Arabidopsis* (Stes et al. 2011). This early effect of Sd could be sufficient to slow down the progression of development and, consequently, ripening, as demonstrated by the delayed accumulation of ctg1823 and ctg57 transcripts, two genes indicated as markers for the S3 and S4 stages of peach fruit growth, respectively (Bonghi et al. 2011). Increases in Sd and Sm content have been positively associated with anabolic processes in Sd-overproducing engineered tomato (Mattoo et al. 2009; Handa and Mattoo, 2010; Nambeesan et al. 2010). Moreover, in

tomato over-expressing a yeast SAMDC, that also accumulated Sd, a stimulation of metabolic activity, a revival in the cellular programme, and increased energy and glucose metabolism were observed in fully ripe fruit (Mattoo et al. 2006; Mattoo and Handa 2008). These findings lend support to the hypothesis that in Sd-treated nectarine differentiation processes and ripening are slowed down due to a 'rejuvenating' effect exerted by the PA.
Another early effect of Sd is the enhancement of the expression of ETR1/ETR2 and ERF2 genes involved in the perception and signal transduction of ethylene, respectively. This does not seem to depend on the up-regulation of ACS1 and ACO1 which, instead occurs at late stages of development (S4), but could be a direct effect of Sd treatment on ethylene signaling. Furthermore, since the expression of ETR2 and ERF2 in peach fruit is induced by auxin (Trainotti et al. 2007), their up-regulation might also be the result of the modulation of auxin metabolism due to the early increase in free PA amount (Cui et al. 2010). In fact, a gene involved in auxin biosynthesis (TRPB; Figure 6A) was also significantly induced by Sd one day after the treatment, thus confirming previous results concerning the positive effect of exogenously applied PAs on auxin levels (Altman 1989). In the SAMDC4-defective *bud2* mutant of *Arabidopsis*, decreased Sd and Sm levels are associated to a bushy and dwarf phenotype (Ge et al. 2006). The mutant also displays defective auxin signaling (hyposensitivity to auxin), as well as altered cytokinin homeostasis and hypersensitivity (Cui et al. 2010) which might be responsible for the CycD3 up-regulation.

6.4.2 Long-term effects of spermidine treatment

In agreement with previous results obtained with late (from S3 onwards) PA treatments (Bregoli et al. 2002; Torrigiani et al. 2004; Ziosi et al. 2006), the most striking effect of Sd observed at ripening, besides an increment in free and conjugated PA levels, was the reduction of ethylene evolution, coherent with its reduced biosynthetic gene expression. This resulted in firmness retention, but no fresh weight loss, indicating that field Sd treatment, even when performed at early developmental stages (S1), influences fruit ripening/softening mainly by affecting ethylene production. The fact that fruit fresh weight was not affected suggests that, when ripe, Sd-treated fruit could be heavier than controls. Late PA treatments (S3/S4) exerted a stronger effect than presently observed on the extent of ethylene emission, but a comparable effect on FF (Torrigiani et al. 2004; Bregoli 2006; Ziosi et al. 2006). Previous work also showed that late Sd application *in planta* or *in vitro* led to a reduction of ACS1 and/or ACO1 transcript amounts, and thus an active role for this PA in modulating ethylene biosynthesis, and, therefore, climacteric fruit ripening is

confirmed. The presently observed reduction in ethylene evolution in Sd-treated nectarines caused, among others, the down-regulation of the ethylene-sensitive ETR2 gene, the most abundant ethylene-sensitive receptor in ripe peaches (Trainotti et al. 2006; 2007), which is in accord with reduced ethylene production. In tomato plants over-expressing a yeast SAMDC causing Sd and Sm accumulation, the observed slowing-down of ripening and better quality traits did not depend on ethylene reduction, which did not occur, but on the presence of higher (Sd and Sm) PA levels (Mehta et al. 2002).

Firmness retention in Sd-treated fruit was most likely caused by the reduced expression of genes coding for cell-wall modifying enzymes and responsible for fruit softening. The role of ethylene-regulated genes involved in cell wall metabolism, such as a PG and a PMEI, and their relation to ethylene evolution has been extensively studied during peach ripening (Trainotti et al. 2003; González-Agüero et al. 2008; Ghiani et al. 2011). Present results show that the expression of both these genes was strongly reduced by Sd. Although a direct role for PAs in counteracting softening by binding to cell wall anionic charges was proposed in apple and plum (Kramer et al. 1991; Valero et al. 2002), present data show that PCA-insoluble conjugated PAs (mostly cell wall-bound; Edreva et al. 2007) did not accumulate at ripening in Sd-treated fruit relative to controls (Table 3). Therefore, firmness retention/softening delay is more likely associated to a general developmental slowing down induced early by Sd, which leads to a less ripe fruit. This hypothesis is also supported by the positive effect exerted by Sd on EXP2 expression. In fact, in ripe peaches, EXP2 is negatively regulated by ethylene, as demonstrated by the application of the hormone (Trainotti et al. 2007) and by the inductive effects exerted by 1-MCP, an inhibitor of ethylene action (Ziliotto et al. 2008).

Present results indicate that, in peach, a reduction of ethylene production was not the only effect on hormone-related genes associated with ripening. In particular, GH3, TIR1 and PIN1, genes involved in auxin conjugation, perception and transport, respectively, were down-regulated in Sd-treated fruit (Figure 6). In control fruit, the expression of GH3, a member of a gene family that conjugates amino acids to IAA (Staswick et al. 2005) and likely serves to dampen the auxin signal by inactivating IAA via conjugation, increased sharply at the S3/S4 transition, and even more at S4, when it was significantly down-regulated by Sd. The fact that this gene is positively regulated by auxin and is considered a marker for free auxin concentration (Trainotti et al. 2007) lends support to the hypothesis that the increase in auxin levels that occurs in ripening peach (Masia et al. 1992; Miller et al. 1987) was counteracted/delayed in Sd-treated fruit. GH3 up-regulation at ripening may, therefore, occur in response to increased auxin levels, possibly occurring during this

developmental phase. Conversely, the lower GH3 transcript levels observed in Sd-treated fruit relative to controls may be possibly related to a lower amount of free IAA. Similarly, PIN1 transcript amounts, very low at S1-S3, increased noticeably during ripening in agreement with previous reports (Trainotti et al. 2007). PIN1 is a member of a multigene family that encodes transmembrane auxin efflux facilitator proteins involved in polar auxin efflux (Paponov et al. 2005). In treated fruit, PIN1 was significantly down-regulated by Sd, possibly because of a decreased level of ethylene; in fact, PIN1 expression is known to be positively regulated by ethylene (Trainotti et al. 2007). Although TIR1 did not respond to auxin (Trainotti et al. 2007) it was also down-regulated at ripening possibly in relation with the altered auxin metabolism.

The expression of two additional ripening-related genes (a GA2-ox and a NCED) involving the actions of two other hormones, namely gibberellins and ABA, was strongly reduced in Sd-treated fruits, thus supporting the notion of a general slowing down of ripening initiation. The up-regulation of GA2-ox in control fruit during ripening may correlate with decreasing gibberellin levels (Srivastava and Handa 2007) which are known inhibitors of ripening processes (Payasi and Sanwal 2008); in Sd-treated fruit GA2-ox down-regulation may lead to a delay in gibberellin catabolism thus maintaining the inhibiting effect of gibberellins on the ripening process. Three NCED genes were cloned from peach and grape fruits. Their transcripts have been described to increase abruptly at ripening in climacteric and non climacteric fruit, and the potential contribution of ABA to the induction of fruit ripening was analyzed in relation to ethylene in both species (Zhang et al. 2009a, b). PpNCED1 (corresponding to ctg2980) and VvNCED1 initiated ABA biosynthesis at the beginning of fruit ripening in peach and grape, respectively, and it was proposed that ABA accumulation preceded the climacteric increase in ethylene production, as exogenous ABA stimulated ethylene production and accelerated fruit ripening (Zhang et al. 2009a, b). Besides being ripening-induced, both ctg544 and ctg2980 are induced by exogenous ethylene (Trainotti et al. 2007) and repressed by 1-MCP (Ziliotto et al. 2008) thus making them probable targets of ethylene. Together, these results indicate that, during fruit ripening the hormonal interplay is rather complex, and confirm that the initiation of the ripening process requires several coordinated hormonal stimuli.

7. Brassinosteroid application to peach fruits at different physiological stages interferes with fruit quality and ripening. Preliminary results.

7.1 Introduction

Brassinosteroids (BRs) have recently been recognized as a new class of phytohormone occurring ubiquitously in the plant kingdom (Clouse and Sasse, 1998). Extensive research over the past two decades has revealed that BRs are essential for normal plant development and regulate a range of physiological processes, such as stem elongation, root growth, vascular differentiation, leaf epinasty and reproductive development (recently reviewed by Clouse, 2011). The potential of BRs to enhance disease resistance of plants has also been investigated (Zhu et al. 2010). Plant ovaries undergo cell division, which is regulated by cyclins (Inzé and De Veylder, 2006), cell expansion, and ripening stages to form fleshy fruits. It has been observed that, besides cytokinins, brassinosteroids (BRs) could increase cell division by increasing CycD3 transcript levels in *Arabidopsis* cell suspension (Hu et al. 2000). Recently, several studies have showed that biosynthesis of BRs is enhanced in the developing seeds or fruits of tomato, pea and Arabidopsis (Nomura et al. 2007 and refs therein). The application of BRs can also accelerate the ripening of tomato and grape fruits (Vardhini and Rao, 2002; Symons et al. 2006). Moreover, exogenous BRs can increase fruit set in a number of crop species (Kamuro and Takatsuto, 1999) and the expression of several cell cycle-related genes, such as cyclins in cucumber (Fu et al. 2008).

Peach is a good model plant to study fruit growth and ripening since much information is now available both on fruit development and ripening (Bonghi et al. 2011 and refs therein). To understand the role of BRs in fruit development and ripening, a preliminary attempt was made to manipulate the BR levels in nectarines, late ripening cv. Fantasia, through the application of exogenous BRs at an early (S1) and a late (S3) fruit developmental stage. The effects of altered BR levels on fruit growth, fruit quality traits and extent of ripeness were subsequently investigated.

7.2 Materials and Methods

7.2.1 Plant material and experimental design

The trial was carried out on 7-years old Flaminia peaches (*Prunus persica* var laevis Gray) trees (3 trees per treatment) grafted on GF677 rootstock (*Prunus persica* x *Prunus*

amigdalus), grown in a private farm, Faenza, Italy. Four branches per plant, homogeneous for size and fruit load (3-4 fruits per branch) were selected for the experiments. For each treatment, 16 branches were sprayed with 24-epibrassinolide (EB; 90% purity; Cat.N. E1641; Sigma-Aldrich). The compound was first diluited in ethanol 100% and after dissolved in an aqueous solution. The complete tree was sprayed. Control branches only received an aqueous solution. The growth curve of Flaminia peach fruit was established in the four typical stages (Figure 1). BR were applied in late S1 and S3; in S1 0.1 mM EB was applied once (1x) or three-times (3x) at 1 week distance both applied 56 dAFfB; in S3 0.5 mM EB was applied twice (2x) at 1 week distance (first application 121 dAFB). Samples of 25 control and 25 treated fruit were collected at commercial ripening (157 dAFB), flesh firmness about 50 N. At ripening, the Index of Absorbance Difference (I_{AD}, Ziosi et al. 2008b) and quality traits were determined on the whole fruit.

7.2.2 Ethylene and fruit quality traits determination

The index of difference of absorbance (I_{AD}; Ziosi et al. 2008b) was measured in one hundred fruit. Flesh firmness (FF) was measured using a pressure tester (EFFE.GI, Ravenna, Italy), and soluble solids concentration (SSC) was measured with an Atago digital refractometer (Optolab, Modena, Italy), as previously described by Bregoli et al. (2002). Total acidity (TA) was determined on 20 ml of flesh juice (titration with 0.25N NaOH) by a semiautomatic instrument (Compact-S Titrator, Crison, Modena, Italy).

7.3 Results and Discussion

Fruit were treated with 24-epibrassinolide in S1, once or three times, and in S3, twice.

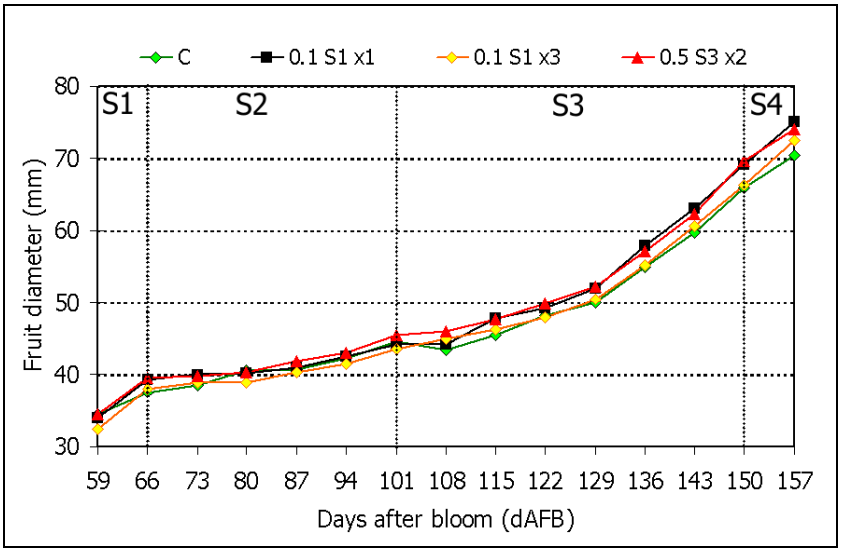

Figure 1. Growth curve of Flaminia peach based on diameter from 59 to 157 days after full bloom (dAFB). Data were collected in 2010 and represent the means of 3 measurements in 3 different trees.

The fruit growth curve is showed in Figure 1. No differences in fruit diameter at harvest was detected between treated and control fruit.

The quality parameters measured at harvest showed control and treated fruit were most abundant in the 75-mm class and, in the latter, JA-treated fruit were more abundant Figure 2A). However fruit quality traits, FF (about 20 N), SSC (about 9°Brix) and TA (about 7 g L^{-1}), were not affected at all by BR (Figure 2B).

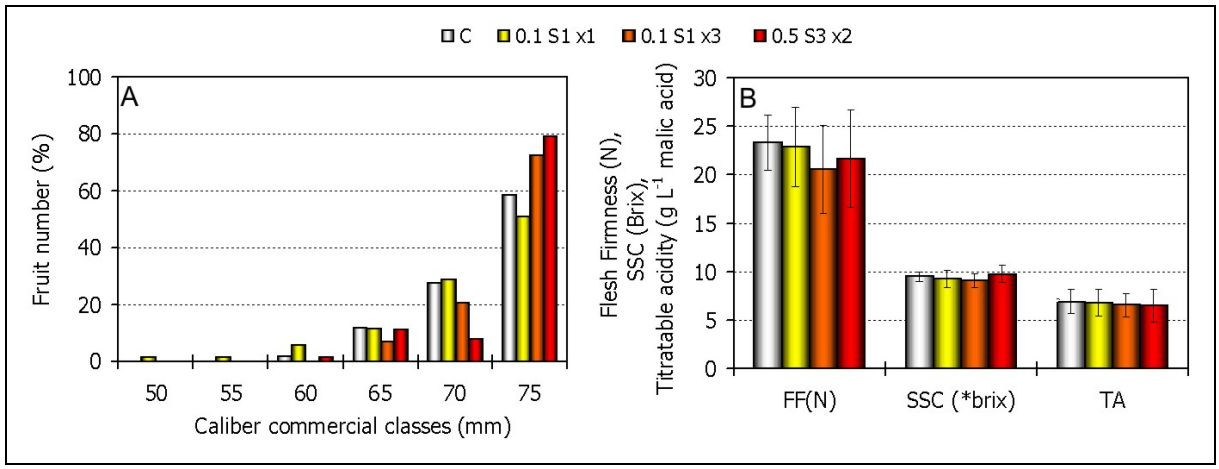

Figure 2. Effects of different 24-Epibrassinolide treatments and application date relative to control, on the frequency of fruit by caliber commercial classes (A) and quality traits of ripen fruit (B) measured at 157 days after bloom. Data are the mean ± SD of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * P<0.05.

There was a slight but not statistically significant increase in fruit weight (Figure 3A) by about 15 g in the treated fruit. The I_{AD} showed that among ripe fruit (I_{AD} 0.8), those treated with JAs were less abundant except for the single treatment in S1 (Figure 3B).

In cucumber (*Cucumis sativus* L.) parthenocarpic growth was induced by exogenous BRs but inhibited by the inhibition of BR synthesis. BRs triggered active cell division associated with increased transcripts of cell cycle-related genes, especially that of cyclin D3 genes indicating that BRs play a regulatory role in early fruit development of cucumber plants (Fu et al. 2008).

Some data suggest that exogenous BRs may promote ripening (via increases in ethylene levels) in tomato, a climacteric fruit, and also in non-climateric fruit like grape. Application of 28-homobrassinolide and 24-epibrassinolide to pericarp discs of tomato resulted in elevated levels of lycopene and lowered chlorophyll levels. In addition brassinosteroid-treated pericarp discs exhibited decreased ascorbic acid and increased carbohydrate contents. Fruit ripening as induced by brassinosteroids was associated with increase in ethylene production revealing the ability of brassinosteroids in accelerating fruit senescence (Vardhini and Rao, 2002). Pattern of gene expression and plant hormone

levels throughout grape berry development indicates that BR levels may influence the process of berry ripening (Symons et al. 2006). Furthermore the manipulation of BR levels via the application of exogenous BR and a BR biosynthesis inhibitor can significantly promote or delay berry ripening.

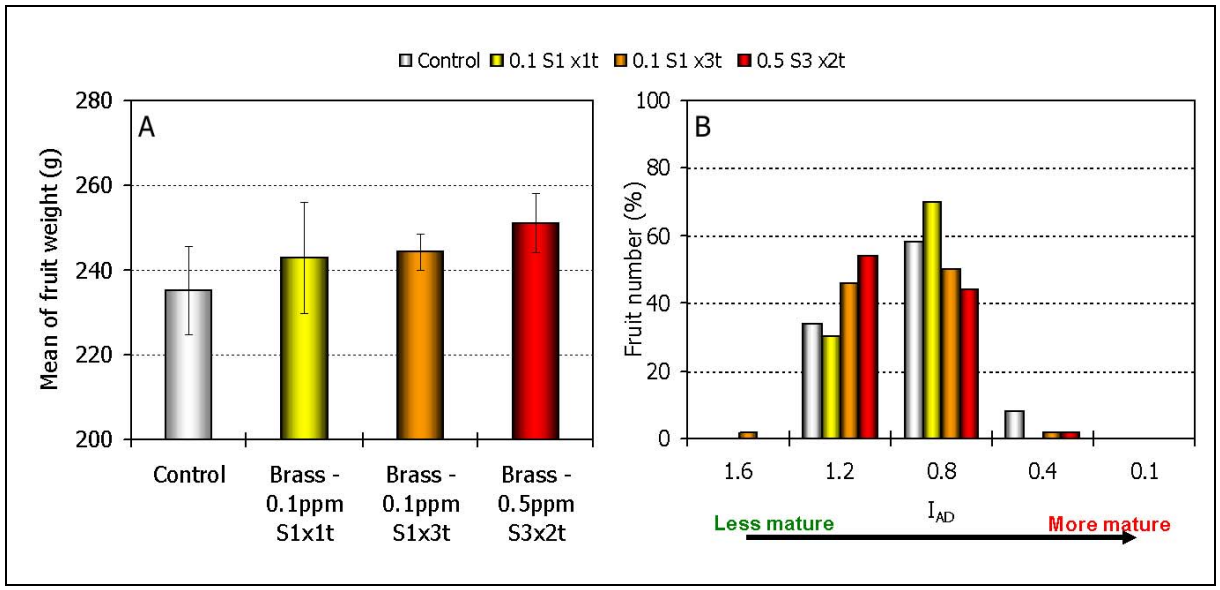

Figure 3. Effects of different 24-Epibrassinolide treatment and application date relative to control, on the mean of fruit weight (A) and distribution of ripen fruit measured with the DA-meter (I_{AD}) (B) at 157 days after bloom. Data are the mean ± SD of three replicates. Statistical significance between treatments and related controls was analyzed using GLM with Tukey as posthoc test; * P<0.05.

Present preliminary results suggest that BR application leads to larger and apparently less ripe nectarines with quality traits comparable with those of controls. This promises a reliable tool in the control of peach fruit ripening.

Conclusions

Present results indicate that the natural plant growth regulators, JAs and Sd, though applied very early during fruit development (late S1), are able to profoundly interfere with seed and/or mesocarp development, ultimately leading to a slowing down of ripening. In JA-treated fruit crucial ripening-related genes are down-regulated in agreement with the I_{AD} and thus the inhibition of ethylene production. This is associated with changes in cell wall architecture and sugar accumulation, cross-talk with other hormones, ABA and IAA, and with defence-related gene expression. Due to a shift ahead in gene expression, most of these genes point to a slowing down of fruit development and thus of ripening. In the seed this delay is particularly evident for the marker genes PRU and LEA. The early changes (one day after application) in gene expression, in treated seed and mesocarp, suggest that JAs are quickly translocated to the seed through the mesocarp. Although MJ and PDJ differentially influence gene expression and phenol accumulation, their effects persist until harvest, about three months later, leading to less ripe fruit and probably less mature seed. Thus, JA/ethylene cross talk occurs both in the mesocarp and seed.

Equally Sd application to young fruit led to a slowing down of ripening as revealed by quality traits. The early increase in free PA levels may prolong the fruit juvenile phase, being the up-regulation of genes involved in cell cycle control (*CYCD3*) and auxin biosynthesis (*TRPB*) either a consequence of the effect or part of it. Later on, when ripening proper starts, fruits still show the effect of the early Sd treatment and a long-term response (ripening delay) is observed. The ripening slowing down in Sd-treated fruit is revealed by the impaired expression of genes known to be good marker for the S3 (ctg1823) and S4 (ctg57) stages, but also of those involved in ethylene biosynthesis and perception, and in cell wall metabolism. Ripening-related IAA metabolism is also substantially influenced by Sd in agreement with the ripening slowing down and evidence for a cross-talk with GA and ABA also occurs. This further supports the contention that PAs and JAs have the potential to modulate important physiological processes, such as fruit ripening, and that their field use promises to be a useful tool to control crop production. These results also support the contention that an intense metabolic networking among different hormones, ethylene, auxin ABA and GA, in the coordination of ripening processes occurs. Present information, arising from hormone application under field conditions contributes to the research for new uses of natural growth regulators in the control of fruit development and ripening.

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And now what? ...now another adventure begins, another research... more fun.

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Appendix S1

oligo for name	sequence of oligo for	oligo rev name	sequence of oligo rev	contig annotation
ctg_469_for	TGTTCAGCTCCCCGACTTTCAC	ctg_469_rev	TCTTGCGGCCGATGTTCACC	1-aminocyclopropane-1-carboxylate synthase ACC1 synthase, ACS1
ctg_64_for	CCCCCATGCGCCACTCCA	ctg_64_rev	CATCACTGCCAGGGTTGTAAAAG	1-aminocyclopropane-1-carboxylate oxidase (ACO1)
ctg_1436_for	AAGCCTATGCTTTGATGGTCTTGA	ctg_1436_rev	TCCCCTTGCCCTCATTGACTCTTCT	ETR1 ethylene receptor
ctg_2025_for	TTCGGGTTGTCTTCGGATAATGG	ctg_2025_rev	ACCGCTGGAATAGTGCCCTCTGA	ETR2 ethylene receptor
ctg_2116_for	AGGGGTTCGAGTTTGGCTTGGTA	ctg_2116_rev	GTTTGGGTGGGAATGTCGTCGTC	Ethylene response factor 2
TF22	CCAAGACCAACAAGGACAACA	TF9	CATGCTCACCTTCACCA	Aux/IAA protein
ctg_1993	AAGAGCGGCAGTTTGAGGAGTT	ctg_1993	CAATGCGGTAAAGATGGGCTAAAA	Auxin-regulated protein GH3 homolog At2g23170 - Arabidopsis thaliana
ctg_1993_for	AAGAGCGGCAGTTTGAGGAGTT	ctg_1993_rev	CAATGCGGTAAAGATGGGCTAAAA	Auxin-regulated protein GH3
ctg_3371	AAGTGCAGGCCTGGATTACCC	ctg_3371	TAGGCCAGTGCATGAGAAGTC	tryptophan synthase beta subunit [Camptotheca acuminata]
ctg_3575	ACAACCGCAATCTGGAAACAT	ctg_3575	TAGGCAATATCATCAGGAGTG	putative indole-3-glycerol phosphate synthase [Arabidopsis thaliana]; PpIGPS
ctg_2713	GGGTGACTGAATCTGGGTTTG	ctg_2713	TGGTTGCCTTGGGTTCATTAT	transport inhibitor response 1 (TIR1) AtFBL1 [Arabidopsis thaliana]
ctg_3721	ATGATGGCGGCTGGGAGGAACT	ctg_3721	TTGCTGGCCCGCCGTGGTAAA	PIN1-like auxin transport protein [Populus tremula x Populus tremuloides]
ctg_4056_for	GGGAGGTGAAGCAGGAAATGTGAT	4056_rev	GGCTGCCTGCAGTTCTATGACCTC	IAA amidohydrolase
ctg_4705	CCAATGGGTATTTGTAAGGGATGC	ctg_4705	TTTTGATTTTATGGTGGGTGTTCG	Putative IAA-Ala hydrolase [Oryza sativa (japonica cultivar-group)]
ctg_544_for	GACACTCGGCTTAACCTCTTCA	ctg_544_rev	AGCTGCTTTCATATTTTCACTGC	Gibberellin 2-oxidase (GA2-oxidase)
ctg_2980_for	GGACGACGGGTACATAATGACTTT	ctg_2980_rev	CCGTACGGGACCCTTGATGG	NCED Nine-cis-epoxycarotenoid dioxygenase2 [Pisum sativum]
AOS_for	GAGCTCACGGGAGGTTACAG	AOS_rev	CTGGAGTGGAACTCGGGTAG	Allene oxide synthase AOS (home made from AOS sequence)
ctg_420_for	GGGGGAGGCCAAGCACTG	ctg_420_rev	TGAACTCCGGGAAACCTGACCA	endo-Polygalacturonidase
DZ09	GACAGACAGGGTAGATTAGGAA	DZ10	GGCTCAGTACCCAGTTAGGT	Expansin (PpExp1)
ctg_941_for	TGGGTCTTCTTGCAATGGTCTCCT	ctg_941_rev	GCTCAGTGCTGCAGTATTTGTCC	Expansin (PPEXP2)
ctg 676_for	GCCTTGCCTCTCTACTCATC	ctg 676_rev	CCATATCCACAAGCCCCACCC	Expansin (PPEXP3)
ctg_938_for	CAGCCAGAGACCTTGCACCAAAAA	ctg_938_rev	TGCACCAGCCATGAGCTTAGGACT	invertase/pectin methylesterase inhibitor
ctg_1024_for	GGGAGCCAGTGCAAAGGGATTCT	ctg_1024_rev	GTTTCAGGGCTGCCACGCTCAT	Catalase
ctg_1026_for	TGACAAACAGGGGCACAGGAG	ctg_1026_rev	GTCGCCACAGTCAACAAAGTCGTA	Pathogenesis-related protein PR-4B precursor
ctg_1540_for	TCCAACGCCCCTCAACTCATCTAC	ctg_1540_rev	TGGCCCTGTTGTCGTCCTTGTTC	Prunin
ctg_1543_for	CGCCCGCAACAACAACAACAAT	ctg_1543_rev	GTGATCGCTGCTGACGTGGAAAAG	Prunin
ctg_2916_for	TGCCTGGGGAATATGAGCAGTAAC	ctg_2916_rev	TGGTCTCGCAGAACTACCTCAAAA	Succinate-semialdehyde dehydrogenase
ctg_3563_for	AGTTGTGGCATGGGCTCTCACTG	ctg_3563_rev	CTCCGGCATGAAGTAGCAGGATGT	LEA (late embryogenesis abundant) protein
ctg_779_for	GGTTCATGTGCTTTCCTTCCATCTGTAGTG	ctg_779_rev	GTCAATTCCGAGAATGCCTAAGAGCTGG	cyclin D3 [Malus x domestica]
ctg_1823_for	CCCGCGACCGCCATACTCTTAC	ctg_1823_rev	TCAACGGAAGGGACTCGCTGTG	Senescence-associated protein SAG102
ctg_2902_for	TGGCCGGCATGGTAGTCACG	ctg_2902_rev	GCCCATCCCGATGGAGAAGAAC	Putative sorbitol transporter
ITS_for	TGACCTGGGGTCGCGTTGAA	ITS_rev	TGAATTGCAGAATCCCGTGA	Annealing to the Internal Transcribed Spacer (ITS) of the ribosomal RNA, to amplify the internal standard
PpN1_for	CCAGGAGAATCGGTGAGCAGAAAA	PpN1_rev	TCGAGGGTGGAGGACTTGAAGAATG	Annealing to the peach putative transcript ppa009483m, orthologous to Arabidopsis AT4G34270
PAL_for	AAGCTGCTGAAAAGGTGCAT	PAL_rev	TCATTTTGGTTGCTGCTCTG	P. persica cultivar Loring phenylalanine ammonia lyase (PAL) gene, partial cds AF206634.1
LDOX_for	AAGTGGGTCACTGCCAAGTGTGTTC	LDOX_rev	GTGGCTCACAGAAAACTGCCCAT	P. persica leucoanthocyanidin dioxygenase (LDOX) gene, complete cds (1521 bp)
F3H_for	TCGGAGGGCAGAGCGAAGAAC	F3H_rev	TTGTGGAGGCTTGTGAGGATTGG	P. persica PpF3H mRNA for flavanone 3-hydroxylase, partial cds (809 bp)
CHS_for	CAGAGATACCCAAAGGTTGGAAGGC	CHS_rev	AACCCATCCTTCCCGACAGCGAT	P. persica PpCHS mRNA for chalcone synthase, partial cds (449 bp)
DFR_for	GGTCGTCCAGGTGAACATACTGCC	DFR_rev	ATTTCTCATGCCATCCATGCCAC	P. persica PpDFR mRNA for dihydroflavonol reductase, partial cds (657 bp)
ANR_for	ACTTCAAGGCTAAGGGCTGCTG	ANR_rev	CCAAGCCAGATAAACGCCAATCAC	P. persica fruit skin mature fruit P. persica cDNA clone Skin41F12, mRNA sequence
LAR_for	CATCCACGGGGAAATTCACCTG	LAR_rev	ACCCTTCCCAGAGTTACCATCACTGA	P. persica fruit mesocarp plus epidermis 30 days after bloom P. persica cDNA clone P-33H4,
UFGT_for	CCGCTGCCTCTCCCAACACTC	UFGT_rev	CCATCAGCCACATCAAACACCTTTAT	PP_YEa0017A04 Peach developing fruit mesocarp Stage S4 Prunus persica cDNA clone PP_YEa0017A04 5-,
CHI_for	TGAAGACCTCAAGGAACTTCTCAATGG	CHI_rev	ACACAGGTGACAACGATACTGCCACT	PU1_plate39_F16 PU1 Prunus persica cDNA similar to similar to (X75963) chalcone isomerase [Vitis vinifera],
MYB for	ACAGCAATCACAGCAGCAAC	MYB rev	TGAGACCATCCCACCTGCATA	MYB transcription factor associated to anticianins biosynthesis and regulation
bHLH for	CTTCAACAAGAAACGGCACA	bHLH rev	AACCAATCTCTTCGCAATCG	bHLH transcription factor associated to anticianins biosynthesis and regulation