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A GENOMIC INVESTIGATION OF THE RIPENING REGULATION IN PEACH FRUIT

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ABSTRACT

The aim of this work is the study of the gene regulation of peach ripening with a genomic approach. The microarray platform µPEACH 1.0 and Real Time PCR experiments have been used in order to find out genes involved in peach ripening (219 genes up-regulated and 188 down-regulated during the transition from stage S3II to stage S4I) and to study the effect of two hormones (auxin and ethylene) and 1-MCP (1-Methylcyclopropene), an inhibitor of ethylene receptors, during ripening. This approach has confirmed that ethylene can affect the expression of many genes and has confirmed its basic role in the regulation of ripening of peach fruit (102 genes induced and 80 repressed by treatment with ethylene associated with the S3II-S4I transition) and, more generally, of climacteric fruit (Alba *et al*., 2005). Also it has been possible to show that auxin is actively involved in the ripening of peaches (43 and 48 genes induced and repressed, respectively, by the NAA treatment and modulated by the transition from pre-climacteric to climacteric stage) but with a role independent from ethylene. Indeed, many genes involved in the biosynthesis, transport, perception and signaling of auxin had increased expression in the mesocarp during ripening. Moreover, there is an important cross-talk between auxin and ethylene, with genes in the auxin domain regulated by ethylene, as ctg3721 encoding a PIN auxin efflux facilitator, and genes in the ethylene domain regulated by auxin, as ACS1 (1-aminocyclopropane-1-carboxylate synthase). The microarray analyses carried out with 1-MCP treated fruit revealed that this chemical modified the expression of 121 genes. Besides inducing ethylene-, auxin- and ripening-repressed genes and repressing ethylene-, auxin- and ripening-induced genes, also genes either induced or repressed by ripening, auxin and 1-MCP were discovered. Thus, blocking ethylene perception with 1-MCP can induce ripening-related effects through a not-yet identified mechanism.

Some of the genes identified as “ripening-related” and “belonging to either auxin or ethylene domains” were mapped with the bin mapping technique. In particular, the *Prunus* reference map constructed with an almond (*Prunus dulcis*) x peach (*Prunus persica*) F_2 population (Howad *et al*., 2005) has been used, namely, the TxE population (*Texas*, almond x *Earlygold*, peach), available in the laboratory of

prof. Pere Arus (IRTA, Barcelona, Spain). Some of these genes are transcription factors, whose expression parallel peach ripening. A functional analysis of these TF genes is a possible way to study the genetic regulation involved in peach ripening. Thus an analysis of the promoters of a gene, named *PpIAA57*, coding for an Aux/IAA protein and of a gene, named *PpbZIP298*, coding for a bZIP transcription factor has been carried out. These genes present a high transcript abundance in ripe fruit and they are up-regulated by the S3II-S4I transition. Nonetheless, their expression is not influenced by treatments with either ethylene or auxin. These promoter sequences have been evaluated with a bioinformatic analysis to discover the presence of *cis*-elements. Progressive deletions of the *PpbZIP298* and *PpIAA57* promoters, fused with the GUS reporter gene, were used for tobacco stable transformation and for agroinfiltration experiments performed on peach fruit. Unexpectedly, the strength of the two promoters resulted to be very weak, despite the length of the used fragments. Anyway, a possible putative regulatory region has been identified in the 5' UTR of *PpbZIP298*.

ABSTRACT

Lo scopo di questo lavoro è lo studio della regolazione genica durante la maturazione della pesca mediante un approccio di tipo genomico. La piattaforma μPEACH 1.0 ed esperimenti di Real Time PCR sono stati utilizzati per scoprire geni coinvolti nella maturazione della pesca (219 geni indotti e 188 repressi durante la transizione dallo stadio S3II allo stadio S4I) e per studiare l'effetto di due ormoni (auxina ed etilene) e di un inibitore dei recettori dell'etilene, 1-MCP (1-Metilciclopropene) durante la maturazione. Questo approccio ha confermato che l'etilene controlla l'espressione di molti geni e ha avvalorato il suo ruolo fondamentale nella regolazione della maturazione della pesca (102 geni indotti e 80 repressi dal trattamento con etilene associato alla transizione S3II-S4I) e, più in generale, dei frutti climaterici (Alba *et al*., 2005). Inoltre è stato possibile dimostrare che l'auxina è coinvolta attivamente nella maturazione delle pesche (43 e 48 geni rispettivamente indotti e repressi dal trattamento con NAA e modulati dalla transizione da stadio pre-climaterico a quello climaterico) ma con un ruolo indipendente dall'etilene. Infatti, molti geni coinvolti nella biosintesi, trasporto, percezione e trasduzione del segnale dell'auxina presentano un incremento di espressione nel mesocarpo durante la maturazione. Inoltre, è presente un importante "cross-talk" tra auxina ed etilene, con geni appartenenti al dominio dell'auxina regolati dall'etilene, come nel caso del ctg3721 codificante un facilitatore di efflusso di auxina di tipo PIN e geni appartenenti al dominio dell'etilene regolati dall'auxina, come ACS1 (acido 1-amminociclopropan-1-carbossilico sintasi). Gli esperimenti di microarray condotti su frutti trattati con 1-MCP hanno rivelato che questo composto chimico modifica l'espressione di 121 geni. Oltre a indurre geni repressi dall'etilene, dall'auxina e dalla maturazione e reprimere geni indotti dall'etilene, dall'auxina e dalla maturazione, è stato scoperto che l'1-MCP può indurre o reprimere geni che sono regolati nello stesso modo dalla maturazione e dall'auxina. Quindi il blocco della percezione dell'etilene con 1-MCP è in grado di indurre degli effetti legati alla maturazione attraverso un meccanismo non ancora identificato.

Alcuni dei geni identificati come “legati alla maturazione” e “appartenenti sia al dominio dell’auxina che a quello dell’etilene” sono stati mappati mediante la tecnica del “bin mapping”. In particolare, è stata utilizzata la mappa di riferimento di *Prunus* costruita incrociando mandorlo (*Prunus dulcis*) con pesco (*Prunus persica*) con lo scopo di ottenere una popolazione F_2 (Howad *et al*., 2005) chiamata popolazione TxE (*Texas*, mandorlo x *Earlygold*, pesco), disponibile presso il laboratorio del prof. Pere Arus (IRTA, Barcellona, Spagna). Alcuni di questi geni sono dei fattori di trascrizione, la cui espressione aumenta durante la maturazione della pesca. Un’analisi funzionale di questi fattori di trascrizione è un possibile modo per studiare la regolazione genica coinvolta nella maturazione della pesca. Quindi è stata condotta un’analisi dei promotori di un gene codificante una proteina di tipo Aux/IAA chiamato *PpIAA57* e di un gene codificante per un fattore di trascrizione di tipo bZIP chiamato *PpbZIP298*. Questi geni presentano un’elevata abbondanza di trascritti nei frutti maturi ed inoltre sono regolati positivamente durante la transizione S3II-S4I. Tuttavia, la loro espressione non è influenzata dai trattamenti con etilene o auxina. Le sequenze di questi due promotori sono state analizzate mediante un’analisi di tipo bioinformatico per scoprire la presenza di *cis*-element. Delezioni progressive dei promotori dei due geni *PpbZIP298* e *PpIAA57*, fuse con il gene reporter GUS, sono state utilizzate per la trasformazione permanente di piante di tabacco e per esperimenti di agroinfiltrazione condotti su pesche. Inaspettatamente, la forza dei due promotori sembra essere molto debole a discapito della lunghezza dei frammenti utilizzati. Comunque, una possibile putativa regione regolativa è stata identificata nel 5' UTR del gene *PpbZIP298*.

1. INTRODUCTION

1.1. THE FRUIT

The fruit is the transformation of the ovary and typically includes carpel tissues in part or in whole. It acts as a protection for seeds during their development and at maturity; it facilitates their dispersal into the environment by animals, wind, water or explosive dehiscence. Fruit growth continues until seeds are developed. There are two different strategies of seed dispersal: the first one leads to develop dry fruit that at maturity scatter seeds by mechanical means. The second strategy consists of the development of fleshy fruit. The ripe phenotype consists of biochemical and physiological changes in order to make available the edible and desirable organ to seed-dispersing animals. Ripening also imparts value to fruit as agricultural products. These changes, although variable among species, generally include conversion of starch to sugars, modification of cell wall structure and texture, alterations in pigment biosynthesis, accumulation of flavor and aromatic volatiles (Giovannoni, 2001).

The dry fruit are composed by dehydrated and sometimes sclerified tissues. They can be classified as dehiscent and indehiscent fruits. Dehiscent fruits set the seeds free by opening of the fruit that remains itself with the mother plant. In contrast, indehiscent fruits and their seeds are a dispersal unit.

In the fleshy fruit, the pericarp is differentiated into three distinct layers: the epicarp, the mesocarp and the endocarp (the outer, median and inner layers, respectively). Fleshy fruit can be divided into two types based on the fact that a peak in the rate of respiration can be measured or not, before the visible onset of the ripening process. In detail, all fruits that ripen in response to ethylene show a characteristic respiratory rise before the ripening phase called climacteric. Such fruits also show a spike of ethylene, recognized as the hormone that accelerates the ripening of edible fruits, produced immediately before the respiration rise. Apples, bananas, avocados, tomatoes and peaches are examples of climacteric fruits. Instead, fruits such as grapes, watermelons and strawberries do not exhibit

the respiration and ethylene production rise and they are called non-climacteric fruits.

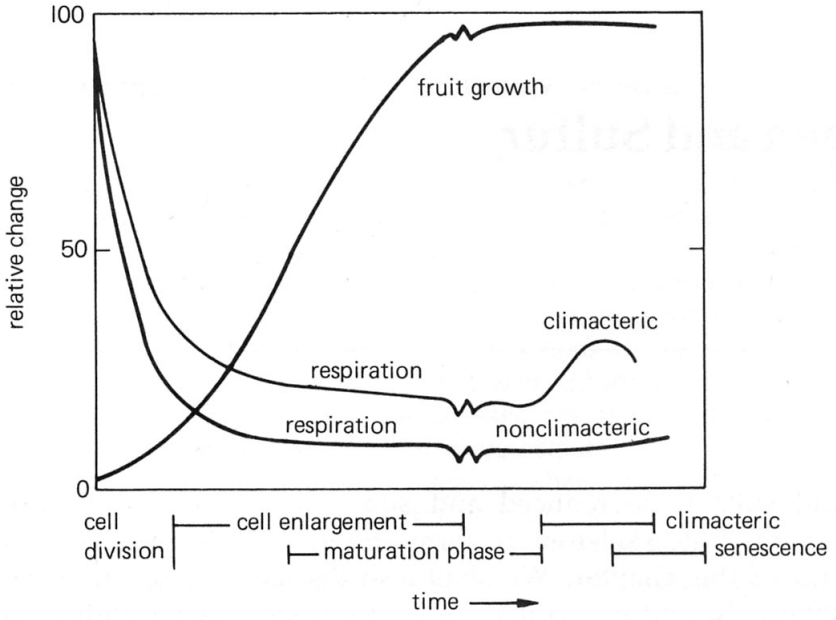


Fig. 1.1. Variation during time of the growth rate and the respiration rate in climacteric and non-climacteric fruits.

1.2. FRUIT RIPENING

The ripening of fruit organs represents the final stage of development in which the matured seeds are released. In *Arabidopsis*, an indehiscent fruit, the seeds dispersal is facilitated by senescence of the mature carpel tissue followed by separation of the valves at an abscission cell layer. In contrast to *Arabidopsis*, at the end of growth, fleshy fruits such as tomato or peach undergo profound physical, biochemical and physiological changes, influencing appearance, texture, flavor and aroma, to make the fruits palatable to animals which carry out the seed

dispersal (Seymour *et al.*, 1993). Although the specific biochemical programs resulting in ripening phenomena vary among species, changes include:

- the alteration of chlorophyll, carotenoid and flavonoid accumulation to modify the fruit colour;
- alteration of sugars, acids and volatile profiles that affect nutritional quality, flavor and aroma;
- textural modification through variation of cell wall structure and metabolism.

Fruit display the ripening program, following these developmental changes, although fruit species are classically defined physiologically on the basis of the absence (non-climacteric) or presence (climacteric) of increased respiration and synthesis of the ethylene hormone at the onset of ripening (Lelievre *et al.*, 1997).
Members of the same species (for example, melon) are reported to include both climacteric and non-climacteric varieties. Even if the specific role of climacteric respiration in fruit ripening remains not very clear, the recruitment of ethylene as a coordinator of ripening in climacteric species likely serves to facilitate rapid and coordinated ripening (Giovannoni, 2004). Recent data of the MADS box regulation of ripening in both tomato and strawberry suggests common regulatory mechanisms working early in both climacteric and non-climacteric species (Vrebalov *et al.*, 2002). The study of the molecular basis of such early and common events represents an active frontier in fruit ripening investigation.

1.3. PEACH RIPENING

Peaches are climacteric fruits. The period of time for the development of *Prunus persica* fruit can be divided into four stages: S1, S2, S3, S4 (Zanchin *et al.*, 1994). The double-sigmoid curve is characteristic of drupe development, where two stages in which growth is slow (S2 and S4) are alternated by two stages in which the growth is fast (S1 and S3) (Fig. 1.2).
In particular:

- in S1 both cellular multiplication and distension occur;

- during S2 the hardening of the endocarp (pith) takes place;
- during S3 the fruit increase is mainly due to cell expansion;
- in S4 the fruit, reached its final dimensions, ripens.

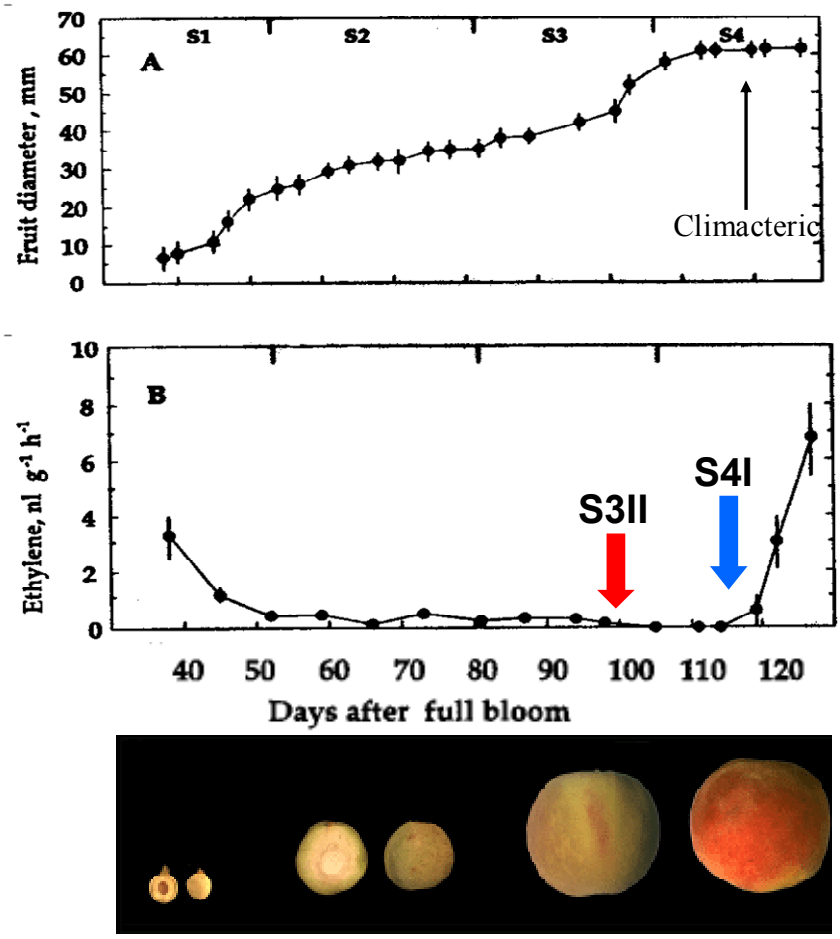


Fig. 1.2. The four stages of peach fruit development and ripening. Fruit diameter (A) and ethylene evolution (B) measured during development (days after full bloom) of cultivar *Redhaven* peach fruit. Stage (S1, S2, S3 and S4) transitions are evidenced in the growth curve (redrawn from Bonghi *et al.*, 1998).

Peaches exhibit a sharp rise in ethylene production at the onset of ripening paralleled by dramatic changes in the transcriptional profile of genes, many of which are regulated by the hormone. Such coordinated and programmed modulation of gene expression leads to several changes, which all contribute to overall fruit quality (Trainotti *et al.*, 2003, 2006). In peach fruit, there is a close

link between on-tree physiological maturity and development of key traits responsible for its quality. A delayed harvest could improve fruit organoleptic characteristics, since sugars and flavor components increase while total acids decrease during late ripening (Vizzotto *et al.*, 1996; Visai and Vanoli, 1997; Etienne *et al.*, 2002). However, melting flesh peaches and nectarines undergo rapid ripening and soften quickly after harvest, leading to losses in the marketing chain. Therefore, fruit are commonly picked at an early stage of ripening to better withstand handling and they do not reach full flavor and aroma.

Flesh firmness (FF), soluble solids content (SSC) and titratable acidity (TA), which are commonly used to evaluate fruit quality and ripeness, can be assessed simply and rapidly, but they might not provide all the information needed to accurately identify fruit ripening stage, especially when individually considered (Costa *et al.*, 2006). Other physiological, biochemical or biomolecular parameters, such as ethylene and aroma volatile compounds emission, respiration, soluble pectins, chlorophyll, carotenoid and flavonoid content and mRNA levels of ripening-related genes could improve the available information as they are strictly related to the progression of ripening (Golding *et al.*, 2005; Carrari *et al.*, 2006).

1.4. HORMONES

Efficient communication among cells, tissues and organs is fundamental for the form and function of multicellular organisms. In higher plants, regulation and coordination of metabolism, growth and morphogenesis, formation and development of several organs, often depend on chemical signals from one part of the plant to another.

Hormones are chemical messengers that are produced in one cell or tissue. They modulate cellular processes in another cell by interacting with specific protein receptors. Plant hormones are signal molecules synthesized in one tissue and act on specific target sites in another tissue at low concentrations. The endocrine hormones are transported to sites of action in tissues distant from their site of

synthesis, while the paracrine hormones operate on cells adjacent to the source of synthesis.

In plant, the six major types of hormones are: auxins, ethylene, cytokinins, gibberellins, abscisic acid and brassinosteroids. A variety of other signaling molecules, such as jasmonic acid and salicylic acid, plays roles in resistance to pathogens and defense against herbivores. Some flavonoids can function as both intra- and extracellular signals while a carotenoid cleavage product is thought to function in long distance signaling. Certainly, the list of plant hormones and hormone-like signaling agents keeps expanding (Taiz and Zeiger, 2006).

1.5. ETHYLENE

Ethylene is C_2H_4 olefin. Ethylene is produced in all higher plants and it is a gaseous hormone; it can be detected and measured by gas chromatography. Senescing tissue and ripening fruits are better sites of ethylene production than young or mature tissues. The precursor of ethylene is the amino acid methionine, which is converted to AdoMet (S-adenosylmethionine), ACC (1-aminocyclopropane-1-carboxylic acid) and ethylene. The rate-limiting step of this pathway is the conversion of AdoMet to ACC, which is catalyzed by ACC synthase. ACC synthase is encoded by members of a multigene family that are regulated in different way in various plant tissues. Ethylene biosynthesis is triggered by various developmental processes, environmental stresses and by auxin, which can increase the level of activity and of mRNA of ACC synthase. The physiological effects of ethylene can be blocked by biosynthesis inhibitors or by antagonists. For example, while AVG (aminoethoxy-vinylglycine) and AOA (aminooxyacetic acid) inhibit the synthesis of ethylene, carbon dioxide, silver ions and methylcyclopropene (1-MCP) inhibit ethylene action.

Ethylene regulates many processes among which some are involved in the fruit ripening, related with leaf and flower senescence, leaf and fruit abscission, root hair development, seedling growth and hook opening. At molecular level, this gaseous hormone regulates the expression of various genes, including ripening-related genes and pathogenesis-related genes.

In climacteric fruits, treatment with ethylene induces the fruit to produce additional ethylene and its action is defined as autocatalytic. In climacteric plants, there are two systems of ethylene production:

- System 1, which acts in vegetative tissue and in which ethylene inhibits its own biosynthesis;
- System 2, which occurs during ripening of climacteric fruit (and in some species involved in senescence of petals) in which ethylene stimulates its own biosynthesis (autocatalytic synthesis) (Taiz and Zeiger, 2006).

One of the primary effects of ethylene signaling is the modification in the expression of various target genes. Ethylene affects the mRNA transcript levels of numerous genes, including those that encode endoglucanases and genes related to ripening and ethylene biosynthesis. EREs (ethylene response elements) are the regulatory sequences and they have been identified among the ethylene-regulated genes. EIN3 family of transcription factors are the key components mediating ethylene effects on gene expression (Chao *et al.*, 1997). In response to an ethylene signal, homodimers of EIN3 or closely related proteins bind to the promoters of genes that are rapidly induced by ethylene to activate their transcription (Solano *et al.*, 1998) such as ERF1 (Ethylene Response Factor 1). This gene encodes a protein that belongs to the ERE-binding protein (EREBP) family of transcription factors. Several EREBPs are rapidly up-regulated in response to ethylene. Thus, the regulation of EIN3 protein stability plays an important role in ethylene signaling.

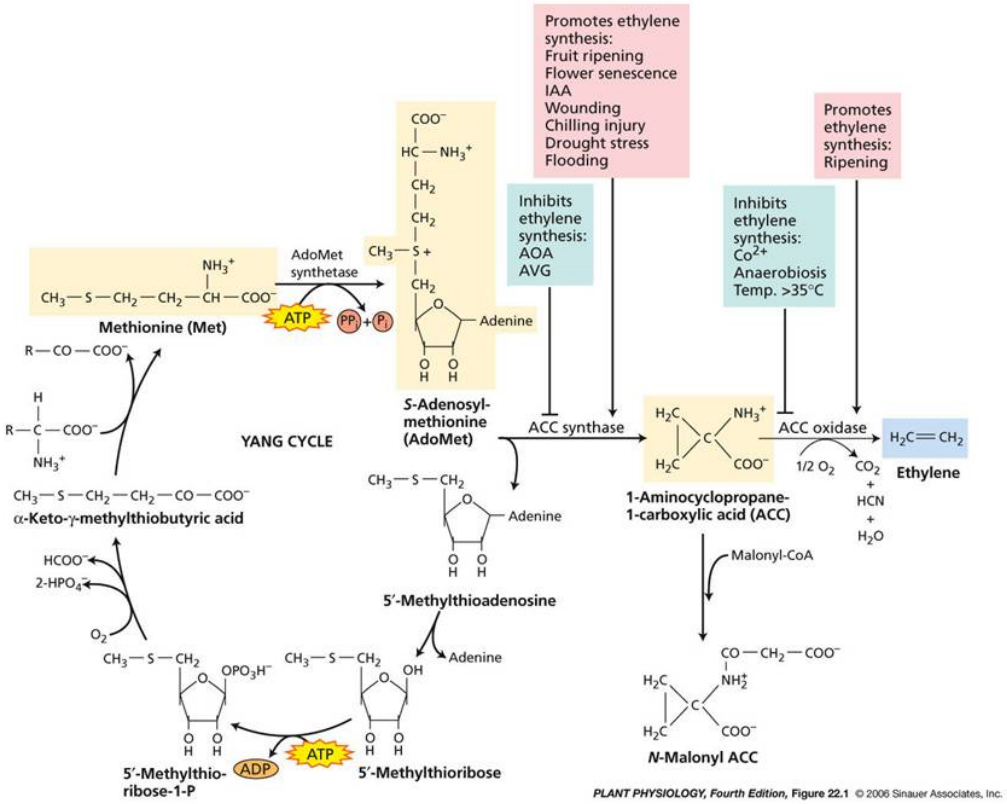


Fig. 1.3. Ethylene biosynthetic pathway and the Yang cycle. The amino acid methionine is the precursor of ethylene. The rate-limiting step in the pathway is the conversion of AdoMet to ACC, which is catalyzed by the enzyme ACC synthase. The last step in the pathway, the conversion of ACC to ethylene, requires oxygen and is catalyzed by the enzyme ACC oxidase. The CH_3-S group of methionine is recycled via the Yang cycle and thus conserved for continued synthesis (from Taiz and Zeiger, 2006).

1.5.1. ETHYLENE AND RIPENING

In climacteric fruits, an increase in ethylene production is observed before the initiation of ripening and ethylene is a trigger of the ripening process; the ethylene-dependent and -independent pathways coexist to co-ordinate the ripening process. Transgenic tomato fruit, in which endogenous ethylene production was reduced by the expression of an antisense ACC synthase, did not soften. In these fruits, treatment with exogenous ethylene reversed the antisense phenotype,

indicating that softening was completely dependent on ethylene (Oeller *et al.*, 1991).

Ethylene is an important plant hormone; also, the appearance of the climacteric ethylene has been considered as the main signal for the regulation of ripening in these fruits. The easy availability of this chemical, in addition to the fact that tomatoes are climacteric fruits, led to an outburst of research dealing with the ripening of climacteric fruits in general, and tomato in particular. It was thus possible to understand many molecular details of the ripening process. Also the availability of mutants (either natural or induced by genetic transformation) [*Nr* (Wilkinson *et al.*, 1995) and *Gr* (Barry *et al.*, 2005; Barry and Giovannoni, 2006) mutants; ACS antisense tomatoes (Oeller *et al.*, 1991); ACO antisense tomatoes (Hamilton *et al.*, 1990)] has helped researchers to understand the mechanisms underlying the ripening process and to confirm the role of ethylene in the ripening of climacteric fruits. However, the very great availability of tomato mutants has also yielded some specimens (mutants *rin*, *nor*, *cnr*) whose fruits are unable to ripen even when treated with exogenous ethylene even though they are not impaired in the hormone signal transduction pathway. The *nor* mutant gene has not yet been made public but it is has been reported to code for a transcription factor (Adams-Phillips *et al.*, 2004). The mutated *cnr* gene has recently been demonstrated to code for an SBP-box transcription factor where a natural epigenetic mutation has occurred (Manning *et al.*, 2006), while the *RIN* gene has been shown to encode a MADS-box transcription factor (Vrebalov *et al.*, 2002). In other cases, MADS-box encoding genes have been found to be expressed in fleshy fruits although their precise role has not yet been defined (Sung *et al.*, 2000; Boss *et al.*, 2001; Busi *et al.*, 2003; Rosin *et al.*, 2003). The characterisation of the *rin*, *nor* and *cnr* mutants is particularly relevant because it demonstrates that other factors, generally named “developmental factors”, act upstream of ethylene and their control of the ripening process is no less important than that played by the hormone (Giovannoni, 2004). In the case of tomato also light has been shown to play a role in the ripening of fruits (Alba *et al.*, 2000).

1.5.2. ETHYLENE AND PEACH RIPENING

Peach fruit ripening relies on changes in expression of several genes, including those encoding ACS and ACO, the key enzymes of the ethylene biosynthetic pathway. Two peach genes encoding ACO, named *Pp-ACO1* and *Pp-ACO2*, have been characterized (Ruperti *et al.*, 2001). Pp-ACO1 is expressed in flower, fruitlet abscission zones, mesocarp and in young fully expanded leaves and its transcripts accumulation strongly increases during fruitlet abscission, in ripe mesocarp and senescing leaves and following treatment with propylene. Differently from Pp-ACO1, Pp-ACO2 mRNA accumulation is detected in fruits only during early development and is unaffected by propylene treatment.
Two peach genes homologous to the *Arabidopsis* ethylene receptor genes ETR1 and ERS1, named *Pp-ETR1* and *Pp-ERS1* respectively, have been isolated and characterized (Rasori *et al.*, 2002). The level of Pp-ETR1 transcripts remained unchanged in all the tissues and developmental stages, whereas Pp-ERS1 mRNA abundance increased in ripening mesocarp, in leaf and fruitlet activated abscission zones and following propylene application. An *ETR2* ortholog has been identified in ripening fruit by means of microarray analysis (Trainotti *et al.*, 2006).

1.5.3. INHIBITORS OF ETHYLENE ACTION

Most of the effects of the ethylene can be antagonized by specific ethylene inhibitors. For example, silver ions (Ag^+) and silver nitrate ($AgNO_3$) are potent inhibitors of ethylene action. Silver is very specific; the inhibition that causes cannot be induced by any other metal ion. Carbon dioxide at high concentrations (in the range of 5 to 10%) also alters many effects of ethylene (in detail, the induction of fruit ripening), although CO_2 is less efficient than Ag^+. This action of CO_2 has often been utilized in the storage of fruits, whose ripening is delayed at elevated CO_2 concentrations. In fact, the high concentrations of CO_2 required for inhibition make it unlikely that CO_2 acts as an ethylene antagonist under natural conditions.

1-methylcyclopropene (1-MCP) binds almost irreversibly to the ethylene receptors (Sisler and Serek, 1997) and effectively blocks multiple ethylene responses. This nearly odourless compound has been marketed under several trade names. EthylBloc® is used in floriculture to increase the shelf life of cut flowers. With the "SmartFresh" name, a commercial formulation of 1-MCP has been registered in several countries to improve fruit storage.

1.5.4. 1-MCP

1-Methylcyclopropene (1-MCP) is used as a plant regulator to inhibit ethylene production in cut flowers, nursery and foliage plants and in stored fruits and vegetables. It is approved for use only in enclosed spaces, such as greenhouse, store-rooms, coolers, enclosed truck trailers, controlled atmosphere food storage facilities and shipping containers. 1-MCP is a gas under normal environmental conditions. It has a non-toxic mode of action, negligible residue and is active at very low concentrations. As a pesticide active ingredient, it is used for prolonging the life of ornamental plants and cut flowers by preventing ethylene from attaching to plant tissues. It is a postharvest tool for counteracting undesirable effects of ethylene on harvested fruits and vegetables during transport and storage. The inhibitor of ethylene perception has resulted in an explosion of research on its effects on fruits and vegetables, both as a tool to further investigate the role of ethylene in ripening and senescence and as a commercial technology to improve maintenance of product quality (Watkins, 2006). The action of 1-MCP is mediated through the inhibition of ethylene perception of plant tissues; in fact, this chemical interacts with the receptor and competes with ethylene for binding sites (Sisler and Serek, 1997, 2003). Moreover, the efficacy of inhibition of ripening and senescence of fruit and vegetables depends on the 1-MCP concentration applied, up to saturation of the binding sites. About the fruit, recovery from 1-MCP-induced inhibition of ripening is often essential in order to provide a ripened product that is acceptable to the consumer. The degree and longevity of 1-MCP action is affected by species, cultivar, tissue and mode of ethylene biosynthesis induction.

The effect related to "concentration×time" is apparent with longer exposure periods required for lower 1-MCP concentrations to obtain the same physiological effects (Sisler and Serek, 1997).

Ethylene production is thought to be inhibited by 1-MCP treatment of peaches and nectarines (Mathooko *et al.*, 2001; Fan *et al.*, 2002; Rasori *et al.*, 2002; Liguori *et al.*, 2004; Bregoli *et al.*, 2005; Girardi *et al.*, 2005, Liu *et al*., 2005), although transient increases in production were detected immediately after treatment in one study. Ethylene production of 1-MCP treated fruit was unaffected at the time of treatment, but was reduced after storage (Dong *et al.*, 2001). Recovery from 1-MCP-induced ripening inhibition resulted in higher production of ethylene than observed in untreated fruit (Rasori *et al.*, 2002). Respiration rates of treated fruit were lower, or not affected by 1-MCP treatment. Fan *et al.* (2002) found that the effects of fruit maturity were greater than those of 1-MCP treatment, with early harvested fruit showing little response to treatment. 1-MCP treatment either did not affect SSC of fruit, resulted in lower SSC, or the increase of SSC during ripening was delayed. Loss of TA was reduced in high acid, but not in low acid cultivars (Fan *et al*., 2002; Liguori *et al.*, 2004; Bregoli *et al.*, 2005; Liu *et al.*, 2005).

Responses of fruit to 1-MCP are affected by concentration and exposure period. Optimal 1-MCP concentrations vary greatly from as low as 0.4 μL L^{-1} to 5 μL L^{-1}, the latter concentration being higher than that registered for use. Inhibition of fruit ripening is temporary in all published studies, but repeated 1-MCP treatment helps maintain suppression of ripening.

In peach, Rasori *et al.* (2002) found that 1-MCP did not affect transcription of the PpETR1 gene, but down regulated that of PpERS1. The increased accumulation of PpERS1 transcripts was associated with recovery from 1-MCP inhibition. .

It is possible that stimulated ethylene production is due to loss of negative feedback regulation of ethylene biosynthesis. The genes encoding two key enzymes of the ethylene biosynthetic pathway, ACS and ACO have been studied in several crops.

In particular, in peach, inhibited ethylene production was associated with reduced activity of ACO and a decrease in PpACO1 and PpACO2 transcript accumulation, but ACC accumulated in treated fruit, and *PpACS1* expression and ACS activity

were not affected by treatment (Mathooko *et al.*, 2001). 1-MCP treated nectarines had lower ACS, ACO1 and ACO2 transcript accumulations than untreated fruit at ambient but not cold storage temperatures (Bregoli *et al.*, 2005).

1.6. AUXIN

Auxin was the first hormone to be discovered in plants. Indole-3-acetic acid (IAA) is the most common naturally form of auxin. Auxin plays central regulatory roles in growth and tropism, in apical dominance, lateral root initiation, leaf abscission, vascular differentiation, floral bud formation, phyllotaxy and fruit development. Commercial applications of auxin include rooting compounds and herbicides. The regulation of elongation growth in young stems and coleoptiles is one of the most important roles of auxin in higher plants. Low levels of auxin are also required for root elongation, although at higher concentrations auxin acts as a root inhibitor.

Several pathways have been implicated in IAA biosynthesis, including tryptophan-dependent and tryptophan-independent pathways and several degradative pathways for IAA have also been studied (Fig. 1.4).

The cytosol and the chloroplasts are two main pools of auxin in the cell. Regulation of growth in plants may depend in part on the amount of free auxin present in plant cells, tissues and organs. Levels of free auxin can be modulated by several factors, such as IAA metabolism, the synthesis and breakdown of conjugated IAA, compartmentation and polar auxin transport.

Polar auxin transport can be divided into two main processes: IAA influx and IAA efflux. In relation to the IAA influx, there are the pH-dependent passive transport of the undissociated form and an active H^+ cotransport mechanism driven by the plasma membrane H^+-ATPase. Auxin efflux is thought to occur preferentially at the basal end of the transporting cells via anion efflux carriers complexes and be driven by the membrane potential generated by the plasma membrane H^+-ATPase. The auxin transport can be directly interrupted by auxin efflux inhibitors by a competition for the efflux channel pore or by binding to regulatory or structural proteins associated with the efflux channel.

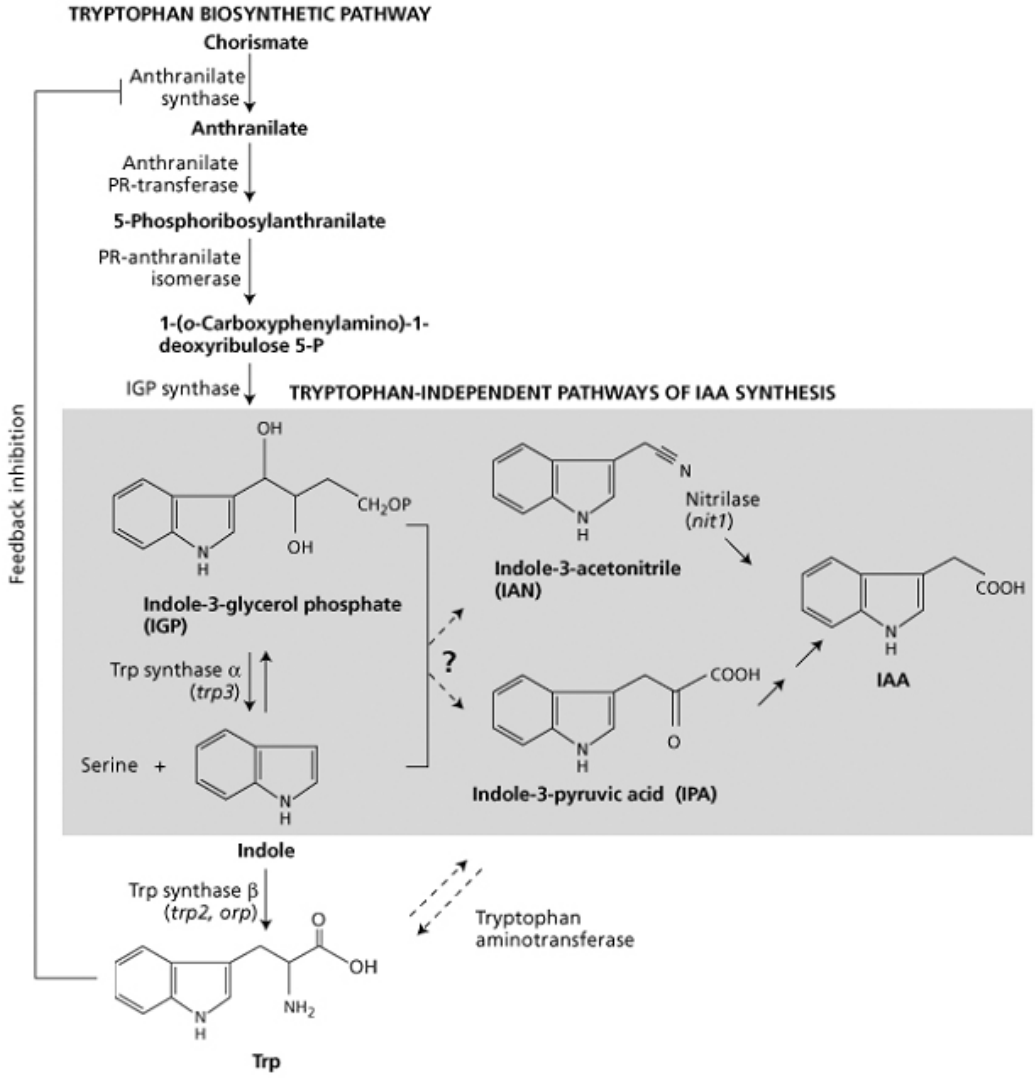


Fig. 1.4. Tryptophan biosynthetic pathway provides precursors for IAA biosynthesis. The branch-point precursor for tryptophan-independent auxin biosynthesis is indole-3-glycerol phosphate (from Taiz and Zeiger, 2006).

TIR1/AFB proteins function as primary auxin receptors and mediate auxin-dependent gene expression. Aux/IAA proteins, together with ARF proteins, mediate auxin-responsive gene expression. Aux/IAA proteins are the immediate targets of auxin-bound TIR1/AFB proteins, which accelerate Aux/IAA proteolytic degradation via an ubiquitin activation pathway.

There are two categories of auxin-induced genes: early and late. Induction of early genes by auxin does not need protein synthesis and is insensitive to protein

synthesis inhibitors. Three functional classes of early genes have been identified: expression of late genes (secondary response genes), stress adaptation and intercellular signaling. The auxin response domains of the promoters of the auxin early genes have a composite structure in which an auxin-inducible response element is combined with a constitutive response element (Taiz and Zeiger, 2006).

1.6.1. AUXIN AND DEVELOPMENT

Much data suggests that auxin is involved in the regulation of fruit development. Auxin is produced in the embryo of developing seeds. Successful pollination initiates ovule growth, which is known as fruit set. Fruit growth, after fertilization, may depend on auxin from developing seeds. The endosperm may contribute auxin during the initial stage of fruit growth and the developing embryo may take over as the main auxin source during the later stages. The influence of auxin produced by the achenes of strawberry on the growth of the receptacle is an example. The strawberry is a false fruit; in fact, it is a swollen receptacle whose growth is regulated by auxin produced by the achenes, the true fruits. The receptacle enlarges and develops its characteristic flavor, sweetness and red colour when the achenes are present. If the achenes are removed, the receptacle fails to develop normally. Spraying the receptacle minus its achenes with IAA restores normal growth and development (Nitsch, 1950).

The production of such seedless fruits is called parthenocarpy. In some plant species, seedless fruits may be produced naturally or they may be induced by treatment of the unpollinated flowers with auxin. In parthenocarpic fruits, auxin may act primarily to induce fruit set, which in turn may trigger the endogenous production of auxin by certain fruit tissues to complete the developmental process. Also ethylene is involved in fruit development and some of the effects of auxin on fruiting may result from the induction of ethylene synthesis.

1.6.2. AUXIN AND RIPENING

Jones *et al.* (2002) report in tomato fruits a differential expression of both ARF (Auxin Response Factors) and Aux/IAA encoding genes which, as their name suggests, are linked to the auxin signaling pathway. This data suggest that auxin might also be part of the mechanisms that control the ripening of climacteric fruits. It is known that plants can produce ethylene by either a System-1 or a System-2 biosynthesis and that the latter is active when climacteric ethylene has to be produced (McMurchie *et al.*, 1972; Oetiker and Yang, 1995). Interestingly, auxin can stimulate the synthesis of more climacteric ethylene (Bleecker and Kende, 2000) through its inductive action on the expression of the key enzyme ACS (Abel and Theologis, 1996). Therefore, in such conditions, any effect of auxin on the ripening process would be indirect and mediated by ethylene.
Evaluations of the auxin contents in *Redhaven* peach fruits were made some years ago in another laboratory (Miller *et al.*, 1987). Those analyses demonstrated that concomitant with the climacteric ethylene production a significant increment of the IAA content could be measured in the mesocarp tissues and that the endogenous IAA concentration and observed ethylene evolution are correlated.

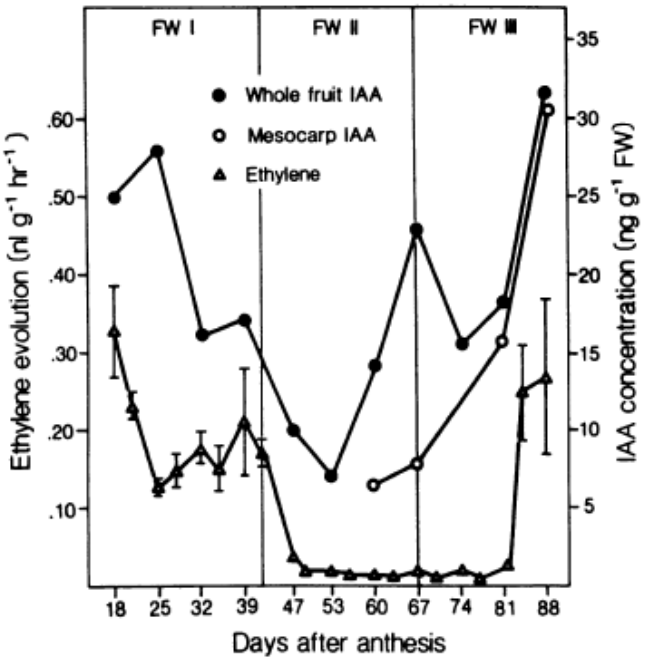


Fig. 1.5. IAA concentration and ethylene evolution of developing *Redhaven* peaches from 18 days after anthesis until ripening (from Miller *et al.*, 1987)

1.6.3. AUXIN-INDUCED ETHYLENE PRODUCTION

In some cases, auxin and ethylene can cause similar plant response that might be due to the ability of auxin to promote ethylene synthesis by enhancing ACC synthase activity. These data suggest that some responses previously attributed to auxin (IAA) are in fact mediated by the ethylene produced in response to auxin. Inhibitors of protein synthesis block both ACC and IAA-induced ethylene synthesis, indicating that the synthesis of new ACS protein caused by auxin gives about the marked increase in ethylene production. Several ACC synthase genes have been recognized whose transcription is elevated following application of exogenous IAA, suggesting that increased transcription is at least partly responsible for the raised ethylene production observed in response to auxin (Nakagawa *et al.*, 1991).

1.7. TRANSCRIPTION

The synthesis of most eukaryotic proteins is regulated at the level of transcription. Transcription is the first step in gene expression and concerns copying DNA into RNA. In eukaryotes, the transcription is characterized by three different RNA polymerases whose activities are modulated by several *cis*-acting regulatory sequences. RNA polymerase II is responsible for the synthesis of pre-mRNA.

In eukaryotes, the term “promoter” is used to describe the region regulating gene transcription. In this region, there are sequences with several functions, including not only the core promoter, sometimes called the basal promoter (the site at which the initiation complex is assembled), but also one or more upstream promoter elements lied upstream of the core promoter. Assembly of the initiation complex on the core promoter can usually occur in the absence of the upstream elements, but only in an inefficient way. Thus, the proteins that bind to the upstream elements include at least some that are activators of transcription and which consequently promote gene expression. During initiation, RNA polymerase, together with the initiation factors, binds to the promoter in a closed complex.

General transcription factors assemble into a transcription initiation complex at the TATA box of the minimum promoter, which lies within 100 bp of the transcription start site of the gene. Two additional regulatory sequences are included in the minimum promoters of eukaryotes: the CAAT box and the GC box. They are the sites of binding of transcription factors, proteins that enhance the rate of transcription by facilitating the assembly of the initiation complex. The *cis*-acting sequences are the DNA sequences that are adjacent to the transcription units they are regulating, while the transcription factors that bind to the *cis*-acting sequences are called *trans*-acting factors, since the genes that encode them are located elsewhere in the genome. Numerous other *cis*-acting sequences located farther upstream of the proximal promoter sequences can exert control over eukaryotic promoters in positive or negative way. These sequences are termed the distal regulatory sequences and they are usually located within 1000 bp of the transcription initiation site. The elements that bind to these sites as a positively acting transcription factors are called activators, while those that inhibit transcription are called repressors.

In multicellular organisms, regulatory sequences can spread thousand of nucleotide from the promoter. Distantly located positive regulatory sequences are called enhancers. Enhancers may be located either upstream or downstream from the promoter.

During formation of the initiation complex, the DNA between the core promoter and the most distally located control elements loops out in such a way as to allow all of the transcription factors bound to that segment of DNA to make physical contact with the initiation complex. In this way, the transcription factor exerts its control over transcription either positively or negatively. Given the large number of control elements that can modify the activity of a single promoter, the possibilities for differential gene regulation in eukaryotes are nearly infinite.

1.8. TRANSCRIPTION FACTORS

Transcription factors bind to a specific sequence of DNA and generally they have three structural features: a DNA-binding domain, a transcription-activating domain and a ligand-binding domain. The DNA-binding domain must have extensive interactions with the double helix through the formation of hydrogen, ionic and hydrophobic bonds. Although the particular combination and spatial distribution of such interactions are unique for each sequence, studies of many DNA-binding proteins have allowed the identification of a small number of highly conserved DNA-binding structural motifs. Most of the transcription factors characterized thus far in plants belong to the basic zipper (bZIP) class of DNA binding proteins. Transcription factor activity in a cell can be regulated by whether or not the factor is synthesized, by environmental signals and by signals (such as hormones) from other cells. They can be classified according to the structure of their DNA binding domains; these include zinc finger proteins, helix-turn-helix proteins, leucine zipper proteins, helix-loop-helix proteins and steroid receptors (Taiz and Zeiger, 2006).

1.8.1. bZIP

Transcription factors play crucial roles in almost all biological processes. Structurally, TFs are usually classified by their DNA-binding domains: basic region/leucine zipper (bZIP) TFs have a basic region that binds DNA and a leucine zipper dimerization motif. In plants, bZIP transcription factors regulate processes including light and stress signaling, pathogen defence, seed maturation and flower development.

Leucine zipper motif combines dimerization and DNA-binding surfaces within a single structural unit. Two long helices form a pincer-like structure that grips the DNA, with each helix inserting into the major groove half a turn apart. Dimerization is mediated by another region within those same helices: in this region, they form a short stretch of coiled coil, wherein the two helices are held together by hydrophobic interactions between appropriately spaced leucine (or

other hydrophobic) residues. Leucine-zipper-containing proteins often form heterodimers as well as homodimers.

1.8.2. AUX/IAA

Two families of transcriptional regulators participate in the TIR1 auxin-signaling pathway: auxin response factors and Aux/IAA proteins. Auxin response factors (ARFs) are short-lived nuclear proteins that bind with specificity to TGTCTC auxin response elements (AuxREs) in the promoters of primary, or early, auxin-response genes. Depending on the particular ARF involved, the binding of ARFs to AuxREs results in the activation or repression of gene transcription. AuxREs appear to be present on the promoters of early auxin genes regardless of the auxin status of the tissue. Aux/IAA proteins are important secondary regulators of auxin-induced gene expression. Aux/IAA proteins regulate gene transcription indirectly by binding to ARF proteins bound to DNA. If the ARF bound to the AuxRE is a transcriptional repressor, then the Aux/IAA protein functions as a transcriptional activator. Conversely, if the ARF is a transcriptional activator, the effect of the Aux/IAA protein is to repress transcription.

Aux/IAA genes encode small nuclear proteins that have a common four domains (I-IV) structure. They are not only subjected to auxin-mediated transcriptional regulation but are also involved in auxin signal transduction. Through their conserved domains III and IV, Aux/IAA proteins can interact with ARFs. They are characterized by an amino-terminal DNA-binding domain (DBD), a long middle region (MR) and domains III and IV near the carboxyl terminus. The DBDs of ARFs bind to conserved promoter elements that confer auxin-responsive gene expression and, depending on the structure of the MR, individual ARFs function as transcriptional activators and repressors. Domains III and IV not only enable interactions between ARF and Aux/IAA proteins but also mediate ARF-ARF dimerization (Berleth *et al.*, 2004).

2. MATERIALS AND METHODS

2.1. SOLUTIONS AND MEDIA

SSC 1X: NaCl 0.15 M
Sodium citrate 0.015 M (pH 7)

TAE 1X: Tris-acetate 40 mM
EDTA 1 mM (pH 8)

DENHARD'T 100X: BSA 2% (w/v)
Ficoll 400 2% (w/v)
Polyvinylyirrolidone 2% (w/v)

CTAB RNA Extraction Buffer: CTAB 2% (Cetyltrimethylammonium bromide)
Polyvinylyirrolidone (PVP) K30 2%
Tris-HCl 100 mM pH 8
EDTA 25 mM pH 8
NaCl 2 M
Spermidine 0.5 g/L
β-mercaptoethanol 2%

Pre-hybridization solution: SSC 5X
SDS 0.1 %
Denhardt's solution 5X
filtrate with a 0.2 µm filter

Hybridization solution: SSC 5X
SDS 0.1 %
Formamide 25%
filtrate with a 0.2 µm filter

LB medium: NaCl 10 g/L
Yeast extract 5 g/L
Tryptone 10 g/L
pH 7.0

YEB medium: Sucrose 5 g/L
Tryptone 5 g/L
Beef extract 5 g/L
Yeast extract 5 g/L
$MgSO_4$ 0.049 g/L

SOC medium: Tryptone 20 g/L
Yeast extract 5 g/L
NaCl 0.5 g/L (10 mM)
KCl 0.19 g/L (10 mM)
$MgCl_2$ 0.95 g/L (10 mM)
$MgSO_4$ 1.2 g/L (10 mM)
Glucose 3.6 g/L (20 mM)

MS: Salts and vitamins MS (Mourashige & Skoog) 4.4 g/L
Sucrose 30 g/L
Agar 0.6%

Infiltration medium: Salts and vitamins MS 4.4 g/L
6BAP (6-benzylaminopurin) 1 mg/L
Vitamins B5 1X
Sucrose 30 g/L
pH 5.6

TAB1 medium: Salts and vitamins MS 4.4 g/L
6BAP 1 mg/L
Indolacetic acid (IAA) 0.2 mg/L
Sucrose 30 g/L

Agar 0.5%
pH 5.6

TAB2 medium: Salts and vitamins MS 4.4 g/L
6BAP 1 mg/L
IAA 0.2 mg/L
Sucrose 30 g/L
Agar 0.5%
Kanamycin 200 mg/L
Cefotaxime 500 mg/L
pH 5.6

TAB3 medium: Salts and vitamins MS 4.4 g/L
Sucrose 30 g/L
Agar 0.5%
Kanamycin 200 mg/L
Cefotaxime 500 mg/L
pH 5.6

MMA medium: MS salts 4.4 g/L
MES 2.13 g/L
Sucrose 20 g/L
Acetosyringone 200 μM
pH 5.6

Protein Extraction Buffer: K phosphate pH 7.8 0.1 M
EDTA pH 8 1mM
Glycerol 10%
Triton X100 0.1%
β-mercaptoethanol 2 μL/mL
PVPP 2%

2.2. PLANT MATERIAL AND TREATMENTS

Plants of *Prunus persica* (L.) Batsch cv. *Redhaven* were grown in a field near Padua. Fruits at various stages of development S1, S2, S3I, S3II, S4I and S4II (Zanchin *et al.*, 1994), corresponding to 40, 65, 85, 95, 115, 120-125 days after full bloom respectively, were collected and used either with or without a hormone treatment. The ethylene treatment was provided by placing whole fruits (attached to a branch for all stages but for the S4s) in a sealed chamber and flushing them with ethylene (10 μL/L) in air at a flow rate of approximately 6 L/h. The auxin treatment was performed by dipping whole fruits in 1-naphthalene acetic acid NAA, 2 mM (added with Silwet L-77 (200 μL/L) as surfactant) for 15 minutes; thereafter, fruits were sprayed with the NAA solution every 12 hours over a period of 48 hours. In the same time, some fruits (samples without treatment) were maintained in air for 48 hours. Flowers were collected at full bloom, most likely after pollination. Leaves were collected in mid summer, fully expanded and without any evident signs of senescence. Both treated and untreated samples were frozen in liquid nitrogen and stored at -80 °C for subsequent use.

Plants of *Stark Red Gold* nectarines were grown at the experimental farm of the University of Bologna and gently provided by prof. G. Costa. The double sigmoid growth pattern was established based on fruit diameter, which was monitored once a week on 40 fruit during the growth cycle. The first derivative was calculated in order to discriminate the four growth stages S1-S4. *Stark Red Gold* nectarines were collected at the S3-S4 transition (corresponding to 123 dAFB). In order to obtain homogeneous fruit at different stages of the ripening process, immediately after harvest fruit were graded into 3 classes by decreasing ranges of the index of absorbance difference:

- class 0: I_{AD} 1.2-0.9;
- class 1: I_{AD} 0.9-0.6;
- class 2: I_{AD} 0.6-0.3.

The I_{AD} is a non-destructive marker of peach fruit ageing which is calculated as the difference in absorbance between two wavelengths near the chlorophyll-*a* absorption peak (670 and 720 nm; Ziosi *et al.*, 2008). Fruit from the 3 classes could be classified as belonging to pre-climacteric (class 0), onset of climacteric

(class 1) and full climacteric (class 2) stages of the ripening process. One hundred fruit per class, homogeneous for size and position on the plant, were placed in two sealed 30-L plastic jars (50 fruit each). SmartFresh^{TM} (AgroFresh Inc., 20 Philadelphia, PA, USA), a commercial powder containing 0.14% (w/w) 1-MCP, was prepared as a 10-fold concentrated stock solution following the technical bulletin of the company and injected as 10 mL of air (final concentration 1 μL/L equivalent to 1 ppm). On the same experimental conditions, 100 fruit belonging to classes 1 and 2 were incubated also with 5 μL/L 1-MCP. The same total number of fruit per class was kept in two sealed jars for 12 h at 25 °C without 1-MCP (air controls). At the end of treatments, temperature, ethylene and CO_2 concentration within the jars were determined. Treated and control fruit were then transferred in a growth chamber at 25°C. At the end of treatment (12 h) and at each following sampling time, ethylene production and flesh firmness were assessed on 20 control and 20 treated fruit. For molecular analyses, mesocarp from a pool of 10 fruits per class was frozen in liquid nitrogen and stored at -80°C until use.

2.3. RNA EXTRACTION

Total RNA was extracted from leaves, flowers and fruits as described in Chang *et al*. (1993). 4 g of fruit sample were grinded in a mortar with liquid nitrogen. The powder was poured in a tube with 20 mL of CTAB extraction buffer preheated at 65°C. After strong agitation, 20 mL of chloroform/isoamyl alcohol (24:1 v:v) were added, the sample was placed on a orbital shaker for 15 minutes and then centrifugated at 4000 x g for 15 minutes. The extraction with chloroform/ isoamyl alcohol was repeated twice. RNA was precipitated overnight with LiCl (2 M final solution). The day after, the samples were centrifuged at 4000 x g for 60 minutes at 4°C. The pellet (containing the RNA) was washed with 5 mL of cold ethanol 80% and then it was resuspended in H_2O mQ DEPC.

2.3.1. DETERMINATION OF THE CONCENTRATION OF NUCLEIC ACIDS

RNA yield and purity were checked by means of UV absorption spectra (Perkin-Elmer UV/Vis Lambda) with the following wavelengths: 230 nm, 260 nm, 280 nm, 320 nm. Readings were carried out in a quartz cuvette and the H_2O mQ was used as blank. The concentration of RNA was expressed in μg/μL:

$$OD_{260} * 40 * \text{dilution factor}$$

The peak of absorption of proteins is 280 nm, while 230 nm is for sugars, so the ratio with OD_{260} allows to understand the purity of the sample. If OD_{260}/OD_{280} is more than1.8, the sample is free of protein contaminations; in the same way, if OD_{260}/OD_{230} is more than 1.8, the sample is free of sugar contaminations. RNA integrity was ascertained by electrophoresis in agarose gel with TAE 1X buffer followed by ethidium bromide staining.

2.4. MICROARRAY EXPERIMENTS

The technique is based on the synthesis and labeling of cDNA from total RNA with the use of oligo dT and a high fidelity retro-transcriptase. At the end of the first strand synthesis a RNA/cDNA hybrid was obtained, so it was necessary to hydrolyse the RNA with incubation in NaOH 0.2 M. After a purification step, the amino-modified cDNA was coupled with a monoreactive, N-hydroxysuccinimidi (NHS)- ester fluorescent dye: the green-fluorescent cyanine3 (Cy3) and the red-fluorescent cyanine5 (Cy5) (Amersham Biosciences, UK). A final purification step removed any unreacted dye and the fluorescently labeled cDNA was ready for hybridization to microarrays.

2.4.1. cDNA SYNTHESIS AND LABELING

Total RNA (15 μg) from peach fruits was converted into target cDNA by reverse transcription using the SuperScript™ Indirect cDNA Labeling System (Invitrogen, USA) following manufacturer instruction, as already described in

Trainotti *et al.* (2006). The SuperScript™ Indirect cDNA Labeling System is a highly efficient system for generating fluorescently labeled cDNA for use on microarrays in gene expression studies.

15 µg of total RNA was prepared in a tube with 2 µL of anchored oligo $(dT)_{20}$. The final volume was 18 µL with H_2O mQ DEPC. The tube was incubated at 70°C for 5 minutes (denaturation of the secondary structure of RNA) and then placed on ice for at least 1 minute. The following components were added:

5X first-strand buffer	6.0 µL
0.1 M DTT	1.5 µL
dNTP mix	1.5 µL
RNaseOUT™ (40 U/µL)	1.0 µL
SuperScript™ III RT (400 U/µL)	2.0 µL
	12.0 µL

The sample was incubated at 46°C for 4 hours. After cDNA synthesis, the hydrolysis reaction was performed to degrade the original RNA. 7.5 µL of NaOH 1 M were added to each reaction tube and then the incubation was placed at 70°C for 10 minutes. 7.5 µL of HCl 1 M were added to neutralize the pH, Tris-HCl 10 mM was added to obtain a final volume of 400 µL and then all was transferred in the Millipore Microcon column to purify after the first strand synthesis. The sample was centrifuged for 15 minutes at 1000 x g, it was washed with 100 µL of H_2O mQ and centrifuged another time for 4 minutes at 1000 x g. The elution was performed with 60 µL of H_2O mQ (the membrane was turned) by a centrifugation of 3 minutes at 1000 x g. The eluate represented the cDNA. The purity and yield of the first strand cDNA synthesis was calculated from the OD values obtained by means of a spectrophotometer at the following wavelengths: 230 nm, 260 nm, 280 nm, 320 nm. The samples containing the cDNA was dried in a *speed-vac*, then the pellet was resuspended in 5 µL of Coupling Buffer 2X. The fluorescent dye (Cy3 e Cy5), resuspended in 5 µL di DMSO, was added to bring the total volume of 10 µL. The incubation was performed over night at room temperature in the dark because the fluorescent dyes were sensible to light.

Then, the labeled cDNA was purified to remove any unreacted dye with the S.N.A.P.^{TM} (Invitrogen) column. In the tube with the dye-coupled cDNA solution, 20 µL of 3 M Sodium Acetate, pH 5.2 were added with 500 µL of Loading Buffer. The sample was mixed well by vortexing and then it was loaded onto the S.N.A.P.^{TM} column. It was centrifuged at 14000 x g at room temperature for 60 seconds. The collection tube was removed and the flow-through was discarded. 700 µL of Wash Buffer were placed onto the S.N.A.P.^{TM} column and then were centrifuged at 14000 x g at room temperature for 60 seconds. The collection tube was removed and the flow-through was discarded. The wash was repeated twice. The S.N.A.P.^{TM} column was placed onto a new amber collection tube. The elution was performed with 60 µL di H_2O mQ DEPC with a centrifugation at 14000 x g for 60 seconds. The flow-through contained purified dye-coupled cDNA. Then it was necessary to carry out the control of the purity and yield of the labeled cDNA with the OD values obtained by means of a spectrophotometer using the formulas reported in the SuperScriptTM Indirect cDNA Labeling System (Invitrogen) instruction manual.

Control of the purity and yield

Absorbance at several wavelengths:

- 230 nm: peak of absorbance for the sugars;
- 260 nm: peak of absorbance for nucleic acids;
- 280 nm: peak of absorbance for proteins;
- 320 nm: background in UV range;
- 555 nm: peak of absorbance for Cy3;
- 650 nm: peak of absorbance for Cy5;
- 750 nm: background in visible range.

To calculate the amount of labeled cDNA and the amount of fluorescently labeled dye, the formulas were:

cDNA (ng) = $(A_{260}-A_{320})$ * 37 ng/µL * volume in µL

Cy3 (pmoli) = $(A_{555}-A_{650})$ / 0.15 * elution volume in µL

Cy5 (pmoli) = $(A_{650}-A_{750})$ / 0.25 * elution volume in µL.

2.4.2. MICROARRAY HYBRIDIZATION

Microarray experiments were carried out using the μPEACH 1.0 platform, developed within the ESTree consortium (ESTtree, see the web site http://linuxbox.itb.cnr.it/ESTree). μPEACH 1.0 is an oligonucleotide microarray carrying 4806 gene-specific probes (70 bases long), selected on cDNA sequences mostly obtained from fruit libraries (ESTree Consortium, 2005; Trainotti *et al.*, 2006). The probes have been synthesized by Operon (from which the Peach Array-Ready Oligo Set is commercially available at www.operon.com). Each of the 4806 oligos, harbouring a 50 amino linker, was deposited onto glass slides (Perkin-Elmer, USA) at CRIBI (University of Padova) using GenpakARRAY spotter (Genetix Inc., Massachusetts, USA) in 32 subgrids (4 columns x 8 rows) with a replicate in the same subgrid. To facilitate image analysis reference spots have been deposited in each first line/column of each subgrid. Distance between spots was 135 μm on either axes and spot average diameter was ranging from 70 to 80 μm.

Pre-hybridizations were carried out by soaking whole glass slides in a solution containing 5X SSC, 0.1% SDS, 5X Denhardt's solution and 100 ng/μL DNA carrier at 48°C for at least 3 h. Then the slides were washed once with a 0.2X SSC solution, rinsed with isopropanol and dried by centrifuging for 2 min at 1000 rpm. Hybridizations were carried out in 250 μL of hybridization solution (5X SSC, 0.1% SDS, 25% formamide), pre-heater at 65°C, containing 100 pmol of Cy3- and Cy5-labeled target cDNAs. The hybridization solution was kept in place by means of a microarray *gene frame* (ABgene, UK), while the glass slides were placed in a hybridization chamber (*Gene Machine*), (*HybChamber*^{TM}, by Genomic Solutions, USA) kept on a rotary oven for at least 36 h at 48°C.

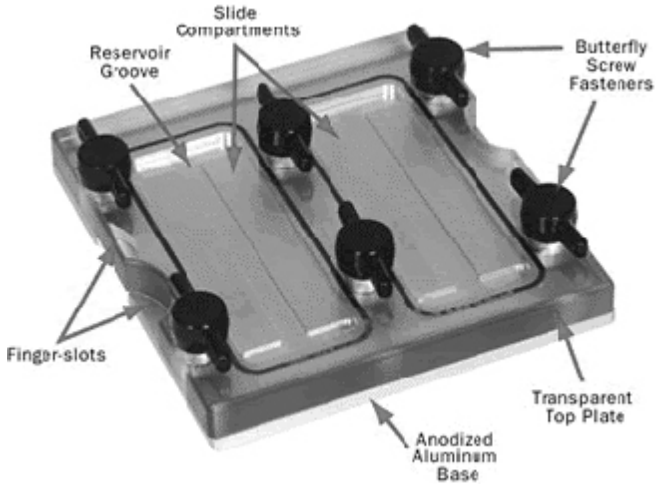


Fig. 2.1. Hybridization Chamber (*HybChamber*™, *Gene Machine)* used to carry out the experiments.

Then several post-hybridization washes were executed:

- a brief rinse with 1 X SSC + 0.1% SDS solution;
- a wash with 1 X SSC + 0.1% SDS solution for 5 min;
- a wash with 0.2 X SSC + 0.1% SDS solution for 5 min;
- a brief rinse with 0.2 X SSC solution;
- two washes with 0.2 X SSC solution for 5 min (to eliminate completely the SDS residues that may cause shadows on the spotted area).

These washes were performed at room temperature before drying the glass slides with a brief centrifugation. The probe design and these hybridization/washing conditions allow the detection of specific genes even within gene families.

2.4.3. DATA ANALYSIS

The microarrays were scanned with a two channel confocal microarray scanner (ScanArray® Lite, Perkin Elmer, USA) using its dedicated software (ScanArray Express 3.0.0, Perkin Elmer). The laser power and the photomultiplier tube (PMT) were set between 75 and 85% of maximum. The excitation/emission

settings were 543/570 nm for Cy3 and 633/670 nm for Cy5. At the first step, the slide was scanned very quickly at a low resolution (30 or 50 µm/pixel). After laser focusing and balancing of the two channels, scans were conducted at a resolution of 5 µm. For any scan, two separate 16-bit TIFF images were produced.

Software from the TM4 (www.tm4.org) package developed at TIGR (*The Institute for Genomic Research,* www.tigr.org, Saeed *et al.*, 2003) was used to analyze microarray data. The images were processed using the *Spotfinder* 2.2.3 software. The program received the two scan images and then it transformed the light intensity in numbers to obtain a text file where the fields were separated by tabulations. A virtual grid was associated with the microarray slide, where each spot was in the middle of the grid square. It was necessary to specify the dimensions of each spot (for this analysis, 15 and 30 pixels). *Spotfinder* executed the *Process all* command, in this way, the program used the Otsu algorithm to process automatically all the grids to extrapolate the fluorescent intensity and the local background for each element. Spots were also visually examined to delete the non-uniform ones.

The expression data extracted by *Spotfinder* were normalized by MIDAS 2.18 (*Microarray Data Analysis System)*. This critical step can help to compensate for variability between slides and fluorescent dyes, as well as other systematic sources of error, by appropriately adjusting the measured array intensities. In this way, data from many individual hybridizations can be treated in a uniform and reproducible manner. MIDAS reads ".tav" files generated by TIGR *Spotfinder*. Normalization was performed using the LOWESS (Locally Weighted Regression Scatter Plot Smoothing, Cleveland, 1979) algorithm with the "block mode", keeping as reference the Cy3 channel. The ratios were transformed to their $\log_2$ values. A $\log_2$ transformation converts the expression values to an intuitive linear scale that represents two-fold differences.

After normalization, data from each slide were split in two, by using Microsoft Excel, since each probe was spotted twice on µPEACH1.0. Thereafter, each spot value was considered independent. Normalized split data were loaded in MeV 3.1 (TIGR Multiexperiment Viewer). It is an application that allows the viewing of processed microarray slide representations and the identification of genes and expression patterns of interest. The data were analyzed with SAM (*Significance*

Analysis of Microarrays, Tusher *et al.*, 2001). This program can be used to pick out significant genes based on differential expression between sets of experiments. A valuable feature of SAM is that it gives estimates of false discovery rate, which is the proportion of genes likely to have been identified by chance as being significant. For the experiments, each comparison was repeated at least twice, there were at least 4 values for each gene to be used in the SAM analyses. Lists of clones with significant changes in expression among at least two experimental samples were identified at delta values that gave a false discovery rate (FDR) of 0% for the S3-S4 transition and hormone treatments, while a FDR of 5% was used in the 1-MCP experiment.

2.5. REAL TIME PCR

To validate some results obtained with microarray analysis, several experiments of quantitative Real Time PCR were conducted. Real-time polymerase chain reaction is a technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted DNA molecule. Real Time PCR is mainly used to provide quantitative measurements of gene transcription. The technology may be used in determining how the genetic expression of a particular gene changes over time, such in response of tissue or different treatments.

The cDNAs were synthesized by means of the "High Capacity cDNA Archive Kit" (Applied Biosystem), which uses random examers as primers. 10 μg of total RNA, pre-treated with 1.5 units of DNaseI, were used as starting template. Reactions were carried out using 25 μL of the “Syber green PCR master mix” (Applied Biosystems), with 0.05 pmoles of each primer, in the “7500” instrument (Applied Biosystems). PCR conditions were as follow:

- 50°C for 2 min
- 95°C for 10 min (incubation to activate the enzyme)
- 40 times: denaturation at 95°C for 15 sec
  annealing at 60°C for15 sec
  extension at 65°C for 34 sec.

At the end of PCR reaction, the dissociation curve was performed at 60°C.

The Internal Transcribed Spacers of the ribosomal RNA were used to amplify the internal standard as housekeeping gene. The primer sequences were as follow:
DZ79: TGACCTGGGGTCGCGTTGAA
DZ81: TGAATTGCAGAATCCCGTGA.
The primer sequences used for each target gene are reported in table 1.
The obtained Ct values were analysed by means of the "Q-gene" software (Muller *et al.*, 2002) by averaging three independently calculated normalized expression values for each sample. The numerical values obtained with these calculations were transformed into graphics by means of the "GraphPad" software (GraphPad Software, USA).

seq name	oligo name	oligo sequence	ctg acronym
42	ctg_42_for	CGGCCGGCTTGAACTACGAC	Aux/IAA
	ctg_42_rev	GGCCCCATCACCATCAGCACA	
57	TF22	CCAAGACCAACAAGGACAACA	Aux/IAA
	TF9	CATGCTCACCTTCACCA	
64	ctg_64_for	CCCCCATGCGCCACTCCA	ACO1
	ctg_64_rev	CATCACTGCCAGGGTTGTAAAAG	
84	ctg_84_for	CAGCAACACCTCCTCCTCCTA	Aux/IAA
	ctg_84_rev	TCTTGCTTGCATGCTGTTTTT	
112	ctg_112_for	CCTGATGGACACCCTCTAAAG	PDC
	ctg_112_rev	CAACCAGCCGGCAATCTCAG	
134	ctg_134_for	CCACAACCACTAACACCCCTTCAA	Unknown
	ctg_134_rev	TTAGCTTTCGCATCACCATCTTCC	
298	ctg_298_for	TGTTTCCTTTGATCTTGGCTGGTC	bZIP
	ctg_298_rev	AAGATGGGTTTGGGGATTTTGA	
356	ctg_356_for	GGCGACCACCTGAATTTTATCCA	ERS1
	ctg_356_rev	GGCAAGTCCAAGTCCAGCACCAT	
358	TF15	GACTCAACCAGCCAAAACAA	Aux/IAA
	TF30	CTTTGATGTAGTGGCCAGAGTT	
420	ctg_420_for	GGGGGAGGCCAAGCACTG	PG
	ctg_420_rev	TGAACTCCGGAAACCTGACCA	
475	ctg_475_for	GACGGGATTTGCATCATTCATTTC	IAA amidohydrolase
	ctg_475_rev	CTTCCAATAGGAGCAGCACTTCAT	
489	ctg_489_for	TGTTCAGCTCCCCGACTTTCAC	ACS1
	ctg_489_rev	TCTTGCGGCCGATGTTCACC	
671	ctg_671B_for	TTGCTTTGTTTGCATTTTCTTTGA	Aux/IAA
	ctg_671B_rev	AGATCCATTGCTCTGTCCTTCCTC	
768	ctg_768_for	CCACCGGATGCTGTTGACGA	Aux/IAA
	ctg_768_rev	CGCTGCAGCTACCACTCTTGA	
938	ctg_938_for	AAGCTTTGTACCTATGCCCTCTCC	PEI
	ctg_938_rev	AATCCTTGACCACCCCTTTCTCG	
941	ctg_941_for	TGGGTCTTCTTGCAATGGTCTCCT	EXP2
	ctg_941_rev	GCTCAGTGCTGCAGTATTTGTCC	

945	ctg_945_for	AAACAGCCTCGCCGAACTTGACA	LHCB2
	ctg_945_rev	ACTACGGATGGGACACTGCTGGAC	
974	ctg_974_for	CAAAGCCGCCACAGGAAAAATC	RD22-like
	ctg_974_rev	TCTCGGGTAACTTGCTGCTTGAAA	
1024	ctg_1024_for	GGGAGCCAGTGCAAAGGGATTCT	CAT
	ctg_1024_rev	GTTTCAGGGCTGCCACGCTCAT	
1151	ctg_1151_for	TCATGGGACAAATCAGAGGAGGAA	ACS3
	ctg_1151_rev	TGGCACAAGCAAAGCATCACC	
1436	ctg_1436_rev	TCCCTTGCCCTCATTGACTCTTCT	ETR1
	ctg_1436_for	AACGCTATGCTTTGATGGTCTTGA	
1505	ctg_1505_for2	GGGAGGAGTTTGTAAACTGTGTTC	ARF
	ctg_1505_rev	CATTAGGCATTCCCACCATCTGAG	
1541	ctg_1541_for	GAGAGCTGTCCTGTTGAGAAG	TIR1
	ctg_1541_rev	GGGTTTGAATCCGTGGGTGTC	
1727	ctg_1727_for	AACATCTCCTACAAGCATCCAATC	Aux/IAA
	ctg_1727_rev	GTTTTTCAGCCCCACTACAGC	
1741	ctg_1741_for	GTCTTGGGTTGAGCCTTGGTGGAG	Aux/IAA
	ctg_1741_rev	GGACGACGAAGAAGCTGATGATGA	
1991	ctg_1991_for	TCACAGCCTCAGACTCAAACTCAG	ARF
	ctg_1991_rev	TCATGGCAGGGACAGCACTT	
1993	ctg_1993_for	AAGAGCGGCACGTTTGAGGAGTT	GH3
	ctg_1993_rev	CAATGCGGTAAAGATGGGCTAAAA	
2025	ctg_2025_rev	ACCGCTGGAATAGTGCCCTCTGA	ETR2
	ctg_2025_for	TTCGGGTTGCTTCGGATAATGG	
2116	ctg_2116_for	AGGGGTTCGAGTTTGGCTTGGTA	ERF2
	ctg_2116_rev	GTTTGGGTGGGAATGTCGTCGTC	
2568	ctg_2568_for	TTTGAAGAACCCAGAAGCCTCCAT	ACS2
	ctg_2568_rev	ATAACAATCCGGTCGGGGTCAAA	
2713	ctg_2713_for	GGGTGACTGAATCTGGGTTTG	TIR1
	ctg_2713_rev	TGGTTGCCTTGGGTTCATTAT	
2902	ctg_2902_for	TGGCCGGCATGGTAGTCACG	ST
	ctg_2902_rev	GCCCATCCCGATCGAGAAGAAC	
3371	ctg3371_for	AAGTGCAGGCCTGGATTACCC	W synt
	ctg_3371_rev	TAGGCCAGTGCATGAGAAGTC	
3575	ctg_3575_for	ACAACCGCAATCTGGAAACAT	IGPS
	ctg_3575_rev	TAGGCAATATCATCAGGAGTG	
3678	ctg_3678_for	TTTGGTGACCGGGAGAATGA	ARF
	ctg_3678_rev	TGGGACAGAAGCCGCACAAT	
3721	ctg_3721_for	ATGATGGCGGCTGGGAGGAACT	PIN1
	ctg_3721_rev	TTGCTGGCCGCCGTGGTAAA	
4040	ctg_4040_for	TTACCAAGCGCAAGGGAATG	ARF
	ctg_4040_rev	TCGAAATGTGATGGGGGTGGAG	
5158	ctg_5158_for	TGTTGCAGGATTCATGTCTCAGTC	ACS4
	ctg_5158_rev	CAGGGTAATATGGTGCAGGAACAA	
ACO2	ACO2_for	CAGCCGGATGGTACCAGAATGTC	ACO2
	ACO2_rev	ACACAAATTTGGGGTAGGCTGAGA	
ACS5	ACS5_for	ATGGCGGAAATGAGGGGTAACA	ACS5
	ACS5_rev	CTGGGTAGTATGGAGTGGGAAGGA	

Table 1. List of the oligonucleotides used in the Real Time PCR experiments.

2.6. BIN MAPPING

A *Prunus* reference map was constructed with an almond x peach F_2 population. On the basis of this map, a set of six plants that jointly defined 65 possible different genotypes for the codominant markers mapped on it was selected. Sixty-three of these joint genotypes corresponded to a single chromosomal region (a bin) of the *Prunus* genome and the two remaining corresponded to two bins each (Howad *et al.*, 2005).

Bin mapping proved to be a fast and economic strategy that could be used for further map saturation, the addition of valuable markers (such as those based on microsatellites or ESTs) and giving a wider scope to, and a more efficient use of, reference mapping populations.

2.6.1. BIN MAPPING IN T X E POPULATION

The population used was the F_2 obtained from selfing a single plant of the cross between "*Texas*" almond and "*Earlygold"* peach. Marker data are available for 88 plants of this population (referred to as the T X E population) and the marker data set used was that of the most recent map (Dirlewanger *et al.*, 2004). The main criterion for selecting the set of plants for bin mapping (the bin set) was for the number of plants included in this set to be minimal. Additional criteria were a good combination of the following: the minimal number of joint genotypes that each corresponds to more than one bin (''duplicate bins''), the smallest maximum bin length and the highest number of bins (minimal average bin length). It was found that fewer than six plants would generate a high number of duplicate bins (Howad *et al.*, 2005). Six was considered a desirable size, because a set of eight individuals (six plants of the F_2 plus the two parents or one parent and the F_1 hybrid) would be enough for bin mapping. Eight is a suitable unit of analysis as the plates used for PCR reactions are usually of 8 X 12 wells or multiples of this number.

2.6.2. PCR AMPLIFICATION AND SEQUENCING

The DNA from *Earlygold* parent, individual F_1 hybrid and the 6 selected individuals of F_2 population was used. After carrying out the PCR (with the proper primers reported in table 2), the products were purified into the plate with Sephadex G-50. The samples were loaded into the plate and centrifuged for 2 minutes at 2000 rpm. Then the purification of PCR products was checked by agarose gel. PCR sequencing was carried out (with BIG DYE of Applied Biosystem kit) and then the products were precipitated (with EDTA 125 mM, Na acetate 3 M and ethanol absolute) and resuspended in formamide HIDI (Applied Biosystem). The plate was loaded into the sequencer ABI PRISM. The sequences were analyzed to discover the segregation of SNPs (Single Nucleotide Polymorphisms) with the *Sequencer* program.

seq name	oligo name	oligo sequence	ctg acronym
39	ctg39_for_map	CCTGATACACTTCGGCTTAC	hypothetical prot
	ctg39_rev_map	CTTTCATTTTTGGTCTTTGG	
42	ctg_42_for	CGGCCGGCTTGAACTACGAC	Aux/IAA
	ctg42_rev_map	TCCAATCCCCATCTCTGTCCTCAT	
57	ctg57_for_map	CCCGAGTGATAAAGAAAGTG	Aux/IAA
	ctg57_rev_map	TTTGGTGGTTTTGGTTTATT	
64	ctg_64_for	CCCCCATGCGCCACTCCA	ACO1
	ctg_64_rev	CATCACTGCCAGGGTTGTAAAAG	
84	ctg84_for_map	TCGATTAAGATGGAAGGGTCAGTG	Aux/IAA
	ctg84_rev_map	CCCAAGCAAAATTAAGGAGTTCC	
85	ctg85_for_map	CGCTTTGGGCGATATGTCTGTC	calcineurin
	ctg85_rev_map	GCTCGTAGCGCTTTAAATGTTCAA	
134	ctg134_for_map	TCATTCATTCCTAGCCCTAA	unknown
	ctg134_rev_map	ACAGCCTCTAGTGCTGCTAC	
298	ctg298_for_map	CTGGTTTTTGTTTCCAGAAG	bZIP
	ctg298_rev_map	ATCTGAGCCATCAAATCATC	
349	ctg349_for_map	CAGGGCTCCTAGTAAGTGTG	PIP
	ctg349_rev_map	GAACCAAGAAAACTGCAAAC	
356	ctg356_for_map	TTGAGCTGGGTAGGACTTTA	ERS1
	ctg356_rev_map	CAGAATAGCAGCATGTGAAA	
358	ctg358_for_map	CATTTAGGAGGCAAACTCTG	Aux/IAA
	ctg358_rev_map	GTTCCTGTTCCTGCACTTC	
475	ctg475_for_map	AAACTTGGGGGAAATTTTAG	IAA amidohydrolase
	ctg475_rev_map	TCTTTATCCCAACAACAAAGA	
489	ctg489_for_map	CAAGACTATCATGGCTTTCC	ACS1
	ctg489_rev_map	TAGATTTCGTCACACACCAA	

544	ctg544_for_map	CGTCTCTACAAACCCTTCAC	GA2 ox
	ctg544_rev_map	TACGACATTTTCCCATTTCT	
671	ctg_671B_for	TTGCTTTGTTTGCATTTTCTTTGA	Aux/IAA
	ctg_671B_rev	AGATCCATTGCTCTGTCCTTCCTC	
768	ctg768_for_map	AACTACTCCTCCTCCAGCTC	Aux/IAA
	ctg768_rev_map	CAGCCTCTGAACTTTTCATC	
974	ctg974_for_map	GAAACCTGAATCAGCAGAAG	RD22-like
	ctg974_rev_map	CAGTTCCTGGCTTAACTTTG	
1068	ctg1068_for_map	CCAAATGGCCAAAGAAGGTTTAG	Aux/IAA
	ctg1068_rev_map	TCAGCCTCTTGCATGACTCAATAA	
1151	ctg1151_for_map	GACCAAGACCAAATTGAAAC	ACS3
	ctg1151_rev_map	GAATTTGCGCAGAACATAAT	
1310	ctg1310_for_map	CAAGAAAAAGTCACCTCTGC	NAM
	ctg1310_rev_map	ATGCGAATTGTTCTTCTTGT	
1357	ctg1357_for_map	TGAGTAGCCAGCAGGAGTAT	MADS box
	ctg1357_rev_map	GACCACTGATATTGGATCGT	
1375	ctg1375_for_map	ACTGCTAAGGACAGCAATGT	nitrilase
	ctg1375_rev_map	AAAGTCACTGGATTTGTTGG	
1436	ctg_1436_rev	TCCCTTGCCCTCATTGACTCTTCT	ETR1
	ctg_1436_for	AACGCTATGCTTTGATGGTCTTGA	
1505	ctg1505_for_map	TTGCACGCTATTCAGGTTATGGTG	ARF
	ctg1505_rev_map	TTCAGCTTCTTTCCACTGCTTGTG	
1541	ctg1541_for_map	GGGGCTTCATCATGTATTGTCTGG	TIR1
	ctg1541_rev_map	CTGCAGCTGGTATACATGGTGGTG	
1727	ctg1727_for_map	TATGGCCTACCAGAAGAAAA	Aux/IAA
	ctg1727_rev_map	TATTGGTGGAGACAAGGAAC	
1741	ctg1741_for_map	GTGCACAGAGTTGCTATGAA	Aux/IAA
	ctg1741_rev_map	CTTTCCAAAAGCTCCAACTA	
1991	ctg1991_for_map	TGTGGCTTCGAGGAGATAATGGAG	ARF
	ctg1991_rev_map	GGAAACGGTGGCAACGAAATAGAA	
1993	ctg1993_for_map	AACTCGGTTTACCGACAAG	GH3
	ctg1993_rev_map	TTTTCATTCTTTCCACGAGT	
2025	ctg2025_for_map	CTTCGGATAATGGAAGTCAG	ETR2
	ctg2025_rev_map	TAGCTTCTCAAGCAGTCTCC	
2116	ctg2116_for_map	CCGACAATTTCTTCGACTAC	ERF2
	ctg2116_rev_map	AAATTGTTGCAATCTGGTTT	
2195	TF 54	GGCACAACAGCAGCAAACAA	MADS box
	TF55	CGCCTCCAAGATTACAGGGA	
2568	ctg2568_for_map	CTATCATGGACTGCCAGAAT	ACS2
	ctg2568_rev_map	TGATTTCTGCAATGCTTATG	
2713	ctg2713_for_map	GGCCCTTTATCTCCCAGTTCTG	TIR1
	ctg2713_rev_map	TTAGATCCAACAGCCTCCAACC	
2797	ctg2797_for_map	ATTCAGGTGGTTTATGATCG	Aux Efflux
	ctg2797_rev_map	TGAAGAGCTTTCCATTTCAT	Car
2902	ctg2902_for_map	CTCGACAAAACCTCAGACTC	ST
	ctg2902_rev_map	TAATACCATGGTCAAGCACA	
2980	ctg2980_for_map	TGTTTCTACCGGAGAGGTAA	NCED
	ctg2980_rev_map	AGTCCAAAAGCTACAAGCTG	
3371	ctg3371_for	AAGTGCAGGCCTGGATTACCC	W synt
	ctg_3371_rev	TAGGCCAGTGCATGAGAAGTC	
3575	ctg3575_for_map	GGCTTGGCAAATCTACTATG	IGPS

	ctg3575_rev_map	GGGTCACTTTGTTTCACAAT	
3678	ctg_3678_for	TTTGGTGACCGGGAGAATGA	ARF
	ctg_3678_rev	TGGGACAGAAGCCGCACAAT	
3721	ctg3721_for_map	GAAGGTCTCAGGGATTGTCGTC	PIN1
	ctg3721_rev_map	GTTGGGGTTTCTGATGAGTTTTCT	
3945	ctg3945_for_map	TTGCAGATGCATATCTCTTG	Subtilisin
	ctg3945_rev_map	ATAATTCAAGGCACAGAGGA	
4040	ctg4040_for_map	GCAAGGGAATGCTAGATATG	ARF
	ctg4040_rev_map	TTTTGGTCAGACTCGAAAGT	
4056	ctg4056_for_map	GCAGCAAAATTACTCCAAAG	IAA
	ctg4056_rev_map	CAGATTCAGGGATCACATTT	amidohydrolase
4705	ctg4705_for_map	CCCCTTCATCTAACATTTTCT	IAA
	ctg4705_rev_map	GAGTCACCTGCATCTTTAGC	amidohydrolase
5158	ctg5158_for_map	ACTGCTGCCATTGAGATACT	ACS4
	ctg5158_rev_map	TAGATAACACCCGCCTTAAA	
ACO2	aco2_for_map	TCATGAAAGAATTTGCAGTG	ACO2
	aco2_rev_map	ATCACCTGGGTTGTAGAATG	
ACS5	acs5_for_map	TCTCTTACGCAATTCCACTT	ACS5
	acs5_rev_map	TAGTATGGAGTGGGAAGGAG	
MADS	mads462_for_map	CTCAGATAGGGAATTTGCAG	MADS box
462	mads462_rev_map	AATTAATTTGGCTGAGACGA	

Table 2. List of the oligonucleotides used in the bin mapping experiments for DNA amplification.

2.6.3. BIN MAPPING ANALYSIS

For the notation, A and B were indicated for homozygotes for female ("*Texas*") and male ("*Earlygold*") alleles, respectively, and H for heterozygotes. The best AHB set included plants 5, 12, 23, 30, 34 and 83 of T X E population. This set detected a total of 67 bins.

Bins of the AHB set were coded with the linkage group number of the bin location, followed by a colon and then a two-digit number, corresponding to the position of the last marker included in the bin according to the map of Dirlewanger *et al.* (2004). For example, bin 8:60 ends with a marker 60 cM from the top of linkage group 8. All genes of interest were mapped in this way and with this notation.

2.7. CLONING OF PROMOTER SEQUENCES

2.7.1. PREPARATION OF PLASMID DNA

A single colony was inoculated in 3 mL of LB with the proper antibiotic and was grown over night at 37°C in rotary incubator. 1.5 mL of culture were put in Eppendorf tube and were centrifuged for 2 minutes at maximum speed. The pellet was resuspended in 200 µL of P1 resuspension solution (100 µg/mL RNAse A; 50 mM Tris-HCl, 10 mM EDTA, pH 8.0). Subsequently, 200 µL of P2 lysis solution (0.2 M NaOH, SDS 1%) were added and the tubes were inverted gently. Quickly 200 µL of cold P3 neutralization solution (3.0 M KAc, pH 5.5) were added; the sample was mixed and centrifuged at 4°C at maximum speed for 10 minutes. The surnatant was drawn and a same volume of phenol: chloroform: isoamyl alcohol (25:24:1, v:v:v) was added. The DNA was precipitated with 2.5 volumes of absolute EtOH incubating the tube at -20°C for 15 minutes. The solution was centrifuged at 16000 x g at 4°C for 15 minutes. The pellet was washed with 1 mL of EtOH 70% and it was centrifuged at 16000 x g at 4°C for 5 minutes. The pellet was dried and then it was resuspended in 50 µL of H_2O mQ.

2.7.2. CLONING IN THE EXPRESSION VECTOR pPR97

For the transient and stable transformations, a pPR97 vector (12.20 kb) that has the kanamicin resistance was used. The promoter sequences were cloned before the GUS reporter gene interrupted by a plant intron (Vancanneyt *et al.*, 1990). The plasmid was cut in the polylinker with the restriction enzymes and the promoter fragments were cloned in the vector with the proper restriction enzymes. The ligation reaction was carried out in a volume of 10 µL with 2 U of T4 DNA ligase (NEB), ligase buffer 1X, insert and vector in the 3:1 molar ratio, with an over night incubation at 14°C. Then the *E. coli* cells were transformed by electroporation with an electric discharge of 1500 V ("Invitrogen Electroporator II", capacity 50 µF). The bacteria were put in 1 mL of SOC at 37°C for 45 minutes and afterward were plated on LB supplemented with 50 µg/mL of

kanamycin. The colonies were controlled for the presence of the correct inserts by means of PCR and sequencing.

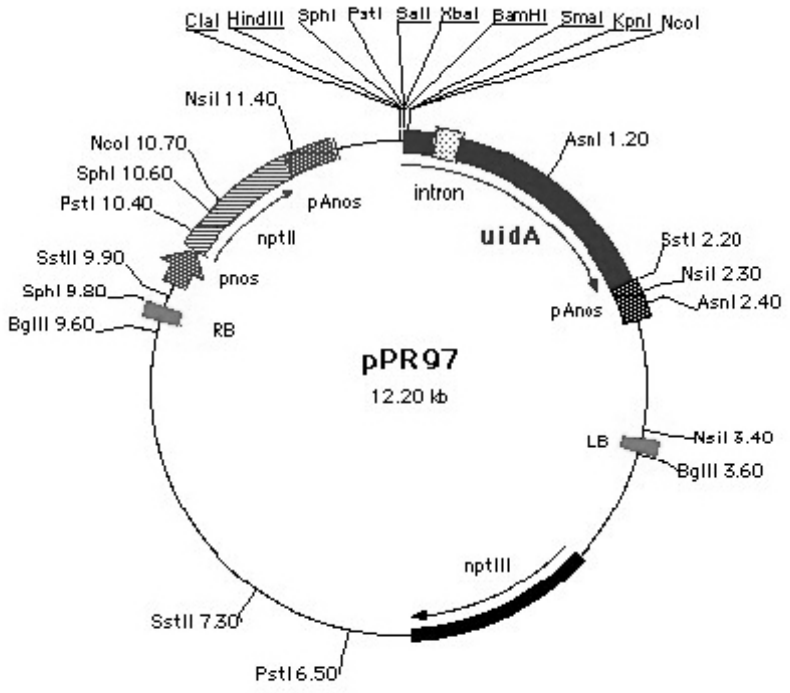


Fig. 2.2. Map of pPR97 vector.

2.7.3. CLONING IN THE EXPRESSION VECTOR ΔpGREEN

Another vector used for the study of the promoter sequences was ΔpGreen that was derived from pGreen (Hellens *et al*., 2000) by modifications carried out in the laboratory of Prof. Livio Trainotti. The ΔpGreen vector contained the GUS reporter gene interrupted by a plant intron driven by the CaMV 35S promoter. The expression of the GUS gene is used to normalize the one of a luciferase-intron reporter gene (LUC, Hanson *et al.,* 1999) driven by the promoter under investigation. The Gateway CC_rfA system allowed to clone the promoter sequences with a simple reaction of recombination because a Gateway LR cassette was present before the LUC gene.

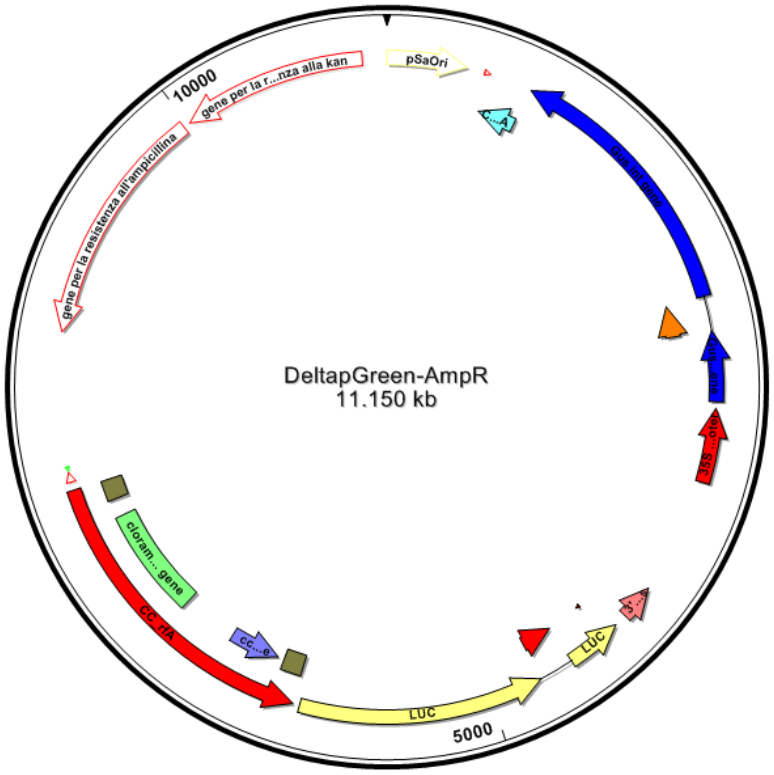


Fig. 2.3. Map of ΔpGreen vector.

2.8. TRANSFORMATION OF *AGROBACTERIUM TUMEFACIENS*

For the transformation, two different strains of *Agrobacterium tumefaciens* LB 4404 and LB 3101 were used. 0.5-1 µg of plasmid DNA were mixed with *A. tumefaciens* and the sample was incubated for 5 minutes on ice, 5 minutes in liquid nitrogen and 5 minutes at 37°C. It was diluted with 1 mL of YEB and it was shaken for 4 hours at 28°C and then the bacteria were plated on YEB medium with proper antibiotics (for strain LB 4404: kanamycin 50 mg/L and streptomycin 100 mg/L; for strain LB 3101: kanamycin 50 mg/L, gentamycin 25 mg/L, rinfampicin 100 mg/L).

2.9. TRANSFORMATION OF TOBACCO PLANTS

For the transformation of tobacco plants, the protocol Fisher and Guiltinan (1995) was used. 20 mL of YEB medium were inoculated and the culture of *Agrobacterium* was grown at 28°C. The sample was centrifuged at 3000 x g for 20 minutes at 4°C and the pellet was resuspended in 20 mL of MS medium. Some green and undamaged leaves were collected from tobacco plants and parallel cuts were realized with a knife on the leaf surface. The petiole was cut off. These leaves were soaked in the *Agrobacterium* culture for 10 minutes, they were dried with chromatography paper and then they were placed in TAB1 co-coltivation medium. After two days of co-coltivation at 25°C in the dark, the leaves were washed by immersion in MS medium and then they were dried on sterile paper. Leaves were transferred in plates with TAB2 medium and were placed in a growth chamber at 25°C with a photoperiod of 16 hours of light and 8 hours of dark until the callus growth. Shoots of 1-3 cm in length were slashed with a cut of 45° and then they were transferred in plates with TAB3 (the rooting medium). Each shoot was called with a serial number. After about 20 days, the plants were moved in soil and they were placed in a greenhouse.

2.10. TRANSIENT TRANSFORMATION OF PLANT MATERIAL

Plasmids used for transformation experiments were co-infiltrated with the pISV2678 vector supplied by Dr M. Schultze, Department of Biology, University of York, UK where the *LUCint* gene (Hanson *et al*., 1999) was cloned under the control of a double 35S promoter.

2.10.1 PLANT MATERIAL

Fruits belonging to cultivar *Redhaven* and *Fantasia* were collected in a field near Padova at various stages of development (S3II and S4I). Other peaches were

collected near Verona. These fruits belonged to cultivar *Tardivo Zuliani* and *Cal Red*.

Also tobacco plants grown in the greenhouse at the Department of Biology in Padova were used for the infiltration of leaves.

2.10.2. PREPARATION OF *AGROBACTERIUM* FOR INFECTION

Growth and induction of *Agrobacterium* was carried out according to Kapila *et al*. (Kapila *et al*., 1997). A culture of *Agrobacterium* GV3101 was grown at 28 °C in YEB medium, buffered with 10 mmol/L MES (2-(*N*-morpholino)ethanesulphonic acid) to pH 5.6 and rifampicin (100 mg/L), gentamycin (25 mg/L), kanamycin (50 mg/L) and acetosyringone (20 μmol/L) were added. When the culture reached an OD_{600} of about 0.8 it was centrifuged and the pelleted bacteria were resuspended (up to a final OD_{600} of 2.4) and incubated 1 hour at 22 °C in MMA medium. For peach fruits and tobacco leaves, after incubation the suspensions of *Agrobacterium* transformed with the two different reporter genes were mixed in a 1 (luciferase) to 3 (GUS) ratio due to the much higher sensitivity of the luciferase assay. Then, the resulting mixture was used in the injection experiments.

2.10.3. INFILTRATION OF PLANT MATERIALS

The *Agrobacterium* suspension was evenly injected throughout the entire fruits by means of a sterile 1 mL hypodermic syringe (Spolaore *et al.*, 2001). The thinness of its needle, besides minimizing the wound damages, allowed very fine control of the injections in fruits with epicarps easy to pierce, such as those used in this work. The outside of the injected fruits was dried to get rid of excess bacteria and the fruits were placed on moistened filter paper at 22 °C for 2 days with a 16 hours light photoperiod. The 2 days incubation time was chosen to be sufficient for detecting significant GUS activity while avoiding the formation of moulds on the fully ripe fruit.

Fig. 2.4. Infiltration of peach fruit with the *Agrobacterium* suspension by means of a sterile 1 mL hypodermic syringe.

In tobacco, the *Agrobacterium* suspension was injected in the lower page of the leaf with a sterile 2 mL syringe without needle.

2.10.4. ASSAY OF GUS AND LUCIFERASE ACTIVITIES

15 g of fresh peach mesocarp were ground in a mixer and protein extracted in 30 mL of extraction buffer (Luehrsen *et al*., 1992). The homogenate was centrifuged twice at 16000 x g for 15 min and the clear supernatant was used for either protein (Bradford, 1976) or reporter activity quantifications.

With tobacco leaves, a wedge for each transformed leaf was made and the samples were ground with a little pestle in a test tube in presence of liquid nitrogen. 1.5 mL of extraction buffer were added and then the homogenate was treated in the same way described above.

The GUS assay was carried out by incubating 300 µL of protein extract with the substrate 4-methylumbelliferyl-ß-D-glucuronide (MUG) at 37 °C. The released 4-methylumbelliferone (4-MU) was quantified with a DTX880 Multimode Detector (Beckman Coulter) according to the manufacturer's instructions. The GUS activity was expressed as nmol 4-MU released min^{-1} μg^{-1} protein (Jefferson *et al*., 1987).

50 µL of reaction were withdrawn at serial time intervals (5 minutes, 1 hour, 3 hours and over night) and the reaction was stopped in Na_2CO_3.

The luciferase assay was carried out on aliquots of 100 µL of the same protein extract used for the GUS assay. The used luminometer (TD-20/20 Luminometer, Turner Design) automatically injected 100 µL of substrate (Luciferase Assay Reagent, Promega) onto 100 µL of fruit protein extract. After 3 seconds incubation, the emitted light was measured over a period of 10 seconds. The luciferase activity was expressed as pg luciferase μg^{-1} protein. The standard consisted of 100 pg of purified luciferase (Sigma) dissolved in 30 µL of a protein extract from fruits transformed with a vector lacking the luciferase gene.

3. RESULTS AND DISCUSSION

3.1. AUXIN AND ETHYLENE EFFECTS ON THE CONTROL OF THE TRANSCRIPTION DURING PEACH RIPENING

3.1.1. MOLECULAR VALIDATION OF A NON-DESTRUCTIVE INDEX FOR MONITORING THE PROGRESSION OF PEACH RIPENING

In fruit and vegetables establishing the optimal harvest time is a crucial issue, since fruit shelf-life potential and quality are closely related to the ripening stage at harvest. The I_{AD}, developed by Prof. Costa's team at the University of Bologna (Italy), is an instrument that, by means of its absorbance properties, allows to measure the chlorophyll content in a fruit. The chlorophyll content in a fruit is a precise index of its ripening state. Thus, the I_{AD} allows to know the ripeness state and the way it works does not depends on the season weather conditions, a factor which influences other kind of measurements such as the brix index. The I_{AD} is a very promising tool both for practical and scientific applications, since it allows monitoring on tree fruit ripening.

Fig. 3.1. Portable spectrometer developed in the laboratory of Prof. Guglielmo Costa. Vis spectroscopy was used to develop an index characterizing ripening changes in peach fruit.

In peach fruit, establishing the optimal harvest time is a crucial issue, since fruit shelf-life potential and quality are closely related to the ripening stage at harvest. In order to develop a non-destructive index for monitoring the progression of ripening, the difference in absorbance between two wavelengths near the chlorophyll-*a* absorption peak (670 and 720 nm; index of absorbance difference, I_{AD}) was related to the time course of ethylene production during on-tree ripening of peaches. The relationship I_{AD}-ethylene production was used to classify fruit at harvest according to their ripening stage (class 0: pre-climacteric; class 1: onset of climacteric; class 2: climacteric). The transition from class 1 to 2 was marked by increased ethylene production and reduced flesh firmness (FF) and titratable acidity (TA). In contrast, fruit quality traits did not discriminate between fruit belonging to classes 0 and 1. Fruit with higher I_{AD} produced lower amounts of ethylene, began to soften later and maintained higher TA than those with lower I_{AD} (Ziosi *et al.*, 2008).

About the correlation of the I_{AD} with changes in ethylene production, in *Stark Red Gold* nectarines, ethylene production was undetectable (pre-climacteric) in fruit characterized by I_{AD} higher than 0.90 (Fig. 3.2); thereafter, ethylene emission levels remained low (onset of climacteric) until I_{AD} reached 0.60. At this I_{AD} value, ethylene production rose sharply, peaked when I_{AD} was 0.45 (climacteric; Fig. 3.2) and decreased thereafter (post-climacteric). A second increase in ethylene emission was registered in senescent fruit showing I_{AD} lower than 0.30. Thus, by correlating the I_{AD} with the changes in ethylene emission, nectarines could be sorted as belonging to pre-climacteric (class 0), onset of climacteric (class 1) and full climacteric (class 2) stages of the syndrome. Fruit graded at harvest according to this relationship were characterized by diverse ripening behaviour during shelf-life and by different quality traits at consumption (Ziosi *et al.*, 2008).

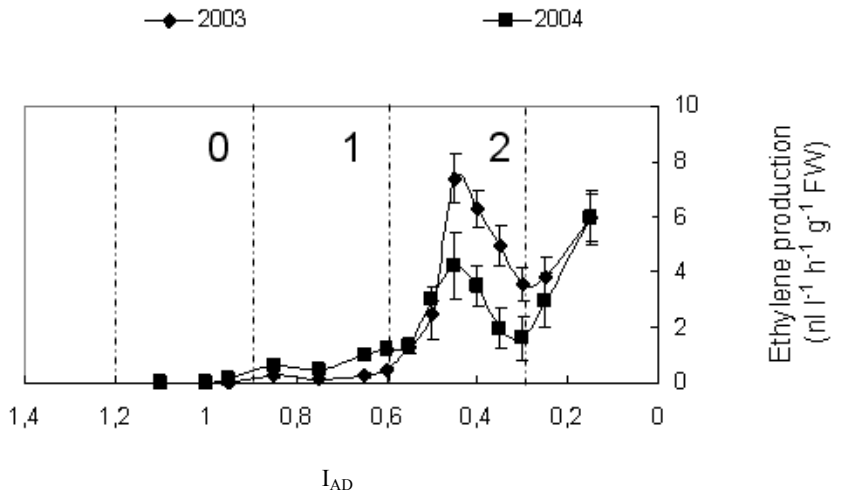


Fig. 3.2. Changes in ethylene production, as a function of the I_{AD}, during ripening of *Stark Red Gold* fruit in the seasons 2003 and 2004. Dashed lines indicate the I_{AD} ranges set in the season 2005 for classifying fruit as belonging to pre-climacteric (0), onset of climacteric (1) and climacteric (2) stages of ripening. Data represent the mean (n = 40)±S.D. Data obtained in the Prof. Costa's laboratory (Ziosi *et al.*, 2008).

The I_{AD} decrease observed in ripening peach fruit reflects the extensive chlorophyll breakdown occurring in the flesh during the establishment of the syndrome (Chalmers and van den Ende, 1975). In fact, a decrease of transcript amounts of ctg945, encoding for a light harvesting chlorophyll *a*/*b* binding protein (Lhcb), was observed in the three selected classes (i.e 0, 1 and 2; Fig. 3.3).

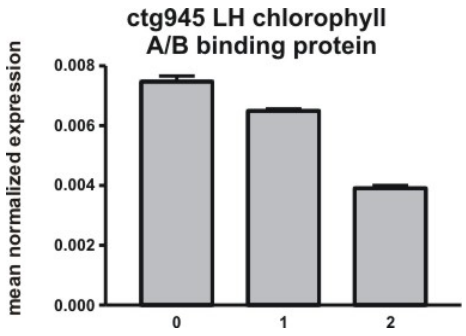


Fig. 3.3. Expression profiles of ctg945 in fruit belonging to classes 0–2 during peach fruit ripening. Values (means of the normalized expression) have been obtained by means of Real Time PCR. Lhcb2: light harvesting chlorophyll *a*/*b* binding protein.

About the correlation of the I_{AD} with transcriptional changes, a validation of the robustness of the I_{AD} was made. mRNA levels of selected marker genes that are either up- (ctg112, PDC; ctg298, bZIP; ctg420, PG; ctg938, PEI) or down-regulated (ctg941, EXP2; ctg974, RD22; ctg1024 CAT; ctg2902, ST) during ripening were monitored in the three classes. Class 0 showed the lowest transcript amounts of the up-regulated genes (Fig. 3.4A-D) and the highest mRNA levels of the down-regulated ones (Fig. 3.4E-H). The opposite situation was observed in class 2. These transcriptional profiles, which are distinctive of fruit at pre-climacteric (class 0) and climacteric (class 1) stages, strongly support the information provided by physiological, such as ethylene production and physico-chemical parameters.

In class 1 fruit, mRNA levels of both up- and down-regulated genes were either similar to those observed in class 0 or intermediate to those detected in classes 0 and 2. Indeed, transcript levels of some genes could clearly discriminate between classes 0 and 1 (ctg420 PG for the up-regulated genes, Fig. 3.4A; ctg2902 ST, ctg974 RD22 and ctg941 EXP2 for the down-regulated ones, Fig. 3.4E-G), while for other genes a clear cut in transcription was detected only between classes 1 and 2 (ctg298 bZIP, ctg938 PEI and ctg112 PDC for the up-regulated genes, Fig. 3.4B-D; ctg1024 CAT for the down-regulated ones, Fig. 3.4H).

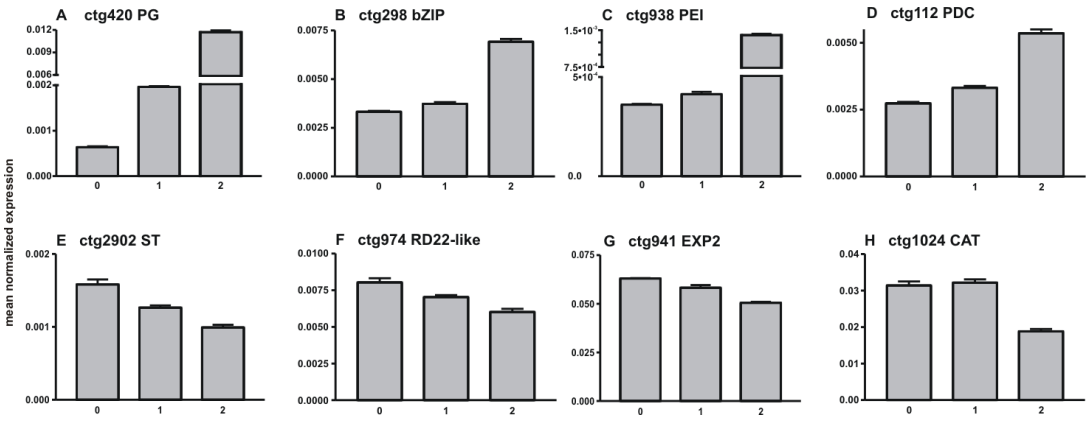


Fig. 3.4. I_{AD} molecular validation by means of expression profiling of ripening-related genes. Expression profiles in fruit belonging to classes 0–2 of some genes either up-regulated (panels A–D) or down-regulated (panels E–H) during peach fruit ripening. Values (means of the normalized expression) have been obtained by means of Real Time PCR. PG: polygalacturonase; bZIP: bZIP

transcription factor; PEI: pectinesterase inhibitor; PDC: pyruvate decarboxylase; ST: putative sorbitol transporter; RD22: dehydration-induced protein RD22-like; EXP2: expansin 2; CAT: catalase. Numbers before gene acronyms indicate the peach contig name. Bars represent the standard deviations from the means.

These data are the first molecular validation of a non-destructive ripening index and demonstrate that the I_{AD} is able to detect both early (transition from pre-climacteric to onset of climacteric stage) and late (transition from onset of climacteric to climacteric stage) physiological changes occurring during on-tree peach fruit ripening, regardless of the fact that they might have or not caused appreciable modifications in fruit quality. In conclusion, the I_{AD} could be very helpful for both practical and scientific applications. In laboratory conditions, the I_{AD} could be used to reduce sample variability, thus allowing to obtain more reliable information on fruit ripening biology. In fact, in this work, the I_{AD} index was used to collect and to treat the peach samples (belonging to the cultivar *Stark Red Gold*) in a precise ripening stage in order to study the transition from pre-climacteric to climacteric stage and to investigate the hormonal regulation during ripening.

3.1.2. MICROARRAY ANALYSIS

Possible independent roles played by the hormones ethylene and auxin during the transition to ripening (i.e. from the pre-climacteric -S3II- to the climacteric -S4I- stage) were studied in peach fruits. To accomplish this, transcriptome changes were first monitored in untreated fruits during the above transition. Then, hormone untreated and treated fruits were compared. The stage S3II of development had previously been identified as the most convenient stage to investigate the hormonal effect on gene expression during the transition from preclimacteric (S3II) to climacteric (S4I) phase (Trainotti *et al.*, 2003; Trainotti *et al.*, 2006). Thus, fruits harvested at the S3II stage (in this case indicated as S3II0) and either kept in air (S3IIair) or treated with either ethylene (S3IIet) or the auxin

analogue NAA (1-naphthalene acetic acid, S3IINAA) were used to prepare the RNA for the microarray study.

The transcriptome variations were analysed by means of the μPEACH1.0 oligonucleodite microarray (Trainotti *et al.*, 2006). The S3II-S4I transition was investigated using a direct comparison experimental design, with 4 repetitions of which one was a dye swap. As each probe was spotted twice on the μPEACH1.0 array, the following SAM (Significance Analysis of Microarrays, Tusher *et al.*, 2001) analysis was performed on a dataset of 8 values for each gene. The hormonal effect on gene transcription was monitored with a double loop experimental design so that the S3II0 and S3IIair samples were the common references. Each comparison was repeated at least twice, so that at least 4 values for each gene were used in the subsequent SAM analyses.

A second group of fruits consisting of nectarine (cv. *Stark Red Gold*) treated with 1-methylcyclopropene (1-MCP) was used to monitor the effects of the chemical on both the ripening physiology and the fruit transcriptome. In order to perform 1-MCP treatments on fruit at a homogeneous stage of ripening, the index of absorbance difference (I_{AD}, Ziosi *et al*., 2008) was used to group nectarines according to their ripening stage. In this case, the strategy employed in the microarray experiments was a direct comparison approach. In particular, class 0 fruit kept in air for 24 hours after a 12 hours 1-MCP treatment were used because they showed the higher retention of flesh firmness compared to control fruit (36 hours in air at room temperature). Setting the false discovery rate (FDR) to 5% yielded 121 genes as differentially expressed (in particular, 58 down-regulated and 63 up-regulated).

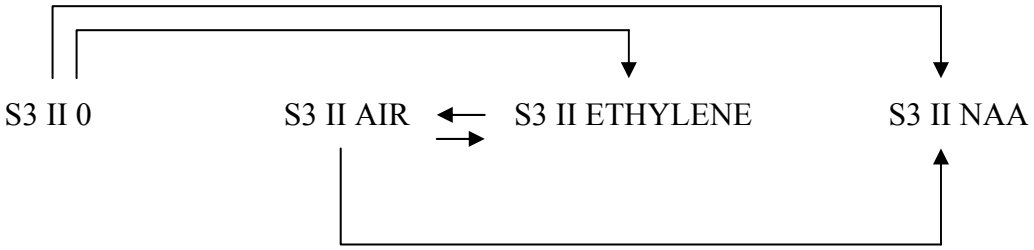
The microarray data obtained from the 1-MCP treatment were crossed with those obtained from the S3II-S4I transition and the hormone treatments (auxin and ethylene).

The expression profiles of selected genes were confirmed with Real Time PCR experiments.

A)

S3 II POINT 0 ⟷ S4 I POINT 0

B)



C)

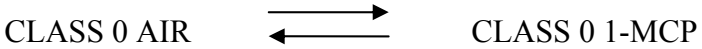


Fig. 3.5. Experimental design of the microarray experiments. The transition from pre-climacteric to climacteric stages (A) and the effects of 1-MCP (C) were investigated with a direct comparison experimental design, while the effects of auxin and ethylene during ripening (B) with a double loop experimental design.

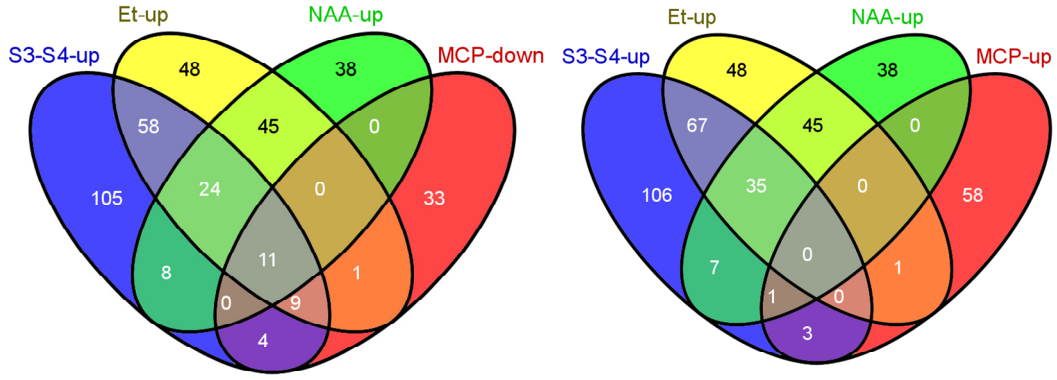
Since the S3II sample used for the comparison with S4I corresponded to the S3II0 used in the hormone experiment, the whole dataset could be subjected to a "multi class" study design with 5 classes:

- S3II/S4I;
- S3II0/S3IIet;
- S3IIair/S3IIet;
- S3II0/S3IINAA;
- S3IIair/S3IINAA.

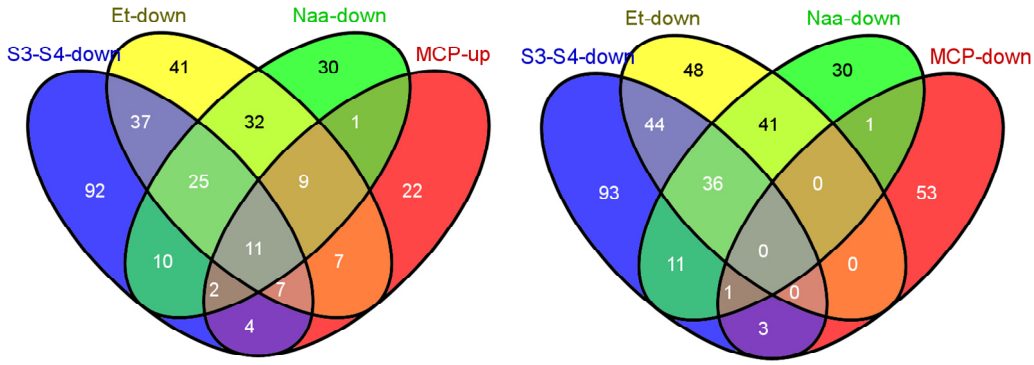
Using a delta of 0.2 and a median false discovery rate (FDR) of 0.00%, the analysis yielded 703 genes as differentially expressed.

The same dataset was also tested with SAM using a "one class" study design. This analysis was carried out on the following 3 subgroups of data: S3II/S4I, S3II/S3et and S3II/NAA because the experiments with the S3II0 and S3IIair reference

clustered together. Keeping the median FDR to 0.00%, 6 groups of genes were selected (the up and down regulated for each of the 3 subgroups of data) and crossed with the 703 genes coming from the multi-class analysis.



(A) Genes up-regulated by ripening



(B) Genes down regulated by ripening

Fig. 3.6. Differential gene expression in peach fruits, as obtained after SAM analysis by using the µPEACH1.0 microarray. The samples analysed were fruits at the S3II-S4I transition, pre-climacteric S3II fruits treated with either ethylene or auxin and class 0 fruits treated with 1-MCP. The Venn diagrams show the numbers of overlapping and non-overlapping genes differentially up-regulated (top) and down-regulated (bottom) by ripening. In detail, in panel A, the genes up-regulated by three conditions (transition S3II-S4I, ethylene or auxin treatments) are crossed with those down-regulated (left) or up-regulated (right) by 1-MCP. The opposite situation is shown in panel B.

The Venn diagrams (Fig. 3.6) summarize the data obtained with the SAM analysis using these conservative parameters which allowed to identify:

- 219 genes up-regulated and 188 down-regulated during the transition from pre-climacteric to climacteric stage (the passage from stage S3II to stage S4I);
- 196 genes induced and 169 repressed following treatment with exogenous ethylene;
- 126 genes induced and 120 repressed by the NAA treatment.

By crossing these data, it was possible to show the different up- and down-regulating effects of the different conditions on the differentially expressed genes:

- a subset of 71 genes are either up- (35) or down-regulated (36) in all the experimental situations (during transition from pre-climacteric to climacteric stage, with the ethylene treatment and with the auxin treatment);
- the S3II–S4I transition showed a specific regulatory effect (apparently not dependent on either ethylene and NAA, but possibly on other signals) on 205 genes: 109 induced and 96 repressed;
- treatments with ethylene associated with the S3II–S4I transition showed an up-regulating effect on 102 genes and a repressive effect on 80 genes. Crossing these data with 1-MCP treatment, it has shown that 20 are the probes induced by both ripening and ethylene and, as expected, repressed by 1-MCP; while 18 are the probes that behave in the opposite way: they are down-regulated by both ripening and ethylene but up-regulated by the 1-MCP treatment. No genes either induced or repressed by ripening, ethylene and 1-MCP together were found;
- treatments with NAA associated with the S3II–S4I transition induced the expression of 43 and the repression of 48 genes, respectively. Crossing this data with those relative to the 1-MCP treatment, it has shown that 11 are the probes induced by both ripening and auxin and repressed by 1-MCP. All these 11 probes fall within the group of the 20 ones induced by ripening and ethylene and repressed by 1-MCP seen above as confirm that their auxin responsiveness is mediated by ethylene. 13 are the probes down regulated by both ripening and auxin but up-regulated by the 1-MCP

treatment. Of these, 11 are in common with the 18 probes down-regulated by ripening and ethylene and up-regulated by 1-MCP, thus confirming that also for these genes their auxin responsiveness is mediated by ethylene. Only one gene was induced by ripening, auxin and 1-MCP and also only one gene was repressed in the three situations;

- by excluding the genes that are also responsive to NAA, it resulted that the ethylene treatment and the transition to ripening specifically up-regulated 67 genes and repressed 44 genes;
- by excluding the genes that are also responsive to ethylene, the auxin treatment and the transition to ripening specifically induced 8 genes and repressed 12 genes.

The data of the microarray experiments for all the 4806 probes present on μPEACH1.0 were reported in the Supplementary Table 2 (available online on the *Journal of Experimental Botany* web site) in a 2007 publication (Trainotti *et al.*, 2007. Genes positive to SAM analysis and belonging to the several groups are labeled. Gene annotations and probe unique identifiers are provided).

Real time PCR experiments were carried out to confirm the data obtained by microarray hybridisations and to investigate the role of ethylene and auxin during peach ripening. These experiments have also demonstrated that a great number of genes appears to be specifically regulated by the S3II-S4I transition. This effect is apparently independent from either ethylene or auxin; in fact, the expression of such genes might be regulated by other factor as yet unknown.

3.1.3. REGULATION OF GENES BELONGING TO THE ETHYLENE BIOSYNTHETIC PATHWAY

ACS1 (Mathooko *et al.*, 2001; Tatsuki *et al.*, 2006) and *ACO1* (Ruperti *et al.*, 2001) code for the key enzymes involved in the ethylene biosynthesis. Climacteric ethylene production is largely dependent on the expression of these genes. The expression of other known members of the two families as *ACS2* and *ACS3* (described in Tatsuki *et al.*, 2006) and *ACO2* (described in Ruperti *et al.*, 2001) does not fit with the model of the transition from System-1 to System-2 described

in tomato (Barry *et al*., 2000). To better understand the regulation of ethylene biosynthesis during the transition from pre-climacteric to climacteric stage in peach, additional ACS genes were searched in the 70000 *Prunus persica* ESTs publicly available. Thus, primers for two additional members of this family have been designed on peach EST sequences found in public database:

- ACS4, ctg5158, corresponds to Acc. no. BU047017, a mesocarp EST;
- ACS5 (absent in the peach microarray μPEACH1.0) corresponds to Acc. no. DW342130, a shoot EST.

The expression profile of these 5 ACS genes was analyzed by means of Real Time PCR experiments during fruit development and ripening and in pre-climacteric fruit treated with exogenous ethylene, auxin and 1-MCP.

The results were the following:

- ACS1 (ctg489) is dramatically induced by the transition from pre-climacteric to climacteric stage. In S3II, NAA is much more effective than ethylene in increasing ACS1 mRNA abundance (Fig. 3.7AII). 1-MCP slackens ACS1 accumulation in class 0 fruit, while strongly induces it in class 1 fruit (Fig. 3.7AIII);
- ACS2 (ctg2568) is relatively abundant only in fully developed leaves (Fig. 3.7BI) and it might be meaningless in S3II stage (Fig. 3.7BII). The expression in nectarines was not detectable so the response to 1-MCP is technically not reliable. Its expression in the leaf could be due to unwanted wounding of the organ in the field (Tatsuki *et al.*, 2006);
- ACS3 (ctg1151) was expressed at low levels and it was detected only in flowers (Fig. 3.7CI);
- ACS4 (ctg5158) was expressed throughout development with a peak in leaves (Fig. 3.7DI). In the pre-climacteric fruit, it was induced by both ethylene and NAA (Fig. 3.7DII), while it was down regulated by 1-MCP in both class 0 and class 1 fruit (Fig. 3.7DIII). ACS4 seems to guarantee basal level of ethylene production;
- ACS5 was expressed as ACS3 at extremely low levels during fruit development and ripening. It was detected in flowers. In the samples treated with hormones and 1-MCP, the expression of this gene was not detectable (Fig. 3.7EI, II, III). ACS 5 and also ACS3 might be involved

in the ethylene production occurring during pollination (Weterings *et al*., 2002) or organ shedding (Clark *et al*., 1997).

The expression profiles of two ACO genes were analyzed and they showed that:

- ACO1 was expressed as previously reported (Fig. 3.7FI e II, Ruperti *et al.*, 2001). The ethylene dependence of ACO1 transcription was strengthened by the fact that in class 0 and class 1 1-MCP-treated fruit the expression level of this gene was less than in air control samples (Fig. 3.7FIII);
- ACO2 was almost constitutive in all samples of the developmental series but it presented a minimum in S1 stage (Fig. 3.7GI); ethylene slightly induced its expression in pre-climacteric fruit (Fig. 3.7GII). Surprisingly, a clear inductive effect of the 1-MCP treatment on ACO2 expression was observed in class 0 and, although to a lesser extent, also class 1 fruit (Fig. 3.7GIII). ACO2 with ACS4 seem to guarantee basal level of ethylene production during fruit development and maturation.

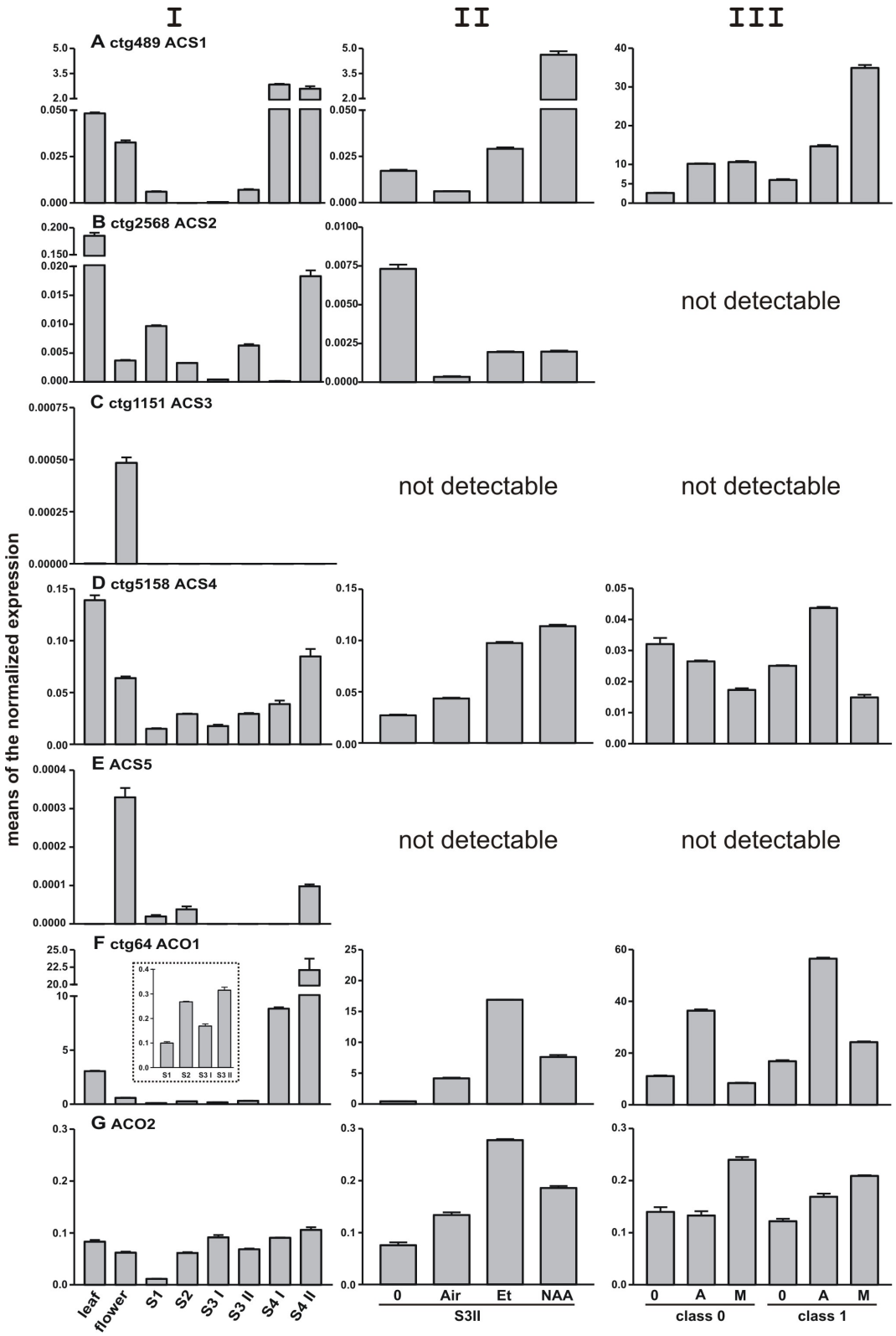


Fig. 3.7. Expression profiles of genes encoding enzymes of the ethylene biosynthesis analysed in different plant tissues and during fruit development and ripening (column I), after hormone treatment of pre-climacteric S3II fruit (column II) and in class 0 and class 1 nectarines following 1-MCP treatments. Values (means of the normalized expressions) have been obtained by means of Real Time PCR.

ACS1 to ACS5: 1-aminocyclopropane-1-carboxylate synthase genes 1 to 5; ACO1 and ACO2: 1-aminocyclopropane-1-carboxylate oxidase genes 1 and 2. Numbers before gene acronyms indicate, when available, the peach contig name. Stages S1 to S4II encompass the development and ripening of peach fruits (column I). Hormone treatments (et: ethylene; NAA: 1-naphthalene acetic acid) lasted for 48 hours (column II). Nectarines have been sampled after 36 h of storage either in air (A, the control) or in 1-MCP (12 h) plus air (24 h in air after the end of the 1-MCP treatment, M; column III). Bars are the standard deviations from the means of three replicates.

3.1.4. REGULATION OF GENES BELONGING TO THE ETHYLENE PERCEPTION AND SIGNAL TRANSDUCTION PATHWAYS

The two ethylene receptors already described by Rasori *et al.* (2002) (ETR1 ctg1436 and ERS ctg356) appeared to be either almost constitutive (ETR1) or expressed at increasing amounts during ripening (ERS1). Despite the difference in expression during fruit ripening, their expression was not significantly affect by treatments with either one or the other of the two hormones. On the contrary, ETR2 ctg2025 showed a fruit-specific, ripening-induced and ethylene-dependent expression (Fig. 3.8A, B, C). The 1-MCP inhibitor has no effect on ETR1 (Fig. 8AIII), it slightly down-regulates ERS1 (Fig. 8BIII), while it strongly suppresses ETR2 transcription in both class 0 and class 1 fruit (Fig. 8CIII), thus confirming other recent findings (Ziliotto *et al.*, 2008).

ERS1 might have a crucial role in sensing ethylene during the initial phase of the transition from System-1 to System-2 but it did not seem to be controlled by either ethylene or auxin, so that it could be considered under developmental control. It could be that the transcription of ERS1 is ethylene independent, because its expression is only slightly repressed by 1-MCP.

Interestingly, a third receptor gene (ETR2; Trainotti *et al.*, 2006) showed a clear ripening-specific expression pattern (Fig. 3.8C). In this case the greatest inductive effect on its expression was yielded by ethylene, while the more limited increase in transcript amount observed following a treatment with auxin was probably indirect and mediated by ethylene.

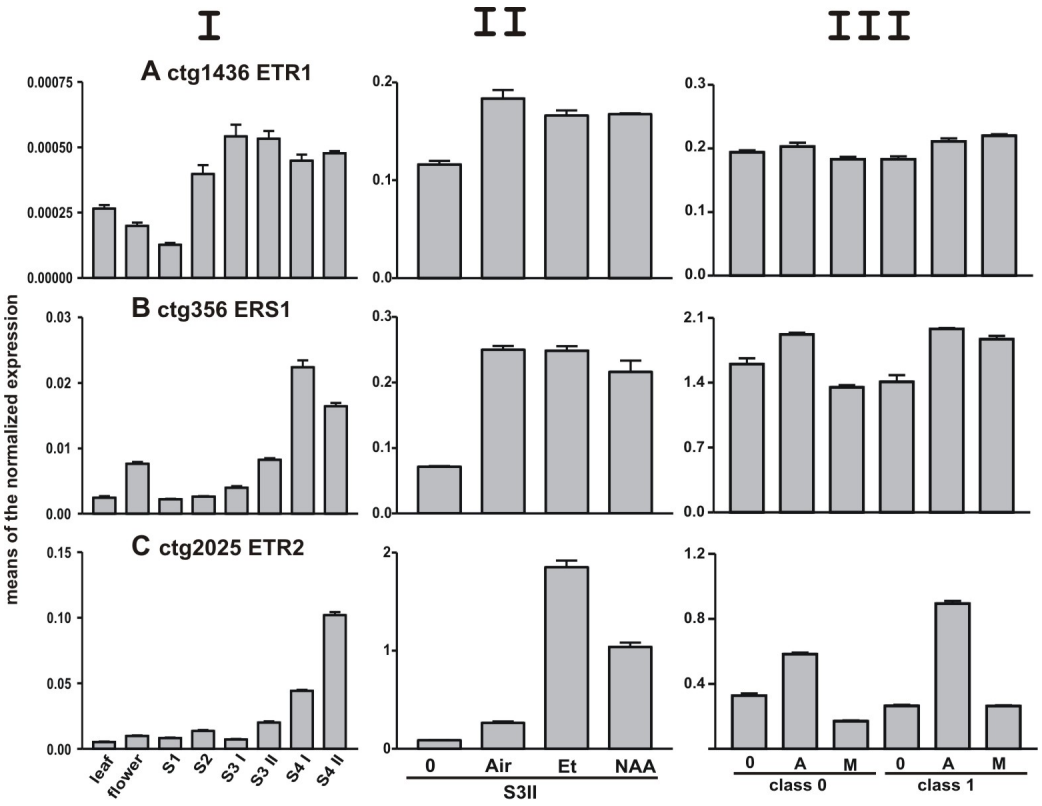


Fig. 3.8. Expression profiles of peach genes involved in ethylene reception analysed in different plant tissues and during fruit development and ripening (column I), after hormone treatment of pre-climacteric S3II fruit (column II) and in class 0 and class 1 nectarines following 1-MCP treatments. Values (means of the normalized expression) have been obtained by means of Real Time PCR. ETR1, Etr1-like ethylene receptor (A); ERS1, ERS1-like ethylene receptor (B); ETR2, Etr2-like ethylene receptor (C). Numbers before gene acronyms indicate the peach contig name. Stages S1 to S4II encompass the development and ripening of peach fruits (column I). Hormone treatments (et: ethylene; NAA: 1-naphthalene acetic acid) lasted for 48 hours (column II). Nectarines have been sampled after 36 h of storage either in air (A, the control) or in 1-MCP (12 h) plus air (24 h in air after the end of the 1-MCP treatment, M; column III). Bars are the standard deviations from the means of three replicates.

Among the genes involved in ethylene transduction, the expression of an ethylene-response-factor (ctg2116) was very interesting. This gene was found to be almost fruit specific. In particular, it showed a dramatic increase during the melting phase S4II (Fig. 3.9I) and, probably because of such a late expression, no variations in gene expression was detected by microarray analysis. Interestingly,

its expression was up-regulated much more by NAA than by ethylene, while its expression was not modulated by 1-MCP (Fig.3.9).

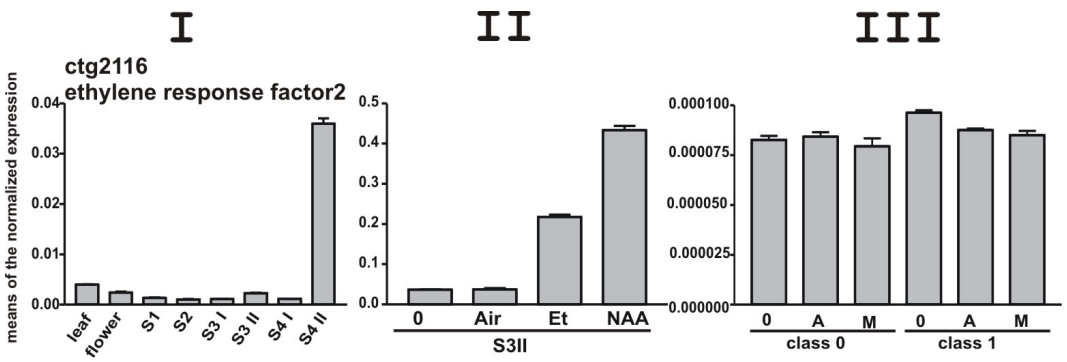


Fig. 3.9. Expression profiles of ctg2116, coding for an ethylene response factor 2, analysed in different plant tissues and during fruit development and ripening (column I), after hormone treatment of pre-climacteric S3II fruit (column II) and in class 0 and class 1 nectarines following 1-MCP treatments. Values (means of the normalized expression) have been obtained by means of Real Time PCR. Stages S1 to S4II encompass the development and ripening of peach fruits (column I). Hormone treatments (et: ethylene; NAA: 1-naphthalene acetic acid) lasted for 48 hours (column II). Nectarines have been sampled after 36 h of storage either in air (A, the control) or in 1-MCP (12 h) plus air (24 h in air after the end of the 1-MCP treatment, M; column III). Bars are the standard deviations from the means of three replicates.

3.1.5. REGULATION OF GENES BELONGING TO THE AUXIN BIOSYNTHETIC AND HOMEOSTATIC PATHWAYS

The fact that some genes related to auxin appeared to be up-regulated during the transition to ripening induced a further exploration of the microarray data. This analysis showed that a number of genes encoding either ARF (Auxin Response Factors) or Aux/IAA proteins (transcriptional modulators of the hormone response), but also a hormone receptor (TIR1), an auxin efflux facilitator, a couple of other proteins possibly involved in the early steps of auxin biosynthesis (indole-3-glycerol phosphate synthase and tryptophan synthase beta subunit) and a couple of proteins involved in auxin conjugation/deconjugation, exhibited increased expression during the transition to ripening (Fig. 3.10).

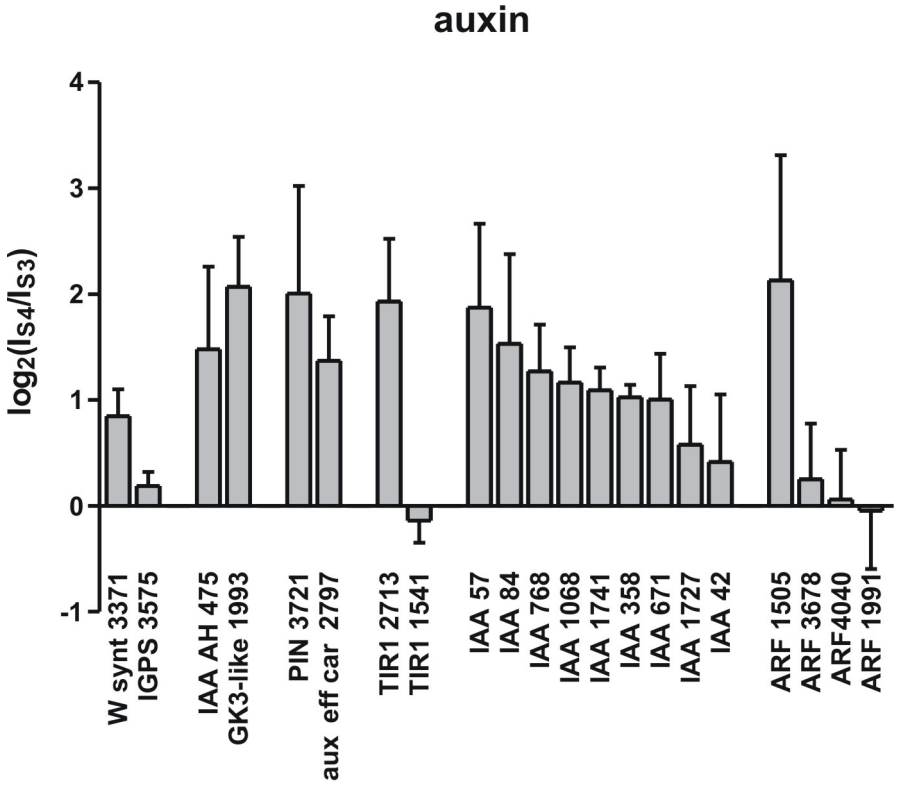


Fig. 3.10. Modulation of gene expression, measured by means of the μPEACH1.0 microarray, during the transition to ripening (from stage S3II to stage S4I). The genes involved in auxin metabolism are: W synt (tryptophan synthase beta subunit); IGPS (indole-3-glycerol phosphate synthase); IAA AH (IAA amidohydrolase); GH3-like (GH3-like protein); PIN (PIN1-like auxin efflux facilitator); aux eff car (auxin efflux carrier); TIR1 (transport inhibitor response 1-like protein); IAA (Aux/IAA); ARF (auxin response factor). Numbers after gene acronyms indicate the peach contig name. Bars are the standard deviations from the means.

To analyse in detail the genes belonging to the "auxin domain", several expression profiles were determined by Real Time PCR experiments.

A gene encoding a putative indole-3-glycerol phosphate synthase (ctg3575, IGPS, belonging to the tryptophan independent auxin biosynthetic pathway, Fig. 3.11A) had increased expression from the end of growth until late ripening and a gene coding for a tryptophan synthase β subunit (ctg3371, belonging to the tryptophan dependent auxin biosynthetic pathway, Fig. 3.11B) appeared to be induced during ripening. However, while ctg3371 showed an NAA stimulated expression, a down-regulation was observed for ctg3575. Tryptophan is known to be the most common IAA precursor and it is interesting to note that the expression of the

above gene appears to be up-regulated by hormone treatments, especially by NAA. 1-MCP was ineffective for both genes.

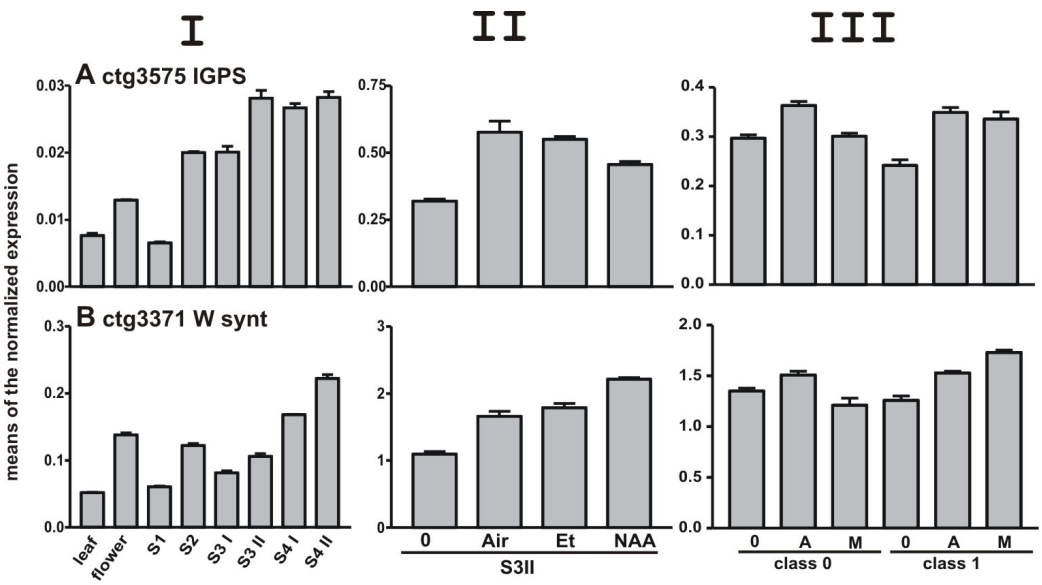


Fig. 3.11. Expression profiles of peach genes involved in auxin biosynthesis in different plant tissues and during fruit development and ripening (column I), after hormone treatment of pre-climacteric S3II fruit (column II) and in class 0 and class 1 nectarines following 1-MCP treatments. Values (means of the normalized expression) have been obtained by means of Real Time PCR. IGPS, indole-3-glycerol phosphate synthase (A); W synt, tryptophan synthase beta subunit (B). Numbers before gene acronyms indicate the peach contig names. Stages S1 to S4II encompass the development and ripening of peach fruits (column I). Hormone treatments (et: ethylene; NAA: 1-naphthalene acetic acid) lasted for 48 hours (column II). Nectarines have been sampled after 36 h of storage either in air (A, the control) or in 1-MCP (12 h) plus air (24 h in air after the end of the 1-MCP treatment, M; column III). Bars are the standard deviations from the means of three replicates.

The expression profiles of some genes, related to auxin conjugation/de conjugation, were studied. Ctg475, coding for an IAA amidohydrolase of the ILR1 (IAA-LEUCINE RESISTANT 1) type, increased during climacteric ripening, was positively regulated by NAA and negatively by ethylene and it was stimulated by 1-MCP in both class 0 and class 1 fruit (Fig. 3.12A). Another gene involved in maintaining auxin homeostasis coding for a GH3 protein (ctg1993) was expressed during peach ripening, induced by NAA in S3II stage and its

mRNA was slightly accumulated in 1-MCP treated fruit (Fig. 3.12B). Probably, the increase of free auxin measured in ripening fruit (Miller *et al.*, 1987) is determined not only by *de novo* synthesis, but also by its release from conjugated forms. In particular, the ctg475 (IAA amidohydrolase) might be involved in the release of IAA from IAA-Leu (reviewed in Woodward and Bartel, 2005) and thus its expression was correlated with free IAA during ripening. In the same time, ctg1993 (GH3 protein) might help to maintain auxin homeostasis by conjugating excess IAA to amino acids (Staswick *et al*., 2005). Both genes (ctg475 and ctg1993) were strongly induced by NAA and expressed almost exclusively in S4 fruit and this kind of profile is very similar to that of ACS1. Thus, the expression of these three genes might be the cause of the peaks of both auxin and ethylene measured in S4 fruit (Miller *et al*., 1987).

The expression of GH3-like genes is often considered diagnostic for increased level of auxin in the tissue and although auxin concentration has not been directly measured in 1-MCP treated fruit, the increased expression of genes as ctg1993 (GH3) and of other auxin-induced genes (such as those encoding Aux/IAA proteins, Fig. 3.14A, D and G) might indicate that a rise in free auxin concentration had probably occurred. This increase is likely to be mediated by the activity of ctg475 (IAA amidohydrolase).

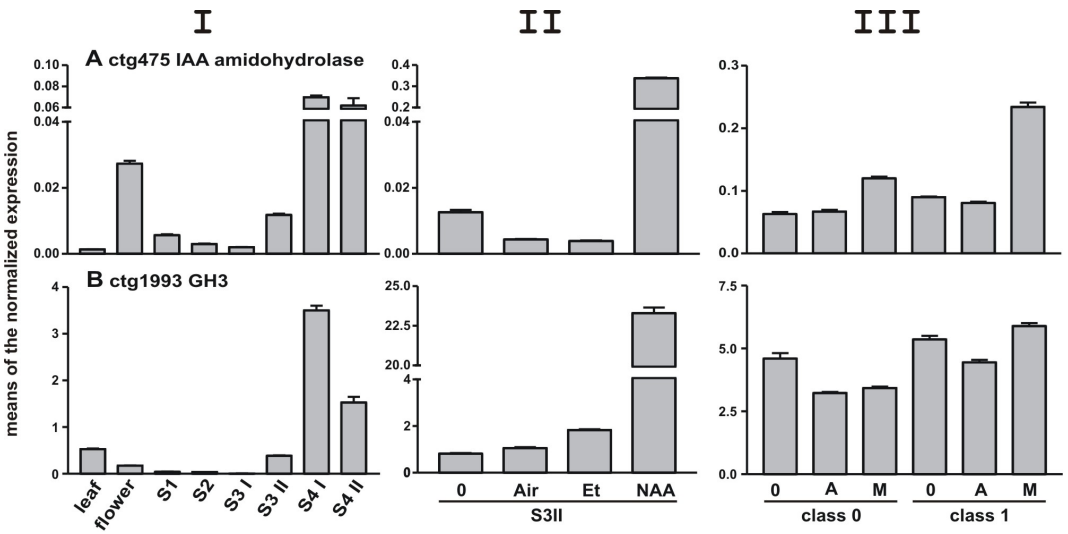


Fig. 3.12. Expression profiles of peach genes involved in auxin conjugation and deconjugation pathway in different plant tissues and during fruit development and ripening (column I), after hormone treatment of pre-climacteric S3II fruit (column II) and in class 0 and class 1 nectarines

following 1-MCP treatments. Values (means of the normalized expression) have been obtained by means of Real Time PCR. Numbers before gene acronyms indicate the peach contig name. Stages S1 to S4II encompass the development and ripening of peach fruits (column I). Hormone treatments (et: ethylene; NAA: 1-naphthalene acetic acid) lasted for 48 hours (column II). Nectarines have been sampled after 36 h of storage either in air (A, the control) or in 1-MCP (12 h) plus air (24 h in air after the end of the 1-MCP treatment, M; column III). Bars are the standard deviations from the means of three replicates.

3.1.6. REGULATION OF GENES BELONGING TO THE AUXIN TRANSPORT, PERCEPTION AND SIGNAL TRANSDUCTION PATHWAYS

For the genes involved in the auxin transport, the ethylene effect was particularly strong on ctg3721, a PIN like gene whose expression was fruit specific and ripening-induced. PIN1, induced more by ethylene than by NAA, in *Stark Red Gold* nectarines responded differently to 1-MCP in the two classes; in particular it was repressed in class 0 and induced in class 1 (Fig. 3.13A).

Two auxin receptors (ctg2713 and ctg1541) have been found to be ripening-related, while the hormone treatments slightly decreased their expression. Also ctg1541 appeared to be induced by the ethylene inhibitor, while the second TIR1 (ctg2713) was repressed in class 0 and induced in class1 (Fig. 3.13B, C).

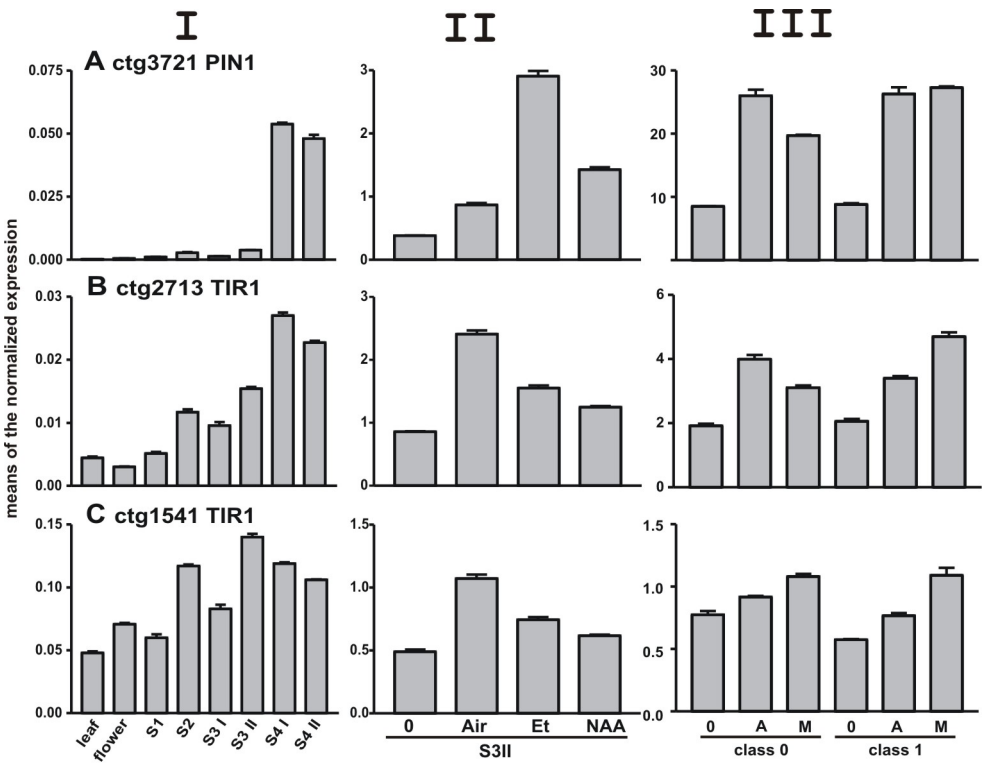


Fig. 3.13. Expression profiles of peach genes involved in auxin perception and transport in different plant tissues and during fruit development and ripening (column I), after hormone treatment of pre-climacteric S3II fruit (column II) and in class 0 and class 1 nectarines following 1-MCP treatments. Values (means of the normalized expression) have been obtained by means of Real Time PCR. PIN, PIN1-like auxin efflux facilitator; TIR1, transport inhibitor response 1-like protein. Numbers before gene acronyms indicate the peach contig name. Stages S1 to S4II encompass the development and ripening of peach fruits (column I). Hormone treatments (et: ethylene; NAA: 1-naphthalene acetic acid) lasted for 48 hours (column II). Nectarines have been sampled after 36 h of storage either in air (A, the control) or in 1-MCP (12 h) plus air (24 h in air after the end of the 1-MCP treatment, M; column III). Bars are the standard deviations from the means of three replicates.

A significant number of genes involved in the "auxin domain" was represented by Aux/IAA (modulators of ARF transcription factors). The Aux/IAA proteins share a four domain structure and domains III and IV allow them to interact either with each other or with similar domains of Auxin Response Factor (ARF) proteins (Berleth *et al*., 2004; Tiwari *et al.*, 2004).

All the Aux/IAA considered in this work (Fig. 3.14) were fruit-specific and ripening-induced (although a few of them showed a decrease in expression during late ripening, S4II), while the hormonal regulation was different.

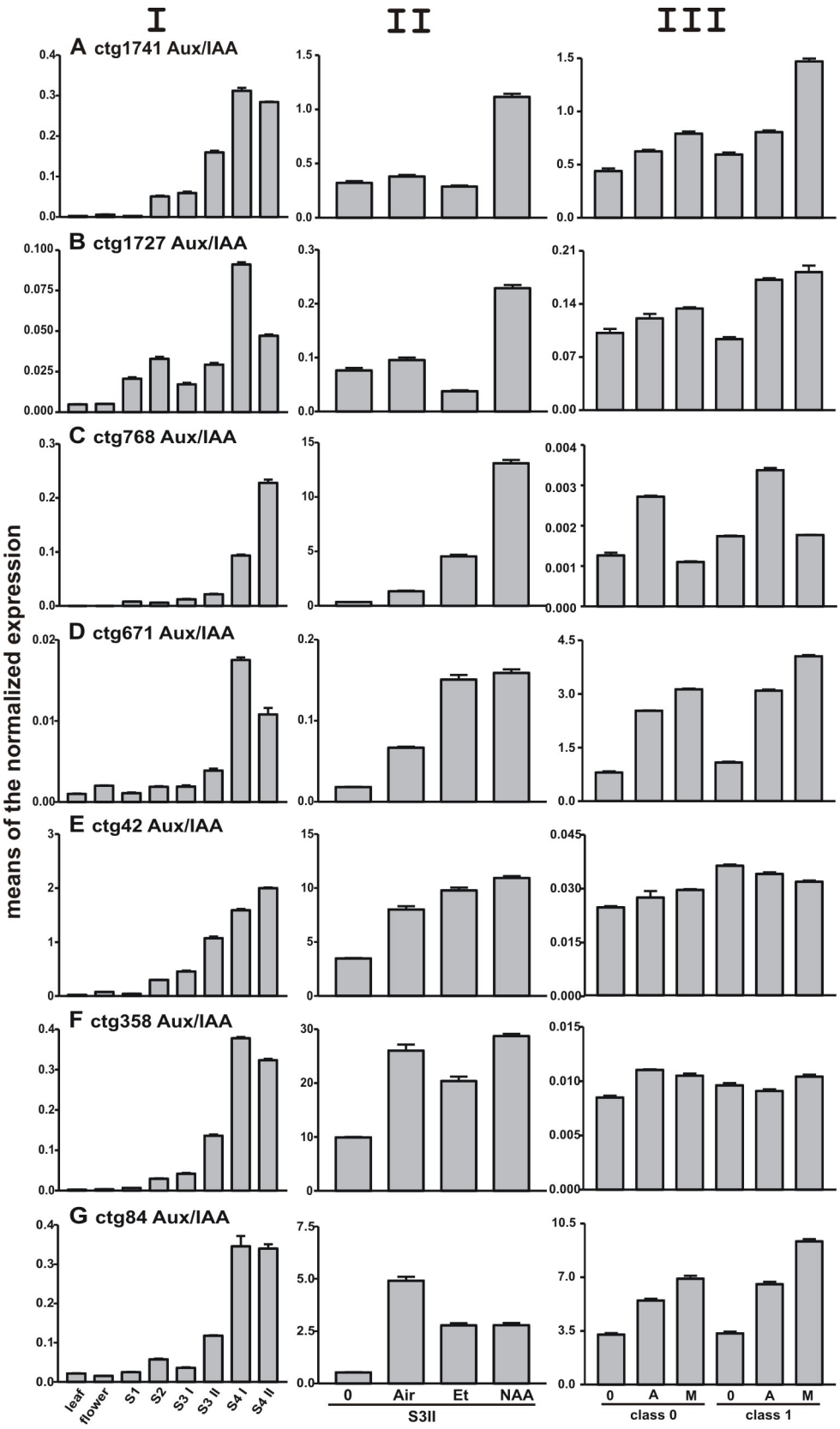


Fig. 3.14. Expression profiles of some peach Aux/IAA genes in different plant tissues and during fruit development and ripening (column I), after hormone treatment of pre-climacteric S3II fruit

(column II) and in class 0 and class 1 nectarines following 1-MCP treatments. Values (means of the normalized expression) have been obtained by means of Real Time PCR. Numbers in the top left of each panel indicate the peach contig name. Stages S1 to S4II encompass the development and ripening of peach fruits (column I). Hormone treatments (et: ethylene; NAA: 1-naphthalene acetic acid) lasted for 48 hours (column II). Nectarines have been sampled after 36 h of storage either in air (A, the control) or in 1-MCP (12 h) plus air (24 h in air after the end of the 1-MCP treatment, M; column III). Bars are the standard deviations from the means of three replicates.

Two Aux/IAA genes (i.e. ctg1741 and ctg1727, Fig. 3.14A, B) were clearly up-regulated only by NAA; one (i.e. ctg768, Fig. 3.14C) was up-regulated by NAA and, to a lesser extent, by ethylene. In other cases, Aux/IAA genes were either up- (i.e. ctg671 and ctg42; Fig. 3.14D, E) or down-regulated (i.e. ctg57, Fig. 3.26 and ctg84, Fig. 3.14G) by both hormones. Finally, ctg358 (Fig. 3.14F) appeared more or less insensitive to the hormone treatments and appeared to be up-regulated mostly by the S3II-S4I transition. About the response to 1-MCP, some genes resulted to be induced: ctg1741, ctg1727, ctg671, ctg84 and ctg57 (Fig. 3.14 A, B, D, G, Fig. 3.26, respectively) while ctg768 resulted to be repressed (Fig. 3.14C). The chemical did not strongly modulate the expression of ctg42 and ctg358 (Fig. 3.14E, F).

ARFs have an N-terminal DNA-binding domain and can thus regulate the expression of auxin-responsive genes, while Aux/IAA proteins have no such domain and can therefore act as transcriptional regulators through their interaction with ARF proteins. In this work, all ARF encoding genes have been found to be expressed in the fruit mesocarp with increasing levels from pre-climacteric to climacteric stage (Fig. 3.15). Ctg1505, ctg3678 and ctg1991 (Fig. 3.15A, B, C) were ripening-induced while ctg4040 (Fig. 3.15D) was almost constitutive (indeed, it did not show any significant variation in expression during ripening and it might be important during the early phase of fruit development). Only ctg1991 expression, which peaked at the melting phase (S4II), was slightly induced by ethylene, while the expression of the other ARFs was reduced by the exogenous hormones.

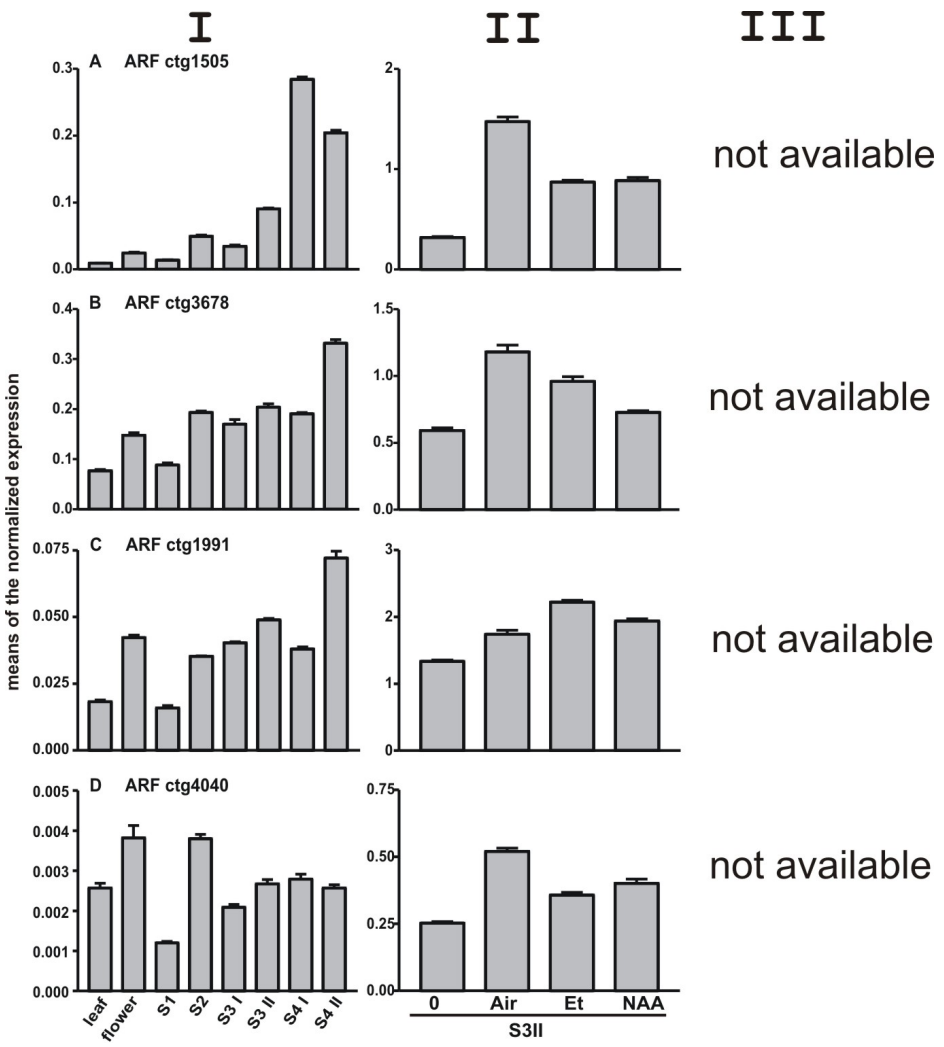


Fig. 3.15. Expression profiles of some peach ARF (auxin response factor) genes in different plant tissues and during fruit development and ripening (column I) and after hormone treatment of S3II fruits (column II). Values (means of the normalized expression) have been obtained by means of Real Time PCR. Numbers in the top left of each panel indicate the peach contig name. Stages S1–S4II encompass the development and ripening of peach fruits. Hormone treatments (et, ethylene; NAA, 1-naphthalene acetic acid) lasted for 48 h. Bars are the standard deviations from the means. Relative expression profiles in class 0 and class 1 nectarines following 1-MCP treatments are not available (column III).

3.1.7. A POSSIBLE AUTONOMOUS ROLE OF AUXIN DURING RIPENING

A significant increase in IAA content had been measured by Miller *et al*. (1987) in the mesocarp of *Redhaven* peaches concomitant with the production of

climacteric ethylene. In connection with a possible autonomous role played by auxin, several genes belonging to the "auxin domain" have been found to have increased expression at the onset of ripening (S3II–S4I transition) and could therefore be regarded as involved in this process. In addition, the embryo that is developing inside the peach stone might be a source of the IAA that is present in the mesocarp. The IAA increment measured by Miller *et al.* (1987) in the mesocarp tissues from 67 d to 88 d after anthesis coincided with a net decrease in the embryo auxin content measured in the same time interval, thus suggesting that the mesocarp might synthesize its own IAA. If the attention is focused on the genes related to the auxin biosynthesis, the expression of the IGPS gene seems to be especially related to the S3II–S4I phase transition in contrast with the expression of W synthase (Fig. 3.11). Besides being a precursor for indole biosynthesis, IGPS can also represent a branch point for IAA synthesis through the tryptophan-independent pathway (Taiz and Zeiger, 2006). In addition, both ctg475 and ctg1993, positively regulated by NAA, are involved in the auxin homeostasis coding the first for an enzyme involved in the release of IAA from IAA-conjugated form and the second for proteins that conjugate free auxin to amino acids. Therefore, the above expression data support the hypothesis that the observed increment in mesocarp IAA content is likely to come directly from the mesocarp.

For two putative TIR1 genes (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005) the expression seems to be particularly up-regulated by the transition from pre-climacteric to climacteric stage while the effect of the two hormones does not appear particularly relevant. So the idea that an active IAA-related metabolism might occur in the fruit mesocarp is further supported by the expression of these two genes and in addition by the expression profile of a gene encoding a putative auxin efflux facilitator protein PIN1 (Paponov *et al.*, 2005) which exhibited a marked increase during the transition (Fig. 3.13).

The genomic approach here used established that the auxin increase is functional to an independent role played by the hormone during ripening of the climacteric peach fruits and adds this function to the well-known auxin role during fruit set (Spena and Rotino, 2001) and early stages of fruit growth (Nitsch, 1950).

3.1.8. ETHYLENE-AUXIN INTERPLAY DURING RIPENING

ACO1 and ACS1 are the two key enzymes in the ethylene biosynthesis. It is known that auxin may stimulate ethylene biosynthesis by increasing the transcription of ACS genes. This also happened in this system, where ACS1 gene (Mathooko *et al.*, 2001) was induced more by NAA than by exogenous ethylene treatments (Fig. 3.7AI e II) and a similar behaviour was shown for ACS4 (Fig. 3.7DI e II). By contrast, the steady-state level of the ACO1 gene (Ruperti *et al.*, 2001) appeared almost insensitive to NAA and was greatly induced by ethylene (Fig. 3.7FI e II). Among the genes involved in the ethylene cascade, the profile of an ethylene-response-factor (ctg2116) was very interesting, because its expression was up-regulated much more by NAA than by ethylene (Fig. 3.9). A similar behaviour, but with an opposite hormonal response, was showed by PIN1 (Fig. 3.13A). This gene, belonging to the "auxin domain", appeared to be significantly up-regulated more by ethylene than by auxin. These data were very interesting because they demonstrated that in peach there is an active cross-talk between auxin and ethylene, with genes in the ethylene domain regulated by auxin and genes in the auxin domain regulated by ethylene.

There was only one gene induced by ripening, auxin and 1-MCP revealed by the microarray analysis: ctg134 (unknown protein). This microarray result was confirmed by Real Time PCR. Ctg134 had increased expression during the S3II-S4I transition, was up-regulated by NAA and repressed by ethylene in pre-climacteric S3II fruit and stimulated by 1-MCP in both class 0 (and thus revealed by microarray) and class 1 fruit (Fig. 3.16). It was a key expression profile that allowed to discover and to investigate other genes, such as ctg475 and ctg1993, selected, at first, for the peculiar features of their overlapping transcription profiles, and, later on, for their involvement with auxin. Their transcriptional regulation is similar to ACS1. Moreover, their expression, and that of Aux/IAA genes, can be considered diagnostic for increased level of auxin in the tissue from which the RNA has been obtained. The idea is that in peach fruit there is a regulatory loop of auxin-ethylene interactions in which hormone levels are reciprocally controlled by yet unknown systems.

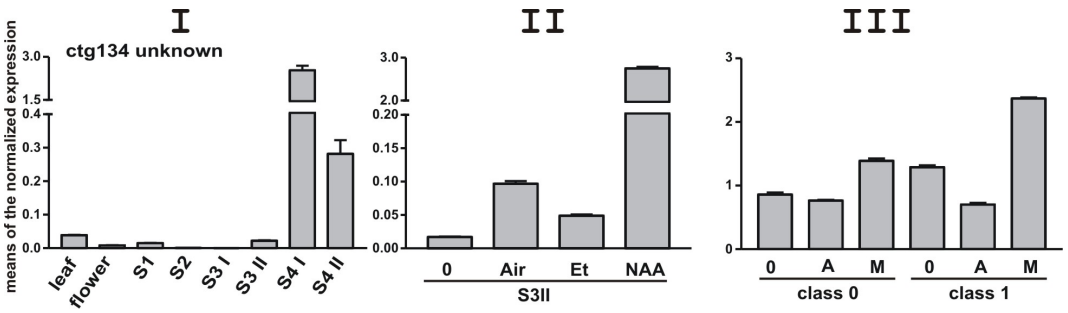


Fig. 3.16. Expression profiles of ctg134, coding for an unknown protein, analysed in different plant tissues and during fruit development and ripening (column I), after hormone treatment of pre-climacteric S3II fruit (column II) and in class 0 and class 1 nectarines following 1-MCP treatments. Values (means of the normalized expression) have been obtained by means of Real Time PCR. Stages S1 to S4II encompass the development and ripening of peach fruits (column I). Hormone treatments (et: ethylene; NAA: 1-naphthalene acetic acid) lasted for 48 hours (column II). Nectarines have been sampled after 36 h of storage either in air (A, the control) or in 1-MCP (12 h) plus air (24 h in air after the end of the 1-MCP treatment, M; column III). Bars are the standard deviations from the means of three replicates.

The genomic approach used in this work has shown that auxin-ethylene interplay is very important for the regulation of ripening, besides the independent roles played by each hormone.

This auxin-ethylene cross talk is also present in tomato fruits (Jones *et al.*, 2002) where an Aux/IAA gene showed a peak of accumulation in early red fruits and had ethylene inducible expression (Chaabouni *et al.*, 2009). Moreover, in ripening tomato fruits, concomitant with the climacteric ethylene production there is an increase in auxin content (Gillaspy *et al.*, 1993), similarly to what has been observed in peach fruit.

3.1.9. EFFICACY OF 1-MCP

The efficacy of the chemical 1-MCP in delaying peach fruit ripening has been controversial until now. In this work it has been shown that the effects are dependent on the ripening stage in which the chemical is applied. The stage of application is critical; in fact, in class 0 fruit the chemical permits the delay of the fruit ripening; on the contrary, in class 2 fruit 1-MCP is ineffective in delaying softening (Fig. 3.17). In class 0 fruit, the basal level of ethylene is very low and

the chemical slightly reduces the amount of hormone produced by fruit during post-harvest storage (Fig. 3.17). In class 1 fruit, on the contrary, 1-MCP anticipates the climacteric ethylene production and then increases its quantity in class 2 fruit. Thus, the effect of the 1-MCP on ethylene biosynthesis also depends on the physiological state of the fruit and this is well identified by means of the I_{AD}.

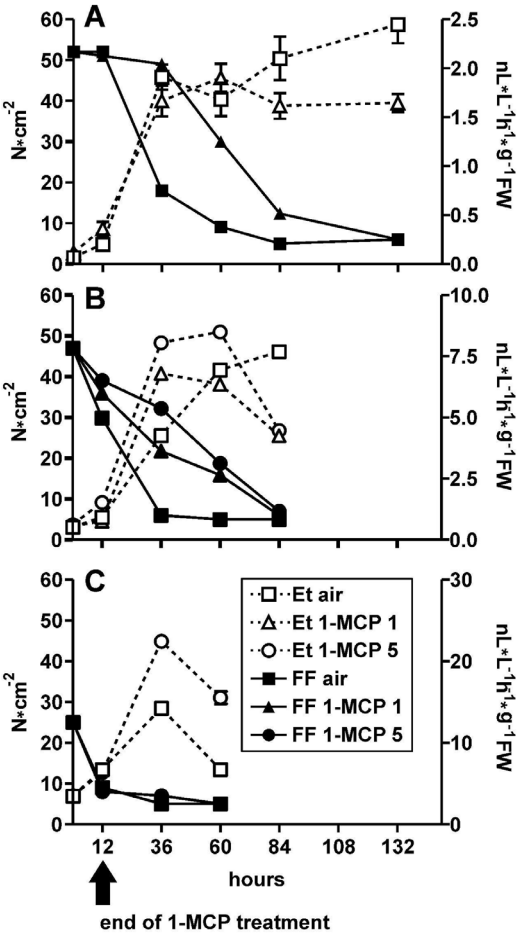


Fig. 3.17. Changes in flesh firmness (solid lines, filled symbols, left Y axe) and ethylene production (dashed lines, open symbols, right Y axe) during post-harvest of nectarines either treated (1-MCP) or not (air) with 1-MCP (1 or 5 ppm). The Y scale is the same in the three panels for FF (left), while it differs for ethylene production (right). I_{AD} has been used group fruit according to their ripening stages: class 0 (pre-climacteric, panel A), class 1 (onset of climacteric, panel B) and class 2 (climacteric, panel C). The arrow at the bottom indicates the end of the 1-MCP treatment in 1-MCP-exposed fruit. Thereafter, fruit have been kept in air at room temperature. Data represent the mean (n = 40)±S.D. Data obtained in the Prof. Costa's laboratory.

3.2. GENE "BIN" MAPPING

3.2.1. T X E POPULATION

This part of the work was conducted in the laboratory of prof. Pere Arus (IRTA, Institut de Recerca i Tecnologia Agroalimentàries: Centre de Cabrils, Departament de Genetica Vegetal) in Barcelona, Spain over a period of 5 months. In particular, the *Prunus* reference map constructed with an almond (*Prunus dulcis*) x peach (*Prunus persica*) F_2 population (Howad *et al.*, 2005) was used; more precisely, the TxE population (*Texas*, almond x *Earlygold*, peach).

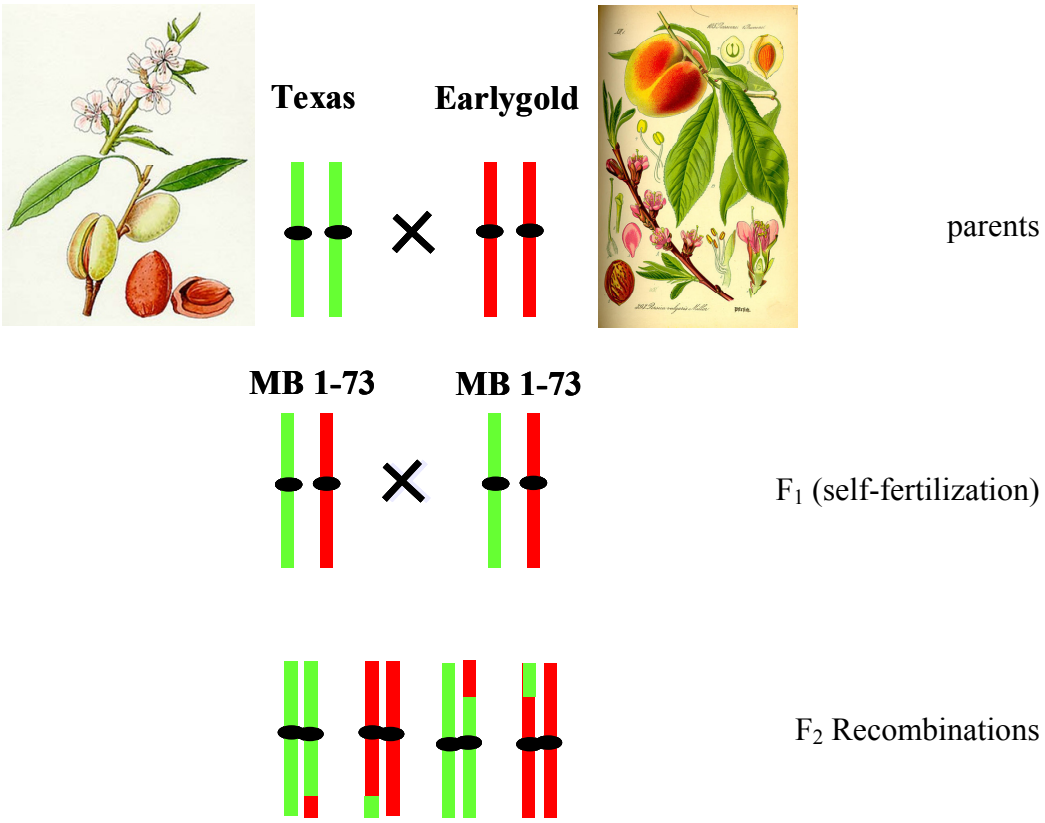


Fig. 3.18. Crosses to achieve the F_2 population. The almond parent (*Texas*) was crossed with the other parent, peach (*Earlygold*). The individuals of F_2 were obtained with the self-fertilization of the F_1 population.

It is very important to explain the concept and the terminology about the bin mapping. In particular:

- bin: it can be defined as a chromosomal interval between one recombination breakpoint or telomere and the next one (within the set of the selected individuals);
- bin genotype: joint individual genotypes of the selected bin mapping set;
- bin terminology: for example, in "bin 2:08", 2 indicates the linkage group (G2) while 08 indicates the position of the last marker in that bin.

About the bin distribution in T x E population, in the laboratory of prof. Pere Arus they obtained the scheme (Fig. 3.19) with the following characteristics:

- 6 individuals of the bin set;
- 68 bins;
- 6-11 bins per linkage group;
- 1.3 cM for the smallest bin;
- 24.7 cM for the biggest bin;
- 2 pairs of duplicated bins.

To describe the bin genotype, there are some particular symbols (Howad *et al.*, 2005):

- A stands for homozygous for the *Earlygold* allele (peach);
- B stands for homozygous for the *Texas* allele (almond);
- H stands for heterozygous like the hybrid.

	TxE plant						BIN
	5	12	23	30	34	83	name
G1	B	H	B	H	H	H	1:14
	H	H	B	H	H	H	1:15
	A	H	B	H	H	H	1:26
	A	H	B	H	H	B	1:28
	A	A	B	H	H	B	1:34
	A	A	B	H	B	B	1:50
	A	A	B	H	B	H	1:52
	A	H	B	H	B	H	1:55
	A	B	B	H	B	H	1:73
	H	B	B	H	H	H	1:78
	H	B	B	C	H	B	1:87
G2	A	B	H	A	A	H	2:08
	A	H	H	A	A	H	2:13
	H	H	H	A	A	H	2:20
	H	H	A	A	A	H	2:25
	B	H	A	A	A	H	2:26
	B	H	A	A	H	H	2:28
	B	H	A	A	H	B	2:34
	B	H	A	H	H	B	2:38
	B	H	H	H	H	B	2:45
	B	H	H	H	H	H	2:50
G3	B	H	H	H	H	B	3:04
	B	H	B	H	H	B	3:06
	H	H	B	H	H	B	3:12
	H	H	B	A	H	B	3:14
	H	H	B	A	H	H	3:22
	H	H	H	A	H	H	3:37
	A	H	H	A	H	H	3:49
G4	H	H	H	H	H	H	4:18
	H	H	A	H	H	H	4:22
	H	H	A	H	B	H	4:27
	H	A	A	H	B	H	4:28
	H	A	A	H	B	B	4:46
	H	A	A	A	B	B	4:63
G5	A	H	H	A	B	H	5:04
	A	A	H	A	B	H	5:05
	A	A	H	A	B	A	5:08
	A	H	H	A	B	A	5:11
	A	H	H	A	H	A	5:13
	A	H	H	H	H	A	5:21
	A	H	H	H	A	A	5:41
	A	H	H	B	A	H	5:46
	A	H	B	B	A	H	5:49
G6	H	A	B	H	H	A	6:00
	H	A	A	H	H	A	6:25
	H	A	A	B	H	A	6:39
	H	H	A	B	H	A	6:45
	H	H	A	B	H	H	6:49
	B	H	A	B	H	H	6:56
	B	H	A	B	H	B	6:65
	B	H	A	B	B	B	6:74
	B	H	H	B	B	B	6:80
	B	B	H	B	B	B	6:84
G7	A	H	H	B	H	B	7:25
	H	H	H	B	H	B	7:31
	H	H	B	B	A	B	7:41
	H	H	B	H	A	B	7:48
	H	H	B	H	A	H	7:56
	H	A	B	H	H	H	7:71
G8	A	B	H	H	H	H	8:11
	A	H	H	H	H	H	8:19
	A	H	A	H	H	H	8:21
	A	H	A	H	H	A	8:23
	A	H	A	H	A	A	8:28
	A	H	H	H	A	A	8:30
	H	H	H	H	A	A	8:41
	H	H	H	B	A	A	8:60
							67

Fig. 3.19. Distribution of the bins in the 8 linkage groups in the T x E population. This scheme was designed in the laboratory of the prof. Pere Arus. In the first column the number of the linkage group was specified, while the bin genotypes and the corresponding bin names were reported in rows.

3.2.2. BIN MAPPING OF THE GENES

In this work, several genes chosen for being ripening related and belonging to either ethylene or auxin domains, were mapped. After PCR amplification, purification and precipitation, the sequences obtained with ABI PRISM 3130xl were analyzed with the *Sequencer* program to select the possible presence of SNPs (single nucleotide polymorphisms). The individuals of bin set are: peach (parent), F_1 individual (hybrid) and the 6 selected individuals of F_2.

For example, the *ctg134* (unknown protein) was mapped in this way:

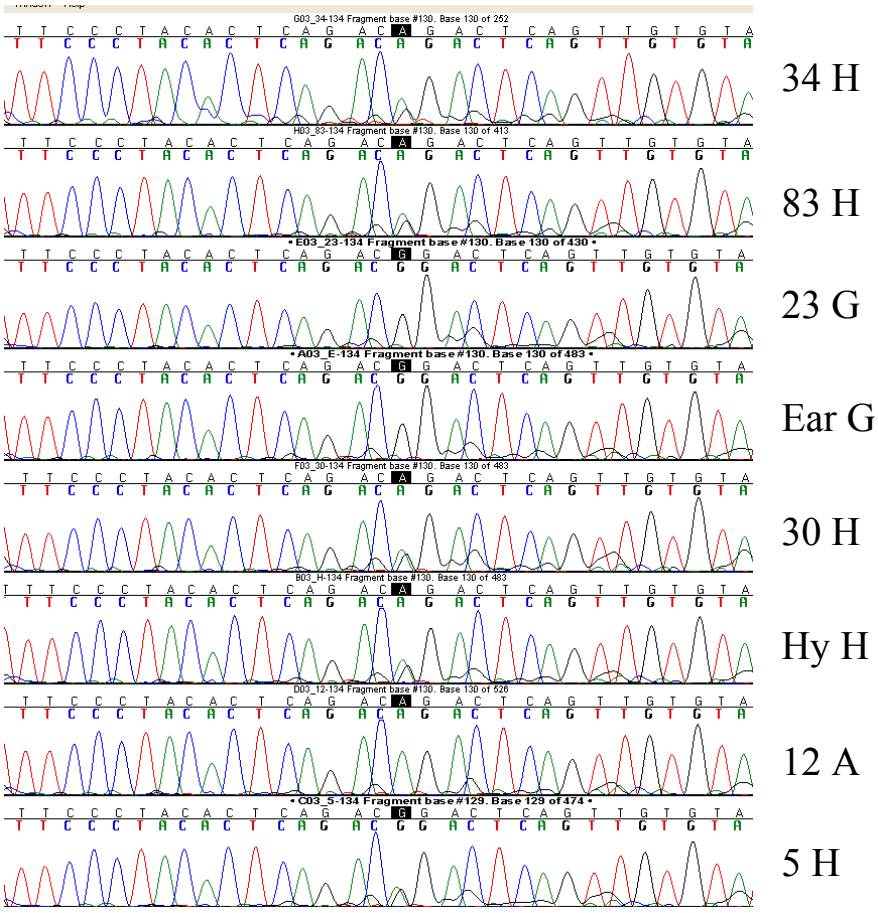


Fig. 3.20. Analysis of the SNPs (single nucleotide polymorphism). The presence of SNP was checked and analyzed for each individual to assign subsequently the bin genotype. The numbers indicates the individuals of F_2, Ear: parent *Earlygold*, Hy: F_1 individual (hybrid).

In this case, individuals 5, 30, 34 and 83 have the same DNA as the hybrid; individual 23 is like the peach parent and thus, for exclusion, individual 12 is like the almond parent.

HABHHH is the bin genotype for ctg134 and thus crossing this result with the distribution of the bins in the T x E population, it has been determined that *ctg134* maps in bin 7:71.

52	B	H	A	B	H	B	6:65	CPPCT047	PC60	6.4	6	8
53	B	H	A	B	B	B	6:74	UDP98-412	Pgl1	2.3	3	5
54	B	H	H	B	B	B	6:80	BC06B	Civ62A	0.8	4	6
55	B	B	H	B	B	B	6:84	CPPCT030	CPPCT021	3.5	5	10
56	A	H	H	B	H	B	7:25	AA12Cd	PC12	24.7	24(1)	36
57	H	H	H	B	H	B	7:31	CPSCT033	AD05D	2.8	8	11
58	H	H	B	B	A	B	7:41	PC34A	AG39B	4.6	5	7
59	H	H	B	H	A	B	7:48	AG60A	PMS2	5.3	7	12
60	H	H	B	H	A	H	7:56	PS8e8	FG42	7.1	7	8
61	H	A	B	H	H	H	7:71	FG27	PS5c3	11.1	11	15
62	A	B	H	H	H	H	8:11	CPSCT018	BF08B	10.9	7	12
63	A	H	H	H	H	H	8:19	AG112A	CPPCT035	5.8	7	17
64	A	H	A	H	H	H	8:21	LY29	UDP96-019	0	3	4
65	A	H	A	H	H	A	8:23	EPDCU3516	CC131A	0	2	2
66	A	H	A	H	A	A	8:28	BPPCT012	AD01A	3.6	4	5
67	A	H	H	H	A	A	8:30	PC101	AB08A	0.8	2	3
68	H	H	H	H	A	A	8:41	AG4A	FG37	10.8	10	15
69	H	H	H	B	A	A	8:60	CPDCT023	PC36	17.1	16	21
70												
71	Genotypes:		B	=	homozygous for the Earlygold allele							
72			A	=	homozygous for the Texas allele							
73			H	=	heterozygous like the hybrid							

Fig. 3.21. Scheme of the ctg134 mapping. Its bin genotype is HABHHH, thus *ctg134* maps in the linkage group number 7, in particular, in bin 7:71.

In some lucky cases, mapping can be resolved without sequencing. An example is the mapping of the ctg4705 (encoding for an IAA amidohydrolase). In this way, the origin of the alleles is clear directly from the agarose gel electrophoresis analysis.

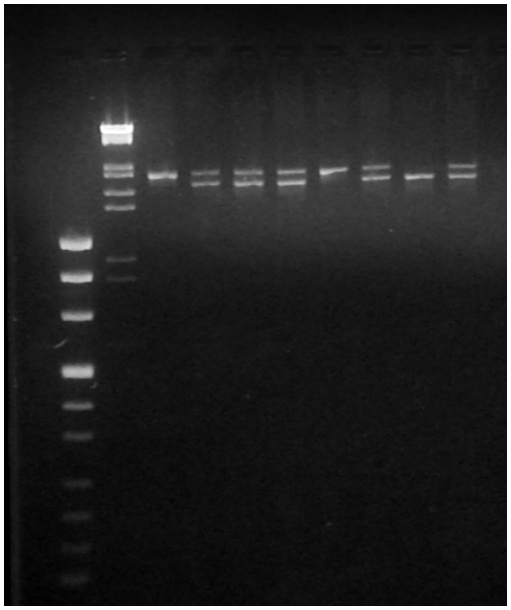


Fig. 3.22. Direct mapping of ctg4705 by agarose gel electrophoresis. The loading order of the DNA samples after the markers (left) are: *Earlygold*, hybrid, 5, 12, 23, 30, 34 and 83 individuals.

The results are: the peach and the individual 23 show the band with the same dimension; the individuals 5, 12, 30 and 83 are like the hybrid, while for exclusion, the individual 34 is homozygous for the almond allele. Thus, the bin genotype is HHBHAH and the *ctg4705* maps in the bin 7:56.

In this project, 49 gene were selected: 38 genes were mapped, 2 genes resulted monomorphics at the analyzed locus (ctg2713 TIR1 and ctg3945 subtilisin like proteinase) and 9 could not be mapped because there were problems with PCR amplifications (in particular, ctg358 Aux/IAA; ctg544 GA2 oxidase; ctg671 Aux/IAA; ctg768 Aux/IAA; ctg1068 Aux/IAA; ctg1375 nitrilase; ctg2902 putative sorbitol transporter; ctg2195 MADS box; MADS 462, MADS box) (Fig. 3.23).

Number of selected CG	designed primer	PCR amplification			mapped CGs	mono CGs
		1 band	0 band	>1 band		
49	49	38	1	8	38	2

Fig. 3.23. Summary of the mapping project. 38 genes were mapped (for all these genes, one single band in PCR was obtained); 2 genes were monomorphic and 9 could not be mapped (for 8 genes more one band was obtained, for one gene the amplification failed).

The results about the mapping of the selected genes have been shown the following table (Fig. 3.24):

ctg name	Annotation	bin	bin set
ctg 1505	ARF	**1:14**	BHBHHH
ctg 1727	Aux/IAA	**1:14**	BHBHHH
ctg 2025	ETR2	**1:14**	BHBHHH
ctg 1357	MADS box protein4	**1:34**	AABHHB
ctg 85	calcineurin B-like protein	**1:52**	AABHBH
ACS5	ACS5	**1:52**	AABHBH
ctg 1436	ETR1	**1:78**	HBBHHH
ctg 489	ACS1	**2:26**	BHAAAH
ctg 42	Aux/IAA	**3:14**	HHBAHB
ctg 64	ACO1	**3:37**	HHHAHH
ctg 3678	ARF	**3:37**	HHHAHH
ctg 3575	IGPS	**3:37**	HHHAHH
ctg 1991	ARF	**4:18**	HHHHHH
ctg 2980	NCED	**4:18**	HHHHHH
ACO2	ACO2	**4:18**	HHHHHH
ctg 3371	trp synthase beta subunit	**4:46**	HAAHBB
ctg 1310	NAM	**4:46**	HAAHBB
ctg 974	RD22-like protein	**4:63**	HAAABB
ctg 349	PIP	**5:21**	AHHHHA
ctg 5158	ACS4	**5:21**	AHHHHA
ctg 2568	ACS2	**5:21**	AHHHHA
ctg 4040	ARF	**5:41**	AHHHAA
ctg 3721	PIN1	**5:46**	AHHBAH
ctg 475	IAA amidohydrolase	**7:31**	HHHBHB
ctg 4056	IAA amidohydrolase	**7:31**	HHHBHB
ctg 2797	auxin efflux carrier protein	**7:48**	HHBHAB
ctg 1151	ACS3	**7:56**	HHBHAH
ctg 4705	IAA amidohydrolase	**7:56**	HHBHAH
ctg 134	unknown protein	**7:71**	HABHHH
ctg 39	hypothetical protein	**8:19**	AHHHHH
ctg 298	bZIP	**8:23**	AHAHHA

ctg 1993	auxin protein GH3	**8:41**	HHHHAA
ctg 84	Aux/IAA	**8:60**	HHHBAA
ctg 1541	TIR1	**8:60**	HHHBAA
ctg 356	ERS 1	**8:60**	HHHBAA
ctg 1741	Aux/IAA	**8:60**	HHHBAA
ctg 2116	ethylene response factor2	**8:60**	HHHBAA
ctg 57	Aux/IAA	**8:60**	HHHBAA

Fig. 3.24. Table with the results of the gene mapping. In the first and the second columns, contig name and annotation are reported, respectively. In third column, the fist number represents the linkage group and the second number represents the bin where the gene maps.

In linkage group number 1, two genes encoding for ethylene receptors are present (*ETR1* and *ETR2*) but in two different bins, 1:78 and 1:14, respectively. The two forms of ACO map in two different linkage groups: *ACO1* in 3:37 while *ACO2* in 4:18. On the contrary, two forms of ACS map in the same bin (5:21). Three of the genes encoding IAA amidohydrolases map in the linkage group number 7 with two of them (*ctg475* and *ctg4056*) falling in the same bin (7:31); another gene of the auxin domain, *ctg2797* (auxin efflux carrier protein), maps on group 7.

In several linkage groups, there is an over-representation of genes belonging to the ethylene domain and to the auxin domain and some of them map even in the same bin. In particular, it is very interesting to observe that in the linkage group number 8, there are genes involved in the ethylene pathway such as *ctg356* (ERS1) and *ctg2116* (ethylene response factor2) and 5 genes involved in the auxin domain such as *ctg1993* (GH3), *ctg84*, *ctg1741*, *ctg57* (Aux/IAA) and *ctg1541* (TIR1). Thus it is possible to suppose a relationship between the genes position, their expression and function and so in the next future this relationship will be further investigated.

At the same time, it will be necessary to analyze the relationship between the position of these genes and those of the traits responsible for fruit characteristics already mapped. In this case, to find these possible correlations, the collaboration with IRTA will continue for the next years (peach bin mapping project). In fact, an important advantage of the bin mapping approach is to facilitate the scientific community access to a reference mapping population and to cooperate in placing new markers or characters by exchanging a limited number of vegetatively propagated plants or DNA samples (Howad *et al.*, 2005).

3.3. STUDY OF THE TRANSCRIPTIONAL REGULATION OF TWO RIPENING-REGULATED GENES

3.3.1. bZIP AND AUX/IAA EXPRESSION

Transcription factors have a key role in the genetic regulation during ripening (Giovannoni, 2004). Eukaryotic genes are primarily modulated by transcriptional regulatory elements as transcription factors. Their activity in a cell can be regulated by environmental signals and by several signals (such as hormones) from other cells. TFs can be classified according to the structure of their DNA binding domains. An example of TF is the bZIP and of transcriptional modulators is the Aux/IAA protein involved in mediating auxin responses (Berleth *et al.*, 2004).

Zinc finger proteins have a peptide sequence that includes two cysteines and two histidines at specific locations in the polypeptide. These amino acids are crosslinked by zinc, producing a loop or "finger" structure, which binds to DNA. Zinc fingers are usually found in pairs, although proteins can have a variable number of fingers in a single polypeptide. The Aux/IAA proteins share a four domain structure and domains III and IV allow them to interact either with each other or with similar domains of Auxin Response Factor (ARF) proteins.

Two of these genes, mapped in the same linkage group (number 8), are the object of the last part of this work. They are the bZIP (ctg298) and the Aux/IAA (ctg57). The complete genomic sequences were available in the laboratory of Prof. Livio Trainotti. As deduced from counting EST clones (*bZIP* gene: 112 sequences; *Aux/IAA* gene: 211 sequences) present in the NCBI database (Fig. 3.25), these genes present a high transcript abundance in ripe fruit. Furthermore, Real Time PCR analyses showed that their expression parallel peach ripening (Fig 3.26).

bZIP (ctg298)

	Ppe.19208	
fruit	2165	109 /50332
leaf	0	0 /2811
shoot	0	0 /1942

Aux/IAA (ctg57)

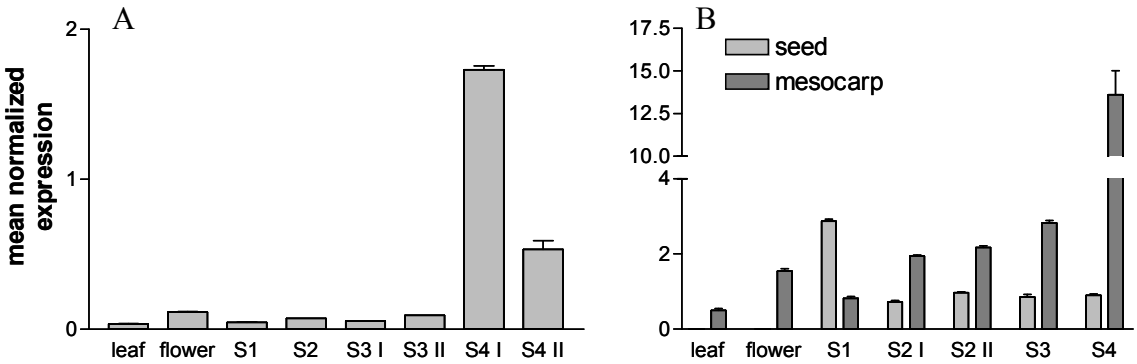
	Ppe.19175	
fruit	4132	208 /50332
leaf	355	1 /2811
shoot	0	0 /1942

Fig. 3.25. High transcript abundance of the two selected genes as deduced from counting EST clones (*bZIP* gene: 112 sequences; *Aux/IAA* gene: 211 sequences) present in the NCBI database (http://www.ncbi.nlm.nih.gov). In the first column the pool name is indicated, in the second column, the transcripts per million, the spot intensity based on the transcripts per million and the gene EST/Total EST in pool are reported, respectively.

bZIP is up-regulated by the transition from pre-climacteric to climacteric stage and it seems to be slightly repressed by treatment with auxin. 1-MCP does not influence the transcription of the gene in class 0 and 1 fruit. When mesocarp and seed are compared, bZIP expression is high in the mesocarp in S4 while in the seed the maximum is in the S1 stage (Fig. 3.26A-D).

As regards Aux/IAA, this gene is up-regulated by the S3II-S4I transition and it is not influenced by treatments with either ethylene or auxin. The treatment with 1-MCP induces its expression in class 0 fruit and, more strongly, in class 1 fruit. Its expression is not detected in seed (Fig. 3.26E-H).

bZIP (ctg298)



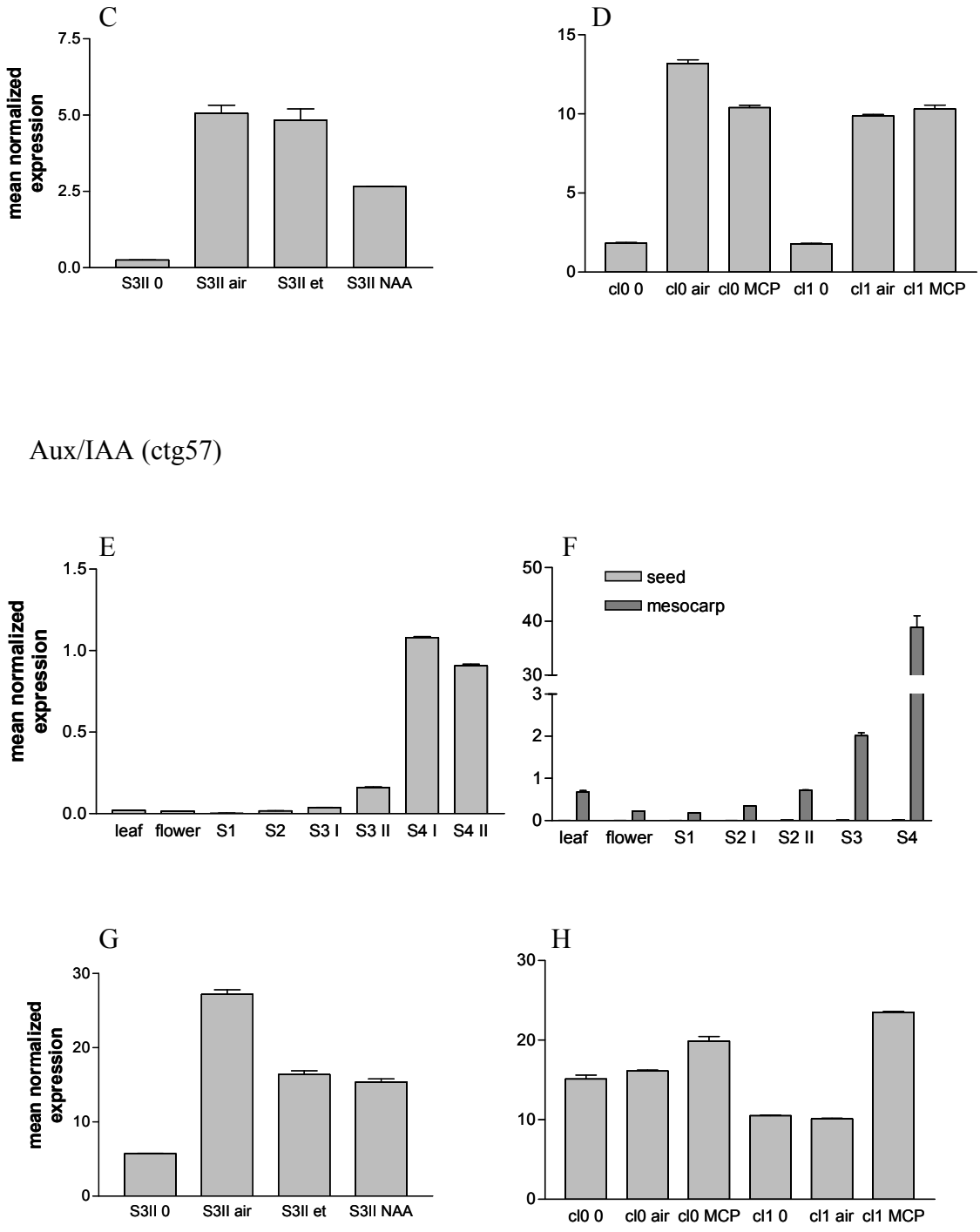


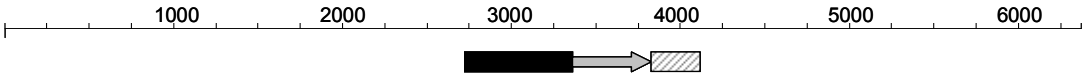
Fig. 3.26. Relative expression profiles of bZIP (ctg298) and Aux/IAA (ctg57) genes analyzed in different samples: in different plant tissues, during *Redhaven* fruit development and ripening (A and E); in different plant tissues, in different stages of seed and mesocarp development (B and F) (samples were from cultivar *Fantasia* and they have been collected by Ramina's coworkers, as part of the ongoing collaboration with Prof. Ramina's laboratory); after hormone treatment of *Redhaven* S3II fruit (C and G); in Class 0 and Class 1 *Stark Red Gold* nectarines following 1-MCP treatments (D and F). Values have been obtained by means of Real Time PCR. Stages S1 to S4II

encompass the development and ripening of peach fruits (panels A and E). Stages S1, S2I, S2II, S3, S4 encompass the development and ripening of seed and mesocarp (panels B and F). Hormone treatments (et: ethylene; NAA: 1-naphthalene acetic acid) lasted for 48 hours (panels C and G). Nectarines have been sampled after 36 h of storage either in air or in 1-MCP (12 h) plus air (24 h in air after the end of the 1-MCP treatment) (panels D and H). Bars are the standard deviations from the means of three replicates.

3.3.2. STRUCTURE OF THE *PpbZIP298* AND *PpIAA57* GENES

bZIP and *Aux/IAA* genes were sequenced to allow the characterization of their regulatory regions. These genes were named *PpbZIP298* and *PpIAA57*, respectively.

PpbZIP298



PpIAA57

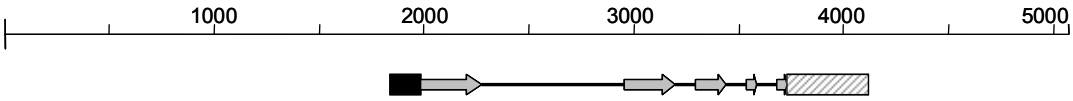


Fig. 3.27. Structures of *PpbZIP298* and *PpIAA57* genes. The 5'UTR is indicated with black box, the 3'UTR with diagonal bars and the exons are represented by grey arrows. Scales over the diagrams are the length, in base pairs.

As regards *PpbZIP298*, a genomic fragment of 6369 bp (Fig. 3.27) revealed that this gene is formed by a single exon, the 5'UTR is formed by 634 bp and the 5'-flanking sequence by 2743 bp.

The sequence (5070 bp) of *PpIAA57* (Fig. 3.27) allowed the isolation of a 5'-flanking sequence of 1826 bp and the 5'UTR of 162 bp. It also revealed that the coding sequence is interrupted by 4 introns, the first one of which, being very long (964 bp), could also contains regulatory region(s); the other 3 introns have short dimensions (177, 110 and 105 bp, respectively).

3.3.3. ANALYSIS OF THE PROMOTERS OF THE *PpbZIP298* AND *PpIAA57* GENES

In this work, for shortness, the term "promoter" indicates the sequence before the transcription start site available for each gene, thus including also the 5'UTRs.
The promoter sequences of the *PpbZIP298* and *PpIAA57* genes were evaluated with a bioinformatic analysis to discover the presence of *cis*-elements. The PlantCARE, a database of plant *cis*-acting regulatory elements, enhancers and repressors and a portal to tools for *in silico* analysis of promoter sequences, (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) was used. Regulatory elements are represented by positional matrices, consensus sequences and individual sites on particular promoter sequences. Upon submission of a promoter sequence by a user, a new implementation presents a dynamic html page with the PlantCARE TF-sites highlighted on the sequence. The PlantCARE database is a partner in the European PlaNet (http://mips.gsf.de/proj/planet/) project to share promoter data with the plant research community.
In the promoter sequence of *PpbZIP298* there are several putative *cis*-elements such as those involved:

- in stresses responsiveness (dehydration, heat, low temperature, salt, drought, anaerobiosis);
- in hormones responsiveness (gibberellins, MeJA, salicylic acid and auxin with a degenerate auxin-response elements TGTCNC);
- in regulation of gene expression (to confer high transcription levels);
- in tissue-specificity (endosperm expression, meristem specific activation and fruit specificity).

The 5'UTR of the *PpbZIP298* gene is longer than that of the *PpIAA57* gene. So the presence of some *cis*-elements in this region might be possible. Indeed, there

are 4 *cis*-acting regulatory elements essential for the anaerobic induction, 2 elements involved in hormones responsiveness (salicylic acid and auxin), one element involved in drought response and 4 *cis*-elements implicated in light response.

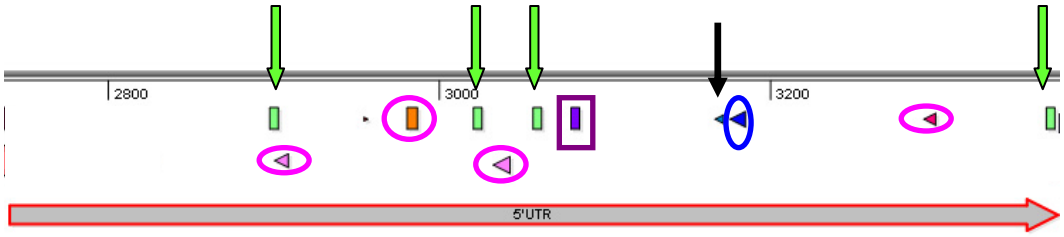


Fig. 3.28. Putative *cis*-elements present in 5'UTR region of the *PpbZIP298* gene. The elements are involved in light response (the pink circle); in auxin responsiveness (purple rectangle); in salicylic acid responsiveness (blue circle); in anaerobic response (green arrows) and drought response (black arrow). Scale over the diagram is the length, in base pairs.

In the promoter sequence of *PpIAA57* gene, putative *cis*-elements are implicated:

- in hormones responsiveness (abscisic acid, salicylic acid, gibberellin, MeJA, ethylene and auxin with a degenerate auxin-response elements TGTCNC);
- in environment-, defence- and stress-responsiveness (light, drought and anaerobic condition);
- in regulation of gene expression (able to confer high transcription levels);
- in tissue-specificity (endosperm-, shoot- and fruit-specific expression).

3.3.4. STABLE TRANSFORMATION OF TOBACCO PLANTS

Tobacco plants are a good system for the stable transformation. The promoter regions consisting of 5'-flanking sequence and 5'UTR of the *PpbZIP298* and *PpIAA57* genes were cloned in the pPR97 plasmid thus being fused with the GUS reporter gene. In this section of the work, 3 different constructs with progressive promoter deletions were set up:

- 2075 bp for *PpbZIP298* gene: fragment of 5'-flanking sequence (1441 bp) plus the 5'UTR region (bZIP 2K);
- 3317 bps for *PpbZIP298* gene: all 5'-flanking sequence available plus the 5'UTR region (bZIP 3K);
- 2000 bps for *PpIAA57* gene: all 5'-flanking sequence available plus the 5'UTR region.

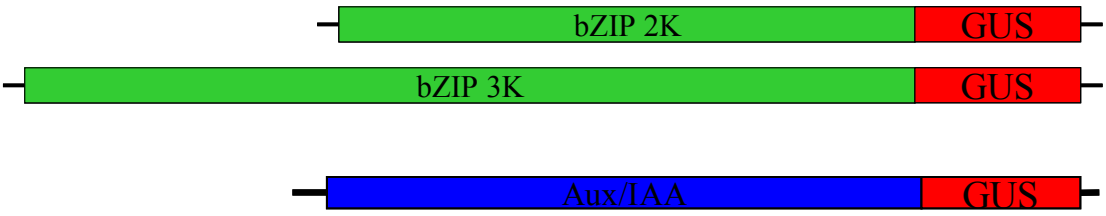


Fig. 3.29. Chimeric promoter-GUS constructs used in the stable tobacco transformation: 2075 bp (abbreviation 2K) and 3317 bp (abbreviation 3K) of *PpbZIP298* promoter sequence and 2000 bp of *PpIAA57* promoter sequence drive the GUS reporter gene (abbreviation Aux/IAA).

For the tobacco plants transformation, the protocol reported in Fisher and Guiltinan (1995) was followed.

In this work, the number of transformed plants obtained for each construct was the following: 20 clones for bZIP 2K, 21 clones for bZIP 3K and 22 clones for Aux/IAA. To control the presence of the transgene, PCR reactions, with proper primers, were made. The plants did not present any particular phenotype.

For each clone GUS enzymatic assay was made. GUS activity was measured from a piece of transformed leaf from each one of the transformants. The GUS activity has been expressed as nmol 4-MU min^{-1} μg^{-1} protein (Jefferson *et al.*, 1987) for all tobacco plants analyzed.

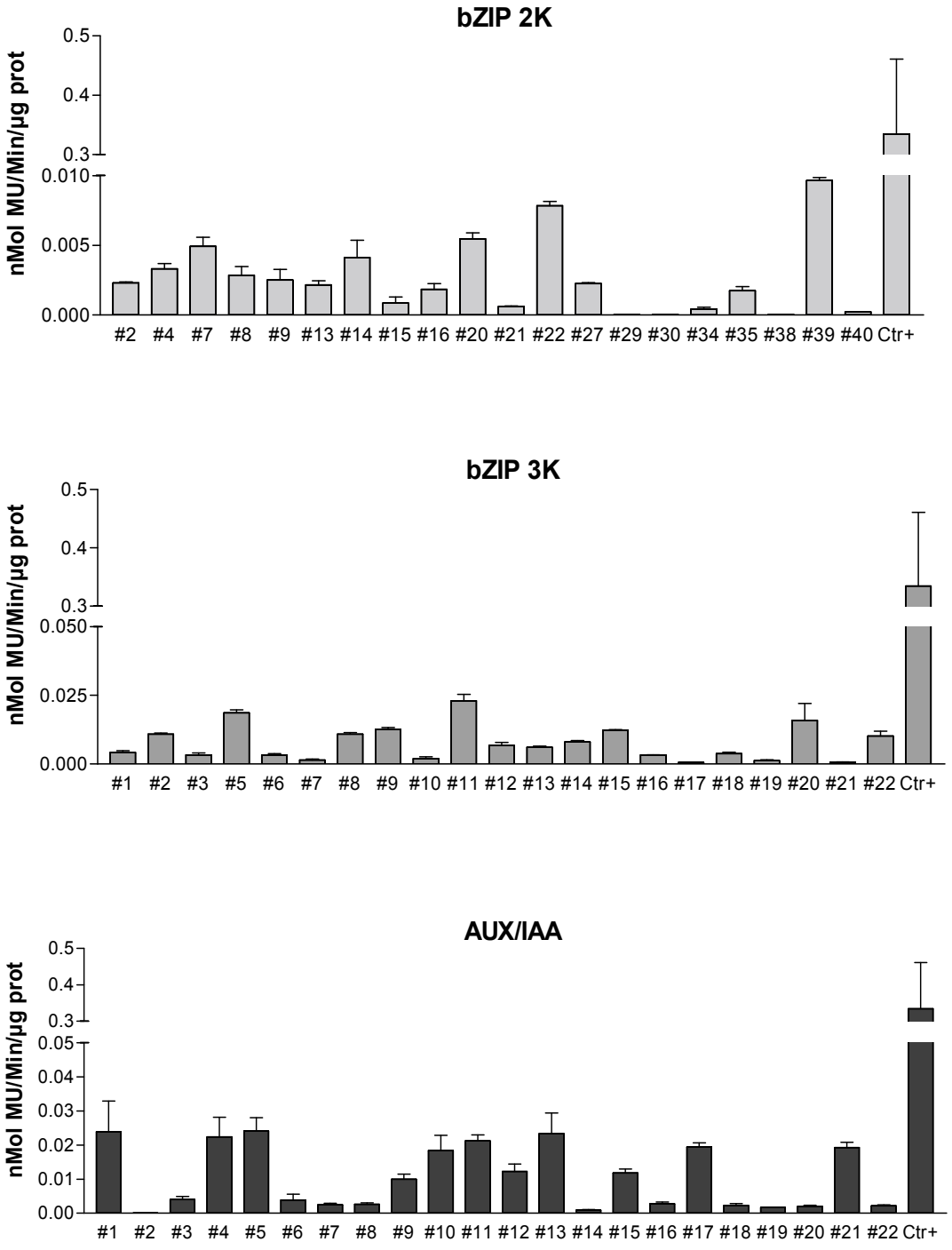


Fig. 3.30. GUS activity expressed in nMol MU/Min/µg of protein obtained for each clone of the tobacco plants transformed in permanent way. bZIP 2K and bZIP 3K: constructs with 2075 and 3317 bp of *PpbZIP298* promoter sequence, respectively; AUX/IAA: construct with 2000 bp of *PpIAA57* fragment sequence; Ctr+: construct with the GUS gene driven by CaMV 35S promoter. Each value is the mean of two assays from two different leaves.

The mean with the 7 clones showing the highest GUS activity for each construct has been used to understand and to compare the strength of the promoter sequences (Fig. 3.31).

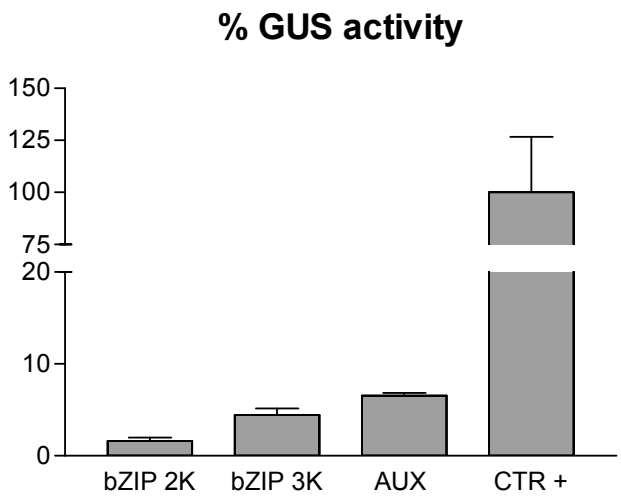


Fig. 3.31. Comparison of the strength of peach-promoter::GUS fusions stably inserted in tobacco plants. GUS activity of the three constructs (means of seven assays, each one from a different tobacco clone) carried out with protein extracts of tobacco leaves. bZIP 2K and bZIP 3K: constructs with 2075 and 3317 bp of *PpbZIP298* sequence, respectively; AUX: construct with 2000 bp of *PpIAA57* sequence; Ctr+: construct with the GUS gene driven by CaMV 35S promoter. The activities measured with these promoter fragments are expressed as a percentage of the activity obtained with the CaMV 35S promoter (Ctr+), set arbitrarily to 100%.

The graphic in Fig. 3.31 has shown that the longer promoter sequence of *PpbZIP298* presents an activity slightly higher than that of 2K, but at the same time, the strength of the promoter region of *PpIAA57* is a little more evident than that of the other two constructs. As it could be expected, in leaves the activities of these promoter sequences are low because these genes are fruit-specific.

3.3.5. TRANSIENT TRANSFORMATION OF PEACH FRUIT

The transient transformation is a good approach because it overcomes the difficulties of those species recalcitrant to the stable transformation and the problem of the regeneration. Moreover, it is fast, so it is possible, with this

method, to analyze the gene promoter regulatory regions of woody species. Fleshy fruit have some particular physiological and anatomic characteristics that could create problems with the application of common transient transformation methods such as the use of the protoplasts or the biolistic method. Thus, in this work the protocol reported in Spolaore *et al.* (2001), based on the mesocarp infection with an *Agrobacterium* culture, was used. The *Agrobacterium* suspension was uniformly injected throughout the entire fruit by means of a sterile 1 mL hypodermic syringe. Two different reporter genes were used, one of them (GUS-INT, Vancanneyt *et al.*, 1990) for the promoter analysis and the other one (LUC-INT, Hanson *et al.*, 1999) to check the transformation procedure and to normalize data. All the GUS values were normalized to the luciferase activity measured in the same protein extract.

The infiltration experiments were conducted with fruit of several peach or nectarine cultivars collected at the S4 stage: *Redhaven*, *Fantasia*, *Tardivo Zuliani*, *Cal Red*.

Fig. 3.32. Peach fruits belonging to the cultivar *Redhaven* (left) collected on 07/14/2009 and to the cultivar *Cal Red* (right) collected on 09/11/09. These fruits were at the stage S4, they were used for the agroinfiltration experiments.

The constructs employed for the transient transformation in the homolog system were the same used for the permanent transformation of tobacco plants (2075 and 3317 bp of *PpbZIP298* promoter sequence, respectively; 2000 bp of *PpIAA57* promoter sequence; GUS gene driven by CaMV 35S promoter as positive control

and Luciferase gene driven by double CaMV 35S promoter as negative control). The *Agrobacterium* culture with the construct containing the GUS reporter gene was mixed with that containing the Luciferase reporter gene at a 3:1 ratio. With *Redhaven* and *Cal Red* cultivars, the GUS activity was detected but it was very low. No activity was detected with fruit of other cultivars.

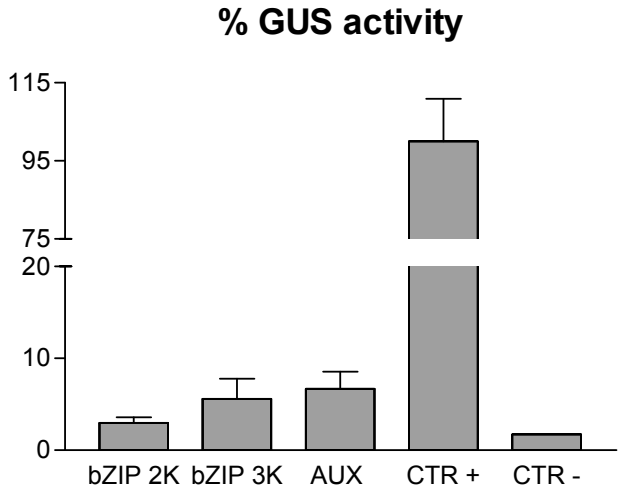


Fig. 3.33. Comparison of the strength of peach-promoter::GUS fusions transiently infiltrated in ripe peach fruit belonging to the *Redhaven* cultivar. GUS activities driven by the promoters of interest (bZIP 2K and bZIP 3K: constructs with 2075 and 3317 bp of *PpbZIP298* promoter sequence, respectively; AUX: construct with 2000 bp of promoter sequence of *PpIAA57*) and CaMV 35S (Ctr +) have been normalized on Luciferase activities deriving from a co-transformed CaMV 35S::Luciferase gene (Ctr -). The activities measured with these fragments are expressed as a percentage of the activity obtained with the CaMV 35S promoter (Ctr+), set arbitrarily to 100%. Values are the means of 4 independently infiltrated fruits. Bars represent standard errors.

The activity of the *PpIAA57* and *PpbZIP298* promoters is similar (Fig. 3.33) and, unexpectedly, it is not high despite the length of the used fragments and although these genes are highly expressed in ripe fruit.

3.3.6. REGULATORY ACTIVITY OF THE *PpbZIP298* 5'UTR IN TOBACCO LEAVES

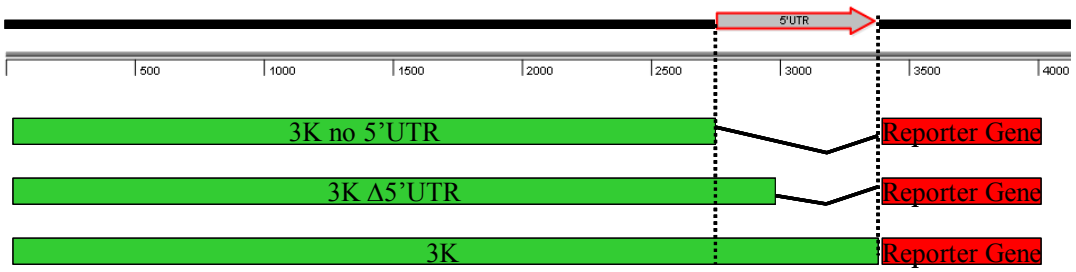
For a deeper investigation of *PpbZIP298* regulatory regions, tobacco leaves were used because peach fruit were not available after October and tobacco leaves are a good system for the agroinfiltration.

In order to generate *PpbZIP298* promoter deletion constructs, specific primers were designed to amplify regions with or without a portion of 5'UTR. After a careful analysis of the 5'UTR of the *PpbZIP298* gene, the constructs used were the following:

- 5'-flanking sequence with a partial deletion (from bp 146 to bp 634) of the 5'UTR region (3K Δ 5'UTR);
- 5'-flanking sequence with a complete deletion of the 5'UTR region (3K no 5'UTR);
- 5'-flanking sequence with all 5'UTR region (3K, as already used for the experiments described above).

The first construct contained 5'-flanking sequence and 145 bp of 5'UTR, in this way the region with all putative *cis*-elements was eliminated. All these constructs were pPR97-derived.

A



B

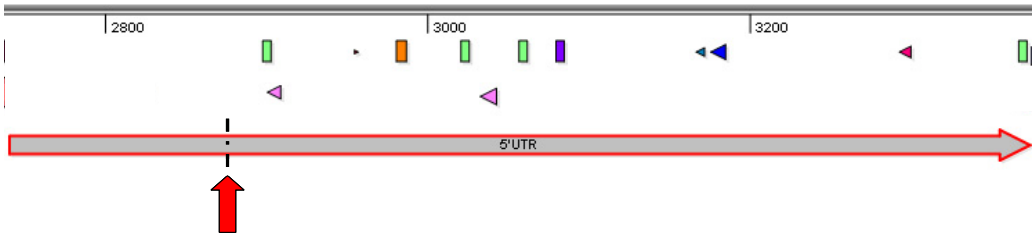


Fig. 3.34. A) Constructs with progressive deletions of *PpbZIP298* promoter sequence. Reporter gene is driven only by 5'-flanking sequence (complete deletion of 5'UTR, 3K no 5'UTR), or by 5'-flanking sequence with a partial deletion of 5'UTR (3K Δ5'UTR) or by 5'-flanking sequence with the complete 5'UTR (3K, the same sequence used in permanent transformation and fruit agroinfiltrations). Broken lines under the 5'UTR arrow indicate the deleted regions.
B) In the construct 3K Δ5'UTR, the starting point of the partial deletion of the 5'UTR is indicated by the red vertical arrow (145 bps of 5'UTR out of 634 are kept): in this way, all the 5'UTR region with the *cis*-elements (indicated with the same symbols of Fig. 3.28) is eliminated.

The protocol used was the same for the agroinfiltration in peach fruit but in this case the injection of the *Agrobacterium* culture was made by a syringe without the needle on the lower page of the leaf.

In the case of *PpbZIP298* promoter with a partial deletion of 5'UTR, whose fragment was named 3K Δ 5'UTR, GUS activity is higher than that of the other two constructs (5'-flanking sequence with and without 5'UTR, Fig. 3.35).

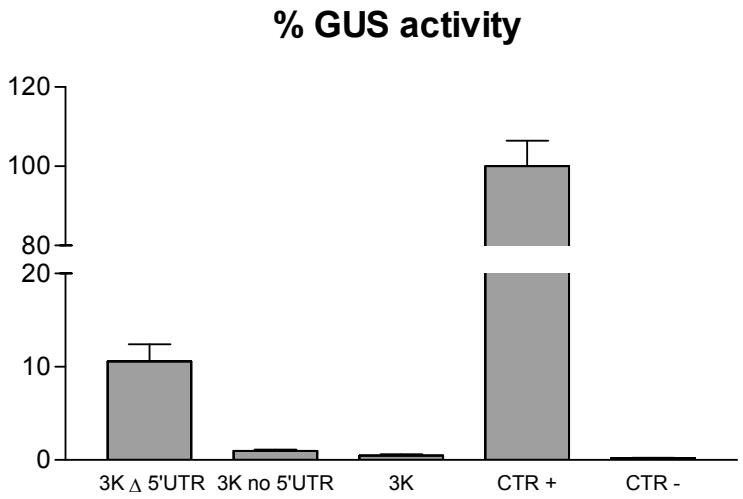


Fig. 3.35. Regulatory role of the *PpbZIP298* 5'UTR tested on tobacco leaves. GUS activity driven by the 5'-flanking sequence of *PpbZIP298* with the complete 5' UTR (3K), or with the 5'UTR either completely (3K no 5'UTR) or partially deleted (3K Δ5'UTR) are compared to that driven by CaMV 35S (Ctr +). All values have been normalized on Luciferase activities deriving from a co-transformed CaMV 35S::Luciferase gene (Ctr -). The activities measured with these fragment sequences are expressed as a percentage of the activity obtained with the CaMV 35S promoter (Ctr+), set arbitrarily to 100%. Values are the means of 4 independently infiltrated tobacco leaves. Bars represent standard errors.

To confirm the results obtained with this last experiment, the same portions of the *PpbZIP298* sequences were inserted in a different plasmid backbone. This last experiment was carried put to verify if the strength of the 3K Δ 5'UTR fragment was independent from the system, the plasmid and the reporter gene. Thus, the three regions (3K Δ 5'UTR, 3K no 5'UTR and 3K) were cloned before the luciferase reporter gene in a pGreen-derived plasmid (Hellens *et al*., 2000). In this vectors, the promoter sequence (object of study) was driving the Luciferase reporter gene while, on the same backbone, the GUS reporter gene was driven by the CaMV 35S promoter. The agroinfiltration experiments were conducted in the same way described above.

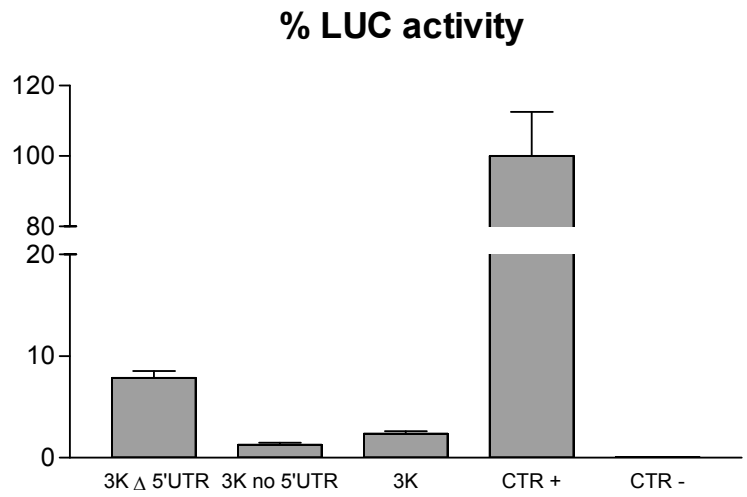


Fig. 3.36. Regulatory role of the *PpbZIP298* 5'UTR tested on tobacco leaves. Luciferase activity driven by the 5'-flanking sequence of *PpbZIP298* with the complete 5' UTR (3K), or with the 5'UTR either completely (3K no 5'UTR) or partially deleted (3K Δ5'UTR) are compared to that driven by NOS promoter (Ctr +). All values have been normalized on GUS activities deriving from the same plasmid. The construct without a promoter driven the Luciferase gene represents the negative control (Ctr -). The activities measured with these fragments are expressed as a percentage of the activity obtained with the NOS promoter (Ctr+), set arbitrarily to 100%. Values are the means of 4 independently infiltrated tobacco leaves. Bars represent standard errors.

The results has shown that the strength of the promoter with the partial deletion of the 5'UTR is again higher than that with the complete UTR (Fig. 3.36) and thus confirm the data obtained with the previous experiment (Fig. 3.35).

This data seem to support the identification of putative *cis*-regulatory region(s) in the 5' UTR of the *PpbZIP298* gene that could contribute to the regulation of the gene transcription.

Further work will be necessary to better analyze and characterize the putative regulatory regions in the 5' UTR of the *PpbZIP298* gene and their possible role(s) in controlling gene expression during peach fruit ripening.

4. CONCLUSIONS

The genomic approach used in this work has shown a novel aspect of the regulatory networks that operate during the ripening of climacteric peach fruits.
These experiments have revealed that a wide number of genes are ripening related; in fact, they are regulated by the transition from pre-climacteric to climacteric stage. This effect is apparently independent from either ethylene or NAA, therefore the expression of such genes might be regulated by other, as yet unknown, factors.
The molecular basis of ethylene synthesis and regulation has been a focal issue of research in fruits. Ethylene has a key role in the regulation of climacteric fruit ripening (Alba *et al.*, 2005): this study has confirmed that ethylene can affect the expression of many genes, as expected since peach is a climacteric fruit. For some ripening-specific genes such as ETR2 (coding for an ethylene receptor), the greatest inductive effect on its expression is caused by ethylene, while the more limited increase in transcript amount observed following a treatment with auxin is probably indirect and mediated by ethylene.
Auxin is a key hormone in fruit development. The IAA increment measured in the mesocarp tissues during ripening coincided with a net decrease in the embryo IAA content measured in the same time interval (Miller *et al.*, 1987), thus probably the mesocarp might either synthesize or release from conjugated forms its own IAA. Genes responsible for the auxin present in the mesocarp during ripening have been identified and their expression profiles characterized.
In this work, the importance of auxin has been investigated; it regulates the expression of some genes directly and with an autonomous way during ripening. It has been discovered that auxin has its own role, independent from ethylene, in the ripening regulation. The idea that an active IAA-related metabolism might occur in the fruit mesocarp is indirect but supported by the expression of some genes belonging to the “auxin domain”. In particular, the expression profiles of Aux/IAA, ARF and genes belonging to biosynthesis, homeostasis, perception, transport has been analyzed in order to study the regulation of peach ripening by auxin.

The investigation of some ripening related genes such as PIN1 (a gene encoding a putative auxin efflux facilitator protein), whose expression appears to be significantly up-regulated by ethylene rather than by auxin, has permitted to show a relationship between auxin and ethylene. In fact, it demonstrates that an active cross-talk between auxin and ethylene is also important for the regulation of ripening, besides the independent roles played by each hormone. The interplay between the two hormones is strengthened by the analysis of the expression profiles of genes belonging to the "ethylene domain". Biosynthetic genes like ACS1 and ACO1 are up-regulated during the transitions S3II-S4I, but it is interesting to observe that while ACO1 is positively regulated by ethylene (Ruperti *et al.*, 2001), the expression of ACS1 gene is more strongly up-regulated by auxin.

The study of the effect of 1-MCP has refined the investigation of regulation in peach ripening and contributed new evidences on the ethylene-auxin cross-talk. The efficacy of 1-MCP in delaying peach fruit ripening has been controversial until now. There are both reports that support an inhibitory action (Mathooko *et al.*, 2001; Rasori et *al.*, 2002) and reports which state that the chemical is (almost) ineffective (Dong *et al.*, 2001; Dal Cin *et al.*, 2006; Ziliotto *et al.*, 2008). In this work, it has been demonstrated that the 1-MCP effects are largely dependent on the ripening stage in which the chemical is applied; in order to select the fruits, it has been used the recently developed non destructive Index of Absorbance Difference (I_{AD}), which can predict the exact fruit ripening stage by means of a computer-assisted spectrophotometric device. The efficacy of 1-MCP is confirmed at the molecular level by changes of transcript abundance that the chemical can induce on genes such as those involved in the autocatalytic ethylene production (ACO1) and in the cell wall rearrangements (PG and PME). The fact that suppressing ethylene perception with 1-MCP leads to the induction of genes related to auxin and, most likely, to an increase in free IAA, further supports the auxin role in peach ripening. The data obtained in this part of the project can confirm that ethylene biosynthesis in peach fruit might be controlled by a regulatory loop of auxin-ethylene interactions in which hormone levels are reciprocally controlled by several signals. For example, high ethylene concentration reduces the mRNA amount of the IAA amidohydrolase but not of

the GH3 gene, thus allowing a balanced auxin homeostasis. When ethylene concentration is low or receptors are blocked by 1-MCP, fruit cells that have completed their maturation feel that ethylene is missing, so its synthesis has to be induced by a release of free auxin (Fig. 4.1).

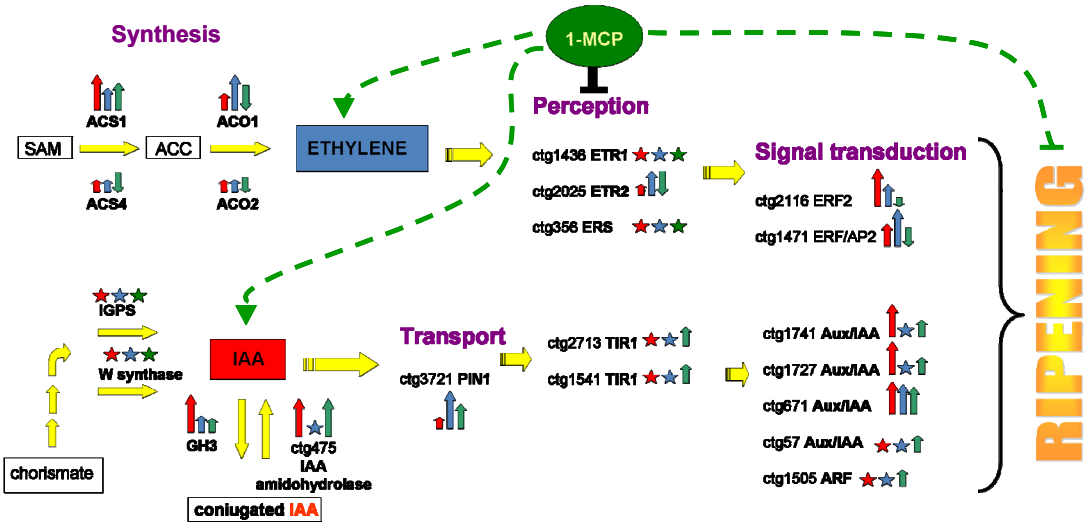


Fig. 4.1. Key genes in the ethylene and auxin pathways. All these genes are ripening-related. Gene induction or repression is represented by upward or downward arrows, respectively. Arrow sizes are proportional to fold changes. Colour codes: red represents the regulation by auxin, blue by ethylene and green by 1-MCP. Stars stand for unchanged expression.

These conclusions are outlined in peach fruit and cannot be extended to other climacteric fruits. However, a possible similar role for auxin could be present in the ripening of tomato fruit, where both ARF (auxin response factors) and Aux/IAA genes are differentially expressed. Also, a cross-talk between auxin and ethylene is shown in tomato fruits, in which an Aux/IAA gene (DR3) presents maximum accumulation in early red fruits and has ethylene-inducible expression (Jones *et al.*, 2002; Chaabouni *et al.*, 2009). Finally, as in peach, in ripening tomato fruits, concomitant with or slightly anticipating climacteric ethylene production there is an increase in free auxin content (Gillaspy *et al.*, 1993).

This work has revealed that also auxin, in close relation with ethylene, participates to the complex network of endogenous and exogenous signals involved in the regulation of peach ripening.

Furthermore, it has been shown that several transcription factors, known to have a key role in the genetic regulation during ripening (Giovannoni, 2004; Alba *et al*., 2005), are highly expressed in peach ripening. The bioinformatic analysis of the 5'-flanking sequence and 5'UTR region of the *PpbZIP298* gene (transcription factor) and the *PpIAA57* gene (transcriptional modulators of the auxin response) has revealed the presence of some interesting putative *cis*-elements involved in environment-, defence-, stress-, hormone-responsiveness and in regulation of gene expression. The behaviour of these sequences has been studied through peach fruit and tobacco leaves transient expression analyses and tobacco stable transformation. The analysis of these sequences has displayed that the activity of the *PpIAA57* and *PpbZIP298* promoters is similar and it is not high although these genes are fruit related. Also, unexpectedly, the strength of the two promoters resulted to be very weak, despite the length of the used fragments and the reason is not yet clear. Perhaps, for *PpIAA57*, it will be necessary to obtain a promoter sequence longer than that available in this study, thus the sequencing of the peach genome carried out by the *Drupomics Project* will help to accomplish this task. As regards *PpbZIP298*, the approach used in this work seems to support the identification of a putative *cis*-regulatory region in the 5' UTR of this gene, which could contribute to the regulation of the gene transcription. The hypothesis is that in this region there are some silencers and, probably, the *cis*-elements implicated in the anaerobic response might be involved in this regulation.

As regards the bin mapping work, some genes map in the same linkage group with few even mapping in the same bin. It will be necessary to carry on the collaboration with IRTA about the "bin mapping peach project" in order to investigate the correlation between the position of these genes and those of the traits responsible for fruit characteristics already mapped and to study the possible interaction between their positions and functions.

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